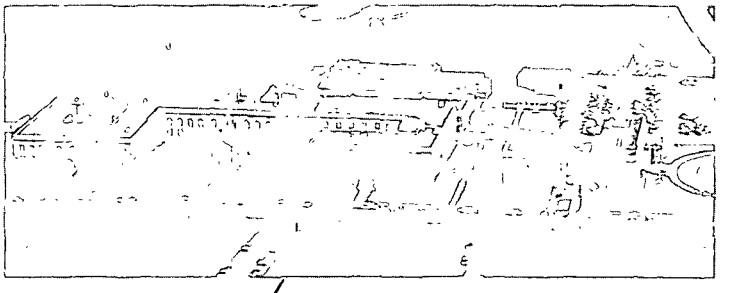
Tech. Pap. Series #34



THE INSTITUTE OF PAPER CHEMISTRY, APPLETON, WISCONSIN

IPC TECHNICAL PAPER SERIES
NUMBER 34

SHOOTS FROM DOUGLAS-FIR CULTURES

LAWSON L. WINTON AND SHIRLEY A. VERHAGEN

MAY, 1976

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SUMMARY

Douglas-fir shoots were produced from several types of plant material using tissue culture techniques. When seed embryos were removed and placed on a simplified agar growth medium, single vigorous shoots appeared on the tip of the swollen seed leaves (cotyledons). Increasing cytokinin levels resulted in the production of multiple shoots from seed leaves.

When the seed leaves from Douglas-fir seeds were removed and placed on a growth medium containing auxin and cytokinin, callus was produced. After subculturing the callus several times (monthly) shoots began to develop from the subcultured callus. Eleven of 35 subcultured sources of seed leaf callus produced shoots. Single shoots were also produced from subcultured needle callus and callus on an isolated stem section (explant) of seed leaf callus origin.

This paper was submitted for publication in the Canadian Journal of Botany.

Canadian Journal of Botany

Shoots from Douglas-fir Cultures

Lawson L. Winton and Shirley A. Verhagen

The Institute of Paper Chemistry, Appleton, Wisconsin 54911, USA

Abstract

Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) embryos were excised from the same seed lot and placed on a simplified medium made without auxin but containing 0.05 mg/l BAP (benzylaminopurine). After 3-4 weeks, usually only one vigorous shoot appeared from the tip of each of the swollen cotyledons. When 0.1 mg/l BAP was added, small multiple shoots appeared all over the cotyledons. When embryos or cotyledons with shoots were transferred from the 0.05 BAP treatment to medium without hormones, the shoots elongated and produced normal appearing needles. However, the small multiple shoots from the 0.1 BAP treatment usually did not elongate normally.

On the other hand, when excised embryos were started on a medium without hormones, and the cotyledons were subsequently excised and placed on medium having both an auxin and a cytokinin, the cotyledons swelled and produced callus that was isolated and subcultured monthly. From 53 seeds we established 35 clonal cultures of subcultured cotyledon callus. Shoots were produced from 11 of the 35 clones, for a frequency of 31%, indicating a high genetic variability among seed genomes in their ability to produce shoots from callus.

Of the many shoots excised and placed in rooting medium, two rooted after treatment with 10 mg/l IBA. In addition to embryo material, one shoot was produced from needle callus subcultured three times and one shoot was produced from callus on a stem explant prepared from a cotyledon callus shoot.

Introduction

At present, the aseptic culture of conifer trees permits the clonal propagation of seeds and seedlings of only a few species, but indicates the future

potential for the mass production of genetically identical ramets from older seedlings and trees of most conifers (3, 7, 14, 21). Methods of propagation from callus will also be necessary for the regeneration of plants from the products of somatic hybridization, involving either the fusion of protoplasts from species of the same genus or from transformed protoplasts which have received genetic information from species of a different genus or family (21, 22).

So far, buds and leafy shoots have been produced from cultures of relatively few conifer tree species (Table 1), and plantlets have been reported only from cotyledons of Pinus palustris (16, 17), hypocotyls of Picea glauca (5), and from cotyledons and subcultured cotyledon callus of Pseudotsuga menziesii (6).

This paper reports methods of shoot production from cotyledons and subcultured cotyledon callus of Douglas-fir different from those cited above, including a measure of variability among seeds to produce shoots from cotyledon callus. We also report the production of leafy shoots from subcultured needle callus and stem explant callus.

Methods and Results

Seeds of Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco), Lot 491-15-1, were received in February, 1974, from Dr. John Rediske, Weyerhaeuser Company, Centralia, Washington, and were surface sterilized either by a quick dip in 95% alcohol or for 15 min in half-strength bleach containing 2.5% sodium hypochlorite. Seeds sterilized in bleach were then rinsed several times and stored overnight in sterile water at 3° C. Seed embryos were excised and placed at 5-7 per Petri dish on an agar medium suitable to the experiment. The dishes were sealed with Parafilm and the embryos were grown for one week in the dark at 27° C, then transferred to an incubator having 2000 lx of cool-white fluorescent light for 16 hr at 24° C, alternating with 8 hr darkness at 18° C.

Shoots Directly from Cotyledons. Seeds were sterilized with bleach, and embryos were excised and placed on medium E-1 or 19 (Table 2) containing 0.05 or 0.1 mg/l

BAP (benzylaminopurine). After one week in the dark, embryos were transferred to the light incubator where they swelled to several times their original diameter and turned green, but did not elongate as did normal seedlings on the same medium without BAP. There was no significant difference between medium E-1 and 19, but a large difference was observed between the two levels of BAP.

On medium with 0.05 mg/l BAP, usually only one shoot appeared at the tip of most cotyledons after 3-4 weeks. Most cotyledons were excised and transferred to medium without hormones, where the shoots elongated and produced normal appearing needles. Figure 1 shows a whole embryo after nine weeks on medium 19 with 0.05 mg/l BAP. This embryo was transferred intact to medium without hormones, to a light source in the laboratory having 4000 lx fluorescent and 400 lx tungsten light, on for 16 hr and dark for 8 hr , at 23-25° C. Fig. 2 shows the embryo three months after transfer, when two shoots had elongated to 2-3 cm.

When excised embryos were placed on medium with 0.1 mg/l BAP, 50-60% produced small multiple shoots all over their cotyledons after 3-5 weeks. About 10% of the embryos were stunted and had very tiny multiple shoots only at the tips of their cotyledons. However, when whole embryos, excised cotyledons, or cotyledon tips were transferred to medium without hormones and placed in fluorescent-tungsten light, the small multiple shoots rarely elongated normally, if at all (Fig. 3).

In this study, many more shoots were produced with the higher level of BAP, but the only shoots to elongate normally were the fewer and more vigorous shoots initiated at the lower level of BAP.

Shoots from Subcultured Cotyledon Callus. Seeds were sterilized with a quick dip in 95% ethanol and the embryos were excised as soon as the alcohol evaporated. Embryos were placed on medium E-1 in the dark, and after one week were transferred to the light incubator where the cotyledons began to elongate and turn green. After 3-4 weeks on medium E-1, the cotyledons were 1-2 cm long and were excised and placed on medium 10 (Table 2) containing both an auxin and a cytokinin.

After a few weeks on medium 10, the cotyledons became covered with soft green callus, which was isolated from the original cotyledon tissue and subcultured monthly to fresh medium 10. Over a period of several passages the callus became darker green in color and firmer in texture, and all callus derived from one embryo was labelled as a separate clonal culture. The amount of callus increased 2-4 times each passage, so that each clone was represented by 1-6 dishes, depending on the number of sulcultures, with 5 pieces of callus per 100 x 15 mm glass Petri dish.

Over a period of 15 months in 1974-5, attempts were made to grow callus from 53 seed embryos. Twenty-three callus clones were lost because of poor growth or contamination, and 35 clones were established as subcultured callus cultures. Of the 35 clones, 11 started to produce shoots after 1-6 sulcultures, giving a frequency of 31% of the surviving clones that produced shoots (Table 2).

Generally only one vigorous shoot appeared on a callus piece and on 1-2 callus pieces per dish of 5 (Fig. 4), but occasionally 2-3 shoots grew from one callus. Shoots were initiated over a period of several months from individual clones, but no attempt was made to accurately record the number of shoots produced. However, a conservative estimate would be 100-200 shoots from all 11 clones.

Many callus pieces were transferred to medium E-1 without hormones, and shoots elongated and produced normal appearing needles. Figure 5 shows one callus piece with several elongating shoots, after several months in fluorescent-tungsten light. Many elongating shoots 1-5 cm tall were excised from callus and placed in rooting medium containing 1-10 mg/l IBA (indolebutyric acid) in the same fluorescent-tungsten light. However, from the 80-100 attempts, only two shoots rooted after treatment with 10 mg/l IBA, indicating that agar may be a poor rooting medium. In the first case, rooting occurred after several months on the rooting medium. In the second case, rooting did not occur until the shoot had successively been transferred to medium with 1 mg/l IBA, medium E-1, 10 mg/l IBA, and to medium

E-1. During this time the shoot produced callus from its base and several new shoots grew from the callus. One vigorous root 2 cm long was initiated from the callus nearly one year after the original shoot was initiated from cotyledon callus.

During this past year we have continued to put out excised embryos and produce cotyledon callus; however, hardly any shoots have been produced from the many established clones. In looking for an explanation, we discovered that all seeds during this past year were sterilized with bleach instead of alcohol. We ran a few tests using the two methods, and found differences in the pattern of shoot initiation directly from cotyledons. We are now testing the hypothesis that bleach sterilization of seeds subsequently inhibits shoot initiation from subcultured cotyledon callus.

Shoots from Needle and Stem Callus. For the past 6-7 years, we have been attempting to produce shoots from subcultured stem callus, initiated from cambial and cortical cells from branches of several conifer species (19-22). After 2-3 years in culture we observed small, nonfunctional budlike structures on callus of some species (Table 1). However, no further shoot organization has taken place in these old cultures, and all except the Douglas-fir callus has been discarded. New cultures of Douglas-fir and loblolly pine are now being used for shoot-initiation studies by our team that includes a biochemist, an electron microscopist, and a tissue culturist (22). However, recent results in our laboratory, and in that of Cheng (6), indicate that the mass production of Douglas-fir may be easier using needle callus than by using stem callus.

A normal seedling was grown from an excised Douglas-fir embryo placed on medium E-1. When the seedling was three months old and 4-5 cm tall, whole needles were excised and placed on medium 10 in 5000 lx continuous fluorescent light at 25° C. From the base of the needles, callus grew and was subcultured three times during four months on medium 10. Eleven days after the third subculture one shoot started to grow from the callus. Figure 6 shows the shoot six months later.

However, it died shortly after it was photographed and did not elongate.

In a similar type of experiment, needle and stem explants were prepared from elongating shoots initiated from subcultured cotyledon callus. Some stem segments were placed on medium 19 with 1 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid). Callus grew rapidly from the explants but was not isolated or subcultured. One vigorous shoot (Fig. 7) grew from the callus of one explant, but when it was isolated from the callus the original explant was not recognizable and we could not determine if the shoot arose from the explant or from callus. We are currently attempting to repeat these results on a larger scale.

Discussion

We have demonstrated propagation methods similar to those reported by Sommer et al. (16, 17) for pine species, Campbell and Durzan (5) for white spruce, and Cheng (6) for Douglas-fir. From whole embryos we obtained a few vigorous shoots from the tips of swollen cotyledons using 0.05 mg/l BAP, but with 0.1 mg/l we observed various degrees of initiation of small and numerous shoots. Campbell and Durzan (5) obtained maximum frequencies of hypocotyl shoot initiation using 2 mg/l BAP and no auxin, but also obtained nearly as good initiation using 2 mg/l BAP and 0.1 mg/l NAA (naphthaleneacetic acid). When we used 2 mg/l BAP and 0.02 NOAA (naphthoxyacetic acid), one of five embryos on one dish became completely covered with small budlike structures, each less than 1 mm in diameter. Unfortunately the embryo was never transferred and eventually died.

Shoot initiation from Douglas-fir cotyledons apparently was controlled by the level of cytokinin added to the medium. Interestingly, we observed the same relationship in cytokinin control of shoot initiation from aspen callus (18). For both aspen and Douglas-fir, a few vigorous shoots were produced by 0.05 mg/l BAP, and small multiple shoots were produced with 0.1 - 0.2 mg/l BAP. For both species, the small shoots did not elongate while on the callus, but only the small shoots of aspen rooted in high frequencies when excised.

On the other hand, our results with subcultured cotyledon and needle callus were different than expected. Callus from both sources was maintained on medium 10 having 0.1 mg/l BAP and 5 mg/l NOAA, and yet the initiation of vigorous shoots was common from cotyledon callus and one shoot was produced from needle callus. This might indicate that either the theory of cytokinin induction of shoots is not valid in callus cultures, or else cytokinin may have been produced endogenously and thus preserved the critically high ratio of cytokinin to auxin. This problem certainly requires a great deal of new information, but perhaps future studies should not be designed to conform strictly to currently popular theories.

Genetic variability was demonstrated in our results with cotyledon callus, but was not surprising because of the variability demonstrated elsewhere by conventional silvicultural and rooting studies. However, by designing specific nutrient media, some day we may be able to screen seedlings for superior characteristics using aseptic culture techniques.

To date, the state of the art has developed to the point where the clonal propagation of seeds can now become a routine practice, either from cotyledons or from subcultured cotyledon callus, depending on the number of ramets needed. For the propagation of seedlings 3-4 years old or trees 10-20 years old, the most promising method seems to be from needle callus. However, for the most efficient, effective, and economical mass production of conifer trees, the eventual method will most probably have to be from suspension cultures. The progress now being made in several laboratories makes this a realistic goal.

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TABLE 1

Conifer tree species producing buds, shoots or plantlets in culture

Species & Reference	Treated Material	Results[a]
Biota orientalis[b] (15)	Cotyledon	Shoots
Cryptomeria japonica (10)	Hypocotyl	Shoots
Picea abies (9, 20)	Stem callus	Shoots
Picea glauca (5)	Hypocotyl	Plantlets
Pinus banksiana (2, 20)	Hypocotyl callus	Shoots
Pinus cembra (2, 20)	Hypocotyl callus	Shoots
Pinus elliottii (17)	Cotyledon	Shoots
Pinus lambertiana (8)	Hypocotyl, hypocotyl callus	Shoots
Pinus palustris (16, 17)	Cotyledon	Plantlets
Pinus ponderosa (20)	Stem Callus	Buds
Pinus rigida (17)	Cotyledon	Shoots
Pinus strobus (17)	Cotyledon, hypocotyl callus	Shoots
Pinus virginiana (17)	Cotyledon	Shoots
Pseudotsuga menziesii (20)	Stem Callus	Buds
(6)	Cotyledon	Shoots
(6)	Cotyledon Callus	Plantlets
(6)	Needle Callus	Plantlets
Sequoia sempervirens (1)	Stem callus	Buds

[a] Buds = nonfunctional structures a few mm long. Shoots = elongating structures with normal appearing needles. Plantlets = excised and rooted shoots.

[b] Genus *Biota* is now genus *Thuja*.

TABLE 2

Nutrient media used to culture Douglas-fir

A. Salts common to all media[a]

Component	Mg/liter	Component	Mg/liter
NH_4NO_3	1650	H_3BO_3	6.2
KNO_3	1900	KI	0.83
$MgSO_4$	370	Na_2MoO_4	0.25
KH_2PO_4	170	$CuSO_4$	0.025
$CaCl_2$	440	$CoCl_2$	0.025
$MnSO_4$	16.9	Fe(EDTA)[b]	5.6
$ZnSO_4$	10.6		

B. Components in mg/liter added to common salts to give specific media

Component	Medium 10[c]	Medium 19	Medium E-1
Nicotinic acid	0.5	-	-
Thiamine	0.1	-	0.4
Pyridoxine	0.1	-	-
Inositol	100	-	100
Asparagine	100	-	-
BAP[d]	0.1	-	-
NOAA[d]	5	-	-
Sucrose	30,000	30,000	40,000
Agar	8,000	8,000	8,000

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[a] Adapted from Brown & Lawrence (4). Water of hydration not given.

[b] 5 ml of stock solution containing 5.57 g $FeSO_4$ and 7.45 g Na_2-EDTA per liter of H_2O.

[c] From Winton (19).

[d] BAP = N^6-benzylaminopurine, NOAA - naphthoxyacetic acid.

TABLE 3

Shoots from Douglas-fir subcultured cotyledon callus

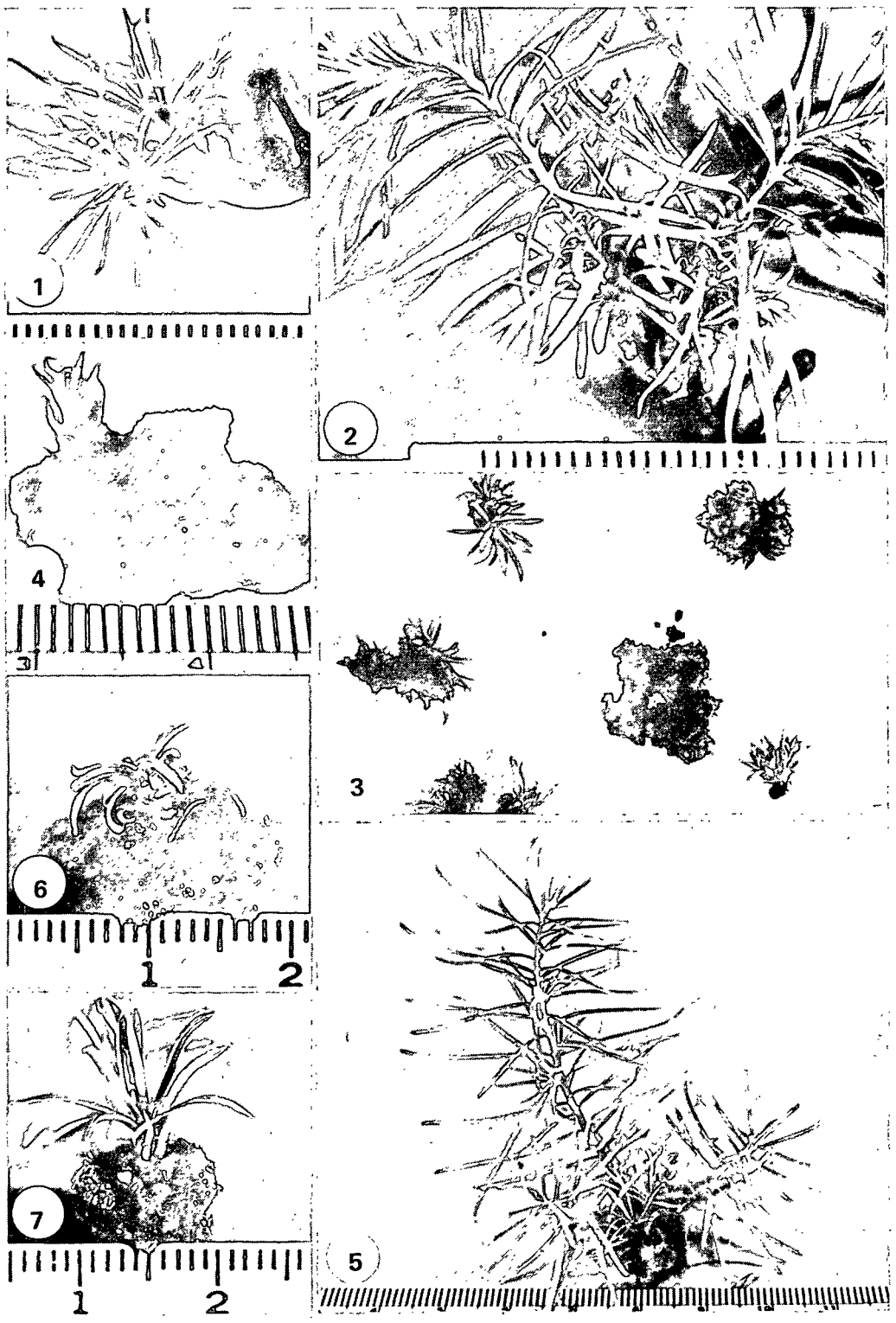
Number	Clone	Embryos Excised	Number of Subcultures[a]	First Shoots	
				1974	1975
1	9	4- 1-74	5	11-18	1-28
2	10	4- 1	4	11-19	
3	34	5-21	4	11-19	
4	42	7-25	2	12- 4	3- 3
5	46	8-26	3		2- 3[b]
6	49	8-26	6		6-13
7	51	10- 5	4		6-4
8	52	10-14	1		1-13
9	58	4-23-75	4		9-26
10	62	4-23	2		8-25
11	67	7- 3	2		11-11

[a] Number of subcultures until shoots first appeared.

[b] First shoots to elongate more than 1 cm while attached to callus.

Figures

Fig. 1. Swollen Douglas-fir embryo after nine weeks on medium with 0.05 mg/1 BAP. Single leafy shoots grew from the tips of most cotyledons. Fig. 2. The same embryo shown in Fig. 1 was transferred to medium without hormones, and is shown here after three months when two shoots elongated to 2-3 cm. Fig. 3. Small multiple shoots were initiated over the surface of excised cotyledons on medium 19 with 0.1 mg/1 BAP, and are shown here after transfer to medium without hormones. Small shoots did not elongate normally. Fig. 4. A small single shoot was initiated from cotyledon callus subcultured three times in late 1974. Fig. 5. When cotyledon callus was transferred to medium without hormones, shoots elongated and produced normal appearing needles. Fig. 6. One shoot was produced from needle callus subcultured three times on medium containing hormones. Fig. 7. One shoot was produced from callus on a stem explant from a cotyldeon callus shoot.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