THE FREQUENCY OF PLANT REGENERATION FROM NORWAY SPRUCE SOMATIC EMBRYOS

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ABSTRACT

Regeneration via somatic embryogenesis holds much promise as a system for mass propagation of genetically improved and/or engineered conifers. Most reports to date on conifer somatic embryogenesis have described initiation of embryogenic callus and in some cases have verified the developmental potential of the somatic embryos. There is little quantitative information on the frequency at which conifer somatic embryos undergo three critical stages of development: 1) the maturation of somatic proembryos to the cotyledonary stage, 2) germination or primary root development, and 3) plant survival (conversion) and growth in soil. This report describes methods for and success rates in stimulating development at each of these stages for somatic embryos derived from immature and mature seed embryos of Norway spruce. Results indicate that the somatic embryo maturation process remains the most difficult and least efficient. Germination frequencies as high as 82% have been achieved, and results concerning efficiency of plant survival in soil are presented. Approaches to improve the "rate-limiting" maturation step are also discussed.

Additional Keywords: Picea abies, somatic embryogenesis

INTRODUCTION

Somatic embryogenesis is the production of embryos vegetatively under test-tube or in vitro conditions. This process holds much promise as a clonal system for mass propagation of plants because the production of embryos is via mitotic division of vegetative cells, rather than the sexual fusion of gametic cells. Furthermore, the process is amenable Co automation. Lastly, because the somatic embryos produced in culture are analogs to seed embryos, it is possible to encapsulate them and form artificial seeds (Redenbaugh et al. 1986). Such somatic seed would interface well with existing nursery production systems in conifers.

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The first reproducible report of somatic embryogenesis in conifers was with Norway spruce (Hakman et al. 1985). Reports of somatic embryogenesis in European larch (Nagmani and Bonga 1985), Radiata pine (Smith et al. 1985), Sugar pine (Gupta and Durzan 1986), loblolly pine (Gupta and Durzan 1987), and white and black spruce (Hakman and Fowlke 1987, Nagmani et al. 1987) have followed recently. In order for somatic embryogenesis to be developed commercially, the process needs to be efficient and repeatable. There is little quantitative information on the frequency at which conifer somatic embryos undergo three stages of development: maturation of immature embryos, emergence and growth of the root ("germination"), and establishment and growth in soil (conversion). We report here results on efficiencies of these stages in establishing somatic embryo plants of Norway spruce. The embryogenic callus used in these studies was initiated from embryos of immature and mature (stored) seed. This is also the first demonstration of overwintering and subsequent spring growth flush of conifer somatic embryo plants.

MATERIALS AND METHODS

Initiation Embryogenic callus was initiated from immature embryos of an open pollinated Norway spruce tree located in Appleton, Wis. in July, 1985 as previously described (Becwar et al. 1987a). A modified MS basal medium (von Arnold and Erickson 1981) supplemented with 2,4-D (2 mg/1) and BA (1 mg/1), hereafter refered to as HM, was used. Embryogenic callus was also initiated from mature embryos of seed purchased from Quality Tree Seed Inc., Brewster, N.Y. The seeds were surface sterilized in 30% H202 for 45 min., rinsed 3 times, and imbibed overnight prior to excision of the embryos. Initiation from mature embryos was on HM and BLG (Amerson et al. 1985), a modified MS medium which replaces the NH4NO3 with 5 mM glutamine. The BLG was supplemented with either NAA or 2,4-D (2 mg/L) and BA (1 mg/L). All cultures were maintained at 23 $^{\circ}$C with 16 hr irradiance (15-50 uE m^{-2} s^{-1}) from cool-white fluorescent and incandescent lights.

Maturation and Germination To obtain somatic embryo development, calli were transferred to basal medium with activated charcoal (1%) lacking growth regulators for seven days, and then to basal medium with IBA and ABA (1 uM each) (Becwar et al. 1987a). In one experiment buthionine sulfoximine (BSO) was added to the above developmental medium to test its effect on maturation (Wann et al. 1986). Individual somatic embryos were removed from the calli when cotyledons appeared distinct and green and hypocotyl elongation had occurred. Maturation frequencies were determined based on the fraction of immature somatic embryos, counted after 21 days on the development medium (Becwar et al. 1987b), which developed to the cotyledonary stage. Three techniques were tested for somatic embryo germination on quarter strength HM medium: 1) radicle of embryo in medium (control), 2) radicle of embryo on medium surface (slant), and 3) cotyledons of embryo in medium with culture vessel inverted (hanging).

Conversion Two experiments were conducted on transferring somatic embryo plantlets, with primary roots of approximately 1 cm, to soil. In the first trial somatic plantlets and zygotic seedlings at an equivalent stage of development were transferred to a Jiffy soil mix in Magenta GA7-3 plastic vessels.

These somatic and zygotic plantlets were grown in the growth chamber as previously described for two months before transfer to the greenhouse. High relative humidity was maintained by controlling the tightness of the GA7-3 vessel lids. In the second trial somatic plantlets were transferred directly from _in vitro_ to greenhouse conditions in Spencer-Lemaire "book planters" containing soil (5:1, Jiffy soil mix:Perlite). High relative humidity was maintained by enclosing the book planter trays in clear plastic bags. All greenhouse plants were fertilized with osmocote (14-14-14) and grown under ambient light supplemented with fluorescent light (16 hr, 100 uE m^{2} s^{-1}) at 20-25°C. Plants which set resting terminal buds were transferred to lower temperatures (5-15°C) and ambient light for 7 weeks. Plants overwintered at -5 to 10°C. On March 1 plants were returned to ambient greenhouse conditions (20-25°C).

Data Analysis Data are presented as mean values among replicated experimental units (e.g., culture plates) and analyzed by ANOVA followed by Duncan's New Multiple Range Test for multiple comparisons of means. Mean values followed by unlike letter are significantly different (P = 0.05).

RESULTS AND DISCUSSION

Initiation Embryogenic callus lines were derived from individual embryo explants. Over 75% of the immature embryo explants collected in early July in Wisconsin initiated embryogenic callus (Becwar, et al. 1987a). Initiation of the desirable embryogenic callus on the HM basal medium dropped off rapidly with time, and by the end of July only a nonembryogenic callus was initiated.

We have been able to extend the window for initiation of embryogenic callus in Norway spruce to mature seed by utilizing a modified basal medium (Table 1). Specifically, by culturing mature embryos on half-strength BLG with either 2,4-D or NAA as auxins, from 21 to 27% of the explants initiated embryogenic callus and over half of the lines were successfully maintained for over four months. Although it was possible to initiate embryogenic callus at very low frequencies from mature embyros cultured on HM, none of these lines could be maintained (Table 1). Furthermore, the change from full strength HM to full strength BLG did not result in initiation from mature embyros. These results point out that levels of other components of the BLG medium, besides absence of ammoniacal nitrogen, are essential for extending the initiation window to mature embryos. We have verified that the somatic embryos initiated on 1/2 BLG develop to cotyledonary stage (Fig. 1A-C) and germinate (Fig. 1D).

Table 1. Effect of modifications in the medium on initiation and maintenance of embryogenic callus from mature embryos of Norway spruce.

Basal medium	Auxin/Cytokinin (mg/L)		Number of explants	Embryogenic callus (% explants)	
	2,4-D/BA	NAA/BA		4-6 weeks	4-5 months
HM	2/1	---	158	3	0
1/2HM	---	2/1	30	0	0
BLG	---	2/1	50	0	0
1/2BLG	2/1	---	40	21	11
1/2BLG	---	2/1	120	27	16

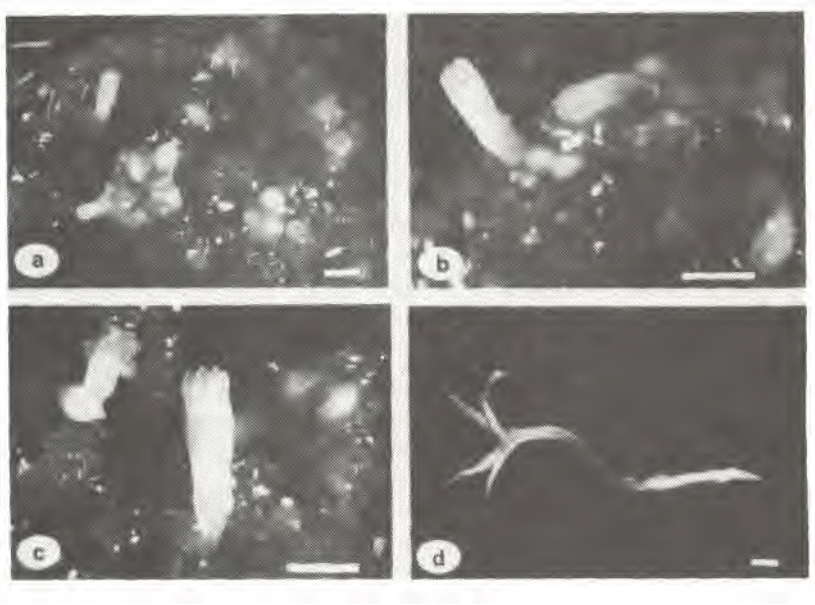

Figure 1. Plantlet regeneration from embryogenic callus initiated from mature embryos of Norway spruce. A: Individual embryos visible after 21 days on development medium. B & C: Maturation of somatic embyros, hypocotyl elongation and cotyledonary development. D: Germination, primary root growth. Scale bars = 1 mm.

Maturation The frequency at which developing somatic embryos reached the mature (cotyledonary) stage is summarized in Table 2. Note that there were differences in maturation frequencies among embryogenic callus lines. Line (NS1)5 had maturation frequencies of 1 to 4%, whereas lines (NS1)8 and (NS1)13 were 12 and 15%, respectively. Each embryo line was initiated from an individual immature embryo explant derived from a single tree. This suggests that selecting for optimum maturation frequency amoung numerous embryogenic lines may be an important step toward developing an efficient regeneration system. The results in Table 2 also illustrate that modifying the development protocol is another way to improve maturation frequencies. The addition of BSO to the development medium doubled the maturation frequency in line (NS1)8. BSO is an inhibitor of reducing agents such as phenolics (Wann et al. 1986).

Germination The results of an experiment designed to test the effects of three culture treatments on somatic embryo germination are summarized in Table 3. Two treatments (slant and hanging) that avoided immersion of the radicle of somatic embryos in agar are compared to the control treatment where radicles were placed directly in the agar solidified medium. The mean frequency of somatic embryo germination was approximately doubled in the slant and hanging treatments over the control, and germination of the hanging treatment was significanity higher than the control. Germination rates of somatic embryos were as high as 82% in both the slant and hanging treatments. More rapid root

growth occurred on the slant treatment than on the control or the hanging treatment.

Table 2. Maturation frequencies of Norway spruce somatic embryos.

Embryogenic callus line no.	Development protocol[1]	Somatic embryo immature density[2]	Somatic embryo maturation frequency
		(no./gr. callus)	(%)
(NS1)5	control	580	2.8
"	"	640	3.6
"	"	800	1.5
(NS1)13	control	150	16.0
(NS1)8	control	400 a	12.0 a
"	+ 10 µM BSO	330 a	25.0 b
"	+ 100 µM BSO	760 b	0.2 c

[1]Control development protocol as described in materials and methods.
[2]Mean of three counts on at least four replicate samples.

Table 3. Effect of three culture treatments on germination of Norway spruce somatic embryos.

Treatment[2]	N[3]	Somatic embryo[1] germination (%) mean	[range]	root length (mm) mean	[range]	Observation
control	60	27a	[0-46]	2.3a	[0.5-4.0]	excessive radicle callus
slant	58	45ab	[20-82]	4.9b	[1.0-8.5]	most vigorous roots
hanging	58	56b	[25-82]	2.0a	[1.0-5.5]	most numerous root hairs

[1]-Percentage of germination and primary root length measured at 21 days.
[2]Refer to materials and methods for description of treatments.
[3]N = number of somatic embryos per treatment.

Conversion A total of 31 somatic embryo plants were transferred to the greenhouse, and nine survived. The frequency of survival was higher when somatic plants were grown in soil in the growth chamber prior to transfer to the greenhouse (trial 1, refer to materials and methods), rather than direct transfer from in vitro to greenhouse conditions (trial [2]). All nine of the somatic plantlets and six of the seven zygotic seedlings set a terminal bud during September. All somatic and zygotic plantlets survived overwintering.

When the plantlets were returned to 20 to 25 °C, enlargement of the terminal buds occurred within 14 days on all somatic plantlets and six of the seven zygotic plantlets.

The growth flush of the terminal buds of the somatic plantlets occurred synchronously with the zygotic seedlings (Table 4 and Figure 2). Several plants began to flush on March 18 and within six days all somatic and six zygotic plants had flushed. The exception was one zygotic plantlet which had not set a resting bud until December (therefore it remained at warm temperatures for about 8 weeks longer than other plants) and did not obtain the same chilling period as the other somatic and zygotic plants. It flushed in mid-May.

Table 4. Initiation of vegetative growth flush from dormant terminal buds of somatic and zygotic plants.

Trial	Embryo origin	Number of plants overwintered	Number of plants initiating vegetative growth flush (3/18 to 3/24/87)	Mean plant height (cm) [range] (5/22/87)
1	somatic	3	3	9.7 a [6.5 - 15.5]
"	zygotic	7	6	13.3 a [8.0 - 19.0]
2	somatic	6	6	3.7 [1.5 - 6.0]

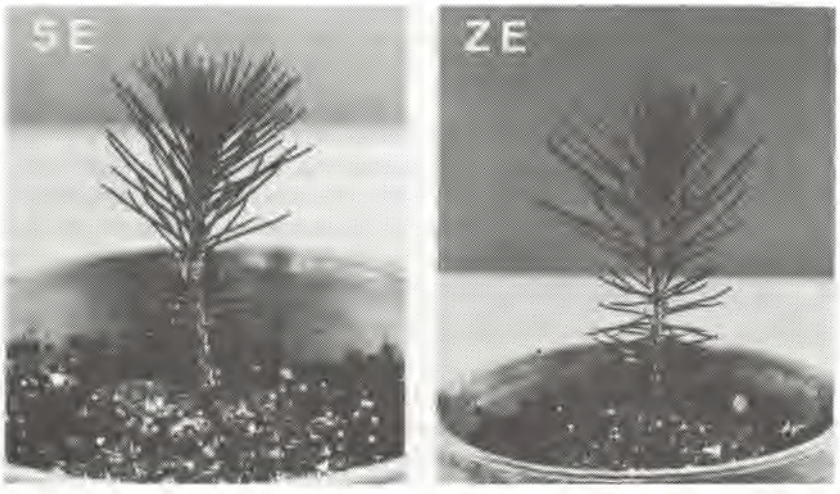

Figure 2. Initiation of secondary vegetative growth from somatic embryo plant (SE) synchronously with zygotic embryo plant (ZE). Photos taken 4/1/87, 14 days after bud break. Plant height, SE = 5.7 cm, ZE = 7.6 cm.

Statistically, there was no significant difference in the mean plant height between the somatic and zygotic plants in trial 1 (Table 4). Rigorous comparison of somatic and zygotic plants, however, is precluded for several reasons. The zygotic seedlings were not derived from the same tree as the somatic plants, and therefore difference could be genetic. Also, the sample size of three somatic plants in trial 1 is undesirably low. Our current efforts are aimed at producing large numbers of somatic plants via the optimum maturation, germination, and conversion techniques previously discussed, and at rigorous quantitative comparisons to zygotic plants derived from the same maternal tree.

In addition, direct comparisons between trial 1 and trial 2 plants are not valid. Trial 1 plants were chronologically older and 2-4 cm in height when transferred to the greenhouse, whereas trial 2 plants were about 1 cm in height when transferred and immediately set dormant terminal buds. Therefore, differences in plant height between trials are due to differences in experimental conditions and age.

CONCLUSIONS

We have utilized Norway spruce as a conifer model system for regeneration of plants via somatic embryogenesis. Our primary objective is to apply our results to conifers of commercial importance to the pulp and paper industry, namely, loblolly pine. Although we have initiated embryogenic callus in loblolly pine and several other commercially important conifers such as eastern white pine and white spruce (Wann et al. 1987, Nagmani et al. 1987, Becwar et al. 1987), we have not completed the regeneration process in these species. In the commercially important conifers the "bottle-neck" in regeneration appears to be the maturation process, similar to what we have reported here for Norway spruce. Therefore, understanding the biochemical basis for the reduced efficiency in spruce, and developing techniques for efficient conversion should be directly applicable to loblolly pine and other commercially important conifers.

Initiation from mature embryos of Norway spruce represents a first step toward utilizing mature explants, an aspect critical to development of "true-to-type" cloning in conifers. Taken collectively, our results on initiation from mature embryos and those reported by other workers (von Arnold and Hakman 1986) demonstrate that changes in and optimization of medium components can significantly effect initiation, and thus play a major role in extending the initiation window to more mature tissues.

The addition of BSO, an inhibitor of reducing agents, showed potential for significantly improving maturation frequency. Also the variation in efficiency among embryogenic callus lines derived from individual explants points out that there may be considerable genetic variation in maturation potential. Selecting for high maturation frequencies among callus lines coupled with refinements in the development protocol should result in considerable improvements.

Based on the highest mean efficiencies obtained for maturation (25%), germination (56%), and survival (conversion) of plants transferred to the greenhouse (29%), an overall efficiency of 4% was attained. Expressed relative to callus weight, about 8 grams of embryogenic callus of line (NS1)8 would be required to regenerate 100 plants. Although this efficiency is low and considerable improvement is needed for any commercial application, we are currently producing a sufficient number of somatic plants for detailed quantitative analysis of uniformity and growth characteristics.

Finally, an encouraging result in terms of the potential utility of embryogenesis in conifers is the complete regeneration of phenotypically *normal* plants from somatic embryos of Norway spruce. In terms of physiological response to changing environment, the somatic embryo plants have thus far responded (set dormant buds, overwintered, and initiated new vegetative growth) strikingly similar to control seedlings. To our knowledge this is the first demonstration of overwintering and initiation of new growth from dormant buds of conifer somatic embryo plants.

LITERATURE CITED

Amerson, H.V., Frampton, L.J., McKeand, S.E., Mott, R.L., and Weir, R.J. 1985. Loblolly pine tissue culture: laboratory, greenhouse, and field studies. In: Tissue Culture in Forestry and Agriculture. Eds., Henke, R.R., et al. Plenum Press, New York. pp. 271-287.

Becwar, M.R., Noland, T.L., and Wann, S.R. 1987a. Somatic embryo development and plant regeneration from embryogenic Norway spruce callus. Tappi J. 70(4):155-160.

Becwar, M.R., Noland, T.L. , and Wann, S.R. 1987b. A method for quantification of the level of somatic embryogenesis among Norway spruce callus lines. Plant Cell Reports 6:35-38.

Becwar, M.R., Wann, S.R., and Kriebel, H.B. 1987. Initiation of embryogenic callus in Pinus strobus (eastern white pine) from immature embyro explants (Abstract). Genetic Manipulation of Woody Plants, Conf. June 21-25. Mich. St. Univ., East Lansing, MI.

Hakman, I. and Fowke, L.C. 1987. Somatic embryogenesis in Picea glauca (white spruce) and P. mariana (black spruce). Can. J. Bot. 65:656-659.

Hakman, I., Fowke, L.C., von Arnold, S., and Eriksson, T. 1985. The development of somatic embryos in tissue cultures initiated from immature embryos of Picea abies (Norway spruce). Plant Sci. 38:53-59.

Gupta, P.K. and Durzan, D.J. 1986. Somatic polyembryogenesis from callus of mature sugar pine embryos. Bio/Tech. 4:643-645.

Gupta, P.K. and Durzan, D.J. 1987. Biotechnology of somatic polyembryogenesis and plantlet regeneration in loblolly pine. Bio/Tech. 5:147-151.

Nagmani, R. and Bonga, J.M. 1985. Embryogenesis in subcultured callus of Larix decidua. Can. J. For. Res. 15:1088-1091.

Nagmani, R., Becwar, M.R., and Wann, S.R. 1987. Single-cell origin and development of somatic embryos in Picea abies (Norway spruce) and P. glauca (white spruce). Plant Cell Reports 6:157-159.

Redenbaugh, K., Paasch, B.D., Nichol, J.W., Kossler, M.E., Viss, P.R., and Walker, K.A. 1986. Somatic seeds: Encapsulation of asexual plant embryos. Bio/Tech. 4:797-801.

Smith, D.R., Singh, A.P., and Wilton, L. 1985. Zygotic embyros of Pinus radita in vivo and in vitro (Abstract). Int. Conifer Tissue Culture Work Group. Abstracts. Ed. Smith, D.R., New Zealand Forest Service, Rotorua, New Zealand.

von Arnold, S. and Eriksson, T. 1981. In vitro studies of adventitious shoot formation in Pinus contorts. Can. J. Bot. 59:870-874.

von Arnold, S. and Hakman, I. 1986. Effect of sucrose on initiation of embryogenic callus from mature zygotic embryos of Picea abies (Norway spruce). J. Plant Physiol. 122:261-265.

Wann, S.R., Feirer, R.P., Johnson, M.A., and Noland, T.L. 1986. Norway spruce as a model system for somatic embryogenesis in conifers. Proc. TAPPI Res. and Devel. Conf., Forest Biotech. Raleigh, NC, Sept. 28-Oct. 1, TAPPI Press, Atlanta, GA. pp. 131-135.

Wann, S.R., Johnson, M.A., Feirer, R.P., Becwar, M.R., and, Nagmani, R. 1987. Biochemical differences between embryogenic an nonembryogenic callus of Conifers (Abstract). Genetic Manipulation of Woody Plants, Conf. June 21-25. Mich. St. Univ., East Lansing, MI.