MICROPROPAGATION OF YELLOW CYPRESS (*CHAMAECYPARIS NOOTKATENSIS*) BY ADVENTITIOUS AND AXILLARY SHOOT MULTIPLICATION

By

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MICROPROPAGATION OF YELLOW CYPRESS (CHAMAECYPARIS NOOTKATENSIS) BY ADVENTITIOUS AND AXILLARY SHOOT MULTIPLICATION

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ABSTRACT

The goal of this study was to develop a micropropagation system for yellow cypress (*Chamaecyparis nootkatensis*) [D.Don] Spach).

Excised mature embryos of yellow cypress were induced to form multiple adventitious shoots when cultured on defined media containing a cytokinin. Among various media evaluated, Schenk and Hildebrandt's medium containing 0.35 mgl^{-1} BA was found to be optimum for shoot induction. Because the optimal BA level was so low on the original Schenk and Hildebrandt formulation containing 1000 mgl^{-1} inositol, the caulogenic effect of 100 mgl^{-1} inositol was compared with 1000 mgl^{-1}. Little difference in the caulogenic response at the two inositol concentrations was observed. Yellow cypress seeds require a long, complex stratification to germinate, therefore the effects of stratification on caulogenesis were studied. Embryos stratified for four weeks at 21°C and eight weeks at 5°C were more caulogenic than unstratified controls. Shoots elongated on basal Schenk and Hildebrandt medium. Approximately 15% of the elongated shoots rooted *in vitro* without the addition of auxin. The best rooting response was obtained under non-sterile greenhouse conditions where 60% rooting was achieved.

Two-year old yellow cypress seedlings (#9777) were the source plants for the axillary shoot multiplication portion of this study. The juvenile explant harvested from the basal

region of the stock plant performed the best *in vitro*. Size of the explant was also important. An overall explant length of 1.5-2.0 cm was the best for axillary shoot induction. The effects of media composition on axillary shoot formation were also tested. Of the three media evaluated, MSK was superior.

Four different explant types the intact, tip, decapitated and lateral were used to test the effects of BA applied both basally through agar-solidified medium and as a liquid pulse to the entire explant. None of the basal applications of BA tested were stimulatory. Those explants receiving a basal medium pulse did better than those that did not. Pulsing with BA [250 uM] had a slight stimulatory effect on only the \`intact´ explant. No apparent trend was evident with any of the explant types for the different pulsing periods tested. Decapitation had a negative effect on the stimulation of axillary shoots.

The auxins IBA and NAA, when applied basally through the agar-solidified medium or via a pulse treatment did little to induce axillary shoot development.

Micropropagules did elongate on MSK basal medium. Those greater than 5 mm were harvested for rooting trials. A 15% frequency of spontaneous rooting was achieved.

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INTRODUCTION

Forestry is Canada's major industry and our timber harvest is the third largest in the world. Canada is the leading exporter of manufactured forest products including newsprint, lumber and pulp. The forest industry has consistently been the major contributor to our trade balance. In 1986, exports exceeded imports by $15.8 billion. Over half of merchantable timber in Canada grows in British Columbia (B.C.) which has 53,015,000 ha of forest land and a current annual harvest of more than 90 million m^3. Forests are this province's and the nation's most valuable resource, generating half of every dollar earned in B.C. and two-thirds of every dollar of foreign exchange. This translates into almost half of every dollar of foreign exchange earned from the export of forest products in all of Canada (Council of Forest Industries, 1986).

In 1986, the value of shipments from B.C. wood industries was $5.5 billion and $3.9 billion from paper and allied industries. Together this accounted for about 45% of the total value of shipments from the manufacturing industries.

In 1986, forest-based industries directly employed about 7% of B.C.'s workers. Many of these were employed in the silviculture program which will produce 200 million planted seedlings in 1988 (J. Russell[1], personal communication). This

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1. John Russell, Research Officer, Cowichan Lake Research Station, Ministry of Forests, Mesachie Lake, B.C.

will reforest about 60% of the areas harvested. The remaining 40% will be restocked through natural regeneration.

The forest industry has attained world class status because there has been an abundant and diverse native forest and because of efficient harvesting. To maintain its major economic contribution to Canada, the forest industry must embrace the achievements of the many new technologies that are evolving, including biotechnology.

Biotechnology is generally viewed as the use of a biological process through which plant or animal cells or their constituents are used to supply critical monogenic or allelogenic traits that cannot physically or economically be introduced by conventional breeding (Durzan, 1985). When researchers talk about forest biotechnology, what they usually mean is tissue culture. The use of tissue culture, which in all its technological and procedural forms includes cell, tissue, embryo and organ culture, holds the greatest promise for forest improvement (Hasnain and Cheliak, 1986).

Biotechnology can potentially have an impact on almost all facets of forest-based industries. This can be achieved by ensuring that this novel technology develops with, and builds upon, the many sound initiatives already underway.

Present-day forestry research is focused on improving and protecting the resource. To improve the resource, classical tree improvement programs direct their efforts towards selecting for increased growth rates, disease resistance, and

improved wood quality for lumber and paper products. Cloning of trees by vegetative propagation can play a significant role in forestry as illustrated by *Cryptomeria* for which cuttings have been used for centuries in Japan (Isikawa, 1987; Toda, 1974). Other examples include *Picea mariana* in Canada (Amerson et al., 1980; Hasnain et al., 1986) and *Picea abies* in Germany (Kleinschmit, 1974), Finland (Lepisto, 1974) and Scandinavia (Bornman, 1987). Vegetative propagation promises to be the basis of a revolution in tree improvement with important effects on forest policy, forest harvesting, silviculture and wood utilization. Cloning allows for the utilization of both the additive and non-additive genetic effects within a population (McKeand and Weir, 1984). This is true in the case of the additive portion where the selection increases the frequency of favorable genes. With the non-additive portion, good specific gene combinations are replicated. However, cloning methods such as tissue culture can capture the maximum genetic gain achievable for individuals in a family (Hasnain and Cheliak, 1986). It has been estimated that the potential for improving forests by exploiting the non-additive genetic gain can be as high as 50% (Zobel and Talbert, 1984). One of the major impacts of biotechnology could be to reduce the long periods required for tree improvement using conventional technology, thereby hastening the incorporation of gains from breeding research into operational silviculture programs.

In traditional tree improvement programs, grafting and rooting of cuttings are the preferred methods for cloning superior trees. Grafting can be performed reliably with most species. However, graft incompatibilities along with the high cost per graft have limited its applications. Grafting is used mostly to move select genotypes for breeding orchards or for increasing the number of select genotypes in seed orchards.

By contrast, stecklings (plantable rooted cuttings) can in some cases be produced for costs similar to those of seedlings. Low cost plus the freedom from delayed mortality of grafts mean that stecklings can be used for research requiring high genetic control and for mass propagation.

Biotechnology offers new methods for cloning of plants through cell, tissue and organ culture. Tissue culture technology offers great potential for aiding traditional methods of tree improvement. This is most notable in the area of genotype evaluation with respect to growth rates, cold hardiness, disease resistance and tolerance to drought and chemicals (Karnosky, 1981). Furthermore, by using in vitro techniques, a desired tree selected on the basis of its past performance, may be vegetatively propagated and cloned at a rapid rate (Bajaj, 1986).

Micropropagation, based on shoot multiplication and rooting has been commercially successful with woody ornamentals (McCowan, 1986). Major micropropagation programs

with *Pinus radiata*, *Pinus taeda*, *Pinus pinaster*, *Pseudotsuga menziesii*, *Sequoiadendron* sp., and *Sequoia* sp., are underway in New Zealand, France and United States (Bonga and Durzan, 1987). Several of these are reaching commercial status (Boulay, 1987).

With an increase in high elevation logging in coastal B.C. there has been greater attention focused on the use of yellow cypress (*Chamaecyparis nootkatensis* [D. Don] Spach) for reforestation. Yellow cypress belongs to the family Cupressaceae which contains a number of other important forest species. These include *Thuja plicata* which produces a valuable timber resistant to insect and fungal attack and is used ideally for roof shakes, buildings and greenhouses; *Thuja occidentalis* used for fencing, poles and roof shingles (Hosie, 1979); *Juniperus polycarpus* which is primarily used for pencil cedar wood and for the production of juniper oil (Ilahi, 1986). In the genus *Chamaecyparis*, two species, *Chamaecyparis nootkatensis* and *Chamaecyparis lawsoniana*, are endemic to the Pacific Northwest (Elias, 1980). A third, *Chamaecyparis obtusa*, is indigenous to Japan (Ishii, 1986). All three species are economically important in that their yellowish-white wood is lightweight, fine grained, hard and very durable. The wood makes excellent lumber as it is easy to work and takes a fine finish (Elias, 1980).

Chamaecyparis nootkatensis is a medium-sized tree. The foliage is medium green, hanging in arched, pendulous sprays.

The trees are largely free of insect pests and diseases (Furniss and Carolin, 1980). Its ability to grow over a wide altitudinal range and regenerate on poor sites, as well as the high foreign demand placed on its wood products, makes it increasingly important to foresters. Yellow cypress is a highly valuable export crop. It has the highest stumpage [$149/m^3] and average log value [$225/m^3] of all coastal species (G. Silvestrini[1], personal communication). Japan receives 75% of yellow cypress exports which are valued at $1500 US/1000 board feet (G. Silvestrini, personal communication). The total product volume (1984) was around 700,000 m^3 and the prognosis indicates that the market will increase. Consequently, there will be increased demands for propagules. The present annual demand for yellow cypress planting stock is around one million. This would be higher if more propagules were available. (J. Russell[2], personal communication). The projected near-future demand is estimated to be in excess of two million propagules per year (G. Dunsworth[3], personal communication).

Seed production by yellow cypress is extremely poor and does not meet current nursery planting needs. Only a few

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3. G. Dunsworth, Research Silviculturalist, MacMillan Bloedel Ltd., Woodlands Services Division, Nanaimo, B.C.

ovules in each cone are fertilized. Consequently, cones usually contain only two viable seeds (Owens and Molder, 1984). The sexual cycle takes two years and few seed cones are produced by reproductive trees. Further problems arise because it is difficult to distinguish first-year cones from second-year cones and seeds from first-year cones cannot germinate. Mature seeds require a long, complex stratification treatment in order to germinate (Owens and Molder, 1984; G. Edwards[1], personal communication). Even then, germination is still not uniform. Finally, cones in seed orchards have experienced premature opening during the first year which could have severe, adverse effects on seed output. These factors combine to make seeds of yellow cypress far more expensive [$2,485/kg] than all other conifer seed (J. Russell[2], personal communication).

As a result of the severe seed shortages and the propagation problems associated with successful germination, a vegetative program based on yellow cypress cuttings (Karlsson, 1982) has been successfully implemented by the British Columbia Ministry of Forests (BCMOF). This program currently produces all of the yellow cypress planting stock (J. Russell[2], personal communication).

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Economic analysis have shown that micropropagation can compete with seed-based and cuttings-based programs in Canada even with low multiplication rates (Hasnain and Cheliak, 1986; Hasnain et al., 1986). This is especially true when the propagules have high intrinsic value. In the case of yellow cypress, each propagule has a value of approximately $0.20 - 0.30. This will increase for select, genetically-improved seed.

The unique set of biological problems which severely limits the number of seeds available for planting makes yellow cypress an ideal candidate for micropropagation. In addition, tissue culture techniques could supplement the present methods of asexual propagation.

The main goal of this study was to develop a micropropagation system for yellow cypress. Two methodologies were used. They included an axillary shoot multiplication system and the production of adventitious shoots from whole, mature embryos. The role of growth regulators in shoot induction, medium composition and strength, stratification along with rooting studies were evaluated.

Careful selection of the primary explant (the organ or piece of tissue used) is of paramount importance in the success of plantlet formation (Aitken et al., 1981; Murashige, 1974; Sommer and Caldas, 1981). The time of year when the explant is collected, its stage of development or age, or physiological status can all have an affect on its subsequent

response in tissue culture. Where the explant source originates from the seed, the age, storage, imbibition time and temperature, degree of stratification, time and conditions of germination can be important (Sommer and Caldas, 1981). As mentioned earlier, stratification of yellow cypress seed is essential for germination to occur (Owens and Molder, 1984). Therefore, the effects of stratification on shoot formation were examined in this study.

Prior work with *Chamaecyparis obtusa* (Ishii, 1986), *Thuja plicata* (Coleman and Thorpe, 1977), *Cupressus lusitanica* (Franco and Schwarz, 1985), *Biota orientalis* (Thomas et al., 1977), and *Juniperus* sp. (Ilahi, 1986) has shown that embryos are good explants for shoot induction with members of the Cupressaceae. Furthermore, embryos have been the most frequently used explants with all conifers (Tran Thanh Van et al., 1987). Organs from germinating seeds have also been successfully used for conifer organogenesis (Aitken et al., 1981; Patel and Thorpe, 1986; Tran Thanh Van et al., 1987).

Plant material will grow *in vitro* when provided with an appropriate medium. A basal medium consists of a solution of salts supplying the macro- and micro-elements necessary for the growth of whole plants, various vitamins and amino acids, and a carbon energy source. Several standard media including SH (Schenk and Hildebrandt, 1972) and LP (=ACLP) (Aitken-Christie, 1984) were tested for their ability to support shoot proliferation and subsequent growth in the absence of

cytokinin. With *Pinus radiata*, LP medium was successfully used for all stages of shoot induction and growth prior to rooting (Smith, 1986; Horgan, 1987). The purpose was to find the simplest culture conditions which promote rapid shoot multiplication and elongation.

Growth regulators are added in variable micro concentrations to the culture medium. The relative levels of the growth regulators determine the type of differentiation obtained in culture. In general, benzyladenine (BA) induces shoot formation with most conifers (Flinn et al., 1986; Minocha, 1987) and has produced satisfactory results with several other members of the Cuspressaceae (Coleman and Thorpe, 1977; Franco and Schwarz, 1985; Ishii, 1986; Thomas et al., 1977; Thomas and Tranvan, 1982). Consequently, it was used for shoot induction and proliferation experiments. BA was applied in one of two ways. For the embryo culture work BA was incorporated into the agar. In the case of the axillary shoot multiplication system, BA was applied through the culture medium as well as by the liquid pulse method devised for *Picea abies* (Bornman, 1983; von Arnold and Eriksson, 1985). The latter was to insure that BA reached the active sites of axillary shoot production.

Micropropagation of conifers has relied on adventitious shoot production. This has led to concerns regarding the genetic fidelity of the resulting plants (Berlyn et al., 1986). While the production of adventitious shoots was one

method used in this study to micropropagate yellow cypress, techniques for the stimulation of axillary shoots was also examined. This method has been routinely used to propagate agricultural and horticultural crops (Zimmerman et al., 1986) and is the basis for the present yellow cypress cuttings program. With gymnosperms, axillary shoot multiplication systems have been successfully used with _Cryptomeria japonica_ (Isikawa, 1987), _Juniperus_ sp., _Sequoia sempervirens_, _Thuja plicata_, _Tsuga heterophylla_ (Amos and McCowan, 1981) and _Pinus pinaster_ (Rancillac, 1981). All of the propagules formed through an axillary shoot multiplication system are considered to be genetically uniform, as they arise directly from pre-existing or newly-formed lateral apices, without any intervening callus stage. In addition, this type of system has produced proven individuals through the successful propagation of older trees (Brown and Sommer, 1982).

Statistical analysis were performed on all data collected. Two tests were used: difference of proportions test (Chi-square) to compare percent response; one-way Anova analysis used to compare the means. The level of significance employed was $p = 0.05$. To avoid confusion in the tables the non-significant results were not included and as a result some tables showed no statistical notations.

MATERIALS AND METHODS

PLANT MATERIAL

Embryo Culture System

Two seedlots, A and B, of yellow cypress (*Chamaecyparis nootkatensis* [D. Don] Spach) were collected in 1975 and 1981, respectively. Both seedlots were obtained from open pollinated stands. Seedlot A was donated by the Pacific Forestry Centre, Canadian Forestry Service, Victoria, British Columbia, Canada, and Seedlot B (#9777) was a gift from the British Columbia Ministry of Forests. To maintain prior storage regimes, Seedlots A and B were stored at 4°C or -15°C, respectively.

Seeds were routinely hydrated by immersion for 48 hours in a Magenta GA7 culture vessel containing 60 ml of sterile water. Imbibed seeds were surface sterilized in 10% commercial bleach (0.52% w/v NaOCl) for 25 minutes and rinsed four times with sterile distilled water. Seeds were immersed in sterile water for no longer than one hour before dissection.

For the stratification experiments, seeds were disinfested as above and then wrapped in sterile 10 x 10 cm cheesecloth squares that had been pre-soaked in sterile distilled water. The cheesecloth sacks, each containing 25 seeds, were then sealed in polypropylene bags for 24 weeks. During the first four weeks all the bags were kept in darkness at 21°C when one sample was removed for culture. Thereafter, the remaining

bags were stratified at 5°C and samples were removed at four week intervals. After each stratification period the seeds were surface sterilized and placed in sterile water prior to dissection.

Wings were aseptically removed from the seed and embryos were then excised and individually placed horizontally on agar slants of culture media.

Axillary Shoot Multiplication System

Two year-old yellow cypress seedlings (lot #9777) ranging in height from 40 - 50 cm were acquired from the B.C. Ministry of Forests and Lands, Victoria.

The limited availability of greenhouse space required the stock plants to be housed in different facilities. Consequently, the plant material was divided into two groups. Group I plants were placed in a greenhouse with an 18 hour photoperiod provided by cool white fluorescent light. The temperature was maintained at 23°C ± 2°C. Group II plants were housed on a growth bench receiving an 18 hour photoperiod of only artificial illumination from cool white fluorescent tubes at 200 - 250 umols $m^{-2}s^{-1}$. Ambient air temperature was 20°C ± 2°C and relative humidity near 20%.

The maintenance program for both blocks of stock plants was identical. Plants were bottom watered to minimize the risk of fungal and bacterial contamination. A bi-weekly application of `Plant Prod' all purpose fertilizer with nitrogen, phosphorus and potassium ratios of 20:20:20 was

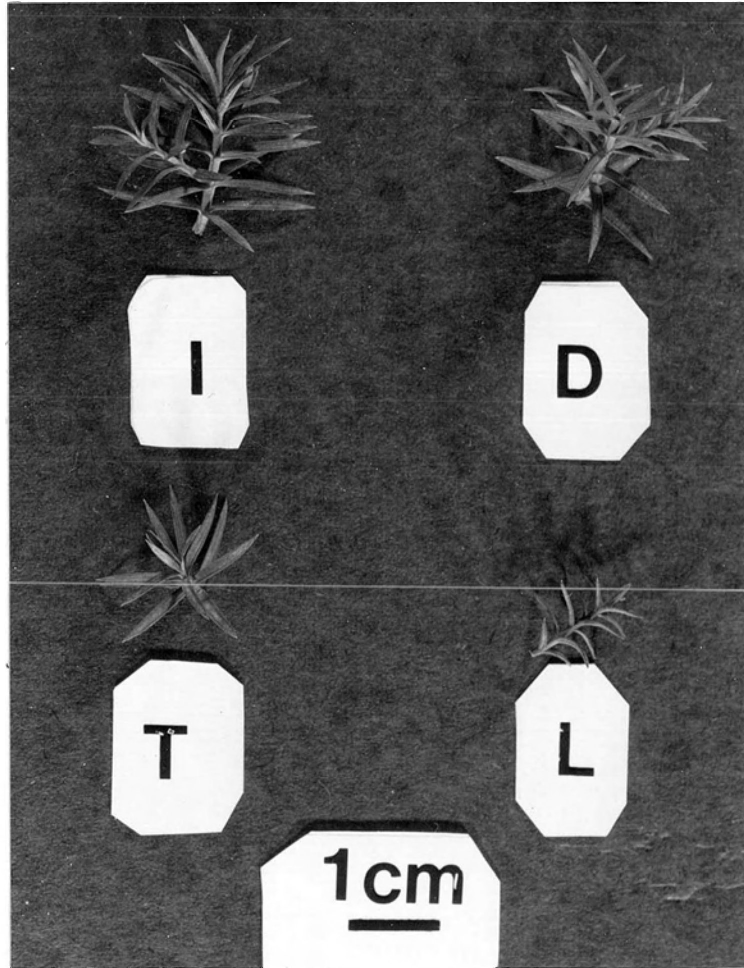
given to ensure a constant supply of fresh growing tips. Top pruning was used to encourage the lateral growth of juvenile foliage.

Explants harvested from Group I were used in studies related to media composition and for the experiments on physiological status of the explant which included age, size and position on the parent plant. Group II plants were primarily used for hormone dose response studies and for examining various methods of growth regulator application. It was necessary to remove the stock plants from the Group II environment to the Group I environment before the conclusion of these latter experiments. This necessitated a Group III classification. Explants acquired from Group III were used to conclude the growth regulator application experiments.

With respect to their shoot system, stock plants from Group I were divided into three regions. These were designated as basal (bottom 1/3 of the plant); the mid-crown (middle 1/3 of the plant); or the top-crown region (top 1/3 of the plant). Furthermore, different explant types along the branching system were identified and tagged. Explants with juvenile foliage (needle-like leaves in whorls of three) and mature type foliage (scale-like decussate leaves arranged such that they formed two opposite rows at 90° to each other) were harvested to test their performance in culture.

Subsequently, shoots with juvenile foliage were the only explant sources used. However, these juvenile explants were further subdivided into four types (Fig. 1). The `intact´

FIGURE 1 Four yellow cypress explant types; intact (I), decapitated (D), tip (T), and lateral (L) used in the axillary shoot multiplication experiments.
Scale bar = 10 mm.


I
D
T
L
1cm

explant was a shoot tip approximately 1.5 - 2.0 cm long which contained the terminal apex and two to three lateral branches. The `tip´ explant was the top 1.0 cm of the intact explant. The tip contained the terminal apex and some associated stem tissue without apparent laterals. `Lateral´ explants were the small lateral shoots from the intact explant which had initiated in the leaf axils. `Decapitated´ explants had the apical portion removed and consisted of the remainder of the intact explant with its attendant laterals.

Disinfestation of the explants began with a 30 minute soap wash with dilute liquinox (5 drops liquinox/50 ml of tap water). A maximum of 20 explants was placed in a 100 ml beaker which was agitated at 125 RPM. After this, shoots were rinsed with tap water for one hour. Throughout the remainder of the surface sterilization shoots were agitated at 125 RPM and rinsed four times with sterile distilled water after each step. A second soap wash was followed by a 30 minute fungicide bath. The fungicide solution consisted of tri-basic copper sulphate (4.050 gl^{-1}) and benomyl (0.607 gl^{-1}) in distilled water. Explants were then placed in 10% commercial bleach (0.52% w/v NaOCl), agitated for 20 minutes and rinsed under the laminar flow hood. Prior to inoculation, shoots were kept in beakers filled with 50 ml of sterile water and covered with sterile petri plates.

CULTURE MEDIA

Seven culture media were tested during this study. The media evaluated were designated as SH (Schenk and Hildebrandt, 1972); MSH (Schenk and Hildebrant, 1972) modified as follows: KNO_3 1.250 gl^{-1}; myo-inositol 0.500 gl^{-1}; thiamine HCl 0.0025 gl^{-1}; nicotinic acid 0.0025 gl^{-1}; pyridoxine HCl 0.0025 gl^{-1}; ACLP (LP medium of Aitken-Christie, 1984); CD (Campbell and Durzan, 1975); CDAA (CD medium modified by Ishii, 1986); CBM, Cupressus Basal medium (Franco and Schwarz, 1985); MSK (Murashige and Skoog, 1962), modified as follows: NH_4NO_3 0.825 gl^{-1}; KNO_3 0.950 gl^{-1}; $MgSO_4$ 0.185 gl^{-1}; $MnSO_4$ 0.008 gl^{-1}; $ZnSO_4$ 0.0043 gl^{-1}; $CuSO_4$ 0.0001 gl^{-1}; Na_2-EDTA 0.0186 gl^{-1}; $FeSO_4$ 0.0139 gl^{-1}. All seven of the aforementioned media were used in the embryo culture work. Based on the explant's performance, in terms of survivability and degree of branching, three of the seven were selected for the axillary shoot culture experiments. These were MSK, CBM and SH. Unless otherwise stated, sucrose was included in all media at 30 gl^{-1} and agar (Sigma) at 5.6 gl^{-1}.

Medium pH was adjusted to 5.6 following the addition of growth regulators. Media for embryo and axillary shoot cultures were dispensed at 10 ml per 25 x 95 mm shell vial or 15 ml per 25 x 150 mm culture tube, respectively. In both cases culture vessels were sealed with clear Magenta 2-way caps and then autoclaved at 121°C and 1.05 kg/cm^2 pressure for 15 minutes. Media were cooled as agar slants. Cultures were

incubated at 23°C ± 2°C with a 16 hour photoperiod of cool white fluorescent light at an irradiance of 25 umols $m^{-2}s^{-1}$.

SHOOT INDUCTION

Embryo Culture System

Regardless of the seed source, excised embryos were exposed to N^6-benzyladenine (BA) for four weeks at concentrations ranging from 1-10 uM. Thereafter, explants were subcultured at four week intervals onto SH basal medium for further shoot development.

In one experiment the degree to which myo-inositol affected embryo performance was evaluated. Based on earlier work by Pollard et al. (1961), Staudt (1984), Steinhart et al. (1962) and White (1963), inositol was shown to have a promotive effect on stimulating cell growth and cell division. Two concentrations of inositol: 0.100 and 1.000 gl^{-1} were tested over a BA gradient ranging from 0.35 - 2.5 mgl^{-1}. The respective inositol levels were maintained during sub-culturing.

After two subcultures on basal medium shoots were separated from the mother explants. Isolated shoots were subcultured until an overall length of 5 mm or greater was attained.

Axillary Shoot Multiplication System

In an effort to stimulate axillary shoot formation hormones were added to the agar-solidified medium, into which

the base of the explant was inserted, or to the entire explant via pulse treatments in liquid medium (von Arnold and Eriksson, 1985). For pulse treatments, explants were agitated at 125 RPM in 125 ml flasks containing 50 ml of liquid medium with or without growth regulators for two, eight or twenty-four hours, respectively. After the pulse treatment the explants were briefly blotted on sterile paper towels before being placed vertically onto agar slants of basal medium. During pulse treatments BA, Indole-3-butyric acid (IBA) and Napthyleneacetic acid (NAA) were applied separately at 250 uM.

Some explants were also exposed to BA for four weeks by its incorporation into the medium at 1.0, 5.0, 10.0 and 25.0 mgl^{-1}. Similarly, both IBA and NAA were applied at concentrations of 0.25, 0.5 and 1.0 mgl^{-1}. In all cases, after hormone treatment, explants were transferred to growth regulator-free medium and subcultured onto fresh basal medium every four weeks.

After the third subculture on basal medium many of the explants had generated flushes of new growth. This new growth made it possible to initiate rooting trials. Explants chosen for rooting consisted of either the original explant left intact, or excised laterals 5 mm or greater in overall length. These explants were classified as micropropagules.

PLANTLET FORMATION AND ROOTING

Embryo Culture System

Microshoots greater than 5 mm in length were selected for rooting. Roots were induced on elongated shoots both *in vitro* and under non-sterile greenhouse conditions. The *in vitro* rooting matrix was 20 ml of vermiculite moistened with 10 ml of 1/2 SH salts containing 2% sucrose and 0.2% activated charcoal (Sigma #C-4386). The rooting medium was dispensed into 25 x 95 mm shell vials and autoclaved for 15 minutes. The rooting treatments were conducted at 23°C ± 2°C under a 16 hour photoperiod with 20 umols $m^{-2}s^{-1}$ cool white fluorescent light. For the greenhouse experiments, microshoots were planted in Spenser-Lemaire #5 cell-paks with 62 cm^3 cavities containing a 1:1 (v/v) peat-perlite mixture. Some of the microshoots were dipped in rooting powder, Stim Root #3 (Plant Products Co. Ltd., Bramalea, Ontario) containing 0.8% IBA prior to planting. Excess powder was removed by gentle tapping. Planted trays were placed in an air-conditioned greenhouse under natural light and were handled like yellow cypress cuttings (Karlsson, 1974). No modifications were made to the environment to accommodate the microshoots. Bottom heat was 20°C with an ambient air temperature of 15°C. Microshoots were misted for 15 - 20 second durations by a Misto-O-Matic watering system and supplemented by hand watering as required. Microshoots were fertilized once a week beginning in April with Green Valley fertilizer (Green Valley

Fertilizer Co.) 10:52:17 at a rate of 0.500 gl^{-1} of water. From May to July Plant Prod (Plant Products Co. Ltd., Bramalea, Ontario) 20:8:20 was alternatively used at 0.500gl^{-1} of water every other week. Fertilization continued until late September. In mid-November microshoots were transferred to a lath house where they are presently being maintained.

Axillary Shoot Multiplication System

Micropropagules greater than 5 mm in overall length were harvested for rooting using either an *in vitro* or *in vivo* approach. The *in vitro* rooting trials were similar to those used for the microshoots. For *in vivo* rooting, micropropagules were planted in 'Greenleaf' potting trays with 2.5 x 4.0 cm cavities containing the same potting mix used with the microshoots and were covered with clear plastic lids. Cultural requirements for the *in vitro* rooting treatments were identical to those used with the microshoot trials. *In vivo* rooting trials were done in a ventilated greenhouse with bottom heat at 25°C and an air temperature of 22°C.

EMBRYO CULTURE SYSTEM

Results

Only 60% of the seeds from Seedlot A contained embryos and only 50% of these embryos germinated on MSK basal medium. Morphologically-normal seedlings developed from these embryos on this medium. Approximately 50% of the embryos cultured on BA showed signs of cotyledonary development. Two types of shoot formation were observed on BA after six to eight weeks of culture. These included, primary shoot development and adventitious shoot formation.

For adventitious shoot production, the cotyledons were the caulogenic portion of the embryo (Fig. 2). After four weeks the cotyledons had become swollen and reflexed and their outer edges had taken on a frilled appearance. The formation of growth centres (tiny green pearl-like nodules) was visible on both cotyledons whether or not they contacted the medium. The radicle produced a translucent callus which later turned brown (Fig. 2). Needle development was evident after six weeks in culture (Fig. 2) and visible shoots arose one to two weeks later. Best shoot formation occurred at 0.5 and 1.0 mgl^{-1} BA (Table 1, Fig. 3). While there were more shoots at these BA levels, the overall size of the shoots was much smaller compared to 0.25 mgl^{-1} BA. Furthermore, an increase in vitrification and callus formation occurred at the higher BA concentrations.

TABLE 1 A comparison of the effects of BA on shoot production by yellow cypress embryos from Seedlots A and B after 12 weeks.

	Medium	BA ($mg l^{-1}$)	N	No. of Shoot-Forming Explants	%Explants with Shoots	Mean No. of Shoots/ SFE[a] [±SEM][b]
Seedlot A	MSK	0.25	24	9	37	2.6±0.4
		0.5	22	5	22	3.6±1.1
		1.0	25	8	32	3.7±0.6
Seedlot B	MSK	0.25	25	16	64	2.6±0.5
		0.5	25	17	68	1.7±0.5
		2.5	25	16	64	1.8±0.5
Seedlot B	CBM	0.25	25	17	68*	1.5±0.2
		0.5	25	17	68*	2.7±0.3
		2.5	25	13	52*	3.2±0.4

[a]SFE = Shoot forming explant

[b]SEM = Standard error of the mean

* = P-value < 0.05 using one-way Anova test

FIGURE 2 A yellow cypress embryo (Seedlot A) after four weeks on SH medium supplemented with 0.5 mgl^{-1} BA and subcultured for two weeks on basal medium showing the formation of primary needles (N) from the cotyledons (C). Note the brown callus (BC) produced from the radicle. Scale bar = 1.8 mm.

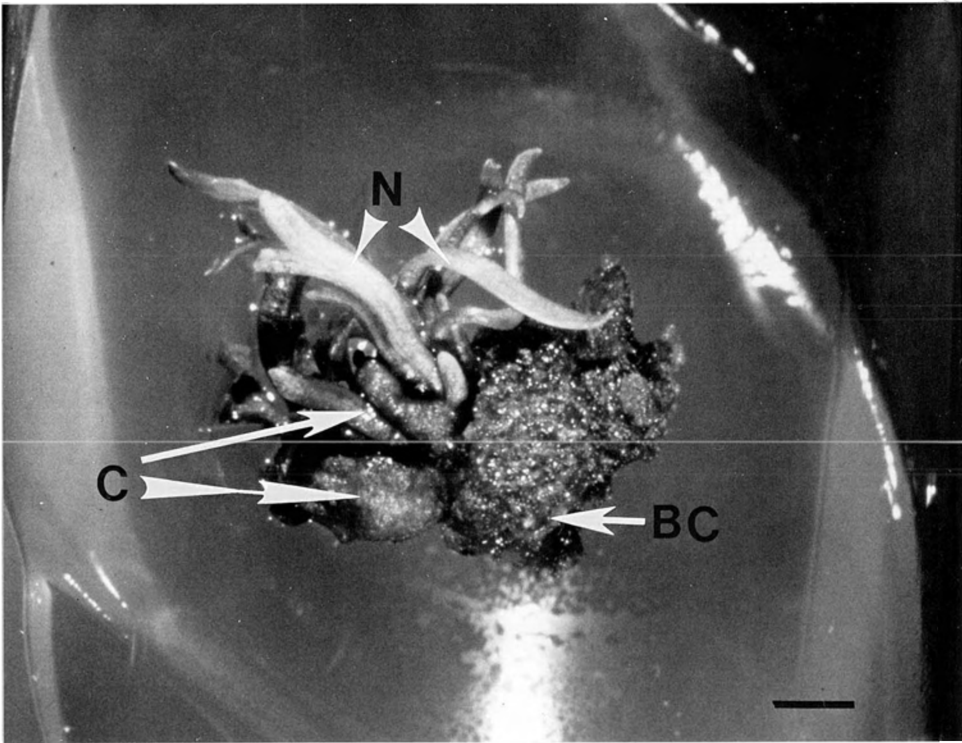
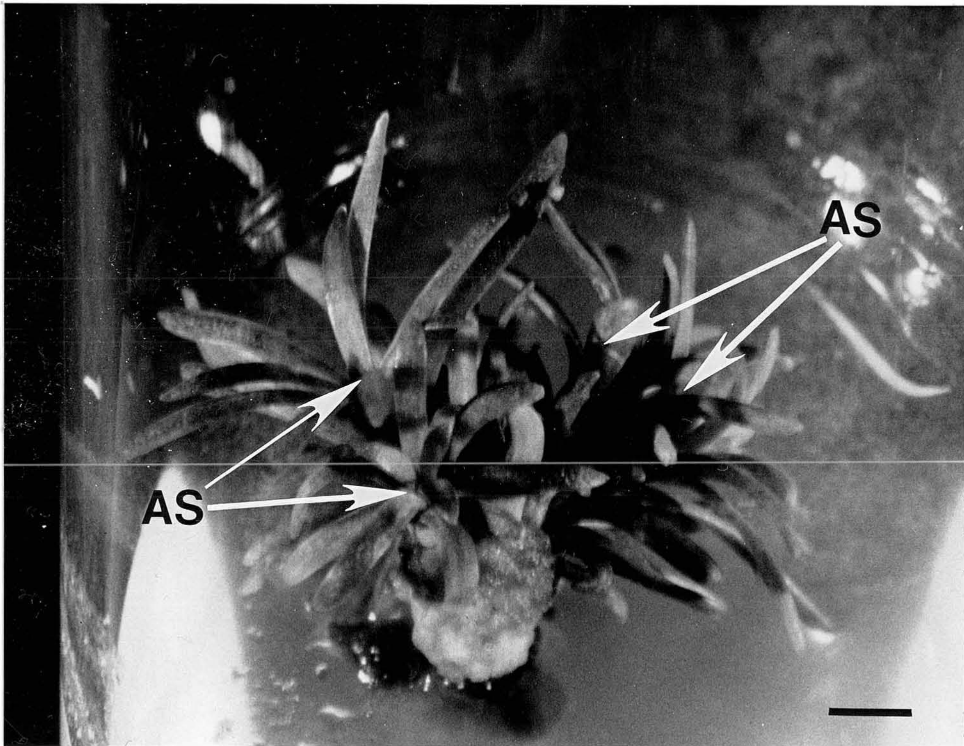

N
C
BC

FIGURE 3 Adventitious shoots (AS) produced by a yellow cypress embryo (Seedlot A) after a four week exposure to 0.5 mgl^{-1} BA on SH medium and four weeks on basal medium. Scale bar = 1.5 mm.


AS
AS

The caulogenic effects of BA with MSK and CBM media were evaluated for Seedlot B (Table 1). In this seedlot 90% of the seeds contained embryos of which 96% germinated on basal medium. On BA, 95% of the embryos developed. Compared to Seedlot A, embryo response occurred more rapidly so that overall development was enhanced by two weeks. However, the average number of adventitious shoots formed was no greater than with Seedlot A (Table 1). Overall, it appeared CBM was slightly superior to MSK for shoot induction.

The caulogenic effects of BA were further evaluated on five media (Table 2). In terms of the frequency and abundance of shoot formation, SH medium was superior and ACLP was the next best. With these two media, best shoot production occurred at low BA levels (0.25 - 0.5 mgl^{-1}) (Tables 2, 3). CBM was superior to either of the CD media. In the latter three, caulogenesis increased as the BA level increased which was opposite to the trend on SH medium. On ACLP there was no real trend and no quantitative difference at the different BA levels. When BA was added at 0.25 and 0.35 mgl^{-1} in SH medium, it gave the highest yield of shoots (Table 2). Although embryos cultured on SH, ACLP and CBM had similar frequencies of shoot formation, the overall number of shoots per explant was higher on SH medium.

SH and MSH media were compared for their caulogenic capacity over a range of BA concentrations (Table 3). Results on SH were similar to those obtained previously (Table 2). However, in this case MSH was better than SH (Table 3). While

TABLE 2 Influence of media and BA on shoot formation by yellow cypress embryos (Seedlot B) after 12 weeks [N=15]

Medium	BA ($mg l^{-1}$)	No. of Shoot Forming Explants	%Explants with Shoots	Ave. No. Shoots/ SFE[a] [±SEM][b]	Range of Shoots/ SFE	SFC[c] Index
SH	0.25	11	73	4.7±1.4	1-14	3.4
	0.35	11	73	5.5±0.5	4-10	4.0
	0.5	8	53	4.6±1.0	1-9	2.4
ACLP	0.25	6	40	4.6±0.4	3-6	1.8
	0.35	7	46	2.0±0.9	1-3	0.9
	0.5	8	53	4.1±0.9	2-8	2.1
CBM	0.25	7	46	2.4±0.5	1-5	1.1
	0.35	8	53	1.8±0.3	1-3	0.9
	0.5	9	60	3.3±0.6	1-7	1.9
CD	0.25	3	20*	2.0±1.0	1-4	0.4
	0.35	4	26*	2.5±0.6	1-4	0.6
	0.5	9	60*	3.0±0.7	1-7	1.8
CDAA	0.25	5	33	1.0±0.0	1	0.3
	0.35	4	26	2.7±0.7	1-4	0.7
	0.5	9	60	2.4±0.5	1-5	1.4

[a]SFE = Shoot forming explant

[b]SEM = Standard error of the mean

[c]SFC Index = Mean number of shoots/explant x percent of shoot forming explants divided by 100.

* = P-value < 0.05 using Chi-square test

TABLE 3 Comparison of the shoot forming capacity of yellow cypress embryos (Seedlot B) on SH medium and MSH medium, [N=20 except for SH at 1.0 and 2.5 mgl^{-1} BA where N=40].

Medium	BA (mgl^{-1})	No. of Shoot Forming Explants	%Explants with Shoots	Ave. No. Shoots/ SFE[a] [±SEM][b]	Range of Shoots/ SFE	SFC[c] Index
SH	0.25	14	70*	3.8±0.4	1-7	2.6
	0.35	8	40*	4.5±0.5	1-7	1.8
	0.5	8	40*	3.5±0.9	1-9	1.4
	1.0	16	40*	2.7±0.4	1-6	1.0
	2.5	8	20*	3.3±0.9	1-10	0.6
MSH	0.35	14	70	4.5±0.3	1-13	3.1
	0.5	14	70	2.7±0.4	1-6	1.8
	1.0	12	60	2.0±0.3	1-5	1.2

[a]SFE = Shoot forming explant

[b]SEM = Standard error of the mean

[c]SFC Index = Mean number of shoots/explant x percent of shoot forming explants divided by 100.

* = P-value < 0.05 using Chi-square test

the mean number of shoots was the same for both media, the frequency of embryo response was higher on MSH. However, in later experiments SH was superior to MSH (Tables 5, 6). Overall, 0.35 mgl^{-1} BA gave consistently good results with both media (Tables 2, 3).

Because unexpectedly low levels of BA stimulated adventitious shoot formation, the effect of inositol concentration (100 and 1000 mgl^{-1}) on the caulogenic capacity of embryos was evaluated over a BA gradient on SH medium (Table 4). In both cases best shoot production occurred at 0.5 mgl^{-1} BA. While there was little difference in the mean number of shoots produced by either of the inositol treatments at this level, this was not the case for the lowest BA concentration of 0.35 mgl^{-1}. Here an increase in caulogenesis was observed at the higher inositol level (Table 4). Overall, despite little difference in the caulogenic response elicited by the two inositol treatments, 1000 mgl^{-1} was somewhat better in that a greater number of shoots per embryo were formed at 0.35 mgl^{-1} BA. Levels of BA higher than 0.5 mgl^{-1} caused a progressive decline in caulogenesis.

The effects of seed stratification on caulogenesis were evaluated in four experiments designated IA and IB on SH medium and IIA and IIB on MSH medium (Tables 5, 6). In both experiments using SH, the time zero controls were more caulogenic compared to controls on MSH. By totaling up the shoot forming index (SFC) values there was a 30% increase in shoot formation with SH compared to MSH (Tables 5, 6).

TABLE 4 Influence of inositol and BA on shoot formation by yellow cypress embryos (Seedlot B) on SH after four weeks exposure to BA followed by eight weeks on basal medium [N=20].

Inositol Treatment (mgl^{-1})	BA (mgl^{-1})	No. of Shoot Forming Explants	%Explants with Shoots	Ave. No. Shoots/ SFE[a] [±SEM][b]	Range of Shoots/ SFE	SFC[c] Index
100	0.35	16	80#	2.3±0.3*	1-6	1.8
	0.5	17	85#	4.0±0.4*	1-9	3.4
	0.75	13	65#	2.9±0.5*	1-8	1.8
	1.0	10	50#	1.3±0.1*	1-2	0.6
	2.5	10	50#	1.4±0.3*	1-4	0.7
1000	0.35	18	90#	3.3±0.4*	1-9	2.9
	0.5	15	75#	4.6±0.8*	1-16	3.4
	0.75	12	60#	2.8±0.7*	1-11	1.6
	1.0	10	50#	2.1±0.6*	1-8	1.0
	2.5	10	50#	1.8±0.2*	1-3	0.9

[a]SFE = Shoot forming explant

[b]SEM = Standard error of the mean

[c]SFC Index = Mean number of shoots/explant x percent of shoot forming explants divided by 100

= P-value < 0.05 using Chi-square test

* = P-value < 0.05 using one-way Anova test

TABLE 5 Effect of stratification on shoot formation by yellow cypress embryos (Seedlot B) from duplicate experiments IA and IB on SH medium after a four week exposure to 0.35 mgl^{-1} BA and eight weeks on basal medium [N=20].

	Treatment (No. of Weeks at RT[a], CT[b])	No. of Shoot Forming Explants	%Explants with Shoots	Ave. No. Shoots/ SFE[c] [±SEM][d]	Range of Shoots/ SFE	SFC[e] Index
IA	0-Control 0/0	14	70	2.5±0.4*	1-7	1.7
	A-4 RT	10	50	1.9±0.2*	1-3	0.9
	B-4 RT/2 CT	10	50	3.6±0.5*	2-8	1.8
	C-4 RT/4 CT	10	50	3.8±0.8*	1-11	1.9
	D-4 RT/8 CT	14	70	5.7±0.4*	2-8	3.9
	E-4 RT/12 CT	10	50	5.0±0.5*	3-7	2.5
	F-4 RT/16 CT	10	50	1.8±0.4*	1-5	0.9
	G-4 RT/20 CT	10	50	2.2±0.6*	1-7	1.1
IB	0-Control 0/0	15	75	3.0±0.4*	1-8	2.2
	A-4 RT	11	55	2.3±0.4*	1-5	1.2
	B-4 RT/2 CT	11	55	1.9±0.2*	1-3	1.0
	C-4 RT/4 CT	11	55	1.9±0.2*	1-3	1.0
	D-4 RT/8 CT	15	75	4.5±0.4*	2-8	3.3
	E-4 RT/12 CT	10	50	2.5±0.4*	1-5	1.2
	F-4 RT/16 CT	12	60	2.5±0.5*	1-6	1.5
	G-4 RT/20 CT	11	55	1.7±0.6*	1-3	0.9

[a]RT = Room temperature (21°C ± 2°C)

[b]CT = Cold temperature (5°C)

[c]SFE = Shoot forming explant

[d]SEM = Standard error of the mean

[e]SFC Index = Mean number of shoots/explants x percent of shoot forming explants divided by 100.

* = P-value < 0.05 using one-way Anova test

TABLE 6 Effect of stratification on shoot formation by yellow cypress embryos (Seedlot B) from duplicate experiments IIA and IIB on MSH medium after a four week exposure to 0.35 mgl^{-1} BA and eight weeks on basal medium [N=20].

	Treatment (No. of Weeks at RT[a], CT[b])	No. of Shoot Forming Explants	%Explants with Shoots	Ave. No. Shoots/ SFE[c] [±SEM][d]	Range of Shoots/ SFE	SFC[e] Index
IIA	0-Control 0/0	15	75	1.8±0.9*	1-4	1.3
	A-4 RT	10	50	1.9±0.6*	1-3	0.9
	B-4 RT/2 CT	10	50	3.8±0.6*	1-7	1.9
	C-4 RT/4 CT	10	50	3.6±0.4*	1-6	1.8
	D-4 RT/8 CT	10	50	3.2±0.5*	1-6	1.6
	E-4 RT/12 CT	11	55	3.8±0.6*	1-8	2.0
	F-4 RT/16 CT	11	55	2.2±0.5*	1-6	1.2
	G-4 RT/20 CT	10	50	1.7±0.3*	1-5	0.8
IIB	0-Control 0/0	11	55	1.8±0.4	1-4	0.9
	A-4 RT	10	50	1.2±0.1	1-2	0.6
	B-4 RT/2 CT	10	50	1.9±0.2	1-3	0.9
	C-4 RT/4 CT	11	55	2.0±0.6	1-6	1.1
	D-4 RT/8 CT	12	60	2.3±0.4	1-5	1.3
	E-4 RT/12 CT	11	55	2.6±0.6	1-7	1.4
	F-4 RT/16 CT	10	50	1.9±0.6	1-4	0.9
	G-4 RT/20 CT	11	55	1.3±0.2	1-3	0.7

[a]RT = Room temperature (21°C ± 2°C)

[b]CT = Cold temperature (5°C)

[c]SFE = Shoot forming explant

[d]SEM = Standard error of the mean

[e]SFC Index = Mean number of shoots/explants x percent of shoot forming explants divided by 100.

* = P-value < 0.05 using one-way Anova test

In IA a definite trend was evident with treatments B through D resulting in a gradual increase in the mean number of shoots per shoot-forming embryo (Table 5). Thereafter, treatments E through G caulogenesis declined back to control levels. This trend was not evident in IB, however, in treatment D caulogenesis increased (Table 5). The remainder of the treatments were not better than the controls.

A similar, but less apparent pattern appears in IIA and IIB (Table 6). In IIA treatments A and B were equivalent while C through E showed an increase in caulogenesis which returned to the original level in treatments F and G. With IIB shoot production remained fairly constant with only a slight elevation in the average number of shoots occurring with treatment E.

Overall, embryo performance was better and more uniform in IA and IB than IIA and IIB (Tables 5, 6). While stratification had a negative effect on the frequency of response, it had a positive effect on the mean number of shoots per shoot-forming embryo. The evidence for this is quite strong in IA but less so in IIA and IIB (Tables 5, 6).

A change in the overall pattern of BA-induced shoot formation occurred with treatment E explants in three out of the four experiments. Shoots were observed arising from the hypocotyl as well as from the cotyledons following stratification (Fig. 4).

Microshoots elongated well on basal SH medium (Fig. 5) and were harvested for rooting trials. The presence of basal

FIGURE 4 A yellow cypress embryo (Seedlot B) stratified for four weeks at 21°C and an additional 12 weeks at 5°C followed by a four week exposure to 0.35 mgl^{-1} BA on SH medium and four weeks on basal medium. Note the distribution of shoots arising from the hypocotyl (H) as well as from the cotyledons (C). Scale bar = 1.8 mm.

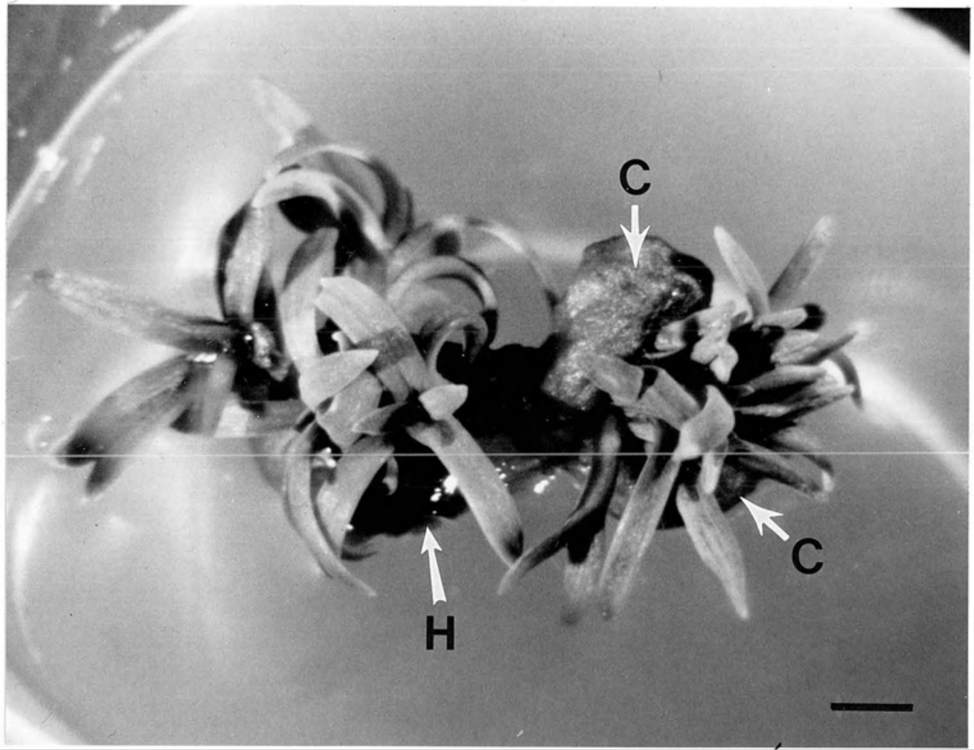

C
C
H

FIGURE 5 Microshoots (MS) isolated from an explant exposed to 0.35 mgl^{-1} BA on SH for four weeks then subcultured on basal medium for a further eight weeks. Scale bar ▫ 2.2 mm.

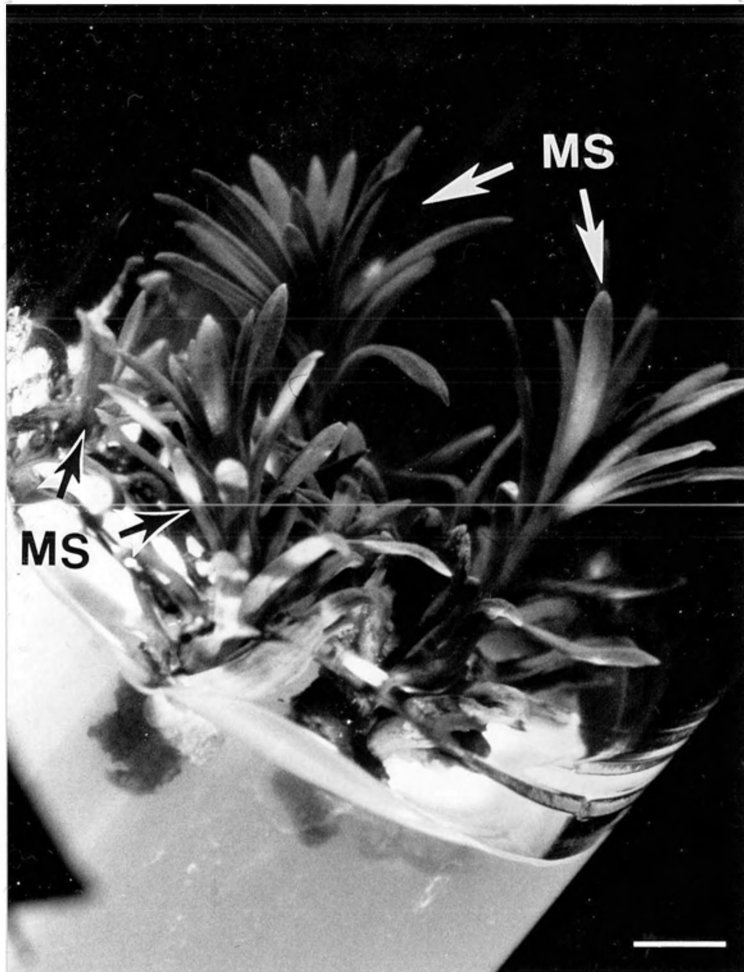
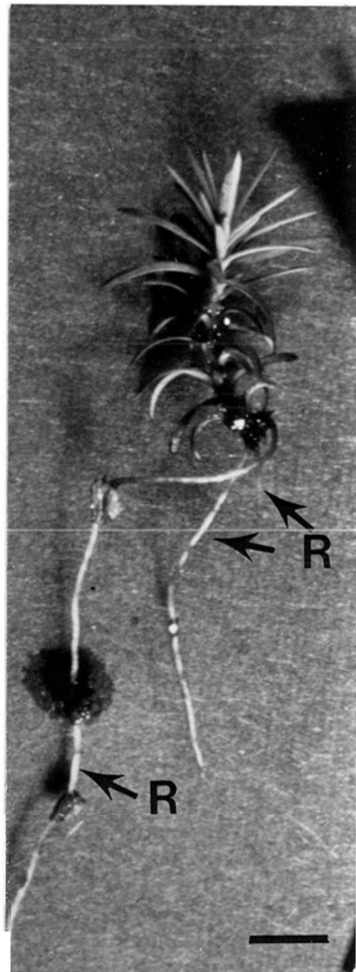

MS
MS

FIGURE 6 Rooted microshoot from *in vitro* rooting trial after 12 weeks. Note the formation of multiple roots (R). Scale bar = 3.0 mm.


R
R

callus was associated with successful rooting *in vitro*. Those microshoots that did not produce a callus turned bluish-green and eventually senesced. Although the frequency of rooting was low (13-15%) those microshoots that did root had a well developed root system (Fig. 6). The best rooting response was obtained under non-sterile greenhouse conditions. Initial rooting trials were evaluated after 12 weeks and were found to have low frequencies of rooting. However, later experiments indicated that a longer period was required for rhizogenesis. Of those microshoots lifted 9 - 12 months after outplanting 59 - 62% had rooted (Table 7, Fig. 7). Average plantlet height over a 12 month period ranged between 10.0 - 13.9 cm with an average caliper (diameter of the stem) of 2.29 - 2.68 mm (Fig. 8). While IBA had no major effect on the frequency of rooting, it did increase shoot caliper (Table 7).

TABLE 7. Rooting and survival of yellow cypress microshoots 9-12 months after outplanting under operational forestry nursery conditions with and without IBA.

	No. of Months in Soil	No. of Microshoots Planted	%Plantlet Survival	Ave. Plantlet ht.[a] (cm) ±SEM[b]	Ave. ht./ 12 Months	Ave. Caliper[c] of Plantlets (mm) ±SEM	Ave. Caliper/ 12 Months
TRIAL 1 (Dec/87)	12						
+IBA		56	69	13.42±0.55	13.42	2.81±0.12	2.81
-IBA		119	59	14.16±0.40	14.16	2.61±0.07	2.61
Total N Value		175	62	13.90±0.32	13.90	2.68±0.06	2.68
TRIAL 2 (Mar/88)	9						
+IBA		95	57	7.12±0.26	9.49	1.78±0.06	2.37
-IBA		80	61	8.12±0.62	10.82	1.65±0.08	2.20
Total N Value		175	59	7.50±0.33	10.00	1.72±0.05	2.29

[a]ht. = Height

[b]SEM = Standard error of the mean

[c]Caliper = The diameter of the stem

FIGURE 7 Rooted yellow cypress microshoots planted in a peat/perlite mix after 12 months under operational forestry nursery conditions.

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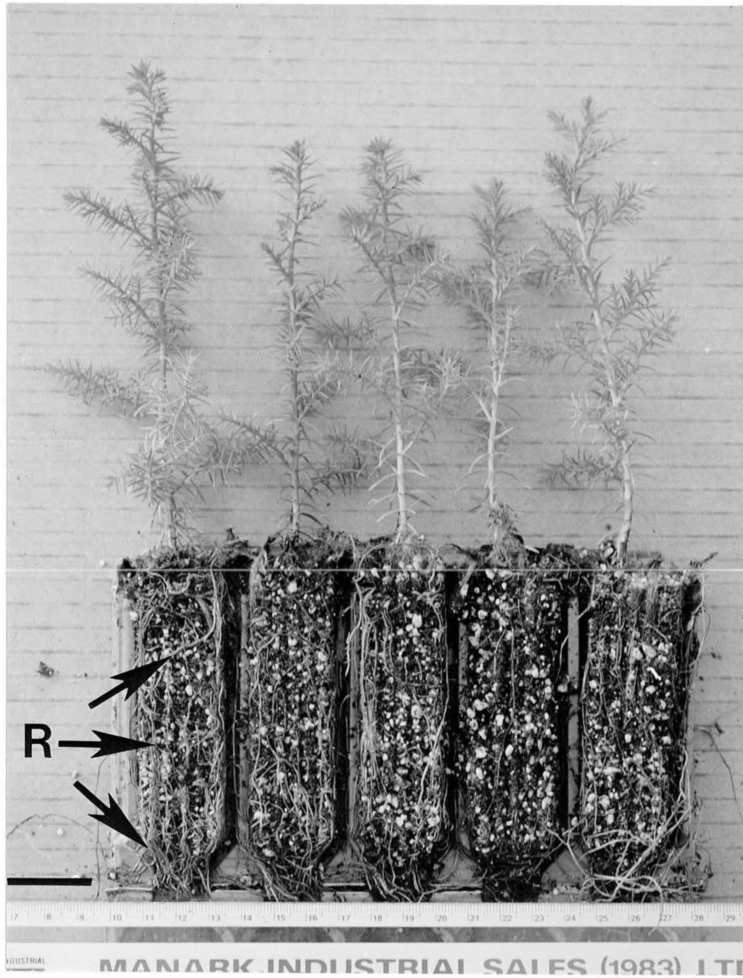
FIGURE 8 Yellow cypress microshoots grown for 12 months under operational greenhouse conditions in a Spenser-Lemaire #5 cell-pak containing a peat/perlite mix. Note the very fibrous and active root (R) systems.


R

DISCUSSION

Seed dormancy is a primary factor which causes poor seed germination in yellow cypress. There are various causes of seed dormancy. These include impermeable seed coats (ectodormancy), physiological inhibitors by tissues surrounding the embryo (paradormancy) and physiological factors within the embryo (endodormancy) which prevent germination (Lang et al., 1987). The stratification procedure required for yellow cypress seed germination (Owens and Molder, 1984) suggests that a complex dormancy mechanism is involved (Poljakoff-Mayber and Mayer, 1982). However, this study has shown that over 90% of cultured yellow cypress embryos germinated and produced normal seedlings after one to two weeks on simple basal medium without any seed pre-treatments. Similar cases have been reported for _Pinus taeda_ (Carpita et al., 1983) and _Pinus lambertiana_ (Barton, 1978) where the embryo was mature, but germination was inhibited by the megagametophyte or seed coat. As our work demonstrates, this type of paradormancy appears to be overcome by culturing embryos on basal medium. This strongly suggests that yellow cypress seed dormancy is probably not due to developmental or physiological factors present within the embryo.

Our results indicate that stratification is not required for caulogenesis as was the case with _Pinus strobus_ (Flinn et al., 1986). However, our data suggests that stratification of the seed enhances the mean number of shoots per shoot-forming

embryo and increases the total shoot formation. Greatest total shoot formation occurred on SH medium after incubation at room temperature for four weeks followed by stratification at 5°C for eight weeks. In *Pinus* sp. seeds a cold stratification also resulted in an increased number of buds per explant (Reilly and Washer, 1977; Smeltzer et al., 1977). Our findings support Murashige's (1974) hypothesis that satisfying dormancy requirements is essential if maximal gains in explant performance are to be achieved.

It is during stratification that the growth substance balance in seeds is changed. In some instances chilling induces the formation of gibberellic acid and or cytokinins (Poljakoff-Mayber and Mayer, 1982). Therefore, it appears that an effect of stratification is to shift the balance between growth inhibitors and growth promotors, in favor of the latter. This could explain the increased frequency and abundance of caulogenesis observed in stratified yellow cypress embryos and the pattern change in shoot distribution.

The cotyledonary origin of adventitious shoots observed here has also been reported for embryonic explants of other Cupressaceae (Coleman and Thorpe, 1977; Franco and Schwarz, 1985; Ishii, 1986; Thomas et al., 1977; Thomas and Tranvan, 1982). However, Harry et al. (1987) found that the epicotyl of *Thuja occidentalis* embryos gave maximum shoot production.

Our results show that BA alone is sufficient to induce caulogenesis in mature embryos of yellow cypress as found for

many conifers (Bornman, 1983; David, 1982; Reilly and Washer, 1977; Rumary and Thorpe, 1984). Furthermore, the frequency and abundance of caulogenesis observed in this study is similar to that described for Chamaecyparis obtusa (Ishii, 1986). With yellow cedar, 68 - 73% of embryos formed shoots with an average of 4.5 - 5.5 shoots per explant. With Chamaecyparis obtusa (Ishii, 1986) these figures were 60 - 87% and 7.9 - 8.3, respectively. However, the optimal BA concentration for caulogenesis of yellow cypress was relatively low, 0.25 - 0.5 mgl^{-1}, compared to other Cupressaceae (Ishii, 1986; Thomas et al., 1977; Thomas and Tranvan, 1982) and other conifers in general (David, 1982). This could possibly be attributed to the high inositol concentration (1000 mgl^{-1}) used in the medium. The inositol level most commonly used in culture of Cupressaceae (Franco and Schwarz, 1985; Ishii, 1986; Thomas et al., 1977) and other conifers (Dunstan et al., 1987; Reilly and Washer, 1977; von Arnold and Eriksson, 1985) is 100 mgl^{-1}. In our work, a higher level of inositol (1000 mgl^{-1}) appeared to have a stimulatory effect on shoot production at 0.35 mgl^{-1} BA. This suggests a synergistic interplay between growth regulators and inositol. Pollard et al. (1961) found that hexitols, especially inositol, promoted cell growth and cell division. Furthermore, Steinhart et al. (1962) and White (1963) found that myo-inositol stimulated the growth of both Picea abies callus and Picea glauca tissue, respectively. Although the

maximum growth response for both spruce species was attained at concentrations ranging between 50 and 100 mgl^{-1} (Steinhart et al., 1962; White, 1963), levels as high as 1000 mgl^{-1} (Steinhart et al., 1962) were not inhibitory.

The strength and composition of the basal medium play an integral role in organogenesis (Bornman, 1983; Flinn et al., 1986; Harry et al., 1987). Of the different standard media evaluated in this study, SH was the best for shoot induction. This finding is in accord with similar work done on _Pinus radiata_ (Reilly and Washer, 1977), _Pinus strobus_ (Flinn et al., 1986), _Picea abies_ (Janssen and Bornman, 1983), and _Picea glauca_ (Rumary and Thorpe, 1984) where SH media has been successfully used. However, our results are contrary to those reported for _Chamaecyparis obtusa_ (Ishii, 1986) and _Thuja occidentalis_ (Harry et al., 1987). With _Chamaecyparis obtusa_ (Ishii, 1986) CD media was superior to SH while with _Thuja occidentalis_ (Harry et al., 1987) QP (ACLP) medium was better than SH. However, with yellow cypress ACLP medium supported good caulogenesis but the CD media gave poor results and supported only a limited amount of shoot induction. In several cases half-strength basal medium was superior to full-strength medium for conifer caulogenesis (Berlyn and Beck, 1980; Ellis and Bilderback, 1984; Flinn et al., 1986; Harry et al., 1987). However, in our work, a dilution of SH was not better than full-strength medium (Table 4). Similar results occurred with _Pinus strobus_ (Flinn et al., 1986). Our results

support the hypothesis that each conifer species has particular nutritional requirements for optimal caulogenesis (Thorpe and Patel, 1984).

Our rooting trials show that micropropagation of *Chamaecyparis nootkatensis* by adventitious shoot production is possible. In this present study, *in vitro* rooting yielded poor results. This contrasts with the work of Ishii (1986) and Harry et al. (1987) who found that addition of auxins was required for rooting and Ishii (1986) found the addition of riboflavin was also necessary.

Our best rooting response (60%) was achieved under non-sterile greenhouse conditions using a peat/perlite mix. Similar results were obtained by Harry et al. (1987), Patel and Thorpe (1986) and Rumary and Thorpe (1984) who found that rooting was improved by placing shoots directly into a potting mix under greenhouse conditions. Microshoots lifted several months after outplanting exhibited a very fibrous and active root system. However, it is not clear at this time as to when actual root development occurred. Further studies would be required to determine this.

Basal application of IBA did not enhance the frequency of rooting but, did increase shoot caliper. This could be due to the effect of auxin on the base of the microshoot. While IBA did not appear to have a major effect in stimulating rooting in yellow cypress microshoots, it has proven successful with

other members of the Cupressaceae (Harry et al., 1987; Ishii, 1986) as well as with other coniferous species (Patel and Thorpe, 1986; Rumary and Thorpe, 1984; Webb et al., 1988).

More importantly, this study demonstrates that yellow cypress microshoots can be integrated into an operational forest regeneration program.

AXILLARY SHOOT MULTIPLICATION SYSTEM

RESULTS

To determine the best explant, shoots were harvested from three designated regions on the stock plants and cultured on basal medium. Selection of the most responsive explant was assessed by determining the percent survivability and degree of branching. In terms of overall performance the juvenile-type explant (Fig. 9) harvested from the basal region was the best explant in terms of survivability (Table 8). However, a progressive decline in juvenile shoot performance occurred from the base to the top-crown region. Juvenile explant performance *in vitro* was more or less equivalent from the base to the apex of each branch and no strong gradient was observed. As a result of this experiment, shoots with juvenile foliage harvested from the basal region were selected as the explants for further work.

To evaluate the role of basal medium composition on axillary shoot production, MSK, CBM and SH were tested. Shoot performance was superior on MSK medium when compared with CBM and SH media (Table 9). While axillary shoots developed on all media, twice as many developed on MSK. Consequently, all subsequent experiments were conducted using MSK medium.

The different juvenile explant types described in the Materials and Methods section, were used to test the effects of BA applied both basally through the agar-solidified culture medium and as a liquid pulse to the entire explant.

TABLE 8 The performance of juvenile and mature yellow cypress explants harvested from the basal, mid-crown and top-crown regions of the stock plant after 12 weeks on MSK basal medium [N=30].

Plant Region	Foliage Type	%Explant Survival	%Explants w. Branching	Ave. No. of Shoots/ SFE[a] [±SEM][b]	SFC[c] Index
Basal	Juvenile	100	71	2.5±0.09	1.77
	Mature	N/A[d]	N/A	N/A	N/A
Mid-Crown	Juvenile	90	60#	1.6±0.08*	0.96
	Mature	70	55#	1.0±0.00*	0.55
Top-Crown	Juvenile	100	55#	1.5±0.08	0.82
	Mature	88	52#	1.4±0.09	0.72

[a]SFE = Shoot forming explant

[b]SEM = Standard error of the mean

[c]SFC Index = Mean number of shoots/explants x percent of shoot forming explants divided by 100.

[d]N/A = Not applicable as no mature foliage found in this region.

= P-value < 0.05 using Chi-square test

* = P-value < 0.05 using one-way Anova test

TABLE 9 Influence of basal media on axillary shoot formation by yellow cypress juvenile shoots after 12 weeks.

Medium	N	No. of Cultures w. ax. Branching[a]	%Explants w. ax. Branching	Ave. No. ax. Shoots/ SFE[b] [±SEM][c]	SFC[d] Index
MSK	40	32	80#	2.5±0.12*	2.00
CBM	40	27	67#	1.5±0.09*	1.00
SH	40	20	50#	1.3±0.09*	0.65

[a]ax. Branching = Axillary branching

[b]SFE = Shoot forming explant

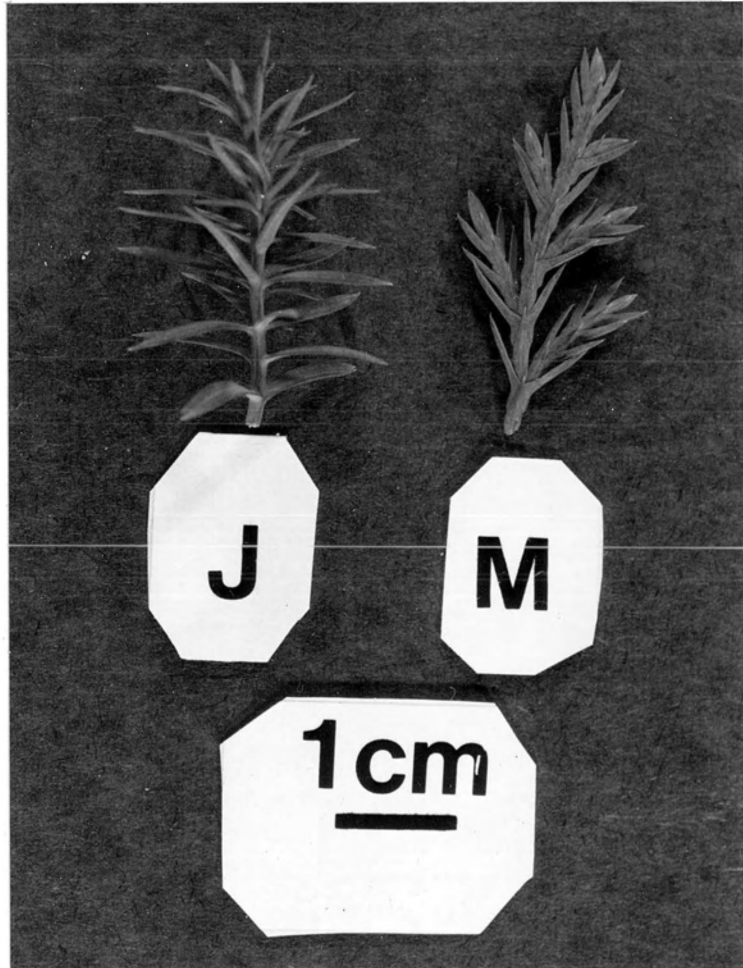
[c]SEM = Standard error of the mean

[d]SFC Index = Mean number of shoots per explant x percent of shoot forming explants divided by 100.

= P-value < 0.05 using Chi-square test

* = P-value < 0.05 using one-way Anova test

FIGURE 9 Two distinct yellow cypress explant types, one juvenile (J) and the other mature (M) collected from a two year old stock plant (#9777). Note the difference in leaf structure and configuration between the two types. Scale bar = 10 mm.


J
M
1cm

Decapitation and its effect on axillary shoot development was also evaluated. Intact, tip and decapitated explants were exposed to BA (1.0, 5.0, 10.0 25.0 mgl^{-1}) through a basal application in agar. None of the BA levels tested were stimulatory. Shoot performance at the lowest BA level (1.0 mgl^{-1}) was equivalent to the control and higher concentrations were inhibitory (Table 10). In terms of the frequency of explant survival and the degree of branching, the intact explant performed the best on both growth regulator-free medium and with 1.0 mgl^{-1} BA (Table 10). Axillary shoot production by the intact explant was greater than by either the tip or decapitated explant by a factor of two and eight, respectively.

Pulse treatments of two, eight or 24 hours were applied to intact, tip and decapitated explants (Tables 11, 12). While many of the explants in the first pulse experiment (Table 11) were lost due to microbial contamination, thus reducing the `n´ number, it was possible to draw some tentative conclusions. The pulse treatment was not detrimental to explant survival and growth (Table 11). Intact explants receiving a basal medium pulse performed better than those that did not. Pulsed explants had better shoot growth, were greener and formed more basal callus.

Although pulsing seemed to stimulate growth there was little stimulation of axillary branching (Tables 11, 12, 13)

TABLE 10 Effect of BA applied basally and decapitation on shoot production by yellow cypress explants after 12 weeks on MSK medium [N=30].

Explant Type	Treatment BA ($mg l^{-1}$)	No. of Cultures w. ax. Shoots[a]	%Cultures w. ax. Shoots	Ave. No. ax. Shoots/ SFE[b] [±SEM][c]	SFC[d] Index
I[e]	0.0	21	70#	2.6±0.13*	1.82
	1.0	20	66#	2.5±0.13*	1.65
	5.0	10	50#	1.5±0.13*	0.75
	10.0	0	0	0.0±0.00	0.00
	25.0	0	0	0.0±0.00	0.00
T[f]	0.0	16	53#	1.6±0.09*	0.84
	1.0	5	16#	1.6±0.30*	0.25
	5.0	4	13#	1.0±0.00*	0.13
	10.0	0	0	0.0±0.00	0.00
	25.0	0	0	0.0±0.00	0.00
D[g]	0.0	1	3.3	1.0±0.00	0.03
	1.0	1	3.3	1.0±0.00	0.03
	5.0	0	0	0.0±0.00	0.00
	10.0	0	0	0.0±0.00	0.00
	25.0	0	0	0.0±0.00	0.00

[a]ax. shoots = Axillary shoots

[b]SFE = Shoot forming explant

[c]SEM = Standard error of the mean

[d]SFC Index = Mean number of shoots/explants x percent of shoot forming explants divided by 100.

[e]I = Intact

[f]T = Tip

[g]D = Decapitated

= P-value < 0.05 using Chi-square test

* = P-value < 0.05 using one-way Anova test

TABLE 11 Effect of a two, eight or 24 hour BA [250 uM] pulse on axillary shoot production by yellow cypress explants after 12 weeks on MSK medium [N=30].

Explant Type	Treatment (No. of Hours)	No. of Cultures w. ax. Shoots[a]	%Cultures w. ax. Shoots	Ave. No. ax. Shoots/ SFE[b] [±SEM][c]	SFC[d] Index
I[e]	NPC[f]	15	50#	1.8±0.13*	0.90
	2 Cont[g]	8	26#	3.4±0.09*	0.88
	2 BA	3	10#	1.6±0.25*	0.16
	8 Cont	4	13#	3.5±0.30*	0.45
	8 BA	6	20#	3.1±0.26*	0.62
	24 Cont	7	23#	2.6±0.18*	0.59
	24 BA	12	40#	3.3±0.15*	1.32
T[h]	NPC	12	40	1.4±0.12	0.56
	2 Cont	9	30	1.6±0.18	0.48
	2 BA	15	50	1.5±0.10	0.75
	8 Cont	12	40	1.7±0.13	0.68
	8 BA	12	40	1.6±0.14	0.64
	24 Cont	4	13	1.5±0.23	0.19
	24 BA	12	40	1.7±0.13	0.68
D[i]	NPC	1	3.3	1.0±0.00	0.03
	2 Cont	5	16	2.4±0.15	0.38
	2 BA	11	36	1.4±0.13	0.50
	8 Cont	5	16	2.4±0.15	0.38
	8 BA	5	16	1.6±0.19	0.25
	24 Cont	7	23	2.0±0.00	0.46
	24 BA	7	23	1.0±0.00	1.23

[a]ax. Shoots = Axillary shoots

[b]SFE = Shoot forming explant

[c]SEM = Standard error of the mean

[d]SFC Index = Mean number of shoots/ explants x percent of shoot forming explants divided by 100.

[e]I = Intact explant

[f]NPC = Non-pulse control

[g]Cont = Basal medium pulse

[h]T = Tip explant

[i]D = Decapitated explant

= P-value < 0.05 using Chi-square test

* = P-value < 0.05 using one-way Anova test

TABLE 12 Effect of an eight hour BA [250 uM] pulse on axillary shoot induction by yellow cypress explants after 12 weeks on MSK basal medium [N=30].

Treatment	No. of Cultures w. ax. Shoots[a]	%Cultures w. ax. Shoots	Ave. No. of ax. Shoots/SFE[b] [SEM][c]	SFC[d] Index
I-NP[e]	10	33#	5.3±0.15*	1.74
I-PC[f]	21	70#	2.2±0.12*	1.54
I-PT[g]	28	93#	4.0±0.09*	3.77
T-NP[h]	21	70	1.7±0.10*	1.19
T-PC	15	50	1.2±0.08*	0.55
T-PT	13	43	1.0±0.00*	0.43
D-NP[i]	8	26	1.2±0.14	0.31
D-PC	5	16	1.0±0.00	0.16
D-PT	7	23	1.0±0.00	0.23
L-NP[j]	4	13	1.0±0.00	0.13
L-PC	5	16	1.2±0.18	0.19
L-PT	0	0	0.0±0.00	0.00

[a]ax. Shoots = Axillary shoots

[b]SFE = Shoot forming explant

[c]SEM = Standard error of the mean

[d]SFC Index = Mean number of shoots/explants x percent of shoot forming explants divided by 100.

[e]I-NP = Intact explant with no pulse

[f]I-PC = Intact explant with basal medium pulse

[g]I-PT = Intact explant with BA pulse

[h]T-NP = Tip explant with no pulse

[i]D-NP = Decapitated explant with no pulse

[j]L-NP = Lateral explant with no pulse

= P-value < 0.05 using Chi-square test

* = P-value < 0.05 using one-way Anova test

TABLE 13 Effect of an eight hour BA pulse [250uM] on axillary shoot formation by yellow cypress intact explants after 12 weeks on MSK basal medium.

Treatment	N	No. of Cultures w. ax. Shoots[a]	%Cultures w. ax. Shoots	Ave. No. ax. Shoots/ SFE[b] [SEM][c]	SFC[d] Index
Pulse-Cont[e]	36	26	72	3.3±0.10	2.37
Pulse-BA	36	29	80	3.5±0.35	2.80

[a]ax. shoots = Axillary shoots

[b]SFE = Shoot forming explant

[c]SEM = Standard error of the mean

[d]SFC Index = Mean number of shoots per explant x percent of shoot forming explants divided by 100.

[e]Pulse-Cont = Basal medium pulse

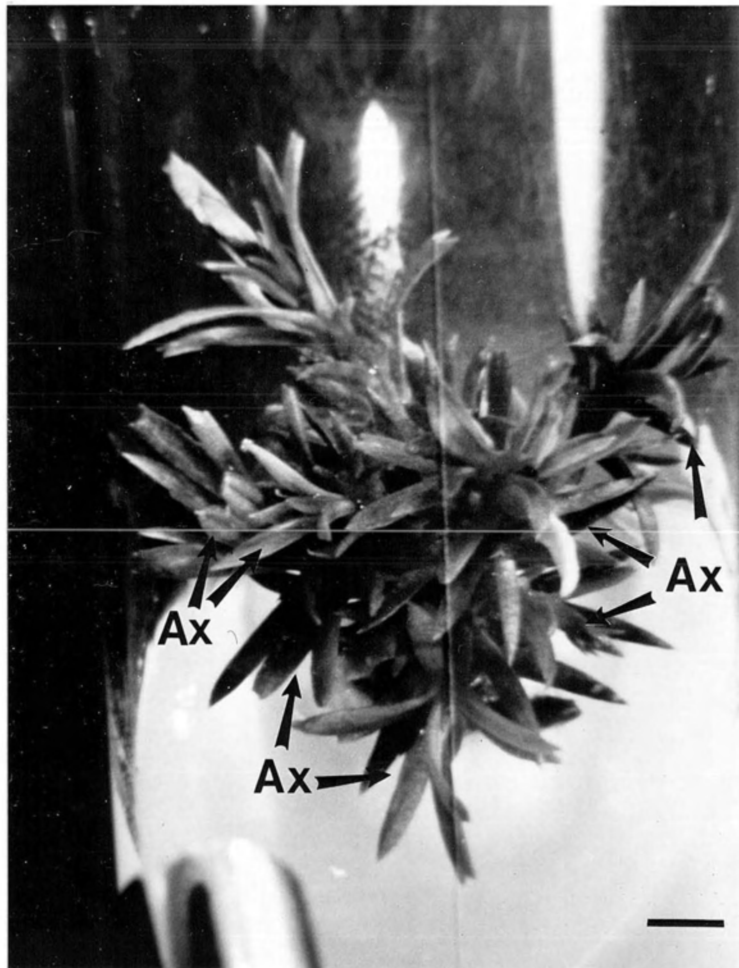
and most of the new shoots developed from the existing terminal and the laterals.

With intact explants pulsing with BA had a slight stimulatory effect on branching (Fig. 10) and did increase explant survival (Tables 11, 12, 13). However, there was no apparent trend with the different pulsing periods (Table 11) and for the sake of convenience, all subsequent pulse experiments used an eight hour treatment.

Axillary shoot production of the intact explant was increased by a factor of two over the growth regulator-free pulsed explants (Table 12). Pulsing with basal medium improved the survivability of the explants over non-pulsed explants. Shoot production was equivalent to the BA pulse but only 33% of the former explants survived and these had lost their terminal shoots. Consequently, this result must be viewed with caution (Table 12). Regardless, overall shoot formation was higher with the BA pulse over either the non-pulse or basal medium pulse because of the higher frequency of explant survival (Tables 11, 12, 13). New growth tended to be abnormal following BA exposure. This foliage took on a normal morphology later.

With tip explants, pulse treatments were not stimulatory (Tables 11, 12). Explants pulsed with BA were more stunted than those pulsed with basal medium. The non-pulsed tip explants had better color and growth than explants pulsed with either basal medium or BA (Tables 11, 12).

FIGURE 10 Axillary shoots (Ax) produced by a yellow cypress intact explant after an eight hour BA [250uM] pulse followed by a further eight weeks of culture on MSK basal medium.
Scale bar = 2.2 mm.


Ax
Ax
Ax
Ax

Decapitation of explants had a negative effect on explant survival and branching as shown by the poor performance of the controls as well as treatments with BA (Tables 10, 11, 12).

Lateral explants did not perform well in culture. No stimulation of axillary shoot growth occurred after a BA pulse (Table 12). However, pulsing with basal medium did provide slightly better results than pulsing with BA (Table 12).

The auxins IBA and NAA reduced the frequency of explant survival when applied basally through the agar-solidified medium. However, 0.25 and 0.5 mgl^{-1} IBA did increase the number of axillary shoots formed (Table 14). Higher IBA levels caused a decline in the number of shoots forming axillary shoots. The best NAA concentration for stimulation of axillary shoots was 0.5 mgl^{-1} (Table 14). Both higher and lower levels were inferior. In addition, there was an increase in basal callus formation and severity of tissue browning at the higher auxin concentrations.

As previously observed in the BA pulse experiments, basal medium pulses were beneficial in stimulating axillary branching (Table 14). However, auxin pulses did little to stimulate axillary shoot formation (Table 14) which is opposite to the trend observed with the BA pulses (Tables 12, 13).

Pulsing with either IBA or NAA for two and eight hours, respectively, had little or no effect on the frequency of explant survival. However, longer exposures to auxin caused a decline in shoot formation (Table 14).

TABLE 14 Effects of IBA and NAA on axillary shoot production by yellow cypress intact explants via basal and pulse applications [N=20, except for control for basal application where N=40].

Treatment		No. of Cultures w. ax. Shoots[a]	%Cultures w. ax. Shoots	Ave. No. ax. Shoots/ [±SEM][c]	SFC[d] Index
IBA	0.00	28	70#	1.2±0.07*	0.84
(mgl^{-1})	0.25	15	75#	2.0±0.13*	1.50
BASAL	0.5	11	55#	2.0±0.16*	1.10
	1.0	8	40#	2.3±0.11*	0.92
NAA	0.25	10	50#	1.2±0.12*	0.60
(mgl^{-1})	0.5	9	45#	2.0±0.11*	0.90
BASAL	1.0	4	20#	1.0±0.00*	0.20
IBA	2hrPC[e]	19	95#	2.8±0.12*	2.66
(250uM)	2hrPT[f]	14	70#	2.2±0.14*	1.54
PULSE	8hrPC	10	50#	2.7±0.13*	1.35
	8hrPT	17	85#	1.5±0.10*	1.27
	24hrPC	14	70#	2.3±0.16*	1.61
	24hrPT	16	80#	1.0±0.00*	0.80
NAA	2hrPC	19	95#	2.8±0.13*	2.66
(250uM)	2hrPT	14	70#	2.2±0.10*	1.54
PULSE	8hrPC	17	85#	3.0±0.10*	2.55
	8hrPT	17	85#	1.5±0.10*	1.27
	24hrPC	13	65#	1.7±0.15*	1.10
	24hrPT	5	25#	1.0±0.00*	0.25

[a]ax. shoots = Axillary shoots

[b]SFE = Shoot forming explant

[c]SEM = Standard error of the mean

[d]SFC Index = Mean number of shoots per explant x percent of shoot forming explants divided by 100.

[e]PC = Basal medium pulse

[f]PT = BA pulse

= P-value < 0.05 using Chi-square test

* = P-value < 0.05 using one-way Anova test

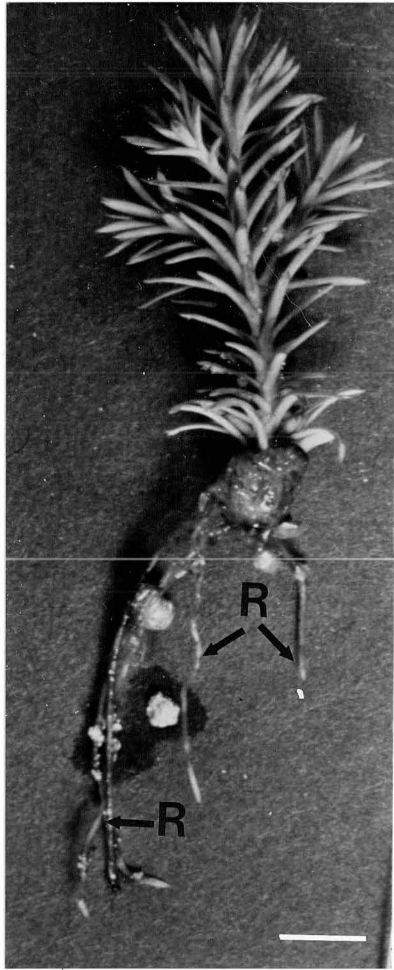
Overall, while pulsing seemed to stimulate growth, the pattern of shoot development observed was similar to previous BA pulse experiments (Tables 11, 12, 13, 14) where shoots arose from pre-existing laterals and not from de novo shoot induction. Both methods of auxin application were not overly beneficial to explant survival and did not enhance axillary shoot production (Table 14).

Micropropagules elongated on MSK basal medium. Those that attained an overall length of 5 mm or greater were selected for in vitro and in vivo rooting trials. As was observed with the microshoots in the embryo culture work, the formation of basal callus was associated with successful rooting of the micropropagules (Fig. 11). However, the frequency of rooting was low (15%). Similar results were obtained for in vitro and in vivo trials.

DISCUSSION

It is widely recognized that success in plantlet formation is dependent to a large extent on the selection of the explant (Murashige, 1974). Our results are in accord with Murashige (1974) and Sommer and Caldas (1981) who found that the more juvenile the tissue, the better it will respond to in vitro treatments leading to de novo primordium initiation and subsequent organogenesis. Our study had shown that juvenile explants from two-year old yellow cypress seedlings are superior to mature explants in all cases. Furthermore, we found that the region from which the explant was harvested was

FIGURE 11 Rooted micropropagule from *in vivo* rooting trial after 12 weeks. Note the formation of multiple roots (R). Scale bar = 2.0 mm.


R
R

also of importance. Explants from the basal region of the stock plant performed better than those from mid-crown or top-crown areas.

Preliminary experiments, which compared the shoot growth and survival of juvenile explants on CBM, SH and MSK media indicated that a limited induction of branching could occur on explants cultured on CBM and SH basal media. However, best shoot growth and survival occurred on MSK medium. This result is contrary to that observed for yellow cypress embryo explants where SH medium was the best and MSK medium was not as good. The difference in explant response could be attributed to the explant type and age, to genotype, environmental conditioning, endogenous phytohormones (Bornman, 1983), preconditioning of donor plants (Jansson and Bornman, 1980), or to the occurrence of inorganic compounds provided in each medium and their subsequent availability to the plant tissue (Mohammed and Dunstan, 1987).

Of the four different juvenile explant types examined, the intact performed the best overall. Our data suggest that explant type and size are an important consideration. Intact explants, with an overall length of 1 - 2 cm were superior to the smaller tip and lateral explants. The larger intact explants also exhibited a higher frequency of shoot survival and branching over the latter two. Work done with *Cryptomeria japonica* (Isikawa, 1987), *Sequoia sempervirens* (Ball, 1987) and *Pinus radiata* (Horgan, 1987) support our finding that

shoot-tip explants measuring between one and three centimetres are the preferred explant and perform best as shoot-tip cultures.

Furthermore, decapitation of yellow cypress explants had a negative effect on shoot survival and branching. Our conclusions agree with Ilahi (1986) who found that decapitation of Juniperus polycarpus explants resulted in the senescence of the explant after four weeks in culture. In contrast, work done with Pinus radiata (Horgan, 1987) has shown that decapitation results in the formation of axillary shoots and, in addition, normal shoot development occurs from the excised terminal portion of the explant. This could be due to the fundamental difference between the branching systems of the genus Pinus and the genera Chamaecyparis and Juniperus. In the genus Pinus, the leaves are needle-like appearing in clusters or bundles of more than one. This may be more tolerant of decapitation than the branching system of the genus Chamaecyparis and Juniperus where the leaves are scale-like and generally closely pressed against the branchlets.

Plant growth regulators traditionally are incorporated into the medium. However, they can also be administered as high concentration pulses (Bornman, 1983; Bornman and Vogelmann, 1984; Misson, 1987; Vogelmann et al., 1984; von Arnold and Eriksson, 1985). Pulsing allows for the direct uptake or contact of BA to the target areas. In our work, when BA was applied as a liquid pulse it was more effective

for shoot growth and survival than application via agar incorporation. This result is in accord with work done on *Thuja plicata* (Misson, 1987) and *Picea abies* (Bornman, 1983; Bornman and Vogelmann, 1984; Vogelmann et al., 1984; von Arnold and Eriksson, 1985) where pulsing with BA was beneficial for enhancing axillary shoot and adventitious bud development. Our data show that while stimulation of axillary branching was evident, axillary shoots developed only from pre-existing lateral apices. Failure to induce *de novo* axillary shoot formation could be attributed to a number of factors. No anatomical work was done to confirm the existence of latent axillary shoot primordia. Therefore, it is not clear at this time as to whether BA did in fact reach these sites and if so was simply not capable of activating them or if there were no primordia present for the BA to act upon. Bornman and Jansson (1983) thought that the xeromorphic nature of the conifer leaf might impose physiological and morphological constraints on its ability to take up BA. This in turn would reduce the availability of meristematic tissues capable of dedifferentiation and, therefore, of morphogenic response. The physiological state of the parent plant can play a decisive role on the explant's behavior in culture (von Arnold, 1981). Genetic uniformity and stage of development or age of the plant are important to consider. The fact that the yellow cypress stock plants were not genetically uniform and

that they were subjected to varying environmental conditions could have caused a delay in shoot development and subsequent loss of morphogenesis. It is also possible that repeated exposures to BA pulsing are required for the stimulation of axillary shoots to occur (McCowan, 1986).

The morphological abnormalities resulting from the BA pulses of intact explants were similar to those observed by John (1983) in vitrified cultures of Picea sitchensis. Needles were dark green and swollen, bent, twisted and somewhat brittle. John and Webb (1987) found that inducing vitrification by submerging Picea sitchensis explants into distilled water and their subsequent reversion, resulted in accelerated shoot growth and development. Furthermore, they discovered that vitrification removed apical dominance resulting in the development of axillary shoots. Whether this abnormal branching and subsequent shoot formation observed with yellow cypress intact explants can solely be attributed to the effect of BA or in part to virtrification, brought about by the pulse treatment, is not clear. Pulsing could work on a similar principle as John and Webb's (1987) vitrification process. By providing for anoxic conditions, thereby changing the physical environment, certain events can be triggered within the explant as evidenced by John and Webb's (1987) work. This suggests that BA may not be the only exogenous factor in the stimulation of axillary shoots (Bornman and Vogelmann, 1984).

Overall, pulsing had a beneficial effect on shoot performance of intact explants. Those receiving a BA and or a basal medium pulse performed better than non-pulsed explants. In contrast, a non-pulse treatment was best for tip and lateral explants and a basal medium pulse better than a BA pulse.

Our results have shown that when BA is incorporated into agar-solidified medium, it has a negative effect on the stimulation of axillary shoots. There was little to no axillary branching evident. Concentrations as low as 1.0 mgl^{-1} BA were detrimental and caused severe browning and senesence. This lends support to Amos and McCowan's (1981) findings that members of the Coniferophyta are more sensitive to BA than woody dicotyledonus species. Furthermore, Amos and McCowan's (1981) work with Juniperus sp., Sequoia sempervirens, Thuja plicata and Tsuga heterophylla showed that while a basal medium supplemented with BA stimulated shoot production, it did so only at very low concentrations. Higher levels of BA were inhibitory to shoot growth.

The pulse experiment examining the effects of IBA and NAA on axillary shoot formation indicated that explants did not benefit from such an application of auxin. Furthermore, auxins applied through the basal medium were also equally poor in stimulating axillary branching. Our conclusions suggest that neither mode of auxin application promotes in axillary shoot development.

Preliminary studies have shown that yellow cypress micropropagules can root under laboratory and greenhouse conditions. In this present study a 15% level of spontaneous rooting occurred. Similar cases have been reported for other conifers (Amerson and Mott, 1982; Patel and Thorpe, 1984; von Arnold, 1982). Amerson and Mott (1982) found that a pulse treatment with auxin had a two-fold increase in the frequency of rooting over those explants receiving a continuous level of auxin in the medium. Improved root growth and the initiation of multiple roots were also favored by pulse treatments. Other workers have also shown pulse treatments to be very effective in stimulating root growth (Aitken-Christie and Thorpe, 1984; Bornman, 1983; Mott and Amerson, 1981; Patel and Thorpe, 1984; Poissonnier et al., 1980). These factors need to be explored with yellow cypress micropropagules.

SUMMARY AND CONCLUSIONS

Yellow cypress (*Chamaecyparis nootkatensis*) is native to coastal B.C. where it grows at mid to high elevations. It is a significant export crop and its logs are two to six times more valuable than Douglas-fir. Unfortunately, seed production cannot meet current or future needs. Fertilization is poor and few seeds are produced. It takes two years for cones to ripen and seeds require a complex series of manipulations to germinate. Seed germination is still unpredictable and can require two seasons. The seed's shape prevents automated planting. Consequently, the province has developed a vegetative propagation program for yellow cypress. Tissue culture techniques offer powerful alternatives to cuttings and have proven successful in horticulture and agriculture (Zimmerman et al., 1986).

The primary goal of this study was to analyze factors responsible for *in vitro* shoot proliferation for yellow cypress by adventitious and axillary shoot multiplication. Mature zygotic embryos and shoot tips from two-year old seedlings were used as explants. The methodology and approaches used in obtaining this goal have included the interaction of media composition and strength and phytohormones. In addition, the effects of seed stratification on caulogenesis were evaluated. Furthermore, dose response studies and varying methods of growth regulator application, as well as experimental studies relating to the

physiological status of the explant (i.e., age of explant, size of explant, proximity of explant in relation to harvesting from stock plant) were examined. In order to complete the micropropagation system, the long term goal of this work focused on assessing the rooting potential of those shoots developed *in vitro*.

EMBRYO CULTURE SYSTEM

Prior work done with other members of the Cupressaceae (Coleman and Thorpe, 1977; Franco and Schwarz, 1985; Ilahi, 1986; Ishii, 1986; Thomas et al., 1977) has shown that embryos are good explants for adventitious shoot induction. Therefore, embryo culture with yellow cypress was initiated. Mature seeds from a mixed population of open pollinated yellow cypress were used for all experiments. Seeds were surface sterilized and embryos were either dissected out directly and placed onto culture media or underwent a stratification of four weeks at room temperature and up to 20 weeks at 5°C prior to dissection.

Excised embryos were cultured on CD (Campbell and Durzan, 1975), CDAA (CD medium modified by Ishii, 1986), CBM (Cupressus Basal Medium, Franco and Schwarz, 1985), SH (Schenk and Hildebrandt, 1972), or ACLP (LP medium of Aitken-Christie, 1984) media. Two additional media, MSK (modified Murashige and Skoog, 1962 (see Materials and Methods)) and MSH (modified SH (see Materials and Methods)) were also used in this study. In all cases the minerals were supplemented with sucrose (30 gl^{-1}).

All experiments were conducted at 23°C ± 2°C and received a 16 hour photoperiod. The cultures were illuminated with cool white fluorescent lights with an irradiance of 25 umols $m^{-2}s^{-1}$.

Because benzyladenine (BA) has promoted adventitious shoot formation in a number of species in the Cupressaceae (Coleman and Thorpe, 1977; Franco and Schwarz, 1985; Ishii, 1986; Thomas et al., 1977; Thomas and Tranvan, 1982) it was chosen for the yellow cypress shoot induction and proliferation experiments. BA was applied basally through the culture medium. Four weeks on SH medium with 0.35 mgl^{-1} was optimal for caulogenesis. Sixty-eight to seventy-three percent of embryos cultured formed adventitious shoots with a mean of five shoots per embryo and a range of 1 - 14 shoots per embryo. Shoots arose primarily from the cotyledons whether they were in contact with the medium or not.

Because the optimal BA level was so low on the original SH formulation containing 1000 mgl^{-1} inositol, the effect of inositol concentration on the caulogenic capacity of yellow cypress embryos was investigated. Work done by Pollard et al. (1961), Steinhart et al. (1962), Staudt (1984) and White (1963) has shown inositol to have a promotive effect on cell growth and cell division. Of the two inositol concentrations (100 and 1000 mgl^{-1}) we tested there was little difference noted in the caulogenic response. However, it appeared that 1000 mgl^{-1} was somewhat better in that a greater number of shoots per shoot-forming embryo were formed at 0.35 mgl^{-1}BA.

Yellow cypress seeds have a prolonged stratification requirement for germination (Owens and Molder, 1982). However, we found that by culturing yellow cypress embryos on basal medium, without any seed pre-treatment, 90% germination occurred. Subsequently, we conducted experiments to test the effects of seed stratification on caulogenesis. Our results indicated that a stratification of four weeks at 21°C followed by eight weeks at 5°C increased the mean number of shoots per shoot-forming embryo over unstratified controls. However, while there was a stimulation of adventitious shoot production with stratified embryos, stratification had a negative effect on the frequency of embryo response. Embryos exposed to an additional four weeks at 5°C exhibited a change in the pattern of adventitious shoot formation. Following stratification shoots were observed arising from the hypocotyl as well as the cotyledons. Overall, our results show that stratification of the seed is not required for caulogenesis, however, our data suggests that stratification enhances the mean number of shoots per embryo and, thus, increases the total shoot formation.

Shoot growth on the explant was promoted by transfer to growth regulator-free SH medium. New shoots continued to develop from the cotyledonary region over the next two monthly subcultures onto basal SH medium at which time the maximum yield per explant was obtained and shoots could be excised

from the original explant. Those microshoots greater than 5 mm in overall size were selected for *in vitro* and *in vivo* rooting studies.

Only about 15% of the elongated shoots rooted *in vitro*. However, a 62% frequency of rooting was achieved by those microshoots planted out under non-sterile operational greenhouse conditions. Microshoots were placed together with yellow cypress cuttings. No modifications were made to the environment to accommodate the microshoots. Those microshoots lifted 9 - 12 months after outplanting had an average shoot height of 10.0 - 13.9 cm with an average shoot caliper ranging between 2.29 - 2.68 mm. There was no apparent difference in terms of rooting frequencies between microshoots treated with rooting powder (0.8% IBA) and those without. However, IBA did increase shoot caliper. Further studies will be undertaken to document the relative nursery performance of these propagules compared to seedlings and cuttings.

AXILLARY SHOOT MULTIPLICATION SYSTEM

Two basic explants, one exhibiting all juvenile foliage and the other with mature leaf morphology, were tested for their ability to form axillary shoots *in vitro*. Our results indicated that 100% shoot survival was obtained by those explants with an all juvenile foliage harvested from the basal region of the stock plant. Shoots, 1.5 - 2.0 cm in overall size, performed the best with a 71% frequency of branching. Regardless of explant type, MSK medium was the best. The

juvenile explant was further subdivided into four separate explant types. They included the `intact´ explant which was a shoot tip approximately 1.5 - 2.0 cm long consisting of the terminal apex and 2 - 3 lateral branches. The `tip´ explant was the top 1.0 cm of the intact with a terminal apex and some associated stem tissue without apparent laterals. `Lateral´ explants were the small lateral shoots from the intact explant. `Decapitated´ explants had the apical portion removed and consisted of the remainder of the intact explant. These explants were used to determine what the best explant type would be and which method of BA application (i.e., basally through agar-solidified medium or as a liquid pulse to the entire explant) was more effective in the stimulation of axillary shoots. Our data showed that no stimulation of _de novo_ branching occurred with those explants receiving a basal application of BA. However, in terms of shoot survival and branching, the intact explant performed the best while the decapitated explant gave the poorest results.

In contrast, pulsing treatments with basal medium were not detrimental to explant survival and growth. Pulsed explants formed basal callus, grew better and were greener when compared to non-pulsed controls. However, shoot production came from pre-existing laterals and not from _de novo_ shoot induction. A BA [250 um] pulse had a slight stimulatory effect on branching but only with the intact explant. Axillary shoot production was increased by a factor of two

over non-pulsed explants in approximately 80% of the cultures. There was no apparent trend evident for the different pulsing periods (2, 8, 24 hours) with any of the explant types tested.

The auxins IBA and NAA, when applied separately through the culture medium, reduced the frequency of explant survival. Only a slight increase in the mean number of axillary shoots formed occurred when IBA was added at either 0.25 or 0.5 mgl^{-1}. Similar results were obtained when NAA was added at 0.5 mgl^{-1}. Auxin pulses did little to induce axillary shoot development. Shoot formation was poor and irregular. While there was no effect on the frequency of explant survival, longer auxin exposures caused a decline in shoot formation. Neither method of auxin application was found to be beneficial.

Micropropagules elongated after several subcultures on MSK basal medium. Those with an overall length of 5 mm or greater were harvested for *in vitro* and *in vivo* rooting trials. In both cases a 15% level of spontaneous rooting occurred.

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