Propagation of *Chrysanthemum in Vitro*. I. Multiple Plantlets from Shoot Tips and the Establishment of Tissue Cultures[1,2]

E. D. Earle and R. W. Langhans[3]
Cornell University, Ithaca, New York

Abstract. Small (0.5 mm high) shoot tips of *Chrysanthemum morifolium* 'Giant #4 Indianapolis White' were grown on Murashige-Skoog medium containing various levels of kinetin, NAA, and GA_3. Formation of roots, single or multiple shoots, plantlets and friable, hard or leafy callus depended on the hormone levels used. Multiple shoots and green leafy callus were produced on medium containing 2.0 mg/l kinetin and 0.02 mg/l NAA. The leafy callus was suitable for subculture and subsequent reorganization of plantlets. Multiple shoots were rooted and grown into normal plants or were used to start new cultures which formed more multiple shoots. This technique will be useful for storage and propagation of *Chrysanthemum* and especially for detection and rapid multiplication of virus-free plants.

Plantlet production from tissue cultures of shoot tips is now possible in many genera including carnation (4), geranium (11), gladiolus (13), orchid ((8) and many others), tobacco (15), and *Chrysanthemum* (2). In our experiments with *Chrysanthemum* cultures, we found that the shoot tips used to start the cultures were capable of a range of interesting responses, including formation of multiple plantlets directly from a single shoot tip. Techniques which produce one plant from one meristem have been widely used horticulturally to obtain virus-free plants (6); production of many plants from one tip without a period of subculture in a partly differentiated form is less familiar (9). This paper describes the growth of small *Chrysanthemum* shoot tips on defined media with varying hormone levels and gives the procedures for plantlet formation and establishment of tissue cultures.

Materials and Methods

Stock plants of *Chrysanthemum morifolium* 'Giant #4 Indianapolis White' were grown under long-day conditions in a greenhouse. One to 4 weeks after pinching, the new axillary shoots were removed, and the basal ends promptly put in a beaker of water. Leaves longer than 5 mm were removed by hand.

Explants for culture were prepared from the shoot tips under a binocular microscope (25X) with dissecting needles and scalpels with #11 blades. Tools were sterilized by flaming after an alcohol dip but the shoots themselves were not surface sterilized. The leaves, including those arching over the apical dome, were broken off with the tools. The sides of the exposed tip were lightly scraped but not cut, and a horizontal cut severed the explant from the shoot.

The explant consisted of the apical dome, either bare or surrounded by 1-2 of the youngest leaf primordia, and a conical piece of subapical tissue stripped of leaf primordia. No attempts were made to grow the apical dome (ca. 0.1 mm high) in isolation. Height of the explants (measured by an ocular micrometer) ranged from a min of 0.2 to a max of 1.0 mm. The usual size was 1.0 mm wide at the base and 0.5 mm high. Except where stated, shoot tip explants were placed on the medium with the apex up.

Media. The basal medium contained the Murashige-Skoog inorganic components (10) plus 0.4 mg/l thiamine HCl, 100.0 mg/l myo-inositol, 30 g/l sucrose and 5 g/l Bacto-agar. Kinetin, α-naphthaleneacetic acid (NAA) and gibberellic acid 3 (GA_3) were added in the amounts stated. The pH was adjusted to 5.6-5.8 before addition of agar. Media were sterilized by autoclaving 15 min at 121°C.

Culture conditions. Two to 6 explants were placed in 100 mm plastic Petri dishes sealed with Parafilm to prevent desiccation. Shoots and plantlets were grown in 6.5 or 8.5 cm screw top vials. Cultures were kept at 24°C under constant fluorescent light of ca. 1000-4000 lux. Plantlets transferred to the greenhouse were potted in 5.7 cm pots in a mixture of

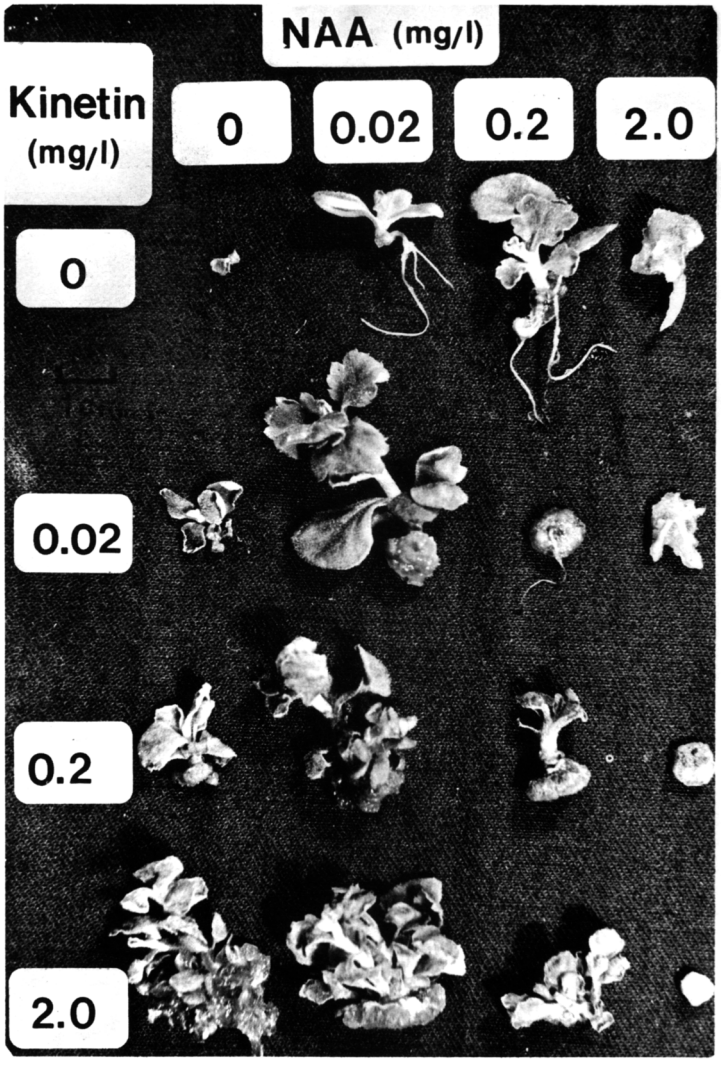

Fig. 1. Shoot tip explants grown 5 weeks on Murashige-Skoog medium with different NAA and kinetin levels.

[1]Received for publication September 25, 1973.
[2]Financial support of the California-Florida Plant Production Corporation, Fremont, CA, is gratefully acknowledged.
[3]Research Associate and Professor, Department of Floriculture.

peatmoss and vermiculite (1:1 v/v) and kept under intermittent mist for 2-4 weeks. They were moved into 12.2 cm pots and grown under long-day conditions until they were 15 cm high. At that time they were given short-day treatment until flowering.

Results

Shoot tips taken from overhead watered plants showed 100% bacterial contamination. All but 3 of the 580 explants from soil watered plants were sterile, even though no surface sterilization was used.

Effects of NAA and kinetin on shoot tip explants. Fifteen to 30 explants were placed on media containing 0, 0.02, 0.2 or 2.0 mg/l NAA and/or kinetin. Figure 1 summarizes the range of responses obtained after 5 weeks. In the absence of NAA and kinetin, only a few very small leaves developed from the top, and there was almost no growth from the base of the explant. With no kinetin and low or moderate levels of NAA, some plantlets or single shoots and increasing amounts of basal callus formed. The highest level of NAA promoted formation of roots and pale friable callus with anthocyanin on the upper surface.

Addition of kinetin with little or no NAA caused formation and growth of leaves, shoots and green basal callus. Root formation was either abnormal or totally suppressed. Explants grown with the lower kinetin levels formed single (occasionally 2 or 3) shoots while those grown with 2.0, 5.0, or 10.0 mg/l kinetin often formed 5 or more shoots and large partially leafy basal callus.

Moderate and high levels of NAA in combination with kinetin reduced root and shoot formation. Explants usually grew into hard green callus.

Shoot tips maintained for a second month in culture showed further development. Adventitious roots usually appeared on shoots that had formed earlier. Vigorous single shoots, probably derived from the original apex, frequently grew up from the center of unorganized hard green callus. Tiny plantlets (embryoids) sometimes appeared in callus produced from small explants.

Effects of GA_3 on shoot-tip explants. Figure 2 shows the results of an experiment in which 10-12 explants were grown with or without 10.0 mg/l GA_3 on media containing either no other hormones or 2.0 mg/l kinetin and 0.02 mg/l NAA. Growth without any hormones was minimal. Shoot tips given GA_3 alone showed definite leaf and shoot development and elongation of the subapical area. Little callus formed. In later weeks of culture, shoot elongation continued, and adventitious roots appeared. In the presence of kinetin and NAA, the effect of GA_3 was less striking but it did enhance shoot development and elongation.

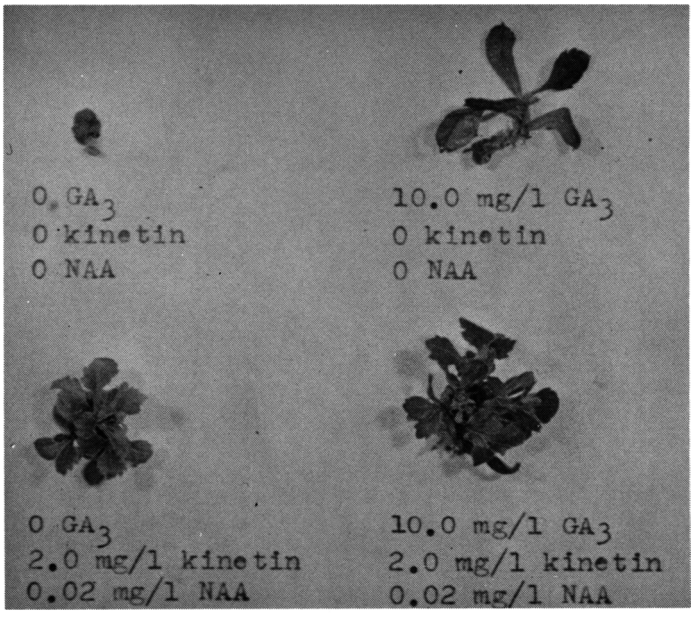

Fig. 2. Shoot tip explants grown 4 weeks on Murashige-Skoog medium with and without kinetin, NAA, and GA_3.

Development on medium containing 2.0 mg/l kinetin and 0.02 mg/l NAA. This medium caused such vigorous callus and multiple shoot formation that it was used for further experiments. Growth of typical explants during the first 4 weeks of culture is illustrated in Fig. 3. Small explants lagged behind; large ones grew more rapidly. In the first week, enlargement from 1 mm to 2-3 mm in diam took place. Dark green areas which developed into shoots, leaves and small leafy areas became increasingly apparent on the top and sides of the explants during the next 2 weeks. The basal callus attained a diam of 10-15 mm. By 4 weeks, shoots started to elongate, and small new leaves were often visible on the basal callus. Transfer of explants to fresh medium in vials allowed further growth of shoots and proliferation of leafy callus.

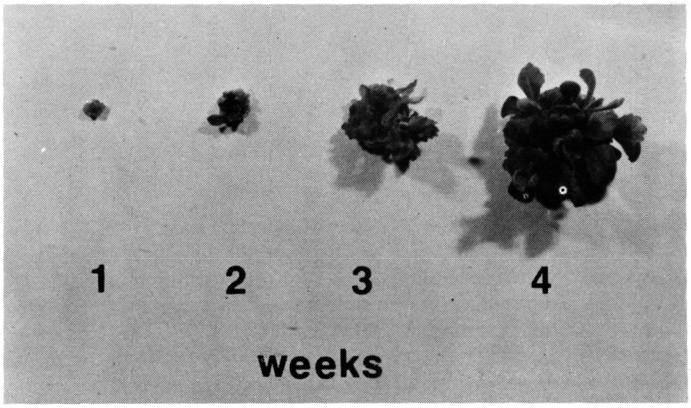

Fig. 3. Shoot tip explants after 1, 2, 3, and 4 weeks growth on medium with 2.0 mg/l kinetin and 0.02 mg/l NAA. X1.

Not all explants formed elongated multiple shoots. Of 119 explants, 65 (55%) formed multiple shoots with notched dark green leaves and leafy basal callus; 54 (45%) formed leafy callus with no shoots. Callus without shoots generally included some clusters of large pale abnormally shaped leaves with tinges of anthocyanin in addition to smaller bright green leaves. Addition of 0.8 mg/l of kinetin and 0.5 mg/l of indoleacetic acid (2) to the basal medium caused similar formation of leafy callus. Multiple shoots developed on 14 of 22 explants.

Placing shoot tips apex down on the agar medium entirely eliminated multiple shoot formation and strongly favored formation of leafy callus with no enlarged leaves (Table 1).

Table 1. Effect of explant orientation on multiple shoot formation.

Orientation	Multiple shoots & leafy callus	Some large leaves & leafy callus	Only leafy callus
Apex up	12	3	0
Apex down	0	3	9

Establishment of tissue cultures. The friable callus that developed on explants grown with 2.0 mg/l of NAA formed cell suspensions in liquid medium of the same composition. Many of the free cells contained anthocyanin.

Tissue cultures used to produce plantlets (as in (2)) were established from more than 20 shoot tips. Small (2-5 mm) pieces of callus with tiny leaves or dark green areas were cut off explants and grown in liquid medium with 2.0 mg/l kinetin and 0.02 mg/l NAA (Fig. 4). Plantlets formed when small pieces of such tissue were returned to agar medium containing kinetin. Details of growth in liquid and the techniques for production of plantlets will be provided in the second paper in this series.

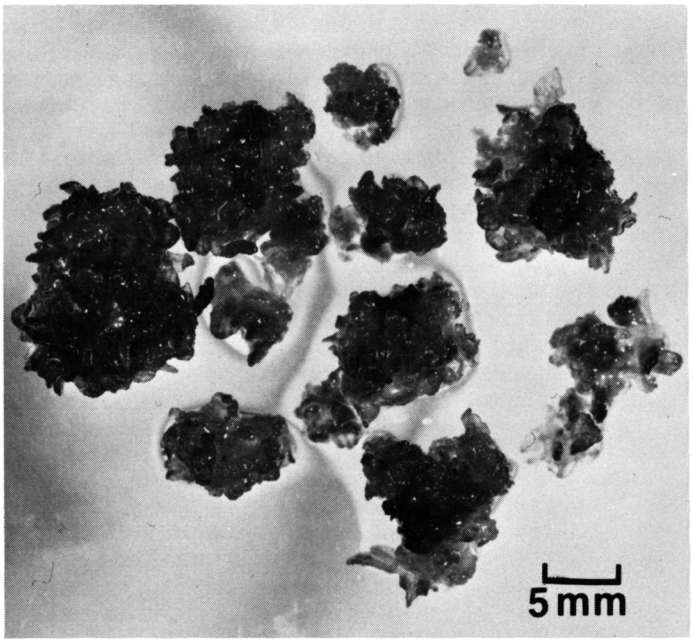

Fig. 4. Partially leafy tissue from cultures grown in liquid medium with 2.0 mg/l kinetin and 0.02 mg/l NAA.

Leafy callus suitable for subculture grew mainly on the outer surfaces of the shoot tips. The inner core of the basal callus consisted of firm unorganized green tissue, which was discarded. Explants whose sides had been trimmed with vertical cuts rather than by light scraping formed less leafy callus, though shoot formation remained good. Similarly small explants with less subapical tissue produced less leafy callus, particularly when shoot formation was vigorous. For subculture, callus without shoots, was easier to handle, however, it was possible to produce both tissue cultures and plantlets from a single meristem tip.

Formation of plantlets from shoot tips. Some plantlets were produced promptly on media containing low levels of NAA; others were formed if roots developed on shoots still attached to basal callus. The quickest way to produce plants from tissue with elongated shoots was to cut the shoots from the callus and place the cut end in medium containing no hormones or 0.02 mg/l of NAA. Under these conditions several adventitious roots with root hairs formed on the shoots in 7-14 days (Fig. 5a & b). Rooted plantlets could either be potted immediately or maintained in vials of basal medium. Some elongated shoots were rooted directly in the greenhouse.

More plantlets were obtained by placing 2 mm tips from shoots formed on explants on fresh medium containing 2.0 mg/l kinetin and 0.02 mg/l NAA, ± 10 mg/l GA_3. Such tips produced 3-6 shoots in 4 weeks (Fig. 5c). Small sections from the nodes of shoots formed on explants also produced new shoots and leafy callus (Fig. 5d).

Growth of shoot tips from other cultivars. Limited attempts to grow shoot tips from other cultivars of *Chrysanthemum* were encouraging. Fourteen explants from 'Fred Shoesmith' plants all produced large numbers of elongated shoots on medium containing 2.0 mg/l kinetin and 0.02 mg/l NAA (Fig. 6). Less leafy callus was formed than on 'Indianapolis' explants, but tissue cultures which produced plantlets were successfully established. Seventeen of 18 explants from 'Deep Ridge' plants also formed multiple shoots and basal callus (again less leafy) on the same medium. Ten plantlets were derived from a single explant within 8 weeks (Fig. 7). Eight 'Indianapolis Bronze' tips responded to media with high levels of NAA or kinetin like 'Giant #4 Indianapolis White' shoot tips.

Growth of plants produced from shoot tips. Eighteen 'Giant #4 Indianapolis White' and 33 'Fred Shoesmith' plantlets or unrooted shoots were moved directly from agar media to pots in the greenhouse. They ranged in height from 0.5 to 7 cm with most between 1 and 4 cm. All but 4 survived. The plants reached the ht of 15 cm in an average of 51 days. Normal white flowers were produced on all plants 7½ to 9 weeks after short days were begun. Four 'Indianapolis Bronze' and 6 'Deep Ridge' plantlets were also potted and grown to normal flowering.

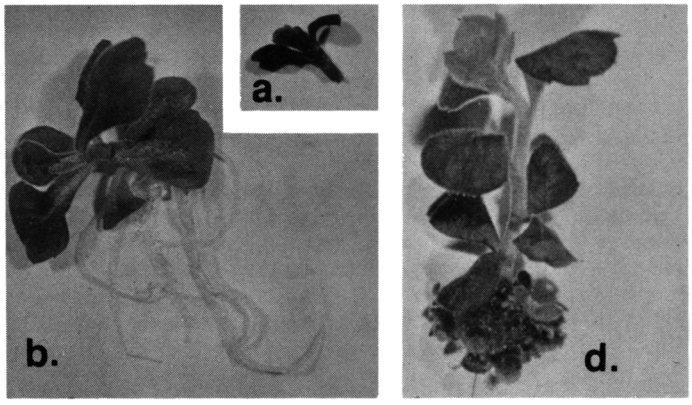

Fig. 5. a. Shoot cut off explant grown 32 days on medium with 2.0 mg/l kinetin and 0.02 mg/l NAA. X1. *b.* Similar shoot with adventitious roots formed after 2 weeks growth on medium with 0.02 mg/l NAA. X1. *c.* New shoots formed by tips taken from multiple shoots. Medium contained 2.0 mg/l kinetin, 0.02 mg/l NAA and 0 or 10 mg/l GA_3. X0.9. *d.* Shoot and leafy callus on nodal section from shoot produced on explant. X1.3.

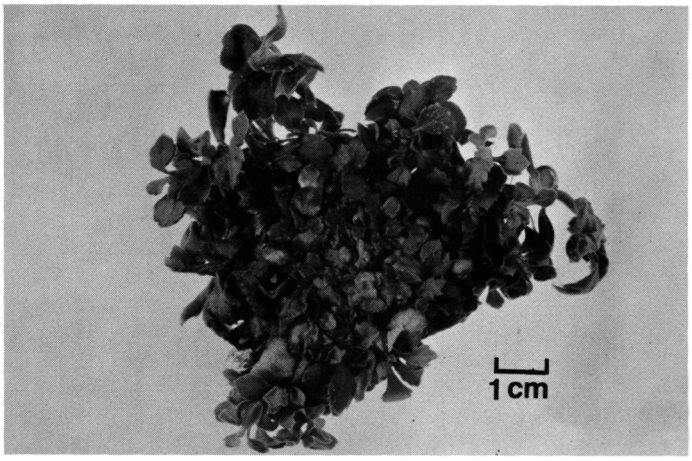

Fig. 6. Multiple shoots produced by 'Fred Shoesmith' shoot tip explant grown 7 weeks on medium with 2.0 mg/l kinetin and 0.02 mg/l NAA.

Discussion

Shoot tip explants formed roots and callus when given high levels of NAA and low levels of kinetin; they formed leaves and shoots when the hormone ratios were reversed. High levels of

both hormones caused the formation of firm unorganized callus. This pattern of growth recalls the familiar response of tobacco pith to the same stimuli (14). However, since the *Chrysanthemum* explants consisted of several types of tissue, changes in hormone ratios may have activated growth of different tissues instead of evoking different responses from a single tissue. Anatomical studies of shoot tips grown on different media are needed to resolve the origin of the new structures formed.

Application of high levels of kinetin to the base of the explant acts as a "chemical pinch," causing the formation of multiple shoots. While removal of leaf primordia in the absence of kinetin did not lead to multiple shoots, leaf excision in combination with kinetin may have facilitated new shoot production. Hackett and Anderson (4) found that carnation meristems from which leaf primordia were removed produced tissue with many short shoots while meristems with intact leaf primordia grew into single shoots.

The stimulatory effect of gibberellic acid on development of explants lacking NAA and kinetin is not surprising because GA is known to promote cell division and elongation in the subapical zone of shoots (12). Moreover, Ball (1) and Morel (7) reported that GA promoted leaf and stem development by isolated *Lupinus* and potato apices. Added GA has no clear cut effect on intact *Chrysanthemum* plants, but compounds such as CCC and Amo-1618 produce marked inhibition of stem growth which can be reversed by applied GA (12). The content or production of GA by very small shoot tips may be too low for growth *in vitro*.

The failure of some 'Indianapolis' apices to form multiple shoots needs further clarification. Both large and small explants were capable of forming multiple shoots. Subtle differences in the preparation of the explants, such as the extent to which leaf bases and epidermal tissue are scraped off or the apex is damaged, may be important. There may also be relevant differences in the physiological state of the initial shoots. The

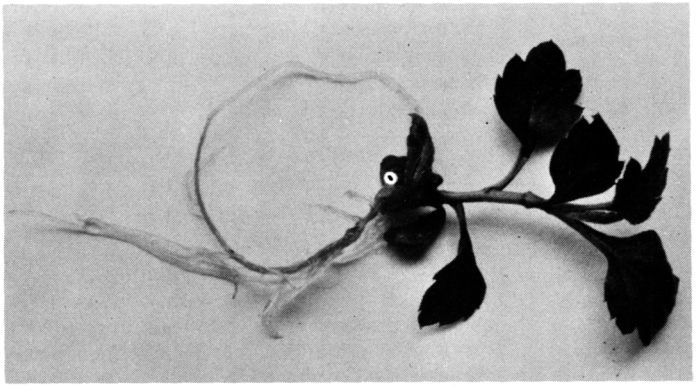

Fig. 7. One of 10 'Deep Ridge' plantlets produced from a shoot tip explant in 8 weeks. X0.9.

fact that 'Fred Shoesmith' and 'Deep Ridge' explants formed elongated shoots much more consistently than 'Giant #4 Indianapolis White' suggests these cultivars are suitable material for propagation via multiple shoots, and indicates that more uniform results with 'Giant #4 Indianapolis White' might be possible. It would be useful to survey more *Chrysanthemum* cultivars to see if the ability of shoot tip explants to form multiple shoots and leafy callus is widespread.

One purpose of this study was to establish leafy *Chrysanthemum* tissue cultures consistently, using fully defined media. Leafy callus suitable for subculture and subsequent reorganization of plantlets was regularly formed when explants prepared as described were placed on media containing 2.0 mg/l kinetin and 0.02 mg/l of NAA. The formation of multiple shoots on many explants grown on this medium suggested an alternate method for *Chrysanthemum* propagation *in vitro*.

Assuming the production of 5 shoots per explant each 4 weeks, excision and subculture of the new shoot tips every 4 weeks and 2 weeks for rooting, about 125 small plantlets would be produced in 3 months, 15,500 in 6 months, and over 200 million per year. Appropriate corrections must of course be made if not all explants form shoots. Hasegawa et al. (5) have made a similar calculation for production of asparagus plantlets from shoot-tips.

While the amount of sterile manipulation involved may make it unrealistic to use this technique for large scale propagation, several applications are both feasible and worthwhile: a) a moderate buildup of plants from new selections could be done in the laboratory under conditions where exposure to pathogens is unlikely. Presence of bacteria and fungi would be quickly seen as contamination in the cultures. b) Plant cultivars not presently needed for propagation could be stored as sterile plantlets in a small amount of space. If plants outgrew their containers, small tips could be cut off and rerooted. Whenever desired, a rapid buildup of plants could be done. c) It is already possible to produce single *Chrysanthemum* plants free of several viruses from tips of infected plants (6). With the procedures described here, it would be possible to obtain many virus-free plants from the same type of explant. Moreover, the culture could be tested for presence of virus, while plantlets were being produced. Viruses in the shoots or basal callus could quickly be detected by the cannula implant method (3). d) The small clonal sterile plants may also be useful for *in vitro* studies of rooting, axillary bud formation, and flowering.

Literature Cited

1. Ball, E. 1960. Sterile culture of the shoot apex of *Lupinus albus*. *Growth* 24:91-110.
2. Ben-Jaacov, J., and R. W. Langhans. 1972. Aseptic clonal propagation of *Chrysanthemum* by meristem tip proliferation. *HortScience* 7:289-290.
3. _____, R. K. Horst, C. A. Geissinger, A. W. Dimock, and R. W. Langhans. 1973. Virus indexing of callus cultures of *Chrysanthemum morifolium* by tissue implantation and mechanical inoculation. *Phytopathology* 63:37-38.
4. Hackett, W. P., and J. M. Anderson. 1967. Aseptic multiplication and maintenance of differentiated carnation shoot tissue derived from shoot apices. *HortScience* 90:365-369.
5. Hasegawa, P. M., T. Murashige, and F. H. Takatori. 1973. Propagation of asparagus through shoot apex culture. II. Light and temperature requirements, transplantability of plants and cyto-histological characteristics. *J. Amer. Soc. Hort. Sci.* 98:143-148.
6. Hollings, M. 1965. Disease control through virus-free stock. *Ann. Rev. Plant Path.* 3:367-396.
7. Morel, G. 1964. La culture *in vitro* du meristeme apical. *Rev. Cyt. Biol. Vegetales.* 27:14-314.
8. _____. 1966. Clonal propagation of orchids by meristem culture. *Cymbidium Soc. News* 20:3-11.
9. Murashige, T., M. N. Shabde, P. M. Hasegawa, F. H. Takatori, and J. B. Jones. 1972. Propagation of asparagus through shoot apex culture. I. Nutrient medium for formation of plantlets. *J. Amer. Soc. Hort. Sci.* 97:158-161.
10. _____, and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant* 15:473-497.
11. Pillai, S. K., and A. C. Hildebrandt. 1969. Induced differentiation of geranium plants from undifferentiated callus in vitro. *Amer. J. Bot.* 56:52-58.
12. Sachs, R. M. 1961. Gibberellin, auxin, and growth retardant effects upon cell division and shoot histogenesis. p. 49-58 *In* Advances in Chemistry, series No. 28.
13. Simonsen, J., and A. C. Hildebrandt. 1971. In vitro growth and differentiation of gladiolus plants from callus cultures. *Canad. J. Bot.* 49:1817-1819.
14. Skoog, F., and C. O. Miller. 1957. Chemical regulation of growth and organ formation in plant tissues culture in vitro. *Soc. Exp. Biol. Symp.* 11:118-131.
15. Walkey, D., and J. Woolfitt. 1968. Clonal multiplication of *Nicotiana rustica* L. from shoot meristem in culture. *Nature* 220:1346.