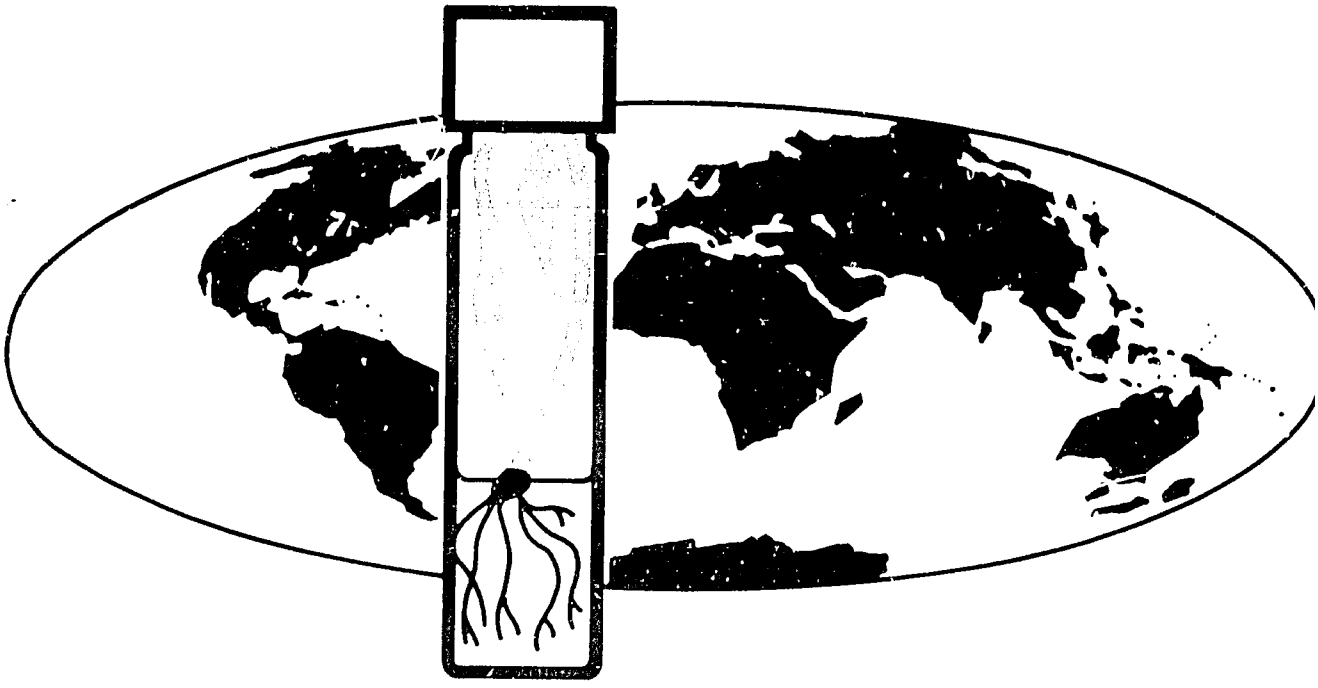

TISSUE CULTURE FOR CROPS

STRESS RESISTANT PLANTS FROM CELL CULTURE

DEPARTMENT OF BOTANY AND PLANT PATHOLOGY
COLORADO STATE UNIVERSITY
AND AGENCY FOR INTERNATIONAL DEVELOPMENT

PROGRESS REPORT

TISSUE CULTURE FOR CROPS PROJECT

Producing Tissue Culture Techniques
for Use
by Plant Breeding and
Agriculture

October 1, 1982

Agency for International Development
Department of State
Washington, D.C.

Department of Botany and Plant Pathology
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Fort Collins, Colorado

Introduction

This progress report summarizes the activities of the Tissue Culture for Crops Project (TCCP) during the first two years of its existence. The project is supported by the U.S. Agency for International Development and is located at Colorado State University, Ft. Collins, Colorado. The overall purpose of the project is to utilize emerging tissue culture technologies to promote the development of stress-tolerant crop plants.

The report is divided into several sections. A **Summary** is followed by a brief discussion of **Project Objectives.** Background information is provided on sodium, aluminum, and drought stress worldwide; on the advisability of modifying crops to fit available environments; and on the reasons for and methods by which tissue culture can contribute to plant improvement. **Project Facilities and Administration** are outlined. **Project Accomplishments** are detailed. Specifics for each species and type of stress are covered in **Methodology and Discussion of Project Accomplishments.** Finally the **Future Experimental Direction and Project Goals** are discussed. The report ends with a listing of **Project Personnel, References,** and a **Project Information Sheet** to be returned by the reader.

Summary

Drought, excess sodium, and excess aluminum are stresses which inhibit crop production on 50% of all arable land. In the face of dwindling water supplies and increasingly expensive irrigation and soil modification it is important to focus plant breeding on efforts to produce varieties of plants with an increased ability to grow in available natural environments with a minimum of energy intensive environmental modification (Fig. 1). Tissue culture techniques are useful in plant improvement because each cell is a potential plant. Thus millions of potential plants can be grown and screened rapidly and economically.

The Tissue Culture for Crops Project (TCCP)[1] seeks to increase sodium, drought, and aluminum resistance in wheat, rice, pearl millet, and oats. During its first two years the project has made substantial progress towards this goal.

1. **A demonstration has been completed that stable NaCl-tolerant cell lines can be selected in cell cultures and that plants regenerated from these cultures retain tolerance and pass it on to subsequent generations.**
2. **Tissue culture methods for cereals have been improved.** In particular, long term, high frequency plant regeneration has been obtained for wheat, rice, pearl millet, proso millet, and oats. To date over 3400 rice plants and 1000 oat plants have been produced from cell cultures and over 200 plants from each of the other species (see Table 1, Fig. 2).

Table 1. Plants Regenerated from Cereal Tissue Cultures by the TCCP

	salt-sensitive cultures	salt-tolerant cultures (1000-9000 ppm NaCl)
Rice	3233	201
Wheat	329	30
Pearl Millet	157	78
Oats	672	374
Proso Millet	85	20

3. **NaCl-tolerant cell cultures have been obtained for each species.** Selection for drought- and aluminum-tolerant cell lines is underway.
4. **Over 200 plants have been regenerated from salt-tolerant cell lines of rice and of oats.** Thirty or more plants have been regenerated from salt-tolerant cell lines of each of the other species. These plants are now undergoing greenhouse testing to determine limits of salt tolerance and to increase seed stock for field tests to follow.

FIGURE 1. Improved varieties of crop plants are needed to increase agricultural production in regions where labor intensive farming, as opposed to energy intensive farming is the rule.

Project Objectives

The TCCP was established in September, 1980, for the purpose of utilizing tissue culture methods to increase the tolerance of rice *(Oryza sativa* L.), wheat *(Triticum aestivum* L.), and pearl millet *(Pennisetum typhoides* [Burm.] Stapf et Hubb) to major soil stresses. Salt (NaCl), aluminum, and drought were selected as the environmental stresses for which resistance should be obtained. It was realized that approximately 50% of the world's arable land is restrained in agricultural production because of one of these stresses. Also it was clear that plant tissue culture had the demonstrated potential (Nabors et al 1980) to produce lines of cells and regenerated plants with increased stress resistance. At the time the project was initiated, the TCCP laboratory, with already eight years of tissue culture experience, had established (Nabors 1981) that tissue culture technology for wheat and oats was developed enough

[1]Project supported by United States Agency for International Development, contract No. AID/DSAN-C-0273.

All reported opinions, conclusions, and recommendations are those of the authors (contractors) and not those of the funding agency or the United States government.

FIGURE 2. The TCCP has produced large numbers of rice, proso millet, oat, wheat, and pearl millet plants from tissue cultures. Rapid propagation of rare, useful cereal plants by tissue culture methods is now possible.

so that selection for stress tolerance could proceed with a reasonable probability of success. This demonstration was important because for over a decade tissue culture of cereals had been hampered by the lack of effective methods for regenerating plants at reasonable frequencies and for long durations from populations of cultured cells. As is detailed in this report the TCCP has continued, during its first two years, to radically improve regeneration technology for cereals. Because of this work, selection for stress-resistant variants has been able to proceed rapidly.

Background

A survey of available data indicates that the chief environmental factors limiting food poduction in the world are:

1. **Drought and excess salts which affect around 25% of the world's arable land.**

   The percentage of the world's arable land which is subject to salt and drought stress is variable according to the estimator but seems to be around 25% (Carter 1975). Even 25% of the world's rice hectarage suffers from drought at sometime during the growing season (O'Toole and Chang 1979, USDA Agricultural Statistics 1976). The usual answer to problems of drought and salinity has been irrigation. At present around 15% of the world's cultivated land is under irrigation (FAO Prod. yearbook. 1977, vol. 31, United Nations) (Fig. 3). This figure is only an average; in some countries 100% of the agriculture comes from irrigated plots. The problem with irrigated agriculture is twofold.

   a. Already, 80% of the readily available water is used in agriculture (Flowers et al 1977). This means that increasing the hectarage currently irrigated will require massive engineering projects to increase the available water supply. Ground water supplies, particularly in dry regions relying heavily on irrigation, are dwindling rapidly. The Ogallala aquifer in the central United States is a particularly well-studied example of a rapidly dropping, soon to be exhausted water table (High Plains Associ-

FIGURE 3. Irrigation systems of various types can increase food production in arid regions. However supplies of water are limited, systems are expensive to install and maintain, and salinity buildup in the soil limits the useful lifetime of any system.

ates 1979). In short, increasing the irrigated hectarage is practical neither in terms of available water nor in terms of the cost required to bring in new supplies.

b. All irrigation systems from those in ancient Sumer over 4000 years ago (Jacobsen and Adams 1958) to those currently in use gradually add salts to the soil (Casey 1972). Careful management can retard salt buildup but not eliminate it. Thus all irrigation systems have a limited useful lifetime.

2. **Soil acidity (bringing aluminum, manganese, and other toxic metals into the soil solution) which affects around 25% of the world's arable land.**

Excess soil acidity is a problem in regions of the world in which drought is not a problem (Christiansen 1979) (Fig. 4). Soils become acidic as a result of CO_2 dissolved in rain water. Thus when rainfall is high, soil acidity increases. This in turn brings usually insoluble ions (such as aluminum) into the soil solution. Also the hydrogen ions tend to displace other cations from binding sites on soil particles. Ultimately, acid soil becomes poor in the nutrients needed by plants and rich in toxic cations such as aluminum. The problem is most often solved by periodic soil liming to reduce acidity. Unfortunately, in large areas of the world this solution is neither practical logistically nor possible financially.

FIGURE 4. In regions of high rainfall excess soil acidity and metal toxicity are important agricultural problems. More tolerant plant varieties are needed for these areas.

3. **An overabundance of crop varieties which require considerable environmental modification (irrigation, fertilization, liming, etc.) in order to yield effectively.**

Modern, mechanized agriculture is very energy intensive (Fig. 5). According to a recent estimate the energy equivalent of 800 liters of gasoline is currently required to produce a hectare of corn in the United States (Pimentel et al 1973). Varietal selection as well as mono-cropping has resulted in an agriculture which requires extensive environmental modification in many regions in order to grow successful crops. Heretofore many developing nations have been unable to afford the cost of this type of agriculture (Fig. 6). Now, increasing energy costs and dwindling supplies have made even developed nations aware of the high cost of energy intensive agriculture based on environmental modification.

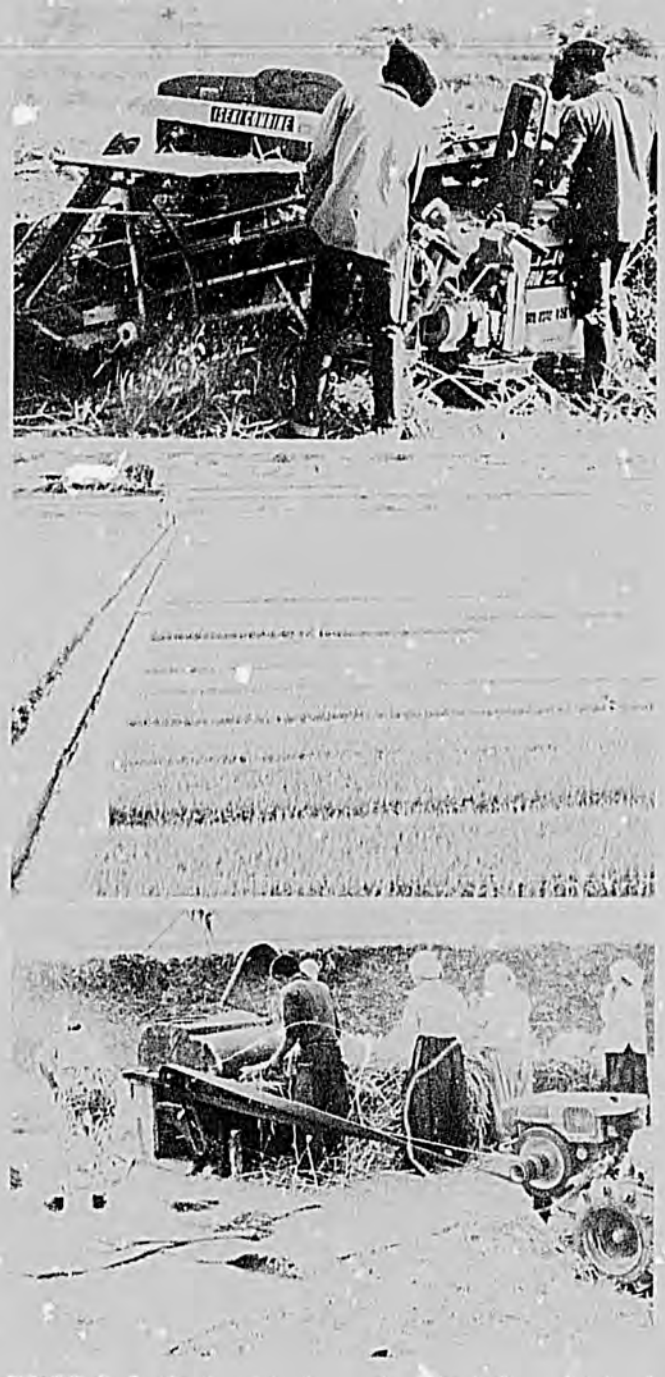


FIGURE 5. Fertilization, irrigation, and mechanically assisted soil preparation, planting, and harvesting are all components of energy intensive agriculture.

The strategy of the TCCP is to approach all of these problems by working to modify the plant to suit available environments. If, for example, the drought tolerance of a crop can be increased then (1) land currently too dry for agriculture can be brought into cultivation; (2) the extent of coverage by available irrigation water can be extended; (3) salt buildup due to irrigation will be slowed because irrigation is reduced with respect to amount and frequency and (4) resources currently devoted to environmental modification and, in some cases, food importation can be redirected. It is our feeling that tissue culture can now make a contribution to plant breeding and that breeding in general should be directed toward the production of energy efficient varieties which are as well adapted as possible to available and unmodified local environments. As an example of the potential economic impact of this strategy it is worth considering the economic value of rice varieties with a 10% increase in production in the face of water stress. One quarter of the world's 141 million hectares (USDA Agricultural Statistics 1976) of rice suffer from drought sometime during the growing season. This hectarage produces roughly 85 metric tons per year (USDA Agricultural Statistics 1976) of rice worth $36 billion (from *Wall Street Journal)* on the world market. Thus a 10% increase in production on these hectares would be worth approximately $3.6 billion. Similarly a 10% increase in wheat production due to the introduction of drought-tolerant varieties could be worth $5 billion. The loss in agricultural production due to salinity in the San Joaquin Valley of California alone is expected to reach $0.5 billion per year (computed from p 279 of the Global 2000 Report to the President).

Having established that it is of importance to produce varieties of crop plants which yield effectively on available and unmodified environments, the role of the plant breeder should be clarified. Despite some accounts to the contrary, it is clear that neither tissue culture nor any other emerging genetic technology offers magical solutions to agricultural problems. Likewise it is obvious to most that the tissue culture worker and the genetic engineer will neither replace the plant breeder nor render him obsolete. Rather, the plant breeder must be seen as the final receiver and the person who makes direct use of techniques, methods, and plants coming from the emerging technologies such as tissue culture. Salt-, drought-, and aluminum-tolerant plants from our cultures may prove to be directly useful in the field. However this usefulness must be demonstrated by plant breeders and agronomists in the field so that the spectrum of field utility can be determined. The major utility of plants produced by the TCCP will probably be in horizontal intraspecific and interspecific (wide) crosses performed by plant breeders utilizing standard methods.

Why does tissue culture technology offer a useful tool to the plant breeder? Basically because it can markedly compress the time, space, and economic requirements involved in selecting new and useful plant types (Nabors 1976). Since each cell represents in genetic terms a potential plant, thousands of potential plants can be maintained in a single vial and billions in a single small room (Fig. 7). Whether this genetic potential is used to clone a single useful plant or to screen for desirable mutant phenotypes, enormous economic, temporal, and spacial savings are realized over conventional reproduction and selection systems. Since many desirable mutations occur very infrequently (perhaps in one of every million plants) the expanse involved in field selection alone is considerable. In the case of selection for a trait like salt tolerance the entire process, for millions of cells, can be performed in a few vials in which

FIGURE 6. In the face of ever increasing costs and dwindling supplies of available energy, all nations are finding mechanized agriculture a costly investment.

FIGURE 7. Since each plant cell has the genetic potential to produce an entire plant, the TCCP culture room (half of which is shown in the top photograph) has the genetic equivalent of 3 billion plants.

uniform application of selection stress is no problem. In the case of selection for a trait like altered nutritional quality, field selection (meaning a chemical analysis on every plant) becomes even more difficult than tissue culture selection (for a particular biochemical reaction). Developing methods for protoplast fusion and intercellular gene movement will make available to tissue culture workers and plant breeders even more powerful techniques for selecting and producing new plant types which might be impossible to produce or find under field conditions alone.

The production of stress-resistant plants using tissue culture methods requires several steps (Fig. 8).

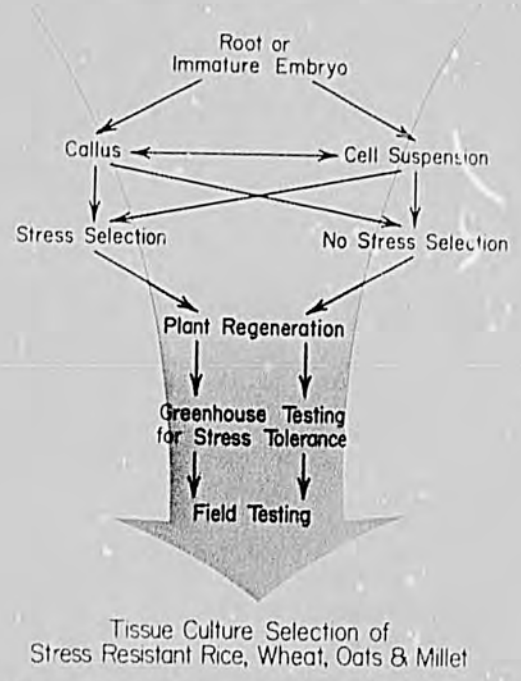
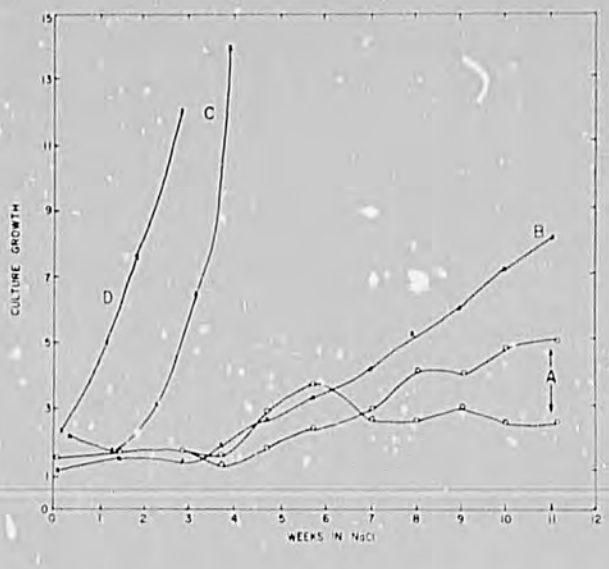


FIGURE 8. A schematic diagram showing how tissue culture is utilized by the TCCP to produce improved plant varieties.

1. **A large population of cells sufficient to contain the desired mutant trait must be produced.** Since desirable phenotypic traits appear spontaneously in one plant out of 100,000 or 1,000,000, at least this many cells must be produced. Mutation induction by radiation or chemicals can increase the frequency at which desirable mutations appear. However, the chance that undesirable mutations will be produced is also increased. For this reason we have elected, after early experiments, not to use mutation induction procedures. We have demonstrated (Nabors et al 1975, Nabors and Kroskey 1982) that desirable mutant cells can be obtained either from cell cultures grown on solid or liquid medium (Fig. 9). Groups of cells on solid medium are referred to as "calluses" while those in liquid medium are referred to as "suspensions." A typical tissue culture vial usually contains a population of around 50,000 cells; a typical suspension flask, around 5,000,000 (Fig. 10). A cell culture of either type is started from a piece of the plant placed on nutrient medium containing hormones which encourage rapid cell division and growth. Typically in our work, mature seeds or immature embryos are used as starting material. A callus arising from such tissue is referred to as "primary callus." After four to six weeks the callus is large enough so that it must be transferred to new medium. At this time the original tissues are removed and the callus is subdivided to become a "secondary callus." Cell suspensions can be obtained from the starting tissue, but it is more effective to obtain them from a callus placed in liquid medium on a shaking machine. Callus can usually be produced with minimal experimentation from any plant, although as will be discussed shortly, several types of callus with differing potentials to regenerate plants can be produced.
2. **A selection procedure designed to a culture of useful mutant cells must be utilized.** For obtaining stress-resistant cells a direct selection procedure is usually most effective. For example, if NaCl-resistant cell lines are needed, cell cultures are grown on or in a medium containing enough added salt to severely retard the growth of non-tolerant cells. Thus if the culture contains one or a few tolerant cells, they will grow and divide more rapidly than non-tolerant cells and slowly come to make up the majority of cells in the population. In our original experiments this type of direct selection was applied in eleven stages, each with an increasing level of NaCl (Nabors et al 1975, see Table 2). The same type of selection can be used to obtain aluminum-resistant cell lines (by adding an aluminum salt like $AlCl_3$ to the medium). To obtain drought-resistant cell lines a high molecular weight chemical (like polyethylene glycol 4000 or dextran) is added to the medium (Heyser and Nabors 1981a). This chemical competes osmotically (in the same manner as dry soil) with the plant cells for available water, thereby selecting for cells with an increased ability to take up water (Fig. 11).



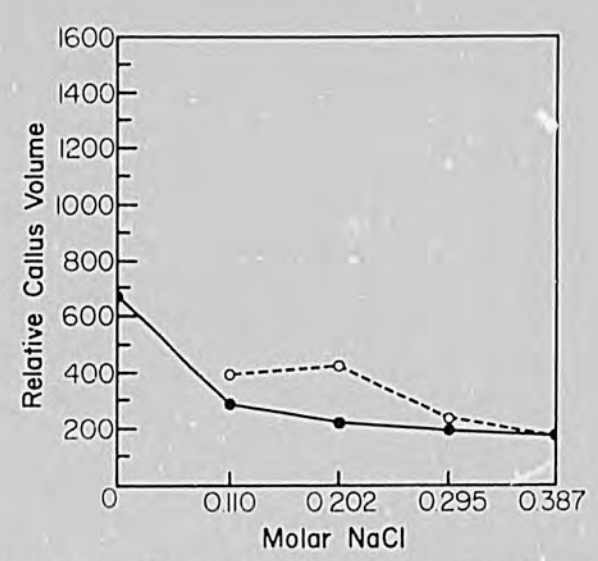


FIGURE 9. Top: Growth of tobacco suspension cultures in medium containing 1.6 g/l NaCl. A. Typical non-mutated cultures. B. Culture exposed to 0.15% EMS for 60 min. C. Typical B culture following 5-way division into fresh medium. Similar results are obtained without the use of mutation enhancing techniques. Bottom: The solid line represents the growth of callus cultures exposed to the indicated concentrations of NaCl for 3 passages following 10 passages on medium containing no NaCl. The dashed line represents the growth of callus cultures exposed to the indicated concentrations of NaCl for 3 passages following 10 passages on medium containing the same salt concentration. Passages were 4 weeks. Each data point represents the 3 passage average growth of 20 calluses. Each point on the dashed line (except at 0.295 molar NaCl) is significantly different than the related point on the solid line.

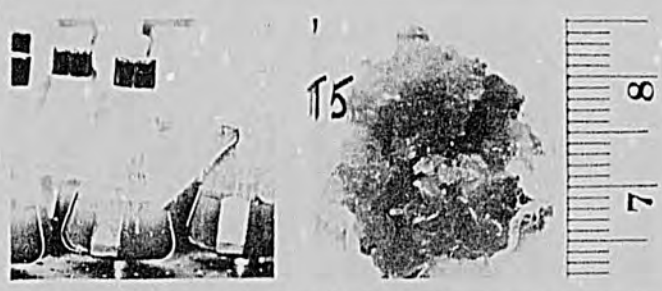


FIGURE 10. A 250 ml flask and a 25 × 70 mm vial are used for plant cell cultures.

Sometimes an indirect form of selection is applied. For example, in nature, salt-tolerant plants are also frequently drought-resistant since these two stresses occur in the same types of environments. This seems to be the case for the plants obtained from our original salt-tolerant cultures. Selecting for salt resistance may well be an indirect means of obtaining drought-resistant plants as well. Also, salt-tolerant plants in nature are frequently shorter than non-salt-tolerant related varieties. We have found this to sometimes be the case for plants regenerated from salt-tolerant cultures of oat cells. Therefore, salt may be an effective agent for selecting plants with potential resistance to lodging.

Although resistant cell lines are frequently referred to as "mutant," it is more appropriate to refer to them as "variant" until they have been shown to be inheritable alterations. Several types of genetic alterations can occur including point mutations, chromosomal mutations (translocations, deletions, duplications, etc.). Some of these alterations, in particular translocations and duplications are frequently unstable (Schimke 1982). It appears from our experiments that variant stability in the absence of stress is related to the length of selection with at least six months of selection required for stability. This is probably a correct observation for two reasons.

a. Short periods of selection do not entirely eliminate non-tolerant cells from the population; thus, reverse selection can occur in the culture in the absence of stress. In addition chimerical plants could result if regeneration occurs from mixed groups of mutant and non-mutant cells.
b. Long periods of selection may allow for the occurrence of cells with two separate genetic mechanisms of tolerance. Such cells would be less affected by reverse selection pressures.

3. **Plants must be regenerated from stress-resistant cultures and from non-resistant cultures (for use as controls).** For most types of cultured cells the rate of plant regeneration gradually declines as the length of culture increases. The decline may occur rapidly, for example, in cereals, or not at all as in the case of some tobacco. Our tobacco cultures are still regenerating large numbers of plants after five years in culture. Prior to the experimental work of recent years in our and other labs, cereal cultures typically lost the limited ability to regenerate plants in a few weeks (Conger 1981). Since selection for stable variants can require up to twelve months it is necessary to have regenerating cultures for at least that period of time. Also the regeneration must be of a reasonably high frequency so that enough plants for testing can be obtained.
4. **Plants obtained from stress-resistant cultures must be shown to retain the resistance and to pass it on in a predictable fashion to subsequent generations.** It is possible that stress-resistance selected in cell cultures will be expressed only in cultures and not in regenerated plants. Our laboratory was the first to

Table 2. Production of Stress-Tolerant Cell Cultures in Stages

1. A culture is obtained which grows rapidly in a medium containing no NaCl. It is transferred to a medium containing 1600 ppm NaCl in which it grows slowly.
2. After several passages in medium containing 1600 ppm NaCl the culture grows rapidly. It is transferred to a medium containing 2200 ppm NaCl in which it grows slowly.
3. After several passages in medium containing 2200 ppm NaCl the culture grows rapidly. It is transferred to a medium containing 2800 ppm NaCl in which it grows slowly.
4. This process is continued until maximum tolerance is obtained. For Samsun tobacco, for example, the maximum tolerance obtained in suspension cultures is 7600 ppm NaCl.
5. Cells adapted to each level of stress can be maintained and plants can be regenerated from each line after various periods of stress. The TCCP maintains several cell lines which have been maintained in medium containing 6400 ppm NaCl for over five years.
6. If cells are grown on solid medium tolerance to higher levels of NaCl can be attained. For example Samsun tobacco can attain tolerance to medium containing 22,600 ppm NaCl. Probably this increased level of tolerance relates to the fact that callus is not in direct contact with the medium whereas suspension cells are surrounded by medium. Similarly, plants regenerated from NaCl-tolerant cultures withstand levels of salt in watering solutions equivalent to those tolerated by cells on solid medium. In the case of whole plants, pot size, soil type, temperature, humidity, and frequency of watering are all variables which affect salt tolerance.

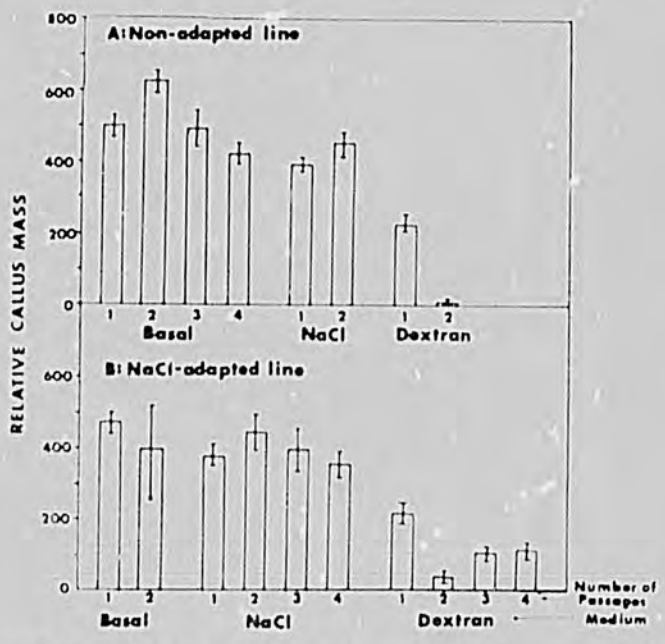


FIGURE 11. Growth of cells subcultured on dextran or 130 mM NaCl for up to four passages. A: Growth of nonadapted cell line. B: Growth of NaCl-adapted cell line. Dextran was iso-osmotic to 130 mM NaCl. The NaCl tolerant cell line is seen to be adapted to chemically-induced drought stress as well.

demonstrate that NaCl-tolerance obtained in cells was also apparent in regenerated plants (Nabors et al 1980) (Table 3, Fig. 12). Since our selection procedure would tend to isolate dominant or co-dominant variants a generation of inbreeding may also be required to obtain a line of plants which inherit the resistant trait in a stable fashion. Some workers have advocated the use of anther-derived cells to aid in the selection of recessive variants. In the first place most cereals are polyploid so such cell lines would be polyhaploid. Secondly androgenic plants are sterile, so additional steps would be required before stress-resistant variants in such plants would be useful in the field.

Testing of potentially stress-resistant plants obtained from cell cultures requires demonstrations that the plants have increased tolerance during germination and the early stages of seedling growth, as mature plants, and during flowering and seed set. Since a single variety typically differs in stress tolerance of these stages all must be examined.

	% alive after 13 weeks			
	F_1		F_2	
	Watering solution		Watering solution	
	26.2 g/l NaCl	0 g/l NaCl	29.8 g/l NaCl	0 g/l NaCl
Original culture #1 resistant to NaCl with NaCl continually present	65	100 (selfed to produce F_2)	100	100
Original culture #2 resistant to NaCl with NaCl not present during regeneration	35	100 (selfed to produce F_2)	90	100
Original culture #3 not resistant to NaCl	20	100 (selfed to produce F_2)	15	100

Table 3. Survival rate under high or no salt stress of salt-tolerant and non-salt-tolerant tobacco plant lines derived from cultured cells. Plants regenerated from tissue cultures were designated the parental generation (P) and were selfed to obtain the F_1 generation. The F_1 plants of each group in the O g/1 NaCl column were selfed to obtain the F_2 generation. F_1 had 43 plants per group: F_2 had 20.

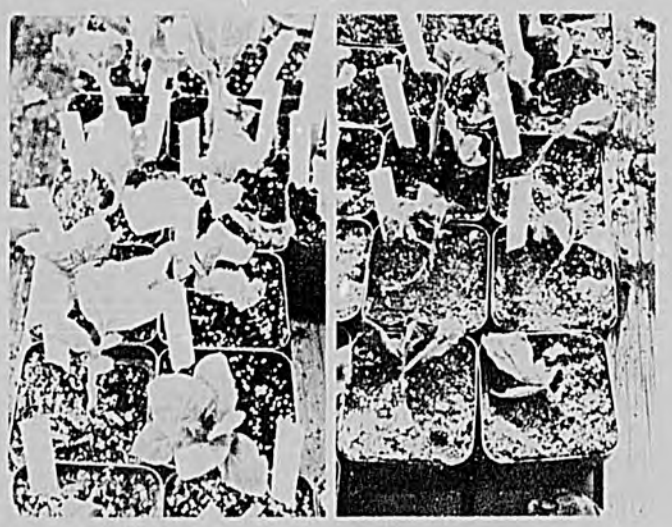


FIGURE 12. Typical progeny of parents regenerated from salt-tolerant (left) and salt-sensitive (right) cell suspensions. Progeny were watered with a solution containing 26.2 g/l NaCl. Clearly salt-sensitive plants do not survive under these conditions.

Also, it is important to determine if resistance is stable in the absence of stress. Plants will be most useful if such stability is found. In the case of NaCl tolerant tobacco obtained from tissue cultures we have found that the resistance is stable through at least two whole plant generations in the absence of stress (Nabors and Kroskey 1982). Cultured cells obtained from regenerated resistant plants also retain tolerance to salt following seven months in culture without exposure to NaCl (Fig. 13).

Finally it must be shown that increasing a particular stress tolerance in a known variety has not unfavorably altered other desirable varietal characteristics. In terms of a single mutation this is equivalent to determining that no detrimental pleiotropic effects occur.

5. **Trials must demonstrate that progeny of regenerated tolerant plants are useful under stress conditions encountered in the field.** Tests of inheritability and stability of tolerance are best carried out under the controlled conditions found in growth chambers or greenhouses. Ultimately, the tissue culture worker and plant breeder must show that plants which appear useful under controlled conditions are useful in the changing environment of the field.

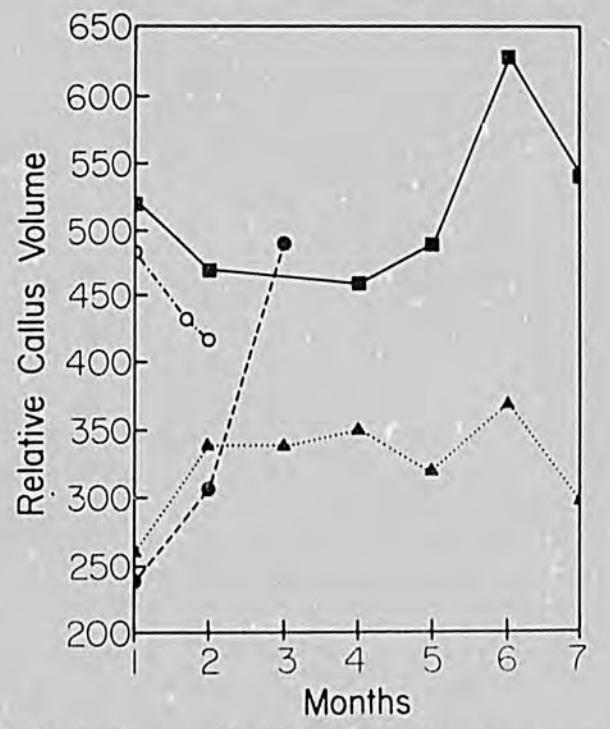


FIGURE 13. Retention of NaCl tolerance in callus during growth in the absence of salt in tobacco cell lines originally obtained from a plant which was the offspring of a plant regenerated from a cell suspension tolerant to 0.110 M NaCl. Triangles represent a callus culture shifted to medium containing 0.295 M NaCl for only the six passages shown. Squares represent a culture after 17 passages on medium containing 0.295 M NaCl. Closed circles represent a similar culture down-shifted for seven passages to medium containing no salt after 17 passages on medium containing 0.295 M NaCl and finally shifted back to salt-containing medium for the four passages shown. Open circles represent growth of the culture represented by closed circles in the three passages prior to down-shifting.

Project Facilities and Administration

The TCCP is housed in the Plant Sciences Building in the main campus of Colorado State University. The project utilizes 2000 square feet of laboratory and office space including a general laboratory, a sterile transfer room and growth chamber facility, and an autoclave-sterilization area (Fig 47, p. 23). Greenhouse facilities for testing of whole plants are located nearby.

Field testing of useful stress-resistant plants is not an in-house goal of the project. Testing of wheat and millet plants can be carried out in Colorado. However, since a variety of natural environments must be considered the project will rely on the International Centers as a primary focus for field testing. Readers of this report who are interested in possible participation in testing germplasm as it becomes available should contact the project director.

Germplasm for our experiments is supplied by the International Maize and Wheat Improvement Center (CIMMYT) and the Agronomy Department at Colorado State University for wheat; by the International Rice Research Institute (IRRI); by Dr. Neil Rutger, Department of Agronomy and Range Science, University of California at Davis and by Dr. M.S. Balal, Ministry of Agriculture, Giza, Egypt for rice; and by Dr. Bill Stegmeir, Kansas State University, Ft. Hays with the advice of the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) for millet.

Project Accomplishments

In its first two years, the TCCP has made significant progress in utilizing plant tissue culture to increase stress resistance in major cereals. Prior to the initiation of the project the following work was completed.

1. **Using tobacco as a model system it was demonstrated that NaCl tolerance could be selected in cell suspensions (Nabors et al 1975).**
2. **Our laboratory was the first in the world to show that salt tolerance obtained in tissue culture was carried into whole plants regenerated from the cultures and their progeny (Nabors et al 1980).** The tolerance is stable in that it remains after two whole plant generations never exposed to NaCl (Nabors and Kroskey 1982)
3. **Tissue culture methods for wheat and oats (as well as other non-cereals) were improved (Nabors 1976, 1979, 1981).** Tobacco was used in the initial work because it is a plant which readily produces cell cultures with high regenerative ability.

Cereals, as noted earlier, are easily used to produce cell cultures, but regeneration from these cultures is typically of low frequency and of short duration. To obtain regeneration at all, hand-dissected immature embryos (a time consuming starting material) had to be used frequently to obtain callus. Therefore the first goal of the project was to obtain methods for high frequency, long term plant regeneration from cells derived from easily obtainable tissues. Progress toward this objective has been rapid, and this phase of the experiment is nearly complete (Heyser and Nabors 1982a, 1982b; Heyser et al 1982; Nabors et al 1982a, 1982b).

1. **For wheat, rice, pearl millet, proso millet, and oats callus cultures have been obtained from seedling roots, from seedling apical meristems, and from immature embryos.**
2. **Callus with high frequency, long duration regeneration has been selected for each plant for callus derived from roots and immature embryos.** Our regeneration frequencies are ten to twenty-five times those reported in the literature. Whereas regeneration typically is difficult to obtain for cereal tissue cultures after two to three months, our cultures maintain suitable regeneration frequencies for over one year and apparently indefinitely. We have concentrated on obtaining regeneration from seedling root callus since seeds are easily obtained and cultured compared to immature embryos. The latter are more used in research reported in the literature because they give rise to callus which will regenerate plants more readily using standard culture conditions.
3. **Callus tolerant to 3000, 6000, and 9000 ppm NaCl has been obtained for each species.** Selection for drought-tolerant and aluminum-tolerant callus is underway for each species.
4. **For rice, over 3400 plants have been regenerated from callus cultures, over 200 of the plants are from cultures tolerant to NaCl (see Table 2).** For wheat and pearl millet over 300 plants have been regenerated, over 30 from NaCl-tolerant cultures. For oats over 1000 plants have been regenerated, over 350 from NaCl-tolerant cultures. For proso millet over 100 plants have been regenerated, over 20 from NaCl-tolerant cultures. In each case, all plants from tolerant cultures and some from non-tolerant cultures are grown to maturity.
5. **As seed is obtained from regenerated tolerant plants, germination tests are performed to determine if increased NaCl tolerance occurs.** Seeds showing increased tolerance are grown to mature plants which are again tested for NaCl tolerance. Plants with increased stress tolerance in both tests are used to obtain seed stock for future field tests. At present germination and whole plant salinity testing is just beginning for rice, wheat, pearl millet, and oats.

Methodology and Discussion of Project Accomplishments

General Methodology

For introductory information on project methods, readers are referred to **Rapid Crop Improvement Using Tissue Culture Technique,** Technical Series Bulletin No. 24, Office of Agriculture, Development Support Bureau, Agency for International Development, Washington, D.C. 20523, April, 1980.

As a basic medium the project uses that of Linsmaier and Skoog (1965). This medium is high in both reduced and oxidized nitrogen; many other similar media would yield suitable results. Readers are referred to two excellent books edited by H.E. Street and T.A. Thorpe (see References) for additional media. Our media are adjusted to pH 5.5 prior to autoclaving. As shown in Figure 14 (D. Hoisington and M. Nabors, unpublished) agar acts as a buffering agent for most media. In initiating tissue culture of any species we reviewed all relevant literature. Then we tested a number of possibly important growth hormones (auxins and cytokinins) at various concentrations to determine the best medium for maximal callus growth and the best medium for growth of callus which regenerates plants (R callus; see next section). Optimal tissue culture media tend to be variety specific. Also the best medium for maximal callus

growth is frequently not the best medium for production of R callus (see Table 4). This fact has retarded the discovery of successful regeneration methods.

For dicots a figure similar to the one in Figure 15 is constructed. The medium which is best for callus growth or for plant regeneration can be easily identified. In Figure 15 two hormones are considered. The introduction of additional substances promoting growth or differentiation considerably complicates the picture. Determining the very best medium for plant regeneration, for example, is a reasonably complex procedure. For grasses a medium containing an optimal concentration of an auxin usually promotes adequate callus growth (Fig. 16). Again, as illustrated in Table 4 this medium may not be optimal for regeneration. In terms of medium selection the goal of the TCCP has been to find a medium which allows callus to regenerate plants at a reasonably high frequency for at least one year.

Callus growth and regeneration frequency are measured by the cross sectional comparison method of Hooker and Nabors (1977). Briefly, callus size is compared to the size (as measured by cross sectional area) of a number of standard clay spheres. By arbitrarily setting the mass of the smallest sphere as 1.00, relative mass determinations can easily be made. We use this method because it eliminates the time-consuming step of weighing individual calluses. Studies in the lab indicate that an experienced technician can obtain comparative measurements with this method which are equal in accuracy to those obtained by actual weight measurements.

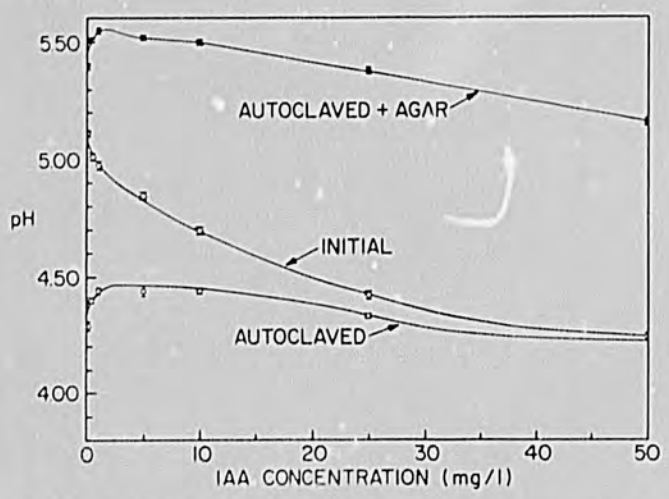


FIGURE 14. The effects of IAA (a weak acid), agar, and autoclaving on pH of Linsmaier and Skoog's medium. Autoclaving lowers the pH by driving off dissolved NH_3.

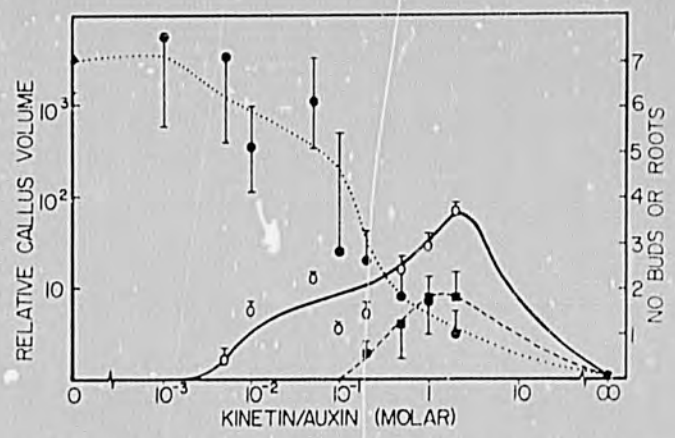


FIGURE 15. The production of callus (open circles), shoots (solid squares), and roots (solid circles) by primary tobacco callus derived from stem sections. Data was taken after six weeks; 25 vials per data point were averaged; standard errors are shown.

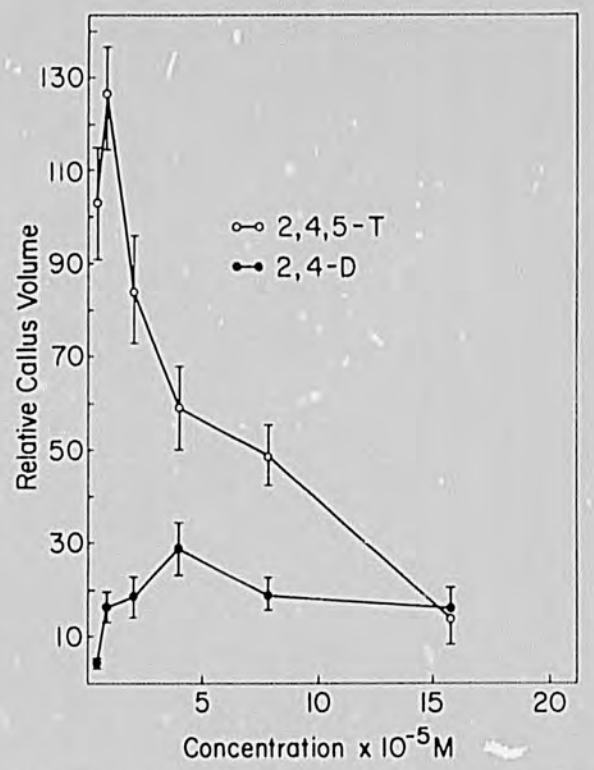


FIGURE 16. The production of callus from germinating seeds in Park oats as a function of the concentration of two artificial auxins.

Table 4. Relative Callus Sizes and Percentages of Calluses With R Regions in Three Millets After Four Weeks on Media Containing Various Hormone Concentrations.

Calluses were initiated from seedling roots. 30 calluses per treatment. Media were selected from those giving maximal callus growth or production of R regions in previous experiments. *'s indicate maximal callus growth or R region percentage. In Senegal Bulk, 147.4 is not significantly different (95% confidence level) from 138.7. For the other two varieties the medium producing maximal callus growth is different from the one producing a maximal percentage of vials with R regions.

Millet Species And Variety	2.5 ppm 2,4-D	2.5 ppm 2,4-D 1.0 ppm IAA 0.5 ppm Kinetin	1.0 ppm 2,4,5-T	1.0 ppm 2,4,5-T 1.0 ppm IAA 0.5 ppm Kinetin
Pearl Millet	16.7%	42.9%*	0%	0%
World Composite	153.5	87.7	129.7	199.6*
Pearl Millet	13.5%	25.7%	10.3%	31.4%*
Senegal Bulk	31.5	91.6	147.4*	138.7
Proso Millet	44.8%*	21.4%	3.3%	0%
Abarr	22.5	10.4	188.6*	150.6

Obtaining high frequency, long term regeneration from cereal callus.

Crucial to the success of the TCCP in regenerating large numbers of cereal plants from tissue cultures has been the recognition that on solid medium at least two types of cells are produced. So-called "embryogenic callus" (Fig. 17) is translucent to opaque in appearance, milky white to yellow or yellow-green in color, and appears as dense, compact regions of non-vacuolated cells which produce small embryos or embryoids that develop into complete plants. So-called "non-embryogenic callus" is crystalline and transparent in appearance, white, tan, brown, or green in color, and appears as loosely packed, friable regions of vacuolated cells which produce roots or less frequently shoots by organogenesis but never complete plants. These two callus types have been noted by a number of tissue culture workers (see Heyser and Nabors 1982a, 1982b; and Heyser et al 1982).

We are the first to quantify the fact that embryogenic callus has a much higher frequency of regeneration than non-embryogenic callus and that it is found in root-derived cultures of several major cereals (Fig. 18).

In terms of basic research several important questions can be asked about the two callus types. First, are there really two callus types or simply two ends of a continuum? Our observations indicate that embryogenic callus tends to become non-embryogenic as the cells become larger and more vacuolated and that the reverse change does not oc-

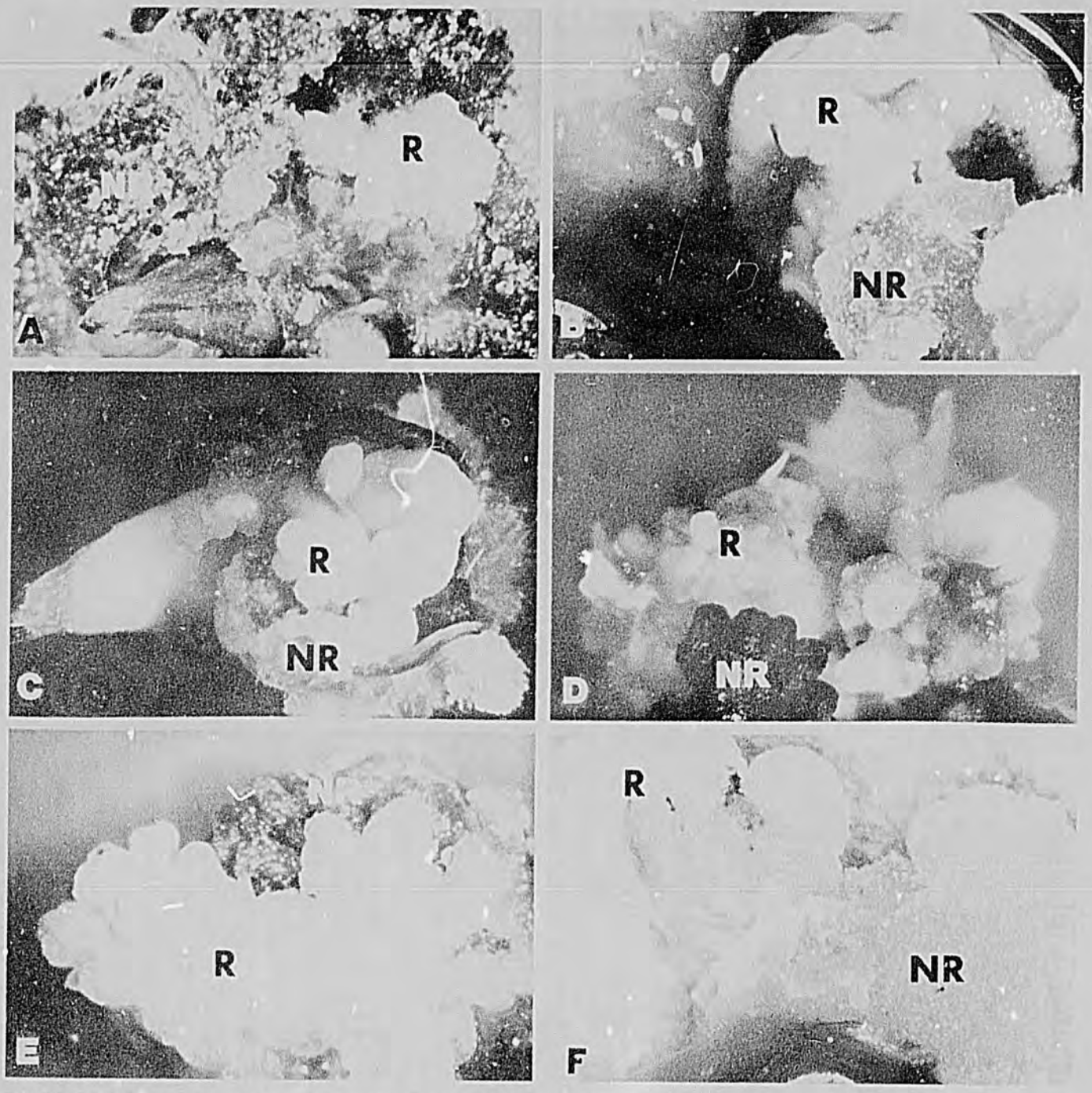


FIGURE 17. Callus cultures with both embryogenic (R callus) regions and nonembryogenic (NR callus) regions. Some calluses have regenerated shoots (S). Callus from (A) seeds of Drought Resistant 7203 pearl millet; (B) seeds of Abarr proso millet; (C) seeds of Park oats; (D) seeds of Giza-159 rice; (E) stem sections of Samsun tobacco *(Nicotiana tabacum)*; (F) seeds of Glennson wheat.

cur. This is in line with the normal developmental process for plant cells although instances in which vacuolated cells give rise, by unequal division to a vacuolated and a non-vacuolated daughter cell are known (see Raghavan 1976). In 1979 H.E. Street proposed that calluses have two types of cells from the time of initiation and that while unequal growth rates can lead to a callus of predominately one type, the two types are basically non-interconvertible. We have observed that the two callus types have different hormone requirement for growth. Thus a callus of predominately one type can be obtained. Also, as noted before we find that embryogenic callus tends to become non-embryogenic; but our observations are not precise enough to distinguish whether this actually occurs or whether small non-embryogenic regions simply outgrow and overgrow the embryogenic regions. Thus the initial question — whether there are two cell types or a continuum of cell types — and the more basic question of interconvertability of embryogenic and non-embryogenic cells remains unanswered at this time. We find that what appears to be totally non-embryogenic callus continues to give rise to embryogenic regions, but these could arise from a few undetected embryogenic cells.

A second series of questions concerns the use of the term "embryogenic" to describe callus that appears to have embryonic stages and to give rise to embryos which develop into complete plants. In a few cases workers have begun to carry out cytological experiments necessary to show that stages characteristic of normal embryogenesis are found in embryogenic callus (Vasil and Vasil 1981). However many workers use the term without that demonstration. While embryogenic callus does appear to consist of lumps representing embryoids the use of the term embryogenic is probably presumptive at this stage. Swamy and Krishnamurthy (1981) feel that for dicots at least, embryos arising from cultured cells bear only superficial anatomical similarity to zygotic embryos and are more closely related to shoot buds. Since our goal is to produce large numbers of plants from callus cultures, we prefer to sidestep these important basic research questions and to use the term "regenerative callus" (R callus) instead of embryogenic callus and "non-regenerative callus" (NR callus) instead of non-embryogenic callus.

Since each cultured cell represents a potential plant, we originally sought to produce a large population of cells as rapidly as possible. This generally resulted in a callus consisting of a larger portion of NR cells with time. The common observation that regeneration in cereal callus falls off rapidly with time results from the fact that most tissue culture media favor the growth and division of NR cells. If small regions of R and NR cells are isolated, the R cells rapidly produce plants while the NR cells never do. Thus the low level of plant regeneration observed in large NR calluses may come from unobserved R regions within these calluses. This understanding resulted in an early redirection of our experiments to discover media which favored the growth and division of R cells.

Typical cereal callus derived from seedling roots consists predominately of NR regions. Between 0 and 25% of

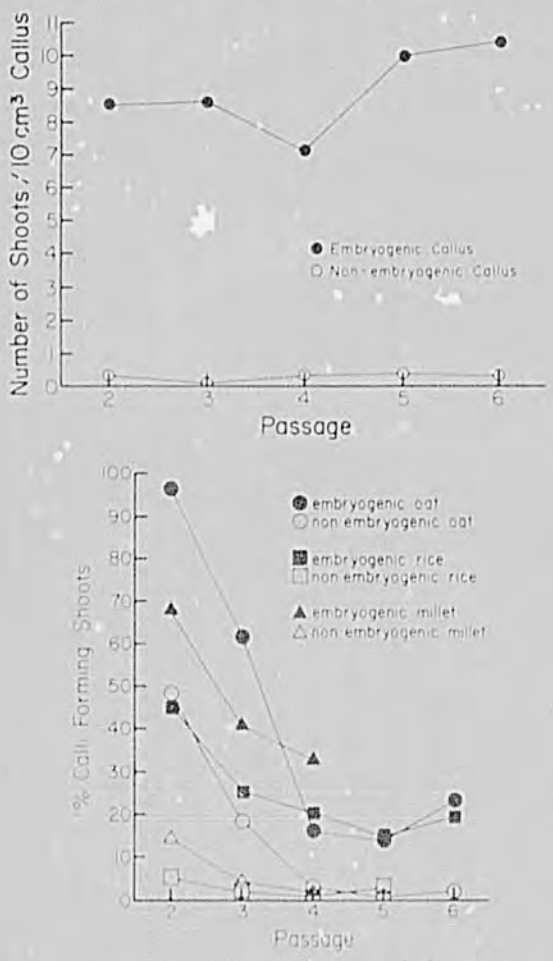


FIGURE 18. Top: Number of shoots per 10cm³ of callus as a function of number of five week passages. The data are combined from four rice varieties (Giza-159, Pokkali, IR-36, and Mahsuri). Average sample size is 102 shoots. A passage averaged five weeks. Bottom: Frequency of shoot formation in vials of embryogenic (R callus) or non-embryogenic (NR callus) tissue derived from seedling roots. A passage averaged five weeks. The first passage was for primary callus induction. Average sample size is 40 vials.

FIGURE 19. Several Mahsuri rice plants regenerating from a white pad of R callus. The shiny gray callus in the lower right is typical of NR cells in Mahsuri. (20×).

culture vials are found to contain R regions which comprise from 1-10% of the total callus. If R regions are removed to appropriate media, plant regeneration results (Fig. 19). No media have been found which allow regeneration from similar NR regions. R callus can be obtained from secondary cultures by (1) maintaining R regions on a medium which favor division and growth of R cells but not regeneration or by (2) continuing to remove R regions as they appear on NR callus masses. Experiments to increase the percentage of culture vials with R regions and to increase the percentage of R cells in a callus are currently in progress.

The ability to produce regenerated plants at a reasonable frequency and for a period of some months is important since selection of desirable mutants in tissue cultures requires time. As discussed earlier, long term mutant selection is necessary to insure that stable cultures and nonchimerical mutant plants are obtained.

FIGURE 21. R and NR callus developed from a germinating Glennson wheat seed. (8.5×).

Wheat Methodology

At present the TCCP is using Pavon, Glennson, Chris, and Chinese Spring cultivars of wheat. Most preliminary and methodological work was carried out with Chris. Chinese Spring is used because of its usefulness in the wide cross program carried out by CIMMYT. In addition preliminary tissue culture experiments are being conducted on Triticale.

Most callus cultures are obtained from seedling roots. Seeds are surface sterilized briefly with 90% ethanol then with 20-50% Clorox for 30 minutes. The Clorox concentration is seed lot dependent. In a few cases the Clorox treatment is followed by a 1-3 minute exposure to .025-.100% mercuric chloride. This treatment is utilized only when surface contamination by bacteria and fungi cannot be satisfactorily reduced with Clorox. Mercuric chloride is taken up and retained by seeds and is thus quite toxic to the seeds both in terms of germination and callus formation. Uptake seems to be more affected by exposure time than by concentration so short exposures of higher concentrations are more effective than longer exposures of lower concentrations. Following surface sterilization the seeds are rinsed six times in sterilized water. When immature embryos are used they are hand dissected following a similar surface sterilization.

Seeds or immature embryos are then placed on a solid medium (1% agar) containing Linsmaier and Skoog's (1965) major and minor salts and vitamins, 4% sucrose, and various concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) or 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). Concentrations of between 1.0 and 10.0 ppm are most effective in producing callus (Fig. 20). The concentration and hormone type are variety dependent. In general more callus forms for wheat in the dark than in the light. However R regions are difficult to identify for wheat (Fig. 21) and this difficulty is increased in dark-grown callus. Methods to increase the amount and proportion of R callus formation in wheat are in the process of being determined. Calluses incubated in the dark are much more effective in producing R callus than are calluses incubated in the light. Hormones other than 2,4-D or 2,4,5-T obscure this difference. The effects of other media additives is variety specific and has not been fully determined. Callus is sized by the cross sectional area method of Hooker and Nabors (1977). In the case of

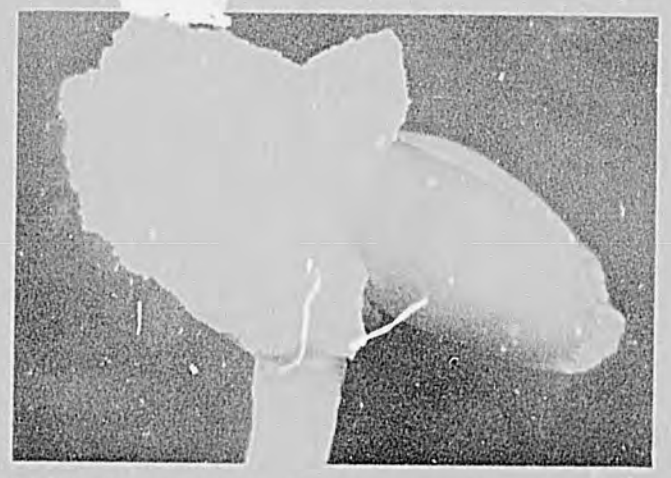


FIGURE 20. Typical Chris wheat callus developed from a germinating seed. (9×).

FIGURE 22. A Chris wheat bud (35×) and several plants (1×) in a culture jar.

selection for stress-tolerance the stressing agent (NaCl, $AlCl_3$, polyethylene glycol, or dextran of a specific MW) is added to the callus induction medium or to medium supporting the growth of callus established in the absence of stress. Callus is transferred to new growth medium every five weeks.

Plant regeneration in wheat is obtained by shifting callus to medium containing no added hormones (Fig. 22). R callus produces regenerated plants within two five-week passages on this medium. Mixed, R and NR callus produces some regenerated plants, but since NR callus grows more rapidly on L and S medium regeneration ceases after 15-20 weeks in culture. Isolated NR callus never produces regenerated plants. R callus does not grow on regeneration medium. After two passages for plant regeneration on this medium it is shifted back to auxin-containing medium for continued growth. Culture conditions which increase the amount of R callus formed and the growth rate of R callus are currently under investigation in wheat.

Plants are removed from attached callus and placed in an open vial containing tap water (changed daily) and perlite for continued root growth. After one-two weeks well-rooted plants are placed in four inch pots in commercial, unsterilized potting soil mixed with 25% perlite. Warm temperatures (around 30 C) seem to promote plant survival during the first three weeks following removal from culture vials.

Wheat plants are grown to maturity in a growth room or greenhouse maintained at 25-30 C (Fig. 23).

FIGURE 23. Wheat plants in the TCCP greenhouse.

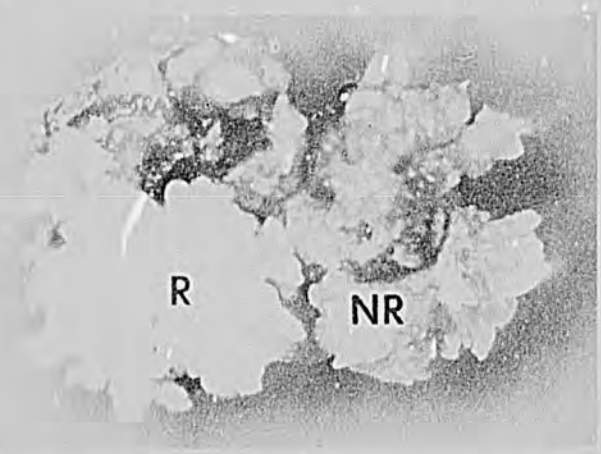


FIGURE 24. R and NR callus from Mahsuri rice seeds. (6×).

Rice Methodology

We are presently using IR-36, Pokkali, and Mahsuri cultivars of Indica rice and Giza-159 and Calrose-76 cultivars of Japonica rice. Conditions for surface sterilizing and culturing seeds and immature embryos to obtain callus are the same as those reported for wheat. In contrast to wheat, R callus regions are easily identified in rice (Fig. 24 and Heyser et al 1982). Rice is quite variety specific with respect to cultural conditions resulting in callus formation in general and R callus formation in particular.

Calrose-76 seeds form maximal callus on medium containing 1.0-5.0 ppm 2,4-D. R regions appear on 20% of the calluses formed at 1.0 ppm and 0% of the calluses formed at 5.0 ppm. If tryptophan (TRY) or indoleacetic acid (IAA) are added to callus initiation medium the percentage of vials containing R regions dramatically increases to as much as 80% (Fig. 25) while growth of NR callus is not promoted. At least 1.0 ppm 2,4-D is necessary for callus initiation in the presence of IAA or TRY. The effect of these additives is most prominent at 1.0 ppm 2,4-D and decreases at 2.0 ppm and 5.0 ppm 2,4-D. After initiation Calrose-76 callus continues to grow for at least a year without the presence of exogenous auxin in the medium. This growth is not enhanced by addition of 1.0 ppm 2,4-D. These results suggest that initiated callus rapidly becomes auxin autotrophic. Plant regeneration occurs only from R regions (Fig. 26) and is enhanced when these regions are placed on a medium containing triiodobenzoic acid (TIBA) as opposed to IAA (Table 5). Since TIBA is an auxin transport inhibitor, regeneration in Calrose-76 rice callus seems to require lower auxin levels than callus initiation and growth.

Giza-159 seeds form maximal callus on medium containing 1.0 ppm 2,4,5-T. Established callus will continue to

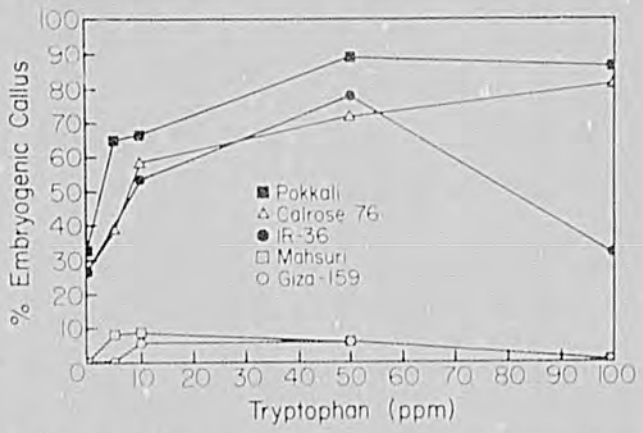


FIGURE 25. A 100% R callus (8×) produced from Calrose-76 rice seed. In some rice varieties R callus production increases dramatically with the addition of tryptophan (an IAA precursor) to the medium. Other varieties do not respond at all.

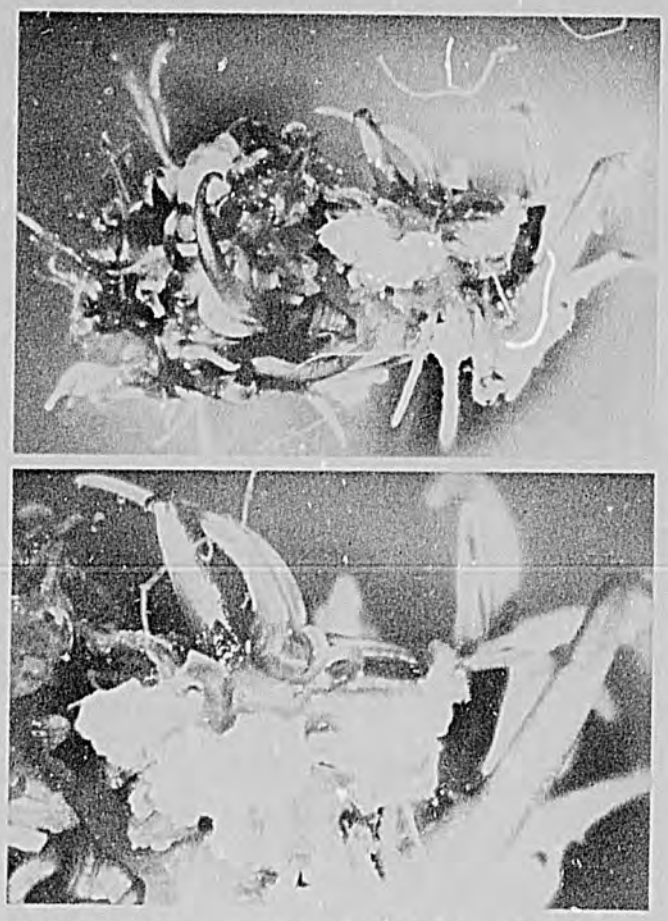


FIGURE 26. Plant regeneration (7× and 12×) from R Calrose rice callus. R callus is clearly visible in some cases and is beneath the plants in other cases.

grow for several passages in the absence of exogenous auxin, although added auxin is required for long term vigorous callus growth. R regions appear on 10-20% of the calluses formed, and added TRY or IAA do not increase this percentage (Fig. 25). Calluses from which R regions have been removed continue to produce R regions for at least 13 months. Most regeneration occurs from R regions placed on medium containing no exogenous auxins.

Mahsuri seeds produce maximal callus on medium containing 0.5 ppm 2,4-D plus 0.5 ppm kinetin. R regions appear on 60-80% of the calluses formed. Addition of 0.5 ppm kinetin to induction medium increases overall callus growth and the amount of R-callus in both primary and secondary passages. Addition of TRY or IAA is without effect (Fig. 25). Mahsuri callus does not grow at all in the absence of exogenous auxin. In fact, low auxin levels such as 0.2 ppm 2,4,5-T are required to keep R regions alive long enough for regenerated plants to form. R regions are produced as long as the callus lives — typically for 7 or 8 passages. Kinetin (0.5 ppm) appears to increase the life span of secondary R callus.

In Mahsuri, R callus can be maintained in a growing, non-regenerating state on a medium containing 1.0-6.0 ppm 2,4-D and 0.1 ppm kinetin (Fig. 27).

A large number of different media containing various auxins, cytokinins, and combinations have been tested for regeneration of plants from R regions. Regeneration of R callus is maximized on a medium containing 0.5 ppm IAA and 0.4 ppm benzylamino purine (BAP) (Fig. 28). Table 6 shows that this medium is far superior to one containing kinetin instead of BAP or 2,4-D instead of IAA. NR callus

Table 5. The Percentage of Calrose 76 Rice Shoots Produced by R Callus in Media Containing 0.5 ppm IAA or 0.5 ppm TIBA.

Cultures were maintained on media containing either 1.0 or 2.5 ppm 2,4-D on the previous passage. Each treatment contained 30 vials. NR callus gave no shoots in an indentical experiment.

	IAA	TIBA
1.0 ppm 2,4-D	41.7%	80.0%
2.5 ppm 2,4-D	22.2%	75.0%

FIGURE 27. Callus from Mahsuri rice. Both vials contain R regions. The vial on the right was placed on regeneration medium.

FIGURE 28. Regenerating R Mahsuri rice callus (0.8× and 1.1×).

Table 6. Plant Regeneration from Mahsuri R Callus as a Function of Three Media. 25 jars per medium.

	0.5 ppm 2,4-D +0.5 ppm kinetin	0.5 ppm IAA +0.4 ppm kinetin	0.5 ppm IAA +0.4 ppm BAP
number of plants per jar after 5 weeks	3	7	14

grows best on a medium containing 1.0 ppm 2,4-D or 2,4,5-T and regenerated plants on no tested medium.

In Mahsuri, if all callus from one seed was maintained and subdivided for 5 passages, approximately 40,000 plants would be produced in the fifth passage from R callus. This projection is based on sixth passage regeneration rates.

IR-36 seeds form maximal callus on a medium containing 2.0 ppm 2,4,5-T. R regions appear on 12-20% of the calluses formed and their rate of appearance is increased by TRY (Fig. 25). Secondary callus is maintained on a growth medium containing 1.0 ppm 2,4-D and 0.5 ppm kinetin. Callus is difficult to keep alive past 6-7 passages unless kinetin is added. Production of R regions and regeneration from them declines gradually throughout the life of the callus. As with Mahsuri, some auxin must be present in the regeneration medium to keep the callus alive. 0.2 ppm 2,4,5-T is a useful concentration for this purpose, however, 0.5 ppm IAA plus 1.0 ppm benzylamino purine (BAP) seems better in preliminary trials.

Pokkali seeds form maximal callus on a medium containing 1.0 ppm 2,4-D. R regions appear on 14% of the calluses, and this percentage is increased by TRY or IAA (Fig. 25). Pokkali callus is easier to keep alive than either IR-36 or Mahsuri callus and its growth is promoted best by 1.0 ppm 2,4-D. Plant regeneration from R regions is promoted by 2,4,5-T and declines gradually for 7-8 passages.

In general, for rice high levels of synthetic auxins (2,4-D or 2,4,5-T) inhibit both callus formation and regeneration. Callus formation requires low levels of these auxins (usually 1.0-2.0 ppm) whereas plant regeneration from R regions requires lower levels (0.1-0.2 ppm) or none. Callus from the two Japonica varieties seems to be somewhat (for Giza-159) to completely (for Calrose-76) auxin autotrophic after it is induced. Higher exogenous auxin levels in callus induction or callus growth media definitely inhibit or delay plant regeneration from R regions maintained on medium containing no exogenous auxins or even TIBA. In contrast, callus from the three Indica varieties does not survive without exogenous auxin which is also required in lower levels to keep R callus alive for plant regeneration. Plant regeneration on optimal media occurs at a higher frequency for Indica than for Japonica varieties.

The cultural conditions (particularly cytokinin effects) favoring production of R callus and plant regeneration from that callus have not been fully determined for any variety. However regeneration rates are of high enough frequency and long enough duration for each variety to allow considerable progress in the selection of stress tolerant cultures and regenerated plants (Table 7).

In terms of auxin levels it seems likely that the rice varieties can be placed in one of two basic groups. Group I contains Calrose-76, IR-36, and Pokkali. The characteristic of this group is that formation of R callus requires auxin levels higher than those necessary for growth of NR callus. Thus added TRY or IAA markedly increased the percentage of vials with R regions. In the case of Calrose-76 endogenously-produced auxin is sufficient for growth of NR callus whereas this is not the case for IR-36. Group II contains Giza-159 and Mahsuri. The characteristic of this group is that the level of auxin required for formation of R callus is lower than that required for formation of NR callus. In the case of Giza-159 the level required for formation of NR callus is produced endogenously so the frequency of formation of R regions is high and added TRY and IAA are without effect. In the case of Mahsuri, endogenous auxin production is low or absent in callus. When exogenous auxin is added, low levels favor R callus; at higher levels NR callus begins growth also.

The effect of NaCl added to callus induction medium is shown in Fig. 29. The Japonica varieties seem to be more salt tolerant than the Indica varieties. Also R callus induction seems to be more sensitive to added salt than NR callus induction.

Regenerated rice plants are removed from callus and placed in a vial containing tap water (changed daily) to promote root growth (Fig. 30). They are then placed in 5 cm pots containing a mixture of commercial potting soil and 25% perlite for 1-2 weeks before transfer to 10 cm pots (Fig. 31). Plants are maintained on long days (16L:8D) until they are 45 cm high at which time they are given a 14 day short day (8L:16D) treatment to induce flowering (Fig. 32). Plants are then moved to a LD growth chamber or greenhouse and fertilized with commercial plant food. Mahsuri and IR-36 appear not to respond to the SD induction treatment. All plants are maintained at 30 C during the day and 25 C during the night.

Table 7. Number of shoots per 10 cm^3 of callus as a function of number of five week passages for four varieties. Average sample size is 25 shoots.

PASSAGE	CALLUS TYPE	SHOOTS/10 CM_3 GIZA-159	IR-36	MAHSURI	POKKALI
2nd	Regenerative	8.45	3.42	25.20	2.02
	Non-regenerative	0.88	0.29	0.94	0.40
3rd	Regenerative	2.43	8.61	23.80	7.68
	Non-regenerative	0.15	0.00	0.00	0.00
4th	Regenerative	0.69	14.00	6.81	3.11
	Non-regenerative	0.17	0.16	2.83	0.18
5th	Regenerative	0.00	11.00	13.40	3.41
	Non-regenerative	0.42	0.16	0.46	0.15
6th	Regenerative	-	11.90	9.41	-
	Non-regenerative	-	0.00	0.75	-

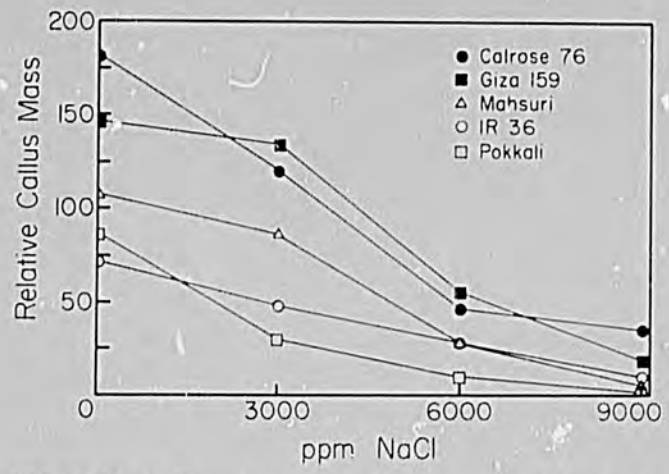


FIGURE 29. NaCl, a typical stressing agent, reduces the rate of callus formation.

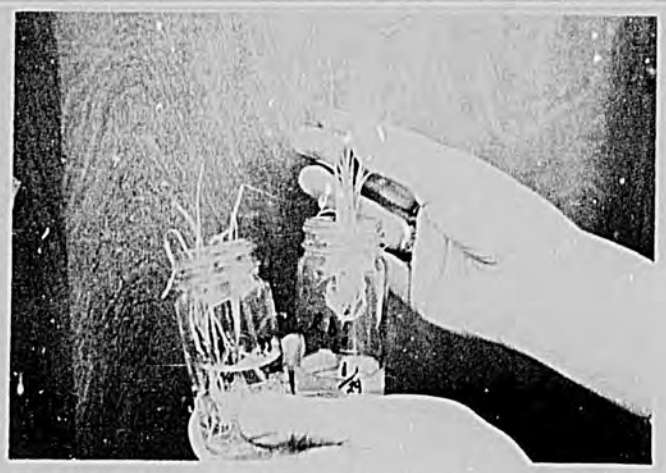


FIGURE 30. Regenerated plants are removed from tissue culture and placed in tap water to increase root formation.

Pearl Millet Methodology

Currently in tissue culture studies we are using the following cultivars: Gahi, Hays Millet Population 559, World Composite C75, Senegal Bulk, and 7203 Drought Resistant. Conditions for surface sterilizing and culturing seeds and immature embryos to obtain callus and for reflecting stress-resistant cell lines are the same as those reported for wheat except that sterilization times are usually less and Clorox concentrations lower. Cultural conditions for pearl millet callus and plant regeneration are much less variety specific than those for rice.

Pearl millet seeds form maximal callus on medium containing 1.0 ppm 2,4,5-T and either 2% or 4% sucrose (Fig. 33). After initiation pearl millet callus continues to grow maximally on the same medium. As is the case with rice, R callus regions are easily identified in pearl millet, and R regions of callus appear in around 0-10% of vials depending on the variety. If 2,4-D replaces 2,4,5-T as the exogenous auxin, overall callus growth decreases but the percentage of vials containing R regions increases in some varieties (Table 4). If low levels of kinetin and IAA are added to the medium in addition to 2,4-D the percentage of vials containing R regions further increases. For example on medium containing 2.5 ppm 2,4-D, 0.5 ppm kinetin, and 1.0 ppm IAA, 25% of calluses contain R regions. Formation of new callus containing R regions does not continue beyond two passages on this medium. The calluses are then shifted to a medium containing 1.0 ppm 2,4,5-T to restore rapid callus growth.

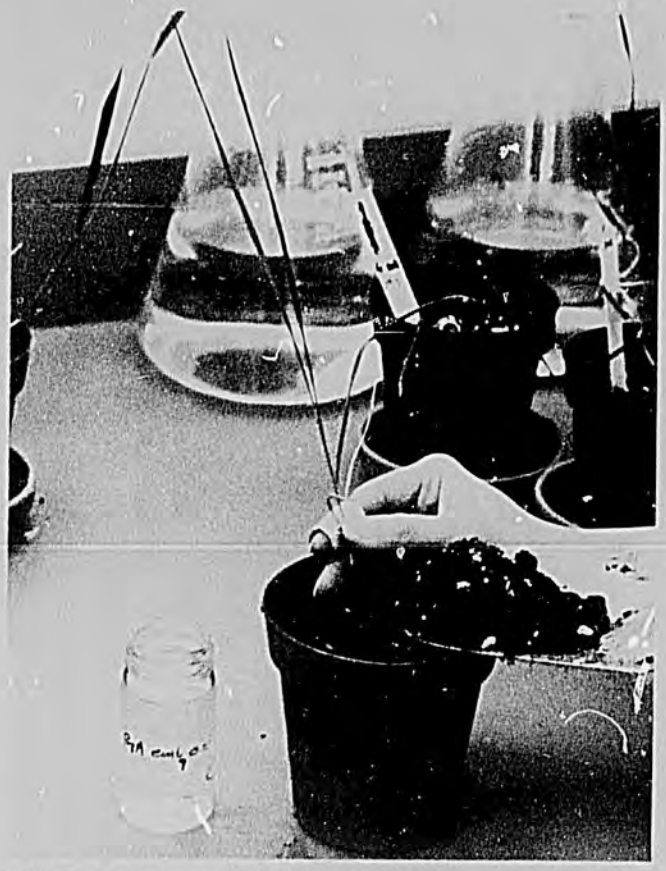


FIGURE 31. Regenerated plants are planted in small plastic pots which are placed in growth chambers or in the greenhouse.

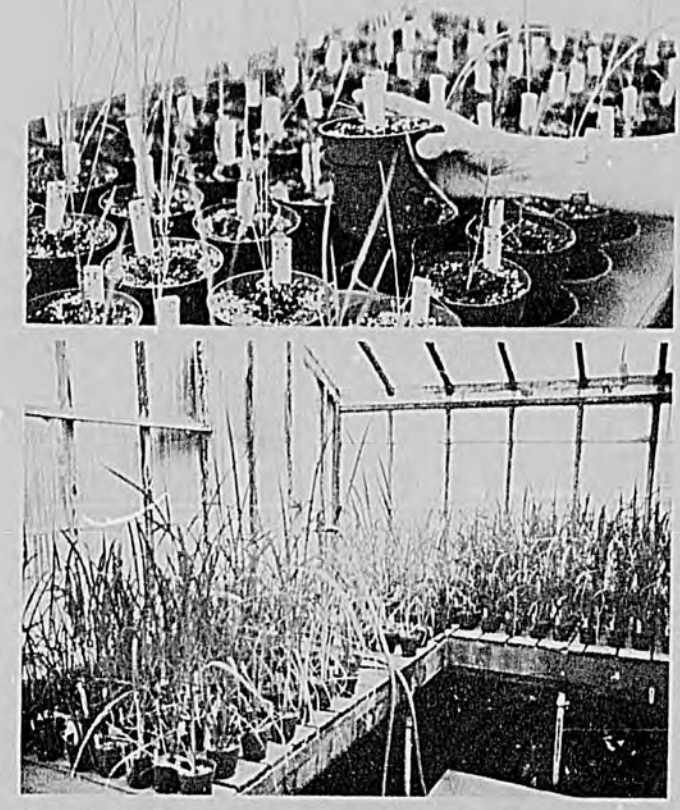


FIGURE 32. Regenerated rice plants in the greenhouse.

R regions of callus are placed on a medium containing 0.2 ppm IAA to stimulate plant formation (Fig. 34). R callus dies if placed on a medium with no exogenous auxin. On media containing low auxin, R callus either forms plants or maintains itself in the R state. The addition of cytokinins to medium appears to have no effect on either callus formation, callus growth, or plant regeneration although some experiments are still underway. Regenerated pearl millet plants are removed from callus and treated exactly as rice plants (Fig. 35).

FIGURE 33. Callus formation (9×) from a germinating Senegal Bulk millet seed.

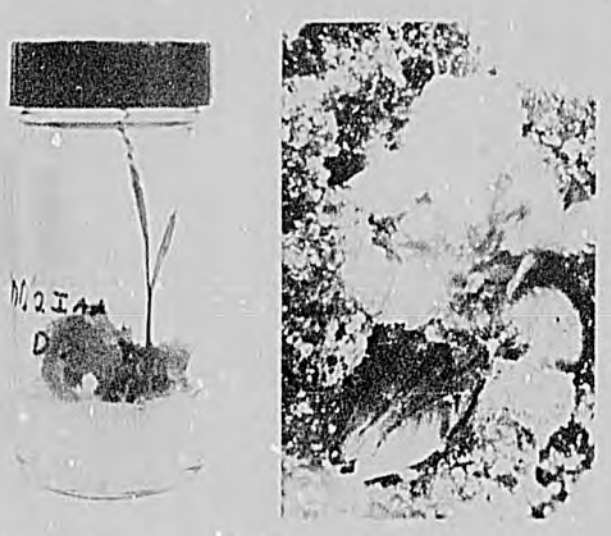


FIGURE 34. Regenerating 7203 Drought Resistant millet plant in a tissue culture jar and a bud forming from 7203 Drought Resistant R callus (7×).

Methodology for Other Millets

The TCCP has completed preliminary experiments with two forage millets — Foxtail *(Setaria italica* 'Golden German') and Proso *(Panicum milaceum* 'Abarr'). For foxtail millet, maximal callus formation was obtained on a medium containing 1.0 ppm 2,4,5-T. The cultural conditions giving rise to R callus regions were not investigated. One plant was regenerated from secondary callus on medium containing 3000 ppm NaCl and one plant on medium containing 9,000 ppm NaCl.

More extensive experiments were carried out for proso millet. Callus was obtained from seedling roots, immature embryos, and mesocotyls as described in Heyser and Nabors (1982b). The 2,4-D or 2,4,5-T concentrations giving the most vigorous callus growth and the highest production of R regions differed for each starting material (Fig. 36). An average of 8% of all calluses produced R regions during the first three passages. Plants were obtained by placing callus on a medium containing no added hormones (Fig. 37). R callus produced plants at a much higher frequency and for a longer duration than NR callus. Culture conditions increasing the frequency of R callus were investigated; 2,4,5-T inhibits its formation compared to 2,4-D. Over 100 plants were regenerated including some from culture media containing NaCl (Fig. 38).

FIGURE 35. Flowering head of a regenerated pearl millet plant.

Methodology for Oats

The TCCP continues to use oats *(Avena sativa* L. 'Park') as a model plant for developing grass tissue culture methods. Oat tissue cultures are obtained primarily from seedling roots although mesocotyls and immature embryos have been used in some experiments. Surface sterilization of starting materials is carried out by the methods described for wheat. Maximal callus production is found on L and S medium containing 1 ppm 2,4,5-T (Fig. 16). The method used for wheat successfully selects stress-resistant calluses. R callus appeared in between 0 and 21% of vials depending on medium, tissue source, and length of culture. Conditions required to increase the frequency of R callus are now under investigation. Media containing 2,4-D produce significantly more R callus than media containing 2,4,5-T. In one experiment, of 628 vials containing callus on medium with 2,4-D, 8.4% produced R regions; for 553 with 2,4,5-T, 2.5% produced R regions.

Media containing 1.0 ppm 2,4,5-T result in more regenerated plants (Fig. 39) than media with more or less 2,4,5-T or 2,4-D. Plants are removed from callus and grown to maturity by the method used for wheat (Fig. 40).

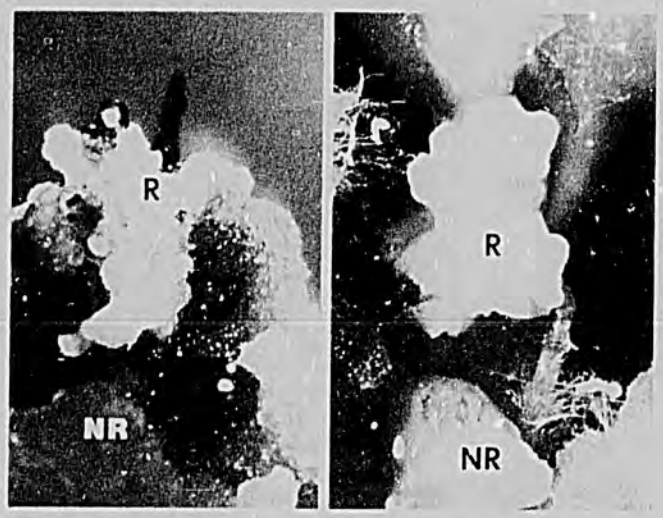


FIGURE 36. R and NR callus from Abarr proso millet (4× left and 6× right). The callus on the left is tolerant to 9000 ppm NaCl.

FIGURE 37. Regenerated Abarr proso millet plants which have been removed from surrounding callus.

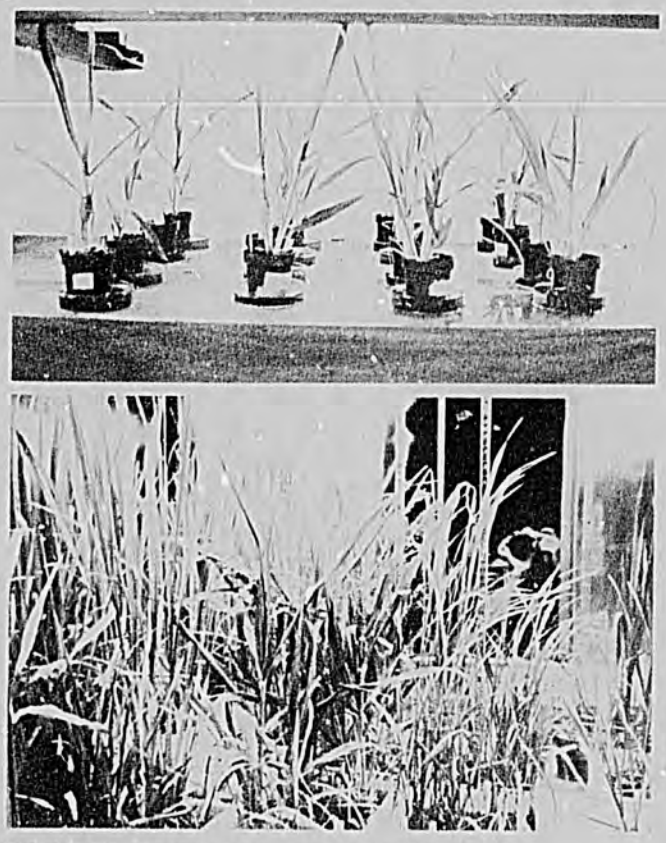


FIGURE 38. Regenerated proso millet plants in pots.

FIGURE 39. A jar containing callus and regenerating Park oat plants (0.8×) and plants regenerating from R callus (6×).

FIGURE 40. Regenerating Park oat plants in a growth chamber.

Data from oat tissue cultures were used to quantify for the first time two important points. (1) Green spots or regions, commonly found in grass calluses (see Nabors et al 1982) are associated with both rapidly growing callus and callus producing regenerated plants. In fact, callus without green spots or regions grows very slowly and never produces regenerants. In mixed callus (containing both R and NR regions) green spot or region production does not diminish for at least 46 weeks but shoot production gradually declines to zero by the 34th week of culture (Fig. 41). Most green spots and regions develop into roots. For these two reasons, the relationship between regeneration and green spots or regions is indirect. (2) R callus produces far more regenerated plants than NR or mixed callus (Fig. 42), Heyser and Nabors 1982a). A logical hypothesis is that all plants arise from R regions which are sometimes difficult to detect. The TCCP is the first laboratory in the world to quantify the differing regenerative potentials of R and NR callus. The suspected relationship between green spots or areas and R and NR callus is shown in Fig. 43. This figure now serves as a hypothetical framework suggesting further research.

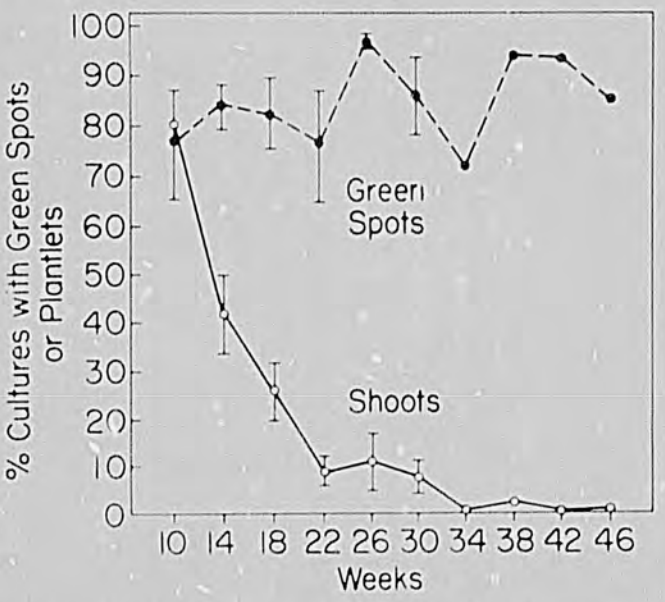


FIGURE 41. Shoot regeneration and green spot formation as a function of callus age. In root callus derived from Park oat seeds calluses were transferred every 4 weeks following a 6 week initiation passage.

Other Crops and Experimental Methods

Our laboratory has experience in tissue culture of corn (Fig. 44), lettuce, tomato, tobacco, sugar beet, and soybean. The laboratory also conducts research on the physiological causes of seed dormancy (see Carpita and Nabors 1981).

In terms of tissue culture, the lab conducts limited research into the biochemical mechanisms of Na^+ tolerance in cultured cells (Heyser and Nabors 1981a, 1981b) and into methods for obtaining herbicide-resistant cells and plants. At present cell cultures resistant to a number of different 2,4-D concentrations have been obtained (Fig. 45). Efforts to obtain and study regenerated plants are now underway.

Future Experimental Direction and Project Goals

During its first two years the TCCP concentrated on perfecting cereal tissue culture methods so that regeneration of reasonably high frequency and long duration could be obtained. Since stress selection inevitably lowers callus

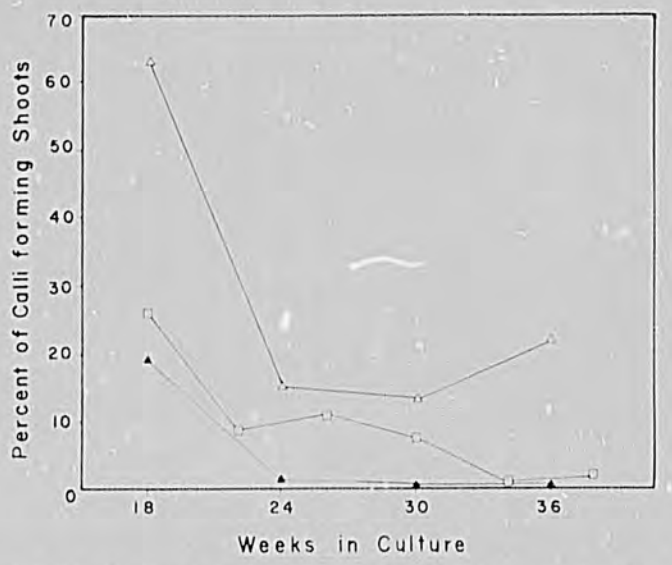


FIGURE 42. Shoot formation as a function of time in culture for Park oat callus derived from seedling roots. Open triangles represent R callus; closed triangles represent NR callus; and open squares represent callus selected for high green spot production. For R callus there were 30-78 samples per data point while NR and green spot callus is represented by 39-759 samples per data point.

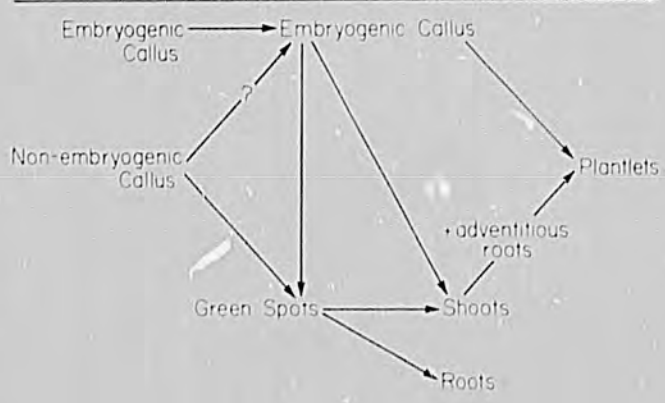


FIGURE 43. A model which relates a possible ontological sequence for the morphogenic structures observed in secondary oat callus.

growth and plant regeneration rates, it is important to utilize methods which will result in suitable rates of plant formation under stress conditions. Also, since selection of stable stress resistance requires a period of some months, regeneration from tissue cultures must persist at least through this period of time.

During its first two years of operation the project has succeeded in achieving both of these goals for wheat, rice, pearl millet, oats, and proso millet. Thus in future years the main experimental thrust of the research will be turned toward the production of stress-resistant cultures and the testing of plants regenerated from such cultures. As noted in earlier sections this process is already underway.

A secondary goal of the project during the coming years will be to continue efforts to improve tissue culture procedures for cereals. These efforts will include a more thorough investigation of the cultural condition favoring development of R callus from primary callus and studies to determine methods for obtaining continued production of R regions in secondary callus. Also, varietal differences in these cultural conditions will be more thoroughly explored. The cultural variables now under study include the effects of 2,4,5-T, 2,4-D, IAA, and tryptophan (all auxins or auxin precursors); of kinetin, BAP, and other cytokinins, of TIBA and other anti-auxins, of abscisic acid (ABA), and of light versus dark. Other cultural variables which have not been studied to date include light quality, photoperiod, the effect of a cell suspension phase, and the use of other plant parts for the initiation of callus. The general strategy employed is to concentrate efforts on the production of plants from stressed cultures in which stress is maintained at several different levels and for as long as possible to insure stability of resistance. As this effort continues, results from smaller experiments designed to improve tissue culture methods are incorporated into standard methods. So, for example, the effect of tryptophan in increasing R callus production in Calrose-76 rice was used immediately to improve the standard culture techniques for that plant. At the same time tryptophan was and is being tested on other varieties and on other species. As noted earlier, a positive effect was obtained with IR-36 and Pokkali rice and no effects were obtained with other rice varieties and pearl millet.

FIGURE 44. A Seneca 60 corn plant regenerating on the underside of callus (1.2×).

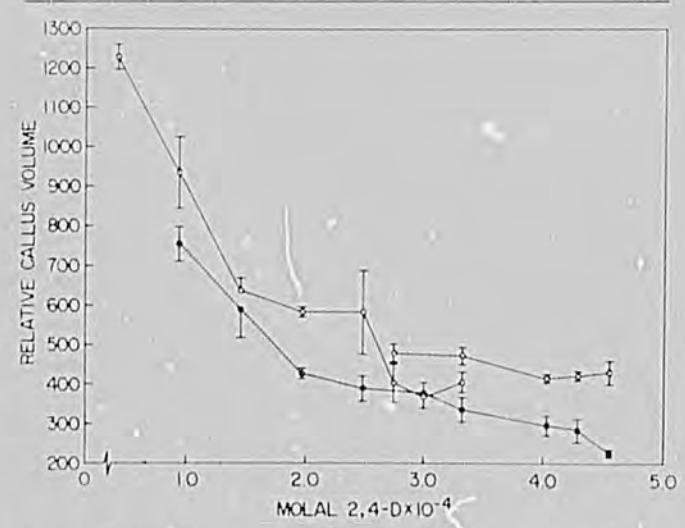


FIGURE 45. Selection for 2,4-D tolerance in tobacco calli. Data presented were collected during three, four week passages on the indicated concentrations. The bottom line (•) represents cultures exposed to the herbicide only during data collection. At the beginning of each passage callus on no herbicide medium was stepped up to medium with the indicated level of 2,4-D. The top line (○) represents cultures exposed continuously to herbicide for 16 passages prior to data collection. Callus on concentrations above 2.44 × 10^{-4} m 2,4-D was obtained by gradual stepups from this level during the 16 week period. Standard error bars are indicated.

The philosophy of the TCCP is that developing technology relating to tissue culture should be integrated into plant improvement and plant breeding programs as it becomes available in an established form. Thus methods for cell fusion are well developed in a few plants and will prove useful in breeding programs once they are extended to useful crop plants and once regeneration methods for the fusion products are obtained. Similarly, methods for isolating single genes and adding them to the genomes of cultured cells via an injection or plasmid mode will be useful to plant breeders provided the effect of the gene can be controlled after insertion and, again, regenerated plants from the augmented cell can be produced. Proposals to transfer multigenic systems involving one or more organisms from one plant to another are regarded by us as being entirely in the realm of basic research at this time. Thus the notion of moving nitrogen fixation from a legume to a non-legume which does not fix nitrogen is at present nowhere near the interface between basic and applied research.

Now, the areas of plant tissue culture which can bridge the interface and move into applied breeding programs are (1) rapid propagation techniques and (2) selection of useful mutant phenotypes. Rapid propagation can occur either by various shoot tipping processes (also useful for obtaining virus-free plants) or by regeneration from callus cultures or cell suspensions. These methods are extremely useful in the reproduction of rare plants encountered in the field and in shortening the time required to introduce new varieties to growers. Such techniques are available for a large number of cultivated plants (see for example, Conger 1981), but are unavailable for others including particularly soybean and many other legumes. The TCCP maintains a computer access file of available regeneration techniques for agricultural plants. Interested workers should contact the project director for information about specific species.

The TCCP encourages the open exchange of information among tissue culture researchers, plant breeders, and other interested persons. Exchange of reprints and other information is encouraged. Those workers with proprietary concerns are urged to participate in this exchange. Secrecy along the interface of basic and applied research retards progress for all concerned.

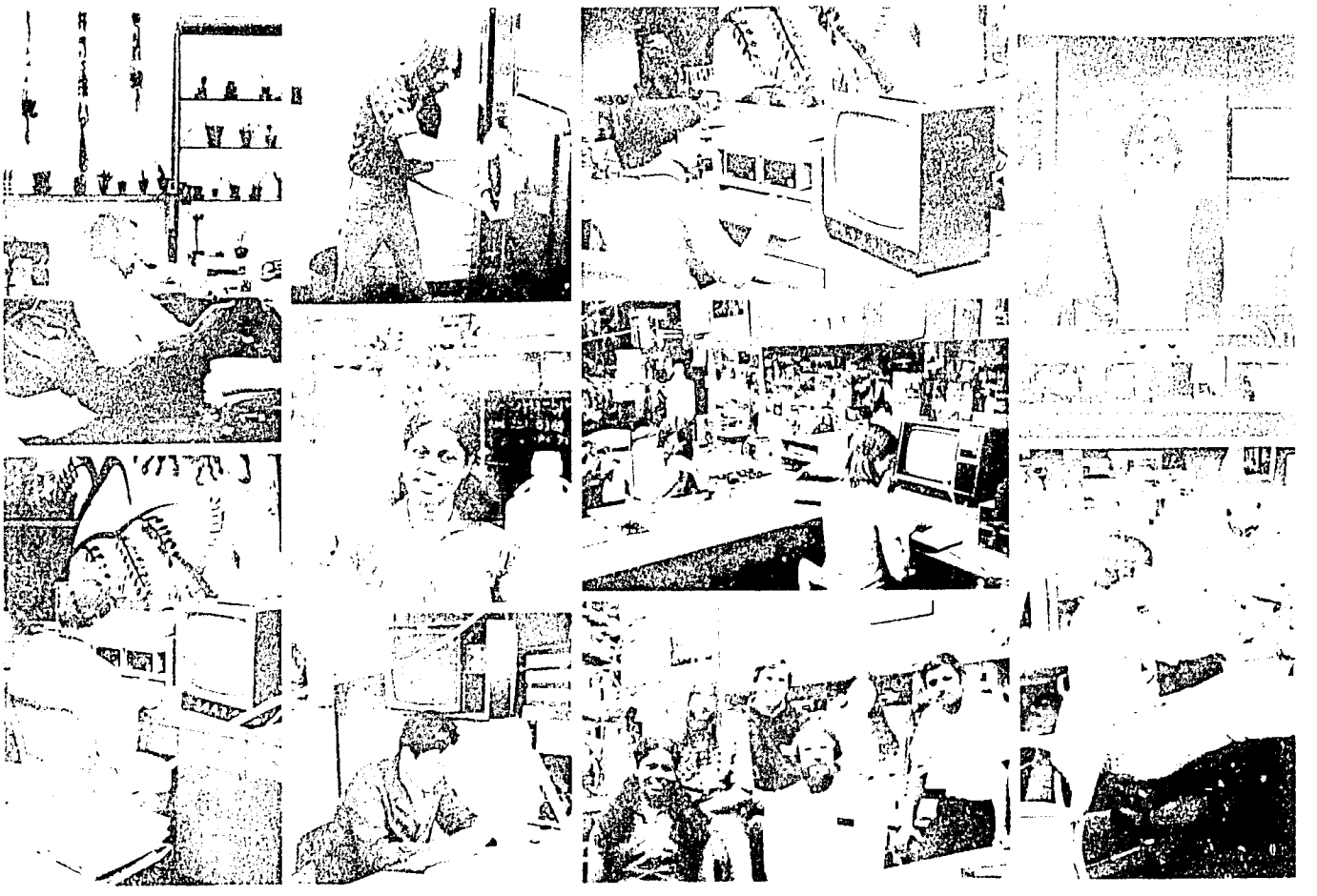


FIGURE 46. Project Personnel

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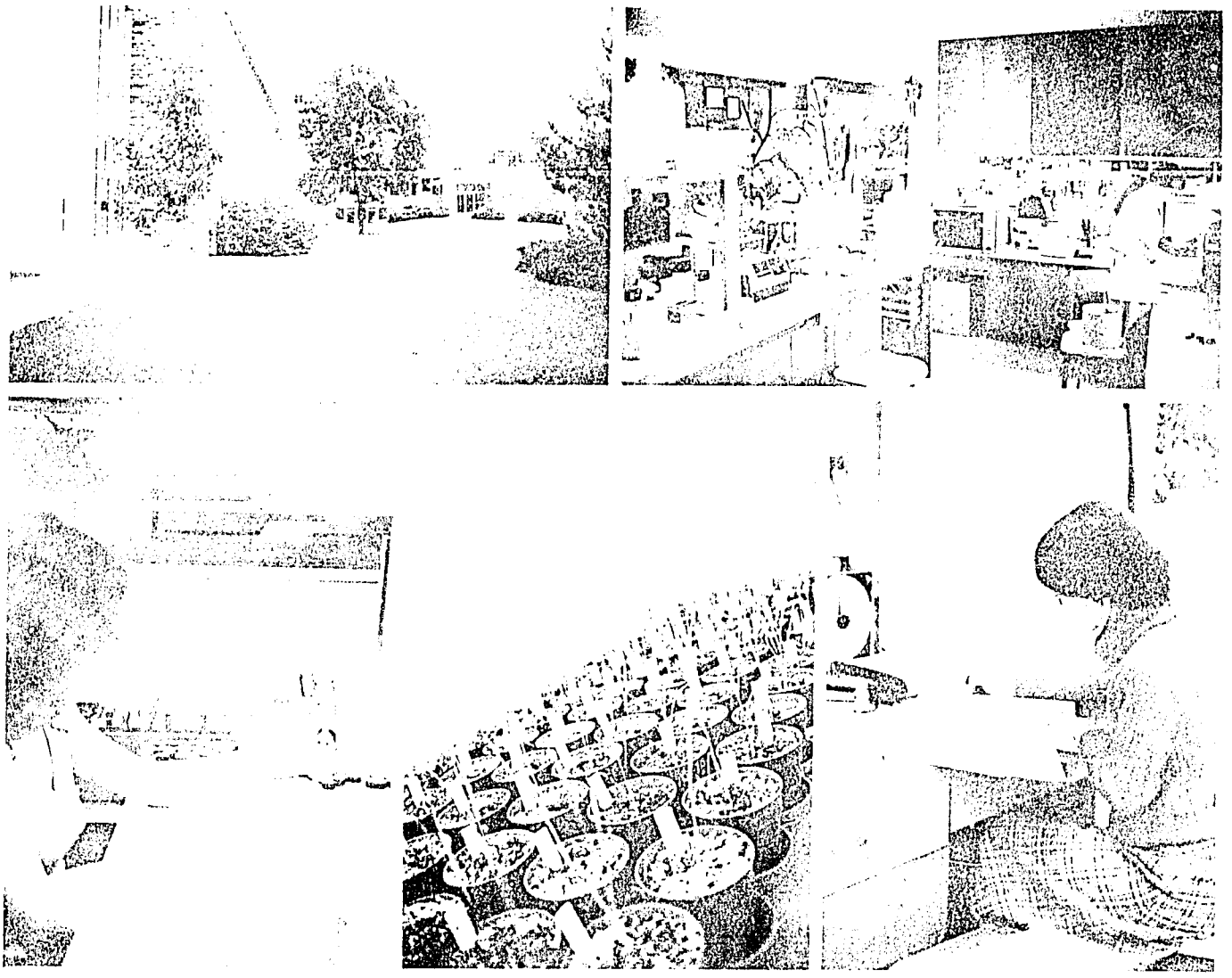

FIGURE 47. ICCP project facilities in Fort Collins

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Feedback Information Sheet

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2. Organization

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5. Comments

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