

Tissue Culture of Mature Ponderosa Pine: Callus Induction and Differentiation

Yiqun Lin and Michael R. Wagner

School of Forestry
Northern Arizona University
Box 15018
Flagstaff, Arizona 86011

Abstract. Callus was induced from needle segments of mature ponderosa pine (*Pinus ponderosa* Doug. ex Laws.). Callus formation was significantly affected ($P \leq 0.01$) by four growth-regulator treatments and the position along needle segments. The highest frequency of callus formation (57%) was obtained with Murashige-Skoog (MS) medium containing 0.5 mg/L BAP (benzylaminopurine) and 1.0 mg/L 2,4-D (2,4-dichlorophenoxyacetic acid). The needle segments located in the middle part of the axis responded best, as measured by callus formation, to the tested treatments. Three callus lines were grown by applying two subcultures for subsequent study of callus differentiation. Semithin sections of callus tissue from the second subculture revealed a vascular nodule (tracheid masses with cambiumlike structure), and meristematic tissue had formed in the lower part of callus cultured in MS medium containing 0.03 mg/L NAA (naphthalene acetic acid) + 0.5 mg/L BAP. Among three callus lines, only line 2—treated with 0.5 mg/L 2,4-D + 1.0 mg/L BAP—developed a vascular nodule and meristematic tissue after transfer to two subculture media.

Key words: Callus formation, meristematic tissue, needles, phytohormones, *Pinus ponderosa*, vascular nodule.

Callus culture is a method by which a mass of unorganized tissue—callus—is induced. Callus can then be transferred to new medium to obtain differentiated callus that would develop into organs or somatic embryos and eventually obtain plantlets (Geissbühler and Skoog 1957; Halperin 1969; Pierik 1987). Callus culture is considered a potential tool to achieve tree regeneration via embryogenesis or organogenesis. Aboel-Nil (1987) estimated that 247,100 ha could be reforested with plantlets produced from 100 L of callus in only 3 months. Callus induction is a first step to achieve tree regeneration. Since 1934, when the first callus was obtained from cambial explants of *Pinus pinaster* Ait. trees 15–50 cm in diameter (Gautheret 1943), many gymnosperms have been successfully

induced to form callus (Harvey and Grasham 1969; Brown and Sommer 1975; John 1983; Tuskan et al. 1990).

Callus differentiation, the second step of callus culture, is critical for achieving regeneration. Vascular nodule and meristematic tissue formation are the two major types of callus differentiation in vitro (Gautheret 1966; Gresshoff 1978). Many studies have been done in angiosperm species (Sterling 1950; Steward et al. 1958; Gautheret 1966; Fosket and Torrey 1969; Meada and Thorpe 1979). In gymnosperms, callus differentiation involved in vascular nodule and meristematic tissue formation is only documented in *Pinus eldarica* Medw. (Wagley et al. 1987), *Pinus radiata* D. Don (Washer et al. 1977), and *Sequoia sempervirens* (D. Don) Endl. (Ball 1950).

Capacity of callus differentiation varied in plant species, organs, and tissues (Halperin 1973). Aging of a plant can also influence the capacity for callus differentiation. The older the plant or tissue is, the more difficult it is to obtain differentiated callus (Mehra-Palta and Thompson 1987). In most instances mentioned above, however, the sources used in the experiments were either from embryos or juvenile materials. Because some superior characteristics are not expressed until trees reach maturity, regeneration from tissues of mature trees is highly desired. For instance, resistance of ponderosa pine to some insects may be recognized only when the trees reach maturity.

With the development of a callus initiation protocol and the study of callus differentiation, we may be able to produce plantlets from resistant, mature ponderosa pine. Our interest in callus culture for mature ponderosa pine and the lack of available information on callus culture for mature gymnosperms prompted our study. In this paper we report the successful induction of callus and the study of callus differentiation of mature ponderosa pine.

Materials and Methods

Callus Induction

Ten current-year branches were collected from each of four open-pollinated mature ponderosa pines (trees 2, 3, 12, and 14, ranging from 20 to 29 years of age) on 15 July 1988. The trees are located south of Parks, Arizona, in the Kaibab National Forest (R4E, T21N, Sections 3, 4, 9, and 10). The trees were a subset of 20 trees used for the past 3 years in a rooting experiment (M. R. Wagner, unpublished data). The branches, including needles that were half-

elongated, were stored along with a wet paper towel in plastic bags inside a refrigerator (0–2° C) for 2 days before the experiments.

The branches with needles were disinfected on the surface by a five-step procedure that included soaking in 1% (w/v) Alconox for 2 min, soaking in 10% (v/v) Clorox for 15 min, soaking in 5% (v/v) H₂O₂ for 15 min, soaking in 2% (w/v) Benlate for 2 min, and finally rinsing with sterilized distilled water.

Difco bacto-agar media were sterilized in an autoclave at 1.4 kg/cm² and 120° C for 20 min. All media were adjusted to pH 5.8 with 1N NaOH and 1N HCl before autoclaving.

Needles were removed from the branches and were transversely cut into 8 segments 8–10 mm long. Segments were numbered from 1 to 8 along the needle axis from proximal to distal and were placed horizontally in petri dishes containing MS nutrient medium with growth regulators. Needle explants were grown in a growth chamber at 25 ± 1° C. Fluorescent white lights (82 μmol/cm²/s) were used with a 16-h photoperiod.

Four growth-regulator treatments were applied to test the effect of auxin-to-cytokinin ratios on callus formation:

1. 0.1 mg/L 2,4-D + 2.5 mg/L BAP (benzylaminopurine);
2. 0.5 mg/L 2,4-D + 1.0 mg/L BAP;
3. 1.0 mg/L 2,4-D + 0.5 mg/L BAP; and
4. 1.0 mg/L 2,4-D + 0.1 mg/L BAP.

The first and second treatments were designed to have auxin-to-cytokinin ratios less than 1. The last two treatments were designed to have the ratio greater than 1.

The effects of phytohormone, positions along needle axes, genotypes (represented by the four trees), and their interactions on callus formation were assessed with three-way ANOVA (Zar 1984). Twenty needles were cultured for each combination of the three factors. Because there were significant differences among the treatments, the Newman-Keuls multiple comparison test (Zar 1984) was used to compare the differences among treatments. Frequency of callus formation was calculated by recording the number of callused explants divided by the total number of callused and noncallused explants for each combination of the three factors after the explants had grown for 4 weeks.

Callus Differentiation

The calli derived from three growth-regulator treatments—MS media containing 1.0 mg/L 2,4-D + 0.5 mg/L BAP; 0.5 mg/L 2,4-D + 1.0 mg/L

BAP; or 1.0 mg/L 2,4-D + 0.1 mg/L BAP—were used in the callus differentiation study and were named callus lines 1, 2, and 3.

After calli grew for 7 weeks, approximately same-sized calli were used for subculturing. Two subculture lines were developed. In the first subculture, 5 calli from each of the four genotypes of all the three callus lines were cultured in two treatments: 2.5 mg/L BAP + 0.03 mg/L NAA (naphthalene acetic acid) and 0.5 mg/L BAP without NAA. After 14 weeks of growth in the above media, the calli were transferred into the second subculture medium containing 0.5 mg/L BAP + 0.03 mg/L NAA. The culture condition was the same as that in callus induction.

Callus tissue was collected after growing for 10 weeks in the second subculture media. The callus was fixed in formalin-aceto-alcohol (FAA) solution, then gradually dehydrated and embedded in paraffin as described by Jensen (1962). The embedded materials were serially sectioned at 10 μ m using a rotary microtome. The sections were stained with safranin and fast green and mounted with resin (Jensen 1962).

Results and Discussion

Callus Induction

Growth Regulator Effect

Vigorous, light green, and fragile callus started forming on the 10th day. The average frequency of callus formation by the four trees (Fig. 1) was taken after 4 weeks.

Three-way ANOVA showed there was a significant difference among the phytohormone treatments ($F = 43.59$, $df = 3,127$, $P < 0.01$). The Newman-Keuls multiple comparison test categorized four treatments into four groups (A, B, C, and D; Table). In terms of the effect, the most effective treatment for callus formation was treatment 3 (group A; 57% callus formation) containing 1.0 mg/L 2,4-D + 0.5 mg/L BAP; followed by treatment 4 (group B; 39% callus formation) with 1.0 mg/L 2,4-D + 0.1 mg/L BAP; then treatment 2 (group C; 29% callus formation) with 0.5 mg/L 2,4-D + 1.0 mg/L BAP; and finally treatment 1 (group D; 18% callus formation) containing 0.1 mg/L 2,4-D + 2.5 mg/L BAP.

An exogenous supply of auxin alone or auxin combining cytokinin is often required to induce callus formation (Pierik 1987). Different species, organs, or tissues have a wide range of growth-regulator requirements (Pierik 1987).

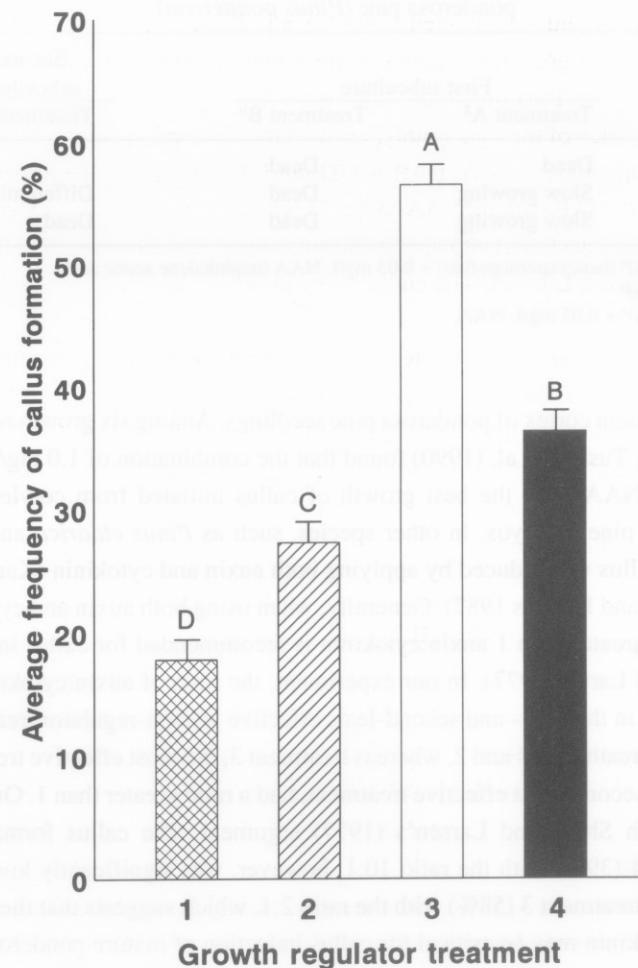


Fig. 1. Effect of phytohormone treatments on callus formation in mature ponderosa pine (*Pinus ponderosa*). The average frequency of callus formation of each treatment was taken after 4 weeks of growth. Bars represent the standard errors. The letter above the bar represents groups categorized by Newman-Keuls test. 1 = 0.1 mg/L 2,4-D + 2.5 mg/L BAP (benzylaminopurine); 2 = 0.5 mg/L 2,4-D + 1.0 mg/L BAP; 3 = 1.0 mg/L 2,4-D + 0.5 mg/L BAP; 4 = 1.0 mg/L 2,4-D + 0.1 mg/L BAP.

Tissues from *Pinus sylvestris* and *Pinus nigra* formed callus when stimulated by auxins (Bogdanovic 1968; Bogdanovic and Jelenic 1968). Harvey and Grasham (1969) reported that 0.5 mg/L NAA was the best treatment for callus

Table. Response of three callus lines to the callus subculture media of mature ponderosa pine (*Pinus ponderosa*).

Callus line	First subculture		Second subculture
	Treatment A ^a	Treatment B ^b	Treatment C ^c
1	Dead	Dead	
2	Slow growing	Dead	Differentiated
3	Slow growing	Dead	Dead

^a2.5 mg/L BAP (benzylaminopurine) + 0.03 mg/L NAA (naphthalene acetic acid).

^b0.5 mg/L BAP.

^c0.5 mg/L BAP + 0.03 mg/L NAA.

growth of stem cortex of ponderosa pine seedlings. Among six growth regulator treatments, Tuskan et al. (1990) found that the combination of 1.0 mg/L BA + 1.0 mg/L NAA gave the best growth of callus initiated from cotyledons of ponderosa pine embryos. In other species, such as *Pinus eldarica* and *Pinus strobus*, callus was induced by applying both auxin and cytokinin (Kaul 1985; Gladfelter and Phillips 1987). Generally, when using both auxin and cytokinin, a ratio of greater than 1 auxin:cytokinin is recommended for callus induction (Sharp and Larsen 1977). In our experiment, the ratio of auxin:cytokinin was less than 1 in the least- and second-least effective growth-regulator treatments, including treatments 1 and 2, whereas treatment 3, the most effective treatment, and 4, the second most effective treatment, had a ratio greater than 1. Our result agrees with Sharp and Larsen's (1977) argument. The callus formation in treatment 4 (39%) with the ratio 10:1, however, was significantly lower than the one in treatment 3 (58%) with the ratio 2:1, which suggests that the ratio of auxin:cytokinin may be critical for callus induction of mature ponderosa pine. Too high an auxin:cytokinin ratio resulted in reduced callus yield.

Position Effect

The position effect along the needle axis on callus formation was significantly different ($F = 27.46$, $df = 7, 127$, $P = 0.000$). The segments (1–8 presenting the needle positions from the proximal to the distal) were categorized into four major groups by Newman–Keuls test (Fig. 2). Segment 4, in the middle part of needles, was determined to callus best. Segment 1, at the bottom of needles, rarely produced callus. The position had no interaction with the other two factors.

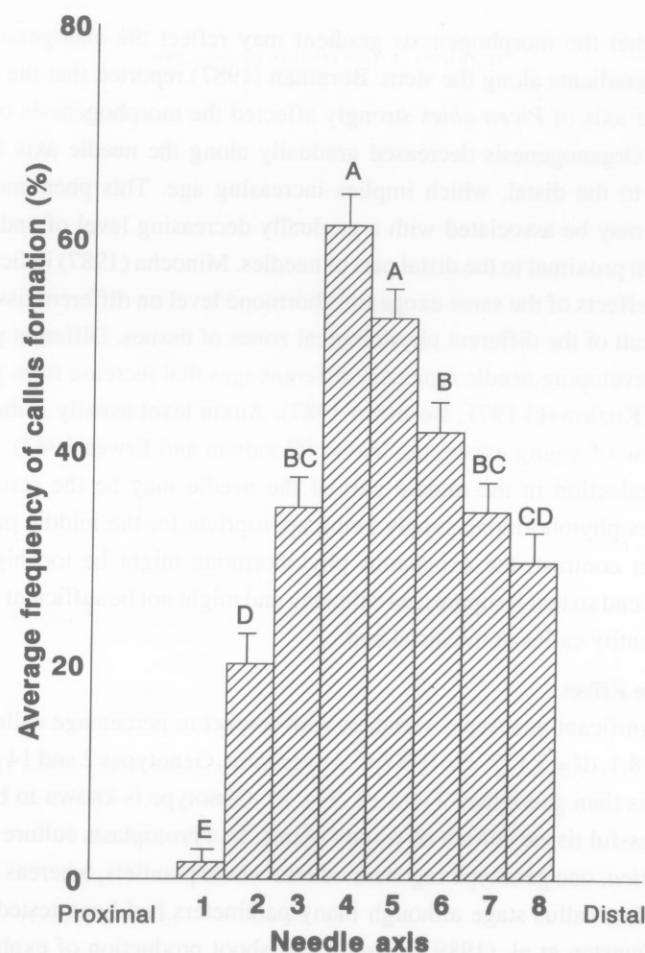


Fig. 2. Effect of explant positions along needle axis on callus formation in mature ponderosa pine (*Pinus ponderosa*). The average frequency of callus formation was taken from each individual segment of four growth-regulator treatments. The bars represent the standard errors. The letter above the bar represent groups categorized by Newman–Keuls test.

The morphogenetic response of explants varies among plants, organs within a plant, and positions within an organ (Minocha 1987). In an in vitro study of *Populus* species, Douglas (1984) found that a gradient of morphogenesis developed along stem internodes cultured on a hormone-free medium. The fourth through seventh internodes produced the most buds and shoots. This

suggests that the morphogenesis gradient may reflect the endogenous plant hormone gradients along the stem. Bornman (1987) reported that the position on the leaf axis of *Picea abies* strongly affected the morphogenesis of needle explants. Organogenesis decreased gradually along the needle axis from the proximal to the distal, which implies increasing age. This phenomenon, he deduced, may be associated with a gradually decreasing level of endogenous auxin from proximal to the distal part of needles. Minocha (1987) indicated that different effects of the same exogenous hormone level on different tissues may be the result of the different physiological zones of tissues. Different positions along a developing needle represent different ages that increase from proximal to distal (Kozłowski 1971; Bornman 1987). Auxin level usually is the highest at leaf base of young expanding leaves (Goodwin and Erwee 1983). The best callus production in the middle part of the needle may be the result of the exogenous phytohormone supply being appropriate for the middle part of the needle. In contrast, the exogenous phytohormone might be too high at the proximal end so that it suppressed callusing and might not be sufficient to induce large-quantity callus at the distal end.

Genotype Effect

Significant genotypic variation with respect to percentage callus formation ($F = 8.1$, $df = 3, 127$, $P = 0.0001$) was present. Genotypes 2 and 14 produced less callus than genotypes 3 and 12 (Fig. 3). Genotype is known to be critical for successful tissue culture of woody plants. In a protoplasts culture of *Malus × domestica*, one genotype regenerated into whole plantlets, whereas the other remained in callus stage although many parameters had been tested (Russell 1993). Dunstan et al. (1989) reported that shoot production of explants from 17-year-old Douglas-fir (*Pseudotsuga menziesii*) varied significantly between provenances and within a provenance. In an in vitro culture experiment of mature *Pinus radia*, the percentage rooting of micropropagated shoots were greatly influenced by plant genotypes (Horgan and Holland 1989). Callus production from cotyledons of ponderosa pine embryos varied among different genotypes (Tuskan et al. 1990). By recognizing genotypic variation, selection of the most responsive genotype would greatly improve success in tissue culture of woody plants.

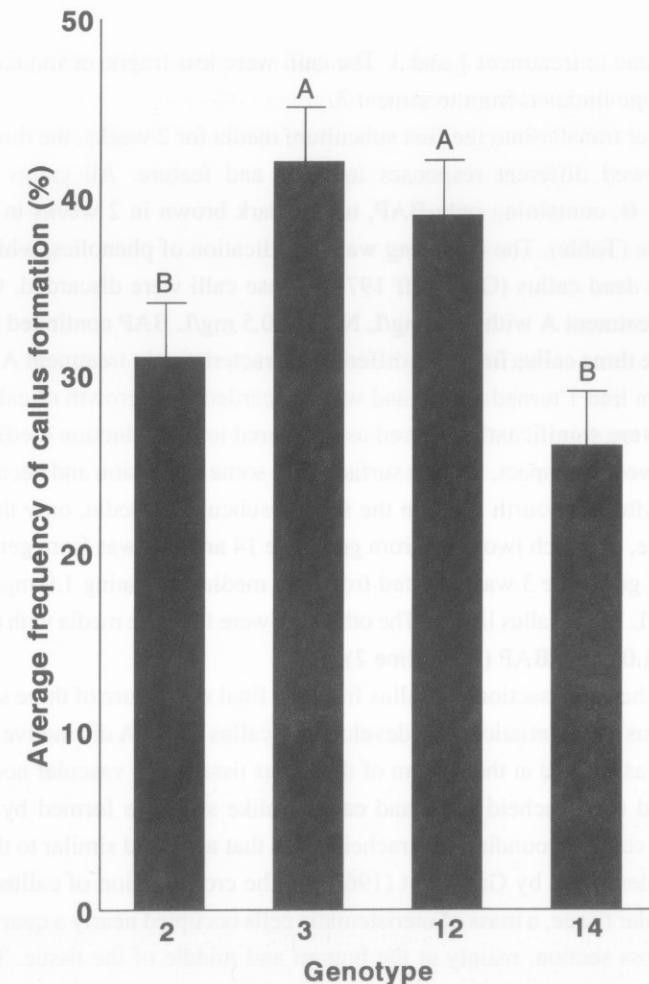


Fig. 3. Effect of genotypes on callus formation in mature ponderosa pine (*Pinus ponderosa*). The average frequency of callus formation of each genotype was taken after 4 weeks of growth. The bars represent the standard errors. The letter above the bar represents groups categorized by Newman-Keuls test.

Callus Differentiation

The external features of callus from the three treatments of the callus initiation media were slightly different. Callus induced from treatment 3 appeared light green and fragile. Callus induced from treatment 1 was yellowish and less proliferated. The color and proliferation of callus from treatment 2 was

intermediate to treatment 1 and 3. The calli were less fragile or more compact than the one initiated from treatment 3.

After transfer into the first subculture media for 2 weeks, the three callus lines showed different responses in color and feature. All callus lines in treatment B, containing only BAP, turned dark brown in 2 weeks in the first subculture (Table). The browning was an indication of phenolics, which often results in dead callus (Gresshoff 1978). These calli were discarded. Only the calli in treatment A with 0.03 mg/L NAA + 0.5 mg/L BAP continued growth.

The three callus lines had different characteristics in treatment A (Table). Calli from line 1 turned brown and were discarded. The growth of callus lines 2 and 3 were significantly reduced as compared to the induction medium. The calli showed a compact, smooth surface with some protrusion and became light brown. After the fourth week in the second subculture media, only three calli were alive, of which two were from genotype 14 and one was from genotype 3. Callus of genotype 3 was initiated from MS media containing 1.0 mg/L 2,4-D + 0.1 mg/L BAP (callus line 3). The other two were from the media with 0.5 mg/L 2,4-D + 1.0 mg/L BAP (callus line 2).

In the serial sections of callus from the final subculture of three surviving calli, callus differentiation only developed in callus line 2. A distinctive vascular nodule was located at the bottom of the callus tissue. The vascular nodule was composed of a tracheid mass and cambiumlike structure formed by rows of flattened cells surrounding the tracheid mass that appeared similar to the cyclic nodules described by Gautheret (1966). In the cross section of callus, next to the vascular tissue, a mass of meristematic cells occupied nearly a quarter of the callus cross section, mainly at the bottom and middle of the tissue. The meristematic cells were characterized by small cells, larger nuclei, and a high density of cytoplasm densely stained by fast green.

Several factors, including genotypes, may have influenced the callus differentiation in our experiment. Among the four genotypes, only genotype 14 showed differentiation. Because of the small sample size, however, we hesitate to conclude that callus differentiation was genotype dependent in our study although genotypes have been demonstrated as having a major influence on plant regeneration (Ernst 1993; Russell 1993).

In this experiment, growth regulators affected callus differentiation in two ways: callus differentiation only developed in the callus line 2 initiated in 0.5 mg/L 2,4-D + 1.0 mg/L BAP; and callus differentiation developed in subculture media containing both BAP and NAA.

Previous studies on the effect of growth regulator on callus differentiation have primarily concentrated on the growth regulator effect on callus subculture media (Sterling 1950; Fosket and Torrey 1969; Bonga and Fowler 1970; Torrey and Fosket 1970; Maeda and Thorpe 1979; Mehra and Annand 1983). The effect of growth-regulator concentrations in callus initiation media, however, is rarely documented in reference to subsequent differentiation. In Christianson and Warnick's study (1983), callus induction media containing different growth-regulator balances had no effect on organogenesis capacity of leaf explants of *Convolvulus arvensis*. In the current study, we found that growth-regulator concentrations in callus initiation medium can be important in determining callus differentiation. Murashige and Skoog (1962) reported that tissue grown in a lower ratio of cytokinin:auxin produced fragile callus, whereas a higher ratio of cytokinin:auxin tended to produce compact callus, which may be considered as the initial step in callus differentiation. The external morphology of callus among the three treatments of initiation media demonstrated this effect. In other words, growth regulators in callus-induction stage may affect the capability of organogenesis of callus during the callus-induction stage.

The result of callus differentiation developing in the second subculture media containing both auxin and cytokinin indicates that the auxin is necessary for callus differentiation. Minocha and Halperin (1974) suggested that differentiation must be involved in cell division that requires auxin. The need for both auxin and cytokinin for vascular nodule formation has been demonstrated (Gautheret 1966; Fosket and Torrey 1969; Torrey and Fosket 1970; Maeda and Thorpe 1979; Wagley et al. 1987). Torrey and Fosket (1970:1072) reported that auxin and cytokinin are necessary for the "initiation of DNA synthesis... for the subsequent division, and finally for their specific cytodifferentiation."

Gresshoff (1978) suggested vascular nodule formation was the initial phase of organogenesis and that further development of vascular nodules would result in organogenesis, such as roots and buds, although Gautheret (1966) indicated that vascular nodule formation would not necessarily result in organogenesis instead of new tissues. Some studies have supported Gresshoff's (1978) suggestion, however. Maeda and Thorpe's (1979) study on tobacco callus culture showed a series of histogenesis events before shoot initiation. They indicated that the developmental sequence of shoots involved "occurrence of meristematic center near the tracheary elements" (Maeda and Thorpe 1979:423), which was similar to our study. In a callus differentiation study of *Sequoia sempervirens*, Ball (1950) observed tracheid cells surrounded by cambium after

the fifth transfer and, after subsequent cultures, adventitious buds were obtained. The same event was also observed by Wagley et al. (1987) in *Pinus ularia* and Washer et al. (1977) in *Pinus radiata*. Steward et al. (1958) found that before roots formed from callus, vascular nodules with lignified elements surrounded by cambiumlike elements were obtained. The callus development process in our study was similar to the above studies suggesting that the callus derived from needles of mature ponderosa pine may have organogenesis potential.

Acknowledgments

We thank the U.S. Department of Agriculture Forest Service, Rocky Mountain Forest and Range Experiment Station for allowing us to use their research facilities. We also thank M. L. Mogensen and T. Zhu for providing photo equipment. R. Tinus reviewed the manuscript. L. Sandoval provided general laboratory assistance. This research was supported by McIntire-Stennis research funds and the Northern Arizona University Organized Research Program.

Cited Literature

- Aboel-Nil, M. M. 1987. Tissue culture of Douglas-fir and western North American conifers. Pages 80–100 in J. M. Bonga and D. J. Durzan, editors. Cell and tissue culture in forestry 3. Martinus Nijhoff, Dordrecht, Boston, Lancaster.
- Ball, E. 1950. Differentiation in a callus culture of *Sequoia sempervirens*. Growth 14:295–325.
- Bogdanovic, M. 1968. The influence of different factors on stem tissue cultures of *Pinus sylvestris* L. Zemljiste i Biljka 17:135–145.
- Bogdanovic, M., and D. J. Jelenic. 1968. The effect of growth substances on the development of initial stem tissue culture of *Pinus nigra* Arn. Zemljiste i Biljka 17:305–313.
- Bonga, J. M., and D. P. Fowler. 1970. Growth and differentiation in gametophytes of *Pinus resinosa* cultured in vitro. Canadian Journal of Botany 48:2205–2207.
- Bornman, C. H. 1987. *Picea abies*. Pages 2–29 in J. M. Bonga and D. J. Durzan, editors. Cell and tissue culture in forestry 3. Martinus Nijhoff, Dordrecht, Boston, Lancaster.
- Brown, C. L., and H. E. Sommer. 1975. An atlas of gymnosperms cultured in vitro: 1924–1974. Georgia Forest Research Council, Macon. 271 pp.
- Christianson, M. L., and D. A. Warnick. 1983. Competence and determination in the process of in vitro shoot organogenesis. Developmental Biology 95:288–293.
- Douglas, G. C. 1984. Formation of adventitious buds in stem internodes of *Populus* species cultured in vitro on basal medium: influence of endogenous properties of explants. Journal of Plant Physiology 116:313–321.
- Dunstan, D. I., T. Bethune, S. Kikcio, and M. S. Lapp. 1989. In vitro shoot formation among 17-year-old Douglas-fir provenances. Canadian Journal of Forest Research 19:1299–1302.
- Ernst, S. G. 1993. In vitro culture of pure species non-aspen poplars. Pages 195–207 in M. R. Ahuja, editor. Micropropagation of woody plants. Kluwer Academic Publishers, London.
- Fosket, D. E., and J. G. Torrey. 1969. Hormonal control of cell proliferation and xylem differentiation in cultured tissues of *Glycine max* var. *biloxi*. Plant Physiology 44:871–880.
- Gautheret, R. J. 1943. Culture du tissue cambial. Comptes Rendus Academic des Science Paris 198:2195–2196.
- Gautheret, R. J. 1966. Factors affecting differentiation of plant tissues grown in vitro. Pages 55–95 in W. Beermann, C. W. Wardlaw, R. J. Gautheret, V. B. Wigglesworth, P. D. Nieuwkoop, and J. A. D. Zeevaert, editors. Cell differentiation and morphogenesis. North-Holland Publishing Company, Amsterdam.
- Geissbühler, H., and F. Skoog. 1957. Comments on the application of plant tissue cultivation to propagation of forest trees. Tappi 40(4):257–262.
- Gladfelter, H. D., and G. C. Phillips. 1987. De novo shoot organogenesis of *Pinus ularia* Medw. in vitro. I. Reproducible regeneration from long-term callus cultures. Plant Cell Reports 6:163–166.
- Goodwin, P. B., and M. G. Erwee. 1983. Hormonal influences on leaf growth. Pages 207–232 in J. E. Dale and F. L. Milthorpe, editors. The growth and functioning of leaves. Cambridge University Press, London.
- Gresshoff, P. M. 1978. Phytohormones and growth and differentiation of cells and tissues cultured in vitro. Pages 1–30 in D. S. Letham, P. B. Goodwin, and T. J. V. Higgins, editors. Phytohormones and related compounds: a comprehensive treatise 2. Elsevier, North-Holland Biomedical Press, Amsterdam.
- Halperin, W. 1969. Morphogenesis in cell cultures. Annual Review of Plant Physiology 20:395–418.
- Halperin, W. 1973. The use of cultured tissue in studying developmental problems. Canadian Journal of Botany 51:1801–1806.
- Harvey, A. E., and J. L. Grasham. 1969. Procedures and media for obtaining tissue cultures of 12 conifer species. Canadian Journal of Forestry 47:547–549.
- Jensen, W. A. 1962. Preparation of the tissue. Pages 55–99 in W. S. Jensen, editor. Botanical histochemistry. Freeman, San Francisco, Calif.
- John, A. 1983. Tissue culture of coniferous trees. Pages 6–21 in J. H. Dodds, editor. Tissue culture of trees. AVI Publishing Company, Inc., Westport, Conn.
- Kaul, K. 1985. Seasonal variation in callus proliferation from explants of mature *Pinus strobus* trees. Page 330 in R. R. Henke, K. W. Hughes, M. J. Constantin, and A. Hollaender, editors. Tissue culture in forestry and agriculture. Plenum Press, New York.
- Kozłowski, T. T. 1971. Growth and development of trees 1. Academic Press, New York. 443 pp.
- Meada, E., and T. A. Thorpe. 1979. Shoot histogenesis in tobacco callus cultures. In Vitro 15(6):415–424.
- Mehra, P. N., and M. Annand. 1983. Callus of *Pinus roxburghii* (Chir pine) and its cytology. Physiologia Plantarum 58:282–286.
- Mehra-Palta, A., and D. G. Thompson. 1987. Tissue culture of eastern North American conifers. Pages 61–79 in J. M. Bonga and D. J. Durzan, editors. Cell and tissue culture in forestry 3. Martinus Nijhoff Publishers, Dordrecht, Boston, Lancaster.

- Minocha, S. C. 1987. Plant growth regulators and morphogenesis in cell and tissue culture of forest trees. Pages 50–66 in J. M. Bonga and D. J. Durzan, editors. Cell and tissue culture in forestry 1. Martinus Nijhoff, Boston.
- Minocha, S. C., and W. Halperin. 1974. Hormones and metabolites which control tracheid differentiation, with or without concomitant effects on growth, in cultured tuber tissue of *Helianthus tuberosus* L. *Planta* 116:319–331.
- Murashige, T., and F. A. Skoog. 1962. A revised medium for rapid growth and bio-assays with tobacco tissue culture. *Physiologia Plantarum* 15:437–469.
- Pierik, R. L. M. 1987. In vitro culture of higher plants. Martinus Nijhoff, Boston. 747 pp.
- Russell, J. A. 1993. Advances in the protoplast culture of woody plants. Pages 67–87 in M. R. Ahuja, editor. Micropropagation of woody plants. Kluwer Academic Publishers, London.
- Salisbury, F. B., and C. W. Ross. 1978. Plant physiology. Wadsworth Publishing Company, Belmont. 747 pp.
- Sharp, W. R., and P. O. Larsen. 1977. Plant cell and tissue culture: current applications and potential. Pages 115–153 in W. R. Sharp, P. O. Larsen, E. F. Paddock, and V. Raghavan, editors. Plant cell and tissue culture. Ohio State University, Columbus.
- Sterling, C. 1950. Histogenesis in tobacco stem segments cultured in vitro. *American Journal of Botany* 37:464–470.
- Steward, F. C., M. O. Mapes, and K. Mears. 1958. Growth and organized development of cultured cells. II. Organization in cultures grown from freely suspended cells. *American Journal of Botany* 45:705–708.
- Torrey, J. G., and D. E. Fosket. 1970. Cell division in relation to cytodifferentiation in cultured pea root segments. *American Journal of Botany* 57: 1072–1080.
- Tuskan, G. A., W. A. Sargent, T. Rensema, and J. A. Walla. 1990. Influence of plant growth regulators, basal media and carbohydrate levels on the in vitro development of *Pinus ponderosa* (Dougl. ex Law.) cotyledon explants. *Plant Cell, Tissue and Organ Culture* 20:47–52.
- Wagley, L. M., H. J. Gladfelter, and G. C. Phillip. 1987. De novo shoot organogenesis of *Pinus elliottii* in vitro. II. Macro- and micro-photographic evidence of de novo regeneration. *Plant Cell Reports* 6:167–171.
- Washer, J., K. J. Reilly, and J. R. Barnett. 1977. Differentiation in *Pinus radiata* callus culture: the effect of nutrients. *New Zealand Journal of Forest Science* 7(3):321–328.
- Zar, J. H. 1984. Biostatistical analysis. Prentice-Hall, Englewood Cliffs, N.J. 718 pp.