

RICE TISSUE CULTURE PLANNING CONFERENCE



INTERNATIONAL RICE RESEARCH INSTITUTE

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1982

INTERNATIONAL RICE RESEARCH INSTITUTE

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FOREWORD

Tissue culture can be used as a plant breeding technique that helps shorten the time needed to develop a true-breeding variety and that enables breeders to make wide crosses, such as the wheat-and-rye cross that produced triticale.

This volume contains issue papers presented at the Rice Tissue Culture Planning Conference, held at the International Rice Research Institute 28-30 April 1980. The conference brought together both applied and basic researchers in plant genetics to plan the cooperative approaches suggested in the conference report.

The authors of these papers explore the potential benefits of applying tissue culture techniques to rice breeding and emphasize the problems that must first be solved. This report particularly addresses the needs for collaborative and cooperative work to incorporate this new plant breeding technique into programs of rice varietal improvement.

The IRRI committee that organized the conference included Dr. M. D. Pathak, Dr. G. S. Khush, Dr. M. C. Rush, and Dr. S. Yoshida. Dr. LaRue Pollard, IRRI visiting science editor, assisted by Ms. Corazon V. Mendoza, assistant editor, and Ms. Emerita P. Cervantes, editorial assistant, edited the proceedings.

Marcos R. Vega
Acting director general

OPENING REMARKS

Workshops and conferences at the International Rice Research Institute (IRRI) have two basic objectives: to bring participants up to date on new development and progress in rice research and, more important, to set future research goals.

We jokingly refer to the first objective as the "show-and-tell" portion of our conferences — informing each other about what we *have* done. We refer to the second objective as the "so what?" sessions — discussing what we *should* be doing, considering what is known.

At this workshop, we hope you contribute to that second objective with specific suggestions on what we should be doing in applying tissue culture to the improvement of rice.

Tissue culture is new. IRRI, while still using traditional plant breeding techniques, now is examining the potential of tissue and pollen culture in speeding the development of improved rice varieties.

Some progress has been made in the tissue-culture propagation of new varieties in several cereal crops. A great deal of work has been done in China on the use of pollen culture to develop improved rice varieties. Some work also has been done in Japan, India, USA, Australia, and other countries. We hope that at this workshop we can share the general information now available on tissue culture.

But we want to take no more time than is absolutely necessary to talk of what is already known in tissue culture. Instead, we want to focus on what should be done next — not just what IRRI should do, but what all of us concerned with rice should do.

Other workshops on other topics have ended with suggestions for the establishment of a network of cooperating scientists in Asia, including cooperators from outside the region. If such a network seems feasible to you, IRRI could bring such a group of collaborating scientists together and could coordinate network activities. Our support could include planning and coordinating survey tours, through which you could visit and observe each other's research programs. We often do this in the International Rice Testing Program. For example, a group of scientists concerned with blast visit research and screening programs in several countries. Then we hold a "so what" conference focused on their observations to develop recommendations for changes.

You can help us decide what should be done by giving us your advice on how we can best carry out research using the techniques about which you are knowledgeable.

We must keep in mind that we want to see scientific expertise, regardless of its source, focused on rice. Rice is by far the most significant crop for the world's low-income countries. The full resources of the best scientists around the world are needed to improve rice yields. In that way, we can improve the well-being of hundreds of millions of the world's poorest farmers and urban consumers.

Thank you for being with us.

Dr. N. C. Brady
Director-General

THE GENETIC EVALUATION AND UTILIZATION PROGRAM AT IRRI

M. D. PATHAK

Rice is the staple food of over half the world's population. Classified primarily as a tropical and subtropical crop, it is cultivated as far north as 49° and as far south as 35° and from sea level to an altitude of 3,000 m. It is grown under diverse water regimes—as a dryland crop, where there is no standing water and rains are the sole source of moisture, and in wetland conditions, where water derived from rain or irrigation systems is impounded in the field. On slopes it is cultivated in terraces, and in valleys or other low-lying areas it is grown in several feet of standing water. It is grown under a wide range of temperature, soil, pest, and disease conditions.

Through 5,000 years of cultivation, rice plants have adapted to these diverse conditions. However, most varieties possessing specialized attributes are tall and leafy and lodge easily when grown under improved agronomic conditions. Their potential for effectively utilizing natural soil fertility or added fertilizers is limited. That discourages farmers from adopting improved agronomic practices. As a consequence, rice yields in the tropics and subtropics range only between 1.5 and 2 t/ha. In temperate areas, where short-statured varieties are more often grown and where improved agronomic practices are followed, yields range between 4 and 6 t/ha.

Scientists in the early 1960s realized these weaknesses of the typical indica varieties. They changed the architecture of the rice plant to a short, stiff-strawed, heavy-tillering plant with moderately upright leaves and insensitivity to changes in day length. This plant type was developed to make more efficient use of sunlight,

water, and nutrients and to reduce the long growth period needed by many varieties.

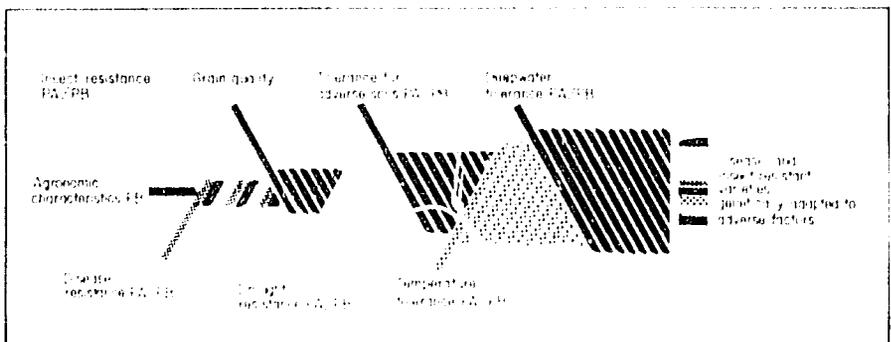
The short, stiff-strawed, nonlodging, nitrogen-responsive, and nonphotoperiod-sensitive varieties, exemplified by IR8, released in 1966, have demonstrated that high yields of rice can be obtained in the tropics and subtropics. Several areas have effected substantial increases in rice production.

Many rice varieties with improved characteristics have been released in the developing countries since IR8. However, much of the rice is grown under such adverse conditions as high incidence of pests and diseases, drought inundation conditions, problem soils, and suboptimum temperatures. Most of the newer rice varieties are not adequately adapted to these stresses. As a consequence, their cultivation has expanded to only about 25-30% of the total rice area in the zones that are apparently better suited to these varieties. Even in those areas, pest and disease problems and uncertainty of normal rainfall conditions cause considerable instability in yields.

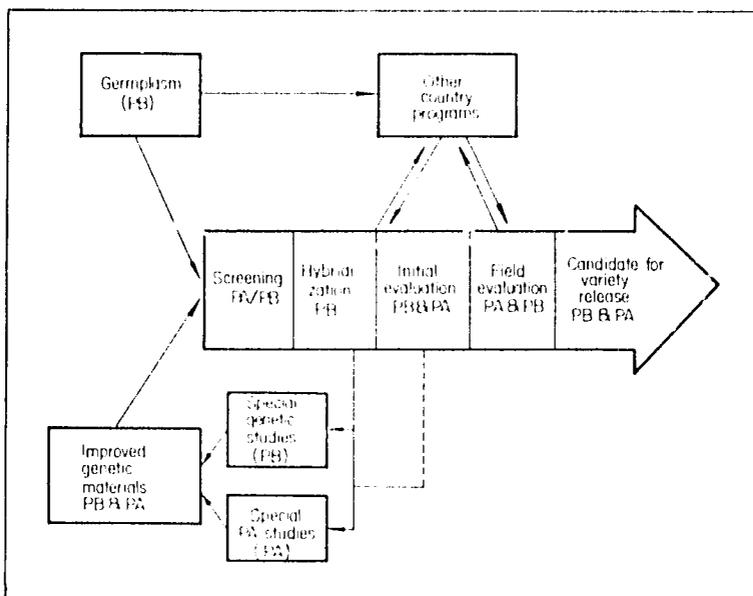
THE GEU PROGRAM

To further expand the use of modern high-yielding varieties and to provide stability at higher yields in current areas of production, it is essential to incorporate into modern varieties genetic resistance to or tolerance for the various adverse conditions in which they are grown. To accomplish that, scientists representing different disciplines must coordinate their efforts. Although IRRI scientists have always worked together in such studies, the interdisciplinary team approach was extended to a formal Genetic Evaluation and Utilization (GEU) program on 19 November 1973. This program embodies an Institute-wide approach to evaluating and utilizing the genetic potential of the rice plant. It is subdivided into various problem areas. An interdisciplinary team consisting of a plant breeder (PB) and one or more problem-area scientists (PA) provides leadership for each problem area (Fig. 1). Team objectives and general operational patterns are:

- planning and review, with continuing coordination to improve rice varieties suitable to different agroclimatic conditions;



1. Plant breeders (PB) and problem area scientists (PA) to gather incorporate genetic components



2. General operational procedure of the Genetic Evaluation and Utilization (GEU) program. PB = plant breeders, PA = problem-area scientists.

- developing methodology;
- maintaining and cataloging the world's rice germplasm;
- screening the world collection to identify parent materials;
- understanding the nature and causes of resistance to and tolerance for various adverse conditions;
- developing biotypes or races of pests capable of survival on resistant plants;
- studying the plant's rice resistance to or tolerance for various problems;
- developing improved sources of resistance to or tolerance for problems;
- producing large-scale hybridization for use by scientists at IRRI and elsewhere;
- screening hybrid materials;
- implementing field evaluation and selection of breeding lines, including yield trials;
- evaluating selected collections and breeding lines of international nurseries;
- disseminating genetic materials;
- developing collaborative experiments with national scientists to identify materials with broad adaptability.

Plant breeders continue to be the common core of the Institute's breeding program. Problem-area scientists contribute primarily through screening and testing and through special host-parasite studies (Fig. 2). The system allows integration of the inputs of various problem-area specialists. And the multidiscipline and multilocation testings act as safeguards against the release of varieties more susceptible to certain problems than existing rices.

GENERAL SCOPE OF GEM PROBLEM AREAS

Germplasm collection and maintenance

Rice germplasm is collected on a worldwide basis to increase seed stocks, characterize and preserve samples, catalog accessions, identify and remove duplicates, provide duplicate storage to other seed banks, rejuvenate seed stocks, and provide seed to researchers throughout the world.

A worldwide germplasm collection is maintained in a modern germplasm bank at IRRI. The capacity of the IRRI facility is 100,000 accessions each for long-, medium-, and short-term storage. Long-term storage in vacuum-sealed cans at -10°C should maintain seed viability for about 100 years. Medium-term storage, at -4°C , is expected to maintain viability for 25 years. Short-term storage of working collections, at 20°C , should maintain viability for 5 years. Samples from the collection are available to agencies in all countries on request. Duplicate samples of all accessions are in storage at the U.S. National Seed Laboratory at Fort Collins, Colorado, as an added precaution.

The collection, conservation, and cataloging of germplasm started in 1962. The bank now has more than 55,000 accessions, which include cultivated varieties, primitive varieties, and species collected from all parts of the rice-growing world. A catalog summarizing the information available on 8,628 accessions was published in 1970. Records on 38 attributes for 40,768 accessions have been completed (Chang 1979). This information is being computerized.

Agronomic characteristics

Agronomic characteristics of the improved rice plant that ensure productivity and adaptability under varying cultural and ecological conditions are studied. The main characteristics investigated are: dwarf and semidwarf statures; high tillering capacity; relatively short, dark-green, and upright leaves; early vegetative vigor; appropriate growth durations; photoperiod response; threshability without grain shattering; and seed dormancy.

Grain quality. Grain size and shape, milling quality and recovery ability of head rice, absence of white areas, appropriate amylose content (waxiness, or low, medium, or high amylose content), gel consistency, and cooking and eating qualities are studied. Physicochemical qualities preferred by consumers and the effect of environmental conditions on these qualities are described. Simple, rapid, reliable tests for these characteristics are developed. The effects of various preharvest and postharvest operations on grain quality (e.g., time of harvest, parboiling) are determined.

Disease resistance. Techniques are standardized and the rice germplasm is evaluated for resistance to common diseases. The nature and causes, inheritance, and sources of resistance are studied. Stability of resistance (horizontal resistance), international disease nurseries to determine pathogen races, and the effect of environmental factors on resistance are investigated.

Insect resistance. Sources of resistance to common insect pests in the rice germplasm collection are identified. The nature (nonpreference, antibiosis, tolerance) and cause (biophysical, biochemical) of varietal resistance, insect biotypes, and genetics

of resistance to current insect populations and to new biotypes are determined. Isogenic and multigenic resistant lines are developed, as are lines resistant to several insect pest species.

Protein content. Improving the protein content of milled rice while maintaining grain yield, grain quality, and other desirable characteristics is attempted. The effects of genotypes and environment and their interactions on protein content are determined. The effect of protein content on grain quality and the inheritance of protein content are studied. At present, studies on the protein content of rice grain are receiving low priority and are limited to the advanced breeding lines with potentials for becoming commercial varieties.

Drought resistance. Varieties are screened for drought resistance at different stages of plant growth. The physiological base of drought escape, avoidance, tolerance, and recovery in different varieties is determined. The effect of drought on plants is studied. The role of root elongation, stomatal spacing, and rolling of leaves and the presence of a waxy layer on leaf surfaces in drought resistance is studied.

Drought-resistant lines are bred and selected lines and breeding materials are evaluated under different agroclimatic conditions. International dryland rice trials are conducted.

Tolerance for adverse soil conditions. Varieties are screened for tolerance for common adverse soils. Plant characters giving tolerance for adverse soils and their inheritance are determined. The effects of agroclimatic conditions on plant tolerance for adverse soils are studied and breeding for tolerance for adverse soils is carried out.

Deepwater and flood tolerance. Factors limiting grain yields of deepwater rice are determined and an improved-plant type for deepwater rice is conceptualized. Rice varieties are screened for ability to elongate with increasing water depth and for submergence tolerance. The mechanism and inheritance of deepwater and flood tolerance are studied and lines are bred for deepwater and flood tolerance. Emphasis will be on developing semidwarf and intermediate-height varieties with elongation genes for growing in 1-5 m water and other desirable attributes, such as appropriate photoperiod sensitivity and resistance to pests, diseases, adverse soils, drought, and other common problems. Much of the work on flood and emergence tolerance is being conducted in Thailand in collaboration with Thai scientists.

Temperate tolerance. Rice varieties are screened at vegetative and reproductive stages for tolerance for high and low temperatures. The effects of nonoptimum temperatures on plant growth, sterility, and other yield components are studied. The inheritance of temperature tolerance is investigated and lines are bred for this character. A collaborative project developed with the government of South Korea facilitates mass screening in Korea of rice collections and breeding lines for cold tolerance at different stages of plant growth.

HOW GEU OPERATES

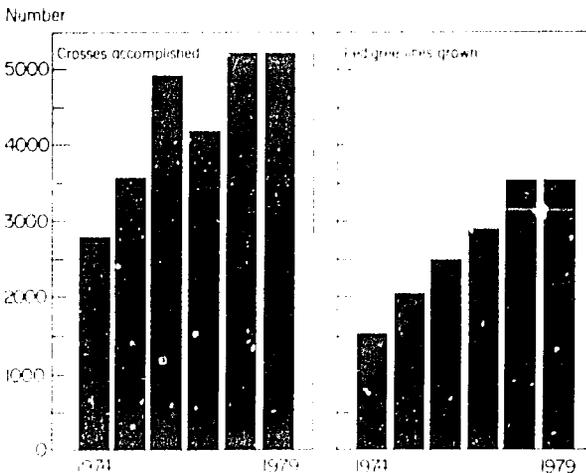
The GEU approach enables scientists of different disciplines, working as a team, to contribute their expertise to the improvement of rice. The plant breeders and geneticists maintain the rice germplasm and provide it to problem-area scientists

Table 1. Characters being incorporated in improved rice plant types.

Grain quality	High protein content
Disease resistance	Drought resistance
Blast	Adverse soil tolerance
Tungro virus	Salinity
Grassy stunt virus	Arid areas
Ragged stunt virus	Coastal areas
Bacterial blight	Zinc deficiency
Bacterial streak	Iron toxicity
Sheath blight	Aerobic soils
Insect resistance	Iron deficiency
Striped borer	Manganese toxicity
Yellow borer	Aluminum toxicity
Brown planthopper	Submergence tolerance
Green leafhopper	Deepwater and flood tolerance
Whitebacked planthopper	Temperature tolerance
Zigzag leafhopper	
Whorl maggot	
Gall midge	

(such as plant pathologists, agronomists, entomologists, soil chemists) for screening against appropriate problems. The screening primarily is done by problem-area scientists, but in close collaboration with plant breeders. All hybridization work is centralized and is done by breeders. Each cross is given a number and the list of crosses is available to all interested scientists upon request. Subsequent screening of breeding materials and specialized studies, such as those on the genetics of various attributes, pests, and pathogen races, are conducted in collaborative efforts (Fig. 2).

The GEU team at IRRI is working on about 30 different attributes of the rice plant in addition to appropriate plant type (Table 1). That requires a large number of crosses, some of which are quite complex. Scientists at IRRI make about 5,000



3. Growth of the IRRI GEU Program

crosses per year, which requires the evaluation of about 50,000 progenies per year (Fig. 3). Such a massive screening requirement is met by evaluating the hybrid materials against problems for which rapid screening procedures are available, such as blast disease, bacterial leaf blight, brown planthopper, and green leafhopper. Selected lines are tested against problems for which screening procedures are slow or more complicated. Finally, they are tested in a series of field experiments at IRRI, in farmers' fields in the Philippines, and under various agroclimatic conditions in collaboration with scientists in other countries. The entire screening is conducted jointly by problem-area scientists and plant breeders.

COMPUTERIZED DATA

The appropriate handling of the diverse and large quantity of data generated by the screening and hybridization procedure is through the use of a computer. All data on the screening of parents and the hybrid materials are sent by scientists to the statisticians, who appropriately code and store them on tape. These data are available to all scientists upon request. To assist scientists in the final selection of promising breeding lines to be grown in yield trials in the field, the statisticians routinely provide computer printouts of the reaction of the test lines to the major problems against which they have been screened. However, any rice scientist may request similar information on the germplasm collection or breeding lines in which he has interest.

OPERATIONS COMMITTEE

A six-person operations committee, including plant breeders, problem-area scientists, and statisticians, coordinates the GEU program, including the crossing and screening and the associated system of data collection, recording, and reporting. The committee arranges for providing (the seed of) breeding materials to concerned scientists, for field planting, and for integrating the overall varietal improvement program.

To facilitate communication among scientists working on GEU, the entire GEU group meets on the first Tuesday of every month to review progress and to plan strategies. Scientists working on different problem areas also meet in smaller groups for similar discussions.

EARLY-GENERATION TESTING AND OTHER COLLABORATIVE EXPERIMENTS

IRRI decided in 1975 to discontinue the practice of naming varieties. That was done primarily to encourage scientists working in different regions to name the varieties best suited to their conditions. Every effort is made to provide them with materials for their breeding programs. The data obtained from these experiments are valuable; they provide information on the broad adaptability of certain rice strains, the suitability of a plant type under diverse agroclimatic conditions, and the occurrence of pest races or biotypes. Some common avenues for providing these materials are:

- Early-generation breeding materials from IRRI P₁ or F₁ seed of appropriate

hybrids is made available to interested scientists. This allows selection of the lines best adapted to various agroclimatic conditions that also possess resistance to the common pest and disease problems. Evaluation of these lines at other locations helps identify lines with broad adaptability.

- Supplying other breeding materials to scientists in national programs. Under a new scheme initiated in 1978, 10-15 rice breeders in selected national programs are invited to participate in the actual selection of breeding materials growing at IRRI. Two such groups of scientists come to IRRI each year—one in April and the other in October-November. These scientists select as many lines as they wish. The seed of the selected lines is sent to them soon after the crop is harvested.
- Collaborative experiments on selected problems. The experiments are designed to determine the stability of resistance or tolerance of select rices under different agroclimatic conditions. The differences in performance could be due to race or biotype variations in pests, chemical composition of soils, or effects of specific environmental conditions on the rice plant. Collaboration with scientists working in developed countries also is sought for detailed studies in identifying these factors.

Rapid generative advance

The GEFU rapid generation advance is an IRRI program based on the principle of exposing photoperiod-sensitive hybrid populations to appropriate day lengths to induce early flowering. It provides early-generation material to national programs on request. It requires less than 2 years to make a cross, grow F_1 plants, and produce the subsequent F_2 and F_3 bulk populations. That gives national program breeders reason to obtain stable material (F_3) from which to start screening and selection program.

Collaborative research

Collaborative research projects have these distinguishing features:

Collaborative projects are concerned with specific research goals that cannot be achieved solely by researchers at Los Baños. These projects may require environments, pests, research expertise, or facilities not present at Los Baños or in the Philippines. They include basic research through which we can gain an understanding of the processes, phenomena, and mechanisms of performance.

Collaborators are carefully selected on the basis of rigorous criteria, such as environment, facilities, staff competency, and support for this type of research. While we do not refuse to collaborate with any scientist who seeks such collaboration, we encourage collaboration with those who satisfy the more rigorous criteria.

Each year, at either the International Rice Research Conference or through some other convenient meeting or mechanism, data on collaborative experiments are reviewed and specific annual work plans are developed in consultation with overseas collaborators.

INTERNATIONAL RICE TESTING PROGRAM (IRTP)

The IRTP serves to effectively link the national and international improvement programs around the world in a testing network of select parents and improved breeding lines. IRRI plays the coordinating role by soliciting materials from various scientists and providing them, along with appropriate methodology and record books, to cooperating scientists. It also compiles and disseminates the data of these tests. Access to a wide array of breeding material helps identify elite breeding lines with broad resistance to various stresses. Screening tests for specific diseases and insects provide information on the strain and biotype variations among the insect pests and pathogens. IRTP provides an excellent testing ground to rice scientists for their own breeding materials because, during a single season, the materials can be subjected to diverse pressures in various environments.

Three broad categories of IRTP nurseries are:

1. yield trials;
2. general observational nurseries;

Table 2. Utilization of entries from 1975 trials in 1976 national programs.

Nursery ^d	Countries (no.)	Entries used in crosses (no.)	Entries (no.) promoted to		
			Station trials	State trials	National trials
Yield					
IRYN-E	6	43	35	12	3
IRYN-M	6	14	42	15	30
IURYN	4	5	12	4	12
Observational					
IRON	8	34	528	268	285
IURON	2	2	21	21	10
Screening					
Diseases:					
IRBN	5	55	23	6	10
IRSHBN	4	14	—	—	—
IRTN	4	14	47	—	—
Insects:					
IRBPHN	3	19	25	—	10
IRGMN	1	—	5	—	—
Other stresses:					
IRSATON	1	—	16	—	—
IRCTN	4	30	30	—	17
IRDWON	1	—	13	—	13

^dIRYN-E = International Rice Yield Nursery-Early; IRYN-M = IRYN-Medium; IURYN = International Upland Rice Yield Nursery; IRON = International Rice Observational Nursery; IURON = International Upland Rice Observational Nursery; IRBN = International Rice Blast Nursery; IRSHBN = International Rice Sheath Blight Nursery; IRTN = International Rice Tungro Nursery; IRBPHN = International Rice Brown Planthopper Nursery; IRGMN = International Rice Gall Midge Nursery; IRSATON = International Rice Salinity and Alkalinity Tolerance Observational Nursery; IRCTN = International Rice Cold Tolerance Nursery; and IRDWON = International Rice Deep Water Observational Nursery.

Table 3. Participants in the GEU training program from 1975 to 1980, by country and discipline.

Country	Breeders	Agronomists	Pathologists	Entomologists	Physiologists	Soil scientists	Cereal technicians	Geneticists	Bio-physicists	Total
Bangladesh	16	1	3	2	4	2	1	0	0	29
Brazil	0	1	0	0	0	0	0	0	0	1
Burma	1	8	0	0	0	0	0	0	0	9
China	7	2	3	2	2	0	0	3	1	20
Cuba	0	0	1	0	0	3	0	0	0	4
India	11	3	6	5	0	1	0	0	0	26
Indonesia	17	5	8	5	2	1	1	0	0	39
Iran	3	0	0	0	0	0	0	0	0	3
Korea	3	0	0	0	0	0	0	0	0	3
Liberia	1	0	0	0	0	0	0	0	0	1
Malaysia	4	1	0	1	0	0	0	0	0	6
Mexico	1	0	0	0	0	0	0	0	0	1
Nepal	0	1	3	1	0	0	0	0	0	5
Pakistan	5	2	2	2	2	0	0	0	0	11
Philippines	0	4	0	0	0	0	0	0	0	4
Sierra Leone	3	0	0	0	0	0	0	0	0	3
Sri Lanka	10	8	2	0	0	0	0	0	0	20
Tanzania	1	0	0	0	0	0	0	0	0	1
Thailand	17	5	0	0	0	0	0	0	0	22
Turkey	1	0	0	0	0	0	0	0	0	1
Total	101	41	28	18	8	7	2	3	1	209

3. specific stress nurseries (environments, diseases, and insects).

The yield trials include promising advanced material and help to identify varieties with wide adaptability and regional suitability. The general observation nursery, composed of several breeding lines from different sources, is subjected to diverse stresses and serves as a potential source for identifying lines with broader resistance that can either be followed up by yield tests or utilized as donors, or both. The specific stress nurseries include both breeding lines and donor varieties. These, besides helping identify potential donors for individual stresses, reveal genetic variation (biotypes and strains) in insect pests and pathogens. Table 2 lists the various nurseries tested in 1978.

Several of the entries in these nurseries have been formally named as varieties in different countries, but a large number are being used in hybridization, for crossing, or for further yield evaluations.

One of the important components of IRIP's activities is the organization of monitoring tours involving groups of rice scientists from different countries who jointly review the international nurseries and national research programs of importance in selected countries. The tours provide an informal forum for the exchange of ideas and interpretation of available data among the scientists. Their recommendations provide an effective supplement to the data from IRIP nurseries in formulating plans.

GEU training program

A formal GEU training program was initiated at IRRI in 1975 with a group of 16 trainees. This course is now offered twice a year with about 25 trainees in each group. It is a multidisciplinary and skills-oriented program that emphasizes the practical aspects of rice improvement and stresses the need for interdisciplinary cooperation. When possible, members of a team from a particular research station—a plant breeder, a pathologist, an entomologist, and representatives of other disciplines—are trained simultaneously. Team members are given a 2-week condensed course on rice production and a 3.5-month training in various theoretical and practical aspects of GEU work. They work together to plan crosses and develop breeding strategies to be implemented when they return to their countries. The training prepares scientists at the local level to utilize the products of the GEU programs and to develop or strengthen their own programs. National programs have shown tremendous enthusiasm for this training.

The numbers of breeders and problem-area scientists who have completed the training courses are shown in Table 3. A total of 209 individual scientists have been trained since 1975. Prior to 1975, many scientists received similar training at IRRI but on a less formal basis.

Liaison scientists

IRRI recently established liaison scientist offices in several parts of the rice-growing world. These offices provide a direct link with national programs and serve a coordinating role for IRRI's resources in given regions or countries. The liaison scientist also provides another invaluable link in the efficient initiation and implementation of the GEU concept in national programs.

TISSUE CULTURE RESEARCH: STATUS AND POTENTIAL

W. R. SCOWCROFT and P. J. LARKIN

Plant tissue culture is still largely an empirical science. The maintenance of sustained protoplast division and subsequent plant regeneration relies on empirical methodology. There are no substantial principles which can be applied to ensure successful regeneration of plants from cell culture.

But the compelling need to maintain and, where possible, expand crop production by the development of supplementary plant breeding technology continues to sustain a vital research interest in plant tissue culture. New and expanded genetic variability for use in plant improvement has been generated.

A HISTORICAL PERSPECTIVE

If success is defined as the ability to adapt plant species to *in vitro* culture conditions, its measure is given by a historical and quantitative perspective (Table 1). All major crop species are included among the 300 or so species for which plants have been regenerated from cell cultures. Significantly, 50% of these cases have been published since 1974.

The heightened interest in cell culture as a fundamental and applied research tool is further supported by the rapid development of protoplast technology (Table 1) and the isolation of cell culture mutants (Table 2). These developments also have

Table 1. A historical and quantitative assessment of developments in plant tissue culture (collated from Murashige 1980, Vasil et al 1979, and Thomas et al 1979).

Development	Species (no.)	Earliest report
Plant regeneration from cell culture	308	<i>Daucus carota</i> (Levine 1947)
Sustained protoplast division	67	<i>Glycine max</i> (Kuo et al 1970)
Plant regeneration from protoplast culture	30	<i>Nicotiana tabacum</i> (Takebe et al 1971)
Androgenetic plants by anther culture	65	<i>Datura innoxia</i> (Guha and Maheshwari 1966)
Somatic hybrids by protoplast fusion		
(a) Intraspecific (all <i>N. tabacum</i>)	7	<i>N. tabacum</i> (Melchers and Labib 1974)
(b) Interspecific (4 cases involve sexually incompatible species)	14	<i>N. glauca</i> + <i>N. langsdorffi</i> (Carlson et al 1972)

occurred within the 1970s, as has the expansion in research on androgenetic haploids (Table 1).

All but six of the species for which plants have been regenerated from protoplasts are members of the Solanaceae. One species of the economically important Gramineae was reported recently. Vasil and Vasil (1980), successfully regenerated protoplasts of pearl millet (*Pennisetum americanum*).

SOMATIC CELL GENETICS

The development of somatic cell genetics in plants requires genetic differences, characterized in terms of origin, mechanism, and inheritance.

Phenotypically distinct cell lines have been selected under in vitro conditions (Table 2). The spectrum of such mutant lines ranges from defined biochemical lesions to undefined gross physiological differences.

The majority of these phenotypically distinct lines can be classified only as presumptive mutants. The biochemical basis of the phenotypic change is largely undefined and the mode of inheritance, as determined by sexual transmission, is unknown.

Phenotypic diversity arising from cell culture

Apart from the phenovariants, which are a consequence of direct selection, phenotypic diversity seems to be a ubiquitous occurrence in cell culture. The diversity is reflected in altered growth regulator responses, changes in growth rate and gross morphology, and alterations in cellular constituents such as anthocyanin. These changes are associated with chromosomal alterations, but a causal relationship is difficult to prove. There is reason to suspect a genetic, or at least an epigenetic, basis

Table 2. Compounds, conditions, or natural products for which resistant, tolerant, or deficient cell lines have been selected (collated from Maliga 1978, Thomas et al 1979, and Brettel and Ingram 1979).

<i>Amino acid analogs</i>	<i>Base analogs</i>
Ethionine	Bromodeoxyuridine
5-methyl tryptophan	Azaguanine
S-2-aminoethyl-cysteine hydroxylysine	5-fluorouracil
Azetidino-2-carboxylic acid	
Hydroxyproline	<i>Pathotoxins</i>
Norleucine	<i>Helminthosporium maydis</i>
p-fluorophenylalanine	<i>Helminthosporium sacchari</i>
<i>Antibiotic drugs</i>	<i>Physiological conditions</i>
Streptomycin	NaCl
Kanamycin	Aluminum
Chloramphenicol	Chilling
Cycloheximide	
Carboxin	<i>Herbicides</i>
Aminopterin	Amitrol
Chlorate	Amulam
	Atrazine
	Propham
	2,4-D
	Picloram

for this variation.

Whatever the cause, this variation also is manifested among plants regenerated from cell culture. *Tissue culture per se appears to be an unexpectedly rich and novel source of genetic variability that already is being utilized in plant improvement.*

SOMAACLONAL VARIATION

Plants derived from callus have been called "calliclones" (Skirvin 1978), those derived from protoplasts "protoclones" (Shepard et al 1980). Plants also can be derived from haploid microspores. It seems appropriate that in vitro regenerated plants be collectively referred to as "somaclones."

In most agronomically important crop species, somaclonal variation is greater than the variation displayed among the seed progeny of the donor (Table 3). Extensive somaclonal variation also was reported in *Pelargonium* (Skirvin 1978). A *Pelargonium* somaclone has been developed as a named cultivar.

The somaclonal variation in sugarcane and potatoes is the most extensively documented and most agriculturally profound among recent findings.

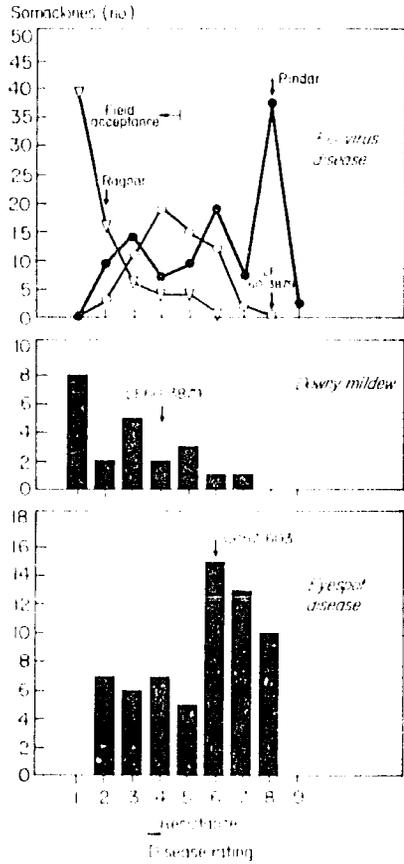
Somaclonal variation in sugarcane

The reaction of sugarcane somaclones to three diseases is shown in Figure 1. Some somaclones derived from susceptible cultivars are highly resistant to both eyespot disease and downy mildew in field conditions. A significant number of somaclones derived from two Fiji virus disease-susceptible cultivars also display field resistance. Somaclonal segregation appears to be in the direction of enhanced resistance. Somaclones derived from a resistant cultivar (Ragnar) appear to retain resistance.

Table 3. Somaclonal variation in economically important plant species.

Species	Source material	Somaclones screened (no.)	Characters displaying enhanced phenotypic variation	Reference
<i>Solanum tuberosum</i>	Leaf mesophyll protoplasts (Russet Burbank)	>10,000	Tuber shape, yield, maturity date, photoperiod requirement; plant morphology; enhanced resistance to <i>Alternaria solani</i> and <i>Phytophthora infestans</i>	Shepard et al 1980
<i>Solanum tuberosum</i>	Suspension culture protoplasts, dihaploid line	<211	Tuber shape	Wenzel et al 1979
<i>Saccharum officinarum</i>	Suspension cells or callus, 10 different cultivars	568	Enhanced resistance to <i>Helminthosporium sacchari</i> , <i>Sclerospora sacchari</i> , Fiji virus, cane and sugar yield	Heinz et al 1977
<i>Saccharum officinarum</i>	Shoot apex, leaf, or inflorescence callus, 8 cultivars	417	Cane and sugar yield; stalk number, length, diameter, volume, and density; fiber %	Liu and Chen 1978
<i>Saccharum officinarum</i>	Leaf base callus, 2 cultivars	102	Enhanced resistance to <i>H. sacchari</i> toxin	Larkin and Scowcroft unpubl.
<i>Zea mays</i>	F ₂ hybrid embryo callus	85	Leaf arrangement, plant height, node number	Green 1977
<i>Oryza sativa</i>	Seed callus, single pure line	1,121	Seed fertility, plant height, heading date, morphology, chlorophyll deficiency	Oono 1978
<i>Hordeum</i>	<i>H. vulgare</i> / <i>H. jubatum</i> triploid embryo culture	43	Relative intensity of isoenzymes, growth habit, head morphology, awicle size, chromosome number and associations	Orton 1980
<i>Lolium</i>	<i>L. multiflorum</i> / <i>L. perenne</i> triploid embryo culture callus	>2,000	Chromosome number and associations, leaf shape and size, floral development, growth, vigor, survival and perenniality	Ahloowalia 1978
<i>Nicotiana tabacum</i>	Dihaploids, 15-generation selfed cultivar (Coker 139)	46	Yield, grade, flowering time, plant height, leaf traits, alkaloids, reducing sugars	Burk and Matzinger 1976

1. Disease reaction of somaclonal segregates of sugarcane. Disease rating on a scale of 1-9: 1 = resistant, 2 = susceptible.

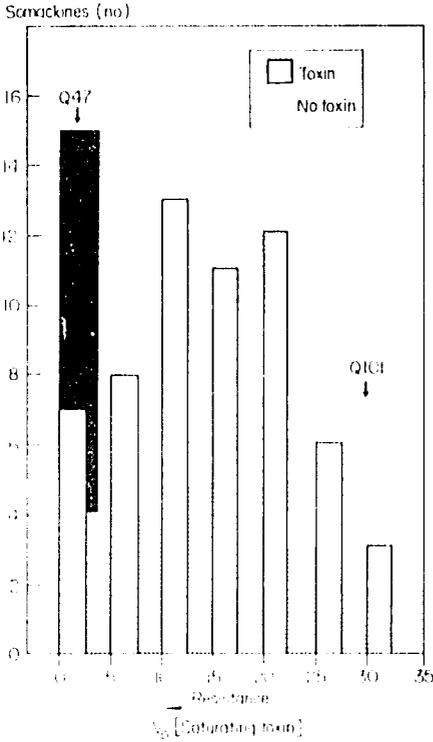


One virus disease-resistant somaclone of Pindar with an equal sugar yield has potential as a commercial cultivar.

Of the 13 sugarcane somaclones evaluated in detail after the initial screening (Liu and Chen 1978), two appeared superior to their donor parent in sugar yield. These somaclones are now being evaluated in advanced yield trials.

Recently initiated research by Larkin on the isolation of *H. sacchari*-resistant lines of sugarcane confirms that somaclonal variability is extensive. The development of a rapid bioassay for *H. sacchari* toxin sensitivity, based on electrolyte leakage from leaf disks following a short exposure to toxin (Larkin and Seowcroft 1981), has enhanced the screening procedure. Figure 2 gives the frequency distribution for the toxin reaction of somaclones derived from both highly susceptible (Q101) and resistant (Q47) cultivars. Somaclones of the susceptible cultivar tend toward enhanced resistance. Preliminary data indicates that somaclones derived from cell lines selected in the presence of toxin do not appear to have a lower frequency distribution for resistance than somaclones derived from unselected callus. A similar result was reported by Heinz et al (1977).

These presumptive *H. sacchari*-resistant somaclones are being evaluated for field



2. Frequency distribution of resistance to *N. sacchari* toxin of somaclonal segregants of a susceptible (Q101) and a resistant (Q47) cultivar of sugarcane. V_0 [saturating toxin] is the initial velocity of the increase in conductivity at saturating toxin concentrations

resistance. A number of somaclones of a cultivar highly susceptible to leaf scald (*Xanthomonas albilineans*), a disease that poses an endemic threat in Australia, also are being tested in field conditions.

Somaclonal variation in potatoes

Heterozygosity is essential to maximizing potato plant vigor. Coupled with tetraploid inheritance and the low fertility, or even sterility, of many cultivars, it makes conventional plant improvement essentially impossible. Shepard et al (1980) argued that it might be simpler to selectively enhance a popular variety than to create a new one. It appears that they have achieved such an improvement through somaclonal variation.

Leaf mesophyll protoplasts were the starting material. With considerable developmental effort, they were induced to divide and form colonies, which in turn were induced to regenerate plants. The procedure did not include any deliberate selection or mutagenesis.

Extreme uniformity among the somaclones was expected. However, considerable phenotypic diversity was apparent in the first 1,000 plants raised in the greenhouse. Some 10,000 plants with extensive variability have now been examined.

As a consequence of shorter internode distances, several somaclones had a more compact habit than the donor parent Russet Burbank. That habit resulted in a fuller

and presumably more efficient canopy. Other populations varied from earlier to later than the parent in the time of onset of tuberization. Russet Burbank requires relatively long photoperiods (16 hours) to initiate flowering. Several somaclones flowered with only a 13-hour photoperiod. Variation in tuber shape, degree of malformation, and skin texture also was observed. Some somaclones also showed potential for outyielding Russet Burbank.

Early blight (*Alternaria solani*) and late blight (*Phytophthora infestans*) are serious diseases of potatoes. A toxin bioassay for early blight revealed presumptive enhanced resistance in 5 of 500 somaclones tested. Of those, four also showed enhanced resistance to fungal infection. For late blight, a complex disease characterized by a number of races, several somaclones (20 from 800 tested) were more resistant than Russet Burbank. Some also had enhanced resistance to infection by a second pathogenic race.

Many of these somaclonal variants have been tested through second and third tuber generations. In many cases, the variant, such as enhanced disease resistance, is retained. In this initial phase, some 60 somaclonal variants have one or more agronomic improvements over the parent. Five are currently being evaluated as potential cultivars.

A further cycle of producing somaclonal variants by culturing protoplasts of several first-cycle somaclones with either improved yield or disease resistance is now in progress. The objective is to select for further enhancement among the regenerants. Successive improvement by recurrent cycles of selection among somaclones might provide a rapid technique for varietal improvement, particularly in asexually reproducing species.

Somaclonal variation in seed-propagated species

The evidence suggesting that somaclonal variation could significantly contribute to plant improvement of seed-propagated species is less substantial. The limited data on maize and the reports in rice (Table 3) seem encouraging. Oono (1978) indicated that 72% of the 762 fertile lines derived from somaclones of a pure breeding line of rice varied from the parent in one or more of the characters examined. Moreover, somaclonal variants were stable through at least one seed generation.

Table 4. Variation among progeny of 46 dihaploids derived from a 15-generation inbred line of tobacco (Burk and Matzinger 1976).

	C139S ₁	Dihaploid		F-tests ^a	
		Mean	Range	Among dihaploids	C139 vs dihaploids
Yield (kg/ha)	2761	2765	1848 - 2934	**	**
Grade index	42.5	40.3	29.7 - 45.8	**	ns
Days to flowering	57.0	54.1	46.5 - 59.2	**	**
Plant ht (cm)	102.7	87.4	75.7 - 98.0	**	**
Alkaloids (%)	2.6	2.96	2.20 - 3.88	**	*
Reducing sugars (%)	13.3	15.3	11.0 - 19.2	**	ns

^a * = significantly different at 0.01 level of probability, ** = significantly different at 0.05 level, ns = not significant.

The data of Burk and Matzinger (1976) are relevant to the potential of somaclonal variation in normally inbreeding species. The differences among 46 dihaploids derived from a 15-generation inbred line of a tobacco cultivar are summarized in Table 4. The variation among the androgenetic somaclones was unexpectedly large.

Somaclonal variation from interspecific hybrids

There is a heightened interest in the use of embryo culture to obtain hybrids between normally incompatible species. Similarly, somatic hybridization by protoplast fusion is seen as a new way of transcending barriers to gene flow between species. The predisposition to allosyndetic pairing during meiosis in such interspecific hybrids largely will preclude recombination between nonhomologous chromosomes.

However, if the hybrid embryo remains as a callus culture (Ahloowalia 1978) or if callus cultures are produced from an interspecific hybrid plant (Orton 1980), somaclones derived from such cultures display extensive variation.

Orton (1980) found considerable variation among 43 somaclones derived from callus cultures initiated from a triploid hybrid plant between *H. vulgare* and *H. tuberosum*. Of particular note was the extensive variation in growth habit and in relative intensity of the several isoenzyme bands of esterase and glutamate-oxaloacetate transaminase. A detailed cytological investigation of only four somaclones demonstrated not only chromosome number variation but also an increased frequency of bivalent and multivalent associations. Orton argues that this could result "from loss or inactivation of an homeologous pairing inhibitor or the selective diploidization of certain chromosome segments of the original complement." Because no chromosome rearrangement was detectable, he favored the loss of homeologous pairing inhibitor hypothesis. If Orton's conclusion is correct, then greater recombinational (mitotic or meiotic) variability will occur in such somaclones than in an interspecific hybrid plant produced directly from a hybrid embryo.

Origin of somaclonal variation

The occurrence of a large amount of apparently stable phenotypic diversity among plants regenerated from cells grown *in vitro* raises the question of mechanism. At best, deliberations are strictly speculative, without appropriate analytical experimentation.

Aneuploidy could account for some variation in polyploid species such as sugarcane and potatoes. Shepard et al (1980) found that 5 promising somaclones had 48 chromosomes each, thereby ruling out pronounced aneuploidy.

Other causes of somaclonal variation might include organelle variability, which could have an opportunity to segregate in somatic cells. Cryptic chromosomal changes, such as small deletions, additions, transpositions, or inversions, which might normally be screened out during meiosis, may persist in somatic cells. Mutation, at least in the classic sense of nucleotide substitution, would have to occur at inordinately high rates to account for the observed variability.

Recent molecular studies of genome organization in eucaryotes indicate that the somatic genome is not static but is highly variable. For example, when murine sarcoma cells are selected in the presence of methotrexate, which inhibits dihydrofolo-

late reductase, the gene coding for the enzyme is amplified up to 200-fold (Schimke et al 1977). There is also evidence for the somatic rearrangement of the genes coding for the constant and variable regions of the immunoglobulin light chain (Brack et al 1978) and for the amplification of specific DNA sequences during chicken cartilage and neural retina differentiation (Strom et al 1978). Similar types and levels of variability are likely to be uncovered in the somatic genome of plants.

Somatic variability in plants should prove an exciting area of research. It may well be one of the major contributions of plant cell culture to the molecular biology of eucaryotes and, most importantly, to the provision of genetic variability for plant improvement.

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PROGRESS AND POTENTIAL OF PROTOPLAST FUSION IN CROP IMPROVEMENT

D. PENTAL and E. C. COCKING

The production of interspecific crosses in crop plants is desirable for two major reasons — the production of new amphiploids and the use of interspecific hybridization for gene transfer. Although rarely of direct value, amphiploids may be used to introduce particular features of related species into cereals, including the insertion of small segments of alien chromosomes by induced translocation. The availability of protoplasts and the ability to fuse protoplasts of different species is providing extra dimension and added stimulus to work in this direction. Progress and problems of protoplast technology in relation to crop plants were fully reviewed in 1976 (Bhujwani et al 1976). This assessment of current progress and potential will emphasize recent work and identify worthwhile fusions.

CURRENT STATUS OF SOMATIC HYBRIDIZATION

Following protoplast fusion and suitable selection of resultant hybrid cells, somatic hybrid plants have been produced between species very difficult or impossible to hybridize sexually, such as *Lycopersicon esculentum* and *Solanum tuberosum*, *Datura innoxia* and *Atropa belladonna*, *Arabidopsis thaliana* and *Brassica campestris*, and, more recently, between the horticulturally important species *Petunia parodii* and *P. parviflora*, which are sexually incompatible (Power et al 1980). Study of the somatic hybrid production between *P. parodii* and *P. parviflora* is particularly relevant to the use of protoplast fusion for crop improvement. An attempt is being made to incorporate new somatic hybrids into a conventional breeding program.

with the aim of introducing new cultivars of *Pennisetum* possessing the highly branched, prostrate growth habit of *P. parviflora*.

The absence of meiotic segregation in somatic hybrids could be profitably exploited, particularly in crops propagated vegetatively. In potato, the suggested procedure is to fuse protoplasts of different dihaploid hybrids. The heterozygous dihaploid would be combined into a completely heterozygous tetraploid plant without meiotic segregation. Propagated vegetatively, the plants could immediately become a stable variety (Wenzel et al 1979).

The formation of cytoplasmic hybrids from the fusion of protoplast has resulted in an ability to directly introduce cytoplasmically based male sterility (Galun and Raveh 1978). The recent indication of the formation of mitochondrial recombinants following interspecific tobacco protoplast fusion (Belliard et al 1979) may also provide a basis for increased genetic variability.

We have identified major areas for the application of protoplast fusion in crop improvement. Progress is slow, particularly because of the limited ability to satisfactorily culture crop species.

FUTURE WORK

Somatic hybridization of legumes

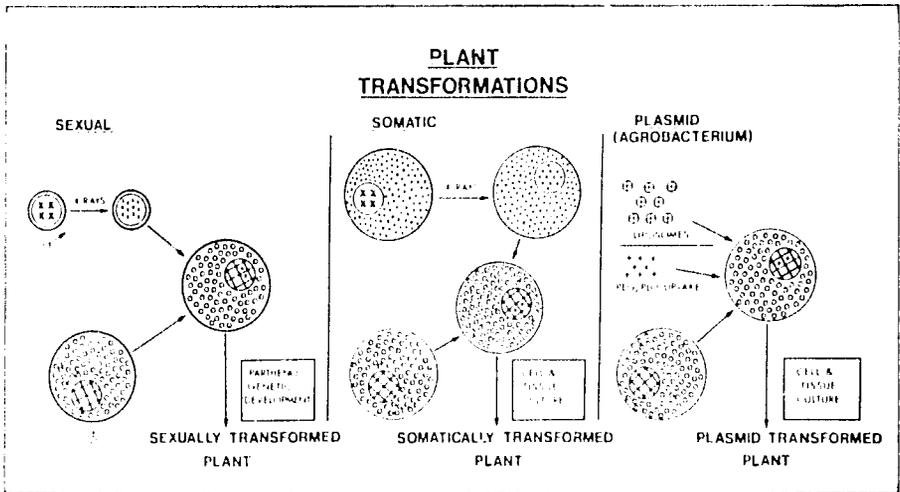
Grain legumes present a major cultural challenge, particularly in relation to plant regeneration. Our efforts have been divided into two major categories—1) forage legumes, such as alfalfa and clover, and 2) grain legumes, such as *Vicia* and *Vigna*.

Some forage legumes now are capable of being adequately cultured. Recently we obtained high-frequency regeneration from leaf protoplasts of whole plants of alfalfa (*Medicago sativa*) via callus and embryoids (dos Santos et al 1980). Protoplasts from cultured cells of *Trifolium repens* also have been regenerated (Gresshoff 1980). We are now attempting somatic hybridization of alfalfa and *Trifolium* with tannin-containing forage legume *Onobrychis viciifolia* (Sainfoin). Presence of leaf tannin reduces bloat problems in grazing animals. Selection procedures will involve culturing individual heterokaryons.

Rice-sorghum somatic hybridization

Recent work on the sexual hybridization of rice and sorghum has reemphasized the need for crosses and highlighted the need for parallel efforts on protoplast fusion (Demming et al 1979). Our main difficulty in setting up somatic hybridization between rice and sorghum has been that at present there is no regeneration of whole plants from protoplasts of either species. We know already that there is no difficulty in the fusion and limited culture of heterokaryons but, until the present cultural difficulties have been overcome, we cannot undertake somatic hybridization. It is likely that regeneration capability is controlled genetically.

Both sexual and somatic hybridization involve extensive intermixing of genes. There often are disadvantages in attempting to combine the total genetic structures of two species but greater advantages in attempting to incorporate some limited, perhaps single, genetic attribute of a donor species into a recipient crop species. There has been a resurgence of interest in the transformation of plants in which gene



1. Schematic representation of the 3 main procedures for plant transformation.

flow is limited.

A major development in whole plants was the demonstration in *Nicotiana* that pollination with highly irradiated (100,000r) killed compatible pollen can cause the transfer of certain genes from the pollen to the egg without proper fertilization (Fig. 1). This phenomenon in effect, transformation of the egg has resulted in egg transformation for the flower color gene and the *S* gene. This has led to an examination of the use of irradiated somatic cells to bring about a limited gene transfer.

We are currently investigating (in collaboration with Dr. A. Muller and his colleagues in Gatersleben, German Democratic Republic) whether somatic transformation can be used to transfer nitrate reductase genes between different species (Fig. 1). We are utilizing the nitrate reductase deficient mutants of tobacco isolated by the Gatersleben Group and fusing them with X-ray irradiated cereal and legume protoplasts. Then, we select for nitrate reductase proficiency among the tobacco cells.

Such markers should facilitate selection for limited gene transfer by protoplast fusion. In future work with fusions—for example, between a nitrate reductase minus legume and a wild-type tobacco—this limited gene flow could result in legumes with improved nitrate reductase capability. The requirement for auxotrophic mutants for these evaluations emphasizes the need for a greater range of such mutants in a large number of species capable of plant regeneration (Cocking 1980).

Maize/sorghum somatic hybridization

Recent work on the embryo culture of hybrids between maize and sorghum at Centro Internacional de Mejoramiento de Maíz y Trigo (James 1980) also reemphasized the need for such crosses and again highlighted the importance of parallel efforts in protoplast fusion. Unfortunately, work in this direction is also being

impeded by an inability to regenerate whole plants from maize protoplasts. There is no difficulty in fusing the protoplasts and obtaining heterokaryons with inherent viability (Brar et al 1980).

PROBLEMS AT THE CULTURE LEVEL

Induction of a fast-growing, friable, unorganized callus from organized explants is a good indication that a culture can be passed through a single cell (protoplast stage). These fundamental attributes of a good callus culture system are lacking in cereals. Much of the early work on the culture of cereals has been on induction of callus from root meristem or mature embryo explants. Shoot differentiation from such callus is sporadic and usually not related to any hormonal combinations (Pental and Grunckel 1979). Consistent shoot regeneration from maize callus induced from immature embryos has been reported (Green and Philipps 1975). Since then, shoot regeneration from immature embryos of other cereals has also been reported.

The use of the embryo as an explant for callus can be criticized. An embryo is a complex mixture of cells, which may have highly variable morphogenetic potentials and variable requirements to pass into an unorganized phase. Since the relatively slow growth rates in cereal calli have led to low explant to callus ratios, callus growth and morphogenesis could be influenced by exudates from the persisting parts of the embryo. There is anatomical evidence that the callus from embryos of maize and other cereals may never pass through an unorganized phase (Cure and Mott 1978, King et al 1978). However, it may still be profitable to use immature embryos of different genotypes of important cereals for a callus that shows good shoot differentiation and is friable enough to produce cell suspensions and protoplasts.

A homogeneous explant like the leaf parenchyma is a more appropriate material for callus initiation. Although much of the work on callus from leaf segments and culture of leaf protoplasts is negative (Potrykus et al 1976), there are reports that the basal region of a barley leaf (Saalbach and Koblitz 1978) and a rice leaf (Henke et al 1978) form callus. Renewed effort is needed to evaluate the possibility of callus induction from leaf tissue. Any part of the leaf tissue that can form callus will be good material for protoplast isolation.

We plan to evaluate different genotypes of rice, corn, and sorghum for their ability to form callus from leaf tissue and immature embryos as a prerequisite to establishing a protoplast system for these plants. Suitable protoplast systems can open the possibility of in vitro genetic manipulation in these important grain crops.

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PROTOPLAST, CELL, AND TISSUE CULTURE IN RICE: PROSPECTS FOR THE FUTURE

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After a decade of intensive tissue culture research on rice and other plants, the uses and limits of tissue culture and related research have been clearly defined. Researchers working with rice have successfully produced callus and plants from single cells, from plated ovaries and anthers, and from isolated pollen (Niizeki and Oono 1968, Chu et al 1975, Oono 1977, Hu 1978, Wang et al 1979, Chaleff 1980, Chen et al 1980, Kucherenko 1980). In addition, protoplasts have been produced and fused, and callus has been developed (Anon. 1976, Deka and Sen 1976, Tsai et al 1978). RNA from soybean was successfully transferred to rice through tissue culture, where it controlled the production of soybean protein in rice plants (Niu et al 1980). Much that was theoretical concerning culture of plant and animal tissues in the 1960s became applied research in the 1970s.

Through tissue culture, it is possible to produce whole plants from single somatic cells, thus extending the techniques of microbial genetics to rice breeding. By using anther and pollen culture techniques, it is possible to produce haploid or homozygous plants from single gametes. When pollen from F_2 or F_3 plants from conventional crosses are cultured, homozygosity is obtained in one step with no further segregation. The population of plants produced has the inherent variation that would have been found in the F_2 or F_3 , but individual plants in the population have a fixed genotype with no further segregation. Characters controlled by recessive genes are immediately apparent in lines produced from tissue culture.

Plants produced through tissue culture often have changes in ploidy or mutations. These may serve as new sources of variation. The mutation rate can be increased by the use of mutagens on cultured cells before regenerating plants (Sung 1976).

Cells from somatic tissues or callus produced through anther culture can be grown in suspension culture and exposed, either directly or plated on solid media, to

deleterious substances such as fungal or bacterial toxins, herbicides, salts, or toxic minerals to select for variant cells with resistance. When resistance to disease or other constraints is mediated by metabolic activities at the cellular level, resistance may be expressed in cultured tissues (Bottino 1975, Chaleff and Parsons 1978, Scowcroft 1978).

Under certain conditions, homozygosity is necessary to reduce experimental error. Examples include differential varieties used to identify races of pathogens and biotypes of insects or varieties and lines used in physiology studies. Varieties can be made absolutely homozygous, barring mutations, through the culture of gametes.

Recently, the technique of protoplast fusion has been developed for making parasexual crosses between species where hybridization through conventional methods has been difficult (Cocking 1972, 1978). Using this technique, intergeneric fusion of protoplasts has been accomplished *in vitro* (Constabel et al 1975, Bhojwani et al 1977). In addition, it has been possible to transfer nucleic acid directly to protoplasts with the concomitant incorporation of genetic control for the production of enzymes and other compounds not previously produced by the variety or species.

Many advances have been made in rice tissue culture in the 1970s. The most significant research has been conducted in Japan, China, and India. Recently, scientists in the United States of America and the Soviet Union also have begun to make significant contributions (Kucherenko 1980, Chaleff 1980).

In September 1979, a pilot tissue culture program was initiated at IRRI. Significant progress has been made in screening rice germplasm for the ability to produce callus and green plants through anther culture (IRRI 1980). Crosses have been made among lines that have exhibited potential in producing green plants through anther

Table 1. Effect of parental efficacy for callus production in anther culture on response of F_1 hybrid.

Material plated ^d	Anthers plated (no.)	Anthers producing callus		Anthers with multiple callus	
		No.	%	No.	%
F_1 (Laipei 309/IR36)	1050	24	2.3	12	1.1
Laipei 309	204	16	7.8	10	4.9
IR36	472	1	0.2	1	0.2
F_1 (Laipei 309/IR8)	540	25	4.6	21	3.9
Laipei 309	204	16	7.8	10	4.9
IR8	368	1	0.3	0	0
F_1 (Laipei 309/IR20)	744	68	9.1	60	8.1
Laipei 309	521	23	4.4	23	4.4
IR20	362	2	0.6	1	0.3
F_1 (Pan-kan-tao/IR20)	579	54	9.3	49	8.5
Pan-kan-tao	138	11	8.0	10	7.2
IR20	362	2	0.6	1	0.3
F_1 (Pan-kan-tao/IR8)	622	18	2.9	18	2.9
Pan-kan-tao	370	26	7.0	24	6.5
IR8	368	1	0.3	0	0

^d Comparative tests with parents and F_1 anthers plated at the same time and maintained under the same cultural conditions.

culture. Comparative platings of anthers from parent varieties and F₁ plants from these crosses are being used to study the heritability of successful plant production. Preliminary data are presented in Table 1. Plants derived by anther culture are in

Table 2. Comparison of F₁ plants with parents for efficacy in anther culture.^a

Variety or F ₁	Anthers producing callus (%)
Taipei 309/IR36	0.8
Taipei	14.4
IR36	0
Silewah/IR28	4.1
Silewah	4.6
IR28	0
Mingolo/D66	2.8
Mingolo	3.9
D66	2.6
Silewah/Taipei 309	19.6
Silewah	4.6
Taipei 309	14.4
Silewah/Taichung no. 65	4.9
Silewah	4.6
Taichung no. 65	1.9
Silewah/IR8	0.2
Silewah	4.6
IR8	0
Silewah/Tainan no. 5	14.6
Silewah	4.6
Tainan no. 5	5.6

^aAnthers of varieties and F₁ plated on Chaloff's R-2 medium at the same time and maintained under the same cultural conditions.

Table 3. Effect of passing varieties through anther culture on subsequent efficacy in anther culture.

Variety	Anthers plated (no.)	Anthers producing callus		Anthers with multiple callus	
		No.	%	No.	%
Mingolo:					
From seed	471	2	0	0.4	0
From anther culture	452	11	1	2.4	0.2
IR9264-324-1:					
From seed	367	1	1	0.3	0.3
From anther culture	407	43	29	10.6	7.1
Mingolo:					
From seed ^a	—	—	3.9	—	—
From anther culture					
Plate no. 1	625	51	8.2	24	3.8
2	424	30	7.1	18	4.2

^aPlating of these anthers was contaminated by fungi. Data are means of many platings of Mingolo during screening.

comparative tests with the variety grown from seed to see if passing the variety through culture improves its efficacy in tissue culture. Preliminary data are presented in Tables 2 and 3.

Other experiments in progress are: a) continued screening of the IRRI rice germplasm collection, b) studies on the improvement of rice tissue culture methods, and c) studies of changes in ploidy and mutations in plant material produced through tissue culture. Preliminary experiments on the use of suspension cultures for screening against biological and physical constraints on rice production are under way. Experiments to study protoplast fusion and related techniques for incorporating foreign nucleic acid into rice are planned.

Scientists in China have made many advances in the application of tissue culture techniques to rice improvement in the decade of the 1970s. Major accomplishments were recently reviewed by Rush and Shao (1981).

RESEARCH NEEDS AND STRATEGIES FOR RICE TISSUE CULTURE

Rice anther and ovary culture have been accomplished many times. However, many problems restrict their use in rice improvement programs. The main problem is unfavorable aspects of the logistics of large-scale culturing of rice gametes, including: the time involved in obtaining, processing, and plating anthers and ovaries; poor production of callus and plants by many varieties and hybrid plants; and a lack of information concerning the reaction of most rice varieties in tissue culture. Adding to the inefficiency of rice anther culture is the high percentage of albino plants produced. Another problem is the failure to produce plants from rice protoplasts. This restricts the use of protoplasts for hybridization and limits studies using protoplasts to examine the uptake of foreign nucleic acid.

IMPROVEMENT OF ANTHER CULTURE EFFICIENCY

Preplating and postplating activities

Additional studies of pollen development and the factors affecting dedifferentiation should be conducted. The developmental stages most favorable for plating in anther culture have been thoroughly studied (Genovesi 1978). However, the effects of chemical or temperature treatments that induce dedifferentiation or that interrupt the normal developmental processes in pollen so that the microspores divide to produce cells instead of continuing to develop into mature pollen have not been sufficiently explored. Auxins and cold-shock treatments of rice anthers before or after plating increase the frequency of callus induction. Recent preliminary research at IRRI suggests that heat treatment of anthers also may increase callus induction (Rush and Shao 1981). A recent study of growth-regulating compounds showed that 2-methyl-4-chlorophenoxyacetic acid (MCPAS) was more effective for inducing callus from anthers than the 2,4-D normally used (Chou et al 1978). Further studies of the effects of growth-regulating substances on callus and green plant induction should be conducted.

Another recent study demonstrated that 2-chloroethylphosphonic acid (Ethrel) caused deviation in pollen development, leading to mitotic divisions in microspores

(Bennet and Hughes 1972). When Ethrel was added to the rice anther culture medium, it increased the callus induction rate by 50-500%, depending on the germplasm plated (Song et al 1978). Reports of effects of preplating temperature treatments on changing microspore development suggest that different rice germplasm respond differently. This area needs further exploration.

Incubation temperature also affects callus and green plant induction (Genovesi 1978, Song et al 1978, Genovesi and Magill 1979, Rush and Shao 1981). In general, the accepted incubation temperatures for both callus induction and plant production range from 24- to 26 °C. However, insufficient studies have been made of the interactions between different germplasm and incubation temperatures.

Media

Many media have been used for rice anther culture. In general, they are variations of the media of Murashige and Skoog (1962) and Miller (1963). Rush and Shao (1981) listed the ingredients of 14 media commonly used for rice tissue culture. Eight were developed in China.

Media constituents are not as important a consideration for callus and plant induction as other factors. If the basic salts (NH₄)₂SO₄ and KNO₃ and sucrose are present, callus induction can occur. A medium developed in China relies on extracts from potatoes for the basic nutrients (Chen et al 1978). This medium compared favorably with complex media in tests of callus and plant induction from anthers.

The literature on the effects of media constituents on anther culture is not clear. Investigations have used different types of F₂ combinations in their studies. Media should be compared at the same time, using selected germplasm of japonica, indica, and japonica-indica hybrids. The same environmental conditions should be maintained throughout the test.

However, different germplasm may respond differently to different media. For screening purposes, a single medium that performs well with most of the germplasm tested may be used. Varieties that perform on the screening medium can be tested on other media to identify the most suitable medium to obtain plants from the F₂.

Additional research is needed to determine the effects of new media constituents on rice anther culture, to relate media effects to germplasm, and to simplify media constituents and the logistics of media preparation, storage, and use.

Variation in plants from anther or ovary culture

Rice tissue culture primarily has been aimed at the production of haploid plants or homozygous diploid plants through the doubling of chromosomes in haploid callus of plants from pollen or ovaries. Niizeki and Oono (1971) regenerated haploid, diploid, tetraploid, and pentaploid plants through anther culture. Since that work, many reports have indicated variation in ploidy in rice plants regenerated through tissue culture. In determinations made on 62 varieties or F₂ combinations from which plants were produced in China during 1972-1977, Chen et al (1978) observed that 36% of the plants produced were haploid, 58% were diploid, and 6% were polyploid. Other observations of ploidy progeny produced through anther culture have indicated that the frequency of spontaneous diploids produced is 40-60%. Homozygous diploids are produced in significant proportions without resorting to

the use of chemicals such as colchicine.

Plants produced through tissue culture with abnormal ploidy may serve as sources of new gene combinations for breeding programs.

There have been reports of mutations in lines from anther culture. This may also serve as a source of variation for breeding programs. Mutations can be increased by treatment *in vitro* with chemical mutagens (Sung 1976, Skirvin 1978). *In vitro* systems can be developed for isolating and identifying mutant types. Little research of this type has been conducted with rice. Such systems may use pathotoxins, herbicides, salts, amino acid analogs, or other constraints.

Homozygosity may be obtained in varieties and breeding lines by passing them through anther culture. This will be useful in certain physiological and genetic studies. The homozygosity of lines produced through anther culture can be used with conventional techniques to screen against pathogens, insects, herbicide damage, damage from salinity, resistance to mineral toxicity or deficiencies, drought, and other constraints to rice production.

Homozygosity in varieties used to differentiate races of plant pathogens or biotypes of insects also would be useful. Development of near-isogenic lines with different sources of resistance to pathogen races should be facilitated by the use of anther culture. These lines could be used to develop multiline cultivars with disease constraints.

Albino plants

The high percentage of albino plants produced in anther culture lowers the efficiency of the system in producing useful plants. Additional anatomical, biochemical, and environmental studies are needed to develop methods for minimizing the development of albino plants. The recent work of Song et al (1978) and Wang et al (1978), showing the effects of incubation temperature on the production of albino plants, was useful and should be extended.

Culture of isolated pollen

One limitation on the production of rice callus and plants from pollen is the necessity of plating anthers. The process is time consuming and only a limited number of anthers can be plated. Culturing of isolated pollen should increase the number of individual calli and plants produced. Recent work in China demonstrated the feasibility of producing rice plants from isolated pollen (Chen et al 1980). Research in this area should be continued to improve the techniques and determine the most suitable media, growth regulators, and environmental conditions. Development of techniques to produce embryoids and plants directly from isolated pollen without production of callus could be a major objective. Direct formation of plants from pollen in anthers has been observed. That suggests the possibility of producing plants directly from isolated pollen, if the germplasm favoring direct formation of embryoids from pollen is identified and used.

Rice protoplasts

Rice protoplasts have been successfully generated and cultured and have developed callus in culture (Isai et al 1978). Unfortunately, the calli had lost totipotency.

Research should be conducted to develop systems for producing protoplasts that will retain their totipotency. Such a system would provide an important tool for genetic engineering. Recent success in China with formation of intergeneric hybrids through conventional crossing techniques suggests that intergeneric hybridization through protoplast fusion also is feasible. A technique for isolating intact nuclei from rice cells was described by App and Granados (1974). It may be possible to develop new germplasm resources by incorporating *Oryza* nucleic acid, or foreign nucleic acid, into protoplasts. The work of Niu et al (1980) suggests that foreign nucleic acid incorporated into rice cells will be retained and will actively code for foreign protein in the rice plants subsequently produced.

Use of rice tissue culture for pathology

Tissue culture research historically has been closely associated with research on host-pathogen interactions and the activities of plant pathologists and plant pathology departments. The techniques and equipment used for tissue culture research and for culturing plant pathogens are similar. The opportunities for using rice tissue culture for pathology-oriented studies are many and varied.

Lines produced from conventional crosses often segregate for disease resistance or susceptibility for many generations. This makes early-generation screening for disease resistance of little use in breeding programs. Lines generated through anther culture are immediately homozygous. Resistance characteristics are fixed and no further segregation will occur. This will be especially useful in transferring disease resistance from plants with undesirable agronomic characteristics to commercial-type varieties. Progeny produced by F_1 plants from wide anther culture crosses can be screened immediately for disease resistance. Lines having resistance and desirable agronomic characteristics can be backcrossed to the commercial parent. Subsequent culturing of anthers from the F_1 should quickly stabilize the progeny and shorten the time required to produce a commercial variety with disease resistance.

Frey et al (1975) suggested that the use of multiline varieties containing near-isogenic lines with different genes for resistance to pathogen races would be a method for stabilizing resistance to major diseases. The primary difficulty encountered with the multiline concept is the development of lines that are similar agronomically, but with different major genes for resistance. It is likely that near isogenic-lines with different resistance genes can be developed by producing F_1 pollen culture from double-crosses or topcrosses.

Homozygosity in varieties used to differentiate races of pathogens is a necessary, but not always present, condition. For example, control of rice blast by monogenic resistance is dependent upon identification of *Pyricularia oryzae* races and genes for resistance (Crill and Khush 1979). The identification of specific races would be facilitated by the development of near-isogenic lines containing monogenes for resistance. It could be accomplished by crossing and back-crossing with different sources of resistance, then producing homozygous lines from pollen culture. Such lines also would be useful in a gene-rotation system for rapidly incorporating genes for resistance into commercial varieties.

In vitro screening for disease resistance in cultured tissues has been successful with certain host-pathogen combinations (Ingram and Robertson 1965, Ingram 1967,

Helgeson et al 1972, Gengenback et al 1977). Early attempts to differentiate between cultured rice tissues susceptible or resistant to *P. oryzae* or *Cochliobolus nivabeomus* did not succeed (Ishii and Yamasaki 1971, 1972; Ishii and Sakamoto 1975; Ogasawara et al 1977). In these studies, pathogens were inoculated directly onto tissue culture plates. All tissues were susceptible in culture, regardless of the level of resistance in the variety. This suggests that pathotoxins or culture filtrates should be used for in vitro screening for resistant cells, rather than the pathogens. Several major rice pathogens are known to produce toxins.

Cultured cells and tissues also may be useful for studying host-pathogen interactions. The observation that pathogenic and nonpathogenic fungi can destroy callus in culture, regardless of their ability to attack whole plants, suggests that an intact epidermis and cuticle or active photosynthesis may play important roles in preventing invasion by fungi. There are many opportunities for future research in this area.

NEW VARIETIES

The ultimate goal of rice tissue culture research at IRRI is the production of new varieties with superior agronomic characteristics and resistance to major production constraints. That this is a reasonable goal is illustrated by the many new varieties already developed, released, and in production in China (Rush and Shao 1981). Rice improvement by tissue culture techniques is already an accomplished fact. The main research thrust in the immediate future must be directed to making present techniques more efficient and to developing new techniques.

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THE SIGNIFICANCE FOR RICE IMPROVEMENT OF STUDYING REGENERATION IN PLANT TISSUE CULTURE

YASUYUKI YAMADA

Tissue culture techniques are a new agronomical development for the improvement of rice productivity. The potential for crop improvement through tissue culture of cereal cells depends on easier and more efficient techniques for selecting cultured cells with desired characteristics than is possible with conventional plant breeding.

Technologically, we culture cells in a liquid medium in shaking flasks, then plate these cells on a specific medium in petri dishes where only tolerant or desired cells can survive. This technique eliminates the use of large field areas for sexual cross breeding. It saves time and space, and requires much less labor than the procedure used in conventional crosses.

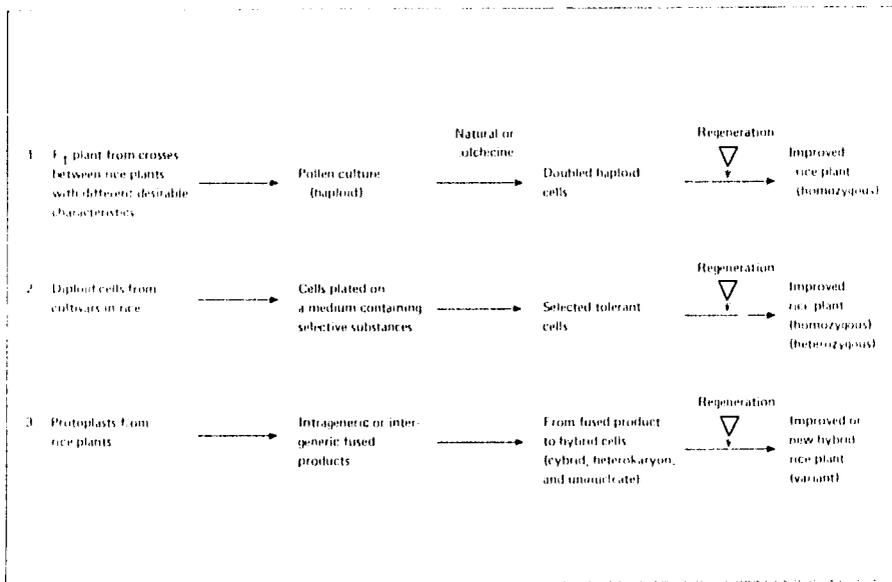
However, we still have some unsolved limitations. One is how we can effectively and efficiently regenerate healthy rice plants from selected cells.

REGENERATING PLANTS FROM CEREAL CELLS

Three basic methods are used (Fig. 1). First, from pollen cultured from anthers of F_1 plants obtained by crossing two different superior plants, we can obtain homozygous diploid cells that have naturally doubled their haploid chromosome sets. After regeneration, the plantlets have the desired characters.

Second, using callus cultures, we can select cells with desirable characteristics through genetic selection, followed by cellular cloning. Genetic selection is probably best done with genetically heterozygous tissue. This tissue would have the variability to contain the desired cells. Alternatively, many homozygous lines from F_1 pollen culture can be tested to identify a desirable plant. After regeneration of plantlets, we obtain cereal plants with specific characteristics.

Third, after somatic fusion of two intrageneric or intergeneric protoplasts, we



1. Possible methods of rice improvement through tissue culture. In all these methods, regeneration is an indispensable step to establish the improvement.

culture the fused products and regenerate hybrid plants from the fused cells.

In all these methods, the key step is plant regeneration. Current techniques are sufficient to regenerate a few plantlets from cultured rice cells, but the percentage of plantlets in relation to the numbers of starting cells is negligible. Also, in rice many differentiated shoots often are albino plants that are practically useless. For these reasons, the development of a more efficient regeneration protocol for cultured rice cells is required. This problem will be discussed from the standpoints of 1) culture condition for regeneration and 2) totipotency of cells.

Culture condition for regeneration

The differentiation of rice shoots and roots from calli and the regeneration of whole plants from callus were among the first successful regeneration experiments using cereals. Nishi et al (1968) obtained calli from rice roots on Linsmaier-Skoog medium containing 10^{-6} M 2,4-D (Linsmaier and Skoog 1965). When this callus was transferred to the same medium without auxin and incubated in the light, the callus differentiated to form shoots and roots and subsequently whole plants. The regenerated plants were diploid, although a few showed peculiar phenotypes (dwarf, twisted, flag leaf shortened, and albino).

Carter et al (1967) also reported that oat calli transferred to an auxin-free medium differentiated into numerous shoots and few roots. Wheat calli showed almost constant root formation and occasional shoot formation on media containing low concentrations of auxin.

Auxins are essential as the inducing compounds for DNA replication (Yasuda and Yamada 1970, Yamada et al 1971, Yasuda et al 1974, Yajima et al 1980). The most effective among the common auxins (2,4-D, NAA, and IAA) used for callus induction in rice is 2,4-D (2,4-dichlorophenoxyacetic acid) (Nishi et al 1973, Sekiya et al 1977).

The concentration of 2,4-D used for callus induction from cereal plants tended to be higher than that used for callus induction from common dicotyledonous plants. No one knows why cereal plant techniques require high concentrations of 2,4-D to induce callus. However, that high concentration of 2,4-D may be the cause of the high numbers of variant plants when cereal plants are regenerated from cultured callus.

For the regeneration of plants or organogenesis of shoots from cultured cereal callus, a decrease in the 2,4-D concentration or the substitution of a weaker auxin, such as IAA or NAA, is essential (Nishi et al 1968, Yamada 1977). For the regeneration of plantlets, exogenously applied cytokinins do not appear to be an essential factor (Nishi et al 1973). If they have a role in regeneration, it may be to accelerate organogenesis.

Usually we transfer cereal callus to a medium containing no auxins when we want to regenerate plantlets from callus (Table I). The first observable signs of regeneration are some green spots on calli cultured in light. These green spots later develop into shoots. When one shoot dominates, other shoots will not grow vigorously (Fig. 2). The relationship of plant hormones in the culture medium and organogenesis in cereal calli is different from that in dicotyledonous plants like tobacco. Furthermore, the shoots regenerated from cereal calli are much fewer than those regenerated from dicotyledonous calli, from which many shoots can continuously differentiate.

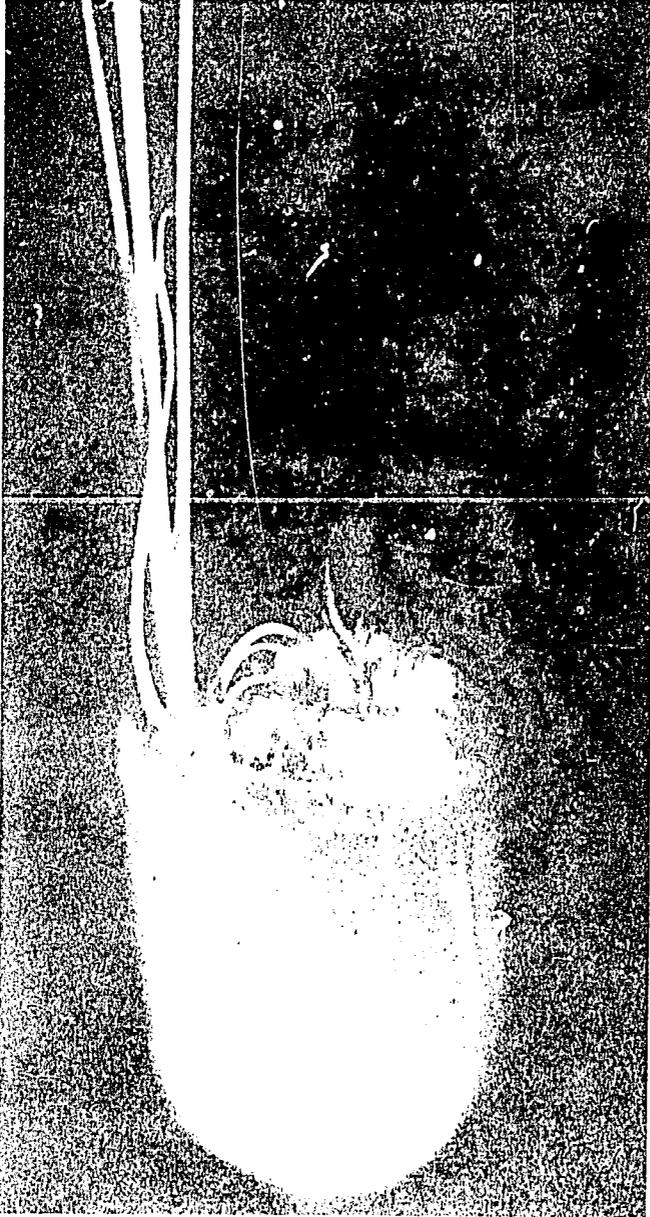
Totipotency of cells

The frequency of shoot regeneration from root callus is much lower than that from embryo callus. After long periods in culture, subcultured calli become more difficult to differentiate into shoots by the method of transferring calli to medium without auxins. Those results indicate that the frequencies of regeneration from calli depend not only on cultural conditions but also on differentiation potentials of individual cells. That means that cultured individual cells are heterozygous and have different potentialities for differentiation.

Table I. Effect of 2,4-D concentration on regeneration of rice callus^a (Nishi et al 1973).

Concn of 2,4-D (M)	Test tubes (no.)	Test tubes with root formation		Test tubes with shoot formation	
		No.	%	No.	%
0	22	20	91	19	86
10 ⁻⁷	22	19	86	16	73
10 ⁻⁶	24	9	38	9	38
10 ⁻⁵	20	0	0	0	0

^aCalli used for the test of regeneration were induced from seed on an agar medium containing 10⁻⁵M 2,4-D at 25 °C in light and were incubated for another 3 months under the same conditions.



2. Plants regenerated from rice callus, (Nishi et al 1968)

Wheat was one of the most difficult cereals from which to regenerate plantlets from calli. Only a very few plants have been obtained sporadically from wheat cultures derived from various organs (Shimada et al 1969, Dudits et al 1975). Shimada and Yamada (1979) recently found that callus cultures from young embryos of wheat plants can regenerate on a medium without auxins and that the

potentialities of the wheat cultures to regenerate have continued even after many subcultures.

The important point is that cultures capable of regeneration can be easily produced and maintained from young embryos. Recently, many regenerated plants also have been obtained from cultures initiated from immature embryos of maize (Green and Phillips 1975), sorghum (Gambore et al 1977, Thomas et al 1977), and oats (Cummings et al 1976). These studies suggest that the totipotency of the cultured cell depends on the explant tissue source.

Table 2 shows that wheat cultures with green spots, which are shoot primordia developing along the periphery of the tissue, were induced from embryos about 14 days old. In cultures obtained from embryos more than 21 days old, green spots were rarely induced. The 14-day-old embryo calli that can develop green spots in the absence of 2,4-D were grown and subcultured on 2,4-D medium. They maintained their potential to differentiate shoots as long as 8 months (Table 3). Once shoot formation from callus has occurred, it is easy to induce root formation. These results indicate that totipotent callus cultures were initiated from immature embryos, not from mature embryos, and suggest that those potential differences among cells depended on developmental variation.

Table 2. Wheat (cultivar Chinese Spring) callus induction from embryos of various ages cultured on Emsmaier and Skoog medium containing 2.0 mg 2,4-D/liter. (Shimada and Yamada 1979).

Embryo age (days)	Embryo size (days)	Embryos inoculated (no.)	Callus induced (no.)	Callus with green spots ^d	
				No.	%
14	1	282	280	149	53
21	1.5-2	33	33	4	12
27	2	23	23	1	4
31	2	37	37	0	0

^dGreen spots mean that shoot primordia developed along the periphery of the tissue.

Table 3. Potential organogenesis in cultures of wheat (cv. Chinese Spring) after 7.5 months of subculture on Emsmaier and Skoog medium containing 2.0 mg 2,4-D/liter (Shimada and Yamada 1979).

Subculturing callus	2,4-D (mg/liter)	Callus inoculated (no.)	Callus differentiation				
			With shoots		With green spots (no.)	With roots (no.)	White callus (no.)
			No.	%			
With green spots	0	21	13	62	0	3	5
	0.2	20	13	65	3	4	0
	2.0	20	0	0	20	0	0
White callus only, without green spots	0	3	0	0	0	0	3
	0.2	3	0	0	0	0	3
	2.0	3	0	0	0	0	3

CONCLUSION

The efficient regeneration of plants from cultured cells is indispensable for rice improvement through tissue culture. Even if we could select cells with desired characteristics, such as resistance to fungal or bacterial toxins, herbicides, drought, or salt, we still have to efficiently regenerate many rice plants with useful characteristics from these cells. At the moment, we can regenerate a few rice plants from calli by culturing rice cells on a medium without auxins. However, to increase the efficiency of regeneration, it is necessary to obtain cells with totipotency.

In rice pollen culture, the frequency of albino plants has been high (Wang et al 1978). That makes the efficiency of green plantlets regeneration very low. As long as we have to culture pollen to obtain homozygous diploid plants, we must seek ways to improve the cultural conditions and the media to resolve the albino problem.

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ANTHER CULTURE OF RICE AND ITS SIGNIFICANCE IN DISTANT HYBRIDIZATION

CHU CHIH-CHING

Several new cultivars of rice have been developed and selected by anther culture in China (Yin et al 1976, Hu 1978). During the past 10 years the Institute of Botany has emphasized increasing the induction frequency of pollen plants in haploid breeding.

INDUCTION OF THE POLLEN CALLUS

Pollen embryos of rice can be grown directly from anthers in a hormone-free medium, but the induction frequency is low (Chu et al 1976). Therefore, rice anther culture is generally divided into two steps: induction of pollen calli and regeneration of the pollen plant.

The factors that affect the initiation of pollen calli have been studied extensively. The genotype of the plant materials used has the greatest influence on the frequency of pollen callus formation. The frequency of pollen callus formation, for example, is much higher in japonica rice than in indicas. The developmental stage of the pollen and the constituents of the culture medium also are important.

Rice anthers ranging from the miduninucleate to the midbinucleate stage can produce pollen calli. Induction frequency is highest in anthers with pollen at the late uninucleate stage, when rice florets have these external morphologic characters:

- The lemma width is almost at mature size but its color is still yellowish; and
- The stamen is 33-50% longer than the glume flower.

Modification of components of the major elements in the medium also increases the induction frequency of pollen calli. We found rice anthers quite sensitive to ammonium salts. Low concentrations of ammonium ions aided pollen calli forma-

Table 1. Constituents of N₆ basic medium.

Component	mg/liter
(NH ₄) ₂ SO ₄	463
KNO ₃	2,830
KH ₂ PO ₄	400
MgSO ₄ · 7H ₂ O	185
CaCl ₂ · 2H ₂ O	166
MnSO ₄ · 4H ₂ O	4.4
ZnSO ₄ · 7H ₂ O	1.5
H ₃ BO ₃	1.6
KI	0.8
Iron ^a	5.57
Glycine	2.0
Thiamine HCl	1.0
Pyridoxine HCl	0.5
Nicotinic acid	0.5
Sucrose	50,000
Agar	8,000
pH	5.8

^a5 ml of solution obtained by dissolving 5.57 g FeSO₄ · 7H₂O and 7.45 g Na₂-EDFA in 1 liter distilled water.

tion, but higher concentrations suppressed it. The best results were obtained when an optimum concentration of ammonium salts was combined with nitrate salts in the medium. On the basis of results of these and other experiments, we established N₆ as an effective medium for calli production (Table 1) (Chu et al 1975). The frequency of pollen callus formation was higher on callus medium than on Miller's or MS medium. In more extensive experiments, the average induction of pollen callus on N₆ medium was 16% in hybrids of rice cultivars and reached 50% in some cases (Yin et al 1976). Hu et al (1978) reported that culturing anthers on liquid N₆ medium increased the induction frequency still further. N₆ medium has been widely used for anther culture of rice in China in the past several years.

Many scientists have studied the hormone requirements for pollen callus formation extensively. So far, scientists have shown that 2 mg 2,4-D/liter is well suited for pollen callus induction and that kinetin is not necessary. The combination of 2 mg naphthylacetic acid (NAA)/liter with 2,4-D was sometimes more efficient in rice anther culture than the use of 3-indoleacetic acid (IAA) (Liang 1978).

Among the organic medium supplements, Wang et al (1974) found that yeast extract and lactoalbumin hydrolysate can help promote the growth of pollen calli. They may be added to the medium separately or in combination. Optimum concentrations are about 500-1,000 mg/liter.

After the medium constituents, the most significant factor in the increase of callus yield is pretreatment with cold temperature (Nitsch and Norreel 1973). Wang et al (1974) suggested that pretreatment of the rice raceme at 10 °C for 2 days would greatly increase the frequency of callus formation. Hu et al (1978) proposed a cold pretreatment in which the anthers were floated on liquid N₆ medium at 8 °C for 4 days, then transferred to agar medium cultured at 30 °C. They found that cold-treated anthers yielded twice as much pollen calli as untreated anthers.

REGENERATION OF POLLEN PLANTS

Liang (1978) studied factors that affect the differentiation of pollen calli, such as the major elements zinc, sucrose, soluble starch, coconut milk, and yeast extract, as well as the concentration and proportion of auxin and kinetin. The complexity of results make a general conclusion difficult but these points may be significant:

1. The age of the pollen callus has a remarkable influence on the differentiation frequency of pollen plants. More than 50% of the pollen calli can produce pollen plants when transferred to the differentiation medium at 10-15 days, but pollen calli older than 50 days produce almost no pollen plants (Wang et al 1974).
2. MS and N6 media induce pollen plants better than Miller's medium.
3. Media supplemented with 0.2 mg IAA/liter and 1 mg kinetin/liter are usually better for differentiation of pollen calli. In a medium devoid of hormones, rice pollen calli can differentiate into plants with an induction frequency approaching that of a medium containing hormone (Chu et al 1976).
4. The frequency of shoot differentiation from pollen callus increases with increasing concentration of sucrose, although a low sucrose concentration is required for initiation of pollen callus. Five percent sucrose is generally appropriate for both induction and differentiation of pollen callus (Chu 1978).

The average induction frequency of green pollen plants, using all effective culture techniques, is more than 5% for japonica varieties and 1% for indicas. In some Chinese laboratories, more than 5,000 green pollen plants have been regenerated in a single season.

DIPLOIDIZATION OF POLLEN PLANTS

It is well known that plants regenerated from pollen callus have different ploidy, including X , $2X$, $3X$, $4X$, and $5X$ plants, but most are haploid and diploid. Huang et al (1978) reported that in 2,496 clusters of regenerated pollen plants of japonicas, 35.3% were haploids and 53.4% were diploids. Yin et al (1976) and Chen and Li (1978) observed that the rate of diploids in pollen plants always exceeded 50%.

Genetic analysis indicated that 90% of the progeny of diploids derived from hybrids were homozygous. Therefore, it can be concluded that diploid pollen plants are caused by spontaneous chromosome doubling during culture and do not originate from somatic anther cells.

For breeding purposes, haploid pollen plants should be diploidized. Chromosome doubling was increased by immersing the roots and tiller nodes in an aqueous solution or in a fine suspension of 0.2% colchicine or 0.1% fumiron (Phenylmercury-P-toluene sulfonamide) (Yin et al 1976). But haploids also can be diploidized by somatic callus culture. We have found that the young spikelets and leaves of haploid rice can readily produce somatic calli on a medium containing 2,4-D. As the result of endomitosis or endoduplication of callus cells, some plants regenerated from somatic callus should be diploid.

ALBINO POLLEN PLANTS IN RICE

Albino plants seem to be a common phenomenon in pollen plants of Gramineae. The frequency of albino pollen plants ranged from 5 to 90%. We found no relationship between the frequency of albinos and medium factors. We have found only two factors related to albino frequency. First, the frequency of albino pollen plants varies among cultivars. For example, albino pollen plants of the cultivar Lien Chiang Mi Tzao are about 70% of those regenerated while those of the cultivar Hung Chi No. 16 are about 10% under similar culture conditions. Second, the culture temperature has an influence. A rise in temperature increases the frequency of albinos (Wang et al 1977).

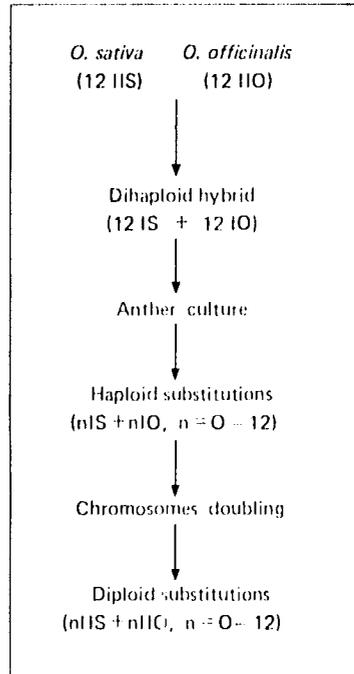
Although albino pollen plants have stimulated widespread interest among anther culture scientists, we do not understand why such high frequencies of albinos occur. We have discovered that protoplasts exist in the leaf cells of albino pollen plants of rice, but they cannot develop into normal chloroplasts because of a lack of ribosomes. Electrophoresis analyses indicated that albino plants have lost their capacity to synthesize fraction I protein. Albino plants also have been shown to differ greatly from green plants in RNA electrophoresis patterns. For example, the 25S and 18S RNA of the cytoplasmic ribosome of albinos were normal but 23S and 16S RNA were absent or rare (Sun et al 1979). The deficiency of plastid rRNA was consistent with the observation under electron microscope that no ribosomes have been found in albino plastids (Sun et al 1974). In albinos of some species, the deficiency of plastid rRNA has been reported to be caused by a variation of plastid DNA (Boener et al 1971). But variation of nuclear DNA also causes the same deficiency (Sprey 1971). The basic cause of albino formation seems to be impaired DNA. We do not yet know whether the impaired DNA occurs in plastids or in nuclei, or in both.

ANTHER CULTURE IN DISTANT HYBRIDIZATION OF RICE

In China, anther culture has been used for conventional hybridization and it is beginning to be used in other breeding methods. I personally feel that the application of anther culture to distant hybridization may be the most promising development in rice improvement. The theoretical potentials of anther culture include:

1. Obtaining stable fertile cultivars from japonica-indica hybrids. F₁ hybrids of japonicas and indicas show partial sterility. The F₁ and succeeding generations segregate vigorously. But anther culture may be used to control such segregation. Hsu (1978) reported that the progenies of pollen plants from japonica-indica hybrids are homozygous diploid and more than 50% are completely fertile. In fact, the early rice cultivar Huateng No. 10 was developed by anther culture of a japonica-indica hybrid. It is grown on more than 333.33 ha in Hupch Province.
2. Heterozygous translocation. If the interspecific hybrids in which chromosomes can be partly paired in meiosis are used for anther culture, some pollen plants with heterozygous translocation chromosomes should be produced. If they are, some genes that confer good grain quality, high yield, and pest resistance can be transferred from one species to another.

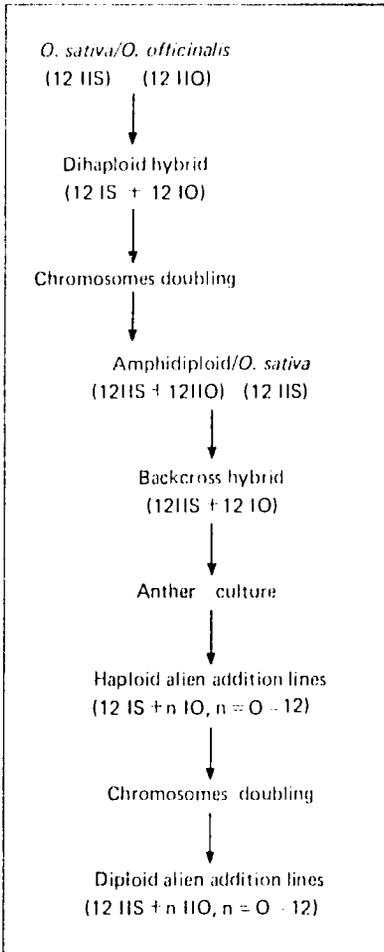
1. An example of a substitution breeding scheme of *Oryza sativa* and *O. officinalis*.



3. Induction of chromosome-substitution and chromosome-addition lines from microspores of interspecific and intergeneric hybrids. A hybrid of *Oryza sativa* and *O. officinalis* explains this experimental scheme. When *O. sativa* is crossed with *O. officinalis*, the F_1 hybrid is dihaploid, showing 12 univalent *O. sativa* chromosomes and 12 univalent *O. officinalis* chromosomes during meiosis. Because of the random distribution of the univalent chromosomes, the microspores of the hybrid should have different numbers of chromosomes, which can be formulated as $nIS + nIO$ ($n = 0-12$). Through anther culture, it should be possible to obtain different chromosome substitution lines from the microspores (Fig. 1).

Anther culture also can be used to induce alien chromosome-addition lines. For example, when amphidiploids that originate from *O. sativa* and *O. officinalis* are backcrossed with *O. sativa*, the reciprocal hybrid should contain 12 bivalent *O. sativa* and 12 univalent *O. officinalis* chromosomes. These pollen plants derived from the microspores of the reciprocal hybrids should be lines adding *O. officinalis* and *O. sativa* chromosomes (Fig. 2).

Nitzsche and Wenzel (1977) reported that 4 albino pollen plants were grown by culturing almost 18,000 anthers of *Festuca pratensis* *Lolium multiflorum* dihaploids, but they could not be used in further breeding work because of a lack of chlorophylls. In 1977, we obtained two pollen calli from a dihaploid by hybridization of *Triticum aestivum* and *Secale cereale*, but they did not differentiate into pollen plants. We also cultured the anthers of hybrids of *Octoploid tritiale* and wheat and obtained lines adding wheat and rye chromosomes (Chu 1978). Similar experiments



2. An example of an addition-line breeding scheme of *Oryza sativa* and *O. officinalis*.

might be conducted more easily with rice than with wheat or Triticale because rice has a higher frequency of induction of pollen plants. Anther culture of distant hybrids in the genus *Oryza* will probably be fruitful and useful in future cytogenetic research and in overall rice improvement.

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APPLICATION OF ANTHER CULTURE TECHNIQUES TO RICE BREEDING

ZHANG ZHEN-HUA

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The production of new cultivars by haploid breeding through anther culture can reduce breeding time, raise the efficiency of selection, and save space and labor in experimental fields (Nizeki and Oono 1968; Chen Yien et al 1974; Chinese Academy of Sciences, Plant Research Institute 1973; Shanghai Academy of Agriculture, Institute of Crop Cultivation, Research Group of Rice, 1976).

But selecting good materials and obtaining ample seedlings are major problems. At present, stress is laid on:

- standardization of haploid breeding techniques by establishing a simplified process for obtaining a large number of pollen plants,
- selection and accumulation of basic materials (parents) that are easy to culture, and
- study of hereditary variation of characters in pollen-plant offspring.

INDUCTION OF RICE POLLEN PLANTS FOR BREEDING

Production of large numbers of pollen-plant lines provides a foundation for obtaining good varieties. Innovation and improvement are needed to make the technique applicable to crop breeding. The hereditary characters of the parents must be taken into account. At the Shanghai Academy of Agricultural Science, anthers are mainly taken from intervarietal hybrids of Keng (japonica) rice and intersubspecific hybrids from Keng and Hsien (indica).

Responses of different genotypic rice pollen to growth hormones

To improve cultural conditions, the effects of growth hormones such as 2,4-D and NAA on inducing calli were tested (Table 1). Different genotypic rice pollen on Miller's medium were supplemented with 2,4-D and NAA. 2,4-D was suitable for calli induction on some materials; NAA was better on others. Selection of suitable plant hormones or cultural conditions may induce calli more easily from certain materials.

Simplification of operations in obtaining pollen plants

Laboratory techniques for plant tissue culture customarily use pure reagents, accurate methods, and strict cultural conditions, but these are not practical for a large-scale breeding operation.

One experiment comparing the effects of double-distilled water, distilled water, well water, river water, and rain water on calli induction showed no obvious differences (Table 2).

Table 1. Differences in genotypic pollen responses to 2,4-D and NAA using Miller's medium.

Material	Component added ^a	Time of culture (days)	Anthers inoculated (no.)	Calli gained	
				No.	%
Mei Zao/Mi Nian F ₂ single line	2,4-D 2 mg/liter	72	1524	38	2.5
	NAA 2 mg/liter	73	1388	50	3.6
Nong Hu 6/7623 F ₁	2,4-D 2 mg/liter	63	1620	191	11.8
	NAA 2 mg/liter	61	819	42	5.1
	NAA 4 mg/liter	76	485	73	15.0
Guang Zuan 78/ Nong Hu 6//Tai Nan 13 F ₁	2,4-D 2 mg/liter	51	1385	182	13.1
	NAA 2 mg/liter	48	687	57	8.3
	NAA 4 mg/liter	51	723	69	9.5
302/A10-4 F ₁	NAA 2 mg/liter	55	271	51	18.8
	NAA 4 mg/liter	54	108	38	35.2
Lai Zhong Yu// Ken Gui/Ai 55 F ₁	2,4-D 2 mg/liter	55	585	22	3.8
	NAA 2 mg/liter	55	232	6	2.4
Ai Huang Zhong/ Ai Pi Nuo F ₁	2,4-D 2 mg/liter	60	1330	89	5.7
	NAA 4 mg/liter	60	313	48	15.4
302/Gong Qin 2γ F ₁	2,4-D 2 mg/liter	70	1229	76	6.2
	NAA 2 mg/liter	68	199	1	0.4

^a2 mg/liter = 6% saccharose concentration, 4 mg/liter = 3% saccharose concentration.

Table 2. The effect on callus induction of water from different sources.

Type of water	Anthers inoculated (no.)	Calli emerged (no.)	Induction rate (%)
Double distilled water	960	31	3.2
Distilled water	1055	45	4.3
Well water	940	55	5.8
River water	553	22	4.0
Rain water	2096	71	3.4

The effects of river water and well water, even though they contained many foreign substances, were no worse than those of double-distilled water. The pH value of the medium was not critical. A pH of 5.4-6.5 was suitable. Purity of ingredients in the medium and precise weighing did not appear necessary. These procedures for large-scale culture were tried:

- Mineral nutrients were prepared as a mother liquor, diluted 10-20 times when used.
- Mineral nutrients were weighed on a common scale with a 1% error allowance.
- Organic compositions were preprepared and stored in a refrigerator.
- Test tubes were cleaned only to get rid of poisonous material.
- Medium was mixed and pH adjusted.
- Agar was dissolved and put into test tubes.
- Batches of 400 test tubes were put in a metal basket and sterilized.

With this standard method, a large-scale culture of 125 combinations from Keng rice intervarietal crosses and from Hsien-Keng intersubspecific crosses were cultured and 5,106 clumps of green seedlings were obtained (each clump had from 1 to 20 plants). With such a large number of pollen plants, a great number of pedigree lines with more stable characters could be selected and evaluated in the second generation.

VIABILITY OF POLLEN-PLANT OFFSPRING AND STABILITY OF THEIR CHARACTERS

Whether the characters of pollen-plant offspring can be maintained with relative stability and uniformity is a major consideration in anther culture rice breeding. More than 2,000 pollen-plant offspring from more than 500 hybrids were observed. Only about 3% showed segregation in the same lines. Most lines had stable and uniform characters.

Pollen-plant offspring from Keng/Hsien hybrids

Distant hybridization has generally been used as a special breeding method to create varieties with pest resistance and good grain quality and to create new plant types. But it is difficult to utilize distant hybrids in large-scale breeding programs because their seed sets are abnormal and their characters are not easy to stabilize.

With pollen plants, some difficulties in distant hybridization breeding can be overcome. Although distant hybrids often are sterile or semisterile, their anthers can be used as the material for culturing pollen plants, most of which normally set seeds.

Pollen plants from the first generation of a Keng-Hsien cross and their offspring normally set seeds. The offspring appeared relatively stable and uniform (Table 3).

Viability of pollen-plant offspring and stability of characters

Genetically, if the characters of second-generation pollen plants are relatively stable and uniform, they can be regarded as homogeneous. But whether these characters are stable and whether viability and growth vigor decrease gradually in later generations are important considerations. Comparisons of selected lines of pollen plants derived from the pollen plant of a first-generation hybrid Ken-Gui (Nong Ken 58-Gui Hua Huang-Ke Qin 3) showed relatively stable and uniform characters

Table 3. Analysis of some characters of pollen-plant offspring from an intermediate-type Hsien/Keng cross.

Experimental material ^a	Plant ht		Ear length	
	X + S	C.V. (%)	X + S	C.V. (%)
Suang Fong 1/Liberate// Qian Chong Lang/Nong Ken 58 F ₁	94.5 + 4.23	4.5	22.1 + 1.64	7.4
Zhong Yin 15/Hsien 762 F ₁	79.5 + 3.87	4.9	21.8 + 1.73	7.9
Ai Nan Zao 1/Nong Ken 58// Guang Tou Len Sui F ₁	58.8 + 6.28	11.3	21.6 + 1.50	6.9
Jia Nong 485/Liberate// Tai Nan 13 F ₁	91.1 + 4.22	4.6	17.0 + 1.18	6.9

^aF₁ = second-generation hybrid.

among individual plants of the same generation and of different generations (Table 4). Their viability remained the same. Once pollen plants were obtained, most became stable lines. The coefficient of variance did not increase with the increase of generations. The fifth generation of a pollen plant planted in the field did not show any segregation at Fu Jian Province, Hai Nan Island, or the Academy. The coefficient of variance of plant height was below 5%.

In hybridization breeding, transfer of characters to the hybrids is often interrupted by dominance and recessiveness. Hybrid vigor decreases progressively from F₂ hybrid offspring, which lose their good qualities, often because of a loss of heterosis and a decrease in viability. Selection is interrupted. But Table 4 shows that among the successive generations there were no characters in which the viability of pollen plants obviously decreased successively. Pollen plants could be selected and evaluated during early generations as a basis for reducing breeding years to speed a breeding program.

USE OF ANTHER CULTURE IN THE REFORM OF BREEDING PROCEDURES

Procedure for rice haploid breeding by anther culture

Breeding procedures to ensure that various measures of breeding are carried out depend on the hereditary variations of breeding materials. The pedigree method of crossbreeding now used requires five nurseries. But in haploid breeding, pollen plants and their offspring possess stable and uniform characters. Several hundred, even several thousand, seeds can be obtained in the first-generation pollen plant. In the second generation, they can be planted in a plot for comparison and evaluation.

The new variety Xin Xiou and the strains 76057 and 77001 were obtained from a haploid breeding experiment begun in 1972 (Table 5). The experiment suggested that the process of haploid breeding could be divided into three stages:

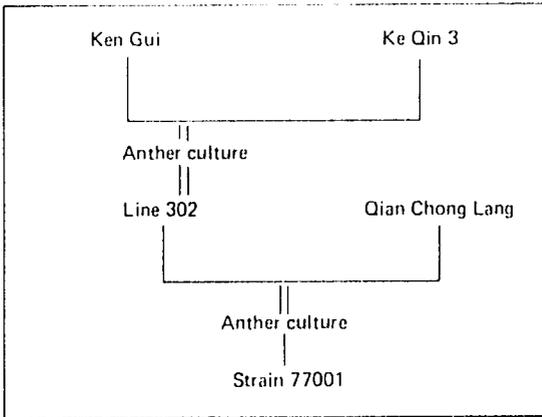
1. preparation of materials and culture of pollen plants,
2. evaluation and selection of pollen-plant offspring, and
3. comparison of varieties.

Table 4. Some characters of pollen-plant offspring from Ken Gui/Ke Qin 3.

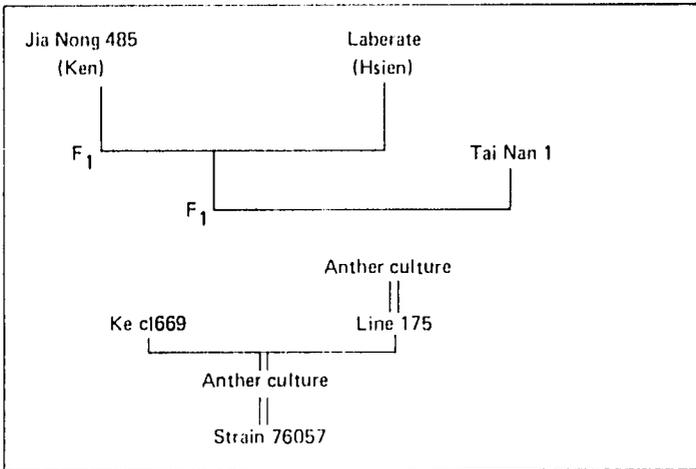
Material	Sowing date	Plant ht		Ear length		Flag leaf		Effective ears (no./hole)	1000-grain wt (g)
		X + S	C.V. (%)	X + S	C.V. (%)	X + S	C.V. (%)		
Pollen plant second generation	23 June 1973	81.3 + 3.01	3.68	15.1 + 1.16	7.68	16.8 + 2.36	14.0		
Second generation	25 June 1974	84.7 + 3.5	4.13	13.8 + 0.74	5.36	13.7 + 2.35	17.15	6.9	27.0
Third generation	25 June 1974	84.7 + 2.24	2.73	13.6 + 0.57	4.19	14.8 + 2.38	16.08	7.2	27.2
Fourth generation	25 June 1974	84.3 + 3.15	3.75	14.1 + 0.74	5.25	15.5 + 2.71	17.48	6.9	28.0
Fifth generation	25 June 1974	82.0 + 2.96	3.62	14.2 + 0.65	4.58	15.6 + 2.45	15.71	6.8	27.0
Fifth generation (in field)	25 June 1974	99.9 + 4.57	4.57	15.3 + 1.14	7.45	21.0 + 4.64	22.1	6.2	27.0

Table 5. Haploid breeding of Xin Xiou.

Year	Experiment
1972	Hybridization -- Ken Gui (Nong Ken 58/Gui Hua Huang//Ke Qin 3)
1972	F ₁ pollen plant obtained from anther culture
1973	Evaluation of second generation of pollen-plant 302
1974	Evaluation of production and comparison of varieties
1975	Field trial at several sites
1976	Demonstration plots
1977	Demonstration plots and dissemination of seed



1. Selection and improvement of strains 77001 and 76057.



Rehybridization and anther culture application in multiple crosses

A multiple cross is effective in combining many good parental characters. But the characters of offspring take a long time to become stable and it is difficult to select at early generations. Because the gametes (pollen and embryo sac cell) are heterozygous, a great many genotypes appear different from phenotypes, which were interrupted by dominance, recessiveness, and heterosis, adding much blindness to the selection.

The pollen plants produced through anther culture techniques were homozygous, with no differences between genotype and phenotype. When only the desirable plants and lines were selected, cross and backcross made, and pollen from hybrids cultured, selection blindness was overcome and the interference of heterosis was eliminated. Anther culture not only increased the correctness of selection of multiple crosses, but significantly reduced the number of breeding years. Recombination of hybrid characters was made by multiple crosses and anther culture. Some of the rice strains obtained are being planted as popular demonstrations (Fig. 1).

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THE DEVELOPMENT OF ANTHUR CULTURE AS A SYSTEM FOR IN VITRO MUTANT SELECTION

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Intensive efforts in recent years have considerably increased the spectrum of plant species for which totipotent cell culture systems have been established. This rapidly expanding ability to regenerate fertile plants from cultured cells introduces new possibilities for genetic experimentation with higher plants. However, these advances have not been realized with many important crop species. Widely heralded expectations for the genetic improvement of crop species by the application of cell culture methodology are largely unfulfilled.

Early successes with anther culture of rice provided the basis for developing an in vitro genetic system with this major cereal species (Niizeki and Oono 1968, 1971; Nishi and Mitsuoka 1969; Iyer and Raina 1972; Guha-Mukherjee 1973; Wang et al 1974). Using this technique, microspores contained within immature anthers are induced to form callus from which plants are regenerated. An important advantage of using this system in mutant selection experiments is that the microspore-derived calli are haploid.

A disadvantage of using rice anthers as a source of cells for genetic studies is that typically callus is produced by fewer than 10% of the anthers cultured (Niizeki and Oono 1968, 1971; Iyer and Raina 1972; Wang et al 1974; Oono 1975). Frequencies of

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When available, names of scientists in China are given in the traditional Chinese order. Surname followed by full given name with no comma. Initials of given names normally are not used. Given names are hyphenated with only the first letter capitalized.

callus formation higher than 20% have been reported in only a few instances (Chu et al 1975, Chaleff et al 1975, Chen and I in 1976, Genovesi and Magill 1979). These studies involved relatively small numbers of anthers.

Moreover, rice calli obtained by anther culture rapidly lose their capacity to form plants during continued propagation in vitro and many of the regenerated plants are albino (Oono 1975). Green plants have been produced at a high frequency from cultured rice anthers in only one case (Chen 1977).

Because mutation is a rare event, the successful isolation of mutants requires the screening of enormous numbers of cells. In addition, a genetic analysis of such mutants is possible only if fertile plants can be regenerated from the cultured cells. However, populations of totipotent rice cells large enough for genetic studies cannot be obtained by subculturing callus because the morphogenetic capacity of such cultures diminishes rapidly. Alternatively, large populations of initially totipotent haploid cells could be produced by increasing the frequencies of both callus and green plant formation from cultured anthers. This strategy provided the basis for the studies reported here.

The recovery of primarily albino plants from microspore-derived calli has been a formidable obstacle to the utilization of rice anther culture. Results from several laboratories have indicated that the proportion of regenerated plants that are albino is genetically determined (Oono 1975, Chen and I in 1976). Attempts to increase the proportion of photoautotrophic plants regenerated by modifying the medium composition or the culture conditions have been unsuccessful (Chaleff, unpubl.).

It appeared that the choice of variety would be critical to insuring the recovery of a high frequency of green plants. Oono (1975) reported the differentiation of primarily green plants from microspore-derived callus of the japonica variety Minchikari. Using Minchikari anthers, culture conditions were varied to define those that would provide the maximum frequency of callus formation. Using this procedure, haploid rice calli capable of forming photoautotrophic plants have been obtained with a reproducibly high frequency.

MATERIALS AND METHODS

Plant material

Seeds of *Oryza sativa* cv. Minchikari were obtained from the International Rice Research Institute (IRRI). Plants were grown in a room maintained at 80-95% relative humidity. Day temperatures were 28-30°C and night temperatures 18-20°C. A mixture of cool-white fluorescent (80%) and incandescent (20%) lamps provided a photosynthetic photon flux density of 350 μ Einstein sec per square meter at the soil surface during a 13-hour day. Plants supported in a mixture of peat and vermiculite were irrigated daily with the culture solution of Yoshida et al (1972), from which minor elements were omitted.

Panicles were harvested prior to emergence from the flag leaf sheath when the base of the flag leaf was 3-5 cm above the base of the penultimate leaf. Cold treatment was performed by sealing panicles in polyethylene bags and refrigerating at 7°C. Then panicles were removed from the leaf sheath, surface-sterilized in a solution of 7% calcium hypochlorite and 0.1% sodium dodecyl sulfate, and tho-

roughly rinsed with sterile distilled water. Individual spikelets were detached from the panicle and dissected.

Flowers containing pollen in the mid- to late-uninucleate (microspore) stage of development could be identified by their characteristic morphology. Extensive cytological studies have correlated external features of the developing flower with the stage of pollen development. This method, although only approximate, was the sole means by which large numbers of anthers of nearly the same developmental stage could be obtained.

Culture medium

The basic medium (designated R3), developed by Einsmaier and Skoog (1965), was modified to contain 5 mM NH₄NO₃, 25 mM KNO₃, 3% sucrose, 2 mg α -naphthalene acetic acid (NAA) liter, and 0.3 mg kinetin liter. Media were solidified by the addition of 0.4% agarose to avoid possible effects of contaminants in agar preparations upon callus formation. Phytohormones were added to the autoclaved medium as concentrated solutions sterilized by filtration. Kinetin, NAA, 2,4-dichlorophenoxyacetic acid (2,4-D), and indole-3-acetic acid (IAA) were used. The addition of 2-chloroethylphosphonic acid (CPA) was accomplished by adding 1 ml of a filter-sterilized solution to a well made in the agar medium with a scalpel. Final concentrations of CPA were calculated by dividing the amount in the 1 ml aliquot by the volume of medium in the petri dish (30 ml).

Statistical analysis

The population of rice flowers being sampled was assumed to be a mixture of two subpopulations: flowers from which no callus formation is possible and flowers for which each anther has a nonzero probability of producing callus (competent flowers). The population fraction of flowers from which callus formation is not possible is denoted θ . If callus formation occurs independently among the six anthers of a competent flower and if the likelihood of forming callus is the same for all competent flowers, then the number of anthers producing callus per competent flower would have a binomial distribution.

If p is the proportion of anthers in the competent subpopulation that produce callus, then the probability distribution of the number of anthers forming callus (out of six) in a flower selected at random from a mixed population would be:

$$(*) f(x) = \begin{cases} \theta + (1-\theta)(1-p)^6 & \text{for } x = 0 \\ (1-\theta) \frac{6!}{x!(6-x)!} p^x (1-p)^{6-x} & \text{for } x = 1, 2, 3, \dots, 6 \end{cases}$$

To test the adequacy of this model, θ and p must be estimated from the data. Maximum likelihood estimators are values of θ and p that maximize the probability of occurrence of the sample actually observed. If anthers from N flowers are plated and f is the number of these flowers from which x anthers form callus ($x = 0, 1, 2, \dots, 6$; and $t = t_0 + f_1 + \dots + f_6 = N$), then the probability of the observed sample is:

$$l(\theta, p) = [0 + (1 - \theta)(1 - p)^6] f_0 + \sum_{x=1}^6 [(1 - \theta) \frac{6!}{x!(6-x)!} p^x (1-p)^{6-x}] f_x$$

With $q = 1 - p$, the maximum likelihood estimation of q is the solution (of which there is exactly one between zero and one) of

$$1 + q + q^2 + q^3 + q^4 + q^5 = \frac{(N - f_0)}{f_1 + 2f_2 + 3f_3 + 4f_4 + 5f_5 + 6f_6}$$

which can be easily obtained numerically. Having found q , the estimation of θ is:

$$\theta = \frac{\frac{f_0}{N} - q^6}{1 - q^6}$$

To test for fit of the model, the estimators of $p (= 1 - q)$ and θ are substituted into the probability distribution (*) and each solution is multiplied by the number of flowers, N , to obtain the expected frequencies. These expected frequencies are compared to the observed frequencies f_0, f_1, \dots, f_6 by a chi-square goodness-of-fit test. The maximum likelihood estimators are such that the expected frequency of flowers from which no anthers will produce callus is f_0 , the expected frequency. Since there are seven classes ($x = 0, 1, 2, \dots, 6$) and two parameters estimated (θ, p), the X^2 statistic has $7-2-1 = 4$ degrees of freedom.

RESULTS

Variability in anther culture system and effect of cold treatment

The conditions under which plants are grown influence the frequency of callus formation from cultured anthers (Dunwell 1976, Foroughi-Wehr and Mix 1979). But most efforts to characterize factors affecting the efficiency of callus or embryoid production from cultured anthers do not utilize plants grown in a controlled environment. As the experimental material is not uniform in such cases, the validity of comparisons between results of experiments performed on different days must be questioned.

In the current studies, plants were grown in a room where illumination, day length, temperature, and humidity were maintained at as constant levels as possible. Despite these measures, callus formation frequencies still proved variable. This variability is apparent in the results of experiments intended to determine the effect of cold treatment duration on callus formation frequency (Table 1).

Although their cause is unknown, these fluctuations in the efficiency of callus production appeared to be seasonal. Among anthers maintained at 7°C for 3 days, the frequency of callus formation was higher in experiments performed in the summer than in experiments performed in the winter. A similar decline in the frequency of callus formation was observed the following winter (data not shown). Callus formation frequency was enhanced by maintenance of flowers at 7°C for periods of 3 days or more and the effect of cold treatment was greater in the winter than in the summer. Although the frequency of callus formation from anthers

receiving a 3-day cold treatment was lower in the winter than in the summer, the maximum frequency obtained in both seasons was nearly the same.

Because of the variability in responsiveness to culture of anthers obtained from plants grown at different times, in all experiments some anthers that had received a 3-day cold treatment were plated on R3 medium to provide a standard for evaluating the effect upon callus formation frequency of varying several parameters of the culture system. By combining data from a number of experiments in which the medium composition varied, the responses of large populations of anthers to a particular medium could be determined and compared with similarly pooled populations that had been plated on the standard R3 medium at the same time. Only by this method could it be concluded with any certainty that differences observed resulted from a particular modification of medium composition or pretreatment conditions rather than from the system's inherent fluctuation.

Effect of hormones

Although supplementation of the medium with NAA increased the frequency of callus formation, some callus also was produced in its absence. NAA concentrations of 2 mg/liter (10.7 μ M) or higher were most effective in promoting callus formation (Table 2). The frequency of callus formation on R3 medium was not increased significantly by the addition of 0.5 mg 2,4-D/liter (2.3 μ M) (Table 3). Callus formation was inhibited by IAA (Table 3) and 2-chloroethylphosphonic acid (Table 4).

Table 1. Effect of cold treatment on callus formation.

Days at 7°C	Anthers plated (no.)	Anthers forming callus	
		No.	%
<i>Summer, 1978</i>			
0	480	149	31.0
2	571	172	30.1
3	1814	681	37.5
4	996	415	41.7
7	599	233	38.9
<i>Winter, 1978-79</i>			
3	892	183	20.5
7	1206	432	35.8

Table 2. Effect of NAA concentration on callus formation.

NAA concn (mg/l)	Anthers plated ^a (no.)	Anthers forming callus	
		No.	%
0	518	52	10.0
1	604	135	22.4
2	1064	357	33.6
3	366	118	32.2
5	1273	438	34.4
6	517	152	29.4

^aMedia contained 0.3 mg kinetin/liter. Flowers pretreated for 3 days at 7°C.

Table 3. Effect of auxins on callus formation.

Hormone composition (per liter)	Anthers plated ^a (no.)	Anthers forming callus	
		No.	%
2 mg NAA	1259	540	42.9
1 mg NAA + 5 mg IAA	601	79	13.1
1 mg NAA + 10 mg IAA	587	30	5.1
2 mg NAA + 5 mg IAA	715	87	12.2
2 mg NAA + 0.5 mg 2,4-D	972	439	45.2
2 mg NAA + 0.5 mg 2,4-D + 5 mg IAA	342	80	23.4

^aFlowers pretreated for 3 days at 7°C.

Table 4. Effect of 2-chloroethylphosphonic acid (CPA) on callus formation.

CPA concn (ppm)	Anthers plated ^a (no.)	Anthers forming callus	
		No.	%
0	1067	238	22.3
1	720	124	17.2
2	802	149	18.6
5	706	106	15.0
10	682	92	13.5

^aFlowers pretreated for 3 days at 7°C.

Table 5. Effect of sucrose concentration on callus formation.

Sucrose concn (%)	Anthers plated ^a (no.)	Anthers forming callus	
		No.	%
<i>On medium solidified with 0.4% agarose</i>			
0	624	0	0
1	360	128	35.6
2	590	207	35.1
3	838	309	36.9
4	789	344	43.6
5	273	97	35.5
6	838	337	40.2
<i>On medium solidified with 0.2% agarose</i>			
1	410	37	9.0
2	275	54	19.6
3	179	53	29.6
4	358	128	35.8
5	383	135	35.2

^aFlowers pretreated for 3 days at 7°C.

Effect of sucrose concentration

Although sucrose is required for callus formation, its concentration in media solidified with 0.4% agarose had little effect on the frequency of callus formation (Table 5). The results do suggest that a sucrose concentration of 4% may be slightly more favorable to callus formation. However, on media containing 0.2% agarose, a

greater dependency of callus formation frequency on sucrose concentration is apparent. A maximum frequency is still realized with 4% sucrose, but the callus formation frequencies are considerably reduced at lower sucrose concentrations.

It is possible that the osmotic pressure of media solidified with 0.2% agarose and containing less than 4% sucrose is below an optimal threshold for callus formation. The effect of sucrose concentration upon the callus formation frequency may be due to its contribution to the osmotic pressure of the medium rather than to its utilization as a carbon source.

To determine if the callus formation frequency was affected by the osmotic pressure of the medium, anthers were plated on media (standard R3 medium solidified with 0.4% agarose) supplemented with varying concentrations of mannitol. The callus formation frequency was maximal on a medium containing 50 mM mannitol and declined with increasing mannitol concentrations (Table 6). This result suggests that the slightly higher callus formation frequency observed on a medium containing 4% sucrose than on a medium containing 3% sucrose is at least partly due to an osmotic effect of the additional sucrose (29 mM). A medium containing 4% sucrose (designated R4) has been adopted for future use.

Effect of other organic additives

Callus formation frequency was depressed slightly by the addition of 0.5% (wt/vol) inositol. On a medium containing inositol, 282 of 764 anthers (36.9%) formed callus. On the standard R3 medium, callus formed from 344 of 817 anthers (42.1%). Callus formation was inhibited more severely by the addition of 0.1% (wt/vol) casein hydrolysate. On a medium supplemented with casein hydrolysate, 370 of 1,387 anthers (26.7%) formed callus. In contrast, 292 of 862 anthers (34.0%) plated on the standard R3 medium formed callus.

Variability of callus formation frequency

Rice flowers were selected for dissection on the basis of certain morphological characteristics. Because these morphological criteria are ultimately subjective, they may not have been applied uniformly in all experiments. The correlation between a particular floral morphology and the microspore stage of pollen development (or other parameter critical to callus formation) that has been used for flower selection may apply only to plants grown under certain conditions. Physiological variation

Table 6. Effect of mannitol concentration on callus formation.

Mannitol concn (mM)	Anthers plated ^d (no.)	Anthers forming callus	
		No.	%
0	679	210	30.9
50	447	154	34.5
100	544	125	23.0
150	299	73	24.4
200	213	36	16.9
250	343	38	11.1
300	231	16	6.9

^dFlowers pretreated for 3 days at 7°C.

among plants could invalidate this correlation and result in the selection of flowers that are not in the developmental stage most favorable to callus formation.

There is ample reason to suppose that the population of rice flowers being sampled represents a mixture of two subpopulations—one consisting of flowers that contain anthers capable of forming callus (competent flowers) and another of flowers that are not capable of forming callus.

Within the subpopulation of competent flowers the probability of callus formation may be the same for all anthers or the probability may vary between flowers. The likelihood of callus formation may be greater for anthers from some competent flowers than from others within the same subpopulation. If callus formation occurs independently among the six anthers of a competent flower and the probability of callus formation is the same for all competent flowers, then the number of anthers forming callus per competent flower will conform to a binomial distribution.

To determine if callus formation obeys these principles, only the subpopulation of competent flowers, not the proportion of the population that are not competent to form callus (designated θ), was tested. To maximize the probability of occurrence of the experimental results, the values of θ and p (the proportion of anthers in the competent population that form callus) were estimated directly from the data. Even using this procedure, the results in Table 7 do not fit a binomial distribution. It is evident that the probability of callus formation is not the same for all anthers from the subpopulation of competent flowers. Anthers from some flowers are more likely to produce callus than are anthers from others.

Origins of callus from microspores

After 10-14 days of culture, many anthers squashed in acetocarmine stain showed darkly staining multicellular callus within an intact pollen exine. Later stages of callus developed from microspores have been observed by scanning electron microscopy (Stelczar, in preparation).

Plant regeneration

Shoots developed spontaneously from most anther-derived calli maintained on R3 medium. Among a sample of 83 calli, 56 gave rise to green shoots, 2 formed albino shoots, and 1 produced both green and white shoots. Shoots did not regenerate from 24 calli (28.9%). All shoots formed roots and developed into plantlets.

Variant selection

Calli from cultured anthers were transferred to an R3 medium supplemented with 1 mM lysine + 0.5 mM threonine. Occasionally, a resistant callus appeared on this selective medium. Approximately half of the resistant calli gave rise to plantlets on the selective medium. Expression of resistance by progeny of six diploid regenerated plants was tested by planting embryos dissected from seeds on a medium lacking plant hormones and supplemented with 2 mM lysine + 1 mM threonine. Seedling development from embryos produced by normal and by variant plants was equally inhibited by lysine plus threonine. Callus obtained by culturing anthers of plants regenerated from two variant calli also was sensitive to a mixture of 1 mM lysine + 0.5 mM threonine.

Table 7. Number of anthers producing callus within individual flowers.^a

	Anthers (no.) from which x number (0-6) formed callus							Flowers (no.)	Anthers forming callus (%)	Estimates		
	0	1	2	3	4	5	6			<i>θ</i>	<i>p</i>	χ^2
Observed	173	103	103	80	47	16	5	527	26.8	0.285	0.375	31.44
Expected	173	81	121	97	44	10	1					

^aFlowers pretreated for 3 days at 7°C.

DISCUSSION

In the present studies, a medium (R4) was developed on which more than 35% of plated rice anthers from flowers in a suitable developmental and physiological state formed callus. Green plants were regenerated from more than 60% of these calli. In the system for efficient production of green plants from cultured rice anthers, medium composition and plant genotype are equally important components. The R4 medium may not promote callus formation as efficiently from anthers of rice varieties other than Minchikari.

A few calculations show that *in vitro* genetic experiments are feasible with this system. One person can easily plate 1,500 anthers/day. Approximately one third, or 500, of these anthers are expected to form callus. As separate calli develop from an average of at least 10 microspores/anther, a conservative estimate of the number of individual clones that can be obtained per day per person is 5,000. If these clones are allowed to grow into small calli of 50-100 cells before transfer to a selective medium, a minimum population of 2.5×10^5 cells is obtained. Applying an effective mutagenic treatment to these calli assures recovery of at least one individual with a mutation in a given locus from a single day's plating.

In the current studies, frequency of callus formation responded significantly to qualitative but not to quantitative changes in the medium composition. Although the callus formation frequency was enhanced by an auxin, and NAA proved more effective than IAA, the concentration of NAA was not critical. Similarly, sucrose was required for callus formation, but on a medium solidified with 0.4% agarose, sucrose concentration had little effect on the frequency of callus formation. This result contrasts with the findings of other investigators (Matsubayashi and Kuranaki 1975, Chen 1978).

A more pronounced effect of sucrose concentration was observed on media containing 0.2% agarose. Since the callus formation frequency on R3 medium was enhanced to approximately the same extent by the addition of 50 mM mannitol as by 29 mM sucrose (4% final concentration), the effect of sucrose concentration upon the frequency of callus formation may be exerted through its contribution to the osmotic pressure of the medium. As the osmotic pressure of a medium containing 0.2% agarose is less than that of a medium containing 0.4% agarose, addition of sucrose to the 0.2% medium is expected to produce a greater effect on the frequency of callus formation.

Addition of 2-chloroethylphosphonic acid has been reported to improve the frequency of callus formation (Wang et al 1974). But the effect of this compound was deleterious in these studies. However, that result is of questionable significance because the rate of ethylene release was not controlled and the concentration of ethylene at any given time was not known.

It also has been reported that the fraction of rice anthers forming callus is increased dramatically by maintaining the flowers at temperatures of 6-13°C prior to dissection (Wang et al 1974, Chaffee et al 1975, Genovesi and Magill 1979). In the current studies, pretreatment of flowers at 7°C had only a small effect on the frequency of callus formation in one series of experiments. A much greater effect of cold pretreatment was observed in a second series of experiments using flowers

harvested at a different time of year. This result suggests that the degree to which the callus formation frequency is influenced by cold pretreatment and even by modifications of the medium composition is determined by the physiological state of the whole plant.

It also is evident from these results that, despite an effort to grow plants under uniform conditions in a chamber, fluctuations (seasonal or otherwise) occurred in the responsiveness of anthers to culture. Since the frequency of callus formation among individual flowers does not fit a binomial distribution, the responsiveness to culture varies even between flowers of a given population. It appears that callus formation frequency could be most improved by devising means of identifying those flowers containing anthers most inclined toward callus formation and by defining optimal plant growth conditions rather than by continuing to explore modifications of the culture medium.

Selection of mutations affecting regulation of the biosynthesis of lysine, methionine, threonine, and isoleucine was attempted by transferring microspore-derived calli to a medium containing an inhibitory concentration of lysine plus threonine. In the past, variant rice cell lines containing elevated levels of free lysine, methionine, and isoleucine were isolated by plating mutagenized diploid cell suspensions derived from seeds on a medium supplemented with the lysine analogue S-2-aminoethylcysteine (SAEC). It was not possible to regenerate plants from these variant cell lines.

However, the greater growth rate differential between normal and variant cells on lysine plus threonine than on SAEC suggested that variants would be selected more efficiently by the amino acid mixture than by the analogue (Chaleff and Carlson 1975). Resistant calli were isolated and plants were regenerated by plating totipotent haploid rice calli on a medium supplemented with 1 mM lysine plus 0.5 mM threonine. Resistance of most of the variant cell lines was stable through successive passages in culture. However, resistance to lysine plus threonine was not expressed by embryos produced by six diploid regenerated plants or by secondary callus cultures derived from anthers of two of these plants. These observations suggested, that in the variants recovered, resistance to lysine plus threonine was effected by epigenetic changes that were expressed in the primary selected cell line but were not retained in the secondary callus cultures nor in embryos obtained from regenerated plants.

Selection for resistance to a mixture of lysine plus threonine has been used to isolate mutants from maize tissue cultures. Maize callus cultures initiated from the scutellum of immature embryos retained their morphogenetic capacity following protracted periods in culture (Green and Phillips 1975). Fertile plants could be regenerated from a cell line capable of growth on a medium containing a normally toxic concentration of lysine plus threonine. Reciprocal backcrosses of these regenerated plants, which presumably were diploid and heterozygous, yielded both resistant and sensitive progeny. A resistant plant obtained from a backcross produced a majority of resistant progeny when selfed and nearly equal numbers of resistant and sensitive progeny when crossed again with a normal plant. These results, in combination with the biochemical data, show that resistance to lysine plus threonine is due to a single semidominant nuclear mutation. Homozygous mutant

kernels contain nearly eightyfold more free threonine, threefold more free serine, and fourfold more free methionine than normal kernels. In heterozygous kernels, the amount of free threonine is increased thirtyfold (Hibberd and Green personal comm.). The discovery of a mutation in maize conferring resistance to lysine plus threonine that affects regulation of the biosynthesis of the aspartate-derived amino acids and the amino acid composition of the seed is exciting. The dream of employing cell culture to achieve the genetic improvement of crop plants is fast becoming a reality.

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TISSUE CULTURE WORK ON RICE IN KOREA

GUN SIK CHUNG

Breeding work utilizing rice anther culture began at the Crop Experiment Station, Suweon, Korea, in 1977. Results obtained so far and progress in breeding work are described here.

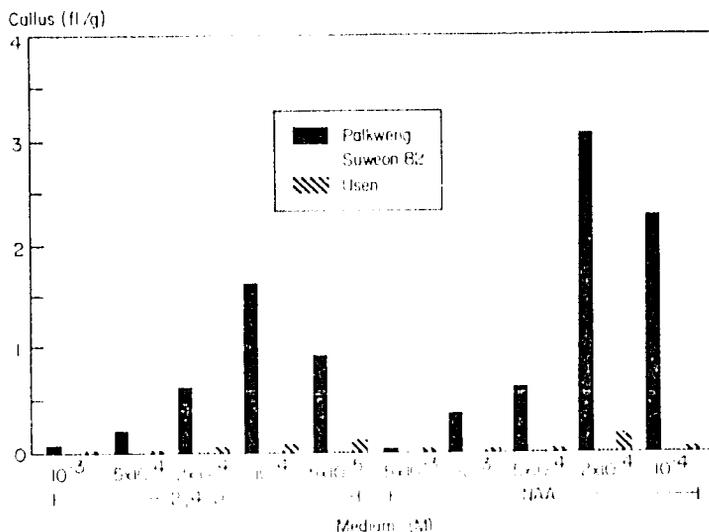
Varietal variation in callus formation

It is well known that auxin should be included in the culture medium for plant tissue culture. Optimum levels of 2,4-D and NAA, popular auxins for callus-inducing media, were studied in 1972. The amount of callus induced from rice seedlings at 30 days after seeding on media differed among three rice varieties and among levels of auxins (Fig. 1). Palkweng, a japonica rice variety, showed the greatest amount of callus at all levels of auxins used and Usen, an indica variety, showed the least. The optimum level for callus formation was 10^{-5} M for 2,4-D and 2×10^{-5} M for NAA. It was considered that media composition should be improved for specific rice varieties like Usen, the lower callus-forming variety.

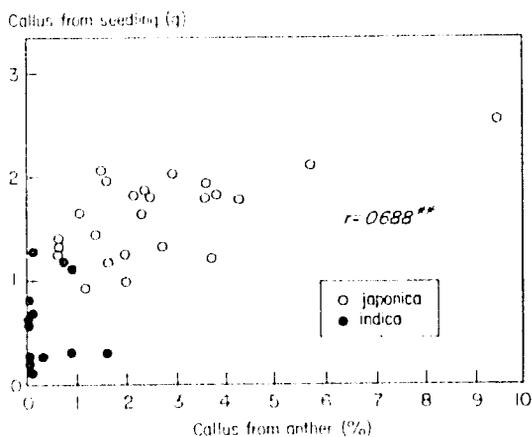
Another experiment also showed great varietal variation in degree of callus formation from seedlings and anthers of 38 rice varieties. Generally, japonica rice varieties were superior to indica varieties in callus formation from both seedlings and anthers (Fig. 2). Callus induced from seedlings and from anthers showed highly significant correlations. The degree of callus formation from diploid plant tissue appeared to equal that from haploid plant tissue.

Callus formation and organogenesis in the F_1

In a study of callus formation and organogenesis in 10 varietal crosses, seed of a



1. Effect of auxin concentration on callus formations induced from rice seedlings.



2. Correlations between callus tissue formation of seedlings and anthers.

Paikweng Usen cross showed a higher degree of callus formation than seed of a Usen Paikweng cross (Table 1). The seed of Tongil IR24, with parents showing a medium degree of callus formation, produced more callus than the seed of either parent. A lower degree of callus formation was generated in seed of crosses whose parents had shown less callus formation. There were no differences in callus formation among reciprocal crosses. The higher degree of callus formation appeared as complete dominance or overdominance in the F_1 hybrid generation (calculated by the formula $(F_1 - P) / D$).

Plant organ differentiation from seedling callus showed great variation among rice varieties. Differences in the degree of plant organ inducement from F_1 seed callus observed in the 10 varietal crosses are shown in Table 2. Plant organ

Table 1. Heterosis in callus formation of hybrid seedlings.

Cross	Callus formation (1-5)				
	P ₁	P ₂	F ₁	D ^a	(F ₁ -P)/D
Palkweng/Usen	5.0	2.0	4.5	3.0	0.8
Usen/Palkweng	2.0	5.0	4.3	3.0	0.8
Palkweng/Tongil	5.0	3.2	5.0	1.8	1.0
Tongil/Palkweng	3.2	5.0	5.0	1.8	1.0
Norin 29/Tongil	4.7	3.2	5.0	1.5	1.2
Tongil/Norin 29	3.2	4.7	5.0	1.5	1.2
Tongil/IR24	3.2	2.5	4.2	0.7	2.4
IR24/Tongil	2.5	3.2	4.0	0.7	2.1
IR781/Usen	1.0	2.0	1.3	1.0	0.3
Usen/IR781	2.0	1.0	1.5	1.0	0.5

^aD = difference between parents.

Table 2. Heterosis of organ differentiation from callus tissue derived from hybrid seedlings.

Cross	Shoots (%)				
	P ₁	P ₂	F ₁	D ^a	(F ₁ -P)/D
Palkweng/Usen	4.0	5.3	16.0	1.3	9.2
Usen/Palkweng	5.3	4.0	24.0	1.3	15.4
Palkweng/Tongil	4.0	12.8	8.0	8.8	0.4
Tongil/Palkweng	12.8	4.0	24.0	8.8	2.3
Norin 29/Tongil	0	12.8	7.9	12.8	0.6
Tongil/Norin 29	12.8	0	12.2	12.8	0.9
Tongil/IR24	12.8	2.0	4.0	10.8	0.4
IR24/Tongil	2.0	12.0	2.0	10.8	0.0
IR781/Usen	0	5.3	0	5.3	0.0
Usen/IR781	5.3	0	4.3	5.3	0.8

^aD = difference between parents.

differentiation was higher in the crosses where the female parent had shown higher plant organ differentiation. Reciprocal crosses differed in the degree of plant organ inducement from F₁ seed callus. The F₁ data showed that a higher degree of plant organ differentiation appeared either as complete dominance or partial dominance in the hybrid generation.

The degree of callus formation from anthers of F₁ plants was varied by cross combinations (Table 3). F₁ anthers of Norin 25 × Tetep, Suweon 224 × Chukei 314, Milyang 10 × Chugoku 56, and Kusabue × Tetep produced more callus than the respective parents. Another group of cross combinations, Suweon 224 × Chugoku 52, Norin 25 × Jinheung, Paltal × Norin 25, Pungok × Kusabue, and Pungok × Jinheung, showed a midparental degree of callus formation from anthers. That meant that a higher degree of callus formation from anthers acted as either overdominance or partial dominance in hybrid generation (F₁).

Table 3. Heterosis of callus tissue formation from hybrid anthers.

Cross	Callus formation (1-5)				
	P ₁	P ₂	F ₁	D ^a	(F ₁ -P)/D
Kusabue/Tetep	1.8	0.9	5.4	0.9	5.0
Norin 25/Tetep	4.4	0.9	6.5	3.5	1.6
Suweon 224/IR1317-266	0.3	0.1	1.3	0.2	6.0
Suweon 224/Chukei 314	0.3	4.3	6.5	4.0	1.5
Suweon 224/Chugoku 52	0.3	4.1	3.8	3.8	0.9
Pungok/Kusabue	5.9	1.8	3.1	4.1	0.3
Pungok/Jinheung	5.9	0.6	2.7	5.3	0.4
Norin 25/Jinheung	4.4	0.6	1.1	3.8	0.1
Paltal/Norin 25	2.1	4.4	2.8	2.3	0.3
Milyang 10/Chugoku 56	2.9	2.3	5.8	0.6	5.8

^aD = difference between parents.

Table 4. Heterosis of organ differentiation from anther callus tissue derived from hybrids and their parent plants.

Cross	Callus formation (1-5)				
	P ₁	P ₂	F ₁	D ^a	(F ₁ -P)/D
Kusabue/Tetep	6	11	7	5	0.2
Norin 25/Tetep	1	11	11	10	1.0
Suweon 224/IR1317-266	0	0	4	0	--
Suweon 224/Chukei 314	0	1	1	1	1.0
Suweon 224/Chugoku 52	0	2	1	2	0.5
Pungok/Kusabue	6	6	2	0	--
Pungok/Jinheung	6	0	3	6	0.5
Norin 25/Jinheung	1	0	2	1	2.0
Paltal/Norin 25	0	1	0	1	0.0
Milyang 10/Chugoku 56	3	0	3	3	1.0

^aD = difference between parents.

The plant organ differentiation from callus of F₁ anthers also showed greater differences between cross combinations (Table 4). F₁ of Norin 25/Tetep showed a higher percentage of plant organ differentiation from callus, but F₁ of Paltal/Norin 25 showed the lowest percentage. On the other hand, F₁ of Suweon 224/IR1317-226 differentiated a plant organ from callus, although neither parent showed stems and leaves. The higher percentage of plant organ differentiation seemed to be a partial dominance in the hybrid generation (F₁).

Anther culture breeding of rice

Cross combinations to improve rice blast resistance have been made and F₁ anthers have been cultured since 1977. The medium for callus inducement has been N6 basal media composition plus 2,4-D at 2 mg/liter and for plant organ differentiation, N6 basal composition plus IAA at 0.2 mg/liter and kinetin at 1.0 μg/liter. Callus formation ranged from 1.4% to 15.7% in cross combinations. LB4, a cross of japonica varieties, showed the highest percentage of callus formation (Table 5). The degree of stem and leaf differentiation from callus varied from 4.7% to 30.3% between cross combinations. HB-4, which showed the highest percentage of callus

Table 5. Callus and organ formation of F₁ plants.

HB	Cross	Anthers (no.)	Callus inducement (%)	Callus tube (no.)	Shoots (%)
HB1	S.278/IR2034	6,917	1.4	47	14.8
HB2	M.30/IR2071//S.258	7,216	4.9	54	6.3
HB3	S.258//M.29/Wx.329	8,917	6.0	97	20.3
HB4	S.235/M.15	14,225	15.7	670	4.7
HB5	S.268/IR1487//M.23/M.29	5,846	5.9	50	14.0
HB6	S.264/IR5533//S.258	6,020	5.2	78	19.2
HB7	S.264/IR5533/M.29	4,520	6.9	89	19.1
HB11	SR821//S.264/IR1544//S.258/ IR5533	3,940	2.2	33	30.3

Table 6. Varietal differences in formation of albino and green plants from anther callus.

HB	Cross	Green plants		Albino	
		No.	%	No.	%
HB1	S.278/IR2034	2	28.6	5	71.4
HB2	M.30/IR2071//S.258	1	20.0	4	80.0
HB3	S.258//M.29/Wx.329	3	60.0	2	40.0
HB4	S.235/M.15	20	57.1	15	42.9
HB5	S.268/IR1487//M.23/M.29	6	85.7	1	14.3
HB6	S.264/IR5533//S.258	8	53.3	7	46.7
HB7	S.264/IR5533/M.29	1	5.9	16	94.1
HB11	SR821//S.264/IR1544//S.258/ IR5533	4	40.0	6	60.0

Table 7. Chromosome number of rice plants regenerated from anther callus.

HB	Cross	n	2n	3n
HB1	S.278/IR2034	3	2	—
HB2	M.30/IR2071//S.258	2	1	—
HB3	S.258//M.29/Wx.329	—	2	—
HB4	S.235/M.15	4	9	2
HB5	S.268/IR1487//M.23/M.29	1	3	—
HB6	S.264/IR5533//S.258	2	3	1
HB7	S.264/IR5533/M.29	3	2	—
HB11	SR821//S.264/IR1544//S.258/IR5533	5	3	2

Table 8. Blast reaction of rice plants induced from anther.

HB	Cross	R ^a	S ^b
HB1 (N)	S.278(S)/IR2034(R)	—	1
(M)	"	—	1
HB2	M.30(S)/IR2071(R)//S.235(S)	1	—
HB4	S.235(R)/M.15(S)	6	3
HB5	S.268(S)/IR1487(R)//M.23(S)/M.29(S)	1	2
HB6	S.264(S)/IR5533(R)//S.235(S)	1	2
HB7	S.264(S)/IR5533(R)//M.29(S)	1	1
HB11	SR821(S)//S.264(S)/IR1544(R)//S.258(S)/IR5533(R)	1	2

^aR = resistant, ^bS = susceptible.

Table 9. Characteristics of diploid plants induced from anther.

Variety	Cross	Days to heading	Culm length (cm)	Grain		Amylose (%)
				WC ^a (0-5)	WB ^b (0-5)	
HB1 (N)	S.278/IR2034	118	100	2	1	20.7
(M)	"	112	64	3	3	20.7
Suweon 278		116	62	0	0	23.6
HB2	M.30/IR2071//S.258	104	59	1	3	20.2
Milyang 30		107	56	1	0	25.4
IR2071		128	49	4	0	25.4
Suweon 258		129	53	1	1	19.3
HB3 (N)	S.258//M.29/Wx329	105	68	0	2	18.5
(M)	"	116	89	1	3	20.2
Milyang 29		119	59	2	0	17.8
HB4	S.235/M.15	115	60	1	1	19.7
Suweon 235		121	71	0	0	19.8
Milyang 15		124	79	0	0	19.3
HB5	S.268/IR1487//M.23/M.29	117	91	0	3	19.1
Suweon 268		110	60	0	0	17.3
Milyang 23		114	66	1	0	19.5
HB6	S.264/IR5533//S.258	121	60	3	0	22.6
Suweon 264		106	58	3	0	18.4
IR5533		128	62	3	0	25.4
HB7	S.264/IR5533//M.29	111	61	0	0	19.3
HB11	SR821//S.264/IR1544//S.258/IR5533	119	64	3	3	18.5
IR1544		122	52	0	0	20.0

^aWC = white center, ^bWB = white belly.

formation, showed the least plant stem and leaf regeneration.

The occurrence of albino plants is one of the great problems in anther culture. Around 50% of the rice plants induced from F_1 anther callus were albinos, with 45 green plants and 56 albinos among 101 plants differentiated from callus in 8 crosses (Table 6). In another study, the frequency of occurrence of albinos was greater in callus of seedlings than in callus of anthers.

Plants from rice anther callus included haploid, diploid, and triploid plants (Table 7). Diploid plants appeared almost 50% of the time. Diploid plants could be obtained by natural chromosome doubling during plant organ differentiation from originally haploid callus tissue. The natural appearance of diploid rice plant from anther callus makes the utilization of anther culture techniques for rice breeding easier, since haploid plants originated from anther callus have poorer vigor.

The blast resistance of F_1 lines derived from F_1 anther culture showed segregation between lines but not within lines in all cross combinations used (Table 8). Agronomic characteristics such as heading date, culm length, grain appearance, and amylose content of rice grain of F_1 lines are shown in Table 9. Genetically, F_1 lines were almost all homozygous.

Three F_1 lines selected from F_1 anther callus originating pedigrees were tested for yield performance in 1979. HB11B exceeded the grain yield of check variety Suweon 264 and showed resistance to blast disease, but its amylose content was too high to be accepted (Table 10).

Table 10. Yield trials for homozygosis lines derived from anther culture.

HB	Days to heading	Culm length (cm)	Blast (0-9)	Grain		Amylose (%)	Yield	
				WC ^a (0-5)	WB ^b (0-5)		t/ha	%
HB7	107	65	6	0	0	19.3	5.7	102
HB11A	119	67	1	3	3	18.5	4.2	93
HB11B	104	64	1	1	0	26.9	6.2	111
Suweon 264	109	57	5	1	0	20.4	5.6	100

^aWC = white center, ^bWB = white belly.

CONCLUSION

F_1 anther culture shortens the breeding cycle of rice. Although techniques to induce more callus from anthers and more green plants from callus should be developed, breeding efficiency can be improved by the process of F_1 plant inducement from anthers; selection for agronomical characters in the F_1 and F_2 in the field, greenhouse, and laboratory; and testing for yield performance in the F_2 and F_3 . Although no new rice variety has been developed using anther culture in Korea, this breeding method is now progressing toward improving blast-resistant varieties.

RICE ANTHHER CULTURE IN TAIWAN

S. C. WOO and C. C. CHEN

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Research in rice anther culture in Taiwan first investigated the mechanism of plantlet regeneration for rice breeding (Woo and Fung 1972, Lin et al 1974). Anthers excised during the booting stage of rice plants were cultured on a basal medium supplemented with plant hormones and complex natural preparations. Microspores in the cultured anthers were developed into plants through an intermediary step of callus formation. Haploid plants were diploidized through colchicine treatment and homozygous lines were secured for breeding.

Material for rice anther culture

Chen and Lin (1976) tested plant production capacity in cultured anthers of five japonica and seven indica rices. They found that the frequencies of both callusing anthers and plant-regenerating calli varied considerably from one variety to another. In general, anthers of japonica rice were more productive than those of indicas. Anthers of japonica cultigen H-124, with a 44.7% callus-forming rate and a 22.4% regenerating rate (Chen 1976), were the easiest to culture. Anthers of indica rices Chianung sen II, I-kung-bau, Ai-chueh-chien, and Taichung Native I formed no calli (Tsai and Lin 1977).

Woo et al (1973) cultured anthers of F_1 plants from IR8 Chianung 242-d. Many hybrid plants involving crosses between japonica and indica varieties were subsequently used for anther culture. The frequencies of callus induction and plant regeneration were low in these hybrid materials. Among 25,000 anthers of IR8 Chianung 242-d. cultured, only 122 (0.49%) formed calli. One haploid plant was

diploidized through colchicine treatment and 14 plants were raised through self-fertilization of the doubled-haploid plants. They were identical in phenotypic appearance and showed the same distribution of peroxidase zymograms. Their progeny proved to be doubled-haploid lines (Woo and Su 1975).

In the culture of hybrid anthers between distantly related rice reported by Woo et al (1978), hybrid plants derived from single crosses and backcrosses between *Oryza sativa* L. (Taichung 65) and *O. perennis* Moench were studied. The anthers of reciprocal single-cross hybrids produced only calli and albino plantlets. Hybrids backcrossed to *O. sativa* developed green and albino plantlets.

Backcrossed hybrids with *sativa* as the original female parent showed enhanced callus induction and plantlet regeneration. The regenerating frequencies of normal green plantlets from backcrossed hybrids were 13.6% with *sativa* (Taichung 65 \times *O. perennis*) as the original female parent and 5.5% with *perennis* (*O. perennis* \times Taichung 65 \times Taichung 65). This may reveal the cytoplasmic effect on tissue growth.

About 43% of the anthers of *O. glaberrima* Steud. grew callus in culture (Woo and Huang 1980). However, only 3.5% of the anthers of reciprocal hybrids from *O. glaberrima* \times Taichung 65 grew callus. Although the rate of callus formation was low, the rate of plantlet regeneration was 16.72%. Albinos appeared twice as often as green plantlets among the regenerated plantlets. Crosses with *O. glaberrima* as the female parent produced more albino plantlets than those with *O. sativa* as the female.

Culture medium

An agar medium (8-10 g/liter) has been used for all these studies. Woo and Fung (1972) reported the use of Nitsch and Nitsch (1969) medium supplemented with 1 g yeast extract/liter, 100 ml coconut milk/liter, and 4 mg 2,4-dichlorophenoxyacetic acid (2,4-D)/liter for callus induction and supplemented with all components except 2,4-D for plantlet regeneration. Lin et al (1974) applied Blaydes' (1966) basal inorganic and organic constituents and added various amounts of indoleacetic acid (IAA), 2,4-D, and kinetin. Chen and Lin (1976) cultured anthers on White's (1963) basal constituent with 6% sucrose, 5 mg *o*-naphthalene acetic acid (NAA)/liter, 2.5 mg kinetin/liter, and 15% coconut milk. Chen (1977) modified MS medium (Murashige and Skoog 1962) by reducing major salts to half strength and adding 2 mg kinetin/liter and 4 mg NAA/liter. Calli were transferred to MS medium containing 4 mg kinetin/liter and 1 mg NAA/liter for plantlet regeneration. Sucrose concentrations of 3, 6, and 9% in the callus induction medium were reported by Tsai and Lin (1977) and Chen (1978). The 9% sucrose concentration promoted callus and organ formation, but the calli initiated differentiated into more albinos than those in 6% and 3% sucrose concentrations.

These media differed slightly in basal constituents and growth factors. Woo et al (1978) used the major medium constituents of MS, B₅ (Gamborg et al 1968), and Nitsch and Nitsch, supplemented with 2 mg 2,4-D or NAA/liter and 0.5 mg kinetin/liter, with and without coconut milk (15% vol/vol). In general, a medium containing coconut milk induced more calli than one without it. A small increase in the amount of inorganic salts MgSO₄·7H₂O, CaCl₂, KH₂PO₄ may help calli induc-

tion. However, the difference in calli induction caused by medium components was insignificant compared with differences caused by the genotype of parental material and the pollen stage of anther explanting.

Chromosome number of callus

Chen and Chen (1980) studied 46 callus cultures of single microspore origin maintained on MS agar medium containing 2 mg 2,4-D liter. Chromosome number determination at the end of the first transfer indicated that 24% of the cultures were nonhaploid, consisting of only diploid ($2n = 24$), polyploid, or two ploidy level cells. Nuclear fusion and endomitosis occurring during the initial stage of microspore development were postulated to account for the formation of nonhaploid callus. A total of 17 cultures were studied cytologically through 19 transfers. The ploidy levels of cells remained unchanged during culture in only one tetraploid and one hexaploid callus. Chromosome numbers in 13 cultures fell into a geometric series (n , $2n$, $4n$, and $8n$), suggesting that the changes induced by in vitro culture was mainly a repeated doubling of the chromosome numbers of cells initially present in the populations.

Since no diplochromosome or quadruple chromosome was observed, it was inferred that endomitosis rather than endoduplication was responsible for the changes. In spite of the tendency toward chromosome doubling, the proportions of cells of different ploidy levels were fixed in the 13 cultures at later transfers. Haploid cells were completely eliminated from all cultures. Diploid cells became predominant in eight cultures and tetraploid cells in five, suggesting a selection for either cell type. Triploids appeared in two cultures which initially did not contain this cell type. Limited cytological information indicated that triploid cells might have originated from a triploid cells through reductional grouping of chromosomes accompanied by multipolar formation.

Origin of callus initiation

Since anthers are cultured intact on agar medium, the cells of both somatic tissue and microspores may proliferate into calli. Because calli derived from these two categories of cells cannot be distinguished by visual observation or microscopy, the use of marker genes to identify the origin of callus becomes necessary. Woo and Tung (1972) cultured anthers of F_1 plants from IR8 (Chianung 242-d). The F_1 plants were identical in normal plant height (120-125 cm). Anthers of the F_1 plants were cultured. An anther plant, either haploid or diploid, of semidwarf stature was considered as having pollen origin. Pollen carrying no semidwarf genes would give normal tall plants. On the other hand, plants derived from the somatic cells of cultured anthers would be considered clonal progeny. The genetics of anther plants would remain identical with the F_1 's and the progeny of anther plants with somatic origin would segregate in all characters as F_1 populations (Mok and Woo 1976).

Using the same principle, Woo and associates (Woo et al 1978, Woo and Huang, 1980) developed heterozygous lines from hybrid crosses between less-related rice species *O. sativa* × *O. perennis* and *O. sativa* × *O. glaberrima*. A number of progeny lines bred true. But progeny-line sterility partially remained. Hsu and Chen (1977) found that 46 diploid plants derived from anthers of a intraspecific hybrid of rice

differed in many agronomic characters. As no further segregation was observed in the subsequent generation, individual plants appeared to be completely homozygous. These results are expected if anther plants have been derived from microspores of hybrid plants. Their chromosome numbers have doubled spontaneously during the process of anther culture.

Induced mutation through anther culture

Induced mutation furnishes a new gene source to enrich the germplasm. However, the frequency of induced mutation is usually low since a large experimental field and extensive field work are required to recover a few mutant plants.

Chen and Chen (1979) selected mutants resistant to 5-methyltryptophan (5-MT) from pollen calli. 5-MT has feedback inhibition against anthranilate synthetase and competition with tryptophan. 5-MT reduces the binding of tryptophan to transfer RNA, blocking protein synthesis and causing cell death. Mutants that are resistant to 5-MT usually contain more tryptophan and may become good breeding stock.

A total of 500 anther calli, each weighing approximately 10 mg, were treated with 1% ethylmethanesulfonate for 1 hour, then grown on MS medium containing 30 mg 5-MT/liter. Sixty plants regenerated from resistant cell lines and three grew to maturity. These morphologically similar plants differed from normal plants in a number of characteristics. Callus cultures regenerated from the somatic tissues of two of these plants were resistant to 5-MT. Callus of the third plant was not available for test. However, its morphological characteristics appeared to be transmittable through self-pollination.

Progeny study and field test

Anther plants developed from either a single parental variety or a hybrid plant may vary considerably in phenotypic appearance. Lin (1980, pers. comm.) found that the agronomic characters of over 300 doubled haploid lines derived from varieties Taichung 6S, Tamam 5, and Taichung Yu 196 differed among lines. Differences derived from anther culture of a single variety could be attributed to genetic disturbance during callus growth or to impurity of donor plants. The yield differences between 87 promising lines planted in 1978 and parent varieties were statistically insignificant.

The transfer of resistance to yellow dwarf virus through anther culture was attempted by Lin (1980, pers. comm.). He cultured anthers of the Kabara IR661-1-127-3-4 hybrid on Blasdale's medium (Table 1). About 12.7% of the anthers developed calli and 25% of the calli regenerated plants. Anther plant YD-80 of

Table 1. Yellow dwarf virus resistance of anther-derived hybrid YD-80.

Variety or line	Origin	Resistance		Plant ht (cm)		Grain fertility (%)	
		%	Rank ^a	I	II	I	II
IR661-1-127-33	IRRI	86.2	HS	87	80	74	72
Kabara	Sierra Leone	0	R	155	128	87	72
YD-80	F ₁ anthers	0	R	96	93	71	63

^aHS = highly susceptible, R = resistant.

Table 2. Yields of anther-derived hybrid compared with those of one parent.

Variety or line	1978 II		1979 I		1979 II	
	t/ha	%	t/ha	%	t/ha	%
771AC-321-59-3	2.81	123.2	4.32	64.4	5.07	82.4
771AC-321-59-4	3.42	150.0	4.74	70.5	5.67	92.1
Tainan 5	2.28	100.0	6.72	100.0	6.15	100.0

semidwarf stature proved resistant but its fertility was somewhat lower than that of its parents.

Woo (1979) tested 15 pollen lines derived from reciprocal crosses of Tainan 5 (semidwarf mutant) and Tainan sen-yu 30. Seven lines were resistant to brown planthoppers while the control variety Tainan 5 was heavily infested with the pest. Pollen line 771 AC-321-59-3 outyielded Tainan 5 by 23% and pollen line 771 AC-321-59-4 outyielded it by 50% in the second rice crop season of 1978 (Table 2).

The two pollen lines showed field tolerance for stress, particularly during the second season when diseases and insect pests were highly prevalent. However, when the environment was ideal, the lines did not outyield the control variety. The low yield was due mainly to a relatively low grain test weight and a low grain setting rate.

Discussion

Taiwan's rice anther culture started with the screening of japonica and indica varieties for easily cultured materials. Inducement of calli and regeneration of plantlets were easier with japonica varieties than with indicas. The African rice *O. glaberrima* also developed calli and plantlets from anthers with more than 50% frequency. The wild species *O. perennis* M.ENCH proliferated calli poorly. As these results were obtained by several workers in different years at different laboratories, no definite conclusion could be drawn.

As hybrid material, anther calli and plantlets from indica-japonica and from *O. sativa*-wild species varied significantly from the parent material. In general, hybrids with japonica rice as the female parent would induce more anther calli and regenerate more plantlets than those with indica or wild species as the female parent. That may indicate the cytoplasmic effect of hybrid materials on the growth of cultured anthers, although no strong cytoplasmic effect on the agronomic character and growth vigor of rice plants was found in indica-japonica crosses.

Most plantlets regenerated from anther calli, either haploid or diploid, were initiated from microspores. The genetics of pollen plants was fixed from the first generation. Anther cultures having pollen later than the miduninucleate stage may proliferate calli from anther walls of somatic origin. The difference in callus origin cannot be identified from a single first-generation plant, although some phenotypic differences may be found among anther plants or even among callus plants derived from a single anther. The differences also may be found among plants from a single callus. Anther plants with somatic origin of a hybrid parent would have genetic and phenotypic segregations in the second generation because the genetic background would be identical with the hybrid parent.

The origin of anther calli could be visually identified by segregation in the second

generation of anther plant progeny.

The bred-true lines obtained through anther culture are genetically homozygous and phenotypically identical because of either endomitosis or nuclear fusion. That solves the major problem of continuously segregating progeny from distant crosses. Another problem is sterility. Some anther plants were found with completely blanking panicles; they, of course, set no seed. Most lines are partially sterile and sterility persists in subsequent generations. No chromosomal aberration has been discovered in meiosis. Whether sterility is caused by nuclear-cytoplasmic interaction has not been established.

It is suggested that anther plants with low fertility be subjected to another cycle of anther culture. Plant fertility may be gradually recovered through a few cycles of anther culture. However, it seems unlikely that improvement could be made from highly homozygous plants, such as double-haploids, unless some minor genes for sterility can be replaced by fertility genes.

Basal media modified from the media of Murashige and Skoog (MS), Miller (ML), Blaydes, White, Gamborg, and Nitsch and Nitsch have been used. They are generally supplemented with the auxin of 2,4-D, NAA, and kinetin to induce calli. Media with little or no auxin content and with a raised level of 2 mg kinetin/liter were specifically prepared for plantlet regeneration. Rice anthers containing microspores of miduninucleate stage could proliferate calli on these media and regenerate plantlets on differentiating media. The callus-induction rate differs with experimental materials and medium components. Nevertheless, the variation stemming from different experimental materials for callus and plantlet development is larger than that caused by chemical components of the medium. A medium highly suitable for all rice materials has not yet been developed. How to identify the most suitable anther at the right miduninucleate stage has become most important in attempts for a successful culture.

POTENTIAL OF ANTHER CULTURE

Effect of recessive genes

Since anther culture aims to utilize the genetic constituents of microspores, anthers are cultured to develop the genotype of pollen into phenotypic performances. Plantlets derived from either haploid or diploid pollens through endomitosis or nuclear fusion would carry the characteristics of recessive genes and genetic fixation. This furnishes a direct approach for analyzing and evaluating the effect of rice genes on genome proportions. The ploidy series also can be obtained through colchicine treatment and the chromosomal complement can be studied under the microscope.

Gene incorporation for stronger growth vigor

Rice breeding usually depends upon varietal hybridization and selection of selfed progeny. In the long run, wide crosses to introduce a series of genes from less-related species, genera, or even families to the rice cultivar could be practical. Incorporation of distantly related or nonrelated genotypes usually brings about a strong growth vigor and occasional high tolerance for environmental stresses, including diseases and insect pests. However, wide crossings would cause a wide and continuous

segregation and low fertility. These defects could be overcome through anther culture and diploidization and be a realistic breakthrough in breeding practices. In the meantime, the methods of backcrossing and selfing also may lead hybrid populations to a homozygous state. The genotype of pure lines would then be close to the recurrent parent. But the likelihood is that no significant gain in growth vigor can be expected.

Induction of cellular mutations for enriching breeding stocks

Cells of callus tissue may serve as an independent unit in induced mutations. Anther calli could be treated with chemical and physical agents and screened for chemical mutants on nutrient medium. A great number of populations could be narrowed down to a manageable one. This screening is somewhat similar to that of microorganisms. This practice would overcome inefficiency in collecting a few mutants from a large nursery field. Applications along this line could be expected using microtoxins to screen germplasm for resistance to such diseases as sheath blight, which is not preserved in the present gene pool.

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CEREAL TISSUE CULTURE AT COLORADO STATE UNIVERSITY

MURRAY W. NABORS

A major problem in cereal tissue culture has been that cultures lose their regenerative abilities in a matter of months. To circumvent this problem for mutation selection, a useful strategy is to rapidly produce a large population of cells. From this large population, spontaneous or induced mutant cells can be selected and plants regenerated.

In our cereal tissue cultures, the first series of experiments attempts to maximize callus production from the germinating root by varying medium constituents. Plant species, type and concentration of auxin, and concentration of sugar appear to be the only significant variables. Alterations in concentrations of macronutrients or vitamins or additions of vitamins, cytokinins, or amino acids have no significant effects.

The medium used for induction of primary callus also caused the best growth of secondary callus. At present, we are attempting to rapidly increase the growth rate of secondary callus by enzymatically or physically dissociating the cells before transferring them to a new medium. Physical methods seem most productive. The closer proximity of medium and cells following callus dissociation seems to increase the rate of cell proliferation in model experiments with tobacco.

PEANUT REGENERATION EXPERIMENTS

For peanut and wheat tissue cultures, we find that shoot regeneration is most readily induced by the standard practice of reducing or eliminating auxin from the medium.

No other tested variable of medium composition increases regeneration. At present, we are testing the effects of photoperiod, light intensity, and differing amounts of red and far-red light on shoot production in callus.

The goals of these experiments are to increase the number of shoots produced and to extend the regenerative lifetime of cultured cells. Our oat and wheat cell lines retain totipotency for about 9 months (compared with more than 4 years for our tobacco), with oats producing more regenerated shoots than wheat. The production of roots on callus shoots is accomplished by transferring the shoots to a new medium without sugar. Transferring rooted plantlets from culture jars to pots is the most critical stage of our experiments. In some cases, 60% of the plants are lost at this stage. We are currently conducting experiments to reduce this percentage to obtain large numbers of mutant and nonmutant regenerated plants for greenhouse studies.

MUTATION SELECTION STUDIES

Our mutation selection procedures have centered on obtaining NaCl- and 2,4-D-tolerant mutants using tobacco as a model system. In the past 2 years we have begun to transfer the tobacco-derived technology to cereals. Our initial mutants were selected from suspension cultures by adding levels of NaCl or 2,4-D, which killed 50-99% of all cells. As soon as a cell line began to grow rapidly in a given selection medium, the level of selection was increased. In tobacco we have subjected several lines of cells to 11 periods of increasing selection pressure. Even after 3 years of selection using suspension cultures, it seems impossible to obtain rapid growth in NaCl concentrations greater than 8,000 ppm.

Recently we have found that selection on a solid medium, although less clear cut, seems to produce variant cell lines (Table 1). For salt stress in tobacco, selection can occur at levels considerably above 8,000 ppm, although levels in agar may not reflect actual tissue concentrations. Our variant cell lines are currently tolerant of 100-ppm levels of 2,4-D, over 20 times the normal level, and selection is continuing.

Mutation selection on a solid medium is an important technique for cereal tissue cultures, since suspension cultures are frequently difficult to initiate and maintain and since a suspension stage wastes valuable time in obtaining regenerated plants from the culture.

For root-derived callus cultures of cereals, we combine the callus induction and

Table 1. Selection for NaCl tolerant mutants in calli grown on solid medium.

NaCl on 20 ppm in solid medium ^a	Relative callus vol. after 4-wk culture ^b			
	Culture #1 1st month	Culture #2 1st month	Culture #3 3rd month	Culture #4 7th month ^c
0	1253	1344	1253	1344
6400	377	255	1019	511
11800	314	218	762	444
17200	224	148 ^c	517	287
22600	189	148 ^c	148 ^c	393

^aSea water is 21,000 ppm NaCl + 14,000 ppm other salts (av. figures). ^bCulture #1 derived from the 0% concentration of culture #3. Culture #2 derived from the 10% concentration of culture #3. ^cNo growth.

Table 2. Survival rate, under high or no salt stress, of salt-tolerant and nonsalt-tolerant tobacco plant lines derived from cultured cells^a

Original culture resistant to NaCl	Plant lines (%) alive after 13 wk			
	F ₁		F ₂	
	Watering solution		Watering solution	
	26.2 g NaCl/liter	0 g NaCl/liter	29.9 g NaCl/liter	0 g NaCl/liter
#1, with NaCl continuously present	65 (selfed to produce F ₂)	100	100	100
#2, without NaCl during regeneration	35 (selfed to produce F ₂)	100	90	100

^aPlants regenerated from tissue culture were designated the parental generation (P) and were selfed to obtain the F₁. The F₁ plants of each group in the 0 g NaCl/liter column were selfed to obtain the F₂. F₁ had 43 plants/group; F₂ had 20.

mutation selection steps. Seeds are placed on a callus induction medium, which also contains the selective agent. Small calli of several hundred cells are slowly produced. If a stress-tolerant mutation is present, a larger callus will appear. If hundreds of callus induction vials are set up, the rarely occurring larger calli are easily noticed and can be subcultured or placed on a regeneration medium. At present, we are comparing this mutation selection method with the more traditional one of producing large numbers of cultured cells and then applying stress for selection to determine which is more efficient.

GREENHOUSE TESTING OF MUTANT PLANTS

In our tobacco model system, we have regenerated plants from NaCl-tolerant and NaCl-sensitive cell cultures. The two groups of whole plants differ in NaCl tolerance in greenhouse tests and that difference is inherited by two subsequent generations (Table 2). The mechanism of inheritance is not Mendelian. We feel that, considering the selection procedure, the salt tolerance may be multigenic in nature. The salt tolerance of regenerated plants in general is higher than that of cultured cells when salt in the watering solution is compared with salt in the tissue culture medium. The regenerated salt-tolerant plants appear to be drought tolerant as well.

With wheat and oats, we have produced several lines of NaCl-tolerant cells. Tolerances for up to 9,000 ppm NaCl have been obtained. Based on the results on tobacco, these values would translate to a whole plant tolerance for 20,000-30,000 ppm. Greenhouse testing of regenerated salt-tolerant and salt-sensitive oat plants is under way.

HAPLOMETHOD ON RICE AT IRAT

MARYSE ASSELIN DE BEAUVILLE

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The Institut de Recherches Agronomiques Tropicales (IPAT) inquiries into a new method for increasing the rice breeding program efficiency started in 1974. The choice from a segregating generation is sometimes difficult with a pedigree method because of uncertain climatic conditions. With the haplomethod, plants can be tested several years before the choice of a segregating population. Haplomethod has good rapidity for exploring crosses and good security in the choice.

ANTHER CULTURE AND PLANT REGENERATION

A temperate variety, Cigalon, pure line, was used to test such experimental conditions as light and induction temperature and culture medium. A modified Miller's medium was chosen, substituting a Morel vitamin for a Fudji one and supplying 4 mg ANA/liter in the embryoid and callus induction medium and 2 mg kinetin/liter and 0.5 g AIA/liter in the regeneration medium.

Anthers of different microspore stages were plated on the culture medium. The best stage for haploid plant induction was the early uninucleate (Table 1). Pollen cells started to divide after 2 days and gave small embryoids 10 or 12 days later. These embryoids come from the generative or the vegetative cell.

After 3 weeks, small calli which differed in color and structure were observed. Only some regenerated plantlets, about 15% of them albino. About 80% haploid, 10% diploid, and 10% triploid and mixoploid plantlets have been obtained.

The doubled haploids have been observed in the field. About 30% of the lines

Table 1. Pollen stage for haploid plant induction.

	Anthers inoculated		Anthers giving callus		Calli		Plantlets	
	No.	%	No.	%	No.	%	No.	%
Early uninucleate microspores	420	—	126	30	684	162	43	10
Middle uninucleate microspores	480	—	94	19.5	530	110	4	0.8
Late uninucleate microspores first pollen mitosis	420	—	8	1.9	21	5	0	—
Total	1320		228		1235		47	

Table 2. Plant frequency obtained by anther culture.

	Anthers (no.)	Plants (no.)	Green plants		
			No.	% ^a	On best medium (%)
<i>F₃ families</i>					
Moroberekan/IRAT113					
A	1780	10	9	0.5	0.9
B	4122	34	21	0.5	0.6
C	1530	34	25	1.6	3.5
D	1940	34	16	0.8	1.8
F	1980	36	17	0.8	1.3
<i>F₄ hybrids</i>					
915/JAC25 and reciprocal cross					
P	4924	162	56	1.1	4.8
R	5200	90	41	0.7	2.9
915/M1265					
M	2720	76	32	1.2	2.3
RI 1031/Lung Sheng 1					
RI	3136	277	65	2.0	4.8
<i>Kiyosawa scale varieties</i>					
Pin 4					
Pi	960	—	56	5.8	10.8
Toride					
Tot	1824	—	105	5.7	9.2
Aishi Asahi					
Ai	2032	—	21	1.0	2.3
Cigalon					
Cp	960	77	33	5.4	16.6

^a plants/no. of plated anthers.

differed from Cigalon (a pure line) in precocity or tardiness and in length or width of seed. This variability could be of some interest in exploring anther culture crosses.

HAPLOMETHOD IN BREEDING

Tropical and temperate plants — five *F₃* families from the cross Moroberekan

Table 3. Frequency of green plants according to callus type.

	Granular translucent	Smooth translucent	Granular white	Smooth white
<i>F₃ families</i>				
Moroberekan/IRAT13				
A	2.8	1.8	2.7	2.5
B	0.7	0	10.0	30.0
C	0.7	14.2	14.2	37.7
D	1.7	15.3	17.6	25.0
E	1.4	11.5	3.3	21.7
<i>F₁ hybrids</i>				
915/IAC25 and reciprocal cross				
P	1.2	5.5	11.5	21.7
R	1.2	3.6	21.4	27.6
915/MI265				
M	1.2	12.1	10.5	25.9
<i>Kiyosawa scale varieties</i>				
Pi n ^a 4				
Pi	2.0	7.1	12.5	39.8
Torride				
Tor	4.0	12.5	5.1	19.8
Aishi Asahi	1.5	2.8	0	16.3

Table 4. Characteristics of doubled haploids from Moroberekan/IRAT 13.

	Heading duration (days)	Ripening duration (days)	Yield (t/ha)	Plant height (cm)	Generation
D 200	102-105	145	2.2	105	HD3
D216	102	145	2.6	105	HD3
Dh 57	98	140	3.0	90	HD2
Bh 238	98-100	140	2.5	107	HD3
B. 13	98-100	140	2.9	105	HD2
B. 403	100	140	2.1	107	HD2
B. (16) check	98	140	2.0	105	F6
IRAT13 check	98	140	2.0	98	

IRAT13, some F_1 hybrids from three different crosses, and some varieties of Kiyosawa scale — were used as anther sources. There was great diversity among the genotypes (Table 2). Sucrose concentration, pH, and mineral nutrient were used in the medium to increase the regeneration of green plantlets. The Chinese medium N6 was slightly better than Miller's; however, the number of albino plants increased.

The relation between callus structure and plantlet regeneration was studied. Calli were separated into four classes: granular translucent, smooth translucent, smooth white, and granular white. Smooth white callus had the highest regeneration rate (Table 3).

Some doubled haploid lines obtained by anther culture from the Moroberekan/IRAT13 cross have been tested in Ivory Coast. Table 4 shows yields of the doubled lines. All are better than the checks, three have been nominated to the IRAT catalog.

Table 5. Green haploid plants obtained from unfecundated ovaries.

	Un- fecundated entries (no.)	Ovaries producing plantlets		Plants	
		No.	%	No.	%
Hybrids					
915/IAC25	117	1	0.8	11	9.4
IAC25/915	78	0	—	—	—
M1265/915	20	0	—	—	—
Doubled haploids					
915 x IAC25	73	4	5.4	38	52.0
IAC25 x 915	21	0	—	—	—
M1265/915	62	2	3.2	42	67.8
Pure lines					
MS	202	0	—	—	—
IRAT13	125	2	1.6	41	32.8
Total	698	9	1.3	132	19.0

HAPLOIDS FROM OVARY CULTURES

Experimentation was started on unfecundated ovaries in 1979. We plated 698 ovaries on 2 media (Miller's and Monnier). Some ovaries (1.3%) inoculated on Miller's medium gave haploids of green plantlets (Table 5). In most cases, the plants were directly induced from the female gametophyte but sometimes a callus was observed first, which then regenerated into many plants. From direct induction, we obtained 132 plants from 9 ovaries (19%).

CONCLUSION

Haplomethod appears to be an interesting way to obtain fixed genotypes. Commercial seed was produced from the Moroberekan IRAT13 cross in only 3 years. However, it will be necessary to establish the number of haploid plants necessary to have real security in the genotype choice.

Culture of nonfecundated ovaries is another interesting procedure. No albino plants were produced in our experiments.

INDUCTION AND CRYOPRESERVATION OF GENETIC VARIABILITY IN RICE

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Genetic erosion, with the regeneration of phenotypically different plants, is a common trait of aging tissue cultures. In prolonged culturing, the cells undergo nuclear and chromosomal changes such as endomitosis, polysomy, and mutations (D'Amato 1977). These conditions, although undesirable for the maintenance of a clone, can be exploited for the selection of desirable variables (Skirvin 1978). Moreover, through manipulation using various drugs and growth regulators, such changes can be induced and useful variants can be isolated, as has been done in sugarcane (Li and Chen 1976, Hemz et al 1977).

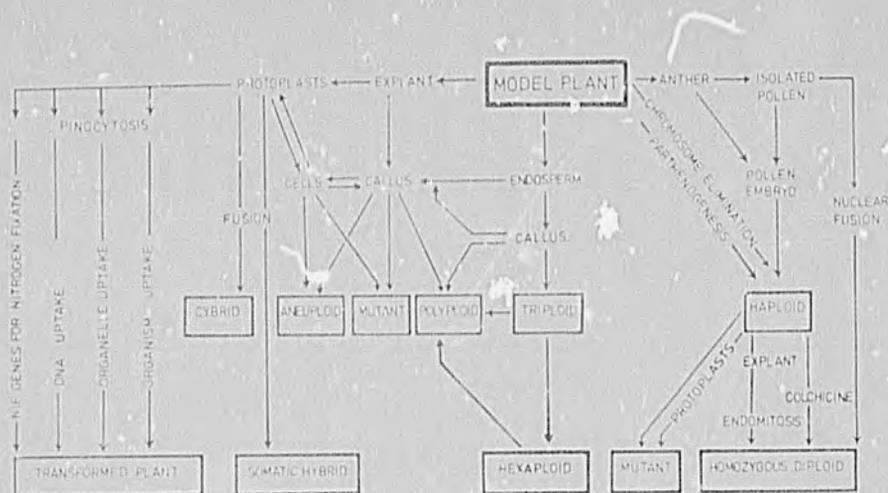
In our laboratory, efforts are being made to employ *in vitro* means to induce genetic variability in rice to broaden its genetic base and to develop cryogenetic methods using cell and tissue cultures for the conservation of rare and important germplasm.

GENETIC VARIABILITY

Methods employed to induce genetic variability in rice are schematically represented in Figure 1.

Embryo- and seedling-derived callus and plants

Nine rice cultivars (Basmati 370, Palman 879, HM95, PR106, Cynthroceros, Chow-sung, Jaya, IR8, and IR 36) were used to establish callus tissue cultures from various tissues and organs and to study their regenerating potentials. Comparative responses of the embryo-derived cells and different segments from *in vitro*-grown seedlings



1. In vitro methods for induction of genetic variability in rice (From Bajaj 1979b)

raised on different media are summarized in Table 1 and Figures 2 and 3.

The nature of the callus, its rate of growth, texture, and color were genotypically oriented (Table 1). Basmati 370 gave the best response and its mesocotyl yielded the maximum callus.

Excised embryos behaved somewhat similarly. Within 5 days, the embryo enlarged considerably, started to proliferate, and formed a callus. It underwent various modes of differentiation, mostly rhizogenesis, but occasional (3-5%) formation of plants was also observed. The embryo-derived callus and the regenerated plants showed a wide range of genetic variability (Fig. 4). A study of 796 dividing cells showed that calli were predominantly diploid ($2n = 24$). However, haploids, polyploids, and aneuploids were observed. The number of chromosomes varied from 11 to 60 (Bajaj and Bidani 1980).

Endosperm-derived callus and plants

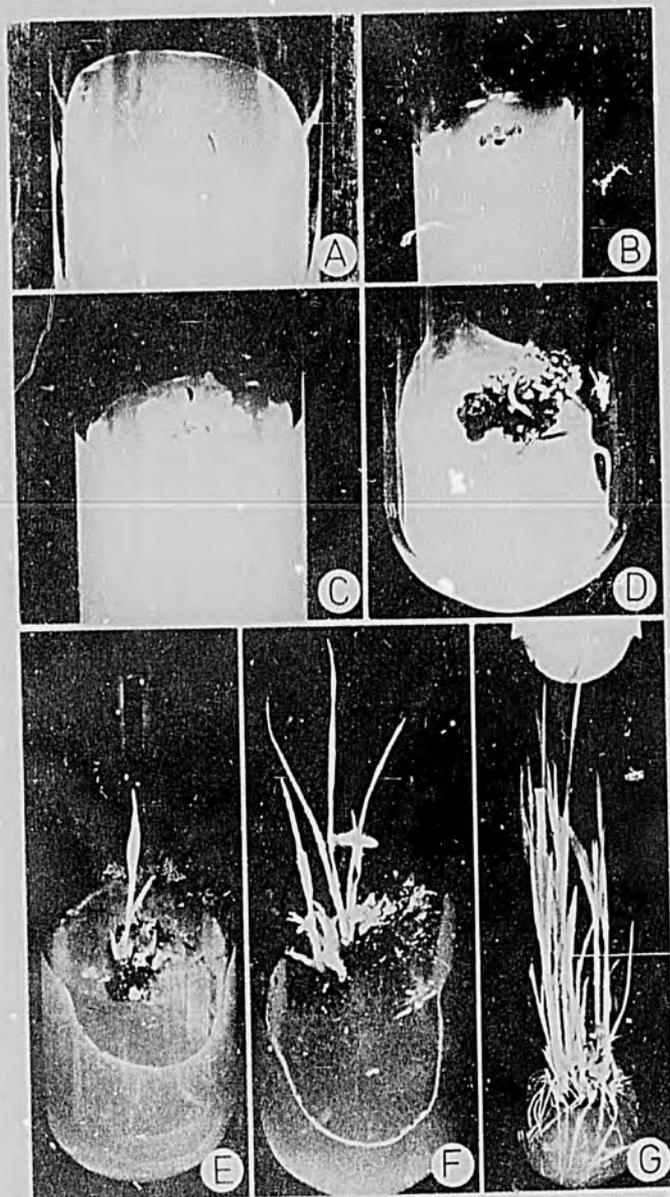
There was a striking difference in the growth response of explants from immature and mature endosperm of various cultivars (Table 2). The endosperm taken from young seeds underwent two modes of differentiation: direct regeneration of plants without an intervening callus phase and indirect regeneration through the differentiation of callus. However, mature endosperm proliferated to form callus, and plants differentiated in 4-6 weeks (Fig. 5).

Segments of mature endosperm reared on MS + 2,4-D started to proliferate in 3-7 days and a mass of callus was formed in 4 weeks. Rhizogenesis was observed occasionally in some cultures, but there was no shoot formation. The callus was mostly nodular, soft, and creamy. When transferred to modified Murashige and Skoog's (1962) medium, designated here as MS₁ medium (MS + IAA [4 mg/liter] + kinetin [2 mg/liter]), it differentiated within 2 weeks and complete plants were obtained in 4-6 weeks. The triploid plants thus produced showed broader leaves, a faster rate of growth, and more tillering than embryo-derived plants.

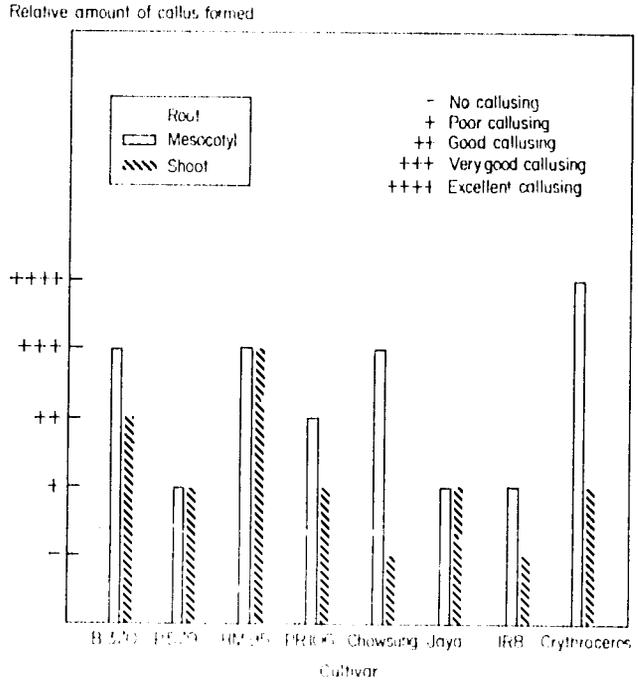
In addition to triploids, a large variation in the number of chromosomes in the

Table 1. Response of embryo-derived callus tissue cultures of rice cultivars on different media (Bajaj and Bidani 1980).

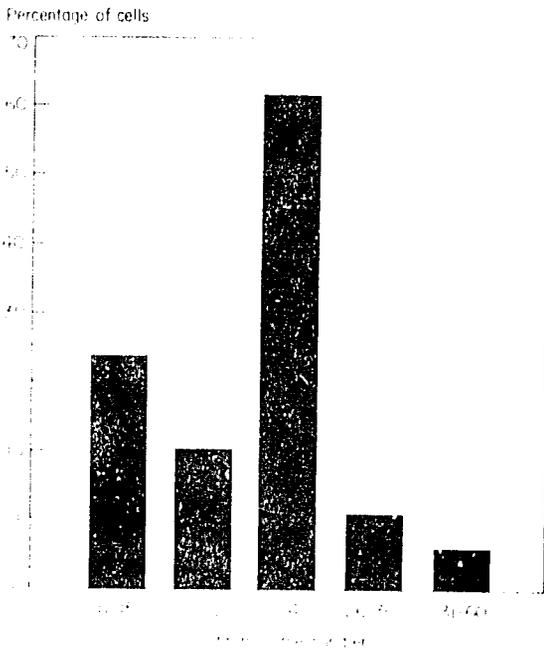
Cultivar	Medium			
	MS + 2,4-D (2 mg/l)	MS ₁	MS ₂	MS ₃
IR56	Callus cream, growth fast	Callus cream, growth moderate, rooting in a week	Callus dark brown, growth very slow	Callus cream, growth fast, rooting within a week, shoots in 2 weeks
Basmati 370	Callus light brown, growth fast	Callus light brown, growth fast, rooting	Callus cream, growth slow, rooting in 10 days	Callus cream, growth very fast, rooting within a week, shoots in 3 weeks
HM95	Callus brownish, growth fast	Callus brownish, growth very fast, rooting in 9 days	Callus light brown, growth very slow	Callus light brown, growth fast, rooting after 2 week



2. Regeneration of plants from callus cultures of rice (*Oryza sativa* L.). Induction of callus from segments of stem (A) and root (B) cultured on MS + 2,4-D (2 mg/liter). C. Mass of callus obtained from an embryo 4 weeks after culture. D. Rhizogenesis in callus on MS + 2,4-D (0.5 mg/liter). E-G. Differentiation of plantlets and multiple shoots from callus subcultured on a medium devoid of 2,4-D (Bajaj and Bidani 1980).



3. Extent of callus formation by various segments of seedlings of different cultivars of rice cultured on B₅ (2 mg 2,4-D/liter) medium (Bajaj and Bidani 1980).

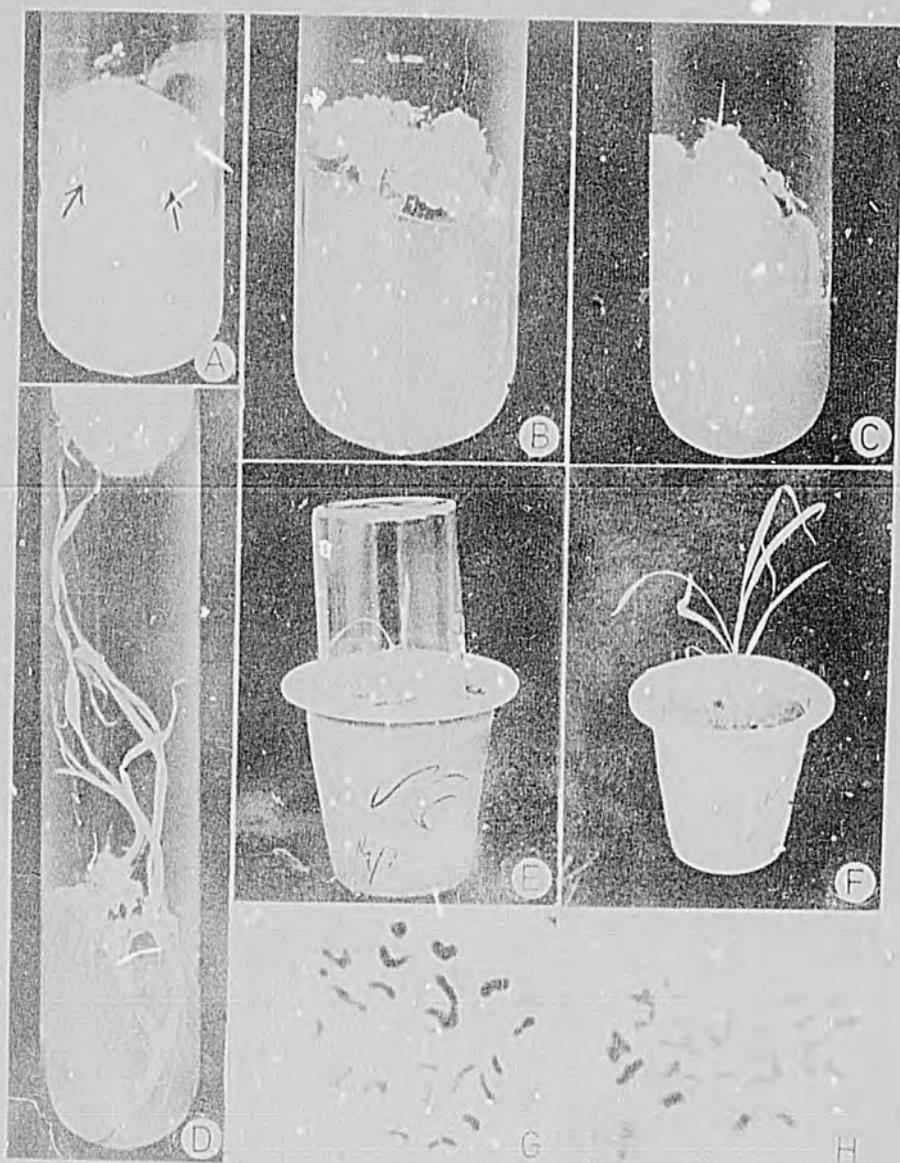


4. Genetic variability in embryo-derived callus of rice -- data based on 796 dividing cells (Bajaj and Bidani 1980)

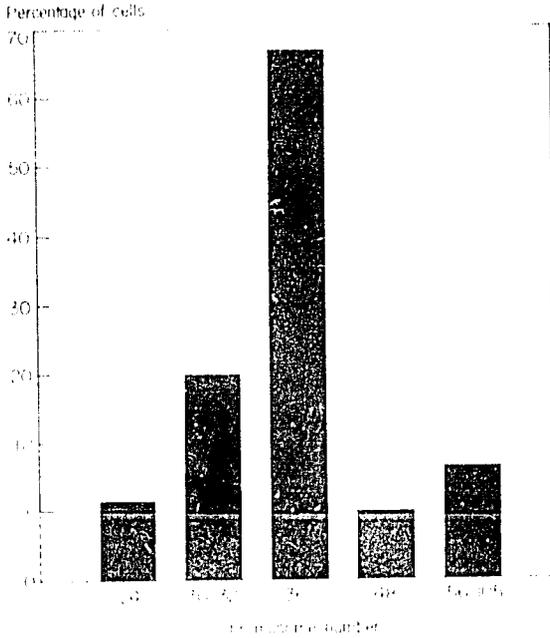
Table 2. Response of immature and mature endosperm from rice cultivars cultured on different media for 5-6 weeks (Bajaj et al 1980).

Medium	Endosperm ^a from					
	Basmati 370		IR36		HM95	
	Mature	Immature	Mature	Immature	Mature	Immature
MS + 2,4-D (2 mg/l)	Callus size increased, small roots, turned brown	Good amount of callus formed, turned brown	Callus size increased, produced many roots	Good amount of friable callus formed, turned brown	Creamy callus formed, no root formation	Callus growth good, brownish
MS + IAA (4 mg/l) + Kinetin (2 mg/l)	Differentiation of plants via callus	Direct and indirect regeneration of plants	Differentiation of plants via callus	Direct and indirect regeneration of plants	Differentiation of plants via callus	Direct and indirect regeneration of plants
MS + YE (4%) + 2,4-D (2 mg/l) + Kinetin (2 mg/l)	Increase in callus size	Initially a small amount of callus formed with no further growth	A small amount of callus formed with no further growth	Callus light brown, growth slow	Increase in callus size, brownish black	No response

^a30-40 endosperm explants in each medium.



5. In vitro regeneration of plants from rice endosperm. A. Endosperm explants, 6 days after inoculation on MS + 2,4-D, showing the initiation of callus (see arrows). B. Profuse proliferation and rooting in callus 3 weeks after subculturing on MS₃. C. and D. Differentiation of plantlets from endosperm-derived callus of cultivar Basmati-370, 3 and 6 weeks after subculturing on MS₃ medium. E. and F. Plantlets, 1 and 5 weeks after transfer to soil. G. Root tip squash from a normal embryo-derived diploid ($2n = 24$) plant. H. Endosperm callus cell with triploid number ($3n = 36$) of chromosomes (Bajaj et al. 1980).



6. Range of genetic diversity in endosperm-derived callus cultures of rice—data based on 850 dividing cells (Bajaj and Bidani 1990)

mature endosperm-derived (via callus) plants was observed. Aneuploids and polyploids were common (Fig. 6). The number of chromosomes varied from 24 to 105 (6n—96).

Anther- and pollen-derived callus and plants

Culturing anthers at the uninucleate pollen stage on a relatively high-sucrose (6-8%) medium results in androgenesis. There were, in general, two modes of development: direct transformation of a pollen into an embryoid (Fig. 7A), which eventually developed into a haploid plant, and indirect differentiation of plants via callus formation (Fig. 7B). Such plants show deviations from the normal haploid and may also be diploids. If there is endomitosis or fusion of the pollen nuclei, such plants are homozygous.

The culturing of anthers, although easy, suffers from one drawback. Plants arise not only from the pollen, but also from various other parts, such as the filament and connective wall of the anther. That results in a mixed population of plants of different ploidy. Although a handicap as far as the production of haploids is concerned, this phenomenon can be advantageous for obtaining mixoploids (Bajaj et al 1978).

Genetic variability through isolated protoplasts

Isolating protoplasts offers a novel tool for increasing genetic variability (Bajaj et al 1978). The technique, when perfected, would facilitate the regeneration of mutants

from cells rich in protein and resistant to salts, diseases, herbicides, and drought. Through the fusion of protoplasts, somatic hybrids and cybrids could be regenerated (Gleba 1979). In such cases, the genetic combinations would then depend on the extent of the mixing of cytoplasm and the fusion of nuclei. An increase in genetic diversity could also be achieved by introducing foreign genetic material into the protoplasts (Fig. 1).

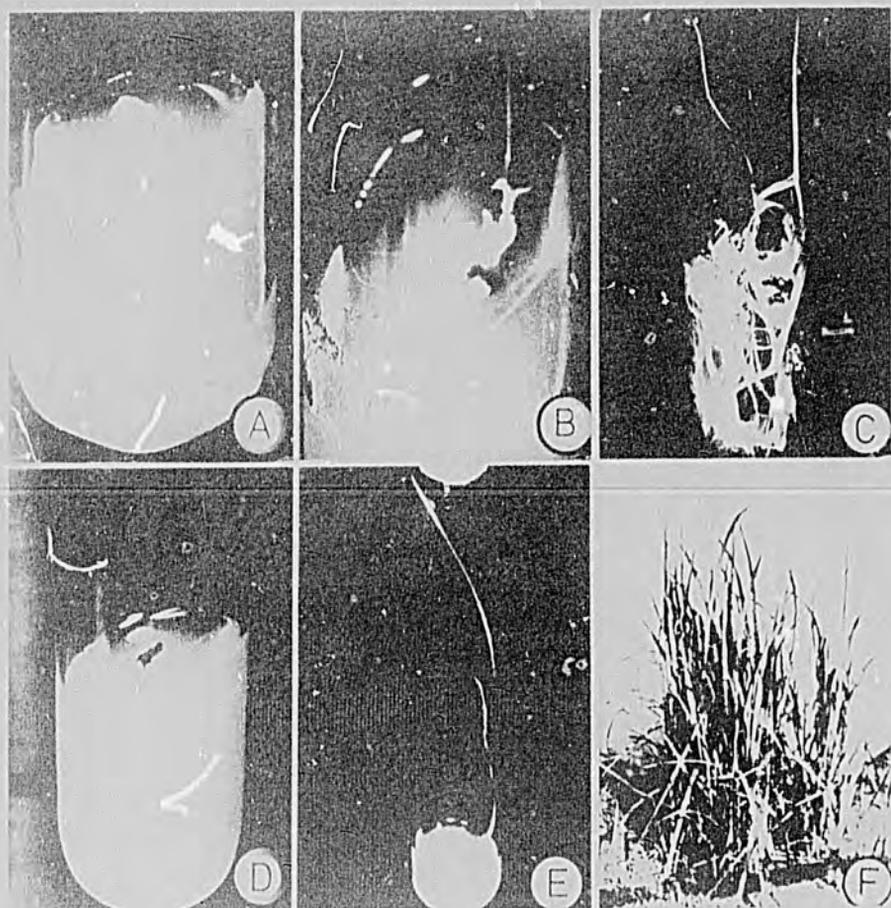
Limited quantities of protoplasts are obtained from actively growing cell suspensions of anther- and endosperm-derived callus of cultivars Basmati 370 and Cytroceros. The fusion of these *Oryza* protoplasts among themselves or with those of legumes such as pea (*Pisum sativum*) or soybean (*Glycine max*), poses no serious problems to forming hybrid cells, which may be designated as *Orysa* and *Oryme* (Bajaj 1981a). We have not yet made any concentrated effort to culture them. This important aspect of tissue culture will yield handsome dividends in wide hybridization programs, which may lead to the regeneration of plants capable of fixing their own nitrogen.



7. Induction of haploid plants from anther culture of rice cultivar Basmati 370. A. A pollen embryo obtained from a frozen anther culture. B. Regeneration of shoots from callus obtained from frozen anthers (Bajaj 1980).

FREEZE-PRESERVATION OF GERMPASM

The technology of freeze preservation of plant cell and tissue cultures has facilitated their storage in liquid nitrogen for varying periods and the subsequent regeneration of entire plants in a number of plant species (Bajaj 1979b). In this connection, our experiments on the anthers, excised embryos, endosperm, and callus cultures of rice frozen in liquid nitrogen have yielded some interesting results (Fig. 7, 8; Table 3, 4).



8. Induction of growth and morphogenesis in excised endosperm and embryos of rice frozen in liquid nitrogen. A. Frozen-thawed segments of endosperm 4 weeks after culture on MS + 2,4-D (2 mg/liter), initiation of callus. B and C. Differentiation of endosperm callus into shoot and plantlets after transfer to MS + IAA (4 mg/liter) + kinetin (2 mg/liter). D. An embryo excised from a frozen seed 3 weeks after culture, showing root formation but suppressed growth of shoot apex. E. Normal plant obtained from a frozen embryo. F. Transfer of test-tube plants to soil (Bajaj 198 b).

Although the revival of retrieved cultures is rather low, it can be improved by manipulating factors affecting cryoability.

Morphogenesis in frozen anthers

The progress made since 1970 on *in vitro* production of haploids in rice and on cryobiology of plant tissue cultures (Bajaj 1979a,b) supports the contention that the technology of freeze preservation may be useful in the conservation of haploid germplasm. Haploid-cell cultures are highly unstable. When subcultured, they undergo various chromosomal and ploidy changes that result in genetic erosions in the stock cultures (D'Amato 1977). In view of the importance of haploid cultures in

Table 3. Effect of sudden freezing (-196 °C) on seeds, excised embryos, and endosperm of rice preserved for 3 weeks in liquid nitrogen (Bajaj 1981b).^a

	Control	Frozen	
		Growth response	Growth (% of control)
Seeds	98% germination	96% germination	98
Dehusked seeds	94% germination	82% germination	87
Excised embryo with a portion of endosperm	86% grew	71% embryos callused and developed shoots	83
Segments of mature endosperm	16% callused	11% proliferated to form callus	68

^aSeeds germinated on moist filter papers in a petri dish. Dehusked seeds, excised endosperm, and embryos cultured on MS + 2 mg 2,4-D/liter. Data based on 350 seeds, 92 dehusked seeds, and 360 cultures of embryos and endosperm.

mutation, biochemical genetics, and haploid breeding programs, it is highly desirable to develop methods by which genetic stocks can be conserved.

Cultured anthers of rice cultivar Basmati 370 undergoing androgenesis (incubated for 3-4 weeks) and subjected to -196 °C in the presence of 5% each of dimethyl sulfoxide, glycerol, and sucrose occasionally revived and resumed further development (Bajaj 1980). Initially, most of the retrieved anthers became soft and spongy, turned brown, and appeared dead. However, a few (5 out of 927 anthers) showed localized proliferation. The anther-derived callus (Fig. 7) was compact, slow-growing, and formed only roots, only shoots, or both. The frozen anthers invariably underwent a lag period and growth was considerably delayed.

These observations show the possibility of selecting cold-resistant cell lines from which to regenerate plants.

Although tobacco plants were regenerated from haploid callus cell suspensions frozen in liquid nitrogen (Bajaj 1976), the possibility of genetic erosions in such cells is not ruled out. It is believed that haploid meristems and pollen embryos would be highly suitable materials for preservation as haploid germplasm.

Morphogenesis in frozen embryos and endosperm

The viability and growth responses of frozen endosperm and embryo cultures and the dehusked seeds are summarized in Table 3. Initiation of proliferation in the retrieved endosperm was delayed compared with controls. In some cases a mass of callus was formed in 5-9 weeks (Fig. 8A). When transferred to MS + kinetin (2

Table 4. Survival of endosperm-derived callus cultures of rice subjected to freezing at -196 °C.

Cryoprotectant	Cell survival (% of control)	
	Basmati	Cryothoceros
Sucrose 15%	21	26
Glycerol 15%	24	29
DMSO 10%	31	34
Sucrose (5%) + Glycerol (5%) + DMSO (5%)	39	42

mg/liter) + IAA (4 mg/liter), the callus underwent morphogenesis to form plantlets in about 3% of the cultures (Fig. 8B,C).

The frozen-thawed embryos, with a portion of the endosperm, started to germinate and initiated callus within a week (Fig. 8D). When transferred to a plain agar medium, the plantlets continued to grow (Fig. 8E), resulting in normal-looking plants (Fig. 8F).

There was practically no difference in viability between the frozen seeds preserved in liquid nitrogen and the controls. However, survival was lower in the frozen embryos and endosperm (Table 3). The capacity to withstand freezing depended on the water content of the seeds. Two-year-old seeds (8-11% water content) showed complete revival; immature and fresh seeds (35% water content) only partly survived.

Revival of freeze-preserved cells (Bajaj 1976, Sala et al 1979) and regeneration of complete plants from retrieved pollen (Bajaj 1980), embryos, and endosperm (Bajaj 1981) suggest the possibility of using the cryogenic method as a meaningful tool for the conservation of favorable and rare germplasm.

Survival of endosperm-derived callus at -196 °C

The effect of cryoprotectants on the survival of endosperm-derived callus (triploid) of two cultivars, Basmati-370 and Cythroceros, is shown in Table 4. The mixture of sucrose (5%) + glycerol (5%) and DMSO (5%) yielded cell survival up to 42% (Table 4). Survival of seed-derived callus (presumably diploid) was reported earlier (Sala et al 1979).

These observations on the survival of cell and tissue cultures of rice frozen in liquid nitrogen are the basis of further explorations of the application of cryogenic methods to elucidate fundamental problems in research programs for the conservation of rice germplasm.

Freeze storage of cells has potential uses in basic and applied research. For instance, a cell line has to be transferred to a fresh medium periodically for maintenance. Freeze storage would suppress cell division and avoid the need for periodic subculturing.

Moreover, tissue cultures often show chromosomal erosions and ploidy changes and are a rich source of genetic variability. Such desired cell lines can be frozen and banked. The germplasm banks of plant tissue cultures would be somewhat like the semen banks responsible for the conservation and distribution of desirable germplasm.

CONCLUSION

The dual problem of the induction of genetic variability and its conservation can be fruitfully tackled by applying various *in vitro* techniques, combined with cryogenic methods. Although the technique of anther culture in rice is more or less refined, the frequency of androgenesis is still low. The successful culturing of cells and protoplasts and the reproducible regeneration of plants from callus tissue cultures remain the vital problems, slowing progress in utilizing these tools to induce mutant plants. Before protoplasts and callus cultures can be exploited to obtain genetically diverse

plants, effort should be concentrated on basic research.

One of the main reasons for delay in the use of cryogenic methods for the long-term storage of cells is the low rate of cell revival in most crop plants, combined with a lack of knowledge regarding any genetic changes taking place during storage. Multifactorial studies need to be undertaken to find the conditions responsible for high survival rates and to ensure that no genetic changes occur during prolonged storage.

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THE APPLICATION OF TISSUE CULTURE TECHNIQUES TO RICE IMPROVEMENT

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Report from the conference

Plant cell culture techniques can assist in the identification, assembly, recombination, and selection of new and novel forms of genetic variability. In vitro methodology already has accelerated the attainment of specific breeding objectives in sugarcane, potatoes, rice, wheat, tobacco, and several horticultural species.

Continuing genetic improvement of rice necessitates new technology. Integrating in vitro techniques into existing breeding programs will contribute substantially to the momentum of rice improvement at IRRI and elsewhere, as it has in other species.

Specific cell culture techniques that appear useful include microspore culture, embryo culture and in vitro fertilization, cellular selection of desirable recombinant and mutant genotypes, rapid screening for disease resistance and physiological traits, and mass regeneration of plants from cell culture.

Microspore (anther) culture in a hybridization program greatly enhances ability to screen many recombinants. Since genes segregate in gametic rather than zygotic proportions, screening efficiency among segregating genotypes can be improved up to six times (depending on the mode of inheritance) more than by conventional techniques.

Microspore culture is relatively efficient in rice, but cultivars vary in their predisposition (genetic or environmental) to produce doubled haploid plants. But recombination and selection for increased efficiency of microspore culture among rice cultivars, particularly those of value to current rice improvement programs, can be successfully achieved. Because of its Genetic Evaluation and Utilization (GEU) program and the Rice Genetic Resources Laboratory, IRRI is the preeminent site for this research.

Parallel research in biochemical and physiological parameters to improve microspore culture techniques should be sustained at IRRI. Collaboration with

similarly motivated research institutes elsewhere should be strengthened. The production of haploid plantlets directly from the cultured anthers, rather than via a callus phase, should have immediate research priority.

The mass regeneration of diploid plants from callus cell lines recently has provided an unexpectedly rich and novel source of variability in sugarcane, potatoes, and rice. The variation displayed among regenerated plants is of agronomic importance affecting such parameters as disease resistance, physiological properties, and plant morphology. Evaluation of this potential in rice would best be integrated into the GEU and ongoing breeding programs at IRRI.

Immature embryo culture and *in vitro* fertilization have a potential in overcoming hybridization barriers between species and possibly genera. Well-established techniques have been applied successfully in several species, including wheat and barley. Specific plant breeding objectives in rice, such as the transfer of cold and drought resistance and disease and insect pest resistance from relatives of cultivated rice, can now employ embryo culture successfully. Collaboration should be established with the People's Republic of China to evaluate the potential of wide hybridization, such as rice-sorghum and rice-wheat, in rice improvement.

Desirable recombinant and mutant genotypes of plants grown under *in vitro* conditions may be rapidly identified and selected because defined chemical and physical conditions are used. There is foreseeable value in utilizing cell culture technology in rice to select for salt, drought, and chilling tolerance and for resistance to pathogens. Cell culture also might facilitate selection of genotypes that produce pathogen inhibitors or pest repellants.

Plant cell culture is a rapidly developing research field. Additional techniques that will have direct application to plant improvement are expected to be developed. IRRI must retain an active liaison and, where appropriate, specific collaborative research projects with other research institutes developing such innovative technology.

Protoplast culture is one such development. The ability to efficiently culture large numbers of protoplasts to mature plants could permit selection beyond that practicable using whole plants. This is particularly significant where cellular and whole-plant responses are correlated. Protoplast research at various institutes is concerned with generating genetic variability, cell fusion between incompatible genomes, introduction of cytoplasmic sterility, and selective gene transfer following the molecular manipulation of specific DNA.

The development of efficient protoplast culture of rice is imperative. IRRI can play a significant role through its GEU program by providing cultivars, relevant information from its cell culture research, and active research support with the integration into GEU of novel genotypes produced by genetic manipulation at the cellular level.

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