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Multiple bud formation in vitro on Larix occidentalis and Pinus ponderosa buds stems and needles

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Multiple bud formation in *vitro* on *Larix occidentalis* and *Pinus ponderosa* buds, stems and needles.

by

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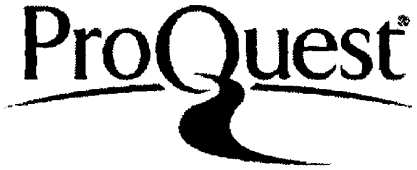

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Multiple bud formation *in vitro* on *Larix occidentalis* and *Pinus ponderosa* buds, stems and needles (47 pp.)

Director: Dr. George M. Blake

The objectives of this study were to produce multiple buds and shoots from buds, stems and needles of "mature" *Larix occidentalis* and *Pinus ponderosa* using tissue culture techniques. All tissues were cultured aseptically on a modified Shenk and Hildebrandt medium containing one of six benzyladenine (BA) concentrations (0, 0.225, 1.125, 2.25, 11.25, and 22.5 mg BA/l medium) and occasionally other hormones. Only western larch buds and stems produced multiple buds in culture. Single needles and needle pieces did not survive in culture. BA was found to be required for multiple bud formation; all concentrations produced multiple buds but more buds and stems formed multiple buds when on the lower three BA concentrations than when on the two higher concentrations. The origin of the multiple buds seemed to be both axillary and *de novo*. Larch buds were also cultured on gibberellin and auxin containing media. Gibberellins were detrimental in culture - within six weeks all buds were dead. The auxins resulted in bud and needle elongation. Ponderosa buds enlarged and elongated in culture, but no multiple buds formed.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Forest tree geneticists have emphasized coniferous tree improvement through selective breeding. However, the length of time required for conifers to reach sexual maturity is long, often twenty years; consequently, considerable time is required before superior progeny are available. Forest trees are heterogeneous and do not breed true. Vegetative propagation is a method of producing many individuals from one parent tree, all with identical genotypes. This can be done in a relatively short time, preserving genetic traits that could be altered or lost in sexual reproduction. In addition both additive and non-additive genetic effects are utilized in vegetative propagation (McKeand and Weir 1984). In sexual reproduction only additive and dominance effects may be utilized because of epistasis. Vegetative propagation is now recognised as a practical and important tool of forest tree improvement.

Grafting, rooting cuttings, and tissue culture are all methods of vegetative propagation. Grafting is the joining of a scion from one genotype to a rootstock from another genotype to form a whole tree. Early cone production, which is highly desirable for seed orchards, often occurrs on grafted trees. Also tree and clone banks may be produced using grafting. However, incompatablities in grafted trees often occur; the conducting tissues do not grow together properly, which eventually results in slow death of the tree. This physiological tissue rejection may not show up for several years (Copes 1971) and is a major problem where large numbers of the grafted trees are needed. Rooting cuttings is a means of circumventing graft incompatabilities;

roots are induced to form directly on stem cuttings. However the rooting of conifer cuttings has proven difficult, slow, and unreliable (Bonga 1974). Tissue culture is the growth of tissue in vitro on a nutrient medium, and by modifying the nutrient and hormone concentration whole trees can theoretically be produced. If tissue culture techniques can be perfected it could provide a means of directly producing tree clones from tissues, i.e. needles, buds, and embryos of individuals. With tissue culture complete plantlets could be produced in about six months, avoiding grafting incompatability, and mature, differentiated tissues incapable of rooting.

Potential of Tissue Culture

Tissue culture techniques could be useful in mass production of improved trees for operational planting and in the production of clones for experimental purposes (to control variation due to genotype). Seed orchards of improved stock could be produced without grafting. Storing material as a "clone bank" would be efficient using tissue culture, because storage of only a few cells, or possibly a callus, would be all that is needed since whole trees theoretically can be produced from a few cells. Cell suspension cultures could be used to produce many embryoids (Karnosky 1981) which would then be grown in culture like normal embryos. Tissue culture could also be used to create new species or hybrids through protoplast fusion of otherwise non-hybridizing species. Adapting techniques of DNA alteration and DNA splicing to forest trees to introduce or modify genetic traits may also eventually be possible using protoplast and tissue culture techniques. Once a

genetically altered individual has been produced and grown to maturity, tissue culture can be used to produce many more if desired. These techniques may only be used once tissue culture methods have been perfected.

OBJECTIVES

The primary objective of this study was to produce multiple buds and shoots using bud and needle tissue from mature individuals of Larix occidentalis and Pinus ponderosa using tissue culture techniques.

Specifically the objectives are as follows:

1. Induce multiple bud formation from bud and needle tissue.
2. Induce shoot elongation from the new buds.
3. Determine seasonal variation, if any, in multiple bud formation.
4. Test effects of different hormones and media concentrations on bud formation.

LITERATURE REVIEW

Plant tissue culture was first used at the beginning of the century by Haberlandt (Konar and Nagmani 1974). The first conifer embryo was cultured in 1924 by A. Schmidt (Wochok and El-Nil 1977). Early conifer culture attempts nearly always resulted in callus formation without any significant differentiation (see Table 1). The only organogenesis reported was from Sequoia sempervirens burl shoots (Ball 1950). High auxin levels were found to stimulate callus growth (Harvey 1967, Cheng 1975, Hussey 1978, Winton 1972), indicating the hormone levels needed to induce differentiation were not discovered.

Most conifer culture work has been done using portions of excised embryos. Differentiation of whole plantlets has originated from embryonic cultures with limited exceptions (Coleman and Thorpe 1977, Ball et al. 1978). The advantage of working with embryos is that the tissue is still in the juvenile growth stage (Mott 1981, ed. Conger). The tissue culture of Pinus radiata has been one of the most successful; its cotyledons and embryos have produced multiple buds in culture and whole plantlets have been produced from these buds (Reilly and Brown 1976, Reilly and Washer 1977, Aitken, Horgan and Thorpe 1981, and Horgan and Aitken 1981). Embryonic portions of many other conifer species have successfully produced buds in culture which were then rooted to produce whole plantlets (see Table 2). Multiple buds but no rooting have also been produced from embryonic parts of other species (see Table 3).

Table 1

Early conifer culture attempts resulting in callus formation.

Species name	Author		Tissue used
Abies grandis Larix occidentalis Picea abies Picea engelmannii Picea pungens Pinus albicaulis Pinus contorta Pinus flexilis Pinus nigra Thuja plicata	Harvey and Grasham	1969	cortex from 3-7 year old seedlings
Cupressus lusitanica	Borchert	1967	wood cambium
Picea spp.	Steinhart et al.	1961	tissue
Pinus banksiana	Chalupa et al.	1976	hypocotyls
	Durzan and Chalupa	1976	
Pinus monticola	Harvey	1967	stem sections
Pinus palustris	Brown and Lawrence	1968	hypocotyls
Pinus radiata	Washer et al.	1977	tissue
Pinus roxburghii	Mehra and Anand	1983	seedlings
Pinus strobus	Minocha	1980	embryos
Pseudotsuga menziesii	Al-Talib and Torrey	1959	buds
	Winton	1972	needles and stems
Sequoia sempervirens	Ball	1950	burl shoots

Table 2

Conifer species producing whole plantlets from embryo portions

Species name	Author		Tissue used
Cryptomeria japonica	Isikawa	1974	hypocotyls
Larix laricina	Bonga and McInnis	1983	embryo sections
Larix occidentalis	Ellis (unpublished)	1984	cotyledons
Picea abies	Von Arnold	1982	cotyledons
	Bornman	1983	and embryos
Picea glauca	Campbell and Durzan	1975,76	cotyledons
	Rumary and Thorpe	1984	and epicotyls
Picea mariana	Rumary and Thorpe	1984	cotyledons and epicotyls
Pinus contorta	Von Arnold and Eriksson	1981	hypocotyls,
	Patel and Thorpe	1984	cotyledons, embryos and shoot tips
Pinus eldarica	Momot	1981	cotyledons
Pinus gerardiana	Konar	1975	embryos
Pinus monticola	Mott and Amerson	1981	embryos
Pinus palustris	Sommer et al.	1975	embryos
Pinus pinaster	Rancillac	1981	shoot tips, and embryos
Pinus pithyusa	Momot	1981	cotyledons
Pinus ponderosa	Ellis (unpublished)	1984	cotyledons
Pinus taeda	Mehra-Palta et al.	1978	cotyledons
	Mott and Amerson	1981	
Pseudotsuga menziesii	Cheng and Voqui	1977	cotyledons
Thuja plicata	Coleman and Thorpe	1976,77	cotyledons
Tsuga heterophylla	Cheng	1976	cotyledons

Table 3

Conifer species producing multiple buds on embryonic tissue in culture.

Species name	Author		Tissue used
Biota orientalis and other Cupressaceae	Thomas et al.	1977	hypocotyls, cotyledons and epicotyls
	Thomas and Tranvan	1982	
	Konar and Oberoi	1965	
Larix dahurica Larix siberica	Momot and Smirnov	1978	embryos and cotyledons
Pinus echinata Pinus elliottii Pinus rigida Pinus sabiniana Pinus virginana	Brown and Sommer	1977	cotyledons
Pinus nigra	Kolevska-Pletibapic et al.	1983	embryos
Pinus pinaster	David et al.	1982	cotyledons
Pinus ponderosa	Momot and Smirnov	1978	embryos and cotyledons
	Ellis and Bilderback	1984	
Pinus pumila	Skripachencho	1982	embryos
Pinus strobus	Minocha	1980	embryos
Pinus sylvestris	Tranvan	1979	embryos and seedlings
	Bornman and Jansson	1980	
	Skripachenko	1982	
Pinus taeda	Cheng	1976	cotyledons
Pinus wallichiana	Konar and Singh	1980	hypocotyls and embryos
Pseudotsuga menziesii	Cheng	1975,77	cotyledons and embryos
	Reilly and Brown	1976	
	Winton and Verhagen	1977	
Sequoia gigantea Cupressus arizonica	Coleman and Thorpe	1976	hypocotyls and cotyledons

In all cases cytokinins were required for bud induction. In Pinus radiata 5.0 mg Benzyladenine (BA) per liter produced the highest number of buds (Reilly and Washer 1977, Horgan and Aitken 1981). In Pseudotsuga menziesii $5x10^{-6}$ M BA + $5x10^{-9}$ M NAA (napthalene acetic acid, an auxin) induced buds, and higher NAA concentrations produced callus (Cheng 1977). Rumary and Thorpe (1984) found that the length of exposure to BA was important in bud induction in black and white spruce. The proper balance between BA and auxins was found to be necessary in a number of cases, for optimum bud production (Cheng 1976, Cheng and Voqui 1977, Cheng 1977, Mehra-Palta et al. 1978, David et al. 1982, Skripachenko 1982). Mehra-Palta et al. (1978) stated that low auxin levels act synergistically with cytokinins but high levels are inhibitory to bud formation. High auxin levels relative to cytokinins have been found to induce callus rather than organogenesis (Bornman 1982). However, Bornman (1982) found that with Picea abies BA and IBA (indole butyric acid, an auxin) had their greatest organogenic effects when applied singly. While older or more differentiated tissues required an increased concentration of BA or increased length of exposure to BA to induce organ formation (Tranvan 1979, David et al. 1982), Pinus radiata required BA during the first few days in culture for bud formation to occur (Biondi and Thorpe 1982).

To elongate initiated buds the tissue required transferring to a medium lacking growth regulators and, occasionally, with decreased sucrose and nutrient concentrations (Cheng 1975, 1976, Reilly and Brown 1976, Reilly and Washer 1977, Mehra-Palta et al. 1978, Horgan and Aitken 1981, Mott and Amerson 1981, Von Arnold and Eriksson 1981, Von Arnold

1982, Rumary and Thorpe 1984). The inclusion of charcoal in the elongation medium has been found to enhance shoot growth (Mehra-Palta et al. 1978, Rumary and Thorpe 1984) since it absorbs inhibitory substances that may be produced by the cultures. Growth regulators and other low molecular weight compounds are also absorbed which prevents the use of charcoal in steps where the exact amount of the growth regulator and other substances in the medium is important (Weatherhead et al. 1978, 1979).

Mature tissue from only a few species of trees has been put in culture and has subsequently differentiated into organized structures. Ball (1950) was the first to report organogenesis in tissues taken from a mature tree. He reported meristem, shoot apices, leaf primordia, and stele formation in callus cultures of Sequoia sempervirens, and eventually induced whole plantlet formation (Ball et al 1978). Whole plantlet formation was also induced from Thuja plicata lateral shoot tips (Coleman and Thorpe 1977) with over 90% of the buds producing shoots but of these only 11% rooted; $5x10^{-5}$ M BA + $1x10^{-7}$ M NAA was used in the medium to induce multiple bud formation and $5x10^{-5}$ M IBA was used to induce rooting. Short shoots from Pinus pinaster were also induced to form plantlets in culture (David et al. 1978, Franclet et al. 1980). Multiple buds were induced on organ cultures from mature tissues of a number of different species of conifers (see Table 4), and BA was required for this to occur.

Table 4

Conifer species producing buds on mature tissues placed in culture.

Species name	Author		Tissue used
Abies balsamea	Bonga	1977, 1981	buds from 15-20 year old trees
Araucaria bernieri	Anderson	1983	shoots
Larix dahurica	Momot and Smirnov	1978	stem apices and needle tufts
Larix decidua	Bonga	1982	immature female strobili stalks
Larix siberica	Momot and Smirnov	1978	stem apices and needle tufts
Picea abies	Jansson and Bornman	1983	buds from 10 yr. old trees, lateral and terminal shoot from 1 yr old seedlings, buds from trees 5 - 50 yrs old, new needles on 5 yr old trees
	Jansson and Bornman	1980	
	Von Arnold and Eriksson	1979a and 1979b	
	Romberger et al.	1970	
Picea glauca	Bonga	1981	buds
Picea pungens	Manandhar and Gresshof	1980	leaves, stems, buds
	Misson et al.	1982	buds
Picea sitchensis	Webb and Street	1977	shoot meristems from 2 year old trees
Pinus pinaster	David et al.	1982	short shoots and elongating needles from 10 year old trees
Pinus sylvestris	Bornman and Jansson	1980	needle fascicles
Pinus taeda	Mehra-Palta et al.	1978	needle fascicles (5 yr, 8 mo. old trees)
Pseudotsuga menziesii	Thompson and Zaerr	1981	buds
	Bonga	1981	

Al-Talib and Torrey (1959) cultured Pseudotsuga menziesii buds but obtained only bud elongation, enlargement and callus growth. Chalupa and Durzan 1973) also only obtained elongation when they cultured Picea glauca dormant vegetative buds but got different amounts of growth on different media, indicating that different nutrient concentrations result in different types and amounts of bud growth. Picea abies apical meristems were also cultured on a number of different media to determine which produced the most overall growth and what secondary products were produced by the bud and exported to the agar (Romberger and Tabor 1975, 1971).

Reports of the successful development of roots on shoots is limited. Reilly and Brown (1976) got less than 1% rooting with Pinus radiata and Pseudotsuga menziesii initally. However, later, Reilly and Washer (1977) obtained roots on Pinus radiata shoots after treating with 1-25 mg/l IBA on a modified Gresshof and Doy (GD) medium. Eventually, Horgan and Aitken (1981) obtained reliable rooting in a solid GD medium with 2% sucrose plus 2 mg/l IBA and 0.5 mg/l NAA; using only shoots with a recognisable stem. Cheng (1977) obtained 80% rooting with Pseudotsuga menziesii when the shoots were treated with 0.25 uM NAA. Loblolly pine (Mehra-Palta et al. 1978, Mott and Amerson 1981) shoots also were rooted using NAA (0.01-0.1 mg/l BA + 0.1 mg/l NAA). Roots were induced on 30% of Pinus monticola shoots on a medium with 4% sucrose and high auxin levels (Mott and Amerson 1981), and rooting was induced on Pinus sylvestris tissue (Bornman and Jansson 1980) using coumarin, BA, and IAA (indole acetic acid, an auxin). Coleman and Thorpe (1977) induced rooting on 50% of Thuja plicata shoots when placed

on a medium with 3% sucrose and $5x10^{-5}$ M IBA; only shoots 5-30 mm high were used. Tsuga heterophylla shoots rooted directly in soil after being watered with a 15 μM solution of NAA (Cheng 1976); 70-100% of Pinus pinaster shoots rooted when cultured on an agar solidified basal nutrient medium with 10^{-6} M NAA for 12 days (Rancillac et al. 1982) and the quality of the roots was found to depend on the length of the auxin treatment. Roots were induced on black and white spruce at the same rate (84%) in sterile as in non-sterile conditions using a dip in commercial rooting powder containing 0.1% IBA (Rumary and Thorpe 1984). Roots from Larix laricina cultures (Bonga and McInnis 1983) were found to have originated at the base of the shoots within the callus, some formed between needles on the elongating stem and within young needles.

Auxins were found to be required for root initiation, but they also inhibited root elongation; therefore, shoots had to be transferred to a medium lacking growth regulators to induce root elongation (Cheng 1977, Mehra-Palta et al. 1978, Mott and Amerson 1981). Sucrose concentration was also thought to influence rooting through osmotic effects (Greenwood and Berlyn 1973).

In all cases a cytokinin was required for bud induction on mature and embryonic tissues. However,the recommended length of time on BA medium, the concentration of BA required and the treatment afterwards varied with author, species, and age of original explant. New buds then required transfer to a medium lacking growth regulators for shoot elongation. Rooting shoots required auxin treatments, then transfer to a medium (or soil) lacking growth regulators for root elongation. Exact techniques of rooting shoots also varied depending on author and

species. The limited number of papers on organogenesis of mature conifer tissues in culture seems to suggest that either few projects are being performed or the results are not being reported.

METHODS

Field Collection and Treatment

Western larch (Larix occidentalis Nutt.) stem tissue was collected from three locations: three year old seedlings at the Lubrecht Experimental Forest in western Montana, the edges of a nine year old ponderosa pine plantation at Lubrecht Experimental Forest (approximately 8 year old material), and a 20 year old cut area at Blue Mountain on the Lolo National Forest. Ponderosa pine (Pinus ponderosa Laws) stem tissue was collected from trees about nine years old at Lubrecht Experimental Forest. Only the terminal buds and stems of lateral branches were harvested and the two-inch stem pieces were stored in plastic bags in a cold room at 0-5°C until the tissue could be placed in culture.

Stem tissues were collected and cultured year round. Resting vegetative buds were used from August to mid-April. Stem and needle pieces from the larch and the apical meristems and needles from the ponderosa were cultured during mid-April to August.

Laboratory Procedures

Excised resting buds were surface sterilized by soaking in a solution of Orthocide Garden Fungicide containing 50% Captan (N-[(trichloromethyl)thio]-4-cylohexene-1,2-dicarboximide) for 10 minutes, rinsing in tap water until the solution was clear, and then soaking in 25% Clorox for 15-20 minutes. If the Clorox solution discolored, then it was changed. Growing tissue such as stems and needles were treated similarly except they received either a 1.5 minute soak in 50% Clorox or a 20 minute soak in 3.5% Clorox. All tissue was

then rinsed 2 or 3 times in sterile distilled water.

Larch buds were excised from the stem by cutting around the base of the bud and slipping off the bud scales. The tiny non-woody green bud with its preformed needle primordia was then cut from the branch aseptically and placed on the chosen sterile medium. When larch stems were used, most of the needles were removed prior to surface sterilization, then only the base of the stem section was removed before placing it in culture. The stem piece was cut into two approximately 1 centimeter long pieces, with the terminal bud on one piece. Needles were sterilized the same way as stems, but no tissue was removed before placing them in culture.

The ponderosa buds were more difficult to expose as each bud scale was attached beneath a different fascicle primordium so the scales could not be removed all at once, but had to be individually removed. All mature needle fascicles were removed before the buds were surface sterilized; buds were excised from the stems after the bud scales had been removed. Apical meristems were dissected from the bud scale coverings, and as much as possible of the greenish young bud scales were removed. Needle fascicles were surface sterilized both with the fascicle sheath intact or removed.

The tissue was cultured on a Shenk and Hildebrandt (SH) medium as modified by Reilly and Washer (1977). Either half or full strength of all constituents except hormones and sucrose were used. The full strength medium contained per liter: 2.5g KNO_3, 0.4g $MgSO_4.7H_2O$, 0.3g $NH_4H_2PO_4$, 0.2g $CaCl_2.2H_2O$, 10 mg $MnSO_4.H_2O$, 5mg H_3BO_3, 1 mg $ZnSO_4.7H_2O$, 1 mg KI, 0.2mg $CuSO_4.5H_2O$, 0.1mg $NaMoO_4.2H_2O$, 0.1 mg $CoCl_2.6H_2O$, 15mg

$FeSO_4.7H_2O$, 20mg Na_2EDTA, 100mg Myoinositol, 5 mg Thiamine-HCl, 0.5mg Nicotinic acid, 0.5 mg Pyridoxin- HCl, 30g sucrose and between 4.5 and 5 grams of Difco Bacto agar. For bud initiation five benzyladenine (BA) levels were used: 10^{-4} M (22.5 mg/l), $5x10^{-5}$ M (11.25 mg/l), 10^{-5} M (2.25mg/l), $5x10^{-6}$ M (1.125 mg/l), 10^{-6} M (0.225 mg/l). No auxins were used in the bud initiation medium. The bud elongation medium was half strength SH with no hormones. All media were adjusted to pH 5.5 using 0.3 N KOH and 1 N HCl. The BA was dissolved in either boiling water or a small amount of 0.3 N KOH.

Media was poured into autoclavable glass bottles with black plastic screw-caps. Approximately 25 ml of a medium was poured into 4 oz bottles, 15 ml into 2 oz and about 7 ml into one oz bottles. All media were autoclaved in the culture jars for 20 minutes at 15 pounds pressure, except when antibiotics were added to the elongation medium. When this occurred, the antibiotic was added after autoclaving (10 ml of a sterile 100 mg streptomycin per liter solution, or 1 mg streptomycin per liter of medium), and the medium was poured into sterile jars.

No more than 5 larch buds and 3 ponderosa buds were placed in each 4 oz jar, with only 1 bud in the 1 and 2 oz jars. The number of tissue pieces per experimental treatment varied from 2 to 20. Most cultures had 20, except those established during the summer. Each treatment was replicated several times during the year.

An experiment was run culturing western larch buds on full strength SH with a Gibberellin mixture, GA_{4+7} (76+24; 76 parts GA_4 plus 24 parts GA_7), and one BA concentration (1.125 mg/l or $5x10^{-5}$ M). Six different combinations were used: 10^{-5} M GA, 10^{-6} M GA, 10^{-7} M GA, 10^{-5}

M GA + BA, 10^{-6} M GA + BA, 10^{-7} M GA + BA. The same experimental design was repeated two times substituting IBA and IAA for the GA, using the same molarities as the GA. Twenty larch buds were placed on each medium with a total of 120 for each hormone tested (360 total). Larch and pine buds were also placed on a full strength SH medium containing 1 mg of both IAA and IBA, as well as on media completely lacking hormones.

Tissue was transferred to half strength SH after being on the bud induction medium. Often this transfer was done before any multiple buds were visible, usually six or eight weeks after the tissue was originally placed in culture. When the tissue was transferred, it was inspected under a dissecting microscope to count multiple buds and note other changes. Dead tissue, if present, was then removed. If any multiple buds were large enough and visible enough, they were separated from the parent tissue and individually placed on the elongation medium. All placing of tissue in culture and transferring of tissue were done aseptically either in a plexiglass glove box or in an open room under a dissecting microscope.

Cultures were placed in one of two growth chambers, with a temperature of $25^{o}C$, a 24 hour photoperiod, and a light intensity of 3 watts/m^2. Humidity within the chambers was maintained by tubs of water on the bottom of the chambers.

Statistical Analysis

Friedmans two-way ANOVA was run using the SPSSX program on the University of Montana computer. Two hypotheses were tested: the probability that there was no difference with season, and that there was no difference between treatments. The data used were number of buds or stems producing multiple buds. Since sample size was not uniform, the data were converted to percentages.

RESULTS AND DISCUSSION

Western Larch

Multiple Bud Formation

Multiple buds were induced on larch buds and stems; no multiple buds formed on needles or needle pieces. All benzyladenine (BA) treatments resulted in multiple bud formation, though fewer buds and stems on the higher BA concentrations (11.25 and 22.5 mg BA/l) produced multiple buds. BA inhibited elongation; only buds on the lowest concentration elongated more than 5 - 7 mm and at this concentration only the needles seemed to elongate. Tissue on media with the other concentrations enlarged overall, retaining the original shape longer. Treatments lacking BA resulted in stem and needle elongation and no multiple bud formation, though the tissue did not elongate as much as if it were left on the tree.

Within any single BA treatment variation was observed: some pieces died, some enlarged and died, some enlarged and produced multiple buds (see Figures 2 - 6), some produced callus and multiple buds (see Figure 7), and some just produced callus. The number of multiple buds produced per tissue piece varied from one to over 30. Figures 2, 7, 8, and 9 show unelongated multiple buds. Figures 3 and 10 show slightly elongated buds and figures 4, 5, and 6 show multiple buds after being on the elongation medium for 1 - 2 months.

Figure 1. Western larch bud before culturing.

Figure 2. Multiple buds on a western larch bud after 4 months 22.5 mg BA/l SH medium.

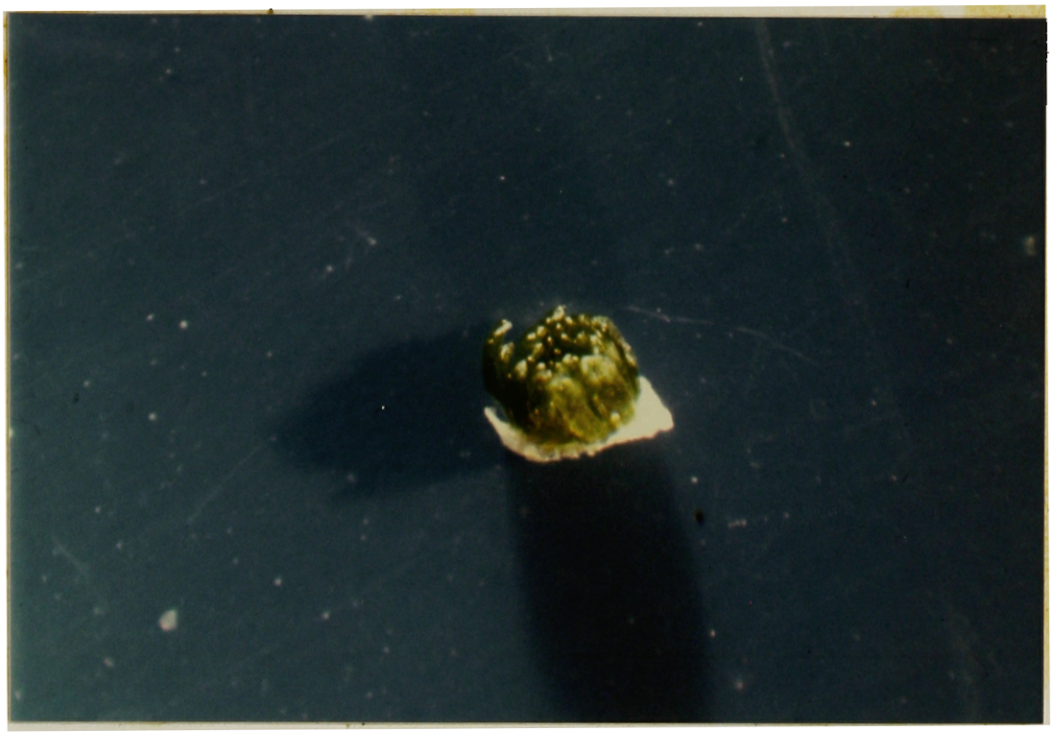
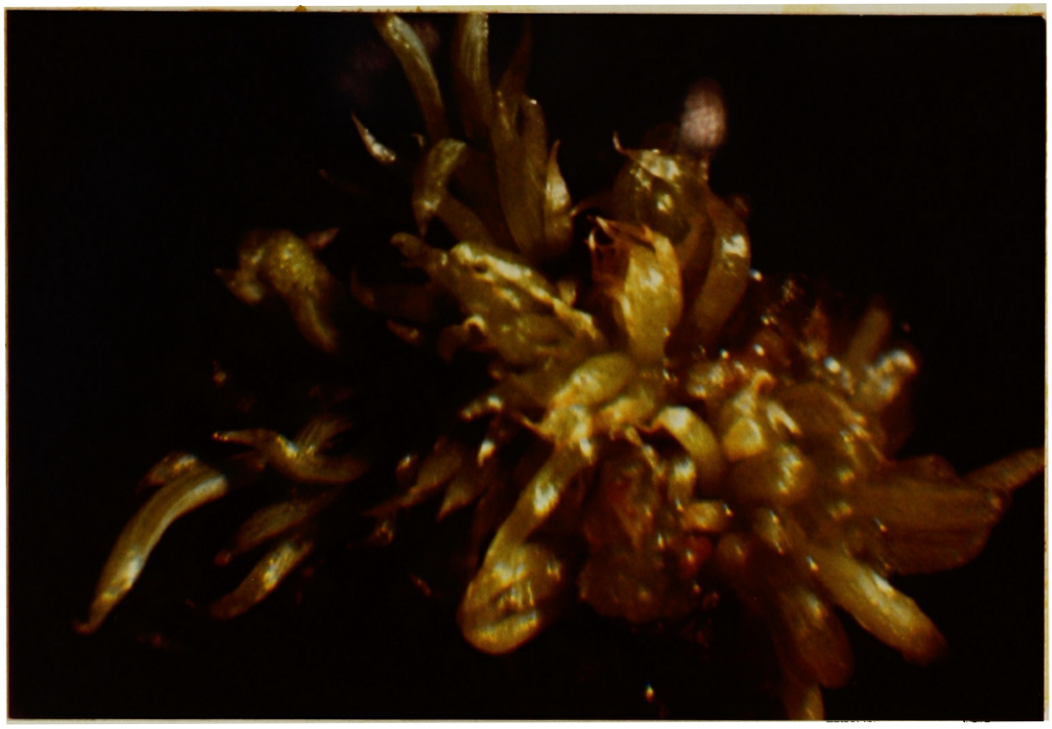


Figure 3. Western larch bud after 3 months on 2.25 mg BA/l medium

Figure 4. Portion of the same bud as Figure 3 one month later after being on the elongation medium for 1 month



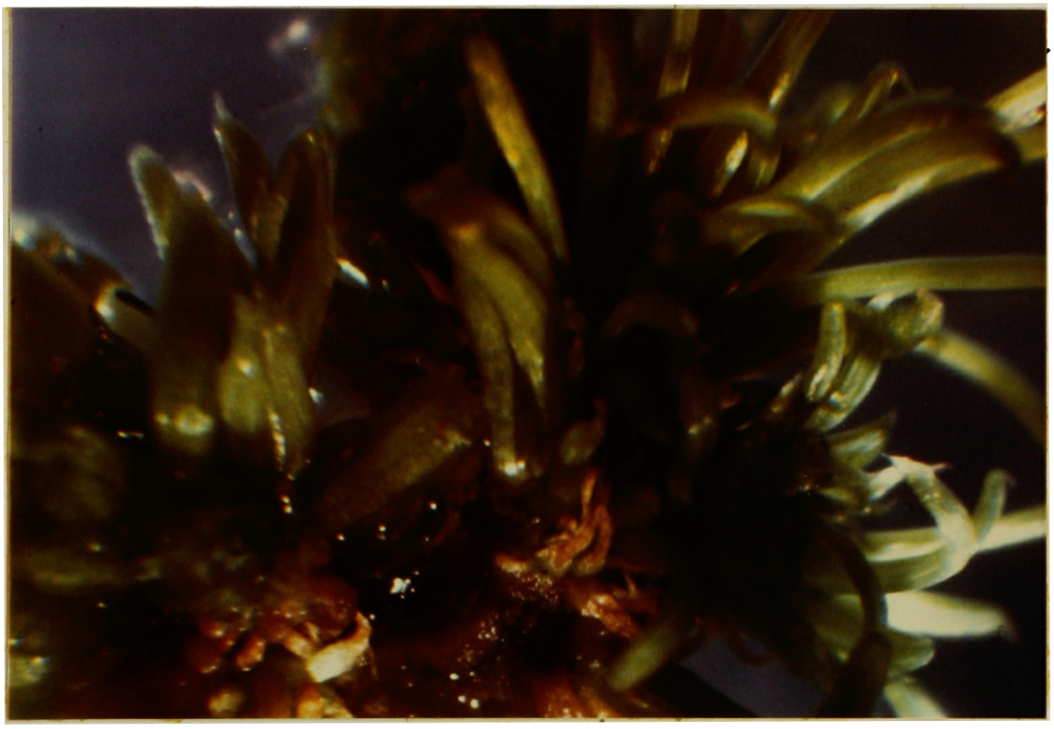
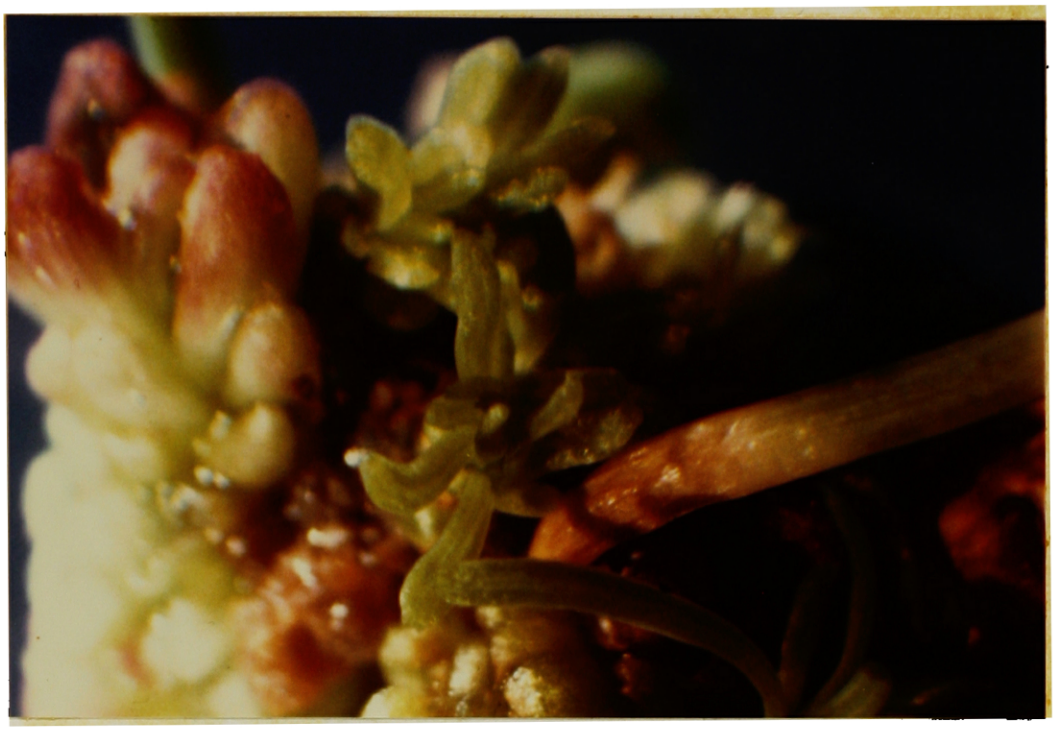


Figure 5. Multiple buds on a larch stem after 3 1/2 months on 0.225 mg BA/l SH medium.

Figure 6. Multiple buds on a larch stem after 2 1/4 months in culture on 0.225 mg BA/l SH medium and 1 2/4 months on the elongation medium.



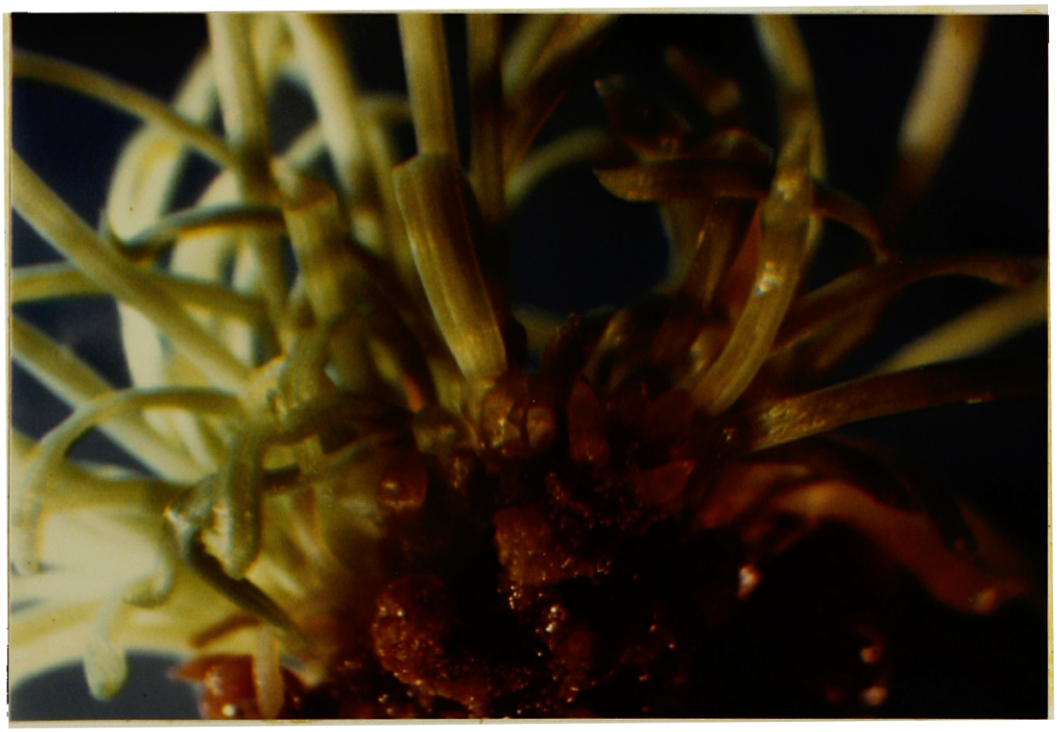


Figure 7. Callus and multiple buds on a larch stem after 2 1/4 months on 11.25 mg BA/l SH medium and 1 2/4 months on the elongation medium.

Figure 8. Axillary bud formation on a larch stem after 2 3/4 months in culture on 2.25 mg BA/l SH medium.

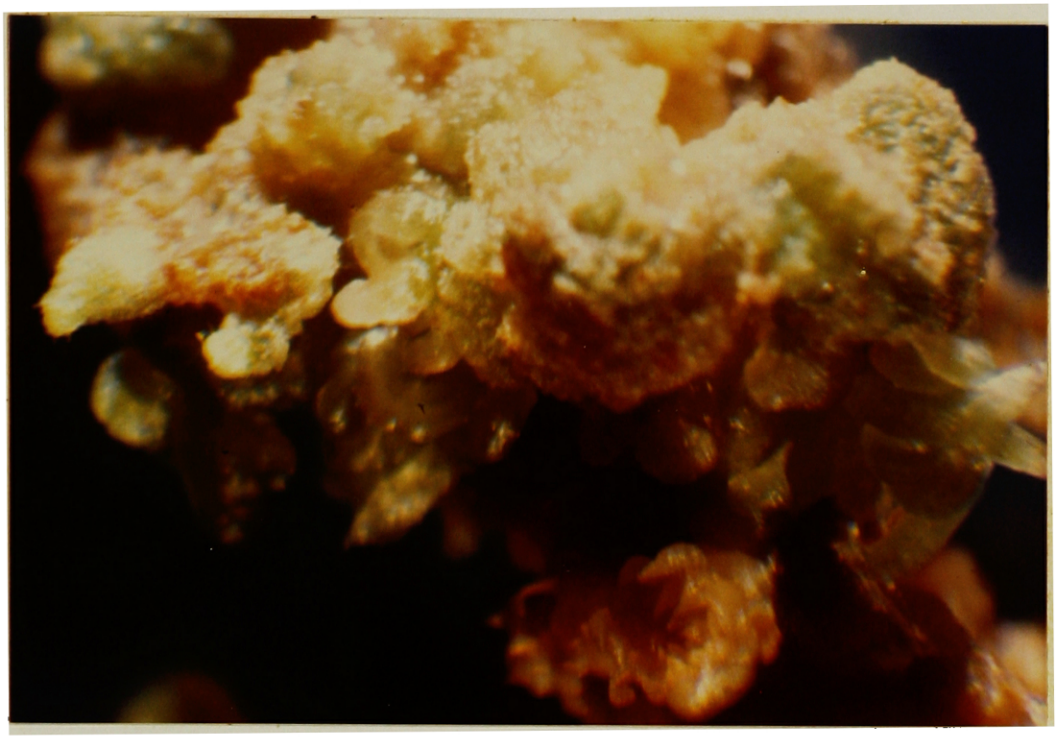
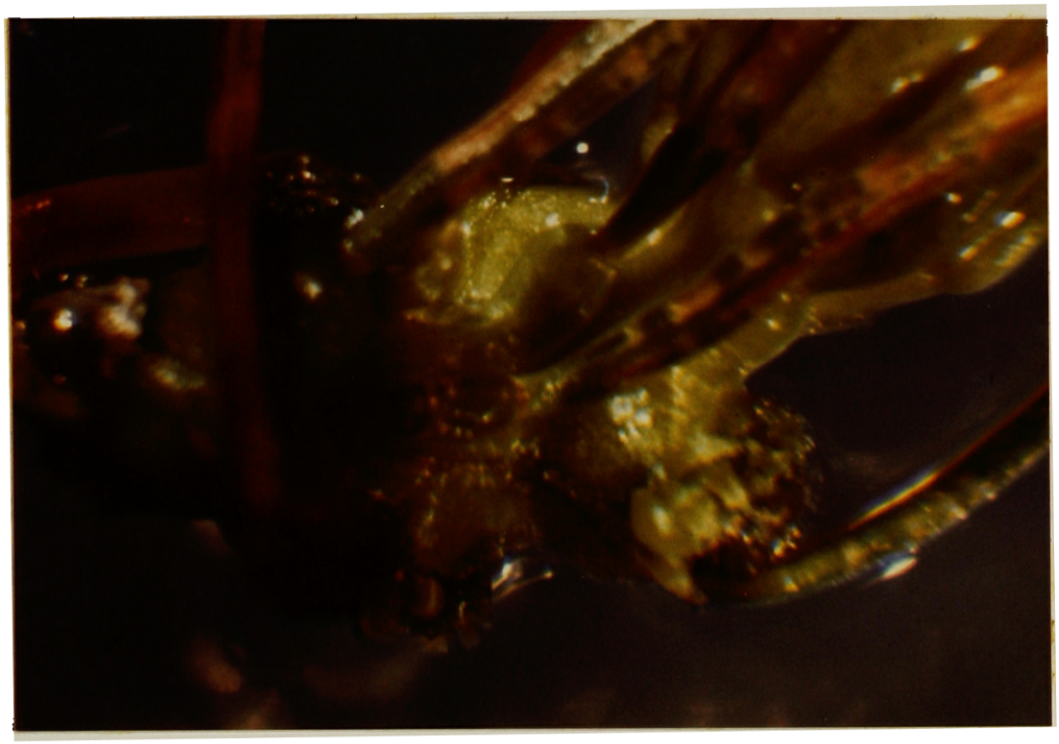
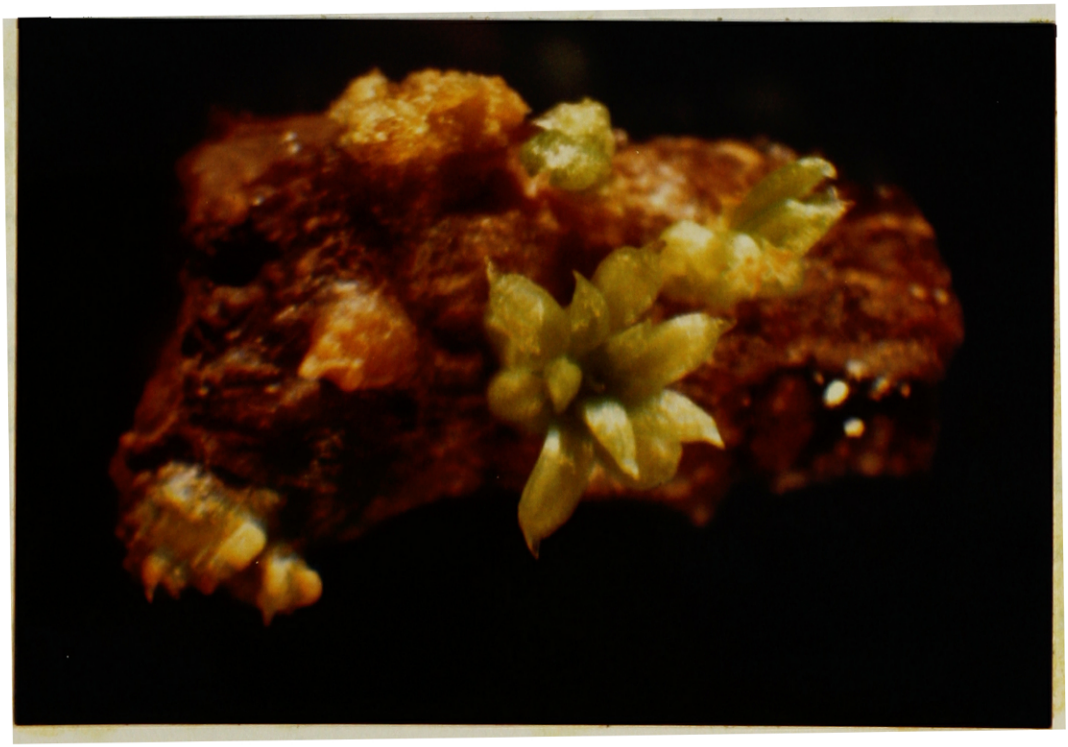


Figure 9. Axillary bud formation on a larch stem after 1 month in culture on 0.225 mg BA/l SH medium.

Figure 10. Axillary bud formation on a larch stem section after 2 2/4 months in culture on 11.25 mg BA/l SH medium and 1 1/4 months on the elongation medium.





The BA concentrations that produced multiple buds from buds and stems of Larix occidentalis are similar to those Momot and Smirnov (1978) used to obtain multiple buds from the buds and apices of mature Larix siberica and Larix dahurica. Thompson and Zaerr (1981) induced buds from Pseudotsuga menziesii to form adventitious buds also by using concentrations of BA similar to those that induced multiple buds on Larix occidentalis.

The multiple buds looked like tiny needle clumps, regardless of location of origin and amount of cytokinin in the induction medium. The number of needles in these clumps varied from 2 or 3 to many (see figures 2 - 10). Bud color varied from light green to dark green and from opaque to more translucent. Translucent buds did not occur frequently, and they usually did not elongate or survive in the elongation medium. One piece of tissue had a bud that may contain anthocyanins (see Figure 5).

It was not possible to determine if the multiple buds produced on excised buds were axillary or de novo. By the time the new bud was large enough to be visible, the original morphology was often completely altered, and the origin was not discernable (see Figures 2, 3, 4). Figure 8 shows buds that developed on a 3 month old bud placed in culture in August on a medium with 2.25 mg BA/l. These were probably axillary buds because of apparent location of origin.

The buds formed on stem pieces were probably both axillary and de novo. Buds formed in needle axils above needle scars; if only one formed, it was assumed to be an axillary bud and if more than one formed in a needle axil then at least one was assumed to be de novo (see

Figures 9 and 10). Lateral branches of Thuja plicata (Coleman and Thorpe 1977) were induced to form multiple buds both in axillary positions as well as de novo at needle bases. Figure 9 shows a stem section after a month in culture on 0.225 mg BA/l medium with multiple buds forming in two needle axils. Figure 10 shows axillary buds on a stem section after 3 months on 11.25 mg BA/l medium. Only stem sections with the terminal meristem survived in culture; they first swelled and enlarged in radius. Eventually on some stems the whole bud was taken over by the multiple buds and stem anatomy was no longer discernable (Figures 5, 6, 7).

Elongation of multiple buds occurred when they were transferred to the half strength SH, no hormone elongation medium, but many buds did not survive or elongate on the medium. Possibly they may had been left on the BA medium too long.

During the summer 3 to 6 weeks elapsed before multiple buds were visible, but during the winter 3 to 4 months were required before multiple buds were visible. Usually buds were visible before transfer to elongation media, but occasionally they were not visible until after the tissue had been on the elongation medium for two weeks.

Statistical Analysis

The results from the Friedman two-way ANOVA for seasonal differences showed that there was no significant difference in bud formation due to treatment date: the probability was 42% that the tissue responded the same regardless of season, or no real differences between collection dates. Since the data used in this test were number

of buds producing multiples not length of time before the new buds were visible or actual number of new buds formed; the number of buds and stems producing multiple buds is about the same year round. The second hypothesis, no difference between the 6 treatments, was rejected at the 3% probability level. When the 5 BA treatments, excluding the control, were analyzed the probability of real differences dropped from 97% to 82%. This drop of only 15% could indicate meaningful treatment effects and suggests further trials.

The variation was so large that the data does not lend itself to statistical analysis. Endogenous hormone levels and genetic differences between trees may have a greater influence on bud formation than treatment. Endogenous differences will continue to be a problem until buds can be reliably produced.

Media and Hormone Effects

Full strength SH medium was used more often than half strength because tissue growing on full strength medium was slightly greener than that growing on half strength. Full strength was used whenever hormones other than BA were tested. Streptomycin did not greatly influence the level of contamination in the transferred cultures. The effects of antibiotics on culture growth were not evaluated but should be further tested and used if contamination is at all decreased.

Gibberellin (GA) did not seem to stimulate bud elongation, and no multiple bud formation was observed. Slight growth occurred in all six of the media used but within six weeks all of the cultured buds were dead. The GA + BA containing media stimulated the tissue to enlarge

slightly from about 3 mm to about 5 mm. Results from the GA only media were similar except the needles elongated slightly. There were no apparent differences between the 3 different GA concentrations and only slight difference between the GA only and the GA + BA treatments - all resulted in culture death relatively quickly.

The auxins, IAA and IBA, stimulated bud elongation when BA was absent. When BA was included in the medium elongation did not occur, only overall enlargement of the bud. The results from the IAA treatments were essentially the same as the IBA treatments. There was more callus growing on the auxin + BA treatments than on the IAA or IBA only treatments. Callus on treatments including BA rapidly turned brown, grew from the lower outer edge of the bud in, eventually taking over the needles. The callus on treatments without BA was greenish, began growing later, was less extensive and grew at the base of the bud, with needles forming callus slightly later. The length of the elongated stems on the auxin only treatments varied from 5 to 15 mm high with more of the shorter stems in the 10^{-7} M concentration, but all 3 concentrations had various stem lengths. Essentially no multiple buds formed under these treatments (2 buds out of 240 formed multiple buds).

The buds that were placed on a medium with 1 mg/l each of IAA and IBA elongated quickly and, after about one month, formed greenish callus, but formed no multiple buds. Forty buds were placed on IAA + IBA medium for 5 days then were transferred to media containing the usual BA concentrations. Only one bud produced any multiple buds (it was placed on 1.125 mg BA/l SH medium and produced 7 buds); all others died. BA seems to be required at the beginning of culture for multiple

buds to form. Shoots elongated on a medium lacking BA have not yet been induced to form buds when transferred to a BA medium.

BA may induce multiple bud formation by masking or overriding auxin effects in the tissue, or it may stimulate bud formation more directly, or even a combination of the two. Differences in numbers of multiple buds per piece of tissue in the same treatment were assumed to be due to differences in concentration of endogenous hormones and other substances. This could account for the large variation observed in response to treatments: anything from tissue death to multiple bud formation.

Obtaining multiple bud production on buds or stems taken from mature larch trees is the first step in vegetatively propagating phenotypically superior trees by tissue culture. In the future the number of buds and shoots produced in culture should be maximized, elongation of shoots and root formation must be reliably induced and new trees aclimatized to the outside environment. Then the performance of tissue culture may be evaluated and improved.

Ponderosa Pine

None of the cultured ponderosa tissue produced multiple axillary or adventitious buds, though buds and apices survived and grew for a while *in vitro*. In the 360 to 400 buds cultured elongation, and swelling occurred, and all growth was about the same regardless of medium concentration and hormone concentration and type. The whole buds grew and elongated to 2 or 3 times their original size, then stopped and died. Some of the horizontally placed buds elongated and grew upright. On two buds a few fascicle primordia elongated and distinct needles were visible - one set elongated about 1.5 cm. These needles did not survive when they were left on the bud or when excised and placed on the medium.

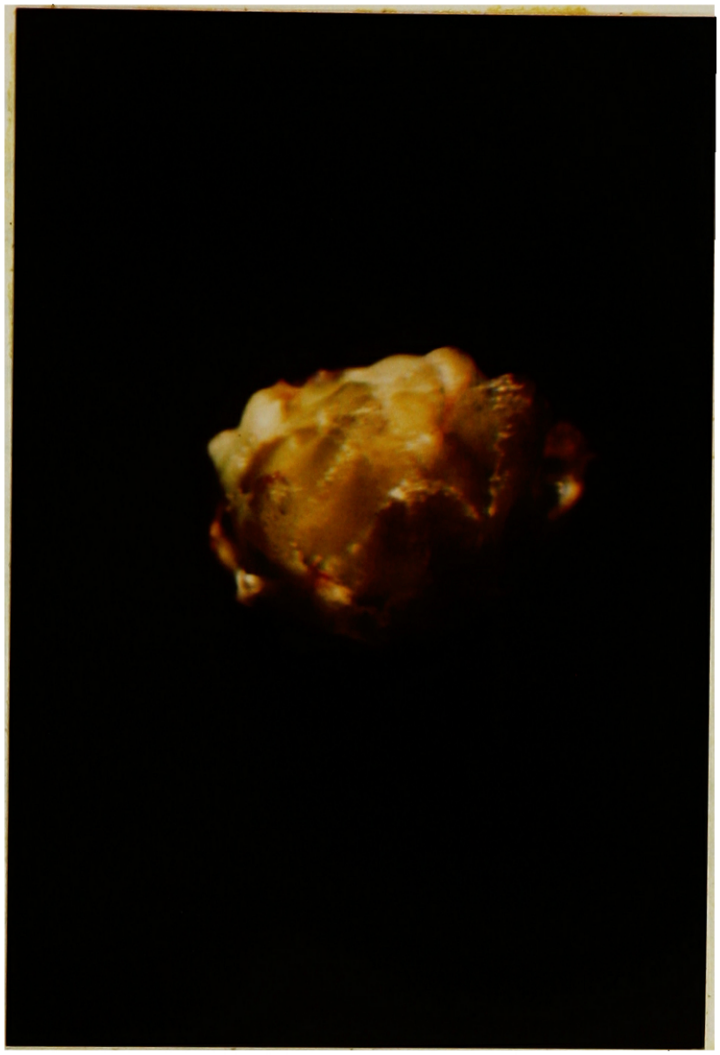
Abies balsamea (Bonga 1977), *Picea pungens* (Misson et al. 1982), and *Picea abies* (Von Arnold and Eriksson 1979, Bonga 1977) all produced adventitious buds from needle primordia of vegetative buds, which might be expected from *Pinus ponderosa* but did not occur. In fact there was no report of multiple buds forming on vegetative buds of any species of *Pinus*. Bonga (1977) reported that embryonic shoots of *Abies balsamea* required pre-soaking in malonic acid solutions for organogenesis to occur, and he (1977) also obtained almost no response from vegetative buds of *Picea glauca*, *Abies balsamea*, and *Pseudotsuga menziesii* unless they were forced before culturing. Both forcing buds and pre-culture soaks may remove inhibitory substances which may be what the ponderosa pine requires for multiple bud production to occur.

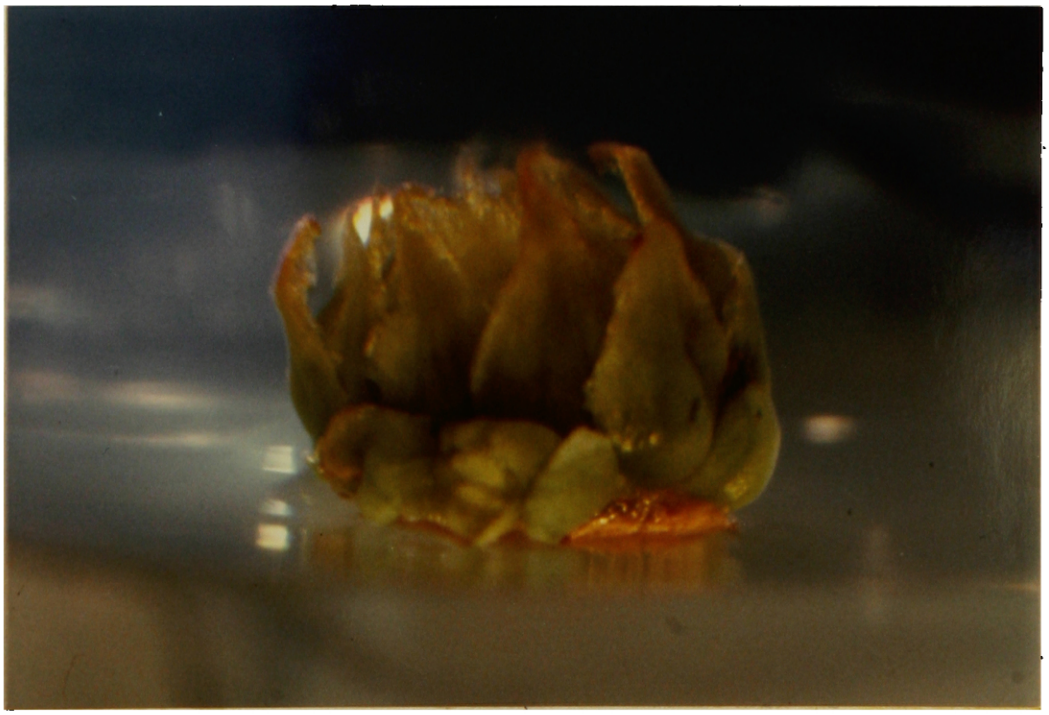
During the summer about 181 apices were dissected from the small residual buds and placed on the usual BA media. Needle fascicles and pieces of young elongating needles were also cultured. The apices grew until they reached a certain size, usually 2 to 3 times the original, then they died. One apex on 1.125 mg BA full SH medium grew rapidly and began forming a whole new bud. Within two weeks in culture, it had doubled in size (Figure 11), and within a month it was beginning to produce green bud scales (Figure 12). One month after that the apex looked like a short but normal ponderosa vegetative bud but unfortunately became contaminated. Some needle pieces and fascicles remained green for a while in culture but did not grow; others died immediately. If fascicle sheaths were not removed the needle tissue was not sufficiently surface sterilized and contamination resulted.

Contamination was a significant problem when working with ponderosa needles and whole buds. Buds seemed to carry endogenous spores between or on the fascicle primordia and spores also lodged between needles and in fascicle sheaths. Young green ponderosa tissue was highly intolerant of Clorox treatments (for surface sterlization) and death occurred even when the tissue was treated with only 3 1/2% Clorox. Therefore, a second sterilization of vegetative buds after the bud scales were removed was not possible; it killed the tissue and did not prevent contamination. A more effective method of surface sterilizing ponderosa tissue is required before any extensive use of ponderosa tissue *in vitro* can occur.

Figure 11. Ponderosa pine apex after 2 weeks on 1.125 mg BA/l SH medium.

Figure 12. The same ponderosa apex after 1 month on 1.125 mg BA/l SH medium.





CONCLUSIONS AND SUMMARY

Multiple bud formation was induced from western larch buds and stems; BA was required for this to occur. More buds formed on the three lower BA concentrations than on the two higher concentration. The buds formed year round from available tissues but they formed 3 to 4 times quicker during the summer than the winter. Shoot elongation was induced on a half strength, no hormone SH medium. Various other hormones were tried in the media: GA killed larch buds while both IAA and IBA stimulated elongation of needles and stems and callus formation. Callus formation increased when BA + auxins were included in the medium. Ponderosa pine tissue did not form multiple buds in culture, it elongated and enlarged, then died. However, with the correct combination of hormones, nutrients and timing, multiple buds could probably be induced on ponderosa tissue. Single needles and needle pieces from both species did not survive in culture.

APPENDIX

Friedmans Statistic

Friedmans formula for randomized block 2-way ANOVA:

$$X^2 = \{12/ab(a+1)\sum^{a}(\sum^{b}R_{ij})^2\} - 3b(a+1)$$

Where a = number of rows and b = number of blocks

Data used : Percent forming buds

mg BA	Jan	Mar	Apr	June	Jul1	Jul2	Aug1	Aug2	Aug3	Sept	Oct	Dec
0.00	00	00	00	00	00	00	00	00	00	00	00	00
0.225	00	05	00	25	21	33	00	00	04	06	06	15
1.125	00	00	10	25	36	43	00	00	00	06	06	10
2.25	00	05	15	25	00	14	11	00	04	09	00	05
11.25	00	00	05	00	21	00	00	00	04	00	00	00
22.5	00	05	00	00	14	00	00	13	00	00	06	00

LITERATURE CITED

Aitken, J., K.J.Horgan, and T.A.Thorpe. 1981. Influence of explant selection on the shoot-forming capacity of juvenile tissue of Pinus radiata. Canadian Journal For. Res. 11:112-117

Al-Talib, K.H. and J.G.Torrey. 1959. The aseptic culture of isolated buds of Pseudotsuga taxifolia. Plant Physiology 34:630-637

Anderson, B. 1983. Tissue culture of Araucaria bernieri. Unpublished. 15pp. Redwood Institute, University of California, Santa Cruz

Ball, E.A., D.M.Morris, and J.A.Rydelius. 1978. Cloning of Sequoia sempervirens from mature trees through tissue culture. Table Ronde-Multiplication "in vitro" d'Especes Ligneuses Gembloux, Belgique 6-8 juin, pp181-226.

Ball, E. 1950. Differentiation in a callus of Sequoia sempervirens. Growth 14:295-325

Biondi, S. and T.A.Thorpe. 1982. Growth regulator effects, metabolite changes and respiration during shoot initiation in cultured cotyledon explants of Pinus radiata. Bot. Gaz. 143(1):20-25

Bonga, J.M. and A.H.McInnis. 1983. Origin and early development of roots in plantlets derived from embryo sections of Larix laricilna in vitro. Can For. Ser. Bimonthly Res. Notes. 3(2);12-14

Bonga, J.M. 1982. Shoot formation in callus from the stalks of young female strobili of Larix decidua. Can. J. Bot. 60:1357-1359

Bonga, J.M. 1981. Organogenesis in vitro of tissues from mature conifers. In Vitro 17(6):511-518

Bonga, J.M. 1977. Organogenesis in in vitro cultures of embryonic shoots of Abies balsamea (balsam fir). In Vitro 13(1):41-48

Bonga, J.M. 1974. Vegetative propagation: tissue and organ culture as an alternative to rooting cuttings. N.Z.J.For.Sci. 4(2):253-260

Borchert, R. 1967. Cultivation of Cupressus lusitanica in vitro. Physiol. Plant. 20:608-616

Bornman, C.H. 1983. Possibilities and constraints in the regeneration of trees from cotyledonary needles of Picea abies in vitro. Physiol. Plant. 57:5-16

Bornman, C.H. and E.Jansson. 1980. Organogenesis in cultured Pinus sylvestris tissue. Z. Pflanzenphysiol. 96:1-6

Brown, C.L. and H.E.Sommer. 1977. Bud and root differentiation in

conifer cultures. Tappi 60(6):72-73

Brown, C.L. and R.H.Lawrence. 1968. Culture of pine callus on a defined medium. For. Sci. 14:62-64

Campbell, R.A. and D.J.Durzan. 1976. Vegetative propagation of Picea glauca by tissue culture. Can. J. For. Res. 6:240-243

Campbell, R.A. and D.J.Durzan. 1975. Induction of multiple buds and needles in tissue cultures of Picea glauca. Can. J. Bot. 53:1652-1657

Chalupa, V., D.J.Durzan, and C.Vithayasai. 1976. Growth and metabolism of cells and tissue of jack pine (Pinus banksiana). 2. The quantitative analysis of the growth of callus from hypocotyls and radicles. Can. J. Bot. 54:446-455

Chalupa, V. and D.J.Durzan. 1973. Growth and development of resting buds in vitro. Can. J. For. Res. 3:196-208

Cheng, T-Y. 1977. Factors effecting adventitious bud formation of cotyledon culture of Douglas-fir. Plant Science Letters. 9:179-187

Cheng, T-Y. and T.H.Voqui. 1977. Regeneration of Douglas-fir plantlets through tissue culture. Science 198:306-307

Cheng, T.Y. 1976. Vegetative propagation of western hemlock (Tsuga heterophylla) through tissue culture. Plant and Cell Physiology. 17:1347-1350

Cheng, T.Y. 1975. Adventitious bud formation in culture of Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco). Plant Science Letters 5:97-102

Coleman, W.K. and T.A.Thorpe. 1977. In vitro culture of western redcedar (Thuja plicata Donn.). I. Plantlet formation. Bot. Gaz. 138(3):298-304

Coleman, W.K. and T.A.Thorpe. 1976. Induction of buds in tissue culture of four different conifers. Plant Physiology 57(supplement):67

Conger,B.V. ed. 1981. Cloning agricultural plants via in vitro techniques. CRC. Press, Inc. Boca Raton, Florida. pp218-254

Copes, D.L. 1971. Interstock trials with grafted coastal Douglas-fir. USDA Forest Service Research Note PNW-151

David,A., H.David, and T.Mateille. 1982 In vitro adventitious budding on Pinus pinaster cotyledons and needles. Physiol. Plant.

56:102-107

David,H., K.Isemukali and A.David. 1978. Obtention de plants de Pin maritime (Pinus pinaster Sol.) a partir de brachyblastes on d'apex caulinaires de tres jeunes sujets cultives in vitro. C.R.Acad.Sc.Paris. Serie D 287:245-248

Durzan, D.J. and V.Chalupa. 1976. Growth and metabolism of cells and tissue of jack pine (Pinus banksiana). 3.Growth of cells in liquid suspension cultures in light and darkness. Can. J. Bot. 54:456-467

Ellis, D.D. and D.E.Bilderback. 1984. Multiple bud formation by cultured embryos of Pinus ponderosa. J. Plant Physiol. 115:201-204

Franclet, A., A.David, H.David and M.Boulay. 1980. Premiere mise en evidence morphologique d'un rajeunissement de meristemes primaires caulinaires de Pin maritime age (Pinus pinaster Sol.). C.R.Acad.Sc.Paris. Serie D 290:927-930

Greenwood, M.S. and G.P.Berlyn. 1973. Sucrose - indole-3-acetic acid interactions on root regeneration by Pinus lambertiana embryo cuttings. Amer. J. Bot. 60(1):42-47

Harvey, A.E. and J.L.Grasham. 1969. Procedures and media for obtainilng tissue cultures of 12 conifer species. Can. J. Bot. 47:547-549

Harvey, A.E. 1967. Tissue culture of Pinus monticola on a chemically defined medium. Can. J. Bot. 45:1783-1787

Horgan, K.(nee Reilly) and J.Aitken. 1981. Reliable plantlet formation from embryos and seedling shoot tips of radiata pine. Physiol. Plant. 53:170-175

Hussey, G. 1978. The application of tissue culture to the vegetative propagation of plants. Sci. Prog., Oxford. 65:185-208

Isikawa, H. 1974. In vitro formation of adventitious buds and roots on the hypocotyl of Cryptomeria japonica. Bot. Mag. Tokoyo 87:73-77

Jansson, E. and C.H.Bornman. 1983. Morphogenesis in dormant embryonic shoots of Picea abies: Influence of the crown and cold treatment. Physiol. Plant. 59:1-8

Jansson, E. and C.H.Bornman. 1980. In vitro phyllomorphic regeneration of shoot buds and shoots in Picea abies. Physiol. Plant. 49: 105-111

Karnosky, D.F. 1981. Potential for forest tree improvement via tissue

culture. Bioscience 31(2):114-120

Kolevska-Pletikapic, B., S.Jelaska, J.Berljak and M.Vidakovic. 1983. Bud and shoot formation in juvenile tissue culture of Pinus nigra. Silvae Genetica 32(3-4):115-119

Konar, R.N. and M.N.Singh. 1980. Induction of shoot buds from tissue cultures of Pinus wallichiana. Z. Pflanzenphysiol. 99:173-177

Konar, R.N. 1975. In vitro studies on Pinus. II. The growth and morphogenesis of cell cultures from Pinus gerardiana. Phytomorphology 25:55-59

Konar, R.N. and R.Nagmani. 1974. Tissue culture as a method for vegetative propagation of forest trees. N.Z.J.For.Sci. 4(2):279-290

Konar, R.N. and V.P.Oberoi. 1965. In vitro development of embroids on the cotyledons of Biota orientalis. Phytomorphology 15:137-140

Manandhar, A. and P.M.Gresshof. 1980. Blue spruce (Picea pungens) tissue and cell culture. Cytobios 29:175-182

McKeand, S.E. and R.J.Weir. 1984. Tissue culture and forest productivity. J. For. April:212-218

Mehra, P.N. and M.Anand. 1983. Callus of Pinus roxburghii (chir pine) and its cytology. Physiol. Plant. 58:282-286

Mehra-Palta, A., R.H.Smeltzer, and R.L.Mott. 1978. Hormonal control of induced organogenesis. Experiments with excised plant parts of loblolly pine. Tappi 61(1):37-40

Minocha, S.C. 1980. Callus and adventitious shoot formation in excised embryos of white pine (Pinus strobus). Can. J. Bot. 58:366-370

Misson, J.P., M.Coumans, P.Giot-Wirgot and T.H.Gaspar. 1982. Induction of adventitious buds on buds of Picea pungens grown in vitro. Z. Pflanzenphysiol. 107:161-167

Momot, T.S. 1981. Differentiation of Pinceana pinyon pine (Pinus pithyusa Stev.) plants in culture in vitro. Tsilol. Genet. 15(6):54-57

Momot, T.S. and A.M.Smirnov. 1978. Organogenesis from various organs of the Siberian and Dahur larch (Larix sibericaMaxim. and L. dahurica Turcz.)and yellow pine(Pinus ponderosa Dougl.) cultured in vitro. Izv. Acad. Nauk. SSSR Ser.B. 5(6):758-761 (trans)

Mott, R.L. and H.V.Amerson. 1981. Tissue culture plantlets produced from Pinus monticola embryonic materials. For. Sci. 27(2):299-304

Mott, R.L. and H.V.Amerson. 1981. A tissue culture process for the clonal production of loblolly pine plantlets. N.C.Agric.Res.Serv.Tech.Bull. No. 271. 14pp.

Patel, K.R. and T.A.Thorpe. 1984. In vitro differentiation of plantlets from embryonic explants of lodgepole pine (Pinus contorta Dougl. ex Loud.). Plant Cell Tissue Organ Culture 3:131-142

Rancillac, M. 1981. Perspectives d'application des cultures d'organes in vitro a la multiplication vegetative du Pin maritime Pinus pinaster Sol. Ann. Sci. Forest. 38(1):55-70

Reilly, K. and J.Washer. 1977. Vegetative propagation of radiata pine by tissue culture: plantlet formation from embryonic tissue. N.Z.J.For.Sci. 7(2):199-206

Reilly, K. and C.L.Brown. 1976. In vitro studies of bud and shoot formation in Pinus radiata and Pseudotsuga menziesii. Georgia Forest Research Paper 86. Georgia Research Council. 9pp.

Romberger, J.A. and C.A.Tabor. 1975. The Picea abies shoot apical meristem in culture. II. Deposition of polysaccharides and lignin-like substances beneath cultures. Amer. J. Bot. 62(6):610-617

Romberger, J.A. and C.A.Tabor. 1971. The Picea abies shoot apical meristem in culture. I. Agar and autoclaving effects. Amer. J. Bot. 58(2):131-140

Romberger, J.A., R.J.Varnell, and C.A.Tabor. 1970. Culture of apical meristems and embryonic shoots of Picea abies - Approach and techniques. USDA For. Ser. Tech. Bull. 1409 30pp.

Rumary, C. and T.A.Thorpe. 1984. Plantlet formation in black and white spruce. 1. In vitro techniques. Can. J. For. Res. 14:10-16

Skripachenko, V.V. 1982. In vitro cultivation of tissues from seedlings of three species of pine. Soviet Plant Physiology 29:162-167

Sommer, H.E., C.L.Brown, and P.P.Kormanik. 1975. Differentiation of plantlets in longleaf pine (Pinus palustris Mill.) tissue cultured in vitro. Bot. Gaz. 136:196-200

Steinhart, C.E., L.C.Standifer, Jr., and F.Skoog. 1961. Nutrient requirements for in vitro growth of spruce tissue. Amer. J. Bot. 48(6):465-472

Thomas, M.J. and H.Tranvan. 1982. Influence relataive de la BAP et de l'IBA sur la neoformation de bourgeons et de racines sur les plantules du Biota orientalis (Cupressacees). Physiol. Plant.

56:118-122

Thomas, M.J., E.Duhoux and J.Vazart. 1977. In vitro organ initiation in tissue cultures of Biota orientalis and other species of the Cupressaceae. Plant Science Letters 8:395-400

Thompson, D.G. and J.B.Zaerr. 1981. Induction of adventitious buds on cultured shoot tips of Douglas-fir (Pseudotsuga menziesii(Mirb.)Franco). From:Colloque international sur la culture "in vitro" des Essences Forestieres.

Tranvan, H. 1979. In vitro adventitious bud formation on isolated seedlings of Pinus sylvestris L. Bio. Plant. 21(3):230-233

Von Arnold, S. 1982. Factors influencing formation, development and rooting of adventitious shoots from embryos of Picea abies(L.)Karst. Plant Science Letters 27:275-287

Von Arnold, S. and T.Eriksson. 1981. In vitro studies of adventitious shoot formation in Pinus contorta. Can. J. Bot. 59:870-874

Von Arnold, S. and T.Eriksson. 1979. Induction of adventitious buds on buds of Norway spruce (Picea abies) grown in vitro. Physiol. Plant. 45:29-34

Von Arnold, S. and T.Eriksson. 1979. Bud induction on isolated needles of Norway spruce (Picea abies L. Karst) grown in vitro. Plant Science Letters 15:363-372

Washer, J., K.J.Reilly, T.R.Barnett. 1977. Differentiation in Pinus radiata callus culture: the effect of nutrients. N.Z.J.For.Sci. 7(3):321-328

Weatherhead, M.A., J.Burdon, and G.G.Henshaw. 1979. Effects of activated charcoal as an additive to plant tissue culture media: Part 2. Z. Pflanzenphysiol. 94:399-405

Weatherhead, M.A., J.Burdon, and G.G.Henshaw. 1978. Some effects of activated charcoal as an additive to plant tissue culture media. Z. Pflanzenphysiol. 89:141-147

Webb, K.J. and H.E.Street. 1977. Morphogenesis in vitro of Pinus and Picea. Acta. Hort. 78:259-269

Winton, L.L. and S.A.Verhagen. 1977. Shoots from Douglas-fir cultures. Can. J. Bot. 55:1246-1250

Winton, L.L. 1972. Callus and cell cultures of Douglas-fir. For. Sci. 18:151-154

Wochok, Z.S. and M.Abo-El-Nil. 1977. Conifer tissue culture. Int.

Plant Prop.Soc.Comb.Proc. 27:131-136