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EXECUTIVE SUMMARY

This Annual Report provides a thorough summary of current Project efforts in a number of areas related to research, training, technical assistance, and cooperative research in tissue culture and plant biotechnology. Between October 1986 and October 1987, the Tissue Culture for Crops Project has made major progress in meeting the objectives of our workplan. Despite a core budget reduction of 10%, the Project has remained on track toward all important goals. Several significant achievements should be noted.

The TCCP Training Program continues to produce well-trained, enthusiastic graduates from the Fort Collins facility. In addition, with the cooperation of CATIE, a joint training course in Spanish was taught for the first time in Costa Rica. Fifty applicants applied to attend the course, and twenty were accepted. This course supplies a major need for plant biotechnology training in Central and Latin America and will be repeated in the coming year.

In January, 1987, the TCCP held its Second International Plant Biotechnology Network (IPBNet) Conference in Bangkok, Thailand. Seventy-nine scientists from eighteen countries attended the week-long conference, presented scientific papers and posters, and participated in discussions about plant biotechnology in the developing world. Plans were begun for the next IPBNet Conference, to be held in Nairobi, Kenya in January, 1989.

Interest in the IPBNet continues to grow. At present, the Network has over 1000 affiliated members from 90 countries. The TCCP fulfills a major need of its members in supplying information about current scientific literature as well as simple, repeatable methods to establish tissue cultures, regenerate plants from them, and obtain stress-tolerant

mutants for field testing.

In terms of research, the TCCP continues to provide important new methodologies in the areas of cereal and legume tissue culture. Major breakthroughs in the regeneration of tropical grain legumes from suspension cultures have been achieved. The lab continues to be the world leader in the development of high-frequency, long-term plant regeneration methods from cereals.

Potentially stress-tolerant TCCP germplasm of millet, sorghum, and rice is now in field tests around the world. For example, rice field testing for salinity tolerance is now entering its second year in the Philippines under a collaborative agreement with the University of the Philippines at Los Baños (UPLB). A number of TCCP lines have been advanced from preliminary testing and look quite promising.

In Georgia, field screening for aluminum tolerance and for resistance to fall armyworm in sorghum have both yielded excellent results from tissue culture-generated, TCCP germplasm. In the case of the fall armyworm, resistance at the level of TCCP material has not been seen in the field before and represents a significant improvement. Following more greenhouse and field testing in the coming year, we hope to release the resistant germplasm to breeders and growers.

Finally, under a new "Special Focus," Project scientists have begun investigating the use of protoplast fusion and the techniques of molecular biology to transfer salt tolerance from halophytic forage-grown and wild cereal relatives to cultivated lines. This work represents an extension of ongoing Project efforts—in cooperation with CIMMYT—to use tissue culture to increase the rate of gene introgression from wild wheat relatives into cultivated wheat.

STATEMENT OF GOALS

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. 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, the five-year Cooperative Agreement was funded. Project Objectives included: 1) Development of methods for establishment of tissue culture and high-frequency regeneration of plants in rice, wheat, millet, corn, sorghum and several tropical legumes; 2) Utilization of *in vitro* selection 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, and 5) Formation of the International Plant Biotechnology Network (IPBNet).

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

RESEARCH HIGHLIGHTS

Research at the TCCP covers a wide range of topics from laboratory to field testing. Recent accomplishments are highly significant breakthroughs unparalleled, to the best of our knowledge, in the world.

Tissue culture of rice and other cereals remains a tough problem. Regeneration can be achieved in most laboratories, but this capability usually is limited to cultures of short duration. At the TCCP, two culture lines continue to produce plants after culturing for nearly five years. The lines are IR-36, which was cultured for 47 passages, and Mahsuri, and indica-japonica hybrid, which was cultured for 66 passages.

Grain legumes are a group of crops which have proven to be extremely difficult to regenerate. This year at the TCCP, several cell lines from three species have been established and plants regenerated for more than one year. The development of this technology is a major achievement since biotechnology can now be applied to these crops. One species, moth bean, has been cultured in 1 and 2% NaCl for one year and plants have been regenerated.

The ultimate proof of the use of *in vitro* systems, of course, is the expression of a new trait under field conditions. Evaluation of R_2 sorghum is showing tolerance to acidic soils with toxic levels of aluminum, to fall armyworm, and to the sorghum midge. The existing germplasm of sorghum contain one line which will survive on acidic soils but it is not a good breeding line. The TCCP-produced, tolerant line can be used as breeding material throughout the world. There is no known line tolerant to either the fall armyworm or sorghum midge. The results of these field studies are beginning to demonstrate the value and effectiveness of tissue culture technologies for plant breeding and culture.

Summary of Research at the TCCP in Grant Year Three.

Crop	*In vitro* research	No. Plants Established in greenhouse from cultures selected on: NaCl	AlCl	Other	Field Sites Evaluation	Organization
Rice	anther culture, media pH, protoplast suspension, screening for rice blast	523	332	100 from suspensions	NaCl	UPLB Philippines
				151 from non-stressed	NaCl	NIAB Pakistan
					seed increase for Al	CIAT Colombia
Wheat spring wheat unless noted	suspension culture, initial culture of 5 winter wheat cultivars, selection levels of AlCl	2721	--	209 from non-stressed cultures	seed increase for NaCl	CIMMYT Mexico
				408 from non-stressed cultures of winter wheat		
Sorghum	initial culture of 2 new cultivars, improvement of callus initiation		171	248 from $KClO_3$	NaCl	U. of Arizona Yuma, AR.
				17 from hydroxy-proline	Al	U. of Georgia Griffin, GA.
				22 from non-stressed cultures	insect	U. of Georgia Tifton, GA.
					drought	INTSORMIL Niger
Millet	initial culture of 3 new cultivars, improvement of callus initiation	141	118	338 from non-stressed cultures	seed increase for drought	Kansas State U. Hays, KS.

Summary of Research at the TCCP in Grant Year Three.
(Continued)

Crop	*In vitro* research	No. Plants Established in greenhouse from cultures selected on:			Field Sites	
		NaCl	AlCl	Other	Evaluation	Organization
Oats	--	--	--	--	seed increase for NaCl	U. of Saskatchewan Canada
Moth bean	suspension culture	89	--	--	--	--
Tepary bean	suspension culture	29	--	--	--	--
Pigeon pea	suspension culture	65	--	--	--	--
Dry bean	suspension culture	--	--	--	--	--
Cowpea	suspension culture	--	--	--	--	--

RESEARCH AND DEVELOPMENT

The research at TCCP includes improvement of tissue culture systems for long-term culture and plant regeneration, use of callus cultures for selection for tolerance to stress, development of suspension and protoplast technology, and biochemical analysis. This research is performed by graduate students, trainees, visiting scientists, and the full-time staff at TCCP. The TCCP also supports, by subgrants, research in these areas in other laboratories.

TECHNOLOGY DEVELOPMENT FOR TISSUE CULTURES, SELECTION, AND PLANT REGENERATION

Basic research to define culture conditions required for development of tissue cultures capable of long-term culture and retention of the ability to regenerate plants is an essential part of the TCCP research. This research includes new technologies such as suspension and protoplast cultures as well as improving existing technologies in callus cultures and developing *in vitro* regeneration procedures for new germplasm.

Improvements in Tissue Culture Technology

For the cereals, most of the basic tissue culture technologies are known for callus cultures; however, there is still a need to improve basic methodologies, and to define conditions for new crops and cultivars. In the legumes, regeneration systems are being developed at TCCP for use in stress selection experiments.

Rice. Many graduate students, full-time staff, and subgrants are associated with rice at TCCP. Research spans from protoplast and anther culture to long-term maintenance and regeneration.

Long-term rice culture. Two sets of long-term rice callus cultures

continue to be maintained and tested for their regeneration capabilities. The cultures of the rice cultivar Mahsuri have been maintained for 66 passages as of October 1987 and IR-36 cultures have been transferred for 41 passages. Both sets of cultures are divided and placed on regeneration media every two passages in addition to subculturing to new maintenance media every passage. Although regeneration rates have continued to slowly decline in the last twelve months, the last regeneration attempt yielded five plants per gram of E callus for both Mahsuri and IR-36. All regenerated plants are grown to maturity in the greenhouse.

Conditioning Factor. There seems to be an optimum pH that is desired by the tissues growing under *in vitro* conditions. The best pH for the callus induction from the scutellum of mature IR-36 seeds is between 4.8 and 4.9. The pH of the medium was buffered in this range using 0.5 mM citric acid along with lowering the sugar level to 1.5% from 3.0%. It has not been possible to maintain the E calli for long periods on this modified medium.

Mature seeds of IR-36 were dehusked, surfaced sterilized with 70% commercial Clorox for 30 min., rinsed three times with sterile de-ionized water and plated singly on 10 ml of solidified LS medium contained in vials. The cultures were incubated under a light regime at 23$\pm^{o}$C. After 28 days, callus was harvested from each vial, separated into E and N callus and Weighed individually. The pH of the medium in each vial was also observed. Embryogenic callus was subcultured again on callus initiation medium.

The results of the experiment conducted to find out the optimum pH for callus initiation has shown that a pH of 4.8 - 4.9 was optimum (see

1987 Progress Report). Although there are noticeable variations between experiments, there was a basic pattern that was observed in all three experiments. Based on this observation, the medium was buffered at this pH range. An observation of pH indicates that the pH increased in sucrose level. The result of these trials are presented in Tables 1, 2, and 3. The result in Table 3 indicates that a combination of citric acid buffer at 50 mM and sugar level at 1.5% produces more E callus than other combinations or the control.

Table 1. Effect of different levels of buffer (citric acid) on the initiation of E callus from the rice cultivar I-36.

Buffer (mM)	E callus (av.-mg.)
0.0	10.8966[a*]
2.5	9.6922[a]
5.0	11.8408[a]
7.5	5.4388[a]
10.0	

* means followed by different letters are significantly different at the .05 level (error d.f. was not large enough to detect any variation).

Table 2. Effect of different levels of sugar on the initiation of E callus from the rice cultivar IR-36.

Sucrose (%)	E callus (av.-mg.)
0.0	3.2428[c*]
0.50	5.2133[bc]
1.00	9.3748[b]
1.50	19.7375[a]
2.00	8.9428[bc]
3.00	8.2711[bc]
4.50	3.3200[bc]

* means followed by different letters are significantly different at the .05 level.

Table 3. Effect of the interaction of citric acid and sucrose on the initiation of E callus from the rice cultivar I-36.

Citric acid	Sucrose Percentage						
	0.25	0.50	1.00	1.50	2.00	3.00	4.50
0.0 mM	6.196	11.347	12.986	24.678	11.469	5.283	4.317
2.5 mM	3.225	3.389	15.561	19.765	4.381	18.038	3.487
5.0 mM	2.916	5.675	8.869	34.550	22.692	4.042	12.425
7.5 mM	1.117	3.567	5.853	11.727	3.550	10.003	2.255
10.0 mM	2.761	3.155	6.104	7.967	2.622	3.989	2.400

There were 5 vials per treatment and the treatments triplicated. The set pH of the media was 4.85. Control yielded 14.560 mg of E callus per vial (average). The pH was 5.5 and there was no buffer. The media was 3.00% sucrose.

When this modified media was used to maintain the callus, ti was not possible to maintain the callus for more than two passages. Experiments are in progress to identify this negative factor.

Anther Culture of Rice. Japonica types of rice are more amenable for anther culture than indica types. There seems to be a genetic potential that could be manipulated for inducing callus from anthers. The results show that callus induction from anthers can be improved by optimizing certain physiological factors such as tiller position, media composition or osmotic stress.

Six rice varieties, IR-36, IR-60, Pokkali (indica types), Mahsuri (indica x japonica hybrid), Calrose and Yamahoushi (japonica types were grown under greenhouse conditions from March to September 1987. The temperature was 24 to 30°C and the relative humidity was 65±5%. IR-36 and IR-60 started producing inflorescence 100 days after planting (DAP), yamahoushi by 110 DAP, Mahsuri and Calrose by 125 DAP. Pokkali did not mature even after 180 DAP.

Florets bearing anthers of the size determined to contain cells at uninucleate stage by chromosome analysis, were surface sterilized for

seven minutes using 100% commercial Clorox and rinsed in sterile deionized water. Twenty-four anthers were plated on 10 ml of solid callus induction medium contained in a vial, and were incubated at 23 $\pm 2^{o}C$ in the dark.

The best response was noticed in Yamahoushi. Calli were noticed on 10% of the anthers plated. Callus initiation in Calrose was 4%, in IR-60 was 2%, in Mahsuri was 1% and in IR-36 it was 0.1%. These results reaffirm that japonica cultivars produce anthers that are more amenable to tissue culture techniques than indica cultivars.

A significant observation was made that most of the anthers that callused could be traced to a few individual plants, regardless of what treatment they were on. This indicates that there could be some gene(s) that control callus induction response from anthers; however, the frequency of anthers callusing was also influenced by the medium. IR-36 responded only on N6 medium, while Ir-60, Calrose, Mahsuri and Yamahoushi produced more calli from anthers plated on B5 medium (Table 4). It is surprising that N6 induced better responses in an indica rather than a japonica cultivar since N6 was developed using a japonica cultivar.

Table 4. Callus induction response from anthers.

Cultivar	% callused N6	B5	L&S
IR-36	0.4	0	0
IR-60	1.9	3.2	0.5
Mahsuri	N.T.	3.6	0.3
Calrose	0.5	7.1	0.9
Yamahoushi	0	10.2	0

N.T. - Not tested.

Another important physiological factor is the effect of the position of the tiller. It is reported that primary tillers are better than

secondary tillers. This was tested in cultivars IR-36, IR-60, Mahsuri and Yamahoushi. The results indicate that primary tillers of IR-60 and yamahoushi do indeed produce more anthers that produce callus, while in IR-36 and Mahsuri the tiller position was not a significant factor. In fact, in the latter two varieties, secondary tillers produced more anthers formed callus. From these results, tiller effect could be genotypic specific.

Cultivar IR-60 was used to study the effect of mild osmotic shock on anthers. The result is tabulated in tAble 5 and it appears that the effect is modified by the media used.

Table 5. Effect of pretreatment of anthers on callus initiation in IR-60

Media	24 h mild osmotic shock			No shock control		
	Plated		Callused %	Plated		Callused %
N6	240	0	0	346	4	1.157
LS	240	1	0.416	358	1	0.279
B5	120	5	4.167	292	0	0

Millet. Obtaining new germplasm of inbred lines of pearl millet and the development of media for those cultivars are the most recent objectives included in this project. To date, TCCP has acquired three new inbred lines of millet that will replace the old cultivars which were random mating populations (HMP-559 and Senegal Bulk). The addition of new seed stock deemed necessary as variability within the old lines was large and few breeders utilized the germplasm.

One inbred line (Tift 23 DBE, obtained from Mr. Bill Stegmeier, Hays, Kansas) is the subject of a sterilization, media, and regenerability study. The two inbred lines of millet (Line #2069, Line #7911, also obtained from Mr. Stegmeier) are presently being grown to maturity

in the greenhouse to 1) increase seed stock, and 2) to obtain cleaner seed for in vitro testing. Culturability studies on these lines will resume when a sufficient amount of seed has been produced, making in vitro experimentation possible.

Culturability Studies. The induction of embryogenic callus by the standard initiation procedure developed for pearl millet cvs. HMP and SB does not have general application to all cultivars of pearl millet. The objective of the initiation studies was to perform media manipulations that would enable the production of sufficient E callus growth for use in stress selection experiments by cell line.

Whole seed was used as the explant source for this trial. Seeds were rinsed in various concentrations of Clorox (standard protocol is 20% Clorox) or exposed to HCl gas. Nine media combinations utilizing varying levels of 2,4-D, IAA, and a constant level of KIN were used. These studies were performed on the Tift 23 DBE and Line # 2069.

The chlorine gas treatment resulted in a high degree of contamination and severely reduced seed viability (Table 6). The most effective treatment for the sterilization of millet seed proved to be a 30-second rinse with soap followed by a 95% ethanol rinse and a 30 minute rinse in Tween 20 and 40% Clorox accompanied with fairly vigorous stirring.

There are three different callus types formed in millet: 1) creamy white, dense, compact, nodular, 2) yellow, nodular, compact, and 3) green, nodular, and compact. As in other cereal crops, the creamy white callus is embryogenic and will produce plantlets. From preliminary investigations, it is known that the yellow callus will also produce

plantlets, but at a greatly reduced rate compared to the true E callus. The production of plantlets from green callus is extremely limited. Studies are being continued to further determine whether the latter two callus types produce enough plantlets to benefit the millet *in vitro* program.

Table 6. Hormonal composition of LS media for embryo initiation from whole seed after 5 months in culture.

Cultivar	Media mg/l	E callus (%)	Green callus (%)	Yellow callus (%)
Tift	*3D1A.3K (1)	0	69.0	27.3
	5D1A.3K (2)	1.3	45.8	52.7
	7D1A.3K (3)	16.6	8.8	74.5
	3D2A.3K (4)	14.6	42.2	44.0
	5D2A.3K (5)	11.3	25.0	63.8
	7D2A.3K (6)	3.8	18.8	78.8
	3D3A.3K (7)	16.6	44.1	39.2
	5D3A.3K (8)	1.5	29.7	53.1
	7D3A.3K (9)	43.9	10.0	46.2
Line #2069	(1)	0	4.7	95.2
	(2)	0	0	100
	(3)	0	0	100
	(4)	12.7	3.1	84.1
	(5)	0	5.8	94.0
	(6)	0	0	100

* D=2,4-D
A=IAA
K=Kinetin

Sorghum. The standard initiation procedure for inducing E callus developed for sweet sorghum cvs. Keller and Rio, and grain cvs. Hegari, Rtx 430, and RTx 700 is not universally applicable to all grain sorghum. The objective of the initiation experiments was to determine what modifications of protocol were necessary in order to reliably produce sufficient amounts of E callus by cell line for use in stress selection experiments.

In these experiments, only mature embryos were used. Seeds were

washed in different concentrations of Clorox (standard protocol utilized 30% Clorox) or exposed to chlorine gas for 1-4 hours. The standard initiation media was used (L salts, 2 mg/l 2,4-D, 0.5 mg/l KIN). The results of efforts with four cultivars, using three treatments, is summarized in Table 7. Although chlorine gas treatment most effectively eliminated contamination, embryo viability was severely impaired. The standard 30% Clorox treatment was most effective if preceded by a ten minute rinse in Tween-20 and a 15 second rinse in 95% ethanol. It is important to completely excise the embryo from the seed coat and endosperm tissue. The addition of activated charcoal to absorb phenolics produced by sorghum callus cultures, does not appear to enhance E callus formation.

Work in progress is designed to further adapt and optimize the initiation of E callus by cell line of four target cultivars: Hegari, SC283, RTx 430, and M-35-1. Embryogenic cell lines generated from this effort will then be utilized in critical levels experiments for $AlCl_3$ and PEG (selective agent for drought-tolerant variants).

Table 7. Initiation of mature sorghum embryos for E callus formation.

Treatment	Cultivar	% Contamination	Callus Formed	E Callus at 3 passages
30% Clorox	RTx430	0	Yes	Yes
	SC283	10	Yes	Yes
	Hegari	0	Yes	Yes
	M-35-1	40	Yes	Yes
50% Clorox	SC283	22	Yes	Yes
Chlorine gas	SC283	0	Limited	Yes
	RTx430	0	None	None
	M-35-1	90	Limited	Yes

Winter Wheat. Winter wheat is supported by an external grant from the Colorado State Experiment Station, USDA. The goal of this project is to select for increased drought tolerance of some important winter wheat cultivars of Colorado, using in vitro selection methods. The specific objectives are as follows: 1) To establish efficient methods of regeneration from callus cultures; and 2) In vitro selection for drought-tolerant cell lines using PEG 8000 as the stress agent.

Five cultivars of Colorado winter wheat (Hawk, Bennett, Baca, Vona, and Tam 101) were screened from regenerability from callus cultures initiated from mature embryo explants. Seeds were sterilized with 20% commercial bleach solution, soaked overnight, and embryos were dissected under sterile conditions and placed on L media solidified with 0.9% agar. The basic medium used was LS supplemented with several different combinations of 2,4-D and BA or KIN. Callus induction on each media type was assessed after culturing the explants for six weeks in continuous light.

These media experiments indicate that the responses observed in callus production is cultivar specific (Figs. 1-3). From the data it is clear that callus growth is greater at 1 mg/l 2,4-D, but 2mg/l may be optimal for callus production in all cultivars except Baca. Baca shows a relatively poor response to callus induction in all media tested. It is also seen that Vona and Hawk show improved callus production when the medium is supplemented with a cytokinin. Kinetin and BA at 0.5 mg/l give enhancements to Hawk and Vona respectively.

Two types of callus from mature embryos have importance in plant regeneration. The initial callus type is referred to as smooth E callus and is identified by its cream color and compact nature. The second

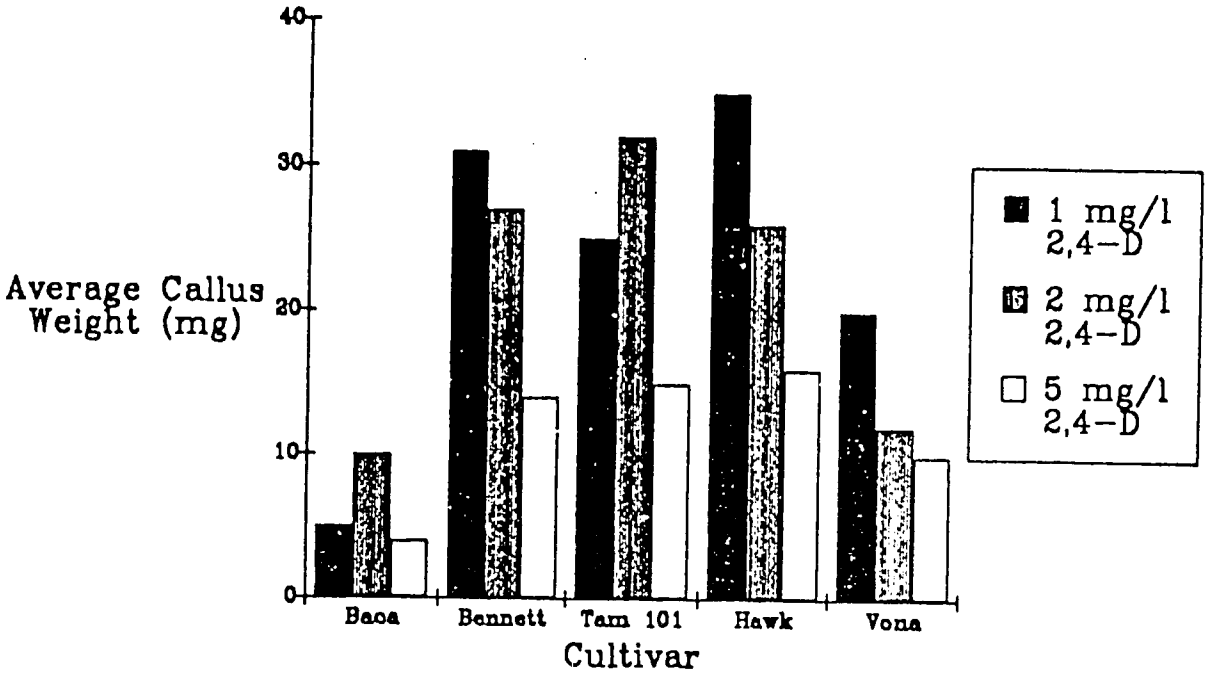


Figure 1. Average callus weights of five winter wheat cultivars with 2,4-D added to the basal media.

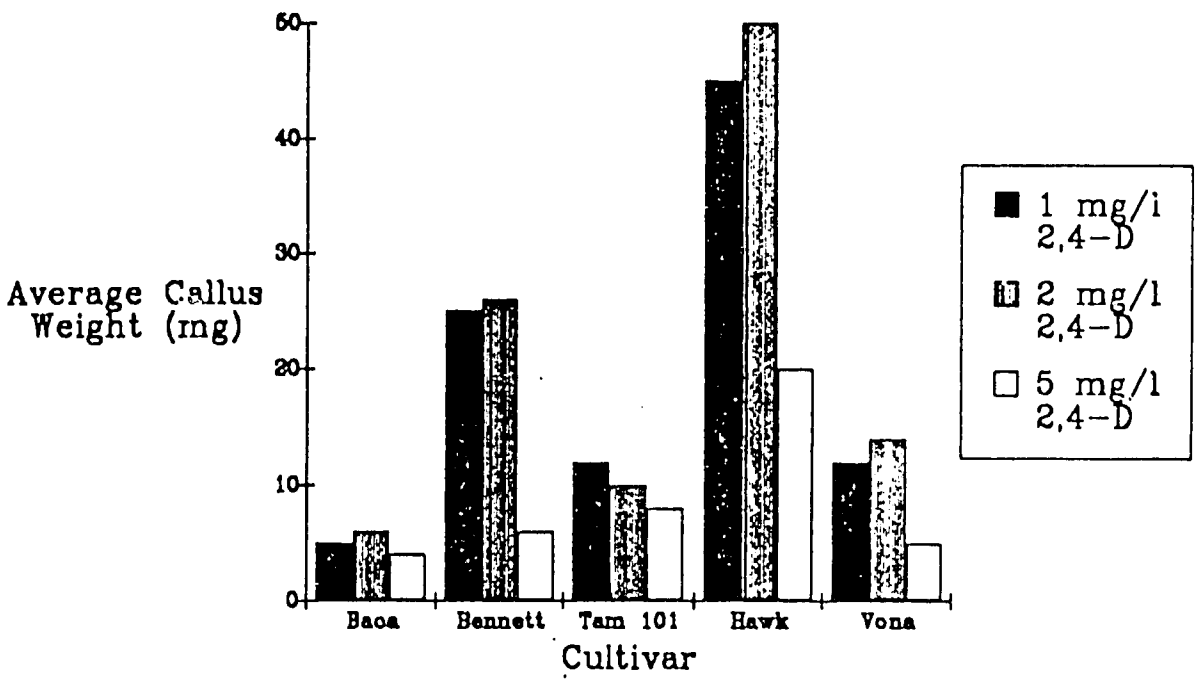


Figure 2. Average callus weights of five winter wheat cultivars with 2,4-D, and 0.5 mg/l KIN added to the basal media.

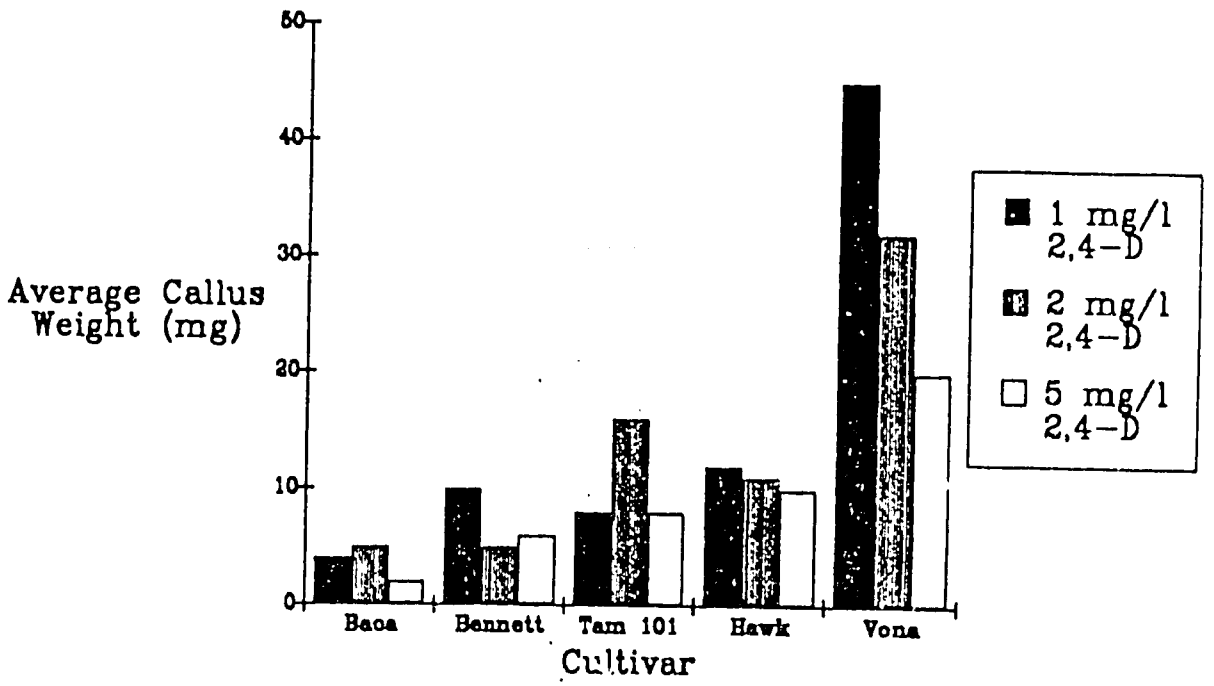


Figure 3. Average callus weights of five winter wheat cultivars with 2,4-D and 0.5 mg/l BA added to the basal media.

type, which appears to be derived from the smooth E, is called green embryoid. This callus type has develop green areas from which plants are regenerated. Individual pieces of callus may have both callus types. Callus type is determined by genotype and growth regulator concentration. Cytokinins, both BA and KIN, increase the proportion of the green embryoid callus type over 2,4-D alone (Table 8). Spontaneous regeneration is limited with increase in 2,4-D concentration. This may be important for developing long-term cultures of E callus. Hawk, Bennett and Tam 101 are all good with 2,4-D alone for their E callus response, while TAM 101 response is improved with the addition of BA. Plant regeneration is enhanced when BA or KIN is added to the maintenance media, especially KIN (Table 9). These plants must go through a four to six week vernalization period at 4°C to induce floral buds for seed set.

Table 8. Percent of cultures with different callus types from different cultivars with 2,4-D alone, or in combination with 0.5 mg/l BA or 0.5 mg/l KIN.*

Cultivar	Smooth Ξ callus			Green Embryoids			Spontaneously Regenerated shoots		
	1D	2D	5D	1D	2D	5D	1D	2D	5D
Hawk	53	100	100	7	30	11	46	8	0
Vona	14	55	41	0	5	6	57	10	0
Bennett	17	71	75	0	0	0	29	0	0
Tam 101	50	100	100	20	63	0	20	9	0
0.5 mg/l BA									
Hawk	0	50	65	53	25	0	6	0	0
Vona	43	69	86	29	31	7	0	0	0
Tam 101	75	0	18	33	7	0	6	0	0
0.5 mg/l KIN									
Hawk	11	18	36	50	23	0	22	0	0
Vona	4	12	27	16	37	0	33	0	0
Bennett	0	0	0	0	0	0	0	0	0
Tam 101	0	28	13	20	0	0	0	0	0

* Data for 2,4-D alone after two passages and for cultures with BA or KIN after one passage.

Table 9. Influence of the maintenance media on plant regeneration on LS media with .1 mg/l IAA or .5 mg/l BA.

Cultivar	Maintenance Media								
				0.5 mg/l BA			0.5 mg/l KIN		
	1D	2D	5D	1D	2D	5D	1D	2D	5D
	Plants/gram								
Hawk	7.5	14	0	39	2	0	68	0	0
Bennett	0	5	0				81	19	0
Baca	0	28	0				267	19	0
Tam 101	24	11	0	12	5	13	89	31	0
Vona	0	0	0	112	13	0	6	0	0

Rice Suspension Culture. Cell and molecular biology offer ways for plant improvement. Cell suspensions can be used as a source for

protoplast isolation, transformation and as a tool for *in vitro* selection. In order for cell suspensions to be efficient, the cells must be able to regenerate into plants.

Cell suspension cultures of *Oryza sativa* L. cv G-159 were initiated from embryogenic callus in January 1986 (TCCP Progress Report, 1986). The suspensions consisted of cells in different shapes and sizes (Figure 4). Cells divided and formed clumps of cells. These clumps were transferred to solid maintenance medium and regeneration medium subsequently. Very few plantlets formed. Attempts to develop embryogenic cell suspension cultures from these suspensions were carried out.

A technique that is frequently used for separating cells based on size is the use of filters. Suspensions were filtered through 500 um mesh filters. One gram of clumps larger than 500 um were inoculated in 20 ml liquid medium. The medium used was Linsmaier and Skoog's basal medium (LS medium) supplemented with 1 mg/l 2,4-D, 0.3 mg/l KIN and 4% sucrose. Clumps that were smaller than 500 um were transferred by inoculating 5 ml aliquots of suspensions to 20 ml liquid medium. Cultures were agitated at 120 rpm, $25 \pm 2^{o}C$ in the dark and subcultures were made every two to three weeks. After one to two subcultures, cells that were filtered through 500 um mesh divided and formed isodiametric cells with dense cytoplasm (Figure 5). Suspension cultures from cells that were bigger than 500 um, formed fewer small cell aggregates.

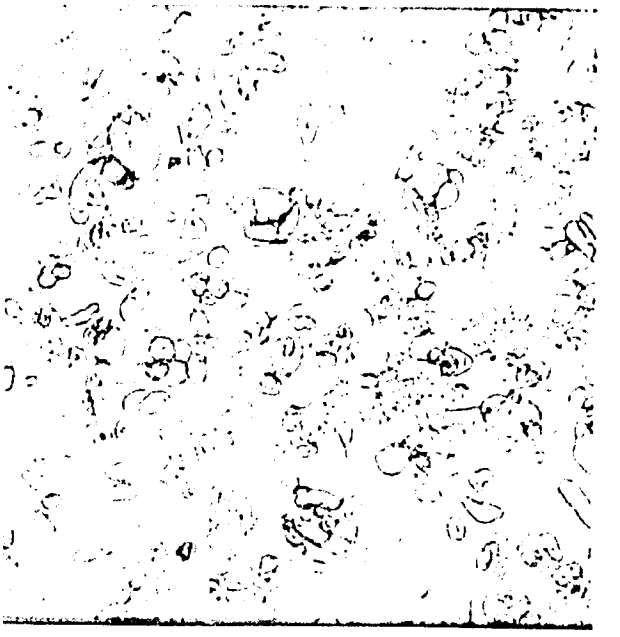


Figure 4. Rice suspension culture of G-159.

Figure 5. Embryogenic rice suspension cultures of G-159.

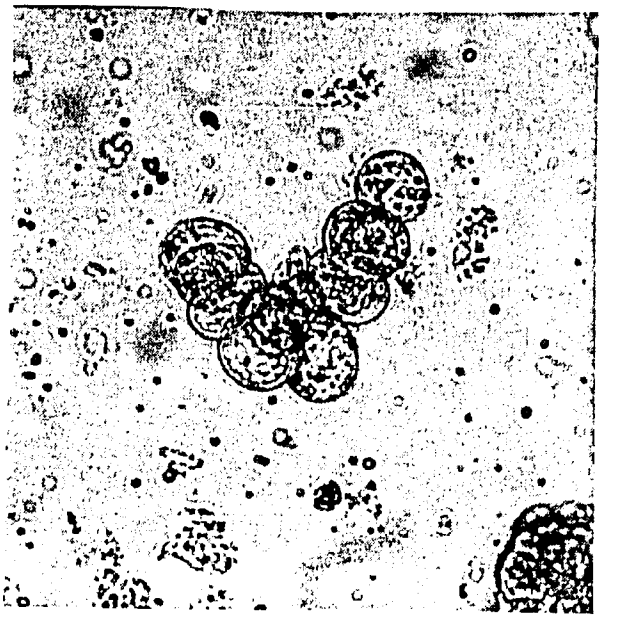


Figure 6. Plants regenerated from suspension cultures of Taipei 309.

Different basal media were used to induce cell division and obtain small aggregates of cells from clumps that were larger than 500 um. Amino acid medium (AA medium) either alone or in combination with protoplast culture medium (1:1 ratio) increased cell division and formed small cell aggregates. Amino acid medium was chosen for maintenance medium because cells did not grow as well in the medium with protoplast culture medium after several subcultures. This medium has also been used for culturing cells that were smaller than 500 um.

Clumps of cells from G-159 suspensions that were larger than 500 um were transferred onto different solid media to induce growth of E callus. Cells divided but failed to form E callus or regenerated into plants. Cells that were smaller than 500 um were transferred onto solid media by pipetting 1 ml aliquots onto the medium. Cells divided and formed NE callus.

After one to two months in suspensions in AA medium with 2.5 mg/l 2,4-D, clumps of Taipei 309 cultured on solid LS medium with or without 0.5 mg/l BA and 0.05 mg/l NAA. Within two to three weeks, greenspots occurred and eventually regenerated into shoots and roots (Figure 6). Plantlets were transferred to jars containing the same medium until they were 10 cm tall. They were then transferred to the greenhouse for maturity.

Wheat Suspension Culture. Wheat suspension cultures were established from excised embryos. The suspensions were filtered through 500, 250, and 125 um mesh to obtain cell suspensions. The suspensions then consisted of many cell sizes and shapes. Cultures were maintained in liquid LS basal medium supplemented with 4 mg/l 2,4-D and 2% sucrose. The cultures were kept on a gyratory shaker at 120 rpm, $25 \pm 2^{C}C$ in

continuous light. Subcultures were made every four weeks. Attempts to obtain E suspensions were carried out by testing different basal media (LS, B5, AA). Different concentrations of 2,4-D are also being tested. Media combinations have not induced the formation of an E culture. The source for initiating the suspensions may also be important. Embryos were used as explant for initiating the suspensions. The suspensions had both NE and E cells. Even though the suspensions were filtered, NE cells divided faster and overtook the population. Rice suspension cultures were established by inoculating E callus in the liquid medium. An experiment is being conducted by using E callus as the explant source. More E clumps are formed in these suspensions. Cells will be observed and tested for regeneration.

Grain Legumes/Pulse Crops. Seeds of grain legumes or pulses are second only to cereals as a source of human and animal food. Nutritionally, they are two to three times richer in protein than cereal grains. The percentage varies from 20 to 40%. The total production of pulses worldwide in 1983 was 64.5 million metric tons.

All legumes have a special function relating to energy conservation. They convert atmospheric nitrogen to ammonia in a symbiotic process through rhizobia bacteria. The total contribution of atmospheric nitrogen (fertilizer) supplied by a legume crop can reach up to 276 kg/hectare/year.

The grain legume program at TCCP has received increased emphasis since mid-1986. Since no reported, reliable technology for plant regeneration except for primary callus in major grain legumes was available, the initial research focused on developing effective procedures. The species in the program were Phaseolus spp. (common bean,

tepary bean), Vigna spp. (moth bean, cowpea), and Cajanus cajan (pigeonpea).

These grain legumes were tested in parallel experimentation. Since the plant regeneration capability is a genetic trait, the chances for success in one species might be considerably better than would be the case for others. Success was achieved in developing a method for plant regeneration in long-term cultures of Phaseolus (tepary bean), pigeonpea, and Vigna aconitifolia (also known in India as moth bean).

The grain legumes and related species lack tolerance to salinity and aluminum toxicity. On the other hand, some species possess exceptional drought and heat tolerance. That is especially true for tepary bean and moth bean. The plants are productive in arid, hot regions on relatively poor soil.

Tepary bean is grown for food in Southwest USA and Northern Mexico and is gaining importance in some parts of Africa. The seed protein content is about 24% and the species is used in breeding programs with common bean as a source of drought and heat tolerance as well as disease resistance. Moth bean is the most drought-and heat-tolerant grain legume in the Indian subcontinent. The seeds contain about 23% protein. Both tepary bean and moth bean have been grown to maturity in Colorado. They have great potential for cultivation in Africa and other regions including the USA, where low or irregular precipitation and high temperatures prevail during the growing season. They also constitute a valuable germplasm for breeding with other grain legumes.

Plant Regeneration from Cell Suspensions Cultures. Procedures were developed for plant regeneration from long-term cell suspension cultures of moth bean and tepary bean. Cell suspensions were initiated from 30-

day-old green, nodular callus derived from leaf tissue. A nutrient medium was developed by replacing some of the inorganic nitrogen with organic nitrogen (casein hydrolysate), using less sulfate and increasing the calcium (L6 medium). After appropriate testing it was observed that sorbitol significantly increased plant regeneration and it was subsequently added routinely. Growth of densely cytoplasmic, E cells were maximum in L-6 medium compared to several other media tested. Filtration of the cell suspensions at each subculture with 125-250 mesh resulted in the production of E cell lines in moth bean (VA 686) and tepary bean (TB 686). The procedure was repeated with one cultivar of moth bean (VA-986) and two cultivars of tepary bean, (TB 986, TB1 387, TB2 387). Growth of the cell lines, VA 986 and TB 986 were monitored by collecting data on packed cell volume and dry weights. Doubling of the cell mass of both the cell lines was approximately three days (Figure 7).

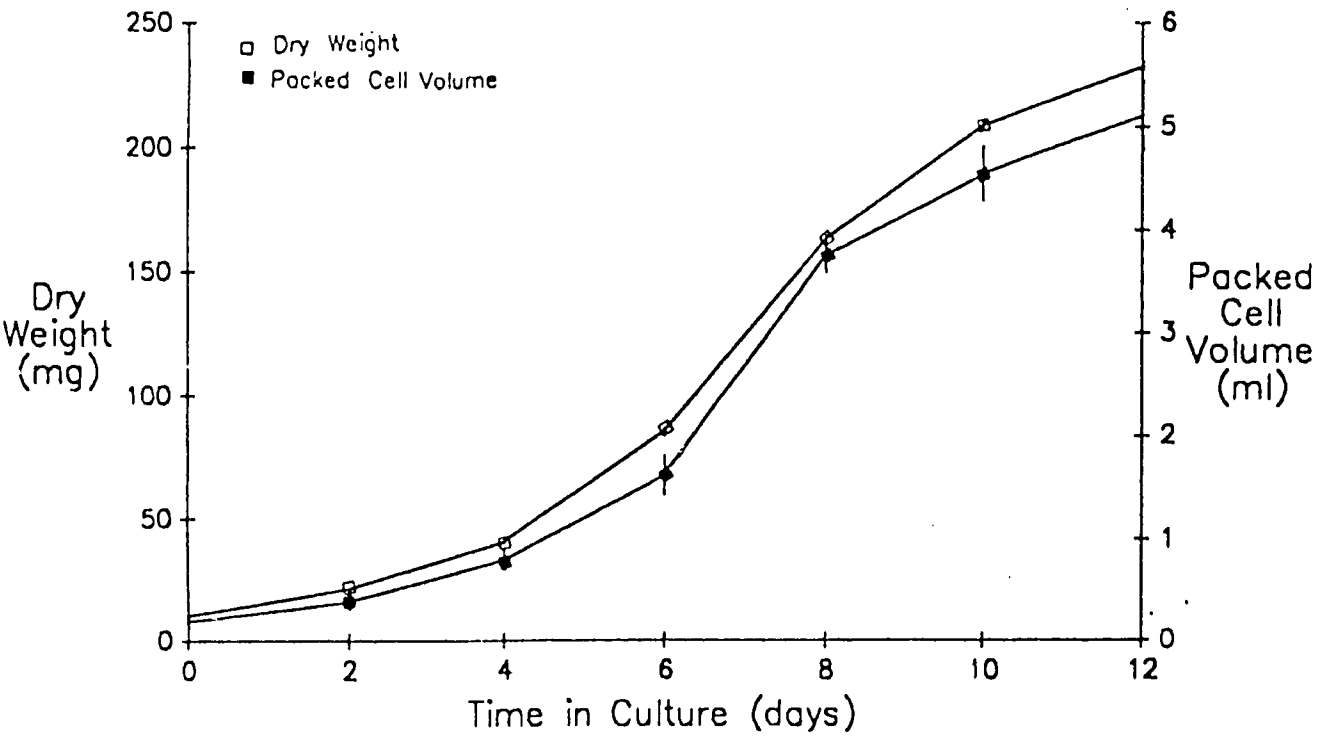


Figure 7. Growth curves of Vigna aconitifolia cell suspension cultured on L-6 medium.

Partially-differentiated embryogenic cell clumps developed when cells from maintenance medium was transferred to L-6 medium, with zeatin for moth bean, and with BA for tepary bean. Embryo-like structures were produced from the cell clumps of moth bean and tepary bean upon plating on L-6 agar medium with cytokinins. Plantlets were regenerated from the embryos of moth bean and tepary bean on L-6 medium with IBA.

The plant regeneration capability in long-term cultures of moth bean and tepary bean remained relatively constant for more than one year (Table 10). The reduction in percent E callus formation and number of plants regenerated after 270 days in tepary bean may be due to the fact that filtration of the cells was done less frequently.

Plants have also been regenerated from cell suspension cultures of pigeonpea for the first time. The cell lines were established from leaf explants of seven-day-old seedlings in L-6 medium with 1 mg/l 2,4-D. Subsequent filtration and high auxin containing medium resulted in the isolation of a fast-growing cell suspension line. The E cell suspension of pigeonpea contained green cell aggregates. Upon transfer to L-6 medium with 5 mg/l of 2,4-D, embryo-like structures were produced. These

Table 10. Efficiency of plant regeneration in long-term cultures of *Vigna aconitifolia* and *Phaseolus acutifolius*.

Age of culture (1)	Percent E-forming plates (2)		Ave. No of plants regenerated (3)	
	Vigna	*Phaseolus*	*Vigna*	*Phaseolus*
120	77.5	83.4	12.3	10.6
150	84.4	79.5	11.6	10.3
180	82.4	68.5	13.5	9.2
210	76.6	79.7	11.6	11.4
240	79.4	67.4	12.6	11.5
270	64.7	53.7	10.5	8.2
300	71.8	57.4	11.3	6.3
330	69.4	49.5	11.0	5.6
360	64.2	47.6	8.7	3.6

1. *Vigna aconitifolia* culture was initiated in June 1986, while *Phaseolus acutifolius* cell suspension was started in Sept. 1986.

2. 50 mg. of the cell clumps from respective induction medium were placed on agar regeneration medium. Data represent an average of 25 plates.

3. Average number of plants regenerated in 25 jars.

structures developed into somatic embryos when plated on L-6 medium with high concentrations of sucrose and sorbitol. After testing several concentrations, the L-6 medium with 2% sucrose and 3% sorbitol was found to be optimal for the development of somatic embryos (Table 11). Plantlets regenerated from the embryos when cultured on L-6 medium with 0.1 mg/l of NAA and 0.05 mg/l of GA_3. The regenerated plants from moth

bean, tepary bean, and pigeonpea were transferred to vermiculite and soil mixtures (1:1) and are being grown to maturity in the greenhouse.

Somatic embryogenesis in dry bean and cowpea suspension cultures. Experiments were conducted to define the conditions to regenerate plantlets from cell suspension cultures of dry bean and cowpea. Cell suspensions of dry bean established from leaf explants were used to isolate suspensions with densely cytoplasmic and isodiametric cells by growing them in various media regimes and by regular filtration. Partially differentiated embryo-like structures were observed in the cell suspensions cultured on modified LM medium with 2,4-D and zeatin. These structures developed into tiny shoots upon plating on a medium containing extracts from zygotic embryos. Complete plantlet regeneration was not achieved on several media combinations from the developing shoots. Cowpea cell suspensions were established from immature embryo cotyledons and from young leaf explants in L-6 medium with 2,4-D and zeatin. Culture of these suspensions on modified L-6 with high calcium and sorbitol helped in the isolation of cell suspension lines with palnt regeneration capability. Cells collected, which passed through a 250 um filter, differentiated into somatic embryos on L-6 medium with KIN and NAA, but did not develop into complete plantlets under those media conditions.

Table 11. Somatic embryo development from cell aggregates developed in suspension cultures of pigeonpea.

Conc. of Sucrose (gm/l)	No. of embryo-like structures (a)			
	Concentration of sorbitol (gm/l)			
	0	10	20	30
10	0	1.8	2.3	1.0
20	0	3.8	4.7	2.1
30	0	6.4	8.2	1.8

(a) Average no. of embryo-like structures developed from 50 mg. of cell clumps, on an average of 10 replicates.

Chickpea Tissue Culture Program (TCCP and ICRISAT). Chickpea (Cicer arietinum L.) is an important grain legume crop and is grown mainly in the Indian subcontinent, parts of Asia, Africa, Europe and South and Central America. It is valued for its high protein nutritive seeds.

The chickpea tissue culture program is a collaborative project between the TCCP and ICRISAT, Patancheru, India, and was set-up to develop efficient procedures of tissue culture techniques for the improvement of chickpea. The main objectives of this program at TCCP are to:

a. develop a reliable procedure for rapid clonal propagation of elite lines,

b. generate interspecific hybrid materials,

c. develop a plant regeneration procedure by somatic embryogenesis, and

d. screen and select variants with increased salt tolerance.

About 2-3 cm long shoot cuttings from mature plants grown in the

Table 12. Effect of indolebutyric acid[1] (IBA) on cultured shoot cuttings[2] of three wild Cicer species.

	Trial 1		Trial 2	
Cicer species	Total explants	Number of shoot cuttings rooted	Total explants	Number of shoot cuttings rooted
C. bijugum (No. 201)	12	0	10	6
C. cuneatum (SL 157)	14	13	10	10
C. judaicum (SL 185)	12	3	10	6

1 = RM (825 mg/l NH_4NO_3, 950 mg/l KNO_3, 185 mg/l $MgSO_4.7H_2O$, 330 mg/l $CaCl_2.2H_2O$, 170 mg/l KH_2PO_4, 700 mg/l $Ca_2(NO_3)_2.4H_2O$, 50 mg/l EDTA Ferric-sodium salt, 15.6 mg/l $MnSO_4.4H_2O$, 8.6 mg/l $ZnSO_4.7H_2O$, 6.2 mg/l H_3BO_3, 0.83 mg/l KI, 0.25 mg/l $Na_2MoO_4.2H_2O$, 0.025 mg/l $CoCl_2.6H_2O$, 0.025 mg/l $CuSO_4.5H_2O$, 10 mg/l Thiamine hydrochloride, 1 mg/l Pyridoxine monohydrochloride, 1 mg/l Nicotinic acid, 100 mg/l myo-Inositol, 30 g/l Sucrose, and 9 g/l agar containing 0.2 mg/l IBA.

2 = 2-3 cm. in length from three-month-old plants grown in the greenhouse.

greenhouse were used as explants. The explants were rinsed first with deionized water using Tween 20 as a surfactant, then with 95% ethanol, and washed with deionized water. The explants were then treated with 10% Clorox (5.25% sodium hypochlorite) for 10 minutes and rinsed five times with sterile deionized water.

Shoot cuttings of the wild Cicer species, C. judaicum (SL 182) were surface sterilized and placed on rooting medium (RM) previously used for tepary bean containing 0, 0.5, 1.0, 2.0, 4.0 or 6.0 mg/l IBA or NAA. Four weeks after culturing, extensive swelling occurred around the tissue

submerged in the medium in all the treatments except the control. Root differentiation occurred in some explants placed on medium with 0 or 0.5 mg/l IBA. Root induction was attempted with low concentrations (0, 0.01, 0.05, 0.1, 0.2, 0.4 or 0.6 mg/l) of IBA. Root development was good in medium with 0.2 mg/l IBA.

Shoot cuttings from four-month-old, wild Cicer species were tested for root induction. The explants from C. bijugum (No. 201), C. cuneatum (SL 157), and C. judaicum (SL 185) were placed on RM containing 0.2 mg/l IBA. The experiment was repeated. In both the trials, maximum explants of C. cuneatum rooted (Table 12).

Very few shoot cutting explants from the chickpea genotypes rooted in medium containing low concentrations (0.01, 0.05, 0.1, or 1.0 mg/l) IBA. The effect of the other auxins such as IAA and NAA and high concentrations of IBA on root induction of the chickpea genotypes was tested (Table 13). Explants from three-month-old plants of two genotypes (K 850 and C 235)were used. In both the trials, few explants rooted in RM containing 0.1 mg/l NAA. In RM containing IBA, and IAA or 0.2 mg/l NAA, the base of the explants produced thick roots and callus.

Shoot tips from 15- to 25-day-old seedlings raised in vitro on MS medium were used for multiple shoot production. Excised shoot tips from four chickpea genotypes, K 850, ICCC 32, Annigeri, and L 550 were placed on L-6 medium (Kumar et al., 1987) containing various concentrations of BA and zeatin with or without IAA, IBA or NAA (Table 14). The average number of shoots per explant of ICCC 32 and K 850 was maximum in medium containing 2.0 or 4.0 mg/l BA and 0.5 mg/l IAA. Callus production was higher in medium with 4.0 or 6.0 mg/l BA--with or without auxin. Excised shoot tips from C. arietinum (K 850 and L 550), C. judaicum (SL 182) and

C. cuneatum (SL 157) were placed on L-6 medium containing 2.0 mg/l BA and 0.5 mg/l IAA. Ten explants were tested for each genotype. The average

Table 13. Effect of auxins on root induction of shoot cuttings[1] from two chickpea genotypes.

	K 850		C 235	
RM + mg/l auxin	Total explants	Number of shoots rooted	Total explants	Number of shoots rooted
Trial 1[2]				
0.1 NAA	5	2	5	3
2.5 IBA	5	4 (c)	5	4 (c)
5.0 IBA	5	5 (c)	5	4 (c)
2.5 IAA	5	4 (c)	5	3 (c)
5.0 IAA	5	5 (c)	5	3 (c)
Trial 2				
0.1 NAA	6	3	6	3
0.2 NAA	6	6 (c)	5	4 (c)
1.0 IBA	6	5 (c)	6	5 (c)
2.0 IBA	7	7 (c)	6	5 (c)

1 = 2-3 cm length from three-month-old plants grown in the greenhouse

2 = RM medium with 500 mg/l instead of 700 mg/l calcium nitrate

c = the base of the explants produced callus

number of shoots produced per explant was 5.8, 4.8, 2.6 and 6.0, respectively.

Interspecific crosses of wild Cicer species (C. bijugum (No. 201), C. cuneatum (SL 157), and C. judaicum (SL 185) with C. arietinum (ICCC 32, K 850, C 235, and L 550) were attempted. Very few hybrid embryos

were produced but none germinated on RM or L-6 medium containing 0 or 0.35 mg/l Gibberellic acid. Medium with suitable hormone concentration was developed to culture immature embryos. Immature embryos (6, 9, 12, and 15-day-old) of the female parent C. arietinum genotypes germinated on L-6 or MS medium containing 2.0 mg/l BA and 0.1 mg/l IBA. The media will be used to culture the hybrid embryos. Attempts to regenerate plants by somatic embryogenesis are in progress.

Table 14. Multiple shoot[1] production from cultured shoot tips of four chickpea genotypes.

	Genotype			
L-6 medium + mg/l hormone	K 850	ICCC 32	Annigeri	L 550
2.0 BA + 0.5 IAA	3.7	9.0	2.7	2.3
4.0 BA + 0.5 IAA	9.0	8.7	4.3	3.7
2.0 BA + 0.5 IBA	2.7	5.0	5.3	2.0
4.0 BA + 0.5 IBA	5.7	6.7	5.7	4.7
1.0 ZEA + 0.01 NAA	2.0	5.0	3.3	1.0
2.0 ZEA + 0.01 NAA	2.0	1.0	2.3	1.3
4.0 BA	3.0	5.3	1.7	2.7
6.0 BA	2.7	3.7	1.7	4.7

1 = the data are an average from three explants per treatment

2 = 1000 mg/l NH_4NO_3, 1000 mg/l KNO_3, 170 mg/l $MgSO_4.7H_2O$, 440 mg/l $CaCl_2.2H_2O$, 170 mg/l KH_2PO_4, 500 mg/l $Ca(NO_3)_2.4H_2O$, 50 mg/l EDTA Ferric-sodium salt, 15.6 mg/l $MnSO_4.4H_2O$, 8.6 mg/l $ZnSO_4.7H_2O$, 6.2 mg/l H_3BO_3, 0.83 mg/l KI, 0.25 mg/l $Na_2MoO_4.2H_2O$, 0.025 mg/l $CoCl_2.6H_2O$, 0.025 $CuSO_4.5H_2O$, 10 mg/l thiamine hydrochloride, 1 mg/l Pyridonine monochloride, 1 mg/l Nicotinic acid, 100 mg/l myo-Inositol, 20g/l Sucrose, 10 g/l Sorbitol, 200 mg/l casein hydrolysate, and 9g/l agar.

Subgrants. The TCCP has supported research in other labs for two reasons: 1) to assist in strengthening their ability to perform tissue culture experiments and 2) to assist in completion of a task as outlined in the Work Plan. Subgrants have been provided to Dr. Nguyen Thanh-Tuyen, Visayas State College of Agriculture (ViSCA), Philippines, Dr. Milton Rush, Louisiana State University (LSU), Ms. Alfinetta Zamora, University of the Philippines at Los Baños (UPLB), for work on corn, rice and wheat, respectively. A fourth subgrant was provided for field evaluation and will be discussed in a later section.

Corn/ViSCA. Initiation of E callus from immature embryos, 1.5 mm long, of three varieties of corn, IPB 2, ViSCA 8311, and ViSCA 8341, were evaluated on six culture media. Nearly 100% of the immature embryos plated produced callus; however, the percentage of explants which produced embryogenic callus varied with genotype and media. Embryogenic callus in corn is classified as Type I or Type II. Type I callus is compact, white and is found in many cultivars but is difficult to maintain for long periods of time. Type II E callus is friable and found in few cultivars but is easily maintained for the time required to conduct *in vitro* selection. The MS medium with 25 mM L-proline was most effective for all varieties tested (Table 15).

Table 15. Percent of embryos plated which formed embryogenic callus and percent increase in callus fresh weight of 3 varieties on MS media with 25 mM L-proline.

Cultivar	Embryogenic Response	Callus Fresh Weight Increase
	%	
IPB 2	92.6	230.3
ViSCA 8311	21.4	303.0
ViSCA 8341	80.0	875.0

The regenerative ability of cultured immature embryos of the three varieties was observed. In terms of percent cultures regenerating shoots and developing plants, average number of shoots, and plants produced per 100 mg callus, IPB 2 and ViSCA 8341 were superior to ViSCA 8311. Cultures of IPB 2, ViSCA 8341 and ViSCA 8311 regenerated plants readily in the first passage. For ViSCA 8341, the callus retained the regenerative ability until the fifth passage, while for ViSCA 8311 and IPB 2 the regenerative ability was lost after the third and fourth passage, respectively.

These observations confirm reports in the literature for many species, including corn, that response in culture is dependent on genotype and there is usually a large genotype media interaction.

The effect of L-proline was investigated in greater depth. A range of proline, 0 to 100 mM, was added to MS medium. A positive effect of proline was observed. Moderate levels of 5 to 25 mM favored Type II E callus while higher levels above 25 mM promoted formation of Type I callus (Table 16).

Table 16. Effect of proline on the percent of embryos forming either Type I or Type II E callus.

	IBP 2		ViSCA 8341	
Proline	Type I	Type II	Type I	Type II
mM	%			
0	0.0	6.7	6.7	13.3
5	6.7	10.0	23.3	30.0
25	26.7	23.3	33.4	23.3
50	23.3	6.7	41.4	6.9
100	23.3	6.7	71.4	3.6

Type I callus culture medium developed by the TCCP with various

levels of sucrose and dextrose were tested. Immature embryos of ViSCA 8341 were used in this experiment. A increase in the percent of the cultures forming Type I E callus from 23% to 61% was observed when the sucrose level was raised from 4% to 12%. An increase was also observed when dextrose was used in place of surcose and the dextrose concentration raised from 4% with a response of 13% to 8% with a response of 40%.

Regenerated plants from all experiments were grown to maturity. Survial was low with no more than 40% of the regenerates surviving. The main causes of death were stem rotting and root insects.

Time to flowering and morphological characters including plant height at flowering and formation of reproductive structures were observed in the regenerated plants. Variation in plant height at flowering was recorded among the R_0 plants of IPB 2 with short stature (i.e. 60-80 cm) being associated often with early flowering (i.e. 34-41 days from potting) and low fertility. Plant height was however more uniform and superior (i.e. 150-180 cm) for the R_1 plants which derived from seeds of controlled pollinated R_0 plants. Likewise, the timespan from planting to flowering of the R_1 plants was also longer (i.e. 52-59 days).

Various abnormal characters of the reproductive structures were observed at flowering, including: 1) occurrence of tassels and silks together in one structure located either at the plant apex or leaf axils, 2) absence of tassels, 3) production of ears without silk, 4) formation of naked ears, and 5) kernels with protruding embryos at maturity. Usually, more than one ear per R_0 or R_1 plant was observed for IPB 2, yet only a few kernels matured. Production of multiple ears at one leaf axil was encountered in one R_0 plant of ViSCA 8311.

Rice/ViSCA. Research on E callus initiation, maintenance and plant regeneration from mature seeds of upland rice varieties, IR 43 and UPLRi 7, was continued after the return of Merlie Dionzon from TCCP's Training Program. In spite of satisfactory performance of cultured seeds of the these varieties at CSU, several problems were encountered and delayed implementation of the planned activities. The problems incurred included: 1) a high rate of contamination, 2) poor callus formation, 3) browning of the callus, 4) lack of callus proliferation on maintenance medium, and 5) no plant regeneration.

The problem of high contamination was recognized as due mainly to storage of the seed and aseptic operations; use of newly harvested seed greatly reduced the contamination rate. Along with using HgCl in place of Clorox for surface sterilization the contamination rate has been reduced to less than 5%.

Several experiments were conducted to test the basal media and growth regulatory concentrations with mature seeds. MS basal salts were superior to B5, Blaydes or Y3. Callus formation requires the addition of 2,4-D; however, concentrations above 2 mg/l caused the callus to brown quickly. The presence of BA up to 1 mg/l was beneficial for E callus formation.

Due to poor E callus formation from mature seed, immature panicles were tested. This procedure was adapted from Dr. M.C. Rush at LSU. E callus formation was better with the immature panicles than from mature seed. Of 120 plated seed of UPLRi 7, about 60 plants were regenerated after four passages. Immature panicles of UPLRi 7 did not regenerate any plants, despite its relatively high E callus formation. UPLRi 5 and UPLRi 3 did produce one plant from immature panicles. Morphological

characteristics and yielding performance of the regenerated plants are being evaluated under screenhouse conditions.

Rice/LSU. This subgrant has three components; 1) enhance the *in vitro* ability to form somaclonal variation; 2) to evaluate the lines resulting from tissue culture for the somaclonal variation and determine their usefulness in breeding programs; 3) to investigate requirement for transportation of *in vitro* material internationally.

More than 10,000 somaclones have been generated at LSU as of August 15, 1987. The only limitation has been technician time and greenhouse space to grow out the R_1 plants. More than 30 types of somaclonal variation have been observed and catoloqued from regenerated cultures without *in vitro* selection. This has included variation in disease resistance and morphological characters. The somaculture system is based on MS medium with 2 to 4 mg/l 2,4-D in the callus induction medium and either a combination of 0.5 mg/l IAA and 0.8 mg/l BA or 2 mg/l NAA and 1 mg/l K in the regeneration medium.

In an effort to increase the amount of somaclonal variation occurring in somaclones was supplemented the MS medium with concentrations of ethyl methanesulfonate (EMS) at 0, 100, 500, 1000, 1500, 2000, and 2500 ppm. Callus was transferred from the immature panicle explants to these media. After 10 days the calli were subcultured on fresh regeneration medium without EMS. The calli were subcultured once per month. Plants were regenerated from media containing EMS at concentrations from 100 to 1000 ppm. Plants from the check treatment and the various EMS treatments were transplanted to the greenhouse to produce seeds for the R_1 generation. About 3000 plants of EMS treated Tetap, Labelle, and Lemont have been regenerated. Obervation

of the R_1 plants suggests that variation was increased in all of the cultivars. Several hundred Tetep somaclones previously produced without exposure to EMS did not show somaclonal variation.

A method has been developed at LSU for regeneration of plants from single cells or small cell clusters from suspension cultures. Although several green plants have been produced and transferred to the greenhouse, there have been a large proportion of albinos and chorophyll-deficient plants. This procedure whould allow for increased variation by generating plants from single cells, by utilizing long-term suspension cultures, and by using in vitro selection schemes. Preliminary work has begun with protoplasts.

In field evaluations, different rice germplasm show different degrees of somaclonal variation. Lines derived from Labelle, Lemont and Zenith show several variations including grain type, hull color, height, pubescence, and resistence to sheath blight. The lines derived from Melrose, Tetep, Taipei 309 and Tadukan showed little or no variation.

Four somaclones have been identified with significantly more resistance to sheath blight than the cultivars they were derived from. Three resistant somaclones are from Labelle and one is from Zenith. All of the resistant somaclones also have significant changes in plant type or morphology. One Labelle somaclone is resistant to sheath blight, blast, and narrow brown leaf spot. This somaclone (Labelle 163) may be registered as elite germplasm. The Zenith somaclone is a semi-dwarf, long-grain with more resistance to sheath blight. The Zenith cultivar is a tall, lodging-prone, medium-grain cultivar that was considered moderately susceptible to sheath blight.

For international tranportation, dehulled seeds or detached embryos

can be disinfected with a five minute treatment of 1000 ppm HgCl. The effectiveness of HgCl can be increased with a 30 minute treatment of a 0.5% Clorox solution. The tissue culture growth media of MS, B5 and Chaleff's without growth regulators supported seed or embryo germination and growth. Transplanting survival percentage after 35 days of in vitro incubation in the dark was high.

Sterilized dehulled seeds and detached embryos of the rice cultivars Bellevue, Calrose 76, Lebonnet, and Newbonnet were air-shipped to IRRI and IITA. Seedlings from germinated seeds or embryos survived the shipment of 36 days to IITA and 11 days to IRRI. One hundred mm disposable petri plates containing Chaleff's medium without growth regulators supported seedling growth during the shipment. The visual detection and elimination of contaminated culture plates on arrival was easily accomplished. The survival rate of germinated seeds or embryos was high during shipment. Techniques developed and improved by this research may prove beneficial for international transfer of microorganism-free plant material as a source of germplasm.

Wheat/UPLB. This subgrant at UPLB with Ms. Zamora, is to develop techniques for selecting wheat calli tolerant to heat stress. The first step was to test TCCP's tissue culture procedures in a different environment to determine culture conditions. Several problems have been investigated by Ms. Zamora's group which will be benefical in other cases.

Cultivars used were Pavon, an unadapted cultivar to the Philippines, and Trigo, which was developed in the Philippines and has heat tolerance. For both cultivars, the LS media used at TCCP promoted the greatest amount of E callus. A concern arose when, in Pavon less than 10% of the embryos developed E callus when at TCCP over 60% formed E callus. The

culture conditions were then investigated. The light conditions of eight hours versus 24 hours had little effect on E callus formation in Pavon. Temperature had a significant effect; the range of 15 to 27°C formed more E callus than high temperatures of 25 to 36° C. Two techniques have increased E callus formation in Trigo 3: 1) cutting through the scutellum when plating the embryos; and 2) orientating the embryo with the embryo axis in contact with the medium.

Immature embryos were tested as to their effectiveness in forming E callus. Immature embryo do form significantly more E callus than mature embryos when the immature embryos when sampled at the milk to soft dough stage.

Plant regeneration can be achieved on several media. The most effective for shoot formation and development seems to be LS medium supplemented with 1 mg/l NAA and 5 mg/l KIN (LSR) (Table 17).

Table 17. Shoot formation from two regeneration media in wheat tissue cultures.

Cultivar	Media	Cultures with Shoots	Number of shoots per culture
		%	
Pavon	MSRa	16.7	1.83
	LSR	60.0	2.80
Trigo 1	MSR	20.8	1.00
	LSR	42.8	2.43
Trigo 3	MSR	38.2	1.68
	LSR	40.9	1.50

a = MS minus biotin with 15% coconut water and 2% sucrose.

Heat selection was studied by placing three, one-half month-old E

callus of Trigo 2 in incubators set at a constant temperature of 17, 20, 26, 28.5 and 39° C. After four weeks callus was moved either to regeneration media or maintenance media under the heat stress. Percent of browning of callus was dependent on the heat treatment. During the first subculture, all of the cultures maintained at 17°C remained healthy. Moreover, the cultures maintained at 20 and 26°C did not show heat stress until the fourth week of incubation. The calli incubated at 26°C exhibited more stress than those at 20°C. The cultures maintained at 28.5 and 39°C showed browning one and one-half weeks after transfer. At the end of four weeks, all of the calli incubated at 39°C were brown. In subsequent passages, the trend for health of cultures in different heat treatment was maintained. After the first passage of heat treatment, the cultures from the 26, 28.5 and 39°C treatments had little E callus. More E callus was formed at 20°C than at any other treatment. Besides callus health, callus size was also observed during the first passage of heat treatment. Those calli maintained at the lower temperatures, 17, 20, and 26°C, were larger than those maintained at higher temperatures. Plants were regenerated from all temperature levels except 39°C (Table 18). Progeny of the regenerated plants will be evaluated in the field for increased heat tolerance during the next season.

Table 18. Number of shoots regenerated from heat stressed cultures.

Temperature	Weeks on Heat	Number of Shoots	Number of Cultures	Shoot/Culture
C				
17	4	32	10	3.2
20	4	36	9	4.0
26	4	25	11	2.3
28.5	4	8	8	1.0
17	9	14	11	1.2
20	9	11	10	1.1
26	9	12	9	1.3
28.5	9	6	8	0.7
17	13	6	7	0.9
20	13	11	9	1.2
26	13	12	8	1.5
28.5	13	26	14	1.9

Selection of in Vitro-Tolerant Cell Lines and Plant Regeneration

Development of germplasm from stress tolerance culture has become a priority at the TCCP. This germplasm will be used to evaluate the *in vitro* selection methods in an attempt to improve these selection techniques.

In the cereals, many plants have now been regenerated from NaCl selected cell lines, with much of this work finishing in the past year. Aluminum and PEG are being tested to determine the selection levels.

Rice. Callus stress selection efforts in rice have made considerable progress, advancing from results produced in long-term, high-frequency regeneration experiments at TCCP since 1980. Both japonica and indica cultivars (G-159 and IR-36, respectively) are represented in the experiments reported here. The general objective of producing regenerated plants with enhanced tolerance to NaCl or aluminum has been approached using several selection strategies: short-term,

high-level stress exposure, long-term, low- to critical-level stress exposure, and recurrent stress selection on cultures initiated from plants regenerated from tissue culture.

Selection for salt tolerance. In two NaCl selection experiments, the desired level of stress was intended to reduce E callus fresh weight over a four to six month period by 90%, yet still allow for regenerated plants to be recovered from the surviving callus. Both cultivars G-159 and IR-36 were included in these studies, with different selective NaCl levels for each cultivar.

In the first experiment, both cultivars were initiated and maintained for three months on media without NaCl stress before stress treatments began. After the initial period of culture, G-159 callus was placed on 13 g/l NaCl for five passages. After three passages on stress medium, G-159 callus from 13 g/l NaCl treatment was placed on 13 and 16 g/l NaCl. IR-36 callus was placed on maintenance media with 9 g/l NaCl for five passages. A second experiment was similarly conducted to repeat the findings. In this experiment, G-159 callus--after three passages on non-stressed media--was placed on both 13 g/l and and 16 g/l for four to six passages. IR-36 callus was once again placed on 9 g/l NaCl. The desired callus reduction (90%) was achieved with 16 g/l NaCl for G-159 and with 9 g/l for IR-36 in both experiments. The total E callus weight, measured at the transfer of callus at each passage in both experiments, is shown in Figure 8 for G-159 and Figure 9 for IR-36. The total number of plants regenerated from the stress treatments in both experiments is summarized in Table 19.

A subsequent, short-term experiment examined a broader range of salt-stress levels; 0, 11, 13 and 15 g/l for G-159 and 0, 8, 10 and 12

g/l for IR-36. Mature embryos were initiated and callus maintained for three passages on non-stress media. Plant regeneration, after three passages on stress media, was achieved on media without NaCl added. The decline of E callus compared to the non-stress control over the period of stress treatment is shown in Figure 10 for G-159 and in Figure 11 for IR-36. A total of 29 plants were regenerated from selected cultures of G-159 and six plants from IR-36. Although E callus weights declined rapidly under high levels of stress in these experiments, plants were recovered from all but the 12 g/l NaCl treatment for IR-36.

Table 19. Total number of plants regenerated from short-term, high level NaCl experiments.

Cultivar	Stress level	Passages on stress	Total plants regenerated
G-159	13 g/L	5	181
	13 g/L	6	7
	16 g/L	2[a]	100
	16 g/L	4	16
IR-36	9 g/L	1	5[b]
	9 g/L	4	11 + 2[b]
	9 g/L	5	65

[a] after 3 passages on 13 g/L
[b] spontaneous regeneration

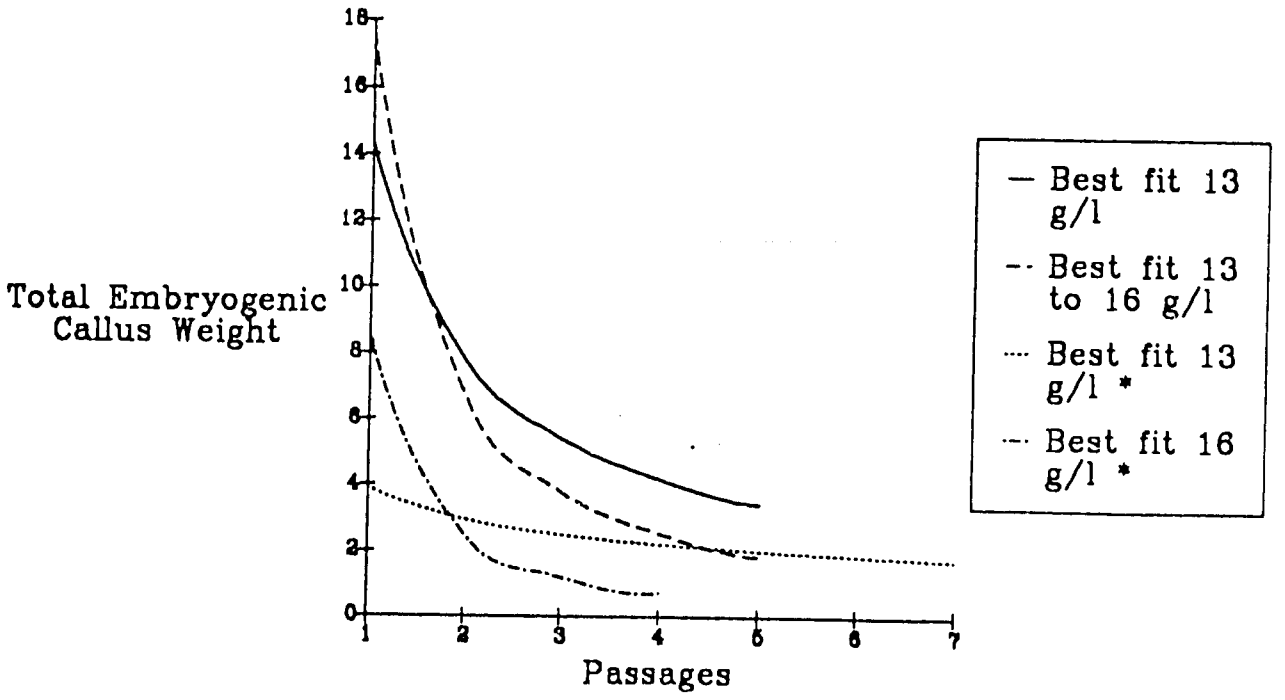


Figure 8. Embryogenic callus weight on G-159 on high NaCl stress.

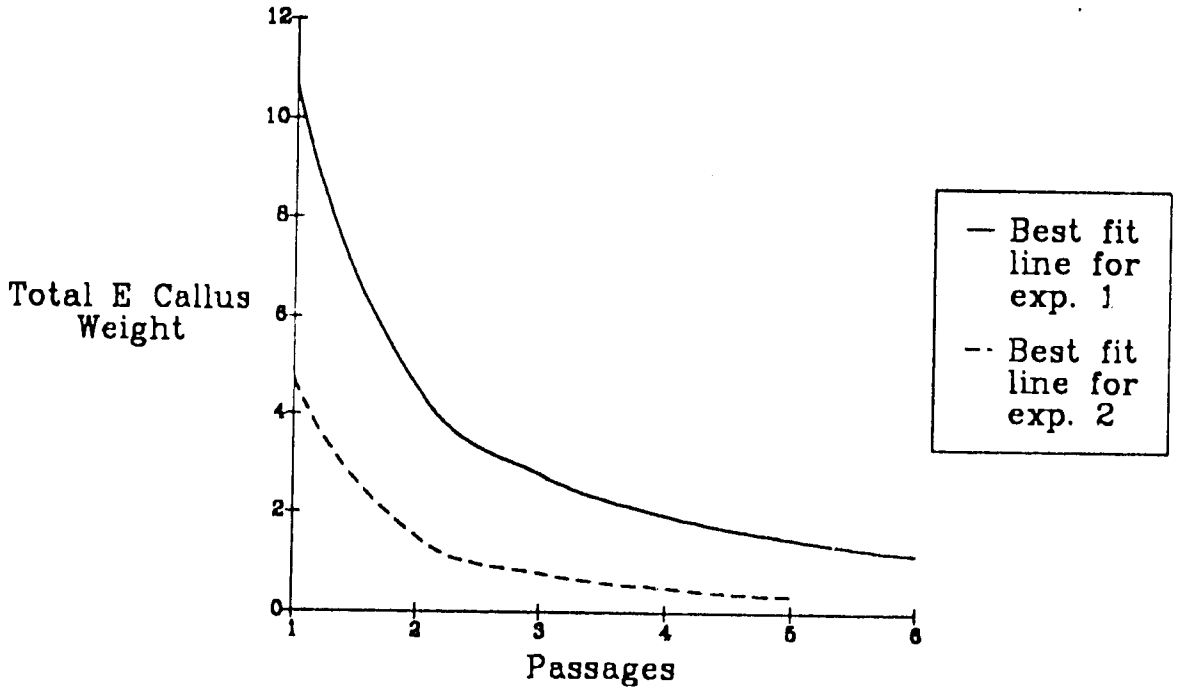


Figure 9. Decline of IR-36 callus on 9 g/l NaCl stress media.

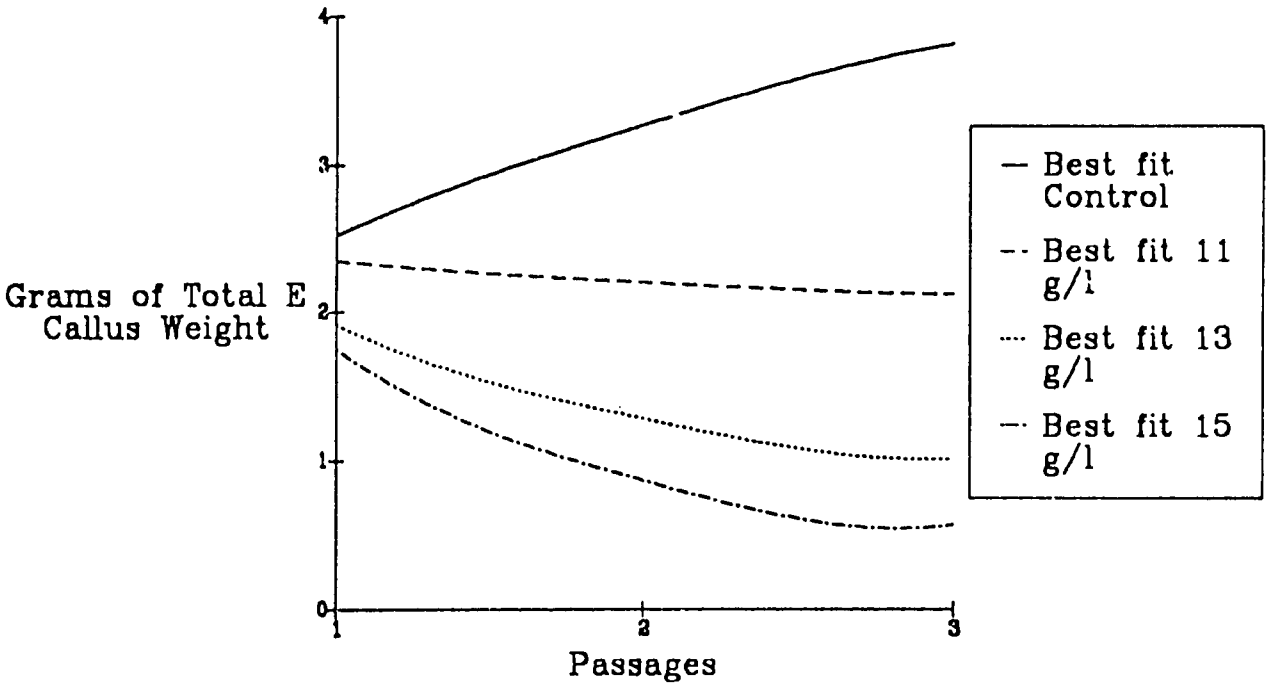


Figure 10. Growth of E callus of G-159 under NaCl selection.

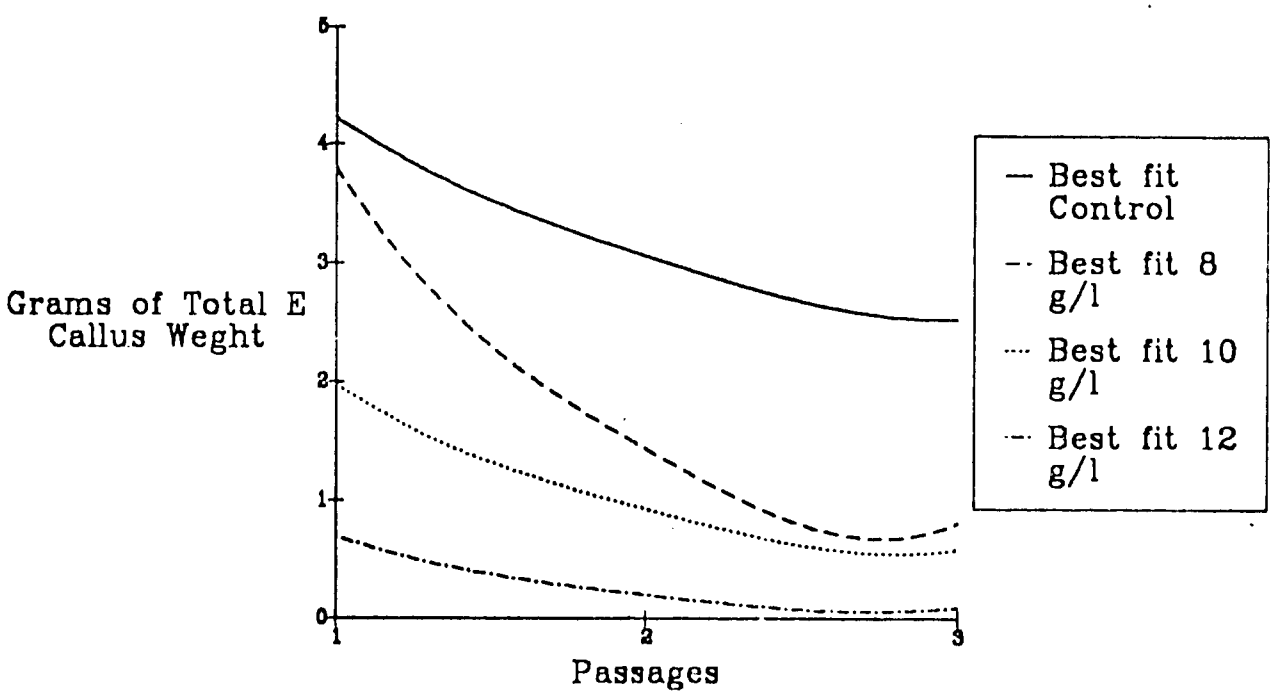


Figure 11. Decline of IR-36 callus on constant NaCl selection pressure.

Recurrent selection. Seed recovered from regenerated tissue culture plants is designated as R_1 seed, indicating the first generation removed from tissue culture. The objective of this experiment was to measure the tolerance of R_1 seed from salt-selected callus, non-selected callus, and non-tissue culture seed to NaCl levels for callus growth and plant regeneration.

Selected R_1 seed was collected from regenerated plants stressed *in vitro* for ten passages on 9 g/l NaCl. The non-selected R_1 seed was collected from regenerated plants that were *in vitro* for seven passages without stress. The original parent seed had never been in culture. All seed was of the cultivar G-159. After two passages *in vitro* on media with no salt, callus from the three seed sources was split and transferred onto media with 0, 9, or 12 g/l NaCl. After four passages on these treatments, all E callus was transferred to regeneration media.

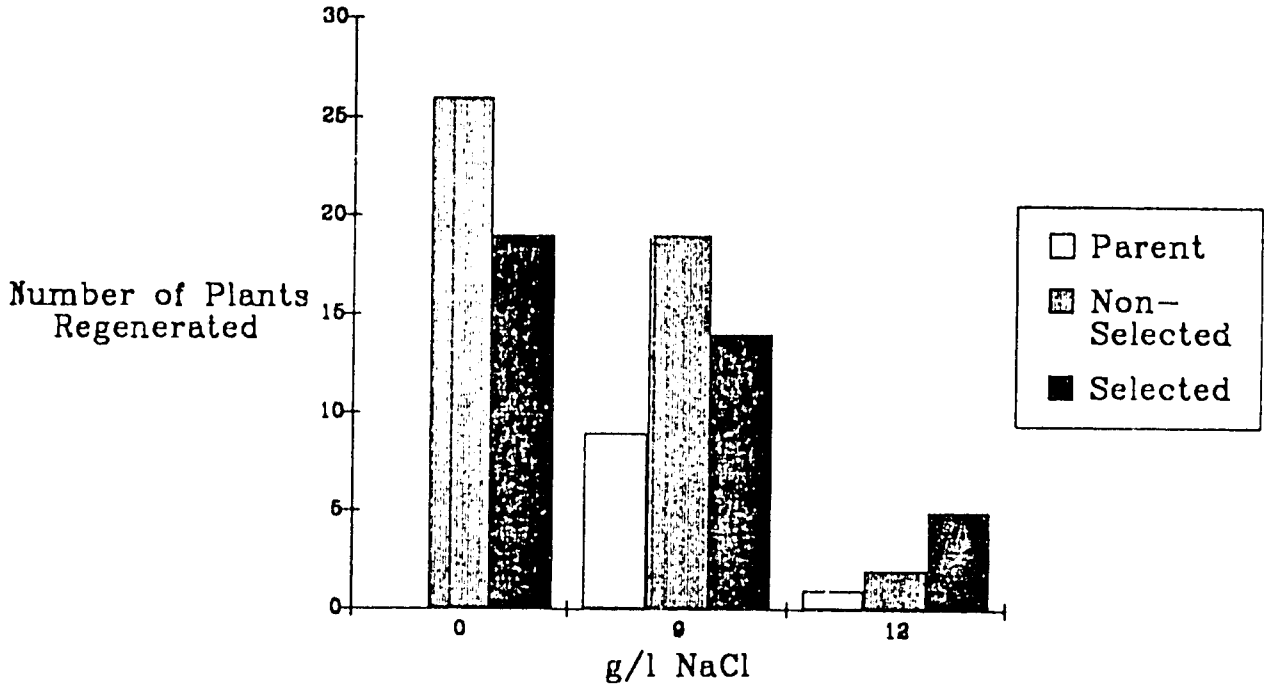


Figure 12. Regeneration after NaCl selection from R_1 and parent seed of G-159.

The total number of shoots produced per treatment from each seed source is shown in Figure 12. The results of this experiment suggest that there may be an adaptive advantage in utilizing R_1 seed in tissue culture stress selection experiments since E callus produced from both non-selected and selected R_1 seed demonstrated greater regeneration capability than E callus from parent seed across all treatments.

Selection for aluminum tolerance in rice callus culture. A preliminary experiment utilizing G-159 callus was conducted to regenerate plants after maintenance on media containing either 200 or 400 ppm $AlCl_3$. The callus on 400 ppm $AlCl_3$ died after five passages and only one plant from this treatment was regenerated. Callus on 200 ppm aluminum was maintained for 17 months and a total of 205 shoots were recovered from this treatment over that time period. A much larger aluminum stress selection effort is now in progress. Two hundred cell lines each of G-159 and IR-36 were initiated and maintained for two or three passages without stress and then transferred onto $AlCl_3$-containing media. G-159 is being challenged with 100 and 200 ppm $AlCl_3$ and IR-36 callus was placed on media containing levels of 75 and 150 ppm $AlCl_3$. Selection levels for IR-36 were too restrictive. Callus declined so rapidly that all IR-36 callus was moved to regeneration media after the third passage of stress (Table 20). G-159 callus remains on stress maintenance and will also be moved to regeneration media again after six and nine passages or as long as the callus remains viable.

The investigation and implementation of different stress selection strategies in rice tissue culture continues to produce both useful information on optimizing in vitro selection and significant amounts of R_1 seed for field testing. Table 21 summarizes the data on the numbers of

stress variants regenerated and established in the greenhouse. As this R_1 seed is distributed and tested under field stress conditions, important correlations between the expression of stress tolerance by regenerated whole plants and callus culture stress tolerance can be seen. It has not yet been determined whether short-term, high stress selection produces more useful variants compared with selection conducted with cultures at low stress over longer periods of time (six months or more). A step-wise approach, where cultures are transferred to increasing stress treatments every three passages and then regenerated, will be compared with constant level stress treatments in both salt and aluminum studies through work which is now in progress. The use of recurrent selection in callus culture to identify tissue culture lines which perform well *in vitro* and regenerate may further optimize the identification and production of useful stress-tolerant variants in large numbers.

Table 20. Plants regenerated after three passages on aluminum stress media, cvs. G-159 and IR-36.

Cultivar	Stress level	Number of plants regenerated
G-159	Control	18
	100 ppm	17
	200 ppm	31
IR-36	Control	10
	75 ppm	40
	150 ppm	46

Table 21. Greenhouse plants established for R_1 seed production from rice culture experiments.

Selection agent	Cultivar	Number of plants grown to maturity
Sodium chloride	G-159	327
Sodium chloride	IR-36	196
Aluminum chloride	G-159	251
Aluminum chloride	IR-36	81
Control - No stress	G-159	65
Control - No stress	IR-36	86

Selection for tolerance to Blast Phytotoxin. Several successful application of callus culture technique for selection of callus lines resistant to phytotoxin have been reported. These successful reports are based on screening of plant calli on a phytotoxin containing medium. This study is to investigate how an *in vitro* screening can be effectively used in selection of rice calli and to regenerate plants with resistant to blast disease.

There are three cultivars of javanica rice used for this study; Rojolele, Ketan Hitam, and Mayang. These cultivars are sensitive to blast disease, especially to the strains of *Pyricularia oryzae* isolated in Indonesia with isolate numbers 24 and 26. Rice seeds were obtained from the Central Research Institute of Agriculture, Bogor, Indonesia.

To determine selection of callus, lines were started from the first step of callus formation and followed by screening callus lines which are resistant to specific phytotoxin treatments.

Mature seeds were callused on media containing the phytotoxin and maintained on selection media. In this study, picolinic acid, one of the two phytotoxins produced by the fungus has been used for selection. The basic composition of the callus formation medium is based on LS medium. Callus formation medium contains 4% sucrose, 2 mg/l 2,4 D and 0.3 mg/l KIN. Preliminary research (Table 22), showed that the appropriate concentrations of picolinic acid for selection are 12.3 to 123 ppm. The concentration of 12.3 ppm seemed to be the lowest since there was not much effect of this treatment on E callus production. Screening for callus lines resistant to the specific concentration of picolinic acid—12.3 ppm, 6.15 ppm and 123 ppm--are now in progress.

Table 22. Percentage of embryos forming E callus on picolinic-containing media.

Cultivar	Concentration of picolinic acid (ppm)			
	0	1.23	12.3	123
Ketan Hitam	92	52	78	92
Rojolele	98	50	96	98
Mayang	90	60	92	96

The next step of this study will be to regenerate plants from selected callus lines and evaluate the resistance of the regenerated plants to the rice blast disease. Tolerance to the other phytotoxin, pyricularin, will also be studied since the susceptibility and resistance of rice plants to the rice blast disease depend essentially on both picolinic acid and pyricularin. Various concentrations of pyricularin and a mixture of picolinic and pyricularin will be used in the future selection experiments.

Sorghum. A system for long-term, high-frequency regeneration in both sweet and grain sorghum was developed at TCCP between 1984 and 1986. Grain sorghum cultivars with desirable agronomic traits have been identified and initiated into this system. Those cultivars which were successfully initiated in sufficient quantities were then challenged <u>in vitro</u> with selection pressures designed to generate stress-tolerant variants for germplasm development. Selection agents utilized include $KClO_3$, hydroxproline, and $AlCl_3$.

<u>Stress selection with callus cultures.</u> Three different types of selection experiments were conducted with the objective of generating useful variants from tissue cultures for use in breeding sorghum for stress tolerance and growth efficiency.

The addition of $KClO_3$ to sorghum callus media was used to select for

variants with an enhanced ability to reduce nitrate to nitrite, the nitrogen form utilized by plants for essential growth and development. $KClO_3$ was added to the media at concentrations of 100, 150, and 200 ppm. The growth of E callus of Hegari on these treatments over four months is shown in Figure 13. Callus growth remained relatively constant on the control while on all levels of $KClO_3$, the callus declined over the four passages. Plants could be regenerated from all chlorate levels and passages (Figure 14).

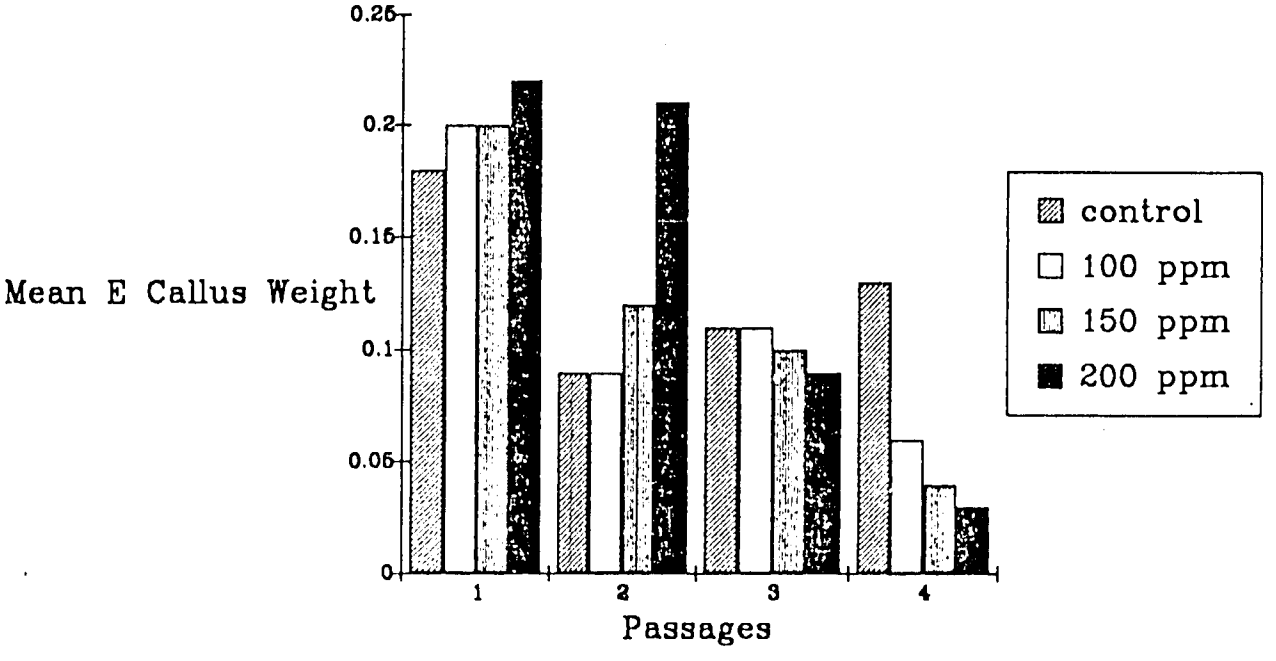


Figure 13. Growth of E callus of Hegari on $KClO_3$-containing media.

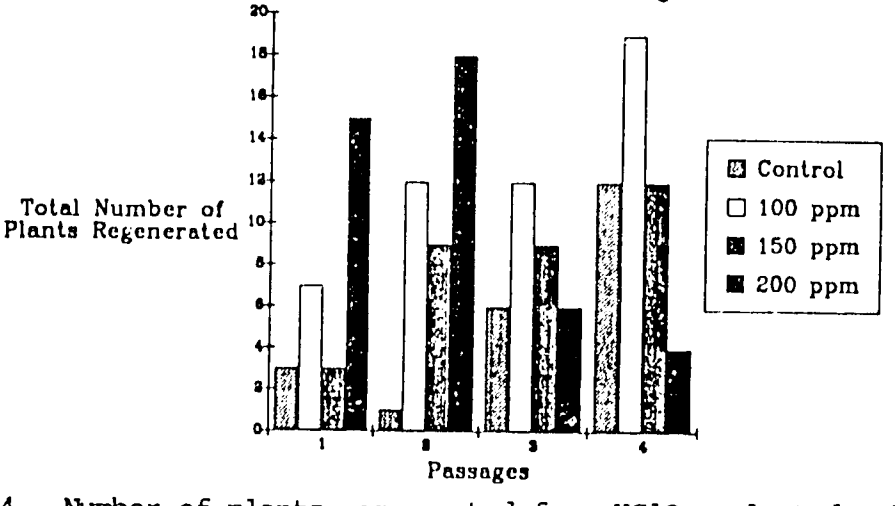


Figure 14. Number of plants regenerated from $KClO_3$ selected callus.

To select for sorghum cell lines which might have an altered proline (amino acid) enzyme system and thereby have enhanced tolerance to osmotic stress, hydroxyproline was added to callus media as a selective agent. Bulked cell lines from the cultivar Hegari were used. This experiment was conducted twice with hydroxyproline levels of 3, 5, and 7 mM. Callus on treatment media declined rapidly and both experiments were terminated after the second passage. Plants were regenerated from each treatment and grown to maturity.

The preliminary examination of the effects of aluminum stress on sorghum callus growth was accomplished through two experiments. In the first effort, aluminum chloride was added to sorghum callus maintenance media after autoclaving to arrive at final concentration levels of 100, 400, and 700 ppm. Levels of 400 and 700 ppm were observed to be extremely toxic (Figure 15). The subsequent experiment was initiated using 100 and 300 ppm as the selective levels of stress (Figure 16). Both experiments were conducted with bulked cell lines of Hegari. Although E callus weights declined significantly, regenerated plants from all stress levels were recovered (Table 23). In addition, a step-wise stress selection experiment was created when E callus surviving five passages on 100 ppm $AlCl_3$ was placed on treatments of 100, 400, and 700 ppm. Growth of E callus on these levels was improved over the original experiment conducted with the same stress levels. This suggests that increasing the stress from low to high levels allows for a greater potential recovery of stress-tolerant variants than does exposing callus to high levels of stress alone. Altogether, the initiation studies and stress selection experiments in sorghum tissue culture produced 464 regenerated plantlets which were successfully established in the

greenhouse for R_1 seed production (Table 24).

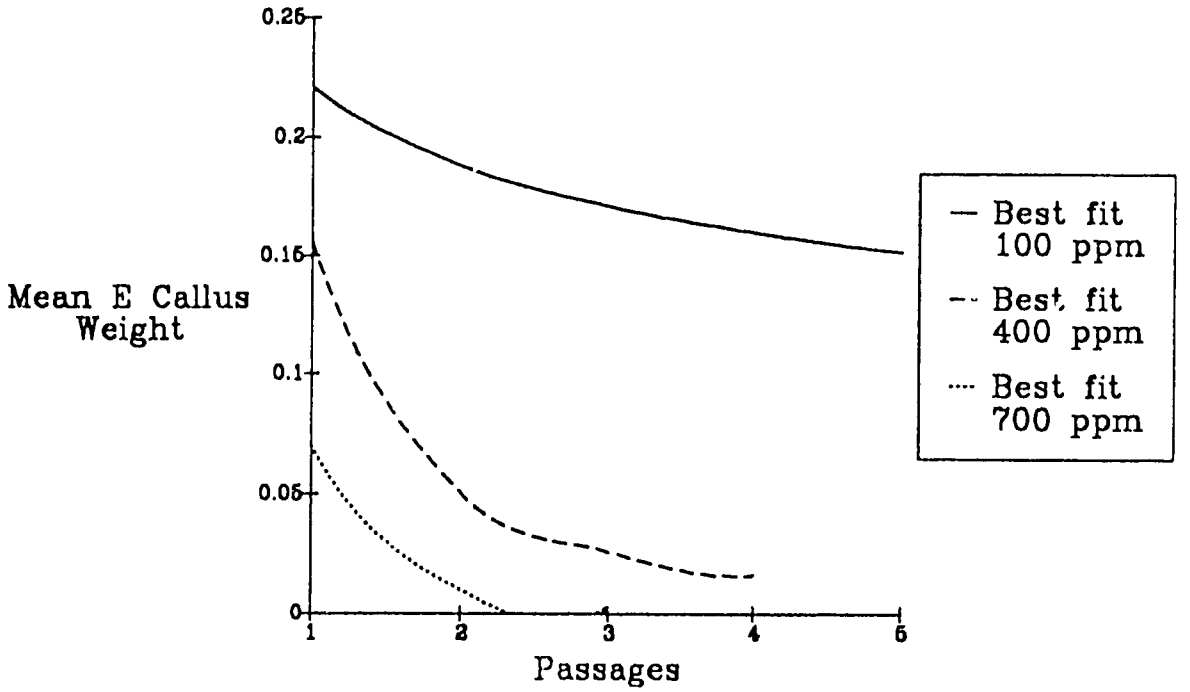


Figure 15. Growth of E callus on $AlCl_3$-containing media.

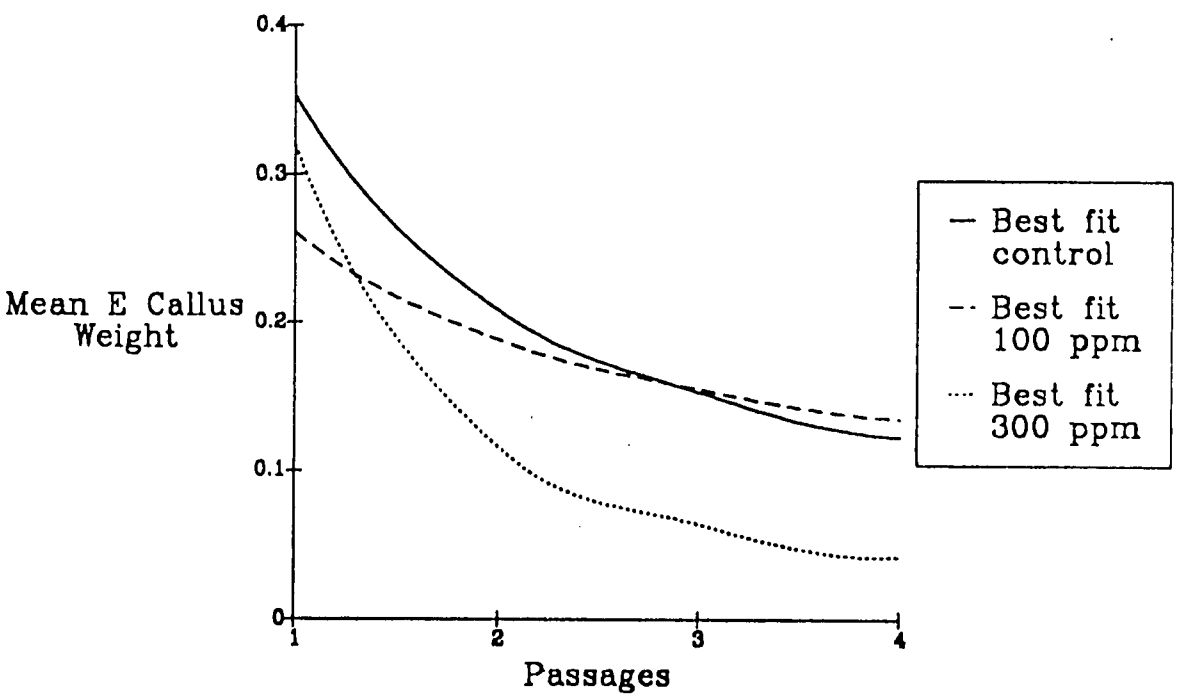


Figure 16. Growth of E callus on $AlCl_3$-containing media.

Table 23. Combined number of Hegari plants regenerated from aluminum stress experiments.

$AlCl_3$ level	Months on stress	Number of plants regenerated
100 ppm	1	15
	2	24
	3	10
	4	11
	5	2
	6	8
	7	2
	8	2
	9	4
	10	12
300 ppm	1	1
	2	5
	3	5
	4	10
400 ppm	1	12
	2	17
	3	22
	4	6
700 ppm	1	9
	2	1

The selection efforts in $KClO_3$, hydroxyproline, and $AlCl_3$ demonstrate that determining an effective range of treatment levels is an essential first step in stress selection *in vitro*. Once critical levels are determined, constant level and step-wise stress selection experiments can be conducted with the expectation that useful variants in significant numbers can be regenerated and established in the greenhouse for R_1 seed production and further whole plant testing.

Table 24. Greenhouse plants established for R_1 seed production from grain sorghum experiments.

Selection agent	Cultivar	Number of plants grown to maturity
Potassium chlorate	Hegari	248
Aluminum chloride	Hegari	171
Hydroxyproline	Hegari	17
Control - No stress	Hegari	22
Initiation study - No stress	SC283	6

Millet. The production of plantlets tolerant to NaCl and $AlCl_3$ and long-term regeneration of plantlets from callus cultures stressed *in vitro* have been primary goals for the millet project at TCCP.

Currently, two cultivars of pearl millet (HMP-559 and Senegal Bulk) are being used for plant regeneration from callus stressed on NaCl or $AlCl_3$. The initial experimentation of the effects of NaCl on millet growth was accomplished through several studies. In the earliest *in vitro* trials with NaCl, the work performed encompassed the use of one level of NaCl plus a control. Data was not taken at that time with the exception of recording number of cultures and total number of plants regenerated. Plants were produced from these earlier *in vitro* studies and were successfully established in the greenhouse (Table 25). With the progression of the project, experimentation was broadened to include a greater range in stress levels and data was taken in much greater detail.

Table 25. Regenerated pearl millet plants which have set seed from NaCl stressed cultures.

Cultivar	NaCl g/L	Range of passages on stress	Range of total passages	Plants regenerated
HMP	0	---	10	18
	8	2-6	13-15	50
SB	0	---	10	15
	8	3-6	12-16	66

The latter group of *in vitro* stress selection experiments proceeded with the addition of NaCl to millet initiation media following eight passages (eight months) on initiation media with no stress. The subsequent experiment was also initiated on media with no stress (for eight passages) followed by the addition of 8 g/l NaCl to the media. Both experiments were conducted by cell line with HMP and SB. At this point, the compilation of data taken shows the percentage of E white callus. This data will provide the necessary information to calculate plants per gram when plantlets have been regenerated. Callus is currently on regeneration media (Table 26). In addition, a step-wise stress selection experiment was also conducted on SB. Six g/l NaCl was inoculated in initiation media and callus was maintained for three passages followed by an increase of NaCl to 8 g/l for two additional passages. Embryogenic callus was severely reduced, and plantlets were not recovered from this experiment.

Table 26. Percentage of cultures containing white, E calli's.

Cultivar	NaCl g/L	Range of passages on stress	E callus (%)	Media mg/L
HMP	0	—	14	*5D 1A .3K
	6	7	26	5D
	8	7	17	5D
	0	—	25	3.5D 1A .3K
	8	3	43	3.5D
SB	0	—	61	5D
	6	6	35	5D
	8	6	43	5D
	0	—	46	3.5D
	6	6	27	3.5D
	8	6	24	3.5D

*D=2,4-D
A=IAA K=KIN

Table 27. Pearl millet plants which have been regenerated from *in vitro* stress selection experiments.

Cultivar	Stress	Range of passages on stress	Range of total passages	Plants regenerated
SB	0	—	15	10
	150 ppm $AlCl_3$	6	10-13	13
	8 g/L NaCl	3	12-16	72
HMP	0	—	9-12	83
	150 ppm $AlCl_3$	3-6	10-13	105
	8 g/L NaCl	2-6	12-15	128
Tift	0	—	5-9	245

Embryogenic callus was cultured on LS basal media containing 150 ppm $AlCl_3$. As with NaCl-stressed cultures, callus was subcultured initially for three passages in the absence of stress. $AlCl_3$ was added to media following the third subculture. A decline of callus growth occurred over time once callus had been subjected to the stress (Figure 17). This study was conducted with HMP and SP and was subcultured by cell line. Plantlets from both cultivars were regenerated from this stress level and grown to maturity in the greenhouse (Table 27).

Within the past year, 656 plantlets have been regenerated and established successfully in the greenhouse for the production of R_1 seed from initiation studies and stress selection experiments. Continuing work is designed to further optimize E callus growth for Line #2069, Line #7911 and Tift 23 DBE. Embryogenic callus obtained from these efforts will be utilized in other stress selection strategies. Both stepwise and

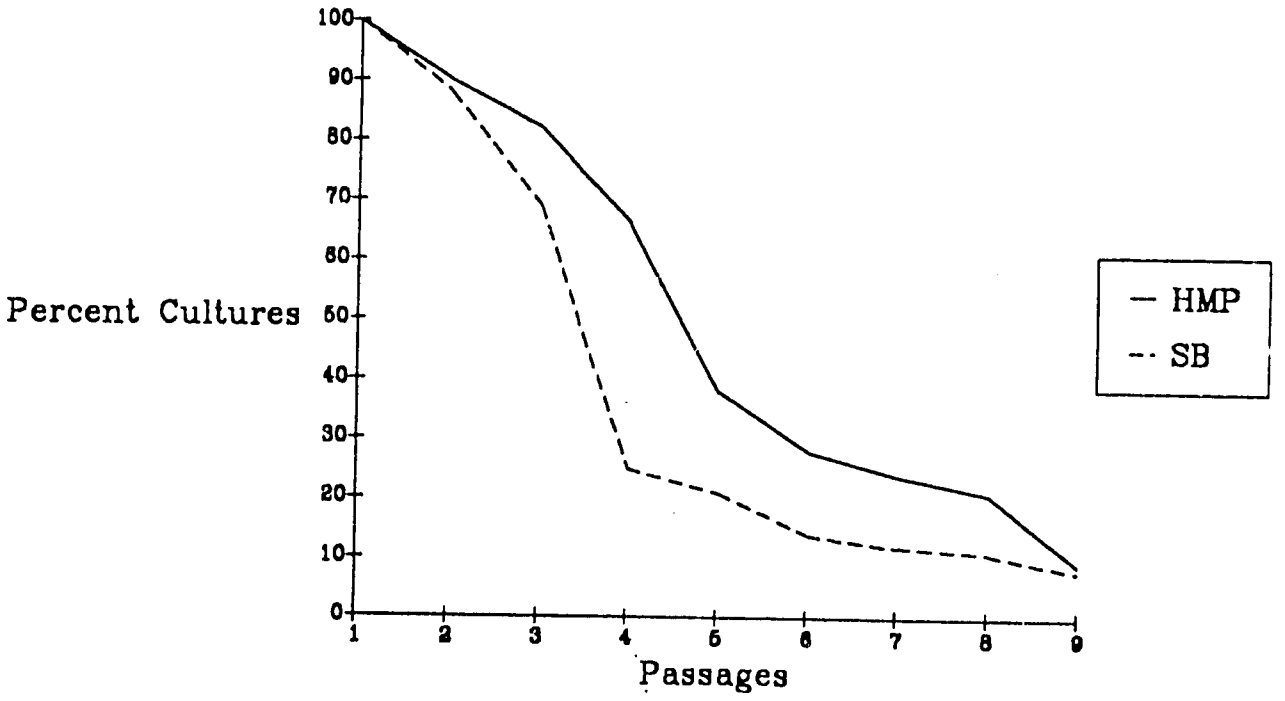


Figure 17. Percentage of cultures lost due to stress on 150 ppm $AlCl_3$.

constant level experimentation will be conducted in the continued effort to produce regenerated plantlets for whole plant testing and the production of R_1 seed.

Spring Wheat. The primary purpose of this project is to establish salt-, aluminum-, and drought-tolerant cultivars through tissue culture methods.

<u>NaCl Selection.</u> Plants from two cultivars of spring wheat (Pavon and Glennson) were regenerated from salt-stressed tissue cultures initiated on LS medium supplemented with 2 mg/l 2,4-D with or without NaCl. The regenerated plants were transplanted into soil in the greenhouse and grown to maturity. Some R_1 seed was sent to Dr. A. Mujeeb-Kazi at CIMMYT for cytogenetic analysis and salt tolerance screening. In other experiments, three cultivars, Pavon, Glennson, and Kharchia, were selected on long-term salt selection experiments on levels

of NaCl.

In the first experiment, 800 mature embryos of Pavon and Glennson were initiated on medium containing 0, 6 or 9 g/l NaCl. The E calli were maintained on their respective salt levels for eight passages and then moved to regeneration medium for plant regeneration. From 6 g/l, 85 plants of Pavon were regenerated and 2585 from Glennson. The quantity of E callus was very low on 9 g/l NaCl and one plant was regenerated from Pavon and 50 from Glennson.

In the second experiment, 800 mature embryos of Pavon and Glennson and 120 embryos of Kharchia, a salt-tolerant line from India, were used for salt tolerance screening. In this experiment, E calli produced on non-stressed media were subjected to salt-stressed medium containing 0, 4, and 8 g/l NaCl. A portion of calli from each stress level were used for plant regeneration after sixth passage (Table 28). The remaining calli were moved to fresh medium, and plant regeneration will be conducted after every three passages.

Table 28. Plants regenerated from NaCl-selected culture of Pavon, Glennson and Karchia

	NaCl level (g/l)		
Cultivar	0	4	8
Pavon	73	63	48
Glennson	58	69	70
Kharchia	78	41	66

Due to space restriction in our greenhouse, only 20 plants from each stressed level were transplanted into soil to grow to maturity.

Aluminum Selection. A study was initiated on Pavon and Glennson for aluminum tolerance. A total of 1000 embryos of both cultivars were

initiated on LS basal medium supplemented with 2 mg/l 2,4-D. The produced E-calli were then moved to the same medium for two more passages. After the third passage, E-calli were weighed and transferred to medium containing [illegible], [illegible], 200, 300, and 400 ppm aluminum. Currently these callus cultures are on their first passage on stress.

Drought Selection. A study to test critical levels of polyethylene glycol (PEG), using 0, 9, 12, 15, and 18% on spring wheat cultivars Pavon and Glennson for drought tolerance has been started. Approximately 500 mature embryos of each cultivar were initiated. Callus which is produced after the second passage will be subjected to PEG-stressed medium and tested for plant regeneration.

Plant regeneration from NaCl-tolerant cell cultures of moth bean, tepary bean and pigeonpea. Plants were regenerated from cell cultures of moth bean and tepary bean that were grown in L-6 medium with 1.0 and 2.0% NaCl. Plants were also regenerated from the callus cultures of pigeonpea that were screened on saline medium for four to five subcultures (Table 29).

For developing NaCl-tolerant cell line in moth bean, the suspension line VA 986 was cultured in high concentrations of NaCl (10 and 20 gm/l) for two weeks. Green cells were then produced after transferring the stressed cells into salt-free maintenance medium. The recovered culture was then transferred to L6 medium with 10 and 20 g/l NaCl, respectively. A salt-tolerant cell line (VA 986 ST) was isolated by repeating the above procedure for four more times. The cell lines have been maintained in 1.0 and 2.0% NaCl medium, respectively, for six months.

Table 29. Number of plants in moth bean, tepary bean and pigeonpea transferred to the greenhouse.

Conc. of salt (NaCl) (gm/l)	Moth bean	Tepary bean	Pigeon pea
0	50 (15)	28 (6)	42 (7)
5.0	-	-	35 (14)
7.5	-	-	22 (9)
10.0	52 (31)	16 (5)	18 (11)
20.0	-	12	25 (18)

1. Number in parenthesis represents the No. of plants established/grown to maturity.

2. " " indicates concentration not tested for that crop.

3. Moth bean and tepary bean plants were regenerated from cell suspension cultures, while in pigeonpea plants were regenerated from callus.

Table 30. Plant regeneration from NaCl-sensitive and tolerant cell lines of Vigna aconitifolia on NaCl-free and saline media.

Cell line	Percent embryo-forming cultures (1)			Ave. No. of plants regenerated (2)		
	Regeneration medium with NaCl					
	0%	1.0%	2.0%	0%	1.0%	2.0%
VA 986 (C)	89.7	26.6	0	10.7	2.4	0
VA 986 ST (1.0%)	67.7	74.2	14.2	7.2	6.9	1.8
VA 986 ST (2.0%)	45.0	54.2	22.4	4.3	3.6	3.2

1. 50 mg. of the cell aggregates were inoculated/jar. Data represent an average of 10 cultures.

2. Number of plants regenerated 150 mg. of embryo-forming callus. Average of 10 jars.

For plant regeneration the tolerant and sensitive cell lines were plated on L-6 agar medium with or without salt. No plants were

regenerated from control cell line on L-6 medium with salt, whereas, tolerant cell lines were grown to plants even on 2% regeneration medium (Table 30). Similarly, in contrast to E callus of the tolerant cell line, the E callus growth of sensitive cell line was almost negligible in salt containing L-6 medium. The response of tolerant cell lines to various salinities and pH regimes were observed by growing cells in respective media for three subcultures.

Cell growth and plant regeneration was also observed from the tolerant cell lines grown on L-6 medium with Na_2SO_4, mixed salts, and different pH regimes ranging from 5.0 to 7.0. Plants regenerated from different salinities are being grown to maturity in the greenhouse.

In tepary beans, we were unable to isolate a NaCl-tolerant cell line that could be induced to plant regeneration on saline medium. Plants were regenerated, however, from the TB 986 cell line which was grown in 1.0 and 2.0% NaCl for six to seven subcultures without NaCl in the regeneration media, and were transferred to the greenhouse. The plants were transferred for seed production. Experiments are also in progress to develop an Al-tolerant cell line in tepary bean.

Since we were able to regenerate plants from long-term callus cultures of pigeonpea, an <u>in vitro</u> screening and selection for NaCl-tolerant cell lines was conducted. Pigeonpea callus was found to be highly sensitive to NaCl. The callus growth was drastically decreased in saline medium as compared to NaCl-free media; however, we were able to regenerate a few plants from the callus stressed on 0.5, 0.75 and 1.0% of NaCl. The regenerated plants were then grown to maturity and the seeds collected will be increased for R_2 screening at ICRISAT. Experiments are in progress to develop a NaCl-tolerant cell suspension line of pigeonpea.

Selection for Biochemical Traits.

Nitrate Reductase. Cells or plants which are able to grow when NO_3 levels are abnormally low might have a nitrate reductase enzyme (NR) with increased activity. This ability would be beneficial in low fertility soils where available nitrogen is a limiting factor. Earlier research at TCCP showed that IR-36 callus will not survive in media with KNO_3 levels lower than what is present in the LS media (1900 mg/l). Therefore, only NH_4NO_3 provides a means for manipulating nitrate levels. IR-36 E calli, were subjected to decreasing NH_4NO_3 levels of 330, 247, 165 and 0 mg/l NH_4NO_3 in the LS medium (1650 mg/l NH_4NO_3 is the normal concentration) 65 - 75 mg in size. There were 20 replicates per treatment. Embryogenic callus died on 165 and 0 mg/l NH_4NO_3 after three passages (Table 31). The lowest level tolerated by the callus was 247 mg/l NH_4NO_3, where weights increased continually up to the tenth passage; however, the lowest level where plants were regenerated after the tenth passage was 330 mg/l NH_4NO_3. There were about 35 plants produced which are presently being grown for seeds. R_1 plants will be evaluated for increased nitrogen uptake.

Table 31. Relative growth of IR-36 embryogenic callus on five NH_4NO_3 treatments for ten passages.

	Passage Number									
	1	2	3	4	5	6	7	8	9	10
NH_4NO_3 mg/l	Embryogenic Callus Growth (%)									
1650	6	14	8	15	49	32	42	28	33	32
330	6	6	16	10	14	24	18	17	31	24
247	5	6	7	6	19	19	31	30	31	40
165	7	5	4	*	*	*	*	*	*	*
0	6	5	4	*	*	*	*	*	*	*

* Callus died

A second method was used to select callus which can grow in the

presence of chlorate, a specific NR inhibitor. Cells or plants that grow in the presence of chlorate might have an NR with altered substrate binding activity. IR-36 callus at an initial inoculum of 65 - 75 mg/10 ml medium were stressed at 0, 10, 20, 30, 40, 50, and 60 ppm $KClO_3$. There were ten replicates per treatment. Callus growth was completely inhibited at 60 ppm $KClO_3$ (Table 32). There was no significant difference between the treatments from one passage to another. The presence of $KClO_3$ in the media allowed survival of callus only up to fourth passage in all treatments. Four plants were regenerated from the 50 ppm $KClO_3$ treatment and are being grown in the greenhouse for seed. R_1 plants will be characterized for increased nitrogen uptake.

Table 32. Relative growth of IR-36 E callus under different $KClO_3$ levels.

$KClO_3$ ppm	Passage Number 1	2	3	4	No. of cultures left after 4th passage
	Embryogenic Callus Growth (%)				
0	25	28	25	15	5
10	20	26	16	18	1
20	29	28	24	13	1
30	23	25	22	17	2
40	13	37	19	13	1
50	21	44	20	29	3
60	20	*	*	*	*

* Callus died

EVALUATION OF REGENERATED PLANTS

Major improvements in the greenhouse facilities and procedures for growing regenerate plants have occurred this past year. Survival rates for the regenerate plants have increased due to the improvements. Major physical upgrades included:

Installation of double poly roof
Cooling system for wheat bay
Seed cleaning area
Propagation bench
Fiberglas headhouse roof
Installation of lighting and humidity systems

Field evaluation increases this past year included plots from Pakistan, Georgia, and Africa. Data from these field plots are beginning to provide information concerning the effectiveness of in vitro techniques.

Greenhouse Activities

Cultivation of Regenerated Plants for Seed Production. A major activity in the TCCP greenhouse is the establishment and cultivation of regenerated plants. Improving establishment percentages and seed yield of regenerates has been a major objective of the TCCP greenhouse staff during this last year. Data taken on all in vitro treatment groups of plantlets includes number of plantlets transferred to the greenhouse, tissue culture background, growing conditions in the greenhouse, dates of planting, flowering and harvest, number of early deaths, and number of sterile plants. This data will be used to evaluate and improve growing techniques of regenerated plants.

Table 33. Number of regenerated plantlets established in TCCP greenhouse, Oct. 1, 1986 - Sept. 30, 1987.

Crop	Plantlets Established
Rice	1002
Spring wheat	3177
Winter wheat	407
Sorghum	473
Millet	666
Pigeonpea	65
Mothbean	89
Tepary bean	11
Sword bean	2

Number of plantlets established is generally limited by actual greenhouse space rather than in vitro productivity. Optimum number of plants is maximized by reducing pot size and hastening maturity.

Establishment and Seed Production Efficiency. Techniques for improving establishment efficiency of regenerates are still developing the TCCP greenhouse. Establishment percentage of the cereal crops has been improved significantly during the last year. Mortality rates in sorghum, for example, have declined from 30% to less than 1%. Much of this improvement has been effected by working closely with in vitro researchers on determining optimum plantlet size and stage for transfer to the greenhouse. This optimum size varies by crop. It can be said in general, however, that very small plantlets and plantlets that have outgrown their vial are difficult to establish.

Improving the greenhouse environment for regenerates has been accomplished in part by constructing plastic chambers in each bay. These chambers are equipped with humidifiers and exhaust fans, and are covered with a layer of shade cloth to reduce direct sunlight. Placing all newly-established regenerates in this high-humidity, reduced-light environment for a period of one to two weeks has significantly increased survival percentage. Other improvements in the growth environment which have improved both survival and yield include: overhead fog systems in each bay, fluorescent lighting on timers to optimize photoperiod, and tank systems for growing rice. Cultural conditions such as pot size, growth media, and fertilizer types and amounts have been altered to improve overall vigor. It is important that each crop and cultivar be investigated individually to determine the optimum combination of growth inputs for maximizing establishment and yield efficiency.

Computerized R_0 and Progeny Inventory System. A new numbering and inventory system was implemented this year to simplify record-keeping of regenerated plants and their progeny. In the past, separate numbering systems were used for the same regenerates in the laboratory, greenhouse, and inventory records. This system led to inefficiencies and excessive paperwork. In order to simplify the record-keeping and allow field testers to more easily design plot plans, a computerized inventory was designed. This system utilizes Symphony database management software and is set-up hierarchically by crop and cultivar. The computer inventory is designed to include all of the information necessary for setting-up field experiments. It can also be used to generate lists of lines from any given tissue culture background and show what field evaluations have been conducted. Eventually, information on all TCCP regenerates and progeny will be entered into this database for permanent record-keeping.

R_0 Screening for Stress Tolerance Selection. Investigations were conducted this year on techniques for screening excess regenerated plantlets for stress tolerance. This was necessitated by overcrowding in the greenhouse due to improvements in regeneration and establishment efficiency. Tests were set-up with sorghum, millet, rice, tropical legumes, and wheat to determine optimum screening techniques for selection at vegetative stages. Variables considered include pot size, growth media, nutrient solutions, stress levels, and duration.

Table 34. Computer database for TCCP inventory.

Example of R_1 Database	Example of Progeny Database
crop: sorghum	crop: millet
cultivar: hegari	cultivar: HMP
line number: 1020105	line number: 1003031
lab experiment number: 102	selection number: 1
total passages *in vitro*: 9	generation: R_2
type of stress: salt	evaluation: drought
type of selection: constant	location Hays, Kansas
highest level of stress: 8	date: 1987
passages on stress: 6	number of selections from this line: 5
harvest date: 6/87	amount of seed: 10
amount of seed: 10.5	as of date: 11/87
as of date: 6/87	

R_0 screens were conducted for NaCl-tolerance levels from 0 to 15 g/l NaCl. It was determined that 9 to 12 g/l is the optimum selection level for sorghum and millet cultivars. Three to five g/l NaCl is the optimum range for wheat, rice, and legumes. The exact stress level which is optimum for selection of tolerance varies by cultivar. Screens for nitrate efficiency of chlorate-selected sorghum lines were tested at 0, 25, 50 and 100% normal nitrogen levels. Drought-tolerance of wheat and sorghum was screened using PEG (max 6000) at 15, 20, 25, 30, and 40% concentrations. Optimum duration of R_0 screens was determined to be approximately 30 days in a 50/50 mixture of perlite and vermiculite. Nutrient solutions varied with stress selection and species.

To date, one NaCl-stressed line of rice and two salt-stressed lines of tepary bean have been selected by this screening system.

Cultivation of Tropical Legumes in Colorado. This summer, performance trials were conducted on pigeonpea (*Cajanus cajan*), mothbean (*Vigna acontifolia*), and tepary bean (*Phaseolus acufolia*) on dry land conditions in Ft. Collins, Colorado. Plots of each species were seeded on June 1, 1987 in a shallow clay loam soil outside the TCCP greenhouse.

A total of 9.98 cm of rainfall were received during the growing period (from June 1 to September 20). No supplemental fertilizers or irrigations were applied.

Moth bean began flowering approximately 45 days after sowing and began to mature within another 20 days. They showed little sign of drought stress and yielded well. Pigeonpea segregated into three distinct height and maturity groups. The earliest group began flowering approximately 50 days after planting. These yielded exceptionally well and never appeared affected by drought stress. Tepary bean took 60 to 90 days to flower and did not have a sufficient growing season to mature this year. Of the three species, tepary bean was most severely affected by drought and cool night temperatures. These tests were useful in determining that these species can be cultivated in Colorado and may lead to local testing and generation advance of regenerated progeny from *in vitro* stress selection.

Greenhouse Screening

Rice. Since October, 1986, three rice saline hydroponic tests have been conducted in the TCCP greenhouse. Two of these have been completed and a third, RGH-106, is on-going.

One whole plant evaluation was developed to test 58 regenerate lines and checks of the cultivars IR-36, G-159, and RD-23 against checks of Pokkali in saline hydroponic conditons, and to select those lines showing salt tolerance comparable that of Pokkali for further testing in the field.

This experiment was conducted in ten, wooden, plastic-lined tanks to provide proper submergence for the growing plants. The seed was germinated in 6" sand-filled pots within the tanks. The experimental

design was completely randomized. Each regenerate or check line was replicated twice per treatment, except the Pokkali checks which were replicated three times. Both the 3 and 5 g/l were replicated twice; the 0 g/l treatment was replicated once. At two weeks after planting, a fertilizer solution (Peter's 20-19-18) supplemented with $CaSO_4$ and FeEDTA, was applied to the plantlets. At three weeks, NaCl was applied. The tanks were changed every two weeks. A vigor rating of 1 - 5, (dead to vigorous) was conducted each month, beginning one month after planting.

At termination of the experiment, the surviving plants were measured for yield. It should be noted that the yield data derived from this experiment reflects seed production in the IR-36 alone (Table 35). By the end of the experiment, none of the Pokkali or RD-23 plants had flowered. Of the G-159, four plants in the control and three plants in one of the 3 g/l tanks had flowered but did not set seed.

A second whole plant experiment was developed to screen seven regenerated Pokkali lines and checks on 0 and 4g/l NaCl, comparing the performance among lines selected in-vitro on 0,6,and 9g/l NaCl for four passages, on 3 and 6g/l NaCl for ten passages, and among selected lines on 6 g/l NaCl for 4, 10, and 14 passages.

This experiment was conducted in six wooden hydroponic tanks. Each treatment was replicated three times and each line replicated three times per treatment. Vigor ratings were carried out monthly. Height, tiller number, pannicle number, and yield data was taken at the termination of the experiment.

A third experiment was developed to screen large numbers of R_1 seed from the TCCP parent collection in a relatively short period of time, and to select survivors showing significant seedling vigor relative to

controls. Seed from the selected plants will then be included in future rice field trials for salinity tolerance.

Table 35. Hydroponic testing of regenerated rice lines in 0, 3, and 5 g/l NaCl.

Line #	*in vitro* NaCl g/l	Total passages	Yield 0 g/l	Yield 3 g/l	Yield 5 g/l
Check	—	—	1.57	5.24	—
300010	0	5	—	3.15	0.26
300041	0	9	—	1.47	—
4183	0	19	13.54	0.16	—
4229	0	9	0.025	—	—
4243	0	22	—	0.045	—
306064	0	9	0.24	0.435	—
4241	0	9	—	0.29	—
308001	8	4	4.36	0.205	—
308002	8	4	4.06	1.54	—
309003	9	2	2.56	—	—
4265	9	6	0.61	1.58	—
4238	10	1	0.91	1.23	—
R_2					
300044	0	8	7.84	1.27	—
306042	6	5	5.7	0.91	0.1
306042	6	5	1.64	—	—
306051	6	12	0.76	0.63	—
309001	9	5	2.8	3.5	—
309002	9	5	4.4	7.23	—

This experiment is an on-going one, with new sets of regenerate and check seed being sown weekly. Four IR-36 and Four Giza-159 lines, which have not been field tested, are selected from the inventory and sown with checks in separate flats of a perlite/vermiculite growing base. Each of the four lines is replicated eight times, each check, four times. The plantlets are fertilized with Peter's 20-19-18 and at three weeks, 3 g/l NaCl is applied to the IR-36 and 4 g/l NaCl is applied to the Giza-159. After eight weeks, the two sets are terminated and data is taken for height, tiller number, and vigor. Plants which have performed better

than checks are tranplanted for maturation.

As of October, 26 sets have been initiated, totaling 208 IR-36 and G-159 R_1 lines or 832 plants (including checks). Approximately 70, IR-36 and 45 G-159 plants have been transplanted.

Tepary Bean and Moth Bean. Experiments were conducted with limited populations of plants regenerated from NaCl-tolerant cell lines. The plantlets were established for 10-12 days in perlite/vermiculite mixtures in 12" pots containing nutrient media. After two to three weeks, nutrient solutions with known NaCl concentrations were applied every five days to the top of the pots. Control plants received NaCl-free nutrient solutions. The data on electrical conductivity of the solutions in the saucers, and on plant height and survival in treatments and controls were recorded every five days after adding the saline solution. Experiments were performed with seedlings as well as the regenerated plants.

The saline nutrient solution was tested at concentrations starting at EC 3.0 and increasing to EC 11.9. Salinity damage of leaf burning and stunted growth was observed within one week at an EC of four or greater (approximately 2 g/l NaCl). The plantlets of moth bean were more sensitive to NaCl than those of tepary bean. The plantlets from seeds were dead when the EC reading reached about five. The regenerated plants of moth bean were killed at the same salinity level.

The tepary bean was somewhat more tolerant. One plant survived at an EC of nine and another at an EC close to 12. These plants were transferred to soil mixtures in pots and grown to maturity for seed production.

Field Screening

Millet. Field evaluation of pearl millet lines derived from *in*

vitro selection began in 1987. Mr. Bill Stegmeier, millet breeder at Kansas State University, collaborated with TCCP in conducting these field trials at the Ft. Hays Experiment Station near Hays, Kansas. This site was chosen because the soil type and rainfall pattern usually provide sufficient drought pressure for selecting early generation material with increased tolerance to dry conditions. Pearl millet is thought to have originated on sandy soils and is generally cultivated on these soil types. The field site at Hays was chosen by Mr. Stegmeier for its clay soil in order to select for lines which have the ability to penetrate heavy soils in search of moisture. The lines with more vigorously penetrating root systems have been shown to have increased drought tolerance.

Sixty plots were planted June 20, 1987 representing five R_1 lines from Senegal Bulk and eight R_1 lines from HMP-559. These lines were derived from *in vitro* NaCl selection at 0, 3, 6, and 8 g/l NaCl (Table 36). Four replications of each line were planted in single row plots, 7.3 m long, on 0.9 m centers. The field was previously wheat fallow and 35.2 kg/ha nitrogen fertilizer was applied. No significant cultural problems were encountered except for some poor stands due to reduced seed quality and a brief hail storm during vegetative growth.

Although this field test was designed to evaluate the performance of R_1 lines under moisture stress, regular rainfall and relatively mild temperatures prevented drought conditions from developing. The longest rainless period fell between July 19 and August 3 and was not severe enough to set-back plant development. The overall vigor of the plots was very good, and although drought tolerance could not be rated this year, the test did allow for observation of a significant amount of variability

both within and between R_1 lines. Morphological characters such as leaf architecture, panicle length and width, seed color, plant height, and growth habit were seen to vary between lines of each cultivar tested. Physiological traits such as vigor, maturity, spikelet fertility, and incidence of leaf spot were also variable. It was not uncommon to see variation for these traits within a line. These variations were noted to occur across replication throughout the field study.

Selections were made and bagged to ensure self-pollination. These were harvested by Mr. Stegmeier and will be used for further testing in the R_2 generation.

Table 36. R_1 Pearl millet lines grown in Hays, KS. field test, 1987.

Cultivar	TCCP line #	Total passages	Stress level (g/l NaCl)	Passages on stress
HMP 559	100002	7	0	0
	01000102	9	0	0
	103003	5	3	5
	103020	6	3	6
	106001	4	6	4
	106006	4	6	4
	106018	5	6	5
	01080103	13	8	6
SB 7203	200001	10	0	0
	02000101	15	0	0
	203010	9	3	9
	203018	11	3	11
	02080111	13	8	3

Rice. In the Philippines, TCCP regenerate rice lines are being field-tested for NaCl tolerance. To date, our collaborators there have been supplied with over 500 R_1 and R_2 lines.

The subagreement between TCCP and the University of the Philippines at Los Baños (UPLB) was entered into on October 1, 1986. The designated Principal Investigators from UPLB were Dr. Edwin L. Javier and Dr. Eduardo P. Paningbatan, Jr.--both with the Agronomy Department. Dr.

Javier has since left UPLB and has been replaced by Dr. Jose E.

Table 37. Summary of lines sent to UPLB for seed increase and saline tolerance evaluations.

Cultivar	*in vitro* NaCl level (g/l)	Range of passages represented	# of R_1 lines sent	# of R_2 lines sent
Calrose	0	3-11	25	2
	3	6-8	17	9
	6	4-7	1	3
G-159	0	7-20	34	1
	3	3-15	28	-
	6	2-11	83	16
	8	10	5	9
IR-36	0	2-14	40	1
	3	4-11	42	-
	6	2-15	33	4
	8	4	2	-
	9	2-5	4	10
Mahsuri	0	4-9	5	1
	3	3-6	29	-
	6	2-3	20	-
	8	3	3	1
	9	3	1	2
Pokkali	0	3-14	13	-
	3	4-5	3	-
	6	39-22	16	-
	9	2-9	3	-

Hernandez (Agronomy). Also involved in the administration of this project is the Philippine Council for Agriculture and Resources Research and Development (PCARRD).

The general objectives of this project are to determine the magnitude and direction of variation among lines of rice generated from *in vitro* NaCl-selection, and to identify NaCl-resistant lines or plants that could be used as parents in hybridization work or entered in preliminary yield testing.

Five cultivars—Calrose-76, Giza-159, IR-36, Mahsuri, and Pokkali—

Table 38. Surviving lines of the 1987 Pampanga saline field trial and their culture histories.

Line #	Passage length on stress	NaCl level (g/l)
Calrose		
Check	—	—
100028	11	0
100029	9	0
103024	6	3
103026	6	3
103031	7	3
103036	7	3
103039	7	3
103041	7	3
103046	8	3
103048	8	3
106001	7	6
106003	7	6
G-159		
Check	—	—
200100	17	0
206060	8	6
206061	8	6
206063	8	6
206099	9	6
206101	9	6
206103	9	6
206106	10	6
206110	10	6
206112	10	6
206123	10	6
206139	11	6
208001	10	8
208002	10	8
208010	10	8
208012	10	8
Mahsuri		
Check	—	—

were included in this project. By January 1987, approximately 387 R_1 lines were planted for increase at IRRI. Sixty-three R_2 lines were transplanted to one saline paddy in Pampanga Province (Table 37). A full characterization was carried-out on the maturing R_1 plants in the IRRI paddies. Environmental, pest and disease infestation, and survival studies were conducted at the Pampanga saline trial.

At that site, EC in the paddy water reached as high as 12 ms/cm by March, and some 35 surviving plants were transplanted to recovery plots at Los Baños (Table 38). No yield data is available on the survivors as this time as this was a non-replicated plot. From the IRRI increase characterization, it was noted that growth habit and tillering ability differed among the IR-36 R_1 lines. Pokkali check plants were observed to be more susceptible to whorl maggot than most of the R_1 lines, and six Pokkali R_1 lines exhibited semi-dwarf stature while the rest were tall. Initial results indicated that mutations had been induced during culture.

In December, 1987, R_2 plants from the IRRI R_1 increase, as well as R_4 plants from R_2 salinity trial survival and R_3 increase will be transplanted into four saline trial plots in Pampanga. Salinities in the separate paddies will be maintained at different levels through controlled inputs from an on-site fresh water well.

Sorghum. Field testing of R_1 and R_2 sorghum lines was conducted in Yuma, Arizona this year in collaboration with Pioneer Hi-Bred and the University of Arizona. The Yuma site provided the first opportunity to field test our sorghum lines in a saline environment. Dr. Dwight Tomes, Pioneer's tissue culture research manager and Dr. Robert Voight, University of Arizona sorghum breeder helped TCCP obtain and set-out the stress selection plots. A total of 1000 plots of sorghum lines derived

from *in vitro* NaCl selection of cultivars Hegari, RTx-430, and Keller were screened for stress tolerance. The test was located at the University of Arizona Agricultural Center near Yuma. This site has a silty clay soil type with an average pH of 8.0. Salinity hazard is increasing in this area due to declining quality of irrigation water from the Colorado River.

The test plots were planted on June 22, 1987. Each plot consisted of a single row 3 m long on 1 m centers. Each regenerate line was replicated two to four times depending on available seed. The field was fertilized with 44 kg N/ha and irrigated a total of five times. Irrigations were spaced to allow for a degree of NaCl stress to build-up. Some drought stress was also evident on both visits to the site by TCCP personnel. Soil and water samples taken from the field site and analyzed at CSU indicated that the salinity hazard of our particular field was not as high as we had originally estimated (Table 39). Electrical conductivity (EC) readings of 2-4 mmhos/cm indicated a low to moderate salinity problem. As a result of these moderate salinity levels, we relied upon a combination of environmental factors to select stress-tolerant individuals.

Table 39. Soil and water data from Yuma plot.

	Date	location	pH	EC	SAB	Lime (est.)
Soil	8/20	top of field	8.1	2.0	3.7	high
Soil	8/20	bottom end	8.1	4.0	4.5	high
Soil	9/15	top	8.0	2.9	5.0	high
Soil	9/15	bottom	7.9	2.9	5.0	high
Water	8/20	irrigation ditch	8.0	1.1	2.3	—

Among these factors were drought, high temperature, salinity, and

insect infestations. Each line and replicate were rated for vigor, growth stage, insect tolerance, and unusual characteristics. Selections for generation advance were made on the basis of stress tolerance and overall vigor for continued R_2 and R_3 testing. These selections were bagged to preclude bird damage and insure self pollination.

The Yuma test was divided into two separate experiments. The first experiment (Exp. 203) was designed to allow observation of specific in vitro selected lines in the R_1 and the R_2 generation for comparison of variation between generations. The second experiment (Exp. 204) was set-up to compare R_1 lines from various levels of in vitro NaCl stress, duration on stress, and total culture time in a field-stressed environment. Due to the moderate level of salinity which occurred in this field, it is difficult to completely satisfy the objectives of these two experiments; however, these tests did increase our knowledge of field screening regenerated material, and we were able to make selections of individuals which performed well to be tested in later generations.

Experiment 203 included R_1 Hegari and RTx-430 lines from 0, 9 and 12 g/l NaCl in vitro stress selection. These were planted in the midst of their R_2 progeny which had been selected the previous year from field trials in Georgia and Texas. R_1, R_2, and non-regenerated checks were replicated and randomized within one or two consecutive ranges in the field plot. This allowed side by side comparison of the generations. One objective of this comparison was to see if selections made on non-stressed environments would represent the variability of the line when subjected to stress in the field. A total of 11 R_1 lines and 48 R_2 selections were compared for vigor and uniformity (Table 40). Non-regenerated Hegari was used as the check comparison.

Table 40. Summary of R_1 and R_2 Hegari lines used in Exp. 203.

TCCP R_1 lines	# of R_2 lines	*in vitro* stress level (g/l NaCl)	Stress passages	Total passages
2007	1	0	--	9
2182	5	0	--	10
2208	5	0	--	11
2413	5	9	5	11
2442	5	9	5	11
2443	4	9	5	11
2639	5	12	3	9
2640	6	12	3	9
2644	4	12	3	9
2649	3	12	4	10
2650	5	12	4	10

At the time of this report, all plots have been harvested, but no yield data has been computed; however, the two site visits by TCCP personnel did net some data and observations of interest. Ratings were assigned for vigor, growth stage, and insect tolerance. Vigor rating (1=excellent, 5=dead) were based on leaf number and color, height, panicle size and number, and stress injury symptoms. Growth stage ratings were based upon the observation that salt and drought stress delay anthesis in susceptible lines. Earliness was therefore considered a useful selection criterion. Insect tolerance ratings were taken because data from the Georgia tests suggested that some TCCP sorghum lines had significant tolerance to fall armyworm and sorghum midge. Some lepidopterous pests were present in the Yuma plots, presenting the opportunity to gather more data on insect tolerance in a second environment. Insect feeding was noted to occur throughout the plots, but at sporadic levels. The overall level and distribution was not uniform enough to allow for significant screening for tolerance. Each plot was scored (1=no damage, 5=severe damage) and some variation was noted.

Whether this variation was due to distribution or tolerance could not be determined.

The relatively moderate salinity levels of this test site did not promote the expression of highly tolerant lines. This may also have obscured some differences between R_1 and R_2 generations. It was generally noted at both vegetative and reproductive stages that the R_1 plots of a given line were less uniform overall than the self-pollinated, single head selections which made up the R_2 lines. Two-hundred thirty-seven selections from Exp. 203 were made on the basis of overall vigor, growth stage, and stress tolerance. The best of them will be re-tested in harsher environments as R_3 material. Table 41 summarizes the nine best and eight poorest lines from Exp. 203 on the basis of field performance.

Table 41. Selected R_2 lines from Exp. 203.

Lines rated	*in vitro* level g/l NaCl	Stress passages	Total passages
very good			
2182-1	0	0	10
-2	0	0	10
-4	0	0	10
2413-8	9	5	11
2442-6	9	5	11
2443-4	9	5	11
2649-7	12	4	10
2650-8	12	4	10
poor			
2007-3	0	0	9
2639-1	12	3	9
-2	12	3	9
-3	12	3	9
-5	12	3	9
-6	12	3	9
2640-5	12	3	9
-9	12	3	9

Experiment 204 was designed to allow for comparisons of sorghum lines of the same parent and generation but from various levels of *in vitro* stress and time in culture. The field plot was laid-out in a completely randomized block design. *In vitro* stress levels included 0, 9, 12, 13, and 15 g/l NaCl. Total culture time ranged from 7 to 13 passages (Table 42). A total of 100 R_1 lines of Hegari and 62 R_1 lines of Keller were included. These entries exhibited a significant amount of variation between lines. Vigor, growth stage, panicle fertility, plant morphology, and insect feeding varied between R_1 lines.

Table 42. Tissue culture background of R_1 Hegari lines included in Exp. 204.

# of R_1 lines	*In vitro* stress level, g/l NaCl	Stress passages	Total passages
7	0	0	7
3	0	0	8
1	0	0	9
14	0	0	10
7	0	0	11
5	0	0	12
3	0	0	13
4	9	4	11
15	9	5	11
15	9	7	13
5	12	1	8
1	12	2	8
2	12	2	9
1	12	2	10
3	12	2	12
3	12	2	13
1	13	1	12
2	15	3	12
1	15	1	12
7	15	2	13

A comparison of mean vigor rating values indicates that intermediate total passage times of seven to nine months are most vigorous (Table 43). *In vitro* stress level of 13 g/l NaCl had the best vigor and growth stage

rating. When separated by total passage in culture, there is no clear trend for vigor, but after nine passages, growth stage is consistently later, indicated by the low rating values. Time on stress did not cause significant change in either vigor or growth stage. Yield data will be analyzed and compared to these data to see if an optimum culture time and level of *in vitro* stress for selecting tolerant cell lines can be established.

Table 43. Mean values of vigor and growth stage ratings for various *in vitro* stress levels and times from Exp. 204.

	Stress Level, g/l NaCl				
	0	9	12	13	15
Vigor*	2.68	2.76	2.62	2.33	3.19
Growth Stage**	2.93	2.73	2.62	3.33	2.54

	Total Passages								
	0	6	7	8	9	10	11	12	13
Vigor	2.66	3.00	2.59	2.56	2.50	2.62	2.85	3.08	3.83
Growth Stage	3.06	2.50	2.91	2.92	3.00	2.83	2.75	2.62	2.68

	Stress Passages					
	0	1	2	3	5	7
Vigor	2.68	2.80	3.04	2.80	2.77	2.75
Growth Stage	2.93	2.60	2.68	2.40	2.81	2.67

* Vigor ratings at anthesis. 1= excellent, 5= dead

** Growth stage rated on 9/14/87. 1= vegetative, 2= boot, 3= anthesis, 4= grain filling to mature

One-hundred and ninety-two selections were made from Exp. 204 which

showed promise as stress-tolerant lines. These were bagged and harvested individually as separate lines for R_2 testing next year. Table 44 lists the line numbers and tissue culture background of some very good and poor performing lines.

Table 44. *In vitro* background of outstanding lines in Exp. 204.

Lines rated	*In vitro* stress level, g/l NaCl	Stress passages	Total passages
very good			
2154	0	0	7
2156	0	0	7
2180	0	0	10
2566	9	7	13
2580	9	7	13
2594	9	7	13
2602	9	7	13
2704	15	2	13
poor			
2281	0	0	11
2694	15	2	13
2703	15	2	13

Aluminum Screening in Georgia. This was the second year for evaluating regenerated sorghum lines on a low pH, high aluminum soil. The previous year, R_1 seed was used previously while this year R_2 seed was available. The R_2 seed was planted on an acidic soil (pH 4.0 to 4.3) as well as on a normal soil (pH 6.1). A summary of the lines planted is in Table 45.

A pH of 4.2 is the lowest pH the acidic soil tolerant sorghum line, SC 283, will grow. Therefore, there were areas in the field were even SC 283 did not survive. The field was blocked by cultivar and within each block every ten rows were a row of non-regenerated check and a row of SC 283. This provided side by side comparisons throughout the field.

Table 45. Summary of R2 lines planted on low pH, high aluminum soil.

Cultivar	Number of Lines	Total Passages	Stress Passages	NaCl Level
Hegari	31	6	-	-
	5	10	-	-
	6	12	-	-
	4	11	4	9
	5	15	4	9
	9	11	5	9
	19	13	7	9
	1	9	3	13
RTX 430	3	3	-	-
	1	7	-	-
Rio	8	3	-	-
	7	7	-	-

Two site visits were conducted. The first, when the plants were 45 cm tall, about 40 days after planting, to make a visual ratings of the plots. At this time the majority of the plots had been eliminated by the stress environment. The lines with the highest ratings of 4 and 5 are listed in Table 46.

Table 46. Highest vigor rating in July of regenerate sorghum lines on low ph high aluminum soils. Seed from the same head selection grown on normal ph soil was sent to Puerto Rico.

	in vitro Information			
Line Number	Total Passages	Stress Passages	NaCl Level (g/l)	Rating
2024-7	6	-	-	5
2043-9	6	-	-	5
2110-2	6	-	-	5
2110-8	6	-	-	5
2131-1	6	-	-	5
2518-4	13	7	9	5
2110-1	6	-	-	4
2118-6	6	-	-	4
2128-5	6	-	-	4
2384-6	15	4	9	4
2471-7	11	5	9	4
2522-10	13	7	9	4
2539-4	13	7	9	4
2539-5	13	7	9	4
2592-6	13	7	9	4

Dr. Duncan has a winter nursery in Puerto Rico and consented to let us use up to 100 plots for a seed increase. The seed had to be sent by the middle of October. The lines which had been rated high (Table 47) were chosen as well as several R_1 lines from aluminum selected cultures (Table 47).

Seed set was hampered, again this year, in Georgia due to a drought which began in mid July through the end of October. There were plants in 30 plots which set seed (Table 48). There was one line, 2024-7-19, which produced significantly more seed than the rest. Of the 30 plots which set seed, 16 of them were rated high in July.

An interesting aspect of this field evaluation is that the regenerated plants are from tissue cultures or tissue cutlures stressed on NaCl. Therefore, we can ask questions concerning somaclonal variation. Of the 30 lines which set seed, 16 are from non-selected callus and 19 from NaCl-stressed cultures. When comparing the 31 lines derived from non-stressed cultures 53% set seed, while from the 50 lines from NaCl-stressed cultures 28% set seed. As might be expected NaCl did not increase the aluminum tolerance in the regenerated lines; however there is tolerance above that of the non-regenerated seed due to be regenerated from <u>in vitro</u> cultures.

Table 47. Number of R1 lines sent to Puerto Rico through Dr. Duncan.

Passages on Stress	$AlCl_3$ Stress Level		
	100	400	700
1	6	3	3
2	5	5	-
3	3	-	-
4	2	-	-
6	3	-	-

Table 48. Lines which had plants which set seed on low pH, high aluminum soil with drought conditions during flowering.

Line Number	in vitro Information: Total Passages	Stress Passages	NaCl Level (g/l)	Seed Yield (g)
2021-4-1	6	-	-	35.0
2024-7-15	6	-	-	4.6
-16				4.0
-17				7.5
-18				4.8
-19				79.7
2034-5-1	6	-	-	3.5
2034-5-2	6	-	-	1.6
2043-9-15	6	-	-	2.2
-16				1.8
2047-3-15	6	-	-	2.2
-16				20.4
2102-8-15	6	-	-	3.9
2110-1-15	6	-	-	14.5
2110-1-15				6.7
-16				28.4
2110-8-15				7.9
-16				7.4
-17				1.9
2113-5-15	6	-	-	36.2
2117-2-1	6	-	-	3.6
-2				9.8
2117-4-15	6	-	-	6.2
-16				11.2
2122-3-15	6	-	-	10.9
2125-3-15	6	-	-	16.2
2128-5-15	6	-	-	0.9
-16				25.4
2131-1-15	6	-	-	5.2
2131-1-16	6	-	-	19.7
-17				28.8
2135-1-15	6	-	-	15.6
-16				1.0
2353-2-15	11	4	9	14.0
2384-6-15	15	4	9	2.6
-16				9.4
2471-7-15	11	5	9	6.1
2473-3-1	11	5	9	7.6
2518-4-15	13	7	9	4.4
2518-4-16	13	7	9	6.5
2522-6-1	13	7	9	18.4
-7-1	13	7	9	2.4
2539-4-15	13	7	9	21.1
2539-5-15				13.9
2541-6-1	13	7	9	2.6
2637-6-15	9	3	12	1.3
-16				1.8

Insect Screening. Nine sorghum entries of regenerated material were planted in replicated tests on two different planting dates at the Coastal Plain Experiment Station of the University of Georgia at Tifton in 1987 (Table 49). The first planting was on May 8 and the second on May 27.

Individual plots consisted of a single row 3 m in length. Sorghum plants were infested with neonate fall armyworms at the early whorl stage using the bazooka-method described by Wiseman et al. (1980) [Fla. Entomol. 63:425-432]. Visual ratings were made to determine the degree of defoliation beginning four days after the last infesting date, with plants checked at least every other day for about one week.

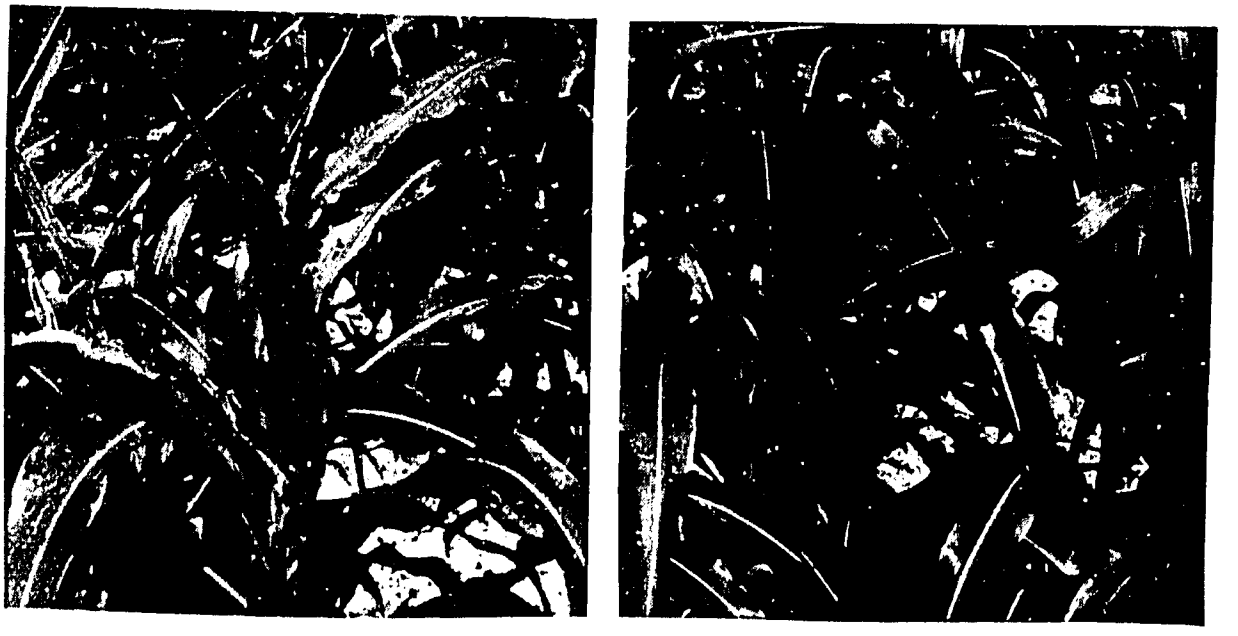
The line showing the most promise as a source of FAW resistance in the whorl stage in the field was 2442 (Table 49, Figure 18). Self-pollinated heads of this line were obtained from several plants for further evaluation in the field, greenhouse and laboratory. The greenhouse screens revealed no evidence of resistance at the seedling stage in plants from remnant R_2 seed or R_3 plants of 2442. This does not mean that whorl stage resistance will not be present, only that FAW resistance is not expressed at the seedling stage. The laboratory and greenhouse studies scheduled for December should answer the question of whorl stage resistance to the fall armyworm in line 2442.

Sorghum midge damage was also considered, with visual ratings being conducted. Heads showing a marked reduction in sorghum midge damage were marked and harvested for seed. Evidence of possible sorghum midge resistance was observed in two lines. Both 2354 and 2435 (Table 50), especially the latter, produced heads of a much higher quality than any of the other lines in this planting. Sorghum midge pressure was very

severe during the season and the lesser degree of head damage for 2354 and 2435 was very distinct. Field studies during 1988 will be conducted to determine if actual sorghum midge resistance exists in these two lines or whether the difference in damage was due to an escape.

Table 49. Lines selected in 1986 and planted at Tifton in 1987 for fall armyworm (FAW) screening.

Cultivar	Number of lines	Total passages	Passages on stress	Stress level	Tolerance
Hegari	2	6	—	—	
	1	12	—	—	
	1	11	4	9	2354 midge
	3	11	5	9	2442 FAW
	2	13	7	9	2435 midge



A. B.

Figure 18. Regenerated sorghum lines. A. Damage caused by FAW to a sensitive line. B. Little damage on lin 2442.

Drought study in Niger. In May, ten lines were sent to Niger for evaluation in a severe environment (Table 50). Seeds were sent through Dr. Eastin at the University of Nebraska and were planted by Dr. John Clark of the Purdue INTSORMIL group stationed in Niger. The seeds were planted on July 2, 1987 in sandy soil. The pH of the soil was about 4.0 with soil temperature up to 50°C. Seed germinated but died soon after due to the high soil temperature, drought conditions, low fertility, and sand blasting.

Table 50. Summary of lines sent to Niger for evaluation.

	in vitro information		
Number of Lines Sent	Total Passages	Passages on Stress	NaCl Level (g/l)
1	8	0	0
2	10	0	0
6	9	3	12
1*	0	0	0

* Non-regenerated check seed.

TRAINING

The primary objective of the TCCP plant tissue culture training course is to assist researchers of Developing Countries to gain skills they require for the execution of various research programs.

In the past year, two training sessions each of six months duration have been conducted at the Tissue Culture for Crops Project at Colorado State University. Eight trainees have received diplomas during this period for having fulfilled all of the course requirements. The current training session (fall 87) hosts five trainees from countries representing India, Malaysia, Mexico and Guatemala.

The Course offers a wide range of topics in plant tissue culture. All trainees regardless of their area of specialization or prior tissue culture experience have the opportunity to gain skills on a multitude of disciplines pertaining to plant tissue culture. Lectures and discussions are conducted by the training program staff consisting of a full-time coordinator, a quarter-time instructor, and three consulting instructors. In addition, four Project personnel serve as guest speakers each semester. Laboratory exercises are performed on model plant systems or on crops used at the TCCP for research. The practical experience further familiarizes the concepts introduced through the lectures.

The integration of theory and practice of plant tissue culture (PTC) by implementing individual research projects is an important feature in the program. A detailed literature survey is made, followed by the completion of a written proposal. All experiments in this proposal are planned and designed by the trainees and supervised by the training staff. Trainees have the choice of selecting a crop of importance to their countries. In the fall 1987 session (current), all trainee

projects are integrated with the TCCP research programs, with two working in cereals and three working on selected legume species. All research is focused on the development of stress selection strategies.

RESEARCH HIGHLIGHTS.

After receiving comprehensive formal instruction in various concepts and protocols of plant tissue culture in the first half of the program the entire latter half of the training period is devoted to the development of an appropriate research portfolio and implementing research on crops of their choice. The results of the research are presented prior to graduation (Table 51).

Table 51. Research projects of trainees in FY 1987

Session	Researcher	Crop	Titles of projects
Fall 1986	R. Avenido Philippines	Mung bean Pigeon pea	Plant regeneration and selection for NaCl tolerance in callus culture of mung bean and pigeonpea
	F. Bakheet Saudi Arabia	Wheat	*In vitro* screening for drought tolerance in spring wheat
	B.D. Gothwal India	Wheat	Callus induction, plant regeneration and *in vitro* selection for salt tolerance in winter and spring wheat cultivars
	E. Rillo Philippines	Coconut	Callus induction, maintenance, and plant regeneration from immature inflorescences and embryo of the coconut palm (*Cocos nucifera* L.)
	O. Benlhabib Morocco	Tomato	Plant regeneration and salt tolerance selection of three lines of *Lycopersicon escelentum* and wild species *L. peruvianum* and *L. cheesmanii*

Table 51 (continued)

Session	Researcher	Crop	Titles of projects
Fall 1986	A. Matibiri Zimbabwe	Tobacco	Study of plant cytoplasmic genomes 1. Chloroplast - mitochondrial recombination 2. Induction and selection for chloroplast mutations
Spring 1987			
	C. Rohrmann Guatemala	Potato	Meristem culture to produce virus free planting material for potato (Solanum tuberosum)
	B. Silayoi Thailand	Banana	Preliminary observations on callus induction and regeneration in some Musa species
Fall 1987			
	G. Casas Mexico	Sorghum	Establish procedures for sterilization, regeneration, and drought tolerance selection.
	M. Mahmood Malaysia	Tepary bean Peanut	in vitro plant regeneration and selection for aluminum tolerance in selected legumes
	M. Purohit India	Millet	In vitro screening for drought tolerance in pearl millet
	I. Singh India	Lentils	Callus induction and plant regeneration in lentils and in vitro selection for aluminum tolerance in moth bean
	R. Bressani Guatemala	Black bean	In vitro screening for drought tolerance in moth bean and plant regeneration from callus cultures of black bean

LABORATORY HANDBOOK.

A laboratory management handbook was compiled during the spring 1987 session. This consists of detailed information on set-up, management, and maintenance of a plant tissue culture facility. The manual will be used

for instructional purposes in future semesters and is intended to provide some guidelines to participants when resuming work upon returning home. Earlier in the Training Program, a similar manual on basic plant tissue culture methods was also compiled and has been used when conducting laboratory exercises for the past four training seesions.

GERMPLASM PRESERVATION.

A short course on germplasm preservation has been added in 1987 to the Program as a topic in the formal instruction. Dr. Leigh Towill of the National Seed Laboratory, Fort Collins, CO., serves as the instructor. Students receive information on the concepts of germplasm preservation and visit the National Seed Laboratory on the CSU campus.

QUESTIONNAIRES.

The progress of graduates is monitored periodically by circulating questionnaires by mail (Table 52). This enhances communication with the trainees after they have returned to their home institutions. In the past, assistance has also been given by sending specific technical information in the form of publications. This is of importance to participants for whom inaccessibility to current literature is a major limiting factor of research.

FUNDING.

One of the major and consistent problems encountered in administering the Training Program is the limited funding available to sponsor participants. In the last year, there were 58 inquiries including 46 applications received (Table 53). Only 13 of the 30 nominees were able to attend the program, primarily due to lack of sponsors (Table 54). Currently TCCP is concentrating its efforts on locating funding sources for potential candidates. A directory of IPBNet

members and possible funding agencies will be published in January 1988.

Table 52. A summary of the current activities of graduates of the fall 86 session (responded to questionnaire).

	Avenido	Benlhabib	Gothwal	Rillo
Nature of present work	Research	Teaching Research	Research	Research
Area of work	Physiology	Agronomy Breeding	Plant Breeding	Plant breeding
Crop	Wheat, Citrus Mungbean	Barley, Wheat	Wheat, Barley Pearl millet	Coconut
TC tech. used most	*In vitro* selection, Endosperm culture, Callus culture			
Extension of training	trained visitors to lab	not yet	informally training colleagues	two assistants
Source of funds	Government, International	Government	Government	Government
Major limitation	Inaccessibility to chemicals and equipment	Funding	Funding	Personnel

Table 53. Information on applicants.

Session	# Applicants (accepted)	Countries	#Participants	# Unable to attend	Reason
Fall 86 (12)	19	Philippines Nepal, India Sierra Leone Saudi Arabia Ethiopia Nigeria, Zimbabwe Morocco	6	6	Lack of funds
Spring 87 (8)	12	Philippines, Nigeria, Egypt Solomon Islands Guatemala, Thailand	2	6	Lack of funds 1 declined
Fall 87 (10)	15	India, Malaysia Mexico, Guatemala Egypt, Tunisia, China, Ethiopia, Nigeria, Ghana, Saudi Arabia, Philippines, Thailand	5	5	Lack of funds
Total (30)	46		13	17	

Table 54. Funding sources which have supported trainees.

Session	Agency	# Individuals Sponsored	Amount
Fall 86	King Abdul Aziz Univ.	1(Saudi Arabia)	S & F & T
	Morocco Project/USAID	1(Morocco)	S & F & T
	Faculty of Agriculture Project		
	Michigan Univ./ Zimbabwe	1(Zimbabwe)	S & F & T
	USAID/India	1(India)	F & T
	TCCP	1(Philippines)	S
	TCCP	1(Philippines)	S & F
Spring 87	IAEA/UNDP	1(Thailand)	S & T & F
	TCCP	1(Guatemala)	F
	Personal funds		S & T
Fall 87	Enviro BioTech, USA	1(India)	S & F & T
	TCCP	1(India)	S & F
	Fulbright		T
	TCCP	1(Malaysia)	S & F
	Personal Funds		T
	TCCP	1(Mexico)	F
	CONACYT		S & T
	Personal Funds	1(Guatemala)	S & F & T

S= Stipend
F = Fees
T = Transportation

6/13 fully funded; 4/13 partially funded by sources other than TCCP; 3/13 fully funded by TCCP.

INTERNATIONAL PLANT BIOTECHNOLOGY NETWORK

Since its inception in early 1985, the International Plant Biotechnology Network (IPBNet) has gained over 1000 affiliated members from 72 developing and 18 developed countries. The Network grew extensively in 1986-1987. Major activities included seeking new and mutually beneficial and strengthening existing collaborations, expanding the computerized literature filing system, distributing publications and reports pertaining to the Project's progress, and sponsoring and coordinating the Second International IPBNet Conference in Bangkok, Thailand, in January, 1987. IPBNet also helped establish a joint tissue culture training course with CATIE.

Expanding IPBNet's growth and effectiveness in 1986-1987 included the continuation and strengthening of ten Memoranda of Agreement (MOA) with institutions in eight countries in Asia, Africa and Latin America. Four of these MOA's are with International Agricultural Research Centers.

Currently, the most active international collaborations are with CATIE, CIAT, CIMMYT, ICRISAT, NIAB, PCARRD, UPLB and ViSCA. These agreements encompass activities ranging from training to field testing (Table 55). There are also several U.S. collaborations which are discussed in the research sections of this report.

In April 1987, CATIE held the first session of a jointly-developed tissue culture training program. The Program, which was patterned after the TCCP Training Course, lasted three months, was taught in Spanish, and emphasized tissue culture of tropical crops which the TCCP is not equipped to handle. The program was a huge success, evidenced by the number of applicants and eventual participants. Fifty persons applied;

Table 55. Current memoranda of agreement; and sub-agreements

Institution	Country	Activity
CATIE	Costa Rica	Regional Training Center
CIAT	Colombia	Possible Aluminum testing in rice
CIMMYT	Mexico	Cooperative research/ wheat, personnel exchange
Chiang Mai Univ.	Thailand	General - graduate studies training
Chulalongkorn Univ.	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
LSU	USA	Sub-agreement, rice research
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

originally, the plan was to accept only 15, but 20 were accepted due to the great demand. All of the participants obtained funding from University, governmental, private, or personal sources in Latin America.

In June 1987, Mr. Thomas A. Dykes, former IPBNet Coordinator, was hired as a consultant to travel to CATIE and assess the program. Some problems became evident, such as the excessive amount of time spent on

formal lecture and the difficulties associated with a group whose experience ranged from none to many years. It also became clear that CATIE had certain expectations of the TCCP which had gone unexpressed and unfulfilled, such as financial and personnel support. The TCCP is currently re-negotiating its agreement with CATIE to the mutual satisfaction and benefit of both parties. Details on the CATIE program and Mr. Dykes' observations and recommendations are contained in his trip report which was sent to AID in September.

The collaborations with CIAT, CIMMYT, ICRISAT, and NIAB are all on research or field testing and are discussed in detail in the research section of this report.

The MOA with PCARRD is for coordination and monitoring of collaborative research (subgrants) in the Philippines. The Agreement was recently re-negotiated to allow for better formal communications. PCARRD oversees all agricultural research in the Philippines and they review and approve all subgrant proposals and reports and control disbursement of all subgrant funds sent to the Philippines. Our relationship with them is crucial to the implementation of research in the Philippines. The details of subgrants with UPLB and ViSCA are discussed in the research section of this report.

U.S. collaborations with the University of Arizona, University of Georgia, and Kansas State University are for field testing of sorghum and millet. Details are included in the research section of this report.

A large part of IPBNet's function is information dissemination. It has been expressed at IPBNet conferences and by participants in the TCCP and CATIE training courses that one of the major limitations of performing research in developing countries is the lack of access to

scientific publications. The TCCP has therefore spent a lot of effort on building a computer library of scientific publications. At their request, IPBNet collaborators can obtain listings of references on any crop, stress, or method. The library currently contains 50,000 entries.

The TCCP also distributes scientific information through newsletters, progress reports, and conference proceedings. In grant year three, at the request of IPBNet members, the format of the newsletters has been changed slightly to include scientific reports and announcements from the members. The newsletters have become a key communication link with scientists in developing countries and are distributed on a regular basis to more than 800 IPBNet members. In addition, several hundred requests for newsletters and other Project information are filled on a yearly basis.

In July, the TCCP distributed its comprehensive 1987 Progress Report. This was the first major report of the five-year Cooperative Agreement made available for external distribution. The Report summarizes the research and networking activities of the TCCP since its inception in September of 1980. The volume contains several methods appendices, and thus provides most of the basic information needed to initiate tissue culture experimentation.

The Second International IPBNet Conference was held in Bangkok, Thailand, January 11-16, 1987 sponsored by the TCCP and Chulalongkorn University. Seventy-nine persons from 18 countries attended including 36 Thai scientists. Keynote speakers included Dr. Roger Beachy, Dr. Paiboolya Gavinlertvatana, and Dr. Colette Nitsch addressing topics of gene movement, rapid propagation, and anther culture, respectively. Thirty-one papers and over two dozen posters were presented. Follow-up

activities included compilation of abstracts for publication (62 total) and incorporating feedback from roundtable discussion groups to improve IPBNet's effectiveness. The idea for the IPBNet Directory, which will contain IPBNet member names and research interests plus a list of funding agencies, was a result of these discussions. The Directory will be distributed in January 1988 along with Newsletter No. 8.

Another important function of the TCCP is technical assistance. In 1986, TCCP was contacted by the National Academy of Science (BOSTID) to perform tasks for the Government of Thailand and USAID/Thailand. The task was to prepare recommendations for development of biotechnology programs for Thailand. Oluf Gamborg spent four weeks in Thailand to obtain the necessary background information. Proposals were then prepared with details on objectives and requirements for personnel, facilities, and costs. The overall objectives were to use the proposed projects as a plan for USAID to enable Thailand to develop technologies for application in their crop improvement programs. The proposed plans would set-up research in tissue culture in each of the major universities in Thailand. The total estimated cost is $2.2 million.

At the time of the IPBNet Conference in Bangkok in January 1987, Drs. Nabors and Gamborg were asked by Thailand Science and Technology Development Board (STDB) to review and make recommendations on nine projects for funding. The projects, which at this time have been approved for funding, were for tissue culture research in oil palm, coconut, and soybean, respectively. One project to establish molecular biology projects to introduce virus resistance by genetic cross protection was also approved. Dr. Gamborg and/or Dr. Nabors are named as consultants in each of the approved projects. The funding is provided through USAID to

the Government of Thailand (STDB).

After the IPBNet Conference in Bangkok, Oluf Gamborg visited India, Pakistan and Bangladesh. At ICRISAT near Hyderabad, India, the final arrangements were made for the collaboration on tissue culture of chickpea and for field testing of legumes. As a result, Ms. Sheila Vijayakumar came to the TCCP to perform research sponsored by ICRISAT. In Delhi, Dr. Gamborg had discussions with Dr. Pollack and Dr. Smith, USAID, and Dr. Abrol, Deputy Director General, IARC, to discuss the participation of the TCCP in the subproject of the INDIA/US Cooperative Scientific Agreement which is on tissue culture for crop improvement and micropropagation. TCCP was involved in defining the research and is now named as a participating US Institution.

In Pakistan, Oluf Gamborg presented invited seminars at the Advanced Molecular Biology Research Institute, University of Lahore, at the Agricultural Research Institute, Faisalabad, and the Nuclear Institute of Agriculture and Biology (NIAB), Faisalabad. The visit also included discussions with USAID and Winrock. One of the principal reasons for visiting Pakistan was to meet with Dr. Naqvi, Director, NIAB, and Dr. Malik, collaborator on an existing joint project with TCCP. Dr. Gamborg reported on the progress in the research to produce hybrids of rice and kallar grass by cell fusion. The research is part of a plan to transfer salinity tolerance from the highly-salt-tolerant kallar grass to Basmati rice. Dr. Zafar from NIAB (sponsored by USAID/Pakistan) has spent time this last year working on the project at TCCP. Arrangements were also made for field testing at NIAB and other institutions in Pakistan as TCCP germplasm becomes available. The field testing of rice began in June, 1987.

The visit to Dhaka, Bangladesh, was at the invitation of the USAID Mission (Mr. A.R. Hurdus, Mr. Kevin Rushing). Strong enthusiasm was expressed by Dr. A. Rohman, Director for crops at BARC, Dr. Ali, Director Genera, BARC, Dr. M.. Mannan, Director General, BRRI and Dr. S. Rohman, Joint Secretary, Ministry of Agriculture to send trainees to the TCCP and to have the TCCP as a resource in the development and application of tissue culture and biotechnology in crops.

In July, Kerri Wright, Network Coordinator, traveled to Nairobi, Kenya to solidify plans for the Third IPBNet Conference. Africa was chosen as the site with plans to improve linkages for field testing in drought and acid tolerance, and to provide opportunities for scientists in preliminary stages of plant biotechnology development to become familiar with applicable research for their region's needs. Kenya was identified as the specific site due to its central location, stable political situation, and linkages which have been established with IPBNet since 1985.

While in Nairobi, Ms. Wright met with Drs. John Thomas and Al Smith of USAID to discuss the design and implementation of Kenya's agricultural research and development program. John Thomas referred Ms. Wright to Dr. William Wapakala, Director of the Kenya Agricultural Research Institute (KARI). John Thomas was very supportive of a IPBNet Conference in Nairobi and a subsequent proposal will be submitted to USAID for possible funding of 10-15 local Kenyan scientists to attend.

A meeting was arranged with Dr. Wapakala and B.N. Majjisu, Head Breeder for KARI. At the time of the meeting the budget for plant biotechnology and KARI's overall plan was still under negotiation. Midwestern International Agricultural Consortium (MIAC) is involved with key research organization and implementation on behalf of the U.S. and

meetings to decide where priorities would be placed were forthcoming. Dr. Wapakala did express an interest in TCCP's six-month Training Program and wanted Kenyan scientists to be able to participate in the Conference as well as assimilate information presented there. As of yet very few Kenyan scientists have actually been trained in tissue culture and plant molecular biology techniques.

Ms. Wright also met with several members of the Crop Science Department at the University of Nairobi, Kabete Campus. Mr. Ben Musyimi, a graduate of TCCP's first training session, is still active in the Crop Science Department under the direction of Dr. Kimani Waithaka. Contact was also made with Dr. B.I. Muruli, a cowpea breeder, who in turn came to visit TCCP during a sabbatical leave in the United States. Dr. Muruli discussed with TCCP staff several options for field testing sorghum, millet and wheat material in the spring of 1988. An agreement is pending with the University of Nairobi for a field testing experiment.

In addition, personnel from eight hotels were interviewed and the Silver Springs Hotel was chosen as the Conference site. The Silver Springs came highly recommended from both John Thomas, and Dr. S.O. Keya, Dean of Faculty of Agriculture at the University. The Conference will be held January 8-12, 1989. Funding sources for conference participants are presently being pursued.

SPECIAL FOCUS.

Three processes will be studied to produce entirely new, NaCl- or other stress-tolerant wheat and rice hybrids which cannot be obtained when standard crop improvement breeding techniques alone are used: 1) Making widecrosses between crop plants and alien species and tissue culturing their embryos to increase frequency of translocation of chromosomes; 2) Isolating protoplasts of stress tolerant species and fusing them with those of cultivated lines; hybrid plants are regenerated and the trait transferred by backcrossing to the crop plant; and 3) Identifying and characterizing the molecular genetic material for stress tolerance among various lines. Using gene cloning, gene splicing and transformation designed to transfer genes responsible for stress tolerance to crop plants.

The resulting germplasm from the three approaches will be tested in the greenhouse and the field. In this effort, the cooperating institutions and the developing countries will include CIMMYT, IITA, ICRISAT, IRRI, Thailand, India, Pakistan, and Indonesia.

HYBRIDS OF PIGEONPEA AND ATYLOSIA PLATYCARPA THROUGH EMBRYO CULTURES

Some of the wild relatives of pigeonpea, for example *A. albicans*, *A. platycarpa* and *A. scarabaeiodes* grow well in stress environments. Such traits could be transferred to pigeonpea if hybridization were possible, thus a crossing program was initiated in the TCCP greenhouse to produce intergeneric hybrids.

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

Gibberellic acid and KN were used independently at concentrations ranging from 3.5 to 27.7 mg/l of GA and 2.1 to 17.2 mg/l of KN and in combinations thereof to prolong the retention of hybrid pods. A hypodermic needle was used to fill the bud cavity surrounding the pistil with hormone solution. The treatments were given at 24 hours and 48 hours after pollination. When A. platycarpa was used as male parent, most of the pollinated pigeonpea buds dropped within two days. In the reciprocal cross, the pollinated buds of A. platycarpa were retained for four days. A treatment of GA_3 at 17.3 mg/l prolonged bud retention for twelve days. The delay in the drop of pollinated buds resulted in the successful rescue of embryos when in vitro culture was employed.

After hormone treatment, 14% of the pollinated buds were retained on the female parent, pigeonpea c.v. ICP-7128, for more than 12 days. On the twelfth day the buds were transferred to L-6 medium with 0.5 mg/l KIN plus 0.01 mg/l GA for plant development. Eight intergeneric hybrid plants were regenerated on this medium. Two of the hybrids were grown to maturity in the greenhouse. The F1 seeds of the plants are being grown in the greenhouse to observe the segregation pattern. Promising selections will be used in backcrosses with the female parent.

WIDECROSSES IN WHEAT.

The TCCP/CIMMYT collaboration was initiated in mid-1985 with research activites to be conducted in Mexico in association with CIMMYT's wheat widecrossing program. A TCCP staff member, Nitschka S. ter Kuile, was posted in CIMMYT in order to initiate the tissue culture collaborative efforts and to obtain wheat widecross, cytogenetics and breeding expertise. Two areas of research are within the scope of this project: 1) Media manipulation conducted for long-term callus cultures and plant regeneration of tetraploid and hexaploid wheat cultivars used

in widecrosses, and 2) introduction of alien genes into wheat by widecrosses.

Several cultivars of Triticum aestivum and T. turgidum were evaluated and five T. aestium and six T.turgidum were selected on the basis of the crossability potential for further study associated with hybridization with alien species. Callus cultures of these cultivars were established by June 1986. Several Aegilops accessions were identified which had resistance to karnal bunt. This alien genus was selected for transferring karnal bunt resistance to wheat via tissue culture. Selected accessions were Ae. umbellulata, Ae. variabilis, Ae. squarrosa, and Ae. ventricosa.

Ae. variabilis has been identified as a near immune species to karnal bunt. It hybridizes satisfactorily with T.aestivum, has substantial N-bands on all chromsomes, posesses some isozyme markers, and in a 35 chromosome F_1 (T. aestivum/Ae. variabilis) hybrid is characterized by low pairing (less than one bivalent per meiocyte). Such hybrids then form an excellent means of evaluating alien transfer responses via tissue culture. Several F_1 hybrids of T. aestivum/Ae. variabilis have been produced, embryos excised and callusing initiated. The regenerated plants from long-term cultures are being maintained in the greenhouse. These plants will be backcrossed to T.aestivum.

Callus culture and regeneration was adequately achieved for the five T. aestivum and six T. turgidum cultivars that will be incorporated in applied crossability studies with Aegilops species. R_0 plants were grown in the greenhouse, spikes were bagged to ensure selfing and production of R_1 seed that were increased to form R_2 progeny. This base-line study will serve as means of field evaluation for heritable

responses.

NACL TOLERANCE THROUGH PROTOPLAST FUSION.

An agreement was reached between the TCCP and the Nuclear Institute for Agriculture and Biology (NIAB), Pakistan in October, 1985 to employ recent biotechnology techniques to develop NaCl-tolerant crops. Protoplast fusion hybridization between two distantly-related plants which cannot be crossed through standard breeding is a novel approach which is being implemented in this joint research. The cereal selected for the project is the indica type, fine grain, scented Basmati (B-370) rice (Oryza sativa). It is a foreign cash crop for Pakistan and is sensitive to NaCl. Kallar grass (Leptochloa fusca), the other parent for fusion, is a halophytic grass and is recommended as a primary colonizer of NaCl-affected soils of Pakistan. This species is a C_4 (high photosynthetic efficiency) and also has associative nitrogen fixation. The plants selected in this research scheme were not explored earlier in any detail. Therefore, experiments were initiated to establish the required basic procedures for cell fusion hybridizations.

Plant regeneration.

The experiments on both plants were initiated simultaneously in September, 1986. The callus initiation in rice was made from excised embryos of mature seeds. The seeds were surface sterilized with 30% commercial bleach (Clorox) for thirty minutes and washed thoroughly with sterilized distilled water. Embryos were excised and placed on LS, B5 and N6 media with three levels of auxins and cytokinins. Calli were observed after two weeks. After four weeks, calli were transferred to initiation media to increase the callus size. E calli (yellowish-white, compact) were later placed on regeneration media and plantlets were

obtained through somatic embryogenesis. It was observed that the N6 medium with 4.0 mg/l NAA, 0.5 mg/l 2,4-D and 2.0 mg/l BA and LS medium with 1.0 mg/l 2,4-D and 0.5 mg/l KIN were equally good for callusing. For regeneration, N6 medium with 0.5 mg/l NAA and 2.0 mg/l BA and LS medium with 0.5 mg/l IAA and 0.4 mg/l BA were observed to be optimum. Calli are maintained on LS (0.5 mg/l 2,4-D and 0.25 mg/l KIN) and N6 media (0.5 mg/l 2,4-D and 0.25 mg/l KIN). Experiments were repeated in the summer of 1987 and similar results were obtained.

One of the basic prerequisites for using callus for mutant selection is the establishment of in vitro cultures with a high frequency of plant regeneration. Experiments were therefore initiated for long-term maintenance of B-370 E callus. Four different media (B5, N6, LS and MS + 1% sorbitol) were being used to select the most suitable one for Basmati rice. The results indicated that regeneration ability declined with the time period. LS medium with 0.5 mg/l 2,4-D and 0.25 mg/l KIN was optimum for maintenance. The experiment was continued for six months.

The surface-sterilized mature seeds of kallar grass were used for initiation of callus. Among the different media (LS, B5, N6, Blaydes, Schenk and Hildebrandt) tested, the B5 medium with 2.0 mg/l 2,4-D was found to be the most suitable. Unlike rice, the growth of kallar grass callus was very slow. Regeneration was achieved in kallar grass in B5 and LS media with 0.5 mg/l NAA and 2.0 mg/l BA (Figure 19). Experiments are presently being repeated by using B5 and modified MS containing 1% sorbitol and less inorganic nitrogen.

The studies carried out so far have resulted in establishing a system for routine regeneration of plants for Basmati rice and kallar

grass by tissue culture.

Figure 19. Plants regenerated from callus of kallar grass.

Somatic embryogenesis of cell suspension.

After establishing the conditions for regeneration on agar media, experiments were initiated for establishing cell suspension cultures of Basmati rice and kallar grass. Cells growing in liquid media provides a good source of protoplasts.

The chopped materials of friable callus of rice or roots and shoots of sterile germinating seedlings were used as the starting materials. An appropriate amount of this material was placed in the following media: (1) B5-1 (200 mg/l casein hydrolysate), (2) N6, (3) LS, (4) amino acid medium (AA), and (5) AA plus protoplast culture medium (PCM) without osmoticum (1:1). Cultures were placed in 125 ml Erlenmeyer flasks and kept on shakers at 100 rpm in continuous light at 20-22°C. Media were replaced every two weeks without reducing the cell

densities. After three months a cell line of Basmati (RB-187) was established. This is the first report of a cell suspension line in Basmati rice. A schematic protocol is given in Figure 20. The cell line is maintained on AA with 2 mg/l 2,4-D (Figure 21). The doubling time of cell line RB-187 was compared in three different media. It was observed that under our conditions the doubling time of RB-187 was three to four days (Figure 22). Cell aggregates > 500 um were transferred to six different regeneration media. Three plants were regenerated in MS medium (0 hormone); however, in the latter transfers only green spots were formed which were unable to grow into plantlets. Ongoing efforts are being made to obtain a method for routine regeneration of plants from cell line of RB-187. Multi-step procedures of transferring the cell clumps to different media are being employed.

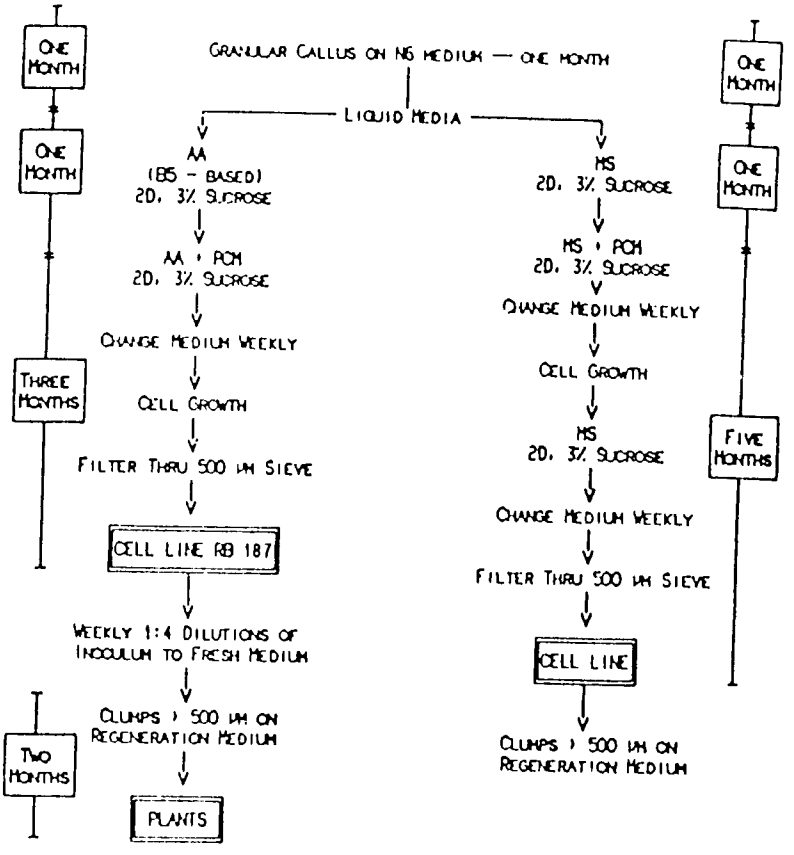


Figure 20. Flow diagram for obtaining cell suspension cultures of Basmati-370 rice.

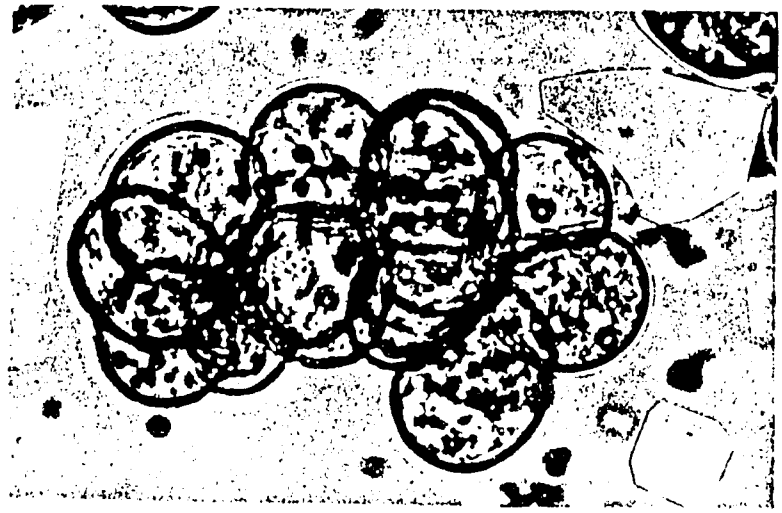
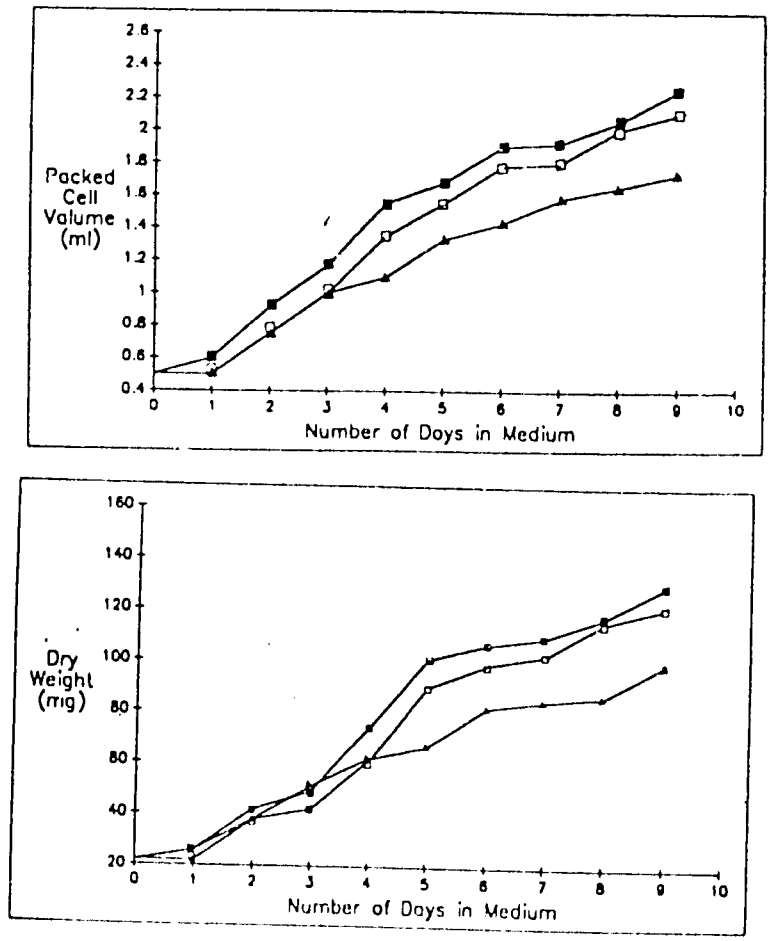


Figure 21. Cells from suspension cultures of Basmati-370 rice.



Amino acid + protoplast culture medium --■
Amino acid (MS-based) medium --□
Amino acid (B5-based) medium --▲

Figure 22. Growth rates of RB 187 on various media.

Work on establishing the cell line of kallar grass was initiated simultaneously with that of rice. The conditions, starting materials and media were as described above. In developing cell lines of kallar grass, some flasks were also placed at higher temperatures/dark condition. The growth rates of some of the cultures are high. The cells are longer and do not resemble "typical" cells of suspension cultures of other crops. The cultures are maintained on AA with 2 mg/l 2,4-D. Cell aggregates of cell line KG-187 were placed on different regeneration media. Only green roots were formed in some plates. Experiments are in progress to standardize the conditions for development of E cell line of kallar grass.

The experiments reported above resulted in development of two cell lines of Basmati rice (RB-187 and RB-387) and one cell line of kallar grass (KG-187). Efforts are continued to define reliable plant regeneration methods using the newly-developed cell suspension lines.

Protoplast isolation.

The isolation of protoplasts leading to regeneration in cereals has met with limited success. In view of the limited reports on effective systems for regeneration of plants from protoplasts of cereals, attempts were first made to obtain protoplasts from the leaves of aseptically-grown young seedlings of Basmati rice and kallar grass. Yield of protoplast was found to be low despite variations in the seedling age and level of enzymes (cellulase, hemicellulase and pectolyase).

Attempts were made to determine the optimum concentrations of cell wall digesting enzymes for the isolation of protoplasts from newly established cell lines of rice (RB-187 and RB-387). The optimum concentrations of cellulase, hemicellulase, and pectolyase were found to

be 300 mg, 200 mg and 10 mg/10 ml, respectively. The other conditions like culture age, isolation procedure and percoll density gradient levels were also fairly well established. The protoplast yield is good.

The attempts to isolate the protoplast from the leaves of young seedlings of kallar grass was also not successful. Efforts are now being made to isolate the protoplast from the cell suspension of Kallar grass.

Salt-tolerant cell suspension cultures.

Plant cells growing in liquid medium are generally more uniform and multiply at a higher rate than callus cells grown on solid agar media. All the cells are immersed and in contact with the medium therefore the selection of stress-tolerant cells may be more stringent in liquid media. Attempts were made to develop salt-tolerant lines of Basmati rice (RB-187 and RB-387) and Kallar grass (KG-187) from the established cell cultures.

The cell lines of rice (RB-187 & RB-387) were grown in AA medium with 1% NaCl. The cultures were transferred every 10 days. The cells turned brown. Transfer of these cells to AA medium minus salt resulted in normal growth of the cells. After repeated inter-transfers between AA medium with and without salt, cell lines of the Basmati rice were established which grow in 1% NaCl.

A fast-growing cell suspension of Kallar grass (KG-187) was grown in AA medium with 1% and 2% NaCl, respectively. Cells turned brown in 2% NaCl, while growth in 1% NaCl was normal. The cells in 2% NaCl were then transferred to AA medium + 1% NaCl. After restoring the growth, the cells were transferred back to AA medium + 2% NaCl, the medium in which the line is being maintained (KG-587-S).

The cell line of kallar grass (KG-587-S) is providing a source material for research on the molecular basis for NaCl tolerance. The NaCl

tolerance trait can also be used as a marker for the selection of somatic hybrids in future experiments of fusion hybridization between kallar grass and rice.

The above reported results look promising and substantial progress has been made in this project; however, there is still considerable work that needs to be done. USAID, Islamabad, Pakistan has agreed to sponsor another person for a period of one year to complete the task. The attainment of the objective of producing hybrids would be a unique and highly significant achievement, scientifically, as well as being a practical application in plant breeding for NaCl tolerance in rice.

PROTOPLAST FUSION HYBRIDIZATION OF DIPLOID POTATOES.

The technique of protoplast fusion of two diploid lines, Gp. tuberosum x Gp. phureja (US-W-9310.3 and 77-9) and three diploid Solanum species, S. bulbocastanum, S. phureja and S. microdontum is being studied. The regenerated hybrid fusion will have bacterial wilt resistance derived from S. tuberosum lines and heat tolerance from Solanum species.

The approaches involve the study of regenerability of plant materials (leaf mesophyll and suspension culture), screening for heat tolerance of Solanum species, establishment of suspension culture of S. tuberosum lines, protoplast fusion, and selection and regeneration technique of fusion hybrids.

Regenerated plants were obtained from mesophyll protoplasts of both S. tuberosum lines which were planted at 6 x 10^5 density (Figure 23). The isolation and culture method was a modification of Kao & Michayluk (1975). Expanded leaves from one-month-old shoot cultures were

incubated in protoplast culture media with 1.25% Onozuka cellulase, 0.5% Sigma hemicellulase and 0.125% Sigma pectolyase, 0.5 M osmoticum with glucose, at 27°C in the dark for three hours. Protoplasts were purified with 20% percoll in culture media followed by centrifugation (Figure 24). Protoplasts were counted in a haemocytometer and then adjusted to the desired density by adding protoplast culture media. They were plated by transferring 0.5 ml onto solidified culture media in a petri dish (50 x 10 mm). The culture condition was 23°C, 12/12 light/dark photoperiod. Callus formed after two months of culture was transferred to induction media for one month and then to regeneration media.

Suspension cultures were established after six months culture from 3-4 gm tuber callus which had been grown in MS supplemented with 6 mg/l NAA, 2 mg/l 2,4-D and 0.2 mg/l BA. A fine suspension culture was obtained after a screening at several subcultures using 250 um mesh. The media was B5 macro- and micro-nutrients, Nitch & Nitch vitamins and organics: 500 mg/l casein hydrolysate, 876 mg/l L-glutamine, 266 mg/l aspartic acid, 174 mg/l arginine and 7.5 mg/l glycine with 1 mg/l 2,4-D and 0.2 mg/l either BA or KIN. The cultures are subcultured every seven days. The growth curve and regenerability of these suspension cultures are being studied.

Four lines of S. bulbocastanum, S. microdontum and S. chacoense were selected after heat screening based on percentage of injury (Kefe and Elfigih, 1983 (Table 56). The electrolyte leakage from leaf disc (1 cm diameter) after treatment at 50°C for thirty minutes were taken from the plants grown in the greenhouse, and then transferred into the growth chamber at 35°C for 24 and 48 hours. These four lines now are being propagated for leaf mesophyll protoplast isolation. The enzyme composition required for both for cell suspension and leaf of these lines

Figure 23. Potato plants regenerated from protoplast.

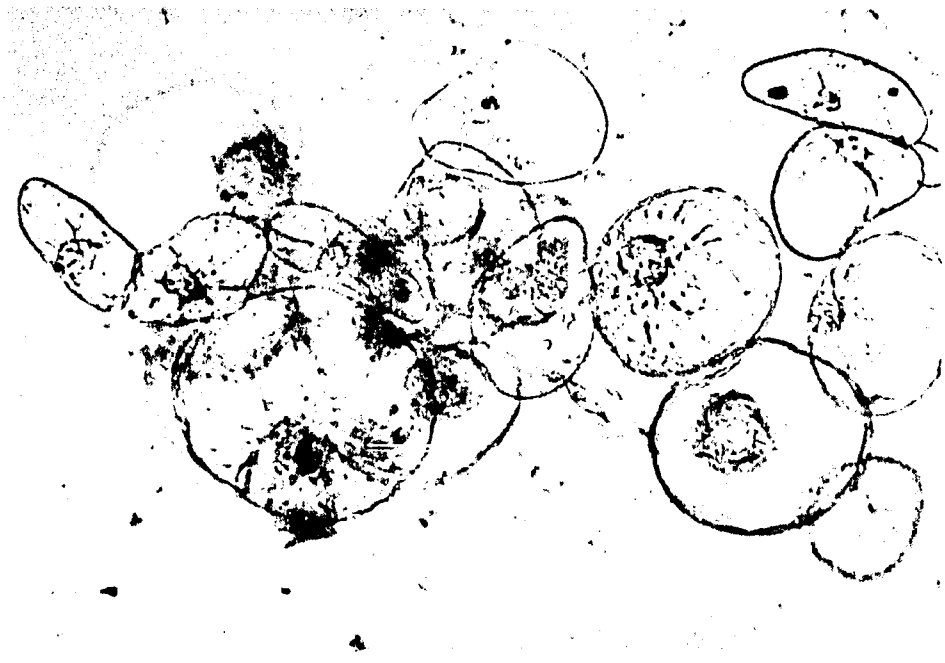


Figure 24. Protoplast from potatoes.

are being studied.

ISOLATION AND CLONING OF STRESS PROTEINS AND THEIR SPECIFIC GENES.

Research is in progress to identify genes which are induced to be expressed in response to NaCl stress. The rationale is that genes which are induced by NaCl stress are likely to play a role in conferring NaCl tolerance. Recent work by others has indicated that specific genes can be induced by salt stress (Singh *et al.*, 1985; Ramagopal, 1987); however, the specific role of any gene product in conferring NaCl tolerance remains to be established.

Table 56. Percentage of injury of four lines *S. bulbocastanum*, *S. chacoense* and *S. microdontum*.

Line No.	Percentage of Injury			
	greenhouse	24 hrs 35°C	48 hrs	35°C
S. bulbocastanum				
	187.21	35.37	79.90	65.64
S. chacoense				
	2	80.09	46.07	24.05
	8	65.72	78.13	69.30
S. microdontum				
	5	70.72	78.97	58.23

The plant material is cell suspension cultures of a salt marsh grass (*Distichlis spicata*). The *D. spicata* system was chosen for the following reasons: 1) Well characterized suspension cultures of *D. spicata* are available; 2) Use of suspension cultures allows more stringent NaCl-stress conditions; 3) Both *D. spicata* plants and suspension cultures exhibit high levels of salt tolerance; and 4) Use of suspension cultures should allow isolation of genes involved with conferring NaCl tolerance specifically at the cellular level as opposed to NaCl tolerance at the organ or whole plant level. The objectives in

this project are: 1) To determine the NaCl concentration and time of exposure that are most effective at eliciting changes in gene expression; and 2) To produce and screen a cDNA library derived from poly(A)$^+$ RNA isolated from cells exposed to the NaCl treatment determined in (1).

Preliminary results obtained by comparing the in vivo polypeptides produced by D. spicata cells growing in LS medium plus 10 g/l NaCl to the in vivo polypeptides produced by cells growing in LS medium (at 0 g/l NaCl) showed no detectable change in the polypeptide pattern at the level of 1-dimensional SDS-polyacrylamide gels or the level of 2-dimensional O'Farrel gels. Subsequently the research was changed to the approach of isolating poly(A)$^+$ RNA, translating the mRNAs in vitro, and analyzing the in vitro produced polypeptides on SDS-polyacrylamide gels. This approach was chosen in order to observe polypeptides which are actively synthesized during salt-stress as opposed to those polypeptides which are relatively abundant.

Initially the effect of growing the cells in LS medium containing 10 g/l NaCl on the polypeptides produced in an in vitro translation system was studied. The results indicated that no mRNAs encoding novel polypeptides were observed to be induced by including 10 g/l NaCl in the culture medium.

In subsequent experiments, NaCl concentrations of 25 g/l and 40 g/l were tested. The assumption was that 10 g/l may not be perceived as a stress by the highly NaCl tolerant D. spicata cells. Initial results indicated the production of a polypeptide at approximately 29 kDA by seven days of salt stress at 40 g/l NaCl. At 40 g/l NaCl the D. spicata cells do not show significant growth (Warren and Gould, 1982; I. John, O. Gamborg, J.T. Colbert, unpublished) but the cells exhibit metabolic

activity as evidenced by their ability to take up ^{35}S-methionine and produce labeled polypeptides *in vivo*.

The effect of 40 g/l NaCl in the culture medium for shorter time periods was investigated. One day after transfer to a medium with 40 g/l NaCl, cells exhibited enhanced levels of mRNAs encoding polypeptides of approximately 70, 54, 39, and 32 kDA (Fig. 25). One or more of these polypeptides may play a role in conferring NaCl tolerance to *D. spicata* cells. Attempts are now being made to confirm these results. Based on current data the plan is to produce a cDNA library derived from poly(A)$^+$ RNA isolated from *D. spicata* cells treated with 40 g/l NaCl for one day in an attempt to obtain cDNA clones corresponding to mRNA species which increase in abundance in response to salt stress.

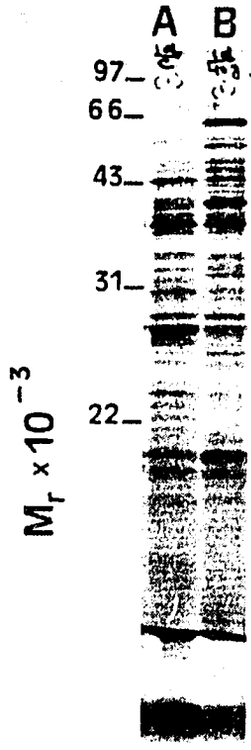


Fig. 25. Induction of specific mRNAs by salt stress. Poly(A)$^+$ RNA was isolated from Distichlis spicata cells which had been subcultured for one day in LS medium (A) or LS medium plus 10 g/l NaCl (B). The isolated poly(A)$^+$ RNA was translated in vitro using ^{35}S-methionine and the rabbit reticulocyte lysate system. Equal TCA-precipitable counts per minute of ^{35}S were applied to each lane (30,000 cpm/lane). After electrophoresis the SDS polyacrylamide gel (12%) was dried and exposed to X-ray film for six days at -70°C. The abundance of polypeptides at approximately 70, 54, 39 and 32 kDa is enhanced in lane B. Positions of the protein molecular mass markers are indicated.

MANAGEMENT AND OPERATIONS

Management of the TCCP has become increasingly effective over the course of the past fiscal year. Much of this stems from experience gained by Management Team members in the first two grant years and is largely a function of a stable workforce.

The "Management Team" consists of nine persons, each with specific areas of responsibility. This "team" works as a unit to decide on policy changes and carries direct responsibility for goal-setting and implementation, reporting, budgeting, and operations of the Project. Areas of responsibility and authority are outlined below:

Murray Nabors, Ph.D. Project Director. Directly accountable to AID for goal achievement. Directs team members to implement goal-related activities.

Julie Ketchum, MBA Operations Director. Responsible for Project Budget, human resources development, editing of reports, newsletters and manuscripts, and general policy development and implementation.

Oluf Gamborg, Ph.D. Associate Director. Responsible for Molecular Biology research program, report and grant writing, and IPBNet development and expansion in India and Pakistan. Primary scientific consultant.

Gary Hanning, Ph.D. Research Coordinator. Responsible for direction and supervision of research, and report and manuscript writing.

Kerri Wright, M.S. Netowrk Coordinator. Responsible for expansion and organization of IPBNet. Partially responsible for sorghum field testing.

Sunitha Siriwardana, M.S. Training Coordinator. Responsible for direction and execution of Training Program. Partially responsible for USDA Winter Wheat program.

Reagan Waskom, M.S. Greenhouse Manager. Responsible for greenhouse

operations and maintenance. Partially responsible for sorghum field testin

Glen Hildreth, B.S. Lab Manager. Responsible for procurement of all lab supplies, management of lab operations, and public relations tours.

Lee Olton, Office Manager. Responsible for procurement of office/computer supplies, billing and payment, travel, and personnel processing.

The core research group consists of six persons at the B.S. and M.S. level. These persons have responsibility for hands-on research and data analysis and are directly accountable to the Research Coordinator and Associate Director.

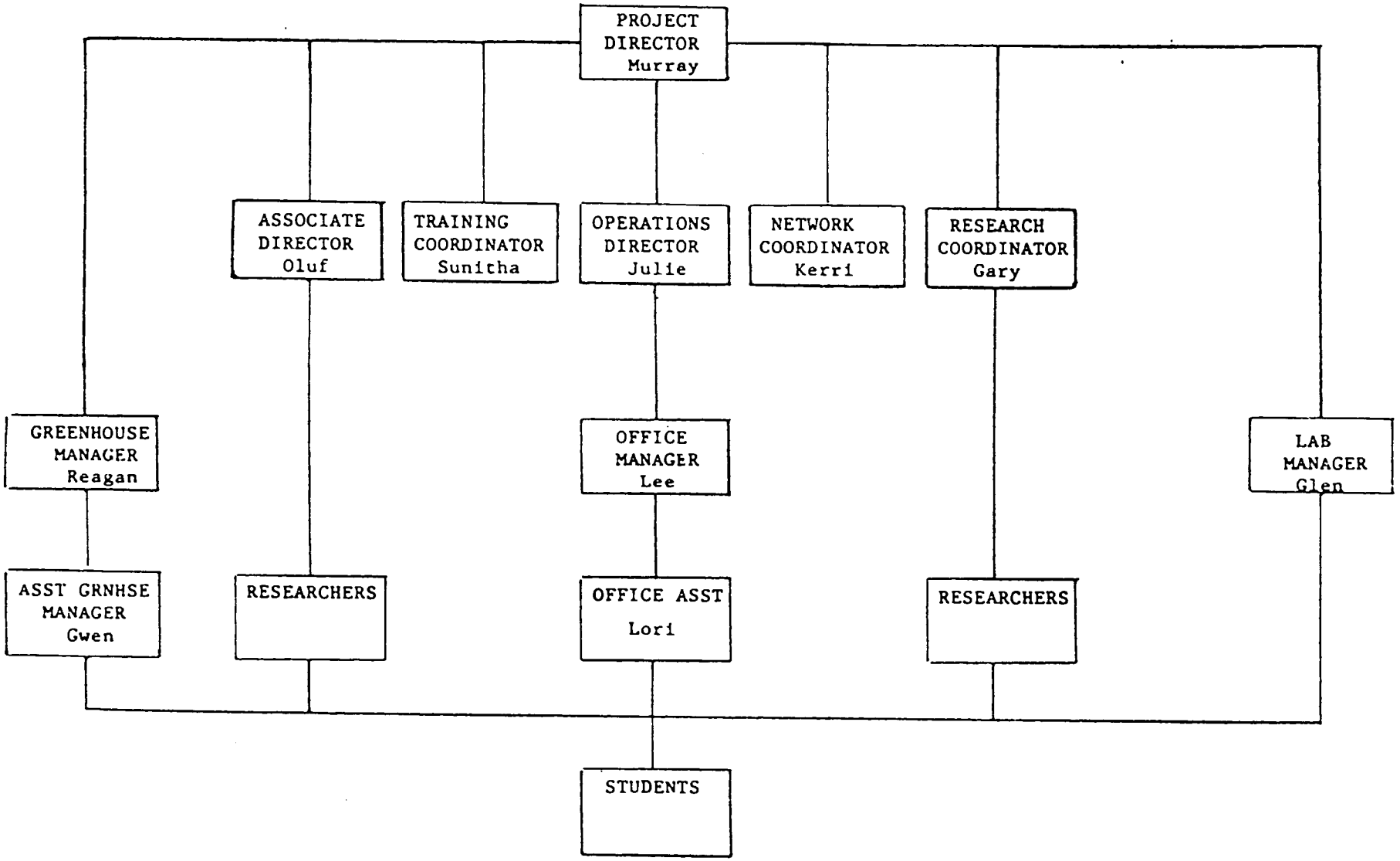
Technical support for the researchers is provided by quarter-time students. Currently, 28 undergraduate students, supervised by researchers and managers, perform the majority of day-to-day culture and plant maintenance. While it is recognized that such a large number of part-time workers is not the most efficient use of time, it is a financial necessity; such students are paid 20% by the Project and 80% by the federal workstudy program.

Graduate students round-out the staff by providing a creative component. Each graduate student works on his/her own project which is directly or indirectly related to Project goals.

FINANCIAL SUMMARY.

Shifts in research priorities and budget cuts forced some changes in the fiscal budget. First of all, the budget was re-calculated by crop area, which is more specific than the budgets included in the original 1985 Work Plan. The new "Special Focus" on molecular biology and widecross was added (in place of) the existing widecross budget. Thus, the "Mol/Wide" budget is a separate, two-year budget totaling $200,000, of which $73,713

TCCP Organizational Chart



was spent in the first fiscal year (86/87). The "Operations" category was also added to account for time spent on purchasing, typing, payroll, budget, and management. The line item "Operating expenses," includes all repair services, telephone and mail charges, computer services, lease of equipment, and rent.

The largest single line item was in personnel. The original third-year budget was $450,006, and was increased to $483,558 (not including nine months of salaries paid by the Gas Research Institute for the sorghum program). Other line items are calculated on a percentage, based on the dollar amount of personnel in each budget area. "Operations" can be spread out over each area, also on a percentage basis, to calculate management effort for all areas.

The areas of training and subgrants were drastically reduced. The Training Program has become largely self-supporting, thus requiring less dollar input. The subgrants budget was reduced by half, the money saved went toward supporting additional personnel at the TCCP lab, travel to field testing and subgrant sites, and operating expenses not budgeted originally.

Contributions from other sources, totaling $122,156, went primarily toward the support of personnel and purchase of materials and supplies in the related area. Contributions from USDA, however, went toward personnel only, and funds from GRI were spread over all line items. The cessation of GRI funding in June resulted in the addition of two, jointly-supported FTE's to the AID budget.

Indirect contributions include tuition and living allowances for graduate students and visiting scientists. These funds, therefore, did not directly support TCCP operations; however, they did partially support the Project-related research efforts of these persons.

Indirect costs amounted to one-quarter of the total budget. The indirect cost rate changed from 38.8% to 39.9% when Project expenditures reached $2,000,000.

APPENDIX I.

REVISED BUDGET: OCTOBER 1986 - SEPTEMBER 1987

TOTAL INPUTS

	OPERATION	NETWORK	TRAINING	RICE	WHEAT	SORGHUM**	LEGUMES	CORN	MILLET	PROTO/SUS	MOL/WIDE	OTHER	TOTAL
PERSONNEL	63699	53041	45647	78689	65595	46340	64824	11311	24186	28642	36339	0	518313
TRAVEL	0	12271	0	9817	4908	4958	0	4908	4908	0	7362	0	49133
MAT & SUP	6132	4818	4380	7884	5694	22539	5694	375	2190	2628	3692	1000	67827
OPERATING EXP	5896	4633	4211	7581	5475	3669	5475	842	2106	2527	3369	0	45783
PRINT/COPY	1043	820	745	1341	969	142	969	149	373	447	596	0	7593
CONSULTING	0	0	1277	0	0	0	0	0	0	0	0	0	1277
TRAINING FEES	0	0	8983	0	0	0	0	0	0	0	0	0	8983
TUITION	0	0	0	6397	0	0	0	0	0	9595	0	0	15992
SUBGRANTS	0	0	0	29150	12500	0	0	6165	0	0	0	0	47815
EQUIPMENT	3399	2671	2428	4371	3157	0	3157	486	1214	1457	1943	0	24282
INDIR COSTS*	30150	29611	25574	52638	33508	30166	30198	9462	13230	13442	20212	388	288579
TOTAL	110320	107864	93246	197867	131805	107915	110316	34199	48206	58738	73713	1388	1075577

OTHER FUNDS - DIRECT SUPPORT

AID/PAKISTAN										1875	1875		3750
GRI						93582							93582
IAEA			4400										4400
ICRISAT							4000						4000
SAUDI ED ATTACHE			4700										4700
USDA					9649								9649
WINROCK												1388	1388
WORLD BANK				687									687
TOTAL													122156

OTHER FUNDS - INDIRECT SUPPORT

AID/PAKISTAN											2740	2740	5480
CSU DEPT BOTANY											7546		7546
FAO/PAKISTAN					12940								12940
IAEA			5400										5400
ICRISAT							4500						4500
LIBYAN GOV'T					12940								12940
SAUDI ED ATTACH			12000										12000
WINROCK												12940	12940
WORLD BANK				12940									12940
TOTAL													81206

*INDIR COSTS = 38.8% ($6499 calc at 39.9%), not calculated on TUITION, EQUIPMENT, OR USDA

**SORGHUM program funded 9 months by the Gas Research Institute

TOTAL INPUTS based on AID Coop Agreement (#4137) funds of $953421 plus total DIRECT SUPPORT

APPENDIX II.

RECENT VISITORS TO THE TCCP FACILITIES

Christine Alang	University of Agriculture, Malaysia
Supat Attathom	Kasetsart University, Thailand
Pierre-Paul Bove	Pasco Industries, Colorado, USA
Ed Bresch	Pasco Industries, Colorado, USA
F. Brinkerhoff	Tegal Sci., Denver, CO., U.S.A.
K.P.S. Chandel	National Bureau of Plant Genetic Resources, New Delhi, India
Ralph B. Clark	University of Nebraska, Lincoln, NE., U.S.A.
Joel Cohen	USAID/Washington, U.S.A.
Yin Congnong	Shandong Provincial Hydrology Service, P.R. of China
H.S. Dhaliwal	Punjab Agricultural University, India
Jay Dowling	Kellogg Corporation, Colorado, USA
Ronny Duncan	University of Georgia, Griffin, Georgia USA
Harold Eddleman	Gunes Tohum Islah A.S., Turkey
Unver Ekmekei	University of the Philippines at Los Baños, Philippines
Liwayway Engle	INTSORMIL, CIAT, Colombia
Catalino I. Flores	USAID, Washington, D.C., USA
Lloyd Frederick	University of Nebraska, Lincoln, NE., U.S.A.
David Galbraith	USAID/Washington, U.S.A.
Tejpal S. Gill	Nat. Sciences Research Institute, Philippines
Saturnina Halos	University of Quagadougou, Burkina Faso
Ditalamane Hebie	Colorado State University, USA
Pedro Hernandez	Somatogenetics Intl., Colorado, USA
Steve Hoffman	CSURF, Colorado State University, USA
Bob Hutchinson	CARI, Sri Lanka
Gerry Jayawardana,	University of Arizona, USA
Hans Jobert	Japan Economic Journal, Sapporo, Japan
Koji Katada	CIMMYT, Mexico
A. Mujeeb-Kazi	MOST, Islamabad, Pakistan
Abdul Quyyum Kazi	University of Wyoming, USA
John Keenan	Colorado State University, USA
Wayne Keim	Danbury USA, Inc., Colorado, USA
Bob Kelley	University of California, Riverside, USA
Iqrar Khan	All in One, Inc., Japan
Masanori Kimura and 19 colleagues	AAAS Office of International Science, Washington, D.C., USA
Lisbeth A. Levey	Plantech, Inc., U.K.
Alastair Lindsay	Agricultural University, Hungary
Gezo Marai	USA
Brad Masi	USA
JoAnne Masi	

S.L. Mehrutra	CSIR, New Delhi, India
David Mok	Oregon State University, USA
Ogonnaya O. Okoro	Forestry Research Institute, Nigeria
P.E. Onnorah	National Center of Genetic Resources and Biotechnology, Nigeria
Suresh Patil	University of Hawaii, USA
Allyson Porter	World Technology Group, Inc., Colorado, USA
Hasan Rhadra	Royal Scientific Society, Amman, Jordan
Mario Salazar	Colorado State University, USA
David Sands	Montana State University, Bozeman, MT., U.S.A.
Charles Scoggin	Somatogenetics Intl., Colorado, USA
M.T. Shirolon	Somali National University, Somalia
Yang Shoufa	Ministry of Water Resources, Northeast Normal Univ., P.R. of China
Hao Shui	Ministry of Water Resources, Northeast Normal Univ., P.R. of China
Khushnood A. Siddiqui	Agricultural Research Center, Tando Jam, Pakistan
Shree P. Singh	CIAT, Colombia
Charles Y. Sullivan	University of Nebraska, Lincoln, NE., U.S.A.
Porntip Thanutong	Khon Kaen University, Thailand
S.A. Tjaja	Batan, Jakarta, Indonesia
Joseph Varner	Washington University, St. Louis, MO. U.S.A.
Marion Verzea	Research Institute for Cereals and Industrial Crops, Romania
Sheila Vijayakumar	ICRISAT, India
Xiao Yuquan	Institute of Water Conservancy and Hydroelectric Power Research, P.R. of China
Yusuf Zafar	Nuclear Institute for Agriculture and Biology, Faisalabad, Pakistan
Hu Zunfeng	Chinese Hydrology Society, P.R. of China

APPENDIX III

TCCP Staff List

Research Associates

Name	Phone	Title/Area	TCCP Funding
Barbara Ashton	1793	Group Leader/Millet	100%
Gwendolyn Bane	6093	Asst. Manager/Greenhouse	100%
Fariha Faizi	1793	Group Leader/Wheat	100%
Lori Frederickson	6996	Tech/Rice Hydroponics Office Asst.	100%
Oluf Gamborg	1176	Assoc. Director	100%
Touba Ghazi-Moore	1793	Group Leader/Cowpea	100%
Gary Hanning	1813	Research Coordinator	100%
Glen Hildreth	1793	Lab Manager	100%
Julie Ketchum	1813	Operations Director	100%
Murray Nabors	1176	Project Director	25%
Carol Ostrom	1130	Tech/Wheat	75% USDA
Catherine Rivera	1793	Group Leader/Sorghum/Rice	100%
Sunitha Siriwardana	1130	Training Coord., Wheat	100%
Reagan Waskom	6093	Greenhouse Manager	100%
Kerri Wright	1176	Network Coordinator	85%

State Classified

Lee Olton	6996	Office Manager	100%

Post Doc

Suresh Kumar	1793	Pigeonpea, Moth Bean, Tepary Bean	100%

Visiting Scientist

Sheila Vijayakumar	1793	Chickpea	100% ICRISAT

Yusuf Zafar	1793	Rice/Kallar Grass Hybridization	100% AID/PAK

Grad Students

Iteu Hidayat	1793	Potato Protoplast Fusion	100% Winrock
Isaac John	5026	Molecular Biology and Salt Tolerance	100% AID/PAK
Ray Ketchum	1793	Protoplasts/Rice and Millet	100%
Doug McMurray	1793	Mung Bean	100% Botany Dept.
Akbar Mohmand	1793	Wheat/Drought	100% FAO
Anna Novero	1793	Rice/NR	100%
Poungpet Poonsapaya	1793	Suspension/Rice	100%
Sathish Puthigae	1793	Rice/Cond. Factor	100%
Gaith Sasi	1793	Triticale	100% Libya
Nanan Widiyanto	1793	Rice/Blast	100% World Bank

Undergrads

Leann Avery	6093	Greenhouse	20%
Barbara Buchnam	6996	Office	20%
Laurie Clauss	1793	Rice	20%
Theresa DeMersseman	6996	Office	20%
Kimbery Ellis	6093	Greenhouse	20%
Jan Emming	6093	Greenhouse	20%
Lauren Fesler	1793	Millet	20%
Hal Gibson	6093	Greenhouse	20%
David Hewitt	1793	Greenhouse	20%
Michelle Hunt	1793	Hormone Detection/HPLC	20%
Jeannette Hut	1793	Hormone Detection/HPLC	20%
Julie Hewitt	1793	Millet	20%

Christa June	6093	Wheat	20%
Larry Klima	1793	Protoplasts/Millet and Rice	20%
Alanna Kulchak	1793	Sorghum	20%
Kristina Muscarella	6093	Greenhouse	20%
Heather Loomis	1092	Office	20%
Elizabeth Orle	1793	Sorghum	20%
Scott Price	6093	Greenhouse/Rice	100%
Greg Pott	1793	Molecular Biology	20%
Stephanie Schaal	1793	Corn	20%
Peter Schuerman	6093	Greenhouse/Rice	20%
Dennis Shippee	6093	Hydroponics/Rice	20%
Nitschka ter Kuile		Widecross/Wheat	100%
Jeffrey Tjaden	1793	Pigeonpea	20%
David Timm	1793	Rice	20%
Michael Tinker	1793	General Lab	20%
Kristin Vanderbilt	1793	Rice	20%

APPENDIX IV

Publications and Reports

Dykes, T. 1987. Report on IPBNet regional training course, Center Agronomico Tropical de Investigacion y Ensenanza, Turrialba, Costa Rica.

Gamborg, O.L., R.E.B. Ketchum and M.W. Nabors. 1985. Tissue culture and cell biotechnology for increased salt tolerance in crop plants. BioSaline Research Workshop, Karachi, Pakistan, September.

Ghazi, T.D., H.V. Cheema and M.W. Nabors. 1986. Somatic embryogenesis and plant regeneration from embryogenic callus of soybean. Glycine max L. Plant Cell Reprots 5:452-456.

Hanning, G.E. 1987. Brief on insect studies in Georgia. TCCP, Colorado State University, Fort Collins, Colorado.

Hanning, G.E. 1987. Brief on regenerate sorghum plants tolerant to acidic soils. TCCP, Colorado State University, Fort Collins, Colorado.

Hanning, G.E. 1987. Brief on results from regenerate rice evaluation of saline soils. TCCP, Colorado State University, Fort Collins, Colorado.

Ketchum, J.L.F., O.L. Gamborg, G.E. Hanning, and M.W. Nabors (Eds.). 1987. Progress Report. TCCP, Colorado State University, Fort Collins, Colorado.

Ketchum, J.L.F. (Ed.). 1987. Newsletter No. 7. TCCP, Colorado State University, Fort Collins, Colorado.

Ketchum, R.E.B., L.J. Klima, F.L. Gutierrez, W.R.S. Warren and M.W. Nabors. 1987. The mechanisms and role of proline accumulation in suspension cell cultures of the halphytic grass Distichlis spicata L., submitted.

Kumar, A.S., O.L. Gamborg, and M.W. Nabors. 1987. Plant regeneration from cell suspension culture of Vigna aconitifolia. Plant Cell Reports, submitted.

MacKinnon, C., G. Gunderson, K.M. Petersen, and M.W. Nabors. 1987. Plant regeneration from salt-stressed sorghum cultures. Life Science Advances 6:No. 2.

MacKinnon, C., G. Gunderson, and M.W. Nabors. 1987. High efficiency plant regeneration by somatic embryogenesis from callus of mature embryo explants of bread wheat (Triticum aestivum) and grain sorghum (Sorghum bicolor). In vitro Cellular & Developmental Biology 23:443-448.

MacKinnon, C., G. Gunderson and M.W. Nabors. 1986. Plant regeneration by somatic embryogenesis from callus cultures of sweet sorghum. Plant Cell Reports 5:349-351.

Siriwardana, S., and G.A. Hildreth. 1987. Handbook for plant tissue culture laboratory organization and management. Colorado State University, Fort Collins, Colorado.

Tissue Culture for Crops Project. 1987. Proceedings of the Second Annual Conference of the International Plant Biotechnology network (IPBNet) Colorado State University, Fort Collins, Colorado.

Wright, K.W. 1987. Trip report—Kenya. TCCP, Colorado State University, Fort Collins, Colorado.