THE PRODUCTION OF AGRICULTURALLY USEFUL MUTANT PLANTS WITH CHARACTERISTICS CONDUCIVE TO SALT TOLERANCE AND EFFICIENT WATER UTILIZATION

by

Murray W. Nabors

October 1979

Completion Report No. 96

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OWRT Grant No. 14-34-0001-6232

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Dr. Murray W. Nabors
Department of Botany and Plant Pathology
Colorado State University

submitted to

Office of Water Research and Technology
U. S. Department of the Interior
Washington, D. C. 20240

October, 1979

The work upon which this report is based was supported (in part) by funds provided by the United States Department of the Interior, Office of Water Research and Technology, as authorized by the Water Resources Research Act of 1964, and pursuant to Grant Agreement No. 14-34-0001-6232.

COLORADO WATER RESOURCES RESEARCH INSTITUTE
Colorado State University
Fort Collins, Colorado
Norman A. Evans, Director

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ABSTRACT

Because of rising energy costs and decreasing reserves, the goals of increasing food production on arid lands and of increasing the efficiency of water usage can best be achieved by producing plant varieties with high yields on available soil and water conditions. The inevitable salination of irrigated soils combined with the gargantuan costs involved in further increasing the extent of irrigation have caused at least one worker to question the validity of the whole irrigation process. Realistically any irrigation system has a very limited lifetime. This lifetime could be considerably extended by salt- and drought-tolerant varieties of crop plants. Such plants would require less water; water of lower ionic quality could also be used. In addition, the goal of irrigating with sea water or partially desalinated sea water is not unreasonable for costal areas if suitable varieties of plants can be produced. Sea water irrigation systems would offer additional benefits since unlimited quantities of water are available and thus increasing soil salinization would not occur.

The research discussed in this project report shows clearly that available tissue culture methodology can be used to rapidly select NaCl-tolerant cell lines and to regenerate whole plants from these lines. The regenerated plants are shown to be salt-tolerant in greenhouse experiments and to pass this tolerance onto two generations of offspring. Thus, salt tolerance, which can be economically selected in tissue culture, is both persistent and inheritable in regenerated plants. To date, salt-tolerant cell lines have been selected in wheat, oats, and tobacco. Salt-tolerant plants have been regenerated from these three species; greenhouse testing is nearly complete for tobacco and is underway for oats. In addition,

considerable advancements in tissue culture methodology were achieved for sugarbeets, tomatoes, and corn; mutation selection could begin now in tomatoes and within a year in corn and sugarbeets.

STATEMENT OF THE PROBLEM

As the world population approaches 4 billion people, the heretofore approachable goal of providing adequate nutrition is rapidly becoming a crash program to avoid wholesale starvation. In future years a dwindling energy supply and the onset of mass famine will probably be dominant political and sociological realities (1, 2). Thus it is not only appropriate but also crucial to critically evaluate all prospects for rapidly improving the quality and quantity of the world's food supply.

Quantity and quality of food production can be increased either by altering the environment to suit the plant or by changing the plant for the environment in which it grows. The thesis of this report is that technological advances--artificial fertilizers, mechanization, irrigation, herbicides, pesticides, and etc.--which in effect change the environment, have made substantial contributions to modern agriculture, but that we have reached the point at which the law of diminishing returns applies. Most varieties of crop plants for instance will not respond to further fertilization (3) or irrigation. In addition, mechanized, technological agriculture requires tremendous expenditures of fossil fuels which are themselves in increasingly short supply.

> 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 (2).

Energy concerns are also increasingly important in considering the cost of irrigation and other environmental modifications.

> The goals of the irrigator are twofold. He must supply enough water for plant consumption (evapotranspiration) and enough additional water for the leaching of salts past the root zone. Balanced against these supply requirements is the need to achieve a reasonably high irrigation efficiency in order to conserve usually scarce and expensive irrigation water, as well as the necessity of avoiding the creation of a high water table. This is a balancing act which is often, but by no means always, accompanied by a fairly wide error margin. In fact, it is fair to say that irrigation agriculture is usually a balancing of offsetting water control measures (4).

For these reasons, improvements in agricultural production may most economically and feasibly come from the development of large numbers of new varieties designed to grow well in specific, already existing environments. The Green Revolution of Norman Borlaug (5) is often thought to be a prime example of changes which can occur following the introduction of new varieties. Unfortunately, green revolution varieties require high energy crop production technology. Energy-efficient varieties for existing environments remains as a goal for the future.

Agricultural productivity is influenced by land and water availability. Of the two, water may be more limiting since a lack of sufficiently high quality water currently limits agricultural production on many land areas. An estimate of the importance of rapid methods for producing water-utilization efficient, drought-, and salt-tolerant plants can be obtained from the following figures (6). 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%, or 40% of all potentially arable land could sustain year-around agriculture were 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 (7). 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 (8). 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.

Salt water occupies over four-fifths of the world's surface, and most crop plants can tolerate only a small fraction of the amount of salt in sea water due to the large component of sodium. In addition a large fraction of the world's land already suffers from problems of salt balance or concentration. For instance 25% of the irrigated land in the western United States suffers from excess salts, particularly NaCl (9). As another example, in West Pakistan, 100,000 acres per year are going out of production because of salinization (4). In 1964 West Pakistan lost 1 ha. of prime agricultural

land to waterlogging and salinity every 17 minutes, while a child was born every 12 seconds (4).

Agricultural problems due to salts can be grouped in two categories: those caused by an excessive concentration of salt ions, regardless of type; and those caused by excesses or deficiencies of particular ionic species. In general, problems of generalized excess concentration are encountered under four conditions:

1. When a source of irrigation water is excessively contaminated; or when semi-brackish or brackish water must be utilized
2. When a land area is arid enough so that salts have not been leached from the soil
3. When periodic irrigation is used, evaporation, seepage and transpiration leads to a concentration of formerly dilute salt solutions into solutions of high ionic strength during the periods following irrigation water applications
4. When excessive irrigation combined with insufficient return flow raises the water table into the root zone bringing with it salts which were formerly below the level of agricultural concern in the soil profile.

The problems posed to growing plants by an excess concentration of ions result chiefly from the fact that ions compete with the plant for the available water. Thus, an irrigation source of relatively high ionic content may cause no problem for plants unless the period between waterings increases so that evaporation, seepage, and transpiration lead to a decrease in soil water and increase in salt concentration. Under these conditions the salt ions may compete osmotically with the roots for water; in addition the force (matrix potential) binding water to ion covered

soil particles may be so great that no water uptake by plant roots can occur. In other words, many apparent cases of salt damage are actually cases of water deficiency and osmotic competition.

This problem can be controlled using more frequent irrigation. However, availability of water as well as cost considerations frequently result in a rigid irrigation regime. The development of plants which could more effectively utilize water of high ionic strength between periods of irrigation would effectively enhance the value of currently unsuitable water and increase the amount of available water.

The problem of overall salt concentration is further complicated by the fact that both agricultural and non-agricultural cycling of water tend to add to the total salt content. For instance, irrigation systems add dissolved salts to the land and where large quantities of salts occur naturally in the soils, additional salts are added to the hydrologic system. If sufficient irrigation occurs, the added salts will be leached through the soil profile into the ground-water, and thus, in a sense, they will return to the source. Thus, overall salt concentration is often increased just because the amount of pure water is reduced and salt is picked up throughout the system. Cities and industries also use considerable quantities of water. In most cases the water is returned with the salt it originally contained plus additional salts. Rebhun (10), for instance, found that one cycle of urban (150,000 population) use produced additional salts resulting in increases in water salinity of 350 ppm. The Colorado River increases in salinity from less than 50 mg/l at the headwaters to over 865 mg/l at the Imperial Dam (11). This figure is projected to be over 1200 mg/l by the year 2000. The general increase in salinity caused

by both agricultural and urban water use has noticeably affected plant productivity in many semi-arid or arid, irrigated agricultural regions. The EPA estimates the current annual damage from excess salinity in the Colorado basins to be $16 million (11). This figure will increase to $51 million by 2010. This salinity increase may be effectively and economically counteracted by utilizing new varieties of plants designed to meet these specific problems.

Problems relating to the excess or deficiency of particular ionic species are commonplace throughout the world.

Excess NaCl is the most widespread chemical condition inhibiting plant growth (12). The presence of sodium ions is particularly inhibitory to plant growth for two reasons:

1. Sodium itself is a particularly toxic ion for most plants. The growth of the majority of plants is generally inhibited if the concentration of sodium chloride in the environment of the root rises above 3,700 ppm (13).
2. If the soil is calcium deficient, plants lose even this tenuous hold on NaCl tolerance because the membranes are not as viable.

For instance, bean plants normally tolerate 1,850 ppm NaCl with no ill effects. However, if calcium was supplied at less than 40 ppm with the same NaCl concentration then massive damage to the plants resulted from the uptake of sodium into the leaves (14). Ordinarily, the sodium concentration in irrigation water would not approach 3,700 ppm--unless semi-brackish or partially desalinated sea water (about 19,000 ppm NaCl) is utilized. However, irrigation water is often quite low in calcium (15). Furthermore, even slight excesses of sodium and other ions can

replace calcium in the soil. Thus, many cases of apparent NaCl damage to agricultural plants are actually the result of calcium deficiency which renders the plants much more sodium sensitive.

The problems of general sodium toxicity and enhanced toxicity due to low calcium can be effectively dealt with by new varieties of plants with either a genetically based sodium tolerance or an enhanced ability to take up available calcium.

It might be mentioned at this point that NaCl tolerant plants really offer two advantages. First, they would alleviate some of the problems inherent in extant irrigation systems. Second, they would enable the use of partially desalinated water in agriculture. Currently, desalination procedures become progressively more expensive as water quality standards are increased. The effective cost of agricultural water would be considerably lowered if partially purified sea water could be readily and effectively utilized.

The rationale for attempting to produce new varieties of plants to counter specific ionic problems is based on the fact that many types of plants have, over evolutionary time periods, become salt tolerant (halophytic). In other words, the genes of such plants have been modified to cope with an originally harsh environmental condition. Unfortunately, agricultural plants have not often been exposed to excess salt and so remain, for the most part, remarkably intolerant to ionic excess. Considering the expense of attempting to modify environments to suit plants, we think it logical to use modern tissue culture techniques to speed up the process of evolutionary adaptation and produce plants to suit specific, already existing environmental situations.

GENERAL OBJECTIVES OF THE PROPOSED RESEARCH

Our principal objective was to produce several new, mutant varieties of useful agricultural plants. These mutants were selected so that they could utilize water supplies which are currently agriculturally unsuitable due to undersirable ionic concentrations. Research in this area has been a heretofore somewhat neglected but potentially quite productive aspect of agricultural research.

> In contrast to the vast efforts invested in reclamation of saline soils, attempts at breeding for salt tolerance have so far been on an exceedingly modest scale (16).

A breakdown of our main objective into its several parts describes the need for the following types of mutant plants.

A. Varieties which are specifically resistant to NaCl.

Such plants could grow in NaCl contaminated irrigation water, partially desalinated sea water, or on unirrigated land on which NaCl concentrations have remained at inhibitory levels due to insufficient rainfall and leaching. NaCl (particularly the Na^+ ions) often act as rather specific inhibitors of plant growth at very low concentrations (often only a few hundred ppm). The presence of excess NaCl in otherwise agriculturally suitable soils and waters is a common enough problem to necessitate the generation of tolerant varieties.

B. Varieties which are highly efficient in taking up calcium from the soil. Calcium is a vital element for the proper functioning of plants and maintenance of cell membranes; calcium deficiencies cause plants to be noticeably more susceptible to NaCl poisoning (15). The problem is compounded by the fact that many sources

of irrigation water contain insufficient calcium and that sodium, when present in high enough quantities, tends to displace calcium from the soil (14), resulting in Ca^{++} deficient, NaCl damaged plants, and Ca^{++} deficient animals. Production on most irrigated land could be markedly improved and much non-arable land could be brought into production if new varieties of plants, highly efficient at calcium uptake, were available. Using these plants, low calcium conditions could be better tolerated.

C. Varieties which can take up water in the face of high ionic strength in the soil (osmotic effect). Between periods of irrigation the ionic strength of soils frequently becomes very high as a result of pure water consumption due to evaporation and transpiration, with an accompanying decrease in total water supply due to drainage. Under such conditions plant growth is frequently inhibited because the roots can no longer pull water away from the salt coated soil particles. The solution to this problem is the use of new varieties of plants with a heightened ability to take up water.

D. Varieties which can utilize irrigation water of inhibitory ionic strength and composition. Water from major drainage systems is frequently inhibitory to plant growth due both to ionic strength and to a particular combination of ionic species. For these major rivers new plant varieties, specifically adapted to their ionic pattern, are needed. In addition, varieties are needed which anticipate future increases in ionic strength or changes in ionic composition.

ACHIEVEMENT OF OBJECTIVES

The research of the past 3 years has succeeded in producing numerous cell lines of tobacco, oats, wheat, sugarbeets, corn, and tomato. Experiments to obtain varieties which can utilize water supplies containing high salt levels were concentrated on tobacco, oats, and wheat. For these plants numerous lines of NaCl-, high osmotic-strength-resistant cells were selected, and plants were regenerated from these cultures. For oats and tobacco, plants were removed from tissue culture vials, placed in pots, and grown to maturity in the greenhouse. At maturity seeds were obtained for future greenhouse testing experiments. In the case of tobacco, considerable greenhouse testing of regenerated plants and their progeny was completed. The salt tolerance selected in tissue culture was found to be passed on to the next two generations (see Appendix I). In addition, tolerance was retained when tissue from F_1 plants (first generation progeny of regenerated plants) was again placed in culture.

Objectives of the original proposal were reduced somewhat in scope since the proposal was funded for $65,000 instead of the $100,000 originally anticipated. Objective B was not included in the actual research, although we intend to test the calcium uptake ability of the salt-tolerant mutants produced.

In addition to the proposed and achieved Objectives, considerable state-funded effort was put into understanding the physiology of salt-tolerant cultured cells and regenerated plants. This work was possible because of the interest of a Ph.D. candidate, James Heyser, in the experiments. His data for example (see Appendix II) show that NaCl-tolerant cells take NaCl (into the vacuole) to balance the osmotic effects of external salt. In contrast, cell lines resistant to

nonpentrating, high molecular weight osmotica offset osmotic effects by taking up potassium and producing a still to be identified, additional substance. These experiments contributed substantially to the OWRT-funded project in that they increased our understanding of the physiological and molecular processes of mutant cells and plants with characteristics conducive to efficient water utilization.

RESEARCH PROCEDURE

A. Plant species utilized. Maximum effort was placed on plants for which tissue culture procedures had already been worked out since this lessened the difficulty in obtaining successful results. A second criterion was that the plant should be widely cultivated throughout the world. Thirdly, we concentrated on major food crops.

Since relatively small amounts of space are required for our procedures, we began selection with several different plants, moving on to additional species as we obtained successful results. We placed considerable effort on wheat because of its nutritional importance in the world food supply and because wheat tissue culture is a well established procedure (17). The second variety chosen for experimentation was oats. Additional plants investigated were tomatoes, sugarbeets, and corn. Tobacco has long been a model plant system for tissue culture experiments. Previously-initiated work on this plant was continued throughout the contract period because of the obvious importance of the results obtained. Corn and soybeans were utilized because no successful plant regeneration technique is known. A minor goal of our research was to provide such regeneration techniques.

B. The first goal was to obtain cell cultures for all plant species to be utilized. On obtaining cultures, we tested plant regeneration procedures to insure a high yield of plants from cultured cells. Unsuitable procedures were the subject of further experimentation.

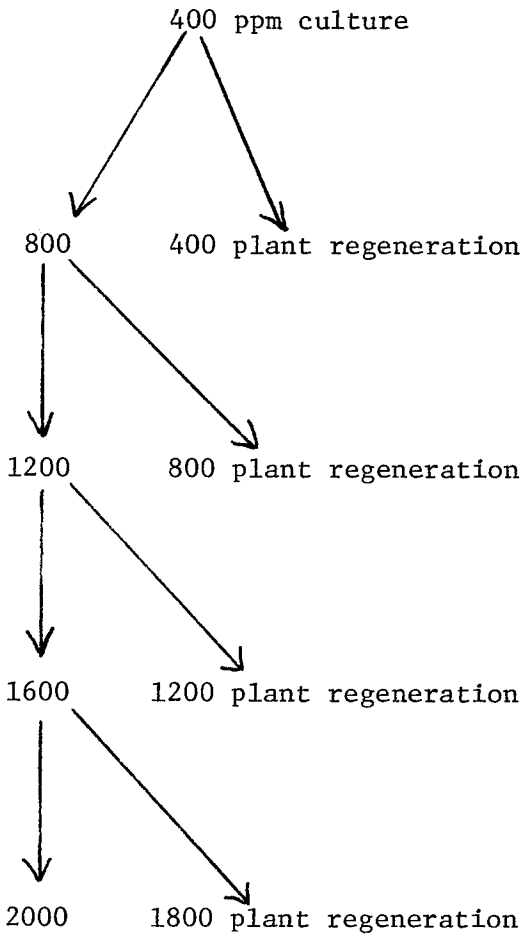
C. Following establishment of cell cultures we instituted selection procedures for naturally occurring NaCl-tolerant mutant cells.

1. NaCl tolerant mutants were selected by adding increasing amounts of NaCl to the medium. Our selection occurred in steps of 400 or 1000 ppm. That is, we added 400 or 1000 ppm NaCl to a rapidly growing culture. When the culture reached maturity, it was subdivided. Some subdivisions were placed in 800 or 2000 ppm NaCl. Others were placed on a different medium for plant regeneration. This process continued until we could no longer achieve successful adaptation to high levels of NaCl. As a result of this technique, we obtained a graded series of plants capable of growing in various levels of NaCl (see Figure 1).

2. Cell varieties resistant to high ionic strength were selected by increasing, in stepwise fashion, the ionic concentration of the medium. Ionic composition can be varied to correspond to the present, or expected future condition of various rivers.

D. Once a desired resistant cell line was established, plant regeneration began. Plant formation was and will be carried out on a limited scale until greenhouse tests have determined the extent of resistance in regenerated plants. Greenhouse tests were and are conducted in soil; the watering solution contains the high NaCl or ionic strength utilized in selecting cells.

Figure 1. Flow sheet for NaCl tolerant mutants (see text)



etc.

E. The goals of this proposal were to produce resistant plants and to carry them through to initial greenhouse testing. We feel that field testing procedures are already well worked out and readily available for trials of any new varieties.

PROJECT RESULTS

A. Production of cell cultures

Numerous cell cultures on solid medium (callus cultures) were produced using wheat, oats, tobacco, sugarbeet, and tomato. Cultures in liquid medium (suspension cultures) were produced using wheat, tobacco, sugarbeets, and tomatoes. In all cases cell cultures from monocots (oats and wheat) were obtained by placing sterilized seeds on a solid (agarized) nutrient medium containing Linsmaier and Skoog's salts (18), sucrose (2% for wheat; 4% for oats), and 2,4-D* (2 ppm for wheat; 5 ppm for oats). Seeds were surface sterilized by a brief exposure to 100% ethanol followed by a 30 minute soak in 20% Clorox plus Tween 80. Seed germination on the solid medium was followed by cell proliferation of root cells into a callus mass. The rate of callus formation from root cells is regulated by the 2,4-D concentration. Cell cultures from dicots (tobacco, sugarbeets, and tomatoes) were produced by culturing either stem sections or sections of the cotyledons on solid medium containing Linsmaier and Skoog's salts, 4% sucrose, 5 ppm IAA†, and varying levels of kinetin (0.1 to 10 ppm).

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*2,4 dichlorophenoxyacetic acid, an auxin.

†Indoleacetic acid, an auxin.

In dicots the rate of callus formation is controlled by the ratio of IAA to kinetin.

Callus produced directly from a differentiated plant part is termed "primary callus." It can be used to produce more ("secondary") callus, or can be induced to differentiate into roots and shoots. The selection of mutant cells requires a large initial population. Thus our general procedure was to produce large amounts of secondary callus before attempting mutation selection. Usually secondary callus is obtained by subdividing the primary callus and placing the subdivisions on new medium. For some plants (oats and wheat) it was found that subcultured callus masses must have a certain critical size for growth to continue: Masses less than 1 cc generally ceased growth on subculturing for oats and wheat but not for tobacco, tomato, and sugarbeets.

In the case of monocot cell cultures the presence of 2,4-D is usually sufficient to prevent differentiation and allow continued callus production. For dicots the IAA to kinetin ratio is varied to obtain roots, shoots, or more callus (Figs. 2 and 3). Callus mass was determined by visually comparing calli to a series of clay spheres. The smallest sphere was arbitrarily given a mass of one; thus the relative masses of the other balls could be obtained as well as the relative mass of similar sized calli. This callus measuring system permits accurate mass determinations to be made without sacrificing the callus by removing it from the vial for weighing. The measuring system

Figure 3. The Production of Callus, Shoots (Buds), and Roots from Tobacco Callus Cultures

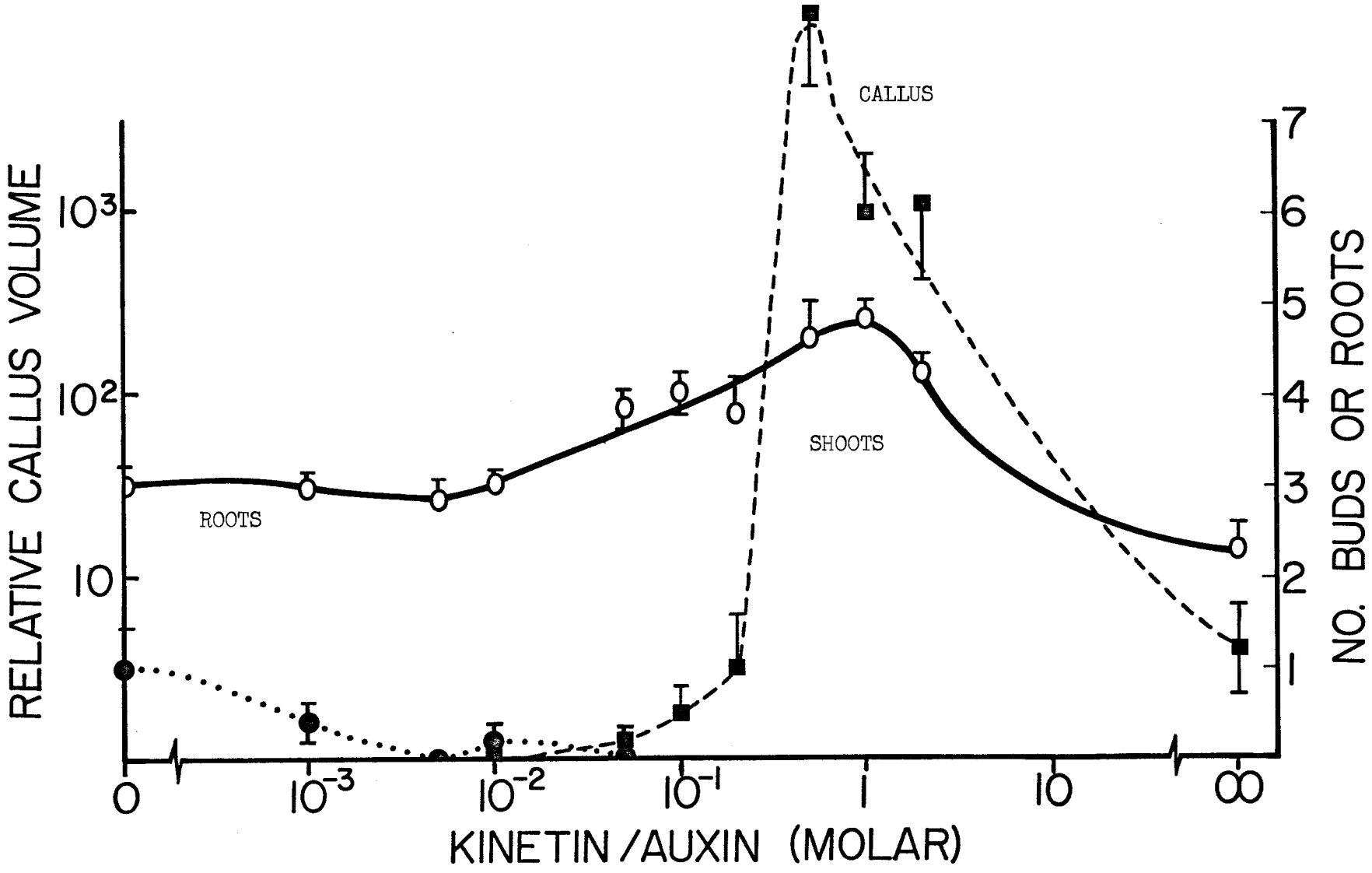
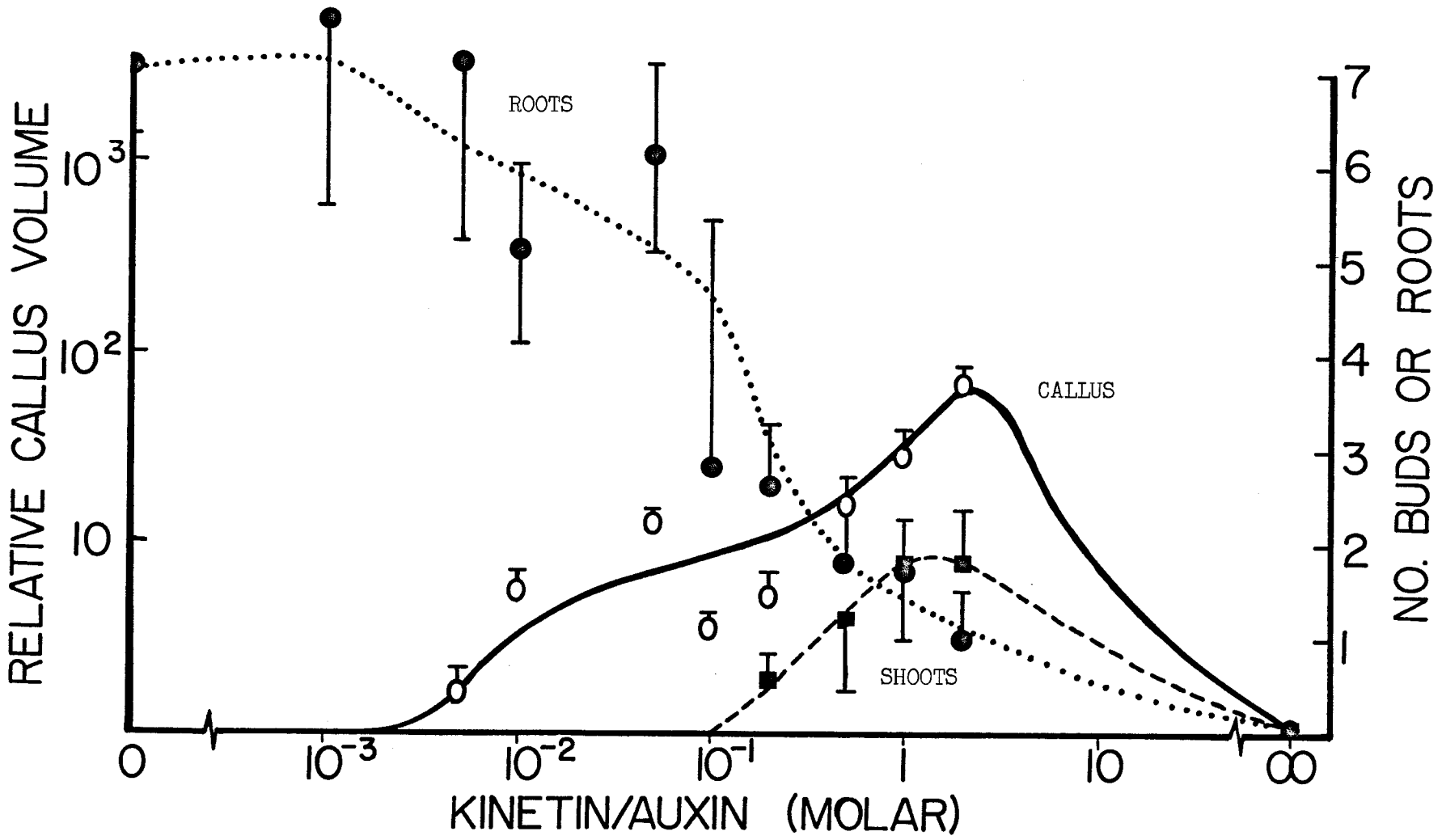


Figure 2. The Production of Callus, Shoots (Buds), and Roots from Tomato Callus Cultures



and the graphic presentation (Figs. 2 and 3) are innovative features of this work not heretofore seen in tissue culture studies.

Considerable experimentation on the production of wheat callus and sugarbeet callus was carried out by Claire Brown and Michael Hooker (Masters degree candidates). Their experiments were carried out to fill gaps in the existing literature and to obtain basic cultural data for the specific varieties we utilized. Copies of their papers may be found in the Appendix of this report.

B. Production of Cell Suspensions

Suspensions were produced by placing calli in liquid media in baffle-bottom flasks. These were placed on a gyrotory shaking machine at 80 opm until the calli broke apart sufficiently into single cells and small clumps. Then the suspensions were transferred to Nephelo flasks (which have a side arm) for routine culture. Growth was measured by filling the side arm with suspension; allowing the suspension to settle; and measuring the column height in the side arm. This height was correlated with cell density in the growing suspension.

C. Selection of NaCl-tolerant Mutants

Large callus masses produced as discussed above, contained around 500,000 cells; 100 ml suspensions contained around 10×10^6 cells. Based on known spontaneous mutation rates (19), cell population of this general size should contain at least one spontaneous mutant for increased NaCl tolerance. In both callus and in suspension cultures these mutant cells were selected by adding enough NaCl to the medium so that normal, salt-sensitive cells were slowed in growth and division rates, allowing salt-tolerant cells to increase in percentage

in the population. After the addition of salt, the growth rate of a culture drops markedly and gradually returns to a normal level. At this point cells can be removed to plant regeneration medium or the selection process can be repeated (Fig. 1). Selection for NaCl-tolerant mutant cell lines using suspension cultures had been achieved previously in this lab (20), and this technique was utilized routinely. Selection on solid medium is theoretically less rigorous since not all cells are in contact with the medium. Experiments with tobacco, wheat, and corn have demonstrated that this method of selection is successful (for example Table 1). Since cultured plant cells gradually lose totipotency with time, a mutant selection method which minimizes cultural steps is highly desirable. Cell suspensions are produced from callus cultures, so a proven method of selection at the callus stage itself is a useful addition to tissue culture technology.

In wheat and oat seeds callus production and mutant selection were placed in individual culture vials. The NaCl concentration of the media was varied from zero to 9000 ppm in steps of 1000 ppm. Germination percentage and rate is much reduced at high salt concentrations. Since callus forms from root cells, callus formation at higher salt concentrations is also markedly reduced (see Fig. 9 in Claire Brown's manuscript in Appendices). At the highest salt concentrations very few seeds produce callus. However in a few vials, those in which a NaCl-tolerant mutant cell become involved in callus formation, large callus masses appeared. These were subcultured and then placed on plant regeneration media.

TABLE 1. Selection for NaCl-tolerant mutants in calli grown on solid medium. Culture #1 derived from the 0 concentration of culture #3. Culture #2 derived from the 0 concentration of culture #4.

NaCl concentration in solid medium in PPM*	Relative callus volume after four weeks culture			
	Culture #1 first month on salt	Culture #2 first month on salt	Culture #3 third month on salt	Culture #4 seventh month on salt
0	1253	1344	1253	1344
6400	377	255	1019	541
11800	314	218	762	444
17200	224	148**	517	287
22600	189	148**	148**	393

*Sea water is 21000 PPM NaCl + 14000 PPM other salts (average figures).

**No growth.

D. Regeneration of Plants from NaCl-Tolerant and NaCl-Sensitive Cultures.

For monocots, regeneration from tissue cultures generally involves placing the cultured cells on a nutrient medium containing no hormones. Published studies on the regeneration of wheat and oats (17, 21) indicate that this general method is applicable to these plants. We found that for both wheat and oats reduction or removal of auxin (2,4-D) from the medium induced the appearance of shoots in subcultured callus. In oats the subcultured (secondary) callus masses continued to produce shoots through the third or fourth transfer. In wheat regeneration seemed to be limited to the first transfer after initial callus formation. Experiments designed to improve regeneration in secondary callus are continuing.

For dicots regeneration from tissue cultures involves placing callus tissue on medium containing a high kinetin/auxin ratio. Figures 2 and 3 indicate the type of experiment performed to determine the optimal ratio for regeneration. Tobacco and tomato regeneration systems are working well, whereas those for sugarbeet, soybeans, and corn will require further experimentation. More specific comments on each plant type will be found below.

In general, "regeneration" as discussed above means shoot regeneration. Callus cultures have the potential to produce either roots or shoots, and the expression of one or both of these potentials depends on the hormone concentration of the medium. Sometimes regenerated shoots will spontaneously form roots; in other cases the shoots must be transferred to a different medium to induce root formation. The problem of obtaining roots on shoots

has been significant for all plant species we have studied. In general, high concentrations of auxins (10-30 ppm of IAA or NAA) and a reduction in available mineral nutrients have been found to be the most effective methods for inducing the appearance of roots on cuttings and on regenerated shoots. We have found that maintenance of regenerated shoots on medium which keeps them green and healthy eventually results in the production of shoots, whereas supposed "rooting media" which ignore shoot nutritional requirements are less effective.

Regenerated shoots which have produced roots were removed from tissue culture vials and placed in soil in clay or plastic pots. This transition involves moving the plantlet from a 100% sterile, 100% humid environment to one which is nonsterile and has variable humidity. Initially 50-100% of the plantlets died during this transition. Control of humidity and a gradual reduction from 100% to lower levels resulted in a considerable increase in numbers of live plantlets following transplanting. During the transfer process an attempt is made to remove as much of the agar medium as possible from the plant roots. The medium contains sugar and other nutrients; its removal considerably reduces the chance for invasion of the plantlet by bacteria or fungi. We are currently investigating the use of agarase (an enzyme which degrades agar) and several other techniques to improve agar removal from and thus survival rate of regenerated plantlets. At present the survival rate of transplanted tobacco and tomato plantlets is about 80%; the survival rate of transplanted oat and wheat plants is about 50%. Each of these rates has increased considerably during the past year.

E. Greenhouse Experiments with Regenerated Plants

Both salt-tolerant and salt-sensitive cell cultures were used for plant regeneration studies. Greenhouse experiments must determine (1) if the salt tolerance selected in culture has carried through into the regenerated plant and (2) if the tolerance is inherited by future generations of plants in a predictable manner. These experiments are crucial because (1) the salt-tolerant strains could represent selection of a mutation or selection of a physiological variant (see Appendix D) (2). A mutation could be selected in tissue culture but the altered gene might not be expressed in the whole plant.

Since relatively small numbers of regenerated plants were utilized in the first greenhouse studies these plants were selfed and seeds were collected so that studies with larger populations could be carried out. A small percentage of regenerated plants proved to be sterile. These were automatically eliminated from further experiments. No cases of sterility were encountered beyond the parental generation. Seeds from regenerated, parental plants were planted to obtain the F_1 generation. Three types of parents were utilized: salt-sensitive; salt-tolerant; and salt-tolerant in which regeneration occurred in the absence of NaCl. The third type was used simply because many more plants could be obtained when regeneration occurred in the absence of NaCl.

The F_1 plants were placed in groups of 43, and each group was watered with a different concentration of salt water (see Appendix D). The plants whose parents had been regenerated from salt-tolerant cell cultures proved to be more tolerant than the plants

from salt-sensitive parents. The F_1 plants were selfed and the same experiment was repeated with F_2 plants in groups of 20. Again, at high salt concentrations plants originating in salt-tolerant cultures tolerated more salt than those originating in salt-sensitive cultures. Thus the salt tolerance obtained in cultures does appear in regenerated plants and is passed on to future generations. The tolerance behaves as a dominant trait with a complex Mendelian pattern of inheritance. Interestingly the tolerance of cells to salt was considerably less than the tolerance of whole plants (see Table I of Appendix D). Cells in suspensions are surrounded by NaCl-containing medium, whereas most cells in a whole plant are quite distant from NaCl in the soil solution. However Jim Heyser's data show clearly that whole plants take up NaCl from the soil to the extent that NaCl concentrations of leaf and soil solution are nearly equal. Thus the increased tolerance of whole plants is at present unexplained. The most work on greenhouse studies has been carried out with tobacco. Sufficient oat plants have now been regenerated, transplanted, and gone to seed so that $F_1 \times F_1$ greenhouse studies for oats are now underway.

In a related experiment, callus was obtained from the three types of F_1 plants. When this callus was subject to various levels of NaCl it was found that the salt tolerance and salt sensitivity of the F_1 whole plants was retained on return to tissue culture. Thus, salt-tolerant cell cultures can give rise to salt-tolerant whole plants which pass this tolerance on to the next generation which in turn retains it both in whole plants and in cell cultures derived from whole plants.

F. Wheat

Common wheat, Triticum aestivum L. var. Chris was used in all experiments. Lots from some varieties were found to be internally comtaminated by fungus and thus could not be effectively surface sterilized for culture. Claire Brown's manuscript (Appendix C, in preparation for publication) contains details on experiments designed to maximize callus production from wheat roots. She found a 2,4-D concentration of 2 ppm and a sugar concentration of 2,000 ppm to be optimal. Her Fig. 3 seems to indicate that, by extrapolation, maximal callus production would occur at 0 ppm 2,4-D; this is not, however, the case since no callus formation (normal germination) occurs in the absence of 2,4-D. Also she found that wheat callus does not subculture successfully unless a minimal-sized piece (at least 1 cc) is used. The same size requirement seems also to apply to shoot regeneration. In the past year, obtaining shoots from callus has not been a problem owing to considerable earlier experimentation.

Numerous salt-tolerant calli of wheat have been selected at various levels of NaCl. Improvements in plant regeneration efficiency, rooting methods, and transplantation techniques now make it possible for wheat to move rapidly into the greenhouse testing phase.

G. Oats

Avena sativa var. Park was used in all experiments. The callus culture methods for oat are similar to those for wheat except that 5 ppm 2,4-D and 4,000 ppm sucrose give maximal callus formation. As in wheat, mutation selection occurred in callus cultures. This

considerably streamlines the selection process by eliminating the suspension culture stage. Shoot regeneration occurs more readily and is retained longer by secondary callus than in wheat. We have now obtained regenerated plants in the greenhouse from a number of salt-tolerant and salt-sensitive cultures. Seeds have been collected and testing of the F_1 plants at various salt concentrations is now underway.

H. Tobacco

Nicotiana tabacum var. Samsun was used in all experiments. For historical reasons tobacco has long served as a model system for tissue culture studies. Our objective has been to use tobacco in this manner and to rapidly transfer tobacco-derived technology to more useful food crop plants. Numerous lines of NaCl-tolerant tobacco have been selected in this laboratory (see for example 20). In each case the upper limit of tolerance which can be selected in cell suspensions appears to be around 8,200 ppm NaCl. Beyond this limit the cultures inevitably die. We theorize that beyond this concentration more than one mutation is required for tolerance in a cell. Since typical mutations appear at frequencies of between 1×10^{-5} (for specific phenotypic changes [19]) to 1×10^{-9} (for changes in specific DNA bases [19]) the chance of two such mutations occuring in the same cell would be between 1×10^{-10} and 1×10^{-18}. Our cell populations have around 1×10^{7} cells and thus are at least three orders of magnitude to small for obtaining such a mutant.

In callus cultures higher levels of NaCl tolerance have been obtained (Table 1). To date tolerance to 22,600 ppm NaCl is the

maximum achieved. The higher tolerance may result from the fact that callus is in direct contact with salt only on its bottom surface whereas all cells in a suspension are in direct contact. Thus the higher tolerance of calli may be apparent rather than actual. Further research is required.

Our tobacco cultures rapidly become auxin autotrophs and do not require an exogenous supply in the nutrient medium. Thus our regeneration medium contains only 0.5 ppm kinetin as a hormonal additive. Rooting of regenerated shoots is accomplished by transfer to a medium containing between 5 and 20 ppm IAA or NAA.* The presence of NaCl markedly reduces regeneration and rooting efficiency. The ongoing greenhouse testing of the progeny of salt-tolerant and salt-sensitive regenerated plants is summarized in a paper which is found in Appendix D. Jim Heyser's Ph.D. research on the physiology of NaCl-tolerant cell lines is summarized in Appendix E.

I. Tomato

Lycopersicon esculentum vars. Marglobe and Rutgers were used in all experiments. This project concentrated on experiments using wheat, oats, sugarbeet, and tobacco. Tomato was utilized mainly in studies on regeneration (Fig. 2). Many workers have been able to produce callus from tomato and to regenerate plants from primary callus. Regeneration from secondary callus has proven to be a difficult problem. We have been able to develop a method for shoot regeneration from secondary callus after one and after several transfers.

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*Naphthalene-acetic acid, an auxin.

The details of this method are now in the form of a manuscript in preparation. Whereas tobacco callus appears green and "healthy" even after years in culture, tomato callus is usually brown and frequently appears "dead" when in fact it is not. We have found that the basic regeneration strategy for dicots of increasing the kinetin to auxin ratio produces regeneration in up to 50% of cultures. Thus tomato is now ready for mutation selection procedures leading to greenhouse experiments.

J. Sugarbeets

Beta vulgaris L. var. Mono-Hy A-1 was used in all experiments. Michael Hooker carried out extensive experiments using sugarbeet in work for his Masters thesis. He found that published methods for low frequency regeneration in this plant by French and by Russian workers were not repeatable possibly due to varietal differences. His publication (Appendix B) details methods for obtaining rapidly growing callus for this plant. In the past callus production was viewed as a major problem. He also made considerable progress toward a suitable regeneration method. This species is of considerable importance in western agriculture in the western United States. We estimate that following a year or two of continuing experimentation methodology should be complete enough so that mutation selection and greenhouse studies can begin.

K. Corn

Zea mays var. Seneca 60 was utilized in all experiments. To date the only published regeneration method for corn utilizes callus obtained from the scutellum of embryos isolated 18 days post

pollination (22). This method is complicated by the time and tissue specificity required. Our objective was to obtain a simpler regeneration method using root callus. Corn roots are quite resistant to callus formation induced by 2,4-D. Whereas oat and wheat roots yield maximal callus at 2 and 5 ppm respectively, corn roots produced maximal yields at 40 ppm. The callus resulting from such treatments is a mixture of true callus undifferentiated cells and shortened thickened roots. This mixture of callus and root production continued growth and differentiated even more roots. After many unsuccessful experiments we began to add anti-auxins (tri-iodobenzoic acid [TIBA]; beta NAA; and idole propionic acid [IPA] to the medium in hopes of reducing the high internal auxin levels required for callus growth to allow shoot formation according to the monocot model in which shoots form when all hormones are removed from the medium. In cultures with 5-20 ppm added TIBA shoot regeneration occurred in 10-30% of cultures. The shoots always formed on the callus base in an upside down position. After some experimentation they could be induced to form roots and behave normally on transfer to pots. Further experimentation on this system has proceeded sporadically since it is not a high priority of the project. We feel, however, that with a year's further progress corn methodology should be complete enough to allow mutation selection to begin.

L. Soybean

Glycine max var. Amsoy was utilized in all experiments. Numerous

laboratories world-wide have worked on tissue culture and regeneration systems for this agronomically important plant. To date, however, regeneration from callus or from suspension cultures has not been achieved. In this laboratory we have tested around 300-400 media for regenerative potential. On several occasions we have appeared to be on the verge of success in the sense that calli have been produced with intensely green areas which often--in other plants--precede bud formation. We feel that the strategy of increasing the kinetin to auxin ratio by including anti-auxins in the medium offers promise for future development of a successful, high frequency regeneration system.

PROJECT APPLICATIONS

The potential applications of the research results are immense and can be viewed on several different levels. Because of rising energy costs and decreasing supplies, the goals of increasing food production on arid lands and of increasing the efficiency of water usage can best be achieved by producing plant varieties with high yields on available soil and water conditions. The inevitable salination of irrigated soils combined with the gargantuan costs involved in further increasing the extent of irrigation (4) have caused at least one worker to question the validity of the whole irrigation process. Realistically any irrigation system has a very limited lifetime. This lifetime could be considerably extended by salt- and drought-tolerant varieties of crop plants. In addition, the goal of irrigating with sea water or partially desalinated sea water is not unreasonable for coastal areas if suitable varieties of plants can be produced. Sea water irrigation

systems would offer additional benefits since unlimited quantities of water are available and thus increasing soil salinization would not be a problem.

With respect to obtaining slat-resistant crop plants with highly efficient water utilization this project has demonstrated some important points:

1. Highly salt-tolerant cell cultures can be rapidly obtained for crop plants by using available tissue culture technology.
2. Plants regenerated from these cell cultures are also salt-tolerant.
3. Salt-tolerant plants regenerated from cell cultures pass this tolerance onto future generations.
4. The level of salt tolerance obtained in cell cultures is considerably increased in whole plants obtained from these cultures.
5. Tissue culture methodology can be rapidly developed in plants for which it is not currently available.

It is also worth noting that tissue culture technology can be utilized in another manner to solve water-related agricultural problems. The availability of plant regeneration procedures for many types of food crop plants means that salt- or drought-resistant individual plants noticed in the field can be rapidly propagated in tissue culture or cloned and released to the market as a new variety in a short period of time. At present, valuable, individual mutant plants must be propagated by traditional procedures which may require several plant generations and considerable time delaying the introduction of a new variety to the market. The plant regeneration methods of tissue culture mean that an individual grower or farmer, or a water specialist anywhere in the world could identify a rare, useful mutant plant, then have it rapidly cloned into millions of individual plants for testing in many different agricultural regions. The power of tissue culture breeding in this respect is illustrated by the fact that one small flask of cell

suspension (100 ml of suspension) contains 10^7 cells, each one a potential plant if tissue culture techniques are correctly applied.

A state-of-the-art publication was produced by this laboratory in 1976 (19). This project report demonstrates the considerable progress which has occurred in the two and a half years since that article appeared. Considering the current critical world energy situation and the absolute dependence of the United States on undependable supplies of foreign energy resources, we feel that tissue culture is very important because of its ability to rapidly and economically develop salt-tolerant, water-utilization-efficient, and therefore energy-efficient varieties of food crop plants.

PROJECT CONTINUATION AND LONG TERM OBJECTIVES

The long term objectives for the research discussed in this project report are:

(1) To establish tissue culture as a standard method in the repertoire of the plant breeder.

(2) To use tissue culture techniques to produce several varieties of food crop plants with increased salt tolerance and water utilization efficiency.

(3) To promote the development of an energy-extensive as opposed to an energy-intensive agriculture in the United States.

(4) To use tissue culture technology to develop several varieties of plants useful in arid regions requiring irrigated agriculture.

In terms of the specific projects mentioned in this report we hope to:

(1) Complete greenhouse testing of NaCl-tolerant oats so that field testing can begin.

(2) Begin greenhouse testing of NaCl-tolerant wheat.

(3) Begin mutation selection studies in tomato cell cultures.

(4) Begin mutation selection studies in corn.

(5) Obtain a suitable, high frequency regeneration method for soybeans and for sugarbeets.

(6) Complete greenhouse tests to determine inheritance pattern for salt-tolerant tobacco plants.

(7) Complete physiological and biochemical experiments to determine the cellular basis of NaCl-tolerance in tobacco cell lines.

PROJECT PERSONNEL

1. Dr. Murray W. Nabors--principal investigator
2. Susan Gibbs--technician
3. James Heyser--graduate student
4. Claire Brown--graduate student
5. Kathy Blanton--undergraduate student
6. Nancy Petretic--technician
7. Mary E. Meis--technician
8. Michael Hooker--graduate student
9. Dr. Anthony Frey--co-investigator
10. Carol Bernstein--technician
11. Touba Ghazi--graduate student
12. Randy Basinger--undergraduate student
13. Cathy Shantz--technician
14. Chloe Weil--technician

PROJECT RELATED PUBLICATIONS

1. M. W. Nabors, A. Daniels, L. Nadolny, and C. Brown. Sodium Chloride Tolerant Lines of Tobacco Cells. Plant Science Letters 4: 115-119 (1975).

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

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

4. 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 U. Agricultural Experiment Station, 1977.

5. B. Nyberg. Searching for Mother Nature's Mistakes. Denver Post Empire Magazine, April 10, 1977.

6. AP/UPI Wire Release, September, 1978.

7. M. W. Nabors, S. E. Gibbs, C. S. Bernstein, and M. E. Meis. NaCl-Tolerant Tobacco Plants from Cultured Cells. In press, Zeits. fur Pflanzen.

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APPENDICES

A. M. W. Nabors. Using Spontaneously Occurring and Induced Mutations to Obtain Agriculturally Useful Plants. BioScience 26: 761-768 (1976).

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

C. C. E. Brown and M. W. Nabors. Culture and Sodium Chloride Tolerance Studies on Wheat (Triticum aestivum L.) in vitro. Manuscript to be submitted for publication.

D. M. W. Nabors, S. E. Gibbs, C. S. Bernstein, and M. E. Meis. NaCl-Tolerant Tobacco Plants from Cultured Cells. In press, Zeits. fur Pflanzen.

E. J. W. Heyser. The Growth and Osmotic Adjustment of Sodium Chloride-Adapted and Non-Adapted Tobacco Cell Cultures and Plants. Dissertation, Colorado State University, 1979. Abstract is included here.

Reprinted from *BioScience*

Appendix A.

Using Spontaneously Occurring and Induced Mutations to Obtain Agriculturally Useful Plants

Murray W. Nabors

As the world population increases in the face of rising energy costs, it is appropriate to evaluate prospects for rapidly and economically improving the quality and quantity of the world's food supply. For developing nations in particular, fiscal and temporal constraints are of crucial importance in attempts to increase agricultural production. Modern agriculture has increased the quantity and ameliorated the quality of agricultural production by using fertilizers, irrigation water, herbicides, pesticides, machines, and improved varieties of plants.

All practices but the last fall under the category of altering the environment to suit the plant. Environmental alteration requires, directly or indirectly, large amounts of fossil fuel (Pimentel et al. 1973). Altering the plant to suit the environment by producing new plant varieties is becoming increasingly important as a means of economically augmenting production on arable land and of bringing new areas into cultivation.

The purpose of this paper is to discuss the selection and potential use of spontaneous or induced mutations by different methods. In producing new varieties, the goal of plant breeders is to incorporate new useful genes into the genotypes of cultivated varieties. To achieve this end, traditional breeding programs often involve hybridizing existing domestic or wild varieties. Such methods can often add several alleles to the best existing genotype.

A second procedure attempts to select individuals in which the desired trait has arisen by spontaneous or induced mutation. This method avoids the several breeding seasons required to obtain a stable cross-varietal product. Its chief usefulness is in adding one allele at a time to an existing genotype.

The author is with the Department of Botany, Colorado State University, Ft. Collins, CO 80523.

In general, spontaneous or induced mutants are easily used to improve self-fertilizing species (e.g., wheat, oats, barley, rice, soybeans, peas, beans, potatoes, and some forage grasses). A desired mutant phenotype is located in a wild or domestic variety. The mutation is established in homozygous form and the resulting plants propagated extensively to yield an altered variety. Frequently, the desirable trait is transferred to other varieties by available hybridization techniques.

In the case of cross-pollinated and, therefore, heterozygous crops (e.g., corn, rye, alfalfa, many clovers, and many forage grasses), the use of spontaneous or induced mutations is more complicated. Corn seed production and breeding involves crossing highly inbred lines so spontaneous or induced mutants can be incorporated into homozygous parents. For the allele to appear phenotypically in the F_1 heterozygote, it must be dominant, quantitative, or incorporated into two separate homozygous parents. In many cross-pollinated crops, such as alfalfa, homozygosity is difficult to obtain due to high self-sterility. When selfing does occur, severe inbreeding depression is common. For such plants, spontaneous or induced mutant alleles are useful if incorporated into a number of cross-pollinating genotypes, or if a single mutant plant is crossed to other selected plants in a specialized breeding program.

DeVries initiated emphasis on the use of mutations for solution of specific agricultural problems with speculation in the early 1900's that induced mutations would be used in the production of new plant and animal varieties (Gustafsson 1963). Research efforts to isolate spontaneous new mutants have been productive over long time periods and, due to low mutation rates, have involved large land and labor commitments. With respect to induced mutations, the publications of the International Atomic Energy Agency (IAEA) give an idea of the expenditures of money and time in this area. As of 1972, the verified list of useful new crop plant varieties produced by induced mutation stood at 68.

Usually, spontaneous mutants are isolated by screening huge numbers of seedlings or plants. Most studies of induced mutation in agricultural plants are initiated by irradiating or chemically treating large numbers of seeds (IAEA 1970, 1972). All treated seeds are germinated; plants are examined for possibly valuable new phenotypes; occasionally, selection for specific phenotypes is imposed.

Recently, it has become apparent that plant cells or pollen grains grown in culture can also serve as mutable material which can be grown into entire and possibly mutant plants (Street 1973a). There have been numerous suggestions that spontaneous or induced mutations of tissue culture materials may provide new varieties (Brock 1971, Carlson 1973a, Delieu 1972, Melchers and Labib 1970, Smith 1974, Street 1973b, Sunderland 1973a). To date only a few workers have produced potentially useful results using the method.

I would like to call attention to the advantages and disadvantages of each method for obtaining agriculturally useful mutant plants. In particular, I shall concentrate on the potential usefulness of tissue culture breeding and on the problems to be overcome in its development.

SPONTANEOUS MUTANTS FROM PLANTS

Naturally occurring mutants are discovered by serendipity or by purposefully examining large numbers of seedlings or plants either with or without the application of selection pressure. For example, Luke, Wheeler, and Wal-

lace (1960) used more than 800 working hours and a large amount of space to screen about 50 million oat seedlings for resistance to *Helminthosporium* blight. They isolated 72 plants which appeared to be natural, resistant mutants, occurring at a frequency of 1.44×10^{-6}. Resistance to this disease is a recessive trait, and since oats are self-fertilizing plants, these mutants probably arose as a result of zygotic or gametic mutations in one to several individual plants with homozygosity following. Their method is noteworthy in that, even though considerable effort was involved, the logistics were simple when compared to procedures involving selection of mature plants in the field.

Nonchimerical plants must arise from either gametic or zygotic alterations. Desirable phenotypes in higher plants could result either from changes in chromosome number (ploidy change or aneuploidy) or structure (translocation, inversion, duplication, and deletion) or from point mutations causing single amino acid changes in a gene product. Examples of the first type of mutation (so called "genome" and "chromosome" mutations) would be (a) alteration or elimination of a regulatory gene, which slows production of a desirable protein, or (b) dysfunctional change in a gene related to production of an undesirable plant product. Examples of point mutations would be (a) an increase in the number of essential amino acid residues in a storage protein or (b) modification of a transport protein's active site so that Na^+ was less readily bound.

For both dominant and recessive changes the basic assumption is that the likelihood of a particular mutation is on the order of 1×10^{-5} per gene copy per generation (Serra 1968). For instance, in corn, mutations in the gene P_r occur with a frequency of 1.0×10^{-5} per gamete. The dominant lethal mutation for retinoblastoma in humans occurs in 2.3 of every 10^5 gametes. Mutation rates are most frequently and easily measured for genes having alleles with obvious, often deleterious, phenotypic effects. Such mutation rates may actually be the sum of a number of separate events, involving one or more genes, each of which causes a particular phenotypic change—often elimination or gross alteration of a particular gene product. Agriculturally useful gene alterations might often involve more subtle structural changes, perhaps a change in one of several vulnerable nucleotides. Therefore, although I will use a standard mutation rate of 1×10^{-5} per gene copy per generation in this paper, it must be understood that the mutation rate for a particular base pair is much lower.

Vogel (1970) has calculated that specific nucleotide mutation rates (the rate at which a given base changes to another specified base resulting in an amino acid substitution) are on the order of 1×10^{-8} or 10^{-9} per gene copy per generation for the hemoglobin molecule. The length of a generation is difficult to specify because mutations occurring throughout the lifetime of the gamete-producing parent could contribute to the total number of mutations. Since crop plant generation times are about 0.4 years instead of 25 years, specific base mutation rates for plants may be lower than for humans. I have used the hemoglobin estimate because it is a eukaryotic system in which all mutations can be recovered, even those with no phenotypic effect.

For a typical diploid organism, then, the chances of a new dominant spontaneous mutation (responsible for a specific, defined phenotype) arising at a particular gene locus are about 2×10^{-5}. If the plant is polyploid, the chances are correspondingly higher. Phenotypic expression of a recessive mutation depends on two or more independent mutational events in the same zygote or on the chance union of mutant gametes. In either case the probability of a particular new phenotype from new mutations is around 1×10^{-5y}, if "y" is the ploidy of the plant.

These calculations are only for plants in which outcrossing predominates and recombination of gametes occurs at random. If the plant is self-fertilizing, mating is not random; then the probability that a zygote will be formed from two identical recessive mutant gametes approaches the basic mutation rate of 1×10^{-5} in the second generation following a gametic mutation.

This analysis has assumed that a particular mutation exists only because of a new event. In fact, the actual frequency of a mutation could be higher because those occurring in past generations would be maintained to an extent that depends on selective value. However, many desirable phenotypic alterations could result from point mutations that occur at a much lower frequency than the one I have used. The actual frequency of a particular mutation depends on the type of genetic alteration, forward and reverse mutation rates, selection pressure, and generations of accumulation (Dobzhansky 1970).

Thus, an investigator screening plants for a particular mutant phenotype must be prepared to examine perhaps 10^5 or 10^{5y} plants. Actually more plants would have to be screened for 95% probability of finding at least one mutant. The time and expense involved in growing and screening large numbers of plants is considerable. Selection methods such as that of Luke et al. (1960), which can use seedlings rather than field-grown plants, significantly improve selection economics. In some cases, however, seedlings will respond to a selective agent in a different way from whole plants. Certain desirable mutant phenotypes (e.g., those involving altered growth habit) are easily identified by field observation. Others, such as improved amino acid profiles, cannot be identified in the field and require chemical analysis of all plants. A third group, such as herbicide-resistant or salt-tolerant mutants, require that plants are subjected to selective pressure. Thus, for each specific desired mutant phenotype the potential cost and time involved in field selection should be estimated and the availability of other selection methods considered.

SPONTANEOUS MUTANTS FROM TISSUE CULTURE

Recent developments in tissue culture techniques offer a possible method for rapidly isolating spontaneously occurring mutant[1] phenotypes. Since a complete review of recent advances in tissue culture has already appeared (Street 1973c), I will briefly summarize: For some time, it has been possible to grow large numbers of plant cells in minimal space under sterile conditions and, by proper medium manipulation, to grow cultured cells into entire plants (Murashige 1974, Street 1973c, Vasil and Vasil 1972). Plant cells may regenerate embryos (embryogenesis) or shoots or roots (organogenesis). Organogenesis of shoots is the usual regeneration method because embryogenesis is uncommon (except in carrot cultures) and because whole plants are not easily regenerated from roots. New shoots are rooted by horticultural techniques, or they form roots spontaneously in the

[1] Although I have used the term *mutant*, it should be understood that this implies inheritability that has not been demonstrated for most variants derived from tissue culture.

tissue culture. Table 1 lists representative regeneration techniques for agricultural plants.

Since 100 ml of a rapidly growing suspension culture of tobacco contain upwards of 1×10^7 cells (Nabors et al. 1975), tissue cultures allow a large number of potential plants to be grown economically in minimal space. This feature of tissue culture alone makes the method valuable for rapid propagation (cloning) of rare variants. For instance, introduction to the market of a rare, disease-resistant plant would be delayed several years by conventional means of propagation (such as cuttings or seeds). Tissue culture cloning could produce unlimited numbers of plants in a matter of months. Problems related to cloning are discussed in the following section.

Mutant cells can be selected either from calli or from cell suspensions. Considering the number of cells in a typical tissue culture and the mutation rate of single genes, it seems quite likely that a 100 ml culture would contain at least one cell possessing a given dominant mutation. A large suspension culture of 100 liters should contain more than 10^{10} cells, and a small possibility exists for recovering even recessive mutant phenotypes in diploid organisms.

Use of haploid cells increases the probability of obtaining recessive mutants (see Sunderland 1973a). If the parent plant is polyploid such cultures are referred to as "polyhaploid" (Kimber and Riley 1963). Since a tissue culture is derived from a portion of a single plant, the chance that the plant will already carry the desired mutation is low and equal to the frequency of the mutation in the population.

Thus, in tissue culture breeding, as opposed to field breeding, only new mutations need to be considered. When selection for a specific phenotype is imposed, an entire culture of mutant cells is obtained. Regenerated plants (forming from either single cells or cell clumps) will thus carry the mutant gene, although its phenotypic expression in whole plants is unpredictable.

Several workers have isolated naturally occurring plant cell mutants resistant to a metabolic inhibitor. Maliga et al. (1973a) obtained 5-bromodeoxyuridine-resistant cell lines from haploid tobacco. The same workers (1973b) also regenerated streptomycin-resistant plants from resistant haploid callus. One mutant was isolated per 10^6 cells. Widholm (1972a, 1972b, 1974) has isolated several types of 5-methyltryptophan-resistant lines of both carrot and tobacco cells. In one case plants were regenerated, and subsequent cell cultures still carried the trait. Heimer and Filner (1970) isolated a line of tobacco cells in which nitrate uptake was no longer inhibited by L-threonine.

Unfortunately, few workers have obtained spontaneous cellular mutants of potential use in agriculture. Nabors et al. (1975) found that suspension cultures of tobacco cells exposed to high levels of NaCl gradually develop tolerance for the salt, apparently due to the selection of naturally occurring mutants. Our mutant cells are now growing in 8,000 ppm NaCl, about 10 times the original tolerance. Dix and Street (1975) have also isolated NaCl-tolerant tobacco cell lines. Since NaCl tolerance is a widespread agricultural problem (Dregne 1963, Gauch 1972, Rains and Epstein 1967, Waisel 1972), such mutants may be useful if the phenotypic trait persists in regenerated plants.

Aside from Maliga et al. (1973b), no one has estimated spontaneous mutation rates in cultured plant cells. This is an important consideration for future study because, in animal cell cultures, mutation rates are sometimes abnormally high and depend on cultural conditions (Cass 1972).

Tissue culture breeding offers the possibility of rapid, economical isolation of specific mutant types with possible agricultural utility. Millions of potential plants can be grown in a single flask, within which selection for mutant phenotypes can occur. Thus, a mutant selection process normally involving huge numbers of whole plants and large commitments of space and labor is tremendously simplified. The power of the technique is that it arranges the normally occurring processes of mutation formation and natural selection into a logistically simple format in which time and space requirements are remarkably compressed.

TABLE 1. Representative procedures for plant regeneration (shoot formation) in some agricultural plants (information relating only to haploids is not included).

Family	Genus	Common name	Source
Amaryllidaceae	*Allium cepa*	onion	Fridborg 1971
Chenopodiaceae	*Beta vulgaris* vars.	beet, sugarbeet	Margara 1970
Compositae	*Lactuca sativa*	lettuce	Doerschug and Miller 1967
Cruciferae	*Brassica oleracea* vars.	Brussels sprouts	Clare and Collin 1974
		cauliflower	Walkey and Woolfitt 1970
		kale	Lustinec and Horak 1970
Cucurbitaceae	*Cucurbita pepo*	pumpkin	Jelaska 1974
Leguminosae	*Medicago sativa*	alfalfa	Saunders and Bingham 1972
	Pisum sativum	pea	Gamborg et al. 1974
Liliaceae	*Asparagus officinalis*	asparagus	Wilmar and Hellendoorn 1968
Poaceae	*Avena sativa*	oats	Carter et al. 1967
	Hordeum vulgare	barley	Cheng and Smith 1975
	Oryza sativa	rice	Nishi et al. 1968
	Saccharum officinarum	sugar cane	Barba and Nickell 1969
	Sorghum bicolor	sorghum	Masteller and Holden 1970
	Triticum sps.	wheat	Shimada et al. 1968
	Zea mays	corn	Green and Phillips 1975
Rosaceae	*Prunus amygdalus*	almond	Mehra and Mehra 1974
Rubiaceae	*Coffea canephora*	coffee	Staritsky 1970
Rutaceae	*Citrus* sps.	citrus fruits	Murashige 1974
Solanaceae	*Lycopersicon esculentum*	tomato	Nabors, unpublished
	Solanum tuberosum	potato	Lam 1975
Umbelliferae	*Daucus carota*	carrot	Murashige 1974

MUTANT PLANTS FROM CELLS—PROBLEMS

At present, four important problems pose a barrier to the use of tissue cultures in agricultural breeding programs.

Absence of Suitable Tissue Culture Techniques

Tissue culture breeding is sometimes criticized for being a tobacco-based technology. It is a fact that most experiments have used tobacco and that extension of the complete method to food crop plants is at present a theoretical construct.

The method involves production of callus on solid medium, cell suspensions in liquid medium (this step is sometimes omitted), selection of mutants, and plant regeneration from mutant cells. For most agricultural plants, callus is easily produced. In dicots, stem, petiole, or cotyledon sections are easily and

frequently used. In monocots, root or embryo callus is easily obtained.

Even though callus production is usually possible, it is still something of a magical art. A medium producing callus for one species may not work for a second or for another variety of the first. Callus production from the stem may be routine, whereas the root may not respond. Also, calli derived from different parts of the same plant may differ markedly in regenerative ability (e.g., Doerschug and Miller 1967).

Finally, a medium satisfactory for callus initiation may not support growth of callus excised from the site of formation. Problems of callus production and growth are resolved by testing as many permutations of medium constituents, their concentrations, and cultural conditions as possible (de Fossard et al. 1974). The ideal situation involves rapidly forming callus with high regenerative ability.

Mutant cells can be selected from callus tissue or from cell suspensions initiated from calli by mechanical or enzymatic disruption. In my laboratory, suspensions are initiated simply by placing callus tissue in a baffle-bottom Erlenmeyer flask containing liquid medium on a gyrotory shaker. Suspensions are subcultured when cell density surpasses a minimal value. Subculturing can be avoided by use of continuous culture methods (King and Street 1973).

As with callus cultures, problems are frequently encountered. Often the best medium for callus growth may not work well, in liquid form, for suspensions. Or a medium may allow suspension formation and some growth, but not continued growth. In such cases, perturbations of all medium components and cultural conditions must be considered, as well as addition of new components or conditions.

The principal difficulty in adapting tissue culture breeding to food crop plants has heretofore been in obtaining reliable plant regeneration techniques. In recent years such methods have been published for a number of different plants (Table 1), especially for a number of monocots. Still, significant gaps exist. For most legumes, in particular soybean and dry beans, regeneration methods are unavailable despite extensive effort. For other plants, available methods need modification. In corn, for instance, regeneration has been reported only from milk stage embryo-derived callus; in sugarbeet, only from floral peduncle-derived callus (Table 1). Techniques using more easily obtainable tissue would be desirable.

Some regeneration techniques are inefficient; rather few plants are regenerated. In some cases, this is undoubtedly due to medium composition or cultural conditions. In other cases, regenerated shoots may interfere with the regenerative ability of nearby cells.

Finally, as with other tissue culture techniques, varietal differences are found in regenerative ability. This is true for tobacco as well as for food crops.

All of these problems can be either resolved or lessened by continued experimentation. Despite these problems, tissue culture methodology is complete enough for some plants (e.g., wheat, oats, barley, and tomatoes) to encourage experimentation in mutation selection and testing of regenerated plants.

Selection of Cellular Mutant Phenotypes

For successful selection, two requirements must be met. First, the desirable phenotype must require alteration of only one allele: Conversion of a C-3 to a C-4 plant would be impossible in the course of one selection process whereas increasing the rate of nitrate reduction should be possible. This is not to say that alterations involving several genes could not be obtained by tissue culture techniques; sequential selections would be required.

Second, a selection procedure suitable for callus or suspension cultures must be devised for each desired mutant phenotype. However, it might be impossible to select for many desirable phenotypes, such as improvements in fruit or grain quality, at the tissue culture stage. There are several indications that this problem is not as serious as it might at first appear.

The goal of increasing protein levels in starchy grains such as corn or wheat might seem unapproachable by mutant selection in cell cultures. The amount of protein accumulated in the grain or vegetation of wheat is directly related to (Croy 1967, Eilrich 1968), although not necessarily caused by, assayable nitrate reductase activity. Traditional breeding experiments can produce corn lines with high, medium, or low activities of nitrate reductase, and the differences in activity are found in all developmental stages (Schrader et al. 1966).

Single cell selection could occur for mutants more efficient in using available nitrate. Such mutants might have an altered nitrate uptake system or increased amounts or activity of nitrate reductase, which might in turn lead to higher protein levels. The mutants could be selected by lowering nitrogen levels in the medium until nonmutant cells could no longer grow efficiently or by adding to the medium various inhibitors of nitrate reductase induction or action (Beevers and Hageman 1969).

The goal of changing low levels of certain essential amino acids, such as lysine, tryptophan, and methionine, in grains or in grain protein also initially seems unadaptable to a tissue culture approach. Carlson's experiments (1973b) show at least that mutant cells with an enhanced amino acid level can be selected and that the trait is passed on to regenerated plants. It remains to be demonstrated that a similar mutant phenotype appears in the seed of a regenerated plant.

Increasing photosynthetic efficiency is another aim of plant breeders that seems unsuitable to the approach of selecting spontaneous mutants in cultured cells. With respect to possible breeding for structural changes in the photosynthetic apparatus (a C-4 instead of a C-3 organization, for instance), this impression is correct. However, tissue cultures are often photosynthetically active (Zelitch 1975), and one worker has produced autotrophic callus (Corduan 1970). By lowering light or CO_2 levels or by including various inhibitors of photosynthesis in such cultures, one could select for any mutants with efficient photosynthesis. Selection of a culture using glycolic acid as a carbon source could result in elimination or reduction of photorespiration (Zelitch 1975).

In general then, clever selection techniques can probably be used to obtain many sorts of useful mutants in the cellular stage. Table 2 gives some examples. As the physiology and biochemistry of various mutant lines are determined, researchers will have a better idea of initial and secondary selection procedures that might prove useful.

Retention of Mutant Phenotype in Regenerated Plants

A third problem with mutants from tissue cultures is that, even though mutants with altered traits such as salt or temperature sensitivity might be selected at the cellular stage, the pheno-

TABLE 2. Some agriculturally useful mutant phenotypes which might be or have been selected at the cellular stage.

Mutant phenotype	Possible/actual selection procedure	Accomplished in cells/ [plants]
NaCl tolerant	(1) Add NaCl to medium	Nabors et al. 1975, Dix and Street 1975
	(2) Reduce Ca in medium (Kelley 1963)	–
Alkali tolerant	Add alkali to medium	–
Tolerant of high ionic strength	Increase ionic strength of medium	–
Resistant to temperature extremes	Grow cultures at extreme temperatures	–
Efficient user of available nitrogen (possible high protein content)	(1) Reduce nitrogen levels in medium	–
	(2) Include inhibitors of nitrate use in medium	–
Rapid growth rate	Measure growth rate; discard slow-growing cultures	–
Drought resistant	Add nonpenetrating osmoticum to medium*	–
Disease resistant	Add toxin or pathogenic organism to culture (successful only for selected diseases)†	–
Inhibitor or herbicide tolerant	Add normally inhibitory amounts of compound	–
Efficient at photosynthesis	(1) Omit carbon sources from medium (see Zelitch 1975)	–
	(2) Add photosynthetic inhibitors to medium	–
Decreased photorespiration	Supply glycolate as the carbon source (Carlson and Polacco 1975)	–
Increased levels of certain amino acids	Add amino acid analogues to medium	Widholm 1972a, 1972b, [Carlson 1973b]

*In an osmoticum, the water potential of plant cells (which is a negative quantity) will be raised toward zero. Thus, selection will occur for cells that have a lower water potential and can grow more rapidly. Many osmotica leak slowly into cells and soon cease to be effective. Polyethylene glycol 4000 or 6000 or Ficoll (MW=400,000) are examples of usually nonpenetrating osmotica.

†Plant diseases can be divided into two types: (a) those caused by pathogenic toxin and (b) those for which no pathogenic toxin has been isolated and which require the presence of the pathogenic organism itself. In both cases, some diseases will prove infective at the tissue culture level and others will not.

typic characteristics may not persist through the various stages of development to be useful in the field. The problem can only be evaluated, and if necessary resolved, by experimentation.

Some plant diseases affect cultured cells as well as whole plants (Gengenbach and Green 1975, Helgeson et al. 1972, Ingram 1967, 1973). Also, Carlson (1973b) has succeeded in obtaining tobacco cells resistant to an analogue of *Pseudomonas tabaci* toxin; regenerated plants showed increased but not full resistance to the disease and passed toxin-analogue resistance on to progeny. Other workers (Maliga et al. 1973b, Márton and Maliga 1975) selected streptomycin-resistant or BUdR-resistant tobacco cells and found the inherited trait persisted in regenerated plants.

Several other considerations are related to the problem of phenotype persistence in regenerated plants. First, the mechanism of mutation inheritance must be demonstrated. Progress in this direction has been made by Carlson (1973b), Maliga et al. (1973b), and Márton and Maliga (1975). However, considerable work remains because several types of noninheritable traits could be selected in cultured cells. Second, it must be shown that useful alleles do not have any deleterious "side effects" (pleiotropisms) in regenerated plants.

Appearance of Cytological Alterations in Cultured Cells

Another potential problem in plant production from tissue cultures is that cultured cells frequently undergo cytological and nuclear changes (Sunderland 1973b) and suffer a progressive loss of totipotency. Such changes are usually considered degenerative in nature. In many respects, though, populations of isolated higher plant cells behave as cultures of a newly created, ill-adapted microorganism. (A similar view of animal cell cultures led T. T. Puck to write a book entitled *The Mammalian Cell As a Micro-Organism* [Holden-Day, San Francisco, 1972].)

Spontaneous mutations or cytological changes occur in cultured cells and may confer a selective advantage or disadvantage. Cells taken into culture may be expected to undergo a long period of genetic adjustment involving phenotypic changes as each potential mutation, or combination of changes, occurs and is subjected to natural selection.

In terms of producing agriculturally useful plants, undesired genetic changes and loss of regenerative ability are to be avoided. They can be avoided by minimizing time spent in tissue culture through use of rapid callus formation and regeneration techniques. Our tobacco suspensions contain totipotent cells after more than four years in culture. This length of time is quite sufficient for mutant selection to occur.

INDUCED MUTANTS FROM PLANTS

In addition to looking for spontaneous mutant phenotypes, researchers can employ a second method: inducing mutations to increase the frequency of novel phenotypes. Mutation induction in seeds offers the possibility of increasing the mutation rate so that several desirable mutations might occur in the same seed, although probably in different cells. Statistically though, deleterious mutations are much more likely to occur than desirable ones.

To induce mutations, large numbers of seeds are exposed to ionizing radiation or to mutagenic chemicals. Then the seeds are germinated; sometimes selection is imposed for desirable phenotypes. For instance, Wallace, Singh, and Browning (1967) used cobalt-60 gamma rays and several chemicals to induce *Helminthosporium* resistance in oats. They found second generation mutant-resistant seedlings with a frequency of about 3.0×10^{-5} and so succeeded in increasing mutant phenotype frequency to about 20 times the spontaneous rate.

The main problem with induced mutations in seeds is that the multicellular nature of the embryo makes it statistically unlikely to find first generation mutant plants that are not chimerical for the phenotype in question (Broertjes 1972, D'Amato 1965, IAEA 1970–pp. 99-104). This point is most obvious when dealing with chloroplast mutations. Even if the entire shoot portion of the plant arises from a plumule of only a few cells, the probability that each of these cells will be mutated in the same gene is vanishingly small. An outside possibility is that the

mutagenic agent could kill all plumule cells except one, which then gives rise to the entire shoot. But there is no evidence that this occurs.

In many cases, such as those involving hormonal modifications or certain types of disease resistance, the mutant chimerical phenotype may average out to provide an apparently uniform phenotype for the entire plant in the first generation following mutation. The problem is that inheritance of chimerical mutants is unpredictable as well as unstable; a few breeding seasons may be needed before pure stock can be obtained. Establishment of homozygous stock from chimerical plants depends (a) on the chance that some mutated sectors of the plant are gamete-forming sectors; (b) on the chance that mutated gametes combine; and (c) on diplontic selection (Broertjes 1972, IAEA 1970–pp. 134-137), a term describing the fact that mutated cells may increase in number more or less rapidly than similar nonmutated cells. Some workers (e.g., Hirono and Smith 1969, Wallace et al. 1967) have found apparently stable new phenotypes in the generation following the mutated one. However, usually two or three generations are required for stabilization (IAEA 1970, p. 1). Still, time is frequently saved over varietal stabilization from hybridization breeding.

To a limited extent one can avoid chimeras by mutating pollen before fertilization (IAEA 1970, p. 134) or by using plants in which various sorts of asexual regeneration from single cells occur (Broertjes et al. 1968). For instance, many plants form leaf buds in this manner. The problem with both these methods is in obtaining large numbers of potential mutant cells. In some plants (e.g., pine or corn) large numbers of pollen grains could be easily collected, but in others this would be difficult. Regenerating leaf buds could be laborious to obtain in large numbers, and in many whole plants leaf buds or similar structures originating from single cells occur only in tissue culture.

The limited and only recent success of useful plant production from induced mutations in seeds can probably be attributed to the multicellular, differentiated nature of seeds, resulting in chimerical mutants, and to the physical outlay and expense involved in carrying large numbers of mutated seeds through several breeding generations to achieve phenotype stability. A 1972 International Atomic Energy Agency (IAEA) publication lists 68 useful induced-mutant varieties of food crop plants released to growers between 1930 and 1971.

INDUCED MUTANTS FROM TISSUE CULTURE

The production of mutant plants by tissue culture techniques and induced mutations can be divided into four steps: (a) production of callus or suspension cultures, (b) mutation induction, (c) selection of desired mutants, and (d) regeneration of mutant plants from callus or suspension cultures.

Having already discussed steps *a*, *c*, and *d* in conjunction with the isolation of spontaneous mutants, I will note that recent efforts have been successful at inducing mutations in cultured plant cells and then selecting various mutant phenotypes. Carlson (1969, 1970) has isolated various amino acid and vitamin auxotrophs in cell lines of tobacco and a fern. He has also induced a line of tobacco cells and regenerated plants partially resistant to a *Pseudomonas tabaci* toxin analogue and containing increased levels of methionine (Carlson 1973b). Complete resistance occurs naturally in another tobacco variety, so there was reason to believe that resistance could be altered easily by mutation. A NaCl-tolerant line of mutant tobacco cells has also been induced (Nabors et al. 1975), as well as a 5-bromodeoxyuridine-resistant cell line in soybean (Ohyama 1974). An auxin-autotrophic line of maple cells has been selected (Lescure 1969). The basic selection technique for spontaneous mutations is to expose cultures to conditions that slow or prevent growth of normal cells while favoring growth of the desired mutant cells.

In many cases, selection for desirable phenotypes could occur in populations of cultured cells. An alternative is that mutation induction would be followed by regeneration of large numbers of plants, which would then be subjected to selection. Even if the nature of the phenotype requires that selection occurs in the second manner, tissue culture methods are possibly more efficient than traditional techniques because the several breeding seasons often required for stabilization of chimeras are avoided. Chimeras are not produced for two reasons: (a) in many and perhaps all cases plants arise from single cells and (b) if selection has occurred in suspension culture all cells will carry the desired phenotype, and even plants arising from cell clumps will be nonchimerical.

Mutation induction increases the frequency at which various desirable mutations appear. Mutation frequency depends on dosage of the mutagenic agent as well as on various treatments preceding or following mutagenesis (IAEA 1970, pp. 44-57). One consideration for plant breeders is that most mutations are deleterious in a particular environment. It is quite possible, therefore, to induce a desirable mutation in one gene, an undesirable one in another gene, and to produce a mutant cell improved in one respect, but worsened in another.

The ideal situation would be to obtain a mutation rate creating an average of one new mutation per cell, in a cell population large enough to ensure appearance of the specific mutation. Assuming a mutation rate of 1×10^{-5} per gene copy per generation for a specific mutant phenotype and 10^4 genes per genome (Strickberger 1968, p. 525), it is easily estimated that 20% of all diploid cells contain one newly arisen spontaneous mutation.[2] Whatever the actual percentage of cells containing new mutants, a culture containing 10 liters (10^9 cells) of cell suspension should have at least one cell carrying a given mutation, even if the mutation occurs at a rate of only 1 per 10^8 cells. This means that for most dominant or co-dominant phenotypes spontaneous mutation approaches a suitable rate.

There is no certain way of predicting whether a particular desired mutation will be dominant, co-dominant, or recessive, or whether the trait is under the control of several different genes or of polygenes. It would seem advisable, if little is known about the inheritance of the desired trait, to begin searching for spontaneous mutants in tissue cultures and, if this fails, to institute mutation induction.

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[2]The chance of a mutation in a gamete is equal to the mutation frequency for single genes times the number of genes, or 1 in 10. A zygote would have a chance of 2 in 10.

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Überreicht vom Verfasser · Nicht einzeln im Buchhandel!
Sonderdruck aus «Zeitschrift für Pflanzenphysiologie», Band 84, Heft 3, Seite 237–246 (1977)
Gustav Fischer Verlag Stuttgart

Department of Botany and Plant Pathology, Colorado State University, Ft. Collins, Colorado 80521, U.S.A.

Callus Initiation, Growth, and Organogenesis in Sugarbeet (*Beta vulgaris* L.)

MICHAEL P. HOOKER and MURRAY W. NABORS

With 7 figures

Received March 3, 1977 · Accepted March 21, 1977

Summary

Using a broad spectrum tissue culture approach, callus was initiated from cotyledons, hypocotyls, and embryos of sugarbeet. The callus was formed under a regime of differing concentrations of several organic constituents, including growth regulators. Casein hydrolysate, GA, and vitamins were found to inhibit or have no reliable affect on callus initiation from cotyledon and hypocotyl tissues. Initiation in response to auxins and cytokinins was complex due to strong interactions between these two classes of growth regulators. In general, high concentrations (10–25 mg/litre) of auxins promoted callus initiation best. However, 2,4-D was an exception, showing inhibitory effects at concentrations greater than 1 mg/litre. The effects of cytokinins were more variable, depending upon the auxin used in combination with them. Cotyledon and hypocotyl tissues either failed to exhibit an effect due to differing concentrations of cytokinins, or showed maximal callus initiation at moderate concentrations (1–10 mg/litre). High concentrations (10–25 mg/litre) of cytokinins were, in almost all cases, inhibitory.

Subsequent growth of subcultured callus was tested under a similar regime of growth regulators. Once again, the results were complex; but growth was generally favored by moderate concentrations (.5–5 mg/litre) of cytokinins in conjunction with low concentrations (.05–.5 mg/litre) of auxins. Bud initiation in cotyledon and hypocotyl callus was promoted by 6-BAP at a concentration of 5 mg/litre in combination with TIBA at .5–5 mg/litre.

Key words: Sugarbeet, Beta vulgaris, callus, tissue culture.

Introduction

Sugarbeet (*Beta vulgaris* L.) is an economically important agricultural plant. Because its importance lies in sucrose production, sugarbeet may eventually lend itself to industrial, *in vitro* production of this metabolite using large batch cultures of photosynthesizing cells. Meanwhile, cell and tissue culture methods may be used to solve practical problems leading to the improvement of sugarbeet as a crop plant (NICKELL and TORREY, 1969; INGRAM and JOACHIM, 1971; BUTENKO and ATANASOV, 1971).

Until recently, there was virtually no information in the literature about the tissue culture of sugarbeet (NICKELL and TORREY, 1969; BUTENKO and ATANASOV, 1971). However, callus has now been initiated from various seedling tissues, root crops, and embryonic buds (BUTENKO and ATANASOV, 1971), with subsequent studies (BUTENKO et al., 1972) on subcultured callus indicating high mineral and sucrose concentrations are optimal for callus growth on a simple medium. IAA did not reliably affect tissue growth, while kinetin was shown to be inhibitory. In another study (WELANDER, 1974), moderate (1.0–5.0 mg/litre) concentrations of IAA in conjunction with low (0.01–1.0 mg/litre) concentrations of kinetin were shown to optimize callus initiation from cotyledon explants. It has further been shown (WELANDER, 1976) that sucrose and nitrogen levels may influence callus growth under some hormonal conditions. Experiments on several other species indicate that, in addition to the effects of these constituents, vitamins, amino acids, and gibberellins may also be necessary for abundant and dependable production of primary callus (GAUTHERET, 1955; YEOMAN, 1973).

As a step toward the effective culture of sugarbeet, broad spectrum experiments similar to that proposed by DE FOSSARD et al. (1974) were performed to give an indication of the type and quantity of organic constituents necessary for optimal initiation and subsequent growth of callus cultures. Previous studies have been extended to include not only the role of vitamins and exogenous amino acids in the culture of sugarbeet, but also the effects of several growth regulators.

Materials and Methods

Tissue

Hypocotyls and cotyledons from sugarbeet seedlings (*Beta vulgaris* L., commercial var. Mono-Hy Al) were used 6 days after seed imbibition. Hypocotyls were dissected into 5 mm segments; cotyledons were used intact after removing the proximal quarter to prevent accidental planting of axillary bud cells.

Explants of petioles and floral stalks were also used in the initial experiments. However, repeated contamination due to an endogenous bacterium necessitated the elimination of these tissues from further experimentation.

Naked embryos (i.e., pericarp removed, with or without seed coat) were obtained from Great Western Sugar Agricultural Research Center, Longmont, Colorado, 80501. Embryos were pinched with forceps prior to placement in culture vials.

In the final experiment concerned with callus growth, soft brown calli from stock cultures were transplanted with an initial mass of approximately 180 mg.

Sterilization

Seeds were soaked in 95 % ethanol for 15 seconds, 50 % Clorox for 45 minutes, then given 3 rinses in sterile water. Embryos were treated similarly, except 20 % Clorox was used for 15 minutes.

Conditions

Tissues were grown in 25 ml vials containing 10 ml of medium under continuous fluorescent light (approximately 250 foot-candles) at 25 °C. Duration of the experiments was 6 weeks for callus initiation and 4 weeks for growth of subcultured callus.

Treatments

The basal medium consisted of minerals according to LINSMAIER and SKOOG (1965), .4 mg/litre thiamine HCl, 100 mg/litre myoinositol, 50 mg/litre Fe-EDTA, 4 % sucrose, and 1 % agar. The pH was adjusted to 5.5 before autoclaving (20 minutes at 120 °C). Four separate experiments were performed. In the first, the concentrations of several organic constituents were varied, including vitamins at 0, .1, and 1 norm according to BUTENKO et al. (1972); casein hydrolysate at 0, 50, and 500 mg/litre; gibberellic acid (GA) at 0 and 2 mg/litre; and kinetin at .1 and 10 mg/litre. These constituents were combined in all possible combinations to yield 36 different media on which hypocotyl and cotyledon tissues were placed in a test for callus initiation.

The results of this experiment were then applied in an expanded effort to determine the effects of several different growth regulators on production of primary callus using these same tissues. These included the auxins indole-3-acetic acid (IAA), α-naphthaleneacetic acid (NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D) as well as the cytokinins 6-benzylaminopurine (6-BAP) and kinetin. Each was present at a concentration of 1, 10, or 25 mg/litre and all possible auxin-cytokinin combinations were tested.

Embryos were also used in a test for callus initiation on media containing several levels of different auxins. IAA, NAA, and 2,4-D were again used, but the concentrations were varied from .2–60 mg/litre. In all cases, kinetin was added at a concentration of .05 mg/litre.

The calli resulting from these three experiments were repeatedly transplanted and were maintained as stock cultures. These were subsequently used in a final experiment to determine callus growth in response to different concentrations of several growth regulators. IAA or 2,4-D, each at a concentration of .05, .5, or 5 mg/litre were added in combination with kinetin or 6-BAP at these same concentrations. 2,35-triiodobenzoic acid (TIBA) at .0 or 5 mg/litre was also added in conjunction with the cytokinins. Consistent with the broad spectrum nature of these experiments, only six repetitions per treatment were cultivated in all cases.

Recording of results

In order that the tissue cultures need not be destroyed in taking data, and to facilitate rapid quantification of results, a system utilizing relative mass based on size was developed for use in this laboratory. Eighteen different clay callus models were made ranging in mass from .019 g to 20.507 g. Using the smallest as a standard, the ratio of masses ranged from 1.0 to 1079.3. Callus size was compared to model size and the corresponding ratio was recorded. A ratio of 1.0 is equivalent to .003 g of soft, friable callus.

Results and Discussion

Callus initiation

The effects of exogenous vitamins, casein hydrolysate, GA, and kinetin can be noted in Figs. 1 and 2. The ordinates show relative mean callus mass per vial using the system discussed previously. Kinetin at a concentration of 10 mg/litre stimulates callus initiation in both tissues to far greater extent than if present at .1 mg/litre. GA is clearly inhibitory in the case of hypocotyl tissue whereas, with cotyledons, its role is somewhat unclear. Except for the single treatment showing exceptionally good growth, however, GA appears to have a generally inhibitory effect here as well. Casein hydrolysate at a concentration of 500 mg/litre is inhibitory using cotyledons. Hypocotyl tissue does not exhibit this effect. The role of vitamins in this broad spectrum experiment is complex: no clear trends emerge. However, there is some

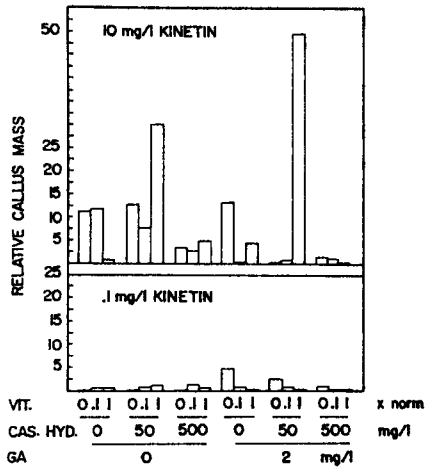

Fig. 1: Effects of exogenously-added organic constituents on callus initiation from cotyledon tissue. See the text in *Materials* and *Methods* for clarification of treatments.

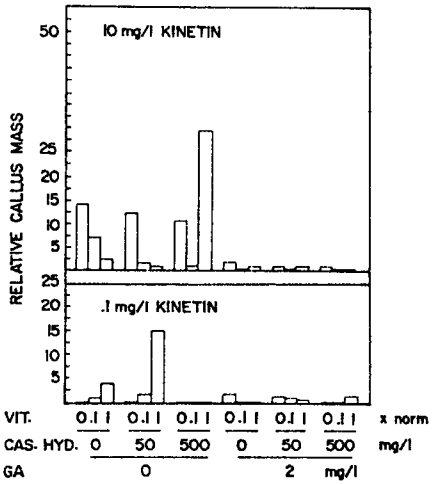

Fig. 2: Effects of exogenously-added organic constituents on callus initiation from hypocotyl tissue. See the text in *Materials* and *Methods* for clarification of treatments.

indication of a synergistic interaction between the vitamins and casein hydrolysate. This is evidenced by the peaks obtained using vitamins at a concentration of 1 norm in conjunction with casein hydrolysate at 50 mg/litre in the case of cotyledons and 500 mg/litre in the case of hypocotyls. Considering this ambiguity in the role of vitamins and based on the other results from this experiment, GA, casein hydrolysate, and vitamins were omitted from the media used in all subsequent experiments.

Figs. 3 and 4 illustrate the effects of several growth regulators on primary callus production. The data clearly indicates auxin- cytokinin interactions which are dependent on both type and concentration of growth regulators. Callus formation in cotyledons is inhibited by 10 and 25 mg/litre of 6-BAP when used in conjunction with NAA or 2,4-D (Fig. 3). If IAA is used, 6-BAP exhibits no such effect. In this case, initiation is maximal at 25 mg/litre of IAA and, in fact, may well increase at higher concentrations. NAA shows a peak at 10 mg/litre while 2,4-D is inhibitory beyond 1 mg/litre.

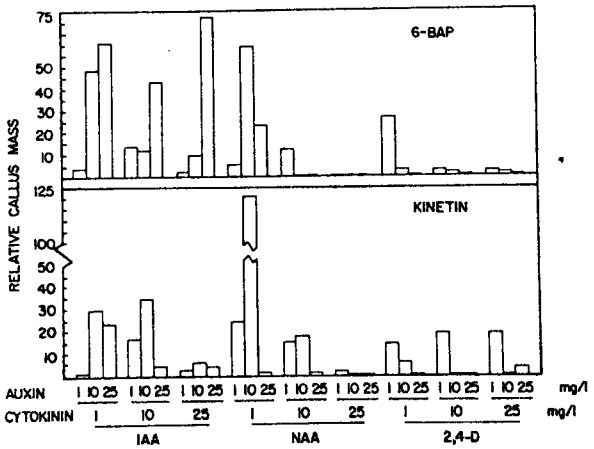

Fig. 3: Effects of differing concentrations of growth regulators on callus initiation from cotyledon tissue.

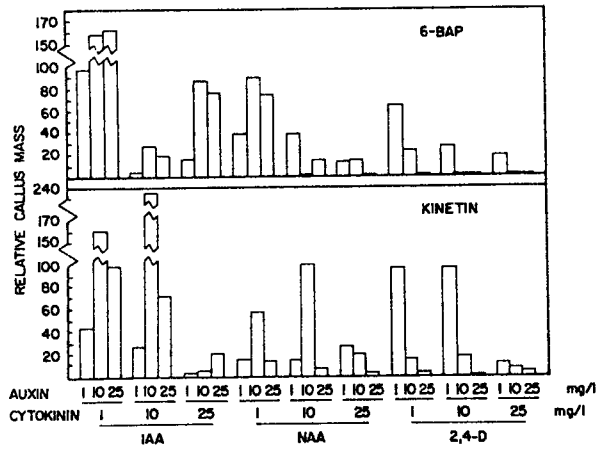

Fig. 4: Effects of differing concentrations of growth regulators on callus initiation from hypocotyl tissue.

Kinetin exhibits quite a different interaction with these auxins. It is inhibitory only at 25 mg/litre, but this effect does not occur in conjunction with 2,4-D. As in the case of 6-BAP, NNA is optimal that 10 mg/litre. Likewise, 2,4-D becomes increasingly inhibitory as the concentration increases from 1 mg/litre. IAA promotes initiation best at a concentration of 10 mg/litre.

Although not completely consistent with these results, hypocotyl tissue behaves in a similar fashion (Fig. 4).

The callus obtained in this experiment was, in nearly all cases, hard, compact, and green (Fig. 5 A). However, kinetin at a concentration of 1 mg/litre in conjunction with NAA at 10 mg/litre produced large amounts of soft brown callus from both cotyledon and hypocotyl tissues (Fig. 5 B).

Results from a separate experiment in which pinched naked embryos were used are shown in Fig. 6. Initiation and growth in response to auxin concentration is consistant with results obtained using hypocotyl and cotyledon tissues. In this experiment, callus formation diminished with increasing concentration of 2,4-D and would likely show a maximum at some concentration less than .2 mg/litre. NAA shows a peak at 20 mg/litre while IAA is optimal at 40 mg/litre.

The embryo-derived calli were hard, compact and heterogeneously colored in all treatments containing auxins at 20 mg/litre or above (Fig. 5 C). Treatments with .2–2 mg/litre of auxin showed sporadic appearance of soft brown callus. All calli formed on the medium containing .2 mg/litre of 2,4-D were of this type (Fig. 5 D).

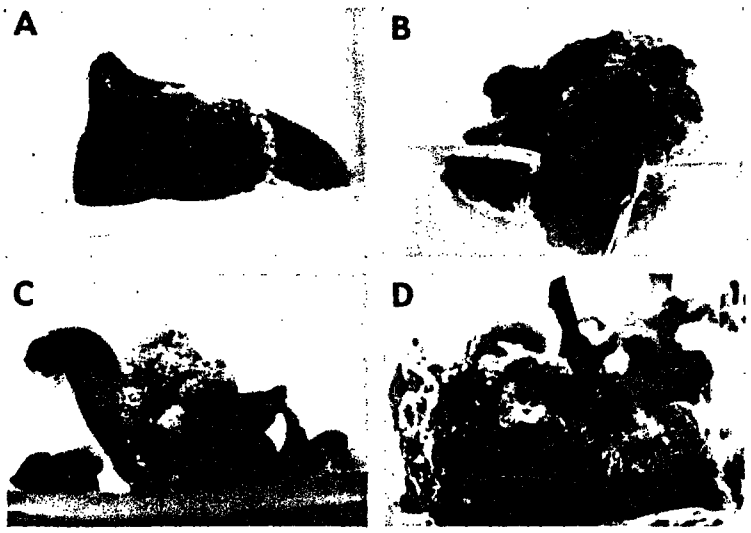

Fig. 5: Characteristic primary calli obtained from different sugarbeet tissues. A: compact green callus from cotyledon tissue; B: soft brown callus from hypocotyl tissue; C: compact heterogeneous callus from embryo tissue; D: soft brown callus from embryo tissue.

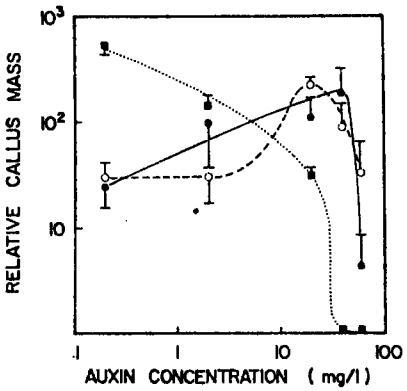

Fig. 6. Effects of differing concentrations of auxins on callus initiation from embryo tissue. IAA (●), NAA (○), and 2,4-D (■) were each used at .2, 2, 20, 40, and 60 mg/litre. Standard errors are shown in one direction only.

Callus growth

Table 1 lists data for callus growth in response to different concentrations of growth regulators. The values are relative mean increase in mass per vial based on all treatments at the concentration shown. These results indicate not only a difference in activity between the two auxins and the two cytokinins but also a difference in growth response due to callus origin. For example, embryo callus exhibits maximal growth at 5 mg/litre of kinetin whereas growth of cotyledon callus is optimal at

Table 1: Effects of differing concentrations of growth regulators on growth of cotyledon, hypocotyl, and embryo callus.

Hormone	Conc. (mg/litre)	Relative Mean Increase In Mass		
		Cotyledon	Hypocotyl	Embryo
Kinetin	0.05	115.2	74.8	153.5
	0.5	157.5	55.9	181.4
	5.0	110.6	71.8	210.2
6-BAP	0.05	95.0	72.8	153.1
	0.5	133.7	67.4	211.6
	5.0	151.0	123.6	86.0
IAA	0.05	99.1	90.8	309.2
	0.5	179.1	81.0	161.9
	5.0	153.1	74.3	160.0
2,4-D	0.05	253.8	80.7	202.2
	0.5	148.8	112.8	142.7
	5.0	26.1	45.9	10.1
TIBA	0.5	87.0	75.7	230.2
	5.0	69.9	60.6	111.6

.5 mg/litre and the response of hypocotyl callus is independent of kinetin concentration. Cytokinin-induced growth of hypocotyl callus appears to be more easily stimulated by 6-BAP than by kinetin, yet may not have reached a peak even at 5 mg/litre of 6-BAP. Growth of embryo callus also seems to be more easily stimulated by 6-BAP but evidently reaches a maximum at .5 mg/litre. Cotyledon callus, on the other hand, appears to be more sensitive to kinetin and exhibits a peak, as stated above, at .5mg/litere. In response to auxins, embryo callus is extremely sensitive and may grow best at a concentration of IAA less than .05 mg/litre. Cotyledon and hypocotyl callus are less sensitive and grow best on a medium containing .05 and .5 mg/litre, respectively, of the much more active 2,4-D. It is interesting to note that, as in callus initiation, 2,4-D becomes increasingly inhibitory even at concentrations as low as .5 mg/litre in the case of coytledon and embryo callus. TIBA inhibits growth of cotyledon callus and has little affect on growth of hypocotyl callus. However, growth of embryo callus is promoted by TIBA at a concentration of .5 mg/litre. Considering the sensitivity of embryo callus to auxins, this response to TIBA would be expected.

Based on Table 1, optimal concentrations of growth regulators for maintenance of each callus type can be defined. It should be kept in mind, however, that each figure includes all treatments in conjunction with that concentration of growth regulator to which it refers. Nonetheless, cotyledon callus would be expected to grow well on a medium containing .5 mg/litre of kinetin along with .05 mg/litre of 2,4-D. Embryo calus, on the other hand, should do well on a medium containing .5 mg/litre of 6-BAP in conjunction with .05 mg/litre of IAA. Hypocotyl callus would be expected to grow well on 5 mg/litre of 6-BAP in conjunction with .5 mg/litre of 2,4-D.

In general, the soft brown nature of the tissue was preserved throughout the incubation period (Fig. 7 A). However, on some media there was a tendency toward greening (Fig. 7 B), root formation (Fig. 7 C), or shoot formation (Fig. 7 D). Table 2 summarizes these results. Although the maintenance of rapidly growing callus of the desired consistency is an important objective of this research, regeneration of plants from established cultures is also highly desirable. MARGARA (1970) has already shown that plants can be regenerated from primary sugarbeet callus. In addition, BUTENKO and ATANASOV (1971) have observed infrequent occurrence of shoot formation in first passage callus. The data in Table 2 indicates a tendency toward shoot formation on a medium containing 5 mg/litre of 6-BAP in combination with TIBA. In each case, budding occured on soft green callus. Green callus of a compact consistency showed no tendency toward budding. In all three tissues, greening is facilitated by increasing the concentration of 6-BAP. Kinetin also exhibits this effect, but to a lesser degree.

Although no generalizations can be made concerning root formation in cotyledon and hypocotyl callus, decreasing the concentration of 6-BAP clearly promotes root initiation in embryo callus.

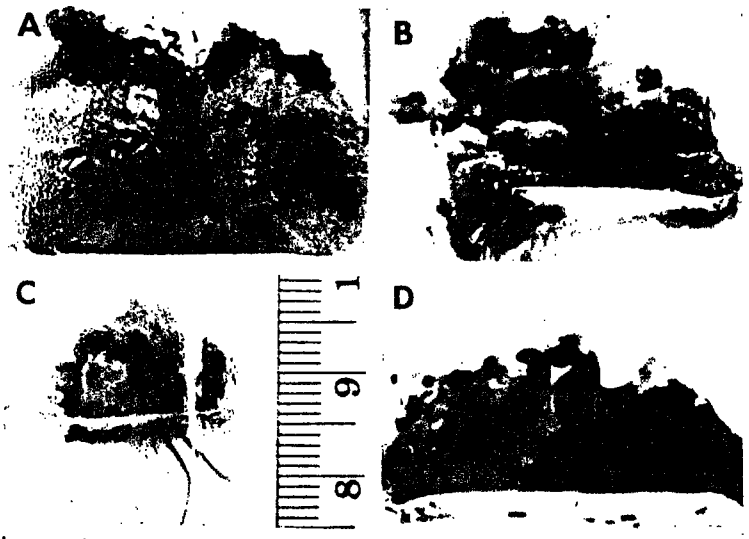

Fig. 7: Characteristic subcultured calli of different tissue origin showing various degrees of differentiation and organogenesis. A: soft brown callus of embryo origin; B: greening callus of hypocotyl origin; C: root formation from callus of cotyledon origin; D: budding on callus of hypocotyl origin.

Table 2: Characteristics of cotyledon, hypocotyl, and embryo callus under a regime of differing concentrations of growth regulators (number of vials, or in the case of roots, total number, per concentration of growth regulator).

Hormone	Conc. (mg/litre)	Characteristics[a] Cotyledon				Hypocotyl				Embryo			
		(1)	(2)	(3)	(4)	(1)	(2)	(3)	(4)	(1)	(2)	(3)	(4)
Kinetin	0.05	40	–	–	–	33	1	2	–	33	–	–	1
	0.5	39	3	–	6	33	3	1	1	37	–	4	4
	5.0	39	1	1	–	37	4	2	–	29	6	7	13
6-BAP	0.05	41	2	–	–	38	2	–	1	36	5	4	31
	0.5	42	2	–	–	37	2	4	7	14	14	16	21
	5.0	35	3*	2	–	29	7*	10	–	5	10	32	2
IAA	0.05	29	4	–	6	25	2	2	–	24	6	4	16
	0.5	33	2	–	–	26	4	2	7	27	6	1	7
	5.0	33	1	–	–	33	–	2	–	23	7	3	21
2,4-D	0.05	36	–	–	–	28	–	2	–	17	6	12	4
	0.5	33	–	2	–	27	4	3	–	7	4	20	–
	5.0	17	–	–	–	26	–	–	–	3	–	14	–
TIBA	0.5	31	2*	1	–	22	5	4	1	29	3	3	23
	5.0	24	2	–	–	20	4*	4	1	24	3	6	1

[a](1) Soft brown callus; (2) Soft green callus; (3) Compact green callus; (4) Roots. * Budding.

In general, the results of broad spectrum experiments such as these should not be taken as conclusive. Their role is to point out areas of promise, which should then be pursued in detail. On the other hand, if the objective is to obtain callus cultures with no interest in optimizing the procedure, broad spectrum experiments are an appropriate technique. The usefulness of such an approach is evident in the established cultures of sugarbeet callus resulting from these experiments.

Acknowledgements

The authors gratefully acknowledge Dr. R. HECKER (U.S.D.A., A.R.S.) and A. FREYTAG (Great Western Sugar) for their generosity in supplying plant materials, as well as their time, during the course of this study.

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Dr. M. W. NABORS, Department of Botany and Plant Pathology, Colorado State University, Ft. Collins, Colorado 80521, U.S.A.

Culture and Sodium Chloride Tolerance Studies on Wheat (*Triticum aestivum* L.) *in vitro*

Claire E. Brown and Murray W. Nabors

Department of Botany and Plant Pathology

Colorado State University

Fort Collins, Colorado 80523

USA

SUMMARY

In attempts to produce a vigorous, friable wheat callus from seed for use in other tissue culture experiments, the influences of several nutrient factors were studied.

A modified Linsmaier-Skoog medium was used in all experiments. For callus initiation and maintenance, the sucrose level found most suitable was 2 per cent.

The auxins NAA (at 10 mg/litre) and 2,4-D(at 2 mg/litre) produced excellent callus initiation and growth. In 2,4-D medium, growth seemed to be stimulated by the addition of exogenous vitamins, or extra amino acids plus casein hydrolysate at 1 g/litre. Growth was inhibited by high levels of casein hydrolysate (10 g/litre), kinetin (5 mg/litre), or 2,4-D (10 mg/litre). Kinetin at .5 mg/litre seemed to have very little influence on primary callus. Growth of primary callus is sigmoid under ideal growth conditions.

The best auxin for secondary callus growth was found to be IAA, at a concentration of 10 mg/litre. NAA alone was poor for secondary callus, and 2,4-D alone was fair. The addition of casein hydrolysate at 1 g/litre plus extra amino acids increased the effectiveness of IAA and NAA. Exogenous vitamins had an inhibitory effect with 2,4-D and IAA. Kinetin in general also inhibited growth with all the auxins.

It was noticed that roots on secondary callus were profuse in the presence of IAA and/or kinetin, and were essentially absent with 2,4-D.

From some studies with media containing NaCl it was found that differences in NaCl concentration were more apparent in primary callus than in secondary callus. 2,4-D at 2 mg/litre was better than at 5 mg/litre for primary callus growth. The varying of 2,4-D level, or the addition of

vitamins, had no noticeable effect on secondary callus. Kinetin at .05 mg/litre seemed to help secondary callus slightly.

Key words: Callus, tissue culture, *Triticum*, wheat

INTRODUCTION

Tissue culture techniques have been developed for many of the higher plants. In general, the culture of dicotyledonous plants has been more successful than that of monocotyledons (Gamborg and Eveleigh, 1968; Trione *et al.*, 1968; Shimada *et al.*, 1969; Sheridan, 1973).

Prior to 1968 there were few reports concerning the tissue culture of wheat. Gamborg and Eveleigh (1968) initiated callus from seedling root sections of wheat (*Triticum aestivum* and *T. monococcum*) and barley on their defined solidified PRL-4 medium. Then these calli were transferred to liquid PRL-4 to form suspension cultures, which were later transferred to their defined liquid B-5 medium. Both of these media contained 2,4-D. Trione *et.al.* (1958) tested callus growth on twenty different media, using somatic tissues and endosperm as source materials. Tissue derived from the cotyledonary node produced callus most readily. Hildebrandt's "D" medium (Hildebrandt *et al.*, 1946), containing 2,4-D and modified to include chelated Fe instead of Fe tartrate, was observed to be most suitable for somatic callus growth. On all the media, endosperm tissue did not grow and intercalary meristem tissue gradually became necrotic. Shimada et al. (1969) observed callus formation from seedling roots of einkorn wheat (*Triticum monococcum*, 2n=14), emmer wheat (*T. dicoccum*, 2n=28) and common wheat (*T. aestivum* 2n=42). White's solid basal medium, supplemented with 2,4-D or IAA, was used, and the best callus growth was noted when coconut milk or casein hydrolysate was added to the medium. Two plants of common wheat were restored to maturity from callus. Examination of the root cells of these plants showed that most

of the cells contained the normal chromosome number (2n=42). Schenk and Hildebrandt (1971) formulated a culture medium suitable for a wide range of monocots and dicots. They found that monocots in general required auxins for callus growth while dicots required low levels of cytokinin. Wheat showed only fair growth on this medium. Sheridan (1973) induced and subcultured callus using Linsmaier and Skoog's basal medium (Linsmaier and Skoog, 1965) with the basal salts modified, and supplemented with 2,4-D. The addition of casein hydrolysate to this medium produced the greatest increase in size while yeast extract was clearly inhibitory, with or without casein hydrolysate. Dudits *et al.* (1975) obtained callus from root tips, seedling shoots, and rachis tissue. Their defined "T" medium and Gamborg's B-5 medium (Gamborg and Eveleigh, 1968) were used. They found that the auxins 2,4-D, Benazolin, Banvel D (Dicamba), and 2,4,5-Trichlorophenoxyacetic acid were effective in callus initiation and growth. Various cytokinins inhibited callus growth but prompted root production. Plantlets were found on some shoot and rachis calli in the presence of auxins plus cytokinins.

One objective of particular interest in this laboratory is the production of mutant crop plants with tolerance to abnormally high levels of sodium chloride. A fast-growing, friable callus is desirable for use in further tissue culture studies toward this objective. The experiments described in this report were aimed toward obtaining optimum wheat callus growth from seed on a solid medium.

MATERIALS AND METHODS

Seeds of *Triticum aestivum* var. Chris were used for callus initiation Seeds were soaked in 95% ethanol for 10 seconds, then in a solution of 20% Clorox, plus a few drops of Tween 20, for 30 minutes. This was followed by three quick rinses in sterile distilled water, then one 10-minute soak in sterile distilled water.

Under sterile conditions the seeds were implanted, groove side down, in 25-ml screw-top vials containing 10 ml of medium. Duration of the primary callus experiments ranged from 34 days to 121 days.

Primary calli from these experiments were used as inoculum for the secondary callus studies. Shoot tissue from seed germination was excised and discarded during transfer. In the experiment involving secondary callus growth on media containing kinetin plus NaCl, initial callus mass was approximately .09 g, and tissue was transferred to the vials with 10 ml of medium. In all other secondary callus experiments, initial mass was approximately .8 g and 70-ml jars with 20 ml of medium were used. Duration of secondary callus experiments ranged from 28 days to 94 days.

All cultures were kept under continuous fluorescent light (approximately 250 foot-candles) at 25 C.

The basal medium consisted of minerals according to Linsmaier and Skoog (1965), .4 mg/litre thiamine HCl, 100 mg/litre myoinositol, 50 mg/litre Fe-EDTA, and 1% agar. Sucrose was 2% in all media unless otherwise noted. The pH was adjusted to 5.5 with NaOH before the medium was autoclaved at 120 C for 20 minutes.

Callus initiation (primary callus) experiments and secondary callus experiments were performed on media with no NaCl and, less extensively, on media containing NaCl. Unless otherwise noted, there were ten replications per treatment.

In earlier experiments using indole-3-acetic acid (IAA) in concentrations of .1 to 40 mg/litre, callus initiation was very poor. IAA was not further investigated in primary callus experiments.

For callus initiation -naphthaleneacetic acid (NAA) was tested at levels of 2, 5, 10, 20, and 40 mg/litre; twenty replications per treatment were used in this experiment. 2,4-dichlorophenoxyacetic acid (2,4-D) was used in various experiments at .1, .5, 1, 2, 5, 10, 20, and 40 mg/liter. One experiment, with twenty replications per treatment, tested 2,4-D at 2, 5 and 10mg/litre, with or without the addition of kinetin at .5 and 5 mg/litre; casein hydrolysate at 1 and 10 g/litre together with extra amino acids (see below); or extra vitamins at 1 and 10 norm (see below). Sodium chloride at 1, 2, 3, 4, 5 and 6 g/litre was added to medium containing 2,4-D at 2 and 5 mg/litre. In three separate experiments which were later compared, callus was initiated on media with 2,4-D at 2 mg/litre plus NaCl at 3, 4, and 5 g/litre. There were 300 replications for each of these.

The extra amino acids consisted of tryptophan, glutamine, asparagine, serine, threonine, and tyrosine, each at 35 mg/litre. The extra vitamins (1 norm) consisted of Vitamin B_6 at .1 mg/litre; nicotinic acid at .5 mg/litre; calcium at 1 mg/litre, and pantothenic acid at 1 mg/litre.

Sucrose was tested at 1, 2, and 4 per cent for both primary callus and secondary callus in 2,4-D medium. There were twenty replications per treatment in both sucrose experiments.

Secondary callus growth was tested in media containing 2,4-D at 2 and 5 mg/litre with or without vitamins at 1 norm. The effects on secondary callus were compared in one experiment using kinetin alone at .5 mg/litre, and the auxins--2,4-D at 2, 5, and 10 mg/litre; and IAA and NAA each at

2, 10, and 20 mg/litre--alone or in combination with vitamins at 1 norm; casein hydrolysate at .5 g/litre with extra amino acids at 1 norm; or kinetin at .5 mg/litre.

For the study of the effect of NaCl on secondary callus, 5 g/litre NaCl was added to medium containing 2,4-D at 2 and 5 mg/litre, with or without vitamins at 1 norm. Also NaCl at 1, 2, 3, 4, 5, and 6 g/litre was added to medium with or without kinetin at .05 mg/litre.

For the collection of callus data in this laboratory, a set of eighteen clay callus models were made, ranging in mass from .019 g to 20.507 g. With the smallest as a standard, the ratio of masses ranged from 1.0 to 1079.3. Each callus, still in its container, was compared with the clay models, and the ratio of the model most nearly the size of the callus was recorded. A ratio of 1.0 is equivalent to .003 g of soft, friable callus.

Secondary calli were measured in this manner, and the increase in size was tabulated and expressed as per cent gain. Thus a 50 per cent gain would be expressed for a callus with an initial mass ratio of 300.0 and a final mass ratio of 450.0.

RESULTS AND DISCUSSION

Callus forms along the roots of the wheat seedling approximately one week after germination. All during growth the callus tends to remain friable and white or cream colored.

Fig. 1 compares the growth patterns of primary callus with 2,4-D at 2 and 5 mg/litre. Fig 2 compares these patterns at sucrose levels 1, 2, and 4 per cent. From these two illustrations it appears that 2,4-D at 2 mg/litre, with sucrose at 2% is the combination producing the best growth rate. During the early parts of callus initiation experiments it was noticed that there was little or no callus formation during the

first seven to ten days after the seeds were planted. With this growth lag in mind, and from Figs. 1 and 2, the conclusion may be drawn that under good growing conditions the primary growth pattern in wheat callus follows a sigmoid curve.

Fig. 3 shows the effects of increasing levels of 2,4-D. Growth is inhibited at high 2,4-D levels (10 mg/litre and higher). At sucrose level 2%, the best 2,4-D level is 2 mg/litre, consistent with Figs. 1 and 2. At 4% sucrose, this graph shows 5 mg/litre 2,4-D to be optimal, thought this is contradicted by Figs. 1 and 2. Since Fig. 2 shows consistently suboptimum growth for 4% sucrose at 5 mg/litre 2,4-D, it is still indicated that 2 mg/litre of 2,4-D is optimal with sucrose at 4% as well as 2%.

The effects of increasing levels of NAA are shown in Fig. 4. Excellent growth is observed with NAA at 10 mg/litre and higher--growth is in fact comparable to growth at the optimal 2,4-D concentration discussed above. It appears that there is little or no change in effectiveness as NAA concentration is increased beyond 10 mg/litre.

Fig. 5 illustrates the effect of adding kinetin, vitamins, or casein hydrolysate plus extra amino acids, to 2,4-D medium. While stimulatory effects are not pronounced, it does appear that growth is promoted somewhat with exogenous vitamins, 1 norm being sufficient. The addition of extra amino acids and casein hydrolysate stimulates growth at 1g/litre casein hydrolysate. growth is inhibited by casein hydrolysate at 10 g/litre, and by kinetin at 5 mg/litre. Kinetin at .5 mg/litre seems to have little or no primary effect on primary callus.

Best sucrose level for secondary callus growth is 2%. (Fig. 6), though the difference shown between 2% and 4% is small.

It was found that with exogenous vitamins at 1 norm, secondary callus growth is clearly inhibited with 2,4-D at 2 mg/litre, and less

so with 2,4-D at 5 mg/litre (Fog. 7).

Fig. 8 compares the effects of adding vitamins, casein hydrolysate plus extra amino acids, or kinetin to the auxins 2,4-D, IAA or NAA. Kinetin alone is also tested. The concentrations of these additions are specified in MATERIALS AND METHODS. For secondary callus the most suitable auxin with no additions seems to be IAA at 10 mg/litre. While NAA stimulates primary callus growth, it is poor for secondary callus. 2,4-D is fairly stimulatory. Exogenous vitamins greatly increase NAA's effectiveness, while growth is impaired with vitamins plus 2,4-D or IAA. There seems to be little or no effect when casein hydrolysate and extra amino acids are added to 2,4-D medium, but the effectiveness of IAA and NAA is increased. Kinetin stimulates growth with 2,4-D at 5 mg/litre, and is ineffective or slightly inhibitory with the other media. Kinetin alone is fairly stimulatory.

Considerable root proliferation was noted on media containing IAA alone (most roots forming at 2 mg/l IAA), kinetin alone, and IAA plus kinetin. Roots were found in moderate amounts with NAA plus vitamins (most roots form0 ing at 2 mg/litre NAA). Roots were almost entirely absent with all 2,4-D media. The roots were long and filamentous with some greening--there was more greening with more root production.

There were no distinct, viable plantlets observed in any of the media.

The results of primary and secondary callus experiments with NaCl are shown in Figs. 9, 10, and 11.

In Fig. 9 it is shown that primary callus growth decreases fairly steadily as NaCl concentration is increased. The detrimental effect of NaCl is more pronounced at 5 mg/litre 2,4-D than at 2 mg/litre 2,4-D.

Fig. 10 shows growth patterns of primary callus with 2,4-D at m mg/litre and NaCl at 3, 4, and 5 g/litre. As would be expected, callus at the lowest (3 g/litre) NaCl concentration follows most nearly the sig-

moid pattern. From this graph it appears that NaCl at 4 and 5 g/litre essentially affects primary callus to the same extent. Before a definite conclusion can be drawn, however, a similar experiment should be performed on media containing NaCl, and the growth observed at several intervals.

In the NaCl experiments with secondary callus, there was little or no difference between 2 mg/litre and 5 mg/litre 2,4-D for callus growth. Exogenous vitamins seemed to have no influence on growth. Kinetin apparently helps callus growth somewhat with NaCl present (Fig. 11) and has no influence, or a slightly inhibitory influence, on growth in the absence of NaCl. The closeness in growth responses of calli from each NaCl series in Fig. 11 suggests that for secondary callus it is the presence of NaCl, more important than the amount, that influences growth.

From these studies it is strongly indicated that 2,4-D at 2 mg/litre and 2% sucrose in the modified Linsmaier-Skoog medium will produce a healthy wheat callus from seed. NAA at 10 mg/litre can replace the 2,4-D with equal or possibly better results.

For maintenance of secondary callus, IAA at 2 to 10 mg/litre with casein hydrolysate at .5 g/litre, or IAA alone at 10 mg/litre, is recommended, based upon evidence presented here.

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LIST OF FIGURES

Figure 1. . . Growth patterns of primary callus at 2,4-D 2 mg/litre and 5 mg/litre. Standard errors are shown in one direction only.

Figure 2. . . Growth patterns of primary callus at 2,4-D 2 mg/litre and 5 mg/litre; sucrose 1%,2%,4%. Standard errors are shown in one direction only.

Figure 3. . . Effects of varying concentrations of 2,4-D on primary callus growth at sucrose 2% and 4%. Standard errors are shown in one direction only.

Figure 4. . . Effects of varying concentrations of NAA on primary callus growth. Standard errors are shaded.

Figure 5. . . Effects of exogenously-added organic constituents on primary callus growth. Standard errors are shaded.

Figure 6. . . Effects of sucrose levels 1%, 2%, and 4% on secondary callus growth.

Figure 7. . . Effects of exogenous vitamins on secondary callus growth at 2,4-D 2 mg/litre and 5 mg/litre.

Figure 8. . . Effects of exogenously-added organic constituents on secondary callus growth. See text in MATERIALS AND METHODS for clarification of treatments.

Figure 9. . . Effects of varying concentrations of NaCl on primary callus growth at 2,4-D 2 mg/litre and 5 mg/litre.

Figure 10 . . Growth of primary callus at NaCl 3 g/litre, 4 g/litre, and 5 g/litre.

Figure 11 . . Effects of varying concentrations of NaCl on secondary callus growth with and without kinetin.

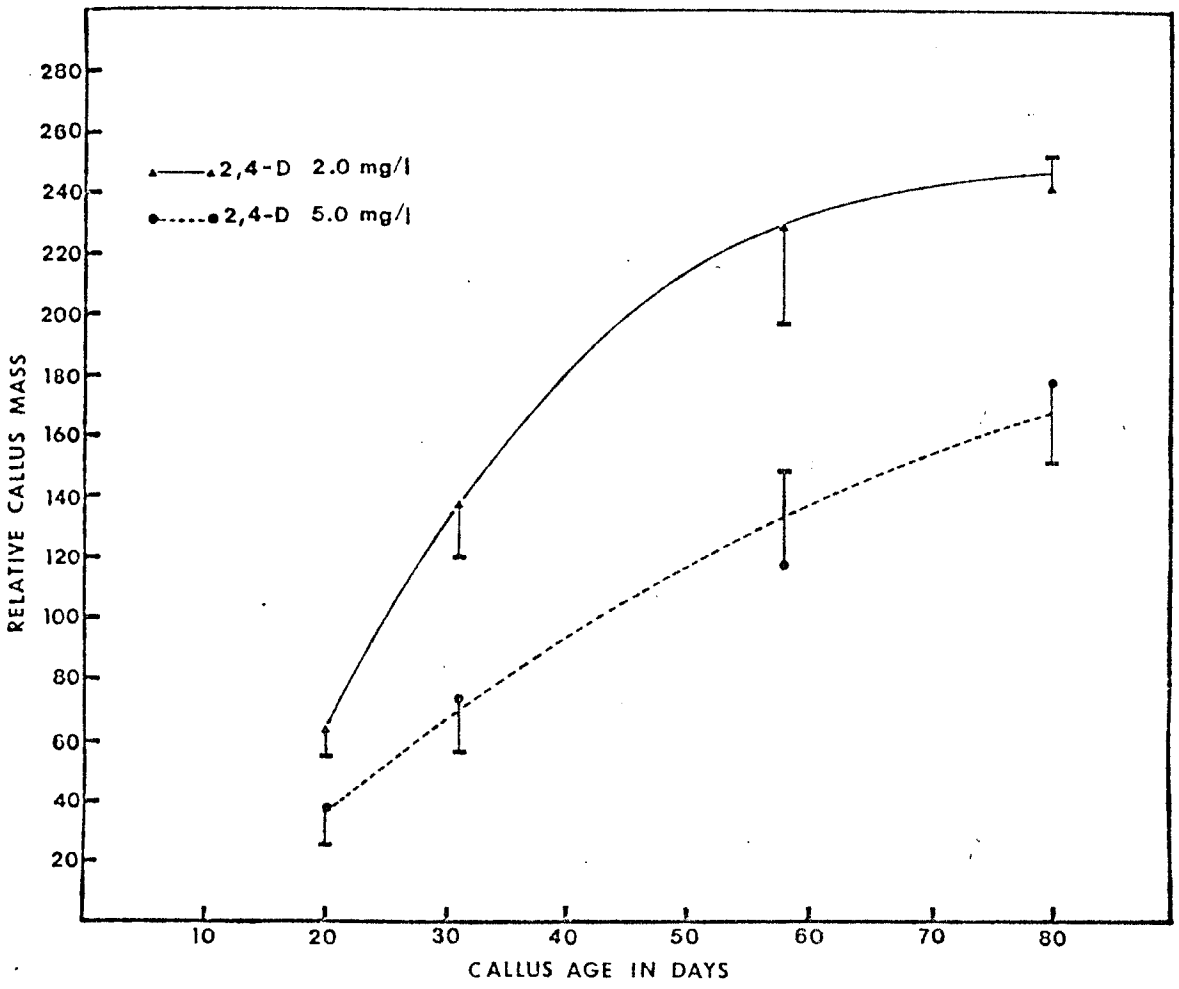

2,4-D 2.0 mg/l
2,4-D 5.0 mg/l
RELATIVE CALLUS MASS
280
260
240
220
200
180
160
140
120
100
80
60
40
20
10
20
30
40
50
60
70
80
CALLUS AGE IN DAYS

Fig. 1

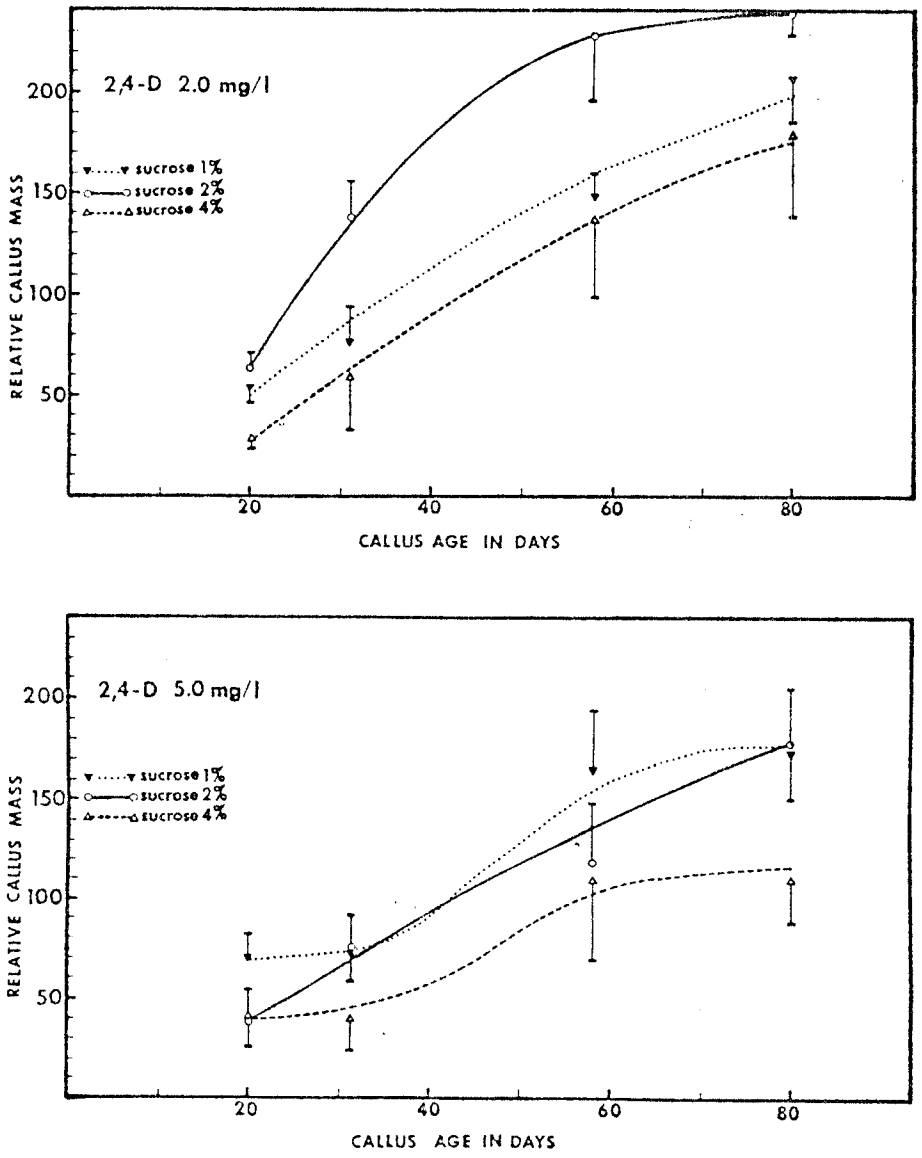

2,4-D 2.0 mg/l
sucrose 1%
sucrose 2%
sucrose 4%
RELATIVE CALLUS MASS
200
150
100
50
20
40
60
80
CALLUS AGE IN DAYS
2,4-D 5.0 mg/l
sucrose 1%
sucrose 2%
sucrose 4%
RELATIVE CALLUS MASS
200
150
100
50
20
40
60
80
CALLUS AGE IN DAYS

Fig. 2

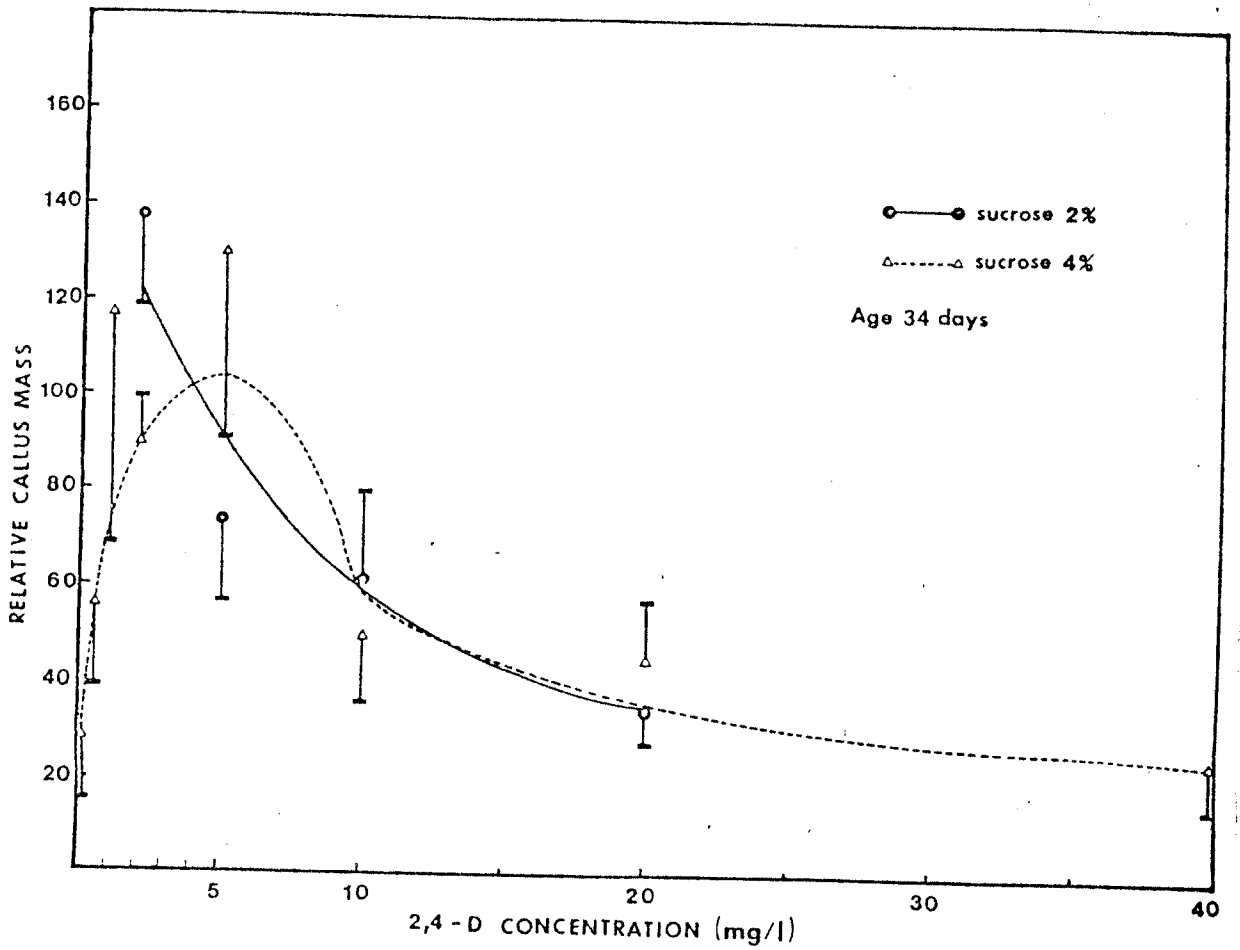

RELATIVE CALLUS MASS
160
140
120
100
80
60
40
20
5
10
20
30
40
2,4 - D CONCENTRATION (mg/l)
sucrose 2%
sucrose 4%
Age 34 days

Fig. 3

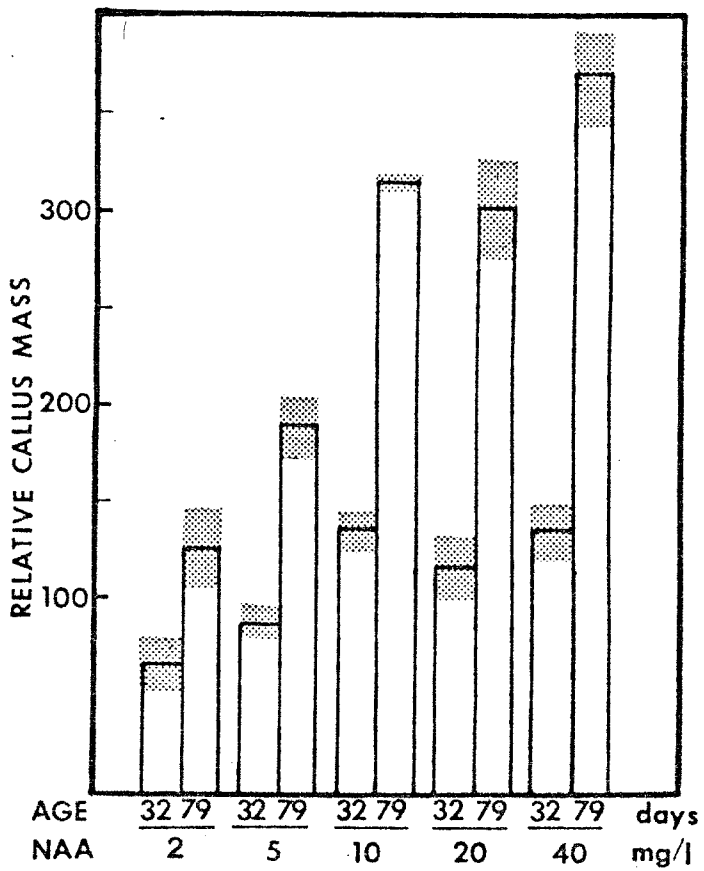

RELATIVE CALLUS MASS
300
200
100
AGE
32 79
32 79
32 79
32 79
32 79
days
NAA
2
5
10
20
40
mg/l

Fig. 4

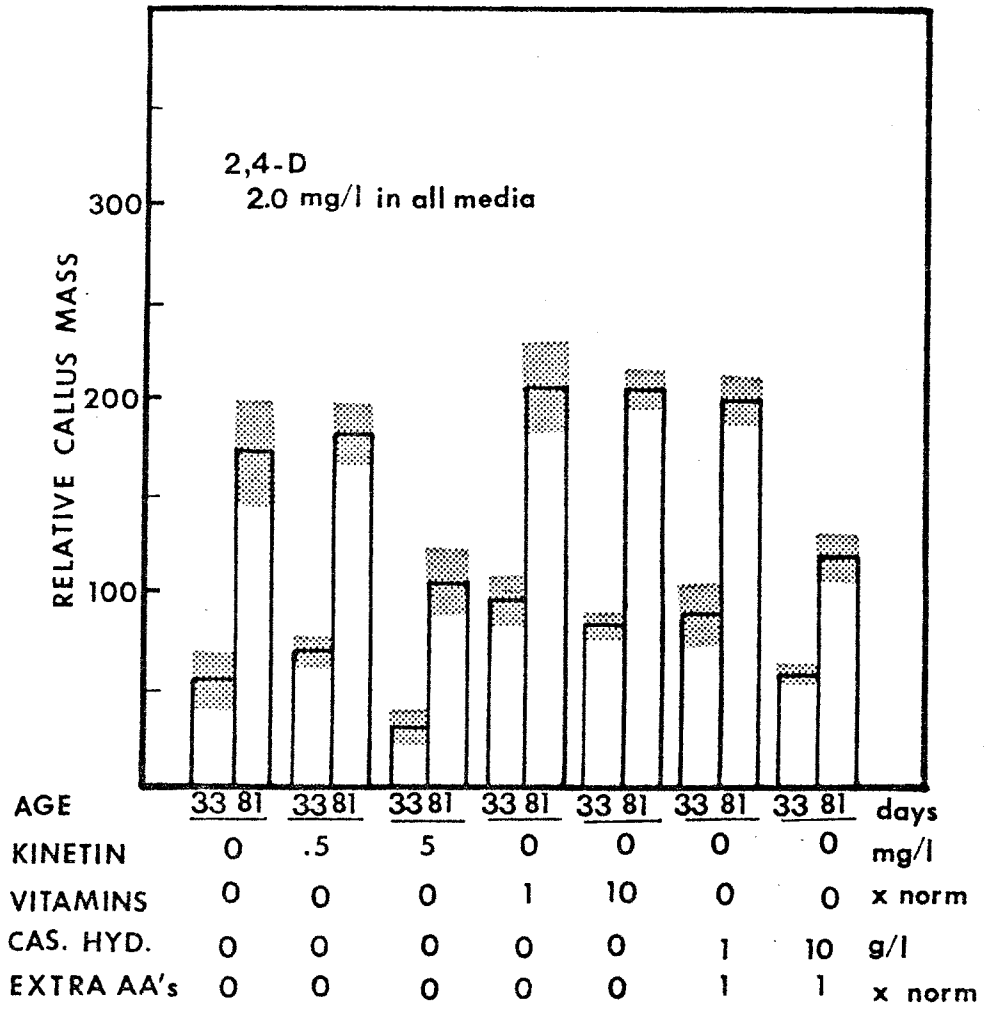

2,4-D
2.0 mg/l in all media
RELATIVE CALLUS MASS
300
200
100
AGE
33 81
33 81
33 81
33 81
33 81
33 81
33 81
days
KINETIN
0
.5
5
0
0
0
0
mg/l
VITAMINS
0
0
0
1
10
0
0
x norm
CAS. HYD.
0
0
0
0
0
1
10
g/l
EXTRA AA's
0
0
0
0
0
1
1
x norm

Fig. 5

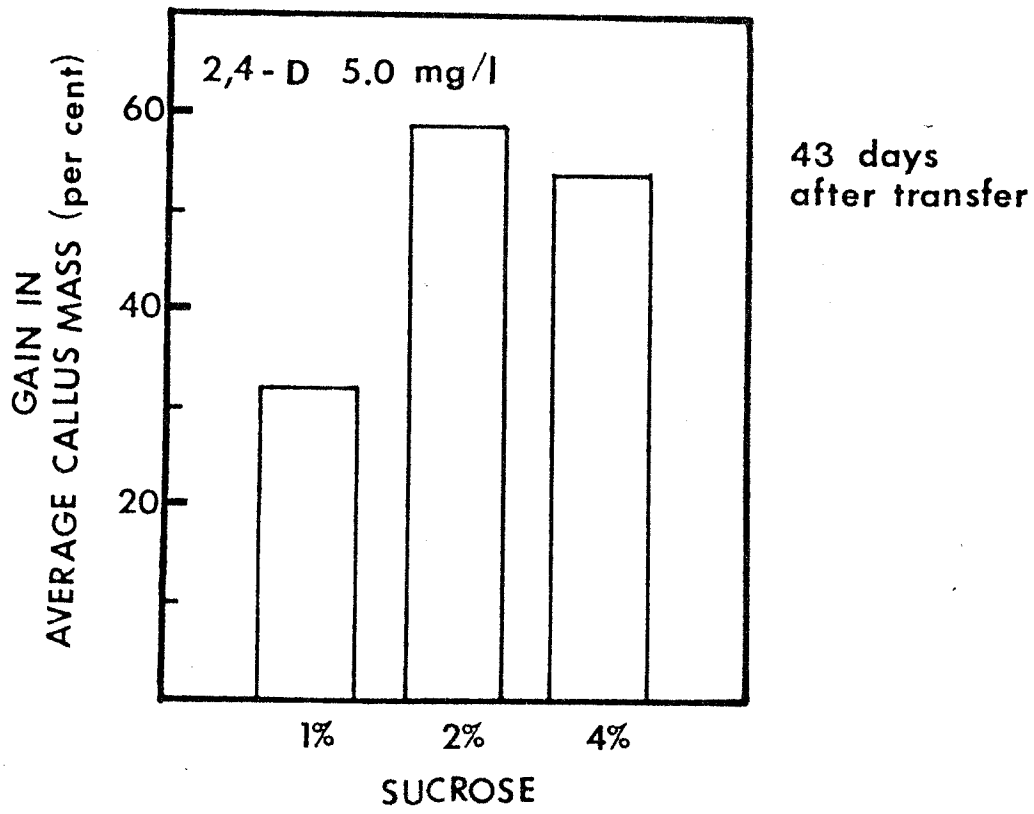

2,4 - D 5.0 mg/l
60
40
20
GAIN IN
AVERAGE CALLUS MASS (per cent)
1%
2%
4%
SUCROSE
43 days
after transfer

Fig. 6

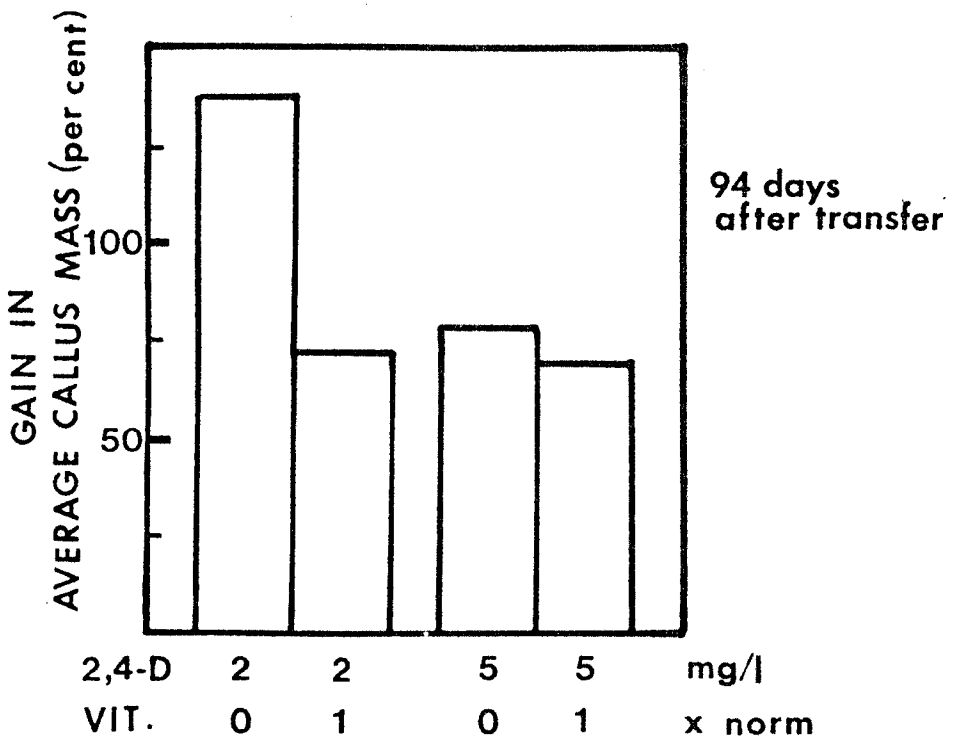
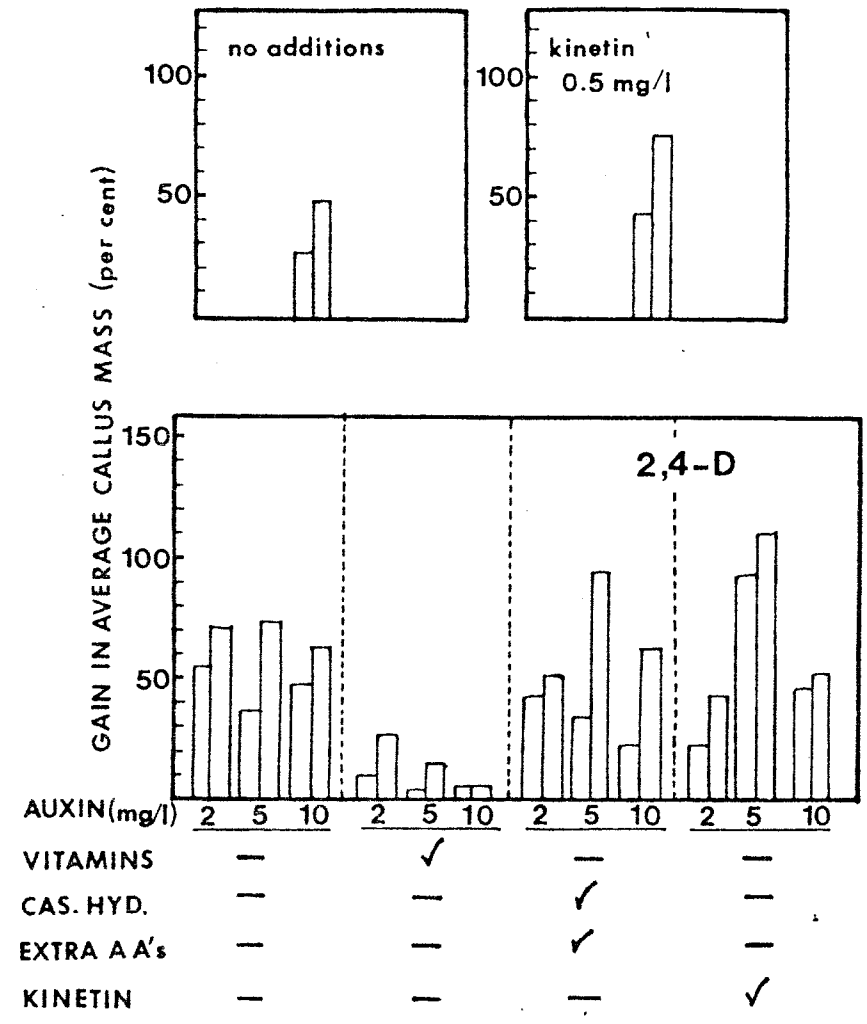
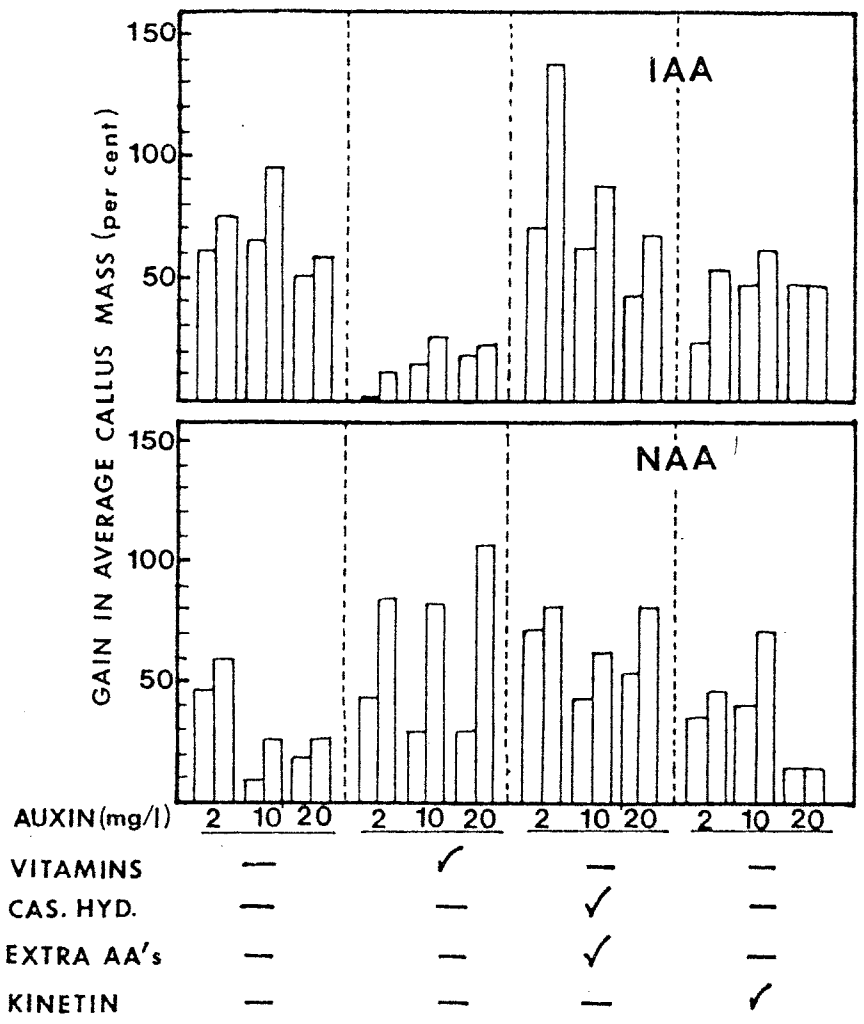

GAIN IN
AVERAGE CALLUS MASS (per cent)
100
50
94 days
after transfer
2,4-D 2 2 5 5 mg/l
VIT. 0 1 0 1 x norm

Fig. 7





NOTE: For each treatment, callus growth is shown at 31 days after transfer and at 67 days after transfer.

Fig. 8

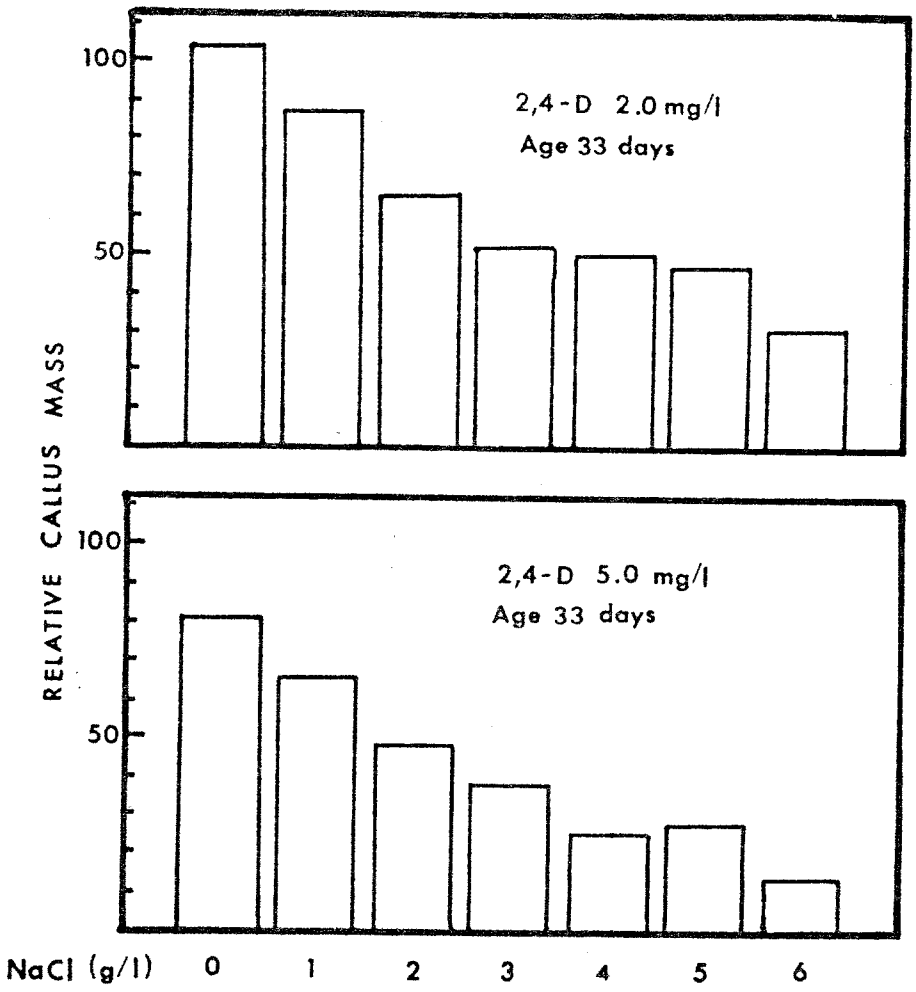

RELATIVE CALLUS MASS
100
50
2,4-D 2.0 mg/l
Age 33 days
100
50
2,4-D 5.0 mg/l
Age 33 days
NaCl (g/l) 0 1 2 3 4 5 6

Fig. 9

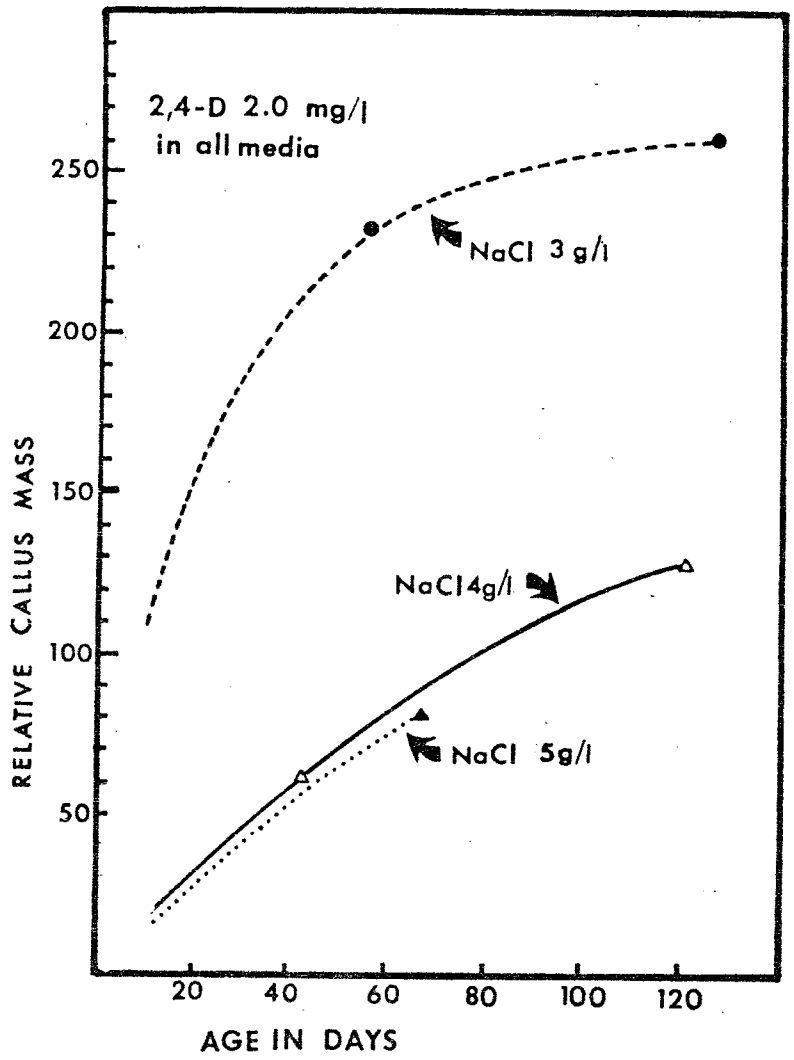

2,4-D 2.0 mg/l
in all media
NaCl 3 g/l
NaCl 4g/l
NaCl 5g/l
RELATIVE CALLUS MASS
250
200
150
100
50
20 40 60 80 100 120
AGE IN DAYS

Fig. 10

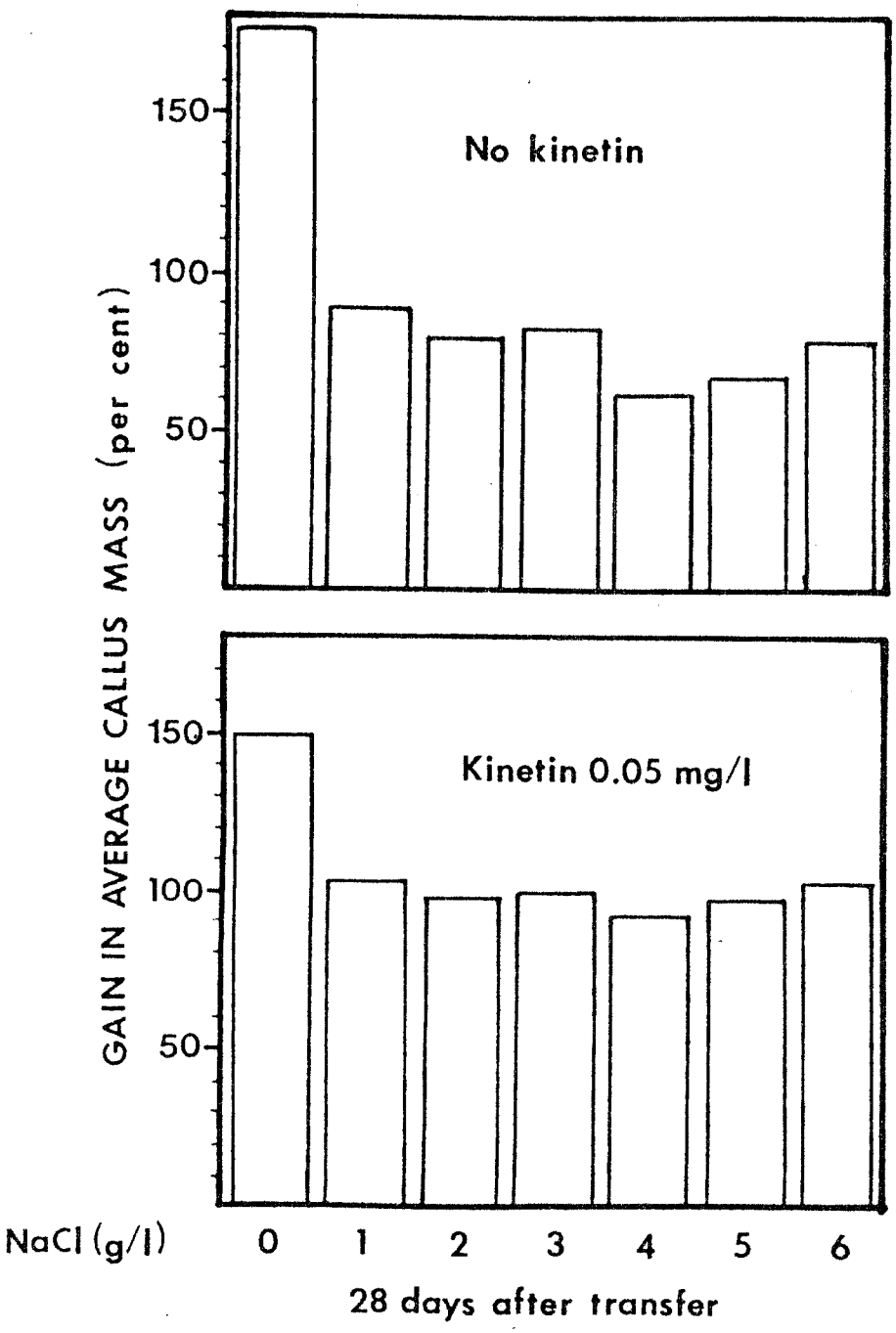

No kinetin
Kinetin 0.05 mg/l
150
100
50
GAIN IN AVERAGE CALLUS MASS (per cent)
NaCl (g/l)
0
1
2
3
4
5
6
28 days after transfer

Fig. 11

NaCl-Tolerant Tobacco Plants From Cultured Cells

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 F_2 plants survive watering with solutions containing more than 15.4 g/l NaCl whereas most tolerant plants survive 33.4 g/l NaCl.

Keywords: tissue culture, salt tolerance, mutation, plant breeding

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. Gengenback 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 world-wide 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. Samsun 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 re-

sistant 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 regneration 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 indileacetic 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 F_1 generation. F_1 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 F_1 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 F_1 plants of the three groups watered with a salt-free solution were planted to obtain the F_2. Groups of F_2 plants were salt-stressed at various levels as for the F_1. Data at two levels of NaCl are shown in Table 1. Figure 1 shows two groups of F_2 plants under various levels of salt stress.

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 F_1 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 nonstressed plants so that both salt-sensitive and salt-tolerant plants survived. Data of our F_1 and F_2 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 F_1 and F_2 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 F_2 plants are noticeably more salt tolerant if seeds are collected from F_1 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 occurred in F_1 plants. (2) A high reverse mutation rate could be promoted by the absence of NaCl. (3) The F_1 plants could be chimerical, composed of salt-tolerant and salt-sensitive cells. Thus selection would still occur in F_1 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 enyyme of some other physiological adptation 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.

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.

Murray W. Nabors

Susan E. Gibbs

Carol S. Bernstein

Mary E. Meis

Department of Botany and Plant Pathology

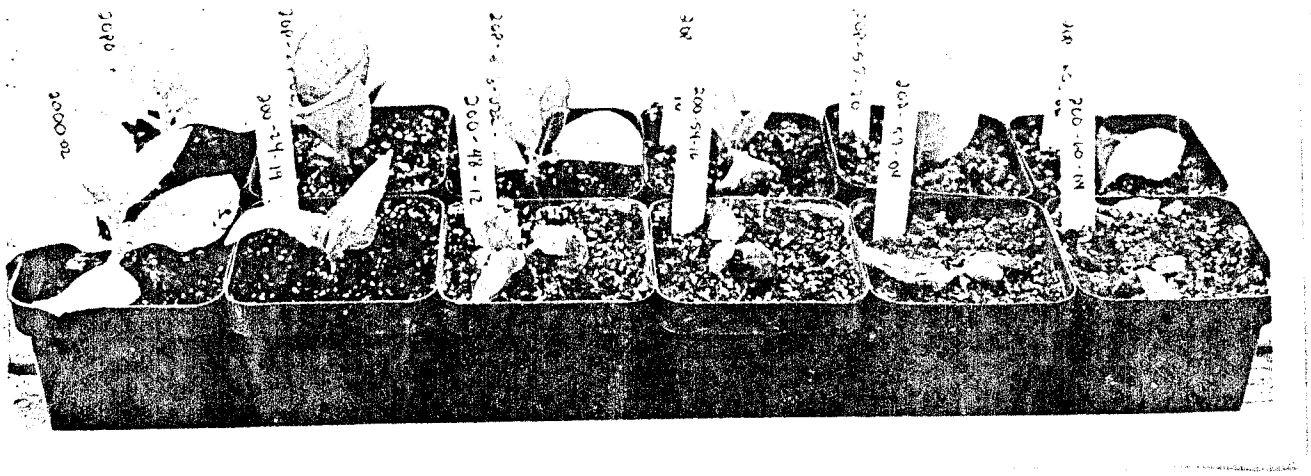
Colorado State University

Ft. Collins, Colorado 80523

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 F_1 generation. The F_1 plants of each group in the o g/1 NaCl column were selfed to obtain the F_2 generation. F_1 had 43 plants per groups. F_2 had 20.

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

Fig. 1. Typical F_2 plants derived from non-NaCl-tolerant cultures (front row) and NaCl-tolerant cultures always exposed to salt (back row). The watering solution contained from 0.0 (left) to 27.8 g/l NaCl. Plants are 13 weeks old.



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Acknowledgements

This work was partially supported by consecutive grants from the Agency for International Development and the Office of Water Resources Technology.

ABSTRACT OF DISSERTATION

THE GROWTH AND OSMOTIC ADJUSTMENT OF SODIUM CHLORIDE-ADAPTED AND NON-ADAPTED TOBACCO CELL CULTURES AND PLANTS

Tobacco cultures (*Nicotiana tabacum* var. Samsum) were grown on increasing levels of NaCl to select mutations for increased salt-tolerance. This dissertation research studied the growth and osmotic adjustment of NaCl-adapted and NaCl-sensitive cell lines. Both cell lines were grown on media with NaCl, KCl, or the non-penetrating solutes polyethylene glycol (PEG) or dextran (15,000 to 20,000 M.W.). Few differences were found between the response of these two cell lines to these salts and organic osmotica. Other data obtained from breeding experiments with the progeny of regenerated plants showed some evidence for salt-tolerant mutations.

The growth of non-adapted cells in suspensions was inhibited when they were shifted to media containing 48 mM or greater NaCl, while cells adapted to growth on lower NaCl levels generally showed less growth-inhibition when shifted to higher NaCl levels. Both NaCl-adapted and non-adapted cell lines showed equal inhibition of growth on 130 mM NaCl on solid media but no inhibition on 130 mM KCl as compared with basal medium. Growth was more inhibited on either PEG 4000 or dextran isismotic to 130 mM NaCl. Growth during the second passage on dextran ceased for the non-adapted cells, while the NaCl-adapted cells grew slowly through five passages. Water contents for both cell lines were 95 percent on basal medium, NaCl, or KCl, and 70 to 88 percent on PEG or dextran.

Concentrations of sugars, Na^+, α-amino-nitrogen, Cl^-, and NO_3^- were identical in the cells and medium. Potassium was accumulated by the cells.

These solutes accounted for 80 to 90 percent of the osmotic potential for the cells of both lines growth on basal medium, NaCl, or KCl. On PEG or dextran, these solutes accounted for 28 to 70 percent of the cellular osmotic potential. The osmotic potential of growing cells was always 1 to 3 bars (40 to 120 milliosmoles/l) more negative than that of the medium.

During the first 10 days of culture on all media, the cells hydrolyzed the 117 mM sucrose in the medium, and the media became more negative by around 3 bars. Growing cells absorbed and metabolized the sugars, NH_4^+, and NO_3^- during the next 25 days, and the osmotic potential of the medium and cells became less negative. The addition of 130 mM NaCl, Kcl or isosmotic PEG or dextran made the media and cells osmotically more negative by 6 bars throughout the growth cycle as compared to cells growing on basal medium.

In whole plants, proline and amino acid levels rose when plants were watered with 260 or 500 mM NaCl. The leaves accumulated Na^+ and Cl^- until these ions represented 62 to 68 percent of the osmotica.

The efflux of solutes into distilled water washes was resolved into two components. The fast component (0.6 to 1.7 minutes half-time) included solutes of the free space and cytoplasm, while the slow component (1.6 to 4.9 hours half-time) represented the vacuolar solutes. No differences between the cell lines were observed in solute efflux.

James W. Heyser
Dept. of Botany and Plant Pathology
Colorado State University
Fort Collins, Colorado 80523
September, 1979