FRIDAY MORNING SESSION

September 4, 1969

VICE-PRESIDENT BRIGGS: After our tours all day yesterday we will again resume our technical sessions. Our first moderator today will be Dr. J. Harold Clarke, Clarke Nursery, Long Beach. Washington. Dr. Clarke:

MODERATOR CLARKE: The first speaker on today's program will be Dr. Jean P. Nitsch, Director of Laboratoire de Physiologie Pluricellaire, Gif-sur-Yvette, France. He will talk to us about his most interesting work on plant reproduction at the cellular level under aseptic conditions. Dr. Nitsch:

PLANT PROPAGATION AT A CELLULAR LEVEL: A BASIS FOR FUTURE DEVELOPMENTS

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Plant biologists have become convinced that each of the numerous cells which form a plant contain the complete genetic information necessary to reproduce a similar individual. Thus, if we could isolate a cell from either the root, the stem, or the leaf of that plant and find a way to make it grow, it would develop into a new plant exactly like the stock plant from which the cell had been isolated.

POSSIBILITIES OFFERED BY SINGLE CELLS

What would be the advantages of such a system? Essentially two main types of application are possible, one concerned with the *multiplication* of a given sport or variety, the other with the *modification* of the hereditary traits of that variety.

(1). *A means to multiply plants.* The culture of cells, and the subsequent development of each of them into a new plantlet apparently allows rapid multiplication of a single sport into millions of copies which would be like the original. One could thus multiply an outstanding plant of carrot, asparagus, lily, orchid, etc.

(2). *A means of modifying plants.* The technique of cell culture could also be used to change certain heritable characters such as height, shape of leaves, shape, size and color of flowers, etc. Various possibilities may be used for this aim, namely: mutation of certain genes, changes in the number of chromosomes, even the mixing of cells of different individuals.

> (*a*) *Cell mutation* — Once cells are well separated and plated on agar media in Petri dishes, it is possible to treat them with chemical mutagens or to irradiate them. Some of these cells will mutate. If per-

suaded to divide and to regenerate a whole plant, these mutants will have the advantage of being spotted easily and to consist entirely of mutated cells. This is not the case when one irradiates the apex of a plant: an apex contains many cells of which only a few may mutate, so that one obtains a mixture of mutated and normal cells. Another drawback in the use of whole apices lies in the fact that the normal cells may outgrow the mutant ones, which leads to the loss of the mutant character.

(*b*) *Change in chromosome number* — The chromosome number of a given cell may be changed either by being multiplied by a given factor (polyploidization) or by having a few chromosomes added or lost (formation of aneuploids). Formation of polyploids (such as tetraploids) can be brought about by applying colchicine, or by the process of *endomitosis* which occurs when undifferentiated calli are grown *in vitro*. Aneuploids may also be formed during the culture of such calli.

(*c*) *Mixing cells of different individuals* — In the past, certain graft-hybrids have become famous; for example, *Cytisus adami*. A French horticulturist, Mr. Adam, grafted *C. purpureus* with purple flowers onto *C. laburnum* with yellow flowers. From the region of the graft union a shoot arose which had regions which looked like *C. purpureus*, others which looked like *C. laburnum*. It was actually a chimera, that is, a mixture of cells of the two types. It could be perpetuated by grafting, although some branches tended to revert to one type, others to the other type. This example shows that cells of two different species can grow as a mixture and form organs such as stems, leaves and flowers, which may be composed partly of cells of one partner, partly of cells of the other, each cell retaining its genetical characters.

Instead of using grafting techniques, one could think of *mixing* cell suspensions obtained from two different plants in an effort to produce buds that would contain cells of both and give rise to composite plants. In fact animal biologists have obtained muscle tissue composed of chicken and rat or rabbit cells. Why should it be impossible to mix cells of a rose bush with those of an apple tree?

One could go even further and attempt to *fuse* two cells into one. Such a prospect seems dim since plant cells are surrounded by rigid cellulosic walls. However, it has recently been possible to digest these walls with enzymes and to obtain "protoplasts". A protoplast is the content of a cell without its cellulosic wall; that is, actually a mass of cytoplasm containing

the nucleus. As soon as they become liberated from their envelopes, protoplasts take a spherical shape. Of course, they are very fragile and may burst easily if not surrounded by a medium with the right osmotic pressure. Under appropriate conditions, protoplasts may remain alive for several days. The nucleus may divide, and a cell wall regenerate. Fusion of two protoplasts may be attempted before wall regeneration takes place. Although fusion of the nuclei in addition to that of two cytoplasms may be much more difficult to achieve, the mere presence in the same cell of two nuclei endowed with different genetical potentialities may already lead to changes in the properties of that cell.

TECHNIQUES OF CELL CULTURE

The process of producing whole plants from single cells comprises at least three main steps: (1) separating the cells, (2) growing the separated cells, and (3) causing the formation of buds and roots.

(1). *Methods for separating the cells.* Among the techniques devised for getting the cells apart, the three following ones seem most promising at the present time:

(*a*) *Mechanical separation after tissue culture* — This technique — the oldest and most widely used at the present time — consists in obtaining first a callus culture from the species which is to be used; a fragment of the cambial region of a root or a stem, or of the apical meristem in the case of monocots. This is grown on a standard mineral medium (2,7,15) enriched with 2% sucrose, an auxin such as naphthalene -1-acetic acid (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D) at concentrations ranging from 0.1 to 1 mg/1, certain vitamins such as thiamin (1 mg/1) and *myo*-inositol (100 mg/1), and a cytokinin, for example benzyladenine (0.1 mg/1). The pH is adjusted to 5.5 with dilute hydrochloric acid or potassium hydroxide solutions, and the medium may be solidified with 0.8 to 1% agar. Naturally one has to operate under aseptic conditions, which means that the medium has to be sterilized in the containers (generally test tubes), by autoclaving at 110° C for 15 minutes. The plant parts are disinfected by plunging them in 70% ethanol for a few seconds and immersing them for 15 minutes in a filtered solution of 7% calcium hypochlorite in water. After rinsing them with sterile (autoclaved) water, one cuts out pieces from the inside of the plant fragments with tools which have been disinfected with ethanol.

Once planted in the test tubes, the cultures proliferate, giving rise to undifferentiated cell masses. If

the auxin level is sufficiently high, the cells do not adhere strongly one to the other, so that they can be shaken loose when placed on a shaker in a liquid medium. The liquid containing free cells can be filtered under aseptic conditions through sieves of appropriate mesh sizes in order to remove the clumps and select single cells of similar caliper.

The method just described requires the preliminary raising of callus cultures, which means a loss of time. In addition, the treatments given during the callus phase may affect the growth of the single cells derived from the calli in a manner which may be difficult to control. For example, the extent of endopolyploidy which may occur during the callus phase depends upon the nature and concentration of the auxin and cytokinin used, the length of the period during which the culture has been growing, as well as the physical constitution of the medium (solid or liquid).

(*b*) *Mechanical separation of leaf cells* — A new technique, developed in our laboratory by Rossini (14), eliminates the drawbacks of the previous method. It consists in taking leaves which — after proper disinfection — are ground lightly in a "Potter" homogenizer in the presence of the liquid medium in which the cells are to grow. The tissue is disrupted, but — with some species at least — the cells are not broken. The grindate is then filtered through sieves of appropriate mesh sizes, and the fraction with single cells plated on nutrient agar in Petri dishes. Among the species which yield single cells in this manner are bindweed (*Calystegia sepium*), peanut (*Arachis hypogea*), apple, pear, blackberry (*Rubus*), etc. However only the two first-mentioned species have produced cells which have divided freely. Paradoxically, it is the mature leaves, rather than the young ones, which give the best results.

(*c*) *Use of enzymes* — Pectinases are capable of digesting the pectic cement which holds young cells together, at least those of young leaves. Japanese workers have reported success with this method in dissociating the cells of young tobacco leaves. Further experiments are necessary in order to know if this procedure may be generalized to other species and, also, if the enzymically dissociated cells are capable of dividing, once plated on nutrient agar. Work of this nature is in progress in our laboratory.

(2). *Methods for growing cells.* Once cells have been separated from each other, the next step consists in getting them to grow and divide. Here a particular situation has to be taken into consideration. If a cell isolated from the others was plac-

ed in too big a volume of medium, it seems as if the cell was loosing some important factors — leaking out of it, as it were. Such a cell fails to divide and finally dies. Muir and co-workers (6) have devised a technique which consists in placing an isolated cell on an actively growing callus, separating it from the callus cells with a piece of moist filter paper. The isolated cell, nourished by what the callus cells give off, divides and forms a new callus. Other authors have found that suspensions of separated cells which would not grow in a given medium, can grow in the same medium if other cells have grown in it previously. These first cells apparently have changed the properties of the initial medium, possibly by diffusing some important substances in it; such a medium is said to have been "conditioned".

However, a conditioned medium is not needed if one inoculates a new medium with the right proportion of cells, for example of cells obtained by grinding leaf tissue of *Calystegia sepium* or *Arachis hypogea.* Apparently these cells condition the medium one for the other, if the distance between the cells plated on nutrient agar is not too great.

Numerous formulae have been devised for nutrient media. At first, the addition of natural extracts, such as coconut milk or yeast extract, was recommended. In most cases, however, it is possible to omit them, provided a strong auxin (such as 2,4-D) and a cytokinin (such as benzyladenine) are present. An organic source of nitrogen, such as glutamine, asparagine, arginine, or urea, is sometimes beneficial. Mineral salts, sucrose (2%), and vitamins complete the list of ingredients. An example of such a medium is that used by Rossini (14).

(3). *How to grow whole plants.* Once the isolated cells have been stimulated to divide, they grow and develop into an unorganized mass called a "callus". In order to cause this callus to regenerate whole plants one can try to get in motion one of two processes, namely: (a) the formation of buds followed by the eventual rooting of such buds, or (b) embryogenesis.

(*a*) *Bud formation* — Bud initiation in tissue culture requires an optimal level of sucrose (1-2%), a low level of auxin (preferably IAA, between 0.01 and 0.1 mg/1), the presence of a cytokinin (preferably zeatin or isopentenyladenine, between 0.05 and 0.2 mg/1) and adenine (40 mg/1) as a synergist (12). Once buds have been initiated, they may be transferred to a similar medium lacking adenine and the cytokinin and having only about 1% sucrose. IAA (0.1 mg/1) may be added to encourage rooting. Once rooted, the new plants can be transplanted into pots and grown in the greenhouse. Commercially, this method is being used to propagate high-yielding clones of asparagus.

(*b*) *Embryo formation* — Embryogenesis, as strange as it may seem, may also be induced in certain types

of tissue cultures, especially in the case of plants belonging to the Umbrelliferae. Such calli may form thousands of little embryos — that is, bipolar units consisting of a bud primordium attached to a root primordium — if first cultured on 2,4-D (1 mg/1) or naphthoxyacetic acid, as shown by Norreel and Nitsch (13), on a medium containing NH_4^+ ions, as shown by Halperin and Whetherell, (5), and subsequently transferred to a medium having litte or no 2,4-D (4). Like normal seed-embryos, the "vegetative" embryos have cotyledons, but they differ from the former ones in that they are all genetically like the mother plant and form one uniform clone.

Many plants can be raised in this manner from a single carrot, water parsnip, etc. An interesting offshoot of this technique has been the formation of embryoids from the mature endosperm of certain species. The endosperm is a triploid tissue; that is, each of its cells contain a triple set of the basic chromosome number of the species instead of the double set which normally occurs in ordinary cells. The production of buds or embryos from such endosperm tissues may become a new means of obtaining triploid plants.

PLANTS FROM POLLEN GRAINS

Among the techniques which have been developed recently, one is particularly notable; this is a procedure which uses the male germ alone to produce a new plant. In 1966 Guha and Maheshwari (3) in India observed that some of the *Datura* stamens they had planted *in vitro* produced embryo-like structures, and that these structures were haploid. In our laboratory, we tried to stimulate the proliferation of pollen grains of various species and observed that stamens of tobacco produced little plantlets which, once transplanted into the greenhouse, developed into flowering plants which had only half the number of chromosomes (1,8,9). These investigations were pursued actively and led to a clarification of the factors necessary for success.

How to obtain plants from pollen grains — Extensive trials with different types of media in which all known hormone types, (auxins, gibberellins, cytokinins, dormins), nucleic acid constituents, many amino acids, vitamins, sugars, etc. were tried, led to the conclusions that: (1) a combination of an auxin and a cytokinin as used by the Indian (and later by Japanese) workers gave rise to rather undesirable effects such as callus formation, and that (2) simple nutrient media were superior. In fact, a sucrose solution alone, solidified with agar, allows pollen grains to develop into embryos, although development does not

proceed beyond the "globular stage". A complete medium has been devised for various species of *Nicotiana* (8). This medium allows the formation of plantlets as early as 4 weeks after planting in nearly 50% of the anthers.

The crucial factor lies not so much in the composition of the medium as in the stage of development at which the anther is excised from the flower bud. Anthers excised too early, for example at the "tetrad stage", do not lead to embryo formation, nor do anthers excised once the pollen grains have become binucleated. The right stage occurs just before mitosis, when the grain is still a *microspore* containing a single nucleus. This moment occurs in *Nicotiana tabacum* when the tips of the growing petals just reach the tips of the sepals. Flower buds having reached that stage are cut off the plant and sterilized superficially. The anthers are excised and laid horizontally on the nutrient medium, four anthers to a tube (25 mm in diameter). The culture tubes are kept under 16-hour days at 28° C (day) and 20°C (night).

Monoploid plants — Using such a technique, we have obtained thousands of haploid — or monoploid — plantlets, that is plants with only one set of chromosomes: 24 in the case of *Nicotiana tabacum,* 12 in that of *N. sylvestris,* as revealed by counts made from root tips. Some of these plants have been grown to full size in the greenhouse and have flowered abundantly, but, of course, without setting any seed. The size of the flowers was about one-fourth smaller than that of the diploid flowers, and the coloration was lighter.

Geneticists are very interested in monoploid plants because mutations, which generally bring about recessive traits, are immediately visible in such plants. We have obtained several types of mutants by subjecting young plantlets derived from pollen to radiation produced by a cobalt source. Some mutants of *N. N. tabacum* 'Wisconsin 38' had strapped, narrow leaves, others had white flowers (instead of pink ones), or flowers with petals of a different shape (11).

Diploidization through tissue culture — Once one has produced the haploid mutant, one wants to make it diploid again in order to obtain seeds. This can be done by treating the buds with colchicine. We have devised a different technique, which — again — makes use of tissue culture. A piece of the stem or leaf petiole of a monoploid plant is cut off and aseptically planted on a medium which favors callus formation (2,4-D and a cytokinin — such as kinetin).

In the cells of this callus, the process of endopolyploidy takes place; that is, there is a doubling of chromosomes in some cells without the division of these cells into two daughter cells.

When this callus is then transferred to a new medium with a low auxin content and containing zea-

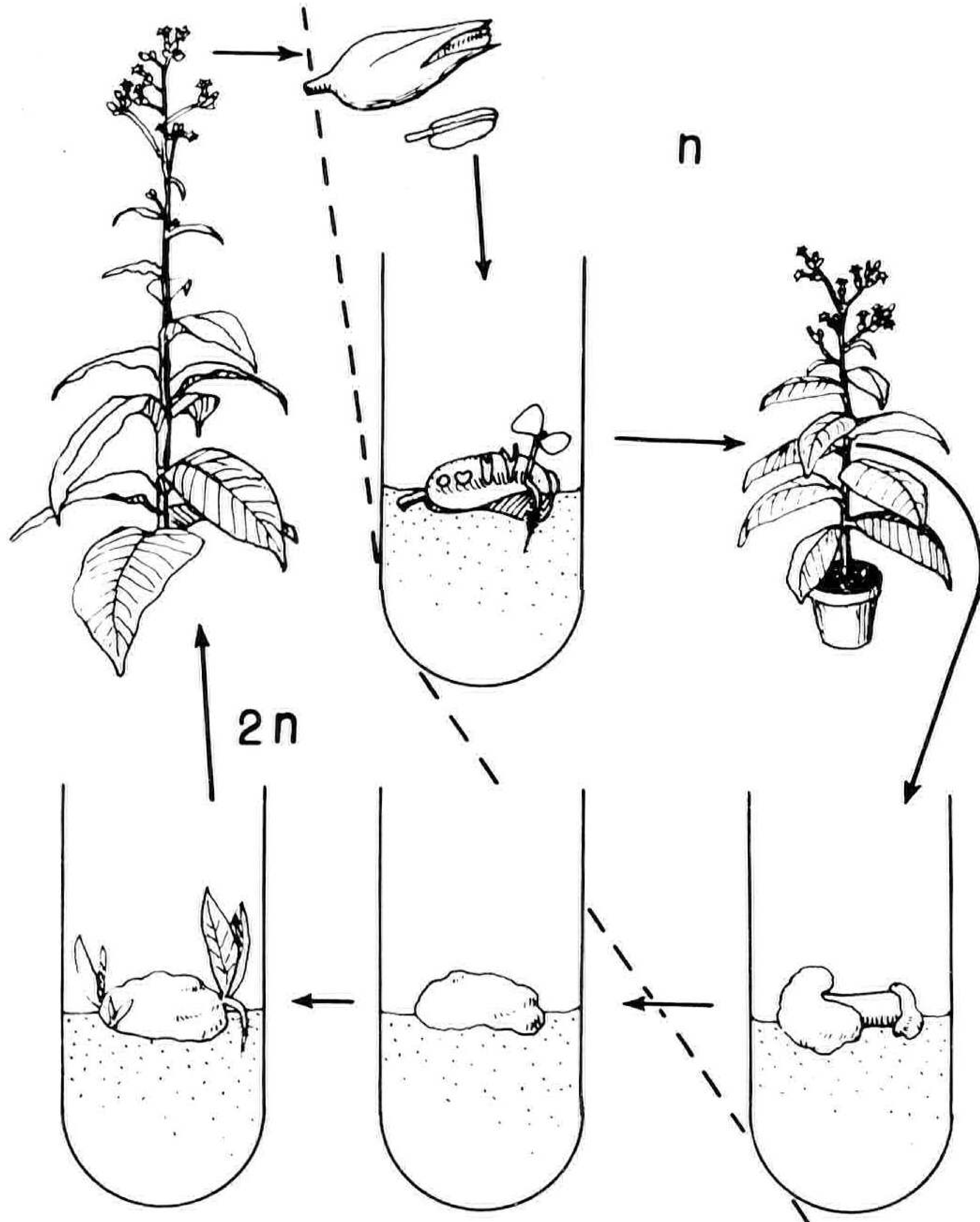

Fig. 1. Procedure for obtaining haploid plants from *Nicotiana tabacum*, then diploid individuals from the haploid ones. A flower bud at the right stage is excised from a flowering plant (*top, left*). The immature stamen is removed and planted aseptically on the proper nutrient medium. Pollen grains at the uninucleated, microspore stage develop into haploid embryos which germinate and form plantlets which are transplanted in pots in the greenhouse. The haploid plants flower abundantly, but do not set seed as they contain only *n* chromosomes per cell. In a second step, stem sections of haploid plants are surface-sterilized and planted aseptically on a nutrient medium which favors the proliferation of a callus (*bottom, right*). This callus can be transferred to the same medium in order to get rid of the initial explant and to let the process of endomitosis produce diploid cells. The callus is then transferred to a new medium which favors the formation of adventitious shoots (*bottom, left*) from which whole plants can be raised, the majority of which are diploid and capable of setting seed.

tin (0.1-0.2 mg/1), plus adenine (40 mg/1) instead of kinetin, buds are produced which now have cells with 2 sets of chromosomes. Once rooted as indicated above, these buds may be transplanted in the greenhouse and raised to form new plants. These plants are now diploid and set seed (10). Thus new mutant strains can be established, as well as non-mutant ones. They are homozygous strains which can be used in crosses in order to bring forth hybrid vigor. Figure 1 summarizes the main steps of our method.

Incidentally, in the course of their culture *in vitro*, certain cells may acquire chromosomal aberrations which may lead to various freaks. Thus, from a red-flowered strain of tobacco, we have obtained plants bearing double flowers with a dark red, velvety appearance. This new strain has been called 'Double Velvet'.

CONCLUSION

The various possibilities which have been presented are at the present time lines of research which present exciting problems to the biologist. They may seem far remote from the daily preoccupations of the plant propagator as they are today. However, as it seems to be the rule, new scientific discoveries sooner or later are being adapted by ingenious minds to yield practical applications. I am confident, therefore, that some of the processes which have been presented here will help, sometime in the future, in creating new varieties and multiplying them rapidly.

LITERATURE CITED

1. Bourgin, J. P., and J. P. Nitsch. 1967. Obtention de *Nicotiana* haploides à partir d'étamines cultivées *in vitro*. *Ann. Physiol. Vég.* 9 :377-382.
2. Gautheret, R. J. 1959. La Culture des Tissus Végétaux. Masson & Cie, E.d., Paris, 863 p.
3. Guha, S., and S. C. Maheshwari. 1966. Cell division and differentiation of embryos in the pollen grains of *Datura* in vitro. *Nature* 212 : 97-98.
4. Halperin, W., and D. F. Wetherell. 1964. Adventive embryony in tissue cultures of the wild carrot, *Daucus carota*. *Amer. Jour. Bot.* 51 : 274-283.
5. Halperin, W., and D. F. Wettherell. 1965 Ammonium requirement for embryogenesis *in vitro*. *Nature* 205 : 519-520.
6. Muir, W. H., A. C. Hildebrandt and A. J. Riker. 1958. The preparation, isolation and growth in culture of single cells from higher plants. *Amer. Jour. Bot.* 45 : 589-597.
7. Murashige, T., and F. Skoog. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol. Plantarum* 15 : 473-497.
8. Nitsch, J. P., and C. Nitsch. 1969. Haploid plants from pollen grains. *Science* 163 : 85-87.
9. Nitsch, J. P., C. Nitsch, and S. Harmon. 1968. Réalisation expérimentale de l' "androgenèse" chez divers *Nicotiana*. *C. R. Soc. Biol.* 162 : 369-372.
10. Nitsch, J. P., C. Nitsch, and S. Hamon. 1969. Production de *Nicotiana* diploides à partir de cals haploides cultivés in vitro. *C. R. Acad. Sci., Paris* 269 (D) : 1275-1278.
11. Nitsch, J. P., C. Nitsch, and M. P. Péreau-Leroy. 1969. Obtention de mutants à partir de *Nicotiana* haploides issus de grains de pollen. *C. R. Acad. Sci., Paris* 269 (D) :1650-1652.

12. Nitsch, J. P., C. Nitsch, L. M. E. Rossini, and D. Bui Dang Ha. 1967. The role of adenine in bud differentiation. *Phytomorphology* 17 : 446-453.
13. Norreel, B., and J. P. Nitsch. 1968. La formation d' "embryons végétatifs" chez *Daucus carota L. Bull. Soc. Bot. France* 115:501-514.
14. Rossini, L. 1969. Une nouvelle méthode de culture *in vitro* de cellules parenchymateuses séparées des feuilles de *Calystegia sepium L. C. R. Acad. Sci.,* Paris 268 (D) : 683-685.
15. White, P. R. 1943. Handbook of Plant Tissue Culture. Jacques Cattel Press, Lancaster, Pa.

MODERATOR CLARKE: We will now hear a review by Dr. Robert Ticknor, of the North Willamette Experiment Station, Aurora, Oregon of work that has been done on the rooting of pine cuttings. Bob.

REVIEW OF THE ROOTING OF PINES

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Propagation of pines by cuttings has received comparatively little attention from horticulturists until recently (13, 19,22) but has been the subject of intensive investigation by foresters at least as far back as 1934 (1). Most of the results reported in this review have been reported since O'Rourke's (19) article in the 1961 Proceedings. Much of the work has been done in non-English speaking countries so that I have had to depend on *Forestry Abstracts* for most of the results reported in this review. Each of the many factors which influence rooting will be discussed separately.

Tree age. Tree age is probably the most important factor in rooting pine cuttings. Watanabe (26) reported 4, 17 and 30 year *Pinus densiflora* rooted 62, 42 and 30% respectively. Kummerow (10), reporting on rooting needle bundle cuttings of *P. radiata,* found average rooting percentages for 1-3 year trees as 24.7%, for 7-9 year trees as 19.5%, and for 28 year trees as none. An even earlier loss of juvenile rooting ability was reported in *P. thunbergiana* by Ogasawara (16) where cuttings from trees 1, 2, 3, 6 and 10 years old rooted 40, 16, 2, 0 and 0%, respectively.

A possible explanation for this observed decrease in rooting with tree age is contained in reports on content of indoleacetic acid (IAA) and growth inhibitors in pine trees. Yim (31) studied the growth substances in the terminal buds of *P. rigida* 1, 10, and 17 years old. Concentrations of IAA were highest in 1 year and very low in 17 year trees. In 10-year trees, IAA was highest in the buds in the lowest third of the crown. Better rooting of cuttings from the lower third of the crown has often been reported (19). Ogasawara (15) found similar results with 1, 2, 8 and 15 year *P. densiflora.* In addition, he found the content of growth inhibitors tended to in-