To the Graduate Council:

I am submitting herewith a thesis written by Angela Dawn Cockrel entitled "*In Vitro* Propagation of Cineraria (*Senecio cruentus* DC.) by Shoot Tip Culture." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Ornamental Horticulture and Landscape Design.

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IN VITRO PROPAGATION OF CINERARIA (*SENECIO CRUENTUS* DC.) BY SHOOT TIP CULTURE

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Angela Dawn Cockrel

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ABSTRACT

Cineraria (*Senecio cruentus* DC.) shoot tips were grown and multiplied *in vitro* on Murashige and Skoog medium (1962) and on the modified Murashige and Skoog medium (1974) containing IAA, kinetin, and organic supplements. Explants were isolated in culture from plants grown from seeds germinated *in vitro*. Germinated seeds produced cotyledons heavily covered with colored trichomes. The early detection of the intensity of flower color was predictable in accordance with color of trichomes on cotyledons. Formation of single or multiple shoots, plantlets, leafy callus and roots depended on the hormone levels and organic constituents used. Shoots cultured on a medium with a high kinetin to auxin ratio produced shoots; whereas, the addition of only an auxin to the medium produced roots and complete plants which could later be transferred to the greenhouse.

The greatest number of shoots per excised shoot tip was obtained when the MS medium was supplemented with adenine sulfate and L-tyrosine, especially when added to the modified MS medium with half the recommended concentration of macroelements. The addition of casein hydrolysate in the MS medium caused shoot suppression and broad thick leaves. Exogenous auxin was not a critical additive to any multiplication media, since shoot tip explants developed plants without an IAA supplement. Shoots originated form axillary and adventitious buds. Root formation

was best on the MS medium containing the supplement casein hydrolysate and half the recommended concentrations of macroelements. Adventitious root growth increased with increasing NAA concentrations in the medium. The addition of adenine sulfate and L-tyrosine to a modified MS medium caused a decrease in frequency of rooted plantlets. The shoots tended to form stubby to no roots with a large percent of callus on the modified MS medium. Rooted shoots grew into normal plants and subsequently flowered after a 23 week period from *in vitro* culture to greenhouse. Growth habits and bloom characteristics of plants originated *in vitro* or in a greenhouse were similar.

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CHAPTER I

INTRODUCTION

The florists' cineraria, Senecio cruentus DC., is an inexpensive pot plant with everlasting, showy flowers. Although its popularity in Europe has grown over the past 40 years, interest in this country has declined, perhaps resulting from a change in public taste.

There appears to be a renewed American interest in the greenhouse cultivation of cineraria. This may be associated with one of the most current issues in greenhouse management--energy. Cineraria is a cool season crop requiring very little heat. It requires a minimum of three to four weeks of 13°C night temperature or lower to initiate flowers, which results in lower heating costs as compared with many other crops. They are also cost-effective relative to their beauty--the flowers remain fresh and showy for an extended period (3 to 4 weeks). The new dwarf hybrid varieties resist wilting more effectively than do the taller strains, although they do not grow well at high temperature regimes. The old style, taller hybrids possessed larger leaves which wilted when temperatures exceeded 21°C. The new hybrids grown in Europe have a shorter stature and smaller leaves. Plants having true blue flowers are greatly desired by the public and are of limited availability among the floricultural crops. Cineraria, with its brilliant blue colored strains, is therefore of great horticultural interest. The range

of blue colored petals of cineraria is caused by variations in proportionate concentration of the two anthocyanidins (cyanidin and delphinidin). According to Ashtakala and Schwartz (1971), the blue flowered phenotypes were found to have more delphinidin than cyanidin; whereas cyanidin was found in greater quantity in lavender flowered phenotypes. The presence of delphinidin in blue strains of cineraria is of interest because of its infrequent occurrence in the Asteraceae family (Ashtakala and Schwartz, 1971).

Although the cineraria could fulfill the public demand for blue flowering plants, this has not been possible since cineraria has such a complex gene pool (Barkley, 1966). Even though propagation has been conventionally by seed, the cultivated cineraria are extremely heterozygous and their seedlings are not uniform for flower color (Canning, 1928; Barkley, 1968). Cineraria seeds can be obtained from self-pollinated plants, but size and quality of the flower will deteriorate after one or two generations unless cross pollination occurs (Canning, 1928). The seeds purchased by growers consist of a variety of colors, a large percentage of which may be less desirable. Vegetative propagation could be considered as another means for obtaining a constant supply of blue flowered cineraria. However, even though cineraria is herbaceous and may be vegetatively propagated by cuttings, asexual propagation yields plants of reduced vigor with smaller flowers; therefore, an alternative method of asexual propagation would seem necessary (Canning, 1928; Barkley, 1966). Tissue culture, a widely used

method for aseptic clonal multiplication, would be the ideal means for obtaining homogeneity in the size and colors of the capitulum (flower head). It would be an alternative method used to increase the commercial number of the blue flowering cineraria.

Objectives of the investigation were to:

(1) Obtain an aseptic cultural method for shoot multiplication.

(2) Regenerate plants using excised shoot tips.

(3) Initiate the proliferation of adventitious roots on cultured shoots.

(4) Acclimatize rooted shoots for transfer to greenhouse.

(5) Reproduce with consistency, the desired characteristics of a selected phenotype, especially those having the blue flower color.

CHAPTER II

LITERATURE REVIEW

Botanical Relationships

Florists' cineraria belongs in the family Asteraceae (formerly Compositae). It is a perennial herb but is grown as an annual in the greenhouse (Larson, 1980; Canning, 1928). Its origin has been of interest. Florists' cineraria has been referred to as *Cineraria cruenta* Mass. (Rolfe, 1888; Canning, 1928) and as *Senecio* x *hybridus* Regal (Hammer, 1980) in the literature; however, Barkleys' (1968) binomial of *Senecio cruentus* DC. will be accepted and will hereafter be used. There are two points of view concerning its origin. The first is that it evolved directly from *Senecio cruenta* Mass. (Rolfe, 1888; Canning, 1928). The strains now in cultivation are different in both habit and appearance, from the original species, *S. cruenta*. Height of the earlier species ranged from 0.91-1.52 meter, compared to the 0.31-0.61 meter tall species of the existing cineraria. The flower structure consisted of small capitula (flower heads) and were lilac in color (Rolfe, 1888). The flower structure of the florists' cineraria consists of capitula similar to its ancestor but of a slightly increased size and larger range of flower colors.

Mr. Francis Masson in 1777 introduced the *S. cruentus*, from the Canary Islands to Kew Gardens in England where horticulturists selected plants which performed well in the cool, moist and cloudy

climate. This may explain the environmental conditions under which it has been able to survive in cultivation (Rolfe, 1888).

Another view of its origin postulates that it is a complex interspecific hybrid formed from S. purpurea Hort., S. heritieri DC., S. cruentus and possibly other distinct species from the Canary Islands (Canning, 1928). The florists' cineraria differentiated from its progenitors as hybridization and selection progressed (Lynch, 1901; Bateson, 1895; Barkley, 1966). According to Focke (1881) the cineraria is a multispecific complex hybrid and consists of an intricate genetically controlled breeding system made up not only of obligate outbreeders but also some self-incompatible and self-fertile species (Lynch, 1901; Barkley, 1968). It has heterozygous expression for flower color in white, yellow, pink, red, magenta, purple, blue and various bicolored patterns.

Tissue Culture

The first records of plant regeneration from small tissue explants were made in the first half of the twentieth century. Haberlandt (1902) set forth a theory of totipotency which postulated that single cells could generate normal development if the environmental conditions and nutritional ingredients were manipulated. Depending on constituents of the medium, it was theorized that cultured plant cells would have the ability to proliferate callus tissue, produce new embryonic tissue, and generate new plants. Although Haberlandt established a fundamental direction for

physiological processes in plant development, it was not until White (1934) grew tomato root tips in liquid media that tissue culture progressed. Later work was concerned with the influence of exogenous hormonal substances on morphogenesis in plant tissue culture.

Skoog and Miller (1957) found that a balanced ratio of auxin and cytokinin was essential for organogenesis and morphogenesis in tissue culture of tobacco. Many experiments have been performed to elucidate the effects of varying concentration ratios of auxins and cytokinins in different media on cellular differentiation and morphogenetic development in vitro. According to Hemple (1979), the same composition of growth regulators in media may cause various responses among different species and cultivars. Similar differences in reactions can occur by placing explants of different organs on the same medium. Shabde and Murashige (1977) speculated that these variations originated from the capabilities of the species and organ fragments in their rate of synthesis of endogenous growth substances, in their differing contents of existing hormonal substances at the time of isolation, and in their variable diffusion rates of substances from and distances between "source to site."

Tissue Culture in Propagating Floriculture Crops

According to Hempel (1979), micropropagation is very popular in the floriculture industry for two reasons: (1) a large proportion of floriculture crops must be vegetatively propagated because of nonuniformity of progeny of plants grown from seed, and (2) vegetative propagation is an expensive procedure.

The first floriculture plant regenerated in tissue culture was dahlia (Morel and Martin, 1952). They found that plants grown in shoot meristem cultures were mostly virus-free. Later Morel (1964) found that orchids could be grown *in vitro* from stem tips to marketable mature plants in mass quantities. This method produced orchids faster than conventional seed propagation.

The shoot apex is an excellent explant of an organ to isolate in culture since many pathogens are not present in high concentrations. This method of utilizing primordial-axillary buds as sources of new plants is not only an effective means for propagation, but also for clonal multiplication (Hackett and Anderson, 1967) and as a method for maintaining pathogen-free plant material (Hollings, 1965).

The commercial importance of carnations for floral arrangements has led to the use of tissue culture for production of multiple adventitious shoots from shoot tip culture (Hackett and Anderson, 1967). Although their method for multiplying carnations was successful, deviations from normal flower color suggested that there probably was a rearrangement of chimeral tissues. This method of shoot tip culture has been used in several cases to produce large numbers of shoots in a small area under controlled environmental conditions (Hackett et al., 1966; Shabde and Murashige, 1977; and Davis et al., 1977). The optimum medium and balance of hormonal substances varied among cultivars. However, there were similarities among methods for culturing explants. Surface sterilized excised

shoot apices, ranging in size from 0.5mm to 2cm long with two to four primordial leaves attached were placed cut edge down on several defined media. Explants then remained in culture for various periods of time ranging from 3-8 weeks (Davis et al., 1977; Shabde and Murashige, 1977; Hackett and Anderson, 1966) and finally cultured shoots were acclimatized to greenhouse conditions.

Phillips (1968) reported that shoot tip growth and survival of carnation apices could be improved by the addition of adenine to the medium; whereas the addition of kinetin could prevent root formation, depending on concentration. In a similar investigation, Pennazio (1975) concluded that there was a point at which the stimulating concentration of kinetin was below that which suppressed apical dominance and inhibited root formation. Davis et al. (1977) showed that carnation shoot tip growth was consistently enhanced by addition of casein hydrolysate in the medium at the rate of 1 g/liter. While casein hydrolysate caused increased growth, many abnormally broad leaves resulted.

The florists' gloxinia can be regenerated from petioles, pedicels, stem pieces, and leaves. Haramaki (1971) tested shoot tips of gloxinia on media containing several different mixtures of indoleacetic acid (IAA) and kinetin and found that a high cytokinin to auxin ratio in the MS medium developed rapid multiple shoots through the enhancement of axillary shoot growth.

Iizuka et al. (1973) reported the varying effects of growth substances on the regeneration of shoots from tubular florets and

from callus of chrysanthemum (Chrysanthemum morifolium Ramat.) and cineraria. The florets differentiated shoots from callus grown in culture for 10 weeks on media containing varying concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) in combination with 6-benzyladenine (6-BA), indolebutyric acid (IBA), and 3-indoleacetic acid (IAA). Although cultures grown in the dark at 25°C developed callus and later differentiated organs, those florets grown under the 16 hour light-8 hour dark cycle died without any callus development.

In vitro propagation for chrysanthemum was first used as a method for eliminating virus diseases from stock plants (Hakkaart and Quak, 1964). Researchers later found that multiple plants could be produced by the formation of adventitious shoots. Hill (1968) reported regeneration of shoots on callus cultures originated from explants of stems, receptacles, and leaves. It was found that isolation of explants on media containing 1-naphthaleneacetic acid (NAA) (2 mg/1) and 6-furfurylaminopurine (kinetin) (1 mg/1) and soya peptone (1 g/1) usually was effective for shoot regeneration. There was a difference in the number of shoots produced in a culture, from many to just one shoot. Hill found that the habit of growth and the bloom characteristics were usually the same as on the motherstock; however, a few dwarf plants did result. According to Hill, this variation in the plants could be the result of shoot morphogenesis while in culture. Matsubara et al. (1971) described the production of adventitious shoots on sections of the

receptacle. Ben Jaacov and Langhans (1970, 1972) reported the formation of adventitious shoots on callus cultures derived from stem tips, but there were some changes in leaf shape, photoperiodic response, and flower color compared with parent cultivars. Bush et al. (1974) showed that shoots could be obtained from callus cultures derived from pith tissue, petal epidermis, and complete petals of chrysanthemum. Roest and Bokelmann (1973) obtained shoots from the capitulum of Chrysanthemum cinerariafolium Vis. which may have originated both axillarily and adventitiously. They also tested segments of flower peduncles for production of adventitious shoots. After 2-3 months, shoots (4.0cm in length) were excised from the explants and transferred to rooting media. The most successful method for root formation was to culture isolated shoots on a Murashige and Skoog (1962) medium modified by the addition of 2% sucrose and IAA at 10^{-4} mg/ml (Roest and Bokelmann, 1975).

Broertjes et al. (1976) described the regeneration of adventitious shoots from irradiated (500 rad X-rays) leaf, capitulum, and pedicel explants for mutation breeding of C. morifolium. These shoots ultimately originated from single cells, which led to the formation of solid, nonchimeral mutants. Earl and Langhans (1974a) described a method for obtaining multiple shoots from explants of shoot tips of 'Giant #4 Indianapolis White' chrysanthemum. Single shoot tips produced multiple shoots on a Linsmaier and Skoog (1965) medium containing 2mg/liter kinetin and 0.02mg/liter NAA. These shoots could be subcultured through the same system so that each

shoot regenerated additional shoots. These shoots grew into normal white-flowered plants. Another method of propagation using a rotating wheel produced leafy callus tissue in a liquid medium (Earle and Langhans, 1974b). The only morphological changes noticed after growth in culture could be related to greenhouse conditions. There were no mutated forms produced by this method.

Pierik et al. (1973) developed an *in vitro* technique to force excised gerbera capitulum explants to form shoots. This technique is based on the activation of tissues from the involucral bracts by an auxin and cytokinin. Their method resulted in the production of normal adventitious shoots. Previous work (Pierik and Segers, 1973) was not successful in regenerating adventitious shoots from differentiated tissues in midrib explants. Adventitious bud formation was not promoted by cytokinin even at high levels. Later, Pierik et al. (1975) developed a method based on a high ratio of cytokinin:auxin to produce shoots from excised capitulum explants.

The regeneration of adventitious shoots by shoot tip culture of gerbera (*Gerbera jamesonii* Hook) was described by Murashige et al. (1974). Complete removal of microorganisms from explants was not possible; however, approximately 20% of the explants survived. These surviving shoot tip explants produced axillary shoots, which were separated and recultured on fresh media. Rapid multiplication of shoots was then accomplished by repeatedly dividing and reculturing branch shoots. Gerbera shoot tips were increased 6-fold in 4 weeks and 12-fold in 8 weeks on a Murashige and Skoog

(1962) medium containing 0.5mg/liter IAA and 10mg/liter kinetin. This process of subdivision could continue indefinitely as long as stock cultures were maintained. Cultures retained their normal diploid number of chromosomes and no morphological abnormalities were observed.

Murashige et al. (1974) compared capitulum explant culture and shoot tip culture and concluded that each method has several advantages and disadvantages. Although losses from infection in capitulum culture were only 10%, the number of shoots per flower (8 to 12 after 8 weeks) was very small (Pierik et al., 1975). Infection in shoot tip culture was much higher (80%) but this method was rapid, with a high percentage of survival and no apparent mutants. They concluded that micropropagation should start with genetically uniform shoot tips and any callus formation as an intermediary step should be avoided since genetic variations frequently result from callus. Other explant sources, such as whole florets (Iizuka et al., 1973), stems, and flower receptacles have been used to proliferate plantlets. However, in each case color mutants were encountered when plants were grown to maturity. Murashige (1974) separated general procedures in clonal multiplication into three stages of development as follows: Stage I, establishment of the aseptic culture; Stage II, multiplication of propagules; and Stage III, preparation for reestablishment of plants in soil.

The purpose of this research was to (1) obtain a suitable explant, free from obvious infection, with an acceptable survival rate and proportionate rapid growth among explants, (2) increase the number of divisions/culture by either adventitious or axillary bud formation, (3) initiate adventitious roots, acclimatize, and prepare for growth under greenhouse conditions, and (4) obtain phenotypically normal blue flowering plants.

CHAPTER III

MATERIALS AND METHODS

Leaf blades, petiole sections, and shoot tips of greenhouse grown cineraria were tested as possible explants for propagation by tissue culture methods. Plant parts were surface sterilized and placed on modified Murashige and Skoog (MS) medium (Murashige et al., 1974) containing 0.5mg/liter IAA and 10ml/liter kinetin as recommended for gerbera tissue culture. Because a consistently high rate of culture contamination resulted from greenhouse grown plant parts, subsequent experiments were performed with shoot tips derived from seedlings grown *in vitro*.

Experiments were performed with the following media to determine the appropriate nutrient solution for seed germination: (1) modified Murashige and Skoog (MS), (2) Whites (1954), and (3) Schnek and Hildebrant (SH) (1972). Explant survival and shoot regeneration were superior on the modified MS medium, and it was selected for further testing. Seedlings obtained from surface sterilized seeds were used as the source of experimental tissue for subsequent investigations.

The following experiments were performed for the vegetative propagation of cineraria *in vitro*: (1) seed germination and shoot initiation, (2) shoot multiplication, and (3) root initiation. The MS (1962) basal medium, containing either the MS organic substances supplemented with casein hydrolysate or the modified

MS medium (1974) containing the organic substances supplemented with L-tyrosine, adenine sulfate, and additional phosphate as $Na_2PO_4 \cdot H_2O$, was used consistently throughout the experiments.

The constituents of the MS medium are shown in Tables I and II. To facilitate comparisons in the experiments, any modifications of the concentration of macroelements will be designated in multiples, such as 1/2 X level = half or 1/1 X level = full strength.

All media were buffered to 5.7-5.8 with 1N NaOH or 1N HCl before the agar was added. The media were heated to boiling, Difco bacto-agar was added, and media were then dispensed into Erlenmeyer flasks (size depending on experiment). Flasks were plugged with cotton, capped with aluminum foil, and autoclaved at 121°C and 15 psi for 20 minutes.

The following media combinations were used for Experiments No. 2 Shoot multiplication by shoot tip culture and No. 3 Root initiation:

(1) MS macroelements 1/1, microelements 1/1, ferric citrate, and modified organic substances (hereinafter called MS-1).

(2) MS macroelements 1/2, microelements 1/1, ferric citrate, and modified organic substances (hereinafter called MS-2).

(3) MS macroelements 1/1, microelements 1/1, ferric citrate and organic substances (hereinafter called MS-3).

Table I. Inorganic nutrients (salt composition) of Murashige and Skoog medium.

Elements	mg/liter
Macronutrient Elements	
KNO_3	1900.000
NH_4NO_3	1650.000
$CaCl_2 \cdot 2H_2O$	440.000
$MgSO_4 \cdot 7H_2O$	370.000
KH_2PO_4	170.000
Micronutrient Elements	
H_3BO_3	6.200
$MnSO_4 \cdot 4H_2O$	22.300
$ZnSO_4 \cdot 2H_2O$	8.600
KI	0.830
$NaMoO_4 \cdot 2H_2O$	0.250
$CuSO_4 \cdot 5H_2O$	0.025
$CaCl_2 \cdot 6H_2O$	0.025
Na_2-EDTA *	37.300
$FeSO_4 \cdot 7H_2O$ *	27.800

*A stock solution of $FeSO_4 \cdot 7H_2O$ and Na-EDTA$\cdot 2H_2O$ was made by first heating 7.45gm Na_2 EDTA in 500ml H_2O to dissolve and then adding 5.57gm $FeSO_4 \cdot 7H_2O$ and 500ml H_2O to the heated solution.

Table II. Composition of Organic Nutrients for Murashige and Skoog (MS) Basal Medium.

Elements	mg/liter	gm/liter
MS (1962) organic constituents		1.0
Casein Hydrolysate	2.00	
Glycine	100.00	
Myo-Inositol	0.50	
Nicotinic acid	0.50	
Pyridoxin·HCL	0.10	
Thiamin·HCL		
MS (1974) organic constituents		
Myo-Inositol	100.00	
Nicotinic acid	10.00	
Pyridoxin·HCL	1.00	
Thiamin·HCL	30.00	
Adenine Sulfate Dihydrate	80.00	
L-tyrosine	85.00	
additional phosphate as		
$NaH_2PO_4 \cdot H_2O$	100.00	
Organic constituents for both media		
Sucrose		45.00
Agar		10.00

pH was set at 5.8

(4) MS macroelements 1/2, microelements 1/2, ferric citrate and organic substances (hereinafter called MS-4).

Sterilization Procedure

Cineraria seeds were placed in a 2% (v/v) aqueous solution of a detergent (Micro, International Products Corp.) and vigorously agitated on a gyrotary shaker for 3 minutes. This was done to remove as much debris and contaminates as possible and to break the surface tension of the gelatinous film surrounding the testa. Following a thorough rinsing (3 times) with sterile distilled water, the seeds were surface sterilized in a 10% (v/v) commercial sodium hypochlorite solution for 3 minutes. Following a second rinsing with sterile distilled water, seeds were then transferred to the seed culture medium. All procedures were performed with standard sterile techniques in a dead air space tissue culture cabinet. Shoot tips, derived from these *in vitro* germinated seed cultures and used in subsequent experiments required no further disinfection after divisions and transfers of shoots were made from one sterile flask to another.

Experiment 1: Seed Germination and Shoot Initiation

This study was conducted to determine both the potential of seeds to germinate *in vitro* and the ability of a single seed to regenerate multiple shoots. Two tests were replicated 10 times with 5 seeds per flask.

Seed germination and plant initiation was induced on MS medium containing macroelements at full strength, modified organic substances (Table II), 0.5mg/liter IAA, and 10mg/liter kinetin and grown in an environmental chamber at 23°C in a 13 hour light-11 hour dark cycle. Multiple shoot development occurred 5 weeks following seed placement. These miniature divisions, or lateral shoots, were separated and subcultured to a freshly prepared medium to continue regeneration of divisions until an adequate supply of shoot tips had been established. These shoot tip divisions were then used in subsequent experiments.

Experiment 2: Shoot Multiplication by Shoot Tip Culture

This study was conducted to determine: (1) whether the additives in the modified MS medium (Murashige et al., 1974) were essential to regeneration and growth of cineraria shoot tips in culture; (2) an optimum auxin level for shoot tip multiplication; and (3) the origin of growth and morphological characteristics of the organs formed from shoot tips in culture.

Shoot tips, consisting of 2-4 primordial leaves, were excised from individual plantlets obtained from selected seed germination cultures (previous experiment). A total of 48 excised shoot tips, 1-2cm long, were transferred to sterile flasks containing the four different media combinations: MS-1, MS-2, MS-3, MS-4. Each combination consisted of 16 flasks with 4 flasks per auxin level

and one shoot tip per flask. Auxin levels were varied to determine the optimum balance for shoot development and multiple shoot regeneration. IAA was added to the four different media combinations at 0.0, 0.25, 0.5 and 1.0mg/liter at each auxin level, except when no auxin was specified.

Cultures were grown at 23°C ± 1°C in a 14 hour light-10 hour dark cycle. Eight weeks following shoot tip placement, the tissue mass was removed from culture and examined. Data was recorded on the number of viable plantlets greater than 4cm long that were produced per shoot tip, the presence of callus, and the occurrence of adventitious bud production.

Experiment 3: Root Initiation

This study was conducted to determine the optimum hormone level to be used to initiate adventitious roots on plantlets obtained from multiple shoot cultures derived from seed germination cultures. Cultures were subdivided and 300 shoots over 2.5cm long with attached callus were transferred to sterile flasks containing the four different media combinations: MS-1, MS-2, MS-3, MS-4. NAA was added to the four media combinations at 0.0625, 0.1250, 0.2500, 0.500, and 1000mg/liter. Kinetin was omitted. The media were distributed among a total of 60 flasks and 5 shoots were inoculated per flask. Each medium combination was distributed among 15 flasks (500 ml) with 3 flasks per auxin level.

Cultures were grown in an environmental chamber at 23°C ± 1°C in a 14 hour light-10 hour dark cycle. Rooted shoots were

removed from culture 4 weeks following placement of plantlets on media. Roots were measured and evaluated qualitatively on a rating scale of 1-10 (Figure 1), according to root mass and length and to the amount of shoot growth. Production of callus was also counted in the rating of rooted plantlets.

Transfer to Soil

Plantlets were transferred to 5.7cm pots containing a commercial potting medium and kept moist. Half of the rooted plantlets were transferred directly to the greenhouse and placed under shade cloth. They were irrigated daily and maintained with Peter's soluble liquid fertilizer (20N-8.8P-16.6K). The other half of the rooted plantlets were given a transition period before being transferred to the greenhouse. These were maintained under glass covers with artificial fluorescent light at 21°C for 3 weeks. Plantlets were then transferred to 12.2cm pots, moved to the greenhouse, placed under shade cloth, and kept under intermittent mist for 3 weeks. They were then allowed to grow until pot bound at which time they were given 3-4 weeks' cold treatment at 15°C-16°C in a cold chamber. The plants remained in the chamber with continuous low light until flower buds were initiated. They were then removed to the greenhouse and maintained in the same manner as the test group.

Histological Procedures

Histological investigations were performed to determine the origin of adventitious shoots obtained in culture. Cultured

Figure 1. Rooted plantlets were visually rated according to the scale depicted above. Measurement of the longest roots and little or no shoot growth were observed in the ratings. A rating scale of 1-10 (descriptions listed below) was given to each plantlet. Scale reads from left to right.

1. No root growth to few stubby roots. No shoot growth to shoot death.
2. Stubby roots to few roots. No shoot growth.
3. Few short roots. Small percent shoot growth with callus present at shoot base.
4. Optimum number of short roots emerging from a callused shoot base. Optimum shoot growth.
5. Large number of medium length roots emerging from a callused shoot base. Optimum shoot growth.
6. Few long roots emerging from a semi-callused shoot base. Shoot growth minimum (dark green vigorous shoot growth).
7. Optimum number of long roots emerging from semi-callused shoot base. Minimum shoot growth (dark green vigorous growth).
8. Optimum number of long roots, no callus present. Shoot growth proliferating (pale green coloration of rapidly growing shoots).
9. Optimum long root growth, no callus present. Maximum shoot growth (dark green vigorous growth).
10. Maximum root growth, no callus present. Maximum shoot growth (dark green vigorous growth).

materials for histological studies were fixed 48 hours in a solution of chromium trioxide, acetic acid and formaldehyde, designated as Craf III (Sass, 1958). The fixed tissue samples were dehydrated by passing them through a graduated series of ethanol and tertiary butyl alcohol (TBA) (Johansen, 1940; Sass, 1958; Jensen, 1962). Tissue samples were embedded in paraffin and sectioned on a rotary microtome according to standard procedures. Sections were mounted on slides and stained with Heidenhain's iron hematoxylin (Jensen, 1962) applied with acidified ferric-ammonium sulfate mordant solution (Lang, 1936).

CHAPTER IV

RESULTS

Sterilization Procedures

The greenhouse grown plants were too contaminated with fungal spores and bacteria to be used for further culture, even following surface sterilization in varying concentrations of sodium hypochlorite/water solutions, ranging from 3% to 15% (v/v). Nearly all cultures obtained from these plants showed fungal contaminates (i.e., primarily Botrytis and Rhyzoctonia). Because of this contamination, seeds were tested as a means for obtaining explants to use for further experiments.

The seeds also were extremely contaminated with fungal spores. Efforts to eliminate this contamination through soaking in various levels of hypochlorite/water solutions (3% to 15% v/v) at varying time intervals of 3 to 60 minutes proved to be phytotoxic to the embryo or failed to remove pathogens from testa, again resulting in contaminated cultures.

Further attempts to sterilize the seeds that had been soaked in the sodium hypochlorite solutions were made by rinsing them in ethanol concentrations ranging from 75%-95% for 30 seconds to 3 minutes. Although this method eliminated much of the contamination, the seeds produced abnormal plantlets in culture.

Investigation of the seed was conducted to determine why contamination was still occurring in spite of sterilization

techniques. Microscopical examination of the seed revealed numerous convolutions (ridges and fissures) in the testa which were difficult to wet with aqueous germicidal fluid. This could explain why simple rinsing in a sodium hypochlorite solution was not effective in eliminating contamination of cultures.

The problem of abnormal growth with the use of alcohol led to closer examination of the seed. A seed was immersed in water and observed microscopically. Imbibition of water occurred within 15 seconds and the seed increased in size two-fold. During this process, a very thick gelatinous coating covered the wetted testa. A drop of 85% ethanol was then placed on the imbibed seed. Within 12 seconds, the imbibed seed was dehydrated and shriveled to its natural size, still maintaining some of the gelatinous coating. It seemed probable that it was the gelatinous coating which prevented penetration of the hypochlorite solution to the surface of the testa, and thus prevented sterilization. Therefore, the seeds were placed in a detergent solution (3% micro detergent) and agitated on the gyrotary shaker for 3 minutes to break up the gelatinous coating which covered the testa. The seeds were then removed from the detergent solution, rinsed several times in sterile water, and sterilized with hypochlorite solution. This procedure yielded 99% contamination-free cultures. The only contaminates observed were penicillin spores (possibly airborne). There were no signs of either Botrytis or Rhyzoctonia in these cultures.

Experiment 1: Seed Germination and Shoot Initiation

Germinated seeds produced cotyledons heavily covered with colored trichomes, which were used to predict the future desired flower colors. Germinated seeds regenerated shoots within 6 weeks of placement in culture. Subdivisions yielded an average of 15 viable plantlets per culture for future use. Shoot multiplication of cineraria was accomplished primarily through enhancement of axillary bud growth; however, some shoots arose from buds produced adventitiously from leaf surfaces. Adventitious shoots were not used for further experimentation because of the possibility for genetic changes.

Experiment 2: Shoot Multiplication

Selected seedlings were grown to mature plantlets from which the shoot tips were removed and transferred to four different media combinations: MS-1, MS-2, MS-3, MS-4. Addition of the supplements L-tryosine and adenine sulfate to the shoot multiplication media was advantageous for growth of cineraria shoot tips, especially when added to the modified MS medium containing half the recommended concentration of macroelements. There were differences in the growth of shoot tips observed among treatments, as shown in Figure 2. Shoot initiation occurred in both of the modified MS media containing 0.0-1.0mg/liter IAA. However, the modified MS medium containing half the recommended concentration of macroelements yielded a higher number of divisions per culture with less variation in growth among auxin levels. Although a large number of shoots

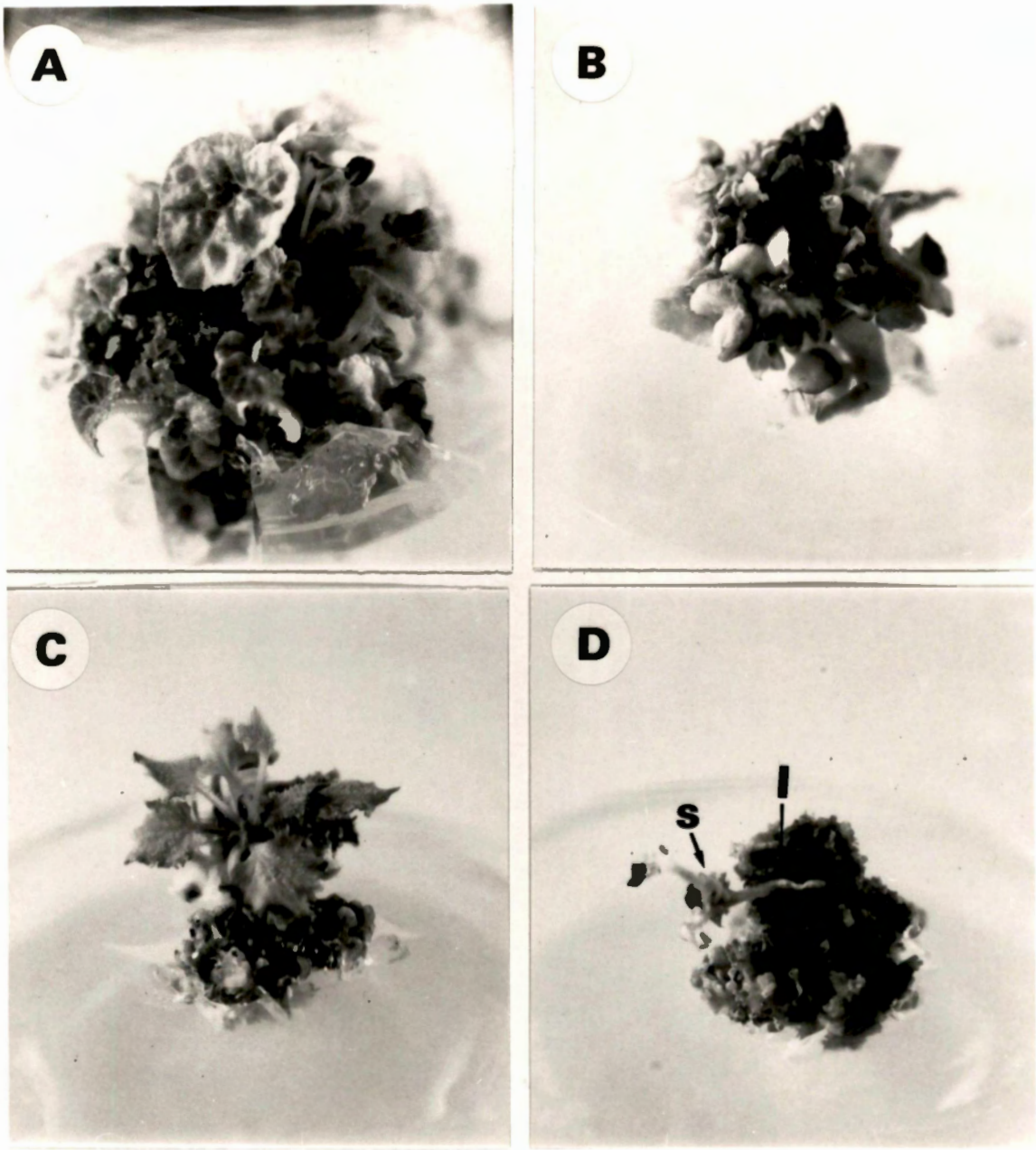
Figure 2. Selected shoot tip cultures showing variation among media and auxin treatments on shoot tip culture growth 8 weeks following incubation.

A. Having proliferation of shoots cultured on the modified MS medium with the recommended concentration of macroelements and containing 0.5mg/liter IAA.

B. Mass of shoots cultured on modified MS medium at half the recommended concentration of macroelements and 1.0mg/liter IAA.

C. Single plantlet growth with unorganized tissue at the base of the plantlet was cultured on modified MS medium with the recommended concentration of macroelements and containing 1.0mg/liter IAA. This type of abnormal growth was sporadic.

D. Adventitious shoots emerged from leaf sections on MS medium at the recommended concentration of macroelements and containing no auxin. The shoots were derived from callus initiated on the leaf of the original shoot tip explant, (l) = original leaf, (s) = shoot apex.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was obtained on the modified MS medium containing the recommended concentration of macroelements and containing a 0.5mg/liter IAA (Figure 2A), consistently better shoot yields were obtained on the modified MS medium containing only half the concentration of macroelements (Figure 2B). Callus was produced sporadically on both modified MS media. Some callus was formed at the base of shoots on modified MS medium that contained 1.0mg/liter IAA (Figure 2C). Shoots began to emerge from the callus mass within 5 weeks following callus production. On both modified media there was no increase in shoot yields with an increase in auxin levels.

The addition of casein hydrolysate to the shoot multiplication media was deleterious to growth of cineraria shoot tips. The shoot tips cultured on the MS media containing casein hydrolysate with half the recommended concentration of macroelements gave consistently low yields; however, the MS media containing casein hydrolysate with the full concentration of macroelements was consistently inferior in supporting growth of the shoot tips. The viability of the shoot tips on both media was 100%. Shoot tips on MS media containing the recommended concentration of macroelements and containing 0.25mg/liter and 0.5mg/liter IAA died within 2 weeks in culture. Growth of plantlets on MS medium having half the recommended concentration of macroelements was often small leafy clusters with some small stunted shoots arising from areas of axillary meristems. The leaves appeared broader and thicker than normal. Shoots cultured on both MS media with the full or half the

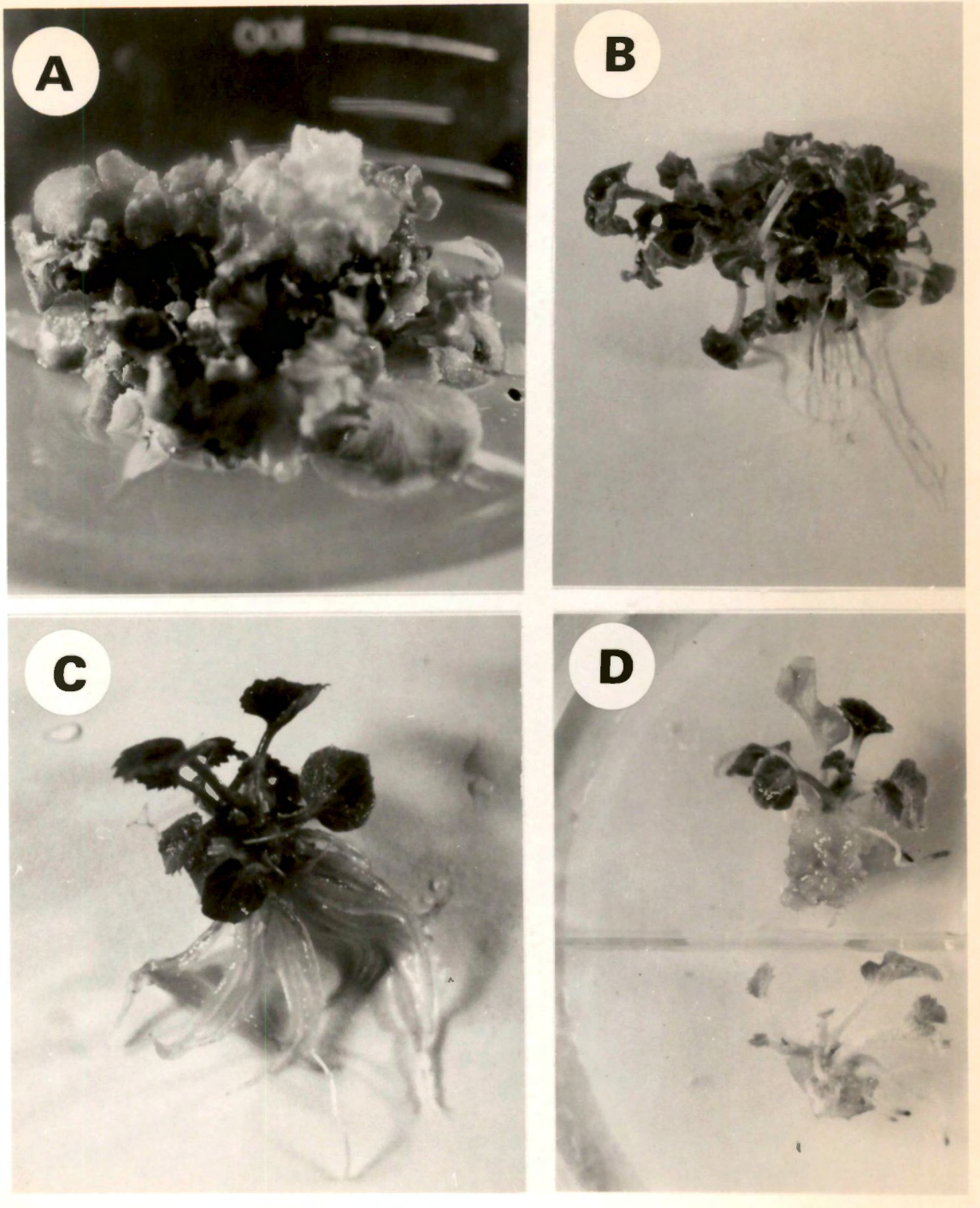
Figure 3. Root initiation cultures showing variation among media and auxin treatments on root initiation and shoot growth 4 weeks following incubation.

A. Proliferation of shoots cultured on modified MS medium containing 0.5mg/liter IAA prior to subdivision.

B. Root initiation on MS medium with the recommended concentration of macroelements and containing 0.25mg/liter NAA produced thin long roots ranging from 2mm-4mm long. Multiple shoot production was not usually common among these cultures as shown.

C. Root initiation on MS medium at half the recommended concentration of macroelements produced a large number of very thick roots in many cultures. Note the dark multiple shoot growth.

D. Extreme callus development on modified MS medium at half the recommended concentration of macroelements and containing 0.0625, 0.25, and 0.5mg/liter NAA caused few to no root production.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The addition of casein hydrolysate to the MS root initiation media aided growth of adventitious roots, especially when half the recommended concentration of macroelements was used. Root growth increased with increasing NAA concentrations. Very little or no callus was produced on either MS media with half of the originally recommended concentration of macroelements at all auxin levels. However, callus was present on MS media having the recommended concentration of macroelements with 0.250mg/liter NAA and also on MS media having half the recommended concentration of macroelements with 0.0625mg/liter NAA added. Both of the MS media containing either the half or originally recommended concentration of macroelements formed thin roots ranging from 4-9mm long at all auxin levels. However, it was observed that when 0.250mg/liter NAA was added to the MS medium containing the originally recommended concentration of macroelements, roots tended to be very short or no roots were produced. Similar results were obtained when 0.0625mg/liter NAA was added to the MS medium containing half the concentration of macroelements. In both MS media, containing half or originally recommended concentrations of macroelements, single plantlets grew in most cases; however, there were instances of multiple shoot production from those cultures which had callus development (Figure 3B). Although most cultures produced long thin root systems, the MS medium containing half the concentration of macroelements produced better yields of roots and generally initiated very thick root systems (Figure 3C).

Addition of the supplements of L-tyrosine and adenine sulfate to the modified MS rooting media was deleterious to growth of adventitious roots. This was most noticeable in cultures when the modified MS medium having half the recommended concentration of macroelements was used, as root growth decreased with increasing NAA concentrations. Root and shoot growth was consistently less on this medium at all auxin combinations. The modified MS media having either the original or half the recommended concentration of macroelements caused callus production at the base of the stem of all cultured shoots. A small number of short or stubby roots were initiated on shoots at most auxin levels, except at 0.25mg/liter NAA in the modified MS media with the recommended concentration of macroelements where roots were very fine, thin, and less than 1.5cm in length. Similar results were obtained when 0.125mg/liter and 1.00mg/liter NAA were added to the modified MS media with half the recommended concentration of macroelements as shown in Figure 3D. In both modified media there was a division of shoots (ranging from 2 to 8 per plantlet) at every auxin level.

It was observed in all four experimental rooting media that separated divisions were more prone to initiate roots than did undivided clusters. This was also the case for shoots which proliferated plantlets while in the rooting media.

Transfer to Soil

The rooted plantlets were transferred to a seedling culture medium in pots. Half were placed directly in the greenhouse in

the winter when conditions should be expected to be most favorable for survival. However, the shoots died within 3 days as a result of heat and water stress to the tender cultured plantlets. The other half were placed under environmentally controlled conditions before they were transferred to the greenhouse. After a 4-week period the acclimatized plants were removed from controlled conditions, repotted, and placed in the greenhouse under shade cloth and intermittent mist for a 3-week period (Figure 4). Following a 3-4 week cold treatment at 15°C-16°C in a cold chamber, these plants subsequently grew and flowered normally (Figure 5). Phenotypically, the growth habit and bloom characteristics were similar to plants grown from seeds in the greenhouse. Except for a single incidence of a double flowered plant, no variation among these plants was observed. Any slight variations in plant structure and flower color were not investigated further.

It was also observed that the colored trichomes on the cotyledons proved to yield the same colored flower. Blue colored trichomes on cotyledons gave the predictable blue colored flower; similarly, the lavender colored trichomes yielded lavender colored flowers.

Histological Results

Shoots taken from the regeneration media were examined microscopically to determine the origin of plant regeneration. Both axillary and adventitious shoot formation occurred (Figure 6).

Figure 4. Plantlets transferred to 12.2cm pots (prior to transfer to greenhouse) after 4 weeks of environmentally controlled conditions.

Figure 5. Blue flowering plants 3 weeks following removal of plants from the cold chamber to the greenhouse. Plant on left is tissue culture grown, compared to greenhouse grown seedling on the right.

Figure 6. Shoot tip cultures showing adventitious shoot formation on modified MS medium 7 weeks following incubation *in vitro*. s = shoot with leaf primordia

A. Shoots arising from the adaxial leaf surface. Note the high concentration of anthocyanin in the structures, giving the lilac coloration.

B. Well developed shoots arising from the adaxial leaf surface.

C. A single meristematic structure is arising from the vascular tissue region of the adaxial leaf surface.

D. Well developed adventitious shoot formed from meristematic regions in callus matrix by organogenesis. Note well developed leaves.

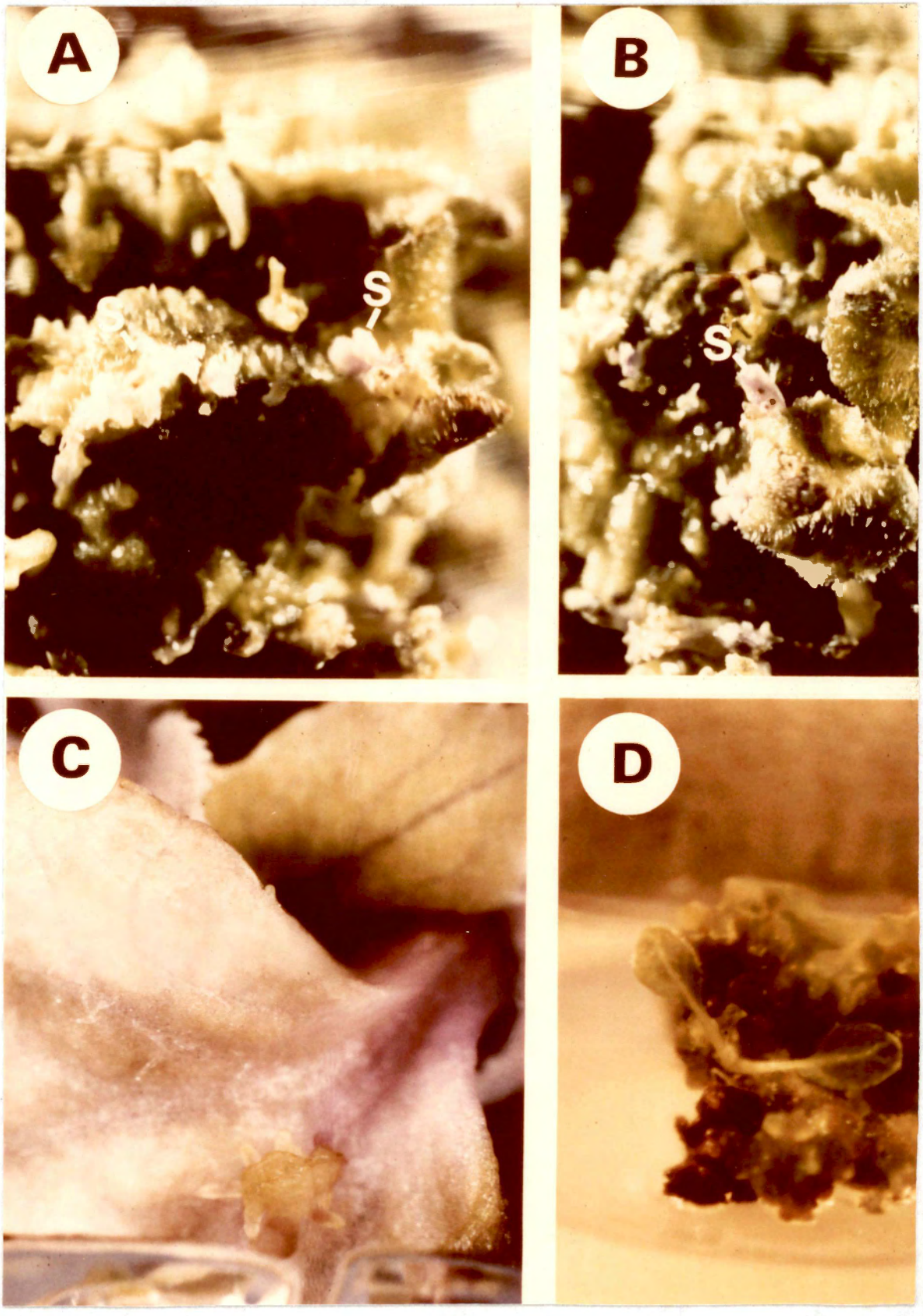

Figure 6

Adventitious shoots were observed to originate from the adaxial leaf surface and leaf margins (Figures 6A and 6B). These adventitious shoots possessed a low chlorophyll pigmentation; however, they were covered in trichomes high in anthocyanin. Shoot formation was found to arise from the xylem and phloem regions of leaves (Figure 6C). Shoots arose from the adaxial leaf surface or margins on those leaves that were still attached to the plant body. Some shoot tips developed callus and regenerated new shoots within 5-6 weeks (Figure 6D). Callus development was probably a result of damage to the shoot apical meristem during excision of the shoot tip explant from the plantlet.

Reference plants and regenerated plant sections from the regeneration experiment were examined histologically to determine the mode of plant regeneration. Sections revealed that shoot formation also occurred by organogenesis. The developmental sequence of shoot initiation began with the appearance of surface cell division activity on leaf surfaces, which led to the development of tiny protuberances (Figure 7). Meristematic activity arose in the vascular parenchyma cells as shown in Figure 7A. These meristematic areas gave rise to shoot primordia, which emerged from both surfaces of leaves. The aggregation of thick-walled cells and densely staining nuclei and cytoplasm were observed (Figure 7B). The nuclei were centrally located and occupied a large proportion of the cell. It was also observed that the meristematic area arose from epidermal tissue as shown in Figure 7C. The vascular strand

Figure 7. Sections of leaf tissue showing meristematic regions arising from the epidermal tissue and the xylem and phloem area by organogenesis. Meristematic regions and meristamoids are in the early stages of development.

A. Well developed meristematic structure resembling a bud from a longitudinal section. 68X

B. Longitudinal section of leaf showing meristamoid structure arising from the xylem and phloem tissue. Note the aggregation of thick-walled cells and densely stained nuclei and cytoplasm. 227X

C. Meristematic structure arising from the epidermal tissue of the leaf section. 34X.

D. Cross section of meristematic structure showing the cells in orderly cell files and the presence of vascular tissue. 227X

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was visible and cells were in orderly cell file (Figure 7D). These meristematic centers or meristemoids are still in early stages of development; however, these cell divisions led to the formation of primordial leaf-like structures with apical meristems among the primordia (Figure 8). Adventitious meristems formed on epidermal tissues on leaf explants cultured in vitro after 3-5 weeks of incubation. Advanced shoot apical meristems and young leaf primordia developing from the adaxial leaf surface of leaf cross sections are shown in Figures 8A and 8C. Well developed vascular tissue on leaf sections are shown in Figures 8B and 8D.

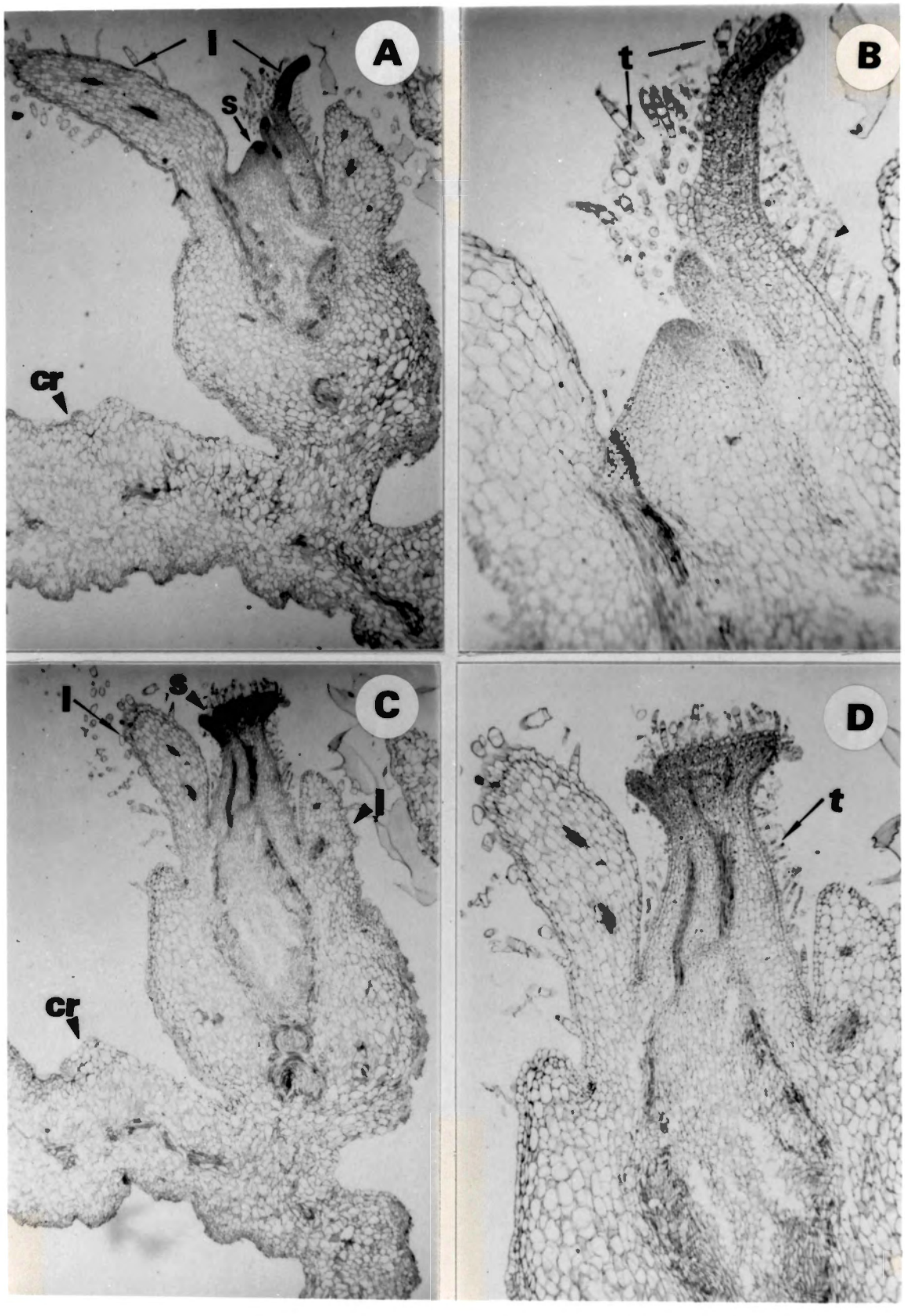
Figure 8. Sections of leaf tissue showing shoot formation from adaxial surface of leaf by organogenesis (s) shoot apical meristem, (1) developing leaves.

A. A meristematic area with 4 shoot primordia and apical region, longitudinal shoot on cross section (cr) of leaf. 34X

B. Close up of 7a. Notice the well developed trichomes (tr) and orderly cell files. 113X

C. A single shoot primordia with 3 developing leaf primordia, longitudinal shoot on leaf cross section (cr). 34X

D. Longitudinal section of a regenerated shoot showing well developed vascular tissue and trichomes (tr). Notice the dark stained meristematic area in axillary bud area of the leaf primordia. 113X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CHAPTER V

DISCUSSION

Consistently sterile seed germination cultures were obtained when seeds were rinsed with detergent solution prior to sterilization with hypochlorite. From these seed cultures numerous divisions or small outgrowths of axillary shoots were obtained.

Germinated seeds produced cotyledons heavily covered with colored trichomes. This early detection of color enabled the determination of the future flower color desired. Explants with blue trichomes yielded blue flowered plants; whereas, explants with lavender trichomes yielded lavender flowered plants. Intensity of flower color was predictable in accordance with trichome color.

Shoot morphogenesis is regulated by limiting and interacting factors such as inorganic substances, organic substances, auxin level, and cultural procedures. Conditions for shoot formation and root initiation of cineraria shoot tips on the modified MS and MS media differed markedly. Shoot tips on the modified MS medium produced axillary shoots at a higher rate of divisions per culture than did with the MS medium, where growth of shoots was limited. Although the MS medium with the recommended concentration of macroelements and containing no auxin developed more shoots than the comparable modified MS medium containing the various auxin concentrations, many were fasciated.

The number of adventitious roots initiated showed a reverse trend on the two media. A higher rate of root growth occurred on the MS medium as compared to the inhibition of root growth on the modified MS medium.

The highest yield of divisions from shoot multiplication was obtained with the modified MS medium with half the recommended concentration of macroelements; whereas, the results were reversed for the rate of adventitious root initiation and growth of plantlets obtained on the MS medium with half the recommended concentration of macroelements. The consistent trend for the increased growth rate of shoot and roots on media containing macroelements at half the recommended concentration could be due in part to the concentration of inorganic substances. Evidently the enhancement of the growth of morphologically normal shoots and roots of cineraria requires a lower concentration of inorganic salt in the medium. This is in conformity with Pierik et al. (1975) who found that gerbera capitulum explants developed optimum shoots and roots on the MS medium having half the recommended rate of macroelements added.

The differences in the yield of divisions per culture from shoot multiplication and the rate of adventitious root initiation were partly caused by the organic substances in the media such as L-tyrosine, adenine sulfate, and casein hydrolysate. Addition of the supplements L-tyrosine and adenine sulfate to the shoot multiplication media aided in the increase of shoots. Similar results

were obtained by Davis et al. (1977) who reported that with increasing concentrations of adenine sulfate there was an increase in the growth rate of carnation shoot tips. The stimulatory effects of adenine sulfate in addition to IAA and kinetin on development of the explants is not surprising because adenine sulfate is known to promote the best cell divisions and growth of Capsella embryos and initiation of morphogenesis (Raghavan, 1965). The addition of adenine sulfate had a reverse effect on the rooting medium where it inhibited the production of adventitious roots on cineraria shoots. There has also been evidence of adenine sulfate causing inhibition of root formation on chrysanthemum explants (Pennazio, 1975). L-tyrosine has also been shown to have a stimulatory effect on shoot formation in work by Lee and Skoog (1965). They suggested that L-tyrosine influenced the level of IAA through the activity of IAA oxidase, thus altering the auxin/cytokinin balance in favor of shoot formation.

The addition of casein hydrolysate was deleterious to growth of cineraria shoot tips. Repression of shoot formation by casein hydrolysate in cineraria shoot tip research was observed. This was in contrast to work on carnation shoot tips by Davis et al. (1977). They found that casein hydrolysate at 1 to 4g/liter enhanced the growth rate of shoot tips; however, omission of casein hydrolysate from the multiplication stage of carnation cultures reduced the number of abnormally broad and thick leaves (Davis et al., 1977), which was also the case in cineraria cultures. According

to Murashige and Skoog (1962) the addition of casein hydrolysate may influence the activities of IAA and kinetin and possibly the ratio of the two substances required for a specific growth pattern during morphogenesis. Casein hydrolysate caused a reverse effect on the rooting media, where it seemed to enhance the initiation of adventitious roots on cineraria plantlets. Any increase or decrease which did result from the addition of casein hydrolysate perhaps is caused by unknown organic factors (Murashige and Skoog, 1962).

Exogenous auxin was not a critical additive since some shoot tip explants developed into plants with no IAA supplement. Nevertheless, the addition of IAA to media improved the vigor of the cultures. Carnation meristem dome explants and gerbera shoot tip explants have also been shown to develop into plants with no IAA supplement (Shabde and Murashige, 1977; Murashige et al., 1974). According to Gautheret (1959) the acquired capacity for increased rates of biosynthesis was responsible for this behavior in his work with auxin. The tissue itself has been postulated to synthesize and release metabolites during its growth, which not only react with particular substances but may modify the physical and physiological properties of the media (Murashige and Skoog, 1962). Therefore, a positive or negative response is likely to reflect the presence in the medium of growth promoting or inhibiting substances.

Many cultures proliferated a mass of plantlets with very short shoots. The addition of a relatively high cytokinin-auxin ratio stimulated the formation of multiple shoots on the modified MS media. This conforms with the production of shoots on tobacco callus cultures by Skoog and Miller (1957). Removal of leaf primordia from cineraria shoot tips may have facilitated the production of many short shoots. This is in agreement with Hackett and Anderson (1967) who found that carnation meristems with excised leaf primordia produced multiple short shoots.

Multiple shoots were often produced from shoots on rooting media lacking kinetin. This growth was possibly caused by the type of organic substances in the media. Also the residual endogenous cytokinin may have been translocated along with the explant into the kinetin-free culture, as was demonstrated by Shabde and Murashige (1977). These researchers postulated that roots formed on a medium containing only auxin eventually became the producer of the source of kinetin for the shoot meristem.

Histological investigation of the developing shoots showed the majority of shoot growth arose from axillary bud growth; while some shoots arose from adventitious buds. This growth may have been regulated by the kind of chemical mechanism observed in tobacco callus cultures by Skoog and Miller (1957).

Because explants placed in culture were derived from different cultures, it is possible that chemical substances (such as auxin and cytokinin) were translocated along with the explant, which

could have had a counter effect on the chemical factors (i.e., organic substances, auxin or cytokinin) in the nutrient media. Thus the variability observed in total yields in response to a given treatment is possibly due to the sensitivity of explants to chemical factors (hormone-like in their action) involved in regulating differentiation and morphogenesis in plants. Variability can be derived from nonuniform stock culture explants. Stock cultures of different size and damage to shoot apices during transfer can yield sub-cultures which differ in both growth rates and yield.

Consistently successful transfers from tissue culture to greenhouse and reestablishment of vigorously growing plants were achieved when the divisions were cultured for 4 weeks on a nutrient medium to produce roots, and acclimatized just prior to transfer to greenhouse. Growth habits and bloom characteristics of plants originated _in vitro_ or in the greenhouse were similar. They were observed to have few obvious variations in leaf shape and flower color, although a double flowering plant did appear. These were not investigated further by performing tests for chromosome aberrations.

CHAPTER VI

SUMMARY AND CONCLUSION

Cineraria shoot tip cultures were obtained that were consistently pathogen-free. The intensity of flower color was predictable in accordance with color of trichomes on cotyledons.

Shoot differentiation was studied in shoot tips of cineraria derived from surface sterilized seeds germinated *in vitro*. The basal medium was revised from that of Murashige and Skoog (1962). Organ differentiation and callus formation varied according to combinations and concentrations of growth substances. The best medium for plantlet regeneration was the modified MS medium (Murashige et al., 1974) containing the supplements: adenine sulfate, L-tyrosine, sodium phosphate, 10mg/liter kinetin, a range of IAA concentrations (0.25-1.00mg/liter), and macroelements at half the recommended concentration. The modified MS medium also produced satisfactory shoot yields with macroelements at the normal recommended concentration having 0.5mg/liter IAA added.

The best medium for adventitious root initiation was MS with macroelements at the normal recommended concentration and supplemented with casein hydrolysate and IAA at 0.0625mg/liter. The MS medium with half the recommended concentration of macroelements, supplemented with casein hydrolysate, and 1.0mg/liter NAA also yielded acceptable root growth.

Differences in survival rate and in organ differentiation on media containing varying organic constituents, inorganic constituents, and auxin concentrations were observed. Variations in morphogenetic development were probably caused partly by inorganic substances and partly by organic substances in the nutrient medium. Depression of shoot growth occurred on media containing casein hydrolysate and/or the normally recommended concentration of macroelements. A change from normal development to severe inhibition of root formation occurred on all plantlets cultured on the modified MS medium. This was a result of the addition of adenine sulfate to the basal medium. A majority of shoots were regenerated from axillary buds. However, histological observations proved that adventitious shoots could originate from epidermal cells and parenchyma vascular tissue of leaf sections. Plants transferred from in vitro cultures were grown to maturity and bloomed in the greenhouse. Plants originated in vitro or according to standard seedling production were generally similar. There was, however, an instance of one double flowering plant. Selection of particular phenotypes, especially leaf shape and blue flower color, has been performed with plants originated in vitro. Plants generated through tissue culture were uniform, grew faster, and produced larger flower heads (capitulum) compared with those obtained by conventional methods of propagation. This difference is a result of selection of good starting plants, clean cultures, and growth factors in the nutrient medium.

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