medium, masses of callus can be formed. This callus is useful for developmental studies of plants from true tissue culture. Herbaceous perennial plants have been developed from callus as *Iris*, *Hemerocallis*, and *Hosta*, in our laboratory. We hope to use callus to propagate large quantities of more woody perennials in a short time. The growth of embryos *in vitro* can be a valuable tool for studies of tissue culture propagation by other methods.

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ADVANCES IN TISSUE CULTURE: RAYFLOWER AND PROTOPLAST CULTURE[1]

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INTRODUCTION

Culture of organs, tissues, single cells and protoplasts has been used to solve many problems including improving propagation time and increasing clones, developing new clones, growth regulator and physiological studies and producing disease-free clones. In our laboratory these techniques have been used for reduction of time for propagation, increasing clones, physiological and growth regulator effects and plant

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improvement of asparagus and freesia; in addition, separating chimeras and protoplast culture are being studied on dahlias. This paper is a preliminary report of our findings, techniques and of investigation underway, together with a brief review of research in tissue culture.

Asparagus: We will discuss asparagus tissue culture in some detail because its development has elements common to tissue culture in many species. Asparagus is a dioecious plant. The yield varies between sexes, male and female plants being morphologically different, and even varies among plants within the same sex (19). Propagation by seed produces populations less suitable for commercial production than asexual propagation, unless male seeds can be readily obtained. However, dividing crowns takes too much time and labor for commercial production. Aseptic culture of asparagus (20,25,30,31,40) appears promising for increasing desirable clones which could be used in commercial plantings.

Various organs and tissues including the meristem (20,25,30,31,40), stem segments with one lateral bud (40), and lateral buds (15) have been cultured successfully on artificial agar media with suitable nutrients, vitamins and growth regulators. Plantlets from callus were eventually transplanted to the field. Our work using lateral bud culture (15) utilizes the following procedures, many of which are common to most tissue culture approaches.

Lateral buds are excised after surface sterilization (20,25,30,31,40) and cultured on agar or liquid medium. Our surface sterilization is a modification of Basile's (3) method. Buds are surface sterilized in aseptic test tubes containing 0.5% NaOCl with a few drops of Tween 20. The solution is moved in and out with a sterile syringe 3 to 4 times for good contact with the tissue, then rinsed in sterile, deionized, distilled water. Three buds are transferred onto 50 ml of modified Murashige and Skoog (24) medium (MMS) in 125 ml Erlenmeyer flasks which were previously autoclaved at 121°C for 20 minutes and the pH adjusted to 5.8 ± 0.1 with either 1N NaOH or 1N HCl after 10 minutes in solution. The tissues are incubated under cool white fluorescent lights of 100 ft-c at 25°C for 16 hours per day.

We studied the effect of auxin and cytokinin on callus formation and organogenesis using all combination (4 × 4 factorial design) of NAA and kinetin at 0, 0.01, 0.1 and 0.5 ppm. We found that both growth regulators play an important role in callus and plantlet formation. The combination of NAA and kinetin at 0.1 mg/l promoted callus formation. If callus was not sub-cultured, shoots and roots would differentiate and form

complete plantlets. NAA and kinetin alone had less effect on callus formation, callus growth and plantlet formation than when in combination (15). Callus could be multiplied by dividing callus into several small pieces before plantlets developed (approximately every 2 weeks) and transferring onto an agar or liquid medium containing 0.10 mg/l each of NAA and kinetin. When liquid medium is used, shaking or rotating provides satisfactory aeration for the tissue.

Plantlets could be induced by directly culturing or subculturing callus on an agar medium for 4 to 6 weeks (Fig. 1). They then could be transplanted to soil after acclimating to the new environment (20,40). An auxin-free medium or incubating under high light intensity (20) before transplanting aided in the success of transplanting. We have transferred asparagus plantlets directly from the flask to moist peat under cool white fluorescent light at room temperature. The first week they are watered with MMS solution without growth regulators, followed by tap water the second week. The plants can then be placed in the greenhouse.

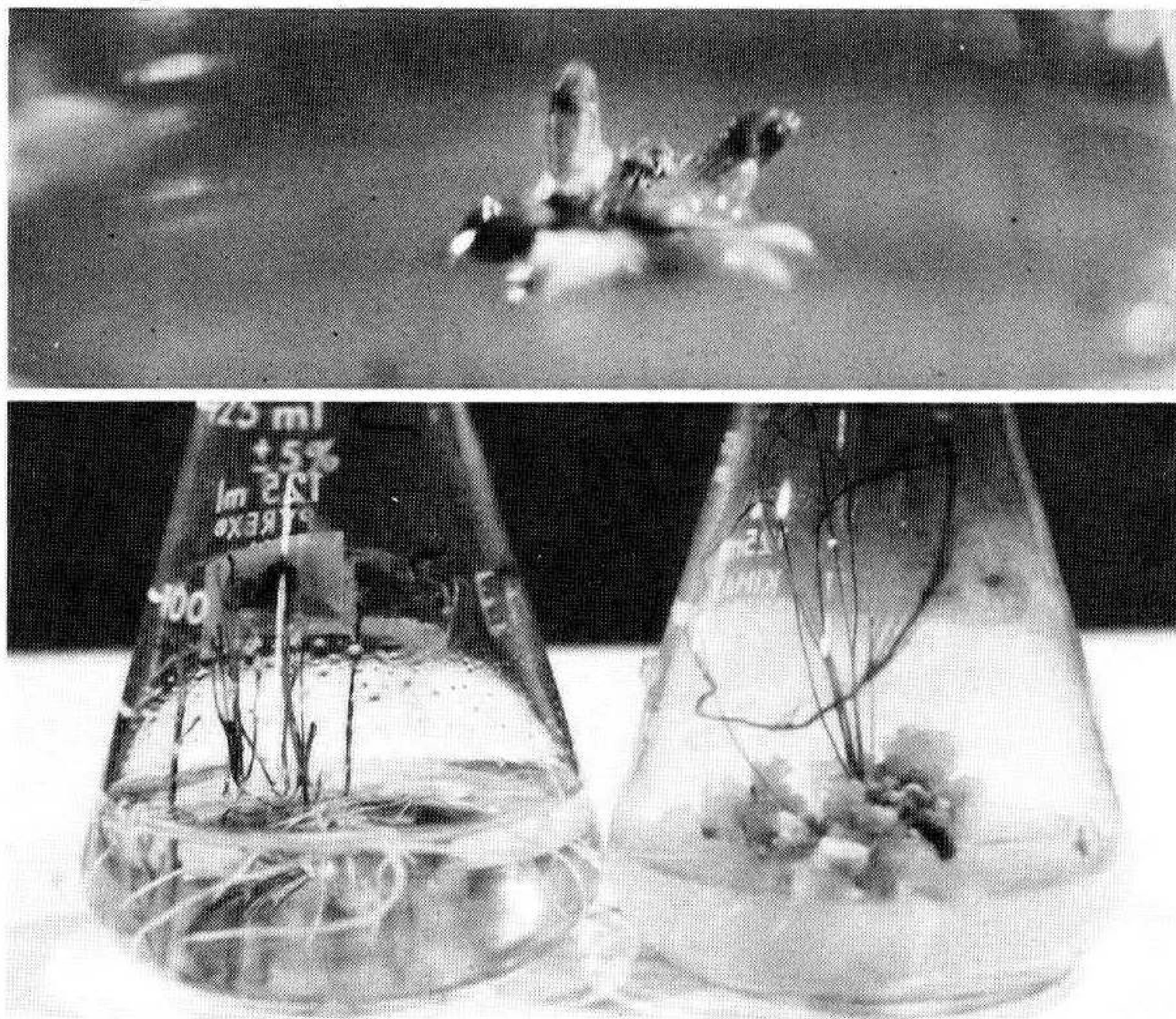

Figure 1. Above: Asparagus plantlets induced from lateral buds cultured on an agar medium.
Below: More advanced plantlets in liquid and agar media (note roots readily visible in liquid medium).

Dahlia: *Dahlia variabilis* L., a species native to Mexico, is a popular garden flower with a wide color range and frequently has natural chimeral mutations which may appear as variegated colors (Fig. 2). Our efforts with dahlia chimeras are aimed at separating chimeras which are evidenced by single cells or cell group differences. In chimeras of this sort the mutated parts cannot be separated as easily as when the mutation affects an

entire organ or part of an organ. Such is the case with variegated flowers and variegated leaves, and we are attempting special methods including surgical techniques and tissue culture to attempt to isolate the components of some of these mutations.

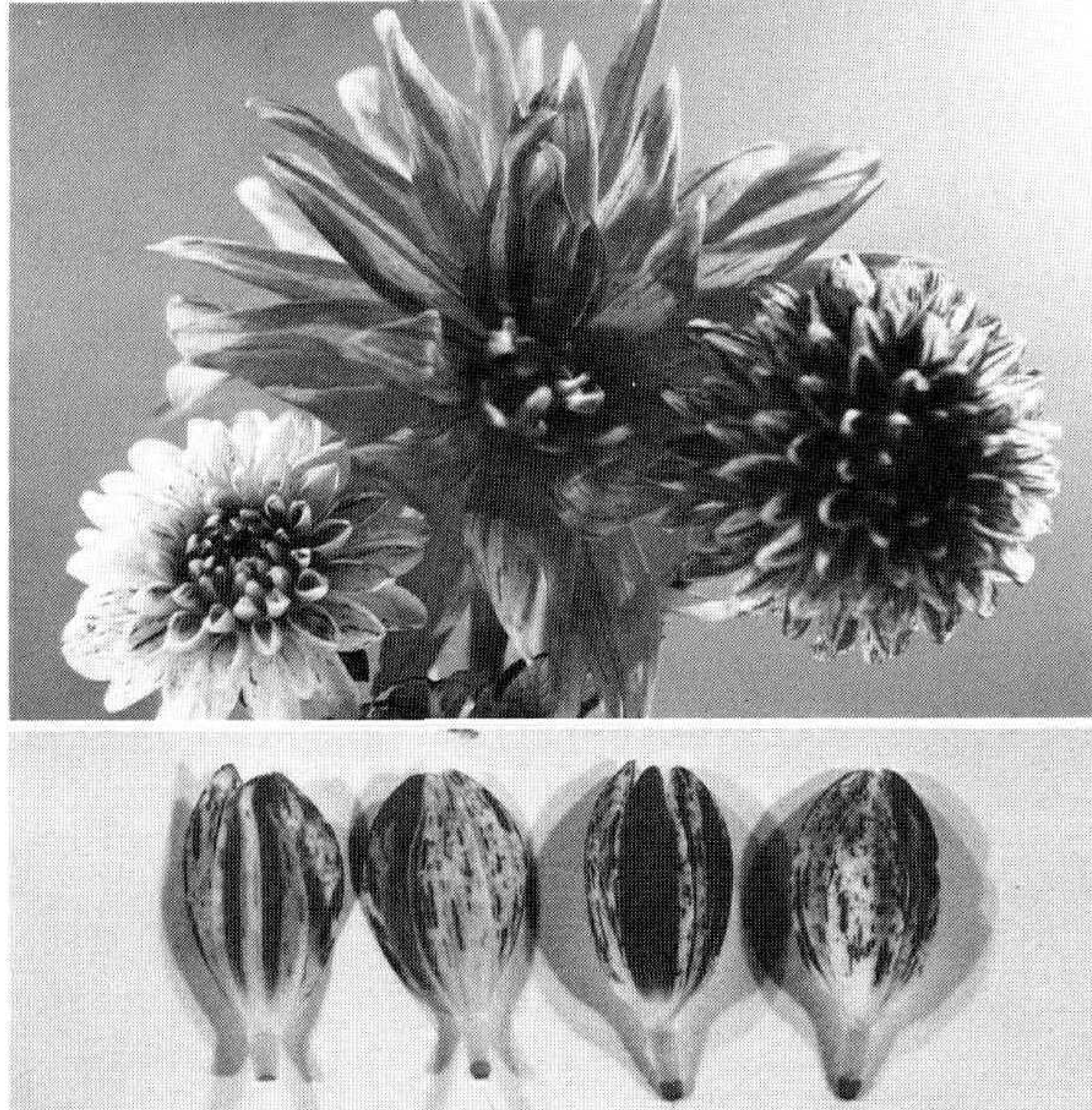

Figure 2. Above: variegated dahlia blooms (heads of ray flowers)
Below: ray flowers removed from a bloom of 'Frosted Plum'.

We are currently attempting this approach with two dahlias, 'Nita' and 'Frosted Plum', which show chimeras occurring in the ray flowers. Fresh sections of ray flowers 30 to 50 microns thick observed under the microscope illustrate that the chimeras occur primarily in the upper and/or lower epidermal layers but do not occur in the mesophyll (middle) layers. Both single cell and cell group chimeras are clearly visible in the sections, the upper epidermal layer usually has more chimeras occurring than the lower epidermal layer. Even though chimeras in plants such as 'Nita' or 'Frosted Plum' do not show variegation in the leaves, it seems possible that the genetic message may be differentially distributed in the leaves. Therefore, our experiments have attempted to use both leaves and ray flowers. Techniques which appear most promising to separate and propagate chimeras include (a) selecting plants from nonselective tissue culture, (b) surgical separation and culture of the mutated tissues *in vitro*, or (c) isolation of single cells and/or protoplasts and subsequent culture.

SELECTING PLANTS FROM TISSUE CULTURE

Leaf epidermal tissue has been demonstrated to differentiate buds and roots in several plant species (5,12,33,34).

When leaf discs were placed onto a suitable medium, callus formed at the cut edge and on the surface of the leaf (33,34). Shoots and roots developed after the callus was formed. The shoot buds were formed from the epidermal cells but the roots originated internally. The shoot and root formation was regulated by auxin and cytokinin levels (5,12,33,34).

Dahlia leaves were prepared using techniques similar to those described for asparagus bud culture (15). Leaf segments 0.5 × 1.0 cm were utilized in a 4 × 4 factorial design of NAA and kinetin added to the MMS high salt medium, as with asparagus, with thiamin HCl 0.5 mg/l, pyridoxin HCl 0.5 mg/l, nicotinic acid 0.5 mg/l, glycine 2 mg/l, myo-inositol 100 mg/l, sucrose 30 g/l and agar 4.0 g/l added. Callus formed initially at the cut surface edge of the leaf segment and subsequently on the surface of the leaf segment after 2 weeks of incubation. Thus callus formation was observed on the medium containing both NAA and kinetin at 1 mg/l. Leaf segments containing midrib tissue formed larger amounts of callus than those without the midrib. Apart from that, there was no effect of selecting the leaf segment from various parts of the leaf, tip, middle, and basal portions responded equally to the growth regulators in the medium. 'Frosted Plum' formed callus earlier and more uniformly than 'Nita'. Roots formed on 'Frosted Plum' leaf segments after 4 weeks but rarely or not at all on 'Nita' leaf segments. No shoots have produced from leaf segment callus of either cultivar, even though a wide range of growth regulator levels have been attempted.

Ray flowers: Ray flower segments and ray flower peels have been cultured successfully in chrysanthemum (5). Bush *et al.* found that the basal portions of chrysanthemum ray flowers were best for callus and plantlet formation. The plants obtained from ray flower culture, whether from segments or peels, were variable and not reproductions of the parent plant. Many different types of plant and flower forms resulted. We have tried to separate chimeras by culturing ray flower segments and peels. These studies have been conducted under both light and dark conditions using procedures similar to those used for leaf segments and asparagus culture with the following modifications. Ray flowers were surface sterilized in 95% alcohol (ETOH) 1.5 min before treating with NaOCl — Tween 20 for 10 min. Tissues were rinsed 3 times with sterilized deionized distilled water and cut into 3 portions, tip, middle, and bottom (not including ovarian tissue) and transferred onto 50 ml of modified Linsmeier and Skoog medium in 125 ml Erlenmeyer flasks. Growth regulators used were all combinations of NAA at 0, 0.3, 3 and 9 mg/l and 6-benzylamino purine (BA) at 0, 0.03, 0.3 and 3 mg/l which were added to the medium before autoclaving and

then placed under conditions of 16 hours light at 100 ft-c or in 24 hours of darkness. Although segments increased in size and began to produce small amounts of callus (rays from open flowers) and large amounts of callus (rays from buds not yet open), no successful plantlet production has resulted to date.

SURGICAL SEPARATION AND CULTURE IN VITRO

The results from our preliminary work showed that the variegation occurred locally in epidermal layers only. Bush *et al.* reported that peels of epidermis of chrysanthemum ray flowers could be cultured and produce plantlets, while shoots and roots from epidermal layers in culture have been recently investigated (33,34). For epidermal layer culture *in vitro* to be successful, sub-epidermal layers must be present (7). The growth regulators used play an important role in organogenesis (7,33,34). We are currently investigating surgical techniques to separate chimeral portions of ray flowers, either using only epidermal layers or epidermal layers with mesophyll layers, using methodology similar to that described for ray flower culture.

ISOLATION AND CULTURE OF SINGLE CELLS OR PROTOPLASTS

Single Cells. If plantlets are obtained from single cells, plantlets usually vary dramatically because of the variation in the location of the cell taken from the parent plant. It is possible that screening these plants could separate chimeral plants from normal plants. Methods of single cell culture have been studied in several plants including asparagus (30,38), sycamore (23) and others. Two main groups of methods have been classified, either mechanical or enzymatic. Mechanical isolation is done by methods such as grinding the tissues (16), microsurgically separating the tissue (2), shaking callus or rotating callus in liquid medium. Enzymatic isolation has been used successfully in some cases (8,32). A more advanced technique has been developed by using EDTA (8), or adding potassium dextran sulfate to the enzyme solution to improve yield of single cells (32). Dahlia callus has been placed on liquid medium (Linsmeier and Skoog) containing 1 mg/l of both NAA and kinetin and placed on a shaker at 100-125 rpm. So far, little isolation of single cells has occurred under these conditions, and our research plans for the future involve selection of different media and utilizing other techniques of isolation of single cells using enzymes such as pectinase.

Protoplasts. Plant protoplasts are intact cells with the cell wall removed. It is difficult to obtain a large amount of protoplasts which are intact and viable. In general, two methods have been used with varying degrees of success for protoplast isola-

tion: mechanical methods and enzymatic methods. Mechanical methods have been relatively complicated and quite time-consuming and therefore are not used to any great extent in modern protoplast culture. However, historically, mechanical methodology was the first technique used for obtaining satisfactory protoplasts for culture. Before 1960, mechanical methods were the simplest and most commonly used. The tissues were first incubated in a hypertonic solution leading to plasmolysis (shrinking of the cells from the cell wall). Protoplasts became spherical in shape when they shrunk from the cell wall, and then when the tissues were cut with a scalpel or sharp razor blade the protoplasts were released. A mini-pipet was utilized to collect the undamaged protoplasts under a microscope. This series of steps was called the micro-surgical process (16,37). Large amounts of protoplasts were damaged during the cutting and collecting but the techniques are reported to be successful using parenchyma or epidermal cells (6,37).

Enzymatic Method. After 1960, Cocking (9) introduced the enzymatic method using crude fungal cellulase, an enzyme which dissolves cell walls. Purified cellulase became commercially available after 1968 which stimulated much research in protoplast culture. A relatively large amount of protoplasts can be obtained from a small amount of plant material in a short period of time using the purified enzymes. A million protoplasts can be readily obtained from 1 gr of leaf tissue (10,11,21). The enzymes can harm protoplasts but cleaning the protoplasts several times with washing solution solves this problem (10,27). The enzymes, cellulase and pectinase, can be used separately or in combination (8,9,10,11). Successful enzymatic isolation is dependent on several factors, including use of an osmotic stabilizer, the right combination of enzymes, or suitable sequential timing of incubation, and the right kinds of enzymes (10,11). Cocking (9,13) has pointed out that the level of osmotic stabilizer has to be high enough to prevent the majority of protoplasts from bursting once the cell wall is weakened and yet not be so high that irreversible damage is done to the protoplasts.

It is apparent that the cells have to be plasmolyzed in early stages of enzymatic digestion. Several researchers have reported successful protoplast isolation by using high concentrations of osmotic stabilizer (13,21,28). Tribe (35) reported that the possible damaging effects to the protoplasts associated with enzyme incubation may be reduced if the cells are plasmolyzed but the cell functions may be adversely influenced (18). Shepard (29), showed that a large quantity of tobacco leaf protoplasts could be isolated under low osmotic stabilizer (0.25 M sucrose). Thus, the osmotic stabilizer appears to be very important for good protoplast isolation.

In our preliminar work on dahlia, the ray flowers and leaves were cut into small strips (0.5-1 mm), placed in solutions containing 0.1% macerozyme R-10), 0.4% cellulase R-10[3] and 0.30, 0.35 or 0.7 M D-mannitol, and incubated for 18 to 21 hours in the dark at room temperature. The osmotic value of the tissue was found to be approximately 0.36 M. We were able to obtain large amounts of good protoplasts in 0.35 M solutions (isotonic) while bursting protoplasts were observed in 0.30 M solutions (hypotonic) and abnormal conditions (systrophae) were observed in 0.7 M solutions (hypertonic). These results would corroborate the belief that for isolation of adequate numbers of good protoplasts it is important to have the osmotic stabilizer at the proper level.

The kind of osmotic stabilizer is also important. Mannitol, sorbitol (10,17), sucrose (28,29), and polyethylene glycol (10,36) have been used successfully. Mannitol is suitable because it is not readily metabolized or taken into the cytoplasm (13,14) while sorbitol is more soluble. Sucrose has been used, but it is difficult to filter sterilize and can be detrimental to protoplasts if used in high concentrations (13,14). The osmotic level and kind of osmotic stabilizer are important not only in isolation but also in culturing the protoplasts. Wallin and Erikkson (38) have shown that the osmotic pressure of the culture medium is of great importance for a good yield of dividing and growing protoplasts. For example, sorbitol at 0.2 M was the optimum concentration for carrot protoplasts to grow and divide while sorbitol at 0.5 M or higher inhibited protoplast division. Sucrose inhibited carrot protoplast growth and division in parallel experiments, indicating the importance of the kind of osmoticum used.

Our current research involves attempting to study the relationship between osmotic level of the isolation solution and the culturing solution together with the osmotic ground value of the tissues which are being used. We also anticipate using this technique as a possible tool for separation of chimeras, since plantlets derived from protoplasts should be individuals representing different characteristics, depending upon from what part of the plant the protoplasts were extracted. This could result in obtaining many totally new kinds of plants.

Freesias. *In Europe, freesias rank 4th among all cut flower* sales. They are normally propagated by corms and by separating cormlets from the mother corm. Propagating freesia by natural corm multiplication is relatively slow and it was considered appropriate to pursue tissue culture methods to hasten multip-

[3] Macerozyme R-10 and cellulase R-10 from Kinki Yakult Mfg. Co., Ltd. 8-21 Shingikan-cho, Nishinomiya, Japan.

lication. Most freesia organs are capable of regenerating roots and shoots (1); organogenesis was dependent on at least three factors: cultivar, light and dark, and auxin to cytokinin ratio (26). Tissue culture techniques show promise of producing a large number of plants in a short time. However, it has taken 16 weeks to produce plantlets (26) which is not as efficient as desired for commercial production. We have attempted to reduce the time of propagation and using aerial and basal corms.

Aerial corms were sterilized in 95% ETOH for 2 minutes and then in 0.5% NaOCl — Tween 20, rinsed 3 times with sterilized deionized distilled water and sectioned transversely into thin pieces about 1 to 2 mm thick. Three sections were randomly transferred onto 50 ml MMS medium as described previously for asparagus tissue culture. NAA and kinetin levels were employed at 0, 0.05, 0.1 and 1 mg/l respectively. The tissues were incubated in growth chambers at 25°C and with no light. Roots and shoots were both formed within 4 weeks of incumbation (Figure 3). NAA at 0.05 to 0.10 mg/l combined with kinetin at 0.05 mg/l promoted roots, while combinations of NAA at 0.1 mg/l with kinetin at 0.05 mg/l was the best balanced medium for shoot and root formation. Higher NAA (1 mg/l) or kinetin at 5 mg/l promoted callus formation but few roots and shoots.

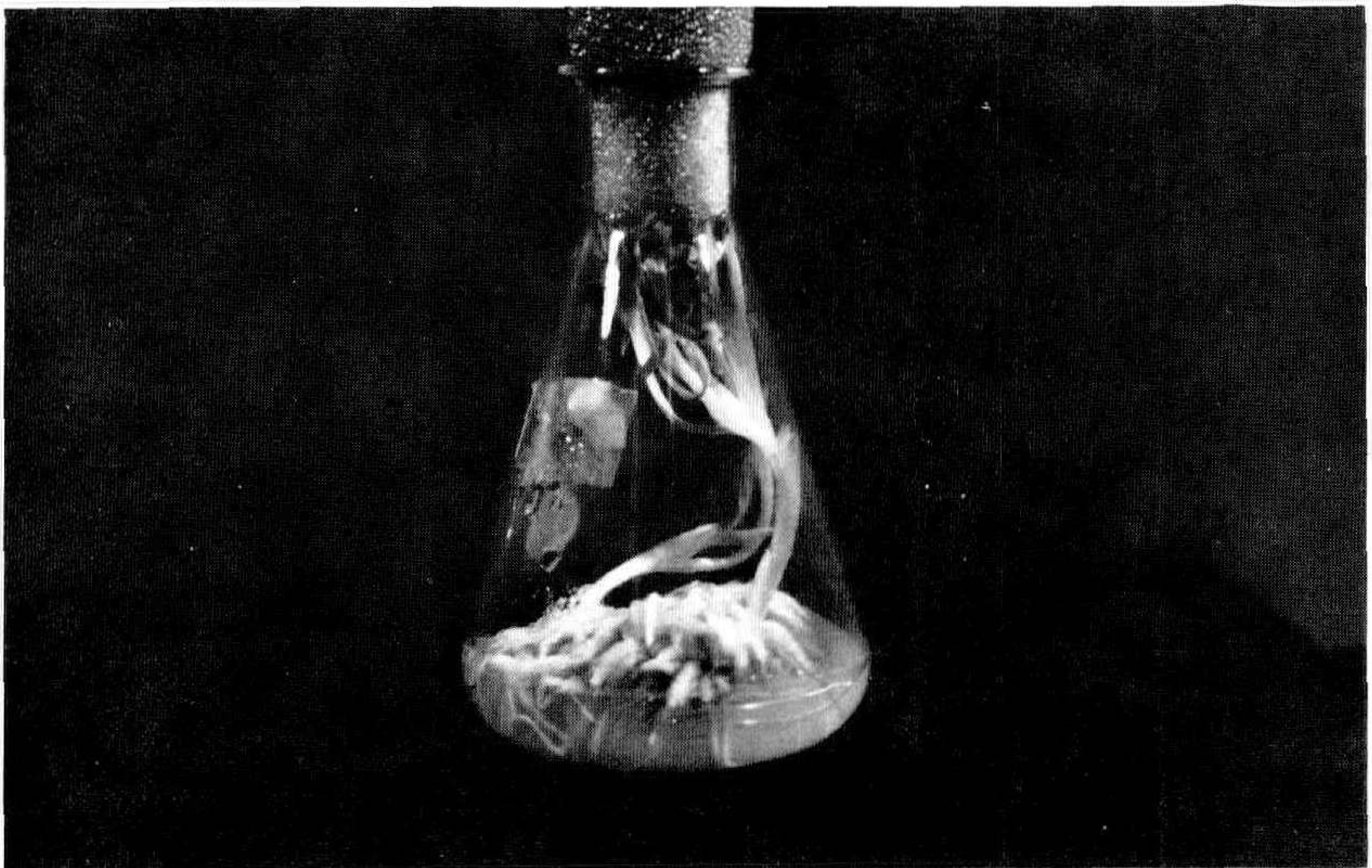

Figure 3. Plantlets of freesia derived from pieces of aerial corms.

After shoots and roots were formed they were incubated 1 to 2 weeks under cool white flurorescent lights of 16-hour duration and 100 ft-c intensity. The plantlets were transplanted to moist peat and placed under cool white fluorescent lights at room temperature. Success of transplanting was dependent on

plants having root to shoot vascular connections. They were then watered with MMS nutrient solution. Tap water was used after the first week. Plants were transplanted to soil and placed in the greenhouse within 7 to 8 weeks after transferring. By this method of transplanting, plants from the flask could be established in the greenhouse 10 to 12 weeks after culture was begun. This represents a reduction of 4 to 6 weeks when compared to published work on freesia tissue culture (26).

We plan to continue research in the areas described, in an effort to improve propagation time, efficiency and to perhaps develop potential new clones.

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CELL-FUSION HYBRIDS

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Abstract. Cell-fusion hybrids were obtained by fusing protoplasts of *Nicotiana glauca* and *N. langsdorffii* in the presence of polyethylene glycol. The hybrid protoplasts were selected out of a mixed population by growing on a culture medium that does not support the growth of parental protoplasts. The cell fusion hybrids had chromosome numbers that were higher (56 to 64) than in the amphiploid ($2n = 42$). Most of these "hyper-aneuploids" were fertile and their progeny retained the characteristic morphology and approximate chromosome number of their hybrid parent.

The technical advance of being able to remove plant cell walls in order to produce viable wall-less or naked cells (the protoplasts) has opened up new avenues of research in plant propagation and improvement. These fall into two main categories of use: 1) fusion of protoplasts to give hybrid somatic cells, thus by-passing the usual sexual techniques of hybridization by cross-pollination; 2) facilitated entry or more rapid uptake of "genetically informed" particles that are generally excluded by the cell wall; for example, foreign macromolecules (DNA and RNA), chromosomes, nuclei, organelles and viruses. The genetic significance in all these cases is that new additional genetic material migrates into the protoplasts and, in so far as it persists through replication and integration, adds significantly to the genetic variability (8).

The successful production of cell-fusion hybrids has been greatly aided by two additional new techniques for handling protoplasts. These are: 1) the use of polyethylene glycol (PEG) to adhere and fuse protoplasts; and 2) the development of selection methods to recover preferentially regenerated fused hybrid protoplasts from a mixed population of protoplasts.