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ORGANOGENESIS IN IN VITRO CULTURES OF EMBRYONIC SHOOTS OF *ABIES BALSAMEA* (BALSAM FIR)

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SUMMARY

Embryonic shoots of 15- to 20-year-old *Abies balsamea* (balsam fir) trees were soaked in (a) water for 15 min or 24 hr and (b) water with 1000 mg per l indolebutyric acid (IBA), N-dimethylaminosuccinamic acid (Alar-85), or 1-phenyl-3-methyl-5-pyrazolone (PPZ), singly or in combination with 100 mg per l caffeic acid, for 15 min. After the soaking, the embryonic shoots were transferred to a nutrient medium. Nonsoaked (control) embryonic shoots elongated and often formed a basal callus but never showed organogenesis. The soaked embryonic shoots formed new apical buds, with or without bud scales, adventitious dwarf needles or shoots, and root- and embryo-like structures. One of the embryos germinated and formed an irregular shoot. No differences were found between the various soak treatments, except that the 15-min water soak was ineffective. The 24-hr water soak was as effective as the 15-min growth regulator treatments.

Key words: Abies balsamea; organogenesis; vegetative propagation.

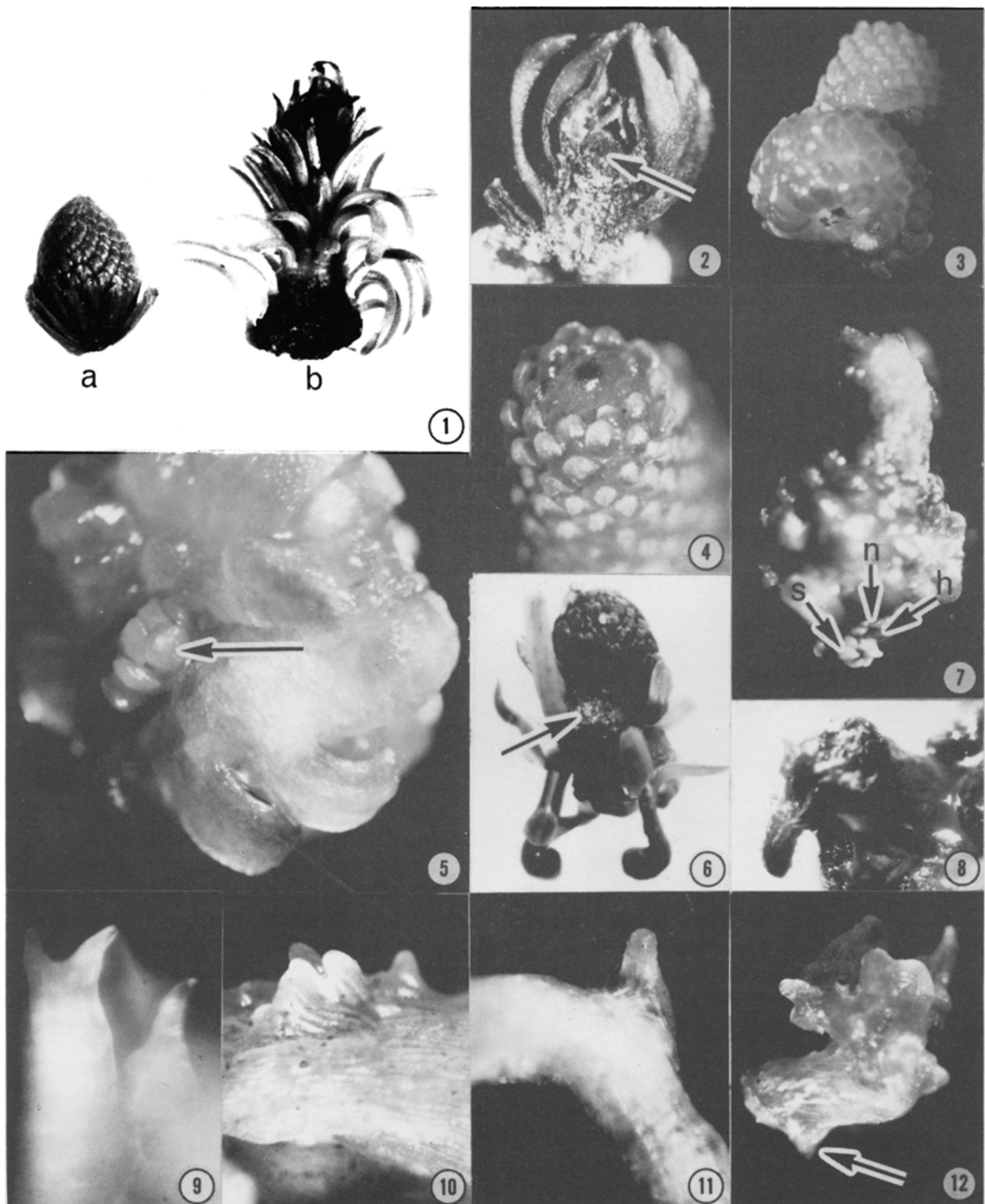
INTRODUCTION

Conifers are mainly propagated sexually because vegetative propagation of trees old enough to have demonstrated their superior characteristics is often difficult (1-3). However, where vegetative, clonal propagation is economically feasible, it is often preferred to sexual propagation because it allows fixing of selected genetic characteristics. Consequently, it is important to develop methods to vegetatively propagate older trees.

To date, the most common method of vegetative propagation of conifers has been rooting of cuttings. However, cuttings often fail to root properly and in these cases tissue and organ culture could be a more promising approach (4,5). Complete or partial vegetative regeneration of conifers from tissue or organ cultures has been achieved for several species from explants taken from either embryos or young seedlings (6-8), but not from explants from older trees.

In our attempts to obtain vegetative propagation from tissues of older trees of *Abies balsamea* L. (balsam fir), embryonic shoots (dormant buds minus bud scales (9)) were used as explants. Several hardwood species have been propagated by in vitro rooting of buds (for review, see Bonga (4)). Also shoot tips and young foliage are frequently used for in vitro vegetative propagation because they or their callus often form adventitious embryos or organs more easily than explants from other parts of the plant (10).

In our propagation attempts, we have, over the past several years, repeatedly tried several basal culture media and numerous combinations of growth regulators. This has resulted in elongation of the embryonic shoots and formation of callus at the base of the stem, but never in the formation of roots or other organs (*unpublished data*). It was not until we started specific soak treatments of the embryonic shoots before their transfer to the culture medium that distinct organogenetic responses were obtained. These soaking experiments were initiated after Cresswell and Nitsch (11) found that soaking the explant before inoculation stimulated the in vitro propagation of *Eucalyptus*. They assumed that inhibitors had been leached from the explant. In this paper, the effects of various soak treatments applied to the embryonic shoots of older *A. balsamea* trees are described.



FIGS. 1-12. Dissecting microscope observations of cultures of embryonic shoots of *A. balsamea*. Figs. 2-12, soaked specimens.

FIG. 1. No-soak treatment. *a*, An embryonic shoot at the time of inoculation; *b*, an elongated shoot with a callus at its base. × 5.

FIG. 2. A new apical bud dissected from an elongated shoot. Half of the bud scales removed to expose the new embryonic shoot (*arrow*). ×20.

FIG. 3. A new embryonic shoot at the apex of an elongated shoot. Note the absence of bud scales on the new shoot. ×10.

FIG. 4. The new embryonic shoot of Fig. 3 with apical dome and regularly arranged primordia. ×20.

Materials and Methods

Dormant twigs were cut from the upper-half of the crowns of 15- to 20-year-old *A. balsamea* trees in a natural stand in central New Brunswick. The twigs were placed in dark plastic bags and stored in the dark at 2° to 5°C for a few days before being processed for in vitro culture.

To obtain the embryonic shoot, the terminal whorl of buds on a twig was dipped in 95% ethanol for a few seconds; the alcohol was then removed by igniting it. The flamed buds were broken from the twig with sterile forceps and placed on filter paper moistened with 70% ethanol in a sterile Petri dish. Bud scales were removed with sterile needles and forceps, and the green embryonic shoot was transferred to a soak solution.

The soak solutions were distilled water, and water with indolebutyric acid (IBA), N-dimethylaminosuccinamic acid (Alar-85), or 1-phenyl-3-methyl-5-pyrazolone (PPZ), singly or in combination with caffeic acid. These chemicals were chosen because they are commonly used for rooting of conifers and other plants (12–14).

In preliminary experiments, soaks in water for 15 min, 24 hr and 48 hr, and in IBA, Alar-85 and PPZ at 10 mg per l for 24 hr and 48 hr, and at 1000 mg per l for 15 min were used. Soaking for 48 hr in water or in any of the growth regulator solutions was too long; many shoots lost their chlorophyll and presumably other essential chemicals, and the survival rate after transfer to the nutrient medium was low. Therefore, 48-hr soaks were discontinued. The 1000 mg per l growth regulator soaks for 15 min were more effective in eliciting a growth response than the 10 mg per l soaks for 24 hr, and therefore in subsequent experiments only the 15-min treatments were used. The caffeic acid was used at 100 mg per l.

For each soak treatment, 10 ml of solution was added to a 25-ml flask covered with aluminum foil, autoclaved and then cooled to room temperature. Ten embryonic shoots were added to each flask, soaked and then transferred to test tubes containing culture medium. A modified Romberger, Varnell, and Tabor's (9) nutrient medium was used; the $FeCl_3$ was replaced by 65 mg per l FeEDTA, the agar concentration was increased to 0.8%, and 1 mg per l IBA was added. The cultures were kept at 21°C and exposed to approximately 540 lux (50 fc) of fluorescent light for 16 hr daily.

The experiment described was carried out with material collected once in December, January and February. From each of the three collections, 40 embryonic shoots were used for each soak treatment and the no-soak control. At the termination of the experiment, 2 to 5 shoots from each treatment were fixed in formalin-acetic acid-alcohol and embedded in wax, using tertiary butyl alcohol as the dehydrating agent (15). In most cases where the needles had produced structures of interest, they were pulled from the shoot and embedded individually. The wax blocks were sectioned at 10 μm; the sections were stained in rose-bengal (16) and mounted in Euperal.

Results

Observations before microtome sectioning. The following terminology is used: "embryonic shoot" refers to the original explant; "elongated shoot" refers to the shoot arising from the embryonic shoot by elongation of its stem and/or needles; and all new, organized structures developing on the embryonic shoot or elongated shoot during culture are denoted by the adjectives "new," "adventitious," "apical" or "dwarf."

Study of the cultures under a dissecting microscope revealed that about half of the embryonic shoots in each treatment elongated for 1 to 3

FIG. 5. A group of dwarf needles (*arrow*) arising from the cortex at the base of an elongated shoot. The pith tissue is hidden under these needles. ×20.

FIG. 6. A new lateral bud (*arrow*) arising from the cortex near the base of an elongated shoot. ×10.

FIG. 7. A dwarf "shoot" (*s*) and dwarf needles (*n*) grouped around a callus hump (*h*) at the base of an elongated shoot. Microtome sections of these structures are shown in Figs. 17 and 18. ×10.

FIG. 8. A rootlike protrusion on a callus near the base of an elongated shoot. A microtome section of this structure is shown in Fig. 23. ×40.

FIG. 9. A needle with a split tip. ×20.

FIG. 10. Regular, transparent protrusions on a needle. Their surface structure is similar to that of the needle. ×40.

FIG. 11. A needle with an opaque embryo-like protrusion (cotyledons attached to needle surface). ×40.

FIG. 12. A shootlike structure which developed from a hypocotyl with cotyledons after removal from a needle and transfer to new culture medium. Note small cone (possibly a root) (*arrow*) at the base of the hypocotyl. ×20.

months and remained alive for several more months. In the controls (no-soak) they elongated up to 3 cm, had fully expanded needles and frequently had a vigorous callus at the base (Fig. 1). There were no signs of adventitious organized growth either on this callus, or elsewhere on the elongated shoot. In contrast, many of the elongated shoots arising from soaked embryonic shoots formed a variety of new organized structures. Several formed new apical structures. A few of these were normal new buds, with bud scales enclosing a well developed new embryonic shoot (Fig. 2), but, in most, the bud scales were lacking, i.e. the new apical structures were well developed, naked new embryonic shoots with a distinct apical dome and well defined needle primordia (Figs. 3, 4), which sometimes elongated into well developed needles.

In many of the elongated shoots, groups of pale green dwarf needles developed, mostly from the cortical tissues around a receding, dark-colored pith at the base of the elongated shoot (Fig. 5). Sometimes the needles were dark green and elongated, but differed from the normal needles on the elongating shoot by being flatter and having a longer taper. The groups of needles did not always arise solely from the cortical tissue at the base of the elongated shoot. Sometimes they arose from the pith and sometimes, not from the base, but from a lateral position on the stem of the elongated shoot. In a few cases, a normal-looking new bud or naked embryonic shoot grew at the base of the elongated shoot (Fig. 6). Often the entire base of the elongated shoot and the basal part of its lower needles callused. Groups of dwarf needles often developed from this callus, particularly around the base of humps on the callus (Fig. 7). Occasionally a rootlike protrusion, i.e. a cylindrical protrusion with a dome-shaped apex, was formed on the callus (Fig. 8).

Many of the elongated needles of the elongated shoots developed split tips (Fig. 9). Often one or more of the lower needles formed regularly shaped lateral protrusions along its surface. Two distinct types of protrusions were observed: (a) green and transparent, with the surface cells being an extension of the needle epidermis and often occuring in groups (Fig. 10); and (b) yellow-green and opaque (Fig. 11), becoming embryo-like with either the cotyledons or the radicle attached to the needle. Sometimes the radicle was missing, in which case the hypocotyl was attached to the needle. One such structure was aseptically removed from the needle when it had reached about the size of a mature embryo and was transferred to a culture medium with sucrose reduced to 2% and without IBA. After 10 days on the new medium, it had doubled in length and had formed several stunted, somewhat swollen, green needles and a small cone (possibly a new root) at the base of the hypocotyl (Fig. 12).

To determine if organogenetic development was associated with specific treatments, an analysis of variance was carried out on the 24-hr water soak and 15-min growth regulator soak treatment. The data of the no-soak and 15-min water soak treatments could not be included in this analysis because in these treatments the response was always zero (zero populations cannot be dealt with statistically). The analysis showed that there

FIG. 13. A new apical shoot (no bud scales) at the apex of an elongated shoot. The needles have started to elongate. The structure of this new apical shoot appears indistinguishable from that of shoots elongating in situ. ×30.

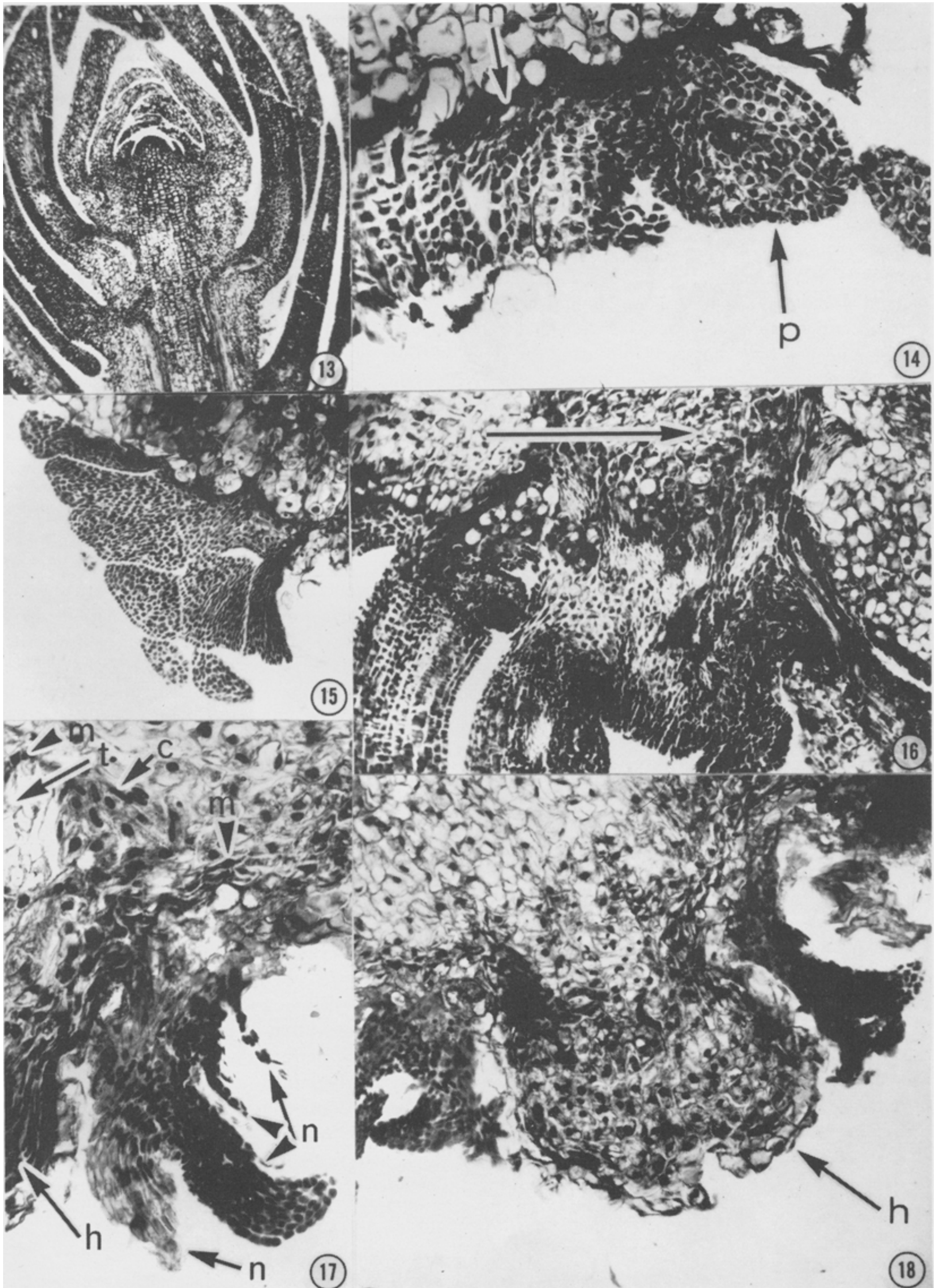
FIG. 14. A meristematic layer (*m*) formed in the callusing cortex at the base of the elongated shoot. This meristem formed rows of small cells in basipetal direction. Dwarf needle primordia (*p*) were formed in this small celled tissue. ×150.

FIG. 15. A dense group of dwarf needles at the base of an elongated shoot. Note the absence of a stem with apical organization. ×75.

FIG. 16. Some of the callusing cortex and all of the surface layers of the pith (*arrow*) have participated in the formation of rows of small cells in basipetal direction. Primordia, which elongated into needles, were formed along the rough surface of this small celled tissue. ×75.

FIG. 17. One of the dwarf "shoots" at the base of a callus hump (*h*) at the base of on elongated shoot. Note that the dwarf "shoot" is more shootlike than the structures in Figs. 15 and 16, its dwarf needles (*n*) being implanted at various levels along a cylinder. This cylinder is homogenously composed of small cells, i.e. it lacks the complete organization of a normal stem. The dwarf "shoots" appeared to be initiated in the meristem layer (*m*) close to the periphery of the callus. Additional meristem layers (*m*) and irregular tracheids (*t*) are found in the interior of the callus. Note the regular cone (*c*) extending into the callus from the base of the dwarf "shoot." This cone presumably is a root primordium. The callus hump shown here is the same as shown in Fig. 7. ×150.

FIG. 18. The same callus hump (*h*) as shown in Fig. 17. Several dwarf "shoots" are grouped around the base of the callus hump. ×75.



FIGS. 13–23. Microtome sections of cultures of embryonic shoots of *A. balsamea*. All specimens soaked.

were no significant differences between the three collection dates, nor between the various soak treatments. However, the observation that 98 (12%) of the 840 soaked specimens formed new organs, while none of the 120 nonsoaked specimens in this experiment nor any of the thousands of nonsoaked specimens in earlier experiments did so, strongly suggests that the soaking had an organogenetic effect.

Observations of sectioned material. The new structures which developed at the apex of the elongated shoots had an anatomy similar to that of buds, embryonic shoots, or expanding shoots in freshly collected field material. In most of the apical structures the needles were in the primordium stage or slightly elongated; a few had elongated considerably (Fig. 13).

The formation of dwarf needles at the base or the side of the elongating shoot proceeded as follows. The cortical tissues at the base of the elongating shoot callused slightly, while the pith tissue darkened and receded. Close to the surface of the callus a meristematic layer developed, which formed rows of small densely staining cells in basipetal direction. Primordia developed in this tissue, mostly along the surface facing the pith (Fig. 14) but occasionally elsewhere on its surface, sometimes in dense groups (Fig. 15). The primordia developed into dwarf needles and occasionally into longer needles.

In many specimens, needle initiation was not confined to tissues originating from the cortex but occurred also in tissues arising from the pith. Like the cortex in Figs. 14 and 15, the pith formed rows of cells in basipetal direction and groups of dwarf needles or elongated needles on the surface of this newly formed tissue (Fig. 16). Sometimes vascular tissues developed in the new tissue, generally as an extension of the vascular tissues of the elongated shoot.

In some of the elongated shoots, all tissues at the base callused. A meristematic layer developed parallel to and close to the surface of this callus, depositing short rows of cells towards the callus exterior (Fig. 17). Additional layers of meristem developed in the interior of the callus, forming nests of short pitted tracheids (Fig. 17). Some of the cells of the meristem close to the surface of the callus developed into primordia which formed new dwarf needles or shoots breaking through the surface layers of the callus. Most of these needles and shoots were found in a ring around the base of many of the humps on the callus (Fig. 18). In some instances, the meristem produced a small cone of small cells pointing towards the center of the callus, each cone being located at the base of a dwarf shoot, and each possibly being a root primordium (Fig. 17). The new shoots were primitive in structure. Their needles and stems were uniformly composed of small densely staining parenchymatous cells and lacked vascular organization (Figs. 17, 18).

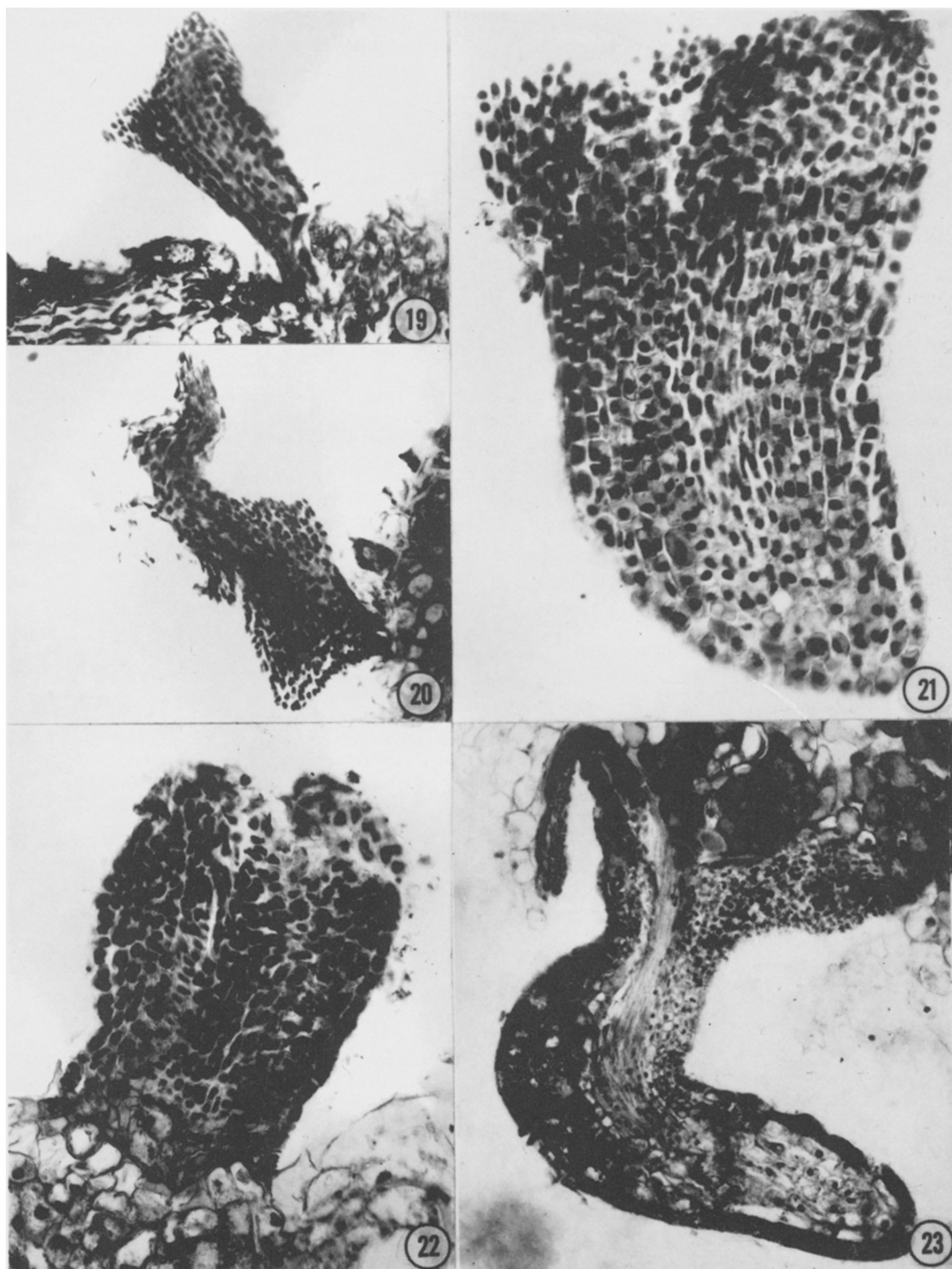
The transparent protrusions on the needles were composed of a few elongated cells and therefore probably were the result of cell elongation rather than cell division. The opaque embryo-like protrusions, however, clearly were the product of cell division, i.e. they were entirely composed of small densely staining cells. Either the radicle (Fig. 19) or a cotyledon (Fig. 20) of the embryo-like structures was attached to the slightly callused needle surface. Occasionally, the embryo-like structures became detached from the needles during the embedding procedures and were found separate from the needle in the microtome sections (Fig. 21). The needle protrusion shown in Fig. 22 resembled an embryo without a radicle (a hypocotyl with cotyledons), and had arisen from the lining (not showing in this figure, but in several of the other serial sections of this specimen) of a resin duct near the surface of the needle. The arrangement of the cells in the hypocotyl suggests that an embryonic cortex and central cylinder had been initiated.

Sections of the rootlike protrusion shown in Fig. 8 revealed that its internal structure also had some of the characteristics of a root (Fig. 23). It had a clearly defined epidermis and vascular system and a dome-shaped apex. However, it lacked a root cap and well defined apical meristem, although the latter may have been present at an earlier stage of development.

FIG. 21. An embryo-like structure that became detached from the needle. ×180.

FIG. 22. A protrusion resembling the hypocotyl and cotyledons of an embryo (no radicle) arising from a needle. The arrangement of the cells in the hypocotyl is suggestive of the initiation of an embryonic cortex and central cylinder. ×180.

FIG. 23. A section of the protrusion shaped like a root shown in Fig. 8. Note the epidermis and the generally regular arrangement of cells. However, a well defined meristematic area and root cap are lacking. ×90.



FIGS. 19–22. Individual needles removed from the elongated shoots, wax-embedded and sectioned individually.

FIG. 19. A needle with an embryo-like protrusion. Note cotyledons. Radicle end of the embryo-like structure is attached to the needle surface. ×90.

FIG. 20. An embryo-like protrusion arising from the slightly callused surface of a needle. One cotyledon attached to the needle. ×90.

Discussion

Embryonic shoots of several conifer species have been cultured in vitro (9,17,18). These shoots, much like the embryonic shoots of *A. balsamea* cultured without soak treatments, showed varying degrees of stem and needle elongation and, sometimes, callus formation at the base of the shoot. In addition, Chalupa and Durzan (18) noted formation of a few small scales in a few of their embryonic shoot cultures of white spruce, and Romberger, Varnell, and Tabor (9) noted formation of needle primordia in apical meristem cultures of *Picea abies*. However, none of the authors reported adventitious structures as varied and as complex as the ones found in the cultures of *A. balsamea* embryonic shoots that had received soak treatments. This, as well as the fact that organogenesis never occurred in the thousands of *A. balsamea* embryonic shoots cultured over the past several years without soak treatment, strongly suggests that soaking was highly effective in inducing organogenesis.

It has not yet been established which of the various soak treatments was the most effective. If water was used alone, a 24-hr soak was effective, but a 15-min soak was too short. However, if water was used with high concentrations of growth regulators, a 15-min soak was sufficient. This indicates that different mechanisms may be involved acting individually or in combination: (a) a 24-hr water soak removed enough inhibitor(s) from the embryonic shoot to allow organogenesis. (b) Exposure of tissues to very high concentrations of growth regulators over a short period of time, i.e. 15 min, has an effect which is different from that of exposure of tissues to much lower concentrations supplied continuously in the nutrient medium. (c) High growth regulator concentrations in water affect the tissue in such a manner that leaching occurs at a much faster rate, i.e. under these circumstances a soaking period of 15 min removes enough inhibitor(s) to allow organogenesis.

With respect to vegetative propagation the most important aspect of the experiments is that embryonic shoots of trees which have reached an age at which propagation by rooting of cuttings becomes very difficult can be stimulated to produce various complex organized structures, including embryo-like structures as large as mature embryos. Although this has not yet resulted in the regeneration of viable new plants, it appears likely that with further refinements of the techniques described here, vegetative propagation of these older *A. balsamea* trees will eventually become possible.

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