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FOREWORD

The principal aim of the international agricultural research system, supported by the Consultative Group on International Agricultural Research (CGIAR), is to increase the productivity, profitability, stability, and sustainability of the major food crop and livestock farming systems in developing countries. The 13 international agricultural research centers or IARCs now functioning under the CGIAR umbrella utilize all the tools science can offer in fulfilling their mandates. They are concerned not only with the development of economical, ecologically sound, and socially acceptable technologies but with the diffusion of benefits from new technologies to all farmers, irrespective of the size of their holdings and their innate input-purchasing and risk-taking capacity. For this purpose they work in close partnership with national agricultural research systems.

An area of great concern in the pattern of diffusion of benefits from new technologies is the bypassing of economically handicapped farmers such as small farmers and sharecroppers. It is now widely accepted that new technologies are scale neutral to farmers with different sizes of holdings. However, they are not resource neutral, because more inputs are needed for increased output. IARCs are approaching this problem in three ways. First, they develop varieties with a broad spectrum of resistance to pests and diseases, tolerance for adverse soil factors, and greater genetic capacity to utilize soil nitrogen. Second, they work on home-grown substitutes for market-purchased inputs such as fertilizers and pesticides. Third, they standardize techniques for maximizing returns from purchased inputs such as mineral fertilizers. Biotechnology offers scope for adding a dimension of resource neutrality in technology development.

On a suggestion received from Dr. Clarence C. Gray 111, former director for Agricultural Sciences of The Rockefeller Foundation, the directors of IARCs decided to organize an Inter-Center Seminar on IARCs and Biotechnology. The seminar was held at IRRI from 23 to 27 April 1984, with generous financial support from The Rockefeller Foundation.

We are grateful to Dr. Gray; Dr. Alva A. App, the Rockefeller Foundation's present director for Agricultural Sciences; and to Dr. Gary Toenniessen, assistant director for Agricultural Sciences, for their guidance and help in organizing the seminar.

We are grateful to the distinguished scientists who participated in the seminar and gave us the benefit of their vast experience and expertise. Particular thanks are due Dr. Nyle C. Brady, former director general of IRRI and now senior assistant administrator of the U.S. Agency for International Development, for delivering the keynote address at the inaugural session. Dr. F.J. Zapata, who served as the convenor of the Organizing Committee, worked exceedingly hard to attend to every detail of the seminar. I also thank the other members of the Organizing Committee, the chairmen of the sessions, the convenors of the working groups, and the secretaries and other staff members of IRRI who worked very hard to make the seminar a worthwhile one.

This volume was edited by Ms. Killara Burn with the assistance of Ms. Corazon V. Mendoza, assistant editor.

It has been said that in many areas of biotechnology and genetic engineering, there are more potential applications than originally conceived. The recommendations clearly bring out the wisdom behind the following statement of Sir Francis Bacon: "It would be an unsound fancy to expect that things which have never yet been done can be done except by methods which have never been tried."

M.S. Swaminathan
Chairman, Organizing Committee

PLANT PROPAGATION AND GERMPLASM CONSERVATION

IN VITRO CLONAL PROPAGATION TO ELIMINATE CROP DISEASES

W. M. ROCA

Diseases caused by viruses 2nd viroids are particularly important in asexually propagated crops because once the crop is infected, its propagula transmit the disease indefinitely, and effect severe yield and quality losses. Shoot – tip culture techniques used to recover healthy cassava and potato clones have resulted in dramatic yield increases in several cassava cultivars. Changes in plant and leaf morphology accompanied yield increase in a local cassava cultivar after in vitro propagation.

One of the most important applications of biotechnological tools has been the recovery of disease – free plants. Disease – free clones have been regenerated from about 55 cultivated species through tissue culture techniques (3, 9, 10): 23 ornamental plants, 11 fruit crops, 8 tuber root crops, 6 vegetables, and 7 industrial crops. The techniques can be applied to all known infectious agents, but are especially valuable in eliminating viruses and similar organisms from vegetatively propagated crops. Viruses disseminate with the crop propagula through successive generations, so that entire crops become infected. The infection may alter plant morphology or remain unnoticed. Viral diseases not only reduce crop yield and quality, but seriously limit international germplasm exchange.

This paper deals with the use of in vitro techniques to produce healthy plants and to restore crop yield and quality. It also presents results from work with cassava and potato.

DISEASE ELIMINATION THROUGH SHOOT – TIPCULTURE

Methods are available (9, 10, 11), or can be readily developed, to grow plants to maturity from shoot – tip explants (0.4 to 0.8 mm) comprising the apical meristem (0.1 to 0.2 mm) and at least one primordial leaf. Because all plants regenerated in vitro are not necessarily pathogen free, the methods should include 1)

Physiologist, Centro Internacional de Agricultura Tropical (CIAT), A. A. 67-13, Cali, Colombia.

identifying the pathogens, 2) applying techniques for eliminating disease, 3) testing regenerated plants for freedom from pathogens, and 4) propagating healthy plants under conditions that prevent reinfection (2).

FACTORS IN DISEASE ELIMINATION

Because virus movement is considerably slower through the symplasm than through the vascular system, and vascular differentiation occurs away from the shoot apical meristem, virus concentration decreases acropetally, and in the vegetative point, virus titer may be nil (5). In addition, mitosis in the meristem cells competes with virus multiplication, as occurs in rapidly dividing callus. The regeneration of healthy plants, through the culture of shoot tips, despite the presence of virus particles in the apical meristem cells (11), suggests that the in vitro condition helps inactivate the virus. Cell injury, caused by excising the shoot tips, may induce metabolic alterations that lead to virus degradation (8). Eliminating disease depends on several interrelated factors, such as the virus to be eliminated, the size of the explant for culture, and physical or chemical treatments applied to the infected plants or to the cultures.

Virus strain

Viruses vary in how difficult they are to eliminate; those restricted to the phloem can be eradicated by shoot-tip culture with little difficulty. The potato viruses are listed according to increasing difficulty of eradication as follows: potato leaf roll virus (PLRV), potato virus A (PVA), potato virus Y (PVY), potato virus M (PVM), potato virus X (PVX), potato virus S (PVS), and potato spindle tuber viroid (PSTV); the cassava virus diseases: frog skin, common mosaic, caribbean mosaic, and latent virus. The cassava African mosaic has been eradicated without much difficulty (4).

Infections with more than one virus can also be eradicated by shoot-tip culture. In several naturally infected andigenum and tuberosum potato cultivars, elimination rates of PVX plus PVY were much higher than those of PVX plus PVS; and PVX alone was somewhat easier to eliminate than PVS alone (Table 1). Similarly, 100% cleaning was possible when cassava stakes infected both with frog skin plus latent virus and frog skin plus bacterial blight were sprouted at high temperature before their shoot tips were excised and cultured (1).

Size of shoot-tip explant

Because of the way virus spreads in a plant, efficiency of virus elimination inversely relates to the size of shoot-tip explants. However, in vitro growth of very small explants (0.1 to 0.2 mm) is quite slow and often leads to callus formation. Because of this, the standard practice has been to culture shoot tips measuring 0.4 to 0.5 mm.

Table 1. Individual and double virus eradication rates from potato cultivars through potato explant thermotherapy and shoot-tip culture.[a]

Subspecies	PVX	PVS	PVX+PVY	PVX+PVS
andigenum				
Cultivars (no.)	6	2	3	3
Elimination (%)	60	50	85	50
tuberosum				
Cultivars (no.)	11	2	5	5
Elimination (%)	66	60	75	54
Total				
Cultivars (no.)	17	4	8	7
X Elimination (%)	63	55	80	52

[a] 36°C (day) and 30°C (night), applied to potted cuttings for 15 days; shoot-tip size used for culture: 0.4 to 0.5 mm; virus indexing with indicator plants and serology (unpublished data from work at Centro Internacional de la Papa, Lima, 1978). PVX = potato virus X. PVS = potato virus S. PVY = potato virus Y.

Explant size greatly influenced the efficiency of frog skin disease eradication in cassava; furthermore, sprouting the infected stakes at high temperature enhanced the rate of cleaning up to 100% when 0.4-mm shoot tips were cultured (Table 2).

Thermotherapy

Heat treatment does not eliminate most viruses, but it reduces virus multiplication and translocation in the plant. For example, Caribbean mosaic symptoms do not show up in new cassava leaves grown at 33°C, but the disease symptoms reappear in plants transferred to 25°C. Frog skin disease symptoms only decreased from severe to mild when the cassava crop was grown from stem cuttings from stakes sprouted at 40°C (day), and 35°C (night) temperature, for 30 days (1). Treating PVX-infected potato plants with high temperature decreased virus infectivity, Dilution point tests of the treated plants suggested a decrease in virus concentration due to heat treatment. As expected, elimination of PVX by shoot-tip culture was much higher from heat-treated than from nontreated plants (Table 3). In practice,

Table 2. Influence of explant size used for culture and sprouting temperature of stakes on frog skin elimination from cassava.

Treatment		Frog skin disease-free plants[c] (%)
Size of shoot tip[a] (mm)	Sprouting temp[b] (°C)	
0.4	26	79
	40/35	100
0.8	26	16
	40/35	85
1.2	26	0
	40/35	65

[a]Height of shoot tip. [b]Day/night temperature, applied to stakes prior to shoot tip culture, for 15 days. [c]Av of 6 plants/treatment. - Disease indexing: grafting and electrophoresis.

Table 3. Potato virus X (PVX) elimination through thermotherapy of infected plants followed by shoot-tip culture.

Potato leaf sap dilution	Relative infectivity of PVX in *G. globosa* leaves inoculated with leaf sap[a] of			
	Clone 800244		Clone 720057	
	Heat treated[b]	Control	Heat treated[b]	Control
1/10	+	+++	+	+++
1/100	–	++	+	+++
1/1000	–	+	–	++
1/10000	–	+	–	+
PVX elimination (%) by shoot-tip culture[c]	90	5	40	2

[a]No. local lesions per half *G. globosa* leaf inoculated at end of heat treatment. [b]36°C (day) and 30°C (night), for 15 days. [c]0.4 - 0.5 mm in size (unpublished data from work done at Centro Internacional de la Papa, Lima, 19771.

thermotherapy permits excising slightly larger explants, which may be devoid of viruses because the virusless region of the shoot tip is bigger (11).

High temperature tends to increase the infectivity of PSTV in potatoes (L. Schilde-Rentschler, pers. comm., 1983). Low temperature (8°C) decreased viroid concentration in the tissues. Culture of apical meristems from low temperature-treated plants resulted in 30% viroid elimination (6). Thus, using low instead of high temperature may help eliminate viroids through in vitro techniques.

Chemotherapy

Applying chemicals to eliminate viral diseases has had limited success. Most compounds tested are phytotoxic, and the virus regains its original concentration after the chemical treatment ends (3, 4, 10). Recent work with the nucleoside analogue virazole seems promising. With cassava, virazole was far more phytotoxic to sand-grown cuttings than to in vitro cultures; therefore, large shoot tips (0.8 to 1.0 mm) were cultured in media containing 40 ppm virazole and after 30 days, the terminal bud was taken from each culture to be grown in virazole-free media: frog skin disease-free plants were recovered (1).

OTHER TISSUE CULTURE METHODS

Decreasing virus multiplication in proliferating callus cultures (13) and uneven distribution of viruses in plant tissues may explain how establishing callus and regenerating plants can recover PVX-free potato (12) and tobacco mosaic virus (TMV)-free tobacco. Using these methods to eliminate viruses is limited to crops that can be regenerated from cell and callus cultures. The instabilities that may be displayed by unorganized cultures and that lead to variant phenotypes also limit these approaches.

On the other hand, in vitro grafting of shoot tips from infected plants onto seedling stocks has produced virus-free citrus, with the advantage that return to juvenility was overcome (9). Eliminating diseases in woody species through somatic embryogenesis using tissues as explants may now be possible (R. Litz, pers. comm., 1984).

PATHOGEN TESTING

As mentioned, not all plants regenerated in vitro are necessarily free of pathogens. Therefore, indexing regenerated plants for the pathogens to be eliminated is important. It is especially necessary for international plant material exchange. Most common indexing techniques involve one or more of the following: sap inoculation, serology, aphid transmission, graft transmission, electron microscopy, and enzyme-linked immunoabsorbent assays (ELISA). Recent developments in biotechnology will permit detecting pathogens by highly specific assays, through monoclonal antibody and nucleic acid hybridization techniques.

RECOVERY OF CROP YIELD AND QUALITY

Yield and vigor of local cultivars may decline because of pathogen accumulation. This decline has been observed in potatoes and other crops infected with viruses (2, 8). Producing clean planting material through in vitro techniques can significantly raise crop yield and quality. Preliminary results of three case studies with cassava will be summarized here.

Cassava caribbean mosaic disease, endemic to northern Colombia, has severely affected the cutlivar Secundina. Clean planting material was produced through thermotherapy followed by shoot-tip culture. Yield in fresh weight and starch content of roots increased by 70%; furthermore, yields of the in vitro-derived Secundina plants and a new hybrid (CM 342-170) developed for the region were similar (7). After 3 years of planting, yields of in vitro-processed Secundina have remained stable, whereas yields of the hybrid abruptly decreased in the third year (2).

Cassava frog skin disease, endemic to an area in southern Colombia can reduce yield up to 80%. Yield in root fresh weight and starch content of the local cultivars Quilcace and M Col 113, cleaned of the disease by in vitro methods, more than doubled. In the second year, the yield of treated material decreased slightly, but was still very high. Planting materials, of both cultivars, were distributed in the area and, after 1 year, farmers' yields increased to 10 t/ha from 0.5 t/ha (2).

The third case deals with cassava varieties that show continued decrease in performance with no apparent disease. Two materials were chosen for the study: Llanera, an old cultivar with poor yields, and CMC-40 known for its recent outstanding performance. Stakes taken from apparently healthy plants were

sprouted at 40°C (day) and 35°C (night); control stakes were sprouted at 26°C. Plants regenerated from 0.4- to 0.5-mm shoot tips were grown in the field for 1 year beside conventionally propagated plants (control). At harvest, stakes were taken from both in vitro-treated and control plants and grown for another year in two locations.

In vitro propagation greatly increased the yield of roots and planting material of both cultivars. Yield and quality continued to increase in the second year, but were lower than in the first (Table 4). In in vitro plants, total HCN content in roots of Llanera was 95 ppm and free HCN was 14 ppm; in the control plants, total HCN was 65 ppm and free HCN 7 ppm. HCN level in CMC-40 roots remained stable. In addition, the point of the stem at which the first branching, i.e. flowering, occurred was strikingly taller in the in vitro-derived plants than in the control, and the magnitude of height increase, in the first and second year, was larger in Llanera than in CMC-40 (Table 4, Fig. la). The part of the stem below the first branching provides planting material (stakes) of the highest quality.

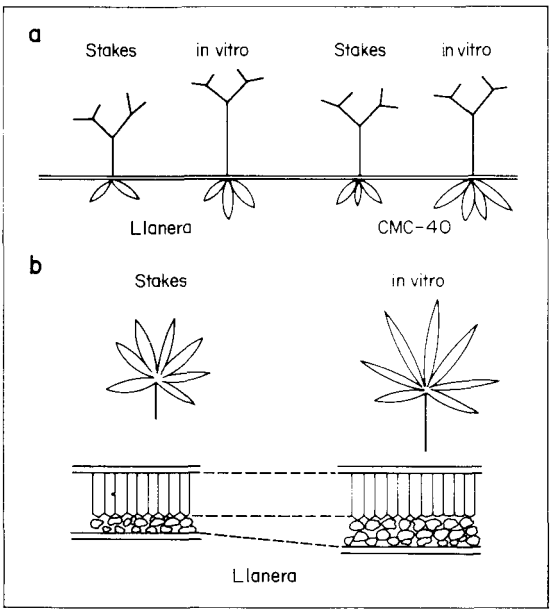
The length-to-width ratio of leaf lobes in all in vitro-derived plants of Llanera, but not in CMC-40, doubled that of control plants (Fig. lb); besides being narrower and more elongated, those leaves were thicker, leading to the formation of 50% thicker spongy mesophyll (Fig. lb); the palisade mesophyll did not change. These changes occurred in all regenerated plants, regardless of heat treatment. Changes in yield, as in morphology, were also evident in the second and third year of field testing, but not as strikingly as in the first year.

Elimination, or dilution, of pathogenic factors may explain the increases in root yield and amount of planting material, especially with an old cultivar like Llanera. Increases of 20%-30% in yield have been obtained recently after in vitro propagation of other four apparently healthy cassava cultivars (2). It is unlikely that the in vitro condition exerts residual effects on the regenerated plants during 2 or 3 years. If

Table 4. Yield increases from in vitro propagation of cassava.

Cultivar	Parameter	Yield increase (%) over control[a]	
		1st year	2nd year
Llanera	Root yield — fresh wt	53	25
	— dry wt	22	10
	Planting material[b]	47	46
	Plant height	25	0
	Height 1st branching	76	26
CMC-40	Root yield — fresh wt	43	29
	— dry wt	29	2
	Planting material[b]	18	23
	Plant height	6	5
	Height 1st branching	19	14

[a]Conventionally propagated plants, without in vitro culture. [b]Number of commercial size stakes. Percentages are averages of 3040 plants/treatment-year.



1. Morphology changes from in vitro propagation of cassava.

wide-lobed leaves are formed in the presence of the pathogenic factor(s), dilution or elimination of such factors by in vitro techniques would give rise to narrow lobed leaves. Thus, a gradual return to the original plant morphology, as well as yield, while the material is propagated in the field, should be expected. Grafting regenerated plants onto conventionally propagated root stocks failed to elicit the wide-lobed leaf even after 6 months. Thus, if any pathogenic factor exists, either it did not pass through the graft or it moved extremely slowly. Interestingly, changes in leaf shape were predominantly evident between 3 and 6 months, the period of active root growth in cassava plants. Whether narrow-lobed leaves have higher photosynthetic efficiency than wide-lobed leaves is to be evaluated.

Viruses and viroids cannot be controlled by applying chemicals to infected plants, and breeding for resistance to viruses is generally difficult and does not allow rehabilitating varieties with already valuable agronomic attributes. This paper has shown that producing clean planting material could be a short-term approach to restoring yield and quality in local cultivars of vegetatively propagated crops.

REFERENCES CITED

1. CIAT (Centro Internacional de Agricultura Tropical). 1983. Cassava tissue culture. Pages 23-50 in Genetic Resources Unit 1982. Cali, Colombia.

2. CIAT (Centro Internacional de Agricultura Tropical). 1984. Pathology section. Pages 1-39 in Cassava program annual report 1983. Cali, Colombia.
3. Hollings, H. 1965. Disease control through virus-free stock. Ann. Rev. Phytopathol. 3:367-396.
4. Kartha, K.K. 1981. Tissue culture techniques for virus elimination and germplasm preservation. Pages 123-138 in Genetic engineering for crop improvement. K.O. Rachie and J.M. Lyman, eds. The Rockefeller Foundation, New York.
5. Limmaset, P., and P. Cornuet. 1949. Recherche de virus de la mosaique du Tabac (Marmor Tabaci Holmes) dans l~es meristems des plantes infectees. C. Red. 228:1971-1972.
6. Lizarraga, R.E., L.E. Salazar, W.M. Roca, and L. Schilde-Rentschler. 1980. Elimination of potato spindle tuber viroid by low temperature and meristem culture. Phytopathology 70:754-755.
7. Lozano, .J.C., B. Pineda, and C. Jayasinghe. 1982. Effect of cutting quality on cassava (Manihot esculenta Crantz) performance. Tropical Root Crops Symposium, Lima, Peru. (in press)
8. Mellor, F.C., and R. Stace-Smith. 1977. Virus-free potatoes by tissue culture. Pages 616-635 in Applied and fundamental aspects of plant cell, tissue and organ culture. J. Reinert and Y.P.S. Bajaj, eds. Springer-Verlag, Berlin.
9. Murashige, T. 1974. Plant cell and organ culture methods for the establishment of pathogen free stocks. Dimock Lecture, Cornell University, Ithaca, New York.
10. Quak, F. 1977. Meristem culture and virus-free plants. Pages 598-615 in Applied and fundamental aspects of plant cell, tissue and organ culture. J. Reinert and Y.P.S. Bajaj, eds. Springer-Verlag, Berlin.
11. Walkey, D.G.A. 1978. In vitro methods for virus elimination. Pages 245-254 in T.A. Thorpe, ed. Frontiers of plant tissue culture 1978. International Association of Plant Tissue Culture, University of Calgary, Alberta, Canada.
12. Wang, P.J., and L.C. Huang. 1975. Callus cultures from potato tissues and the exclusion of potato virus X from plants regenerated from stem tips. Can. J. Bot. 53:2565-2567.
13. Wu, J.H., A.C. Hildebrandt, and A.J. Ricker. 1960. Virus-host relationships in plant tissue culture. Phytopathology 50:587-594.

RESEARCH ON LONG-TERM STORAGE AND EXCHANGE OF IN VITRO PLANT GERMPLASM

L. A. WITHERS and J. T. WILLIAMS

Short-term and long-term methods of storing in vitro cultures exist. The short-term methods are most effective for shoot cultures, the long-term for cell suspensions. Effective storage techniques for the other culture systems, however, are not available. Germplasm can be exchanged internationally as shoot cultures; such exchange requires phytosanitary procedures. Other aspects of in vitro conservation, including acquisition, disease indexing, genetic stability, and characterization, must be developed. The International Board for Plant Genetic Resources (LBPGR) has established an Advisory Committee on In Vitro Storage, reviewed in vitro conservation techniques, and recommended increased attention to crops other than model systems and to specific methods. IBPGR has also initiated major research into priority crops and key techniques, and is coordinating a worldwide survey and information service on in vitro conservation.

The Consultative Group on International Agricultural Research (CGIAR), the major international provider of funds, currently mobilizes well over $14 million annually, for work on plant germplasm (4). The mandate of IRPGR (one of the 13 International Agricultural Research Centers (IARCS) within CGIAR) is to develop and coordinate an international program for conserving the genetic resources of economic plants for breeding (14). The conservation program includes all major staples and many industrial crops important in rural development (13).

Germplasm of many crops can be conserved as seeds, but several important species produce short-lived (recalcitrant) seeds or are vegetatively propagated. Even if the latter do produce seeds that can be stored for long periods, their clones cannot be. That is the reason for the interest in in vitro alternatives (36).

Technical secretary, The International Board for Plant Genetic Resources (IBPGR) In Vitro Committee, University of Nottingham, School of Agriculture, Department of Agriculture and Horticulture, Sutton Bonington, Loughborough, Leics. LE12 5RD. UK; and executive secretary, IBPGR, FAO, Via delle Terme di Caracalla, 00100 Rome, Italy.

In vitro culture and germplasm storage are a critical area within biotechnology. The conservation of plant germplasm as in vitro cultures defies conventional agricultural practice. If in vitro mass propagation, crop improvement, and biosynthesis are to succeed, they will require stable, fully characterized "feed-stock" cultures and a storage facility.

The objective of in vitro culture work is to develop simple storage, propagation, and exchange methods for germplasm for which these activities are otherwise difficult or impossible. Sequestration in culture isolates the germplasm from potential pathogens (but see the section "Disease indexing and quarantine") and major environmental hazards, but culture media are complex, and contain balanced nutrients and hormones. They must be aseptic; their physical environment must be carefully controlled; and frequent transfer to fresh culture medium is necessary (several days to several weeks, depending on the system). Storage and exchange of cultures growing under standard conditions are therefore costly, impractical, and, because of their complexity, risk culture contamination, deterioration, and genetic instability.

Slowing culture growth rates extends subculture intervals to as much as 1 yr and reduces some of the required inputs and the risks, but this method is not ideal. Theoretically, cryopreservation does permit indefinite in vitro storage, providing base collections for long-term conservation but not for exchange. Material in limited growth storage must be considered active collections, i.e., the working collections for breeders and other plant researchers, (15).

Vegetative, cultured material can include protoplasts, cells, shoots, embryos, and plantlets. Somaclonal variation may occur more in cell and protoplast culture, so these will be used more in biotechnology than in genetic conservation, where shoots, embryos, and plantlets will be favored (25, 26). Although only storage, acquisition, and exchange are considered here, in vitro conservation also involves clonal propagation, disease indexing and eradication, genetic characterization, and crop evaluation.

IBPGR ACTIVITIES

In 1982, the IBPGR established an international committee to assess scientific research and advise on in vitro germplasm storage. The increasing need to accelerate genetic conservation techniques for several clonally propagated crops had been widely recognized.

IBPGR then commissioned a technical report on storage techniques (30), cosponsored (with IUBS and IGF) an international workshop on genetic conservation involving recalcitrant seeds and in vitro cultures (36), and included in vitro storage in the 1981 FAO/UNEP/IBPGR technical conference. The Advisory Committee has now met twice in full (15, 17) and once as a subcommittee (16). As a result of these meetings, IBPGR has already initiated several research programs on key crops.

Among the tasks of the Advisory Committee is identification of crops and techniques relating to in vitro storage that most need IBPGR attention. For most high-priority crops -- cacao, Citrus, coconut, Musa and sweet potato -- there is a lack of culture techniques generally applicable to a wide range of genotypes for use with in vitro gene banks. For cacao and coconut new acquisition techniques are also needed; and for Musa and sweet potato, disease indexing. For two crops, Allium (second global priority) and temperate fruits (third global priority), information on the level of in vitro technique development requiring research attention must be obtained. This information will be available in 1985.

Little attention has been given to cytological or biochemical monitoring of genetic stability in in vitro cultures and regenerated plants. Stability in vivo and in vitro should be compared. IBPGR has commissioned expert reports on these areas. No in vitro base collections of frozen cultures exist, although some active collections are being developed. Better communication between research workers in these areas is needed (34).

INFORMATION RESOURCES

In vitro propagation and storage have been extensively reported (10, 33, 36), but such practical matters as plant materia] availability and technique application to untried crops have not. IBPGR has, therefore, Commissioned a survey on in vitro conservation.

The survey replies received through 1982 have been published in report form (31), and are being computerized with replies received from 1983 onward. Such information as where cryopreservation work on a particular crop is being done or what problems researchers have encountered in international culture exchange will be available. Literature references on in vitro propagation, conservation, genetic characterization, and priority crops are also being computerized. Requests for information and searches are welcome (7). A new survey will be conducted in 1985.

STORAGE TECHNIQUES

General principles

In short-term storage, growth is slowed by low temperature or by hormonal or osmotic inhibitors. Reduced pressure or oxygen tension and partial desiccation may also work, but must he tested further.

For cryopreservation, the biological material is taken to an ultralow temperature (e.g. - 196°C, the temperature of liquid N) at which metabolism, and therefore growth, ceases. In growth limitation, the risk of stress exists for the duration of storage, but in cryopreservation freezing and thawing are the risky stages. Except for routine checks to ensure an adequate supply of liquid N, cryopreservation requires little monitoring.

Avoiding injury in freezing or thawing cultures for cryopreservation normally requires pretreatment with cryoprotectant compounds and then careful choice of cooling or warming rates. Cooling may be slow (or stepwise) to permit protective dehydration and removal of potentially damaging freezable water from the cell, or very rapid, so that cellular water freezes in the cytoplasm and vacuoles, but forms small, innocuous ice crystals. In the latter case, warming is always rapid to avoid injurious ice recrystallization. In the former case, warming may theoretically be slow (occasionally this is advantageous), but most procedures use rapid thawing.

In recent years, attention has turned to culture pregrowth and recovery in cryopreservation. In early studies, any culture viability during the early post-thaw period was cause for satisfaction; now the pattern of recovery in organized culture systems is monitored, and confirmation of totipotency and stability is sought (see 19, 30, 33, 36).

Storage of shoot cultures, shoot tips, and embryos

Growth limitation in shoot cultures. More than 20 species have been kept in short-term storage as shoot cultures (leaf-bearing stem cultures) (Table 1). Many genotypes of potato and cassava are in active in vitro collections at CIP and CIAT (5, 6, 10).

Henshaw, Westcott, and colleagues (10, 11, 12) have stored shoot cultures of several *Solanum* species at reduced temperature (12°C day/6°C night) or, with mannitol (6%) and ABA (20 mg/litre) to inhibit growth, for more than 1 yr. Mix (21) used medium containing n-dimethyl-succinamic acid (B-nine; 50 mg/litre) and storage at 10°C. Various other storage regimes have suited shoot cultures and 'minitubers' of this crop (see 30, 31), but some variation in sensitivity to storage conditions has been found among potato genotypes (11). Cassava cultures cannot tolerate prolonged exposure to the apparently benign temperature of 18°C, but can survive more than 2 yr at temperatures reduced from 30° to 22° or 20°C (Roca, pers. comm; 5). Thus, storage requirements (temperature, sucrose concentration, inhibitors, subculture interval, etc.), must be carefully determined.

Cryopreservation of shoot tips. The first shoot tips (shoot apical meristem plus leaf primordia) to be successfully exposed to storage in liquid N were of *Dianthus caryophyllus* (27). Since this study (1976), over 15 more species have been cryopreserved as shoot tips, with varying success (Table 1). Composite cryopreservation procedures are presented in Figure 1.

As in growth limitation, requirements and responses vary among species, necessitating examination of each. Kartha and colleagues found cassava did not respond to methods successful with other species (*Pisum sativum* and *Fragaria* sp.; 20, 33) and it appears that shoot tips need to be placed in droplets of cryoprotectant solution on a metal foil sheet, rather than in an ampoule. Even then, percentages of recovery range from 14% with no plant regeneration to 80%, of which 20% could regenerate plants. Overall recovery was as high as 95% in the other species.

Table 1. A summary of successful attempts at storage and exchange of in vitro plant material. In published reports, original sources may be traced through references 30 and 36. Entries in parentheses are from the IBPGR Survey (see 7,31). The latter include only those submissions describing storage or exchange as "reproducible or successful." Survey entries duplicating the literature are not included.

Species[a]	Crop[b]	Storage[c]		Exchange of germplasm[c]
		Growth limitation	Cryopreservation[d]	
Acer pseudoplatanus			C	
Actinidia chinensis	Chinese gooseberry	(S)		(S)
Allium ampeloprasum	Levant garlic	(B)		
Ananas comosus	Pineapple			(S)
Arachis hypogaea	Groundnut		S	
Asparagus officinalis	Asparagus		S	
Atropa belladonna	M	C	A PE	(U)
Berberis dictyophylla			C	
Beta vulgaris	Beet	S		
Brassica napus	Rape		CS	
Bromus inermis			P	
Bupleurum falcatum		Ca		
Capsicum anuum	Cayenne/paprika	Ca	C	
Catharanthus roseus	M		C	
Cicer arietinum	Chickpea		S	
Cinchona ledgeriana	Quinine			(Ca) (S)
Citrus spp.	Citrus	(Ca)		(Ca) (S)
Coffea spp.	Coffee			(U)
Coptis japonica	M		(C)	(C)
Corydalis sempervirens			C	
Cryptocoryne spp.		S		
Cymbidium sp.		Pc		
Dactylis sp.	F	S		
Datura spp.	M		C	
Daucus carota	Carrot	Ca SE	PCSE	
Dianthus caryophyllus			CS	
Digitalis lanata			C	
Dioscorea deltoides	M		C	(Ca)
Elaeis guineensis	Oilpalm		ZE SE Ca	
Emmenopteris henrii				(S)
Eucalyptus spp.				(S)
Festuca spp.	M	S		
Fragaria spp.	Strawberry	S	S	(S)
Freesia cv.				(S)
Gerbera jamesonii				(S)
Glauciurn flavum			C	
Glycine max	Soyabean		PC	
Gossypium spp.	Cotton		A Ca	
Hevea brasiliensis	Rubber	(U)		(SE)
Hordeum vulgare	Barley		ZE	
Hyoscyamus muticus	M		C	
Ipomoea spp.	Sweet potato	S		(S)
Lactuca sativa	Lettuce		S	
Lavandula sp.			Ca	
Lithospermum erythrorhizon		Ca		
Lolium spp.	F	S	S	
Lycopersicon exulentum	Tomato		S ZE	(S)

Continued on next page

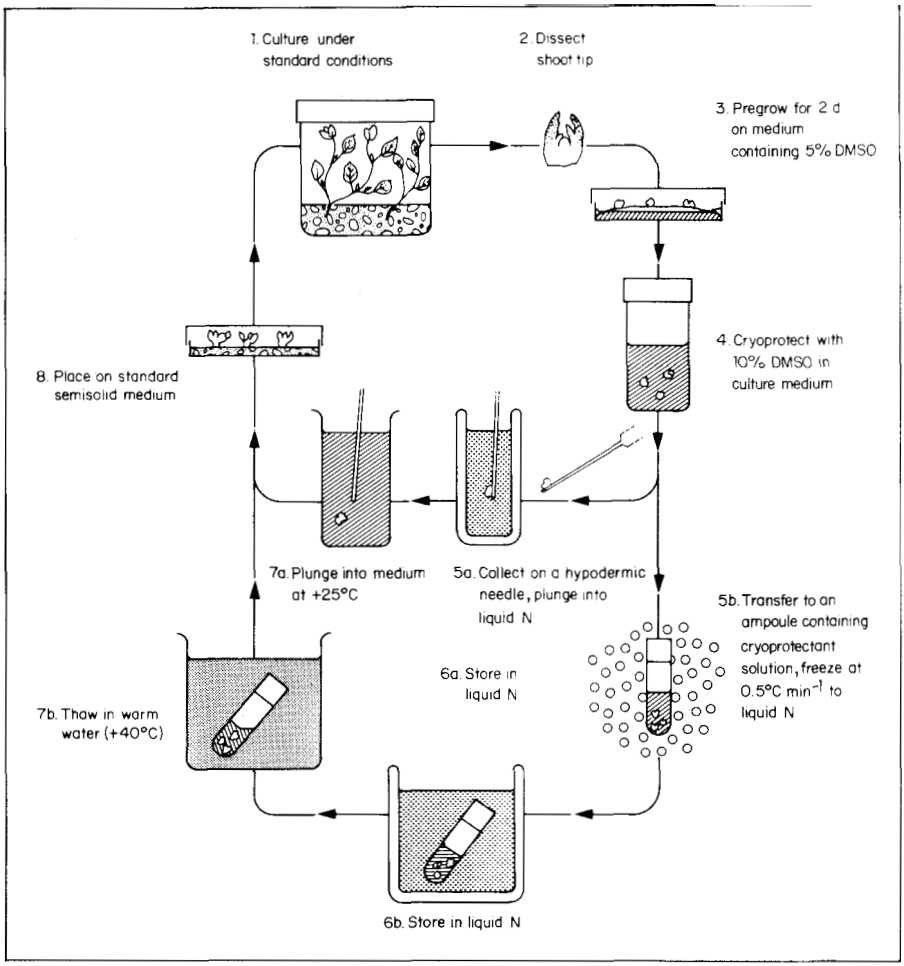
Table 1 continued

Species[a]	Crop[b]	Storage[c]		Exchange of germplasm[c]
		Growth limitation	Cryopreservation[d]	
Mallotus japonicus		Ca		
Malus spp.	Apple	S (Ca)	S	(S)
Manihot spp.	Cassava	S	S	S
Medicago sativa	F	(U)	C	(Ca) (S)
Musa spp.	Banana/plantain			(S)
Nicotiana spp.	Tobacco	Ca S	A PE C	(Ca)
Olea europea	Olive	(U)		
Onobrychis viciifolia	F		C	
Oryza sativa	Rice		C Ca ZE P	
Panax ginseng	M		C	(Ca)
Pennisetum americanum	Pearl millet		C	
Petunia hybrida			A	
Phleum sp.	F	S		
Phoenix dactylifera	Date	(Ca)	Ca SE	(S) (Ca)
Phytolacea americana		Ca		
Pisum sativum	Pea		S ZE P	
Populus spp.		(S) (R)	C Ca	
Primula obconica			A	
Prunus spp.	Plum/cherry	(S) (Ca)		
Pseudotsuga menziesii		(Ca)	C	
Pyrus sp.	Pear	S	S	
Rauwolfia serpentina	M			(Ca)
Rhazya spp.			C	
Ribes sp.	Currants		S	
Rosa cv. Paul's Scarlet			C	
Rubus sp.	Raspberry	(S)	S	
Saccharum spp.	Sugarcane	(U)	C Ca	(Ca) (S)
Salix babylonia		(S)		
Sambucus racemosa			C	
Solanum spp.	Potato	S	S C	S
Sorghum bicolor	Sorghum		C	
Tectona grandis				(S)
Trifolium spp.	F	S		
Triticum spp.	Wheat		C ZE P PE	
Vitis spp.	Grape			(S)
Zea mays	Maize		C Ca ZE P	

[a]Genera indicated by "spp" frequently include several named species in the original source. [b]Minor species are identified as M = medicinal plant, F = forage. [c]Culture systems are identified as follows: P = protoplast, C = cell, Ca = callus, SE = somatic embryo, ZE = zygotic embryo, S = shoot culture, shoot-tip or in vitro plantlet, R = root, PE = pollen embryo. A = anther, B = bulbil, Pc = protocorm, U = unspecified. [d]Refers to storage in liquid N, not at higher temperatures.

This example illustrates two areas requiring procedure improvement for shoot tips: 1) handling at the freezing stage to permit secure specimen enclosure (Fig. 1; 9, 11, 20); and 2) achieving satisfactory recovery and organized development.

Apical dome damage may be massive and adventitious regeneration common (33), though this may be evident only microscopically. In fact, no categorical example of nonadventitious recovery can be found. This has potentially serious consequences for genetic stability and the extent of



1. Cryopreservation procedures for shoot tips. Alternative treatments: (1) Cold-harden parent material; (2) None; (3) Pregrow for 1 day only; pregrow on plain medium; omit pregrowth and transfer freshly dissected shoot-tip to cryoprotectant; (4) Cryoprotect with 5 or 15% DMSO; (5a) None; (5b) Enclose in a foil envelope; dispense in droplets of cryoprotectant on foil sheet in a petri-dish; freeze at a faster or slower rate; (6a, b) None; (7a) None; (7b) Thaw slowly (for material in foil envelopes); wash in fresh medium; (8) Recover on semi-solid medium containing growth hormones or activated charcoal; recover on filter paper in contact with liquid medium;graft onto host plant (9, 10, 20, 24, 33).

somaclonal variation in cryopreserved shoot-tip cultures should be tested.

Henshaw and colleagues (12; see also 30, 33) have found that supplementing the recovery medium with hormones can increase the percentage of shoot tips undergoing organized recovery. Withers and Jarvis (unpubl.) have found that brief exposure of potato shoot tips to the hormones will promote organized outgrowth of the shoot apex and minimize callusing. Another way to restore the cryopreserved shoot tip to normal

growth and accelerate germplasm use may be by grafting (24).

Embryo cultures. Seed-embryo storage is not considered growth limitation and, as culture storage, remains virtually unexplored. However, both zygotic and somatic embryos have been successfully cryopreserved. Immature zygotic embryos of barley and wheat have been frozen by dry freezing, a technique first developed for somatic carrot embryos (33, 36). This involves cryoprotection with 10% dimethylsulfoxide (DMSO), draining the embryo free of the cryoprotectant, and enclosing it in a foil envelope, then freezing it at a rate of 1°C min^{-1} to about -40°C before plunging it into liquid N. Slow or rapid thawing is effective. Pollen-derived embryos and anthers undergoing androgenesis have been frozen by many methods (l), none of which are clearly superior. (These specimens differ from pollen stored for fertilization, which is useful in breeding some crops).

Storage of cell suspensions, callus cultures, and protoplasts

Some attention has been given to limited-growth storage of callus cultures, but in general it does not suit unorganized cultures. Cryopreservation should be used for their short- and long-term conservation.

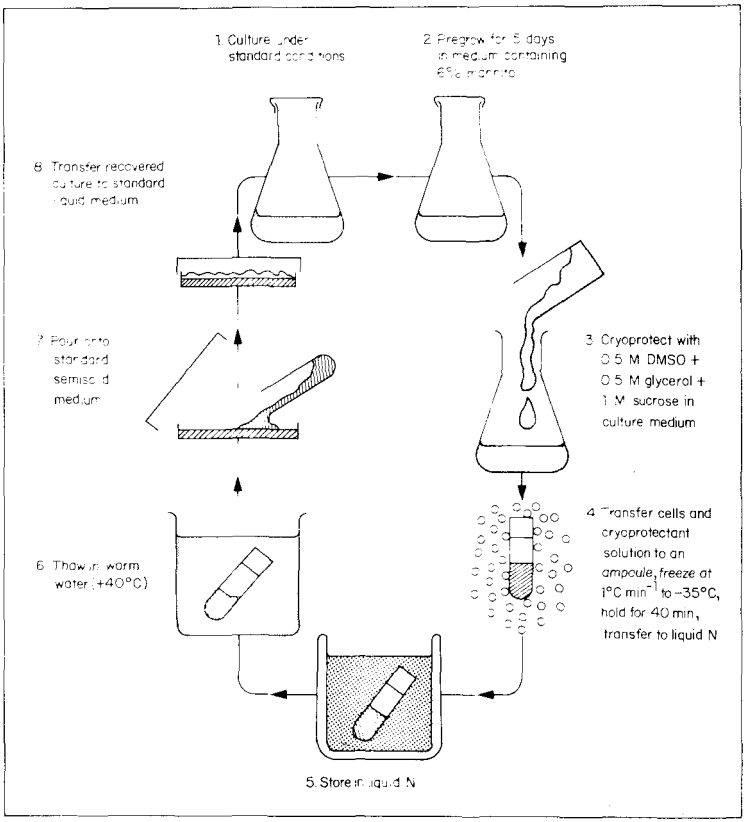
Cell suspension cultures. In 1980, Withers and King (35) published a report on a cryopreservation method for cell cultures, now proven successful for more than 20 species and routinely used (Fig. 2). Subsequent studies show a consistency of approach but suggest minor procedural changes that may broaden its applicability (32, 33; Fig. 2).

Post-thaw viabilities as high as 80% and recovery of an actively growing culture within about 2 wk can be expected, given some fine-tuning for each species. Rapid recovery depends particularly upon careful handling during the early post-thaw period, when cells must recover from ultrastructural damage (29), membrane lesions (leakiness and susceptibility to deplasmolysis injury), and impairment of such physiological processes as respiration (3).

During the early post-thaw period, growth kinetics may change, but normal behavior is subsequently restored. For example, cryopreserved rice cells display the same rate of increase in sedimented cell volume as controls (3). Cryopreserved morphogenic cultures of several species have demonstrated totipotency (33). (However, there are a few examples of failure to regenerate plants attributable to physical damage rather than to genetic instability). A more critical test lies in the stability of cells' response to antimetabolites (e.g., resistance to DL-5 methyl tryptophan in tobacco), or capacity to synthesize (e.g., authocyanins in carrot or cardenolides in *Digitalis lanata* (32, 33). No evidence suggests that cryopreservation is selective, except, perhaps, for a freeze-tolerant morphology.

Callus cultures. Little is known of the cryopreservation requirements in callus cultures. Special pregrowth treatments have not usually been applied, but procedures are otherwise similar to those in Figure 2. Work with callus cultures has demonstrated that removing cryoprotectants with a warm wash may be superior (8).

Protoplasts. Even less attention has been paid to protoplast cryopreservation. Takeuchi and colleagues (28), who have carried out the most extensive study with freshly isolated protoplasts, recommend the following procedure: 1) enzymic digestion and protoplast isolation; 2) cryoprotection with 5% DMSO plus 10% glucose; 3) freezing at rates of about 0.8° - 3°C min^{-1} to a transfer temperature of -30° to −40°C; 4) plunging into liquid N; 5) thawing rapidly; and 6) washing with fresh liquid medium. Bajaj (2) has attempted to cryopreserve fused protoplasts of wheat or rice with pea, but survival was never more than 18% and recovery was insignificant.



2. Cryopreservation procedure for cell cultures. Alternative treatments: (1) None; (2) Pregrow in standard medium; pregrow in medium containing 70% proline or 1M sorbitol; (3) Substitute 1M proline for 1M sucrose; omit proline from mixture; (4) Transfer directly to liquid nitrogen from -35°C or a lower temperature; (5) None; (6) Thaw slowly; (7, 8) Wash in liquid medium before plating; culture in liquid medium on a shaker; pour into static liquid medium containing 1M sorbitol for 3 days before transfer to plain liquid medium on a shaker (32, 33, 34).

Equipment

Limiting growth by modifying the culture medium requires no special equipment. Reduced temperature simply requires a culture room or incubator with appropriate temperature control.

Cryopreservation requires a controlled freezer of some kind (32, 33) and a liquid N refrigerator with coolant. Most consumable items, chemicals, and equipment other than these are standard to the in vitro laboratory or are readily obtainable.

Establishing laboratories for in vitro work is relatively inexpensive. The IBPGR is currently drawing up recommendations for in vitro gene banks.

ACQUISITION AND EXCHANGE OF PLANT GERMPLASM IN VITRO

De novo acquisition of germplasm

Until very recently, the use of in vitro techniques to collect primitive and wild genotypes in the field attracted little interest. *Theobroma cacao* proved a useful model for preliminary experiments (Withers and Yidana, unpubl.) Clearly, conventional laboratory culture inoculation cannot be directly translated to the field, but a simplified procedure using nodal cuttings sterilized by water purification tablets (which require no washing) has been devised. The cuttings were inoculated, outside a sterile work station and with unsterilized instruments, into tubes of culture medium supplemented by a fungicide.

Viability has been retained up to 6 wk. During this time, microbial overgrowth is contained and axillary buds develop. We are trying to optimize surface sterilization treatments, improve the culture medium, and develop procedures for transferring the collected germplasm either to continued culture or to in vivo growth in field gene banks (clonal repositories, living collections, orchards, plantations, etc.; 15).

Exchange of established cultures

For as long as in vitro propagation has been routine for vegetatively propagated species and specialized cultures of others, ad hoc experiments on the transportation of growing cultures have been conducted. Although little of this information is published, it was revealed in the IBPGR survey (7, 31; Table 1). Exchange seems easy (about 90% successful), but without rigorously controlled experimentation across a range of species, it is uncertain. Guidelines for such research can be drawn from attempts to exchange potato shoot cultures from CLP (6, 23, 31) as detailed below.

Shoot cultures are inoculated into small tubes containing solid agar medium. They are cultured for 2-3 wk to induce rooting and to reveal any microbial contamination. For transit, the tubes are packed with necessary documentation into polystyrene boxes and mailed. Cultures have been distributed

worldwide, staying in transit for an average of 10-12 d (maximum of 25 d). Survival levels after in vivo plants were established ranged from 10 to 93%. Inoculation into a culture passage before potting appears to enhance the survival rate. Training recipient personnel is essential to successful transfer of in vitro material. Cultures of cassava clones have been distributed similarly from CIAT (Roca, pers. comm.).

Disease indexing and quarantine

Meristem culture can eradicate pathogens and be combined with conventional therapies (22). Furthermore, inoculation into culture removes superficial contaminants; pest infestation in established cultures will normally be detected, although systemic pathogens compatible with relatively normal growth can escape detection unless carefully screened. The IBPGR Survey (7, 31) suggests hardly any attention is given to such considerations.

Promoting in vitro cultures as ideal propagules for distributing disease-free material internationally is not premature but requires detailed phytosanitary consideration. Certainly it requires recommendations as detailed as those for distributors of conventional forms of germplasm (18). Quarantine regulations need revising. These measures should standardize procedures, permit more rapid release of cultures from quarantine, and prevent accidents that would unduly restrict the movement of in vitro material. Some of the current international conventions for distributing microbial organisms should be considered.

CONCLUSIONS

Short-term storage is available for shoot cultures of many crops but not for unorganized cultures. Cryopreservation can meet both short- and long-term requirements for cell cultures but methods for protoplasts, anthers, and callus cultures are lacking. Shoot-tip cryopreservation has progressed considerably in recent years, but cannot yet be applied to a wide range of crops, and often involves poor recovery.

Implementation of available techniques (even in centers of excellence) for germplasm storage in conservation and genetic manipulation lags well behind their development. In vitro techniques for collecting hold great promise. Exchange of growing in vitro material appears to present few problems, but has not been tested across a range of species and under extreme conditions. Exchange of frozen plant cultures is unexplored.

Phytosanitary and quarantine aspects of germplasm exchange in vitro require clarification and review.

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REFERENCES CITED

1. Bajaj, Y.P.S. 1977. Survival of Nicotiana and Atropa pollen embryos frozen at -196°C. Curr. Sci. (India) 46:305-307.
2. Bajaj, Y.P.S. 1983. Survival of somatic hybrid protoplasts of wheat x pea and rice x pea subjected to -195°C. Indian J. Exp. Biol. 21:120-122.
3. Cella, R., R. Columbo, M.G. Galli, E. Nielsen, F. Rollo, and F. Sala. 1982. Freeze-preservation of Oryza-sativa L. cells: a physiological. study of freeze-thawed cells. Physiol. Plant. 55:279-284.
4. CGIAR (Consultative Group for International Agricultural Research). 1982. Integrative Report, CGIAR Secretariat, World Rank, Washington DC.
5. CIAT (Centro Internacional de Agricultura Tropical). 1981. Cassava Program. ClAT Annual Report, Cali, Colombia.
6. CIP (Centro lnternacional de la Papa). 1980. Disease-free potatoes by tissue culture. CIP Circular VIII.
7. FAO (Food and Agriculture Organization) and IRPGR (International Board for Plant Genetic Resources). 1983. Information on in vitro culture. Plant Genetic Resources Newsl. 55:16. (Details of computer data-base searching service available from IBPGR Secretariat, FAO, Via delle Terme di Caracalla, 00100 Rome, Italy).
8. Finkle, E.J., and J.M. Ulrich. 1982. Cryoprotectant removal temperature as a factor in the survival of frozen rice and sugarcane cells. Cryobiology 19:329-335.
9. Grout, B.W.W., and G.G. Henshaw. 1978. Freeze-preservation of potato shoot-tip cultures. Ann. Bot. 42:1227-1229.
10. Henshaw, G.G., and J.F. O'Hara. 1983. In vitro approaches to the conservation and utilization of global plant genetic resources. Pages 219-238 in Plant biotechnology. S.H. Mantell and H. Smith, eds. SEB Sem. Ser. 18, Cambridge University Press.
11. Henshaw, G.C., J.F. O'Hara, and R.J. Westcott. 1980. Tissue culture methods for the storage and utilization of potato germplasm. Pages 71-76 in Tissue culture methods for plant pathologists. D.S. Ingram and J.P. Helgeson, eds. Blackwell., Oxford.
12. Henshaw, G.G., J.A. Stamp, and R.J. Westcott. 1980. Tissue culture and germplasm storage. Pages 277-282 in Developments in plant biology, volume 5, Plant cell cultures: results and perspectives. F. Sala, R. Parisi, R. Cella, and O. Cifferi, eds. Elsevier, North Holland, Amsterdam.
13. IBPGR (International Board for Plant Germplasm Resources). 1981. Revised priorities among crops and regions. Publ. 81/34, Rome.
14. IBPGR (International Board for Plant Germplasm Resources). 1983. Facts about the IBPGR. Rome.

15. IBPGR (International Board for Plant Germplasm Resources). 1983. Report of the first meeting of the IBPGR Advisory Committee on In Vitro Storage. IBPGR Publ. 82/84, Rome.
16. IBPGR (International Board for Plant Germplasm Resources). 1984. The potential for using in vitro techniques for germplasm collection. Report of the meeting of a Sub-committee of the IBPGR Advisory Committee on In Vitro Storage. IBPGR Publ. 83/108.
17. IBPGR (International Board for Plant Genetic Resources). 1985. Report of the second meeting of the IBPGR Advisory Committee on In Vitro Storage. (in press)
18. Kahn, R.P. 1977. Plant quarantine: principles, methodology and suggested approaches. Pages 289-307 in Plant health and quarantine in international transfer of genetic resources. W.B. Hewitt and L. Chiarappa, eds. CRC Press Inc., Boca Raton, Florida.
19. Kartha, K.K., ed. 1985. Cryopreservation of plant cells and organs. CRC Press Inc., Boca Raton, Florida. (in press)
20. Kartha, K.K., N.L. Leung, and L.A. Mroginski. 1982. In vitro growth responses and plant regeneration from cryopreserved meristems of cassava (Manihot esculenta Crantz). Z. Pflanzenphysiol. 107:133-140.
21. Mix, G. 1982. In vitro preservation of potato material. Plant Genetic Resources Newsl. 51:6-8.
22. Roca, W.M. 1985. In vitro clonal propagation to eliminate crop diseases. Pages 5-10 in Biotechnology in international agricultural research. International Rice Research Institute, Los Banos, Philippines.
23. Roca, W.M., J.E. Bryan, and M.R. Roca. 1979. Tissue culture for the international transfer of potato genetic resources. Am. Potato J. 56:l-10.
24. Sakai, A., and Y. Nishiyama. 1978. Cryopreservation of winter vegetative buds of hardy fruit trees in liquid nitrogen. HortScience 13:225-227.
25. Scowcroft, W.R. 1984. Genetic variability in tissue culture: impact on germplasm conservation and utilization. Expert Report Commissioned by IBPGR In Vitro Storage Committee. IBPGR Publ. 84/152. Rome.
26. Scowcroft, W.R., S.A. Ryan, R.I.S. Brettel, and P.J. Larkins. 1985. Somaclonal variation in crop improvement. Pages 99-109 in Biotechnology in international agricultural research. International Rice Research Institute, Los Banos, Philippines.
27. Seibert, M. 1976. Shoot initiation from carnation shoot apices frozen to -196°C. Science 191:1178-1179.
28. Takeuchi, M., H. Matsushima, and Y. Sugawara. 1982. Totipotency and viability of protoplasts after long-term freeze-preservation. Pages 797-798 in Plant tissue culture 1982. A. Fujiwara, ed. Maruzen, Tokyo.
29. Withers, L.A. 1978. A fine-structural study of the freeze-preservation of plant tissue cultures. II. The thawed state. Protoplasma 94:235-247.

30. Withers, L.A. 1980. Tissue culture storage for genetic conservation. IRPGR Publ. 80/8, Rome.
31. Withers, L.A. 1982. Institutes working on tissue culture for genetic conservation. IBPGR Publ. 82/30, Rome.
32. Withers, L.A. 1984. Freeze-preservation: cells. Pages 608-620 in Cell culture and somatic cell genetics of plants. I. I.K. Vasil, ed. Academic Press, New York.
33. Withers, L.A. 1985. Cryopreservation. In Cell culture and somatic cell genetics of plants. II. I.K. Vasil, ed. Academic Press, New York. (in press)
34. Withers, L.A. 1984. Germplasm conservation in vitro: present state of research and its application. Pages 138-157 in Crop genetic resources, conservation, and evaluation. J.H.W. Holden and J.T. Williams, eds. George Allen and Unwin, London.
35. Withers, L.A., and P.J. King. 1980. A simple freezing unit and cryopreservation method for plant cell suspensions. Cryo-Letters 1:213-220.
36. Withers, L.A., and J.T. Williams, eds. 1982. Crop genetic resources -- the conservation of difficult material. International Union of Biological Sciences/ International Board for Plant Genetic Resources, Paris, IUBS Publ. Ser. B42.

TISSUE CULTURE OF OIL PALM AND COCONUT: CURRENT RESEARCHES AT IRHO[1] AND ORSTOM[2]

C. PANNETIER, J. M. NOIRET, and P. DUBLIN[3]

Micropropagation of oil palm through somatic embryogenesis from leaf tissue has been achieved; several 1aboratories have been set up to produce plantlets for clonal trials. Studies are made on the culture of young coconut leaf tissues; Structures exhibiting characteristics of somatic enbryos were obtained from young and adult trees. Culture of coconut zygotic embryos exercised at different stages or maturity has led to the development of plants that have been successful1y transferred to soil. Haploid embryos have been obtained from anther culture of coconut. The first successful cryopreservation of oil palm somatic embryos has been reported.

SIGNIFICANT OF TISSUE CULTURE

For breeding and varietal production of oil palm and coconut, tissue culture can lead to numerous applications (3).

Oil palm and coconut seeds for commercial use are those of crosses between heterozygous parents. These crosses vary widely: the best trees produce up to 50% more than the average of the cross they belong to. Moreover, it is possible to create individuals assembling interesting characters. Because there is no conventional method for vegetative propagation, tissue culture is one of the only possible means to create uniform clones of elite palms.

The length of one generation (10-12 yr) makes the creation of inbred lines long, difficult, and expensive. Haploids that can be doubled to obtain homozygous individuals can be produced by culture of gametic cells.

With respect to conservation and exchange of germplasm, embryo culture can greatly simplify the storage and transport of coconut seeds (which are naturally bulky and which germinate rapidly).

Tissue culture will permit the creation of germplasm collections otherwise impossible because of the size of the

[1]Institut de Recherches pour les Huiles et Oleaglneaux, 11 Square Petrarque, 75116 Paris, France, [2]Institut Francais de Recherche Scientifique pour le Developpement en Cocoperation, 24 rue Bayard, 75008 Paris, France. [3]Laboratoire de Physlologie Vegetale S. S. C. ORSTOM, Route d'Aulnay 93140, Bondy, France; Departement Selection, IRHO/GERDAT, BP 5035, 34032 Montpellier Cedex, France; and Laboratoire Culture In Vitro GERDAT, BP 5035, 34032 Montpellier Cedex, France.

palms. Long-term collections will be possible through cryopreservation of zygotic or somatic embryos.

Research on regeneration from protoplast cells and use of vectors for genetic manipulation of oil palm is under way.

TISSUE CULTURE OF OIL PALM

The choice of vegetative propagation method depends on certain morphological and genetic characteristics. Micropropagation will be applied to adult trees whose yields are known. The ortet must be kept alive, and the only vegetative bud is the apex, which builds the whole tree. These limitations have led to research on plant production through neoformation on calluses.

Somatic embryogenesis from leaf tissue of seedlings cultivated in vitro was reported in 1976 by Rabechault and Martin (7). Later studies led to a reliable and repeatable method, applicable to adult trees.

Young leaves are cut without wounding the apex. Leaflet fragments are seeded; proper explant preparation and culture medium will result in formation of calluses on an average of 40% of the leaf explants. The calluses obtained can be subcultured satisfactorily and, under certain culture conditions, somatic embryos are formed. These embryoids multiply actively and shoots develop. For mass production of uniform plantlets, the organized structures on calluses should be propagated, not the calluses themselves (4). The clonal material consists of clumps of embryoids at different stages of development. Young plantlets can be isolated and embryoids repropagated at any subculture phase. A rooting treatment is applied to the shoots to obtain homogeneous groups of vigorous plantlets for transfer to the natural environment. Our transfer technique results in more than 90% of the plantlets' developing in prenursery.

An experimental unit has been installed on the La Me IRHO station in Ivory Coast to apply this method to many ortets. The conformity and variability of the clonal material produced are studied in field trials. Sampling of 110 different trees selected for their agronomic characteristics has led to some improvement of the techniques (Arthuis, unpubl.). Two other laboratories were set up in Malaysia and Indonesia to produce in vitro plantlets for clonal trials.

In a laboratory of the Centre National de la Recherche Scientifique, research is conducted on cryopreservation of somatic embryos. Young embryoids have been successfully cooled to liquid nitrogen temperature, and some have started to develop after thawing. Under certain culture conditions, somatic embryos have multiplied after freeze preservation (1).

TISSUE CULTURE OF COCONUT

In vitro culture techniques have been used for vegetative propagation, development of plants from excised zygotic embryos, and creation of haploids from gametic cells.

Vegetative propagation

Vegetative propagation has mainly been attempted from leaf tissue culture. Explant behavior in coconut is very different from that in oil palm, particularly in the tissues' response to auxins. We have succeeded in selecting and maintaining healthy explants with little or no browning symptoms. Through use of auxins and activated charcoal in the culture media, we have obtained nodular calluses that are of internal origin and appear on the secondary veins of the lamina, on both nursery seedlings and adult trees (the average of callus-bearing explants on adult trees is 30%). Three or four months of culture could yield many nodular calluses on one explant; their subculture resulted in organized structures with somatic embryo characteristics. We have obtained the proliferation of these structures, and their development into plantlets is being attempted (5, 6).

Culture of zygotic embryos

Excised embryos, aseptically cultured, yielded plantlets that were successfully transferred to soil (Ahee and Guenin, unpubl.). Moreover, zygotic embryos excised at different stages of maturity (from 8 mo after pollination) have developed into plantlets.

In vitro production of haploids

Gynogenesis. The experiments on gynogenesis have met several practical problems: in the dissection of ovaries and ovules, in the size of ovaries, and in the limited number of ovules per ovary.

Androgenesis. The following points have been studied: stages of inflorescence, development of microspores, pretreatment conditions, and culture media. The correlation between browning of the anther tissues and survival of the microspores has been observed. Some haploid structures exhibiting embryo characteristics were obtained from anthers of the hybrids West African Tall/Malayan Yellow Dwarf and West African Tall/Rennell Tall (2).

REFERENCES CITED

1. Engelman, F., Y. Duval, and J. Dereuddre. 1984. First successful cryopreservation of oil palm somatic embryos. Paper presented at the 41st Conference in the Easter School Series in Agriculture Science. Plant Tissue Culture and its Agricultural Applications, 17-21 Sep, University of Nottingham School of Agriculture.
2. Monfort, S. 1984. Androgenesis of coconut. First embryos from anther culture. Zeitschirft fur Pflanzenzuchtung. (in press)
3. Noiret, J.M. 1981. Application de la culture in vitro a l'amelioration du materiel clonal chez le palmier a huile. Oleagineux 36(3):123-126.

4. Pannetier, C., P. Arthuis, and D. Lievoux. 1981. Neoformation de jeunes plants d'Elaeis guineensis a partir de cals primaires obtenus sur fragments foliaires cultives in vitro. Oleagineux 36(3):119-122.
5. Pannetier, C., and J. Buffard-Morel. 1982. Premiers resultats concernant la production d'embryons somatiques a partir de tissus foliaires de Cocotier, Cocos nucifera L. Oleagineux 37(7):349-354.
6. Pannetier, C., and J. Buffard-Morel. 1982. Production of somatic embryos from leaf tissues of coconut, Cocos nucifera L. Proceedings 5th International Congress of Plant Tissue and Cell Culture. International Association for Plant Tissue Culture. Tokyo.
7. Rabechault, H., and J.P. Martin. 1976. Multiplication vegetative du palmier a huile (Ealaeis guineensis Jacq) a l'aide de culture de tissus foliaire. C.R. Acad. Sci. Paris, Ser. 283:1735-1737.

APPLICATION OF TISSUE CULTURE TO TUBER CROPS AT IITA

S. Y. NG and S. K. HAHN

The international germplasm exchange for tuber crops is hampered by the risks of introducing, through vegetative materials, diseases and pests to noninfected areas. Since tuber crops are vegetatively propagated, their conservation as germplasm is also a problem. At IITA, we have used meristem culture to eliminate disease in tuber crops: disease-free plants can be distributed in vitro to requesting national programs. Disease-free sweet potato has been distributed to more than 40 countries throughout the world, and cassava to over 30 countries in Africa. Minimal growth storage is used at IITA to conserve germplasm of sweet potato and yams. Over 600 clones of sweet potato germplasm are held in vitro. Arrangements for in vitro tuber crop germplasm exchange are described.

Root and tuber crops (cassava, yam, sweet potato, cocoyam) are major food sources in Africa, the Caribbean, South America, India, the Pacific Islands, and Southeast Asia. In some regions, these crops constitute the principal human staples. They are also used for animal feed and fuel.

The International Institute of Tropical Agriculture (IITA) established its Root and Tuber Improvement Program in 1971. IITA has global responsibility for the genetic improvement of sweet potato, yam, and cocoyam; and regional responsibility for improving cassava. It has been able to assemble and introduce germplasm of these crops in seed form. Tuber crops are vegetatively propagated and their seeds are usually heterogeneous. In some cases, they may not flower even under natural conditions. Most diseases of tuber crops persist in the vegetative plant parts, which are used for propagation. The infected plant parts disseminate the disease very effectively. For that reason, the Inter-African Phytosanitary Council of the Organization of African Unity prohibits international exchange of vegetative material. As a consequence, germplasm exchange is difficult.

During the past 10 yr, IITA has developed, through hybridization, high-yielding varieties of cassava, sweet potato,

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Plant tissue culture scientist and plant breeder, International Institute of Tropical Agriculture (IITA), Oyo Road, PMB 5320, Ibadan, Nigeria.

and yam with resistance to major diseases and pests, wide adaptability, and other desirable agronomic characteristics (8).

In the past, germplasm exchange was limited to true seeds. Only recently has clonal material been permitted across quarantine barriers.

Tissue culture research at IITA began in 1973. The objectives are to eliminate disease, multiply clones, distribute improved material, and conserve tuber crop germplasm. Cassava receives first priority, then sweet potato, yams, and cocoyams. Remarkable progress has been made. Virus researchers at IITA have been characterizing and producing antisera and have developed reliable virus indexing methods.

This paper summarizes the 5-yr tissue culture research at IITA.

MERISTEM/SHOOT-TIP CULTURE AND VIRUS INDEXING

Meristem culture to eliminate disease greatly depends on virus indexing. Only reliable and sensitive methods can ensure that a culture is virus free and expedite its acceptance by quarantine officers.

Meristem culture was first successfully used to free dahlias of virus infection (23). It is now widely used in horticulture.

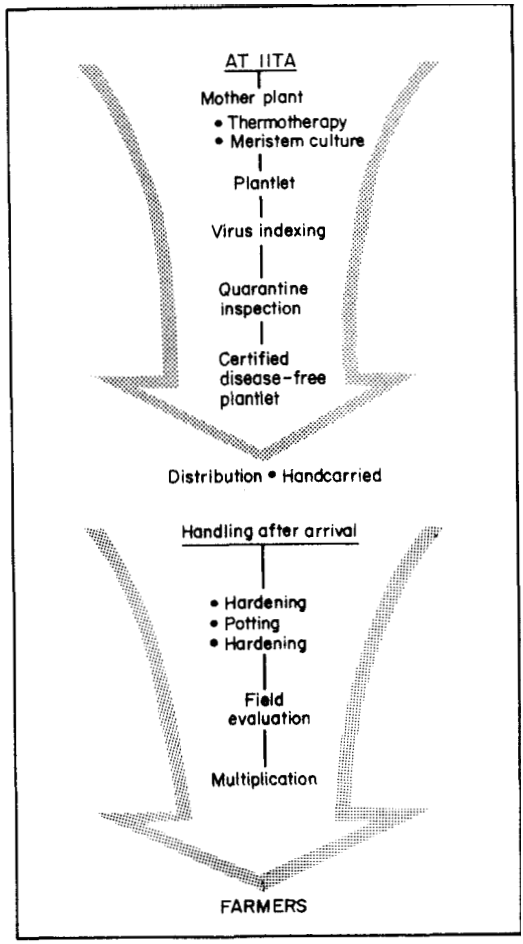
Meristem culture has helped eliminate several virus diseases from sweet potato (2, 6, 24, 29, 30), cassava (1, 5, 13, 18), yams (20, 28), aroids (10, 17), and other plant species (31, 39).

Success in obtaining virus-free plants by meristem culture depends on virus type, host plant species, and size of meristem used for culture. In general, the number of virus-free plants produced is inversely proportional to the size of the meristem used. The scheme for producing, distributing, and evaluating disease-free plants is shown in Figure 1.

CASSAVA (*MANIHOT ESCULENTA*)

During 1978, we started cassava meristem culture work to free our improved cassava varieties from cassava mosaic virus (CMV). Kartha and Gamborg's medium (18), with the addition of 100 mg inositol/litre and 80 mg adenine sulfate/litre, was found suitable for meristem growth and development. Meristems taken directly from field-grown cassava could not effectively eliminate disease. Only 25% of the regenerated plantlets were CMV free. In some cases the CMV symptoms were readily detected in vitro. To increase the CMV-free plant percentage, the young cassava plants were subjected to thermotherapy (35°C day and night temperature) for 4 wk. Mother plants with CMV symptoms initially lost their symptoms after 4 wk of heat treatment. Meristems obtained from heat-treated plants resulted in a higher percentage of CMV-free plants (11). Almost all plants obtained through meristem culture of heat-treated mother plants were CMV free (12).

Apical dominance in cassava plants is strong; their lateral buds are usually tiny. Unless the apical bud is removed early in the season, the lateral buds will often not develop.



1. Scheme for producing disease-free plants at IITA and distributing them to the national program.

Meristems from the apical buds downward to the 11th lateral bud were cultured and compared for plantlet regeneration rates. Because the apical meristems consistently showed the highest plantlet formation rate (Table 1), only apical buds were used.

Thirty improved cassava varieties were regenerated through meristem culture. The plantlet formation rate usually ranged from 20 to 90%. One IITA variety yielded no plantlet in several trials. Later a prolonged heat treatment (more than 2 mo) improved the plantlet formation rate. Plantlets obtained from meristem culture were transplanted to sterile soil In pots and protected from reinfection. Virus indexing was based on symptoms on both cassava and the indicator plant *Nicotiana benthamiana.* The cassava plants were kept at a temperature range of 20-25°C, which favors symptom expression of CMD (11). The observation period was 8 mo. *N. benthamiana* was mechanically inoculated with sap from the cassava shoot apex.

Table 1. Growth and development of meristems obtained from different buds (according to their location on the cassava plant).

	Formation rate (%) in meristem from lateral bud										
	Apical bud (1st)	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th
Plantlet	86.7	0	13.3	40.0	20.0	13.3	26.7	26.7	33.3	13.3	6.6
Callus	13.3	0	13.3	6.6	0	0	13.3	6.6	33.3	13.3	0
Root	0	0	0	0	0	0	0	6.6	0	0	0
Green growth	0	0	0	13.3	6.6	33.3	0	0	0	0	0
No growth	0	100	73.3	40.0	73.3	53.3	60.0	60.0	33.3	73.3	93.3

Serological diagnosis of the disease is more reliable than the test plant method and symptom observation. Antiserum was produced at IITA in 1982; but its titer was low. The virus can be detected in cassava by enzyme-linked immunosorbent assay (ELISA) (37). Antiserum recently produced at IITA showed some reaction with ELISA (G. Thottappilly, pers. comm.).

SWEET POTATO (*IPOMOEA BATATAS*)

Meristem culture medium for sweet potato was developed in 1979 (12). Effects of different sucrose concentrations and light intensities were examined in 1980. Optimal meristem response was observed with 3-4% sucrose in the culture medium and at about 5,000 1x.

Both apical and lateral buds of sweet potato were used for meristem isolation. The plantlet formation rates ranged from 20 to 100%; in most varieties they ranged from 60 to 80%.

Over 100 sweet potato varieties were regenerated through meristem culture. About 80% of the plants were indexed as free from sweet potato virus disease (SPVD), the only sweet potato virus disease reported in Nigeria. Heat treatment was not needed.

SPVD has been described by Schaefers and Terry (33), who concluded that it is caused by two viruses. The first virus is a nonpersistant virus and is transmitted by aphids; it is latent in sweet potato, but produces marked vein clearing in *I. setosa*. The second virus is transmitted by whitefly; it is also latent in sweet potato, but produces mild chlorosis and stunting in *I. setosa*. When the two viruses are both present in sweet potato, they produce severe symptoms.

Several methods are used to detect SPVD in sweet potato plants. For virus indexing, the plantlets are transplanted into sterile soil and kept in an insect-free isolation room. The indexing method is as follows:

1. The plants are individually grafted to *I. setosa* seedlings 3 times at 2-mo intervals.
2. Antiserum for the aphid component is produced and ELISA and serological-specific electron microscopy (SSEM) performed (14).

3. Cuttings of tissue-cultured plants are approach-grafted separately to cuttings of a susceptible sweet potato clone preinfected with either the whitefly component or the aphid component. Either component in the plant can therefore be easily detected (13, 14).

YAM (*DIOSCOREA ROTUNDATA* AND *D. ALATA*)

Most of IITA's past efforts were devoted to cassava and sweet potato, primarily because of national programs' demand for improved materials, and because virus-indexing methods for cassava and sweet potato had been developed. Yam meristem culture medium had been developed by 1983. The requirements of the two yam species are quite different. *D. rotundata* (white yam) developed into a complete plantlet 5 mo after being cultured on the cassava meristem culture medium with 50 mg L-cysteine/litre and transferred once to fresh medium. *D. alata* (water yam) did better with a higher concentration of gibberellic acid. The small size of the meristem and blackening of the explant material and culture media may have slowed down the plantlet development.

Several varieties of white yam have been regenerated from meristem culture, and the plantlets easily established in soil. Yam mosaic virus is the only virus disease to occur in white yam in Nigeria. Although the symptom expression is quite different among clones, the isolated virus was morphologically and serologically identical (15). It is a potyvirus, easily detectable by electron microscopy, and readily transmitted to *N. benthamiana* (36). Reliable indexing methods, including inoculation to *N. benthamiana*, the ELISA test, and SSEM, are available. Although there has been some success in water yam meristem culture, no reliable virus indexing method has been developed.

COCOYAM (*XANTHOSOMA SAGITTIFOLIUM* AND *COLOCASIA ESCULENTA*)

Cocoyam has the lowest priority among the four root crops mentioned, but in 1982-1983, cocoyam meristem growth and development under different physical culture conditions were studied. Plantlet formation of *C. esculenta* (taro) in liquid culture medium on a rotary shaker is favored, but that of *X. sagittifolium* (tannia) is not (27).

Four cultivars from each of the two cocoyam species were successfully reproduced through meristem culture, and the plants easily established in soil.

In Nigeria the only virus disease occurring in cocoyams is dasheen mosaic disease (DMV). By mechanical inoculation of DMV-infected materials into *Philodendron selloum* (38) and into cocoyam seedlings (32), disease symptoms can be easily induced. Serological studies showed that this virus is closely related or identical to the DMV occurring in the US. Therefore, the preferred indexing methods are test plant inoculation and serology.

MULTIPLICATION

In vitro propagation technique is the most advanced application of plant tissue culture. It involves using smaller propagules and a substantially faster multiplication rate than the conventional field and greenhouse multiplication methods. Node cuttings or shoot tips are the explant material for micropropagation of food yams (16, 21, 19), cassava (3, 13, 26, 35), sweet potato (6, 13, 22), and cocoyams (25). Tuber discs can serve as propagules in sweet potato (7) and yam (16).

In vitro multiplication of the IITA-mandated root crops usually uses node cuttings or shoot tips as explant material either through the formation of shoot and roots or multiple shoots. The propagation rates obtained at IITA are 4.2/mo for sweet potato, 4/mo for cassava, 3/mo for yam, 10/mo for taro, and 5/mo for tannia. The media are solid, except in taro, for which the preferred medium is liquid.

Among these four root crops, yams are the most problematical. The plant material is not available all year round. The tuber is dormant for 3 – 4 mo, and the blackening of the explants and media from phenol oxidation can be very severe in some clones. In addition to node cuttings, white yam tuber discs of about 1 cm^3 were induced to form meristems and then roots and shoots. With one variety, where tubers harvested the same year were used, the result was promising. However, response was very poor with another variety and with a different harvest of the promising variety.

Low field multiplication rate has limited yam production. Seed yam can be produced through in vitro propagation. Once the clone has been established in vitro, its normal growth pattern would not affect multiplication.

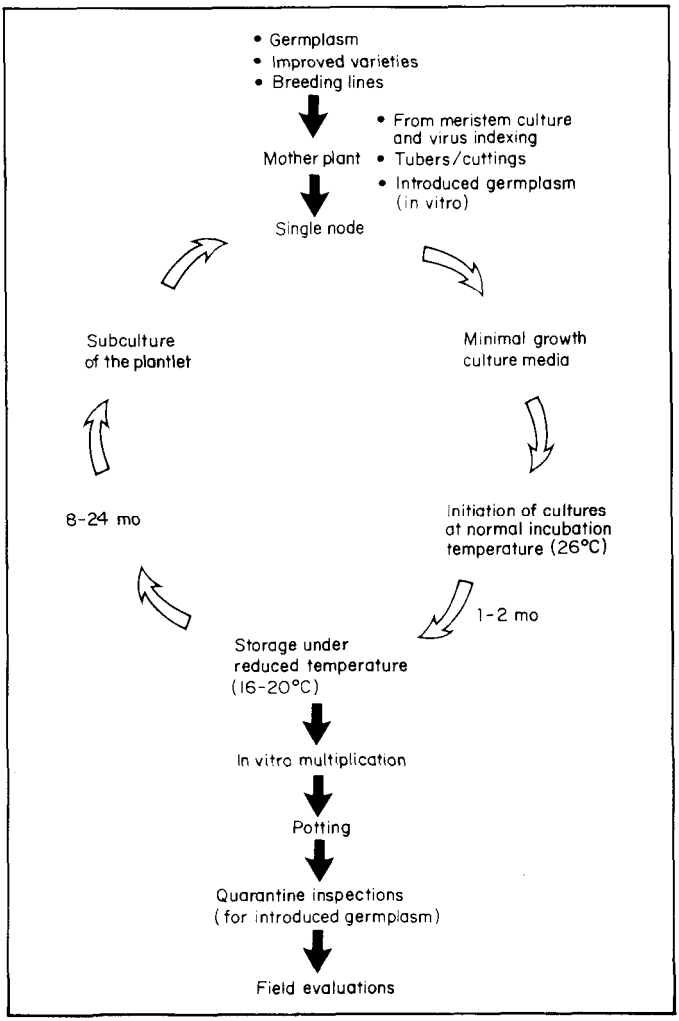
In experiments to eliminate or minimize the blackening of explants, different antioxidants were tried. The method used included soaking the explants in the various antioxidants and incorporating the antioxidants in the culture medium.

Cysteine appeared the most effective antioxidant in yams. It reduced the blackening, as does frequent transfer of the explant to fresh culture medium. In some cases, however, the whole explant turned black overnight and died.

At IITA, several selected yam clones have been multiplied in vitro, and the plants are being transplanted to the field for evaluation.

GERMPLASM CONSERVATION AND EXCHANGE

Conserving the germplasm of vegetatively propagated crops presents a serious problem, which in vitro technique can overcome. Minimal growth storage is an immediate option. Like IITA, some international agriculture research centers are using this method. Cryopreservation is a long-term option. However, research efforts in cryopreservation have concentrated on very few crops and plant recovery rate after freezing is still low. Potato (9) and cassava (3, 4) shoot tips have been freeze-stored.



2. In vitro germplasm conservation and use at IITA.

IITA has been designated by the International Board for Plant Genetic Resources (IBPGR) to hold world collections of sweet potato germplasm. The scheme for germplasm conservation of sweet potato and other root crops is shown in Figure 2. The minimal growth storage method is used for sweet potato and yam (14); by adding 3% mannitol to the culture medium and maintaining a low incubation temperature (16-20°C), sweet potato germplasm can be maintained for 8 to more than 24 mo.

IITA now maintains more than 600 sweet potato lines in this manner. A few yam collections have been maintained in vitro at reduced temperature for more than 1 yr. Yam germplasm collections are being transferred from the field to be conserved in vitro.

In vitro meristem culture and virus indexing have become very important tools for the safe international exchange of valuable vegetative germplasm. IITA collaborates with the Nigerian Plant Quarantine Services (PQS), for inspection and export certification of the in vitro materials. The Inter-African Phytosanitary Council approves these methods.

To ensure that in vitro plant materials arrive in good condition, they are handcarried to requesting countries. Cassava has been sent to 31 countries in Africa in about 60 consignments. Sweet potato has been distributed to 47 countries throughout the world in more than 80 consignments. To facilitate distribution, the IITA disease-free cassava clones are deposited at the Kenya Agriculture Research Institute (KARI), and the disease-free sweet potato clones are deposited at the Instituut Voor Plantenziektenkundig Onderzoek (IPO), Netherlands. KARI will assist IITA in distributing cassava germplasm in East Africa. IPO has been assisting IITA in distributing sweet potato germplasm by mail since 1980. The disease-free clones are also being checked for virus at IPO.

PQS also allows importing in vitro disease-free sweet potato, yam, and cocoyam from all over the world. The imported plant materials must be maintained in vitro at IITA. Quarantine inspections are required before field release of such material.. Since IITA is responsible for conserving world germplasm collections of sweet potato. IPO assists in disease elimination and virus indexing before the materials are introduced to IITA. The arrangements for germplasm exchange are represented in Figure 3. Collaborators without tissue culture facilities can send their germplasm as tubers or cuttings to IPO, where meristem culture and virus indexing will be performed. Disease-free germplasm can then be sent to IITA for conservation and eventual use.

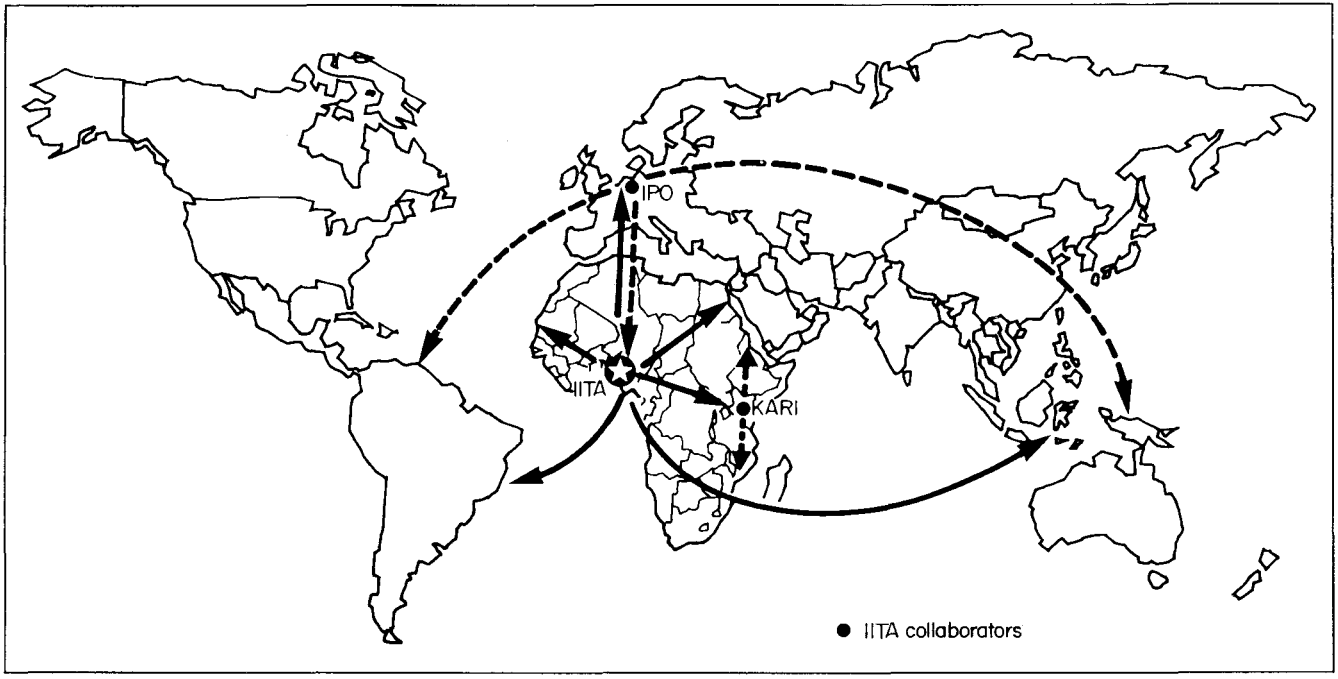
ANTHER CULTURE AND EMBRYOGENESIS

Haploid plants are valuable for genetic, cytological, and breeding research. Their phenotype is the expression of a single set of chromosomes, because there is no dominance to mask traits. When haploid plant chromosomes are doubled, either naturally or by colchicine treatment, homozygous diploids result in only one generation. Haploid plants can be obtained by anther or pollen culture. The microspores are induced to produce plantlets directly or to produce callus, which then differentiates into plantlets.

At IITA, calluses were obtained from cassava anthers, but no plantlets were obtained (15). Embryogenesis has been reported in callus culture of cassava (34).

FUTURE PLANS

Tissue culture work at IITA will concentrate on elimination of disease, and on multiplication, distribution, and germplasm



3. Germplasm exchange system arranged by IITA.

conservation of cassava, yam, sweet potato, and cocoyam. Plantain will also be included. Germplasm collections are expected to increase in the coming years and anther culture research will continue.

Collaboration with universities in the use of ultralow temperature storage for sweet potato, yam, and cocoyam should begin. Selection of stress-resistant plants from cell culture could be included. Protoplast fusion studies in breeding yam should be considered since flowering in yams is rare.

REFENRENCE CITED

1. Adejare. G.O., and K.H.A. Coutts. 1981. Eradication of cassava mosaic disease from Nigeria cassava clones by meristem-tip culture. Plant Cell Tissue Organ Culture 1:25-32.
2. Alconero, R., A.G. Santiago, F. Morales, and F. Rodriguez. 1975. Meristem tip culture and virus indexing of sweet potatoes. Phytopathology 65:769-773.
3. Bajaj, Y.P.S. 1977. Clonal multiplication and Cryopreservation of cassava through tissue culture. Crop Improv. 4(2):198-204.
4. Bajaj, Y.P.S. 1983. Cassava plants from. meristem culture freeze-preserved for three years. Field Crops Res. 7:161 – 167.
5. CIAT (Centro Internacional de Agricultura Tropical). 1979. Annual report, 1979. Cali, Colombia.
6. Frison, E.A., and S.Y. Ng. 1981. Elimination of sweet potato virus disease agents by meristem tip culture. Trop. Pest Manage. 27(4):452-454.
7. Gunkel, J.E., W.R. Sharp, B.W. Williams, W.C. West, and W.O. Drinkwater. 1972. Root and shoot initiation in sweet potato explants as related to polarity and nutrient media variations. Bot. Gaz. 133(3):254-262.
8. Rahn, S.K., E.R. Terry, and K. Leuschner. 1980. Breeding cassava for resistance to cassava mosaic disease. Euphytica 29(3):673 – 683.
9. Tienshaw, G.C., J.F. O'Hara, end R.J. Westcott. 1980. Tissue culture methods for the storage and utilization of potato germplasm. Pages 71-76 in Tissue culture methods for plant pathologists. D.S. Ingram and J.P. Helgeson, eds. Blackwell Scientific Publication, Oxford.
10. Hartman, R.D. 1974. Dasheen mosaic virus and other phytopathogens eliminated from caladium, taro and cocoyam by culture of shoot tips. Phytopathology 64:237-240.
11. IITA (International Institute of Tropical Agriculture). 1978. Annual report, 1978. Ibadan, Nigeria.
12. IITA (International Institute of Tropical Agriculture). 1979. Annua1 report, 1979. Ibadan, Nigeria.
13. IITA (International Institute of Tropical Agriculture). 1980. Annual. report, 1980. Ibadan, Nigeria.

14. IITA (International Institute of Tropical Agriculture). 1981. Annual. report, 1981. Ibadan, Nigeria.
15. ITTA (International Institute of Tropical Agriculture). 1982. Annual report, 1982. Ibadan, Nigeria.
16. IITA (International Institute of Tropical Agriculture). 1983. Annual report, 1983. Ibadan, Nigeria.
17. Jackson, G.V.H., E.A. Ball, and J. Arditti. 1977. Tissue culture of taro, Colocasia esculenta (L.) Schott. J. Hortic. Sci. 52:373-382.
18. Kartha, K.K., and C.L. Gamborg. 1975. Elimination of cassava mosaic disease by meristem culture. Phytopathology 65:826-828.
19. Mantell, S.H. 1979. A rapid propagation system for yam. Yam Virus Project Bull. 1. 18 p. CARDI, Trinidad.
20. Mantell, S.H. 1980. Apical meristem tip culture for eradication of flexous rod viruses in yams (Dioscorea alata). Trop. Pest Manage. 26 (2):170-179.
21. Mantell, S.H., S.Q. Haque, and A.P. Whitehall. 1978. Clonal multiplication of Dioscorea alata L. and Dioscorea rotundata Poir. yams by tissue culture. J. Hortic. Sci. 53:95-98.
22. Moran, M., and M. Quoirin. 1983. In vitro plant regeneration from apical meristem and stem internodes of sweet potato (Ipomoea batatas Lam.) Sixth Symposium of the International Society for Tropical Root Crops. 20-25 February 1983, CIP, Lima, Peru.
23. Morel, G.M., and (J.C. Martin. 1953. Guerison de dahliaa attients d'une maladie a virus. Co. R. Acad. Sci., Paris, 235:1324-5.
24. Mori, K., E. Hamaya, T. Shimomura. and Y. Ikegami. 1969. Production of virus-free plants by means of meristen culture. J. Cent. Agric. Exp. Agric. Stn. 13:79-110.
25. Mostafa, N., Abo El-Nil, and F.W. Zettler. 1976. Callus initiation and organ differentiation from shoot tip cultures of Colocasia esculenta. Plant Sci. Lett. 6:401-408.
26. Ng, S.Y. 1982. Tissue culture and rapid multiplication: Techniques and importance in distributing improved cassava clones. Paper presented at the International Workshop on Biological Control and Host Plant Resistance to Control the Cassava Mealybug and the Green Spider Mite in Africa, 6-10 December 1982, IITA, Ibadan, Nigeria.
27. Ng, S.Y. 1983. Tissue culture of cocoyams. Paper presented at Symposium on Tissue Culture of Economically Important Plants, 28 August to 2 September, 1983, University of Nuskka, Nigeria.
28. Ng, S.Y. 1983. Special techniques assist in quality seed production of sweet potato, yam, and cocoyam. Paper presented on the Global Workshop on Root and Tuber Crops Propagation, September 12-16, 1983, CIAT, Cali, Colombia.
29. Nielsen, L.W. 1960. Elimination of the internal cork virus by culturing apical meristems of infected sweet potatoes. Phytopathology 50:841-842.

30. Over De Linden, A.J., and R.F. Elliot. 1971. Virus infection in Ipomoea batatas and a method for its elimination. N.Z.J. Agric. Res. 14:720-724.

31. Quak, F. 1977. Meristem culture and virus free plants. Pages 598-615 in Applied and fundamental aspects of plant cell, tissue and organ culture. J. Reinert and Y.P.S. Bajaj, eds. Springer-Verlag.

32. Rossel, H.W., and G. Thottappilly. 1982. Latest research results on root and tuber crop viruses and their indexing. Paper presented at the Workshop on Tropical Root and Tuber Crops Germplasm Distribution and Plant Quarantine Regulations, 28-30 April 1982, IITA, Ibadan, Nigeria.

33. Schaefers, G.R., and E.R. Terry. 1976. Insect transmission of sweet potato disease agents in Nigeria. Phytopathology 66:624-645.

34. Stamp, J.A., and G.H. Henshaw. 1982. Somatic embryogenesis in cassava. Sonderdruck aus Zeitschrift fur Pflanzenphysiologie 105(2):183-187.

35. Tamin, M.S., and P. Debergh. 1979. Regeneration of cassava plants from axillary meristems. Med. Fac. Landbouwwetenschappen, Rigksuniv. Gent. 44:1109-1112.

36. Terry, E.R. 1977. Incidence, symptomathology and transmission of a yam virus in Nigeria. Pages 170-173 in Proceedings 4th symposium of the International Society for Tropical Root Crops, Cali, Colombia 1976.

37. Thouvenel, J.C., D. Fargette, C. Fauquent, and A. Monsarrat. 1983. Serological diagnosis of African cassava mosaic by immunoenzymatic method. Paper presented at the Sixth Symposium of the International Society for Tropical Root Crops, 20-25 February 1983, CIP, Lima, Peru.

38. Volin, R.B., G. Thottappilly, and H.W. Rossel. 1980. Isolation of a dasheen mosaic-like virus and screening cocoyam seedlings populations of resistance. Ann. Proc. Am. Phytopathol. Soc. 1980.

39. Walkey, D.G.A. 1980. Production of virus-free plants by tissue culture. Pages 109-117 in Tissue culture methods for plant pathologists. D.S. Ingram and J.P. Helgeson, eds. Blackwell Scientific Publications, Oxford.

IN VITRO CULTURE OF *SABA BANANA* [*Musa balbisiana* cv. Saba (BBB)]

D. P. DAMASCO and R. C. BARBA

Corm sections and shoots derived from plants in culture formed multiple shoots on Murashige and Skoog's (MS) medium supplemented with 10 mg benzyl adenine/litre. Subculture of shoot tips in fresh medium of the same composition increased the number of shoots produced with each culture cycle. Shoots cultured 1 mo in MS medium without growth regulators initiated roots and were readily transplanted in soil. With subculture every 2 mo, 200 000 plantlets can be derived from an explant in 10 mo.

Banana is the most important fruit in the Philippines in both hectarage and commercial value. Aside from the large plantations in Mindanao that are largely for export, most plantings are backyard or small scale.

Saba, a cooking banana, is the most popular cultivar grown in the Philippines. It is a staple food and an important carbohydrate source for the rural population. Saba is consumed in many forms: prepared as vegetable or snack (dried, boiled, fried, or fresh); or processed into banana puree, flour, dried chips, alcohol, or vinegar. Although saba has great potential, it is hardly grown on a commercial scale.

Tissue culture, which can rapidly propagate even the slow-to-propagate species, has been used to accelerate plantation development, to reduce the breeding cycle, and to rapidly multiply and disseminate limited materials of new varieties. It also avoids the problem of bulky corm transport. Having certified cultures in a germplasm exchange minimizes dissemination of unwanted pests and diseases.

Tissue culture of several banana species has been reported (1, 2, 3, 4, 6).

MATERIALS AND METHODS

Shoot explants were obtained from developing suckers of saba grown in the field. The roots and outer tissues of the suckers were removed and the remaining portion was rid of adhering soil. The

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Science research specialistand professor of Horticulture; and researcher, Instituteof Plant Breeding, University of the Philippines at Los Baños, College of Agriculture, College, Laguna, Philippines.

corms were surface-disinfested in pure commercial bleach (a.i. 5.25% sodium hypochlorite) for 45 min. Outer leaves were removed until the shoot measured 1.5 cm wide at the base. The shoot was then sectioned into four and each section served as an initial. explant. For subculture, shoot explants about 10 mm in height were excised from in vitro cultures.

The banana tissues were transferred onto a culture medium containing Murashige and Skoog's salts and vitamins (5) supplemented by Fe-sequestrene (0.025 g/liter), coconut water (100 ml/litre), sugar (20 g/Iitre), and agar (Bacto-agar, 5 g/litre). Growth regulators (BA, kinetin! were added as required.

The pH of the media was adjusted to 5.7 ± 0.1 and the media were sterilized at 0.106 kg/cm^2 or 15 min.

Cultures were maintained in an air-conditioned storage room, 8 h in light and 16 h in darkness. White flourescent lamps were the light sources.

Observations for all experiments were made 8 wk after culture.

RESULTS

Shoot proliferation

Shoots proliferated from sectioned corm tissues and tissue culture-derived shoots grown on media supplemented with a cytokinin such as benzyl adenine (BA) (Fig. 1). The optimum

1. Maximum shoot proliferation of in vitro-derived single shoot (left) and initial explant (right) on a medium containing 10 mg BA/litre.

concentration of BA for shoot proliferation was 10 mg/litre for both cultured materials (Table 1). More shoots were observed in the sectioned corm tissues than in the in vitro-derived shoots. In the absence of a cytokinin, proliferation was not observed.

Kinetin-supplemented media also promoted shoot proliferation from subcultured shoots, but were not as effective as BA (Table 2). Kinetin, however, enhanced root formation in cultured shoots.

The shoots that proliferated from subcultured shoots increased with number of culture cycles on medium supplemented with 10 mg EA/litre (Table 3).

Table 1. Effect of different concentrations of BA on the number of shoots proliferated from 2 types of explant.

BA (mg/litre)	Shoots[a] (no.)	
	Initial explant	Single shoot
0	1.0 d	2.3 d
1	2.9 d	2.6 d
5	19.2 b	6.7 c
10	37.4 a	11.2 c
25	17.9 b	1.8 d
50	9.9 c	1.4 d

[a] Separation of means by Duncan's multiple range test at the 5% level.

Table 2. Effect of BA and kinetin on the average number of shoots, degree of bud proliferation, and percentage of cultures with roots 8 wk after subculture of banana shoots.

Cytokinin (mg/litre)	Shoots[a] (no.)	Bud proliferation[b]	Cultures with roots (%)
Control (0)	1.1 c	0	100
BA			
5	8.1 b	0.83	0
10	10.1 a	3.27	0
Kinetin			
5	3.4 c	0.67	86.77
10	4.2 c	0.85	61.77

[a] Separation of means by Duncan's multiple range test at the 5% level. [b] Rating scale. 0 = no bud formation, 1 = poor, 2 = slight, 3 = fair. 4 = good, 5 = excessive.

Table 3. Effect of culture cycles on average number of shoots proliferated from 1 initial shoot on medium supplemented with 10 mg BA/litre.

Culture cycle	Shoots produced[a] (no.)
Initial culture	11.22 b
1st subculture	12.51 b
2nd subculture	16.78 a
3rd subculture	17.78 a

[a] Separation of means by Duncan's multiple range test at the 5% level.

Root formation and transplant survival

Shoots subcultured on medium without growth regulators produced roots. The plantlets were initially established in a mixture of manure and garden soil (1:1) and were later grown under field conditions, where they grew normally.

DISCUSSION

With an average of 10 shoots originating from 1 shoot per subculture every 2 mo, an increase in propagation material from 1 sucker to 200 000 plantlets in 10 mo can be proiected, assuming an 80% survival (see Table 4.) Shoots formed when subcultured on medium without growth regulators. Resulting plants readily survived when transplanted in garden soil.

Table 4. Increase in planting materials from 1 sucker.[a]

Time		Increase (no.) when survival was	
Wk	Mo	90%	80%
8	2	54	48
16	4	486	384
24	6	4 374	3 072
32	8	39 366	24 576
40	10	354 294	196 608

[a]One sucker or corm would produce 4 initial explants, each with an average of 15 shoots. Each single shoot would then produce an average of 10 shoots in 2 mo.

REFERENCES CITED

1. Berg, L.A., and M. Bustamante. 1974. Heat treatment and meristem culture for the production of virus-free bananas. Phytopathology 64:320-322.
2. De Guzman, E.B., C. Decena, and F.M. Ubalde. 1980. Plantlet regeneration from unirradiated and irradiated banana shoot tip tissues cultured in vitro. Philipp. Agric. 63:140-146.
3. Ma Su Chien, and Chou-Tou Shii. 1972. In vitro formation of adventitious buds i.n banana shoot apex following decapitation. J. Kortic. Soc. China 1:135-142.
4. Ma Su Chien, and Chou-Tou Shii. 1974. Growing banana plantlet from adventitious buds. J. Hortic. Soc. China 20:6-12.
5. Murashige, T., and F. Skoog. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. 15:473-497.
6. Vessey, J.D., and J.A. Rivera. 1981. Meristem culture of bananas. Turrialba 31:162-163.

THE USE OF TISSUE CULTURE IN PLANT BREEDING IN VIETNAM

NGUYEN VAN UYEN

The potentials of plant tissue culture in modern plant breeding have been outlined by several authors. Since 1975 several plant tissue culture laboratories have been established in Vietnam. Their researchers focus on applying tissue culture to rice breeding and to the maintenance and rapid multiplication of asexually propagated plants.

ANTHER CULTURE IN RICE BREEDING

The use of anther culture in rice breeding is a top research priority in Vietnam, where rice, the major food crop, is grown on more than 2 million ha. Haploid plantlets were successfully regenerated from anther culture of more than 20 indica varieties, mostly local varieties. A modified N6 medium was selected; it was supplemented with 1 mg IAA/litre and 4-8 mg NAA/litre. The formation of green plants largely depends on genotype; an average of 10-12% of anthers cultured formed plantlets. After being transplanted into soil in pots, the roots of the haploid seedlings were treated with colchicine to obtain doubled haploids.

Compared with original varieties, the doubled haploids showed the following characteristics:

- In most cases, the growing period was reduced by 7-10 d.
- The field canopy of the doubled haploids was highly homogeneous.
- The morphogenetic potential (capacity to form calluses and green plantlets in vitro) was much higher.

Anther culture techniques should therefore be used for

- selecting early varieties (very important in multiple cropping),
- shortening the breeding cycle, and
- establishing a doubled haploid bank (which ensures that the starting genetic materials are the purest possible and varietal differences, minimal).

Institute for Experimental Biology, Ho Chi Minh City, Vietnam.

THE MULTIPLE-SHOOTSYSTEM (MSS)

Single-cell and protoplast techniques in rice breeding have been tried by several authors. Until recently, however, successful reports have been limited.

Generally rice calluses lose their morphogenetic capacity after three to four subcultures. Although continuous single-cell culture has been reported in rice, successful plating and plant regeneration from specific, mutated cell lines have not. The situation is the same in rice protoplast research.

Another approach is to reduce the rice plant to a size small enough that it can be multiplied and handled with microbiological techniques but still retain its morphogenetic capacity. Multiple shoots, masses of microscopic shoots of a monocot such as rice, established in vitro by hormonally induced excessive tillering of the initial explant, exemplify this approach.

We have investigated the MSS of rice in several varieties at both haploid and diploid levels. To obtain the haploid MSS of a variety, the regenerating haploid shoots from anther culture were put on medium supplemented with 0.1 mg BA/litre and 1 mg IBA/litre. With monthly subcultures on the same medium, the MSS could be established within 3-4mo.

After working 3 yr on this system, we observed that it has the following advantages:

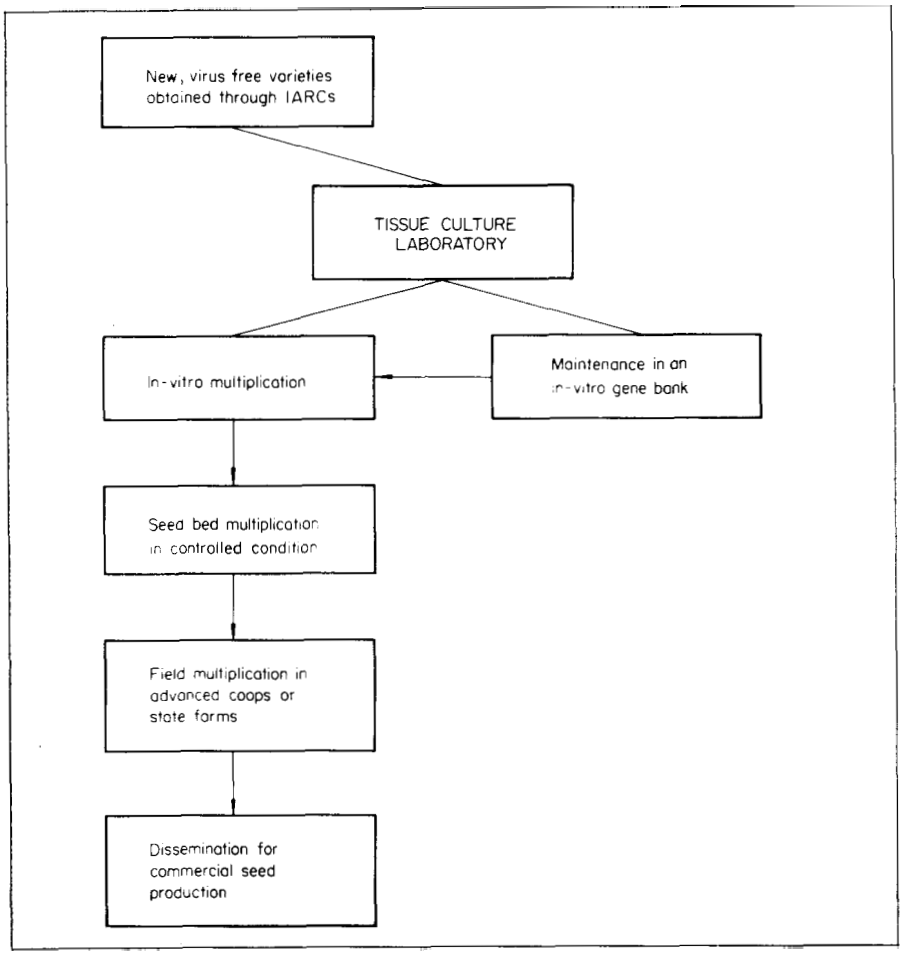
- The MSS of rice can be obtained and handled at different ploidy levels.
- It can be rapidly multiplied up to any population size on solid agar medium and in liquid medium using a shaker at low speed. Multiplication in liquid medium produces a suspension of single microshoots that can be plated on petri dishes for selection.
- The system can persist a long time without losing its morphogenetic capacity.
- At any time during the selection cycle, complete green plants can be regenerated by subculturing the microshoots onto a hormone-freemedium.

In 1983 we used MSS to select salt-tolerant rice. A MSS line proliferating on MS medium supplemented with 10 g NaCl/litre was obtained from an original line whose growth is affected at only 3 g NaCl/litre. Seeds have been harvested from the regenerated plants and resultant seedlings show much higher vigor than the control. We are now testing the second generation for salt tolerance.

USE OF TISSUE CULTURE ON ASEXUALLY PROPAGATED PLANTS

The following problems have hampered the selection and distribution of super varieties of asexually propagated plants.

- The plants have an extremely low natural multiplication rate.
- Seeds, in most cases, are too large for easy dissemination.



1. Scheme for potato preservation and multiplication.

- Disease-free maintenance of the germplasm is impossible.
- International diffusion of new varieties is limited by quarantine barriers.

Developing countries depend heavily on potato seed materials imported from Europe and North America, which cost them several million dollars per year. In Vietnam, we are trying to avoid this cost by developing methods for long-term preservation and rapid multiplication of new potato varieties. Tissue culture has been used extensively for this purpose (see Fig. 1). It ensures a continuous supply of good seed tubers for production in Vietnam. As the in vitro techniques have been simplified over the last 2 yr, at the Central Highlands City of Dalat, several family-size tissue-culture laboratories have been set up by farmers wishing to profit from producing seed potato. These farmers call themselves "satellites" and complement the State Tissue Culture Laboratory. One satellite at Dalat can produce

100 000 potato plants/mo, so CIP (International Potato Center) varieties can be diffused very rapidly. Three CIP clones (Atzimba, CFK-691, and B-71-240.2), received as single test-tube plants in the Fall of 1981, have been multiplied by both the state laboratory and its satellites. Several hundred hectares of these clones were planted by Dalat farmers in a year.

The economies of developing countries heavily depend on the exportation of agricultural products, most of which are asexually propagated crops. The Green Revolution for asexually propagated plants has not begun. Tissue culture, a powerful tool for germplasm exchange and maintenance, for selecting new varieties, and for rapid propagation of elite seed materials, can help effect the Green Revolution for these important crops.

Our Dalat experience shows that if in vitro techniques are simplified to a particular level, not only the sophisticated modern laboratories but also the farmers can enjoy the new achievements in biotechnology. This lesson is particularly important to developing countries.

HAPLOIDS IN PLANT BREEDING

SOME PLANT BREEDING PROBLEMS NEEDING BIOTECHNOLOGY

G. S. KHUSH and S. S. VIRMANI

Plant breeders have always used the best available technology to develop new varieties with stable yield, high quality, and genetic resistance to various stresses. During the past decade, a number of in vitro techniques (viz., tissue culture, cell culture, protoplast fusion) and recombinant DNA technology have shown promise as plant breeding tools for creating genetic variability, or increasing selection efficiency, or both, These tools should be used where conventional breeding techniques are less effective or efficient. Biotechnology is needed in 1) breeding for increased biomass and, hence, yield; 2) breeding for protein quantity and quality; 3) breeding for disease resistance; 4) identifying bacterial, fungal, and viral strains; 5) selecting for tolerance for soil salinity and aluminum toxicity; 6) increasing genetic variability and inducing novel variability by producing cybrids; 7) incorporating nitrogen fixation (nif) genes into cerea1s; 8) clonal propagation of horticultural and forest tree species and some vegetatively propagated crop plants; 9) in vitro induction of flowering and hybrid production for breeding tree crops with a long life cycle; and 10) preserving germplasm of asexually reproducing species.

Plant breeding has been practiced for about 10 000 yr. Crop improvement started with the domestication of the species. Cereals were probably domesticated at the dawn of agriculture; several other important crops, sugar beets, for example, were domesticated only a few hundred years ago. A comparison of modern cultivars with their wild relatives reveals the progress made in developing productive genotypes through plant breeding.

Plant breeding consists of two phases: the evolutionary phase, creating genotypic variability; and the evaluation phase, selection among genotypes.

Until the middle of the 19th century, when hybridization use began, selection was primarily for natural variability. During

Plant breeder and head, and plant breeder, Plant Breeding Department, International Rice Research Institute, P. O. Box 933, Manila, Philippines,

the present century, advances in various scientific disciplines, particularly genetics, have permitted creating new variability and selection procedures, and provided new tools for the plant breeder, though none will replace conventional plant breeding.

- In the first decade of this century, rediscovery of Mendel's laws provided the scientific basis for plant breeding by elucidating the mechanism of segregation and the laws of inheritance.
- In the second decade, Morgan and his colleagues mapped Drosophila chromosomes, and thereby explained linkage and recombination of traits.
- Vavilov's collection of germplasm of cultivated species and their wild relatives and establishment of a gene bank, in the third decade, are the forerunners of modern germplasm banks, which are the backbones of crop breeding programs.
- Developments in cytogenetics during the fourth decade, considered the golden era of cytogenetics, improved plant breeders' understanding of chromosome structure and function, and mechanisms of recombination.
- The discovery of colchicine in the fifth decade raised hopes of producing new varieties or crops through polyploidy. Although these hopes were not fulfilled, colchicine has proved a powerful tool in overcoming some of the barriers to interspecific gene transfer.
- The role of X-rays in generating new variability was the greatest discovery of the sixth decade. Various mutation induction techniques have proved powerful tools for the plant breeder.
- The seventh decade will be known for developments in biometrical and quantitative genetics which have helped the plant breeder formulate selection strategies and conduct stability analyses.
- The in vitro techniques developed during the eighth decade are extensively used in plant breeding to obtain homozygosity and fix dominance variance rapidly, and as selection tools.
- The ninth and tenth decades will probably see great crop improvement through recombinant DNA technology and other new biotechnologies.

Plant breeders have always used the best new technology leading to new productive varieties with stable yield, high quality, and genetic resistance to various stresses. Yields in such major crops as wheat, rice, maize, sorghum, and soybean have substantially increased and are still doing so.

Some of the new biotechnology techniques likely to be used in plant breeding have been reviewed by Swaminathan (47), Sybenga (48), Barton and Brill (2), and Kosuge et al (27). In this paper we have identified some plant breeding problems that need biotechnology.

MAJOR PLANT BREEDING OBJECTIVES

Generally, major plant breeding objectives include

- yield improvement;
- quality improvement;
- incorporation of genetic resistance to biological stresses, e.g., diseases, insects, nematodes;
- incorporation of genetic tolerance for environment stresses, e.g., low or high temperature, soil toxicities, moisture stress through excess or deficiency, or nutritional imbalance;
- incorporation of novel traits from alien species; and
- streamlining of current breeding methods and selection procedures.

We have identified some areas in which further improvement through conventional breeding techniques is difficult or where the efficiency of conventional methods is inadequate. New techniques of biotechnology must do what conventional means cannot (35).

Yield improvement

Increasing yield is the principal objective of any plant breeding program. During the past 50 yr, yields of all the crops studied seriously by plant breeders, except those grown in harsh environments, have steadily increased. Improved genotypes (5 or plant breeding effects) and improved environments (E or husbandry effects) are equally responsible for the improved yields. The GE interaction has been more important than is generally realized (40). There is no evidence of a yield plateau for any crop (16).

Yield is a function of total dry matter and harvest index. According to several plant scientists (49, 11, 1, 20, 42, 14), increase in the genetic yield potential of crops has stemmed from an increase in harvest index rather than in dry matter. How much further harvest index can be increased is not known, but 63% in wheat (1) and 60% in rice (Yoshida, unpubl.) have been speculated.

Dry rather production involves leaf area development; leaf area index; the rates of photosynthesis, respiration, and photorespiration; nutrient uptake; nitrate assimilation; and water use. Factors influencing harvest index include investment in unharvested organs, growth duration of the harvested organ, rate of photosynthate storage in the harvested organ, sink capacity, and competitiveness by the storage organs (14). Genetic variability in many of these characteristics at intraspecific, specific, and generic levels has been established, but control of many of these traits is not known, though they are probably not controlled by major genes, and E and GE factors are probably more important. Besides, crop physiologists are not in

a position to specify desirable single characters that are touchstones for greater yield and are genetically simple enough for the ready application of biotechnology tools (14). Photosynthetic pathways may provide many areas for potential improvement. Transfer of more efficient Calvin cycle enzymes (the pathway responsible for CO_2 fixation) between plant varieties may lead to higher rates of C fixation.

Frey (17, la), using wide hybridization, increased biomass and yield potential by introducing alien germplasm into advanced breeding lines in oats, barley, sorghum, and pearl millet. Embryo rescue and protoplast fusion should help obtain hybrids between sexually incompatible species for wider genetic diversity. The mechanisms for gene introgression in wide hybridization and biomass and yield increase, as reported by Frey, are unknown, but results indicate that introducing small random segments of alien DNA into high-yielding, adapted cultivars may further raise crop biomass and yield. Until this is possible, we should explore using irradiated pollen of sexually compatible wild species to cross with superior varieties and transfer only segments of the wild genome (23).

Anther culture. Yield is a trait with low heritability and the selection efficiency in the early generations (F_2-F_4) is low with current breeding procedures. Doubled haploid lines can give more additive genetic variation expression, and mean the elimination of dominance variation (Table 1). The environmental variance between F_2 individuals (V_{EI}) may be greater than that between F_3 or doubled haploid plots (V_{EP}), which comprise families of genetically similar individuals. Furthermore, replications can reduce or eliminate V_{EP} so that individual breeding lines can be more accurately assessed. In the F_3 and F_4 lines both additive and dominance effects will contribute to phenotypic differences while doubled haploid progeny variation is due only to microenvironment effects (43). Consequent ly, anther culture-derived plants are genetically identical from one generation to the next, and their selection efficiency is likely to be higher when dominance variation is significant. Anther culture has already been used in the self-pollinated crops rice, tobacco, and wheat. In barley, variety Mingo was developed through haploids and produced through chromosome elimination.

Different filial. generations can serve as parent for haploidization. The choice depends on breeding objectives. The

Table 1. Expectations of phenotypic variances in different generations (43).

Generation	Variance[a]
F_2	$V_A + V_D + V_{EI}$
F_3 family means	$V_A + 1/2\ V_D + V_{EP}$
F_1 derived DH means	$2V_A + V_{EP}$

[a]V_A = Additive component of variation.
V_D = Dominance component of varition.
V_{EI} = Environmental variance between F_2 individuals.
V_{EP} = Environmental variance within F_3 /dihaploid progenies.

most common system is to use F_1 hybrids. Simpson and Snape (41) proposed the "selected F_3 system" used with winter barley. The F_1 doubled haploid system can be extended to allow recurrent selection in inbreeding species. Superior doubled haploids of one cycle provide the next generation parents in successive intercrossing, doubled haploid production, and selection. Varieties can be extracted at any generation and population performance is expected to improve gradually. This system would have more selection efficiency and a higher frequency of desirable genotypes than an F_1 derived population. Choo et al (9) have described the genetic consequences of such a system, and in spring barley, for example, each cycle of production and evaluation took 2 yr (24).

Doubled haploid lines developed from different filial generations can be used to detect and estimate the components of genetic variation, to detect linkage disequilibrium, and to estimate the number of effective factors segregating in a cross (44, 45). Even such complexly inherited traits as yield can be improved through anther or pollen culture. In-vitro screening would help segregate materials for efficient N assimilation (viz., nitrate reductase and NH_4 assimilation) which may be correlated with biomass production and yield.

Quality improvement

Total food production and food production per capita have improved, yet about 500 million people in the world suffer from hunger and malnutrition. To eliminate widespread malnutrition, developing countries should double their food production by the end of the century (46).

Eight foods, rice, wheat, maize, sugar, sorghum, millet, cassava, and animal products, provide most of the nutrients for people in developing countries. They account for about three quarters of the energy intake and two-thirds of the protein consumed, and, in the poorest populations, may account for 85-90% of the energy and protein. Nutritional quality is an important objective of any plant breeding program.

There is still wide genetic variation in content among food crops, and breeding for higher yield does not mean lowering nutritional value.

Protein content and quality are important traits that conventional plant breeding has not significantly improved. The cereals have poor amino acid profiles, and they constitute the bulk of the human diet in the developing countries. Improving the level of such limiting amino acids as lysine will greatly help solve malnutrition. In rice lysine content does not vary. We hope that such biotechnology tools as induction and selection of amino acid-overproducing mutants at cellular level can help improve the lysine content of rice.

Resistance to biological stresses

All responsible for solving the world's rapidly increasing need for agricultural produce are gravely concerned about the

remarkable ability of many parasites to adapt to introduced resistances (and to pesticides).

The plant breeding efforts to incorporate pest and disease resistance into various crops and horticultural species have been highly successful, but breeding for resistance to biological stresses remains a struggle for plant breeders, pathologists, and entomologists. To be effective, plant breeders have to be ahead of the parasites; they need to develop resistant host plants faster. Pathogen-produced toxins may be useful in selecting disease-resistant-individuals in genetically modified plants in vivo or tissues, cells, and protoplasts in vitro (5, 13, 21).

HB-toxin, a confirmed pathogen produced by the fungus Cochliobolus victoriae, can help identify resistant oak plants (50). Use of such toxins in cell and tissue culture should further increase selection efficiency to identify resistant genotypes. Liu (27) described the recovery of smut (Ustilago scitaminea) resistance by tissue culture in sugarcane. Table 2 lists disease resistance studies in which whole plants were recovered and tested against toxins or pathogens.

Table 2. Disease-resistant mutant plants isolated from cultured cells (53).

Species	Explant	Mutant phenotypes	Mode of inheritance	Reference
Nicotiana tabacum	Haploid protoplasts and cells	Resistance to methionine sulfoximine	Two cases of a semi-dominant nuclear allele and one case of two recessive nuclear alleles	7
Zea mays	Young embryos	Resistance to *Helminthosporium maydis* race T toxin	Maternal	19 and 6
Saccharum officinarum	Young leaves	Resistance to Fiji disease	Stable but unknown	22
		Resistance to eyespot disease	Stable but unknown	
		Resistance to dewy mildew	Stable but unknown	
Solanum tuberosum	Young leaves	Resistance to *Phytophtora infestans* toxin	Unknown	3
	Leaf protoplasts	Resistance to *Phytophthora infestans* toxin	Unknown	38
	Leaf protoplasts	Resistance to *Alternaria solani* toxin	Unknown	38
Brassica napus	Haploid leaf mid-ribs and flower stems	Resistance to *Phoma lingam* toxin; inhibitory action on growth of *Phoma lingam*	Heritable	37

To incorporate specific resistance genes plant breeders always must identify physiological races of fungal, bacterial, and viral diseases. Race identification is not always easy and precise. The monoclonal antibody technique (25) should help identify viral and bacterial strains more precisely than antisera, and more quickly than cross-infection techniques (12).

Plant breeders frequently must transfer a wild species resistance gene to a host that may be sexually incompatible. Wide hybridization among unrelated species may result in genotypes immune to the particular disease, e.g., the incorporation of rust resistance genes from *Aegilops* and *Agropyron* species into the cultivated wheats. In genus *Oryza*, several wild species cross-incompatible with cultivated rice are highly resistant to brown planthopper; embryo culture or protoplast fusion techniques might overcome this incompatibility. Recombinant DNA techniques might help transfer genes of species whose genome normally does not pair and recombine with that of the cultivated species.

Tolerance for environmental stresses

Conventional breeding methods have little improved genetic tolerance for soil salinity and aluminum toxicity. Tissue culture could help here. Salinity, perhaps the most widespread soil constraint in agriculture, affects 380 million ha, 240 million ha of which are not strongly saline (28). It affects 33% of the world's irrigated land (8). Aluminum toxicity is a major problem on acid upland and acid sulfate soils. By impairing root growth, it makes plants less efficient at exploiting soil nutrients, and more susceptible to drought.

Tissue culture has produced only two salt-tolerant species, i.e., *Nicotiana tabacum* (32, 33) and *Oryza sativa* (36, 10). The tobacco work has produced convincing results, but work on rice is inconclusive.

Tissue culture has been used in selecting for aluminum tolerance in tomato (29, 30), carrot (34), and rice (53). It holds promise, but only if the problems of loss of regeneration capacity in prolonged cultures and lack of variant expression at plant level are resolved.

Novel traits from alien species

The success of plant breeding largely depends on the extent of genetic variability available. Interspecific sexual hybridization has been important both in crop evolution (39) and in developing new varieties. Several cultivated crops are allopolyploids originally derived through sexual hybridization, chromosome doubling, and diploidization. Interspecific hybrids have also transferred genes into cultivated crops, viz, tomato, tobacco, barley, wheat, potato, rice, and sugarcane. Novel variation (expressed in neither parent species) such as cytoplasmically controlled male sterility in wheat, tobacco, and rice, and improved pigmentation in tomato, has been observed in some interspecific sexual hybrids.

Protoplast fusion has produced previously unavailable germplasm (14). The variability in somatic hybrid plants derived from protoplast fusion can be due to nuclear incompatibility, mitotic recombination, somaclonal variation, and organelle segregation. Evaluation of a number of somatic hybrids suggests that although two types of cytoplasm are initially mixed during protoplast fusion, cytoplasmic segregation usually occurs (14). This produces classes of hybrid plants differing in organelle DNA content. Recombinant plastomes, particularly mitochondrial. genomes, may be recovered after fusion (4). Hence, with organelle segregation and recombination, the range of nuclear-cytoplasmic genetic combinations is wider than in comparable sexual hybrids.

Protoplast fusion can expeditiously produce cytoplasmic, male-sterile lines for developing F_1 hybrids in several crop plants. Conventional techniques require five to seven backcross generations to develop such lines.

Perhaps most important, protoplast fusion can make cybrids, and thereby help elucidate cybrids' influence on recombination frequency, gene expression, and species stability.

To use somatic hybridization in plant breeding (14),

- more species' protoplasts must be regenerated (only 35 have been);
- somatic hybrids must be made capable of sexual reproduction;
- intergenomic recombination or chromosome substitution between parental genomes must be achieved; and
- as protoplast fusion results in plants with the summation chromosome number, chromosome number must be manipulated.

The transfer of phaseolin gene from bean to sunflower via tumor-inducing plasmid (31) and its expression in sunflower tissue exemplifies how gene transfer could improve crops. Expression and transmission at plant level must first be achieved, however.

Incorporating nitrogen-fixing (Nif) genes into cereals is the most ambitious genetic engineering project (47), because at least 17 genes are involved in nitrogen fixation. We still do not know if manipulating so many genes will be possible. Besides, transfer of these genes into non-nitrogen-fixing plants will certainly demand energy from the host species and hence reduce yield.

Streamlining breeding methods

In several horticultural and forest species, clonal propagation has been achieved. Tissue culture is most valuable in highly heterozygous species with longer life cycle and low multiplication rate, i.e., those difficult to improve conventionally. Mass propagation of coconut, oil palm, pineapple, rubber tree, teak tree, Douglas Fir, loblolly pine, desirable apple root stocks, hybrid carrot, and parental lines of asparagus should be a priority. In such forest trees as teak,

where the tree can be judged only after 50-100 yr, clonal propagation from elite trees would be highly desirable.

In some dicotyledonous species, such as pigeon peas, culturing of cotyledons can induce flowering. Flowers are small but complete, and they produce viable pollen grains and fertile ovaries for making crosses. Similar success in tree crop species could shorten the 15-25 yr normally required to produce hybrids to a few months.

Cryopreservation, particularly for asexual species difficult to maintain by seed, will, when the problem of regeneration ability loss is solved, greatly streamline breeding techniques. Cryopreservation will also help preserve rare genotypes, control genetic erosion, and increase the genetic variability available to breeders through international exchange of healthy materials.

CONCLUSIONS

During the past eight decades plant breeders have developed improved germplasm with high yield. yield stability, good quality, and genetic resistance to various biological and environmental stresses for various plant species.

The past two decades have unveiled biotechniques (e.g., cell and tissue culture, protoplast fusion, genetic engineering) that could improve plants even more, but plant breeders have used them little for these reasons:

- Most of the in vitro techniques are still experimental and genotype specific.
- Manipulation of polygenes, which control most economically importact traits, has not been clearly demonstrated.
- There is a wide communication gap between plant breeders and the molecular geneticists and plant physiologists who develop these techniques.

Philips (35) suggested that emerging biotechnologies would not begin to increase US maize yields before 1990 and would not have a major impact until 2000. The impact of conventional plant breeding, however, should persist. World population will likely double in the next 40 yr, though, and conventional plant breeding will be insufficient to meet that demand. Conventional methods need help from biotechnology in

- breeding for increased biomass and, hence, yield;
- breeding for protein quantity and quality;
- selection of disease-resistant genotypes at plant and cellular level under pressure of pathogen-produced toxins;
- breeding for genotypes immune to certain pathogens through hybridization among sexually incompatible species;
- positive and expeditious identification of bacterial, viral, and fungal strains using monoclonal. antibodies;
- selection for tolerance for soil salinity and aluminum toxicity;

- increasing genetic variability and inducing novel variability by producing cybrids;
- incorporating nitrogen fixation (Nif) genes into cereals;
- clonal propagation of horticultural and forest tree species and some vegetatively propagated crop plants;
- in vitro induction of flowering, and making hybrids for breeding tree crops with a long life cycle; and
- preserving germplasm of asexually reproducing species.

REFERENCES CITED

1. Austin, R.B., J. Bingham, R.D. Blackwell, L.T. Evans, M.A. Ford, C.L. Morgan, and M. Taylor. 1980. Genetic improvement in winter wheat yields since 1900 and associated physiological changes. J. Agric. Sci. 94:675-689.
2. Barton, K.A., and W.J. Brill. 1983. Prospects in plant genetic engineering. Science 219:671-675.
3. Behnke, M. 1979. Selection of potato callus for resistance to culture filtrates of Phytophthora infestans and regeneration of resistant plants. Theor. Appl. Genet. 55:69-71.
4. Belliard, G., F,. Vedel, and G. Pelletier. 1979. Mitochondrial recombination in cytoplasmic hybrids of Nicotiana tabacum by protoplast fusion. Nature 281:401-403.
5. Brettle, R.I.S., and D.S. Ingram. 1979. Tissue culture in the production of novel disease-resistant crop plants. Biol. Rev. 54:329-345..
6. Brettle, R.I.S., and E. Thomas. 1980. Reversion of Texas male-sterile cytoplasm maize in culture to gene fertile, T-toxin resistant plants. Theor. Appl. Genet. 58:55-58.
7. Carlson, P.M. 1973. Methionine sulfoximine-resistant mutants of tobacco. Science 180:1366-1368.
8. Cassey, H.E. 1972. Arid Lands Resource Inf. Paper No. 1, Univ. Arizona Tucson, Arizona, USA. (cf. Yoshida and Ogawa, 1983).
9. Choo, T.M., B.R. Christic, and E. Reinbergs. 1979. Doubled haploids for estimating genetic variances and scheme for population improvement in self-pollinating crops. Theor. Appl. Genet. 54:267-271.
10. Croughan, T.P., S.J. Stavarek, and D.W. Rains. 1981. In vitro development of salt resistant plants. Environmental Exp. Rot. 21(314):317-324.
11. Duncan, W.G., D.E. McCloud, R.L. McGraw, and K.J. Boote. 1978. Physiological aspects of peanut yield improvement. Crop. Sci. 18:1015-1020.
12. Duvick, D.N. 1983. Genetic engineering in plants. An agricultural prospective. Round table discussion on research and priorities. Pages 482-484 in Genetic engineering of plants. An agricultural perspective. T.

Kosuge, C.P. Meredith, and A. Hollaender, eds. Plenum Press, New York.

13. Earle, E.D. 1981. Application of plant tissue culture in the study of host-pathogen interactions. Pages 45 – 62 in Application of plant cell and tissue culture to agriculture and industry. D.T. Tomes, B.E. Ellis, P.M. Harvey, K.J. Kasha, and R.L. Peterson, eds. Univ. Guelph Press, Canada.
14. Evans, L.T. 1983. Raising the yield potential: by selection or design. Pages 371 – 389 in Genetic engineering of plants and agricultural perspective. T. Kosuge, C.P. Meredith, and A. Hollaender, eds. Plenum Press, New York.
15. Evans, D.A. 1983. Agricultural applications of plant protoplast fusion. Bio-technology 1(3):253 – 261.
16. Frey, K.J. 1971. Improving crop yields through plant breeding. Pages 15 – 33 in Moving off the yield plateau. J.D. Eastin and R.D. Munson, eds. American Society of Agronomy, Madison, Wisconsin.
17. Frey, K.J. 1981. Capabilities and limitations of conventional plant breeding. In Genetic engineering for crop improvement. K.O. Rachie and J.M. Lyman, eds. RockeFeller Foundation, New York
18. Frey, K.J. 1983. Genes from wild species for improving cereal production. Pages 119 – 120 in XV International Congress of Genetics, New Delhi, India. Dec. 12 – 21, 1983 Symposia Session: Newer approaches to plant genetics and breeding. (Abstr.).
19. Gengenback, B.C., C.E. Green, and C.M. Donovan. 1977. Inheritance of selected pathotoxin resistance in maize plants regenerated from cell cultures. Proc. Natl. Acad. Sci. USA 74:5113 – 5117.
20. Gifford, R.M., and L.T. Evans. 1981. Photosynthesis, carbon partitioning and yield. Ann. Rev. Plant Physiol. 32:485.
21. Gracen, V.E., M.L. Forster, K.D. Sayre, and C.O. Grogan. 1971. Rapid method for selecting resistant plants for control of southern corn leaf blight. Plant Dis. Rep. 55:469 – 470.
22. Heinz, D.J., M. Krishnamurthi, L.G. Nickell, and A. Maretzki. 1977. 1. Cell, tissue and organ culture in sugarcane improvement. Pages 3 – 17 in Applied and fundamental aspects of plant cell, tissue, and organ culture. J. Reinert and Y.P.S. Bajaj, eds. Springer-Verlag, Berlin.
23. Jinks, J.L., P.D.S. Caligari, and N.R. Ingram. 1981. Gene transfer in *Nicotiana rustica* using irradiated pollen. Nature 291:586 – 588.
24. Kasha, K.J., and E. Reinberg. 1981. Recent developments in the production and utilization of haploids in barley. In Proceedings fourth int. barley genetics symposium, Edinburg.
25. Kohler, G., and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of predefined

specificity. Nature (London) 256:495-497.

26. Kosuge, T., C.P. Meredith, and A. Hollaender. 1983. Genetic engineering of plants. An agricultural perspective. Plenum Press, New York. 499 p.
27. Liu, M.C. 1981. In vitro methods applied to sugarcane improvement. Pages 299-323 in Plant tissue culture. T.A. Thorpe, ed. Academic Press, New York.
28. Massound, F.I. 1974. Salinity and alkalinity as soil degradation hazards. FAO/UNDP Expert Consultation on Soil Degradation, F.A.O. Rome (of. Yoshida and Ogawa, 1983).
29. Meredith, C.P. 1978. Response of culture tomato cells to aluminum. Plant Sci. Lett. 12:17-24.
30. Meredith, C.P. 1978. Selection and characterization of aluminum-resistant variants from tomato cell cultures. Plant Sci. Lett. 12:25-34.
31. Murai, N., D.W. Sutton, M.G. Murray, J.L. Slightom, D.N. Merlo, N.A. Reichert, C. Sengupta-Gopalan, C.A. Stock, R.F. Barker, J.D. Kemp, and T.C. Hall. 1983. Phaseolin gene from bean expressed after transfer to sunflower via tumor inducing plasmid vectors. Science 222:475-481.
32. Nabors, M.W., A. Daniels, L. Nadolny, and C. Brown. 1975. Sodium chloride tolerant lines of tobacco cells. Plant Sci. Lett. 4:155-159.
33. Nabors, M.W., S.E. Gibbs, C.S. Bernstein, and M.E. Meis. 1980. NaC1-tolerant tobacco plants from cultured ce1l.s. Z. Pflanzenphysiol. 97:13-17.
34. Ojima, K., Y. Umino, H. Takashima, and K. Ohira. 1982. Selection and its redifferentiation of culture cells resistant to aluminum tolerance in carrot. Abstract of 1982 Meeting, Japanese Society of Soil Science and Plant Nutrition 28:70.
35. Philips, R.L. 1983. Genetic engineering of plants: some perspectives on the conferences, the present, and the future. Pages 453-465 in Genetic engineering of plants. An agricultural perspecitve. T. Kosuge, C.P. Meredith, and A. Hollaender, eds. Plenum Press, New York.
36. Rains, D.W., T.P. Croughan, and S.J. Stavarek. 1980. Selection of salt-tolerant plants using tissue culture. Pages 279-292 in Genetic engineering of osmoregulation: impact on plant productivity for food, chemicals, and energy. D.W. Rains, R.C. Valentine, and A. Hollaender, eds. Plenum Press, New York.
37. Sacristan, M.D. 1982. Resistance responses to Phoma lingam of plants regenerated from selected cell and embryonic cultures of haploid Brassica napus. Theor. Appl. Genet. 61:193-200.
38. Shepard, J.F., D. Bindey, and E. Shahin. 1980. Potato protoplasts in crop improvement. Science 208:17-24.
39. Simmonds, N.W. 1976. Evolution of crop plants. Longman, London. 339 p.

40. Simmonds, N.W. 1981. Genotype (G) Environment (E) and GE components of crop yields. Exp. Agric. 17:355-362.
41. Simpson, E., and J.E. Snape. 1981. The use of doubled haploids in a winter barley program. Proceedings of Fourth International Barley Genetics Symposium. Edinburgh.
42. Sinha, S.K., P.K. Aggarwal, G.S. Chaturvedi, K.R. Konndal, and R.A. Khanna Chopra. 1981, Comparison of physiological and yield characters in old and new wheat varieties. J. Agric. Sci., Cambridge Univ. Press.
43. Snape, J.W. 1981. The use of doubled haploids in plant breeding. In Induced variability in plant breeding. Centre for Agricultural Publishing and Documentation, Wageningen.
44. Snape, J.W., and E. Simpson. 1981. The genetical expectations of doubled haploid lines derived from different filial generations. Theor. Appl. Genet. 60:123-218.
45. Snape, J.W., and E. Simpson. 1981. The use of doubled haploid lines for genetical analysis in barley. Proceedings Fourth International Barley Genetics Symposium, Edinburgh.
46. Sneep, J., A.J.T. Hendriksen, and O. Holbek. 1979. Plant breeding perspectives. Centre for Agricultural Publishing and Documentation, Wageningen. 435 p.
47. Swaminathan, M.S. 1982. Biotechnology research and Third World agriculture. Science 218:967-972.
48. Sybenga, J. 1983. Genetic manipulations in plant breeding: somatic versus generative. Theor. Appl. Genet. 66:179-201.
49. Van Dobben, W.H. 1962. Influence of temperature and light conditions on dry matter distribution, development rate, and yield in arable crops. Neth. J. Agric. Sci. 10:377.
50. Wheeler, H.E., and H.H. Luke. 1955. Mass screening for disease-resistant mutants in oats. Science 122:1229.
51. Yoder, O.C. 1981. Asay. Pages 45-78 in Toxins in plant disease. R.D. Durbin, ed. Academic Press, New York.
52. Yoder, O.C. 1983. Use of pathogen produced toxins in genetic engineering of plants and pathogens. Pages 335-353 in Genetic engineering of plants. An agricultural perspective. T. Kosuge, C.P. Meredith, and A. Hollaender, eds. Plenum Press, New York.
53. Yoshida, S., and M. Ogawa. 1983. The application of tissue culture-induced mutagenesis to crop improvement. Food Fert. Techno1 Cent. Tech. Bull. 73.

ANTHER CULTURE IN CROP PLANTS

G. WENZEL, B. FOROUG-HI-WEHR, W. FRIEDT, and F. KÖHLER

Cell and tissue culture techniques are no longer a monopoly of research laboratories: they have penetrated breeding institutes and private companies. Using haploids in potato, rape seed, wheat, and barley breeding programs is routine. Our results with these crops, especially those on resistance to viruses and fungi, are outlined. Particular emphasis is given to potato viruses and to barley yellow mosaic virus. Results from incorporating a haploid step into other important species are summarized. From isolated microspores in a liquid culture medium conditioned by ovaries, our regeneration rate of functional barley plants was high enough to use this system for selection. In our first attempt we applied fusaric acid (an unspecific toxin of several Fusarium species) as selective agent. Callus surviving on 0.2mM fusaric acid could be selected. In this system microspore culture combines the advantages of a single-cell system with those of haploidy. The influence of an unspecific selection during anther culture on the characters of androgenetic offspring is discussed.

A major reward in cell culture work with plants, not present in that with animals, is that a functional plant can be regenerated from a single cell and this plant can be haploid, which is very interesting for breeding programs. As early as 1922 Blabeslee et a1 (2) suggested using haploids, but sufficient numbers were unavailable for practical progress. Today haploids can be produced parthenogenetically or androgenetically. The parthenogenetic approach is older and has already been successfully applied (18). In maize, haploid seeds can be selected through the use of color markers. Haploids can be produced (11, 18) using the ig gene in maize or the hap gene in barley. With such haploids the Hordeum bulbosum chromosome elimination technique used on Hordeum vulgare has made possible substantial progress in barley breeding (13). The cultivar Mingo was produced by this technique.

Federal Biological Research Centre for Agriculture and Forestry, D-8059 Gruenbach, Federal Republic of Germany.

Since its discovery by Guha and Maheshwari (9), androgenesis has prompted trials to understand its process physiologically, to find ways to control its induction, and to use it in crop breeding.

Cell and tissue culture is expanding beyond model plants in Iaboratories to crops in the fields. Genetic engineering, too, is expanding rapidly, so that tissue culture approaches are already considered old-fashioned. Scientific and financial decisions are up to the breeders. This means haploid techniques may be overtaken by newer approaches before their potential has been realized. This paper aims to demonstrate their value, for one in the increased efficiency during recombination and selection. Table 1 summarizes economically important crops for which androgenetic haploidization works.

METHODS AND RESULTS

The world's most important crops are the cereals; and we have concentrated on barley, rye, and wheat (24). They are an unwieldy group in biotechnological methods such as protoplast work, so we also concentrate on potato and rape seed, where most cell and tissue culture techniques do work. This permits a combined breeding scheme incorporating haploid, protoplast, and classical techniques.

Secale cereale

Under optimal preculture conditions, up to 10% of the plated rye anthers develop macroscopic structures on a modified N6 medium (3). Of these, however, less than 10% form plantlets (Table Z), most of them albino. From more than 2 million anthers we regenerated a total of about 300 green lines, only a third of which were vigorous enough for field tests. The best responsive genotype is still an F_1 hybrid between Secale cereale and S. vavilovii.

Anther culture produced self-compatible inbred lines when initiated from heterozygous self-compatible F_1 anther donors.

Table 1. First report of important haploid crop plant regeneration using anther culture.

Species	Year	Reference
Avena sativa	1983	(20)
Brassica campestris	1975	(14)
Brassica napus	1975	(22)
Hordeum vulgare	1971	(4)
Oryza sativa	1968	(17)
Pisum sativum	1972	(10)
Secale cereale	1974	(26)
Solanum tuberosum (4x)	1973	(5)
Solanum tuberosum (2x)	1977	(7)
Triticale	1973	(27)
Triticum aestivum	1973	(19)
Zea mays	1975	(1)

The unimpressive quality of most of them probably was attributable to their close relation to the wild species S. vavilovii and not to their inbred nature. In addition to their direct hybridization, five of the androgenetic lines were tested for combining ability by H.H. Geiger, Hohenheim. The topcross progeny of doubled-haploid lines can yield as high as the best selected inbreds. Because the anther-derived lines lodged severely, their average performance at three locations, measured by a general breeding index, was not comparable to that of conventionally selected inbred lines (8).

Hordeum vulgare

Experiments were started with spring barley varieties and hybrids (Table 2). Because of the increasing importance of winter barley hybrids and varieties, we concentrated on them (Table 2). In northern Europe, they have been frequently damaged by the soil-borne barley yellow mosaic virus (BaYMV) in recent years.

Table 2. Regeneration frequencies in anther culture of crop plants.

Donor	Number of				%
	Anthers plated	Regenerants			
		Albino	Green	Total	
Spring wheat					
Atys 2	7 970	2	21	23	0.3
Atys 3	8 485	2	16	18	0.2
Orofen	8 691	4	2	6	0.02
Winter barley					
Franka	4 488	1	3	4	0.07
Igri	9 138	292	220	512	2.4
Franka x Igri	12 807	248	175	432	1.4
Spring barley					
Trurnpf x VM 260	7 863	236	34	269	0.4
Trurnpf x Villame	3 987	71	12	83	0.3
Trurnpf x 9541/2916	4 254	115	14	129	0.3
Winter rye					
Hellkorn x S. vavil.	15 141	63	110	173	0.7
L 281 x L 282	15 966	6	2	8	0.01
L 282 x 4470	4 950	–	–	–	0.0
Potato (2n)					
H 78.01127	3 765	–	2 564	2 564	68.1
H 80.108818	1 682	–	149	149	8.8
H 81.100114	2 107	–	41	41	1.9
Potato (4n)					
632.07	300	–	4	4	1.3
77/7	300	–	8	8	2.7
22/71	300	–	4	4	1.3
Rape seed					
KWS 24/72	48 222	–	43	43	0.1
R 500 x Torch (15)	1 068	–	535	535	50.1
Tower (16)	240	–	386	386	160.8

So we started a resistance breeding program using haploids. Because the virus cannot be controlled by chemicals, yield losses (up to 100%) can be prevented only by growing resistant cultivars.

Through anther culture, more than 500 androgenetic lines were recovered from crosses of the resistant cultivar "Franka." Of 292 A-lines tested, 65% proved BaYMV resistant. This demonstrates that a recessive character such as BaYMV resistance can efficiently and rapidly be combined with other favorable traits through a haploid step (6). Table 3 shows that segregation for resistance in the anther-derived progeny is approximately 1:1 (resistant:susceptible), that in the F_2 is normal (1:3).

Data on more than 5 years' evaluation of anther-derived spring barley material show that most doubled-haploid lines, in such characters as grain yield and thousand kernels, are less valuable than the midparent of both parents. This can be explained by a linkage disequilibrium for each character studied. There seems to be excessive repulsion linkage in some crosses and coupling linkage in others (6), which can influence even the best starting material. If linkage groups should be kept together, and if few recombinations are desired, as in crosses between adapted varieties, one should start with F_1 material. If, however, many recombinations are necessary to break repulsion linkage, the starting material should be no earlier than F_3.

Triticum aestivum

Our recently started wheat program aims to accelerate incorporation of fungal resistance into breeding lines. We could repeat the French results (19). The regeneration rates are given in Table 2. Two findings were encouraging:

- Wheat microspores produce hardly any albino plants.
- More than 90% of the regenerants are stably haploid, making further confirmation of the homozygous nature of spontaneously doubled material unnecessary.

And our best barley anther culture media works well for wheat anther ,culture, so both procedures can use the same medium, a strong advantage, especially in an applied program.

Table 3. BaYMV reactions of segregating F_2 plants and corresponding doubled haploid lines (DH).[a]

Cross		Lines (no.)		
		Total	Susceptible	Resistant
Franka x	F_2	128	100	28
LP 8.34218	DH	64	19	45
Franka x	F_2	129	95	34
Igri	DH	129	53	76

[a] Expectations: 3:1 for F_2, 1:1 for DH.

Solanum tuberosum

Potato cultivars differ from cereals in that they are autotetraploid. This means their ploidy level can be reduced two times. The first reduction from 4x results in dihaploids (2n=2x =24). These are predominantly produced parthenogenetically by crossing with special clones of the primitive species Solanum phureja. The efficiency reaches 46% (25). As this frequency can hardly be exceeded by anther culture, we restrict our microspore experiments to extracting monohaploids (2n=x=12) from dihaploids. We produced nearly 6,000 primary dihaploid clones and started to select for fertility and for interdihaploid production. As much as 68% of the plated anthers from these dihaploids and interdihaploids formed plantlets (Table 2).

Transferring plants from sterile culture into the greenhouse could be simplified by inducing tuber formation in the petri dish, which occurs spontaneously in some clones and requires higher cytokinin concentrations in others (23). Such tubers could be stored and used later to form vigorous plants.

Stepwise reduction of ploidy to simplify inheritance is generally accepted for qualitatively inherited characters. We concentrate on potato clones carrying extreme resistance to potato virus X or potato virus Y or both, and field resistance to potato leaf roll virus. Clone testing for resistance after ploidy reduction showed extremely resistant clones that were hypersensitive and clones that were field resistant only and were susceptible after grafting on infected tomato stocks. We also found resistance-type changes: primarily field-resistant clones became extremely resistant; extremely resistant ones showed only field resistance (Table 4). This means that qualitatively and quantitatively inherited resistances (and probably other characters) are kept during haploidization. Having passed the haploid level, a quantitative resistance can become homozygous and, like a qualitative trait, can be transferred, at least to the next generation.

Another useful point is that haploids give more information about the starting material. Especially in such aspects as parent selection for crosses, haploids reveal characters invisible in the parents. Furthermore, in a haploid step, genomes are purified from sublethal alleles, as these either

Table 4. Shifts of resistance types[a] during haploidization in 2x potato.

	PVX[b]				PVY[c]				PLRV[d]
	ER		FR		ER		FR		FR
Donor clones (no.)	7		11		5		11		7
	ER	FR	ER	FR	ER	FR	ER	FR	FR
2x clones (no.)	1	1	1	1	2	1	1	2	5

[a]ER = extreme resistance, FR = field resistance. [b]PVX = potato virus X. [c]PVY = potato virus Y. [d]PLRV = potato leaf roll virus.

segregate to wild type or to lethal type. Consequently, despite their being inbreeding, doubled haploids are vigorous.

The homozygotes permit breeders to propagate potato using true seeds and to produce hybrid seed.

Regenerating protoplasts (from dihaploid potatoes) and fusion products expands synthetic breeding possibilities (25). Parthenogenesis yields sufficient dihaploids for anther culture and subsequent doubling to make homozygous dihaploids. Classical breeding combines these into hybrids from which protoplasts are isolated and fused. The final product is a heterozygous tetraploid potato, in which characters of several starting clones are pooled. As potato can be propagated vegetatively, this rapidly made fusion product can become a new variety.

Brassica napus

One of the most striking aspects of rape seed anther culture, the regeneration rates of which are given in Table 2, is strong selection. When we screened microspore genotypes for glucosinolate content, the androgenetic offspring behaved peculiarly: most plantlets contained much more glucosinolate than the starting material. Only 6 lines contained less than 10 µM/g seed and these were from stable haploids. High glucosinolate, then, favors spontaneous doubling, an in vitro selection process that might be used for screening low-glucosinolate plants (12). The result indicates that strong selection exists in microspore culture. Often the selection goes in the direction wished by the breeder, but this cannot be guaranteed.

Isolated microspore culture

Selection within microspore populations will be useful in breeding programs when it can be controlled. One prerequisite for this is a technique to separate and subsequently regenerate selected microspores. In dicots isolated microspore culture is difficult, as the exine is thick and microspore development cannot be monitored without fixation. Cereal microspores, with their transparent exine, are an easier system. In rye some anther-isolated microspores start to develop in simple media, but stop at the 8- to 16-cell. stages. In barley, regeneration could be enhanced by conditioning the culture media either with anthers (21), or with ovaries. In media conditioned with 10 ovaries/ml for 7 days, the 40 anthers plated released about 50 000 microspores. Of the total microspores 0.2% developed into microcalluses and 0.06% into visible calluses. From these, however, plants regenerated only occasionally.

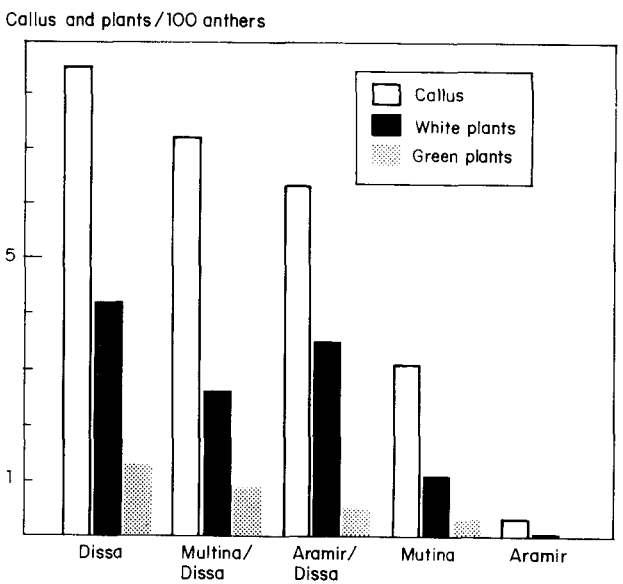
We are trying to identify the conditioning factor, and as we know it is not unique to spring barley, but also helps in winter barley, its benefit in other cereals is being explored. With this system microspores regenerate so well that we could start to select for such desirable traits as toxin resistance. In a first trial fusaric acid was applied at 0.1 mM. Four of every 1 000 calluses were resistant. We are trying to regenerate surviving structures.

DISCUSSION

One general problem in applying cell culture to breeding programs is its specificity. Only some genotypes respond satisfactorily in vitro. Two principal approaches can overcome this problem: 1) look for better media, or 2) genetically combine responsive genotypes. Responsive genotypes can be combined through breeding and selection, a generally accepted strategy, particularly in potato and barley, in which the mode of inheritance is not too complicated (Fig. 1). We always start our tissue culture work by testing the line for regeneration capacity. If it has none, we combine the line or clone with a genotype that has, preferably as homozygous. An additional problem in cereals is albino formation. The frequency of green or albino plantlets varies with the genotype (6). In potato even regeneration by embryo or organogenesis depends on the genotype. All these genes must be considered.

Cell regeneration, in general, is linked to internal phytohormones. Genotypes that do not require a specific phytohormone combination, or that are flexible, plastic clones or lines, respond more often to an unspecified external phytohormone combination in the medium. These genotypes may also be the most vigorous, and therefore interesting for application. Here, in vitro selection and breeding have the same aim. On the other hand, regeneration of all microspore types might be desired. The first (physiological) approach also cannot guarantee this, as developing an optimal medium for each microspore genotype is probably impossible.

Physiology, genotype, and the environment during preculture and culture dramatically influence haploid plantlet regeneration. Basic research on these influences is badly needed. Biochemical



1. Influence of variety and hybrid genotype in barley anther culture (24).

regulation of the higher plant must be understood for successful gene technology. Genetic engineering may enhance cell and tissue culture, but it may be expensive. Crop plants have been improved by haploids, especially during the last 3 yr. In vitro techniques have been simplified enough that breeders routinely use haploids and will increasingly do so. Tissue culture techniques are affordable. It would be wasteful to prematurely divert funds away from them to the perhaps too speculative gene technology.

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REFERENCES CITED

1. Anonymous. 1975. Primary study on induction of pollen plants of Zea mays. Acta Gen. Sin. 2:142-143.
2. Blakeslee, A.F., J. Belling, M.E. Franham, and A.D. Bergner. 1922. A haploid mutant in the Jimson weed, Datura stramonium. Science 55:646-647.
3. Chu, Ch.-Ch. 1978. The N6 medium and its application to anther culture of cereal crops. Pages 43-50 in Proceedings of symposium on plant tissue culture. Science Press, Peking.
4. Clapham, D. 1971. In vitro development of callus from the pollen of Lolium and Hordeum. Z. Pflanzenzuechtg. 65:285-292.
5. Dunwell, J.M., and N. Sunderland. 1973. Anther culture of Solanum tuberosum L. Euphytica 22:317-323.
6. Foroughi-Wehr, B., and W. Friedt. 1984. Rapid production of recombinant barley yellow mosaic virus resistant Hordeum vulgare lines by anther culture. Theor. Appl. Genet. 67:377-382.
7. Foroughi-Wehr, B., H.M. Wilson, G. Mix, and H. Gaul. 1977. Monohaploid plants from anthers of a dihaploid genotype of Solanum tuberosum L. Euphytica 26:361-367.
8. Friedt, W., B. Foroughi-Wehr, V. Lind, S. Zuechner, H. Walther, and G. Wenzel. 1983. The value of inbreeding lines derived from Secale cereale x S. vavilovii via classical inbreeding and androgenetic haploids. Z. Pflanzenzuechtg. 91:89-103.
9. Guha, S., and S.C. Maheshwari. 1964. In vitro production of embryos from anthers of Datura. Nature 204:497.
10. Gupta, S., K.K. Gh0osal, and V.N. Gadgil. 1972. Haploid tissue culture of Triticum aestivum var. Sonalika and Pisum sativum var. B 22. Indian Agric. 16:277-278.
11. Hagberg, A., and G. Hagberg. 1980. High frequency of spontaneous haploids in the progeny of an induced mutation in barley. Hereditas 93:341-343.
12. Hoffman, F., E. Thomas, and G. Wenzel. 1982. Anther culture as a breeding tool in rape. II. Progeny

analysis and induced mutants from haploid cultures. Theor. Appl. Genet. 61:225-232.

13. Kasha, K.S., and E. Reinbergs. 1980. Achievements with haploids in barley research and breeding. Pages 215-230 in The Plant genome. D.R. Davies and D.A. Hopwood, eds. John Innes Charity, Norwich.
14. Keller, W.A., K.C. Armstrong, and A.J. de la Roche. l983. The production and utilization of microspore derived haploids in Brassica crops. Pages 169-183 in Plant cell culture in crop improvement. S.K. Sen and K.L. Giles, eds. Plenum Press, New York.
15. Keller, W.A., T. Rajhathy, and J. Lacapra. 1975. In vitro production of plants from pollen in Brassica campestris. Can. J. Genet. Cytol. 17:655-666.
16. Lichter, R. 1982. Induction of haploid plants from isolated pollen of Brassica napus. Z. Pflanzenphysiol. 105:427-434.
17. Niizeki, H., and K. Oono. 1968. Induction of haploid rice plant from anther culture. Proc. Jpn. Acad. 44:554-557.
18. Nitzsche, W., and G. Wenzel. 1977. Haploids in plant breeding. Paul Parey, Berlin and Hamburg.
19. Picard, E., and J. De Buyser. 1973. Obtention de plantules haploides de Triticum aestivum L. a partir de culture d antheres in vitro. C.R. Acad. Sci. Paris 277D31463-1466.
20. Rines, H.W. 1983. Oat anther culture - genotype effects on callus initiation and the production of a haploid plant. Crop. Sci. 23:268-272.
21. Sunderland, N., and Z.H. Xu. 1982. Shed pollen culture in Hordeum vulgare. J. Exp. Bot. 33:1086-1095.
22. Thomas, E., and G. Wenzel. 1975. Embryogenesis from microspores of Brassica napus. Z. Pflanzenzuechtg. 74:77-81.
23. Wenzel, G. 1980. Anther culture and its role in plant breeding. Pages 68-78 in Plant tissue culture genetic manipulation and somatic hybridization of plant cells. P.S. Rao, M.B. Heble, and M.S. Chadha, eds. BARC, Bombay.
24. Wenzel, G., and B. Foroughi-Wehr. 1984. Anther culture of cereals and grasses. Pages 311-325 in Cell culture and somatic cell genetics. I. I.K. Vasil, ed. Academic Press, New York.
25. Wenzel, G., and B. Foroughi-Wehr. 1984. Anther culture of Solanum tuberosum. Pages 293-301 in Cell culture and somatic cell genetics of plants. I. I.K. Vasil, ed. Academic Press, New York.
26. Wenzel, G., and E. Thomas. 1974. Observation on the growth in culture of anthers of Secale cereale. Z. Pflanzenzuechtg. 74:77-81.
27. Ying, Y.W., S.C. San, W.C. Chu, and C.N. Feu. 1973. The induction of pollen plantlets of Triticale and Capsicum annuum from anther culture. Sci. Sin. 16:147-151.

USE OF HAPLOIDS IN CROP IMPROVEMENT

HU HAN

Since 1921, when Bergner discovered haploid plants in Datura stamonium scientists have developed many techniques for inducing haploidy. Induction methods in vitro include anther (pollen) culture, unpollinated ovary culture, and chromosome elimination, of which anther culture is the simplest and most efficient. This technique has been refined and its use in fundamental and applied genetics and in crop improvement is extensive. To increase the induction frequency of green pollen plants, various factors influencing anther culture were investigated. Genetic and cytogenetic theories of wheat, maize, and rice pollen plants were also examined. These include plant-regenerating ability, genetic stability and variability of pollen plants, and plant-level gamete analysis. Chinese scientists have extensively used anther culture in plant breeding: many new varieties of rice, wheat, and maize inbred lines of pollen plant's have been released.

Since A.D. Bergner discovered haploid plants in Datura stramonium in 1921, many techniques for inducing haploidy have been developed. These are mainly classified as in vivo and in vitro. In vivo techniques include spontaneously occurring haploids from polyembryony, pseudogamy, semigamy, and androgenesis. In vitro methods include anther (pollen) culture, unpollinated ovary culture, and chromosome elimination (the bulbosum method). In vitro anther culture induction is the most simple and efficient. Haploids have been induced by anther or pollen culture in 247 species from 88 genera and 34 families (5).

During the last 10 yr, much progress has been made in anther culture for crop improvement. To increase the induction frequency of green pollen plants, the response of anther in culture was systematically investigated. Theoretical aspects of genetics and cytogenetics of wheat, maize, and rice were explored. In China many new varieties and strains from rice, wheat, and maize inbred

Institute of Genetics, Academia Sinica, Beijing, China.

lines of pollen plants have been developed and released. French and American strains of wheat pollen plants have also been evaluated.

FACTORS INFLUENCING ANTHERS IS CULTURE

The induction frequency of green pollen plants has been steadily increasing. The following factors were found important in inducing pollen plantlets: 1) genotype of donor plant, 2) stage of microspore development, 3) culture medium, and 4) physical conditions.

Genotype of donor plant

Recently Ouyang et a1 (7) found that the induction frequencies of pollen callus and green pollen plantlets were heritable characteristics, quantitatively controlled by many genes. The induction frequency of hybrid vigor only existed in heterozygous F_1, indicating that the expression of this characteristic is determined by genotype of the anther wall, not of the pollen.

Stage of microspore development

Although the pollen-derived calluses could be induced in cultured anthers inoculated at different stages from pollen mother cell to mature pollen, usually only the anthers containing miduninucleate to late-uninucleate microspores were inoculated. At these stages the callus induction frequencies were highest.

Culture medium

The culture medium is the principal factor controlling the induction and development of intact plantlets.

Raising sucrose concentration. Sucrose may not only regulate the osmotic pressure of the medium, but also may be the best carbohydrate source. Nine percent sucrose is optimum for anther culture of wheat; 12%, for maize, rapeseed, and barley.

New media. Several culture media developed by Chinese scientists are now used widely in China and abroad. Potato-IT medium contains 10% aqueous potato extract, ½ macroelements of W4 medium, and the Fe-salt and thiamine levels of MS medium. The frequency of callus induction was much higher in this medium than in others. N6 medium has a low ammonium ion concentration and the optimal ratio of ammonium nitrogen to nitrate nitrogen for efficient anther culture of rice and other cereals.

Physical conditions

Physical factors of culture, e.g. temperature, markedly affect the dedifferentiation of pollen, and influence the induction frequency of pollen callus and embryoids.

Pretreatment temperature. A cold treatment before inoculation increases callus induction frequency in rice, wheat, and other cereals.

Incubation temperature. When incubation temperature varied, callus induction of wheat and rapeseed increased. When wheat anthers were cultured at 33°C for some days and then transferred to 24°C, not only the callus induction frequency increased, but also the frequency of differentiating green plantlets increased significantly.

Float culture of anthers. Liquid media have resulted in better anther culture of some species, including tobacco, *Datura*, wheat, and barley. Float culture was also used to increase the induction frequency of rice. But the callus differentiation frequency was far lower than with a solid medium (agar). The best callus differentiation was obtained with the potato medium sterilized by filtration. Differentiation frequency, of green plantlets reached 50% (9).

At present, induction frequencies of green plantlets are more than 10% (of anther number) for *Oryza sativa* L. subsp. Keng. In our laboratory, the frequencies averaged 5% and reached more than 10% in most wheat and triticale strains tested.

POLLEN PLANTS FOR CROP IMPROVEMENT

To use pollen haploids to improve crops, the genetic characteristics of anther culture and haploidy, i.e. pollen plants, must be understood. These include genetic stability and variability of pollen plants and gamete expression in pollen-derived plants.

Stability

Unselected populations of pollen plants in wheat, rice, maize, and tobacco over a period of several years had genetic uniformity in about 907 of their dihaploid lines (2). Stable, homozygous diploid strains can be developed through chromosome .doubling of haploids. Honozygous dihaploids may be obtained in one generation by anther culture, which markedly improves the selection efficiency in breeding, and shortens the breeding cycle.

Variability

Cultured cells of both animal and plant tissues are unstable in chromosome number and structure, a phenomenon common also to pollen-derived plants. The instability or variability includes gene (locus) mutations and chromosome variations and expresses mianly in ploidy level and fertility.

Production of aneuploids and heteroploids of common wheat (*T. aestivum*). In general, nullisomic monosomic, trisomic, and tetrasomic plants originate from haploids or from the progeny of hybrids between plants of different ploidy levels, or from physical or chemical treatments (3). Pentaploids, octoploids, and mixoploids of wheat are obtained through anther culture, as are about 90% of haploids and homozygous diploids (2).

Anther culture aneuploid production was further verified from 1981 to 1983, using spring wheat Orofen. The root tip cells

of 472 pollen-derived plants and PMCs of some plant lines were cytologically examined and identified. These plants can be classified into four types: euploids, aneuploids, heteroploids, and mixoploids (Table 1). Among 472 pollen-derived plants, euploids comprised 85.8%; aneuploids, 9.3%; heteroploids, 1.7%; and mixoploids, 3.2%. Eighteen telocentrics and enuploids with chromosome aberrations were obtained, 3.8% of the total. One stable 1B long arm deletion strain was identified.

Chromosome variants may, then, be produced without hybridization. These aneuploids, especially telocentrics and chromosome aberrations, are very useful for genetic studies.

Variability in culture. Oono (6) reports that pollen-derived rice plants sometimes show homozygous and sometimes heterozygous mutations. If all the variation preexisted in the microspores (pollen) all mutants would be homozygous. The heterozygous mutants must originate from events following chromosome doubling. Riley and Kimer (8) estimated the frequency of aneuploids in four varieties of bread wheat as 1.08% and in one cultivated oat 1.27%. Because the frequency of aneuploidy from a variety without tissue culture is much less than that from pollen-derived plants, the observed variation must have occurred during the culture phase.

Variant frequency seems to increase with increased culture duration. In our previous work (l), we used two regenerated Hl pollen plants from the same callus, but with different redifferentiation time. Table 2 shows that the frequency of chromosome doubling of plant no. 72-1-11-1 (pollen-derived plant.) was only 7.3%, whereas that of 75-1-14 was 23.4% (significant at 1%), a difference due to the prolonged culture duration of 75-1-14. Culture durations from regeneration of plantlet to transfer into pots (64 d for 72-1-11-1 and 69 d for 75-1-14) were not statistically different. But from appearance of callus to regenerated plantlets, the durations were quite different (64 d). This difference markedly influences the frequency of chromosome doubling and the variability of pollen-derived plants.

High temperature after anther inoculation can also affect the chromosome doubling and fertility of pollen-derived wheat. The root-tip cells of pollen-derived inbred winter wheat Kedong 58 were cytologically examined. The frequency of chromosome-doubled plants after heat treatment was 27.98%; but 14.58% in the control, the difference significant at 5%. In, another experiment, high inoculation temperature affected the fertility of pollen-derived Orofen. The frequency of fertile pollen-derived plants after heat treatment was 34.72%, but 22.51% in the control, the difference significant at 1% (t = 3.0974).

Gamete expression

Since haploid plants contain only one set of chromosomes, the recessive characters can be expressed in the F_1, which means they can be efficiently selected and reproduced, and are valuable for mutation breeding and investigation.

Diversity. Since pollen grains from F_1 hybrids are heterozygous, different gene combinations of both parents of a

Table 1. Ploidy status of pollen plants from wheat variety Orofen (1981-1983).

	Euploids		Aneuploids							SAC[a] and telos	Heteroploids					Mixoploids					Total
	3x (21)	6x (42)	20	22	23	40	41	43	44		5x	7x	9x	11x	12x	20 21 22	40 41 42	43 44	3x 6x	8x 12x	
No.	217	188	1	3	2	9	3	4	4	18	1	1	2	1	3	3	6	2	3	1	472
%	46.0	39.8	0.2	0.6	0.4	1.9	0.6	0.9	0.9	3.8	0.2	0.2	0.4	0.2	0.6	0.6	1.3	0.4	0.6	0.2	100

[a] Spontaneous aberrations in chromosomes.

Table 2. Effect of callus culture duration on chromosome doubling of H_1 wheat pollen plants.

No.[a]	Culture duration from callus appearance to regenerated plant (d)	Culture duration from regenerated plantlet to plantlet transfer into pots (d)	Cells examined (no.)	Doubling cell		*t* value
				No.	%	
72-1-11-1	31	64	69	5	7.3	
75-1-14	95	69	94	22	23.4	2.7419**

[a] 72-1-11-1 and 75-1-34 are 2 regenerated H_1 plants derived from the same callus which originated from F_1 anthers of a cross of spring wheat (India X Hongtu). **Significant at 1%.

cross occur in every grain. If pollen grains of F_1 hybrids are induced into pollen plants by anther culture, the plants (H_1) show various phenotypes of both parents. Diversity of pollen progeny has been observed in experiments with rice, wheat, and tobacco.

Gamete types expressed. Wang (10) cultured hybrid (F_1) anthers of hexaploid triticale Rosner and hexaploid common wheat Kedong 58 and cytologically analyzed the regenerated plants. Hybrids (F_1) between hexaploid triticale and common wheat have the chromosome constitution AABBDR. There are two sets of univalent DR, each of which, at meiosis, will distribute into daughter cells in one of seven possible ways, and result in diversified chromosome compositions. A total of 12 aneuploid and haploid plants were identified by Giemsa C-banding technique: there are 2-7 rye chromosomes and 18-21 wheat chromosomes. Table 3 indicates 11 different types of chromosome compositions. If the five rye chromosomes in plants no. 36 and 63 were different, these should be of 12 different genotypes. The chromosomes of mixed DR, then, coexisted in these pollen-derived plants. The types of pollen-derived plants were theoretically the same as the types of chromosome composition formed by F_1 hybrid meiosis. That means the possible recombinant types of F_1 hybrid pollen, i.e., recombined gamete, might, by anther culture, be fully expressed at plant level.

Table 3. Chromosome composition of pollen plants from (triticale Rosner × wheat Kedong 58) F_1.

Plant (no.)	Chromosome (no.)	Rye chromosomes (no.)	Wheat chromosomes (no.)
68	21	2	19
32	22	3	19
22	23	3	20
37	23	4	19
38	23	5	18
69	24	4	20
11	24	3	21
36	25	5	20
63	25	5	20
56	25	4	21
24	26	7	19
46	27	7	20

In conventional breeding, in which hexaploid triticale is crossed with hexaploid common wheat, several generations of self-pollination and selection might yield plants with different genotypes. But because of natural selection, combining some gamete types into one zygote is difficult. Anther culture of this same material yielded 10 types of pollen plants, but conventional method produced only 4 types of F_2 plants (Fig. 1). In conventional breeding, self-pollination, especially in distant hybridization, can lead to gamete selection, and loss of the initial gamete types. In addition, fusion between female and male gametes with the same chromosome composition is very improbable. Anther culture can produce different kinds of hybrids between common wheat and hexaploid triticale in a much shorter time and from a much smaller population than can conventional methods.

APPLICATION OF POLLEN-DERIVED PLANTS

Transfer of desirable alien chromosomes into cultivars

Table 4 shows the number of gamete chromosomes and their probabilities using heterozygous materials with one mixed genome, e.g., DR mixed genome. There are 15 chromosome types and many different compositions because the number of DR chromosomes ranges from 0 to 7. Therefore, the pollen-derived plants possess a large number of genotypes. About 1/5 of them have 21 chromosomes, and about 1/300 have 7A7B6DlR genome. After chromosome doubling, the alien substitution lines would be obtained directly.

Hexaploid triticale
Rosner
14W"7R" AABBRR
Gamete 14W'7R' ABR

×

Common wheat
Kedong 58
21W" AABBDD
Gamete 21W' ABD

AABBDR
Gamete ABD (0-7) R (0-7)

Self-pollinated (F_2)		Anther culture (H_1)	
Plants (no.)	Chromosomes (no.) of regenerated plants	Plants (no.)	Chromosomes (no.) of regenerated plants
28	42	1	21
1	44	1	22
4	46	3	23
1	49	3	24
		4	25
		1	26
		1	27
		4	46
		4	Haplo-mixoploid
		5	Diplo-mixoploid

1. Chromosome composition of pollen-derived plants (H_1) and F_2 plants.

Table 4. The numbers and probabilities of gamete chromosomes.[a]

Gamete chromosomes (no.)	Probability
14	1/16 384
15	1/170
16	1 /180
17	1/45
18	1/16.4
19	1/8.18
20	1/5.46
21	1/4.77
22	1/15.46
23	1/8.18
24	1/16.4
25	1/45
26	1/180
27	1/1170
28	1/16 384

[a]R-genome pairs (no.): 7 6 5 4 3 2 1 0
D-genome pairs (no.): 0 1 2 3 4 5 6 7
Possible chromosome combinations: (no.): 1 7 21 35 35 21 7 1
The probability of the gamete with 7A7B6DIR genome is $(7/128)^2 \approx 1/300$.

Recently the F_1 hexaploid triticale Beagle/Jinghua No. 1 and Beagle/Kedong 58 was used for anther culture. Of the more than 800 green pollen-derived plants produced, 555 plants were examined cytologically. Among these, 410 were euploid and aneuploid; the remainder were heteroploid and mixoploid. Table 5 shows the chromosome number of euploid and aneuploid pollen plants: there were 11 types of chromosome number in aneuploids and the numbers of chromosomes ranged from 17 to 27. These results were expected (Table 4). Various types of F_1 pollen could, then, be fully expressed at homozygous plant level by culturing hybrid anthers of hexaploid triticale x hexaploid wheat. Chromosome breaks and reunions are likely during anther culture. Anther culture can, therefore, be used to enhance the frequency of exchange required in sexual hybrids for introgressing desirable alien genes. Also, new substitution, addition, and translocation lines can be obtained,

Development of new varieties

The anthers of hybrid progeny are excellent breeding materials. With them, the good complementary characteristics of two parents car, be combined in pollen plants and, in one generation, result in desirable homozygous plants, shortening the conventional breeding cycle by three to four generations.

Eighty-one rice varieties and strains have been developed through anther culture. For example, Hua Yu No. 1 possesses high yield (7,500 kg/ha), resistance to bacterial. blight, to1erance for salt, and wide adaptability. It has been released in the suburbs of Beijing and Tianjin. The new rice variety Xin Xiu, developed by Z.H. Zhang, has been grown over 100,000 ha in

Table 5. Chromosome number of euploid and aneuploid pollen plants.

Chromosomes (no.)	Plants	
	No.	%
17	1	0.2
18	1	0.2
(38)	4	0.9
20 (40)	7	1.7
21 (42)	40	9.8
22 (44)	50	12.2
23 (46)	133	32.5
24 (48)	106	25.9
25 (50)	40	9.8
26 (52)	20	4.9
21	8	1.9
Total	410	100.0

eastern China. Breeding of high-yield rice varieties with high blast resistance in the rice-growing region of North China has traditionally been by backcross, which is time consuming. Through anther culture, the successful introduction of the resistance gene pi-z^t in Toride No. 2 into Zhonghua No. 8 and No, 9, with high yield and good quality, has taken only 2 yr, whereas a similar Japanese project by conventional means (reciprocal backcross) took 12 yr (4).

Up to June 1982, Chinese scientists had developed more than 20 new wheat varieties and strains through anther culture. Spring wheat Huapei No. 1 (developed by Kunming Institute of Agriculture and Institute of Genetics, Academia Sinica) has several desirable characters, including vigorous tillering, cold resistance, early maturity, and good grain quality. Its cultivation covers about 700 ha in Kunming suburb. The new winter wheat Jinghua No. 1, developed by D.F. Hu and others, is the best example of anther culture's potential in plant breeding programs. Through anther culture combined with complex crosses, it was developed over 7 yr, including 4 yr of field trials, regional and general performance tests, and multiplication of seeds. It is high-yielding, disease resistant, and short statured, and is widely adaptable. In the autumn of 1983 it covered about 7,000 ha.

Improving hybrid rice

Because hybrid rices possess good genetic backgrounds, breeding them through anther culture is an important achievement. Six strains have been selected from pollen plants derived from F_1 anther culture of hybrid rice Shan You No. 2. These strains can replace hybrid rice Shan You No. 2 and avoid the trouble of producing hybrid seed.

Developing inbred maize

Since 1975, many maize pollen-derived plants have been obtained in China. An important one, with good combining ability, is a

line named Qun Hua, selected by J.L. Wu and others. The cross 411/Qun Hua was included in corn regional yield tests from 1982 to 1983. The test included 13 different cross combinations, with two crops each year. At the end of 1983, 411/Qun Hua, having shown high yield and disease resistance, was identified as one of the three recommended superior hybrids and was named Huayu No. 1. It will be propagated in 1984 and put into farm production in 1985.

Developing asexual lines of rubber tree and sugarcane

Pollen plants can improve varieties of rubber tree and sugarcane: we have obtained a pollen rubber tree of more than 6 m and a pollen sugarcane with high sugar content and tall stem.

REFERENCES CITED

1. Hu H., T.Y. Hsi, and S.E. Chia. 1978. Chromosome variation of somatic cells of pollen calli and plants in wheat (Triticum aestivum L.). Acta Genet. Sin. 5(1):23-30.
2. Hu, H. 1983. Genetic stability and variability of pollen-derived plants. Pages 145-157 in Plant cell culture in crop improvement. S.K. Sen and K.L. Giles, eds. New York and London, Plenum Press.
3. Khush, G.S. 1973. Cytogenetics of aneuploids. Academic Press, New York.
4. Li, M., P. Ni. Y. Chen, and J. Shen. 1983. Studies on anther culture for breeding varieties resistant to rice blast. Acta Agron. Sin. 9(3):173-179.
5. Maheshwari, S.C., A. Rashid, and A.K. Tyagi. 1983. Antherlpollen culture for production of haploids and their utility. IAPTC Newsl.. 41:2-7.
6. Oono, K. 1981. In vitro methods applied to rice. Pages 273-298 in Plant tissue culture. T.A. Thorpe, ed. Academic Press, New York.
7. Ouyang, J., S. Zhou, and S. Jia. 1983. The response of anther culture to culture temperature in Triticum aestivum. Theor. Appl. Genet. 66:101-109.
8. Riley, R., and G. Kimber. 1961. Aneuploids and the cytogenetic structure of wheat varietal populations. Heredity Lond. 275-290.
9. Tian, W., and Y. Chen. 1983. The factors affecting induction and differentiation of callus in rice anther float culture. Acta Genet. Sin. 10(5):362-368.
10. Wang, X.Z. 1984. Cytogenetical study on pollen plants from F_1 hybrid between hexaploid triticale and common wheat. Acta Genet. Sin. 11(1):33-38.

RICE ANTHER CULTURE AT IRRI

F. J. ZAPATA

The effects on androgenesis of several factors, such as the stage of microspore development and physical conditions before and during anther culture, have been studied. From the results, a standard anther culture procedure, now used to produce double haploid lines from F_1 sexual crosses, was established.

Anther culture-derived lines from crosses have been screened for cold tolerance at seedling stage. Some lines whose parents are both cold susceptible were found cold tolerant, probably because of gene complementation or induced mutation during anther culture. Five lines regenerated from Taipei 309 and selected by stressing the calluses with high concentrations of S^2-amino ethyl cysteine, an analog of lysine, had increased contents of lysine or total protein, or both, in the grain. One line had 13.9% more lysine and 9.3% more protein than the parents.

Gametoclonal variation in agronomic characteristics among anther culture-derived lines from Taipei 309 was observed in field experiments. The uniformity in height and number of days to maturity shown by plants of individual anther culture-derived lines from F_1 sexual crosses indicates their nonsegregating, homozygous characters, thus confirming that homozygosity is attained just by passing the anthers of F_1 crosses through anther culture. Double-haploid Lines from the anther culture of F_1 hybrids have shown combined characteristics from both parents, which suggests that they originated from different recombinants.

We have started a collaborative project with Korea and cultured anthers of japonica/japonica crosses one or both of whose parents are cold tolerant. In seven crosses, 3,275 anthers were plated in 1 d and at least 1,572 plants were regenerated.

When rice anther culture at IRRI started in 1980, its immediate objective was to identify genotypes with anther culture capacity (the ability to produce callus and regenerate plants) (2).

Associate plant physiologist, Tissue Culture Facility. International Rice Research Institute, P. O. Box 933, Manila, Philippines.

Japonica and indica varieties were used in studies of the proper stage of microspore development and the effects of physical conditions such as cold, light intensity, and different culture media on androgenesis.

In 1981 four highly androgenetic varieties were used in establishing a standard anther culture procedure. In this procedure, anthers at the miduninucleate to early binucleate stage of development are plated in a callus-production medium, subjected to cold shock for 8 days at 8°C, and transferred to 25°C in the dark. After 6 wk, calluses begin to form. The calluses are then plated on an M-shaped paper bridge in a test tube containing liquid plant-regeneration medium (9, 7). Four wk later, the growing calluses are plated in a semisolid medium to regenerate plants. Using this procedure, we started to work with F_1 sexual crosses. In one experiment, anthers from 18 F_1 reciprocal crosses of indica/indica, indica/japonica, japonica/japonica, and japonica/indica rice varieties were cultured. The main objectives in using these crosses (8) were

- to improve plant type (e.g., to transfer Suweon 290's good plant type, high-yield capacity, and waxy characteristic into other genotypes);
- to increase yield capacity (e.g., to transfer BG90-2's high-yielding capacity and semidwarf characteristic);
- to increase cold tolerance (e.g., the cold-tolerant varieties Tatsumi mochi and Taipei 309 were crossed with susceptible indica varieties);
- to produce early-maturing lines (e.g., to cross the early-maturing Tatsumi mochi with later maturing lines); and
- to incorporate anther culture capacity (e.g., to transfer Taipei 309's anther culture capacity to other genotypes).

The calluses were plated in a regeneration medium and 502 green plants were regenerated (Table 1).

The current objectives of anther culture research at IRRI are

- to reduce the time required to produce cold-tolerant rice varieties from F_1 sexual crosses between japonica/japonica, japonica/indica, indica/indica, and indica/japonica rices;
- to increase total protein and lysine content in rice grains of anther culture-derived plants regenerated from calluses stressed with amino acid analogs;
- to study yield and yield components of plants regenerated from anther culture of F_1 sexual crosses;
- to examine the variability in adverse soils and environmental stresses among anther culture-derived plants under field and screenhouse conditions; and
- to conduct studies on increasing plant regeneration.

BREEDING FOR COLD TOLERANCE THROUGH ANTHER CULTURE

Low temperature is one of the major environmental factors affecting yield. It affects all stages of the rice plant's

Table 1. Green plant regeneration from pollen culture of 18 F_1 reciprocal crosses. IRRI, 1982.

Parents[a]	Anthers plated (no.)	Calluses plated for green plant regeneration[b] (no.)	Green plants regenerated (no.)
BG90-2/Tatsumi mochi (1/J)	274	64	132
Tatsumi mochi/BG90-2 (J/I)	238	23	29
BG90-2/1R48 (I/I)	180	8	3
IR48/BG90-2 (I/I)	192	20	40
BG90-2/Suweon 290 (I/I)	82	6	6
Suweon 290/BG90-2 (I/I)	383	48	35
BG90-2Daipei 309 (I/J)	1085	445	80
Taipei 309/BG90-2 (J/I)	193	15	10
Tatsumi mochi/Suweon 290 (J/I)	341	79	58
Suweon 290/Tatsumi mochi (I/J)	68	0	0
IR48/Suweon 290 (I/I)	609	171	43
Suweon 290/1R48 (I/I)	100	12	13
IR48/Taipei 309 (I/J)	214	16	1
Taipei 309/IR48 (J/I)	240	111	21
Suweon 290/Taipei 309 (I/J)	484	19	0
Taipei 309/Suweon 290 (I/J)	106	0	0
Taipei 309/Tatsumi mochi (J/J)	171	103	30
Tatsurni mochi/Taipei 309 (J/J)	58	48	1
Total	5018	1188	502
Av		66	27.89

[a]J - japonica, I - indica. [b]The number of calluses plated for plant regeneration is only a part of the total number of pieces of calluses actually obtained from pollen grains.

development, from seedling to flowering, and causes low germination, reduced height, incomplete panicle exsertion, and, most important, reduced yield. In South and Southeast Asia alone, 7 million ha of rice is damaged by cold water or cold weather.

Modern indica rice varieties, which are semidwarf and high yielding, have not done well in low-temperature areas because they are cold susceptible. Japonica rice varieties are excellent sources of cold-tolerant genes, and could introduce cold tolerance into the high-yielding indica varieties. However, their use in breeding programs has been hampered by the high sterility in japonica/indica and indica/japonica crosses. In addition, the progeny of many crosses involving cold-tolerant parents show little or no cold tolerance. Backcrossing seems effective, but is very slow. Anther culture may fix cold tolerance in doubled haploid lines and hasten the production of cold-tolerant materials.

Seeds of plants regenerated from anther culture of F sexual crosses were harvested and handed to IRRI plank physiologists in the cold tolerance research group for cold tolerance screening of seedlings (Table 2). After 12 d in cold-water tanks at 12°C, the seedlings were scored as follows: 1, seedlings dark green; 3, seedlings light green; 5, seedlings yellow; 7, seedlings brown; 9, seedlings dead (6). Through anther culture, it is possible to obtain from cold-susceptible parents, lines with increased cold tolerance, such as the AC-derived lines of the IR48/Suweon 290 cross, because of gene

Table 2. Cold tolerance response of anther culturederived plants.

Mother plant	Cold tolerance score[a]	Cross	Plants tested (no.)	Seedlings (no.) that received a score of				
				1	3	5	7	9
IR20	9	IR48/BG90-2	6	0	0	0	1	5
IR30	9	IR48/Suweon 290	31	0	0	11	20	0
IR36	9	Tatsumi mochi/ Suweon 290	25	0	1	14	10	0
IR48	9	Tatsumi mochi/ IR48	1	0	0	0	1	0
Taipei 309	3	Tatsumi mochi/ Taipei 309	1	0	0	1	0	0
Tatsumi mochi	3	Tatsumi mochi/ BG90-2	22	0	1	4	10	7
BG90-2	9	Taipei 309/1R20	1	0	0	1	0	0
M148	5	Taipei 309/1R30	1	0	0	0	1	0
Pokkali	7	Taipei 309/1R36	1	0	0	0	1	0
Suweon 290	9	Taipei 309/ Tatsumi mochi	27	0	11	12	4	0
Silewah	3	Taipei 309/BG90-2	1	0	1	0	0	0
Taichung 65	3	Suweon 290/ BG90-2	8	0	0	0	7	1
		BG90-2/Tatsumi mochi	4	0	0	0	3	1
		BG90-2/Taipei 309	36	3	10	0	23	0
		MI48/Pokkali	3	0	0	3	0	0
		Silewah/Taichung 65	1	0	1	0	0	0
		IR36870	1	0	0	0	1	0
		IR36781	7	0	0	1	6	0
Total			177	3 (1.7%)	25 (14.1%)	47 (26.6%)	88 (49.7%)	14 (7.9%)

[a]1 = seedlings dark green, 3 = seedlings light green, 5 = seedlings yellow, 7 = seedlings brown, 9 = seedlings dead.

complementation in the recombinants or because mutation may occur during anther culture.

Another way to regenerate cold-tolerant lines is to culture anthers of F_1 crosses bred specifically for cold tolerance. We have started a collaborative project with Korea and cultured anthers of indica/japonica and japonica/japonica crosses whose parent or parents were cold tolerant. The materials were plated in September 1983 and the regenerated plants then grown to maturity in the screenhouse. The tested materials will be planted in Korea for cold tolerance screening at the vegetative and reproductive stages.

In the studies mentioned earlier, selection for cold tolerance is performed after plant regeneration. Another system selects for cold tolerance at the cellular level: calluses are exposed to 8°C for 90 d. Calluses that survive this selection pressure are placed in a regeneration medium, and plants regenerated from them are presumed cold tolerant. Such plants will also be screened to confirm their cold tolerance.

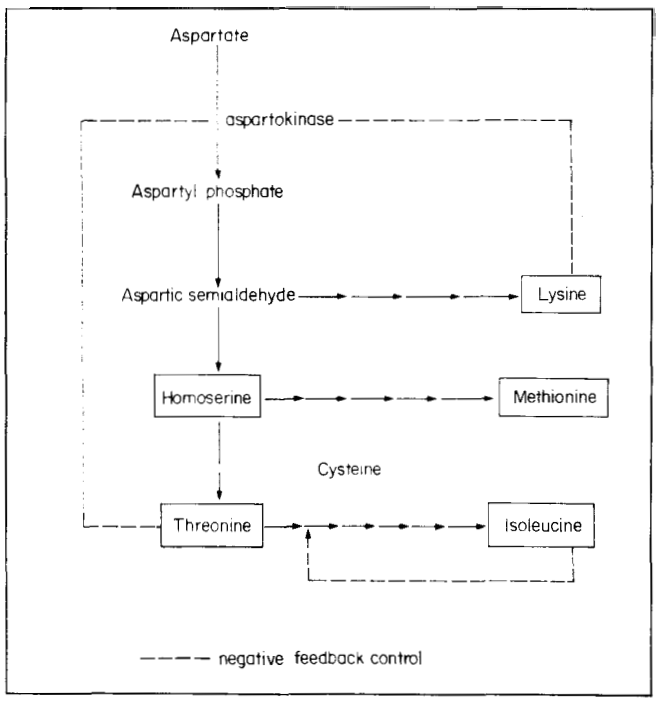
SELECTION OF VARIANTS WITH INCREASED PROTEIN AND LYSINE CONTENT

Rice is one of the major staple foods in the world; unfortunately, because of low lysine content, its grain is nutritionally poor in protein. It is therefore a continuing goal of plant breeders and geneticists to breed and select plants with higher lysine content. One way to produce high-lysine plants is by using amino acid analogs and anther culture techniques in model rice varieties.

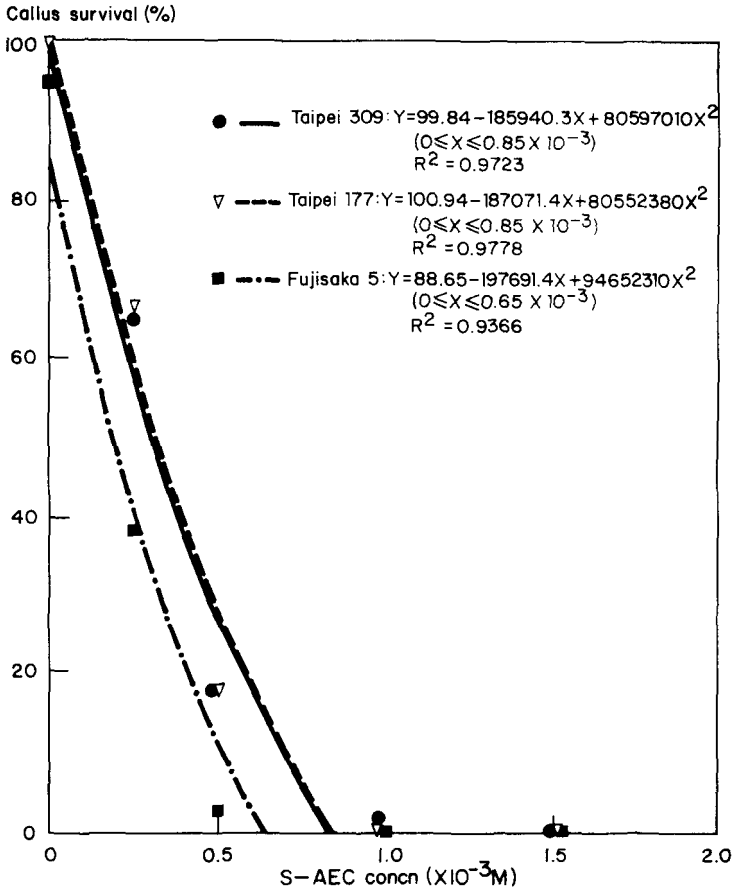
Lysine production is regulated by the aspartate pathway (Fig. 1). The first enzyme, aspartokinase, is sensitive to feedback control by lysine. Enzyme sensitivity is increased in the presence of the analog S,2-aminoethyl-L-cysteine (S-AEC) (4).

Aspartokinase activity in cell-free extracts of rice shoots was sensitive to feedback inhibition by S-AEC (l), suggesting that resistance to S-AEC could be used to select rice mutants in which lysine biosynthesis is released from regulatory control. Increasing lysine in rice through anther culture and S-AEC has been reported; the percentage of lysine was increased by 10% and that of total protein by 6% (5).

Variants with increased lysine content are selected as follows. Calluses from anthers of rice variety Taipei 309 are stressed with 1 x 10^{-3} M S-AEC, a concentration that kills



1. Hypothetic scheme for the biosynthetic pathway of methionine, lysine, threonine, isoleucine, and their regulatory mechanism in rice callus tissue.



2. Relationship between S-AEC concentration and percentage of callus survival,

normal cells (Fig. 2). Haploid calluses are stressed for 90 d, after which the surviving callus is plated on a regeneration medium. Seeds of the regenerated plants are analyzed for lysine content by the dye-binding capacity method and for total protein by the Kjeldahl digestion method (3) by the IRRI Chemistry Department.

In rice, total protein and total lysine normally correlate negatively. This is also true in AC1033-1 and AC1040-1 (Table 3). Increases in total protein and lysine content in AC1040, AC1040-6, and AC1040-6A show that high-protein variants are obtainable through anther culture techniques. In AC1040-6A, total protein was increased by as much as 9.3% over that of the parent, and total lysine, by 13.9%. Second and third generations are being grown in a glasshouse for seed production to determine if this change can be carried genetically to the next generation.

Table 3. Percentage lysine and total protein in brown rice from firstgeneration seeds of anther culture-derived plants of rice variety Taipei 309.

AC plant no.	Lysine[a] (%)	Protein[a] (%)
Taipei 309 parent	3.6	7.5
AC1033-1	3.5 (-2.8)	8.8 (+ 17.3)
AC1040	3.8 (+5.6)	8.3 (+10.7)
AC1040-1	4.0 (+11.1)	6.9 (-8.0)
AC 1040-6	3.7 (+2.8)	8.0 (+6.7)
AC 1040-6A	4.1 (+13.9)	8.2 (+9.3)

[a] Numbers in parentheses represent increase (+) or decrease (–) in % lysine and % protein over the parent.

FIELD EXPERIMENTS

Varieties

Using varieties in anther culture not only allows the establishment of a standard procedure for triggering callus production and green plant regeneration, but also helps in the comparison and analysis of any variation in agronomic characteristics and yield between mother plants and regenerants. Agronomic characteristics of selected lines regenerated from Taipei 309 have been tested in the field (Table 4). Each line was planted adjacent to the parents at 1 seedling/hill in 2 rows, each with 25 hills spaced 20 cm apart. The fertilizer application rate was 100-50-50 (N-P-K) kg/ha.

The anther culture-derived lines tend to be taller than the parent, because they have a longer culm. In most cases, their panicles were shorter than the parent's, although AC96, AC331, and AC356 have significantly more panicles, which may indicate a potential for higher yield. In percentage of fertility, most of the AC lines were the same as the parent.

Gametoclonal variation in agronomic characteristics is evident. In most cases, the change in a certain character is genetically stable: the change in characteristics of plants from both the first- and second-generation seeds of a regenerated plant was consistent.

Besides the mentioned variations, variability in grain size (some offspring have bigger grains than the parent; others have smaller) and in reaction to soil stress (e.g., regenerated plants from iron-susceptible varieties show increased iron tolerance) was observed in anther culture-derived lines.

F_1 sexual crosses

Plant breeders rely on sexual propagation to produce new rice varieties. They aim to assemble in one plant all the desirable characters of both parents, and then to rapidly achieve homozygosity. Homozygosity can be reached in one generation

Table 4. Mean comparison of agronomic characteristics between Taipei 309 and plants from first-generation (1) and second-generation (2) seeds of anther culture-derived plants of the same variety. 1982 DS. Masapang, Victoria.[a]

AC	Panicles (no.)		Fertility (%)		Flagleaf length (cm)	
	1	2	1	2	1	2
Taipei 309 (parent)	10.30		93.88		45.15	
96	14.80 a	13.57 a	92.91	93.34	43.03	40.58 b
133	13.53 a	11.63	68.94 b	91.56	36.57 b	39.17 b
152	12.25 a	10.97	89.92	91.97	42.95	40.98 b
157	11.96 a	11.13	90.44	91.12	41.24 b	41.88
171	11.23	10.30	89.73	88.98 b	40.75 b	43.52
182	10.03	10.60	85.86 b	89.81	41.68	40.93 b
184	11.40	9.50	89.42 b	63.44 b	41.82	41.27 b
196	10.23	9.77	89.85	93.99	35.25 b	42.17
204	12.40 a	10.40	87.03 b	92.53	41.88	43.13
185	11.03	10.50	91.19 b	91.64	40.92 b	39.57 b
33 1	13.17 a	11.80 a	86.04 b	90.34 b	37.87 b	37.87 b
356	13.93 a	15.53 a	88.58 b	92.63	38.98 b	43.65
318	9.62	9.93	90.88	9 1.86	40.58 b	39.58 b
386	10.00	15.27 a	93.39	95.33	32.33 b	38.95 b

	Culm length (cm)		Panicle length (cm)		Plant ht (cm)	
	1	2	1	2	1	2
	83.85		24.48		107.83	
96	89.08a	90.17 a	24.20	23.68	113.28a	113.85a
133	94.52 a	86.62	22.93 b	22.83 b	117.45 a	109.45
152	94.20 a	89.80 a	24.09	23.57 b	118.28 a	113.37 a
157	93.67 a	89.08 a	22.72 b	22.93 b	116.39 a	112.02 a
171	92.42 a	90.25 a	23.00 b	24.23	115.42 a	114.48 a
182	90.22 a	89.25 a	22.92 b	24.00	113.13 a	113.25 a
184	94.28 a	88.85 a	22.95 b	23.22 b	117.23 a	111.77
196	87.90 a	86.08	23.85	22.92 b	111.75 a	109.00
204	91.70 a	88.73 a	23.52	23.46 b	115.22 a	112.20 a
185	94.45 a	90.83 a	23.30 b	23.10 b	117.75 a	113.93 a
33 1	85.22	83.15	22.98 b	23.10 b	108.20	106.25
356	85.87	86.80 a	23.05 b	23.73	108.92	110.53
318	87.38 a	85.25	22.42 b	22.85 b	109.79	108.10
386	88.19 a	84.18	20.79 b	22.00 b	109.98	106.18

[a]Means followed by a are significantly higher than the mean of the parent at 5% by t-test. Means followed by b are significantly lower than the mean of the parent at 5% by t-test. Means without letters are not significantly different from the mean of the parent.

through anther culture techniques; each plant thus obtained can be a new variety. The advantages of anther culture over conventional breeding are rapid attainment of homozygosity and increased selection efficiency.

Plants of individual anther culture-derived lines from F_1 sexual crosses showed uniformity in height and in days to maturity, which indicates their nonsegregating, homozygous character. This was confirmed by F-test. Data on agronomic

characteristics such as culm length, panicle length, percentage of fertility, panicle number, and flagleaf length were gathered. An F-test compared the standard deviation of the parents' agronomic characteristics with that of the anther culture-derived plants'. The range of variability among the plants of the anther culture-derived lines was within the limits of variability of their respective parents.

Doubled haploid lines derived from anther culture of F_1 hybrids have shown mixed characteristics from both parents, which suggests they originated from different recombinants. The agronomic characteristics of selected anther culture-derived lines were studied in a replicated yield trial (Table 5). Days to flowering may be shorter than in both parents, as in AC594 — 3; intermediate, as in AC683 and 700; or longer, as in AC655 and AC721 — 1. Plant height may be shorter than in both parents, as in AC721 — 1, AC594 — 3, and AC683; intermediate, as in AC655; or taller, as in AC700 and AC32. Flagleaves may be shorter than in both parents, as in AC700 and AC32, or longer, as in AC683, AC721 — 1, and AC594 — 3.

The yields of these selected lines were studied in a replicated yield trial. AC700 yielded higher than both parents and the rest lower, but the differences were not significant at 5% by Duncan's multiple range test (Table 6).

Reactions to strong acid soils (containing toxic amounts of iron) of anther culture-derived lines from F_1 crosses were also studied. Taipei 309 and Tatsumi mochi were both highly susceptible but one line from the anther culture of the F_1 cross of these two varieties was highly iron toxicity-tolerant. In this case, variability due to mutation induced by in vitro culture could have combined with gene complementation.

Table 5. Comparison of agronomic characteristics among anther culturederived double haploids and their respective parents in a replicated yield trial experiment, Victoria, Laguna, Philippines. 1983 DS.

Entry	Days to flower after seeding (no.)	Culm length[a] (cm)	Panicle length[a] (cm)	Plant height[a] (cm)	Flagleaf length[a] (cm)
IR30	82	70.900 b	25.340 a	96.24 b	28.740 a
AC32	92	79.887 a	23.473 b	103.36 a	24.873 b
Suweon 290	94	78.733 bc	23.893 c	102.63 b	25.620 b
BG90-2	102	76.740 c	27.833 a	104.57 b	26.500 b
AC683	99	69.100 d	25.087 b	94.19 c	30.167 a
AC700	97	82.213 ab	28.013 a	110.23 a	24.440 b
IR48	110	91.747 a	27.213 a	118.96 a	26.120 a
BG90-2	102	76.740 c	27.833 a	104.57 c	26.500 a
AC655	112	83.230 b	28.020 a	11 1.25 b	25.612 a
AC721-1	112	63.213 d	25.813 b	89.03 e	26.993 a
AC594-3	101	76.664 c	23.743 c	99.41 d	26.934 a

[a]Means followed by a common letter within each cross or variety are not significantly different at 5% by Duncan's multiple range test (DMRT).

Table 6. Yield comparison between anther culture-derived plants and their parents in a replicated yield trial experiment. Victoria, Laguna, Philippines. 1983 DS.

Cross/variety	Parent/ACP no.	Yield[a] (g/m^2)
IR48/BG90-2	IR48	780.10 a
	BG90-2	799.64 a
	655	595.31 a
	721-1	600.00 a
	594-3	654.51 a
Suweon 290/BG90-2	Suweon 290	826.41 a
	BG90-2	799.64 a
	683	722.28 a
	700	831.04 a
IR30	IR30	775.60 a
	32B18	657.23 a

[a]Means followed by a common letter within each cross or variety are not significantly different at 5% by Duncan's multiple range test (DMRT).

REFERENCES CITED

1. Halsall, O., R.D. Brock, and J.B. Langridge. 1972. Selection for high lysine mutants. CSIRO Division of Plant Industry, Genetics Rep. 31.
2. IRRI (International Rice Research Institute). 1981. Annual report 1979. p. 159-160.
3. Juliano, B.O. 1983. Rapid, simple testing for nutrients as an aid to plant breeding. Pages 515-525 in Chemistry and world food supplies: the. new frontiers. CHEMRAWN II. L.W. Shemilt, ed. Pergamon Press, Oxford.
4. Miflin, B.J., S.W.J. Bright, S.E. Rognes, and J.S.H. Kueh. 1983. Aminoacids, nutrition and stress: the role of biochemical mutants in solving problems of crop quality. In Genetic engineering of plants: an agriculture perspective. T. Kosuge, C.P. Meredith, and A. Hollaender, eds. Plenum Press, London.
5. Schaeffer, G.W., and F.T. Sharpe, Jr. 1981. Lysine in seed protein from S-aminoethyl-L-cystein resistant anther-derived tissue culture of rice. In Vitro 17(4):345-352.
6. Vergara, B.S. et al. 1976. Screening of the rice germplasm for low temperature tolerance at different stages of growth. SABRAO J. 8(2):97-104.
7. Zapata, F.J., G.S. Khush, J.P. Crill, M.H. Heu, R.O. Romero, L.B. Torrizo, and M. Alejar. 1983. Rice anther culture at IRRI. Pages 27-46 in Cell and tissue culture techniques for cereal crop improvement. Science Press, Beijing, China.
8. Zapata, F.J., M.H. Neu, and G.S. Khush. 1982. Anther culture research for rice breeding at IRRI. In International Rice Research Conference, 1982. Los Baños, Philippines. Paper 31.

9. Zapata, F.J., L.B. Torrizo, R.O. Romero, and M.S. Alejar. 1982. Androgenesis in Oryzae sativa. In Plant tissue and cell culture 1982. A. Fujiwara, ed. The Japanese Association for Plant Tissue Culture, Tokyo, Japan.

SOMACLONAL VARIATION AND MUTANT ISOLATION

SOMACLONAL VARIATION IN CROP IMPROVEMENT

W. R. SCOWCROFT, S. A. RYAN, R. I. S. BRETTEL, and P. J. LARKIN

Somaclonal variation -- the enhanced genetic variability in plants regenerated from tissue culture -- is widespread and has been observed in most major crop species.

In wheat somaclonal variation comprises genetic changes affecting many morphological and biochemical characteristics, including some of potential agronomic importance, such as height, presence of awns, seed proteins, yield, and disease resistance. In vitro tissue culture and plant regeneration permit screening and selecting of somaclonal progeny for useful variants.

Chromosomal rearrangements are more frequent in plants derived from tissue culture, which may facilitate the introgression of alien genes from wild relatives into crop species, and thereby broaden the germplasm base of crop plants.

The yield potentials of such crops as wheat, barley, oats, maize, and soybean have steadily increased in many countries (8) and, possibly, except in the case of soybean, will continue to do so. Plant breeding efforts explain about 50% of the increase, but several misgivings have been expressed about future plant breeding. Many argue that plant breeding for most of our major crops rests on a precariously narrow genetic base, a problem exacerbated by the fact that most modern varieties are carefully engineered genetic entities. Broadening the genetic base through hybridization risks destroying the plant breeder's genetic improvements. On the other hand, wild relatives have genes for disease and stress tolerance, and more efficient photosynthate use, which, if transferred to our cultivars, could further improve yields.

Tissue culture and somaclonal variation may permit exploiting the wild relative germplasm for desirable traits and enhanced genetic variation without risking the genetic damage likely through conventional hybridization.

Commonwealth Scientific and Industrial Research Organization, Division of Plant Industry, G. P. O. Box 1600, Canberra, A. C. T. 2601, Australia.

SOMACLONAL VARIATION

Recognized as ubiquitous only in 1981 (15), somaclonal variation refers to the genetic variability in plants from tissue and cell culture. Its widespread nature has been extensively documented (4, 1.5, 27); it occurs in most economically important crop species (Table 1). Many of the traits for which genetic variability is generated during culture can significantly improve a plant's agronomic value.

Table 1. Somaclonal variation in economically important plant species.

Species	Explant	Variant characters[a]	Transmission[b]
Monocots			
Avena sativa	Immature embryo, apical meristem	Plant ht, heading date leaf striping, awns	S
Triticum aestivum	Immature embryo	Plant ht, spike shape, awns, maturity, tillering, leaf wax, gliadins, a-amylase	S
Oryza sativa	Seed embryo	Tiller no., panicle size, seed fertility, flowering date, plant ht	S
Saccharum officinarum	Various	Eyespot, Fiji virus, downy mildew, culmicolous spot disease; auricle length, esterase isozymes, sugar yield	A
Zea mays	Immature embryo	Endosperm and seedling mutants, *D. maydis* race T toxin resistance, mt-DNA sequence rearrangement	S
Dicots			
Solanum tuberosum	Protoplast, leaf callus	Tuber shape, yield, maturity date, plant habit; stem, leaf and flower morphology; early and late blight resistance	A
Lycopersicon esculentum	Leaf	Male sterility, jointless pedicel, fruit color, indeterminate growth	S
Nicotiana	Anthers, protoplasts, leaf callus	Plant ht, leaf size, yield grade index, alkaloids, reducing sugars; specific leaf chlorophyll loci	S
Medicago sativa	Immature ovaries	Multifoliate leaves, petiole length plant habit, plant ht, dry matter yield	A
Brassica spp.	Anthers, embryos, meristems	Flowering time, growth habit, waxiness glucosinolates, *Phoma lingam* tolerance	S

[a] Characters of possible economic importance. [b]S= sexual transmission, A= transmission through vegetative propagules.

Maize

Among 85 somaclones derived from immature maize embryo cultures morphological irregularities appeared in such characters as height, node number, ear arrangement, and stalk number (10).

Edallo et a1 (5) identified 17 classes of defective endosperm or seedling mutants among progeny of 77 plants regenerated from maize tissue cultures. Classification of a somaclone progeny variant as a mutant was based on its conformation to Mendelian segregation ratios. The somaclones analyzed were derived from two different genotypes, and progeny analysis revealed each somaclone carried an average of one simply inherited mutation. Some plants carried more than one mutation and when more than one plant was regenerated from a callus, they often carried different mutations.

Among the progeny of 51 initial (SC_1) maize regenerants, 8 segregated for recessive kernel mutations and one, for a mutation that caused premature wilting (19). Segregation for some mutants was not observed until the third (SC_3) generation, suggesting that the male and female inflorescence on. the progenitor SC_1 plant differed genetically. Alternatively, an event during the culture phase that causes somaclonal variation my persist after regeneration.

Somaclonal variation in maize also affects the mitochondria. The difference between T-cytoplasm (male-sterile, D. maydis race, T toxin susceptible) and N-cytoplasm (male-fertile, toxin resistant) is encoded in the mitochondrial genome. The reversion of T-cytoplasm to a toxin-resistant male-fertile type among maize somaclones is associated with mitochondrial. genetic changes (3, 13). Analysis of the reverted somaclone mt-DNA by restriction endonuclease demonstrated that fragment lengths had changed. Almost invariably the loss of one. particular mt-DNA fragment was associated with toxin tolerance (3, 28, 37).

Rice

Variation was dramatic in rice somaclones from a true breeding dihaploid derivative of the cultivar Norin 10 (23). In the progeny of more than 1,000 somaclones examined over 3 generations, heritable changes affecting chlorophyll development, plant height, heading date, maturity, and grain yield were observed. Many of the variants were true breeding within two generations of selfing. Progeny of about 70% of the somaclones differed from the parent by at least one heritable mutation.

Schaeffer (29) has confirmed that somaclonal variation occurs in rice: doubled haploid regenerants from the cultivar Calrose 76 varied in height, seed number and size, panicle size, leaf morphology, and tiller cumber and length.

Mutations may occur independently and sequentially in rice (9). Among the second-generation (SC_2) progeny of 12 plants derived from the callus of a single seed, 4 recessive mutations (early heading, albina, short culm, and sterility) were observed. Because of the pedigree relationship of these plants, one genetic event was assumed responsible for each mutation and it followed

that the mutational sequence was early heading, albina, short culm, and sterility.

Wheat

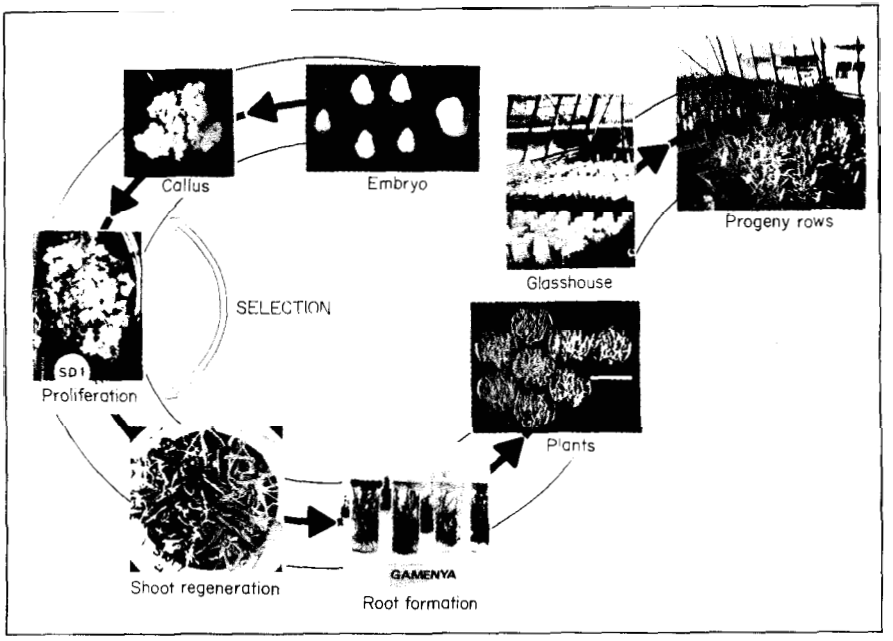
Plants may now be regenerated from tissue cu1ture of wheat with reasonable efficiency (1, 18, 25, 30). Our recent research to improve wheat indicates that many cu1tivars can be tissue-cultured, and plants can be regenerated from callus derived from immature embryos (see Fig. 1). Although more than 200 plants have been recovered from a single embryo, 20 plants from each immature embryo is the average. These SC_1 plants display some phenotypic variation, but their progeny reveal the true extent of the genetic changes induced during culture (14). Progeny of 142 somaclones of the Mexican double dwarf accession Yaque 50E through two generations segregated for increased height, reduced height, maturity, tiller number, presence or absence of awns, glume color, grain color, clavate head shape, and leaf waxiness. Segregation ratios for some of the qualities were Mendelian. Plant height had an estimated heritability of 0.67. The spectrum of height variation among the SC_3 plants of sone of the Yaque 50E somaclones is shown in Figure 2.

Seed of the progeny of some of these somaclones vary extensively in the electrophoretic pattern of their gliadin proteins. Some protein bands were lost, and "new" bands were observed; and the intensity of other electrophoretic fractions changed. This contrasts with the striking uniformity of the gliadin patterns among seed of the parent. Many, of the variant patterns breed true in subsequent generations, hut sone continue to segregate.

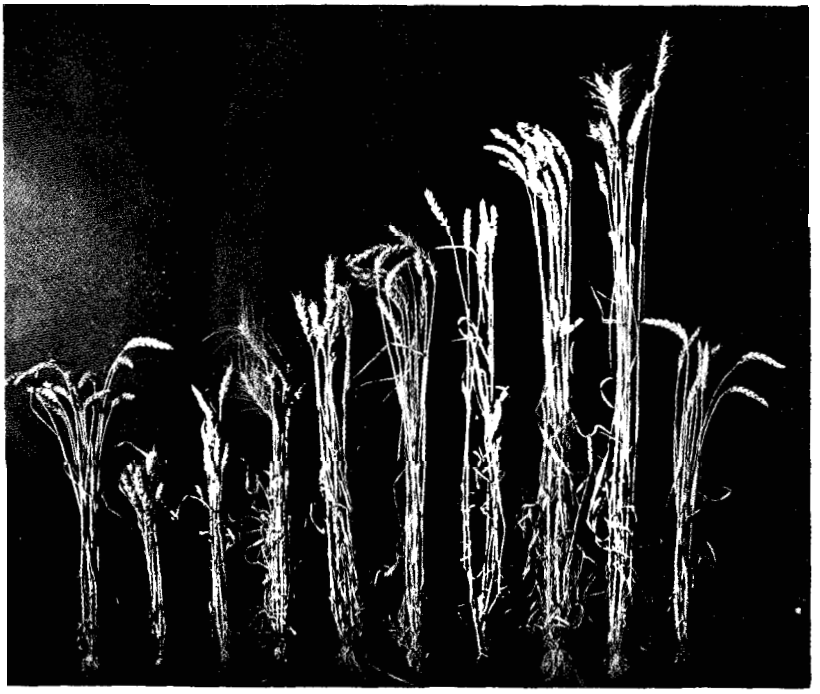
Gibberellic acid (GA_3)-induced synthesis of a-amylase varies in. response both to GA_3 induction and to abscisic acid repression of GA_3 a-amylase synthesis. Some of these variants transmit the altered response to their progeny.

Our research on somaclonal variation in wheat has shown that

- variation occurs in both morphological and biochemical characters and in traits under simple genetic control (gliadins, grain color) and polygenic control (height, heading date);
- a single somaclone can be variant for several traits which, in progeny analysis, appear to assort independently;
- both heterozygous and homozygous mutants are recovered in the primary regenerant. One somaclone car, contain both states at different loci;
- aneuploidy occurs but is not the primary cause of variation; rather it reflects genetic instability during culture;
- mutations affect characters for which major gene loci are located on all sever homologous groups; and
- somaclonal mutants can be recessive (awns, grain color), dominant (glume color, awn inhibition), or codominant (gliadins).



1. Wheat cell cultures are initiated from immature embryos, and cultured as disorganized callus. Shoots and roots are induced by altering hormones in the medium.



2. Variation in plant height in SC_3 progeny of somaclones from a double dwarf wheat variety. Parental plants are at extreme left and right.

Potato

Shepard and his colleagues (31, 32, 34) have described the extensive variation among plants regenerated from cultured protoplasts of the cultivar Russet Burbank. (This old but particularly valuable cultivar has effectively been excluded from potato improvement programs because it is sterile.) In their initial somaclone (protoclone) experiments, some 1,700 plants were evaluated for overall morphological variation. In 2 subsequent growing seasons this population was reduced to 65 clones possessing acceptable vigor, and vine and tuber characteristics. The clones were then evaluated under replicated field conditions for 35 characters including preplant sprouting, vine quality, maturity, morphology, and yield. Differences were statistically significant for 22 of the 35 characters, and each of the 65 somaclones differed from its parent in at least one trait. The modal class of 15 somaclones differed from Russet Burbank in 4 characters, and 1 plant was distinguished from the parent by no less than 17 traits. Shepard concluded that cell culture-generated variation provides enough variability for the selective improvement of Russet Burbank.

The potato somaclones were also screened for resistance to both late and early blight, to which the parent Russet Burbank is highly susceptible. The more than 800 plants tested varied widely in their reaction to late blight (*Phytophthora infestans*) (34). About 2% of the somaclones displayed enhanced resistance, which was transmitted through subsequent tuber generations. The form of resistance recovered in the somaclones appeared different from the common R gene type derived from *Solanum demissum*. Similarly, several disease-resistant somaclones were recovered from 500 plants screened for resistance to early blight (*Alternaria solani*). On the basis of a toxin assay, five plants were initially identified as resistant. Four of then subsequently displayed resistance in the field.

Somaclonal variation has been confirmed in several other important potato cultivars, including Maris Band (36) and the widely grown European variety Bintje (35). In these experiments and those of Shepard and colleagues, protoplast culture was used to produce somaclonal variants. Variation among potato somaclones is not, however, a consequence of the protoplast technique. That plants derived from a single callus vary indicates that variation arose during callus growth (36). Moreover, somaclonal variation has been observed in plants regenerated from leaf discs and rachis and petiole explants of the cultivar Desire (38). The recovery of Variation in potato without protoplast culture is important, because plant regeneration from leaf or other tissue explants is far simpler than that from protoplast culture.

Other crops

Somaclonal variation in sugarcane, particularly its role in selecting for disease resistance, has been extensively documented (11, 16, 17, Table 1). Several experiment stations have begun to use somaclonal. variation routinely in plant breeding.

In forage species, variation generated during tissue culture is beginning to directly improve plants. In alfalfa (Medicago sativa) somaclonal variants that have improved yield or enhanced protein quality are being incorporated into breeding programs, (26; Bingham, pers. comm.). Research using somaclonal variation in such inportant tropical forage legumes as Stylosanthes has begun.

Among vegetable crops amenable to tissue culture, tomato and lettuce reveal somaclonal variation. Some 13 different nuclear mutations have been recovered among the progeny of on 230 regenerants of tomato (7). These mutations, which had simple Mendelian inheritance, affected fruit color, pedicel jointediness, male fertility, chloropbyll formation, and growth habit. Plants regenerated from. protoplasts of lettuce also possess frequent mutations (6; Mickelmore, pers. comm.).

MECHANISMS IN SOMACLONAL VARIATION

Extensive genomic flux occurs in eukaryotes, e.g., repeated DNA, sequences can readily be amplified or reduced in copy number, many genes are organized in multigene families, altered methylation patterns can be inherited. DNA replication is disturbed by altered nucleotide pools and active gene can be silenced or silent genes activated by mutations in associated noncoding sequences. Nucleotide sequence insertions resulting from DNA tranposition cause heritable changes in gene expression in several ways. The known consequences of DNA transposition include insertional inactivation of structural genes, altered regulation of a gene by either reducing or increasing its level of expression, coincident transfer of unique sequence genes to new positions where expression and developmental timing may be different, and generation of sequence duplications and deficiencies.

There is no molecular proof that these mechanisms cause somaclonal variation, but recent evidence does indicate that "genomic shock" at the chromosomal level rises significantly during tissue culture and that this is reflected in regenerated plants. Mitotic identification of aneuploid somaclones and meiotically identifiable chromosome rearrangements have heen documented in barley (24), Haworthia (22), oats (20), wheat (12), triticale (2), and garlic (21). They include reciprocal and nonreciprocal interchanges, inversions, partial chromosome loss, and duplications.

There is little evidence correlating chromosomal aberrations and gene mutations among somaclones and their progeny. In our analysis of somaclonal variation in wheat (14), many mutants have "normal" mitotic and meiotic karyotypes. We believe that chromosomal rearrangements among plants regenerafed from culture reflect somaclonal variation, and are not caused by gene mutations.

Alien gene introgression

The increase in genome rearrangement during tissue culture provides a new opportunity for alien gene introgression, which

can help widen the crop germplasm base, particularly in wide crosses where crop and alien chromosomes cannot replicate through meiosis.

Introgression of genes may be better achieved by imposing one or more cell culture cycles on interspecific hybrid material. The enhanced somatic genome exchange would likely produce regenerants where part of the alien genome has been somatically "recombined" into the chromosomes of the crop species. Repeated cell culture plant regeneration cycles coupled with in vitro or in vivo screening could provide plants with fewer contaminating alien genomes than routine backcrossing.

Already several laboratories use tissue culture of wide cross hybrids in cereals and grasses to obtain novel genotypes. The crosses include *Lolium perenne/L. multiflorum*, *L. multiflorum/Festuca arundinacea*, *H. vulgare/Secale cereale*, *Triticum crassum* /*H. vulgare*, and *Saccharum/Zea* and triticales.

Protoplast fusion, at least in some dicot families, is increasingly recognized as a means to widen our major crops' germplasm base (33). Somaclonal variation will doubtless be important in somatic hybridizations to transfer useful genes from alien species into crop plants.

CONCLUSION

Somaclonal variation is a novel way of generating potentially useful genetic variability. Research with major crop species has already shown that many somaclonal variants have agronomic value. However, somaclonal variation is not a panacea for plant improvement: it must be combined with efficient in vitro or in vivo selection and plant breeding programs. Perhaps one of the greatest benefits of somaclonal variation will be the broadening of the genetic base of our crop plants.

REFERENCES CITED

1. Ahloowalia, B.S. 1982. Plant regeneration from callus culture in wheat. Crop Sci. 22:405-410.
2. Armstrong, K.C., C. Nakamura, and W.A. Keller. 1983. Karyotype instability in tissue culture regenerants of triticale (X *Triticosecale* Wittmack) cv. 'Welsh' from 6-month-old callus cultures. Z. Pflanzenzuchtg 91:233-245.
3. Brettell, R.I.S., M.F. Conde, and D.R. Pring. 1982. Analysis of mitochondrial DNA from four fertile maize lines obtained from a tissue culture carrying Texas cytoplasm. Maize Genet. Coop. Newsl. 56:13-14.
4. Earle, E.D., and Y. Demarly, eds. 1982. Variability in Plants Regenerated from Tissue Culture. Praeger, New York.
5. Edallo, S., C. Zucchinali, M. Perenzin, and F. Salamini. 1981. Chromosomal variation and frequency of

spontaneous mutation associated with in vitro culture and plant regeneration in maize. Maydica 26:39-56.

6. Engler, D.E., and R.G. Grogan. 1983. Isolation, culture and regeneration of lettuce leaf mesophyll protoplasts. Plant Sci. Lett. 28:223-229.

7. Evans, D.A., and W.R. Sharp. 1983. Single gene mutations in tomato plants regenerated from tissue culture. Science 221:949-951.

8. Evans, L.T. 1983. Raising the yield potential: by selection or design? Pages 371-389 in Genetic Engineering in Plants. Kosuge, T., C.P. Meredith, and A. Hollaender, eds. Plenum Press, New York.

9. Fukai, K. 1983. Sequential occurrence of mutations in a growing rice callus. Theor. Appl. Genet. 65:225-230.

l0. Green, C.E. 1977. Prospects for crop improvement in the field of cell culture. Hortsci 72:7-10.

11. Heinz, D.J., M. Krishnamurthi, L.G. Nickel, and A. Maretzki. 1977. Cell, tissue and organ culture in sugarcane improvement. Pages 3-17 in Applied and fundamental aspects of plant, cell, tissue and organ culture. Reinert, J., and Y.P.S. Bajaj, eds. Springer, Berlin.

12. Karp, A., and S.E. Maddock. 1984. Chromosome variation in wheat plants regenerated from cultured immature embryos. Theor. Appl. Genet. 67:249-255.

13. Kemble, R.J., R.B. Flavell, and R.I.S. Brettell. 1982. Mitochondrial DNA analysis of fertile and sterile maize plants from tissue culture with the Texas male sterile cytoplasm. Theor Appl. Genet. 62:213-217.

14. Larkin, P.J., S.A. Ryan, R.T.S. Brettell, and W.R. Scowcroft. 1984. Heritable somaclonal variation in wheat. Theor. Appl. Genet. 67:443-456.

15. Larkin, P.J., and W.R. Scowcroft. 1981. Somaclonal variation - a novel source of variability from cell cultures for plant improvement. Theor. Appl. Genet. 60:197-214.

16. Larkin, P.J., and W.R. Scowcroft. 1983. Somaclonal variation and eyespot toxin tolerance in sugarcane. Plant Cell Tiss. Org. Cult. 2:111-121.

17. Liu, M.C. 1981. In vitro methods applied to sugar cane improvement. Pages 799-323 in Plant tissue culture. T.A. Thorpe, ed. Academic Press, New York.

18. Maddock, S.E., V.A. Lancaster, R. Risiott, and J. Franklin. 1983. Plant regeneration from cultured immature embryos and inflorescences of 75 cultivars of wheat (Triticum aestivum). J. Exp. Bot. 34:915-926.

19. McCoy, T.J., and R.L. Phillips. 1982. Chromosome stability in maize (Zea mays) tissue cultures and sectoring in some regenerated plants. Can. J. Genet. Cytol. 24:559-565.

20. McCoy, T.J., R.L. Phillips, and H.W. Rines. 1982. Cytogenetic analysis of plants regenerated from cat (Avena sativa) tissue cultures; high frequency of partial chromosome loss. Can. J. Genet. Cytol. 24:37-50.

21. Novak, F.J. 1980. Phenotype and cytological status of plants regenerated from callus cultures of Allium sativum L. Z. Pflanzenzucht. 84:250-260.
22. Ogihara, Y. 1981. Tissue culture of Haworthia. Part 4: Genetic characterisation of plants from callus. Theor. Appl. Genet. 60:353-363.
23. Oono, K. 1981. In vivo methods applied to rice. Pages 273-298 in Plant Tissue Culture. T.A. Thorpe, ed. Academic Press, New York.
24. Orton, T.J. l980. Haploid barley regenerated from callus cultures of Hordeum vulgare x H. jubatum. J. Hered. 71:780-782.
25. Ozias-Akins, P., and I.K. Vasil. 1982. Plant regeneration from cultured immature embryos and inflorescences of Triticum aestivum L. (wheat): evidence for somatic embryogenesis. Protoplasma 110:95 – 105.
26. Reisch, B., and E.T. Bingham. 1961. Plants from ethionine – resistantalfalfa tissue cultures: Variation in growth and morphological characteristics. Crop Sci 21:783 – 788.
27. Scowcroft, W.R., and P.J. Larkin. 1984. Somaclonal variation, cell selection and genotype improvement. In Comprehensive biotechnology. Vol. 3. Robinson, C.W., and H.J. Howells, eds. Pergamon Press, Oxford (in press)
28. Scowcroft, W.R., P.J. Larkin, and R.I.S. Brettell. 1983. Genetic variation from tissue culture. Pages 139 – 162 in The use of tissue culture and protoplasts in plant pathology. B.J. Deverall, and J. Helgeson, eds. Academic Press, Sydney.
29. Schaeffer, G.W. 1982. Recovery of heritable variability in anther – derived doubled – haploid rice. Crop Sci. 22:1160-1164.
30. Sears, R.G., and E.L. Deckard. 1982. Tissue culture variability in wheat: callus induction and plant regeneration. Crop Sci. 22:546 – 550.
31. Secor, G., and J.F. Shepard. 1981. Variability of protoplast derived potato clones. Crop Sci. 21:102 – 105.
32. Shepard, J.F. 1981. Protoplasts as sources of disease resistance in plants. Ann. Rev. Phytopathol. 19:145 – 166.
33. Shepard, J.F., D. Bidney, T. Barsby, and R. Kemble. 1983. Genetic transfer in plants through interspecific protoplast fusion. Science 219:683 – 688.
34. Shepard. J.F., D. Bidney, and F. Shahin. 1980. Potato protoplasts in crop improvement. Science 208:17 – 24.
35. Sree Ramulu, K., P. Dijkhuis, and S. Roest. 1983. Phenotypic variation and ploidy levels of plants regenerated from protoplasts of tetraploid potato (Solanum tuberosum L. cv. 'Bintje'). Theoret. Appl. Genet. 65:329 – 338.
36. Thomas, E., S.W.J. Bright, J. Franklin, V.A. Lancaster, B.J. Niflin, and R. Gibson. 1982. Variation amongst

protoplast derived potato plants (Solanum tuberosum cv. "Maris Bard"). Theor. Appl. Genet. 62:65-68.

37. Umbeck, P.E., and B.G. Gengenbach. 1983. Reversion of male-sterile T-cytoplasm maize to male fertility in tissue culture. Crop Sci. 23:584-588.

38. Van Harten, A.M., H. Bouter, and C. Rroertjes. 1981. In vitro adventitious bud techniques for vegetative propagation and mutation breeding of potato (Solanum tuberosum L.). II. Significance for mutation breeding. Euphytica 30:l-8.

CELL CULTURE ISOLATION AND CHARACTERIZATION OF AGRONOMICALLY USEFUL MUTANTS OF HIGHER PLANTS

P. MALIGA

Advances of the past decade in cell culture selection for agronomically useful mutants are reviewed. The mutations include resistance to herbicides or diseases and significantly increased levels of free amino acids. Early predictions concerning the requirements for successful cell culture selection are evaluated. The significance of unexpected findings such as tissue culture-induced variability and isolation of stable cytoplasmic mutants is discussed. We describe the rescue by cell fusion of nuclear and cytoplasmic mutations, from tissue culture lines into fertile plants, a method to be applied in crops in the future. We need to improve the cell culture systems in crops and further define selection criteria in culture to modify agronomic traits.

About 10 yr ago, there was much enthusiasm about using cultured plant cells for mutant selection (11, 29). We must now ask: To what extent was that enthusiasm warranted? What can we expect from the future? This paper focuses on producing genetically altered plants through selection in culture. Examples are mentioned only to illustrate the principles. Mutant selection in cultured plant cells has been reviewed elsewhere (12, 30, 33). Specific problems including the sources and nature of heritable variation in cell culture (36), tissue culture-induced variability for crop improvement (13, 27), and protoplast culture application in mutant selection (5, 32) have also been discussed.

CELL CULTURE SELECTION 1974-1984

Selection in single-cell culture

Protoplast suspensions, a true single-cell system for mutagenesis, were considered the best choice for screening variant phenotypes in culture (11). Most variants, however, including all with a potential commercial value, were obtained in other systems (12,

Advanced Genetic Sciences, Inc., 6701 San Pablo Ave., Oakland, California, USA.

30, 33). For cereals, there is no established system for regenerating plants from protoplasts: in maize, diploid embryo-derived morphogenic calluses were screened for dominant mutant traits (21, 22), and in barley, M2 embryo cultures were tested for recessive mutations (37). For convenience, screening was primarily carried out in suspension culture cells (12, 30, 33). Selection in protoplast cultures was essential, however, to isolate recessive nutritional auxotrophic mutants.

Screening of large numbers – recovery of rare phenotypes

Screenings of millions of cells are today routine, and they permit recovery of rare mutants. Nitrate reductase-deficient line appearing in Nicotiana tabacum at the frequency of about 10^{-7} are a good example (39). The lack of nitrate reductase activity was the result of two independent mutations; its low frequency may explain why the mutants could not be obtained at plant level (33).

Isolation of a range of auxotrophic phenotypes was not expected since only thiamine and arginine auxotrophs could be obtained at the intact-plant level (42). Phenotypes isolated in culture included requirement for adenine (24), histidine, tryptophan, nicotinic acid (19, 20, 45), isoleucine (47), leucine, and uracil (46). The cell culture isolates have an absolute nutritional requirement whereas the auxotrophs isolated at plant level were leaky. Screening in haploid protoplast cultures and effective mutagenesis were the key factors in the cell culture work. Any colony not of single-cell origin and containing wild-type cells would behave as wild type in the tests used. The limited success at plant level could be attributed to the small numbers tested. Also, the auxotrophic embryos may have died from insufficient metabolites in the vascular sap. In cell culture this problem does not exist because the embryos can take metabolites directly from the medium. Seedling screening was indirect, based on chlorophyll deficiency, and not on nutritional requirements.

Sexual transmission of traits selected at the cellular level

Phenotypic changes in culture may be based on mutation or epigenetic changes; the latter are not transmitted sexually. Initial concern that most altered phenotypes selected in cell culture would be epigenetic, and would not be transmitted through meiosis was unwarranted. The proven epigenetic cases are few compared with the number of lines in which inheritance studies confirmed mutation as the origin of phenotypic change (33, 36). The mutants satisfactorily characterized genetically are listed in Table 1. Lack of sexual transmission in most cases can be explained by improper experimentation (33). An additional complicating factor is that novel phenotypes observed in tissue culture are not necessarily expressed in plants (54).

Table 1. Genetically characterized mutants isolated in cell culture.

Tissue culture phenotype	Species	Mode of inheritance[a]	Reference
Resistance to methionine sulphoximine	*Nicotiana tabacum*	Mendelian, R. S	10
Resistance to *Helminthosporium maydis* toxin	*Zea mays*	Maternal	6, 21
Chlorate resistance	*N. tabacum*	Mendelian, R	39
	N. plumbaginifolia	Mendelian, R	41
Resistance to valine	*N. tabacum*	Mendelian, D. S	4
Resistance to lysine+threonine	*Zea mays*	Mendelian, D	22
Glycerol utilization	*N. tabacum*	Mendelian, D	15
Resistance to streptomycin	*N. tabacum*	Maternal	34,52
	N. sylvestris	Mendelian, R	31
Resistance to lincocymicn	*N. plumbaginifolia*	Maternal	16
Resistance to picloram	*N. tabacum*	Mendelian, D. S	14

[a]D = dominant, R = recessive, S = semidominant.

UNPREDICTED FINDINGS

Genetic variability induced in tissue culture

Genetic variability is more frequent, or more readily detectable, in tissue culture regenerates than in seed-grown plants. Plants regenerated from old culture may be different from and inferior to plants from which the culture was initiated, but most plants regenerated from new cultures are exact copies of the originals. Some of the tissue culture-induced changes may be valuable (27), although the range of new, useful phenotypes recoverable in this way is probably limited (33). The spectrum of tissue culture-induced phenotypes is currently being investigated in a number of crops.

Isolation of stable plastid mutants

The unicellular alga Chlamydomonas, in which plastid-coded antibiotic resistance mutations have been obtained, has only a single chloroplast. Flowering-plant cells, however, contain several plastids (there are about 80-100 plastids in a tobacco leaf mesophyll cell). Because of this, the possibility of

isolating stable, plastid-coded mutations in flowering plants was doubted. Nicotiana has, however, yielded stable, plastid-coded streptomycin and lincomycin resistance mutants (16, 34, reviewed in 33).

Plastid mutants resistant to triazine herbicides were found in weed populations, but are extremely rare (28). Herbicide spraying on a scale leading to resistant weeds is unacceptable. Successful isolation of the antibiotic resistance mutations suggests that cell culture selection is the best system for this.

RESCUE OF NEW MUTATIONS INTO NORMAL PLANTS

Many of the regenerated plants exhibit tissue culture-induced chromosome abnormalities, which result in slower growth, abnormal morphology, partial or full sterility, and irregular marker transmission (12, 33, 36). This problem can be reduced by screening for new mutations in new cultures. Alternatively, the mutant chromosome can be backcrossed into a normal nuclear background. Backcrossing will probably be a normal practice since traditional breeding should be more economical than isolating the mutations over and over again in each cultivar. In crops in which only specific genetic lines can be handled in culture, backcrossing has no alternative.

If the cell lines lost their ability to regenerate, or were able to regenerate but produced only sterile flowers, cell fusion could rescue the new mutations. The case of the nitrate reductase-deficient Nicotiana plumbaginifolia lines is an example (35). Mutant protoplasts were fused with recessive pigment mutants (regenerating) of the same species. In somatic hybrid cultures, the regeneration ability was restored. In the seed progeny of the somatic hybrids, segregation was shown for both the pigment mutation and the nitrate reductase deficiency.

Cell fusion also permits rescuing cytoplasmic mutations. Through it lincomycin resistance from sterile Nicotiana sylvestris plants was transferred into Nicotiana plumbaginifolia (17).

MUTANTS WITH POTENTIAL AGRONOMIC VALUE

Herbicide resistance mutants

Selection for herbicide resistance in cell culture allows the production of new forms of existing cultivars identical to the original plants except their newly acquired herbicide resistance.

Selection for resistance to picloram in N. tabacum (14) resulted in the isolation of three dominant (PmR1, PmR2, PMR7) and one semidominant (PmR6) alleles. The mutations were located in three linkage groups. Plants were tested for resistance after calluses were initiated from the seedlings; resistance was expressed in in vitro-grown plants. Paraquat-tolerant cell lines were also isolated in N. tabacum (38). Paraquat tolerance could

be demonstrated in regenerated plants and in their seed progeny; and it correlates with tolerance for diquat, another putative free radical inducer (23). Paraquat-tolerant lines were also isolated in tomato callus culture (50). Paraquat tolerance was expressed in plants when sprayed; it was seed transmitted and dominant.

Amitrole (3-amino-1,2,4-triazole) is a broad-spectrum herbicide toxic to nonphotosynthetic cultured cells (1, 48). The plants' tolerance for the herbicide can be rated by the extent of chlorosis in new growth (48). Amitrole-tolerant tobacco lines have been isolated in culture (1, 48). Plants regenerated from a diploid suspension were also tolerant, and inheritance could be followed through two sexual generations. Inheritance was biparental, but no simple Mendelian ratios were observed (48).

Increased free amino acids

Improving crop nutritional value by increasing their concentration of specific free amino acids was the goal of research on amino acid and amino acid analog-resistant mutants (12, 30, 53). The regeneration of plants from the overproducing cell lines and the study of free amino acid accumulation in plants developed from that work.

A lysine+threonine (L+T)-resistant maize line, LT19, was selected in tissue culture and regenerated into plants (22). Biosynthesis of lysine-threonine-methionine-isoleucine (aspartate family) is subject to feedback inhibition by L+T. Altering aspartate pathway regulation resulted in a 75- to 100-fold increase over L+T-sensitive plants in free threonine content of homozygous Ltr*-19 plant kernels. Free threonine overproduction increased the total threonine content in homozygous Ltr* by 33-59%. L+T resistance was inherited as a single dominant nuclear gene (22).

Nature M_2 barley embryos were screened under aseptic conditions (37). Because the embryos contain little reserve protein, the young plants are forced to synthesize amino acids de novo and are thus very susceptible to externally supplied inhibitors. The L+T-resistant mutants R3004, R3202, and R2501 partially or completely lost feedback regulation of one or the other of two aspartate kinase isoenzymes (separate unlinked dominant genes) by lysine plus adenosylmethionine. R2501 and R3004, but not R3202, accumulated threonine in the seeds (7, 9, 43). A greater than 12-fold increase in the threonine content in the free amino acid pool occurred; it corresponded to a 7% increase in the total (free amino acid plus protein) seed threonine (7). The proline content was increased three- to six-fold in the leaves of 4-hydroxy-L-proline barley plants, possibly because of altered feedback regulation (25, 26).

Cell culture selection could, then, be used to increase the concentration of specific free amino acids in plants; it should be useful in crops in which increase in one or a few amino acids is desired. To what extent tissue-specific expression may interfere with such a program is not yet clear, but there has been no problem in maize. Differential gene expression occurred in a 5-methyltryptophan-resistant (tryptophan-overproducing) tobacco

line: altered feedback control of tryptophan biosynthesis could be shown in callus but not in the regenerated plants. In callus initiated from the plant, however, the altered enzyme was expressed again (54). Another example is the organ-specific expression of a mutation in an uptake system in barley (8).

Salt and water stress tolerance

Improved salt and water-stress tolerance would be of obvious value, and will be treated in a separate lecture (40).

Disease tolerance

Plant diseases are caused by different organisms (fungi, bacteria, viruses). Through selection for tolerance for chemicals and toxins, rational systems to isolate tolerant lines have been designed. Host-specific toxins are the most promising for tissue culture selection for disease resistance: however, the number of well-characterized toxins of this type is limited (18).

The first paper to describe isolation of disease-resistant plants was by Carlson (10), who obtained tobacco mutants resistant to wildfire disease by selecting cell lines resistant to methionine sulfoximide. This compound produces the same chlorotic haloes on tobacco leaves as does the bacterial wildfire toxin. The regenerated plants were resistant to the *Pseudomonas tabaci* infection, and inherited the resistance as a single semidominant trait. Selection for tolerance for toxins from *Pseudomonas syringae* pv. *tabaci* (causing wildfire disease) and from *Alternaria alternaria* pathotype tobacco (causing brown spot) has recently been reported (49). Surprisingly, close to 1% of the protoplast-derived calluses were toxin resistant after 2 selection cycles. Of the plants regenerated from the resistant calluses, about 10% were resistant to the pathogen itself.

Selection for tolerance for *Helminthosporium* T-toxin (a host-specific toxin) in maize cultures was reported by Gengenbach and colleagues (21). Embryogenic maize callus was exposed to the toxin; from the toxin-susceptible and male-sterile plants, toxin-tolerant male-fertile forms were obtained. Both traits were inherited maternally. It is possible to obtain tolerant, fertile plants from susceptible sterile plants after their calluses have been exposed to T-toxin (6). However, a significant proportion [more than 30% (6), and 5% (51)] of the regenerates were T-toxin tolerant even if no selection pressure had been applied. The value of T-toxin selection in maize cultures is, therefore, questionable. Stable cytoplasmic reversion to male fertility and T-toxin tolerance has not been documented for sexually propagated, T-cytoplasm maize.

Selection for tolerance for the culture filtrate of *Phoma lingam* resulted in resistant *Brassica napus* plants (44). Again, resistant lines were obtained in the nonselected material as well.

The nonspecific toxin of *Phytophtora infestans* (the potato late blight pathogen) was used for tolerance selection in *Solanum tuberosum* culture. Potato callus tolerant of the culture filtrate was selected and regenerated into plants (2). The plants themselves had an improved tolerance of the pathogen (3).

FUTURE DIRECTIONS

No tissue culture-selected mutation has been incorporated into commercial cultivars. Why? The mutations are recent and few; most of them were isolated in model species in which they have no agronomic value. This lack of practical applicability may explain why no field testing of the herbicide-tolerant isolates was reported. For practical application, cell culture screening must be preceded by marketing research.

We must define selection criteria that, from cultured cell behavior, permit predicting the properties of regenerated plants. Some cases may require understanding the metabolic or structural basis of a phenotype. Some differentiated, complex functions are not manifested at cellular level (such as yield), and selection for such functions may not be directly feasible.

We must increase the number of crops in which cell culture selection can be performed. Screening for dominant traits in such relatively simple systems as embryo-derived callus is feasible in most crops, including the cereals. Further improvement of tissue culture systems in crops, however could permit more sophisticated manipulations, e.g., rescue of mutations by cell fusion or selection in haploid protoplast cultures.

REFERENCES CITED

1. Barg, R. and N. Umiel. 1977. Development of tobacco seedlings and callus cultures in the presence of amitrole. 2. Pflanzenphysiol. 83:437-447.
2. Behnke, M. 1979. Selection of potato callus for resistance to culture filtrates of Phytophthora infestans and regeneration of resistant plants. Theor. Appl. Genet. 55:69-71.
3. Behnke, M. 1980. General resistance to blight of Solanum tuberosum plants regenerated from callus resistant to culture filtrates of Phytophthora infestans. Theor. Appl. Genet. 56:151-152.
4. Bourgin, J.P. 1978. Valin-resistant plants from in vitro selected tobacco cells. Mol. Gen. Genet. 161:225-230.
5. Bourgin, J.P. 1978. Protoplasts and the isolation of plant mutants. Pages 43-50 In Protoplasts 1983. I. Potrykus, C.T. Harms, A. Hinnen, R. Hutter, P.J. King, R.D. Shillito, eds. Basel, Birkhauser.
6. Brettel, R.I.S., E. Thomas, and D.S. Ingram. 1980. Reversion of Texas male-sterile cytoplasm in culture to give fertile T-toxin resistant plants. Theor. Appl. Genet. 58:55-58.
7. Bright, S.W.J., J.S.H. Kueh, J. Franklin, S.E. Rognes, and B.J. Miflin. 1982. Two genes for threonine accumulation in barley seeds. Nature 299:278-279.
8. Bright, S.W.J., J.S.H. Kueh, and S.E. Rognes. 1983. Lysine transport in two barley mutants with altered uptake of

basic amino acids in the roots. Plant Physiol. 72:821-824.

9. Bright, S.V.J., B.J. Miflin, and S.E. Rognes. 1982. Threonine accumulation in the seeds of a barley mutant with an altered aspartate kinase. Biochem. Genet. 20:229-243.
10. Carlson, P.S. 1973. Methionine sulfoximine-resistant mutants of tobacco. Science 180:1366-1368.
11. Carlson, P.S. 1973. The use of protoplasts for genetic research. Proc. Natl. Acad. Sci. USA 70 598-602.
12. Chaleff, R.S. 1981. Genetics of Higher Plants, Applications of Cell Culture. Cambridge University Press, New York.
13. Chaleff, R.S. 1983. Isolation of agronomically useful mutants from plant cell cultures. Science 219:676-682.
14. Chaleff, R.S., and M.F. Parsons. 1978. Direct selection in vitro for herbicide resistant mutants of Nicotiana tabacum. Proc. Natl. Acad. Sci. USA 75:5104-5107.
15. Chaleff, R.S., and M.F. Parsons. 1978. Isolation of a glycerol-utilizing mutant of Nicotiana tabacum. Genetics 89:723-728.
16. Cseplo, A., and P. Maliga. 1982. Lincomycin resistance, a new type of maternally inherited mutation in Nicotiana plumbaginifolia. Curr. Genet. 6:105-109.
17. Cseplo, A., F. Nagy, and P. Maliga. 1983. Rescue of a cytoplasmic lincomycin resistance factor from Nicotiana sylvestris into Nicotiana plumbaginifolia by protoplast fusion. Pages 126-27 in International Protoplast Symposiun Poster Proceedings. Basel.
18. Earle, E.D. 1982. Application of plant tissue culture in the study of host-pathogen interaction. Pages 45-62 in Application of plant cell and tissue culture to agriculture and industry. D.T. Tomes, B.E. Ellis, P.M. Harney, K.J. Kasha, and R.L. Petersen, eds. Univ. Guelph, Guelph.
19. Gebhardt, C., V. Schnebli, and P.J. King. 1981. Isolation of biochemical mutants using haploid mesophyll protoplasts of Hyosciamusmuticus. II. Auxotrophic and temperature sensitive clones. Planta 153:81-89.
20. Gebhardt, C., K. Shimamoto, G. Lazar, V. Schnebli, and P.J. King. 1983. Isolation of biochemical mutants using haploid mesophyll protoplasts of Hyoscyamus muticus III. General characterisation of histidine and tryptophan auxotrophs. Planta 159:18-24.
21. Gengenbach, B.G., C.E. Green, and C.M. Donovan. 1977. Inheritance of selected pathotoxin-resistance in maize plants regenerated from cell cultures. Proc. Natl. Acad. Sci. USA 74:5113-5117.
22. Hibberd, K.A., and C.E. Green. 1982. Inheritance and expression of lysine plus threonine resistance selected in maize tissue culture. Proc. Natl. Acad. Sci. USA 79:559-563.
23. Hughes, K.W., D. Negrotto, M.E. Daub, and R.L. Meeusen. 1984. Free-radical stress response in paraquat-sensitive and resistant tobacco plants. Environ. Exp. Bot. 24:151-157.

24. King, J., R.B. Horsch, and A.D. Savage. 1980. Partial characterization of two stable auxotrophic cell strains of Datura innoxia (Mill.). Planta 149:480-484.
25. Kueh, J.S.H., and S.W.J. Bright. 1981. Proline accumulation in a barley mutant resistant to trans-4-hydroxy-proline. Planta 153:166-171.
26. Kueh, J.S.H., and S.W.J. Bright. 1982. Biochemical and genetical analysis of three proline-accumulating barley mutants. Plant Sci. Lett. 27:233-241.
27. Larkin, P.J., and W.R. Scowcroft. 1981. Somaclonal variation - a novel source of variability from cell cultures. Theor. Appl. Genet. 60:197-214.
28. LeBaron, H.M. and J. Gressel, eds. 1982. Herbicide resistance in plants. Wiley, New York. 401 p.
29. Maliga, P. 1976. Isolation of mutants of cultured plant cells. Pages 227-231 in Cell genetics in higher plants. D. Dudits, G.L. Farkas, and P. Maliga, eds. Hungary Academic Science, Budapest.
30. Maliga, P. 1980. Isolation, characterization, and utilization of mutant cell lines in higher plants. Int. Rev. Cytol. Suppl. 11A:225-250.
31. Maliga, P. 1981. Streptomycin resistance is inherited as a recessive nuclear trait in a Nicotiana sylvestris line. Theor. Appl. Genet. 60:l-3.
32. Maliga, P. 1983. Protoplasts in mutant selection and characterization. Int. Rev. Cytol. Suppl. 16:161-167.
33. Maliga, P. 1984. Isolation and characterization of mutants in plant cell culture. Ann. Rev. Plant Physiol. 35:519-542.
34. Maliga, P., A. Breznovits, and L. Marton. 1975. Non-Mendelian streptomycin resistant mutant with altered chloroplasts and mitochondria. Nature 255:401-402.
35. Marton, L., G. Biasini, V. Sidorov, and P. Maliga. 1983. Nitrate reductase deficient plants in the progeny after selfing complemented somatic hybrids of Nicotiana plumbaginifolia mutants. Pages 100-101 in International protoplast symposium Poster Proceedings, Basel.
36. Meins, F., Jr. 1983. Heritable variation in plant cell culture. Ann. Rev. Plant Physiol. 34:327-346.
37. Miflin, B.J., S.W.J. Bright, S.E. Rognes, and J.S.H. Kueh. 1982. Amino acids, nutrition and stress: the role of biochemical mutants in solving problems of crop quality. Pages 391-414 in Genetic engineering of plants. C.P. Meredith and A. Hollaender, eds. Plenum, New York.
38. Miller, O.K. and K.W. Hughes. 1980. Selection of paraquat-resistant variants of tobacco from cell cultures. In Vitro 16:1085-1091.
39. Muller, A.J. 1983. Genetic analysis of nitrate reductase deficient tobacco plants regenerated from mutant cells. Evidence for duplicate structural genes. Molec. Gen. Genet. 192:275-281.
40. Nabors, M.W. 1983. Increasing the salt and drought tolerance of crop plants. In Current topics in plant biochemistry and physiology. D.D. Randall, ed. 2:165-84. University of Missouri Press, Columbia.

41. Negrutiu, I., R. Dirks, and M. Jacobs. 1983. Regeneration of fully nitrate reductase-deficient mutants from protoplast culture of Nicotiana plumbaginifolia (Viviani). Theor. Appl. Genet. 66:341-347.
42. Redei, G.P. 1975. Arabidopsis as a genetic tool. Ann. Rev. Genet. 9:111-127.
43. Rognes, S.E., S.W.J. Bright, and B.J. Miflin. 1983. Feedback insensitive aspartate kinase isoenzymes in barley mutants resistant to lysine plus threonine. Planta 157:32-38.
44. Sacristan, M.D. 1982. Resistance to Phoma lingugm of plants regenerated from selected cell and embryogenic cultures of haploid Brassica napus. Theor. Appl. Genet. 61:193-200.
45. Shimamoto, K., and P.J. King. 1983. Isolation of a histidine auxotroph of Hyosciamus muticus during attempts to apply BUdR enrichment. Molec. Gen. Genet. 189:69-72.
46. Sidorov, V.A., and P. Maliga. 1982. Fusion-complementation analysis of auxotrophic and chlorophyll-deficient lines isolated in haploid Nicotiana plumbaginifolia protoplast cultures. Molec. Gen. Genet. 186:328-332.
47. Sidorov, V.A., L. Menczel, and P. Maliga. 1981. Isoleucine-requiring Nicotiana plant deficient in threonine deaminase. Nature 294:87-88.
48. Singer, S.R., and C.N. McDaniel. 1984. Selection of amitrole tolerant tobacco calli and expression of this tolerance in regenerated plants and progeny. Theor. Appl. Genet. 67:427-432.
49. Thanutong, P., I. Furusawa, and M. Yamamoto. 1983. Resistant tobacco plants from protoplast-derived calluses selected for their resistance to Pseudomonas and Alternaria toxins. Theor. Appl. Genet. 6:209-215.
50. Thomas, B.R., and D. Pratt. 1982. Isolation of paraquat-tolerant mutants from tomato cell cultures. Theor. Appl. Genet. 63:169-176.
51. Umbeck, P.F., and B.G. Gengenbach. 1983. Reversion of male-sterile T-cytoplasm maize to fertility in tissue culture. Crop Sci. 23:584-588.
52. Umiel, N. 1979. Streptomycin resistance in tobacco: III. Test on germinating seedlings indicates cytoplasmic inheritance in the St-R701 mutant. Z. Pflanzenphysiol. 92:295-301.
53. Widholm, J.M. 1977. Selection and characterization of amino acid analog resistant plant cell cultures. Crop Sci. 17:597-600.
54. Widholm, J.M. 1980. Differential expression of amino acid biosynthetic control isozymes in plants and cultured cells. Pages 161-166 in Plant cell cultures: results and perspectives. F. Sala, R. Parisi, R. Cella, O. Ciferri, eds. Elsevier/North Holland, Amsterdam.

TISSUE CULTURE OF CEREAL CULTIVARS WITH INCREASED SALT, DROUGHT, AND ACID TOLERANCE

M. W. NABORS and T. A. DYKES

The Tissue Culture for Crops Project at Colorado State University has produced NaCl-tolerant tobacco, oat, and rice plants. In each case, the selected tolerance is inheritable and stable in regenerated plant progeny. The project has also developed high-frequency, long-term plant regeneration methods from tissue cultures of wheat, rice, pearl millet, proso millet, and oats. From one starting embryo of rice, we can produce up to 127 500 plants in 6 mo. Over 4 000 plants have already been regenerated from NaC1-tolerant tissue cultures of rice, wheat, and pearl millet.

NEED FOR STRESS-TOLERANT CULTIVARS

Of the world's available land area (14 billion ha), only around 3.2 billion ha are arable or potentially arable (with mininal environmental modification) (26). Approximately 25% of this arable land is subject to excess salinity, a problem both in arid regions and in some areas near the ocean (26). Another 25% of arable land suffers from excess rainfall and, therefore, soil acidity (27). Rain water contains dissolved carbon dioxide in the form of carbonic acid. As hydrogen ion concentrations build up in soils, other more useful cations are leached from the soil particles; in addition, normally insoluble salts, such as aluminum, come into the soil solution.

Of the 1.4 billion ha currently cultivated, 14% are irrigated and produce half of the world's food (2). Prospects for markedly increasing the extent of irrigation are not bright, since 80% of the world's readily available fresh water is already used in agriculture. Also, all irrigation systems, no matter how carefully managed, gradually add dissolved salts to the soil, thereby limiting their useful lifetimes.

The financial burden of stressed soils in modern agriculture can be appreciated by noting the economic impact of salination in a single river basin. If it is assumed that 25% of the world's cultivated land lies in extremely arid regions, the Colorado River in the United States drains approximately one sixtieth of that arid land. It begins in the Rocky Mountains and, after flowing through the southwestern United States, enters Mexico,

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Professor and research associate, Department of Botany and Plant Pathology, Colorado State University, Fort Collins, Colorado 80523, USA.

where it drains into the Gulf of California. Dissolved salts at the headwaters are about 50 ppm. At the Imperial Dam in California, the salinity has already reached more than 1,000 ppm. Of this salt loading, 47% is natural, 49% due to irrigation, and 4% due to industry and municipal sources (4).

The combined salt loading makes the water at the Imperial Dam considerably more saline than the 500 ppm salinity standard for drinking water set by the US Environmental Protection Agency (EPA) and the 800 ppm generally recognized as a threshold for agricultural damage. The current yearly agricultural damage in the Colorado River Basin (calculated in 1984 US dollars) is almost $119 million (23). Worldwide (using the one-sixtieth figure mentioned earlier), the amount is more than $7 million. These figures ignore nonagricultural damage and the damage by salinity from brackish water contamination near the ocean.

Energy-intensive modern agriculture utilizes considerable economic resources needed either to further increase agricultural production or to augment other sectors of national economies. It is currently estimated that energy inputs per hectare of maize production are equivalent to 757 litres gasoline (21). Agriculture can be viewed either as an energy-intensive enterprise designed to modify the environment to suit the plant, or as an exercise in genetics that modifies the plant to suit available environments.

The goal of the Tissue Culture for Crops Project, sponsored by USAID at Colorado State University, is to use plant tissue culture to increase the stress tolerance in rice, wheat, and pearl millet (15). Plants with increased salt, drought, or acid tolerance would have several important effects:

- They would allow production increases on currently arable but stressed land, while lowering costs by decreasing the amounts of irrigation and soil liming required.
- They would allow production on currently nonarable land requiring more soil amelioration than can currently be provided.
- They would free financial and human resources for use in other areas vital to local and national economies.
- They would allow subsistence farmers to produce additional food for their own use, for use by others in the area, or for sale as cash crop.

Tissue culture can increase the stress tolerance of plants because most plant cells contain a complete species genome and thus are totipotent. Provided individual cells can produce at least shoots, large cell populations can be maintained and selected for agriculturally useful mutants. A cubic centimeter of cell suspension or callus (a solid disorganized mass of plant cells) contains perhaps 50 000 cells. Thus tissue culture miniaturizes and streamlines the selection process.

METHODS FOR OBTAINING SALT-TOLERANT CELLS AND REGENERATED PLANTS

Our work on developing salinity tolerance began in 1972 with tobacco. Tobacco is tissue culture's laboratory white rat. By 1975, we were able to show how salt-tolerant lines of cells could be obtained (17). As has been well documented in recent years (8), all cells can physiologically adapt to various stresses. Thus any method for obtaining salt-tolerant cells must be able to distinguish between cells adapted to their environment, and those that are true genetic variants (and will pass the adapted trait to their progeny). The distinction is accomplished in two ways:

1. The selection medium must stress the cells sufficiently so that physiological adaptation is not useful as a long-term survival mechanism: The medium should kill more than 95% of all cells or prevent their growth and division.
2. Selection must last long enough to allow rare genetic variants to repopulate the entire culture.
3. Once an adapted cell line is obtained, it should be reselected at even higher stress levels.

Our first selections used NaCl as a stressing agent. We repeated the selection process with nine increasing levels of NaCl. Each higher level eliminated growth and division of most of the cells and was followed by still further selection only when the growth of the cell lines had returned to a high rate. The total selection process required more than 18 mo.

In 1980, we described plants regenerated from the tolerant cultures (18). The plants coming directly from cultured cells were designated as parents (P). They were selfed to obtain the F_1 which was again selfed to obtain the F_2, etc. Both the F_1 and F_2 showed substantially increased tolerance for NaCl (at sea water concentrations). In addition, we found that the salt tolerance obtained was stable in the absence of NaCl for at least 7 mo in cultured cells and for 2 whole plant generations in entire plants (16).

Calculations on apparent mutation rates for increased NaCl have shown that our mutations behave as dominant alleles appearing in about one of every 500 000 cells. In whole plants, the tolerance is inheritable and does not fit a single Mendelian allele model for either the diploid or the tetraploid assumption (tobacco is an allotetraploid). That is not surprising considering the length of selection and the number of NaCl levels used. We are currently trying to determine the mode of transmission.

NaCl is the principal salt in saline soils but by no means the only one (1, 5). We began with only NaCl to avoid selecting for cells with more than one simultaneous genetic change. Our culture room at Colorado State University now has a capacity of 4

billion cells. At the time of our initial selections, however, our capacity was considerably smaller. One change occurring in 500 000 cells was relatively easy to find; two such simultaneous changes would have required examining 500 000 x 500 000 or 250 billion cells.

Some workers are concerned about the possibility of obtaining chimerical plants from tissue culture regeneration. This possibility exists only if plants arise not from single cells but from clumps of unrelated cells. In cereals, whether regenerated plants have a single or multicellular origin is still debated. For our purposes this debate is of theoretical interest only; a lengthy selection procedure ensures that all cells of the population are salt tolerant and that chimerical plants, regardless of origin, cannot result.

Plant regeneration from stress-tolerant cell cultures cannot be assumed to require the same media and cultural conditions as that from normal cultures. Considerable manipulation of our NaC1-tolerant tobacco cultures was required before large-scale, continual plant regeneration could be achieved. In some early cases, cultures were shifted back to saltless media to encourage the regeneration process (18). Our present lines of NaC1-tolerant tobacco cells have been in culture for more than 11 yr. Shoot regeneration is still frequent, hut obtaining rooted plants has recently become a problem requiring further experimentation with the media.

Selection for drought tolerance in our laboratory is accomplished in one of two ways:

1. testing for drought tolerance all plants with a salt tolerance increase, because many salt-tolerant plants are also naturally drought tolerant. (The salt-tolerant tobacco shows more tolerance for desiccation.)
2. adding to the medium high molecular-weight chemicals (such as polyethylene glycol 6,000 or various dextrans), which compete with the cells for available water in much the same manner as dry soil, as proposed by Nabors (15). While it appears that cultivars with differing degrees of drought tolerance also differ in ability to produce rapidly growing calluses on the media described, the value of this selection method has yet to be demonstrated.

To elicit tolerance for acid or aluminum, hydrogen or aluminum ions are added to the medium in much the same way as in NaCl selection. For both hydrogen and aluwinum, selection is easily accomplished in a cell suspension on liquid medium. Solid medium is unsatisfactory because the usual gelling agent (agar) does not function correctly with even moderate decreases in acidity (induced by either H+ or Al+++). (This problem can be solved by either microinjecting the cation into an already gelled medium and allowing it to diffuse throughout, or adding the cation to the medium just before final solidification.)

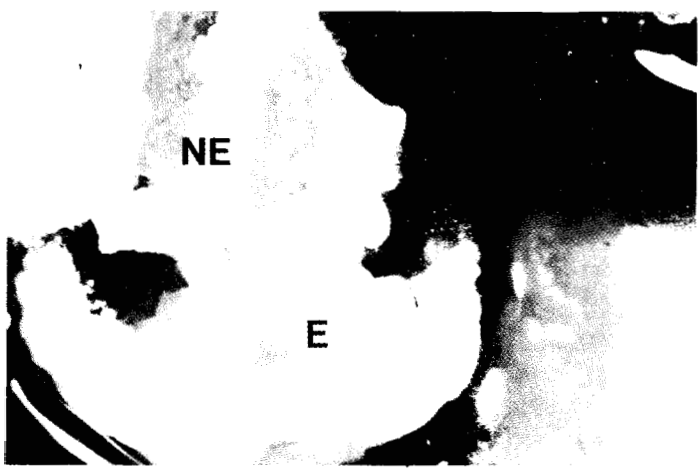
Whole-plant tolerance for NaCl and for other soil stresses can be based either on cells or on structural plant modifications

(23). While cell-based tolerance can be selected for in cell culture (assuming that the same genes functioning in cell culture function in the entire plant), tolerance based on structural modification may not he. If the biochemical basis of the structural modifications is known, cellular selection methods could be devised.

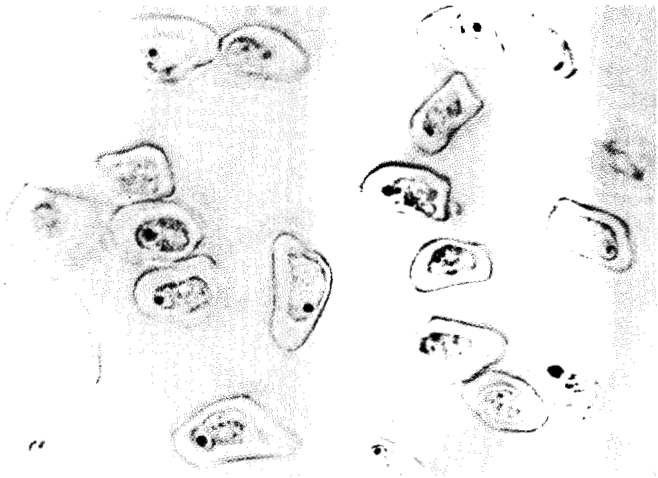
PLANT REGENERATTON FROM CEREAL TISSUE CULTURES

Having demonstrated that tobacco tissue culture can produce salt-tolerant cells and regenerated plants, we turned to the important food crops, the cereals. In the late 1970s plant regeneration from tobacco was routine, but that from cereal tissue cultures was sporadic and infrequent, and the required media and cultural conditions were ill defined (3). At the time of a 1980 IRRI planning conference, for example, no suitable rice plant regeneration methods from somatic cells existed, and the methods for plant regeneration from haploid pollen tended to produce albino plants (14).

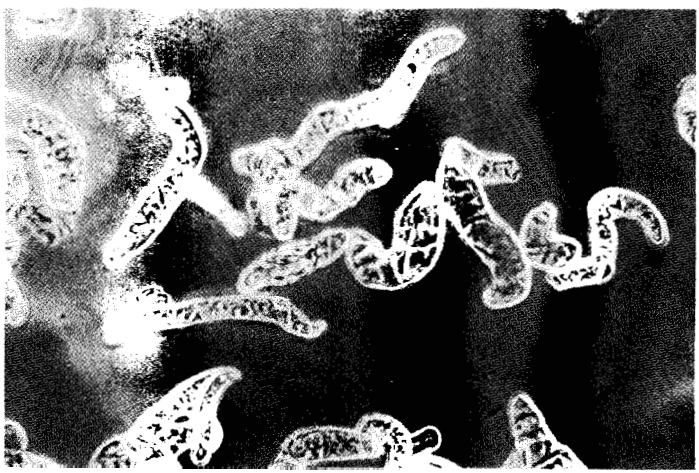
Many workers gradually realized that cereal tissue cultures can produce at least two cell types (Fig. 1) and that most available methods tended to produce cells with a very low capacity for plant regeneration (19). We have referred to the principal cell types as embryogenic (E) and nonembryogenic (NE). E cells (Fig. 2) are small and isodiametric with a diameter of around 50 cm. They resemble typical nonvacuolated or small vacuolated neristematic cells. NE cells (Fig. 3) are elongated (typically 50 mm by 400 mm or so) and contain large vacuoles easily distinguished by their refraction of light under a microscope.



1. Embryogenic (E) and nonembryogenic (NE) callus is clearly distinguishable on this proso millet callus,



2. Individual E cells are isodiametric and around 50 μm in diameter.



3. individual NE cells are elongated and around 50 × 400 μm.

Over the past 3 yr, we have been developing two types of media for wheat, rice, pearl millet, proso millet, oats, and maize (11, 10, 9, 12, 15, 20, 16, 19, 22, 23, 24, 25). First, we have tried to obtain a medium that favors the growth and division of E cells over NE cells. In all cases we have succeeded; some cases have found media that allow for E cell production exclusively. Second, we have tried to obtain a medium that allows rapid regeneration of E cells into plants. We have never found a medium permitting more than minimal plant regeneration from NE callus. For all cereal cultivars plant regeneration from E callus is frequent and long-lasting, whereas that from NE callus is essentially zero.

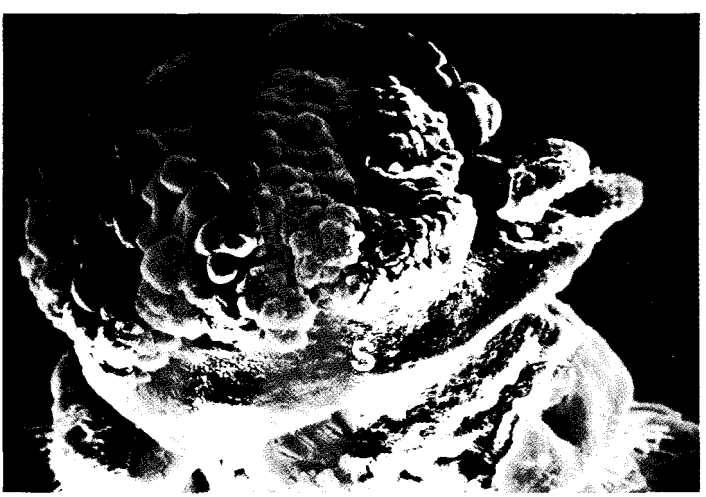
The best media for promoting E callus formation and plant regeneration in cereals tend to differ from species to species

and also from cultivar to cultivar. However certain generalizations can be made.

- An auxin such as 2,4-dichlorophenoxyacetic acid (2,4-D) or 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) is required to initiate callus formation from a seed or seed section, meristem, or leaf base.
- Auxins such as NAA or IAA are not effective in this initiation process.
- The concentration of 2,4-D or 2,4,5-T itself can affect not only overall callus formation but also the ratio of E to NE callus. In many cases, this ratio will be quite small; i.e., hardly any E callus will appear. In some cases, no observable E callus is formed for 6 or 8 wk.
- Adding other compounds to the medium markedly influences the rate of E callus formation. Promotive compounds include cytokinins (kinetin and benzylaminopurine), amino acids (tryptophan, glutamine, and proline [6]), other auxins (indoleacetic acid, IAA), ammonium (28), and abscisic acid (13).
- Plant regeneration from E callus is promoted by media without 2,4-D or 2,4,5-T but with IAA, cytokinins, proline, and ammonium.

Determining optimal media is a complex process since not only the concentrations, but also the interactions, of individual additives are important. We will discuss this process in more detail later for both rice and wheat.

Early successful cereal plant regeneration used immature embryos as starting material (7). When these embryos were placed with the scutellum facing away from the medium, the scutellum would produce a highly regenerative embryogenic callus consisting of many embryos. A scanning electron micrograph of a rice embryo with E callus on the scutellum is shown in Figure 4. We began to



4. Scanning electron micrograph of a rice embryo with embryogenic callus forming on the surface of the scutellum (S). The E callus consists of embryos at various stages of development.

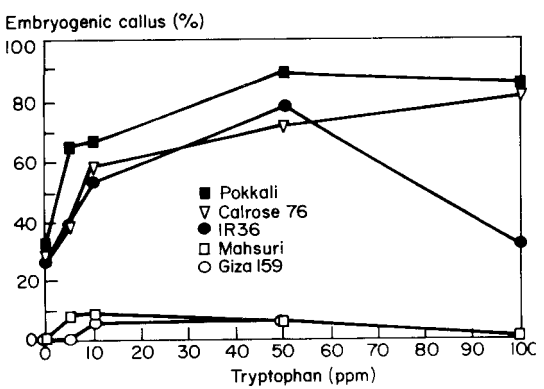
try to develop regeneration methods that could start with seeds instead of immature embryos. Seeds are more easily obtained and manipulated than immature embryos. Embryos require not only more time and manipulative skill but also the year-round maintenance of reproductive plants. Since our charge was to develop methods easily exported to less technologically developed laboratories, we needed the simplest and most repeatable methods possible.

We have been able to increase cereal plant regeneration rates from essentially zero to more than 100 000 plants from 1 seed in 6 mo. Also, our methods for all the cereals we use now permit maintaining regenerable cultures for more than 1 yr.

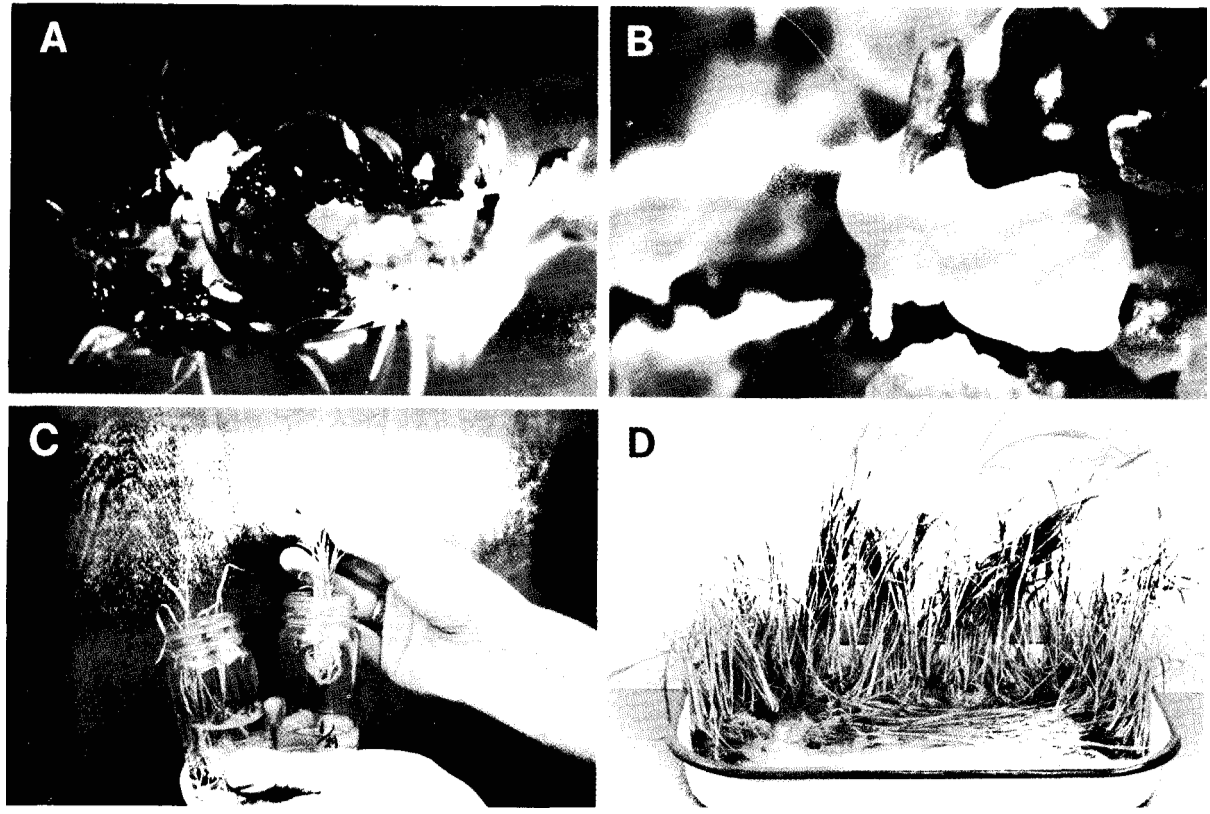
CEREAL TISSUE CULTURE METHODS FOR RICE

We use rice cultivars IR36, Mahsuri, Pokkali, Giza-159, and Calrose-76. In all cases, extremely low, nonpersistent E callus formation is obtained with media containing 2,4-D or 2,4,5-T as the only growth regulator. All our optimal E callus production media now contain a cytokinin whose optimal concentration is cultivar specific. For some cultivars the addition of tryptophan is important (Fig. 5). For Calrose-76, IAA can mimic the tryptophan effect (25), whereas for Pokkali it cannot (24). Light is an important variable affecting the amount and proportion of E callus obtained from a mature seed. In some cases the photo condition optimal for callus initiation must be altered for best growth and maintenance. We have not yet tested the effects either of proline or of increased ammonium levels. Abscisic acid appears to function by preventing cell elongation, which in effect transforms them from E to NE cells. This was noted by Green in maize suspensions and it appears to be the case in our work as well (6).

For maximal plant regeneration of E callus in rice (Fig. 6), a medium containing a cytokinin and possibly IAA is essential. Recently we have discovered that the ratio of callus to medium volume is critical. (24). In addition, regenerating rice callus produces a factor, secreted into the medium, which markedly



5. Tryptophan promotes the formation of E callus in some cultivars of rice.



6. A. Embryogenic Mahsuri rice callus with a number of regenerating plants. B. A single embryo of Giza-159 rice showing the shoot and root regions enveloped by the scutellum. C. Regenerating plants are removed from culture and placed in vials of distilled water to encourage root elongation. D. A tray containing several hundred regenerated Pokkali rice plants.

increases regeneration of callus subsequently placed on the same medium (24). The conclusions from these experiments are summarized in Table 1. Again, we have not tested the effects of either proline or increased levels of reduced nitrogen on plant regeneration. The E callus production and plant regeneration rates achieved are more than sufficiently high to produce stress-tolerant variants from rice tissue cultures.

To date we have regenerated almost 4,000 rice plants from NaC1-tolerant cultures of all cultivars used, including IR36. These parental plants are grown to maturity in our greenhouses. The seeds are collected and catalogued as to how long and at what salt concentration the cultured cells were selected. The seeds are germinated, and the seedlings evaluated for salt tolerance by having their roots placed in a series of hydroponic solutions containing various concentrations of NaC1. Control plants are F_1 seedlings whose parents were regenerated from tissue cultures not exposed to NaC1, as well as field-produced seeds of the cultivar being tested. These experiments are just now beginning in Fort Collins.

CEREAL TISSUE CULTURE METHODS FOR WHEAT

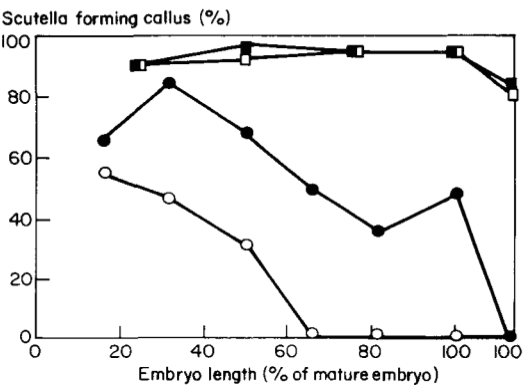
We currently use wheat cultivars Glennson, Pavon, and Chris as well as Caborca triticale. The cereals we have investigated seem to fall into two groups according to how easily mature seeds can produce E callus. For rice, and probably for millet, immature and mature embryos produce E callus from the scutellum with equal facility. Maize and wheat, on the other hand, produce E callus readily -- if the correct medium is used –– from immature scutella but not from mature scutella (Fig. 7). The physiological reason for the decreasing ability of maturing scutella to produce E callus in these species is not known, but it may involve changes in endogenous hormone levels in the developing embryos. We are investigating these changes.

Mature wheat embryos ordinarily produce no callus from the scutellum. They produce NE callus from the root axis of the embryo (Fig. 7), which appears to be the source of most NE callus in grass tissue culture, although the scutellum has the potential

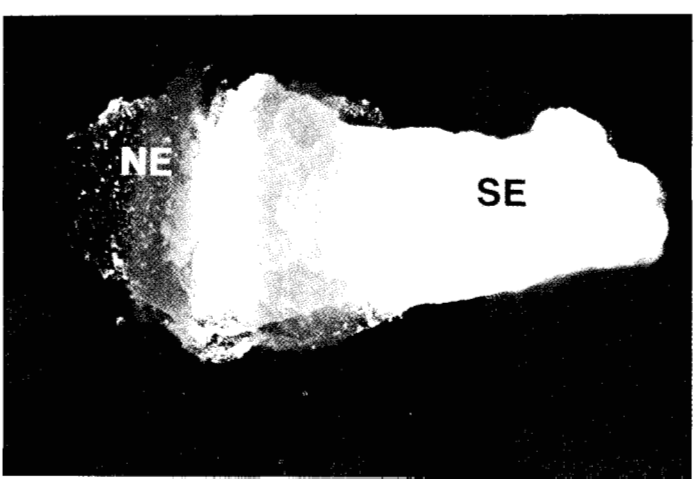
Table 1. Improvement in plant regeneration from Pokkali rice callus after the indicated changes in tissue culture procedure.

Procedure	Plants regenerated from one seed or meristem in 6 mo (no.)
NE callus	0
E callus	4 881
E callus on improved medium	25 049
E callus, maximum callus/medium volume	69 417
E callus on conditioned medium	127 500
E callus plus factor	250 118

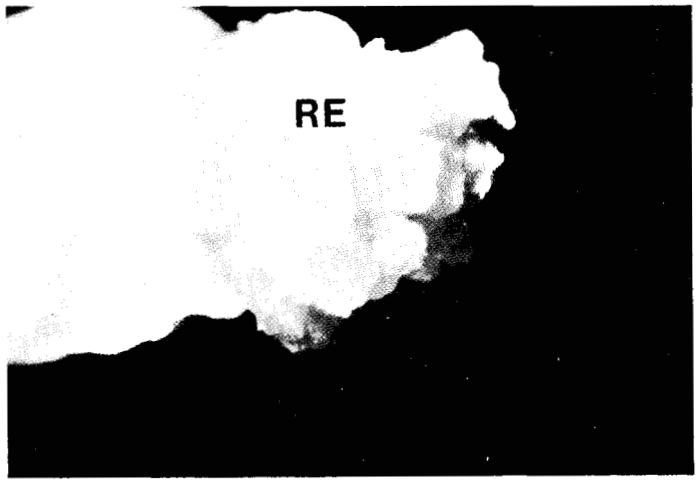
to produce NE callus as well. In wheat, the shoot apex of a mature embryo gives rise to a type of E callus (Fig. 8) we call smooth E (SE) (12). SE callus differs from ordinary E callus (sometimes called rough E or RE for comparison) in that whereas both types are composed of small, nonvacuolated, isodiametric cells; only RE callus forms masses of embryoid-like structures, giving the callus surface a convoluted, rough appearance (Fig. 9). SE callus can develop into RE callus. We are now investigating the media and cultural conditions that favor this transition. It appears that SE callus must first differentiate into RE callus before it gains the high plant regeneration potential of the latter type. Our current plant regeneration system for wheat uses mature embryos and the RE callus originating indirectly from the shoot apical region, as well as immature embryos producing scutellar RE callus.



7. Formation of callus by rice (squares) and wheat (circles) scutella. Open symbols Indicate E callus, Closed symbols indicate total callus.



8. Smooth embryogenic callus that develops from the shoot apical region of mature wheat embryos.



9. Rough embryogenic callus that develops from the scutellum of immature wheat embryos or from smooth embryogenic callus.

We have produced over 300 plants from salt-tolerant cell cultures of wheat. These plants are producing F_1 and F_2 in our greenhouses and at CIMMYT, in research directed by Dr. Mujeeb Kazi. Dr. Kazi finds that approximately 18% of the F_1 progeny of regenerated plants have abnormal numbers of chromosomes; plants from field-grown seed have effectively 0% chromosome number anomalies. The well-known but not well-understood ability of tissue culture media or conditions to increase both point and chromosomal mutation rates gives rise to the increased frequency of somaclonal variants discussed by other participants in this seminar.

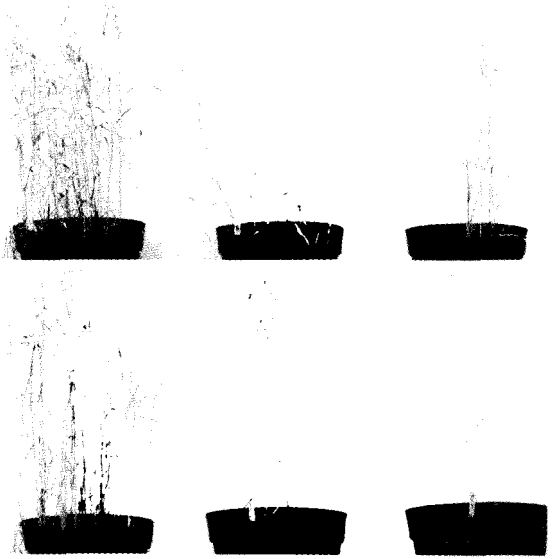
STRESS-TOLERANT CEREALS FROM TISSUE CULTURE

Our project has continued earlier experiments with tissue culture of Park oats (10, 20, 16). Salt-tolerant cell cultures have been made; plants regenerated from them and from nonselected cultures have been produced. Their progeny are now in F_5 of whole-plant testing in the greenhouse. Figure 10 shows that inheritable, stable salt tolerance has been achieved. In addition, one of the tested, salt-tolerant lines flowers and sets seed 2 wk earlier than the unaltered cultivar (Fig. 11). This trait is obviously important for a crop such as oat grown in cold temperate regions. We are trying to determine patterns of inheritance.

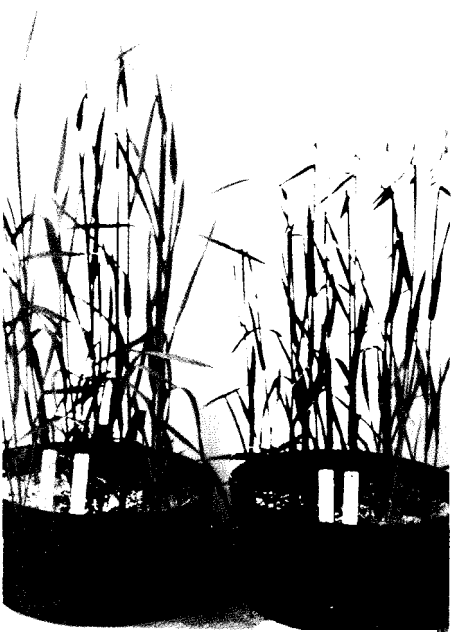
In hydroponic greenhouse testing, rice seedling progeny regenerated from salt-tolerant cell cultures of IR36 and Lahore 76 show greater tolerance than do the controls. Only 6 of 4,000 such potential lines have been tested.

FUTURE PLANS

We have sought to improve selected tolerances for cultivars with demonstrated field utility. Table 2 summarizes our progress in



10. F_3 oat plants. The parental (P) generation was regenerated from NaCl-tolerant cultures (top row) or NaCl-sensitive cultures (bottom row). Plants were watered with tap water containing 0 g NaCl/litre (center) or 10 g NaCl/litre (right).



11. F_3 oat plants. The salt-tolerant plants (right) flower and set seed 2 wk earlier than salt-sensitive plants (left).

Table 2. Timetable for obtaining salt-tolerant plants from tissue cultures of rice. Project initiated, Fall, 1980.

Problem	Current progress (1983)	Future progress	Available technology for IARCs
Plants cannot be regenerated from somatic tissue cultures of rice with high frequency and long duration. Lack of this methodology renders selection of mutants or somaclonal variants nearly impossible. (1980)	Most tissue culture media produce non-embryogenic callus. Cultivar-specific media are devised to produce E callus and to regenerate plants. Callus-to-medium volume ratio is maximized. A medium conditioning factor produced by regenerating callus is discovered. Regeneration rates rise from nearly 0 to at least 127 500 plants obtainable from one starting seed after 6 mo of tissue culture.	Conditioning factor will be chemically identified and tested on other cultivars of rice and other cereals. E callus production media and plant regeneration media can be further improved.	Rare, useful plants can be rapidly cloned for increased availability to breeders. Regeneration technology available makes possible massive production of somaclonal variants in conventional breeding programs.
How can stable salt-tolerant callus be obtained for rice? (1980)	Over 4000 cell lines tolerant of 3000, 6000, or 9000 ppm NaCl have been obtained. Stable tolerance is linked to long-term selection at high levels of salt. Salt-tolerant rice callus is demonstrated to have stable tolerance.	Increased salt tolerance will be obtained by further selection of already tolerant lines. Selection for drought tolerance, acid tolerance, and increased efficiency of fertilizer use is underway.	Stable cell lines useful for studying the cellular basis of salt tolerance are now available.
Can plants be regenerated from salt-tolerant callus? (1980)	Over 3500 rice plants from salt-tolerant cultures of IR36, Pokkali, Mashuri, Giza-159, and Calrose have already been produced and have been or are being grown to maturity in the greenhouse.	Plant regeneration will be increased from tissue cultures exposed to and tolerant of high concentrations of salt for long durations.	A variety of plants resistant to different concentrations of salt should be available for breeding experiments and release to growers.

Problem	Current progress (1983)	Future progress	Available technology for IARCs
Do plants regenerated from salt-tolerant callus show increased tolerance in greenhouse tests? Is tolerance inheritable? What is the mechanism of inheritance? (1982)	A comparison of soil-based, hydroponic, and aeroponic testing methods demonstrates wide variability. Hydroponic testing is deemed most reliable if proper controls are used.	A data bank of response to salinity of many rice cultivars will be developed.	Plants passing greenhouse tolerance tests with known tolerance levels and mechanisms of inheritance will be useful in many breeding programs.
	Salt-tolerant oats from tissue culture have passed greenhouse tests and are now in the fourth whole-plant regeneration from tissue culture-regenerated plants. Modified cultivar has inheritable, stable salt tolerance and is 2 wk earlier	Mechanism of inheritance for oats is under investigation.	
	Salt-tolerant rice plants are being tested and have passed initial tests.		
Do progeny of regenerated plants show increased salt tolerance in field tests? Are they useful and available to plant breeders? (1983)	Oat seeds to be released for 1985 season.	Rice seeds to be released in late 1984.	Correlations among tissue culture selection procedures, greenhouse tests, and field trials will establish tissue culture as one useful element in an overall breeding strategy for rice improvement.

producing salt-tolerant rice in the past 3 yr.

Our charge is to produce wheat cultivars with increased tolerance for salt, drought, and aluminum; rice cultivars with increased tolerance for salt and aluminum; and pearl millet cultivars with increased salt tolerance. At present, we are well ahead of schedule.

We have also begun experiments to further develop two important methods for future use.

1. We have produced embryogenic cell suspensions of rice (Fig. 12) containing examples of all the expected embryogenic stages. Embryogenic suspensions have been produced for other cereals (24). This improved method is an important advance toward more efficient tissue culture for cloning rare plants, producing virus-free populations, and obtaining useful mutants with stress tolerance, disease resistance, etc.

12. Stages of rice embryogenesis obtained from cell suspension culture.

2. We have obtained living protoplasts (cells with the cell wall removed) from several sources of embryogenic cereal callus. We are working on obtaining high-frequency plant regeneration from these protoplasts. Only two groups (Vasil's at University of Florida and Heyser's at Los Alamos National Laboratory) have regenerated whole plants from protoplasts (24); Heyser was a graduate student and postdoctoral fellow with our project.

Reliable methods for plant regeneration from cereal protoplasts are required before we can directly transfer genes from one cell to another.

ACKNOWLEDGMENTS

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REFERENCES CITED

1. Casey, H.E. 1972. Salinity problems in arid lands irrigation: a literature review and selected bibliography. Arid Lands Res. Inf. Pap. 1. Univ. of Arizona Office of Arid Lands Studies, Tucson.
2. Christiansen, M.N. 1982. World environmental limitations to food and fiber culture. Pages 1-11 in Breeding plants for less favorable environments. M.N. Christiansen and C.F. Lewis, eds. Wiley, New York.

3. Conger, B.V. 1981. Agronomic crops. Pages 165-215 in Cloning agricultural plants via in vitro techniques. B.V. Conger, ed. CRC Press, Boca Raton, Florida.
4. Eldridge, R.L. 1982. Transborder salinity and drought management: U.S./Mexico unresolved issues. International Symposium on Hydrometeorology, American Water Resources Association.
5. Flowers, T.J., P.F. Troke, and A.R. Yeo. 1977. The mechanism of salt tolerance in halophytes. Ann. Rev. Plant Physiol. 28:89-121.
6. Green, C.E., C.L. Armstrong, and P.C. Anderson. 1983. Somatic cell genetic systems in corn. In Advances in gene technology: molecular genetics of plants and animals. Miami Winter Symp. Ser. 20. A. Fazelahmad, K. Downey, J. Schultz, R.W. Voellmy, eds. Academic Press, New York.
7. Green, C.E., and R.L. Phillips. 1975. Plant regeneration from tissue cultures of maize. Crop Sci. 15:417-421.
8. Handa, A.K., R.A. Bressan, S. Fanda, and P.M. Hasegawa. 1983. Clonal variation for tolerance to polyethylene glycol-induced water stress in cultured tomato cells. Plant Physiol. 72:645-653.
9. Heyser, J.W., T.A. Dykes, K.J. DePlott, and M.W. Nabors. 1983. High frequency, long term regeneration of rice from callus culture. Plant Sci. Lett. 29:175-182.
10. Heyser, J.W., and M.U. Nabors. 1982. Long term plant regeneration, somatic embryogenesis and green spot formation in secondary oat (Avena sativa) callus. Z. Pflanzenphysiol. 107:153-160.
11. Heyser, J.W., and M.W. Nabors. 1982. The regeneration of proso millet from embryogenic calli derived from various plant parts. Plant Physiol. 68:1454-1459.
12. Heyser, J.W., M.W. Nabors, C. MacKinnon, T.A. Dykes, K.J. DeMott, D.C. Kautzam, and A. Mujeeh-Kazi. 1984. Long-term, high-frequency plant regeneration and the induction of somatic embryogenesis in callus cultures of wheat (Triticum aestivum L.). A. Pflanzenzuecht. (in press)
13. Inoue, M., and E. Maeda. 1981. Stimulation of shoot bud and plantlet formation in rice callus cultures by two-step culture method using abscisic acid and kinetin. Jpn. J. Crop Sci. 50(3):318-322.
14. IRRI (International Rice Research Institute). 1982. Rice tissue culture planning conference.
15. Nabors, M.W. 1982. Tissue culture for crops: stress resistant plants from cell culture. Progress Report, Tissue Culture for Crops Project.
16. Nabors, M.W. 1983. Increasing the salt and drought tolerance of crop plants. Pages 165-184 in Current topics in plant biochemistry and physiology. Vol. 2. D.D. Randall, ed. University of Missouri, Columbia.
17. Nabors, M.W., A. Daniels, L. Nadolny, and C. Brown. 1975. Sodium chloride tolerant lines of tobacco cells. Plant Sci. Lett. 4:155-159.

18. Nabors, M.W., S.E. Gibbs, C.S. Bernstein, and M.E. Meis. 1980. NaC1-tolerant tobacco plants from cultured cells. Z. Pflanzenphysiol. 97:13-17.
19. Nabors, M.W., J.W. Heyser, T.A. Dykes, and K.J. DeMott. 1983. Long-duration, high-frequency plant regeneration from cereal tissue cultures. Planta 157:385-391.
20. Nabors, M.W., C.S. Kroskey, and D.W. McHugh. 1982. Green spots are predictors of high callus growth rates and shoot formation in normal and in salt stressed tissue cultures of oat (Avena sativa L.). Z. Pflanzenphysiol. 105:341-349.
21. Pimentel, D., L.E. Hurd, A.C. Bellotti, M.J. Forster, I.N. Oka, O.D. Sholes, and R.J. Whitman. 1973. Food production and energy crisis. Science 182:443-449.
22. Raghava Ram, N.V., and M.W. Nabors. 1984. Cytokinin mediated long-term, high-frequency plant regeneration in rice tissue cultures. Z. Pflanzenphysiol. 113:315-323.
23. Raghava Ram, N.V., and M.W. Nabors. 1985. Salinity tolerance. Biotechnology handbook. Butterworth. (in press)
24. Raghava Ram, N.V., and M.W. Nabors. 1984. In preparation.
25. Siriwardana, S., and M.W. Nabors. 1983. Tryptophan initiated somatic embryogenesis and plant regeneration in rice. Plant Physiol. 73:142-146.
26. Thorne, W. 1970. Agricultural production in irrigated areas. Pages 31-56 in Arid lands in transition. H.E. Dregne, ed. American Association for the Advancement of Science, Washington, D.C.
27. Van Wambeke, A. 1976. Formation, distribution and consequences of acid soils in agricultural development. Pages 15-30 in Plant adaptation to mineral stress in problem soils. M.J. Wright and S.A. Ferrari, eds. Cornel1 University, Ithaca, New York.
28. Walker, K.A., and S.J. Sato. 1981. Morphogenesis in callus tissue of Medicago sativa: the role of ammonium ion in somatic embryogenesis. Plant Cell Tissue Organ Culture 1:109-121.
29. Worthington, E.B. 1977. Arid land irrigation in developing countries: environmental problems and effects. Pergamon Press, Oxford.

HAPLOIDS, PLOIDY MANIPULATION, AND MEIOTIC MUTANTS IN POTATO BREEDING

M. IWANAGA

Ploidy manipulation takes advantage of two cytological tools, haploids and first-division restitution (FDR) 2n pollen (pollen with the same chromosome number as the parent). Using haploids (2n=2x=24) of cultivars (2n=4x=48) is the direct approach to germplasm transfer. The desirable traits of haploids and the many 2x relatives of the potato are combined at the 2x level, and can be efficiently transferred into 4x progeny by 4x-2x crosses using diploid male parents with FDR 2n pollen. This paper reports progress in 1) producing a large number of haploids from selected tetraploids; 2) screening haploids for specific traits (e.g., pest resistance), male fertility, and FDR 2n pollen production; and 3) hybridizing the haploids to related 2x species with specific resistance and then screening the hybrids for the specific resistance and FDR 2n pollen production. Tissue culture techniques in ploidy manipulation are also discussed.

Three biological features distinguish the potato from other crops:

1. Potatoes are asexually propagated: unlike cereals, they can instantly fix elite heterozygous genotypes after proper selection and asexual maintenance.
2. Potatoes are autotetraploid (2n=4x=48) with tetrasomic inheritance, a serious disadvantage for breeding since it means a slow response to selection (34). Maximizing heterozygosity under tetrasomic polyploid conditions is very important for higher potato tuber yield (18, 19, 35).
3. Modern varieties have a narrow genetic background (20, 32, 35), and broadening the genetic base of breeding populations by introducing alien germplasm is urgently needed (10, 33, 28). The wild and cultivated tuber-bearing relatives of potatoes represent a large source of valuable germplasm with such desirable traits as disease and insect resistance. Any long-term potato breeding strategy should aim for maximum use of these species and maximum heterozygosity in new varieties.

The International Potato Center (CIP), Apartado 5969, Lima, Peru.

THEORETICAL BACKGROUND

Haploids

Haploids of higher plants provide several opportunities for breeding and genetic studies. For breeding crops (rice, maize, wheat), however, only two uses of haploids have been emphasized:

1. instant production of homozygous lines; and
2. selection of genetic variants (induced or spontaneous) in the haploid stage.

For potato breeding, haploids have a different use. Since potatoes are autotetraploid, haploids extracted from them are not x but 2x (9).

Although many important traits for potato breeding are available in the world germplasm collection of the International Potato Research Center (CIP), efficient use of the collection has been limited by two factors: 1) Potatoes are tetraploid but most (approximately 74%) of the relatives are diploid. 2) The use of a wild species as parent may introduce undesirable traits whose elimination through breeding is very slow at the 4x level. Haploids can overcome these problems. They can be easily crossed with most of the numerous 2x species (9). The hybrid population can then be efficiently improved through simple disomic inheritance to combine specific desirable traits of related species with good agronomic characters of cultivated tetraploid potatoes. Genomic differentiation between cultivated potatoes and most related species occurs little, if at all, and there is no cytological barrier for incorporating useful genes from the related species into the cultivated genome. For example, a wild species, S. chacoense, with many desirable traits (resistance to bacterial-wilt, root-knot nematodes, and insects) can be easily crossed with potato haploids and, assuming normal genetic recombination, their 2x hybrids will have completely normal pairing.

Meiotic mutants

Mutants that alter meiosis have been reported in many plant species (1). Their use in plant breeding, however, has been very limited. Control of homoeologous chromosome pairing in wheat and its relatives (6, 26, 29, 30, 31) is probably the only well-known example in crop breeding, but two types of meiotic mutants are already used in potato variety breeding (25).

Mutants that give rise to 2n gametes (gametes with the same chromosome number as the parent. 2n gametes occur naturally in many plant species (7) and simple genetic control of their production has been identified in 2x potatoes (21). Genetic theory and application of 2n gametes is well documented in potatoes (5, 16, 18, 22, 23). Of the several mechanisms by which 2n gametes may be produced (not including some types associated with apomictic seed production), first-division restitution (FDR) and FDR-type 2n gametes are preferred. These mechanisms maximize heterozygosity, which generally corresponds to increased yield of

potatoes. They transmit all of the heterozygosity from the centromere to the first crossover, and half of that beyond the first crossover. In potatoes, where there is only one chiasma or two chiasmata per bivalent, it is estimated that FDR 2n gametes transfer intact 80% of the diploid genotype to the tetraploid progeny 4x - 2x and 2x - 2x crosses (17). The meiotic mutant characterized by parallel spindles (ps) is an FDR mechanism that accomplishes this transfer in the production of 2n pollen (22).

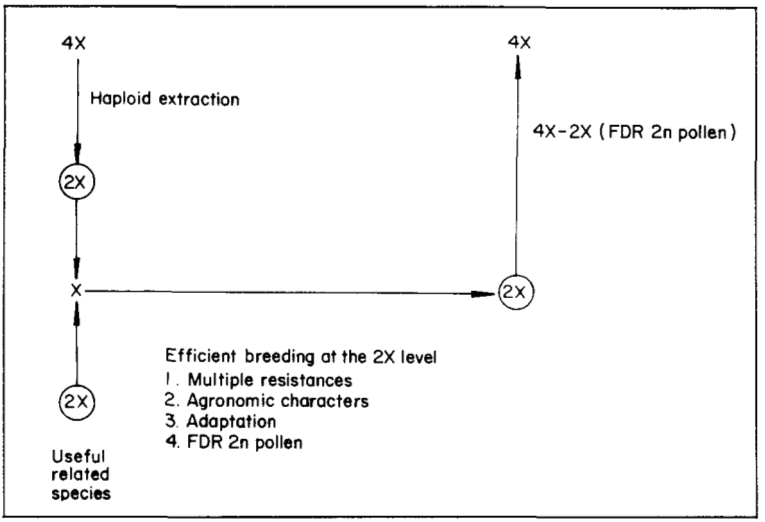
Synaptic mutants. Synaptic mutants, meiotic mutants that influence the extent of meiotic pairing (27), have been reported in many plant species (14). The use of related species, haploids, and FDR 2n gametes in potato breeding has become increasingly important since Okwuagwu and Peloquin (24) proposed combining the synaptic mutant with ps. A synaptic mutant affects synapsis in microporogenesis, resulting in mainly univalents at metaphase I and high male sterility. However, when a synaptic mutant is combined with the ps mutant, fertile 2n pollen is produced. The genetic significance of this combination is that it permits more heterozygosity that can be transmitted to the diploid offspring. If a synaptic mutant is completely asynaptic (i.e., no pairing and no crossing over), 100% of the heterozygosity and epistasis of the parent can be transmitted to the offspring. Even though a synaptic mutant is only partially asynaptic, the transmission rate of the heterozygosity of the diploids is higher than that of FDR 2n pollen produced by normal ps. Thus, meiotic mutants provide a powerful breeding method that maximizes heterozygosity and epistasis, and transfers to tetraploids diploid germplasm with desired characteristics.

Synaptic mutants are rather common in potatoes. Iwanaga and Peloquin (12) first reported a synaptic mutant (syl) and emphasized the importance of a further search for meiotic mutants for potato breeding. Since then, three new synaptic mutants, sy2 (13), sy3 (23), and sy4 (11) have been reported. All the mutants were discovered accidentally, so an intensive search for synaptic mutants would likely have good results. A synaptic mutant with stable and complete asynapsis is needed.

Ploidy manipulations

Manipulating ploidy (Fig. 1) is based on the analytic breeding scheme of Chase (3), and uses haploids and FDR 2n pollen. It involves three steps: 1) extracting haploids from selected tetraploids; 2) hybridizing haploids with useful 2x species and subsequently breeding for multiple resistance, agronomic characteristics, adaptation, and 2n pollen production in wide genetic backgrounds. Population breeding at the 2x level (disomic inheritance) is much more efficient than traditional breeding, which is at the 4x level (tetrasomic inheritance); and 3) transferring the improved 2x germplasm into 4x via EDR 2n pollen.

The 4x progeny produced by 4x - 2x crosses would be excellent not only in a clonal selection program but also as true potato seed (TPS) cultivars. The use of TPS instead of traditional seed tubers for commercial potato production has been



1. Scheme of ploidy level manipulations.

a major research objective at CIP and other institutions (4). Uniformity in agronomic characters, high yield, and multiple resistance are the most important traits for TPS use in developing countries. The 4x - 2x cross approach would produce TPS families with better traits than normal 4x - 4x crosses. The genetic homogeneity of FDR 2n pollen assures uniformity of the 4x TPS families; heterozygosity of FDR 2n pollen (produced by heterozygous diploids) results in highly heterozygous and high-yielding 4x progeny; and multiple resistances combined at the 2x level are efficiently transmitted to the 4x progeny by FDR 2n pollen.

PROGRESS THROUGH PLOIDY MANIPULATION

Haploids from selected tetraploids were extracted through 4x - 2x crosses using the diploid clone IvP-35, a good haploid inducer with an embryo spot marker (8). Pollen stainability, 2n pollen production, and cytological mechanisms of 2n pollen production have been studied.

From 76 tetraploids, we obtained 2,928 haploids with different attributes and genetic backgrounds (Table 1). Extracting a large number of haploids from late-blight-resistant tetraploids was necessary, since only a few of the haploids were expected to be highly resistant. Because the haploids were from many tetraploids having tuberosum, andigena, and phureja in their genetic backgrounds, we expected haploid use would not lead to inbreeding unless the useful clones became scarce.

Pollen stainability of 501 haploids was studied. About two thirds of the haploids had poor pollen stainability and were considered either male sterile or to have low male fertility (Table 2). The remaining third (36%) of the haploids with more than 30% stainable pollen were expected to have adequate pollen fertility. Some of them were used for 2x - 2x crosses as male

Table 1. Number of haploids extracted from selected tetraploids.

Resistance of tetraploid parent	Resistant parents (no.)	Genetic background	Extracted haploids (no.)
Late blight	21	*tbr, adg*	2,019
PLRV	10	*tbr, adg*	236
PVX/PVY	16	*tbr, adg*	312
Cyst nematode	17	*adg*	118
Heat	12	*tbr, adg, phu*	243
	76		2,928

Table 2. Distribution of pollen stainability values in extracted haploids.

Pollen stainability (%)	Haploids	
	No.	%
<5	179	36
5-30	144	29
30-50	70	14
> 50	108	22

parents, and high seed sets were obtained. The results are very encouraging, since the use of haploids as male parents permits circumventing cytoplasmic-genic male sterility in hybrids with other 2x germplasm.

Screening for 2n pollen production among 322 haploids from 19 tetraploids with more than 5% pollen stainability resulted in 151 (about half) FDR 2n pollen haploids. FDR 2n pollen production appears common in haploids, provided the haploids are not male sterile.

Many stress-resistant diploid populations have been developed at CIP (Table 3). Where wild species were the resistance sources, they were hybridized with cultivated germplasm. FDR 2n pollen was identified in many groups of the diploids resistant to bacterial wilt, soft rot, late blight, early blight, root-knot nematodes, cyst nematodes, potato tuber moth, and heat. Female 4x parents are being crossed to the resistant diploids with 2n pollen to evaluate 4x progeny for resistance and agronomic character. Diploids with different resistances are being intermated and hybrids are being screened for combined resistance.

TISSUE CULTURE TECHNIQUES IN PLOIDY MANIPULATION

A breeding scheme using conventional and tissue culture techniques, proposed by Wenzel et al (37), consists of six steps (Fig. 2): 1) extracting haploids from selected 4x parents, 2) selecting the extracted haploids for desired traits such as disease resistance, 3) producing homozygous 2x plants by

Table 3. Diploid groups selected for specific resistance: their genetic background and 2n pollen production.

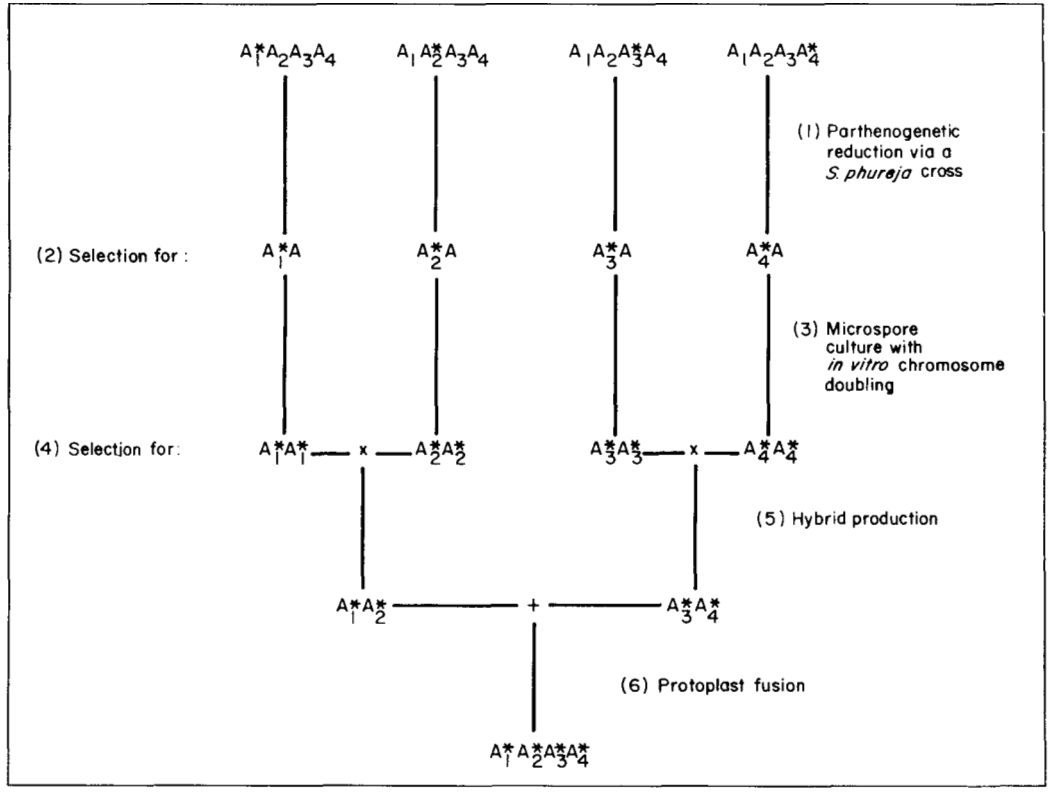
Selected resistance	Genetic background	Production of 2n pollen
Late blight	*tbr, adg*	Yes
Root-knot nematodes	*chc, spl, mcd, phu, stn, tbr, adg*	Yes
Bacterial wilt	*chc, spl, phu, stn, tbr, adg*	Yes
Erwinia spp.	*phu, stn*	Yes
Early blight	*phu, stn*	Yes
Potato tuber moth	*spl, phu, stn, tbr, adg*	Yes
Heat	*stn, phu, chc, tbr*	Yes
Cyst nematodes	*1) spl, chc, phu, stn, tbr, adg*	Yes
	2) adg	Not studied
Potato viruses X and Y	*tbr, adg*	Not studied
Potato leafroll virus	*tbr, adg*	Not studied

microspore culture and spontaneous in vitro chromosome number doubling, 4) selecting homozygous 2x plants for desired traits, 5) hybridizing unrelated homozygous 2x plants with different attributes, and 6) producing highly heterozygous 4x plants by protoplast fusion of unrelated heterozygous 2x hybrids with different attributes. The first step (extracting haploids) can be done by the 4x - 2x cross method; 3 and 6 involve tissue culture.

To apply this scheme in practical breeding, step 3 must produce a large number of homozygous 2x plants. Wenzel (36) reported differences in genotype ability to produce plantlets from microspores and identified highly capable clone genotypes as having "tissue culture ability." If such genetic factors exist and they can be introduced into other useful breeding materials, many useful homozygous 2x plants with different genetic attributes could be efficiently produced.

Dr. S. Tanaka of Meijo University, Japan, currently on sabbatical leave at CIP, is collaborating with two CIP scientists on microspore culture. Both Dr. Tanaka and CIP independently tried microspore culture of 2x potatoes but obtained very disappointing results, presumably because they used genotypes without good tissue culture ability. Hybridization between one of Dr. Wenzel's clones and a CIP 2x clone with resistance to root-knot nematodes has been done. The hybrid progeny and Dr. Wenzel's clones have been checked for their microspore culture capability to learn if the tissue culture ability exists and can be transferred. Although the experiments are not completed, Dr. Wenzel's clones do have excellent capability to produce plantlets from microspores, and most of the hybrid progeny have an equal capability.

Step 6, if successful, would provide a special opportunity in potato breeding, not because protoplast fusion permits combining two sexually incompatible species, but because it combines two unrelated but selected genotypes. All the genetic variation of the donor diploids would be transmitted to a hybrid tetraploid, which is not normally possible. Successful protoplast fusion between different species such as potato and



2. Scheme for an analytical synthetic breeding procedure combining tissue culture techniques and conventional breeding steps (37). A = potato genome. * = favorable genes with qualitative inheritance (e. g. resistance).

tomato (15) and cultivated potato and wild species (2) has been reported. However, combining two similar genotypes has not succeeded, partly because of the lack of methods for selecting protoplast fusion hybrid materials. Most current selection schemes depend on some genetic difference between the parents, such as albino complementation or nutritional requirement. These methods are not useful for hybridizing and selecting any suitable breeding materials. The need to develop "universal" selection systems for fusing any materials together and selecting the hybrids is urgent (38).

CIP does not directly work on protoplast fusion, but monitors the activities of other institutions. Protoplast fusion could produce new improved varieties when diploid protoplasts with desirable traits are used. Many fusion combinations of different diploids should be evaluated. If practical 2x fusion and hybrid regeneration techniques become available, CIP would immediately adapt the technology to produce elite potato varieties. Its work at the 2x level has been successful. The 2x breeding materials developed from haploids and related species are being used for 4x - 2x crosses via FDR 2n gametes. These will be the best materials for protoplast fusion.

REFERENCES CITED

1. Baker, B., A.T.C. Carpenter, M.S. Esposito, R.E. Esposito, and L. Sandler. 1976. The genetic control of meiosis. Ann. Rev. Genet. 10:53-134.
2. Butenko, R., A. Kuchko, and I. Komarnitsky. 1982. Some features of somatic hvbrids between Solanum tuberosum and S. chacoense and its F_1 sexual progeny. Pages 643-644 in Proceedings 5th International congress on plant tissue and cell culture. A. Fujiwara, ed. Tokyo.
3. Chase, S.A. 1963. Analytic breeding in Solanum tuberosum L. A scheme utilizing parthnotes and other diploid stocks. Can. J. Genet. Cytol. 5:359-363.
4. CIP (International Potato Center). 1982. Report of the planning conference on the production of potatoes from true seed. Manila, Philippines.
5. De Jong, H., and G.C.C. Tai. 1977. Analysis of tetraploid-diploid hybrids in cultivated potatoes. Potato Res. 20:111-121.
6. Dover, G.A., and R. Riley. 1972. Variation at two loci affecting homoeologous meiotic chromosome pairing in Triticum aestivum x Aegilops mutica hybrids. Nature (New Biology) 235:61-62.
7. Harlan, J.R., and J.M.J. de Wet. 1975. On O Winge and prayer: the origin of polyploidy. Bot. Rev. 41:361-390.
8. Hermsen, J.G.T., and J. Verdenius. 1973. Selection from Solanum tuberosum group Phureja of genotypes combining high-frequency haploid induction with homozygosity for embryo-spot [potatoes]. Euphytica 22(2):244-259.
9. Hougas, R.W., and S.J. Peloquin. 1958. The potential of

potato haploids in breeding and genetic research. Am. Potato J. 35:701-707.

10. Howard, H.W. 1963. The significance of breeding in improving quality and yield. Pages 292-302 in The growth of the potato proceedings. J.D. Ivins and F.L. Milthorpe, eds. Butterworths, London.
11. Iwanaga, M. 1984. Discovery of a synaptic mutant in potato haploids and its usefulness for potato breeding. Theor. Appl. Genet. (in press)
12. Iwanaga, M., and S.J. Peloquin. 1979. Synaptic mutant affecting only megasporogenesis in potatoes. J. Hered. 70:385-389.
13. Johnston, S.A., R.E. Hanemann, and R.W. Rhude. 1981. Identification of an asynaptic variant in Solanum commersonii. Am. Potato J. 58:506.
14. Koduru, P.R.K., and M.K. Rao. 1981. Cytogenetics of synaptic mutants in higher plants. Theor. Appl. Genet. 59:197-214.
15. Melchers, G., M.D. Scristan, and A.A. Holder. 1978. Somatic hybrid plants of potato and tomato regenerated from fused protoplasts. Carlsber Res. Commun. 43:203-218.
16. Mendiburu, A.O., and S.J. Peloquin. 1979. Gene-centromere mapping by 4x-2x matings in potatoes. Theor. Appl. Genet. 54:177-180.
17. Mendiburu, A.O., and S.J. Peloquin. 1977. The significance of 2n gametes in potato breeding. Theor. Appl. Genet. 49:53-61.
18. Mendiburu, A.O., S.J. Peloquin, and D.W.S. Mok. 1974. Potato breeding with haploids and 2n gametes. Pages 249-258 in Proceedings I international symposium on "haploids in higher plants." K.J. Kasha, ed. University of Guelph, Ontario, Canada.
19. Mendoza, H.A., and F.L. Haynes. 1974. Genetic basis of heterosis for yield in the autotetraploid potato. Theor. Appl. Genet. 45:21-25.
20. Mendoza, H.A., and F.L. Haynes. 1974. Genetic relationship among potato cultivars grown in the United States. Hortscience 9:328-330.
21. Mok, D.W.S., and S.J. Peloquin. 1975. The inheritance of three mechanisms of diplandroid (2n pollen) formation in diploid potatoes. Heredity 35:295-302.
22. Mok, D.W.S., and S.J. Peloquin. 1975. Three mechanisms of 2n pollen formation in diploid potatoes. Can. J. Genet. cytol. 17:217-255.
23. Mok, D.W.S., and S.J. Peloquin. 1975. Breeding value of 2n pollen (diplandroids) in tetraploid x diploid crosses in potatoes. Theor. Appl. Genet. 48:307-114.
24. Okwuagwu, C.O., and S.J. Peloquin. 1981. A method of transferring the intact parental genotype in the offspring via meiotic mutants. Am. Potato J. 58:512-513.
25. Peloquin, S.J. 1982. New approaches to breeding for the potato of the year 2000. Pages 32-34 in Research for

the potato in the year 2000. W.J. Hooker, ed. International Potato Center, Lima, Peru.

26. Riley, R., V. Chapman, and R. Johnson. 1968. The incorporation of alien disease resistance in wheat by genetic interference with the regulation of meiotic chromosome synapsis.

27. Riley, R., and C.N. Law. 1965. Genetic variation in chromosome pairing. Adv. Genet. 13:57-107.

28. Ross, H. 1979. Wild species and primitive cultivars as ancestors of potato varieties. Pages 237-245 in Proceedings Conference Broadening Genetic Base Crops, Wageningen, 1978. Pudoc, Wageningen.

29. Sears, E.R. 1973. Agropyron - wheat transfers induced by homoeologous pairing. Pages 191-199 in Proceedings 4th international wheat genetic symposium.

30. Sears, E.R. 1976. Genetic control of chromosome pairing in wheat. Ann. Rev. Genet. 10:31-51.

31. Sears, E.R. 1977. An induced mutant with homoeologous pairing in common wheat. Can. J. Genet. Cytol. 19:585-593.

32. Simmonds, N.W. 1962. Variability in crop plants, its use and conservation. Biol. Rev. 27:442-465.

33. Simmonds, N.W. 1969. Prospects of potato improvement. Annu. Rep. Scott. Plant Breed. Stn. 48:18-38.

34. Stebbins, G.L. 1971. Chromosomal evolution in higher plants. Edward Arnold (Publishers) Ltd., London.

35. Tai, G.C.C. 1976. Estimation of general and specific combining abilities in potato. Can. J. Genet. Cytol. 18:463-470.

36. Wenzel, G. 1980. Recent progress in microspore culture of crop plants. Pages 185-196 in Proceedings of the 4th John Innes Symposium and Second International Haploid Conference. Norwich.

37. Wenzel, G., O. Schieder, T. Przewozny, S.K. Sopory, and G. Xelchers. 1979. Comparison of single cell culture derived Solanum tuberosum L. plants and a model for their application in breeding programs. Theor. Appl. Genet. 55:49-55.

38. Widholm, J.M. 1982. Selection of protoplast fusion hybrids. Pages 609-612 in Proceedings 5th international congress on plant tissue and cell culture. A. Fujiwara, ed. Tokyo.

SOMATIC CELL CULTURE AT IRRI

W. M. ABRIGO. A. U. NOVERO, V. P. CORONEL,
G. S. CABUSLAY, L. C. BLANCO, F. T. PARAO,
and S. YOSHIDA

Screening techniques for selecting salt- and aluminum-tolerant lines were developed.

Selected cells from seed-derived callus culture showed higher relative growth in the presence of NaCl. Of 522 entries screened at seedling stage, 6% were tolerant, 14% moderately tolerant, and 80% susceptible.

R2 plants of Taichung 65 were tested for aluminum toxicity tolerance. The plants showed a wide range of variation, the number of variants increasing with the duration of callus culture.

Variations in both visual and agronomic traits were found in field-grown plants.

Salinity and aluminum (Al) toxicity are major soil constraints to rice production. In South and Southeast Asia, there are approximately 60 million ha of saline but otherwise cultivable soil, about 50% of which are in coastal areas (1). Likewise, nearly 2.6 billion ha of strongly acid soils, mostly in humid regions, are currently uncultivated but have food-producing potential (2).

Tissue culture is now recognized as an important means to generate genetic variability (3), and inducing mutation has already improved various agronomic and grain characteristics of rice (9, 10). Increasing rice's salt tolerance can significantly increase production. Similarly, improving the A1 tolerance of modern varieties can improve rice yields in Al-toxic soils.

SEED-DERIVED CALLUS CULTURE

The culture (C) medium for callus induction and proliferation was Murashige and Skoog's basic salts (7) supplemented with casein hydrolysate and yeast extract, each at 2 g/litre; sucrose at 30 g/litre; and 2,4-D at 2 mg/litre. For the selection (S) medium, NaCl (0, 0.5, 1.0, 1.5, 2.0, and 3.0 wt/vol) was added. The

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Research assistant, (Tissue Culture), research aide, senior research assistant, research assistant, research assistant, assistant scientist, and plant physiologist (deceased 24 Jan 1984), Plant Physiology Department, International Rice Research Institute, P. 0. Box 933, Manila, Philippines.

regeneration (R) medium (same basic composition as C medium) was supplemented by 5 x 10^{-5} M kinetin and 2 x 10^{-6} M NAA (instead of 2,4-D). The sucrose concentration was increased to 70 g/litre.

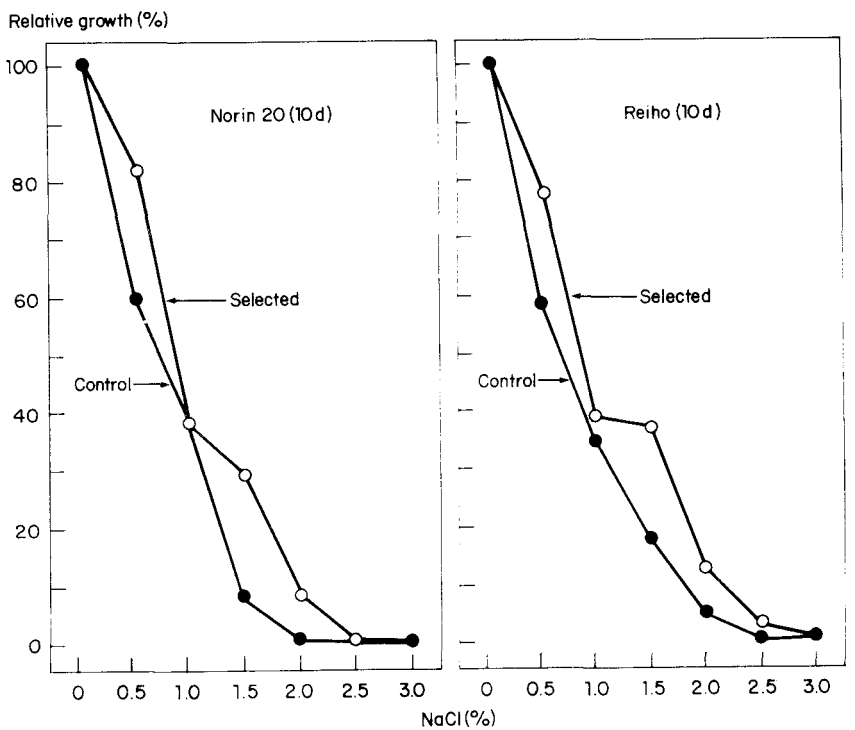
Dehulled seeds (mature and immature) were kept on C medium for 4 wk. The induced calluses were proliferated on fresh C medium and transferred onto S medium. The cultures were then kept in the dark at 26°-29°C.

After the desired passages on S medium, the calluses were transferred to R medium. These cultures were kept in a lighted room at 28°C.

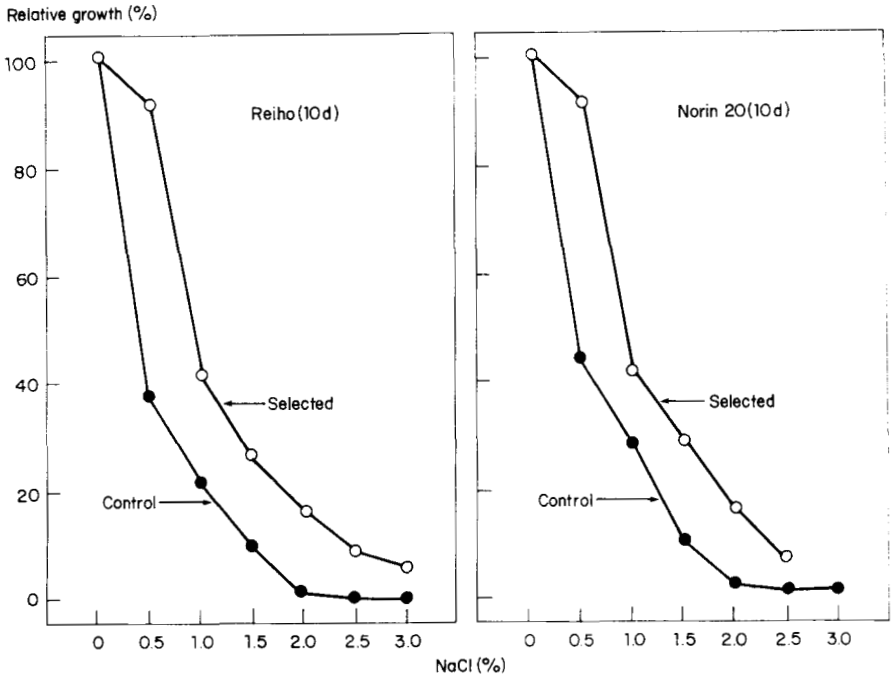
NACL TOLERANCE OF SELECTED CELLS

Selected cell lines of Reiho (10 d) and Norin 20 (10 d) continuously grown on S medium for 24 and 52 wk were used. In nonselected lines, increasing the concentration of NaCI impaired growth. In the selected cell lines, the growth curve was slightly different: it showed bending at 1% NaCl, indicating that the selected lines are more tolerant than the nonselected (Fig. 1, 2).

Tal et a1 (12) found that calluses derived from the salt-tolerant wild species Lycopersicon peruvianum and Solanum pennelli grew better than those derived from the cultivated



1. Growth curve of calluses cultured for 24 wk on media with or without 1.5 NaCl in response to a gradient of NaCl concentration in the medium. Calluses were induced from 10-d-old seeds of Norin 20 (left) and Reiho (right).

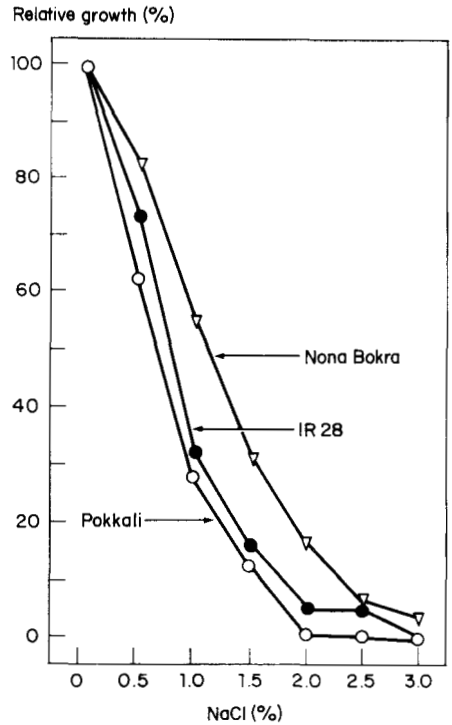


2. Growth curve of calluses cultured for 52 wk on media with or without 1.5 NaCl in response to a gradient of NaCl concentration in the medium. Calluses were induced from 10-d-old seeds of Reiho (left) and Norin 20 (right).

species. Smith and McComb (11) showed that calluses of salt-susceptible Phaseolus vulgaris grew poorly when salinity was increased, and the callus of Beta vulgaris (salt-tolerant) showed the same tolerance to salt as did the whole plant. However, callus cultures of halophytes grew poorly with increasing NaCl, suggesting that the Beta vulgaris callus has a mechanism of salt tolerance operating at the cellular level. To learn the difference in salt tolerance at the cellular level between calluses from tolerant and susceptible varieties, their growth at different NaCl concentrations was investigated. The salt-tolerant varieties used were Pokkali and Nona Bokra; the susceptible variety was IR28. The growth curves of Pokkali and IR28 were similar at different NaCl concentrations (Fig. 3); Nona Bokra was more tolerant. These results suggest that at the cellular level, the salt tolerance of Pokkali is similar to that of IR28. So, calluses derived from salt-tolerant plants are not necessarily tolerant at the cellular level. Pokkali and Nona Bokra exhibit this phenomenon.

PLANT REGENERATION

The calluses transferred to R medium were kept in a lighted room. When the regenerated plantlets were fully developed and ready for transfer to soil, their roots were kept on 0.1% nicotinamide solution for 1 h. Transferred to soil, the plants were grown



3. Effect of NaCl on the growth of callus derived from 3 rice varieties.

until mature, and their panicles were harvested individually.

Different kinds of variations in visible plant traits were observed in R1 and R2. In R1 plants of Taichung 65, abnormal glumes, awns, dwarfs, sterility, stripes, and tetraploids were observed. (Tetraploid in rice is characterized by somewhat stunted growth, fewer panicles, thicker and coarser leaves, thicker culms, poor panicle exsertion, larger grain size, awn, and high percentage of sterility [6]).

Several kinds of chlorophyll mutants -- albino, lethal yellow, pale green, and stripes -- were observed at the seedling stage of R2 plants. The pooled frequency of 1,259 lines was 8.8/100 R2 lines, a value as high as that obtained by irradiation, but lover than that induced by chemical mutagens. According to Mikaelsen et a1 (5), chlorophyll mutation frequencies by gamma rays and by ethyl methane sulfonate (EMS) treatment were about 10 and 30/100 M2 panicles. Much higher frequencies of 40-70/100 M2 panicles were obtained through treatment of fertilized egg cells with N-methyl-N-nitrosourea (MNU) (10). Thus, tissue culture cycle is as effective as irradiation but less effective than chemical mutagens in inducing chlorophyll mutations.

Variations in height, sterility, heading date, chlorophyll mutations, and various morphological traits were observed in field-grown R2 plants (Table 1). About 54% of R2 lines of Taichung 65 exhibited variations in one or more visible plant traits; 17.5% of R2 lines showed variations in more than one

Table 1. Various kinds of morphological variations observed in the field.

Part of plant	Variant traits
Glume or grain	Long, big, small, round, small, and round, awned
Panicle	Clustered, dense
Leaf	Rolled, short, narrow, erect
Tiller	Compact tiller arrangement

trait. Seeds of these variants were used in screening for salt tolerance.

SCREENING FOR SALINITY TOLERANCE

A screening technique for selecting salt-tolerant mutants was developed. One-week-old (3-leaf stage) seed1ings were transplanted to a Styrofoam board with 60 holes. The plants were grown in standard C medium and left overnight. The following day, they were transferred to standard C solution plus 0.5% NaCl. Electrical conductivity of the solution was about 10.50 mmho/cm. Taichung 65, a susceptible variety, was included in every set. The dead plants were counted when only 3 of 30 Taichung 65 plants survived.

Healthy mutants (R2 plants) that survived salinization were transferred to full-concentration nutrient solution for recovery. After 1 wk, the plants were transferred to soil and grown to maturity. The R3 seeds obtained were designated as ST (salt-tolerant) and again tested for salt tolerance.

From 1982 to 1983, a total of 463 japonica mutants and 83 Nona Bokra mutants were screened for salinity tolerance. Selected ST lines or R3 mutants totaled 370.

A scoring system based on percentage of dead plants was devised; 61 – 100% survival was rated tolerant, 31-60% moderately tolerant, and 0-30% susceptible. Of 522 entries screened, 31 (6%) were rated tolerant, 72 (14%) moderately tolerant, and 419 (80%) susceptible.

Among R3 mutants of japonica varieties, only one was tolerant of salinity, 16 were moderately tolerant. Twenty-six Nona Bokra mutants were salt tolerant, 43 moderately tolerant.

PLANT TYPE IMPROVEMENT OF NONA BOKRA VARIANTS

Somaclonal variation reported in rice (4, 8, 13) can be used to improve morphological traits of the traditional rice varieties that adapt better to physiological disorders. Nona Bokra is tall, weak strawed, droopy leaved, and photosensitive. Though a poor yielder, it is popular among farmers in coastal areas because it is highly adapted to saline conditions.

Twenty-one R2 field-grown Nona Bokra plants were selected for plant height, leaf type, fertility, heading date, and tiller type (open, semiopen, or compact) and regenerated in the screenhouse. Ten R3 lines were harvested by hill and grown at 30- x 30-cm spacing in the field in January 1982.

Except for NB 35-20, R4 variants were uniform in stand (Table 2). Most of them had slightly erect leaves. One variant, NB

Table 2. Agronomic traits of Nona Bokra and its mutants grown in the field. IRRI, 1983 dry season.

Mutants (R4)	Height (cm)	Tillers (no./hill)	Panicles (no./hill)	Tiller angle	Stand	Leaf	Sterility[a]	Days to flower	Disease[c]
Nona Bokra (NB)	125	24	22	Open	Uniform	Droopy	3	62	Leaf blast (BI)
NB 35-20	-	-	-	Variable	Not uniform	Variable	Variable	Variable	*b*
NB 138-13	86	98	83	Compact	Moderately uniform	Erect	5	71	BI
NB 65-2	138	30	29	Open	Uniform	Droopy	5	62	BI
NB 353-3	128	48	40	Open	Uniform	Slightly erect	7	75	BI
NB 360-13	152	21	21	Open	Uniform	Slightly erect	5	75	BI
NB 360-18 (1)	159	23	20	Open	Uniform	Slightly erect	5	75	BI
NB 360-18 (3)	157	23	18	Open	Uniform	Slightly erect	5	75	BI
NB 388-4 (1)	120	27	25	Open	Uniform	Slightly erect	7	68	BI
NB 388-4 (2)	122	25	23	Open	Uniform	Slightly erect	5	62	BI
NB 388-4 (3)	117	26	22	Open	Uniform	Slightly erect	5	67	BI

[a]Based on the 1980 Standard Evaluation System for Rice 1-9 scale for spikelet fertility. [b]Some hills showed least infection by leaf blast, others were relatively unaffected. [c]BI = blast.

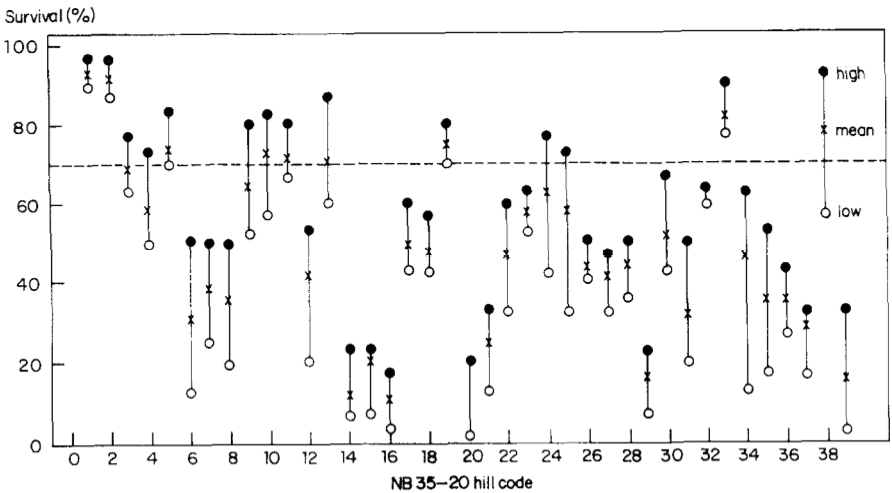
Table 3. Character variability in fieldgrown R4 Nona Bokra 35-20. IRRI, 1983 dry season.

Plant character	Variability	% of 44 hills
Plant height (cm)	110 or	18
	111 - 125	14
	125	68
Color, brown rice	Red	66
	Light red	9
	White	25

135-13, was much shorter than the parent, tillered profusely, but in most cases was unproductive. Like the parent, almost all variants were susceptible to leaf blast. The variant NB 35-20 was heterogeneous in height, tillering type, maturity, fertility, and reaction to leaf blast. On the basis of fertility and resistance to blast, 44 NB 35-20 plants were individually harvested and observed (Table 3). Selected NB 35-20 lines reacted variably to salinity (Fig. 4). While 90% of Nona Bokra parent seedlings were alive at 0.5% NaCl for 10 d, survival of NB 35-20 lines ranged from 0 to 57%. These changes in visible characteristics and reaction to salinity suggest that mutation occurs in both morphological and biochemical traits.

To purify good plant-type NB 35-20 lines and keep their salt tolerance, R4 lines surviving the salinity test were cultivated in replicated pots. Shorter seedlings were preferred.

Table 4 shows plant characteristics of surviving seedlings at maturity. Lines 1 and 33, which showed high salinity tolerance, were taller than, and seemed as photosensitive as, the parent. Lines 35 and 36 in two replications were shorter and headed earlier, but were less resistant to salinity, than the control. Variation appears to extend to the fifth generation. Lines 35 and



4. Diversity map in tolerance of salinity in selected hills of R4 NB 3520. Phytotron, 1983. (Note: Plants at 3.3-leaf stage salinized with 0.5% NaCI for 10 d.)

Table 4. Morphological characters of some promising Nona Bokra regenerates (5th). Screenhouse, 1983 (planted day).

Regenerates/parent	Replication			Mean
	I	II	III	
		Plant ht (cm)		
NB 35-20-1	178	215	200	198
NB 35-20-33	168	158	178	168
NB 35-20-35	128	120	145	131
NB 35-20-36	124	127	–	126
Parent Nona Bokra	170	168	167	168
		Days to flower		
NB 35-20-1	89	89	94	91
NB 35-20-33	93	113	128	111
NB 35-20-35	95	86	132	104
NB 35-20-36	84	84	–	84
Parent Nona Bokra	97	98	99	98
		Plant survival (%) at 0.5% NaCl in 10 d		
NB 35-20-1	93	97	90	93
NB 35-20-33	77	90	80	82
NE 35-20-35	37	53	17	36
NB 35-20-36	43	37	27	36
Parent Nona Bokra	90	87	87	88

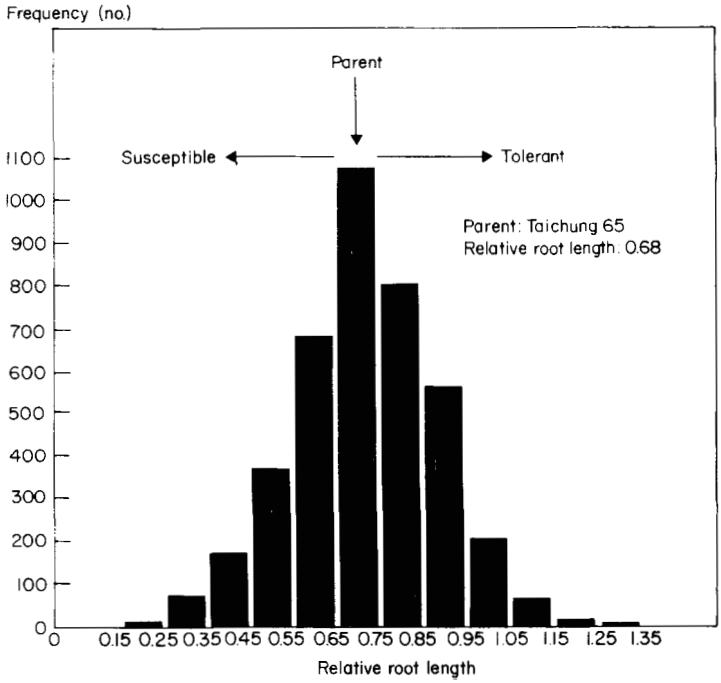
36 in two replications were regenerated by panicle in the screenhouse in January 1984. Line 35 showed uniform height but differed in maturity. Line 36 was highly homogeneous in both plant height and heading date. To isolate highly salt-resistant materials, these lines would again be subjected to salinity test. Lines 1 and 33 were planted in the field in the 1984 dry season for further plant-type observation.

ALUMINUM TOLERANCE OF RICE VARIANTS

Aluminum toxicity is a major problem in acid and acid-sulfate soils. It impairs root growth, thereby making plants less efficient in soil nutrient use and more susceptible to drought.

We tested the R2 plants of Taichung 65 for Al tolerance by measuring the root growth of 2-wk-old seedlings in a culture solution containing 30 ppm Al. The frequency distribution of the relative root lengths of 3,991 R2 plants from 472 lines is shown in Figure 5. The parent, Taichung 65, had 32% shorter roots at 30 ppm Al. The R2 plants' relative root length varied widely in both directions. Some highly A1-tolerant variants grew roots that at 30 ppm Al were even longer than those of the parent at 0 ppm Al.

The Al-tolerant variants increased with the age of the callus culture (Table 5). Their frequency increased from 18% for a 4-wk culture to 46% for a 20-wk culture; the average was 34%. The Al tolerance of these variants should be studied under field conditions.



5. Frequency distribution of the relative root lengths of 3,991 seedlings from 472 rice lines at 30 ppm Al.

Table 5. Frequency of Al-tolerant variants in R2 plants regenerated from Taichung 65 calluses.[a] IRRI, 1982.

Passages (no.)	R2 lines tested (no.)	R2 plants tested (no.)	Frequency of variant production[b] (%)	
			Lines	Plants
1	69	624	36	18
2	76	674	50	17
3	23	84	70	46
4	118	1093	87	43
5	60	506	87	46
Overall	346	2981	66	34

[a]Subjected to repeated cycle of subculture without selection pressure, [b]Variants have roots longer than average plus 3 X standard deviation of the parent in the presence of 30 ppm Al in culture solution.

REFERENCES CITED

1. Akbar, M., and F. Ponnamperuma. 1982. Saline soils of South and Southeast Asia as potential rice lands. In Rice Research Strategies for the Future. The International Rice Research Institute, Los Baños, Philippines.
2. Dudal, R. 1976. Inventory of major soils of the world with special reference to mineral stress hazards. In Proceedings Workshop on Plant Adaptation to Mineral Stress in Problem Soil. Beltsville, Maryland.

3. Larkin, P., and W. Scowcroft. 1981. Somaclonal variation - a novel source of variability from cell cultures for plant improvement. Theor. Appl. Genet. 60:197-214.
4. Larkin, P., and W. Scowcroft. 1982. Tissue culture research: Status and Potential. In Rice Tissue Culture Planning Conference. The International Rice Research Institute, Los Baños, Philippines.
5. Mikaelsen, K., K. Karunakaran, and I. Kiss. 1971. Mutagenic effectiveness and efficiency of gamma rays, fast neutrons, and ethylmethane sulfonate in rice. In Rice Breeding with Induced Mutations III. International Atomic Energy Agency, Vienna, Austria.
6. Morinaga, T., and F. Fukushima. 1937. Cyto-genetical studies on Oryza sativa L. III. Spontaneous autotetraploid mutants in Oryza sativa L. Jpn. J. Bot. 3:71-94.
7. Murashige, T., and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.
8. Oono, K. 1983. Genetic variability in rice plants regenerated from cell cultures. In Cell and Tissue Culture Techniques for Cereal Crop Improvement. Science Press, Beijing and IRRI.
9. Rutger, J., and M. Peterson. 1981. Research tool uses of rice mutants for increasing crop productivity. In Induced Mutations A Tool in Plant Research. IAEA, Vienna.
10. Satoh, H., and T. Omura. 1979. Induction of mutation by the treatment of fertilized egg cell with N-methyl-N-nitrosourea in rice. J. Fac. Agric. Kyushu Univ. 24(2):165-174.
11. Smith, M., and J. McComb. 1981. Effect of NaCl on the growth of whole plants and their corresponding callus cultures. Aust. J. Plant Physiol. 8:267-275.
12. Tal, M., H. Heikin, and K. Dehan. 1978. Salt tolerance in the wild relatives of the cultivated tomato: Response of callus tissue of Lycopersicon esculentum, L. peruvianum, and Solanum pennelli to high salinity. Z. Pflanzenphysiol. 36:231-240.
13. Yoshida, S., and M. Ogawa. 1983. The application of tissue culture-induced mutagenesis to crop improvement. Food and Fertilizer Technology Centre. Tech. Bull No. 73:1-20.

PLANT IMPROVEMENT THROUGH TISSUE CULTURE AT ENEA

G. ANCORA

Some of the research at ENEA's laboratories using in vitro culture techniques is reported, with emphasis on the various achievements of the plant improvement programs.

Clonal propagation, very important in the optimization of plant production, can also be useful in mutation breeding of vegetatively propagated plants.

The results obtained in the interspecific and intergeneric hybridization of tomato and cereals illustrate the use of embryo culture. In tomato particularly, this work has introduced new lines interesting for several of their traits. The cereals work produced a quantity of genetic material used in the triticale breeding programs.

To learn more about plant regeneration and haploid induction, polyploidization has been investigated in *L. peruvianum* and *N. tabacum* cultures. Polyploidization seems correlated to the medium's composition and to the culture's length of stay in vitro.

Finally, some interesting effects of the interaction of *Agrobacterium rhizogenes* with the plant cell genome are reported. The bacterium, which could become a vector for the transfer of genetic material into plant cells, inserts into the plant genome T-DNA which, through meiosis, is transmitted to the progeny of the transformed plants.

ENEA's interest in agriculture began about 25 yr ago. At that time, its researchers focused on the use of nuclear energy in agriculture and, in particular, on using ionizing radiations to improve plants. Much basic and applied work has since been conducted on several species. The principal crops studied have been the cereals (in particular durum and bread wheat), some fruits (cherry, peach, olive, and grape), and some vegetables (tomato, pea, and pepper); but ENEA's activities have extended to most of the main aspects of agricultural research and technology. Recently, energy-saving in agriculture has received more emphasis.

Divisione Tecnologie Biogoicheed Agrarie, Dip, FARE, ENEA, CRE Casaccia, Rome, Italy.

Plant tissue culture is relatively new to plant breeding and it has always involved a small group. This particular work was begun by Prof. Devreux, the first in Italy to obtain haploid tobacco plants through in vitro anther culture (15, 18). His research was made possible through a contract with the European Community and, thanks to his efforts, several other Italian laboratories soon took an interest in tissue culture.

Increasing worldwide attention to the biotechnologies, including tissue culture, has stimulated ENEA to work in these areas. This paper will describe some of the in vitro work of our laboratory, particularly some of Casaccia's successes in plant improvement.

CLONAL PROPAGATION

Among in vitro methodologies, micropropagation is the most widely applied. At Casaccia, conditions for in vitro propagation have been established for several vegetatively propagated plants. The species that have received the greatest attention have been, among flowers, the orchids (Cattleye, Cymbidium, Phalaencpsis) (12), iris (22), and gerbera; among fruits, several apple and pear varieties (3, 5, unpubl.) and the cherry rootstock F12/1 (6); and among vegetables, the potato (29) and globe artichoke (4). At the outset, interest in micropropagation was due to its importance in optimizing plant production. We now believe it may also be useful. in mutation breeding, especially when, as for most fruits, other methods for in vitro plant regeneration are unavailsble.

Rapid propagation of mutagenized material should help isolate induced somatic mutations, either in a periclinal chimera or solid, which represent the main difficulty in the mutation breeding of vegetatively propagated plants. Depending on the plant species, the V3 generation normally needed to isolate nonchimeral mutants can be detected in vitro after 2-3 mo. In vivo, on the other hand, the process takes a long time. A possible additional advantage is that shoot primordia of plantlets growing in vitro are generally smaller than buds in vivo: the chances to form shoots and thereby originate a mutated sector are greater.

After in vitro-grown potato plantlets were irradiated with X-rays, solid mutants were found in plants that underwent only two cycles of in vitro propagation (29). In cv. Desirée, after treatment with 3,000 rads of X-rays, we obtained mutants for anthocyanin absence in the stem and tubers, for plant habitus (short type), and for flower color (Sonnino and Ancora, in preparation).

The cherry rootstock F12/1 also yielded positive results (6). After approximately 5 mo of in vitro multiplication of buds of an unstable mutant formed by irradiation, we isolated some stable variants. The variants greatly differed from the control in leaf morphology and growth habit; one also differed in phyllotaxis. In micropropagated control plants, only true-to-type plants were observed. Irradiation experiments have also been recently performed on gerbera, iris, and pear; these materials are now in the propagation phase.

Micropropagation can indirectly improve some hard-to-multiply, vegetatively propagated plants. The globe artichoke, a very important vegetable in Italy, offers an interesting example of such improvement. Its cultivation is undergoing difficulties on account of the plants' poor health (because of attack by Verticillum, Fusarium, and certain viruses) and their difficult propagation (which also hinders the diffusion of improved clones of the breeding programs).

We worked out a method of in vitro propagation (7), which in 4 wk gives us 4-5 shoots from 1 plant. After rooting, virtually 100% of the plants can be successfully transferred to the soil, if watered during the first 10 d after transplanting. Agronomic trials (26) have also shown that micropropagated plants are more productive than the controls. If transplanted to the field during the correct period (under our conditions, the first week of September), the micropropagated plants, after one season, yield comparably to a 2-yr-old plant. Conventional propagation generally produces a commercially valuable yield only in the second year after the offshoots have been planted.

A project to determine plantlet cost and to evaluate the economic convenience of micropropagation for industry is under way. In any case, our method will prove very useful in obtaining virus-free plants, mother plants to be used as a source of selected material, and in propagating selected lines.

EMBRYO CULTURE

In vitro culture is 2 valuable method for obtaining plants from immature embryos or from dry seeds that germinate with difficulty. In particular, after interspecific or intergeneric crosses, immature embryos which would otherwise degenerate because their endosperm is unsuited to further development, may be used. The embryo culture can also produce additional generations in 1 year and thereby shorten required breeding time.

Cereal embryos isolated from seeds 15-20 d after fertilization can produce plantlets ready for potting after only 1 wk of in vitro culture. Similarly, through in vitro culture of immature tomato embryos, we obtained in a year one generation more than conventional greenhouse cultivation produces.

Interspecific hybrids between the tomatoes L. esculentum and L. peruvianum were obtained through embryo culture (13) in work that was part of a program to transfer valuable wild traits to the cultivated tomato. In vitro techniques were needed not only to obtain hybrid plants, but also to then obtain plants from the FlBCl seeds that had failed to germinate under normal conditions. The FlBCl plants obtained in this way have, after several backcrosses and selection, yielded very interesting and diverse lines. Some of the lines isolated are interesting for high yield, contemporaneous ripening, high sugar content, brown seeds, and resistance to TMV, to Fusarium oxysporum (race l), and to Verticillium dahliae (race 1) (27).

We also routinely use embryo culture to rescue immature embryos. This forms part of a research program to introduce into cultivated cereals alien variation from wild relatives. In this

program we use a mutant of durum wheat that causes homologous pairing after interspecific or intergeneric hybridization, thus favoring genetic recombination (20, 21).

Embryo culture has greatly enhanced the development of new primary triticales. These triticales, crossed with Triticum aestivum, secale, and secondary triticales, have produced a great quantity of genetic material. The Casaccia triticale breeding used this variability and entries from CIMMYT, and has produced the first triticale variety in Italy, now cultivated on approximately 10 000 ha (25).

ANTHER CULTURE

The potential of haploids in plant breeding and mutation breeding is well known. We have attempted haploid production in tobacco (15, 16), tomato (9, 32), strawberry (24), and pepper (28, 33). We have succeeded with several varieties of tobacco. Their diploidization, generally through in vitro regeneration from stem internodes, has produced several isogenic lines (18). Agronomic trials with isogenic lines of the cultivars Burley G.R., Xanthy-Yaka, Perustiza, and Erzegovina and their respective controls have shown great uniformity within lines; morphological differences among lines; and, on the whole, superiority to the respective controls (16). Crosses between isogenic lines showed no heterotic effect in the F1 progeny (17).

In L. esculentum we failed to induce haploid regeneration, while in L. peruvianum, plants were regenerated from calluses produced by anther culture (9, 32). These plants were not haploid though microdensitometer analysis of nuclear DNA did show haploid cells in the calluses. In strawberrv, too, haploid plants were never found among the regenerated plants (24).

On the other hand, several Italian varieties of pepper (Corrida, Venus, Quadrato d'Asti, and Toro) have yielded, by the method of Dumas de Vaulx et a1 (19), haploid and diploid plants (33). Diploid plants from microspore (the regeneration occurs without callus formation) are very useful in pepper, considering their difficult regeneration from somatic tissues and the difficulty of chromosome duplication by colchicine treatment of plant shoots.

With the collaboration of an Italian seed company, a program for developing pure lines is now under way. Its aims are to maintain the purity of pepper varieties and to develop hybrids.

PLANT REGENERATION

Much of the in vitro work of our laboratory has been regenerating a variety of plants from tissues. We have achieved shoot regeneration from different plant tissues in N. tabacum (10, 18), in L. esculentum (1), in L. peruvianum (2, 8, 9, 32), in Pisum (14), in Solanum (unpubl.), and in Mentha (unpubl.). The instability of plant cells in vitro (particularly polyploidization) is one of the investigated aspects that determine the type of plants regenerated.

In the tobacco studies, we performed cytophotometric analysis on the nuclear DNA of the stem and pith tissue cells, which differ consistently in the ploidy level, and in the calluses they produced during 6 mo of in vitro culture (23).

At the end of the experimental period, we observed great similarity in the frequency distribution of the different classes of ploidy in the calluses from both explants. The 4C cells were dominant, followed by 8C and 16C cells. Compared with the original explants, there was more ploidy in the calluses from the stem, and fewer highly polyploid cells (32C) in the calluses from the pith.

An increase of ploidy has also been observed in the calluses derived from anthers and from stem internodes of *L. peruvianum* (9, 32). Here the increase was effected by the prolongation of in vitro culture and by the media composition, and especially by a high kinetin concentration.

Our regeneration experiments have sometimes profited from polyploid cells in differentiated tissues and from in vitro polyploidization. The diploidization of haploid tobacco plants has always been achieved by regeneration from stem internodes. Similarly, in *L. esculentum* and in *L. peruvianum,* tetraploid plants have come from diploid plants and hexaploid plants from triploid, as part of some work on self-incompatibility and unilateral cross-incompatibility (1, 8, 32). Furthermore, the analysis of the ploidy level in the different histogenetic layers of regenerated *L. peruvianum* has shown that out of 81 adult plants originating from the in vitro culture of diploid genotypes, 47 were diploid, 21 tetraploid, and 13 cytochimeras (32), which proved that shoot regeneration can originate from more than one cell.

Propagation by microcuttings, plant regeneration from somatic cells, and plant regeneration from protoplasts will be compared for practicality and efficiency in mutant induction and selection in a new potato breeding program at Casaccia.

PLANT CELL TRANSFORMATION

At Casaccia attention is now being focused on the capacity of *Agrobacterium rhizogenes* to transform plant cells. This bacterium is known for its peculiar ability to induce rooting in most dicotyledonous plants; it inserts into the plant genome a specific region of a large plasmid; this plasmid is responsible for the bacterium's virulence (34). The process shares many characteristics with the tumor causing of the much more studied *A. tumefaciens,* but while the crown gall tumors regenerate with difficulty, plant regeneration is easily induced from hairy root cultures.

Our laboratory has been collaborating in this research with Prof. Constantino's molecular biology group at the University of Rome. In studying the interaction of the bacterium with the plant cells, we have observed that tobacco plants regenerated from calluses derived from hairy roots undergo a series of permanent alterations through the insertion of T-DNA into their nuclear DNA (11, 31). The rhizogenic potential of the hairy root cells is

maintained throughout in vitro plant regeneration (11). In fact, leaf explants taken from transformed plants regenerate roots in vitro, under culture conditions in which normal tissues would yield only shoots. This process can be inhibited by adding kinetin to the culture medium, which suggests that the T-DNA modified the plants' hormone metabolism. The T-DNA also influences isoperoxidase enzyme regulation. In T-DNA-modified leaf tissues, the pattern of these enzymes appears similar to the one in the roots; in normal plants, this is not the case (10). In addition, the transformed plants synthesize agropine, an aminoacid normally derived from bacteria.

The rhizogenic potential, the characteristic isoperoxidase pattern, and the synthesis of agropine are traits that have also been detected in the selfed progeny of the hairy root regenerant (10). The transmission of T-DNA through meiosis has been demonstrated by Southern blot analysis (11).

While the molecular structure of T-DNA is being analyzed, we are trying, with tobacco and potato protoplasts, to establish efficient methods of in vitro infection. We hope to eventually use A. rhizogenes as a vector for DNA transfer.

CONCLUSION

In certain cases, in vitro technologies have already contributed to plant improvement. Efficient methods of in vitro selection could greatly stimulate mutation breeding, but the isolation of variants can require growing large numbers of plants, which involves much space, time, and work. The plant breeding programs may benefit more from a wider use of embryoculture (especially after interspecific and intergeneric hybridization) and of haploids regeneration.

On the other hand, micropropagation can help improve the cultivation of vegetatively propagated plants. It can also be useful in mutation breeding experiments, when adventitious shoot regeneration is not available.

The greatest hopes are set on the genetic manipulation of plant cells through recombinant DNA technology. This could become a sort of directed mutagenesis: placing selected genes into a specific genetic background. This work will require efficient vectors and a better understanding of the genetic and molecular structure of the plant genome.

REFERENCES CITED

1. Ancora, G. 1979. In vitro plant regeneration from leaf cultures of Lycopersicon esculentum Mill. TGC Rep. 29:18.
2. Ancora, G. 1979. In vitro plant regeneration from stem internode cultures of hybrid, Lycopersicon esculentum Mill. X L. peruvianum Mill. TGC Rep. 29:18-19.
3. Ancora, G. 1981. In vitro propagation of varieties and rootstocks of fruit trees for the induction and isolation of mutations. Genet. Agric. 35:48-49.

4. Ancora, G., M.L. Belli-Donini, and L. Cuozzo. 1981. Globe artichoke plants obtained from shoot apices through in vitro micropropagation. Sci. Hortic. 14:207-213.
5. Ancora, G., M.L. Belli-Donini, and L. Cuozzo. 1981. Propagazione di fruttiferi mediante la coltura in vitro di apici vegetativi. L'ottenimento di piante autoradicate nella cv. di melo "Annurca". Riv. Ortofruttic. Ital. 65:59-66.
6. Ancora, G., E. Benvenuto, G. Roselli, B. Donini, and L. Cuozzo. 1982. Micropropagation of cherry rootstock F12/1 clones originated from irradiation: the isolation of solid mutants. Riv. Ortofruttic. Ital. 66:231-238.
7. Ancora, G., F. Saccardo, M. Cappadocia, and K. Sree Ramulu. 1981. Backcross progenies from Lycopersicon esculentum L. X Hybrid (L. esculentum X L. peruvianum Mill.). Z. Pflanzenzuchtg. 87:153-157.
8. Ancora, G., and K. Sree Ramulu. 1981. Plant regeneration from in vitro cultures of stem internodes in self-incompatible triploid Lycopersicon peruvianum Mill. and cytogenetic analysis of regenerated plants. Plant Sci. Lett. 22:197-204.
9. Ancora, G., K. Sree Ramulu, and M. Devreux. 1977. In vitro culture anthers and stem internodes of L. peruvianum: nuclear DNA determination in calli and cytological analysis of regenerated plants. Z. Pflanzenphysiol. 82:377-388.
10. Benvenuto, E., G. Ancora, L. Spano, and P. Costantino. 1983. Morphogenesis and isoperoxidase characterization in tobacco "hairy root" regenerants. Z. Pflanzenphysiol. 110:239-245.
11. Costantino, P., L. Spano, M. Pomponi, E. Benvenuto, and G. Ancora. 1984. The T-DNA of Agrobacterium rhizogenes is transmitted through meiosis to the progeny of hairy root plants. J. Molec. Appl. Genet. 2:465-470.
12. Dalla Rosa, M., and U. Laneri. 1977. Modification of nutrient solutions for germination and growth in vitro of some cultivated orchids and for the vegetative propagation of cymbidium cultivars. Am. Orchid Soc. Bull. 813-820.
13. de Nettancourt, D., M. Devreux, U. Laneri, M. Cresti, E. Pacini, and G. Sarfatti. 1974. Genetic and structural aspects of self- and cross-incompatibility in interspecific hybrids between self-compatible Lycopersicon esculentum and self-incompatible L. peruvianum. Theor. Appl. Genet. 44:278-288.
14. Devreux. M. 1970. Regeneration of numerous shoots by in vitro culture of young pea embryos. Pisum Newsl. 2:12.
15. Devreux, M. 1970. La coltura delle cellule vegetali in vitro: nuove prospetive. Euro-spettra 9:105-110.
16. Devreux, M., and U. Laneri. 1974. Anther culture, haploid plant, isogenic line and breeding researches in Nicotiana tabacum L. Pages 101-107 in FAO/IAEA Eucarpia Conf-Polyploidy and induced mutation in plant breeding. Bari.

17. Devreux, M., and F. Saccardo. 1972. Ibridazione di linee isogeniche di tabacco ottenute da coltura in vitro di antere. Genet. Agric. 26:143-146.
18. Devreux, M., F. Saccardo, and A. Brunori. 1971. Plantes haploides et lignes isogeniques de Nicotiana tabacum obtenue par culture d'antheres et de tiges in vitro. Cariologia 24:141-148.
19. Dumas De Vaulx, R., D. Chanbonnet, and E. Pochard. 1981. Culture in vitro de antheres de piment (Capsicum annuum L.):amelioration de taux d'obtention de plantes chez differents genotypes par des traitements a +35°C. Agronomie 10:859-864.
20. Giorgi, B., F. Barbera, and O. Bitti. 1981. Intergenetic hybrids, meiotic behaviour and amphiploids involving a ph mutant of Triticum turgidum ssp. durum. Pages 96-100 in Symposium Eucarpia, induced variability in plant breeding. Wageningen.
21. Giorgi, B., and L. Cuozzo. 1980. Homoelogous pairing in a pH mutant of tetraploid wheat crossed with rye. Cereal Res. Commun. 8(3):485-490.
22. Laneri, U., and S. Lucretti. 1982. Tissue culture in tall bearded iris:results and perspectives. Atti del XXVI convegno della S.I.G.A:82-83.
23. Marchetti, S., G. Ancora, and A. Brunori. 1976. Time course of polyploidisation in calli derived from stem and pith explants of Nicotiana tabacum studied on isolated nuclei. Z. Pflanzenphysiol. 78:307-313.
24. Rosati, P., M. Devreux, and U. Laneri. 1975. Anther culture of strawberry. Hortscience 10(2):119-120.
25. Rossi, L. 1981. Mizar:prima varieta italiana di triticale iscritta ne1 Registro nazionale. L'informatore Agrar. 37(25):16197-16202.
26. Saccardo, F., and G. Ancora. 1984. Contributo della micropropagazione a1 miglioramento della coltura del carciofo. Giornata di studio della S.O.I. su "Recenti acquisizioni del miglioramento genetico Italiano in Orticoltura e Floricoltura", Ferrara, Italy:24-26.
27. Saccardo, F., G. Ancora, and K. Sree Ramulu. 1981. Transfer of useful characters from Lycopersicon eruvianum to L. esculentum. Pages 235-242 in Proceedings of the meeting of Eucarpia Tomato working group, Avignon, France.
28. Saccardo, F., and M. Devreux. 1974. In vitro production of plantlets from anther culture of Capsicum annuum. Pages 49-50 in Proceedings of the meeting of Eucarpia, Genetics and Breeding of Capsicum, Budapest.
29. Sonnino, A., and G. Ancora. 1983. Haploids and micropropagation for mutation breeding of potato. Genet. Agrar. 37(1,2):216-217.
30. Spano, L., and P. Costantino. 1982. Regeneration of plants from callus cultures of roots induced by Agrobacterium rhizogens on tobacco. Z. Pflanzenphysiol. 106:87-92.
31. Spano, L., M. Pomponi, P. Costantino, G.M.S. Van Slogteren, and J. Tempe. 1982. Identification of T-DNA in the

root inducing plasmid of the agropine type Agrobacterium rhizogenes 1855. J. Plant Molec. Biol. 1:291-300.

32. Sree Ramulu, K., M. Devreux, G. Ancora, and U. Laneri. 1976. Chimerism in Lycopersicum peruvianum plants regenerated from in vitro cultures of anthers and stem internodes. Z. Pflanzenzuchtg. 76:299-319.

33. Tavazza, R., G. Ancora, and F. Saccardo. 1982. The effect of gamma radiations on the male gametophyte of Capsicum annum in vivo and in vitro. Capsicum Newsl. 1:23-25.

34. White, F.F., and E.W. Nester. 1980. Hairy root: plasmid encodes virulence traits in A. rhizogenes. J. Bacteriol. 141:1134-1141.

PLANT REGENERATION FROM SOMATIC CELL CULTURES OF SOME IR VARIETIES OF RICE

D. S. BRAR, D. H. LING, and S. YOSHIDA

The optimum level of 2,4-D for plant regeneration from somatic cell cultures of some IR varieties was found to be 0.5-1.0 mg/litre; higher levels of 2,4-D (more than 2 mg/litre) were inhibitory. Addition of cytokinins such as BAP or kinetin was essential in promoting plant regeneration from cultured cells. Regenerative callus cultures could be induced in IR36, IR58, and IR28 from mature seeds on MS medium supplemented by 0.5 mg 2,4-D/litre, 9.3 mg NAA/litre, and 5.6 mg BAP/litre having either 3 or 6% sucrose. Plantlets were easily obtained upon transfer of hard, compact callus with organized cell growth to MS medium free of 2,4-D but supplemented by 0.93 mg NAA/litre, 2.5 mg BAP/litre, and 3% sucrose. The regenerated plantlets were transferred to auxin-free MS medium having half the amount of inorganic substances for rooting. About 1 100 plants have been regenerated from 6- to 10-wk-old callus cultures of IR36, IR58, IR54, and IR28 varieties. Somatic embryoids could be obtained and maintained in IR36 and IR54 varieties; however, such cultures in the regeneration medium mainly showed green spots and had poor plant regeneration. Some variation, including chlorophyll chimera, nodal branching, and in chromosome number was observed in regenerated plants (Rl). In a preliminary experiment, a few plants were regenerated from callus cells of IR54 grown for 4 wk in salt-enriched medium (1.5% NaCl).

Tissue and cell culture techniques are being explored as an innovative breeding method in the genetic modification and improvement of plants. These techniques include in vitro propagation of desirable genotypes, anther culture, embryo rescue, ovule culture, in vitro fertilization protoplast fusion, induction, and selection of mutants at cellular level; somaclonal variation, genomic modification by uptake of exogenous DNA, isolated chromosomes, chloroplasts, mitochondria, bacteria, etc.; microinjection of DNA, and insertion of foreign genes through

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Senior research fellow, research fellow, and plant physiologist (deceased 24 Jan 19841, Tissue Culture Facility, International Rice Research Institute, P. O. Box 933, Manila, Philippines.

recombinant DNA technology. To apply these techniques in crop improvement, efficient plant regeneration methods are required. Plants may regenerate forming supernumerary adventitious or axillary buds through the de novo organization of shoot and root meristems, or through somatic embryogenesis. Somatic embryogenesis has recently been demonstrated in a few cereal species such as millet, wheat, and maize (9). Nabors et al (7) obtained embryogenic callus cultures in rice, oats, and wheat, and successfully regenerated plants from long-duration cultures of rice by growing E-callus in successive subcultures. Ling et a1 (5) reported somatic embryogenesis and plant regeneration from cultured cells of Oryza sativa/O. latifolia; no IR varieties have yielded such results.

Cereals, in general, are difficult to manipulate in vitro and response of cultured somatic cells to plant regeneration and division of protoplasts is genotype specific (2, 1). Also, regeneration is drastically reduced in successive subcultures. The problem is more acute with indica rices, particularly IR varieties (improved varieties of rice developed at IRRI), whose regeneration is extremely poor -- 0.0% in IR36, IR30, IR26, and IR8 -- but as high as 61.8% in Taichung 65, a japonica rice (8). The objective of this investigation was to improve plant regeneration from somatic cell cultures of promising IR varieties for eventual somaclonal variation and induction and selection of mutants at cellular level for salinity tolerance and other desirable traits.

MATERIALS AND METHODS

The experimental material consisted of IR5, IR8, IR28, IR36, IR50, IR52, IR54, IR58, and other rice varieties: Nona Bokra (traditional salt-tolerant indica rice), Basmati 370 (fine aromatic variety of indica), and Taichung 65 (japonica rice).

Callus induction

Callus cultures were initiated from dehulled seeds following the procedure of Suenaga et a1 (8).

In a few experiments, young panicles were also used for callus induction. The explant consisted of 5- to 25-mm panicles from the initiation of second rachis to the formation of stamens and pistils. The suitable boots were washed in running tap water and cleaned with cotton soaked with 70% alcohol. The boots were continually peeled until the leaf sheath and node emerged, then they were soaked in 70% alcohol for 3 min and rinsed 3 times in sterilized water, treated with 0.1% mercuric chloride for 10-15 min, and washed again 3 times in sterilized water. Later, the immature panicles were carefully dissected from the boots in petri dishes.

Subculture of callus and plant regeneration

Small (50- to 150-mg) callus pieces were transferred during subculture onto the proliferation medium and, for

differentiation, onto the regeneration medium. The details of this procedure are given by Suenaga et al (8).

During subculture and regeneration of callus, hard, compact calluses with organized growth of cells were used and separated by stereomicroscope from the wet, friable, loosely packed callus regions. Organized growth of cells was occasionally verified under the light microscope. In a few experiments, callus pieces with somatic embryos were separated and used for subsequent subculturing and regeneration.

RESULTS AND DISCUSSION

Callus induction and plant regeneration

The results of different experiments on callus induction and plant regeneration are given in Tables 1-5.

Effect of 2,4-D, zeatin, and BAP. Callus cultures were initiated from dehulled seeds of different varieties. In the first experiment, the regenerating ability of the medium used by Suenaga et a1 (8) was determined. Regeneration was poor in IR5, IR36, and Nona Bokra (Table 1). Several other varieties, such as IR8, IR50, IR52, IR54, and Basmati 370, also responded poorly to this medium. The callus induction frequency is high and proliferation and growth of callus are extensive; however, the medium is unsatisfactory for regenerating plants. The poor response may be due to the lack of any cytokinin in the callus induction medium, and to a sudden change in sucrose level from 3%

Table 1. Plant regeneration from callus cultures of rice varieties.

Medium[a]	No. of seeds (%)	Calluses (%)	Calluses (no.) cultured at				Plants regenerated[b] (%) from calluses aged			
			4 wk	12 wk	16 wk	20 wk	4 wk	12 wk	16 wk	20 wk
				IR36						
MS1	132	78.0	42	63	-	47	0.0	0.0	-	0
MS2	111	77.5	43	-	42	36	0.0	0.0	0.0	0
MS3	138	53.6	32	69	45	74	3.1	2.9	0.0	0
MS4	138	56.5	89	71	43	36	7.8	12.7	0.0	2.8
				IR5						
MS1	-	-	43	39	52	-	0.0	0.0	0.0	-
MS2	150	58.0	57	-	47	59	7.0	-	4.2	3.4
MS3	252	40.9	58	57	50	50	5.9	15.8	8.0	2.0
MS4	105	20.0	52	56	52	40	7.7	5.4	0.0	0.0
				Nona Bokra						
MS1	129	94.6	73	80	-	95	2.8	0.0	-	0.0
MS2	150	99.3	64	-	37	45	10.9	-	2.7	6.6
MS3	210	94.3	51	39	36	40	15.7	12.8	2.8	10.0
MS4	138	78.3	60	80	55	41	30.0	28.8	12.7	2.1

[a] MS:1 Induction and regeneration medium (S).
MS2: MS + 2 mg 2,4-D/litre + zeatin 1 mg/litre.
MS3: MS t 2 mg 2,4-D/litre + BAP 1 mg/litre.
MS4: MS + 0.5 mg 2,4-D/litre + NAA 9.3 mg/litre + BAP 5.6 mg/litre.
[b] Regeneration media for MS2, 3,4, same as MS2, 3, 4 respectively but without 2,4-D

to 7% in the regeneration medium. The regeneration was better in the medium supplemented by cytokinin, either zeatin or BAP (Table 1). The regeneration was further enhanced by the medium (MS-4) with a low level of 2,4-D (0.5 mg/litre) and supplemented by 9.3 mg FAA/litre, and 5.6 mg BAP/litre. The regeneration was reduced in the successive passages of three varieties.

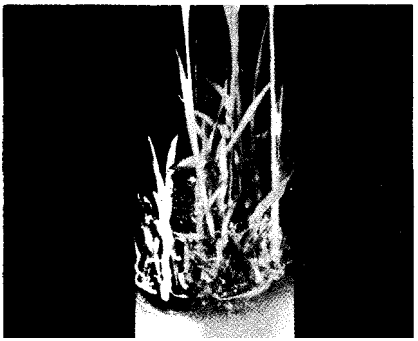
The effect of different levels of 2,4-D and BAP on callus induction and plant regeneration in IR58 is shown in Table 2. Callus could be induced by using a wide range of 2,4-D (0.5-10.0 mg/litre); however, a higher level of 2,4-D combined with 5 mg/litre reduced callus formation considerably (7.2%). Plant regeneration was severely inhibited at higher levels of 2,4-D (more than 2 mg/litre). The addition of cytokinin (BAP) promoted plant regeneration significantly at 0.5-1.0 mg 2,4-D/litre. The promotive effect of kinetin on plant regeneration was also observed in IR36 and IR54. Low levels of 2,4-D (0.5-1 mg/litre) in combination with RAP (1-5 mg/litre) seem optimal for plant regeneration. It seems that rice responds like sorghum to the exogenous addition of cytokinin for plant regeneration (3, 2).

Separation of hard, compact callus regions with organized growth of cell. Induction of callus on the MS medium consisting of 3% sucrose, 0.5 mg 2,4-D/litre, 9.3 mg NAA/litre, and 5.6 mg BAP/litre, followed by regeneration on MS medium without 2,4-D and supplemented by 0.93 mg NAA/litre and 2.52 mg BAP/litre was adequate to regenerate plants from 6- to 10-wk-old callus cultures of IR36 and IR58 (Fig. 1, 2, Table 3). The separation of callus regions from the cultures grown on this medium was difficult. A similar medium (medium 2, Table 3) resulted in low frequency of callus induction; however, hard, compact callus regions with organized cell. growth could be differentiated and

Table 2. Effect of 2.4-D and BAP on callus induction and plant regeneration in IR58.

MS + supplements (mg/litre)		Callus induction[a]			Plant regeneration[b] (5-wk callus)		
2.4-D	BAP	Seeds (no.)	Calluses (no.)	Calluses (%)	Calluses (no.)	Regenerating calluses (no,)	Regeneration (%)
0	0	50	0	0.0	–	–	–
0.5	0	60	9	15.0	7	1	14.3
0.5	1	78	13	16.6	12	4	33.3
0.5	5	78	8	10.2	11	4	36.4
1.0	0	75	39	52.0	30	1	3.3
1.0	1	75	53	70.7	35	5	14.3
1.0	5	75	35	46.7	30	9	30.0
2.0	0	69	46	66.4	40	1	2.5
2.0	1	72	28	38.9	20	0	0.0
2.0	5	75	27	36.0	24	1	4.2
5.0	0	72	24	33.3	20	0	0.0
5.0	1	63	32	50.7	20	0	0.0
5.0	5	75	17	22.2	15	0	0.0
10.0	0	60	39	65.0	34	0	0.0
10.0	1	75	35	46.7	31	0	0.0
10.0	5	69	5	7.2	5	0	0.0

[a]Callus induction medium: MS + 3% sucrose + different levels of 2.4-0 and BAP. [b]Regeneration medium: MS t 3% sucrose + BAP (1 mg/litre).



1. Regenerating callus culture of IR58.

2. Plants regenerated from somatic cells of IR36 and IR58.

separated from the loosely packed and wet callus regions. As a result of separation of such callus sections, and subsequent subculturing on the optimum medium, upon transfer to regeneration medium, 40-60% of the 6-wk-old callus cultures showed plant regeneration and 15-30% in the first passage (10-wk callus cultures). Higher level of sucrose (62) in the induction medium decreased the frequency of callus formation but increased plant regeneration.

Growing the cells in an optimum medium and then separating the callus cells very effectively improved plant regeneration. More than 1,100 plants have been regenerated from 6- to 10-wk callus cells of IR36, IR58, and IR28 varieties. The origin of these plants should be investigated further. Also, more efforts are needed to regenerate plants from long-term callus cultures of these varieties. Nabors et a1 (7) obtained embryogenic calluses in rice and successfully regenerated plants in a high frequency from long-duration cultures.

Table 3. Callus induction and plant regeneration in IR varieties.

Medium[a]	Callus induction			Plant regeneration[b] from calluses aged					
				6 wk			10 wk		
	Seeds (no.)	Calluses		Calluses transferred (no.)	Regenerating calluses		Calluses transferred (no.)	Regenerating calluses	
		No.	%		No.	%		No.	%
				IR36					
1	150	79	52.1	52	7	13.5	40	6	15.0
2	310	93	30.0	55	23	41.8	37	6	16.2
				IR58					
1	150	94	62.7	40	8	20.0	30	5	16.6
2	348	126	36.2	80	48	60.0	32	10	31.2
				IR28					
1	90	46	51.1	–	–	–	–	–	–
2	150	52	34.7	28	11	39.3	16	3	18.1

[a]Medium 1: MS + 3% sucrose + 2.4-D 0.5 mg/litre + NAA 9.3 mg/litre + BAP 5.6 mg/litre. Medium 2: same as Medium 1 but with 6% sucrose. [b]Regeneration medium: MS + 3% sucrose + NAA 0.93 mg/litre + BAP 2.52 mg/litre.

Effect of abscisic acid and kinetin (two-step culture). The effect of ABA in the preculture medium and kinetin in the last culture medium was studied in callus cultures derived from mature seeds and young panicles. The regeneration was 0% in IR36, IR52, and IR54, and 2% in Taichung 65 and Nona Bokra; however, IR50 had 6% plant regeneration. Inoue and Maeda (4) obtained high frequency of shoot bud and plant regeneration in japonica rice variety Aichi asahi from the two-step culture method. The results of our study showed no improvement in plant regeneration; probably different materials were involved.

Induction and regeneration of embryogenic callus. In this experiment, we obtained callus cultures with somatic embryoids in lR36 and IR54 by using mature seed and young panicles (Fig. 3, Table 4). Other varieties, such as IR8, IR50, and IR52, and "elite" IRRI lines (IR3646-8-1-2, IR6023-10-1-1, IR15429-268-1-2-1, lR17488-2-3-2-2-2-3), also showed somatic embryoids from cultured cells. Vasil et a1 (9) reported somatic embryogenesis in cereal and such grasses as *Pennisetum americanum*, *P. purpureum*, *P. americanum*/*P. purpureum*, *Panicum maximum*, *Triticum aestivum*, and *Zea mays*. We enhanced the frequency of E-callus in IR54 by supplementing the medium with casein hydrolysate and yeast extract (Table 5). The E-callus could be maintained during successive passages in both IR36 and IR54. Such callus cultures upon transfer to regeneration medium usually showed green spots with limited plant regeneration (Fig. 4). Although the frequency of E-callus was 35% in second and 30% in third passage of IR36, no plants could be regenerated. Similarly, E-callus of IR54 in 4th passage showed no plant regeneration. Nabors et al (7) could successfully regenerate plants from E-callus of rice from long-duration cultures. Ling et al (5) could maintain E-callus from cultured cells of an interspecific hybrid of *Oryza* for 12 passages. More experimentation on optimum conditions is needed to regenerate plants from long-duration cultures of IR varieties.

In a preliminary experiment, callus pieces (100-150 mg) of IR54 were grown on MS medium supplemented by 1.5% NaCl, 6% sucrose, 1 mg 2,4-D/litre, 9.3 mg NAA/litre, and 5.6 mg BAP/litre. Of the 114 calluses grown on salt-enriched medium for 4 wk, only 18. survived, and those with only limited growth. The surviving, calluses were proliferated and transferred to regeneration medium, but only one callus regenerated. It resulted in 11 plantlets.

3. Callus showing somatic embryoids.

Table 4. Induction and regeneration of embryogenic (E) calluses.

Variety	Seeds or panicles cultured (no.)	E-calluses[a]				
		No.	%	Transferred (no.)	Regenerated	
					No.	%
IR36						
Seed	72	20	27.7	56	2	3.6
Young panicles	42	21	50.0	56	3	5.3
IR54						
Seed	72	11	15.3	60	7	11.7
Young panicles	40	12	30.0	64	4	6.2

[a]Callus induction: MS + 3% sucrose + kinetin (3 mg/litre) + NAA (2 mg/litre) + 2,4-D (1-2 mg/litre). Subculture: MS + 3% sucrose + 2,4-D (0.5 mg/litre) + ABA (0.1 mg/litre). Regeneration medium: MS + 3% sucrose + NAA (2 mg/litre) + kinetin (2 mg/litre).

Table 5. Induction, subculture, and regeneration of embryogenic calluses in successive passages.

Variety	Induction[a]			E callus (%) in different subculture[b]				Plant regeneration[c] (%)			
	Seeds (no.)	E-calluses									
		No.	%	1st passage	2nd passage	3rd passage	4th passage	1st passage	2nd passage	3rd passage	4th passage
IR36	60	18	30.0	45.6	35.0	30.0	–	7.5	0.0	0.0	0.0
IR54	60	33	55.0	62.0	75.0	65.0	60.0	33.3	16.0	6.2	0.0

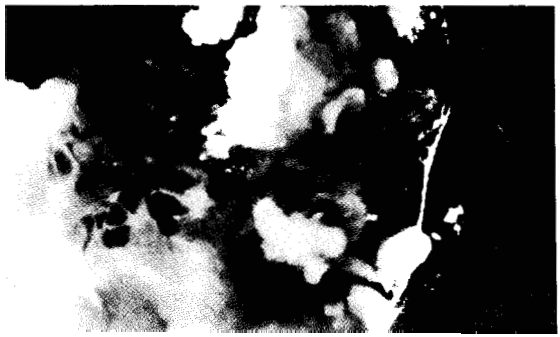
[a]Medium: MS + 3% sucrose + casein hydrolysate 300 mg/litre + yeast extract 1300 mg/litre + 2.4-D 2 mg/litre + NAA 2 mg/litre + kinetin 3 mg/litre. [b]Medium MS + 3% sucrose + 2.4-D 0.5 mg/litre + ABA 0.1 mg/litre. [c]Medium: MS + 3% sucrose + NAA 2 mg/litre + kinetin 2 mg/litre.

Variation such as chlorophyll chimera, nodal branching, and in chromosome number was observed in the regenerated plants (R1). The 1,100 descendents (R2) of the tissue culture-derived plants will be tested for somaclonal variation for salinity tolerance and other desirable traits.

CONCLUSIONS

Higher levels of 2,4-D (>2 mg/litre) inhibit plant regeneration from somatic cell cultures of IR58. Addition of cytokinins such as BAP promotes plant regeneration from such cells. The optimum level of 2,4-D for regenerative callus in IR36, IR58, and IR28 is 0.5 mg/litre supplemented by NAA (9.3 mg/litre) and BAP (5.6 mg/litre). The medium used by Suenaga et al (8) was unsatisfactory for plant regeneration of IR36, IR58, IR54, IR50, IR52, and Basmati-370.

Selection of hard, compact callus with organized growth of cells increased the plant regeneration frequency, but friable, loosely packed, and wet callus cultures proliferated rapidly, yet had only sporadic and poor regeneration ability.



4. Embryogenic callus showing green spots.

About 1,100 plants have been regenerated from 6- to 10-wk-old cultures of IR36, IR58, IR54, and IR28. Some variation such as chlorophyll chimera, nodal branching, and in chromosome number was observed in regenerated plants (Rl). A few plants have been regenerated from callus cells of IR54 grown for 4 wk on salt-enriched medium (1.5% NaC1).

Somatic embryos could be obtained in IR8, IR36, IR50, IR52, and IR54 varieties; however, callus cultures, upon transfer to the regeneration medium, show mainly green spots and poor plant regeneration.

Our attempts to regenerate plants from long-term callus cultures of IR varieties, and from those cultures showing green spots, have not been very successful.

REFERENCES CITED

1. Brar, D.S., S. Rambold, F. Constabel, and O.L. Gamborg. 1980. Isolation, culture, and fusion of sorghum, and corn protoplasts. Z. Pflanzenphysiol. 96:269-275.
2. Brar, D.S., S. Rambold, O.L. Gamborg, and F. Constabel. 1979. Tissue culture of sorghum and corn. Z. Pflanzenphysiol. 95:377-388.
3. Gamborg, O.L., J.P. Shyluk, D.S. Brar, and F. Constabel. 1977. Morphogenesis and plant regeneration from callus of immature embryos of sorghum. Plant Sci. Lett. 10:67-74.
4. Inoue, M., and E. Maeda. 1981. Stimulation of shoot bud and plantlet formation in rice callus cultures by two-step culture method using abscisic acid and kinetin. Jpn. J. Crop Sci. 50:318-322.
5. Ling, D.H., W.Y. Chen, M.F. Chen, and Z.R. Ma. 1983. Somatic embryogenesis and plant regeneration in an interspecific hybrid of Oryza. Plant Cell Reports 2:169-171.
6. Murashige, T., and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

7. Nabors, M.W., J.W. Heyser, T.A. Dykes, and K.J. Demott. 1983. Long-duration, high-frequency plant regeneration from cereal tissue cultures. Planta 157:385-391.
8. Suenaga, K., E.M. Abrigo, and S. Yoshida. 1982. Seed derived callus culture for selecting salt-tolerant rice. Part I. Callus induction, plant regeneration, and variations in visible plant traits. IRRI Res. Pap. Ser. 79. 11 p.
9. Vasil, I.K., V. Vasil, C. Lu, P. Atkins, Z. Haydu, and D. Wang. 1982. Somatic embryogenesis in cereals and grasses. Pages 3-21 in Variability in plants regenerated from tissue culture. E. Earle and Y. Demarly, eds. Praeger Press, New York.

SOMATIC HYBRIDIZATION AND WIDE CROSSES

PROTOPLAST FUSION AND ITS APPLICATION IN AGRICULTURE

E. C. COCKING

Species hybridization is a valuable tool for plant breeding: it may extend the available range of heritable variation. Until recently hybridization was only possible sexually; protoplast technology is now an additional method, which further augments heritable variation.

Procedures for isolating plant protoplasts are now well established (5). As long as plant cell and tissue culture remains largely an empirical science, sustained division of cell regenerated from protoplasts, and whole plant regeneration from callus or somatic embryos will probably remain difficult. Steady success has, however, been achieved in the production of plants from protoplasts (5), although the grasses still present major difficulty (34).

Protoplasts can usually be satisfactorily fused into homokaryons and heterokaryons through use of chemical fusogens (23); and if selection and plant regeneration are satisfactory the potentials of somatic fusions can be explored for plant improvement.

At the 1980 IRRI Rice Tissue Culture Planning Conference, the progress and the potential of protoplast fusion in crop improvement were surveyed (20). This conference focuses on broader aspects, 2nd it will therefore be appropriate to discuss the more general. implications of in vitro culture for protoplast technology, and also to give examples for applications of protoplast fusion.

CLONING OF PLANTS FROM PROTOPLASTS

In recent years it has been repeatedly emphasized that plant tissue culture per se seems an unexpectedly rich source of genetic variation (14); it has stimulated attempts to find out whether such genetic variation can be enhanced by protoplast cloning, e.g., using potato protoplasts (29). In studies using a diploid tobacco (Nicotiana sylvestris), protoplast culture induced a high frequency of recessive mutations affecting qualitative and quantitative characters of plant morphology or plant growth (24). Riley (25) believes, however, that we need evidence that this variation produces dominant alleles and that the variants are not just aneuploids.

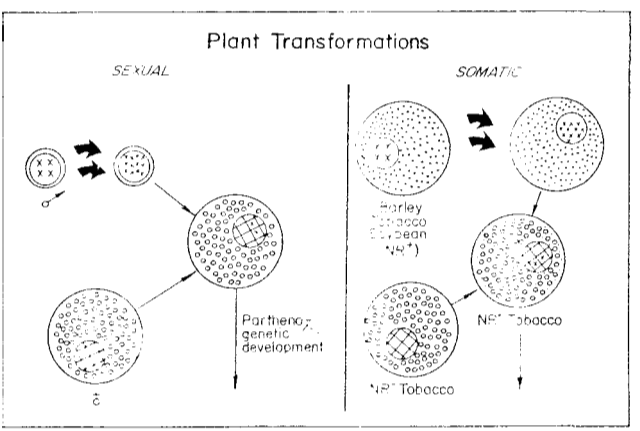
Plant Genetic Manipulation Group, Department of Botany, University of Nottingham, Nottingham, U. K.

An important point is that after fusion, protoplasts are also subjected to in vitro culture, and single gene mutations have been detected in the regenerated plants, providing good evidence that plant cell and tissue culture can be mutagenic (6). Mobile genetic elements (28) may be involved, and in vitro culture may accentuate these tendencies. The opportunity for mutagenesis presented by the in vitro culture of heterokaryons and homokaryons will undoubtedly lead to new genetic variation.

SOMATIC HYBRIDIZATION BY PROTOPLAST FUSION

Sybenga (33) has concluded that although classical methods will remain the basis of plant breeding, a number of new somatic genetic manipulation techniques will be useful in special. situations. The challenge is to identify these special situations, and to know the special attributes of the somatic systems. An attractive potential of recombinant DNA technology is the transfer (through suitable vectors) and expression of agronomically improving genes in plants; protoplasts may be useful in effecting this. Protoplast fusion also provides the opportunity for such transfers. Particularly relevant here is recent work in Nicotiana showing that irradiation of pollen can help effect limited sexual gene transfer between genotypes (11, 16). In wheat (31) and in barley (22), irradiated pollen has facilitated limited gene transfer. It may be possible to obtain limited gene transfer by protoplast fusion even between sexually isolated species, if one of the fusion partners is suitably irradiated (see Fig. 1). With suitable selection, this would be equivalent to direct genetic transfer; it avoids the need to identify the plant-improving genes and could be readily used in polygenic transfers.

The cytoplasmic mix obtained from protoplast fusions offers the opportunity for producing cybrids and forming mitochondrial



1. Irradiated pollen can help transfer a few genes sexually. Currently a nitrate reductase (NR^-) mutant of tobacco is being used to determine whether similar limited gene transfer can result from the fusion of irradiated protoplasts.

recombinants (4). We have made important advances in our understanding of the segregation of cytoplasmic traits in higher plant somatic fusion hybrids (7). Hybrid vigor is well known in sexual hybridization, and somatic hybridization may produce an even greater vigor in hybrids (3), a trait that could enhance yields in many crops.

PROTOPLAST TECHNOLOGY

Rice improvement

Since half the world's population depends mainly on rice for food, it is understandable that the importance of biotechnology to rice improvement has been highlighted (32). Before many of the ideas can be implemented, however, we need the ability to regenerate whole plants from isolated protoplasts in a range of rice varieties. The University of Nottingham and IRRI are jointly undertaking research on protoplast fusions in rice. These fusions will not only be restricted to the transfer of cytoplasmic male sterility but will also embrace fusions with wild rice species to enhance disease resistance and salt tolerance.

The possibility of using the water fern Azolla with its associated blue-green alga, as a nitrogen-fixing green manure crop for rice culture, has been recognized by many. Sexual crossing of Azolla species to improve strains and enhance biomass has not been successful. The University of Nottingham and IRRI are now engaged in collaborative attempts to cross Azolla species by protoplast fusions.

Brassica improvement

Schenk and Robbelen (27) have shown that resynthesis *Brassica napus* from its ancestral diploids (*B. oleracea* and *B. campestris*) is possible through somatic protoplast fusion.

Pelletier et a1 (19) are investigating intergeneric cytoplasmic hybridization in the Cruciferae by protoplast fusion, which could produce plants useful for *Brassica* hybrid seed production. *B. napus* plants were regenerated after fusion of protoplasts bearing cytoplasms of different genera. One type of cybrid produced had *B. napus* chloroplasts and cytoplasmic male sterility (cms) from *Raphanus sativus*; another type had chloroplasts of a triazine-resistant *B. campestris* and cms from *Raphanus sativus*, with the nucleus of *B. napus*.

Tobacco improvement

Nicotiana tabacum, the most widely grown commercial nonfood plant in the world, is susceptible to bacterial wild fire disease and fungal black shank. *Nicotiana rustica* carries resistance to both diseases, but its sexual crosses with *N. tabacum* lack fertility. Protoplast fusions can produce amphiploid somatic hybrids between the two species. Leaf protoplasts of wild-type *N. tabacum* can be fused with wild-type suspension culture protoplasts and directly

isolated heterokaryons (17), and cultured to regenerate whole, fertile somatic hybrids (18). Fertile somatic hybrids can also be produced by fusing wild-type N. rustica cell suspension protoplasts with leaf protoplasts of the double mutant of N. tabacum that is both NR and SR (10). In this somatic hybrid, the black ovary wall gene from N. rustica can be readily demonstrated; the influence of irradiation is being assessed on the degree of gene transfer between the two species and to determine the readiness with which disease resistance genes could be transferred from N. rustica without backcrossing.

Solanum improvement

Use of protoplast fusion to improve Solanum species has been stimulated by advances in their protoplast cultural capability. Kowalczyk et a1 (12) have regenerated plants from Solanum viarum and Solanum dulcamara leaf mesophyll protoplasts. Solanum viarum has been suggested as the main alternative source of steroid drug precursors to the medicinal yams. It is an annual prickly undershrub widely distributed throughout India. The stereoidal glycoalkaloid solasodine is contained in its mature fruits. Improvements required are: 1) a spineless strain, to facilitate harvesting; 2) an increase in the berry solasodine content; and 3) resistance to vascular wilt disease caused by the fungus Fusarium oxysporum. Protoplast technology could improve the agronomic character of Solanum viarum through protoplast cloning and somatic fusions with other alkaloid-producing species such as Solanum dulcamara. Irradiation of the S. dulcamara protoplasts could facilitate the limited gene transfers for the required improvements.

Commercial potato cultivars cannot be crossed directly with many related Solanum species. Shepard et al. (30) have suggested that since many primitive Solanum species possess broad-spectrum resistance to disease, protoplast fusion might permit introduction of resistance genes from such sexually isolated species into potato germplasm pools. Since many of our established potato varieties are sterile, fusion of protoplasts, even from different varieties, could produce new genetic variation and improve potato genotypes.

Legume improvement

A bloat-safe white clover (Trifolium repens) and alfalfa (Medicago sativa) are needed. Protoplast fusion may provide a means of introducing characteristics for leaf tannin production, which acts as the antibloat factor, into these key western forage legumes (9). White and Bhojwani (35) have suggested that bloat-safe white clover might also be produced by interspecific protoplast fusion with the annual clover T. arvense, which accumulates condensed tannins in its leaves. (Sexual incompatibility, within the genus Trifolium and other forage legumes, prevents the introduction of such desirable characters by conventional means.)

Sainfoin (Onobrychis viciifolia) is a forage legume combining persistency, drought resistance, and high palatability with valuable protein and nonbloating properties (8). It is one of the

few Leguminosae with tannins in the vegetative parts as well as in the seed coat, which are potentially valuable in protecting proteins in ruminant digestion (26). It is ideal for somatic hybridization with clover and alfalfa (1), and for direct introgression assessment using irradiation. While somatic hybrids have not yet been produced, good cultural capability with regeneration of plants from protoplasts, exists in all these species, including the bloat-safe Lotus corniculatus (2). Protoplast fusion between these species is satisfactory. Mutants are not available for complementation selection, but direct isolation of heterokaryons is (17). (This isolation is likely to be automated using fluorescence-activated, cell-sorted technology for obtaining adequate numbers of heterokaryons.)

The use of protoplast technology to improve grain legume is still young. Although callus from several varieties of soybean (Glycine max) cotyledon protoplasts has been formed (13), reproducible regeneration of whole plants has not been reported. Hybridizations between soybean and wild perennial relatives are needed to produce diverse germplasm for soybean breeding programs. Hybrid plants have been obtained between soybean and a wild perennial relative, Glycine tomentella Hayata, through in vitro ovule culture (15), but, as might have been expected, they were sterile, and no seed was recovered. Somatic hybridization between soybean and wild perennial relatives is likely to broaden the range of hybrids available. The direct production of tetraploids by fusion of somatic diploids might also restore fertility.

CONCLUSION

The great success of conventional plant breeding tends to obscure the need for other methods of genetic manipulation. But, as we have seen, a number of specific applications require an alternative source of enhanced gene flow. Protoplast fusion fulfills that need.

REFERENCES CITED

1. Ahuja, P.S., D.Y. Lu, E.C. Cocking, M.R. Davey. 1983. An assessment of the cultural capabilities of Trifolium repens L. (White clover) and Onobrychis viciifolia Scop. (Sainfoin) mesophyll protoplasts. Plant Cell Rep. 2:269-272.
2. Ahuja, S., S. Hadiuzzaman, M.R. Davey, and E.C. Cocking. 1983. Prolific plant regeneration from protoplast derived tissues of Lotus corniculatus L. (Birdsfoot trefoil). Plant Cell Rep. 2:101-104.
3. Bingham, E.T. 1983. Maximizing hybrid vigour in autotetraploid alfalfa. Pages 130-143 in Ciba Foundation Symposium 97. Better crops for food:130-143.
4. Cocking, E.C. 1981. Opportunities from the use of protoplasts. Philos. Trans. R. Soc. Lond. B. 292:557-568.

5. Davey, M.R. 1983. Recent developments in the culture and regeneration of plant protoplasts. Pages 19-29 in Lecture proceedings 6th international protoplast symposium. I. Potrykus, ed. Exp. Suppl 46:19-29.
6. Evans, D.A., and W.R. Sharp. 1983. Single gene mutations in tomato plants regenerated from tissue culture. Science 221:949-951.
7. Fluhr, R. 1983. The segregation of organelles and cytoplasmic traits in higher plant somatic fusion hybrids. Pages 85-92 in Lecture Proceedings 6th international protoplast symposium. I. Potrykus, ed. Exp. Suppl. 46.
8. Goplen, B.P. 1969. Forage legumes. Pages 225-259 in Canadian forage crops symposium 1969. Modern Press, Saskatoon, Canada.
9. Goplen, B.P., R.E. Howarth, S.K. Sarkar, and K. Leskins. 1980. A search for condensed tannins in annual and perennial species of Medicago, Trigonella, and Onobrychis. Crop Sci. 20:801-804.
10. Hamill. J.D., D. Pental, E.C. Cocking, and A.J. Muller. 1983. The culture of manually isolated heterokaryons of Nicotiana tabacum and Nicotiana rustica. Heredity 50:197-200.
11. Jinks, J.L., P.D.S. Caligari, and N.R. Ingran. 1981. Gene transfer in Nicotiana rustica using irradiated pollen. Nature 291:586-588.
12. Kowalczyk, T.P., I.A. Mackenzie, and E.C. Cocking. 1983. Plant regeneration from organ explants and protoplasts of the medicinal plant Solanum Rhasianum C.B. Clarke var. chatterjeeanum Senpupta (Syn. Solanum viarum Dunal). Z. Pflanzenphysiol. 111:55-68.
13. Lu, D.Y., S. Cooper-Bland, D. Pental, E.C. Cocking, and M.R. Davey. 1983. Isolation and sustained division of protoplasts from cotyledons of seedlings and immature seeds of Glycine max L. Z. Pflanzenphvsiol. 111:389-394.
14. Meins, F. 1983. Heritable variation in plant cell culture. Ann. Rev. Plant Physiol. 34:327-346.
15. Newell, C.A., and T. Fymowitz. 1982. Successful wide hybridization between the soybean and a wild perennial relative, G. tomentella Hayata. Crop Sci. 22:1062-1065.
16. Pandey, K.K. 1975. Sexual transfer of specified genes without gametic fusion. Nature 256:310-313.
17. Patnaik, G., E.C. Cocking, J. Hamill, and D. Pental. 1982. A simple procedure for the manual isolation and identification of plant heterokaryons. Plant Sci. Lett. 24:105-110.
18. Patnaik, G., J.D. Hamill, D. Pental, and E.C. Cocking. 1984. The culture of manually isolated heterokaryons of Nicotiana tabacum and Nicotiana rustica. Golden .Jubilee number, Proceedings Indian Academy of Science (Plant Science).
19. Pelletier, G., C. Primard, F. Vendel, P. Chetrit, R. Remy, P. Rouselle, and M. Renard. 1983. Intergeneric

cytoplasmic hybridization in Cruciferae by protoplast fusion. Mol. Gen. Genet. 191:244-250.

20. Pental, D., and E.C. Cocking. 1982. Progress and potential of protoplast fusion in crop improvement. Pages 25-29 in Rice tissue culture planning conference. International Rice Research Institute, Los Baños, Philippines.
21. Pental, D., J.D. Hamill, and E.C. Cocking. 1984. Somatic hybridization using a double mutant of Nicotiana tabacum. Heredity. (in press)
22. Powell, W., P.D.S. Caligari, and A.M. Hayter. 1983. The use of pollen irradiation in barley breeding. Theor. Appl. Genet. 65:73-76.
23. Power, J.B., P.K. Evans, and E.C. Cocking. 1978. Fusion of plant protoplasts. Cell Surface Rev. 5:369-385.
24. Prat, D. 1983. Genetic variability induced in Nicotiana sylvestris by protoplast culture. Theor. Appl. Genet. 64:223-230.
25. Riley, R. 1983. Discussion. Pages 188-189 in Ciba Foundation Symposium 97, Better crops for food.
26. Rodgers, H.H. 1975. Forage legumes (with particular reference to lucerne and red clover). Pages 22-27 in Plant Breeding annual report.
27. Schenck, H.R., and G. Robbelen. 1982. Somatic hybrids by fusion of protoplasts from Brassica oleracea and B. campestris. Z. Pflanzenzuechtg. 89:278-288.
28. Shapiro, J.A. 1983. Mobile genetic elements. Academic Press, New York.
29. Shepard, J.F., D. Bidney, and E. Shahin. 1980. Potato protoplasts in crop improvement. Science 208:17-24.
30. Shepard, J.F., D. Bidney, T. Barsby, and R. Kemble. 1983. Genetic transfer in plants through interspecific protoplast fusion. Science 219:683-688.
31. Snape, J.W., B.B. Parker, E. Simpson, C.C. Ainsworth, P.I. Payne, and C.N. Law. 1983. The use of irradiated pollen for differential gene transfer in wheat (Triticum aestivum). Theor. Appl. Genet. 65:103-111.
32. Swaminathan, M.S. 1982. Biotechnnlopy research and third world agriculture. Science 218:967-972.
33. Sybenga, J. 1983. Genetic manipulation in plant breeding: somatic versus generative. Theor. Appl. Genet. 66:179-201.
34. Vasil, I.K. 1983. Isolation and culture of protoplasts of grasses. Int. Rev. Cytol. Suppl. 16:79-88.
35. White, D.W.R., and S.S. Bhojwani. 1981. Callus formation from Trifolium arvense protoplast-derived cells plated at low densities. Z. Pflanzenphysiol. 102:257-261.

THE APPLICATIONS OF EMBRYO RESCUE IN AGRICULTURE

V. RAGHAVAN

The importance of various embryo rescue operations in obtaining viable seedlings from interspecific and intergeneric crosses is discussed. One of the widely used methods is to excise and culture the undifferentiated hybrid embryo in a specified medium. Limited success has also been obtained by implanting the embryo on a nurse tissue of a normal endosperm or by culturing the hybrid ovule. In certain cases, seed lethality is overcome by regenerating plants through organogenesis from a hybrid embryo callus. Mutant embryo culture permits analysis of the causes of seed genetic lethality.

Crosses between distantly related species of plants have not yielded any agriculturally beneficial hybrids primarily because in the transfer of beneficial alien genes across interspecific or intergeneric barriers, too many deleterious genes that are difficult to eliminate are also introduced. The resulting disturbance in equilibrium between the growth processes of the maternal tissues, embryo, and endosperm leads to embryo lethality and seed collapse. In an extensive and thoughtful review of embryo-endosperm relations in plants, Brink and Cooper (6) listed six types of genetic manipulations that might cause embryo abortion in hybrids:

1. enforced self-fertilization in a normally cross-fertilizing species;
2. crosses involving parents of the same ploidy level but belonging to different species and even genera;
3. crosses between parents of the same species but differing in ploidy;
4. crosses between parents of different species and ploidy levels;
5. unbalanced chromosome condition, especially aneuploidy in the endosperm; and
6. maternal genotype causing embryo arrest irrespective of the source of the male gamete.

Department of Botany, The Ohio State University, Columbus, Ohio 43210, USA.

For effective hybridization, union of the egg and sperm from members of the same species with identical chromosome numbers appears a basic necessity.

Hybrid seeds suffer disturbances during embryogenesis and endosperm formation. A recent analysis of seed development in a cross between Trifolium ambiguum and T. repens exemplifies the numerous developmental studies published on inviable hybrids (31). The hybrid embryo grew faster than the control T. ambiguum embryo only during the first 2 d after pollination. In most of the hybrid seeds the embryo did not differentiate beyond the heart-shaped stage; it began to disintegrate soon after attaining that stage. A comparative ultrastructural study of embryo development in Hibiscus costatus and in H. costatus/H. aculeatus and H. costatus/H. furcellatus hybrids has attempted to relate embryo abortion in the latter to cellular changes (1). In contrast to the normal embryo cells, which have many organelles, hybrid embryo cells have few organelles and extensive vacuolation. This suggests that postfertilization developmental blocks prevent hybrid embryos from reaching their genetically determined size in the seed.

Because developing embryos are heterotrophic and depend upon the nutritional supplies of the endosperm, attention has been focused on the endosperm development in seeds of unsuccessful crosses. In interspecific and intergeneric crosses embryo collapse is preceded by the disintegration of the endosperm, which deprives the embryo of nutrients. This is clearly shown in the analysis of seed development in Trifolium ambiguum x T. repens hybrids referred to earlier (31). Although division of the endosperm nucleus ceased at different stages of hybrid seed development, in no case did the endosperm proceed beyond the 128-nucleate level; in many hybrids endosperm development ceased even earlier. The endosperm reached maximum size and then disintegrated. In contrast, the control seed had a typical cellular endosperm with characteristic haustoria. The frequent correlation between embryo abortion and the onset of endosperm deterioration in inviable crosses suggests two possibilities: (1) the endosperm malfunction is one of several events that trigger embryo abortion; (2) endosperm behavior may stimulate embryo abortion caused by something else.

EMBRYO RESCUE IN BREEDING

Rescuing hybrid embryos requires different kinds of approaches. The special nutritional relationship between the embryo and the endosperm suggests that the hybrid embryo would complete its development if grown in an artificial milieu with its customary endosperm nutrients. As early as 1925, Laibach (13) showed that progeny from crosses between Linum perenne and L. austriacum can be rescued by excising and culturing embryos before they disintegrate. Since this pioneering work, embryo culture methods have been widely used to obtain many transplantable seedlings from seeds of interspecific and intergeneric crosses previously considered incapable of germination. In certain instances, the

hybrid embryo continues to grow when implanted on a normal endosperm which is then cultured, thus initiating a nurse tissue culture. Several hybrid embryos have been rescued by culturing ovules. Organogenesis and somatic embryogenesis from an embryogenic callus are also potentially useful in obtaining viable seedlings from unsuccessful crosses.

Embryo culture

Numerous examples of hybrid embryo rescue attempts that succeeded in surmounting crossability barriers in plants have been reviewed (17, 18). A few attempts to obtain interspecific and intergeneric hybrids from crop plants by embryo culture were made recently.

In many plants embryo culture is neither simple nor straightforward. It requires isolating the embryo from the ovule without injury, concocting a suitable nutrient medium, and then inducing continued embryogenic growth and seedling formation. Since embryos are lodged in the sterile environment of the ovule, their surface sterilization is not necessary; instead, entire ovules or seeds are sterilized and embryos aseptically removed. Splitting open the seeds and transferring embryos to the nutrient medium is perhaps the simplest technique that can be used with seeds. The small embryos of inviable hybrids are best isolated under a dissecting microscope. Although nutrient media for hybrid embryo culture vary with the species, small hybrid embryos generally require vitamins, amino acids, growth hormones, and natural endosperm extracts (such as coconut milk), and, to foster continued embryogenic development, perhaps an osmoticum. After the embryos have grown into plantlets in vitro, they are transferred to sterilized soil or vermiculite and grown to maturity in the greenhouse.

Table 1 lists some recent successes in rearing seedlings from interspecific hybrids of legume genera by excising and culturing embryos. In cereals, too, hybrid embryos have been rescued through embryo culture; recent successes involve seedlings from *Triticum aestivum/Aegilops speltoides* hybrids (7), various lines of *Triticum durum/Secale cereale* hybrids (2), *Hordeum vulgare/Secale cereale* (4), and *Aegilops squarrosa/Triticum boeoticum* (9) hybrids.

Embryo implantation

Nutrient media formulated for hybrid embryo culture vary with the species because of the inherently different chemical compositions of hybrid ovule endosperms. Transplanting the embryo into a normal endosperm overcomes the limitations of artificial media.

A standard method of embryo transplantation to rescue hybrids from the crosses *Hordeum/Triticale*, *Hordeum/Agropyron*, and *Hordeum/Secale* was described by Kruse (12). The embryo is removed from each dehulled seed and the endosperm is extruded from the pericarp. The endosperms, either singly or in groups of two to four, are then aseptically transferred to the surface of a solidified mineral salt medium. Hybrid embryos are generally

Table 1. Embryo culture of interspecific legume hybrids.

Hybrids	Medium composition	Seedling characteristics	Reference
Phaseolus vulgaris/ P. ritensis	White's medium with 4% sucrose and 0.1% casein hydrolyzate; transferred to a medium lacking casein hydrolyzate after 4 wk.	Transplantable seedlings	5
Phaseolus vulgaris/ P. lunatus; P. vulgaris /P. acutifolius; P. acutifolius/ P. vulgaris	Murashige-Skoog medium; survival rate of embryos enhanced by addition of glutamine	Normal plants	14
Phaseolus coccineus/ P. vulgaris	Murashige-Skoog medium	Normal plants; plants from underdeveloped embryos have low survival rate	24
Arachis hypogaea/ A. villosa	Murashige-Skoog medium with IAA (4 mg/litre) and kinetin (2 mg/litre)	Normal seedlings with chromosome number intermediate between parents	3
Arachis hypogaea/ A. monticola, A. glabrata		Normal plants	21
Vigna mungo/ V. radiata	Murashige-Skoog medium, with IAA (1 mg/litre), kinetin (0.2 mg/litre), coconut milk (70 ml/litre) or casein hydrolyzate (500 mg/litre)	Plants intermediate between parents with respect to leaf color and other morphological characters	10

implanted in groups of 5 to 10 on the cultured endosperm. Yield of hybrid plants and ability to induce growth in relatively small embryos were best in donor endosperms cultured in a medium containing 25% coconut milk. Hybrid embryos from pasture legume genera Trifolium, Lotus, and Ornithopus have been rescued similarly (30). In this work hybrid embryos excised at early to late heart-shaped stages did not grow when cultured directly on the surface of a nutrient medium. But, when they were enveloped in a nurse endosperm, dissected from normally developing ovules, and cultured, embryo growth, differentiation, and recovery rates were high. Culture of endosperm with the implanted embryo determined the success or failure of embryo rescue operations. Generally, €or improved growth, early heart-shaped-stage embryos required a medium containing casein hydrolysate and vitamins; advanced-stage embryos required a medium with less complex additives.

The wider use of embryo implantation technique is hampered by the delicacy of the operation: field trials are particularly exasperating. Still, the technique is a promising alternative for obtaining progeny from crosses of unrelated parents.

Ovule culture

Two deterrents to the culture of hybrid embryos are the complexity of formulating a nutrient medium and the difficulty of

isolating embryos from the ovule. Ovule culture has proved to be an alternate method for rearing embryos from unsuccessful crosses. Even before the use of ovule culture, ovules excised at the stage of the fertilized egg or early-division-phase embryos have been shown to develop completely in culture to the stage of viable seeds. Nutrient requirements for ovule growth and embryo differentiation appeared surprisingly simple. Generally, they include amino acids and hormone (19).

One crop plant in which ovule culture has been successfully used to rescue hybrid embryos is cotton. Among cultivated cottons. *Gossypium arboreum* and *G. herbaceum* are diploids; *G. barbadense* and G. *hirsutum* are allotetraploids. According to the embryological studies of Weaver (28, 29) and Pundir (16), when the maternal genome was from tetraploid cotton, malformed growth of the endosperm led to an aborted embryo; on the other hand, both endosperm and embryo growth of a diploid/tetraploid pollen cross were abnormal. While hybrid embryos from tetraploid-diploid crosses have been grown to maturity in a specified culture medium, embryos have been rescued from reciprocal crosses only sporadically.

After their success in inducing normal embryo development in cultured ovules of *G. hirsutum* at the zygote stage (25), Stewart and Hsu (26) obtained triploid hybrid plants by ovule culture from all possible crosses between diploid G. *herbaceum* and G. *arboreum* and tetraploid *G. hirsutum* and *G. barbadense,* except the cross *G. herbaceum*/*G. barbadense*. Ovules were cultured 2-4 d after anthesis in a modified Murashige-Skoog medium containing 4% sucrose. Germinated embryos were subsequently removed and allowed to root and form seedlings in a low-salt medium before being transplanted into soil. Although cultured ovules of G. *hirsutum* yielded well-developed hybrid embryos in the basal medium, those of *G. barbadense*, *G. arboreum*, and *G. herbaceum* required the addition of IAA for favorable yields. In crosses between diploid and tetraploid genomes, propensity for callus proliferation from the integuments of the cultured ovule also contributed to low embryo yields. Reed and Collins (20) found that seeds from crosses between several wild species of *Nicotiana* (*N. stocktonii*, *N. nesophila*, and *N. repanda*) and diploid *N. tabacum* did not germinate, but that seedlings were obtained from all the three desired hybrids by culturing ovules and recovering germinated embryos.

Another technique is based on the culture of ovaries, instead of ovules, following wide crosses. In an extensive investigation of crosses obtained from various cultivars of *Brassica campestris* and *B. oleracea*, Inomata (11) obtained seeds with well-developed embryos protruding when ovaries were explanted 4 d after pollination into White's medium containing casein hydrolyzate. Embryos subsequently subcultured in the same medium developed into plantlets with roots and leaves.

Both ovule culture and ovary culture should be useful in raising plants from wide crosses in other crops where embryo culture and embryo implantation techniques are unsatisfactory. Various explanations for the growth of hybrid embryos within cultured ovules must be considered:

- The extraembryonal tissues of the ovule may supply nutrients for the enclosed embryos' growth, or nutrients absorbed by the ovule or ovary may easily be translocated to the embryo.
- Injury to the embryo may be avoided in the ovule or ovary culture methods.
- These methods prevent the osmotic shock young embryos experience when removed from the embryo sac.

Plant regeneration from callus

Inducing undifferentiated callus from an explanted organ and regenerating plants from the callus by organogenesis or by embryogenesis have been a cornerstone of morphogenesis in recent years. Efficiency of embryo rescue is handicapped by the need to culture many embryos or ovules for a seedling population sizable enough for further analysis. More plants of the same genetic composition are obtained from crosses between related species through plant regeneration from a hybrid embryo callus than through other embryo-rescue methods.

Although obtaining hybrid plants from an embryo-derived callus is generally hampered by the slow growth of the callus and the death of seedlings, a few attempts have succeeded. In one case, isolated parts of seedlings generated from cultured ovules from crosses between Nicotiana stocktonii/N. tabacum and N. nesophila/N. tabacum formed a callus, and plants regenerated from the callus by organogenesis grew to maturity (20). In another study, a cultured embryo from a cross between Hordeum distichum and Secale cereale formed a friable callus which spontaneously regenerated roots, albinos, and green shoots. Clones of hybrid plants were established from the green shoots (8). In the third study, hybridization between Lycopersicon esculentum and 4. peruvianum (27) resulted in a callus when undeveloped hybrid seeds were cultured in a hormone-enriched medium. With manipulation of the medium composition, plantlets subsequently regenerated from the callus. Although the callus was formed from cultured seeds, the regenerated plants displayed morphological traits, ploidy values, isozyme banding patterns, and fertility relations of embryo-derived hybrids. Overall these results are encouraging. Current techniques to regenerate normal plants from cultured cells and tissues may lead to the production of a large number of hybrid plants from rescued embryos.

EMBRYO RESCUE FROM GENETIC LETHALITY

Embryo or ovule culture can be used to overcome not only seed lethality from endosperm failure, but also genetic lethality of embryos. Spontaneous lethal mutants causing different degrees of malformations in the embryo and endosperm of plants such as maize have occurred for some time. Recently similar mutations were induced in normal maize plants pollinated with a pollen suspension treated with a chemical mutagen. Included in the recovered mutants were several auxotrophs whose embryos grew into

normal plants when cultured in an enriched medium. A mutation induced by culturing embryos in media supplemented with proline and its intermediates was traced to a genetic block in embryo proline biosynthesis (15, 22, 23). Analyses of other nutritionally impaired embryo mutants might elucidate the metabolic systems activated at different stages of embryogenesis and the nutrient requirements for culturing undifferentiated hybrid embryos.

CONCLUSIONS

We need a better understanding of the physiology and genetics of pollination, fertilization, and seed formation in angiosperms to obtain viable seeds and seedlings from interspecific and intergeneric crosses in plants. Recent advances in tissue culture have facilitated recovery in the laboratory of a number of offspring from certain distant crosses. The low survival rate of rescued hybrid embryos and seedlings is a major problem in extending tissue culture techniques to a large number of plants in the field. We hope that future research will identify the isolation conditions and nutrient media needed for mass isolation and culture of hybrid embryos and regeneration of seedling plants in quantity.

REFERENCES CITED

1. Ashley, T. 1972. Zygote shrinkage and subsequent development in some Hibiscus hybrids. Planta 108:303-317.
2. Bajaj, Y.P.S., K.S. Gill, and G.S. Sandha. 1978. Some factors enhancing the in vitro production of hexaploid Triticale (Triticum durum x Secale cereale). Crop Improv. 5:62-72.
3. Bajaj, Y.P.S., P. Kumar, M.M. Singh, and K.S. Labana. 1982. Interspecific hybridization in the genus Arachis through embryo culture. Euphytica 31:365-370.
4. Bajaj, Y.P.S., M.M. Verma, and M.S. Dhanju. 1980. Barley x rye hybrids (Hordecale) through embryo culture. Curr. Sci. 49:362-363.
5. Braak, J.P., and E. Kooistra. 1975. A successful cross between Phaseolus vulgaris L. and P. ritensis Jones with the aid of embryo culture. Euphytica 24:669-679.
6. Brink, R.A., and D.C. Cooper. 1947. The endosperm in seed development. Bot. Rev. 13:423-541.
7. Chueca, M., Y. Cauderon, and J. Tempi. 1977. Technique d'obtention d'hybrides ble tendre x Aegilops par culture in vitro d' embryons immatures. Ann. Amelior. Plant. 27:539-547.
8. Cooper, K.V., J.E. Dale, A.F. Dyer, R.L. Lyne, and J.T. Walker. 1978. Hybrid plants from the barley x rye cross. Plant Sci. Lett. 12:293-298.
9. Gill, B.S., J.G. Waines, and H.C. Sharma. Endosperm

abortion and the production of viable Aegilops squarrosa x Triticum boeoticum hybrids by embryo culture. Plant Sci. Lett. 23:181-187.

10. Gosal, S.S., and Y.P.S. Bajaj. 1983. Interspecific hybridization between Vigna mungo and Vigna radiata through embryo culture. Euphytica 32:129-137.
11. Inomata, N. 1978. Production of interspecific hybrids in Brassica campestris x B. oleracea by culture in vitro of excised ovaries. I. Development of excised ovaries in the crosses of various cultivars. Jpn. J. Genet. 53:161-173.
12. Kruse, A. 1974. An in vivo/vitro embryo culture technique. Hereditas 77:219-224.
13. Laibach, F. 1925. Das Taubwerden von Bastardsamen und die kiinstliche Aufzucht fruh absterbender Bastardembryonen. Z. Bot. 17:417-459.
14. Mok, D.W.S., M.C. Mok, and A. Rabakoarihanta. 1978. **Interspecific hybridization of Phaseolus vulgaris with** P. lunatus and P. acutifolius. Theor. Appl. Genet. **52:209-215.**
15. Neuffer, M.G., and W.F. Sheridan. 1980. Defective kernel mutants of maize. I. Genetic and lethality studies. Genetics 95:929-944.
16. Pundir, N.S. 1972. Experimental embryology of Gossypium arboreum L. and G. hirsutum L. and their reciprocal crosses. Bot. Gaz. 133:7-26.
17. Raghavan, V. 1976. Experimental embryogenesis in vascular plants. Academic Press, London.
18. Raghavan, V. 1977. Applied aspects of embryo culture. Pages 375-397 in Applied and fundamental aspects of plant cell, tissue, and organ culture. J. Reinert and Y.P.S. Bajaj, eds. Springer-Verlag, Berlin.
19. Raghavan, V. 1980. Embryo culture. Int. Rev. Cytol. Suppl. 11B:209-240.
20. Reed, S.M., and G.B. Collins. 1978. Interspecific hybrids in Nicotiana through in vitro culture of fertilized ovules. N. stocktonii x N. tabacum; N. nesophila x N. tabacum; N. tabacum; N. repanda x N. tabacum. J. Hered. 69:311-315.
21. Sastri, D.C., and J.P. Moss. 1982. Effects of growth regulators on incompatible crosses in the genus Arachis L. J. Exp. Bot. 33:1293-1301.
22. Sheridan, W.F., and M.G. Neuffer. 1980. Defective kernel mutants of maize. 11. Morphological and embryo culture studies. Genetics 95:945-960.
23. Sheridan, W.F., and M.G. Neuffer. 1982. Maize developmental mutants. Embryos unable to form leaf primordia. J. Hered. 73:318-329.
24. Shii, C.T., A. Rabakoarihanta, M.C. Mok, and D.W.S. Mok. 1982. Embryo development in reciprocal crosses of Phaseolus vulgaris L. and P. coccineus Lam. Theor. Appl. Genet. 62:59-64.

25. Stewart, J.M., and C.L. Hsu. 1977. In-ovulo embryo culture and seedling development of cotton (Gossypium hirsutum L.). Planta 137:113-117.
26. Stewart, J.M., and C.L. Hsu. 1978. Hybridization of diploid and tetraploid cottons through in-ovulo embryo culture. J. Hered. 69:404-408.
27. Thomas, B.R., and D. Pratt. 1981. Efficient hybridization between Lycopersicon esculentum and L. peruvianum via embryo callus. Theor. Appl. Genet. 59:215-219.
28. Weaver, J.B. 1957. Embryological studies following interspecific crosses in Gossypium. I. G. hirsutum x G. arboreum. Am. J. Bot. 44:209-214.
29. Weaver, J.B. 1958. Embryological studies following interspecific crosses in Gossypium. 11. G. arboreum x G. hirsutum. Am. J. Bot. 45:lO-16.
30. Williams, E.G., and G. De Lautour. 1980. The use of embryo culture with transplanted nurse endosperm for the production of interspecific hybrids in pasture legumes. Bot. Gaz. 141:252-257.
31. Williams, E., and D.W.R. White. 1976. Early seed development after crossing of Trifolium ambiguum and T. repens. N.Z. J. Bot. 14:307-314.

WILD SPECIES IN CROP IMPROVEMENT

J. P. MOSS

Wild relatives are useful sources of characters desired to improve crop. Many of these species are resistant to pests and diseases or adapted to adverse environments. Some have higher yields than their cultivated relatives. Genes from some wild relatives can undergo meiotic recombination, producing segregants from which the plant breeder can select improved types; these genes can usually function effectively in cultivars. The limit to utilization of wild relatives depends on the breeder's ability to produce hybrids, but hybrids may be sterile. Ploidy differences between crops and wild relatives are frequent, but manipulating ploidy level can improve gene transfer. Triploids are often sterile, but progeny from triploids include recombinants that may not occur at other ploidy levels. The Groundnut Cytogenetics Unit at ICRISAT has transferred desirable genes from diploid wild species into tetraploid lines crossable with groundnut, enabling breeders to incorporate wild species genes into locally adapted material. Triploids, hexaploids, amphiploids and autotetraploids have been produced and backcrossed with the cultivated groundnut. Some lines have disease resistance and good agronomic traits, including high seed yield, and have been entered in All India Coordinated Oilseeds Project trials.

The aim of the plant breeder is to change one or more genes in a cultivar to better adapt it to its environment or to its intended use. These genes come from a range of sources. Most plant breeders use other cultivated germplasm. Few attempt to transfer genes from more distantly related germplasm even though wild relatives have many desirable attributes. Wild species have often survived pests and diseases, and have many resistance genes. They may grow in a wider range of environments, including harsh ones. There is now evidence that they can also contribute to yield increases.

All this can also be claimed for more exotic germplasm, but wild relatives have advantages over unrelated material. There may

Principal cytogeneticist, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru P. O., 502 324,A. P., India.

be genomes or parts of genomes common to cultivated and wild species, facilitating gene transfer. Wild genes may function in the genetic background of the cultivar; exotic genes may not.

There have been a number of recent reviews and books on wild species utilization (5, 27, 32), but relatively few people currently transfer genes from wild species.

This conference looks at new technology to make genes available to the breeder. This paper focuses on transferring genes Of wild relatives to crops, emphasizing the work at ICRISAT using wild Arachis species to improve groundnut.

LIMITS TO THE GENE POOL

The difference between cultivated and wild is arbitrary. Many wild species are freely crossable with cultivars; indeed, some wild species scarcely differ from cultivated, but survive in the wild because of a single gene difference such as rachis fragility. One wild Arachis species, A. monticola, freely crossable with A. hypogaea, has been used to develop the cultivar Spancross (4). The next section of the gene pool is not readily accessible. Its limits generally follow taxonomic divisions; it may be restricted to one species, or span even several genera. The gene pool for wheat has increased greatly in recent years, and the breeder has a wide range of potential gene donors. Isolation may be due to crossability barriers and the inability to produce hybrids, or to hybrid sterility, preventing further gene flow. When fertile hybrids have been produced, lack of recombination may mean that undesirable characters are always expressed in the desired selections (28). When the gene has been transferred, gene interaction or pleiotropy may prevent selection of the desired new phenotypes. Alternatively, transferred genes may produce effects not present in the wild species (15). Only one of these barriers may be effective, but in some cases many may occur, and the more distantly related the species, the more problems are likely. Where a gene has been introduced and has undergone recombination within the cultivated genome, it can then recombine and be used by any breeder. A gene introduced by other means may not be amenable to traditional plant breeding.

This section of the gene pool comprises many species. Table 1 shows crop diversity in ploidy levels and numbers of wild relatives. Some wild species are represented by few accessions; others have been extensively collected.

SELECTION OF PARENTS

Having decided on the gene pool, assemble all knowledge of those species. Usually desirable genes will have first priority (31); if possible, the knowledge should extend to the nature and number of the genes in each species, and whether the gene is the same in all species. This knowledge can come from genetic studies, but they take time and assume easy crossability and full hybrid fertility; genetic ratios from interspecific hybrids will not be valid if meiosis is not normal. Another approach is to study mechanisms and components of resistance (30). Even where

Table 1. Major crops of the IARCs and their wild relatives.

Cultivated species	2n =	Ploidy level	Wild relatives
A. Sexually reproduced			
Phaseolus sp.	22	2x	Many; diploids - many cultivated species, and wild forms of cultivated species
Vigna unguiculata	22	2x	Many; diploids
Cajanus cajan	22	2x	Few; diploids
Vicia sp.	12	2x	Very many; different basic chromosome numbers, no interspecific hybrids involving *V. faba*
Glycine max	40	4x	Few; diploids, tetraploids
Cicer arietinum	16	2x	Few; diploids
Oryza sativa	14	2x	Many; mostly diploids, some tetraploids
T. aestivum	42	6x	Many wild relatives; diploids, tetraploids
T. durum	28	4x	and hexaploids. Widely crossable. Elymus, Sitanion, Haynaldia,Aegilops,Agropyron, Secale, Hordeum now part of gene pool
Zea mays	20	2x	Related genera, with different basic numbers and different ploidy levels
Sorghum vulgare	20	4x	Many; diploids, tetraploids
Pennisetum americanum	14	2x	Few; x=7 diploids, tetraploids Many; x=9 diploids, tetraploids
Arachis hypogaea	40	4x	Many; diploids, Few; tetraploids
B. Vegetatively reproduced: may be highly polyploid, triploid, aneuploid, also sterile and produce few seeds which germinate poorly.			
Solanum tuberosum	48	4x	Very many, at different ploidy levels
Ipomoea batatas	90	6x	Many, 2x. 4x
Xanthosoma spp.	26	2x	Few; (tetraploids occur)
Colocasia esculenta	28	2x	–
Manihot esculenta	36	4x	Few; tetraploids. Interspecific hybrids have been produced

resistance occurs in the cultivated species, resistance in the wild species may be controlled by different genes (23) that can be combined to give a more stable resistance. There is a very wide choice of cultivated germplasm (13); cultivars to be used as parents should be selected for crossability with the wild species as well as for local adaptation.

THE WILD SPECIES

A wild species consists of a number of plants in their natural habitat, but for the plant breeder it is the gene pool at his disposal: the number of accessions he can collect from the wild or acquire from gene banks. These accessions may differ from one another. The breeder should screen all of them for the desired character, bearing in mind that resistant selections may differ in genotype, or in resistance components (30). Crossability may differ, as it often does in the cultivated species (8, 9, 17, 24). A study of allozymes indicates the degree of variability in species (16), and karyotype studies will indicate similarities and differences between genomes (20). The variability may lead to

differences in chromosome behavior and hybrid fertility, which can result in changes from homologous to homoeologous pairing (2, 3) or differences in chiasma distribution.

About 1,000 collections from the wild have been made in *Arachis*, and a collection program supported by the International Board for Plant Genetic Resources is still active. Although not all collections have been maintained, ICRISAT has 181 accessions of wild *Arachis* species (14), and there is an active program to assemble all living accessions at ICRISAT. All the accessions are being screened for resistance to major pests, diseases, and drought (1, 29, 31).

Effective screening techniques are essential to wild species utilization, not only to screen accessions of wild species, but also to identify segregants with the desired characters. The infector row technique has been valuable in screening for resistance to rust (*Puccinia arachidis*) and late leafspot (*Cercosporidium personatum*).

The genus Arachis

There are seven sections in the genus. *Arachis hypogaea*, the cultivated groundnut, is in section Arachis. It is a tetraploid; *A. monticola* is also a tetraploid freely crossable with, and considered by some a subspecies of, *A. hypogaea*. All the other species in section Arachis are diploid, and crossable with *A. hypogaea*, but *A. hypogaea* will not cross with species in any other section, unless hormone treatment and embryo culture are used (18, 19). Diploid species in section Arachis can be crossed with species in other sections, but gene transfer by bridge crossing has not been possible (28).

ICRISAT has therefore emphasized work to overcome barriers to intersectional hybridization (18) and to utilize diploid wild species in section Arachis, especially those with disease resistance, by producing large populations of hybrids and backcrosses.

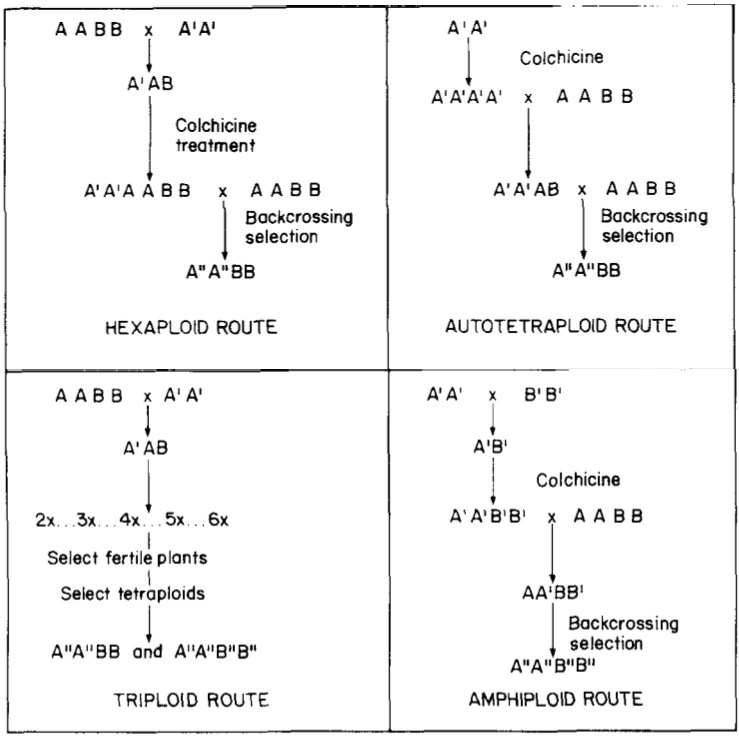
A knowledge of crossability and genomic constitution of the species is necessary in planning its utilization; most of this knowledge comes from hybrid production and analysis but knowledge of genomes can come from karyotype studies, DNA studies, and taxonomic studies.

Barriers to hybridization

The available gene pool is limited by the capability and resources of the breeder. Many species can be crossed by conventional means, but the use of mentor pollen, hormone treatment, and embryo culture can extend the pool (19).

Ploidy differences

Many crop species are polyploid, with diploid or polyploid wild relatives, or both. Existing ploidy differences impede gene transfer, as hybrids are often sterile, but induced ploidy can be used to overcome crossability barriers and to manipulate genome ratios to alter gene doses, and to effect gene transfer (22).



1. Use of related diploid species in improvement in *Arachis.* A, B = cultivated genomes, A', B' = wild genomes, A": B" = introgressed genomes.

Ploidy level differences between species can be adjusted before or after crossing (Fig. 1). A diploid wild species can be rendered tetraploid before crossing with the tetraploid crop (autotetraploid route) (6), or the tetraploid crop can be rendered dihaploid before crossing at the diploid level. Two diploid relatives, where available, can be crossed and the hybrid can be rendered tetraploid before crossing with the cultivated tetraploid (amphiploid route). The second diploid species may contribute another resistance (leafspot resistant A. cardenasii/rust resistant A. batizocoi)/A. hypogaea, or confer cytological stability and fertility on the tetraploid hybrids, thereby facilitating transfer of the desired gene. In Arachis, we have concentrated on eight diploid species, most of them with A. hypogaea as female parent; of the 72 possible species combinations we have produced 56; we have also crossed diploid hybrids with a third wild species and produced complex hybrids.

Often direct hybrids without ploidy manipulation are sterile. Many triploid hybrids produced in Arachis are totally or partially sterile (11, 12). Colchicine treatment produces fertile hexaploids (26). Wild and cultivated genomes are combined, but after treatment each genome is homologous, and there may be no pairing between wild and cultivated chromosomes. If the genomes are related, intergenomic pairing may occur, resulting in multivalents (25). Backcrossing alters genome ratios, removes

homologous partners, and encourages wild and cultivated chromosome pairing; but the recombinants may be released slowly, needing many generations for backcrossing and screening. After an initial backcross, chromosome numbers may continue to decline in selfed progenies, but there may be strong selection for balanced gametes, and pentaploids may give mostly tetraploids and hexaploids on selfing. Recombination may have occurred at the pentaploid level; pentaploid-derived tetraploids and hexaploids would then contain chromosomes with both wild and cultivated segments.

Though triploids are usually sterile, some seeds can be produced from anaphase distribution, giving balanced, viable gametes, or from restitution to produce triploid gametes. Chromosome numbers in progeny from such partially fertile triploids range from diploid to hexaploid (7, 21); tetraploid or near-tetraploid progeny are doubly valuable as restoring chromosome numbers to cultivated level requires little time and no colchicine or backcrossing, and recombination between wild and cultivated chromosomes, which may not occur at other ploidy levels (22), has been possible.

Recombination

The key to successful utilization of wild species is recombination; induced transfer of chromosome segments can be used, but producing a large number of hybrids in which meiotic recombination leads to large segregating populations gives more opportunity for the selection of desirable plants. Usually maximum recombination occurs in the first-generation hybrids. The difficulty in producing hybrids often results in small populations; a common report is that "a hybrid was produced; of the few F_3 plants obtained, only one was fertile." Vegetative reproduction of F_1 plants is essential in such cases to ensure acceptable population sizes. Tissue and cell culture at this stage may be valuable to introduce somaclonal variation (10). Where ploidy manipulation and the elimination of wild species characters are planned, changes in chromosome number or the occurrence of deletions, duplications, or translocations that affect chromosome pairing, chiasma frequency, or position (and hence the number and nature of recombinants in future generations) may be more important than the genetic changes that occur.

Backcrossing

It is hoped that the initial hybrids contain the desired gene. Usually because they combine both wild and cultivated genomes, they contain a number of undesirable wild characters also. Such characters can be eliminated by backcrossing to the cultivated species, although backcrossing to the wild species may be desirable. Hexaploids can be backcrossed to parental diploid wild species to regain tetraploidy and also increase the dosage of wild genes (4).

Backcrossing to the cultivated species restores cultivated chromosome number, where necessary, and also restores the genotype of the cultivated parent. Backcrossing to a nonparental cultivar

can introduce alien genes; it will also give rise to variation in the background genotype in which the introduced gene is to express itself. This has been used extensively in the Groundnut Cytogenetics crossing program at ICRISAT. Long-season hexaploids with genomes from perennial Cercosporidium-resistant wild species and from Nigerian cultivars were backcrossed to short-season Indian cultivars; and resistant short-season selections were backcrossed to a Groundnut Rosette Virus-resistant cultivar from Malawi.

Cytological screening

The need for large populations precludes cytological screening of all plants. We currently grow about 7 ha of wild species derivatives each rainy season at ICRISAT, and have grown 12 ha in one season. Chromosome counting and checking for regular meiosis is restricted to key hybrids and any plants with unexpected morphology or reduced fertility.

Cytological stability

Ploidy differences and intergenomic pairing are useful in the early generations, but not in the finished product. Many generations of backcrossing may be necessary to select stable lines with the desired character and no deleterious wild species genes. Selected plants can be selfed, and progeny rows grown for several generations until uniform lines are produced. Chromosome numbers are counted and meiosis is checked at this stage. Lines for release as new germplasm must be checked for crossability with the cultivars and for regular meiosis in the hybrids produced.

Table 2. Disease reaction (1-9 scale) and yield of selected wild species derivatives in *Arachis* (8 × 8 triple lattice, plot size 16 m^2), ICRISAT Center and Bhavanisagar, 1982 rainy season.

Entry no.	Rust resistance	Leaf spot[a] resistance	Pod yield (kg/ha)	Estimated kernel yield (kg/ha)	Oil content (%)	Estimated oil yield (kg/ha)	Haulm yield (kg/ha)
13	2.3	2.3	3150	1960	42	820	4570
30	2.7	2.3	2640	1700	44	760	6540
46	6.7	2.3	2540	1740	44	770	4490
6	2.7	2.3	2150	1360	43	580	3700
11	2.7	2.0	2520	1750	44	770	5620
23	3.0	3.0	2140	1410	44	620	5060
39	9.0	2.3	2130	1370	45	610	3270
SE	±0.42	±3.55	±134	±87	±0.7	±39	±350
Check							
Robut 33-1	9.0	6.3	1830	1320	41	510	1560
TMV2	9.0	9.0	1240	850	41	350	1630
SE	±0.0	±0.24	±80	±60	±0.3	±24	±53
Site mean[b]	4.2	5.5	1950	1280	43	550	4050
CV (%)	17	17	12	12	3	12	15

[a] Results from trial at Bhavanisagar. [b] Mean of 64 entries (ICRISAT Center) 36 entries (Bhavanisagar).

ACHIEVEMENTS

Many fertile, cytologically stable derivatives with desirable characters from wild species have been selected (7). The numbers of segregants produced from crosses have permitted selection for agronomic traits (growth, habit, yield, earliness) as well as for disease resistance. The most advanced lines have been grown in replicated trials for a number of seasons at ICRISAT center and other locations (Table 2). The best lines have been entered in All India Coordinated Oilseeds Research Project trials. Two foliar disease-resistant lines have been selected for a second year of testing, and one high-yielding line has been advanced to regional trials.

REFERENCES CITED

1. Amin, P.W. 1984. Resistance of wild species of groundnut to insect and mite pests. ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). Proceedings of the international workshop on cytogenetics of Arachis. 31 Oct-2 Nov 1983, India.
2. Dover, G.A. 1973. The genetics and interactions of 'A' and 'B' chromosomes controlling meiotic chromosome pairing in the Triticinae. Proc. 4th Int. Wheat Genetics Symposium, Missouri Agric. Exp. Stn., Columbia, Mo., USA.
3. Dover, G.A., and R. Riley. 1972. Prevention of pairing of homoeologous meiotic chromosomes of wheat by an activity of supernumary chromosomes of Aegilops. Nature 240:159 – 161.
4. Hammons, R.O. 1970. Registration of Spancross peanuts, Crop Sci. 10:459.
5. Harlan, J.R. 1976. Genetic resources in wild relatives of crop plants. Crop Sci. 16:329 – 333.
6. ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). 1980. Annual report 1978 – 79. India.
7. ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). 1983. Annual report 1982. India.
8. Jalani, B.S., and J.P. Moss. 1980. The site of action of the crossability genes (Krl and Kr2) between Triticum and Secale I. Pollen germination, pollen tube growth and number of pollen tubes in common wheat. Euphytica 29:571 – 579.
9. Jalani, B.S., and J.P. Moss. 1981. The site of action of the crossability genes (Krl and Kr2) between Triticum and Secale II. Proportion of different parts of the pistil containing pollen tubes in common wheat. Euphytica 30:105 – 112.
10. Larkin, P.J., and W.R. Scowcroft. 1981. Somaclonal variation -- a noval source of variability from cell cultures. Theor. Appl. Genet. 60:197 – 214.
11. Moss, J.P. 1980. Wild species in the improvement of groundnuts. Pages 525 – 535 in Advances in legume

science. Vol. 1. J. Summerfield and A.H. Bunting, eds. Proc. Int. Legume Conf. Kew, England 1978.

12. Moss, J.P., and I.V. Spielman. 1976. Interspecific hybridisation in Arachis. Proc. American Peanut Research and Education Association 8:88. (Abstr)

13. Plucknett, D.L., N.J.H. Smith, J.T. Williams, and N. Murthi Anishetty. 1983. Crop germplasm conservation and developing countries. Science 220:163-169.

14. Rao, V.R., and A.K. Sadasivan. 1984. Wild Arachis Genetic Resources at ICRISAT. ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). Proceedings of the international workshop on cytogenetics of Arachis. 31 Oct-2 Nov 1983, India.

15. Rick, C.M. 1967. Exploiting species hybrids for vegetable improvement. Proc. XVII Int. Hortic. Congr. 3:217-269.

16. Rick, C.M., J.F. Fobes, and S.D. Tanksley. 1979. Evolution of mating systems in Lycopersicon hirsutum as deduced from variation in electrophoretic and morphological characters. Plant Syst. Evol. 132 (4):279-298.

17. Riley, R., and V. Chapman. 1967. The inheritance in wheat of crossability with rye. Genet. Res. Camb. 9:259-267.

18. Sastri, D.C., and N. Mallikarjuna. 1984. Techniques for overcoming incompatibility in wide crosses, Inter-Center Seminar on IARCs and Biotechnology, 23-27 April, IRRI, Manila.

19. Sastri, D.C., and J.P. Moss. 1982. Effects of growth regulators on incompatible crosses in the genus Arachis L. J. Exp. Bot. 33:1293-1301.

20. Singh, A.K., and J.P. Moss. 1982. Utilization of wild relatives in genetic improvement of Arachis hypogaea L. 11. Chromosome complement of species of section ARACHIS. Theor. Appl. Genet. 61:305-314.

21. Singh, A.K., and J.P. Moss. 1983. Utilization of wild species in genetic improvement of A. hypogaea L. VI. Fertility in triploids. Cytological basis and breeding implications. Peanut Sci. (in press)

22. Singh, A.K., and J.P. Moss. 1984. Interspecific breeding through ploidy manipulations. Bangladesh J. Bot. (in press)

23. Singh, A.K., P. Subrahmanyam, and J.P. Moss. 1983. Utilization of wild species in genetic improvement of Arachis hypogaea VII. A note on the dominant nature of resistance to Puccinia arachidis in wild Arachis species. Peanut Sci. (in press)

24. Snape, J.W., V. Chapman, J. Moss, C.E. Blanchard, and T.E. Miller. 1979. The crossabilities of wheat varieties with Hordeum bulbosum. Heredity 42(3):291-298.

25. Spielman, I.V., A.P. Burge, and J.P. Moss. 1979. Chromosome loss and meiotic behaviour in interspecific hybrids in the genus Arachis L. and their implications in breeding for disease resistance. Z. Pflanzenzuchtung. 83:236-250.

26. Spielman, I.V., and J.P. Moss. 1976. Techniques for chromosome doubling in interspecific hybrids in Arachis. Oleagineux 31:491-494.

27. Stalker, H.T. 1980. Utilization of wild species for crop improvement. Adv. Agron. 33:111-147.
28. Stalker, H.T., J.C. Wynne, and M. Company. 1979. Variation in progenies of an Arachis hypogaea x diploid wild species hybrid. Euphytica 28(3):675.
29. Subrahmanyam, P., A.M. Ghanekar, B.L. Nolt, D.V. Reddy, and D. McDonald. 1984. Resistance to groundnut disease in wild Arachis species. ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). Proceedings of the international workshop on cytogenetics of Arachis. 31 Oct-2 Nov 1981, India.
30. Subrahmanyam, P., D. McDonald, R.W. Gibbons, and P.V. Subba Rao. 1983. Components of resistance to Puccinia arachidis in peanuts. Phytopathology 73(2):253-256.
31. Subrahmanyam, P., J.P. Moss, and V.R. Rao. 1983. Resistance to peanut rust in wild Arachis species. Plant Dis. 67(2):209-212.
32. Zeven, A.C., and A.M. van Harten. 1979. Proceedings of the conference on broadening the genetic base of crops. Wageningen, Netherlands 3-7 July 1978. Pudoc, Wageningen.

OVERCOMING INCOMPATIBILITY IN WIDE CROSSES

D. C. SASTRI and M. MALLIKARJUNA

Wild species of crop plants attract much attention as a valuable gene pool. A few successes in improving crop plants have resulted from crossing wild taxa with cultivated ones. Several other taxa are not crossable with their cultivated relatives and are therefore unavailable for sexual gene transfers. Methods for breaking these barriers to interspecific hybridization and hybrid production have been developed.

The literature on exotic germplasm abounds with examples of genetic introgression from wild taxa conventionally crossed with their cultivated relatives. But there is a wealth of germplasm that cannot be crossed with cultivated taxa.

The barriers to hybridization were known even before the present range of germplasm became available. During the last five decades, interest in incompatibility has increased to encompass phylogenetic-taxonomic purposes and the genetic improvement of crop plants. While emerging somatic methods show promise, sexual manipulation is still the first choice, and sexual methods have contributed significantly to improving crops.

The germplasm amenable to sexual manipulation is difficult to quantify. It may be an insignificant proportion of existing germplasm. Even in the well-worked genus Nicotiana, of which about 65 species are known, only a little more than 300 hybrids have been realized. About 90% of the crosses in this genus have not. The situation is similar for most other crops. A number of review articles and books on incompatibility and methods to break it are available (9, 15, 16, 17, 18, 41, 49, 51).

CAUSES OF SEED FAILURE IN INCOMPATIBLE CROSSES

The characteristic prefertilization barriers and postfertilization breakdown of zygote or embryo in incompatible crosses have been extensively described. Inhibition of pollen germination on stigma and pollen tube growth through the style,

Cytogeneticist and research associate, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru P. O. 502 324, A. P., India.

failure to fertilize, and zygote growth cessation at some stage of development are common to many failing crosses (41, 49, 56). Unfortunately, their molecular causes are little understood. Even so, the fragmentary information available has led to development of methods for breaking crossability barriers in a few taxa.

METHODS

Among the several methods used are recognition pollen, hormones, immunosuppressants, and temperature. Early surgical methods are also important (28, 37, 41). Delayed and bud pollinations have overcome both sporophytic (*Brassica* spp.) and gametophytic (*Petunia* spp.) self-incompatibilities in a few taxa, but not any interspecific incompatibility. Their success depends on the stigma's receptiveness, i.e., buds of *Arachis hypogaea* even a day before anthesis are not receptive even to compatible pollen; so bud pollination does not work in *A. hypogaea* (43).

Growth hormones

The discovery of growth hormones in plants inspired investigations on their potential in overcoming incompatibility, which is not mentioned in books and conferences on plant growth hormones. The influence of exogenous and endogenous hormones on developing fruit has been investigated in normal compatible (self) crosses but rarely in incompatible crosses. The effects of different growth hormones on free pollen grains in in vitro cultures have been extensively studied (25, 26, 51). The effects of hormones on compatible and self-incompatible pollen tube growth in pistils of *Lilium longiflorum* (14) and a few other species (9, 41) have been reported but the studies are too few for conclusions on self-incompatible taxa, let alone interspecific ones. Hormone applications have, however, been found to enhance a few incompatible fruit and seed set crosses.

Crane and Marks (7) and Brock (5) first used hormones to delay floral abscission in incompatibly pollinated flowers with short life. Interest in use of hormones in incompatible crosses subsequently grew. The latest hormone-aided hybridizations are interspecific crosses in *Arachis*. In a few cases application of gibberellic acid to the bases of incompatibly pollinated flowers stimulates the initiation and geotropic elongation of the peg, the aerial phase of *Arachis* fruit (Table 1; 43, 44, 45). Subsequent application of IAA, NAA, or kinetin increases the number of pods (42) and cultured ovules and embryos (29).

Over 20 interspecific or intergeneric hybrids have resulted from exogenous application of natural or synthetic plant growth hormones (41). We have yet to understand how hormones stimulate hybrid development from incompatible crosses. A comparison of hormonal balances in ovaries between compatibly and incompatibly pollinated flowers is called for and specific effects of exogenous hormones in successful cases need study. Nessling and Morris (32) found that endosperm from an interspecific *Phaseolus* cross does not develop normally and has much less cytokinin than

Table 1. Peg and pod production after gibberellin treatment in some intersectional crosses.

Cross	Pollinations (no.)	Pegs (%)	Pods/pegs (%)
Section *Arachis*/Section *Triseminale*			
A. duranensis (2n=20)/*A. pusilla* (2n=20)	33	79	39
A. hypogaea cv. Robut 33-1/*A. pusilla* (2n=20)	78	46	0
Section *Arachis*/Section *Extranervosae*			
A. hypogaea cv Robut 33-1/*A. villosulicarpa* (2n=20)	39	59	3
A. hypogaea cv MK 374/*A. villosulicarpa* (2n=20)	9	89	11
Section *Extranervosae*/Section *Triseminale*			
A. villosulicarpa (2n=20)/*A.* pusilla (2n=20)	24	54	46
Section Arachis x Section *Rhizomatosae*			
A. hypogaea cv Robut 33-1/*Arachis* sp. Coll. No. 9649	82	44	6
A. hypogaea cv Robut 33-1/*Arachis* sp. Coll. No. 9797	46	57	2
A. hypogaea cv Robut 33-1/*Arachis* sp. Coll. No. 9806	26	62	0
A. hypogaea cv TMV2/*Arachis* sp. PI 276233	408	76	20
A. hypogaea cv TMV2/*Arachis* sp. Coll. No. 9649	11	73	0
A. hypogaea cv MK 374/*Arachis* sp. PI 276233	648	68	32
A. hypogaea cv MK 374/*Arachis* sp. Coll. No. 9649	26	42	15
A. hypogaea cv M 13/*Arachis* sp. PI 276233	75	56	5
A. hypogaea cv Chico/*Arachis* sp. PI 276233	58	66	9
A. hypogaea cv Chico/*Arachis* sp. Coll. No. 9649	26	73	19

that from selfed pollinations. Such studies not only help elucidate hormonal regulation of fruit morphogenesis, but also assist in formulating strategy for further work with incompatible crosses.

Recognition pollen

Since Tsinger and Petrovskaya-Bananova (62) showed "pollen grain walls [to be] physiologically active substances," interest in postpollination changes in pollen grains and pistil has grown. It is now believed that extracellular (wall) pollen proteins communicate with extracellular (surface) stigma papillae proteins: pollen grains are thereby recognized as either compatible or incompatible (16, 17, 18). The existence of pollen grain walls (16) and stigma surfaces (19, 20) with a protein component has been demonstrated in a number of taxa, and in a few, even stigmas of immature flowers are lined with proteins (52, 53, 50).

Knox et a1 (27) exploited this information to produce hybrids from an interspecific cross in Populus. Earlier Stettler (57) had achieved interspecific hybridization in Populus by mixing live incompatible pollen grains with gamma-irradiated (killed) compatible pollen grains called recognition or mentor pollen (27). Mentor pollen stimulated incompatible pollen grains to produce fruit and seed.

Subsequently this method was tested in other taxa and in other systems of self-incompatibility and interspecific incompatibility. Sporophytic self-incompatibility was overcome in Cosmos bipinnatus (21), Brassica oleracea (40), and Raphanus sativus (48), but not in Brassica campestris (48). Gametophytic self-incompatibility was overcome in apple (8), Petunia hybrida

(47, 48) and Nicotiana alata (55), but not in Oenothera organensis (55). Recognition pollen did not work in other interspecific crosses in Sesamum (46), Arachis (43), Ipomoea (13), Trifolium (58), and Cucumis (10).

Sastri and Shivanna (48) have suggested that the method of killing the compatible pollen grains determines success. The recognition pollen can be prepared by storage at normal temperature, repeated freezing and thawing, and gamma-ray irradiation until pollen grains lose their ability to germinate. In a few instances the recognition pollen function could be effected by leachates of compatible pollen grains (21, 48).

Pandey (33, 34, 35) found that in Nicotiana gene transfers could be effected with just gamma-irradiated compatible pollen grains. Jinks et a1 (22) have confirmed this. Recently Shuzikuda et al (54) combined this method with ovule culture to transfer an incomplete chromosome complement of Nicotiana tabaccum into N. rustica, two species which are not otherwise crossable.

Immunosuppressants

About 10 yr ago Bates and his coworkers began using in wide hybridization experiments animal immunosuppressants including E-aminocaproic acid (EACA), chloramphenicol, acriflavin, salycylic acid, and gentisic acid. The success rate varied, but EACA was the most effective: with EACA, hybrids were obtained in crosses between barley and rye, durum wheat and barley, and bread wheat and barley (3, 4). EACA also stimulated embryo development in crosses between Triticum turgidum and Secale cereale (60, 61).

In another interspecific cross, Vigna radiata/V. umbellata, a foliar spray of EACA (100 ppm) effected twice as many hybrids as those that resulted from the unsprayed controls (1). Baker et a1 (2) obtained optimum results in the same cross by injecting 250 ppm of EACA. Chen et a1 (6) observed in two cultivars that foliar EACA spray for 14 d starting no later than the premeiotic stage of flower development delayed but did not prevent embryo abortion. EACA application to florets on 4 consecutive d after pollination reduced embryo recovery from 30 to 19%, but increased the number of ovaries forming embryo and endosperm in Triticum timopheevi/Secale cereale (31). It was not effective in Festuca arundinacea/Dactylis glomerata (30).

OTHER METHODS

Embryo, ovule, and ovary cultures have yielded hybrids in more than 50 cross combinations with early abortion of embryo or endosperm, or both (see 36, 41). According to Johnston et al. (24), when there are ploidy differences between the parents, an abnormal endosperm is caused by a deviation of the maternal and paternal genome ratio from 2 to 1 in the endosperm itself. By assigning a specific number (endosperm balance number) to the endosperm of each parent irrespective of its ploidy, and by

manipulating these numbers, Johnston and Hanneman (23) have succeeded in crossing some diploid Solanum species.

Ploidy manipulations are not new and a species with higher ploidy is generally better as a female parent. (Even when the parents have the same ploidy, one of them is raised to a higher level through colchicine treatment.) In other situations, a third species, itself crossable with each incompatible parent, is used as a bridge. Cultivar crossability differences indicate the need for a range of cultivars. Genes for crossability, such as Krl and Kr2 in wheat, should be investigated in other taxa. Most of these aspects have been reviewed by Rees et a1 (38), Thomas (59), Driscoll (11), and Riley et a1 (39).

CONCLUSIONS

Several means for hybridization between incompatible species have been found. Until the somatic methods (e.g., 12) can find wider applications, these methods, with or without modifications, must be exploited. In some instances combining methods with ovule or embryo culture is needed. For example, in a few interspecific crosses of Arachis, applying gibberellin to incompatibly pollinated flowers stimulated normal development of the pegs, and a few pods (Table 1) up to certain stages. Only ovules in the pods were found to be immature, with poorly differentiated embryos (29, 43, 44, 45). Ovules and embryos had to be cultured in vitro to yield plantlets (29, 43, 44). Similarly, in interspecific crosses in Sesamum (46) and Cucumis, (10) mentor pollen effected only part of the desired response. For further development, another method must be adopted.

Incompatibility must be better understood before it can be overcome and the wild germplasm exploited for further improvement of crops.

REFERENCES CITED

1. AVRDC (Asian Vegetable Research and Development Center). 1976. Progress report for 1975 (Mungbean). Shanhua, Taiwan, Republic of China. p. 35-40.
2. Baker, L.R., N.C. Chen, and H.G. Park. 1975. Effect of an immunosuppressant on an interspecific cross of the genus Vigna. Hortic. Sci. 70:325-333.
3. Bates, L.S. 1974. Wide crosses. In Proceedings: worldwide maize improvement in The 70s and the role for CIMMYT 5, IB-7B.
4. Bates, L.S., and C.W. Deyoe. 1973. Wide hybridization and cereal improvement. Econ. Bot. 27:401-412.
5. Brock, R.D. 1954. Hormone-induced pear-apple hybrids. Heredity 8:421-429.

6. Chen, N.C., J.F. Parrot, T. Jacobs, L.R. Baker, and P.S. Carlson. 1978. Interspecific hybridization of food grain legumes by unconventional methods of breeding. Pages 247-252 in International mungbean symposium. Asian Vegetable Research and Development Center, Taiwan.
7. Crane, M.B., and E. Marks. 1952. Pear-apple hybrids. Nature (London) 170:1017.
8. Dayton, D.F. 1974. Overcoming self-incompatibility in apple with killed compatible pollen. J. Am. Soc. Hortic. Sci. 99:190-192.
9. de Nettancourt, D. 1977. Incompatibility in Angiosperms. Springer-Verlag, Berlin.
10. Den Nijs., A.P.M., and E.H. Oost. 1980. Effect of mentor pollen on pollen pistil incongruities among species of Cucumis L. Euphytica 29:267-272.
11. Driscoll, C.J. 1981. Perspectives in chromosome manipulation. Philos. Trans. R. Soc. London 292:535-546.
12. Evans, D.A., C.E. Flick, S.A. Kut, and S.M. Reed. 1982. Comparison of Nicotiana tabacum and N. nesophila hybrids produced by ovule culture and protoplast fusion. Theor. Appl. Genet. 62:193-198.
13. Guries, R.P. 1978. A test of the mentor pollen technique in the genus Ipomoea. Euphytica 27:825-830.
14. Henny, R.J., and P.D. Ascher. 1973. Effect of auxin (3-indole acetic acid) on in vivo compatible and incompatible pollen tube growth in detached styles of Lilium longiflorum Thunb. Incompatibility Newsl. 3:14-17.
15. Heslop-Harrison, J. ed. 1971. Pollen: development and physiology. Butterworths, London.
16. Heslop-Harrison, J. 1975. The physiology of pollen grain surface. Proc. R. Soc. London 190B:275-299.
17. Heslop-Harrison, J. 1978. Genetics and physiology of angiosperm incompatibility systems. Proc. R. Soc. London 202B:73-92.
18. Heslop-Harrison, J. 1978. Recognition and response in the pollen-stigma interaction. Pages 121-138 in Cell-cell recognition. Vol. 32. A.S.G. Curtis, ed. Society for Experimental Biology, Cambridge, England.
19. Heslop-Harrison, Y. 1981. Stigma characteristics and angiosperm taxonomy. Nord. J. Bot. 1:402-420.
20. Heslop-Harrison, Y., and K.R. Shivanna. 1977. The receptive surface of the angiosperm stigma. Ann. Bot. 41:1233-1258.
21. Howlett, B.J., R.B. Knox, J.B. Paxton, and J. Heslop-Harrison. 1975. Pollen-wall proteins: physico-chemical characterization and role in self-incompatibility in Cosmos bipinnatus. Proc. R. Soc. London 188B:167-182.
22. Jiaks, J.L., P.D.S. Caligari, and N.R. Ingram. 1981. Gene transfer in Nicotiana rustica using irradiated pollen. Nature (London) 291:586-588.

23. Johnston, S.A., and R.E. Hannenman, Jr. 1982. Manipulations of endosperm balance number overcome crossing barriers between Solanum species. Science 217:446-448.
24. Johnston, S.A., T.P.M. den Nijs, S.J. Peloquin, and R.E. Hannenman, Jr. 1980. The significance of genic balance to endosperm development in interspecific crosses. Theor. Appl. Genet. 57:5-9.
25. Johri, B.M., D.C. Sastri, and K.R. Shivanna. 1977. Pollen viability, storage and germination. Pages 120-139 in Advances in pollen spore research. P.K.K. Nair, ed. Vol. 2. Today and Tomorrow Printers and Publishers, New Delhi.
26. Johri, B.M., and I.K. Vasil. 1961. Physiology of pollen. Bot. Rev. 27:325-381.
27. Knox, R.B., R.R. Willing, and L.D. Pryor. 1972. Interspecific hybridization in poplars using recognition pollen. Silvae Genet. 21:65-69.
28. Maheshwari, P. 1950. An introduction to the embryology of angiosperms. McGrawhill Book Co. Inc., New York.
29. Mallikarjuna, N., and D.C. Sastri. 1983. In vitro culture of ovule and embryos from some interspecific crosses in the genus Arachis L. In Proceedings international workshop on cytogenetics of Arachis. (in press)
30. Matzk, F. 1981. Successful crosses between Festuca arundinacea Schreb. and Dactylis glomerata L. Theor. Appl. Genet. 60:119-122.
31. Mujeeb-Kazi, A. 1981. Triticum timopheevii x Secale cereale crossability. J. Hered. 72:227-228.
32. Nessling, F.A.V., and D.A. Morris. 1979. Cytokinin levels and embryo abortion in interspecific Phaseolus crosses. Z. Pflanzenphysiol. 91:345-358.
33. Pandey, K.K. 1975. Sexual transfer of specific genes without gametic fusion. Nature (London) 256:311-312.
34. Pandey, K.K. 1978. Gametic gene transfer by means of irradiated pollen. Genetica 49:53-69.
35. Pandey, K.K. 1980. Further evidence for egg transformation in Nicotiana. Heredity 45:15-29.
36. Raghavan, V. 1977. Applied aspects of embryo culture. Pages 375-397 in Applied and fundamental aspects of plant cell, tissue and organ culture. J. Reinert and Y.P.S. Bajaj, eds. Sprinper-Verlag, Berlin.
37. Rangaswamy, N.S. 1963. Control of fertilization and embryo development. Pages 327-353 in Recent advances in the embryology of angiosperms. P. Maheshwari, ed. International Society of Plant Morphologists, University of Delhi, Delhi.
38. Rees, F., R. Riley, E.L. Rreese, and C.N. Law, eds. 1981. The manipulation of genetic systems in plant breeding. Philos. Trans. R. Soc. London 292B:401-609.
39. Riley, R., C.N. Law, and V. Chapvan. 1981. The control of recombination. Philos. Trans. R. Soc. London 292:529-534.
40. Roggen, H.P. 1975. Stigma application of an extract from

rape pollen (Brassica napus L.) effects self-incompatibility in Brussels sprout (Brassica oleracea var. "gemmifera"). Incompatibility Newsl. 6:80-84.

41. Sastri, D.C. 1984. Incompatibility in angiosperms significance in crop improvement. Adv. Appl. Biol. 10:71-111.
42. Sastri, D.C., and N. Mallikarjuna. 1983. Methods for utilization of incompatible species: current options and their limitations. In Proceedings international workshop on cytogenetics of Arachis. (in press)
43. Sastri, D.C., and J.P. Moss. 1982. Effects of growth regulators on incompatible crosses in the genus Arachis L. J. Exp. Bot. 33:1293-1301.
44. Sastri, D.C. J.P. Moss, and M.S. Nalini. 1982. The use of in vitro methods in groundnut improvement. Pages 365-370 in Proceedings international symposium on plant cell culture in crop improvement. S.K. Sen and K.L. Giles, eds. Plenum Press, New York.
45. Sastri, D.C., M.S. Nalini, and J.P. Moss. 1981. Tissue culture and prospects of crop improvement in Arachis hypogaea and other oilseed crops. Pages 42-57 in Proceedings symposium on tissue culture of economically important plants in developing countries. A.N. Rao, ed. National University of Singapore, Singapore.
46. Sastri, D.C., and K.R. Shivanna. 1976. Attempts to overcome interspecific incompatibilitv in Sesamum using recognition pollen. Ann. Bot. 41:891-893.
47. Sastri, D.C., and K.R. Shivanna. 1976. Recognition pollen alters incompatibility in Petunia. Incompatibility Newsl. 7:22-24.
48. Sastri, D.C., and K.R. Shivanna. 1980. Efficacy of mentor pollen in overcoming intraspecific incompatibility in Petunia, Raphanus and Brassica. J. Cytol. Genet. 15:107-112.
49. Shivanna, K.R. 1982. Pollen-pistil interaction and control of fertilization. Pages 131-174 in Experimental embryology of vascular plants. B.M. Johri, ed. Narosa Publ House, New Delhi.
50. Shivanna, K.R., Y. Heslop-Harrison, and J. Heslop-Harrison. 1978. The pollen stigma interaction: bud pollination in the Cruciferae. Acta Bot. Neerl. 27:107-119.
51. Shivanna, K.R., B.M. Johri, and D.C. Sastri. 1979. Development and physiology of angiosperm pollen. Today and Tomorrow Printers and Publishers, New Delhi. 117 p.
52. Shivanna, K.R., and D.C. Sastri. 1976. Stigma surface proteins and bud pollinations in Petunia: a correlative study. Incompatibility Newsl. 7:16-21.
53. Shivanna, K.R., and D.C. Sastri. 1981. Stigma surface esterase activity and stigma receptivity in some taxa characterized by wet stigma. Ann. Bot. 47:53-64.
54. Shuzikuda, N., K. Yamamoto, and T. Nakajima. 1983. Sexual transfer of an incomplete chromosome complement from

Nicotiana tabacum L. to N. rustica. Jpn. J. Breed. 33:15-22.

55. Sri Ramulu, K., G.M.M. Bredemeijer, P. Dijkhuis, D. de Nettancourt, and H. Schibilla. 1979. Mentor pollen effects on gametophytic incompatibility in Nicotiana, Oenothera and Lycopersicum. Theor. Appl. Genet. 54:215-218.

56. Stebbins, G.L. 1958. The inviability, weakness, and sterility of interspecific hybrids. Adv. Genet. 9:147-215.

57. Stettler, R.F. 1968. Irradiated mentor pollen: its use in remote hybridization of black cottonwood. Nature (London) 219:746-747.

58. Taylor, N.L., R.F. Quarles, and M.K. Anderson. 1980. Methods of overcoming interspecific barriers in Trifolium. Euphytica 29:441-550.

59. Thomas, M. 1981. Interspecific manipulation of chromosomes. Philos. Trans. R. Soc. London 292:519-528.

60. Tiara, T., and E.N. Larter. 1977. Effects of d-amino-n Caproic acid and L-Lysine on the development of hybrid embryo of triticale (x-triticale-Secale). Can. J. Bot. 55:2330-2334.

61. Tiara, T., and E.N. Larter. 1977. The effects of variation in ambient temperature alone and in combination with d-amino-n-caproic acid on development of embryos from wheat rye crosses (Triticum turgidum var. durum cv. gora x Secale cereale. Can. J. Bot. 55:2335-2337.

62. Tsinger, N.V., and T.P. Petrovskaya-Baranova. 1961. The pollen grain wall: a living physiologically active structure. Dokl. Akad. Nauk SSSR 138:466-496.

CIMMYT's WIDE CROSS PROGRAM FOR WHEAT AND MAIZE IMPROVEMENT

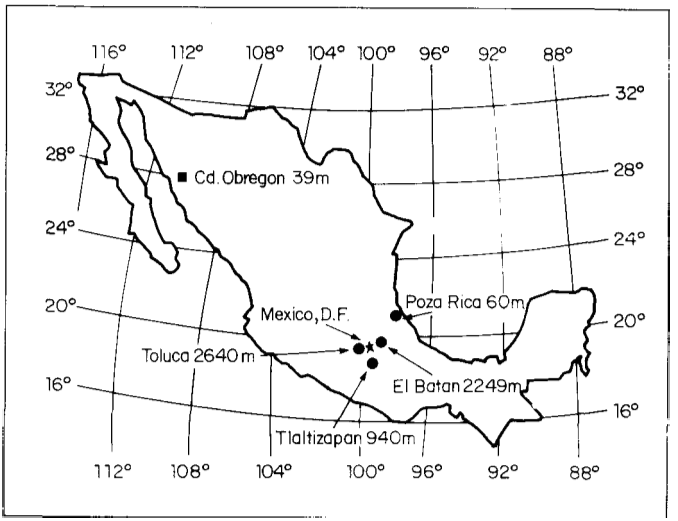
A. MUJEEB-KAZI and D. C. JEWELL

CIMMYT is in its fourteenth year of wide cross research in wheat and maize, and the program is an important part of the Center's over-all research effort. Traditional breeding techniques and wide cross research are seen as complementary. Progress in the former is predictable and dependable, while progress in the latter is less so, but has the potential to make quantum leaps. Recently developed techniques may allow wide cross research to proceed much more rapidly than it has to date, and permit the relatively quick development of germplasm with superior resistance to diseases and insects, as well as improved tolerance to salt, heat, drought, and aluminum. Specifically, CIMMYT is modifying prepollination and postpollination techniques, and experimenting with embryo-rescue techniques and novel cytogenetic systems (e.g., the 5B effect). The potentials of both tissue culturing and a recently. developed transforming DNA technique are also being investigated. CIMMYT is optimistic that such research will, in the years to come, furnish superior germplasm for use in conventional breeding programs,

CIMMYT was established as an international agricultural research center in 1966, having evolved from a collaborative research program begun in 1943 between The Rockefeller Foundation and the Mexican Ministry of Agriculture.

Research is done at five experiment stations in Mexico (Fig. l), four of which are operated by CIMMYT and one by the Mexican National Institute for Agricultural Research (INIA). The five stations range in altitude from near-sea level to 2,640 m and differ markedly in average temperature, moisture, and solar radiation. Within Mexico, then, experimental germplasm can be exposed to many of the environmental conditions of the developing world. The wide cross program in wheat and maize is a crucial part of CIMMYT's overall breeding work.

Centro Internacional de Mejoramiento de Maize y Trigo, A. C. (CIMMYT), Apdo. Postal 6-641, Col. Juarer, Delg. Cuahutemoc, 06600 Mexico, D. F., Mexico.



1. Locations and elevations of principal stations in Mexico at which CIMMYT conducts research (Cd. Obregon Station of the Instituto Nacional de Investigaciones Agricolas).

WIDE CROSS RESEARCH AT CIMMYT

Most of the work on wide crosses, started in 1972, was collaboration between CIMMYT and other centers. An early priority in CIMMYT's wheat wide cross research was to introduce high protein and high lysine into bread wheat (Triticum aestivum I,.) from barley (Hordeum vulgare L.). To produce relevant hybrids and develop methods for transferring genes from related species to wheat for general crop improvement were also program objectives.

In maize, the priority was to develop hybrids with Tripsacum, a related species with genes for disease and insect resistance. Because available close relatives of maize are much fewer than those of wheat, however, the Maize Wide Cross Program is also investigating crosses between maize and more distant relatives such as sorghum.

The early efforts at CIMMYT did not result in many applied benefits; so when we joined CIMMYT, program priorities were changed. At first, in-house activities were intensified, and collaborative efforts with other institutions reduced.

Eventually, however, the wheat program focused on crosses, and the progeny of crosses, between wheat and Elymus spp. and between wheat and Agropyron spp. In the maize program, efforts were again directed to maize/Tripsacum spp. following intense but unsuccessful attempts to isolate sexually a waize-sorghum hybrid. By conservative estimates, this hybrid combination has a probability of less than one in three million of being isolated from sexual crosses (1).

The following criteria are now used to define wide cross research at CIMMYT:

1. A wide cross should, as in normal hybridization, result in an offspring (F_1) with chromosome complement of

both parents (the maternal complement may he haploid or diploid);

2. For CIMMYT to he interested in the resulting F_1, it should possess a phenotype intermediate between the parents;
3. The F_1 should he male sterile.

These hybrids usually require specialized crossing and embryo rescue techniques. Hybrids that can he produced normally and are self-fertile, can be evaluated and selected as part of CIMMYT's ongoing breeding programs, and the wide cross programs need not be involved. This research should result in the accession of genetic information on species related to maize and wheat, not to form new species hut to improve cultivated maize and wheat crops. Introducing genetic variability for traits with little or no known variability into the existing maize and wheat germplasm is emphasized.

THE WHEAT WIDE CROSS PROGRAM

The Wheat Wide Cross Program emphasizes bread wheat (T. aestivum I,.) improvement, specifically the incorporation of

1. resistance to Helminthosporium sativum (known to cause seed decay, root rot, and leaf spots);
2. resistance to Fusarium graminearum (the pathogen of head blight and leaf spot); and
3. stress tolerance to salt, drought, heat, aluminum, and copper.

Several related genera are excellent sources of resistance or tolerance. They include several of the Aegilops, Agropyron, Clymus, Haynaldia, and Secale species. Until recently, however, the success rate for hybridizing wheat with related species has been alarmingly low, primarily because of problems associated with crossability barriers and poor embryo development.

We have now lowered the crossability barriers such that we can produce intergeneric hybrids. In general, prepollination or postpollination techniques, or both, together with manipulation of the embryo culture media may influence 1) pollen tube growth, 2) gynoecia longevity, 3) micropylar harriers, 4) delivery of male gametes, 5) initiation and assistance of seed set, and 6) embryo development.

Other more easily defined factors have contributed to overcoming crossabilitv harriers. They include 1) cross direction, 2) varietal choice, 3) polyploid level of the related parent, 4) presence or absence of recombination in the F_1, 5) production of amphiploids from the F_1, 6) cytological differences in the backcross (BC) progeny, and 7) restoration of self-fertility by continued backcrossing.

CIMMYT currently maintains 80 intergeneric hybrid combinations with wheat, the majority of which are new. Where possible, these hybrids have been made using commercially grown spring wheat varieties. In most of these F_1 hybrids, no

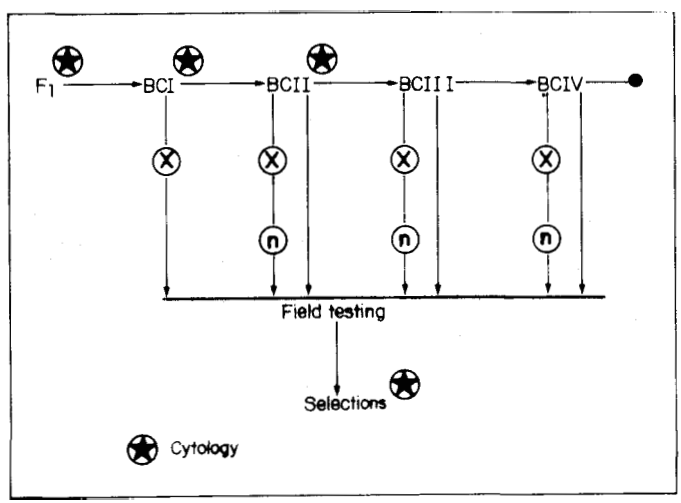
recombination takes place between the wheat and alien chromosomes (chromosomes from the related species); in several cases, the BCl progeny is cytologically abnormal (3, 4). Approximately 27,000 lines, derived from 5 of the 80 cytologically analyzed intergeneric combinations, have been field tested. Figure 2 illustrates the methodology used to advance an F_1 to field testing. Selection is based on plant type, on resistance to leaf rust, stem rust, H. sativum, and F. graminearurn, and on cytological analysis.

About 60% of our effort in wheat wide cross program resources are devoted to advanced progeny from crosses with Elymus giganteus (2n=4x=28) (5, 7), a species moderately salt tolerant, free from leaf-spotting diseases, and resistant to F. graminearurn. The chromosomes of this species are uniquely marked by chromosome banding and it is relatively easy to identify the chromosomal complement in the advanced progeny.

Lines have been tentatively identified for resistance to H. sativum or to F. graminearum. The advanced offspring have good plant type, but have not yet been tested for stress tolerance.

Fourteen different addition lines (normal wheat chromosome complement plus an additional chromosome from the related species) are possible with the tetraploid species E. giganteus. Five of them have been confirmed; seven others have been tentatively isolated; and one of the remaining two chromosomes has been substituted for a wheat chromosome. Only one chromosome has yet to be isolated in a wheat background. Specific resistances or tolerances have not yet been ascribed to specific E. giganteus chromosomes. Our objective is to introduce only the beneficial segments of these chromosomes.

Subtle gene transfers are essential for the germplasm to be useful to breeders in conventional breeding programs. They are facilitated by 1) suppression of the 5B effect, 2) irradiation, 3) tissue culture, and 4) pentaploid-induced translocation (all



2. This scheme represents the movement of germplesm from the intergeneric F1s to field testing.

recently incorporated into the wheat wide cross program). To produce new germplasm for breeders, CIMMYT and collaborators have evolved the following steps:

- identify CIMMYT wheat with highly crossable genes,
- transfer the 5B chromosome stocks to these cultivars,
- develop a monosomic series in one of these cultivars,
- ascertain their suitability for tissue culturing (callusing and regeneration), and
- remake some of the hybrid combinations using lines adapted to tissue culturing and having the desired crossability genes.

Ideally small pieces of the alien chromosomes should be inserted into closely related chromosomes; however, insertions of alien genetic material into less closely related chromosomes can be equally valuable. The benefits breeders have derived through introgression of alien genetic material are best exemplified by CIMMYT's IB/IR wheat varieties. These have been released globally because of their wide adaptation, yield stability, aluminum tolerance, and resistance to *Septoria tritici* (8). Significant improvements in wheat through translocations made by institutions other than CIMMYT include

- stem rust resistance: *A. elongatum* and the wheat chromosome 6A;
- leaf rust resistance: *A. intermedium* and the wheat chromosome 7A;
- leaf rust resistance: *Ae. umbellulatum* and the wheat chromosome 6B;
- leaf rust and powdery mildew resistance: *S. cereale* and the wheat chromosome 4A; and
- green bug resistance: *S. cereale* and the wheat chromosome 1A.

Significant practical success from the wheat wide cross program is expected within the next decade, particularly since hybrid production has been simplified and the methods for successful hybrid exploitation, well documented. Once the desired variation has been recognized in the wild species, and its expression in a hybrid established, the choice of method to introduce the alien variation follows logically from the relative affinity of the chromosomes involved. The introduction of alien variation can only increase the range of variation for specific traits from which plant breeders can select (6).

The genes introduced into wheat cultivars are not entirely different from the genes already present; and their expression will be affected by the same factors affecting the genes normally found in cultivated wheat. Two situations exist: mutable and nonmutable. An example of a mutable situation is the introduction of genes conferring resistance to disease. The pathogen is free to mutate, and the resistance is expected eventually to fail. The plant breeder must then identify a different source of resistance. Examples of nonmutable situations include tolerance for salt, drought, low copper, and high aluminum. An alien gene or genes conferring tolerance for these conditions should be very long lasting and could

substantially contribute to food production in areas where the lack of such tolerance is a limiting factor.

The practical potential of wide hybridization is probably greater with wheat than with most other crops because 1) wheat hybridization is easy; 2) the species cytogenetics are wel1 understood; and 3) bread wheat, being a hexaploid (2n=6x=42), has a large amount of genetic material so it is well buffered against introgression of alien material. The introduction of genetic material from species with relatively close evolutionary ties to wheat has great potential and will continue to be exploited by CIMMYT

THE MAIZE WIDE CROSS PROGRAM

Current emphasis in the maize wide cross program is on maize/ *Tripsacum* and the resulting progeny. *Tripsacum* possesses wide adaptability, tolerance for waterlogging and drought, and resistance to several foliar diseases and insect pests. Several approaches are being used to incorporate genes from this relative into maize.

We currently maintain more than 130 F_1 hybrids between maize and *Tripsacum*. These involve 5 different maize pools in combination with 17 different collections representing 6 taxa of *Tripsacum*. They include both diploid (2n=2x=36) and tetraploid (2n=4x=72) species.

One of the techniques by which we obtain a maize population with *Tripsacum* genes was developed by Harlan and de Wet (2, 9). It requires sequential backcrossing of the F_1 with maize, together with maintenance of the F_1 chromosome number. After several generations of maintaining this F_1 chromosome number the backcross plants with the normally expected chromosome number are selected and backcrossed again. Plants are then selected for near-normal maize chromosome number and for phenotypic differences. The selected plants are recombined to form a population that can be tested for desirable traits. This method is applicable only to tetraploid *Tripsacum* spp. because maintaining the F chromosome complement for several generations is not possible with the diploid *Tripsacum* spp.

The main difficulties in using this procedure are

1. time (the F_1 and recovered F_1s take about 18 mo to flower);
2. difficulty in obtaining backcross progeny, because a dominant gene in some *Tripsacum* spp. prevents backcrosses to maize without a similar gene; and
3. applicability to only the tetraploid *Tripsacum* spp.

We are therefore continuing efforts to isolate new F_1 hybrid and backcross progeny for single addition lines that could, perhaps by irradiation, be used to incorporate the beneficial genes into the maize chromosome complement. The Harlan and de Wet method (2, 9) is being used, where possible, for the F_1 hybrids involving tetraploid spp.

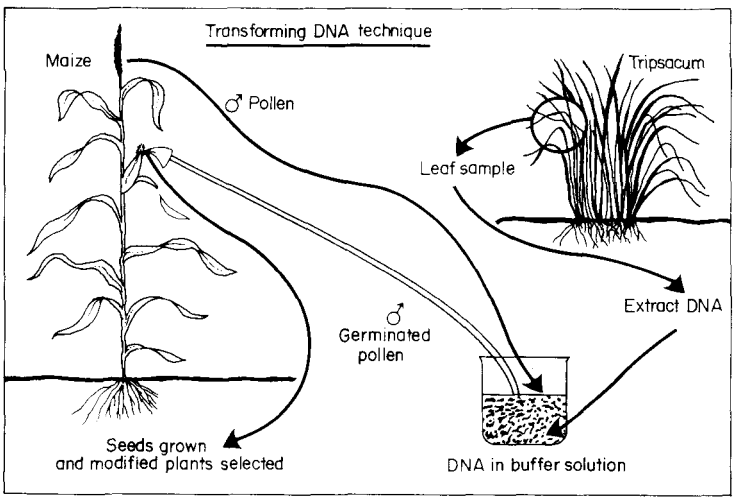
We are also initiating collaboration in tissue culture of *maize/Tripsacum* F_1 s in an attempt to use somaclonal variation to

recover annual tripsacoid maize plants from the perennial F_1s more rapidly than by the classical method.

A subset of the existing tripsacoid maize was obtained from the University of Illinois and has been crossed to CIMMYT's breeding material. The resultant progeny is being selected for resistance to Southwestern Corn Borer (Diatraea grandiosella); it shows promise and may be screened for other beneficial traits.

We have also been evaluating other techniques. In collaboration with The Plant Breeding Institute, Cambridge, we are attempting to determine whether or not maize and sorghum nuclei can coexist following microinjection. of the pollen nuclei into the micropyle. This should help in determining future strategies. For example, if maize and sorghum chromosomes can coexist in the same cell, even for a short time, protoplast fusion, followed by irradiation, might be used to transfer sorghum genes to maize.

We are just completing 18 mo of collaboration with the University of Illinois to evaluate a "transforming DNA technique" for incorporating small segments of alien DNA into maize. This involves extracting DNA from a donor species and soaking maize pollen in a solution of this DNA before pollination, to incorporate small donor DNA segments into the developing embryo (see Fig. 3). This technique has been used successfully to transfer genes for cob color and rust resistance from one maize inbred to another (de Wet, pers. comm.). Tripsacoid plants have been recovered, both at the University of Illinois and at CIMMYT, using maize as the male and female and Tripsacum as the DNA donor. The technique has tremendous potential, as it may allow us to quickly introduce genetic material from both diploid and tetraploid Tripsacum spp., and it may permit introducing genes from other, more distantly related genera. The program intends to expand its efforts with this new technique, not only to svnthesize tripsacoid maize populations, but alsot to test the technique's potential to incorporate sorghum genes into maize.



3. Diagram of the method used for incorporating allen material by the transforming DNA technique.

EXPECTATIONS

Nearly all maize and wheat breeding in the world today is of the conventional type, where progress is, of course, still possible. And success in wide crosses is expected to be slow, proceeding from plants that are near relatives to plants that are more distantly related.

However, in the next decade, wide cross research is expected to provide new sources of genetic variation for conventional breeding. Thus wide cross research should, in the near term, significantly contribute toward feeding the world's growing population. In the longer term, the quantum leaps referred to at the beginning of this paper are well within the realm of possibility.

REFERENCES CITED

1. Bernard, S.E.C., and D.C. Jewell. 1984. Crossing maize with sorghum, Tripsacum and millet: the products and their level of development following pollination. (in preparation)
2. Harlan, J.R., and J.M.J. de Wet. 1977. Pathways of genetic transfer from Tripsacum to Zea mays. Proc. Natl. Acad. Sci. USA 74(8):3494-3497.
3. Jewell, D.C., and A. Mujeeb-Kazi. 1982. Unexpected chromosome numbers in backcross I generations of F_1 hybrids between Triticum aestivum and related alien genera. Wheat Inf. Serv. 55:5-9.
4. Mujeeb-Kazi, A., and M. Bernard. 1982. Somatic chromosome variations in backcross I progenies from intergenetic hybrids involving some Triticeae. Cereal Res. Commun. 10:41-45.
5. Mujeeb-Kazi, A., M. Bernard, G.T. Bekele, and J.L. Miranda. 1983. Incorporation of alien genetic information from Elymus giganteus into Triticum aestivum. Sixth Int. Wheat Genet. Symp., Kyoto, Japan. (in press)
6. Mujeeb-Kazi, A., and G. Kimber. 1984. Increased variability and the use of wide crosses in wheat. (in preparation)
7. Mujeeb-Kazi, A., and R. Rodriguez. 1981. An intergeneric hybrid of Triticum aestivum L. x Elymus giganteus. J. Hered. 72:253-256.
8. Rajaram, S., C.E. Mann, G. Ortiz-Ferrara, and A. Mujeeb-Kazi. 1983. Adaptation, stability and high yield potential of certain IB/IR CIMMYT wheats. Sixth Int. Wheat Genet. Sympo., Kyoto, Japan. (in press)
9. Stalker, H.T., J.R. Harlan, and J.M.J. de Wet. 1978. Genetics of maize-Tripsacum introgression. Caryologia 31(3):271-282.

ORGANOGENESIS IN VITRO: STRUCTURAL, PHYSIOLOGICAL, AND BIOCHEMICAL ASPECTS

T. MURASHIGE and LI-CHUN HUANG

The status of plant regeneration through adventitious organogenesis and embryogenesis in vitro is reviewed briefly, with principal focus on the single-cell origin of organs and embryos, the confinement of manipulability to meristemoids, the chemical and physical variables that are employable, and the progress of biochemical investigations on meristemoid differentiation and organogenic induction.

Recoverability is the attribute of plants that renders plant cell and tissue culture valuable in biotechnology and agriculture. The in vitro approach is already extensive1y used in propagating clones and establishing pathogen-free stock. Its other potential uses are in germplasm storage and dissemination, and in parasexual genetic crop improvement.

Plants can be reproduced in vitro by several methods. But the more important genetic uses of in vitro plant reproduction rely on adventitious organogenesis and embryogenesis. The organogenic method requires 1) formation of shoots and 2) rooting of shoots. Plant regeneration by embryogenesis does not require separate differentiation steps, since shoots and roots are normally initiated simultaneously on the same structure.

Plant regeneration by either method, although performed in hundreds of species, is still not universally achievable. The regenerating facility varies with genotype and even among cells within the same plant. We briefly review the progress of research into the structural, physiological, and biochemical aspects underlying initiation of organized development in vitro. Information has been included both for adventive organogenesis and embryogenesis, inasmuch as data obtained for one process are frequently applicable to the other.

STRUCTURE

Structural considerations of organogenesis in vitro might include details of the final morphology, the sequence of histological events, or the fine structure of cells giving rise to organs and

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Professor of Horticultural Science and plant physiologist, Department of Botany and Plant Sciences, University of California, Riverside, California, U. S. A.. and associate research fellow, institute of Botany. Academia Sinica, Nankang, Taipei, Taiwan,

embryos. We have focused on two other aspects: 1) the cellular origin of adventitious shoots and embryos and 2) the confinement of organogenesis and embryogenesis to cells identified as meristemoids.

CELLULAR ORIGIN OF ORGANS AND EMBRYOS

Adventitious shoots, roots, and embryos arise directly from explants or through an intermediary callus. The concern in each instance is whether the organ or embryo originated in a single cell. If adventive organs and embryos emerge directly from organ or embryo explants, a second question arises: must organogenesis in these species be restricted to preexisting organ initials? These concerns are not important when employing tissue cultures in clonal propagation or establishment of pathogen-free stocks, but they may be critical. when applying protoplasts and single cells to parasexual genetic crop improvement.

Adventitious shoots may or may not originate in single cells. Observations of organogenesis in chimeras revealed that a multiple of cells are involved in forming the shoot primordium (57). Any nonchimeral shoot probably arose from a group of cells with a common initial.

Although development does not proceed directly from a single cell, the single-cell origin of callus embryos has been documented for many species (24). In several cereal callus cultures observed, the differentiation of adventitious shoots and embryos was confined to partially organized regions (14, 15, 80). Such regions were carried over from embryo explants or were associated with aberrant roots that differentiated in vitro. We are hopeful that continued experimentation will permit plant regeneration from newly produced callus cells, even in cereals, inasmuch as Lu et al (42) achieved embryogenesis in callus of protoplasts from suspension-cultured cells of the grass Panicum.

MERISTEMOIDS AS MORPHOGENICALLY COMPETENT CELLS

It is reasonable to assume that all plant cells possess totipotential, or inherent capacity to become plants. However, the expression of that potential is limited to relatively few cells, identified by Torrey (77) as "meristemoids." These are the morphogenically "competent" cells: only they respond to variations in milieu to differentiate shoots, roots, or embryos. Thus, to regenerate plants, the cultured tissue must contain meristemoids or cells that are readily transformed into meristemoids.

Meristemoids become recognizable in callus when they have produced, by repeated divisions, compact clusters of small, fairly isodiametric, thin-walled cells with prominent nuclei, dense cytoplasm, and minimal vacuolation. Apical meristems and immature embryos contain cells with the same appearance, and those of some species respond in the same way to hormonal treatments (3, 6, 83).

Meristemoids are scarce. Meins et a1 (46) estimated one meristemoid per 1 000-10 000 tobacco callus cells. Nevertheless, their frequency in cell cultures can be increased, particularly by choosing and preparing explants judiciously and by continual selection in vitro.

In choosing and preparing explants, the following characteristics should he recognized:

- For each plant, the meristemoid content varies among tissues and organs.
- Morphogenically competent cells are more likely to he found in younger organs of younger plants than in older organs of flowering plants (5, 21).
- With woody perennials, regenerability is found in the juvenile phase but rarely in adults (52).
- Any seasonal dormancy requirements must be satisfied before explants are removed for culture (52).

Repeated selection at each subculture will enrich the population of competent cells (11); it will also free the meristemoids from the repressive effects of nonmeristemoid cells.

PHYSIOLOGY

This discussion is applicable only to meristemoids and nonmeristemoids that are readily transformed into meristemoids. Without them no manipulations of nutrient medium or culture environment are expected to permit plant regeneration.

BASIS OF HORMONAL REGULATION OF ORGANOGENESIS

It is possible to initiate and direct the organogenesis of a given culture of responsive cells by varying their chemical and physical environments. The pioneering experiments of White (81) and of Skoog (66) revealed that adventitious shoots could be induced in tobacco callus by keeping the tissue submerged in liquid medium. The manipulation of organogenesis by balancing growth factor supplements began with the discovery by Skoog and Tsui (68) that shoot formation in tobacco callus was inhibited by auxin and restored by adenine or inorganic phosphate. Miller and Skoog (47) subsequently showed that the progressively greater repression of shoot initiation by increasing levels of IAA could be reversed through corresponding increases of adenine sulfate.

The current basis of chemical regulation of shoot and root formation in plant tissue cultures was established nearly 30 yr ago by Skoog and Miller (67). Their experiments with tobacco callus disclosed the need to provide both auxin and cytokinin for sustained callus growth. In turn, the relative supplies of the two substances determined whether shoots or roots developed from the callus. A medium high in auxin and low in cytokinin stimulated rooting, and one with the reverse balance promoted shoot formation.

Balances between auxin and cytokinin also underlie other morphogenetic processes leading to plant regeneration. Development of plants, or of roots or shoots only, in isolated shoot apical meristems is determined by the nutrient medium concentrations of auxin and cytokinin (64). The enhanced axillary branching in shoot-tip explants by high concentrations of cytokinin is presumably due to a suppressive action by the cytokinin on apical dominance, a manifestation attributable to auxin (82).

A formerly widely held view that hormones, particularly auxin, were not involved in the development of embryos from callus, although they were needed for callus growth, emerged because embryos formed only when the callus was removed from an auxin-supplemented medium to one with less or no auxin. It was strengthened by observations that embryogenesis was enhanced by supplements of auxin-antagonists 110).

More recent evidence suggests that auxin induces embryogenesis (18, 30, 53): a high dosage of auxin appears critical during the initial period of embryogenesis. Subsequent development and emergence of embryos, however, is suppressed by the same dosage that causes induction.

Gibberellins have principally negative effects. Exogenous gibberellins depress all adventitious shoot (51), root (76), and embryogenesis (30, 36). However, rooting is stimulated in dark-grown tissue cultures (76). Simultaneous use of gibberellin antagonists may counteract the depressed embryogenesis (36). And the calluses of embryogenic anise and carrot contain lower endogenous levels of GA_1 and higher levels of GA_4 and GA_7 than their nonembryogenic counterparts (56).

ABA may stimulate the formation of adventitious shoots (65) and reverse the inhibition by gibberellin (72). It can also enhance somatic cell embryogenesis (36). More significantly, it prevents precocious germination and minimizes abnormalities frequently associated with adventive embryos (1).

Ethylene inhibits adventitious shoot formation (13, 27, 40). It inhibits during early shoot initiation and it stimulates during the later stages (27). The stimulation is enhanced by CO_2 (13) and negated by ethane (40). Although ethylene is generally regarded as a promoter of rooting, it represses root regeneration in some tissues (12). It has also shown ambivalent effects on somatic cell embryogenesis (36, 74).

ACCESSORY SUBSTANCES

Substances other than hormones play significant roles in organized development. Many phenolic derivatives enhance shoot differentiation (39) and root initiation (61). Derivatives that stimulate IAA oxidase enhance shoot differentiation and those with auxin-sparing tendencies promote rooting.

Nitrogen enrichment of nutrient media has been critical during induction of somatic cell embryogenesis and in shoot differentiation. For embryogenesis of some plants, e.g., Medicago (79), only the NH_4^+ form has been effective. But for others, a combination of NO_3^- and one or more amino acids or amides

has been satisfactory (31). In fact, carrot embryogenesis can occur with glutamine as the sole nitrogen source (31). Amino acids and amides also improve adventitious shoot and root initiation (32, 67).

A K^+ requirement has been established for somatic cell embryogenesis in carrot callus (8). It is clearly distinguishable from that for cell growth. Whereas a 1-mM level suffices for cell proliferation, a substantially higher concentration, 20 mM, is required for maximum yield of embryos.

A variety of sugars, many of which are ineffective in sustaining callus growth, support adventitious shoot initiation and somatic cell embryogenesis (9, 37, 71, 78). The effectiveness of galactose and galactose-yielding sugars, usually toxic to plant cells, is surprising.

Tissue cultures release several substances that inhibit their morphogenetic activities as well as their cell growth. Phenylacetic and benzoic acid derivatives have been identified as inhibitors produced by carrot cells (17). Citron cells liberate ethylene and ethanol, both of which are potent repressors of somatic cell embryogenesis (75). It is possible to reduce the adverse effects of some inhibitors, not ethanol, by including activated charcoal in the nutrient media (2, 17). Alcohol effects can be removed only through improved gas exchange or more frequent transfers to fresh medium,

TEMPERATURE AND ILLUMINATION

Species differences in temperature optima for organogenesis are as expected (16, 33, 66). More interesting have been the promotive effects of low-temperature pretreatments. Prior exposure of Begonia and Heleniopsis leaves and stems to 15°-18°C improved the regeneration of adventitious shoots and roots in their tissue cultures at 21°-25°C (16, 33). A very short preexposure to a lower temperature, 4°C, was more effective for shoot and root differentiation in Arabidopsis callus (54). Low-temperature stresses also enhance androgenesis in pollen cultures 155). Chilling may be prerequisite to complete development and germination of somatic embryos and other propagules obtained in vitro (60, 69).

Spectral quality, period of daily exposure, and intensity of illumination should also be considered to maximize organogenesis. The effective wavelengths for shoot initiation in tobacco callus have been in the near-ultraviolet (371 nm) and blue (419 nm) regions; red (660 nm) and far-red (750 nm) regions were totally ineffective (62). In contrast, shoot formation in Douglas fir (28) and lettuce (29) cotyledons responded favorably to red light, but not to blue or near-ultraviolet. The enhancement of lettuce by red light was reversible by far-red light, suggesting a role of phytochrome in the caulogenic process. Adventitious roots al.so form best in red light, less effectively in blue light (41).

Photoperiod control of organogenesis has been evident in some species. For example, plant regeneration in Crassula leaf sections appears to be a short-day process, with an 8-h day length or constant darkness being optimum (59). An appropriate

photoperiod treatment of donor plant further enhances organogenesis in Crassula leaf cultures.

The observed differences in response to day length in vitro do not invariably signal photoperiodism. Frequently, organogenesis depends on the total daily dosage of radiant energy. Thus, for example, shoot and root differentiation occurs under short daily illumination in tobacco callus exposed to high light intensities; longer illumination periods are required in cultures given low levels of illumination (38, 52).

Using constant illumination with white light as standard, different-intensity optima can be observed for different morphogenetic processes. That of rooting in Jerusalem artichoke is 500-600 lx (20), whereas that of somatic cell embryogenesis in tobacco is considerably higher, 10 000-15 000 1x (25).

BIOCHEMISTRY

A primary goal of biochemical studies has been to disclose information permitting more extensive manipulation of plant regeneration in cell cultures, especially by transforming nonmorphogenic cells into meristemoids. Unfortunately, biochemical studies of organogenesis have been limited. At best, we now have some markers to distinguish the organogenic cells or tissues from the nonorganogenic. And we have confirmed that biochemical activities in morphogenic processes are as expected. At the current rate of biochemical investigation, and without the involvement of skilled molecular biologists, we will not soon attain our goal. Meanwhile, we must continue with our rather primitive, circumventive approach to plant regeneration.

MARKERS ASSOCIATED WITH ORGANOGENESIS

Increase of peroxidase activity and alterations of its isozymes have been correlated with adventitious shoot formation and somatic embryogenesis (35, 43, 49, 70). In culture of shoot-forming tobacco callus, fast-migrating anodic isozymes disappear and all other peroxidases increase (43, 70). A cathodic isozyme is found only in embryo-forming orange callus (35).

Root formation in carrot callus is associated with elevated polyphenoloxidase activity (23). Enzymes of the shikimate pathway are stimulated by culture variables promoting tobacco shoot formation (4). Very interestingly, two unique antigens have been found in shoot-differentiating tobacco callus (48).

Starch accumulation is the first visible biochemical activity common to shoot, root, and embryo initiation (7, 26, 73). It occurs principally in cells that eventually differentiate into meristemoids. Gibberellin treatments suppressing organogenesis also prevent starch accumulation (71, 73). These observations simply underscore the high energy needs of organogenetic processes (34, 58, 71).

EVIDENCE OF GENE DEREPRESSION IN ORGANOGENESIS

The expected rise in template activity, signifying new gene expression, has been observed in chromatin preparations from cells

undergoing organogenesis or embryogenesis (19, 22, 45). The predominance of m-RNA among the newly synthesized RNA has also been confirmed (63). The template activity, may be related to nonhistone chromosomal proteins. Natsumoto et al (45) were able to reverse histone-inhibited transcription with a nonhistone protein preparation from embryogenic carrot cells. In tobacco cells, Guerri et al (22) observed during very early shoot initiation, a decrease of high-molecular-weight nonhistone chromosomal proteins. This was followed by an increase of low-molecular-weight nonhistone proteins. The low-molecular-weight proteins also increase during adventitious bud initiation of Douglas fir cotyledon explants (84).

Each of these observations simply confirms the view that new genes are being expressed and that they are probably derepressed by nonhistone proteins. The mechanism specifying the derepressed genes and the sequence of their derepression remains unclear. The appearance of certain polyamines in conjunction with organ and embryo initiation processes suggests that these substances may help regulate that mechanism (44, 50).

CONCLUSIONS

With few exceptions, plants can be regenerated in vitro without preexisting initials. And, although the plant may not develop directly from a single cell, it seems to originate in single-cell initials. Even adventitious shoots can arise from cell groups with common initials.

Organogenetic manipulation are possible only with meristemoids or cells readily transformed into meristemoids. The manipulations involve varying the hormonal contents of nutrient media. Adventitious roots and shoots are initiated in response to auxin and cytokinin balances. Somatic embryos are induced by high doses of auxin, but their subsequent development is inhibited by the same dosage. Similarly critical roles have not been evident with other homones. Phenolic derivatives enhance the initiation of shoots or of roots, depending on their enhancing or suppressing effects on IAA oxidase. Reduced N and K^+ are critical in somatic embryo induction. Temperature optima differ with the species. Preexposure of explant sources to cold temperature can, enhance organogenesis of some plants. A cold treatment may also improve germination and permit in vitro propagules to complete development. Shoot formation of some species requires near-ultraviolet or blue, whereas that of others needs red light. Adventitious roots are produced in red light and, to a lesser extent, in blue. Organogenesis of some species is photoperiodically controlled. Photoperiod-insensitive plants also respond to day lengths, according to the intensity of incident illumination.

Biochemical studies have produced markers to distinguish organogenic cells from nonorganogenic. These markers include perioxidases, polyphenoloxidase, and other proteins. Starch accumulation is the earliest visible sign common to shoot, root, and embryo initiation. Template activity is elevated in chromatin

of organogenic cells, the main product being m-RNA. The transcription is possibly regulated by nonhistone chromosomal proteins. Polyamines have also been implicated in transcription regulation. In the absence of major breakthroughs, it is unlikely that we will be able to manipulate totipotential of nonmeristemoid cells in the reasonable future.

REFERENCES CITED

1. Ammirato, P.V. 1977. Hormonal control of somatic embryo development from cultured cells of caraway. Plant Physiol. 59:579-586.
2. Anagnostakis, S.L. 1974. Haploid plants from anthers of tobacco -- enhancement with charcoal. Planta 115:281-283.
3. Ballade, P. 1972. Etude experimentale de l'organogenese axillaire chez Nasturtium officinale R. Br. Comportement d'aisselles isolees ou de parties d'aisselles cultivees in vitro. C.R. Acad. Sci. Paris 274:1282-1285.
4. Beaudoin-Eagan, L.D., and T.A. Thorpe. 1983. Shikimate pathway activity during shoot initiation in tobacco callus cultures. Plant Physiol. 73:228-232.
5. Bhatt, P.N., D.P. Bhact, and I.M. Sussex. 1979. Organ regeneration from leaf disks of Solanum nigrum, S. dulcamara and S. khasianum. Z. Pflanzenphysiol. 95:355-362.
6. Bonnett, H.T., Jr., and J.G. Torrey. 1966. Comparative anatomy of endogenous bud and lateral root formation in Convolvulus arvensis roots cultured in vitro. Am. J. Bot. 53:496-507.
7. Brossard, F. 1977. Root organogenesis from foliar discs of Crepis capillaris L. Wallr. cultured in vitro: cytochemical and microspectrophotometric analysis. New Phytol. 79:423-429.
8. Brown, S., D.F. Wetherell, and D.K. Dougall. 1976. The potassium requirement for growth and embryogenesis in wild carrot suspension cultures. Physiol. Plant. 37:73-79.
9. Button, J. 1978. The effects of some carbohydrates on the growth and organization of Citrus ovular callus. Z. Pflanzenphysiol. 88:61-68.
10. Chandra, N., T.H. Lam, and H.E. Street. 1978. The effects of selected aryloxyalkanecarboxylic acids on the growth and embryogenesis of a suspension culture of carrot (Daucus carota L.). Z. Pflanzenphysiol. 86:55-60.
11. Chin, J.C., and K.J. Scott. 1977. The isolation of a high-rooting cereal callus line by recurrent selection with 2,4-D. Z. Pflanzenphysiol. 85:117-124.
12. Coleman, W.K., T.J. Huxter, D.M. Reid, and T.A. Thorpe. 1980. Ethylene as an endogenous inhibitor of root regeneration in tomato leaf discs cultured in vitro. Physiol. Plant. 48:519-525.
13. Cornejo-Martin, J.J., A.M. Mingo-Castel, and E. Primo-Millo. 1979. Organ redifferentiation in rice callus: effects

of C_2H_4, CO_2 and cytokinins. Z. Pflanzenphysiol. 94:117-123.

14. Cure, W.W., and R.L. Mott. 1978. A comparative anatomical study of organogenesis in cultured tissues of maize, wheat and oats. Physiol. Plant. 42:91-96.

15. Deambrogio, E., and P.J. Dale. 1980. Effect of 2,4-D on the frequency of regenerated plants in barley and on genetic variability between them. Cereal Res. Commun. 8:417-423.

16. Fonnesbech, M. 1974. Temperature effects on shoot and root development from Begonia x cheimantha petiole segments grown in vitro. Physiol. Plant. 32:282-286.

17. Fridborg, G., and T. Eriksson. 1975. Effects of activated charcoal on growth and morphogenesis in cell cultures. Physiol. Plant. 34:306-308.

18. Fujimura, T., and A. Komamine. 1980. Mode of action of 2,4-D and zeatin on somatic embryogenesis in a carrot cell suspension culture. Z. Pflanzenphysiol. 99:l-8.

19. Fujimura, T., A. Komamine, and H. Matsumoto. 1980. Aspects of DNA, RNA and protein synthesis during somatic embryogenesis in a carrot cell suspension culture. Physiol. Plant. 49:255-260.

20. Gautheret, R.J. 1969. Investigations on the root formation in the tissues of Helianthus tuberosus cultured in vitro. Am. .T. Bot. 56:702-717.

21. Green, C.E., and R.L. Phillips. 1975. Plant regeneration from tissue cultures of maize. Crop Sci. 15:417-421.

22. Guerri, J., F. Cukianez, E. Primo-Millo, and E. Primo-Yufera. 1982. Chromatin changes related to dedifferentiation and differentiation in tobacco tissue culture (Nicotiana tabacum L.). Planta 155:273-280.

23. Habaguchi, K. 1977. Alterations in polyphenol oxidase activity during organ redifferentiation in carrot calluses cultured in vitro. Plant Cell Physiol. 18:181-189.

24. Haccius, B. 1978. question of unicellular origin of non-zygotic embryos in callus cultures. Phytomorphology 28:74-81.

25. Faccius, R., and K.K. Lakshmanan. 1965. Adventiv-embryonen aus Nicotiana-kallus, der bei hohen lichtintensitaten kultiviert wurde. Planta 65:102-104.

26. Xalperin, W. and D.F. Wetherell. 1964. Adventive embryonv in tissue cultures of the wild carrot, Daucus carota. Am. J. Bot. 51:274-283.

27. Huxter, T.J., T.A. Thorpe, and D.M. Reid. 1981. Shoot initiation in light- and dark-grown tobacco callus: the role of ethylene. Physiol. Plant. 53:319-326.

28. Kadkade, P.G., and H. Jopson. 1978. Influence of light quality on organogenesis from the embryo-derived callus of Douglas fir (Pseudotsuga menziesii). Plant Sci. Lett. 13:67-73.

29. Kadkade, P., and M. Seibert. 1977. Phytochrome-regulated organogenesis in lettuce tissue culture. Nature 270:49-50.

30. Kamada, H., and H. Harada. 1979. Studies on the organogenesis in carrot tissue cultures. I. Effects of growth regulators on somatic embryogenesis and root formation. Z. Pflanzenphysiol. 91:255-266.
31. Kamada, H., and H. Harada. 1979. Studies on the organogenesis in carrot tissue cultures. TI. Effects of amino acids and inroganic nitrogenous compounds or somatic embryogenesis. Z. Pflanzenphysiol. 91:453-463.
32. Kamada, H., and H. Harada. 1979. Influence of several growth regulators and amino acids on in vitro organogenesis of Torenia fournieri Lind. J. Exp. Bot. 30:27-36.
33. Kato, Y., and N. Ozawa. 1979. Adventitious bud format-ion on leaf 2nd stem segments of Heloniopsis orientalis grown at various temperatures. Plant Cell Physiol. 20:491-497.
34. Kessell, R.H.J., C. Goodwin, and J. Philp. 1977. The relationship between dissolved oxygen concentration, ATP and embryogenesis in carrot (Daucus carota) tissue cultures. Plant Sci. Lett. 10:265-274.
35. Kochba, J., S. Lavee, and P. Spiegel-Roy. 1977. Differences in peroxidase activity and isoenzymes in embryogenic and non-embryogenic 'Shamouti' orange ovular callus lines. Plant Cell Physiol. 18:463-467.
36. Kochba, J., P. Spiegel-Roy, H. Neumann, and S. Saad. 1978. Stimulation of embryogenesis in citrus ovular callus by ABA, ethephon, CCC and Alar and its suppression by GA_3. Z. Pflanzenphysiol. 89:427-432.
37. Kochba, J., P. Spiegel-Roy, S. Saad, and H. Neumann. 1978. Stimulation of embryogenesis in Citrus tissue culture by galactose. Naturwiss. 65:261-262.
38. Larrieu, C., and V.S. Trippi. 1979. Etude du vieillissement d'Anagallis arvensis L.: action regulatrice de la 1umiere, du saccharose et de l'acide indolyl-acetique sur la capacite rhizogeniqce des feuilles et des rameaux axillaires. Biol. Plant. 21:336-344.
39. Lee, T.T., and F. Skoog. 1965. Effects of substituted phenols on bud formation and growth of tobacco tissue cultures. Physiol. Plant. 18:386-402.
40. Lefebvre, R. 1978. Actions comparees de l'ethylene et de l'ethane sur le bourgeonnement. C.R. Acad. Sci. Paris 287:25-27.
41. Letouze, R., and G. Eeauchesne. 1969. Action d'eclairements monochromatizues sur la rhizogenese de tissus de topinambour. C.R. Acad. Sci. Paris 269:1528-1531.
42. Lu, C.-Y, V. Vasil, and I.K. Vasil. 1981. 1solation and culture of protoplasts of Panicum maximum Jacq. (Guinea grass): somatic embryogenesis and plantlet formation. Z. Pflanzenphysiol. 104:311-318.
43. Mader, M., P. Munch, and M. Bopp. 1975. Regulation und bedeutung der peroxidase-musteranderungen in sprossdifferenzierenden kalluskulturen von Nicotiana tabacum L. Planta 123:257-265.
44. Malfatti, H., J.C. Vallee, E. Perdrizet, M. Carre, and C. Martin. 1983. Acides amines et amines libres

d'explants foliares de Nicotiana tabacum cultives in vitro sur des milieux induisapt la rhizogenese ou la caulogenese. Physiol. Plant. 57:492-498.

45. Matsumoto, H., D. Gregor, and J. Reinert. 1975. Changes in chromatin of Dacus carota cells during embryogenesis. Phytochemistry 14:41-47.

46. Meins, F., Jr., R. Foster, and J. Lutz. 1982. Quantitative studies of bud initiation in cultured tobacco tissues. Planta 155:473-477.

47. Miller, C., and F. Skoog. 1953. Chemical control of bud formation in tobacco stem segments. Am. .J. Bot. 40:768-773.

48. Moiseeva, N.A., A.D. Volodarskii, and R.G. Butenko. 1979. Clarification of tobacco stem meristem marker antigens. Fiz. Rast. 26:479-484.

49. Moncousin, C., and T. Gaspar. 1983. Peroxidase as a marker for rooting improvement of Cynara scolymus L. cultured in vitro. Biochem. Physiol. Pflanzen. 178:263-271.

50. Montague, M.J., J.V. Koppenbrink, and E.C. Jaworski. 1978. Polyamine metabolism in embryogenic cells of Daucus carota. I. Changes in intracellular content and rates of synthesis. Plant Physiol. 62:430-433.

51. Murashige, T. 1963. The role of gibberellin in shoot differentiation in tobacco tissue culture. Pages 321-330 in Proceedings international conference plant tissue culture. P.R. White and A.R. Grove, eds. McCutchan Publ.,Berkeley.

52. Murashige, T. 1977. Manipulaticn of organ initiation in plant tissue cultures. Rot. Bull. Acad. Sin. 18:31-24.

53. Nabors, M.W., J.W. Heyser, T.A. Kykes, and K.J. DeMott-. 1983. Long-duration, high-frequency plant regeneration from cereal tissue cultures. Planta 157:385-391.

54. Negrutiu, I., and M. Jacobs. 1978. Factors which enhance in vitro morphogenesis of Arabidopsis thaliana. Z. Pflanzenphysiol.90:3423-430.

55. Nitsch, C., and B. Xorreel. 1973. Effet d'un choc thermique sur le pouvoir enbryogene du pollen de Datura innoxia cultive dans l'anthere ou isole de l'anthere. C.R. Acad. Sci. Paris 276:303-306.

56. Noma, M., J. Huber, D. Ernst, and R.P. Pharis. 1982. Qyantitation of gibberellins and the metabolism of [H] gibberellin A_1 during embryogenesis in carrot and anise cell cultures. Planta 155:369-376.

57. Norris, R., R.H. Smith, and K.C. Vaughn. 1983. Plant chimeras used to establish de novo origin of shoots. Science 220:75-76.

58. Patel, K.R., and G.P. Berlyn. 1983. Cytochemical investigations on multiple bud formation in tissue cultures of Pinus coulteri. Can. J. Bot. 66:575-585.

59. Paterson, K.E., and T.L. Rost. 1979. Effects of light and hormones on regeneration of Crassula argentea from leaves. Am. J. Bot. 66:463-470.

60, Rajasekaran, K., J. Vine, and M.G. Mullins. 1982. Dormancy in somatic embryos and seeds of Vitis: changes in

endogenous abscisic acid during embryogeny and germination. Planta 154:139-144.

61. Rucker, W., and C. Paupardin. 1969. Action de quelques acides-phenols sur la rhizogenese des tissus de tubercules de topinambour (variete violet de Rennes) cultives in vitro. C.R. Acad. Sci. Paris 268:1279-1281.

62. Seibert, M., P.J. Wetherbee, and D.D. Job. 1975. The effects of light intensity and spectral quality on growth and shoot initiation in tobacco callus. Plant Physiol. 56:130-139.

63. Sengupta, C., and V. Raghavan. 1980. Somatic embryogenesis in carrot cell suspension. 11. Synthesis of ribosomal-RNA and poly(A)$^+$RNA. J. Exp. Bot. 31:259-268.

64. Shabde, M., and T. Murashige. 1977. Hormonal requirements of excised Dianthus caryophyllus I.. shoot apical meristem in vitro. Am. J. Bot. 64:443-448.

65. Shepard, J.F. 1980. Abscisic acid-enhanced shoot initiation in protoplast-derived calli of potato. Plant Sci. Lett. 18:327-333.

66. Skoog, F. 1944. Growth and organ formation in tobacco tissue cultures. Am. J. Bot. 31:19-24.

67. Skoog, F., and C.O. Miller. 1957. Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Symp. Soc. Exp. Biol. 11:118-131.

68. Skoog, F., and C. Tsui. 1948. Chemical control of growth and bud formation in tobacco stem segments and callus cultured in vitro. Am. J. Bot. 35:782-787.

69. Takayama, S., and M. Misawa. 1980, Differentiation in Lilium bulb-scale grown in vitro. Effects of activated charcoal, physiological age of bulbs and sucrose concentration on differentiation and scale leaf formation in vitro. Physiol. Plant. 48:121-125.

70. Thorpe, T.A., and T. Gaspar. 1978. Changes in isoperoxidases during shoot formation in tobacco callus. In Vitro 14: 522-526.

71. Thorpe, T.A., and D.D. Meier. 1972. Starch metabolism, respiration, and shoot formation in tobacco callus cultures, Physiol. Plant. 27:365-369.

72. Thorpe, T.A., and D.D. Meier. 1973. Effects of gibberellic acid and abscisic acid on shoot formation in tobacco. callus cultures. Physiol. Plant. 29:121-124.

73. Thorpe, T.A. and T. Murashige. 1970. Some histochemical changes underlying shoot initiation in tobacco callus cultures. Can. J. Bot. 48:277-285.

74. Tisserat, B., and T. Murashige. 1977. Effects od ethephon, ethylene, and 2,4-dichlorophenoxyacetic acid on asexual embryogenesis in vitro. Plant Physiol. 60:437-439.

75. Tisserat, B., and T. Murashige. 1977. Probable identity of substances in citrus that repress asexual embryogenesis. In Vitro 13:785-789.

76. Tizio, R., R. Saussay, and R. Gautheret. 1970. Action de quelques gibberellines sur la rhizogenese de fragments de rhizomes de topinambour cultives in vitro. C.R. Acad. Sci. Paris 270:2088-2092.

77. Torrey, J.G. 1966. The initiation of organized development in plants. Adv. Morphog. 5:39 – 91.
78. Verma, D.C., and D.K. Dougall. 1977. Influence of carbohydrates on quantitative aspects of growth and embryo formation in wild carrot suspension cultures. Plant Physiol. 59:81 – 85.
79. Walker, K.A., and S.J. Sato. 1981. Morphogenesis in callus tissue of Medicago sativa: the role of ammonium inn in somatic embryogenesis. Plant Cell Tissue Organ Culture 1:109 – 121.
80. Wernicke, W., I. Potrykus, and E. Thomas. 1982. Morphogenesis from cultured leaf tissue of Sorghum bicolor -- the morphogenetic pathways. Protoplasma 11:62-74.
81. White, P.R. 1939. Controlled differentiati-on in a plant tissue culture. Bull. Torrey Bot. Club 66:507 – 513,
82. Wickson, M., and K.V. Thimann. 1948. The antagonism of auxin and kinetin in apical doninanre. Physiol. Plant. 11:62 – 74.
83. Wochock, Z.S., and I.M. Sussex. 1976. Redetermination of cultured root tips to leafy shoots in Selaginella willdenovii. Plant Sci. Lett. 6:185 – 192.
84. Yasuda, T., P.M. Hasegawa, and T.-Y. Cheng. 1980. Analysis of newly synthesized proteins during differentiation of cultured Douglas fir cotyledors. Physiol. Plant. 48:83-87.

THE NEED FOR INTERSPECIFIC CROSSES IN AZOLLA

I. WATANABE

Interspecific crosses are needed in azolla to improve its agronomically important traits such as heat tolerance, biomass yield, insect and fungus resistance, N_2-fixing rate, and P requirement. Because azolla sexual organs are not easily formed (nor have they been artificially induced), sexual crossing has been impossible. Somatic hybridization could solve this problem, but reinoculation of the hybrid with its symbiont Anabaena may still be impossible. Tissue culture and cybrid techniques may facilitate this inoculation.

The water fern azolla can grow in nitrogen-deficient conditions and still have high nitrogen content because of the N_2-fixing activity of its blue-green algae (BGA) symbiont, Anabaena azollae. Azolla, which has long served as an aquatic green manure crop for wetland rice in southern China and northern Vietnam, is now widely used (5). Until the 1970s, the value of azolla in many tropical countries was unnoticed. Since 1975, IRRI has been studying azolla use in the tropics and organizing training courses, workshops, and network trials around it (International Network on Soil Fertility and Fertilizer Evaluation for Rice).

Unfortunately, environmental constraints, such as high temperature and humidity year-round, and severe drought during the dry season, have prevented wide-scale adoption of azolla as a green manure in the Philippines. The success of azolla in the inland area of South Cotabato of Mindanao was unexpected; in 1980 – 1981, azolla was spread across 5,000 ha of rice field without the sophisticated labor-intensive care by farmers (1) it normally requires.

Azolla's high potential as an organic fertilizer cannot be realized without some improvement of such important traits as its heat tolerance, N_2-fixing rate, biomass yield, P requirement, and insect and fungus tolerance.

Azolla has six species; their agronomically important characteristics are summarized in Table 1. No trial to cross different azolla species has been reported, although ideal

Soil microbiologist, International Rice Research Institute, P.O. Box 933, Manila, Philippines,

Table 1. Some characteristics of azolla species.

Azolla species	Heat tolerance	Cold tolerance	Maximum biomass	Sensitivity to P deficiency
A. nilotica	Intermediate	Low	Intermediate	?
A. *pinnata* var pinnata	High	Low	Low	?
A. pinnata var. imbricata	High	Intermediate	Low	Low
A. microphylla	High	Low	Intermediate	?
A. mexicana	High	Low	Low	High
A. caroliniana	Intermediate	Intermediate	Intermediate	High
A. filiculoides	Low	High	High	High
Reference[a]	(4) (9)	(4)	(4) (9)	(7)

[a]Including author's own observation.

candidates for crossing exist: A. filiculoides (because it grows erect) produces high biomass, but is the most sensitive Azolla to heat. A. pinnata is heat tolerant, but its biomass is lower because it is flat. A cross of these two species might obtain heat-tolerant, erectly growing (and therefore high-biomass) plants.

There are, however, technical difficulties. First, the sexual organs, microsporocarps and macrosporocarps, are not easily formed. It is not known how to induce sporocarps artificially. Somatic hybridization may permit wide crosses in azolla; however, even when somatic hybridization and regeneration of cybrids are possible, another obstacle remains. Unlike in rhizobia-legume symbiosis, there has been no report of successful reinoculation of Anabaena to BGA-free azolla. Laboratory-grown A. azollae strains supposedly isolated from azolla are not likely to be symbiont. Immunological studies showed no cross reaction between A. azollae isolated from azolla and laboratory-grown A. azollae (3).

A. azollae cells accompany host cells from sporocarps to sporophyte (2). During the macrospore germination, Anabaena cells in macrosporocarps may be trapped by the growing shoot apex. In vegetatively growing azolla, Anabaena cells are trapped in the youngest leaf meristem and embedded in the leaf cavity very early in development (6).

This suggests young leaf tissue can initiate symbiosis with Anabaena cells. Hence, it is not unrealistic to assume that young embryos from protoplasts or cybrids might initiate symbiosis with free-living Anabaena. Because high temperature inhibits heterocyst development and N_2-fixation, heat tolerance may be an important character of symbiotic algae, but not of the host plant (8). Artificial symbiosis between nonhomologous host and algae may, then, change temperature response.

Tissue culture and cybrid techniques may provide ways to 1) wide-cross azolla species, and 2) artificially combine host and symbiont algae.

REFERENCES CITED

1. Kikuchi, M., I. Watanabe, and L.D. Haws. Economic evaluation of Azolla use in rice production. Pages 569-592 in Organic matter 2nd rice. International Rice Research Institute, Los Banos, Philippines.
2. Konar, R.N., and R.K. Kapoor. 1974. Embryology of Azolla pinnata. Phytomorphology 24:228-261.
3. Ladha, J.K., and I. Watanabe. 1982. Antigenic similarity among Anabaena azollae separated from different species of Azolla. Biochem. Biophys. Res. Comun. 109:675-682.
4. Li, Z, X., S.X. Zu, M.F. Mao, and T.A. Lumpkin. 1982. Studies on the utilization of 8 Azolla species in agriculture. I. Shangguo Nongye, Kexue 1982-1:19-27.
5. Lumpkin, T.A., and D.L. Plucknett. 1982. Azolla as green manure: Use and management in crop production. West View Trop. Agric. Ser. 5.
6. Peters, G.A., R.E. Toia, Jr., D. Raveed, and N.J. Levine. 1978. The Azolla Anabaena azollae relationship. VI. Morphological aspects of the associations. New Phytol. 80:583-593.
7. Subudhi, B.P.R., and I. Watanabe. 1981. Differential phosphorus requirement of Azolla species and strains in phosphorus limited continuous culture. Soil Sci. Plant Nutr. 27:237-247.
8. Tung, H.F., and I. Watanabe. 1983. Differential response of Azolla-Anabaena associations to high temperature and minus phosphorus treatments. New Phytol. 93:423-431.
9. Watanabe, I., and N.S. Berja. 1983. Growth of four species of Azolla as affected by temperature. Aquat. Bot. 15:175-185.

MOLECULAR BIOLOGY AND GENETIC ENGINEERING

MOLECULAR AND BIOCHEMICAL TECHNIQUES IN PLANT BREEDING

R. B. FLAVELL

Some applications of molecular biology to plant breeding are discussed. The complement of repeated DNA sequences in a genome often differs considerably even between closely related species. These sequences are thus useful probes for detecting DNA incorporated from one species into another in wide crossing programs. An example is given in which a repeated DNA sequence cloned from rye chromosomes is used in a hybridization assay on squashed root tips, to distinguish wheat plants carrying the short arm of a rye chromosome from pure wheat genotypes. A similar assay has been devised to distinguish male-sterile and fertile cytoplasms of maize. The use of DNA sequences for mapping chromosomes is highlighted. Chromosome maps constructed using phenotypic, biochemical, and DNA markers are likely to be useful in providing markers closely linked to agronomically important genes. New markers will be created by the insertion of DNA sequences from the test tube into plants. DNA copies of RNA viral genes have been constructed and used as probes to detect viruses in sap samples. This technique is in routine use in a potato breeding program to identify lines resistant to viral infections.

The handling of individual plant genes in test tubes is in its infancy. However, our knowledge about plant gene and chromosome structure is expanding so rapidly that there is every reason for confidence that over the next two decades molecular biology will impinge significantly on plant breeding.

Molecular biology is concerned with gene isolation, detailed analysis of gene structure and regulation, the directed modification of genes, and the insertion of new genes into plants. The methods are extremely powerful and are already turning over many of our long-held beliefs about gene and chromosome structure. Still, the subject is very young and few molecular biologists have addressed the opportunities or needs of the plant breeder.

Plant Breeding institute, Trumpington, Cambridge CB2 2LQ. England.

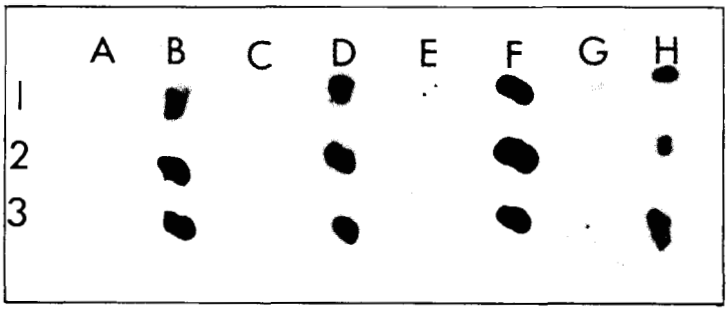
In 1984, we cannot expect that the complex, polygenically controlled traits handled by the plant breeder be analyzed or manipulated by the molecular biologist. However, research with purified DNA has already opened up specific opportunities for plant breeders and geneticists. In this short paper I would like to describe some opportunities we have developed at the Plant Breeding Institute. These serve not only as examples useful today, but also as examples of approaches for international institutes in the future.

SCREENING GERMPLASM USING PURIFIED DNA SEQUENCES AND ELECTROPHORESIS

Plant breeders often have sufficient variation within a species to solve many problems, but screening their germplasm for desirable genotypes is often difficult, time consuming, and expensive. When chromosomal segments from an alien species are incorporated into a breeding program, such as expected in the maize-tripsacum wide cross program at Centro Internacional de Mejoramiento de Maiz y Trigo, purified repeated sequences from alien species can detect the alien DNA. The reason is that plant genomes usually have thousands or millions of species-specific repeats dispersed throughout the DNA (6). We have characterized many repeated DNA sequences in rye not found in wheat (2). These sequences are useful for detecting rye chromosomal segments in wheat and triticale (2, 8, 14).

Many European wheat cultivars and, of course, the material in wheat breeding programs, contain the short arm of chromosome IR^S from rye (23), which has been selected by breeders because of its beneficial disease resistance alleles (23). Several breeders now also wish to develop elite material with a complete wheat chromosome 1B instead of the rye DNA. They wish to reduce the uniformity of these particular disease resistance alleles in agriculture. Also, the rye DNA may reduce grain breadmaking quality. To identify material lacking the DNA segment by conventional procedures is not easy. We have therefore developed a simple "squash dot" procedure, using a purified rye repeated DNA sequence as the probe (13). Root tips of the germinating seeds are squashed onto nitrocellulose filter paper and the released DNA is denatured and baked onto the paper. The paper is then incubated with the ^{32}P-labeled DNA probe under conditions that allow hybridization of the probe to any rye DNA extruded from each root tip. Hybridized probe DNA is detected by placing the filter paper next to X-ray film. Results on some progeny from a cross of a wheat + $1R^S$ x wheat are shown in Figure 1. The screening test is easily applied to hundreds or thousands of plants and the results are obtained in a few days. The desired plants can then be selected for planting.

A similar test for distinguishing the different male-sterile cytoplasms of maize has been developed (13, 7). The classical test is to cross the relevant plant as female with tester stocks that contain known nuclear restorer genes. Each cytoplasm can be distinguished by its response to a specific restorer gene. The



1. Recognition of wheat lines carrying the short arm of rye chromosome 1R by the 'squash dot' method.

Three root tips (1-3) from each seed of progeny from a cross between wheat containing the short arm of rye chromosome 1R x a pure wheat line were squashed onto nitrocellulose paper. The extruded DNA was denatured by floating the paper on 0.5 M NaOH for 5 min, neutralized in 1M Tris-HCI for 5 min, then in 1.5 M NaCI, 0.5 M Tris-HCI pH 7.5 for 5 min. The filter was then baked at 80°C for 2 h (13).

The filter was then incubated at 60°C for 16 h 5XSSC (SSC = 0.15 M sodium chloride 0.0015 M sodium nitrate) 5 x Denhardt's solution, 30 mM sodium phosphate pH 6.5 and denatured salmon sperm DNA (200µg/ml) containing 10^6 cpm/ml ^{32}P labeled repeat DNA cloned from rye. After hybridization, the filter was washed with 2XSSC, 1XSSC, and 0.5XSSC at 60°C for 1 h each wash. Filters were dried and exposed to X ray film (13).

A = root tips from wheat

B-H = progeny root tips from the cross

progeny are then grown to observe pollen production, so the test takes two generations. In contrast, the "squash dot" procedure takes a few days to score even thousands of plants. The squashed root tips with the different cytoplasms are hybridized with a purified DNA sequence present in many copies in the mitochondrial genomes of S cytoplasm, in two copies in the mitochondrial genomes of the fertile N cytoplasm, but absent from T and C cytoplasms. These cytoplasms can also be distinguished by purifying the mitochondrial DNA, treating it with a restriction endonuclease, and separating the fragments by electrophoresis (15, 4). The fragment pattern is different for each cytoplasm.

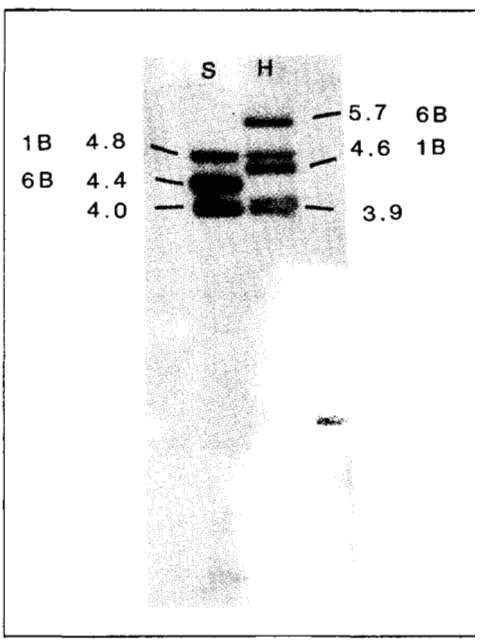
From these examples it is clear that rapid screening procedures can be devised using DNA sequences. Whenever the genetic differences to be detected involve repeated DNA sequences differing in concentration between genotypes, then the "squash dot" is applicable. To develop a "squash dot" test, it is necessary to identify an appropriate repeated sequence and purify a copy of it by molecular cloning.

Using purified DNA sequences is not the only way of screening germplasm rapidly: the rapid screening of proteins by polyacrylamide and starch gel electrophoresis has been available for many years (22).

If there is variation at a locus (a "reporter" locus) easily screened by any method in large populations, and this locus is tightly linked to a gene controlling a phenotypic trait the breeder finds difficult or cumbersome to score, then the

"reporter" locus is a useful marker. Most of the plants selected by the reporter gene will also carry the desired allele. This kind of opportunity illustrates the need to develop as many screening techniques for as many loci as possible in our crop species.

The numbers of easily assayable proteins that show frequent genetic variation is insufficient to provide reporter loci for all regions of the chromosomes. There is much more variation in the DNA, however. Many base changes, small insertions, or deletions in and around genes cannot be detected in any phenotypic character. These differences are often recognized easily by digesting the DNA with a restriction endonuclease, fractionating the DNA pieces by electrophoresis, transferring the DNA to nitrocellulose, and hybridizing with a specific radioactive probe to identify the sequences at the locus in question. Variation at the Nor (Nucleolus organizer) locus on wheat chromosomes 1B and 6B is shown in Figure 2. The variation in DNA



2. Variation at a gene locus revealed by restriction endonucleases.

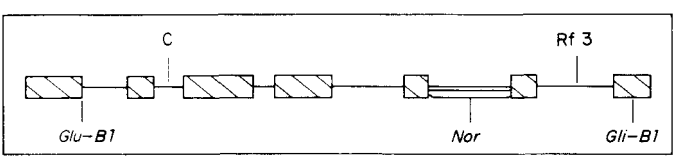
DNA was extracted from two wheat varieties, Sportsman (S) and Highbury (H), and cleaved with the restriction endonucleases EcoRI and Bam HI. The fragments were separated by electrophoresis through agarose and transferred to nitrocellulose (21). They were then hybridized with ^{32}P-labeled ribosomal DNA purified by molecular cloning. The excess labeled DNA was removed by washing and the wheat rDNA fragments were revealed by autoradiography (2). The numbers represent the length of the DNA fragments in kilobase pairs. The designation of the bands to the *Nor* loci on chromosomes 1B and 6B was concluded from a separate genetic analysis.

fragment length depicted in the figure is due to different amounts of DNA in a regulatory region of the gene. The potential for obtaining many markers covering the whole genome by detecting DNA fragment length polymorphisms in any crop is very great. One of this method's drawbacks for screening large numbers of plants is the investment necessary to make DNA from each plant. Rapid methods enabling one person to purify 30-40 DNA samples per day have recently been devised.

This method was used to map the Nor locus (containing the ribosomal DNA) relative to other loci specifying other phenotypic and biochemical markers and permitted the physical mapping of wheat chromosome 1B (Fig. 3; 18, 20). At the Gli-B1 and Glu-B1 loci variation between genotypes is extensive and the gliadin and glutenin proteins can be assayed by simple polyacrylamide gel electrophoresis of endosperm segments (19). The method is therefore useful for distinguishing plants carrying particular alleles tightly linked to the Gli-B1 or Glu-Bl loci. Many more genes are being mapped onto this arm (unpubl.). When many easily screened DNA, protein, or morphological markers are available, to find one tightly linked to agriculturally important loci on this arm should not be too difficult. Electrophoretic analysis of Gli-B1 and other alleles on this arm can substitute for the rye repeated DNA sequence in detecting the rye or wheat 1B chromosome arms (Fig. 1).

There is obvious merit for future plant breeding in having very detailed chromosome maps with DNA and protein markers, but we will not have to rely on the markers from natural variation. It will be possible to insert into plant chromosomes DNA sequences that specify selectable or screenable phenotypic traits. For example, antibiotic resistance genes have already been inserted into tobacco plants (3, 10, 12), and such plants can easily be recognized by their ability to survive high antibiotic concentrations. Many different selectable markers could be inserted into our elite germplasm at various sites, closely linked to genes critical for consistent, efficient crop performance.

As research progresses, specific gene products responsible for valuable plant traits will be recognized. Easier germplasm screening procedures will probably result. One example of this is the correlation between specific glutenin subunits in wheat grains and the viscoelasticity of dough (17). Combined genetic, biochemical, and dough-making analyses established the correlations. Wheat breeders attempting to improve breadmaking



3. Physical map of the short arm of the wheat chromosome 1B.

The *Gli-B7* and *Glu-67* loci specify gliadin and glutenin genes respectively (18). Nor = nucleolus organizer and contains the genes for ribosomal RNAs. Rf = restorer gene of cytoplasmic male sterility (20). C = centromere. The hatched areas depict the sites of heterochromatin, which are made up, at least in part, of tandem arrays of repeats (5).

quality can now choose parents on the basis of rapidly determined glutenin subunit patterns (17, 9). They can also screen large numbers of progeny by electrophoresis to select those with the most favorable combinations of subunits. These opportunities are of real benefit to grain improvement programs.

SCREENING GERMPLASM FOR PRESENCE OF VIRUSES AND VIROIDS

Little needs to be said to emphasize the losses viruses and viroids can cause in crops, especially in those propagated vegetatively. Many breeding programs, therefore, seek genotypes resistant to viruses, and adopt management practices that reduce the possibility of viruses' spreading to commercial lines. Plant breeders' most common assay for viruses is to grow the plant and look for the characteristic virus symptoms. This method is not always reliable, and it is time consuming and expensive. In our potato breeding program, resistance to potato virus X, potato virus Y, and potato leaf roll virus is required, so assays for all three viruses are included in the program. The assay for virus X is simple and can be applied to the large number of clones handled in the second year of the program. The assays for virus Y and leaf roll, however, are much more demanding and are therefore used later, when the number of clones has been reduced to a few hundred. The clones to be assayed are planted close to infected plants to ensure that aphids transmit the viruses to representatives of each clone. In the following year, three tubers from four representative plants of each clone are grown and scored for viral symptoms. Our objective has been to devise an assay technique that would shorten the test period to 1 year.

The technique developed involves detecting viral RNA in sap samples on nitrocellulose and is similar to the method used to detect rye chromosome 1R DNA in Figure 2 (13, 1). It was first necessary to synthesize a probe nucleic acid molecule. DNA copies of the viral RNA molecules were prepared using reverse transcriptase and the RNA/DNA hybrids separated. A double-stranded DNA molecule was constructed from the single DNA strand, using DNA polymerase, and the DNA molecules were ligated into a plasmid and transformed into *E. coli* (1). Replication of the plasmid in *E. coli* cultures produced a large supply of DNA complementary to the viral RNA. This DNA is radioactively labeled and used to detect viral RNA in potato sap by the method outlined in Figure 2. The results from this test agree with those from visual inspection of the plants and from an enzyme-linked immunosorbent assay (ELISA) which uses an antibody to detect the viral protein (11).

Only small volumes of sap are required for the new testing procedure, so sap from tubers, sprouts, or leaves can be extracted rapidly. Transfer of the sap to nitrocellulose is simple and 80-150 samples per hour can be processed. Thereafter, all the samples are handled together on one or a few sheets of nitrocellulose and the screening of hundreds of plants for three different viruses need only take a few days. The positive and

negative results appear similar in format to those shown in Figure 1. The radioactive "sap spot" assay is very sensitive. About 2 ng virus can be detected by autoradiography overnight. With a longer time for autoradiography, considerably less virus can be detected. This procedure requires fewer manipulations and less sap than ELISA, and the graphic answer is interpreted more rapidly. Furthermore, it is possible to make clones of complementary DNA (cDNA) to viruses that have not been purified or characterized.

The "sap spot" test should make virus detection more efficient and less costly. Because many more plants can be surveyed easily, virus resistance could be sought early in a breeding program, when genetic variation is still large. A nondestructive rapid assay should make it easier for plant breeders to establish whether material in yield trials is in fact virus-free, and should also enable batches of seed potatoes to be more reliably certified as virus free. It should facilitate rapid assessment of imported germplasm; at present this germplasm is held in quarantine and has to be proved virus free before it is released to breeders. This kind of method can also detect viroids in potatoes. Owens and Diener (16) have shown that, with DNA copies of potato spindle tuber viroid, the viroid can be conveniently detected in sap samples. These sets of methods, emerging from molecular biology, are ready for use in plant breeding programs.

REFERENCES CITED

1. Baulcombe, D., R.B. Flavell, R.E. Boulton, and G.J. Jellis. 1984. The sensitivity and specificity of a rapid nucleic acid hybridisation method for the detection of potato virus X and other plant RNA viruses in crude sap samples. Plant Pathol. 33:361-370.
2. Bedbrook, J.R., J. Jones, M. O'Dell, R.D. Thompson, and R.B. Flavell. 1980. A molecular description of telomeric heterochromatin in Secale species. Cell 19:545-560.
3. Bevan, M.W., R.B. Flavell, and M.D. Chilton. 1983. A chimaeric antibiotic resistance gene as a selectable marker for plant cell transformation. Nature 304:184-187.
4. Borck, K.S., and V. Walbot. 1982. Comparison of the restriction endonuclease digestion patterns of mitochondrial DNA from normal and male sterile cytoplasms of Zea mays L. Genetics 102:109-128.
5. Dennis, E.S. W.L. Gerlach, and W.J. Peacock. 1980. Identical polypyrimidine polypurine satellite DNA in wheat and barley. Heredity 44:345-366.
6. Flavell, R.B. 1980. The molecular characterisation and organisation of plant chromosomal DNA sequences. Annu. Rev. Plant Physiol. 31:569-596.

7. Flavell, R.B., R.J. Kemble, R.E. Gunn, A. Abbott, and D. Baulcombe. 1983. Application of molecular biology in plant breeding: the detection of genetic variation and viral pathogens. Pages 198-209 in Better crops for food. Ciba Foundation, London.

8. Flavell, R.B., M. O'Dell, J. Raimpau, and D.B. Smith. 1978. Biochemical detection of alien DNA incorporated into wheat by chromosome engineering. Heredity 40:439-455.

9. Flavell, R.B., P.I. Payne, R.D. Thompson, and C.N. Law. 1984. Strategies for the improvement of wheat grain quality using molecular genetics. In Biotechnology and genetic engineering. Vol. 2. G.W. Russell, ed. Intercept Press, Newcastle, England. (in press)

10. Fraley, R.T., S.G. Rogers, R.B. Horsch, P.R. Sanders, J.S. Flick, S.P. Adams, M.L. Bittner, L.A. Brand, C.L. Fink, J.S. Frey, G.R. Galluppi, S.B. Goldberg, N.L. Hoffman, and S.C. Woo. 1983. Expression of bacterial genes in plant cells. Proc. Natl. Acad. Sci. USA 80:4803-4807.

11. Gugerli, P. 1979. Le immuno enzymatique (ELISA) et son application pour le diagnostique rapide des viroses de la pomme de terre. Rev. Suisse Agric. 11:253-260.

12. Herra-Estrella, L., M. De Block, E. Messens, J.-P. Hernalsteens, M.J. van Montagu, and J. Schell. 1983. Chimeric genes as dominant selectable markers in plant cells. EMBO J. 2:987-995.

13. Hutchinson, J., A. Abbott, M. O'Dell, and R.B. Flavell. 1984. A rapid screening technique for the detection of repeated DNA sequences in plant tissues. Theor. Appl. Genet. (in press)

14. Jones, J.D.G., and R.B. Flavell. 1982. The mapping of highly repeated DNA families and their relationship to C bands in chromosomes of Secale cereale. Chromosoma (Berl) 86:595-612.

15. Kemble, R.J., R.E. Gunn, and R.B. Flavell. 1980. Classification of normal and male-sterile cytoplasms in maize. II. Electrophoresis analysis of DNA species in mitochondria. Genetics 95:451-458.

16. Owens, R.A., and T.O. Diener. 1981. Sensitive and rapid diagnostic of potato spindle tuber viroid disease by nucleic acid hybridisation. Science 213:670-672.

17. Payne, P.I., K.G. Corfield, L.M. Holt, and J.A. Blackman. 1981. Correlation between the inheritance of certain high molecular weight subunits of glutenin and breadmaking quality in progenies of six crosses of bread wheat. J. Sci. Food Agric. 32:51-60.

18. Payne, P.I., L.M. Holt, J. Hutchinson, and M.D. Bennett. 1984. Development and characterisation of a line of bread wheat; Triticum aestivum which lacks the short arm satellite of chromosome 1R and the Gli-B1 locus. Theor. .Appl. Genet. 68:327-334.

19. Payne, P.I., L.M. Holt, E.A. Jackson, and C.N. Law. 1983. Wheat storage proteins: their genetics and their potential for manipulation by plant breeding. Philos. Trans. R. Soc. London, Ser, B 304:3549-3571.

20. Snape, J.W., R.B. Flavell, M. O'Dell, W.G. Hughes, and P.I. Payne. 1984. Intrachromosomal mapping of the nucleolar organiser region relative to three marker loci on chromosome 1B of wheat (Triticum aestivum). Theoret. Appl. Genet. (in press)
21. Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Molec. Biol. 98:503-517.
22. Tanksley, S.D., and J.J. Orton. 1983. Isozymes in plant genetics and breeding. Elsevier, The Netherlands.
23. Zeller, F.J., and E. Fuchs. 1983. Cytologie und Krankheitsresistenz einer IA/lR und mehrerer 1B/1R-Weizen-Roggen-Translokationssorten. Z. Pflanzen-zuecht. 90:285-296.

APPROACHES TO THE TRANSFORMATION OF CROP PLANTS

W. L. GERLACH, E. S. DENNIS, and W. J. PEACOCK

Transfer DNA (T-DNA) can transform dicotyledonous plants, but a system for transforming monocotyledonous species has not been developed. Monocotyledon transformation is being attempted with methods similar to those established for yeast, Drosophila, and animals. Direct transformation or microinjection of naked DNA is being tested. It requires cells capable of regeneration, or specialized recipient cells such as germinating pollen or developing ovules. Plant protoplast-bacterial spheroplast fusion may also provide a delivery system, but requires appropriate monocot cell culture systems. We are adapting transposable genetic elements from maize for transforming DNA into plant genomes.

Gene transfer requires the ability to isolate specific genes. Conventional approaches have relied upon cDNA cloning. New methods to isolate genes follow isolation of small amounts of protein, or use "transposon mutagenesis" for genes of which we can only monitor phenotype.

Understanding gene expression is important for the developmental regulation of transformed genes. In animal genes, "enhancers" and "promoters," oligonucleotide sequences that regulate and induce gene activity, have been characterized. Plant genes are structurally similar to animal genes, but nothing is known of their control.

The recent knowledge explosion in plant molecular biology stands ready for exploitation in the genetic engineering of major crops. It will provide the plant breeder with novel phenotypes and genetic material. It will break species barriers and even introduce genes from animals and bacteria into plants. Isolated genes can already be mutated in vitro and reintroduced into plants. The introduction of the new or modified genetic information will be rapid and will not suffer the coincident introduction of undesirable genetic backgrounds that currently occurs with, for example, wide crossing breeding programs.

Division of Plant Industry, Commonwealth Scientific and Industrial Research Organization, G. P. O. Box 1600, Canberra, A. C. T., Australia 2601.

However, the regular application of this technology to plant breeding requires

1. isolation of genes for specific characters,
2. reintroduction of genes into plant species, and
3. understanding of the mechanisms of gene regulation.

In this paper we have outlined current work on these aspects, emphasizing the experimental approaches of our laboratory. We discuss gene-transfer techniques already developed in animals, yeast, and Drosophila, and the T-DNA system used with dicotyledons.

DETECTION AND ISOLATION OF SPECIFIC GENES

The traditional approach to molecular isolation of plant genes has been to prepare cDNA clones from the cell types in which the gene is abundantly expressed. For example, seed storage protein genes from several plant species have been isolated initially as cDNA from developing endosperm in which their transcripts constituted the majority of mRNA (28, 39). It has been straightforward to prepare clones and identify them by hybridization-release-translation. Other abundant messages so cloned have included those for genes encoding the photosynthetic proteins for small subunit Fraction 1 of ribulose-bisphosphate-carboxylase (1).

Another method used to isolate tissue or developmental-specific genes uses differential hybridization to form clones from mRNA of tissues in which the desired gene is being expressed. We have used this approach to isolate the Adhl, Adh2, and other anaerobically induced genes in maize (17). cDNA clones were isolated from anaerobically treated maize seedling roots in which ADH protein and mRNA were induced. The anaerobic-specific genes were better detected by the greater hybridization with a cDNA probe from anaerobic tissue mRNA, than by the hybridization level of a cDNA from aerobic tissue mRNA. Hybridization-release-translation was used to identify the Adh1 gene.

While these approaches are useful for cloning genes expressed and represented at reasonable levels in messenger RNA populations, they are not suitable for isolating genes encoding rare messages or those whose protein product we do not know. For such genes we need new approaches.

An extension of traditional cDNA cloning that can be used for rare cDNAs is "RNA subtraction." The technique involves preparing first strand cDNA using an mRNA population from a tissue in which the desired gene is being expressed. If this cDNA population is then hybridized with excess mRNA from tissue in which the gene is not expressed, we can "subtract" the bulk of the sequences as cDNA-mRNA hybrids. cDNAs that do not hybridize with the mRNA are then cloned as a basis for isolating the specific gene. An example of this approach is found in Hedrick et a1 (21).

Another method produces nucleic acid primers using amino acid sequence information from the protein encoded by the gene to he isolated. Of course, this method requires the ability to isolate

small amounts of the proteins. If an oligonucleotide tract of 5-6 consecutive amino acids can be identified for the particular protein, then, from knowing the genetic code and something about eukaryote codon usage, a suitable 15-18 base oligonucleotide tract encoding that protein can be synthesized. The sequence can be radioactively labeled and used to probe cDNA or gene libraries for the gene sequence. This approach is increasingly used and through it many animal genes have been cloned (5).

An alternative approach, applicable when small amounts of protein from the desired gene can be isolated, involves production of a monoclonal antibody specific to the protein. RNAs from tissues in which the gene is expressed are used to prepare cDNA libraries and then inserted into *Escherichia coli* expression vectors. Libraries of transformed *E. coli* colonies can be immunologically assayed using the antibody preparation. Colonies expressing the desired gene's cDNA as a protein product can thereby be detected and the clone subsequently purified (22).

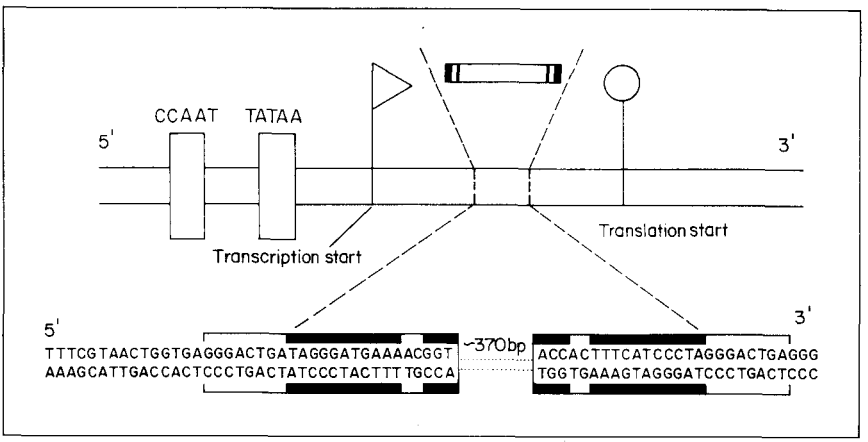
Finally, transposable genetic elements may serve as molecular tags to isolate eukaryotic genes just as "transposon mutagenesis" has been used to isolate bacterial genes. This approach should permit the isolation of genes about whose transcription or translation products we know nothing but whose phenotypic changes caused by DNA insertion we can detect. The strategy uses a transposable element to mutate the locus we are seeking. If the transposable element does mutate the locus, and this must be checked genetically in any recovered mutant, the cloned transposable element can serve as a probe to isolate the mutated gene from a genomic library of the mutant line. The sequences surrounding the transposable element in the clone will include the desired gene. This method has already isolated eukaryotic genes; the first was the *white* eye locus of *Drosophila melanogaster* (2). With the recent cloning of Ac-Ds (Fig. 1) and Mu elements from maize (12, 14, 29, 38, 40), we have plant-transposable elements adaptable for these purposes.

TRANSFORMATION IN EUKARYOTES

Molecular transformation of plants

The T-DNA system is the only systen available for transforming plants (see 6, 7 for reviews). It uses the tumor-inducing plasmid of the soil bacterium *Agrobacterium tumefasciens*, the agent responsible for crown gall disease in dicotyledonous plants. A portion of the Ti plasmid called T-DNA induces tumor formation and is found transferred to the plant genome in established tumors. This T-DNA has been manipulated in vitro to remove the tumor-causing genes and insert foreign DNA. Modified Ti plasmids of this type can serve as vectors for transferring foreign genes into plants and have already been used to transfer and express bacterial and heterologous genes in dicots.

However, the T-DNA system is restricted to dicot plants. Whether it can be adapted for transformation of monocots (wheat, rice, maize and other major crop species) remains to be seen.



1. Features of the Ds controlling element sequence insertion in the *Adh1-FM335* mutation of maize. The progenitor *Adh*1 allele (top line) has 100 bases of 5' noncoding DNA. TATA and CCAAT are seen upstream of the transcription start site. The Ds element within the dotted lines is a 405 bp insertion sequence which is flanked by a duplication of nucleotides +38 to +45, presumably formed during its integration. It has characteristic termini composed of 11 bp and 3 bp inverted repeat sequences separated by a 2 bp direct repeat as shown. Internally the Ds element is 78% AT. We have isolated other similar Ds elements from maize and it appears that there is a family of these Ds insertion sequences in all lines of maize and its wild relatives.

Approaches to monocot transformation

Direct plant transformation using DNA is being attempted. That naked DNA can transform plant protoplasts has been demonstrated by poly-L-ornithine and Ca^{++}/PEG transformation of petunia and tobacco with Ti plasmid DNA (13, 26). Its efficiency is low, less than 10^{-4}; and application of direct DNA transformation to monocots awaits the development of efficient protoplast regeneration systems. Efficient, direct transformation of animal cell cultures has been developed. In these experiments the DNA incorporates into apparently random genome sites, often in multiple copies. In yeast transformation, on the other hand, sequence homology is required for inserting DNA into the genome (41). Therefore, repetitive DNA, or rRNA genes in yeast, greatly enhances transformation efficiency by extending the target size.

Microinjection of DNA into embryos has proven a very efficient means to transform Drosophila (31, 36) and mice (9, 19). DNA-injected Drosophila embryos yield a high proportion of progeny containing the transforming DNA stably incorporated into their genome. Microinjection of DNA into the pronuclei of fertilized mouse eggs, then reimplantation of these embryos into surrogate mothers, also produces many stably transformed offspring. In Drosophila transformation, gene location may not be very important in control of gene expression development. Different transformants, which have genes incorporated at different chromosomal sites, seem to retain tissue regulation of gene expression (18, 33, 37). However, chromosomal location may affect gene activity when genes are introduced into mammals (see 15).

An essential component in the efficiency of Drosophila transformation has been the transposable P-element. P-elements are natural Drosophila transposons capable of excising and inserting into DNA of Drosophila strains that lack them. Complete and defective P-element DNAs have been characterized (27).

A number of transposable genetic elements cloned from maize may prove similarly useful for efficient monocot transformation. As mentioned previously, Ac and Ds elements from the Ac-Ds controlling element system have been cloned (12, 14, 29, 40) and characterized; Mu (38) and Cin (34) elements have also been cloned. These elements all have some features in common with other eukaryotic transposable elements. They have variable length and inverted repeat sequences at their termini. Indeed, little or no internal homology appears between different families of Ds-controlling elements, and the short terminal inverted repeats seem to be the only sequences required for transposition in response to the Ac activator element. In our laboratory, potential vector molecules based on these sequences are being constructed.

Another approach to DNA delivery into monocot cells is the fusion of protoplasts with bacterial spheroplasts containing the genes to be introduced. This method has been successful in introducing Ti-DNA into dicots (20): *Vinca rosea* protoplasts were transformed with Ti-DNA by fusion with *A. tumefasciens* spheroplasts. In the absence of a suitable monocot protoplast system capable of regeneration, an available maize protoplast line (8) can be developed for assays. We are attempting to introduce maize genes and other constructs into these cells by direct DNA transformation and bacterial spheroplast fusions. Using maize *Adh1* has value in that it is a homologous system involving a gene about whose sequence, expression, and developmental control we know much.

Yet another approach for transforming monocots is the introduction of DNA by pollen uptake. Pollen takes in macromolecules (23, 24) and its transformation using DNA has been suggested although tests for the presence of transforming DNA could not be undertaken in these experiments. The procedure in our laboratory involves in vitro germination of Adh^- null pollen in DNA containing a cloned $ADH1^+$ gene. The germinated pollen is used to fertilize plants that are $Adh1^-$ null, and the progeny are tested for *Adh* activity. Optimal conditions for maize pollen germination and maintenance of intact pollen tubes on artificial media have been determined. Conditions that facilitate and optimize DNA uptake into germinated pollen are being investigated.

Transformation of cotton by microinjection of DNA into the area of newly fertilized ovules has been reported (43). However, it has relied upon the recipient line's acquiring new phenotypic characters, and requires defined DNA segments for unequivocal demonstration. Monocot transformation may also be achieved by microinjection of DNA into egg cells, especially in those species where fertilized ovules can be dissected and cultured (16).

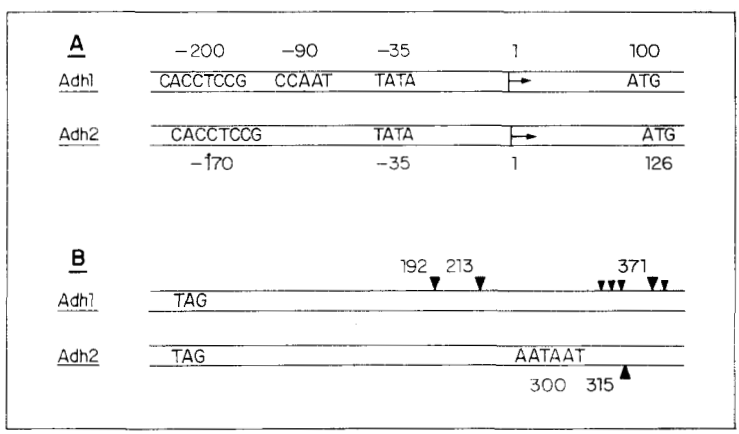
EXPRESSION OF PLANT GENES

A number of plant genes have now been cloned as both cDNA and entire genomic sequences. For many of them full sequence information has been determined and analyzed for clues about signals responsible for transcriptional activity (expression) and developmental control of the genes.

Many plant genes appear similar in expression to animal genes: they have TATA and CCAAT boxes in positions -30 and -80 bp upstream of the transcription start point. These sequences have functional activity in many animal genes, determining the actual transcription start point and, to some extent, gene activity. We have determined the full sequences of the Adh1 and Adh2 genes of maize (10, 11). Both have TATA boxes but only the Adh1 gene has a CCAAT box sequence (Fig. 2).

Structural features of introns in plant genes appear similar to those in animal genes, with GT ...AG boundaries being invariant. The consensus sequences at splice junctions have been determined in detail for maize Adh1 (10) and bean phaseolin (35), and are similar to animal intron counterparts. The maize Adh1 and Adh2 genes each have nine introns at the same locations in the coding sequence, indicating that they evolved from a common ancestral gene (11).

Polyadenylated RNAs make up the majority of plant mRNA populations. Polyadenylation may facilitate mRNA movement from nucleus to cytoplasm or provide for mRnA stability. As in animal genes, the polyadenylation site in many plant genes has the sequence AATAAA or a near relative of it 10-20 bp upstream. By analogy with animal genes, this signal may be a determinant for the polyadenylation site except that, not all plant genes have it. In the maize Adh2 gene, the sequence is present 15 bp upstream of the single polyadenylation site. However, in the maize Adh1 gene



2. A comparison of 5' and 3' noncoding regions of the *Adh1* and *Adh2* genes of maize. The features shown are: A) 5' region (numbers refer to nucleotide positions relative to transcription start point +1) and E) 3' regions showing major (▼) and minor (▾) polyadenylation sites, Numbers refer to nucleotide positions beyond termination codon.

where mapping has precisely located a number of polyadenylation sites (32), AATAAA or any other consensus sequence cannot be found upstream of these sites. To determine the polyadenylation signal in the Adh1 gene, we need a transformation or accurate in vitro transcription system for mutation analysis.

Nothing is known about the developmental regulation of these plant genes. For a number of animal genes, sequences responsible for tissue-specific or developmental control have been determined. The signal sequences are oligonucleotides usually located in the immediate 5' vicinity of the structural gene. For example, in Drosophila the response of the heat shock genes is due to a CT-GAA-TTC-AG sequence located 50-300 bp upstream of each gene. That this oligonucleotide sequence is the sole requirement for induction by heat shock was demonstrated by chemically synthesizing the sequence, locating it in front of a thymidine kinase gene, and transforming the engineered gene into Drosophila. This modified gene was then found, to be environmentally controlled by heat shock (30). The metallothionin promoter sequence has also been characterized in animals. In response to heavy metal ions, another short oligonucleotide sequence is responsible for switch-on of metallothionin genes (4).

In maize the Adh1 and Adh2 genes are both induced by anaerobiosis, and we have therefore searched for homologies in their 5' regions. Some sequence homology is seen; the best parallel is an 8-bp sequence CACCTCCC conserved in the -170 and -200 bp region (Fig. 2). Other anaerobically induced cDNAs have been isolated (17) and it will be interesting to determine whether their genes contain this oligonucleotide. However, only a transformation system using mutated gene segments will finally demonstrate whether such oligonucleotides are responsible for the anaerobic response.

"Enhancers" are among the eukaryotic promoter elements that increase transcriptional activity (25). The first enhancer was isolated from the virus SV40 and is a cis-acting element more than 100 bp upstream of the gene. It is a 72-bp sequence that markedly enhances gene activity when associated with other genes as well as its own specific gene. It can be associated upstream or downstream of the transcription start point, its orientation is not important, and it can exert its influence over as much as 10 kbp of DNA. A number of viral enhancers have now been isolated. It is thought that natural enhancers are present in animal genomes and may have a role in tissue-specific control of gene expression. For example, an enhancer isolated from an immunoglobulin gene is active in the spleen but not in the liver of transgenic mice (3). It also appears that there are tissue-specific enhancers near the 5' end of insulin and chymotrypsin genes (42). We await isolation of enhancer sequences from plant genes.

CONCLUSIONS

Genetic engineering of plants has been accomplished, with bacterial and plant genes already transferred and expressed in dicot plants using T-DNA model systems. However, if gene transfer

into plants is to provide a range of new phenotypes to plant breeders, a number of areas must be developed:

1. We must be able to use a number of approaches for isolating specific genes from plants and other eukaryotes.
2. We must be able to introduce these genes hack into plants. T-DNA transfomation can be used for dicots, but we must develop systems for monocots, which include the major crop species.
3. The genes that we isolate, perhaps modify in vitro, and then transfer, must be developmentally regulated.

For these challenges, we need a greater understanding of the structure of plant genes and their developmental control.

REFERENCES CITED

1. Bedbrook, J.R., S.M. Smith, and R.J. Ellis. 1980. Molecular cloning and sequencing of cDNA encoding the precursor to the small subunit of chloroplast ribulose-1,5-biphosphate carboxylase. Nature 287:692-697.
2. Bingham, P.M., R. Levis, and G.M. Rubin. 1981. Cloning of DNA sequences from the white locus of E. melanogaster by a novel and general method. Cell 25:693-704.
3. Brinster, R.L., K.A. Ritchie, R.E. Hammer, R.L. O'Brien, B. Arp, and U. Storb. 1983. Expression of a microinjected immunoglobulin gene in the spleen of transgenic mice. Nature 306:332-336.
4. Brinster, R., H.Y. Chen, R. Varren, A. Sarthy, and R.D. Palmiter. 1982. Regulation of metallothionein-thymidine kinase fusion plasmids injected into mouse eggs. Nature 296:39-42.
5. Capetanaki, Y., J. Ngai, C.N. Flytzanis, and E. Lazarides. 1983. Tissue specific expression of two mRNA species transcribed from a single rimentin gene. Cell 35:411-420.
6. Caplan, A., L. Herrera-Estrella, D. Inze, E. van Haute, M. van Montagu, J. Schell, and P. Zambryski. 1983. Introduction of genetic material into plant cells. Science 222:815-821.
7. Chilton, M. 1983. A vector for introducing new genes into plants. Sci. Am. 248:36-45.
8. Chourey, P.S., and D.B. Zurawski. 1981. Callus formation from protoplasts of a maize cell culture. Theor. Appl. Genet. 59:341-344.
9. Costantini, F., and E. Lacy. 1981. Introduction of a rabbit ß-globin gene into the mouse germ line. Nature 294:92-94.
10. Dennis, E.S., W.L. Gerlach, A.J. Pryor, J.L. Bennetzen, A. Inglis, D. Llewellyn, M.M. Sachs, R.J. Ferl, and W.J. Peacock. 1984. The Adhl gene of maize. Nucleic Acids Res. 12:3983-4000.

11. Dennis, E.S., M.M. Sachs, W.L. Gerlach, E.J. Finnegan, and V.J. Peacock. 1984. Alcohol dehydrogenase 2 (Adh2) gene of maize. (Submitted to EMBO J.)
13. Doring, H.P., E. Tillmann, and P. Starlinger. 1984. DNA sequence of the maize transposable element Dissociation. Nature 307:127-130.
13. Draper, J.M., R. Davey, J.P. Freeman, E.C. Cocking, and B.J. Cox. 1982. Ti plasmid homologous sequences present in tissues from Agrobacterium plasmid-transformed Petunia protoplasts. Plant Cell Physiol. 23:451-458.
14. Fedoroff, N., S. Wessler, and M. Shure. 1983. Isolation of the transposable maize controlling elements Ac and Ds. Cell 35:235-242.
15. Flavell, A. 1983. Drosophila takes off. Nature 305:96-97.
16. Flavell, R., and R. Mathias. 1984. Prospects for transforming monocot crop plants. Nature 307:108-109.
17. Gerlach, W.L., A.J. Pryor, E.S. Dennis, R.J. Ferl, M.M. Sachs, and W.J. Peacock. 1982. cDNA cloning and intrduction of the alcohol dehydrogelase gene (Adh1) of maize. Proc. Natl. Acad. Sci. USA 79:2581-2985.
18. Goldenberg, D., J. Posakony, and T. Maniatis. 1983. Correct developmental expression of a cloned alcohol dehydrogenase gene transformed into the Drosophila germ line. Cell 34:59-73.
19. Gordon, J.W., and F.H. Ruddle. 1981. lntegration and stable germ line transmission of genes injected into mouse pronuclei. Science 214:1244-1246.
20. Hasezawa, S., T. Nagata, and K. Syono. 1981. Transformation of Vince rosea protoplasts mediated by Agrobacterium tumefasciens spheroplasts. Molec. Gen. Genet. 182:206-210.
21. Hedrick, S.M., D.I. Cohen, E.A. Nielsen, and M.M. Davis. 1984. Isolation of cDNA clones encloding T cell-specific membrane associated proteins. Nature 308:149-153.
22. Helfman., D.M., J.R. Feramisco, J.C. Fiddes, G.P. Thomas, and S.H. Hughes. 1984. Immunological screening of cDNA expression libraries. Focus 6:l-5.
23. Hess, D., H. Lorz, and E. Weissert. 1974. Uptake of bacterial DNA into swelling and germinating pollen grains of Petunia hybrida and Nicotiana glauca. Z. Pflanzenphysiol. 74:52-63.
24. Hess, D., P.M. Gresshoff, U. Fielitz, and D. Gleiss. 1974. Uptake of protein and bacteriophage into swelling and germinating pollen of Petunia hybrida. Z. Pflanzen-physiol. 74:371-376.
25. Khoury, G., and P. Gruss. 1983. Enhancer elements. Cell 33:313-314.
26. Krens, F.A., L. Molendijk, G.J. Wullems, and R.A. Schilperoort. 1982. In vitro transformation of plant protoplasts with Ti-plasmid DNA. Nature 296:72-74.
27. O'hare, K., and G.M. Rubin. 1983. Structures of P transposable elements and their sites of insertion and

excision in the Drosophila melanogaster genome. Cell 34:25-35.

28. Park, W.D., E.D. Lewis, and I. Rubenstein. 1980. Heterogeneity of: Zein mRNA and protein in maize. Plant Physiol. 65:98-106.

29. Peacock, W.J., E.S. Dennis, W.L. Gerlach, D. Llewellyn, H. Lorz, A.J. Pryor, M.M. Sachs, D. Schwartz, and W.D. Sutton. 1983. Gene transfer in maize, controlling elements and the alcohol dehydrogenase genes. Pages 311-315 in Advances in gene technology: molecular genetics of plants and animals. A.B. Downey, ed. Academic Press, New York.

30. Pelham, H.R.B., and M. Bienz. 1982. A synthetic heat shock promoter element confers heat-inducibility on the herpes simplex virus thymidine kinase gene. EMBO J. 1:1473-1477.

31. Rubin, G.M., and A.C. Spradling. 1982. Genetic transformation of Drosophila with transposable element vectors. Science 218:348-353.

32. Sachs, M.M., E.S. Dennis, W.L. Gerlach, and W.J. Peacock. 1984. Two alleles of maize Adhl exhibit 3' structural and poly (A) addition polymorphisms. (Manuscript in preparation).

33. Scholnick, S., B. Morgan, and J. Hirsch. 1983. The cloned Dopa decarboxylase gene is developmentally regulated when reintegrated into the Drosophila genome. Cell 34:37-45.

34. Shepherd, N.S., Z. Schwarz-Sommer, J. Blumberg vel Spalve, M. Gupta, U. Wienand, and H. Saedler. 1984. Similarity of the Cin1 repetitive family of Zea mays to eukaryotic transposable elements. Nature 307:185-188.

35. Slightom, J.L., S.M. Sun, and T.C. Hall. 1983. Complete nucleotide sequence of a French bean storage protein gene: Phaseolin. Proc. Natl. Acad. Sci. USA 80:1897-1901.

36. Spradling, A.C., and G.M. Rubin. 1982. Transposition of cloned P elements into Drosophila germ line chromosomes. Science 218:341-347.

37. Spradling, A.C., and G.M. Rubin. 1983. The effect of chromosomal position on the expression of the Drosophila xanthine dehydrogenase gene. Cell 34:47-57.

38. Strommer, J.N., S. Hake, J. Bennetzen, W.C. Taylor, and M. Freeling. 1982. Regulatory mutants of the maize Adh1 gene caused by DNA insertions. Nature 300:542-544.

39. Sun, S.M., J.L. Slightom, and T.C. Hall. 1981. Intervening sequences in a plant gene -- comparison of the partial sequence of cDNA and genomic DNA of French bean phaseolin. Nature 289:37-41.

40. Sutton, W.D., W.L. Gerlach, D. Schwartz, and W.J. Peacock. 1984. Molecular analysis of Ds controlling element mutations at the Adhl locus of maize. Science 223:1265-1268.

41. Szostak, J.W., and R. Wu. 1980. Insertion of a genetic marker into the ribosomal DNA of yeast. Plasmid 2:536-544.

42. Walker, M.D., T. Edlund, A.M. Boulet, and W.J. Rutter. 1983. Cell-specific expression controlled by the 5'-flanking region of insulin and chymotrypsin genes. Nature 306:557-561.

43. Zhou, G., J. Weng, Y. Zeng, J. Huang, S. Quian, and G. Liu. 1983. Introduction of exogenous DNA into cotton embryos. Methods Enzymol. 101C:433-482.

MANIPULATING PLANT AND RHIZOBIUM GENES TO IMPROVE SYMBIOTIC NITROGEN FIXATION

D. P. S. VERMA

Root-nodule symbiosis is a partnership between plant and Rhizobium genomes. It can be affected by mutation in either of them or by incompatibility. Agriculturally important tropical legumes generally form symbiosis with slow-growing strains of Rhizobium. These strains appear to differ from fast-growing ones in genetic organization and in the reputation of symbiotic genes, including those coding for nitrogenase. The symbiotic genes in slow-growing strains, unlike those in the fast-growing strains, are not plasmid borne and may be dispersed on the chromosome. Recently mutants have been obtained through transposon mutagenesis In these strains of R. japonicum. A novel vector system has been developed to introduce transcription fusion for identification and eventual isolation of symbiotic genes. The involvement of a specific set of host genes in symbiotic nitrogen fixation has been demonstrated. Induction of these genes before the onset of nitrogenase activity in nodules suggest their importance in the early stages of nodule development. Six of the host genes have been isolated from soybean. These genes may serve as molecular probes in screening germplasm for improving the symbiotic nitrogen fixation trait in soybean.

Use of chemical nitrogen fertilizers has brought about a large part of the increases in world agricultural production during the last 3 decades. High energy costs, however, have limited the use of this approach to increase food production, particularly in developing countries. Other methods for increasing crop yields must therefore be found.

An estimated 20% of the world's food comes from legumes. Legumes use atmospheric nitrogen fixed by their closely associated bacteria, which makes them autotrophic for nitrogen. Because legumes have such a high nutritional value, their cultivation has increased by 32% per year during the last 10 yr

Professor, Genetic Manipulation Research Group, Biology Department, McGill University, 1205 Docteur Penfield Avenue, Montreal, Quebec, Canada HSA 1B1.

and is expected to continue increasing (7). Progress in biological nitrogen fixation is therefore expected to dramatically improve world agriculture. But before symbiotic nitrogen fixation can be manipulated and improved, its molecular biology must be understood.

Symbiotic fixation of atmospheric nitrogen is a joint endeavor of plant and microorganism. It is affected by mutations in either of these organisms, as well as by soil conditions, temperature, and microelements (1, 24, 30, 31). Significant progress in the biochemistry, genetics, and molecular biology of nitrogen fixation has been made in *Rhizobium*, but the role of the host is poorly understood. Transferring nitrogen fixation genes from bacteria directly to the plant is impossible in the near future. However, existing symbiotic associations can be improved and new associations developed by altering plant and microbial genomes. For this, specific bacterial and plant host genes controlling nitrogen fixation must first be identified.

A number of host and *Rhizobium* genes affect legume nodule development and their ability to fix nitrogen (21, 31). In soybean, several host gene products, termed nodulins, which appear important in symbiotic nitrogen fixation, have been identified through molecular techniques (19). The presence of nodulins has also been demonstrated in other legumes, specifically pea (3) and lupin (8). Two other groups of host genes exist. One group is modulated by auxins; the other directly or indirectly influences symbiotic nitrogen fixation (27, 31). Since all these genes are induced before nitrogenase appears in nodules (29), the relationships between their structure and function, and their mode of regulation must be understood before symbiotic nitrogen fixation can be improved.

RHIZOBIUM GENETICS

Agriculturally important tropical legumes (e.g., *Glycine max*, *Vigna* spp., *Arachis hypogaea*, and *Cicer*) are nodulated generally by slow-growing strains of Rhizobium. These strains differ significantly from the fast-growing *Rhizobium* spp., which nodulate primarily temperate legumes. The slow-growing strains of *Rhizobium* have been placed in a new genus, *Bradyrhizobium* (14). The understanding gained in the last 5 yr about nitrogen fixation and symbiotic genes in fast-growing species cannot be directly applied to the slow-growing strains. This is partly because conjugative gene transfers in the slow strains are too infrequent for chromosome mapping (16, 23). The transposon mutagenesis technique widely used for the fast-growing strains has not worked for slow-growing strains. Moreover, slow-growing strains in one host can differ so much in their DNA sequences (cf. *R. japonicum*, strain 61A76, and USDA 110) that they probably represent different species (13).

One group of fast-growing *Rhizobium* strains can form nodules in some plants normally hosting slow-growing strains (5). However, the nodules formed by fast-growing and slow-growing

Rhizobium strains have not been compared for their nitrogen-fixation efficiency. In many cases, the association is ineffective. The genomic arrangements of Sym (symbiotic) and nif genes, unique to slow-growing strains, may be critical to symbiosis with tropical legumes. Intraspecific variation in the nif gene arrangement has also been demonstrated for two R. japonicum strains used in commercial inoculants for North American soybeans (11). It appears that R. japonicum contains two highly divergent groups of bacteria with symbiotic genes able to nodulate soybeans.

IDENTIFYING SYMBIOTIC GENES OF RHIZOBIUM

Through transposon mutagenesis of fast-growing strains of Rhizobium followed by screening of the mutants for symbiotic defects, several genes affecting nodule development and effectiveness have been identified (20). Since most of the symbiotic genes are not expressed in free-living rhizobia, a new approach to identify them and determine their pattern of expression was developed recently. Through a conjugative suicide vector pGS6 (25) and a defective mu phage containing part of a lac operon, random transcription fusions in Rhizobium japonicum have been created. These should allow detection of gene types coding for

- Rhizobium survival (soil, pH, and salinity),
- growth (C, N, and P),
- competition among bacteria,
- attachment to root hair,
- host specificity,
- the infection process,
- systemic regulation in host (preclusion of secondary infections),
- efficiency of nitrogen fixation,
- duration of nitrogen fixation, and
- maintenance of the bacterioid state.

Expression of some of the above genes can be assayed ex planta by measuring 6-galactosidase activity under different conditions.

ORGANIZATION OF SYMBIOTIC GENES IN FAST- AND SLOW-GROWING RHIZOBIUM SPP.

Symbiotic genes in fast-growing Rhizobium, including those encoding nitrogenase, are located on extrachromosomal elements called plasmids, some of which are very large (greater than 300 megadaltons) and cannot be eliminated from the strain, such as the megaplasmid in R. meliloti. Large plasmid linkage of Sym genes holds true even for fast-growing strains forming nodules with tropical legumes generally nodulated by Bradyrhizobia (5). No Sym plasmid harboring nif and symbiotic genes has been

observed in any slow-growing Rhizobium strains. These genes may be organized quite differently in Bradyrhizobium spp. (11). Symbiotic genes in slow-growing Rhizobium may have been integrated into chromosomes during evolution, and their organization and regulation may also be different. For example, nifH in fast-growing Rhizobium is closely linked to nifK and D, while in R. japonicum and in cowpea strains it is not (H. Hennecke, E. Zurich, and A. Szalay, Cornel1 University, pers. corn.). Furthermore, nif genes in some slow-growing strains can be induced ex planta under microaerophilic conditions; this is not the case in fast-growing strains of Rhizobium.

A common 'Nod' region of about 3 kb containing symbiotic genes has been identified in R. meliloti (15), it carries genes involved in root-hair curling, a very early step in nodule formation. However, our evidence indicates that root-hair curling genes of slow-growing R. japonicum are located away from the common nodulation region and appear to occur in multiple copies. Sutton et a1 (26) have isolated from R. japonicum strain USDA 122 and 110 genomic libraries, two sequences able to confer characteristic root-hair curling on wild soybean when transferred to other bacteria (e.g., Pseudomonas) (Fig. 1a,b). In fact, these two sequences are present in each strain, but whether the phenotype is caused by the expression of only one kind of sequence in a given strain is not known.

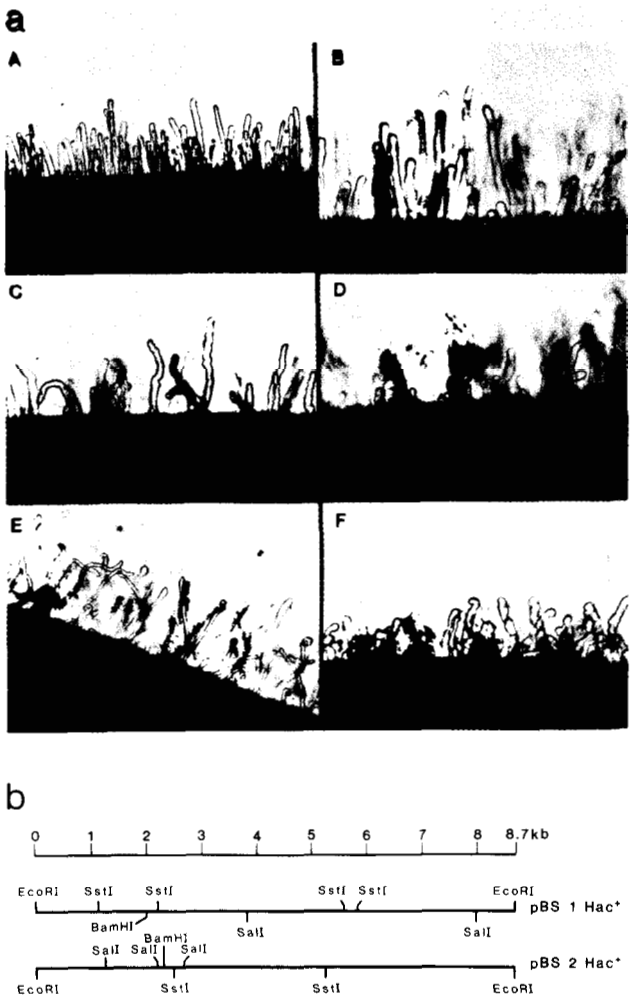
HOST GENETICS

The host's importance in symbiotic nitrogen fixation has been known for over 5 decades. However, classical breeding has wrought little improvement of nitrogen fixation, primarily because few phenotypic markers are available for selecting better hosts. (The total nitrogen content or yield of a plant cannot be used, because it is compounded by other factors.)

The host plant's involvement in symbiosis is apparent at all levels (21, 30). The two partners apparently coevolved to accomplish symbiosis (31). The host plant must provide all the energy for nitrogen fixation and the survival of the Rhizobium endosymbiont. It must protect the nitrogenase system, and must suppress any indigenous defense response against the invading (rhizobia) pathogen. Factors controlling photosynthesis and carbon and nitrogen metabolism must, therefore, also influence symbiosis.

Numerous studies have demonstrated that host plant inheritable characteristics are involved in various aspects of symbiotic nitrogen fixation. These characteristics include nodule number and size, timing of appearance, nodule morphogenesis, and rate of nitrogen fixation (6, 12, 21, 30, 32). The activity of hydrogenase, a rhizobia1 enzyme involved in oxidizing hydrogen (a by-product of nitrogen fixation) is also controlled by the host (4). In addition, the following properties of bacterioids may be affected by a given host:

- chemotaxis,



1. a. Root-hair curling of *G. soja* 1-3 d after inoculation. (A) Uninoculated control; (B) *Pseudomonas putida;* (C) *R. japonicum* USDA 122; (D) *P. putida* transconjugant containing pBS1; (E) *R. japonicum* USDA 110; (F) *P. putida* transconjugant containing pBS2. b. Physical maps and phenotypic analysis of pBS1 and pBS2 inserts. The 8.7 kb *Eco*RI fragments from strains USDA 122 (contained in pBS1), and USDA 110 (contained in pBS2) express soybean root-hair curling (Hac^+) in *P. putida* transconjugants (26).

- host specificity,
- attachment to root hair,
- infection process (mode of invasion),
- transformation to the bacterioid state,
- Hup/Nif gene expression,
- efficiency of nitrogen fixation, and
- survival.

Verma and Nadler (31) listed host genes identified by classical genetics and variant selection. Most of these genes are recessive and strain specific. More than one may be involved in a given symbiotic phenotype (22). The precise lesion caused by any of these mutations and the biochemical processes they affect are unknown. However, through recombinant-DNA technology, some of these genes can be cloned and their function in symbiosis identified. Host mutants defective in symbiosis must be studied extensively and molecular probes developed to explore this complex process.

IDENTIFYING SPECIFIC HOST GENES

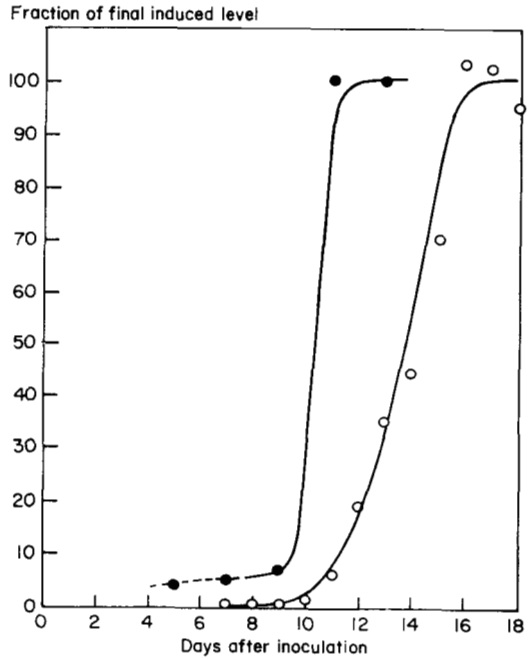
Two major groups of nodule-specific host gene products, leghemoglobins and nodulins, are induced during symbiotic nitrogen fixation (28, 29). While the structure and function of leghemoglobins are comparatively clear, little is known about nodulins.

On the basis of their possible functions, nodulins have been divided (31) into 1) those that are like leghemoglobins and common to all legumes (c-nodulins) and 2) those that are species specific (s-nodulins). A previously identified nodulin, nodulin-35 (18), functions as a nodule-specific uricase involved in ureide biosynthesis and is localized in the nodule's uninfected cells (2). In tropical legumes, fixed nitrogen is primarily transported as ureides; in temperate, it is transported as amides (glutamine or asparagine).

Through molecular cloning five nodulin sequences have been isolated from soybean (9). They encode 100, 44, 27, 24, and 23.5 K dalton MW nodule-specific polypeptides (nodulins). Genomic sequences corresponding to these clones have been isolated from soybean lambda libraries, and are being characterized. They are not linked with those encoding for leghemoglobins, which are arranged on the soybean chromosome in four loci, only two of which are functional (17). (The arrangement of leghemoglobin genes is different in Phaseolus [J. Lee and D.P.S. Verma, in prep.] and appears to be simpler.)

INDUCTION OF NODULINS AND THEIR RELATION TO NODULE EFFECTIVENESS

Individual nodulin mRNA levels at different times after infection were measured following dot-blot hybridization to the nodulin cDNA probes (10) to determine the roles of nodulins in symbiosis. The accumulation of each nodulin mRNA appears to reach a steady state 10 d after infection, when nitrogen fixation commences (Fig. 2). Moreover, growing uninfected plants in the presence of 15 mM $(NH_4)_2NO_3$ does not affect the level of nodulin mRNA sequences, indicating that accumulation of nodulin and leghemoglobin mRNAs is specific to infected root tissue and not related to the assimilation of externally applied inorganic nitrogen.

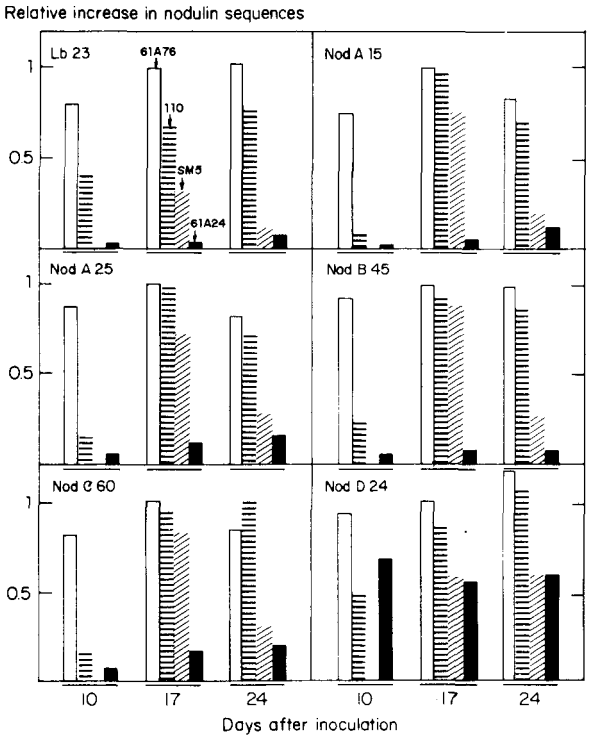


2. Relative accumulation of nodulin mRNAs (■) and nitrogen fixation activity (●) during nodule development, Nodulated roots from 7- to 24-d plants and control roots were assayed for acetylene-reduction activity (o). Relative levels of nodulin mRNAs at different times were determined by dot-blot analysis and quantified by densitometry (10).

Nodulin mRNA levels in root nodules formed by effective and ineffective Rhizobium strains were measured to determine whether they correlated with the nodules' nitrogen-fixing ability. The results (Fig. 3) suggest that fractional accumulation of leghemoglobin and that of individual nodulin mRNAs are similar. Nodules formed by another effective strain, USDA 110, accumulated more slowly than all nodulin mRNAs. The ineffective strain 61824 induced all nodulin mRNAs, but at 10-25% of levels found in wild type (61A76) nodules. On the other hand, accumulation of these mRNAs in SM5 (an ineffective derivative of 61A76)-induced nodules was nearly normal up to 17 d, but then it declined. These studies suggest that nodulin sequences, accumulating in a strain-specific manner, regulate the effectiveness of symbiosis. More studies are needed to identify suitable nodulins to serve as molecular probes for monitoring the functional state of symbiosis.

CONCLUSION

Plant-*Rhizobium* interactions occur throughout the life of nodules; they comprise subtle plant responses to bacterial invasion and host influences on the *Rhizobium* as it passes



3. Effect of different *R. japonicum* strains on the accumulation of leghemoglobin and nodulin mRNAs. Total polysomal RNAs were isolated from nodules produced by effective strains 110 and 61A76 and by ineffective strains SM5 and 61A24. Nodules were harvested 10, 17, and 24 d after planting. Isolated RNAs were dot-blotted and hybridized to nick-translated nodulin DNAs. Autoradiographic results were quantified by densitometry (10).

through the infection thread. The interactions may or may not allow expression of the bacterial genes necessary for symbiotic nitrogen fixation. A strain effective on one host can make ineffective nodules on another, and two strains differing in their genomic composition can nodulate the same host. The sequence of events during nodulation and the effect on this process of various mutations in both the plant and Rhizobium must be studied at the molecular level. Several host gene products supporting symbiosis have been identified. Some of them are present in the infected cells; others, in uninfected cells, suggesting that nodule metabolism is further compartmentalized. For significant progress in improving symbiotic nitrogen fixation, a combination of plant and Rhizobium strain has to be selected and improved in the field. Identifying and characterizing the host genes needed to develop and maintain symbiosis may permit their eventual transfer to novel hosts and enable them to form new associations with naturally occurring or

novel nitrogen-fixing microbes. In the short term, these host genes can serve as molecular probes to select new varieties for legume breeding programs.

ACKNOWLEDGMENTS

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REFERENCES CITED

1. Bergersen, F.J. 1977. Physiological chemistry of dinitrogen fixation by legumes. Pages 519-556 in A treatise in dinitrogen fixation. R.W.F. Hardy and W.S. Silver, eds. Wiley-Interscience, London.
2. Bergmann, H., E. Preddie, and D.P.S. Verma. 1983. Nodulin-35: a subunit of nodule-specific uricase (uricase II) induced and localized in uninfected cells. the EMBO J. 2: 2333-2339.
3. Bisseling, T., C. Been, J. Klugkist, A. van Kammen, and K. Nadler. 1983. Nodule specific host proteins in effective and ineffective root nodules of Pisum sativum. The EMBO J. 2:961-966.
4. Brewin, N.J. 1984. Hydrogenase and energy efficiency in nitrogen fixing symbionts. In Genes involved in plant-microbe interactions. D.P.S. Verma and T. Hohn, eds. Springer-Verlag, Wein New York. (in press)
5. Broughton, W.J., N. Heycke, H. Meyer Z.A., and C.E. Pankhurst. 1984. Plasmid-linked nif and "nod" genes in fast-growing rhizobia that nodulate Glycine max, Psophocarpus tetragonolobus and Vigna unguiculata. Proc. Natl. Acad. Sci. (in press)
6. Caldwell, B.E., and H.G. Vest. 1977. Genetic aspects of nodulation and dinitrogen fixation by legumes: the macrosymbiont. Pages 557-575 in A treatise on dinitrogen fixation. Sec. III. R.W.F. Hardy and W.S. Silver, Wiley-Interscience, London.
7. FAO (Food and Agricultural Organization). 1981. FAO report. Rome.
8. Farnden, K.J.F., N.S. Fernando, M.R. Grant, A.L. Johnson, V.S. Nyoni, and C.W. Ronson. 1983. Protein synthesis in the plant fraction of lupin and pea nodules. Page 593 in Advances in nitrogen fixation research. C. Veeger and W.E. Newton, eds. Nijhoff/Junk Pub., Pudoc, Wageningen.
9. Fuller, F., P. Kunstner, T. Nguyen, and D.P.S. Verma. 1983. Soybean nodulin genes: analysis of cDNA clones reveals four plant genes which are expressed specifically in

nitrogen-fixing root nodules. Proc. Natl. Acad. Sci. 80:2594-2598.

10. Fuller, F., and D.P.S. Verma. 1984. Appearance and accumulation of nodulin mRNAs and their relationship to the effectiveness of root nodules. Plant Molec. Biol. 3:21-28.

11. Haugland, R., and D.P.S. Verma. 1981. Interspecific plasmid and genomic DNA sequence homologies and localization of Nif genes in effective and ineffective strains of Rhizobium japonicum. J. Molec. Appl. Genet.l:205-217.

12. Holl, F.B., and T.A. LaRue. 1976. Genetics of legume host plants. Pages 391-399 in Proceedings 1st international symposium on nitrogen fixation. Vol. II. W.E. Newton and C. Nyman, eds. Washington State University Press, Pullman, Washington.

13. Hollis, A.B., W.E. Kloos, and G.M. Elkan. 1981. DNA:DNA hybridization studies of Rhizobium japonicum and related Rhizobiaceae. J. Gen. Microbiol. 123:215-222.

14. Jordan, D.C. 1982. Transfer of Rhizobium japonicum Buchanan 1980 to Bradyrhizobium gen nov., a genus of slow-growing root nodule bacteria from leguminous plants. Int. J. Syst. Bacteriol. 32:136-139.

15. Kondorosi, E., Z. Banfalvi, and A. Kondorosi. 1984. Physical and genetic analysis of a symbiotic region of Rhizobium meliloti: identification of nodulation genes. Molec. Gen. Genet. (in press)

16. Kuykendall, L.D. 1979. Transfer of R factors to and between genetically marked sublimes of Rhizobium japonicum. Appl. Environ. Microbiol. 36:862-866.

17. Lee, J., G.G. Brown, and D.P.S. Verma. 1983. Chromosomal arrangement of leghemoglobin genes in soybean. Nucl. Acid Res. 11:5541-5553.

18. Legocki, A., and D.P.S. Verma. 1979. A nodule-specific plant protein (Nodulin-35) from soybean. Science 205:190-193.

19. Legocki, A., and D.P.S. Verma. 1980. Identification of "nodule-specific" host proteins (Nodulins) involved in the development of Rhizobium-legume symbiosis. Cell 20:153-163.

20. Meade, H.M., S.R. Long, G.B. Ruvkun, S.E. Brown, and F.M. Ausubel. 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of Rhizobium meliloti induced by transposon Tn5 mutagenesis. J. Bacteriol. 149:114-122.

21. Nutman, P.S. 1981. Hereditary host factors affecting nodulation and nitrogen fixation. Pages 194-204 in Current perspectives in nitrogen fixation. A.H. Gibson, and W.E. Newton, eds. The Australian Academy of Science, Canberra.

22. Peterson, M.A., and D.K. Barns. 1981. Inheritance of ineffective nodulation and on-nodulation trait in alfalfa, Crop Sci. 21:611-616.

23. Pilacinski, W.P., and E.L. Schmidt. 1981. Plasmid transfer within and between serologically distinct strains of Rhizobium japonicum using antibiotic resistance and auxotroohs. J. Bacteriol. 145 – 1025 – 1030.
24. Rolfe, B.G., and J. Shine. 1984. Rhizobium--1eguminosae symbiosis: the bacterial point of view. Pages 95 – 128 in Genes involved in plant-microbe interactions. D.P.S. Verma and T. Hohn, eds. Springer-Verlag, Wein, New York.
25. Selvaraj, G., and V.N. Iyer. 1983. Suicide plasmid vehicles for insertion mutagenesis in Rhizobium meliloti and related bacteria. J. Bacteriol. 156:1292 – 1300.
26. Sutton, B.C.S., J. Stanley, M.G. Zelechowska, and D.P.S. Verma. 1984. Isolation and expression of cloned DNA from Rhizobium japonicum encoding an early soybean nodulation function. J. Bacteriol. 158:920 – 927.
27. Verma, D.P.S., D. Bewley, S. Auger, F. Fuller, S. Purohit, and P. Kunstner. 1983. Host genes involved in symbiosis with Rhizobium. Pages 236 – 245 in Genetic engineering: application to agriculture. L.D. Owens, ed. Granada Pub/Romman and Allanhead Pub., London, New Jersey.
28. Verma, D.P.S., J. Lee, F. Fuller, and H. Bergmann. 1983. Leghemoglobin and nodulin genes: two major groups of host genes involved in symbiotic nitrogen fixation. Pages 557 – 564 in Advances in nitrogen fixation research. C. Veeger and W.E. Newton, eds. Nijhoff/Junk Pub., Pudoc, Wageningen.
29. Verma, D.P.S., R.P. Legocki, and S. Auger. 1981. Expression of nodule-specific host genes in soybean. Pages 205 – 208 in Current perspectives in nitrogen fixation. A.H. Gibson and W.E. Newton, eds. The Australian Academy of Science, Canberra.
30. Verma, D.P.S., and S. Long. 1983. The molecular biology of Rhizobium -legume symbiosis. Pages 211 – 245 in Int. Rev. Cytol. Suppl. 14. K. Jeon, ed. Academic Press, New York.
31. Verma, D.P.S., and K. Nadler. 1984. Legume-rhizobium symbiosis: host's point of view. Pages 57 – 93 in Genes involved in plant-microbe interactions. D.P.S. Verma and T. Hohn, eds. Springer-Verlag, Wein New York.
32. Vincent, J.M. 1980. Factors controlling the legume- Rhizobium symbiosis. Pages 103 – 129 in Nitrogen fixation. Vol. II. W.E. Newton and H.H. Orme-Johnson, eds. University Park Press, Baltimore.

RECOMBINANT DNA RESEARCH FOR VIROID AND VIRUS IDENTIFICATION

L. F. SALAZAR, R. A. OWENS, and T. O. DIENER

Identification of the potato spindle tuber viroid (PSTV) illustrates how recombinant DNA technology can be applied in viroid and virus research. Hybridization of highly radioactive PSTV complementary DNA to PSTV-RNA bound to a solid support, with subsequent detection of the DNA-RNA hybrids by autoradiography is more sensitive than all other methods available for PSTV diagnosis. Nonradioactive detection is also highly probable. Applying the technique to the detection of other viroids and viruses and related problems is discussed.

Virus and viroid diseases of plants are very important because they dramatically affect crop yield and quality. Since they are controlled by prevention rather than cure, highly sensitive methods to identify their causal agents are needed.

Immunological techniques are very important for detecting viruses, and techniques with improved sensitivity, such as the enzyme-linked immunosorbent assay (ELISA), are now widely used. The development of monoclonal antibodies has improved the sensitivity and reliability of the serological approach.

Because viroids do not have a protein coat, they cannot be detected by immunological techniques. Other methods, such as bioassay on indicator hosts or electrophoresis, are not suitable for large-scale detection because their sensitivity is insufficient and they are laborious and expensive.

Recombinant DNA technology has overcome these disadvantages in detecting the potato spindle tuber viroid (PSTV), and it is being applied to other viroids and viruses. As originally described by Owens and Diener (9), the procedure calls for hybridizing highly radioactive DNA complementary to PSTV (PSTVcDNA), with, PSTV bound to a solid support, and subsequent detection of DNA-RNA hybrids by autoradiography. This paper briefly reports the advances made with this technique to detect PSTV, and evaluates a similar procedure to identify viruses.

Virologist, International Potato Center, P. O. Box 5959, Lima, Peru; research chemist, and research plant pathologist, Plant Virology Laboratory, Agricultural Research Services (ARS), U. S. Department of Agriculture (USDA), Beltsville, MA 20705.

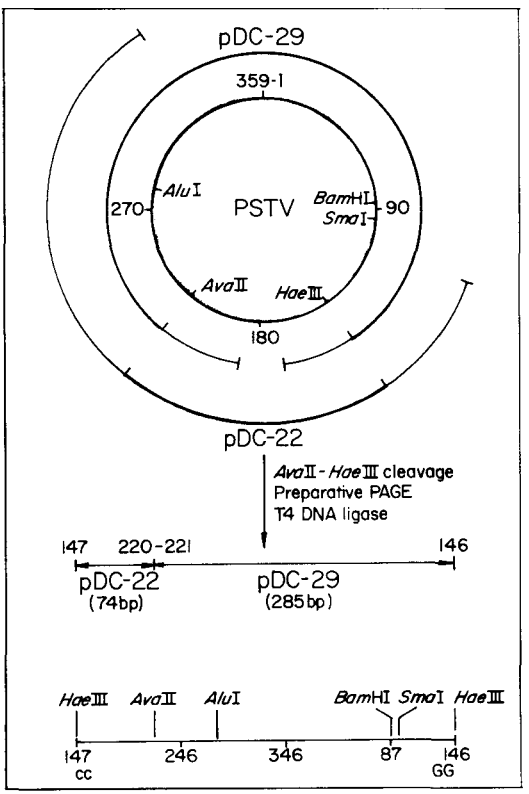
DETECTION OF PSTV BY NUCLEIC ACID HYBRIDIZATION

Molecular cloning of PSTVcDNA

The determination of the 359 nucleotide sequence of PSTV (3) facilitated the molecular cloning and characterization of a double-stranded (ds) PSTVcDNA. Owens and Cress (8) synthesized ds PSTVcDNA from a polyadenylated linear PSTV template and inserted it into the Pst I endonuclease site of plasmid pBR322, using the oligo (dC)-oligo (dG) tailing procedure. Since recombinant DNA clones did not contain the complete copy of PSTV, Owens and Cress (8) constructed one by ligating fragments from two partially overlapping clones at the Ava II site. To the resulting linear ligate, Hind III oligodeoxynucleotide linkers were added to the Hae III blunt ends and, following digestion with Hind III, this fragment was cloned in the Hind III site of pBR322 (Fig. 1). Digestion of the recombinant DNA with Hae III releases a 359 bp fragment, which is a full-length ds PSTVcDNA.

PSTV diagnosis

According to Diener et a1 (l), two conditions had to be met before hybridization with PSTVcDNA could be developed into a

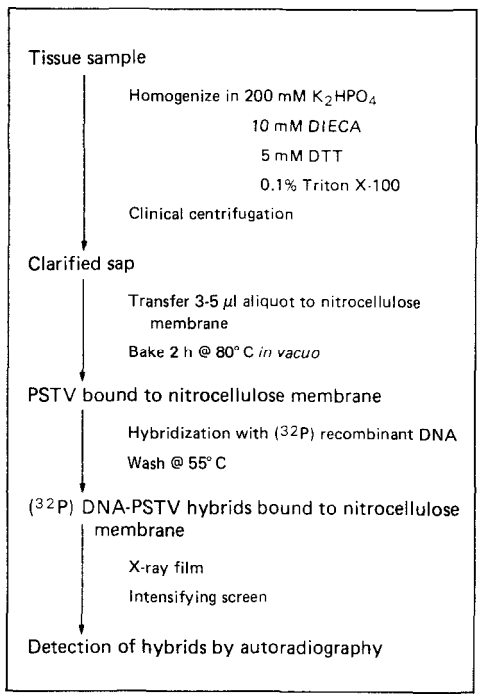


1. Strategy used for constructing a full-length ds PSTV-cDNA.

practical PSTV diagnostic test: 1) PSTVcDNA had to be available in unlimited quantities, and 2) the sample preparation had to be simple and efficient. To be universally applicable, however, it was soon found that an additional condition had to be met: the marker molecule to label the PSTVcDNA must be readily available, easy to handle, and reliable.

The first condition was resolved by cloning the PSTVcDNA in Escherichia coli; the second, by developing a highly ionic extraction buffer to release PSTV from the nucleolus and by adding diethyldithiocarbamate to inhibit enzymatic polyphenol oxidation in the samples. The sample preparation and test procedure are summarized in Figure 2. The hybridization reaction is performed for 24 h at 55°C in 10% dextran sulfate and ^{32}P-labeled, nick-translated recombinant PSTVcDNA (1 – 2.5x 10 cpm/ml). Autoradiography is carried out for 24 to 48 h at – 70°C with X-ray film and intensifying screen (1).

Owens and Diener (9) could detect the PSTV directly in tuber sprouts and foliage. Even though the presence of sap reduces PSTV binding to the nitrocellulose membrane almost tenfold, 83 – 250pg of PSTV can be detected after hybridization with radioactive recombinant DNA. Diener et al (1) showed that in some cases PSTV is unevenly distributed, and is present in some but not all tubers from the same plant. Comparative studies showed the nucleic acid spot hybridization is more



2. Outline of the nucleic acid spot hybridization procedure for detecting PSTV. DIECA, diethyldithiocarbamate; DDT, dithiothreitol.

sensitive and reliable than electrophoresis (12). Martinez and Salazar (7) could not detect PSTV by silver nitrate-staining of polyacrylamide gels as well in electrophoresis as in nucleic acid hybridization, since more than 62 ng of PSTV were required for positive detection. The nucleic acid hybridization test proved even more sensitive than the tomato bioassay because it allowed detecting PSTV in extracts from 16 true potato seeds in which only one was infected (12; Table 1; Fig. 3).

The third requirement to make the nucleic acid hybridization a practical diagnostic method was a highly sensitive, reliable, and easily available marker to label the cDNA probe. Although ^{32}P is highly sensitive, its value is limited because it has a short half-life and is difficult to obtain in some countries. Biotin-labeled nucleotide analogs, which can be incorporated in polynucleotides, may fulfill this requirement. In conjunction with immunological or affinity detector systems, these reagents can effectively replace radioisotopes (5). The strong interaction between biotin and avidin seems the best, since avidin coupled to an indicator molecule (Fig. 4) can detect minute amounts of biotin. This approach has made it possible to detect biotin-labeled DNA probes after hybridization to DNA or RNA immobilized on nitrocellulose filters (6).

The nucleic acid hybridization test will be valuable for detecting other viroids such as citrus exocortis, chrysanthemum stunt, and coconut cadang-cadang.

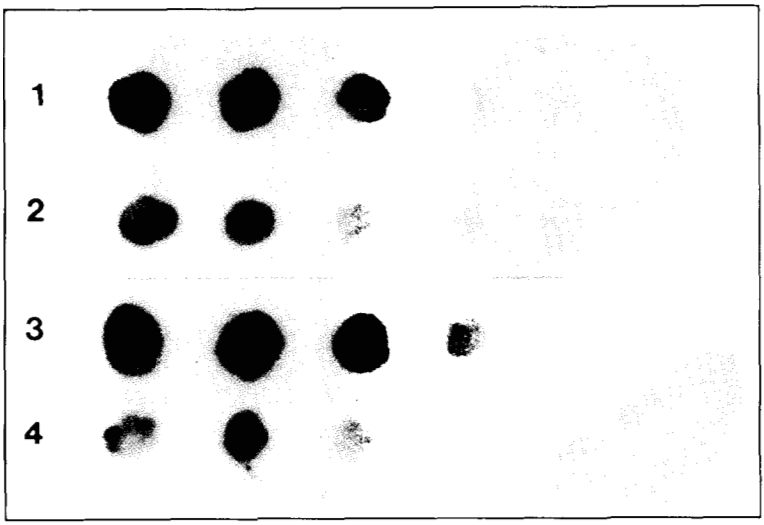
NUCLEIC ACID HYBRIDIZATION TO DETECT VIRUSES

The nucleic acid hybridization test can more accurately detect viruses in the following situations:

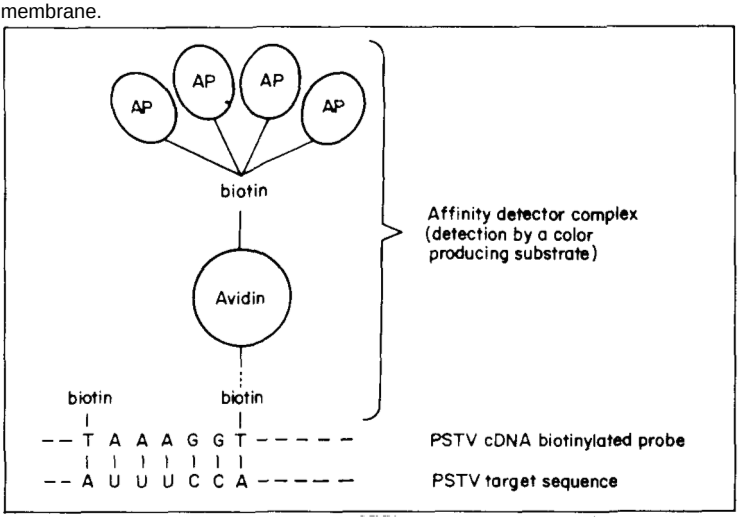
1. viruses whose infective nucleic acid genome is not encapsiolated in a protein coat, i.e., tobacco rattle virus, NM isolates, in which case neither serological methods nor electron microscopy can identify them, but the cDNA test proved sufficiently sensitive and reliable to detect them in field-grown plants (4);
2. viruses occurring in such a low concentration in the host that the serological tests (i.e., ELISA) can only detect them after purification and concentration (this

Table 1. Detection of PSTV in healthy and infected composite true potato seed samples (from Diener et al (1)).

Seed ratio (infected: healthy)	Symptoms on tomatoes	PAGE of tomatoes	cDNA of potato seeds
0: 16	–	–	–
1: 15	–	–	+
2: 14	–	–	–
4: 12	–	+	+
8: 8	–	+	+
16: 0	–	+	+



3. Detecting of PSTV in true potato seed by nucleic acid spot hybridization. First and third rows, extract from PSTV-infected tomato leaves, undiluted and diluted 1:5, 1:25, 1:125, and 1:625 with extract from uninfected tomato leaves (left to right), Second and fourth rows, extracts of seed from PSTV-infected potato plants, undiluted and diluted 1:3, 1:9, 1:27, and 1:81 with extract of seeds from uninfected plants (left to right), The sample at extreme right in each row is undiluted extract from uninfected tissue. Samples in the third and fourth rows were extracted with phenol and chloroform before being applied to the nitrocellulose membrane.



4. Suggested approach for enzymatic detection of PSTV (RNA)-biotynilated DNA hybrids. AP, polymerized alkaline phosphatase molecule.

occurs, for instance, in PLRV-infected tomatoes or in some potato genotypes carrying genes for PLRV resistance (unpubl.)).

Nucleic acid hybridization also seems best suited for studying virus relationships, especially those in which

immunological techniques provide insufficient differentiation to account for other observed differences. Luteoviruses are good examples of this, and such work has been done with other plant virus groups (2, 10, 11).

Nucleic acids of cryptic viruses or nonencapsiolated nucleic acids (such as RFs and RIs) present during replication of viruses may be target molecules for a cDNA probe.

Recombinant DNA techniques will undoubtedly be applied to many problems, including screening for such specific genes as those for resistance to pests, diseases, and other plant stresses.

REFERENCES CITED

1. Diener, T.O., L.F. Salazar, R.A. Owens, and D.E. Cress. 1983. New potato spindle tuber test: implications for the future. Pages 71-76 in Research for the potato in the year 2000. Proceedings international congress. International Potato Center, Lima, Peru.
2. Gonda, T.J., and R.H. Symons. 1978. The use of hybridization analysis with complementary DNA to determine the RNA sequence homology between strains of plant viruses: its application to several strains of cucumoviruses. Virology 88:361-370.
3. Gross, H.J., H. Domdey, C. Lossow, P. Jank, M. Raba, H. Alberty, and H.L. Sanger. 1978. Nucleotide sequence and secondary structure of potato spindle tuber viroid. Nature (London) 273:203-205.
4. Harrison, B.D., and D.J. Robinson. 1983. Use of complementary DNA to detect tobacco rattle virus in potato foliage. Pages 93-94 in Research for the potato in the year 2000. Proceedings international congress. International Potato Center, Lima, Peru.
5. Langer, P.R., A.A. Waldrop, and D.C. Ward. 1981. Enzymatic synthesis of biotin-labeled polynucleotides: novel nucleic acid affinity probes. Proc. Natl. Acad. Sci. USA 78:6633-6637.
6. Leary, J.J., D.J. Brigati, and D.C. Ward. 1983. Rapid and sensitive colorimetric method for visualizing biotin-labeled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose: bio-blots. Proc. Natl. Acad. Sci. USA 78:6633-6637.
7. Martinez, E., and L.F. Salazar. 1984. A rapid and sensitive staining procedure of polyacrylamide gels for detection of potato spindle tuber viroid. Submitted to Can. J. Plant Pathol.
8. Owens, R.A., and D.E. Cress. 1980. Molecular cloning and characterization of potato spindle tuber viroid cDNA sequences. Proc. Natl. Acad. Sci. USA 77:5302-5306.
9. Owens, R.A., and T.O. Diener. 1981. Sensitive and rapid diagnosis of potato spindle tuber viroid disease by nucleic acid hybridization. Science 213:670-672.

10. Palukaitis, P., and R.H. Symons. 1980. Nucleotide sequence homology of thirteen tobacco virus RNAs as determined by hybridization analysis with complementary DNA. Virology 107:354-361.
11. Rosner, A., I. Ginzburg, and M. Bar-Joseph. 1983. Molecular cloning of DNA sequences of citrus tristeza virus (CTV) RNA [abstr.]. Phytopathology 73:793.
12. Salazar, L.F., R.A. Owens, D.R. Smith, and T.O. Diener. 1983. Detection of potato spindle tuber viroid by nucleic acid spot hybridization: evaluation with tuber sprouts and true potato seed. Am. Potato J. 60:587-597.

RICE VIRUS DISEASE PROBLEMS AND NEED FOR MONOCLONAL ANTIBODY TECHNOLOGY FOR DIAGNOSIS AND EPIDEMIOLOGY

H. HIBINO

In the tropics, virus diseases, already a major threat to stable rice production, are becoming increasingly important. Varietal resistance is the most practical and economical control measure. Development of new virus strains and virus variation among locations are the worst problems associated with the cultivation of virus-resistant varieties. More basic research on resistance and epidemiology and on the potentials of serological tests and monoclonal antibody techniques is needed to solve these problems.

Before the 1960s virus-like diseases broke out occasionally in the tropics, but most of them were not recognized as transmissible entities. Now, virus diseases are known major threats, and ever increasing ones, to stable rice production in the tropics (23). The viruses have generally increased where rice cultivation has been improved, and where rice is grown throughout the year. Outside these problem areas, virus diseases are sporadic. Once an outbreak in an improved area occurs, thousands of hectares of rice are lost if the varieties grown are not resistant. Under such high pressure, insecticide is not effective. Monoclonal antibody technology would be a great aid in the basic research needed to fight rice virus diseases.

RICE VIRUS DISEASES IN THE TROPICS

Throughout the world, 19 rice viruses, mycoplasmas, and virus-like diseases are known; 13 of them occur in the tropics (Table 1). Tungro (29), grassy stunt (30), and ragged stunt (13, 24) are the most important diseases in South and Southeast Asia. Hoja blanca (25) is a serious problem in Central and South America, and yellow mottle (2) is the worst in Africa. All these rice viruses are insect-borne (except one, which is transmitted by a soil-borne fungus) and the problem of their control is also a problem of vector insect control.

Plant virologist, International Rice Research Institute, P. O. Box 933, Manila, Philippines.

Table 1. Rice virus, mycoplasma, and virus-like diseases in the tropics.

Virus	Size (nm)	Vector	Geographical distribution
Dwarf virus	70	Leafhopper	China, Japan, Korea, Nepal
Gall dwarf V.	65	Leafhopper	China, Malaysia, Thailand
Ragged stunt V.	65	Planthopper	South and Southeast Asia, China, Japan, Taiwan
Yellow mottle V.	32	Beetle	East and West Africa
Tungro spherical V.	30	Leafhopper	South and Southeast Asia, China, Japan
Tungro bacilliform V.	30-35 x 100-200	Leafhopper	South and Southeast Asia, China
Transitory yellowing V.	96 x 180-210	Leafhopper	China, Japan, Taiwan, Thailand
Necrosis mosaic V.	14 x 257	Fungus	India, Japan
Grassy stunt V.	6-8 x 1000-1300	Planthopper	South and Southeast Asia, Japan, Taiwan
Hoja blanca V.	Filamentous	Planthopper	South and Central America
Yellow dwarf	Mycoplasma	Leafhopper	Throughout Asia
Orange leaf	Mycoplasma ?	Leafhopper	South and Southeast Asia, China, Sri Lanka
Chlorotic streak	?	Mealybug	India

Rice tungro virus complex

Tungro is transmitted by leafhopper species (23). A green leafhopper, Nephotettix virescens, is its most important vector. It can transmit the tungro agent immediately after acquisition feeding on an infected plant and can retain its infectivity for 3-6 d (22). On tungro-susceptible varieties, even under a relatively low leafhopper population, the disease can spread very fast.

Tungro is associated with two other viruses, rice tungro bacilliform virus (RTBV) and rice tungro spherical virus (RTSV) (12, 28) (RTSV was once cited as rice tungro virus) (6). RTSV causes no symptoms, but RTBVcauses typical tungro symptoms (9, 12). RTSV can be transmitted alone, but RTBV depends on RTSV for its transmission by the vector leafhopper (8, 12). Rice waika virus in Japan may actually be RTSV or certain1.y a close relative (32).

Rice grassy stunt virus

Grassy stunt is transmitted by the brown planthopper Nilaparvata lugens and two related species (19). The planthopper becomes infective at 1.east 5 d after acquisition access to grassy stunt-infected plants, and remains infective until death. The virus agent has only recently been clearly demonstrated: circular thread-like filaments isolated from infected plants are thought to be the rice grassy stunt virus (16, 17).

Rice ragged stunt virus

Ragged stunt virus (RRSV) was first reported in Indonesia and the Philippines in 1977 – 1978 (13, 24). It is transmitted by the brown

planthopper in the same manner as is RGSV (13). RRSV is polyhedral (14) and contains 10 segments of double-stranded RNA (27). Generally, both RGSV and RRSV occur together in the same field and the planthopper can transmit them either separately or together.

Rice hoja blanca virus

Rice hoja blanca virus (RHBV) is transmitted by the planthopper Sogatodes oryzicola in the same manner as RCSV is transmitted by the brown planthopper (1). RHRV is also transmitted from infective planthopper females to their progeny through eggs (7). RHEV is a thread-like filament (26) and may be in the same virus groups as RGSV (unpubl.).

Rice yellow mottle virus

Rice yellow mottle virus can be transmitted by mechanical means and also by adult beetles (2). The virus was first reported in Kenya in 1966 and then observed in Nigeria, Sierra Leone, Liberia, Ivory Coast, and Ghana (31).

CONTROLLING RICE VIRUS DISEASES

Rice virus diseases in the tropics are often not correctly diagnosed, or other unrelated problems are wrongly attributed to the viruses. Current diagnosis is based on symptoms and on transmission tests using suspected vector insects. Diagnosis based on symptoms is often inconclusive, as the symptoms of each virus are not always typical and physiological disorders or other pests can cause similar symptoms. The transmission test is laborious, takes almost 1 mo, and can be practised at only the few institutions where greenhouse and laboratory facilities are available. Serological tests will give conclusive diagnoses.

Rice virus diseases are controlled through the use of resistant varieties, insecticide application, and special cultivation methods. In the tropics, varietal resistance is the most practical and economical control measure. Insecticide is not always effective for virus control, and may also not be economical.

In 1981, a new strain of grassy stunt (RGSV 2) occurred in Laguna and Mindanao, Philippines (3, 10). RGSV 2 causes symptoms similar to those of tungro. Since the early 1970s, grassy stunt had been successfully controlled in the Philippines and other countries through the cultivation of improved resistant varieties (21). However, those varieties are not resistant to RGSV 2 (3), and thus far, no variety resistant to it has been identified. RGSV 2 can be controlled only by using brown planthopper-resistant varieties. It has become predominant at the IRRI farm; and similar strains have been reported in Taiwan. (4) and Thailand (5), and observed in Indonesia. A survey of RGSV 2 using a strain-specificantibody is urgently needed. RGSV 2 antiserum has

been produced, but is not specific to the strain (10).

Eight institutions in six countries have collaborated in a greenhouse study of rice variety reactions to tungro and the vector leafhopper. Results showed remarkable differences among locations, indicating the possible existence of different virus strains and vector biotypes (18, Table 2). Resistant varieties bred at IRRI may not be resistant outside the Philippines. Therefore, national tungro programs should be enhanced as much as collaborative programs on tungro-associated viruses and vectors.

AREAS NEEDING SEROLOGY

Diagnosis and assessment

Serological tests are invaluable for conclusive rice virus diagnoses and for reliable disease assessment. Traditionally, an experienced specialist must visit the problem area or the test field to diagnose or assess existing diseases. Serological tests, however, can be applied to dried leaf samples in the laboratory, even those mailed from fields outside the country (H. Hibino, unpubl.). At the moment, quarantine regulations in many countries preclude the import of fresh diseased materials. The serological test diagnosis by mail would help the country that lacks specialists.

Strain identification requires serological tests, so the mail service would permit a thorough and much-needed survey of virus strains.

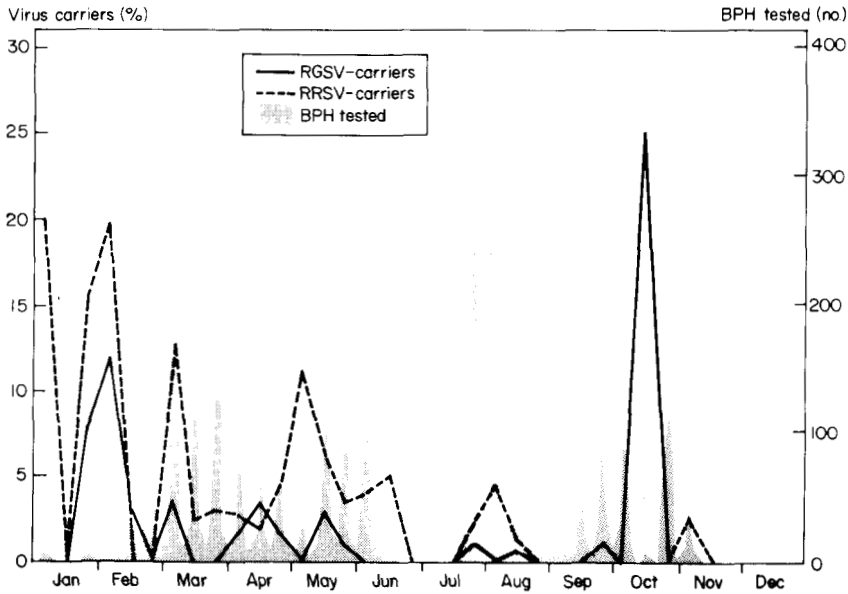
Monitoring virus-carrying insects

Vector insects must be monitored for virus disease outbreak predictions. In South and Southeast Asia, the brown planthopper is itself one of the most important rice pests and is also an important vector of RGSV and RRSV. The planthopper is a long-distance migrator (20); in tropical Asia, as it migrates throughout the year, it spreads the two viruses (Fig. 1).

Table 2. Varietal reaction to tungro virus (left letter) and tungro vector (right letter) at 8 locations in 6 countries (RTV collaborative project 1978-1983, IRRI).

Country	Station	Ambe-mohar	Gam Pai	Habi-ganj	IR26	IR36	Katarib-hog	Lati-sail	Pankhari 203	Ptb 18	TN1
India	AICRIP [a]	II	IR	II	II	II	II	II	II	II	SS
	BCKVV[b]	IS	II	RI	SI	II	RS	SS	RI	IR	SS
	CRRI[c]	SS	SI	RS	SS	SR	RS	SS	RS	RR	SS
Bangladesh	BRRI[d]	IS	II	RS	IS	II	RS	SS	RS	RR	SS
Thailand	RPB[e]	II	II	II	II	SI	SI	II	IR	IR	SS
Malaysia	MARDI [f]	II	II	RS	SS	II	RS	IS	RR	IR	SS
Indonesia	MORIF [g]	II	II	RS	SS	IR	II	IS	RI	RR	SS
Philippines	IRRI[h]	SS	IR	RS	SI	IR	IS	II	RI	IR	SS

[a]All India Coordinated Rice Improvement Project. [b]Bidan Chandra Krishi Viswa Vidyalaya. [c]Central Rice Research Institute. [d]Bangladesh Rice Research Institute. [e]Rice Pathology Branch, Department of Agriculture. [f]Malaysian Agricultural Research and Development Institute. [g]Maros Research Institute for Food Crops. [h]International Rice Research Institute.



1. Variation of RGSV and RRSV carriers in migrating brown planthoppers caught by high beam trap in 1983 at IRRI, Laguna, Philippines. Individual planthoppers were tested for the presence of both viruses in ELISA.

Planthoppers collected with nets or light traps are never tested for viruses, because they die before they are retrieved. Serological tests bypass this problem because they can detect the viruses even in dead planthoppers (11).

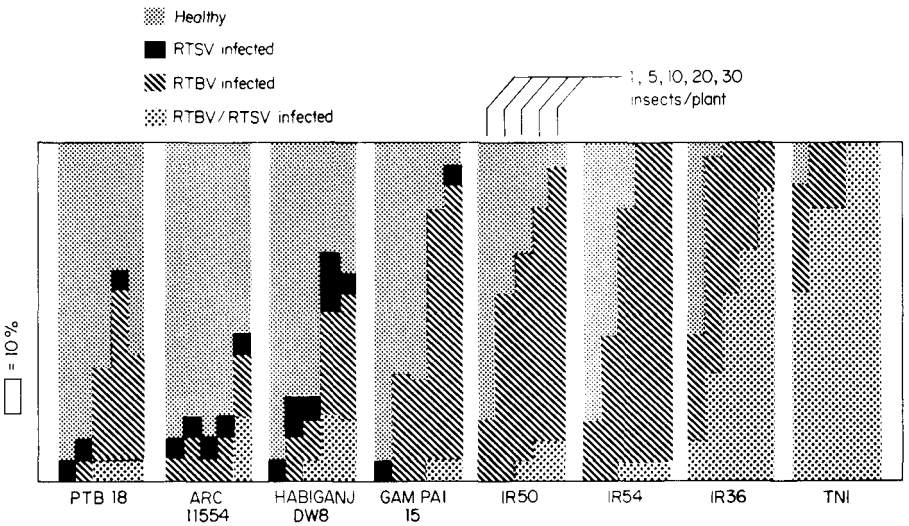
Serological tests generally identify virus carriers more efficiently than do infectivity tests, and can therefore monitor virus-carrying insects in the field. However, the tungro viruses and the yellow mottle virus are difficult to identify through serologic tests because they are not propagated within the vector insects.

Evaluation of varieties

Virus-resistant varieties, the most important virus control measure in the tropics, are generally evaluated for percentage of infection from visible symptoms. In tungro, this method is not desirable; instead, the varieties must be evaluated by serological test for the presence of RTBV and RTSV (Fig. 2).

MEED FOR MONOCLONAL ANTIBODY TECHNOLOGY

Since 1982, serological tests have been regularly applied at IRRI in diagnosis and disease assessment, epidemiology, tungro-resistance evaluation, and basic research (10, 15). National institutions have now asked IRRI for antisera and expect it to strengthen the collaborative research network for rice viruses and varietal resistance. Purification of rice viruses is



2. Reaction of tungro-resistant varieties to rice tungro virus complex when inoculated by varying num bers of viruliferous insects per plant, IRRI. IR36 is an intermediate check and TN1 a susceptible check Leaf samples were tested for the presence of rice tungro bacilliform (RTBV) and rice tungro spherical (RTSV) viruses by latex test using antisera.

difficult, and only a few institutions have produced antisera usable in diagnosing diseases. IRRI produces, for its own use, conventional rice virus antisera but because the amount is limited, all requests cannot be met. In addition, conventional antisera present two major difficulties for international use: they differ in quality, depending on the batch, and they cannot be strain specific. Monoclonal antibody technology can solve these problems, and lead to better control of rice virus diseases.

REFERENCES CITED

1. Acuna, J., L. Ramos, and V. Lopez. 1958. Sogatoda oryzicola Muin, .vector de la enformeded virosa hoja blanca del arroz en Cuba. Agrotecnica 13:23-24.
2. Bakker, W. 1970. Rice yellow mottle, a mechanically transmissible virus disease of rice in Kenya. Neth. J. Plant Pathol. 76:53-63.
3. Cabauatan, P.Q., and H. Hibino. 1983. Unknown disease of rice transmitted by the brown planthopper in the Philippines. Int. Rice Res. Newsl. 8(2):12-13.
4. Chen, C.C., and R.J. Chiu. 1982. Three symptomatologic types of rice virus diseases related to grassy stunt in Taiwan. Plant Dis. 66:15-18.
5. Disthaporn, S., D. Chettanachit, and M. Putta. 1983. Unknown virus-like rice diseases in Thailand. Int. Rice Res. Newsl. 8(6):12.

6. Galvez, G.E. 1968. Purification and characterization of rice tungro virus by analytical density-gradient centrifugation. Virology 25:418-426.
7. Galvez, G.E. 1969. Transmission of hoja blanca virus of rice. Pages 155-163 in The virus diseases of the rice plant. Proceedings of a symposium at the International Rice Research Institute, April, 1907. The Johns Hopkins Press, Baltimore, Maryland.
8. Hibino, H. 1983. Transmission of two rice tungro-associated viruses and rice waika virus from doubly or singly infected source plants by leafhopper vectors. Plant Dis. 67:774-777.
9. Hibino, H. 1983. Relations of rice tungro bacilliform and rice tungro spherical viruses with their vector Nephotettix virescens. Ann. Phytopathol. Soc. Jpn. 49:545-553.
10. Hibino, H., and P. Cabauatan. 1983. Purification and serological properties of rice grassy stunt virus. 2. Int. Rice Res. Newsl. 8(6):11-12.
11. Hibino, H., and I. Kimura. 1982. Detection of rice ragged stunt virus in insect vectors by enzyme-linked immunosorbent assay. Phytopathology 72:656-659.
12. Hibino, H., M. Roechan, and S. Sudarisman. 1978. Association of two types of virus particles with penyakit habang ltungro disease) or rice in Indonesia. Phytopathology 68:1412-1416.
13. Hibino, H., M. Roechan, S. Sudarisman, and D.M. Tantera. 1977. A virus disease of rice (kerdil hampa) transmitted by brown planthopper, Nilaparvata lugens Stal., in Indonesia. Contrib. Cent. Res. Inst. Agric. 35. 15p.
14. Hibino, H., N. Saleh, and M. Roechan. 1979. Reovirus particles associated with rice ragged stunt diseased rice and insect vector cells. Ann. Phytopathol. Soc. Jpn. 45:228-239.
15. Hibino, H., E.R. Tiongco, and R.C. Cabunagan. 1983. Rice tungro virus complex in tungro resistant IR varieties. Int. Rice Res. Newsl. 8(4):12-13.
16. Hibino, H., T. Usugi, and T. Omura. 1983. Morphology and serological relationship of grassy stunt-associated filamentous nucleoprotein and rice stripe virus. Int. Rice Res. Newsl. 8(1):9-10.
17. Hibino, H., T. Usugi, T. Tsuchizaki, M. Iwasaki, and S. Izumi. 1983. Purification and properties of grassy stunt-associated filamentous particles. Int. Rice Res. Newsl. 8(2):11.
18. IRRI (International Rice Research Institute). 1981. Annual report for 1980. Los Baños, Philippines. 467 p.
19. Iwasaki, M., M. Nakano, and A. Shinkai. 1980. Transmission of rice grassy stunt disease by Nilaparvata muiri and N. bakeri. Ann. Phytopathol. Soc. Jpn. 46:411. (Abstract in Japanese).
20. Kishimoto, R. 1976. Synoptic weather conditions inducing long-distance immigration of planthoppers, Sogatella

furcifera Horvath and Nilaparvata lugens Stal. Ecol. Entomol. 1:95-107.

21. Khush, G.S., and K.C. Ling. 1974. Inheritance of resistance to grassy stunt virus and its vector in rice. J. Hered. 65:134-136.
22. Ling, K.C. 1966. Nonpersistence of the tungro virus of rice in its leafhopper vector, Nephotettix impicticeps. Phytopathology 56:1252-1256.
23. Ling, K.C. 1972. Rice virus diseases. International Rice Research Institute, Los Banos, Philippines. 142 p.
24. Ling, K.C., E.R. Tiongco, and V.M. Aguiero. 1978. Rice ragged stunt, a new virus disease. Plant Dis. Rep. 62:701-705.
25. Malaguti, G., H.C. Diaz, and N. Angeles. 1957. La virosis "hoja blanca" del arroz. Agron. Trop. 6:157-163.
26. Morales, F.J., and A.I. Niessen. 1983. Association of spiral filamentous virus-like particles with rice hoja blanca. Phytopathology 73:971-974.
27. Omura, T., Y. Minobe, I. Kimura, H. Hibino, T. Tsuchizaki, and Y. Saito. 1983. Improved purification procedure and RNA segments of rice ragged stunt virus. Ann. Phytopathol. Soc. Jpn. 49:670-675.
28. Omura, T., Y. Saito, T. Usugi, and H. Hibino. 1983. Purification and serology of rice tungro spherical and rice tungro bacilliform viruses. Ann. Phytopathol. Soc.: Jpn. 49:73-76.
29. Rivera, C.T., and S.H. Ou. 1965. Leafhopper transmission of "tungro" disease of rice. Plant Dis. Rep. 49:127-131.
30. Rivera, C.T., S.H. Ou, and T.T. Iida. 1966. Grassy stunt disease of rice and its transmission by the planthopper Nilaparvata lugens Stal. Plant Dis. Rep. 50:453-456.
31. Rossel, H.W., G. Thottappilly, and I.W. Buddenhagen. 1982. Occurrence of rice yellow mottle virus in two important rice-growing area of Nigeria. FAO Plant Prot. Bull. 30 (3/4):137-139.
32. Saito, Y. 1977. Interrelationship among waika disease, tungro and other similar diseases of rice in Asia. Trop. Agric. Res. Ser. 10.

BIOTECHNOLOGY AT ILRAD

V. M. NANTULYA

ILRAD's target diseases, East coast fever (ECF) and trypanosomiasis, are believed responsible for the deaths of 3 million cattle a year. In Africa alone, they restrict livestock from 7 million km^2 of land that would probably support 120 million cattle and equivalent numbers of small ruminants. Through monoclonal antibody technology, tissue culture, and molecular biology, fundamental understanding of many aspects of these two diseases has been significantly expanded. In addition, development of a vaccine against ECF seems likely.

The growth of world population exerts increasing pressure on the livestock industry for more proteins and other animal products. Yet livestock production already suffers numerous drawbacks, including disease. The parasitic diseases African trypanosomiasis and theileriosis are considered responsible for the deaths of 3 million cattle a year. A form of theileriosis, East Coast fever (ECF), alone kills an estimated 0.5 million cattle a year in East Africa.

African trypanosomiasis is encountered throughout tropical Africa wherever the tsetse fly vector is present. This area of more than 7 million km^2 stretches across the middle of Africa between latitudes 12° North and 20° South. It is almost devoid of a viable livestock industry, and is agriculturally underutilized as well.

ECF (like trypanosomiasis) is caused by a protozoan parasite, *Theileria parva*. It occurs in Kenya, Uganda, Tanzania, Rwanda, Burundi, Malawi, Zambia, Zaire, and Mozambique, and puts approximately 20 million cattle at risk. The brown ear tick vector *Rhipicephalus appendiculatus* extends over an even wider area, including Zimbabwe, Botswana, and much of Southern Africa, and its further spread is possible. Introduced high-grade cattle are particularly susceptible to ECF, while indigenous breeds often show significant innate or acquired immunity. The presence of ECF frustrates the efforts to upgrade local breeds- by introducing improved cattle, because the morbidity and mortality rates in the fully susceptible breeds approach 100%.

Head, Parasitology, International Laboratory for Research on Animal Diseases, P. O. Box 30709, Nairobi, Kenya.

Approximately one third of tropical Africa is infested to some degree by one or more species of the tsetse fly. These flies occupy extensive light woodland and savannah with good rainfall, and prevent the development of the areas for livestock production and mixed farming enterprises. Better control of the tsetse fly would mean better control of trypanosomiasis and would result in markedly increased food-production opportunities. The present cattle population in Africa, located mainly in tsetse-free areas, is approximately 160 million. It has been estimated that the region could support a further 120 million cattle and many more sheep and goats if tsetse flies and trypanosomiasis could be controlled.

Actual cattle mortality figures caused by ECF in Eastern and Central Africa are not available, but in Kenya, where the cattle population is 11 million, about 70 000 animals over 1 year old succumb to the disease annually. The disease also reduces productivity and growth rate in many of the animals surviving an attack.

Neither the vectors of trypanosomiasis and ECF nor the parasites can be completely eliminated from the reservoir hosts, but the International Laboratory for Research on Animal Diseases (ILRAD) is trying to develop immunological control measures for the two diseases.

Monoclonal antibody technology, molecular biology, and tissue culture have helped make prospects for a vaccine against ECF brighter.

PROGRESS IN DEVELOPING A VACCINE AGAINST EAST COAST FEVER

Cattle that recover, spontaneously or by chemotherapy, acquire resistance against reinfection (21). Serum from animals recovering from a tick-transmitted infection contain antibodies that neutralize, in vitro, the sporozoites of the infecting strain (14). Cross-testing sera from animals infected with different strains has shown that the antibodies against sporozoites of one strain also neutralize the infectivity of sporozoites of several other *T. parva* strains isolated from such diverse geographical localities as Uganda, Kenya, and Tanzania, including the buffalo strain *T. parva lawrencei* (15). This cross-reactivity was confirmed in vivo (15).

In subsequent studies using hybridoma technology, monoclonal antibodies against sporozoites of one strain were raised and tested against sporozoites of other *T. parva* strains. All the monoclonal antibodies neutralized sporozoites of the several *T. parva* strains so far examined, and by competitive inhibition enzyme-linked immunosorbent assay (ELISA), all the monoclonal antibodies reacted to the same antigenic epitope on the sporozoite surface (15). These . results have led to the conclusion that a common protective, antigenic determinant exposed on the surface of all *T. parva* strains exists. This information is critical for developing a vaccine. We are immunizing cattle with sporozoite lysates and will identify and biochemically characterize the relevant asntigen(s). *Theileria*

parva genes are being cloned in microbial systems for large-scale production of protective antigen(s). In addition, studies aimed at translating T. parva mRNA in both reticulocyte and wheat germ for in vitro antigen synthesis are under way.

This approach has yielded vaccines against a variety of diseases of man and animals, particularly viral diseases (3), but also parasitic diseases (5, 7). One example is foot-and-mouth disease (FMD) (1, 2, 3, 4, 12), a highly contagious, economically important disease affecting domestic and wild ruminants throughout South America, Africa, and the Far East. Although the mortality it causes seldom exceeds 5% in adult animals, it can reach 50% in young cattle and swine, in which it causes myocarditis. FMD also often induces deformities of the feet, abortion, and mastitis.

Several serotypes and subtypes of the FMD virus exist. The disease is primarily controlled through the slaughter and sanitary disposal of infected or exposed herds, or through intensive vaccination programs. Vaccines are produced primarily in the country where they will be used. Inactivating virus grown in baby hamster kidney cells or bovine tongue epithelium, and attenuated virus have been used to produce them. FMD outbreaks are frequently linked to incompletely inactivated vaccine or to escape of virus from research and production facilities. Whole-virus vaccine preparations are unstable and require cold storage.

Progress in developing a subunit FMD vaccine started with Bachrach et al's observation (2) that an isolated capsid protein of the FMD virus could elicit large amounts of neutralizing antibody and protect cattle and swine against challenge exposure. Later, by recombinant DNA technology, the gene coding for this FMD protective surface protein was cloned into E. coli. The protein synthesized by the bacteria had similar protective properties (12).

The FMD subunit vaccine is the first effective vaccine produced through gene splicing for any human or animal disease. The initial cloned vaccine was for one FMD virus serotype, but the same strategy is being modified and applied to others.

Another method to prepare new subunit vaccines for animals is chemical peptide synthesis. A 13-kilodalton (kd) fragment cleaved by cyanogen bromide from native FMD virus protein can also protect immunized livestock (3). A polypeptide corresponding to the 13-kd fraction was synthesized (4) and it protected test animals against live FMD virus. Most synthetic vaccines are purified by monoclonal antibodies. Chemically synthesized peptides may help screen for peptides that act as test vaccines; the DNA corresponding to these fragments can then be cloned for large-scale vaccine production in microbial systems.

APPLICATION OF HYBRIDOMA TECHNOLOGY

To diagnose most infectious diseases, the causative organism or a specific immune response to the causative organism must be recognized; for many diseases, this is not possible. Monoclonal

antibodies are particularly useful in these cases in assays for unique antigens on particular organisms. Besides, a suitable monoclonal antibody can be indefinitely reproduced, and provide a standardized reagent. Unlike polyclonal antisera this does not require periodic immunization of different batches of animals to obtain more sera nor subsequent absorption to remove cross-reactivity.

Effort has therefore been made to raise species-specific monoclonal antibodies against three African trypanosomes species infective to livestock, and design assays to detect species-specific antigens in the mammalian host and in the infected vector. In addition, the species-specific monoclonal antibodies are being used to identify, isolate, and purify the corresponding antigens for use in serodiagnosis. Our ultimate goal is to prepare enough relevant antigens, biochemically or otherwise, to provide standardized reagents for other investigators. Anti-idiotypic monoclonal antibodies against the species-specific monoclonal antibodies will be made for possible substitution of the antigens with anti-idiotypic monoclonal antibodies.

Monoclonal antibody technology has also been used to type bovine lymphocyte populations and subpopulations (18, 19, 20). The reagents have helped characterize the type of cell infected by *Theileria parva* (17). Furthermore, monoclonal antibodies against *T. parva* intralymphocytic developmental stages (the macroschizonts) have revealed that distinct strains of *T. parva* exist (11, 17). Finally, monoclonal antibodies are being used to analyze metacyclic trypanosomes (16).

IN VITRO PROPAGATION OF AFRICAN TRYPANOSOMES

After the pioneering work of Hirumi et al (8) on *Trypanosoma brucei*, complete developmental cycles of *T. congolense* and *T. vivax* have been established in vitro (9, 10).

In vitro propagation of *T. congolense* has been attempted 1) to establish a standardized in vitro method for semilarge-scale production of *T. congolense* metacyclics; 2) to compare the biological and immunological properties of the in vitro-derived metacyclics with those from tsetse; and 3) to develop technology for continuous in vitro propagation of *T. congolense* bloodstream forms.

An in vitro system that supports the semilarge-scale production of *T. congolense* metacyclics has been developed and standardized (10). Eight lines of metacyclic-producing cultures have been established: six lines from three cloned and three uncloned stocks. Biological and immunological characterization of the in vitro-derived metacyclics revealed that they are morphologically identical to those grown in tsetse flies; retain the ability to induce local skin reactions in cattle and goats; have a surface coat; and are infective to cattle, goats, and mice. The in vitro-derived metacyclics also displayed the antigenic composition of tsetse-derived metacyclics (revealed by

indirect immunofluorescence neutralization and cross-protection) (10).

In vitro-derived metacyclics were successfully transformed to hematozoic trypomastigotes when transferred to a bovine endothelial cell culture, using HEPES-buffered MEM supplemented with 15% FBS and 5% fetal goat serum, and they continued to multiply for over 90 d at 28°C. They were transformed into bloodstream forms and lost the ability to produce local skin reaction by day 7. However, they retained infectivity to goats and mice, were coated with surface glycoproteins, were neutralized by antiserum raised against tsetse-derived metacyclics, and displayed the antigenic composition of tsetse-derived metacyclics up to 55 d in vitro (10).

An in vitro system allowing continuous growth of animal-infective hematozoic T. congolense trypomastigotes has been recently established (9). The cultures were begun by inoculating wells (Costar Tissue Culture Cluster^{24}, 24-wells/ /plate) with bloodstream forms derived from infected mouse blood (1 x 10^6 trypanosome/ml per well). The trypanosomes were continuously cultivated in the presence of bovine endothelial cells using a HEPES (25 mM)-buffered RPMI 1640 with 20% heat-inactivated adult goat serum. From day 20, trypanosomes were transferred to T-25 plastic culture flasks containing bovine endothelial cells, and maintained in the same medium. Trypanosomes grown under these conditions multiplied for over 90 d and retained all the characteristics of hematozoic trypomastigotes examined.

We have achieved complete cyclic development of Trypanosoma vivax in vitro (10).

APPLYING MOLECULAR BIOLOGY TO ANTIGENIC VARIATION IN AFRICAN TRYPANOSOMES

Unlike T. parva, African trypanosomes have an elaborate method of escape from the immune responses of the infected host. They change their surface coats, which carry the protective antigens, apparently without limit. Antibodies against one surface coat type do not confer protection against other surface types. We do not know how many different antigenic types can evolve from a single trypanosome, but it may be more than 100. The regulation and control of trypanosome gene expression is being investigated to learn the mechanism that triggers the surface coat synthesis (6, 13, 22-27).

Attention has also been focused on generating molecular hybridization reagents for epidemiological studies of T. congolense and T. vivax. Complementary DNA (cDNA) copies of mRNA isolated from four different clones of T. congolense have been synthesized for a library.

These VSG cDNAs are being used in genomic blot analyses to study antigenic variation in T. congolense. The T. congolense cDNA library was also screened for sequences similar to the T. brucei miniexon. One cDNA has been identified, and may be a

useful hybridization probe to identify T. congolense, T. brucei, and T. vivax. Combined with certain restriction enzymes, this cDNA probe can differentiate these trypanosome species since it hybridizes to different size fragments in each. Such a probe would be of taxonomic value in analyses of trypanosome field isolates.

GENETIC RESISTANCE TO AFRICAN TRYPANOSOMIASIS

Because of the ability of the African trypanosome to undergo antigenic variation, alternatives to control by vaccine must be considered. Increasing attention is, therefore, being paid to the genetic basis for the better tolerance in some cattle breeds and the wild bovidae for the pathogenetic effects of trypanosoma1 infections. The ultimate goal here is for laboratory assays to identify resistance genes and then ultimately transfer them into the cattle germ line. In addition to screening local (East African) cattle breeds, ILRAD has recently acquired, through embryo transfer, the reportedly resistant West African Ndama.

REFERENCES CITED

1. Bachrach, H.L. 1982. Recombinant DNA technology for the preparation of subunit vaccines. J. Am. Vet. Med. Assoc. 181:992-999.
2. Bachrach, H.L., D.M. Moore, P.D. Mc Kercher, and J. Polatnick. 1975. Immune and antibody responses to an isolated capsid protein of foot-and-mouth disease virus. J. Immunol. 115:1636-1641.
3. Bachrach, H.L., D.O. morgan, P.D. Mc Kercher, D.M. Moore, and B.H. Robertson. 1982. Foot-and-mouth disease virus: immunogenicity and structure of fragments derived from capside protein VP_3 and of virus containing cleaved VP_3. Vet. Microbiol. 7:85-96.
4. Bittle, J.L., R.A. Houghten, H. Alexander, T.M. Shinnick, J.G. Sutcliffe, R.A. Lerner, D.J. Rowlands, and F. Brown. 1982. Protection against foot-and-mouth disease by immunization with a chemically synthesized peptide predicted from the viral nucleotide sequence. Nature 298:30-33.
5. Chochrane, A.H., F. Santoro, V. Nussenzweig, R.W. Gwadz, and R.S. Nussenzveig. 1982. Monoclonal antibodies identify the protective antigens of sporozoites of Plasmodium knowlesi. Proc. Natl. Acad. Sci. USA 79:5651-5655.
6. Donelson, J.E., P.A.O. Majiwa, and R.O. Williams. 1979. Kinetoplast DNA minicircles of Trypanosoma brucei share regions of sequence homology. Plasmid 2:572-588.

7. Ellis, J., L.S. Ozaki, R.W. Gwadz, A.H. Cochrane, R.S. Nussenzweig, and G.N. Godson. 1983. Cloning and expression in E. coli of the malarial sporozoite surface antigen gene from Plasmodium knowlesi. Nature (London) 302:536-538.
8. Hirumi, H., J.J. Doyle, and K. Hirumi. 1977. African trypanosomes: cultivation of animal-infective forms of Trypanosoma brucei in vitro. Science 196:992-994.
9. Hirumi, H., and K. Hirumi. 1984. T. congolense bloodstream form culture. Ann. Trop. Med.Parasito1. (in press)
10. Hirumi, H., K. Hirumi, R.T. Nelson, V. Nantulya, S.K. Moloo, R. Dwinger, A. Musoke, G.W.O. Akol, and M. Murray. 1983. Present status of the in vitro cultivation of African trypanosomes. In Current status of unresolved problems in parasitic diseases: U.S.A. - Japan Joint Symposium, July 18-19, 1983. N.I.H., Bethesda, Maryland (unpubl. proc.)
11. Irvin, A.D., D.A.E. Dobbelaere, D.M. Mwamachi, T. Minami, P.R. Spooner, and J.G.R. Ocama. 1983. Immunization against East Coast fever: correlation between monoclonal antibody profiles of Theileria parva stocks and cross immunity in vivo. Res. Vet. Sci. 35:341-346.
12. Kleid, D.G., D. Yansura, B. Small, D. Dowbenko, D.M. Moore, M.J. Grubman, P.D. Mc Kercher, D.O. Morgan, B.H. Robertson, and H.L. Bachrach. 1981. Cloned viral protein vaccine for foot-and-mouth disease: responses in cattle and swine. Science 214:1125-1129.
13. Laurent, M., E. Pays, E. Magnus, N. van Meirvenne, G. Matthyssens, R.O. Williams, and M. Steinert. 1983. DNA rearrangements linked to expression of a predominant surface antigen gene for trypanosomes. Nature 302:263-266.
14. Musoke, A.J., V.M. Nantulya, G. Buscher, R.A. Masake, and B. Otim. 1982. Bovine immune response to Theileria parva: neutralizing antibodies to sprozoites. Immunology 45:663-668.
15. Musoke, A.J., V.M. Nantulya, F.R. Rurangirwa, and G. Buscher. 1984. Evidence for a common protective antigenic determinant on sporozoites of several Theileria parva strains. Immunology 52. (in press)
16. Nantulya, V.M., A.J. Musoke, S.K. Moloo, and J.M. Ngaira. 1983. Analysis of the variable antigen composition of Trypanosoma brucei brucei metacyclic trypanosomes using monoclonal antibodies. Acta Trop. 40:19-24.
17. Pinder, M., and R.S. Hewett. 1980. Monoclonal antibodies detect antigenic diversity in Theileria parva parasites. J. Immunol. 124:l000-1001.
18. Pinder, M., A.J. Musoke, W.I. Morrison, and G.E. Roelants. 1980. The bovine lymphoid system. 3. A monoclonal antibody specific for bovine cell surface and serum IgM. Immunology 40:359-365.
19. Pinder, M., T.W. Pearson, and G.E. Roelants. 1980. The bovine lymphoid system. 2. Derivation and partial characterization of monoclonal antibodies against

bovine peripheral blood lymphocytes. Vet. Immunol. Immunopathol. 1:303-316.

20. Pinder, M., and G.E. Roelants. 1981. The nature of the lymphocyte subpopulation transformed by Theileria parva. Pages 348-350 in Advances in the control of theileriosis. A.D. Irvin, M.P. Cunningham, and A.S. Young, eds. Martinus Nijhoff Publishers, The Hague.
21. Radley, D.E., A.S. Young, C.G.D. Brown, M.J. Burridge, M.P. Cunningham, F.L. Musisi, and R.E. Purnell. 1975. East Coast fever. 11. Cross-immunity trials with a Kenya strain of Theileria lawrencei. Vet. Parasitol. 1:43-50.
22. Williams, R.O., K.B. Marcu, J.R. Young, L. Rovis, and S.C. Williams. 1978. A characterization of mRNA activities and their sequence complexities in Trypanosoma brucei: partial purification and properties of the VSSA mRNA. Nucleic Acids Res. 5:3171-3182.
23. Williams, R.O., J.R. Young, and P.A.O. Majiwa. 1979. Genomic rearrangements correlated with antigenic variation in Trypanosoma brucei. Nature 282:847-849.
24. Williams, R.O., J.R. Young, and P.A.O. Majiwa. 1982. Analysis of genomic rearrangements associated with two variable antigen genes of Trypanosoma brucei. Nucleic Acids Res. 10:803-819.
25. Williams, R.O., J.R. Young, P.A.O. Majiwa, J.J. Doyle, and S.Z. Shapiro. 1980. Analyses of variable antigen gene rearrangements in Trypanosoma brucei. Am. J. Trop. Med. Hyg. 29:1037-1042.
26. Williams, R.O., J.R. Young, P.A.O. Majiwa, J.J. Doyle, and S.Z. Shapiro. 1981. Contextual genomic rearrangements of variable-antigen genes in Trypanosoma brucei. Cold Spring Harbor Symposia on Quantitative Biology 45:945-949.
27. Young, J.R., J.S. Shah, G. Matthyssens, and R.O. Williams. 1983. Relationship between multiple copies of a T. brucei variable surface glycoprotein gene whose expression is not controlled by duplication. Cell 32:1149-1159.

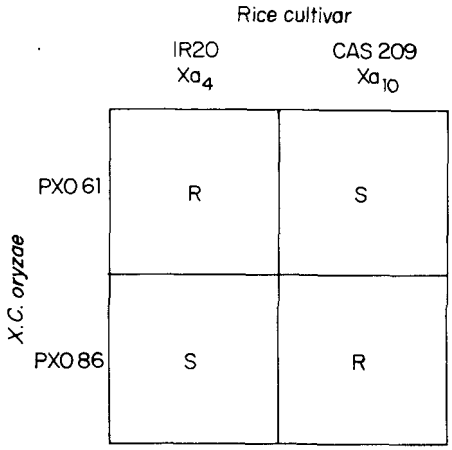
GENETIC ENGINEERING FOR DISEASE RESISTANCE: THE MOLECULAR BIOLOGY OF PATHOGENICITY IN BACTERIAL BLIGHT OF RICE

J. A. CALLOW

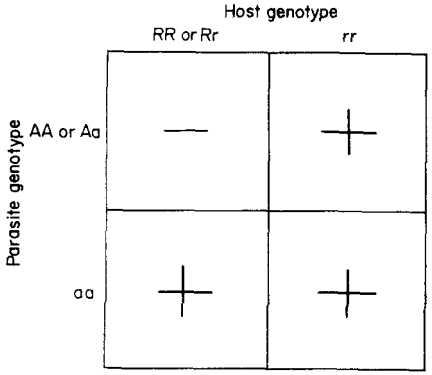
Despite considerable attention in recent years, the primary products of host genes for resistance and parasite genes for avirulence and the way in which these products interact to initiate resistance are essentially unknown. One of the priorities of fundamental research in agriculture and plant molecular biology must therefore be to determine gene function and expression in the resistant interaction. Recent advances in molecular genetics, notably recombinant DNA technology, now permit the phytopathologist to approach plant disease from an entirely new perspective. This paper describes how techniques of gene manipulation may be applied to the interaction between cultivars of rice and Xanthomonas campestris pv. oryzae; first, to isolate and clone the genes controlling cultivar specificity in the pathogen; and second, to determine the products of those genes. This is an important first step in isolating the products of complementary host genes for resistance, which will permit the isolation and cloning of these genes and, ultimately, their use as resistance sources in crop plants.

Bacterial blight is an important rice disease caused by the gram -ve bacterium Xanthomonas campestris pv. oryzae (9). Like most other phytopathogens, natural isolates fall into groups usually distinguished only by their reaction on certain host plant cultivars or "differentials." In our studies, the following pattern of interactions prevails (Fig. 1). A much wider pattern of differential interactions between cultivars and bacterial isolates has been studied at IRRI (9) and this type of evidence has been taken to suggest that there is a gene-for-gene relationship in this interaction, i.e., for every host gene controlling resistance or susceptibility, there is a corresponding, or complementary, gene in the parasite controlling virulence or avirulence. This relationship is expressed in the well-known quadratic check (Fig. 2), in which it is clear that resistance gene expression is conditional on the expression of the

Mason Professor of Botany and Head, Department of Plant Biology, University of Birmingham, P. O. Box 363, Birmingham, P. O. Box 363, Birmingham B15 2TT.



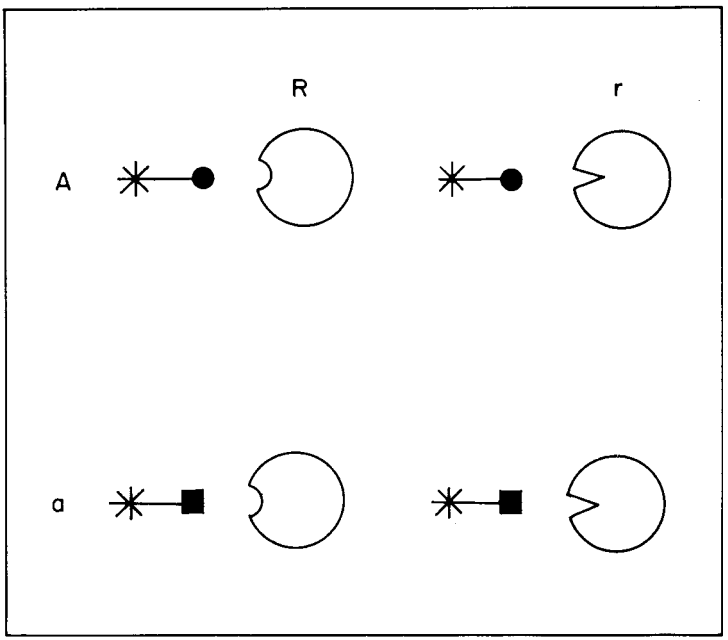
1. pattern of interactions between *X. campestris* pv. *oryzae* races (PX061 and PX086) and rice cultivars (IR20 and CAS209). and host resistance genotypes. R = resistant interactions, S = susceptibility.



2. The quadratic check in gene-for-gene interactions. R and r represent host genes for resistance (dominant) and susceptibility (recessive). A represents genes for avirulence (dominant) and a, genes for virulence (recessive) in a diploid or dikaryotic parasite. Compatible response is denoted by + and incompatible by –.

allele for avirulence in the parasite and that incompatibility is the positive function, resulting from the expression of the dominant genes for resistance (R) and avirulence (A) and the association of their gene products. Another way of putting this is that specific recognition between host and parasite is for incompatibility; a simple molecular model that expresses this is shown in Figure 3. (See Ellingboe (7) and Day (4) for further elaboration of this theme.) At present, despite all efforts to understand the biochemical basis of resistance and of the recognition mechanisms that control it, we do not understand what the primary products of genes for resistance and avirulence are. I believe that future attempts to create novel sources of resistance in plants through genetic engineering will require this detailed molecular information, and it is widely acknowledged that a major aim of fundamental agricultural research is to elucidate the molecular basis of specificity and its genetic control.

How are we to go about this? There are in effect two basic approaches. The first, the "outside-in" approach, requires that we biochemically isolate the products of the genes for avirulence and resistance. Using this widely adopted approach the researcher may, for example, attempt to isolate from the pathogen surface molecules which, when applied to the host plant, elicit the appropriate resistance response (I). Despite intense effort by many, this approach has yet to provide clear answers. Many parasite molecules able to induce host defense reactions have been examined, but not one of them possesses the specificity of action required by the gene-for-gene relationship. Another approach is to examine structural variability in those molecules believed to play a role in pathogenicity. I would like to exemplify this approach by referring to some of our own studies on the extracellular polysaccharide of pathovars of Xanthomonas campestris, because it highlights an area where the tools of biotechnology could make a dramatic impact.

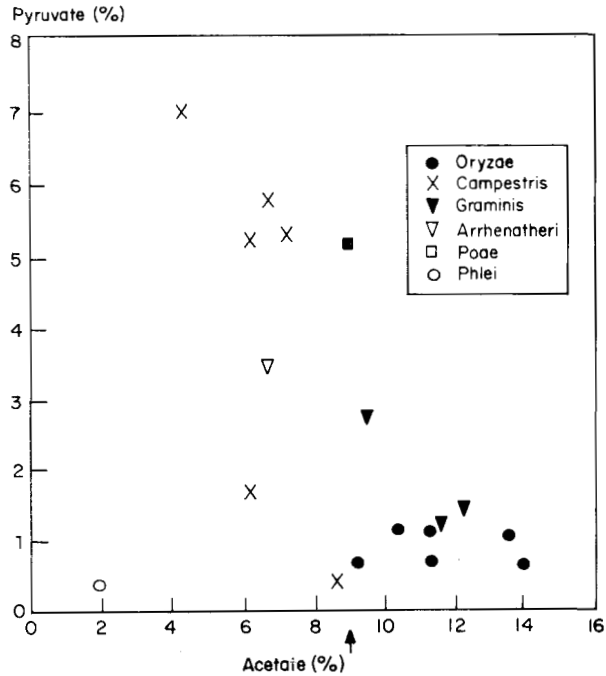


3. Simple lock-key interpretation of the molecular implications of the gene-for-gene concept. Products of host R/r alleles are shown as surface-localized receptors with defined binding-site configurations. Products of parasite avirulence/virulence alleles are shown as molecules with a substantially common structure but specific recognized termini. Only in the case R/A can correct binding and recognition take place, triggering a resistant response.

CHEMICAL VARIATION IN XANTHAN POLYSACCHARIDE

The EPS of Xanthomonads is known as xanthan. The basic molecular organization of xanthan (11) is that of a ß1,4-linked, cellulosic backbone; glucosyl residues carry trisaccharide side chains containing two mannosyl and one glucuronic acid residue. The two mannosyl residues are variably substituted with either acetate (proximal mannose to backbone) or pyruvate (distal mannose residue). The pattern of pyruvylation and acetylation determines to a large extent both the tertiary, conformational structure of the xanthan molecule, i.e. its "shape," and its potential association with polymers in the plant cell wall (11).

We have attempted to examine structural variation in xanthans from different pathovars of X. campestris, and from different strains of the pathovar specific for rice, in terms of both chemical and immunochemical variation (12). Briefly, between pathovars there is no variability in the cellulosic backbone, nor in the nature of the trisaccharide side chains. What seems to vary is the degree of pyruvylation and acetylation (Fig. 4). The xanthans of all oryzae strains examined to date are characterized by a high acetate and low pyruvate xanthan. In fact, the degree of acetylation is such that a second acetylation on some trisaccharide side chains has to be postulated. Now, what



4. Degrees of pyruvylation and acetylation in the xanthans of different pathovars and strains of *Xanthomonas campestris* The arrow indicates the level of acetylation at which a 2nd acetate group per side chain needs to be postulated. (Data include information from Parry [12].)

we are not saying is that the relative contents of acetate and pyruvate in the EPS of different pathovars correlate precisely with pathological specificity, and indeed there are pathovars such as pv. graminis that have xanthans with similar levels of substitution, but quite different biological specificity. Of course, the measurement of gross levels of acetate and pyruvate does not provide detailed views of this aspect of structural variation between pathovars in that there could be, for example, several different distributions of the pyruvate ketal. While it is possible to improve polymer 'definition' by chemical means, we have adopted an immunological approach.

Antibodies to xanthans of several pathovars have been raised. All antisera so raised have been pathovar specific, i.e., antisera raised against an oryzae xanthan only reacts with oryzae xanthan antigen; it does not cross-react with xanthans of other pathovars, even if they have a similar gross pyruvate and acetate content. This has been tested in a reciprocal manner (12). However, what we have been unable to detect using polyclonal antisera is any immunological specificity between races of the pathovar oryzae. Now it could be that there is no intrapathovar variation and that the interpathovar variation observed merely indicates pathogenic specificity at the level of EPS structure. If so, intrapathovar specificity presumably resides in some other feature or activity

of the pathogen. However, it is equally possible that there is structural variation between races of oryzae, but because we are using polyclonal antisera, it is masked. The implication of this, and where biotechnology is important, is that monoclonal antisera might reveal the extent of variation at this finer level of specificity. Furthermore, if xanthan is involved in determining specificity through variation in its fine structure, those enzymes controlling, say, pyruvylation patterns, are the products of specificity-determining genes. What is urgently required is an analysis, by the procedures of molecular genetics, of the genes that control the fine structure of this polysaccharide.

MOLECULAR GENETICS OF XANTHOMONAS

The second general approach to elucidating the molecular basis of the expression of specificity genes may be termed the "inside-out" approach. Instead of examining suspected virulence determinants such as toxins and polysaccharides, this approach assumes no knowledge of the molecular expression of the genes for specificity and calls for the procedures of recombinant DNA technology and molecular genetics to isolate and clone the gene (or genes) determining specificity. Such procedures are limited by the necessity for DNA cloning systems in the pathogen; the diseases caused by gram -ve phytopathogenic bacteria have certain advantages in this respect over relatively poorly developed fungal systems. In the past 2 yr, some progress has been made in isolating and cloning specificity genes for a number of gram -ve bacteria (Table 1). Bacterial blight of rice offers a number of distinct advantages for gene cloning studies, not the least of which is that some of the molecular technologies used for genetic manipulation of Rhizobium have been shown to be applicable to Xanthomonas spp. at the John Innes Institute, Norwich, U.K. We at the University of Birmingham are collaborating with Dr. M. Daniels at the John Innes Institute in an attempt to isolate and clone the

Table 1. Cloning of specificity genes.

Gram -ve bacterium	Description
Pseudomonas savastanoi Comai and Kosage (2)	Cloned 2.75 kb fragment containing gene for tryptophan 2-mono-oxygenase. Introduction of fragment into avirulent mutant restored indoleacetamide synthesis and avirulence.
Pseudomonas solanacearum Staskawicz (13)	Cloned 3.2 kb fragment from Tn5-induced nonfluidal (-EPS), avirulent mutant. Fluidal, virulent revertants lack sequence.
Pseudomonas syringae pv. glycinea, Staskawiez (13)	Cosmid clones from avirulent race mobilized into virulent race to complement pathogenecity.
Rhizobium spp. Downie (6)	Nodulation genes isolated from *R. meliloti, R. leguminosarum, R. trifolii, R. parasponium,* cloned and will transfer plant-host determinants between different spp. of *Rhizobium.*

genes controlling race specificity in X. campestris pv. oryzae. (Progress in this general area for other plant-bacterium associations is shown in a seminal paper by Staskawicz [13]). We used the following strategy.

1. A genomic library of pathogen DNA is made by isolating restriction DNA fragments of one of the strains, say, PX086, and cloning them into Escherichia coli using a suitable vector. The vector of choice in this case is the wide host range "cosmid" pLAFR1, which can accommodate large inserts (up to 20 kb). The library is made in E. coli because it is easy to manipulate and has a high transformation rate. The same vector system has been used to construct genomic libraries of Pseudomonas glycinea (13). The number of individual E. coli clones necessary to encompass the Xanthomonas genome is approximately 1,000.
2. By simple mating procedures, the DNA library is then transferred to the heterologous race of X. campestris pv. oryzae, i.e., if the library is prepared from PX086, it is transferred to PX061, and vice versa. The cosmid pLAFRl will replicate in both E. coli and Xanthomonas spp. (Daniels, pers. comm.), but to mobilize the cosmid into Xanthomonas, a helper plasmid pRK2013 is necessary, as shown for Rhizobium meliloti by Ditta et a1 (5).
3. The transformed clones of Xanthomonas can be selected through the tetracycline resistance conferred by pLAFR1, and each new clone is tested individually on the differential rice cultivars to detect the clones with modified specificity. Any such changes in specificity should result from incorporating the heterologous DNA fragment, which in effect contains the gene for host specificity.
4. Steps 1-3 are done in a reciprocal manner, which eliminates consideration of dominance and epistasis.
5. Assuming now that we have a clone of Xanthomonas with specificity altered by the presence of a new insert of DNA transferred from a heterologous race, we will aim to localize the specificity gene in the inserted DNA more precisely to allow the gene itself to be cloned. This could be done in a number of ways. The most promising is transposon mutagenesis with Tn5, which has been used by Staskawicz (13) for Pseudomonas solanacearum and by Daniels et a1 (3) for certain species of Xanthomonas, as follows:
   - The DNA insert from the appropriate clone can be randomly mutagenized with the transposon Tn5 that carries a tetracycline-resistance marker. Insertion of this transposon into the heterologous DNA conferring a change in specificity will inactivate that gene function, thus returning that clone to a pattern of specificity identical to that before cosmid transfer.
   - These mutants will then be used as a source of Tn5-carrying molecular probes, since digestion of

the Tn5-carrying DNA with the restriction nuclease EcoR1 yields the complete Tn5 probe plus flanking portions of the target DNA.

- The Tn5-containing, EcoR1-cut DNA can then be ligated into a plasmid and transformed into E. coli for cloning. Thus, at this stage the mutated gene ha;, in effect, been cloned.
- To isolate the nonmutated version of this gene, the Tn5-target gene-plasmid complex can be labeled by nick-translation with ^{32}P and used to screen the wild-type libraries prepared in step 1, for a colony containing a cosmid clone with DNA sequence homology.
- As a check, the appropriate cosmid clone should be reintroduced into the Tn5-mutagenized strain. If the wild-type gene is present in the clone, the mutation should be complemented and the phenotype restored to the original wild-type condition.

Assuming progress is made along the lines indicated, how does it help us analyze the molecular basis of gene function in determining specificity? When a specificity gene has been cloned, a wide range of possibilities present themselves:

- comparison of gene structure from the different races through restriction mapping and DNA sequencing;
- study of the primary gene products of the specificity genes through in vitro translation, and comparison with candidate gene products;
- transfer of the gene(s) to other pathovars of X. campestris to examine the control of host range at the species level; and
- biochemical studies on the potential interaction of bacterial gene product with, for example, host receptor site potentially encoded by the host resistance gene.

The elucidation of pathogenicity and host range in the bacterium is an important initial step to understanding the expression of resistance genes in the higher plant host. Referring back to a point made earlier (and strongly emphasized by Ellingboe [7]), the genes of avirulence and resistance are complementary. If this complementarity extends to an association of gene products (as shown in Fig. 3), then the procedures outlined in this paper produce one-half of the molecular association. It is potentially feasible, then, to isolate through biochemical procedures the complementary gene product or receptor site for host resistance encoded by the host R gene. If that product (presumably a protein) can be isolated, then the molecular procedures are available to permit isolation of the gene specifying that protein, in effect the gene for resistance! Let me expand on this possibility. Let us assume that we have two near-isogenic rice cultivars, one containing the dominant allele for resistance and, presumably, expressed; the other containing the recessive allele for susceptibility. The presumption would be that this genetic difference is reflected in the spectrum of polypeptides

synthesized by the two plants. It should therefore be possible to prepare cDNA library clones reflecting the transcriptional products unique to the R genotype. Considerable advances have been made in this area of plant molecular biology, where genes for discrete plant proteins are being isolated and cloned (10). But in the context of plant disease resistance, the problem remains that until the particular polypeptide produced by a gene for resistance is known, the isolation and cloning of the R gene that creates a resistant phenotype is still intransigent since the difference spectrum of polypeptides synthesized by R and S genotypes is unlikely to be limited to a single polypeptide.

Although considerable progress has been made in identifying mRNAs involved in the various metabolic changes that result in the expression of resistance, such as those controlling the early steps of phytoalexin synthesis (8), the genes controlling these steps are not the genes controlling the initial recognition and discrimination of the avirulent pathogen. This therefore emphasizes the importance of probing the complementary molecular interaction implicity in the gene-for-gene relationship. Once the gene products determining avirulence in the pathogen have been isolated, the gene products of the host encoding resistance may be isolated, thus permitting the cloning and identification of the genes themselves from libraries of host-genomic DNA or from cDNAs. The possibilities of transferring resistance genes into crop plants from widely divergent genetic backgrounds then assume more realistic proportions.

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REFERENCES CITED

1. Callow, J.A. 1984. Cellular and molecular recognition between higher plants and fungal pathogens. Encycl. Plant Physiol. N.S. 17:213-237.
2. Comai, L., and T. Kosage. 1982. Cloning and characterisation of iaaM, a virulence determinant of *Pseudomonas savastanoi.* J. Bacteriol. 149:40-46.
3. Daniels, M.T., P.C. Turner, C.E. Barber, W.G. Cleary, and G. Reed. 1983. Pages 340-344 *in* A. Puhler, ed. Molecular genetics of the bacteria-plant interaction. Springer-Verlag, Berlin.
4. Day, P.R. 1984. Genetics of recognition systems in host-parasite interactions. Encycl. Plant Physiol. N.S. 17:134-147.
5. Ditta, G., S. Stanfield, D. Corbin, and D.R. Helinski. 1980. Broad host range cloning system for gram -ve bacteria: construction of a gene bank of *Rhizobium meliloti.* Proc. Natl. Acad. Sci. USA, 77:7393-7351.
6. Downie, A. 1983. Molecular genetics, out of the laboratory into the field. Nature 306:639.

7. Ellingboe, A.H. 1982. Genetical aspects of active defense. Pages 179-192 in Active defense mechanisms in plants. R.K.S. Wood, ed. Plenum, New York.
8. Hahlbrock, K., C.J. Lamb, C. Purwin, J. Ebel, E. Fautz, and E. Schafer. 1981. Rapid response of suspension-cultured parsley celles to the elicitor from Phytophthora megasperma var. sojae. Induction of the enzymes of general phenylpropanoid metabolism. Plant Physiol. 67:768-773.
9. Mew, T.W., S.Z. Wu, and O. Horino. 1982. Pathotypes of Xanthomonas campestris pv. oryzae in Asia. IRRI Res. Pap. Ser. 75. 7 p.
10. Miflin, B., and P.J. Lea. 1984. The genetic manipulation of crop plants. Nature 308:498-499.
11. Morris, E.R., D.A. Rees, M.D. Walkinshaw, and E. Darke. 1977. Order-disorder transition for a bacterial polysaccharide in solution. A role for polysaccharide conformation between Xanthomonas pathogen and its plant host. J. Mol. Biol. 110:l-16.
12. Parry, R.W.H. 1984. Xanthomonas infections of rice; the involvement of bacterial extracellular polysaccharide. Ph D thesis, University of Leeds, United Kingdom.
13. Staskawicz, B.J. 1983. Molecular genetics of plant disease. Pages 199-213 in Biochemical plant pathologv. J.A. Callow, ed. J. Wiley, London.

BIOCONVERSION AND BYPRODUCTS UTILIZATION

BIOTECHNOLOGY PROGRAM AT THE UNITED NATIONS UNIVERSITY

R. KOKKE

According to its charter, the United Nations University (UNU) is mandated to be an international community of scholars engaged in research, postgraduate training, and dissemination of knowledge. It is funded by endowment, which helps to ensure its autonomy, academic freedom, and objective research. It has much more flexibility than any other UN agency and, with due respect to governments, it can work with institutes and investigators directly. The UNU's administrative headquarters is in Tokyo, but its collaborators and associated institutions are located all over the world. UNU research is devoted to problems of human survival, with attention to the social sciences and humanities, as well as to pure and applied natural sciences.

After a meeting on biotechnology and microelectronics held in Tokyo in 1981, University Rector Mr. Soedjatmoko was advised by an international group of scientists to emphasize those two issues more, for if their research in these areas is not well planned now, the less developed countries, despite the wealth of biomass resources and traditional technologies in many of them, will become, also in these areas, dependent on the industrialized ones.

The UNU is mobilizing biotechnology scientists everywhere to strengthen their research through cooperation. It wants to ensure that biomass resources are used conservatively. Biotechnology is the technique for optimum and balanced biomass development. It must preclude biomass domination by capital-intensive large-scale projects. Biotechnologists must exploit traditional and new knowledge for applications that integrate feasibility, economic viability, and human need.

We have chosen four projects, not so much for solving problems, but as models for cooperation in biotechnology research:

1. Nitrogen fixation in the root system of rice;
2. Solid-state fermented foods;
3. Vaccines and diagnostics against tropical diseases (brucellosis); and
4. Legal, social, and ethical aspects of biotechnology.

If more funds become available, we hope to initiate projects on improving the use of 1) aquatic photosynthesis, 2) hydrogen through biomembranes, and 3) biogas.

All these projects have well-defined practical goals; they use traditional knowledge and the most advanced science; and they all require multidisciplinary cooperation.

Senior programme officer, The United Nations University, Shibuya-ku, Tokyo 150, Japan.

UNU established the cooperative research projects by

1. creating the above-mentioned international networks;
2. funding fellowships, visiting scholars, consultants, training; and
3. financially supporting selected cooperative institutes.

For efficient and productive cooperation, the number of institutes participating in one network project should be limited to not more than 15, including about 10 from developing countries.

We offer cooperation and we receive cooperation. My participation in this conference is very much due to Dr. Swaminathan's cosupport of our nitrogen-fixation project. In that project soil microbiologists, plant tissue culturists, and molecular biologists will work together. Only through cooperation of researchers at the bench, do such biotechnology projects have a chance. Communication among those scientists, with others, and with data banks is a matter of life or death in such an information-intensive science as biotechnology. Therefore UNU is exploring the possibility of computer communication links among participants in its networks.

In short, we want to support scientists in selected areas of biotechnology and create stimulating conditions for their research to the benefit of their institutes, their countries, and, ultimately, all people.

PROGRESS AND PROSPECTS OF NEW BIOMASS INDUSTRIES

H. R. BUNGAY

Prospects for early commercialization of biomass processes continue to improve despite shifts in government support. Refining shows particular promise for providing a mix of high-value products. Lower value uses of biomass, as a feedstock for producing gaseous, liquid, or solid fuels, should be phased in after more profitable but small-market uses have justified the research and development on handling, pretreatment, and processing.

In spite of the complacency that has set in because there is no longer a critical shortage of petroleum, biomass programs throughout the world continue. The United States Government has redirected support toward longer range research deemed too risky for private industry, and it expects that worthwhile short-term programs will be commercialized by the private sector. A number of other nations, notably Sweden, Canada, and Italy, support research and development on biomass for energy. New York, Florida, and California have agencies for funding biomass programs; the Electric Power Research Institute and the Gas Research Institute also contribute significantly.

Political and emotional issues such as world hunger and the need for environmental protection impede the development of new uses for biomass. Some people immediately dismiss using biomass for fuel and chemicals because they fear insufficient land will be devoted to food. If feeding people overshadows all else, however, a purely agrarian society with few of the benefits of an advancing civilization may result. Farmers in developing nations deserve the maximum return on their efforts whether these be toward food or biomass production.

It has been said that when people are neither starving nor worried about health care, they will have smaller, more prosperous families. This premise has a fatal flaw -- the time lag for implementing food and health programs. Population growth tends to outstrip increases in food, and the intervals when developing countries have no urgent nutrition problem are brief.

In a detailed analysis of alternatives, energy balances, land-use patterns, land quality, and other factors affecting

Rensselaer Polytechnic institute, Troy, NY 12181 U. S. A.

biomass energy in developing nations, Barnard (2) points out that if improving agricultural productivity were a simple matter, so many millions would not be on the edge of starvation. Areas where pressures on land resources for food are severe may not be the place to use land for fuel production. But in some areas, fuel for cooking now costs more than food, so food and fuel costs can be separated only for foods eaten raw; even then, energy of some kind is needed for farming. (Research and management for biomass resources are discussed by Swaminathan [10].)

Unfortunately, it is impossible to separate the politics from the technology. Many environmentalists oppose anything but preserved forests. A dispute over soil damage from clear cutting of trees is used against biomass. Even if disproven, the old accusations may plague the development of biomass for many years.

Some developing nations are currently trying to derive alcohol fuels from biomass. The efforts in Brazil have augmented that country's energy resources and international trade (6). Sugar prices would have been much lower if Brazil and other nations had not diverted much of their sugar to the manufacture of alcohol. The US has new projects for fermenting grain to ethanol, but commodity prices are currently so high that profitability is questionable. In any event, the real opportunities lie not with plants that produce sugar or starch, but with cellulose, the world's most abundant carbohydrate. This paper will comment on commercialization of some processes based on cellulose.

GENERAL OPTIONS

There is little new on direct use of biomass as combustion fuel. Boiler design has improved and efficiency has increased through drying or pelletization of fuel.. A plus for biomass is that it recycles carbon dioxide, whereas the combustion of fossil fuels simply releases it. The possible accumulation of toxic dioxin formed in trace amounts by combustion is a new worry of environmentalists and it comes from biomass as well as other fuels. Various thermochemical processes can produce convenient, clean fuels from biomass, but their economics are not favorable. This paper premises that biomass has some high-value uses that will be more profitable than most uses requiring thermochemical conversion.

Biomass rich in starch or sugars is easily fermented to valuable products, but it may be worth more as food. Most residues and wastes from agricultural operations have relatively low value, but can prevent erosion or provide organic nutrients for the next growing period. They can be used more profitably if inexpensive substitutes can be found for their low-value applications. Species of trees poorly suited for lumber or fiber may best serve as feedstocks; many individual trees or crop plants are too deformed, diseased, or spoiled for their traditional uses. The quantity of readily available biomass can meet only a part of the enormous demand for fuels, but there is plenty for manufacturing chemicals. Success with producing chemicals from biomass should provide investment capital for developing other biomass industries.

TECHNOLOGY

Anaerobic digestion employs elective microbial cultures in crude equipment. The main products are impure methane gas and a sludge that is often used as fertilizer or soil conditioner. Anaerobic digestion of cattle manure provides a sludge that could be fed to cattle and have more value than the methane. Unfortunately, sludge is lower in nitrogen, phosphorus, and potassium than are other fertilizers, and feeding cattle with digester sludge results in poor weight gain and meat quality (11). When disposal of a waste biomass is a problem, anaerobic digestion can be profitable. Small-scale anaerobic digestion has been tried by several nations, with some success. The methane must be purified to enter gas pipelines, but impure methane containing 30-45% carbon dioxide may be burned locally. Small digesters providing only enough gas for a small stove and one or two gas-mantle lights are not appealing in a developed country because the rewards are minuscule. In a developing nation these modest rewards do warrant the efforts required with small digesters.

Lignin, an aromatic phenolic polymer, is a major component of spent sludge, but recovery of good lignin from digester residue has not been reported. However, purified lignin is a very useful specialty chemical. It can substitute for expensive phenolformaldehyde in adhesives for chipboard or plywood; it can thicken muds for drilling oil wells; it can even substitute for the asphalt in highways. In fact, the markets for lignin could match those for ethanol (8). (Coughlin et a1 [5] provide an extensive review of the processing and uses of lignin.)

The hydrolysis of cellulose, an important way to obtain glucose, is greatly improved by pretreatment. At the very least, the biomass must be ground, chipped, or shredded so that chemicals or enzymes can reach the cellulose. Certain pretreatments hydrolyze the hemicellulose to soluble sugars that are easily washed away. These sugar solutions can be fed to animals or be used to make valuable chemicals. Organic solvent pretreatments simultaneously remove the lignin and the hydrolysis products of hemicellulose to leave solids rich in cellulose. When the cellulose fibers are relatively undamaged by the pretreatment, their best uses may be as paper fiber or as a source of chemical derivatives of cellulose. The byproducts lignin and hemicellulose may also yield valuable products.

The Soviet Union and some other nations have large factories for acid hydrolysis of lignocellulosic materials. Acid hydrolysis of cellulose is rapid at elevated temperatures with sulfuric acid, but the resulting glucose reacts further to yield hydroxymethyl furfural and resins. The usual yield is about 55% of the theoretical glucose yield, but certain pretreatments can yield 70%. Other acids function at milder temperatures and approach 100% of the theoretical yield. These acids (e.g., hydrechloric, hydrofluoric, or nitric) are expensive, and their recycling, therefore, crucial. But recovery of acid after hydrolysis is not easy, and complexes can form with the carbohydrates.

Enzymatic hydrolysis of cellulose gives almost no side products and can give excellent yields, but rates are slow and the

cost of the required enzymes, high. Direct fermentation of cellulose shows great promise (1); aspects of employing thermophilic organisms are presented by Carreira et a1 (4).

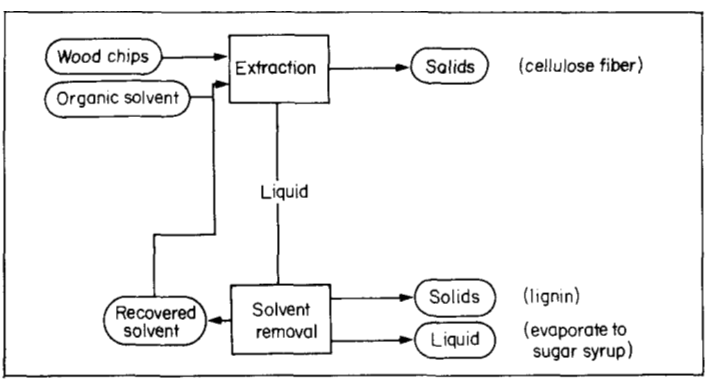
STATUS OF VARIOUS PROCESSES

In a review of anaerobic fermentation, Klass (7) concludes that conventional digestion is not competitive for manufacturing methane. Of course, there are many large units for anaerobic treatment of wastes, and the side-product methane is used locally or sold. Wise (13) covers many recent developments in digester design and methane production.

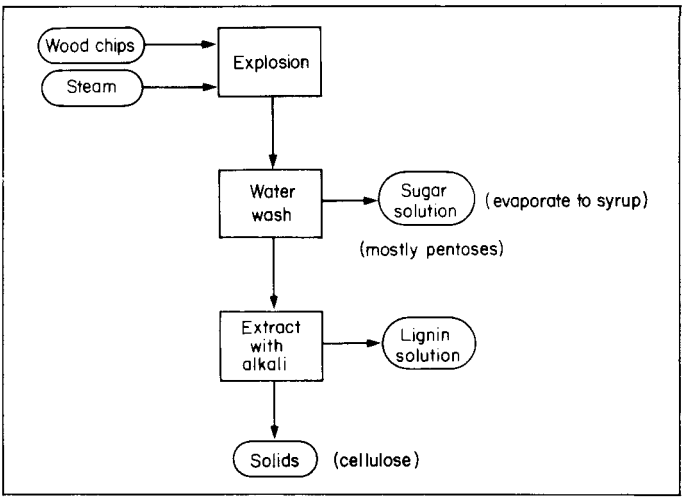
Biotechnology for liquid fuels is reviewed by Phillips and Humphrey (9). Items whose costs must be lowered for successful commercialization are feedstock; biomass harvesting; acid recovery for acid hydrolysis; enzyme production for enzymatic hydrolysis; and recovery of products that are at low concentrations in fermentation broths.

White (12) has assessed producing ethanol from cellulose. Environmental consequences of new biomass refining processes can be foreseen because the operations are the same or similar to those in the chemical process or paper industries. Although alcohol from cellulose is technically feasible, it is not yet competitive with older processes. Acid and enzymatic hydrolysis each have advantages and disadvantages, though acid hydrolysis may be more economic.

Solvent refining is shown in Figure 1. Wood chips are extracted with a solvent (e.g., butanol, ethanol, phenol, or formic acid) containing some water with acid or base. While lignin is dissolved, hemicellulose is hydrolyzed to sugars. The liquid is removed and treated to yield solid or resinous lignin and a solution of sugars. (The solution is then concentrated and used as animal feed or fermentation substrate.) The cellulose residue is fibrous and may be used in paper or as a feedstock for cellulose derivatives. Rough estimates of values starting with 1 t of dry wood chips at $30 to $50/t are lignin (0.2 t), $300-600/t; fiber (0.45 t), $250-$450/t; and syrup (0.2 t),



1. Solvent refining of biomass.



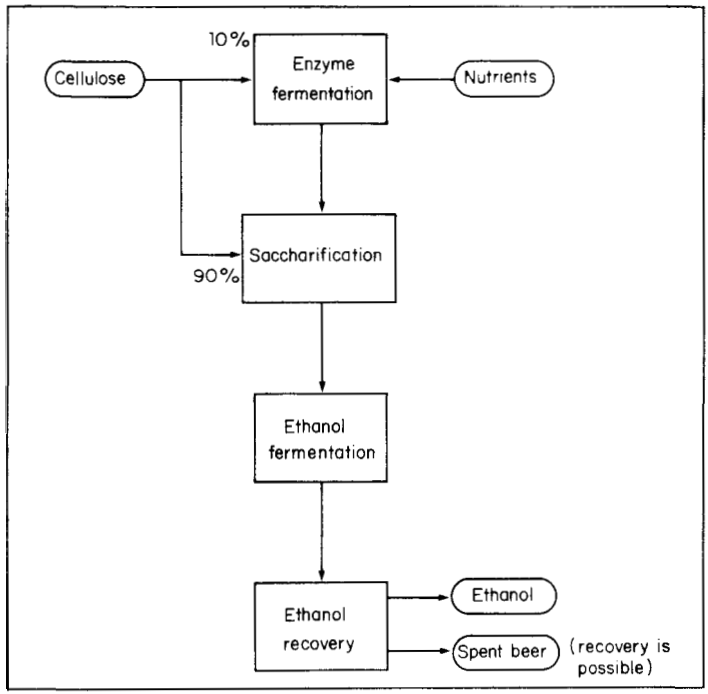
2. Biomass refining via steam explosion.

$30-90/t. If the cellulose serves as fiber, the potential profit is good, but if the cellulose is considered only a source of glucose, processing costs are too high. (Note that there are no biological steps in this scheme.)

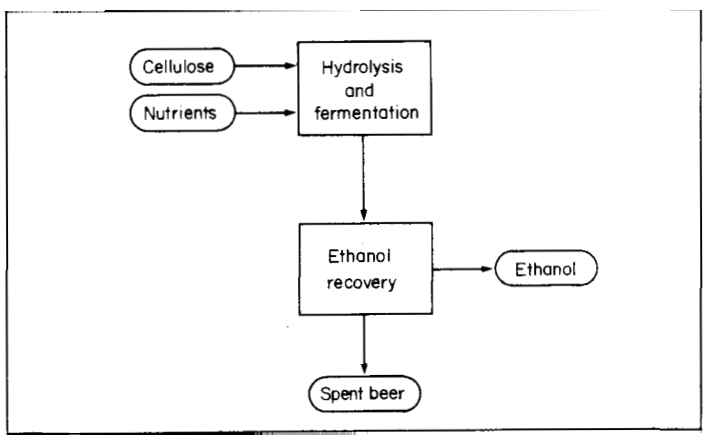
Steam explosion is outlined in Figure 2. Wood chips are impregnated with high-pressure steam and explode when pressure is released suddenly. This shatters the cellular structure, hydrolyzes all the hemicellulose, and degrades some of the resulting sugars. The next step is a water wash to remove the sugars and the various impurities and inhibitors of subsequent steps. As with solvent processing, the extracts can be concentrated to a syrup, but the other substances formed during steam explosion lower the syrup's value. Lignin extracted from the solid residue is as valuable as lignin from solvent processing. The cellulose remaining after lignin removal is more than 90% pure, but the fibers are too weak for use in paper and the degree of polymerization is too low for making most cellulose derivatives. This cellulose can be a cheap filler material or a feedstock for bioconversion. The main advantage of steam explosion over solvent processing is its lower cost. Feedstock and product values are the same as before except that the cellulose may be worth only $60-$100/t.

Direct fermentation of cellulosic biomass is shown in Figure 3. The preferred feedstock at present is maize stover, because it is easily shredded to an acceptable particle size and is rich in carbohydrates. (Note that all the lignin is lost in the spent solids.) The products are ethanol and boiler fuel. The process seems inferior because the product values are low. However, processing is simple and costs should be very low. As a route to ethanol, this one-step method is much more attractive than the costly steps for producing enzymes, for saccharification, and for fermentation to ethanol shown in Figure 4.

There are good reasons for handling hemicellulose sugars separately. The best available organisms for ethanol fermentation



3. Stepwise production of ethanol from cellulose.



4. Direct fermentation of cellulose.

tend not to accept the pentoses that predominate in hemicellulose. Furthermore, organisms that can ferment both pentoses and glucose use the glucose first. Ethanol tolerance is not good in these organisms, so early ethanol accumulation from fermenting glucose slows the fermentation rate of the other sugars.

Partly purified cellulose from a steam explosion process should ferment well. Strains developed at Massachusetts Institute of Technology showed promise, but did not use all the cellulose (Wang, D.I.C., unpubl.). This is characteristic of direct

fermentation, but strains with more active enzymes can probably be found.

Solvent refining of biomass requires sophisticated technology and depends on the paper or cellulose chemical companies to purchase the major cellulose product -- both potential problems in a developing nation. On the other hand, steam explosion and its subsequent steps are familiar and straightforward. When direct fermentation of cellulose to ethanol is perfected, steam explosion refining should be attractive for any nation with biomass resources (3). The syrups for cattle feed aid the agricultural sector; the lignin, the construction industry; and the ethanol can fulfill needs for liquid fuel.

CONCLUSIONS

Wood fuel, methane from anaerobic digestion, and thermochemical conversion have merit, but biomass refining can yield more valuable products. Food and fiber are the highest priority biomass products. However, some biomass has little or no food value and poor fiber quality. Solvent refining of inferior wood may yield a fiber of high value, but steam explosion refining greatly weakens the fiber. On the other hand, steam explosion is a rapid process and leads to easy separation of biomass constituents. The key to biomass refining is high-value lignin. At present, the specialty markets for lignin are small and undeveloped: They are upstaged by the enormous potential market for ethanol from cellulose as a motor fuel additive. All countries should consider the high-value uses of biomass. Experience in its handling and processing should translate into lower costs and higher profits for other biomass alternatives.

REFERENCES CITED

1. Avgerinos, G.C., and D.I.C. Wang. 1980. Direct microbial conversion of cellulose to ethanol. Ann. Rep. Ferment. Proc. 4:165-191.
2. Barnard, G.W. 1984. Liquid fuel production from biomass in the developing countries -- an agricultural and economic perspective. Pages 163-248 in Bioconversion systems. D.L. Wise, ed. CRC Press, Inc., Boca Raton, Florida.
3. Bungay, H.R. 1983. Industrial biotechnology for developing countries. In New Frontiers in Technology Applications: integration of emerging and traditional technologies; proceedings. E.U. von Weizsacker, M.S. Swaminathan, and A. Lemma, eds. Tycooly International Publishing Ltd., Dublin, Ireland.
4. Carreira, L.H., J. Weigal, and L.G. Ljungdahl. 1983. Production of ethanol from biopolymers by anaerobic, thermophilic, and extreme thermophilic bacteria: I. Regulation of Carbohydrate Utilization. Biotechnol. Bioeng. Symp. 13:183-191.
5. Coughlin, R.W., D.W. Sundstrom, H.E. Klei, and E. Avni. 1984. Conversion of lignin to useful chemical products.

Pages 41-58 in Bioconversion systems. D.L. Wise, ed. CRC press, Inc., Boca Raton, Florida.

6. Goldemberg, J. 1984. Energy problems in Latin America. Scier.ce 223:1357-1362.

7. Klass, D.L. 1984. Methane from anaerobic fermentation. Science 223:1021-1028.

8. Muller, P.C., and W.G. Glasser. 1983. Lignin's potential contribution to the feasibility of biomass conversion to ethanol. Biotechnol. Bioeng. Symp. 13:481-494.

9. Phillips, J.A., and A.E. Humphrey. 1983. Process biotechnology for the conversion of biomass into liquid fuels. Pages 65-95 in Liquid fuel developments. D.L. Wise, ed. CRC Press, Inc., Boca Raton, Florida.

10. Swaminathan, M.S. 1982. Perspectives in biotechnology research from the point of view of third world agriculture. Pages 38-63 in Priorities in biotechnol.ogy research for international development. National Academy Press, Washington, D.C.

11. Ward, R.W. 1983. Utilization of biomass digester effluents as soil conditioners and feeds. Pages 161-188 in Fuel gas developments. D.L. Wise, ed. CRC Press, Inc., Boca Raton, Florida.

12. White, H.M. 1983. Progress in the production of ethanol from cellulose. DOE/CE/50305-1 (1983) National Technical Information Service.

13. Wise, D.L., ed. 1983. Fuel gas developments. CRC Press, Inc., Boca Raton, Florida.

AGRICULTURE OF TOMORROW — GREATER PRODUCTIVITY, EFFICIENCY, AND DIVERSITY

H. K. JAIN

The evolution of agriculture, especially in the last 50 yr, is traced and the directions for the next 50 yr are suggested. Recommendations are made for concentrating research on renewable energy resources such as biological N fixation, exploiting advances in genetics and molecular biology to increase rates of photosynthesis and increase crop dry matter production, and reestablishing in cultivars the genetic diversity they require to withstand diseases, pests, and other stresses.

Many people are now asking whether tomorrow's agriculture can be both more productive and more efficient. The concern about production efficiency is understandable. Modern agricultural technology attempts to exploit favorable genotype-environment interactions. This means synthesizing genotypes that will benefit most from improved agronomic management, which depends heavily on energy-intensive chemical fertilizers, pesticides, and farm machines. The strategy worked well especially while fossil fuels were cheap and readily available. In the developed countries, this model is still valid, because despite the energy crisis, agriculture accounts for only a small fraction of the energy budget. The industrial sector is the greatest energy user, but it is so well developed that it can subsidize agriculture, even when agricultural production is inefficient.

ENERGY, UNIFORMITY, AND YIELD CEILINGS

In the short term, even the developing countries have no alternative to high-energy agriculture; in the long term, however, many of them require that high-energy inputs be replaced with renewable ones. Both the developed and the developing countries should exploit the new agricultural technology developing from advances in basic sciences.

The development of high-yielding varieties, particularly in crops such as rice and wheat, has led to erosion of genetic diversity, especially in the last 15 yr. In India, for example,

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Director, Indian Agricultural Research Institute, currently senior research fellow, International Service for National Agricultural Research, The Hague, Netherlands.

until recently as many as 30,000 different rice varieties have been grown; by the end of the century, there may be only 100 varieties. Of these, no more than 20 may cover 50-60% of the total rice area. Uniformity of this kind may predispose our agriculture to disease and pest epidemics, some of which have occurred in the past 10 yr. The last epidemic in India, resulted from a buildup in pearl millet of downy mildew, and was traceable to the male-sterile line, common to all the newly developed high-yielding hybrids. The maize leaf blight epidemic in the us is a similar example.

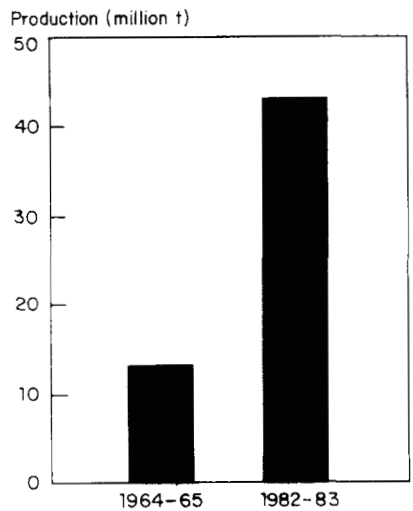
Finally, yield improvement in some important cereals and other crops seems to be reaching its limits.

The answer to these problems is not a return to traditional agriculture. Continuous growth of population pressures requires that advances in molecular biology and other disciplines be used to widen the scientific base of present agricultural technology.

GENETIC RECONSTRUCTION FOR REPARTITIONING OF DRY MATTER

Classical genetic changes, such as Vogel and Borlaug's wheat reconstruction, profoundly affected agriculture (2, 5). In India in the last 18 yr, wheat production has increased from 12 million t to more than 44 million t with the advent of the dwarf varieties (Fig. l), and in many other countries major production gains have been achieved. The case of the irrigated rice crop is similar.

The basic change in these crops is in repartitioning the dry matter so that more photosynthates are diverted to the grains. Dry matter production has not increased; but the harvest index or the proportion of grain to straw in wheat, rice, maize, sorghum, and many other crops has. This has been the most effective



1. Increase in wheat production in India with the advent of dwarf varieties in the mid-1960s.

yield-increasing mechanism used by plant breeders since Mendel's discoveries. But can the harvest index increase indefinitely?

Consider the evolution of wheat since 1900. An analysis of wheats evolved in the United Kingdom, United States, and India during the last 75 yr shows that grain yield improvement has never been associated with any significant change in dry matter production (Fig. 2, 3, 4; 1, 3, 4). In fact, total dry matter production in some of the latest wheat varieties has decreased. Even when grain yields increased, productivity of total photosynthates remained constant. The grain yield increase in all three countries is associated with a reduction in plant height and increase in harvest index. This may also be the case in most other crops (Fig. 5, 6).

At IRRI, breeders have repartitioned the dry matter of traditional tall indica varieties to develop dwarf types with a high harvest index and a grain-straw ratio of nearly 50:50. In some of the more traditional crops, such as pigeonpea in India, the traditional varieties have been similarly restructured.

Soyabean, now a dominant crop in America, has also been genetically reconstructed. The soyabean plant not only directs much of its dry matter to pods and seeds, but also gives a high harvest index of protein. Expressed in calories per hectare per day, the yield of soyabean, with 42% protein and nearly 18% oil, is much higher than that of most other crops (including improved wheat and rice).

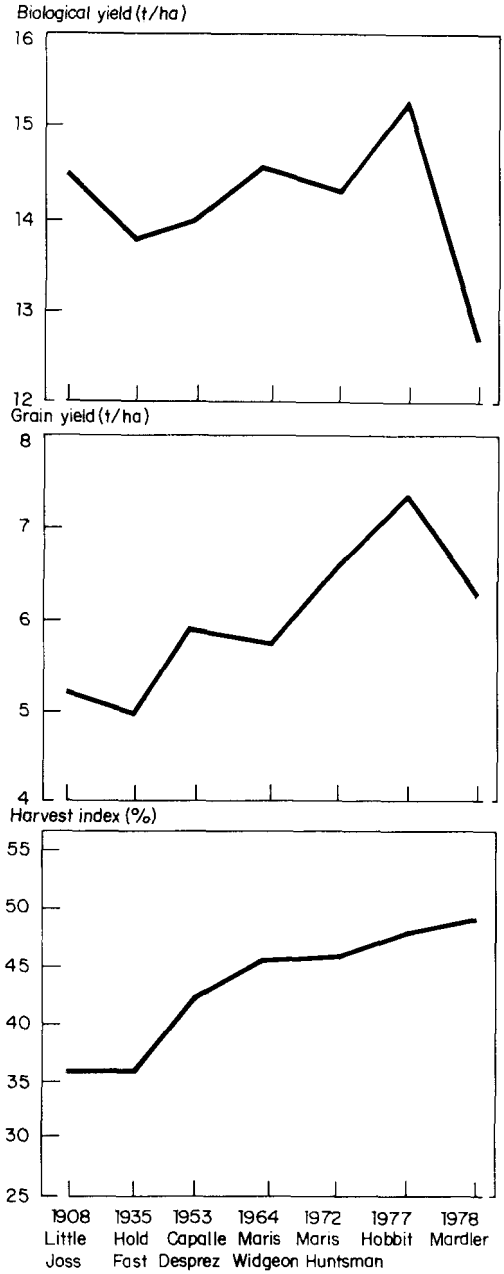
SELECTION PRESSURES

What are the selection pressures in crop plants in a low-management agriculture? Plants require adaptation to stress from weed competition, pest and pathogen attack, and exhausted soil fertility, and, above all, to moisture stress. Under such conditions plants are selected more for their ability to survive than for high grain yields. During 10,000 yr of domestication, selection has resulted in crop plants with a large capacity to produce dry matter.

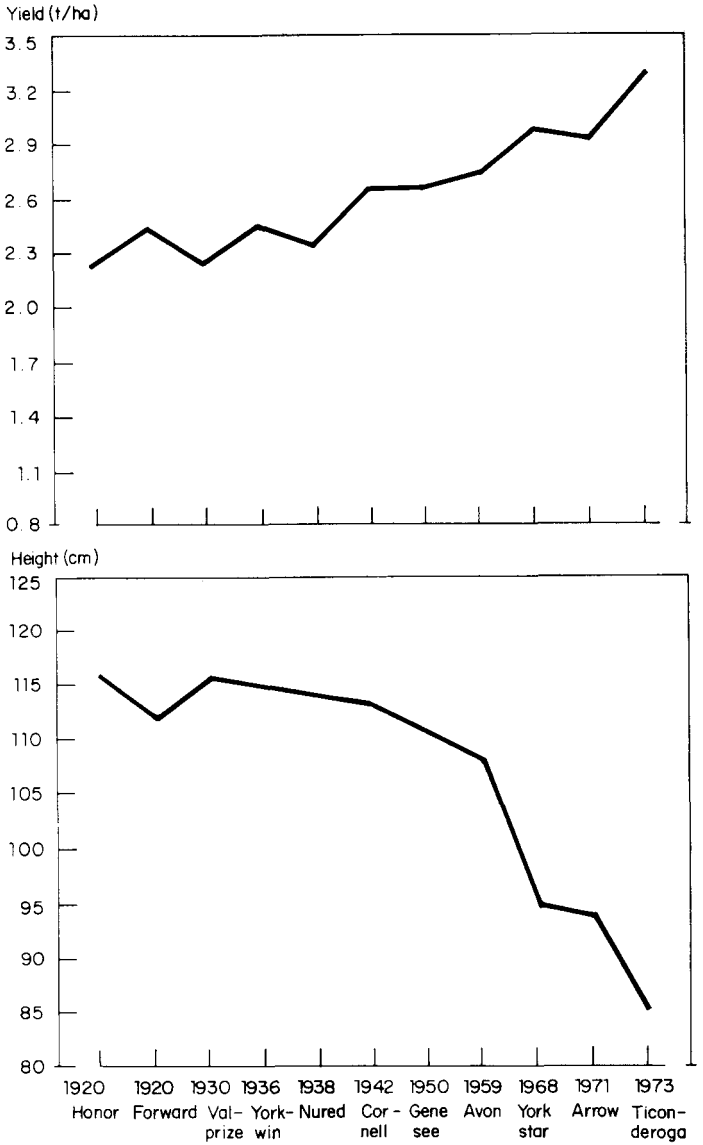
Recent changes in crop plants have been brought about by a different kind of selection pressure. Plant-type genes, already used to improve harvest index in wheat and rice, should be found in most other crops; hence germplasm collection and evaluation is particularly important. The plant-type genes help accelerate crop evolution, so breeders do not have to depend on polygenic variation.

PHOTOSYNTHESIS

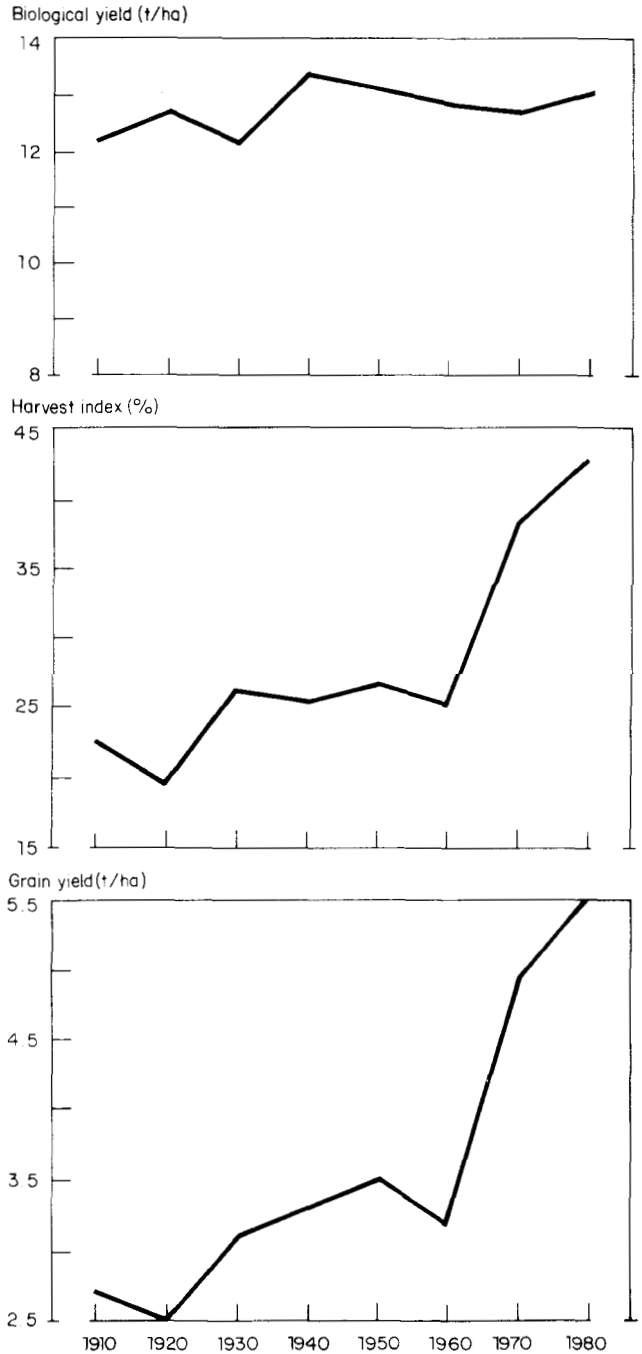
The harvest index maximum in some crops may have been reached. In others, such as the grain legumes and oilseeds, increased harvest index may increase yields in the next 15 – 20 yr, but eventually in these crops, too, the harvest index will reach saturation. If crop yields are to increase consistently, dry matter production itself must be considered. Can dry matter production be increased as it was through natural selection



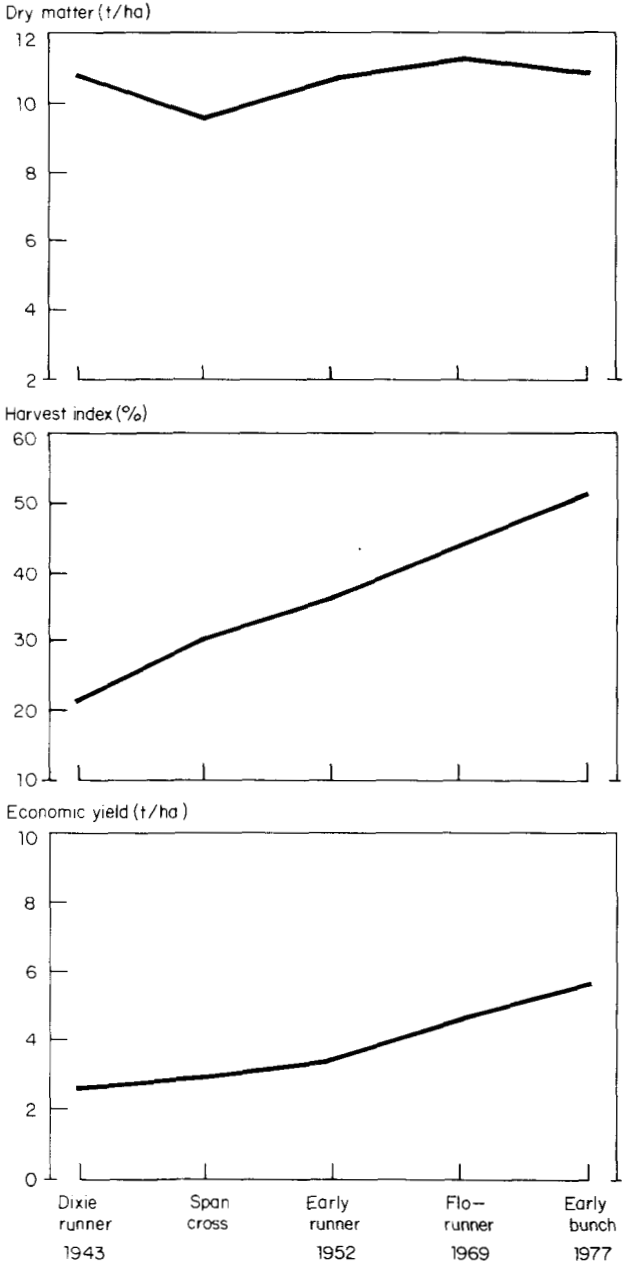
2. Grain yield, biological yield, and harvest index of some of the wheat varieties released in the UK since the early years of this century.



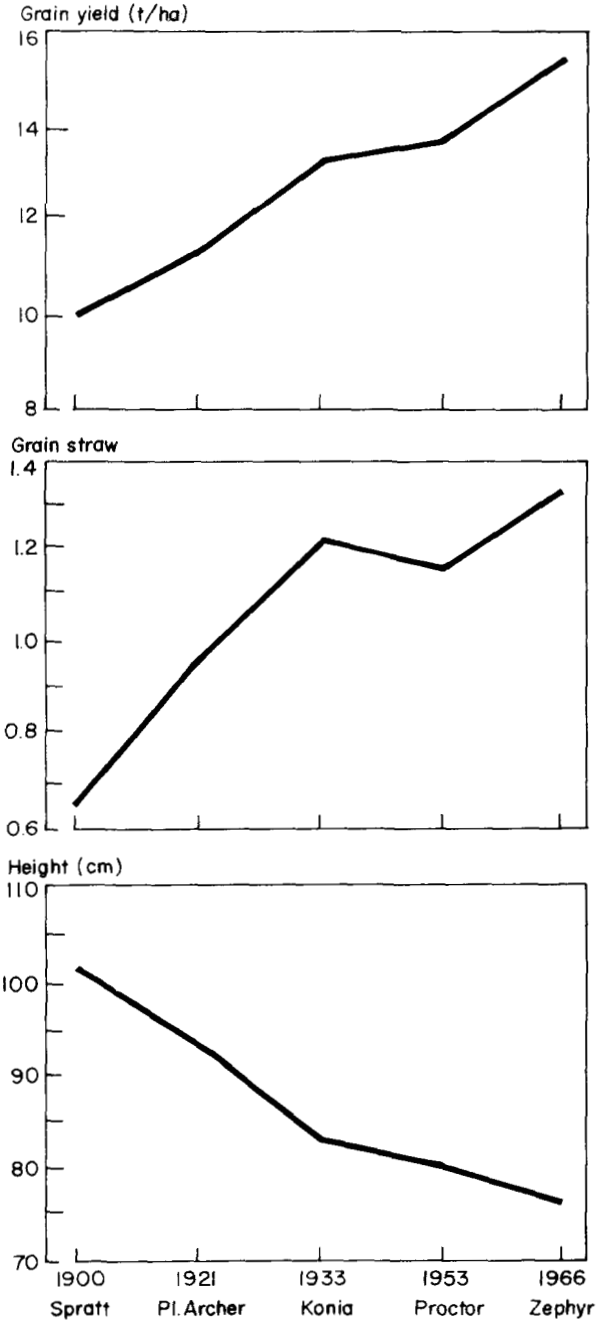
3. Wheat varieties released in New York since 1920. Additional data now shows that in US wheat varieties, reduced plant height has been associated with increased harvest index.



4. Grain yield, biological yield (dry matter production), and harvest index of wheat varieties developed in India since the early years of this century. The data are averages of two or more varieties released in each decade starting in 1910.



5. Dry matter production, economic yield, and harvest index of improved groundnut varieties released in the US since the early 1940s.



6. Grain yield, plant height, and grain-straw ratio of barley varieties released in UK since the beginning of this century (data from Welsh Plant Breeding Station).

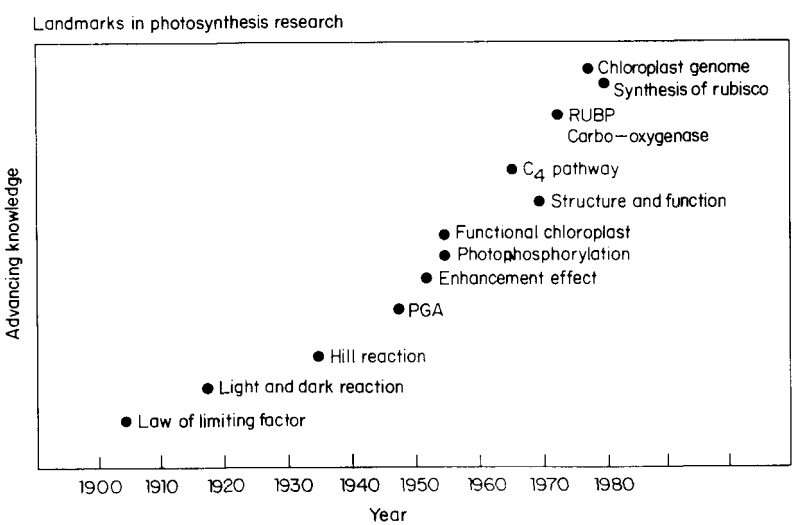
during the 10,000-yr evolution of crop plants in traditional agriculture? In other words, can the photosynthetic rate be genetically manipulated? The key enzyme involved in photosynthesis fixes not only carbon dioxide, but also oxygen. Loss of some photosynthates makes the system inefficient.

A surprisingly large number of important discoveries about photosynthesis have been made only in the last 20 yr or so (Fig. 7). Can plants now be genetically induced to fix more carbon dioxide? In the long term, this area should probably receive the highest priority from molecular biologists.

The importance of photosynthesis to high crop yields can be better appreciated when the grain yields of some recently evolved Eritish wheat varieties (14 t/ha) are compared with those of the improved Indian wheat cultivars (7 t/ha). The ripening wheat plant in Britain receives 17 h of daylight, and the temperatures favor low rates of respiration. In India, on the other hand, the ripening period coincides with high temperatures and fewer daylight hours. Also, of course, most British wheats are of the winter type and have a much longer growth period.

BIOLOGICALLY FIXED NITROGEN

In countries such as India it takes about 2 million Kcal of energy to produce a wheat crop that yields about 4 t/ha. More than 60% of this energy cost is for chemical fertilizers. Can we replace some of the chemical fertilizer nitrogen with biologically-fixed nitrogen? This seminar should more sharply focus on such possibilities. Understanding the molecular basis of nitrogen fixation is very important now, but applied programs cannot yet be very ambitious. The practical possibilities in the next 15-20 yr will probably be in inducing many microorganisms to

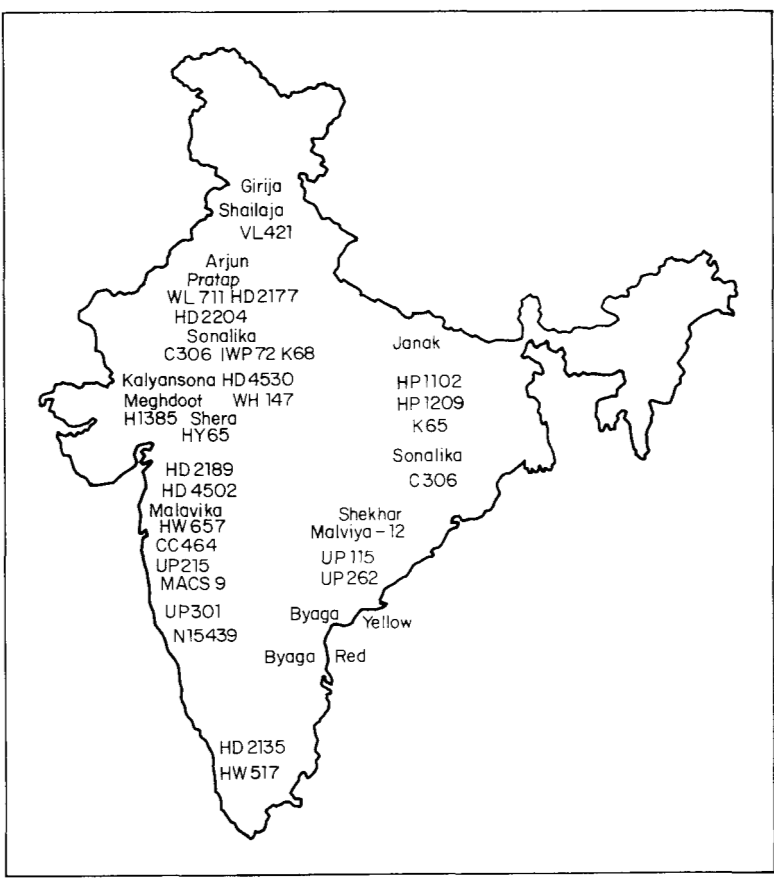


7. A chronological mapping of important discoveries about photosynthesis since the early years of the century (after S. K. Sinha, IARI).

fix nitrogen better. Rhizobia1 bacteria, through their symbiosis with legumes, already fix enormous quantities of nitrogen in the soil. Other microorganisms, such as the Azotobacter and Azospirillum, fix small quantities on their own. The blue-green algae should also receive greater attention in the short term. The compulsion for studies on nitrogen fixation is great. Even India, the world's fourth largest producer and consumer of fertilizer nitrogen will need three to four times its current nitrogen production to meet its food needs at the end of the century.

GENES FOR DIVERSITY

The plant-type genes and broad adaptability of some of the dwarf wheats and rices have been valuable in improving agriculture. They have also brought in a great deal of genetic uniformity. Agriculture cannot be organized around broadly adapted varieties of this kind. The genetic base of our crop varieties must be diversified. In India genetic barriers against diseases and



8. Distribution of wheat varieties recommended for different parts of India helps to diversify the genetic base of the country's wheat crop.

pests have been erected through a "multilineal complex of genetically diverse varieties" distributed in space and time to control disease and pest epidemics (Fig. 8). The next stage should be to locate genes for disease and pest resistance. The deployment of these genes in different genetic backgrounds in varieties of wheat, rice, and other crops will help restore some of the much-needed genetic variability now being lost through intensive international and national breeding programs for wider adaptability.

REFERENCES CITED

1. Austin, R.B., J. Bingham, R.D. Blackwell, L.T. Evans, M.A. Ford, C.L. Morgan, and M. Taylor. 1980. Genetic improvements in winter wheat yields since 1900 and associated physiological changes. J. Agric. Sci.(Camb.) 94:675 – 689.
2. Borlaug, N.E. 1968. Wheat breeding and its impact on world food supply. Pages 1 – 36 in Proceedings 3rd International wheat genetic symposium, Australian Academy of Science, Canberra.
3. Jenson, N.F. 1978. Limits to growth in world food production. Science 201:317 – 320.
4. Kulshrestha, V.P., and H.K. Jain. 1982. Eighty years of wheat breeding in India: Past selection pressures and future prospects. Z. Pflanzenzuecht. 89:19 – 30.
5. Vogel, O.A., R.E. Allen, and C.J. Paterson. 1963. Plant and performance characteristics of semi-dwarf winter wheats producing most efficiently in the Eastern Washington. Agron. J. 397 – 398.

NATIONAL STRATEGIES AND COLLABORATIVE RESEARCH

THE NETWORK APPROACH: UNITED NATIONS DEVELOPMENT PROGRAMME IN AGRICULTURE

W. T. MASHLER

A number of participants representing national, international, and other aid agencies in this seminar are already involved in networks created to promote international scientific collaboration for development. The IARCs, sponsored by the Consultative Group on International Agricultural Research (CGIAR), represent the first major effort in scientific research to meet the needs of the developing world. Dr. Swaminathan felt, therefore, that many of the scientists meeting here today might benefit from a description of how their efforts fit into the broader scheme of development support.

I am particularly pleased to address the subject of cooperative networks, since I have helped develop many of them. To give you an insight into UNDP involvement, I would like to briefly describe the Global and Interregional Programme.

In January 1970, UNDP decided to support the first global scientific project: to help develop high-protein maize, a food crop important to millions throughout the developing world. This project, basic scientific research into new solutions for old problems, was a new form of development support for UNDP.

In the past 13 years, CNDP's Global Programme has grown into a worldwide collaborative research effort of the highest scientific quality. A complementary program of interregional activities disseminates research results and assists developing countries in other ways. The combined program spans five areas: food and agriculture; health, including drinking water and sanitation; international economic relations and cooperation among developing countries; energy; and human resources. More than 100 developing countries currently participate in our projects.

Two convictions underlie the Global/Interregional Programme:

1. Every serious development effort must be accompanied by a constant search for new approaches to cure not only the symptoms, but also the causes, of underdevelopment.
2. Many problems of developing countries can only be solved through global cooperation to pool and exchange knowledge, skills, and experience. Over the years, the Global and Interegional Programme has proved an important vehicle in such cooperation, both among developing countries and among UNDP, other international donors, and recipient countries. The Programme's "multiplier effect" has been substantial: between 1971

Senior director, Division for Global and Interregional Projects, United Nations Development Programme, 1 United Nations Plaza, New York, NY 10017, USA.

and 1982, $104.2 million of UNDP's global and interregional funds helped generate an additional $1,1816.3 million from other sources, an amount nearly 17.5 times the UNDP contribution.

Another great strength of the Programme has been its ability to pioneer. Few other activities supported by international systems of assistance have better fulfilled the Preamble of the UN Charter ". . . to employ international machinery for the promotion of the economic and social advancement of all peoples."

Seventy-two percent of the program's resources is committed to agricultural research, 24% to health research. The agricultural research is on the food crops (rice, maize, sorghums, millets, root crops, and tuber crops) typically grown by small farmers and important to lower income groups; and on such smallholder farming practices as nitrogen fixation.

Two features of UNDP's global activities set them apart from the typical UNDP-supported project:

1. They are research-oriented and involve fundamental research, field testing or clinical trials, further research to adapt results to diverse conditions, training of large numbers of national scientists and technicians in applying results, and strengthening agricultural and health services to achieve effective delivery. By its nature, scientific research requires long-term support. Whenever new scientific opportunities are offered, even generations away, they must be recognized and seized.
2. They represent collaboration among UNDP, many other donors, and the developing countries themselves. To mobilize the needed sources, UNDP has helped develop several major donating consortia, the most prominent of which is the CGIAR.

The very organizations investing in UNDP are those that like minimum risk and maximum safety. They have learned quickly that investment risks taken in scientific research can pay handsome dividends, particularly when the investments are made in such excellent institutions as the IARCs.

The personalities who created the system are for the most part gone, but those who now represent their donor organizations are no less supportive of the system: we have achieved a long-term network of donor support for scientific research.

LINK WITH NATIONAL PROGRAMS

In agriculture, we have tried to strengthen national capabilities by increasing training opportunities, conferences, workshops, and, in certain cases, small grants for collaborative research at national institutions. The UNDP-financed International Rice Testing and Improvement Program (IRTP) and the West African Sorghum and Millet Improvement Programme typify the effort to strengthen our links with national institutions. Expanded training of developing-country nationals remains integral to all

our projects and is, in fact, the backbone of scientific expansion in national programs. In training, links have sprung up spontaneously because hundreds of scientists trained in international centers maintain professional ties between national programs and the centers. However, a much larger effort is needed by donor agencies and developing countries themselves to mobilize resources for national institutions and meet the agricultural needs of growing populations.

IRTP, implemented by IRRI in collaboration with some 80 developing countries, has become an indispensible vehicle for the exchange of improved materials, knowledge, and experience among rice scientists around the world. IRRI's biological nitrogen-fixation research, supported by UNDP, has led to similar initiative at International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), International Institute of Tropical Agriculture (IITA), International Center for Agricultural Research in the Dry Areas (ICARDA), and Centro Internacional de Agricultura Tropical (CIAT). Research on biological nitrogen fixation in developed and developing countries must be closely coordinated, a task performed in large part by the UNDP project advisory committees, which include internationally known scientists from developed and developing countries.

In all of our projects with the IARCs, we have encouraged interinstitutional cooperation within and among disciplines. For example, since we became involved with CIMMYT in research to improve maize protein. quality, I have encouraged crop-oriented IARCs to more closely interact in nutritional and crop sciences to define parameters for nutritional breeding of food crops. In that spirit, we helped bring together concerned IARCs to the recent nutrition workshop at International Livestock Center for Africa (ILCA).

In contrast to agriculture, where there is already cooperative action, tropical medicine research has only recently entered the international arena. Using our experience in fostering the IARCs of the CGIAR, unquestionably the most successful institutional innovation of the past two decades in development assistance, we led a concerted attack on tropical and diarrhoeal diseases, poor environments, and inadequate nutrition. This program involves close collaboration with the World Health Organization (WHO), the World Rank, United Nations International Children's Emergency Fund (UNICEF), many bilateral donors, several foundations, and research laboratories and health institutes of developed and developing countries. Another example of our health involvement is the International Center for Diarrhoeal Diseases Research in Bangladesh, one of the most important enterprise in its field. One more example of our efforts to link agriculture with health is our role in reshaping the mandate of the International Centre of Insect Physiology and Ecology (ICIPE) in Kenya from primarily basic entomology to practical pest problems.

Several of the IARCs are already engaged in biotechnology research financed by UNDP, e.g., nitrogen fixation at IRRI, ICRISAT, ICARDA, IITA, and CIAT; tissue and cell culture at IRRI and International Laboratory for Research on Animal Diseases (ILRAD); protoplast fusion and recombinant DNA technology at

ILRAD. Many of these programs coordinate with national programs in developing and developed countries. To accelerate the benefits of these programs, inter-institutional cooperation between developing and developed countries should be enhanced. Support for training developing-country scientists, for supplies and equipment, and for consultants should increase. The recently established International Organization for Chemical Sciences in Development (IOCD), originally sponsored by UNESCO, is an example of a network providing a variety of services to developing countries (e.g., training scientists in applied chemistry, developing new drugs, exchanging information). UNESCO has developed a similar one in biological sciences. As a consequence of the USAID-sponsored 1982 Conference on Biotechnology and the UNDP-supported IRRI conference on Chemistry in Agriculture, collaboration in applying biotechnology is being attempted. Much biotechnology research can be undertaken through the existing cooperative networks. We do not need a new international center.

As the IARCs have grown and matured, they have upheld their mandate of interaction: they respond to each other's needs as well as to those of national programs.

The Southern Africa Development Coordination Conference (SADCC), which had the enthusiastic support of all center directors, is an extension of the networking the centers already practice with each other. The recent agreement between CIMMYT and IITA on maize research in Africa and the joint rice-wheat integrated production trials of IRRI and CIMMYT are just two of the many successful such joint endeavors.

The centers are uniquely positioned to anticipate in scientific and technical terms the projected needs and demands of an ever-changing world. They have the advantage of being able to make political decision makers aware of their responsibilities in ensuring that scientific opportunities are properly used to fully benefit their constituents.

We in the donor community are truly grateful to you for improving the conditions of mankind. When we see how much time, money, and effort is wasted, we can only marvel at how much is being achieved here with so little. Too few in our world know, much less appreciate, your splendid achievements, which transcend political borders, cultural differences, and even conflict.

All the network examples I have cited in this paper illustrate the basic need for harmony of intent; for realistically formulating institutional structures; for shrewd and sensitive international leadership and quality executive and secretarial support; and for good scientific and financial housekeeping. In international research, political chasms must be bridged by scientific fraternity, initiative, and commitment. You scientists and we who help fund your research can take great pride in our joint endeavor to improve the lives of vast numbers of poor people. Indeed, it leads the way toward eliminating intolerable inequities, and in so doing, lays the foundations for peace.

BIOTECHNOLOGY RESEARCH IN INDONESIA

M. A. RIFAI and D. S. SASTRAPRADJA

Very little biotechnological work has been conducted in Indonesia, but in 1981 a 5-yr biotechnology research program was begun. This paper describes that plan and outlines the hopes it has generated and its potential handicaps, the most important of which is lack of trained manpower.

Although for centuries microbes have been used in Indonesia to prepare traditional fermented foods and beverages such as tempe, oncom, tapai, terasi, kecap, brem, and tuak, there are no large-scale industries in these biotechnological processes. As in many other developing countries, these foods have been produced through home-based operations, the techniques and equipment recycled from generation to generation. Understandably, production processes have scarcely changed, much less improved. Modern science has not entered into these traditional food production methods, although they could become important industrial resources (4). Biotechnology, if properly channeled, could vastly improve the diet and the lives of poor Indonesians. The Indonesian Government has already taken steps to ensure that Indonesians will benefit from biotechnology.

BIOTECHNOLOGY-RELATED RESEARCH IN INDONESIA

The Indonesian private sector has not undertaken biotechnological work, but Indonesian researchers at many universities and governmental research institutions have approached it. During the Third National Microbiological Congress, 46 contributions out of 147 dealt with aspects of food fermentation (l), and 5 works presented had used modern biotechnology methods. The remaining papers reported research results in agricultural, veterinary, and medical microbiology. Attempts to improve the microbial strains used in food fermentation have had some success, but no impact on industry.

Indonesian tissue culture researchers are attempting to produce large quantities of plantlets of the "tanduk" banana,

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Assistant director (Research), National Biological Institute-LIPI, Bogor, Indonesia; and deputy chairman, Indonesian Institute of Sciences (LIPI), Jakarta, Indonesia,

taro, begonia, orchid, and valuable trees, such as sandalwood, illipe nut, and Bouea macrophylla. In vitro germplasm conservation is an important objective of this work. Embryo culture of sugarcane is also being tried.

Many Indonesian soil microbiologists have focused on Rhizobium and Azolla. Their goal is to incorporate the attributes of these organisms into crop breeding programs.

Indonesia is an agrarian country, so we have extensive vegetable waste. A few research institutes are therefore trying, with the help of microorganisms, to convert this agricultural waste into biogas, animal feed, or other useful products.

Although Indonesian research activities are diverse and numerous, results have not been promising. This is mainly due to a lack of qualified manpower (3). Moreover, most of these projects are conducted in poorly equipped and insufficiently financed laboratories all over Indonesia.

CIBINONG BIOTECHNOLOGY CENTER

In early 1981, the Indonesian Government considered setting up a national biotechnology program. The Indonesian Institute of Sciences established a feasibility study team to evaluate technological, managerial, and economic aspects and to formulate short- and long-term program goals.

This team has suggested that Indonesia focus on food, feed, medicinal and industrial chemicals, and energy (2) and that resources now scattered all over the country be linked into a national network. Furthermore, a center and clearing house for the national network should be established. The Indonesian Institute of Sciences (LIPI) might serve this function, keeping the remaining institutes in close collaboration with it.

Two hundred hectares of government land in Cibinong (a suburb of Bogor) has been committed for the site of the network center. The government will build the required laboratories as it is doing for the National Science and Technology Center in the Bogor suburb of Serpong.

The first phase of the program consists of assigning to it a group of scientists from the Indonesian Institute of Sciences (e.g. the National Biological Institute and the National Chemical Institute). Laboratory, pilot plant, and site development should be started at the same time. Within 2 or 3 yr after the program begins, the special laboratories for microbiology and biochemistry, a general purpose pilot plant, and greenhouses should be completed. Research equipment and other facilities should be made available as they are needed. Priority should be given to developing oral contraceptives and corticosteroids based on sitosterol and diosgenin, which can be formed from agricultural waste extraction and conversion by microorganisms.

Manpower recruitment and training in biochemistry, microbial genetics, chemical technology, microbial fermentations, genetics, plant breeding, and animal reproduction are a top priority of the 5-yr plan. Many recruitees are already studying in universities abroad; by the end of 1989, at least 20 Ph Ds and 40 graduate technicians will implement the project.

Establishing such a sophisticated program in Indonesia will require cooperation and assistance from the international scientific community. Agricultural development in Indonesia has enjoyed much success; we are optimistic about the new challenge biotechnology presents.

REFERENCES CITED

1. Josodiwondo, S., R. Utji, and V.C. Warsa. 1983. Mikrobiologi di Indonesia. Perh. Mikrob. Indon., Jakarta. 619 p.
2. Sastrapradja, D.S., ed. 1984. Laporan studi kelayakan program bioteknologi Cibinong. LIPI, Jakarta.
3. Sastrapradja, S., S. Saono, M.A. Rifai., and Rustamsyah. 1983. Biotechnology: Indonesian country report. Paper read at The ASEAN-EEC Seminar on Biotechnology 14-18 November 1983 in Singapore.
4. Steinkraus, K.H. 1982. Traditional food fermentation as industrial resources. In Traditional food fermentation as industrial resources in ASCA countries. S. Saono, F.G. Winarno, and D. Karyadi, eds. LIPI, Jakarta. 259 p.

TISSUE CULTURE RESEARCH AT NATIONAL INSTITUTE OF AGROBIOLOGICAL RESOURCES, JAPAN

T. SHIGA

The National Institute of Agrobiological Resources (NIAR) started in December 1983 at Yatabe, Tsukuba, Ibaraki, Japan. Plant tissue culture research by its Department of Cell Biology includes work on conservation of sweet potato germplasm, meristem culture, and organogenesis of mulberry tree, anther culture of rice, improving anther culture conditions, variation in regenerated rice plants, variation in regenerated pineapple plants, in vitro selection, and isolation and culture of protoplasts.

The National Institute of Agrobiological Resources (NIAR) consists of four research departments: Genetic Resources, Molecular Biology, Cell Biology, and Applied Physiology. It has 219 staff members, including 124 researchers. The Department of Cell Biology aims to develop in vitro culture techniques for plant breeding and to create new crops with novel characteristics. It has 7 laboratories (Wide Hybridization, Heterosis, Cell Physiology, Cell Genetics, Cell Manipulation, Morphogenesis, and Cell Breeding), and 23 researchers. Three of the laboratories originated from the former Division of Genetics, National Institute of Agricultural Sciences; three, from the Sericultural Experiment Station; and one, from the National Institute of Plant Virus Research. Plant tissue culture had already started in Cell Genetics, Morphogenesis, and Cell Breeding laboratories before the transfer. In the other laboratories, plant tissue culture started only afterwards. This paper reviews the plant tissue culture research carried out by the Cell Biology Department before the transfer,

SWEET POTATO GERMPLASM CONSERVATION

After several tests on in vitro germplasm conservation of sweet potato, Kobayashi (2, 3) concluded that meristem culture is a promising tool for germplasm conservation. More than 100 accessions of the cultured materials have been multiplied. After

Director, Department of Cell Biology, National Institute of Agrobiological Resources, Yatabe, Tsukuba, Ibaraki, 305 Japan.

repeated isolation and subculture of the stem tip, virus symptoms on leaves usually disappeared.

Five-mm-long stem tips were suitable for first-passage culture; for successive culture, stem tips from transplants were 2-3 cm long. There were no significant growth differences among basal media such as those of Murashige and Skoog (MS) (5), White (38), or Bonner and Devirian (1). In general, plant growth substances were not necessary. The materials from first passage culture of fourth subculture were maintained under 12-h illumination of about 1,500 Lx (or natural length) at 28°C. (Long-term storage of sweet potato germplasm calls for 20°C.)

Calluses covered some culture stem tips. Their formation depends on plant species and strain, or size of stem tips. To prevent it on cultured stem tips, any of the following treatments is recommended: 1) diluting the basal medium by half, 2) diluting the NH_4NO_3 and KNO_3 of MS medium (5) by one-tenth, 3) using White medium (38) instead of MS medium (5), or 4) adding 0.5% charcoal to MS medium with the tip of the subcultures cut off every 2 or 3 mo.

Apical meristem culture of sweet potato and related species showed no variation during 3 yr of research and may therefore be recommended for germplasm conservation of those plant species.

MERISTEM CULTURE AND ORGANOGENESIS IN MULBERRY TREE

Mulberry tree (*Morus alba* L.) is a woody feed crop for silkworm (*Bombyx mori* L.). The conventional method of propagating it is by grafting and cutting. Interest in tissue culture to propagate and breed it has been increasing. Ohyama (71, Ohyama and Oka (l0), and Oka and Ohyama (13, 14, 17, 18, 19, 20) have attempted in vitro culture of various buds of mulberry trees. Nondormant winter buds without scales, terminal buds in the growing season, and axillary buds at various stages of development were aseptically isolated and cultured on MS medium containing growth substances. Excised buds developed shoots only with 1 mg benzyladenine (BA)/litre added to the medium. BA was more effective than zeatin, while kinetin was not effective at any concentration tested. Some varieties developed shoots in the presence of either sucrose or fructose, while others did so only in the presence of fructose.

Shoot meristem from bud cultures could be repeatedly subcultured. Subcultured explants subsequently grew on medium without growth substances, provided they already had roots.

Despite intense effort by scientists, mulberry callus has not regenerated plantlets. Oka and Ohyama (21) cultured leaf explants from mulberry seedlings aseptically grown on MS (5) medium. Adventitious buds formed either on leaves excised from the subcultured shoot tips or on the first leaves of the seedling cultured on medium containing 1 mg BA/litre.

Ohyama and Oka (12) cultured hypocotyl segments of mulberry on MS (5) medium supplemented with N-(2-chloro-4-pyridyl)-N'-phenylurea (4PU). In a few days, both ends of the explants began

to swell. However, multiple buds were produced only at the apical end. When transferred to g-napthalene acetic acid (NAA) at 0.1 mg/litre medium, these buds rooted and formed whole plants. We are studying the histology of multiple shoot formation.

Ohyama and Oka (11), and Oka and Ohyama (15, 16, 22) also succeeded in forming shoot from hypocotyl or callus in the woody plants, Broussonetia kazinoki and Eucalyptus globulus.

RICE ANTHER CULTURE

Niizeki and Oono (6) and Oono (23) reported producing haploid rice from pollen-derived callus. They used basal media developed by Miller (1963) and Murashige and Skoog (5), or a modification of Miller's medium. 2,4-dichlorophenoxy acetic acid (2,4-D) or NAA induced pollen callus. Pollen calluses were induced in cultivars and hybrids of indica and japonica and their interorigin hybrids.

Callus formation rates differed among cultivars and hybrids. Cells of pollen callus 50-55 d after inoculation exhibited a wide range of chromosome numbers (8-114). The medium with high yeast extract concentration, sucrose, and low NAA concentration enhanced regeneration of plants from pollen calluses. Addition of cytokinin (benzyladenine) was only slightly effective. Plant regeneration rates were about 20% on the average, and 57.8% at the maximum.

Plant regenerated from pollen callus showed chromosome numbers of x, 2x, 3x, 4x and 2x+1. Forty percent were haploid and 48.5%, diploid. Many of the regenerated plants were chlorophyll-deficient as albina (80%), striata, and maculata. Sometimes, normal and chlorophyll-deficient plants were simultaneously regenerated from one pollen callus.

Diploid plants of several cultivars and their progeny were studied genetically. The progeny of about two-thirds of the diploid plants were homozygous. They were uniform in flowering time, plant height, and other agronomic traits. Any differences between them and their parents were insignificant.

Wakasa and Watanabe (36), and Wakasa (32) cultured anthers from rice species of the A or A^g genome group, Oryza sativa, O. glaberrima, O. perennis perennial type, O. perennis annual type, O. perennis American form, and O. breviligulata. Callus formation frequency differed considerably with type, concentration, and combination of auxin and cytokinins. Calluses could be induced from pollen by anther culture of interspecific hybrids of rice.

The shoot differentiation rate varied among species, as did the regeneration rate of albino plants from rice pollen calluses. Regardless of the different culture conditions required among species for callus formation, calluses originally developed on medium with 2,4-D differentiated shoots on medium with BA. The regeneration rate of green plants varied with rice cultivar and species. O. sativa showed a higher rate than O. glaberrima; and

other related wild rices, except O. perennis annual type, regenerated albino plants.

IMPROVING ANTHER CULTURE CONDITIONS

Wakasa (32) reported that the culture conditions for regenerating plants from rice pollen calluses could be improved. In the rice cultivars Norin 8 and Fujisaka 5, which differed in the rates of shoot and albino plant regeneration, several conditions increased the shoot differentiation rate to more than 80%:

1. supplying 2-10 mg BA/litre;
2. increasing the sucrose concentration to 60 g/litre;
3. transferring the young callus tissue to regeneration medium; and
4. preventing prolonged growth of calluses on BA.

These media conditions did not affect the rate of albino plant differentiation characteristic for each cultivar. Only high temperature during callus and shoot formation increased the albino plant differentiation frequency. There was no correlation between shoot and root differentiation.

Regeneration from rice anther culture can produce haploid and diploid plants derived from pollen grain. Anther culture has been used for rapid generation in the rice breeding programs at Kamikawa and Hokuriku Agricultural Experiment Stations.

VARIATION IN REGENERATED RICE PLANTS

Many chlorophyll-deficient and polyploid plants regenerate from pollen calluses (23, 32) and Oono (23) suggested that gene mutations occur during culture.

Oono (24, 25, 26, 27) confirmed, through analysis of regenerated plants from homozygous diploid rice callus, that mutations were easily induced in cultured cells. Calluses were induced from the seeds of spontaneous diploids from spontaneous haploids, and plants were regenerated from them without subculture. Seventy-five seeds were used, and 1,121 plants (D_1) were regenerated; 83 of these were albino. Mutation in about 800 D_1 and 11000 of their D_2 and D_3 progeny was analyzed.

The seed fertility of D_1 plants decreased to 58.7%, and some of the plants were not genetic mutants. The mutation rate of chlorophyll mutants in D_2 was 8.4% (comparable to the rates from X-ray and g-ray irradiation). Analysis for mutations in chlorophyll, flowering, plant height, fertility, and morphology in D_1, D_2, and D_3 generations revealed that only 28.1% of regenerated plants were normal in all these characters. The mutation frequencies appear to depend partially on medium condition.

Fukui and Kobayashi (2) successfully regenerated 12 rice plants from callus induced from one Nihonbare rice seed.

Calluses were induced on N6 medium with 2,4-D and PFP. In the following year, seeds (D_2) from 12 regenerated plants (D_1) were sown in the field. Eight of the twelve lines (D_2) showed characteristics including albinism, early maturity, short culm, and low fertility.

Wakasa (32) also reported variation among regenerated plants from rice anther. Calluses were induced from anther of rice cultivars Norin 8, Fujisaka 5, and Sasanishiki, and the 46 plants regenerated were propagated vegetatively in the field. Among the 46 clones, 2 were classified as mixploid; 10, as haploid; 6, as diploid; 24, as triploid; and 4, as tetraploid. Phenotype variation, including short culm and low seed fertility, occurred among the regenerated haploid and diploid plants.

The diploid plants in D_1 were selfed and plants in the D_2 generation were cultivated in the field and their agronomic characters evaluated. Fifty offspring from six diploid plants (D_1) with short culm retained their character. This variation was confirmed to be due to mutation.

VARIATION IN REGENERATED PINEAPPLE PLANTS

In vegetatively propagated plants, tissue culture is useful for propagating clones of the same genotype, producing virus-free plants, and conserving germplasm. Wakasa (31, 32) investigated variation in regenerated plants from tissue culture of pineapple (*Ananus comosus* [L.] Merr.) Syncarp, crown, slip of syncarp, sucker, and axillary bud were cultured on basal MS (5) medium with NAA and BA. Nodular bodies or globular bodies induced from an organ regenerated into plants when transferred to the regeneration medium. Leaf color, wax secretion, foliage density, and spine variants were observed. Variants were frequent in regenerated plants from syncarp and slip, but not in those from crown and axillary.

Culture of bud from plants is suitable for propagating clones of the same genotype and cultivar, while cells and organs exhibiting variation are probably good variant producers.

SELECTION IN VITRO

If high frequencies of gene mutations were induced in the tissue culture, in vitro breeding would be more efficient and productive.

Oono and Sakaguchi (28), after 6-12 mo of subculturing, isolated Norin 8 rice callus lines resistant to 1% NaC1. Seventy-two D_1 plants were regenerated from these. The regenerated plants were albino-like in the NaCl regeneration medium but became normal green plants upon transfer to NaC1-free medium. Nineteen D_2 plants from lines showing normal seed fertility were examined for their germination in 1% NaCl medium. Three of the nine D_2 lines regenerated from these callus clones segregated into resistant and susceptible types. The resistant

type was inherited by the D_3 generation, but the resistant population was smaller than that in D_2, and fixed lines were not obtained.

Using suspension cell lines from mesophyll protoplast of Nicotiana tabacum, Wakasa (33) selected calluses resistant to aminoethylcysteine (AEC) or threonine and regenerated plants from these. Nine AEC-resistant cell lines and three cell lines resistant to threonine regenerated many plants. The resistant tobacco lines, were not genetically analyzed, because no self seed was obtained and D_2 seedlings did not show clear resistance. However, two lines were probably mutants.

Wakasa and Widholm (37) selected 5-methyltryptophan (5MT)-resistant cells using 50-day-old calluses from rice seeds. Calluses that grew on MS medium with 2,4-D, NAA, and kinetin were divided and inoculated on the 5MT medium. Surviving calluses were transferred to the regeneration medium. Only 1 out of over 1000 calluses grew rapidly on 5MT medium, and regenerated into 6 plants. Calluses from the second generation (D_2) showed 5MT resistance. Three out of six lines were mutant plants exhibiting resistance to 5MT. Seedlings from third-generation seeds (D_3) were inoculated on culture medium with 5MT. Susceptible D_2 plants produced only susceptible seedlings. On the other hand, resistant D_2 plants produced both resistant and susceptible seedlings. Resistant calluses had increased phenylalanine content. The mechanism causing this is not clear, so the genetics are being analyzed.

Wakasa et al (34) successfully isolated chlorate-resistant rice calluses from the nitrate reductase-deficient cell lines. Calluses were produced from anther culture of plants obtained from offspring of g-irradiated plants. Chlorate resistance was selected four times on MS medium with amino acid and sodium chlorate. The 67 selected calluses were transferred to 3 media with different N sources. Only two calluses could not grow on the NO_3 medium. They lacked the enzyme activity of nitrate reductase, and may serve as markers in somatic cell genetics.

From seed of Norin 8, Sano et al (29) produced rice callus lines on MS medium with 2,4-D. One hundred sixty-seven plants were regenerated and selfed. Total protein content in the endosperm of clean D_1 seeds was analyzed. Protein content per gram of endosperm varied up to a maximum of 40%.

From seeds of Nihonbare, Sato et al also isolated rice callus with methionine resistance. Surviving calluses were repeatedly cultured on MS medium containing methionine. Twenty-seven plants were regenerated (D_1) and selfed. Amino acid content per gram of endosperm from cleaning seeds of 197 plants (D_2) and the 167 mentioned above were determined. Two plants had high lysine content. All of these are being genetically analyzed.

PROTOPLAST ISOLATION AND CULTURE

In 1971 Takebe et al reported that protoplast isolated from tobacco leaves could be induced to divide in culture and form

calluses that could regenerate into plants. Mass populations of protoplast can be isolated, made to take in foreign genetic material, and induce protoplast fusion between species. Many recent reports indicate protoplast isolation and regeneration into plants may become a powerful new plant-breeding tool.

Ohyama and Nitsch (8) enzymatically isolated protoplasts from haploid tobacco leaves and cultured them in synthetic media. Cultured protoplasts divided within a few days after inoculation and subsequently developed into small colonies. After several subcultures, these colonies differentiated into shoots and roots, and finally resulted in haploid flowering plants.

Ohyama and Oka (9) studied cell and protoplast culture in mulberry and other species. Protoplasts prepared from the leaves and callus cultivars of the papar mulberry have been induced to divide and develop into cell colonies.

Wakasa (30) isolated intact protoplasts in 28 plants, including woody and succulent ones. From intact leaves, cells or protoplasts were isolated through macerozyme followed by cellulase, which worked for most dicotyledons, or through a mixture of macerozyme and cellulase for monocotyledons and certain dicotyledons. Zymolyase was very effective in isolating protoplast from pollen tetrads or pollen mother cells.

Wakasa (32) investigated nurse cultures of Antirrhinum majus protoplasts. Nurse culture for fresh stem segments or callus and conditioned medium for fresh stem segments stabilized protoplast division and clone formation.

Wakasa et al (35) selected nitrate reductase-deficient rice mutants. The protoplasts from these cell lines were easily isolated with enzyme solution consisting of 0.3M manitol, 1% macerozyme R 10, and 4% Cellulase Onozuka RS. A 1-g callus yielded 5 x 10^7 protoplasts, which divided and propagated into colonies at frequencies of 0.1 to 1%.

A large-scale project (sponsored by MAFF, Japan) to use cell fusion in plant breeding started in 1982. More than 30 researchers are now investigating protoplast isolation and culture in crops including rice, soybean, potato, vegetables, taro, yam, fruit trees, marine algae, mushroom, and yeast. Their objective is to produce somatic hybrid plants. Five laboratories are involved in the project, carrying out basic research on culture, regeneration, and fusion of protoplasts.

REFERENCES CITED

1. Bonner, J., and P.S. Devirian. 1939. Growth factor requirements of four species of isolated roots. Am. J. Bot. 26:661-665.
2. Fukui, K., and M. Kobayashi. 1981. Sequential occurrence of the mutation in a growing rice callus [in Japanese]. Jpn. J. Breed. 31 (Suppl. 2):198-199.
3. Kobayashi, M. 1982. Applications of plant tissue culture in the improvement of sweet potato. 1. Maintenance and multiplication of germplasm [in Japanese]. Jpn. J. Breed. 32 (Suppl. 2):66-67.

4. Kobayashi, M. 1983. Applications of plant tissue culture in the improvement of sweet potato. 2. Suppressing effect of charcoal powder on dedifferentiation of stem tips [in Japanese]. Jpn. J. Breed. 33 (Suppl. 2):90-91.
5. Murashige, T., and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.
6. Niizeki, H., and K. Oono. 1968. Introduction of haploid rice plants from anther culture. Proc. Jpn. Acad. 44:554-557.
7. Ohyama, K. 1970. Tissue culture in mulberry tree. JARQ 5(1):30-34.
8. Ohyama, K., and J.P. Nitsch. 1972. Flowering haploid plants obtained from protoplasts of tobacco leaves. Plant Cell Physiol. 13:229-236.
9. Ohyama, K., and S. Oka. 1975. Isolation of protoplasts from mesophyll cells of Morus alba L. [in Japanese]. Proc. Crop Sci. Soc. Jpn. 44(1):121-122.
10. Ohyama, K., and S. Oka. 1976. Regeneration of whole plants from isolated shoot tips of mulberry tree [in Japanese, with English summary]. J. Sericult. Sci. Jpn. 45:115-120.
11. Ohyama, K., and S. Oka. 1980. Bud and root formation in hypocotyl segment of Broussonetia Kazinoki Sieb. in vitro. Plant Cell Physiol. 21(3):487-492.
12. Ohyama, K., and S. Oka. 1982. Multiple shoot formation from mulberry (Morus alba L.) hypocotyls by N-(s-chloro-4-pyridyl)-N'-phenylurea. Pages 149-150 in Plant tissue and cell culture 1982. A. Fujiwara, ed. The Japanese Association for Plant Tissue Culture, Tokyo, Japan
13. Oka, S., and K. Ohyama. 1973. Induction of callus and effects of constituents of media on callus formation in mulberry tree [in Japanese, with English summary]. J. Sericult. Sci. Jpn. 42(4):317-324.
14. Oka, S., and K. Ohyama. 1974. Studies on in vitro culture of excised bud in mulberry tree. I. Effects of growth substances on the development of shoot and organ formation from winter bud [in Japanese, with English summary]. J. Sericult. Sci. Jpn. 43 (3):230-235.
15. Oka, S., and K. Ohyama. 1974. Shoot formation from callus of Broussonetia Kazinoki Sieb [in Japanese]. Proc. Crop Sci. Soc. Jpn. 43(2):289-290.
16. Oka, S., and K. Ohyama. 1975. Effects of constituents of media on the growth of Broussonetia callus tissue [in Japanese, with English summary]. Proc. Crop Sci. Soc. Jpn. 44(1):147-152.
17. Oka, S., and K. Ohyama. 1975. Studies on in vitro culture of excised bud in mulberry tree. II. Effects of growth substances on the development of shoots from axillary bud [in Japanese, with English summary]. J. Sericult. Sci. Jpn. 44(6):444-450.
18. Oka, S., and K. Ohyama. 1976. A study of the medium for the subculture of mulberry callus [in Japanese, with

English summary]. J. Sericult. Sci. Jpn. 45(5):385-391.

19. Oka, S., and K. Ohyama. 1978. Studies on in vitro culture of excised buds in mulberry tree. III. Effects of agar concentration, pH and sugars of medium on the development of shoots from winter buds [in Japanese, with English summary]. J. Sericult. Sci. Jpn. 47(1):15-20.
20. Oka, S., and K. Ohyama. 1978. In vitro culture of excised mulberry bud and dormancy analysis with it. Pages 136-141 in Long term preservation of favourable germ plasm in arboreal crop. T. Akihama and K. Nakajima, eds. Fruit Tree Research Station, MAF., Japan.
21. Oka, S., and K. Ohyama. 1981. In vitro initiation of adventitious buds and its modification by high concentration of benzyladenine in leaf tissue of mulberry (Morus alba). Can. J. Bot. 59(1):68-74.
22. Oka, S., E.C. Yeung, and T.A. Thorpe. 1982. Shoot formation in Eucalyptus globulus hypocotyl explants. N.Z. J. For. Sci. 12(2):501-509.
23. Oono, K. 1975. Production of haploid plants of rice (Oryza sativa) by anther culture and their use for breeding [in Japanese, with English summary]. Bull. Natl. Inst. Agric. Sci., Ser. D, 26:139-222.
24. Oono, K. 1978. Test tube breeding of rice by tissue culture. Trop. Agric. Res. Ser. 11:109-124.
25. Oono, K. 1981. In vitro methods applies to rice. Pages 273-298 in Plant tissue culture. T.A. Thorpe, ed. Academy Press, New York.
26. Oono, K. 1982. Characteristics of mutation in cultured rice tissue. Pages 409-410 in Plant tissue and cell culture 1982. A. Fujiwara, ed. The Japanese Association for Plant Tissue Culture, Tokyo, Japan.
27. Oono, K. 1983. Genetic variability in rice plants regenerated from cell culture. Pages 95-104 in Cell and tissue culture techniques for cereal crop improvement. Gordon and Breach, Sci. Pub., New York.
28. Oono, K., and S. Sakaguchi. 1980. Induction of mutation in tissue culture and their use for plant breeding. III. characteristics of regenerated plants from saline resistant calluses of rice [in Japanese]. Jpn. J. Breed. 30 (Suppl. 2):10-11.
29. Sano, H., F. Takaiwa, and K. Oono. 1983. Induction of mutation in tissue culture and their use for plant breeding. IX. Variation of seed protein and amino acid (Lysine) in regenerated plants [in Japanese]. Jpn. J. Breed. 33 (Suppl. 2):84-85.
30. Wakasa, K. 1973. Isolation of protoplast from various plant organs. Jpn. J. Genet. 48:279-289.
31. Wakasa, K. 1979. Variation in the differentiated from the tissue culture of pineapple. Jpn. J. Breed. 29:13-22.
32. Wakasa, K. 1982. Application of tissue culture to plant breeding-----method improvement and mutant production [in Japanese, with English summary]. Bull. Natl. Inst.

Agric. Sci., Ser. D., 33:121-200.

33. Wakasa, K. 1983. Mutation selection in vitro in rice and tobacco. Contributed paper. Page 23 in International genetics symposium on gene structure and function in higher plants. Hyderabad, India.

34. Wakasa, K., H. Kamata, and M. Kobayashi. 1983. In vitro selection of mutants. 3. Nitrate reductase deficient callus of rice [in Japanese]. Jpn. J. Breed. 33(Suppl. 1):192-193.

35. Wakasa, K., M. Kobayashi, and H. Kamata. 1983. Colony formation from rice protoplast of nitrate-reductase-deficient mutant. Abstr. contributed paper. Page 770 in 15th International congress on genetics. New Delhi, India.

36. Wakasa, K., and Y. Watanabe. 1979. Haploid plant of Oryza perennis (spontanea type) induced by anther culture. Jpn. J. Breed. 29:146-150.

37. Wakasa, K., and J.M. Widholm. 1983. In vitro selection of mutant. 4. Some characters of 5MT-resistant mutant in rice [in Japanese]. Jpn. J. Breed. 33 (Suppl. 2):82-83.

38. White, P.R. 1963. The cultivation of plant and animal cells. The Ronald Press Co., New York. 288 p.

PARENT STATUS OF BIOTECHNOLOGY AT SPANISH NATIONAL INSTITUTE OF AGRICULTURAL RESEARCH

A. CADENAS and J. V. TORRES

Impelled by the potential usefulness of biotechnology for improving crops and livestock, the Spanish Institute of Agricultural Research (INIA) supports several projects involving in vitro culture of meristems and embryos, and has initiated research on haploid culture, monoclonal antibodies, and recombinant DNA technologies (13, 18, 22).

CLONAL PROPAGATION

In vitro meristem culture to obtain virus-free citrus material is INIA's most advanced biotechnology project. All the tristeza virus-free plant material currently used by Spanish farmers was produced in INIA, and clonal propagation is yielding other virus-free vegetable and ornamental plant material as well.

EMBRYO CULTURE

Through embryo culture, viable seedlings are obtained from hybrids between Triticum and related genera by introgressing leaf rust resistance genes from Agropyrum into Triticum and by hybridizing with taxonomically related genera.

Introgression of leaf rust resistance genes from Agropyrum into Triticum

The major aim is to obtain meiotically stable genotypes of Triticum aestivum that have incorporated genes of Agropyrum intermedium coding for Puccinia recondita resistance. The starting material was additior, or substitution lines of Triticum-Agropyrum obtained by Cauderon's method (2). To promote homologous pairing, these lines were crossed with Aegilops mutica or Aegilops speltoides (3); then the embryos were cultured in vitro (4, 16). Resulting plants were repeatedly backcrossed to T. aestivum and the progeny selected for resistance to P. recondita and for meiotic stability. After backcrossing, 350 offspring with 42 chromosomes and leaf-rust resistance were obtained. These genotypes have now been included in breeding programs as donors of resistance and of desired agronomic traits.

Direccion Technica de Relaciones Cientificas. Instituto Nacional de Investigaciones Agrarias. Jose Abascal, 56. Madrid-3, Spain.

Obtaining new wheat and Triticale germplasm

Genetic variability in wheat and triticale is increased by hybridization with taxonomically related genera. Through in vitro embryo culture with several synthetic media the hybrids' difficulty in developing viable seeds is overcome. The hybrid seedlings obtained were treated as follows:

1. Chromosome duplication by colchicine (several hexaploid or octoploid primary triticales were obtained by this method); or
2. Self-pollination or backcrossing of the hybrid. The following plant materials were obtained in this way:

   A) Genotypes at different selection steps, derived from:

   [T. turgidum x S. cereale] x Triticale (6x) or Triticale (6x) x T. aestivum

   B) F_1 or G_1 plants from Triticale (6x) x T. turgidum

   C) F_1 plants from the crosses:

   [T. aestivum x S. cereale] x Triticale (8x).
   [T.. aestivum x S. cereale] x Triticale (6x).
   [T. aestivum x S. cereale] x T. aestivum.

Morphologic, agronomic, cytogenetic, and biochemical characteristics of some of these genotypes have been reported (9, 10, 21).

HAPLOID CULTURE

Haploid culture accelerates obtaining new homozygous barley genotypes. The technique used is based on the poorly understood chromosome elimination phenomenon observed in hybrids from several Hordeum species (23).

H. vulgare (2n = 14) F_3 plants are crossed with H. bulbosum (2n = 14), the ears are treated as described by Jensen (a), and the embryos cultured in vitro in the Kao and Kasha medium (12). The haploid seedlings are then treated with colchicine; and the homozygous diploids obtained are agronomically evaluated.

The steps of this technique have already been established, but some minor adjustments in humidity and temperature are required to optimize the yield of haploid seedlings.

IN VITRO GERMPLASM CONSERVATION

INIA has recently initiated research on citrus cryoconservation and in vitro germplasm exchange.

MONOCLONAL ANTIBODIES

In January 1983 INIA began a project comprising several studies on monoclonal antibodies against African swine fever virus (ASFV). The project's main objectives are:

- To obtain monoclonal antibodies against ASFV by fusion of myelome cells with splenic cells from swine inoculated with different ASFV partially purified proteins;
- To use these monoclonal antibodies in diagnoses and to identify ASFV strains;
- To locate immunopathological lesions using labeled monoclonal antibodies against ASFV as well as T and B cells;
- To purify ASFV antigenic proteins by affinity chromatography using monoclonal antibodies linked to chromatographic supports; and
- To study the immunogenic potential of these purified proteins as a vaccine.

At present we have three monoclonal antibodies against ASFV major proteins and are using them in neutralization assays and other biological tests. We have recently obtained anti-ASFV hybridomas; these are now being characterized.

Our institute uses monoclonal antibodies to make diagnosis kits for several plant viruses. Kits for citrus tristeza virus and for potato X, Y, and leaf roll viruses are now available.

RECOMBINANT DNA

INIA supports three recombinant DNA projects. The goal of the first is to improve *Streptococcus lactis*.

Improvement of *Streptococcus lactis*

S. lactis is the principal starter in the dairy industry. Ability to metabolize lactose and hydrolize casein [plasmid-coded traits (5)] have been the traditional selection criteria for *S. lactis* starters. Extrachromosomal codification of these physiological functions causes the high instability observed in industrial *S. lactis* strains.

INIA has available a bank of lactic bacteria, screened for several traits, to obtain genotypes suitable for starter production. To overcome the instability of some of these traits we are using new genetic techniques. In September 1983 we initiated a project with the following main objectives:

- to ascribe biochemical functions to *S. lactis* chromosome or plasmid DNA;
- to transfer these functions by conjugation or transformation;
- to isolate the DNA sequences coding for these functions; and
- to transfer these DNA sequences.

Genotypes now available are:

- strains of S. lactis selected for resistance to Erythromycin (E+) or Novobiocin (Nb+). (These can act as markers in conjugation or transformation experiments.)
- S. lactis strains that have lost the ability to metabolize lactose (Lac-) after plasmid curing.
- E+ Lac⁻ strains obtained from conjugating E+ and Lac⁻ S. lactis strains by the method of Gasson and Davies (6).

Cloning of DNA coding for wheat storage protein

Although the endosperm of wheat is a major protein source for man (19), wheat proteins have an unbalanced aminoacid profile and a low nutritional value (12). Knowing the gene organization and expression could help improve wheat's food value (7, 17). Molecular-cloning and DNA sequencing techniques can elucidate gene structure and expression (1, 15).

INIA supports a cereal quality breeding program. New biological techniques can help conventional breeding programs (20), so we have recently established cooperation with the United States Department of Agriculture (Western Regional Research Center, Berkeley, California) for a scientist training project with the following proposed objectives:

1. To determine the structure of wheat messenger RNA coding for storage protein components by
   - extracting and characterizing mRNA in wheat and related species of Triticum and Aegilops,
   - studying the in vitro translation of the mRNA,
   - fractionating mRNA to obtain the components coding for storage proteins,
   - preparing nNA(cDNA) complementary to specific mRNA,
   - cloning cDNA through bacterial or viral amplification, and
   - sequence-selecting cDBA clones.

2. To determine the structure and organization of genes in the wheat genome by
   - preparing genomic DNA from wheat,
   - developing methods for cloning DNA fragments large enough to include complete genes or clusters of genes,
   - with cDNA probes, separating and identifying genomic DNA clones that include genes for wheat storage or other proteins important for wheat quality, and
   - carrying out DNA sequencing on selected clones to determine gene structure, organization, and structure of noncoding DNA.

3. To study the expression of storage protein genes at various stages of kernel development by
   - obtaining genomic DNA corresponding to genes being

- transcribed at various stages of development and using cDNA and other clones to examine the nature of genes expressed at various stages of development.

Cloning of African swine fever virus DNA

Hind II and Cla I restriction fragments of ASFV's DNA have been cloned in pACYC 177. To express these clones in E. coli, an expression plasmid using the Tn 10 promoter was used (14).

REFERENCES CITED

1. Bedbrook, J.W. Gerlach, S. Smith, J. Jones, and R. Flavell. 1980. Chromosome and gene structure of plants: a picture deduced from analysis of molecular clones of plant DNA. Pages 49-62 in Genome organization and expression in plants. C.J. Leaver, ed. Plenum Press, New York.
2. Cauderon, Y. 1966. Etude cytogénétique de l'évolution due materiel issue de croisment entre Triticum aestivum et Agropyron intermedium. I. Crèation de types d'addition stables. Ann. Amelior. Plantes 16:43-70.
3. Chueca, M.C., and Y. Cauderon. 1977. Induction d'appariements homeologues entre les chromosomes du Blétendre et un telosome d' Agropyron intermedium par croisement avec des Aegilops. Ann. Amelior. Plantes 27:135-150.
4. Chueca, M.C., y. Cauderon, and J. Tempe. 1977. Technique d' obtention d' hybrides Ble tendre X Aegilops par culture in vitro d' embryons immatures. Ann. Amelior. Plantes 27:539-547.
5. Efstathiou, J.D., and L.L. Mac Kay. 1976. Plasmids in Streptococcus lactis: evidence that lactose metabolism and proteinase activity are plasmid linked. Appl. Environ. Microbiol. 32:38-44.
6. Gasson, M.J., and F.L. Davies. 1980. High-frequency conjugation associated with Streptococcus lactis donor cell aggregation. J. Bacteriol. 143:1260-1264.
7. Greene, F.C. 1981. In vitro synthesis of wheat (Triticum aestivum L.) storage proteins. Plant Physiol. 68:778-783.
8. Jensen, C.S. 1975. Barley genetics III. Pages 316-345 in proceedings of the 3th international barley symposium, Garching.
9. Jouve, N., D. Montalvo, and C. Soler. 1980. Differential heterochromatic regions in homologous chromosomes of wheat. Cereal Res. Commun. 8:599-603.
10. Jouve, N., D. Montalvo, and C. Soler. 1982. C-banding in cytogenetics of 6x-Triticale X Triticum aestivum L. hybrids. Z. Pflanzenzuecht. 88:311-321.
11. Kao, K.N., and K.J. Kasha. 1969. Haploidy from interspecific crosses with tetraploid barley. Pages

82-88 in Barley genetics 2. Washington State University Press, Washington.

12. Kasarda, D.D., J.E. Bernardin, and C.C. Nimmo. 1976. Wheat proteins. Pages 158-236 in Advances in cereal science and technology. Y. Pomeranz, ed. American Association of Cereal Chemists. Saint Paul, Minnesota.
13. Lewis, L. 1982. Agriculture overview. Pages 147-158 in Priorities in biotechnology research for international development. National Academy Press, Washington, D.C.
14. Martin, E., E. Tabares, G. Santurde, J.M. Escribano, and M.G. Notario. 1982. In Proc. IV Reunion Virologia. Noviembre 1982. Avila-España. Sociedad Espanola de Microgiologia, ed. Madrid.
15. Messing, K., D. Geraghty, G. Heidecker, N.T. Hu, J. Kridl, and I. Rubenstein. 1983. Plant gene structure. Pages 211-227 in Genetic engineering of plants. An agricultural perspective. T. Kosuges, C.P. Meredith, and A. Hollaender, eds. Plenum Press, New York.
16. Monnier, M. 1976. In vitro culture of the immature embryo of Capsella bursa-pastoris Moench (Fre). Rev. Cytol. Biol. Veget. 39:l-120.
17. Okita, T.W., and F.C. Greene. 1982. Wheat storage proteins. Isolation and characterization of the gliadin messenger RNAs. Plant Physiol. 69:834-839.
18. Phillips, R.L. 1983. Genetic engineering of plants: some perspectives on the conference, the present, and the future. Pages 453-465 in Genetic engineering of plants. An agricultural perspective. T. Kosuges, C.P. Meredith, and A. Hollaender, eds. Plenum Press, New York.
19. Reitz, L.P. 1967. Wheat and wheat improvement. K.S. Quisenberry and L.P. Reitz, eds. American Society of Agronomy, Inc., Madison, Wisconsin.
20. Simmonds, N.W. 1983. Plant breeding: the state of the art. Pages 5-25 in Genetic engineering of plants. An agricultural perspective. T. Kosuges, C.P. Meredith, and A. Hollaender, eds. Plenum Press, New York.
21. Soler, C., D. Montalvo, and N. Jouve. 1980. Secondary association of univalent hexaploid triticale and rye and wheat. J. Hered. 71:408-410.
22. Swaminathan, M.S. 1982. Perspectives in biotechnology research from the point of view of third world agriculture. Pages 38-63 in Priorities in biotechnology research for international development. National Academy Press, Washington, D.C.
23. Symko, S. 1969. Haploid barley from crosses of Hordeum bulbosum (2x) X Hordeum vulgare (2x). Can. J. Genet. Cytol. 11:602-608.

STRATEGIES TO DEVELOP BIOTECHNOLOGY IN THE PHILIPPINES

W. G. PADOLINA

In the Philippines, biotechnology is used to "industrialize" agriculture. The country's biomass resources are vast and varied. In 1982 30 million tons of biomass was harvested. Efforts to biologically convert biomass into useful products are promising. BIOTECH (The National Institutes of Biotechnology and Applied Microbiology), based at the University of the Philippines at Los Baños, has research programs on biofuels, food fermentation, nitrogen fixation and mycorrhiza, antibiotics, microbial insecticides, and genetic engineering.

In such developing countries as the Philippines, the most immediate use of biotechnology is in the industrialization of agriculture. Industrializing agriculture means improving primary and downstream processing of food and producing valuable fuels and chemicals from postharvest biomass. The national strategy is to transform biomass biologically into food, fuel, fertilizers, and chemicals.

BIOTECHNOLOGY's RAW MATERIALS

In the Philippines, the raw materials for use in biotechnology are the crops, the forest, and possibly the sea.

In 1982, of the 15 million ha of arable land, more than 12.2 million ha was cultivated (4). Rice, corn, and coconut each occupied roughly a third of 9.9 million ha (Table 1). Of the 30 million t of crops produced, 22 million t consisted of food and 8 million t, of commercial crops. In such major food crops as rice, corn, banana, and pineapple, a significant amount of biomass remains after harvest. The residue may be solid or liquid, saccharine, starchy, or lignocellulosic (Table 2).

Data on these biomass quantities are incomplete, but a ton of rough rice should leave a ton of rice straw. In 1982, 8 million t rice straw would have been available. That same year, rice hull estimated at 1.3 million t was produced in 1982 (10).

Associate professor of Chemistry and executive deputy director, National Institutes of Biotechnology and Applied Microbiology, University of the Philippines at Los Baños, College, Laguna, Philippines.

Table 1. Quantity, hectarage, and mean yield of some major Philippine crops in 1982 (4).

Crop	Quantity (thousand metric tons)	Area (thousand ha)	Mean yield (t/ha)
Palay	8107.9	3432.8	2.36
Corn	3290.2	3360.7	0.98
Coconut	3785.5	3162.0	1.20
Sugarcane	3402.7	470.8	7.23
Banana	4076.8	331.4	12.30
Pineapple	1242.1	60.0	20.70
Root crops	3173.4	479.7	6.62

Table 2. Processing biomass residues of some major crops.

Crops	Saccharine	Starchy	Lignocellulosic
Rice	–	Bran Broken grains	Straw Hull
Corn	–	Broken grains	Stalks Cobs Husk
Banana	Ripe banana fruit rejects	Banana fruit rejects	Stalk Leaves Peelings
Pineapple	Juice from processing wastes	–	Processing wastes
Coconut	Water	–	Coir dust Husk Plant parts Meat residue
Sugarcane	Molasses	–	Bagasse
Root crops	–	Processing wastes	Plant parts

Every year 80 000 t of bananas are thrown away because they fail to meet export standards (1). A dried banana is approximately 75% carbohydrate and 5% protein (8).

The biomass residues from the processing of coconut and sugarcane are considerable (5, 6).

One interesting waste product from coconut is coconut water (liquid endosperm). Each nut contains around 0.3 litre coconut water; all the nuts produced in 1981 would have yielded 4.3 billion litres coconut water (7). One-third of the nut's weight is that of the husk, which is mainly lignocellulose and is used to manufacture coir fiber. The 1982 UNIDO (10) estimate for coconut husk was 3.19 million t. Other parts of the coconut tree such as the leaf blades, the petiole, the trunk, and the shell can have various applications; and coconut sap can serve as a fermentation substrate (7).

The most valuable by-products of sugarcane are molasses and bagasse. In 1982, around 885 000 t of molasses was produced in the Philippines (Hoshiai, K.F. Engineering Co., Ltd., Tokyo,

Japan, pers. comm., 1984) and around 544 000 t of bagasse remained as surplus even after its use as fuel in sugar mills and refineries (10).

Around 3.1 million t of wood wastes was estimated for 1982 (10).

CURRENT STATUS OF BIOTECHNOLOGY-BASED INDUSTRIES

Biotechnology-based industries in the Philippines comprise mainly alcoholic beverage producers. Nineteen distilleries use molasses as their main raw material; they can produce 391 700 litres/day of 189-proof alcohol (9). San Miguel Brewery has a total rated capacity of 9.2 x 10^8 litres/year (2).

Vinegar is produced both on a large scale and on a cottage industry scale. The biggest vinegar producer, Lazatin Vinegar Company, has a capacity of 30 000 litres/day and uses ethanol as feed (2).

Union Ajinomoto uses molasses to produce 8,000 t monosodium glutamate/yr (2). BIOPHIL Inc. produces 2,000 t RNA concentrate/yr (2).

Baker's yeast is produced locally, but we have no production figures. Magnolia Dairy Products Corporation produces cheese, but again no data is available.

Export of the fermented foods fish sauce (patis) and soy sauce earned $800 000 for the country in 1981. Other fermented foods produced here include fish paste, shrimp paste, and the edible microbial polysaccharides nata de coco and nata de pina.

One distillery in southern Philippines can produce 30 000 anhydrous ethanol litres/day. The ethanol produced is mixed with gasoline at 15% levels to make alcogas, which is being tested as a transport fuel on two major Southern Philippine islands.

One of the world's largest commercial biogas facilities is at Maya Farms, an hour's drive from Metro Manila Using dung from 25 000 pigs, the facility produces 7 500 m^3 biogas/day. It was established to control pollution, but has been expanded to fuel almost all the farm's units, including a food processing plant (3).

RESEARCH AND DEVELOPMENT

BIOTECH, a unit of the University of the Philippines at Los Baños (UPLB), is mandated to lead biotechnology research and development in the Philippines. It was established in 1979 as a joint project of the Ferdinand E. Marcos Foundation, the Ministry of Energy, and UPLB. Following are BIOTECH's major research interests:

- biofuels from agricultural crops and residues,
- rural food fermentation,
- nitrogen-fixation and enhancement of soil-nutrient availability in crops and reforestation species (including mycorrhiza),

- molecular biology (recombinant DNA work),
- hydrocarbon-like oils from plants,
- antibiotics,
- animal vaccines,
- microbial insecticides,
- mushroom production, and
- plant biomass conversion.

Biofuels research at BIOTECH is focused on developing new technologies to produce alcohol from saccharine, starchy, and cellulosic substrates. This means designing new fermentors that reduce capital costs, screening and breeding new highly productive microbial strains with high alcohol tolerance at around 37°-40°C, and using distillery wastes and crop residues (rice straw, coconut coir dust, bagasse, coconut water) to produce methane.

BIOTECH's Food Fermentation Program is initially focused on fish sauce and fish paste, particularly the shortening of their fermentation time. Another high-priority research area is the natural fermentation of cereals and legumes to improve their nutritive values when they are used in preparing traditional foods.

BIOTECH's Nitrogen Fixation and Mycorrhiza Program is important because it directly affects agricultural production; work is focused on isolating and testing local strains of Rhizobia for their potential to improve food and agro-forest legumes; associative bacteria and blue-green algae for cereals especially rice and corn; and actinomycetes, for nonleguminous forest species. Results have been significant; many local strains are being field-tested. Ectomycorrhizal fungi have been isolated for important reforestation species and are now being packaged as tablets for field application. The inoculants are being applied to such food legumes as soybean, mungbean, and peanuts.

A Molecular Biology Laboratory, funded by the National Science and Technology Authority, is being established at BIOTECH to house genetic engineering research. The BIOTECH Microbial Culture Collection Unit stores and preserves microbial strains collected by BIOTECH researchers.

BIOTECH also supports several other projects. The antibiotics project isolates local bacterial strains for producing Streptomycin on local substrates. The animal vaccines project aims to develop vaccines against hemorrhagic septicemia.

The microbial insecticides project is field-testing a local isolate of *Bacillus thuringiensis* for effectiveness against mosquito larvae. *Bacillus thuringiensis* strains which may be used for the control of corn borer are also being evaluated. This project has received support from the World Health Organization, the Philippine Council for Health Research and Development, the Palawan Integrated Area Development Project, and the International Foundation for Science.

The mushrooms project is developing techniques for producing mushrooms under tropical conditions, using new and improved species.

The plant biomass conversion project (supported by the Philippine National Development Company) processes the plant Euphorbia tirucalli for its hydrocarbon-like oil, which may serve as an alternative fuel.

BIOTECH is housed in a laboratory building donated by the Ferdinand E. Marcos Foundation, and is gradually acquiring additional facilities needed for research and large-scale testing of results. It was established without external assistance and is supported solely by the Philippine Government and the Ferdinand E. Marcos Foundation.

Other UPLB units also conduct biotechnological research and development; they include:

- the Institute of Food Science and Technology,
- the Institute of Chemistry,
- the Institute of Biological Sciences,
- the Dairy Training and Research Institute, and
- the College of Engineering and Agro-Industrial Technology.

Outside UPLB, several government institutions engage in similar research and development:

- The National Institute of Science and Technology;
- within the University of the Philippines at Diliman, the Natural Science Research Institute, the Department of Chemistry, and the Department of Food and Nutrition; and
- The Philippine Atomic Energy Commission.

The University of Santo Tomas, a private university in Manila, conducts microbiological studies.

CONCLUSION

The high-priority areas for biotechnology in the Philippines are biofuels, microbially based fertilizers, food fermentation, and genetic engineering. If given sustained support, biotechnology promises to help fulfill many of the country's needs.

REFERENCES CITED

1. del Rosario, E.J., and O.N. Gonzalez. 1982. Fermentation technology applied to the utilization of food waste materials in the Philippine. Country report presented at the First ASEAN Workshop on Fermentation Technology Applied to the Utilization of Food Waste Materials, 22 – 24Feb. 1982, Kuala Lumpur, Malaysia.
2. del Rosario, E.J., L.M. Joson, and G.L. Enriquez. 1983. Philippine country paper -- national strategies and developments in biotechnology. Paper presented in the

seminar on Biotechnology sponsored by EEC under the ASEAN-EEC Scientific and Technological Cooperation Programme, November 1983, Singapore.

3. Maya-Farms. 1982. Biogas and waste recycling at Maya Farms. Manila, Philippines.

4. NEDA (National Economic Development Authority). 1983. Philippine statistical yearbook. Manila, Philippines.

5. Padolina, W.G. 1983. Biotechnology and the coconut industry. Paper delivered at the Integrated Chemists of the Philippines Annual Convention, Oct 1983, Bacolod City.

6. Padolina, W.G. 1983. Biotechnology and the sugar industry. Paper delivered at the Integrated Chemists of the Philippines Annual Convention, Oct 1983, Bacolod City.

7. Padolina, W.G. 1983. Coconut water and coconut sap: suggestions research and development. Paper delivered during the Miniworkshop on Coconut Water and Sap Utilization, sponsored by PCARRE and PCRDF, 20 Sep 1983, Manila, Philippines.

8. Pontiveros, C.R., J.A. Alcantara, and E.J. del Rosario. 1978. Acid saccharification and alcohol fermentation of unripe banana fruits. (*Musa sapientum* L.). Philipp. J. Crop Sci. 3:153-158.

9. Samaniego, R., and V.V. Garcia. 1982. Extension and technology transfer in alcohol distilleries in the Philippines. Project Report - BIOTECH-UPLB.

10. UNIDO-Technical Research Centre of Finland. 1983. Techno-economic study or production of ethanol from cellulosic materials in the Philippines. Report of UNIDO Contract No. 82/47 MK.

THE INTERNATIONAL PROGRAM OF THE JOINT FAO/IAEA DIVISION WITH SPECIAL REFERENCE TO BIOTECHNOLOGY

B. SIGURBJÖRNSSON

Since 1964, the Joint Division of the Food and Agriculture Organization of the United Nations (FAO) and the International Atomic Energy Agency (IAEA) in Vienna, Austria, has helped developing countries apply isotope and radiation techniques in their agricultural research and development programs. Its problem-oriented programs have increasingly involved nuclear methods and related advanced research efforts, primarily within biotechnology, such as in vitro techniques in plant breeding and propagation, improving symbiotic nitrogen fixation, immunoassay techniques for hormonal studies and disease diagnosis, polyclonal and monoclonal antibody techniques, animal vaccine production, rumen microbial breakdown of roughages, biological control of pests, conversion of biomass into energy and animal feeds, and microbial degradation of agricultural chemicals and their residues. The programs are primarily carried out through coordinated research and technical cooperation in the developing countries, and are assisted by research and training services from the FAO/IAEA Agricultural Biotechnology Laboratory near Vienna, Austria.

The Joint Division of the Food and Agriculture organization (FAO) of the United Nations (UN) and the International Atomic Energy Agency (IAEA) is 20 yr old. As part of a worldwide effort to harness atomic energy for peaceful purposes, it promotes the use of various nuclear techniques in food and agricultural research and development.

At the outset, because much of the promotion was pursued by overzealous nuclear scientists, the nuclear techniques frequently received excessive emphasis. A more balanced approach was achieved when FAO and IAEA joined in applying nuclear techniques to agriculture. Both organizations are represented in the division's staff, and their financial resources are pooled. Both approve the division's programs, and the activities are carried out in their names.

The activities of the Joint FAO/IAEA Division are problem oriented; they are aimed at using nuclear techniques in cases

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Director, Joint FAO/IAEA Division, Vienna, Austria.

where they are the most efficient means of solving food and agriculture problems. Although the division emphasizes isotopes and radiation, its programs are not exclusively bound to these techniques. Other techniques include nonradioactive isotopes, atomic absorption, nuclear magnetic resonance, the enzyme-linked immunosorbent assay (Elisa), and chemical mutagens.

IAEA is unique among UN organizations in that it operates its own research and treining facility. This laboratory is located at the Austrian Research Center some 30 km south of the IAEA Vienna headquarters near the town of Seibersdorf. It has four units. One deals with safeguards, another with nuclear physics and chemistry, a third with applications to biology and medicine, and the fourth with food and agriculture. The programs of the Joint FAO/IAEA Division are primarily research oriented, but also emphasize training. Most of them and nearly all laboratory activities at Seibersdorf are of a biotechnological nature.

In 1983 the administrations of FAO and IAEA designated the agricultural part of the IAEA Seibersdorf Laboratory as the FAO/IAEA Agricultural Biotechnology Unit. The activities of the laboratory directly support corresponding FAO/IAEA internationally coordinated research and development programs and include biotechnology research and training and laboratory service projects in soil science, plant nutrition, plant breeding and genetics, animal science, entomology, and agrochemicals and residues. The division operates a training facility in food preservation in the Netherlands.

MODE OF OPERATIONS

The most important activity of the Joint FAO/IAEA Division is its research program. The Division now operates some 32 coordinated research programs in different subjects comprising more than 400 research contracts and agreements in more than 50 countries. Consultants are called upon to identify an area in which nuclear techniques can be useful. They outline a program and submit it to IAEA and FAO authorities. Potential participants are invited to submit research proposals. Contracts involving moderate financial support are awarded to participating institutes in developing countries. Participating institutes in developed countries do not receive financial support from the division. Participants coordinate chiefly at annual or periodic meetings at which research plans are drawn up and results reported. At the end of most programs the results are published. Each coordinated program normally lasts 5 yr and a thorough review is required after the first 3 yr. Such coordinated research programs have been supervised by the Division for 20 yr and most have been highly successful. Almost all have been supported by work at the Seibersdorf laboratory, where methods can be tested and essential services, such as isotope analyses, radiation treatments, and calibration of equipment, can be provided (see Table 1).

Table 1. FAO/IAEA coordinated research programs.

Title	Period	Participating countries
Soil fertility, irrigation, and crop production		
Nuclear techniques in development of fertilizer and water management practices for multiple cropping systems	1979-1985	Bangladesh, Ghana, Indonesia, Mexico, Panama, Tanzania, Thailand, Turkey, United States, Zambia
Nuclear techniques to improve crop production in salt-affected soils	1984-1989[a]	Bangladesh
Nuclear techniques in improving pasture management	1981-1986	Brazil, China, Colombia, Cyprus, Greece, Iceland, India, Kenya, Malaysia, New Zealand (2), Panama, Peru, Spain, Sri Lanka, Sudan, Switzerland, United Kingdom (2), Uruguay, United States (2)
Isotopic studies of nitrogen fixation and nitrogen cycling in. *Azolla* and blue green algae	1982-1987	Austria, Bangladesh, Belgium, Brazil, China (2). Hungary, India, Indonesia, Malaysia, Pakistan, Philippines (2), Senegal, Sri Lanka, Sudan, Thailand, United States
To improve yield and N_2 fixation of grain legumes in South America and Africa with the aim of increasing food production and saving N-fertilizer	1984-1989	
To improve yield and N_2 fixation of grain legumes in South East Asia and the South Pacific Region with the aim of increasing food production and saving N fertilizer	1984-1989	
Plant breeding and genetics section		
Use of induced mutations for improvement of grain legume production in S. E. Asia	1976-1986	Bangladesh, India (2). Indonesia (2), Korea, Malaysia, Pakistan, Philippines, Sri Lanka, Thailand
Improvement of leguminous food crops in Africa and the Near East through induced mutations	1981-1986	Egypt (3), Federal Republic of Germany, Ghana, Greece, Kenya, Poland, Uganda
Improvement of leguminous and oil seed crops in Latin America through induced mutations	1981-1986	Brazil (2). Chile, Guatemala, Israel, Italy, Peru (2), Uruguay
Improvement of root and tuber crops and similar vegetatively propagated crop plants in tropical countries by induced mutations	1983-1988	Austria, Canada, Ghana, Italy, Japan, Korea, Mexico, Nigeria, Pakistan, Thailand (2), Uganda, United Kingdom, United States
Improvement of basic food crops in Africa through plant breeding including the use of induced mutations	1984-1989	

Continued on next page

Table 1 continued

Title	Period	Participating countries
In vitro technology for mutation breeding	1983-1988	Australia, Belgium (2), Brazil, Czechoslovakia, France, Federal Republic of Germany (3). Hungary, India, Ireland, Japan, Netherlands, Philippines Switzerland, Thailand, United Kingdom, United States
Evaluation of semidwarf cereal mutants for cross-breeding	1979-1986	Australia, Bulgaria, Hungary, India, Italy, Japan, Korea, Sweden, United Kingdom, United States (2)
Semi-dwarf mutants for rice improvement in Asia and the Pacific	1981-1986	Bangladesh (2), India, Indonesia, Korea, Malaysia, Pakistan (2), Philippines, Thailand
Tissue culture applications through mutation breeding to increase resistance in rice against adverse soil factor	1984-1989a	
Evatuation of semi-dwarf cereal mutants for cross-breeding	1980-1985	Australia, Bulgaria, Hungary, India, Italy, Japan, Poland, Sweden, United Kingdom, United States (2)
Semi-dwarf mutants for rice improvement in Asia and the Pacific Region	1981-1986	Bangladesh (2), India (2), Indonesia, Pakistan (2). Philippines, Thailand
Animal production and health section		
Isotope-aided studies on NPN and agroindustrial by-products utilization by ruminants with particular reference to developing countries	1980-1986	Australia, Bangladesh, Brazil, Canada, Cyprus, Egypt, Federal Republic of Germany, Korea, Malaysia, Nigeria, Peru, United Kingdom, United States
Optimizing grazing animal productivity in the Mediterranean and North African regions with the aid of nuclear techniques	1982-1987	Australia (2). Cyprus (2), Egypt (7), Israel, Italy (2), Morocco (3). Niger, Romania, Sudan, Turkey, United Kingdom, United States, Yugoslavia (2)
Improving sheep and goat productivity with the aid of nuclear techniques	1982-1987	Australia (2). Canada, China (2), Czechoslovakia, Kenya (2), Malaysia (2), Netherlands, Nigeria, Sri Lanka, Sudan, Thailand, United Kingdom, Zimbabwe
The application of radioimmuno-assay to improve the reproductivity of large ruminants	1982-1987	Australia, Canada, Egypt, Iceland, India, Indonesia, Israel, Kenya, Korea, Malaysia (2). Portugal, Sri Lanka, Sweden, Thailand, Uganda, Zambia

Continued on opposite page

Table 1 continued

Title	Period	Participating countries
Use of nuclear techniques to improve domestic buffalo production in Asia — Phase II	1983-1988	Australia (3), Bangladesh (2), China, India, Indonesia, Japan, Malaysia (3). Pakistan, Philippines, Sri Lanka (2). Thailand (2)
Regional network for improving the reproductive management of meat and milk producing livestock in Latin America with the aid of radioimmunoassay techniques	1983-1988	Argentina, Brazil (3). Chile, Colombia, Costa Rica, Ecuador, Mexico (3), Peru (2), Uruguay, United States (3). Venezuela
Use of nuclear techniques in the study and control of parasitic diseases of farm animals	1981-1986	Argentina, Australia (2), Colombia (2), Ethiopia, Kenya, Morocco, Nigeria, New Zealand, Poland, Sri Lanka, Sudan, United Kingdom, Uruguay, Yugoslavia
Improving the diagnosis and control of infectious and parasitic diseases of livestock in developing countries with the aid of radioimmunoassay and related techniques	1985-1988[a]	
Insect and pest control section		
Development of sexing mechanisms in fruit flies through manipulation of radiation Induced conditional lethals and other genetic measures	1981-1987	Argentina, Greece, Israel, Italy, Kenya (2), Mexico (2). Netherlands, United Kingdom, United States (2)
Standardization of Medfly trapping for use in the Sterile Insect Technique programme	1984-1989	
Radiation-induced F_1 sterility in lepidoptera for area-wide control	1984-1989	
Development of methodologies for the application of the Sterile Insect Technique (SIT) for tsetse eradication or control	1984-1989	Austria, Belgium, Czechoslovakia, France, Federal Republic of Germany, Kenya (2) Nigeria (6), Tanzania, Uganda, United Kingdom (3), United States, Zambia, Zimbabwe
Agrochemicals and residues section		
Isotopic tracer-aided studies of unextractable or "bound" pesticide residues in soil, plants and food	1979-1987	Canada, Costa Rica, Egypt, Federal Republic of Germany, India, Pakistan, Philippines, Poland, Sudan, United Kingdom, United States (2)
Coordinated studies of agricultural chemical residues in meat, milk and related products of livestock with the aid of nuclear techniques	1980-1986	Brazil, Canada, Egypt, Pakistan, Philippines, Sudan, United States, Yugoslavia

Continued on next page

Table 1 continued

Title	Period	Participating countries
The fate of persistent pesticides in the tropics, using isotope techniques	1982-1987	Brazil, Costa Rica, Ecuador Egypt, India, Kenya, Panama, Philippines, Sri Lanka, Sudan, Thailand, United States
Isotopic tracer-aided studies of pesticide residues in stored products	1982-1987	Brazil, Canada, Egypt, Ghana, Malaysia, Panama, Philippines, Turkey, United Kingdom, Yugoslavia
Use of isotopes in studies of pesticide residues in rice-fish ecosystems	1983-1987	Egypt, India, Korea, Malaysia, Philippines (2), Thailand, United States
Radiotracer studies of fungicide residues in food plants	1984-1989	Brazil, Canada, Egypt, India, Lebanon, Panama, Philippines, United States
Development of improved rural methane production from biomass utilizing nuclear techniques	1980-1986	Argentina, Egypt, Greece, Italy, Pakistan, Turkey, United States (2). Yugoslavia
To develop and evaluate controlled-release formulations of pesticides to reduce residues and increase efficacy, utilizing radioisotopes	1982-1987	Belgium, Egypt, Federal Republic of Germany, Hungary, India, Indonesia, Pakistan, United Kingdom
Food preservation section		
Asian Regional Cooperative Project on Food Irradiation	1978-1987	Bangladesh (2), India, Japan, Philippines (2). Sri Lanka, Thailand (2)
Pre-commercial scale radiation treatment of food	1980-1986	Argentina, Ecuador, Egypt, France, Hungary (2), Netherlands, Pakistan, Philippines, Poland, Thailand, Yugoslavia (2)
Insect disinfestation of food and agricultural products by irradiation	1981-1986	Bangladesh, Chile, Egypt, Hungary, Indonesia, Iraq, Philippines, Singapore, Thailand, United States
Use of irradiation as a quarantine treatment of food and agricultural commodities	1984-1987[a]	Australia, United States (2)
Factors influencing the utilization of food irradiation process	1980-1986	Bulgaria, Ghana, India, Indonesia, Poland, Thailand (2). Turkey, United States, Yugoslavla

[a] Being initiated.

The Division is also responsible for many individual technical cooperation projects in developing countries. These agricultural projects make up about 20% of the IAEA technical cooperation budget and many are also supported by UNDP and by bilateral sources. They vary in size and scope: some, especially development projects, cost several million dollars;

others simply consist of sending an expert to an institute, of awarding a fellowship, or of providing needed equipment or instruments. The division's training program awards fellowships to young scientists from developing countries to go to selected institutes, including our own laboratory in Austria. Training courses (many of which are conducted at Seibersdorf) are held in various subjects.

RANGE OF ACTIVITIES

Some highlights of current activities and future trends in the division's program follow. The ongoing coordinated research programs are shown in Table 1.

Soil fertility, irrigation, and crop production

The five ongoing coordinated research programs in soil fertility, irrigation, and crop production include studies on multiple cropping systems aimed at a better understanding of the fate of applied fertilizer and of efficiency of water and fertilizer use when different crops are grown in sequence or together. The main emphasis in the last few years has been on nitrogen fixation. Isotope use has made it possible to distinguish atmospheric nitrogen fixed in legumes from soil and fertilizer nitrogen. Management practices that would result in the best growth of different legume-grass mixtures in pastures and still maintain optimum nitrogen fixation are being ought. The methods in these programs use the heavy isotope ^{15}N to measure the nitrogen fixed in the symbiotic relationship between legumes and the bacterium Rhizobium. One of the programs involves the water fern Azolla, which thrives in rice paddies and can fix nitrogen at 335-670 kg/ha per year. But many problems continue to limit the use of these organisms. There is no convenient method for assessing the effects of different agricultural practices on nitrogen fixation, and the availability to the rice crop of the nitrogen fixed is unknown. The studies within this program will include ^{15}N substrate labeling techniques developed for grain legumes and adapted to aquatic systems to measure nitrogen fixation. The Seibersdorf laboratory is trying to further develop the ^{15}N methods of measuring nitrogen fixation (using ^{15}N for this purpose was conceived in that laboratory). Isotope use in studies of nitrogen fixation was the subject of an international training course held in the Seibersdorf laboratory in June and July 1984 and will be repeated in the summer of 1985. Photosynthesis using ^{14}C and phosphate metabolism using ^{32}P will also be studied and included in the training program.

The division has initiated studies of crop production in salt-affected soils in accordance with recommendations of an advisory group meeting last year. The studies will analyze different salt-affected systems and delve deeper into the original salinity so that management practices that minimize salinization and optimize crop production under these adverse conditions can be established.

Plant breeding and genetics

Activities in plant breeding and genetics emphasize using induced mutations for crop improvement. Induced mutations have successfully improved cultivars in many crop species, but most of such work has been with cereals and a few other seed-propagated annual crop species. The division is shifting its efforts to asexual species and vegetatively propagated crop plants to learn the most appropriate mutagen treatments of seeds, pollen, and plant organs. It is also moving toward using mutation induction in combination with tissue culture techniques. A newly completed tissue culture laboratory at Seibersdorf is being used in training courses on tissue culture and induced mutations. Gene transfer techniques using high radiation doses on pollen grains -- techniques that have been called the poor man's genetic engineering -- are also being developed. The division looks forward to initiating cooperation with IRRI in using tissue culture with induced mutations and other technology to improve rice's resistance to adverse soil conditions. Inadequate mutant screening techniques are considered the greatest obstacle in mutation breeding, restricting its practical applications to relatively few and easily recognized plant characters, such as flowering time and short culm. Efforts to improve the technology for mutant selection for more traits are planned.

The division attempts to foster international cooperation in the application of mutation breeding in crop plants that have improved little through classical plant breeding. In grain legumes of the tropics and subtropics, alterations in plant architecture and physiology are needed but are often difficult to obtain from existing germplasm. Also, improving disease resistance and quality often appears unlikely from cross breeding within available adapted germplasm. This is also true for certain industrial crops and oil seeds (e.g., jute, sesame). Efforts will be made to support inducing mutations to genetically improve vegetatively propagated tropical root and tuber crops otherwise difficult to improve.

Abundant genetic variation can be generated as easily by mutation induction as by cross breeding, but it is the specific and desired variation that counts. The division will therefore evaluate mutants for their breeding value, and provide the plant breeder with more useful germplasm. It is running two such programs; one evaluates semidwarfs in rice, and the other, semidwarfs in wheat. It has recently held research coordination meetings on these topics at IRRI and at Centro Internacional de Mejoramiento de Maiz y Trigo (CIMMYT).

Animal production and health

The program aimed at improving the health and productivity of grazing animals in the tropics and subtropics, emphasizes the animals' nutrition, reproductive efficiency, and disease status. Isotopic methods to examine the nutritive value of locally available feedstuff and to determine the best means to upgrade and supplement them with cheap nonprotein and protein nitrogenous

materials are used. Radioimmunoassay techniques measure levels of reproductive hormones in body fluids down to 10^{-12} g/ml. Since these levels can indicate the onset of sexual maturity, pregnancy, and ovarian function, radioimmunoassays are a powerful means to assess reproductive status and the likelihood for success of any efforts to improve reproductive efficiency. In animal diseases, there are three areas of interest in the division:

- irradiation-attenuated vaccines to control diseases (e.g. lung-worm and babesia infections);
- isotope use to examine the immune response and the pathogenesis of helminth and protozoal infections;
- immunoassay methods to diagnose diseases.

In a program on nonprotein nitrogen and agroindustrial by-product use by ruminants, isotopes are used to measure digesta flow and composition. Nutritional studies are an integral component of coordinated programs on animal productivity in the Mediterranean and North African regions, on sheep and goat productivity in the Middle East, on buffalo production in Asia, and on cameloids in Latin America. In all of these programs the interest is in interactions between the animal and its environment, and therefore the stress is on the multidisciplinary approach.

The facilities at the laboratory section in Seibersdorf include a rumen-simulator (Rusitec), which, with gas chromatography and emission spectrometer, will permit measuring fermentation end-products and microbial output, digestion of feeds, and fate and effect of additives. These techniques will also be used to study the degradation of fibrous feeds; radioisotopes can be used here more safely and economically and with easier waste disposal than in work with animals.

The laboratory is also used to develop and test analytic techniques for application in developing countries. Exotic feeds, by-products, and additives supplied by research contract and agreement holders can be tested.

The division is establishing an immunoassay technology program at the Seibersdorf laboratory to prepare and distribute labeled antigens and antibodies, antisera, and standards; to provide a quality control service; to refine and improve existing techniques and develop new ones; and to focus training in enzyme and radio-immunoassays.

Insect and pest control

The insect and pest control program primarily researches the biological control of insects using the sterile insect technique (SIT) against various species of the tsetse-fly and the Mediterranean fruit fly (medfly). In addition, the division has a program to use isotopes in research on integrated management of pests, particularly rice insects.

To use SIT for medfly control, emphasis is on improving mass rearing; developing a genetically engineered sexing method so that only males can be released; and assisting large field

programs in Egypt, Peru, and elsewhere. The development programs are being carried out at Seibersdorf in our new experimental rearing facility for medfly. In the use of SIT against tsetse flies, emphasis is on improving mass-rearing technology, especially in vitro, including artificial diets for feeding tsetse flies. A large-scale field program in Nigeria has released sterile flies in a 1,500-km^2 area to eradicate Glossina p. palpalis. Other projects on tsetse are planned in Tanzania, Kenya, and possibly Zambia. The SIT successfully eradicated the medfly from Mexico and is now being used there as a control and quarantine measure on the Mexico-Guatemala border. A very large project in Egypt aims to eradicate the medfly within 4-5 yr. A medfly factory near Alexandria is being planned. It will have the capacity to produce 1 billion medflies per week (about 7 t of medflies). This project (estimated cost, some $50 million) will release sterile insects in overwhelming numbers. The principles for rearing these flies were developed at the Seibersdorf laboratory and used in Mexico.

Isotopes can aid in ecological studies to improve integrated pest management programs. A project on isotope use in insect physiology studies just started at the International Centre for Insect Physiology and Ecology (ICIPE), and more such programs are expected to begin. The division plans to start work on mosquitoes and hopes to carry out a research and development project on the island of Mauritius. It is also initiating work at the Seibersdorf laboratory and in field programs using induced F-1 sterility in Lepidoptera species that are harmful pests of the major cereals and of many other crop plants.

Agrochemicals and residues

The current research includes investigating agricultural chemical residues in meat, milk, and related products from livestock; studying the degradation rates and environmental fate of organochlorine pesticide chemicals under tropical conditions for much needed data on their dissipation rates; studying the magnitude and fate of residues of fumigants and other chemicals applied to agricultural products after harvest; and investigating pesticide residues bound to plants, soils, and other substrates from which they cannot be conventionally extracted. The "bound" residues program focuses on the needs of regulatory and research agencies concerned with the potential toxicity to mammals, plants, or other organisms of such residues.

A research program on controlled-release formulations of pesticides has been established. New formulations to be evaluated by research contract and agreement holders for effectiveness against pests of rice and other crops have been prepared with the Seibersdorf laboratory's support. A program involving field and model ecosystem experiments, which could be useful in promoting guidelines for improving pest control practices in rice-fish culture, has been started. A major problem in pheromone use to suppress or to monitor insect populations is designing the proper formulation to release the active ingredient. New control strategies based on these

techniques are being investigated. In chemotherapy to control trypanosomiasis in cattle, the limited number of drugs available must be optimized. A new program at the Kenya Trypanosomiasis Research Institute in Nairobi has been initiated; the Seibersdorf laboratory is developing the analytical methods needed.

Microbial biotechnology for using agricultural wastes to produce fuel and animal feeds is being investigated. Isotopically labeled substrates are ideal for studying the enzymic and microbial mechanisms of these reactions. The potential of mutant strains of organisms induced by gamma radiation to increase the rate of waste degradation is being investigated. Microorganisms capable of degrading lignocellulose may be obtainable from such unconventional sources as African termites. A program of cooperative research with ICIPE was recently initiated to explore the enzymic potential of microflora from the gut of various African termite species.

Food preservation

National public health authorities and the World Health Organization (WHO) show great interest in irradiation of food, especially to disinfest fruits, vegetables, and spices. The recent ban on some of the most important fumigants has increased interest in irradiation. The Codex Alimentarius Commission has ruled that doses as high as 1 million rads are safe. The use of irradiation for disinfesting grain, preventing potatoes from sprouting, and sterilizing meat is being seriously contemplated. The technology is well known; many developments in this field have resulted from the division's program and its extensive training activities in Wageningen, Netherlands.

I have recently visited some of the international agricultural research institutes and discussed with their scientists possibilities for collaboration between them and our division and laboratory. Several areas in which such cooperation could be very fruitful have been identified. As mentioned earlier, the division's program is oriented more and more toward biotechnical applications that include isotopes, radiation, and related technology. The division and the institutes will surely benefit from such collaboration.

SUMMARY

The Joint FAO/IAEA Division will continue to work on nuclear applications in biotechnology, i.e., the use of isotopes, radiation, and related technology, to find solutions to food and agricultural problems.

The activities of the division are problem oriented, not technique oriented: The division is not interested in elegant applications of nuclear tools and techniques if no real practical agricultural need for them exists.

Although the division works within nuclear technology, its program development is not restricted to it. It will use related scientific approaches that are needed and likely to achieve

progress. These approaches lie primarily within biotechnology. The division intends to closely follow developments in biotechnology in its widest sense to be able to assist our collaborators in developing countries and to promote international cooperation and coordination in the use of nuclear and related technologies to increase, stabilize, and protect food and agricultural production.

In this task the division is anxious to maintain close and effective cooperation and coordination with the international agricultural research institutes and with its sister divisions in FAO and other international organizations.

THE IARCs AND THE DEVELOPMENT AND APPLICATION OF BIOTECHNOLOGIES IN DEVELOPING COUNTRIES

F. H. BUTTEL, B. KENNY, and J. KLOPPENBURG, JR.

Major aspects of the socioeconomic context of the emergence of biotechnology in international agricultural research and development are discussed. It is argued that we are witnessing the early stages of an institutional structure of international agricultural research that will differ fundamentally from that which characterized the "Green Revolution." The most crucial differences will relate to the private character of biotechnology in the developed countries. The private character of applied agricultural biotechnology research by the private firms of the developed countries will indirectly benefit the IARCs by speeding the rate of technology transfer. But at the same time, private dominance of research and development will create challenges for the IARCs, such as possible direct competition with private firms, lack of access to scientific information, debates over research priorities, and lack of resources with which to embrace new research thrusts while maintaining old ones.

It is now barely more than a decade and a half since the euphoria about the "miracle seeds" of the so-called Green Revolution yielded to criticism of the IARC role in international agricultural development. The IARCs responded cautiously to these criticisms, but over the past several years have demonstrated their commitment to research geared toward the technical needs of the peasant smallholders: they are exploring methodological approaches (e.g., farming and cropping systems research) and agronomic strategies (e.g., integrated pest management, low-input agricultural systems) that are most likely to meet smallholder needs. Today the defenders and critics of the IARCs remain nearly as polarized by the disagreements of the past decade as they were at the peak of debate in the mid-1970s. But one can see that the two sides have moved closer together: Many critics of the Green Revolution acknowledge the need for increased agricultural productivity, and many defenders recognize that technologies based

Associate professor, Department of Rural Sociology and Program on Science, Technology and Society, Cornell University; research associate, Department of Rural Sociology, Cornell University, and Ph D candidate in Development Sociology. Cornell University, USA.

on high-yielding varieties can have socially undesirable consequences. In fact, the goals of the Green Revolution critics, on one hand, and of the Green Revolution defenders, on the other, have moved closer together than either side acknowledges.

That such a convergence of hitherto disparate goals has occurred might have merely been a scholarly curiosity were it not for the rapid emergence of biotechnology on the agendas of public agricultural research institutions in the developed countries (DCs) and of the quasipublic institutions of the CGIAR and the IARCs. Most simply put, the emergent issue is no longer whether land-grant and IARC science will focus on large farm- or "landlord"-biased technologies, but whether public agricultural research will exist at all over the next 20 – 40 yr. It is ironic that the era during which criticisms of public-sector agricultural research became most strident (1973 – 1980) vas also the era during which forces developed that could eventually reduce the viability of public agricultural research everywhere.

These challenges to public agricultural research do not imply the demise of these systems. But changes in the structure and function of agricultural inputs manufacturers in the DCs and of the global agricultural economy have created major pressures for change in the division of labor between public and private agricultural research. Some of these prospective changes are positive for public agricultural researchers and for the diverse populations of the developed and less developed worlds (8). But others threaten public agricultural research oriented to the needs of the minority segments of the global agricultural economy. These future pressures must be broadly understood PO that the positive aspects can be set in motion without delay and the negative ones planned for and dealt with.

The various socioeconomic issues relating to the role of biotechnology in development are extremely complex. This complexity, the number of crosscutting possibilities, and the rapidity of research breakthroughs make any projections tentative. Yet the tremendous importance of biotechnology justifies attempts at assessing its potential social impacts. Its future will he shaped by many social forces over the next several years, so the public-sector policymakers should understand biotechnology's possibilities and pitfalls now.

FROM GREEN REVOLUTION TO BIOREVOLUTION

We noted earlier that the seeds of a second "Green Revolution" or "Biorevolution" were being sown at precisely the same time that the first Green Revolution was being debated so intensely (4). After the discovery of DNA and intensive research into its relationship to protein synthesis in the 1950s and 1960s, several key discoveries in the 1970s made genetic-manipulative biotechnology a scientific and commercial possibility. In the early 1970s, molecular biologists perfected cloning technology and rapidly began to understand the complex chemical reactions in living cells. Most significantly, DNA sequences from one species were successfully removed and recombined with the DNA of a different species. Nearly simultaneously, hybridoma techniques,

protoplast fusion, cell tissue culture, and other biotechnologies were being perfected. The exciting potentials of recombinant DNA stimulated the massive private investment boom in biotechnology in the late 1970s. Several firms with agricultural biotechnology research interests (Cetus, Agrigenetics, Genentech, Molecular Genetics) were among the earliest and most prominent among the new biotechnology start-up firms (6).

While the initial investments were largely confined to small firms, large multinational companies (MNCs) -- many of them major agroinput suppliers -- rapidly began to invest in biotechnology during the early 1980s. These investments occurred after a decade-long change in the structure and function of chemical and pharmaceutical MNCs in general, and of agrochemical and agricultural pharmaceutical firms in particular (2). These formerly distinct sectors were already becoming more integrated. Many large agroinput firms had become subsidiaries of general chemical and pharmaceutical MNCs. Then passage of the 1970 Plant Variety Protection Act (PVPA) in the US further stimulated this integration and set into motion a decade-long seed company acquisition movement. Since the early 1970s, the multiregional and national independent seed companies (except Pioneer Hi-Bred) in the USA and several other advanced industrial countries have become subsidiaries or joint-venture partners of MNCs. DeKalb, in its joint venture with Pfizer, is a partial exception. Numerous small, generally regional seed companies do still exist in the DCs, but the vast bulk of field crop seed sales is by Pioneer Hi-Bred (a diversified MNC) and by the seed company subsidiaries of the larger chemical and pharmaceutical MNCs of North America and Europe.

PVPA led to the purchase by MNCs of seed companies because, in giving patent-like protection to sexually propagated plant varieties, it made private plant breeding seem much more profitable. At the same time, tightening world grain markets of the early 1970s increased the attractiveness of agriculturally related investments.

Thus, while biotechnology did not initiate the trend toward producing various inputs, from pesticides and fertilizers to seeds and agropharmaceuticals, within a single large MNC, it has helped justify that integration (see below). In many cases, the fact that MNCs already owned seed companies increased the incentive to make in-house investments in agricultural biotechnology research and development (R&D). Most major MNCs with agroinput lines invest in all or most of the following: 1) in-house biotechnology investments, 2) purchase of seed company subsidiaries, 3) investments in university-based biotechnology research, and 4) investments in biotechnology start-up companies.

While most observers of agricultural biotechnology consider the smaller scientifically expert start-up companies to be the leaders in the industry, MNCs are certain to eventually dominate because the start-up sector lacks products and cash flow and depends on infusions of external capital. This lack of products and cash flow is especially evident in the agricultural biotechnology sector, where major commercial products may not emerge for decades. Indeed, most start-up companies in the United

States have turned to MNCs for capital investments and to commercialize their new technologies (6). This is not to suggest that the start-up sector will disappear entirely or soon. It is likely that basic molecular and cell biology research will stay primarily within universities and that start-up firms will continue to "transfer" university-based knowledge and expertise to MNCs for commercialization. MNCs have become the principal customers of the start-up companies, because these larger firms have enormous financial resources and established marketing networks. In addition, the overhead costs of MNC marketing networks require constant new products.

Seeds are a logical vector for bringing biotechnology to the farmer. Of course, inoculation with nitrogen-fixing bacteria and application of bacterial pesticide are other such vectors. But seeds are potentially the most inportant, especially since biotechnology has increased the options available for the formulation of various "packages" of proprietary seeds and chemicals, for example, Ciba-Geigy's "safener" for herbicide applications to sorghum, several firms' emphasis on the genetic engineering of herbicide resistance, and Plant Genetics Inc.'s "artificial seed" (a pregerminated celery embryo encapsulated by a gel coating to which plant-protecting chemicals) may be added. The "logic of integration" should not, however, be overemphasized. Effective "packages" of seeds and chemicals require expensive, long-term R&D that many private firms may be reluctant to sustain.

Most of the major biotechnology MNCs are now striving for prospective customers in less-developed countries (LDCs), especially because of the recession-caused stagnation of DC agroinput markets. Given the potentially enormous agroinput markets in the LDCs, MMCs will. place particular emphasis on being leaders in international agricultural R&D (4).

These R&D arrangements greatly differ from those "encountered by CIMMYT and IRRI early in the Green Revolution. Even the institutional structures of the Green Revolution and the current Biorevolution are profoundly different. The principal reason for this difference is the pervasively private character of the Biorevolution (4). The PVPA, the Paris UPOV convention, and the extension of industrial patent law to novel life forms have all increased the profit potentials of agricultural biotechnologies (1, 3, 4, 6). Massive private-sector R&D investments and the fact that molecular and cell biology programs are generally of second-tier status in US 1and-grant institutions (2, 5) have led to private sector dominance of applied agricultural biotechnology in the US. This seems true in most other DCs, and means that, unlike during the Green Revolution, the IARCs will not have access to scientifically unrivaled public-domain technologies. International agricultural researchers will likely turn from US land-grant universities to public agricultural research institutions in other countries.

Table 1 summarizes several other important respects in which the biorevolution will differ from its predecessor. These important differences between the Green Revolution and the Biorevolution -- differences that can mean <u>both</u> opportunity and threat to the IARCs -- reflect some of the unresolved issues in

Table 1. A comparison of the institutional structures of the green revolution and biorevolution.[a]

Characteristics	Green Revolution	Biorevolution
Crops affected	Wheat, rice, maize	Potentially all crops, including vegetables, fruits, agroexport crops (e.g., oil palms, cacao), and specialty crops (E.g. spices, scents)
Other products affected	None	Animal products, pharmaceuticals, processed foods, energy
Areas affected	Some locations in some LDCs (i.e., if accompanied by irrigation, high quality land, transport availability, etc.)	All areas including marginal lands (characterized BY drought, salinity, Al toxicity, etc.)
Technology development and dissemination	Largely public or quasi-public sector	Largely private sector (multi-national corporations and start-up firms, with the former pre-dominating commercializing)
Proprietary considerations	Patents and plant variety protection generally not relevant	Processes and products patentable and protectable
Capital costs of research	Low	High
Research skills required	Conventional plant breeding and parallel agricultural sciences	Molecular and cell biology expertise plus conventional plant breeding skills
Crops displaced	None (except the germplasm resources represented in traditional varieties and landraces)	Potentially any

[a] Source: Kenney and Buttel (6).

current biotechnology and international R&D policy-making. We will explore several of the issues central to discussions within CGIAR and the IARCs about the role of the international public sector in R&D for LDC smallholders.

The private sector seems committed to R&D for the LDCs, but public-sector agricultural research institutions must ensure that the needs of all. LDCs are met equally. Indeed, the private sector-led Biorevolution will likely increase the range of responsibilities of the IARCs.

Many of the dilemmas that will emerge in LDC-oriented biotechnology efforts will not be entirely novel. The new division of labor between public and private research in the Third World caused by the private sector-led biotechnology transfer to the LDCs is already clearly on the agenda of agricultural research politics in the US and elsewhere (5). The need to carefully select breeding goals to make the most of scarce public research funds for applied biotechnology will become ever more acute.

There is, however, one crucial difference between the biotechnology research policy issues that will affect the DCs' and LDCs' public agricultural R&D institutions. While DC farmers' purchased inputs come almost exclusively from private firms, LDC farmers obtain many of theirs from public and quasipublic organizations. This could change over the next decade as MNCs

further penetrate LDC markets, and will do so most rapidly in countries with plant variety protection laws (1). Plant variety protection legislation and the UPOV convention will protect private firms' breeding lines and investments in promotion and marketing. (We are grateful to John Barton for pointing out that one should not overestimate the role of plant breeder's rights.) In the US, Section 101 patent protection is generally preferred over PVPA, since Section 101 patenting is more restrictive than PVP and patents are recognized by many more countries. It is likely that the most attractive commodities to MNCs over the long-term will be precisely those LDC commodities that have heretofore received the greatest IARC research effort: maize, wheat, and rice. This may mean direct competition between private seed firms and the national seed improvement agencies. A number of developing countries either lack the plant-breeding infrastructure for plant variety protection or do not provide an attractive market for MNC agroinput firms, so the IARCS will likely continue to make advanced breeding lines and finished varieties available to LDC governments. Although it will, of course, be largely up to national governments to negotiate the public/private division of labor, there may be contentious conflicts over the public and private division of Labor in R&D in a number of LDCs.

This is not to suggest that the goals of private agroinput firms apd TARCs are inherently incompatible, but their relationship will also not be symbiotic. Most important for the purposes of this paper, the potential bases of competition and conflict may limit IARC access to biotechnological advances made in private-sector firms.

CGIAR/IARC BIOTECHNOLOGY STRATEGIES

There are three major categories of biotechnology policy issues important to CGIAR/IARC policymakers. The first concerns access to technologies; the second, the resources for R&D; and the third, priorities or research goals.

IARC/LDC ACCESS TO BIOTECHNOLOGIES

The issue of IARC, and ultimately LDC, access to agricultural biotechnology advances has two major interrelated roots. First, the private biotechnology investment surge in the DCs has been partly induced by the probability that technological advances can be effectively privatized and denied to competitors. Second, the IARCs can no longer call upon the unrivaled expertise of public-sector agricultural scientists in the DCs, because more of these scientists are becoming corporate employees or owners, or both. Indeed the public agricultural R&D institutions of the DCs are facing their own "access" issue: uncertainties about access to biotechnology start-up and MNC agroinput firms' university research funds, most of which have beer! going to nonland-grant universities with superior programs in basic molecular and cell biology.

The IARCs will probably continue to emphasize primarily applied research and, therefore, will require external sources of fundamental biotechnological information. There are four major potential sources of such information. The first is the DC biotechnology start-up firms, many of which need investment and operating capital and which welcome remunerative joint ventures with Third World groups, The start-up firms are moving increasingly rapidly toward application-ready techniques, and, although they will restrict diffusion of proprietary information, they are more likely than their MNC counterparts to share technologies with others (6). In large part this is because most start-up firms have no realistic hope, at least in their early years, of marketing their products. They will be particularly willing to sell technology to LDCs and LDC-oriented institutes because these will not directly compete in the same markets. And the start-ups will likely continue to have the latest information, especially since their principal scientists will probably continue to be senior university researchers.

The second source will be DC public-sector agricultural research institutions. While these institutions have lost their earlier hegemony over agricultural R&D, there will be three to five land-grant universities in the USA with excellent plant and animal molecular and cell biology research programs. An additional five to seven land-grant universities will have considerable expertise in certain areas of agricultral biotechnology. However, even if the land-grant institutions in the US and their counterparts in other DCs were to have world-class programs, their potential contribution to IARC efforts would be limited because their biotechnology programs would have strong connections with private sector funders. Another increasingly important source of agricultural' biotechnology information will be the nonland-grant universities such as MIT, Harvard, and the University of California, San Francisco, although all of these information sources are becoming inexorably privatized.

University molecular and cell biology programs are depending increasingly upon private research funding. Noreover, private sponsors of university research are being granted unprecedented conditions including exclusive licensing of patented technologies, protection of trade secrets, and delays in publication. Nevertheless, the maintenance of international public research in the IARCs depends upon viable public research institutions in the DCs.

The restoration of excellence in the DCs' public agricultural research institutions will be pivotal if the third potential source of biotechnological information to the IARCs -- UNIDO's proposed International Centre for Genetic Engineering and Bio-Technology (ICGEB) -- does not materialize. The world's two leading biotechnology powers -- the USA and Japan -- have been striving to prevent its establishment, which reflects the increasingly intense international competition over high technology (2, 3). The future of ICGEB (or a counterpart successor), however, is one of the most important long-term factors in the viability of LDC-oriented biotechnology research.

ICGEB could provide nonproprietary information to the IARCs and information and postdoctoral training for national biotechnology and agricultural research programs. It may be so important to the IARCs that the CCIAR network and the UN system should work toward the estab1ishment of the ICCEB or toward a revised version focused on agriculture. (John Barton has suggested an elaboration of the collaborative research model of the World Health Organization in vaccine research, or developing large international research agreements between counterpart universities and scientists in DCs and LDCs.)

A fourth potential source of IARC biotechnological information, the emergent biotechnology institutes of certain LDCs, is one that has often been neglected (6). Over the past few years a number of promising national biotechnology programs have been established, These include efforts in India, Mexico, Cuba, Brazil, China, and the Philippines. Assuming these programs keep to their targets of emphasizing LDC-relevant applications (rather than reproducing the high-status science of DC institutions), they will likely contribute significantly to the IARCs. Many of these institutes face major problems in recruiting personnel and establishing an R&D infrastructure, but their very numbers ensure that several will be successful.

While the IARCs will probably encounter unprecedented restrictions on accessing current technological knowledge (especially knowledge applicable over a short time), they will have a number of viable options. However, no single option can be relied upon over the long term. The IARCs would thus be advised to diversify their information from the outset, and the CGIAR to consider formal association with ICGEB.

RESOURCES

Our initial work on biotechnology led us to the opinion that biotechnology R&D is extraordinarily expensive and may be beyond the capability of all but the most sophisticated research institutions in the DCs 17). We believe now that the contrary is true; biotechnology is more "information intensive" than it is capita1 intensive. Dependable access to information (or the ability of the IARCs and counterpart institutions to generate their own information) is more of a barrier than are initial investments in facilities and annual operating expenses (6).

While the pivotal input into biotechnological innovation in LDC-oriented agricultural research, in general, and IARC research, in particular, is information rather than capital, significant financial and human resources will also be necessary. Some broad parameters of resource needs and options that should be discussed by CGIAR and IARC policymakers follow.

We assume that the bulk of biotechnological information eventually used by individual IARCs now in existence will be largely external in origin. Qualitative budget increases for cooperation with external institutions (especially biotechnology start-up companies and DC public agricultural and molecular biology research institutions) will therefore be needed. In-house capability will also be needed in at least certain areas --

especially cell and tissue culture and monoclonal antibody work, and perhaps DNA recombination. (Of course, some IARCs already conduct biotechnology research, for example, ILRAD's monoclonal antibody research on animal diseases and IRRI's rice tissue culture work.) In-house capabilities will be necessary to apply knowledge now geared principally for DC applications to the often quite different conditions of LDCs. The development of in-house capability will require expenditures not only for new facilities, but also for new staff and for retraining of many current staff members.

Assuming that CGIAR decides to pursue agricultural biotechnology, tough decisions will be needed on both securing additional resources and allocating available ones. Should the bulk be allocated to in-house R&D, or should the IARCs rely primarily on external contracts? Should the CGIAR system eventually add another IARC devoted largely to molecular and cell biology to supply other IARCs with fundamental and applied biotechnological knowledge? If so, should such a new CGIAR center have a general mandate (i.e., to conduct biotechnological research relating to both the agricultural and nonagricultural sectors), or should the center's activities be confined to agriculturally oriented research? All of these courses of action will require substantial additional funding for the CGIAR system or reductions of existing programs, or both.

RESEARCH PRIORITIES AND BREEDING GOALS

The first question policymakers must address is: for what purposes and goals is biotechnology to be employed? The CGIAR and IARCs are, of course, no strangers to discussions of this sort. However, biotechnology is likely to open up a now long-standing debate over priorities. The dominant thrust of biotechnology R&D in the DCs -- and, hence, biotechnological information -- tends to be startlingly like the philosophy, "betting on the strong," a philosophy that has slowly been rejected by the IARCs in favor of helping the weak. Put somewhat differently, biotechnology R&D in the DCs is mainly directed toward the technical problems (e.g., pesticide toxicity, saline soils) of highly capitalized producers on high-productivity lands (see 9). On the other hand, the tremendous promise of biotechnology for LDC agriculture is that the new technologies may enhance the productivity of marginal agroecosystems (8). Indeed, some observers are beginning to argue that while biotechnology may initially have the effect of increasing technical disparities between high-productivity, capital-intensive agroecological zones, on the one hand, and low-productivity, labor-intensive zones, on the other, its long-term impact will be to decrease these disparities.

This argument assumes that R&D effort can be allocated to the problems of peasant smallholders on suboptimal lands. We feel that it is essential for the IARCs to consider strategies for this task, since it is unlikely that private firms, even those strongly committed to LDC markets, will work for cassava improvement for Colombia or potato improvement for Peru. These firms will be developing and commercializing products for which there are

substantial markets. The task of providing new technologies for peasant smallholders will thus remain that of the IARCs.

This sounds relatively straightforward, but the very promise of biotechnology -- its potentially broad application to an extraordinary range of commodities -- will make setting research priorities very difficult. Should the IARCs increase their emphasis on typical "peasant crops" (root crops and pulses) or continue their emphasis on cereal grain crops (e.g., by increasing the adaptability of the cereal grains to the stresses and climatic extremes of marginal agroecosystems)? Should the IARCs greatly increase their emphasis on capital-input-minimizing cropping systems or place equal emphasis on input-intensive systems? Should the IARCs emphasize research goals neglected by the private firms of the DCs or strive to compete directly with these firms? We believe, as does the CGIAR, that the future of the TARCs lies in targeting the services of these centers to the needs of peasant smallholders. However, peasant smallholders across the LDC continents are an extremely diverse group. No single CGIAR strategy can meet all their needs, but if priorities are carefully selected, biotechnology opens up unprecedented opportunities to ensure that the "second Green Revolution" will have broad beneficial effects.

CONCLUSION

We have attempted to demonstrate in a number of previous papers that biotechnology will have important socioeconomic impacts -- some beneficial, some adverse -- on LDCs. Debate concerning the future implications of biotechnology has tended to be contentious and has ranges between the voices of "biohype" or one hand, and of "biodoom" on the other. Our results have alternately disappointed both promoters and critics of biotechnology. The messengers of biohype tend to be oblivious of the profound dilemmas and potentially adverse socioeconomic impacts of biotechnologies. The advocates of biodoom ignore biotechnology's broad promise to equitably improve productivity and living standards. We have tried to identify both the promise and the pitfalls of biotechnology.

We have given equal attention to biotechnology issues in developed and developing countries. As agricultural R&D -- indeed, agriculture -- has become an increasingly global system, it becomes increasingly important to understand the interrelations among the agricultural systems of the diverse countries in the world economy. In particular, LDC-oriented agricultural researchers and administrators can learn a great deal through the policy issues of DC biotechnology efforts. For example, the "brain drain" from public sector research institutes to the private sector in the DCs may well affect LDC-oriented research institutes. (Scientists who straddle the public-private interface in LDC-oriented research institutes may, as in the DCs, tarnish the image of the public research system even though their efforts increase the speed of technology transfer.) Likewise, private-sector firms may try to influence LDC-oriented research institutes away from direct competition. How these issues are resolved in

the DCs will affect decision-making in international research institutions.

The present paper has emphasized two major points. First, because of the predominantly private character of biotechnologies, their development and transfer create unprecedented problems and challenges. Second, the maintenance of viable public agricultural research institutions is essential to ensure that biotechnologies result in the broadest possible benefits. The pervasively private character of biotechnologies would be relatively benign were it not for the fact that private sector dominance inherently threatens public agricultural R&D. Careful planning will be required to ensure IARC-LDC access to biotechnologies and to carve out a public/private division of labor consistent with the needs of peasant smallholders and the rural and urban poor in developing countries.

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REFERENCES CITED

1. Barton, J. The international breeder's rights system and crop plant innovation. Science 216:1071-1075.
2. Buttel, F.H., J.T. Cowan, M. Kenney, and J. Kloppenburg, Jr. 1984. Biotechnology in agriculture: the political economy of agribusiness reorganization and industry-university relationships. In Research in rural sociology and development. H.K. Schwarzweller, ed. JAI Press, Greenwich, Connecticut. (in press)
3. Buttel, F.H., M. Kenney, and J. Kloppenburg, Jr. 1984. Biotechnology and the third world: toward a global political-economic perspective. Politics and the Life Sciences 3:160-164.
4. Buttel, F.H., M. Kenney, and J. Kloppenburg, Jr. 1984. From green revolution to biorevolution: some observations on the changing technological bases of economic transformation in the third world. Econ. Dev. Cultural Change. (in press)
5. Buttel, F.H., J. Kloppenburg, Jr., M. Kenney, and J. T. Cowan. 1983. Genetic engineering and the restructuring of agricultural research. The Rural Sociologist 3:132-144.
6. Kenney, M., and F.H. Buttel. 1984. Biotechnology: prospects and dilemmas for third world development. Development and Change. (in press)
7. Kenney, M., F.H. Buttel, J.T. Cowan, and J. Kloppenburg, Jr. 1982. Genetic engineering and agriculture: exploring the impacts of biotechnology on industrial structure,

university-industry relationships, and the social organization of U.S. agriculture. Department of Rural Sociology, Cornell University, Ithaca, New York, Cornell Rural Sociology Bull. 125.

8. National Research Council. 1982. Priorities in biotechnology research for international development. National Academy Press, Washington, D.C.

9. National Research Council. 1984. Genetic engineering of plants: agricultural research opportunities and policy concerns. National Academy Press, Washington, D.C.

PRACTICAL APPLICATIONS OF GENETIC ENGINEERING IN AGRICULTURE[1]

V. L. CHOPRA[2]

The XV international conference on genetics took place in New Delhi in December 1983. In the field of agriculturally applicable genetic engineering, symposia included work on clonal propagation and embryo culture; haploids; protoplast fusion; gene transfer, especially transfer on nitrogen-fixing genes; and monoclonal antibody and hybridoma techniques. This paper is a summary of the presentations in these areas, excluding those made by authors published in this volume.

CLONAL PROPAGATION AND EMBRYO CULTURE

Dr. V. Jagannathan of the National Chemical Laboratory, Poona, India, described the application of plant tissue culture in agriculture and forestry, specifically, in the use of micropropagation in plant breeding. Table 1 lists plant species for which methods of micropropagation by tissue culture have been standardized. The following are contributions from India:

- In Eucalyptus and Pomegranate, the tissue-raised plantlets overcome juvenility but retain characteristics of the mature trees from which explants were derived.
- Field tests with tissue culture-generated, virus-free sugarcane gave 20% more yield than did virus-infected seed material.

Table 1. Plants for which micropropagation methods have been standardized.

Plantation crops	Fruit crops	Medicinal plants	Ornamentals	Forest trees[a]
Sugarcane	Citrus	Dioscorea	Rose	Teak
Turmeric	Pineapple	Glycorrhiza	Bougainvillea	Eucalyptus
Ginger	Pomegranate			Bamboo
Rubber	Almond			Sandal
Mustard	Banana			Rosewood
Cardamon	Apple			Pine

[a]Multiplication by meristem culture.

[1]This paper is based on presentations made at the XV International Congress of Genetics. [2]Professor of Genetics and president, International Genetics Federation. Division of Genetics, 129 Nuclear Research Laboratory, Indian Agricultural Research Institute. New Delhi-110012.

- Plantlets from subcultured wheat callus showed genetic variation.
- Among the somaclonal variants, turmuric mutants with high curcumin content have been obtained and propagated.
- By overcoming, through embryoculture, F_1 infertility of the cross between Carica papaya and Carica cauliflora, virus-resistant or -tolerant recombinants have been obtained.

HAPLOID CELL CULTURE

Plant haploids provide material for varied uses in basic and applied genetics. Androgenic haploids, first reported in India, have been applied in China. Dr. Hu Han reported the Chinese work on standardizing culture conditions and on the behavior of crop haploids. He also underlined the success of the varieties developed using haploids in China.

Eighty-one varieties of rice have been developed through anther culture. These varieties possess characteristics such as high yield (7500 kg/ha), resistance to bacterial blight, and wide adaptability. Large areas in China have been planted to them: as much as 100 000 ha for the variety Xin Xiu. Yields of the anther culture varieties are up to 22.57 higher than conventionally produced rice hybrids. Through use of such varieties the problems of hybrid seed production are avoided. They will soon replace the hybrid rice now widely grown.

Until mid-1982, 20 wheat varieties from anther culture had been released for cultivation. These varieties have vigorous tillering, cold resistance, early maturity, and good grain quality. Like rice, the anther culture-developed wheats cover large areas in regions of China where they have been released.

Pollen-raised plants have been produced to develop inbreds for hybrid production and improved varieties of maize, rubber, and sugarcane.

The anther culture technique has also been used to obtain material for chromosome engineering. Monosomics, telocentrics, and restrustured chromosomes containing alien genes have been produced for use in crop improvement.

PROTOPLAST FUSION AND GENE EXPRESSION

Development of techniques for protoplast fusion and for organelle uptake permits manipulation, through somatic cells, of the plant genome. These techniques also elucidate the regulation of gene expression both in cultured cells and in redifferentiated hybrid plants. Dr. D. Dudits from the Hungarian Academy of Sciences presented work on gene expression patterns in somatic hybrid cell lines.

Intraspecific and interspecific hybrid plants developed from fused protoplasts possess diploid genomes of both parental species, and express morphological characters and isoenzyme

markers similar to those of the sexual hybrids. (Somatic hybrids are here identified on the basis of complementation between nuclear chlorophyll-deficient mutants.) In higher plants, patterns of gene expression are linked with morphogenetic status. For example, in carrot the cyclohexamide resistance is a differentiation-dependent function: it is expressed in differentiated tissue, but not in unorganized calluses. When protoplasts of a mutant line, which expresses cyclohexamide resistance in callus cultures, were fused with chlorohexamide-sensitive protoplasts, the differentiated callus of the somatic hybrids did not express the resistance phenotype. Somatic segregants, however, retained cyclohexamide resistance.

Studies with somatic hybrids have shown that chromosomal elimination is not an inevitable consequence of protoplast fusion between taxonomically distant parents; even in some intergeneric somatic hybrids, no extensive chromosomal elimination is observed. The reason for the large number of fusion experiments with unsuccessful hybrid regeneration may be the expression of somatic incompatibility. Transferring only a part of the genome could prevent the loss of fusion products in those cases. Plant mutant cell lines sometimes lose their morphogenetic capabilities and are unable to differentiate into mature flowering plants. In such cases somatic cell hybridization is the only method of genetic analysis.

GENE TRANSFER AND EXPRESSION

Through the use of plasmids in *Agrobacterium tumefaciens* (Ti) and *Agrobacterium rhizogenes* (R_i), gene transfer and expression can be effected in plants. These bacteria transfer a defined DNA fragment (T-DNA) and covalentally integrate it into chromosomal DNA. No function within the T-region is required for either transfer or integration. The plasmid-derived T-DNA consists of a number of well-defined units that are transcribed by the host polymerase II and code for different functions, such as inhibiting plant differentiation and synthesizing opine. The groups at the Max Planck Institute, Koeln and the Laboratorium Genetika, Gent, have shown that by combining in vivo and in vitro recombinant DNA techniques, it is possible to eliminate each of the differentiation-controlling one genes by deletion mutations. Such studies indicate that

- the genes that code for shoot inhibition pleitropically stimulate root formation;
- reciprocally, the genes coding for root inhibition simultaneously stimulate shoot formation; and
- removing tumor-controlling genes does not affect DNA transfer or integration. It is thus possible to design modified Ti plasmids that can insert foreign genes in plant cells from which normal plants can then be regenerated. These plants will express the foreign genes and sexually transmit them with normal Mendelian segregation ratios.

Several plasmid-introduced foreign genes failed to express in the plants. To test whether this failure is due to the absence of certain specific sequences, 5' upstream sequences of genes that do express in plants were used to promote the foreign genes' expression. The two promotors used were the 5' promotor region of the nopaline synthetase gene and the 5' promotor region of the small subunit of RuBis Co. Different coding sequences were fused to these promotor sequences to construct chimeric genes, which were then introduced into plants. It was demonstrated that

- the nopaline synthetase promotor permits the expression of neomycine phosphotransferase, chloramphenicol transacetylase, and Mtx-resistant dihydrofolate reductase of *Escherichia coli*;
- RuBis Co promotor permits the light-inducible expression of the chloramphenicol transacetylase coding sequence.

A full session was devoted to the properties and expression of genes concerned with N fixation in symbiotic and asymbiotic bacteria and their host plants. Dr. P. Gresshoff reported his group's work on bacteria infecting legumes and a nonlegume (*Parasponia*):

- Sequence analysis of the nitrogenase genes *nif* HDK of Rhizobium strain ANU 289, which forms nodules on cowpea, siratro, and Parasponia, shows that while certain regions are strongly conserved, others are diversified;
- *Nif* H is separated from *nif* DK, where *nif* HDK is linked in *Rhizobium trifolii*, *meliloti*, and *Klebsiella*;
- Parasponia symbiosis differs from legume symbiosis in 1) nodule ontogeny, 2) the failure of bacteria to be released from infectious thread, and 3) the absence of high leghemoglobin content;
- The Parasponia leghemoglobin, despite its low overall concentration and oxygen dissociation constant, has a physiological role in regulating oxygen. The specific activities of Parasponia and siratro bacterioids are very similar under optimal oxygen conditions. This similarity, coupled with the fact that the bacterioid density of invaded tissue in Parasponia is about one-third that seen in siratro, demonstrates the efficiency of nonlegume-Rhizobium symbiosis.

Dr. A.W.B. Johnston reported on work to delineate the genetic elements in nitrogen fixation by *Rhizobium leguminosarum*:

- mutants defective in nitrogen fixation map in two clusters;
- mutants defective in nodulation map between the two clusters of nitrogen-fixing genes;
- the bacterium's symbiotic region spans about 50 kbp. (The genes involved in nodulation and determining host range in *R. leguminosarum* may thus be limited in number and we may soon have a detailed description of their organization and their function.);

- in R. leguminosarum and R. phaseoli, the region responsible for nodulation and host range determination is restricted to only 10 kbp DNA. Because so little Rhizobium DNA appears involved, most of the information determining specificity may be present in the host plant.

Dr. E. Kondorosi and his group have been studying bacterial genes coding for symbiotic nitrogen fixation in the Rhizobium meliloti - alfalfa (Medicago sativa) association. In R. meliloti, most of the symbiotic genes, including those coding for early functions in nodulation, the nif structural genes, and adjacent fix (probably nif) genes, are located on a megaplasmid DNA region of about 40 kbp. The nif gene organization and regulation are similar to those in K. pneumoniae. The nodulation genes of R. meliloti are arranged in two clusters. One cluster is conserved in many Rhizobium species and codes for common nod functions. The other cluster contains genes that determine specificity for the plant host. Mutations in nod genes of both clusters affect the very early steps of nodulation, such as root hair curling. Although the biochemical mechanisms of recognition and infection are still obscure, researchers believe gene products from both regions probably interact at the same stage of nodule development.

There are fix genes or other megaplasmid regions and also on the chromosome, but very little is known about these genes and their functions in symbiotic N fixation. Most of the symbiotic genes identified on the nod-nif region are expressed specifically during nodule development or in the developed nodules. It is not known which genes code for the later steps in nodule development, nor whether fix genes located in other parts of the Rhizobium genome are also nodule specific; i.e., do they evolve to function only during symbiotic fixation? Further molecular analysis of these genes would help answer these questions, and thereby aid in the future manipulation and improvement of symbiotic N fixation.

Dr. C.K. Kennedy presented the work of the Nitrogen Fixation Group of Sussex University on nif structure and regulation in Azotobacter:

- Genes homologous to Klebsiella pneumoniae nif H, nif D, and nif K are contiguous in Azotobacter chroococcum.
- While Klebsiella pneumoniae nif A activates, the nif L does not repress the Azotobacter nif expression.
- Azotobacter vinelandii is able to express Klebsiella nif L promotor but not nif H promotor.
- Azotobacter shares with K. pneumoniae certain features of nif regulation despite the many taxonomic differences between these two organisms.

Dr. W. Klingmuller and his group have aimed to elucidate the genetics of N fixation in associative Enterobacteria forming a substantial part of the inner rhizosphere of some grasses and cereals. They have successfully integrated Klebsiella nif genes into Enterobacter cloacae, an associative of Festuca. The transfer was mediated through the plasmid pRD_1 in Escherichia coli. Nif integration has also been achieved using bacteriophage

Mu. The integration hybrids thus obtained have been stable nif positive over many cell generations. Through the agency of nif plasmid cointegrate pCE1:: Mu cts from Escherichia coli, the nif genes have also been incorporated into the chromosome of Enterobacter agglomerans.

MONOCLONAL ANTIBODIES AND HYBRIDOMA TECHNIQUES

Hybridomas are fusion products of a cell, capable of indefinite multiplication with a cell to make an antibody. Dr. G.P. Talwar summarized work on hybridomas in his own and other laboratories. Hybridoma-produced monoclonal antibodies are used in immunodiagnostics, imaging of metastasis, and immunotherapy. They have also served as vehicles for drug delivery, to control fertility, and, as anti-idiotypes, in immunizations. Work so far has mainly emphasized the diagnosis and control of human diseases. However, since farm animals encounter diseases similar to those of humans, the techniques have great potential in agriculture.

UNIDO's WORK TO BUILD BIOTECHNOLOGY CAPABILITY IN DEVELOPING COUNTRIES[1]

B. SIGURBJÖRNSSON[2]

Convinced that biotechnology can help solve the critical shortages of food, energy, and health care in developing countries, UNIDO is helping establish the International Center for Genetic Engineering and Biotechnology (ICGER), sensitizing developing-country policy makers to biotechnology's potential benefits and limits, and facilitating contact between developing and developed countries, partly through its newsletter Genetic Engineering and Biotechnology Monitor.

Since its inception in 1967, UNIDO's chief objective has been to further industrialize developing countries. The 1975 Lima Declaration called for raising the developing countries' share in world industrial production to 25% by the year 2000. UNIDO was given much of the responsibility for achieving this goal. Biotechnology and genetic engineering are an important part of industrial production, and a critical one to the developing world: UNIDO Executive Director Abd-E1 Rahman Khane stated in 1981 that biotechnology ''could galvanize and broad-base the industrial structure of developing countries and impart new directions to industry, agriculture, and energy."

In February 1981 UNIDO invited leading scientists and technologists to discuss biotechnology's implications for the developing nations. The group concluded that biotechnology could help solve some of such pressing problems as disease control, food shortages, and energy shortfalls. They recommended that a group of experts be formed to gauge interest among national decision-makers and the international scientific community in an international center to promote research and development in biotechnology.

Soon after the February meeting UNIDO set up this group "to explore the official and professional reactions in selected countries to the idea of such a center, its scope and functions, the needs it could meet, and especially the support it would give to national efforts." Scientists in the group included leader Carl Goran Heden, A. Chakrabarty, H. Boyer, R. Wu, S. Narang, and S. Riazuddin.

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[1]Delivered by author on behalf of United Nations Industrial Development Organization (UNIDO).

[2]Development and Transfer of Technology Branch, Division for industrial Studies, UNIDO, Vienna, Austria.

During the summer and fall of 1981 the members of the group began a series of trips to meet with senior government officials, scientists, and technologists in 16 nations and 4 international organizations. (The group communicated throughout its work with UNDP.) At the end of the travel period, the group convened in Vienna to discuss members' findings. A project report including six recommendations was issued; the one pertaining to the proposed center is particularly important. It states that an International Center for Genetic Engineering and Biotechnology (ICGEB) with the following characteristics should be established:

- It should be a high-quality institute where scientists from developed and developing nations will interact.
- It should have a training program to build scientific manpower in the third world by offering fellowships at the Center and sponsoring workshops in different countries.
- It should promote cooperation between nations through exchange programs, collaboration, and promotion of communications among national and regional institutions engaged in biotechnology research.
- It should advise national decision-makers and help them formulate policy on biotechnology.
- It should promote the flow of information by making scientific journals and books available to the developing countries.

While the expert group was still formulating its recommendations, several nations expressed interest in hosting the center, including Mexico, Sweden, and Belgium. To resolve potential technical problems in setting up the ICGEB and to consider the national offers to host it, the Government of Yugoslavia and UNIDO jointly convened a meeting in Belgrade on 13-17 December 1982. Official delegations from 28 nations, observers from 9 international organizations, an observer from industry, and several eminent scientists attended the meeting.

General consensus favored the Center's establishment and the general characteristics previously elaborated by the UNIDO experts and Secretariat:

1. Goal-oriented, high-quality research and development will focus on the needs of developing countries, including enhanced oil recovey, production of energy and fertilizers from biomass, the improvement of fermentation techniques, the development of human and animal vaccines, and improvement of agriculturally important crops.
2. The Center will provide scientific and technological training, on its premises and at affiliated centers, to individuals with the potential to enhance industrial activity in their home countries. The target is to admit 100 persons for 2 rears at the ICGEB and to arrange for the training of an additional 300 persons in affiliated institutions.
3. The ICGEB will facilitate the international flow of information and biotechnological advances from the

developed countries to the developing ones. It will be linked with existing national and regional centers and will thereby be able to encourage scientists and institutions in joint efforts to solve common problems. It will assist individual centers with problems they are incapable of solving by themselves.

4. The Center will help set up affiliated regional and national centers for particular biotechnology research and development important to their regions or nations.

The ICGEB's Division of Research and Development will probably have six departments or sections:

1. The Department of Molecular Biology and Biochemistry will undertake research on expression, vector development, DNA/RNA sequencing, polynucleotide synthesis, protein Sequencing and, as necessary, the manufacture of restriction enzymes.
2. The Department of Microbiology and Molecular Genetics may establish a sizable culture collection and also concentrate on biomass conversion, improving bacteria and yeast useful in single-cell protein production, and perhaps work on enhanced oil recovery.
3. The Department of Immunology and Infectious Diseases will concentrate on vaccine development and monoclonal antibody research.
4. The Department of Genetics, Cell Biology, and Biochemistry of Plants will form a seed collection and plant gene bank and develop vectors for genetically manipulating plants.
5. An Election Microscope Facility will be available to all researchers.
6. The Department for Process Development and Manufacturing will include pilot plant operations and will research such production processes as immobilizing enzymes and cells, adsorption and filtration methods, and developing efficient purification methods.

This is, of course, a wish list, but the ICGEB will probably include these departments and thereby have the broad, interdisciplinary base necessary for training. Resources available will limit research to between three and six projects at any one time.

During the Belgrade Meeting several countries expressed a wish to host the Center. Belgium, Canada, Cuba, India, Pakistan, and Thailand presented offers but asked for time to prepare proposals. Italy and Spain also indicated this wish, but will formalize it after consultations with their governments. Brazil, Indonesia, Mexico, Nigeria, Sweden, and Venezuela would like affiliated centers within their borders.

To help decide the Center's location, a Selected Committee of scientists was formed. The Governments of Argentina, China, Hungary, Indonesia, Nigeria, Sweden, and Yugoslavia each nominated one scientist, and UNIDO expert Dr. David McConnell from the University of Dublin was chosen as Committee Chairman.

By the deadline for submitting proposals to host the Center, UNIDO had received eight offers: one each from the Belgian regions of Brussels, Flanders, and Wallonia, and from Cuba, India, Italy, Pakistan, and Thailand. Four more were received later from Argentina, Bulgaria, Spain, and Tunisia, but the Selected Committee could only consider the first eight. Each of the offers met the basic requirements for land and buildings suggested by UNIDO, including approximately 372 000 m^2 of and as site for 3 buildings -- a main building with 11 625 m^2 of floor space for laboratories, offices, and storage facilities; a second building of 1 500 m^2 to house a pilot plant and maintenance workshop; and a third building for a greenhouse of 2 400 m^2. All offers also included such other facilities as guesthouses, hostels, staff housing, conference centers, and parking lots.

The Committee members visited each site and evaluated each proposal. The most important factors were an infrastructure sufficient to support and nurture the Center; a scientific community to interact with the Center's personnel; a good communications system; and an environment attractive to scientists and others from all over the world. The Selected Committee concluded unanimously that Belgium, Italy, and Thailand were suitable.

It became clear that another meeting was necessary. At the invitation of the Government of Spain, a Ministerial-Level Plenipotentiary Meeting was held in Madrid during 7-13 September 1983. Forty-three nations were formally represented and observers from other nations, international organizations, and private industry also attended. At the Madrid Meeting the ICGEB was established legally when 76 countries signed its statutes. However, a site was not chosen. Intense competition to host the Center had developed. The offers of potential host countries were generous:

- Belgium offered a site in Brussels and a financial package worth US$18.5 million.
- India offered a site near New Delhi and a package worth US$17.6 million.
- Italy offered a site in Trieste and a package worth US$40 million, including US$10 million to help set up affiliated centers in developing countries.
- Spain offered a site near Madrid and a package worth US$38.5 million, including an interest-free loan to the ICGEB of US$15 million.
- Thailand offered a site at the Mahidol University in Bangkok and a package worth US$19 million.

Delegates wondered whether other organizational forms of the ICGEB could better take advantage of the offers and serve the needs of developing countries for biotechnology. National representatives decided to postpone selecting a site, and to set up a Preparatory Committee, consisting of one delegate from each of the countries that signed the ICGEB statutes, to reexamine proposals for sites and consider several options on the organization of the ICGEB. The options analyzed included: 1) establishing two centers, one in a developed and the other in a

developing country; and 2) a form resembling a university with several branches; in other words, the Secretariat would be housed in one country and branches would be located in other countries.

In January 1984 the Preparatory Committee chose the two-component ICGEB form and recommended that Trieste, Italy, and New Delhi, India, be the sites. This was accepted by the reconvened Ministerial Meeting on 4 April 1984, and 10 countries signed the amended statutes. Another 5-10 countries are expected to sign soon.

The Preparatory Committee will meet in September to discuss starting activities. The Italian authorities have indicated their willingness to provide temporary facilities in Trieste near the future ICGEB site. In fact, Italian scientists and decision-makers believe it is possible to begin ICGEB-directed research and development in early 1985.

In addition to establishing the ICGEB, UNIDO is trying to sensitize national decision-makers in the third world to the potentials and limits of biotechnology and to help them strengthen their nations' biotechnological capabilities. Those nations whose decision-makers know biotechnology's possibilities, and can link those possibilities with existing national resources will benefit most from the ICGEB and, probably, from biotechnology.

UNIDO also encourages contacts between developed and developing countries and provides an information service, in the form of a quarterly newsletter, Genetic Engineering and Biotechnology Monitor, first issued in February 1982. The newsletter monitors biotechnological advances throughout the world and is distributed free to industrialists, government workers, and scientists in developing countries.

In conclusion, UNIDO's interest in biotechnology is in applied research and the industrialization of its results. UNIDO believes that its wide-ranging program in biotechnology is timely: biotechnology applications are just now appearing in the developed nations. By the tine developing-country decision-makers appreciate biotechnology's potentials, and the ICGEB is operating, the Center and its trainees will be ready to apply biotechnology to fulfilling the needs of developing countries.

RECOMMENDATIONS

The research histories and mandates of institutes in developed countries and the International Agricultural Research Centers (IARCs) are often different. Whereas the former have pioneered in basic aspects of biotechnology research (as have several national institutes in developing countries), the latter have unique experience in the improvement of crops for which the greatest human gains may result from use of all tools science offers. Future collaborative research should draw upon and balance these complementary strengths. A major aim of the Inter-Center Seminar on IARCs and Biotechnology was to identify methods of promoting such symbiotic linkages between IARCs and advanced institutions with relevant expertise both in developed and developing countries. The conclusions and the recommendations of the participants at the seminar are grouped under the various categories of research applications discussed.

I. IN VITRO PROPAGATION, DISEASE ELIMINATION, GERMPLASM CONSERVATION AND EXCHANGE

A. CLONAL PROPAGATION

In vitro propagation, a modern extension of conventional vegetative propagation, has wide applications in both clonally and sexually propagated crops. The main routes to plant regeneration through in vitro culture comprise shoot development and rooting by adventitious and nonadventitious means and somatic cell embryogenesis.

Since 1968 there has been a dramatic increase in the number of species propagated in vitro (30 species by 1968, more than 300 by 1978) and there is the prospect of a further tenfold increase by 1988. Most of the IARC-mandated crops are included. In vitro propagation is a basic requirement for the successful implementation of all aspects of plant biotechnology, with the possible exception of some by-product applications.

Problem areas

In vitro propagation remains problematic for many crops. Much of the progress has been from empirical experiments. Future progress, particularly on crops such as cereals and grain legumes, will require intensified crop-specific work and an

understanding of the biochemical basis for plant regeneration and its control.

In vitro propagation may release instability manifested as somaclonal variation. This appears to be related to the species and the route to regeneration. Although of potential value in plant breeding, somaclonal variation is disadvantageous in the clonal propagation of elite genotypes and must be controlled to maintain genetic integrity of propagating material.

Recommendations

1. The IARCs and national programs should build up a nucleus of in vitro expertise to facilitate the rapid transfer and implementation of biotechnological methods as they become available through developments elsewhere.
2. Criteria to evaluate the phenotypic stability of cultures and regenerated plants should be developed. This should take place in the appropriate IARC for each crop.
3. Economic technologies for mass propagation appropriate to developing countries should be researched. Such countries could form collaborative projects on transfer and monitoring of the technology being carried out by the IARCs in cooperation with national programs.
4. Implementation of clonal propagation in vitro through high-efficiency mass-propagation systems will require development of appropriate mechanization (encapsulation or fluid drilling of somatic embryos or sowing of artificial seed). This research should be carried out in an advanced institute in collaboration with the IARCs.

B. DISEASE ELIMINATION

Several in vitro methods to eliminate diseases from crop plants are available. The techniques are valid for all known pests and pathogens, but are especially valuable for eliminating virus-type pathogens from vegetatively propagated crops.

Methods currently available include

- culture of the shoot-apex comprising the apical meristem plus 1- to 2-leaf primordia,
- culture of the apical meristem,
- grafting of the shoot tip onto seedling rootstocks, and
- adventitious organogenesis or embryogenesis in special tissues such as nucellar tissue.

The last two methods are applicable to crops, such as woody plants, for which shoot-tip culture is not possible. All the methods are normally linked to thermotherapy and chemotherapy before or during culture.

In vitro disease elimination methods are particularly valuable because 1) crop yields can be increased significantly, and 2) international exchange of germplasm is facilitated.

Not all in vitro-produced plants are automatically pathogen-free. Therefore regenerated plants should be tested for pathogens. Rigorous indexing is especially necessary if the plant material is for international exchange.

Problem areas

Adequate disease-indexing methods are available for some crops, but are poorly developed or lacking for others.

All chemotherapeutic agents tested so far against viruses have proved highly phytotoxic.

Reinfection of clean stocks may occur unduly rapidly because of inappropriate agricultural practices by farmers.

Recommendations

1. Virological research, particularly on the development of indexing techniques, should be strengthened in all IARCs concerned.
2. Indexing methods for specific crop diseases should be developed. Emerging biotechnological tools such as nucleic acid hybridization and the use of monoclonal antibodies should be investigated. The technology should be developed in advanced institutes and tested in the IARCs.
3. Research should be carried out to develop more efficient and less phytotoxic antiviral chemotherapeutic agents.
4. Attention should be giver to monitoring reinfection and its control by appropriate integrated methods.

C. GERMPLASM STORAGE

In vitro methods being developed for the conservation of vegetatively propagated and recalcitrant seed-producing crops include short- to medium-term storage by slow growth for active collections and cryopreservation for base collections.

In vitro methods can provide a safe means of storing germplasm collected in the wild and from less secure field gene banks (clonal repositories, orchards, plantations, etc.). Genotypes produced in vitro will require secure storage in vitro.

In vitro storage will provide protection against pest and disease attack and may retard time-related genetic changes.

Problem areas

Research into the development of in vitro storage methods is inadequate and underfunded.

Good cryopreservation methods for the long-term storage of shoot, embryo, and callus cultures and pollen are lacking. The possibility of in vitro storage (especially by cryopreservation) inducing a degree of genetic instability has not been considered or studied adequately.

Recommendations

1. Programs of in vitro conservation, including those involving IARC mandated crops, are best carried Out in collaboration with the International Board for Plant Genetic Resources (IBPGR) and its advisory committees. Every effort should be made to evaluate and document material committed to storage in vitro and the collaboration of breeders should be sought in the selection and use of such material.
2. IBPGR recommendations for in vitro gene banks should cover a) the number of accessions that can be handled in vitro, b) necessary duplication of accessions, and c) unit costs of storage by slow growth and cryopreservation.
3. Technologies available now for short-term storage by slow growth, and emerging cryopreservation technologies should be tested on a wide range of genotypes for each crop. That would strengthen and promote the wider use of short-term and long-term in vitro preservation in the IARCs.
4. Pollen storage should be examined in species for which that technology does not yet exist.
5. The feasibility of using in vitro techniques to revive aged seeds of the orthodox type should be explored.
6. The possibilities of generating genetic instability in organized cultures as a result of cryopreservation, and the control of this instability by manipulating storage conditions should he examined in a suitable clonal crop. Storage techniques should be developed in an advanced institute and stored material should be evaluated in both that institute and the relevant IARC.

D. GERMPLASM EXCHANGE

The use of in vitro methods to collect permplasm holds great promise. Some IARCs have transferred newly collected germplasm and distributed elite clones in vitro. In vitro distribution reduces the risk of disseminating disease and permits rapid and efficient distribution of material.

Problem areas

Adequately detailed procedures on in vitro exchange of germplasm for a range of crops are not available.

Quarantine procedures have not kept pace with developments in germplasm distribution in vitro.

There are no routine procedures for transporting frozen in vitro plant cultures.

Recommendations

1. In vitro stored material that has not been tested for pathogens should not be used for international exchange.

2. Indexing methodologies must be strengthened and implemented (including in quarantine stations).
3. The IBPGR should collect and publish available information on germplasm exchange in vitro covering a) culture preparation, b) packaging and methods of transport, c) handling upon receipt, and d) appropriate quarantine considerations.
4. Quarantine authorities should be requested to review present regulations on the handling of in vitro material and to a) educate quarantine personnel on the advantages and limitations of in vitro techniques, b) advise scientists sending and receiving in vitro material on procedures to safeguard the cultures and satisfy control regulations.

11. HAPLOIDY, SOMACLONAL VARIATION, AND MUTANT ISOLATION

A. HAPLOIDS

Haploidy is desirable for crop improvement programs. Through haploidy it is possible to achieve

- rapid homozyposity,
- rapid incorporation of new genes after recombinations or mutagenesis,
- increased selection efficiency,
- selection at the haploid level for autopolyploid species, and
- specific objectives in wide crossing such as production of alien addition lines and genome analysis.

Methods for incorporating haploidy are available now for rice (anther culture), wheat (anther culture, alien species crosses), barley [anther culture, chromosome elimination (*H. bulbosum*), *hap* gene], maize (anther culture, genetic induction), and potato (haploids, alien species crosses!.

For those crops the major constraint to haploidy for crop improvement is genotypic differences, which affect the efficiency of haploid production and plant regeneration.

Recommendations

1. Haploid methods for some crops such as legumes are not currently available. For these crops, existing technologies should be applied to achieve efficient haploid production.

   In particular, haploidy would be useful in groundnut, which is a tetraploid, and in beans.
2. Efforts should be exerted to develop efficient haploid production in recalcitrant genotypes such as indica rices. This could be achieved by direct effort within the IARCs along with collaborative research in other institutes.

B. SOMACLONAL VARIATION

Somaclonal variation is enhanced genetic variability in plants regenerated from tissue culture. Active research on somaclonal variation attempts to understand

- quantitative aspects such as relative mutation frequencies,
- qualitative aspects and mechanisms of origin (are variants and the mutation-generating process different from that for spontaneous or induced mutants and does somaclonal variation generally cause mutations in organelle genomes?),
- potential value in intravarietal improvement, and
- use of somaclonal variation to enhance alien gene introgression in wide crosses.

In crop species where the creation of genetic variability through sexual reproduction is difficult, somaclonal variation along with mutagenesis is the most likely way to generate variability for crop improvement. In sexually reproducing crops where an in vitro screening procedure is available or can be readily designed, somaclonal variation can be useful, particularly for highly adapted varieties.

Recommendations

Where tissue culture methods are being employed by IARCs, somaclonal variation should be considered as a breeding option.

C. MUTANT ISOLATION

In vitro techniques now allow efficient recovery of mutant genotypes. In many cases the selected mutation conditions useful agronomic changes at the whole-plant level. Examples include disease resistance and herbicide and salinity tolerance.

Research to extend this technology to recover additional types of mutants of agronomic importance (tolerance for metal toxicity, temperature, and water stress) is being pursued.

The advantages of in vitro mutant isolation Over conventional field screening include

- application of greater selection intensities, and
- substantial reduction in time, expense, and resources use.

Problem areas

In vitro selection is limited to crop species in which efficient tissue culture and plant regeneration already exists.

In vitro selection can be applied only to agriculturally useful traits, which are correlated with cell culture response.

Recommendations

1. In IARCs that employ tissue culture methods, in vitro selection should be evaluated as a method for recovering agriculturally useful phenotypes.
2. We strongly urge the IARCs to establish research linkages with other institutes so that they will be in a position to rapidly exploit haploidy, somaclonal variation, and mutant isolation as these are developed for specific crops.
3. In crops where such technologies are not currently available, initiatives to stimulate research should be taken by the IARCs without diluting or dropping current plant improvement programs.

III. WIDE CROSSES, SOMATIC HYBRIDIZATION AND EMBRYO CULTURE IN PLANTS, AND MOLECULAR BIOLOGY AND GENETIC ENGINEERING IN PLANTS AND ANIMALS

The mandate of the IARCs is to organize and carry out applied research to increase food production in developing countries. Food crops research concentrates on developing improved germplasm characterized by high crop yields, resistance to diseases and pests, and stable performance under a wide range of agronomic management regimes. In animal production programs, the emphasis is on developing control measures for some of the most serious diseases, particularly those endemic in the African continent.

The centers must harness all scientific advances that would make their technology more productive and more efficient. Genetic engineering involving molecular biology and collateral methodologies, in particular, requires a critical mass for efficient pursuit of the research. We recommend enhanced collaboration between IARC scientists and relevant scientific groups in advanced laboratories.

A. WIDE CROSSING

Importance and potentials

Interspecies crossing, even across wide taxonomic separations, has long been used by plant breeders to introduce genetic variation in related species that was not present in the crop. The purpose is to introduce into a crop plant species the parts of an alien genotype necessary to incorporate beneficial and stable phenotypic change, or to make combinations of genomes that create effectively a new crop species such as Triticale.

Problem area

A problem in wide crossing is the difficulty of screening for the presence of genetic material of different origins in the

derived plants. In addition to the traditional technologies such as cytology and isozyme markers, molecular biology can provide a solution to this problem because each species possesses repeated sequences of DNA that are specific to it, sequences that are distributed throughout the chromosome complement. Probes can be prepared to test unknown plant material for the presence of DNA that is unique to any of the potential contributing species. Such tools will increase the speed and precision with which some wide cross derivatives can be handled.

About 5% of the centers' efforts are currently devoted to wide cross programs.

The state of the art is good using traditional techniques. Not all of the desired traits are available in now crossable germplasm. A large number are available, however, and crossability barriers continue to be overcome.

Recommendation

It is suggested that if a Center has problems in handling wide cross plant material, the necessary DNA probes should be prepared in a developed country laboratory (public or private). These probes could then be used in the Centers after their staff have acquired the necessary competence. Subsequently if the demand is high, a facility for new probe production could be established in the IARC system.

B. EMBRYO RESCUE

Seed failure associated with embryo abortion and endosperm disintegration is common in intervarietal, interspecific, and intergeneric crosses in plants. Isolation and culture of embryos from defective seeds before embryo abortion sets in continue to be the primary means of obtaining viable seedlings from such crosses. Refinements of this technique should be encouraged and transferred to IARCs. Embryo culture also eliminates postzygotic incompatibility problems encountered in certain types of crosses. In some cases, culture of isolated ovules or even ovaries may prove advantageous, especially when the hybrid embryo is too small to be rescued without recourse to micromanipulative techniques.

Problem area

We wish to emphasize that until reasons for embryo failure in wide crosses are understood cytologically, physiologically, and genetically, success in attempts to develop specific crosses and enhance crop biomass will continue to be limited.

Recommendation

The potential of excised hybrid embryos to produce a callus and the subsequent induction of plants from the callus by

organogenesis or by embryogenesis should be explored. This method might be particularly useful in raising hybrid seedlings in quantity for field trials. Although no major technical problems are associated with ovule, ovary, and callus culture, use of those methods to rescue embryos has not been appreciated.

C. PROTOPLAST FUSION

Protoplast fusion can overcome barriers to sexual incompatibility between sexually isolated species. It allows transfer of cytoplasm between sexually isolated species and the crossing of sterile plants. Fusions are also possible between bacterial and fungal protoplasts and plant protoplasts, and even with animal cells.

In species such as potato, the fusion of dihaploid protoplasts to produce tetraploids combined with cell culture to regenerate whole plants is a realistic field of investigation. Forage legume protoplasts can be readily regenerated into plants. Fusions are being investigated in relation to specific breeding objectives. These studies emphasize the use of fusions for limited gene transfer (by irradiation of one of the fusion partners) between different species and, as in the case of Brassicas, the transfer of cytoplasmically based factors such as male sterility and triazine resistance.

The new biotechnological procedures could be used to explore limited gene transfer for disease resistance and salinity tolerance in rice, as well as to produce male sterile and triazine resistant lines. Applications for crop improvement also clearly exist from protoplast fusion procedures in wheat, maize, barley, in several of the legume pulses, and in other species. Interspecies Azolla protoplast fusions are also required.

Problem area

The major deficiency that prevents the implementation of the protoplast fusion program in the IARCs is that it is not now possible to regenerate plants from protoplasts of many of the species of interest, among them rice, wheat, maize, barley, and the major legume pulses. If transformation of monocots is shown to be facilitated by the use of protoplasts (by uptake or micro-injection of DNA), then it will be essential to have reproducible regeneration of plants from rice, wheat, maize, and barley protoplasts.

Recommendations

To create necessary technologies, advanced laboratories should be encouraged to undertake the necessary research on protoplast fusion because of its potential to extend genetic variation. Because regeneration is closely linked to genotype, this work will greatly benefit from links with IARCs possessing germplasm for research.

D. RECOMBINANT DNA

Current uses in plant breeding

The ability to isolate, purify, and characterize specific pieces of DNA has a place in plant breeding programs of the IARCs today. Specific DNA fragments can be used in screening germplasm to detect alien DNA introduced by wide crossing programs and to distinguish different male sterile cytoplasms. The methods are rapid, inexpensive, and often can be adapted for screening large numbers of plants.

Recombinant DNA molecules are already used in breeding programs to detect plants with virus resistance. Segments of viral and viroid pathogens purified by recombinant DNA methods provide excellent probes for rapidly and effectively screening plant populations for the presence of pathogens. Programs in the IARCs involving wide crossing and the selection of virus-resistant plants in potato, cassava, legumes, and groundnut are suitable for the incorporation of DNA probes.

Transformation

Wide crosses will continue to be the principal means of increasing the germplasm base. In the long run gene transfer will also broaden the variability available to breeders. Gene transfers will provide for the introduction into established cultivars of specific characters controlled by one or a few genes, without perturbing the adapted genetic background. Gene transfer will enable the copy number of a given gene to be varied, the expression of a gene to be varied, and genes from other species to be inserted.

Problem areas

Identification and isolation of genes important to agriculture. There is an urgent need to translate an economically advantageous trait into DNA segments that can confer the trait. We have the tools, but not the knowledge. We need a much better knowledge of plant molecular biology. IARCs should establish a dialogue between their staff scientists and plant molecular biologists who express the appropriate interest in agricultural problems.

Single genes to be used for transformation should be sought now. A promising approach is insertional mutagenesis using transposable elements. This work will require close cooperation of breeders and molecular biologists.

At the next level, DNA sequences underlying multigenic traits must be identified and isolated. Knowledge of the physical basis of multigenic traits is almost completely lacking, and research using insertional mutagenesis should be started as soon as possible. This, too, will require close cooperation between breeders and molecular biologists.

The transposable elements identified in maize have already proved to be of tremendous importance for this crop.

The elements change their position in the chromosomes and when one of them inserts itself into a dominant single gene, its effect on the plant phenotype can be observed. The molecular biologist can isolate the gene into which the element has inserted itself by isolating the transposable element from its new position and characterizing the DNA adjacent to the element.

To develop a library of genes important for the plant breeder, it will be necessary for

- specific genes to be nominated by the breeder,
- active transposable elements to be inserted into or characterized in the appropriate plant genotypes,
- large plant populations to be screened to recognize individuals carrying the appropriate mutation, and
- gene mutated by the transposable element to be isolated.

Because large numbers of plants need to be screened using assays often available only to the breeder, the project must be seen as a collaboration between IARC and molecular biologists. Before such a project can begin, transposable elements must be transferred to the appropriate plant genotypes. For maize this is readily possible with conventional breeding methods. However, for crops other than maize the elements must be inserted into the crop or indigenous elements located. The mobilization of maize transposable elements into dicots is being explored. Once gene transfer, gene substitution, and insertional mutagenesis are available for our crop species, their genetics and manipulation will enter a completely new phase.

Breeders at IARCs may recognize unstable genetic characters in the germplasm. If so, they should save the plants and consider setting up a collaborative program with a molecular biologist so that the transposable element responsible for the instability can be isolated and characterized. This research is important for one of the long-term goals of both molecular biologist and crop breeder.

Organelle genomes. Chloroplasts are the sites of photosynthesis and are prime targets for genetic modifications that may improve dry matter production. Amyloplasts are the sites of starch production and accumulation. They are targets of genetic manipulations that can lead to improvements in seed starch characteristics and the nutritious quality of seed materials. Cytoplasmic male sterility appears to be associated with properties of mitochondrial genomes and, in some cases, plastid genomes.

Both plastids and mitochondria are composed of products of organelle and nuclear genes. Genetic engineering of plastids and mitochondria will require

1. detailed knowledge of the nuclear and organelle genes whose expression leads to mature differentiated forms of these organelles,
2. understanding of mechanisms by which expression of these genes are regulated, and

3. techniques for transforming the nuclear and plastid genomes with foreign DNA.

Recommendations

The efficient application of recombinant DNA techniques requires access to sophisticated technology not available in the IARCs. In most cases the work will be performed initially in collaboration with well-established advanced centers. Basic expertise subsequently could be built in the centers that feel they are competent and in the right environment.

The familiarity of the centers with crop plant germplasm makes them ideal partners in informing molecular biologists about cultivars that are likely best suited for working out of gene transfer methods. Knowledge of the germplasm will play an even more crucial role in identifying cultivars that could be used to attempt the cloning of a particular gene that specifies a desirable trait.

For organelles to be manipulated within 5 yr or so, much additional information will be required. Rapid accumulation of basic information for species not now being studied should attract more workers to the subject. Nuclear transformation techniques are needed for other manipulations as well, but the importance of organelle transformation attempts should be emphasized. The work can be focused initially on developmental and functional features of agricultural importance through close consultation between advanced laboratories outside of the centers and IARC staff.

E. PLANT-BACTERIA INTERACTIONS AND CROP YIELD

A large number of microbes interact with crop plants. Most of them are deleterious, but some are highly beneficial. Because these relationships are mutually interdependent, efforts must be made to improve germplasm with respect to a given interaction using state of the art technologies. Research is urgently needed to help us understand these interactions at the molecular and cellular levels and to exploit them to increase crop productivity. Such research will have particular relevance for crop improvement in developing countries in a relatively short time.

Recommendations

Beneficial microbes

1. Microbial samples from various tropical zones should be collected, identified, and screened for their ability to enhance the growth of crop seedlings. Attempts should be made to mutagenize the strains and select for the most competitive ones. The use of modern techniques for screening the strains is recommended.
2. Plant breeding programs to improve plant-bacteria associations (particularly nitrogen-fixing ones) should

be introduced for legume crops. The germplasm should be screened for efficiency with an improved inoculum.

3. Anabaena-azolla associations should be further exploited.
4. IARCS can play an important role in the development and testing of improved inocula of useful microorganisms (Rhizobium, azolla, associative nitrogen-fixing organisms). Development of those inocula should consider the soil conditions and the specific germplasm.

Pathogenic microbes. Interactions caused by fungi and bacteria constitute major limitations to crop yield. Data on the genetics of pathogenicity and resistance strongly indicate the high degree of complementarity between the two organisms. It is generally accepted that genetic resistance is likely to be the major route for controlling diseases.

There is essentially no information available on the ways in which resistance genes are expressed, yet this information is invaluable if we are to

- make maximum use of available sources of resistance,
- understand the nature of race structure and differentiation in the pathogen, and
- explore the nature of genetic change accompanying the breakdown of resistance.

1. International collaborative efforts to explore the fundamental basis of pathogenicity/symbiosis should be intensified. The IARCs should stimulate interest and develop momentum in pathogenicity/symbiosis research through collaborative international programs in developed countries. For this work, IARCs can provide the most suitable sources of germplasm and develop near-isogenic lines containing different resistance genes in a common genetic background. Those lines will be essential to the isolation and study of genes for resistance through molecular biology.
2. The IARCs could undertake some of the extensive testing of the recombinant strains that will be generated through sophisticated programs on isolating genes for pathogenicity.

F. PLANT VIRUSES AND VIROIDS

Viruses (including virus-like diseases, or viroids) are insidious pathogens, and considerable effort is devoted to overcoming their effects. Biotechnological research to provide efficient screening procedures for viruses, to increase our understanding of their interactions with crop plants, and to control infection of crop plants should be encouraged. The detailed structure and biology of some viruses and viroids are understood from the application of molecular and cell biology.

Recommendations

1. Plant viruses and viroids relevant to IARC programs should be identified and characterized. The research should cover pathological and epidemiological aspects.
2. Because serological approaches to viral detection need improvement, emphasis should be on research for the development and utilization of improved methods such as indirect ELISA, monoclonal antibodies, etc.
3. Recombinant DNA clones should be developed for the identification and detection of viroids and viruses for which the serological approach is not sufficiently sensitive or reliable. Advancements in nonradioactive nucleic acid detection should be implemented as soon as practicable.
4. Viruses and viroids of particular importance should be suggested for study by the IARCs, which should spell out the specific needs of each program. Links should then be made with advanced laboratories to strengthen the research and to achieve the synthesis of relevant antibodies and recombinant DNA clones.

G. CHROMOSOME MAPPING

A detailed working knowledge of the chromosome map of a plant can play a small but useful role in plant breeding. For example, if a single gene trait is difficult, expensive, or time-consuming to assay, recognition at the seedling stage of a gene closely linked to it can be very helpful. Morphological characters are rarely useful because the appropriate variation is not in the elite germplasm of the breeder. However, there is often variation at the loci of specifying multiple isoenzymes. Hundreds of individual plants can be screened per day and usually only very small pieces of tissue are required.

Isoenzyme assays are well established in biochemistry laboratories and could be easily done in the IARCs in response to specific needs. To develop a useful system, many isoenzyme assays need to be carried out on genotypes in which the agriculturally important trait is segregating (to establish genetic linkage). Thus this research needs to be located at an IARC or must depend upon efficient collaboration between an IARC and a biochemistry laboratory.

The number of isoenzymes suitable for mapping is limited. Many more chromosome markers can be generated as a consequence of polymorphism in DNA sequences throughout the chromosomes. These polymorphisms are easily detected by digestion of DNA with restriction endonucleases, and hence are known as restriction fragment length polymorphism (RFLP).

Polymorphisms of DNA restriction fragment lengths serve as genetic markers, which segregate as codominant Mendelian markers. RFLP mapping would serve as an additional breeders' tool screening for the presence of desirable traits once a RFLP has been located close to the gene of interest.

When many DNA probes are available for mapping of specific chromosome segments in the relevant crop, RFLP mapping could be easily introduced in the IARCs. Development of the unique sequence probes, and mapping of the chromosomal sequences identified by the probe would be most efficiently done first by a molecular biology laboratory in a developed country.

Where simply assayed isoenzymes are not available to routinely tag a gene required by the breeder, then it will be possible to insert such a gene tag. Antibiotic resistance genes have already been inserted into dicot plants, allowing those plants to be selected from plants that do not possess the marker gene.

DNA marker genes will be inserted into the chromosomes of a line nominated by an IARC. Analysis of subsequent crosses will isolate lines in which a marker gene has been inserted close to the gene nominated by the breeder. The breeder can then make selections throughout the breeding program with the line having the marker gene and thus the single gene trait.

This work can be extended over time to enable polygenically determined traits to be selected. When different selectable or visual markers can be inserted into elite germplasm, then the combination of markers that cosegregate with a phenotypic character can be recognized and used in subsequent selection for the characters.

IV. ANIMAL DISEASES AND LIVESTOCK PRODUCTIVITY

Short-term programs

Diagnosis. Because the most commonly used diagnostic techniques are imprecise and cumbersome, monoclonal antibody technology and recombinant DNA technology should be used to devise the requisite probes. Hybridization should be visualized with nonradioactive means.

Vaccine development. Where conventional means of identification and production of relevant antigenes for vaccines cannot be applied because of difficulties in antigen identification or insufficient amounts of antigens available by conventional technologies, recombinant DNA, and synthetic and monoclonal antibody technologies offer unprecedented opportunities for solving these problems. Careful selection of recombinant DNA technologies can result in improved antigen with enhanced immunological activity.

Characterizing elements of the immune system. An understanding of the role of innate or acquired resistance to diseases in livestock requires characterization of elements of the immune system and an understanding of their roles in innate or acquired immunity. Although such analyses are more easily carried out in inbred populations such as the mouse, definition of the mouse immune system has taken more than 20 years and much labor and is not yet complete.

The economically important species of domestic livestock are, of course, outbred populations that are little studied in

this regard. Monoclonal and recombinant DNA technology can increase the rate of acquisition of knowledge of the immune system of domestic livestock. Monoclonal antibodies are being used to identify relevant cell surface markers, antibody classes, and antibody ideotypes.

Recombinant DNA probes generated to identify products of the immune system in mouse and man species can be used to identify analogues in some species of domestic livestock. Biologically active molecules such as interferon and interleukin 11, which are well characterized in mouse and man, can be sought in the immune systems of domestic livestock by the use of comparative biotechnology techniques. Such active molecules may also prove to be important adjuvants in vaccine administration and in therapy.

Long-term programs

Specific gene transfer. The potential for increasing productivity and disease resistance in domestic livestock by adaptive gene transfer should not be overlooked. This will require

- identification of gene products relevant to productivity or disease resistance,
- subsequent identification of genes responsible for such products,
- isolation and characterization of such genes,
- eventual functional transfer of such genes into a new host genome, and
- expansion of the population carrying the newly introduced genes by embryo transfer and embryo splitting.

Mammalian gene transfer. Mammalian gene transfer is currently possible in mice and rats. Clone viral, human, and hybrid genes can now be directly transferred into embryos by microinjection. Integration into the germline is obtained in 20-30% of cases. Gene expression has been obtained in several cases. The use of hybrid genes with correct signals should improve the percentages. Embryo transfer is now possible in most livestock species. The use of frozen embryos allows genetic manipulation in existing laboratories instead of on-site.

In cases where genes have important tissue and time specificities, obtaining expression at the proper time and in the proper tissue will be difficult because control of gene expression during development is not yet understood. Gene expression has generally been very low.

Another major problem is the identification, isolation, and construction of genes to be transferred.

Recommendations

Because inbred lines are largely unavailable in most livestock, genes should be introduced in a controlled manner.

This applies to genes transferred within a species as well as to genes from other natural sources and hybrid genes. IARCs must first identify the traits they wish to transfer into a new population and then by themselves or in collaboration with other laboratories, identify the gene products responsible for such traits. Identification of such products will subsequently allow identification of relevant genes.

Cloning and construction of hybrid genes can be carried out in collaborative studies with advanced laboratories. Subsequent gene insertion and embryo transfer can be carried out at the IARCs. This requires IARC competence in embryo transfer technologies.

Role of IARCs in improved control of animal diseases and increased productivity of domestic livestock

Animal diseases. IARCs are situated in areas affected by important animal diseases and provide a unique opportunity for studying such diseases. Veterinary regulations in developed countries preclude the introduction of important disease agents into such countries and effectively prevent their laboratories from studying these diseases. Where certain infectious agents such as trypanosomes can be studied in laboratory rodents, developed countries permit experimentation on infections in the natural hosts (in this case domestic livestock) only under very sophisticated conditions. The IARCs therefore can provide a focal point where the expertise of the IARCs and the expertise from the developed countries can be used to investigate diseases important in less developed countries (LDCs).

Animal production. The productivity of domestic livestock under the widely different conditions of animal husbandry in the LDCs can be assessed only in those countries. Expertise available in the developed countries can be utilized in solving the problems of the LDCs through the IARCs, which have the expertise to define the problems requiring solutions, can select and modify methods as required, and can transfer the technology to national programs.

V. BIOCONVERSION AND BY-PRODUCTS UTILIZATION

Future research by IARCs should stress the use of the whole plant for maximum return to the producer. Useful products should be developed for the parts of the plant other than the grain or the fruit. Much of the effort, however, may have to be outside of the agricultural sector because the proposed products in most cases will need extensive market development.

Problem areas

Certain general considerations are important in planning and implementing priority research programs in bioconversion and by-products utilization by IARCs.

Technology and information transfer. Emphasis should be on low-investment, relatively simple technology that can be implemented at an industrial level in LDCs, except for a few processes such as composting and fermentative upgrading of wastes to fodder. Processes at the farm level should be assigned a low priority because sufficient research and development are already underway throughout the world. Large-scale, complicated technology should be studied in the developed nations that can attract capital investment for such projects. However, some methods of bioconversion are suited to agribusiness and could be integrated into regional agriculture.

A catalog of institutions doing strong research on specific fields of biotechnology should be prepared. It should include the institutions in each country, names of scientists, fields of interest in biotechnology, crops or products of interest, and national programs in biotechnology related to IARC concerns. Such information will facilitate interchange of ideas, experimental materials, and possibly scientists.

A mechanism within IARCs must be provided to allow them to subcontract projects not within their capabilities, but are suited to established groups or laboratories.

Close links with animal scientists must be established when assessing the nutritive values of animal feed from bioconversion.

Institutional linkages. Coverage of new developments should be thorough and IARCs should establish contact with organizations and institutions that have new technology. Scientific exchanges and collaboration should be considered, samples should be requested, and particularly attractive proprietary technology should be purchased or licensed. Topics for collaboration include

- local testing of devices or processes such as wood chippers for very soft or very hard biomasses,
- testing products of bioconversion within the agricultural sector, and
- research at IARCs to complement projects of a proprietary nature (perhaps in exchange for a license).

IARCs must establish administrative linkage to promote the exchange of scientists among the centers, to allow optimum use of personnel resources, and to focus on high-priority biotechnology research.

Research such as microbial improvement using recombinant DNA and other sophisticated biochemical techniques may be a matter for collaborative research between institutions.

Inventory of feedstocks. High priority should be given to the inventory and assessment of feedstock availability. A systematic and updated inventory of agricultural residues that can be converted to higher valued products should be undertaken on a country or regional basis. Present uses of these residues should be monitored and their current value estimated. This will help in assessing the costs of diverting them to other uses.

Recomendations

1. High priority should be given to processes for microbial protein. Separation of biomass into fractions based on cellulose, hemicellulose, and lignin will provide novel feedstocks for bioconversion. Compared to existing feedstocks, these new materials will be more easily fermented, will be much more uniform, and will be essentially homogeneous. Furthermore, the protein can be used at the farm level to close the marketing loop so that a complex interplay with other sectors of the economy will be avoided. Specific activities required are
   - search for organisms that thrive on the new materials,
   - development of low-technology bioconversion,
   - nutritional testing of the products,
   - genetic improvement of amino acid patterns and food quality, and
   - genetic changes in the microorganism to aid processing, for example vigor to resist contamination and strains that can withstand higher temperature.

2. There should be additional research and development on enzymatic hydrolysis of cellulose. Such studies increase fundamental understanding of biochemistry. That is important regardless of whether there are separate or simultaneous steps for processing cellulose. Specific projects should be
   - matching organisms and their enzymes to the individual materials (processing of biomass alters cellulose so that a different mix of enzymatic activities may be more effective),
   - genetically improving organisms to get fast-growing organisms because the present fermentations to produce cellulose enzymes are complex technology (rugged, hardy organisms raise the possibility of low-technology fermentation), and
   - modifying pretreatment to facilitate enzymatic hydrolysis.

High priority should be given to direct fermentation of cellulose. Fractionated biomass should be easier to process because of homogeneity, absence of inhibitors, and removal of physical barriers to enzymatic action. Direct fermentation avoids the crippling expenses of separate fermentation to produce the enzyme and a separate hydrolysis step. Suggested products are microbial protein, solvents such as ethanol, acetone/butanol, and specialty chemicals such as citric acid, gluconic acid, and 2,3-butanediol.

3. New uses for lignin in the agricultural. sector should be sought. This major product of biomass refining will be available in relatively large quantities and has a wide

range of potential uses. Lignin could be used as an adhesive in waferboard, chipboard, and lumber composites; as a coating or substitute for or extender of asphalt; and as an intermediate in the manufacture of plastics, insecticides, copolymers, and the like.

4. Medium priority should be assigned to bioconversion of common carbohydrate materials such as molasses. Although such research has a long history, the economic impact of alternative uses in LDCs in times of surplus is so great that continued search for new processes is fully justified. Furthermore, the processes would probably be applicable to carbohydrate materials derived from lignocellulosic feedstocks. Suggested topics are
   - novel designs of bioreactors, especially those with high rates, immobilized organisms or enzymes, for low technology;
   - fermentation to polymers for adhesives, thickening agents, and biodegradable packaging materials; and
   - development of fermented foods such as tempeh.

VI. GENERAL RECOMMENDATIONS FOR IARCs

Intensification of cooperative research

The tempo of progress in harnessing the knowledge and techniques emerging from biotechnology research for the improvement of Third World agriculture can be greatly accelerated if IARCs will expand and deepen collaborative linkages between their scientists and scientists of appropriate institutions in developed and developing countries. These linkages could be organized along the following lines:

1. Twinning of institutions. An IARC and an appropriate advanced laboratory working in the same field of research could develop a joint research program to solve specific applied problems. This is already being done by several IARCs with great benefit.
2. Trilateral linkages. An IARC could serve as a bridge between institutions in developing and developed countries, and thus promote more active collaboration in frontier areas of science relevant to food and agriculture.
3. Scientist-to-scientist interaction. Individual scientists in IARCs can be encouraged and assisted to work with peers in biotechnology research in appropriate universities and research institutes.
4. Networks. Cooperative networks can be organized in research in which progress can be fast if scientists in different countries working on a similar problem can jointly plan and implement a research program. For example, in material exhibiting somaclonal

variation for salinity tolerance in tissue culture, screening could be done jointly by scientists from regions where soil salinity is a major constraint to crop production.

5. Exchange of scientists. IARCs should promote greater exchange of scientists working in a common area of concern. Visiting scientists and postdoctoral fellows should be invited to fill critical gaps in the internal competence of IARCs. One example would be the use of diagnostic tests with DNA probes and monoclonal antibodies, which can be immediately adapted to suit specific needs.

Institutional arrangements

1. Every 3 yr, IARCs should organize an intercenter seminar in biotechnology along the lines of the one held at IRRI in April 1984. This will enable them to both measure the progress made and identify the problems faced in taking advantage of new research tools.
2. Each IARC that is interested in fostering research on biotechnology applications to solve specific problems and to advance the frontiers of crop and livestock production and productivity should set up a Biotechnology Working Group. The group should be organized at the institute level and consist of scientists working in biotechnology and representatives of the administration. For this area of research to receive the attention it deserves, the group should be chaired by the director general of the institute. Scientists from collaborating institutions could be invited to serve on the working group. Where appropriate, eminent scientists engaged in research areas of interest to the IARC could also be requested to serve on the group.
3. CGIAR should include an outstanding molecular or cell biologist as a member of TAC. Such scientists should also be invited to serve on the boards of trustees of IARCs in which biotechnology applications hold considerable promise for the speedy solution of complex problems.
4. Training courses in biotechnology for the benefit of developing countries should become an integral part of the training activities of IARCs. Guest lecturers could be invited to assist in organizing such training programs. IARCs should also provide adequate funds in their budgets both for training their own staff and for providing the necessary infrastructure for research and education. Joint training programs with appropriate advanced institutions need to be fostered. Training could also be organized jointly with relevant regional and international centers such as the international genetic engineering centers in New Delhi and Trieste that are sponsored by the United Nations Industrial Development Organization, and the FAO-IAEA

(International Atomic Energy Agency) biotechnology laboratory in Vienna.

Forward edge

Each IARC should develop a 5-yr plan to assimilate the new developments in biotechnology. The program should aid IARC personnel in acquiring an appreciation of the newly developing areas of science 2nd in gaining a sophisticated understanding and working knowledge of biotechnology. This will also help them understand the potentials and limitations of the new tools and to avoid arousing premature expectations. IARC scientists will then be able to combine in a meaningful manner conventional and new techniques, and to refrain from worshipping a technique just because it is new and has become fashionable. The CGIAR and TAC should continuously analyze the implications of patent rights and regulations from the view of ensuring free exchange of knowledge and material relevant to Third World agriculture.

PARTICIPANTS

Ahmed, K.A.
Genetics Department
Faculty of Agriculture
University of Cairo
Giza, Egypt

Alton, A.K.
Molecular Biology Laboratory
BIOTECH, College, Laguna
Philippines

Ancora, G.
ENEA-Laboratory on Biological
Advanced Methodologies
Viale Regina Margherita 125
00198 Rome, Italy

App, A.A.
Agricultural Sciences
The Rockefeller Foundation
1133 Avenue of the Americas
New York, NY 10036
USA

Barba, R.C.
Institute of Plant Breeding
College, Laguna
Philippines

Barton, J.H.
1340 Harwalt Drive
Los Altos, CA 94022
USA

Bogorad, L.
The Biological Laboratories
Harvard University
Cambridge, MA 02138
USA

Boulter, D.
Department of Botany
University of Durham
Durham, DH1 3LE
England

Bozzini, A.
Plant Production and
Protection Division
FAO of the UN
Via delle Terme di Caracalla
00100 Rome, Italy

Brady, N.C.
Bureau of Science and
Technology
Room 4942 NS
A.I.D.
Department of State
Washington, DC 20523
USA

Bungay, H.R.
Department of Chemical and
Environmental Engineering
Rensselaer Polytechnic
Institute
Troy, NY 12181
USA

Buttel, F.
Department of Rural Sociology
Cornell University
Ithaca, NY 14853
USA

Callow, J.
Department of Plant Biology
University of Birmingham
P.O. Box 363
Birmingham B15 2TT
U. K.

Cantrell, R.
Maize Program
CIMENT
Apartado Postal 6-641
Londres 40, Mexico 6, D.F.

Chopra, V.L.
International Genetics
Federation
Division of Genetics
129-Nuclear Research Laboratory
Indian Agricultural Research
Institute
New Delhi 110012
India

Cocking, E.C.
Department of Botany
University of Nottingham
University Park
Nottingham NG7 2RD
U. K.

Coffman, W.R.
Department of Plant Breeding
and Biometry
Cornell University
Ithaca, NY 14853
USA

Crouch, R.A.
Apartado 77-2
Santo Domingo
Dominican Republic

Curtis, B.
CIMMYT
Apartado Postal 6-641
Londres 40, Mexico 6, D.F.

Doyle, J.J.
ILRAD
P.O. Box 30709
Nairobi, Kenya

Dublin, P.
In Vitro Culture Laboratory
GERDAT
42, Rue Scheffer
75116 Paris, France

Dykes, T.
Department of Botany
Colorado State University
Fort Collins, CO 80523
USA

Enriquez, G.L.
Department of Zoology
University of the Philippines
Diliman, Quezon City
Philippines

Erskine, W.
ICARDA
P.O. Box 5466
Aleppo, Syria

Flavell, R.
Plant Breeding Institute
Mari's Lane, Trumpington
Cambridge CB2 2LQ
U. K.

Frederick, L.R.
Office of Agriculture
Bureau of Science and
Technology
USAID
Washington, DC 20523
USA

Friedrichsen
GTZ
D-6236 Eschborn 1
Postfach 51 80
Dag-Hammarskjold-Weg 1
Federal Republic of Germany

Gerlach, W.
Division of Plant Industry
CSIRO
G.P.O. Box 1600
Canberra, A.C.T. 2601
Australia

Hahn, S.K.
IITA
Oyo Road, PMB 5320
Ibadan, Nigeria

Goldsby, R.
Hybridoma Center
Amherst College
Amherst, MA 01003
USA

Halos, S.
Forest Research Institute
College, Laguna
Philippines

Hu Han
Institute of Genetics
Academic Sinica
Beijing, China

Harahap, Z.
AARD
Jalan Ragunan 29
Pasar Minggu, Jakarta Selatan
Indonesia

Hulse, J.H.
Research Programs
IDRC
P.O. Box 8500
Ottawa, Canada K1G 3H9

Iwanaga, M.
CTP
Apartado 5969
Lima, Peru

Jain, H.K.
ISNAR
P.O. Box 93375
2509 AJ The Hague
The Netherlands

James, C.
CIMMYT
Apartado Postal 6-641
Londres 40, Mexico 6, D.F.

Janssen, W.
USAID Mission
India

Javier, E.L.
Department of Agronomy
UPLB, College, Laguna
Philippines

Javier, E.Q.
UPLB, College, Laguna
Philippines

Jewell, D.
CIMMYT
Apartado Postal 6-641
Londres 40, Mexico 6, D.F.

Kauffman, H.E.
INTSOY
College of Agriculture
University of Illinois at
Urbana-Champaign
113 Mumford Hall
1301 West Gregory Drive
Urbana, TL 61801
USA

Kokke, R.
Development Studies Division
The United Nations University
Toho Seimei Bldg.
15-1, Shibuya 2-chome
Shibuya-ku, Tokyo 150
Japan

Maliga, P.
Advanced Genetic Sciences, Inc.
P.O. Box 1373
Manhattan, KS 66502
USA

Manguiat, I.J.
BIOTECH
College, Laguna
Philippines

Mashler, W.T.
UNDP
1 United Nations Plaza
New York, NY 10017
USA

Meer, S.A.
American Embassy
New Delhi, India

Miles, J.W.
CIAT
Cali, Colombia

Van Montagu, M.
Rijksuniversiteit Gent
Laboratorium Genetika
K.L. Ledeganckstraat 35
R-9000 Gent (Belgium)

Morales, C.L.
Philippine Sugar Commission
La Granja, Negros Occidental
Philippines

Moss, J.P.
ICRISAT
Patancheru P.O.
Pin 502 324, A.P.
India

Mujeeb-Kazi, A.
CIMMYT
Apartado Postal 6-641
Londres 40, Mexico 6, D.F.

Murashige, T.
Department of Botany and
Plant Sciences
University of California
Riverside, CA 92521
USA

Nabors, M.
Tissue Culture for Crop Plants
Department of Botany and
Plant Pathology
Colorado State University
Fort Collins, CO 80523
USA

Nantulya, V.M.
ILRAD
P.O. Box 30709
Nairobi, Kenya

Ng, S.Y.
IITA
Oyo Road, PMB 5320
Ibadan, Nigeria

Nitayangkura, S.
Department of Biology
Mahidol University
Thailand

Nyindo, M.B.A.
ICIPE
P.O. Box 70772
Nairobi, Kenya

Padolina, W.
BIOTECH
College, Laguna
Philippines

Patena, L.F.
Tissue Culture Laboratory
Institute of Plant Breeding
College, Laguna
Philippines

Plucknett, D.
CGIAR
1818 H. St., N.W.
Washington, DC 20433
USA

Quisumbing, E.C.
Ministry of Agriculture
Quezon City, Philippines

Qureshi, A.
PARC
L — 13,Almarkaz, F-7
Post Box 1031
Islamabad, Pakistan

Raghavan, V.
Department of Botany
The Ohio State Unviersity
Columbus, OH 43210

Ramachandran, S.
National Biotechnology Board
Technology Bhavan
New Mehrauli Road
New Delhi 110016
India

Rifai, M.A.
National Biological Institute-
LIPI
Jalan Raya Juanda 18
Bogor, Indonesia

Rimando, T.J.
Department of Horticulture
UPLB, College, Laguna
Philippines

Riley, R.
Agricultural Research Council
160 Great Portland St.
London W1N 6DT
U. K.

Roberts-Pichette, P.
Technical Advisory Committee
FAO of the UN
Via delle Terme di Caracalla
00100 Rome, Italy

Roca, W.
CIAT
Cali, Colombia

del Rosario, A.
Department of Horticulture
UPLB, College, Laguna
Philippines

Rush, M.C.
Department of Plant Pathology
and Crop Physiology
302 Life Sciences Building
Louisiana State University
Agricultural Center
Baton Rouge, LA 70803-1720
USA

Rutger, J.N.
Crops Pathology and Genetics
Agronomy and Range Science
University of California
Davis, CA 95616
USA

Rutter, W.J.
School of Medicine
Department of Biochemistry
and Biophysics
University of California
San Francisco, CA 94143
USA

Salazar, L.F.
CIP
P.O. Box 5969
Lima, Peru

Sastri, D.C.
ICRISAT
Patancheru P.O. 502 324,
A.P., India

Sattar, M.A.
Institute of Nuclear
Agriculture
Mymensingh, Bangladesh

Schmidt, B.L.
Department of Agronomy
Ohio State University
Columbus, OH 43210
USA

Scowcroft, W.
Division of Plant Industry
CSIRO
G.P.O. Box 1600
Canberra, A.c.T. 2601
Australia

Shiga, T.
Department of Cell Biology
National Institute of
Agrobiological Resources
Yatabe, Tsukuba, Ibaraki 305
Japan

Sigurbjornsson, B.
Joint FAO/IAEA Division of
Isotope and Radiation
Applications of Atomic
Energy for Food and
Agricultural Development
Nagramerstrasse 5, P.O. Box 100
A-1400 Vienna, Austria

Srinives, P.
Department of Agronomy
Kasetsart University
Thailand

Sudarisman
AARD
Jalan Ragunan 29
Pasar Minggu, Jakarta Selatan
Indonesia

Szabados, L.
CIAT
Cali, Colombia

Tahadi, S.
AARD
Jalan Ragunan 29
Pasar Minggu, Jakarta Selatan
Indonesia

Tahir, M.
ICARDA
P.O. Box 5466
Aleppo, Syria

Thi Thanh Tuyen, N.
VISCA
Leyte, Philippines

Toenniessen, G.H.
The Rockefeller Foundation
1133 Avenue of the Americas
New York, NY 10036
USA

Torres, J.V.
Instituto Nacional de Investigaciones Agrarias
Subdireccion de Relaciones Cientificas
Jose Abasca, 56, Madrid-3
Spain

Uyenco, F.R.
Department of Botany
University of the Philippines
Diliman, Quezon City
Philippines

Valmayor, H.
Department of Horticulture
UPLB, College, Laguna
Philippines

Van Uyen, N.
Institute for Experimental Biology
1 Mac Kinh Chi
Ho Chi Minh City
Vietnam

Verma, D.P.
Department of Biology
Genetic Manipulation Research Group
McGill University
1205 Avenue Docteur Penfield
Montreal, P.Q. Canada H3A 1B1

Villareal, R.
Institute of Plant Breeding
College, Laguna
Philippines

Wenzel, G.
Biologische Bundesanstalt fur Land-Und Fortswirtschaft
Institut fur Resistenzgenetik
Graf-Seinsheim-StraBe 23
8059 Bockhorn
Federal Republic of Germany

Withers, L.A.
Department of Agriculture and Horticulture
University of Nottingham
School of Agriculture
Sutton, Bonington, Lougborough
Leics, LE12 5RD
U. K.

Zamora, A.B.
Tissue Culture Laboratory
Institute of Plant Breeding
College, Laguna
Philippines

IRRI

Abrigo, E.
Tissue Culture Facility

Aldemita, R.
Tissue Culture Facility

Bajet, N.
Plant Pathology

Brar, D.S.
Plant Physiology

Cabuslay, G.
Plant Physiology

Chang, T.T.
International Rice Germplasm Center

Coronel, V.
Plant Physiology

Encarnacion, G.
Tissue Culture Facility

Greenland, D.J.
Deputy Director General

Hibino, H.
Plant Pathology

Juliano, B.O.
Cereal Chemistry

Khush, G.S.
Plant Breeding

Ladha, J.K.
Soil Microbiology

Ling, D.H.
Plant Physiology

Magaling, L.
Tissue Culture Facility

Novero, A.
Plant Physiology

Ohkawara, R.
Plant Physiology

Parao, F.
Plant Physiology

Pathak, M.D.
Research and Training

Salcedo, E.
Plant Physiology

Swaminathan, M.S.
Director General

Torrizo, L.
Tissue Culture Facility

Vergara, B.S.
Plant Physiology

Virmani, S.S.
Plant Breeding

Wanatabe, I.
Soil Microbiology

Wood, D.M.
Training and Technology Transfer

Zapata, F.J.
Tissue Culture Facility

Zhang, Z.H.
Tissue Culture Facility