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EFFECTS OF CYTOKININS AND AN AUXIN ON ORGANOGENESIS IN VITRO OF FRASER FIR (*ABIES FRASERI* (PURSH) POIR.)

A Thesis

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Degree

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ABSTRACT

Abies fraseri (Pursh) Poir., Fraser fir, grows in the southern Appalachian region of the United States and is important in the Christmas tree industry. Natural stands of Fraser fir are rapidly dwindling. This decline has been attributed to the exotic pest balsam woolly adelgid [Adelges piceae (Ratz.)]. In this investigation, Abies fraseri embryos have been cultured in vitro with the main objective of micropropagation through adventitious bud induction. Sucrose concentrations in the medium of 0 to 8% and three medium formulations [Schenk and Hildebrandt (1972), Cheng (1977), and Medium for conifer morphogenesis of Bornman and Jansson (1982)] were tested on the growth of Abies fraseri embryo explants in an attempt to optimize conditions for explant growth. Two to 4% sucrose and Cheng medium formulation produced the best growth of explants exhibited by cotyledon length and fresh weight. Induction of adventitious buds was accomplished with the addition of cytokinin to the medium. Basal medium (Cheng with 3% sucrose) was supplemented with N_6-(2-isopentenyl)amino purine (2iP), kinetin (KN), or N_6-benzylaminopurine (BA) at the concentrations of 10^{-7}M, 10^{-6}M, and 10^{-5}M, respectively. No adventitious buds were formed on explants left on cytokinin-containing medium for six weeks. Adventitious buds were formed on explants cultured on cytokinin-containing medium for two or four weeks then recultured on basal medium without growth regulators. Adventitious buds were produced on explant hypocotyls and varied

in number with each treatment. The average number of adventitious buds formed per explant ranged from 0 to 0.52. No greater than 36% of the cultured embryos formed adventitious buds. Data analysis indicated that each cytokinin tested was equally effective in inducing adventitious buds. In general, the explants with a four-week incubation on a medium containing cytokinins had a significantly greater production of adventitious buds as compared to explants with a two-week exposure to cytokinins. Cytokinin levels of 10^{-6}M and 10^{-7}M induced significantly more adventitious buds on explants with a four-week exposure to cytokinin-containing medium than did 10^{-7}M cytokinin. The addition of 10^{-9}M or 10^{-7}M NAA to the medium containing KN or BA (at 10^{-6}M or 10^{-5}M) did not increase the number or improve the form of adventitious buds produced when embryos were cultured on this medium for four weeks then transferred to basal medium without growth regulators.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BA	N_6-benzylaminopurine
IAA	Indole-3-acetic acid
IBA	Indolebutyric acid
KN	Kinetin (6-furfurylaminopurine)
MCM	Medium for conifer morphogenesis
NAA	Naphthaleneacetic acid
SH	Schenk and Hildebrandt
2,4-D	2,4-dichlorophenoxyacetic acid
2iP	N_6-(2-isopentenyl)amino purine

CHAPTER I

INTRODUCTION

Abies fraseri (Pursh) Poir., Fraser fir, is an upright conifer highly valued as a Christmas tree species and utilized as a landscape specimen in the southern Appalachians. Fraser fir has a limited natural range of less than 25000 ha occurring at elevations above 1200 meters in Virginia, Tennessee, and North Carolina. Most native stands exist in national parks and forests where Fraser fir has an aesthetic importance (Hinesley and Blazich 1981). Presently, Abies fraseri is propagated entirely by seed (Wise et al. 1986) generally obtained from natural stands (Hinesley and Blazich 1981). The rapid depletion of natural Fraser fir stands by the exotic balsam wooly adelgid [Adelges piceae (Ratz.)] is of concern to the National Park Service as well as the southeastern Christmas tree industry. Serious decline in the Fraser fir population is occurring and it is predicted that the balsam wooly adelgid will eliminate mature Fraser fir from most of its natural range within the next several decades (White 1984).

In recent years, the demand for Fraser fir seed for use in Christmas tree production has increased while seed availability remains low. Seed shortages may be due to the disappearance of seed-producing individuals in native stands and poor seed-producing years when only a small quantities of collectable seed can be harvested (Hinesley and Blazich 1981).

Two objectives exist for this investigation: to create a model system for Abies fraseri in which the hormonal control of organogenesis can be studied and to begin to develop a micropropagation scheme for Abies fraseri utilizing mature embryos as explant material. Potential benefits of such a micropropagation system are several: (a) to allow for the clonal multiplication of genetically superior seed (Aitken et al. 1981) in a breeding program, (b) to reduce demand for seed-grown Fraser fir planting stock thereby decreasing the need for a large seed supply (Wise et al. 1985, Hinesley and Blazich 1981), (c) to provide clonal material for use in physiological and pathological studies to assist in the elimination of genetic variability encountered in such studies (Thompson 1984), and (d) to allow for the propagation of balsam woolly adelgid-tolerant genotypes if such trees were identified. This would have an advantage that more trees may survive to maturity producing seed for the Christmas tree industry and assist in the preservance af Fraser fir in its natural environment (Hinesley and Blazich 1980).

Generally, there are three approaches to achieve vegetative propagation of plants through cell and tissue culture (Minocha 1980, Thorpe and Patel 1984):

1. induction of axillary bud development into shoots,
2. somatic embryogenesis from callus or suspension culture, and
3. production of adventitious buds.

The approach chosen for the micropropagation of Abies fraseri in this investigation is the production of adventitious buds. This method is more common than somatic embryogenesis and has a greater potential for mass clonal propagation of plants than does propagation from axillary buds (Thorpe and Patel 1984). For the development of buds to be useful in micropropagation, a complete plantlet that can grow outside of controlled in vitro conditions must be produced. Usually four distinct stages are necessary in plantlet formation via organogenesis: (1) induction of adventitious shoot buds, (2) development of shoot buds into shoots, and (4) transfer of shoot to rooting mixtures and acclimatization of plantlet to outside conditions.

In this investigation, sucrose concentration and medium composition were varied in an attempt to optimize conditions favorable to the induction of adventitious buds on embryo explants of Abies fraseri. The effects of cytokinins and an auxin on organogenesis of embryo explants were also studied.

CHAPTER II

LITERATURE REVIEW: ORGANOGENIC CULTURES OF CONIFERS

Introduction

Conifer species have been used successfully as experimental material for studies in organogenesis as early as 1950 when Ball (1950) achieved adventitious shoot formation in callus cultures of Sequoia sempervirens (D. Don.) Endl.. An interest in the application of tissue culture to propagate forest trees existed in the 1950's (Geissbuhler and Skoog 1957, Haissig 1965) and continues through current research. Numerous successful attempts to induce adventitious organ formation in conifers have been made in the past several decades.

Explant Selection

In studies concerned with in vitro organ formation, there exists a general objective to apply a method whereby cells and tissues can be reversed "to an 'embryonic' condition leading to controlled organogenesis" (Brown and Sommer 1977). It is important to select plant cells and tissues with which organogenesis can be accomplished. In many instances, seeds are chosen to directly provide embryonic explant material because of their availability (Thompson 1982).

Asceptically excised embryos have been employed as explant material and have been induced to form adventitious shoot buds and shoots in 19 species of Pinus, 3 species of Picea, and in Pseudotsuga menziesii (Mirbel) Franco, Table 1.

Adventitious buds usually arise on the cotyledons of the cultured embryo as opposed to the hypocotyl. Utilizing embryo explants, adventitious buds have also been produced from an intervening phase of callus as in the following species: Picea abies (L.) Karst. (Chalupa 1977), Pseudotsuga menziesii (Cheng 1975), Pinus wallichiana (Pinus excelsa Wall. ?) (Konar and Singh 1980), and Pinus radiata D. Don (Reilly and Brown 1976).

Juvenile tissues are commonly utilized in organogenic studies of conifers. Whole or portions of cotyledons have been used as explants from plants of several conifer species, exempli gratia, Pinus oocarpa Schiede (Franco 1983), Pinus pinaster Sol. (David et al. 1982), Thuja plicata Donn (Coleman and Thorpe 1977), and Pseudotsuga menziesii (Cheng 1979). Segments from different parts of the cotyledon have shown distinct levels of reponse in culture. Cheng cultured Pseudotsuga menziesii cotyledon segments derived from cotyledons cut into thirds creating segments that originated in the basal, middle, and distal portions of the cotyledon in segments from two- to eight-week-old seedlings. A higher morphogenic response as exhibited by adventitious buds was observed in the basal segments (81%) than in the middle (69%) and distal (52%) segments. Franco (1983) observed a different response with Pinus oocarpa cotyledons.

Table 1. Studies describing adventitious bud and shoot formation in cultures of embryo explants of conifer species.

Conifer species	Reference
Picea abies	von Arnold and Eriksson 1978
Picea smithiana Boiss	Mehra and Verma 1981
Picea stichiensis (Bong.) Carriere	Webb and Street 1977
Pinus brutia Ten.	Abdullah et al. 1985
Pinus caribaea var. *hondurensis* Mortlet	Webb and Santiago 1983
Pinus contorta Dougl. Ex Loudon	von Arnold and Eriksson 1981a Patel and Thorpe 1984
Pinus echinata Mill.	Brown and Sommer 1977
Pinus elliottii Engelm.	Brown and Sommer 1977
Pinus monticola Dougl. Ex D. Donn	Mott and Amerson 1981
Pinus nigra Arnold	Kolevka-Pletikapic et al. 1983
Pinus oocarpa	Franco 1983
Pinus palustris Mill.	Sommer et al. 1975
Pinus pinaster	Rancillac 1981
Pinus ponderosa Dougl. Ex P. and C. Lawson	Brown and Sommer 1977 Ellis and Bilderback 1984
Pinus radiata	Reilly and Brown 1976 Reilly and Washer 1977 Aitken et al. 1981
Pinus rigida Mill.	Brown and Sommer 1977
Pinus sabiniana Dougl. Ex D. Don	Brown and Sommer 1977
Pinus strobus L.	Brown and Sommer 1977 Minocha 1980a
Pinus sylvestris L.	Tranvan 1979
Pinus taeda L.	Brown and Sommer 1977 Smeltzer et al. 1977
Pinus virginiana Mill.	Brown and Sommer 1977
Pinus wallichiana	Konar and Singh 1980
Pseudotsuga menziesii	Sommer 1975 Winton and Verhagen 1977

Whole cotyledon explants from 14-day-old seedlings produced adventitious buds almost entirely at the distal ends. Adventitious buds predominantly formed also on the distal portions of the cotyledons from seven- and ten-day-old seedlings, however, buds could be found along the entire cotyledonary surface. Other juvenile tissues utilized as explants for studies in conifer organogenesis are listed in Table 2. Much of the conifer seedling can be employed as explants for the formation of organs in sterile culture.

Areas where adventitious buds form on juvenile explants appear to correspond to cellular areas that retain their mitotic abilities (Vazart et al. 1979, Jansson and Bornman 1981, David et al. 1982). While Jansson and Bornman (1981) state the futility in attempting to induce the formation of organs in needles longer "than a critical maximum" length in Picea abies, Brown and Sommer (1977) found it possible to produce adventitious buds on secondary needles of Pinus radiata. These secondary needles had been given a one- to four-week soak in half-strength SH with 5 mg/liter BAP or 2iP. Cheng (1977), working to estimate the influence of age on the regenerative capabilities of Pseudotsuga menziesii cotyledons, observed no difference in adventitious bud production for cotyledon explants of two-week-old or two-month-old plants. Franco (1983), with Pinus oocarpa, observed a reduction in ability to produce adventitious buds with increasing age of cotyledon explants from seven-, ten- and fourteen-day-old seedlings.

Table 2. Other types of explants from conifer seedlings employed in organogenic studies.

Explant Type	Species	Reference
cotyledon-hypocotyl segment	*Pinus strobus*	Kaul 1987
hypocotyl	*Cryptomeria japonica* D. Don	Isikawa 1974
	Picea glauca	Campbell and Durzan 1975, 1976
	Picea abies	Chalupa 1977
	Pinus taeda	Mehra-Palta et al. 1978
	Pinus contorta	Patel and Thorpe 1984
plumules or shoot tips	*Pinus sylvestris*	Bornman and Jansson 1980
	Pinus radiata	Horgan and Aitken 1981
	Picea abies	Bornman and Vogelmann 1984
fascicles or short shoots	*Pinus sylvestris*	Bornman and Jansson 1980
apical meristem slices	*Pinus sylvestris*	Bornman and Jansson 1980
needles	*Picea abies*	von Arnold and Eriksson 1979 Jansson and Bornman 1981

Explants from mature trees are preferred over explants from juvenile material when utilizing micropropagation in the improvement of tree species. This is to insure that clonal multiplication is made utilizing genotypes with proven growth potentials. Mature tissues, however, are presently difficult to obtain organogenic responses from (Bonga 1982a). Explants of mature conifers in which organogenesis in sterile culture has been observed include (a) fascicles from Pinus pinaster (David et al. 1982), (b) immature female strobili of Larix decidua Mill. (Bonga 1982b, 1984), (c) dormant buds of Picea abies (von Arnold and Eriksson 1979), and (d) shoot tips of Abies balsamea, Picea glauca, Pseudotsuga menzesii (Bonga 1981), and Thuja plicata (Coleman and Thorpe 1977). Bonga (1981) found it necessary to soak explants in treatments such as malonic acid at 1 mg/liter or buffered water at pH 2 to 4 in order to achieve favorable organogenesis in shoot tips of several conifer species. Von Arnold and Eriksson (1979) did not discover any improvement in organogenic performance in resting Picea abies bud explants with soak treatments of BA or IBA (5×10^{-5} M to 5×10^{-3} M).

Although callus tissue does have specific utilities in conifer regeneration such as in the in vitro formation of plants from pollen, anther, megagametophyte, or suspension cultures (Thompson 1982), callus is sometimes not considered desirable as an intermediate stage in conifer organogenesis (Thorpe 1978, Bornman and Jansson 1980). Callus is an undersirable intermediate stage in organogenesis possibly because the chromosome

constitution of callus cells is unstable in many plants and that critical analysis of the molecular events leading to meristem formation is complicated by the considerable amount of non-organogenic cells to potentially organogenic cells (Hicks 1980). Several studies describe callus as an intermediate step in adventitious organ formation on several conifer species (Table 3). Konar (1975) has also successfully cultured Pinus gerardiana Wall. cells which formed callus when transferred to solid nutrient agar and were induced to form roots and shoots.

Genotypes of explants within a given species appear to affect the outcome of an organogenic study. Jansson and Bornman (1983) chose three distinct ten-year-old clones of Picea abies as sources for explant material. One clone could not be induced to produce organs in vitro. The other clones responded organogenically but to different extents. A similar response was noted by Cheng (1977) with Pseudotsuga menziesii cotyledon explants. In 80% of the population sampled there was an organogenic response. However, no adventitious organs were produced by the other 20%. Variations in degree of organogenesis also occurred in the responsive explants. A diversity of genotypes may be desired in the sample population to achieve an organogenic response in conifer cultures.

The environment to which the explant source is exposed prior to culturing and the timing of explant sampling are important considerations in morphogenic studies. Jansson and Bornman (1980)

Table 3. Organogenesis in conifer cultures in vitro with a intervening callus phase.

Conifer Species	Original Explant	Reference
Sequoia sempervirens	burl shoots	Ball 1950
Pinus wallichiana	embryo and hypocotyl segments	Konar and Singh 1980
Pinus radiata	embryo and primary needles	Reilly and Brown 1976
Pinus pumila (Pall.) Rgl.	apical segments	Skripachenko 1982
Picea abies	embryo	Chalupa 1977
Pseudotsuga menziesii	embryo	Cheng 1975
Pinus eldarica (Med.)	various juvenile explants	Herrera and Phillips 1985

suggested that "the environmental and physiological prehistory" and original stem position of a Picea abies needle explant affect the organogenic response. Bonga (1981) and von Arnold and Eriksson (1979) found that seasonal timing of source material collection was important in the induction of adventitious buds on shoot tip cultures from mature conifers.

Culture Media Selection

Growth Regulating Substances

Several studies, (Jansson and Bornman 1980 and Thompson 1982), indicate the necessity of a proper auxin to cytokinin balance in the culture medium to obtain an organogenic response in conifer cultures. While the inclusion of both auxin and cytokinin in the culture medium is commonly required to obtain adventitious organ formation, some studies show adventitious bud formation with the application of either auxin alone, exempli gratia, Ball 1950, Konar 1975, and Bonga 1981, or with cytokinin alone, exempli gratia, Jansson and Bornman 1983, Horgan and Aitken 1981, and Kolevska-Pletikapic et al. 1983. Levels of auxin employed in the formation of adventitious buds in conifer cultures range from low micromolar to nanomolar amounts. Cytokinins were generally employed in low to middle micromolar amounts.

In this type of study, the auxins most frequently utilized are IAA, NAA, and IBA. Occasionally, 2,4-D is employed. The cytokinin most often utilized is BA. Other cytokinins giving

favorable results separately or in combination with BA are KN, zeatin, and 2iP. Other growth regulators also may be useful in the induction of adventitious organs. Isikawa (1974) used abscisic acid to facilitate the formation of adventitious buds on Cryptomeria explants.

Levels and types of growth regulators appropriate for organogenesis vary with the species tested, explant type, and the method of growth regulator application, id est, exposure of growth regulating substances through solidified medium, liquid medium, vacuum infusion, or high concentration soak treatment. Auxin concentrations required to induce organogenesis in vitro will vary due to the substantial differences that exist in stability, growth promotion ability, and influence on organ formation (Cheng 1977). The synthetic auxins, NAA and 2,4-D, are used primarily for their excellent stability when compared to the weaker natural auxins and analogues, exempli gratia, IAA and IBA (Cheng 1975, 1977, Thompson 1982).

Medium Composition

Growth regulating substances are not the only factors in the culture medium contributing to the organogenic ability of conifer explants. The concentration and composition of micronutrients, macronutrients, and organic compounds within the medium have considerable importance. David et al. (1982) observed the importance of the potassium ion to ammonium ion ratio for adventitious bud induction in Pinus pinaster cotyledons. Thompson

(1982) found that adventitious buds would form in shoot tip cultures of Pseudotsuga menziesii only if the nitrogen (NH_4^+) level was one-half that normally utilized in MS medium. Brown and Sommer (1977) experimented with two culture media differing only in their concentrations of nitrogen, magnesium, and phosphorus and observed significant differences in the organogenic response of Pseudotsuga menziesii embryos. Occasionally, only one or two media are reported to have been used in a study such as in Coleman and Thorpe (1977) and in von Arnold and Eriksson (1978). In these studies, the medium reported is not likely to be optimal for organogenic cultures unless several different medium formulations have been tested (vide Mehra and Verma 1981, Bornman 1983, Ellis and Bilderback 1984). Reports (von Arnold and Eriksson 1981, Koleveska-Pletikapic et al. 1983) exist in the literature that conifer tissues respond better when media contain reduced concentrations of inorganics, especially during the bud elongation step such as in Cheng (1975), Mott and Amerson (1982), and Patel and Thorpe (1984). Half-strength macronutrients and/or micronutrients plus full-strength organic constituents are often utilized in organogenic cultures of conifers (Ball 1950, Chalupa 1977, Ellis and Bilderback 1984). Few studies report on the effects of the organic components of media on organogenesis. Bilderback and Ellis (1985) reveal that there is no absolute requirement for vitamins in initiating multiple adventitious buds

on cultured embryos of Pinus ponderosa, and that inositol appears inhibitory to the induction of these buds.

A nutrient medium commonly employed in organogenic studies, directly or somewhat modified, is MS medium (Murashige and Skoog 1962) which was originally developed for rapid growth of tobacco tissue. This medium has been used in various modified forms by several investigators studying conifer adventitious bud induction including Thomas et al. (1977), Tranvan (1979), Minocha (1980a), Konar and Singh (1980), and Bornman and Vogelmann (1984). Schenk and Hildebrandt (1972) medium, originally formulated for callus cultures of monocotyledonous and dicotyledonous species, has been widely used for conifer cultures often in modified form with favorable results as in Aitken et al. 1981 and Jansson and Bornman 1983). A modified Romberger et al. (1970) medium was employed by Bonga (1977) to achieve organogenesis in explants of Abies balsamea (L.) Mill.. A few media have been created specifically for growth of conifer cells or tissues in sterile culture. Important examples of these media are (a) Litvay et al. (1981) medium formulated to allow growth of suspension cultures of Pseudotsuga menziesii and Pinus taeda, (b) Medium for conifer morphogenesis (Bornman and Jansson 1982) designed to meet several growth requirements of conifer tissues, and (b) Cheng (1977) medium optimized for clonal mass propagation of Pseudotsuga menziesii. There are other nutrient media formulations which have been employed, however infequently, in the literature of conifer cell and tissue culture. Other nutrient media formulations

include: (a) modified Knop's medium (Ball 1950), (b) Campbell and Durzan medium (Campbell and Durzan 1975, Bonga 1982b), and (c) modified Wolter and Skoog medium (Isikawa 1974, Chalupa 1977).

Media are commonly solidified by the addition of agar or Gelrite gellan gum. Use of liquid medium has also been reported (Horgan and Aitken 1981). Von Arnold and Eriksson (1979) demonstrated that adventitious bud induction on buds of Picea abies took less time in liquid medium than on solidified medium. A significant reduction in the percentage of explants that formed buds, however, was noted when liquid medium was used. A similar occurrence was reported by Reilly and Brown (1976) who compared the amount of bud differentiation utilizing solidified versus liquid medium.

Culture Conditions

Culture conditions such as illumination, temperature, and explant positioning are important to the organogenic response of the explant in vitro. Optimization of these conditions, however, are not often specifically mentioned in published investigations. Webb and Street (1977) reported that dark treatment has stimulated adventitious bud formation in Pinus contorta embryo cultures. Bornman (1983) found it advantageous to keep Picea abies cotyledon explants in the dark for the first few weeks in culture if the formation of roots are desired. Mission et al. (1982) reported the necessity of placing Picea pungens Engelm. bud cultures in darkness for eight days to achieve adventitious

bud formation. Adventitious bud appearance in Cryptomeria japonica hypocotyl explants, however, occurred preferentially in illuminated cultures (Isikawa 1974). Illumination of conifer cultures throughout the culture period is the most common practice observed in investigations dealing with the induction of adventitious buds. Abdullah et al. (1985) noted that the photoperiod that Pinus brutia cultures were exposed to had affected the quantity of adventitious bud initiation. Continuous illumination of these cultures resulted in a lower quantity of buds produced than did a 16 hr photoperiod.

There is little information on the effect of temperature on organogenic cultures of conifer tissue. Most studies have reported favorable results when the culture temperature ranged from 19 to 24°C. Restool (1957) observed that normal buds develop slowly on isolated stem segments of Sequoia sempervirens grown at 6°C and observed the most rapid but abnormal growth at temperatures near 22°C. A temperature increase from 20°C to 27°C in the culture of Pinus contorta embryos shortened the amount of time required to induce the formation of adventitious buds (Patel and Thorpe 1984).

The manner of physical contact the explant has with the medium can affect the morphogenic response of the explant (Haissig 1965). The amount of contact between conifer tissue and medium has been related to the quantity of adventitious buds formed (Aitken et al. 1981, Bornman and Vogelmann 1984).

Positional effects were tested on the ability of Picea abies explants, consisting of cotyledons, apical meristem, and 1 to 3 mm of hypocotyl from 14-day-old seedlings, to form adventitious buds (Bornman and Vogelmann 1984). These explants were cultured either in an upright position with only the hypocotyl was in contact with the medium or in a position where cotyledons and hypocotyl rested against the medium. A significantly greater number of adventitious buds formed on the explant which had cotyledons flattened onto the medium permitting complete surface contact (Bornman and Vogelmann 1984). It was suggested that the increase in adventitious buds was due to a differential uptake of BA into cotyledons of the explants in the two positions. Cotyledons in contact with the medium had accumulated less BA than did the hypocotyls. In explants where cotyledons did not touch the medium, equal amounts of BA were found in the cotyledons and hypocotyl portions. It was surmised that BA was taken up selectively by "'target' cells", id est, cells of developing stomatal complexes. In the upright explants, the investigators speculate that evapotranspirational losses may lead to an even distibution of BA within these explants. In Pinus ponderosa embryo explants, Ellis and Bilderback (1984) revealed that adventitious buds would form on cotyledons in contact with the with the medium's surface but would not form on those above or beneath the semi-solid nutrient medium. Reilly and Washer (1977) and Aitken et al. (1981) discovered that in Pinus radiata adventitious buds would form from areas of cotyledons directly

touching the medium and did not form where no contact was made. Contactual effects such as this were also found by Patel and Thorpe (1984) working with Pinus contorta. The quantity of adventitious buds produced in organogenic conifer cultures can be affected by explant positioning and culture medium contact.

CHAPTER III

MATERIALS AND METHODS

Explant Source

The seed source utilized for investigations on the effects of sucrose concentration and medium compositions on the growth of embryos was open-pollinated seed from a private Fraser fir seed orchard, Carter County, Tennessee, collected in the fall of 1984. All other experiments utilized seed also collected in the fall of 1984 from tree number 45 of the same private Fraser fir seed orchard.

Medium Preparation and Sterilization

All media were adjusted to pH 5.8 with HCl or NaOH and solidified with 0.8% Difco Bacto Agar. Media were autoclaved at 121°C with 15 psi for 20 minutes before the addition of growth regulators (where required) or organic constituents of the medium for MCM (Bornman and Jansson 1982). Growth regulators, or organic components of MCM, were added with stirring to sterile media by sterile filtration in a laminar flow hood after the media had cooled to approximately 60°C. Media were dispensed in 20 ml portions into 150 mm x 25 mm culture tubes, sealed with cotton plug and Bellco Kap-uts, and solidified on a slant (at an approximately 45° angle).

Seed Sterilization and Embryo Excision

Outer scales of seeds were manually removed and seeds were surface sterilized by agitation in 20% (v/v) commercial bleach (5.25% sodium hypochlorite) solution with 1% Tween^{R}20 for 15 minutes. Seeds were then immersed in a 0.01N HCl solution followed by two rinses in sterile distilled water.

After surface sterilization, seeds were placed in sterile petri dishes. Under a dissection microscope the seed coat and endosperm were cut away to remove the embryo. Excised embryos were oriented with cotyledons upward on slanted medium.

Culture Conditions

Embryos were placed in a growth chamber at 20 ± 2^{o}C with a photoperiod of 16 hr light: 8 hr dark. Illumination was supplied by cool white fluorescent tubes (80 μE m^{-2} sec^{-1}).

Experimental Descriptions

The following is a list of experiments undertaken and their descriptions:

A. Sucrose concentration on embryo growth. Excised embryos were cultured to test the effect of sucrose concentration on the growth of embryos and development into seedlings.

B. Medium composition on embryo growth. Three culture medium compositions were tested for their effects on growth and development of embryo explants.

C. Adventitious bud induction on embryos exposed to cytokinins. Three cytokinins were tested at different concentrations for their effects, specifically adventitious bud induction, on excised embryos. Length of explant incubation with cytokinins was also varied to test the effects of cytokinin exposure on induction of adventitious organs.

D. Effect of auxin on adventitious bud induction. Naphthaleneacetic acid was used in combination with KN or BA in the culture medium to test auxin effects on cultured embryos.

Sucrose Concentration on Embryo Growth

Excised embryos were placed on Knop's medium (modified according to Steeves et al. 1955; Appendix A) supplemented with 0, 2, 4, 6, or 8% sucrose. Embryos were cultured for a period of 15 days and measured for average cotyledon length, hypocotyl length, and fresh weight. Dry weight was obtained after placing embryos in a drying oven at 60°C for 24 hours. At least 23 embryos were cultured per treatment. The Tukey-Kramer method of mean comparison at the 0.05 error level (Sokal and Rohlf 1981) was performed on the data to indicate significant differences in cotyledon length, hypocotyl length, fresh weight, and dry weight that may have existed due to varying sucrose levels.

Medium Composition on Embryo Growth

Excised embryos were placed on either of three medium formulations: SH (1972; Appendix B), Cheng (1977; Appendix C), or

MCM (Bornman and Jansson, 1982; Appendix D). All media were supplemented with 3% sucrose. Embryos were cultured for a period of 20 days and measured for average cotyledon length and fresh weight. Dry weight was obtained after placing embryos in a drying oven at 60° C for 24 hours. At least 28 embryos were cultured per treatment. Data were subjected to a single classification ANOVA and a Tukey-Kramer comparison of means at the 0.05 level of error (Sokal and Rohlf 1981) to indicate any significant differences in cotyledon length, fresh weight, and dry weight that may have existed due to different media formulations.

Adventitious Bud Induction on Embryos Exposed to Cytokinins

Excised embryos were placed on Cheng (1977) medium supplemented with the cytokinins 2iP, KN, or BA at 0.0M, 10^{-7}M, 10^{-6}M, or 10^{-5}M. All media contained 3% sucrose. Embryos were cultured on cytokinin-containing medium for either two weeks, four weeks, or eight weeks. Embryos cultured for two or four weeks on cytokinin-containing medium were transferred to basal medium [Cheng (1977) medium with 3% sucrose and no added growth regulators] after two or for weeks, respectively. Explants that were exposed to cytokinins for two or four weeks were cultured for a total of ten weeks. Weekly, measurements of embryo length and width were made and observations on explant color (by comparison with ISCC-NBS centroid color charts -- Standard Sample No. 2106, National Bureau of Standards 1965), presence or absence

of adventitious organs, and presence or absence of callus was recorded. The location of adventitous buds was noted by a visual division of the explant hypocotyl into nine sections from cotyledonary node to root beginning. From 22 to 26 embryos were cultured per treatment. Data were subjected to a three-way ANOVA, when applicable, and a Tukey-Kramer means comparison at the 0.05 error level (Sokal and Rohlf 1981) to indicate any significant differences in adventitious bud production that may have existed due to cytokinin treatments.

Effect of Auxin on Adventitious Bud Induction

Excised embryos were placed on Cheng (1977) medium supplemented with the cytokinins KN or BA at 10^{-6}M or 10^{-5}M in combination with NAA at 10^{-9}M or 10^{-7}M. All media contained 3% sucrose. Embryos were cultured on medium containing growth regulators for four weeks, then transferred to basal medium and cultured for an additional six weeks. Weekly measurements of embryo length and width were made, and observations on explant color, presence or absence of adventitious organs, and presence or absence of callus were recorded. Adventitious bud locations were noted as in the previous experiment. At least 23 embryos were cultured per treatment. Data were analyzed with a three-way ANOVA (Sokal and Rohlf 1981) at the 0.05 level of error to indicate possible differences in adventitious bud formation due to growth regulator treatment.

Histological Sections

Tissues were fixed with Craf III (Sass 1958) and dehydrated by ethanol/tertiary butyl alcohol series, then embedded in paraffin. 10 μm sections were made by rotary microtome and stained with Gill's Hematoxylin stain (No. 2 from Fisher Scientific Company, Fair Lawn, New Jersey).

CHAPTER VI

RESULTS

Sucrose Concentration on Embryo Growth

Greening of the embryos occurred within five days in culture. By the sixteenth day in culture cotyledons increased in length from about 1 mm to approximately 3.4 mm in most treatments. Primary roots grew unhampered through the medium, with the exception of the 0 and 2% sucrose treatments. Embryos grown on medium with no sucrose often developed into curled seedlings with short white-tipped cotyledons and relatively reduced root lengths.

The range of average cotyledon lengths among embryos cultured on medium containing 2 to 8% sucrose was approximately 14.3 to 18.3 mm. Embryos cultured on 0% sucrose had an average cotyledon length of 1.39 mm which was significantly lower than the other treatments (Table 4).

Average hypocotyl length in the 0% sucrose treatment was the shortest, at 8 mm, and differed significantly from all of the other sucrose concentrations tested (Table 4). The longest hypocotyls were observed in the cultures grown on 2 and 4% sucrose, which averaged 18.33 and 18.32 mm respectively.

In both fresh weight and dry weight of embryos, the 0% sucrose treatment demonstrated the lowest value when compared to all other sucrose treatments. The means for embryo fresh weight among

Table 4. Growth of Abies fraseri embryos after fifteen days in terms of average cotyledon length, hypocotyl length, fresh weight, and dry weight on medium with varying sucrose concentrations.

Sucrose Concentration (%)	Mean Average Cotyledon Length (mm)[a]	Mean Hypocotyl Length (mm)[a]	Mean Fresh Weight (mg)[a]	Mean Dry Weight (mg)[a]
0	1.39^{A}	7.89^{A}	4.33^{A}	0.60^{A}
2	3.52^{B}	18.33^{B}	8.31^{B}	1.57^{B}
4	3.49^{B}	18.32^{B}	8.71^{B}	2.01^{C}
6	3.16^{B}	15.45^{C}	7.99^{B}	2.47^{D}
8	3.60^{B}	14.27^{C}	7.18^{B}	2.57^{D}

[a]Means marked by the same superscript letter are not significantly different at the 0.05 level of error.

the 2 to 8% sucrose treatments did not differ significantly. the embryos cultured on medium containing 6 and 8% sucrose treatments demonstrated significantly higher dry weights than embryos cultured at lower sucrose concentrations. The 4% sucrose treatment embryos displayed a significantly greater dry weight than those cultured on 2% sucrose, but was not as high as observed in the 6 or 8% sucrose treatment.

Media Composition on Embryo Growth

Root growth was poor on all media during the 20-day culture period. Approximately one-third of the cotyledons of the embryos cultured on SH medium were shorter and thinner at the end of the culture period than those cultured on Cheng or MCM media. Cotyledons considered to be normal in length, id est, 4.5 mm, were observed in two-thirds of the embryos cultured on SH medium and in nearly all of the embryos cultured on Cheng and on MCM media. Cotyledon length of embryos cultured on the Cheng medium was significantly greater than embryos cultured on the other media (Table 5). Embryo fresh weight was also greater for embryos cultured on Cheng medium. This value was not significantly different, however, from the fresh weight of embryos cultured on MCM. No significant difference was observed between the media for embryo dry weights.

Table 5. Growth of Abies fraseri embryos after twenty days in terms of average cotyledon length, fresh weight, and dry weight on three medium formulations.

Medium Formulations	Average Cotyledon Length (mm)[a]	Mean Fresh Weight (mg)[a]	Mean Dry Weight (mg)[a]
Schenk and Hildebrandt	4.01^{A}	19.14^{A}	3.34^{A}
Cheng	6.74^{B}	31.69^{B}	4.76^{A}
MCM	5.19^{A}	25.42^{AB}	3.63^{A}

[a]Means marked by the same superscript letter are not significantly different at the 0.05 level of error.

Adventitious Bud Induction on Embryos Exposed to Cytokinins

Embryos On Medium With Cytokinins For Eight Weeks

Color

Observations on color of embryos in culture revealed that the greening of tissues occurs immediately after placement in culture. Excised embryos begin as yellow white (No. 92, vide National Bureau of Standards 1965) and turn pale green yellow (No. 104) by the second day in culture. By the third day of culture the embryos are a light green yellow (No. 101) to brilliant green yellow (No. 98). Cotyledons and hypocotyls turn a combination of strong yellow green (No. 117) and or deep yellow green (No. 118) by the second week in culture. There was essentially no color change in the cultured embryos after the second week except for some browning (to strong brown No. 55) near the end of the six week culture period. Browning occurred only on media containing BA or KN, where hypocotyls were in contact with the medium. Some embryos (5 to 50%) in all of the culture treatments displayed a reddened lower hypocotyl and/or cotyledon tips during the first or second week of culture, which either disappeared or decreased in intensity with time.

Size and Form

Cotyledons were unexpanded and parallel to the embryonic axis in newly excised embryos. While in culture, the cotyledons of embryos in the control treatment (no added cytokinins)

remained straight but grew in length and cotyledon apices grew away from the original embryonic axis. This did not occur in embryos on most of the cytokinin treatments (2iP at 10^{-5}M, BA and KN at 10^{-5}M, 10^{-6}M, 10^{-7}M). Embryos in those treatments show various degrees of cotyledon elongation, cotyledon growth away from the embryo axis, and cotyledon curling.

The approximate size of an embryo was 5.0 to 6.0 mm long and 1.0 mm wide before being placed in culture. After six weeks, embryos ranged in length from 7.0 to 17.5 mm in cultures containing cytokinin (Figure 1.a). The embryos were approximately 25 mm long in the control treatment. In general, the embryos cultured on 10^{-6}M and 10^{-7}M cytokinin levels grew longer than on the higher 10^{-5}M concentration by the end of the culture period.

Some curvature of hypocotyls was observed, occuring early in culture (by the first or second week). Hypocotyls were moderately curled, id est, with a curvature of 180^{o} in the control treatment. Embryos cultured on treatments of 2iP at 10^{-6}M and 10^{-7}M were very curled (curvatures of up to 440^{o}), making measurement of embryo length difficult. Hypocotyls of embryos cultured on KN at 10^{-6}M and 10^{-7}M were slightly curled (curvatures up to 90^{o}), and those cultured on 10^{-5}M remained straight. In the 2iP at 10^{-5}M treatment, approximately two-thirds of the hypocotyls were curled. No hypocotyl curvature was observed in cultures grown on BA.

Figure 1. Average length of Abies fraseri embryo explants after a period in culture.

(a) Average length of explants after six weeks on cytokinin medium.

(b) Average length of explants with a two-week exposure to cytokinin medium. Data taken after nine weeks in culture.

(c) Average length of explants with a four-week exposure to cytokinin medium. Data taken after nine weeks in culture.

(d) Average length of explants after ten weeks with a four-week exposure to NAA and KN or BA.
Legend:

A. NAA 10^{-9}M + KN 10^{-6}M

B. NAA 10^{-9}M + KN 10^{-5}M

C. NAA 10^{-7}M + KN 10^{-6}M

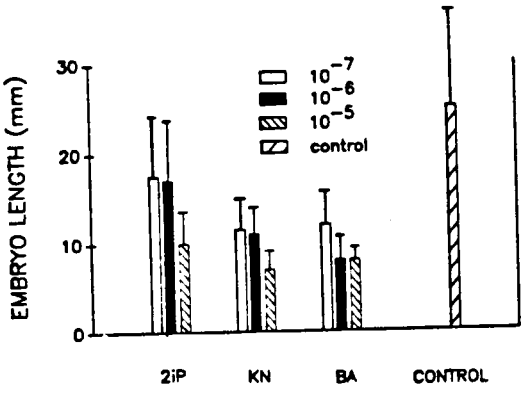
D. NAA 10^{-7}M + KN 10^{-5}M

E. NAA 10^{-9}M + BA 10^{-6}M

F. NAA 10^{-9}M + BA 10^{-5}M

G. NAA 10^{-7}M + BA 10^{-6}M

H. NAA 10^{-7}M + BA 10^{-5}M


EMBRYO LENGTH (mm)
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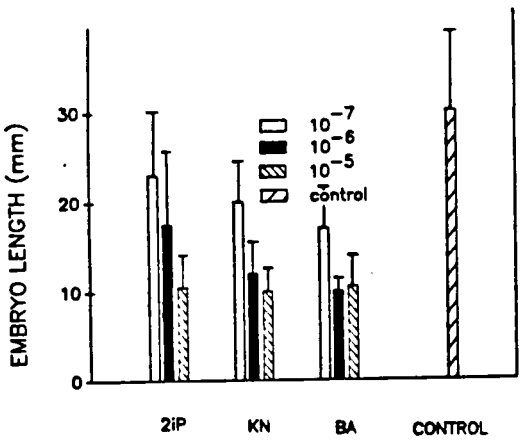
10
0
10^{-7}
10^{-6}
10^{-5}
control
2iP
KN
BA
CONTROL

(a)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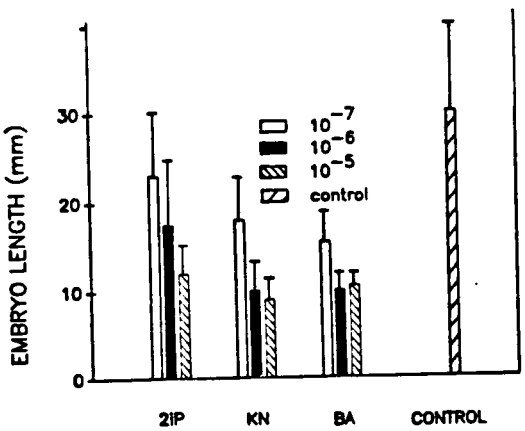
(b)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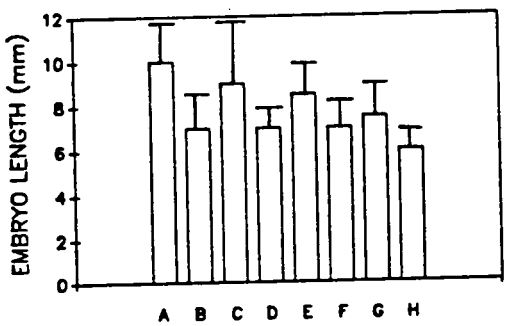
BA
CONTROL

(c)


EMBRYO LENGTH (mm)
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10
8
6
4
2
0
A
B
C
D
E
F
G
H

(d)

Other Observations on Appearance

Splits, (shallow cleavages often vertically-oriented), were observed in hypocotyls of explants in every treatment except in 2iP at 10^{-7}M and in the control. Most frequently splits occurred in the lower one-third of the hypocotyl or up and down the entire hypocotyl. Splits in the cotyledons occurred less frequently and were present primarily in embryos cultured on four cytokinin treatments (2iP at 10^{-6}M and 10^{-7}M, KN at 10^{-7}M, and BA at 10^{-7}M) and in the control.

Vitreous (glassy, water-logged look to tissues) embryos or hypocotyls appeared infrequently. Vitrification occurred in all of the cytokinin treatments except 2iP at 10^{-5} and 10^{-7}M, and BA at 10^{-5}M. Organs that appeared vitreous often changed to give a normal appearance during the culture period.

Needles formed in all cytokinin treatments at the level of 10^{-7}M, and additionally in the 10^{-6}M 2iP and the control treatment.

Presence of Adventitious Organs

No adventitious organs were present in any of the cultured embryos at the end of the sixth week. Bumps (low rounded bulges from the surface of the explant) and protrusions (extensions of tissue protruding from the surface of the explant at a steeper angle than those of bumps) occurred infrequently on hypocotyls in several cytokinin containing cultures. The treatments in which bumps arose were 2iP at 10^{-7}M and 10^{-5}M, KN at 10^{-5}M, and BA at

10^{-6}M. A protrusion resembling a needle arose in one culture on BA at 10^{-7}M. On one cotyledon of an embryo in the 10^{-7}M KN treatment, a protrusion resembling a trichome appeared. No adventitious buds or roots were produced in this experiment.

Presence of Callus

A small amount of callus formation was observed in all cytokinin treatments, although it failed to proliferate in culture. Callus was often associated with splits on hypocotyls or on cotyledons. Callus, often a brilliant yellow green (No. 116) occassionally turned brown by the sixth week in culture.

Embryos On Medium Containing Cytokinin For Two Or Four Weeks

Color

Patterns of coloration of cultured embryos were very similar to the patterns of coloration reported for embryos exposed to cytokinins for an eight week period. Browning of embryos usually began at the lower hypocotyl and apically progressed. Browning was observed by the eighth week in all culture treatments.

Size and Form

Growth of cotyledons and curvature of hypocotyls followed patterns of growth and curvature reported for embryos exposed to cytokinins for an eight week period. Occasionally, a differential elongation of cotyledons was observed, where cotyledons farthest from the agar surface were more elongated than those in contact with the agar. Increases in length (Figure 1.b and 1.c) and width

of embryos were similar to those in the previous experiment. Partially shrunken cotyledons and/or hypocotyls (portions of the explants which appear shrivelled) were observed at a low frequency in all of the treatments, including controls, with the exceptions of the KN at 10^{-5}M (two- and four-week exposure) and 2iP at 10^{-6}M (four-week exposure) treatments.

Other Observations on Appearance

Splits were common in all of the cytokinin and control treatments. Splits usually occurred in the lower one-third of or throughout the entire hypocotyl as well as in portions of the cotyledons.

Vitreous embryos were observed in 10% or less of the explants in the KN (all levels of concentration), the BA (10^{-6}M and 10^{-7} M), and the two-week control treatments. In cultures of embryos exposed to cytokinins for a four-week period, vitreous embryos occurred occasionally, (between 10% and 40%), in all but the 2iP at 10^{-7}M treatment.

Needles were evident on 4 to 24% of the embryos in all of the cytokinin treatments with the exception of 10^{-5}M and 10^{-6}M KN or BA. Needles were also observed in 36% and 50% of the embryos in the two-week control and the four-week control embryos, respectively.

Presence of Adventitious Organs

Adventitious buds were formed by the tenth week of culture on embryos in all of the cytokinin treatments except for 2iP at 10^{-7}M (two-week exposure) and BA at 10^{-7}M (four-week exposure) (Table 6). All adventitious buds formed on hypocotyls of cultured embryos. Most adventitious bud primordia were visible by the fifth week of culture and the majority had developed into adventitious buds by the seventh week in culture. adventitious buds occurred most commonly in the lower one-third of the hypocotyl.

Protrusions from cultivated embryos that superficially resembled bud-like forms were considered adventitious buds (Plate 1, Figures 1 and 2). Histological sections of these protrusions revealed internal bud-like organization (Plate 1, Figures 3, 4 and 5). Adventitious buds did not form synchronously and many appear to have stopped development before the end of the ten-week culture period. Adventitious buds did not elongate into shoots.

The analysis of the data indicated that the type of cytokinin employed had no influence on adventitious bud production. The level of cytokinin utilized and the length of explant exposure to cytokinin was important in the induction of adventitious buds. A Tukey-Kramer means comparison indicated that explants exposed to 10^{-7}M cytokinin in the medium performed significantly poorer in terms of adventitious bud production than explants on either 10^{-6}M or 10^{-5}M cytokinin in the medium. In addition, there was a significant increase in the number of

Table 6. Adventitious bud formation after ten weeks on embryos cultured with a two-week or four-week exposure to cytokinin medium.

Cytokinin Treatment		Explants Forming Buds (%)	Mean Number of Buds/Explant (Number)[a]	Range of Number of Buds/Explant Forming Buds (Number)
Embryos cultured for two- weeks on cytokinin medium				
2iP	10^{-7}M	0	0.00^{A}	--
	10^{-6}M	36	0.36^{A}	1
	10^{-5}M	15	0.23^{A}	1-3
KN	10^{-7}M	8	0.08^{A}	1
	10^{-6}M	8	0.08^{A}	1
	10^{-5}M	4	0.04^{A}	1
BA	10^{-7}M	8	0.12^{A}	1-2
	10^{-6}M	4	0.04^{A}	1
	10^{-5}M	18	0.23^{A}	1-2
Embryos cultured for four weeks on cytokinin medium				
2iP	10^{-7}M	4	0.04^{A}	1
	10^{-6}M	17	0.33^{B}	1-3
	10^{-5}M	27	0.31^{B}	1-2
KN	10^{-7}M	12	0.12^{A}	1
	10^{-6}M	17	0.21^{B}	1-2
	10^{-5}M	25	0.33^{B}	1-2
BA	10^{-7}M	0	0.00^{A}	--
	10^{-6}M	36	0.52^{B}	1-2
	10^{-5}M	25	0.29^{B}	1

[a]Means marked by the same superscript letter are not significantly different at the 0.05 level of error.

Plate 1. Adventitious buds on explants of Fraser fir.

Figure 1. Adventitious bud on explant with four-week exposure to 10^{-5}M BA. Photograph taken after ten weeks in culture. (80x).

Figure 2. Adventitious bud on explant with two-week exposure to 10^{-7}M BA. Photograph taken after eleven weeks of culture. (100x).

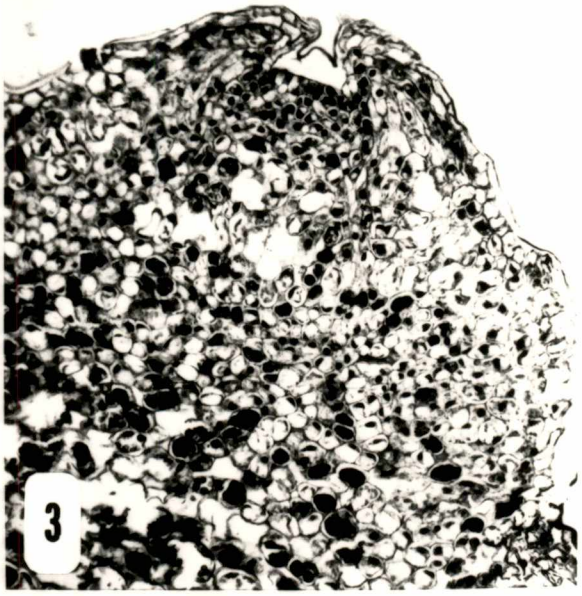
Figure 3. Section through adventitious bud. Explant from two-week 10^{-5}M KN treatment. (160x).

Figure 4. Section through adventitious bud showing rudimentary vascular system. Explant from two-week 10^{-7}M KN treatment. (160x).

Figure 5. Section through adventitious bud. Explant from two-week 10^{-6}M BA treatment. (70x).


1


2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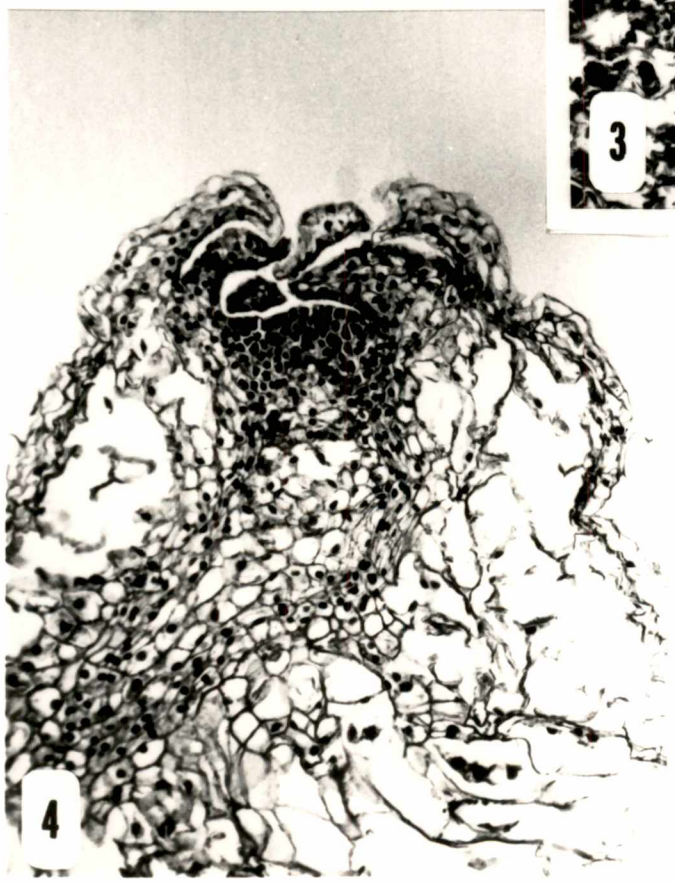
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adventitious buds formed in those explants exposed to cytokinin medium for four weeks, rather than two weeks, before reculture.

Anomalous protrusions (protrusions with irregular form and of uncertain classification as adventitious organs) were also formed on hypocotyls of cultured embryos. Occasionally, these protrusions occurred on the same explants that formed adventitious buds. Anomalous protrusions were generally of two types, classified by their outward form. One type of anomalous protrusion was often wider than any of the other protrusions and was flat-topped (Plate 2, Figures 5 and 6). This type of protrusion was observed in less than 25% of the explants exposed to 2iP at 10^{-5}M or BA at 10^{-5}M or at 10^{-6}M for four weeks. The second type of anomalous protrusion appeared globular and was usually smaller than anatomically normal adventitious buds. Globular protrusions were frequently evident in the 2iP at 10^{-6}M and 10^{-7}M (four-week exposure), with 54 to 80% of the explants forming one globular protrusion. They were less frequent in the 2iP at 10^{-7}M (two-week exposure) treatment occurring on an average of 8% of the explants. These protrusions did not show any internal morphological indication of bud formation (Plate 2, Figure 7).

Another type of protrusion was observed in this experiment and was needle-like in form, having the same shape, size, and color as leaves formed on the cultured embryos. This type of

Plate 2. Anomalous protrusions and an adventitious bud.

Figure 6. Photograph illustrating form of a flat protrusion. Explant from 10^{-9}M NAA plus 10^{-6}M BA treatment.(16x).

Figure 7. Section through flat protrusion. Explant same as in Figure 6. (50x).

Figure 8. Section through globular protrusion. Explant from four-week 10^{-7}M 2iP treatment. (80x).

Figure 9. Section through globular protrusion. Explant from four-week 10^{-7}M 2iP treatment. (100x).

Figure 10. Section through globular protrusion. Explant from four-week 10^{-5}M 2iP treatment. (125x).

Figure 11. Adventitious bud with good bud-like form. Explant from 10^{-9}M NAA plus 10^{6}M BA treatment. Photograph taken near the seventh week in culture. (Approximately 25x).


6


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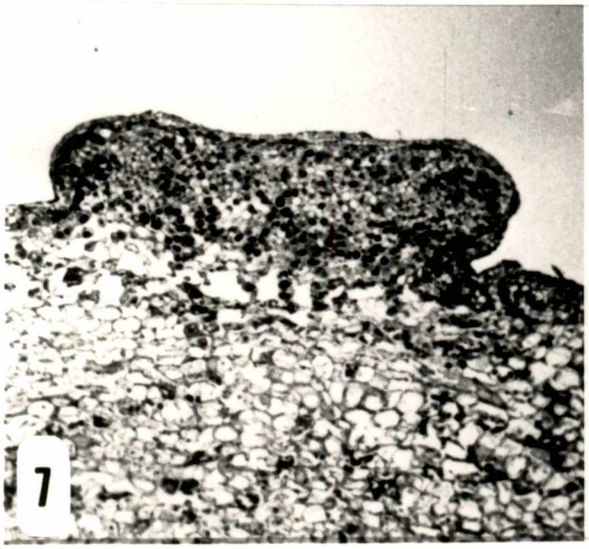
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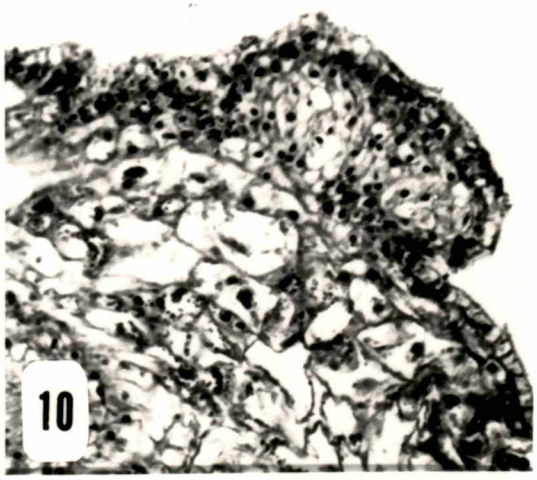
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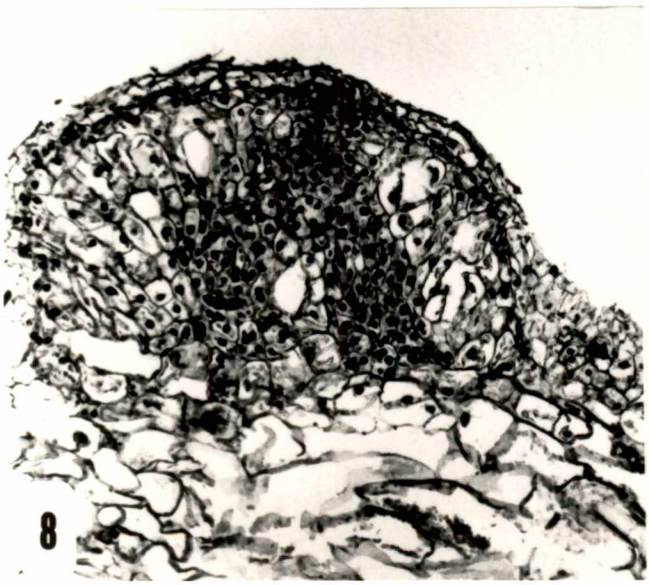
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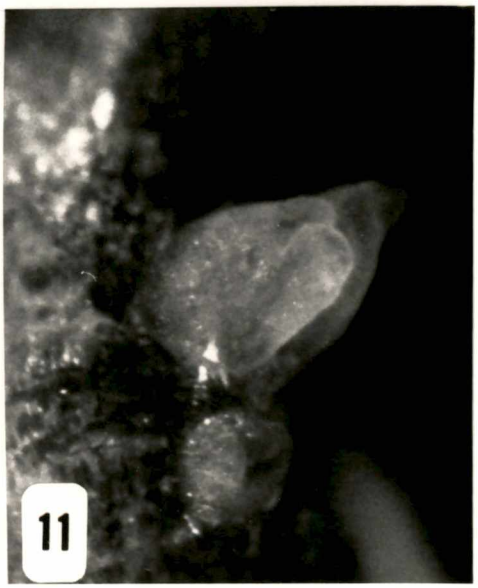
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protrusion occurred in only one instance in the 2iP at 10^{-6}M four-week exposure treatment and was classified as an adventitious needle.

Presence of Callus

Callus was present infrequently in small quantities in 2iP (all concentrations) and two-week control treatment. Embryos of the four-week control treatment and those exposed to 10^{-5}M KN and 10^{-5} and 10^{-6}M BA also produced small quantities of callus infrequently. Callus did not proliferate during the culture period of ten weeks. In approximately 70% of the instances where callus was observed, it protruded from splits in hypocotyls or cotyledons. Callus that was observed in the control treatments arose from cotyledons and was colorless. Most of the callus was brown by the eighth week of culture.

Effect of Auxin on Adventitious Bud Induction

Color

Color patterns observed for cultured embryos were very similar to the color patterns reported for embryos exposed to cytokinins for an eight-week period. Browning of explants occurred in all of the treatments except for the NAA at 10^{-9}M treatment (these explants remained green). Browning was observed by the eighth week in culture and commonly began in areas where the embryo came in contact with the medium.

Size and Form

After ten weeks in culture, explants ranged from approximately 6 to 10 mm in length (Figure 1.d, page 32) and from 1.25 to 2 mm in width for culture treatments containing both auxin and cytokinin. In treatments with NAA only, embryos grew longer (from 27.5 to 32.5 mm in length) but were not as wide (about 1 mm in width). The embryos of the control treatments grew to an average of 37 mm long and 1 mm wide.

Curvature of hypocotyls was not observed in embryos cultured on medium containing NAA with BAP and was not observed in any other treatments. Embryos cultured on medium containing (a) NAA with KN, (b) NAA at 10^{-9}M, or no growth regulators displayed slightly curved hypocotyls. Moderately curved hypocotyls were evident in the NAA at 10^{-7}M treatment.

Other Observations on Appearance

Splits in explants appeared occasionally in all of the treatments. Superficial splits in epidermal layers of the hypocotyls in explants cultured on medium without growth regulators or with NAA at 10^{-9}M.

Vitreous embryos were not commonly observed during the ten weeks in culture. Vitrification was evident only in the treatment of NAA at 10^{-7}M in less than 20% of the explants.

Partially shrunken hypocotyls and cotyledons were occasionally observed in all treatments except that of NAA at

10^{-7}M. Partially shrunken areas did not appear until after the seventh week of culture.

Needles arose only on the embryos cultured on medium containing no growth regulators or NAA (at 10^{-9}M and 10^{-7}M).

Presence of Adventitious Organs

Adventitious buds formed in every treatment except those of NAA (10^{-9}M and 10^{-7}M) and NAA at 10^{-9}M with KN at 10^{-6}M (Table 7). All adventitious buds were produced on hypocotyls of cultured embryos. The majority formed on the upper seven-nineths of the hypocotyls. Adventitious bud primordia were visible by the fourth or fifth week in culture and the majority of adventitious buds were visible by the seventh week of culture. There was no significant difference in the number of buds produced among any of the NAA plus cytokinin treatments.

Adventitious buds generally do not appear to be further developed than those described for embryos cultured on cytokinin medium for two or four weeks before transfer to basal medium. One exception is a single adventitious bud produced in the NAA at 10^{-9}M with BA at 10^{-6}M treatment. This structure displayed a more developed bud-like form when compared when compared to other bud-like structures (Plate 2, Figure 8, page 42). No adventitious buds expanded into shoots during the ten-week culture period.

Two types of anomalous protrusions were evident on the hypocotyls of the cultured embryos. The flat-topped and often wide protrusion, as previously described for embryos cultured on

Table 7. Adventitious bud formation after ten weeks on embryos cultured for four weeks on medium containing NAA and KN or BA.

Growth Regulator Treatment (M)		Explants Forming Buds (%)	Mean Number of Buds/Explant (Number)[a]	Range of Number of Buds/Explant Forming Buds (Number)
NAA	KN			
10^{-9}	10^{-6}	0	0.00^{A}	--
10^{-7}	10^{-6}	4	0.04^{A}	1
10^{-9}	10^{-5}	12	0.24^{A}	1-3
10^{-7}	10^{-5}	8	0.08^{A}	1
NAA	BA			
10^{-9}	10^{-6}	12	0.15^{A}	1-2
10^{-7}	10^{-6}	19	0.27^{A}	1-2
10^{-9}	10^{-5}	15	0.19^{A}	1
10^{-7}	10^{-5}	8	0.08^{A}	1
NAA				
10^{-9}		0	0.00	--
10^{-7}		0	0.00	--

[a]Means with the same letter are not significantly different at the 0.05 level of error.

cytokinin medium for two and four weeks, was observed infrequently (less than 10%) of explants in treatments where NAA was in combination with BA and in the NAA (10^{-9}M) plus KN (10^{-6}M) treatment. Another type of anomalous protrusion was observed; a root-like protrusion emerging slightly below the mid hypocotyl region in one embryo cultured on medium containing NAA at 10^{-9}M with BAP at 10^{-5}M. No globular types of protrusions were observed in the auxin plus cytokinin treatments.

Presence of Callus

Callus seldom formed and did not proliferate. Callus was primarily associated with cotyledonary splits in the NAA at 10^{-7}M treatment. Browning of existing callus took place as other parts of the explant became brown near the seventh week in culture.

CHAPTER V

DISCUSSION AND CONCLUSIONS

Whole Fraser fir embryos were found to grow best on medium with a sucrose concentration of 2 or 4% on the basis of cotyledon length, length of hypocotyl, fresh weight, and dry weight. In most organogenic cultures, the energy requirement is commonly provided with 2 to 4% (w/v) sucrose (Thorpe and Patel 1984). Fraser fir embryo explants demonstrated a significantly better growth in terms of cotyledon length and fresh weight on Cheng (1977) medium than on either MCM or SH medium formulations. A medium composition optimized for growth and development of embryo explants, however, does not assure the medium is also favorable for explant organogenesis (Webb and Santiago 1983). Cheng (1977) medium was chosen on the basis of cotyledon length and fresh weight for use in subsequent experiments involved with adventitious bud induction.

Fraser fir embryos can be induced to form adventitious buds in vitro. In only two other studies, as far as the author is aware, have adventitious buds formed on Abies explants -- both with Abies balsamea (vide Bonga 1977). Cytokinin is necessary for the induction of these buds. Embryos of other conifer species also require explant incubation cytokinins for adventitious buds to be produced, exempli gratia, in Ellis and Bilderback (1984)and Abdullah et al. (1985). The cytokinins 2iP, KN, and BAP were equally effective in promotion of adventitious bud formation.

Embryos must be removed from the exogenous cytokinin source for adventitious buds to develop. Length of exposure of embryo explants to cytokinin-containing medium is important to the number of adventitious buds produced. This has also been reported to occur in adventitious bud-producing cultures of Picea abies (von Arnold and Eriksson 1978), Pinus radiata (Biondi and Thorpe 1982), and Pinus contorta (Patel and Thorpe 1982). A four-week exposure to 10^{-6}M cytokinin exhibited a greater formation of adventitious buds than did any of the other cytokinin levels and periods of cytokinin exposure. The lowest level of cytokinin tested in the medium (10^{-7}M) was not able to induce as many adventitious buds on embryos as the higher levels (10^{-6}M and 10^{-5}M).

Auxin (NAA) alone was unable to induce adventitious buds on embryo explants and did not demonstrate an improvement in the number or form of adventitious buds when applied in combination with cytokinin. These results were consistent with those of Flinn et al. (1986) utilizing Pinus strobus embryos and Biondi and Thorpe (1982) with cotyledon explants of Pinus radiata.

Adventitious buds developed only along the hypocotyl of cultured Fraser fir embryos. This is contrary to most reports where adventitious buds arise primarily from developing cotyledons and occasionally, but only secondarily, on explant hypocotyls. Only in cultures of Picea abies embryos of von Arnold and Eriksson (1978, 1981b) do the majority of adventitious buds

appear to arise from hypocotyls. Thompson (1982) suggested that IAA may play a role in determining the location of adventitious buds formed in cultured conifer tissues. Jansson and Bornman (1980) noted that the locations of adventitious bud formation on needle explants of Picea abies was determined by the amount of exogenous auxin applied. As the level of auxin applied to the cultured needles increased, formation of adventitious buds occurred closer to the tip of the needles. If a similar situation occurs in embryo explants, then a decrease in cytokinin to auxin ratio obtained by the addition of increasing auxin levels would shift adventitious bud formation towards the base of the hypocotyl. In this investigation, adventitious buds were produced predominantly near the base of the hypocotyl when cultured with cytokinin alone. With the addition of increasing levels of NAA to the cytokinin-containing medium, however, embryo explants formed the majority of adventitious buds near the middle of the hypocotyl not at the base of the hypocotyl. Perhaps Fraser fir does not react in the same manner to exogenously supplied auxin in vitro as other conifer species. A more detailed investigation would be necessary to ascertain if this assumption is correct.

Adventitious buds observed in this investigation did not elongate into shoots during the culture period. Usually in caulogenic cultures of conifers, adventitious buds can be stimulated to elongate with a reduction or elimination of growth regulators from the culture medium. An addition of 1% activated charcoal has been utilized to encourage elongation of

adventitious buds into shoots, exempli gratia, Mott (1978) and Abdullah et al. (1986), because it will absorb growth regulators (Constantin et al 1977). The development of adventitious buds into shoots can also be enhanced by the separation and culture of buds from the original explant (Abdullah et al. 1985, von Arnold 1978). These techniques may be useful in possible future studies on the elongation of adventitious buds of Fraser fir.

Some of the adventitious buds formed on Fraser fir embryos appeared to cease in development during the culture period. Mott (1981) notes that it may possible for tissue cuture conditions themselves to cause a bud to go into a repressed state of growth under apical dominance or become dormant. An arrest in development of some lateral buds in vivo in Abies balsamea was noted by Powell (1974) and were termed latent buds. Latent buds retained the capacity to develop and demonstrated full development after decapitation of branch. Decapitation of the embryo explant, id est, removal of the apical bud or growth area, may assist in the restoration of development in adventitious buds of cultured Fraser fir embryos.

Anomalous protrusions observed in this investigation differed from morphological structures seen in organogenic conifer cultures reported by Jansson and Bornman (1980) and Coleman and Thorpe (1977). Globular protrusions observed in this investigation are similar in description of visual characteristics to the pseudobulbil structures observed by

Jansson and Bornman (1980). Globular protrusions, however, differed from pseudobulbils in that no adventitious buds, fully or partially formed, ever formed from them. In this respect, the globular protrusions were similar to phymas (Bornman 1983), but never grew as large (to 3 mm) as those structures. Flat protrusions observed in this experiment are not comparable to any morphological structures described by Jansson and Bornmann (1980) or meristemoid domes discussed by Coleman Thorpe (1977). No embryo-like structures as described by Bonga (1977) with Abies balsamea were formed on explants in this investigation.

Callus formed only in small quantities and did not proliferate on Fraser fir embryos in any of the culture treatments. Reports of callus formation in Abies species are several, id est, in Abies pectinata DeCandolle (Gautheret 1934), A. nephrolepis Maxim, A. sibirica Ledeb (Bytchenkova 1963), A. grandis Lindl. (Harvey and Grasham 1968), A. concolor (Gord. and Glend.) Lindl. (Olson and Eiser 1972), and A. sachalensis Masters (Shibakusa and Kimata 1976). In many instances these studies utilized stem explants, glucose as the carbon source, and an undefined medium. A comparison of callus formation cannot directly be made because of the large disparity in culture conditions that exist between those studies and this investigation. The callus eventually turned brown during culture. When tissues turned brown, they did not increase in size or produce any more adventitious structures. Browned tissues appeared as if they were no longer alive. Some of the browning

may have been prevented by more frequent reculture or perhaps by the addition of antioxidants to the medium (Zimmerman 1985).

Vitreous explants were seldom encountered and tended to recover normal appearance before the formation of adventitious buds occurred. Therefore, vitreous explants were not as common in this investigation as in a study by von Arnold (1982) where approximately 70% of the adventitious shoots formed on embryos of Picea abies were vitrified. Vitrification of adventitious buds or shoots can become a difficulty in a micropropagation scheme. Affected shoots or plantlets lose water too quickly outside of in vitro conditions and are highly susceptible to infection by pathogens (von Arnold and Eriksson 1984). Vitrification can be overcome by an increase in the medium's gelling agent, however, this may reduce the number of adventitious buds that conifer explants produce (Bornman and Vogelman 1984, von Arnold and Eriksson 1984). Vitrification, however, is only a problem if it persists. In this investigation, no adventitious organs were vitreous and the small number of explants that revealed any vitrification appeared normal by the end of the culture period. This investigation has accomplished the first step, shoot bud induction, in a micropropagation scheme for Fraser fir. Protocols, however, require refinement in order to be utilized in a practical Fraser fir multiplication scheme. Studies should be undertaken to achieve synchronous adventitious bud formation and

development allowing for minimal manipulation in selecting buds for elongation.

The low number of adventitious buds produced per explant can indirectly result in extremely low numbers of plantlets. Multiplication rates in a micropropagation scheme can be greatly reduced at either the shoot elongation, root induction, or transfer to soil step (Thomas 1984). It is important to improve the numbers of adventitious buds produced on embryo explants so that potential losses encountered in subsequent steps would have a minimum impact on the total number of plantlets formed.

In this investigation, seed to seed organogenic responses varied. Mott (1978) states that these genotypic differences are present under marginal conditions and tend to disappear when culture conditions are optimized. Optimization of culture conditions for adventitious bud induction may be required for maximal adventitious bud production. Efforts should also be made to complete the micropropagation steps of: (a) shoot formation from adventitious buds, (b) root induction on shoots, and (c) transfer of plantlet to soil and acclimatize to conditions outside of in vitro culture. The entire micropropagation process from embryo explant to plantlet acclimatization should be completed in a relatively short time period to reduce the chance of somaclonal variation (Brown and Sommer 1977). The commercial use of Fraser fir dictates that plantlets must prove true to type when planted out in field test conditions.

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APPENDICES

APPENDIX A

MODIFIED KNOP'S MEDIUM (STEEVES ET AL. 1955)

Compound	mg or ml/liter
KNO_3	125 mg
$MgSO_4\ 7H_2O$	125 mg
$Ca(NO_3)_2\ 4H_2O$	500 mg
KH_2PO_4	125 mg
$MnCl_2\ 4H_2O$	12.5 mg
H_2SO_4	0.00025 ml
H_3BO_3	10 mg
$ZnSO_4\ 7H_2O$	0.25 mg
$CuCl_2\ 2H_2O$	0.075 mg
$Na_2MoO_4\ 2H_2O$	0.125 mg
$CoCl_2\ 2H_2O$	0.15 mg
$FeC_6H_5O_7\ 5H_2O$	10 mg

APPENDIX B

SCHENK AND HILDEBRANDT (1972) MEDIUM

Compound	mg/liter
KNO_3	2500
$MgSO_4$ $7H_2O$	400
$NH_4H_2PO_4$	300
$CaCl_2$ $2H_2O$	200
$MnSO_4$ H_2O	10
H_3BO_3	5
$ZNSO_4$ $7H_2O$	1
KI	1
$CuSO_4$ $7H_2O$	0.2
Na_2MoO_4 $2H_2O$	0.1
$CoCl_2$ $6H_2O$	0.1
FeEDTA	15
$FeSO_4$ $7H_2O$	15
myo-inositol	1000
thiamine HCl	5
nicotinic acid	5
pyridoxine HCl	0.5

APPENDIX C

CHENG (1977) MEDIUM

Compound	mg/liter
KNO_3	950
$MgSO_4\ 7H_2O$	185
NH_4NO_3	825
$CaCl_2\ 2H_2O$	220
KH_2PO_4	85
$MnSO_4\ 4H_2O$	11.15
H_3BO_3	3.1
$ZnSO_4\ 7H_2O$	5.25
KI	0.4
$CuSO_4\ 5H_2O$	0.013
$Na_2MoO_4\ 2H_2O$	0.15
$CoCl_2\ 6H_2O$	0.013
Na_2EDTA	7.2
$FeSO_4\ 7H_2O$	6
myo-inositol	250
thiamine HCl	2.5

APPENDIX D

MEDIUM FOR CONIFER MORPHOGENESIS (MCM)

Compound	mg/liter
KNO_3	2000
$MgSO_4\ 7H_2O$	250
$(NH_4)_2SO_4$	400
$Ca(NO_3)_2\ 4H_2O$	500
KH_2PO_4	270
KCl	150
NaFe EDTA	37.5
H_3BO_3	1.5
$ZnSO_4\ 7H_2O$	3.0
KI	0.025
$CuSO_4\ 5H_2O$	0.025
$Na_2MoO_4\ 2H_2O$	0.25
$CoCl_2\ 6H_2O$	0.025
myo-inositol	90
thiamine HCl	1.7
nicotinic acid	0.6
pyridoxine HCl	0.5
glycine	2
pantothenate	1.2
folic acid	1.1
biotin	0.125
urea	150

VITA

Nancy J. Zygmont was born in the Northeast, within the tempering climactic influence of the Long Island Sound. During her early years, she developed her fondness for raspberries, blueberries, and Morris dancing. She spent several years after her high school graduation sampling colleges in the Northeast, eventually earning her B.S. in Biology. She went on to graduate study, earning her M.S. in Botany from the University of Tennessee. Disillusioned, she plans to return to civilization as soon as possible.