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Research note

Direct shoot organogenesis from needles of three genotypes of *Sequoia sempervirens*

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Abstract

Using *in vitro*-grown needles of *Sequoia sempervirens* (D. Don.) Endl., direct shoot organogenesis was induced. The effects of three genotypes and two cytokinins, N^6-benzyladenine (BA) and *N*-benzyl-9 (2-tetrahydropyranl) adenine (BPA), in combination with 2,4-D were investigated. Among tested cytokinins, BPA produced the highest frequency of shoot organogenesis from all three genotypes tested. Adventitious shoots were induced directly from explants without intervening callus within 5 weeks following incubation. Shoots were elongated on a 1/2 Wolter and Skoog (WS) medium supplemented with activated charcoal but without growth regulators. Later, elongated shoots were transferred to a 1/4 WS medium, but without activated charcoal and free of plant growth regulators to promote continued shoot growth. These shoots rooted spontaneously.

Abbreviations: BA – N^6-benzyladenine; BPA – *N*-benzyl-9 (2-tetrahydropyranl) adenine; 2,4-D – 2,4-dichlorophenoxyacetic acid; WS – Wolter and Skoog (1966) medium

Sequoia sempervirens (D. Don.) Endl., coast redwood, is an important conifer species as it is the tallest tree on earth with a high volume of standing biomass, in some stands exceeding 3500 metric tons/hectare. Moreover, it grows very rapidly along the pacific coastal area of North America, beginning in southern Oregon and ending just south of Monterey, California.

There are few *in vitro* studies on micropropagation of this coniferous plant using different sources of explants (Boulay, 1987; Fouret et al., 1988; Thorpe et al., 1991; Sul and Korban, 1994). Other studies have indicated that induction of adventitious shoots or somatic embryos in *S. sempervirens* is possible, but only from callus tissues derived from zygotic embryos or from cotyledons and hypocotyls of *in vitro* germinated seedlings (Ball, 1987; Bourgkard and Favre, 1988), and then at low frequencies (ranging from 3 to 14%).

In general, most reports on induction of organogenesis and/or embryogenesis in conifers involve culture of zygotic or seed tissues (Attree and Fowke, 1993; Pullman et al., 2003; Stasolla and Yeung, 2003). These sources of explants are highly heterozygous, and therefore regenerants are likely to exhibit variability. In order to maintain trueness-to-type of elite clones or superior genotypes of conifer species having desirable characters (e.g., resistance to diseases or insects, wood quality, or growth characteristics, among others), explants for micropropagation should be derived from somatic tissues of trees old enough to have demonstrated their value, and not from zygotic

tissues. Moreover, the micropropagation protocol should involve minimal or no callus development in order to reduce the likelihood of induction and recovery of variants.

There are only few reports on direct induction of organogenesis from somatic tissues of conifer species. Using needles of 14-day-old seedlings *of Pinus sylvestris*, Bornman and Jansson (1980) reported that adventitious shoot buds were induced in these explants but only when the entire short shoot or dwarf spur was retained in the explant. von Arnold and Erickson (1979a) induced adventitious bud primordia from needles of *Picea abies* L. Induction of somatic embryogenesis has been successfully achieved in numerous conifer species (Sutton, 2002; Stasolla and Yeung, 2003), but in most cases these have been derived from zygotic rather than somatic tissues. In this paper, we report on the induction of direct organogenesis from needles of three genotypes of *S. sempervirens,* and investigate the influence of various media components on frequency of shoot organogenesis and mean number of shoots per needle for each of these genotypes.

Fully expanded green healthy needles (~1 cm in length and ~0.2 cm in width) were collected from *in vitro*-grown shoots of three *S. sempervirens* genotypes, designated B, D and E, and used for shoot induction experiments. These proliferating cultures were initially obtained from stem segments collected from 2-year-old trees grown in the greenhouse, and has been described in detail in an earlier publication (Sul and Korban, 1994).

A basal medium containing Wolter and Skoog (1962) (WS) salts, Staba vitamins (Staba, 1969), 100 mg l^{-1} myo-inositol, and 20 g l^{-1} sucrose was supplemented with various plant growth regulators. The medium was solidified with 6 g l^{-1} Difco Bacto-agar. The pH of the medium was adjusted to 5.6 with 0.5 N KOH or 0.5 N HCl prior to autoclaving for 15 min at 121 °C.

A factorial experiment, arranged in a completely randomized design, was designed using three genotypes (B, D, and E), two cytokinins (BA and BPA) (Sigma-Aldrich, St. Louis, MO), each at five different concentrations including 0, 5, 10, 15 and 20 μM, and four 2,4-D (Sigma-Aldrich) concentrations (0, 0.1, 0.5 and 1 μM). Each treatment consisted of three petri plates (100 × 15 mm), and each plate contained 10 needles. This experiment was replicated three times. Plates were wrapped with parafilm, incubated in a culture room in the dark at 22 ± 1 °C for 3 weeks, and then transferred to a 16-h photoperiod at low-light intensity (15–20 μmol m^{-2} s^{-1}) provided by cool-white fluorescent tubes at 22 ± 1 °C. Following induction, explants were transferred to fresh 1/2 WS medium containing 2 g l^{-1} activated charcoal, but free of plant growth regulators (PGRs) to promote further growth of induced shoot buds.

After 8 weeks in culture, data were collected on the number of explants producing adventitious shoots and the number of shoots/explant. All percent data were transformed into arcsin, and ANOVA was conducted using a SAS statistical package (SAS Institute, 1985). Means were compared using a least significant difference test at the 5% probability level.

Incubating needle explants in the dark was necessary for inducing adventitious organs for all genotypes and for all treatments. Moreover, *in vitro*-grown needles of *S. sempervirens* incubated on a WS medium, but free of PGRs (control) died within 2–3 weeks following culture. After 1 week in the dark and except for control treatment, small dome-shaped structures began to form along the needles (observed under an Olympus SZ-PT stereomicroscope). After two additional weeks in the dark, most *S. sempervirens* needles expanded and developed small organized structures along their surfaces. Adventitious organs were clearly visible to the naked eye 1 week following transfer of explants to light conditions (4 weeks after *in vitro* culture). Maintaining needle explants under low-light intensity (15–20 μmol m^{-2} s^{-1}) may have resulted in additional shoot induction (Figure 1a). All adventitious organs were derived directly from needles without an intervening callus stage, thus reducing the likelihood of inducing somaclonal variation.

After four weeks of growth under low-light intensity, organized structures developed into adventitious buds (Figure 1b). Significant differences for induction frequency and mean number of shoots per explant were observed among genotypes ($p = 0.01$), cytokinin treatments ($p = 0.01$), 2,4-D concentrations ($p = 0.05$), and for genotype × cytokinin ($p = 0.001$), cytokinin × 2,4-D ($p = 0.05$), and genotype × cytokinin × 2,4-D interactions ($p = 0.001$). Thus, mean comparisons were conducted separately for individual genotypes and for each of the two cytokinins at each 2,4-D concentration used for the two parameters analyzed.

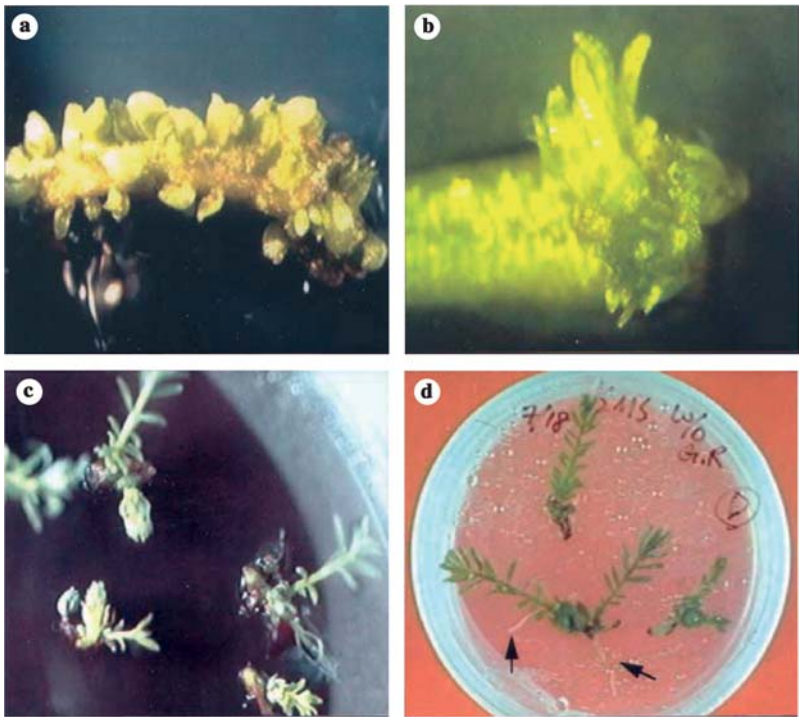

Figure 1. Shoot organogenesis and somatic embryogenesis from needles of *Sequoia sempervirens*. (a) Induction of adventitious buds after 4 weeks of culture of explants; (b) growth of adventitious shoots after 5 weeks of culture; (c) elongation of adventitious shoots on a growth-regulator medium with activated charcoal; (d) spontaneous rooting of elongated shoots (note arrows).

For genotype B, the highest percent shoot organogenesis was observed at 0.1 μM 2,4-D for both BA and BPA (Figure 2a). A shoot induction frequency of 90% adventitious was observed on explants grown in a medium containing 5 μM BPA (Figure 2a). Among the tested BA concentrations, 47% shoot organogenesis frequency was observed on explants grown on 5 μM BA (Figure 2a). The highest mean number of adventitious shoots/explant (2 shoots/explant) was observed on explants grown on a medium containing 5 μM BPA and 0.1 μM 2,4-D (Figure 2a). A mean of one shoot per explant was obtained from explants grown on 5 μM BA and 0.1 μM 2,4-D (Figure 2a).

Unlike genotype B, genotype D showed a different induction response to the two cytokinins tested. Shoot organogenesis was observed on needle explants grown on media containing either BA or BPA. For BA treatment, concentrations of 5, 15, and 20 μM combined with 0.1 μM 2,4-D showed a high frequency of shoot organogenesis (97–87%) (Figure 2b). For BPA treatment, all four concentrations combined with either 0.1 or 0.5 μM 2,4-D produced almost a 100% shoot induction frequency (Figure 2b). As for mean number of shoots per explant, the highest numbers of shoots/explant were obtained at 10 or 15 μM BA with or without 0.1 μM 2,4-D and at 10 or 20 μM BPA with 0.1 or 0.5 μM 2,4-D (Figure 2b). The presence of 2,4-D along with cytokinins in the medium was necessary for optimizing the shoot organogenesis frequency and number of adventitious shoots per explant (Figure 2b).

Compared to genotypes B and D, genotype E produced the highest frequency of shoot organogenesis for almost all cytokinins and concentrations tested. Again, shoot organogenesis was observed on explants grown on media containing either BA or BPA. For all BA concentrations, a frequency of 100% shoot organogenesis was observed with 0.1 or 0.5 μM 2,4-D (Figure 2c). Similarly, for all BPA concentrations with 0.1 or 0.5 μM 2,4-D, >95% frequency of shoot organogenesis was observed (Figure 2c). Mean number of adventitious shoots/explant was highest (5.4) for explants grown on media containing 5, 10, 15 and

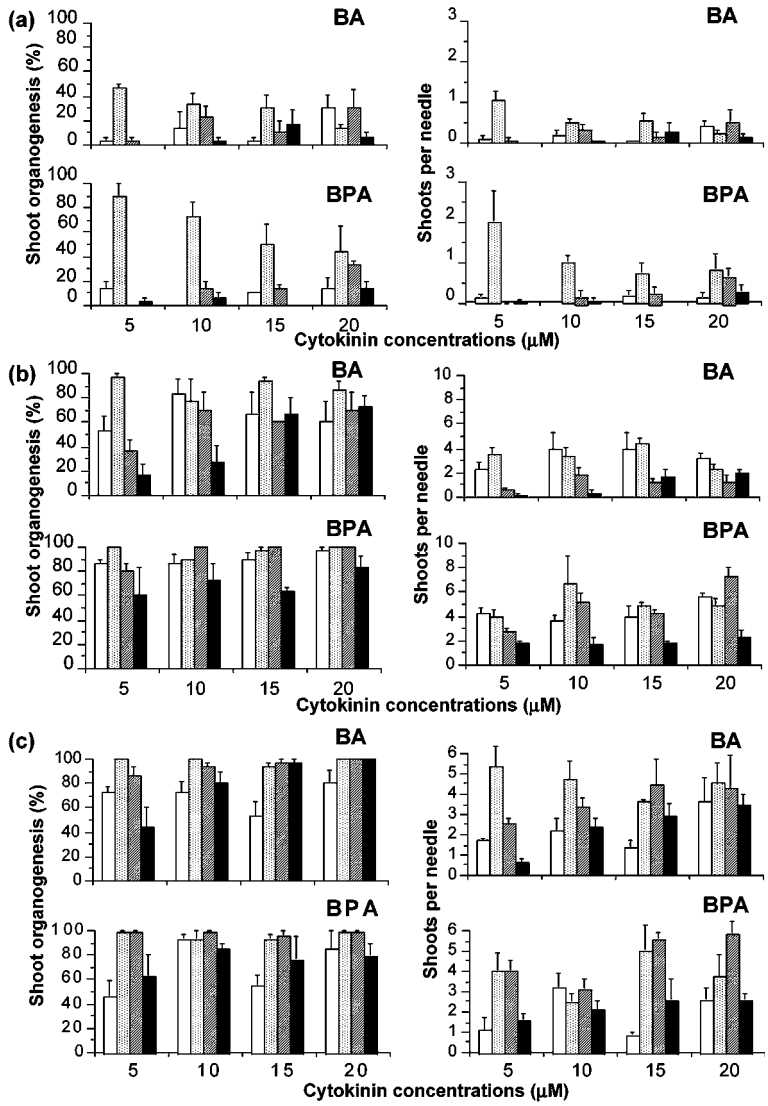

Figure 2. Effect of BA and BPA at four levels along with four concentrations of 2,4-D on frequency of shoot organogenesis (on the left) and mean number of shoots per explant (on the right) from *in vitro*-grown needles of *Sequoia sempervirens* genotypes B (panel a), D (panel b), and E (panel c). Lines correspond to the standard error of the mean (bar). For each treatment combination, $n = 30$, and this was replicated three times. Data were collected after 8 weeks of culture. □ = 0; ⬚ = 0.1; ▨ = 0.5; and ■ = 1.0 μM 2,4-D.

0.5 μM 2,4-D (Figure 2c). Explants grown on media containing either 15 or 20 μM BPA with 0.5 μM 2,4-D produced 5.6 and 5.9 shoots/explant, respectively (Figure 2c).

Overall, shoot organogenesis frequencies as well as mean number of shoots per explant were significantly higher for genotypes D and E than for genotype B (Figure 2a–c). Genotype D showed a higher mean number of shoot per explant than genotype E. Adding 2,4-D to the induction medium induced a high frequency of organogenesis and mean number of adventitious shoots. The use of BPA combined with 2,4-D showed a higher frequency of shoot organogenesis and mean number of shoots per explant than that of BA.

Adventitious shoots were induced directly from needles of all tested genotypes. However, significant genotypic variation was observed for frequency of shoot organogenesis and number of shoots per explant. This was similar to reports on

shoot organogenesis from other conifers including *Picea abies* (von Arnold and Erickson, 1979a, b), *Pinus eldarica* (Gladfelter and Phillips, 1987), *Pinus radiata* (Thorpe, 1988), and *Pinus taeda* (Tang and Guo, 2001), among few others. Genotype E showed the highest response for adventitious shoot induction, and this was followed by genotypes D and B (Figure 2a–c). For most organogenic culture systems of conifers, BA has been found to be superior to other cytokinins in the shoot induction phase (Bonga and Aderkas, 1992). In this study, BPA, a tetrahydropyranyl form of BA, was superior to BA in inducing shoot organogenesis from needles of *S. semperivrens* ($p = 0.05$). BPA has been used previously to induce shoot organogenesis in anthers of strawberry, but it was not found to be better than BA when either BPA or BA were combined with either α-naphthaleneacetic acid (NAA) or indoleacetic acid (IAA) (Owen and Miller, 1996). Previously, we evaluated the effect of BPA, among other cytokinins, on inducing shoot organogenesis in zygotic embryos of *Pinus sylvestris*, and found that it was either comparable or less efficient than BA, depending on the concentrations used (Sul and Korban, 1998). Therefore, it is likely that the interaction of BPA with 2,4-D in this study may have contributed to the enhanced frequency of shoot organogenesis observed in Sequoia needles.

When adventitious shoots, induced from needles of all genotypes tested from the different treatments, were excised and transferred to a 1/2 WS medium containing 2 g l^{-1} activated charcoal, all shoots continued to grow and elongate (Figure 1c). After 2 weeks, developing shoots were then transferred to a fresh 1/4 WS medium free of PGRs and without activated charcoal to promote further shoot elongation. Within 6–7 weeks, shoots were >2 cm in length and over 75% of these shoots (~200 shoots) spontaneously developed roots (Figure 1d). This latter rooting phenomenon was also observed in our earlier work with proliferating shoots induced from axillary buds of stem segments of *S. sempervirens* (Sul and Korban, 1994). Some of these plantlets were later acclimatized, and transferred to the greenhouse.

For most organogenic and embryogenic culture systems in various conifers, the addition of such auxins as NAA (for organogenesis) or 2,4-D (for embryogenesis) has been recommended (Bonga and von Aderkas, 1992). In this study, the presence of 2,4-D in the culture medium was necessary to induce organogenesis from needles of *S. sempervirens* regardless of the genotype and cytokinin used. Interestingly, callus development was not observed on needle explants incubated on any of the 2,4-D concentrations used in combination with BA or BPA that induced shoot organogenesis. Whether somatic embryogenesis was also induced in these cultures could not be fully confirmed as embryo-like structures were also observed, but none of these underwent any further phenocritical development (data not shown).

In summary, although earlier studies have reported on induction of adventitious shoots (Ball, 1987; Boulay, 1987) or somatic embryos from callus tissues of *S. sempervirens* (Bourgkard and Favre, 1988), this is the first report on inducing adventitious shoots directly from needles of *S. sempervirens* and without an intervening callus phase. Adventitious shoots were elongated and rooted spontaneously *in vitro*.

Acknowledgements

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References

Attree SM & Fowke LC (1993) Embryogeny of gymnosperms: advances in synthetic seed technology of conifers. Plant Cell Tiss. Org. Cult. 35: 1–35

Attree SM, Pomeroy MK & Fowke LC (1995) Development of white spruce [*Picea glauca* (Monech.) Voss] somatic embryos during culture with abscisic acid and osmoticum, and their tolerance to drying and frozen storage. J. Exp. Bot. 46: 433–439

Ball EA (1987) Tissue Culture Multiplication of *Sequoia*. Cell and Tissue Culture in Forestry; Case Histories: Gymnosperm, Angiosperms and Palms, Vol. 3. (pp 146–158). Martinus Nijhoff, Dordrecht

Bonga JM & Von Aderkas P (1992) In Vitro Culture of Trees. Kluwer Acad Publ, Dordrecht

Bornman CH & Jansson E (1980) Organogenesis in cultured *Pinus sylvestris* tissue. Z. Pflanzenphysiol 96: 1–6

Boulay (1987) *In vitro* propagation of tree species. In: Green CE, Somers DA, Hackett WP & Biesboer DD (eds) Plant Tiss. Cell Cult. (pp. 367–382). Alan R. Liss Inc., NY

Bourgkard F & Favre JM (1988) Somatic embryos from callus of *Sequoia sempervirens*. Plant Cell Rep. 7: 445–448

Carrier DJ, Cunningham JE, Taylor DC & Dunstan DI (1997) Sucrose requirements and lipid utilization during germina-

tion of interior spruce (*Picea glauca engelmannii* complex) somatic embryos. Plant Cell Rep. 16: 550–554

Fouret Y, Larrieu C & Arnaud Y (1988) Rajeunissement *in vitro* chez la Sequoia sempervirens (endl.). (pp. 55–82). Ann Rech Sylv, AFOCEL

Gladfelter HJ & Phillips GC (1987) *De novo* shoot organogenesis of *Pinus eldarica* Medw. *in vitro*: I. Reproducible regeneration from long-term callus cultures. Plant Cell Rep. 6: 163–166

Gupta PK, Pullman G, Timmins R, Kreitinger M, Carlson WC, Grob J & Welty E (1993) Forestry in the 21st century; the biotechnology of somatic embryogenesis. Bio/Tech 11: 454–459

Owen HR & Miller AR (1996) Haploid plant regeneration from anther cultures of three North American cultivars of strawberry (*Fragaria* × *ananassa* Duch.). Plant Cell Rep. 15: 905–909

Park YS, Pond SE & Bonga JM (1993) Initiation of somatic embryogenesis in white spruce (*Picea glauca*): genetic control, cultural treatment effects, and implications for tree breeding. Theor. Appl. Genet. 86: 427–436

Pullman GS, Zhang Y & Phan BH (2003) Brassinolide improves embryogenic tissue initiation in conifers and rice. Plant Cell Rep. 22: 96–104

SAS Institute (1985) SAS User's Guide: Statistics. 5th edn. SAS Institute Inc., Cary, NC

Sato S, Toya T, Kawahara R, Whittier RF, Fukuda H & Komamine A (1995) Isolation of a carrot gene expressed specifically during early-stage somatic embryogenesis. Plant Mol. Biol. 28: 39–46

Staba EJ (1969) Plant tissue culture as a technique for the phytochemist. Recent Adv. Phytochem. 2: 75–102

Stasolla C & Yeung EC (2003) Recent advances in conifer somatic embryogenesis: improving somatic embryo quality. Plant Cell Tiss. Org. Cult. 74: 15–35

Sul I-W & Korban SS (1994) Effect of different cytokinins on axillary shoot proliferation and elongation of several genotypes of *Sequoia sempervirens*. In Vitro Cell Dev. Biol. 30P: 131–135

Sul I-W & Korban SS (1998) Effects of media, carbon sources and cytokinins on shoot organogenesis in the Christmas tree Scots pines (*Pinus sylvestris* L.). J Hort. Sci. Biotech. 73: 822–827

Sutton B (2002) Commercial delivery of genetic improvement to conifer plantations using somatic embryogenesis. Ann. Forest Sci. 59: 657–661

Tang W & Guo Z (2001) *In vitro* propagation of loblolly pine via direct somatic organogenesis from mature cotyledons and hypocotyls. Plant Grow Regul. 33: 25–31

Thorpe TA (1988) Physiology of bud induction in conifers in vitro. In: Hanover JW Kethley & DE (eds) Genetic Manipulation of Woody Plants (pp. 167–184). Plenum Press, New York

Thorpe TA, Harry IS & Kumar PP (1991) Application of micropropagation to forestry. In: Debergeh PC & Zimmerman RH (eds) Micropropagation: Technology and Application (pp. 331–336). Kluwer Acad Publ, Dordrecht

von Arnold S & Eriksson T (1979a) Bud induction on isolated needles of Norway spruce (*Picea abies* L., Karst.) grown *in vitro*. Plant Sci. Lett. 15: 363–372

von Arnold S & Eriksson T (1979b) Induction of adventitious buds on Norway spruce (*Picea abies*) grown *in vitro*. Physiol. Plant 45: 29–34

Wolter KE & Skoog F (1966) Nutritional requirements of *Fraxinus* callus cultures. Am. J. Bot. 53: 263–269