DATE: October 17, 1979

memorandum

PD-ABA-462

REPLY TO ATTN OF: DS/AGR/TSWM, T. Gill

SUBJECT: Approval for the Revised Project Statement on, "Utilization of Tissue Culture to Increase Food Production"

TO: See Distribution

Please review the attached project statement and indicate by phone (235-8877) or in writing whether it meets your approval.

During the May 29, 1979 DSB Project Review Committee meeting it was agreed that the proposal has merit and should be funded. However, it was suggested that before the proposal is submitted to RAC, the Office of Agriculture should take a number of actions. These actions called for a revision of the project statement based on narrowly defined objectives and prioritized scope of work. In our opinion the attached project statement has fully taken in account the committee's suggestions.

The adjusted scope of work will reduce the original project budget of $1,953,000 by about $800,000 over a five year's period. The project will retain the following combinations of crop vs. stress tolerant studies:

wheat - salt, aluminum, high temperature and drought,
rice - salt and aluminum,
millet - salt and drought.

The following studies were deleted from the project:

rice - low temperature,
sorghum - salt and drought,
regeneration studies - cowpeas, soybeans and corn.

As a result of reduction in the scope of work, there is no need for subcontractors i.e., the Universities of Cornell and Michigan State. Budget information on cost of individual crop vs. stress items is given in attachment 1. List of equipment required, their use and justification is given in attachment 2. Equipment cost has been reduced by $36,000 in the revised statement.

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PD-ABA-462

PROJECT STATEMENT

Utilization of Tissue Culture to Increase Food Production

(Project # 931.4055)

DS/AGR/TSWM

October 1979

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Figure (1) Mutant Selection Flow Chart
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PROJECT STATEMENT

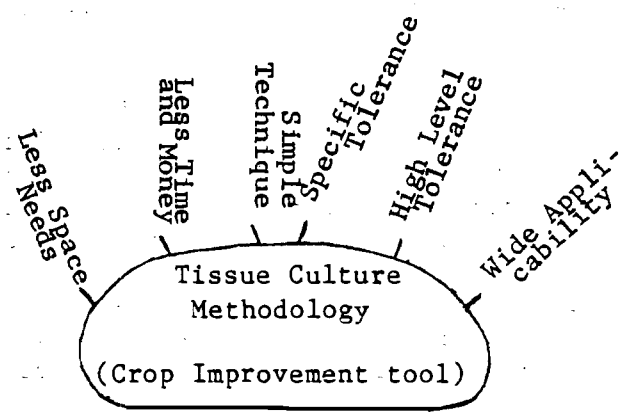
I. Project Summary

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
Duration:	About Five Years(Est. Cost: $1,157,900)
Initial Three Years Funding:	First Year: $312,750 Second Year: $188,675 Third Year: $206,250 TOTAL : $707,675
Project Manager:	Dr. T. S. Gill, DS/AGR

B. Graphic

(i) Possible Advantages:



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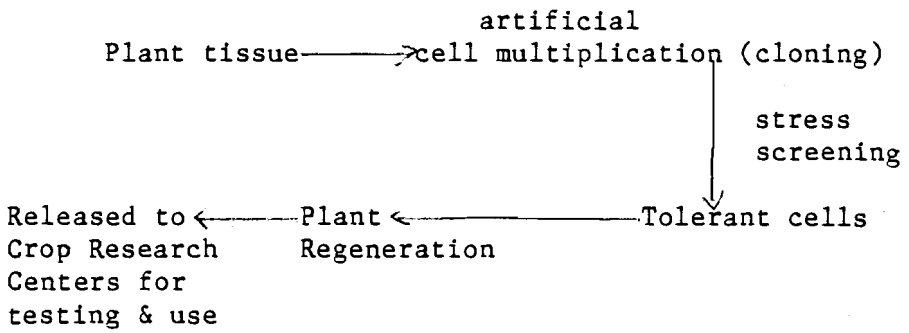
(ii) Flow of Activities:



(iii) Out-puts: Scope of work

- o Wheat variety(s) tolerant to salt concentration, aluminum toxicity, drought conditions and high temperature.
- o Rice variety(s) tolerant to salt concentration and aluminum toxicity.
- o Millet variety(s) tolerant to salt concentration and drought.

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 test 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 alleviate 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, conservativly 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 worldwide 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 cost effective technique 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.

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

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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 test the use of tissue culture as a cost effective technique to add stress tolerance characteristics to wheat, rice and millet.

C. Objectives:

1. To utilize tissue culture procedures to obtain Na^+ tolerant, Al^{+++} tolerant, high temperature tolerant, and drought tolerant alleles cell lines of useful cultivars of three food crop plants-wheat, rice, and millet.

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.

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.

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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.

> 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 are now 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 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.

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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).

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).

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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 IARCs personnel. Schedule of student and personnel exchanges between tissue culture labs at CSU, and International Centers is established.

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 $AlCl_3$ to growth medium.

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

(3) High temperature tolerance: Grow cultures at high temperature 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 Agricultural Research Centers.

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.

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 $AlCl_3$ to the medium. Selection will occur in a stepwise fashion (Fig. 1). 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 temperature tolerance.

These mutants will be selected by growing cultures at high temperature 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.

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 IARCs 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) on wheat, rice, and millet. Begin stress selection for Na^+, Al^{+++}, and drought, and high temperature tolerance.

(d) 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.

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

(f) 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 rice, and millet.

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

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

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

(f) 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^+, and Al^{+++}, all lines of rice and Na^+ and drought-tolerant cell lines of millet. Determine maximum tolerances obtainable in tissue cultures.

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

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

(d) Initiate short visits and personnel exchanges between International Centers and developing nations.

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

(2) Expected product.

(a) Na^+-, and Al^{+++}-, tolerant cell lines of rice, and Na^+ and drought-tolerant cell lines of millet.

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

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

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

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

(f) 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^+-, and Al^{+++}-, tolerant cell lines of rice and Na^+ and drought-tolerant cell lines of millet.

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

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

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

(e) 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^{+++}, and drought-tolerant cell lines of rice, and millet.

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

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

d. Fourth year of contract (1983).

(1) Work to meet research objectives.

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

(b) Begin field testing of one Na^{+}-tolerant line of wheat.

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

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

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

(2) Expected product.

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

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

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

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

(e) Attended national 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^{+}, and Al^{+++}, tolerant lines of rice and Na^{+} and drought-tolerant lines of millet.

(b) Continue field testing of one Na^{+}-tolerant lines of wheat. Release varieties to ICARs and National Crop Centers.

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

(d) Continue personnel exchanges with International Breeding Centers.

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

(2) Expected product.

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

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

(c) One Na^{+}-tolerant line of wheat released to IARCs and NARCs.

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

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

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

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

(i) Submit a final report to A.I.D.

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

IV. Project Analysis

A. Technical, Economical and Social Soundness

a. Tissue culture breeding has successfully produced improved varieties.

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 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

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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.

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).

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.

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 countries 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.

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.

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.

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 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

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.

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

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."

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 Responsibility

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 develop close working relationships with sutiable 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

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 (2 mos./yr.)	4,457	4,858	5,295	5,705	6,271	26,586
J. Welsh (1 mo./yr.)	2,743	2,990	3,259	3,552	2,872	16,416
2 technicians (24 mos./yr	18,720	20,404	22,241	24,240	26,424	112,029
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	26,932	29,359	31,996	34,807	36,994	160,083
C. Fringe Benefits 10.64% of B	2,865	3,123	3,404	3,703	3,936	17,031
D. Post docs/graduate stu.						
3 grad. students (36 mos./yr.)	14,400	15,696	17,108	18,648	20,326	86,178
2post 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	80,197	86,333	94,522	101,738	100,634	463,424
II. Equipment						
electric autoclave	25,000					25,000
laminar flow hoods	8,000					8,000
shaking machines	7,000					7,000
New Brunswick fermentors	90,000					90,000
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. Total Direct Costs	250,197	121,333	132,522	145,738	146,634	796,424
VII. Indirect Costs (78% of I.F.)	62,554	67,340	73,727	79,356	78,495	361,472
VIII. TOTAL COSTS	312,751	188,673	206,247	225,094	225,129	1,157,896

Figure (1) Mutant Selection Flow Chart

population of non-NaCl tolerant
cultured cells (callus or suspension)

800 ppm NaCl tolerant culture —— plant regeneration —— greenhouse testing

stress selection in 1600 ppm

1600 ppm NaCl tolerant culture —— plant regeneration —— greenhouse testing

stress selection in 2400 ppm

2400 ppm NaCl tolerant culture —— plant regeneration —— greenhouse testing

stress selection in 3200 ppm

3200 ppm NaCl tolerant culture —— plant regeneration —— greenhouse testing

stress selection in 4000 ppm

4000 ppm NaCl tolerant culture —— plant regeneration —— greenhouse testing

stress selection in 4800 ppm

4800 ppm NaCl tolerant culture —— plant regeneration —— greenhouse testing

etc.

AID 1020-28 (1-72)

PROJECT DESIGN SUMMARY
LOGICAL FRAMEWORK

Life of Project:
From FY 1980 to FY 1984
Total U.S. Funding $1,157,900
Date Prepared: 10/79

Project Title & Number: Utilization of Tissue Culture to Increase Food Production (#931.4055)

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 environments.	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 test the use of tissue culture as a technique to add stress tolerance characteristics to wheat, rice and millet.	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.
Outputs: 1. Wheat variety(s) tolerant to salt, aluminum toxicity, drought and high temperature. 2. Rice variety(s) tolerant to salt and aluminum toxicity. 3. Millet variety(s) tolerant to salt and drought. 4. Linkages between tissue culture labs and worldwide plant breeding personnel.	Magnitude of Outputs: 1. At least one wheat variety tolerant to salt, aluminum toxicity, drought and high temperature respectively, released to IARCs and NARCs for testing and use. 2. At least one rice variety tolerant to salt and aluminum toxicity released to IARCs and NARCs. 3. At least one millet variety tolerant to salt and drought released to IARCs and NARCs. *see 4 below.	1. Laboratory, greenhouse, and field-testing progress reports. 2. On site visits. 3. Scientific publications. 4. Verification from IARCs & NARCs.	Assumptions for achieving outputs: 1. Specific outputs and assessments of anticipated technical problems are not unsurmountable. 2. Past progress rates are indicative of future rates.
Inputs: 1. Expertise and facilities in tissue culture breeding at Colorado State University. 2. Expertise in plant breeding at the CSU. 3. Physical plants and administrative staffs at CSU. 4. Financial support from A.I.D.	Implementation Target (Type and Quantity) 1. Project approved by RAC - January 1980. 2. Project funded by AID/ISTC - March 1980. 3. Staff and Facilities in place - June 1980. * 4. Linkages with CIMMYT, IRRI, ICRISAT and at least 3 national agricultural research centers (IARCs)	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. IARC personnel are interested in tissue culture input. 3. University support is forthcoming. 4. A.I.D. support and monitoring provided.

APPENDIX A

CURRICULUM VITAE OF MURRAY W. NABORS

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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. 111. 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).

BIOGRAPHICAL DATA

James R. Welsh
Professor of Agronomy
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Education - B.S. in Agronomy, North Dakota State University, 1956.
Ph.D., Plant Genetics, Montana State University, 1963.
Post Doctoral, Biological Statistics, North Carolina State University, 1963 summer.

PUBLICATIONS:

1. Welsh, J.R., and E.R. Hehn. 1964. The effect of chromosome ID on hexaploid wheat flour quality. Crop Sci. 4:320-323.

2. Joppa, L.R., F.H. McNeal and J.R. Welsh. 1966. Pollen and another development in cytoplasmic male sterile wheat. Crop Sci. 6:296-297.

3. Welsh, J.R., C.A. Watson, and C.W. Green. 1968. Chromosomal control of flour properties in three substitution sets of common wheat (Triticum aestivum L.). Crop Sci. 8:81-82.

4. Violin, R.B. and J.R. Welsh. 1970. Serological Evaluation of winter wheat aneuploids. Crop Sci. 10:13-14.

5. Welsh, J.R., V.R. Stewart, E.L. Sharp, G.A. Taylor and E.R. Hehn. 1970. Registration of crest wheat (Reg. No. 483) Crop Sci. 10(4):462.

6. Calpouzos, L., M.E. Madson, and J.R. Welsh. 1971. The effect of harvest operation on yield of wheat from experimental plots with and without stem rust. Phytopathology 61:1022.

7. Welsh, J.R. and A.R. Klatt. 1971. The effects of temperature and photoperiod on Spring Wheat Pollen viability. Crop Sci. 11:864-865.

8. Diehl, A.L. and J.R. Welsh. 1972. Yield components of semi-dwarf and tall spring wheat. Wheat Information Service 35:14-17. Sept.

9. Welsh, J.R. and R.M. Normann. 1972. Use of the Modified Pelshenke Test for Early Generation Wheat Quality Screening. Crop Sci., 12:80-81. January-February.

10. Lorenz, K., J. Welsh, R. Normann, and J. Maga. 1972. Comparative Mixing and Baking Properties of Wheat and Triticale Flours. Cereal Chem. 49:187-193.

11. Keim, D.L., J.R. Welsh, and R.L. McConnell. 1973. Inheritance of photo-periodic heading response in winter and spring cultivars of bread wheat. Can. J. Plant Sci. 53:247-250.

12. Welsh, J.R. G. Ellis, R. Normann, G. Hinze, H. Mann and H.D. Moore. 1973. Registration of Baca Wheat. Crop Science 13:540-542.

13. Lorenz, K. and J.R. Welsh. 2974. Food product utilization of Colorado grown triticales. In: Triticale: First Man-made Cereal. The American Association of Cereal Chemists. P. 243-251. Cho. C. Tsen (Ed).

14. Welsh, J.R. and K. Lorenz. 1974. Environmental effects on utilization and agronomic performance of Colorado grown triticales. In: Triticale: First Man-Made Cereal. American Association of Cereal Chemists. St. Paul, Minn. p. 252-260. Cho. C. Tsen (Ed).

15. Welsh, J.R., H.D. Moore, S.L. Shafer, G.H. Ellis and R.N. Normann. 1974. Registration of Colano spring wheat. (Reg. No. 540) Crop Sci. 14:777.

16. Lorenz, K., J. Welsh, R. Normann, G. Beetner and A. Frey. 1974. Extrusion Processing of Triticale. Journal of Food Sci. 39:572-576.

17. Pirasteh, B., and J.R. Welsh. 1975. Monosomic analysis of photo-periodic response in wheat. Crop Science. 15:503-505.

18. Lorenz,K., J. Maga, C. Siger, and J.R. Welsh. 1975. Variability in the limiting amino acid and fatty acid composition of winter wheats and triticales. Agri. and Food Chem. 23(5):932-938.

19. Calleros, R.J., and J.R. Welsh. 1976. Relationship of Dwarfing and Hybrid Wheat Mechanisms. Crop Science. 16:241-243.

20. Welsh, J.R. 1976. Semidwarf Wheats - Their Strengths, Weaknesses. Crops and Soils 28:10-11.

21. Lorenz, K. and J.R. Welsh. 1976. Alpha-amylase and Protease Activity of Maturing Triticale and Its Parental Species. Food Science and Technology 9:7-10.

22. Calpouzos, L., A.P. Roelfs, M.E. Madson, F.B. Martin, J.R. Welsh, and R.D. Wilcoxson. 1976. A New Model to Measure Yield Losses Caused by Stem Rust in Spring Wheat. U. of Minn. Agr. Exp. Station Tech. Bul. 307.

23. Welsh, J.R., C. Ellis, R. Normann, G. Hinze and H. Mann. 1977. Registration of Lindon Wheat. Crop Science. 17:346.

24. Cholick, F.A., James R. Welsh, and C. Vernon Cole. 1977. Rooting Patterns of Semi-dwarf and Tall Winter Wheat Cultivars Under Dryland Field Conditions. Crop Science 17(4):637-639.

25. Lorenz,K. and J. Welsh. 1977. Agronomic and Baking Performance of Semi-Dwarf Triticales. Cereal Chemistry 54(5):1049-1055.

26. Welsh, J.R., G. Ellis, R. Normann, W.S. Ball, G. Hinze and H. Mann. 1978. Registeration of Vona Wheat. Crop Sci. 18 (4):694-695.

NaCl-Tolerant Tobacco Plants from Cultured Cells

by

M. Nabors, S. Gibbs, C. Bernstein and M. Meis

Colorado State University

(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.

References

Alt, F.W., R.E. Kellems, J.R. Bertino, and R.T. Schimke: J. Biol. Chem. 253, 1357-1370 (1978).

Carlson, P.S.: Science 180, 1366-1368 (1973).

Casey, H.E.: Salinity problems in arid lands irrigation: a literature reveiw and selected bibliography. Arid Lands Resource Inf. Paper No. 1, 1972. Univ. Arizona Office of Arid Lands Studies, Tucson, Arizona, U.S.A. 85719.

Chaleff, R.S., and M.F. Parsons: Proc. Natl. Acad. Sci. U.S.A. 75: 5104-5107 (1978).

Gengenbach, B.G., C.E. Green, and C.M. Donovan: Proc. Natl. Acad. Sci. U.S.A. 74: 5113-5117 (1977).

Linsmaier, E.M., and F. Skoog: Physiol. Plant. 18: 100-127 (1965).

Nabors, M.W., A. Daniels, L. Nadolny, and C. Brown: Plant Sci. Lett. 4, 155-159 (1975).

Smith, H.H.: Adv. Genet. 14: 1-54 (1968).

Strickberger, M.W.: Genetics, 524-525, Macmillan, New York, 1968.

Vogel, F.: Spontaneous mutation in man. In. Vogel,F., and G. Rohrborn (Eds.): Chemical Mutagenesis in Mammals and Man, 16-68, Springer-Verlag, New York, 1970.

Wadleigh, C.H.: USDA Misc. Pub. 1065, 112 (1968).

Tissue Culture Program
($,000)

Attachment 1

NO.	Crop	Stress	Var.	Personnel Yr. 1	Yr. 2	Yr. 3	Yr. 4	Yr. 5	Sub. T	Equip, Supplies, Travel, etc Yr. 1	Yr. 2	Yr. 3	Yr. 4	Yr. 5	Sub. T	G. Tot
1.	Wheat	Na+	1st	35	30	35	11	-	111	64	9	9	2	-	84	195
	Wheat	Na+	2nd	9	10	11	11	-	41	-	2	2	1	-	5	46
2.	Wheat	Al+++	1st	15	13	15	20	13	76	20	4	4	5	2	35	111
	Wheat	Al+++	2nd	10	10	10	16	12	58	-	2	2	2	2	8	66
3.	Wheat	Drought	1st	9	10	10	16	26	71	10	2	2	3	6	23	94
4.	Wheat	High temp	1st	18	19	22	27	12	98	20	4	4	4	3	35	133
5.	Rice	Na+	1st	13	15	16	17	27	88	10	2	3	5	7	27	115
	Rice	Na+	2nd	5	5	5	10	20	45	10	1	1	2	4	18	63
6.	Rice	Al+++	1st	18	14	16	18	13	79	-	2	2	2	2	8	87
7.	Millet	Na+	1st	18	19	21	27	39	124	10	4	4	6	6	30	154
8.	Millet	Drought	1st	9	10	10	16	26	71	10	2	2	4	5	23	94
G. TOTAL		4	11	159	155	171	189	188	862	154	34	35	36	37	296	1,158

Equipment Need for the Tissue Culture Project

	Items	Purpose	To purchase #Pieces	To purchase Total Cost ($,000)	Now Available at CSU # pieces
(a)	Electric Autoclave	Sterilize Medium and equipment	1	25	1
(b)	Laminar Flow Hood	Provide sterilized Area	3	8	2
(c)	Shaking Machines	Preparing and maintain culture	4	7	4
(d)	Fermenters	Provide Continuous Environments for growing cells	8	67	2(Small)
(e)	pH meter & Balance	Adjusting acidity of medium and weighing chemicals	1+1	5	1+1

If the project proposal is approved and funded, CSU will provide a separate laboratory for the studies. At that time from among the items listed in the column - "Available at CSU", only item (a) will not be moved to the new facility. This item (electric autoclave) is used by twenty(20) other teachers in the Department. Thus the studies will be severely handicapped without another electric autoclave. Items (b), (c) and (e) are occasionally used for teaching purposes by the department staff (labs/classes) and are removed as needed. Thus an independent supply of these items is needed. Item (d), the two small fermenters, are extremely inadequate for the project studies.

Additional fermenters(8) can possibly be substituted by additional shaking machines and additional technician time. Over a five year's period the substitution cost of equipment and time is estimated to be $14,000 per fermenter. Thus the substitution cost will be about $45,000 over the cost of the fermenters.

Thus items (a) to (e) listed under the column, "To purchase" are needed and are justifiable for efficient progress of the project.

TO: See Distribution

DATE: June 21, 1979

FROM: DAA/DS/FN, Tony Babb

SUBJECT: Official Minutes of the DSB Project Committee Review Meeting on, "Utilization of Tissue Culture to Increase Food Production", May 29, 1979.

The subject minutes are attached for your files.

Distribution:
PPC/RD, D. Caton
PPC/PB, A. Silver
ASIA/TR, M. Doyle
NE/TECH/AD, W. Fitzgerald
LAC/DR/RD, B. Allen
DS/PO/RES, M. Rechcigl
DS/PO/RES, G. Belt
DS/AGR, D. Peterson
F. Williams
K. Byergo
G. Corey
T. Gill
M. Mozynski

Minutes of the DSB Project Review Meeting on the "Utilization of Tissue Culture to Increase Food Production"

DAA/DS/FN, Mr. Tony Babb chaired the review which was held on May 29, 1979. The following participants attended the review:

DAA/DS/FN, Tony Babb, Chairman
PPC/RD, Doug Caton
PPC/PB, Art Silver
ASIA/TR, Mary Aloyse Doyle
NE/TECH/AD, Worth Fitzgerald
LAC/DR/RD, C. Blair Allen
DS/PO/RES, Mike Rechcigl
DS/PO/RES, G. H. Belt
DS/AGR, Dean Peterson
Floyd Williams
Keith Byergo
Gil Corey
Tejpal Gill
Mary Mozynski

The minutes of the TPCA sub-committee were distributed and Mr. Babb asked if there were any additional written comments. There were none. Dr. Corey opened the meeting by describing the project proposal. He stated that the proposal had been received in November from the Department of Botany and Plant Pathology, Colorado State University and had already gone through several stages of review. It had been reviewed internally by the scientists in DS/AGR, had been sent to 4 International Agricultural Research Centers, and had been discussed with the 4 Regional Bureaus. Dr. Gill stated that as a result of the original reviews and suggestions the proposal had been revised by Colorado State University and the scope scaled down.

On May 22, 1979, the sub-committee for Soils Management had reviewed the project statement based on the revised proposal and had made several recommendations which are included in the minutes.

The 4 International Agricultural Research Centers who reviewed the proposal are: IITA, CIAT, IRRI, and CIP. Mr. Babb questioned why the proposal had not been sent to CIMMYT for its reaction. Dr. Williams stated that CIMMYT does not have plant physiologists in the specific areas required for the review. Dr. Williams indicated that it would be some time before the products of the project will need to be tested by the International Centers and developing countries.

There followed a general discussion on the project design and the need to revise the project paper. Mr. Fitzgerald stated that the purpose of the project must be obtainable within the scope set forth in the paper and that the outputs must be something that can be achieved. Dr. Williams stated that

the purpose of the project should be: 1) to test the hypothesis that cultivars of wheat and rice that are tolerant to relatively high levels of sodium and aluminum can be developed by selecting tolerant cells from diploid somatic cell cultures and regenerating normal, but tolerant plants from them; and 2) to develop techniques that will allow the regeneration of normal maize, sorghum, millet and cowpea plants from single diplois somatic cells (or callus tissue).

Ms. Doyle stated that the project appeared to be basic research and that it should be funded by ISTC.

Dr. Williams stated that the wheat lines could be obtained from CIMMYT and could be tested for tolerance to stresses, etc. The method used in Tobacco could be applied to other crops.

Dr. Rechcigl stated that the contractor should be allowed the flexibility to work at its own pace. This process is a proven method for speeding up the process to test aluminum toxicity in certain grains.

Mr. Fitzgerald stated that he was for the project, but the project paper did not describe clearly what should be accomplished under the project.

In response to a question about the views of the crops scientists, Mr. Byergo stated that DS/AGR/FCP enthusiastically endorsed the project.

Dr. Caton stated that he questioned whether the problem was being addressed in the proper perspective or should DS/AGR be funding alternative methods of increasing crop production; i.e., utilization of alternative soils or using existing techniques. This project is a long term undertaking and costing $2.0 million; shouldn't the funds be used for a short-term undertaking which has been proven successful. In Brazil, North Carolina State University has been treating aluminum toxicity in Brazil for over 12 years and has been unable to move the results into Central Africa although they should be applicable there.

Dr. Gill stated that to solve the aluminum toxicity problem NCSU was using the technique of adding lime to the soil. Lime is expensive to apply and the roots go only up to the lime level. Lime is not always available to the farmers. Brazilian farmers need plants that are tolerant to aluminum toxicity and this project will give the plant breeders a new tool to obtain such material.

Dr. Gill stated that the tissue culture method speeds up the process for obtaining plant strains that are tolerant to aluminum toxicity and other soil stresses. The method has not been pushed in the U.S. because we are not using marginal lands but poor farmers in LDCs are plagued with poor soils. The USDA has proposed initial tissue culture studies on some crops within their own shop. Work is being done by the Universities of California and Oregon and in Canada Canada, but these are relatively small.

Dr. Caton stated that he would favor a small pilot project in wheat and rice. Dr. Williams stated that the budget should be looked at very closely as there were certain items, equipment, and level of personnel which may not be necessary. Mr. Babb indicated that he thought that the budget was high.

Mr. Allen expressed the view that the project appeared to be high risk and there were varying views on the probability of chance for success.

Mr. Babb questioned what the reaction was to reducing the number of crops to 2. Dr. Gill replied that while any level of activity can be proposed and perhaps be worked at, a critical mass is essential for any activity to be effective. The level of the scope of work as indicated in the project statement regarding the number of crops and the stress situations for study are based on the suggestions obtained from reviews within DS/AGR, Regional Bureaus and the International Centers. It is his considered opinion that the present scope of work is just about right. This project will benefit U.S. as well as the LDCs.

Dr. Gill suggested that the main objective of the project is not to produce, per se, stress tolerant strain (whether one or ten) of one crop or another, but rather fill an educational need (familiarization) of plant and soil scientists, on a world-wide basis, the potential and problems of a new tool that can select desirable germ plasm at the cellular level with a considerable efficiency. Crops included are more as test crops. Stress tolerant strains produced are more as by-products. To expose and convince people with a new, promising tool the project must include studies of varying levels of complications. This is why the project is designed the way it has been.

In summary, stress resistant strains of wheat and rice might be developed fairly soon because regeneration techniques are in hand. For maize, sorghums and millet regeneration is still to be accomplished, but should be straight forward. The work on legumes is less well developed. The discussion ranged around how many of these crops should be included and what the incremental cost would be to add each crop beyond wheat and rice.

It was agreed that the proposal has merit and should be funded. However, before the project is submitted to the RAC the following actions should be taken:

1. The contractor is to be contacted regarding the budget:

   - Need for all the equipment
   - Need for the 10 full time people
   - The relative cost of testing each crop; i.e., if soybeans and legumes are dropped, what would be the savings in the budget.

2. The project paper is to be rewritten to clearly define the purpose and outputs of the project.

3. Priorities will be identified for the contractor regarding the crops to be tested and the methods for plant regeneration from tissue culture.

Mr. Babb stated that after the revised project paper is prepared and reviewed by DS/AGR, the decision would be made on whether it would be necessary to hold another DSB project review meeting. The revised project paper will be distributed to the Regional Bureau representatives for comment.