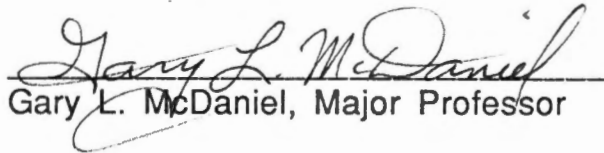
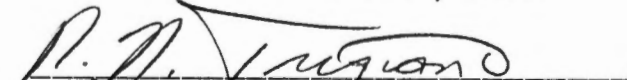
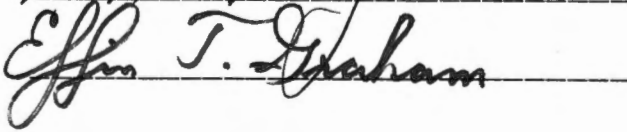


To the Graduate Council:

I am submitting herewith a thesis written by Kathleen Renick Malueg entitled "Regeneration of *Senecio x hybridus* Hyl. (Florists' Cineraria) by Somatic Embryogenesis." 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.


Gary L. McDaniel, Major Professor

We have read this thesis
and recommend its acceptance:

Accepted for the Council:


Vice Provost
and Dean of The Graduate School

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirement for a Master's degree at The University of Tennessee, Knoxville, I agree that the Library shall make it available to borrowers under rules of the Library. Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgment of the source is made.

Permission for extensive quotation from or reproduction of this thesis may be granted by my major professor, or in his absence, by the Head of Interlibrary Services when, in the opinion of either, the proposed use of the material is for scholarly purposes. Any copying or use of the material in this thesis for financial gain shall not be allowed without written permission.

Signature Kathleen Renick Maling
Date November 30, 1990

REGENERATION OF
SENECIO X HYBRIDUS HLY. (FLORISTS' CINERARIA)
BY SOMATIC EMBRYOGENESIS

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Kathleen Renick Malueg
December 1990

AG-VET-MED.

THESIS
90
.1258

ACKNOWLEDGEMENTS

Many people have graciously assisted me in the completion of this degree. In particular I would like to express my gratitude to Dr. Gary McDaniel, for serving as my major professor and giving me the opportunity and the freedom to learn how to begin to think for myself. His knowledge, patience, kindness, friendship, and encouragement were critical to the completion of this endeavor.

I would also like to thank Dr. Effin Graham for serving on my graduate committee, his constant knowledgeable and wise council, his mature example of how science should work and most of all for his endearing friendship. Thank you for being such an excellent role model for my present and future professional responsibilities in science.

I would also like to thank Dr. Robert Trigiano, for his friendship, skilled guidance, emotional support, much needed constructive suggestions, use of his laboratory facilities, and patient instruction in plant tissue culture techniques.

I would also like to thank three wonderful friends who helped me in countless ways: Noel Gerahty for his endless energy, cheerful helping attitude and incredible computer skills; Ann Stodola for her

emotional support and friendship; and Dr. Priyavadan Joshi for valuable suggestions and help with photography.

Finally, I would like to thank my husband Leon and my children, Scott, Dan, Andrea, John, Gina and Missy for managing to accept gracefully all that I have asked of them.

ABSTRACT

The somatic embryogenic potential of *Senecio x hybridus* Hyl (Florists' Cineraria) was explored. Somatic embryos were initiated on semi-solid MS basal media amended with 3 mg/l 2,4-D and 1 mg/l BA (induction medium). An inverse relationship existed between sucrose concentration in the induction medium and globular embryo formation when sucrose exceeded 3%. The first and second leaves, from 13-18 and 20 days post-sown seedlings, respectively, were more responsive explants than cotyledons from the same source and sampling times.

Somatic embryo development beyond the globular stage was greatly delayed or prevented by remaining on auxin-rich induction medium; therefore several two or three media transfer systems using either 0.5% or 1.0% AC were attempted. The two media system using (1) induction medium and a transfer to (2) MS basal medium with 1.0% AC, but without hormones, caused explant death or decline by 28 days post-inoculation to induction medium. The three media transfer system using (1) induction medium, (2) MS basal medium without hormones, supplemented with 0.5% AC for three days followed by a transfer to (3) MS basal medium without hormones and AC produced mature somatic embryos by 28 days post-inoculation to induction medium; however, embryos germinated precociously. Occasionally, explants that were not transferred from the initial

induction medium (controls) developed mature embryos that did not germinate precociously after two-four months. Somatic embryos converted to plants and were grown to maturity; however, most plants were multi-stemmed. Histological examination of mature somatic embryos produced verification of embryo anatomy.

TABLE OF CONTENTS

SECTION 1	1
Introduction.....	2
Literature Review.....	11
Somatic Embryogenesis in Asteraceae.....	14
Tissue Culture Propagation of Cineraria.....	16
Complex In Vitro Requirements for Somatic Embryogenesis.....	18
Somatic Embryo Anatomy.....	22
Literature Cited.....	23
SECTION 2	31
Somatic embryogenesis and plant regeneration of Florists' Cineraria, <i>Senecio x hybridus</i> Hyl. (Asteraceae).....	32
Abstract.....	32
Introduction.....	33
Materials and Methods.....	34
Results and Discussion.....	36
Literature Cited.....	46
A transfer system to optimize somatic embryogenesis of Florists' Cineraria, <i>Senecio x hybridus</i> Hyl. (Asteraceae).....	48
Abstract.....	48
Introduction.....	49
Materials and Methods.....	51
Results and Discussion.....	54
Literature Cited.....	68
VITA	70

LIST OF FIGURES

FIGURE	PAGE
1 Possible methods of plant propagation.....	5
2 Somatic embryo and zygotic embryo similarities and organogenesis and somatic embryogenesis comparison.....	7
3 Somatic embryo verification criteria (Ammirato, 1985).....	9
4 2,4-D and BA chemical structures and effects	13
5 Sucrose concentration and explant age effect on cineraria globular embryo formation.....	37
6 Conversion of cineraria somatic embryo.....	40
7 Cineraria plantlet.....	42
8 Regenerated mature cineraria plant.....	44
9 Effect of time on induction medium and comparison of no transfer and transfer to activated charcoal on cineraria somatic embryogenesis.....	56
10 Cineraria somatic embryo.....	57
11 Effect of transfer to MSO or MSO+1& AC on cineraria somatic embryogenesis.....	60
12 Cineraria somatic embryo anatomy	61
13 Active meristematic shoot region	63
14 Quiescent shoot region	66



SECTION 1

INTRODUCTION

The florists' cineraria, *Senecio x hybridus* Hyl., (Asteraceae), is a daisy-like flowering pot plant with a wide range of flower colors and patterns. Brilliant and beautiful flower colors, including white, pink, red, purple, maroon, magenta and striking hues of blue, are its greatest attribute (Barkley, 1966; Canning, 1928; Cockrel, et al., 1986; Hammer, 1980).

Cineraria is a perennial grown as an annual and is well-suited to the low light intensities of winter production in the Northern Hemisphere. Most cultivars require a low temperature induction treatment to flower (Hildrum, 1969). Plants are propagated from seeds August through November and marketed during late winter and early spring. Because of the requirement for night temperatures of 13-18°C, cineraria is an inexpensive, cool season crop for wintertime greenhouse production (Hammer, 1980; Larsen, 1985).

The cultivation of cineraria began in the late 18th century when Francis Masson, a plant collector for Kew Gardens, gathered several insular endemic *Senecio* species from the Canary Islands. These plants were later hybridized with other *Senecio* species from the Azores and Madeira (Barkley 1966; Rolfe, 1888). Exactly when

or precisely what actions caused the cineraria to differentiate from its progenitors is unknown, but by the early 1800's the florists' cineraria encompassed numerous cultivars, and exhibited great diversity of form and flower characteristics. The available evidence indicates that the modern florists' cineraria is of hybrid origin, involving *Senecio cruentus* and *S. heritieri* and possibly other species; however, a complete factual record of its origin and development is lacking (Barkley, 1966; Canning, 1928; Lynch, 1901).

Commercial production of cineraria is for the flowering pot plant market. Since most cineraria are self-sterile (Barkley, 1966, 1968; Canning, 1928; Lynch, 1901), current propagation is from heterozygous seed developed by cross-pollination, which results in several flower color phenotypes in the commercial crop. Occasionally seeds from self-pollination are produced but plant size and flower quality deteriorates after one or two generations without cross pollination (Barkley, 1968).

Although many flower colors are exhibited by cineraria, the blues are particularly striking, rare in floriculture crops and preferred by consumers. Since commercial seed is heterozygous for flower color, the ratio of desirable blue flowering plants to the less desirable plants in production is unknown until the crop flowers. Therefore, the commercial floriculture industry would benefit from a large scale, reliable, economic method for producing clones propagated from elite mature plants.

Many methods exist for propagating plants (Figure 1), however only a clonal propagation method will ensure a blue flowering cineraria crop. Conventional vegetative propagation has been tried in an effort to produce the desired flower colors, but because of the growth habit of cineraria this method is not feasible for large scale production. Only a limited number of cuttings can be taken from a single plant due to the rosette arrangement of leaves. In addition, clones produced by this method usually have inferior habit and flower quality which becomes more pronounced with each successive vegetatively propagated generation (Barkley, 1966; Canning, 1928).

When conventional vegetative methods fail to provide a solution, micropropagation using plant tissue culture techniques is often attempted. These techniques have become useful in horticultural plant production systems, especially ornamentals, when no efficient, high volume conventional clonal propagation system exists. It has also been useful for disease detection and elimination, germplasm preservation, investigations of morphogenesis, production of secondary products, production of haploid plants by androgenesis, induction of mutations especially multiple branching, and overcoming breeding incompatibilities (Chu, 1986; Gautheret, 1983; Griesbach et al., 1988; Lutz et al., 1985). The first plants clonally propagated using tissue culture techniques were ornamentals (Morel, 1965).

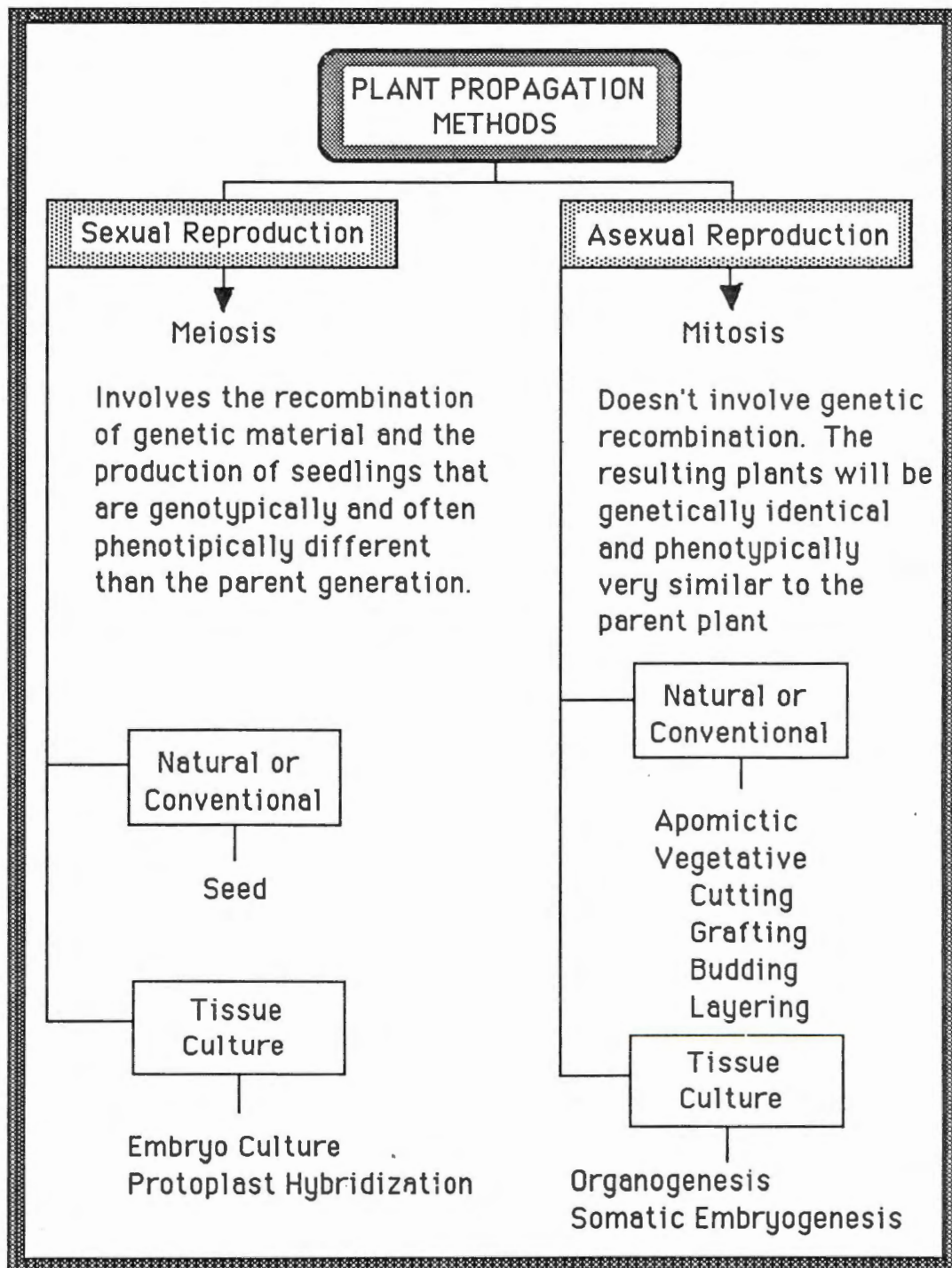


Figure 1: Possible methods of plant propagation

Plant tissue culture is a term that has evolved to embody all types of *in vitro* plant culture including growth of protoplasts, cells, tissues, organs, embryos and plantlets (Tisserat, 1985). Plant tissue, cells or protoplasts in culture are usually moved through a series of media and the morphogenesis that results follows either the organogenic or somatic embryogenic developmental pathway (Ammirato, 1985; Tisserat, 1985).

Organogenesis (Figure 2) is the process that results in the formation of a unipolar structure that has a vascular connection to the explant tissue (Tisserat, 1985). Three organogenic modes for plant regeneration are the following: (1) adventitious organ production from callus, (2) adventitious organ production directly from the explant without callus formation, and (3) axillary bud proliferation (Tisserat, 1985). Axillary bud proliferation is the most commonly used method for commercial clonal propagation (Debergh, 1987; Lutz et al., 1985; Rowe, 1986).

Somatic embryogenesis (Figure 2) is the process that results in the formation of bipolar, embryo-like structures from haploid or diploid somatic cells (Tisserat, 1985; Williams and Maheswaran, 1985). Unlike zygotic embryos that result from fertilization, no genetic recombination takes place (Pierik, 1987; Stuart and Redenbaugh, 1987). Like zygotic embryos, somatic embryos are independent structures, with both root and shoot meristems that are not attached by vascular tissue to the donor explant (Ammirato,

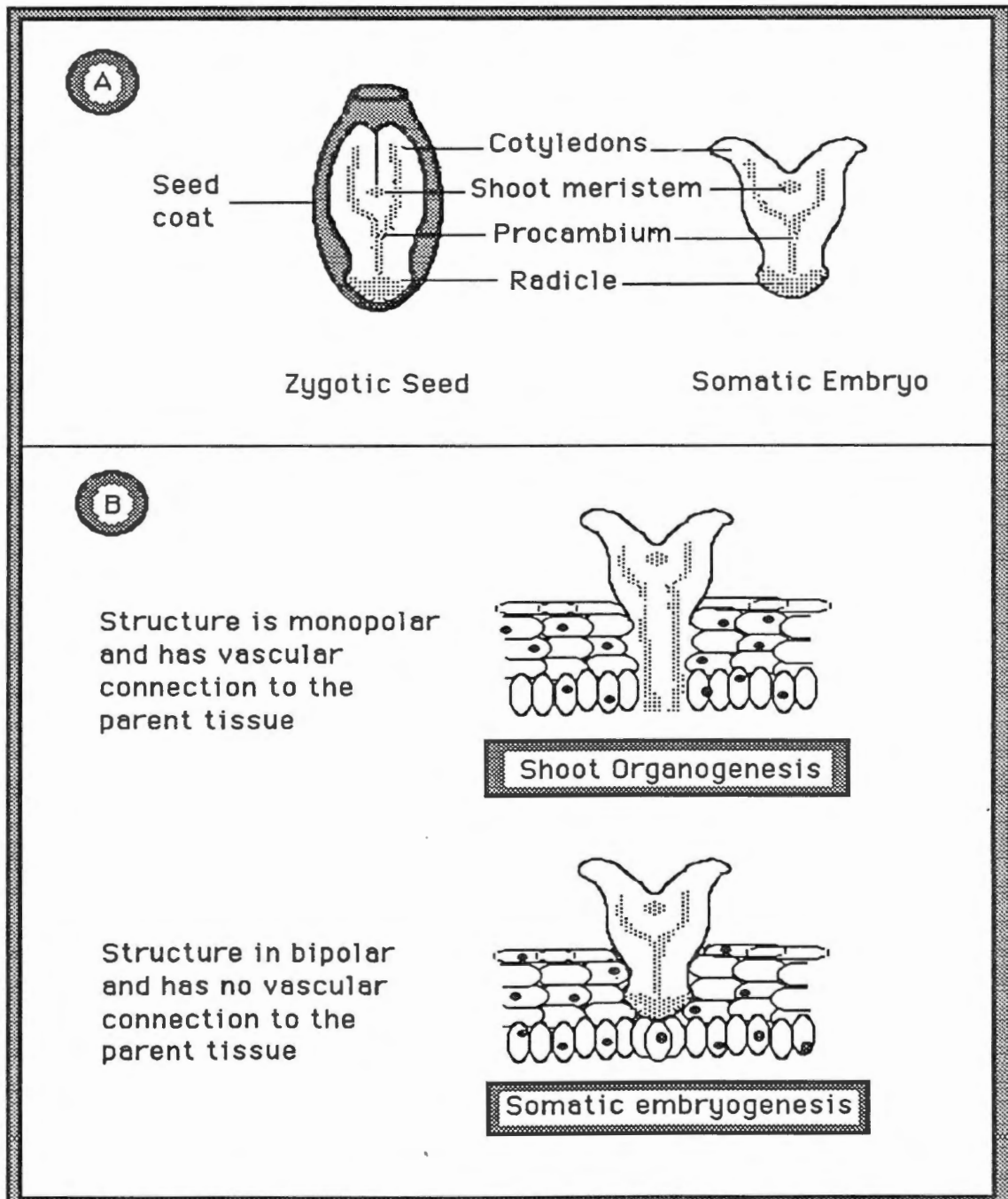


Figure 2: Somatic embryo and zygotic embryo similarities and organogenesis and somatic embryogenesis comparison

- (A) Zygotic embryo and somatic embryo comparison
 (B) Somatic embryogenesis and organogenesis comparison

1983; Haccius, 1978; Tisserat, 1985). They are biochemically similar to zygotic embryos and accumulate embryo-specific storage proteins (Crouch, 1982; Goldberg et al., 1989; Roberts et al., 1990) and can further develop and germinate into plantlets in a manner similar to zygotic embryos (Ammirato, 1983; Stuart and Redenbaugh, 1987). However, a somatic embryo lacks a seed coat and endosperm (Stuart and Redenbaugh, 1987). Somatic embryos are thought to arise from a single cell (Haccius, 1978); however, this has not been substantiated in all cases (Williams and Maheswaran, 1986), and may develop either directly or indirectly. In direct somatic embryogenesis, somatic embryos develop from the explant tissue without an intervening callus stage. In indirect somatic embryogenesis, somatic embryos develop from callus that has formed from the explant tissue (Tisserat, 1985). Verification that a structure is really a somatic embryo can involve three areas of evidence: morphological, anatomical and chemical (Figure 3). Somatic embryos are sometimes referred to as adventive embryos, non-zygotic embryos, embryo-like structures, adventitious embryos, vegetative embryos and embryoids.

Somatic embryogenesis as a clonal propagation system offers several advantages. It has the potential to produce tremendous numbers of plants (Ammirato, 1987; Lutz et al., 1985; Rowe, 1986). If dormancy induction and encapsulation techniques can be developed, somatic embryos could be stored or germinated as "artificial seeds" (Ammirato, 1987; Redenbaugh et al., 1987;

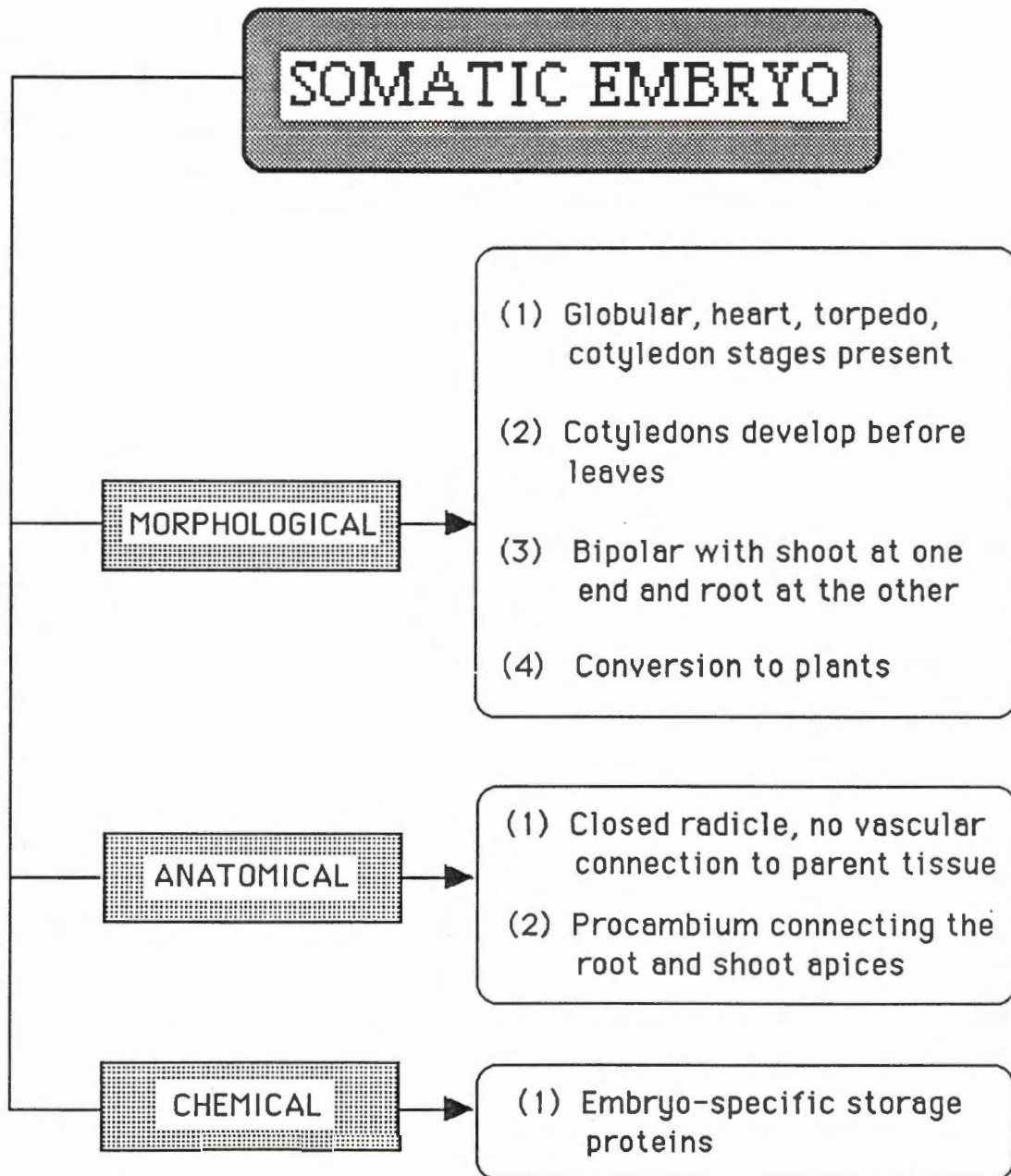


Figure 3: Somatic embryo verification criteria (Ammirato, 1985)

Tisserat et al., 1979). Mechanization possibilities exist, which could increase efficiency and reduce costs (Chu, 1986; Lutz et al., 1985). In addition, somatic embryos might be useful for germplasm preservation (Marin, 1988), a basic research tool to answer questions regarding plant development (Cocking, 1987), for exclusion of pathogens and production of secondary products (Stuart and Redenbaugh, 1987).

Propagation of cineraria by organogenesis has been reported (Cockrel et al., 1986; Gertsson, 1986, 1988a, 1988b, 1988c); however, this method is thought to be less labor and cost efficient than propagation by somatic embryogenesis (Chu, 1986; Lutz et al., 1985). This suggests that regeneration by somatic embryogenesis may be the preferred method of propagation.

The fundamental goal of this research project was to regenerate *Senecio x hybridus*, Hyl. by somatic embryogenesis. Therefore, the objective of this study was to optimize culture conditions that will allow somatic embryo induction, maturation and germination, and to verify with morphological and anatomical evidence that the structures produced are somatic embryos.

The format of this thesis will be as follows: Section 1 will consist of the Introduction, Literature Review and Literature Cited; Section 2 will be two journal articles written in the format suitable for submission to Plant Cell Reports.

LITERATURE REVIEW

Plant Tissue Culture History and Current Status

The history of plant tissue culture began in 1838-1839 when T. Schwann and M. Schleiden independently stated the cellular theory which essentially states that cells are the smallest biological unit with the inherent capability of forming all the cell types found in the adult organism (Gautheret, 1983; Pierik, 1987). This cellular characteristic is today referred to as totipotency (Gautheret, 1983; Schaeffer, 1990). However, no experimental evidence at that time provided confirmation for this theory. Haberlandt (1902, English translation by Krikorian and Berqum, 1969), convinced of the validity of cell totipotency, attempted to culture plant tissues. Even though his results were inconclusive, his experimental methods and careful interpretations laid the foundation for modern plant tissue culture techniques (Gautheret, 1983; Krikorian and Berqum, 1969; Murashige and Huang, 1987).

Advances in plant tissue culture were largely impeded until the discovery of plant hormones. The first plant hormone discovered was an auxin. The pioneering work was begun in the 1880's by

Charles and Francis Darwin while investigating the causes of phototropism. The first auxin was determined to be a chemical substance by Frits Went in 1926 and identified as indole 3-acetic acid by F. Kogl, A. Haagen-Smit and M. Erxleben in 1934 . The work of Folke Skoog and his colleagues during 1945-1955 led to the discovery of the first natural cytokinin, zeatin (isolated from corn), which was isolated by Carlos Miller in 1961 and characterized by D. Letham and co-workers in 1964 (Gautheret, 1983; Goodwin and Mercer; 1983, Raven et al., 1986).

Plant hormones are defined as organic substances, produced by the plant in one location and translocated to another, are active in very small amounts, and result in the inhibition or promotion of growth (Pierik, 1987; Raven et al., 1986). In addition to natural hormones, synthetic hormones have been developed and collectively these compounds are called plant growth regulators. 2,4-D (2,4-dichlorophenoxyacetic acid) and BA (6-benzylamino purine), used exclusively in this research, are plant growth regulators often used in plant tissue culture (Figure 4).

Plant tissue culture techniques rapidly advanced following the discovery of plant growth regulators and improvements in culture nutrient media. However, it was not until 1958 that somatic embryos were first reported (Steward, et al., 1958; Reinert, 1958) and not until 1965 was it shown by H. Kohlenback that they could be produced from cultures derived from isolated single leaf cells. In 1958 Steward and Reinert independently produced somatic embryos

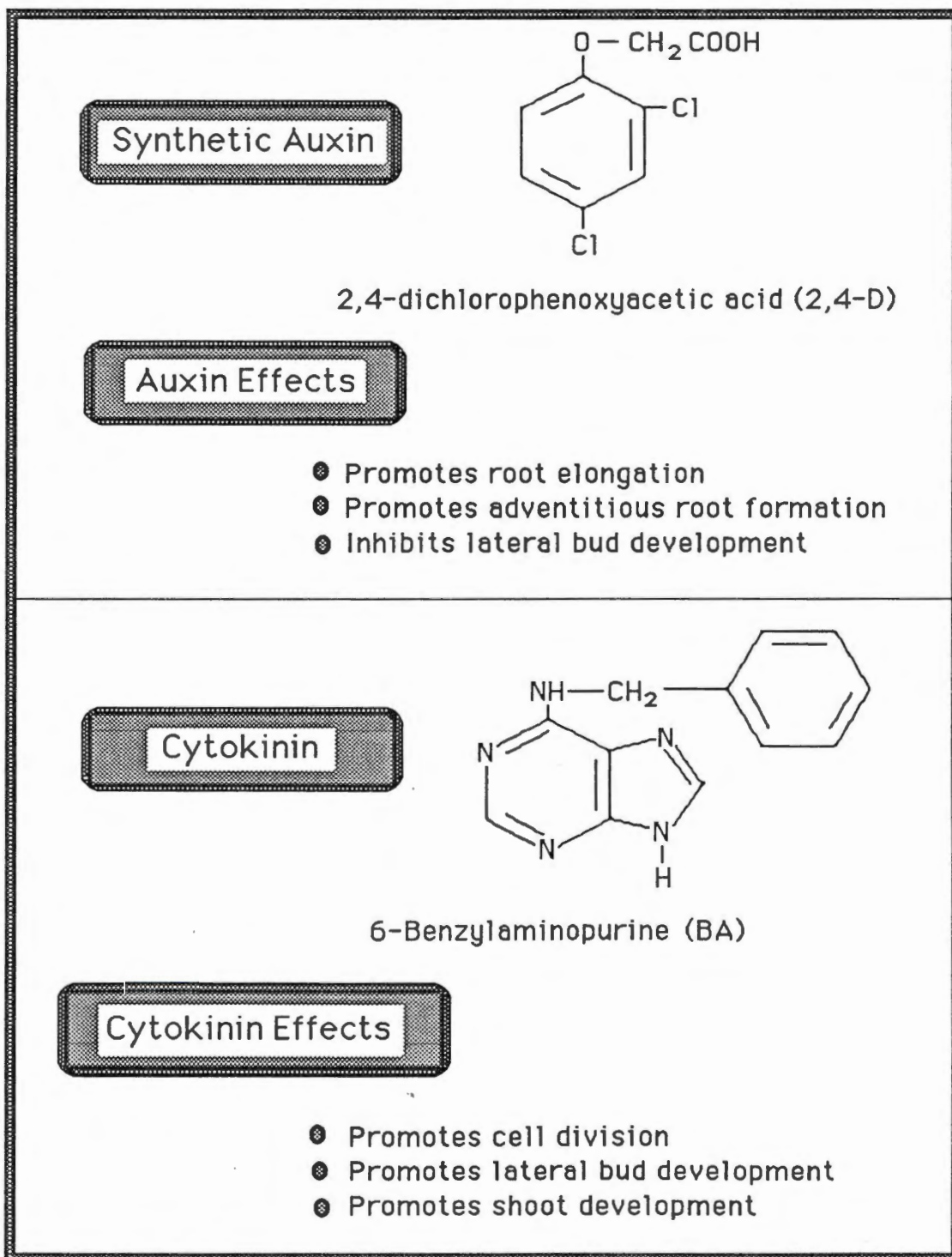


Figure 4: 2,4-D and BA chemical structures and effects

with callus and suspension cultures of carrot, *Daucus carota*, when cultured in basal medium supplemented with coconut milk (CM, liquid endosperm) and auxin. Callus forms when a plant is wounded or cells are grown in culture and consists of a mass of unorganized cells. In 1959, Reinert showed somatic embryos could be induced on synthetic medium. In 1970, additional evidence of cell totipotency was provided by D. Backs-Husemann and J. Reinert when they published a photograph showing the transformation of a single somatic cell into an embryo (Gautheret, 1983; Pierik, 1987).

The technology of plant tissue culture for propagation has more recently become well developed for commercial or research applications. The number of universities, industries, government laboratories and botanical gardens that are currently using tissue culture technologies has steadily increased, with over 200 commercial micropropagation facilities currently operating worldwide (Chu, 1986).

Somatic Embryogenesis in Asteraceae

Only six species of Asteraceae have been reported to produce somatic embryos (Table 1): *Helianthus annuus* L., sunflower (Paterson and Everett, 1985; Finer, 1987; Finer and Norris, 1988; Prado and Berville, 1990; Pélissier et al., 1990), *Cichorium intybus* L., chicory (Heirwegh et al., 1985), *C. endivia* L., endive (Vasil et al., 1964; Vasil and Hildebrandt, 1966), *Chrysanthemum morifolium*

Table 1: Reports of somatic embryogenesis in Asteraceae

PLANT SPECIES	EXPLANTS	HORMONE	MEDIA	REFERENCE
<i>Pterotheca falconeri</i> Hook	root, hypocotyl, stem, leaf, petiole, cotyledon, shoot	IAA	MS, 15% coconut water (semi-solid)	Mehra and Mehra (1971)
<i>Brachycome lineariloba</i> (DC) Druce	leaves, buds	NAA kinetin	Millers B liquid	Gould (1978)
<i>Cichorium endivia</i> Linn	callus derived from zygotic embryos	kinetin	inorganic salts, coconut milk	Vasil, Hildebrandt and Riker (1964)
	Callus derived from zygotic embryos		modified MS (liquid)	Vasil and Hildebrandt (1966)
<i>Cichorium intybus</i> L. var. 'Carolus'	roots	2,4-D BA	MS (semi-solid)	Heirwegh, Banerjee, Nerum and Langhe (1985)
<i>Helianthus annuus</i> L.	hypocotyls	NAA BA GA3	MS (semi-solid)	Paterson and Everett (1985)
	zygotic embryos	Dicamba or 2,4-D	MS (semi-solid)	Finer (1987)
	hypocotyls	2,4-D	liquid	Finer and Norris (1988)
	hypocotyls	2,4-D	MS (liquid)	Prado and Berville (1990)
	hypocotyls	NAA BA	MS (liquid)	Pelissier et al. (1990)
<i>Chrysanthemum morifolium</i> Ramat.	leaf	2,4-D BA	MS (semi-solid)	May and Trigiano (1991)

Ramat., chrysanthemum (May and Trigiano, 1991), *Brachycome lineariloba* (DC) Druce, (Gould, 1978), and *Pterotheca falconeri*, Hook (Mehra and Mehra, 1971). Propagation of cineraria by somatic embryogenesis has not been reported.

Tissue Culture Propagation of Cineraria

Several reports of *in vitro* propagation of cineraria through organogenesis have been published. Iizuka et al. (1973) attempted to regenerate plants from callus obtained from tubular florets; however, few shoots and no roots were produced.

Cockrel et al. (1986) reported shoot multiplication from seedling shoot tips using MS gerbera medium (Murashige et al., 1974) supplemented with 2.8 μ M indole-3-acetic acid (IAA). Root formation was induced using modified MS medium (Murashige and Skoog, 1962) with 1.3 μ M 1-naphthalenacetic acid (NAA). Rooted plants were successfully grown to maturity in soilless medium. Trichome color observed in the explant tissue proved to be an accurate indicator of flower color.

Gertsson (1986) experimented with cineraria explant orientation, hormonal, and media effects on shoot and root formation using petioles from four month old plants with flower buds. Her results indicated that shoot formation was best on either MS (Murashige and Skoog, 1962) or Welander (Welander, 1985) medium

containing BA and IAA, whereas root formation was best on these same nutrient media containing 2iP ($N_6-(\Delta_2\text{-isopentenyl})$ adenine) or zeatin. She also found that explants formed more shoots when placed with their morphological base either downward or sideways in the culture vessel, but more roots were formed when the explant was placed with its base up.

A large scale micropropagation system, using petiole explants, was also reported by Gertsson (1988a). The most successful initial culture medium consisted of MS macronutrients (Murashige and Skoog, 1962), Welander micronutrients (Welander, 1979), Bourgin and Nitsch (1967) organics, supplemented with 4-8 mg/liter IAA concentrations and 1 mg/liter BA. Shoot initiation was successful, but shoot multiplication required a transfer to a medium lower in IAA. Rooting of the shoots was induced by a third transfer onto a medium with reduced BA. Micropropagated plants flowered normally, but some plants had multiple shoots and plant quality was not assessed.

Gertsson (1988b) also investigated the influence of BA and culture temperature on the development of clones from mature seedling plants. She reported that flower color remained consistent, but foliage height, developmental time and number of shoots, leaves and flowers were influenced by BA concentrations. She suggested that BA might be useful for regulating the growth of a crop.

In an effort to improve shoot formation, Gertsson (1988c) also investigated the effects of varying MS macronutrient concentrations and ratios of $\text{NH}_4:\text{NO}_3$, potassium concentrations, TIBA (2,3,5-triiodobenzoic acid, a polar auxin transport blocker), and dark treatments on shoot production. Lowering the nitrogen concentration to 30 mM and potassium to 15 mM, dark treatment immediately after explant inoculation for a two week period, and 1.0 μM TIBA resulted in the highest number of explants forming shoots.

Complex *In Vitro* Requirements for Somatic Embryogenesis

The conditions that will support induction, development and maturation of somatic embryos are often elusive and difficult to determine. This is due to the complexity and interactions of the numerous key variables. Explant choices include donor tissues, orientation in the culture vessel, size and physiological state of maturity of explant tissue. Growth regulator choices include selection of natural or synthetic compounds, ratio of one regulator to another and concentration of each. Optimum basal medium, concentrations, additives and carbohydrate sources must be determined, while keeping in mind that sequential transfers to different media at varying time intervals may be necessary. The addition of activated charcoal to the culture medium may enhance or inhibit somatic embryogenesis. Optimal environmental conditions, including light/dark regimes and temperature, must be provided. The

physical state of the medium, usually semi-solid or liquid, must be chosen before beginning experiments. Culture vessel type, which can include flasks, jars, test tubes, filter paper, bridges, and petri dishes, must be chosen while considering vessel effect on the accumulation of morphogenic substances in the vessel or dissolved medium and the subsequent effect on the cultures (Gould and Murashige, 1985). In addition to all of the above, explant sterilization techniques that will yield axenic cultures while allowing survival of the majority of the explant cells need to be determined empirically (Ammirato, 1983; Evans et al., 1981; Tisserat, 1985).

Sucrose is most commonly used as a carbon source for the explant cells although other carbon sources have been used successfully (Verma and Dougall, 1977, Kinnersley and Henderson, 1988). Many researchers have also found it to be useful as an osmoticum. Raising the sucrose concentration of the initial induction medium to 12% enhanced the formation of embryogenic callus in corn, *Zea mays* L., (Lu and Ozias-Akins., 1982). Precocious germination was prevented in carrot, *Daucus carota*, and water parsnip, *Sium suave*, somatic embryos by increasing the sucrose concentration of the medium (Ammirato and Steward, 1971). In studies of *in vitro* pea, *Pisum sativum*. L., embryo development, prevention of precocious germination was accomplished by raising the sucrose concentration to 10% (Cook, et al. 1988). Additionally, inclusion of 3% mannitol in celery, *Apium graveolens* L., cell

suspensions, which also contained 3% sucrose, produced more singular somatic embryos (Nadel et al., 1989).

Activated charcoal is often used for the absorption of inhibitory compounds, darkening of the medium and absorption of auxins and cytokinins (Pierik, 1987). Inclusion of activated charcoal in the maturation medium has proven advantageous for somatic embryo development and maturation in many species (Buchheim et al., 1989; Fridborg et al., 1978; Litz and Conover, 1980; Tisserat and De Mason, 1980), but deleterious to others (Fridborg et al., 1978; Smith and Krikorian, 1990). Activated charcoal absorbs many substances, including phenol-like compounds, 5-hydroxymethylfurfural (a degradation product from autoclaving sucrose), benzoic acids and considerable amounts of auxins and cytokinins (Ammirato, 1983; Pierik, 1987). A recent report demonstrated that the inclusion of 2.5 g/l activated charcoal in 0.6% semi-solid agar medium with 2×10^{-5} M 2,4-D absorbed all but approximately 4% of the auxin in 1 day and only 2% of the auxin remained at the end of 10 days (Ebert and Taylor, 1990).

Sequential transfers to different media are often necessary to promote induction, maturation and germination of somatic embryos (Ammirato, 1983; Steward et al., 1967). These transfers involve varying media components, such as different types, ratios or concentrations of growth regulators (Evans et al., 1981), or the exclusion of growth regulators and/or the inclusion of abscisic acid (Ackerson, 1984; Ammirato, 1977; Qureshi et al., 1989; Johri and

Rao, 1984). An elaborate sequence of media may be necessary to optimize somatic embryo development.

The requirement for auxin or auxin-like compounds in the primary induction medium for somatic embryo initiation, while its complete absence promoted maturation, was demonstrated for many plant species early in somatic embryogenesis research (Halperin, 1966; Halperin and Wetherell, 1964; Johri, and Rao, 1984; Steward et al., 1967). The most commonly used auxin for somatic embryogenesis is 2,4-D (Evans et al., 1981), however this auxin has been implicated as being responsible for morphological abnormalities, inhibition of somatic embryo development and lack of high frequency conversion in soybean somatic embryos (Buchheim et al., 1989).

Cytokinins are considered important in fostering somatic embryo maturation and cotyledon development (Ammirato, 1983). The stimulation of mitosis may be the major effect of cytokinins on cells in culture; however, a second effect might be the alteration of cell polarity and division planes. In work with white clover, *Trifolium repens*, zygotic embryos cultured in the presence of BA, the first sign of somatic embryo induction was a shift from anticlinal divisions to irregular periclinal and oblique divisions (Williams and Maheswaran, 1985).

Somatic Embryo Anatomy

Cells capable of developing into somatic embryos have many features which are characteristic of rapidly dividing meristematic cells: small size, densely staining cytoplasm, large nuclei containing prominent nucleoli, and the lack of prominent vacuoles. Histochemical analysis suggests intense RNA synthesis and metabolic activity (Johri and Rao, 1984; Williams and Maheswaran, 1985).

In general, zygotic embryogenesis and somatic embryogenesis are very similar processes from the globular stage to maturity; however, there may be differences in cell numbers, cell expansion, presence or absence of suspensor, and shape and number of cotyledons (Williams and Maheswaran, 1985). Populations of somatic embryos may include a mixture of single, twin, fused and clusters of embryos, many of which exhibit premature cell enlargement, precocious vacuolation and differentiation, continued cell divisions and delayed initiation of cotyledons and shoot apex (Ammirato, 1987). Many of the morphological and anatomical abnormalities observed in somatic embryos are believed to be the result of deviations from the normal sequence of cellular organization (Ammirato, 1985; 1987).

LITERATURE CITED

- Ackerson, R. C. (1984). Regulation of soybean embryogenesis by abscisic acid. *J. of Expt. Bot.* 35: 403-413.
- Ammirato, P. V. (1977). Hormonal control of somatic embryo development from cultured cells of caraway. *Plant Physiol.* 59: 579-586.
- Ammirato, P. V. (1983). Embryogenesis. In: *Handbook of Plant Cell Culture*, D. A. Evans, W. R. Sharp, P. V. Ammirato and Y. Yamada eds., (New York: Macmillan Publishing Co.), pp. 82-123.
- Ammirato, P. V. (1985). Patterns of development in culture. In: *Tissue Culture in Forestry and Agriculture*, R. R. Henke, K. W. Hughes, M. J. Constantin and A. Hollaender eds., (New York: Plenum Press), pp. 9-28.
- Ammirato, P. V. (1987). Organizational events during somatic embryogenesis. In: *Plant Tissue and Cell Culture*, C. E. Green, D. A. Somers, W. P. Hackett and D. D. Biesboer eds., (New York: Alan R. Liss, Inc.), pp. 57-81.
- Ammirato, P. V., and Steward, F. C. (1971). Some effects of the environment on the development of embryos from cultured free cells. *Bot. Gaz.* 132: 149-158.
- Barkley, T. M. (1966). A review of the origin and development of the florists' cineraria, *Senecio cruentus*. *Econ. Bot.* 20: 386-395.
- Barkley, T. M. (1968). Observations of the compatibility system in the florists' cineraria, *Senecio cruentus* D. C. (Compositae). *Phyton.* 25: 135-140.

- Bourgin, J. P. and Nitsch, J. P. (1967). Obtention de *Nicotiana haploides* a partir d'etamines cultivees *in vitro*. Annales de Physiologie Vegetale. 9: 377-382.
- Buchheim, J., Colburn, S. and Ranch, J. (1989). Maturation of soybean somatic embryos and the transition to plantlet growth. Plant Physiol. 89: 768-775.
- Canning, E. J. (1928). Cineraria. In: The Standard Cyclopedia of Horticulture, L. H. Bailey ed., (New York: Macmillan and Co.), pp. 771-772.
- Chu, I. Y. E. (1986). The application of tissue culture to plant improvement and propagation in the Ornamental Horticulture Industry. In: Tissue Culture as a Plant Production System For Horticultural Crops, R. H. Zimmerman, R. J. Griesback, F. A. Hammerschlag and R. H. Lawson eds., (Boston: Martinus Nijhoff Publishers), pp. 15-51.
- Cocking, E. C. (1987). Plant cell biology in the 21st century: The needs of plant cell and tissue culture. In: Plant Tissue and Cell Culture, C. E. Green, D. A. Somers, W. P. Hackett and D. D. Biesboer eds., (New York: Alan R. Liss, Inc.), pp. 3-15.
- Cockrel, A. D., McDaniel, G. L. and Graham, E. T. (1986). In vitro propagation of florists' cineraria. HortScience 21: 139-140.
- Cook, S. K., Adams, H., Hedley, C. L., Ambrose, M. J. and Wang, T. L. (1988). An analysis of seed development in *Pisum sativum* VII. Embryo development and precocious germination in vitro. Plant Cell Tissue Organ Cult. 14: 89-101.
- Crouch, M. L. (1982). Non-zygotic embryos of *Brassica napus* L. contain embryo-specific storage proteins. Planta. 156: 520-524.
- Debergh, P. (1987). Recent trends in the application of tissue culture to ornamentals. In: Plant Tissue and Cell Culture, C. E. Green, D. A. Somers, W. P. Hackett and D. D. Biesboer eds., (New York: Alan R. Liss, Inc.), pp. 383-393.

- Ebert, A. and Taylor, H. (1990). Assessment of the changes of 2, 4-dichlorophenoxyacetic acid concentrations in plant tissue culture media in the presence of activated charcoal. *Plant Cell Tissue Organ Cult.* 20: 165-172.
- Evans, D., Sharp, W. and Flick, C. (1981). Growth and behavior of cell cultures: embryogenesis and organogenesis. In: *Plant Tissue Culture: Methods and Applications in Agriculture*, T. A. Thorpe ed., (New York: Academic Press, Inc.), pp. 45-61.
- Finer, J. and Norris, B. (1988). Development of an embryogenic suspension culture of sunflower. *In Vitro.* 24: 59a.
- Finer, J. (1987). Direct somatic embryogenesis and plant regeneration from immature embryos of hybrid sunflower (*Helianthus annuus* L.) on high sucrose-containing medium. *Plant Cell Rep.* 6: 372-374.
- Fridborg, G., Pedersen, M., Landstrom, L. and Eriksson, T. (1978). The effect of activated charcoal on tissue cultures: adsorption of metabolites inhibiting morphogenesis. *Physiol. Plant.* 43: 104-105.
- Gautheret, R. J. (1983). Plant tissue culture: a history. *Bot. Mag. Tokyo.* 96: 393-410.
- Gertsson, U. E. (1986). Influence of explant orientation and media composition on organ formation, growth and nitrogen content in petiole explants of *Senecio x hybridus* Hyl. *J. of Hort. Sci.* 61: 121-128.
- Gertsson, U. E. (1988a). Large-scale in-vitro propagation of *Senecio x hybridus* Hyl. *J. of HortSci.* 63: 131-136.
- Gertsson, U. E. (1988b). Development of micropropagated plants from different clones of *Senecio x hybridus* in relation to BAP concentration and temperature in vitro. *J. of Hort. Sci.* 63: 489-496.
- Gertsson, U. E. (1988c). Influence of macronutrient composition, TIBA and dark treatment on shoot formation and nitrogen content in petiole explants of *Senecio x hybridus*. *J. of Hort. Sci.* 63: 497-502.

- Goldberg, R. B., Barker, S. J. and Perez-Grau, L. (1989). Regulation of gene expression during plant embryogenesis. *Cell*. 56: 149-160.
- Goodwin, T. W. and Mercer, E. I. (1983). Phytohormones and related compounds. In: *Introduction to Plant Biochemistry*, (New York: Pergamon Press), pp. 567-626.
- Gould, A. R. (1978). Diverse pathways of morphogenesis in tissue cultures of the Composite *Brachycome lineariloba* ($2n=4$). *Protoplasma* 97: 125-135.
- Gould, J. and Murashige, T. (1985). Morphogenic substances released by plant tissue cultures. *Plant Cell Tissue Organ Cult.* 4: 29-42.
- Griesbach, R. J., Semeniuk, P., Roh, M. and Lawson, R. H. (1988). Tissue Culture in the Improvement of Eustoma. *HortScience* 23: 790-791.
- Haberlandt, G. (1902). Culturversuche mit isolierten pflanzenzellen. *Sitz-Ber. Mat.-Nat. Kl. Kais. Akad. Wiss. Wien*. 111: (1), 69-92. (Experiments on the culture of isolated plant cells. English translation by A.D. Krikorian and D. L. Berqum. (1969). *Bot. Rev.* 35: 68-88).
- Haccius, B. (1978). Question of unicellular origin of non-zygotic embryos in callus cultures. *Phytomorph.* 28: 74-81.
- Halperin, W. (1966). Alternative morphogenetic events in cell suspensions. *Amer. J. Bot.* 53: 443-453.
- Halperin, W. and Wetherell, D. (1964). Adventive embryony in tissue cultures of the wild carrot, *Daucus carota*. *Amer. J. Bot.* 51: 274-283.
- Hammer, P. A. (1980). Other Flowering Pot Plants. In: *Introduction to Floriculture*, R. A. Larson, ed., (New York: Academic Press), pp. 466-469.
- Heirwegh, K., Banerjee, N., Nerum, K. and Langhe, E. (1985). Somatic embryogenesis and plant regeneration in *Cichorium intybus* L. (Witloof, Compositae). *Plant Cell Rep.* 4: 108-111.

- Hildrum, H. (1969). Factors affecting flowering in *Senecio Cruentus* D.C. Acta Hort. Int. Soc. Hort Sci. 14: 11-123.
- Iizuka, M., Matsumoto, E., Doi, A., Madrigal, R. and Fukushima, A. (1973). Tubular floret culture of chrysanthemum and cineraria in vitro. Jap. J. Genet. 48: 79-87.
- Johri, B. M. and Rao, P. S. (1984). Experimental Embryology. In: Embryology of Angiosperms, B. M. Johri ed., (N.Y.: Springer-Verlag), pp. 735-802.
- Kinnersley, A. and Henderson, W. (1988). Alternative carbohydrates promote differentiation of plant cells. Plant Cell Tissue Organ Cult. 15: 3-16.
- Krikorian, A. D. and Berquam, D. L. (1969). Plant cell and tissue cultures: The role of Haberlandt. Bot. Rev. 35: 59-88.
- Larsen, R. (1985). Temperature and Flower Induction of *Senecio x hybridus* Hyl. Swed. J. Agric. Res. 15: 87-92.
- Litz, R. and Conover, R. (1980). Somatic embryogenesis in cell cultures of *Carica stipulata*. HortScience 15: 733-734.
- Lu, C. and Ozias-Akins, P. (1982). Somatic embryogenesis in *Zea mays* L. Theor. Appl. Genet. 62: 109-112.
- Lutz, J. D., Wong, J. R., Rowe, J., Tricoli, D. M. and Lawrence, R. H., Jr. (1985). Somatic embryogenesis for mass cloning of crop plants. In: Tissue Culture in Forestry and Agriculture, R. . Henke, K. W. Hughes, M. J. Constantin, A. Hollaender and C. M. Wilson eds., (New York: Plenum Press), pp. 105-116.
- Lynch, I. R. (1901). Hybrid cinerarias. J. Royal Hort. Soc. 24: 269-274.
- Marin, M. L. and Duran-Vila, N. (1988). Survival of Somatic embryos and recovery of plants of sweet orange (*Citrus sinensis* (L.) Osb.) after immersion in liquid nitrogen. Plant Cell Tissue Organ Cult. 14: 51-57.

- May, R. and Trigiano, R. (1991). Factors affecting somatic embryogenesis and plant regeneration from leaves of *Chrysanthemum x morifolium* Ramat. J. Amer. Soc. Hort. Sci. 116: (In press).
- Mehra, P. and Mehra, A. (1971). Morphogenetic studies in *Pterotheca falconeri*. Phytomorph. 21: 174-191.
- Morel, G. M. (1965). Producing virus-free *Cymbidiums*. Amer. Orchid Soc. Bull. 29: 495-497.
- Murashige, T. and Huang, L. C. (1987). Cloning Plants by Tissue Culture: Early Years, Current Status and Future Prospects. Acta Hortic. 212: 35-42.
- Murashige, T., Serpa, M. and Jones, J. (1974). Clonal Multiplication of Gerbera through Tissue Culture. HortScience 9: 175-180.
- Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.
- Nadel, B., Altman, A. and Ziv, M. (1989). Regulation of somatic embryogenesis in celery cell suspensions. Plant Cell Tissue Organ Cult. 18: 181-189.
- Paterson, K. and Everett, N. (1985). Regeneration of *Helianthus annuus* inbred plants from callus. Plant Sci. 42: 125-132.
- Pélissier, B., Bouchefra, O., Pepin, R. and Freyssinet, G. (1990). Production of isolated somatic embryos from sunflower thin cell layers. Plant Cell Rep. 9: 47-50.
- Pierik, R. L. M. (1987). Vegetative Propagation. In: In Vitro Culture of Higher Plants, (Boston: Martinus Nijhoff), pp. 13-230.
- Prado, E. and Berville, A. (1990). Induction of somatic embryo development by liquid culture in sunflower (*Helianthus annuus* L.). Plant Sci. 67: 73-82.
- Qureshi, J., Kartha, K., Abrams, S. and Steinhauer, L. (1989). Modulation of somatic embryogenesis in early and late-stage embryos of wheat (*Triticum aestivum* L.) under the influence of

- (+)-abscisic acid and its analogs. *Plant Cell Tissue Organ Cult.* 18: 55-69.
- Raven, P., Everet, R. and Eichhorn, S. (1986). Regulating growth and development: The plant hormones. In: *Biology of Plants*, (New York: Worth Pub., Inc.), pp. 475-492.
- Redenbaugh, K., Slade, D., Viss, P. and Fujii, J. A. (1987). Encapsulation of somatic embryos in synthetic seed coats. *HortScience*. 22: 803-809.
- Reinert, J. (1958). Morphogenese und ihre Kontrolle an Gewebekulturen aus Carotten. *Naturwissenschaften*. 45: 344-345.
- Roberts, D. R., Flinn, B. S., Webb, D. T., Webster, F. B. and Sutton, B. C. (1990). Absciscic acid and indole-3-butyric acid regulation of maturation and accumulation of storage proteins in somatic embryos of interior spruce. *Physiol. Plant*. 78: 355-360.
- Rolfe, R. A. (1888). The Cineraria. *Gardeners' Chronicle* III. 3: 654.
- Rowe, J. W. (1986). New technologies in plant tissue culture. In: *Tissue Culture as a Plant Production System for Horticultural Crops*, R. H. Zimmerman, R. J. Griesbach, F. A. Hammerschlag and R. H. Lawson eds., (Boston: Martinus Nijhoff Publishers), pp. 35-51.
- Schaeffer, W. I. (1990). Terminology associated with cell, tissue and organ culture, molecular biology and molecular genetics. In *Vitro*. 26: 97-101.
- Smith, D. and Krikorian, A. (1990). Somatic proembryo production from excised, wounded zygotic carrot embryos on hormone-free medium: evaluation of the effects of pH, ethylene and activated charcoal. *Plant Cell Rep.* 9: 34-37.
- Steward, F., Kent, A. and Mapes, M. (1967). Growth and organization in cultured cells: Sequential and synergistic effects of growth regulating substances. *Ann. N. Y. Acad. Sci.* 144: 326-334.
- Steward, F. C., Mapes, M. O. and Mears, K. (1958). Growth and organized development of cultured cells. II. Organization in

cultures grown from freely suspended cells. Amer. J. Bot. 45: 705-708.

- Stuart, D. A. and Redenbaugh, M. K. (1987). Use of somatic embryogenesis for the Regeneration of Plants. In: Biotechnology in Agricultural Chemistry, H. LeBaron, R. Mumma and D. Honeycutt eds., (Washington, D.C.: American Chemical Society), pp. 87-96.
- Tisserat, B. (1985). Embryogenesis, organogenesis and plant regeneration. In: Plant Cell Culture, R. A. Dixon ed., (Oxford, England: IRL Press), pp. 79-105.
- Tisserat, B. and De Mason, D. (1980). A histological study of development of adventive embryos in organ cultures of *Phoenix dactylifera* L. Ann. Bot. 46: 465-472.
- Tisserat, B., Esan, E. B. and Murashige, T. (1979). Somatic embryogenesis in angiosperms. Hort. Rev. 1: 1-78.
- Vasil, I., Hildebrandt, A. and Riker, A. (1964). Endive plantlets from freely suspended cell and cell groups grown *in vitro*. Science. 146: 76-77.
- Vasil, I. and Hildebrandt, A. (1966). Variations of morphogenetic behavior in plant tissue cultures I. *Cichorium endivia*. Amer. J. Bot. 53: 860-869.
- Verma, D. and Dougall, D. (1977). Influence of carbohydrates on quantitative aspects of growth and embryo formation in wild carrot suspension cultures. Plant Physiol. 59: 81-85.
- Welander, T. (1985). Micropropagation of *Begonia x lorraine* hybr. cv Astrid. Report 39. Swed. Univ. of Agric. Sci., Depart. of Hort. Sci.
- Welander, T. (1979). Influence of medium composition on organ formation in explants of *Begonia x hiemalis in vitro*. Swed. J. of Agric. Res. 9: 163-168.
- Williams, E. G. and Maheswaran, G. (1986). Somatic embryogenesis: Factors influencing coordinated behavior of cells as an embryogenic group. Ann. of Bot. 57: 443-462.

SECTION 2

Somatic embryogenesis and plant regeneration of Florists' Cineraria, *Senecio x hybridus* Hyl. (Asteraceae)*

Abstract

Somatic embryos of *Senecio x hybridus* Hyl., (florists' cineraria) were initiated on semi-solid MS basal media amended with 3 mg/l 2,4-D and 1 mg/l BA. Globular embryo formation was inversely proportional to the sucrose concentration in the induction medium when sucrose exceeded 3%. The first and second leaves, from 13-18 and 20 days post-sown seedlings, respectively, were more responsive explants than cotyledons. Mature somatic embryos seldom formed until two-to-four months after inoculation onto induction medium and most were malformed. Somatic embryos transferred to MS medium without hormones converted to plants and were grown to maturity; however, most plants were multi-stemmed.

* Kathleen R. Malueg, Gary L. McDaniel, Robert N. Trigiano, and Effin T. Graham, Department of Ornamental Horticulture and Landscape Design, The University of Tennessee, Knoxville, 1990. Paper to be submitted for publication.

Abbreviations

2,4-D, 2,4-dichlorophenoxyacetic acid; BA, 6-benzylamino purine.

Introduction

The florists' cineraria is a compact, low temperature-requiring, pot plant that exhibits a wide range of flower colors, including brilliant hues of blue. Blue flowers are rare in floriculture crops and therefore desirable. Since nearly all cineraria are self-sterile (Barkley, 1968), seed is produced from crosses which maintain heterozygosity for flower color in the commercial crop. Recently it has become possible to purchase seed that will produce blue flowering plants, although it is expensive. Conventional clonal propagation has been attempted in an effort to produce the desired blue flowers; however, this method is not efficient for large-scale production because of the rosette arrangement of leaves. Additionally, clones produced by this method usually have inferior habits and flower quality diminishes with each successive vegetatively-propagated generation (Barkley, 1968; Canning 1928). The development of an in vitro clonal propagation system would be advantageous to the floriculture industry for production of large quantities of selected clones.

Propagation of the florists cineraria by organogenesis has been accomplished using shoot tips from in vitro grown seedlings (Cockrel et al., 1986) and petioles from four month old plants (Gertsson, 1986, 1988). No report of plant regeneration by somatic embryogenesis has been reported. Propagation by somatic embryogenesis may offer an efficient method of clonal propagation due to the potentially large number of plantlets regenerated (Ammirato, 1987).

In this study we report (1) the effects of varying sucrose on somatic embryo induction, (2) the effect of maturity of the leaf or cotyledon explant on the formation of globular somatic embryos, and (3) the conversion of somatic embryos to plants.

Materials and Methods

1. Plant Materials

Explants for all experiments were from seedlings and plants of the 'Hansa' cultivar (George W. Park Seed Co., Greenwood, SC.). Seeds were sown in soilless potting medium (Premier Pro-Mix, Cassco, Montgomery, AL), covered with translucent plastic wrap and allowed to germinate in a flat at approximately 21°C. Seedlings were grown at approximately 25°C and a 16 hour photoperiod with 50 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ of fluorescent light. These and all other measurements of light

were made with a 21X micrologger (Campbell Scientific, Inc.) and LiCor Quantum sensor (LiCor, Inc., Lincoln, NE) .

Four types of explants were used in this study: (1) cotyledons, (2) not yet fully expanded first leaves, and (3) second leaves removed from seedlings 13-20 days post-sowing; and (4) 3-4.25 cm long leaves from 1.5 month old plants. The first three types of explants were bisected longitudinally, and the fourth type was cut to yield 6-12 explants from center portions of each leaf.

2. Media

The effects of sucrose on induction of somatic embryos was investigated using Murashige and Skoog (1962) basal medium amended with 3%, 6%, 9%, 12%, or 15% sucrose (w/v), 3 mg/l 2,4-D and 1 mg/l BA, 0.8% Phytagar® (Gibco Laboratories, Grand Island, NY), and adjusted to pH 5.8 before autoclaving. Media were poured into 12 well dishes or 60mm petri dishes, and wrapped with Parafilm®. The induction medium for the investigation of explant age was identical to the above medium except only 3% sucrose was used.

Two-to-four months following plating onto induction medium, white somatic embryos were removed from the original cultures and transferred to 60mm petri dishes containing MS medium without hormones with 3% sucrose, (maturation medium) and placed in a lighted incubator. The resulting plantlets were placed into GA7 boxes (Magenta Corp., Chicago, IL) on fresh maturation medium and

eventually transplanted to soilless potting medium (Premier Pro-Mix, Cassco, Montgomery, AL).

3. Surface Sterilization and Culture Incubation

Explants were surface sterilized in a 10% (v/v) commercial bleach (0.525% NaHOCl) solution supplemented with 0.1% Triton X-100 for 5-12 minutes with gentle agitation followed by three rinses with autoclaved water. Cultures were incubated for 2 weeks in the dark at 22°C, moved to a lighted incubator with 25 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of light (cool white fluorescent tubes) for 10-15 days and returned to the dark incubation. Assessment of globular embryo formation was made with the aid of a stereoscope 28 days after culture initiation.

Results and Discussion

The sucrose concentration of the induction medium affected somatic embryo formation. In experiments using 15-16 days post-sown cotyledons and first true leaves, increased sucrose concentration in the induction medium resulted in a decreased number of explants forming globular embryos (Figure 5-A, cotyledons and first leaves). This inverse relationship was also observed with leaf explants from 6 week-old plants (Figure 5-A, juvenile leaves). These results are dissimilar to reports of two other members of Asteraceae: *Chrysanthemum x morifolium* Ramat. somatic embryos formed on medium containing 9 to 18% sucrose,

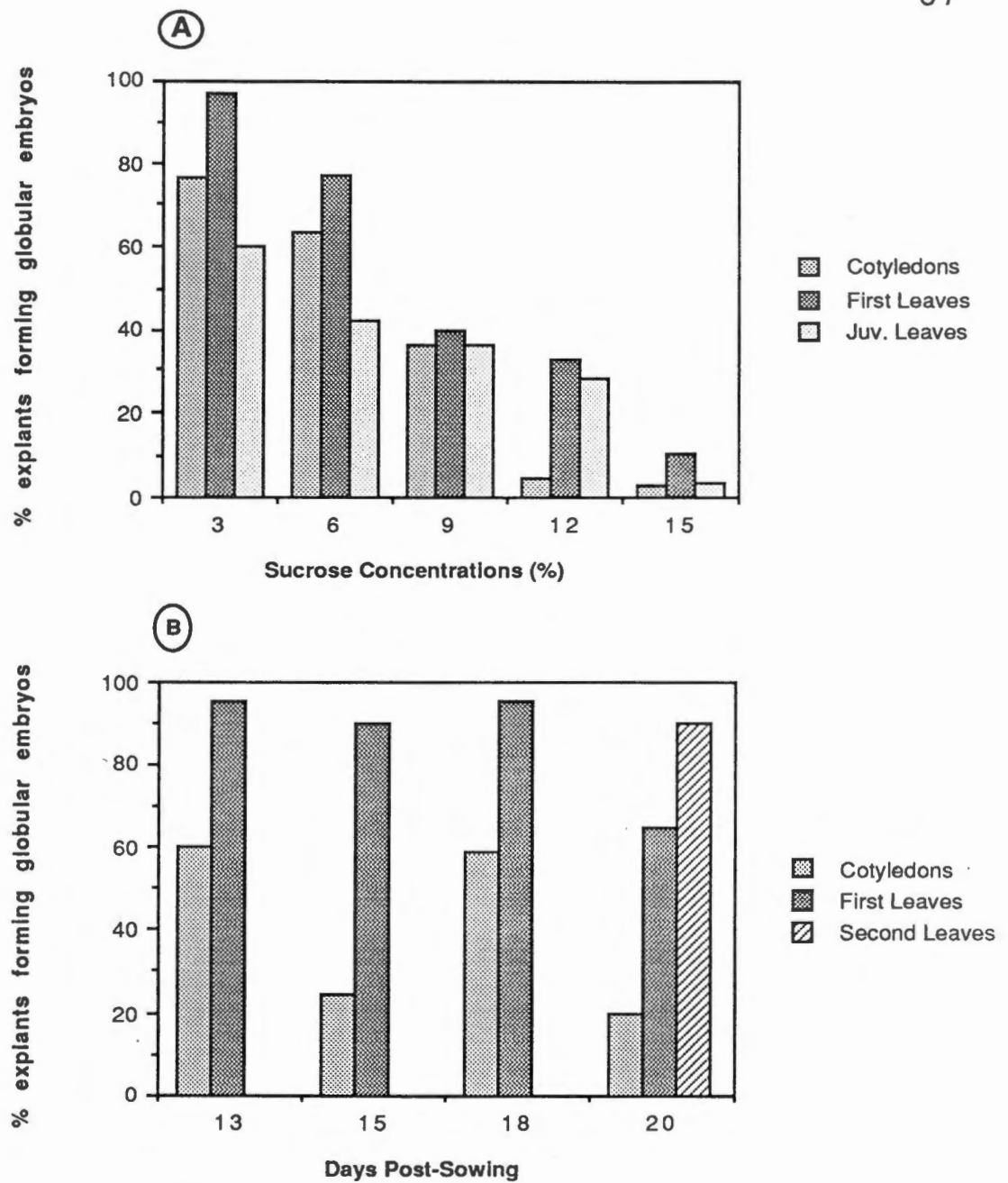


Figure 5: Sucrose concentration and explant age effect on cineraria globular embryo formation

(A) Effect of varying sucrose concentration on globular embryo formation

(B) Effect of explant age on globular embryo formation

with 12 or 15% sucrose being the most efficient concentrations (May and Trigiano, 1991) and *Helianthus annuus* L. required 6 or 12% sucrose containing medium for somatic embryo induction (Finer, 1987).

The physiological maturity of the explant has a direct effect on regenerative capacity of the resulting culture. Explants from vegetative plants generally respond with higher frequency than explants from adult plants. Embryonic, meristematic, young vegetative material, and reproductive tissues are often excellent choices for somatic embryogenic cultures (Ammirato, 1983). In this study, age of explants was found to affect somatic embryo formation (Figure 5-B). First leaf explants 13-18 days post-sowing showed a high capacity for forming globular embryos; however, this declined by 20 days post-sowing. Second leaf explants (20 days post-sowing) were almost as capable of forming globular embryos as the first leaf explants 13-18 days post sowing. Explants from cotyledons were the least responsive explants. These results indicate that first and second leaves are good explant choices, but cotyledons are not the optimal explant at 13 or more days post-sowing.

In all experiments, globular embryos were the most common type present 28 days after initial plating; however, more mature stages were occasionally present. Two-to-four months post inoculation onto induction medium (with no transfer), more heart-shaped and torpedo-shaped embryos were observed; however, most

embryos were pluricotyl, and appeared larger than zygotic embryos. When somatic embryos were transferred to MS medium without 2,4-D and BA, most converted to plants (Figures 6-8). Few explants yielded mature somatic embryos, which might be a result of remaining on 2,4-D-rich induction medium. 2,4-D has been implicated as being responsible for morphological abnormalities, inhibition of somatic embryo development and the lack of high frequency conversion in *Glycine max* L. Merr. somatic embryos (Buchheim et al., 1989).

The majority of regenerated plants from somatic embryos did not have a single basal stem, a normal characteristic of cineraria. This may be caused by too high a concentration of cytokinin or remaining too long on cytokinin-rich medium, since cytokinins strongly promote axillary bud initiation.

This study demonstrated that somatic embryos can develop from leaf and cotyledon explants of the florists' cineraria, and that explant age and sucrose concentration affected somatic embryo formation. More research is needed to further refine culture conditions in an effort to increase efficiency and reduce abnormalities.

Figure 6 : Conversion of cineraria somatic embryo



Figure 7: Cineraria plantlet



Figure 8: Regenerated mature cineraria plant



Literature Cited

- Ammirato, P. V. (1983). Embryogenesis. In: Handbook of Plant Cell Culture: Techniques for Propagation and Breeding, D. Evans, W. Sharp, P. Ammirato and Y. Yamada eds., (New York: MacMillan Pub. Co.), pp. 82-122.
- Ammirato, P. V. (1987). Organizational events during somatic embryogenesis. In: Plant Tissue and Cell Culture, C. E. Green, D. A. Somers, W. P. Hackett and D. D. Biesboer eds., (New York: Alan R. Liss), pp. 57-81.
- Barkley, T. M. (1968). Observations of the compatibility system in the florists' cineraria, *Senecio cruentus* D.C. *Phyton* 25: 135-140.
- Buchheim, J., Colburn, S. and Ranch, J. (1989). Maturation of soybean somatic embryos and the transition to plantlet growth. *Plant Physiol.* 89: 768-775.
- Canning, E. J. (1928). Cineraria. In: The Standard Cyclopedia of Horticulture, L. H. Bailey ed., (New York: Macmillan and Co.), pp. 771-772.
- Cockrel, A. D., McDaniel, G. L. and Graham, E. T. (1986). In vitro propagation of florists' cineraria. *HortScience*. 21: 139-140.
- Finer, J. (1987). Direct somatic embryogenesis and plant regeneration from immature embryos of hybrid sunflower (*Helianthus annuus* L.) on high sucrose-containing medium. *Plant Cell Rep.* 6: 372-374.
- Gertsson, U. E. (1986). Influence of explant orientation and media composition on organ formation, growth and nitrogen content in petiole explants of *Senecio x hybridus* Hyl. *J. of Hort. Sci.* 61: 121-128.
- Gertsson, U. E. (1988). Large-scale in-vitro propagation of *Senecio x hybridus* Hyl. *J. of Hort. Sci.* 63: 131-136.

- May, R. and Trigiano, R. (1991). Factors affecting somatic embryogenesis and plant regeneration from leaves of *Chrysanthemum x morifolium*. *Ramat. J. Amer. Soc. Hort. Sci.* 116: (In press).
- Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473-497.

A transfer system to optimize somatic embryogenesis of Florists' Cineraria, *Senecio x hybridus* Hyl. (Asteraceae)*

Abstract

Development of somatic embryos of *Senecio x hybridus* Hyl. beyond the globular stage was greatly delayed or prevented by remaining on auxin rich induction medium; therefore, two or three media transfer systems using activated charcoal (AC) were tested. The two media system using (1) MS supplemented with 3 mg/l 2,4-D and 1 mg/l BA for varying lengths of time followed by transfer to (2) MS basal medium without 2,4-D and BA supplemented with 1.0% AC caused explant death by 25-28 days post-initial plating. A three media transfer system using: (1) MS basal medium supplemented with 3 mg/l 2,4-D and 1 mg/l BA for varying lengths of time followed by transfer to (2) MS basal medium without 2,4-D and BA supplemented with 0.5% AC for three days and a final transfer to (3) MS basal medium without 2,4-D, BA or AC produced mature somatic embryos by 28 days post-initial plating; however, embryos germinated precociously. Mature embryos that did not germinate precociously developed after approximately 7 weeks in culture

* Kathleen R. Malueg, Gary L. McDaniel, Robert N. Trigiano, and Effin T. Graham, Department of Ornamental Horticulture and Landscape Design, The University of Tennessee, Knoxville, 1990. Paper to be submitted for publication.

without transfer from the initial medium. Verification of embryo anatomy was accomplished by histological examination of mature somatic embryos.

Abbreviations

AC, activated charcoal; BA, 6-benzylamino purine; CrAF III, chromium trioxide, acetic acid, and formaldehyde; 2,4-D, 2,4-dichlorophenoxyacetic acid

Introduction

Blue flowers are rare in floriculture crops, therefore the floriculture industry would benefit from a clonal propagation system that could produce commercial quantities of selected elite blue flowering Florists' cineraria. Recent advances in molecular genetics have made plant transformations effecting flower pigmentation possible (Meyer et al., 1987). However, a successful regeneration system is necessary to make transformed plants available commercially. Somatic embryogenesis potentially could be the means to supply large numbers (Chu, 1986) of naturally blue or transformed blue clones.

The necessity for auxin or auxin-like compounds in the primary induction medium for somatic embryo initiation, while its complete absence promoted maturation, was demonstrated early in somatic

embryogenesis research (Halperin, 1966; Halperin and Wetherell, 1964, Steward et al., 1967). The most commonly used auxin for somatic embryogenesis is 2,4-D (Evans et al., 1981). This auxin, however, has been implicated as being responsible for morphological abnormalities, inhibition of somatic embryo development and lack of high frequency conversion in soybean somatic embryos (Buchheim et al., 1989).

Sequential transfers to different media are often necessary to promote induction, maturation and germination of somatic embryos (Ammirato, 1983, Steward et al., 1967). These transfers involve varying media components such as different types, ratios or concentrations of growth regulators (Evans, 1981) or the exclusion of growth regulators and/or the inclusion of ABA (Ackerson, 1984; Ammirato, 1977; Qureshi et al., 1989). An elaborate sequence of media may be necessary to optimize somatic embryo development.

Activated charcoal (AC) is often used in primary and/or maturation media for the absorption of inhibitory compounds, darkening of the medium and absorption of auxins and cytokinins. Inclusion of a AC in the maturation medium has proven advantageous for somatic embryo development and maturation in several species (Buchheim et al., 1989; Litz and Conover, 1980; Tisserat and De Mason, 1980), but deleterious to others (Smith and Krikorian, 1990). AC is thought to absorb many substances, including phenol-like compounds, 5-hydroxy-methylfurfural (a degradation product of autoclaving sucrose), benzoic acids and considerable amounts of

auxins and cytokinins (Ammirato, 1983). Ebert and Taylor reported that the inclusion of 2.5 g/l activated charcoal in 0.6% semi-solid agar medium with 2×10^{-5} M 2,4-D absorbed all but approximately 4% of the auxin in 1 day and only 2 % of the auxin remained at the end of 10 days (Ebert and Taylor, 1990). However, AC also will absorb certain iron chelates, thereby sometimes interfering in the transition from globular to heart-shaped embryos (Heberle-Bors, 1980).

In this study we report on the following: (1) the beneficial effects of a three media transfer system, (2) the precocious germination of embryos produced by this method, (3) the appearance of somatic embryos on aged cultures that do not precociously germinate, (4) the effect of varying time on induction medium on globular embryo formation, and (5) somatic embryo anatomy.

Materials and Methods

1. Plant Materials

Seeds of the 'Hansa' cultivar (George W. Park Seed Co., Greenwood, SC) were sown in soilless potting medium (Premier Pro-Mix, Cassco, Montgomery, AL), covered with translucent plastic wrap and allowed to germinate in a flat at approximately 21°C. Seedlings were grown at approximately 25°C and a 16 hour photoperiod with $50 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ of fluorescent light. Explants consisted of not yet fully expanded first leaves 11-18 days post-germination (less than

1 cm in length). All leaves were bisected longitudinally and both halves placed together on the induction medium.

2. Media

The induction medium used for all experiments was MS basal medium (Murashige and Skoog, 1962) amended with 3% sucrose (w/v), 3 mg/l 2,4-D and 1 mg/l BA, 0.6%-0.8% (w/v) Phytagar® (Gibco, Cat # 670-0675 IN) adjusted to pH 5.8. Media were autoclaved at 121°C and 1.06 Kg/cm² of pressure for 20 minutes, poured into 60 mm petri dishes and wrapped with Parafilm®.

Transfer media consisted of (1) MS basal medium without 2,4-D and BA (MSO), (2) MS basal medium without 2,4-D and BA amended with 0.5% AC (MSO+0.5% AC) and (3) MS basal medium without 2,4-D and BA amended with 1.0% AC (MSO+1.0% AC).

3. Surface Sterilization

Explants were surface sterilized in a 10% (v/v) commercial bleach (0.525% NaHOCl) solution supplemented with 0.1% Triton X-100 for 5-10 minutes with vigorous agitation followed by three rinses with sterile, distilled water. A 30 second immersion in 70% EtOH followed by three rinses with autoclaved water prior to the standard NaHOCl treatment was added to the above procedures for

the final experiments, which resulted in a reduction of culture contamination.

4. Transfer Times

In order to determine the most efficient timing and media for somatic embryogenesis, explants remained on induction medium 4-15 days, with transfers being made daily or on alternate days to one of the three transfer media defined above. Experiments included transfer systems with only one transfer and some with two transfers. In the final experiment, explants were transferred at varying times from induction medium to MSO+0.5% AC for three days, then transferred to MSO.

5. Culture Incubation and Assessment

Cultures were incubated in the dark at approximately 22°C. Assessment of embryo formation was made with a stereoscope at 25-28 days post-inoculation onto induction medium. In each experiment, control leaves remained on induction medium for comparison to transfer treatments. Number of explants forming globular, heart, and torpedo stages were counted along with numbers of each embryo type per treatment.

6. Histological Procedures

Explants and somatic embryos were fixed in CrAF III (Sass, 1958), degassed in a vacuum chamber, dehydrated in a graded ethanol/t-butanol series and embedded in Paraplast+® (Sherwood Medical, St. Louis, MO). Sections (10 µm) were cut on a rotary microtome, expanded on a water pool on glass slides, and dried on a warming tray. Sections were deparaffinized in Americlear® and stained in (1) 0.1% toluidine blue for 15-30 minutes or (2) 0.1% safranin O for 1-24 hours, 0.1% crystal violet for 15-30 minutes and 0.01% fast green FCF for 15-30 minutes. The stained sections were dehydrated in ethanol, cleared with Americlear® and cover glasses were affixed with Pro-Texx® resin.

Results and Discussion

Most explants began to curl after four days on induction medium. After six days, explants developed a crystalline-like gloss on all surfaces viewed and globular embryos could be seen on many explants 13 days post-plating to induction medium.

In an effort to determine optimum length of time on the induction medium, while also improving embryo maturation and survival, a three media transfer system, with reduced AC concentration and length of exposure (0.5% for three days) was developed (Figure 9-A). After 11 or more days of exposure to

induction medium, explant response steadily improved, with increased globular, heart and torpedo stages observed; however, the optimal time to remain on the induction medium could be beyond 14 days (Figure 9-A). Embryo formation was retarded by high auxin concentrations on the control explants (no transfer from induction medium). Several mature somatic embryos developed on control plates (no transfer from induction medium) approximately 12 weeks following initial plating (Figure 10). The appearance of mature embryos on aged cultures is probably due to the gradual degradation of the hormones in the induction medium over time, thereby allowing the embryos to mature. These embryos did not germinate precociously, perhaps because of the more dehydrated conditions of the culture vessel environment.

A comparison was made between explants transferred at varying intervals to MSO+1.0% AC and explants remaining on auxin-rich induction medium (Figure 9-B). Explants transferred to MSO+1.0% AC after 4 or 6 days on induction medium did not form globular embryos, indicating the necessity for a longer exposure to the induction medium for embryo initiation if AC is present in the transfer medium. Six weeks after initial plating, two explants (one transferred to AC after 13 days on induction medium, and one transferred to AC after 15 days on induction medium) formed white somatic embryos. Seven weeks after plating, two control plates

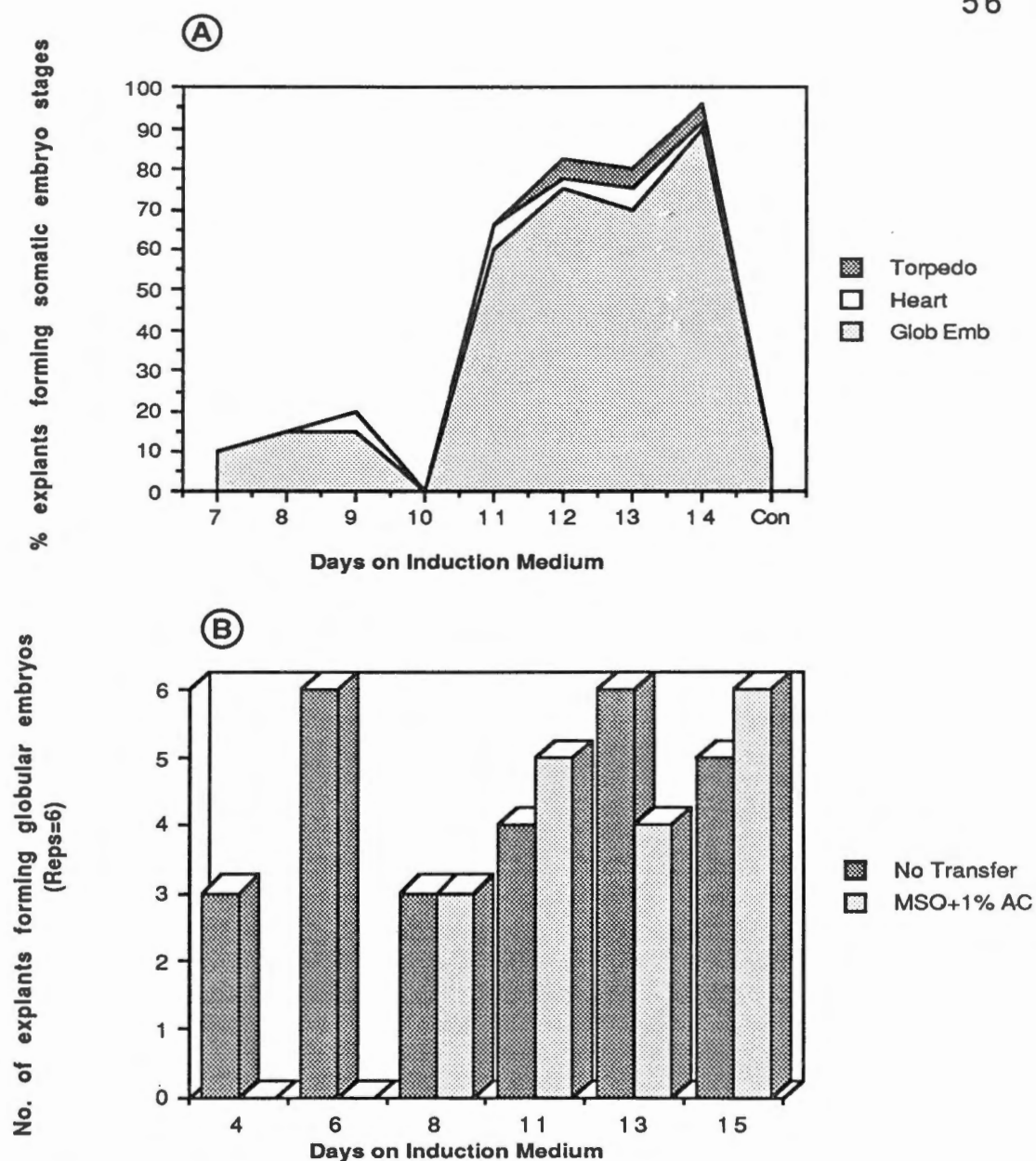
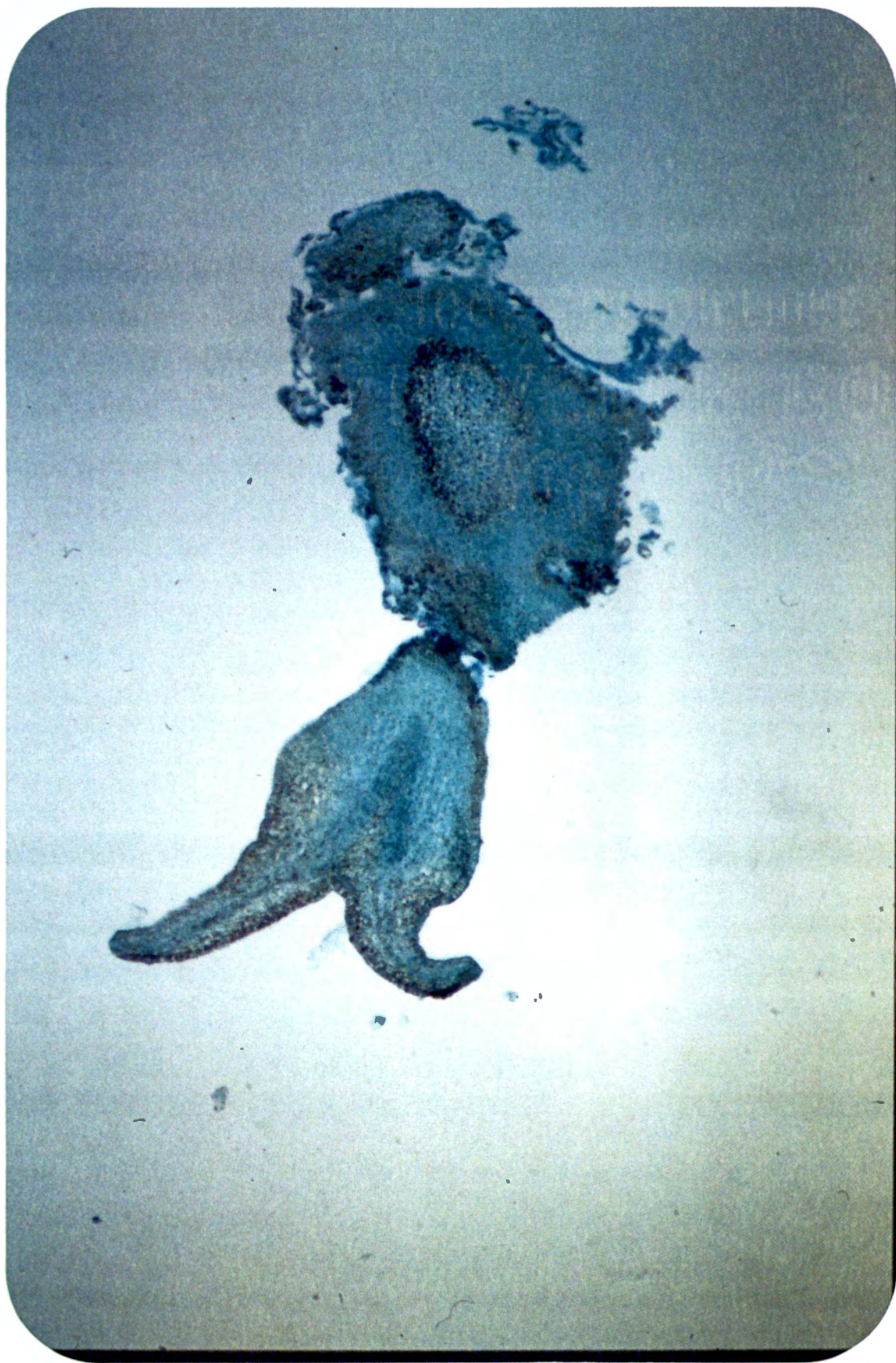


Figure 9: Effect of time on induction medium and comparison of no transfer and transfer to activated charcoal on cineraria somatic embryogenesis

- (A) Effect of time on induction medium on number of explants forming globular, heart and torpedo stage embryos
- (B) Effect of no transfer and transfer to 1.0% activated charcoal on globular embryo formation

Figure 10: Cineraria somatic embryo



(no transfer from induction medium) had one white somatic embryo each. All somatic embryos exhibited abnormal cotyledonary growth. After 7 weeks in culture almost all of the explants transferred to MSO+1.0% AC were dead or browning. Somatic embryos germinated precociously after transfer from induction medium, while remaining on induction medium repressed further development of most globular embryos.

The comparison of the effects of transfer to MSO and MSO+1.0% AC is shown in Figure 11. More mature somatic embryos formed on explants transferred to MSO than those transferred to MSO+1.0% AC (Figure 11-A). However, when assessing all stages of embryo development occurring on explants, the two transfer media produced similar responses (Figure 11-B). Somatic embryos germinated precociously on both transfer media. The majority of the explants that remained on induction medium as controls only formed globular embryos, suggesting that remaining on auxin rich medium prevented embryo development beyond the globular stage (data not shown).

Histological examination of mature somatic embryos produced by the three media transfer system showed evidence of normal embryo anatomy: both root and shoot apices were well organized, no vascular connection to the parent tissue was evident, and a procambium connection of the root and shoot apices was visible (Figure 12). Examination of these embryos showed evidence of an active meristematic shoot region (Figure 13) while embryos that

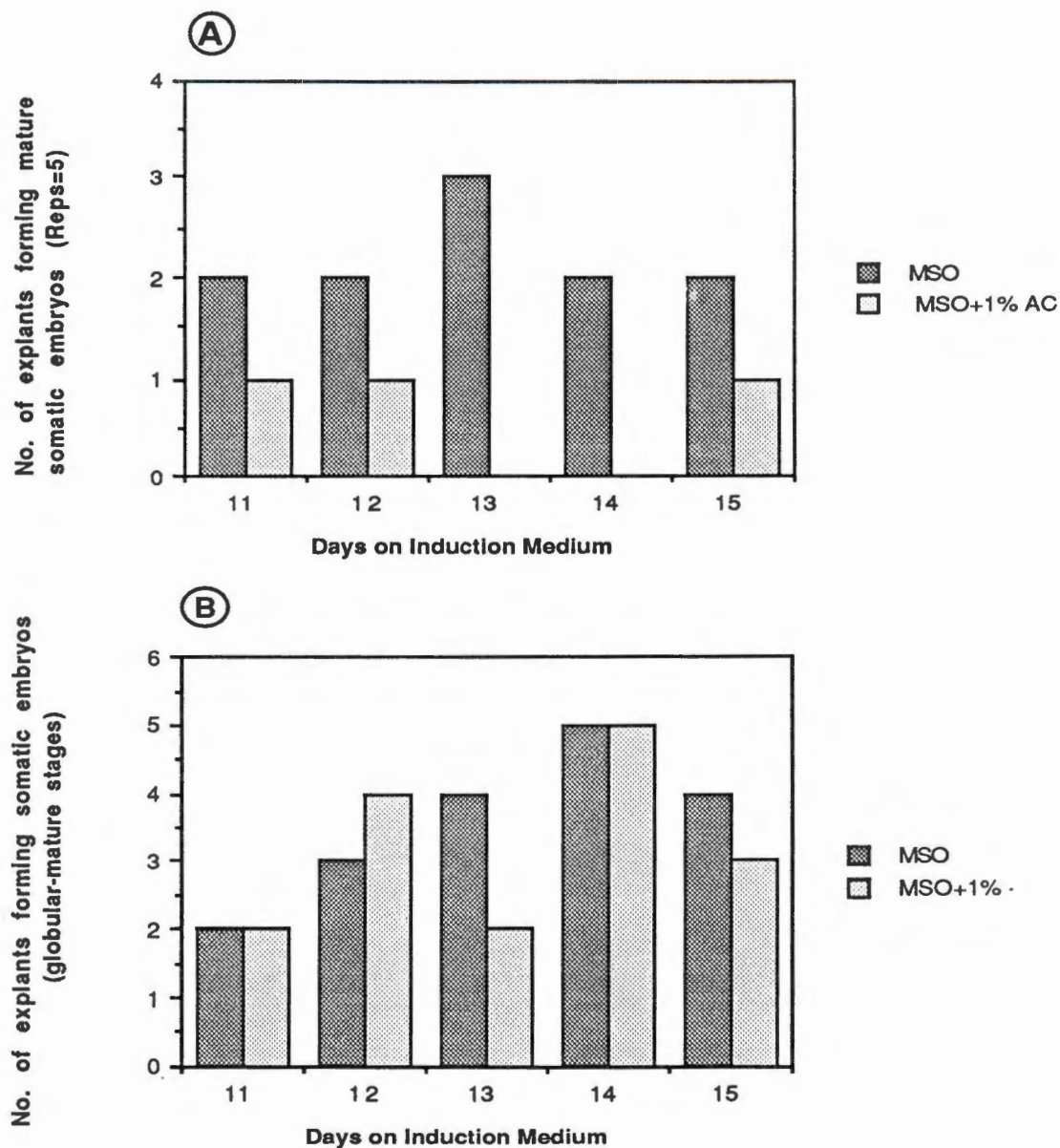


Figure 11: Effect of transfer to MSO or MSO+1% AC on cineraria somatic embryogenesis

- (A) Effect of transfer to MSO or MSO+1% AC on number of explants forming mature somatic embryos
- (B) Effect of transfer to MSO or MSO+1% AC on number of explants forming globular-to-mature embryo stages

Figure 12: Cineraria somatic embryo anatomy

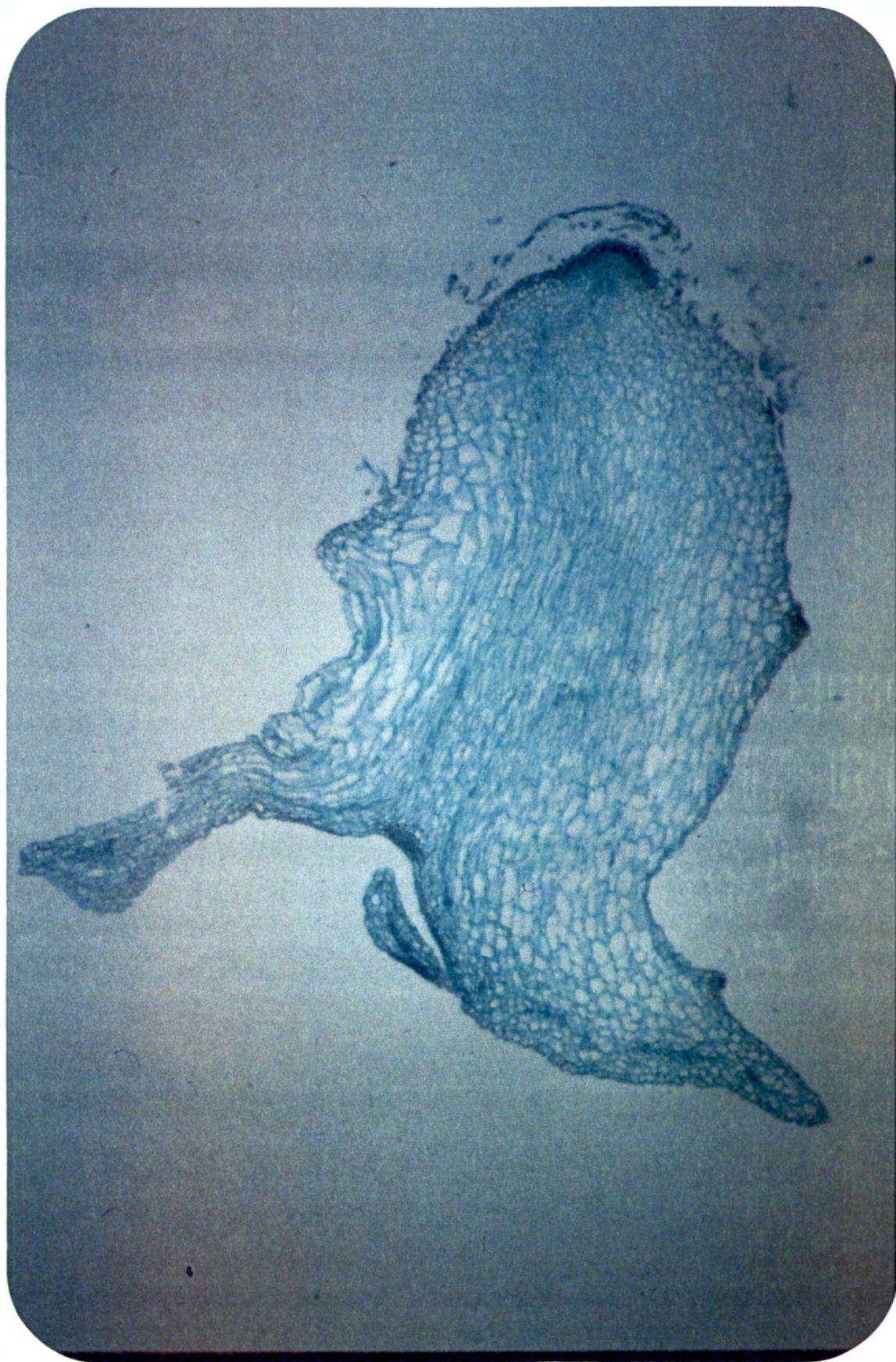
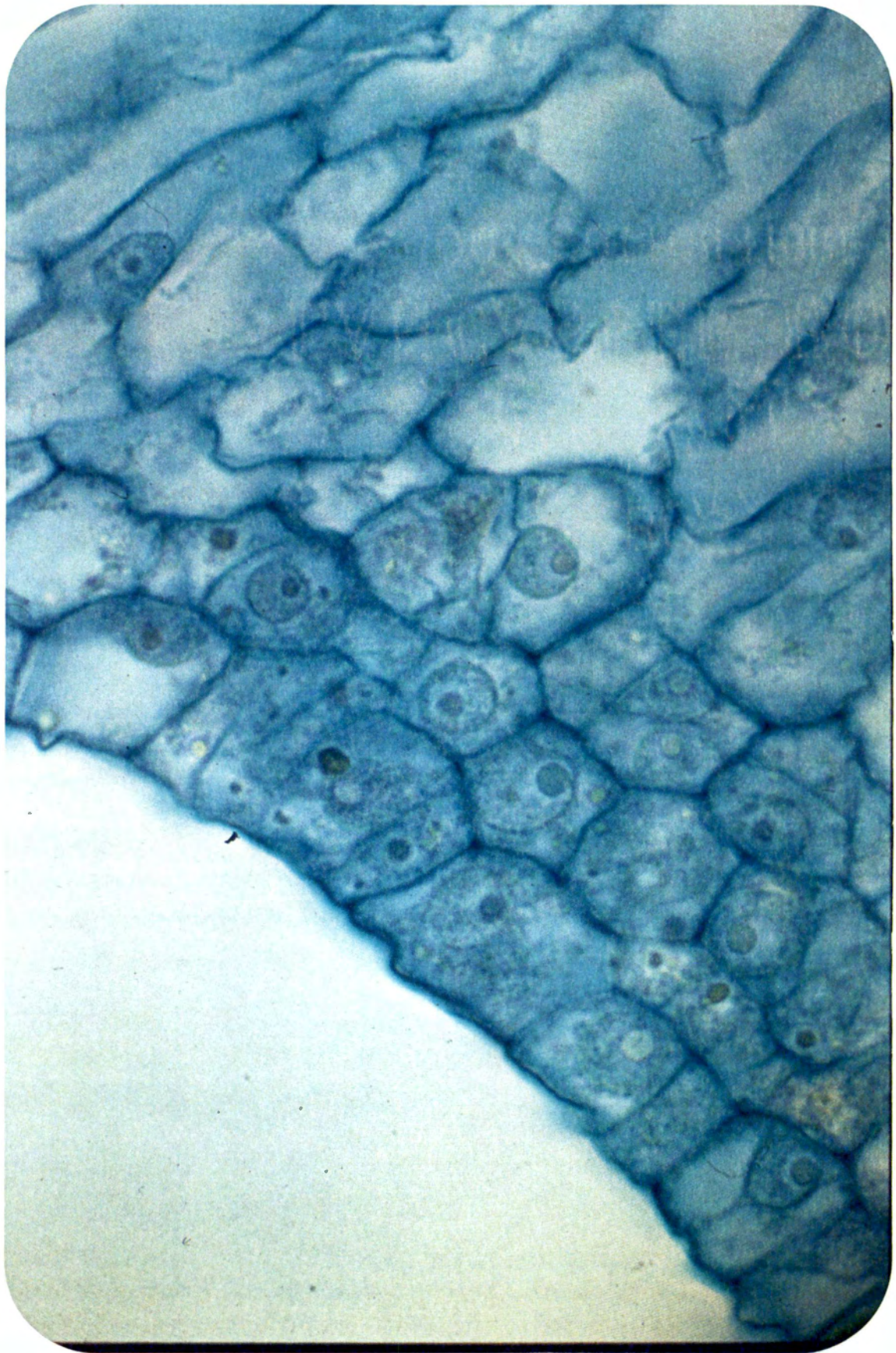


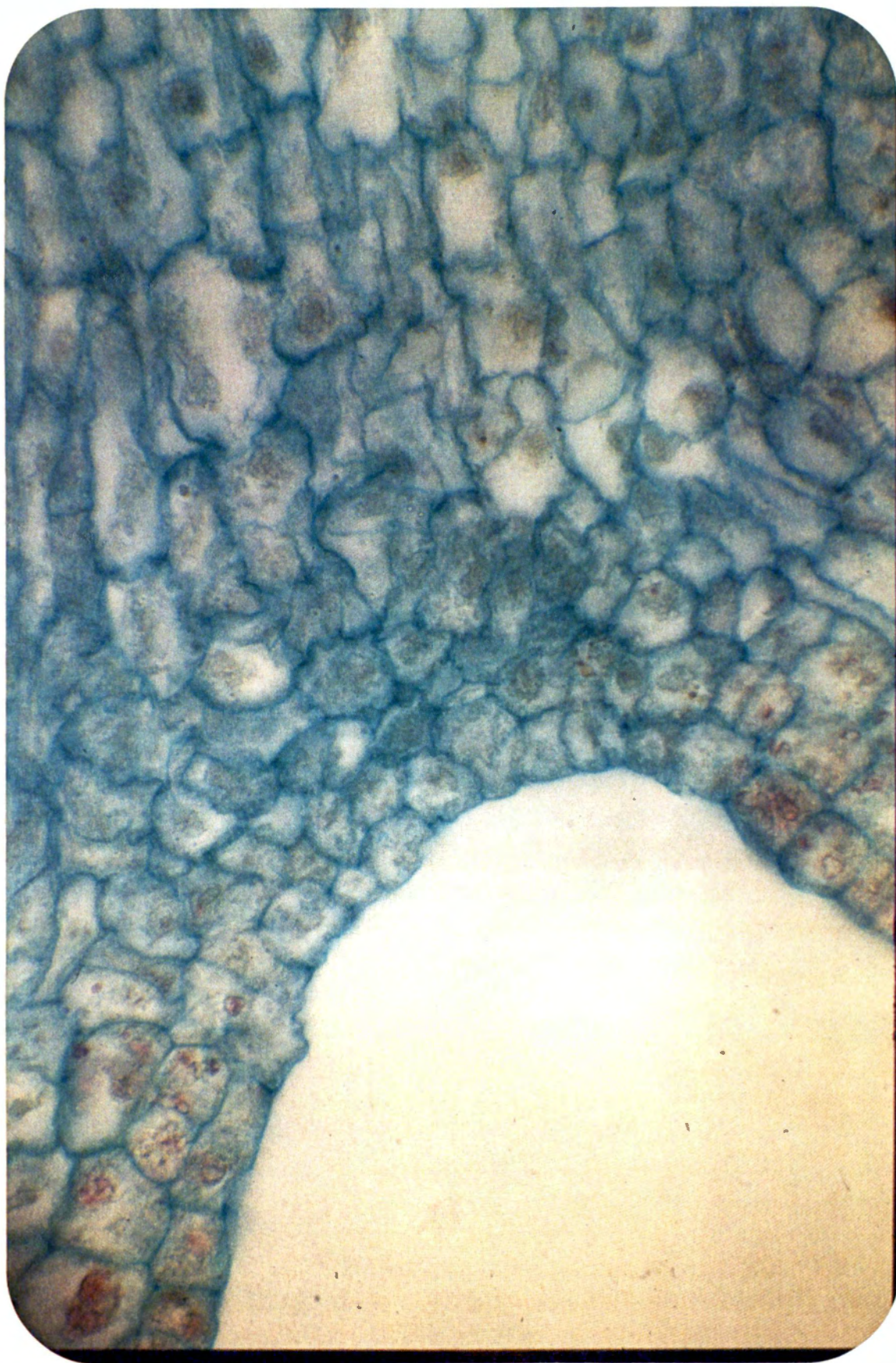
Figure 13: Active meristematic shoot region



developed on aged cultures more closely resembled a quiescent embryo (Figure 14).

This study demonstrated that somatic embryo development from leaf explants of the florists' cineraria was delayed or prevented by remaining on the auxin-rich induction medium, whereas embryo development could proceed if explants were transferred to a nutrient medium without plant regulators. The use of a two media transfer system, consisting of the induction medium and MSO+1.0% AC, indicated that exposure to 1.0% AC for an extended period of time was deleterious to explant and embryo survival. Use of a three media transfer system, consisting of induction medium, MSO+0.5% AC for three days and MSO, was beneficial for somatic embryo development. The three media transfer system reduced somatic embryo maturation from 2-4 months to approximately one month. Further research on media components and timing of transfers will be necessary to improve somatic embryo development, prevent precocious germination and improve efficiency of embryo production in cineraria.

Figure 14: Quiescent shoot region



Literature Cited

- Ackerson, R. C. (1984). Regulation of soybean embryogenesis by abscisic acid. *J. of Expt. Bot.* 35: 403-413.
- Ammirato, P. V. (1977). Hormonal control of somatic embryo development from cultured cells of caraway. *Plant Physiol.* 59: 579-586.
- Ammirato, P. V. (1983). Embryogenesis. In: *Handbook of Plant Cell Culture*, D. A. Evans, W. R. Sharp, P. V. Ammirato and Y. Yamada eds., (New York: Macmillan Publishing Co.), pp. 82-123.
- Buchheim, J., Colburn, S. and Ranch, J. (1989). Maturation of soybean somatic embryos and the transition to plantlet growth. *Plant Physiol.* 89: 768-775.
- Chu, I. Y. E. (1986). The application of tissue culture to plant improvement and propagation in the Ornamental Horticulture Industry. In: *Tissue Culture as a Plant Production System For Horticultural Crops*, R. H. Zimmerman, R. J. Griesback, F. A. Hammerschlag and R. H. Lawson eds., (Boston: Martinus Nijhoff Publishers), pp. 15-51.
- Ebert, A. and Taylor, H. (1990). Assessment of the changes of 2, 4-dichlorophenoxyacetic acid concentrations in plant tissue culture media in the presence of activated charcoal. *Plant Cell Tissue Organ Cult.* 20: 165-172.
- Evans, D., Sharp, W. and Flick, C. (1981). Growth and behavior of cell cultures: embryogenesis and organogenesis. In: *Plant Tissue Culture: Methods and Applications in Agriculture*, T. A. Thorpe ed., (New York: Academic Press, Inc.), pp. 45-61.
- Halperin, W. (1966). Alternative morphogenetic events in cell suspensions. *Amer. J. Bot.* 53: 443-453.
- Halperin, W. and Wetherell, D. (1964). Adventive embryony in tissue cultures of the wild carrot, *Daucus carota*. *Amer. J. Bot.* 51: 274-283.

- Heberle-Bors, E. (1980). Interaction of activated charcoal and iron chelates in anther cultures of *Nicotiana* and *Atropa belladonna*. Z. Pflanzenphysiol. 99: 339-347.
- Litz, R. and Conover, R. (1980). Somatic embryogenesis in cell cultures of *Carica stipulata*. HortScience. 15: 733-734.
- Meyer, P., Heidmann, I., Forkmann, G. and Saedler, H. (1987). A new petunia flower colour generated by transformation of a mutant with a maize gene. Nature. 330: 677-678.
- Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.
- Qureshi, J., Kartha, K., Abrams, S. and Steinhauer, L. (1989). Modulation of somatic embryogenesis in early and late-stage embryos of wheat (*Triticum aestivum* L.) under the influence of (+)-abscisic acid and its analogs. Plant Cell Tissue Organ Cult. 18: 55-69.
- Sass, John E. (1958). Botanical Microtechnique. Iowa State University Press, Ames, Iowa.
- Smith, D. and Krikorian, A. (1990). Somatic proembryo production from excised, wounded zygotic carrot embryos on hormone-free medium: evaluation of the effects of pH, ethylene and activated charcoal. Plant Cell Rep. 9: 34-37.
- Steward, F., Kent, A. and Mapes, M. (1967). Growth and organization in cultured cells: Sequential and synergistic effects of growth regulating substances. Ann. N. Y. Acad. Sci. 144: 326-334.
- Tisserat, B. and De Mason, D. (1980). A histological study of development of adventive embryos in organ cultures of *Phoenix dactylifera* L. Ann. Bot. 46: 465-472.

VITA

Kathleen Renick Malueg was born in Seattle, Washington and attended schools in Washington, Florida and Alabama. She received her Bachelor of Science degree in Ornamental Horticulture and Landscape Design from The University of Tennessee in the Spring 1988. She began her Master's degree studies in June, 1988 and was awarded this degree in December 1990.

The author is a member of Phi Kappa Phi, Pi Alpha Xi, Gamma Sigma Delta, Alpha Zeta, American Association for the Advancement of Science, and the American Society for Horticultural Science. She is currently employed as a senior research assistant in plant histology by the Department of Ornamental Horticulture and Landscape Design, The University of Tennessee, Knoxville, Tennessee.