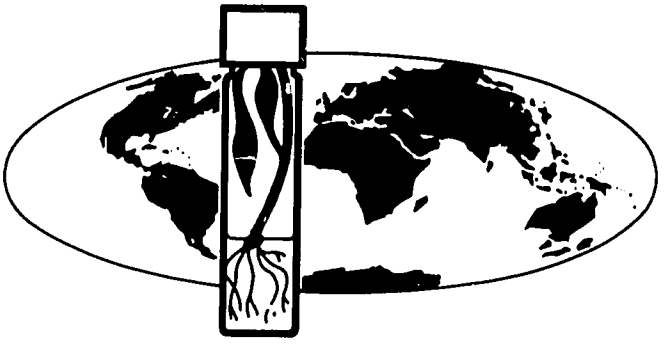
Tissue Culture for Crops Project Progress Report 1987



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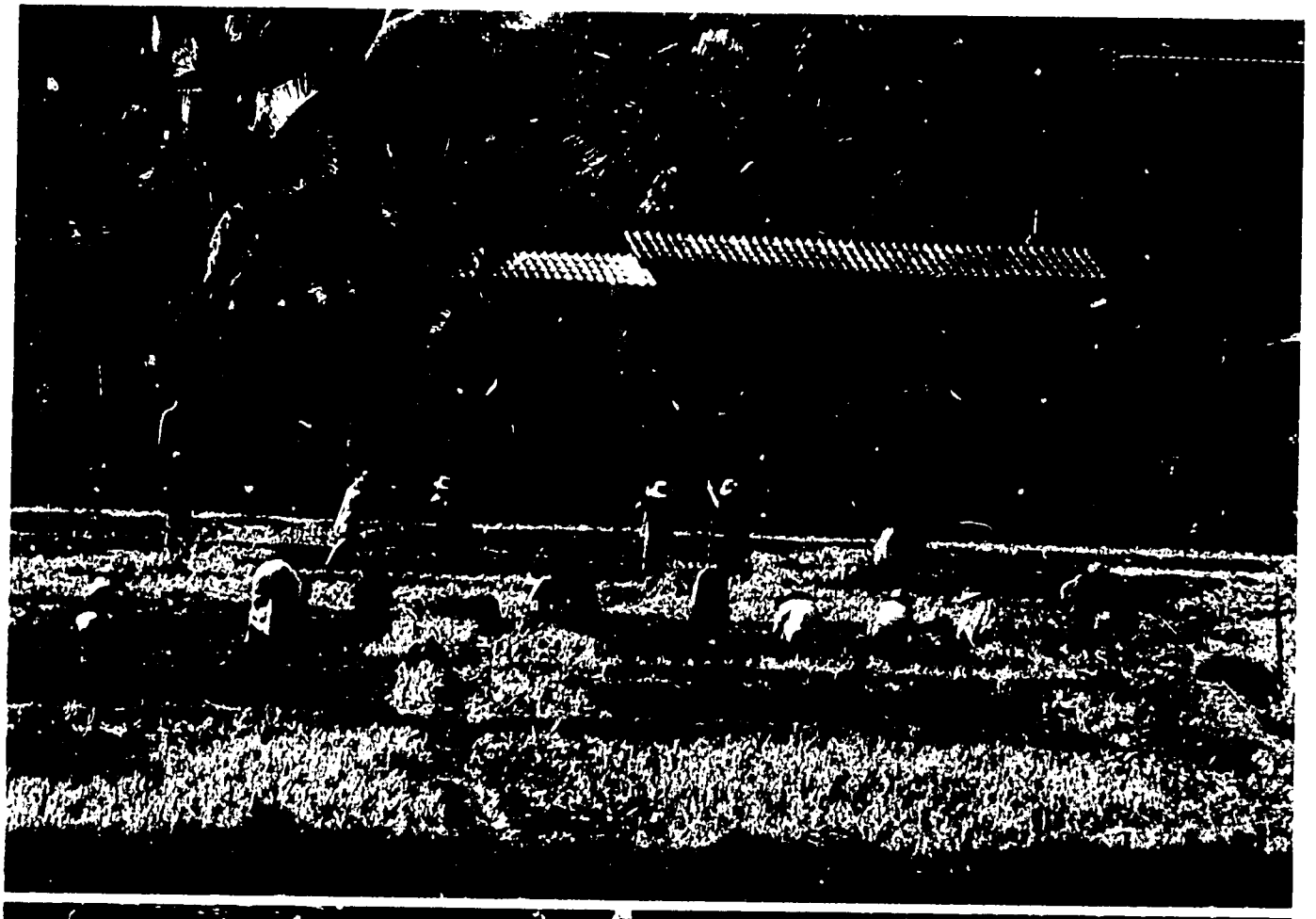


Introduction

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

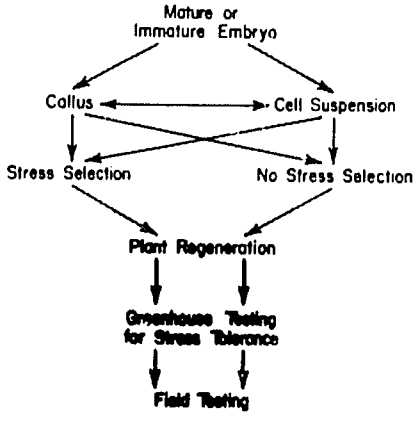
The TCCP was established in September, 1980 for the purpose of utilizing tissue culture methods to increase tolerance of rice, wheat, and pearl millet to the major soil stresses. Plant tissue culture had already demonstrated the potential (Nabors *et al.*, 1980), using tobacco as a model plant, to produce lines of cells and regenerated plants with increased stress resistance. Since drought, salinity, and excess aluminum had been shown to affect a large portion of arable land, these stresses were selected as those for which tolerant lines of crop plants would be obtained.

The goal of the TCCP is to help alleviate these problems by generating germplasm which will thrive in areas of stress. By altering the plant, the need for a continual flow of funds into soil-stress management will be reduced. In addition, land not currently cultivated because of stress factors can be brought into cultivation, current yields on stressed land can be increased, and resources currently used for environmental modification can be redirected.

In 1984, Project Objectives were expanded to include:

1. Completion of the development of methods for establishment of tissue cultures, selection of mutants, and high-frequency regeneration of plants in rice, wheat, millet, corn, sorghum and several tropical legumes.
2. Utilization of these methods to produce plants with increased tolerance to soil salinity, acidity, and drought.
3. Greenhouse and field testing of potentially useful, tolerant plants.
4. Establishment of a six-month tissue culture training program.
5. Formation of an international plant biotechnology network.

In 1986, Project Objectives were expanded again to include the use of widecross breeding, protoplast fusion, and direct gene isolation to move useful genes for stress tolerance from wild relatives into crop plants.



Tissue Culture Selection of
Stress Resistant Rice, Wheat, Oats & Millet

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

Executive Summary

Tissue Culture and World Agriculture

For the past six and one half years, the Tissue Culture for Crops Project has worked to define and demonstrate the usefulness of plant tissue culture in improving world agricultural production. In particular, our mandate has been to increase the tolerance of cereals and legumes to important soil stresses such as salinity, acidity, and drought. Together, these stresses limit production on over 60% of arable land and a larger percentage of potentially cultivated hectarage. The small landholders and tenant farmers of developing nations are most affected, for they often have the poorest land and most need not only food sufficiency but also excess production to feed the teeming cities and for export to other countries.

In 1980, as the TCCP began, tissue culture was by no means a proven technology. If anything it was overpromoted, and seen — by laboratory scientists with no real knowledge of or appreciation for the methods of and problems facing agronomists and plant breeders — as the quick solution to all the world's agricultural problems. Six years later that situation has begun to change. Plant tissue culture has shown its usefulness in producing virus-free plants and as a source of rapid propagation for many species which are difficult to propagate by other means. Embryo culture is now widely used to insure the survival of many "difficult" crosses, and anther culture is a proven method for stabilizing the recombinant variety and useful traits of F_1 hybrid plants of many types.

In other areas, the ability of tissue culture to serve agriculture is rapidly developing. Six years ago plant regeneration techniques for cereals were rudimentary and those for legumes were non-existent. Today, through work at the TCCP laboratory and other labs worldwide, indica and japonica rice, grain and sweet sorghum, wheat, corn, millet, and oats can be regenerated at high frequency from tissue cultures of short or long duration. At the TCCP, Mahsuri rice callus still regenerates plants after 50 months in culture. Using one seed of Pokkali rice as a starting explant, we have the technology to produce over 127,500 plants in six months, and this number may soon be doubled. In legumes like soybean, cowpea, pigeonpea, and chickpea, regeneration techniques are available or are rapidly developing where none at all were available six years ago.

By no means are all the problems of tissue culture solved or the needs of field workers met. Dr. S.S. Virmani at IRRI can still produce just as many hybrid rice progeny by short-term tillering as we can by tissue culture techniques. Plant regeneration from protoplast and cell suspension cultures of cereals and legumes are still woefully inadequate or non-existent. And identification of the genes controlling resistance to stress and our ability to isolate and move them are primitive technologies at best.

The first tractors were little better than draft animals at turning the soil efficiently and were certainly more difficult to care for. Still tractors are not the equipment of choice in many situations. Likewise, we view plant tissue culture and molecular biology as developing, useful tools. But the development will take time, and the usefulness will not be universal. In the coming years, the realization of plant breeders and agronomists that plant tissue culture is a valuable tool will increase. It is our hope at the TCCP that a working partnership between field and laboratory scientists at many levels and in many countries will be realized. It is toward this goal that our research is directed.

Research Progress at TCCP

The Laboratory

During its first six years, the TCCP has made substantial progress toward obtaining increased stress resistance in major cereals using tissue culture methods. This section briefly summarizes the major accomplishments.

Prior to the beginning of this Project in 1980, the following results were shown:

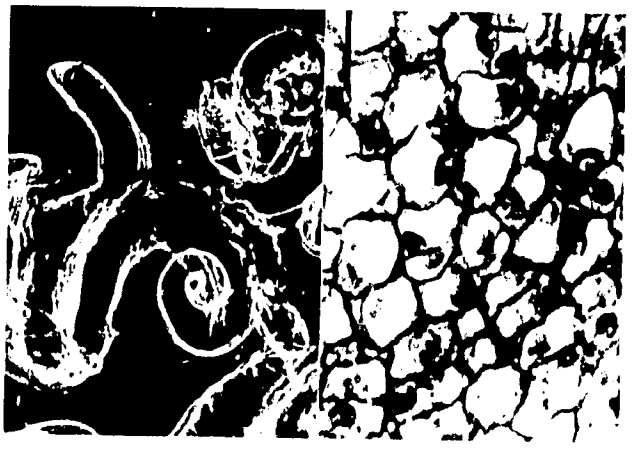
1. Using tobacco as a model system, it was demonstrated that NaCl tolerance could be selected in cell suspensions (Nabors *et al.*, 1975).
2. The TCCP laboratory was the first in the world to show that salt tolerance obtained in tissue culture was carried into whole plants (and their progeny) regenerated from those cultures (Nabors *et al.*, 1980). The tolerance is stable in that it is still expressed after two generations of whole plants never exposed to NaCl (Nabors, 1983). Tobacco was used in the initial work because it is a plant which easily produces cell cultures with high regenerative ability.
3. Tissue culture methods for wheat, oats, and sugarbeets (as well as other non-cereals) were improved (Nabors, 1976a, 1976b, 1979, 1981).

At Project inception, callus cultures could be obtained from cereal explants with few problems; however, regeneration from these had uniformly been of short duration and low frequency. To obtain reliable plant regeneration from cereal tissue cultures, it was usually necessary to use immature embryos (placed scutellum up on the medium) as explants in order to form a regenerable callus (Conger, 1981). Hand-dissecting immature embryos is a time consuming process and requires a continual supply of blooming plants. One of the first goals of the Project was to develop methods whereby easily obtainable tissues could be used to form callus which had high-frequency, long-term regeneration. Improvement of existing methods to fulfill this goal has proceeded quickly, and development of reproducible methods of tissue culture and efficient plant regeneration has been achieved (Nabors *et al.*, 1982, 1983, Nabors and Dykes, 1984; Heyser and Nabors, 1982a, 1982b; Heyser *et al.*, 1983; Raghava Ram and Nabors, 1984a, 1985, MacKinnon *et al.*, 1986a, 1986b). Specifically, the following has been accomplished in cereal tissue culture:

1. For wheat, rice, sorghum, pearl millet, proso millet, corn, and oats, a highly regenerative form of callus has been identified and media have been developed to produce it preferentially from seeds, seedling apical meristems, and immature embryos.
2. High-frequency, long-term regeneration has been achieved from callus derived from seeds and immature embryos. Regeneration frequencies are 10 to 25 times those previously reported in the literature. Regeneration, typically difficult to maintain in cereal tissue cultures for more than a few months, is still occurring at high efficiency in cultures over four years old. The Project has emphasized and developed routine methods to obtain regeneration from callus produced by mature seeds since this is a convenient source to obtain and manipulate.

The goal of developing reproducible tissue culture and plant regeneration methods for cereal callus culture was the application to *in vitro* stress selection. Embryogenic callus has been selected on various growth-restricting levels of NaCl, PEG, and $AlCl_3$. Specifically:

1. Callus tolerant to various levels of NaCl has been obtained for rice, wheat, sorghum, millet, and oats. Aluminum- and PEG-tolerant cultures of rice have been obtained and regenerated. Selection for drought and aluminum-tolerant callus has begun for wheat, sorghum and millet.
2. In rice, over 17,000 plants have been regenerated from callus cultures up to 50 months old. Over 4400 of these plants are from cultures resistant to NaCl and 525 from cultures resistant to $AlCl_3$. Over 2700 wheat plants have been regenerated, at least 20 from NaCl-resistant cultures. Oat and millet NaCl-resistant cultures have regenerated over 700 plants. For selection experiments, a sample of plants were regenerated from non-stressed and stressed cultures and grown to maturity.
3. Plant regeneration has been achieved from soybean and pigeonpea cultures. Plants of soybean have been regenerated from salt-stressed cultures. The work in pigeonpea has just begun, with most *in vitro* work dealing with long-term callus growth and plant regeneration.
4. Initial progress has been reported in isolation of proteins expressed by a halophytic grass when under salt stress. Further work will be done to isolate the gene for this protein. This work is being done in collaboration with Dr. James Colbert at Colorado State.



A highly regenerable form of callus called E callus produces a high frequency of regenerated plants from tissue culture. E callus consists of non-vacuolated, meristematic cells in contrast to NE callus which consists of highly-vacuolated, elongated cells.

5. Suspension cultures capable of plant regeneration of *Distichlis*, rice and millet have been developed and will be used as sources of protoplasts for future somatic hybridization experiments.
6. Seeds have been collected from regenerated plants obtained from initial stress selection investigations. The seeds have been given a TCCP identification number and put into storage. Using the original seed source, plus some seed obtained from regenerates, a hydroponic screening system for NaCl stress was designed. From preliminary results, it appeared that some progeny of regenerated rice and oat plants had enhanced tolerance to salt stress (Nabors, 1983, Nabors & Dykes, 1984; TCCP Newsletter No. 3, July, 1984).

Greenhouse and Field Testing

In the past year, the major thrust at the TCCP has been whole plant testing. Recognizing that results in the laboratory were of little value unless they could be transferred to the field, the Project has focused on demonstrating the viability of stress-tolerant plants in the field.

1. The TCCP has developed simple, easily maintained and replicated hydroponic growth systems for rice, wheat, sorghum, millet, and oats. These systems use simple nutrient media and make it possible to readily grow plants to maturity and to compare the effects of various levels of stressing agents.
2. Cultivars used in laboratory experiments at the TCCP have been compared and grown to maturity in hydroponic experiments to determine the relative sensitivity of each cultivar to salinity and, in some cases, to acidity in the nutrient medium. Non-regenerated seed is always used as a control when testing tissue culture-derived materials.
3. Field tests for regenerate lines of oats, rice, and sorghum were planted in 1986. Several R_3 lines of oats were selected for early flowering and vigor under salt conditions in Canada. A second field test is being planned. Rice lines from tissue culture were sent to Texas A & M University and Louisiana State University (LSU) for generation advancement, then sent to the University of the Philippines at Los Baños (UPLB) for evaluation of salt tolerance. The evaluation will begin in early 1987. Regenerated sorghum lines were evaluated for tolerance to acidic aluminum soils in 1986 in Georgia. Several of the lines showed promise and will be re-evaluated in 1987.
4. IR-36 and Calrose 76 rice derived from tissue cultures under 0, 3, 6, and 9 g/l salt stress have been tested hydroponically at the seedling stage. These experiments indicate that rice callus, selected for salt tolerance *in vitro*, can produce salt-tolerant whole plants, when given a selection period of at least five months. If tolerance had not been incorporated into the R_1 progeny of plants regenerated from salt-stressed tissue cultures, it would be expected that 50% of the progeny tested would do worse than the control average, and 50% would do better. In these experiments, 61% of the R_1 seedlings derived from callus cultures stressed at 3 g/l NaCl, 71% of the R_1 seedlings derived from callus stressed 6 g/l NaCl, and 92% of the R_1 seedlings derived from callus stressed at 9 g/l NaCl did better than R_1 plants derived from callus that was never stressed. These data tend to suggest that total stress in culture — and resulting tolerance — is a function of both the level of NaCl in the medium and the length of the selection period. This is consistent with previous findings for tobacco.
5. Rice seed from five regenerated plants of Giza 159 and two of IR-36, as well as non-tissue culture checks, were grown in hydroponics with 0, 3, 5 and 7 g/l NaCl. All plants grown at 7 g/l NaCl died. Plants in 5 g/l NaCl had to be removed from the salt for seed set. One IR-36 regenerate line from 9 g/l NaCl in tissue culture out-yielded the non-tissue culture check after the 3 g/l NaCl hydroponic tests. Also, a high-yielding Giza 159 line was identified from the control hydroponic tank. Seed from these selected lines are being tested in a second hydroponic test and field study.
6. Field testing of lines of regenerated rice has been carried out at UPLB, at LSU, and at the Texas A & M Agricultural Research Station. Results indicated that R_1 progeny of plants regenerated from tissue culture show considerable variation for both useful and non-useful agronomic traits in the field. Variations in height, tillering, and the presence of awns were commonly observed. Resistance to rice blast has been found in two regenerated plants and is under further investigation. Individual plants with tolerance to salinity have been observed, and progeny seed are being collected for further experimentation. Both somaclonal and selected variation was observed, and correlations have not yet been made between the length and strength of tissue culture selection and observed field tolerance.
7. Salt-tolerant R_3 oat plants were tested in potted soil by watering with solution containing 0, 5, or 10 g/l. Plants whose genetic heritage began with salt-tolerant cell lines were considerably more tolerant than those from salt-sensitive lines. In addition, the salt-tolerant R_4 progeny flower had set seed a full two weeks earlier than plants from salt-sensitive cultures. R_1 data show that individual plants from salt-tolerant tissue culture backgrounds are either more tolerant than, or equally tolerant to control plants. These oat data represent the second demonstration in the literature and the first for cereals that (1) traits selected in cell cultures also appear in regenerated plants, (2) these traits are stable in the plants in the absence of stress, and (3) these traits are inherited by subsequent generations.
8. Subsequent work with regenerated oat plants has shown that plants regenerated from salt-tolerant tissue cultures are more tolerant than plants regenerated from salt-sensitive cultures; however, plants regenerated from salt-sensitive cultures are, on the average more salt-tolerant than plants from field-grown seeds. Thus the tissue culture process itself, regardless of stress selection, induces useful, so-called "somaclonal" or "mutaclonal" variants. Also, the desired variations do not occur in field grown seeds at a high level as a result of recombination of stress-tolerant alleles within the cultivar.
9. Field testing of 300 lines of regenerated sorghum plants has been carried out at Texas A & M University and at the University of Georgia. Seventeen lines showed increased drought and aluminum tolerance. Nine were selected as having some resistance to fall armyworm. Selected lines will be re-evaluated this year.
10. Plans for the coming year include testing of 450 R_1 and R_2 lines of regenerated sorghum at three locations, 700 R_1 and R_2 lines of regenerated rice at three locations, 1000 R_1 lines of regenerated wheat at one location, 13 R_1 lines of regenerated millet at one location, and 14 R_1 lines of regenerated oats at one location.

International Plant Biotechnology Network (IPBNet)

"Networking" is a commonly used concept these days and describes any number of activities. In general, networks seek to provide channels of communication which would not otherwise exist. Through these channels, scientists can communicate with each other on similar interests, capabilities, and problems. Experience has revealed that such communication is sadly lacking in terms of plant biotechnology, even within countries, regions, and institutions not to mention between them. In many cases, a lack of scientific information or knowledge of others working on similar or identical projects slows progress and dampens enthusiasm. Competition invariably clouds the picture and can prevent scientists from working together for the achievement of mutual goals and benefits.

To further research goals and enhance communication among tissue culturists worldwide, the TCCP has formed IPBNet. From the beginning in early 1985, when responses were received to an earlier distributed survey, it was clear that interest in and need for such an organization was great. Over 150 surveys were returned that year. And in the last two years, the TCCP staff has responded by visiting hundreds of scientists and institutions in over 20 countries. As a result, formal, cooperative links with eight countries have been established.

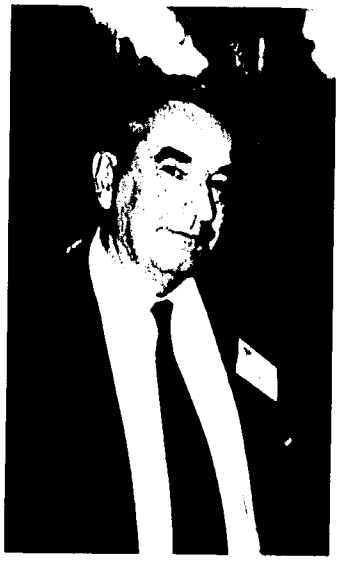
Through the Network, the TCCP has sought to provide technical assistance, training, and support systems that would be of mutual benefit. In the fall of 1985, the first Tissue Culture Training Program was held in Fort Collins. This proved to be an invaluable experience for trainees and staff alike, and the Training Program has continued to evolve through input from its 17 participants. Feedback from the first three sessions is now being solicited and received, and the results show that it has been a successful vehicle for technology transfer to labs and institutions in developing countries. Graduate studies are another way the TCCP provides technical assistance and technology transfer. The Project currently has 13 graduate students from eight developing countries and the U.S. The majority of these students will return to other countries and direct research in university and government institutions. Their ability to teach others is crucial to the spread of technical knowledge around the world.

Another activity vital to communication among Network members is the IPBNet Conference. These events are held periodically as a forum for Network members to present and discuss their current research. The first conference, held in Fort Collins in October of 1985, drew 94 participants from 21 countries; the second conference was held in Bangkok, Thailand in January 1987 and was attended by 79 persons from 18 countries.

In the process of forming and expanding the Network, the TCCP has had to expand its own capabilities for providing information. The Project library and computer literature system have almost doubled in size (to include over 10,000 entries), and searches of a multitude of tissue culture and related methods on a wide variety of tropical, cereal, grain and vegetable crops can be made in a matter of minutes. Few small labs in developing countries have such resources, which are provided upon request at no charge. In addition to scientific references, the TCCP is in the process of compiling a data base and directory of Network members so that scientists working on similar projects can communicate and benefit from each other's knowledge. The Project is also working on a directory of funding institutions which will provide information on support for a variety of activities including training, graduate education, travel, and research.

As the Network grows and expands, our concept about what it needs to provide will change and evolve. For example, many workers are eager to participate in IPBNet but lack even minimal funds. We are working in many countries at this time to facilitate the flow of funds so that tissue culture scientists can attend Network meetings and participate in other activities. At the same time, TCCP's ability to meet the needs of qualified graduate student applicants and visiting scientists are strained. As a result, we are now moving to initiate training programs of various sorts in the laboratories of other Network members. A first step in this direction is the establishment of a regional Training Program at CATIE in Costa Rica, under the direction of Dr. Ludwig Müller. Beginning in April 1987, a full-scale training course in Spanish will be underway.

Since "network" implies interaction — a sort of web-like connection between members — IPBNet cannot exist without the continued input and support of scientists and administrators outside of the TCCP. The TCCP will continue to organize and encourage such input for the benefit of all IPBNet members.



Stresses Limiting Crop Production

Drought, excess sodium, and excess aluminum are stresses which limit crop production on more than 50% of all arable land and much unarable land as well. In the past, man has dealt with these problems by altering the environment through irrigation, careful management, and soil liming; however, because of dwindling water supplies and the increasing expense of environmental modification, these methods are becoming less useful, especially in economically developing countries.

Plant breeders are realizing with increasing enthusiasm that the answer to these problems is to alter the plant to suit the environment. Tissue culture has the potential to considerably shorten the time required by plant breeders to produce improved cultivars. Not only by using tissue culture to rapidly clone existing useful plants but also by regenerating plants selected for tolerance to certain stresses, plant breeders can reduce farmers' costs of irrigation and crop losses, and increase their yields and profits. More importantly, subsistence farmers in areas plagued by drought, salinity and excess aluminum will be able to increase crop yields.

Drought

Webster's Third New International Dictionary defines drought as "a period of dryness especially protracted and causing extensive damage to crops or preventing their successful growth." Drought is a problem on 40% to 60% of world cultivated land. Droughts do not need to be prolonged to affect crop yields. Water stress for one day will have some effect if the stress is large enough or comes at a critical period of the plant's life cycle. Even 25% of the world's rice crop is subject to drought at some time during the growing season (O'Toole and Chang, 1979, USDA Agric. Statistics, 1976).

Throughout history, humans have dealt with the problem of drought by irrigating. It has been currently estimated that, worldwide, 201 million hectares are irrigated, representing approximately 14% of cultivated land, which produces nearly one half of total world food (Christiansen, 1982). Irrigation, however, is not always a viable solution to the drought problem. Water is scarce in many areas of the world, and when water is available, it is energy intensive and prohibitively expensive to obtain. Worldwide, 80% of readily available water is already used in agriculture (Flowers, 1977). Many underground aquifers are rapidly disappearing. In addition, even the most carefully designed and managed irrigation systems exacerbate salinity problems by gradually increasing the concentration of salt already in the soil. Because of this, all irrigation systems have a limited useful lifetime (Casey, 1972).

Salinity

Salinity problems in agriculture are primarily found in the coastal, arid, and semi-arid regions of the world, which comprise more than 25% of the earth's surface (Carter, 1975). Salinity poses problems in almost every irrigated area of the world as well as in coastal regions.

Salt concentrations become a problem as water evaporates from the soil. The salt concentration in the soil after evapotranspiration (the combined water loss from transpiration and direct evaporation from soil) may become four to ten times the salt concentration in irrigation water (Carter, 1975). A level of 800 ppm salt is generally recognized as the threshold of severe agricultural and non-agricultural damage. Based on detailed studies of the Colorado River Basin, world agricultural and non-agricultural damage due to salinization of rivers has been estimated at $54.6 trillion per year (TCCP Newsletter No. 3). Approximately half the Colorado River's salinity is due to natural salt loading from rocks and soil while the other half is related to human activity.

Acidity

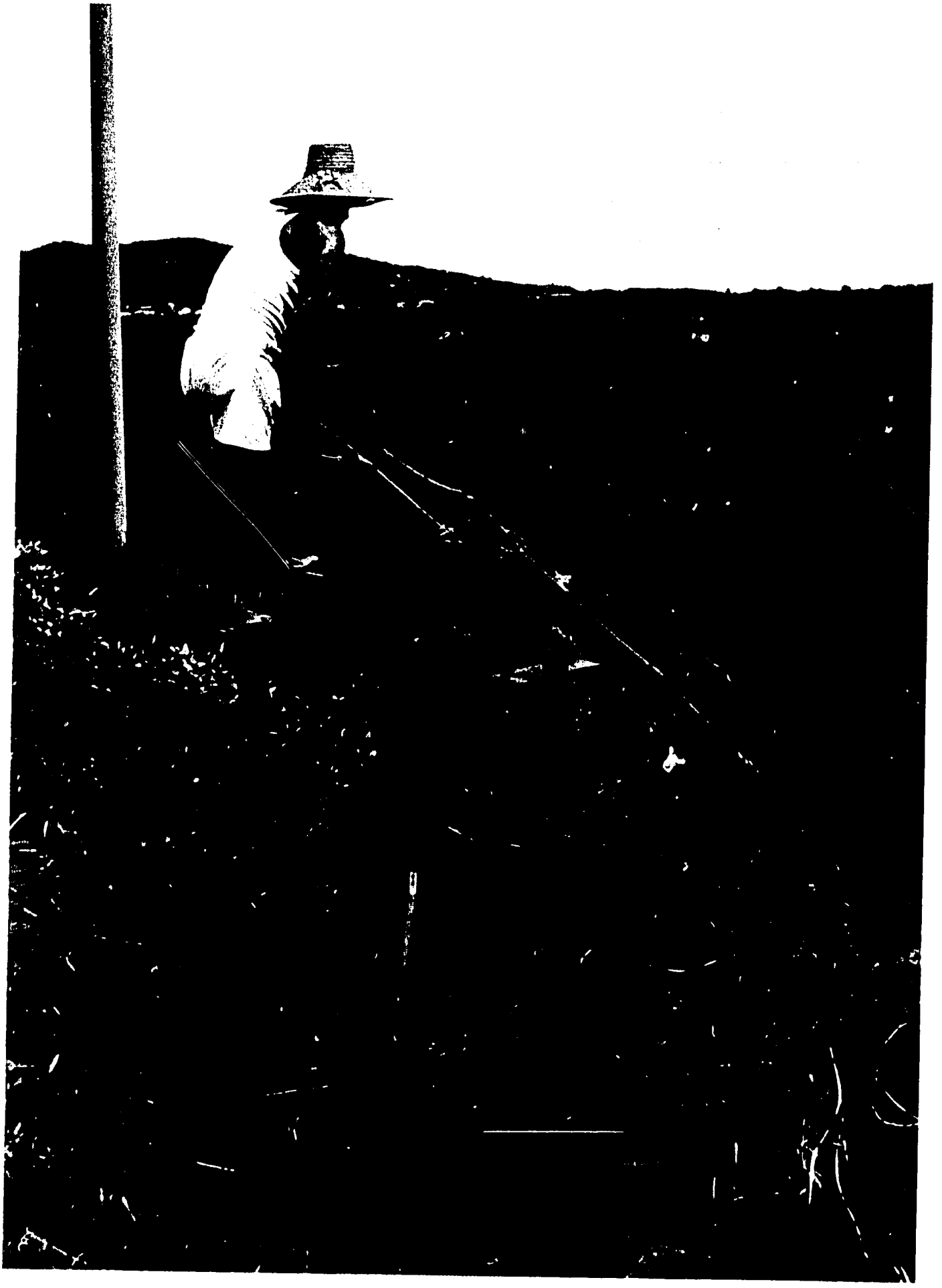
Excess acidity is a problem which affects approximately 25% of arable and, land which is typically unaffected by drought. Usually occurring in areas of high rainfall, excess acidity is the result of dissolved CO_2 (*i.e.* H_2CO_3) which acidifies the soil and brings normally insoluble ions like Al^{+3} into the soil solution. Acid soil eventually becomes poor in nutrients needed by plants and rich in toxic elements such as aluminum. This is because H^+ and Al^{+3} are effective in dislodging other ions from soil particles and into ground water. The problem is most often solved by periodic soil liming; however, this is a costly method which is infeasible in many areas of the world.

It is clear from the previous discussion that the amount of environmental modification (irrigation, desalinization, liming, fertilization, etc.) needed to produce effective crop yields is becoming prohibitive. Modern, mechanized agriculture is energy intensive and many countries cannot afford to utilize these techniques. For example, a typical center pivot in Nebraska requires 500 liters (133 gallons) of diesel fuel per hectare to apply one foot of water per year (Sloggett, 1981). This figure is even higher if the *total* cost of production is converted to energy. Pimental *et al.*, in 1973, reported that 800 liter equivalents of gasoline were required to produce a hectare of corn in the United States.

Disease and Weather Fluctuation

Aside from soil mineral stresses, disease and weather are the major stresses limiting crop productions. The impact of disease is difficult to assess, but overall yields are estimated by pathologists to be reduced 25% or more by plant pathogens.

Thompson (cited in Christiansen, 1982) says that 60-80% of the variability in crop production is due to weather fluctuations, chiefly variability in temperature and moisture. The recent droughts in northern Africa have provided dramatic demonstrations of the agricultural and sociological effects of weather fluctuations. Crops with greater latitude to grow under changing conditions are clearly needed.



Research Accomplishments and Progress

Rice

Rice is grown in more than one hundred countries and on every continent of the world (except Antarctica) and produces more calories and carbohydrates per hectare than any other cereal (Lu and Chang, 1980). This grain, second only in economic importance to wheat, is the staple food for one-half to two-thirds of the world's population. According to Lu and Chang (1980), by the end of this century, "the number of people dependent on rice for their staple food will be greater than the present world population."

Long-term, High-frequency Regeneration

When TCCP began experiments with rice tissue culture in 1980, plant regeneration from rice callus cultures was of low frequency and short duration. It was also restricted to japonica cultivars. Now, high-frequency, long-term regeneration from both japonica and indica cultivars can be achieved.

Cultivars for use in tissue culture experiments were suggested by Dr. Ronnie Coffman (then of IRRI). They represent a mixture of indica and japonica varieties, of salt-tolerant (Pokkali and Giza 159) and salt-sensitive (IR-36 and Mahsuri) cultivars, and of green revolution cultivars (IR-36) and native land races (Pokkali). Calrose 76 and Giza 159 are japonica cultivars, Pokkali and IR-36 are indica, and Mahsuri is a japonica x indica hybrid.

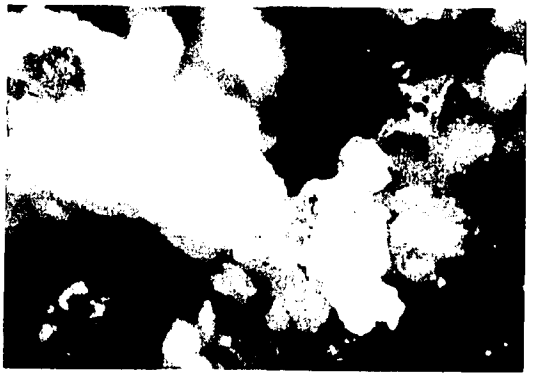
Mature seed has been a very successful explant for establishing E callus cultures as the scutellum and occasionally the shoot apical meristem give rise to embryoids (Figs. 1a-f). Unlike wheat and corn, the potential of the rice scutellum to produce somatic embryos in tissue culture remains high as the embryo matures (Fig. 2).

Optimal media and growing conditions differ for each cultivar and do not seem to be completely consistent within japonica or indica groups (Table 1). For example, Calrose 76 seeds form maximal E callus in the dark on LS medium supplemented with 1 mg/l 2,4-D and 0.2 mg/l KIN. If TRP is added to the medium at 100 mg/l, the proportion of E to NE callus increases significantly (Table 2). It was also reported that IAA had a similar effect (Siriwardana and Nabors, 1983). This is true if data is taken on the percentage of cultures containing E callus, however if callus masses are used, TRP enhances the production of E callus more than IAA. Therefore, TRP may not be acting as an IAA precursor in this system. Following initiation, it is not clear whether or not TRP is helpful in maintaining E callus because results have not been consistent. It is apparent that the maintenance of E callus of Calrose 76 is more successful if the cultures are placed in the light, on LS media containing 2 mg/l 2,4-D and 0.2 mg/l KIN. Regenerable callus has been maintained in culture for 15 months.

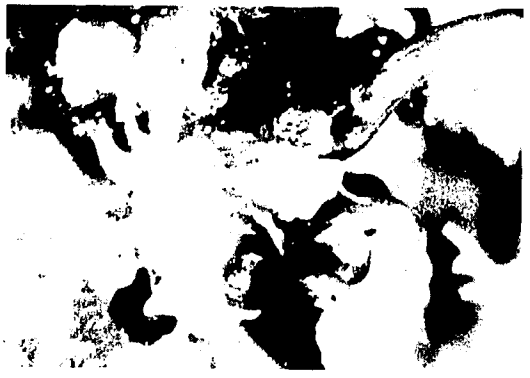
Plant regeneration occurs only from E callus. A higher cytokinin to auxin ratio is required for plant regeneration than for E callus formation (Table 3). This appears to be attained by the addition of more cytokinin (BAP) or by the inhibition of auxin transport (by TIBA).

Cultures of Giza 159 were initiated and maintained in the dark on LS medium containing 1 mg/l 2,4-D and 0.3 mg/l KIN. Callus has been maintained for 23 months and is still providing efficient levels of regeneration. Typically, 30 to 45% of the callus is E at the end of each passage; NE callus is removed before transfer. Tryptophan has no effect on E callus formation in Giza 159. Regeneration medium contains 0.4 mg/l BAP and 0.5 mg/l IAA. Much of the plantlet formation does not occur until the second passage on regeneration medium.

Fig. 1a. A single somatic embryo on Giza 159 callus.



1b. RD-23 E callus at second passage.



1c. Shoots forming on E callus of Mahsuri at third passage.

1d. Embryos forming on the scutellum.

1e. Scanning EM micrograph of scutellum.

1f. Close-up of scutellum in le.

IR-36 cultures are initiated in the light on medium with 1 mg/l 2,4-D, 0.5 mg/l KIN and 50 mg/l TRP. The callus is maintained on 0.5 mg/l 2,4-D and 0.25 mg/l KIN, also in the light. Tryptophan enhances E callus formation but does not help in its maintenance. If the auxin concentration is not decreased in maintenance media, the cultures die in four or five months. The rice cultivar IR-36 has been maintained in culture for up to 29 months as of October, 1986. Regeneration was accomplished with medium containing 0.5 mg/l IAA and 0.2 mg/l BAP. The callus was put on regeneration medium for two passages to obtain the maximum number of plantlets as many small embryoids will continue to develop into plants during the second passage. Initiation, maintenance, and regeneration media have been determined for some other IR lines. These media are similar to those determined as optimal for IR-36 in that the same types of hormones are used, but the concentrations differ for each cultivar (Table 4).

Mahsuri seeds produce the most E callus when cultured on initiation medium containing 0.5 mg/l 2,4-D and 0.5 mg/l KIN in the light. Maintenance medium is identical to initiation medium, and callus is kept in the light. Regeneration medium contains 0.5 mg/l IAA and 0.4 mg/l BAP. As of October, 1986, Mahsuri callus has been in culture for 50 months. Although the number has slowly fallen off with time, plants are still being regenerated. One important procedure to note is the transfer technique. If the E callus is clearly separated from the NE and necrotic tissue, regeneration is high; if, however, the dark, dying callus is not carefully removed at each passage, very little or no regeneration will subsequently occur.

Pokkali seeds, grown in the dark, form approximately 80% E callus on a medium containing 0.5 mg/l 2,4-D, 0.2 mg/l KIN and 50 mg/l TRP. Callus maintenance is best when placed in the light on medium containing 0.5 mg/l 2,4-D and 0.4 mg/l KIN. If the callus is maintained on medium with 0.2 mg/l KIN it will continue to grow, but regeneration rates will decline faster through successive passages. Plants have been regenerated from callus which has been in culture 22 months. Regeneration of Pokkali is best accomplished on medium containing 0.5 mg/l BAP. Work on this variety shows an optimal callus mass to media volume ratio which aids in callus growth and regeneration. Regeneration can also be increased through the use of "conditioned" medium and optional callus to regeneration

medium volumes. Over 127,500 regenerated plants *could* be produced from one seed if callus were multiplied for six passages and plants regenerated during the seventh (Raghava Ram and Nabors, 1985; TCCP Newsletter No. 3; Table 5).

Studies with a Thai rice cultivar, RD-25, were conducted concurrently at the TCCP and the laboratory of Mrs. Montakan Vajrabhaya at Chulalongkorn University (CU), Bangkok, Thailand. A Colorado State technician worked at CU for six months while a Thai graduate student worked at Colorado State. The results from these investigations have shown that callus initiation rates were significantly higher when LS medium with 2.0 mg/l 2,4-D, 10% CW and 3% sucrose was used instead of a TCCP combination of LS medium with 1.0 mg/l 2,4-D, 0.4 mg/l KIN and 4% sucrose (Fig. 3).

The Colorado State regeneration medium (LS + 0.5 mg/l IAA + 0.3 mg/l BAP + 4% sucrose) produced more plants per gram of callus than the CU regeneration medium (modified White's major salts with LS minor salts and vitamins + 1.0 mg/l IAA + 3.0 mg/l KIN + CW, (Fig. 4).

In experiments done at both locations, the culture jar used at Chulalongkorn was much less effective in callus initiation experiments than the culture jar used at Colorado State. The CU jar has a tight fitting, pliable, plastic cap whereas the Colorado State jar has a loose fitting, rigid Bakelite cap.

Callus initiation and plant regeneration was always less at the CU location than at Colorado State. It was hypothesized that this difference was due to higher concentrations of hydrocarbons (such as ethylene) in the air in Bangkok, a large, metropolitan city, as opposed to Fort Collins, a small, rural city. But confirmation of this hypothesis requires further testing.

It was discovered that exact replications of experimental methods in two different locations is difficult. Chemical and water supplies as well as containers and culture room conditions are different. Also, simple procedural differences may profoundly affect results. For instance, the investigators at CU were able to obtain initiated callus which was 100% E cells. This had never been possible at Colorado State. The CU investigators had developed the method of removing germinated root and shoot meristems from the cultures after two weeks of incubation whereas Colorado State investigators did not do this until the first transfer to new medium at four weeks.

These experiments show the importance of complete descriptions of methods. They also emphasize the importance of personnel and information exchange between laboratories engaged in similar research.

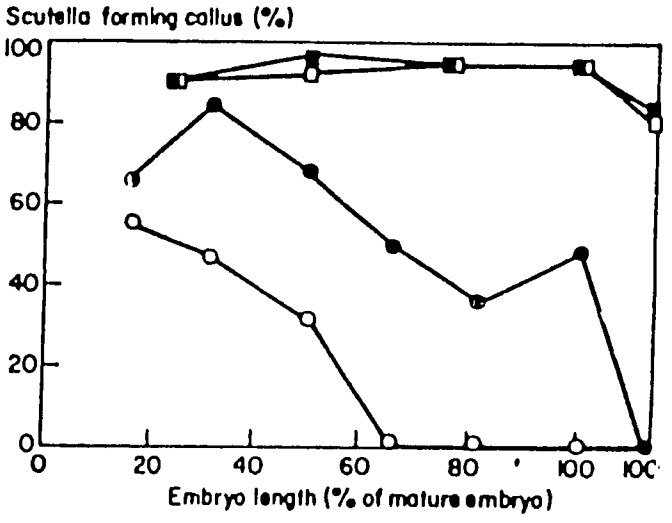


Fig. 2. Formation of callus by rice (squares) and wheat (circles) scutella. Open symbols indicate E callus. Closed symbols indicate total callus.

Table 1. *Optimal media and growing conditions.*

Japonica	Initiation	Maintenance	Regeneration (Light)
Calrose 76	1D+.2K+100TRP (d)*	2D+.2K (l)	.5 TIBA
Giza 159	1D+.3K (d)	1D+.3K (d)	.5A+.4B
Indica			
IR-36	1D+.5K+50TRP (l)	.5D+.25K (l)	.5A+.2B
Pokkali	.5D+.2K+50TRP (d)	.5D+.4K (l)	.5B
(hybrid) Mahsuri	.5D+.5K (l)	.5D+.5K (l)	.5A+.4B

D = 2,4-D
K = KIN
B = BAP
N = NAA
A = IAA

Numbers preceding hormones denote concentration in mg/l
*Culture conditions: callus grown in the light (l) or dark (d)

Table 2. *Effect of tryptophan concentrations on average E and NE callus (in mg/seed) and E/NE ratio of Calrose 76, Pokkali and IR-36. Media contained 2,4-D or 2,4,5-T. Results are for 20 seeds/sample.*

TRP	Calrose 76			Pokkali			IR-36		
mg/l	E	NE	E/NE	E	NE	E/NE	E	NE	E/NE
0	22.6 ± 14.6	207.5 ± 11.1	.109	38.0 ± 50.2	147.6 ± 19.8	.256	13.9 ± 5.6	160.0 ± 8.2	.085
50							24.4 ± 4.4	213.7 ± 8.7	.114
100	86.1 ± 10.6	258.5 ± 13.5	.333	262.1 ± 99.2	206.3 20.9	1.271			

Table 3. *Regeneration capacity of Calrose 76 E and NE callus on media containing one of three different growth regulators callus was initiated on medium containing 1 mg/l 2,4-D and 100 mg/l tryptophan. Sample size was 20 vials each containing 40 mg E callus.*

Concentration	BAP		TIBA		IAA	
	E	NE	E	NE	E	NE
mg/l			% vials with shoots			
0	25.0	0	25.0	0	25.0	0
0.1	53.0	0	60.0	0	20.0	0
0.2	68.8	0	73.3*	0	36.4	0
0.5	81.3*	0	75.0*	0	36.4	0

*Significantly different from control (2 × 2 contingency test) at 0.05 level of probability

Table 4. *Maintenance and Regeneration media of Several IRRI and Thai Cultivars*

	Initiation	Maintenance	Regeneration (Light)
IR-54	.5D+.5K (l)*	.5D+.5K (l)	.5A+.2B
IR-6	1D+.2K (d)	1D+.2K (d)	.5A+.5B
IR-60	1D+.2K (d)	1D+.2K (d)	.5A+.2B
IR-62	1D+.2K (l)	1D+.2K (l)	.5A+.2B
RD-23	1D+.3K (d)	1D+.3K (l)	.5N+1.6B+10% CW
RD-25	1D+.4K (d)	1D+.2K (l)	.5A+.3B

D = 2,4-D
K = KIN
B = BAP
N = NAA
A = IAA

Numbers preceding hormones denote concentration in mg/l
*Culture conditions: callus grown in the light (l) or dark (d).

Table 5. *Pokkali rice plants that could be regenerated from one seed under culture conditions if E callus is initiated from one seed in the dark on a medium containing 0.5 mg/l 2,4-D, 0.2 mg/l K, and 50 mg/l TRP, and maintained for 6 passages (after 1 passage on initiation medium) in light on 0.5 mg/l 2,4-D+0.4 mg/l K. The amount of E callus that could be produced at the end of the 6th passage is 257.83 g. For the 7th passage, all the E callus is transferred to a regeneration medium containing 0.5 mg/l BA as the only hormonal additive.*

Culture condition	Plants regenerated at the end of 7th passage	Total number of plants regenerated (including those on maintenance medium)
NE Callus		0
E Callus unimproved media		4,881
E Callus improved media	20,167	25,049
Callus to medium ratio (mg/ml) adjusted to 6.5	64,535	69,417
Regeneration medium conditioned for 2 weeks at optimal conditions	122,618	127,500
Utilizing the conditioning factor directly (proposed)	245,236	250,118

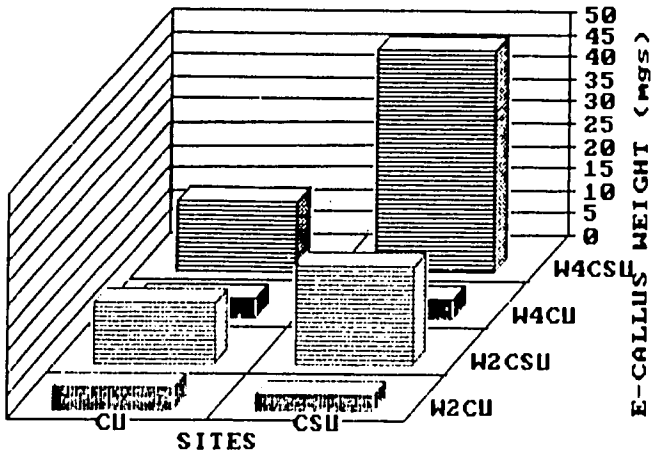


Fig. 3. *Callus initiation using CU and Colorado State methods at both sites.*
W2CU = Two week culture using CU method.
W2CSU = Two week culture using Colorado State method.
W4CU = Four week culture using CU method.
W4CSU = Four week culture using Colorado State method.

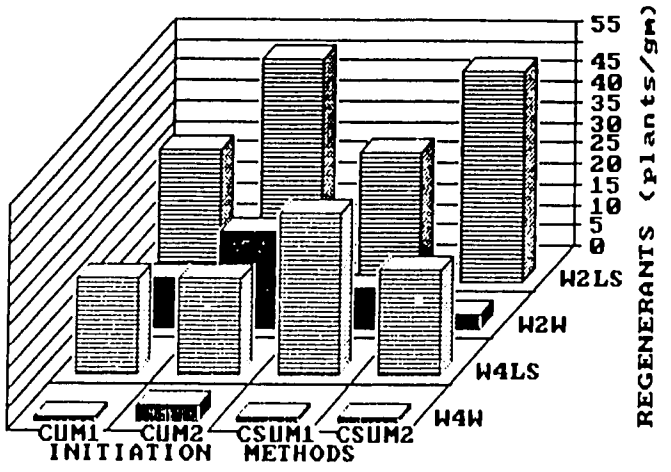


Fig. 4. *Plant regeneration in CU and Colorado State methods at Colorado State.*
*CUM1 = CU initiation medium using CU method.**
*CUM2 = Colorado State initiation medium using CU method.**
*CSUM1 = CU initiation medium using Colorado State method.**
*CSUM2 = Colorado State initiation medium using Colorado State method.**
W2W = Two week old calli in CU regeneration medium.
W2LS = Two week old calli in Colorado State regeneration medium.
W4W = Four week old calli in CU regeneration medium.
W4LS = Four week old calli in Colorado State regeneration medium.

**Each method was used for both initiation and regeneration.*

The number of regenerants from each treatment are significantly different at 0.01 probability level.

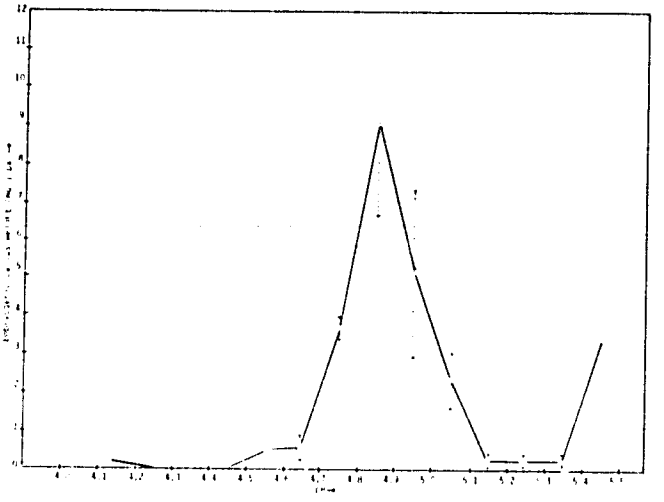


Fig. 5. Distribution pattern for the amount of E callus initiated from mature seeds of Oryza sativa *cv. IR-36 and the final pH of the medium after the initiation period of 28 days. Results were compiled from 3 replications, conducted over a period of 6 months, with each replication having more than 60 samples. Bars represent standard errors of the mean.*

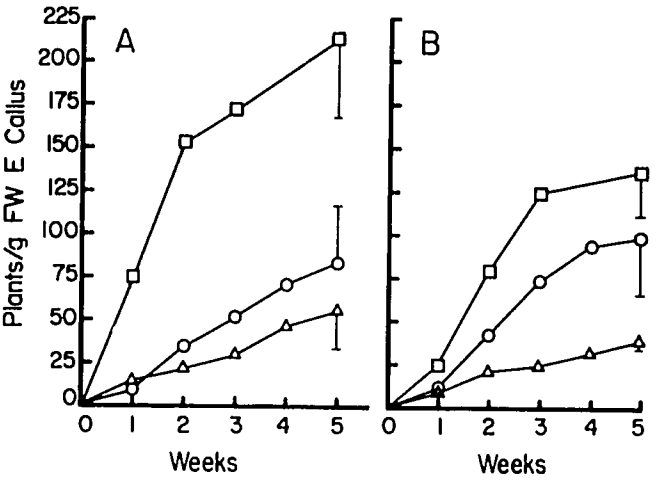


Fig. 6. Conditioning of medium. A: initial inoculum size was 65 mg. B: initial inoculum size was 500 mg. (triangle); no conditioning of medium. (circle); medium conditioned for 1 week. (square); medium conditioned for 2 weeks. In all these cases, after conditioning, the medium was reinoculated with 65 mg E callus. The regeneration from this callus in 5 weeks is evaluated. Standard errors are shown. Sample size was 20 containers.

Inoculum Size/Conditioning Factor

Rice has been used in experiments to determine if there is an optimal callus mass/medium volume ratio. An inoculum size which is too small will result in callus death; if too much callus is transferred, initial growth is high, but declines by the fourth week in culture. Similarly, the mass of callus placed on regeneration medium has a dramatic effect on the number of shoots produced per gram. In experiments in which either the inoculum size or the medium volume was varied, an optimal ratio was 65 mg callus per ml medium for Pokkali rice (Raghava Ram & Nabors, 1985). If the piece of callus inoculated was too small, death or very poor regeneration occurred. With a large inoculum size, plant regeneration was reduced while more callus growth occurred.

The media used at TCCP is pH'd to 5.50 prior to autoclaving. Due to autoclaving, the pH drops to 5.40 or 5.45. The pH drops further after four weeks in culture. The greatest E callus growth occurs when the final pH of the media is 4.85 (Fig. 5). Buffering the media at 4.85, at the start of the experiment, markedly improves E callus production.

Regeneration rates can also be increased in Pokkali through the use of conditioned media. If a piece of callus was used as an inoculum for one or two weeks to condition the medium and then replaced by another piece of callus, the regeneration rate from the second piece of E callus was nearly four times that of a control placed on non-conditioned medium (Fig. 6). If roots had grown into the medium, the agar was softened in a 70°C water bath to allow easier removal of callus and plantlets. The medium was then resolidified and inoculated with fresh E callus. The heating of the medium prior to resolidification did not appear to have any effect on the conditioning.

Liquid Cultures

The principal difficulty in the development of cereal suspension culture technology is the lack of reliable regeneration. It is possible, however, to regenerate callus grown in liquid cultures, and for selection purposes, short-term liquid cultures have immediate application.

Embryogenic callus of Giza 159 has been successfully cultured in liquid media for up to 20 weeks, although the regeneration capacity is not as great as on solid media (Fig. 7). Embryogenic callus from fourth passage Giza 159 was cut into fine pieces and added to liquid LS media with 1 mg/l 2,4-D + 0.3 mg/l KIN. After one week on a rotary shaker at 100 rpm in dark conditions, the mixture was centrifuged and the packed cells were added to new media. This procedure repeated for up to three months until the suspension contained small, individual yellow clumps; cultures were then transferred by pipetting every two weeks

A few plants were regenerated after individual clumps of cells from the suspension were picked out and placed in the dark on LS solid media with 1 mg/l 2,4-D, 0.3 mg/l KIN and 1.6% agar. After four weeks, the E callus was separated and placed in the light on regeneration media containing 0.5 ppm IAA + 0.4 ppm BAP. Healthy, green callus was transferred to a second passage on regeneration media after four weeks. Plants were regenerated on both passages.

The rice cultivars IR-36 and Giza 159 have been tested using liquid saline selection. NaCl levels were chosen so that 90% reduction in E callus fresh weight increase was achieved. In this scheme, 0.5 gm E callus was placed in 250 ml Erlenmeyer flasks containing 50 ml liquid saline maintenance media with 11 g/l NaCl. The flasks were placed on a rotary shaker at 100 rpm. After 20 days, calli were removed and placed on regeneration media. At 56 days (after introduction of stress), all remaining callus material was placed on regeneration media. Plants were obtained at both regeneration attempts and seeds produced for future whole plant testing.

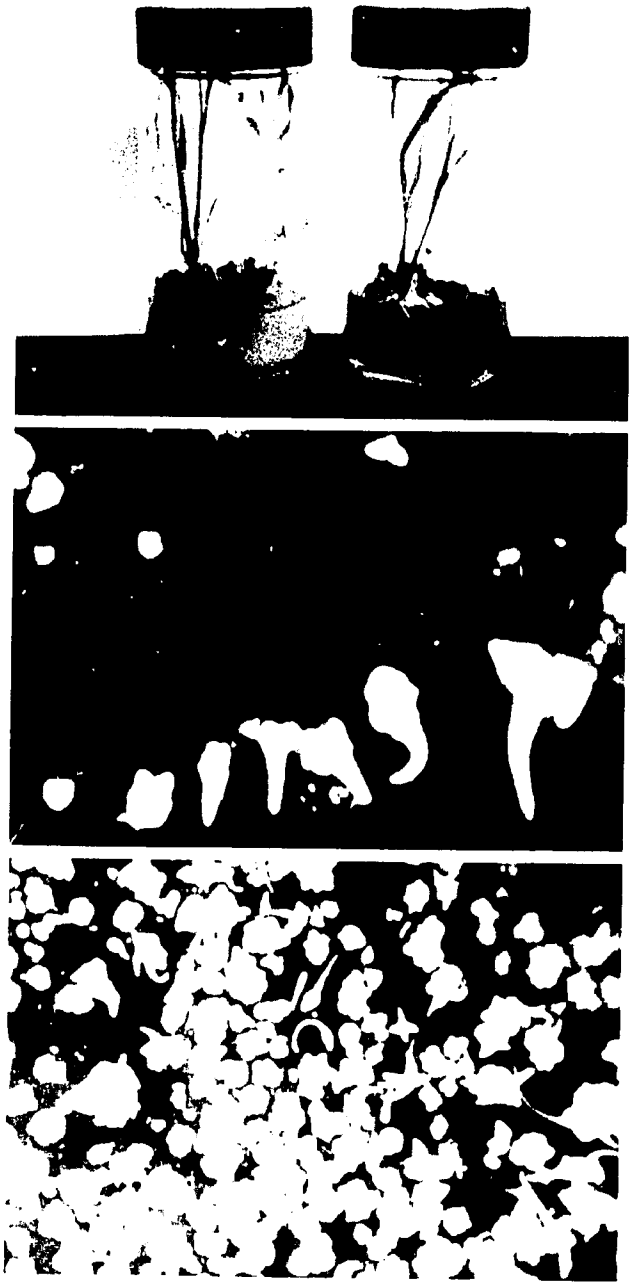


Fig. 7. Giza 159 regenerated from suspension culture.

In Vitro Selection — NaCl

Several long-term selection studies have been conducted with E callus of the cultivars Calrose, Giza 159, IR-36, Mahsuri and Pokkali. From these studies, 81 plants of Calrose, 535 of Giza 159, 519 of IR-36, 258 of Mahsuri and 146 of Pokkali have been regenerated and grown to maturity (Table 6). Many more were regenerated but not grown to maturity due to greenhouse space limitations. These cultures were started on their respective levels of NaCl and were maintained on that level until the callus was moved to regeneration media. Cultures have been maintained on 6 g/l NaCl for at least seven passages for all cultivars tested and have retained regeneration ability to maturity and seed set. On higher salt levels cultures did not survive seven passages.

Long-term cultures, those of more than 12 passages under NaCl stress, are possible with Giza 159 and Mahsuri at 3 g/l NaCl, Pokkali at 3 and 6 g/l and IR-36 at 6 and 9 g/l.

Table 6. *Selected salt levels and their influence on length of culture and number of plants regenerated and grown to maturity in the greenhouse. More plants were regenerated but due to limited space, only a few were kept.*

Cultivar	Salt level g/l	Maximum total passage length in culture	# plants which set seed	Total
Calrose	0	11	29	
	3	8	47	
	6	7	5	81
G-159	0	20	108	
	3	18	130	
	6	11	183	
	8	10	13	
	9	5	38	
	11	10	63	535
IR-36	0	22	258	
	3	11	173	
	5	2	1	
	6	21	70	
	8	5	7	
	9	15	7	
	10	2	3	519
Mahsuri	0	22	15	
	1	4	3	
	3	6	209	
	6	8	26	
	8	4	4	
	9	3	1	258
Pokkali	0	13	31	
	3	13	38	
	6	22	65	
	9	7	12	146

In Vitro Selection — PEG/$AlCl_3$

Preliminary investigations have been conducted to determine the PEG and $AlCl_3$ concentration ranges which support reduced callus growth sufficient to obtain tolerant mutants. In experiments with PEG, the most successful concentration, in terms of allowing callus growth, were 10, 20 and 50 g/l. Six plants which set seed have been regenerated from PEG stressed cultures of Pokkali; two plants were regenerated from Giza 159 (Table 7).

The concentrations tested for $AlCl_3$ were 50, 100, 200, 250, 500, 1000 and 2000 mg/l. Plants have been recovered from each level after a minimum of four months in culture for Pokkali, Giza 159 and IR-36. A total of 170 fertile plants from IR-36, 38 from Giza 159 and 1 from Pokkali (Table 8). All future experiments with IR-36 and Giza 159 stressed on $AlCl_3$ should use concentrations between 200 and 400 mg/l since lower levels tend not to be restrictive enough to growth, and higher levels are usually fatal.

Table 7. Plants regenerated from PEG-stressed cultures.

Cultivar	PEG level g/l	Longest passage length in culture	# regenerate plants which set seed
G-159	50	9	2
Pokkali	10	6	6

Table 8. Plants regenerated from various aluminum levels.

Cultivar	Al stress level mg/l	Longest total passage length in culture	# plants which set seed	Total
G-159	200	11	28	
	250	8	8	
	500	5	2	38
IR-36	50	9	34	
	100	11	57	
	200	11	51	
	250	9	28	170
Pokkali	50	4	1	1

Nitrate Efficiency

Enhancing the fertilizer efficiency of crops would reduce one of the major costs of farming. The approach used by the TCCP is to select for altered nitrate reductase with increased substrate specificity or reduction capacity. This can possibly be accomplished by growing cells in the presence of chlorate, a competitive substrate. Nitrate reductase will chemically reduce the chlorate to chlorite, which is lethal to cells; however, variant cells with increased levels of nitrate reductase or with altered nitrate reductase — having increased specificity for the substrate nitrate — will not be affected by the chlorate, and will thus preferentially grow.

In initial chlorate experiments conducted with IR-36, it was determined that 50 mg/l chlorate selectively inhibited callus growth to about a tenth of control growth. Surviving cultures from chlorate screens were subsequently cultured on media containing one-half, one-quarter, and one-tenth concentrations of LS nitrate; however, none of these chlorate-selected calli grew as well on the reduced nitrate media as the controls. Because selection time was short and pressure was low for this experiment, additional chlorate selections are planned to generate chlorate resistant calli, and to screen calli on minimal nitrate media.

Table 9. Relative salt tolerance at seedling stage of two rice cultivars, selected for tolerance in callus culture at various levels of NaCl. Scale range: 0=dead, 6=completely healthy. Seedlings were placed in test solutions containing 0, 3, 6, 9, 12, or 15 g/l NaCl, 20 days after germination. Data was collected 5 days after plants were placed in test solutions.

	in vitro		hydroponic seedling test of R_1 plants		
Cultivar	*in vitro* stress level: NaCl(g/l)	selection period (mos)	Avg. score of plants grown in 0 g/l NaCl	Avg. score of plants at all NaCl levels tested (0-15 g/l)	Avg. score of plants grown in 15 g/l NaCl
Calrose	0	7	4.00	1.72	0.83
	3	7	5.20	2.37	1.00
	3	8	4.30	2.54	1.80
	6	7	5.00	2.92	1.67
IR-36	0	5	6.00	2.01	0.67
	3	5	5.50	2.46	0.67
	6	5	6.0	2.96	1.75
	9	5	5.50	4.16	2.00

Whole Plant Greenhouse Evaluation

To date, plants from three rice cultivars, Calrose 76, Giza 159 and IR-36, have been evaluated. Calrose 76 was initially selected for salt tolerance in tissue culture for seven or eight months, on media containing 3 g/l or 6 g/l NaCl. As a control, some callus was also grown for seven months on a medium without added NaCl. IR-36 and Calrose 76 were initially selected for salt tolerance in tissue culture for five months on media containing 0, 3, 6, or 9 g/l NaCl (Fig. 8). At the end of the *in vitro* selection period, plants were regenerated, grown under greenhouse conditions, and seed was collected. These seeds were then germinated and placed into beakers containing one-half strength Hoagland's solution and 0, 3, 6, 9, 12, or 15 g/l NaCl. After five days in the NaCl solutions, plants were rated for vigor on a 0-6 scale, ranging from death (0) to perfectly healthy (6).

Calrose seedlings were stressed somewhat by the testing system itself; that is, plants placed in 0 g/l NaCl averaged only 4.0 on the 0-6 scale (Table 9). The average score of plants in all test solutions (0-15 g/l NaCl) increased steadily with an increase in either the *in vitro* stress level, or the selection period. R_1 seedlings derived from callus that had been selected for tolerance at 6 g/l NaCl were approximately 70% more tolerant than the R_1 progeny of plants derived from callus cultures which were not selected for salt tolerance (averaged over the entire range of NaCl test solutions). At the highest level of salt tested (15 g/l NaCl), the R_1 progeny of callus selected at 6 g/l NaCl were approximately 100% more tolerant than the controls.

IR-36 plants grew well in solutions containing no NaCl, averaging a 6.0 rating out of a possible 6.0. There was again a steady increase in seedling tolerance across the entire range of NaCl test solutions, correlating with the level of *in vitro* selection pressure. R_1 plants regenerated from callus selected for tolerance at 9 g/l NaCl did approximately 100% better than R_1 plants derived from non-selected callus. With 15.0 g/l NaCl in the test solution, R_1 progeny from the 9 g/l NaCl-selected cultures did almost 200% better than the controls. It is interesting to note that, at a low *in vitro* selection pressure (3 g/l NaCl), tolerance of the R_1 progeny does not appear to be significantly greater than the controls when the selection period was seven months or less. When the selection period was

more than seven months, however, some NaCl tolerance was expressed in the R_1 progeny.

These experiments indicate that rice callus, selected for salt tolerance *in vitro*, can produce salt-tolerant seedlings when given a selection period of at least seven months. In this experiment, 61% of the R_1 seedlings derived from callus culture stressed at 3 g/l NaCl, 71% of the R_1 seedlings derived from callus stressed at 6 g/l NaCl, and 92% of the R_1 seedlings derived from callus stressed at 9 g/l NaCl did better in seedling tests than did R_1 plants derived from callus that was never stressed. The data tend to suggest that the total stress in culture is a function of both the level of NaCl in the medium and the length of the selection period. This is consistent with our previous findings for tobacco.

A salt threshold level hydroponic test for whole plants has been developed. Seeds were planted in six-inch pots containing sand. Thirty-six pots were placed in large wooden tanks lined with plastic (Fig. 9). Liquid nutrient solution was added to the tanks. Once the rice plants were 10-15 cm tall, the level of the hydroponic solution was raised to above the top of the pots. At that time NaCl was added over the next seven to ten days until the final concentration was reached.

In the first experiment, seeds from regenerated Giza 159 and IR-36 plants were evaluated. The R_1 lines came from callus selected for ten passages on 9 g/l for Giza 159 and for IR-36 for five passages on 9 g/l. Non-tissue cultured seeds were used as checks. Four levels of NaCl were tested: 0, 3, 5, and 7 g/l. Percent survival was 100, 89, 31 and 0%, respectively (Fig. 10). One IR-36 line out-yielded its check at 3 g/l and one regenerate line of Giza 159 out-yielded its check significantly in the control tank. The IR-36 line is now in a second hydroponic test and the Giza 159 is in field tests to confirm its yielding ability.

A second, whole plant, salt hydroponic test was started in February, 1987, with 58 regenerate lines of IR-36, Giza 159 and RD-23 as well as non-tissue culture checks of each cultivar and Pokkali, a salt-tolerant cultivar. Vigor ratings taken after five weeks of salt stress are summarized in Table 10. From the regenerated lines of IR-36 and Giza 159, one to four and one to two lines were rated higher than both Pokkali and their respective check seeds.

Fig. 8. Pokkali seedlings in beaker hydroponics.

Fig. 9. R_1 IR-36 plants in 5 g/l NaCl.

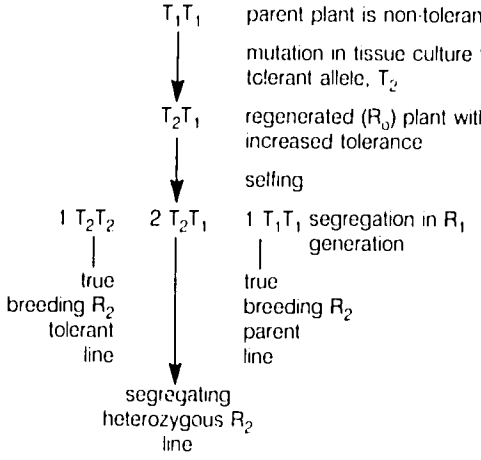
Table 10. Summary of vigor rating of second whole plant hydroponic test. Vigor rating 1 to 5 (dead to healthy). Plants were 8 weeks old and had been stressed for 5 weeks.

	0	3 g/l		5 g/l	
IR-36		1	2	1	2
Pokkali check*	5.0	2.7	3.0	2.7	2.7
IR-36 check	4.0	3.7	2.0	3.0	1.3
Ave. of tank	3.9±.2	3.1±.1	2.3±.2	2.4±.2	1.8±.2
# of lines in tank	31	17	17	18	18
# of lines better than both Pokkali and IR-36 checks	0	1	4	3	2
G-159					
Pokkali check*	5.0	3.7	2.0	2.3	2.7
G-159 check	5.0	3.0	3.4	3.7	3.0
Ave. of tank	4.1±.2	3.1±.2	2.7±.2	2.4±.2	2.5±.1
# of lines in tank	18	15	19	17	28
# of lines better than both Pokkali and G-159 checks	0	0	2	0	1

*Check is seed not derived from tissue culture experiments.

Fig. 10. Whole plant hydroponic evaluation showing R_1 Giza 159 in control (background) and in 3 g/l NaCl (foreground).

Dominant or co-dominant mutant alleles for tolerance are selected in tissues cultures and, after one generation of inbreeding, produce homozygous true breeding lines with increased tolerance.



Field Testing

In a collaborative research project, Dr. Milton C. Rush of the Louisiana State University Rice Research Station has investigated somaclonal variation. Through November 1, 1986, somaclonal variation has been observed in regenerated plants in the characters of leaf blade angle, flag leaf angle, culm angle, leaf color, leaf pubescence, leaf senescence, culm strength, disease resistance, panicle type and size, panicle exertion, sterility, seedling height, mature plant height, sheath color, tiller number, leaf blade length, blade width, days to heading, panicle number, panicle weight, 100-seed weight, grain-type, grain quality, yield, apiculus color, and hull color.

Somaclonal variation was observed in regenerated lines from Labelle, Zenith and Lemont during both the 1985 and 1986 growing seasons; however, the cultivars Melrose, Tetep, Taipei 309, and Tadukan showed little or no variation. It is clear that different rice germplasms show different responses as far as development of variation from culture is concerned. The cultivars Tetep and Tadukan have shown no variation to date. If only these cultivars would have been studied, the conclusion would have been that rice does not show somaclonal variation.

In a yield trial during the 1986 season, the line Labelle #215 out-yielded the parent variety Labelle and the high-yielding, commercial variety Lemont; however, grain quality was seriously reduced due to breakage of the larger grain during milling. Lemont line #726 had out-yielded Lemont in a 1985 yield test, but yielded less than Lemont in the 1986 test.

In 1985, two Labelle lines were identified that were resistant to sheath blight. These lines were also resistant in 1986, although one line appeared to be segregating for resistance. In 1986, two more Labelle somaclones and one Zenith somaclone with apparent increased resistance to sheath blight were identified. Many Labelle lines appeared to vary in resistance to the rice blast disease when exposed to natural infection. Some lines appeared more resistant than the parent cultivar and some appeared to be more susceptible. About thirty lines have been sent to Dr. M.A. Marchetti at the Texas A & M Rice Research Station in Beaumont, Texas for evaluation of their blast resistance under controlled conditions.

In May of 1986, approximately 100 regenerant lines and check seeds from TCCP of cultivars Calrose, Giza 159, IR-36, Mahsuri, and Pokkali were sent to Dr. Charles N. Bollich at the Texas A & M Agricultural Research Station and to Dr. Milton C. Rush at LSU for generation advance. Variation in flowering date, number of tillers and morphological characteristics were observed (Figs. 11a and b).

In early August, 1986, seeds from 387 regenerant lines of Calrose, Giza 159, IR-36, Pokkali and Mahsuri were delivered to Dr. Dely P. Gapasin at the Philippine Council for Agriculture and Resources Research and Development (PCARRD) in Los Baños, Philippines, for use in structured field tests in saline soils. The testing is part of a collaborative program with Dr. Edwin Javier of UPLB. Fifty-eight R_2 lines from the Louisiana and Texas field increases greenhouse hydroponics tests, and sixty additional R_1 lines from the TCCP rice inventory were also sent to the Philippines for these new field tests. A total of approximately 500 lines are now in the Philippines for testing. R_1 lines were increased and will be tested in saline soils and the R_2 seed from Texas and Louisiana were evaluated on the saline soil in early 1987.

Based on visual observations after six weeks in salt, several R_2 lines looked promising either in comparison to their bag seed check or by having outstanding individuals within the R_2 lines. From field increased R_2 lines, six of fourteen Calrose, five of nineteen Giza 159 and one of four Mahsuri lines looked promising. After selecting in hydroponics, two of seven Giza 159 and four of nine IR-36 lines showed promise (Table 11). Final data will be taken in late April or early May, 1987

Table 11. R_2 *Lines in Salt Evaluation Trial in the Philippines*

Seed from Texas Seed Increase Cultivar	Salt Stress	Passage	Number of Lines Sent	Promising
Calrose	0	9	1	0
		11	1	0
	3	6	2	0
		7	4	3
		8	3	1
	6	7	3	2
Giza 159	0	17	1	1
	6	8	1	2
		9	3	1
		10	7	1
		11	2	1
	8	10	2	1
IR-36	0	8	1	0
	6	5	3	0
		12	1	0
	9	5	1	0
Mahsuri	0	9	1	0
	6	3	1	1
	8	3	2	0
Seed from hydroponic test				
Giza 159	8	10	7	2
IR-36	9	5	9	4

Taxonomic Terms

Genus A systematic unit consisting of a group of species which are of common phytogenetic origin and appear to be clearly differentiated from other such groups.

Species Groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups.

Population A community of potentially interbreeding individuals which share a common gene pool.

Land race Also referred to as a geographic race, is a population which occupies a geographical subdivision within the range of a species.

Variety A group of individuals within a species which are distinct in form or function.

Genetic Terms

Genotype The sum total of the genetic information (genes) contained in the chromosomes.

Phenotype The observable properties of an organism, produced by the interaction between its genetic potential and the environment it is in.

Breeding Terms

Family In breeding a group of individuals directly related by descent from a common ancestor.

Line In breeding referred to as an inbreed line produced by continued self-fertilization. At TCCP has been used to denote the R_1 seed.

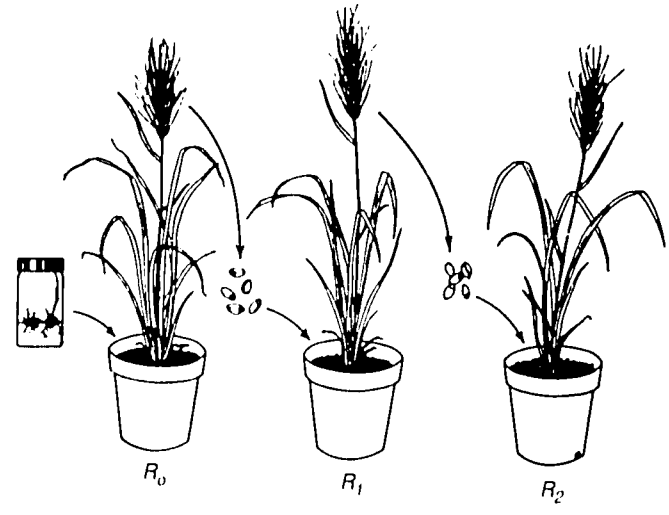
Cultivar A variety of plant that has been produced only under cultivation.

Field Plot Term

Entry At TCCP used to denote a line, which may be replicated, in a field evaluation.

Fig. 11a. *R_1 lines in Texas showing variation in heading date.*

11b. *R_1 seed increase in the Philippines.*



Nomenclature used at TCCP for identification of progeny derived from tissue culture regenerated plants.

Wheat

Wheat is the largest of the cereal crops in terms of both hectares and production and a staple food for over one third of the world's population (Reitz, 1967). Wheat contributes more calories and protein to the world's diet than any other food crop (Hanson, *et al.*, 1982). It is nutritious, concentrated, readily stored and transported, and easily processed. The uniqueness of wheat stems from the fact that it contains gluten which allows dough to rise.

There are two primary types of wheat: bread wheat *(Triticum aestivum)* and durum wheat *(Triticum durum)*. Bread wheat covers about 90% of world wheat area and produces 94% of the total harvest. At present, the TCCP is using two bread wheat cultivars obtained from CIMMYT: Glennson 81 and Pavon 76. Preliminary tissue culture and regeneration experiments have been conducted with spring wheat cultivars Chris and Chinese Spring and also with Triticale. A winter wheat program, funded by the Colorado State University Experiment Station, is also in operation.

Long-term, High-frequency Regeneration

Cereal callus which is highly regenerable (E callus) is composed of somatic embryos. In wheat, such callus was much more difficult to recognize than in rice and other cereals. In initial reports, it was observed that a smooth-compact callus was produced from seeds in culture (Fig. 12), and preliminary evidence indicated that this callus was moderately regenerable (Nabors *et al.*, 1983).

When the smooth-compact callus is cultured on maintenance media, it continues to "differentiate" into a bumpier callus (Fig. 13). It was thought that this bumpy callus represented embryoid primordia; however, all attempts to regenerate at high efficiency the smooth-compact callus and its derivative bumpy callus failed, although more than 20 hormone combinations were tested in regeneration media (MacKinnon, 1985).

This dilemma of sporadic plant regeneration from wheat callus cultures was resolved when studies were conducted on the production of E callus from developing scutella. Scutella of immature wheat embryos have the capacity to develop somatic embryos only during early stages of embryo development (Fig. 14; Dykes, Wahler, and Nabors, in preparation). From these experiments, the morphological appearance of embryoids derived from immature scutella was a guide, and all callus cultures established from seeds and isolated mature embryos were carefully examined. It was discovered that cultures containing smooth-compact callus often had somatic embryos, although microscopic observation was necessary to see them. Experiments with seeds and mature embryos provided evidence that the smooth-compact callus was competent to produce embryoids within the first few subcultures; at that time, the smooth-compact callus developed root primordia and was capable of limited plant regeneration.

In order to maximize the amount of the smooth-compact callus produced in culture from seed, a series of complex media experiments were implemented. It was determined that 5 mg/l 2,4-D plus 20 mg/l TRP produced the maximum amount of smooth-compact callus, and minimal amounts of NE callus (Heyser *et al.*, 1985). It has since been determined that TRP does not consistently enhance E callus amounts in wheat, and it has thus been eliminated from initiation and maintenance media. Isolated mature embryos will also produce callus with embryos. In Pavon, 2 mg/l 2,4-D is better than 5 mg/l 2,4-D at most KIN concentrations for the percent of cultures with embryos (Table 12; MacKinnon, 1985).

Formation of E callus takes one to two months when immature embryos are used, as compared to two to four months when mature embryos are the explant source. The composition of the regeneration medium does not appear to be critical for long-term, high-frequency plant regeneration from the E callus. It was found that embryos were able to develop into plants on media devoid of any hormone, but more vigorous shoot and root formation occurred on media with hormones (MacKinnon, 1985). A critical factor for optimal regeneration was determined to be the inoculum size cultured on regeneration media. If less than 0.01 gm of E callus was used on regeneration medium (LS + 0.1 mg/l IAA + 0.5 mg/l BAP), plant formation was diminished (about 60% of the cultures formed plants); however, if larger pieces were used, approximately 90% of the cultures regenerated. From the results of these experiments, it was concluded that maximal regeneration can be obtained using an inoculum mass of 0.02 gm of E callus per 10 ml of medium (MacKinnon, 1985). The majority of regenerates obtained from long-term cultures were phenotypically normal, and reached maturity under greenhouse conditions.

In summary, the success of callus initiation medium for wheat depends more upon the developmental maturity of the

explant than on the cultivar. When whole seeds are used as explants, 5 mg/l 2,4-D is required to maximize the amount of smooth-compact callus and minimize the amount of NE callus. If isolated mature embryos are used, smooth-compact callus readily forms at 2 mg/l 2,4-D and produces significant amounts of E callus when maintained at this concentration (MacKinnon, 1985). While the majority of embryos are formed during the first passage they are not, because of their small size (generally less than 1 mm), usually detectable. Thus, E callus is not evident in quantity until the second passage. With improved techniques, regenerative callus of spring wheat has been maintained for 22 passages and average regeneration rates of over 100 plants per gram (TCCP Newsletter No. 7, 1987).

Fig. 12. *Smooth E. callus regenerated from Pavon.*

Fig. 13. *"Bumpy" callus from Glennson.*

Fig. 14. *E callus from immature embryos of Pavon.*

In Vitro Selection – NaCl

Preliminary research with salt selection of the spring wheat cultivars has established the parameters for the minimal levels of salt stress as well as the minimal culture period *in vitro*. Since salt selection in cereals must be done with E callus if regeneration is to be achieved, the relative sensitivities of E callus versus other types needed to be assessed. Several experiments investigated the effects of NaCl on long-term callus growth. Fresh weight measurements revealed that total callus amounts grew reasonably well on media containing 9 gm/l NaCl; however, E callus appeared more sensitive to salt in that fresh weight amounts decreased over time. In addition to reduced growth rates, E callus did not survive as long as other callus types on media containing NaCl.

For further screening, cultures were initiated from mature embryos of spring wheat cultivars Pavon and Glennson. In the first experiment with Pavon, cultures were initiated and maintained on 2 mg/l 2,4-D with 3, 6, or 9 g/l NaCl. Green and yellow, compact E calli were maintained for five passages on their respective selection media (Figs. 15a and b). When transferring to the sixth passage, one half of the E callus from each group was divided into small pieces and placed on regeneration medium containing 0.1 mg/l IAA and 0.5 mg/l BAP. The other half was contained in the stress culture. The callus cultures were handled as described for the sixth to ninth passage. A total of 99 plants were regenerated from 3 g/l, 21 plants from 6 g/l and 0 plants from 9 g/l NaCl (Fig. 16).

In a second experiment on Pavon, cultures were maintained for four passages on non-stress media then moved to 12 g/l NaCl for four additional passages. After a total of eight passages, all of the calli were moved to regeneration media and 22 plants were regenerated.

In similarly designed experiments with Glennson, after four passages on non-stressed media the cultures were moved to either 10 or 11 g/l NaCl. From both salt levels, one plant was regenerated from each. In the past year, over 2200 plants have been regenerated from cultures containing NaCl and over 1650 from control cultures.

Fig. 15a. Third passage E callus from mature embryos of Pavon on 0 g/l NaCl.

In Vitro Selection – PEG/$AlCl_3$

Screening for drought tolerance generally involves the use of PEG. This polymer will interfere with the solidification of the agar media unless sepcial techniques are used (see Appendix IV). A filter paper bridge method has been used with higher concentrations of osmotica. This technique uses liquid media with the callus supported by the paper bridges. To make the bridges, a double layer filter paper (Whatman No. 1) is cut and folded to make a 130 mm by 30 mm strip. This strip is folded into three sections with the sections being 50 mm long and the center being 20 mm. The bridges are placed in jars (75 mm tall by 35 mm in diameter; Fig. 17) and 30 ml of medium containing the PEG is added prior to autoclaving. Callus grows more rapidly on filter paper bridges, but set-up time is considerably lengthened.

$AlCl_3$ stress studies for wheat have progressed to the initial screening stage. Mature embryos were cultured on media containing 100 mg/l $AlCl_3$, but none survived to form callus. Having identified a lethal concentration of $AlCl_3$, further experiments will employ concentrations ranging from 10 to 75 mg/l $AlCl_3$.

Whole Plant Evaluation

Seeds from all regenerated plants are sent to CIMMYT for seed increase, cytological analysis, and field testing. In one experiment, an analysis of root tips revealed abnormal chromosomes in 18% of the increased plants regardless of whether they were from stressed cultures or controls (Nabors and Dykes, 1984). Hydroponic testing of plants from control and 3 g/l salt stressed cultures did not show any increased salt tolerance in the whole plants. Plants regenerated from higher levels of salt will be used in future testing. In addition, plants regenerated from non-stressed cultures will be tested for the presence of stress tolerance and somaclonal variants.

Widecross – Collaborative Research Program

Collaborative research arrangements have been made between the TCCP and Dr. A. Mujeeb-Kazi at CIMMYT to apply several tissue culture techniques to the widecross program. Additionally, production and field-testing of progeny from plants regenerated from tissue cultures will be conducted at CIMMYT.

A widecross, or alien-addition, is a type of hybrid in which one or more pairs of chromosomes from a wild parent species has been added to the chromosome complement of a cultivated species, or a segment of the wild-type chromosome is translocated to a chromosome of the cultivated species.

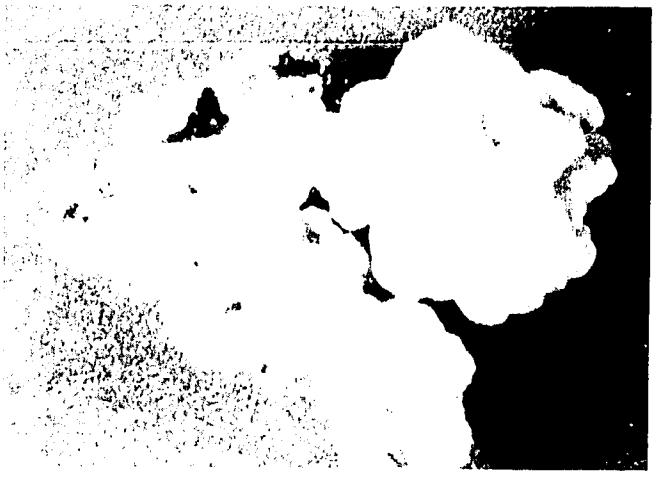


Fig. 15b. Third passage E callus from mature embryos of Pavon on 9 g/l NaCl.

Fig. 16. Pavon plants regenerated on 0, 3, and 6 g/l NaCl.

Table 12. Effects of KIN, change of 2,4-D level, and selective transfer on percent of cultures with embryos, cultivar Pavon.*

	Passage						
	1	2		3		4**	
Auxin mg/l	5D	5D	2D	5D	2D	5D	2D
Kinetin mg/l							
0	4	30	37	64	88	38	100
0.1	4	41	25	73	100	63	100
0.3	0	42	50	81	100	77	100
0.5	7	25	30	89	100	50	100
1	4	44	37	75	93	89	100
2	10	54	93	80	100	89	100

*Percentages within treatments at some passages varied significantly.
**At the start of fourth passage, only vials with embryos were transferred.

In the widecross program at CIMMYT, *Triticum aestivum* cv. Chinese Spring will be used as the cultivated species, and *Agropyron junceum*, *Haynaldia villosa*, and *Aegilops variabilis* as one of the wild parent species. *Aegilops variabilis* particularly is used because of its resistance to Karnal bunt and rust, and its tolerance to aluminum. It also has good N banding, providing an opportunity to follow the progress of recombination events cytologically. Repeated back-crosses of the hybrid with the cultivated species are to be performed which may result in monosomic alien addition lines in which the alien chromosome(s) are unpaired within the cultivated genome. These monosomic alien-addition lines can be doubled via colchicine to become disomic addition lines in which the alien chromosome are paired. This doubling results in a fully fertile disomic alien-addition line. Fertile alien-addition lines will be tested hydroponically to see if desirable traits for saline, drought, or disease resistance have been incorporated into the cultivated genome.

Media manipulation experiments were conducted for long-term callus cultures and regeneration potential of tetraploid and hexaploid wheat cultivars. Callus from several cultivars of *Triticum aestivum* and *T. turgidum* was maintained until the seventh, monthly passage. At each passage, some E callus was regenerated and plants were transplanted to the greenhouse.

The greenhouse conditions were set at 22°C/14°C day/night temperatures, 15 hours natural light and approximately 55 to 60% relative humidity. Bread wheats *(T. aestivum)* were more effective in producing E callus and yielded a higher number of regenerated plants (370) than durum wheats *(T. turgidum)* (82) (Table 13). There were several spike developmental abnormalities (Figs. 18a to d) that at the R_0 stage, may well have been transient changes. The heritable changes will be analyzed in advanced generations.

Somatic root tip cytology was done on 255 bread wheat and 45 durum wheat plants with some spikes being randomly sampled for meiotic analyses. Somatic root-tip chromosomes generally had a count of 42 for *T. aestivum* and 28 for *T. turgidum* (Figs. 19a and b). The range for *T. aestivum* chromosome number varied from 39 to 45 and from 28 to 56 for *T. turgidum* with 1 to 2 telocentric chromosomes. There was a single *T. turgidum* plant with 56 chromosomes attributed to spontaneous doubling. The plant was male sterile but female fertile and has set seed upon backcrossing to *T. turgidum*. The meiotic analyses of a few 42 and 28 chromosome plants provided evidence of cytological variations where apart from the normal bivalent formation, meiocytes possessed several univalents, trivalents and quadrivalents. All R_0 derivatives will be advanced because there exists the potential for selecting variants in advanced generations with the observed intrinsic R_0 meiotic changes.

Seeds of the R_0 plants have been cut into an endosperm half and the embryo portion. The latter material is being advanced one generation under greenhouse conditions whereas the corresponding endosperm portion has been used for electrophoretic analyses.

Several tissue culture techniques will be utilized in this program. Meristems culture will be used for inducing microtillering of rare F_1 *Triticum* x *Agropyron* hybrid embryos to increase the frequency of F_1 and backcross progeny. Several dozen different media have been tested with bread wheat immature embryos for maximal induction of tillering. Preliminary results indicate that media with 0.25 mg/l 2,4-D plus 0.25 mg/l BAP give at least one tiller, but only in about 20% of the total cultures. More experimentation with hormone types and concentrations, as well as photoperiod, is in progress. Anther culture will be used to obtain polyhaploids in the F_1 of intergeneric hybrids. *In vitro* screening of callus is also in progress to find the relative tolerance of existing, useful cultivars and to select variant lines with increased salt tolerance.

Embryo rescue is also being used for multiplying rare F_1 hybrids. Immature embryos of Triticale (wheat x rye) have been tested as a preliminary screen before other widecross embryos are cultured. It has been observed that hybrid embryos are often developmentally abnormal, and a high percentage of them die in culture; however, some do survive and develop E callus. This E callus can be regenerated into whole plants, thus multiplying the rare hybrid.

Micropropagation is in progress using E callus derived from F_1 hybrids of *Triticum* and *Agropyron*, *Haynaldia*, and *Aegilops*. Immature embryos were cultured on LS media plus 2 mg/l 2,4-D with approximately 40% of them forming callus, although the formation of E callus was sporadic. Plants will be regenerated from the E callus at the end of the second, four-week passage. These plants will be grown to maturity and used in backcross experiments. This cloning procedure is also being used with Triticale F_1 embryos.

Embryogenic cultures of Glennson have been maintained for two years. Cultures greater than one year old have been regenerated. Cytogenetical analyses are planned with these regenerates to document the extent of chromosome aberrations. It is especially desirable to obtain monosomics, which are of particular value to breeding programs.

Table 13. *Regenerated* T. aestivum *and* T. turgidum *plant data from callus maintained up to seven months.*

Varieties	Months in callus 3	4	5	6	7		Total
	Number of Plants Regenerated						
BREAD WHEATS							
MRL"S"/BUC"S"	21	33	26	6	0		86
BOW"S"	1	17	12	0	0		30
ALD"S"/PVN"S"	24	36	21	15	11		107
GH"S"	30	68	33	6	7		144
PAVON	0	0	3	0	0		3
						Total	370
DURUM WHEATS							
CHEN"S"	0	8	0	0	0		8
LARU"S"	12	12	4	10	0		38
CNDO/R143/ / ENTE"S"/MEXI"S"	9	0	0	0	0		9
MEMO"S"/MEXI"S"	22	3	0	0	0		25
ROK"S"/KMLI"S"	2	0	0	0	0		2
						Total	82
						Grand Total	452

Fig. 17. *Testing for drought tolerance in wheat using filter paper bridge.*

Fig. 18a-d. Spike morphology variations in R_0 plants of Triticum aestivum.

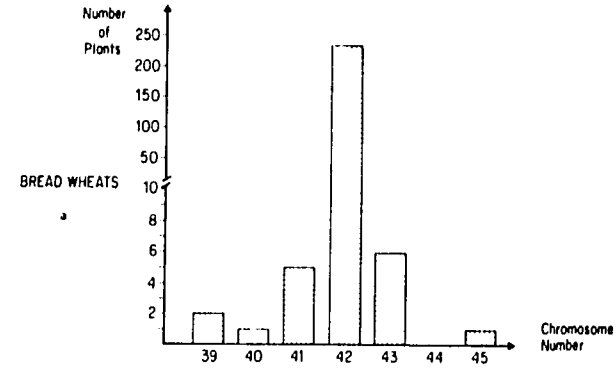


Fig. 19a. Number of Triticum aestivum *regenerated plants and their somatic chromosome numbers.*

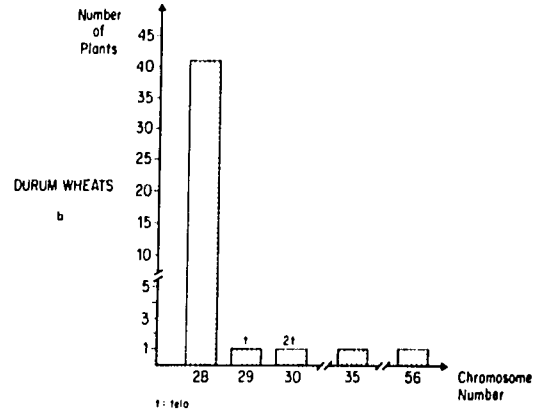


Fig. 19b. Number of T. turgidum regenerated plants and their somatic chromosome number (t = telocentric chromosomes).

Sorghum

Sorghum (*Sorghum bicolor* R. Moench) is a staple food crop in Africa, Asia and parts of Latin America and a main feed grain crop in the U.S., Mexico, Argentina, and South Africa. *Sorghum bicolor* includes grain sorghum, sweet sorghum, broomcorn, and Sudan grass and may have been domesticated in Africa by as early as 3000 B.C. (Archeampong *et al.*, 1984).

Sorghum yield on marginal land is a primary focus of research. Sorghum's ability to survive and maintain yields under heat, drought, aluminum and saline soil conditions is part of the reason it is significant as a food crop in developing countries.

Long-term, High-frequency Regeneration

A separate in-house study, supported by the Gas Research Institute of Chicago, Illinois, has resulted in the development of reliable methods for the induction of E callus in U.S. cultivars of both grain and sweet sorghum, and the achievement of long-term, high-frequency plant regeneration. Seeds of three grain sorghums, Hegari, RTx-430, RTx-7000, and two sweet sorghums, Keller and Rio were supplied by Dr. Fred Miller of Texas A & M University.

Both immature and mature embryos were tested as explant sources. Mature seeds were surface sterilized using 30% Chlorox and soaked for 20-24 hours in sterile distilled water to facilitate embryo excision. Mature embryos were dissected from imbibed seeds, freed from extraneous endosperm, and randomly placed with respect to scutellar orientation on basal media. Opaque, immature embryos, 1-2 mm in length, were excised from plants and grown under greenhouse conditions approximately 21 days after the emergence of the inflorescence when the endosperm is in the late milk to early dough stage. Immature seeds were sterilized as above, omitting the overnight soaking. Immature embryos were planted with scutella facing away from media.

A standard initiation and maintenance medium was developed containing LS salts and vitamins, 2% sucrose, 1.1% agar, 2 mg/l 2,4-D plus 0.5 mg/l KIN. The medium was adjusted to pH 5.5 prior to autoclaving (MacKinnon *et al.*, 1986b). Embryos were placed on this media and subcultured for one, five-week passage in the dark. All additional passages consisted of four weeks in the light. During the first and second passages callus production was low: less than 0.05 grams total callus per embryo. Visual selection of E callus did not occur until the third or fourth passage when callus growth increased.

Fig. 20. *E callus from third passage Hegari.*

Optimal callus quantity to medium volumes have not been investigated; however, all experiments were done using inocula size of 0.1 gm of E callus per 20 mls of medium. This procedure resulted in long-term growth, maintenance and regeneration.

The major callus type produced by sorghum is a watery, sometimes crystalline, friable, large-celled, NE callus. A second type of NE callus formed in association with the friable callus is densely compact, nodular and creamy yellow in color. This second type of NE callus should not be confused with E callus because only roots can be regenerated from this cell type. Embryogenic callus is distinguished by being very compact, nodular, often cleft, and always creamy white in color (Fig. 20). This callus type, produced by both immature and mature embryos is recognized as E in grain sorghum (Wernicke and Brettel, 1980) and other cereals (Nabors *et al.*, 1983). Occasionally, in sweet sorghum cultures, a smooth, intensely green compact nodular callus is produced in close association with creamy white E callus from which regeneration can also be obtained (Fig. 21).

Plant regeneration from E callus readily occurs on LS media supplemented with 1.0 mg/l IAA plus 0.5 mg/l BAP. It appears that the absolute amount of IAA is more critical for plant regeneration than the ratio of IAA to BAP. Growth regulators are essential for the occurrence of long-term, high-frequency plant regeneration. Occasionally, some E callus requires an additional passage on regeneration media before developing into plants. Regenerated sorghum plantlets are subcultured on a medium containing 3.0 mg/l IBA to complete root formation, (MacKinnon *et al.*, 1986a).

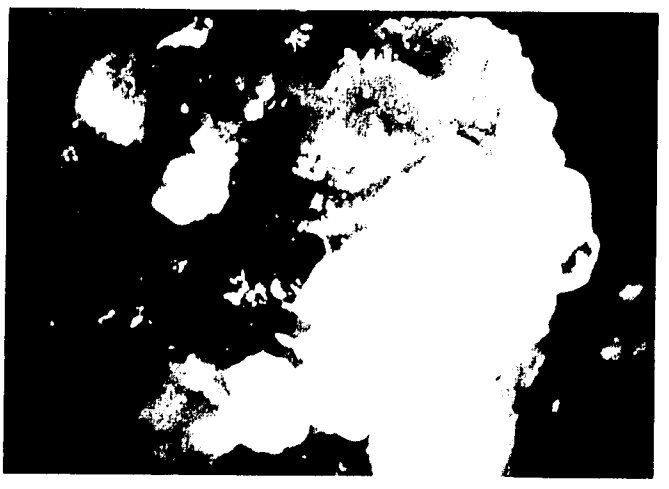


Fig. 21. *Green, compact E callus from third passage RTx-430.*

In Vitro Selection — NaCl

After cultural procedures and basal media compositions for callus induction, maintenance and regeneration were developed, *in vitro* stress selection for variants was started. The selective agents used thus far have been NaCl, chlorate, and hydroxyproline. NaCl was chosen as a primary selective agent.

For all cultivars tested except RTx-7000, within a passage, E callus weights decreased as salt levels increased from 9 to 15 g/l (Table 14). Also, E callus weights decreased from the first to the second passage with one exception of Keller at 15 g/l. For RTx-430 and Keller on 10 and 12 g/l NaCl, E callus weights increased in the third passage compared to the second passage.

In further experiments with Hegari, several selection strategies were followed. Embryogenic callus of grain and sweet sorghums (cvs. Hegari, Rio and Keller) were stressed *in vitro* using four different selection strategies:

1. Direct selection on 9 g/l NaCl in solid media for four months;
2. Direct selection on 12 g/l NaCl in solid media for up to 3.5 months;
3. Step-wise selection: 2-3 months on 6 g/l NaCl, then stepped up to 9 g/l NaCl for 3-4 months;
4. Liquid-agar selection: callus cultured in liquid media containing 1.5, 3, 6, or 9 NaCl, either continuous, or step-wise selection for up to two weeks.

Embryogenic callus produced on basal media was subsequently cultured and plants were regenerated from media containing NaCl. Callus which was stressed on 12 g/l NaCl decreased in fresh weight over time, with trace amounts of callus remaining at the end of the three passages (Table 15). Callus cultured on 9 g/l NaCl also lost over 90% of its fresh weight after four passages; however, callus stressed first on 6 g/l NaCl, then stressed on 9 g/l NaCl decreased in fresh weight by only about 50%. Over 1500 plants have now been regenerated from Hegari, Keller, Rio, RTx-430, and RTx-7000 from NaCl-stressed cultures (Table 16).

To test whether or not E callus could be successfully grown in liquid cultures, liquid media (35 ml in 250 Erlenmeyer flasks) were inoculated with 0.5 gm of E callus per culture. Every two to three days, medium was poured off and fresh medium added. At each media change some callus was collected and plated onto solid regeneration medium. The callus recovered from liquid cultures was frequently completely black, but within a few days formed regions of E clusters and, eventually, regenerated plants. The greatest number of plants were recovered after the first few days in liquid culture; however, plants were recovered from callus cultured for up to 12 days in liquid media (MacKinnon *et al.*, 1986b).

Having demonstrated that E callus could be cultured in liquid media and retain its regeneration capacity, experiments were initiated with liquid saline cultures. Generally, callus recovered from liquid saline cultures was completely black. When plated directly on solid regeneration media, some calli produced E regions which regenerated into plants (5 plants from *ca.* 0.4 gm callus). If the callus was plated first on solid saline media, and then cultured on regeneration media, fewer E calli were recovered, hence regeneration was poor (one plant from *ca.* 0.15 mg callus). In one experiment, however, callus recovered from liquid saline media was cultured on media containing 9 g/l NaCl, then placed on 10 g/l NaCl. Upon transfer to regeneration media, 24 plants from 0.3 gm callus were recovered (Table 15).

Sorghum callus is somewhat unique to cereals in that phenolic compounds are exuded by some cultivars of sorghum. Hegari, a grain type, produces a purplish-black exudate, while Rio, a sweet sorghum, exudes a reddish-orange color. The phenols do not appear to interfere with E callus production or development; however, there is a correlation between the amount of phenols produced and level of stressing agents in media.

The callus stress response associated with successively higher NaCl concentrations could be seen by the extent of phenolics exuded into the medium. Rarely did callus growing on salt-containing media fail to exude phenolics. It has been suggested that the phenolics produced by the calli indicate stresses associated with the differentiated state of tissue (Oberthur *et al.*, 1983). And it appeared in the present study that salinity exacerbated these stresses. Callus stressed on 6 g/l or higher concentrations of NaCl, eventually became intensely pigmented with the black phenolics, and it was difficult to determine if the callus was still viable. Once transferred to regeneration media, some of the stressed calli produced regions of embryoid clusters (Fig. 22). These embryoid regions subsequently regenerated into plants.

Table 14. *Average E callus weight (in grams) after each passage when 0.1 g of E callus was used to initiate each passage.*

Cultivar	Culture Period (mo)	-----NaCl Concentration (g/l)----- 9	10	12	15
Hegari	1	—	0.145	0.105	0.024
	2	0.148	—	0.031	0.018
RTx-430	1	0.562	0.124	0.104	0.037
	2	0.448	0.107	0.070	0.036
	3	—	0.113	0.082	—
RTx-7000	1	0.197	—	0.900	—
	2	0.021	—	0.044	—
Keller	1	0.147	0.075	0.078	0.024
	2	0.130	0.054	0.042	0.027
	3	—	0.074	0.061	—
Rio	1	0.173	0.081	—	0.092
	2	0.075	0.043	—	—

Table 15. *NaCl selection strategies, and whole plant regeneration obtained with cultivar Hegari.*

Selection Scheme g/l NaCl	Culture Period	Total qm E Callus Inoculated	Total qm E Callus Surviving	Total # Plants Regenerated
Liquid/agar:				
1.5-3	9-10 days	2.0	0.3	24*
3-6	9 days	3.0	0.66	1**
Agar:				
6-9	5 mos.	2.0	1.1	348
6-9	7 mos.	2.0	1.0	99
9	4 mos.	9.6	0.44	92
12	2 mos.	3.9	2.0	34
12	3 mos.	5.2	0.2	1
12	3.5 mos.	1.0	0.1	4

*callus recovered from liquid cultures was subcultured on solid saline (9 g/l NaCl) maintenance media for four-week passage, solid saline (10 g/l NaCl) maintenance for a second four-week passage, then cultured on solid regeneration media.

**callus recovered from liquid cultures was subcultured on solid saline (9 g/l NaCl) maintenance media for one four-week passage, then cultured on solid regeneration media.

Table 16. Number of sorghum plants regenerated from salt-stress experiments which matured and set seed.

Cultivar	Range of total passages	NaCl g/l	Range of passages on stress	Number of plants regenerated
Hegari	4-23	0	—	284
	11-15	9	4-7	301
	12	10	1	2
	8-22	12	1-4	55
	7-14	15	1-3	36
Keller	3-18	0	—	177
	10	9	4	7
	13-14	10	2-6	23
	13-16	12	1-5	74
	13-15	15	1-3	65
Rio	3-15	0	—	178
	21	9	2	11
	13-14	10	3-4	21
	13-18	12	1-2	51
	14	15	1	132
RTx-430	3-8	0	—	132
	14	10	2	1
	15-16	12	3-4	5
RTx-7000	4-5	0	—	22

Fig. 22. Third passage Hegari showing embryoid clusters and phenol-blackened callus.

In Vitro Selection — Hydroxyproline

An indirect method for *in vitro* selection of enhanced stress tolerance was attempted by altering to select for increased concentrations of proline, an amino acid involved with osmoregulation and essential to cell survival. Hydroxyproline, a proline analog, was chosen as the selective agent because it causes feedback inhibition of proline biosynthesis. Cells that survive may have an altered proline enzyme system that might also have enhanced tolerance through cellular adaptation to osmotic stress.

A preliminary study for selection of proline accumulating mutants was conducted. Hegari E callus was inoculated onto maintenance media containing 0, 2, 5 and 10 mM hydroxyproline. After first passage, 10 mM hydroxyproline was determined to be severely growth restrictive (Fig. 23). Upon testing the cultivars Keller, Rio and Hegari at 10 mM hydroxyproline, differences in callus growth were observed between cultivars. The number of regenerated plants are summarized in Table 17.

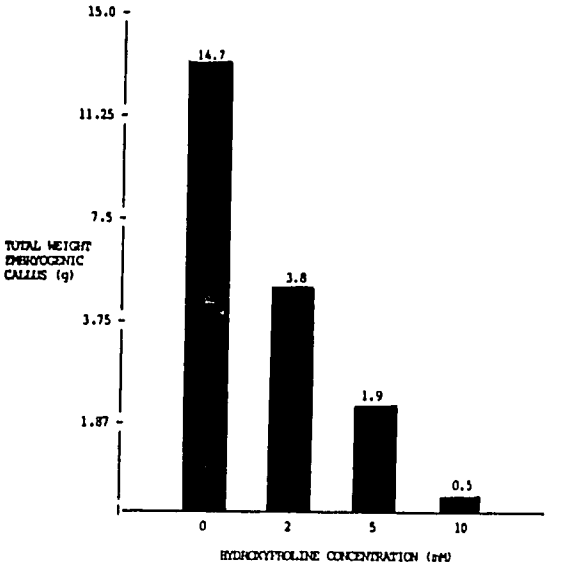


Fig. 23. Sorghum (cv. Hegari) E callus response to differing hydroxyproline levels after a 4-week subculture interval. All treatments were initiated using 3.3 g embryogenic callus.

In Vitro Selection — Chlorate

Potassium chlorate was chosen as a selective agent to obtain efficient nitrate reducers using another indirect *in vitro* selection method. Nitrate reductase (NR) is an enzyme that reduces nitrate to nitrite; however, NR will also reduce other compounds chemically similar to nitrate, *i.e.* chlorate. When chlorate is reduced by NR it is changed to chlorite, a lethal compound to plant cells. Variants having an altered NR that does not reduce chlorate could have enhanced efficiency or concentration of nitrate reductase.

Embryogenic calli of four sorghum cultivars, Keller, Rio, Hegari, and RTx-430, were screened for tolerance to $KClO_3$. Potassium chlorate was added to the maintenance media at a rate of 50 mg/l. Results indicate a significant decrease in callus growth after one month (Table 18). The cultivar Rio shows a similar growth response after exposure to chlorate. Regeneration rates of sweet sorghums (Keller and Rio) show a different response to chlorate stress as compared to the grain sorghums (Hegari and RTx-430). Regenerated plants are now being grown to maturity in the greenhouse. Nitrate reductase efficiency will be evaluated utilizing hydroponic testing.

Whole Plant Evaluation

During the growing season of 1986, three field plots were planted and evaluated. Two plots were on non-stress soil, one in Texas and the other in Georgia. The third plot was also in Georgia on a high aluminum soil (Fig. 24).

The lines planted in non-stress environments were observed for somaclonal variation. The lines represented five cultivars, three NaCl levels, and between three and fifteen passages in culture (Table 19). Little variation was seen within an R_1 line, but some variation on plant height maturity and insect resistance was noted. In Georgia, resistance to fall armyworm was observed. A larger trial for insect resistance is planned for 1987 with nine selected lines.

Some of the lines planted in the non-stress environment in Georgia were also planted on a high aluminum soil (Table 20). Of the 123 lines planted, 17 survived the aluminum soil and the unusually dry conditions, although only one of the 17 actually set seed. Further field evaluations on aluminum in Georgia and salt/drought in Arizona are planned for 1987.

Table 17. Number of plants which regenerated and set seed from 1 passage on 10 mM hydroxyproline

Cultivar	Total # of Passages	Number of Plants
Hegari	21	1
Keller	13	2
	15	1
Rio	15	7

Table 18. Response of sorghum E callus and regeneration to one passage on 50 mg/l $KClO_3$.

Cultivar	Culture Period (mo)	Mean Weight E Callus Inoculated	Recovered	# plants/g	Total # of plants
Hegari	1	3.4	0.33	2	9
	2	8.0	0.17	2	15
	3	11.2	0.19	1	10
RTx-430	1	0.2	0.09	0	0
	2	0.1	0.05	0	0
Keller	1	1.0	0.23	15	74
	2	1.5	0.05	16	58
	3	1.8	0.09	10	44
Rio	1	0.4	0.22	45	11
	2	0.7	0.23	19	57
	3	0.6	0.16	10	17

Table 19. Number of lines planted in non-stress environment in Texas and Georgia divided by in vitro NaCl level.

Cultivar	*in vitro* NaCl level g/l	Range of passages	Number of lines planted Texas	Georgia
Hegari	0	4-12	117	87
	9	11-15 (4-7)*	8	104
	12	9-10 (3-4)	0	12
RTx-430	0	3-6	99	24
RTx-7000	0	4-5	16	5
Keller	0	3-4	3	10
Rio	0	3	14	40
			257	282

*passages on NaCl

Table 20. Lines placed in a high aluminum soil field evaluation in Georgia and the number of lines which survived.

Cultivar	Number of lines planted	*in vitro* stress level g/l NaCl	Number of lines with survivors
Hegari	17	0	2 (12)*
	48	9	8 (17)
	8	12	1 (13)
Keller	3	0	0
Rio	22	0	3 (14)
RTx-430	18	0	3 (17)**
RTx-7000	7	0	0
	123		17

*Percent of lines planted which survived
**One line set seed

Fig. 24. Hegari plants on high aluminum soil.

Millet

Pearl millet grows on all the food-producing continents and occupies an estimated 20 million hectares of the earth's surface. It is the most important cereal crop in the hot, arid sections of the world. It will grow to maturity on sandy or rocky soils too acid, too dry and too infertile for sorghum or maize. It is a summer cereal that is robust and quick-growing with large stems, leaves and heads. It has exceptional grain- and fodder-yielding potential and can produce more forage than either sorghum or maize; however, the best sorghum and maize hybrids out-yield pearl millet grain produced under optimum growing conditions (Burton, 1983).

Pearl millet is used mainly for cereal and is one of the most nutritious of the grain crops (Rachie and Majmudar, 1980). Less than 5% of millet is planted for fodder, and it is used in the U.S., to a limited degree, for forage.

Long-term, High-frequency Regeneration

The TCCP has in the past worked on various pearl millet varieties: Gahi, World Composite-C75, Hays Population (HMP) 559, Senegal Bulk (SB), and inbred line 7203. Preliminary experiments have been conducted for callus initiation for foxtail millet.

More extensive experiments were carried out for proso millet. It was in this cereal that TCCP scientists first noticed and quantified the regenerability difference between E and NE callus. Callus was obtained from seedling roots, immature embryos, and mesocotyls as described by 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 E regions differed for each starting material. Initially, available tissue culture media formulations allowed the production of only very small, almost indistinguishable regions of E callus. An average of 8% of all calli produced E regions during the first three passages. Plants were obtained by placing E callus on a medium containing no added hormones. E callus produced plants at a much higher frequency and for a longer duration than NE callus. Culture conditions increasing the frequency of E 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.

Currently, two cultivars of pearl millet (HMP-559 and SB) are being used to develop methods for long-term, high-frequency plant regeneration from callus stressed on NaCl or $AlCl_3$. Following initiation, a friable, clear, watery callus (Fig. 25) is produced and will form E callus.

Embryogenic callus produced in pearl millet cultures is similar to E callus produced by other cereal crops in culture (Nabors *et al.*, 1983); it is characteristically dense, compact, nodular and creamy white in color (Fig. 26). Another type of callus commonly seen in millet appears yellowish in color and is also compact and nodular (Fig. 27). It is not yet known whether the latter callus has the ability to regenerate.

Early media experiments have determined that LS media supplemented with 5 mg/l 2,4-D, 1 mg/l IAA and 0.3 mg/l KIN plus 4% sucrose resulted in maximal E callus production from seed. Embryogenic callus production during the first subculture is low, and tends to occur inside the callus mass, therefore, the entire mass of callus minus the shoot growth and the original explant source is subcultured for two additional four-week passages after which time callus types can be differentiated. Embryogenic callus can then be separated from NE and is subcultured. This type of selective subculturing is necessary to maintain long-term cultures of E callus.

To obtain plantlets, the E callus is placed on LS media with either 0.2 mg/l IAA and 0.5 mg/l BAP for HMP or 0.5 mg/l BAP for SB to obtain shoots. Callus is subcultured for a minimum of two passages to allow the development of shoots. Following the formation of well-developed shoots they are transferred to LS media containing 3 mg/l IBA to induce root formation. Usually one, four-week passage is sufficient for a well-established root system, although two passages may be required at times.

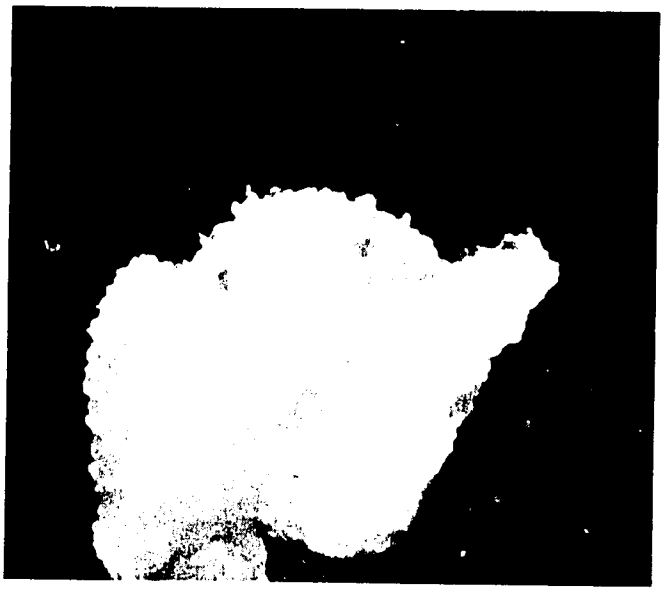


Fig. 25. Friable, watery pearl mill· ·allus showing small patch of E callus (top).

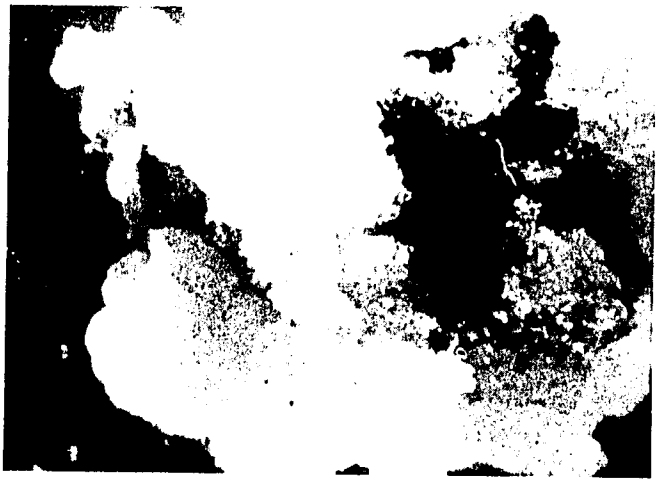


Fig. 27. Yellow, compact nodular callus from Pearl millet.

In Vitro Selection — NaCl

Recent work has been directed toward HMP-559 and SB and their tolerance to stress. Once long-term plant regeneration was obtained and optimal media composition were determined for culture, addition of NaCl and $AlCl_3$ were tested. The number of cultures initially stressed on 6 g/l NaCl and then stressed on 8 g/l NaCl were drastically reduced from 250 cultures in the first passage to 43 in the second passage. A continual decline in the number of cultures was observed until tenth passage when those remaining turned brown and necrotic. Callus stressed on 9 g/l NaCl also showed a decrease in growth over time until it was transferred to regeneration media without NaCl. After the callus was removed from the stress, the growth rate of E callus remained almost constant.

Plants which have regenerated and set seed are summarized in Table 21. Cultures of non-stressed millet can be maintained for 9-15 months. This time is reduced to 8-11 months when the cultures are started on media containing NaCl.

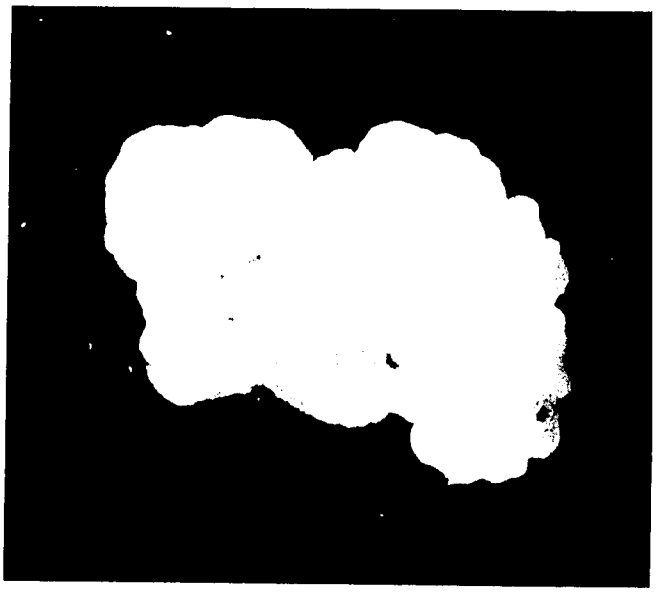


Fig. 26. Pearl millet E callus.

Table 21. Regenerated pearl millet plants which have set seed from NaCl stressed cultures.

Cultivar	Range of total passage	NaCl level g/l	Range of passages on stress	Plants regenerated
HMP-559	5-9	0	—	29
	4-6*	3	—	21
	4-8*	6	—	15
	13-14	8	2-6	4
	3*	9	—	2
SB	9-15	0	—	19
	9-11*	3	—	7
	7*	6	—	3
	12-15	8	3	13

*Cultures started on media containing NaCl

In Vitro Selection — $AlCl_3$

Embryogenic callus is being cultured on LS basal media containing 150 ppm $AlCl_3$. As with NaCl-stressed cultures, E callus is subcultured on fresh media every four weeks following approximately three passages on initiation media. Embryogenic callus is stressed on $AlCl_3$ for a minimum of three passages, then removed from stress and subcultured on regeneration media. This callus must also be subcultured on shoot-inducing as well as rooting media. A slow decline of callus growth occurs over time once the callus has been stressed. To date, there are 10 plantlets of SB from $AlCl_3$-stressed cultures which have set seed.

Whole Plant Evaluation

Field evaluation of pearl millet in 1987 will be on a heavy clay, drought soil in Kansas. The field research will be conducted by Dr. Bill Stegmeier of Kansas State University during the growing season of 1987. New inbred lines have also been obtained from Kansas and will be tested in culture in 1987.

Corn

America is the largest producer of corn in the world, where its major use is for livestock and poultry feed (USDA Economic Research Service, 1984). In many other countries, however, such as those in Latin America, central and southern Africa, and parts of China and southeast Asia, corn is a major part of the human diet. The highest per capita consumption of corn is in Mexico and Central America, where 142 kg per person is consumed each year (Watson, 1977).

In Vitro Culture

The long-term tissue culture and regeneration of corn callus has been a challenge for many investigators. Part of the difficulty with corn culture is the inability of mature explants to yield regenerable callus. Immature embryos will yield E callus, but long-term maintenance and regeneration is not readily achieved (Chang, 1983, Green *et al.*, 1983, Green & Phillips, 1975, and Hodges *et al.*, 1986).

Plant regeneration capability has been demonstrated with several hybrid cultivars and inbred lines of corn. When immature embryos of corn are cultured, the tissue gives rise to two separate types of callus. Plant regeneration is achieved by somatic embryogenesis from what is identified as Type II callus after a period of up to four months. Type II callus is very friable, almost thread-like, with well-defined somatic embryos on suspensor-like structures (Fig. 28). It is formed from immature embryos cultured on LS media plus 1 mg/L 2,4-D, 1 mM L-asparagine, and 2% sucrose. Type II callus forms at low frequency and has not been the primary focus at the TCCP. Type I callus is more abundant and grows at a more rapid rate, but plant regeneration is not successful beyond a two-month culture period (Fig. 29).

As part of a separate, in-house project funded by Pioneer Hi-Bred of Johnston, Iowa, tissue culture parameters of corn callus culture have been investigated. Research has been conducted with whole seeds and immature embryos of hybrid cultivars toward extending regeneration from Type I callus.

Whole seeds, when cultured on LS media containing 2-10 mg/l 2,4-D, produced masses of roots; if callus was produced, it was NE (Fig. 30). Experiments with 20 mg/l 2,4-D resulted in fewer roots and more compact callus, but this compact callus was not E or regenerable except in one experiment in which a regeneration medium containing 0.5 mg/l TIBA produced several plants. It was observed that 10 mg/l 2,4-D plus 20-100 mM proline produced results comparable to those obtained with 20 mg/l 2,4-D, but no regeneration was observed. Since the quality of callus obtained from mature embryos was poor, no further experimentation with whole seeds is planned.

The best responding explant source is immature embryos. To obtain the embryos, seeds are planted in large pots approximately nine inches in diameter. In two to three months, depending on the time of year and the cultivar, ears will appear. The ears are covered so that cross-pollination between lines does not occur when the silks (stigmata) appear. Two to three days after the silks emerge, the tops are cut off to form an even brush of silks. Pollination can begin the next day.

The pollen is gathered by placing the bag over the tassels and shaking them so that pollen falls into the bag. The bag is then shaken over the silk brushes so there is enough for all the silks to be pollinated. This is done for three days in a row, preferably before 10:00 am. The embryos are usually ready ten to fourteen days after pollination.

The size of the embryos is checked by cutting the side of the ear and removing a kernel with a scalpel blade. The embryo is removed by cutting off the top of the kernel and squeezing out the endosperm and embryo. If the embryo is between one and two mm, the ear is ready to be used. The ears are taken off and refrigerated overnight if necessary, but the embryos do best if initiated immediately.

Once the ears have been removed, they are sterilized before the embryos are excised and initiated. The ears are cut into two or more segments, approximately 4 cm each in width. They are soaked 5 to 15 seconds in 95% alcohol and rinsed with sterile distilled water. The segments are then covered with a solution of 30% Chlorox and two drops of Tween 20. They are placed on an automatic stirrer for 30 minutes. In a laminar flow hood, the ear pieces are rinsed four to six times in sterile distilled water.

The embryos are excised by cutting off the tops of the

kernels with a sharp scalpel blade while the kernels are still on the cobs. The endosperm is squeezed out from the kernel with a pair of forceps. Sometimes the embryo will come out with the endosperm, other times it will stay in the kernel. If the ear is young and the embyros are less than 1 mm long, they may be the same transparent color as the endosperm and therefore difficult to find. The excised embryo is placed scutellum up on the media and grown in the dark for four to six weeks until callus develops.

The Project has been successful in extending regeneration from Type I callus to over five months. To obtain regeneration from Type I corn callus, five different types of media are needed: initiation, maintenance, budding, regeneration, and rooting. If the experiment is going to be short-term, the maintenance media may be deleted, but for a long-term experiment (longer than four months) all five media are needed to achieve regenerated plantlets.

All media is adjusted to a pH of 5.8. Vials with 10 ml of media are used for second passage. From third passage on, jars with 20 ml of media are used. The subculture time is two weeks until the fourth month of culture when the subculture time is changed to three weeks. Subculturing at three weeks at this time does not reduce callus growth or regeneration.

Type I callus is translucent, convoluted, and compact callus similar to the E callus found in rice, millet and sorghum. It is produced from immature embryos cultured in small petri dishes (five per dish) on 15 ml of LS media plus 1 mg/L 2,4-D, and 12% sucrose. Maintenance of the callus is on 2 mg/l 2,4-D with 4% sucrose. Calli has been successfully maintained for up to 90 days before moving them to the budding media. This media contains 2 mg/l 2,4-D, 0.3 mg/l KIN and 4% sucrose. Only after buds are formed (Fig. 31) are they moved to regeneration media containing 1 mg/l BAP and 0.3 mg/l IAA and 4% sucrose. The shoots (Fig. 32) are then placed on a "rooting" media of LS plus 3 mg/l IBA and 4% sucrose. Purple prop roots, no matter how many, will not support the plant; the roots must be white. A total of 49 plants have been regenerated by the program with seven plants set selfed seed. Two plants have been regenerated after five months in culture. Many abnormalities were observed in the R_0 plants, such as tassels where ears should be, ears where tassels should be, stunted growth and bushy growth habits. No R_1 plants have been studied to determine the heritability of these traits; many reports of corn regeneration mention abnormal growth of the R_0 plant, but few abnormal traits in the R_1 plants.

Further research on corn will be conducted by Dr. Nguyen T. Thanh-Tuyen at Visayas State College of Agriculture (ViSCA) in the Philippines. That project will deal with white corn varieties grown locally.

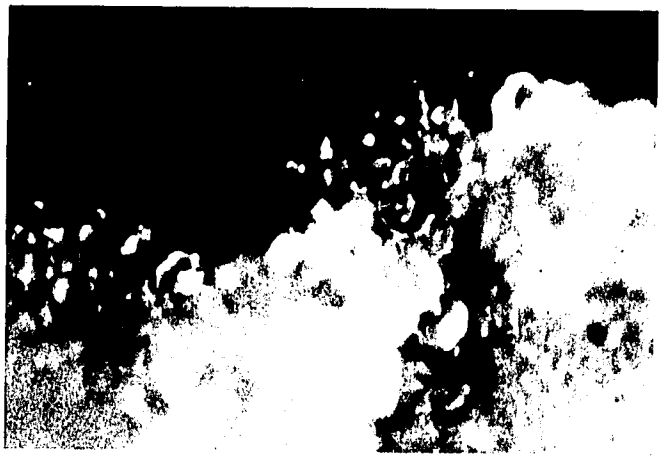


Fig. 28. Type II corn callus from first passage.

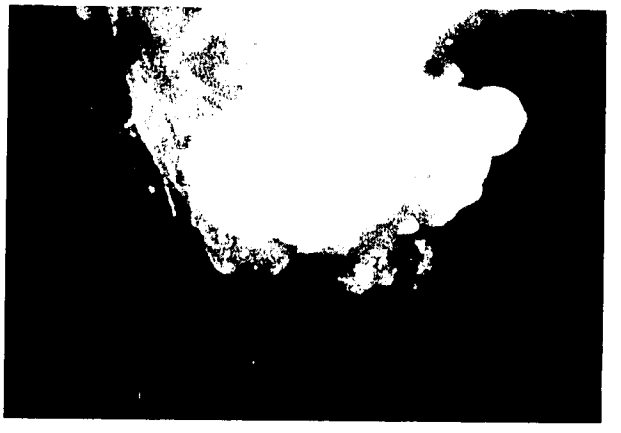


Fig. 29. Type I corn callus.

Fig. 30. NE callus.

Fig. 31. Formed buds from Type I E callus.

Fig. 32. Shoots from buds.

Oats

Oats rank fourth among cereals in world production, behind wheat, rice, and corn. The diversity of varietal types makes it possible to cultivate oats over a wider range of non-tropical climatic conditions than any of the other cereals (Coffman, 1961).

Long-term, High-frequency Regeneration

The TCCP has used oats (*Avena sativa* L. cv. Park) as a model plant for developing grass tissue culture methods. Experiments were supported by funds from the Colorado State University Experiment Station and the Office of Water Resources, Technology, Department of the Interior.

Oat tissue cultures were primarily obtained from mature seeds, although mesocotyls and immature embryos were used in some experiments. In earlier reports (Nabors *et al.*, 1982; Heyser and Nabors, 1982a) it was mentioned that if seeds were used as explants, the callus was derived from the root region. In subsequent investigations with rice and wheat, it was recognized that callus derived from roots is exclusively NE callus. Thus, oat callus which was observed to be regenerable was most likely derived from the scutellum or shoot regions of mature seeds.

Embryogenic callus in oat cultures resembles that produced in other cereal and grass cultures. It is opaque, creamy white, and convoluted in appearance (Fig. 33). Early media experiments with oat callus revealed that the greatest amount of total callus was produced on LS medium containing 1 mg/l 2,4,5-T, when cultures were grown in the light; however, media containing 2,4-D produced significantly more E callus than media containing 2,4,5-T (Heyser and Nabors, 1982a). No extensive investigations were made with cytokinins. Also, no specific regeneration media were tested, although it was found that media containing 1 mg/l 2,4-D or 2,4,5-T resulted in more plants than other concentrations tested. Regenerated plants were easily established in soil, and grown to maturity in the greenhouse.

Two important aspects of cereal tissue culture were determined from oat tissue culture data. The first was the recognition of green spots, often found in grass calli cultured in the light. These green spots were associated with rapidly growing callus and subsequent plantlet regeneration (Fig. 34). In oats, callus without green spots grew very slowly and never produced regenerated plants. In cultures of mixed E and NE callus, green spot production did not diminish for at least one year, but shoot production declined to zero by the ninth passage. As for other cereals, long-term maintenance of E callus is possible only if NE callus is removed from the culture. Green spots are not necessarily E regions since many green spots develop into roots.

The second observation was that E callus produces far more plants than either NE callus, or callus with both E and non-E regions (Fig. 35; Heyser and Nabors, 1982a). In light of the current understanding of the role of E callus in regeneration of whole plants, this second observation seems obvious; however, at the time this research was conducted, this relationship was not fully understood. In fact, prior to 1982, cereal regeneration was widely regarded as a major, unsolved problem of tissue culture. Regeneration, when it occurred, was of low frequency and short duration.

Fig. 33. Embryogenic callus in oat cultures. Somatic embryos forming on first passage.

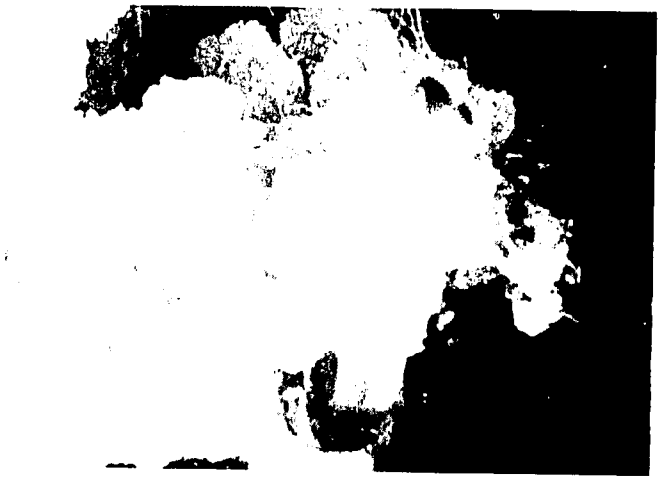


Fig. 34. Green spots and elongating shoots from embryogenic callus.

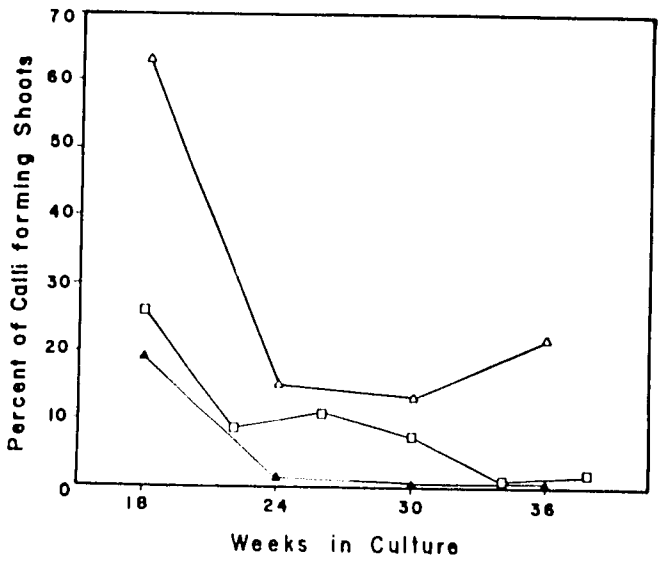


Fig. 35. Shoot formation over time in culture for: A. Embryogenic callus (open triangle), B. Non-embryogenic callus (closed triangle), and C. Callus selected for high green spot production (open square). For embryogenic callus there were 30 to 78 samples per point while non-embryogenic callus is represented by 39 to 759 samples per point.

Fig. 36a. R_3 oats watered with 0, 5, and 10 g/l NaCl solution. Front row: plants from non-tolerant cultures. Back row: plants from cultures tolerant to 3 g/l NaCl. Twenty-three seeds planted per pot.

Fig. 36b. R_4 plants whose parents were regenerated from tissue cultures tolerant (right) and non-tolerant (left) to NaCl. Note that tolerant plants are setting seeds whereas non-tolerant plants set seed after photograph was taken. Tolerant plants are two weeks earlier than non-tolerant plants.

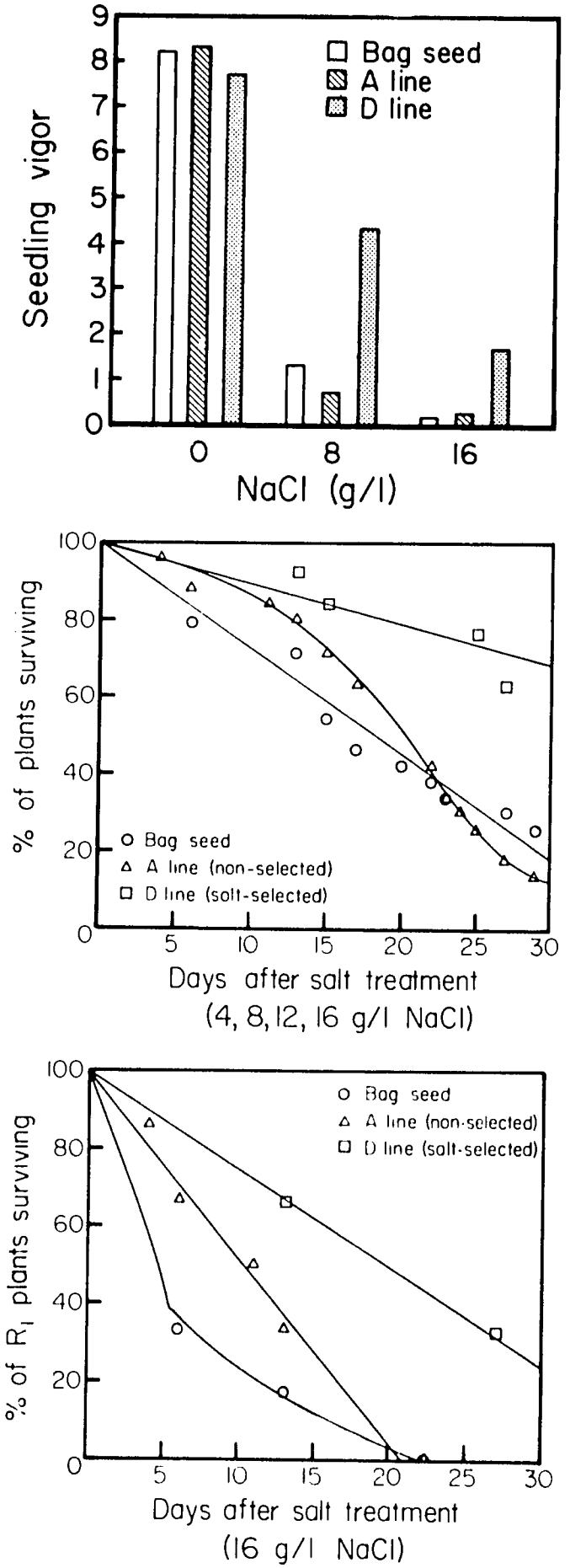


Fig. 37. *Data from hydroponic studies of regenerated oats.*

In Vitro Selection — NaCl

Oat tissue cultures were used as the model cereal system to select *in vitro* for NaCl-resistant variants. In initial experiments, it was observed that as the NaCl concentration increased, the quantity of callus initiated decreased; however, callus formation occurred on media containing up to 10 gm/l NaCl. In several later experiments, additional callus cultures were stressed on media containing 3 and 6 gm/l NaCl for periods of one to five subcultures. Several hundred plants were regenerated from some of the short-term, salt-stressed cultures, and grown to maturity.

Whole Plant Evaluation

Plants from the R_3 generation (Fig. 36a) whose genetic heritage began with salt-tolerant cell lines were considerably more tolerant in pot experiments than those from salt-sensitive lines. In addition, the salt-tolerant R_4 progeny flowered and set seed a full two weeks earlier than plants from salt-sensitive cultures (Fig. 36b).

Seed from regenerated plants (R_1) showed that individual plants from salt-tolerant backgrounds are either more tolerant than, or equally tolerant to control plants. The sample size was too small to confirm that these data indicate segregation.

The oat data represented the second demonstration in the literature, and the first for cereals, that (1) traits selected in cell cultures also appear in regenerated plants (2) are stable in the plants in the absence of stress, and (3) are inherited by subsequent generations. This does not mean that all cell-selected stress tolerance will behave identically. In fact, whole plant testing of such selected traits will continue to be a necessity.

The early flowering and seed-set of the salt-tolerant oats is of interest for several reasons. First of all, this phenotypic trait could be of considerable value since oats are a cold weather crop and are frequently subject to production losses due to early frost. Secondly, the genetic relations between tolerance and earliness needs to be determined; they could be pleiotropic effects. In the latter case, earliness would represent a somaclonal variation not selected for. There is some concern among breeders that selections in cell culture might produce the desired alteration in a cultivar as well as other undesired changes. While this is a clear danger, it can largely be controlled by close scrutiny of the plants produced.

The first experiments, summarized above, to test for salt tolerance in plants regenerated from salt-stressed cultures were carried out in soil watered with solutions containing 0, 5, 10, or 15 g/l NaCl. Greenhouse pot cultures mimic field conditions to the extent that both soil matrix potential and salt concentrations vary with soil moisture.

A hydroponic system, similar to that described for rice, was developed to determine exact levels of salt tolerance. Data from hydroponic studies was used to answer a question frequently posed by plant breeders: Does tissue culture selection simply pick-out stress-tolerant recombinants naturally occurring in field grown seed? Salt tolerance, for example, could arise by mutation or by meiotic recombination. Most cereal cultivars are highly inbred and homozygous for selected agronomic traits, but not necessarily for stress tolerance. Preliminary data from hydroponic experiments (Fig. 37) indicates that, in Park oats at least, salt tolerance does not occur to a high degree in field-grown seed.

Soybean

Soybean is the most important grain legume crop in the world with the total number of hectares close to 80 million (Hymowitz, 1976). The largest producer is the United States. For many developing countries the production of soybean is increasing because of its value as an efficient source of oil and proteins used in the diet. This is particularly true for the peoples of Asia. The mature soybean is used as a liquid, powder or curd, and the immature beans are consumed in large quantities. Soybean plants show little tolerance to salinity and drought (Sammons *et al.*, 1979); however, there is evidence that mutations have occurred as some cultivars have increased salt tolerance (Lauchlii and Lieneke 1978). Tissue culture technology is potentially a valuable approach for the development of soybean germplasm with increased stress tolerance.

Development of procedures for regenerating soybean plants from cultured cells has eluded investigators for many years, despite considerable effort. The TCCP has conducted research into developing efficient, reliable methods for regenerating plants from soybean tissue cultures and more recently from cultures of tropical grain legumes such as pigeonpea and cowpea. Soybean was chosen for the earlier studies because it is the most important of the grain legumes and because most of the work reported in the literature was on this species. Thus, there was a greater likelihood for a breakthrough in the methodology. At the same time, many other laboratories were working on the procedures with different types of soybean. The achievement of a plant regeneration procedure was reported nearly simultaneously by a number of groups (Barwale *et al.*, 1986; Lippmann and Lippmann, 1984; Ranch *et al.*, 1985; Li *et al.*, 1985; Lazzeri *et al.*, 1985).

In Vitro Culture

At the TCCP, the induction of somatic embryogenesis and plant regeneration from immature embryos of soybean, *Glycine max* (L.), cv. Prize, was achieved (Ghazi *et al.*, 1986). Subsequent investigations used the procedure in screening for increased salt tolerance. In this research, two morphological types of E callus, the origin and duration of their differentiation, and subsequent plant regeneration from soybean cultures are described.

Seeds were surface sterilized in 75% ethanol followed by immersion in 8% Chlorox plus two drops of Tween 20. The solution was stirred under vacuum for 15 minutes. The sterilization solution was removed by three to four rinses with sterile distilled water. Germination occurred on a solid basal medium containing LS major and minor salts, vitamins and 2% sucrose. The hypocotyls of germinated seedlings 1, 3 and 5 cm long, were removed from 1 to 5 mm below the cotyledonary attachments and sectioned to lengths of 3 mm.

Whole, immature embryos were obtained from pods 2 to 10 cm long produced on plants grown in the greenhouse. Embryos were 2 to 10 mm long. Cotyledons and hypocotyls were excised. The shoot-tips were discarded and the hypocotyl and cotyledons were retained. The whole embryos, hypocotyls and cotyledons, were sterilized in a manner similar to the mature seeds; however, only ten minutes of stirring was necessary.

Hypocotyls (from seedlings), embryos and cotyledonary segments were planted on a solid basal medium (0.7% agar) containing LS major and minor salts, vitamins, 2% sucrose and various concentrations of growth regulators. The pH was adjusted to 5.8 prior to autoclaving. Cultures were kept under continuous light at 28°C.

Variables investigated included hormone type and concentration, nitrogen source, proline requirement and develop-

Regenerating "difficult" plants

The question of why a successful regeneration procedure for soybean took so long to be developed is interesting since an answer should facilitate the development of tissue culture methods for other "difficult" crops. In part, current tissue culture methods and thinking developed from a tobacco-based technology and mentality. By in large, the production of callus and regenerated plants in tobacco can be accomplished by one medium (Fig. 38). Optimization of the cytokinin to auxin ratio — as first accomplished in Skoog's lab — was seen as the method of choice to accomplish regeneration in dicots. Application of this method to cereal tissue cultures was unsuccessful and delayed for some years the development of high-frequency, long-term regeneration procedures. For both cereals and soybeans, the key to obtaining plant regeneration is the realization that several different types of callus can be produced depending on choice of explant and hormone regimen in the medium. Some callus types are highly regenerable and others are not; furthermore, some callus types readily produce somatic embryos and others do not. Using a variety of hormone levels and explant sources, it is seen that soybean produces a wide variety of callus types — these are readily seen under the dissecting scope — which are rapidly overgrown by a friable, crystalline, and very *non-embryogenic* callus. Most earlier investigators probably failed to achieve reliable regeneration because they did not observe callus closely and microscopically, particularly during the early stages of formation and growth, and because they used a limited selection of hormone types and concentrations.

mental stage of the explant. Various concentrations of IAA or 2,4-D in combination with BAP were tested for in culture initiation media. In addition, 2,4-D and proline combinations, nitrogen source (NH_4NO_3 and NH_4Cl), GA, zeatin, IBA and ABA were tested as components of maintenance and regeneration media (Table 22).

At the end of the four-week initiation passage, callus obtained from different sizes of cotyledonary segments, cultured on media containing 10 mg/l 2,4-D or 10 mg/l 2,4-D in combination with 0.132 to 0.264 mg/l ABA, were transferred to fresh medium of the same composition. In addition, 15 mg calli were transferred to a series of media for studies (Table 22) in which the effect of nitrogen source and proline on callus maintenance was tested.

Table 22. *Hormonal composition of LS media for embryo initiation, maintenance and plant regeneration from various explants of soybean cv. Prize.*

Initiation media (mg/l)			
1.	1 IAA	11.	2 2,4-D
2.	1 IAA+0.5 BAP	12.	2 2,4-D+0.5 BAP
3.	1 IAA+5 BAP	13.	2 2,4-D+5 BAP
4.	1 2,4-D	14.	10 2,4-D+0.132 ABA
5.	1 2,4-D+0.5 BAP	15.	5 IAA
6.	1 2,4-D+5 BAP	16.	5 IAA+0.5 BAP
7.	10 2,4-D	17.	5 IAA+0.5 BAP
8.	2 IAA	18.	5 2,4-D
9.	2 IAA+0.5 BAP	19.	5 2,4-D+0.5 BAP
10.	2 IAA+5 BAP	20.	5 2,4-D+1 BAP
		21.	10 2,4-D+0.264 ABA

Maintenance media (mg/l)				
22.		+0.2 2,4-D	+0	Proline
23.		+0.2 2,4-D	+25 mM	Proline
24.		+0.5 2,4-D	+0	Proline
25.		+0.5 2,4-D	+25 mM	Proline
26.	minus	+0.2 2,4-D	+0	Proline
27.	NH_4NO_3	+0.2 2,4-D	+25 mM	Proline
28.		+0.5 2,4-D	+0	Proline
29.		+0.5 2,4-D	+25 mM	Proline
30.	minus NH_4NO_3	+0.2 2,4-D	+0	Proline
31.	plus 800	+0.2 2,4-D	+25 mM	Proline
32.	NH_4Cl	+0.5 2,4-D	+0	Proline
33.		+0.5 2,4-D	+25 mM	Proline

Regeneration media (mg/l)
34. No growth regulators (control)
35. 0.104 GA
36. 0.103 GA+0.132 ABA
37. 0.103 GA+0.132 ABA+0.102 IBA
38. 0.110 zeatin
39. 0.110 zeatin+0.132 ABA
40. 0.110 zeatin+0.132 ABA+0.102 IBA
41. 0.110 zeatin+0.132 ABA+0.103 GA

Table 23. *Formation of smooth-shiny E callus from developmentally different cotyledonary segments on media containing 10 mg/l 2,4-D.*

Immature embryos lengths (mm)	Cultures with embryos (%)	Embryos per culture (No.)
2	—	—
3	6	0.06
4	36	2.04
5	59	2.00
6	39	2.42
7	38	1.23
8	44	2.00
9	25	1.00
10	13	0.13

The growth regulator selected for maintenance media in this latter series was 2,4-D since previous observations indicated its effectiveness in producing E callus. Ten cultures (10 ml vials, one explant per vial) were scored for each set (21 sets) of conditions. For callus obtained from hypocotyls and embryos, visual observations were made every four weeks and cultures were transferred to fresh media. Callus obtained from cotyledons was checked in two to three weeks and transferred when the embryos had a smooth epidermis and fully developed cotyledons; embryos past this stage did not develop into plants. All experiments were repeated three times.

A total of 33 different initiation and maintenance media have been tested. It was observed that callus from all explant sources produced on media containing 2,4-D was friable and creamy in color. Callus initiated from whole embryos, cotyledons, hypocotyl segments and hypocotyls from germinated seedlings on media containing IAA was greenish and more compact. These four explant sources, on media containing NH_4Cl as a nitrogen source, produced more vigorous growing callus, compared with media containing NH_4NO_3. Proline had no apparent effect on callus growth.

Two types of E callus were observed. One type appeared smooth-shiny (Fig. 39a) and was produced from cotyledonary segments on media containing 10 mg/l 2,4-D in combination with 0, 0.132 or 0.264 mg/l ABA. ABA was not required for embryo formation. The other type of E callus was consistently rough as shown in Fig. 39b, and was produced on cotyledons, hypocotyls, and hypocotyls from germinated seedlings on all initiation media except those containing 10 mg/l 2,4-D. The smooth-shiny, E callus was greenish and translucent while the rough E callus was opaque and creamish in color. The greatest amount of smooth-shiny E callus was produced from cotyledons 6 mm long (Table 23). When 2,4-D was used in combination with 0.264 mg/l ABA, the best development of somatic embryos from smooth-shiny E callus occurred.

Smooth-shiny, E callus was derived from callus obtained from cotyledonary segments. When this callus type was incubated in the dark in regeneration medium for two to three weeks, three different patterns of developing embryos were observed (Fig. 40a-c). A few plantlets (Fig. 41), were also produced in some of the cultures. In the medium supplemented with 0.104 mg/l GA or 0.110 mg/l zeatin, some of the cultures differentiated and produced roots or shoots or complete plantlets. Moreover, shoots or roots alone were obtained from these cultures on media containing ABA plus either GA or zeatin (Table 24). When such shoots were transferred to B5 medium (Gamborg *et al.*, 1968) supplemented with 0.122 mg/l IBA or LS media combined with 5 mg/l IBA, rooting occurred and whole plants were obtained.

The plantlets were transferred to three-inch pots containing vermiculite and placed in a growth chamber. After establishment, plants were transferred to larger pots of soil in the greenhouse. To date, 30 plants have been successfully established in the greenhouse (Fig. 42), some from salt-stressed cultures.

There was an inverse relationship between induction frequency of rough E callus and length of hypocotyl segments obtained from seedlings. Low concentrations of 2,4-D produced less rough E callus and the callus required a longer time to develop into discrete structures. The higher the concentration of 2,4-D, the more smooth-shiny E callus was produced, and the less time was required for its subsequent development (Fig. 43).

Table 24. Percentage of cultures containing shoots, roots, and plantlets initiated from smooth-shiny E callus form cultured cotyledonary segments within two to three weeks. Each treatment involved thirty somatic embryos.

Growth regulators*	Shoots (%)	Roots (%)	Plantlets (%)
Zeatin+ABA+GA	50	10	—
Zeatin+ABA	60	20	10
Zeatin	60	20	20
Zeatin+IBA+ABA	65	—	—
GA+ABA+IBA	70	—	—
GA+ABA	55	20	—
GA	55	25	20
Control	30	—	—

*See Table 22, regeneration media.

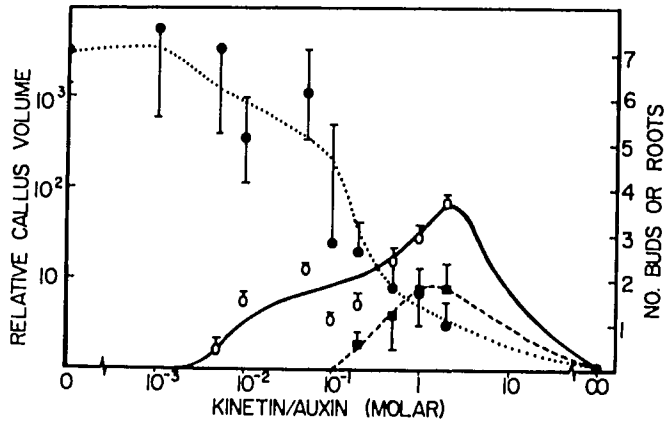


Fig. 38. Production of callus (O), shoots (■), and roots (●) in Samsun Tobacco.

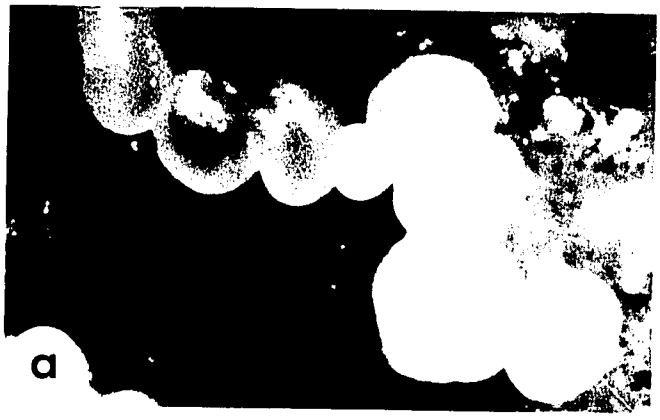
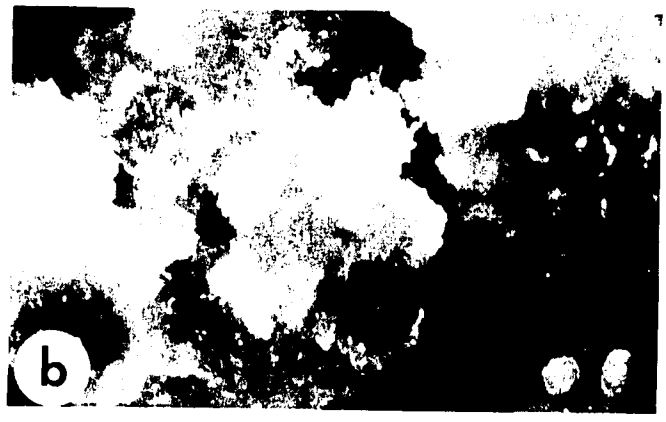


Fig. 39a. Smooth-shiny E callus from cotyledonary segments, formed on media containing 10 mg/l 2,4-D.



39b. Rough E callus appeared in cultures as compacted callus.

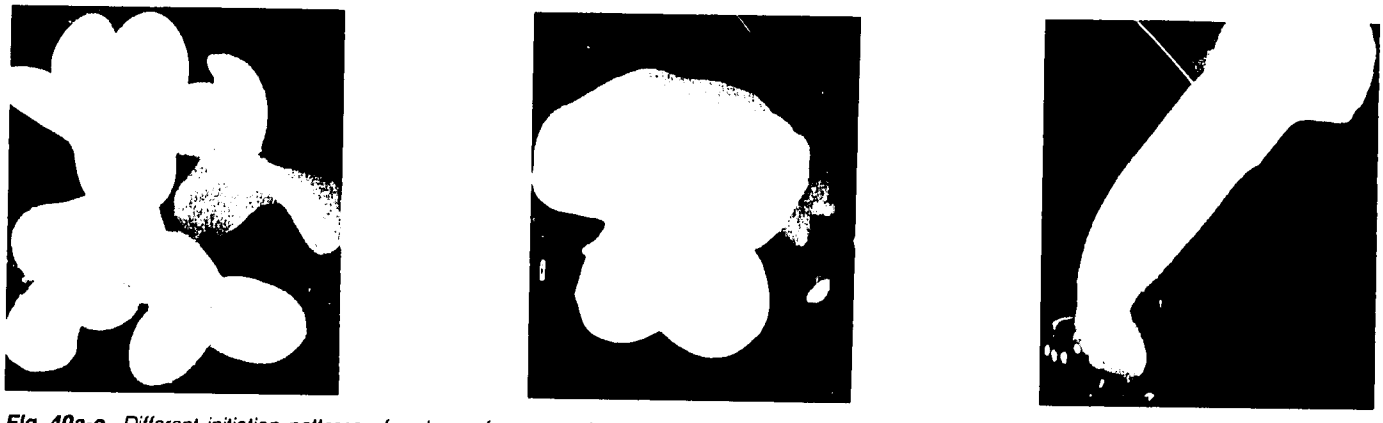


Fig. 40a-c. Different initiation patterns of embryos from smooth-shiny E callus.

The following research on soybeans was a collaborative effort with Dr. Harvinder Cheema of Punjab University in India. When immature leaves of FFR 335 (a line from India), were cultured on 2,4-D media, the E callus remained at the globular stage without further development. After four months on a media with 0.1 mg/l 2,4-D and 1 mg/l KIN, shoots differentiated in 4% of the cultures but further development did not occur. Stem segments, including nodes, were cultured on media with 4 or 5 mg/l 2,4-D. Callus production was observed on both ends and small, proembryonic structures appeared.

Fig. 41. Shoot differentiated simultaneously with root formation.

Fig. 42. Regenerated plants.

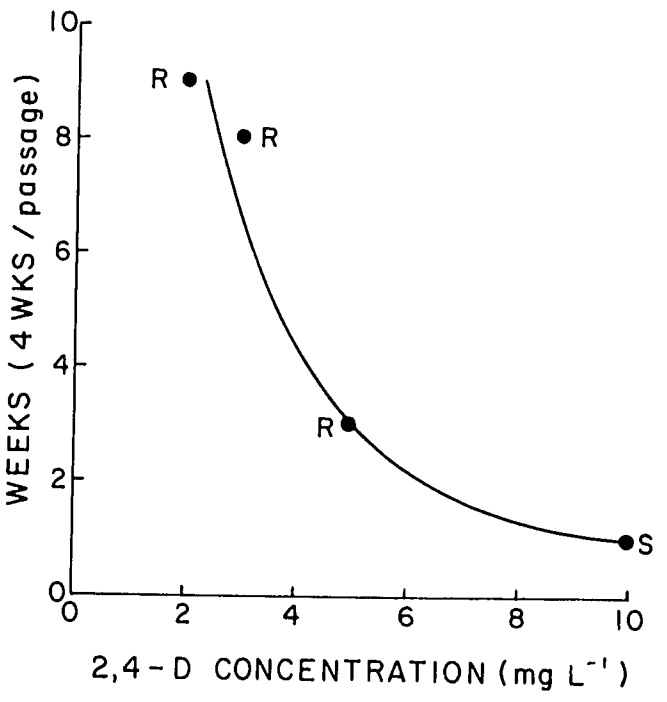


Fig. 43. Relationship between time interval for E callus formation and 2,4-D concentration. Each coordinate represents at least 30 cultures. "S" represents smooth-shiny and "R" represents rough.

In Vitro Selection – NaCl

Seedlings were obtained from seeds of Prize cultivar. Immature embryos were obtained from pods of plants growing in the greenhouse. Pod segments, 1 to 2 cm long, each containing a single immature embryo, were surface sterilized in 75% ethanol followed by immersion in 5% Chlorox plus two drops of Tween 20. The solution was stirred under vacuum for 10 minutes. The sterilant was removed by three to four rinses with sterile deionized water. Embryos 4 to 10 mm long were removed from each pod segment. Cotyledons were excised as segments of the embryo (Ghazi *et al.*, 1986) and planted on a semi-solid basal medium (0.7% agar) containing LS major and minor salts, vitamins, 2% sucrose, and 10 mg/l 2,4-D, with NaCl at 1, 3, 5, 7 or 9 g/l.

Within two to three weeks, embryo-like structures were derived from callus obtained from cotyledonary segments plated on an initiation medium containing 10 mg/l 2,4-D with 1, 3 or 5 g/l NaCl. These structures were counted and transferred to a regeneration medium consisting of 0.103 mg/l GA + 0.132 mg/l ABA + 0.102 mg/l IBA and the same amounts of NaCl as the initiation medium. After two to three weeks incubation in the dark, shoots were produced. These shoots were subcultured on LS medium with half-strength nutrient salts plus 3 or 5 g/l NaCl together with 1.22 mg/l BAP + 1.00 mg/l GA for two weeks. The shoots were then transferred to this medium without the GA for elongation and root formation.

Rooted plantlets were transferred to pots containing vermiculite, soil and sand and they were established in a growth chamber. After establishment, the plants were transferred to larger pots of soil in the greenhouse. NaCl concentrations remained constant from initiation to plant regeneration.

A "decay curve" type response to increasing NaCl concentration was obtained (Fig. 44). Since the F test was highly significant for the regression, the negative influence of salt on production of embryo-like structures is suggested. The curve also shows that when Prize cultivar is used in future studies, NaCl concentrations greater than 3 g/l are unnecessary. An optimum explant size of 6 mm resulted in maximum production of embryo-like structures; however, as salt concentration increased, influence of explant size on the number of embryo-like structures disappeared (Fig. 45). Thus a certain amount of salt tolerance is accomplished by choosing embryos (explants) at an intermediate stage of development.

Callus weight increased exponentially as explant size increased while increasing NaCl levels resulted in an exponential decrease in callus weight.

Twenty-five mature plants originated from 30 cultures, each culture contained 1 g/l NaCl. The five plants obtained from 30 cultures containing NaCl at 3 g/l, did not survive. At 5 g/l, a few embryo-like structures were produced but plants did not develop. Seven and 9 g/l of NaCl failed to yield embryo-like structures.

Following these experiments, the TCCP discontinued research on soybean. The major focus is now on pigeonpea and cowpea. Regeneration procedures are being established and applied in stress tolerance screening research.

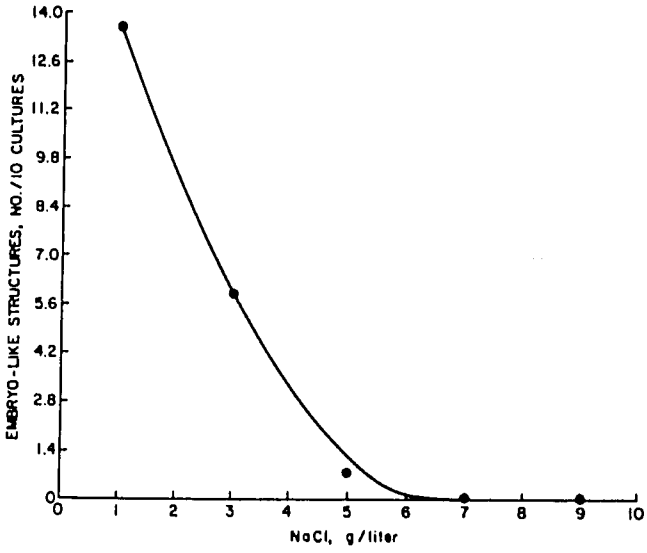


Fig. 44. Influence of NaCl concentration on production of embryo-like structures, based on 2100 cultures. The F value obtained for regression, 191, was highly significant with 2 and 4 degrees of freedom.

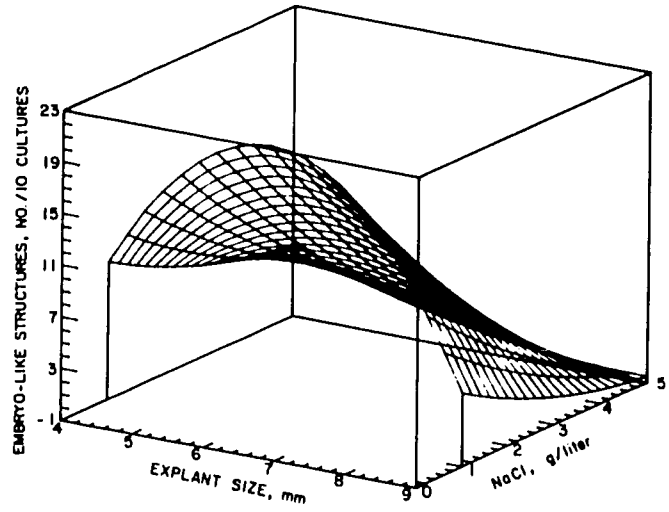


Fig. 45. Influence of NaCl concentration and explant size on production of embryo-like structures, based on 1620 cultures.

Pigeonpea

Pigeonpea is the principal, high-protein grain legume of semi-arid tropics and is grown chiefly in India. This seed legume is also very important is Burma, Uganda and Kenya, the West Indies, Puerto Rico and the Dominican Republic. Interest in this crop is growing in many other countries because of its multiple uses as a source of food, feed, fuel and fertilizer. Although pigeonpea has a wider adaptability to different climates, high temperatures and drought than many other grain legumes, it is sensitive to saline conditions (Joshi and Nimbalker, 1983). Increases in production are expected to come from improved productivity in existing areas and from extending its adaptation into arid and semi-arid zones. Extension of pigeonpea cultivation to salt-affected soils holds great promise. During the last six months, greenhouse and tissue culture experiments were conducted at the TCCP to determine the salt tolerance level of different pigeonpea cultivars. Experiments included: (1) Screening of promising genotypes for their relative tolerance to salt mixtures; (2) producing inter-varietal and intergeneric hybrids by sexual hybridization; (3) defining conditions for efficient plant regeneration by embryogenesis; (4) defining conditions for plant regeneration after long-term culture; (5) screening and selection of cell lines with increased tolerance to salinity; and (6) greenhouse screening for salt tolerance of regenerated plants.

Greenhouse Screening of Germplasm

Before the tissue culture screening was initiated, the seeds of the five promising pigeonpea cultivars representing India, Sri Lanka, West Indies, Uganda and Puerto Rico were screened for salt tolerance. Experiments were conducted during germination and early seedling growth to determine the relative tolerance of the genotypes to NaCl, Na_2SO_4 and mixed salts containing NaCl, and $MgCl_2$ and $CaCl_2$.

The method of Ayers and Hayward (1948) was used to test the effects of salinity on seed germination. It includes moistening the soil with known amount of salts, intermittent mixing of the soil over a two-week period, planting the seeds in saline soils, and recording the germination over a specified period.

Treatment solutions of NaCl, Na_2SO_4 and saline mixture consisting of NaCl, $CaCl_2$ and $MgCl_2$ in the proportions of 1.0M, 0.3M and 0.2M, respectively (concentrations typical to those of saline delta soils), with EC values 2, 4, 6, 8, 10 and 15 mmhos/cm^2, respectively, were prepared from stock solutions by diluting with water. Greenhouse soil was weighed in each pot, then brought approximately to field capacity by the addition of treatment solutions. One lot of the soil was moistened with de-mineralized water as a control. Random samples of soils were taken from each pot to determine moisture content, electrical conductivity, pH, and concentrations of Na, Ca, Mg and K.

Fifty seeds from each of five pigeonpea genotypes, ICP-7128, ICP-6917, ICP-7182, ICP-28 and ICP-7035, were planted in the pots, at a depth of 1 cm, in control and in pre-salinized organic soil. The experimental design included five genotypes, 18 salt levels and two replications. Germination counts were made daily over a period of fifteen days. The data on seedling height was collected on the 30th day after germination.

In the seedling experiment, ten seeds from each of five pigeonpea genotypes were planted in pots containing organic soil mixture. The plants were grown in the greenhouse for 30 days with a single watering. After 30 days, the plants were watered with increasing concentrations of NaCl, Na_2SO_4 and saline mixture with EC values 2, 4, 6, 10 and 15 mmhos/cm^2. Each pot received three liters of the specified salt and the control pot was irrigated with equal amounts of de-mineralized water. Data on plant height and percent of survival were recorded on the 30th day after treatment. The EC values and chemical composition of saline soil are presented in Table 25. Percent of seedling emergence after 6, 8 and 12 days from planting, seedling height in control, and salinized treatments are presented in Table 26. Maximum seed germination was observed in the control within six days of planting, whereas germination in saline soils was delayed up to twelve days. The percent of germination on the 12th day ranged from 98% (in controls) to 16% at 12 mmhos/cm^2. In general, increasing levels of salinity not only delayed germination but also affected the final germination count. The saline mixture treatment had the most adverse effect on seed germination and early seedling growth.

Analysis of variance for seedling germination revealed significant differences for salinity, cultivar, and interactions between salinity and cultivar. All five genotypes were equally sensitive to high saline levels; however, cultivars ICP-7035 and 7182 exhibited more sensitivity, even at low and medium salinity levels, with significant reduction in germination above 4 mmhos/cm^2, whereas the germination rate of ICP-26 and ICP-7128 were not significantly affected until 8 mmhos/cm^2. Cultivar ICP-6917 was the most tolerant among the five and germinated well even at 12 mmhos/cm^2.

Plant height was also adversely affected at higher concentrations of three different salts. Again, mixed salts followed by NaCl was found to be more deleterious. In general, reduction in plant height increased with increasing salt concentrations; however, marginal increase in plant height was observed at low concentrations of salt (Figs. 46a-c). Early seedling development was found to be much more sensitive to salt than was seed germination. Some of the seedlings died within two weeks after germination.

In the second experiment, where the 30-day-old plants of pigeonpea were subjected to increasing concentrations of salinity of NaCl and Na_2SO_4, the symptoms of salinity were visible 15 days after the treatment. At 2 mmhos/cm^2 of salinity, plant vigor was significantly increased and increasing salinity up to 6 mmhos/cm^2 did not significantly decrease plant height; however, reduction in plant height above 6 mmhos/cm^2 increased with the increasing concentration of salt. The salinity effects above 15 mmhos/cm^2 were so acute that most of the plants failed to survive.

Table 25. *Chemical composition of salinized soil. The saline soils were prepared by adding different concentrations of treatment solutions consisting of NaCl $CaCl_2$, Na_2SO_4 and saline mixture consisting of NaCl, $CaCl_2$ and $MgCl_2$ in the proportions of 1M; 0.3M; and 0.2M, respectively.*

Treatment EC (mmhos cm^2)	pH	Na	Ca	K	Mg	Cl
		meg/l*				
NaCl						
control	7.0	8.3	59.9	3.0	25.0	7.10
2.0	6.9	30.30	61.60	3.7	25.70	16.20
4.0	6.7	52.30	71.20	6.7	26.10	23.32
6.0	6.7	60.28	76.94	7.4	26.83	29.59
8.0	6.7	79.16	84.56	9.2	27.12	37.09
10.0	6.6	94.07	91.74	10.2	27.46	44.59
15.0	6.6	314.16	107.36	11.8	28.80	57.63
Na_2SO_4						
2.0	7.4	84.85	62.70	3.90	25.70	18.10
4.0	6.8	161.10	79.76	4.76	25.92	11.89
6.0	6.7	233.76	90.40	6.20	26.24	16.64
8.0	6.4	316.12	98.70	7.48	26.86	22.90
10.0	6.2	406.73	116.30	7.95	27.24	39.52
15.0	6.1	425.89	134.20	9.50	28.80	42.75
Saline mixture (with NaCl, $CaCl_2$, and $MgCl_2$ in proportions 1.0M; 0.3M; 0.2M)						
2.0	6.7	18.7	5.1		25.10	25.26
4.0	6.7	29.1	5.4		25.72	43.10
6.0	6.7	39.6	5.8		26.28	59.95
8.0	6.8	50.6	6.6		27.52	80.30
10.0	6.6	65.4	8.5		28.86	101.65

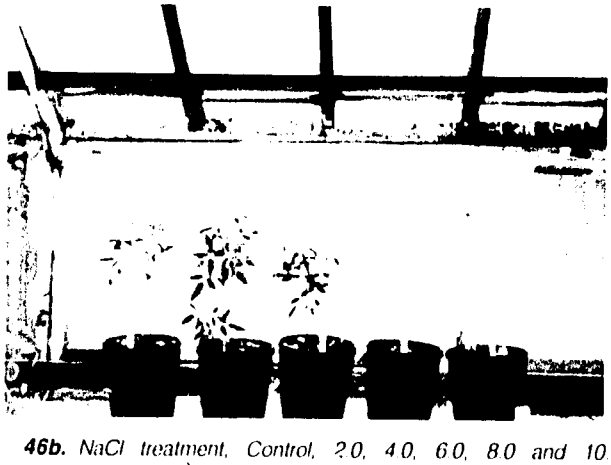
*meg/l + milli-equivalence l^{-1} of saturated soil extract

Table 26. *Influence of salinity on seed germination and plant height. Seeds of ICP 6917 were germinated in salinized soils and data were collected on 30th day after germination.*

Treatment EC (mmhos/cm²)	Percent Emergence 4th day	8th day	12th day	Seedling Height
NaCl				
control	68.0	98.0	98	37.0 + 3.74
2.0	72.0	92.0	96	39.2 + 0.62
4.0	22.0	79.4	90	36.7 + 1.19
6.0	6.0	68.0	82	26.3 + 2.38
8.0	2.4	42.0	84	21.5 + 5.12
10.0	—	38.0	41	17.3 + 4.27
15.0	—	9.0	24	8.7 + 0.76
Na_2SO_4				
2.0	78.0	96.0	99	34.62 + 2.84
4.0	64.0	94.0	95.5	39.46 + 3.56
6.0	14.0	74.0	92.7	32.47 + 1.24
8.0	8.6	58.0	81.0	24.67 + 5.14
10.0	3.2	42.5	38.5	29.30 + 3.92
12.0	—	14.0	27.0	11.74 + 6.10
Mixed Salts				
2.0	70.0	94.0	95.0	34.20 + 2.60
4.0	30.0	72.0	84.5	35.10 + 13.4
6.0	34.0	52.0	70.4	31.00 + 3.20
8.0	22.0	40.6	62.1	24.50 + 8.40
10.0	10.5	24.0	29.0	11.20 + 2.20
12.0	—	4.7	6.5	—

Fig. 46. *Effect of NaCl, Na_2SO_4 and mixed salts respectively, on growth of pigeonpea. Photo taken on 30th day after treatment.*

46a. *Na_2SO_4 treatment, Control, 2.0, 4.0, 6.0 and 8.0 mmhos/cm² of Na_2SO_4.*



46b. *NaCl treatment, Control, 2.0, 4.0, 6.0, 8.0 and 10.0 mmhos/cm² of NaCl.*

46c. *Mixed salts, Control, 2.0, 4.0, 6.0 and 8.0 mmhos/cm² of mixed salts.*

Widecrosses of Pigeonpea and Atylosia

As some of the wild relatives of pigeonpea, *e.g. A. albicans* and *A. scarabaeiodes*, grow well in stress environments, and differential response was observed among different lines of pigeonpea, a crossing program was initiated in the TCCP greenhouse to generate inter-varietal, inter-specific and inter-generic hybrid material with different degrees of tolerance to salt and drought. The F_1 hybrids will be used by ICRISAT scientists in backcross experiments to transfer useful traits to pigeonpea.

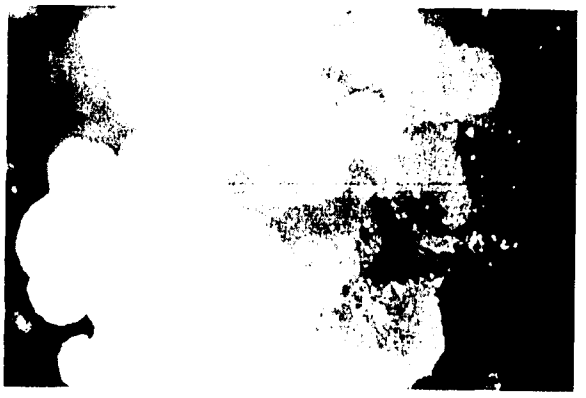
Five lines of pigeonpea *viz.*, ICP-7128, ICP-6917, ICP-7182, ICP-7035, ICP-7186 and six species of *Atylosia (A. lineata, A. platycarpa, A. serecia, A. cajanifolia, A. albicans, A. scarabaeiodes* and *A. grandiflora)* were sown in pots with two to five seeds/genotype (Table 27). Some of the Atylosia species were clonally propagated to increase the number of plants in the greenhouse. For clonal propagation, the surface sterilized seeds were germinated axenically on MS basal medium. The shoot tips and epicotyls from seven-day-old seedlings were excised and implanted on MMS media with 4.5 to 4.6 mg/l BAP. The cultures were grown under continuous, cool, white fluorescent light at 25 ± 2°C.

Some of the important attributes of seven Atylosia species and multiple shoot formation are presented in Table 27. All the shoots from different species were planted on MMS media with 0.5 mg/l NAA for rooting. All the plantlets will be transferred to the greenhouse and grown to maturity.

A crossing program has been initiated among three lines of pigeonpea and *A. platycarpa*. The flower buds of appropriate size on the female parent were emasculated between 8:00 a.m. and 9:00 a.m. and their stigmas immediately dusted with pollen from the male parent.

Gibberellic acid and KIN were used independently at concentrations ranging from 3.5 to 27.7 mg/l for GA and 2.1 to 17.2 mg/l for KIN and in combinations thereof to prolong the retention of hybrid pods. A number 22 hyperdermic needle was used to fill the bud cavity surrounding the pistil with hormone solution. The treatments were given twice at 24 hours and 48 hours after pollination. Only three varieties of pigeonpea and *A. platycarpa* have started flowering so far, ICP-7128, ICP-26 and ICP-6917. The rate of success in inter-varietal crosses varied from 65 to 85% with an average of 74%. When *A. platycarpa* was used as male parent, most of pollinated buds dropped within two days. In the reciprocal cross, the pollinated buds were retained on the female parent for four days. A treatment of GA at 17.3 mg/l prolonged bud retention for seven days. The delay in the drop of pollinated buds helps in successful rescue of embryos in *in vitro* culture. So far, eight buds with four to five ovules were placed on Blaydes' medium with 0.5 mg/l KIN + 0.01 mg/l GA and 100 ppm CH for embryo rescue. The experiments are still in progress.

*Fig. 47. Plant regeneration in pigeonpea (*Cajanus cajan *L.).*



***47a.** 110 day-old cotyledonary callus of ICP 6917 on MMS medium with 1.1 mg/l NAA.*

***47b.** Shoot regeneration from 110 day-old cotyledonary callus.*

***47c.** Acclimatization of regenerated plantlets in greenhouse.*

***Table 27.** Species of Atylosia to be used in crossing program and multiple shoot induction from shoot tips cultured on Blaydes' medium with 2.25 mg/l of BAP.*

Species	Habit	Agronomic traits	No. shoots/ shoot tip
A. lineata	Erect shrub	SMR, High SPC, BS, HPI	5.6
A. sericea	Erect shrub	BR, SMR, High SPC, HPI, HSI	4.2
A. scarabaeiodes	Herbaceous creeper	Variable degree of resistance to SM, blight, source of sterile cytoplasm, DT	12.0
A. volubilis	Perennial climber	SMR,high trypsin & chemotrypsin inhibitors, HPI, HSI	8.0
A. platycarpa	Herbaceous creeper	BR, High SPC, high methionine & tryptophan	10.6
A. albicans	Perennial climber	SMR, high SPC, highly DT, HPI, PBR	4.0
A. cajanifolia	Erect shrub	SMS, BS, PBR	11.0
BDN-1	Erect shrub	SMS, BS, LPC	8.6

For No. of shoots/shoot tip data represents an average of 15 cultures.

SMR = Sterility Mosaic Resistance
BR = Blight Resistance
HPI = High Pod Index
SPC = Seed Protein Content
DT = Drought Tolerant
SMS = Sterility Mosaic Susceptible
BS = Blight Susceptible
LPC = Low Protein Content
HSI = High Seed Index
PBR = Pod Borer Resistant

***Table 28.** The proportion of E callus to total callus induced on MMS medium with 1.1 mg/l BAP + 200 mg/l CH and 2% sucrose.*

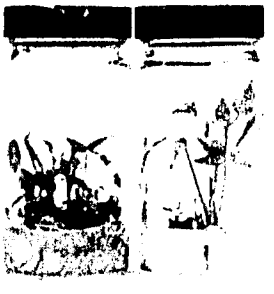
Media type	Total weight of callus (mg)	Weight of E callus
MMS-1*	436.00	22.40
MMS-1 + 1% Mannitol	421.06	124.36
MMS-1 + 2% Mannitol	439.50	92.40
MMS-1 + 1% Sorbitol	468.00	172.20
MMS-1 + 2% Sorbitol	412.00	84.00

Data represents an average of ten cultures.
*MMS-1: modified MS medium with 1.1 mg/l BAP + 200 mg/l CH and 2% sucrose.

Fig. 48. Plant regeneration by somatic embryogenesis in pigeonpea after salt selection.

48a. E callus on maintenance medium with 10 g/l NaCl.

48b. Somatic embryo.



48c. Regenerated plantlet.

48d. Mature regenerated plant of pigeonpea.

Plant Regeneration and Long-Term Cultures

In an earlier study, a reliable regeneration system was developed from primary explant and first passage callus of seedling leaves and excised cotyledons of pigeonpea (Kumar *et al.*, 1983); however, no efforts were made to induce and identify E callus in pigeonpea and to regenerate plants from long-term cultures. A systematic approach has been undertaken to produce E callus and to achieve high-frequency of plantlet regeneration from long-term cultures of pigeonpea.

The mature and immature embryos and excised cotyledons of three genotypes of pigeonpea (ICP-7182, ICP-6917 and ICP-7128), obtained from the germplasm collections of ICRISAT, were used for tissue culture studies. For mature excised cotyledon, the seeds were surface sterilized by exposing to chlorine gas for two hours, washed thoroughly and soaked for three hours in sterile distilled water. The mature embryos and excised cotyledons were separated from seeds and placed on MMS medium containing various concentrations and combinations of auxins (2,4-D, NAA, Dicamba, Picloram and ABA and IAA) and cytokinins (BAP, Zeatin and KIN) with or without ABA.

The cotyledons from mature embryos were excised from sterilized immature pods and placed on MMS media supplemented with growth regulators. All the cultures were incubated under cool white fluorescent light at 25 ± 2°C. Ten cultures from each combination were cultured in the dark at the sarne temperature.

When cultured on MMS medium containing auxin, callus was initiated from both embryo and cotyledonary segments within a week of inoculation. At the end of three weeks, the E and NE callus were separated. Brown, loose callus was produced from the explants cultured on MMS media with 0.1 to 4.0 mg/l 2,4-D, Picloram, Dicamba or NAA. This callus turned dark brown and became necrotic within one sub-culture on the media tested. A whitish green, nodular callus was observed on MMS media either with 1.8 mg/l 2,4-D or 1.1 mg/l NAA. Three-week-old callus was sub-cultured onto maintenance media (MMS with 1.1 mg/l NAA or 1.8 mg/l 2,4-D), which in turn produced organogenic callus. This organogenic capacity of the callus was retained until third passage. The whitish green, nodular callus turned brown after three subcultures and plant regeneration did not occur. To prevent browning, media was incorporated with polyvinylpyrrolidone, charcoal, or ascorbic acid at 0.1 to 1.0 g/l. The callus was also subjected to cold treatment at 4-6°C for several hours. The above treatments failed to inhibit browning in the callus; however, increasing sucrose concentration and organic nitrogen sources of either amino acids or CH was observed to prevent browning of callus until fourth passage.

Plantlet regeneration by organogenesis is a two-step process involving regeneration of shoots, followed by rooting. To induce shoot regeneration, the primary and sub-cultured calli were first transferred to MMS basal medium for four to five days before being placed on regeneration medium.

Shoot bud regeneration was observed from primary and subcultured callus up to fourth passage initiated and maintained on MMS medium with 1.1 mg/l NAA or 1.8 mg/l 2,4-D, and transferred onto the same medium supplemented with 5.6 mg/l BAP, 0.2 mg/l NAA and 0.03 mg/l GA. The frequency of regeneration from embryo and cotyledonary calli varied from 38 to 42% with four to nine shoots per 0.1 gm of callus. Further growth of the shoot buds occurred on MMS medium with 0.2 mg/l NAA and 0.02 mg/l KIN. The regenerated shoots were rooted on Blaydes medium containing 1.1 mg/l NAA and 0.1 mg/l KIN within three weeks. The regenerated plantlets were established in pots containing vermiculite and soil mixture. Different stages of plant regeneration from fourth passage callus is depicted in Figs. 47a-d.

All the auxins used in the experiment at concentrations of 1.0 to 25.0 μm, either alone or in combination with cytokinins, failed to induce somatic embryos either from explants or from callus; however, two to three pro-embryos developed on the surface of shiny green callus induced on MMS medium with 1.1 mg/l BAP + 200 mg/l CH (Fig. 48a). In four weeks of culture, the somatic embryos were further differentiated with distinct radicles, hopocotyls and cotyledons (Fig. 48b). These embryos developed into plantlets on hormone-free MMS medium (Fig. 48c) and the regenerated plantlets were established in the greenhouse (Fig. 48d).

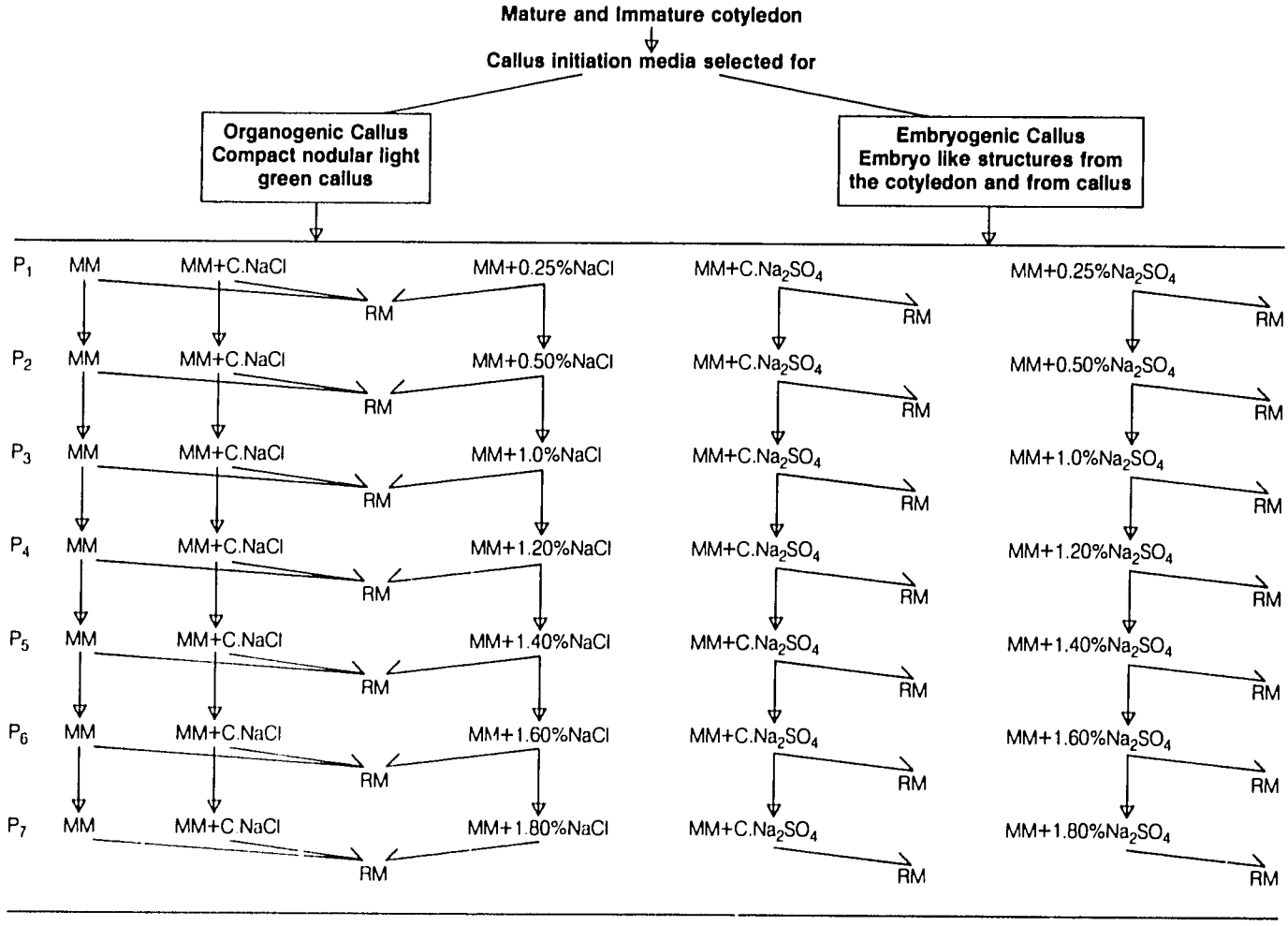
To increase the number of embryos per unit mass of callus, experiments were conducted with different sources of nitrogen, increasing sugar concentration, using mannitol and sorbitol and reducing the sulfate concentration. The MMS media with reduced $MgSO_4$ (70.7 mg/l) + 2.2 mg/l BAP + 200 mg/l CH, 1 and 2% of mannitol or sorbitol was found to be optimum to induce pro-embryos from excised cotyledon and first and second passage callus (Table 28). Further growth of these pro-embryos into fully differentiated embryos was observed on MMS medium with 1.1 mg/l BAP + 200 mg/l CH. The proportion of E callus to total callus is given in Table 28. The proportion of E callus was highest in MMS with 1.0% sorbitol, and lowest without mannitol or sorbitol. Experiments are in progress to enhance E callus production and to retain the E potential in long-term cultures.

In Vitro Selection – NaCl

Since we are able to regenerate plantlets from third passage callus, *in vitro* screening and selection for salt-tolerant cell lines has been initiated. Cultures are grown on media supplemented with NaCl, Na_2SO_4 and mixed salts. The schematic representation of the strategy used for screening is attached (Fig. 49).

First passage callus was placed on saline MMS maintenance medium with 2.5 to 30 g/l of NaCl, 2.5 to 30 g/l Na_2SO_4 and mixed salts (17 to 68 mM Cl salts). In the initial experiments the pigeonpea callus was found to be highly sensitive to salt, callus became brown on the MMS saline medium with more than 10 g/l of NaCl or Na_2SO_4. At salt concentrations of 2.5 to 5 g/l, however, the callus remained green and nodular, hence, this callus was subcultured to MMS media with 25 to 5 g/l of salts and by increasing the concentrations of salt at 0.50 to 1.0 g/l for every passage. At the end of every passage the salt-stressed callus was placed on regeneration media. Attempts are being made to retain the regenerating potential of callus until seventh or eighth passage on increasing concentrations of salts.

Figure 49. *Schematic representation of the salt selection strategy to be followed in Pigeonpea tissue culture*



C. = Same concentration of salt in sequential cultures ranging from 1.0 gm to 10.0 gm/l.
MM = Maintenance medium
RM = Regeneration medium

Molecular Biology

Molecular Basis of Salt Tolerance

Research is in progress using a cell suspension culture of the salt marsh grass *Distichlis spicata* as a model system in which to study the molecular basis of salt tolerance and to obtain useful genes for transfer to crop plants. *Distichlis spicata* cell suspension cultures can grow in the absence of NaCl or in NaCl concentrations as high as 40 g/l. The research aims to detect genes which show an increase in expression in response to salt treatment. Such genes would be candidates for the genes required to confer salt tolerance at the cellular level.

The strategy for identifying salt stress-induced proteins is the following. *Distichlis spicata* cells growing in medium without NaCl are subcultured into medium containing 40 g/l NaCl. After seven days the cells are harvested and polyadenylated mRNA is isolated. The mRNA fractions from salt-stressed and non-stressed cells are translated *in vitro* using the rabbit reticulocyte lysate system in the presence of ^{35}S-methionine. The *in vitro* labeled polypeptides are visualized by direct autoradiography and electrophoretically resolved on SDS-polyacrylamide gels. Preliminary results indicate the appearance of an mRNA which encodes a specific polypeptide. The identity and role of this polypeptide remain to be established but it could be important in allowing *Distichlis spicata* cells to tolerate high NaCl concentrations. Future research will focus on further characterization of proteins and their respective genes.

Proline Mechanisms and Accumulation

The mechanism and physiology of adaptation of halophytic plants to an environment with a high salt concentration has been studied extensively at the level of the whole organism (Flowers *et al.*, 1977). Recent data have shown that in some plants and cell lines there is a cellular basis for tolerance to high levels of NaCl, apart from the role of specialized functions or structures such as salt glands or ion exclusion at the root (Stewart and Lee, 1974). Proline, glycinebetaine, and other organic solutes have been implicated as possible osmoregulants in some halophytes (Stewart and Lee, 1974), as well as in adapted glycophyte cell lines (Handa *et al.*, 1986).

At the TCCP, the accumulation of proline was studied in protoplasts and vacuoles isolated from *Distichlis spicata* cells grown in liquid media with 200 mM NaCl. Tests using the t distribution provide evidence in support of the hypothesis that isolated vacuoles contain no appreciable amount of the proline in the cell (Ketchum *et al.*, submitted).

One of the unexpected and significant observations during the experiment was the increase of free proline in the medium which caused an apparent discrepancy between cell proline levels and protoplast proline levels. Proline levels in the intact cell did not change markedly in the four-hour incubation period of protoplast isolation when cells were subjected to a change in the osmotic environment. The data have shown that, during isolation, protoplasts leak large amounts of free proline. The reasons for this leakage are unknown, although the cause may be related to the net movement of cellular water and solutes into the hypotonic isolation medium.

Initially, protoplast volume estimates were made and were to be compared with vacuole volume estimates, in an attempt to determine the cytoplasmic concentration of proline in the protoplast. This technique makes the common assumption that one protoplast produces one vacuole. In the present study, it was observed that numerous vacuoles arise from a single *D. spicata* protoplast and has also been observed in protoplasts of rice. Consequently, it was not possible, using the protoplast/vacuole volume comparison technique to obtain a meaningful exact proline concentration in the cell cytoplasm. Experiments are currently underway to determine cytoplasmic volume of cells exposed to NaCl by sectioning stressed cells and determining vacuole volumes from the electron micrographs.

Proline was synthesized and accumulated in suspension cultured cells of *D. spicata* from a basal level in the NaCl-free medium of 10.5 pg/cell to a concentration at the end of 24 hours in 200 mM NaCl of 93.4 pg/cell. This represents a ninefold increase in the amount of proline in the cell in a 24 hour period. Proline is synthesized at a more reduced rate after the initial rapid increase in the first 24 hours, for the duration of the culture period, but has essentially stabilized by 48 hours. The phenomenon of rapid synthesis and stabilization of a solute in response to environmental stress is common among known halophytes, becoming measurable within hours after induction of stress, and stabilizing within two to three days. This contrasts with patterns of proline accumulation usually observed in mesophytes. In cases in which a stress was rapidly applied, proline accumulation was measured in terms of days after applying the stress, and usually continued to increase, unchecked, until the stress was removed or the tissue was dead. The mechanisms for proline accumulation in mesophytes and halophytes thus appear to be different. Apparently, it is not the ability of the plant to accumulate proline or other solutes but the kinetics of that accumulation that determines adaptability to a stressful environment.

The inhibition of protein synthesis by cycloheximide, and subsequent inhibition of proline production, together with the lack of an effect on proline synthesis by actinomycin D, suggests that proline accumulation control is post-transcriptional. It is not known if there is a persistent mRNA that is translated to produce unique proline synthesis enzymes in response to osmotic stress, or if it is simply the conventional enzymes responsible for proline synthesis in *D. spicata*.

Network Activities and Accomplishments

The International Plant Biotechnology Network (IPBNet) has been developed for the purpose of establishing working partnerships among researchers worldwide with mutual interest in using plant biotechnology to accelerate the development of stress-tolerant crops.

The Network emphasizes information exchange and equal partner collaboration with laboratories worldwide whose research goals relate to Project objectives. Agricultural goals of developing countries are stressed through the development of various suitable linkages between the Project and universities, national programs and CGIAR International Centers.

Since its inception in early 1985, the International Plant Biotechnology Network has gained over 1000 affiliated members from 72 developing and 18 developed countries. The TCCP currently has ten signed Memoranda of Agreement (MOA) with institutions in eight countries in Africa, Asia and Latin America including four international centers (Table 29). These agreements outline the terms of current and potential collaboration and include guidelines on exchange of information, field testing, germplasm exchange, technical assistance, training, graduate education, and personnel exchange.

The following paragraphs provide a synopsis of the individual collaborations and agreements:

1. In Africa the signed Memorandum of Agreement is with IAV Hassan, Morocco. Initial contacts have also been made with five other countries in Africa, and potential field testing sites have been identified for drought tolerance testing sorghum and millet.
2. In Asia a number of MOA's exist in the southeast. In Thailand, collaborations with two universities have been initiated. A graduate student exchange program exists between Colorado State and Chulalongkorn University and the TCCP hosted the first exchange student in the fall of 1985. Chulalongkorn has worked actively with the TCCP on rice regeneration experiments; in the fall of 1985, the TCCP sent a technician to Thailand to assist in this research. Results were presented in a poster at the Second Annual IPBNet Conference in January 1987. In the Philippines, two MOA's have been signed; one with PCARRD and one with IRRI. Collaborations include sub-agreements for research in rice and corn tissue culture, heat tolerance selection in wheat, and field testing for salt tolerance in rice at ViSCA and UPLB, respectively.
3. Additional agreements in Asia have been established in Pakistan with the Nuclear Institute of Agriculture and Biology (NIAB), and in India with ICRISAT. Both organizations have sent scientists to the TCCP to perform collaborative research. The agreement with NIAB includes a project on producing a salt-tolerant rice/grass hybrid by protoplast fusion. The agreement with ICRISAT is a project which applies tissue culture techniques for the improvement of chickpea.
4. In Central America, three MOA's exist with CIMMYT, CIAT and CATIE. The collaboration with CIMMYT involves the development of tissue culture techniques with application to the CIMMYT widecross program on wheat. Since January of 1986, a member of the TCCP staff has been living and working at CIMMYT to assist with this research. The Project's potential collaboration with CIAT lies in field testing for acid tolerance in rice. Finally, the extent of participation and collaboration with CATIE has led to establishment of a regional tissue culture training program, which combines the course material used in the TCCP's Training Program with the necessary Spanish translation instruction.

In addition to the various internationally focused collaborations, the TCCP has agreements with various North American universities: (1) rice regeneration field testing and seed in-

crease at Louisiana State University; (2) sorghum field testing for aluminum and drought tolerance and seed increase at the University of Georgia and Texas A & M University; and (3) oats salt tolerance field testing at the University of Saskatchewan, Canada.

An important part of the Network is training and education. The TCCP has established a formal, six-month training course designed to train scientists and technicians, primarily from developing countries, and equip them with the necessary skills to utilize the technology in individual research programs. The course includes general lecture and laboratory instruction on sterile technique, callus and cell culture, plant regeneration, meristem and embryo culture, virus indexing, stress selection, anther and protoplast culture and laboratory management. The greater part of the course involves individual research on crops of particular interest to each person. Seventeen students have graduated to date (Table 30). Two students have remained at the TCCP to pursue graduate degrees and one other student is pursuing an undergraduate degree in Colorado State's Department of Horticulture.

Presently, the TCCP supports graduate candidates from India, the Philippines, Thailand, and the U.S. and hosts graduate candidates from Indonesia, Jordan, Libya, Mexico, Pakistan and the U.S. — 13 students in all (Table 31). Another student is hosted by Dr. James T. Colbert, Assistant Professor of Botany and a TCCP Training Program instructor, and works in his laboratory on a TCCP collaborative molecular biology project. The TCCP has also hosted visiting scientists from China, India, and Pakistan. Visiting scientists come for six months to two years to work on projects of mutual interest.

Exchange of information among scientists is vital to promote mutually beneficial research and training interests. One way this is accomplished is by an up-to-date, computerized literature filing/access system. This system, explained in more detail in the "Project Facilities" section, holds over 10,000 entries. Entry lists on particular subjects are available to all Network members as requested. The Project currently answers approximately half-a-dozen requests per week.

Network collaborations are highlighted in IPBNet newsletters and at IPBNet conferences. The IPBNet Newsletter is published every six to twelve months by the TCCP. It includes current information on Project research, training, and networking activities, as well as information on collaborators, training courses, funding, and publications. The TCCP has published seven newsletters to date. The conferences provide a unique opportunity for Network collaborators, members and interested scientists to gather to exchange ideas and concerns. The conferences are small — usually limited to 100 participants — and provide the opportunity for more discussion than is usually afforded at larger conferences. Papers and posters are presented on a wide range of plant biotechnology topics, but the emphasis is on small group discussion and planning. It is during these conferences that the TCCP gains valuable feedback on what is needed to make IPBNet a more effective program.

The First Annual IPBNet Conference was held in Fort Collins, Colorado, USA, October 21-25, 1985. Ninety-four persons from twenty-one different countries attended. Speakers included Drs. Trevor A. Thorpe, E.C. Cocking, and Karen Hughes covering the topics of tissue culture, protoplast culture, and mutation selection and transformation, respectively. The Second Annual IPBNet Conference was jointly sponsored by the TCCP and Chulalongkorn University, and held in Bangkok, Thailand January 11-16, 1987. Seventy-nine persons from eighteen countries attended, including 36 persons from Thailand. Keynote speakers, Drs. Roger Beachy, Paiboolya Gavinlertvatana, and Colette Nitsch, addressed the subjects of gene movement and protoplast technologies, rapid propagation, and anther culture and somatic embryogenesis, respectively. Conference III is in the planning stages at this time.

Table 29. *Current Memoranda of Agreement and Formal Collaborations*

Institution	Country	Activity
CATIE	Costa Rica	Regional Training Center
CIAT	Colombia	Aluminum testing in rice
CIMMYT	Mexico	Cooperative research/ wheat, personnel exchange
Chiang Mai University	Thailand	General — graduate studies, training
Chulalongkorn University	Thailand	Cooperative research/rice, graduate student exchange
IAV Hassan II	Morocco	General agreement
ICRISAT	India	Cooperative research/ chickpea, visiting scientist
IRRI	Philippines	Rice field testing
NIAB	Pakistan	Cooperative research/rice, visiting scientist
PCARRD	Philippines	Cooperative research
UPLB	Philippines	Sub-agreements (2), field testing/rice, wheat research
ViSCA	Philippines	Sub-agreement, corn research

Table 30. *Trainee Program Participants September 1985-February 1987*

Name	Country	Crop(s) of Interest
Mr. Renato Avenido	Philippines	Mungbean, Wheat
Mr. Raed Bakheet	Saudi Arabia	Wheat
Ms. Ouafae Benlhabib	Morocco	Tomato
Ms. Olivia Damasco	Philippines	Wheat, Corn
Ms. Merlina Dionzon	Philippines	Corn, Rice
Dr. Bishamber Gothwal	India	Wheat, Barley
Mr. Francisco Gutierrez	Mexico	Sweet Potato
Ms. Abeer Abu Hassan	Jordan	Potato, Citrus
Ms. Iteu Hidayat	Indonesia	Potato
Dr. Flordeliza Javier	Philippines	Apple
Mr. Rachmat Kartapradja	Indonesia	Tomato
Mr. Andrew Matibiri	Zimbabwe	Tobacco
Mr. Ben Musyimi	Kenya	Cassava
Mr. Simon Ndirangu	Kenya	Tea
Ms. Mussarat Raza	Pakistan	Peanut, Mungbean
Ms. Erlinda Rillo	Philippines	Coconut
Mr. Takaniko Ruabete	Fiji	Sweet Potato, Taro

Table 31. *Graduate Students at the TCCP*

Name	Country	Degree/Project
Francisco Gutierrez	Mexico	Ph.D./Physiology of salt tolerance
Iteu Hidayat	Indonesia	M.S./Potato protoplast fusion
Isaac John	Pakistan	Ph.D./Salt tolerance genes
Raymond Ketchum	USA	Ph.D./Rice/millet protoplast regeneration
Douglas McMurray	USA	Ph.D./Mung bean regeneration
Amani Mnayer	Jordan	Ph.D./Wheat regeneration
Akbar Mohmand	Pakistan	Ph.D./Drought tolerance in wheat
Anna Novero	Philippines	M.S./Nitrate reductase in rice
Pounggpet Poonsapaya	Thailand	Ph.D./Rice regeneration from suspension cultures
Sathish Puthigae	India	Ph.D./Conditioning factor in rice media
Gaith Sasi	Libya	Ph.D./Triticale regeneration
Nanan Widiyanto	Indonesia	Ph.D./Rice blast resistance
Kern Wright	USA	M.S./Rice field testing

IPBNet is Interested in Your Cooperation

The Network Can Offer:

1. A Newsletter with summary reports on the activities of Network members.
2. A Resource Directory (in preparation) detailing plant biotechnology-related interests of laboratories and investigators worldwide.
3. A six-month, comprehensive Tissue Culture Training Program in English and a three-month Training Program (at CATIE) in Spanish.
4. A Network Conference every 15 to 24 months in various locations worldwide.
5. The opportunity for graduate and postgraduate training and cooperative research.
6. Access to a computer-based listing of current tissue culture literature, primarily accessible by crop.

The Network Cannot Provide:

1. Direct research grants except in a few cases and only when directly related to TCCP research objectives.
2. Financing for graduate or post-graduate education or training. The Network can *assist* you in obtaining funding for these activities.
3. Transportation money.

How You Can Participate

1. Send us information on your research interests and current program for summary publication in the Newsletter and for listing in the Resources Directory.
2. Contact us if you have questions about participation or Network services.
3. Fill out and mail back the Network Questionnaire.

Network Interest Questionnaire

1. Name

2. Position

3. Organization/Address: (Telex, phone number, cable number)

4. What tissue culture research are you presently involved with? Please be specific as to crops, research goals, etc. (Please attach sheet for any additional information).

5. Are you aware of any current national, USAID or other outside agency funding for plant biotechnology projects in your country? a) yes b) no

   If yes, please describe the nature and source of funding.

6. Are you aware of a need for or interest in plant tissue culture training in your country? a) yes b) no

   If yes, please name interested organization(s) and person(s) to contact.

7. Does a formalized research exchange system or organization exist for plant tissue culture and genetic engineering in your country? a) yes b) no

   If yes, please give names of people/organization.

8. Would you like to be a member of IPBNet?

9. Comments:

10. Please return survey form to: Network Coordinator
    Tissue Culture for Crops Project
    Department of Botany
    Colorado State University
    Fort Collins, Colorado 80523 USA

Future Goals

Research and development during the first six years of the Tissue Culture for Crops Project has resulted in the establishment of efficient and reliable procedures for plant regeneration in tissue culture. The methods have been developed for several cultivars of rice, spring and winter wheat, sorghum, millet and corn, and have been applied to screening for selection of variant cell cultures with tolerance to salt, aluminum and drought. The results have fully demonstrated a capability not previously reported by other research centers — that plants can be regenerated from cultures of the major grains after lengthy culture periods of one to two years or more. In addition, an international network (IPBNet) and Tissue Culture Training Program have been established to encourage and strengthen relationships among scientists with interest in using plant biotechnologies to increase food production.

The research and development is progressing into several areas in accordance with those detailed in the "Introduction." Increased emphasis has been placed on the following areas:

1. field testing and greenhouse screening;
2. developing and applying tissue culture technologies to tropical grain legumes;
3. applying widecross hybridization methods to wheat;
4. using plant protoplast technology and molecular biology to isolate and transfer the genes for stress tolerance traits;
5. strengthening Network collaborations among members and between the TCCP and members; and
6. providing information and assistance on funding for research, training, education and travel.

Field Testing and Greenhouse Screening

The primary future goal of the TCCP is to transfer potentially stress-tolerant germplasm from tissue culture to the field, thus the field testing program is expanding continuously. During the coming year, the plans are to perform field tests in seven locations in North America. Testings will consist of seed increase and screening for a stress tolerance. There will be three locations for sorghum, and one each for rice, millet and oats. In the developing countries, there will be one site in the Philippines and one at CIAT, Colombia. Negotiations are in progress to include additional sites at specific locations in south Asia and Africa. The tests are being set-up as collaborative projects with established plant breeders at particular sites. Testing will expand as more germplasm becomes available from the tissue culture and greenhouse screening operations. The testing is focused principally on selection for lines with increased tolerance to salinity, aluminum toxicity and drought. Lines with increased tolerance will be incorporated into the breeding programs of the respective collaborators. Results should provide necessary information on the stability of the stress trait and the agronomic characteristics of the line.

Greenhouse screening of large numbers of regenerated plants is expected to provide a preliminary indication of the number of plants with increased tolerance that have been selected in the tissue culture screening. Greenhouse screening is expected to facilitate procedures and reduce the number of plants that are grown to maturity, thus maximizing available greenhouse space.

Grain Legume Research

Since work on soybean is completed, the Project is concentrating on some of the food legumes of importance to tropical and subtropical regions. Methods have been developed for plant regeneration in pigeonpea, and tissue culture screening is being applied to select for increased salt tolerance. This will be followed by greenhouse screening and field testing. A similar program is being implemented for salt tolerance selection in cowpea. Two species of legumes, which are closely related to major food legumes, are being explored as sources of drought and salt tolerance. The tepary bean is a *Phaseolus* species related to dry bean and moth bean is a *Vigna* species related to cowpea and the grains. Both of these are extremely drought-tolerant. The current research is designed to demonstrate the degree of salt tolerance and to select for lines with increased salt tolerance. Any lines of these species with increased salt tolerance could then be crossed with plants of the particular grain legume for the transfer of the drought and salt tolerance traits.

Widecross Hybridization

The production of germplasm with increased stress tolerance includes hybridization of crop plants and alien species. In wheat, the hybridization is being performed with several grass species which are the sources of stress tolerance and disease and pest resistance. The procedure of hybridization requires an embryo culture step to insure hybrid plant development. Similar hybridizations are being performed with pigeonpea and *Atylosia* spp. The F_1 hybrids now being produced are expected to be selfed and the F_2 generation screened for the stress tolerance traits. Such hybrids are highly sought by breeders at ICRISAT.

The program to produce germplasm with increased salt tolerance also includes crosses between incompatible species. The transfer of salt tolerance to rice is difficult because highly salt-tolerant species are incompatible with *Oryza* spp. Research is in progress to produce hybrids between cultivated rice — specifically Basmati — and Kallar grass. Kallar grass is highly-tolerant to alkaline salinity. The plants also have high photosynthetic efficiency and associative nitrogen fixation.

Since the plants are from two widely different genera, the hybrids will be produced by protoplast fusion and tissue culture procedures. The research is a collaborative project between the Nuclear Institute of Agriculture and Biology (NIAB) in Faisalabad, Pakistan, and TCCP. The research projects consist of developing the necessary protoplast and plant regeneration technology of the two species, then performing the fusion hybridization and producing the hybrid plants. The hybrid plants then will be backcrossed to rice.

Isolation and Transfer of Salt Tolerance Genes

Tolerance to stress conditions varies between species. The variability is due to differences in heritable traits. Plants have been observed to grow in extremes of salinity combined with alkalinity or some other adverse condition. Such highly-tolerant species have potential as germplasm for introducing tolerance to crop plants. Highly salt-tolerant species (halophytes) are potential sources of genes for the salt tolerance trait. The halophytes have the genetic, biochemical, and structural features for coping with high salinity. Any information on the genetics of the salt tolerance traits, particularly at the molecular level, would be highly valuable in attempts to transfer salt tolerance systematically across incompatibility barriers in plants. The information also would be the avenue towards an understanding of salt tolerance genes and their regulation. The technology and methods of molecular and cellular biology (biotechnology) now permit the search for genes and gene products which relate directly to salt tolerance.

There is evidence for salt stress response proteins in crop species, but no report that they are directly related to stress tolerance. Research has been initiated to identify salt stress proteins, using plant materials from species which are halophytic and thus have the biochemical and genetic characteristics to tolerate highly saline conditions. The initial experiments are being performed with cell suspension cultures of the halophytic species. These cultures grow rapidly both in saline and normal culture media.

The initial steps of the research involve the identification of polypeptides produced in response to salt stress. The respective mRNA can then be used to prepare the cDNA and ultimately identify the gene for the protein(s). Research will include attempts to demonstrate a salt stress tolerance function of the proteins. The program also includes setting-up a gene transfer and expression system in plants. The anticipated plant materials include rice.

The gene transfer procedure will involve the use of protoplasts. Marker genes of different types available from outside sources are used in the development of the gene transfer and expression systems. The system will then be used to determine the functions of the salt stress protein/tolerance genes. This research is a collaborative project with Dr. James T. Colbert, Botany Department, Colorado State University.

Network and Training Activities

Since the Network is constantly changing and adapting to the needs of its members, it is difficult to make specific plans for the future; however, from the feedback received at IPBNet Conferences, a few goals can be outlined.

In the next year, the TCCP will publish a Network Member Directory. The Directory will include names and addresses of members, as well as specific information on current research interests. The Directory will be mailed to every correspondent member (over 1000 persons) and will serve to stimulate communication among members, not just between members and the TCCP.

Another important goal of the Network is to assist in seeking funding for the activities of its members, including support for training and education, research grants, and travel to conferences, workshops, and other laboratories. The Project is also interested in facilitating the formation of regional and single-country plant biotechnology networks. A list of funding agencies is being compiled at this time, and when available it will assist members in looking to appropriate sources to fulfill their funding needs. The TCCP itself will reduce the amount of funding it currently provides for these activities, thus being able to funnel this money into other areas.

Along these same lines, the TCCP staff will work to make the Training Program self-supporting. The Training Program will be expanded to include at least one other regional training program in Africa or Asia.

Overall, the Network will expand much more slowly, focusing on strengthening current collaborations. IPBNet members will be encouraged to branch out and to seek and strengthen new and existing relationships within their own geographic regions.

Project Facilities and Operations

The TCCP is currently located in its own 638 square meter building on the main campus of Colorado State. Two general laboratories of 70 square meters each are available to TCCP researchers and trainees. The laboratories are adjacent to a 111 square meter culture room with a capacity of approximately 150,000 cultures (equivalent to 6 billion cells). A separate culture room equipped with day-length controls is also available. The culture rooms are temperature-controlled by a specially-designed, computer-operated system. The remaining area of the building is divided into a sterile transfer room, instrument room, autoclave-sterilization area and office space.

A 765 square meter greenhouse is attached to the TCCP building. The greenhouse is used to multiply seed, raise explant sources, and grow regenerated plants to maturity. Preliminary screening of experimental material is done by hydroponic testing within the greenhouse.

The TCCP also houses an extensive collection of tissue culture literature. The literature access system currently holds over 10,000 entries which have been selected from *Current Contents* by members of the TCCP staff and entered into a computer using the Article Filing System. The system was recently transferred to a hard disk unit, increasing the potential capacity to 175,000 entries.

Entries are categorized according to subject matter and applicable keywords. Crop names and research areas are typical categories; for example, rice, wheat, corn, drought, salt, fusion, protoplast. The Artfile System allows access using any part of the entry, *i.e.* author's name, keyword, journal date. Literature searches of the system are made as requested by Network members and interested parties. In addition to the literature file, a large collection of tissue culture and related biotechnological books is maintained.

Germplasm for TCCP experiments is supplied by several sources. Wheat and corn seeds are provided by CIMMYT and the Department of Agronomy at Colorado State. Seeds of rice cultivars are supplied by IRRI and by Dr. Neil Rutger, Department of Agronomy and Range Science, University of California at Davis. Millet is supplied by Dr. Bill Stegmeir, Kansas State University, Fort Hays. Sorghum is supplied by Dr. Ronny Duncan, University of Georgia, Dr. Fred Miller, Texas A & M University, and ICRISAT. Legume seeds are provided by ICRISAT and IITA. The TCCP also relies on the International Centers and various individual investigators worldwide to field test promising experimental lines under various local conditions.

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Mungbean Regeneration

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Colombia
Costa Rica
Ecuador
Egypt
India
Indonesia
Jordan
Kenya
Liberia
Mexico
Morocco
Pakistan
Panama
Philippines
Sri Lanka
Thailand
Togo
Zimbabwe

List of Abbreviations

Abscisic acid	ABA
Benzylaminopurine	BAP
Casein hydrolysate	CH
Coconut water	CW
2,4-Dichlorophenoxyacetic acid	2,4-D
Embryogenic	E
Gibberellic acid	GA
Indole-3-acetic acid	IAA
Indolebutyric acid	IBA
2-Isopentenyl adenosine	2ip
Kinetin	KIN
Linsmaier & Skoog	LS
Murashige & Skoog	MS
Modified Murashige & Skoog	MMS
Naphthaleneacetic acid	NAA
Non-embryogenic	NE
Polyethyleneglycol-4000	PEG
2,4,5-Trichlorophenoxyacetic acid	2,4,5-T
2,3,5-Tri-iodobenzoic acid	TIBA
Tryptophan	TRP

Appendix I: Methods for Obtaining Stress-Tolerant Plants

Crop species and cultivars are generally bred for maximum yields and productivity on optimum or ideal soils and environments. There is an increasing interest and need to develop crop plant cultivars which can be productive under one or more conditions of adverse soils or environments.

The presence of tolerance to a particular environmental or soil stress varies between species and within cultivars of a species. The variability is due to genetic differences. Thus it is possible by selection methods to obtain variants with greater than normal tolerance. Some non-cultivated plant species have adapted to survive under extreme conditions of soil salinity, alkalinity, acidity, drought or temperature extremes. Such species provide a germplasm resource for use in systematic attempts by crossing procedures to introduce tolerance into crop species. The following sections outline strategies and methods for obtaining crop cultivars with increased stress tolerance.

Germplasm Evaluation

The search for valuable agronomic traits for crop cultivar development routinely includes an evaluation of available germplasm resources. The search for stress tolerance traits is no exception; however, it appears to have been performed to a relatively limited extent using a variety of different, and often uncomparable systems. Reports indicate that the more comprehensive evaluations have been carried out for wheat (*Triticum* spp.), (Kingsbury and Epstein, 1984), wheat and barley (Srivestava and Jasa, 1984) and rice (Akbar and Seshu, 1986) on tolerance to saline conditions. Other evaluations have been concerned with identifying drought-resistant lines of rice (DeDatta and Seshu, 1982).

Conventional Breeding

Conventional breeding methods are being used in attempts to produce cultivars with increased stress tolerance. These methods include production of F_1 hybrids, backcrossing, progeny testing and selection. The most extensive and successful work to date is on rice and wheat breeding for salt tolerance. The breeding for salt, drought or other stress tolerance is usually carried out in conjunction with introduction of one or more desirable agronomic traits, such as disease and insect pest resistance, which relate to yields.

In rice, the understanding of the genetics of salt tolerance is increasing. From several genetic studies, it has been determined that there are three groups of genes which control Na and Ca content in the leaves at seedling stage (Akbar *et al.*, 1985). Another three pairs of genes code for resistance to salinity-induced panicle sterility (Akbar and Yabuno, 1977).

The results of conventional plant breeding approaches in the development of cultivars with stress tolerance have been significant but very limited in scope. The cultivars of major crop species designated as tolerant to a particular stress appear to be productive only within relatively narrow limits of the particular stress. Very few highly-tolerant crop cultivars have been reported, partly because most of them are local land races developed by the farmers.

Selection of Stress-Tolerance Traits

Field Selection

Stress tolerance is a multigenic trait, but changes in a single allele can still increase tolerance. Variations in tolerance occur within a plant species, and they can also be induced

through mutations. Selection for variants with increased stress tolerance is usually accomplished through field screening. This is a routine procedure and also a regular part of conventional plant breeding procedures. Variability in response to stress occurs not only within a species, but the tolerance can also vary during the different phases of development. Standard tests are based on the performance of a particular plant during the entire life cycle. Field tests and screening are the ultimate steps in selection of promising variants.

Tissue Culture Selection

The advent of plant tissue culture methods has introduced several valuable features that can be integrated into total plant breeding and substantially amplify and accelerate the process. The most important features at this stage are the variations that occur during tissue culture and the facility of the methods to screen large populations in a small area. Plant cells from any species can be cultured aseptically in defined media. Millions of cells can be grown for several months and then many can be induced to produce complete plants. The TCCP found that, using presently available methods, over 127,000 Pokkali rice plants could theoretically be produced from tissue culture of one seed after six months (TCCP Newsletter No. 3, 1984). This figure probably represents only a small percentage of possible production if methodological refinement were continued.

Another important benefit of tissue culture is that it drastically increases the mutation rate of plant cells. Such mutations are referred to as "somaclonal" variants (Scrowcroft and Larkin, 1983). Up to 20% of tissue culture-regenerated plants have new mutations (Evans *et al.*, 1983), compared to less than 0.1% of plants from seeds. Since desirable mutants occur infrequently in field situations, the increased mutation rate from tissue culture is extremely useful in selecting for stress tolerance. The strategy being used is to grow large populations under the influence of stress such as salt. The surviving cells are tolerant and plants grown from such cells are potentially tolerant. Such procedures have been successful for obtaining plants with increased stress tolerance, disease resistance and useful agronomic traits.

Widecross Breeding

While attempts are being made to transfer stress tolerance traits by conventional breeding methods, most plant sources with high stress tolerance are often distantly related and incompatible with crop species. Widecross breeding methods are thus an alternative. The best documented example of wide-cross breeding is the production of triticale which is a hybrid between wheat *(Triticum)* and rye *(Secale)*. The chromosomes in the hybrid are duplicated to produce a plant with 42 wheat chromosomes and 14 rye chromosomes. Such a plant is called octaploid triticale. Eventually, the rye chromosomes will be lost due to differences in the cell cycle between wheat and rye chromosome sets. During the time in which chromosome sets from both species reside in the same nucleus translocations of chromosome segments occur. Thus the final plant with 42 chromosomes — called hexaploid triticale — is different from wheat. The chromosomal differences can be demonstrated by chromosomal banding techniques. Triticale shows increased disease and stress tolerance while maintaining many of the useful traits of wheat.

Many other widecrosses have been and can be made. Widecross breeding thus can be a valuable tool in introducing more stringent stress tolerance as well as obtaining information on the genetics of stress tolerance.

Haploid Breeding

In haploid breeding, a hybrid is produced by conventional means, then anther culture is carried out to obtain haploid or polyhaploid plants. These are treated with colchicine to induce chromosome duplication, and thus homozygous diploid plants are obtained. Since recombination occurs during meiosis in the original hybrid, the homozygous progeny obtained vary widely in the combinations of genes they have from the original parents. This variation can be screened for derived traits such as stress tolerance.

Somatic Cell Fusion Hybrids

The various breeding and selection approaches now in use may not be adequate for all major crops to develop cultivars with adequate stress tolerance. The incompatibility barriers between the crop species and the stress-tolerant plant source may be too great to permit any type of sexual crossing. Other methods are now available which permit production of desirable hybrids of crop species with distantly related plants. These include somatic cell fusion and transfer by genetic transformation.

The fusion of plant cells of any two species has become a reliable, useful technology, but is limited to the species which can be regenerated from protoplasts. Protoplasts (cells with the walls removed) of two plant sources can be fused and give rise to hybrids, which can be grown into complete plants. These plants express traits derived from both parental species. Such plants can in turn be backcrossed to the crop species, and thus permit the transfer of the trait. This approach also can be expected to be used in the transfer of desirable salt tolerance to crop plants from halophytes, for example, which through centuries have acquired the characteristics for efficient growth on saline soils. In all cases, however, the protoplast to plant technology must be developed prior to the use of the fusion procedures.

Transgenic Plants

A technology is emerging which permits the transfer of single genes for particular traits from one plant to another. The use of the method necessitates that the gene(s) are known. Gene cloning has become standard procedure, and can be used for the isolation and cloning of genes for stress tolerance. The isolation of genes for heat shock proteins (HS), which provide protection against high temperature damage, is an example of how the methods are being used. The HS protein genes have been transferred from one plant to another and expressed (Key *et al.*, 1981). Similar approaches are being used at the TCCP to identify salt shock (SS) proteins and cloning of the respective genes. The reported research has not progressed so far as to demonstrate that the SS proteins had any salt stress tolerance function. The gene transfer technology has been demonstrated in several crop species to give transgenic plants which express the single gene that was transferred (Powell, 1986). The progeny of the transgenic plants also expressed the injected gene. One of the best documented examples is the transfer and expression of a gene that confers resistance to a herbicide (Kosuge *et al.*, 1986).

Appendix II: Tissue Culture Technology

To increase stress tolerance in crop plant cultivars, the tissue culture process involves several steps:

1. Callus initiation and culture. A large population of cells sufficient to contain desired mutants or produce desired numbers of regenerated plants must be obtained. This is accomplished by removing a small portion of the plant (an explant), and placing it in liquid or on solid medium. These media contain various nutrients and hormones which allow the cells to divide and reproduce, eventually forming a large collection of cells (suspensions, if liquid medium; calli, if solid medium). After one to four months, depending on species and genotype, this primary suspension callus is large enough to be subdivided into identifiable cell types, and transferred to a second medium where stress selection can take place. Many callus cell types of varying essential regenerability, are produced.

2. Screening. Stress-tolerant, mutant (variant) cells must be identified. Once a sufficiently large callus has been produced, it is ready to be subjected to a specific stress. In the case of salt tolerance, the callus will be subdivided and placed on a medium containing a level of NaCl sufficient to severely retard the growth of non-tolerant cells (overall growth reduction should be to about 10% of controls). If mutant cells are present in the callus, they will grow and divide more rapidly than non-tolerant cells and will eventually comprise the majority of the population (Simons *et al.*, 1984). If the level of NaCl is not sufficient to eliminate non-tolerant cells, these cells may divide and regenerate along with tolerant cells.

During the selection process, other beneficial mutations might be identified. In nature, many salt-tolerant plants are also drought-tolerant (*e.g.*, *Distichlis spicata* and *Distichlis stricta*). Thus, if one is selecting for salt-tolerant mutants in culture, one might also discover drought-tolerant mutants. In addition, salt-tolerant plants in nature tend to be shorter than related, non-tolerant varieties. Therefore, in selecting for salt tolerance, one might also be selecting for a resistance to lodging.

Crucial to the effectiveness of plant tissue culture as a means of decreasing environmental alterations is the genetic stability of variant cells. Several types of alterations are possible, for example point mutations and chromosomal alterations, and some of these, especially translocations and some duplications, are unstable (Schimke, 1982). Variant stability in the absence of stress seems to be related to the length of selection, with at least six months selection required for stability, although increased time in culture also increases the amount of chromosomal alterations.

It seems clear from experimentation at the TCCP that short periods of stress-selection and low selection pressures do not entirely eliminate non-tolerant cells from the population; thus, both tolerant and non-tolerant plants, as well as theoretical considerations over whether or not regenerated plants arise from single cells, are irrelevant if selection is continued until 100% of the population consists of tolerant cells. Chimerical plants could result from regeneration of mixed groups of tolerant and non-tolerant cells.

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. Recurrent selections at increasing levels of stress may be necessary to obtain high enough levels of stress tolerance to be practically useful (Nabors, *et al.*, 1980).

3. Plant regeneration. To validate the usefulness of *in vitro* manipulations, plants must be regenerated from long-term, stress-selected cultures as well as from control cultures. In tobacco, cultures have been regenerated after ten years or more in culture; however, it has been widely accepted that, for most types of cultured cells, the rate of plant regeneration gradually declines as the time in culture increases. When the

TCCP began work, this was the number one tissue culture problem for both cereals and legumes (Nabors *et al.*, 1983). Results from TCCP experiments have revealed that, in wheat, rice, and millet, the length of time plants are regenerable is dependent on the ability to maintain E callus in culture. In the case of wheat, regeneration has been obtained after twenty months in culture. Some rice cultivars still produce plants at high frequency after four years in culture. Long-term regeneration is important because selection for stable variants can require up to twelve months of growth *in vitro*. In addition, such regeneration must be of a high enough frequency to ensure that enough plants are produced for seed production and subsequent whole plant screening.

Undesirable chromosomal abnormalities and chromosome loss also increases with lengths of time in culture, thus, the correct period of selection before plant regeneration must be long enough to ensure stable variants and short enough to maintain agronomic chromosomal integrity.

4. Testing of regenerated plants and progeny. Plants obtained from stress-tolerant cultures must be shown to retain the tolerance and to consistently pass it on to future generations. The TCCP lab was the first to demonstrate that NaCl tolerance obtained in cells was also apparent in regenerated plants (Nabors *et al.*, 1980); however, it is possible that other resistance, shown in other cultures, will not be passed on to regenerated plants. Most *in vitro* selection procedures isolate dominant or co-dominant variants, therefore, a generation of inbreeding may be required to obtain a stable, tolerant line of plants.

It must be demonstrated, through whole plant testing, that stress-tolerant plants obtained from cell cultures also show increased tolerance during germination, seedling growth, maturity, flowering and seed set. In addition, it is important to determine that tolerance is stable in the absence of stress. The Project has found that NaCl-tolerant oat plants obtained from cultures retain the resistance through at least four whole plant generations in the absence of stress. Cultured cells obtained from regenerated, resistant plants also retain salt tolerance after sixteen months in non-NaCl-stressed cultures.

Finally, it must be demonstrated that an increase in stress tolerance has not unfavorably altered other desirable characteristics. The plants must be returned to the breeder with as many good characters as were originally present.

Appendix III: Review of General TCCP Tissue Culture Methods

Equipment

A tissue culture laboratory requires several specialized pieces of research equipment in addition to standard lab supplies. Standard lab supplies include glassware and chemicals used in preparing the media. A pH meter and an accurate (0.001 gm) balance are also necessary for media preparation. Special equipment should include an autoclave for the sterilization of media and utensils, a laminar flow hood equipped with forced air circulation and filters designed to remove small (0.3 μm), contaminating objects. Culture vessels are needed, such as jars or vials, and petri plates or Erlenmeyer flasks. A culture cabinet, ideally a room, with controlled temperature and light conditions is also required. Unless seeds are the only explant source utilized, a growth chamber or greenhouse is needed for growing donor plants.

Explant Sources

In legume and cereal tissue culture, mature seeds are the most convenient explant source to obtain and culture. Either the entire seed can be used as in rice, or the seed can be soaked over night and the mature embryo removed. With a few plants (*e.g.* corn and soybean), developmentally immature embryos, immature leaves, or immature inflorescences are the best explants for establishing regenerable cultures. It is important that plants grown for explants be carefully monitored since environmental influences upon the donor plant can significantly (positively or negatively) affect responses *in vitro*. Growth conditions *in vivo* of the donor plant are especially important if one wishes to compare genotypic responses *in vitro* because differences observed in culture may be reflections of environmental, and not necessarily genetic, factors.

General Formulations

Most TCCP experiments use a solid culture medium containing LS (Linsmaier and Skoog, 1965) major and minor mineral salts (Table 32), plus 40 mg/l thiamine, 10 g/l myo-inositol, 50 mg/l Fe-EDTA, 2-4% sucrose, 1% agar, and different growth hormones, *e.g.*, 2,4-D, KIN, IAA, etc. Stock solutions are prepared ahead to facilitate making media (Table 33). This medium is high in both oxidized and reduced nitrogen. Other similar media may yield comparable results. Linsmaier and Skoog medium is the same as MS except that the vitamin formulation is simpler. (LS contains only Thiamine HCl while MS has Thiamine HCl, Pyroxidine HCl, Nicotinic acid and Glycine). Deionized water is used in all media preparation and rinsing of laboratory equipment. This water has passed through a 5 micron filter, then a series of resin-filled, ion-exchange tanks, to free the water of debris and "contaminating" ions, and then a 2 micron filter.

Unused media kept longer than one week should be stored in a refrigerator to prevent desiccation. This will keep media in good condition for up to one month. The media are adjusted to pH 5.5 with 0.1 N NaOH (or 0.1 N HCl) before adding and dissolving the agar and autoclaving. In addition to solidifying the medium, agar acts as a buffering agent for most media as shown in Fig. 50 (TCCP Progress Report, 1982).

Table 32. Salts used in Linsmaier and Skoog (LS).

Major Salts	
Compound	Final Concentration
NH_4NO_3	1650 mg/l
KNO_3	1900
$MgSO_4.7H_2O$	370
$CaCl_2.2H_2O$	440
KH_2PO_4	170
FeEDTA	50
Minor Salts	
Compound	Final Concentration
$MnSO_4.H_2O$	15.6 mg/l
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CoCl_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.025
Organics	
Compound	Final Concentration
Thiamine-HCl	0.40 mg/l
Myo-Inositol	100.0
Sucrose	20-40 g/l*

*Concentration varies on crop and cultivar. See individual sections for specific concentrations

Table 33. Preparation of stock solutions.

Stock Solutions	Concentration	Preparation	Storage
Major salts	See Table 32	dissolve in water	room temp.
Minor salts	See Table 32	dissolve in water	room temp.
thiamine	40 mg/l	dissolve in water	refrigerate
myo-inositol	10 g/l		
Fe-EDTA	5 g/l	dissolve in water	refrigerate
2,4-D	150 mg/l	dissolve in hot water, stir	room temp.
KIN	25 mg/l	autoclave*	room temp.
IAA	50 mg/l	dissolve in hot water, stir	this stock is prepared daily to prevent dimmerization
IBA	100 mg/l	dissolve in hot water, stir	room temp.
NAA	50 mg/l	dissolve in hot water, stir	room temp.
BAP	50 mg/l	autoclave or dissolve chemical in a few drops of IN KOH and then add water	room temp.
TRP	400 mg/l	dissolve in hot water	refrigerate

*Stock solutions of greater than 50 mg/l will not stay in solution reliably.

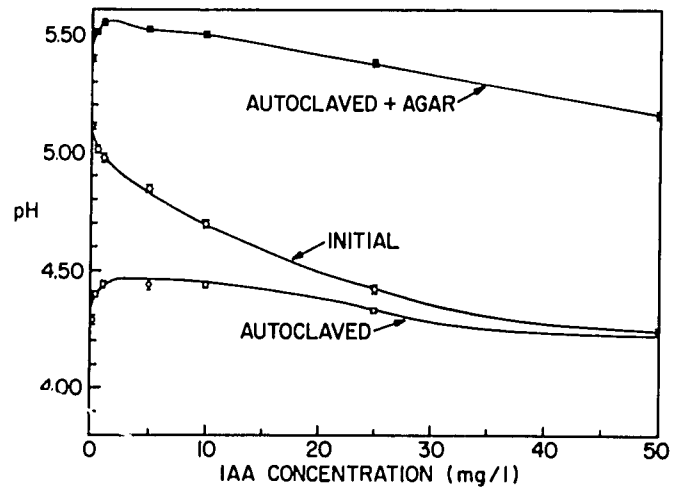

Fig. 50. The effects of IAA (a weak acid), agar, and autoclaving on pH of Linsmaier and Skoog's medium. Autoclaving lowers the pH by driving off dissolved NH_3.

Sterilization Procedures

General surface sterilization methods are as follows: (1) explants are placed in 90% ethanol for 30 seconds, (2) followed by a two-minute wash in a mild soap solution (2-4 drops liquid dishwashing soap/100 ml), (3) rinsed thoroughly with distilled or deionized H_2O, and (4) followed by a 10-30 minute wash (stirred vigorously with a magnetic stir bar, under vacuum) in a 20-50% commercial bleach solution (Chlorox) containing 5.25% sodium hypochlorite. Tween 20, or other detergent, is added as a surfactant. The length of sterilization is critical as is complete exposure of the surface area of the explant (which is facilitated by carrying out sterilization under vacuum). If seed lots are heavily contaminated with fungal spores, then a 0.05% (w/v) mercuric chloride rinse for 0.5-3.0 minutes (under vacuum, with stirring) is used following the bleach wash. Under sterile conditions, the explants are then rinsed three to six times with sterile, distilled water. If mature embryos are to be dissected out of imbibed seeds, then the sterilized seeds are left to soak in sterile distilled water for 12-24 hours.

Determining Optimal Culture and Regeneration Media for a Specific Crop

Before initiating tissue cultures of new species, the current literature should be reviewed keeping in mind that different cultivars of the same species frequently require different hormone concentrations. If the information available is insufficient, or not reproducible, then it is necessary to determine which growth hormones and concentrations give optimal callus growth and regeneration. It is essential that a systematic and comprehensive approach be used. In determining an "optimal" medium it

is necessary to know if growth of total callus, promotion of a particular callus type or differentiation is the goal. The results from one experiment are seldom sufficient since tissue culture is an inherently variable biological system. Replicate treatments/experiments are essential for assessing with reasonable confidence what medium is best.

Determining optimal media and cultural conditions for callus growth or plant regeneration is best viewed as an N-dimentional matrix problem in which each axis represents a different medium constituent of cultural conditions. Media experiments should be designed to test one factor at several concentrations, or at most, a few factors in combination, each at a few concentration levels. Large, complicated experiments with greatly variable biological systems are seldom efficient producers of reliable results.

Although optimal media are often cultivar specific, once a growth regulator is identified as best it can usually be applied successfully to other cultivars with minor adjustments in concentrations. Different media formulations are required for callus initiation, callus maintenance, plantlet regeneration, and root initiation.

For some model dicots, a medium which is best for callus growth and plant regeneration can be easily identified. In cereals, legumes, root crops, etc., the introduction of additional substances promoting growth or differentiation complicates the picture, but may be necessary for determining optimal media. The following compounds, growth regulators, and cultural conditions, are commonly regarded as likely candidates to increase growth rates or initiate differentiation:

1. auxin (*e.g.*, 2,4-D, 2,4 5-T, NAA, IBA, IAA)
2. cytokinin (*e.g.*, KIN, BAP, 2ip, zeatin)
3. light vs. dark
4. temperature
5. amino acids, particularly glutamine, proline and tryptophan
6. ascorbic acid
7. light quality
8. photoperiod
9. gibberellins
10. vitamins
11. ethylene
12. sugar (*e.g.*, sucrose)
13. callus to medium volume ratio
14. medium conditioning by previous callus

For growth regulation, both the type and concentration of growth factors must be considered. Indoleacetic acid, 2,4-D, 2,4,5-T, NAA, and IBA frequently give quite different effects at equal concentrations. Usually, one of these auxins at a specific concentration is optimal for callus induction. In practice, determining auxin and cytokinin type and concentration is the most critical step in successful initiation experimentation. In TCCP investigations, 2,4-D at low concentrations (0.5 to 2.0 ppm) was found to give the best callus growth and response. In addition, the effect of cytokinin (KIN and BAP) on callus formation is investigated in combination with the best auxin. For many cereals, media manipulations for initiation and maintenance of certain callus types are necessary only during the first few subcultures.

Callus Types

As previously mentioned, the decision for an optimal medium depends upon which factor needs to be promoted by media manipulations. In some early TCCP experiments, it was believed that an increase in total callus mass was the desired result, and optimal media were chosen on the basis of overall growth enhancement. It was soon recognized, however, that the callus quantity was not the only critical factor; callus quality was equally important. Hence, media were selected for the promotion of particular callus types.

The major callus types recognized by TCCP researchers are "embryogenic" (E) and "non-embryogenic" (NE) (Fig. 51). Embryogenic callus is translucent to opaque in appearance and milky white to yellow or yellow green in color. It appears as compact, nodular regions of non-vacuolated cells which produce small somatic embryos. Embryos further develop into whole plants when cultured on appropriate media. The surface of E callus is often shiny. In contrast, NE callus is crystalline and transparent in appearance, white, tan, brown, yellow or green in color, and appears as loosely-packed, friable regions of vacuolated cells which produce more callus, roots, or rarely, shoots. These two callus types have been noted by a number of tissue culture workers (Heyser and Nabors, 1982a, 1982b; Heyser *et al.*, 1982). The TCCP was the first to quantify the fact that E callus has a much higher frequency of regeneration than NE callus (Nabors *et al.*, 1982).

In earlier reports with wheat (Nabors *et al.*, 1982; Heyser *et al.*, 1985) the term "embryogenic" referred to the capacity for complete plant formation, and was not necessarily meant to imply that the mode of regeneration was via germination of somatic embryos; however, from the overwhelming evidence of TCCP results and those of others, it appears that embryogenesis, and not organogensis, is the plant regeneration route for cereals.

In some experiments, the percentage of cultures with E callus was the factor analyzed; in other experiments, fresh weight amounts of callus types were determined. For some investigators, the frequency of occurrence is more important than the quantity produced; for others, the reverse is true. For any media determination experiment for production of particular callus types, both factors should be scored and analyzed.

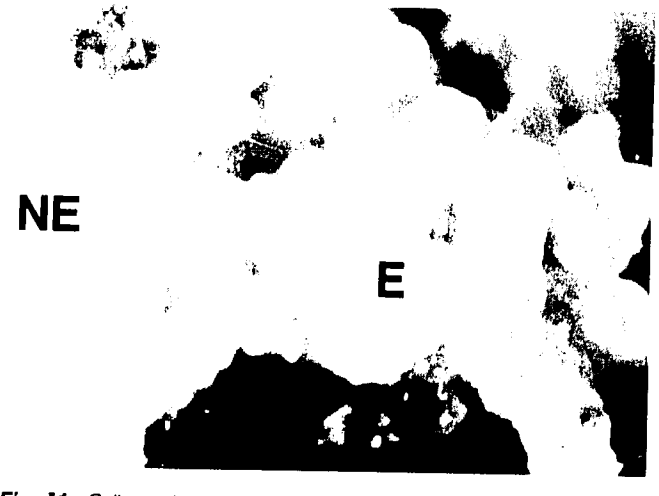

Fig. 51. *Callus culture showing both friable NE callus and compact E callus.*

Table 34. *Data quantification using relative callus mass based on size (eighteen different clay callus models ranging from .019 g to 20.507 g were made. With the smallest as a standard, the ratio of masses ranged from 1.0 to 1079.3. A ratio of 1.0 is equivalent to .003 g of soft, friable callus).*

Size	Ratio of Callus Mass	Size	Ratio of Callus Mass
1	1.0	13	365.7
2	3.2	14	431.9
3	4.8	15	438.4
4	7.0	16	580.3
5	16.8	17	817.0
6	20.4	18	1079.3
7	29.1	19	1439.0
8	58.8	20	1918.7
9	76.9	21	2558.3
10	142.7	22	3411.1
11	199.4	23	4548.0
12	261.3		

All the stock solutions are stirred during their preparation. The concentrations used are those which have been determined to provide maximal solubility without precipitation. Most of these stock solutions will keep for several weeks. Hormones and organic supplements are more susceptible to heat, light, and time so may not keep for as long a time.

Growth Measurements

For many TCCP studies, callus growth was measured by the cross-sectional comparison method of Hooker and Nabors (1977) (Figure 52; Table 34). More recent experiments have used fresh weight measurements of callus. In the first method, 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 be made easily. This method is used in many TCCP experiments because it eliminates the time consuming step of weighing individual calli, and minimizes contaminations risks. Informal studies at the TCCP indicate that an experienced technician can obtain comparative measurements with this method which are equal in accuracy to those obtained by actual weight measurements for tissues of consistent density.

Sub-Culture Intervals

Once callus cultures have been established, the frequency of transfer and size of the inoculum in relation to the amount of medium can be important factors. Size of the inoculum is particularly important in determining whether or not regeneration will occur. In general, for all tissue culture processes — from initiating callus and suspensions to obtaining regeneration — too little or too much inoculum are equally ineffective.

As mentioned earlier, callus type is often the most important factor to consider, especially with regard to regeneration potential. It has been determined in TCCP experiments that the selective sub-culture of a particular callus type, especially E callus, is essential to the long-term maintenance of that callus type. If callus types are not separated, then the faster-growing calli can literally take-over a culture or even exert morphogenic influences which can cause one callus type to deteriorate into a less desirable type.

Environmental Conditions for Cultures

In the TCCP laboratory, a large (40 × 30 feet) room is available for housing cultures. Continuous light is provided to all cultures (unless otherwise stated) by four 40 Watt, wide-spectrum fluorescent bulbs (36 inches in length) mounted on shelving units. The light sources are approximately 24 cm (9.5 inches) from the shelf, and provide approximately 2000-5000 lux of light intensity. Air circulation is provided by individual fan units, or a larger, central air conditioning unit. It has been found that air circulation is important for maintaining constant temperatures. The culture room is maintained between 25-28°C. Higher temperatures are very detrimental to callus growth.

Cleanliness in a culture room is an important but often overlooked factor. The culture room should be kept relatively free of dust, organic matter, and unnecessary materials. Physical isolation from other laboratory space is desirable, and traffic through this room should be kept to a minimum. Contaminated cultures should be immediately removed from the room or cabinet, and sterilized before being opened. If a sudden "bloom" of contamination occurs, inspection for carriers such as mites, gnats, etc. should be performed. Mites are difficult to see even microscopically and are usually first noticed because of the fungal spores they carry when moving from vial to vial. They can enter even apparently tightly-sealed containers and reproduce vigorously and rapidly. If contamination carriers are identified, shelf paper impregnated with mild, but effective pesticides can be used, provided that air circulation is not impeded. Spraying with insecticides in some areas is a necessary, if unpleasant, preventive measure for maintaining a culture area free from carriers.

If contaminated cultures occur in large numbers and "carriers" are not proven to be the cause, transferring techniques need to be examined. All objects in the hood should be sprayed with 70% alcohol. The immediate working area should be kept clean and uncluttered and care taken to prevent breathing on or touching the callus with a non-sterile surface (hands, hair, counter, etc.). Instruments used in the TCCP laboratory are no longer flame sterilized, which dulls and ruins forceps and scalpels, but are autoclaved in surgical trays wrapped with heavy cloth.

Plantlet Establishment in the Greenhouse

Each crop species has unique growth requirements and therefore specific establishment techniques must be refined for individual crops; however, a general plantlet establishment technology has been developed in the TCCP greenhouse which has application to a wide number of species.

Regenerated plantlets must have developed sufficient root and shoot growth in order to survive in a greenhouse environment. This varies by species, but as a general rule the roots should be a minimum of 2 cm in length before transplanting will be uniformly successful.

Tepid water is added to vials of plantlets to begin softening the agar. This is allowed to stand for about 30 minutes. The softened agar is then loosened using a dull spatula and the plantlets are gently removed from the vials. It is important to thoroughly rinse all agar away from plantlet roots with tepid water as any agar left on the roots could be a source of contamination. Plantlets are carefully separated from the callus taking care not to detach the shoots at the crown. Once individu-

al plantlets are separated, any stressed leaf or root tissue and any residual callus is trimmed away before transferring to soil.

Plantlets of hardy crops with well-developed root systems can be planted directly into pots of a soil or soilless mixture. Best results are obtained by placing these plants in a shaded area and keeping them moist for a period of five to ten days before moving into direct sunlight. Difficult to establish species or plantlets with poorly developed root systems are initially transferred into small pots of vermiculite and watered with a dilute nutrient solution including NAA and IBA (Vitastart® 1 T/gal). These are placed in a shaded, high humidity environment for one to two weeks and are kept moist until good root growth is established. After this time, the plantlets can be transplanted into a soil or soilless mix. Most of the difficult to establish species benefit from being placed back into a shaded, high humidity area for about one additional week. Fertilization is generally begun after the plants are moved into full sunlight.

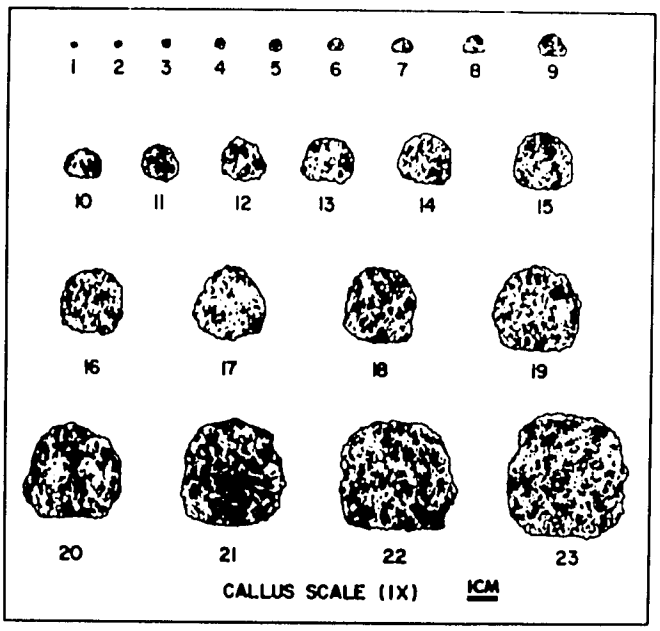

Fig. 52. Clay models used to estimate callus size.

Appendix IV: Stress Selection *In Vitro*

Plant cell cultures are ideal systems for selecting variants because of several unique characteristics: (1) a large population of cells can be readily screened; (2) screening generally requires a small space and less time than that required for whole plant screening; (3) the nutritional and physical environments can be strictly controlled; (4) selective agents are easily administered; (5) the relatively non-differentiated state of culture systems avoids the complications of different responses associated with developmental stages; and (6) several systems — callus, cell, protoplasts, pollen, meristem culture — can be employed in selection schemes.

Another important and now widely recognized characteristic is that plant tissue culture systems are inherently variable. Spontaneous or "natural" mutations pre-existing in the explant donor tissue are one source of variation; the mutagenic influence of the culture environment is another. The genetic variability observed in cultured plants regenerated from cells has been defined as mutaclonal or somaclonal variation. (Gametoclonal variation is the genetic variability observed in plants obtained from cultured haploid cells.) The degree of variation observed is largely determined by the genetic constitution of the particular cell population which is regenerated, as well as the recovery of the variation in regenerated plants. For example, callus cultures can exhibit extensive chromosomal observations, but most regenerated plants (from short-term cultures) are phenotypically normal. This implies that either the variability in the callus is not always recovered during the regeneration stage because normal cells are the ones with regenerative capacity, or that observed chromosomal changes do not adversely affect regenerated plants.

At this point, it is not resolved whether the somaclonal variants recovered out of tissue culture are different from mutations previously observed by breeders in whole plants. Some authors have reported the expression of novel mutations in regenerated plants (Evans and Sharp, 1986), but the majority of published reports describe mutations due to, chromosomal number variation, single gene mutations including dominants, semi-dominants, and recessives, mutations resulting from mitotic crossing over, and mutations in chloroplast DNA (Larkin *et al.*, 1985).

Despite reports of extensive amounts of heritable somaclonal variation, mass production of regenerates — some of which might be variant — might not be the most efficient means of selecting variants with specifically altered traits. A more appropriate means of obtaining mutants is selection *in vitro;* that is, applying some selective agent that permits the preferential survival and/or growth of a desired phenotype in an otherwise lethal environment.

Suspension vs. Callus

Ideally, suspension cultures are best suited for selection procedures since virtually every cell is in contact with the selective agent. In cereal systems, however, reliable regeneration of liquid cultures has not yet been achieved, partly because E cells have not been maintained in liquid cultures. While suspensions are relatively easy to initiate, the lack of regeneration is a severely limiting factor in their use in selection systems.

The alternate system is callus cultures. Most callus cultures are established using diploid tissues (*e.g.*, embryos, leaf segments), but pollen cultures are also possible. Since pollen culture is a haploid system, recessive traits can be immediately recovered in the regenerated plant. In diploids, the progeny of regenerates must be screened in order to observe recessives.

Selection Schemes

The length of selection is an important criterion. Since selection agents allow for the preferential growth of variants, several generation times are required for the few mutant cells to become the dominant cell type in the population. In callus cultures, this usually involves 10-12 months. In suspensions, in which cell doubling times are usually faster and the selective agent is more extensively distributed, three to four months is minimum. Some investigators have combined both liquid and solid phase selection systems in order to take advantage of the more dispersed state of the selective agent in liquids, and the regenerative capacity of the callus culture. Long-term cultures can be maintained on one concentration level or selective agent, or increased step-wise over time. The time required before the selective agent is increased in concentration is usually determined by the time required for doubling of fresh weight of the callus mass.

An alternate selection route is short-term exposure to very high, essentially lethal concentrations of selective agents. This procedure allows for the survival of those cells which possess an altered genotype, and minimizes the risks of selecting cells with physiologically adapted phenotypes. Acute exposure also minimizes the chromosomal aberrations that accumulate with long-term culture.

The level of a selective agent used must be determined empirically; that is, for any crop, E callus must be initiated/maintained on a level of stress which reduces growth. The initial reduction in growth should be at least half of the controls, with 80-90% decrease in growth ideal. In some cereal systems that require two to three months for production of significant quantities of embryogenic callus, it may be more efficient to initiate callus first, then transfer callus to selective media.

Growth Measurements

Selection for tolerant callus cultures can be measured by callus weight or by percent callus weight of controls. Since plant regeneration is important, the total number of plants regenerated or plants regenerated per gram of callus should also be considered. Visual assessment of the callus is also an important criteria. Embryogenic callus must be maintained during the selective process if regeneration is to be ultimately achieved. Cells which are resistant to very high levels of stress but cannot be regenerated are of little use to the breeder; the breeder wants plants, not cells!

Direct vs. Indirect Selection

For most mineral stress selection studies, the mineral can be added directly to the culture medium (*e.g.*, NaCl, $AlCl_3$, $ZnCl_2$). Drought tolerance may be co-selected with salt tolerance, but otherwise needs to be approached indirectly using a compound which simulates osmotic stress, for example, polyethylene glycol (PEG). Similarly, selection for chlorate tolerance may be an indirect selection for altered nitrate reductase metabolism. Chlorate is a toxic analogue (or substitute) for nitrate, which is metabolized (chemically reduced) by the cells to chlorite or "bleach", and is thus fatal to cells. Cells which have an altered nitrate reductase metabolism — in that only the substrate nitrate is very specifically reduced — will not reduce chlorate and thus those cells will survive.

Proline analogues can be used for selecting cells with altered proline synthesis. Proline synthesis is controlled in a feedback fashion. A proline analogue can mimic proline in a sense that it will feedback to the synthesis cycle the information that sufficient amounts of proline-like material are available, and this will shut down the synthetic processes; however, proline is an essential amino acid, and analogues will not meet specific physiological needs, thus cells will die due to lack of proline. Cells with an altered proline feedback system will continue synthesizing proline, even in the presence of analogues which would normally shut down synthesis, and thus these cells will live in the presence of lethal amounts of analogues. The selection of cells which over-synthesize or accumulate proline can be used for salt or drought stress studies. Since proline accumulation has been associated with salt tolerance, this selection method allows for an indirect way of obtaining salt-tolerant mutants.

Salt Tolerance

Halophytes ("salt lovers") are able to grow in environments where the salt levels normally restrict the growth of glycophytes ("non-salt lovers"). Salt tolerance by halophytes is divided into those which accumulate salt and those which can regulate the ratio of salt to water to maintain the plants proper water potential. In the plants which accumulate and store salt there is an exclusion of salt to some extent, probably by the endodermal layer in the roots. Those plants which regulate the osmotic potential do so by swelling to store more water, by accumulating organic compounds, such as proline, in the cytoplasm, or by exuding the salt through the leaves (Salisbury and Ross, 1978).

Drought Tolerance

Jones *et al.*, (1981) have identified three main classifications of drought resistance: (1) those plants that escape drought by completing their life cycle while moisture is available; (2) those plants which tolerate drought with high tissue culture potential by maintaining water uptake to meet the demands or by reducing water loss with stomata control or waxy covering of the leaves; and (3) those plants which are drought-tolerant and have low water potentials in the tissue because they accumulate organic solute or resist protoplast desiccation.

Aluminum Tolerance

In soil and liquid solutions, Al^{+3} lowers pH by displacing H^+ ions from binding sites into the solution. The mechanism of toxicity in plants appears to be more complex than this, since many plants can withstand low pH but not Al^{+3}. Theoretically, aluminum ions are taken up by the root and lower the pH in the cytoplasm, thereby causing cell death.

Temperature Stress Tolerance

Chilling and freeze injury must be dealt with by tolerance mechanisms. These mechanisms are poorly understood, but the cell wall as well as the plasma membrane play important roles. High temperature stress can be "avoided" somewhat by plants, for example, by decreasing respiration or decreasing absorption of radiation energy. Tolerance mechanisms are known in that plants will synthesize heat shock proteins which appear to "buffer" the effects of high temperature. Temperature stresses can be selected for directly by applying cold/hot temperatures to cultures.

Stress Selection Strategies

The majority of stress selection experiments conducted at the TCCP have involved NaCl stress, although investigations

with $AlCl_3$ and PEG have also been accomplished. For any of the stressing agents utilized, initial experiments involved a stress selection strategy of long-term culture on growth-restricting levels of agent. Assessment of callus growth in long-term cultures is difficult because callus quantities fluctuate. Also, plants regenerated from long-term cultures have increased rates of chromosomal aberrations. Thus, to ensure a comprehensive approach to selecting variants, several strategies were developed for *in vitro* use at the TCCP. Each scheme has a termination point dictated by the regenerability of E callus. It is important that plants are regenerated from stressed cultures, grown to maturity in the greenhouse, and progeny screened as soon as possible since whole plant screening of regenerate progeny is vital for feedback to any *in vitro* method for stress selection.

Long-term Stress Selection

Embryogenic callus is repeatedly subcultured on medium containing a minimum of 9 g/l NaCl. Regeneration is attempted after six passages if the selection pressure is 9 gm/l NaCl, earlier if higher concentrations are used. Total E callus fresh weight is assessed each passage; an approximate equal fresh weight amount is subcultured onto fresh medium. Experiments are continued only as long as regeneration is possible.

Long-term, Step-wise Stress Selection

Embryogenic callus is repeatedly subcultured on medium containing salt. Step-wise increases can be done in two ways: (1) salt concentration can be increased every second-third passage. This approach requires minimal quantification since only total embryogenic fresh weights are measured at the end of each passage, and an approximate equal fresh weight amount subcultured; or (2) salt concentration is increased only when a culture doubles its fresh weight. This approach requires more extensive effort in tracking cell lines, and individually weighing cultures. For either method, selection is continued only as long as E callus remains regenerable. The minimal initial salt level used is 6 g/l, with 2-3 gm increases used.

Killer Level of Stress Selection

Embryogenic callus is plated on medium containing lethal salt levels (1-2% NaCl). Callus may be small clumps of embryoids or chopped into small pieces. Selection is continued as long as E callus remains regenerable. To date, regeneration has been achieved after up to four passages on medium containing 1.2% NaCl.

Liquid/Agar Combination Selection

Embryogenic callus is added to liquid saline culture medium. Usually 0.5 g of E callus (separated into small clumps, but not chopped) is used to initiate a culture. Every two to four days, clumps of callus are plated out on either regeneration medium (with or without salt), or saline agar medium (at the same or higher salt concentration used in liquid culture). In liquid culture, medium is poured off and fresh medium added every two to four days. The salt concentration in liquid culture is stepped-up 1-2 g every second medium change. To date, callus clumps generally do not remain regenerable past three weeks in liquid culture; 9 g/l NaCl has been the highest salt concentration tested in liquid culture.

Suspension Selection

Embryogenic callus is used to initiate suspensions. Suspensions are then maintained at a constant or step-wise level of stress. Since long-term regeneration from cereal suspensions is not often possible, experiments are maintained as long as the cultures survive. Quantification of cell growth is by fresh weight or packed cell volume.

Media Preparation

NaCl

Although environmental salt stress results from a mixture of excess ions, NaCl is used as the selective agent in TCCP experiments because Na^+ is usually the toxic ion in abundance. Media containing NaCl are easily prepared by adding NaCl directly to the basal medium prior to autoclaving.

$AlCl_3$

The readily available form of aluminum, $AlCl_3$, is used in TCCP experiments. If $AlCl_3$ is added directly to the basal medium, however, solidification by agar cannot occur because the agar is hydrolyzed by the high sterilization temperatures and low pH caused by the $AlCl_3$. If the medium is not buffered prior to autoclaving, the aluminum will precipitate out of the medium. If the basal medium is autoclaved, and allowed to cool slightly, $AlCl_3$ can be added without the agar being hydrolyzed, and the medium will gel. The addition of $AlCl_3$ to basal medium requires that certain steps be followed. First, a stock solution 100 times more concentrated than that required in the final medium is prepared. The basal medium is then prepared and autoclaved along with the separate aluminum stock solution. Following sterilization, either before or after the media have set, the aluminum is dispensed into the culture vessels at the rate of 0.1 ml per 10 ml. If addition of the aluminum is done before the medium solidifies, the cultures are ready to use; however, if it is added after the agar has gelled, the medium must set for 2-3 days to allow the aluminum to diffuse through it. Analysis of agar slices confirms that diffusion of $AlCl_3$ occurs.

PEG

Selection for drought tolerance is accomplished by using a high molecular weight osmoticum as the stressing agent. Polyethylene glycol (PEG), of approximately 4000 molecular weight, is the osmoticum of choice because it competes with the callus tissue for available water in the medium without penetrating the tissue. High concentrations of PEG also inhibit agar from gelling.

A two-step method has been developed to prepare PEG in solidified media. The first step involves mixing the major minor mineral salts, vitamins, sucrose, and agar. The PEG is dissolved in water to make-up the remaining volume. Both components are separately autoclaved, cooled slightly, and poured together. Aliquots are dispensed into individual culture containers. The PEG makes the medium very viscous and dispensing is difficult. Very large, pore pipettes must be used, or the media can be hand poured into vials previously marked for volume.

Appendix V: Publications

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