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Vegetative propagation is most effective when individuals of demonstrated superior characteristic can be propagated. For this reason, a method for the induction of adventitious buds on cultured Douglas-fir shoot tips from 20-25 year old trees was developed. Douglas-fir shoot tips failed to survive and grow on a full strength Murashige and Skoog (M&S) mineral salt medium without growth regulators but would if the KNO_3 and NH_4NO_3 levels were reduced to one-fourth original strength. On a medium containing 10^{-4}M N_6-benzylaminopurine (BAP) and 0.0 or 10^{-7}M napthaleneacetic acid (NAA) adventitious buds formed on the needle primordia of the shoot tips. These adventitious buds formed only when the basal medium contained one-fourth strength NH_4NO_3 suggesting that the NH_4 ion level in the medium affected adventitious bud formation. Other

cytokinins, including kinetin and zeatin when applied at 10^{-4}M would also induce adventitious bud formation, while N_6-isopentenylpurine (2iP) even when applied at 10^{-3}M did not. Only a two to three week culture period on the high cytokinin medium was necessary to induce adventitious bud formation.

The regeneration of adventitious buds from unorganized callus cultures of Douglas-fir was also studied. Douglas-fir callus cultures were induced from aseptically grown seedlings placed on a one-half strength M&S (macro and microelements) containing 10^{-5}M NAA and 10^{-6}M BAP. Adventitious buds arose on subcultured callus placed on a medium containing 10^{-5}M BAP and either 0.0 or 10^{-7}M NAA. Other cytokinins including kinetin, zeatin and 6-benzyl-9-tetrahydropyrone adenine (SD 8339) were all able to induce adventitious bud formation when applied at 10^{-5}M, however, 2iP induced bud formation only when applied at 10^{-4}M. NAA levels greater than 10^{-6}M inhibited adventitious bud formation. Callus cultures formed adventitious buds on a one-half strength M&S medium, and again the NH_4 ion concentration was found to affect bud formation. The ability to form buds declined the longer callus was maintained in culture and it was completely lost after one year in culture.

In an attempt to understand the role of IAA in bud formation in Douglas-fir tissues in culture, a method for the rapid isolation and quantitation of endogenous IAA levels was developed. This involved extraction of the IAA in methanol, followed by purification of the extract by column chromatography and trace enrichment, separation on HPLC and detection by the natural fluorescence of IAA. The system

is sensitive enough to detect picogram amounts of IAA in milligram tissue samples.

No large differences were found in the endogenous IAA levels in bud forming and non-bud forming cultures. Estimates for IAA in Douglas-fir shoot tips collected before, during and after bud break in the spring showed that endogenous IAA levels increase dramatically just before bud break in the springand then begin to decline slowly. There were also no large differences in the endogenous IAA levels in Douglas-fir shoot tips forming and not forming adventitious buds. Because no large differences were found in either the callus or the shoot tip cultures forming or not forming buds, the role of endogenous IAA in regulating bud formation may be questioned. The results also show that this method of IAA analysis can be used to detect and quantify endogenous IAA levels in tissues and tissue cultures of Douglas-fir to aid in our understanding of the role of IAA in plant development.

Effect of Auxins, Cytokinins and Nutrition on Adventitious Bud Formation in Tissues of Douglas-fir Grown in Culture

by

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EFFECT OF AUXINS, CYTOKININS AND NUTRIENTS ON ADVENTITIOUS BUD FORMATION IN TISSUES OF DOUGLAS-FIR GROWN IN CULTURE

INTRODUCTION

Forest trees, because of their long life span typically may not express all of their genetic traits until the plant has reached a certain age. The only way to ensure that a propagule of a tree will retain the desired characteristics of the original tree is to propagate it vegetatively. Unfortunately traditional methods of vegetative propagation are most successful with young material in the so called juvenile growth phase before the tree is able to form flowers. Thus, by the time a tree may have demonstrated that it contains a certain desired trait or traits, conventional methods of vegetative propagation are unsatisfactory. The exact reason for this change is not understood.

Because of this problem, interest has developed in alternative methods of plant propagation. Ever since the early days of plant tissue culture when unorganized masses of tobacco cells could be induced to regenerate shoots or roots by treatment with certain plant growth regulating substances (Skoog and Miller, 1957) there has been interest in applying these techniques to propagate forest trees (Geissbuhler and Skoog, 1957; Haissig, 1965). One of the advantages of a tissue culture propagation system would be that the cost of propagating plants by this method would be offset by the large number of plants that could be produced. As an example Murashige et al.

(1974) have estimated that over one million plants could be propagated from one original explant in one year. Tissue culture propagation might also avoid some of the "aging" problems encountered with woody plants.

Some progress has been made in the propagation of conifers in tissue culture in that we are now able to regenerate complete plants from parts of seed embryos and very young seedlings (Sommer et al. 1975). However, the trees propagated by this method are of unknown and untested phenotypes so no selection of superior individuals can take place. Also these techniques work only with very young tissues and are not successful with seedlings even several months old, let alone mature trees. Only very recently have techniques been developed for the propagation of mature trees by inducing the formation of adventitious buds on cultured shoot tips of conifers (von Arnold and Eriksson 1979, Thompson 1980).

While the advantages of plant tissue culture as an alternative propagation method for forest trees are attractive, plant tissue culture techniques offer much more than a method of plant propagation. The culture of naturally haploid tissues of the plant such as pollen, anthers and female gametophyte tissue of seeds can be used to regenerate haploid plants which contain only one set of chromosomes. Thus, there are no hidden recessive traits and all the genetic information of the plant is expressed. Such plants would be useful in plant breeding programs. By exposing plant cells to treatments designed to cause mutations, new genotypes could be induced and selected from among millions of cells contained in a single flask.

Differences in growth habit and disease resistance have already been observed in potato plants regenerated from protoplasts (plant cells with the surrounding cell wall removed) (Shepard et al. 1980).

Rare and valuable germplasm for future breeding programs could be stored by freezing cells and tissues to liquid nitrogen temperatures and later thawing and regenerating plants. Perhaps the most promising application is in the "genetic engineering" of plant cells to manipulate and insert selected genetic characteristics into plant protoplasts much like current work with bacterial cells.

The limiting factor in all of this is our inability to regenerate trees from unorganized cells and tissues. It is possible to regenerate shoots from embryonic and young seedling tissues and from cultured shoot tips of old trees, but we cannot do the same thing with unorganized cells and tissues like pollen, cell suspension or callus cultures. This is why the above mentioned applications of tissue culture to forest trees have not been accomplished.

One of the advantages of plant tissue culture is that plant parts or tissues can be grown isolated from the influences of the other parts of the plant. Thus,at least in theory single factor experiments can be done to determine the effect of one treatment on the growth of differentiation of the plant part or tissue. In this way the roles of some of the plant growth regulators on plant growth and differentiation were first identified (Skoog and Miller 1957).

Most of our knowledge of organ formation in plants arises from work done with herbaceous plants grown in culture. The basic principle of the chemical control of organ formation was done in

tobacco callus tissue over 25 years ago (Skoog and Miller 1957). They demonstrated that organ formation could be controlled by the ratio of two of the five classes of known plant growth regulators, the auxins and cytokinins. The auxins typically stimulate cell elongation while the cytokinins stimulate cell division. Both also have numerous other effects on plant cells. Skoog and Miller observed that a high ratio of auxin to cytokinin promoted root formation in tobacco callus while a reversal in the ratio promoted shoot formation. High levels of both auxin and cytokinin promoted the growth of unorganized callus tissue. This is the basic hypothesis for the regulation of organ formation in plants and it has been demonstrated in a large number of plant species.

Because we can control most of the factors that affect growth and differentiation in tissue culture, we believe that we have a good understanding of the factors that control organ formation. Unfortunately while we know the amounts and types of growth regulators we apply to tissue cultures, we are ignorant of the natural endogenous levels of auxin, cytokinins and other growth regulators in the tissues and how they may be affecting the response of the tissue.

One explanation as to why some tissues do not respond to treatments known to induce shoots or roots in other plants may be that the endogenous ratios of levels of growth regulators makes the exogenous growth regulator treatment unfavorable for organ formation. The common observation that tissues in culture tend to lose the ability to form organs the longer they are maintained in culture, has been attributed to increasing endogenous auxin levels in these tissues,

however, there is no direct evidence for this hypothesis (Negrutiu et al. 1978). Because conifer tissues grown in culture are notoriously reluctant to form either shoots or roots in culture, such tissues would appear to be useful in testing this hypothesis. Slight changes in endogenous auxin levels may result in changes in the location of induced adventitious buds. Thus, the role of endogenous auxin in the control of organ formation in Douglas-fir tissues grown in culture was examined as part of the present study.

The objectives of this study were threefold. First, to develop a propagation system using shoot tips from selected mature trees of desired characteristics. Second, to study the effect of exogenously applied plant growth regulators on shoot formation in unorganized callus cultures of Douglas-fir. Third, to measure the endogenous levels of indole-3-acetic acid, the major natural plant auxin in both cultured shoot tips and callus cultures of Douglas-fir.

INDUCTION OF ADVENTITIOUS BUDS ON CULTURED SHOOT TIPS OF DOUGLAS-FIR

ABSTRACT

Shoot tips of flowering Douglas-fir trees 25-years-old or more were grown on a modified Murashige and Skoog mineral salt medium. A reduction in the level of the NH_4NO_3 and the KNO_3 to one-fourth of the original strength was important for the growth and survival of the cultured shoot tip. Adventitious buds were formed on shoot tips grown on 10^{-4}M benzylaminopurine with either no or very low (10^{-7}M) napthaleneacetic acid. Adventitious buds could be identified after six weeks on the bud inducing medium, but only a two-three week exposure to the high cytokinin treatment was necessary. Kinetin and zeatin also induced adventitious bud formation, but 2-isopentylpurine did not, even when applied at 10^{-3}M. Adventitious buds formed only on a medium containing one-fourth of the original strength NH_4NO_3, thus implicating the NH_4^+ in the regulation of bud formation.

Introduction

Vegetative propagation offers methods for the selection and multiplication of individuals that have demonstrated superior characteristics. Conventional methods such as the rooting of cuttings (which declines with increasing age of the tree) and grafting (graft incompatibilities between scion and stock can occur) have not been used successfully on a large scale. Plant tissue culture techniques offer an alternative to the conventional methods of vegetative propagation, however, they have been confined to the multiplication of plants from embryonic or very young seedling tissues of unknown phenotypes. Thus, a method for the multiplication of mature trees selected for their demonstrated superior characteristics would be of importance both for forest genetics research and tree improvement programs.

Shoot tip culture (defined here as the culture of the shoot apical meristem including several leaf primordia contained in the bud) has been successful in the large-scale vegetative propagation of many horticulturally important plants as well as a few woody horticultural crops (Murashige 1978). The culture of shoot tips of conifers is not a new idea, for it was first attempted over 20 years ago with Douglas-fir (Al-Talib and Torrey 1959). Shoot tips of other conifers have been cultured over the years for various reasons (Chalupa and Durzan 1973, Coleman and Thorpe 1977, and Shibakusa 1977), however, the induction of adventitious and axillary buds on cultured shoot tips has only recently been reported (von Arnold and Eriksson 1979, Thompson 1980). The objective of this study was to develop a

method for the clonal propagation of selected mature individuals of Douglas-fir through the induction of adventitious and axillary buds on cultured shoot tips of mature trees.

Materials and Methods

Plant Material

Terminal resting buds on stem segments 10-12 cm long from 20-30 year old flowering trees were collected in the vicinity of Corvallis, Oregon. These stem segments could be stored in plastic bags at 1°C for up to six months before use, however, most were cultured immediately after collection. The needles were removed and the terminal resting bud was dipped in 70 percent ethanol for several seconds (but not flamed) and the bud scales were removed with sterile forceps. The bud scales were removed in one step by cutting through the bud scales at the widest point of the bud and sliding the bud scales off over the shoot tip. This exposed the entire shoot tip (the shoot apical meristem and all the needle primordia contained in the bud) which was excised and cultured.

Culture Media

The basal mineral medium used in these experiments was a modified Murashige and Skoog (1962) (M&S) mineral salt formula (see results) with both the KNO_3 and NH_4NO_3 reduced to one-fourth the original strength. The salt formula was supplemented with 100 mg/l myo-inositol, 0.5 mg/l pyridoxine, 0.5 mg/l nicotinic acid and 0.4 mg/l thiamine·HCL. All growth regulators were dissolved in several drops of 1N NaOH and then diluted with distilled deionized water to produce the growth regulator stock solutions. The pH of the medium was adjusted with

either 1N HCl or 1N NaOH to pH 5.7. Sucrose was added at 30 g/l and the medium was solidified with 10 g/l Difco Bacto agar. Twenty-five ml of medium was dispensed into 25 X 150 mm glass culture tubes and capped with Bellco Kaputs. All components of the culture media were autoclaved together at 121°C for 20 minutes and cooled on a slant. Ten culture tubes of each treatment were used in each experiment. All experiments were repeated at least twice.

Culture Conditions

Cultures were grown in a growth chamber at a constant 20°C under a 20 Wm^{-2} mixture of fluorescent and incandescent light on a 16 hour photoperiod. The shoot tips were grown for eight weeks before they were examined for the formation of adventitious buds.

Results

Effect of Mineral Nutrients

Shoot tips collected in the fall of 1977 did not have a high survival rate when placed on a medium consisting of full strength M&S salts without growth regulators. Those shoot tips that did survive failed to develop normal needles. For this reason, the effect of mineral nutrients on shoot tip survival, growth and development was examined. The concentrations of KNO_3 and NH_4NO_3 were reduced because they are present at the highest levels of any of the salts in the M&S medium. Both KNO_3 and NH_4NO_3 were applied at four levels, full, one-half, one-fourth and one-eighth strength, resulting in a total of 16 treatments. Higher survival rates and better development of the needle primordia into small needles was possible if both the KNO_3 and NH_4NO_3 levels were reduced to one-fourth original strength (475 mg/l KNO_3 and 412.5 mg/l NH_4NO_3). This medium was used as the basal mineral medium throughout the remainder of this study, unless stated otherwise.

Effect of Exogenous Growth Regulators

Because auxins and cytokinins have been demonstrated to regulate shoot and root formation in many plant tissues in culture (Murashige 1974), these were the first two classes of growth regulators examined. As an auxin, napthaleneacetic acid (NAA) and as a cytokinin

N_6-benzylaminopurine (BAP) were selected because of their stability during autoclaving of the medium and during the eight week culture period. Both of these growth regulators were applied together at levels of 0.0M, 10^{-7}M, 10^{-6}M, 10^{-5}M and 10^{-4}M which resulted in a total of 25 treatments.

After eight weeks in culture, the shoot tips grown on either no or very low (10^{-7}M) NAA and BAP treatments exhibited the most normal development of the needle primordia. Callus increased to a maximum at 10^{-5}M NAA but was reduced above that concentration. Increasing levels of BAP had little effect on the development of the shoot tip, other than a reduction in the development of needle primordia into needles. At the highest cytokinin treatment (10^{-4}M) with either no or very low (10^{-7}M) NAA the tips of some of the needle primordia rather than becoming the tip of a needle were transformed to shoot apices complete with needle primordia of their own (Figures 1,2). Of the 25 growth regulator combinations tested, adventitious shoots were found only in treatments containing 10^{-4}M BAP with either 0.0M or 10^{-7}M NAA. Between 40 and 80 percent of the shoot tips in these two treatments developed adventitious buds. They could be identified as adventitious buds after six weeks on the high cytokinin, low auxin medium.

Shoot tips collected at any time of the year between late August and just before bud break in the spring could be induced to form adventitious buds. There usually was a period of poor survival of the shoot tips during the late fall and early winter (November-December), perhaps due to the presence of some endogenous growth inhibitor in the shoot tip at the time of culture. Usually by late winter (January-

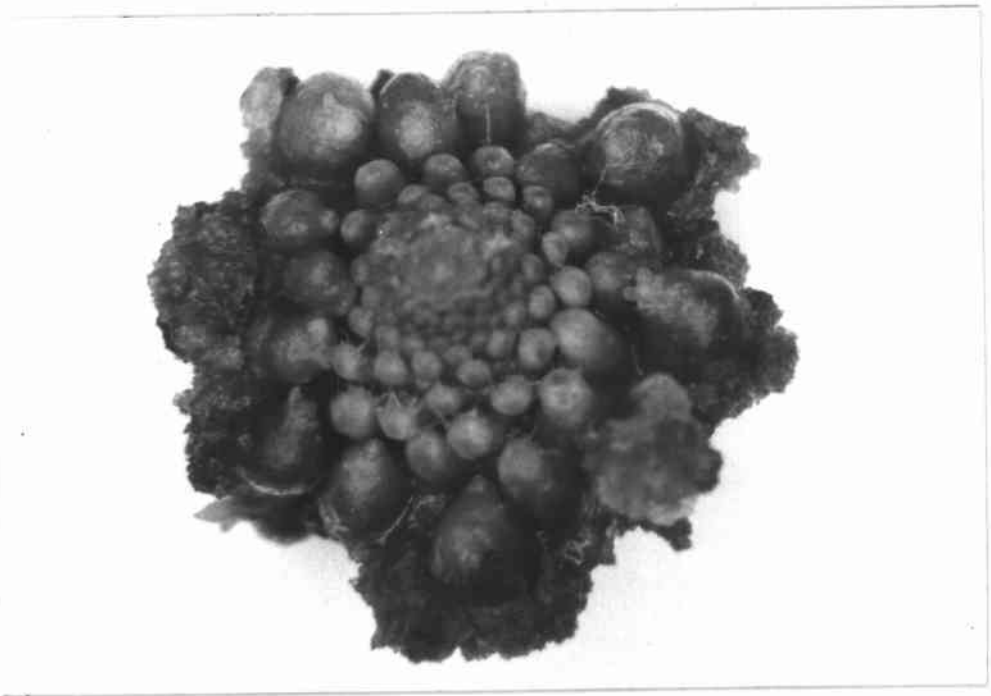

Figure 1. Douglas-fir shoot tip forming adventitious buds on the tips of the needle primordia.

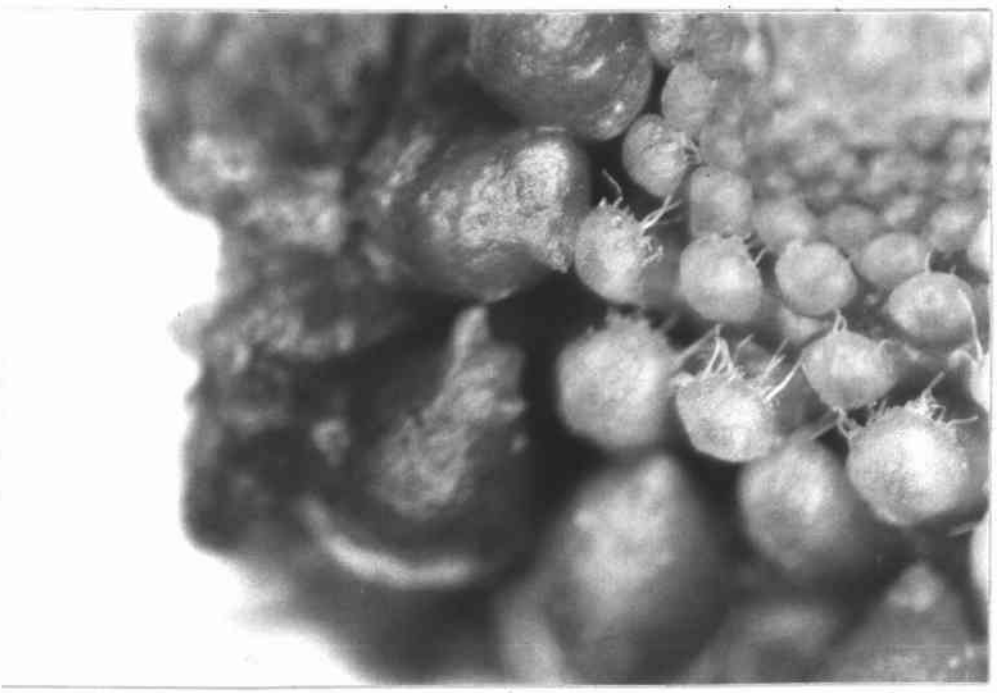

Figure 2. Enlargement of the tip of a needle primordium converted to an adventitious bud.

February) adventitious buds could again be induced on the cultured shoot tips. The best growth and development of the shoot tips and the highest rate of adventitious bud formation was on shoot tips collected several weeks before bud break (March-April). Growth and survival of the shoot tips also appeared to be affected by the severity of the winter, with mild winters producing many shoot tips that turned brown and died in culture and cold winters producing many shoot tips that grew well in culture.

The location of these adventitious buds varied apparently with the time of year when the shoot tips were collected. In late summer and early fall as well as in late winter and early spring the majority of the adventitious buds formed at the tips of the needle primordia (Figures 1,2). In late spring just prior to bud break these buds formed occasionally on the sides of the needle primordia (Figure 3) and more commonly at the base of the needle primordia (Figure 4). The buds at the base of the needle primordia may be axillary buds rather than true adventitious buds.

Effect of Mineral Nutrition

The effect of the basal mineral medium on the formation of the adventitious buds was profound. If shoot tips were grown on full strength M&S medium or one-half strength M&S (Both macro and micro-elements) with 10^{-4}M BAP and either no or 10^{-7}M NAA no adventitious bud formed (Table I). On the same growth regulator treatment, but with the level of the KNO_3 and NH_4NO_3 reduced to one-fourth strength adventitious buds formed. Results of both the KNO_3 and NH_4NO_3 applied at

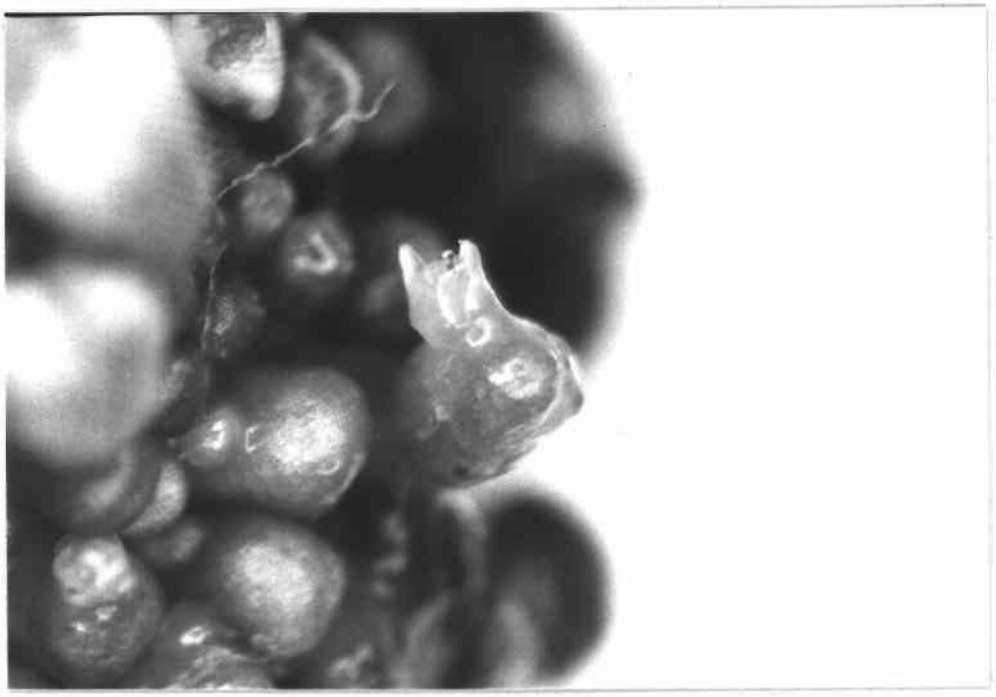

Figure 3. An adventitious bud forming on the side of a needle primordium.

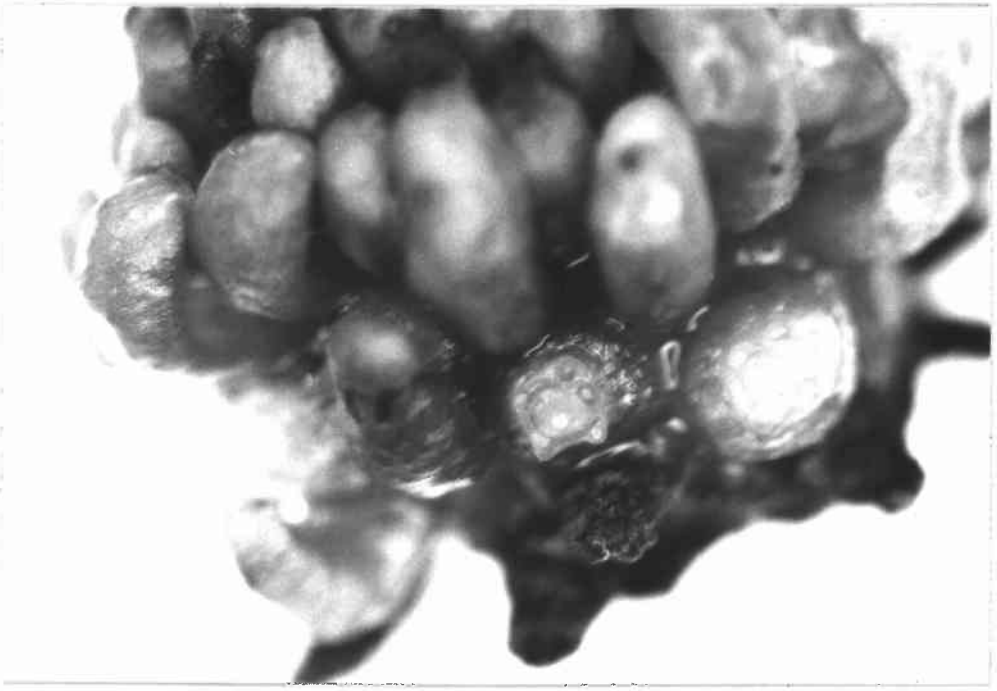

Figure 4. Adventitious buds forming at the base of needle primordia.

TABLE I. EFFECT OF MINERAL SALTS ON BUD FORMATION (n=10)

Medium	Number of buds formed	
	0.0M NAA 10^{-4}M BAP	10^{-7}M NAA 10^{-4}M BAP
full M&S	0	0
1/2 M&S	0	0
1/4 KNO_3 1/4 NH_4NO_3	6	7

full, one-half and one-fourth strength resulting in nine treatments, adventitious and axillary buds formed only in the treatments containing one-fourth strength NH_4NO_3. These results suggest that the high NH_4NO_3 level in the full strength M&S medium and in particular the high level of ammonium ions, inhibits adventitious buds formation (Table II).

Effect of Other Cytokinins

Test of cytokinins other than BAP showed that 10^{-4}M kinetin and 10^{-4}M zeatin also induced adventitious bud formation on cultured shoot tips at rates similar to those produced with BAP (40-80 percent). N_6-(isopentyl) purine (2iP), however, did not induce adventitious bud formation even when applied at 10^{-3}M.

Levels of BAP higher than 10^{-4}M (5 X 10^{-4}M and 10^{-3}M) were found to induce adventitious bud formation, but because of the high cytokinin levels involved, the mortality rate among the shoot tips was high

TABLE II. EFFECT OF REDUCED LEVELS OF KNO_3 AND NH_4NO_3 ON BUD FORMATION (n=10).

number of shoot tips forming buds

Strength of NH_4NO_3	Strength of KNO_3: full	1/2	1/4
full	0	0	0
1/2	0	0	0
1/4	6	7	6

(Table III).

Several other plant growth regulators were applied to cultured Douglas-fir shoot tips. These included gibberellic acid (GA_3) carbonyl cyanide m-chlorophenyl hydrazone (CCC), a gibberellin antagonist, and triiodobenzoic acid (TIBA) an auxin transport inhibitor. Each compound was tested over a range of concentrations (10^{-7}M, 10^{-6}M, 10^{-5}M and 10^{-4}M). None of these growth regulators affected the growth and development of the shoot tips, except that (10^{-4}M) GA_3 and CCC caused the death of the shoot tips. None of these compounds induced the formation of adventitious buds of stimulated the elongation of the shoot tip.

Effect of Exposure Time to Bud Inducing Conditions

Although the standard culture period for the shoot tips was eight

TABLE III. EFFECT OF BAP LEVELS BELOW AND ABOVE 10^{-4}M ON THE FORMATION OF ADVENTITIOUS BUDS (n=10).

number of shoot tips forming buds

conc. of BAP	conc. of NAA: 0.0M	10^{-7}M	5 X 10^{-7}M
5 X 10^{-5}M	5	6	4
10^{-4}M	6	7	5
5 X 10^{-4}M	0	2	2
10^{-3}M	0	1	1

weeks, adventitious buds could be identified after six weeks on the bud inducing medium. An experiment was conducted to determine how long the shoot tip had to be exposed to the high cytokinin treatment to induce adventitious bud formation. One hundred shoot tips were placed on each of the two bud inducing media (one-fourth KNO_3 and NH_4NO_3, 10^{-4}M BAP and either 0.0M or 0.$^{-7}$M NAA) at the same time and each week for the next eight weeks, ten shoot tips from each of the two treatments were transferred to the basal medium but without exogenous growth regulators. A two-three week exposure period to the high cytokinin treatment was sufficient in inducing adventitious bud formation (Table IV).

Other auxins were tested for their effect on the induction of adventitious buds. In addition to NAA, indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) were tested. Adventitious buds were formed at low concentrations of each of the auxins tested (Table V).

TABLE IV. EFFECT OF EXPOSURE TIME OF THE SHOOT TIPS TO THE BUD INDUCING MEDIUM (n=10).

number of weeks on bud inducing medium	number of shoot tips forming buds	
	0.0M NAA 10^{-4}M BAP	10^{-7}M NAA 10^{-4}M BAP
1	1	1
2	8	4
3	6	4
4	7	5
5	9	6
6	9	9
7	6	8
8	7	8

TABLE V. EFFECT OF NAA, IAA AND IBA ON ADVENTITIOUS BUD FORMATION (n=10).

conc.	number of shoot tips forming buds					
	10^{-9}M	10^{-8}M	10^{-7}M	10^{-6}M	10^{-5}M	10^{-4}M
NAA	8	6	7	0	---	---
IAA	8	8	7	6	7	5
IBA	8	7	7	8	6	2

However, at levels higher than 10^{-7}M NAA the formation of buds was inhibited, while inhibition of bud formation was less with the weaker auxins such as IAA and IBA.

Elongation of Adventitious Buds into Shoots

Once the adventitious buds were formed they failed to elongate further unless they were removed from the high cytokinin medium. Transfer to the modified M&S medium without exogenous growth regulators resulted in development of the buds into shoots over a period of several months.

Application of growth regulators including auxins, cytokinins, gibberellins, antigibberellins and antiauxins did not stimulate shoot elongation. In fact, the best development of the buds into shoots took place in the absence of exogenous growth regulators. The addition of 0.5 or 1.0 percent activated charcoal to a medium free of exogenous grwoth regulators appeared to stimulate shoot elongation of the original shoot tip as reported by Boulay (1979). Experiments are currently in progress to determine if activated charcoal will stimulate elongation of adventitious buds.

Discussion

This work is comparable to a similar study by von Arnold and Eriksson (1979) in which adventitious buds were induced on cultured shoot tips of Picea abies trees up to 50 years old. The mineral salt medium used by von Arnold and Eriksson (1979) also seemed to be important for the growth and survival of the cultured shoot tips. After testing several standard plant tissue culture media they selected one that they had developed for the culture of pea protoplasts (von Arnold and Eriksson 1977). This medium had slightly less NH_4NO_3 than full strength M&S medium (1200 mg/l compared to 1650 mg/l in the M&S), but it was not as much of a reduction as necessary for Douglas-fir shoot tips (412.5 mg/l). However, a reduction in the concentration of NH_4NO_3 from full strength to one-fourth strength was found to be beneficial for the multiplication of rhododendrons by shoot tip culture (Anderson 1978). Because both Douglas-fir and the native wild rhododendrons of the Pacific Northwest commonly grow on the same sites, perhaps they share a sensitivity to high ammonium levels.

The cytokinin range required to induce adventitious bud formation in Picea is much wider than those required by Douglas-fir. Either BAP or 2iP between 10^{-6}M and 10^{-4}M were found to induce adventitious buds in Picea (von Arnold and Eriksson 1979), while only 10^{-4}M BAP and perhaps higher levels induced bud formation in Douglas-fir shoot tips. 2iP was found ineffective in stimulating adventitious bud formation in Douglas-fir even when applied at 10^{-3}M. This difference suggests

that Douglas-fir tissue may have some mechanism for degrading or metabolizing these cytokinins faster that Picea tissues.

Low levels of auxin (10^{-6}M to 5 X 10^{-6}M IAA or IBA and 10^{-7}M NAA in Douglas-fir) were found to slightly stimulate the development of needle primordia. Lower levels of auxin (5 X 10^{-10}M to 10^{-8}M NAA or IBA) were found to permit adventitious bud formation in Picea while levels higher than 10^{-7}M inhibited adventitious bud formation in both species. Treatments with GA_3 were without effect on adventitious bud formation or shoot development in both species.

Adventitious buds could be induced on shoot tips of both species collected from late summer until just prior to bud break. No winter dormancy was observed in Picea cultured shoot tips while in Douglas-fir there does appear to be a period during mid-winter when the growth and survival of the buds is reduced.

The effects of different temperature and light intensities on adventitious bud formation in Picea abies were systematically studied by von Arnold and Eriksson (1979). They found that a temperature of 20°C and a light intensity of 20 Wm^{-2} were optimal for adventitious bud formation. Although these factors were not systematically studied in this work with Douglas-fir, very similar culture conditions were used.

The adventitious buds appear to form at meristematic regions of the needle primordia on the shoot tip. The tip as well as the sides and base of the needle primordia contained meristematic tissue (Owens 1968). The observation that the sites of adventitious bud formation changes with the time the buds are collected can be explained

perhaps by changes in the endogenous auxin level of the tissues. Janssen and Bornman (1980) have demonstrated that the location where adventitious buds form on cultured cotyledons of Picea abies is determined by endogenous auxin levels. The higher the amount of exogenous auxin applied to the cotyledons, the farther down the cotlyedon the adventitious bud form from the tip. Thus, in theory, as the auxin level of the bud increases as the buds get closer to bud break in the spring, the farther down the needle primordia the adventitious buds should be found and this is what is observed. Unfortunately, there have not been any direct measurements of endogenous auxin in buds before, during and after bud break to demonstrate that auxin levels do increase.

The results of these experiments demonstrate that shoot tips of mature Douglas-fir trees can be induced to form adventitious buds in culture. This system is similar to the shoot tip propagation method used to propagate many herbaceous horticultural plants (Murashige 1974) in that a high cytokinin level with low levels of auxin are necessary to induce bud formation.

Further work is necessary to stimulate elongation of these buds into shoots and then to root them. Because the original shoot tip fails to elongate into a small shoot in culture as it would if left on the tree, this suggests that some factor or factors are missing in the medium. Little is known about the factors involved in shoot elongation in the conifers, but it appears that plant growth regulators, and in particular the auxins and gibberellins play a major role in this process (Pharis 1976). The use of activated charcoal appears to be a promising treatment to encourage shoot elongation. Rooting of

shoots of old trees is known to be difficult (Roberts and Moeller 1978) and remains to be studied in this system.

THE EFFECT OF AUXINS, CYTOKININS AND NUTRIENTS ON THE INDUCTION OF ADVENTITIOUS BUDS IN SEEDLING CALLUS OF DOUGLAS-FIR

ABSTRACT

Douglas-fir callus cultures initiated from asceptically grown seedlings could be induced to form adventitious buds on a one-half strength (macro and microelements) Murashige and Skoog medium containing 10^{-5}M N_6-benzylaminopurine (BAP)and 10^{-7}M napthaleneacetic acid (NAA) . Other cytokinins such as kinetin, zeatin and 6-benzyl-9-tetrahydropyrone adenine were also effective in inducing bud formation when applied at 10^{-5}M. N_6-isopentenylpurine was effective in inducing adventitious buds only when applied at 10^{-4}M. NAA levels higher than 10^{-6}M inhibited adventitious bud formation. Bud formation took place only when the NH_4NO_3 was applied at one-half strength, suggesting that the NH_4^+ concentration may be influencing bud formation. The ability of callus to form buds declined in culture and after one year it was lost completely.

THE EFFECT OF CYTOKININS, AUXINS AND NUTRIENTS ON THE INDUCTION OF ADVENTITIOUS BUDS IN SEEDLING CALLUS CULTURES OF DOUGLAS-FIR.

Introduction

Numerous applications of plant tissue culture techniques to forest trees have been anticipated for many years (Geissbuhler and Skoog 1957, Haissig 1965, Durzan and Campbell 1974 and Karnosky 1981). However, current conifer tissue culture systems are limited to the induction of shoots on organized tissues such as cultured seed embryo or very young seedling tissues (Sommer et al. 1975, Cheng 1975) and only recently cultured shoot tips from mature trees (von Arnold and Eriksson 1979, Thompson 1980). These currently developed systems allow only for the propagation of selected material. Other facets of plant tissue culture offer much more than only a method for the propagation of plants.

Plant tissue culture techniques can be used for the regeneration of plants from pollen, anther or megagametophyte tissues to produce haploid plants for use in breeding programs. Mutant individuals with desired characteristics can be induced and selected in cell suspension cultures. Rare and valuable genotypes can be stored in cryogenic germplasm banks for future regeneration of plants. Modification of the genetic information in plant cells through "genetic engineering" is possible through the production, culture and manipulations of plant protoplasts. Unfortunately all of these techniques depend on the ability to regenerate plants from unorganized cells

and tissues in culture, which we are presently unable to do with the conifers.

The ability to regenerate plants from unorganized cells and tissues of conifers in culture would not only expand our understanding of the chemical control of organ formation in these species, but would also make the above mentioned applications of plant tissue culture to forest trees possible.

Among the over 100 papers cited in the most recent review of gymnosperm tissue culture (David and Thomas 1979), only eight papers have reported the induction of shoots in callus cultures of conifers. A comparison of the growth regulator treatments reported to induce shoot formation (Table I) shows that a cytokinin level between 0.1 and 2.0 mg/1 (depending on the cytokinin used) and an auxin level of between 0.0 and 25.0 mg/1 were necessary for bud formation. The wide range in auxin concentrations used relates to their differences in activity and stability in culture. Thus, low levels (1-2 mg/1) of the synthetic auxins such as napthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D), while high levels of less effective and less stable auxins such as indolebutyric acid (IBA) and the natural auxin indoleacetic acid (IAA) were necessary for bud formation.

Despite the fact that there are differences in the species used in these studies, all of the cultures were initiated from either cultured seed embryos or very young seedling tissues. The only exception is Sequoia where shoots from a burl were used. All of

TABLE I. REPORTED SHOOT FORMATION IN CONIFER CALLUS CULTURES.

Author	Species	Explant	Cytokinin mg/l	Auxin mg/l
Ball, 1950	*Sequoia sempervirens*	shoots from burls	----	IAA 0.175
Konar, 1972	*Pinus gerardiana*	embryos	Kinetin 0.5	NAA 2.0
Cheng, 1975	*Pseudotsuga menziesii*	embryos	Kinetin 0.86 & BAP 0.9	IBA 0.812 & NAA 0.744
Ramawat & Arya, 1976	*Ephedra foliata*	embryos	Kinetin 1.0	----
Winton & Huhtinen	*Picea abies*	seedling stems	Kinetin 0.5	IAA 25
Chalupa, 1977	*Picea abies*	embryos	Kinetin 0.5	IAA 25
Winton & Verhagen, 1977	*Pseudotsuga menziesii*	embryos	BAP 0.1	NOAA 5
Konar & Singh, 1979	*Ephedra foliata*	embryos	Kinetin 2	2,4-D 1

these tissues are considered to be juvenile and are known to form adventitious roots more easily than do more mature tissues (Roberts and Moeller 1978). In none of these cases was mature tissue from a mature tree cultured and induced to form buds. This is not because tissues from mature trees cannot be cultured (Risser and White 1964) but rather because seeds are readily available, easier to surface sterilize and thus easier to use to induce cultures than tissue from mature trees. However, the ultimate application of plant tissue culture techniques to forest trees would be to use tissue from selected mature trees with desired characteristics.

The objective of the present study was to examine the influence of exogenously applied plant growth regulators, in particular auxins and cytokinins, as well as the effects of nutrition on bud formation in cultured tissues of Douglas-fir.

Materials and Methods

The basal mineral salt medium used in these studies was a modified Murashige and Skoog (1962) medium (M&S) composed of one-half strength macro and microelements (see results). It was supplemented with 100 mg/1 myo-inositol, 0.5 mg/1 pyridoxine, 0.5 mg/1 nicotinic acid, and 0.4 mg/1 thiamine·HCL. All growth regulators were dissolved in several drops of 1N NaOH and then diluted with distilled deionized water to produce stock solutions. The pH of the medium was adjusted with either 1N HCl or 1N NaOH to pH 5.7. The sucrose (30 g/1) and Difco Bacto agar (10 g/1) were added and dissolved by heating the medium in an autoclave. Twenty-five ml of medium was dispensed into each 25 x 150 mm culture tube which were capped with Bellco Kaputs. All components of the culture medium were autoclaved together at 121°C for 20 minutes and then cooled on a slant. Ten culture tubes of each treatment were used in each experiment.

Initiation of Cultures

Stratified seeds of Douglas-fir were surface sterilized with a ten percent chlorox solution for 12 minutes and then rinsed three times with sterile distilled water. The seed coat was removed from the surrounding female gametophyte tissue. The sterile embryo was placed on a medium consisting of the Murashige and Skoog salt formulation (one-half strength macro and microelements with full vitamins) and 10^{-5}M napthaleneacetic acid (NAA) and 10^{-6}M N_6-benzylaminopurine (BAP) to stimulate callus growth. Callus was maintained on this

medium and was subcultured every six to eight weeks.

Culture Conditions

All callus cultures were maintained in growth chambers under a 20 Wm^{-2} mixture of incandescent and fluorescent lights with a 16 hour photoperiod. The temperature was maintained at a constant 20°C. All cultures were grown for eight weeks before they were harvested and examined for adventitious bud formation.

Results

Effect of Exogenous Auxin and Cytokinin on Callus Growth

Approximately 50 mg of callus tissue was placed on medium containing one of the 25 possible combinations of both NAA and BAP applied at the levels 0.0M, 10^{-7}M, 10^{-6}M, 10^{-5}N and 10^{-4}M. Callus was grown for eight weeks at which time it was examined for any signs of organ formation and then its fresh weight was recorded. Fresh weight increases (Figure 1) showed that with increasing auxin levels up to 10^{-5}M NAA callus growth increased, but beyond that it decreased. Increasing cytokinin levels also increased callus growth but not as dramatically as the auxin. Also, above 10^{-5}M BAP callus growth decreased. A medium containing 10^{-5}M NAA and 10^{-6}M BAP was selected as the standard medium to induce and maintain callus cultures.

Of the 250 cultures in this experiment, only one exhibited any form of organ formation. This culture formed several shoot buds on the growth regulator treatment containing 10^{-5}M BAP with no exogenous auxin.

Effect of Mineral Nutrition on Callus Growth

Growth of subcultured Douglas-fir callus was adequate on full strength M&S medium, however, Washer et al. (1977) have suggested that a reduction in macroelement and sucrose levels in M&S medium from full strength to one-half strength was necessary for vascular nodule formation in Pinus radiata callus. In their work the microelement level did not seem to play an important role. To examine this hypothesis, Douglas-fir callus was grown on both full strength M & S

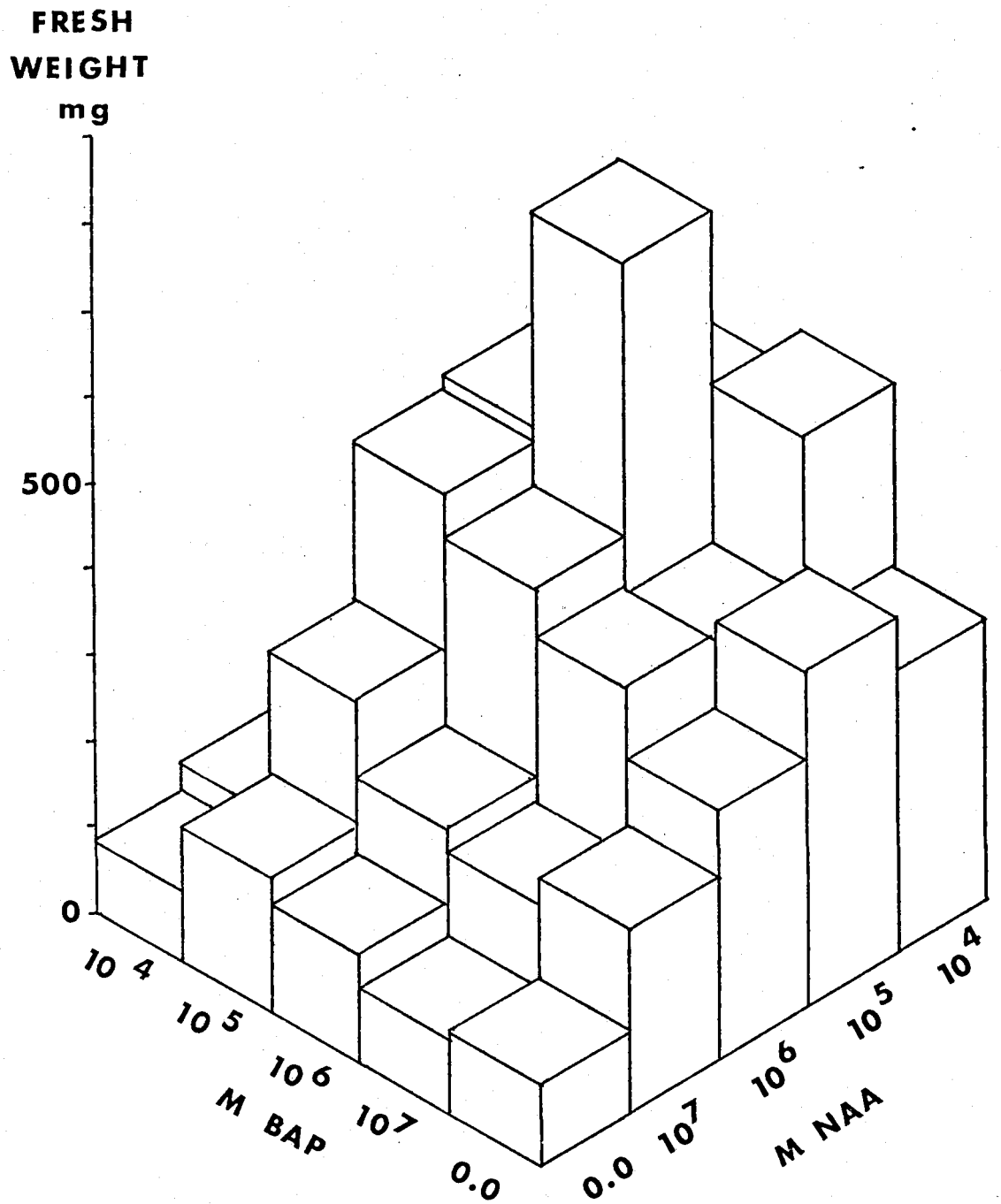

Figure 1. Growth (fresh weight gain) of Douglas-fir callus grown on different levels of NAA and BAP.

and one-half strength M&S (both macro and microelements with full vitamins). Fresh weight increased (Table II) faster on one-half strength M&S than on the full strength medium. This suggests that some component or components are present at supraoptimal levels in the full strength M&S medium that reduces growth of Douglas-fir callus. To maximize callus growth, one-half strength M&S medium (both one-half macro and microelements with full strength vitamins) was used as the basal mineral medium in these studies unless otherwise stated.

Effect of Exogenous Cytokinins on Bud Formation

A previous experiment testing different levels of auxin NAA and BAP on callus growth suggested that 10^{-5}M BAP (2.25 mg/1) induced bud formation in Douglas-fir callus tissue. Also, the reports in the literature on bud formation on conifer callus cultures suggested that this was near the optimum cytokinin level for bud formation (Table I). Levels of BAP above and below 2.25 mg/l were tested for their bud forming ability. BAP was applied at 1.0, 2.0, 3.0 and 4.0 mg/l without any exogenous auxin. Adventitious bud formation was observed only in the 2.0 mg/l BAP treatment (Table III).

Effect of Different Cytokinins on Bud Formation

Because BAP was found to be effective in inducing bud formation in Douglas-fir callus cultures, other cytokinins including kinetin, zeatin, isopentenylpurine (2iP), 6-benzyl-9-tetrahydropyrone adenine (SD 8339) and BAP were tested. All cytokinins induced bud formation when applied at 10^{-5}M except 2iP which was effective

TABLE II. COMPARISON OF DOUGLAS-FIR CALLUS GROWTH ON FULL AND ONE-HALF STRENGTH (MACRO AND MICROELEMENTS) M&S MEDIUM (n=10) (10^{-5}M NAA and 10^{-6}M BAP).

medium	Full Strength M&S	One-Half Strength M&S
fresh weight (mg)	587.9	897.4

TABLE III. EFFECT OF BAP CONCENTRATION ON BUD FORMATION (n=10). (no exogenous auxin).

conc. BAP (mg/l)	1.0	2.0	3.0	4.0
number of cultures forming buds	0	4	0	0

only when applied at 10^{-4}M (Table IV).

The 10^{-4}M 2iP treatment appeared to the most effective of all cytokinins in inducing bud formation. It also produced buds which grew rapidly into small shoots, while buds produced with 10^{-5}M BAP required several weeks on a growth regulator free medium for elongation into shoots.

Effect of Exogenous Auxin on Bud Formation

Previous experiments showed that buds could be formed on a medium containing a cytokinin without any exogenous auxin. Douglas-fir callus was placed on a medium containing 10^{-5}M BAP with varying levels of NAA (0.0M, 10^{-7}M, 10^{-6}M, and 10^{-5}M). With increasing levels of NAA, the number of buds formed declined until at the 10^{-5}M NAA treatment bud formation was completely inhibited (Table V). At 10^{-5}M NAA,

bud-like structures were found on the surface of the callus, but they failed to develop into buds even if the callus were transferred to a medium free of growth regulators.

Because the 0.0M NAA treatment resulted in the largest number of buds formed of any of the auxin treatments, levels of NAA below 10^{-7}M were tested. NAA was applied at 10^{-9}M, 10^{-8}M, 10^{-7}M and 10^{-6}M. It was observed (Table VI) that NAA levels below 10^{-7} may improve bud formation.

Among the cytokinins tested, 2iP appeared to be more effective in inducing buds that would elongate into small shoots, thus, the interaction of 2iP with NAA was studied. The 2iP was applied at 10.0, 20.0 and 30.0 mg/l (10^{-4}M 2iP-20.3 mg/l) and NAA at 10^{-9}M, 10^{-8}M, 10^{-7}M

TABLE IV. EFFECT OF DIFFERENT CYTOKININS ON BUD FORMATION (n=10) (no exogenous auxin).

cytokinin	Kinetin	zeatin	SD 8339	2iP	2iP	BAP
conc.	10^{-5}M	10^{-5}M	10^{-5}M	10^{-5}M	10^{-4}M	10^{-5}M
number of cultures forming buds	2	2	2	0	4	2

TABLE V. EFFECT OF NAA LEVELS ON BUD FORMATION (n=10) (10^{-5}M BAP).

NAA conc.	0.0M	10^{-7}M	10^{-6}M	10^{-5}M
number of cultures forming buds	5	2	1	0

TABLE VI. EFFECT OF NAA LEVELS BELOW 10^{-5}M ON BUD FORMATION (n=10) (10^{-5}M BAP).

NAA conc.	10^{-9}M	10^{-8}M	10^{-7}M	10^{-6}M
number of cultures forming buds	2	3	2	0

and 10^{-6}M. 2iP was found to be effective in forming buds both at 20.0 mg/l and 30.0 mg/l in conjunction with 10^{-7}M or 10^{-8}M NAA. Both lower 2iP levels and lower and higher NAA levels did not induce bud formation (Table VII).

Effects of Mineral Nutrition on Bud Formation

The observation that Douglas-fir callus grew better on one-half strength M&S medium as compared to full strength macro and microelement medium suggested that some factor or factors were present at too high a concentration. An experiment was conducted to determine the effect of reducing the three most concentrated salts in the M&S medium and their effect on bud formation. KNO_3, NH_4NO_3 and $CaCl_2$ were all applied at full and one-half strength resulting in a total of eight treatments. Buds were found only in treatments containing one-half strength NH_4NO_3 (Table VIII).

Further Development of the Adventitious Buds

Once the adventitious buds could be recognized on a callus culture, they could be removed from the callus mass and cultured on a one-half strength M&S medium (one-half macro and microelements with full vitamins) without any exogenous growth regulators. On this medium the buds slowly formed small needles from the needle primordia of the bud and they began to elongate into a small shoot. They would continue to elongate until a small shoot about four-five cm formed. Buds induced with the 2iP cytokinin treatment typically formed small shoots while still on the high cytokinin containing medium. They also tended to

TABLE VII. EFFECT OF 2iP AND NAA ON BUD FORMATION (n=10).

2iP conc.	NAA conc. 10^{-9}M	10^{-8}M	10^{-7}M	10^{-6}M
10.0	0	0	0	0
20.0	0	2	3	0
30.0	0	2	3	0

TABLE VIII. EFFECT OF KNO_3, NH_4NO_3 and $CaCl_2$ LEVELS ON BUD FORMATION (n=10) (10^{-4}M 2iP 10^{-7}M NAA)

Full Strength $CaCl_2$

conc. NH_4NO_3	conc. KNO_3 1	1/2
1	0	0
1/2	2	2

Number of Cultures Forming Buds

One-Half Strength $CaCl_2$

conc. NH_4NO_3	conc. KNO_3 1	1/2
1	0	0
1/2	2	2

grow into small shoots quite easily. Experiments to induce roots on these shoots is currently in progress.

Decline in Organogenetic Potential in Callus

The callus used in this study was initiated in July, 1980 and was used in experiments to induce bud formation from November, 1980 through May, 1981. During that time, the ability of the callus to form shoots steadily declined (Table IX). This decline in morphogenetic potential is not unique to Douglas-fir callus, but has been reported in most callus cultures maintained in culture (Smith and Street 1973).

TABLE IX. DECLINE IN THE ABILITY TO FORM BUDS IN DOUGLAS-FIR CALLUS (n=10).

number of months in culture	number of cultures forming buds
4	5
6	3
8	1
10	1

Discussion

The results of this study agree with previously published reports of adventitious bud formation in callus cultures of conifers. Cytokinin levels near two mg/l are necessary to induce bud formation in Douglas-fir seedling callus cultures. The synthetic cytokinins such as BAP, kinetin, and SD 8339 as well as the naturally occuring cytokinin zeatin induced adventitious bud formation when applied at 10^{-5}M, while another naturally occuring cytokinin 2iP was found to be effective only when applied at a level of 10^{-4}M. 2iP was reported by Cheng (1977) to be the least effective cytokinin tested in inducing adventitious bud formation on cultured cotyledon segments of Douglas-fir. It may be that this cytokinin is more susceptible to degradation or metabolism in Douglas-fir than the other cytokinins. Despite the high levels necessary to induce bud formation, the buds produced with 2iP were the most normal appearing and rapidly developed into small shoots even while the callus was on the high cytokinin containing medium.

Auxin levels also play an important role in the formation of adventitious buds in Douglas-fir callus cultures. Concentrations of NAA between 0.0M and 10^{-6}M may stimulate the formation of adventitious buds while NAA concentrations higher than 10^{-6}M inhibit bud formation. At these higher auxin levels bud-like structures form, but they fail to develop into buds.

These results support the hypothesis presented almost 25 years ago by Skoog and Miller (1957) to explain the regulation of shoot and root formation in tobacco callus cultures. A high cytokinin to auxin

ratio stimulates bud formation, while a high auxin to cytokinin ratio stimulates root formation. Thus, conifer callus cultures follow the same general pattern found in most angiosperms.

This study shows that mineral nutrition plays an important regulatory role in the adventitious bud formation process. In particular, the level of the NH_4^+ appears to regulate bud formation. Only a few other recently published studies have examined the influence of mineral nutrition on organogenesis in callus cultures. Of the components of the mineral medium that appear to be important, nitrogen levels and in particular NH_4^+ levels seem to be the determining factor. Low (5mM) levels of amino acids (alanine, asparagine and glutamic acid) were reported by Kamada and Harada (1979) to stimulate bud formation in callus of Torenia fournieri. They accounted for this by suggesting that an increase in NH_4^+ levels might increase endogenous cytokinin levels as had been reported by Peterson and Miller (1976) in Vinca crown gall callus. Negrutiu and Jacobs (1978a) also found that bud formation in callus of Arabidopsis thaliana was promoted by the addition of glutamine, but that $(NH_4)_2SO_4$ markedly reduced bud formation. Shoot regeneration in leaf callus of Lycopersicon esculentum was found to be affected by the total amount of nitrogen as well as the ratio of NH_4^+ to NO_3^- nitrogen (Behki and Lesley 1980). A reduction in the NH_4^+ level and an increase in the NO_3^- level resulted in increased bud formation. Evans et al. (1976) observed a similar relationship between total nitrogen and the form of the nitrogen in the regeneration of roots from callus of Glycine max. Pierik (1976) found that a reduction in the level of NH_4NO_3

(from full strength M&S) was essential for the induction of adventitious buds in callus of Anthurium andraeanum and that an increase in the NH_4NO_3 level from 206 to 825 mg/l completely inhibited bud formation in most of the genotypes tested. This NH_4 effect may also occur only in certain cultivars as shown by Welander (1979) who reported that only one out of three cultivars of Begonia X hiemalis exhibited reduced bud formation as a result of increased nitrogen levels.

Despite all of these reports on the influence of nitrogen levels and particularly NH_4 levels on bud formation, there seems to be no explanation as to how it affect bud formation. It is interesting to note that the plant species studied in some of the papers such as Glycine and Lycopersicon are typically some of the most difficult genera from which to regenerate plants in tissue culture. Perhaps only after standard auxin and cytokinin ratios fail to result in shoot or root formation on a standard mineral medium are the effects of the mineral elements examined.

The loss of the ability of a culture to form organs the longer it is maintained in cultures is a familiar characteristic of most tissues grown in culture (Smith and Street 1973). Two hypotheses have been proposed to explain the loss of morphogenetic potential over time in culture (Thomas and Street 1970). The "genetic" or "mutational" hypothesis is based on the observation that cells in culture tend toward increasing ploidy levels and accumulate chromosomal abnormalities. It is believed that only diploid cells free of chromosomal abnormalities are capable of regenerating organs. Thus, the longer a culture is maintained, the fewer the

number of normal diploid cells capable of forming organs.

The alternative "physiological" hypothesis suggests that the environmental conditions for the growth of cells in culture may not be optimal for their differentiation into organs. This has also been expressed as a loss of an essential factor or factors that was present in the original explant, but that has not been synthesized by the cultured cells resulting in the loss of the ability to differentiate.

At this time there does not seem to be any strong evidence to support or reject either of the hypotheses. Polyploid cell cultures have been reported to form shoots and there is no good evidence that there is any factor or factors present in the original explant that is lost in the tissue when grown in culture. Thus, about all that can be said is that morphogenetic potential tends to decline with increasing time in culture. Culture on media without exogenous growth regulators before attempting to induce organogenesis has been suggested as a means for restoring the morphogenetic potential perhaps by altering unfavorable endogenous growth regulator levels (Negrutiu and Jacobs 1978b), but there is no evidence to support this hypothesis.

Now that the factors important for bud formation in seedling callus cultures of Douglas-fir such as cytokinin, auxin and ammonium levels have been identified, this information should be applied to tissues from old trees. There may be some minor changes in the growth regulator and nutritional requirements for bud formation in older tissues, but these should not be radically different from

those of seedling callus tissue. The maximum benefit from plant tissue culture in forestry will come only when trees old enough to have demonstrated their superior characteristics can be used.

RAPID ISOLATION AND QUANTITATION OF INDOLE-3-ACETIC ACID

ABSTRACT

A method is described for the rapid quantitation of low levels of indole-3-acetic acid in milligram amounts of plant tissue. The method relies on column chromatography, trace enrichment, separation on HPLC and detection by fluorescence.

Introduction

Attempts to correlate changes in auxin levels with plant development has led to a proliferation of techniques for the measurement of indole-3-acetic acid (IAA) in plants (Yokota et al. 1980). These techniques, however, lack the specificity, sensitivity and rapidity to make practical a large number of analyses on small tissue samples. We report here a method for the rapid measurement of IAA in small tissue samples by means of column chromatography, trace enrichment (Morris et al. 1976), separation by High Performance Liquid Chromotagraphy (HPLC) and detection and quantitation by on line fluorimetry (Crozier et al. 1980). This method has the advantages of rapidity (sample processing time for HPLC is less than one hour and several samples can be processed simultaneously), selectivity (it can separate IAA from other native indole auxins), and sensitivity (it can detect picogram levels of IAA).

Materials and Methods

One gram of plant tissue was ground in 10 ml of a grinding solution consisting of 79 ml HPLC grade methanol (Baker) and 20 ml of 0.1 M NH_4oAC buffer at pH 6.5. To this, 20 mg of diethyldithiocarbamic acid (dieca) (Sigma) and 1.0 ml of 2-mercaptoethanol (Sigma) were added as antioxidants. Butylated hydroxytoluene (BHT) (Sigma) was added to protect IAA from oxidation (Iino et al. 1980) at a rate of 20 mg per 10 ml of grinding solution.

Tissue was ground in this solution for 10-20 seconds with a Polytron (Brinkman Instruments) in a 30 ml silanized Corex tube wrapped in aluminum foil to exclude light and under a stream of nitrogen to replace oxygen.

The sample was then centrifuged at 23,500 RCF for three minutes after which the supernatant was diluted with 20 ml of distilled, deionized water and 17.5 ml of 0.01 M NH_4oAC buffer at pH 6.5. The sample was processed by column chromatography that was conducted in a series of disposable plastic syringe barrels fitted with porous polyethylene frits to retain the packing material. The diluted grinding solution was passed through a 50 cc syringe barrel column containing a three ml bed of cellulose phosphate (CP) (Sigma) below a five ml bed of polyvinylpyrrolidone (PVP) (Sigma). The CP was washed first with 1N HCl and then 1N NaOH for one hour each followed by repeated washes with distilled, deionized water. The PVP was washed with 1N HCl for one hour and then washed with distilled deionized water until the pH stabilized. Both the CP and PVP were stored

suspended in 0.1 M NH_4oAC buffer at pH 6.5 at 1°C in one percent sodium azide. The PVP-CP column was connected directly to a six cc syringe barrel containing a two ml bed of diethylaminoethyl cellulose (DEAE) (Whatman DE-52). The DEAE had been suspended in 0.1 M NH_4oAC buffer at pH 6.5 and the pH was adjusted until it stablized at 6.5. The DEAE was stored suspended in the buffer at 1°C in one percent sodium azide. The sample was followed by a wash of the PVP-CP and DEAE columns with 10-20 ml of 0.01 M NH_4oAC buffer at pH 6.5. The DEAE column was separated fom the PVP-CP column and connected to a six cc syringe barrel containing one g octyldecylsilane (C_{18}). The DEAE was eluted with ten ml of one M HoAC buffer at pH 3.1 containing 20 mg of BHT. The C_{18} column was then washed with ten ml of distilled, deionized water and the IAA was eluted with three ml of HPLC grade methanol containing six mg of BHT.

The IAA in the three ml methanol wash of the C_{18} was collected in a six ml silanized glass test tube. This sample could either be stored for later analysis, in which case the methanol was evaporated in a Speed Vac concentrator (Savant) in the dark, or the sample could be analyzed immediately by injecting an aliquot of the three ml of methanol directly on the HPLC.

Sample aliquots dissolved in methanol (usually $1/100^{th}$ of the sample volume) were separated on a liquid chromatograph (Varian model 5000) with a 5 u ODS column (Shandon Hypersil). The HPLC solvent program ran from injection to 6 minutes isocratically at 45 percent methanol: 55 percent 20mM HoAC adjusted to pH 3.5 with triethylamine. From six to nine minutes the methanol concentration increased linearly

to 80 percent. The solvent flow rate was 1.0 ml per minute. Retention time of IAA under these conditions was 7.8 minutes.

The IAA was detected on a fluorescence spectrophotometer (Perkin-Elmer model 650-10S) with the excitation wavelength set at 293 nm and the emission wavelength at 357 nm. Both slit widths were set at 10 nm. A Hewlett-Packard model 3390A integrator was used to measure peak areas.

Results and Discussion

Traces of the fluorimeter output yielded quantifiable peaks associated with IAA and the other indole auxins used as internal standards (Figure 1). Douglas-fir tissue contained several hundred nanograms of IAA per gram of fresh weight. Endogenous IAA levels were similar in both vegetative and female buds of Douglas-fir, but were dramatically higher in the male buds (Figure 1). Significant differences in the size of the unidentified peak just before the IAA peak were seen in comparing vegetative and male buds with female buds. The nature of this compound is not known other than it is acidic and fluorescent.

The initial extraction work which involved rotary evaporation and solvent partitioning yielded poor recoveries of ^{14}C labeled IAA (Table I). Mann and Jaworski (1970) also reported large losses of IAA during rotary evaportation. Solvent partitioning and rotary evaporation were also the most time consuming steps in this extraction process. As a result the present extraction method was developed to avoid both of these steps (Figure 2).

The PVP in the first column removed phenolic materials which would degrade IAA during tissue processing. The cellulose phosphate which is a cation exchange material removed cations from the extract. Solvent partitioning to remove plant pigments was replaced by the DEAE anion exchange column. Most of the pigments as well as the IAA were retained on the DEAE. A one M HoAC wash of the column eluted the IAA while most of the plant pigments remained on the DEAE. Because the DEAE column was most efficient at low methanol

and low salt concentrations, both the methanol and salt concentrations were lowered by diluting the ten ml of grinding solution with 20 ml distilled, deionized water and 17.5 ml of 0.01 M NH_4oAC buffer. This dilution step avoided the need for rotary evaporation of the extract. The C_{18} column trace enriched the unionized and hydrophobic compounds in the extract including the IAA.

Fluorimetric detection of the indole auxins was more sensistive than UV absorbance detection. This on-line fluorimetric method has been reported to detect low picogram levels of IAA (Crozier et al. 1980).

Several precautions must be taken to protect IAA from degradation during processing of the sample. The addition of BHT to the grinding solution improved the recovery of IAA and it further improved IAA recovery if added to the HoAC wash of the DEAE and methanol elution of the C_{18} (Table II). Protection from light by working in foil wrapped silanized glassware was important, however, the rapidity of this extraction process also reduced the time IAA was subject to photodegradation. Any heating of the extract such as during rotary evaporation should be avoided.

In this study indole-3-propionic and indole-3-butyric acids were used as internal standards. Some form of labeled IAA would be the best compound to determine IAA losses, but ^{14}C-IAA was found to have too low a specific activity and tritiated IAA was not available. If tritiated IAA were available it would be the internal standard of choice.

Preparation of the extract for HPLC with this system was rapid,

requiring less than one hour and several samples could be processed simultaneously. Analysis time on the HPLC was also rapid. A complete HPLC run including clean-up of the HPLC column before the next sample required only 30 minutes. Analysis could be done on one HPLC run thus avoiding longer analysis times and complex column switching devices required for multiple HPLC runs. A tissue sample could be extracted and analyzed in two to three hours.

In this work one gram of tissue was routinely processed, but only $1/100^{th}$ of the sample volume was necessary to detect and quantitate IAA. Thus, measurement of IAA levels in samples as small as several hundred milligrams was possible. This technique makes possible studies on changes in IAA levels at different stages of plant development that until now have not been feasible.

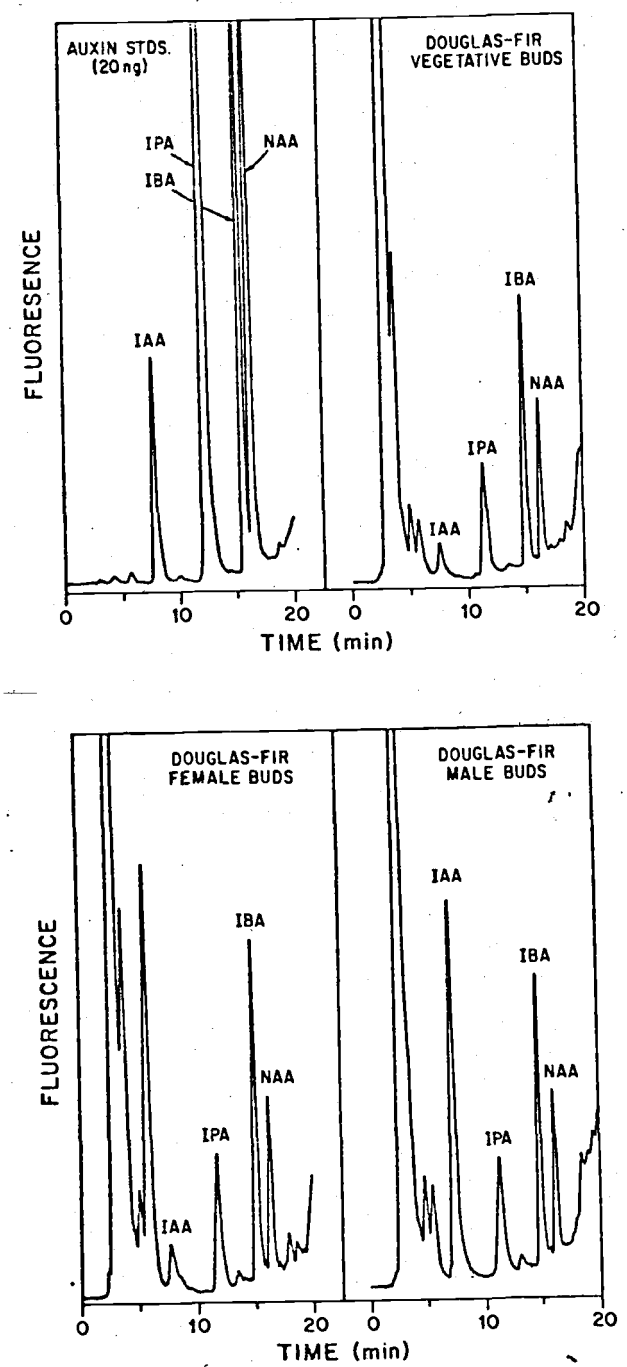

Figure 1. Comparison of endogenous IAA levels in Douglas-fir vegetative, female and male buds.

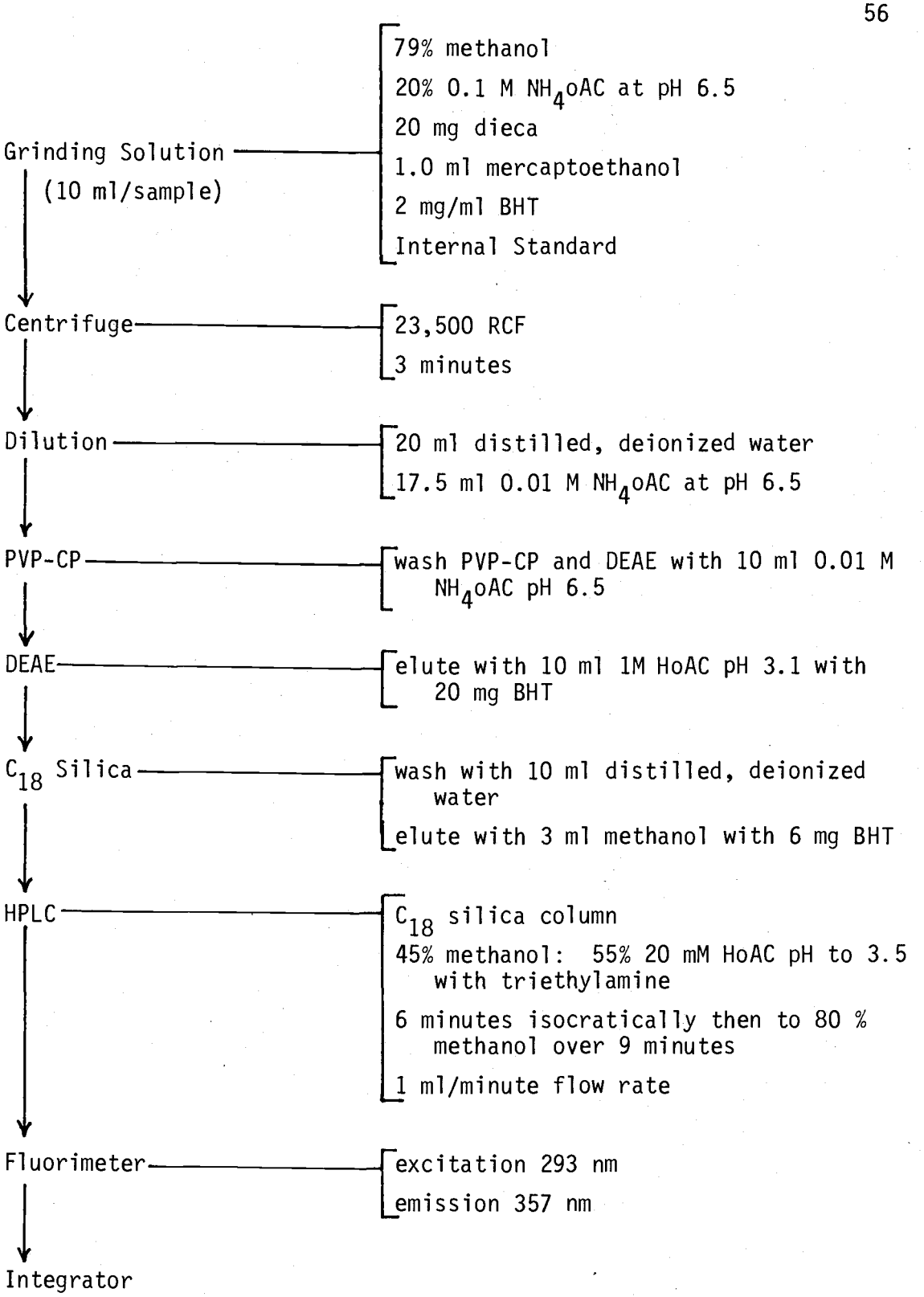

Figure 2. Flow diagram of extraction, separation and detection of IAA.

TABLE I. RECOVERIES OF ^{14}C-IAA AT VARIOUS STEPS IN A TYPICAL IAA EXTRACTION PROCEDURE INVOLVING ROTARY EVAPORATION AND SOLVENT PARTITIONING.

Step	Recovery
Grinding Solution	100%
After Rotary Evaporation	50%
After Partitioning Against Toluene	36%
In Toluene	11%
After PVP	34%
After C_{18}	33%

TABLE II. EFFECT OF ADDITION OF BHT ON RECOVERY OF IAA, IPA AND IBA WHEN ADDED TO THE GRINDING SOLUTION, THE HoAC WASH OF THE DEAE AND THE METHANOL ELUTION OF THE C_{18}.

	% Recovery		
	IAA	IPA	IBA
0 BHT	42%	61%	46%
1 mg/ml BHT	48%	70%	55%
2 mg/ml BHT	50%	78%	54%
3 mg/ml BH%	50%	78%	56%

THE ROLE OF ENDOGENOUS INDOLE-3-ACETIC ACID IN ADVENTITIOUS BUD FORMATION IN DOUGLAS-FIR

SHOOT TIPS AND CALLUS CULTURES: A TEST OF A METHOD AND SOME PRELIMINARY MEASUREMENTS.

ABSTRACT

A method for the rapid detection and quantitation of endogenous IAA levels was applied to Douglas-fir shoot tips collected before, during and after bud break as well as shoot tip and callus cultures forming and not forming adventitious buds. Estimates of the endogenous IAA levels show that IAA levels increased in the buds just prior to bud break and then begin to decline slowly. No differences in endogenous IAA levels could be found between either Douglas-fir shoot tips or callus cultures forming or not forming adventitious buds.

Introduction

Indole-3-acetic acid (IAA) is one of the two major classes of plant growth regulators believed to be involved in the control of organ formation in plants. Skoog and Miller (1957) over 20 years ago proposed a hypothesis to explain the chemical control of organ formation based on the ratio of two types of plant growth regulators, the auxins and the cytokinins. Among other responses, auxins typically stimulate cell elongation and cytokinins stimulate cell division in plants. Low levels of both auxins and cytokinins resulted in little growth in unorganized tobacco callus cultures. By increasing the auxin to cytokinin ratio, roots were formed and the reverse ratio promoted the formation of buds from the callus. High levels of both auxin and cytokinin resulted in only the growth of unorganized callus without the formation of either buds or roots. This regulation of organ formation by ratios of auxin and cytokinin has now been demonstrated in a large number of plant tissues grown in culture.

Besides stimulating root formation, high auxin levels may actually inhibit shoot formation (Skoog and Miller 1957). Direct measurements of endogenous IAA levels in callus cultures, other than by bioassay, have not been reported. Until recently there was only indirect evidence to suggest that high endogenous auxin levels in callus cultures may be inhibiting shoot formation. Negrutiu (Negrutiu et al. 1978) reported low peroxidase levels, one of which is the enzyme IAA oxidase which is believed to regulate endogenous IAA levels, in cultures that did not form shoots, suggesting there was a high endogenous IAA level in this callus tissue. Kevers et al. (1981) provided the first direct

evidence of the role of endogenous IAA levels in organ formation. In a comparison of organ forming and non-organ forming habituated (not requiring exogenous auxin and cytokinin treatments to grow) sugarbeet callus they found that the organ forming callus line contained a high endogenous peroxidase level. They were also able to directly measure endogenous IAA levels in the two tissue lines by combined gas chromatography-mass spectrometry and found that the organ forming line did contain a lower endogenous IAA level than the non-organ forming line.

Similar studies of the peroxidase levels in Douglas-fir callus, a tissue known for its unresponsiveness to auxin-cytokinin treatments to induce organ formation, have been reported (Johnson and Carlson 1977). In this work they reported that Douglas-fir seedlings contained a higher level of peroxidase than Douglas-fir callus cultures. This would suggest that Douglas-fir callus cultures would contain a high level of endogenous IAA that would act to inhibit bud formation (Johnson 1976). However, there was no direct measurement of the endogenous IAA level in the tissues.

IAA may also play a role in determining where adventitious buds may form on a cultured tissue. Janssen and Bornman (1980) found that the position where adventitious buds formed on cultured cotyledons of Picea abies was controlled by the amount of exogenous auxin applied. With no exogenous, but only endogenous auxin, buds formed on the tip of the cotyledon while with increasing exogenous auxin levels the location of the adventitious buds shifted towards the base of the cotyledon. In Douglas-fir shoot tips cultured at different times of the year on a medium containing a high cytokinin level to induce

adventitious bud formation, buds were formed at the tips of the needle primordia in late winter (January-February), but were found closer to the base of the needle primordia the closer the shoot tips were collected to the time of bud burst in the spring. This may reflect a similar change in adventitious bud formation as the endogenous IAA level increased in the shoot tip the closer they were collected to bud break.

There is also evidence that endogenous IAA levels are affected by the application of different exogenous plant growth regulators. Elliott et al. (1979) have reported that Acer pseudoplatanus suspension cultures, when grown on a medium containing 2,4-Dichlorophenoxyacetic acid (2,4-D) synthesized IAA at different rates during the growth phase of the tissue. There is also evidence that 2,4-D can affect IAA oxidase levels, thus affecting endogenous IAA levels (Lee 1972).

The objective of this study was twofold. The first was to test a new method for the rapid isolation and quantitation of IAA (Thompson et al. 1981) in shoot tip buds and in cultured shoot tips and callus cultures, both forming and not forming adventitious buds. The second objective was to obtain some preliminary estimates of endogenous IAA levels in these tissues.

Materials and Methods

IAA Analysis Technique

Endogenous IAA levels were performed by the method of Thompson et al. (1981) involving extraction of the IAA in methanol, clean-up by column chromatography and trace enrichment followed by separation on HPLC and detection and quantitation by the natural fluorescence of IAA. Tissue samples of 250 mg were used and four samples of each tissue were processed simultaneously. Known amounts of indolepropionic acid were added to the extraction solution as an internal standard.

Callus Cultures

Callus cultures of Douglas-fir were initiated from aseptically germinated Douglas-fir seed and grown on a one-half strength (macro and microelement) Murashige and Skoog (1962) mineral medium containing 100 mg/1 myo-inositol, 0.5 mg/1 pyridoxine, 0.5 mg/1 nicotinic acid and 0.4 mg/1 thiamine·HCl. Growth regulators were dissolved in several drops of 1N NaOH and then diluted with distilled, deionized water to produce stock solutions. The pH of the medium was adjusted to pH 5.7 with either 1N HCl or NaOH. Thirty grams of sucrose were added and the medium was solidified with 10 g/1 of Difco Bacto agar. Callus was induced on a medium containing 10^{-5}M napthaleneacetic acid (NAA) and 10^{-6}M N_6-benzylaminopurine (BAP). This growth regulator treatment was used to maintain unorganized callus tissue.

Callus cultures were grown in growth chambers under a 20 Wm^{-2} mixture of fluorescent and incandescent light under a 16 hour photoperiod at a constant 20°C. In addition to growth on a medium containing NAA and BAP Douglas-fir callus could be grown on a medium containing 5.0 mg/l 2,4-D as the sole exogenous growth regulator. For adventitious bud formation in callus cultures, callus was grown on a medium containing N_6-isopentenylpurine (2iP) at 10^{-4}M with 10^{-7}M NAA. Cultures were grown for eight weeks before they were harvested.

Crown Gall Cultures

Six month old Douglas-fir seedlings were wounded on the stem and inoculated with suspensions of several Agrobacterium tumifaciens lines. The seedlings were grown for an additional 5-6 months in a greenhouse, after which those seedlings forming tumors were collected, the tumors excised, surface sterilized and placed on a one-half strength M&S medium containing no exogenous growth regulators. Tumor tissues were grown on under the same temperature and photoperiodic conditions as the normal Douglas-fir callus cultures.

Shoot Tip Cultures

Shoot tips (defined here as the shoot apical meristem and all the needle primordia contained in the bud) were excised and cultured on a modified M&S medium with the KNO_3 and NH_4NO_3 levels reduced to one-fourth their original strength containing the same vitamin, sucrose and agar levels as the callus medium. Shoot tips cultured on a medium containing 10^{-4}M BAP with either no or very low (10^{-7}M) NAA

formed adventitious buds on the needle primordia of the original shoot tip. Cultures were grown under the same light, photoperiod and temperature conditions as the callus cultures for eight weeks.

Shoot Tip Material

Shoot tips of mature, flowering Douglas-fir trees were collected in the vicinity of Corvallis, Oregon during March and April just before bud break, just after bud break and during early elongation of the shoot. Bud scales were removed from the shoot tips and they were frozen in liquid nitrogen and stored at -70°C until extracted for IAA analysis.

Results

Comparison of Estimates of IAA Levels in Light and Dark Grown Douglas-fir Callus

Douglas-fir callus grown in the light had a much greater final fresh weight than did callus of the same line grown on the same medium and under the same conditions except that it was grown in the dark (Table I) Estimates of endogenous IAA levels were higher in the light grown callus as compared to the dark grown callus (Table II). IAA levels were low in the dark grown callus and was not detectable in two of the samples.

Estimates of Endogenous IAA Levels in Callus Cultures Grown on 2,4-D

Douglas-fir callus cultures grown on a medium containing 2,4-D had higher levels of endogenous IAA than those grown on a medium containing NAA and BAP (Table III).

Estimates of Endogenous IAA Levels in Douglas-fir Crown Gall Tissues

Tissues of tumors of Douglas-fir incited by the bacterium Agrobacterium tumifaciens that were grown on a medium free of any exogenous auxins or cytokinins contained high endogenous IAA levels (Table IV). These tumor tissues grew rather slowly and required a culture period of 12-16 weeks as compared to an 8 week culture period for normal tissues grown on a medium containing NAA and BAP. There also appeared to be differences in the estimates of the endogenous IAA levels between the two crown gall tumor lines, due to differences in the strain of Agrobacterium used to incite the tumors.

TABLE I. COMPARISON OF FRESH WEIGHT GAIN IN LIGHT AND DARK GROWN DOUGLAS-FIR CALLUS CULTURES (GROWN ON ONE-HALF STRENGTH M&S MACRO AND MICROELEMENTS WITH 10^{-5}M NAA AND 10^{-6}M BAP) (n=10).

	Dark Grown	Light Grown
Fresh Weight	391.5 mg	772.6 mg

TABLE II. ESTIMATES OF ENDOGENOUS IAA LEVELS IN DOUGLAS-FIR CALLUS CULTURES GROWN IN THE LIGHT AS COMPARED TO CALLUS GROWN IN THE DARK (MEANS AND STANDARD ERROR OF THE MEANS SHOWN).

Light Grown	Dark Grown
16	0
8	12
72	0
24	12
$\bar{X}=30.0 \pm 14.3$ ng/g FW	$\bar{X}=6.0 \pm 3.5$ ng/g FW

TABLE III. ESTIMATES OF ENDOGENOUS IAA LEVELS IN DOUGLAS-FIR CALLUS GROWN ON 2,4-D.

256
268
336
156
$\overline{X}$=254.0 $\pm$ 37.1 ng/g FW

TABLE IV. ESTIMATES OF ENDOGENOUS IAA LEVELS IN TWO DOUGLAS-FIR CROWN GALL TUMOR LINES.

Crown Gall Line #38	Crown Gall Line #28
240	784
232	512
264	417
224	384
$\overline{X}$=240 $\pm$ 8.6 ng/g FW	$\overline{X}$=450.0 $\pm$ 119.8 ng/g FW

Estimates of Endogenous IAA Levels in Bud Forming and Non-Bud Forming Douglas-fir Callus Cultures

Douglas-fir callus cultures could be induced to form adventitious buds if grown on a medium containing 10^{-4}M 2iP and 10^{-7}M NAA. Not all of the cultures of the same callus line formed adventitious buds under these conditions. A comparison of estimates of the endogenous IAA levels in several different callus cultures, both forming and not forming adventitious buds did not show any significant differences between the cultures forming buds and those that did not (Table V).

Estimates of Endogenous IAA Levels in Douglas-fir Shoot Tips Collected Before, During and After Bud Break in the Spring

Shoot tips collected in March and April before the buds swelled contained several hundred nonograms of endogenous IAA (Table VI). As the buds began to swell and the size of the shoot tip increased, the endogenous IAA levels appeared to increase dramatically. This was followed by slow decline in the endogenous IAA levels as the shoot elongated.

Estimates of Endogenous IAA Levels in Cultured Douglas-fir Shoot Tips Forming and Not Forming Adventitious Buds

A comparison of estimates of endogenous IAA levels in cultured shoot tips both forming and not forming adventitious buds, like the comparison of Douglas-fir callus cultures forming and not forming adventitious buds did not show any significant differences in the endogenous IAA levels (Table VII).

TABLE V. COMPARISON OF ESTIMATES OF ENDOGENOUS IAA LEVELS IN DOUGLAS-FIR CALLUS CULTURES FORMING ADVENTITIOUS BUDS AND THOSE NOT FORMING BUDS.

Not Forming Buds	Forming Buds
454	647
445	338
503	688
576	568
$\bar{X}$=494.5 $\pm$ 30.0 ng/g FW	$\bar{X}$=572.7 $\pm$ 66.4 ng/g FW

Difference not significant at p = 0.05

TABLE VI. ESTIMATES OF ENDOGENOUS IAA LEVELS IN DOUGLAS-FIR SHOOT TIPS COLLECTED BEFORE, DURING AND AFTER BUD BREAK.

Before Bud Break (March)	Before Bud Break (April)	Just Before Bud Break	Just After Bud Break	During Shoot Elongation
588	480	4883	1388	1220
580	544	5292	2812	1832
632	428	2104	3608	1450
524	352	7284	3848	1663
$\bar{X}$=581.0 $\pm$ 22.1 ng/g FW	$\bar{X}$=451.0 $\pm$ 40.6 ng/g FW	$\bar{X}$=4890.7 $\pm$ 1066.7 ng/1 FW	$\bar{X}$=2914.0 $\pm$ 554.7 ng/g FW	$\bar{X}$=1541.2 $\pm$ 132.5 ng/g FW

TABLE VII. ESTIMATES OF ENDOGENOUS IAA LEVELS IN DOUGLAS-FIR SHOOT TIPS FORMING AND NOT FORMING ADVENTITIOUS BUDS.

Not Forming Buds	Forming Buds
354	511
556	289
204	167
$\bar{X}$=371.3 $\pm$ 101.9 ng/g FW	$\bar{X}$=322.3 $\pm$ 100.7 ng/g FW

Difference not significant at p = 0.05

Discussion

These results demonstrate that this technique for the detection and quantitation of endogenous levels of IAA can be applied to small quantities of plant tissue. Although the amounts of IAA reported in this study are only estimates of the endogenous IAA levels, perhaps some comments on the patterns that were observed are appropriate. A larger number of samples than were available for this study would be necessary to support any conclusive statements about the levels of endogenous IAA in these tissues.

It appears that endogenous IAA levels are quite low in callus cultures maintained on NAA and BAP. The reason for this is not known. It has been shown that exogenous applications of NAA (Simola and Sopanen 1971), cytokinins (Lee 1971) and even 2,4-D (Lee 1972) can effect IAA oxidase levels in cultured tissues, thus altering endogenous IAA levels. Perhaps the differences in endogenous IAA levels observed in this study are a result of differences in the levels of IAA oxidase resulting from these different growth regulator treatments.

The fact that there were no large differences in the estimates of endogenous IAA levels between Douglas-fir callus cultures and shoot tips forming buds and those that did not, suggests that IAA may not play an important role in determining which cultures will form buds and which will not. However, this may also be a result of the method used to distinguish the bud forming from the non-bud forming cultures. The only way to determine if a culture is forming buds

is to examine it after it has been grown on the bud inducing growth regulator treatment for eight weeks. If a bud or small shoot can be identified, then the culture was considered to be a bud forming culture. There is no way of knowing how far along the developmental pathway the non-bud forming cultures may have progressed before something stopped the process, so there may be no satisfactory way to separate bud forming and non-bud forming cultures unless we had lines that either would or would not form buds consistently.

Another problem is that the number of cells involved in the bud forming process is only a fraction of the total number of cells in either a callus mass or a cultured shoot tip. Thus, any differences in the endogenous IAA levels in these few cells may be hidden by the IAA levels in the surrounding, non-bud forming cells.

Kevers et al. (1981), however, found dramatic differences in the endogenous IAA content of organ forming as compared to non-organ forming cultures of habituated sugarbeet callus. The organ forming cultures had a high peroxidase level and was estimated to contain 198.4 ± 75.8 ng/g fresh weight IAA while the non-organ forming line contained a low peroxidase level and 1241.7 ± 296.1 ng/g fresh weight IAA. No such dramatic differences were found in either Douglas-fir callus or shoot tip culture forming and not forming adventitious buds.

Estimates of endogenous IAA levels in buds of conifers has been attempted several times in the past, including two studies with Douglas-fir. Both of these studies included only one or two sampling dates. Caruso et al. (1978) reported IAA levels in Douglas-fir

shoots collected in June from a mature flowering tree of about 40 years old to be 1600 ng/g fresh weight. Measurements of endogenous IAA in shoot tips of 15 year old Douglas-fir trees reported by De Yoe and Zaerr (1976a) were 705 ng/g fresh weight in periods of active growth and less than 5 ng/g fresh weight during winter dormancy. De Yoe and Zaerr (1976b) also reported levels of 1380 ng/g and 2360 ng/g fresh weight in the spring of 1972 and 1971 respectively. While the highest levels of endogenous IAA reported in this study are higher than either of these two previous reports, they are not unreasonable because there seem to be significant changes in the endogenous IAA level depending on the time when the sample was collected further more, De Yoe and Zaerr (1976b) have shown there appear to be differences in the endogenous IAA levels from year to year.

Although samples were not collected early enough in the spring to answer the question as to whether endogenous IAA levels increase slowly from mid-winter to just before bud break which may affect the site of adventitious bud formation on cultured Douglas-fir shoot tips, it appears that based on the mid-winter measurement of IAA of De Yoe and Zaerr (1976a) of 5 ng/g fresh weight which increases to several hundred or thousand nanograms during bud break, this hypothesis does not seem unreasonable.

This technique for the measurement of endogenous IAA levels hold great promise for future studies on the role of IAA in plant development. The fact that only several hundred milligrams of tissue is required for the analysis makes measurements of single callus masses possible as well as pooled samples of tissues such as shoot

tips. With the development of similar sensistive techniques for the other plant growth regulators at hand, our understanding of the roles of these compounds in plant growth and development is certain to improve.

CONCLUSIONS

The three main aspects of this study, the induction of adventitious buds on cultured shoot tips of Douglas-fir, the induction of adventitious buds on callus cultures of Douglas-fir and estimations of endogenous IAA levels in shoot tip and callus cultures have been combined to try to better understand the role of growth regulators in organ formation in these tissues. The fact that adventitious buds in both cultured shoot tips and seedling callus cultures of Douglas-fir were formed under a high cytokinin to auxin ratio is in agreement with the hypothesis of Skoog and Miller (1957). Thus, it appears that conifers are not unique in this regard. Low auxin levels permitted the formation of adventitious buds in both cultured shoot tips and callus cultures, but increases in the exogenous auxin levels resulted in inhibition of bud formation.

The shoot tip system presently offers the only method for the propagation of mature conifers in tissue culture. If elongation of the adventitious buds could be stimulated and roots could be induced on these shoots this system could be applied directly to forestry problems. A relatively high cytokinin treatment (10^{-4}M) is necessary for bud formation and several cytokinins were able to induce bud formation. The exposure time required to induce buds appears to be only two to three weeks on the high cytokinin treatment. The fact that only no or very low (10^{-7}M) levels of exogenous auxin permitted adventitious bud formation suggests that the shoot tip may already contain adequate levels of IAA. The present study indicates that

several hundred nanograms per gram fresh weight may be present in these shoot tips at the time of culture.

The observation that a reduction in the mineral salt levels, in particular the ammonium level of the M&S medium, is essential for adventitious bud formation raises some interesting questions. The role of mineral nutrition in organ formation has only received attention in tissues that do not respond to different auxin-cytokinin treatments on standard plant tissue culture media. Exactly how the ammonium ion concentration influences the ability of a culture to form adventitious buds is not known.

Douglas-fir seedling callus tissues demonstrated similar responses when grown in culture. A high (10^{-5}M) cytokinin treatment was necessary to induce adventitious bud formation in callus and all of the cytokinins tested were effective in inducing buds to form. As with cultured shoot tips, an increase in the exogenous auxin level inhibited adventitious bud formation. The effect of mineral nutrition, especially the ammonium ion concentration, had a profound effect on bud formation. A useful application of this technique would be to extend it to callus cultures induced from mature trees. This would allow for the development of such techniques as haploid plant production, where plants would be regenerated from unorganized tissues.

The method for the quantitation of endogenous IAA in plant tissues was successful when applied to both shoot tips and callus cultures of Douglas-fir grown in culture. Between 50-60 percent of the added indolepropionic acid used as an internal standard was

routinely recovered. Preliminary measurements of endogenous IAA levels in small quantities of these tissues was successful.

Differences in the pattern of endogenous fluorescent acids (including IAA) in Douglas-fir vegetative, male and female buds raised some interesting questions. The fact that the male buds contained such a large amount of IAA is interesting, but the differences in the patterns of the fluorescent acids that are more polar than IAA between the vegetative and male buds and the female buds opens up a new area for investigation. Little is known about the role of IAA and other related indole compounds in bud determination and flowering in conifers.

Endogenous IAA levels did appear to increase in Douglas-fir vegetative buds just prior to bud break and then slowly declined after bud break and during shoot elongation. The estimates of endogenous IAA levels in these buds were in general agreement with reports in the literature.

It appears that there are no substantial differences in the endogenous IAA levels between both shoot tips and seedling callus cultures of Douglas-fir either forming or not forming adventitious buds. While there is some evidence to show that low peroxidase levels in callus cultures may be associated with high endogenous IAA levels in callus lines that do not form adventitious buds (Kevers et al. 1981) and because low peroxidase levels have been reported in Douglas-fir callus cultures (Johnson and Carlson 1977), high endogenous levels of IAA were expected in Douglas-fir callus tissues.

However, this was not found in the present study. This result might suggest that IAA may not play as large a role in regulating bud formation in callus cultures as has been believed. Cytokinin levels in these tissues may be the key to morphogenesis and should be examined. This has not yet been done.

Plant cells and tissues grown in culture depend heavily on the exogenous levels of auxins and cytokinin for their growth and development. The measurement of endogenous levels of plant growth regulators will improve our understanding of the role they play in morphogenesis and will allow us to predict more accurately the exogenous growth regulator treatments required produce the desired type of organ formation. The interaction between endogenous growth regulator analysis and tissue culture will allow us to better understand how these growth regulators function in forest trees and will aid in our ability to multiply selected forest trees.

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