To the Graduate Council:

I am submitting herewith a thesis written by Olga Ruiz-Marín entitled "Heavy metal, salts and osmotic pressure effect on shoot regeneration from leaf explants of *Dendranthema grandiflora*". 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.

Robert N. Trigiano, Major Professor

We have read this thesis and recommend its acceptance:

Accepted for the council:

Associate Vice Chancellor and
Dean of the Graduate School

HEAVY METAL SALTS AND OSMOTIC PRESSURE AFFECT SHOOT REGENERATION FROM LEAF EXPLANTS OF DENDRANTHEMA GRANDIFLORA

A thesis

presented for the

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Degree

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Olga Ruiz-Marín

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In memory of my father Augusto Ruiz

and my brother Jairo

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ABSTRACT

Shoot organogenesis was induced in two recalcitrant varieties of *Dendranthema grandiflora* Tzvelev 'Adorn' and 'Goldmine'. Regeneration of shoots from 'Goldmine' leaf explants was obtained on the following media: Murashige and Skoog (1962) (MS) medium amended with 0.1 μM BA, 11.5 μM IAA and 4.2 μM $CoCl_2$ and transferred after 5 weeks to medium with the same concentration of BA and $CoCl_2$, but without IAA. Schenk and Hildebrandt (SH) medium also was suitable for shoot induction in this variety when amended with 5% sucrose and 0.1 μM BA and 11.5 μM IAA. Explants were incubated for 2 weeks before being transferred to medium without growth regulators and the same concentration of sucrose. Poor shoot regeneration was obtained from leaf explants of 'Adorn' initially cultured on MS medium amended with 0.1 μM BA, 11.5 μM IAA and 0.58 μM $AgNO_3$ and subsequently transferred after 7 weeks to medium with the same concentration of $AgNO_3$, but without growth regulators.

Mannitol was used to evaluate the osmotic effects on shoot organogenesis of 'Iridon'. Shoot organogenesis was inhibited by all concentrations of mannitol-sucrose relative to similar concentrations of sucrose. High concentrations of sucrose (greater than 6%), significantly inhibited the organogenic response from leaf explants of 'Iridon' and 15% sucrose completely inhibited shoot formation.

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INTRODUCTION

The first report of cultivated chrysanthemum or mums (*Dendranthema grandiflora* Tzvelev) [syn. *Chrysanthemum morifolium* Ramat.] (Anderson, 1987) dates from about 500 B. C. Chrysanthemum was first cultivated in China and brought to Japan in 386 A. D. via Korea (Dowrick, 1953). In Japan, it was selected to increase variation in form and color. The world trade of mums involves millions of dollars each year; the production of standard pompon, potted and potted hardy garden chrysanthemums in the United States was valued at 165.41 million dollars in 1994 (USDA, 1995).

The garden chrysanthemum is a hexaploid species and is normally self-incompatible (Drewlow et al., 1973; Ronald and Ascher, 1975; Langton, 1981). Therefore, in breeding programs, crosses between selected varieties either fail to produce seeds or produce only a few seeds (Drewlow et al., 1973). Lack of viable seeds and progeny result in traditional breeding being a slow process. Mutation breeding (De Jong and Custers, 1986; Broertjes et al., 1976) and genetic engineering offer the possibility of introducing new traits into a range of commercial cultivars (Kaul et al., 1990). Characteristics such as disease (Fletcher, 1992), herbicide (Mullineaux, 1992) and insect resistance (Peferoen, 1992) and flower color or plant morphology (Robinson and Firoozabady, 1993) could be modified using selective breeding, tissue culture and mutation induction. Transfer of genes for desirable traits by co-cultivation with *Agrobacterium* species, chemically mediated DNA uptake, electroporation, microinjection, microprojectile bombardment or electric discharge particle acceleration methods (Lal and Lal, 1993) are also ways for obtaining new varieties with desired characteristics without relying on traditional breeding techniques.

The method of regeneration of plants (organogenesis or somatic embryogenesis) and the explant type are frequently critical to recovery of transformed plants (Robinson and Firoozabadi, 1993) and appropriate tissue culture methods need to be developed before attempting gene transfer by *Agrobacterium* species (Lu et al., 1990; Kaul et al., 1990; Urban et al., 1994) or microprojectiles (Klein et al., 1988). Tissue culture methods are also useful for regeneration and selection of salt tolerant lines (Dalsou and Short, 1987; Nabors et al., 1980), selection of low temperature tolerant lines (Huitema et al., 1987) and for obtaining pathogen free plants (Ahmed and Andrea, 1987).

For most plant species, including chrysanthemum, expression of totipotency is genotype specific (Khalid et al., 1989). Therefore, the development of micropropagation protocols for specific recalcitrant cultivars is critical for studies in transformation and breeding. Different approaches exist for organogenesis in chrysanthemum (Trigiano and May, 1994), but some varieties are recalcitrant to all current culture methods. The cultivars 'Adorn' and 'Goldmine' are recalcitrant to in vitro conditions previously evaluated, whereas 'Iridon' shows a high morphogenetic capability (Trigiano and May, 1994). The objective of the first part of this work was to devise a protocol for shoot organogenesis from leaf explants of the cultivars 'Adorn' and 'Goldmine'.

The second part of this work addressed the effect of medium osmotic potential on in vitro organogenesis of the variety 'Iridon'. This variety was chosen for this study because of its demonstrated highly organogenic response (Trigiano and May, 1994). Sucrose is a major component of most tissue culture media and functions as both an energy source and an osmotic agent (Verma and Dougall, 1977). Since organogenesis is a process that requires high energy (Thorpe and Meier, 1972), a continuous supply of carbohydrate from the medium

is necessary for normal shoot development (Brown et al., 1979) and morphogenesis can be enhanced by sucrose or by combinations of sucrose with mannitol or sorbitol (Leva, 1986). Water exchange between the explant and its environment in vitro plays a major role in controlling explant metabolism (Ettiene et al., 1991) and therefore, affects the morphogenic response of the explant to the medium. In this study, different concentrations of sucrose were used to evaluate the organogenic response of 'Iridon' leaf explants. These results were compared with those from a study of 3% sucrose medium amended with mannitol at concentrations that produced the equivalent osmotic potential of the various concentrations of sucrose in MS medium solidified with agar.

This research project is divided in two parts. The objective of the first part was to develop a protocol for micropropagation of two cultivars 'Adorn' and 'Goldmine' of *D. grandiflora*, which are recalcitrant to the typical regeneration protocols. The second part addressed preliminary studies of osmotic relations during shoot organogenesis from leaf sections of the cultivar 'Iridon'.

I. LITERATURE REVIEW

In vitro organogenesis of Chrysanthemum

The formation of shoot buds or roots from tissue cultures or suspension cultures is called organogenesis (Street, 1977). This process can occur via callus (indirect organogenesis) or without callus formation (direct organogenesis).

Many studies of in vitro shoot organogenesis of chrysanthemum (*Dendranthema grandiflora* Tzvelev) [syn. *Chrysanthemum morifolium* Ramat.] (Anderson, 1987) using different kinds of explants and growth regulators have been published. The first attempts to obtain organogenesis from chrysanthemum were made by Hill (1968). Flower receptacles and stem explants of cultivar 'Bronze Pride' were used to produce callus on four different media. This callus was cultured onto medium B_2 (Hill, 1967) amended with 10.7 μM of 1-naphthalene-acetic acid (NAA) and 3.7 μM kinetin and then subcultured onto a medium containing 1 mg/L Soya peptone. This sequence of two media was found to be the most effective for shoot organogenesis.

Shoots were regenerated from tubular florets of cultivars 'Bonnie Jean', 'Yellow Tuneful' and 'Gilt Glow' by Iizuka et al. (1973) using modified Murashige and Skoog (1962) medium (MS) amended with 0.1 μM 2.4-dichlorophenoxy acetic acid (2,4-D) and 10 μM of each indol butyric acid (IBA), indol acetic acid (IAA) and 6-benzyladenine (BA). Differences in organ differentiation among cultivars were found; only explants from 'Gilt Glow' produced roots and shoots and there was little root differentiation on 'Yellow Tuneful'.

Experiments directed toward rapid multiplication of chrysanthemum were conducted by Ben Jaacov and Langhans (1972). Shoot apices of cv.'#4 Indianapolis White' were grown on a semi-solid MS medium supplemented with 10% coconut milk or 13.8 mM inositol, 3.7 μM kinetin and 2.85 μM IAA. After six weeks, selected cultures were transferred to liquid media. When callus was transferred to semisolid medium of similar composition, shoot initials elongated.

Rapid proliferation from shoot apices of cultivar 'Giant #4 Indianapolis White' was obtained with MS medium amended with 9.3 μM of kinetin and 0.1 μM of NAA by Earle and Langhans (1974). Explants of the varieties 'Fred Shoesmith' and 'Deep Ridge' consistently formed more elongated shoots than 'Giant #4 Indianapolis White' under the same conditions. These early experiments may indicate that the ability to form shoots and the rate of proliferation may be influenced by genotype.

Shoots were regenerated from explants of young flower pedicels of varieties 'Super Yellow' and 'Bravo' on modified MS medium supplemented with 4% sucrose, 44 μM BA and 0.57 μM IAA (Roest and Bokelmann, 1975). Other cultivars were evaluated and shoots were obtained, but the conditions for shoot organogenesis were not optimized. The effect of explant length on regeneration was also evaluated. The number of regenerated microshoots was not influenced by the size of the explant, but the variety 'Bravo' produced increased number of transferable shoots when the size of the explant was increased.

Regeneration of plants from petal segments, petal epidermis and shoot tips of six cultivars of the 'Indianapolis' series was obtained by Bush et al. (1976). Explants were induced to produce callus on Linsmaier and Skoog (1965) medium (LS) with 46.4 μM kinetin and 5.4 μM NAA. The basal portion

consistently produced more callus and shoots than distal areas of the petal. After three or four months, callus was transferred to a liquid LS medium with 9.3 μM kinetin and 0.1 μM NAA. A third transfer to solidified LS medium containing 9.3 μM kinetin, 0.1 μM NAA and 28.8 μM gibberellic acid (GA_3) was required to obtain plantlets. Plants regenerated from petal cultures exhibited more abnormal morphology than plants derived from shoot tip cultures (Bush et al., 1976).

Shoots from leaves of 'Bronze Bornholm' cultured on MS medium supplemented with 18.6 μM kinetin, 10.7 μM NAA and 273 μM parafluorophenylalanine (PFA) were obtained by Slusarkiewicz-Jarzina et al. (1982). Plantlets developed from callus one month after culturing leaf explants. The addition of kinetin and NAA in a ratio of 2:1 played a very important role in the induction of organogenesis. When PFA was added, shoots were formed on the entire leaf surface, whereas without PFA only a few plantlets regenerated at the base of the leaf. Therefore, PFA not only induced the development of callus, but also favored the formation of buds.

Studies to increase genetic variation for yield in *C. morifolium* cv. 'White Spider' were conducted by De Jong and Custers (1986). Irradiated pedicel segments and petal epidermis regenerated adventitious buds on MS medium amended with 1 g/L casein hydrolysate, 0.7% oxoid agar, 4.4 μM BA, 0.57 μM IAA and 87 mM sucrose. The aim of this research was to select 'White Spider' plants for number of flowers, weight and height of plants. There was an increase in flower number, although it was often combined with reduced height and weight. The genotype 'White Spider' seems variable for flower number only and not for weight or height.

Khalid et al. (1989) regenerated plants either directly from leaves and petals of cv. 'Early Charm' or indirectly by callus to obtain somaclonal variants.

Explants were incubated on MS medium supplemented with 5.4 μM NAA, 2.8 μM kinetin, 87 mM sucrose and 0.8% agar. Novel variation was induced by regenerating plants from leaf and petal explants. The frequency of somaclonal variation obtained was in excess of that generated by natural sporting (Khalid et al., 1989).

Direct plant regeneration was obtained by Lu et al. (1990) with 'Royal Purple' on MS basal medium supplemented with 2.2 μM BA and 5.4 μM NAA. After three weeks, explants were transferred to medium without growth regulators for elongation of shoots. Direct shoot organogenesis was obtained and regenerated plants were uniform for morphology and flower color.

Shoots from stem and leaf explants of some chrysanthemum cultivars were regenerated when explants were incubated on MS medium supplemented with 5 μM of each BA and NAA (Kaul et al., 1990). Differences in shoot regeneration frequency were found. Three cultivars were recalcitrant to the combination of growth regulators applied. Histological studies showed that shoots on stem explants seemed to originate from cortical cells.

Experiments on varieties 'Hekla', 'Rave', 'Adorn', 'Goldmine' and 'Iridon' were conducted by Trigiano and May (1994). Leaf explants of 'Iridon' produced the greatest number of shoots and the least number of roots on induction medium containing 11.5 μM IAA and either 0.1 or 1.0 μM BA. Fewer shoots were produced on explants of 'Hekla' and 'Rave'. Mainly roots and an occasional shoot were produced on 'Goldmine' and 'Adorn' leaf segments. Explants were incubated 14 days on medium with growth regulators, then transferred to MS medium lacking growth regulators, and shoots developed after 3 weeks.

Direct shoot organogenesis in cultivar 'Spinder' was obtained by Aribaud et al. (1994) on MS medium supplemented with 0.55 mM inositol, 0.68 mM

glutamine, 87 mM sucrose and 20 μM BA. Polyamine metabolism was studied during the organogenic process. Formation of buds was associated with a decrease of polyamine conjugates, suggesting that high levels of polyamine conjugates inhibit cell multiplication and suppress differentiation.

Shoot organogenesis from leaf explants of 'Early Charm' and 'Tone Maid' was stimulated by the addition of the non-ionic, co-polymer surfactant, Pluronic F-68® (Poloxamer 188) (Khehra et al., 1995) added to MS-based medium amended with 4.4 μM BA and 2.7 μM NAA. The concentration of Pluronic F-68® that stimulated the greatest production of shoots was 0.01% (w/v) for 'Tone Maid' and 0.001% (w/v) for 'Early Charm'.

Effect of thidiazuron on shoot regeneration

Thidiazuron (TDZ) is an urea derivative that stimulates the conversion of cytokinin nucleotides to nucleosides, leading to an uncoupling of the normal inhibition of cytokinin biosynthesis (Mok et al., 1982). It has also been found to exhibit cytokinin-like activity in bioassay systems and to promote growth in cytokinin-dependent callus cultures in beans (*Phaseolus vulgaris* L.) (Capelle et al., 1983), soybean (*Glycine Max* (L.) Merr.), tobacco (*Nicotiana tabacum* L.) (Thomas and Katterman, 1986) and several woody species (Kerns and Meyer, 1986; Van Nieuwkerk et al., 1986). Formation of short, hyperhydric (vitrified) and fasciated microshoots are some of the problems sometimes associated with the use of TDZ as a growth regulator (Huetteman and Preece, 1993). Transfer of shoot cultures to a secondary medium often lacking TDZ or with a different balance of plant growth regulators can overcome problems associated with shoot elongation (Fasolo et al., 1989; Preece and Imel, 1991) and hyperhidricity

(Gribaudo and Fronda, 1991). The effect of TDZ on shoot organogenesis of chrysanthemum was evaluated by Trigiano and May (1994). Shoots were produced on leaf explants of 'Iridon' and 'Hekla' but 'Goldmine' produced only roots.

Effect of heavy metals on shoot regeneration

Some heavy metals have been used in order to induce morphogenesis in different species; salts of heavy metals such as $CoCl_2$, $NiCl_2$, $CuCl_2$, $ZnCl_2$, $CdCl_2$ (Kiyosue et al., 1990) can be used to induce somatic embryogenesis in carrot. Plant regeneration can be enhanced by addition of silver nitrate to culture medium (Burnett, et al., 1994; Escalettes and Dosba, 1993; Palmer, 1992; Chi et al., 1991; Chraibi et al., 1991; Chi et al., 1990; Chi and Pua, 1989; Purnhauser et al., 1987). However, addition of silver nitrate had the opposite effect by reducing shoot regeneration in sugarcane (*Saccharum* spp. hybrids), *Brassica campestris* L. (Hachey et al., 1991) and *Petunia hybrida* Vilm. (Dimasi-Theriou et al., 1993), or had no effect as in musk melon (*Cucumis melo* L., Niedz et al., 1989).

Silver nitrate is an inhibitor of ethylene action (Beyer, 1976), although it also can promote ethylene production in *Saccharum* spp. (Taylor et al., 1994). Since ethylene may act either as a promoter or inhibitor of morphogenesis in tissue culture (Biddington, 1992; Dimasi-Theriou et al., 1993), the incorporation of $AgNO_3$ to the medium can improve organogenesis. Silver nitrate may also function as a stress agent (Kong and Yeung, 1995) and treatments by heavy metals, including silver nitrate, induced morphogenesis in carrot (Kamada et al., 1989; Kiyosue et al., 1990).

Silver nitrate promoted shoot regeneration in wheat (*Triticum aestivum* L.) and tobacco (*Nicotiana plumbaginifolia* Viv) (Purnhauser et al., 1987) tissue cultures. Ethylene production induced by auxin might be responsible for the suppression of shoot regeneration. The addition of Ag^+ inhibited the action of ethylene and therefore was thought to enhance shoot organogenesis.

High frequency shoot regeneration of chinese cabbage (*Brassica campestris* L. spp. *chinensis*) was obtained by Chi and Pua (1989) when Ag^+ was added to the medium, whereas explants growing on medium without Ag^+ showed poor regeneration. Shoot organogenesis was associated with explant age, growth regulators and the presence of Ag^+ in the medium. Poor regeneration may be associated with a negative effect of ethylene. Ionic silver inhibited ethylene and therefore enhanced shoot regeneration (Chi and Pua, 1989).

Enhanced shoot regeneration of eight recalcitrant genotypes of *B. campestris* and *B. juncea* L. was obtained by Chi et al. (1990). The ethylene inhibitors $AgNO_3$ and aminoethoxyvinylglycine (AVG) promoted shoot regeneration. AVG and $AgNO_3$ had little effect on root initiation but 5 or 10 μM of AVG inhibited root elongation and $AgNO_3$ reduced root induction in one cultivar. The recalcitrance of cells and tissues of both *Brassica* species might be associated with ethylene production (Chi et al., 1990). Shoot regeneration from cotyledons of sunflower (*Helianthus annuus* L.) was stimulated by the addition of the ethylene inhibitors, silver and cobalt ions (Chraibi et al., 1991). Addition of $AgNO_3$ at concentrations of 10-25 μM increased plant regeneration in the presence or absence of an exogenous source of ethylene. Cobalt chloride ($CoCl_2$) inhibited ethylene production and increased shoot regeneration. It was suggested that ethylene

inhibited shoot organogenesis from cotyledon cultures of *H. annuus* (Chraibi et al., 1991).

Shoot regeneration from cotyledon explants of *B. campestris* genotype R500 was enhanced by the addition of 30 or 60 μM $AgNO_3$ to the medium (Palmer, 1992). $CoCl_2$ at 20 and 30 μM increased cotyledon shoot regeneration but not with the same efficiency as $AgNO_3$. Removal of explants from $AgNO_3$ after 12 days increased the number of shoots per explant whereas the number of shoots was not affected by removal from medium containing $CoCl_2$. A period of growth without $AgNO_3$ and cytokinin might be required in order to maximize shoot production. Since shoot regeneration was enhanced by the addition of ethylene inhibitor $AgNO_3$, the poor regenerative capacity of this genotype might be related to biosynthesis or metabolism of ethylene (Palmer, 1992).

High frequency of adventitious shoot regeneration from leaf explants of *Prunus* spp. was obtained by Escalettes and Dosba (1993) with the addition of $AgNO_3$. Silver nitrate enhanced regeneration from explants when added at concentrations of 30 or 40 μM.

Cobalt ions are considered inhibitors of ethylene production (Biddington, 1992; Pius et al., 1993; Chraibi et al., 1991; Meijer, 1989; Roustan et al., 1989; Grover and Purves, 1976; Lau and Yang, 1976; Kang and Ray, 1969). Cobalt ions interfer with the conversion of methionine to ethylene (Lau and Yang, 1976) by blocking the conversion of 1-aminocyclopropane carboxylic acid to ethylene (Yang and Hoffman, 1984).

Osmotic Potential

Studies on water potential of in vitro plants

There are some reports on studies of water potential of in vitro plants. A correlation between callus water status and occurrence of somatic embryogenesis in rubber (*Hevea brasiliensis*. Muell. Arg.) was reported by Ettiene et al. (1991). Changes in the medium to favor an elevated water potential (Ψ) were apparently important for initiating somatic embryogenesis and a decrease in osmotic potential (Ψ_π) corresponded to the loss of embryogenic potential of the callus.

The effect of agar concentration on water status and growth of rose plants cultured in vitro was evaluated by Gashghaie et al. (1991). At relatively low agar concentrations (less than 0.75 %), shoot proliferation was promoted and the water potential of the medium and the number of shoots decreased linearly with increasing agar concentration. These changes were attributed to agar-mediated modifications in cytokinin concentrations (Debergh, 1983) and ion uptake in the medium (Singha, 1982).

In chrysanthemum, osmotic adjustment and growth responses of cv. 'Bright Golden Anne', 'Deep Luv' and 'Lucido' using mannitol, sucrose or sorbitol concentrations were studied by Shibli et al. (1992). Shoot growth was inhibited by sorbitol and mannitol in the proliferation medium, whereas increasing levels of sucrose tended to enhance shoot growth, even at high concentrations. Sorbitol and mannitol are suitable agents for inducing artificial osmotic stress on plants in

vitro and sucrose is used by the plant as a carbon source, lessening its suitability for use as an osmoticum (Shibli et al., 1992).

The effects of sucrose concentration on embryogenesis of 'Iridon' and 'Goldmine' were evaluated by May and Trigiano (1991). Explants of cultivar 'Iridon' produced only shoots and roots on MS medium amended with 1 mg/L 2,4-D and 0.2 mg/L BA, with either 3 or 6% sucrose, whereas organogenesis and embryogenesis was obtained on medium that contained 9% sucrose. 'Goldmine' produced some somatic embryos with 9% sucrose but organogenesis did not occur at 3 or 6% sucrose. In all cases, embryogenesis was enhanced by sucrose concentration greater than 9% but less than 10%. Direct somatic embryogenesis from chrysanthemum was influenced by light, sucrose concentration in the medium and genotype.

Osmotic requirement for in vitro morphogenesis

The osmotic requirements for shoot formation in tobacco callus were investigated by Brown et al. (1979). Cultures grown on a low concentration of sucrose produced a high number of shoots only when the medium was supplemented with mannitol to give the same Ψ as 3% sucrose. Values of Ψ_π were not reported. This concentration of sucrose supported the formation of the greatest number of shoots. Mannitol could not replace the sucrose requirement for growth; part of the sucrose was acting as an osmoticum. Metabolic intermediates were unable to act as supplementary sources of energy or carbon. Shoot formation occurred when sucrose was partially replaced by mannitol in osmotically equivalent levels.

The osmotic potential of soybean tissue cultures was reduced by addition of mannitol, sorbitol, glucose or sucrose to a standard medium (Kimball et al., 1975). Maximum callus growth occurred when the $\Psi\pi$ of the culture medium was lowered 8 to 12 bars. Decreasing osmotic potentials by 2 to 20 bars did not increase the frequency of adventitious bud differentiation. Regulation of Ψ_π of a medium might be useful in controlling cell conformation and callus production, but did not appear to enhance differentiation.

Using tobacco callus, Hammersley-Straw and Thorpe (1988) studied the effect of osmotic inhibition on shoot formation. Tobacco callus was transferred from shoot-forming medium (3% sucrose) to medium containing 10, 12, or 15% sucrose or vice versa. Shoot inhibition occurred when high concentrations of sucrose were added to the medium with changes in Ψ_π from -0.56 MPa to -1.42 MPa or -0.54 MPa to -1.73 MPa. When the tissue was transferred from shoot formation medium to high levels of sucrose, histological studies showed a reduction of the preferential cell division zones, disorganization of meristemoids into parenchymatous tissue and the accumulation of starch. These types of studies could be useful for attempting to dissect the process of de novo organogenesis into various stages and to study the causal events in morphogenesis.

Different concentrations of glucose or sucrose were used by Mukherjee et al. (1991) to study the osmotic requirements for shoot regeneration of Black nightshade (*Solanum melongena* L.). When the concentration of sucrose was increased up to 22 mM, shoot organogenesis increased but over this concentration of sucrose there was a reduction in the number of shoots and an increase in callus formation. More shoot meristems were produced when the Ψ_π was increased by the addition of 22 mM mannitol at a sucrose level of 22 mM,

but values of Ψ_π were not reported. When sugar concentration was lower than 22 mM, the efficiency of shoot organogenesis was not altered if mannitol was added. A possible complex interaction between the sucrose and the level of osmoticum in the medium may have occurred.

II. MATERIALS AND METHODS

Micropropagation of two recalcitrant varieties of *D. grandiflora* cultivars 'Adorn' and 'Goldmine'.

Rooted cuttings of *D. grandiflora* c.v. 'Iridon', 'Adorn' and 'Goldmine' were obtained from Yoder Brothers, Inc. (Barberton, Ohio). Plants were maintained on a laboratory bench at 23°C light (18 hours) with about 50 μmol.m^{-2}s^{-1} of light provided by fluorescent tubes and incandescent bulbs and 20°C dark (6 hours). Cultivars were grown at a density of 4 cuttings, in 15 cm diameter pot using Pro-Mix (Premier Brands Inc., Pennsylvania) and watered twice a week with 300 ppm of Peter's® 20–10–20 soluble fertilizer (Grace Sierra Horticultural products, California). Shoot tips were removed every two weeks in order to stimulate branching and formation of new shoots. All stock plants were restarted every 10 weeks. The new cuttings were taken from the original plants, and rooted in cell packs (Hummert International, Missouri) watered with Peter's® solution and covered with a plastic cover (Hummert International, Missouri) to avoid loss of humidity and to accelerate rooting. Cuttings were rooted after ten days in the closed chamber. The newly rooted cuttings were grown in the same manner as the ones received from Yoder Brothers.

Expanding 1.5 to 2.0 cm leaves were taken from 2 week shoots and surface desinfested under sterile conditions in a horizontal laminar air flow cabinet with 10% (v/v) commercial bleach (0.525% NaOCl) plus 0.1% (v/v) Triton X-100 for 6 minutes with agitation. The bleach solution was removed with four changes of sterile distilled water. Four sections per leaf were cut along the midrib and explanted onto culture media for evaluation.

Basal medium was composed of either Murashige and Skoog (1962) (MS) or Schenk and Hildebrandt (1972) (SH) macro and micronutrient salts. Murashige and Skoog basal medium was amended with 0.55 mM myo-inositol, 4 μM nicotinic acid, 2.4 μM pyridoxine-HCl and 0.3 μM thiamine-HCl. Schenk and Hildebrandt basal medium was amended with 5.5 mM myo-inositol, 15 μM thiamine-HCl, 40.6 μM Nicotinic acid, 2.4 μM pyridoxine-HCl. Media were adjusted to pH 5.8 by using 1 N KOH prior to the addition of 0.7% (w/v) Phytagar® (Gibco Laboratories, Grand Island, NY). All media were steam sterilized at 121°C, 1.06 kg/cm^2 of pressure for 20 minutes. Growth regulators were added according to the experimental design after the sterilized media had cooled, but not hardened using a Bioexpress® (Intermountain Scientific Corporation, Utah) 0.45 μm syringe filters.

Twelve ml of culture medium were dispensed into each sterile 60 x 15 mm plastic petri dish. After culturing the explants, petri dishes were sealed with Parafilm®. Cultures were maintained in a Precision® incubator (Precision Scientific Group, Illinois) at 24°C with a photoperiod of 16 hours light with 25 μmol.m^{-2}.sec^{-1} irradiation, supplied by two cool white, 34 watt fluorescent tubes and 8 hours of dark.

Statistical analyses was conducted on data obtained from a balanced incomplete randomized experimental design. The rationale for this was to have the units in each block as uniform as possible so that observed differences could be attributed to treatments and not to differences in the ability of the leaves to respond. Each treatment appears an equal number of times and the variability among units in different blocks is supposed to be greater on the average than variability among units in the same block if no treatments are to be applied (Steel and Torrie, 1980). One explant per petri dish was cultured using 6 to 10

replicates per treatment depending on the particular experimental design. The number of shoots produced per explant was determined for statistical purposes. Data were subjected to ANOVA using SAS program (SAS Institute Inc., North Carolina).

Effect of thidiazuron on shoot organogenesis from leaf explants

The effect of thidiazuron on shoot organogenesis was evaluated using MS medium amended with 0.0001 to 10 μM TDZ and explants were maintained from 1 to 14 days on this medium before changing to MS medium without TDZ. This experiment was conceived as a randomized incomplete block with 36 treatments, 5 replicates per treatment and 4 treatments per block. The experiment was repeated twice.

Effect of heavy metals on shoot organogenesis from leaf explants

The first experiment was intended to evaluate the effect of some heavy metals on shoot organogenesis from leaf explants of 'Adorn' and 'Goldmine', since these cations can induce morphogenesis from somatic cells (Kiyosue et al., 1990). All induction media (MS) were amended with 11.5 μM IAA, 0.1 μM BA (Trigiano and May, 1994). MS and MS half-strength with 3 or 5% sucrose were evaluated. Different concentrations of Cobalt chloride ($CoCl_2$): 4.2, 21 and 42 μM, silver nitrate ($AgNO_3$): 5, 29.4 and 58.8 μM and manganese chloride ($MnCl_2$): 5, 25 and 50 μM were compared. The experimental design was a randomized incomplete block with 36 treatments, 5 replicates per treatment and 4 treatments per block. Cobalt chloride, silver nitrate and manganese chloride

solutions were filter sterilized before adding to autoclaved medium using a Bioexpress® (intermountain Scientific Corporation) 0.45 μM syringe filter.

A second experiment was conducted with 'Goldmine'. Concentrations of $CoCl_2$ ranged from 0 to 25 μM and leaf explants were transferred to medium with 0 to 0.5 μM of BA after 2 weeks of incubation on induction media. The experimental design consisted of 20 treatments, 8 replicates per treatment and 4 treatments per block.

A third experiment was designed using induction medium amended with concentrations of 0, 0.42, 2.1, 4.2, 6.3 and 8.4 μM $CoCl_2$ and leaf explants were transferred after two weeks to MS medium with or without cobalt chloride and amended with BA 0.1 μM, but without IAA. The experimental design was an incomplete randomized design consisting of 12 treatments, 8 replicates per treatment and 4 treatments per block.

In order to evaluate the time necessary for induction of shoot organogenesis from leaf explants of 'Goldmine', a fourth experiment was designed. Explants were incubated on MS induction medium amended with 4.2 μM $CoCl_2$ for 2, 3, 4, 5, 6 or 7 weeks and then transferred to medium with or without 4.2 μM$CoCl_2$ and amended with 0.1 μM BA. The experiment consisted of 12 treatments, 8 replicates per treatment and 4 treatments per block.

The effect of different concentrations of $AgNO_3$ on shoot organogenesis from leaf explants of 'Adorn' was evaluated. The first experiment was designed as a randomized incomplete block with 20 treatments, 8 replicates per treatment and 4 treatments per block. Concentrations from 0 to 11.7 μM silver nitrate and transfer of explants to medium with 0 to 0.5 μM BA after 2 weeks of incubation were the treatments evaluated. These concentrations of silver nitrate were chosen because in a previous experiment shoots were obtained on medium

amended with 5.8 μM of $AgNO_3$. The objective was to evaluate if a continuous presence of BA on the medium was necessary for the production of shoots.

In order to evaluate the time necessary on induction medium amended with silver nitrate on shoot organogenesis from 'Adorn' leaf explants, a second experiment was conducted. Explants were maintained on induction media amended with 0.58 μM $AgNO_3$ during 2, 3, 4, 5, 6 or 7 weeks before transferring to medium with or without 0.58 μM silver nitrate. The experiment consisted of 12 treatments, 8 replicates per treatment and 4 treatments per block.

Effect of low concentration of salts of SH medium on shoot organogenesis from leaf explants

The effect of low concentration of salts on regeneration of shoots from leaf tissues was evaluated in 'Adorn' and 'Goldmine'. Initially, the effect of SH medium on shoot organogenesis was compared with half-strength salts of MS medium with 0 to 9% sucrose. Each medium was amended with 0.1 μM BA and 11.5 μM IAA (Trigiano and May, 1994). Leaf explants were maintained on these media 2 weeks before transfer to medium without growth regulators. The experimental design consisted of 18 treatments, 5 replicates per treatment and 4 treatments per block. A second experiment was designed using 4 to 10% sucrose concentration and 0.1, 2.5 and 3 μM BA. The experiment was designed as a randomized incomplete block with 18 treatments, 5 replicates per treatment and 4 treatments per block.

In order to determine the effect of time on induction medium on shoot production, explants were maintained from 2 to 7 weeks on SH medium amended with 0.1 μM BA, 11.5 μM IAA (Trigiano and May, 1994) and 5 - 6%

sucrose. The experiment consisted of 12 treatments, 8 replicates per treatment and 4 treatments per block

A third experiment was conducted in order to evaluate if ammonium ion concentration had some effect on shoot regeneration from leaf explants of 'Goldmine' and 'Iridon'. Concentrations of ammonium from 2.6 to 20.6 mM were evaluated with MS and SH medium with 12 treatments, 8 replicates per treatment and 4 treatments per block. Ammonium concentration in SH medium was increased using ammonium sulfate ($(NH_4)_2SO_4$). Ammonium nitrate concentration in MS medium was lowered to obtain the concentrations of ammonium evaluated and KNO_3 was added to compensate the amount of NO_3^- decreased. All media were amended with 0.1 μM BA and 11.5 μM IAA (Trigiano and May, 1994).

The effect of osmoticum on shoot organogenesis from leaf explants of *D. grandiflora*

Determination of methodology for measuring osmotic potential of solid medium.

Initial experiments were conducted in order to establish the appropriate methodology to measure the Ψ_π of MS semisolid agar medium. The determination of this methodology is important because many of the culture media used for organogenesis are solid and if liquid medium is used, the response of the plant could be changed.

The first experiment was intended to establish the time needed to establish equilibrium between Ψ_π of solid medium and Ψ_π of filter paper discs

inserted into MS medium amended with 1 to 10% mannitol or 3 to 10% sucrose. Medium was solidified with 0.7% Phytagar® (Gibco Laboratories, Grand Island, NY). Filter paper discs of 6 mm (Wescor Inc., Utah) were manipulated with a forceps and quickly introduced into the agar diagonally until the discs were completely immersed into it. The filter paper discs were equilibrated into the agar for different lengths of time (3, 5, 15, 30 or 60 min.). Osmotic potential was measured with a 5500 vapor pressure osmometer Model # M2448-2 (Wescor Inc., Logan). Data are reported as the mean of four replicates per treatment.

In a second experiment the values of Ψ_π from solid medium measured with a thermocouple psychrometer (SC-10A Decagon devices Inc. Pullman, WA) were compared with the Ψ_π of same medium measured with a vapor pressure osmometer. For the osmometer, two procedures were evaluated. The first, filter paper discs were placed on the surface of the agar and allowed to saturate, and in the second they were immersed into the agar. The objective was to evaluate the reliability of the Ψ_π values obtained with the vapor pressure osmometer and to evaluate the effect of immersion or simple surface contact of the filter paper discs on Ψ_π values.

Changes of Ψ_π among and between plates were evaluated in order to assure that Ψ_π was constant across the plates. MS medium amended with 3 to 10% sucrose were evaluated using 6 petri dishes per concentration and 4 filter paper discs per petri dish.

The fifth experiment was intended to evaluate the changes on Ψ_π values between solid and liquid medium. MS medium was amended with either 3 to 10% sucrose or 3% sucrose + mannitol (to give the equivalent Ψ_π of that concentration of sucrose). Medium was solidified with 0.7% Phytagar® and no agar was added to liquid medium.

Effect of osmoticum on shoot organogenesis from leaf explants of *D. grandiflora* 'Iridon'

In order to determine the amount of mannitol needed to replace the Ψ_π produced by sucrose, MS medium was amended with either 1 to 10% mannitol or 3 to 10% sucrose, solidified with 0.7% agar and dispensed into 60 x 15 mm petri dishes. The relation MPa sucrose : MPa mannitol media between two concentrations of sucrose was calculated. 'Iridon' was chosen because of its highly morphogenic response. The influence of MS medium amended with 0 to 17% sucrose or 3% sucrose and mannitol concentration necessary to produce the equivalent Ψ_π produced by sucrose on shoot organogenesis was evaluated. Each medium was amended with 0.1 μM BA and 11.5 μM IAA (Trigiano and May, 1994). The experiment was designed as an incomplete randomized block with 6 replicates per treatment and 4 treatments per block and repeated 3 times.

III. RESULTS

Micropropagation of two recalcitrant varieties of *D. grandiflora* 'Adorn' and 'Goldmine'

Effect of thidiazuron on shoot organogenesis from leaf explants

The organogenic response of leaf explants obtained from each cultivar to TDZ was different. 'Goldmine produced few shoots, 'Iridon' produced more shoots than 'Goldmine', and 'Adorn' did not produce shoots. Short shoots (0.1 to 0.5 cm) were produced in all the treatments.

'Goldmine' explants produced small shoots on MS medium amended with 0.01 to 0.0001 μM TDZ after 5 to 14 days of incubation on induction medium (Table I). Calli were produced with higher concentrations of TDZ and roots were observed primarily on the lowest concentrations (0.0001 μM).

Small hyperhydric shoots (Debergh et al. 1992) were produced on leaf explants of 'Iridon' in most of the treatments (Table II). Analyses of variance showed significant differences among treatments at 0.05 level. The greatest number of shoots was produced when leaf explants were maintained for 10 days on MS medium amended with 0.11 μM TDZ but shoots were hyperhydric. Shoot fasciation was observed in some regenerated shoots of this variety. Fasciation is a phenomenon in which shoots differ from normal shoots, appearing as if the shoots were fused together to form a flattened stem (Huetteman and Preece, 1993). These two abnormalities were not observed in regenerated plants of 'Goldmine'.

Table I. Effect of thidiazuron on shoot organogenesis from leaf explants of *Dendranthema grandiflora* 'Goldmine'.

TDZ	Mean of number of shoots per treatment					
μM	**1 day**	**3 days**	**5 days**	**7 days**	**10 days**	**14 days**
10 μM	callus	callus	callus	callus	callus	callus
1 μM	0	0	0	0.2	callus	callus roots
0.1 μM	0	0	0	0	0	0
0.01 μM	0	0	0.2	0.4	0.4	0.8
0.001 μM	0	0	0	0	0.2	roots
0.0001 μM	0	roots	0	roots	0.4	roots

Data are shown as a mean number of shoots per treatment. N=5

Table II. Effect of thidiazuron on shoot organogenesis from leaf explants of *Dendranthema grandiflora* 'Iridon'.

TDZ	Mean of number of shoots per treatment					
µM	1 day	3 days	5 days	7 days	10 days	14 days
10 µM	2.8 c*	1.4 c*	0.4 c*	0.4 c	1.4 c*	0.4 c*
1 µM	0.6 c	1.0 c*	2.2 c*	5.6 a*	3.4 bc*	1.2 c*
0.1 µM	0 c	0 c	2.4 c	3.4 bc*	8 a*	7.2 ab*
0.01 µM	0 c	0 c	0 c	0 c	0.8 c	3.6 bc*
0.001 µM	0 c	0 c	0.4 c*	1 c	2.2 c*	0.8 c*
0.0001 µM	0 c	0 c	0.4 c	0.2 c*	0 c	2.4 c

Data are shown as a mean of number of shoots per treatment. N=5. Means followed by the same letter are not significantly different at p=0.05.
(*): hyperhydric.

Effect of heavy metals on shoot organogenesis from leaf explants

Shoot proliferation from leaf explants of 'Goldmine' was slightly stimulated by 21 μM cobalt chloride ($CoCl_2$) (Table III), followed by 50 μM manganese chloride ($MnCl_2$) in MS medium full strength with 3% sucrose. Shoots induced by $MnCl_2$ were very small and therefore, this treatment was not used for the following experiments.

Analyses of variance showed statistical differences among treatments in the second experiment. Explants cultured on MS medium amended with 0.1 μM BA, 11.5 μM IAA (induction medium: IM), supplemented with 4.2 μM $CoCl_2$ and transferred after 2 weeks to a medium with 0.1 μM BA (Table IV) produced more shoots than the other treatments. Shoots in this treatment showed normal phenotype.

In the third experiment few shoots were produced. Significant differences among treatments were not found but well developed shoots were produced with 4.2 μM $CoCl_2$ (Figure I). These were transferred after 2 weeks to a fresh medium with the same concentration of cobalt chloride and 0.1 μM BA. The time necessary for induction of shoot organogenesis from leaf explants of 'Goldmine' was 5 weeks (Figure II) and then transferred to a medium with same concentration of cobalt and with 0.1 μM BA, but without IAA.

Explants of 'Adorn' showed less shoot organogenic response to the initial treatments with different heavy metals than 'Goldmine' and analyses of variance indicated no statistical differences among treatments in this variety. Initially, only 0.6 shoots per explant were obtained with induction medium (MS + 0.1 μM BA + 11.5 μM IAA) amended with 5.8 μM silver nitrate ($AgNO_3$).

Table III. Effect of heavy metals on shoot organogenesis from leaf explants of *Dendranthema grandiflora* 'Goldmine'

MEDIUM	$CoCl_2$ μM			$AgNO_3$ μM			$MnCl_2$ μM		
	4.2	21	42	5	29.4	58.8	5	25	50
MS	0.2 b	2.2 a	0 b	0 b	0 b	0 b	0.4 b	0.2 b	1.8 b
MS / 2	0 b	1 b	0 b	0 b	0.2 b	0 b	0.4 b	0 b	0 b
MS + 5% sucrose	0 b	0.2 b	0 b	0 b	0 b	0 b	0 b	0 b	0.2 b
MS/2 + 5% sucrose	1 b	1 b	0 b	0.2 b	0 b	0 b	0.2 b	0 b	0.8 b

Data are shown as mean of number of shoots per explant. N=5. Means follower by the same letter are not significantly different at p=0.05.

Table IV. Effect of different concentrations of $CoCl_2$ and changes to medium with or without BA on the production of shoots from leaf explants of *Dendranthema grandiflora* 'Goldmine'.

Change at 2 weeks to medium with BA (μM)	$CoCl_2$ concentration (μM)				
	0	4.2	16.8	21	25
0	0.25 abc	0.62 ab	0.12 bc	0.12 bc	0 c
0.05	0.5 abc	0 c	0 c	0.25 abc	0.25 abc
0.1	0.12 bc	0.75 a	0.12 bc	0.25 abc	0 c
0.5	0.12 bc	0.37 abc	0 c	0.25 abc	0 c

Data are shown as mean of number of shoots per explant. N=5. Means followed by the same letter are not significantly different at p=0.05.

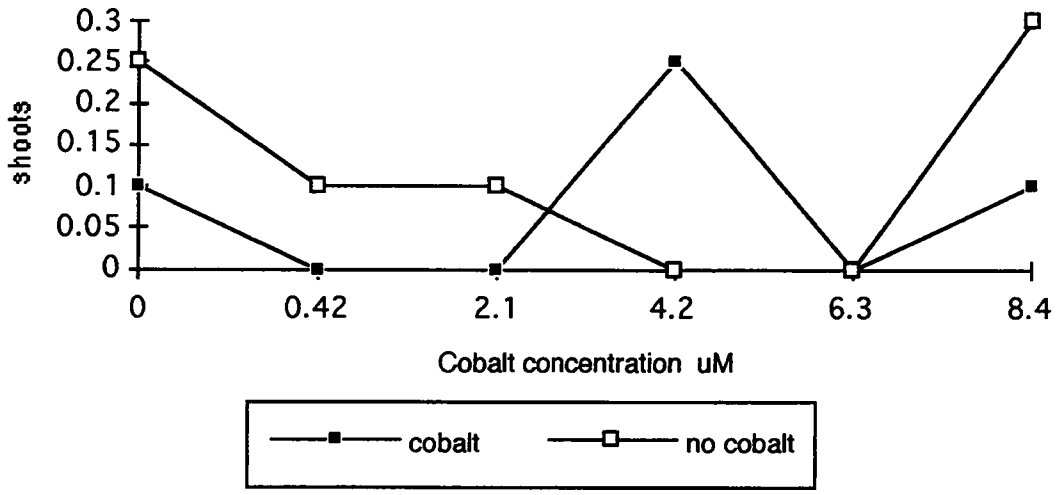

Figure I. Effect of different concentrations of $CoCl_2$ and changes to medium with or without cobalt on shoot organogenesis from leaf explants of *Dendranthema grandiflora* 'Goldmine'. N=8. There were no differences among treatments.

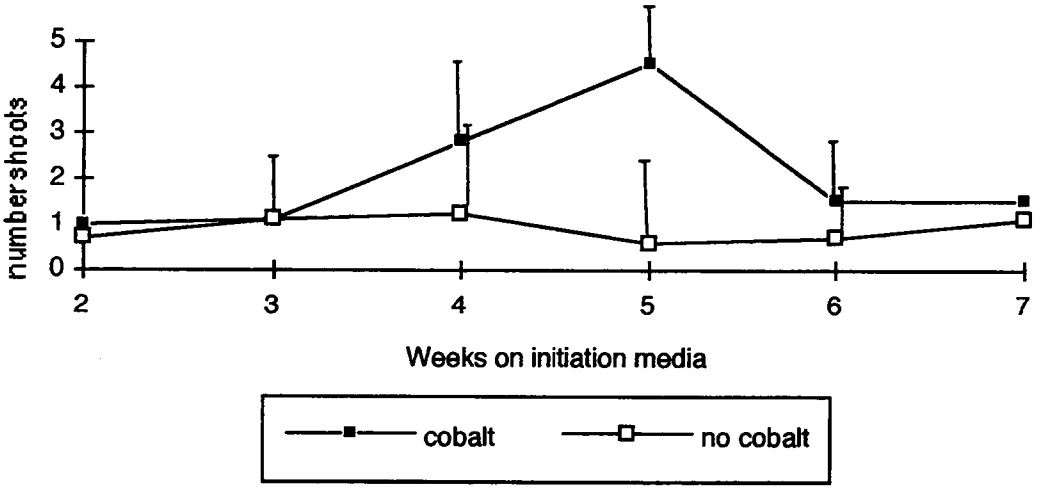

Figure II. Effect of incubation time on medium amended with 4.2 μM $CoCl_2$ and transfer to medium with or without cobalt on shoot organogenesis from leaf explants of *Dendranthema grandiflora* 'Goldmine'. N=8. Bars = standard deviation.

Few shoots were produced from explants cultured on medium containing 0.58 μM $AgNO_3$ (Table V) when explants were transferred to a medium without BA or with 0.1 μM BA. The best induction time seems to be 6 weeks followed by transfer to medium without silver nitrate, or 7 weeks and transferred to a medium with 0.58 μM silver nitrate (Figure III), however, few shoots were produced in this variety. Analyses of variance indicated no statistical differences among treatments.

Effect of low concentration of salts from SH medium on shoot organogenesis from leaf explants

Few shoots were produced in the first experiment and the greatest number was produced by explants cultured on SH medium containing 6% sucrose (Figure IV) and amended with 0.1 μM BA and 11.5 μM IAA. Analyses of variance indicated statistical differences among treatments in the second experiment and medium SH amended with 6% sucrose, 0.1 μM BA and 11.5 μM IAA produced more shoots per explant than the other treatmens (Table VI). Good development of shoots was obtained with 5 and 6% sucrose, whereas at higher concentrations of sucrose, the shoots that developed were very small. Higher concentrations of BA apparently did not have a positive effect on shoot organogenesis from tissue of 'Goldmine', therefore 0.1 μM BA was used for subsequent experiments.

Significant differences among treatments at 0.05 level were found when the effect of time of exposure on shoot organogenesis of 'Goldmine' was evaluated. Vigorously growing shoots were produced with 5% or 6% sucrose and incubation of 2 weeks. (Figure V). Analyses of variance showed no

Table V. Effect of different concentrations of $AgNO_3$ and changes to medium with or without BAP on shoot organogenesis from leaf explants of *Dendranthema grandiflora* 'Adorn'.

	$AgNO_3$				
Change at 2 weeks to medium with BA (μM)	**0 μM**	**0.58 μM**	**2.9 μM**	**5.8 μM**	**11.7 μM**
0	0	0.3	0	0	0
0.05	0	0	0	0	0
0.1	0.2	0.2	0	0	0
0.5	0	0	0	0	0

Data are shown as a mean number of shoots per explants. N=8. there were no significant differences among treatments.

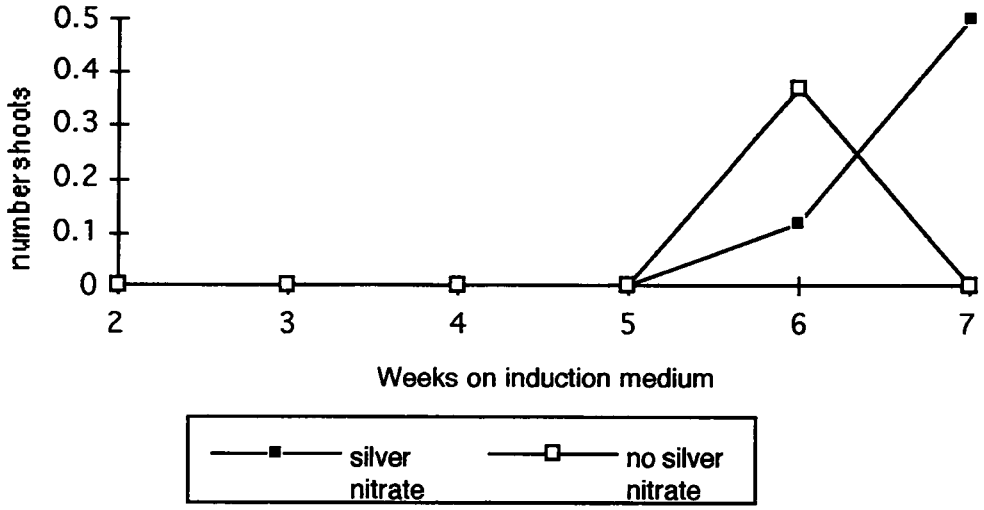

Figure III. Effect of incubation time and change to a medium with or without silver nitrate on shoot organogenesis from leaf tissue of *Dendranthema grandiflora* 'Adorn'. N=8 There were no significant differences among treatments.

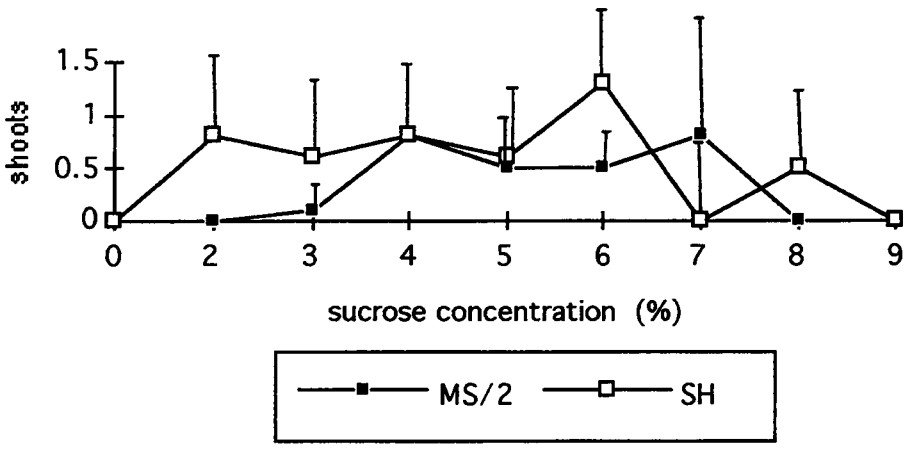

Figure IV. Effect of sucrose and low concentration of salts on shoot organogenesis from leaf tissue of *Dendranthema grandiflora* 'Goldmine'. Medium: MS half-strength (MS/2) and SH. N=8. Bars = Standard deviation.

Table VI. Effect of SH medium, different concentrations of BA and sucrose concentration on shoot organogenesis from leaf explants of *Dendranthema grandiflora* 'Goldmine'

Medium BA (μM)	**Concentration of sucrose**					
	4%	**5%**	**6%**	**7%**	**8%**	**10%**
0.1	0 d	1.1 bc	2 a	0 d	1.3 ab	0 d
2.5	0 d	0 d	0 d	0.1 d	0.3 d	0 d
3	0.1 d	0 d	0 d	0 d	0.8 cd	0 d

Data are shown as mean number of shoots per explant per treatment. N=5. Means followed by the same letter are not significantly different at p=0.05.

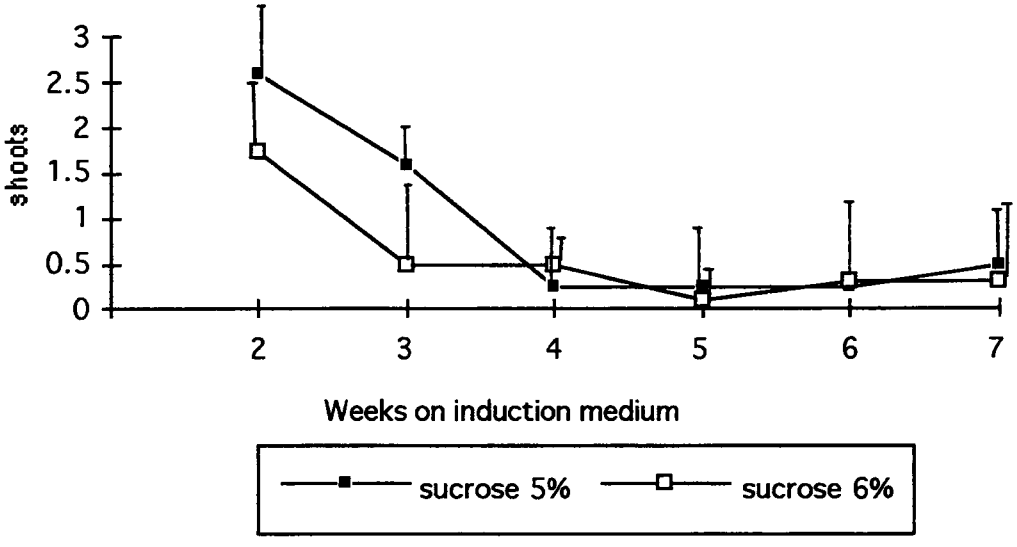

Figure V. Effect of time of exposure on SH medium with 5 and 6% sucrose on shoot organogenesis from leaf tissue of *Dendranthema grandiflora* 'Goldmine'. N=8. Bars = Standard deviation.

statistical differences between these two treatments. Rooting of these shoots was obtained 15 days after transfer to medium without growth regulators and plants were successfully acclimatized to greeenhouse conditions.

When the effect of ammonium concentration was evaluated (experiment 3, Figure VI), statistical differences among treatments were found. Well developed shoots were produced on medium containing 2.6 mM ammonium nitrate (SH medium). Treatments with MS medium did not produce shoots; treatments with SH medium and higher concentrations of ammonium supported the formation of small number of shoots.

Shoots formed on leaf explants of 'Iridon' in all the treatments. (Figure VII). Analyses of variance indicated statistical differences among treatments. The greatest number of shoots was produced on MS medium amended with 10.6 mM and 20.6 mM of ammonium when means were compared with Duncan's multiple range test. Shoot production with SH medium was almost the same regardless the ammonium concentration.

The effect of osmoticum on shoot organogenesis of *D. grandiflora*

Determination of methodology for measuring osmotic potential of solid medium.

The first experiment suggests that sample discs can be kept for 3 minutes in agar before measuring Ψ_π (Tables VII and VIII). The values obtained for Ψ_π of each concentration of mannitol and sucrose were not significantly different

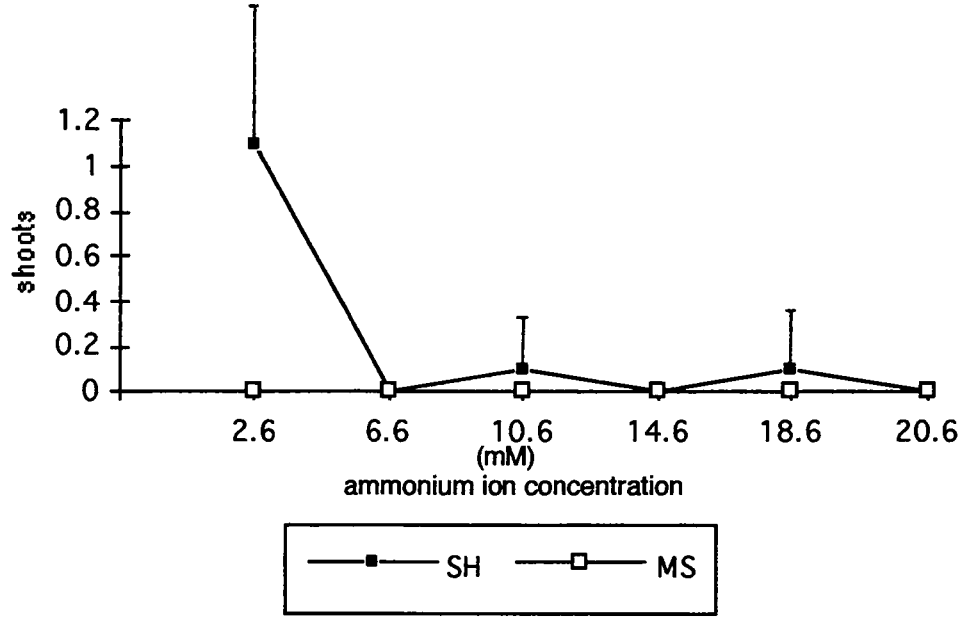

Figure VI. Effect of ammonium ion concentration on shoot organogenesis from leaf explants of *Dendranthema grandiflora* 'Goldmine'. N=8
Bars = Standard deviation

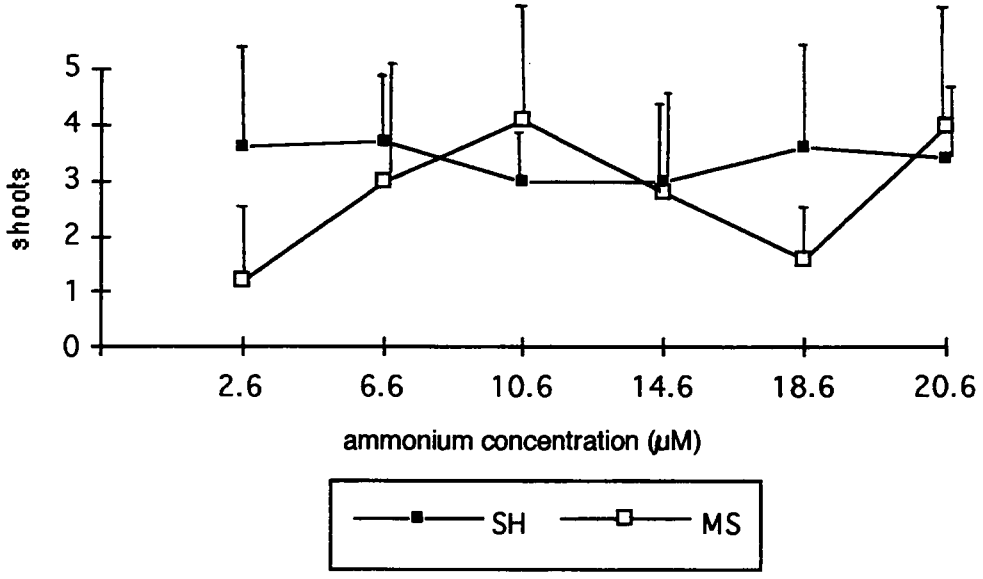

Figure VII. Effect of ammonium concentration on shoot organogenesis from leaf explants of *Dendranthema grandiflora* 'Iridon' N=8
Bars = Standard deviation.

Table VII. Equilibration time between Ψ_π of MS solid medium and $\Psi\pi$ of filter paper discs inserted into medium amended with different concentrations of mannitol. N=4

Conc.	**Mannitol - Ψ_π**						
	3 min.	**5 min.**	**15 min.**	**30 min.**	**60 min.**	**mean**	**sd**
	MPa	MPa	MPa	MPa	MPa		
1%	-0.34	-0.33	-0.35	-0.38	-0.38	-0.36	±0.023
2%	-0.58	-0.58	-0.57	-0.58	-0.56	-0.57	±0.008
3%	-0.71	-0.71	-0.69	-0.72	-0.71	-0.71	±0.010
4%	-0.85	-0.86	-0.85	-0.85	-0.83	-0.85	±0.010
5%	-1.02	-1.03	-1.02	-1.02	-1.01	-1.02	±0.007
6%	-1.18	-1.19	-1.18	-1.18	-1.19	-1.18	±0.005
7%	-1.35	-1.35	-1.32	-1.36	-1.33	-1.34	±0.016
8%	-1.64	-1.64	-1.59	-1.65	-1.67	-1.64	±0.029
9%	-1.83	-1.83	-1.83	-1.83	-1.83	-1.83	±0
10%	-1.84	-1.84	-1.86	-1.84	-1.84	-1.84	±0.008

Table VIII. Equilibration time between Ψ_π of MS solid medium and $\Psi\pi$ of filter paper discs inserted into medium amended with different concentrations of sucrose. Discs were placed in the medium. N=4

conc.	Sucrose - Ψ_π						
	3 min.	**5 min.**	**15 min.**	**30 min.**	**60 min.**	**mean**	**sd**
	MPa	MPa	MPa	MPa	MPa		
3%	-0.54	-0.55	-0.52	-0.55	-0.54	-0.54	±0.012
4%	-0.63	-0.64	-0.63	-0.63	-0.62	-0.63	±0.007
5%	-0.81	-0.81	-0.81	-0.79	-0.82	-0.81	±0.010
6%	-0.84	-0.84	-0.84	-0.84	-0.84	-0.84	±0.000
7%	-0.95	-0.95	-0.95	-0.96	-0.94	-0.95	±0.007
8%	-1.16	-1.16	-1.15	-1.15	-1.17	-1.17	±0.008
9%	-1.29	-1.29	-1.30	-1.28	-1.28	-1.29	±0.008
10%	-1.30	-1.32	-1.30	-1.31	-1.30	-1.30	±0.009

among the different times. The standard deviation of the mean for each concentration of osmotic agent is low (Tables VII and VIII) for both mannitol and sorbitol experiments.

When Ψ_π was measured using sample discs placed into the agar, the osmotic values obtained were more accurate than those obtained when discs were kept over the agar surface (experiment 2, Table IX). Significant differences were not found in Ψ_π of discs placed into the agar. the values of Ψ_π among and within plates (Table X) and the standard deviation in both cases was low. The Ψ_π of liquid medium tends to be higher than that of solid medium (Table XI).

Effect of osmoticum on shoot organogenesis from leaf explants of *D. grandiflora* 'Iridon'.

In order to determine the amount of mannitol needed to replace the Ψ_π produced by 1% sucrose in solid medium, the increment between two consecutive concentrations of sucrose and mannitol were calculated (Table XII) and the relation -0.11 MPa sucrose : -0.16 MPa mannitol was obtained. The Ψ_π produced by 1% sucrose could be replaced by 0.6875 grams of mannitol.

The addition of mannitol to replace the Ψ_π produced by a concentration of sucrose was done begining concentration of 4% sucrose. Production of shoots when mannitol was added was highly affected. More shoots were produced on media amended only with sucrose. The greatest number of shoots was produced with 4% sucrose, but these shoots were smaller than those produced with 3% sucrose (Figure VIII).

Table IX. Osmotic potential of mannitol and sucrose compared between psychrometer and osmometer readings. Values obtained with sample discs placed into the agar(*) and floated on the surface (**) N=5

concentration	Mannitol			Sucrose		
	psychrometer	osmometer		psychrometer	osmometer	
	MPa	MPa	MPa	MPa	MPa	MPa
1%	-0.45	-0.35*	-0.27**			
2%	-0.69	-0.57*	-0.30**			
3%	-0.86	-0.71*	-0.30**	-0.42	-0.54*	-0.38**
4%	-1.07	-0.85*	-0.30**	-0.73	-0.63*	-0.40**
5%	-1.08	-1.02*	-0.31**	-0.98	-0.80*	-0.42**
6%	-1.21	-1.18*	-0.34**	-1.01	-0.84*	-0.43**
7%	-1.35	-1.34*	-0.33**	-1.08	-0.95*	-0.48**
8%	-1.75	-1.64*	-0.34**	-1.35	-1.16*	-0.54**
9%	-1.90	-1.83*	-0.33**	-1.34	-1.29*	-0.62**
10%	-1.95	-1.84*	-0.35**	-1.45	-1.31*	-0.64**

Table X. Changes of osmotic potential among and between plates. N=6

Concentration	Ψ_π			
	Between plates		Among plates	
	MPa	sd	MPa	sd
3%	-0.52	±0.013	-0.51	±0.005
4%	-0.60	±0.006	-0.60	±0.002
5%	-0.70	±0.013	-0.70	±0.004
6%	-0.76	±0.010	-0.76	±0.005
7%	-0.89	±0.010	-0.89	±0.002
8%	-1.0	±0.006	-1.00	±0.000
9%	-1.08	±0.008	-1.08	±0.006
10%	-1.18	±0.013	-1.18	±0.003

Table XI. Osmotic potential of solid and liquid medium in different concentrations of mannitol and sucrose. (*): Differences among treatments at p = 0.05. N=5

concentration	Mannitol		Sucrose	
	solid medium	liquid medium	solid medium	liquid medium
1%	-0.35	-0.35		
2%	-0.57	-0.47*		
3%	-0.71	-0.66	-0.54	-0.45
4%	-0.85	-0.80	-0.63	-0.57
5%	-1.02	-0.96*	-0.80	-0.65
6%	-1.18	-1.12*	-0.84	-0.75
7%	-1.34	-1.25*	-0.95	-0.83
8%	-1.64	-1.44*	-1.16	-0.91
9%	-1.83	-1.58*	-1.29	-1.03
10%	-1.84	-1.74*	-1.31	-1.10

Table XII. Increment of osmotic potential per concentration of sucrose and mannitol solid medium. N=5

concentration	**MPa**		**MPa**	
	Mannitol		**Sucrose**	
	Ψ_π	**increment**	Ψ_π	**increment**
1%	-0.35			
2%	-0.57	0.22		
3%	-0.71	0.14	-0.54	
4%	-0.85	0.14	-0.63	0.09
5%	-1.02	0.17	-0.80	0.17
6%	-1.18	0.16	-0.84	0.04
7%	-1.34	0.16	-0.95	0.11
8%	-1.64	0.30	-1.16	0.21
9%	-1.83	0.19	-1.29	0.13
10%	-1.84	0.01	-1.31	0.02
mean		0.165 ±0.076		0.110 ±0.067

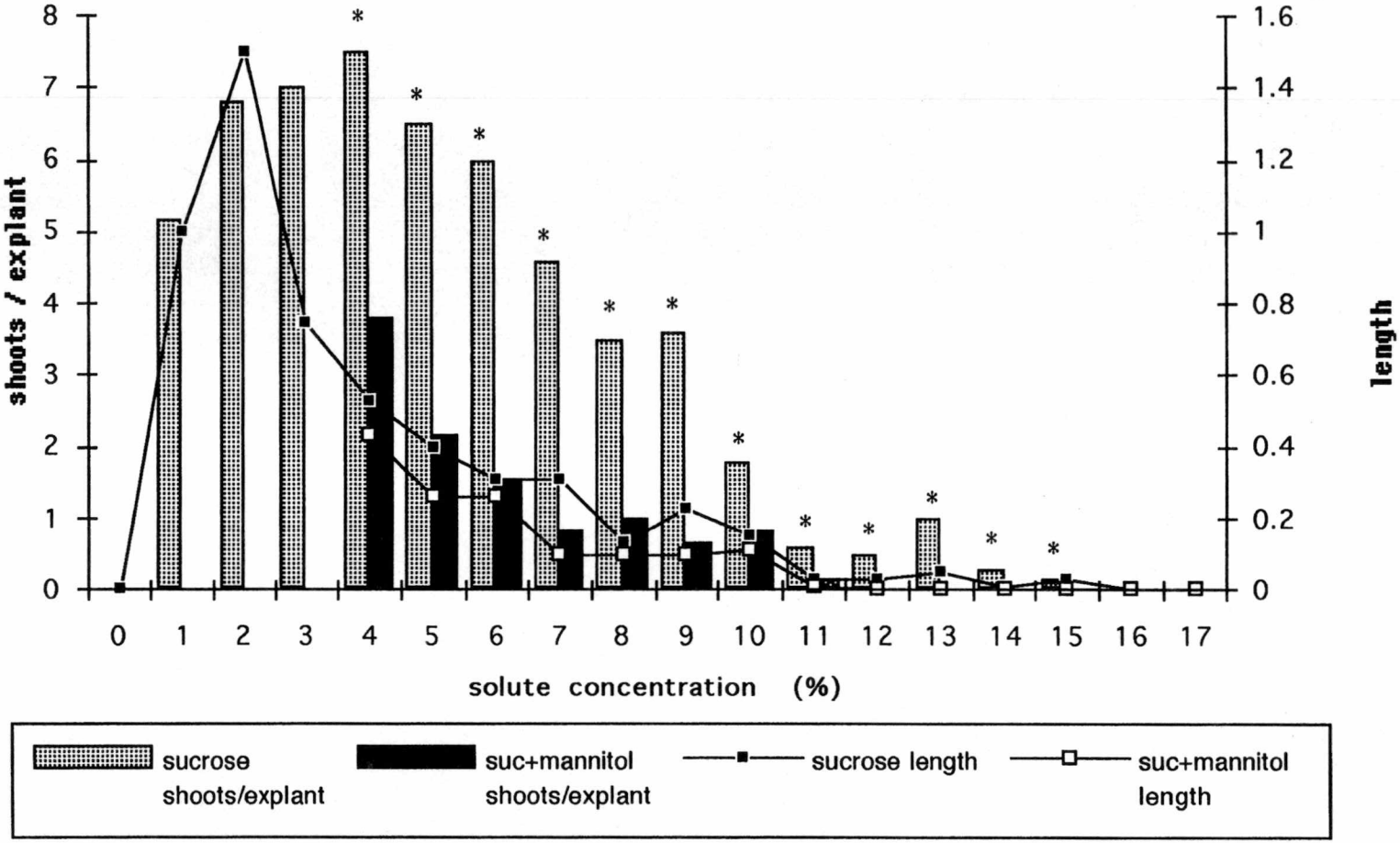

Figure VIII. Effect of sucrose and sucrose + mannitol on shoot organogenesis from leaf explants of *Dendranthema grandiflora* 'Iridon'. N=8. (*): Significantly different at p=0.05.

The addition of mannitol to replace the Ψ_π produced by a concentration of sucrose was done begining concentration of 4% sucrose. Production of shoots when mannitol was added was highly affected. More shoots were produced on media amended only with sucrose. The greatest number of shoots was produced with 4% sucrose, but these shoots were smaller than those produced with 3% sucrose (Figure VIII).

Analyses of variance indicated statistical differences among treatments for the variables number and length of shoots. When sucrose treatments were evaluated for the variable number of shoots, Duncan's multiple range test showed no differences among 1 to 6% sucrose treatments (Figure VIII). The greatest length was obtained in treatments 2, 1, 3 and 4% sucrose, in this order (Figure VIII) when compared with Duncan's test.

For sucrose + mannitol treatments, the greatest number of shoots was obtained with 1 to 3% (Figure VIII) and differences among these treatments were not found with Duncan's comparison. For the variable length 1 and 2% sucrose + mannitol (Figure VIII) showed the best response when compared with Duncan's multiple range test.

Since the optimum concentration of sucrose for shoot production from leaf explants of 'Iridon' was 3%, mannitol was added beginning with a concentration of 4% sucrose. Production of shoots when mannitol was added was highly affected. More shoots were produced on media amended only with sucrose. The greatest number of shoots was produced with 4% sucrose, but these shoots were smaller than those produced with 3% sucrose (Figure VIII). When sucrose treatments were evaluated for the variable number of shoots, Duncan's multiple range test showed no differences among 1 to 6% sucrose treatments. The

longest shoots were obtained with medium amended with 2, 1, 3 and 4% sucrose, in this order (Figure VIII) when compared with Duncan's test.

For sucrose and mannitol treatments, the greatest number of shoots was obtained with 4% (Figure VIII). For the variable length 4 and 5% sucrose and mannitol produced shoots more elongated than the other treatments when compared with Duncan's multiple range test.

IV. DISCUSSION

Micropropagation of two recalcitrant varieties of *D. grandiflora* cultivars 'Adorn' and 'Goldmine'

The production of shoots from leaf explants of each cultivar was different. 'Goldmine produced few shoots, 'Iridon' produced more shoots than 'Goldmine', but not as much as the standard protocol (Trigiano and May, 1994) and 'Adorn' did not produced shoots. The different response of cultivars 'Adorn' and 'Goldmine' to tissue culture may be related to genotype (Williams et al, 1990; Bergmann and Stomp, 1994; May and Trigiano, 1991), in vitro environment (Cuello et al., 1992), physiological conditions of the source explant or the age of explant (Ficcadenti and Rotino, 1995).

In order to explain why some cells of explants or subcultures are incapable of regenerate organs, Halperin (1986) proposed the following 3 major possibilities: (1) Lack of totipotency due to genetic features; (2) epigenetic block, involving stable, but potentially reversible constraints on the functioning of genes required for morphogenesis, and (3) cells may be genetically capable of responding to morphogenetic signals, but the environmental signals required are lacking (i.e., types and concentrations of hormones, culture conditions, etc.).

The poor response of 'Adorn' could be due to the third possibility. Since some shoots were obtained, albeit at a very low rate, it suggests that the poor organogenic response is not due to the genetic make-up of this cultivar or epigenetic changes. The lack of organogenesis may result from the failure of cultures to acquire competence for induction (Christianson and Warnick, 1988). Competence in a developmental process is the ability to recognize hormonal or

other signals that trigger a particular developmental pathway (Halperin, 1986; Meins, 1988). 'Goldmine' cells were more competent under the conditions in these experiments than 'Adorn' cells and therefore, the response to treatments for induction of shoot organogenesis was better.

Effect of thidiazuron on shoot organogenesis from leaf explants

TDZ is a plant growth regulator that exhibits cytokinin-like activity (Mok et al., 1982) and is used commercially as a cotton defoliant (Arndt et al., 1976). TDZ has been shown to be effective not only for micropropagation of woody species (Van Nieuwkerk et al., 1986; Kerns and Meyer, 1986), bean (Malik and Saxena, 1992), soybean, carnation (*Dianthus caryophyllus* L.) (Nugent et al., 1991), geranium (*Pelargonium* x *hortorum* Bailey) (Qureshi and Saxena, 1992), radish (*Raphanus sativus* L.) and tobacco (Thomas and Katterman, 1986) among others. TDZ stimulates the conversion of cytokinin nucleotides to nucleosides (Capelle et al., 1983) and encourages the synthesis (or inhibits the breakdown) of purine cytokinins (Thomas and Katterman, 1986).

TDZ seems not to enhance shoot proliferation from leaf tissue of either 'Goldmine' or 'Iridon'. Shoots produced from explants of both varieties were small and 'Iridon' produced hyperhydric shoots under these experimental conditions. The production of short shoots in 'Goldmine' and 'Iridon' in the presence of TDZ has been reported also in *Ixia flexuosa* L. (Meyer and Van Staden, 1988), *Hibiscus rosa sinensis* L. (Preece et al., 1991), *Malus domestica* Borkh (Van Nieuwkerk et al., 1986) and *Quercus robur* L. (Chalupa, 1988). Callus production at high concentrations of TDZ observed on 'Goldmine' explants has been also reported for many woody species (Huetteman and Preece, 1993).

Shoot number and length decrease at higher concentrations of TDZ (Thomas and Katterman, 1986; Gribaudo and Fronda, 1991), but when lower concentrations of TDZ were evaluated in 'Goldmine' and 'Iridon', improvement in shoot length was not observed.

Abnormalities such as hyperhydricity and fasciation, in regenerated shoots from 'Iridon' were sometimes associated with the use of TDZ as a growth regulator. TDZ has been shown to have some effect on hyperhydricity in azaleas (Briggs et al., 1988) and red raspberry (Cousineau and Donnelly, 1991). In these experiments, *D. grandiflora* cv. 'Iridon' regenerated shoots were hyperhydric when the culture medium was amended with different concentrations of TDZ.

Shoot fasciation, a phenomenon sometimes associated with use of TDZ for long terms, has been reported also in silver maple (Preece et al., 1991), apple (*Malus domestica* Borkh) (Van Nieuwkerk et al., 1986) and redbud (*Cercis canadiensis* L.) (Yusnita et al., 1990). TDZ-induced fasciation could also be associated with genotype (Huetteman and Preece, 1993) and in the case of chrysanthemum, was only observed in regenerated plants of 'Iridon', but not from 'Goldmine'. However, few shoots were produced from 'Goldmine' leaf explants and more experiments should be done in order to establish a relationship between fasciation, genotype and TDZ in chrysanthemum shoot regeneration.

Effect of heavy metals on shoot organogenesis from leaf explants

The response of 'Adorn' and 'Goldmine' to the heavy metal treatments were different. 'Adorn' explants produced few shoots on media amended with

silver nitrate and 'Goldmine' tissue had a positive response with cobalt chloride, silver nitrate and manganese chloride; however, the best production of shoots was obtained with cobalt chloride.

Shoot regeneration from leaf explants of 'Goldmine' can be enhanced by addition of cobalt (Chraibi et al., 1991; Pius et al., 1993). The positive response of 'Goldmine' to cobalt chloride may have been due to the induction of stress responses (Kiyosue et al., 1990) or to the inhibition of ethylene by cobalt ions (Wenzel et al., 1995; Pius et al., 1993; Biddington, 1992; Chraibi et al., 1991; Yang and Hoffman, 1984; Adams and Yang, 1979; Yu and Yang, 1979; Lau and Yang, 1976; Grover and Purves, 1976; Kang and Ray, 1969) by blocking the conversion of 1-aminocyclopropane carboxilic acid to ethylene (Roustan et al., 1989; Wenzel et al., 1995).

The small number of shoots obtained when explants were incubated only 2 weeks could be due to the insufficient induction time. Induction is a process by which a developmental signal acts on competent cells to alter their developmental fate (McDaniel et al., 1992). The induction time necessary for 'Goldmine' cells to become determined seems to be 5 weeks on MS medium amended with 0.1 μM BA, 11.5 μM IAA and 4.2 μM $CoCl_2$. A continuous supplement of cobalt chloride and BA seems to be necessary for shoot production for this cultivar.

Few shoots were produce on leaf explants of 'Adorn'. The positive effect of $AgNO_3$ on shoot organogenesis of cultivar 'Adorn' may have been due to a stress response (Kong and Yeung, 1995; Kiyosue et al., 1990) of this cultivar to silver ions or to an inhibition of ethylene production (Burnett et al., 1994; Chraibi et al.,1991; Palmer, 1992; Purnhauser et al., 1987; Beyer, 1976) or to an increase in ethylene production (Taylor et al., 1994). Silver nitrate stimulated

shoot regeneration in *T. aestivum*, *N. plumbaginifolia* (Purnhauser et al, 1987), *Zea mays* (Songstad et al., 1988), *B. campestris* (Chi et al., 1990; Palmer, 1992), *B. Juncea* (Chi et al., 1990), *H. annuus* (Chraibi et al., 1991) and *Prunus* spp. (Escalettes and Dosba, 1993). Ethylene production was not evaluated in these experiments. In order to evaluate if $AgNO_3$ has some effect on ethylene production and if this stimulates shoot organogenesis from leaf explants of 'Adorn', it is recommended to evaluate ethylene production in this cultivar. Ethylene may act either as a promoter or inhibitor of organogenesis, depending on the species (Biddington, 1992).

Effect of low concentration of salts from SH medium on shoot organogenesis from leaf explants

The most notable difference between MS and SH media is the ammonium ion concentration (Appendix 2), which in MS medium is 20.6 mM supplied as ammonium nitrate (NH_4NO_3) and in SH medium is 2.6 mM provided by ammonium phosphate ($(NH_4)H_2PO_4$).

The incubation of leaf explants 2 weeks on SH medium amended with 0.1 μM BA, 11.5 μM IAA and 5% or 6% sucrose seems to promote shoot organogenesis. Higher concentrations of sucrose support the formation of very small shoots from leaf explants. Reduction on plant height due to high osmoticum has been reported also in cauliflower and chrysanthemum (Short et al., 1987).

Shoots were not produced from leaf explants of 'Goldmine' on MS based media when the effects of ammonium concentration were evaluated. This suggest that another component of MS medium, other than ammonium ion

influenced organogenesis. The production of shoots on SH medium decreased as the ammonium concentration increases. Ammonium ion can inhibit cell division in protoplasts of some asteraceae (*Artemisia vulgaris*, *Chrysanthemum indicum* and *C. zawadskii*) (Okamura et al., 1984).

Nitrate and ammonium ions are the two common nitrogen sources used in tissue culture (Gamborg, 1970) and the balance between nitrate (NO_3^-) and ammonium (NH_4^+) can affect the response of a tissue to in vitro culture (Grimes and Hodges, 1990; Leblay et al. 1991) by regulating the differential absorption of the other ions by the leaf (Leblay et al., 1991; Davis et al., 1989). Good production of shoots from 'Goldmine' explants were achieved with a low ammonium:nitrate ratio. This ratio favors the use of C and N (Chen and Liao, 1993; Leblay et al., 1991).

'Iridon' explants produced shoots on both MS and SH media, with the greatest number of shoots produced with MS medium with 10.6 and 20.6 μM ammonium ion. The production of shoots on SH medium was almost constant regardless the ammonium ion concentration. The high organogenic capability under various culture conditions of this variety is clearly shown in this experiment.

The mechanisms of action of phytohormones and nitrogen compounds promotive of morphogenesis in cell and tissue cultures are still unknown (Reinert et al., 1977). There is no clear knowledge of gene expression associated with meristemoid induction on shoot apical meristem formation (Hicks, 1974) and the fundamental processes of early organogenesis are still poorly understood (Lyndon and Francis, 1992; Coleman and Ernst, 1990).

Effect of osmoticum on shoot organogenesis from leaf explants of *D. grandiflora* 'Iridon'

Differences betwen Ψ_π of agar and Ψ_π of the filter paper disc equilibrated with agar could be due to the matric component of the medium produced by agar (Debergh et al., 1981). Osmotic potential (Ψ_π), pressure potential (Ψ_p) and matric potential (Ψ_m) are the principal components of water potential (Kramer, 1983). In the agar medium the pressure potential does not exist and therefore can be omitted from the equation, but there is matric potential produced by the agar (Deberg et al., 1981; Beruto et al., 1995). Since Ψ_m is negative, psychrometer values (Ψ) should be more negative than osmometer values (Ψ_π). This was true for mannitol and sucrose, except for 3% sucrose but this could be due to errors in manipulations of the samples or to errors of reading the values on psychrometer.

The osmotic potential varied negligibly among and between plates, but it is recommended to stir frequently during the process of pouring the culture media into the petri dishes to avoid changes produced by more concentration of agar in the last petri dishes dispensed. The osmotic potential of liquid medium is more positive than the osmotic potential of solid medium because of the matric potential of solid medium and solute impurities of the gelling agent (Beruto et al., 1995).

Carbohydrates are required for meristemoid and shoot primordia formation (Thorpe and Murashige, 1970; Brown et al., 1979). The number of shoots formed generally decrease with higher concentrations of sucrose. With high concentrations of sucrose explants began to show necrosis, this is probably due to an increase of the rate of senescence (Hirsch, 1975).

The rate of shoot production from leaf explants when Ψ_π was partially replaced by mannitol was lower than shoot production with sucrose alone. This suggests that shoot production was affected by carbohydrate supplied. Mannitol is the most frequent sugar alcohol found in plants (Loescher et al., 1992) and is used as an experimental osmoticum because it is very slowly taken up by a wide range of plant cells (Thimann et al., 1960; Trip et al., 1964; Nadel et al., 1989). For this purpose it is recommended only for short term studies (hours) (Thompson et al., 1986).

Mannitol for this experiment was provided during the entire process of shoot organogenesis from explants of 'Iridon' (5 weeks) and this long exposure could be the cause of the inhibition of organogenesis. Mannitol can be absorbed by cells even if it is not used for growth (Dracup et al., 1986); melibiose does not penetrate the plasmalema, but it may be transported in small quantities to the leaves via apoplast and then accumulate in cell walls and is recommended as an effective osmoticum for long term studies (Dracup et al., 1986), however, the cost may be prohibitive. More experiments involving melibiose are recommended in order to evaluate the osmoticum effect of sucrose on shoot organogenesis of *D. grandiflora* 'Iridon'.

Higher concentrations of sucrose or sucrose+mannitol caused a decrease in mean plant height and number of shoots. High osmoticum strength reduced plant height and slowed growth in tissue culture of cauliflower and chrysanthemum (Short et al., 1987). A critical turgor pressure is necessary before growth and cell expansion can occur (Cleland, 1971; Zimmermann, 1978), but high levels of sucrose or mannitol would might have precluded the formation of that critical turgor pressure (Brown et al., 1979) and therefore,

inhibit shoot formation. However, in salinity studies, increased solute content of the culture medium can actually result in increased tissue Ψp while growth is inhibited (Bressan et al., 1982).

V. CONCLUSIONS

Leaf tissue from 'Adorn' showed less organogenic response than 'Goldmine'. No statistical differences among treatments were found in the experiments conducted with 'Adorn' but there was a slightly positive response and therefore it was considered as significative from the biological point of view. TDZ did not stimulate shoot proliferation from leaf explants of any of the varieties at any of the concentrations evaluated. The phenomena of fasciation and hyperhydricity observed on 'Iridon' could be due to long exposure to TDZ. Shoot fasciation was observed only in 'Iridon', but not in 'Goldmine'.

Silver nitrate stimulated shoot organogenesis from leaf explants of 'Adorn' when explants were incubated on MS medium amended with 0.1 μM BA, 11.5 μM IAA and 0.58 μM $AgNO_3$ during 7 weeks and then transferred to a medium with same concentration of $AgNO_3$ but without growth regulators. Cobalt chloride induced shoot organogenesis from leaf tissue of 'Goldmine' and the greatest number of shoots was obtained after 5 weeks of culture on medium with 0.1 μM BA, 11.5 μM IAA and 4.2 μM $CoCl_2$. Tissues were transferred to a medium with 0.1 μM BA and 4.2 μM $CoCl_2$.

Since evaluations of ethylene production were not made for the varieties 'Goldmine' and 'Adorn', it is not possible to determine if $CoCl_2$ and $AgNO_3$ stimulated shoot organogenesis of 'Goldmine' and 'Adorn' by inhibiting ethylene production or if these heavy metals produce a stress response that indeed stimulates shoot organogenesis. It is recommended to measure this parameter in future experiments.

Another approach for micropropagation of 'Goldmine' was evaluated and shoots were produced when leaf explants were incubated on SH medium

amended with 0.1 μM BA, 11.5 μM IAA and 5% sucrose during 2 weeks. High concentrations of salts of MS medium and high concentration of ammonium on SH medium seem to inhibit shoot organogenesis of 'Goldmine'. 'Iridon' is not affected by the concentration of ammonium in culture media and produces shoots on medium with high and low concentrations of salts. This observation is in accordance with the high organogenic capability of this variety.

A method for measuring osmotic potential of solid medium was evaluated. Sample discs were introduced forming a 45 degree angle with the agar until completely immersed into the agar. The measurement of the osmotic potential can be determined after 3 minutes of incubation of the sample disc into the agar. The differences among values obtained with psychrometer and osmometer could be due to the matric potential of the agar medium; the osmotic potential of liquid medium is more positive than the solid medium.

Shoot production and length of shoots decreases as a sucrose concentration increases over 4% sucrose. Rate of shoot production from leaf explants with sucrose osmotic potential being replaced by mannitol is lower than shoot production with sucrose alone. Mannitol seems to inhibit shoot organogenesis from leaf tissue of 'Iridon'. More experiments should be conducted in order to evaluate if mannitol is being metabolized by chrysanthemum.

LITERATURE CITED

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Adams, D. and S. F. Yang. 1979. Ethylene biosynthesis: Identification of 1-aminocyclopropane-1carboxylic acid as an intermediate in the conversion of methionine to ethylene. Proc. Nat. Acad. Sci. USA 73: 170-174.

Ahmed, H. A. and M. Andrea. 1987. Effect of heat treatment on acceleration chrysanthemum multiplication by meristem-tip culture. Acta Hort. 212: 99-106.

Anderson, N. O. 1987. Reclassification of the genus Chrysanthemum L. HortScience 22: 313.

Aribaud, M., M. Carré and J.Martin-Tanguy. 1994. Polyamine metabolism and 'in vitro' cell multiplication and differentiation in leaf explants of *Chrysanthemum morifolium* Ramat. Plant Growth Regul. 15: 143-155.

Arndt, F. R., R. Rusch, H. V. Stillfried, B. Hanisch and W. C. Martin. 1976. SN 49537, A new defoliant. Plant Physiol. 57: 99.

Bearce, B.C. and H.C. Kohl. 1970. Measuring osmotic pressure of sap within live cells by means of a visual melting point apparatus. Plant Physiol. 46: 515-519.

Ben-Jaacov, J. and R. W. Langhans. 1972. Rapid multiplication of *Chrysanthemum morifolium* plants by stem-tip proliferation. HortScience 7: 289-290.

Bergmann, B. A. and A. M. Stomp. 1994. Effect of genotype on in vitro adventitious shoot formation in Pinus radiata and correlations between pairs of phenotypic traits during in vitro shoot development. Plant Cell, Tiss. Org. Cult. 39: 185-194.

Beruto, D., M. Beruto, C. Ciccarelli and P. Debergh. 1995. Matric potential evaluations and measurements for gelled substrates. Physiol. Plant. 94: 151-157.

Beyer, E.M. 1976. A potent inhibitor of ethylene action in plants. Plant Physiol. 58: 268-271.

Biddington, N.L. 1992. The influence of ethylene in plant tissue culture. Plant Growth Regul. 11: 173-187.

Bressan, R. A., A. K. Handa, S. Handa and p. M. Hasegawa. 1982. Growth and water relations of cultured tomato cells after adjustment to low external water potentials. Plant Physiol. 70: 1303-1309.

Briggs, B. A., S. M. McCulloch and L. A. Edick. 1988. Micropropagation of azaleas using thidiazuron. Acta Hort. 227: 330-333.

Broertjes, C., S. Roest and G. S. Bokelman. 1976. Mutation breeding of Chrysanthemum morifolium Ram. using in vivo and in vitro adventitious bud techniques. Euphytica 25: 11-19.

Brown, D. C. W., D. W. M. Leung and T. A. Thorpe. 1979. Osmotic requirement for shoot formation in tobacco callus. Physiol. Plant. 46: 36-41.

Burnett, L., M. Arnoldo, S. Yarrow and B. Huang. 1994. Enhancement of shoot regeneration from cotyledon explants of *Brassica rapa* ssp. *oleifera* through pre treatment with auxin and cytokinin and use of ethylene inhibitors. Plant Cell, Tiss. Org. Cult. 37: 253-256.

Bush, S. R., E. D. Earle and R. W. Langhans. 1976. Plantlets from petal segments, petal epidermis, and shoot tips of the periclinal chimera, *Chrysanthemum morifolium* 'Indianapolis'. Amer. J. Bot. 63: 729-737.

Capelle, S. C., D. W. S. Mok, S. C. Kirchner and M. C. Mok. 1983. Effects of thidiazuron on cytokinin autonomy and the metabolism of N^6-(Δ_2-isopentenyl) [8-^{14}C] adenosine in callus tissues of *Phaseolus lunatus* L. Plant Physiol. 73: 796-802.

Chalupa, V. 1988. Large scale micropropagation of *Quercus robur* L. using adenine-type cytokinins and thidiazuron to stimulate shoot proliferation. Biol. Plant. 30: 414-421.

Chen, J. J. and Y. L. Liao. 1993. Nitrogen-induced changes in the growth and metabolism of cultured potato tubers. J. Amer. Soc. Hort. Sci. 118: 831-834.

Chi, G.L. and E.C. Pua. 1989. Ethylene inhibitors enhanced de novo shoot regeneration from cotyledons of *Brassica campestris* spp. in vitro. Plant Sci. 64: 243-250.

Chi, G.L., D.G. Barfield, G.E. Sim and E.C. Pua. 1990. Effect of AgNO3 and aminoethoxyvinylglycine on in vitro shoot and root organogenesis from seedling explants of recalcitrant *Brassica* genotypes. Plant Cell Rep. 9: 195-198.

Chi, G.L., E.C. Pua and C.J. Goh. 1991. Role of ethylene on de novo shoot regeneration from cotyledonary explants of *Brassica campestris* spp. *pekinensis* (Lour) Olsson in vitro. Plant Physiol. 96: 178-183.

Chraibi, K. M., A. Latche, J.P. Roustan and J. Fallot. 1991. Stimulation of shoot regeneration from cotyledons of *Helianthus annuus* by the ethylene inhibitors, silver and cobalt. Plant Cell Rep. 10: 204-207.

Christianson, M. L. and D. A. Warnick. 1988. Organogenesis in vitro as a developmental process. HortScience 23: 515-519.

Cleland, R. 1967. Cell wall expansion. Annu. Rev. Plant Physiol. 22: 197-222.

Coleman, G. D. and S. G. Ernst. 1990. Shoot induction competence and callus determination in *Populus deltoides*. Plant Sci. 71: 83-92. 1990.

Cousineau, J. C. and Donnelly, D. J. 1991. Adventitious shoot regeneration from leaf explants of tissue cultured and greenhouse-grown raspberry. Plant Cell, Tiss. Org. Cult. 27: 249-255.

Cuello, J. L., P. N. Walker and C. W. Heuser. 1992. Controlled in vitro environment for stage II micropropagation of chrysanthemum. Trans ASAE 35: 1079-1083.

Dalsou, V. and K. C. Short. 1987. Selection for sodium chloride tolerance in chrysanthemums. Acta Hort. 212: 737-740.

Davis, J. M., W. H. Loescher, M. W. Hammond and R. E. Thornton. 1989. Response of potatoes to nitrogen form and to change in nitrogen form at tuber initiation. J. Amer. Soc. Hort. Sci. 111: 70-72.

Debergh, P. C., Y. Harbaoui and R. Lemeur. 1981. Mass propagation of globe artichoke (*Cynara scolymus*), evaluation of different hypotheses to overcome vitrification with special reference to water potential. Physiol. Plant. 53: 181-187.

Debergh, P., J. Aitken-Christie, D. Cohen, B. Grout, S. Von Arnold, R. Zimmerman and M. Ziv. 1992. Reconsideration of the term 'vitrification' as used in micropropagation. Plant Cell, Tiss. Org. Cult. 30: 135-140.

Debergh, P. C. 1983. Effects of agar brand and concentration on the tissue culture medium. Physiol. Plant. 59: 270-276.

De Jong, J. and J. B. M. Custers. 1986. Induced changes in growth and flowering of Chrysanthemum after irradiation and in vitro culture of pedicels and petal epidermis. Euphytica 35: 137-148.

Dimasi-Theriou, K., A.S. Economou and E.M. Sfakiotakis. 1993 Promotion of petunia (*Petunia hybrida* L.) regeneration in vitro by ethylene. Plant Cell, Tiss. Org. Cult. 32: 219-225.

Dowrick, G. J. 1953. The chromosomes of chrysanthemum, II: garden varieties. Heredity 7: 59-72.

Dracup, M., J. Gibbs and H. Greenway. 1986. Melibiose, a suitable, non-permeating osmoticum for suspension-cultured tobacco cells. J. Expt. Bot. 37: 1079-1089.

Drewlow L.W., P. D. Ascher and R. E. Widmer. 1973. Genetics studies of self incompatibility in the garden chrysanthemum, *Chrysanthemum morifolium* Ramat. Theor. Appl. Genet. 43: 1-5.

Earle, E. D. and R. W. Langhans. 1974. Propagation of *Chrysanthemum morifolium* in vitro. I. Multiple plantlets from shoot tips and the establishment of tissue cultures. J. Amer. Soc. Hort. Sci. 99: 128-132.

Escalettes, V. and F. Dosba. 1993. In vitro adventitious shoot regeneration from leaves of *Prunus* spp. Plant Sci. 90: 201-209.

Ettienne, H., A. Berger and M. P. Carron. 1991. Water status of callus from *Hevea brasilensis* during induction of somatic embryogenesis. Physiol. Plant. 82: 213-218.

Fasolo, F., R. H. Zimmerman and I. Fordham. 1989. Adventitious shoot formation on excised leaves of in vitro grown shoots of apple cultivars. Plant Cell Tiss. Org. Cult. 16: 75-87.

Ficcadenti, N. and G. L. Rotino. 1995. Genotype and medium affect shoot regeneration of melon. Plant Cell, Tiss. Org. Cult. 40: 293-295.

Fletcher, J.T. 1992. Disease resistance in protected crops and mushrooms. Euphytica 63: 33-49.

Gamborg, O. L. 1970. The effects of amino acids and ammonium on the growth of plant cells in suspension culture. Plant Physiol. 45: 372-375.

Gashghaie, J., F. Brenckmann and B. Saugier. 1991. Effects of agar concentration on water status and growth of rose plants cultured in vitro. Physiol. Plant. 82: 73-78.

Gribaudo, I. and A. Fronda. 1991. Effects of thidiazuron on grapevine axillary buds cultivated in vitro. HortScience 26: 1083.

Grimes, H. D. and T. K. Hodges. 1990. The inorganic NO_3^-:NH_4^+ ratio influences plant regeneration and auxin sensitivity in primary callus derived

from immature embryo of indica rice (*Oryza sativa* L.). J. Plant Physiol. 136: 362-367.

Grover, S. and W. K. Purves. 1976. Cobalt and plant development. Plant Physiol. 57: 886-889.

Hachey, J.E., K.K. Sharma and M. M. Moloney. 1991. Efficient shoot regeneration of *Brassica campestris* using cotyledon explants cultured in vitro. Plant Cell Rep. 9: 549-554.

Halperin, W. 1986. Attainment and retention of morphogenetic capacity in vitro. In: I. K. Vasil. Cell culture and somatic cell genetics of plants. Vol. 3. Plant regeneration and genetic variability.

Hammersley-Straw, D. R. H. and T. A. Thorpe. 1988. Use of osmotic inhibition in studies of shoot formation in tobacco callus cultures. Bot. Gaz. 149: 303-310.

Hicks, G. S. 1994. Shoot induction and organogenesis in vitro: A developmental perspective. In vitro Cell. Dev. Biol. 30P: 10-15.

Hill, G. P. 1967. Morphogenesis in stem-callus cultures of *Convolvulus arvensis* L. Ann. Bot. 31: 437-446.

Hill, G. P. 1968. Shoot formation in tissue cultures of *Chrysanthemum morifolium* 'Bronze pride'. Physiol. Plant. 21: 386-389.

Hirsch, A. M. 1975. The effect of sucrose on the difference of excised fern leaf tissue into either gametophytes or sporophytes. Plant Physiol. 56: 390-393.

Huetteman, C. A. and J. E. Preece. 1993. Thidiazuron: a potent cytokinin for woody plant tissue culture. Plant Cell, Tiss. Org. Cult. 33: 105-119.

Huitema, J. B. M., G. C. Gussenhoven, J. De Jong and J. J. M. Dons. 1987. Selection and in vitro characterization of low temperatures tolerant mutants of *Chrysanthemum morifolium* Ramat. Acta Hort. 197: 89-96.

Iizuka, M., E. Matsumoto, A.Doi, R.Madrigal and A. Fukushima. 1973. Tubular floret culture of Chrysanthemum and Cineraria in vitro. Jpn. J. Genet. 48: 79-87.

Kamada, H., K. Kobayashi, T. Kiyosue and H. Harada. 1989. Stress induced somatic embryogenesis in carrot and its application to synthetic seed production. In Vitro Cell. Dev. Biol. 25: 1163-1169.

Kang, B. G. and P. M. Ray. 1969. Ethylene and carbon dioxide as mediators in the response of the bean hypocotyl hook to light and auxins. Planta 87: 206-216.

Kaul, V., R.M. Miller, J.F. Hutchinson and D. Richards. 1990. Shoot regeneration from stem and leaf explants of *Dendranthema grandiflora* Tzvelev (syn. *Chrysanthemum morifolium* Ramat.). Plant Cell, Tiss. Org. Cult. 21: 21-30.

Kerns, H. R. and M. M. Meyer. 1986. Tissue culture propagation of *Acer* x *freemanii* using thidiazuron to stimulate shoot tip proliferation. HortScience 21: 1209-1210.

Khalid, N., M. R. Davey and J. B. Power. 1989. An assessment of somaclonal variation in *Chrysanthemum morifolium*: The generation of plants of potential commercial value. Sci. Hort. 38: 287-294.

Khehra, M., K. C. Lowe, M. R. Davey and J. B. Power. 1995. An improved micropropagation system for chrysanthemum based on Pluronic F-68-supplemented media. Plant Cell, Tiss. Org. Cult. 41: 87-90.

Kimball, S.I., W.D. Beversdorf and E.T. Bingham. 1975. Influence of osmotic potential on the growth and development of soybean tissue cultures. Crop Sci. 15: 750-752.

Kiyosue, T., K. Takano, H. Kamada and H. Harada. 1990. Induction of somatic embryogenesis in carrot by heavy metal ions. Can. J. Bot. 68: 2301-2303.

Klein, T. M., E. C. Harper, Z. Svab, J.C. Sanford, M. E. Fromm and P. Maliga. 1988. Stable genetic transformation of intact Nicotiana cells by the particle bombardment process. Proc. Natl. Acad. Sci. U.S.A. 85: 8502-8505.

Kong, L. And E.C. Yeung. 1995. Effects of silver nitrate and polyethylene glycol on white spruce (*Picea glauca*) somatic embryo development: enhancing cotyledonary embryo formation and endogenous ABA content. Physiol. Plant. 93: 298-304.

Kramer, P.J. 1983. Problems in water relations of plants and cells. Intl. Rev. of Cytology 85: 253- 286.

Lal, R. and S. Lal. 1993. Genetic engineering of plants for crop improvement. CRC press. Boca Raton. FL. 246 p.

Langton, F. A. 1981. Constraints on the selection of superior genotypes in initial trials of chrysanthemum progeny. Acta Hort. 125: 21-29.

Lau, O. L. and S. F. Yang. 1976. Inhibition of ethylene production by cobaltous ion. Plant Physiol. 58: 114-117.

Leblay, C., E. Chevreau and L. M. Raboin. 1991. Adventitious shoot regeneration from in vitro leaves of several pear cultivars (*Pyrus communis* L.) Plant Cell, Tiss. Org. Cult. 25: 99-105.

Leva, A. R. 1986. Test on organogenesis from callus of *Actinidia chinensis*. Acta Hort. 179: 883-884.

Linsmaier, E. M., and F. Skoog. 1965. Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. 18: 100-127.

Loescher, W. H., R. H. Tyson, J. D. Everard, R. J. Redgwell and R. L. Bieleski. 1992. Mannitol synthesis in higher plants. Plant Physiol. 98: 1396-1402.

Lu, C. Y., G. Nugent and T. Wardley. 1990. Efficient, direct plant regeneration from stem segments of Chrysanthemum (*Chrysanthemum morifolium* Ramat. cv. 'Royal purple'). Plant Cell Rep. 8: 733-736.

Lyndon, R. F. and D. Francis. 1992. Plant and organ development. Plant Mol. Biol. 19: 51-68.

Malik, K. A. and P. K. Saxena. 1992. Regeneration in *Phaseolus vulgaris* L.: High frequency induction of direct shoot formation in intact seedlings by N6-benzylaminopurine and thidiazuron. Planta 186: 384-389.

May, R. A. and R. N. Trigiano. 1991. Somatic embryogenesis and plant regeneration from leaves of *Dendranthema grandiflora*. J. Amer. Soc. Hort. Sci. 116: 366-371.

McDaniel, C. N., S. R. Singer and S. M. E. Smith. 1992. Developmental states associated with the floral transition. Dev. Biol. 153: 59-69.

Meijer, E. G. M. 1989. Developmental aspects of ethylene biosynthesis during somatic embryogenesis in tissue cultures of *Medicago sativa*. J. Expt. Bot. 40: 479-484.

Meins, F. 1988. Cell commitment and determination in plants. In: J. K. Setlow (ed). Genetic engineering principles and methods. Vol. 10. Plenum Press. N. Y. p. 141-153.

Meyer, H. J. and J. Van Staden. 1988. In vitro multiplication of *Ixia flexuosa*. HortScience 23: 1070-1071.

Mok, M.C., D. W. S. Mok, D. J. Armstrong, K. Shudo, Y. Isogai and T. Okamoto. 1982. Cytokinin activity of N-phenyl-N'1,2,3-thiadiazol-5-yl urea (thidiazuron). Phytochemistry 21: 1509-1511.

Mukherjee, S. K., B. Rathinasabapathi and N. Gupta. 1991. Low sugar and osmotic requirements for shoot regeneration from leaf pieces of *Solanum melongena* L. Plant Cell, Tiss. Org. Cult. 25: 13-16.

Mullineaux, P. M. 1992. Genetically engineered plants for herbicide resistant. In: Gatehouse, A. M. R., V. A. Hilder and D. Bouter (Eds.) Plant genetic manipulation for crop protection. CAB International. Wallingford. U. K. 266 p.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Nabors, M. W., S. E. Gibbs, C. S. Bernstein and M. Meis. 1980. NaCl-tolerant tobacco plants from cultured cells. Z. Pflanzenphysiol. 97: 13-17.

Nadel, B. L., A. Altman and M. Ziv. 1989. Regulation of somatic embryogenesis in celery cell suspensions. Plant Cell, Tiss. Org. Cult. 18: 181-189.

Niedz, R. P., S. Schiller-Smith, K.B. Dunbar, C.T. Stephens and H.H. Murakishi. 1989. Factors influencing shoot regeneration from cotyledonary explants of *Cucumis melo*. Plant Cell, Tiss. Org. Cult. 18: 313-319.

Nugent, G., T. Wardley-Richardson and C. Y. Lu. 1991. Plant regeneration from stem and petal of carnation (*Dianthus caryophyllus* L.). Plant Cell Rep. 10: 477-480.

Okamura, M., T. Hayashi and S. Miyazaki. 1984. Inhibiting effect of ammonium ion in protoplast culture of some asteraceae plants. Plant Cell Physiol. 25: 281-286.

Palmer, C. E. 1992. Enhanced shoot regeneration from *Brassica campestris* by silver nitrate. Plant Cell Rep. 11: 541-545.

Peferoen, M. 1992. Engineering of insect-resistant plants with *Bacillus thuringiensis* crystal protein genes. In: Gatehouse, A. M. R., V. A. Hilder and D. Bouter (Eds.) Plant genetic manipulation for crop protection. CAB International. Wallingford. U. K. 266 p.

Pius, J., L. George, S. Eapen and P. S. Rao. 1993. Enhanced plant regeneration in pearl millet (*Pennisetum americanum*) by ethylene inhibitors and cefotaxime. Plant Cell, Tiss. Org. Cult. 32: 91-96.

Preece, J. E. and M. R. Imel. 1991. Plant regeneration from leaf explants of Rhododendron 'P. J. M. hybrids'. Sci. Hort. 48: 159-170.

Preece, J. E., C. A. Huetteman, W. C. Ashby and P. L. Roth. 1991. Micro and cutting propagation of silver maple. II. Genotype and provenance affect performace. J. Amer. Soc. Hort. Sci. 116: 149-155.

Purnhauser, L., P. Medgyesy, M. Czakó, P.J. Dix and L. Márton. 1987. Stimulation of shoot regeneration in *Triticum aestivum* and *Nicotiana plumbaginifolia* Viv. tissue cultures using the ethylene inhibitor $AgNO_3$. Plant Cell Rep. 6: 1-4.

Qureshi, J. A. and P. K. Saxena. 1992. Adventitious shoot induction and somatic embryogenesis with intact seedling of several hybrid seed geranium (*Pelargonium* x *hortorum* Bailey) varieties. Plant Cell Rep. 11: 443-448.

Reinert, J., Y. P. S. Bajaj and B. Zbell. 1977. Aspects of organization-organogenesis, embryogenesis, cytodifferentiation. In: Street, H. E. (Ed.). Plant Tissue and Cell culture. California Press. Berkeley. 614 p.

Robinson, K. E. P. and E. Firoozabady. 1993. Transformation of floriculture crops. Sci. Hort. 55: 83-99.

Roest, S. and G. S. Bokelmann. 1975. Vegetative propagation of *Chrysanthemum morifolium* Ram. in vitro. Sci. Hort. 3: 317-330.

Ronald, W. G. and P. D. Ascher. 1975. Self compatibility in garden *Chrysanthemum* [*morifolium*]: occurrence, inheritance and breeding potential. Theor. Appl. Genet. 46: 45-54.

Roustan, J. P., A. Latche and J. Fallot. 1989. Stimulation of *Daucus carota* somatic embryogenesis by inhibitors of ethylene synthesis: Cobalt and Nickel. Plant Cell Rep. 8: 182-185.

Salisbury, F.B. and C.W. Ross. 1985. Plant Physiology. Third edition. Wadsworth Publishing. California. 540 p.

Schenk, R. U. and A. C. Hildebrandt. 1972. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot. 50: 199-204.

Shibli, R. A., M. A. L. Smith and L. A. Spomer. 1992. Osmotic adjustment and growth responses of three *Chrysanthemum morifolium* Ramat cultivars to osmotic stress induced in vitro. J. Plant nutr. 15: 1373-1381.

Short, K., J. Warburton and A. Robert. 1987. In vitro hardening of cultured cauliflower and chrysanthemum plantlets to humidity. Acta Hort. 212: 329-334.

Singha, S. 1982. Influence of agar concentration on in shoot proliferation of *Malus* sp. 'Almay' and *Pyrus communis* 'Seckel'. J. Amer. Soc. Hort. Sci. 107: 657-660.

Slavík, B. 1974. Methods of studying plant water relations. Springer-Verlag. Berlin. 449 p.

Slusarkiewicz-Jarzina, A., M. Zenkteler and B. Podlewska. 1982. Regeneration of plants from leaves of *Chrysanthemum morifolium* Ram. cv. 'Bronze Bornholm' in in vitro cultures. Acta Soc. Bot. Poloniae. 51: 173-178.

Songstad, D. D., D. R. Duncan and J. M. Widholm. 1988. Effect of 1-aminocyclopropane 1-carboxylic acid, silver nitrate, and norbornadiene on plant regeneration from maize callus cultures. Plant Cell Rep. 7: 262-265.

Spanner, D.C. 1951. The Peltier effect and its use in the measurement of suction pressure. J. Expt. Bot. 2: 145-168.

Steel, R. G. D. and J. H. Torrie. 1980. Principles and procedures of statistics a biometrical approach. Second ed. McGraw-Hill. N. Y. 633 p.

Street, H. E. (Ed). 1977. Plant tissue and cell culture. Second edition. University of California press. Berkeley.

Taylor, P.W.J., H. L. Ko, T. A. Fraser, N. Masel and S. W. Adkins. 1994. Effect of silver nitrate on sugar cane cell suspension growth, protoplast isolation, ethylene production and shoot regeneration from cell suspension cultures. J. Expt. Bot. 45: 1163-1168.

Thimann, K. V., G. M. Loos and E. W. Samuel. 1960. Penetration of mannitol into potato discs. Plant Physiol. 35: 848-853.

Thomas, J. C. and F. R. Katterman. 1986. Cytokinin activity induced by thidiazuron. Plant Physiol. 81: 681-683.

Thompson, M. R., T.J. Douglas, H. Obata-Sasamoto and T. A. Thorpe. 1986. Mannitol metabolism in cultured plant cells. Physiol. Plant. 67: 365-369.

Thorpe, T. A. and D. E. Meier. 1972. Starch metabolism, respiration and shoot formation in tobacco callus cultures. Physiol. Plant. 27: 365-369.

Thorpe, T. A. and T. Murashige. 1970. Some histological changes underlying shoot initiation in tobacco cultures. Can. J. Bot. 48: 277-285.

Trigiano, R. N. and R. A. May. 1994. Laboratory exercises illustrating organogenesis and transformation using chrysanthemum cultivars. HortTechnology 4: 325-327.

Trip, P., G. Krotkov and C. D. Nelson. 1964. Metabolism of mannitol in higher plants. Amer. J. Bot. 51: 828-835.

Tyree, M.T. and H.T. Hammel. 1972. The measurement of the turgor pressure and the water relations of plants by the pressure-bomb technique. J. Expt. Bot. 23: 267-282.

United States Department of Agriculture. USDA. 1995. Economics and Statistics System: Reports. Internet. Floriculture Crops (#2FC-BB).

Urban, L. A., J.M. Sherman, J. W. Moyer and M. E. Daub. 1994. High frequency shoot regeneration and *Agrobacterium*-mediated transformation of chrysanthemum (*Dendranthema grandiflora*). Plant Sci. 98: 69-79.

Van Nieuwkerk, J.P., R. H. Zimmermann and I. Fordham. 1986. Thidiazuron stimulation of apple shoot proliferation in vitro. HortScience 21: 5l6-518.

Verma, D. C. and D. K. Dougall. 1977. Influence of carbohydrates on quantitative aspects of growth and embryo formation in wild carrot suspension cultures. Plant Physiol. 59: 81-85.

Wenzel, A. A., H. Schlautmann, C. A. Jones, K. Küpopers and H. Mehlhorn. 1995. Aminoethoxyvinylglycine, cobalt and ascorbic acid all reduce ozone toxicity in mung beans by inhibition of ethylene biosynthesis. Physiol. Plant. 93: 286-290.

Williams, J., D. A. C. Pink and N. L. Biddington. 1990. Effect of silver nitrate on long-term culture and regeneration of callus from *Brassica oleraceae* var *gemmifera*. Plant Cell, Tiss. Org. Cult. 21: 61-66.

Yang, S.F. and N.E. Hoffman. 1984. Ethylene biosynthesis and its regulation in higher plants. Annu. Rev. Plant Physiol. 35: 155-189.

Yu, B. and S. F. Yang. 1979. Auxin-induced ethylene production and its inhibition by aminoethoxyvinylglycine and cobalt ion. Plant Physiol. 64: 1074-1077.

Yusnita, S., R. L. Geneve and S. T. Kester. 1990. Micropropagation of white flowering eastern redbud (*Cercis canadiensis* var. alba L.). J. Environ. Hort. 8: 177-179.

Zimmermann, U. 1978. Physics of turgor and osmoregulation. Annu. Rev. Plant Physiol. 29: 121-148

APPENDICES

APPENDIX 1

Methods for measuring osmotic potential

Different techniques have been developed to measure osmotic potential (Ψ_π), directly in the cells or by first obtaining the sap from tissues and then determining the Ψ_π of the sap. Cell methods are used to measure $\Psi\pi$ *in situ* in cells and includes the limiting plasmolysis, plasmometric, visual melting point method and the pressure-bomb technique.

The limiting plasmolysis method uses a series of test solutions to find the solution at which about half of the cells of a living tissue undergo incipient plasmolysis (Slavík, 1974) This solution concentration corresponds to the zero pressure potential. The Ψ_π of this solution is equal to the Ψ_π of the cell vacuolar sap. Samples of tissue are put in a series of test solutions. The time necessary for plasmolysis to occur is determined by the point at which the protoplast just separates from the cell-wall. This is called limiting or incipient plasmolysis. If there is plasmolysis in some cells, about 100 cells are examined and the number of cells in which plasmolysis has just started is determined. The results are plotted with percentage of plasmolysed cells against molar concentration of the solution. The value at which 50% of the cells are plasmolized corresponds to the zero pressure potential. The accuracy of the values obtained depends on how closely the solutions are graded (Slavík, 1974).

The plasmometric method can be used only where the volume of the protoplast and of the cell can be calculated from measurements made under a microscope (Slavík, 1974). The tissue is placed in a hypertonic solution until equilibrium is reached and then removed and cells large enough for accurate

measurement are examined under the microscope. The Ψ_π is expressed by the following equation:

$$\Psi_{(\text{limiting plasmolisis})} = \Psi_\pi \text{ (solution)} \frac{V \text{ protoplast}}{V\text{cell}}$$ (Slavík, 1974)

This method can be used to determine the Ψ_π of a single cell. Cells must have large vacuoles, using this type of cells this method is more accurate than the limiting plasmolisis method (Slavík, 1974).

The pressure-bomb technique can be used for measuring the Ψ_π of a leaf or branch of a plant previously hydrated (Tyree and Hammel, 1972). The branch or leaf tissue for evaluation is enclosed inside a pressure chamber except for the cut end of the petiole that protrudes through an air-tight seal into the open air. The pressure of the bomb is increased until fluid is forced out of the petiole. The pressure is then lowered until fluids neither flow in nor out. The process is repeated and each time there is an increment in volume and a new balance pressure is found. A plot of 1/p against volume is constructed. This is the pressure-volume curve. From this curve the Ψ_π of the cells of the original hydrated tissue and the volume of liquid originally present in the cells can be interpolated (Salisbury and Ross, 1985).

Visual melting point technique can be used to determine Ψ_π with an accuracy of ±1.2 atmospheres (Bearce and Kohl, 1970). The freezing-slide apparatus can be used for visual observation of freezing water and melting ice within plant cells. The apparatus consists of a microscope slide glued into a Plexiglas jacket through which cold 90% ethanol is pumped at varying rates for temperature control. Temperature is recorded by a thermocouple connected to a recording potentiometer. With this methodology, osmotic pressures of cell sap of

guard and adjoining epidermal cells of chrysanthemum and geranium (*Pelargonium hortorum* Bailey) can be measured (Bearce and Kohl, 1970)

Methods for determination of Ψ_π of cell sap have been developed. It is assumed that the Ψ_π of the expressed cell sap is similar to that of the vacuolar solution in situ. These methods give values of Ψ_π for cells in the normal state of turgor and are appropriate for reliable measurement of Ψ_π of sap released (Slavík, 1974). Some of these methods are cryoscopy, vapor-pressure method and psychrometric methods.

Cryoscopy is an accurate method of measuring the freezing point of solutions. Highly accurate mercury thermometers and thermocouples are available for determining the freezing point of solutions. The osmotic pressure developed by a 1.0 molal solution of nonionizing solute at zero centigrade in a perfect osmometer is 2.27 MPa, and its freezing point (Δf) is -1.86°C (Salisbury and Ross, 1985). Since the freezing point is a function of the mole fraction and hence of the Ψ_π, and can be estimated as follows:

$$\frac{\Psi\pi}{\Delta_f} = \frac{\text{-2.27 MPa}}{\text{-1.86°C}}$$

or Ψ_π (in Mpa) = $1.22\Delta f$ (Δf in degrees C) (Salisbury and Ross, 1985).

The vapor-pressure method can be used for measuring Ψ_π in expressed cell sap. The pressure in the plant cell must be eliminated before doing any kind of measurement and this can be accomplished by rapid freezing that produces ice crystals and therefore ruptures all the membranes. The vapor-pressure method is based on equilibration of Ψ_π from the air and Ψ_π of the tissue when a tissue is placed in a small chamber. A thermocouple is the instrument used to measure the Ψ_π of the cell sap after equilibrium with the air inside of the chamber. This measurement is possible because of the Peltier effect; when two

metals are placed in contact and a current is passed across the junction, there is a liberation or absorption of heat at the point of union (Spanner, 1951). The difference of electrical potential assumed by the two metals is called Peltier coefficient. When a current is applied to cool the junction of the thermocouple below the dew-point, there is a condensation of moisture on the junction and the thermocouple will become potentially a delicate 'wet-bulb' thermometer (Spanner, 1951). The device is calibrated using solutions of known concentration of solutes in order to measure the Ψ_{π} of expressed cell sap.

APPENDIX 2

Composition of Murashige & Skoog (1962) (MS) and Schenk & Hildebrandt (1972) (SH) macro and micronutrients media for plant tissue culture.

SALTS	concentration	
	MS	SH
macronutrients	mM	mM
NH_4NO_3	20.6	
KNO_3	18.8	25.0
$CaCl_2.2H_2O$	3.0	1.4
$MgSO_4.7H_2O$	1.5	1.6
KH_2PO_4	1.2	
$(NH_4)H_2PO_4$		2.6
micronutrients	μM	μM
KI	5.0	6.0
H_3BO_3	100.0	80.0
$MnSO_4.4H_2O$	100.0	
$MnSO_4.H_2O$		60.0
$ZnSO_4.7H_2O$	30.0	3.5
$Na_2MoO_4.2H_2O$	1.0	0.4
$CuSO_4.5H_2O$	0.1	0.8
$CoCl_2.6H_2O$	0.1	0.4
Fe-EDTA chelate	100.0	55.0

VITA

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