SALT- AND DROUGHT-TOLERANT CROP PLANTS FOR WATER CONSERVATION

by

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by

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ABSTRACT

The potential of tissue culture to aid agriculture has been realized and promoted for around fifteen years. This report (and two earlier reports, Nos. 73 and 96) summarizes our research efforts to actualize this potential. The purpose of this report is to summarize the accomplishments of the past two years in addition to the progress already discussed in the earlier reports. The Appendices contain copies of papers and manuscripts prepared during the entire period of OWRT support.

Tissue culture has been on the boundary between basic and applied research for some time. We believe our results demonstrate substantial movement toward actual application. We also hope that this report can serve not only as a current summary statement but also as a basis for future progress in rapidly obtaining varieties of crop plants with improved water utilization characteristics.

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 (4) 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 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 occur.

Tissue culture has the potential to provide a methodology for rapidly incorporating various types of stress tolerance into agriculturally useful crop plants. Large numbers of cells can be economically grown in a very small volume. Since each cell has the genetic potential to produce an entire plant, stress tolerance selection procedures can easily produce plants which can be tested for persistence and inheritability of tolerance.

Some people have promoted the idea that tissue culture can serve as a source of magic solutions for agricultural problems. This is certainly not the case, and an over optimistic promotion of tissue culture has already led to disappointment and skepticism on the part of many. On the other hand it is clear from the work reported here as well as from the efforts of other workers that systematic investigations in tissue culture can provide tools of extreme value to workers interested in water conservation. In the long run, altering the plant to suit available environments makes far more sense than the inevitably energy intensive attempts to alter the environment to suit available plants. It is increasingly obvious that the ability of tissue culture to grow millions of plants and to select desirable variants in a single small flask will be a useful tool in the effort to utilize water more efficiently.

The purpose of the research was to perfect tissue culture methods for several plants and to demonstrate that they can, in fact, be used to produce useful plants with increased levels of salt and of drought tolerance.

The research discussed in this report and in the two previous reports (Nos. 73 and 96) clearly demonstrates the following points.

1. Available tissue culture methodology can be used to rapidly select NaCl-tolerant cell lines and to regenerate whole plants from these lines in tobacco, oats, and wheat.

2. Salt tolerant cell lines have been selected in oats and tobacco.

3. Plants can be regenerated from these cell lines.

4. In tobacco, the regenerated plants are salt tolerant and pass this tolerance onto two generations of offspring.

5. In tobacco, regenerated tolerant plants give rise to tolerant callus if returned to tissue culture.

6. In tobacco, salt tolerance selected in tissue culture is stable in the absence of NaCl for periods of up to six months.

7. Greenhouse testing to demonstrate 4-6 is nearly complete for tobacco.

8. In oat tissue cultures regeneration is correlated with the appearance of green spots on populations of cultured cells (calli) and can be considerably enhanced if such populations are used in subculturing procedures.

9. Regenerated oat plants require a short period of exposure to high temperature (30C) to properly establish themselves in pot culture. They then require exposure to lower temperatures (around 20C as is well known) during the remainder of the life cycle to set seed.

10. Greenhouse testing of oat and wheat plants regenerated from salt tolerant cultures is now underway.

11. Initial investigations on the mechanism of salt tolerance in cultured cells and regenerated plants have been published.

In summary, this research has demonstrated the actual usefulness of tissue culture for selecting stress tolerance in crop plants. Available methodology has been improved and a considerable body of new methodology has been established. The movement of tissue culture's possibilities from the potential to the actual has begun. Continued research should rapidly result in useful field applications.

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.

In the world as a whole sufficient food is unavailable because of several factors: (1) lack of crop varieties which can yield suitable returns on available arable land; (2) lack of energy and money to modify soil and water conditions (as well as other environmental variables such as disease) so that suitable production can occur; (3) a genuine, absolute lack of water; (4) unsuitable soil conditions; particularly excesses of sodium (in dry regions) and aluminum and other metallic ions (in wet regions); (5) inequitable political systems and the lack of equitable land ownership. In the western United States an excess of sodium and a lack of water are the predominant physical realities limiting agricultural production.

At first glance it is unclear why investigators interested in efficient water utilization should not concentrate on irrigation improvement and other directly water related technologies in terms of the agricultural aspects of water use. A little thought, however indicates that the 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. Dwindling water supplies in many underground aquifers also results in increased production costs to pump available water and the very real threat of decreased production in the future. 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 Imperal 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 or to specific selection for salt or drought tolerance 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 situation.

GENERAL OBJECTIVES OF THE 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 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.

C. 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 continuing research of the past two years has succeeded in demonstrating conclusively the following points:

1. A trait related to efficient water utilization--namely NaCl tolerance--can be selected in populations of cultured cells. And the cells can be regenerated into whole plants which also carry the trait and which pass it on to future generations. We were the first laboratory in the world to report in detail on the selection of salt-tolerant cell lines (a passing reference to salt tolerant cell lines was made in 1974 the year before our first paper--see Project Related Publications #1). We were the first laboratory in the world to achieve regeneration of plants from salt tolerant cultures and to show that the plants and their progeney were also salt tolerant (see Appendix I).

This research was carried out with tobacco because of the ease and convenience with which this plant is tissue cultured. Following this work we defined the tobacco system somewhat more (see below) and then rapidly moved on to tissue culture applications in food crop plants for which tissue culture and regeneration methods are more difficult.

2. NaCl-tolerance selected in cultured cells is stable--that is to say it remains in cultured cells and regenerated plants--even when there is no exposure to NaCl for a period of six months. It is important to demonstrate this point since the literature reveals that variant traits selected in cell cultures are not always stable in the absence of the selective factor. Variants selected in cell cultures can be of several types--point mutations, chromosomal mutations of several types, or gene duplications. None of these are inherently stable in the absence of

selective pressure. We feel that our NaCl tolerance is stable because (a) selection occurred for nearly a year and in some cases nearly two years; and (b) selection occurred at ever increasing levels of NaCl. Both of these factors would tend to "lock in" any genetic alteration by allowing the occurrence of secondary or additional alterations increasing the stability and the level of salt tolerance. The stability of our NaCl mutant tobacco cells and plants is a fact; the causes of stability are speculation and the subject of future research efforts. In terms of practical utility for agriculturalists and water conservationists it is of only periferal importance whether or not a salt tolerant variety retains tolerance in the absence of NaCl. If it does not, then breeding experiments and seed production in the field would always have to take place under saline conditions. Some of the experiments relating to the stability of salt tolerance selected in cultured cells are discussed in Appendix II.

3. Salt tolerant plants regenerated from tolerant cell populations are not salt requiring. In some cases cultured tolerant cells grow even more rapidly than non-tolerant controls in the absence of salt. These points are discussed in more detail in Appendices I and II. The second point has important possible applications for water utilization: If salt tolerant cells grow even more rapidly than salt sensitive cells when no salt is present for either group it should be determined if this characteristic carries over to whole plants. If so, then selection for salt tolerance at a high level would result in plants with increased vegetative yield in all conditions--no matter what the level of salt in soil or irrigation water. We hope to make this determination a subject of future research.

4. Experiments to determine the physiological and biochemical basis of NaCl tolerance in cells and plants have begun. Appendices III and IV detail these experiments. Of course a large literature exists on the mechanisms of salt tolerance, but few papers have delt with tolerance selected in cultured cells. Several important findings come out of this work to date. First, it is clear that tolerant cells adjust to the osmotic challenge of NaCl by taking it up into the vacuole but excluding it from the cytoplasm. Non-tolerant cells apparently take up NaCl into both regions of the cell, and thus do not survive. Further research is needed to conclusively demonstrate that this conclusion is correct (see Appendix III for current progress). Second "drought resistant" cell populations differ in several important ways from NaCl resistant populations. "Drought resistance" in cell cultures is obtained by exposing cells to high concentrations of a non-penetrating osmoticum like PEG-4000 or dextran. These osmotica compete with the cell for available water; thus, a cell which survives has an increased ability to take up water. This competition does not occur when NaCl is an osmoticum because the small molecular size of NaCl allows cells to rapidly take it up. Osmotically induced drought resistance has important possible applications if regenerated plants retain the characteristic. In nature drought resistance and salt tolerance are often found in the same plant, since both traits are useful in typical arid environments. There are some circumstantial indications that our NaCl tolerant plants are also drought resistant. This needs to be investigated in some detail in future research.

5. Salt tolerant cell populations can be obtained from an agriculturally useful grass (oats--Avena sativa) and potentially salt tolerant plants can be regenerated in large numbers for further testing. As mentioned above, tobacco has been frequently utilized in tissue culture studies because of the ease with which it is cultured and regenerated. Unfortunately, although of economic importance, tobacco is not a useful food crop plant. The most economically important food crops are all grasses, and until recently grasses have been somewhat difficult to use in tissue culture selection particularly because regeneration of plants was of short duration and low frequency. We felt it important, therefore, to improve tissue culture methods for a typical grass and to demonstrate that useful plants, in terms of water utilization efficiency, could be obtained. Apendices V and VI detail current progress to obtain salt tolerant oats from tissue cultures. First of all we have shown that the low frequency, short duration regeneration from tissue cultures which is characteristic of grasses can be regarded as a solved problem. High frequency, long term regeneration can be obtained either from callus cultures with green spots or with embryogenic regions. This means that a NaCl tolerance selection period sufficient to allow for stable variants can now be used to obtain useful variants from oat tissue cultures. Such regenerated plants are now in the greenhouse testing phase in our laboratory.

In 1976 we published a state-of-the-art report on the use of tissue culture to obtain agriculturally useful plants (see Appendix VII). This report is, in general terms, a similar report for 1981.

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) contain 10^7 cells, each one a potential plant if tissue culture techniques are correctly applied.

FUTURE RESEARCH AND LONG TERM OBJECTIVES

Future research should concentrate on the development and implementation of tissue culture methods to obtain increased efficiency of water utilization for agriculturally useful plants. This research should contain both practical and basic components to insure (1) that useful methods are pressed into practical applications as rapidly as possible and (2) that a continual supply of new techniques is available.

In terms of techniques currently available for utilization, the ability to obtain long term, high frequency regeneration from grasses should be utilized as rapidly as possible to obtain salt tolerant and drought tolerant varieties. Practically speaking this means increasing the stress tolerance of currently useful varieties which are deficient in these respects.

In terms of the development of new techniques, basic research should concentrate on determining whether populations of cells resistant to high molecular weight osmotica yield regenerated plants with improved drought tolerance. Also, experiments should continue to determine the physiological and biochemical bases for salt tolerance in cell populations and in regenerated plants.

In addition available tissue culture methods could be used to obtain heat tolerant variants and variants with increased efficiency of fertilizer utilization. Both mutant types would have utility in terms of water conservation, the first in terms of water utilization itself, and the second in terms of decreased water pollution due to increased efficiency and thus decreased application of fertilizers.

PROJECT PERSONNEL

1. Dr. Murray W. Nabors--Principal Investigator
2. James Heyser--graduate student, Post Doctoral Fellow
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8. Kelley O'Brien--technician
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11. Kim Bushnell-technician
12. David Kroskey--technician
13. Tom Dykes--technician
14. Karen Crane--undergraduate student

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. Zeits. fur Pflanzen. 97: 13-17 (1980).

8. J. W. Heyser and M. W. Nabors. Osmotic Adjustment of Cultured Tobacco Cells (Nicotiana tabacum var. Samsun) Grown on Sodium Chloride. Plant Physiol. 67: 720-727 (1981).

9. J. W. Heyser and M. W. Nabors. Growth, Water Content, and Solute Accumulation of Two Tobacco Cell Lines Culture on Sodium Chloride, Dextran, and Polyethylene Glycol. Plant Physiol. 68: 1454-1459 (1981).

REFERENCES AND FOOTNOTES

1. L. E. Brown. Global Food Insecurity. The Futurist 8: 56-64 (1974).

2. D. Pimentel, L. E. Hurd, A. C. Bellotti, M. J. Forester, I. N. Oka, O. D. Sholes, and R. J. Whitman. Food Production and the Energy Crisis. Science 182: 443-449 (1973).

3. I. Zelitch. Plant Productivity and the Control of Photorespiration. Proc. Nat. Acad. Sci. USA 70: 579-584 (1973).

4. H. E. Casey. Salinity Problems in Arid Lands Irrigation: A literature Review and Selected Bibliography. Arid Lands Resource Information Paper No. 1. Univ. Arizona Office of Arid Lands Studies. Tucson, AZ 85719.

5. N. E. Borlaug. The Green Revolution, Peace and Humanity. Speech delivered upon receipt of the 1970 Nobel Peace Prize. Reprint and Translation Series, No. 3. International Maize and Wheat Improvement Center, Mexico City, 1972.

6. W. Thorne. Agricultural Production in Irrigated Areas. In Arid Lands in Transition, H. E. Dregne, Ed. AAAS, Washington, 1970, pp. 31-56.

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

8. T. J. Flowers, P. F. Troke, and A. R. Yeo. The mechanism of salt tolerance in halophytes. Ann. Rev. Plant Physiol. 28: 89-121 (1977).

9. H. E. Dregne, in Aridity and Man, C. Hodge, Ed., AAAS, Washington D.C., 1963, p. 222.

10. M. Rebhun. Salinity Increments in Municipal Sewage, Their Significance and Sources and Methods of Reduction. Int. J. Air Wat. Poll. 9: 253-261 (1965).

11. Environmental Protection Agency. The Mineral Quality Problem in the Colorado River Basin. 1971.

12. D. W. Rains and E. Epstein. Preferential Absorption of Potassium by Leaf Tissue of the Mangrove, Avicennia marina: An Aspect of Halophytic Competence in Coping with Salt. Aust. J. Biol. Sci. 20: 847-857 (1967).

13. T. J. Flowers. Salt Tolerance in Suaeda maritima (L.) Dum. J. Exp. Bot. 23: 310-321 (1972).

14. P. A. LaHaye and E. Epstein. Salt Toleration by Plants: Enhancement with Calcium. Science 166: 395-396 (1969).

15. W. P. Kelley. Use of Saline Irrigation Water. Soil Science 95: 385-391 (1963).

16. E. Epstein and R. L. Jefferies. The Genetic Basis of Selective Ion Transport in Plants. Ann. Rev. Plant Physiol. 15: 164-184 (1964).

APPENDIX I

Überreicht vom Verfasser · Nicht einzeln im Buchhandel!
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NaCl-Tolerant Tobacco Plants from Cultured Cells

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With 1 figure

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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.

Key words: tissue culture, salt tolerance, mutation, plant breeding, tobacco.

Introduction

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

Specificion 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 resistant to as much as 8.8 g/l NaCl. Resistance to higher levels is not obtainable in our system. Shoots were regenerated from both NaCl-tolerant and NaCl-sensitive cultures by placing cell suspension aliquots on solid regeneration medium. Our regeneration medium consists of LINSMAIER and SKOOG's (1965) basic medium supplemented with 0.5 mg/l kinetin. Shoot regeneration is noticeably restricted on medium containing NaCl. Shoots were rooted by transfer to an identical medium that contained in addition either 5 or 10 mg/l indoleacetic acid (IAA). Reducing the level of major salts to half or tenth normal does not promote rooting in our system. Again, rooting is noticeably less vigorous in NaCl-containing medium.

Regenerated plants were removed from culture vials, potted in soil, and hardened in the lab for several weeks before transfer to the greenhouse. Three groups of plants were used: (1) Plants regenerated from NaCl-sensitive cultures and watered with solutions containing no salt; (2) NaCl-tolerant plants selected from cultures tolerant to 6.4 g/l NaCl, regenerated in the presence of NaCl, and watered with solutions containing the same level of salt; (3) Plants like those in group 2 but regenerated and rooted in medium containing no salt. Seeds from each group were collected and planted to obtain the 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

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 0 g/l NaCl column were selfed to obtain the F_2 generation. F_1 had 43 plants per group F_2 had 20.

	% alive after 13 weeks			
	F_1		F_2	
	Watering solution		Watering solution	
	26.2 g/l NaCl	0 g/l NaCl	29.8 g/l NaCl	0 g/l NaCl
Original culture #1 resistant to NaCl with NaCl 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

(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.

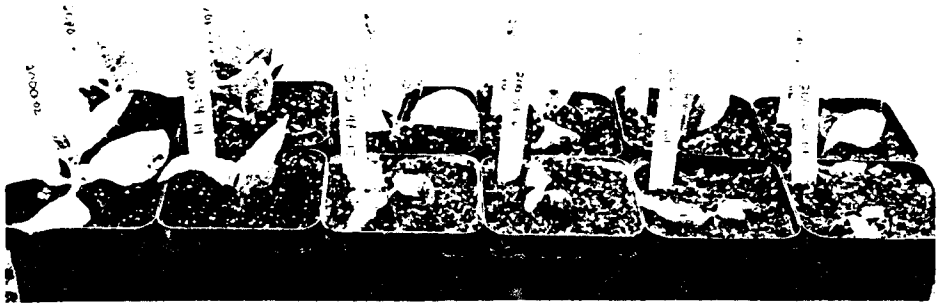

Fig. 1: Typical F_2 plants derived from non-NaCl-tolerant cultures (front row) and NaCl-tolerant cultures always exposed to salt (back row). The watering solution contained from 0.0 (left) to 27.8 g/l NaCl. Plants are 13 weeks old.

This kind of genetic analysis probably does not apply to salt tolerance in these plants for two reasons. First, the ratio of healthy to nonhealthy (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 enzyme or some other physiological adaptation maintained by the presence of NaCl. ALT et al. (1978) have reported on such a system involving duplication of a particular gene in cultured animal cell lines, although their system does not involve regenerated organisms. We consider the first and last possibilities to be most likely and thus candidates for future research.

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.

Acknowledgements

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References

ALT, F. W., R. E. KELLEMS, J. R. BERTINO, and R. T. SCHIMKE: J. Biol. Chem. *253*, 1357–1370 (1978).

CARLSON, P. S.: Science *180*, 1366–1368 (1973).

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

CHALEFF, R. S. and M. F. PARSONS: Proc. Natl. Acad. Sci. U.S.A. *75*: 5104–5107 (1978).

GENGENBACH, B. G., C. E. GREEN, and C. M. DONOVAN: Proc. Natl. Acad. Sci. U.S.A. *74*: 5113–5117 (1977).

LINSMAIER, E. M., and F. SKOOG: Physiol. Plant. *18*: 100–127 (1965).

NABORS, M. W., A. DANIELS, L. NADOLNY, and C. BROWN: Plant Sci. Lett. *4*, 155–159 (1975).

SMITH, H. H.: Adv. Genet. *14*: 1–54 (1968).

STRICKBERGER, M. W.: Genetics, 524–525. Macmillan, New York, 1968.

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

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

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APPENDIX II

Generalized Tissue Culture Methods for Tobacco and Tomato and the Use of These Methods to Select NaCl- and 2,4-D-Tolerant Tobacco Cell Lines

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Running Title: Tobacco and Tomato Cell Culture and Selection for NaCl and 2,4-D Tolerance

Abbreviations: 2,4-dichlorophenoxyacetic acid (2,4-D); 2,4,5-trichlorophenoxyacetic acid (2,4,5-T); naphthaleneacetic acid (NAA); indole-3-acetic acid (IAA); benzyladenine (BA); isopentyladenine (IPA)

Acknowledgments

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Abstract

A general method for callus culture and plant regeneration from tobacco and tomato is detailed which includes a nondestructive method for data collection. In the case of tobacco, methodology is complete enough to allow substantial progress in the area of variant selection. For tomato, significant regeneration in secondary callus occurs at high levels of cytokinins, but these levels are toxic to callus growth, and regeneration does not continue beyond the fourth passage (18 weeks in culture).

Tobacco callus cultures resistant to as much as 0.39 molal NaCl or 4.62×10^{-4} molal 2,4-D are easily obtained by repeated subculture on media containing the respective stressing agent. Cultures with significantly higher growth rates than controls are obtained at several concentrations of NaCl and 2,4-D.

In the case of salt stress, persistence of tolerance in the absence of salt is demonstrated in two manners. (1) Resistance is retained by calli after seven, one-month passages in media containing no salt. (2) Calli isolated from plants whose parents were regenerated from salt-tolerant suspension cultures retain tolerance even though the parent plants and their progeny were never exposed to salt.

Key words: mutation, salt, tissue culture, tobacco, tomato, 2,4-D

Introduction

If tissue culture is to fulfill its potential to aid in the production of agriculturally useful plants it must be shown that (1) potentially useful mutations can be obtained by standardized procedures; (2) the mutations appear in regenerated plants and are inherited; and (3) the mutant traits are reasonably persistent in the absence of stress conditions. This report focuses on the use of callus culture techniques to obtain stress-resistant mutants, in particular those resistant to NaCl or 2,4-D, and on the persistence of NaCl resistance in the absence of stress.

In several laboratories tissue culture techniques have been used to select cell lines resistant to various levels of NaCl. Zenk (1974) was able to select "haploid" cells of Nicotiana sylvestris which were resistant to 0.17 m NaCl although he did not report details of the selection process. Nabors et al. (1975) reported obtaining salt-tolerant cell suspensions from Nicotiana tabacum var. Samsun. Tolerance to salt levels as high as 0.09 m were reported when selection was carried out in suspension culture for 20 weeks. Subsequently we reported (1980) that selection periods of almost a year resulted in cell lines resistant to 0.15 m NaCl and that additional resistance could not be obtained with increased selection periods.

Dix and Street (1975) selected NaCl resistant colonies of Nicotiana sylvestris and Capsicum annum from cell suspensions added to liquid agar which was then allowed to harden. Selection for six weeks resulted in the growth of some colonies at up to 0.17 molal NaCl. Selection was also carried out in cell suspensions for up to 210 days. Variants capable

of growth in 0.17 m and 0.34 m were obtained. Cell lines resistant to 0.17 m retained tolerance after three passages (63 days) without salt stress. Croughan and co-workers (1978) selected callus from alfalfa (Medicago sativa) for resistance to 0.17 m NaCl for eight months. The selected line grew poorly in the absence of NaCl. Tyagi et al. (1981) obtained NaCl resistant calli of Datura innoxia by exposing callus to 0.17 molal NaCl for some months. Resistance was retained after one month's growth in NaCl-free medium. Orton (1980) showed that salt tolerance levels were correlated and similar when Hordeum vulgare and H. jubatum seedlings were compared with callus derived from the immature ovary wall of each plant.

Production of NaCl-tolerant plants under in vitro conditions has been reported by Nabors et al. (1980) and by Mathur et al. (1980). The former group regenerated plants from tolerant and sensitive cultures of Nicotiana tabacum and found that salt-tolerance persisted into the second generation following regeneration. The latter group regenerated plantlets of Kickxia ramosissima from shoot buds produced on cultured internodal segments and leaves without the production of noticeable callus. Salt tolerance was defined as growth on a medium supplemented with 120 mM NaCl and persisted during 27 weeks without salt stress.

Tyagi et al. (1981) regenerated Datura innoxia plants from cell lines resistant and sensitive to 0.17 m NaCl. The sensitive lines would not produce regenerates in the presence of salt. Resistant lines produced plants either in the presence or absence of salt. Furthermore callus obtained from the latter group in the absence of salt showed marked salt

tolerance whereas callus obtained from sensitive plants showed no tolerance. This indicates that salt tolerance was stable during the five week period required for plant regeneration.

With respect to 2,4-D resistance selected in tissue culture, Zenk (1974) utilized N. sylvestris suspension cultures to select resistance to as much as 10^{-3}m 2,4-D, a level which killed wild type cells. He presents few details other than noting that selection occurred by gradually increasing 2,4-levels over 18 months and that 2,4-D was the only auxin in the medium.

Oswald et al. (1975) pretreated white clover (Trifolium repens L. cv. Regal Ladion) cell suspensions for five days with 1.8×10^{-5}m 2,4-D. This pretreatment was correlated with significantly increased growth compared to controls (grown on medium containing 2.3×10^{-6}m 2,4-D) during a subsequent 40 h incubation in medium containing the pretreatment concentration 2,4-D.

Swanson and Tomes (1980) report the isolation of 2,4-D resistant calli from Lotus corniculatus. They show that within the cultivar Leo, calli initiated from different plants can be segregated into fast or slow growing lines on medium containing either 4.5×10^{-6}m or 1.8×10^{-4}m 2,4-D. During a 95 day selection period both lines maintain growth on the lower level of 2,4-D but only the fast growing line maintains growth on high 2,4-D. It is difficult to discern if selection has really occurred since the fast and slow calli exhibited growth rate differences from the beginning of selection. Callus at the end of selection was not statistically compared in terms of growth to callus which had not been through the selection process.

Work on the tissue culture and regeneration of tomato is reviewed by Meredith (1979). Her experiments are the first to study regeneration in secondary tomato callus which is free of substantially differentiated tissue. Of nine established lines regeneration was only obtained with one (VFNT Cherry). Shoot formation was quite variable with respect to cytokinin type and concentration.

This paper discusses a standardized method, applicable to many dicots, for selecting NaCl and 2,4-D resistant callus cultures from tobacco. We feel that an emphasis on methodology is important both in evaluating published work and in charting a course for future research in deriving variants from tissue culture.

Materials and Methods

1. Production of callus and measurement of callus growth. Tobacco (Nicotiana tabacum var. Samsun) callus is produced by culturing 1 mm stem or cotyledon segments on solid, sterile medium containing Linsmaier and Skoog's salts (1965), 4% sucrose, 2.30×10^{-6}m kinetin and 2.85×10^{-5}m IAA acid. Tomato (Lycopersican esculentum var. Marglobe) callus is produced using similar sections on basal medium containing 4% sucrose, 4.64×10^{-5}m kinetin, and 2.85×10^{-5}m IAA. Callus growth is measured by visually comparing calli to a set of clay spheres whose cross sectional areas are shown in Fig. 1. Assuming that the smallest sphere has a weight of one unit, relative weights of the other spheres and thus the calli can be obtained. We find that this system is more accurate than the traditional +, ++, or +++; and more convenient than obtaining actual fresh weights for each vial. Our experience demonstrates that different trained observers obtain the same average relative volumes for groups of calli using the system. Thus it provides a rapid, new, and statistical method for collecting data on callus growth.

The optimal hormone concentration for callus formation is determined by a series of experiments in which one auxin (IAA, 2,4-D, 2,4,5-T, or NAA) concentration is compared to several cytokinin (kinetin, BA, or IPA) concentrations in terms of callus growth. Figures 2-4 illustrate the effect of two cytokinins when IAA is held at 2.85×10^{-5}m, and the effect of similar cytokinin to auxin ratios on tobacco and tomato.

In our experiments medium pH is adjusted to 5.5 prior to autoclaving. At high auxin concentrations reasonable care must be taken to adjust the

pH because of the acidity contributed by the auxin. Figure 5 shows that this problem is not as serious as it first appears because agar acts as a buffer to maintain medium pH near 5.5. Nevertheless slight changes in pH can result in unsolidified medium, particularly at high auxin concentrations.

Callus is normally subcultured after 6 weeks with size 10 (see Fig. 1) being the standard transfer size. Primary callus is grown in 25 x 70 mm glass vials with lightly tightened plastic screw caps and 10 mls of medium. Secondary callus is grown in 38 x 80 mm glass jars with plastic screw caps. Cultures are placed 28 cm from 2, constantly lit 40 watt cool white fluorescent bulbs. The culture room is maintained between 27-30C.

2. Shoot regeneration from calli. To determine the best medium for shoot production several concentration ratios of cytokinin and auxin are compared in the now classical method (Skoog, 1957) for demonstrating the developmental plasticity of dicot callus cells. Figures 2-4 illustrate typical experiments in which the differing effects of IPA and kinetin on primary callus, shoot, and root production are easily noted. We define "primary callus" as that forming on the isolated plant part within 6 weeks and to which the plant part remains attached.

In practice we find that secondary tobacco callus gradually loses its IAA requirement for either growth or shoot regeneration. Thus after six months or so newly isolated callus strains are shifted to a medium supplemented with 2.30×10^{-5}m kinetin only. This medium at once supports reasonably rapid callus growth and high frequency regeneration for at least 4 years. Secondary tomato callus continues maximal growth on

a medium containing 2.85 x 10^{-5}m IAA at a variety of kinetin concentrations. Shoot regeneration through the fourth subculture is sporadic but persistent particularly at higher levels of cytokinins (Table 1).

3. Root formation on regenerated shoots. Figures 2-4 show that root formation is readily obtained on primary callus but that the hormone ratio favoring root production may or may not overlap that favoring shoot production. Also, such roots are seldom directly attached to regenerated shoots.

In our cultures, rooting of tobacco shoots is easily obtained in calli which are less than a year removed from initiation. Beyond one year and if the calli have been stressed with NaCl or 2,4-D, rooting of shoots is considerably more difficult to obtain. In the case of highly salt-stressed cultures, rooting is not obtained by using 0.5 or 0.1 strength basal medium, by increasing or decreasing sucrose concentrations, or by dipping stems in talc-NAA mixes. A combination of a talc-NAA dip (.01% NAA, but not 0.1% or 0.001%) plus implantations on full strength basal medium lacking all organics results in successful rooting of these shoots after several passages.

4. Transfer of rooted shoots to greenhouse conditions. Vials containing rooted shoots are cracked with a hammer, and excess agar is removed from the roots. Plants are potted in a sterilized mixture of potting soil and perlite (3:1 v:v). Further precautions to insure sterility and to prevent stress due to lack of moisture are unnecessary provided plantlets are grown under the same conditions as callus cultures.

5. Selection of stress-resistant tobacco callus cultures. Secondary tobacco callus cultures are exposed to media containing the stressing agent (NaCl or 2,4-D) for repeated passages of four weeks duration. The initial concentrations are 0.109 m for NaCl and 0.271 x 10^{-5}m for 2,4-D. When normal growth rates are achieved in stressed media, cultures are shifted to higher stress levels. For NaCl the stress is increased in 0.900 molal steps, and for 2,4-D the stress is increased in 0.271 x 10^{-5}m steps. The maximum stress levels are 0.386 m for NaCl and 4.62 x 10^{-4}m for 2,4-D.

Results

Our methods for obtaining calli and regenerated plants for tobacco and tomato are reported fully in the Materials and Methods because of the innovations in data collection and presentation which were utilized. A complete investigation of the callus forming potential and plant regenerative potential requires a two-dimensional design utilizing various concentrations of auxins as well as cytokinins. The complete data are not presented because the main experimental goal was an investigation of stress-tolerant mutants obtained in tissue cultures.

Selection of salt tolerant callus. Previous work from this laboratory has shown that NaCl tolerant cell lines can be selected from tobacco suspensions (Nabors et al. 1975). Figure 6 shows the results of an experiment to determine if similar selections can occur using callus cultures instead of suspensions. The cell line utilized was started from stem sections in April 1974, was placed in suspension in June 1974, and was transferred back to solid medium in April 1976. The data presented represent the average callus volumes for three, 4 week (mid 1979) passages on the salt concentrations indicated. The lower portion of each bar (dotted or solid) represents calli which were salt stressed for only one passage before data was taken. The callus on no salt was then utilized to begin a new "step up" passage on the indicated NaCl concentration. The upper clear portion of each bar represents "continued" calli which were maintained continuously on salt for several passages before data was taken and for three passages during which data was obtained.

Thus the lower portion of the bars represent calli transferred from a standard NaCl concentration of 0.00 (dotted bars) or 0.110 m (solid bars) to the indicated concentration for only one passage whereas the upper portion of the bars represent calli transferred from the standard to the indicated salt concentration passages. Selection for salt tolerance is indicated if the clear upper bar portion is significantly higher than the lower dotted or solid bar portion.

The clear portions of the first dotted bars for each concentration represent a callus line never exposed to salt until the 3 passages at the indicated concentration before the data were collected. Since the clear portion of the bars is not higher than the dotted portion no significant selection for salt tolerance occurred at any salt concentration. The clear portions of the second dotted bars represent a callus line exposed to the indicated concentrations for 6 passages before data collection. The data indicate that statistically significant selection occurred at 0.202 m NaCl. The clear portions of the first solid bars represent a callus line maintained in 0.110 m NaCl for 58 passages, then at the indicated concentrations for 3 passages prior to data collection. The clear portions of the second solid bars represent a similar callus line stressed for 4 instead of 3 passages prior to data collection. The data indicate that statistically significant selection for both lines represented by solid-clear bars occurred at 3 NaCl concentrations.

These results show that selection for NaCl tolerance can occur in callus culture. By comparing the second bar at 0.110 and 0.202 m NaCl to the first it can be seen that the length of continuous salt stress

seems to be related to the development of tolerance. Also, a long culture period at 0.110 m NaCl concentration (solid-clear bars) seems to predispose the culture to selection for tolerance when compared to controls (dotted-clear bars). Cultures maintained for 58 generations on 0.110 m NaCl before additional stress selection (solid bars) do not show increased growth at this NaCl level when compared to cultures maintained for 58 generations on no salt (dotted bars).

Selection of 2,4-D tolerant callus. A line of callus was initiated from stem sections and maintained on medium containing 2.71×10^{-5}m 2,4-D. After three passages one portion of the line was placed on 2,4-D concentrations ranging from the basal level to 2.44×10^{-4}m. Calli were maintained on these concentrations for 16 passages during which higher concentrations were added irregularly using tissue from the then highest available concentration. The other portion of the original callus line was maintained at 2.71×10^{-5}m 2,4-D with occasional one passage step-ups to the higher concentrations. Figure 7 demonstrates the growth of the two calli types during passages 18-20. It can be seen that at most 2,4-D concentrations up to 4.62×10^{-4}m the growth of the continuously stressed line was significantly higher than that of the control line. Plant regeneration from both lines has been achieved.

Retention of NaCl tolerance following periods in which NaCl was absent. Several cell lines with demonstrated NaCl tolerance at either 0.202 or 0.295 m were shifted to medium lacking NaCl for seven passages before being placed again on medium containing the original concentration of NaCl.

The results are presented in Figures 8 and 9. The data in Figure 8A show clearly that NaCl tolerance to 0.295 m NaCl is rapidly regained by

the shifted line when compared to a line in which NaCl selection has just been initiated. NaCl tolerance in the shifted line was initially attained by a period of 17 passages on the indicated level of salt. The cell line was originally obtained from stem sections of a salt tolerant plant, itself the offspring of a plant regenerated from a cell suspension tolerant to 0.110 m NaCl.

For comparison, Figure 8B shows data from a similar cell line derived from a nontolerant plant. This line was maintained on medium containing no salt prior to 12 passages on 0.295 m NaCl and seven passages on no salt. In this line no selection for tolerance occurred during the original selection period. When the same cell line was stressed similarly at 0.202 m NaCl Figure 9B shows that tolerance was attained initially and maintained during the step-down period in medium containing no NaCl.

Figure 9A shows that partial tolerance was retained by a line maintained for 17 transfers on no salt followed by 16 transfers on medium containing 0.295 m NaCl before being shifted to no salt.

Retention of NaCl tolerance in callus from the progeny of regenerated tolerant plants. Figure 10 shows the results of experiments in which callus cultures from progeny (F_1) of various types of regenerated plants were stressed at several levels of NaCl. The clear, upper portions of the bars represent the averaged data of three passages following 10 passages on the indicated level of NaCl. The dotted, solid, or slashed lower portions of the bars represent the average growth of three, one month step-ups to the indicated salt concentration for callus never previously exposed to salt. Prior to stressing, the callus from progeny plants was maintained on an initiation medium for four passages.

The dotted bars represent callus from plants whose parents were regenerated from tissue cultures never exposed to salt. Solid bars represent callus from plants whose parents were regenerated from tissue cultures tolerant to 0.110 m NaCl with the salt present throughout the regeneration process. Slashed bars represent similar callus from plants whose parents were regenerated from tissue cultures tolerant to 0.110 m NaCl with the salt absent throughout the regeneration process. In all three cases the progeny plants were grown in the absence of NaCl in the watering solutions.

The data show that selection for increased NaCl tolerance occurred for most cultures at three salt levels. This is indicated whenever the clear portion of a bar is significantly higher than the nonclear portion.

By comparing the heights of the dotted, solid, and slashed bars it is seen that at 0.110 and 0.202 m NaCl the callus from salt tolerant progeny plants has retained this tolerance. We have previously shown (Nabors et al. 1980) that progeny plants (F_1's) reflect tolerance developed originally in tissue culture and that their progeny (F_2's) also reflect tolerance compared to controls.

The data bars for medium with no salt show that callus from salt tolerant progeny plants has significantly increased growth compared to callus from salt sensitive progeny plants.

Discussion

Our use of tissue culture techniques to produce stress-tolerant mutant plants depends on (1) the ability to produce large numbers of cells in a short time; (2) the availability of techniques for selecting induced or spontaneous variants of the desired type; (3) the ability to regenerate plants from both stressed cultures containing variants and nonstressed cultures containing controls; and (4) demonstrations that regenerated plants from variant cultures retain the desired trait and pass it on to future offspring in a predictable fashion.

For NaCl and herbicide tolerance the selection methods are straight forward and present no difficulties. The first and third criteria however are typically met in the literature by rather haphazard, unstandardized procedures. For some plants, especially grasses but also many dicots, it is imperative that available techniques for rapid callus production and for plant regeneration be improved before significant progress can occur toward the selection of useful mutant phenotypes. Thus, we have outlined in some detail our methods for determining the best medium for rapid callus production and our methods for rapidly, accurately, and nondestructively recording and displaying data.

In the case of tomato, it is clear from Fig. 4 that initiation of callus and the formation of shoots and roots from primary callus is easily standardized. For secondary callus however, shoot formation (after the second passage) requires higher cytokinin concentrations than are generally utilized for other dicots (Table 1). Shoot formation in tomato is promoted at cytokinin concentrations which are toxic for tobacco regeneration and for growth of the secondary tomato callus itself. Regenerated shoots frequently appeared on calli which were not growing and might ordinarily have been discarded. Regeneration does not continue beyond the fourth passage (18 weeks in culture), and callus growth becomes progressively more difficult to maintain (data not presented).

The data demonstrate conclusively that selection for NaCl tolerant cell lines can occur in callus (Fig. 6 and 10) as well as in suspensions (Nabors et al. (1975). The length of selection is related to the degree of tolerance developed. Specifically, our results indicate that exposures of three, four-week passages to NaCl do not result in significant selection for tolerance. After six passages significant tolerance develops for some lines. Finally, exposure to NaCl for many passages (58 passages in the data presented) predisposes the culture to significant selection at several NaCl concentrations after three or four passages. The length of time for tolerance to develop should be a function of the doubling time for tolerant cells found in a culture and to the number of these cells present. The data presented are representative. Results from other cell lines support the conclusions presented.

We have not directly compared variant selection in suspension and in callus culture. From indirect comparisons, however, it is seen that using our cell line in suspension, tolerance to more than 0.110 m NaCl is difficult to obtain no matter how long the length of selection (Nabors et al. 1980). For selection in callus, tolerance to 0.295 m NaCl is obtained, but this concentration refers to the solid medium and not to the actual concentration of NaCl reaching the cells. In this sense callus selection is less controlled than suspension selection since different regions of callus may well be exposed to different concentrations of NaCl.

It is curious and unexplained that cell lines from cultures maintained on 0.110 m NaCl for 58 passages grow significantly less rapidly than no salt controls on medium containing 0.110 m NaCl (Fig. 6). This is not the case with other cell lines (e.g. Fig. 10), and is not the case with this cell line throughout most of its history in culture.

Successful selection for 2,4-D tolerance in callus cultures (Fig. 7) is another example of the general utility of the methods discussed in

this paper. Again, a reasonably long period of selection (16 passages) appears essential. Significant tolerance at levels of 2,4-D up to 4.62×10^{-4}m (102 ppm) have been achieved. It is our belief that investigations on rapidly achieved low levels of tolerance (e.g. to 1.09×10^{-4}m) may not be equivalent to investigations on slowly achieved high levels of tolerance.

Our results (Figs. 8 and 9) demonstrate that NaCl tolerance attained after periods of selection ranging from 12 to 17 four-week passages was retained after seven passages in a medium containing no NaCl. These data parallel those of Dix and Street (1975)(who selected for 210 days then eliminated salt for 63 days), but are in contrast to those of Hasegawa et al. (1980). These workers found that cells selected on 0.170 m NaCl for 64 days (40 cell doublings) did not retain salt tolerance during a downstep period of five cell doublings.

The limited evidence from NaCl tolerant variants indicates that stability of tolerance in the absence of salt is related to the length of selection. Certainly, this hypothesis is in need of further experimental confirmation. NaCl tolerant variants could arise by point or chromosomal mutation or by gene amplification (see Skokut and Filner, 1980) among other mechanisms. In either case the stability of tolerance would depend on the relative selective advantage of each phenotype in the presence and absence of stress and on the phenotype reversion rate.

In the case of short term selection, stability would also be greatly influenced by the relative proportions of tolerant and nontolerant cells in the population. During long term selection the population would come to consist entirely of tolerant cells. Stability in the absence of NaCl would then relate to the phenotype reversal rate (including

mutations, translocations and other mechanisms) and to the relative selective advantage of each phenotype. Another factor to consider is that during long term selection the possibility of additional genetic or nongenetic changes increasing salt tolerance is enhanced. Thus long term selection might tend to result in increased stability of the variant phenotype due to the inclusion in cells of more than one salt-tolerance-enhancing mechanism. It is important to realize that the stability of any variant trait should depend on the length of selection as well as selective interactions between phenotypes and environments. Further discussion of the variables involved in NaCl tolerance would be considerably enlightened if the physiological and genetic nature of tolerance were known. To date (see Heyser and Nabors 1980, 1981) this question is still under investigation.

An important point, then, is that variants selected in tissue culture or in the field will probably never be totally stable in the absence of selective pressure. This is to say that all the conditions required for the Hardy-Weinberg equilibrium to be maintained will probably not be met for single cause variations with a finite reversion frequency. Long term selection could increase stability by allowing selection of additional variants relating to maintenance of the selected phenotype. Correlatively, it is becoming clear that variants resulting from gene amplification are not always less stable in the absence of stress than those resulting from point or chromosomal mutations of other types. Schimke's group (Kaufman et al. 1979) has shown that gene amplification can result in either stable or unstable methotrexate-resistant Chinese

hamster cell lines. Stable amplification is correlated with chromosome-based gene duplications whereas unstable amplification is correlated with the presence of small paired extrachromosomal elements denoted "double minute chromosomes." Other workers (Biedler and Spengler 1976), however, have correlated loss of methotrexate resistance with the diminution in size of homogeneously staining regions within chromosomes.

In an earlier paper we demonstrated that NaCl tolerant cell lines gave rise to plants which retained this tolerance and passed it on to two subsequent (F_1 and F_2) generations. In Figure 10 it is demonstrated that callus cultures obtained from stem sections of NaCl tolerant F_1 plants are significantly more tolerant than similar sections obtained from nontolerant F_1 plants. The increased tolerance is apparent at the NaCl level to which tolerance was originally obtained (0.110 m NaCl), at higher levels of salt, and at no salt. In fact, it is in media containing no salt that the calli from tolerant plants shows the greatest growth differential. This indicates that the NaCl tolerance selected in this line is not salt requiring. In terms of practical applications such a variant phenotype, if apparent in whole plants would have extremely interesting agricultural potential. This possibility needs further investigation since tolerant and sensitive F_1 plants themselves did not show noticeable growth differences when grown in soil and watered with tap water.

In terms of the persistence of NaCl tolerance in the absence of salt stress, the callus was obtained from F_1 plants which were not exposed to salt subsequent to removal from tissue culture vials in one case (solid bars) and subsequent to growth in suspension cultures in

another case (striped bars). Thus the plants retained salt tolerance during an entire life cycle in the absence of NaCl.

REFERENCES

Biedler, J.L., Spengler, B.A. (1976) Quantitative relationship between a chromosome abnormality (HSR) and antifolate resistance associated with enzyme overproduction. J. Cell Biol. 70, 117a

Croughan, T.P., Stavarek, S.J., Rains, D.W. (1978) Selection of a NaCl tolerant line of cultured alfalfa cells. Crop Sci. 18, 959-963

Dix, P.J., Street, H.E. (1975) Sodium chloride-resistant cultured cell lines from Nicotiana sylvestris and Capsicum annuum. Plant Sci. Lett. 5, 231-237

Hasegawa, P.M., Bressan, R.A., Handa, A.K. (1980) Growth characteristics of NaCl-selected and nonselected cells of Nicotiana tabacum L. Plant Cell Physiol. 21, 1347-1355

Heyser, J.W., Nabors, M.W. (1981) Osmotic adjustment of cultured tobacco cells (Nicotiana tabacum var. Samsun) grown on sodium chloride. Plant Physiol. 67, 720-727

Heyser, J.W., Nabors, N.W. (In press) The growth, water content, and solute accumulation of two tobacco cell lines cultured on sodium chloride, dextran, and polythylene glycol. Plant Physiol.

Kaufman, R. J., Brown, P.C., Schimke, R.T. (1979) Amplified dihydrofolate reductase genes in unstably methotrexate-resistant cells are associated with double minute chromosomes. Proc. Natl. Acad. Sci. USA 76, 5669-5673

Linsmaier, F.M., Skoog, F. (1965) Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. 9, 100-127

Mathur, A.K., Ganapathy, P.S., Johri, B.M. (1980) Isolation of sodium chloride-tolerant plantlets of Kickxia ramosissima under in vitro conditions. Z. Pflanzenphysiol. 99, 287-294

Meredith, C.P., (1979) Shoot development in established callus cultures of cultivated tomato (Lycopersicon esculentum Mill.). Z. Pflanzenphysiol. 95, 405-411

Nabors, M.W., Daniels, A., Nadolny, L., and Brown, C. (1975) Sodium chloride tolerant lines of tobacco cells. Plant Sci. Lett. 4, 155-159

Nabors, M.W., Gibbs, S.E., Bernstein, C.S., Meis, M.E. (1980) Nacl-tolerant tobacco plants from cultured cells. Z. Pflanzenphysiol. 97, 13-17

Orton, T.J. (1980) Comparison of salt tolerance between Hordeum vulgare and H. jubatum in whole plants and callus cultures. Z. Pflanzenphysiol. 98, 105-118

Oswald, T.H., Smith, A.E., Phillips, D.V. (1977) Herbicide tolerance developed in cell suspension cultures of perennial white clover. Can. J. Bot. 55, 1351-1358

Skokut, T.A., Filner, P. (1980) Slow adaptive changes in urease levels of tobacco cells cultured on urea and other nitrogen sources. Plant Physiol. 65, 995-1003

Skoog, F., Miller, W. (1957) Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Symp. Soc. Exp. Biol. 11, 118-131

Swanson, E.B., Tomes, D.T. (1980) Plant regeneration from cell cultures of Lotus corniculatus and the selection and characterization of 2,4-D tolerant cell lines. Can. J. Bot. 58, 1205-1209

Tyagi, A.K., Rashid, A., Maheshwari, S.C. (1981) Sodium chloride resistant cell line from haploid Datura innoxia. Protoplasma 105, 327-332

Zenk, M.H. (1974) Haploids in physiological and biochemical research. In: Haploids in higher plants, pp. 339-353, Kasha, K.J., ed. University of Guelph, Guelph

Table 1. Number of shoots per vial of callus for tomato as a function of passage and cytokinin concentration.

Kinetin x 10^{-5}m	Initiation	1st passage	2nd passage	3rd passage
0.5	.40 ± .27	---	---	---
2.3	2.67 ± .78	.24 ± .11	0	---
4.6	1.79 ± .38	.42 ± .15	0.46 ± .04	---
6.9	4.50 ± .97	.51 ± .18	0.23 ± .14	---
7.3	7.00 ± .16	1.21 ± .36	0.08 ± .13	.396
11.6	3.86 ± 1.86	---	---	---
13.9	---	---	0	---
18.6[a]	---	---	0.11 ± .07	0.37
27.9[a]	---	---	0	0.14
37.2[a]	---	---	1.00 ± .60	0.07
46.5[a]	---	---	0.76 ± .31	---

[a]Total composed of equal concentrations of kinetin and benzyladenine of kinetin and isopentyladenine.

Figure Legends

Figure 1. Cross-sectional area of clay models used to quantify callus growth.

Figure 2. Primary callus formation from tobacco cotyledons after 6 weeks in culture. ○ callus; ● roots; ■ shoots. Standard errors are shown for sample sizes of 10 vials. Auxin was IAA at 2.85×10^{-5}m.

Figure 3. Primary callus formation from tobacco cotyledons after 6 weeks in culture. ○ callus; ● roots; ■ shoots. Standard errors are shown for sample sizes of 10 vials. Auxin was IAA at 2.85×10^{-5}m.

Figure 4. Primary callus formation from tomato cotyledons after 6 weeks in culture. ○ callus; ● roots; ■ shoots. Standard errors are shown for sample sizes of 10 vials. Auxin was IAA at 2.85×10^{-5}m.

Figure 5. The effects of added IAA in liquid, autoclaved liquid, and autoclaved solid Linsmaier and Skoog's (1965) medium. Standard error bars are shown for three samples.

Figure 6. Selection for NaCl tolerance in tobacco calli. Data presented were collected during three, four week passages on the indicated concentrations. Dotted and solid bars represent cultures which were maintained on media containing no NaCl except for three, one month passages. At the beginning of each passage, callus on no salt medium was stepped up to medium with the indicated level of salt. Clear bars represent cultures maintained on NaCl-containing medium for various lengths of time prior to data collection. Standard error bars are indicated for three, twenty vial samples.

Figure 7. Selection for 2,4-D tolerance in tobacco calli. Data presented were collected during three, four week passages on the indicated concentrations. The bottom line (●) represents cultures exposed to the herbicide only during data collection. At the beginning of each passage callus on no herbicide medium was stepped up to medium with the indicated level of 2,4-D. The top line (O) represents cultures exposed continuously to herbicide for 16 passages prior to data collection. Callus on concentrations above 2.44×10^{-4}m 2,4-D was obtained by gradual stepups from this level during the 16-week period. Standard error bars are indicated. Standard error bars are indicated for three, twenty vial samples.

Figure 8. A. Retention of NaCl tolerance during growth in the absence of salt in cell lines originally obtained from a plant which was the offspring of a plant regenerated from a suspension tolerant to 0.110 m NaCl. ▲ represents a callus culture shifted to medium containing 0.295 m NaCl for only the 6 passages shown. ■ represents a culture after 17 passages on medium containing 0.295 m NaCl. ● represents a similar culture downshifted for 7 passages to medium containing no salt after 17 passages on medium containing 0.295 m NaCl and finally shifted back to salt-containing medium for the 4 passages shown. O represents the ● culture's growth in the three passages prior to downshifting.

B. Similar data for cell lines obtained from a nonsalt tolerant plant. In this case the original selection period was for 12 (cf. 17) passages, and selection for NaCl tolerance did not occur. Standard error bars are indicated for twenty vial samples

Figure 10. Retention of NaCl tolerance in callus from the progeny of regenerated NaCl tolerant plants. Dotted bars represent the average growth of three passages for calli from plants whose parents were regenerated from cultures never exposed to NaCl. Solid bars represent similar cultures from plants whose parents were regenerated from cultures tolerant to 0.110 m NaCl with the salt present throughout the regeneration process. Slashed bars represent similar cultures from plants whose parents were regenerated from tissue cultures tolerant to 0.110 m NaCl with salt absent throughout the regeneration process. Clear portions of all bars represent additional average growth during three periods following 10 passages on media containing the indicated concentrations of NaCl. Standard error bars are indicated for three, twenty vial samples.

Figure 9. Retention of NaCl to tolerance during growth in the absence of salt in cell lines originall obtained from nonsalt tolerant plants. A. ▲ represents a callus culture shifted to medium containing 0.295 m NaCl for only the 3 passages shown. ■ represents a culture after 17 passages on medium containing 0.295 m NaCl. ● represents a similar culture downshifted for 7 passages to medium containing no salt after 17 passages on medium containing 0.295 m NaCl and finally shifted back to salt containing medium for the 3 passages shown. O represents the ● culture's growth in the three passages prior to downshifting. B. ▲ represents a callus culture shifted to medium containing 0.202 m NaCl for only the 3 passages shwon. ■ represents a culture after 12 passages on medium containing 0.202 m NaCl. ● represents a similar culture downshifted for 7 passages to medium containing no salt after 12 passages on medium containing 0.202 m NaCl and finally shifted back to salt-containing medium for the 3 passages shown. O represents the ● culture's growth in the three passages prior to downshifting. Standard error bars are indicated for twenty vial samples.

FIG. 1

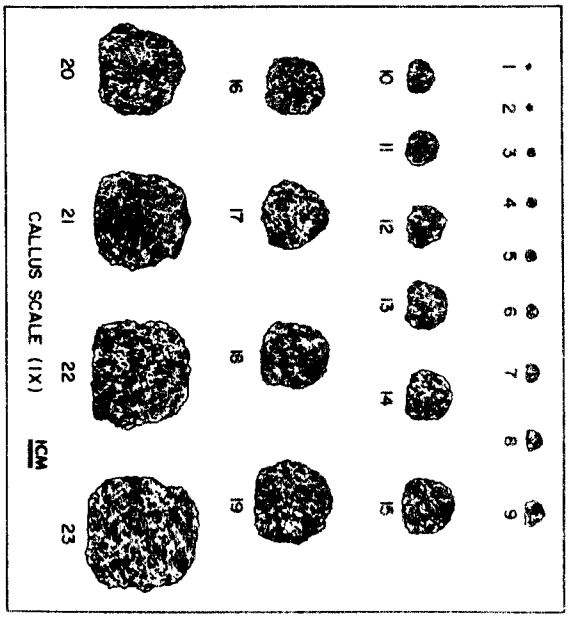

1 2 3 4 5 6 7 8 9
10 11 12 13 14 15
16 17 18 19
20 21 22 23
CALLUS SCALE (1X)
1CM

FIG. 4

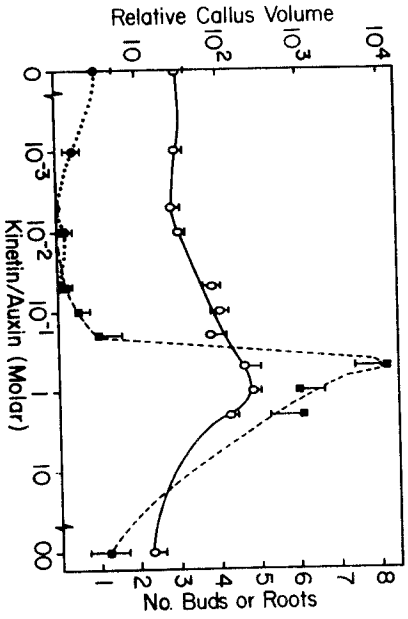

Relative Callus Volume
10
10²
10³
10⁴
0
10⁻³
10⁻²
10⁻¹
1
10
∞
Kinetin/Auxin (Molar)
1
2
3
4
5
6
7
8
No. Buds or Roots

FIG. 3

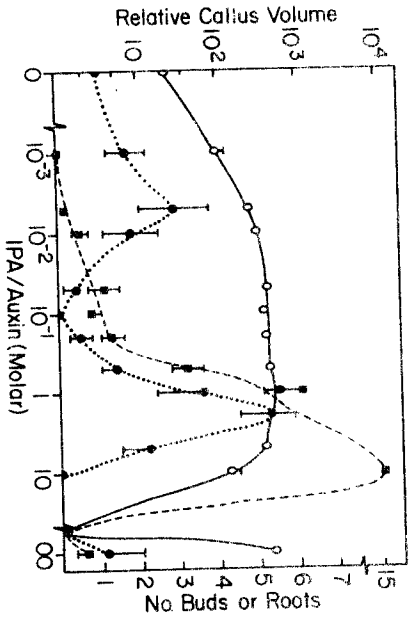

Relative Callus Volume
10
10²
10³
10⁴
0
10⁻³
10⁻²
10⁻¹
1
10
∞
IPA/Auxin (Molar)
1
2
3
4
5
6
7
15
No. Buds or Roots

FIG.4

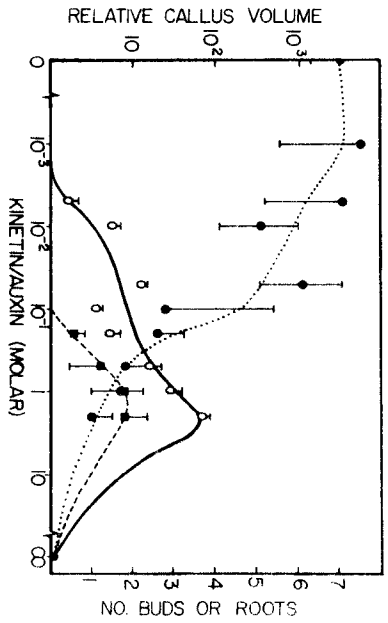

RELATIVE CALLUS VOLUME
10
10²
10³
0
10⁻³
10⁻²
10⁻¹
1
10
100
KINETIN/AUXIN (MOLAR)
1
2
3
4
5
6
7
NO. BUDS OR ROOTS

FIG. 5

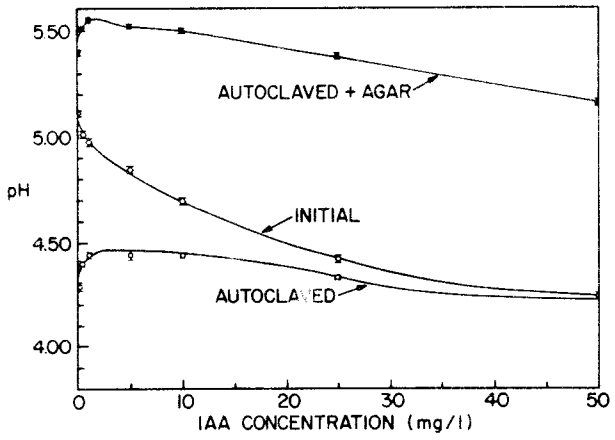

5.50
5.00
4.50
4.00
pH
AUTOCLAVED + AGAR
INITIAL
AUTOCLAVED
0
10
20
30
40
50
IAA CONCENTRATION (mg/l)

FIG. 6

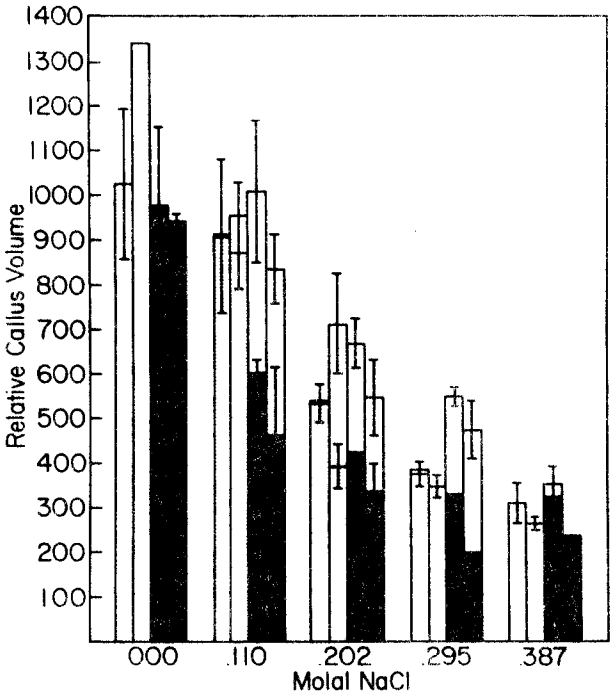
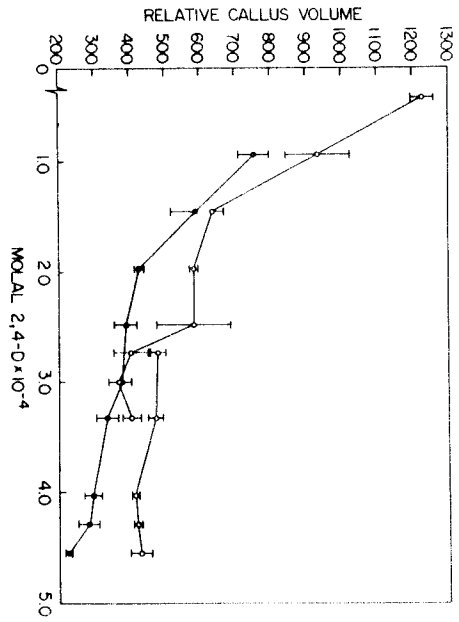

1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
Relative Callus Volume
000
.110
.202
.295
.387
Molal NaCl

FIG. 7


RELATIVE CALLUS VOLUME
0
200
300
400
500
600
700
800
900
1000
1100
1200
1300
MOLAL 2,4-D x 10⁻⁴
1.0
2.0
3.0
4.0
5.0

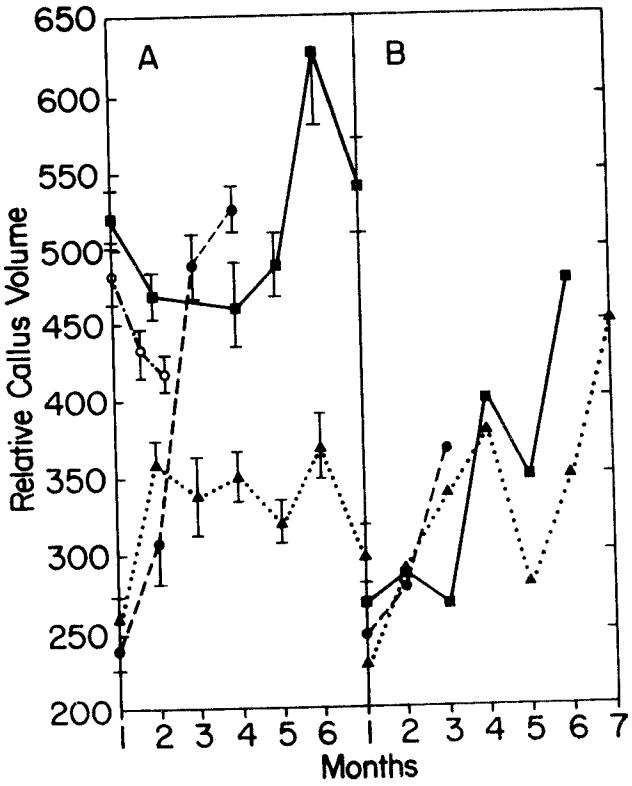

650
600
550
500
450
400
350
300
250
200
Relative Callus Volume
A
B
1 2 3 4 5 6 1 2 3 4 5 6 7
Months

FIG. 4

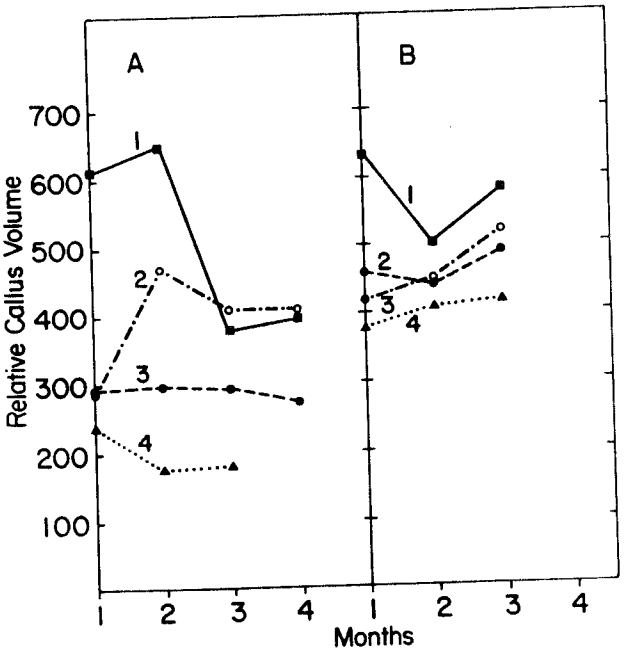
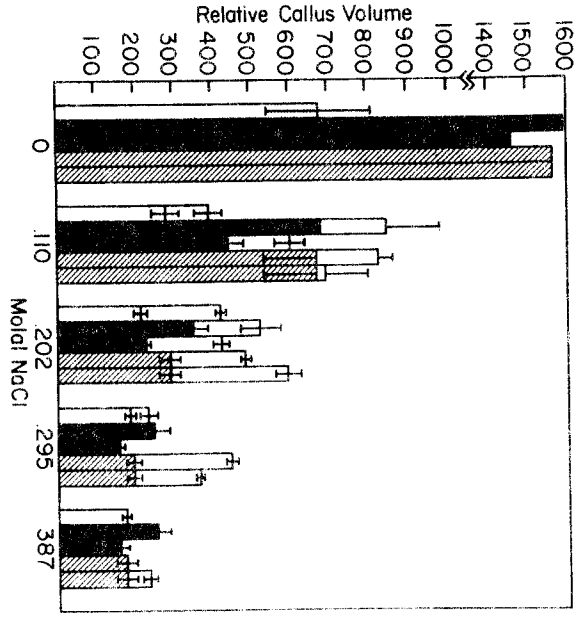

A
B
700
600
500
400
300
200
100
Relative Callus Volume
1
2
3
4
1
2
3
4
1
2
3
4
Months

FIG. 10


Relative Callus Volume
100
200
300
400
500
600
700
800
900
1000
1400
1500
1600
0
110
202
295
387
Molal NaCl

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Osmotic Adjustment of Cultured Tobacco Cells (*Nicotiana tabacum* var. Samsum) Grown on Sodium Chloride[1]

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ABSTRACT

Tobacco cell cultures (var. Samsum) were grown on increasing levels of NaCl to select variants for increased salt tolerance. The osmotic adjustment of NaCl-adapted and nonadapted cell lines was studied. Both cell lines were grown on modified Linsmaier and Skoog medium with or without NaCl. Few differences were found in the response of adapted and nonadapted lines to NaCl.

The concentrations of sugars, Na^+, Cl^-, and NO_3^- were identical in the cells and medium. Potassium and amino acids were accumulated by the cells. All of the above solutes accounted for 80 to 90% of the osmotic potential for both cell lines when grown on basal medium with or without NaCl. The osmotic potential of growing cells was always 1 to 3 bars more negative than that of the medium. During the first 10 days culture, the cells hydrolyzed the 117 millimolar sucrose present in the fresh media, and the media became more negative by 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 media and cells became less negative. The addition of 130 millimolar NaCl made the media and cells osmotically more negative by 6 bars throughout the growth cycle, as compared with cells growing on basal medium.

The efflux of cellular solutes during distilled H_2O 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, whereas the slow component (1.6 to 4.9 hours half-time) represented the vacuolar solutes. Sodium and Cl^- were present in the vacuole. No differences were observed in the solute efflux between the adapted and nonadapted cell lines.

Over 40% of the earth's land surface is arid or semiarid (11). These lands tend to have salinity problems (2, 14). Seasonal or infrequent rains only partially leach accumulated salts out of the soil as rainwater evaporates and dissolved salts remain. Irrigation is essential for crop production in arid areas but also leads to increased salinization (3, 19). Just as rainwater evaporates and leaves a salt residue, irrigation water, which often contains high but tolerable levels of salt, evaporates and salts remain. Salinization at present is reduced in irrigated fields by flushing them with sufficient water to leach out accumulated salts.

An alternative solution to problems of increased salinization in irrigated fields would be to produce crop plants with an increased tolerance to salt. It may be possible to select mutations for increased salt tolerance at the cellular level using tissue culture. Techniques exist for the selection of mutant diploid cells in culture, which may be regenerated into fertile mutant plants (6). Nabors *et al.* (21) reported the selection of a NaCl-tolerant line of tobacco cells in 1975. More recently, Croughan *et al.* (8) isolated NaCl-tolerant alfalfa cells. Nabors *et al.* (22) have reported preliminary findings which show inherited differences in the NaCl tolerance of the F_2 progeny from NaCl-adapted and nonadapted cell lines. Only traditional plant breeding experiments will unequivocally determine if tissue culture has selected for a NaCl-tolerant mutation.

A mutant cell line might show obvious qualitative differences in its osmotic response to NaCl, or increased NaCl tolerance might be due to minor quantitative differences in the accumulation and/or production of solutes. An understanding of cellular osmotic adjustment is helpful in isolating other salt tolerant mutants. Experiments on the osmotic adjustment to NaCl by NaCl-adapted and nonadapted tobacco cells are reported.

MATERIALS AND METHODS

Plant Source. Internodal sections (5 cm) of tobacco stems (*Nicotiana tabacum* L. var. Samsum) were washed 30 s in 95% ethanol. They then were washed 12 min in a 20% (v/v) solution of Clorox bleach, rinsed three times with sterile distilled H_2O, and cut into approximately 5-mm cylinders. These were divided into four to six wedges. Each wedge was placed in a vial of presterilized medium. After callus formation, the calli were transferred to 25 ml liquid medium, which was gradually increased to 100 ml.

Medium. The basal medium was that of Linsmaier and Skoog (15). Unless noted, all cultures were grown on medium E which contained Linsmaier and Skoog medium plus 6 μg/ml 2,4-D, 0.5 μg/ml kinetin, 0.1% (w/v) yeast extract, and 0.1% (w/v) malt extract. Yeast and malt extracts were not essential, but promoted better growth. In a few experiments, Linsmaier and Skoog medium plus 5 μg/ml IAA and 0.5 μg/ml kinetin was used as a basal medium.

Cell Lines. Three cell lines were used. The nonadapted cell lines were started from stem sections, and developing calli then were transferred to their respective media. These cell lines on medium E or Linsmaier and Skoog plus 5 μg/ml IAA and 0.5 μg/ml kinetin were maintained for 1 year on basal medium in suspensions. Periodically, cells from these two lines were transferred to the same basal media plus various NaCl concentrations up to 170 mM. The NaCl-adapted cell line was initiated from internodal stem sections on solid medium E. After callus formation, calli were transferred to medium E with 27 mM NaCl. On the following passage, these calli were shifted to 38 mM NaCl, which was followed by a passage on 48 mM NaCl. Each passage took approximately 6 weeks. The calli were subcultured again on 48 mM NaCl and then shifted to 68 mM NaCl in suspensions. Gradually, this line was shifted up 20 mM at a time to 130 mM NaCl plus medium E and maintained 10 months at this NaCl level.

Cell Growth. Cell suspensions were grown in Nephelo flasks. When these flasks were swirled and tilted toward the sidearm, a

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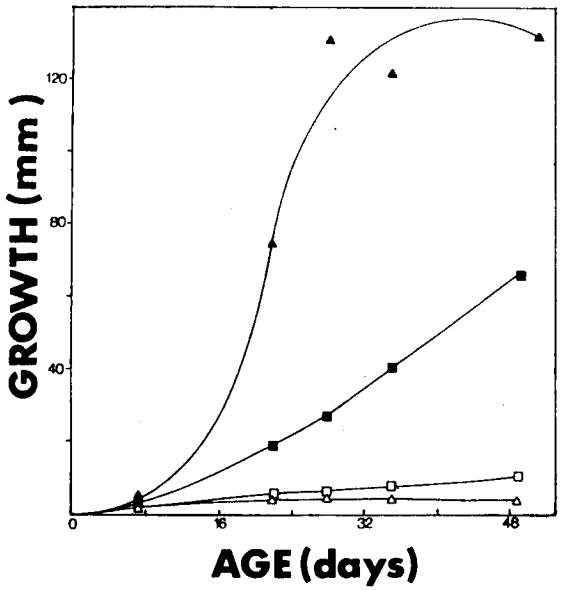

FIG. 1. Growth of cells in four flasks at 0 or 79 mM NaCl. Nonadapted cells grown on medium E: (▲), fast-growing culture; (△), slow-growing culture. NaCl adapted cells with fast growth cultured on Linsmaier and Skoog medium plus 5 μg/ml IAA, 0.5 μg/ml kinetin, and 79 mM NaCl (■). Nonadapted cells with slow growth cultured on Linsmaier and Skoog medium plus 5 μg/ml IAA, 0.5 μg/ml kinetin, and 79 mM NaCl (□).

compact mass of cells settled in the sidearm in 10 to 15 min (18). The settled cell height in the sidearm was proportional to the dry weight of the cells.

Osmotic Potential. The freezing point depression method was used for the determination of the osmotic potential of the cell sap and liquid media. The instrument used was the Osmette produced by Precision Instruments, Sudbury, MA. The Van't Hoff equation was used to convert the readings taken in mosmol/l to the bars at 25 C.

Extraction of Cell Sap. One or more grams of fresh tissue were frozen and then thawed. The thawed tissue was homogenized by 15 strokes in a size A tissue grinder with a Teflon grinding head and a smooth glass vessel (A. H. Thomas Co., Philadelphia). The resulting slurry was centrifuged for 20 min at 23,500*g* in a Sorvall (SS-3) centrifuge with a 11-cm radius head. The supernatant was decanted and saved.

Extraction for Chemical Analysis. The fresh tissue was frozen and thawed as above and then placed in the tissue grinder. Water in a predetermined ratio was added to the grinding vessel. The mixture was homogenized 15 strokes and centrifuged as above. Usually, 9.0 ml double-distilled H_2O was added/g fresh weight.

Chemicals. Inorganic chemicals were purchased from Fisher Scientific Company, St. Louis, MO, or J. T. Baker, Philipsburg, NJ. Organic reagents and enzymes were purchased from Sigma. The Glucostat Reagent Kit for glucose analysis was obtained from Worthington Biochemical Co., Freehold, NJ.

Sugars. Reducing sugars were analyzed by a modified Nelson (23) and Somogyi (30) method. Sucrose was measured after it was hydrolyzed by an invertase obtained from Sigma. Glucose was measured by the Glucostat Reagent Kit. Fructose was not measured.

Amino Acid. Total amino acids and ammonium were analyzed by the ninhydrin method of Rosen (25) which measures α-amino nitrogen. L-Leucine in water was used as the standard.

Nitrate. Nitrate was measured colorimetrically as nitrite after its partial reduction by nitrate reductase (16). The nitrate reductase used was a disassimilatory enzyme from *Escherichia coli* sold as a lyophilized powder by Sigma (34).

Cations. The cations Na^+, K^+, Ca^{2+}, and Mg^{2+} were measured by a Perkin-Elmer 305 atomic absorption spectrophotometer against chloride salt standards.

Chloride. Chloride was measured by the titration method of Schales and Schales (27), using 0.010 N mercuric nitrate as the titration solution.

L-Malic Acid. L-Malic acid was determined by the enzymic assay of Gutman and Wahlefeld (13).

Washing Cells for Efflux Analysis. To analyze for efflux kinetics, a flask of cells was harvested by filtration through Miracloth followed by vacuum filtration on Whatman No. 1 filter paper. The cells were weighed to the nearest 0.01 g and then divided into four to eight equal aliquots. One sample was not washed and was analyzed to obtain the initial cellular solute concentration. A second aliquot was placed on a moistened Whatman No. 1 filter paper in a Büchner funnel. An amount of water usually equal to 10 times tissue mass was added to the funnel with the tissue and immediately removed by vacuum filtration. This wash took from 10 s to 2 min. Other samples washed for longer times were washed as follows: the tissue and distilled H_2O were placed in a glass beaker and shaken at approximately 120 rpm on a gyrorotary shaker. At the appropriate time, the sample was removed and vacuum-filtered to separate cells and wash water. Both were frozen and saved for further analysis. Distilled H_2O was used in the wash solution instead of 0.5 mM $CaSO_4$ as was done by Epstein (9) and others (10, 12, 29). Both NaCl-adapted and nonadapted cells were grown in 3.0 mM Ca^{2+} instead of the 0.1 mM Ca^{2+} often used in ion uptake and efflux experiments. The cells were not prewashed with distilled H_2O before they were placed in distilled H_2O at the start of the efflux experiments, as others have done with excised roots (9) and rice cells (10).

Kinetic Analysis for Localization of Solutes. The efflux analysis which was first applied to plant cells by MacRobbie and Dainty (17) was used. There are three major compartments containing solutes identifiable by kinetic analysis: a free space, a cytoplasmic phase, and the vacuolar compartment. The term compartment refers to the contribution of each compartment to the total solutes that leaked from the cells after a wash (4, 24).

Half-times for the fast efflux component were obtained from linear plots of the remaining cellular solute concentration *versus* the time of wash. The amount of solute present in the slow component was first determined by extrapolating the slope of the slow component back to zero time. This intercept value was subtracted from the initial solute concentration present in the cells. The value representing half of the initial solute concentration present in the fast component was determined. The time of wash at which the efflux curve value equaled this value was the half-time for the efflux of the fast component.

RESULTS

Effect of NaCl on Growth. All cells growing in suspension underwent three phases of growth similar to that of microorganisms (Fig. 1): a lag phase of several days, a log phase from around 8 to 30 days, and a stationary phase. The addition of NaCl to basal media equally reduced the growth of both NaCl-adapted and nonadapted cells. Although not precisely quantified here, the data in Figure 1 are representative of the amount of growth reduction caused by NaCl in adapted and nonadapted cell lines. Considerable variation occurred in cell growth in individual flasks inoculated from the same parent culture. The data in Figure 1 show the extremes of "good" and "slow" growth of inocula from parental cultures with good growth.

Osmotic Potential and Solute Changes in Media. Sucrose accounts for 57% of the osmotic potential in fresh Linsmaier and Skoog medium, whereas major inorganic ions contribute 38% and trace elements and organic supplements add 5%.

When fast- and slow-growing cells were periodically monitored

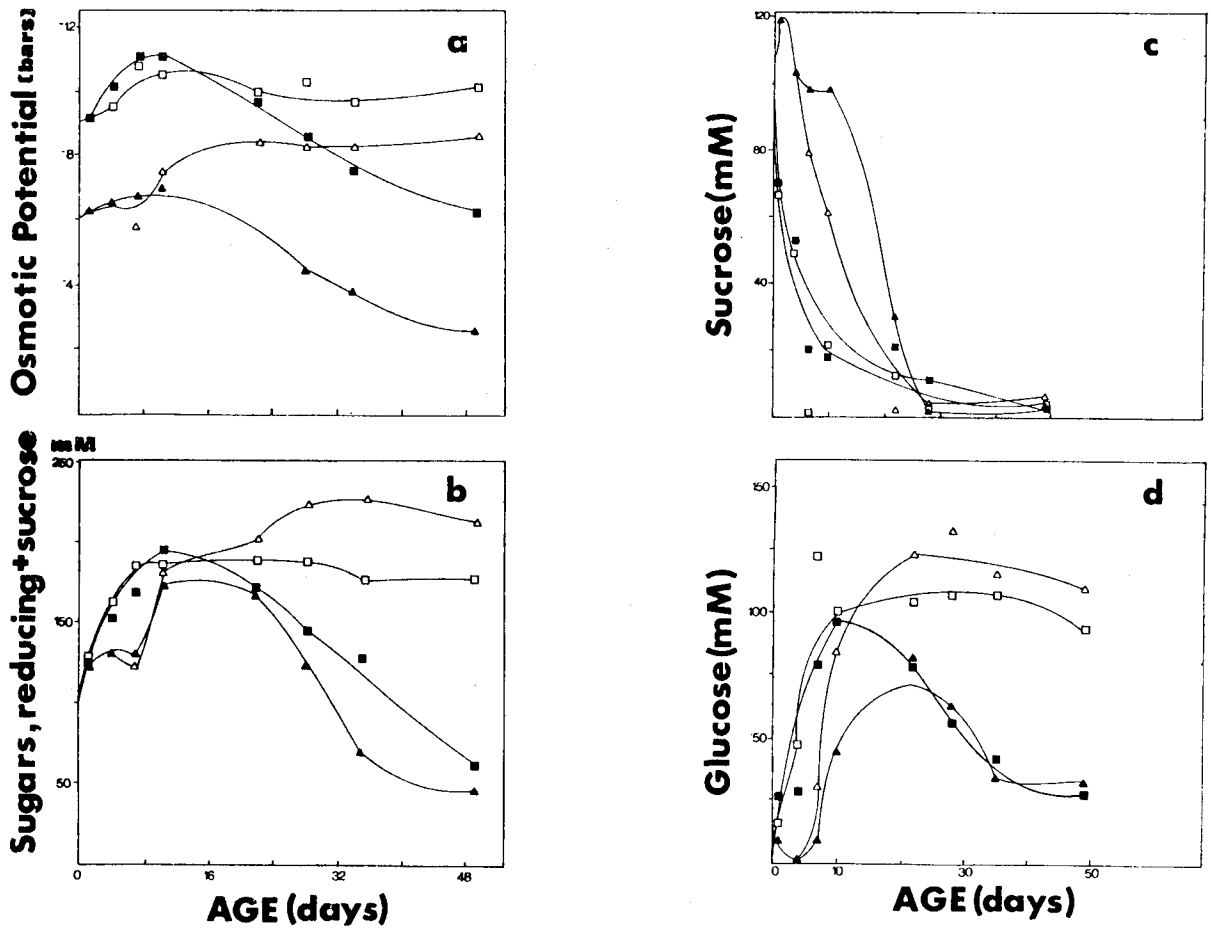

FIG. 2. Osmotic potential of medium (a), sugars (reducing and sucrose) (b), sucrose (c), and glucose (d) in medium *versus* time of culture for four flasks shown in Figure 1. (▲), Medium E in a flask with fast growth; (△), medium E in a flask with slow growth; (■), 79 mM NaCl plus basal medium in a flask with fast growth; (□), 79 mM NaCl plus basal medium in a flask with slow growth.

on 0 and 79 mM NaCl, a characteristic decrease of 2 to 3 bars (100 to 150 mosmol/l) occurred in the medium osmotic potential (Fig. 2a), followed by a gradual increase as solutes were taken up and metabolized. In flasks with little growth, the osmotic potential became more negative by 2 to 3 bars and then remained constant during the rest of the culture period. Most of the decrease in medium osmotic potential was accounted for by changes in the total sugar concentration (Fig. 2b) which increased by 100 mM during the first 10 culture days. During this time, the sucrose concentration declined from 110 to 10 mM (Fig. 2c), whereas the glucose concentration rose from 0 to 100 to 110 mM (Fig. 2d). From 10 to 50 days culture, total sugars and glucose gradually declined in the flasks with growth at 0 and 79 mM. These levels remained constant after the initial increase in the flasks with little growth.

Nitrate levels remained static for 10 to 20 days in all flasks and then were gradually reduced to about 10 mM in the flasks with growth at both NaCl levels (Fig. 3a). Media chloride levels remained constant in flasks with good and slow growth at 0 and 79 mM NaCl (Fig. 3b). The organic anion, malic acid, was present in only small amounts (Table I).

Sodium levels in the media were also constant at both NaCl levels and growth rates (Fig. 4a). The fast growing cells on E medium gradually reduced the K^+ concentration in the medium after 20 days of culture, whereas K^+ remained constant in all other flasks (Fig. 4b). α-Amino nitrogen was also removed by growing cells at equal rates on both NaCl levels (Fig. 5). α-Amino nitrogen rose slightly during the first 10 days of culture in all flasks, presumably due to a leakage of amino acids from the cells and it then gradually declined in the growing flasks at both 0 and 79 mM NaCl, whereas it continued to increase until day 20 in flasks with slow-growing cells. α-Amino nitrogen was removed at the same rate of NO_3^- for the first 15 days culture by fast-growing cells on either 0 or 79 mM NaCl. After that time, NO_3^- removal proceeded at a faster rate. In slow growing cells, NO_3^- was removed from the medium with 79 mM NaCl but not by cells growing slowly on medium E.

Comparison of Cell and Media Osmotic Potentials. The osmotic potentials of the cells were generally 1 to 2 bars more negative than those of the medium at both NaCl levels (Fig. 6). The osmotic potentials became more positive in the cells as media solutes were taken up and metabolized. The osmotic potential of slow growing cells remained more negative by around 3 bars over that of the fresh media after the hydrolysis of the sucrose. Cells grown on 130 mM NaCl had an osmotic potential 6 bars more negative than those grown on basal medium. The Na^+ and Cl^+ concentrations accounted for this difference. The osmotic potentials in cells growing on basal medium and 130 mM NaCl varied similarly during the growth cycle except for the added osmotic component due to NaCl.

Solute Levels in Unwashed and Washed Cells. When unwashed and briefly washed cells were analyzed for major ions and organic compounds (Table I), all solutes except for Na^+ and Cl^- were at similar levels in cells grown in basal medium or 109 mM NaCl. The concentrations of K^+, proline (imino-N) and L-malic acid were higher in the cells on 0 and 109 mM NaCl levels than in their

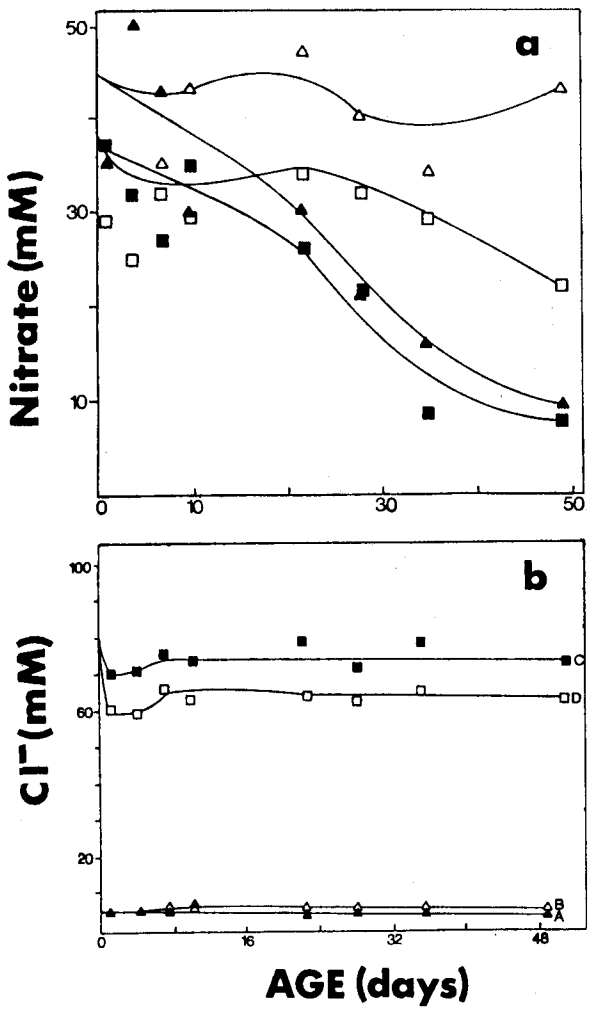

FIG. 3. Nitrate (a) and Cl^- (b) in medium *versus* time of culture for four flasks growing under the following conditions; (▲), medium E, fast growth; (△), medium E, slow growth; (■), 79 mM NaCl, fast growth; and (□), 79 mM NaCl, slow growth.

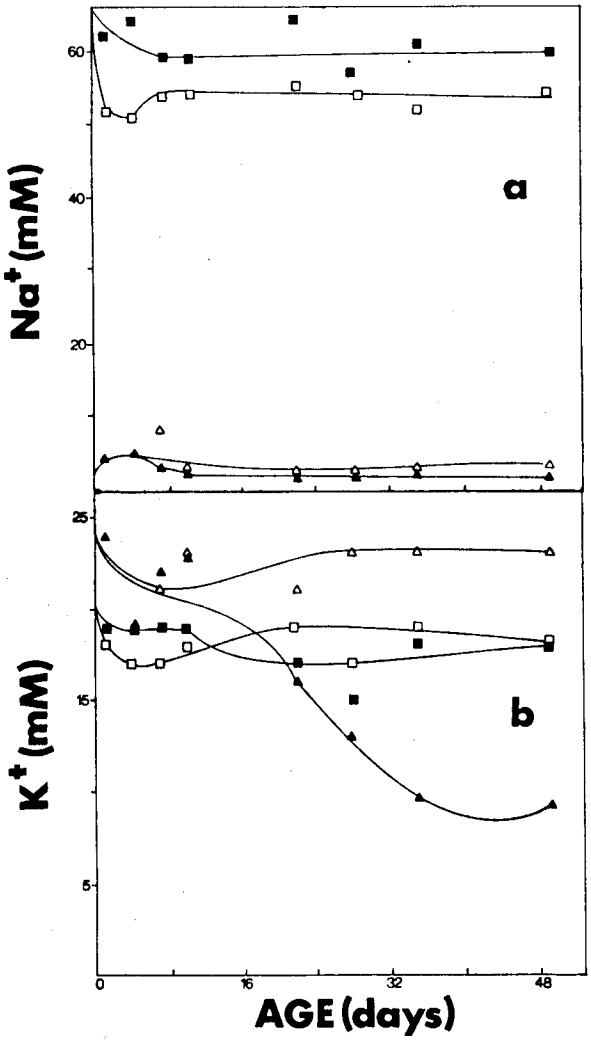

FIG. 4. Na^+ (a) and K^+ (b) in medium *versus* time of culture for four flasks growing under the following conditions: (▲), medium E, fast growth; (△), medium E, slow growth; (■), 79 mM NaCl, fast growth; and (□), 79 mM NaCl, slow growth.

respective media except for slow-growing cells in 109 mM NaCl.

When the contribution of the measured solutes to cellular osmotic potential was determined, the organic compounds (primarily sugars, only sucrose and glucose were measured) contributed 50% of the osmotic potential in both unwashed and briefly washed cells grown on basal medium, whereas measured ions contributed 31 to 33% (Table II). For the cells grown on 109 mM NaCl, the Na^+ and Cl^- ions contributed 46% of cellular osmotic potential in unwashed cells and 44% in washed cells. Organic compounds here contributed 22% of the osmotic potential in unwashed cells and 33% in washed cells (Table II).

The sum of measured cations and anions was approximately equivalent in unwashed and washed cells (Table III). There was a slight accumulation of cations in cells over the media at both NaCl levels for unwashed cells, whereas, on 109 mM NaCl, the medium and washed cells had similar sums of concentrations of cations ($Na^+ + K^+$). The ions and organic compounds in the media at both NaCl levels accounted for 81 to 89% of the cellular osmotic potential (Table I).

Analysis of Solute Efflux from Cells. When Na^+, K^+, Cl^-, total sugars, and amino acids were measured at various times during a distilled H_2O wash, a typical efflux curve was obtained (Fig. 7). The curve contained a fast efflux component which washed out within the first 10 min wash and a distinct second component which took several hours to remove. The average half-times of efflux for the fast component of Na^+, K^+, Cl^-, sugars, amino acids, and proline were 1.6, 0.90, 0.60, 1.7, 0.6, and 0.8 min, respectively (Table IV). Given experimental variation, all of these solutes had similar efflux half-times. The efflux half-times for the slow components of Na^+, K^+, Cl^-, and sugars were 2.2, 1.6, 2.3, and 4.9 h, respectively (Table V).

DISCUSSION

Adapted cells grown on basal medium plus 79 mM NaCl showed reduced growth when compared with similar cells maintained on basal medium (Fig. 1). This reduction of growth on NaCl agrees with the reports of Bernstein (1, 2) that the major effect of NaCl on plant growth is a general stunting of all plant parts.

Salt damage can be divided into two general categories. These are toxic effects caused by specific ions and negative effects caused by a lowered external water potential. Strogonov (32) favored the idea that negative salt effects are primarily due to specific ion toxicities, whereas Bernstein (1) and Bernstein and Hayward (3) identified negative osmotic effects of salt as the primary cause of damage. As the cells accumulated the Na^+ and Cl^- to at least the same concentrations as the medium, negative osmotic effects due to an inability of the cells to reduce their osmotic pressure suffi-

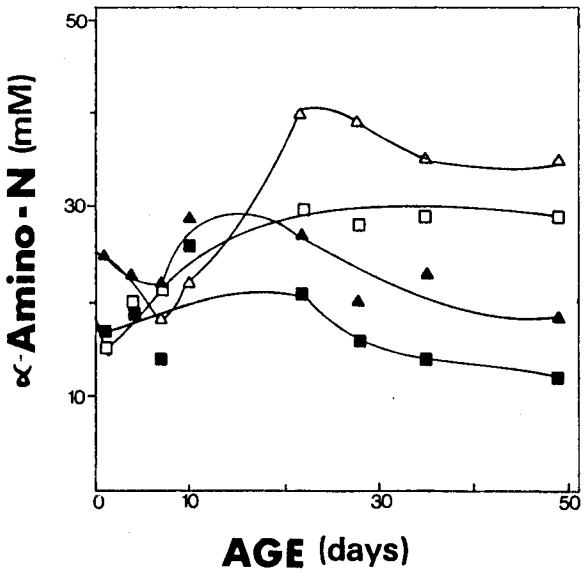

FIG. 5. α-Amino nitrogen (which includes ammonium ion and amino acids) in medium *versus* the time culture for four flasks growing under the following conditions: (▲), medium E, containing cells with fast growth; (△), medium E, containing cells with slow growth; (■), 79 mM NaCl, containing cells with fast growth; and (□), 79 mM NaCl, containing cells with slow growth.

ciently may be questioned. A growth reduction due to a reduction in pressure potential of the cells was not observed (unpublished data). Thus specific toxic effects are the probable cause of the NaCl inhibition of growth in NaCl-adapted and nonadapted cells.

Osmotic Adjustments. The Na^+ and Cl^- were neither removed from nor concentrated in the media (Figs. 3b and 4a) as the cells increased in mass and decreased the medium volume through water uptake. Other media solutes were equally removed by uptake and metabolism at 0 and 79 mM NaCl, except for K^+ which was removed only by fast-growing cells on basal medium. The sucrose in the medium was presumably hydrolyzed by an extracellular invertase. Thorpe and Meier (35) reported such invertase activity for tobacco callus. Soluble invertase activity was found in the medium during the first 10 to 12 days of culture. Extracellular invertase activity was also reported for sycamore cells (7). In this work, only sucrose and glucose were measured. Clearly, the mechanism of osmotic adjustment for cells grown on readily penetrating ions, such as Na^+ and Cl^-, is through their accumulation.

The cells growing on 0 and 130 mM NaCl maintained about the same osmotic potential difference between the cells and media (Fig. 6). When various solutes were measured in the media and cells, only K^+ was clearly accumulated in both washed and unwashed cells (Table I). Sugars are the primary organic solutes in the cells during lag and exponential growth (Fig. 2; Table II) and are at the same concentration in cells grown on basal medium and on NaCl (Table I). The greater increase of α-amino nitrogen in media with slow growing cells could be due to a lysis of dead cells or to a greater leakage of amino acids from stationary cells. There was no K^+ accumulation in slow-growing cells as occurred in fast-growing cells at 0 and 109 mM NaCl (Table I). Senescing cells have increased membrane permeability, which could account for a lack of solute accumulation in the slow-growing cells (33). Ammonium ions and amino acids are not distinguished from each other by the ninhydrin test (25). Precise determination of NO_3^- *versus* NH_4^+ use would require specific measurement of the NH_4^+ ion such as by the assay used by Cataldo *et al.* (5).

A slight accumulation of several compounds and ions instead of a larger accumulation of one or two solutes may account for

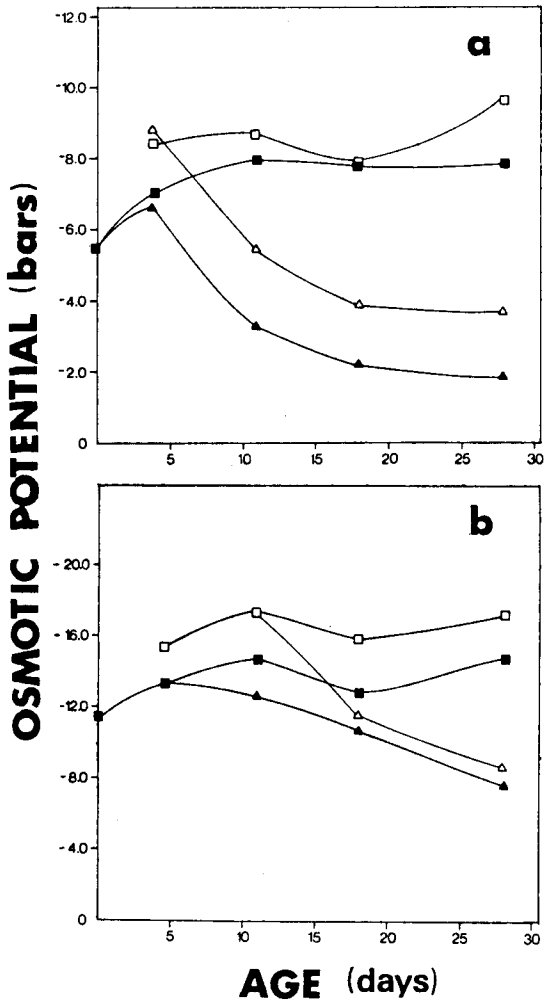

FIG. 6. Osmotic potential of cells and medium *versus* age of culture for nonadapted fast and slow growing cells in medium E (a) and NaCl-adapted fast- and slow-growing cells in 130 mM NaCl (b): (■), slow-growth medium; (□), slow-growing cells; (▲), fast-growth medium; and (△), fast-growing cells.

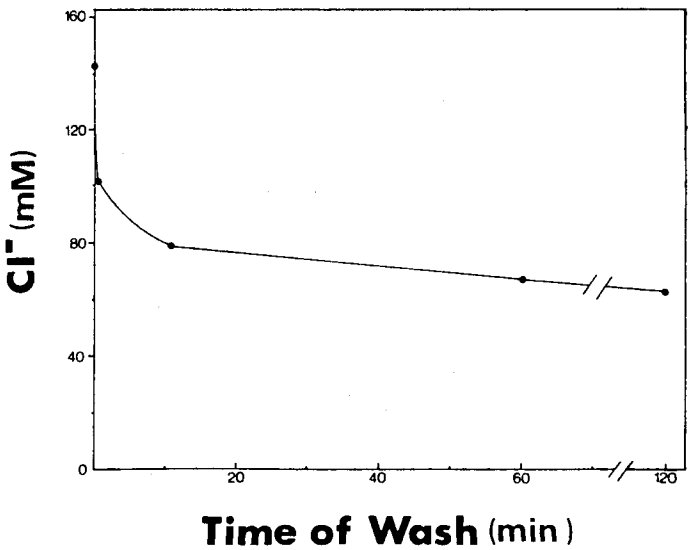

FIG. 7. Efflux curve of Cl^- from cells grown on 109 mM NaCl plus medium E. Cells were washed with 20 ml distilled H_2O/g fresh weight.

Table I. *Analysis of Major Ions and Organic Compounds and Their Contribution to Osmotic Potential of Unwashed Cells, Washed Cells, and Growth Medium*

Conditions were: A, cells with fast growth on medium E; B, cells with slow growth on medium E; C, cells with fast growth on 109 mM NaCl plus medium E; and D, cells with slow growth on 109 mM NaCl plus medium E.

Cells	Water	Osmotic Potential	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	NO_3^-	amino-N	imino-N	Malic acid	Glucose	Sucrose	Total Reducing Sugars	Sum of Measured Compounds	Osmotic Potential
	%	*mosmol*	*bars*						*mM*							%
A. Fast growth, medium E																
No wash	95	232.0	−5.7	2.8	34	1.8	3.3	8.5	21	20	2	7.5	42	21	70	192	83
15 s wash		205.5	−5.0	2.8	31	1.2	2.1	8.8	21	20	2	7.6	55	13	58	168	82
Medium		187.9	−4.6	2.3	11	1.6	2.4	8.9	21	12	0	0.0	63	0	106	165	87
B. Slow growth, medium E																
No wash	93	347	−8.5	3.2	18	1.6	1.8	12	26	17	0	0	132	0	210	290	84
15 s wash				3.9					0.81	0	0	0	0	4	2.5		
Medium		369	−9.1	1.8	16	1.8	2.6	8.8	44	22	0.9	0	147	5.5	227	329	89
C. Fast growth, 109 mM NaCl																	
No wash	95	436.7	−10.7	85	35	2.0	2.6	115	14	17	10	8.0	29	26	37	352	81
30 s wash		313.9	−7.7	67	25	1.5	1.4	72	9.3	21	14	8.8	23	25	34	279	89
Medium		316.1	−7.8	76	17	1.8	2.1	117	15	3.6	2.6	0	21	28	35	298	94
D. Slow growth, 109 mM NaCl																	
No wash				63	14	0.6	0.5	84	39	14	11	0	88	17	159	402	
Medium		543.1	−13.4	71	20	2.1	2.1	103	30	19	0.75	0.15	110	24	165	437	80

Table II. *Relative Contribution of Measured Inorganic Ions and Organic Compounds to Cellular Osmotic Potential of Suspensions Growing on Medium E plus or minus 109 mM NaCl at Two Different Growth Rates*

Cells and/or Medium	Osmotic Potential	Sum Measured Compounds and Ions	Osmotic Potential	Sum Inorganic Ions	Osmotic Potential	Sum Organic Compounds	Osmotic Potential	Sum of Na^+, Cl^-	Osmotic Potential
	mosmol	*mM*	%	*mM*	%	*mM*	%	*mM*	%
Cells									
Fast growth, medium E									
No wash	232	192	83	71.4	31	120.5	52	11.3	5
15 s wash	205.5	168	82	67	33	101	49	11.6	6
Slow growth, medium E									
No wash	347	290	83	63	18	227	65	15.2	4
Fast growth, 109 mM NaCl									
No wash	436.7	352	81	254	58	98	22	200	46
30 s wash	313.9	279	89	176	56	103	33	139	44
Slow growth, 109 mM NaCl									
No wash		402		201		201		147	
Medium									
Medium E									
Fast growth	187.9	165	87	47	25	118	63	11.2	6
Slow growth	369.0	329	89	75	20	255	69	10.6	3
109 mM NaCl									
Fast growth	316.0	298	94	229	72	69	22	193	61
Slow growth	543.1	437	80	228	42	209	38	174	32

the 2-bar osmotic potential difference between the cells and media. Ions should be considered the solutes which contribute to the osmotic potential difference. Cation concentrations were consistently greater in unwashed cells than in medium (Table III). Washed cells could have had their contents diluted by water uptake, whereas a slightly greater ion concentration in the cells could be masked by the still greater cation concentration in the cell walls of unwashed cells. The kinetic efflux analysis, although preliminary, still indicated that most Na^+ and Cl^- are located in the vacuole (Fig. 7; Table V). A certain fraction of the Na^+ and Cl^- was located in the free space (Table IV) and possibly in the cytoplasm. As indicated under "Materials and Methods," the filtered but unwashed cells were placed in distilled H_2O instead of 0.5 mM $CaCO_4$ for efflux analysis. Erdei and Zsoldos (10) found that K^+ ($^{86}Rb^+$) efflux from preloaded rice tissue cultures was greater after a 10-min wash of cells with a minus Ca^{2+} solution than for cells washed in 1 mM $CaCl_2$. They used a 5-min pretreatment of the suspension cells with 1 mM KCl before the start of the

Table III. *Comparison of Cation and Anion Concentrations in Cells Grown on Medium E Plus or Minus 109 mM NaCl at Fast and Slow Growth Rates*

	Sum of Cations		Difference (Cells − Medium)	Sum of Anions		Difference (Cells − Medium)
	$Na^+ + K^+$ in cells	Sum $Na^+ + K^+$ in medium		$Cl^- + NO_3^-$ cells	$Cl^- + NO_3^-$ medium	
	mM			*mM*		
Medium E, fast growth						
No wash	37	13	24	30	30	0
15 s wash	34	13	21	30	30	0
Medium E, slow growth						
No wash	21	17	3	38	53	−15
109 mM NaCl, fast growth						
No wash	120	93	27	129	132	−3
30 s wash	92	93	−1	81	132	−51
109 mM NaCl, slow growth						
No wash	77	91	−14	123	133	−10

Table IV. *Half-times of Efflux for Fast Component which Was Washed from Cells with Distilled H_2O*

The cells were grown on 0 to 151 mM NaCl or 89 or 109 mM KCl. Wash volumes are indicated.

Salt Concentration in Medium		Volume of Wash	Efflux $t_{1/2}$					
			Na	K	Cl	sugars	amino-N	imino-N
	mM	*ml H_2O/g fresh wt*				*min*		
NaCl	0	9.0	1.0			0.80		
	0	20		2.0	0.80			
	27	6.0		0.40	0.20	0.20		
	79	20	1.0	2.0	0.80			
	89	3	3.2	0.80			1.3	1.7
	89	10	0.50	0.3			0.3	0.4
	89	30		0.2			0.2	0.2
	130	6.0	0.6	0.6		1.2		
	130	9.0	3.3	0.50	1.2	1.2		
	151	20	1.7	1.0	1.0			
KCl	89	9.0		1.6	0.80	5.0		
	109	20		0.40	0.40			
Average			1.6	0.90	0.60	1.7	0.6	0.8

Table V. *Half-times of Efflux of Slow Component Which Was Washed from Cells by Distilled H_2O*

The cells were grown on 0 to 151 mM NaCl or 89 or 109 mM KCl. Wash volumes are indicated.

Salt Concentration in Medium		Volume of Wash	Efflux $t_{1/2}$			
			Na	K	Cl	Sugars
	mM	*ml H_2O/g fresh wt*		*h*		
NaCl	0	9.0				3.0
	89	3.0	2.6			
	89	30	1.7	0.77		
	130	6.0	2.2	0.50		4.6
	130	9.0		1.3	2.1	
	151	20			1.3	
KCl	89	9.0		3.6	3.4	7.2
	109	20		2.0	2.5	
Average			2.2	1.6	2.3	4.9

efflux period.

A comparison of NaCl-adapted tobacco cells washed with distilled H_2O for 10 min *versus* those cells washed in 0.5 mM $CaSO_4$ for 15 min showed a slightly greater efflux of both Na^+ and K^+ from the cells washed with distilled H_2O (unpublished data).

These data suggest that a Ca^{2+}-free efflux solution could alter the efflux kinetics (which are based upon the permeability of the plasmalemma and tonoplast) in less than 10 min. A loss in differences in efflux rates between the plasmalemma of Na-adapted and nonadapted cells due to a general increase in permeability caused by Ca^{2+} loss was not considered to be a serious problem for the following reasons. (*a*) Cells in these experiments were grown on 3.0 mM Ca^{2+} instead of the 0.1 mM Ca^{2+} commonly used for solutions in which seeds are germinated and used to provide the material on which root efflux experiments are performed. (*b*) The measured Ca^{2+} level in the cells did not decline during a wash period of 150 min (unpublished data). (*c*) No prewash treatment of cells was done before the start of the efflux experiments. This further reduced Ca^{2+} loss.

An efflux component from the cytoplasm, which was not detected, would have had a half-time of 10 to 20 min (4), which is before serious changes in membrane permeability due to Ca^{2+} deficiency could have obscured differences in cytoplasmic efflux rates between the two cell lines.

The toxic effects of NaCl would be better understood if the Na^+ and Cl^- concentrations in the cytoplasm were determined. A cytoplasmic location in adapted cells would suggest that NaCl tolerance requires tolerant cytoplasmic enzymes, especially in the

competition of Na^+ with K^+ at active sites. Exclusion of these ions from the cytoplasm would imply both a mechanism of NaCl-tolerance in NaCl-adapted cells, and indirect proof that these ions are toxic to essential metabolic processes. The cytoplasm accounts for approximately 4% of the total cellular volume in mature plant cells (26) and the unequivocal location of ions in the cytoplasm is difficult. As only two components instead of three (Fig. 7) were identified in the efflux analysis, an assumption must be made as to the compartmental sources of these two components. It was concluded that the fast efflux component contained solutes of both the free space and cytoplasm for the following reasons: cells in suspensions have leaky membranes (36) and, in suspensions, the cytoplasm is a thin layer pressed against the plasmalemma, whereas a large surface area of each cell is exposed to the external medium. Because of the obvious presence of medium solutes in the free space, no definite statement could be made as to the cytoplasmic source of Na^+ or Cl^- in the first efflux component.

The role of tissue culture in plant breeding programs remains to be determined on an individual basis for each crop species. A review of the possible contributions of mutant selection through tissue culture to agriculture has been previously made (20). The procedures for selection of biochemical mutants (increased amino acid content, more efficient nitrogen use, herbicide tolerance, etc.) at the cellular level using tissue culture techniques are clear. The cells are exposed to a stress which selects for cells with a specifically altered metabolism and those cells which grow under the stress conditions then are used to regenerate plants which can then be tested for that particular stress tolerance.

It is not at first clear how selection for many important whole plant attributes (yield, grain quality, plant height, etc.) might be made at the cellular level (31). Ultimately changes in phenotype will depend on changes in the genome and resultant cellular metabolism. Selection for changes in the biochemistry of a cell may lead to desirable changes in phenotype (20).

A basic problem which awaits solution in biology is how a single totipotent cell can direct its cell division and differentiation to produce a multicellular organism with a characteristic morphology.

An alternative empirical use can be made of tissue culture techniques. Mutant production in culture using either spontaneous rates or with the use of ionizing radiation or mutagenic chemicals can be made and followed by regeneration of large numbers of potentially mutant plants. Screening then can be made at the whole plant level for desirable changes in whole plant phenotype. Shepard *et al.* (28) have obtained variant phenotypes of potato in plants regenerated from single protoplasts obtained from leaf mesophyll cells. Some variants showed increased disease resistance, altered plant morphology, and photoperiod sensitivity.

No major differences were observed in cells previously adapted to NaCl *versus* that of cells just shifted to NaCl media. Other differences were found between NaCl-adapted and nonadapted cells when both were exposed to the nonpenetrating solutes polyethylene glycol and dextran (unpublished data). If a mutation for increased NaCl tolerance was selected, it was probably a quantitative mutation as no obvious qualitative differences were found in the osmotic adjustment of NaCl-adapted and nonadapted cells to NaCl. The most definite work on the genetic basis for salt tolerance will be obtained from plant breeding experiments with the regenerated progeny from nonadapted and NaCl-adapated cell lines.

LITERATURE CITED

1. Bernstein L 1963 Osmotic adjustment of plants to saline media. II. Dynamic phase. Am J Bot 48: 360–370
2. Bernstein L 1975 Effects of salinity and sodicity on plant growth. Annu Rev Plant Pathol 13: 295–312
3. Bernstein L, H Hayward 1958 Physiology of salt tolerance. Annu Rev Plant Physiol 9: 25–46
4. Cameron A 1978 Carbon dioxide and potato wound metabolism. MS thesis. Colorado State University, Ft. Collins
5. Cataldo DA, LE Schrader, VL Young 1974 Analysis by digestion and colorimetric assay of total nitrogen in plant tissues high in nitrate. Crop Sci 14: 854–856
6. Chaleff RS, MF Parsons 1978 Direct selection *in vitro* for herbicide-resistant mutants of *Nicotiana tabacum*. Proc Natl Acad Sci USA 75: 5104–5107
7. Copping L, HE Street 1972 Properties of the invertases of cultured sycamore cells and changes in their activity during culture growth. Plant Physiol 26: 346–354
8. Croughan T, S Stravarek, DW Rains 1978 Selection of a NaCl tolerant line of cultured alfalfa cells. Crop Sci 18: 959–963
9. Epstein E 1961 The essential role of calcium in selective cation transport by plant cells. Plant Physiol 36: 437–444
10. Erdei E, F Zsoldos 1977 Potassium absorption by rice at different levels of organization. I. Effects of temperature and calcium on K^+ fluxes and content. Physiol Plant 41: 99–104
11. Fisher RA, NC Turner 1978 Plant productivity in the arid and semiarid zones. Annu Rev Plant Physiol 29: 277–317
12. Foote BD, JB Hanson 1964 Ion uptake by soybean root tissue depleted of calcium by ethylenediaminetetraacetic acid. Plant Physiol 39: 450–460
13. Gutmann I, A Wahlefeld 1974 L-(−)-Malate determination with malate dehydrogenase and NAD. *In* HU Bergmeyer, ed, Methods of Enzymatic Analysis, Ed 2 Vol 3 Section D. Academic Press, New York. pp 1585–1599
14. Haywood H, C Wadleigh 1949 Plant growth on saline and alkali soils. Adv Agron 1: 1–38
15. Linsmaier FM, F Skoog 1965 Organic growth factor requirements of tobacco tissue cultures. Physiol Plant 8: 100–127
16. Lowe R, T Hamilton 1967 Rapid method for determination of nitrate in plant and soil extracts. J Agr Food Chem 15: 359–361
17. Macrobbie EAC, J Dainty 1958 Ion transport in *Nitellopsis obtusa*. J Gen Physiol 42: 335–353
18. Manasse R 1972 A new culture flask for plant suspension cultures. Experientia 28: 723–724
19. Marx JL 1979 Plants: Can they live in salt water and like it? Science 206: 1168–1169
20. Nabors MW 1976 Using spontaneously occurring and induced mutations to obtain agriculturally useful plants. BioScience 26: 761–768
21. Nabors NW, A Daniels, L Nadolny, C Brown 1975 Sodium chloride tolerant lines of tobacco cells. Plant Sci Lett 4: 155–159
22. Nabors MW, S Gibbs, C Bernstein, M Meiss 1980 NaCl-tolerant tobacco plants from cultured cells. Z Pflanzenphysiol 97: 13–17
23. Nelson M 1944 A photometric adaptation of the Somogyi method for the determination of glucose. J Biol Chem 153: 375–380
24. Pitman MG 1963 The determination of the salt relations of the cytoplasmic phase in cells of beetroot tissue. Aust J Biol Sci 16: 647–668
25. Rosen H 1957 A modified ninhydrin colorimetric analysis for amino acids. Arch Biochem Biophys 67: 10–15
26. Salisbury F, CW Ross 1978 Plant Physiology, Ed 2. Wadsworth, Belmont, CA, p 4
27. Schales O, S Schales 1941 A simple and accurate method for the determination of chloride in biological fluids. J Biol Chem 140: 879–884
28. Shepard JF, D Bidney, E Shahin 1980 Potato protoplasts in crop improvement. Science 20: 17–24
29. Smith IK 1978 Role of calcium in serine transport in tobacco cells. Plant Physiol 62: 941–948
30. Somogyi M 1952 Notes on sugar determinations. J Biol Chem 195: 19–23
31. Sprage CF, CF Alexander, JW Dudley 1980 Plant breeding and genetic engineering. Bioscience 30: 17–21
32. Strogonov BP, ed 1964 Physiological basis of salt tolerance of plants (as affected by various types of salinity). In Israel Program for Scientific Translations, Chap 2. Daniel Davey, New York, pp 16–25
33. Suttle JC, H Kende 1980 Ethylene action and loss of membrane integrity during petal senescence in *Tradescantia*. Plant Physiol 65: 1067–1072
34. Taniguchi S, E Itagaki 1960 Nitrate reductase of nitrate respiration type from *E. coli*. Biochim Biophys Acta 44: 263–279
35. Thorpe T, D Meier 1973 Sucrose metabolism during tobacco callus growth. Phytochemistry 12: 493–497
36. Wareing P, I Phillips 1970 The control of growth and differentiation in plants. Pergamon Press, Elmsford, NY, pp 152–154

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Growth, Water Content, and Solute Accumulation of Two Tobacco Cell Lines Cultured on Sodium Chloride, Dextran, and Polyethylene Glycol[1]

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ABSTRACT

Simulated drought tolerance was compared for an NaCl-adapted and a nonadapted cell line of tobacco (*Nicotiana tabacum* var. Samsum) to determine the relationship of salt and drought tolerances. The osmotic adjustment and growth of these two lines was followed when cultured on solid media which contained isosmotic concentrations of NaCl, KCl, polyethylene glycol (PEG) or dextran. One line was adapted to growth on 130 millimolar NaCl, but the other was not.

The growth of NaCl-adapted and nonadapted cell lines was equally inhibited (61 per cent of control) by 130 millimolar NaCl. Growth inhibition was greater on PEG or dextran than on NaCl. Growth ceased on the second passage of dextran for the nonadapted cells, while the NaCl-adapted cells grew slowly for four passages on dextran. Water contents for both cell lines were 95 per cent on NaCl or KCl and 70 to 88 per cent on PEG 1540 or 4000 or dextran after the second passage on these media.

On dextran or PEG 4000, 46 to 89 per cent of the cellular osmotic potential was produced by the solutes initially present in the medium. Similarly, on NaCl, almost 100 per cent was attributable to solutes in the medium. It was concluded that cells grown on the nonpenetrating solutes had a more negative osmotic potential than those grown in the absence of added solute due to partial dehydration, greater uptake of external ions, and possibly the production of unidentified osmotica. Adjustment to growth on penetrating solutes may have enabled the adapted line to overcome the osmotic stress produced by nonpenetrating dextran.

Salt damage in plants can be divided into two general categories: toxic effects caused by specific ions, and detrimental effects caused by a lowered external osmotic potential due to the salt. Strogonov and others (2, 8, 29) favor the idea that toxic effects of specific ions account for most salt damage. Bernstein and co-workers (3, 4, 6) attribute most of the detrimental effects of salt to osmotic stress.

When nonpenetrating or less readily penetrating solutes are used, the more negative osmotic potential of the external medium due to these solutes can only be counter-balanced by uptake of other external solutes, tissue dehydration, or the synthesis of organic osmotica. PEG has been used as a nonpenetrating osmoticum (1, 20), although several reports suggest that it may be taken up by plants (20, 30) and that it causes detrimental effects other than osmotic ones (14, 17, 18). Kaufmann and Eckard (13) reported that PEG 6000 produced changes in plant water relations similar to those caused by drying soil at the same water potential. This was not true for PEG 400 which had other detrimental effects. Dextran was selected as a nonpenetrating osmoticum by Strogonov (29), who reported no external lesions and hardly any decrease in the growth rate, but somewhat delayed flowering in corn. It was not absorbed by the roots.

A previous paper (9) discussed the means of osmotic adjustment of a NaCl-adapted and a nonadapted cell line of tobacco to NaCl. Both of these cell lines accumulated NaCl or KCl to the same level as in the medium. This paper reports experiments which show the response of these cell lines to the nonpenetrating osmotica, PEG and dextran.

MATERIALS AND METHODS

The source of cell lines of *Nicotiana tabacum* L. var. Samsum, basal media, and most of the chemical assays used herein were reported in earlier papers (9, 21). The NaCl-adapted cell line was grown 10 months in suspension on 130 mM NaCl plus medium E. Medium E contained Linsmaier and Skoog solutes, 0.1% (w/v) each of yeast and malt extracts, 6 μg/ml 2,4-D, and 0.5 μg/ml kinetin. The 130 mM NaCl was the highest level of NaCl on which there was continuous growth. The non-adapted cell line was grown 1 year in suspension on medium E (9) and each line was subcultured every 4 to 6 weeks. Cells from both lines were cultured on solid media for four 2-month passages during the experiments reported here.

Osmotic Potential. The freezing point depression method was used for the determination of the osmotic potential of the cell sap and agar solidified media (9). Sap was obtained by centrifugation of thawed cells at 23,500*g* for 20 min. The instrument used was the Osmette produced by Precision Instruments, Sudbury, MA. Readings were taken in mosm/l(mosm) which could be converted to bars by the Van't Hoff equation (16, 25).

Chemical Analysis. Calli were extracted with water as in previous work (9). The cations Na^+ and K^+ were measured by a Perkin-Elmer 305 atomic absorption spectrophotometer against chloride standards. Chloride was measured by the titration method of Schales and Schales (26) with mercuric nitrate. Nitrate was measured colorimetrically as nitrite after its partial reduction by a commercial nitrate reductase (15) obtained from Sigma. Amino acids were determined by the ninhydrin method of Rosen (24). Reducing sugars were analyzed by a modified Nelson (23) and Somogyi (27) method. Sucrose was measured after it was hydrolyzed by a commercial invertase.

Isosmotic Concentrations of PEG and Dextran. Standard curves of osmotic potential versus solute concentration were developed for PEG 1540, PEG 4000, and dextran (15,000–20,000 mol wt) by measuring freezing point depression. The values obtained were similar to those of Steuter *et al.* (28) who compared freezing-point depression and vapor pressure methods for determination of water

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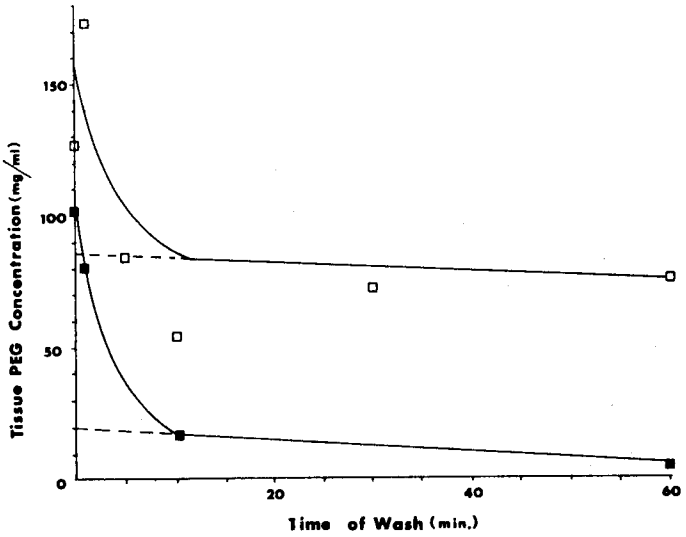

FIG. 1. Efflux of PEG from calli after two months growth on PEG osmotically equivalent to 130 mM NaCl. Cells were washed with 9.0 ml distilled H_2O/g fresh weight. PEG 1540 (□), PEG 4000 (■).

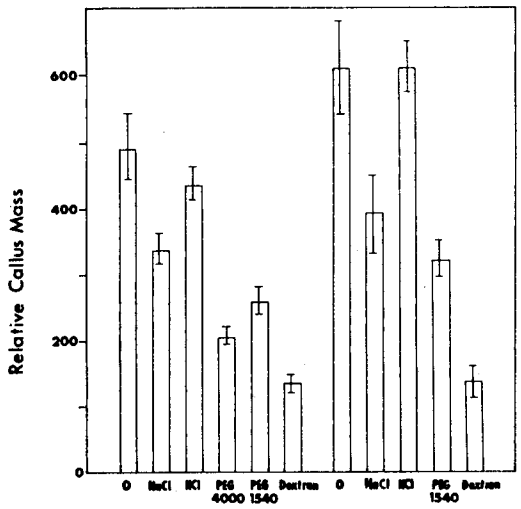

FIG. 2. Relative callus growth on medium E or medium E plus isosmotic concentrations of KCl, PEG 4000, PEG 1540, or dextran. A, inocula from nonadapted cell line previously grown on control medium, and B, inocula from NaCl-adapted cell line. Standard errors are indicated.

potential of PEG solutions. From these curves the appropriate concentrations isosmotic to 130 mM NaCl solutions were determined.

Growth of Cells. One per cent agar-solidified media (medium E plus isosmotic concentrations of NaCl, PEG[2] 1540, or dextran) were used for all treatments except isosmotic concentrations of PEG 4000, which prevented agar solidification. For this treatment, liquid medium containing the desired concentration of PEG 4000 was used. To insure callus growth in this treatment, filter paper (Whatman No. 1) was cut into rectangular strips of approximately 2 by 10 cm, the strips were folded to form a cube with two open ends, and the cube was placed in a 22-ml vial with 10 ml of medium. The filter paper acted as a support for the callus above

[2] PEG was purchased from Baker Chemical Co., Phillipsburg, NJ 08865. PEG from this source has no measurable toxic effects on cultured cells or germinating seeds.

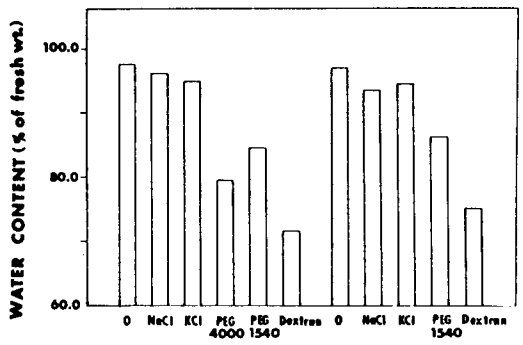

FIG. 3. Water content of cells after 2 months growth on control medium plus isosmotic concentrations of NaCl, KCl, PEG 4000, PEG 1540, or dextran. A, inocula from nonadapted cell line, and B, inocula from NaCl-adapted cell line.

the medium and as a wick which supplied the callus with medium. For all treatments, callus size was determined by the visual assay of Hooker and Nabors (10). Fourteen to 18 samples were used to determine one point for which standard errors are given.

Water Content. A sample of callus tissue weighing around 0.3 g was weighed to the nearest 0.0001 g. The sample was transferred to a 5-cm Petri dish, dried for 3 days at 70°C, and reweighed to determine water content.

Extraction of Solid Media. Approximately 0.5 g agar-solidified medium was weighed as above and placed in a tissue grinding vessel (9). Nine ml of water per gram of solid medium were added to the vessel. After 15 grinding strokes, the sample was transferred to a 15-ml Corex tube. The sample was centrifuged for 20 min at 23,500*g* in a Sorvall (SS-3) with a 11-cm radius head. The supernatant was saved for analysis.

Callus Harvest and Extraction. Calli were removed from the growth medium, weighed to the nearest 0.01 g, and frozen. Prior to extraction they were thawed and extracted with 9.0 ml water/g fresh weight by the procedure used before (9).

For the determination of the intracellular PEG content, calli were washed for varying periods as before (9). The efflux analysis of MacRobbie and Dainty (19) was used to estimate the intracellular concentration of PEG.

PEG Assay. The turbidimetric method of Hyden (11) was used. A water extract of calli was first cleared of protein and sulfate by treatment with $BaCl_2$, $Ba(OH)_2$, and $ZnSO_4$ followed by 10 min centrifugation as above. Trichloroacetic acid then precipitated the PEG which remained as a turbid suspension. The turbidity of the suspension was proportional to the PEG 4000 concentration from 0.20 to 1.0 mg PEG/assay (0.016 to 0.083 mg PEG/ml assay solution).

RESULTS

PEG in Cells. PEG 1540 and PEG 4000 were routinely used as nonpenetrating solutes. To test the degree of penetration after 2 months of callus growth, PEG efflux from the calli was determined. The efflux curves (Fig. 1) showed that PEG was mostly located in the fast efflux component which is assumed to be free space (9). It took 1.9 min for half of the initial PEG 4000 to move from the cells to the wash water. The concentration of PEG 4000 inside the cells was around 18 mg/ml; as judged from the extrapolated concentration of the slow efflux portion of the curve. This concentration had an osmotic potential of 0.4 bar. Likewise, PEG 1540 had an intracellular concentration of 86 mg/ml which contributed 1.4 bars of osmotic potential.

First Experiment. Calli of the nonadapted and NaCl-adapted line (each suspension line was first grown for two passages on

Table I. Comparison of the Major Osmotica Found in Calli Grown on Solid Media Which Contained Isosmotic Concentrations of 130 mM NaCl, Dextran or PEG 4000 SEMs are given.

	Na^+	K^+	Cl^-	NO_3^-	Imino	Amino	Sucrose	Reducing Sugars	Osmotic Potential	Sum of solutes	Osmotic Potential
				mM					*mosm*	*mM*	%
Nonadapted cells											
E	11 ± 2	13 ± 3	7.1 ± 0.1	6.8 ± 0.3	5.1 ± 2.6	17 ± 4	0 ± 0	0 ± 0	81 ± 4	60 ± 6	74
E + NaCl	151 ± 4	24 ± 0	161 ± 30	8.6 ± 0.5	22 ± 4	31 ± 6	0 ± 0	0 ± 0	380 ± 11	398 ± 36	105
E + Dextran	14 ± 4	27 ± 3	19 ± 6	19 ± 2	0.4 ± 0.4	32 ± 4	5.2 ± 4.7	81 ± 69	223 ± 44	198 ± 81	89
NaCl-adapted cells											
E	26 ± 2	19 ± 2	30 ± 2	7.4 ± 0.2	15 ± 0	16 ± 0.5	0 ± 0	2.5 ± 2.5	116 ± 2	116 ± 3	100
E + NaCl	156 ± 6	24 ± 2	178 ± 10	14 ± 6	12 ± 0	23 ± 4	0 ± 0	17 ± 11	377 ± 10	428 ± 27	112
E + PEG 4000	27 ± 4	13 ± 0	28 ± 3	12 ± 4	8.7 ± 1.3	10 ± 2	20 ± 6	99 ± 39	472 ± 50	218 ± 44	46
E + Dextran	34 ± 6	44 ± 1	28 ± 10	28 ± 2	9.6 ± 2.4	28 ± 3	0 ± 0	19 ± 2	250 ± 0	191 ± 17	76
Medium from nonadapted cells											
E	4.8 ± 0.7	14 ± 0	12 ± 1	6.0 ± 0.5	1.5 ± 1.5	15 ± 2	2.1 ± 0	0 ± 0	68 ± 1	55 ± 1.5	81
E + NaCl	110 ± 11	20 ± 0.5	156 ± 8	8.4 ± 0.3	0.4 ± 0.4	7.5 ± 3.5	2.4 ± 0.5	3.6 ± 1	373 ± 43	308 ± 21	83
E + Dextran	4.1 ± 0.9	8.6 ± 0.9	8.4 ± 0.4	9.2 ± 3.8	0.2 ± 0.2	5.7 ± 4.3	13 ± 11	102 ± 60	296 ± 134	151 ± 81	51
Medium from NaCl-adapted cells											
E	19 ± 3	17 ± 1	29 ± 5	9.8 ± 0.2	0 ± 0	17 ± 2	0 ± 0	3.6 ± 3.6	120 ± 9	95 ± 15	79
E + NaCl	118 ± 10	20 ± 3	162 ± 10	10.3 ± 0.7	5.5 ± 1.5	7.6 ± 0.7	1.8 ± 1.8	6.5 ± 6.5	365 ± 43	332 ± 18	91
E + PEG 4000	17 ± 4	11 ± 3	18 ± 2	16 ± 8	5.8 ± 0.6	11 ± 8	1.2 ± 1.2	188 ± 107	603 ± 99	268 ± 126	44
E + Dextran	14 ± 0.5	6.6 ± 0.4	20 ± 1	11 ± 2	3.5 ± 2.1	2.1 ± 0.2	4 ± 4	84 ± 4	351 ± 12	145 ± 6	41

Table II. *Ratios of Osmotic Potential, Na^+, K^+, Cl^-, NO_3^-, and the Sum of Ions in Unwashed Cells to Those Found in Respective Media When Nonadapted Cell Line and NaCl-Adapted Cell Line Were Grown on Solid Medium E, 130 mM NaCl or Isosmotic Concentrations of Dextran or PEG 4000*

	Na^+ Cells / Na^+ Medium	K^+ Cells / K^+ Medium	Cl^- Cells / Cl^- Medium	NO_3^- Cells / NO_3^- Medium	Sum Cells / Sum Medium	o.p. Cells / o.p. Medium
Nonadapted cell line						
0 mM	2.3	0.9	0.6	1.1	1.0	1.2
130 mM	1.4	1.2	1.0	1.0	1.2	1.3
Dextran	3.4	3.1	12.3	2.1	2.6	1.3
NaCl-adapted cell line						
0 mM	1.4	1.1	1.0	0.7	1.1	1.2
130 mM	1.3	1.2	1.1	1.4	1.2	1.3
PEG 4000	1.6	1.2	1.6	0.7	1.3	0.8
Dextran	2.4	6.7	1.4	2.5	2.6	1.3

Table III. *Comparison of the Cation (Na^+, K^+) and Anion (Cl^-, NO_3^-) Concentrations in the Unwashed Cells of the Nonadapted Cell Line, and NaCl-Adapted Cell Line When Grown on Solid Medium E, or Medium E Plus 130 mM NaCl or Isosmotic Concentrations of Dextran or PEG 4000*

SEMs are given.

	Cations Cells	Cations Medium	Anions Cells	Anions Medium
		mM		
Nonadapted cell line				
0 mM	24 ± 2	18 ± 0.5	14	17 ± 0.5
130 mM	176 ± 5	131 ± 10	170 ± 29	164 ± 8
Dextran	42 ± 8	12 ± 2	38 ± 9	17 ± 4
NaCl-adapted cell line				
0 mM	46 ± 4	36 ± 4	37 ± 2	39 ± 5
130 mM	180 ± 8	138 ± 13	192 ± 4	172 ± 11
PEG 4000	40 ± 4	28 ± 8	40 ± 8	34 ± 6
Dextran	79 ± 6	21 ± 0	57 ± 8	32 ± 0.5

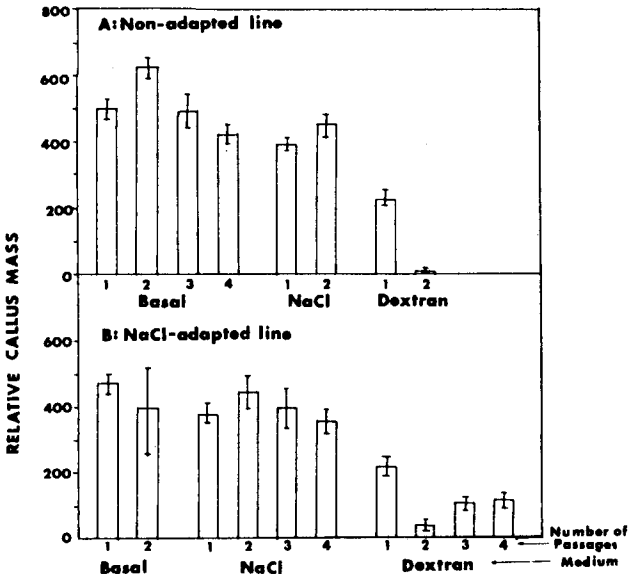

FIG. 4. Growth of cells subcultured on dextran or 130 mM NaCl for up to four passages. A, growth of nonadapted cell line, and B, growth of NaCl-adapted cell line. Dextran was isosmotic to 130 mM NaCl.

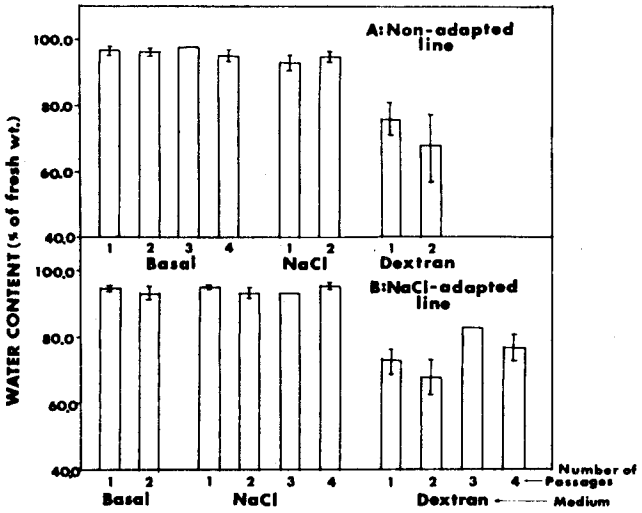

FIG. 5. Water content of cells subcultured on dextran or 130 mM NaCl for up to four passages, A, nonadapted cell line, and B, NaCl-adapted cell line. Dextran was isosmotic to 130 mM NaCl.

respective agar media) were cultured on solid media containing either no extra solutes (medium E) or NaCl, KCl, PEG, or dextran concentrations osmotically equivalent to 130 mM NaCl. A much greater reduction in growth occurred on media with the non-penetrating solutes (Fig. 2) than on media containing penetrating solutes. In both cell lines, growth on medium E (control) and KCl was greater than that on NaCl (67% control). Growth on dextran (25% control) was less than that on PEG 4000 or PEG 1540 (47% control). The water contents of the calli on medium E, and medium E plus 130 mM NaCl or KCl were 90 to 97% (Fig. 3). Calli from both lines grown on E medium plus PEG or dextran had water contents between 70 and 88%.

On medium E plus or minus NaCl, the measured ions and organic solutes initially present in the media accounted for 74 to 100% of the cellular osmotic potential for both cell lines (Table I). On dextran and PEG medium, 46 to 89% of the cellular osmotic potential was attributable to solutes initially present in the medium. The ionic ratios (cell concentration/medium concentration) indicated that both cell lines had a greater accumulation of Na^+, K^+, and NO_3^- over that of the medium in those calli grown on dextran than in those of other treatments (Table II). Cations and anions approximately balanced each other in the cells of both lines grown on all tested media (Table III).

Second Experiment. Calli derived from both nonadapted and NaCl-adapted cell lines were subcultured several times on solid medium E plus and minus 130 mM NaCl or dextran (Figs. 4 and 5). When the calli of both cell lines were subcultured a second time on dextran, growth of the nonadapted line ceased (Fig. 4), and occurred slowly in the adapted line. For the adapted line, growth improved on the third and remained constant on the fourth passage (Fig. 4). Growth on the NaCl media was unaffected

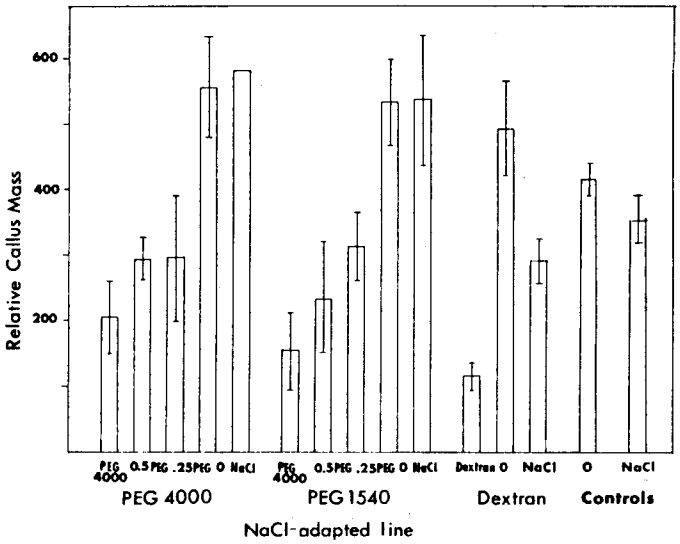

FIG. 6. Growth of callus shifted from PEG 4000, PEG 1540, or dextran to osmotic concentrations of these solutes equivalent to 130, 65, or 32.5 mM NaCl, respectively, *i.e.* PEG 4000, 0.5 PEG 4000, or 0.25 PEG 4000. The calli adapted for growth on PEG or dextran were also shifted to control medium and 130 mM NaCl. Calli came from the NaCl-adapted cell line.

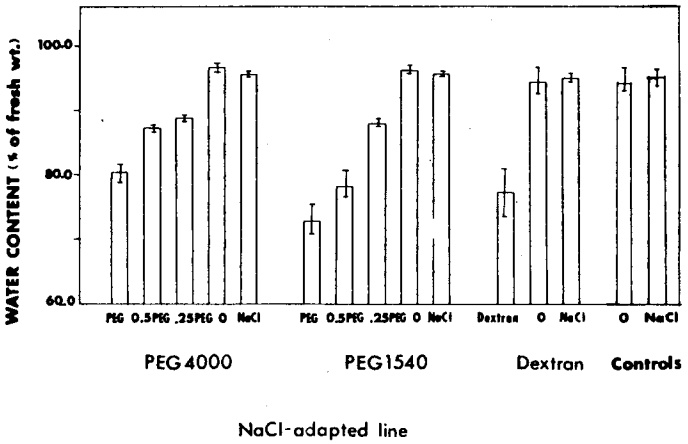

FIG. 7. Water content of (A) calli shifted from PEG 4000 to lower concentrations of PEG 4000, control medium or 130 mM NaCl, (B) calli shifted from PEG 1540 to three PEG 4000 concentrations, control medium or 130 mM NaCl, and (C) calli from dextran medium shifted to control medium or 130 mM NaCl.

by subculturing (Fig. 4). Water contents showed no significant change during subculturing in all treatments (Fig. 5).

Third Experiment. Calli of the NaCl-adapted line grown for three passages on PEG media were transferred to media containing one-half or one-quarter of the original osmotic concentration of PEG, 130 mM NaCl, or no added osmoticum (Fig. 6). NaCl-adapted calli grown for three passages on dextran were transferred to dextran medium (osmotically equivalent to 130 mM NaCl), medium E, or 130 mM NaCl medium. Cell growth appeared to increase as the osmotic stress was reduced to one-half and one-quarter the original PEG concentration, but this was not statistically significant as shown by the standard error bars. Growth increased in the cells shifted to medium E or 130 mM NaCl.

When the water contents of cells grown on various concentrations of PEG was measured (Fig. 7), a stepwise increase in water content was observed as the osmotic stress imposed by PEG and dextran was removed (Fig. 7). In general, an increase in osmotic stress caused by PEG or dextran correlated with a much greater reduction in growth than in water content (Figs. 6 and 7).

DISCUSSION

Efflux from the cells of one-half of the initial PEG 4000 concentration into distilled H_2O required 1.9 min. This short half-time was taken as evidence for the extracellular location of most of the PEG 4000 (9). The cells had a greater uptake of PEG 1540.

The reduced growth that occurred when cells were grown on solid media containing NaCl confirms the reports of Bernstein (4, 5) that the major effect of NaCl on plant growth is a general stunting of the growth of all plant parts. Also, since NaCl reduced growth but KCl did not, the former may have had more toxic effects. Both cations entered the cells readily (9).

The further reduction in growth for cells of both cell lines when they are cultured on the nonpenetrating solutes PEG or dextran clearly indicate that osmotic stress from nonpenetrating solutes is very effective in reducing growth. When the osmotic stress was relieved by external uptake of solutes such as Na^+, K^+, Cl^-, and sugars, minor growth reduction occurred only in the case of Na^+ due to toxic effects. Since the water contents of cells from both lines were equally reduced when cells were shifted to the nonpenetrating solutes (Figs. 3, 5, and 7), the major effect of the nonpenetrating solutes was to produce a dehydration which reduced the availability of water and thus turgidity and growth.

There have been reports of toxic contaminants associated with unpurified PEG (13, 17). However, in earlier work in which PEG was used as an osmoticum for germinating lettuce seeds (7, 22), no such effects were found.

In general, the osmotic adjustment of cells grown on NaCl or KCl depended on the uptake of these salts until the cellular level approximated that of medium. Unwashed cells had higher Na^+ and K^+ contents than the medium (Tables I, II, and III), but these excess cations may have been in the cell wall (9). In the cells grown on the nonpenetrating solutes, 46 to 89% of the osmotic potential was accounted for by measured ions and organic solutes, primarily sugars, and therefore other organic osmotica must have contributed to the osmotic potential of these cells. In his work with pepper and bean plants grown in PEG 400 for 2 to 6 days, Janes (12) described these unknown osmotica as soluble solids, probably carbohydrates.

As both cell lines took up NaCl or KCl from the medium, the induction of a permanent osmotic stress by these salts was unlikely. The data presented support the hypothesis that growth inhibition by the nonpenetrating osmotica was greater and due to osmotic stress (Figs. 2 and 6) as opposed to the stress of ion toxicity imposed by NaCl. The nonadapted cell line ceased growth on the second passage on dextran while the NaCl-adapted line continued to grow slowly (Fig. 4). Selection of salt tolerance may involve selection for an altered response to specific ion toxicities. It is not clear from these experiments how this selection may also confer increased tolerance to osmotic stress produced by nonpenetrating osmotica. Direct selection for drought tolerance by callus culture on nonpenetrating osmotica may be the preferred strategy.

LITERATURE CITED

1. APPELGATE HG 1960 Freezing-point depressions of Hoagland's 'carbowax' systems. Nature 186: 232-233
2. BABAEVA ZA, RG BUTENKO, BP STROGONOV 1968 Influence of salinization of the nutrient medium on the growth of isolated carrot tissues. Soviet Plant Physiol 15: 75-82
3. BERNSTEIN L 1961 Osmotic adjustment of plants to saline media. I. Steady state. Am J Bot 48: 909-918
4. BERNSTEIN L 1963 Osmotic adjustment of plants to saline media. II. Dynamic phase. Am J Bot 50: 360-370
5. BERNSTEIN L 1975 Effects of salinity and sodicity on plant growth. Annu Rev Phytopath 13: 295-312
6. BERNSTEIN L, HE HAYWARD 1958 Physiology of salt tolerance. Annu Rev Plant

Physiol 9: 25–46
7. CARPITA NC, MW NABORS, CW ROSS, NL PETRIC 1979 The growth physics and water relations of red-light-induced germination in lettuce seeds. III. Changes in the osmotic and pressure potential in the embryonic axes of red- and far-red-treated seeds. Planta 144:217–214
8. GOLLEK B (ed) 1973 Structure and Function of Plant Cells in Saline Habitats. Israel Program for Scientific Translations, John Wiley, New York
9. HEYSER, JW, MW NABORS 1980 The osmotic adjustment of cultured tobacco cells (*Nicotiana tabacum* var. Samsum) grown on sodium chloride. Plant Physiol 67: 720–727
10. HOOKER M, MW NABORS 1977 Callus initiation, growth, and organogenesis in sugarbeets (*Beta vulgaris* L.). Z Pflanzenphysiol 84: 237–246
11. HYDEN S 1956 A turbidimetric method for the determination of higher polyethylene glycols in biological materials. Uppsala Ann Roy Agric Col Sweden 22: 139–145
12. JANES BE 1966 Adjustment mechanisms of plants subjected to varied osmotic pressures of nutrient solution. Soil Sci 101: 180–188
13. KAUFMANN MR, AN ECKARD 1971 Evaluation of water stress control with polyethylene glycols by analysis of guttation. Plant Physiol 47: 453–456
14. LAGERWERFF JV, G OGATA, HE EAGLE 1961 Control of osmotic pressure of culture solutions with polyethylene glycol. Science 133: 1486–1487
15. LOWE R, T HAMILTON 1967 Rapid method for determination of nitrate in plant and soil extracts. J Agr Food Chem 15: 359–361
16. LANG ARG 1967 Osmotic coefficients and water potentials of sodium chloride solutions from 0 to 40°C. Aust J Chem 20: 2017–2023
17. LAWLOR DW 1970 Absorption of polyethylene glycols by plants and their effects on plant growth. New Phytol 69: 501–513
18. LESHEM B 1966 Toxic effects of carbowaxes (polyethylene glycols) on *Pinus halepensis* Mill. seedlings. Plant and Soil 24: 322–324
19. MACROBBIE EAC, J DAINTY 1958 Ion transport in *Nitellopsis obtusa*. J Gen Physiol 42: 335–353
20. MEXAL J, JT FISHER, J OSTERYOUNG, CPP REID 1975 Oxygen availability in polyethylene glycol solutions and its implications in plant-water relations. Plant Physiol 55: 20–24
21. NABORS MW, A DANIELS, L NADOLNY, C BROWN 1975 Sodium chloride tolerant lines of tobacco cells. Plant Sci Lett 4: 155–159
22. NABORS MW, A LANG 1971 The growth physics and water relations of red-light-induced germination in lettuce seeds. I. Embryos germinating in osmoticum. Planta 101: 1–25
23. NELSON M 1944 A photometric adaptation of the Somogyi method for the determination of glucose. J Biol Chem 153: 375–380
24. ROSEN H 1957 A modified ninhydrin colorimetric analysis for amino acids. Arch Biochem Biophys 67: 10–15
25. SALISBURY FB, CW ROSS 1978 Plant Physiology, Ed 2, Chap 2. Wadsworth, Belmont, CA, p 21
26. SCHALES O, S SCHALES 1941 A simple and accurate method for the determination of chloride in biological fluids. J Biol Chem 140: 879–884
27. SOMOGYI M 1952 Notes on sugar determinations. J Biol Chem 195: 19–23
28. STEUTER AA, A MOZAFAR, JR GOODIN 1981 Water potential of aqueous polyethylene glycol. Plant Physiol 67: 64–67
29. STROGONOV BP 1964 Physiological basis of salt tolerance of plants (as affected by various types of salinity). Israel Program for Scientific Translations, Daniel Davey and Co., New York
30. WOOLLEY DG 1963 Effects of nutrition, osmotic pressure, and temperature of the nutrient solution on plant growth and chemical composition. I. Spring wheat at the four and six leaf stage. Can J Plant Sci 43: 44–50

APPENDIX V

Callus, Green Spot, and Shoot Formation in Tissue Cultures of Oat (*Avena sativa* L.)

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Summary

A general method for rapidly producing callus from the roots of germinating oat seedlings is discussed. Shoot regeneration occurs through the 38th week of culture. The occurrence of green spots in secondary callus is positively correlated with shoot regeneration although only regeneration decreases with time. Callus with green spots grows more rapidly and produces many more new green spots than plain callus. Continued green spot and shoot production requires auxin. A period of high temperature (30C) is necessary for the establishment of regenerated plants in greenhouse or growth chamber conditions. Several hundred such plants have been produced. Cool temperatures (20C) are required for seed set. The selection of calli tolerant to as much as 0.15 m NaCl is discussed.

Key Words: Avena sativa, callus, regeneration, tissue culture

Abbreviations: 2,4-dichlorophenoxyacetic acid (2,4-D); 2,4,5-trichlorophenoxyacetic acid (2,4,5-T); naphtnaleneacetic acid (NAA); indole-3-acetic acid (IAA).

Introduction

If tissue culture is to play an important role in cereal breeding several conditions must be met. (1) Methods for producing callus cultures and for regenerating large numbers of plants must be available. (2) Techniques for obtaining desired variant phenotypes must be perfected. (3) It must be demonstrated that these variants are inheritable and reasonably stable in future generations. With respect to NaCl tolerance we have demonstrated these points in tobacco (Nicotiana tabacum var. Samsun; Nabors et al. 1975, 1980, 1981). However, tissue culture of grasses presents additional problems particularly in terms of obtaining high frequency plant regeneration over long periods of culture.

Carter et al. (1967) obtained root callus from one variety of germinating oat seeds and found that formation and continued growth was dependent on the presence of an auxin (2,4-D or IAA) but not a cytokinin. Cummings et al. (1976) established callus cultures from immature embryos of 23 oat varieties. Callus typically formed from the radicle. Again, auxin was required for callus initiation and growth. Plants were regenerated from 12 varieties by placing callus on a medium containing no auxin. For one variety (Lodi) 18 month old callus retained the ability to produce regenerated shoots. In all, 133 plants were grown to maturity and "hundreds" of other regenerated shoots were obtained. Lorz et al. (1976) also produced callus (from the hypocotyl of mature embryos) and regenerated plants from three oat varieties. They utilized a complex mixture of hormones for shoot induction and produced several complete plants.

The occurrence of green spots in grass callus cultures has been frequently noted. Ogura and Shimada (1978) observed a probable correlation between the occurrence of green spots and shoot formation in wheat root callus although their sample size was quite small. They also noted that both green spot and shoot formation decreased in older calli. Shimada and Yamada (1979) found the same correlations using a larger sample in callus initiated from immature wheat embryos. They also observed that in subcultures only tissue containing a green spot produced more green spots and shoots. Finally, Inoue and Maeda (1980) studied the formation of green spots in callus produced from rice seeds. During seven, one month passages the numbers of green spots and regenerated shoots continuously declined. No shoots were produced after the fourth passages and no green spots were produced after the seventh passage. In two cases (Shimada and Yamada, 1979; and Inoue and Maeda, 1980) green spots were observed to occur in the periphery of the callus. The latter group observed that the green spots and later shoots arose de novo from callus areas which first developed radially arranged cells.

We present results concerning the initiation of calli, the induction and frequency of shoot regeneration, and the relationship between shoot regeneration and the occurrence of green spots in root derived callus formed in the presence and in the absence of NaCl.

Materials and Methods

Callus cultures were initiated from the germinating roots of Park oat seeds. The seeds were dehusked and then sterilized with 100% ethanol followed by 20% commercial bleach solution (containing a final concentration of 1% sodium hypochlorite) for 30 minutes. Several drops of Tween 80 were added to the bleach solution. Sterilized seeds were placed on solid medium containing Linsmaier and Skoog's salts (1965), 4% sucrose, 1% agar, and various concentrations of auxins (NAA, 2,4-D or 2,4,5-T).

Callus was subcultured after six weeks for primary and four weeks for secondary cultures. Primary callus was grown in 25 x 70 mm glass vials with lightly tightened plastic screw caps and 10 mls of medium. Secondary callus was grown in 38 x 80 mm glass jars with plastic screw caps. Cultures were placed 28 cm from two, constantly lit 40 watt cool white fluorescent bulbs.

Callus growth was measured by visually comparing calli to a set of clay spheres whose cross sectional areas are shown in Fig. 1. Assuming that the smallest sphere has a weight of one unit, relative weights of the other spheres and thus the calli can be obtained. We find that this system is more accurate than the traditional +, ++, or +++; and more convenient than obtaining actual fresh weights for each vial. Our experience demonstrates that different trained observers obtain the same average relative volumes for groups of calli using the system.

Shoot regeneration and rooting of regenerated shoots was accomplished by transferring the callus or shoots to appropriate medium as discussed in the results.

Salt resistant callus was obtained by placing NaCl in the callus induction medium. After six weeks any callus obtained was transferred to jars containing medium with the same level of salt

Results and Discussion

1. Formation of callus. Figure 2 demonstrates that maximal callus formation for this lot of Park oats occurs at 7.8×10^{-6}m 2,4,5-T. NAA did not promote callus formation at concentrations up to 7.8×10^{-5}m. Similar results show that 4% sucrose gives maximal callus growth. Callus formation occurs at similar rates in light and in dark. We have found that auxin type and concentration and sucrose concentration are the only significant variables for primary callus induction in oats. However these determinations need to be repeated not only for different oat varieties but also for different lots of the same variety.

Figure 3 shows the effect of increasing concentrations of NaCl on callus initiation after 13 weeks. The percentage of seeds which did not germinate and therefore produced no callus is indicated for each data point. These seeds were included in the plot of overall callus formation.

Figure 3 shows the effect of increasing concentrations of NaCl on callus initiation after 13 weeks. The percentage of seeds which did not germinate and therefore produced no callus is indicated for each data point. These seeds were included in the plot of overall callus formation.

2. Induction of regenerated shoots. For Park oats, regeneration is maximal on medium containing 7.8 or 3.9×10^{-6}m 2,4,5-T. Equivalent concentrations of 2,4-D also promote high levels of regeneration, but since 2,4-D is less effective in promoting callus growth, the total amount of callus and the total number of regenerated plants is reduced. Regeneration also occurs on medium containing no auxins; however callus growth ceases and so regeneration does not continue.

3. Induction and importance of green spots. Oat callus develops green spots in our experiments following transfer to secondary culture. We find no green spots so long as the seed and seedling remain attached to the developing (primary) callus. Secondary calli containing green spots attain an average size 4.4 times that of calli containing no green spots during a four-week growth or rapidly growing callus tends to produce green spots.

We have never observed shoot production on calli containing no green spots. This observation has been made by other workers (e.g. Shimada and Yamada, 1979, for wheat). Furthermore, all shoots arise directly from green spots. In rice, Inoue and Maeda (1980) showed that both shoot and green spot production decreased with time and that there were always many more green spots than shoots. For oats Figures 4-7 show that on medium containing 3.9×10^{-6}m 2,4,5-T the percentage of jars containing green spots remains relatively constant for many passages whereas the percentage of jars containing shoots is gradually reduced to zero. Secondary passages are set up using calli of size 12 (Fig. 1) and typically at the end of a four-week passage contain dozens of green spots and zero to twelve shoots.

These data can be interpreted in two ways: Either two types of green spots occur, and only one of these has shoot forming potential; or shoots arise from green spots but have initiation requirements--probably nutritional or hormonal--not met by the medium.

The induction of green spots and shoots is correlated with the auxin content of the medium. Table 1 indicates that calli on 3.9 or 7.8×10^{-6}m 2,4,5-T maintain relatively constant green spot production whereas calli

on medium containing no auxin gradually lose the ability to produce green spots and to grow. The total number of calli continued provides an indication of callus growth rate. Table 2 shows that calli with green spots tend to give rise to subcultured calli with green spots whereas calli without green spots tend to give rise to calli without green spots. Oat calli contain numerous meristematic regions which originate from root meristem proliferation during primary callus formation. In some cases these pre-existing meristems seem to give rise to green spots. In other cases green spots occur in regions which appear (under a dissecting microscope) to contain no meristematic regions. Whether this observation relates to the two types of green spots theorized earlier is unclear at present.

4. Rooting and care of regenerated shoots. Root formation occurs spontaneously on some shoots. Usually, however, roots are induced by removing shoots from the callus and transferring them to Linsmaier and Skoog's medium without myoinositol or thiamine. Originally sucrose was also omitted from the rooting medium to reduce contamination on removal from tissue culture. However it was discovered that rooting is best on medium containing 4% sucrose, average on medium containing 2%, and poor if no sucrose is included.

Complete plants are removed from jars, excess agar is removed from the roots, and plants are potted in small pots containing a mixture of sterilized potting soil and perlite (3:1). Since Park oats is a cool weather plant, the potted regenerates were placed in a plant growth room maintained between 20-25C. Under these conditions most plants died,

and fungal and bacterial infections were prevalent. We have found that 100% of potted plants can be grown to maturity if they are placed in a 30C growth chamber for a week subsequent to removal from tissue culture. However at this temperature plants will flower but will not set seed. Thus our regenerated plants in pots are now placed at 30C for a week and then transferred to a growth chamber with 20C for 16h (light) and 15C for 8h (dark). Several hundred plants have now been grown to maturity. Around fifty plants have been grown to maturity from cultures containing 0.05 and 0.10 m added NaCl (see Figs. 5 and 6). These plants are now being tested for salt tolerance and for inheritance of salt tolerance.

5. Salt tolerance. Cultures resistant to NaCl were selected by adding salt to callus induction medium. Figure 3 shows that callus formation occurs up to 0.17 m added NaCl. The basic procedure is to set up a large number of induction vials containing one seed each. After six weeks an callus produced is combined on jars containing secondary medium with 3.4×10^{-6} m added 2,4,5-T. This auxin concentration is used because it seems to result in increased green spot formation in some experiments (see Table 1) when compared to the induction medium. At higher concentrations of salt most seeds produce a very small amount of callus; a few seeds produce larger amounts, hopefully from salt tolerant cells.

Around 75 plants have been grown to maturity from cultures containing 0.05 m and 0.10 m added NaCl (see Figs. 5 and 6) for various lengths of time. Seeds from these plants will be used in experiments to test for the persistence and inheritance of salt tolerance.

Acknowledgments

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References

Carter, O., Y. Yamada, and E. Takahashi: Nature 214, 1030 (1967).

Cummings, D.P., C.E. Green, and D.D. Stuthman: Crop Sci. 16, 465 (1976).

Inoue, M., and E. Maeda: Jap. J. Crop Sci. 49, 167 (1980).

Linsmaier, F.M., and F. Skoog: Physiol. Plant. 8, 100-127 (1965).

Lörz, H., C.T. Harms, and I. Potrykus: Z. Pflanzenzüchtg. 77, 257 (1976).

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

Nabors, M.W., S.E. Gibbs, C.S. Bernstein, and M.E. Meis: Z. Pflanzenphysiol. 97, 13 (1980).

Nabors, M.W., C.S. Kroskey, R. Brownson, and D.W. McHugh: In Press, Planta, (1981).

Ogura, H., and T. Shimada: Wheat Inf. Ser. 45-46, 26 (1978).

Shimada, T., and Y. Yamada: Jap. J. Gen. 54, 379 (1979).

Table 1. Continued Green Spot Production Requires the Presence of an Auxin. 1 = 3.9×10^{-6}m; 2 = 7.8×10^{-6}m.

Passage #[b]	2	3	4	5	6	7
2,4,5-T concentration	0	0 2	0 2	0 2	0 2	0 2
% cultures with green spots	22	50 57	0 50	0 100	0 100	0 0
Total number of cultures	70	10 7	2 2	0 2	0 2	0 0
2,4,5-T concentration	1	0 1	0 1	0 1	0 1	0 1
% cultures with green spots	70	74 81	67 71	24 69	40 85	33 83
Total number of cultures	70	27 26	27 38	29 39	15 27	6 29
2,4,5-T concentration	2	0 2	0 2	0 2	0 2	0 2
% cultures with green spots	63	94 86	90 78	38 93	100 93	0 80
Total number of cultures	70	32 29	40 40	40 42	15 42	15 59

[a] Callus of size 12 (Fig. 1) was transferred every 4 weeks. Only calli with green spots were transferred. Medium contained 0.08 m NaCl.

[b] Passage 1 is callus induction.

Table 2. Callus with Green Spots Gives Rise to Callus with Green Spots. Medium Contains 7.8×10^{-6} m (2T); 3.9×10^{-6} m (1T); or no 2,4,5-T. A. medium contains no salt; B. medium contains 0.08 m NaCl.

		SECOND PASSAGE	
	A.	Green spots	No green spots
FIRST PASSAGE	Green spots	79 (2T) 41%	21 (2T) 11%
		128 (1T) 54%	40 (1T) 17%
		48 (0T) 35%	40 (0T) 29%
	No green spots	17 (2T) 9%	76 (2T) 39%
		15 (1T) 6%	53 (1T) 22%
		9 (0T) 6%	42 (0T) 30%
	Total Percent Green Spots	2T = 63%	
		1T = 70%	
		0T = 20%	
	B.	Green spots	No green spots
FIRST PASSAGE	Green spots	45 (2T) 59%	8 (2T) 11%
		46 (1T) 66%	8 (1T) 11%
		13 (0T) 19%	43 (0T) 63%
	No green spots	3 (2T) 4%	20 (2T) 26%
		3 (1T) 4%	13 (1T) 19%
		1 (0T) 1%	11 (0T) 16%
	Total Percent Green Spots	2T = 50%	
		1T = 67%	
		0T = 41%	

[a] 41% of all 2T vials, etc.

Figure Legends

Figure 1. Cross sectional areas used to estimate relative callus volume.

Figure 2. Callus initiation as a function of auxin concentration. Cultures were 6 weeks old. Each sample consisted of 40 vials.

Figure 3. Callus initiation as a function of NaCl concentration when 2,4,5-T concentration was 7.8×10^{-6}m. Each sample consisted of 20 vials. The no growth percentages are 0, 0, 0, 4, 8, 21, and 36 for data points from left to right.

Figure 4. Shoot regeneration and green spot formation as a function of callus age. Calli were transferred every 4 weeks following a 6 week initiation passage. Medium contained no salt.

Figure 5. Shoot regeneration and green spot formation as a function of callus age. Calli were transferred every 4 weeks following a 6 week initiation passage. Medium contained 0.05 m NaCl.

Figure 6. Shoot regeneration and green spot formation as a function of callus age. Calli were transferred every 4 weeks following a 6 week initiation passage. Medium contained 0.10 m NaCl.

Figure 7. Green spot formation as a function of callus age. Calli were transferred every 4 weeks following a 6 week initiation passage. Medium contained 0.15 m NaCl. No shoots were formed during this experiment. In other experiments limited shoot formation has occurred at this salt concentration.

References

Carter O., Yamada Y., Takahashi, E.: Tissue culture of oats. Nature 214, 1029-1030 (1967).

Cummings D. P., Green C. E., Stuthman D. D.: Callus induction and plant regeneration in oats. Crop Science 16, 465-470 (1976).

Inoue M., Maeda E.: Effects of auxins and cytokinins on the occurrence of green regions in rice callus cultures. Jap. J. Crop Sci. 49, 167-174 (1980).

Linsmaier F. M., Skoog, F.: Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. 8, 100-127 (1980).

Lorz H., Harms C. T., Potrykus I.: Regeneration of plants from callus in Avena sativa L. Z. Pflanzenzüchtg 77, 257-259 (1976).

Nabors M. W., Daniels A., Nadolny L., Brown C.: Sodium chloride tolerant lines of tobacco cells. Plant Sci. Lett. 4,155-159 (1975).

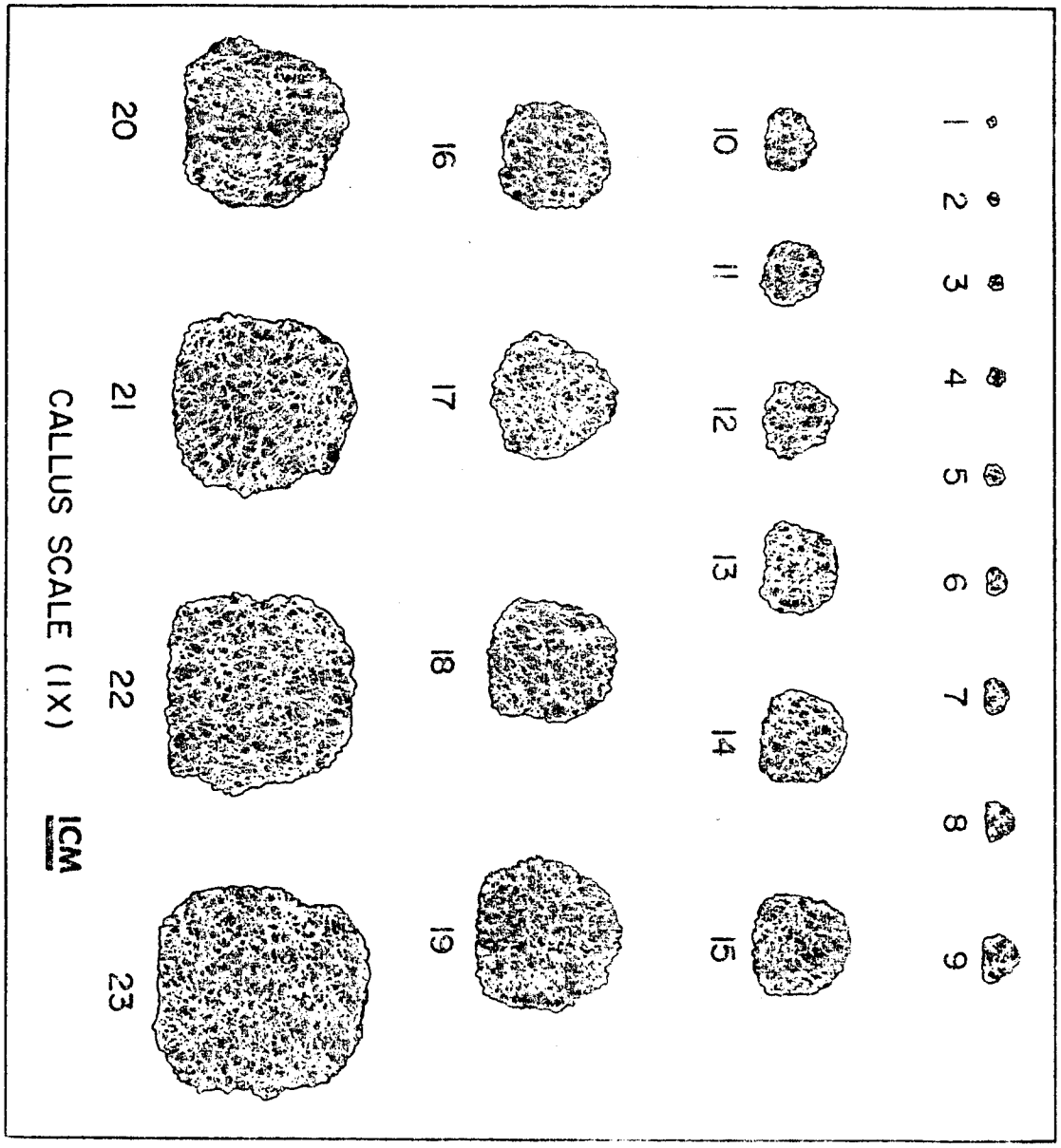
Nabors M. W., Gibbs S. E., Bernstein C. S., Meis M.E.: NaCl tolerant tobacco plants from cultured cells. Z. Pflanzenphysiol. 97, 13-17 (1980).

Nabors M.W., Kroskey C. S., Naumann T. S., Collins P.D., Hoisington D. A., Daniels A.: Generalized tissue culture methods for tobacco and tomato and the use of these methods to select NaCl- and 2,4-D-tolerant tobacco cell lines. In Press, Planta, (1981).

Ogura H., Shimada T.: Preliminary report on shoot redifferentiation from wheat callus. Wheat Inf. Serv. 45, 46, 26-28 (1978).

Shimada T., Yamada Y.: Wheat plants regenerated from embryo cell cultures. Jap. J. Gen. 54, 379-385 (1979).

Figure 1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23
CALLUS SCALE (1X)
ICM

Figure 2

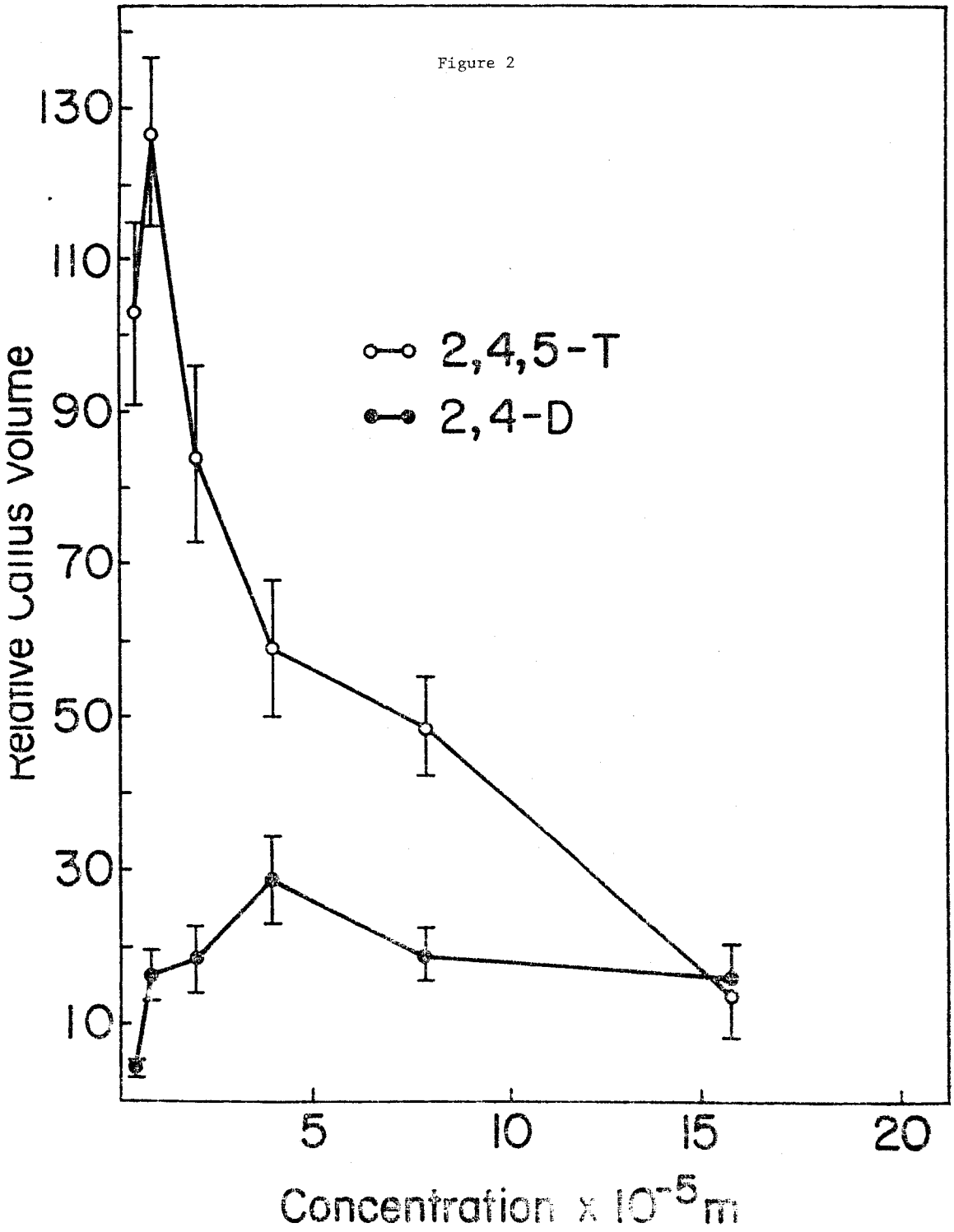

130
110
90
70
50
30
10
Relative Callus Volume
2,4,5-T
2,4-D
5
10
15
20
Concentration x 10^{-5} m

Figure 3

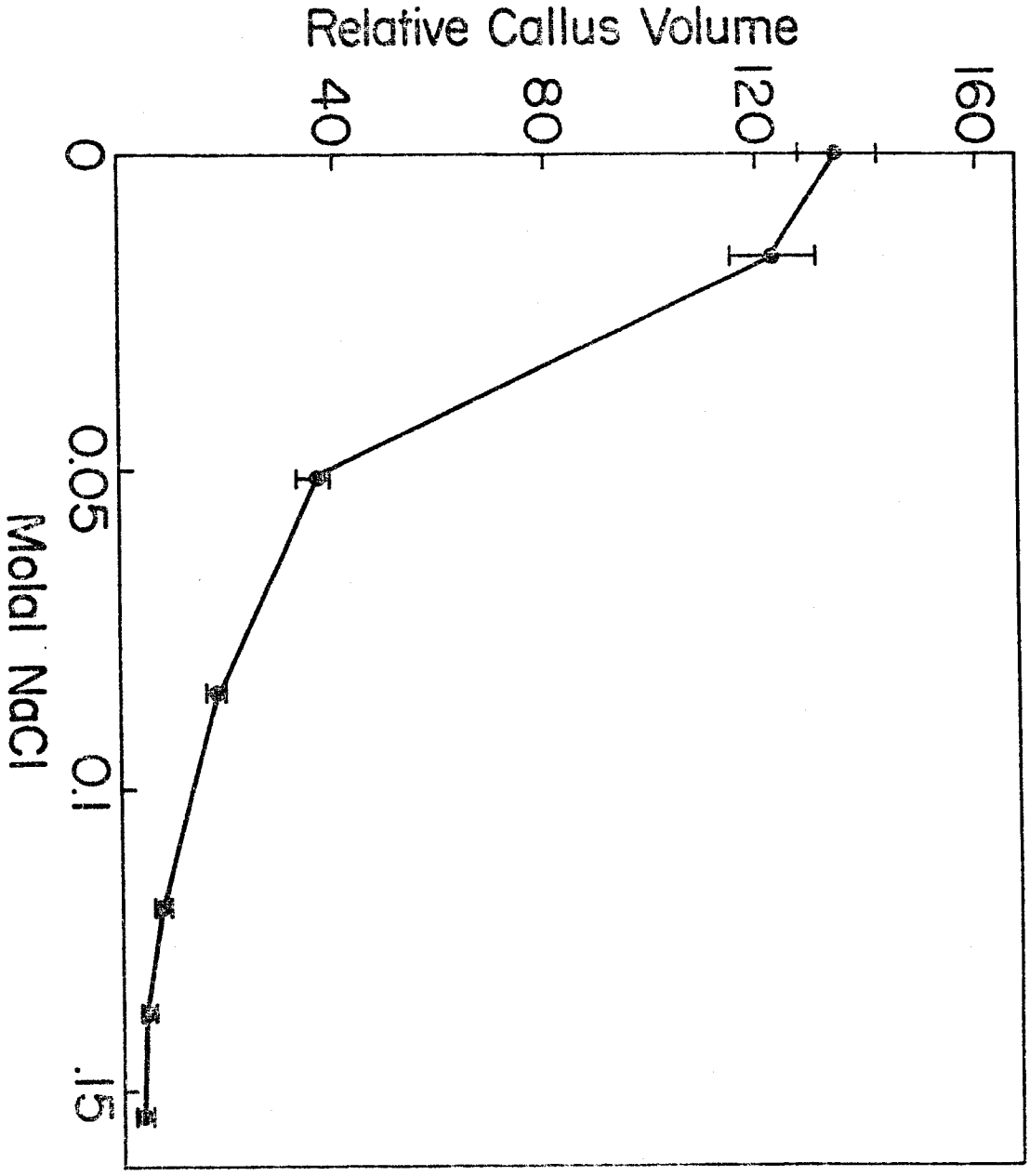
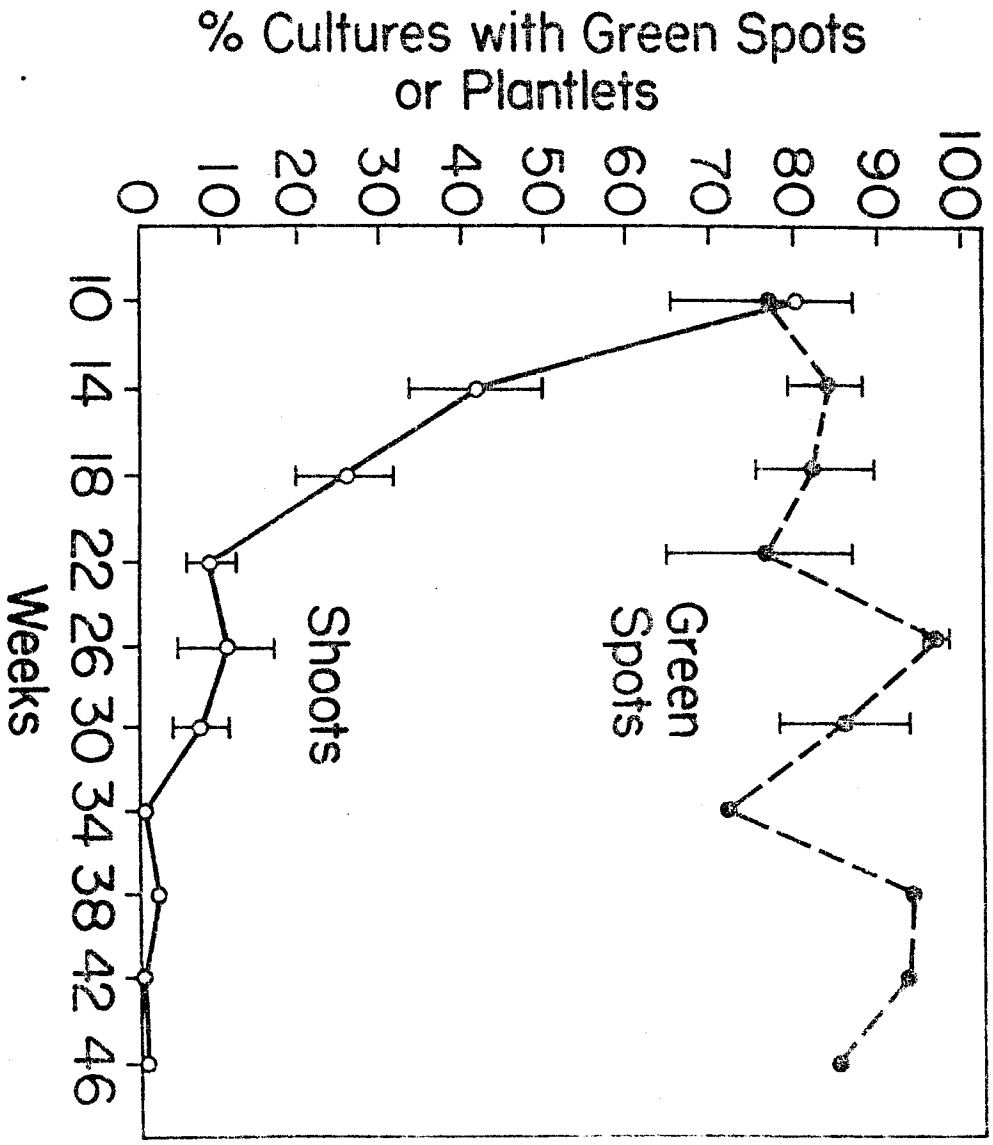


Figure 4


% Cultures with Green Spots or Plantlets
0
10
20
30
40
50
60
70
80
90
100
Weeks
10
14
18
22
26
30
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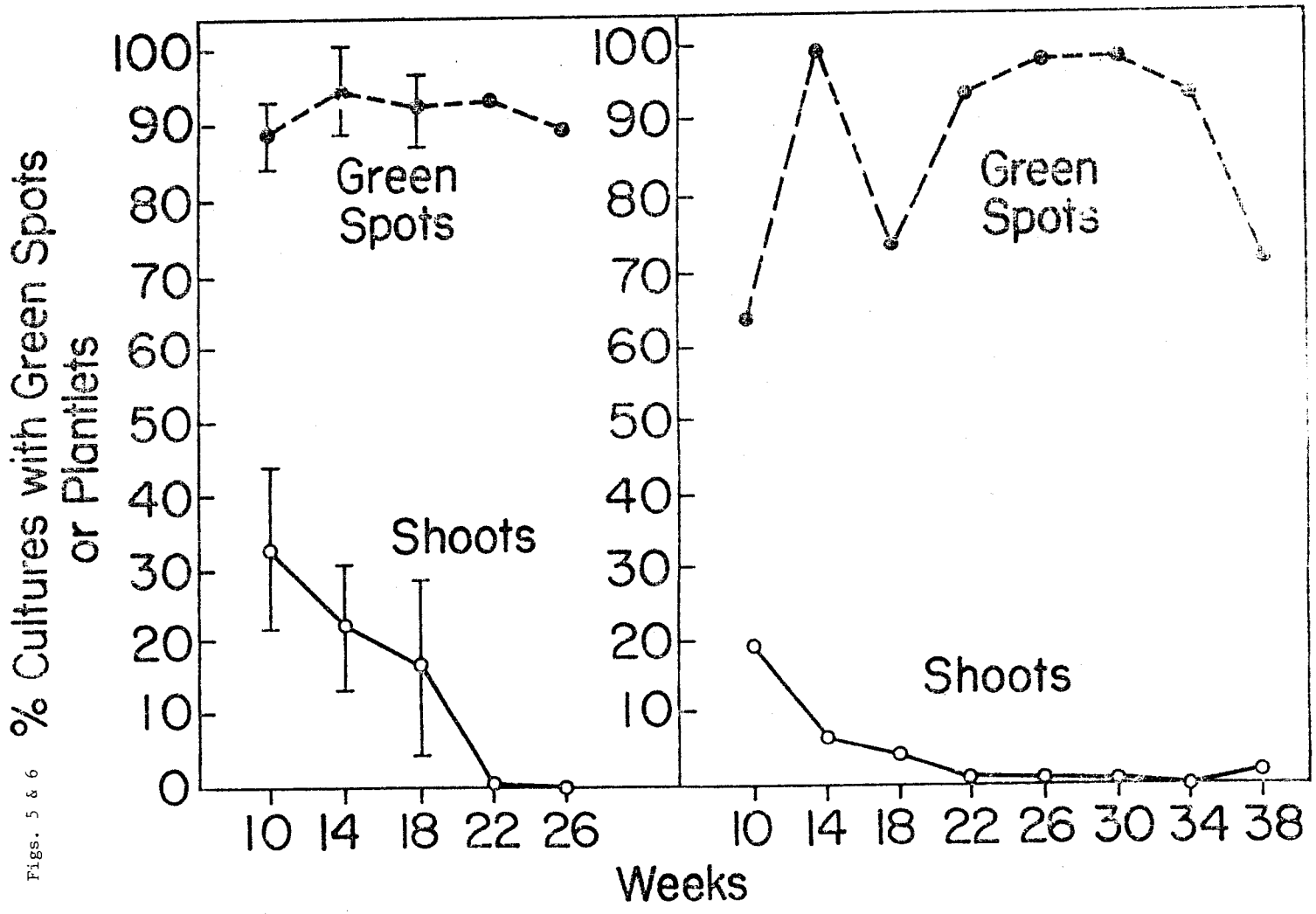
38
42
46
Shoots
Green Spots


% Cultures with Green Spots or Plantlets
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Green Spots
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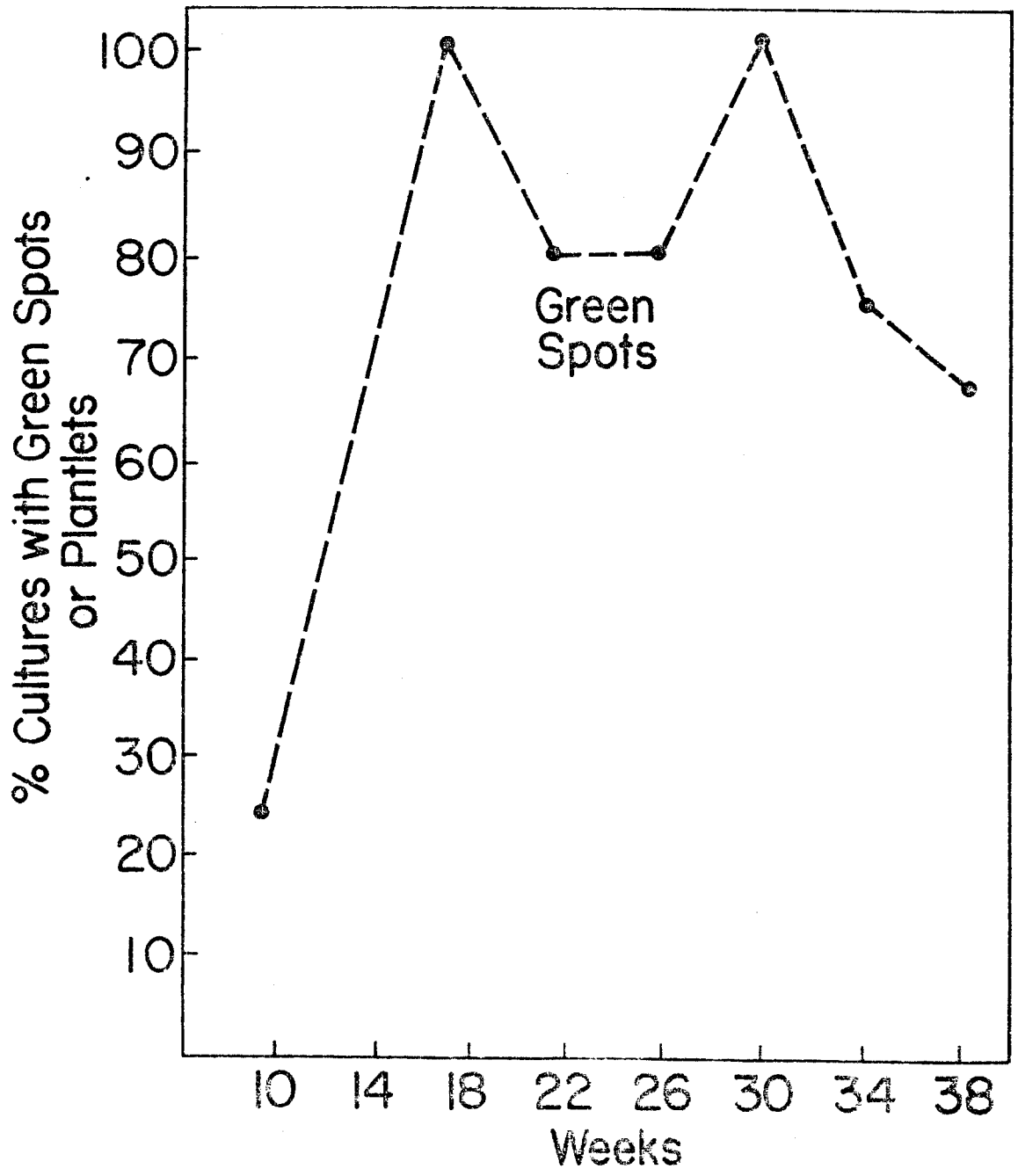
Weeks

Figs. 5 & 6

Figure 7


% Cultures with Green Spots or Plantlets
100
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20
10
Green Spots
10
14
18
22
26
30
34
38
Weeks

APPENDIX VI

The Relationship between Long Term Plant Regeneration, Somatic Embryogenesis and Green Spot Formation in Secondary Oat Callus (*Avena Sativa*)

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SUMMARY

Calli initiated from mature seeds, mesocotyls, and immature embryos of oat (Avena sativa) produced embryogenic and non embryogenic callus and green spots. Embryogenic callus is white, opaque and often convoluted while non-embryogenic callus is rough and yellow to translucent in appearance. Non-embryogenic callus can produce shoots or roots. Embryogenic callus can produce shoots, roots, or complete plantlets. Shoot and plantlet production occurs at much higher frequencies and for longer durations in embryogenic callus. After 36 weeks in culture, 20 percent of embryogenic calli initiated shoots or plantlets while non-embryogenic calli formed no shoots.

Elongated regions called green spots are produced by non-embryogenic callus and have a distinct structure which resembles a root apex. New green spots are formed adjacent to but not in direct contact with existing green spots. Very few green spots are initiated in calli dissected free of green spots.

A model which explains the ontological relationship between observed calli types and green spots is given. The use of embryogenic callus offers a significant advance in the control of regeneration in oats.

Key Words: oat, Avena sativa, tissue culture, green spots, somatic embryogenesis, shoot initiation.

Abbreviation list: 2,4-D, 2,4-dichlorophenoxy acetic acid; 2,4,5- T, 2,4,5-trichlorophenoxy acetic acid.

Acknowledgments

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INTRODUCTION

In cereal tissue cultures, totipotency is unstable and often lost within a few months after callus initiation (Rice et al. 1979; Vasil and Vasil 1981). Maintenance of totipotency for prolonged periods in cereal cultures is a necessary first step in the production of mutant plants from cultures which may need six months to one year for stable mutant selection (Nabors et al. 1982B).

For oats, long term totipotent tissue cultures have been obtained for a few varieties. Cummings et al. (1976) reported that one variety of 23 produced shoots after 18 months in culture. Lorz et al.(1976) found that one of three varieties had a high rate of shoot and root production after one year in culture. Cure and Mott (1978) considered the proliferation of oat tissue in culture to be by growth of aberrant root-like tissues which had the external appearance of callus. Occasional shoot production in secondary cultures was considered by them to be a carryover from the primary culture which included meristems derived directly from the explant.

Green spots or regions have often been observed in cereal tissue cultures. In oat callus, elongated bright green nodules were produced by callus independent of tested growth factors (Brenneman and Galston 1975). When examined histologically these nodules had meristematically active cells arranged along an axis of elongated cells which included tracheids. Further development of these nodules resulted in a "many armed starfish-like body" which was green and rigid. In rice there was a close relationship between green regions and shoot formation (Nakano and Maeda 1979). A high to kinetin to auxin ratio led to the earlier appearance and greater number of green spots (Inoue and Maeda 1980). Both green regions and shoot formation

declined greatly with time in culture. Green spot formation was observed in wheat callus by Ogura and Shimada (1978), and green meristematic regions were noted by Gresshoff and Doy (1973) in Zea mays before the development of shoot primordia.

An earlier paper (Nabors et al. 1982A) examined the relationship between green spot formation and shoot production by oat calli over time in culture. Only callus with green spots grew and produced shoots. Shoot production ceased after 38 weeks in callus selected for high green spot production.

In the literature cell lines which give rise to complete plantlets (embryogenesis) as opposed to shoots or roots (organogenesis) are referred to as embryogenic. Embryogenic tissue cultures have been obtained for the following cereals: pearl millet (Vasil and Vasil 1981), proso millet (Heyser and Nabors 1982), and sorghum (Wernecke and Brettell 1980, Thomas et al.1977). In pearl millet (Vasil and Vasil 1981) and proso millet (Heyser and Nabors 1982) two distinct types of callus were observed: embryogenic and non-embryogenic.Embryogenic callus was characterized by a smooth, white , and knobby appearance while non-embryogenic callus was wet, yellow or translucent, and rough in appearance (Heyser and Nabors 1982). Embryogenesis declined with time in culture.

In grasses the factors controlling embryogenic callus formation are poorly understood. The normal pattern for carrot embryogenesis on inductive medium is for maximum embryogenesis to occur around 20 weeks and to disappear after 60 weeks in culture (Meyer-Teuter and Reinert 1973). Smith and Street (1973) considered the decline in embryogenic potential in carrot to be due to the selective advantage of non-embryogenic cells over embryogenic cells in a mixed culture even though both cell types may have

had similar growth rates. Embryogenic potential was restored in carrot cultures by culture on casein hydrolysate or under low temperature (Syono 1975) or by sequential transfer to NAA and coconut milk media (Steward 1967). Embryogenic potential could be maintained by culture on a non-inductive medium or under low temperature. A higher rate of cell division caused more rapid loss of totipotency. Regardless of growth rate, the end of the period of maximal embryogenesis was determined by the number of cell generations in culture.

Embryogenic callus similar in appearance to that found in the millets has been observed in secondary oat callus. The relationship of embryogenic callus, and green spots to shoot production and retention of totipotency is covered in this paper.

MATERIALS AND METHODS

Callus from seeds, mesocotyls, and immature embryos of oat (Avena sativa L.var. Park) was used. Initiation of callus from the roots of germinating seeds, callus subculture, and the determination of the optimum auxin level for shoot and green spot formation were described earlier by Nabors et al. (1982A). Callus was initiated from 7 to 8 day dark grown-mesocotyls and immature embryos plus endosperm excised 10-20 days after pollination. Both were light cultured. Once initiated, calli were subcultured every six weeks on Linsmaier and Skoog (1965) medium plus 1 percent (w/v) agar. Glass vials 25 x 70 mm were used for all cultures. In earlier work (Nabors et al.1982), 3.9×10^{-6}M 2,4,5-T was the best auxin concentration for callus growth, green spot and shoot production.

Embryogenic callus was detected by its white, opaque, and convoluted appearance which was similar to embryogenic callus observed in proso and pearl millets. When cultured on low auxin (less than 4×10^{-6}M) it produced complete plantlets which developed from a central axis initiated on the surface of embryogenic callus. Green spots were distinguished by their green color and distinct meristematic structure with a central cylinder. Excised green spots were cultured in 0.1 to 2.0 ml of liquid medium with 0 to 2×10^{-5} M auxin in multiwell tissue culture plates with either 24 or 96 wells (produced by Linbro Division, Flow Laboratories, Hamden, Connecticut, U.S.A.).

RESULTS AND DISCUSSION

Figure 1 Regular or non-embryogenic callus is shown in Figure 1A. It is rough and yellow to translucent in appearance, produces shoots without roots, and was the type of callus used to obtain the correlation of oat shoot regeneration with the presence of green spots in callus reported by Nabors et al. (1982A). Embryogenic callus has an opaque, smooth white and often convoluted appearance (Fig. 1B). Shoots without roots (Fig. 1C) and complete plantlets (Fig. 1D) are regenerated from embryogenic callus cultured on low auxin (less than 4.0×10^{-6} M 2, 4-D or 2,4,5-T).

Embryogenic and non-embryogenic areas are commonly found in the same callus. Small regions of embryogenic callus appeared on up to 21 percent of the non-embryogenic calli in the second through fourth passages initiated
Table 1 by mature seeds, mesocotyls, and immature embryos (Table 1). Callus which initiated embryogenic areas was subcultured, and embryogenic areas were observed to disappear and reform on the surface of non-embryogenic callus during four subsequent passages. Both complete plantlets with no visible attachment to embryogenic callus (Fig. 1E) and shoots without visible roots (Fig. 1F) were produced by this mixed callus. The factors controlling the formation of embryogenic callus in grasses are unknown. Embryogenic callus often originates in desiccated non-embryogenic callus, so the effect of osmotic potential on its formation should be investigated.

Elongated green regions called green spots have been observed in non-
Figure 2 embryogenic oat callus by Nabors et al. (1982A) and Brenneman and Galston (1975) (Fig. 2A). In non-embryogenic callus their presence is required for callus growth, shoot formation, and root formation (Nabors et al. (1982A). When excised and examined, green spots have a distinct structure which

resembles root apices (Fig. 2B-C). Both unbranched (Fig.2B-C) and branched (Fig. 2A, G,H) green spots are found. They are unconnected to each other and are distributed throughout secondary callus.

Green spots removed by microdissection are surrounded by a layer of tightly adhering translucent callus. When this callus layer is removed by further dissection, the liquid cultured green spot produces another layer of translucent callus (Fig.2H). This layer occasionally develops projections that become meristems which develop into new, unattached green spots (Fig. 2B-C). Apparently pre-existing green spots serve as the required stimulus to induce differentiation of translucent callus into green spots. Of 400 oat calli dissected free of green spots, less than 2 percent produced new green spots (Fig. 2D) and subsequent roots (Fig. 2E). None produced shoots. Green spots may thus produce localized hormonal gradients which indirectly stimulate meristem initiation in adjacent translucent callus rather than serving as a direct source of new, secondary meristems. The distribution of green spots throughout a callus may be explained by the greater growth of dedifferentiated callus which spacially separates the newly initiated green spot and the originally adjacent green spot.

Branched green spots observed by Brenneman and Galson (1975) and by us arise either by the growth of multiple buds on an unbranched green spot or by the fusion of new green spots initiated by translucent callus adjacent to a pre-existing green spot. As noted, green spots commonly give rise to roots and occasionally to shoots. Shoots initiated in callus containing green spots but no visible embryogenic callus may be obviously attached to green spots and roots within the callus(Fig. 2F) or appear as isolated shoots (Fig. 1F). Clearly a histological study of green spot

formation and development is needed to determine if the observations reported in this paper are correctly interpreted.

Shoot formation declined in both embryogenic and non-embryogenic calli over the length of time in culture (Fig. 3). Callus without green spots did not grow or initiate shoots while mixed callus with green spots lost the ability to regenerate shoots after 38 weeks in culture (Nabors et al. 1982A). In contrast selected embryogenic callus also has a decline in shoot production after 18 weeks in culture (Fig. 3), but shoot formation still occurred in 15 to 20 percent of the calli after 36 weeks in culture. Multiple shoots (2-5 per callus) were common in those calli which did regenerate shoots after 30 weeks so that 30 to 100 shoots could be obtained per 100 vials of calli. Calli lacking embryogenic regions did not form shoots after 30 weeks in culture.

Figure 3

A model which explains the ontogeny of observed morphogenic structures is given in Figure 4. Secondary callus can produce more secondary callus, green spots, or embryogenic callus. Embryogenic callus regenerates complete plantlets (Fig. 1D) and may turn green before it initiates shoots. These green areas formed by embryogenic callus may have been confused with green spots in earlier work (Nabors et al. 1982A). Thus selection for green spots would have included embryogenic green regions and partially selected for embryogenic callus. Green regions of embryogenic callus have been observed to develop into green spots and roots.

Figure 4

Figure 3 shows that selection for embryogenic callus is a better method for retaining totipotency than selection for green spots as Nabors et al. (1982A) reported. In general embryogenic calli were much smaller than non-embryogenic calli when transferred to regeneration medium. Regeneration rates for embryogenic calli could be as much as ten times higher if regeneration per unit mass of callus were reported instead of regeneration per vial. The observation of embryogenic callus in oats, pearl millet, proso millet, rice, and wheat (unpublished data) suggests that it may be a general type of persistently totipotent callus produced by several species of monocotyledons for which long term, high frequency totipotency is sought.

References

Brenneman, F.B. and A. W. Galston: Experiments on the cultivation of protoplasts and calli of agriculturally important plants. I.Oat (Avena sativa L.). Biochem. Physiol. Pflanzen. 168, 453-471 (1975).

Cummings, D.P., Green, C.E., and D.D. Stuthman: Callus induction and plant regeneration in oats. Crop Sci. 16, 465-470 (1976).

Cure, W.W. and R.L. Mott: A comparative anatomical study of organogenesis in cultured tissues of maize, wheat and oats. Physiol. Plant. 42, 91-96 (1978).

Gresshoff, P.M. and C. H. Doy: Zea mays: Methods for diploid callus culture and the subsequent differentiation of various plant structures. Aust. J. Biol. Sci. 26, 505-508 (1973).

Heyser, J.W. and M.W. Nabors: The regeneration of proso millet from embryogenic calli derived from various plant parts. Submitted for publication (1982).

Inoue, M.I. and E. Maeda: Effects of auxins and cytokinins on the occurrence of green regions in rice callus cultures. Japan. J. Crop. Sci. 49, 167-174 (1980).

Linsmaier, F.M. and F. Skoog: Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. 8, 100-127 (1965).

Lorz, H., Harms, C.T., and I. Potrykus: Regeneration of plants from callus of Avena sativa L..Z. Pflanzenzuchtg. 77,257-259 (1976).

Meyer-Teuter, H. and J. Reinert: Correlation between rate of cell division and loss of embryogenesis in longterm tissue cultures. Protoplasma 78, 273-283 (1973).

Nabors, M.W., Kroskey, C.S., Brownson, R. and D.W. McHugh: Green spots are predictors of high callus growth rates and shoot formation in normal and salt-stressed tissue cultures of oat (Avena sativa). Z Pflanzenphysiol. (In press 1982A)

Nabors, M.W., Kroskey, C.S., Nauman, T.S., Collins, P.D. Hoisington, D.A., and A. Daniels: Generalized tissue culture methods for tobacco and tomato and the use of these methods to select NaCl-and 2,4-D tolerant tobacco cell lines. (Submitted for publication 1982B).

Nakano, H. and E. Maeda: Shoot differentiation in callus of Oryza sativa L.. Z. Pflanzenphsiol. 93, 449-458 (1979).

Ogura, H. and T. Shimada: Preliminary report on shoot redifferentiation from wheat callus. Wheat Inf. Ser. 45-46, 26-28 (1978).

Rice, T.B., Reid, R.K., and P.N. Gordon: Morphogenesis in field crops. In: Hughes, K.W., Henke, M.J. and M.J. Constantin (eds.) Propagation of higher plants through tissue culture. pp 262-277. (CONF-7804111). Information Center, US-DOE, Springifield, Va.(USA) (1979)

Smith, S.M. and H.E.Street: The decline of embryogenic potential as callus and suspension cultures of carrot (Daucus carota L.) are serially subcultured. Ann Bot. 38,223-241 (1974).

Steward, F.C.: Totipotency of angiosperm cells. Its significance for morphology and embryology. Phytomorphology 17, 499-507 (1967)

Syana, K.: Changes in organ forming capacity of carrot root calluses during subculture. Plant & Cell Physiol. 6, 403-419 (1965).

Thomas, E., King, P.J. and I Potrykus: Shoot and embryo-like structure formation from cultured tissues of Sorghum bicolor. Naturwissenschaften 64, 587 (1977).

Vasil, V. and I.K. Vasil: Somatic embryogenesis in cereals. BioSci. 31, 246-248 (1981).

Wernicke, W. and R. Brettell: Somatic embryogenesis from Sorghum bicolor leaves. Nature 287, 138-139 (1980).

Table 1. Formation of embryogenic callus by non-embryogenic callus during the first five passages in culture (30 weeks total) by calli derived from mature seeds (root callus), mesocotyls, and immature embryos.

Explant Source	NUMBER OF EMBRYOGENIC CALLI	TOTAL CALLI	PERCENT EMBRYOGENIC CALLI
PASSAGE			
Seeds (Root Callus)			
1	0	559	0%
2	30	270	11%
3	8	192	4%
4	26	124	21%
5	0	111	0%
Mesocotyl Callus			
1	0	200	0%
2	19	148	13%
3	2	117	2%
4	4	60	7%
5	0	60	0%
Immature Embryos			
1	0	14	0%
2	3	14	21%
3	0	10	0%

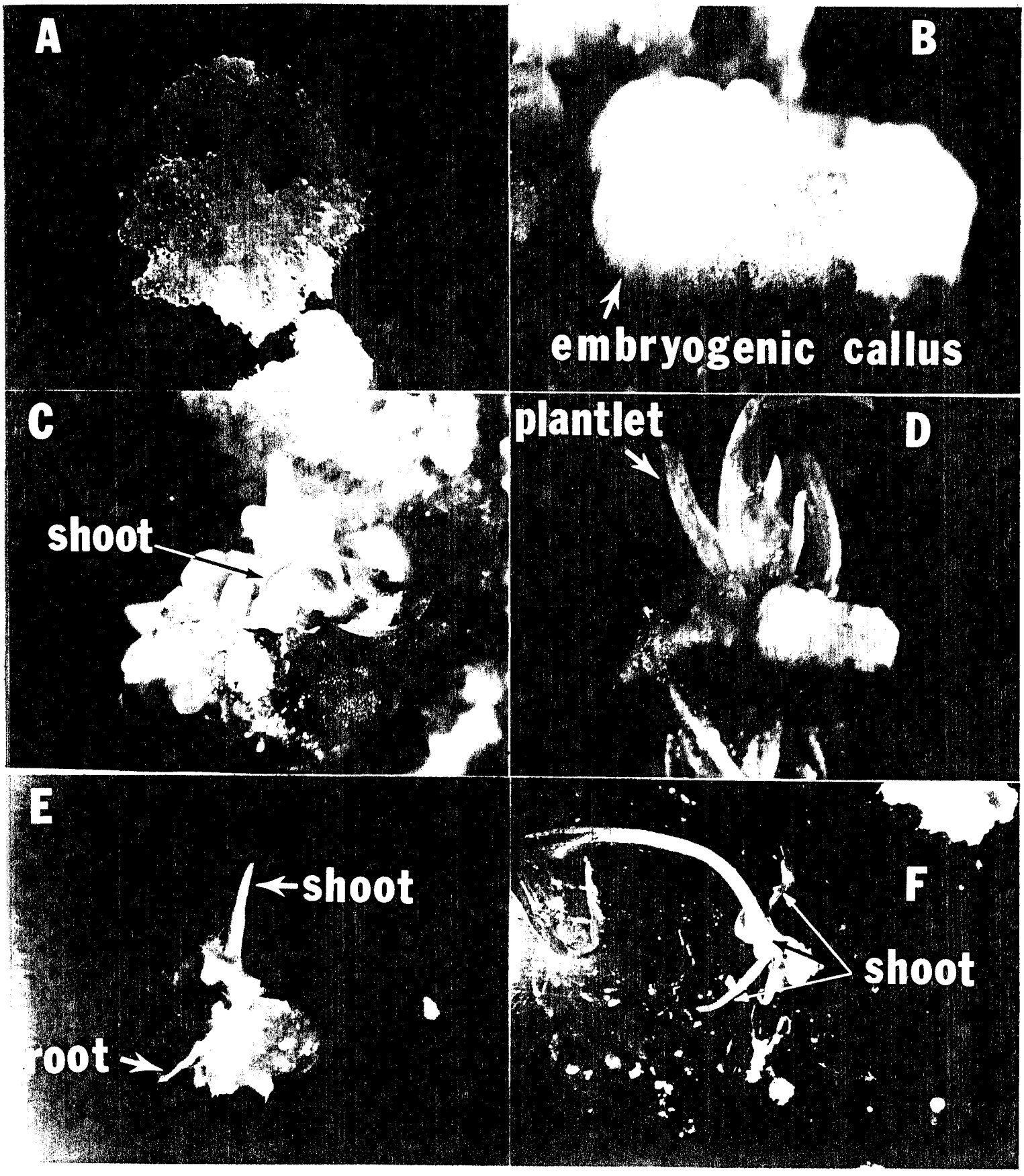
Legends of Figures

Figure 1. Regular and embryogenic callus and plantlet formation. A. Regular or non-embryogenic callus (10x). B. Embryogenic callus (30x). C. Shoot initiation by embryogenic callus (20x). D. Plantlet formation by embryogenic callus (10x). E. Plantlet formation without visible attachement to embryogenic callus (10x). F. Shoot initiated in non-embryogenic callus (5x).

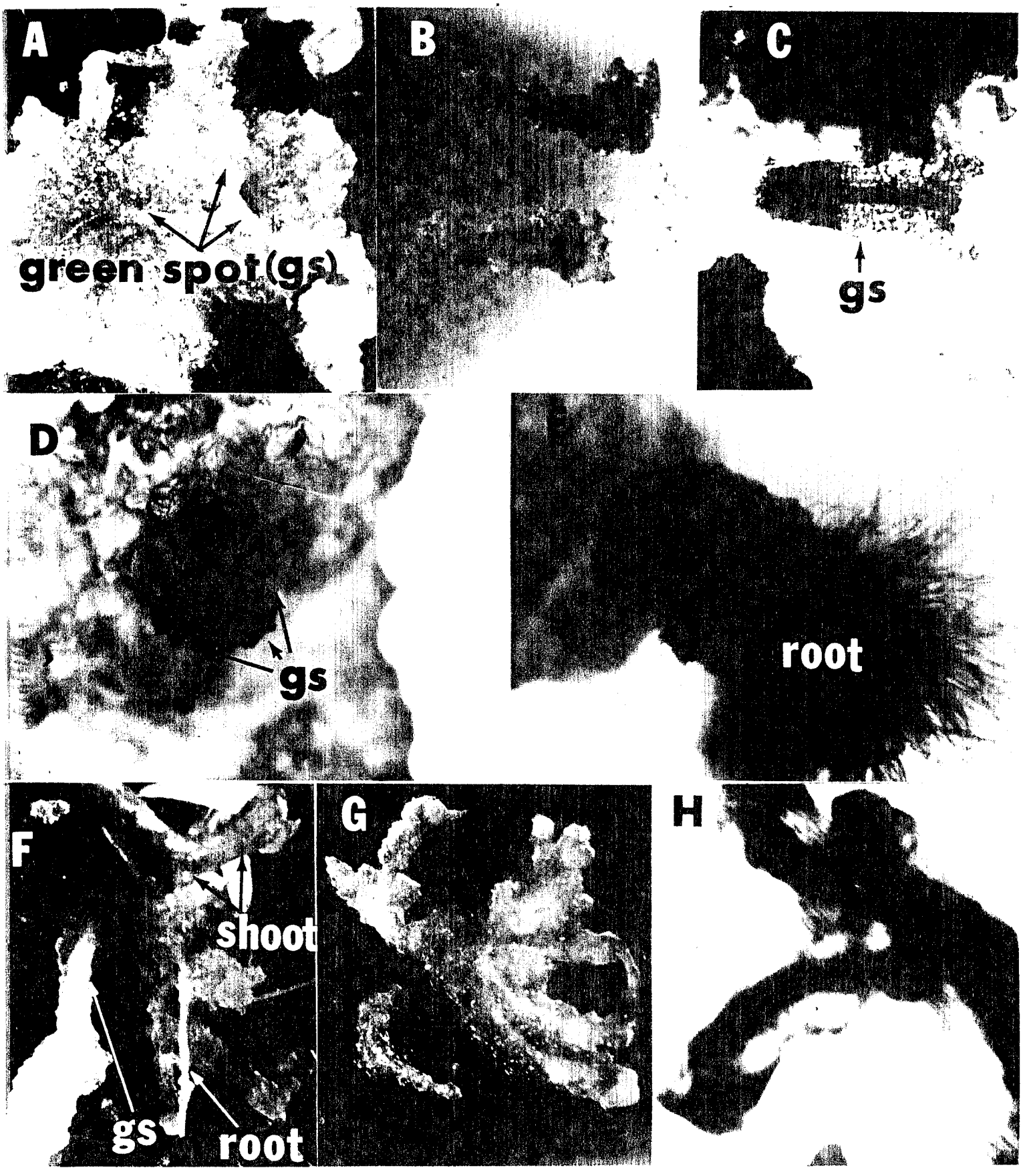
Figure 2. Green spots in secondary callus. A. Non-embryogenic callus with branched green spot (10x). B. Excised unbranched green spots (20x). C. Green spot showing organized central cylinder (40x). D. Green spot initiation in callus in which all green spots were excised (20x). E. Root formation from green spot initiated in callus devoid of green spots (20x). F. Plantlet attached to green spot (5x). G. Branched green spot excised from callus (10x). H. Cultured green spot which has initiated a surrounding layer of translucent callus and bumps which can develop into new meristems (40x).

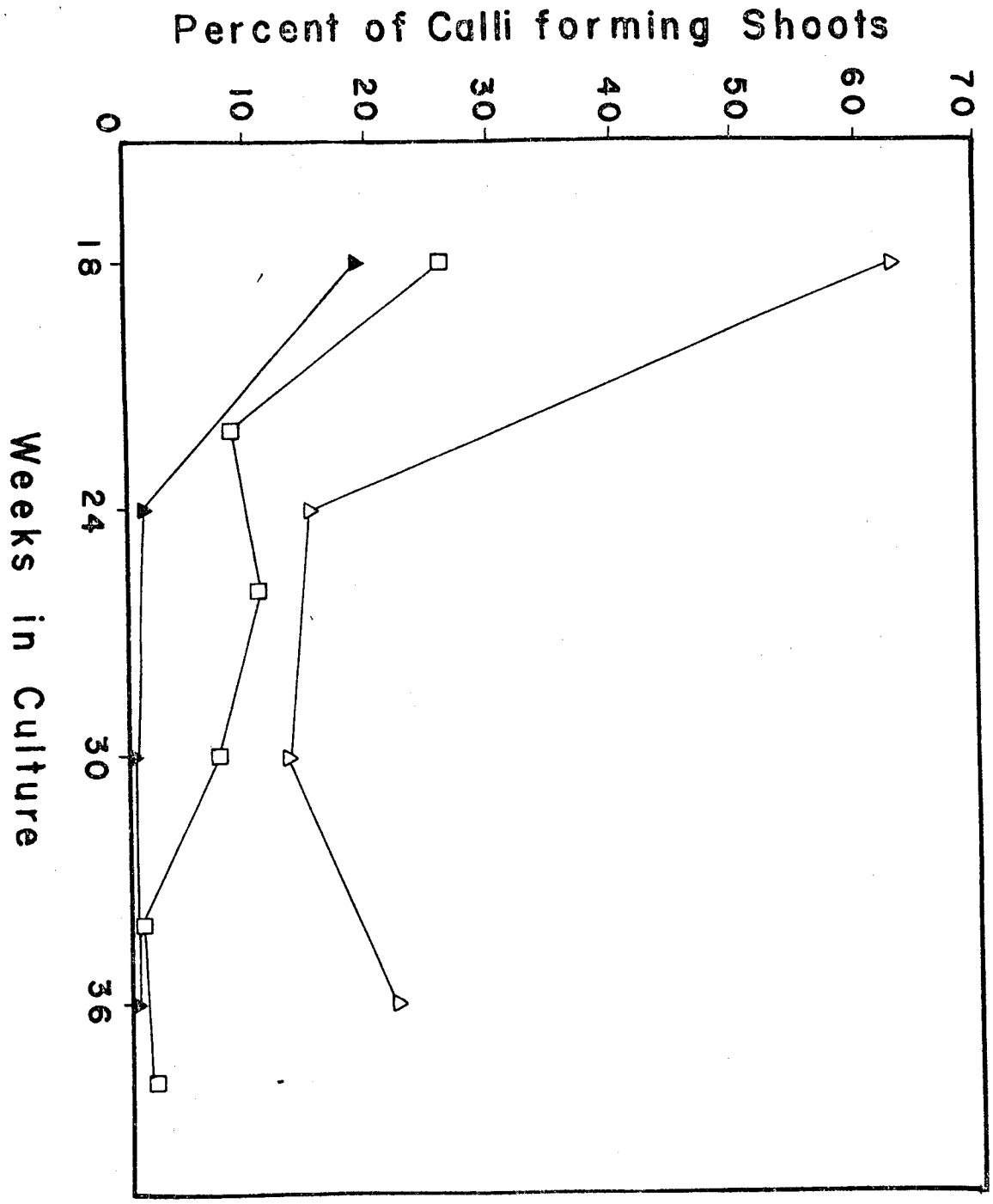
Figure 3. Shoot formation over time in culture for: A. Embryogenic callus (△). B. Non-embryogenic callus (▲). and C. Callus selected for high green spot production (□). For embryogenic callus there were 30 to 78 samples per point while non-embryogenic callus is represented by 39 to 759 samples per point.

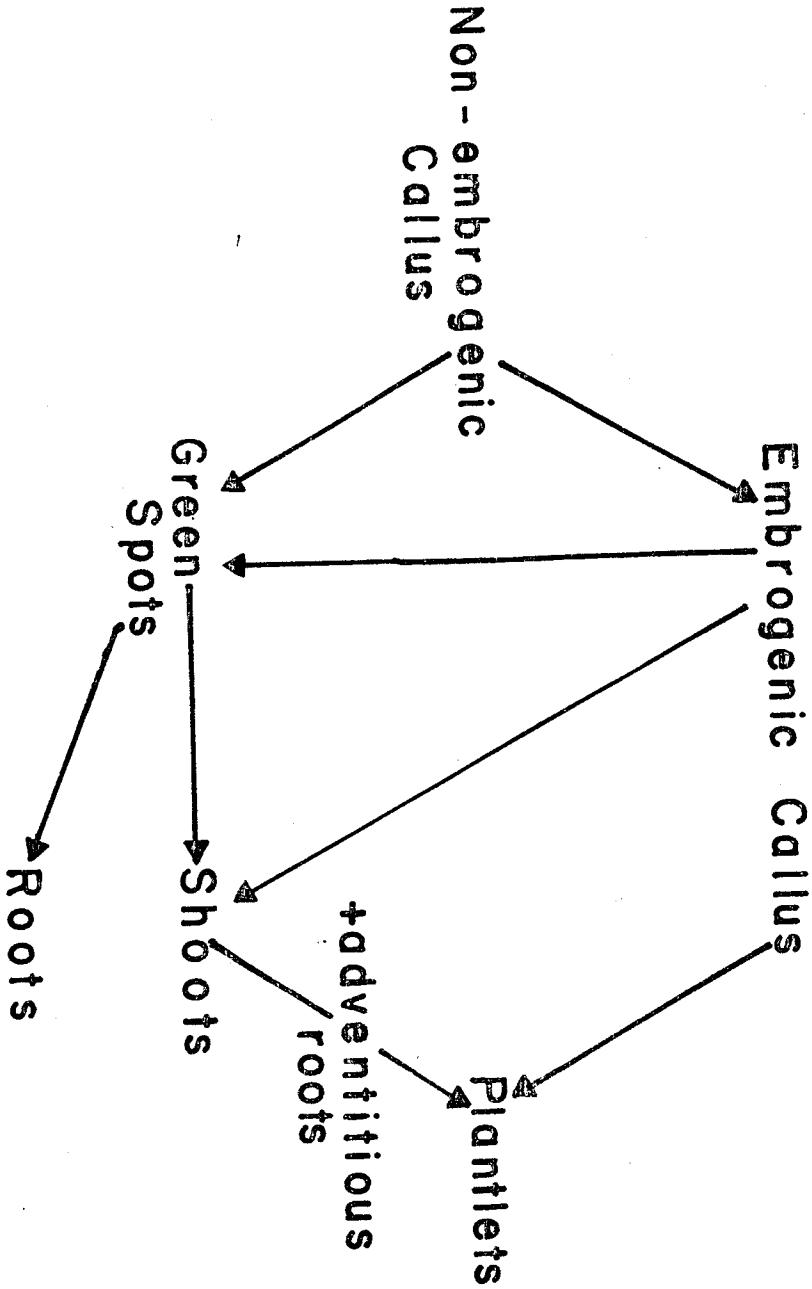
Figure 4. A model which relates a possible ontological sequence for the morphogenic structures observed in secondary oat callus.


A
B
embryogenic callus
C
shoot
plantlet
D
E
shoot
root
F
shoot

ors Fig 2




Percent of Calli forming Shoots
0
10
20
30
40
50
60
70
Weeks in Culture
18
24
30
36


Non-embrogenic Callus
Green Spots
Embrogenic Callus
Roots
Shoots
+adventitious roots
Plantlets

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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.

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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 x 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.

REFERENCES CITED

Barba, R., and L. G. Nickell. 1969. Nutrition and organ differentiation in tissue cultures of sugar cane, a monocotyledon. *Planta* 89: 299-302.

Beevers, L., and R. H. Hageman. 1969. Nitrate reduction in higher plants. *Annu. Rev. Plant Physiol.* 20: 495-522.

[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.

Brock, R. D. 1971. The role of induced mutations in plant improvement. *Rad. Bot.* 11: 181-196.

Broertjes, C. 1972. Page 293 in *Induced Mutations in Plant Improvement.* International Atomic Energy Agency, Vienna, Austria.

Broertjes, C., B. Haccius, and S. Weidlich. 1968. Adventitious bud formation on isolated leaves and its significance in mutation breeding. *Euphytica* 17: 321-344.

Carlson, P. S. 1969. Production of auxotrophic mutants in ferns. *Genet. Res.* 14: 337-339.

———. 1970. Induction and isolation of auxotrophic mutants in somatic cell cultures of *Nicotiana tabacum. Science* 1968: 487-489.

———. 1973a. The use of protoplasts for genetic research. *Proc. Natl. Acad. Sci. USA* 70: 598-602.

———. 1973b. Methionine sulfoximine-resistant mutants of tobacco. *Science* 180: 1366-1368.

Carlson, P. S., and J. C. Polacco. 1975. Plant cell cultures: genetic aspects of crop improvement. *Science* 188: 622-625.

Carter, O., Y. Yamada, and E. Takahashi. 1967. Tissue culture of oats. *Nature* 214: 1029-1030.

Cass, C. E. 1972. Density-dependent resistance to puromycin in cell cultures. *J. Cell. Physiol.* 79: 139-146.

Cheng, T., and H. H. Smith. 1975. Organogenesis from callus culture of *Hordeum vulgare. Planta* 123: 307-310.

Clare, M. V., and H. A. Collin. 1974. The production of plantlets from tissue cultures of brussels sprout (*Brassica oleracea* L. var. *gemmifera* D.C.). *Ann. Bot.* 38: 1067-1076.

Corduan, G. 1970. Autotrophe gewerbekulfuren von *Ruta graveolens* und deren $^{14}CO_2$ markerungsprodukte. *Planta* 91: 291-301.

Croy, L. I. 1967. Nitrate reductase in wheat and its relationship to grain protein and yield. Ph.D. thesis, University of Illinois, Urbana. (Mentioned in Beevers and Hageman 1969.)

D'Amato, F. 1965. Chimera formation in mutation-treated seeds and diplontic selection. *Rad. Bot.* 5(suppl.): 303-316.

de Fossard, R. A., A. Myint, and E. C. M. Lee. 1974. A broad spectrum tissue culture experiment with tobacco *(Nicotiana tabacum)* pith tissue callus. *Physiol. Plant.* 30: 125-130.

Delieu, H. 1972. The combination of cell and tissue culture with mutagenesis for the induction and isolation of morphological or developmental mutants. *Phytomorphology* 22: 283-296.

Dix, P. J., and H. E. Street. 1975. Sodium chloride-resistant cultured cell lines from *Nicotiana sylvestris* and *Capsicum annuum. Plant Sci. Lett.* 5: 231-237.

Dobzhansky, T. 1970. *Genetics of the Evolutionary Process.* Columbia University, New York, pp. 100-104.

Doerschug, M. R., and C. O. Miller. 1967. Chemical control of adventitious organ formation in *Lactuca sativa* explants. *Am. J. Bot.* 54: 410-413.

Dregne, H. E. 1963. Soils of the arid west. Pages 215-238 in C. Hodge, ed. *Aridity and Man.* AAAS, Washington, D.C.

Eilrich, G. L. 1968. Nitrate reductase in wheat and its relationship to grain protein production as affected by genotype and spring application of calcium nitrate. Ph.D. thesis, University of Illinois, Urbana. (Mentioned in Beevers and Hageman 1969).

Fridborg, G. 1971. Growth and organogenesis in tissue culture of *Allium cepa* var. *proliferum. Physiol. Plant.* 25: 436-440.

Gamborg, O. L., F. Constabel, and J. P. Shyluk. 1974. Organogenesis in callus from shoot apices of *Pisum sativum. Physiol. Plant.* 30: 125-128.

Gauch, H. G. 1972. *Inorganic Plant Nutrition.* Dowden, Hutchinson & Ross, Stroudsburg, Pa., pp. 373-393.

Gengenbach, B. G., and C. E. Green. 1975. Selection of T-cytoplasm maize callus cultures resistant to *Helminthosporium maydis* race T pathotoxin. *Crop Sci.* 15: 645-649.

Green, C. E., and R. L. Phillips. 1975. Plant regeneration from tissue cultures of maize. *Crop Sci.* 15: 417-421.

Gustafsson, Å. 1963. Mutations and the concept of viability. Pages 89-104 in E. Åkerberg, A. Hagber, G. Olsson, and O. Tedin, eds. *Recent Plant Breeding Research.* John Wiley and Sons, New York.

Heimer, Y. M., and P. Filner. 1970. Regulation of the nitrate assimilation pathway of cultured tobacco cells. II. Properties of a variant cell line. *Biochim. Biophys. Acta* 215: 152-165.

Helgeson, J. P., J. D. Kemp, G. T. Haberlach, and D. P. Maxwell. 1972. A tissue culture system for studying disease resistance: the black shank disease in tobacco callus cultures. *Phytopathology* 62: 1439-1443.

Hirono, Y., and H. H. Smith. 1969. Mutations induced in Arabidopsis by DNA nucleoside analogs. *Genetics* 61: 191-199.

International Atomic Energy Agency (IAEA). 1970. *Manual on Mutation Breeding.* IAEA, Vienna, Austria.

———. 1972. *Induced Mutations and Plant Improvement.* IAEA, Vienna, Austria.

Ingram, D. S. 1967. The expression of R-gene resistance to *Phytophthora infestans* in tissue cultures of *Solanum tuberosum. J. Gen. Microbiol.* 49: 99-108.

———. 1973. Growth of plant parasites in tissue culture. Pages 392-421 in H. E. Street, ed. *Plant Tissue and Cell Culture.* University of California, Berkeley.

Jelaska, S. 1974. Embryogenesis and organogenesis in pumpkin explants. *Physiol. Plant.* 31: 257-261.

Kelley, W. P. 1963. Use of saline irrigation water. *Soil Sci.* 95: 385-391.

Kimber, G., and R. Riley. 1963. Haploid angiosperms. *Bot. Rev.* 29: 480-531.

King, P. J., and H. E. Street. 1973. Growth patterns in cell cultures. Pages 269-337 in H. E. Street, ed. *Plant Tissue and Cell Culture.* University of California, Berkeley.

Lam, S. 1975. Shoot formation in potato tuber discs in tissue culture. *Am. Potato J.* 52: 103-106.

Lescure, A. M. 1969. Mutagenesis and cell selection of *Acer pseudoplatanus* L. cultivated *in vitro. Physiol. Veg.* 7: 237-250.

Luke, H. H., H. E. Wheeler, and A. T. Wallace. 1960. Victoria-type resistance to crown rust separated from susceptibility to Helminthosporium blight in oats. *Phytopathology* 50: 205-209.

Lustinec, J., and J. Horak. 1970. Induced regeneration of plants in tissue cultures of *Brassica oleracea. Experientia* 26: 919-920.

Maliga, P., L. Márton, and A. S. Sz.-Breznovits. 1973a. 5-bromodeoxyuridine-resistant cell lines from haploid tobacco. *Plant Sci. Lett.* 1: 119-120.

Maliga, P., A. S. Sz.-Breznovits, and L. Márton. 1973b. Streptomycin-resistant plants from callus culture of haploid tobacco. *Nat. New Biol.* 244: 29-30.

Margara, J. 1970. Néoformation de bourgeons in vitro chez la betterave sucrière. *C. R. Acad. Sci. Paris* 270D: 698-701.

Márton, L., and P. Maliga. 1975. Control of resistance in tobacco cells to 5-bromodeoxyuridine by a simple Mendelian factor. *Plant Sci. Lett.* 5: 77-81.

Masteller, V. J., and D. J. Holden. 1970. The growth of and organ formation from callus tissue of sorghum. *Plant Physiol.* 45: 362-364.

Mehra, A., and P. N. Mehra. 1974. Organogenesis and plantlet formation *in vitro* in almond. *Bot. Gaz.* 135: 61-73.

Melchers, G., and G. Labib. 1970. Die Bedeutung haploider höheren Pflanzen fur Pflanzenphysiolozie und Pflanzenzüchfung. *Ber. Dtsch. Bot. Ges.* 83: 129-150.

Murashige, T. 1974. Plant propagation through tissue cultures. *Annu. Rev. Plant Physiol.* 25: 135-166.

Nabors, M. W., A. Daniels, L. Nadolny, and C. Brown. 1975. Sodium chloride tolerant lines of tobacco cells. *Plant Sci. Lett.* 4: 155-159.

Nishi, T., Y. Yamada, and E. Takahashi. 1968. Organ redifferentiation and plant restoration in rice. *Nature* 219: 508-509.

Ohyama, K. 1974. Properties of 5-bromodeoxyuridine-resistant lines of higher plants in liquid culture. *Exp. Cell Res.* 89: 31-38.

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

Rains, D. W., and E. Epstein. 1967. Preferential absorption of potassium by leaf tissue of the mangrove, *Avicennia marina:* An aspect of halophytic competence in coping with salt. *Aust. J. Biol. Sci.* 20: 847-857.

Saunders, J. W., and E. T. Bingham. 1972. Production of alfalfa plants from callus tissue. *Crop Sci.* 12: 804-808.

Schrader, L. E., D. M. Peterson, E. R. Leng, and R. H. Hageman. 1966. Nitrate reduc-

ase activity of maize hybrids and their parental inbred. *Crop Sci.* 6: 169-173.

Serra, J. A. 1968. *Modern Genetics*, vol. 3. Academic Press, New York, p. 436.

Shimada, T., T. Sasakuman, and K. Tsunewaki. 1968. *In vitro* culture of wheat tissues. I. Callus formation, organ redifferentiation, and single cell culture. *Can. J. Genet. Cytol.* 11: 294-304.

Smith, H. H. 1974. Model systems for somatic cell plant genetics. *BioScience* 24: 269-275.

Staritsky, G. 1970. Embryoid formation in callus tissues of coffee. *Acta Bot. Neerl.* 19: 509-514.

Street, H. E. 1973a. Single-cell clones. Pages 191-204 in H. E. Street, ed. *Plant Tissue and Cell Culture.* University of California, Berkeley.

———. 1973b. Old problems and new perspectives. Page 422 in H. E. Street, ed. *Plant Tissue and Cell Culture.* University of California, Berkeley.

———. 1973c. *Plant Tissue and Cell Culture.* University of California, Berkeley.

Strickberger, M. W. 1968. *Genetics.* Macmillan, New York.

Sunderland, N. 1973a. Pollen and anther culture. Pages 205-239 in H. E. Street, ed. *Plant Tissue and Cell Culture.* University of California, Berkeley.

———. 1973b. Nuclearcytology. Pages 161-190 in H. E. Street, ed. *Plant Tissue and Cell Culture.* University of California, Berkeley.

Vasil, I. K., and V. Vasil. 1972. Totipotency and embryogenesis in plant cell tissue cultures. *In Vitro* 8: 117-127.

Vogel, F. 1970. Pages 35-36 in F. Vogel and G. Röhrborn, eds. *Chemical Mutagenesis in Mammals and Man.* Springer-Verlag, New York.

Waisel, Y. 1972. *Biology of Halophytes.* Academic Press, New York.

Walkey, D. G. A., and J. M. G. Woolfitt. 1970. Rapid clonal multiplication of cauliflower by shake culture. *J. Hort. Sci.* 45: 205-206.

Wallace, A. T., R. M. Singh, and R. M. Browning. 1967. Monograph. Erwin Baur Memorial Lecture IV. German Academy of Science, East Berlin, pp. 45-57.

Widholm, J. M. 1972a. Anthranilate synthetase from 5-methyltryptophan-susceptible and resistant cultured *Daucus carota* cells. *Biochim. Biophys. Acta* 279: 48-57.

———. 1972b. Cultured *Nicotiana tabacum* cells with altered anthranilate synthetase which is less sensitive to feedback inhibition. *Biochim. Biophys. Acta* 261: 52-58.

———. 1974. Cultured carrot cell mutants: 5-methyltryptophan-resistance trait carried from cell to plant and back. *Plant Sci. Lett.* 3: 323-330.

Wilmar, C., and M. Hellendoorn. 1968. Growth and morphogenesis of asparagus cells cultured *in vitro*. *Nature* 217: 369-370.

Zelitch, I. 1975. Improving the efficiency of photosynthesis. *Science* 188: 626-633.