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

Emily E. Chesick

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Multiple bud formation in *vitro* on *Larix occidentalis* and

*Pinus ponderosa* buds, stems and needles.

by

Emily E. Chesick

B.A., Carleton College, 1981

Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

UNIVERSITY OF MONTANA

1985

Approved by:

[Signatures]

Chairman, Board of Examiners

[Signature]

Dean, Graduate School

[Signature]

Date

May 23, 1985
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/1 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.
ACKNOWLEDGEMENTS

This research was supported by a McIntire-Stennis grant at the University of Montana. The growth chambers were loaned by the USDA Forest Service at Moscow Idaho.

Special thanks go to the members of my committee: Dr. George Blake for unfailing support and encouragement; Dr. David Bilderback for his technical help and knowledge, and the use of his lab; and Dr. Rustem Medora for freely giving of advice and time.

David Ellis has been invaluable for his advice and knowledge. Thanks also go to Bruce Anderson, computer statistics god, for advice and amusement, and to Elizabeth Easterling for friendship and moral support throughout the duration of the project. Thanks to my cat, Carob, for my sanity.
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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 occurs on grafted trees. Also tree and clone banks may be produced using grafting. However, incompatibilities 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 incompatibilities;
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 incompatibility, 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.

<table>
<thead>
<tr>
<th>Species name</th>
<th>Author</th>
<th>Tissue used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abies grandis</td>
<td>Harvey and Grasham 1969</td>
<td>cortex from</td>
</tr>
<tr>
<td>Larix occidentalis</td>
<td></td>
<td>3-7 year</td>
</tr>
<tr>
<td>Picea abies</td>
<td></td>
<td>old seedlings</td>
</tr>
<tr>
<td>Picea engelmannii</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Picea pungens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pinus albicaulis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pinus contorta</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pinus flexilis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pinus nigra</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thuja plicata</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cupressus lusitanica</td>
<td>Borchert 1967</td>
<td>wood cambium</td>
</tr>
<tr>
<td>Picea spp.</td>
<td>Steinhart et al. 1961</td>
<td>tissue</td>
</tr>
<tr>
<td>Pinus banksiana</td>
<td>Chalupa et al. 1976</td>
<td>hypocotyls</td>
</tr>
<tr>
<td></td>
<td>Durzan and Chalupa 1976</td>
<td></td>
</tr>
<tr>
<td>Pinus monticola</td>
<td>Harvey 1967</td>
<td>stem sections</td>
</tr>
<tr>
<td>Pinus palustris</td>
<td>Brown and Lawrence 1968</td>
<td>hypocotyls</td>
</tr>
<tr>
<td>Pinus radiata</td>
<td>Washer et al. 1977</td>
<td>tissue</td>
</tr>
<tr>
<td>Pinus roxburghii</td>
<td>Mehra and Anand 1983</td>
<td>seedlings</td>
</tr>
<tr>
<td>Pinus strobus</td>
<td>Minocha 1980</td>
<td>embryos</td>
</tr>
<tr>
<td>Pseudotsuga menziesii</td>
<td>Al-Talib and Torrey 1959</td>
<td>buds</td>
</tr>
<tr>
<td></td>
<td>Winton 1972</td>
<td>needles and stems</td>
</tr>
<tr>
<td>Sequoia sempervirens</td>
<td>Ball 1950</td>
<td>burl shoots</td>
</tr>
</tbody>
</table>
Table 2

Conifer species producing whole plantlets from embryo portions

<table>
<thead>
<tr>
<th>Species name</th>
<th>Author</th>
<th>Year</th>
<th>Tissue used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryptomeria japonica</td>
<td>Isikawa</td>
<td>1974</td>
<td>hypocotyls</td>
</tr>
<tr>
<td>Larix laricina</td>
<td>Bonga and McInnis</td>
<td>1983</td>
<td>embryo sections</td>
</tr>
<tr>
<td>Larix occidentalis</td>
<td>Ellis (unpublished)</td>
<td>1984</td>
<td>cotyledons</td>
</tr>
<tr>
<td>Picea abies</td>
<td>Von Arnold</td>
<td>1982</td>
<td>cotyledons</td>
</tr>
<tr>
<td></td>
<td>Bornman</td>
<td>1983</td>
<td>and embryos</td>
</tr>
<tr>
<td>Picea glauca</td>
<td>Campbell and Durzan</td>
<td>1975</td>
<td>cotyledons</td>
</tr>
<tr>
<td></td>
<td>Rumary and Thorpe</td>
<td>1984</td>
<td>and epicotyls</td>
</tr>
<tr>
<td>Picea mariana</td>
<td>Rumary and Thorpe</td>
<td>1984</td>
<td>cotyledons</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>and epicotyls</td>
</tr>
<tr>
<td>Pinus contorta</td>
<td>Von Arnold and Eriksson</td>
<td>1981</td>
<td>hypocotyls, cotyledons,</td>
</tr>
<tr>
<td></td>
<td>Patel and Thorpe</td>
<td>1984</td>
<td>embryos and shoot tips</td>
</tr>
<tr>
<td>Pinus eldarica</td>
<td>Momot</td>
<td>1981</td>
<td>cotyledons</td>
</tr>
<tr>
<td>Pinus gerardiana</td>
<td>Konar</td>
<td>1975</td>
<td>embryos</td>
</tr>
<tr>
<td>Pinus monticola</td>
<td>Mott and Amerson</td>
<td>1981</td>
<td>embryos</td>
</tr>
<tr>
<td>Pinus palustris</td>
<td>Sommer et al.</td>
<td>1975</td>
<td>embryos</td>
</tr>
<tr>
<td>Pinus pinaster</td>
<td>Rancillac</td>
<td>1981</td>
<td>shoot tips, and embryos</td>
</tr>
<tr>
<td>Pinus pithyusa</td>
<td>Momot</td>
<td>1981</td>
<td>cotyledons</td>
</tr>
<tr>
<td>Pinus ponderosa</td>
<td>Ellis (unpublished)</td>
<td>1984</td>
<td>cotyledons</td>
</tr>
<tr>
<td>Pinus taeda</td>
<td>Mehra-Palta et al.</td>
<td>1978</td>
<td>cotyledons</td>
</tr>
<tr>
<td></td>
<td>Mott and Amerson</td>
<td>1981</td>
<td>cotyledons</td>
</tr>
<tr>
<td>Pseudotsuga menziesii</td>
<td>Cheng and Voqui</td>
<td>1977</td>
<td>cotyledons</td>
</tr>
<tr>
<td>Thuja plicata</td>
<td>Coleman and Thorpe</td>
<td>1976</td>
<td>cotyledons</td>
</tr>
<tr>
<td>Tsuga heterophylla</td>
<td>Cheng</td>
<td>1976</td>
<td>cotyledons</td>
</tr>
</tbody>
</table>
### Table 3

Conifer species producing multiple buds on embryonic tissue in culture.

<table>
<thead>
<tr>
<th>Species name</th>
<th>Author</th>
<th>Tissue used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biota orientalis and other Cupressaceae</td>
<td>Thomas et al. 1977</td>
<td>hypocotyls,</td>
</tr>
<tr>
<td>Larix dahurica</td>
<td>Thomas and Tranvan 1982</td>
<td>cotyledons and</td>
</tr>
<tr>
<td>Larix siberica</td>
<td>Konar and Oberoi 1965</td>
<td>epicotyls</td>
</tr>
<tr>
<td>Pinus echinata</td>
<td>Momot and Smirnov 1978</td>
<td>embryos and</td>
</tr>
<tr>
<td>Pinus elliottii</td>
<td>Brown and Sommer 1977</td>
<td>cotyledons</td>
</tr>
<tr>
<td>Pinus rigida</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pinus sabiniana</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pinus virginiana</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pinus nigra</td>
<td>Kolevska-Pletibapic et al. 1983</td>
<td>embryos</td>
</tr>
<tr>
<td>Pinus pinaster</td>
<td>David et al. 1982</td>
<td>cotyledons</td>
</tr>
<tr>
<td>Pinus ponderosa</td>
<td>Momot and Smirnov 1978</td>
<td>embryos and</td>
</tr>
<tr>
<td>Pinus ponderosa</td>
<td>Ellis and Bilderback 1984</td>
<td>cotyledons</td>
</tr>
<tr>
<td>Pinus pumila</td>
<td>Skripachencho 1982</td>
<td>embryos</td>
</tr>
<tr>
<td>Pinus strobus</td>
<td>Minocha 1980</td>
<td>embryos</td>
</tr>
<tr>
<td>Pinus sylvestris</td>
<td>Tranvan 1979</td>
<td>embryos and</td>
</tr>
<tr>
<td>Pinus taeda</td>
<td>Bornman and Jansson 1980</td>
<td>seedlings</td>
</tr>
<tr>
<td>Pinus wallichiana</td>
<td>Skripachenko 1982</td>
<td></td>
</tr>
<tr>
<td>Pseudotsuga menziesii</td>
<td>Cheng 1976</td>
<td>cotyledons and</td>
</tr>
<tr>
<td>Sequoia gigantea</td>
<td>Konar and Singh 1980</td>
<td>hypocotyls and</td>
</tr>
<tr>
<td>Cupressus arizonica</td>
<td>Coleman and Thorpe 1976</td>
<td>cotyledons and</td>
</tr>
</tbody>
</table>
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* $5 \times 10^{-6}$ M BA + $5 \times 10^{-9}$ 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 Voquil 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).

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.

<table>
<thead>
<tr>
<th>Species name</th>
<th>Author</th>
<th>Tissue used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abies balsamea</td>
<td>Bonga</td>
<td>buds from 15-20 year old trees</td>
</tr>
<tr>
<td>Araucaria bernieri</td>
<td>Anderson</td>
<td>shoots</td>
</tr>
<tr>
<td>Larix dahurica</td>
<td>Momot and Smirnov</td>
<td>stem apices and needle tufts</td>
</tr>
<tr>
<td>Larix decidua</td>
<td>Bonga</td>
<td>immature female strobili stalks</td>
</tr>
<tr>
<td>Larix siberica</td>
<td>Momot and Smirnov</td>
<td>stem apices and needle tufts</td>
</tr>
<tr>
<td>Picea abies</td>
<td>Jansson and Bornman</td>
<td>buds from 10 yr. old trees</td>
</tr>
<tr>
<td></td>
<td>Jansson and Bornman</td>
<td>trees, lateral and terminal shoot from 1 yr old seedlings</td>
</tr>
<tr>
<td></td>
<td>Von Arnold and Eriksson</td>
<td>trees 5 - 10 yrs old, new needles on 5 yr old trees</td>
</tr>
<tr>
<td></td>
<td>Romberger et al.</td>
<td>buds from 10 yr. old trees</td>
</tr>
<tr>
<td>Picea glauca</td>
<td>Bonga</td>
<td>buds</td>
</tr>
<tr>
<td>Picea pungens</td>
<td>Manandhar and Gresshof</td>
<td>leaves, stems, buds</td>
</tr>
<tr>
<td></td>
<td>Missen et al.</td>
<td>buds</td>
</tr>
<tr>
<td>Picea sitchens</td>
<td>Webb and Street</td>
<td>shoot meristems from 2 year old trees</td>
</tr>
<tr>
<td>Pinus pinaster</td>
<td>David et al.</td>
<td>short shoots and elongating needles from 10 year old trees</td>
</tr>
<tr>
<td>Pinus sylvestris</td>
<td>Bornman and Jansson</td>
<td>needle fascicles</td>
</tr>
<tr>
<td>Pinus taeda</td>
<td>Mehra-Palta et al.</td>
<td>needle fascicles (5 yr, 8 mo. old trees)</td>
</tr>
<tr>
<td>Pseudotsuga menziesii</td>
<td>Thompson and Zaerr</td>
<td>buds</td>
</tr>
<tr>
<td></td>
<td>Bonga</td>
<td>1981</td>
</tr>
</tbody>
</table>
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* initially. 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 $5 \times 10^{-5}$ 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 KNO₃, 0.4g MgSO₄·7H₂O, 0.3g NH₄H₂PO₄, 0.2g CaCl₂·2H₂O, 10 mg MnSO₄·H₂O, 5 mg H₃BO₃, 1 mg ZnSO₄·7H₂O, 1 mg KI, 0.2 mg CuSO₄·5H₂O, 0.1 mg NaMoO₄·2H₂O, 0.1 mg CoCl₂·6H₂O, 15mg
FeSO₄·7H₂O, 20 mg Na₂EDTA, 100 mg Myoinositol, 5 mg Thiamine-HCl, 0.5 mg
Nicotinic acid, 0.5 mg Pyridoxin-HCl, 30 g 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 (2.25 mg/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, GA₄₇ (76+24; 76 parts GA₄ plus 24 parts GA₇), 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°C, a 24 hour photoperiod, and a light intensity of 3 watts/m². Humidity within the chambers was maintained by tubs of water on the bottom of the chambers.
Statistical Analysis

Friedman's 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/1) 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/1 SH medium.
Figure 3. Western larch bud after 3 months on 2.25 mg BA/1 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/1 SH medium.

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

Figure 12. The same ponderosa apex after 1 month on 1.125 mg BA/1 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:

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

Where \( a \) = number of rows and \( b \) = number of blocks

Data used: Percent forming buds

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