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RAPID CROP IMPROVEMENT
USING TISSUE CULTURE TECHNIQUE[1]

A new method for the production of stress tolerant, energy efficient crop plants

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SUMMARY

Plant cell tissue culture techniques have contributed greatly to our understanding of basic problems in plant physiology, biochemistry, and development. We are able to cultivate large numbers of plant cells from many different plant species and to induce these cells to develop into complete plants. In the past tissue culture has been a tool for use only by the basic researcher, but now the technique offers the possibility of making a contribution to plant breeding by a) cloning rare, useful plants so that they can be rapidly introduced to growers or b) adding new useful alleles to existing food crop varieties.

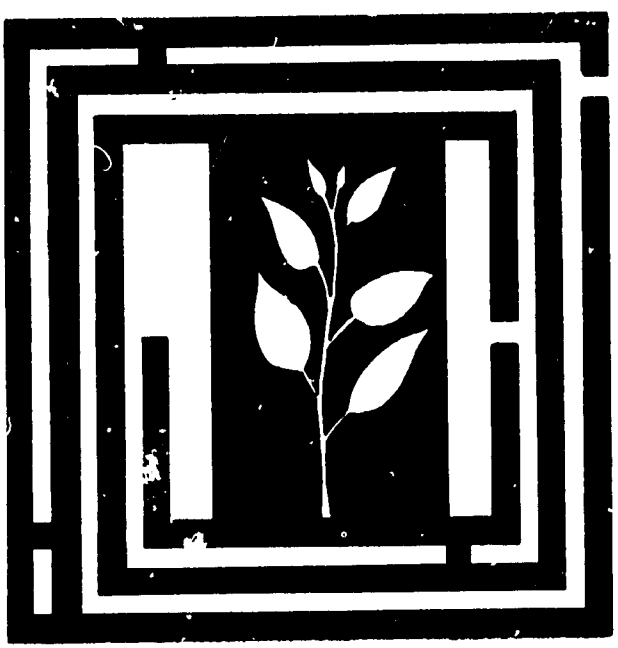
The fundamental power of cell culture as a plant breeding tool is that it arranges the process of mutation and natural selection into a logistically simple format. Essentially, it compresses in the laboratory, in a highly controlled and ordered way, the lengthy and random evolutionary mechanisms of plants. The technique can be used to select specific, beneficial mutations which occur naturally or for the artificial selection of new phenotypes. Traditional breeding methods utilize the same properties of mutation and selection, but tissue culture significantly reduces commitments of time, space, and labor (fig. 1).

Tissue culture breeding is uniquely suited for providing food crop varieties with genes conferring resistance to various environmental stress factors. Plants which are modified for specific environmental conditions will outperform unadapted varieties while requiring less resource and energy input. The inherent properties of tissue culture make it economically feasible to produce plant varieties which are tailor made for specific, local environments.

The purpose of this bulletin is to inform plant breeders of the

Figure 1. Selection of Stress Tolerant Plants

Artificial selection using traditional breeding



1) Uncontrolled
2) Random
3) Slow

Artificial selection using tissue culture

1) Controlled
2) Ordered
3) Fast

potential use of cell culture techniques for augmenting their breeding programs and to report on specific methods we have used to select NaCl-tolerant plants. The method of tissue culture, current results, and future prospects are described. It is hoped that tissue culture will be considered as a viable breeding method to be used in conjunction with traditional techniques. We welcome inquiries for further information on tissue culture methods available for various species.

INTRODUCTION

Over the last three decades, the major advances in food crop production have been accomplished by the use of fertilizers, irrigation, pesticides, herbicides, mechanization, and improved plant varieties. All of these practices except the last involve environmental alteration to suit the plant, and have been so fully employed in some parts of the world that agricultural improvement by such methods has apparently plateaued. In addition, the rising cost of energy puts severe constraints on their use in less developed countries, and in the future even industrialized nations may eventually find their cost prohibitive. A way to reduce these costs and to continue to expand food production is to develop new plant varieties which require less environmental modification and are specifically tailored for local environments.

Plant breeders produce new plant varieties by the incorporation of new useful alleles or allele combinations into the gene pool of cultivated varieties. Traditionally, this has been accomplished by cross breeding existing domestic or wild varieties, or by the selection of spontaneous or induced mutants carrying a desired trait. Once the particular trait has been isolated in a plant by either method, the ease with which it can be used for crop improvement will depend upon the fertilization process of the particular plant species. In self-fertilizing species, the trait is easily established in large numbers of propagated plants. In cross-pollinating species, the introduction of a new trait can be a slow and arduous process.

Cross breeding domestic or wild varieties can potentially add several new genes to the best existing genotype, but several breeding seasons are

required to stabilize the cross varietal product. Moreover, it is not uncommon that neither a domestic nor wild variety can be found carrying the desired trait. The selection of spontaneous or induced mutants can obviate the stabilization requirement and is primarily used for the addition of one gene at a time to an existing genotype. However, there are several logistical and economic disadvantages with these procedures as well.

Due to low mutation rates (one in 100,000 to 100,000,000 plants will be a spontaneous mutant possessing a particular desired trait), the isolation of spontaneous mutants from field-grown plants requires the examination of large numbers of plants. This has typically involved large commitments of time, land, and labor--all at high expense. Selection methods which utilize seedlings rather than field-grown plants reduce such commitments; however, seedlings will respond to a selective agent in different ways than mature plants. In addition, certain desirable traits (e.g., growth habits) are easily identified by field observation whereas ones such as amino acid profiles are not. Others such as herbicide-resistant or salt-tolerant mutants require that plants are subjected to selective pressure.

Inducing mutations in seeds or pollen increases the frequency of novel phenotypes, thereby reducing the number of plants to be screened. Statistically though, the likelihood of a deleterious mutation is greater than an advantageous one. In the event of a beneficial mutation in a seed, the main problem is that the multicellular nature of the embryo makes it statistically unlikely to find all cells of the first generation of the mutant plants to have the same mutations in the same gene. This creates a chimerical inheritance problem and a few breeding seasons may

be required before a pure stock can be obtained. Mutating pollen before fertilization presents problems with obtaining large numbers of potentially mutant plants. The limited, expensive, and only recent success of useful plant production from induced mutations in seed can be attributed to the great expense and physical outlay in carrying a large number of seeds through several breeding generations to stabilize a particular trait.

For these reasons, it is appropriate to consider other developing technologies which reduce or eliminate many of these obstacles. Recent advances in plant cell culture offer a possible method for the rapid isolation of desirable mutations in crop plants. Millions of potential plants can be grown in a single flask or vial within which selection for mutant phenotypes can occur. Plant cell culture significantly reduces the time, space, and labor required to produce a new plant variety, and offers more flexibility in the selection of desirable traits in crop plants (fig. 2).

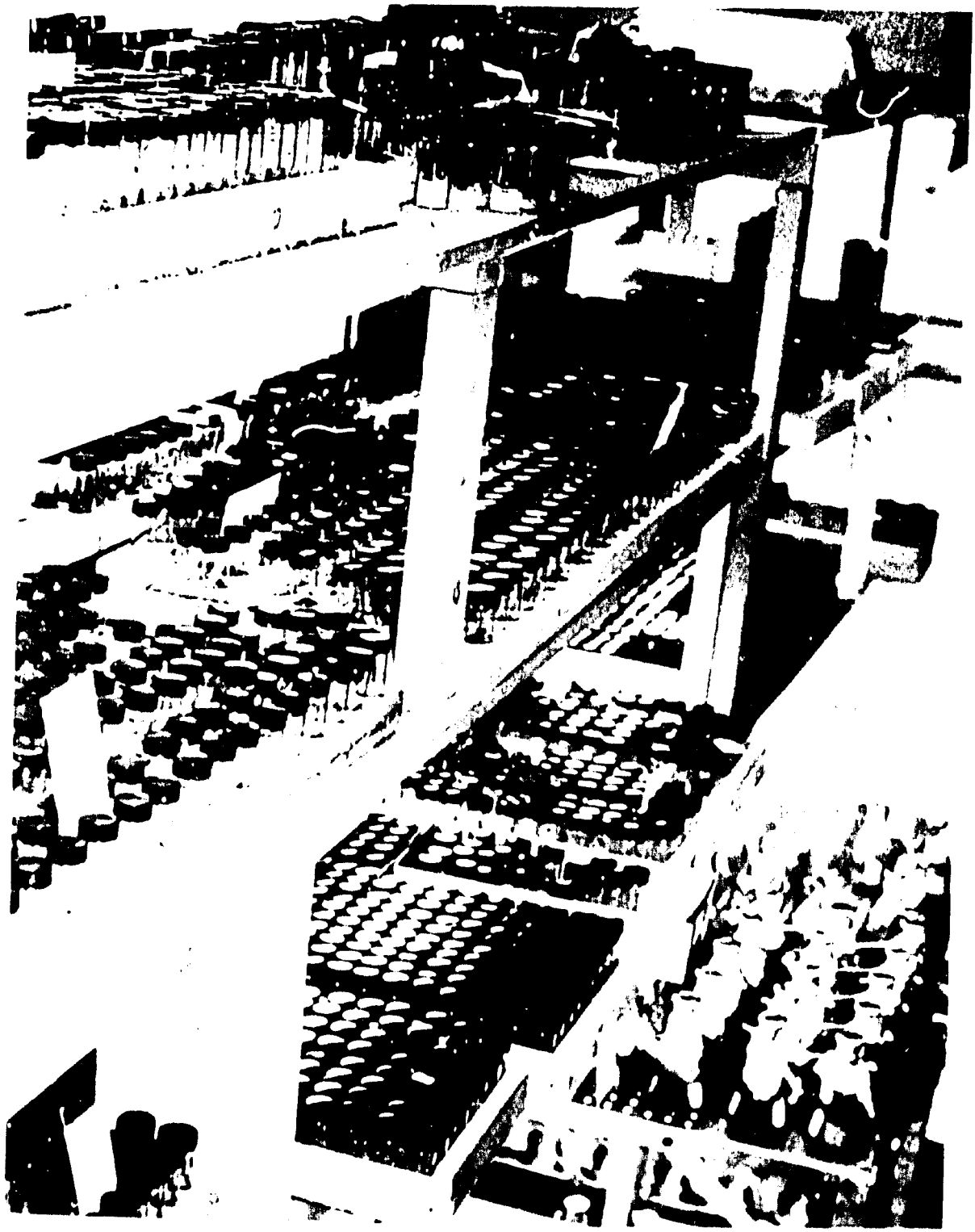


Figure 2. Growth room where cell cultures are kept at a constant temperature (25°C) under continuous illumination. This room contains the genetic equivalent of 1.5 thousand acres of wheat. Selection of stress tolerant mutants is tremendously simplified by growing large numbers of plants in such a small space.

CELL CULTURE TECHNIQUES

The production of mutant plants by cell culture techniques can be divided into four basic steps: a) initiation of the cell culture, b) selection of the desired mutant cell phenotype, c) regeneration of mutant cells into whole plants and d) greenhouse and field testing. This discussion summarizes the general method and details specific methods used in our lab to obtain salt tolerant plants (fig. 3).

In the first step, groups of plant cells are removed from a plant and cultured aseptically on either a solid or liquid medium (fig. 4). Typically, a portion of the plant (a root, stem or leaf section) is surface sterilized with 20% Clorox for 10-20 min. and placed in a vial or test tube containing a solid agar medium with known concentrations of sugar, mineral nutrients, vitamins, and plant growth hormones (fig. 5). A typical and quite useful basal medium is that of Linsmaier and Skoog (1965).

A. Inorganic salts

1.	NH_4NO_3	1650 mg/liter of medium
2.	KNO_3	1900
3.	$CaCl_2 \cdot 2H_2O$	440
4.	$MgSO_4 \cdot 7H_2O$=	370
5.	KH_2PO_4	

B. Trace elements

6.	$MnSO_4$	16.9 mg/liter of medium
	$ZnSO_4 \cdot 2H_2O$	10.3
	H_3BO_4	6.2
	KI	0.83
	$Na_2MoO_4 \cdot 2H_2O$	0.25
	$CuSO_4 \cdot 5H_2O$	0.025
	$CoCl_2 \cdot 6H_2O$	0.025

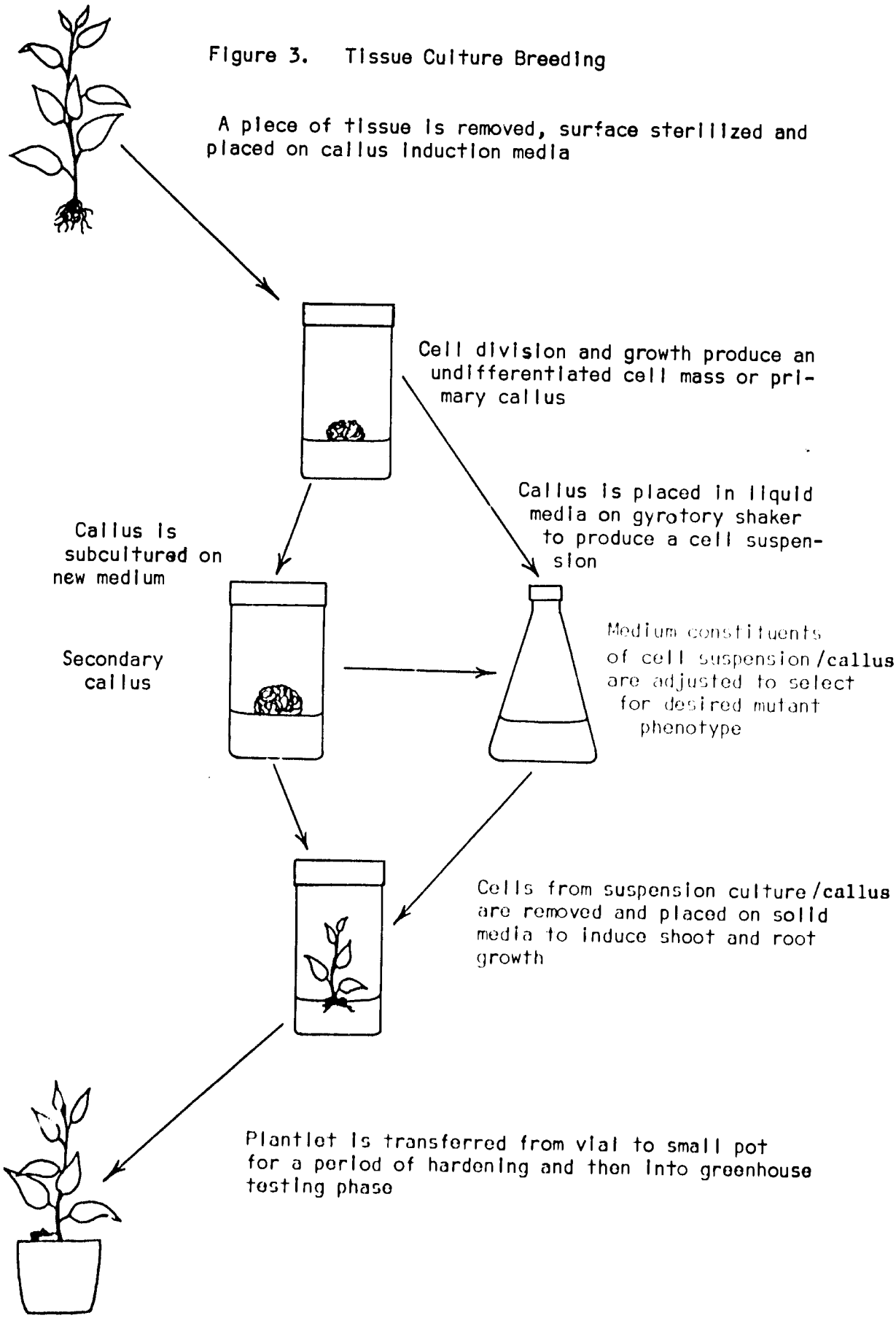
C. Organic supplements

7.	Thiamin HCl	0.4 mg/liter of medium
	myo-inositol	100.0

D. Iron

8.	EDTA-Ferric salt	50.00 mg/liter of medium

Figure 3. Tissue Culture Breeding



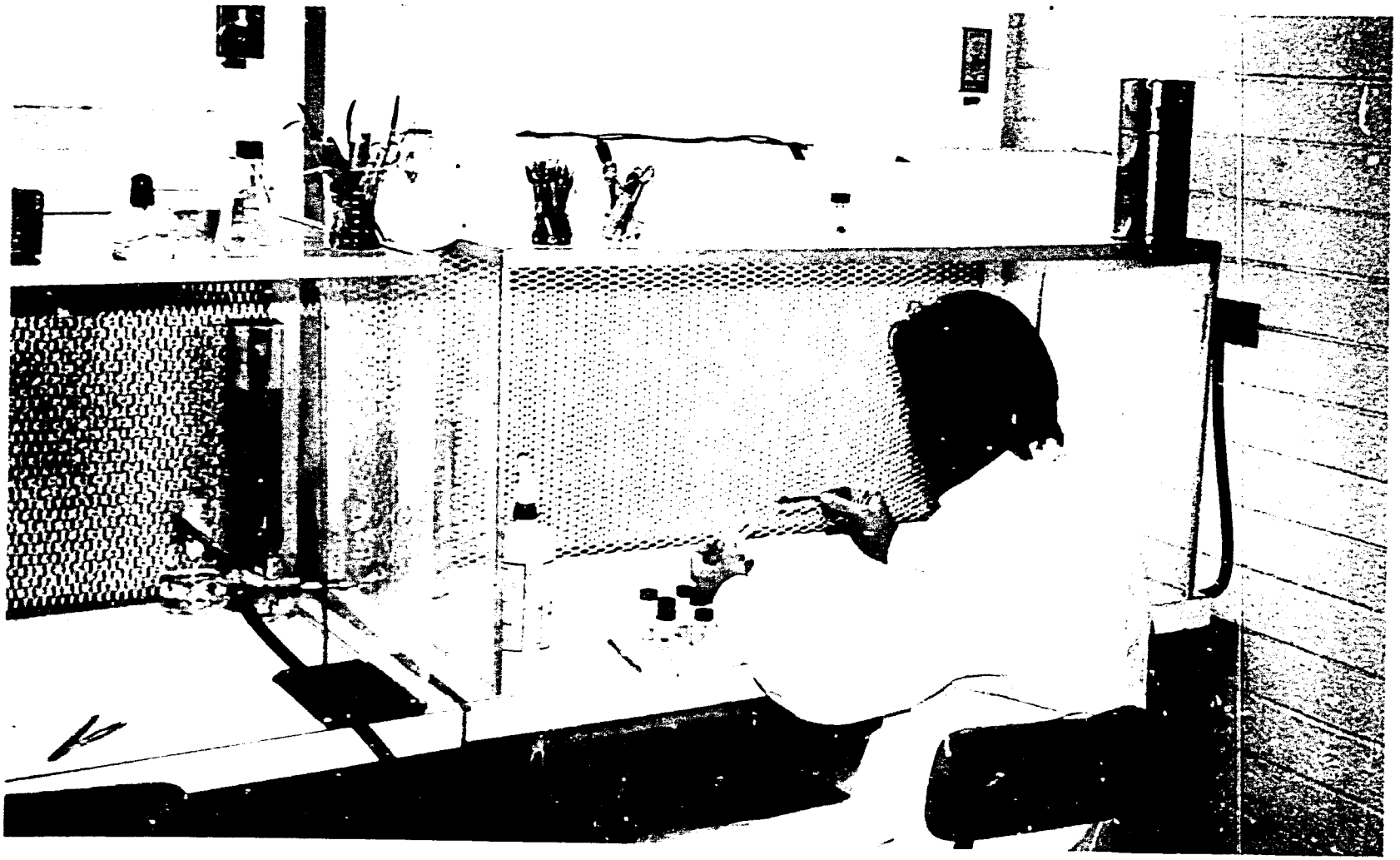


Figure 4. All manipulations of cell cultures are done under a sterile transfer hood to prevent bacterial and fungal contamination of cultures.

Figure 5. Transfer of oat callus to new nutrient medium under a sterile transfer hood.

E. Sugar

9. Sucrose 40 gm/liter

F. Agar 10 gm/liter

G. Medium pH 5.5

The hormonal composition of the medium is adjusted to stimulate the proliferation of the cells into an undifferentiated mass of cells called a "callus" (fig. 6). This is usually accomplished by using high levels (2-40 mg/ℓ) of an auxin (a plant hormone) which encourages cell division but inhibits the formation of shoots and roots. The specific auxin used as well as the concentration varies from species to species and sometimes variety to variety.

In the case of experiments designed to obtain salt-tolerant tobacco, oats, and wheat, cell lines are obtained from tobacco stem sections and from oat and wheat seeds. In the case of tobacco stem sections, cells grow and divide to produce a thumbnail-sized callus containing around 50,000 cells in 2 to 3 weeks. The medium inducing cell proliferation in Samsum tobacco contains 5 mg/ℓ indole-acetic acid plus 0.5-5.0 mg/ℓ kinetin. In the case of wheat and oats the seed is germinated on basal medium containing 2.0-5.0 mg/ℓ 2,4-dichlorophenoxyacetic acid. Cells of the germinating root are induced to grow and divide by the auxin resulting in a callus mass. For all three plants primary callus derived from the plant part can be subcultured to obtain secondary callus. When a sufficient amount of callus has been obtained, portions of callus are sometimes then placed in a flask containing liquid medium on a gyrotory shaker (Fig. 7). The mechanical motion of the shaker disrupts the callus into small clumps of cells and single cells called a "cell suspension." Cell suspensions are subcultured when cell density reaches a maximum value,

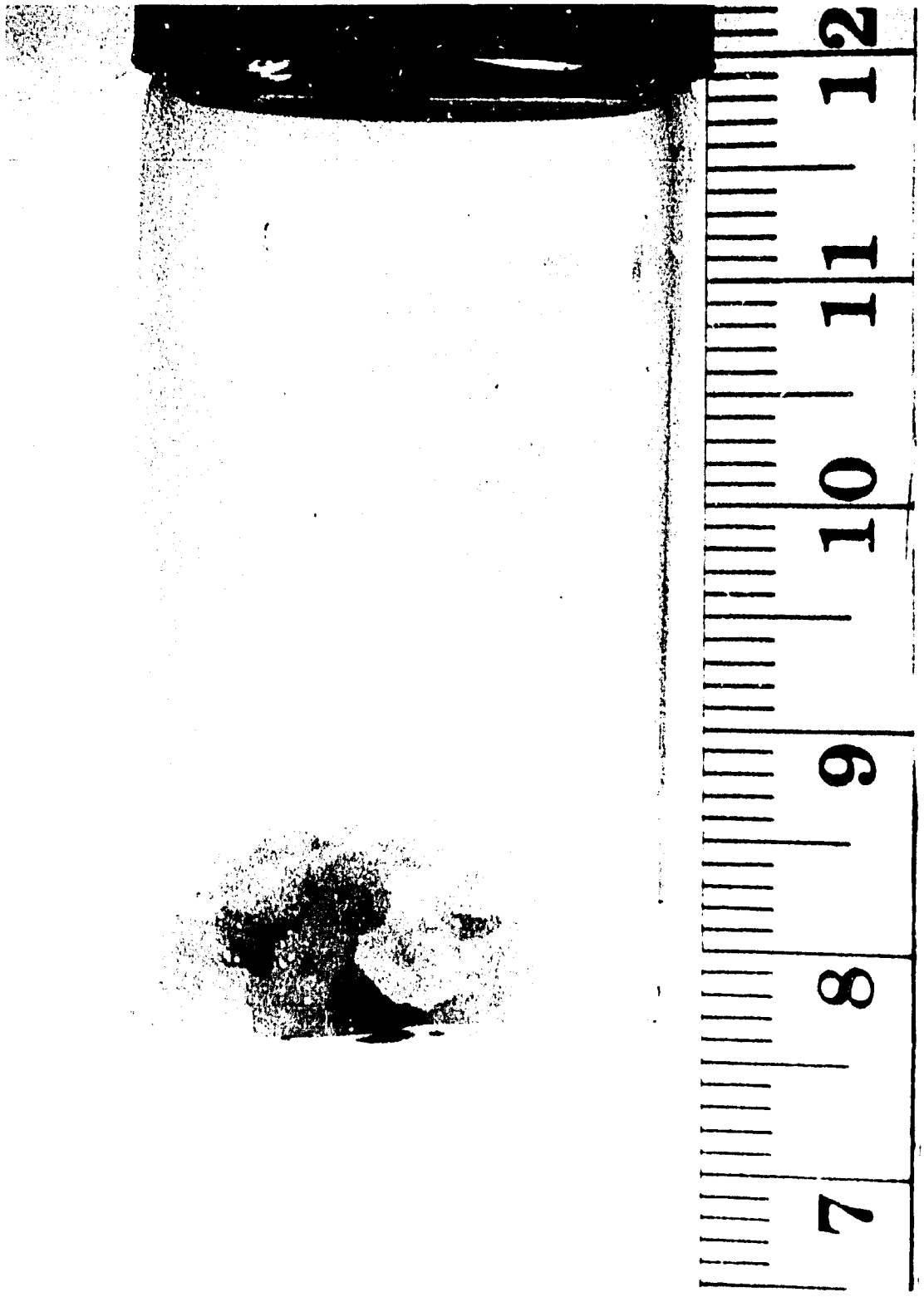


Figure 6. Oat callus on callus induction and growth medium. Selection of mutant phenotypes can take place on solid or liquid medium by altering medium constituents.

Figure 7. Callus is placed in liquid medium on a gyrotory shaker to produce a cell suspension. High salt concentrations have been added to this media to select for salt tolerant mutant cells. A typical cell suspension will contain 10^5 cells/ml, with each cell being a potential plant.

Cell suspensions contain large numbers of cells. A suspension culture of 100 mls can contain as many as 10^7 cells with almost every cell having the genetic capability to produce an entire plant. Although each cell is genetically identical and would give rise to genetically identical plants, specific spontaneous mutations occur in at least one cell in a hundred million. In the second step of the cell culture technique, these spontaneous mutations are selected by exposing cell suspensions or callus cultures to conditions that slow or prevent growth of normal cells while favoring the growth of desired mutant cells. For example, by gradually increasing the NaCl level in a tobacco suspension culture in 600 mg/ℓ steps, NaCl tolerant cells are selected because their growth and cell division are favored. Thus, an entire culture (10^7 cells in 100 mls) of potential plants with NaCl tolerance is obtained in several months. In tobacco cultures NaCl tolerances approaching 8000 mg/ℓ have been obtained in several cell lines. Tolerance beyond this level seems impossible to obtain in suspensions. This may reflect a genetic inability of the cells to adapt to salt concentrations above this level. Salt tolerant tobacco cell lines can also be selected by adding NaCl to solid medium supporting callus growth. Table 1. shows representative data illustrating this type of selection. For oats and wheat, selection of NaCl-resistant cell lines is accomplished on solid medium using 1000 mg/ℓ steps of increased NaCl. Tolerance to 9000 mg/ℓ has been obtained in both species.

In the final step, mutant cells are regenerated into whole plants. Cells are removed from the suspension culture or vial and placed on a solid medium which induces shoot and root growth (fig. 8 and 9). For some species one medium is used to induce shoots and another is used to

Figure 8. Shoot and root growth can be induced from callus or from cells from a suspension culture. Figure 8 shows a salt-tolerant tobacco plantlet growing from mutant cells taken from a suspension culture.

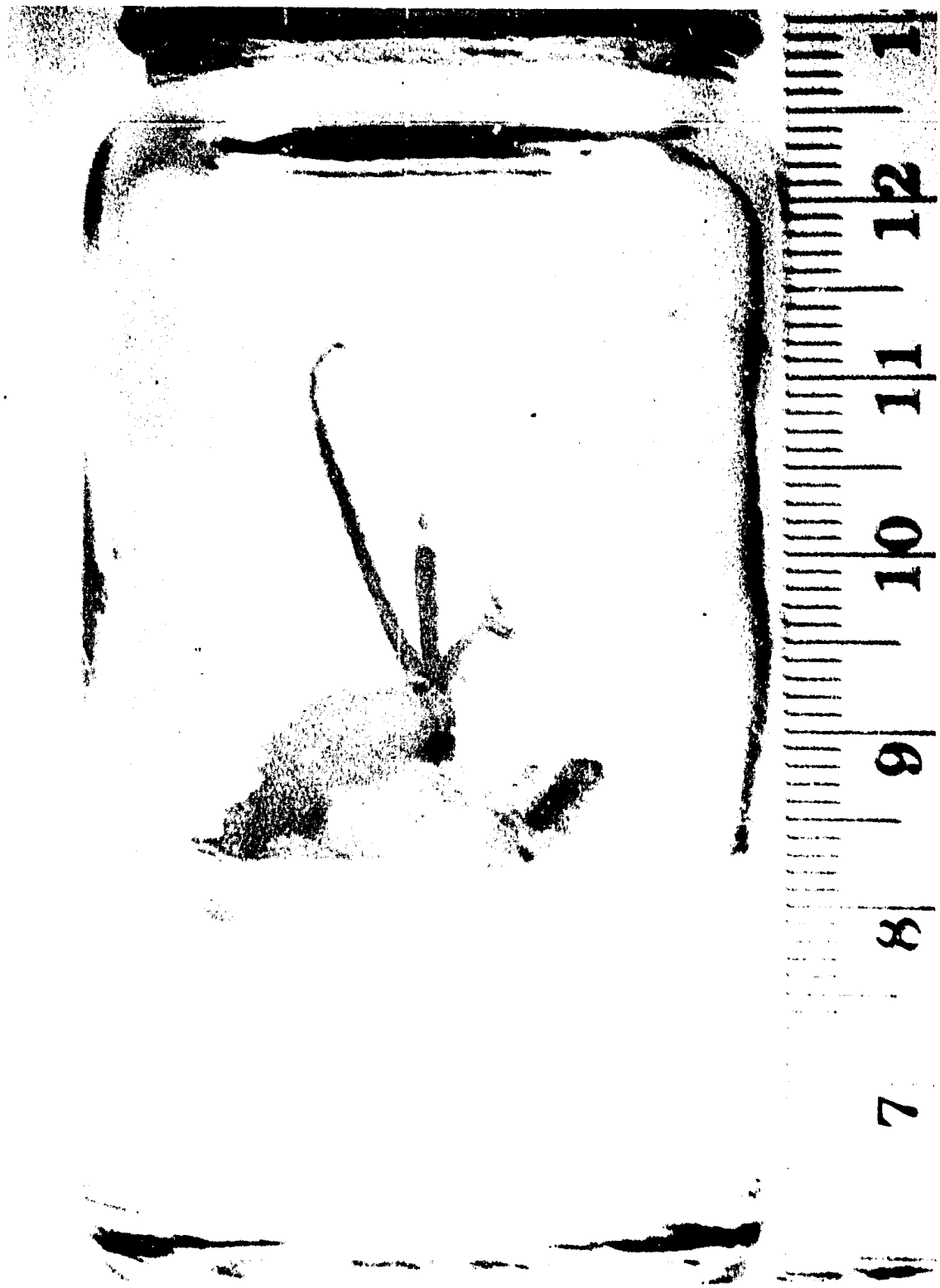


Figure 9. Shoot induction in an oat callus.

TABLE 1. Selection for NaCl-tolerant mutants in calli grown on solid medium. ***

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

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

**No growth.

***Culture #1 derived from the 0 concentration of culture #3. Culture #2 derived from the 0 concentration of culture #4.

Figure 10. A tobacco plantlet transferred from culture vial to a small pot containing soil mixture watered with a nutrient solution. At this stage, plantlets must be grown under controlled conditions for a period of "hardening."

induce roots. This may take from 4 to 6 weeks depending upon the particular plant species. Following the formation of complete plantlets (small, young plants), they are transferred to pots containing a soil mixture and placed in a controlled environment for a period of "hardening" before transfer to the greenhouse (fig. 10). In the greenhouse, regenerated plants are tested for phenotype persistence and inheritability of the mutation. Standard breeding procedures can then be used to further incorporate the new mutation into established cultivars.

For our studies on NaCl-tolerant tobacco, 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 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. Figures 11 and 12 show tobacco plants from group 1 (salt-sensitive) and 3 (salt-tolerant) after 3 weeks watering with a 32.8 g/ℓ NaCl solution.

Plant survival rates at 13 weeks at two levels of NaCl are shown in table II. 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 (fig. 13). 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 2. Figure 14 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.

Mutation induction can be applied at the cell suspension stage to increase the appearance of mutations. Mutation frequency depends on the dosage of the mutagenic agent as well as on various treatments preceding or following mutagenesis. After mutagenesis the selection for desirable phenotypes is accomplished in the same manner as spontaneous mutations.

TABLE 2. Survival rate under high or no salt stress of salt-tolerant and non-salt-tolerant tobacco plant lines derived from cultured cells.*

	% 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

* 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.

Figure 11. F_1 generation of salt-sensitive tobacco plants watered with 32.8 mg/ℓ NaCl solution. Almost all plants are dead.

Figure 12. F_1 generation of salt-tolerant tobacco plants watered with 32.8 mg/ℓ NaCl solution for 13 weeks. Plants are alive and growing indicating that salt-tolerance selected in tissue culture is inherited through regenerated parent plants into the F_1 generation.

Figure 13. Lab technician collecting seeds from the F_1 generation of salt-tolerant tobacco plants.

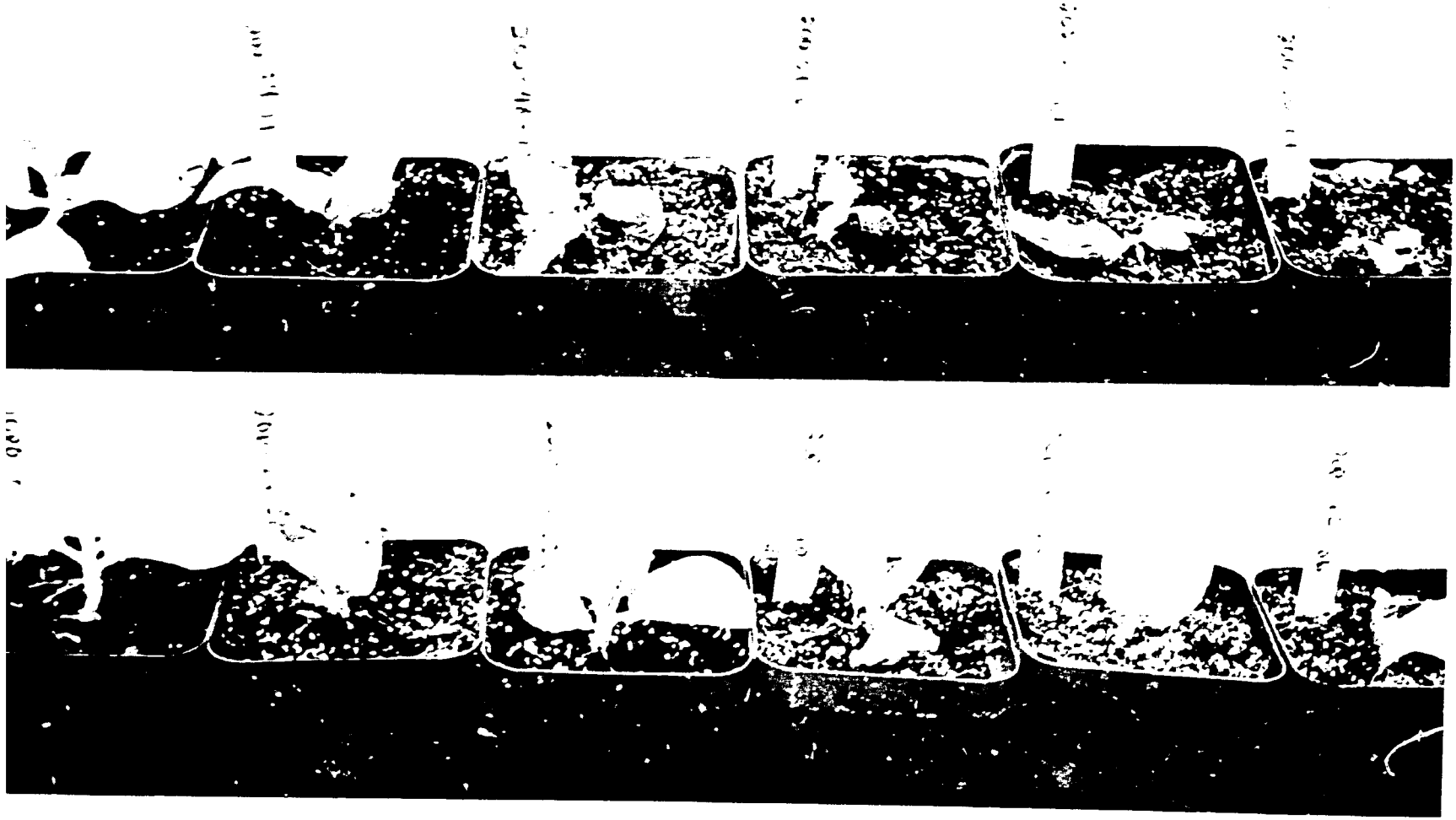


Figure 14. F_2 generation of salt tolerant (bottom) and non-tolerant (top) tobacco plants. Plants were watered with nutrient solutions containing increasing salt concentrations from left to right.

Alternatively, mutation induction can be followed by regeneration of large numbers of plants which would then be subjected to selection. The advantages of mutation induction in cell culture over that in seeds is that several breeding seasons are not required for stabilization of the phenotype. This is due to the fact that most regenerated plants arise from single cells and if selection has occurred in suspension culture all cells will carry the desired phenotype. It should be noted that, as in seeds, most induced mutations in cell culture are deleterious in a particular environment. Therefore, searches should be made for spontaneous mutations before attempting mutation induction.

The ease with which tissue culture breeding can be applied to food crop plants has been obtaining reliable regeneration techniques. For some legumes (soybean, dry bean), regeneration methods have not yet been developed. Despite these problems, complete methodology is available today for incorporation of tissue culture breeding into existing breeding programs of wheat, oats, corn, tomatoes, and barley (Table 3).

A common criticism of tissue culture breeding is that even though mutant cell lines with altered traits might be selected at the cellular stage, the phenotypic characteristics may not persist through the various stages of development to be useful in the field or they may carry deleterious side effects. These questions can only be resolved by experimentation; however, plant cell culture techniques have been successfully used to obtain whole mutant tobacco plants from mutant cell lines resistant to streptomycin, and to an analog of *Pseudomonas tabaci* toxin, and to the herbicide Picloram; corn plants resistant to *Helminthosporium* have also been selected. In all instances, resistance was shown to be maintained in regenerated whole plants and to be inheritable. Currently, as

TABLE 3 Procedures for plant regeneration (shoot formation) in some agricultural plants *

Family	Genus	Common name
Amaryllidaceae	Allium cepa	onion
Araceae	Colocasia esculenta	taro
Chenopodiaceae	Beta vulgaris vars.	beet, sugarbeet
Compositae	Lactuca sativa	lettuce
Cruciferae	Brassica oleracea vars.	Brussels sprout cauliflower Kale
Cucurbitaceae	Cucurbita pepo	pumpkin
Leguminosae	Medicago sativa Phaseolus vulgaris Pisum sativum	alfalfa bean pea
Liliaceae	Asparagus officinalis	asparagus
Linaceae	Linum usitatissimum	flax
Poaceae	Avena sativa Eleusine coracana Hordeum vulgare Lolium sps. Oryza sativa Panicum milaceum Paspalum scrobiculatum Pennisetum typhoideum Saccharum officinarum Sorghum bicolor Triticum sps. Zea mays	oats Indian millet barley ryegrass rice common millet Indian millet Indian millet sugarcane sorghum wheat corn
Rosaceae	Prunus amygdalus	almond
Rubiaceae	Coffea cenephora	coffee
Rutaceae	Citrus sps.	citrus fruits
Solanaceae	Lycopersicon esculentum Nicotiana tabacum Nicotiana sylvestrus Solanum tuberosum	tomato tobacco potato
Umbelliferae	Daucus carota	carrot

* See Nabors, 1976 for references

discussed earlier our laboratory has produced highly NaCl-tolerant tobacco plants from mutant cell lines,and greenhouse testing has shown this trait to be inheritable in the F_2 generation.

No matter whether the source of mutants is tissue culture or traditional sources, tissue culture methods can substantially shorten the time required for introduction of new varieties to growers. For example, if a single drought tolerant plant is located in the field by a breeder,a period of years would normally be required before sufficient seed could be acquired for field introduction. With tissue culture, large numbers of plants could be rapidly propagated in a matter of months.

FUTURE PROSPECTS

Tissue culture breeding offers the possibility of the rapid, economical production of plants resistant to a variety of soil and environmental stresses. Table 4 lists some agriculturally useful mutant phenotypes which might be selected at the cellular stage. For most of these mutants, relatively straightforward selection procedures can be used. For others, the proposed procedures will be modified by continued experimentation. As more knowledge of plant physiology and biochemistry is gained, selection procedures for other agronomically important traits will be developed.

Cell culture techniques have been developed to the point where integration into existing crop improvement programs is feasible. Significant gaps in methodology exist with respect to some major food crops, but for others workable cell culture techniques are available. Tissue culture represents an approach different from that normally taken by plant breeders, but the unique advantages of the method warrant attention. These advantages are briefly summarized in Table 5.

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

Mutant phenotype	Possible/actual selection procedure
NaCl tolerant	Add NaCl to medium
Alkali tolerant	Add alkali to medium
Tolerant of high ionic strength	Increase ionic strength of medium
Resistant to temperature extremes	Grow cultures at temperature extremes
Efficient user of available nitrogen	Reduce nitrogen levels in medium
Increased nitrate reduction	Include inhibitors of nitrate reductase 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 (only for selected diseases)
Inhibitor or herbicide tolerant	Add normally inhibitory amounts of compound
Efficient photosynthesis	Omit carbon sources from medium
	Add photosynthetic inhibitors to medium
Decrease photorespiration	Supply glycolate as the carbon source
Increased levels of certain amino acids	Add amino acid analogs to medium

*In both corn and wheat the total grain yield of specific cultivars has been correlated with nitrate reductase activity (Johnson et al., 1976).

TABLE 5. Advantages of tissue culture breeding

1) Millions of potential plants can be grown in a single flask. Rare useful plants can thus be cloned rapidly.

2) Selection of mutants is economically performed in single flasks. Large commitments of field space and labor are unnecessary.

3) Selection results in a population which consists of 100% mutant individuals. Each cell can potentially be regenerated into a whole mutant plant.

4) Single alleles can be rapidly added to a specific cultivar thus further enhancing its agricultural usefulness.

5) Many types of mutations can be selected.

6) Mutations not found in nature in whole plants can be obtained.

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