Histology of In Vitro Adventitious Bud Development on Cotyledons and Hypocotyls of Fraser Fir

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Abstract. Cotyledons and hypocotyls of Fraser fir [Abies fraseri (Pursh) Poir.] were excised from seeds treated with H_3O_3 for 9 days and placed on bud induction medium containing 10 mg BA/liter and 0.01 mg NAA/liter or medium without growth regulators. Although adventitious buds did not develop, cotyledons exposed to growth regulators responded differently than cotyledons placed on medium lacking growth regulators. Cotyledons and hypocotyls responded similarly to growth regulators during the initial phase in culture, but cell divisions ceased in cotyledons, thus preventing meristemoid and subsequent bud development. After 3 days on medium containing growth regulators cell divisions were localized in epidermal and subjacent layers of hypocotyls, whereas similar cell divisions were' not observed in hypocotyls placed on medium without growth regulators. Cell clusters consisting of two to five cells (promeristemoids) were present after 7 days on hypocotyls placed on bud induction medium. In hypocotyls placed on medium without growth regulators, stomata continued to develop and cells within the cortex became vacuolated during the first 2 weeks in culture. All explants were transferred to secondary medium after 3 weeks. Cell clusters continued to enlarge into meristemoids on hypocotyls initially placed on bud induction medium. Gradually, meristemoids developed into buds and cataphylls were observed covering bud meristems. Chemical names used: N-(phenylmethyl)-1 H-purine-6-amine (BA), 1-naphthaleneacetic acid (NAA).

Adventitious buds can be produced in vitro on hypocotyls of Fraser fir (Saravitz et al., 1987; Saravitz et al., 1991; Zygmont and Schwarz, 1987), but the response is less pronounced compared to other conifers when embryonic tissues are used as explants (Bonga and Durzan, 1987). Also, buds form only on hypocotyls of Fraser fir and very rarely on cotyledons (Saravitz et al., 1991). When placed on medium containing BA and NAA, cotyledons of Fraser fir appear to respond, but buds do not develop (Saravitz et al., 1991). Conversely, adventitious buds produced on hypocotyls elongate into shoots. These shoots can be rooted, but survival of plantlets following transfer to growing medium has not been successful (Saravitz et al., 1991).

In vitro adventitious bud differentiation on embryonic explants has been investigated for several conifers, including Douglas fir [*Pseudotsuga menziesii* (Mirb.) France] (Kirby and Schalk, 1982), eastern white pine (*Pinus strobus* L.) (Flinn et al., 1988), loblolly pine (*Pinus taeda* L.) (Mott and Amerson, 1981), Monterey pine (*Pinus radiata* D. Don.) (Thorpe, 1988), Norway spruce [*Picea abies* (L.) Karst.] (von Arnold and Hakman, 1988), and black (*Picea mariana* Mill.) and white [*Picea glauca* (Moench) Voss] spruce (Rumary et al., 1986). Generally, these studies have shown that cell divisions leading to adventitious bud development are stimulated by various growth regulators, most notably cytokinins, and the divisions are restricted to epidermal and subjacent cells of the explants. Initial cell divisions

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²Professor. ³Associate Professor, Dept. of Forestry. occur resulting in cell clusters termed promeristemoids (Flinn et al., 1988; Villalobos et al., 1985). Further development of promeristemoids results in meristemoids that gradually give rise to buds. It is intriguing to speculate whether similar histologicalchanges occur in hypocotyls of Fraser fir cultured in vitro. In addition, anatomical research with Fraser fir might explain why adventitious bud formation on hypocotyls is generally low and virtually nonexistent on cotyledons. Therefore, we examined the histological changes that occur when excised cotyledons and hypocotyls of Fraser fir are placed on bud induction medium.

Materials and Methods

Explant source, seed germination, and culture conditions. Seeds were collected in 1981 by the North Carolina Forest Service from native trees at Roan Mountain (36°01' N latitude, $82^{\circ}05'$ W longitude, elevation = 1900 m), dried to a moisture content of 4% to 6%, and stored in a polyethylene bag at -17C. Following removal from storage, seeds were immersed in 70% ethanol for 30 sec to remove surface resin, nicked at the micropylar end with a scalpel, and initially placed in 1% (v/v) H₂O₂ for 3 days. On the 4th day, seeds were transferred to 0.03% (v/ v) H₂O₂ and the concentration remained constant thereafter. Regardless of concentration, H₂O₂ solutions were changed each day. As a further measure to prevent contamination, seeds were treated daily, beginning on day 4, with either 6% (v/v) H_20_2 for 5 min or 70% (v/v) ethanol for 30 sec. These procedures were alternated daily and were used in addition to the H_2O_2 treatment (Saravitz et al., 1991). During H₂O₂ treatment, seeds were maintained at an 8/16 h thermoperiod of 30/20C and were subjected daily during the 8-h cycle to irradiation from a combination of cool-white fluorescent lamps and incandescent bulbs. The lamps and bulbs provided a photosynthetic photon flux [PPF (400 to 700 nm)] of 17.9 μ mol·m⁻²·s⁻¹ (1.3 klx). This and all other light measurements were recorded with a LI-COR LI 185A quantum/radiometer/photometer (LI-COR, Lincoln, Neb.).

Following 9 days of treatment with H_2O_2 , seedlings were

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surface sterilized with 70% (v/v) ethanol for 2 min, 0.5% (w/ v) Ca(OCl), for 10 min, and rinsed three times with sterile distilled water (a quick rinse, a 10-min soak, and another quick rinse). Cotyledons and hypocotyls were excised aseptically and placed horizontally into 60×20 mm plastic petri dishes containing 10 ml of bud induction medium. Bud induction medium consisted of Brown and Lawrence (BLG) medium as modified by Amerson et al. (1985) containing 3% sucrose, 0.8% agar, and the following growth regulators, as determined by Saravitz et al. (1991): 10 mg BA/liter and 0.01 mg NAA/liter. Cotyledons and hypocotyls placed on BLG medium without growth regulators served as a control. The pH of the media was adjusted to 5.5 before autoclaving. Explants remained on the initial medium for 3 weeks followed by transfer to secondary medium. Secondary medium consisted of Gresshoff and Doy (GD) medium, as modified by Mott and Amerson (1981), containing 3% sucrose, 1% activated charcoal, and 0.8% agar. The pH of the medium was adjusted to 5.5 before autoclaving. Cultures were transferred every 4 weeks to 60×20 mm plastic petri dishes containing 10 ml of secondary medium. Cultures were maintained at 22C under continuous irradiation provided by a combination of cool-white fluorescent lamps and incandescent bulbs [PPF (400 to 700 nm) = 47 μ mol·m⁻²·s⁻¹(3.3 klx)].

Histological procedures. Cotyledons and hypocotyls placed on bud induction medium were sampled at various times for histological examination (0, 3, 7, 14, 21, 28, 35, 42, 49, and 56 days). Cotyledons and hypocotyls placed on medium lacking growth regulators were taken at the same times, with the last time being at 35 days. Tissue samples were fixed for 24 h in formalin-acetic acid-70% ethanol (FAA) (Jensen, 1962). After fixation, all samples were dehydrated in a series of ethanol and tertiary butyl alcohol (Jensen, 1962), infiltrated and embedded in Paraplast (Monoject Sci., Div. Sherwood Medical Industries, St. Louis), sectioned serially on a rotary microtome at 12 μ m thickness, affixed to slides using Haupt's adhesive (Jensen, 1962), and stained with safranin, crystal violet, and fast green following the procedures of Gerlach (1969).

Results

At the time of excision from the embryo and before placement on bud induction medium (time 0), mitotic activity and visible cell divisions were not localized within all explants as shown in hypocotyl explants (Fig. 1 A and B). Nuclei were prominent. The cytoplasm of the epidermis was stained densely and, to a lesser extent, the cortical cells. Cotyledons on medium without growth regulators. Mitotic activity ceased in cotyledons by day 3 on medium without growth regulators. Parenchyma cells of the cotyledons began to enlarge, but palisade and spongy mesophyll were not differentiated (Liu, 1971). Enlargement of the parenchyma cells continued with an accompanying increase in intercellular space. By weeks 4 to 5 in culture, palisade and spongy mesophyll were observed.

Cotyledons on medium containing BA and NAA. Cotyledons became stunted and swollen in response to exposure to BA and NAA. Clusters of cells were present throughout the mesophyll after 7 days in culture (Fig. 2A). Epidermal and subjacent cells remained compact, whereas the inner parenchyma cells slowly became more vacuolated during the following 4 weeks in culture. By the fifth week in culture, clusters of cells with prominent nuclei and darkly stained cytoplasm were localized in the epidermal and subjacent layers (Fig. 2B). Clusters of cells within the cotyledon never progressed beyond this stage of development. Cotyledons began to senesce and, by week 8 in culture, $\approx 75\%$ of cotyledons had turned brown.

Hypocotyls on medium without growth regulators. No mitotic activity was observed in epidermal and subjacent layers of the hypocotyl by day 3, and cortical cells were more vacuolated than at the time of excision. During the second week in culture, cortical cells of hypocotyls became vacuolated and well developed stomata were present.

Hypocotyls on medium containing BA and NAA. After 3 days on bud induction medium, mitotic divisions were mainly in the epidermal and the subjacent cell layers of the hypocotyl (Fig. 3A). Most of the cells in the cortex were vacuolated and stomata were not observed (Fig. 3A). Clusters consisting of two to five cells were present directly below the epidermis and divisions continued in this layer for at least 7 days (Fig. 3B). Cell clusters continued to enlarge during the second week (Fig. 3C), and protrusions were visible on the surface of hypocotyls by week 3 (Fig. 3D). At this time, hypocotyls were transferred to medium lacking growth regulators.

Bud primordia were visible after 4 weeks in culture (Fig. 4A), but most meristematic areas were still undifferentiated and some never developed into buds. One or more bud scales or cataphylls covered some bud meristems by 5 weeks in culture (Fig. 4B). Upon expansion, cataphylls contained cells that were vacuolated. Undeveloped meristematic areas and buds were present on the same hypocotyl (Fig. 4C). After 7 weeks in culture, needle primordia were observed and cataphylls covered buds (Fig. 4D).



Fig. 1. Photomicrographs of transverse sections of control explants of Fraser fir in vitro. Scale bars = 50 μ m. (A) Cross section of a hypocotyl after 9 days of H₂O₂ treatment (time 0). (B) Cell divisions in a section of an excised hypocotyl after 9 days of H₂O₂ treatment (time 0). Arrows indicate dividing cells.



Fig. 2. Photomicrographs of transverse sections of cotyledons of Fraser fir cultured on bud induction medium containing BA and NAA. Scale bars = 50 μ m. (A) Cross section of a cotyledon after 1 week in culture. (B) Cell clusters in a cross section of a cotyledon after 5 weeks in culture. Arrows indicate cell clusters.



Fig. 3. Photomicrographs of transverse sections of hypocotyls of Fraser fir cultured on bud induction medium containing BA and NAA and illustrating early cell divisions. Scale bars = 50 μm. (A) Epidermal and subjacent cell divisions in a section of a hypocotyl after 3 days in culture. Arrows indicate dividing cells. (B) Promeristemoids in a section of a hypocotyl after 1 week in culture. Arrows indicate cell clusters.
(C) Meristemoid in a section of a hypocotyl after 2 weeks in culture. Arrows indicate meristemoid. (D) Meristemoid in a section of a hypocotyl after 3 weeks in culture.

Discussion

Results suggest that cotyledons of Fraser fir do not contain competent cells for adventitious bud development when placed on medium containing BA and NAA nor do such cells develop. Conversely, hypocotyls contain competent cells as evidenced by the formation of adventitious buds when placed on bud induction medium. However, the number of competent cells is apparently less than for other conifer species, such as Douglas fir (Kirby and Schalk, 1982), eastern white pine (Flinn et al., 1988), loblolly pine (Mott and Amerson, 1981), Monterey pine (Thorpe, 1988), Norway spruce (von Arnold and Hakman, 1988), and black and white spruce (Rumary et al., 1986).

Response of cotyledons to BA and NAA. Villalobos et al. (1985) divided in vitro shoot formation in cotyledons of Monterey pine into two developmental phases. Phase one included induction and differentiation and phase two consisted of primordia development. Cotyledons of Fraser fir did not complete phase one. Cell clusters formed in epidermal and subjacent lay-



Fig. 4. Photomicrographs of transverse sections of hypocotyls of Fraser fir cultured in vitro on bud induction medium containing BA and NAA and illustrating adventitious bud development. Scale bars = $100 \ \mu m$. (A) Bud meristem after 5 weeks in culture. (B) Bud covered with a cataphyll (c). (C) Meristemoid (m) and bud (b) after 6 weeks in culture. (D) Bud with needle primordia (n) and covered by cataphylls (c).

ers of cotyledons, but these cells did not differentiate (Fig. 2B). Meristematic areas in cotyledons of Fraser fir may not have the capability to develop into shoots or, possibly, another stimulus is needed either in conjunction with or following treatment with BA and NAA.

Response of cotyledons to BA and NAA was slow. Several weeks of culture were necessary before localized cell clusters were visible. However, meristematic activity in hypocotyl explants was restricted to epidermal and subjacent layers within 3 days in culture. Although the conditions described herein promote adventitious bud formation on hypocotyls of Fraser fir, a longer exposure time to growth regulators may enhance bud formation on cotyledons. Also, slow response of cotyledons may complicate the process of bud initiation by allowing loss of competence (Flinn et al., 1988).

Bud development in hypocotyls. Confinement of cell divisions to the epidermal and subjacent layers of the explant in response to BA has been observed in other conifers, including Norway spruce (von Arnold and Grönroos, 1986) and Monterey pine (Villalobos et al., 1985). Yeung et al. (1981) suggested that epidermal and subjacent cells are not fully' 'determined" at the time of excision, leaving these cells responsive to cytokinin treatment. Villalobos et al. (1985) and Bornman (1983) observed formation of organized groups of cells in embryonic explants of Monterey pine and Norway spruce, respectively. Villalobos et al. (1985) referred to these cell groups as promeristemoids. Promeristemoids were also present in hypocotyls of Fraser fir (Fig. 3B).

By 3 weeks in culture, meristematic areas were visible on the surface of hypocotyls cultured on bud induction medium (Fig. 3D). Gradually, these areas began to differentiate into buds. Even on the same hypocotyl, buds did not differentiate at the same time. In our previous study (unpublished) the number of buds increased up to 15 weeks in culture. von Arnold et al.(1988) also noted that synchrony of bud formation was lost during development of meristemoids into bud primordia and adventitious buds on zygotic embryos of Norway spruce.

In many cases, buds were covered by one or more cataphylls (Fig. 4B). Bud scales are present during normal development of buds of *Abies* species (Powell, 1977) and have been observed on buds of Norway spruce (von Arnold and Hawes, 1989) and white spruce (Campbell and Durzan, 1975) forming in vitro.

Many cells in hypocotyls of Fraser fir divided into cell clusters or promeristemoids, but only a few continued to develop into meristemoids. Meristemoid formation was greater than ac-

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tual bud formation on hypocotyls of Fraser fir. Possibly, continued development of meristemoids into buds is suppressed by levels of endogenous hormones in the hypocotyl or inhibited by further development of surrounding meristemoids. Bornman (1987) suggested that gradients of endogenous growth regulators along the length of the explant affect bud formation. He reported that the level of auxin, in addition to cytokinin, optimal for promoting bud induction on the upper half of a needle of Norway spruce inhibited bud initiation at the base of the needle. von Arnold and Hawes (1989) noted that many meristemoids of Norway spruce did not develop into shoots. 'They proposed that further maturation of meristemoids into buds maybe inhibited by "abnormalities" that occur during bud development. This may also be the case in Fraser fir.

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