ENHANCEMENT OF SHOOT ORGANOGENESIS IN CONIFERS

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Abstract. Successful genetic modification of conifers through gene transfer and transgenic plant production is dependent upon effective plant regeneration systems. Plantlets from a large number of conifer species have been successfully propagated via organogenesis using cotyledon explants and they may be potenially amenable to gene transfer technology. However, the primary limitation for cotyledonary explants is the

between initiation and plantlet formation. Shoot production can be enhanced by providing more optimal hormone conditions in the shoot induction medium. Abscisic acid (ABA), in addition to auxins and cytokinins, enhances shoot formation from conifer explants. ABA, enhances the morphogenic area of cotyledon explants from two *Pinus* species. The degree of enhancement is related to the seed source and the length of exposure. Shoot production is completely blocked by fluridone (FLUR), an inhilitor of endogenous ABA synthesis. Abscisic acid appears to be an important hormone in shoot morphogenesis.

Keywords: ABA: Abscisic acid, BA: 6-Benzylaminopurine, cotyledon explants, FLUR: fluridone, GD: Gresshoff and Doy (197:) nutrient medium, NAA: a-Naphthaleneacetic acid, *Pinus taeda* L., *Pinus virginiana* Mill., Shoot primordia.

INTRODUCTION

Successful genetic modification of conifers through gene transfer and transgenic plant production is dependent upon effective plant regeneration systems (Sederoff et al. 1987). Plantlets from a large number of conifer species have been successfully propagated via organogenesis using various explants (Campbell and Durzan 1975, Sommer et al. 1975, Mott et al. 1977, Reilly and Washer 1977, Mehra-Palta et al. 1978, Karnosky 1981, Kaul and Kochhar 1985, Patel and Thorpe 1984, 1986, Gladfelter and Phillips 1987, Perez-Bermudez and Sommer 1987, Kaul 1987, Jain et al. 1988a). Cotyledon explants in conifers appear to be amenable to gene transfer technology.

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However, the primary limitation for cotyledonary explants is the low number of regenerated plantlets and the long timespan between initiation and plantlet formation. Larger number of shoots produced per cotyledon explant in a shorter period of time will give a better opportunity for producing a large number of genetically modified propagules after gene transfer.

Shoot production could be enhanced perhaps by providing more optimal hormonal conditions in the shoot induction medium. In addition to auxins and cytokinins (Mott and Amerson, 1981), another plant hormone such as abscisic acid (ABA) may increase shoot production from conifer explants. Abscisic acid could possibly have such a role because previous studies have indicated that it is associated with embryo maturation and development. For example, ABA has been shown to: 1) increase the accumulation of storage proteins in developing embryos of *Glycine* (Bray and Beachy 1985), *Brassica* (Crouch et al. 1985), *Gossypium* (Galau et al. 1986) and *Triticum* (Willamson et al. 1985); 2) stimulate growth of zygotic seeds in *Glycine* (Ackerson 1984) and *Zea* (Fong et al. 1983); 3) influence normal somatic embryo development in *Picea* (Becwar et al. 1987, von Arnold and Hakman 1988, Jain et al.1988b) and *Daucus* (Kamada and Harada 1981); and 4) induce embryogenesis in *Carum* (Ammirato 1974) and to enhance somatic embryogenesis from leaf explants in *Pennisetum* (Rajasekaran et al., 1987).

Abscisic acid also appears to influence morphogenesis. Root formation as well as root growth was increased in *Zea* callus (Abou-Mandour and Hartung, 1986). In this paper, we show that ABA enhances shoot production from pine cotyledon explants and that shoot production is blocked by fluridone (FLUR), an inhibitor of ABA synthesis via carotenoid metabolism (Fong et al. 1983).

MATERIALS AND METHODS

The first experiment was performed with half-sib seeds of *Pinus taeda* L. (Texas Forest Service BA3R13-41 x Open, collected in 1983) from Bastrop, Texas. Seeds were surface sterilized with H_2O_2 for 20 min, washed in tap water for 24 h and pretreated 5 to 6 d in 1% H_2O_2 at room temperature (22°C) to stimulate germination (Ching and Parker 1958). Seeds were again surface sterilized with 15% (v/v) clorox (5.25% sodium hypochlorite) for 5 min followed by 3 rinses of sterile water. Embryos were aseptically removed from the seeds, and the cotyledons were excised and transferred to GD (Gresshoff and Doy 1972) nutrient agar medium supplemented with BA (44 µm) and NAA (0.05 µM) before autoclaving. Abscisic acid (10^{-7} M) or FLUR (50 mg l^{-1}) was filter sterilized prior to its addition to the autoclaved medium.

Cotyledon explants (5 to 9) from 25 seeds for each treatment were placed in 5 Petri dishes containing nutrient agar as described above. Explants from 5 seeds were placed in each dish and incubated under continuous light (250 µmol $m^{-2} s^{-1}$) at 25°C. After 3 weeks of incubation the cotyledons were observed with light microscopy and scored for the percentage of cotyledon area per seed having shoot primordia which indicated shoot

initiation. Each seed (5 to 9 cotyledons) was regarded as a whole (100%) and the percent area having shoot primordia on individual cotyledons was scored (e.g., when an individual seed has 5 cotyledons, each cotyledon is 20%; 5 cotyledons/seed X 20%/cotyledon = 100% seed). Scoring was done for all 25 seeds at 4 weeks. For FLUR-treated cotyledons, the fresh weight after treatment was determined.

In a second experiment, cotyledons were exposed to ABA or FLUR for 4 weeks, scored for shoot initiation and then transferred to 0.5-strength GD medium (without NAA, BA or ABA) for 4 more weeks for shoot elongation (Mott and Amerson 1981). Controls were scored and transferred at the same time. The number of shoots which were 2 mm or greater in height were then counted and the totals compared between ABA-treated cotyledons and the controls.

In a third experiment with cotyledon explants of *Pinus virginiana* Mill. obtained from the Texas Forest Service, ABA (7.6 µM) was added to a full strength GD agar medium supplemented with BA (22.20 µM) and NAA (0.05 M). The control treatment was the same medium without the ABA. All explants were obtained from seeds germinated in 1% H_2O_2 for 7 days. The control treatment consisted of 72 cotyledon explants with 24 inoculated in each of 3 Petri dishes. The ABA treatment consisted of 120 cotyledon explants inoculated in groups of 24 in 5 Petri dishes. Dormant cotyledons with no evidence of shoot meristem activity were discarded.

RESULTS AND DISCUSSION

In our first experiment with seeds originating from Bastrop, Texas the cotyledon explants showed typical development after 4 weeks on the control medium (GD+BA+NAA). The cotyledons had a distinctive green color and showed a very bumpy appearance indicating cell proliferation at their surfaces. When ABA was present in the medium (GD+BA+NAA+ABA), the cotyledons greatly increased in size and they had a bright green color. The regions of cell proliferation appeared similar to those seen in control cotyledons, differing only in that there was an increased area with shoot primordia in 10^{-7} M ABA-treated tissues (Table 1). The percent of total cotyledon surface area with shoot primordia was assessed after 4 weeks. ABA-treated cotyledon explants had significantly larger surface areas with shoot primordia than the control explants.

Because 10^{-7} M ABA was shown to be effective in enhancing morphogenesis in our preliminary experiments, this concentration of ABA was used. Other workers had shown that it takes 4 weeks for *Pinus* cotyledon explants to respond fully to BA+NAA for shoot initiation (Mott and Amerson 1981), and it appeared from our preliminary studies that a prolonged treatment with ABA did not increase the response significantly. Therefore, the effect of ABA was compared to controls after 4 weeks of treatment.

Table 1. Enhancement of shoot production by ABA from *Pinus taeda* cotyledon explants

Concentration* of ABA (µM)	Percent of Cotyledon Surface Area with Shoot Primordia (%)
0	17.7
0.1	37.4*

*BA = 10 mg 1^{-1}, NAA = 0.1 mg 1-1
**Significant at the 0.05 level

Endogenous ABA synthesis is inhibitied by FLUR via inhibition of carotenoid synthesis (Fong et al. 1983). Fluridone-treated cotyledons showed some very small proliferations of growth on the surface and they were white in color. There was no measureable increase in fresh weight (Table 2). The fresh weight of the cotyledon explants at the time of inoculation was 1 mg. After 4 weeks of FLUR treatment, the fresh weight remained at 1 mg, indicating no growth or shoot production (Table 2).

Table 2. Fresh weight of cotyledon explants of loblolly pine (*Pinus taeda* L.) after treatment with FLUR for 4 weeks.

Concentration (mg 1^{-1})	Fresh Weight (mg)
0	15
50	1

In our second experiment, the objective was to determine the effect of ABA on the number of shoots produced. Because the shoots were at various developmental stages, only those shoots that were 2 mm or more in height after being subjected to the shoot elongation treatment for 8 weeks were counted (Table 3). A 56% increase in number of shoots produced from Bastrop cotyledon explants was observed when they had been exposed to 10^{-7} M ABA for 4 weeks. No shoots were produced on cotyledons treated with FLUR. After 4 weeks on elongation medium there was no evidence of shoot production, whereas many shoots were present on the control cotyledons. These data indicate that FLUR completely blocked shoot production.

Table 3. Enhancement of shoot production by ABA from *Pinus taeda* cotyledon explants after 8 weeks on shoot elongation media*

Concentration of ABA (AM)	No. of Shoots ^ 2mm	% Increase
0	225	
0.1	522	56

*(Mott and Amerson, 1981)

We have also observed an ABA-enhanced increase in surface area with shoot primordia on cotyledon explants of other *Pinus* species. In *Pinus virginiana,* we observed a 65% increase in shoot primordia surface area of cotyledon explants after 4 weeks of ABA exposure (Table 4).

Table 4. Enhancement of shoot primordia production by ABA from *Pinus virginiana* cotyledon explants

ABA Concentration* (MM)	Number of Cotyledons Observed	Percent of Cotyledon Surface Area with Shoot Primordia (%)
0	53	26.3
7.6	68	43.1**

*BA = 22.2 AM, NAA = 0.05 AM.
**Significant at 0.001 level

The shoot response to ABA appears to be dependent upon the length of exposure time as well as the seed source. Exposures longer than four weeks appeared to halt shoot development, indicating that higher than optimal levels of ABA may be present in the tissue. This may also explain why ABA addition to callus in both *Pinus strobus* and *Pinus echinata* showed no increase in frequency of somatic embryogenesis or organogenesis (Kaul and Kochhar 1985). Different seed source responses to exogenous ABA may be due to different levels of endogenous ABA in explant tissues. As expected for a hormone mediated event, there is an optimal ABA concentration for shoot enhancement with more effectiveness at 10^{-7}M than at either 10^{-6}M or 10-8M for loblolly pine. Similar optimum ABA concentration dependent responses were reported for callus generated from *Pinus spp.* (Kaul and Kochhar, 1985).

At this time, it is not clear whether ABA enhances shoot production primarily by increasing shoot induction or shoot maturation and development, or whether it *is* increasing both. Our studies with FLUR, an inhibitor of

ABA biosynthesis (Fong et al. 1983), showed a complete inhibition of shoot initiation in loblolly pine cotyledon explants, suggesting that ALA may have a role in shoot induction (Ackerson 1984). Furthermore, Bastrop explants produced significantly more shoots than the controls when treated with ABA, which may indicate shoot induction. On the other hand, studies with other plant systems such as *Glycine* (Bray and Beachy 1985), *Brassica* (Crouch et al. 1985), *Gossypium* (Galau et al. 1986), *Triticum* (Willamson et al. 1985), *Picea* (Becwar et al. 1987, Jain et al. 1988) and *Daucus* (Kamada and Harada 1981) all suggest that ABA has a role in tissue maturation and development.

Environmental stresses have been shown to increase ABA levels in stressed plant tissues. Stresses such as osmotic shock (Jain et al. 1988b), water stress (Liu and Lai 1985) and salt stress (Litz 1986), all stimulate somatic embryo development in embryogenic culture. Osmotic stress increased root formation from *Zea* callus (Abou-Mandour and Hartung, 1986). Water stress has been shown to induce transcriptional cellular processes leading to the production of ABA (Guerrero and Mullet, 1986) suggesting that ABA synthesis in response to stress may lead to subsequent tissue development in morphogenesis. In addition to embryogenesis, the present study shows for the first time that ABA also has a role in *Pinus taeda* organogenesis. Conifer explant tissues seem to be responsive to ABA, because we have observed similar responses in two pine species.

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