PD-ABA-461

PROJECT STATEMENT

Utilization of Tissue Culture to Increase Food Production

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PROJECT STATEMENT

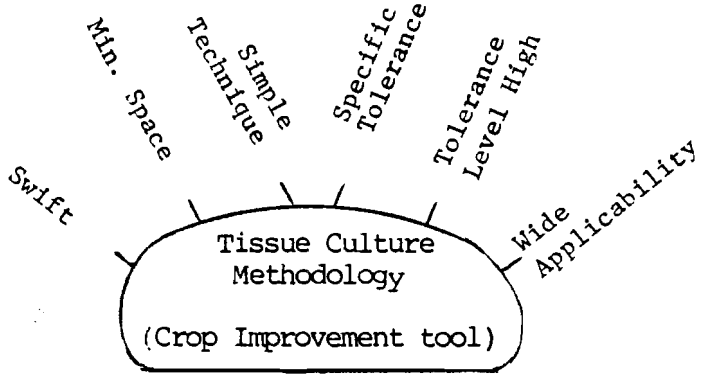
I. Project Summary

A. Statistical

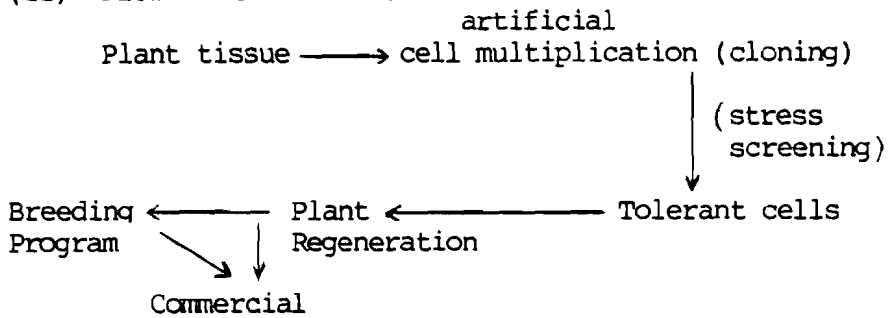
Project Title:	Utilization of Tissue Culture to Increase Food Production
New or Extension:	New
Contractor:	Colorado State University Department of Botany and Plant Pathology Fort Collins, Colorado 80523
Principal Investigators:	Dr. Murray W. Nabors
Co-Investigators:	Dr. P. S. Carlson (Mich. St. Univ.) Dr. R. S. Chaleff (Cornell Univ.)
Duration:	About Five Years
Total Estimated Cost (5 Years):	$1,952,660
Initial Three Years Funding:	First Year: $471,740 Second Year: $337,900 Third Year: $359,000
Project Manager:	Dr. T. S. Gill, DS/AGR

B. Graphic

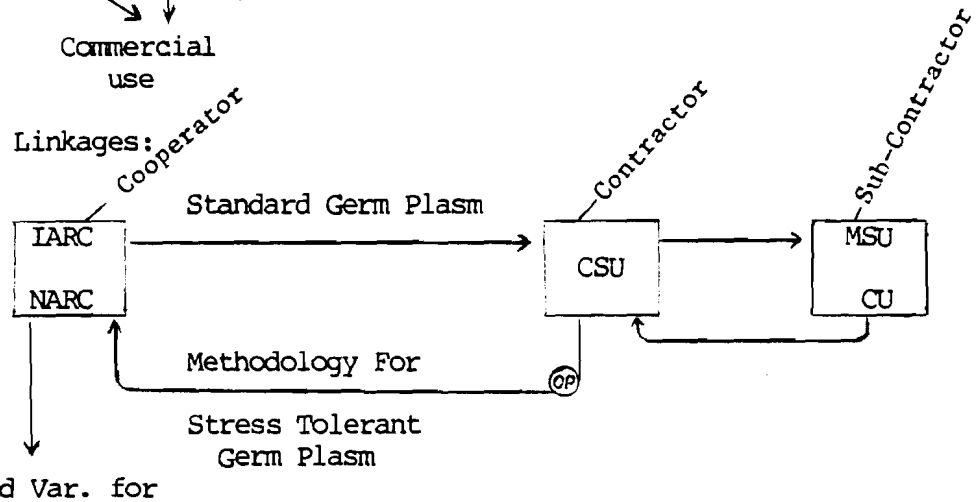
(i) Possible Advantages:



(ii) Flow of Activities:



(iii) Linkages:



Adapted Var. for Farmer Use

(iv) Out-puts (OP): Scope of work

a. Methodology for Soil Stress tolerance in:

- Rice: Na^{+}, Al^{+++} and L-Temp
- Wheat: Na^{+}, Al^{+++} and H-Temp
- Sorghum/Millet: Na^{+} and drought

b. Plant re-generation techniques for

- Corn and cowpeas

Symbols:

IARC:	International Agri. Research Centers
NARC:	National Agricultural Research Centers
CSU:	Colorado State University
MSU:	Michigan State University
CU:	Cornell University
Na^{+} :	Sodium
Al^{+++} :	Aluminum
L-Temp:	Low temperature
H-Temp:	High temperature

C. Descriptive:

This proposal pursues A.I.D. objectives to expand the technology on management of tropical soils to increase food production. It has one principal objective: to promote the use of plant tissue culture as an efficient technique to obtain varieties of food crop plants that have specific tolerance to enviornmental stresses. In the tropical and sub-tropical world, over two billion hectares experience stresses such as mineral deficiencies or toxicities, low and high temperature, drought conditions, etc.

Current approach to help the situation is to change or amend the soil to fit the plant needs or in a few instances fit the plant to the enviornment. However, certain soil conditions either cannot economically be corrected with the current technology or a viable system is not available to deliver an adapted technology to the small farmer in LDC.

Estimate of annual expenditure in the tropics on crop improvement and soil management studies, conservativelyd speaking, is over $120 million. Generally these studies concentrate on producing pest resistant and high yielding varieties that are bred under adequate soil fertility and moisture conditions. Very limited attempts have been made to develop varieties that can stand adverse soil situations. On the other hand studies in this area suggest existance of slight to significant amount of tolerance among varieties for soil stress conditions. Search for such tolerance via world-wide collection of domesticated and wild relatives, of various crop species, however, would be time consuming and an expensive proposition and may not even meet the needs.

It can be visualized that the problem of worldwide soil stress situations is large but so is the potential benefit if suitable solutions could be found. It is to this challenge that this project addresses. It is a new approach, an innovative and an inexpensive short-cut to speed the process of plant adaptation to adverse environments.

The approach briefly stated is as follows: a group of cells are removed from a plant and cultured aseptically on either soild or liquid medium containing relatively high level of an auxin as well as standardized concentration of ions and vitamins. This encourages proliferation of the cells (clones/callus). The callus is then turned into a cell suspension. A 100 mls. of the suspension in a flask may contain 10 million cells. Selection is applied at this time. For example, by increasing salt (sodium chloride) level in the suspension culture, the salt tolerant cells are selected because their growth and cell division is favored. Such selection procedures can rapidly produce the desired level of tolerance among the cells. These cells are regenerated into whole plants. The tolerance thus established is carried genetically from one generation to another. From here on standard procedures of plant breeding are followed as necessary.

x CALLUS a thickening of skin or bark
mass of epithelial and connective tissue

Specifically the project will develop laboratory methodology for developing soil stress tolerance (against high sodium and aluminum, drought; low and high temperature) among the following crops: rice, wheat, sorghum and millet. Not all stresses against all crops will be studied. Regeneration techniques for corn and cowpeas will be also studied.

The project will be intimately linked with the international and national agricultural research centers with regards to germ plasm and utilization of the technique.

The project is expected to last five years and will trigger the utilization of this tool, worldwide, for the purpose of crop improvement and food production. Simplicity of the technique should help resolve local crop adaptation problems.

II. Project Proposal

A. Goal: To improve the efficiency of food production in the developing nations through increased utilization of land resources, especially by growing crop varieties more suited to the prevailing environments.

B. Purpose: To promote the utilization of tissue culture as a technique to add desired characteristics in a crop variety—e.g., adaptation to adverse environmental conditions.

C. Objectives:

1. To utilize tissue culture procedures or initiate procedural development to obtain Na+ tolerant, Al+++ tolerant, temperature extreme tolerant, and drought tolerant alleles in cell lines of useful cultivars of major food crop plants—wheat, rice, corn, sorghum, millet, soybeans and cowpeas.

2. Regenerate stress-tolerant plants from the cell lines and test these plants in green house and the field to determine stress limits and inheritance pattern.

3. Help the utilization of these stress tolerant plants in the national and the international crop improvement programs.

D. Benefits: Feeding the world's population is a much discussed problem with identifiable components. These include:

1. increasing production on currently arable land
2. bringing non-arable land into production
3. dealing with problems of distribution and storage

The first two problems can be approached either by environmental modification (fertilization, irrigation, use of pesticides, mechanization, etc.) or by plant modification (new plant varieties with increased yields

on unmodified enviornments). The first group of solutions has worked well in already developed nations but is energy-intensive and, therefore, frequently of limited utility in developing nations. In addition, the increasingly acute world energy shortage means that an agriculture based on enviornmental modification is becoming more costly for developed nations as well.

adaptation

Because of rising energy costs and decreasing supplies the first two goals above can be best achieved by producing plant varieties with high yields on available soil and water conditions. Currently utilized crop varieties frequently do poorly in such conditions. Stress-resistant varieties would be area specific (e.g. for dry, saline soils; or wet, acidic, aluminum-containing soils) and would increase the capabilities of developing nations to feed themselves utilizing their own resources, without overdependence on imported, expensive agricultural practices and inputs.

Plant breeders have been quite successful over the years in producing new varieties of plants with specific improved traits. Many of these improved plants are themselves dependent on energy-intensive agricultural practices.

low energy ?

> Green revolution agriculture uses high energy crop production technology, . . . While one may not doubt the sincerity of the U.S. effort to share its agricultural technology so that the rest of the world can live and eat as it does, one must be realistic about the resources available to accomplish this mission. In the United States we are currently using an equivalent of 80 gallons of gasoline to produce an acre of corn. With fuel shortages and high prices to come, we wonder if many developing nations will be able to afford the technology of U.S. agriculture.1

> Recently breeders have placed increased emphasis of varieties which yield in available environments and are thus economical users of energy. The international plant improvement centers of the Consultative Group on International Agricultural Research (CGIAR) are now turning more of their over $64 million yearly budget away from energy-intensive green revolution crops and toward" . . . developing plant types and practices to benefit the small farmer, who must operate without the

1 D. Pimentel, L.E. Hurd, A.C. Bellotti, M.J. Forster, I.N. Oka, O.D. Sholes, and R.J. Whitman. Food production and the energy crisis. Science 182: 443-449 (1973).

benefit of controlled irrigation or much help from fertilizers and pesticides."2

It is conservatively estimated that plant breeding programs on major food crop plants currently cost $200 million per year ($50 million in the U.S.) and employ 5400 people.3

An estimated 480 new or improved varieties are released to growers each year at a development cost of over $400,000 per variety. Eight to ten years breeding research are required per variety.

It is suggested that the incorporation of tissue culture techniques into plant breeding programs for stress resistance can considerably economize and expedite the release of new or improved varieties to increase food production.

III. Project Design and Scope of Work

A. Experimental design

Various alleles for resistance to soil, water, and environmental stresses will be selected in cell cultures of varieties of food crop plants. Table 1 summarizes the plant species to be used and the specific stress tolerances to be selected. For each plant and stress condition the following procedure will be utilized:

a. Crop varieties and stress limits are selected by AID and CGIAR personnel. Schedule of student and personnel exchanges between tissue culture labs at CSU, MSU, and Cornell and International Centers is established (see Fig. 1, p.).

b. A cell culture is established for each food crop variety.

c. Cell proliferation occurs until a large population (millions) of cultivated cells is obtained.

d. Cultured cells on solid medium or in liquid suspension are stressed to a level which inhibits growth of normal cells but favors the growth of stress tolerant cells. Stress conditions will be established as follows:

(1) Na+ or Al+++ tolerance: Add NaCl or AlCl3 to growth medium.

2 CGIAR, New York, 1976. U.N. Development Programme, One United Nations Plaza, New York, N.Y. 10017.

3 Revised and expanded data from Science and Education in Agriculture Current Research Information System (CRIS).

(2) Drought tolerance. Add NaCl or high molecular weight, osmotically active compounds to growth medium.

(3) High and low temperature tolerance: Grow cultures at temperature extremes which retard the growth and division of non-tolerant cells.

e. Plants are regenerated from cultured cells.

f. Stress tolerance and inheritance of stress tolerance are tested in greenhouse experiments.

g. Stress tolerant plants are field tested at International Centers.

h. Stress tolerant varieties are released to growers.

The proposed research will cover a five-year period. Figure 2 summarizes the tentative schedule and accomplishments over that time. In general, 2 years are required for the establishment of mutant cell lines; 1 1/2 years for plant regeneration, and 1 1/2 years for greenhouse testing. Work is proposed or has already begun in establishing cell cultures or mutant cell lines for the following plants:

a. Wheat. Tissue culture procedures for this plant have been almost perfected and selected for NaCl-tolerant strains in the callus stage has already begun in Dr. Nabors' lab. Greenhouse testing of plants regenerated from mutant cells will begin in mid-1979.

b. Rice. Dr. Chaleff's lab has considerable expertise in the tissue culture of rice. His procedures will be used to establish cell lines and to regenerate plants.

c. Soybeans and Cowpeas. Procedures for obtaining callus and suspension cultures for soybean and cowpea can be considered routine. The present problem in adapting tissue culture breeding to these plants is that suitable regeneration techniques are still not developed. In general this situation exists for other grain legumes as well. Dr. Carlson's and Dr. Nabors' labs (as well as others) are now trying intensively to resolve these problems. Another consideration with these plants is their association with the root nodule bacteria, Rhizobium.[4] Selection for increased stress tolerance in these plants should be attempted in pure and in innoculated cell cultures since the symbiotic association is affected by environmental factors. Thus our work to develop plant regeneration techniques will utilize both pure and innoculated cultures.

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4 W.R. Scowcroft and A.H. Gibson. Nitrogen fixation by Rhizobium associated with tobacco and cowpea cell cultures. Nature 253: 351-352 (1975).

d. Corn. The only published regeneration technique for this plant utilized callus derived from embryos in the milk stage. While this method is quite workable it is complicated. In Dr. Nabors' lab we are currently attempting to simplify tissue culture methods for this plant by obtaining a successful regeneration procedure from root-derived callus.

e. Sorghum and Millet. Published procedures will be modified in our labs and used to establish cell lines and to regenerate plants from these species. Tissue culture and regeneration methods for proso millet (Panicum miliaceum) are already established and will be utilized. Tissue culture procedures for foxtail millet (Setaria italica), pearl millet (Pennisetum glaucum), and Japanese millet (Echinochloa frumentacea) are not developed and will be established if consultation with the International Breeding Centers shows it to be a desirable goal of the proposed research.

In summary, of the plants we propose to utilize for stress tolerance selection, wheat, millet, rice, corn, and sorghum have a tissue culture methodology which is complete enough to allow the initiation of tissue cultures breeding. Selection for NaCl tolerance is already underway for wheat in Dr. Nabors' lab. Soybean and cowpeas all lack suitable procedures for regenerating plants from cell cultures, and we are continuing and proposing work to develop such procedures.

Table 1. Accomplished and Proposed Research
(parentheses indicate location of proposed work)

	Tissue Culture Methods	Tissue Culture Selection					Methods for Plant Regeneration from Tissue Culture (from our labs or literature)	Green-house Testing	Field Utiliza-tion
		Na^+ Tolerant	Al^{+++} Tolerant	Drought Tolerant	Hi Temp. Tolernat	Low Temp. Tolerant			
Wheat	Yes (CSU)	Complete (CSU)	Proposed (CSU)	--	Proposed (CSU)	--	Yes (CSU)	Winter 1979 (CSU)	Proposed (CGIAR)
Millet	(CSU)	Proposed (CSU)	--	Proposed (CSU)	--	--	(CSU)	Proposed (CSU)	Proposed (CGIAR)
Sorghum	Yes (CSU)	Proposed (CSU)	--	Proposed (CSU)	--	--	Yes (CSU)	Proposed (CSU)	Proposed (CGIAR)
Rice	Yes (Cornell)	Proposed (CSU)	Proposed (Cornell)	Proposed (Cornell)	--	Proposed (Cornell)	Yes (Cornell)	Proposed (Cornell)	Proposed (CGIAR)
Corn	Yes (CSU)	*	*	*	*	*	Yes but too complex proposed (CSU)	* --	--
Soybeans	Yes (MSU)	*	*	*	*	*	No Proposed (MSU)	--	--
Cowpeas	Yes (MSU)	*	*	*	*	*	No Proposed (MSU)	---	--

Figure 1. Proposed Linkages Between Tissue Culture Research Laboratories and CGIAR International Agricultural Research Centers.

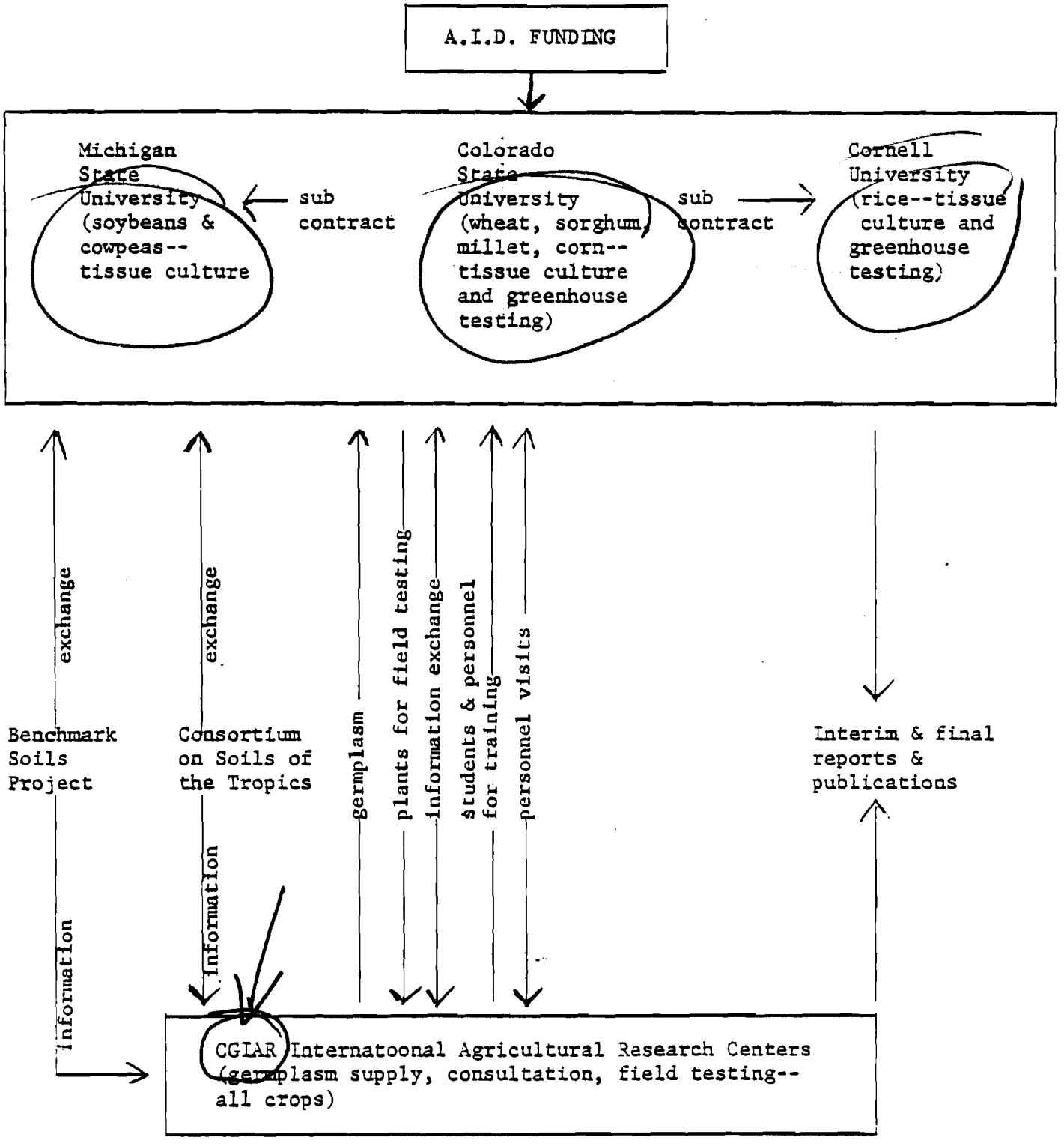
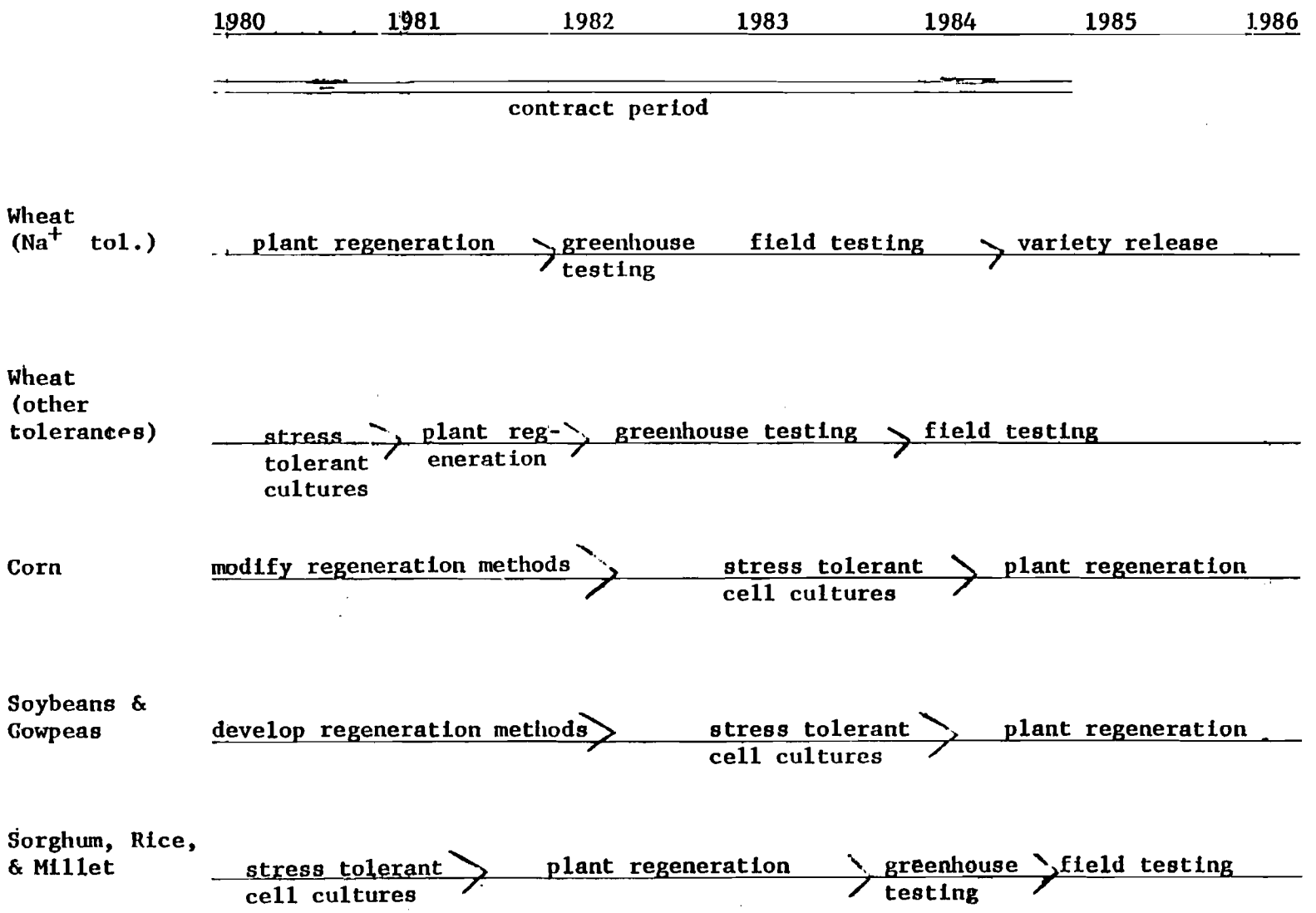


Figure 2. Timetable for Proposed Work Plan



B. Research methodologies and techniques.

The production of mutant plants by tissue culture techniques can be divided into four steps.

a. Production of callus or cell suspension cultures of plant cells.

b. Selection of desired mutants.

c. Regeneration of mutant plants from callus or suspension cultures.

d. Testing of regenerated plants in the greenhouse for phenotype persistance and inheritability of the mutant trait.

A callus is a mass of growing, dividing, but undifferentiated cells. The production of callus can be considered routine for most plants. Typically a small section of stem, root, or cotyledonary tissue is placed in sterile culture on a solid nutrient medium containing known concentrations of sugar, mineral nutrients, and plant growth hormones. The composition of the medium is adjusted to stimulate the proliferation of cells. This is usually accomplished by using high levels of an auxin which encourages cell division and growth, but inhibits the formation of shoots and roots. When a sufficient amount of callus is obtained, cell suspensions are initiated simply by placing callus tissue in a baffle-bottom Erlenmeyer flask containing liquid medium on a gyrotory shaker. The mechanical motion of the shaker disrupts the callus into small groups of cells. Cell suspensions are subcultured when cell density reaches a minimum value.

Plant hormone

Mutant cells are selected by exposing cell cultures to conditions that slow or prevent the growth of normal cells. Specific stress tolerant mutants will be selected as follows:

a. Na+ or Al+++ tolerance.

Mutants tolerant to these specific cations are selected by adding increasing concentrations or NaCl or AlCl3 to the medium. Selection will occur in a stepwise fashion (Fig. 3). At first low levels of a particular salt are added to a rapidly growing culture. The salt will tend to inhibit growth and division of normal, non-tolerant cells while allowing or perhaps encouraging growth and division of tolerant cells. A single culture will have millions of non-tolerant cells and perhaps only one or two individual tolerant cells. When the culture reaches maturity it is subdivided with some subdivisions placed on medium with higher salt concentrations and others on plant regeneration medium. This process continues until the cultures can no longer successfully adapt to high levels of the toxic ion. As a result of this technique we will obtain a series of cell lines resistant to higher and higher concentrations of the toxic ion. For tobacco, wheat, and oats we have already obtained a series of NaCl resistant cell lines. In this regard it should be noted that although a tolerance of

3000 ppm may be desired, a variety with tolerance to 6000 ppm might be more useful in terms of maximizing productivity at the lower concentration.

This selection process can be carried out in any one of three manners:

(1) Mutant cell lines can be selected in callus cultures by incorporating the salt into the solid medium. This selection method works well in practice although the exact concentration of salt in contact with cellus tissue is difficult to determine accurately.

(2) Mutant cell lines can be selected in suspension cultures which are subdivided into new culture medium when mature growth is obtained. This selection system works well, and one knows exactly the salt concentration to which the cells are exposed. However, frequent subdivision of the cultures requires time and effort, and opening the cultures allows the possibility of bacterial or fungal contamination.

(3) Mutant cell lines can be selected in continuous suspension cultures maintained in fermentors. In this selection system new medium with increasing levels of the selective agent is gradually added at a rate which allows a maximal growth rate to be maintained. A new medium is added, suspension is removed for plant regeneration. This selection method requires the most expensive equipment but is most satisfactory in that technician time is minimized, and the culture is never exposed to possible contamination.

Na^+ toxicity is frequently associated with calcium-deficient soils. It may thus be necessary for some environments to obtain plants which have a heightened efficiency of Ca^{++} uptake as well as or in place of Na^+ resistance. Such mutants would be selected by exposing cultures to an abnormally low level of Ca^{++}.

Al^{+++} toxicity is always associated in nature with soils of low pH. Even in the lab chelated Al^{+++} solutions may well lower cellular pH after Al^{+++} enters the plant. Thus it might be useful to select cell lines resistant to low pH for physiological comparisons to those resistant to Al^{+++}.

b. Drought tolerance.

Na^+-tolerant plants are often drought tolerant. This can be seen in our lines of Na^+-tolerant tobacco plants as well as in nature. Thus Na^+-resistant plants will also be tested for drought resistance. Drought-tolerant mutants will also be selected by inhibiting water uptake in cultured cells. This is accomplished by lowering the water potential of the culture medium with increasing ionic concentrations or by the addition of a non-penetrating osmoticum such as PEG 4000 or dextran. Only mutant cells with a lower than normal water potential will grow under these conditions. Selection of increasingly drought-tolerant mutants will be achieved in a stepwise manner, similar to the Na^+ and Al^{+++} mutant selection process. That such osmoticum-resistant cell lines result in drought-tolerant plants remains to be tested.

c. High and low temperature tolerance.

These mutants will be selected by growing cultures at temperature extremes which retard growth and division of non-tolerant cells.

Once the desires stress tolerant cell lines have been established, plant regeneration will begin by placing stress-tolerant cells on regeneration medium. In general monocots are regenerated by removing all hormones from the medium while dicots are regenerated in a medium containing a high cytokinin to xauxin level. However, regeneration media may vary from species to species, and even from variety to variety. The regeneration strategy is complicated if the cultured cells are themselves producing significant quantities of hormones. Regeneration may take place on one medium, or on a series of media in which roots and shoots are formed sequentially. The period of plant regeneration requires approximately 4-6 weeks depending on the experiment. Following the formation of complete plantlets they are transferred to pots and placed in a controlled environment for a period of "hardening" before transfer to the greenhouse.

In the greenhouse, stress-tolerant traits will be tested for persistance and for inheritability pattern in whole plants. This is accomplished in several stages:

(1) Seeds are collected from stress-tolerant and non-stress tolerant regenerated plants.

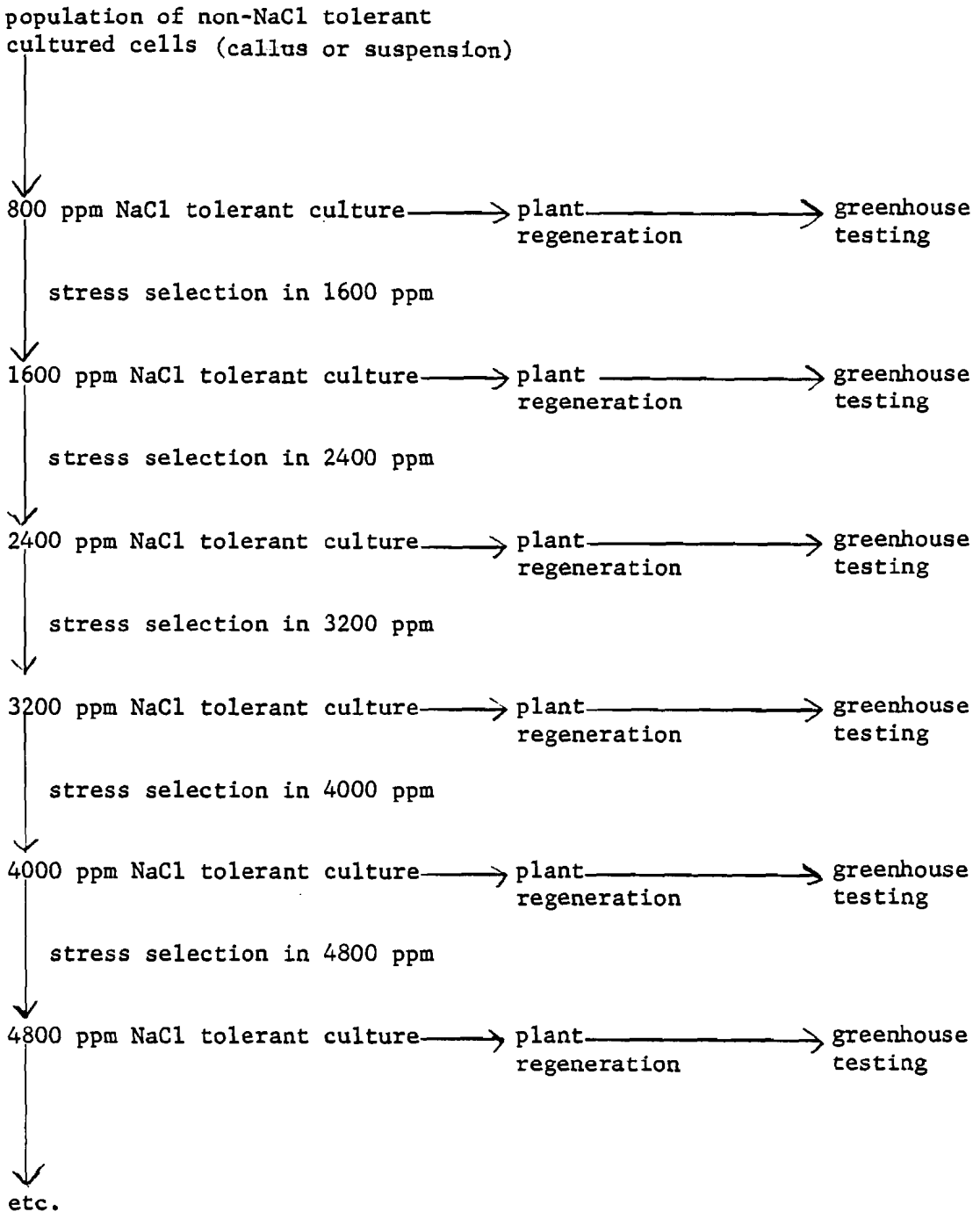
(2) The seeds are planted and the resulting plants (F1 generation) are subjected to a graded series of increasing stress. Growth rates, death rates, and other production indices are recorded.

(3) Seeds of F1 plants are collected and an F2 generation is produced and similarly tested. Also, F1 plants are back crossed to parental stock.

(4) In each test mutant and non-mutant plants all originally derived from tissue cultures are grown side by side for comparison.

Special problems to be encountered will vary with species and stress condition. For example, oat cultures regenerate shoots readily whereas in wheat callus buds frequently remain juvenile unless hormone composition of the medium is varied. Regenerated shoots from neither plant produce roots readily, so again medium composition experiments are in order. Soybean rapidly produces callus and suspensions, but regeneration has proven elusive. Sugar beets produce callus and suspensions slowly, and medium composition is much more critical than for other species. The general process of solving problems of medium composition necessary for achieving a certain desired product in tissue culture will be approached in a manner similar to that discussed in the following paper. A large number of media are screened initially. Those which appear to promote the desired process are utilized in further tests.

Figure 3. Mutant Selection Flow Chart



C. Scope of work.

a. First year of contract (1980).

(1) Work to meet research objectives.

(a) Communicate with plant breeding personnel in A.I.D. and at International and National Agricultural Research Centers (IARC'S and NARC'S) to obtain recommendations for stress tolerances to be obtained and varieties to be utilized. Obtain germplasm to be used in tissue culture stress selection.

(b) Establish linkages with CGIAR centers and developing nations to initiate student and personnel training in tissue culture laboratories.

(c) Establish and maintain cell lines (on solid and in liquid medium) or sorghum, rice, and millet. Note that CSU has already established numerous lines of rice. Begin stress selection for Na^+, Al^{+++}, drought, and low temperature tolerance (see Table 1, p. 9).

(d) Continue experiments to develop suitable or improved regeneration techniques for soybeans, cowpeas, and corn. Utilize several varieties.

(e) Continue plant regeneration studies from one already established Na^+-tolerant cell line of wheat. Initiate cultures of varieties supplied by CGIAR personnel, and begin selection for Na^+, Al^{+++}, and high temperature tolerance.

(f) Compile a bibliography of useful tissue culture methodology for specific plants for use by plant breeders wishing to include tissue culture in their breeding strategies.

(g) Attend national and international tissue culture and plant breeding meetings.

(h) Initiate contact with soil scientists and agricultural engineers interested in soil and water management. Add these people to mailing list compiled in 1978.

(2) Expected product.

(a) Initial contacts made with interested worldwide plant breeding personnel. Mailing list initiated. Personnel and student exchange plans initiated.

(b) Cultured, stress-resistant cell lines under production for sorghum, rice, and millet (see Table 1, p. 9 for specific stress resistances to be obtained).

(c) Na^+-tolerant wheat plants ready to begin greenhouse testing phase for one variety; Na^+-tolerant cell lines established for other varieties; Al^{+++}- and high-temperature-tolerant cell lines established for wheat.

(d) Cell lines of soybeans, cowpeas, and corn established; regeneration studies underway.

(e) Tissue culture bibliography for each crop plant available to interested breeders.

(f) National and international tissue culture and plant breeding meetings attended.

(g) Contacts initiated with soil scientists and agricultural engineers interested in soil and water management. Names added to mailing list.

b. Second year of contract (1981)

(1) Work to meet research objectiveds.

(a) Obtain desired tolerance levels in Na^+-, Al^{+++}-, low temperature-, and drought-tolerant cell lines of sorghum, rice, and millet. Determine maximum tolerances obtainable in tissue cultures (see table 1, p. 9).

(b) Continue experiments to obtain suitable regeneration techniques from cell cultures of soybeans, cowpeas, and corn.

(c) Begin greenhouse testing phase for one variety of Na^+-tolerant wheat plants. These experiments will determine whole plant tolerance levels and tolerance inheritance patterns.

(d) Begin plant regeneration from other Na^+-tolerant wheat lines. Begin plant regeneration from Al^{+++}- and high-temperature-tolerant lines of wheat.

(e) Initiate short visits and personnel exchanges between International Centers and tissue culture of students and personnel from developing nations and International Centers.

(f) Complete progress report to assist A.I.D. review process.

(2) Expected product.

(a) Na^+-, Al^{+++}-, low-temperature, and drought-tolerant cell lines of several varieties of sorghum, rice, and millet (see Table 1, p. 9).

(b) Good possibility of suitable plant regeneration methods for soybeans, corn, and sugar beets.

(c) Preliminary results from greenhouse testing of Na^+-tolerant wheat showing limits of tolerance and pattern of inheritance through F1 generation for one variety.

(d) Complete plant regeneration studies of other Na^+-tolerant wheat lines and of Al^{+++}- and high-temperature-tolerant wheat lines.

(e) Initial short visits between International Breeding Centers' personnel and tissue culture labs. Information exchange initiated. Student and personnel training initiated.

(f) National and international tissue culture and plant breeding mettings attended.

(g) Possible research publication on soybean, corn, or cowpea regeneration techniques. All publications to be distributed to interested people on mailing list.

(h) Progress report submitted to A.I.D.

c. Third year of contract (1982).

(1) Work to meet research objectives.

(a) Begin plant regeneration from Na^+-, Al^{+++}-, low temperature-, and drought-tolerant cell lines of sorghum, rice, and millet.

(b) Continue cell lines of soybean, cowpeas, and corn, and begin selection for Na^+-, Al^{+++}-, and drought-tolerant mutant cell lines if regeneration techniques are available.

(c) Conclude greenhouse testing of one Na^+-tolerant wheat variety.

(d) Begin greenhouse testing of Al^{+++}-, Na^+-, and high-temperature-tolerant wheat plants.

(e) Continue information and personnel exchanges with International Breeding Centers.

(f) Attend national and international plant breeding and tissue culture meeting to discuss program and data and present papers.

(2) Expected product.

(a) Some regenerated plants from Na^+-, Al^{+++}-, low temperature-, and drought-tolerant cell lines of sorghum, rice, and millet.

(b) Na^+, Al^{+++}, and drought-tolerant cell lines of soybeans, cowpeas, and corn.

(c) A research publication detailing greenhouse testing of Na^+-tolerant wheat plants of one variety and the use of tissue culture to select these plants.

(d) Preliminary results from greenhouse testing of Al^{+++}, Na^+, and high-temperature-tolerant wheat.

d. Fourth year of contract (1983).

(1) Work to meet research objectives.

(a) Complete plant regeneration from Na^+, Al^{+++}, low-temperature, and drought-tolerant cultures of sorghum, rice, and millet. Begin greenhouse testing.

(b) Complete selection of Na^+, Al^{+++}, and drought-tolerant cell lines of soybeans, cowpeas, and corn.

(c) Begin field testing of one Na^+-tolerant line of wheat.

(d) Complete greenhouse testing of Al^{+++}, Na^+, and high-temperature-tolerant lines of wheat.

(e) Continue personnel exchanges between International Breeding Centers and tissue culture labs.

(f) Attend national and international plant breedings and tissue culture meetings and present findings.

(2) Expected product.

(a) Na^+, Al^{+++}, low-temperature-, and drought-tolerant sorghum, rice, and millet plants obtained from cell cultures.

(b) Na^+, Al^{+++}, and drought-tolerant cell lines of soybeans, cowpeas, and corn.

(c) Preliminary results on field testing of one Na^+-tolerant wheat variety.

(d) Final results on greenhouse testing of Al^{+++}, Na^+, and high-temperature-tolerant lines of wheat.

(e) A research publication on greenhouse testing results using Al^{+++}, Na^+, and high-temperature-tolerant lines of wheat.

(f) Attended antional and international plant breeding and tissue culture meetings and presented findings.

e. Fifth year of contract (1984).

(1) Work to meet research objectives.

(a) Complete greenhouse testing of Na^+, Al^{+++}, low-temperature, and drought-tolerant lines of sorghum, rice, and millet.

(b) Begin plant regeneration from Na^+, Al^{+++}, low-temperature, and drought-tolerant cell lines of soybeans, cowpeas, and corn.

(c) Continue field testing of one Na^+-tolerant line of wheat. Release varieties to growers when field testing is completed.

(d) Begin field testing of Al^{+++}, Na^+, and high-temperature-tolerant lines of wheat.

(e) Continue personnel exchanges with International Breeding Centers.

(f) Attend national and international plant breeding and tissue culture meetings and present findings.

(2) Expected product.

(a) Na^+, Al^{+++}, low-temperature-, and drought-tolerant lines of sorghum, rice, and millet ready for field testing.

(b) Research publication detailing results of greenhouse testing of Na^+, Al^{+++}, low-temperature-, and drought-tolerant sorghum, rice, and millet.

(c) Plants regenerated from Na^+, Al^{+++}, and drought-tolerant lines of soybeans, cowpeas, and corn.

(d) One Na^+-tolerant line of wheat released to growers.

(e) Al^{+++}, Na^+, and high-temperature-tolerant lines of wheat partially field tested.

(f) Research publication on production of field-tested, Na^+-tolerant wheat derived from tissue culture.

(g) A review publication covering progress during the five-year contract period.

(h) Attended national and international plant breeding and tissue culture meetings and presented papers.

(i) A second Technical Bulletin detailing methodological progress during the five years since publication of the first bulletin.

f. One year beyond contract (1985).

(1) Expected product.

(a) Publication of Final Report to A.I.D.

(b) Completion of field testing of Na^+, Al^{+++}, low-temperature-, and drought tolerant sorghum, rice, and millet varieties.

(c) A research publication on the field testing in (b).

(d) Completion of plant regeneration studies on Na^+, Al^{+++}, and drought-tolerant soybeans, cowpeas, and corn.

(e) Al^{+++}, Na^+, and high-temperature-tolerant lines of wheat released to growers.

(f) Continued cooperation with International Breeding Centers.

(g) Formulation of new objectives based on progress during the five-year contract period.

Selection

IV. Project Analysis

A. Technical, Economical and Social Soundness

Other factors than those selected

a. Tissue culture breeding has successfully produced improved varieties.

IR-8 example

Several workers (including three investigators in this proposal) have already demonstrated the usefulness of tissue culture for selecting mutant plants: Carlson selected tobacco cell lines resistant to methionine-sulfoximine (a compound with effects similar to the toxin from the xcausal agent of tobacco wildfire disease). Plants regenerated from these cell lines also showed resistance which was inherited into the F2 generation.5 Similarly, Chaleff's lab has obtained picloram-resistant plants (picloram is an herbicide).6

5 P.S. Carlson. Methionine-sulfoximine-resistant mutants of tobacco. Science 180: 1366-1368 (1973).

6 R.S. Chaleff and M.F. Parsons. Direct selection in vitro for herbicide-resistant mutants of Nicotiana tabacum. In Press in Proc. Natl. Acad. Sci. U.S.A.

Nabors" lab has obtained NaCl-resistant plants.7 Other workers have produced corn plants resistant to Helminthosporium toxin.8 In all these cases the resistances selected in cell culture have persisted in regenerated plants and have been shown to be inheritable.

b. Tissue culture breeding confers specicity to the mutant selection process.

Tissue culture enables us to specify that a certain type of desired mutant plant will be selected in a desired location (our laboratory flask), at a specific time (when we need it), and in a desired plant variety. This type of selection specificity is almost never available to a plant breeder using whole plants. Breeders frequently screen available varieties in hopes of finding a desired trait. Often the needed characteristic is found in an otherwise undesirable variety necessitating expensive and time-consuming cross-breeding. Many times the desired trait cannot be located in domesticated cultivars, and out-crossing to wild stock must be considered. Too frequently the needed characteristic cannot be located in any available germ plasm lines.

For example, mutations for salt tolerance arise in nature. Perhaps one in ten million plants will show a genetically-based increased level of salt tolerance. An investigator has no way of knowing the location of a desired mutant plant, and time of its production or whether it is found in the variety he desires to improve. Imposing field selection (watering with salt water) to discover the plant can be time consuming and expensive. Sometimes selection using seeds or seedlings instead of adult plants can be used. Tissue culture always enables the investigator to specifically, rapidly, and economically obtain the plant he desires and then to return to the field for whole plant studies.

Also, tissue culture methods enable an investigator to produce a large number of a desired stress-resistant plant whereas field selection might produce one such plant. For example we have imposed NaCl stress on populations of cultured cells. Most cells are killed. However, the few salt-tolerant mutant cells continue to grow and divide until they make up the entire culture. Thus we can produce dozens, hundreds, or thousands of identical, salt-tolerant plants (a clone) by plant regeneration from cell cultures whereas field selection in the same period of time might produce one or a few individual plants.

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7 M. Nabors, S. Gibbs, C. Bernstein, and M. Meis. NaCl-tolerant tobacco plants from cultured cells. Submitted for publication. Manuscript attached as Appendix to this proposal.

8 B.G. Gengenbach, C.E. Green, and C.M. Donovan. Inheritance of selected pathotoxin resistance in maize plants regenerated from cell cultures. Proc. Nat. Acad. Sci. U.S.A. 74: 5113-5117 (1977).

Why not these use ~~tissue~~ skills selection / Reproduction / re-test

c. Tissue culture can be used to rapidly clone mutant plants located by conventional means.

Rare, stress-resistant plants encountered in the field can be rapidly reproduced by the tissue culture process. For example, a single drought-resistant wheat plant might be discovered by a breeder in Mexico. By the normal process of collecting seeds and replanting to increase seed stock, several years would be required to obtain sufficient seed for varietal testing and introcution. Tissue culture could shorten this process considerabldy by producing thousands of identical plants within a year.

d. A combination of tissue culture and traditional breeding methods would considerably reduce the cost of producing new varieties.

Tissue culture compresses space and time requirements for selecting stress-resistant plants. In addition, it adds a desired specificity to the selection process. For these reasons the incorporation of tissue culture techniques could considerably reduce the time and money required for introcution of new plant varieties. A new variety presently requires around $400,000 and 8-10 years for introduction to growers. We estimate that utilizing tissue culture techniques could easily save 2-3 years and $150,000 in many such programs. If tissue culture were utilized in all breeding programs at a considervative savings of $50,000 per variety the yearly return realized would be (480 varieties x $50,000 =) $24 million in the breeding programs alone. This dollar figure is miniscule compared to that realized by the introduction of stress-resistant varieties could be used to further agricultural production or other projects requiring input of capital or labor.

e. Potential for effective utilization of research results in developing countreis worldwide and suggested extension strategies.

Developing nations can all effectively utilize energy-efficient plant varieties designed for specific environmental conditions. Such plants would lower fuel and other energy costs of food production and would enable individual farmers to successfully and economically utilize existing soil and water conditions.

Consultative Group

Worldwide A.I.D. personnel and scientists and growers in CGIAR Breeding Centers would participate in identifying the most critical stress conditions and levels inhibiting food production for particular crops in their region. They would also select varieties which have agronomically desirable traits, but which lack a desired level of stress resistance. For the tissue culture selection and greenhouse testing phases of the research, students and scientific personnel from developing nations and from CGIAR Centers would be funded for short visits and for extended training periods to our three tissue culture laboratories during the five year project duration. Following the addition or augmentation of specific stress resistances in varieties by tissue culture breeding and greenhouse testing of the resulting plants, the new cultivars would be field tested in the particular region for which they were developed by CGIAR personnel.

f. Possible effects of proposed work on other specific concerns of A.I.D.

In general the proposed work should be a step toward the economic independence of developing nations and of the individual farmers within these nations. Crops which require less than normal enviornmental modification are more economically and easily grown. Yields on currently arable land should increase; land formerly non-arable can be brought into cultivation; years with marginal environmental factors should be less damaging; and the willingness of individual farmers to plant should increase. The net result of energy-efficient crop plants which increase food production will be to release A.I.D. and other funds currently encumbered in both plant breeding and in environmental modification in energy-intensive agriculture for other desirable projects.

For example, considerable effort and resources are now expended by A.I.D. and other agencies worldwide to understand and control soil salinity, particularly in irrigated regions.[9,10] Some of this work is managed by Colorado State University. Production of NaCl-tolerant varieties is mentioned by many workers as one primary method for increasing the efficiency of irrigation and salinity management efforts. Thus our use of tissue culture to rapidly incorporate salt tolerance into useful varieties will interface directly with A.I.D. concerns in water quality management.

An estimate of the importance of rapid methods for producing drought- and salt-tolerant plants can be obtained from the following figures.[11] The world has approximately 3.2 billion hectares of arable land of which 1.2 billion hectares are currently cultivated. Of this 1.2 billion, 14% or 0.174 billion hectares are currently irrigated. However, it is estimated that 60% of potentially arable land has six months or more per year of moisture deficit. Two-thirds of this 60% of 40% of all potentially arable land could sustain year around agriculture where sufficient moisture available. Forty per cent of all potentially arable land is 1.15 billion hectares on which agricultural production would be increased by irrigation. Of this land only 0.17 billion hectares are currently irrigated. Thus there are approximately one billion hectares or about one third of all

9 For example see FAO Soils Bulletin #31. Prognosis of Salinity and Alkalinity. Food and Agricultural Organization of the United Nations, Rome, 1976.

10 Also see published papers from an A.I.D.-sponsored symposium, Plant Adaptation to Mineral Stress in Problem Soils. Ed. M.J. Wright. Cornell U. Exp. Sta., 1977.

11 W. Thorne. Agricultural production in irrigated areas. In Arid Lands in Transition, H.E. Dregne, ed. American Association for the Advancement of Science, Washington, 1970, pp. 31-56.

potentially arable land on the earth's surface which could profit agriculturally from either irrigation or drought- and salt-tolerant plant varieties. The cost of providing irrigation facilities varies from $100 to $3000 per hectare (1970 estimate). Taking $1500 as an average current estimate it would require $1500 billion or 1.5 trillion dollars to maximize agricultural production using irrigation.12 This estimate assumes sufficient water is available, and it is not since four fifth's of man's current total fresh water utilization (during the growing season) is already in agriculture. Projects to increase fresh water availability include elimination of high water loss in current irrigation systems, and increase in basic availability. The latter, in particular, involves massive engineering projects and consequent increases in the projected expenditures noted above.

For these and other reasons the role of the plant breeder becomes a crucial factor in increasing agricultural production. Since tissue culture has now the proven capacity to rapidly produce plants with salt tolerance and efficient water utilization. Its incorporation into plant breeding contributes directly to all A.I.D. concerns relating to food production.

B. Review by International Centers

The proposal was reviewed and endorsed by all four international agricultural research centers to which it was sent. The excerpts of the comments from these centers is given here under:

IITA (NIgeria). "IITA finds the proposal technically very sound and logical . . . IITA interested in this work as relates to maize, rice, cowpeas and soybeans with references to sodium and aluminum toxicity and drought stress. Similar work crops with limited genetic diversity such as yam and cocoyams will be of interest . . . IITA very interested prospects screening for virus resistance at cellular stage and in rapid propagation techniques tuber crops, yam in particular."

CIAT (Colombia). "I am pleased to forward, with my endorsement these comments: . . . see a direct application of this type of research to CIAT's Beef Program. Selection for resistance to aluminum toxicity in forage legumes, both among species and among varieties of the given species, would be of great value in developing suitable pastures in the Llanos. . . . the general idea is attractive since the tissue culture system would have the advantage over convential selection/breeding methods in its case of handling large populations of individual cells instead of large numbers of intact plants."

12 This figure is for installation only, not maintenance or utilization.

IRRI (Philippines). " . . . may I say that this proposal is of great interest to us at IRRI. As a matter of fact for a period of about two years, we have had discussions with the scientists concerned in the development of this project and with administrators from their universities. We strongly support this research effort and would do what we could to cooperate with the scientists in supplying appropriate genetic materials and in reproducing materials which come from their research efforts."

CIP (Peru). "We feel an attempt to use tissue culture techniques as an additional tool for breeding is highly welcomed and should be supported. . . . We all want to see this develop on a solid basis without the overplay that was done with irradiation concerning the potentials . . . work should be conducted in association with good breeders who are using conventional methods and working on the same crop plants."

C. Administrative Feasibility

This project will be administered by Colorado State University (CSU). Dr. Murray W. Nabors, Associate Professor of Botany and Plant Pathology will be the principal investigator. CSU will sub-contract some of the work to Cornell and Michigan State University. Dr. R. S. Chaleff, Department of Agronomy at Cornell and Dr. P. S. Carlson, Department of Crop and Soil Sciences at Michigan State will be the co-investigator. The universities of Cornell and Michigan State were selected by CSU for: 1) their strong interest in the tissue culture as a tool to improve agricultural traits of crops and 2) their expertise on tissue culture in rice and grain legumes.

CSU will convene a meeting of three universities immediately after the project is funded to develop detailed work plans and specify responsibility of each party. CSU will coordinate and evaluate the work of the sub-contractors. The three universities will develop close working relationships with suitable international and national crop research centers to obtain germ plasm and to disseminate outputs of this project.

A.I.D. technical office (DS/AGR) will monitor the progress of the project and will interact on a continuing basis with the contractor. It will schedule on-site evaluation as deemed necessary.

D. Environmental Impact

The activities of this project fall into the area described in environmental procedure regulations paragraph 216.2(c) "Analysis, studies, academic or investigative research, workshops and meetings." These classes of activities will not normally require the filing of an Environmental Impact Statement or the preparation of an Environmental Assessment. It is possible that an output of this project will be a set of procedures, guidelines or research results which when used would require such as assessment. However, the project itself only proposes research and directly supportive activities.

Under these guidelines, this activity clearly qualified for a Negative Determination at the time when a threshold decision is determined.

E. Women in Development

It is the goal of this project to encourage the widest possible participation of women workers. The past small research contract AID/ta-C-1223 on tissue culture employed women professionals in the Department of Botany and Plant Pathology to support the research work. A continuation of these strategies under this proposed research project is assumed. Colorado State University has an aggressive affirmative action program to assure opportunities of employment for qualified personnel, including minorities and females, in compliance with Title IX. Colorado State University has an officer responsible for program implementation.

Overall thrust of the program to advance knowledge in crop production particularly on marginal lands holds great promise for increased involvement of poor farmer, especially women, who have traditionally played a major role in such areas.

V. Evaluation Plan

The project will be reviewed by DS/AGR, the A.I.D. Regional Bureaus, A.I.D. Missions, LDC countries and the international crop research centers. Highly qualified outside consultants may also be included in the review process, if A.I.D. considered such assistance desirable.

The review process will involve study of written annual progress reports and other technical papers and on-site campus visits. A comprehensive team review will be conducted after about a two-year period to determine whether project should be extended beyond the initial possible three-year approval period. A terminal team review may also be scheduled to assess progress and determine future utilization activities in the tissue culture field.

VI. Budget

1. Colorado State University's provisional overhead rate on proposals is 56.6% of salaries, wages, annuities, sick leave, holidays and vacation. Audit was performed and the above rate approved by the Department of Health, Education, and Welfare, Denver Regional Center, May 20, 1977.

2. No direct financial contributions are expected from other sources.

Budget For Colorado State University Including Subcontracts

BUDGET ITEM	year 1 1980	year 2 1981	year 3 1982	year 4 1983	year 5 1984	TOTAL 1980-84
I. Personnel Costs						
A. Faculty/Staff						
M. Nabors (6 mos./yr.)	13,371	14,574	15,885	17,315	18,873	80,018
J. Welsh (1 mo./yr.)	2,743	2,990	3,259	3,552	2,872	16,416
3 technicians (36 mos./yr	28,080	30,607	33,362	36,364	39,637	168,050
J. Mestas (0.5 mo./yr.) (bookkeeper)	631	687	749	817	890	3,774
secretary (0.5 mo./yr.)	381	415	452	493	537	2,278
B. Subtotal	45,206	49,273	53,707	58,541	63,809	270,536
C. Fringe Benefits 10.64% of B	4,810	5,243	5,714	6,229	6,789	28,785
D. Post docs/graduate stu.						
3 grad. students (36 mos./yr.)	14,400	15,696	17,108	18,648	20,326	86,178
2 post doc. (24 mos./yr.)	24,000	26,160	28,514	31,080	33,878	143,362
E. Hourly @ $4/hr.	12,000	12,000	13,500	13,500	13,500	64,500
F. Total B, C, D, E	100,416	108,372	118,543	127,998	138,302	593,631
II. Equipment						
electric autoclave	25,000					25,000
laminar flow hoods	8,000					8,000
shaking machines	10,000					10,000
New Brunswick fermentors	100,000					100,000
pH meter, Mettler balance	5,000					5,000

BUDGET ITEM		year 1 1980	year 2 1981	year 3 1982	year 4 1983	year 5 1984	TOTAL 1980 -84
III.	Supplies	25,000	25,000	25,000	30,000	30,000	135,000
IV.	Travel	5,000	5,000	7,000	8,000	10,000	35,000
V.	Printing/Publication/Mailing /Public Awareness Activities	5,000	5,000	6,000	6,000	6,000	28,000
VI.	Subcontracts						
	Michigan State University Dr. P.S. Carlson	40,000	40,000	40,000	40,000	40,000	200,000
	Cornell University Dr. R.S. Chaleff	70,000	70,000	70,000	70,000	70,000	350,000
VII.	Total Direct Costs	393,416	253,372	266,543	281,998	294,302	1,489,032
VIII.	Indirect Costs (78% of I.F.)	78,324	84,530	92,464	99,838	107,876	463,032
IX.	TOTAL COSTS	471,740	337,902	359,007	381,836	402,178	1,952,663

Budget For Subcontract to Michigan State University, Dr. P.S. Carlson.

BUDGET ITEM		year 1 1980	year 2 1981	year 3 1982	year 4 1983	year 5 1984	TOTAL 1980 -84
I.	Personnel Costs						
	Graduate students (45 mos./ yr.) Hourly	17,803 2,520	17,803 2,520	17,803 2,520	17,803 2,520	17,803 2,520	89,015 12,600
	Subtotal	20,323	20,323	20,323	20,323	20,323	101,615
II.	Supplies	5,654	5,654	5,654	5,654	5,654	28,270
III.	Indirect Costs (69% of Salaries and Wages	14,023	14,023	14,023	14,023	14,023	70,115
IV.	TOTAL COSTS	40,000	40,000	40,000	40,000	40,000	200,000

Budget For Subcontract to Cornell University, Dr. R.S. Chaleff.

BUDGET ITEM		year 1 1980	year 2 1981	year 3 1982	year 4 1983	year 5 1984	TOTAL 1980-84
I.	Personnel Costs						
	Technicians (24 mos. for year 1; 15 mos. for years 4 and 5)	19,000			15,750	17,500	52,250
	Post docs (12 mos. for years 1,4,&5; 24 mos for 2&3)	12,000	24,000	26,000	14,000	14,000	90,000
	Subtotal	31,000	24,000	26,000	29,750	31,500	142,250
	Fringe Benefits (24.5% of Sub Total)	7,595	5,880	6,370	7,289	7,717	34,851
II.	Supplies	10,976	22,804	18,446	11,756	8,375	72,357
III.	Travel		1,000	1,500	1,000	1,000	4,500
IV.	Publication Costs		500	550	600	650	2,300
V.	Total Direct Costs	49,571	54,184	52,866	50,395	49,242	256,258
VI.	Indirect Costs (65.9% of Salaries & Wages)	20,429	15,816	17,134	19,605	20,758	93,742
VII.	TOTAL COSTS	70,000	70,000	70,000	70,000	70,000	350,000

APPENDIX.

This section contains:

Manuscript of a paper submitted for publication. This paper shows that NaCl-tolerant cell lines give rise to plants which pass salt tolerance to future generations.

AID 1020-28 (1-72)

PROJECT DESIGN SUMMARY
LOGICAL FRAMEWORK

Life of Project:
From FY 1980 to FY 1984
Total U.S. Funding $1,952,660
Date Prepared: 4/79

Project Title & Number: Utilization of Tissue Culture to Increase Food Production

NARRATIVE SUMMARY	OBJECTIVELY VERIFIABLE INDICATORS	MEANS OF VERIFICATION	IMPORTANT ASSUMPTIONS
Program or Sector Goal: The broader objective to which this project contributes: 1. To improve the efficiency of food production with developing nations through increased utilization of land resources, especially by growing crop varieties more suited to the prevailing enviornments.	Measures of Goal Achievement: 1. Greater use of marginal lands with minimum inputs. 2. Higher crop yields in marginal lands. 3. Increased food production in the developing nations.	1. Government records. 2. Spot checking for use of areas and crop yields.	Assumptions for achieving goal targets: 1. A.I.D. and developing nations view plant breeding for stress resistance as an important alternative to energy-intensive agriculture involving environmental modification.
Project Purpose: 1. To promote the utilization of tissue culture as a technique to add desired characteristics in a crop variety, e.g. adaptation to adverse environmental conditions.	Conditions that will indicate purpose has been achieved: End of project status. 1. Cell cultures tolerant to the various stresses. 2. Regenerated plants from these cultures. 3. Greenhouse testing of these plants for stress tolerance and inheritability of stress resistance. 4. Field testing of the resistant plants.	1. International centers and national centers start using the tissue culture technique in their breeding programs.	Assumptions for achieving purpose: 1. Tissue culture technology is now sufficiently developed to make a practical contribution to plant breeding. 2. Colorado State, Michigan State, and Cornell University personnel represent a unique resource combination to effect this technology transfer.
Outputs: 1. Na^{+}-, Al^{+++}-, drought- and temperature extreme-tolerant varieties of major crop plants. 2. Tested methods for streamlining plant breeding by using tissue culture techniques. 3. Tested methods for rapidly cloning rare, useful plants by tissue culture techniques. 4. Increased linkages & networks between tissue culture & worldwide plant breeding personnel.	Magnitude of Outputs: 1. Na^{+}-tolerant wheat released. 2. Al^{+++}-, Na^{+} and drought tolerant wheat in field testing phase; sorghum, rice millets beginning field testing. 3. Temperature-extreme-tolerant wheat beginning field testing. 4. Na^{+}-, Al^{+++}-, and drought-resistant corn, cowpeas and soybeans regenerated.	1. Laboratory, greenhouse, and field-testing progress reports. 2. On site visits. 3. Scientific publications. 4. Verification from CGIAR centers.	Assumptions for achieving outputs: 1. Specific outputs and assessments of anticipated problems are reasonable. 2. Past progress rates in our labs are indicative of future rates.
Inputs: 1. Expertise and facilities in tissue culture breeding at Colorado State, Michigan State, and Cornell Universities. 2. Expertise in plant breeding at the 3 universities & worldwide. 3. Physical plants and administrative staffs of the 3 universities. 4. Financial support from A.I.D.	Implementation Target (Type and Quantity) 1. Project proposal approved. 2. Facilities adequate for amount of research. 3. Fiscal and temporal production allowances accurate.	1. Annual reports. 2. On-site visits 3. Technical publications. 4. Reports to interested breeders. 5. Cooperation with interested breeders.	Assumptions for providing inputs: 1. Qualified investigators and technical staff continue with project. 2. CGIAR personnel are interested in tissue culture input. 3. University support is provided. 4. A.I.D. support and monitoring provided.

APPENDIX A

CURRICULUM VITAE OF MURRAY W. NABORS

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Colorado State University
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(303) 491-6996

Education: B.S. in Biology, Yale University, June 1965; Ph.D. in Botany, Michigan State University (Atomic Energy Commission/Plant Research Lab), 1970, under Dr. Anton Lang, member National Academy of Sciences.

Publications:

1. E. Epstein, M. Nabors, and B.B. Stowe, Origin of Indigo of Wood, etc. Nature 216:547-549 (1967).

2. M. Nabors, and A. Lang, The Growth Physics and Water Relations of Red-Light-Induced Germination in Lettuce Seeds. I. Embryos Germinating in Osmoticum. Planta 101:1-25 (1971).

3. M. Nabors, and A. Lang, The Growth Physics and Water Relations of Red-Light-Induced Germination in Lettuce Seeds. 11. Embryos Germinating in Water. Planta 101:26-42 (1971).

4. M. Nabors, The Growth Physics and Water Relations of Red-Light-Induced Lettuce Seed Germination, Thesis at Michigan State University, 1970.

5. M. Nabors, A Density Gradient Method for Determining Osmotic Potential of Plant Cells, The American Biology Teacher 35:463, 464, 469 (1973).

6. M. Nabors, P. Kugrens, and C. Ross, Photodormant Lettuce Seeds: Phytochrome-Induced Protein Degradation, Planta 117:361-365 (1974).

7. M.W. Nabors, A. Daniels, L. Nadolny, and Claire Brown, Sodium Chloride Tolerant Lines of Tobacco Cells. Plant Science Letters 4:115-159 (1975).

8. N. Carpita and M. Nabors, Effects of 35C Heat Treatments on Photo-

sensitive Grand Rapids Lettuce Seed Germination, Plant Physiology 57:612-616 (1976).

9. M. Nabors, The Use of Spontaneously Occurring and Induced Mutations to Obtain Agriculturally Useful Plants. BioScience 26:761;767 (1976).

10. M.P. Hooker and M. Nabors. Callus Initiations, Growth, and Organogenesis in Sugarbeet (Beta vulgaris L.) Zeits. fur Pflanzen 84:237-246 (1977).

11. D. Hoisington and M. Nabors, pH of Tissue Culture Medium: Effects of Agar and of Autoclaving, Submitted for publication.

12. D. Hoisington and M. Nabors, Callus Formation and Plant Regeneration from Cotyledons of Some Dicots. In preparation.

13. C. Ross, M. Nabors, and A.K. Huff. Solute Production, Osmotic Potential, and Plant Cell Growth, Submitted for publication.

14. M.W. Nabors. The use of plant tissue cultures to produce altered varieties of agriculturally useful plants. In Plant Adaptation to Mineral Stress in Problem Soils; ed. M.J. Wright. Cornell University Agricultural Experiment Station. 1977.

15. N.C. Carpita, M.W. Nabors, C.W. Ross, and N.L. Petretic. The Growth Physics and Water Relations of Red-Light-Induced Germination in Lettuce Seeds. III. Osmotic and Pressure Potential Changes. Planta 144: 217-224 (1979).

16. N.C. Carpita, M.W. Nabors, C.W. Ross, and N.L. Petretic. The Growth Physics and Water Relations of Red-Light-Induced Germination in Lettuce Seeds. IV. Biochemical Changes. Planta 144: 225-233 (1979).

CURRICULUM VITAE

Peter S. Carlson

306 Plant Biology Building
Michigan State University
East Lansing, MI 48824

Present Appointment

John A. Hannah Distinguished Professor in Agriculture and Natural Resources, Associated with the Department of Crop and Soil Sciences, Michigan State University

Education

A.B. (Magna cum laude), Kenyon College, 1966.
Ph. D., Yale University, 1971.

Publications

Selected Scientific Papers and Review Articles (A total of 40 published scientific articles)

Carlson, P.S. and M.O. Chiscon. 1978. Ann. Rev. Microbiology. (In preparation).

Radin, D. and P.S. Carlson. 1978. In Situ recovery of mutants in higher plants. Genetical Research. (In press).

Carlson, P.S. and T.B. Rice. 1978. Visualization of enzyme phenotypes in higher plants. Plant Science Letters. (In press).

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CURRICULUM VITAE

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APPENDIX C

NaCl-Tolerant Tobacco Plants from Cultured Cells

by

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(submitted for publication)

Summary

We have obtained NaCl-tolerant cell lines by exposing tobacco cell suspensions to increasing levels of NaCl. Tolerance to 8.8 g/l NaCl is the maximum obtainable in cell suspensions. Normal lines are tolerant to about 1.6 g/l NaCl. Plants regenerated from resistant cell lines transmit tolerance to two subsequent generations. The level of NaCl-resistance in regenerated plants is higher than that of cell cultures. Few nontolerant F2 plants survive watering with solutions containing more than 15.4 g/l NaCl whereas most tolerant plants survive 33.4 g/l NaCl.

Introduction

The prospects of utilizing tissue culture to increase food production are bright, but depend on demonstrations that agriculturally desirable traits, selected in cell cultures, persist in regenerated plants and are inheritable. Carlson (1973) has shown persistence and inheritability for methionine-sulfoximine (MSO) resistance in tobacco. MSO causes effects much like those of the toxin causing tobacco wildfire disease. Chaleff and Parsons (1978) have provided a similar demonstration for herbicide (picloram) resistance in tobacco. Gengenbach et al. (1977) have shown resistance to *Helminthosporium* toxin in corn.

Specific ion toxicity in soil and water is probably the largest single environmental factor restricting agricultural production. In particular, Na+ toxicity currently affects 50% of the irrigated land in the western United States and restricts crop production on 25% of this land (Wadleigh, 1968). Casey (1972) cites estimates that 33% of irrigated land worldwide is salt-affected. He questions the usefulness of irrigation schemes in general and suggests that breeding for salt tolerance is highly desirable. We believe this to be the first report that NaCl resistance obtained in cultured cells is retained and inherited by subsequent generations of regenerated plants.

Materials and Methods

Cultured cell lines of *Nicotiana tabacum* var. Samson resistant to various levels of NaCl were obtained as previously reported (Nabors et al., 1975). Both spontaneous and induced (using ethyl methane sulphonate) lines have been obtained. Populations of 10 million cells contain salt-tolerant cells in about 90% of cases tested. Thus the spontaneous mutation rate would be not less than 1×10^{-7} per six months (the time required to obtain a cell suspension before selection begins). We have now produced lines resistant to as much as 8.8 g/l NaCl. Resistance to higher levels is not obtainable in our system. Shoots were regenerated from both NaCl-tolerant and NaCl-sensitive cultures by placing cell suspension aliquots on solid regeneration medium. Our regeneration medium consists of Linsmaier and Skoog's (1965) basic medium supplemented with 0.5 mg/l kinetin. Shoot regeneration is noticeably restricted on medium containing NaCl. Shoots were

rooted by transfer to an identical medium that contained in addition either 5 or 10 mg/l indoleacetic acid (IAA). Reducing the level of major salts to half or tenth normal does not promote rooting in our system. Again, rooting is noticeably less vigorous in NaCl-containing medium.

Regenerated plants were removed from culture vials, potted in soil, and hardened in the lab for several weeks before transfer to the greenhouse. Three groups of plants were used: (1) Plants regenerated from NaCl-sensitive cultures and watered with solutions containing no salt; (2) NaCl tolerant plants selected from cultures tolerant to 6.4 g/l NaCl, regenerated in the presence of NaCl, and watered with solutions containing the same level of salt; (3) Plants like those in group 2 but regenerated and rooted in medium containing no salt. Seeds from each group were collected and planted to obtain the F1 generation. F1 plants were potted in soil in 4 inch pots and groups were watered with various concentrations of salt water containing from 0.0 to 19.0 g/l NaCl. When it became apparent that the tolerance of whole plants was considerably higher than the tolerance of cultured cells, the concentrations of the watering solutions were adjusted upwards to contain from 0.0 to 32.8 g/l NaCl.

Results and Discussion

Plant survival rates at 13 weeks at two levels of NaCl are shown in Table 1. Seeds from the three groups of F1 plants were collected whenever possible. Flowering and seed set are considerably more sensitive to NaCl than plant survival, so seeds were not obtained from all plants. Seeds from F1 plants of the three groups watered with a salt-free solution were planted to obtain the F2. Groups of F2 plants were salt-stressed at various levels as for the F1. Data at two levels of NaCl are shown in Table 1.

Our results demonstrate persistence of tissue-culture-selected NaCl resistance in plants one and two generations beyond regeneration. Since we have been able to repeatedly select spontaneous NaCl-tolerant cell lines from cultures of 10^7 diploid cells we believe the resistance results from a dominant or co-dominant allele. Given typical eukaryotic mutation rates of 1×10^{-5} (for specific phenotypic changes [Strickberger, 1968]) to 1×10^{-9} (for changes in specific DNA bases [Vogel, 1970]), the probability of obtaining two or possibly four (since tobacco is an allotetraploid [Smith, 1968]) recessive alleles in the same cell is vanishingly small.

If a single dominant allele is responsible for the observed salt tolerance a 3:1 ratio of tolerant to sensitive plants would be expected in the F1 assuming the parent plant (regenerated from a salt-tolerant cell culture) was diploid and heterozygous. Continued self-fertilization would lead to an increasing proportion of both salt-sensitive and salt-tolerant homozygotes. This assumes that seeds for each succeeding generation were collected from non-stressed plants so that both salt-sensitive and salt-tolerant plants survived. Data of our F1 and F2 generations (Table 1) support neither this hypothesis at the 95% level nor a similar hypothesis based on a tetraploid parent.

This kind of genetic analysis probably does not apply to salt tolerance in these plants for two reasons. First, the ratio of healthy to non-healthy (or dead to alive) plants for any salt-sensitive or salt-tolerant population is dependent on (1) stress level and (2) duration of stress as well as genetic factors. Second, our method of stress selection involves application of high salt levels to populations of 10 million cells. This leads to a period of no growth followed by slow adaptation of the culture to the stress, presumably (but not necessarily) by growth and division of the few naturally occurring NaCl-resistant cells in the population. Following a resumption of normal or near normal growth rates even higher stress is applied. Thus, a stepwise selection pattern of stress application and accommodation results. Because of this pattern our mutants may well be multiallelic or multigenic and not suitable for a Mendelian-based analysis of inheritance. Backcrosses and other F1 and F2 crosses are being made to provide further data on the inheritance pattern.

Two lines of evidence indicate that phenotype persistence is in some manner dependent on or enhanced by the presence of NaCl. First, Table 1 shows that plant lines derived from NaCl-tolerant cell cultures are noticeably less tolerant if salt was omitted during the original regeneration process. This omission was made as noted earlier to improve regeneration and rooting rates. The observation could be explained if different mutations are involved in each plant line. This interpretation is supported by the fact that under salt stress the two lines have somewhat differing morphologies with one being more compact in growth habit than the other.

Second, F2 plants are noticeably more salt tolerant if seeds are collected from F1 plants that were exposed to salt (data not shown). This observation cannot be explained by different mutations since only one plant line is involved. The results could be explained in one of several ways. (1) Further selection, for pollen with even higher salt tolerance for example, could have occured in F1 plants. (2) A high reverse mutation rate could be promoted by the absence of NaCl. (3) The F1 plants could be chimerical, composed of salt-tolerant and salt-sensitive cells. Thus selection would still occur in F1 plants exposed to salt. This explanation seems unlikely to us since the salt-tolerant cell lines which gave rise to parental plants were exposed to high salt levels for almost a year prior to regeneration. After this period of selection all cells in the culture should have been salt-tolerant. (4) Both observations could be explained if the observed salt tolerance were not genetic but the result of increased levels of a particular enzyme or some other physiological adaptation maintained by the presence of NaCl. Alt et al. (1978) have reported on such a system involving duplication of a particular gene in cultured animal cell lines, although their system does not involve regenerated organisms. We consider the first and last possibilities to be most likely and thus candidates for future research.

Table 1. Survival rate under high or no salt stress of salt-tolerant and non-salt-tolerant tobacco plant lines derived from cultured cells. Plants regenerated from tissue cultures were designated the parental generation (P) and were selfed to obtain the F1 generation. The F1 plants of each group in the 0 g/l NaCl column were selfed to obtain the F2 generation. F1 had 43 plants per group. F2 had 20.

	% alive after 13 weeks			
	F1		F2	
	Watering solution		Watering solution	
	26.2 g/l NaCl	0 g/l NaCl	29.8 g/l NaCl	0 g/l NaCl
Original culture #1 resistant to NaCl with NaCl continu ally present	65	100 (selfed to produce F2	100	100
Original culture #2 resistant to NaCl with NaCl not present during regeneration	35	100 (selfed to produce F2)	90	100
Original culture #3 not resistant to NaCl	20	100 (selfed to produce F2)	15	100

Our findings that NaCl-resistant cell lines give rise to plants that transmit resistance to future generations has many potential practical implications. Incorporation of Na+ tolerance is desirable in many cultivars of crop plants, particularly those grown in arid, irrigated regions with salt accumulation problems. Utilizing typical field selection techniques, salt tolerance is time and space consuming as well as expensive to obtain. The time-space compressibility and the selection specificity inherent in tissue culture techniques when used in conjunction with traditional methods should remarkably improve the efficiency of the plant breeding process.

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