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ORGANOGENESIS IN VITRO OF TISSUES FROM MATURE CONIFERS

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SUMMARY

Shoot and embryolike structures were obtained in cultures of various tissues of mature conifer trees. Morphogenesis occurred in haploid and diploid tissues of female cones of *Pinus mugo* and *Picea abies*, although only rarely. Vegetative shoots dissected from early spring buds of *Abies balsamea*, *Picea glauca*, and *Pseudotsuga menziesii* were more responsive, with most of the organized growth occurring at the base of young needles. The following sequence of treatments was most effective: (a) collect twigs with buds shortly before budbreak, (b) cold store for at least 1 month, (c) force buds to break before excision of the vegetative shoots (keep submerged in water during excision), and (d) soak explant in malonic acid, 1 g/l, for 15 min before transfer of the shoots to the nutrient medium.

Key words: mature conifers; vegetative propagation.

INTRODUCTION

Many coniferous species have been propagated vegetatively by culturing tissues of embryos or young plants. However, because the genetic potential for traits such as height growth, stem taper, branching, and crown shape cannot be evaluated until the tree matures, it is important to have methods to propagate, vegetatively, mature conifers rather than embryos or seedlings. Propagation using tissues of mature conifers is difficult and presently is possible only when the trees have stump sprouts or other sources of juvenile tissue available (1). In most conifers juvenile tissues are not present in mature trees, and explants from such trees have shown only a limited morphogenetic aptitude (2-4).

This report describes some chemical and physical factors that are involved in the initiation of organized growth in mature tissues of various conifers.

MATERIALS AND METHODS

Male and female cones in various stages of development were used because diploid tissues near the site of meiosis could be more morphogenetically active than other tissues (5). Male cones were used also because they contain a large mass of healthy, green pith, which, in vegetative buds, sometimes produced organized structures (2). Most of the work was carried out with young vegetative shoots, excised from winter and spring buds (2). Young vegetative shoots were chosen because their shoot tips and young foliage are known often to have a higher potential for regeneration than other plant parts (6).

Collection and Storage

Immature female cones. The female cones of most pine species in eastern North America mature over two summers. Cones of *Pinus mugo* var. *mughes* Zenari (Mugho pine), *Pinus banksiana* Lamb (Jack pine), and *Pinus sylvestris* L. (Scots pine), in their first summer of development, were collected weekly for 1 month, starting the 1st wk of June when the small purple or green cones were about 5 mm long.

Immature and mature seeds as source of female gametophytes. Cones with immature seeds were collected biweekly in June and July from Mugho pine and *Pinus nigra* var. *austriaca* Aschers and Graebn. (Austrian pine). Commercially obtained seeds of *Picea abies* (L.) Karst. (Norway spruce) and *Picea glauca* (Moench) Voss (white spruce) provided mature gametophytes.

Male cones as source of pith. Male cones of *Pinus resinosa* Ait. (red pine) and Mugho pine

were collected weekly, starting with cones about 10 mm long and ending at pollen shedding about 4 wk later.

Branches as source for young vegetative shoots. Branches with vegetative buds were collected in the fall, winter, and early spring from upper halves of crowns of 15 to 25-yr-old *Abies balsamea* (L.) Mill. (balsam fir) and white spruce trees in wild stands and from lower halves of crowns of 40-yr-old plantation grown *Pseudotsuga menziesii* (Mirb.) Franco (Douglas fir) trees.

Storage. Immature female cones and branches with vegetative buds were stored in plastic bags in the dark at 4° C. Older female and male cones were stored in paper bags at 4° C.

Preparation of Cultures

Immature female cones. After 1 to 4 wk of cold storage, the small cones were surface sterilized by dipping in 95% ethanol, followed by ignition of the alcohol to remove it. The cones were cut transversely, under water, into disks 1 to 2 mm thick. The disks were placed on Brown and Lawrence's modification of Murashige-Skoog's nutrient medium, supplemented with various levels of growth regulators (7).

Immature and mature female gametophytes. Gametophytes were removed from cones or seeds as described previously (7). They were cut longitudinally and all embryonic tissue was removed. During the first few years of the investigation many basal formulations were tested, but eventually Brown and Lawrence's medium was chosen as standard. The growth regulating effects of many chemicals were investigated, but for routine purposes 1 or 5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) or indolebutyric acid (IBA) was used.

Pith tissue. The male cones were surface sterilized by dipping in 95% ethanol and flaming. They were quartered longitudinally under water to expose the pith. To avoid excessive injury to the pith, it was not separated from the other tissues of the cones. The tissue was cultured on Brown and Lawrence's medium supplemented with various growth regulators.

Vegetative shoots. After a few days to several months of cold storage, the shoots were excised either immediately or after the buds had been forced. Before removing vegetative shoots from nonforced buds, twigs with buds were dipped in 95% alcohol and flamed. In most experiments conducted before 1978, the flamed buds were broken from the twigs with sterile forceps and placed on moist filter paper in petri dishes. The bud scales were removed and the green shoots were excised for further treatments. After early 1978 the shoots were excised while submerged in sterile water in petri dishes.

To obtain vegetative shoots from forced buds, twigs about 10 cm long were placed in beakers with about 1 cm of water, Ottawa solution (8), or ethylenediaminetetraacetic acid (EDTA) solutions (0.1, 0.01, 0.001 g/l in water). Ottawa solution was used because it has antiwilting effects and promotes water uptake in cuttings (8). EDTA was tested because it increases the water permeability of cell membranes (9). The twigs were exposed to about 500 lux of fluorescent (Sylvania, Gro-Lux) light for 16 h/d at 20° C until green needle tips emerged between the separating bud scales.

The forced buds could not be surface sterilized by dipping in alcohol and flaming because the emerging needles, lacking the protection of bud scales, dessicated with such treatment. Instead, stem sections with buds were agitated in a beaker containing 6% (wt/vol) sodium hypochlorite for 10 min, using a magnetic stirrer. The stem sections were then stirred in 70% ethanol for 2 min and washed three times with sterile distilled water (4). While keeping the sections under water, the buds were separated from the stems and the vegetative shoots were excised by removing the bud scales.

Before transfer to the nutrient medium, all excised shoots were soaked, generally for 15 min, in water or in growth regulator solutions (2,5). Most soaking was carried out at room temperature in petri dishes with 10 shoots/dish. Sometimes the shoots were soaked in water for 24 h in test tubes rotated at 1 rpm.

Since most of the auxins and herbicides used in the soak treatments were weak acids, part of their action could be a pH effect (10). For example, a solution of IBA (1 g/l), a soak frequently used, had a pH of 3.7. Therefore, a separate experiment was carried out in which white spruce, balsam fir, and Douglas fir shoots were treated with citric acid:phosphate buffer, with or without 1 g/l IBA, at pH's ranging from 2 to 8 at 1 pH unit intervals.

Many of the common conifer nutrient media, and modifications thereof, were used. However, most experiments were performed with modified Romberger et al. (2) medium prepared as described previously.

Incubation Environment

All cultures were in 18-mm test tubes, each containing 15 ml agar medium and capped with "Morton" stainless steel closures. The tubes were held in racks (40 tubes/rack) inside clear plastic bags. Extensive preliminary experiments had shown that enclosure in plastic bags did not alter growth and differentiation of the cultures; apparently the plastic was sufficiently permeable to gases to enable proper gas exchange between the cultures inside and atmosphere outside the bags. The bags greatly reduced dessication and contamination of the cultures.

Several light and temperature regimes were tested, but most cultures were kept at constant 21° C and exposed to 500 lux fluorescent (Gro-Lux) light, 16 h/d.

RESULTS

Immature Female Cones

Most cone disks, regardless of species and collection date, produced a slow-growing green callus. In 1977, the first year of the investigation, disks from only one mugho pine tree from one collection, June 15, showed morphogenetic development. The callus produced small domes that were much darker and greener than the surrounding callus; they resembled shoots with their needle primordia arranged in a phyllotaxy similar to that of primordia in vivo (Fig. 1). Except for one shoot that carried a few well-developed bud scales on one flank (Fig. 2) the shoots lacked bud scales. The experiment was repeated the following year with a large sample, but at that time organized structures did not appear.

Immature and Mature Female Gametophytes

The most rapid haploid callus growth of immature and mature gametophytes was obtained on Brown and Lawrence's medium supplemented with 1 or 5 mg/l 2,4-D or IBA. The gametophytes showed only a low morphogenetic response. The immature gametophytes, or their callus, never produced adventitious organs or embryos. The mature gametophytes of Norway spruce formed a few haploid plantlets once on Brown and Lawrence's medium with 0.2 mg/l indoleacetic acid as auxin (11). Similar results with female gametophytes of Norway spruce have been reported by other investigators (12,13). This experiment was repeated many times on several culture media, but the organized growth could not be reproduced.

Pith Tissue

The pith of the male cones remained relatively inactive, producing little callus but no organized growth.

Vegetative Shoots

The effects of season, forcing, and cold storage. Earlier observations (2) had indicated that material collected in the spring often produced organized structures in the form of embryo or shoot-like protuberances from the basal region of the needles ("needle organs") more readily than fall or winter collections. This seasonal effect was examined further with material collected between October 1976 and April 1977 (Table 1). This experiment confirmed that the incidence of needle organs was highest in the spring.

The possibility of obtaining comparable results by cold storing and subsequent forcing of fall and winter buds was examined by comparing forced and nonforced shoots excised and cultured within 2 d after collection with those excised from twigs that had been cold stored for 6 wk (Table 2). There was practically no response in either non-cold stored or cold stored nonforced material. Forced buds, especially if cold stored and forced in EDTA solution, showed a higher incidence of needle organ formation. The forced buds exhibited a seasonal trend similar to those shown in Table 1. This suggested that shoots excised from forced fall and winter buds were not physiologically comparable to those of spring buds.

Excision effects. At first, shoots were excised on sterile moist filter paper. However, this practice resulted in many shoots turning brown within a few days or weeks. To increase the survival rate, an experiment was performed with balsam fir and white spruce shoots, comparing effects of excision on filter paper dampened with water or antioxidant solutions, and excision completely submerged in water (Table 3). With shoots excised on filter paper all antioxidants improved survival. Excision under water was as effective as on filter paper dampened with antioxidant solution, suggesting that avoiding exposure of cut surfaces to air was critical in assuring high survival. In many consequent experiments, the excision and soaking steps were combined by excising the shoots in the soak solution.

Soaking effects. A large number of auxins, cytokinins, growth inhibitors, herbicides, antioxidants, and other chemicals were included in soak solutions. Most of the chemicals tested had little effect. The only treatment that led consistently to development of needle organs was a soak in 1 g/l malonic acid. In one experiment, shoots from forced, spring-collected buds of balsam fir were excised under water, soaked 24 h in water on a roller drum, then soaked further in malonic acid

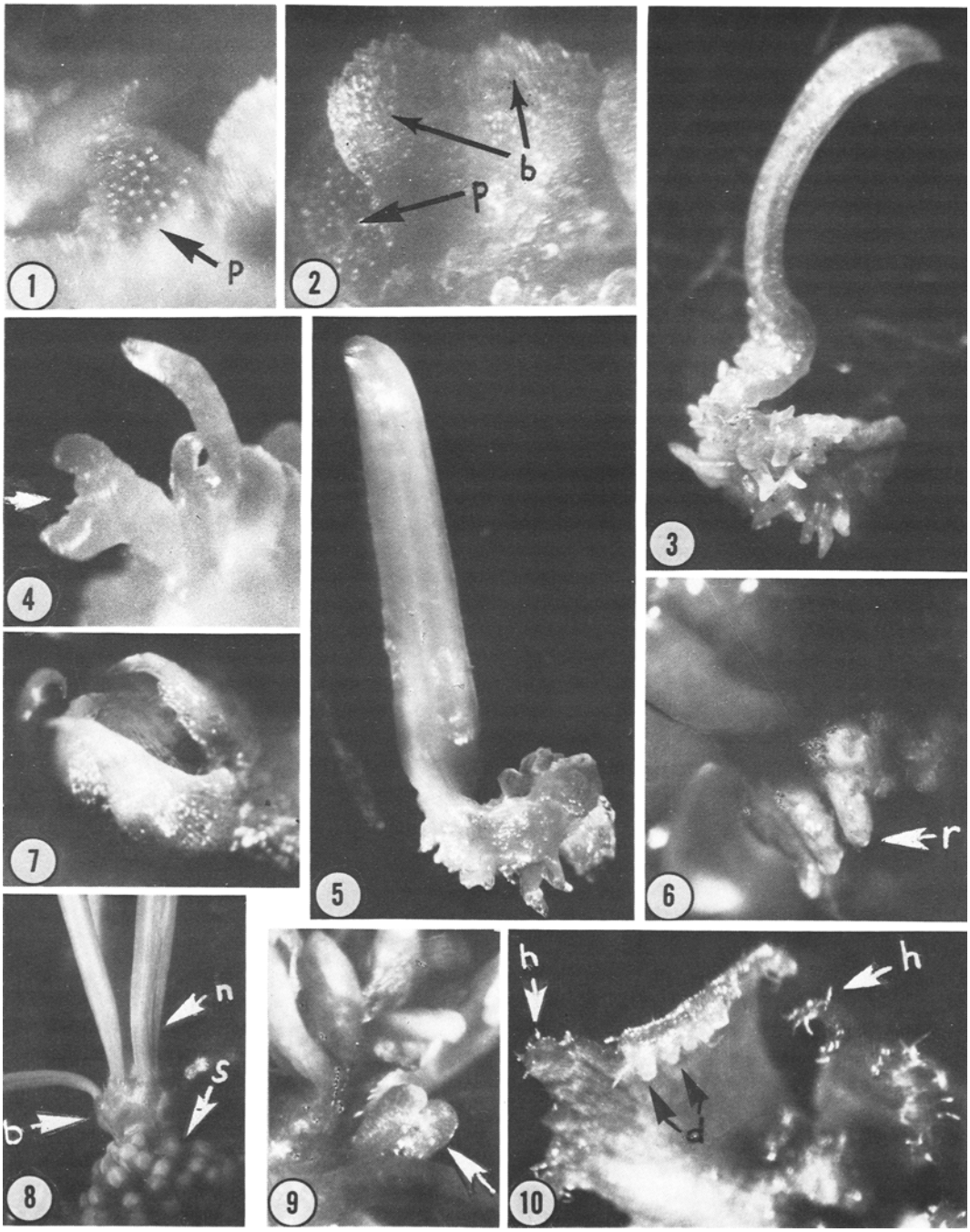

TABLE 1

EFFECTS OF COLLECTION DATE AND PRECULTURE SOAKS (15 MIN EACH) ON NUMBER OF BALSAM FIR SHOOTS THAT FORMED NEEDLE ORGANS (40 SHOOTS/TREATMENT

Collection Date	Preculture Soaks							
	IBA[a] 1	Alar 1	PPZ 1	DMSO 10	Tordon 1	Tordon 0.1	BHT 0.01	Water
Oct.	0	0	0	0	0	0	0	0
Nov.	0	0	0	0	0	0	0	1
Dec.	0	0	0	0	0	0	0	0
Feb.	0	0	0	0	0	0	0	0
Mar.	0	0	5	0	0	0	1	0
Apr.	0	2	1	0	3	5	7	3

[a] IBA = Indolebutyric acid, Alar = dimethylaminosuccinamic acid, PPZ = 1-phenyl-3-methyl-5-pyrazolone, DMSO = dimethylsulfoxide, Tordon = 4-amino-3,5,6,-trichloropicolinic acid, BHT = butylated hydroxytoluene. All concentrations are in grams per liter. All chemicals are dissolved in water, except BHT, which was dissolved in 1% DMSO.

for 15 min before transfer to the nutrient medium. The needles of 8 of 40 explants thus treated formed needle organs (Fig. 3).

In one experiment, organized growth (Fig. 4) was induced by soaking explants in water buffered at pH 2 to 4, but not in water at pH 5 to 8. This suggested that part of the growth regulator effect could have been a pH effect.

Nutrient media. Of the many culture media tested, best growth or morphogenesis occurred on Romberger medium. None of the many combinations and concentrations of growth regulators added to this medium gave a better morphogenetic response than the basal growth regulator combination. Whereas malonic acid was consistently beneficial when used as a preculture soak, it was ineffective when included in the culture medium.

Types of development. Most needle organs developed as clusters of bright green needles or as small shoots near the base of the needles. This type of growth was observed in balsam fir (Fig. 3), Douglas fir (Fig. 5), and less frequently in white spruce. In one experiment, a balsam fir shoot explant produced several small roots around

TABLE 2

EFFECTS OF COLD STORAGE AND FORCING OF BUDS ON THE NUMBER OF BALSAM FIR SHOOTS THAT FORMED NEEDLE ORGANS (60 SHOOTS/TREATMENT)[a]

	Not cold stored Collection Date				Cold stored 6 wk Collection Date		
	Nov.	Dec.	Feb.	Apr.	Nov.	Feb.	Apr.
Nonforced	0	0	1	0	1	0	0
Forced in							
Water	0	0	0	1	2	7	5
EDTA 1 mg/l	0	0	0	0	1	5	0
10 mg/l	0	0	0	2	3	5	21
100 mg/l	0	0	0	5	2	2	11

[a] All dissected shoots soaked in water 15 min before transfer to nutrient medium.

FIGS. 1-2. Morphogenesis in disks cut from immature female cones of *Pinus mugo*. 1. Primordial vegetative shoot (*p*) without bud scales. Note regular arrangement of needle primordia. ×10. 2. Primordial vegetative shoot (*p*) (out of focus) with two bud scales (*b*) on one flank. ×15.

FIGS. 3-10. Morphogenesis in cultures of vegetative shoots from mature trees of *Abies balsamea*, *Picea glauca*, and *Pseudotsuga menziesii*. 3. Emergence of small shoots and clusters of small needles from *Abies balsamea* needle base. ×10. 4. Shootlike structure (*arrow*) arising from the stem between the needles of *Picea glauca*. ×15. 5. Needle base of *Pseudotsuga menziesii* with small shoots and bunches of small needles. ×10. 6. *Abies balsamea* shoot with several small roots (*r*). ×15. 7. *Picea glauca* lateral bud with serrated green scales. ×15. 8. *Pseudotsuga menziesii* vegetative shoot(*s*), which did not elongate but formed a new apical bud with bud scales (*b*) and elongating needles (*n*). ×10. 9. Bilateral structure (*arrow*) originating from the stem between the needles of a shoot of *Pseudotsuga menziesii*. ×15. 10. Young needles of *Pseudotsuga menziesii* that have expanded and flattened into green scales with hairs (*h*) and a ridge with regular domes (*d*). ×15.

TABLE 3

EFFECTS OF EXCISION METHOD ON NUMBER OF SHOOTS THAT SURVIVED AFTER 1 MONTH IN CULTURE (100 SHOOTS/TREATMENT)

	Excision on Filter Paper Moistened With									Excision Submerged in Water
					PVP					
	Water	Cys[a]	Asc	Cle	K15	K60	K90	Asc Cit	BHT	
Balsam fir[b]	24	85	59	71	89	81	67	70	73	57
White spruce[b]	62	91	93	95	94	97	84	84	98	94

[a] Cys = Cysteine 0.1 g/l, Asc = ascorbic acid 1 g/l, Cle = Cleland's reagent 0.15 g/l, PVP = polyvinylpyrrolidone 10 g/l, Asc Cit = ascorbic acid 1 g/l + citric acid 1 g/l [modified from Miller and Murashige (14)]. BHT = Butylated hydroxytoluene 0.001 g/l. Cleland's reagent was filter sterilized; all other chemicals were autoclaved. All chemicals were dissolved in water, except BHT, which was dissolved in 1% DMSO.

[b] Collected February 1978; cold stored 1 month.

the pith at the base of the shoot (Fig. 6). As reported previously (2), scaleless or normal lateral or apical buds often appeared on the shoots, and once, lateral buds composed mostly of serrated green scales were formed (Fig. 7). In a few Douglas fir cultures, the stem and needles of the explant did not elongate, but formed a new apical bud, the primordia of which elongated into full-sized needles (Fig. 8). In some shoot explants, small bilaterally symmetrical structures arose from stem tissues between the needles (Fig. 9). These structures resembled the cotelydonary end of an embryo, except that there were only two "cotyledons" instead of the large number found in sexually generated embryos. In some explants, the needle primordia first expanded into needles, then flattened into irregularly shaped green scales, often with long hairs and sometimes with small regular domes (Fig. 10) along their margins.

Many of the new apical and lateral shoots that developed on the cultured shoots were excised and subcultured on fresh medium. These shoots behaved like the original shoots, except that they elongated less. Sometimes, this second generation of shoots again produced apical or lateral buds, thus providing a third generation of shoots for subculture. None of the subcultured shoots rooted in culture.

DISCUSSION

Haploid and diploid tissues of the female cone were able to form adventitious shoots or embryo-like structures, although this ability was only rarely expressed. More consistent organogenesis was obtained with vegetative shoots. The main factors that determined morphogenesis in these shoots, particularly at the needle base, were: (a) collection in the spring just before budbreak, (b) cold storage followed by forcing, and (c) presoaking of explants in malonic acid before transfer to nutrient medium.

Under field conditions, the 3 to 4-wk period preceding budbreak is morphogenetically an active one, characterized by the initiation of cataphylls and nodal and lateral bud primordia (15,16). It appears that the physiological conditions of shoots during that period not only favor initiation of primordia in situ, but also of needle organs and other organized structures in vitro.

Shoots forced after exposure to cold storage were more morphogenetic than those forced after exposure to merely low winter temperatures in the field. This suggests that the cold storage effect was more than only a low temperature effect. The twigs in cold storage, even though kept in plastic bags, were probably becoming increasingly moisture stressed; they were continuously in the dark, and limited gas exchange caused exposure to high CO_2, ethylene, alcohol, terpenes, and other volatiles.

Cold stored, forced shoots were superior to cold stored, nonforced shoots. This may not have been entirely the result of a physiological stimulus produced by forcing. During cold storage the primordial shoots gradually lost their viability. Often this was not detectable when the primordial shoots were being excised from nonforced buds, resulting in the transfer of large numbers of shoots of reduced vigor to the culture medium. In contrast, during forcing only the most vigorous shoots elongated and broke through the bud scales, and these were selected for in vitro culture.

Of the many chemicals tested, only malonic acid in a preculture soak consistently initiated morphogenesis. Malonic acid inhibits succinic dehydrogenase, thus causing an accumulation of

succinate and other TCA cycle acids (17), and glutamate and glutamine (18) reduced nitrogen compounds important in somatic embryogenesis (19).

It has been reported that malonic acid increases alcohol levels in cells (20,21). However, it is not known if soaking of shoot explants in malonic acid increases alcohol in the shoot tissues, but, if so, it would be difficult to explain why it had a morphogenetic effect; alcohol is a strong inhibitor of morphogenesis (22). Possibly the high level of alcohol in the cells induced a rise in the level of alcohol dehydrogenase (23), which, after removal of the shoots from the malonic acid soaks, could have temporarily reduced the level of alcohol to a below normal level. Conceivably, such a temporary low level of alcohol could have initiated the observed morphogenesis.

The inhibition of the TCA cycle by malonic acid may also have had another effect. It may have switched a large part of the oxidative metabolism to the pentose phosphate pathway (PPP) (24), and this may have initiated the formation of primordia in the shoot cultures (25,26).

Finally, malonic acid may have affected morphogenesis in ways other than by inhibition of the TCA cycle. For example, through malonyl CoA it may have become involved in lipid and membrane synthesis (27), thus influencing cell division and differentiation.

The tissues that exhibited the highest morphogenetic activity were those in the basal parts of the young needles. One of their characteristics was that they contained large quantities of monoterpenes, precursors for gibberellins, sterols, and carotenes, in the epithelial cells of the resin ducts (28). These epithelial cells are important because the origin of some needle organs was traced back to them (2). The needle base is also high in lipids (29) and enzymes such as acid phosphatase, succinic dehydrogenase (the enzyme inhibited by malonic acid) and cytochrome oxidase (29,30). The young needle, before budbreak, has only rudimentary chloroplasts (31), and the needle base lacks chlorophyll (28). This is of interest because chlorophyll degradation by dark treatment has been found to stimulate morphogenesis in cultures of conifers (32) and other plants (33).

The needle organs were similar in appearance to the early stages of shoots induced on sections of cotyledons or hypocotyls of embryos and young seedlings in vitro (34,35). These adventitious shoots on juvenile explants readily developed into viable plants. This raises the expectation that, once needle organs become obtainable consistently and in large numbers, methods for vegetative propagation of mature conifer trees can be developed.

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