

Initiation of embryogenic callus and suspension cultures of eastern white pine (*Pinus strobus* L.)*

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Received March 17, 1989/Revised version received May 4, 1989 – Communicated by G. C. Phillips

Abstract. Embryogenic callus and suspension cultures of eastern white pine (*Pinus strobus*) have been obtained. The whole female gametophyte was plated on a medium containing 50 mg/l glutamine, 500 mg/l casein hydrolysate, 3% sucrose, 2 mg/l 2,4-D, 1 mg/l BA and 0.2% Gelrite as a solidifying agent. Embryogenic calli could be seen as early as 5 days following culture. Histological studies indicate proliferation of pre-existing embryogenic tissue in the corrosion cavity followed by extrusion of embryogenic callus through the micropylar end of the gametophyte. Embryogenic suspension cultures were obtained by placing embryogenic callus into liquid medium. Embryogenic suspension cultures were subcultured weekly and proliferated as early-stage embryos with attached suspensors. Embryo development was obtained following transfer of the embryogenic tissue to an auxin-free medium containing 50 mM glutamine, 38 μ M abscisic acid, and 6% sucrose. Although embryo development could be consistently obtained, whole plants have not yet been recovered from these somatic embryos.

Abbreviations: 2,4-D = 2,4-Dichlorophenoxyacetic acid, ABA = Abscisic acid, BA = 6-Benzyladenine

Introduction

Over the past few years, much progress has been made in the area of somatic embryogenesis in conifers. Somatic embryogenesis and plant regeneration have been reported for loblolly pine (*Pinus taeda* L.) (Gupta and Durzan, 1987), sugar pine (*Pinus lambertiana* Lamb.) (Gupta and Durzan, 1986), Douglas-fir (*Pseudotsuga menziesii*) (Durzan and Gupta, 1987), Norway spruce (*Picea abies* (L.) Karst) (Hakman and von Arnold, 1985), and both black spruce (*Picea mariana* (Mill.) BSP.) and white spruce (*Picea glauca* (Moench) Voss.) (Hakman and Fowke, 1987a). The explant source in all except one of these reports was the excised, immature or mature zygotic embryo. In one study, Gupta and Durzan (1987) reported use of the whole female gametophyte with attached suspensors and embryos for initiation of embryogenic callus (embryonal-suspensor masses) of loblolly pine. In that study, embryonal suspensor-masses formed in 9 to 10% of the total explants.

The origin of proliferative embryogenic tissue in conifers varies with explant tissue and plant type. Although histological studies on the initiation process have not been performed, the source of embryogenic tissue has been reported to be suspensor tissue in sugar pine (Gupta and Durzan, 1986) and epidermal or subepidermal tissue of the hypocotyl in spruce (Nagmani *et al.*, 1987). Although plant

regeneration from somatic embryos of some conifers can be consistently obtained, the efficiency of somatic embryo initiation and development is often very low, especially for pines. This paper describes a system for high frequency initiation of embryogenic callus in eastern white pine (*Pinus strobus*) and the source of that proliferative embryogenic tissue. In addition, development of an embryogenic suspension culture of eastern white pine is described.

Materials and methods

Initiation and Subculture of Embryogenic Callus: Seed cones of eastern white pine (*Pinus strobus* L.) were collected weekly for 6 weeks starting in June of 1988 and continuing to August of 1988. Trees were located near Wooster, Ohio and cones were collected from different eastern white pine trees. The cones were stored in paper bags at 4°C for a maximum of three months prior to explanting. Immature seeds were removed from the cones and surface sterilized for 20 minutes in a 15% commercial bleach solution (0.8% sodium hypochlorite) containing 0.5% Tween-20. After 3 rinses with sterile, distilled water, gametophytes and embryos were excised for culture. The gametophyte refers to the female gametophyte with the intact immature zygotic embryo(s) and suspensor tissue. Small embryos, which were prone to desiccation, were kept moist by performing manipulations in small amounts of liquid medium. Twenty-five explants were placed into each of three 100 x 25 mm Petri dishes containing 35 ml of DCR-m medium which consisted of DCR basal medium (Gupta and Durzan, 1985) with 50 mg/l glutamine, 500 mg/l casein hydrolysate, 3% sucrose, 2 mg/l 2,4-D, 1 mg/l BA, and 0.2% Gelrite as a solidifying agent. All medium treatments were performed in triplicate and explants were evaluated for production of embryogenic callus weekly for 8 weeks. Cultures were initiated and maintained at 23°C in the dark. Once embryogenic callus cultures were established, cultures were subcultured biweekly.

Histology: Immature gametophytes from 2 collection dates (July 5 and 11, 1988) were placed on DCR-m medium as described above and removed following 0, 2, 3, 4, 7, 9, and 11 days of culture for histological studies. For each time point and collection date, 25 gametophytes were randomly selected. The gametophytes were fixed in a Formalin-acetic acid-alcohol solution (Berlyn and Miksche, 1976), dehydrated in a tertiary-butyl alcohol/ethanol series and embedded in paraffin according to Johansen (1940). Paraffin sections were cut 13 to 40 μ m thick and stained using Johansen's (1940) quadruple stain.

* Salaries and research support were provided by State and Federal funds appropriated to OSU/OARDC. Journal Article No. 62–89
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Embryogenic Suspension Culture: For initiation of embryogenic suspension cultures, 10 to 100 mg of embryogenic tissue was placed into 35 ml of liquid DCR-m in a 125 ml deLong flask. Suspension cultures were agitated at 150 rpm and maintained at 23°C in the dark. Liquid cultures were subcultured every 3 to 4 days to biweekly depending on the quantity of tissue used for subculture. For subculture, 35 ml of fresh medium was added to the flask to be subcultured (total volume now 70 ml). The flask was swirled and 35 ml of the diluted suspension was removed with a pipet and placed in a new flask. If a low inoculum of tissue was used, then subculture was biweekly. Higher inoculum subculture required a 3 to 4 day subculture.

Embryo Development: Embryo development was obtained following transfer of the tissue first to liquid and then solid auxin-free media. For early development in liquid media, cells were first allowed to settle for 5 min in deLong flasks and the old supernatant medium was removed and discarded. The cells were washed 2 times with auxin-free DCR-m medium containing 50 mM glutamine, 38 μ M ABA, and 12% sucrose and resuspended in this auxin-free medium. Developing embryos were washed in this liquid development medium weekly for 2 weeks before plating on solid medium.

For further embryo development on solidified media, developing embryos and embryogenic tissues obtained as described were utilized. Clumps of developing embryos (1 to 4 mm) were placed on auxin-free Gelrite-solidified (0.2%) DCR-m media containing all combination of 1, 3, 6, and 12% sucrose; 0, 15, and 50 mM glutamine; and 0, 19 and 38 μ M ABA. Twenty-five clumps of embryogenic tissue were placed in each of three Petri dishes at 23°C in the dark. One month after transfer to the solid development medium, the number of developing embryos on each clump of embryogenic tissue was recorded.

Results and Discussion

Initiation of Embryogenic Callus: The gametophyte was clearly the superior explant source for initiation of embryogenic tissue in eastern white pine (Table I). An average of 54% (in one case, 72%) of the gametophytes formed embryogenic callus. For this reason, the gametophyte was used as the explant for all subsequent experiments. In an earlier study on initiation of eastern white pine embryogenic callus, Becwar *et al.* (1988) obtained a maximum of 2.7% of excised eastern white pine embryos that formed embryogenic callus. It was reported that only precotyledonary embryos formed embryogenic callus and the origin of the callus was the suspensor region (Becwar *et al.*, 1988). Although the gametophyte was used as the explant source for initiation of embryogenic tissue in loblolly pine (Gupta and Durzan, 1987), a comparison between gametophytes and excised embryos as explant sources was never made. In that study, 9 to 10% of the gametophytes formed embryogenic callus.

Table I Percent of eastern white pine explants forming embryogenic callus after 8 weeks of culture. Cones for this experiment were collected on July 11, 1988.

Explant	Percent explants forming embryogenic callus \pm SE
Excised zygotic embryo	3.0 \pm 3.3
Whole gametophyte	54.0 \pm 10.8
Corrected gametophyte*	63.8 \pm 15.0

*value is percent of gametophytes forming embryogenic callus divided by the percent gametophytes containing at least one nonaborted embryo.

Zygotic embryos in gametophytes from the July 11 collection date ranged from 0.1 to 0.3 mm in length and cotyledon development was apparent in less than 50% of the embryos. Ten to twenty percent of the gametophytes did not contain visible embryos and were considered to be aborted. Since some of the gametophytes did not contain embryos, a corrected value for the gametophyte based on the presence of at least one nonaborted embryo is presented in Table I. This corrected gametophyte value may be used to better compare the values obtained for gametophyte versus excised embryos. The maximum frequency of 72% reported earlier translates to a corrected gametophyte value of 89%.

Embryos which were excised for culture from cones from later collection dates (July 18, July 25, August 1, 1988) produced embryogenic callus at a frequency of less than 1%. Embryos from the July 11, 1988 collection date were less than 0.3 mm in length and therefore very difficult to excise. The advantages of using the whole gametophyte for establishment of embryogenic cultures of eastern white pine are two-fold. First, the frequency of embryogenic callus initiation is much higher than that obtained with the excised embryo (Table 1) and second, it is simply much easier to culture the gametophyte and not attempt dissection of a very small embryo.

Embryogenic callus was observed protruding from the micropylar end of the gametophyte as early as 5 days following culture. The embryogenic callus was mucilaginous and translucent (Fig. 1) as is typical of embryogenic tissue of other conifers (Hakman and von Arnold, 1985). Stage 2 somatic embryos (Hakman and Fowke, 1988) were only rarely observed on the embryogenic callus. Numerous embryo initials, which consisted of 4 to 20 cells and were attached to elongated suspensor cells, were observed protruding from the callus. Approximately two-thirds of the embryogenic calli proliferated following removal from the gametophyte. The other one-third may represent suspensors and zygotic embryos which were simply extruded through the gametophyte.

The influence of embryo age or state of maturation on initiation of embryogenic callus is shown in Table II. Gametophytes which were taken from cones that were harvested on the earliest collection date gave the lowest percent of embryogenic callus. We estimate on the basis of previous research (Kriebel, 1972) that fertilization date for these cones was between June 16 and June 24, 1988. The highest percentage of initiation of embryogenic callus was obtained from the cones with the more mature (but still young) gametophytes that contained mostly precotyledonary zygotic embryos.

Table II Effect of embryo age on initiation of embryogenic callus from whole gametophytes of eastern white pine.

Cone Collection Date*	Percentage gametophytes forming embryogenic callus
June 20, 1988	6.0 \pm 2.0 (\pm SE)
June 27, 1988	32.0 \pm 5.7
July 5, 1988	48.0 \pm 2.8
July 11, 1988	54.0 \pm 10.8

*Estimated fertilization date = June 20 \pm 4 days.

Histology: Determination of the origin of somatic embryogenic tissue in eastern white pine gametophytes was difficult due to the large number of zygotic embryos present in the gametophyte before culture. In one case, 8 small zygotic embryos were observed in a single fertilized female gametophyte (Figs. 2 and 3 show 2 different sections of the same gametophyte containing 8 small zygotic embryos). It is not unusual in pine to have a large number of zygotic embryos although usually only one develops

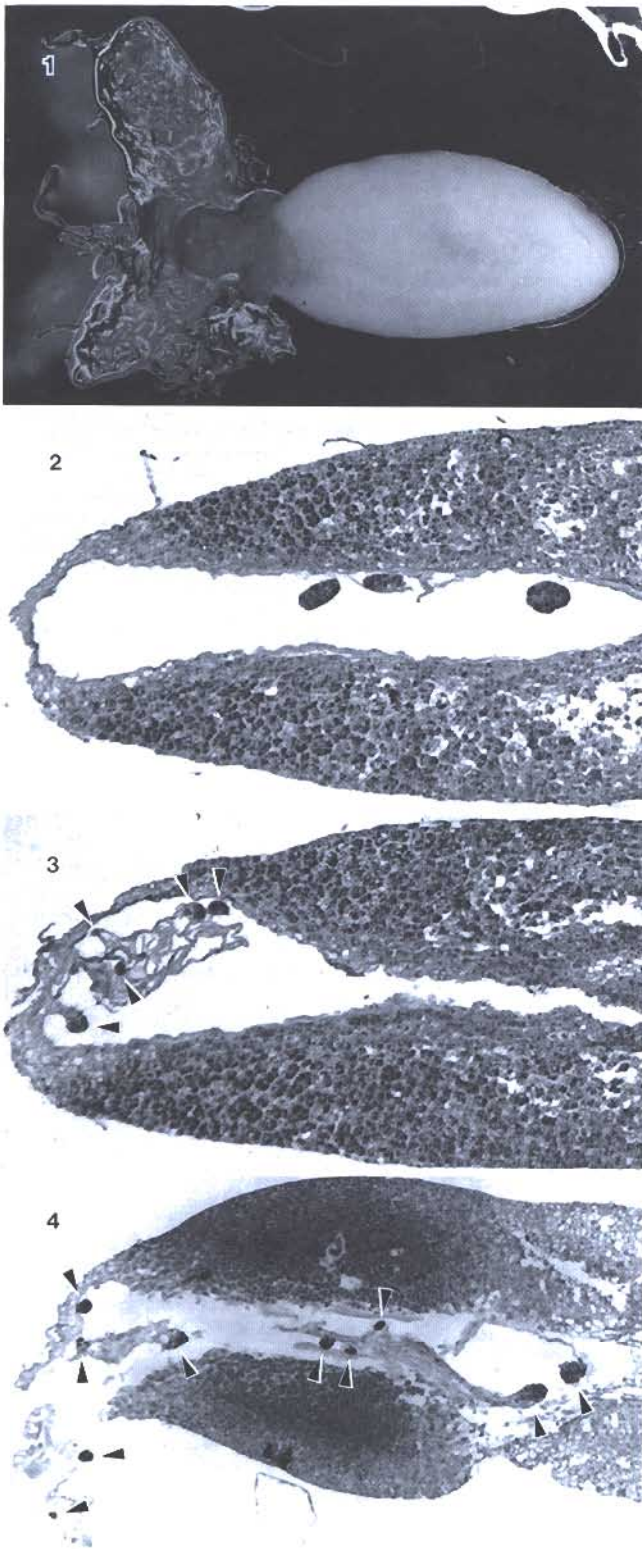


Fig. 1 Production of embryonic callus through micropylar end of female gametophyte (X 7.2). Fig. 2 Section through female gametophyte before culture showing 3 zygotic embryos (X 37). Fig. 3 Different section through gametophyte shown in Fig. 2 showing presence of 5 additional zygotic embryos (arrows) (X 38). Fig. 4 Section of gametophyte placed in culture for 9 days showing extrusion of embryonic callus from the micropylar end of the female gametophyte. Ten embryos (arrows) can be seen in this section (X 19).

to maturity (Buchholz, 1918). Cleavage polyembryony can result in as many as 48 zygotic embryos in fertilized immature seed of pine (Buchholz, 1918).

The staining properties of zygotic and somatic embryos were similar using the quadruple staining procedure of Johansen (1940). It was not possible to distinguish between the small zygotic embryos and proliferating somatic embryos in the gametophyte based on this histological study. Figure 4 shows a section of a gametophyte which had been placed in culture for 9 days. Embryogenic callus can be seen protruding from the micropylar end of this gametophyte and 10 embryos can be observed in this single section. The first somatic embryos in eastern white pine may simply result from proliferation of smaller zygotic embryos, which would otherwise abort during development under natural conditions. The low percentage initiation of embryogenic callus resulting from use of single excised zygotic embryos (Table I) may be a result of discarding the smaller zygotic embryos. It was not possible to separately culture the smaller abortive embryos and the large surviving zygotic embryo.

Embryogenic Suspension Cultures: Embryogenic suspension cultures of eastern white pine were not difficult to initiate and maintain in DCR-m medium. Due to the presence of long suspensor cells (Fig. 5) on the embryonic tissue, tissue in suspension culture appeared almost fibrous in nature. The embryo proper consisted of a small cluster of densely cytoplasmic cells that appeared yellow-brown when viewed on an inverted microscope. The suspensors were highly elongated and vacuolated. As reported in other studies (Hakman and Fowke, 1987b), the suspensors were attached to small embryos and were observed to occur as single strands or in groups of parallel strands.

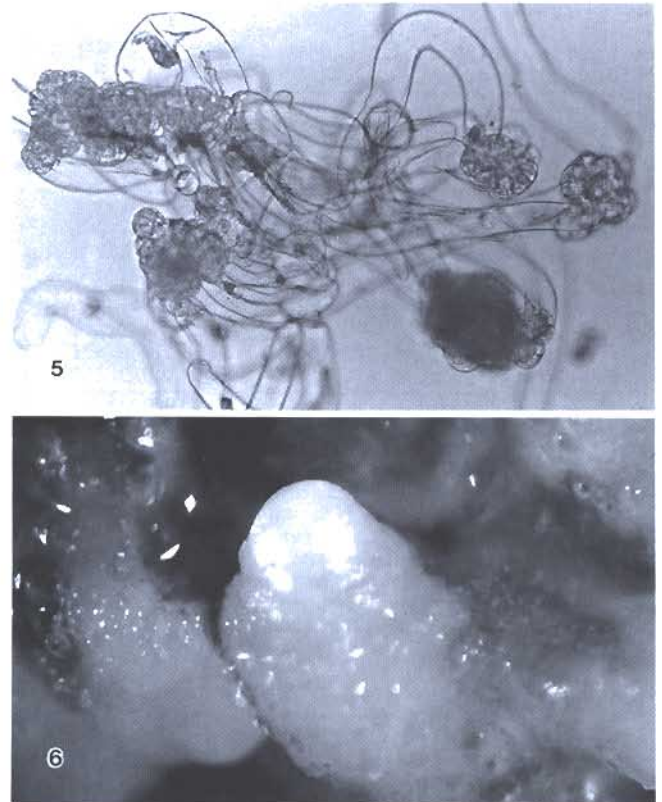


Fig. 5 Embryonic suspension culture tissue of eastern white pine (X 66). Fig. 6 Stage 2 somatic embryo on solid medium (X 34).

Liquid cultures have been maintained for 6 months using low density subculture. As little as one clump (~2 mg fresh weight) of embryogenic tissue could be used for subculture. Low inoculum subculture has been used successfully for maintenance of embryogenic suspension cultures of cotton (Finer, 1988), soybean (Finer and Nagasawa, 1988), and Chinese yam (Nagasawa and Finer, 1989). If subculture was delayed or the density of the cells became greater than 0.5 g fresh weight/35 ml medium, the quality of the embryogenic suspension culture rapidly declined. The necessity of frequent subculture of embryogenic tissue of pine has previously been reported (Gupta and Durzan, 1987).

Somatic Embryo Development: Following transfer of embryogenic suspension culture tissue to a liquid auxin-free DCR-m medium containing 50 mM glutamine, 38 μ M ABA, and 12% sucrose, the embryos underwent early development. Clumps of stage 1 embryos (Hakman and von Arnold, 1988), that were easy to transfer with a forceps, were formed in 2 weeks. These clumps of early-staged developing embryos were used as a uniform inoculum for development studies.

In all cases, clumps of embryogenic tissue that were plated on solidified media lacking glutamine proliferated as embryogenic callus. This embryogenic callus contained no stage 2 embryos. Stage 2 embryos (Fig. 6) were formed following the addition of 50 mM glutamine, 6% sucrose, and 38 μ M ABA (Table III). These embryos were light-yellow to cream colored and were supported by suspensor tissue.

Table III Number of eastern white pine somatic embryos formed per plate after 30 days. (\pm SE)

Sucrose and glutamine concentrations	ABA concentration (μ M)		
	0	19	38
1% Sucrose			
0 mM glutamine	0	0	0
15 mM glutamine	0.7 \pm 0.9	2.7 \pm 1.2	0.3 \pm 0.3
50 mM glutamine	1.0 \pm 0.8	4.3 \pm 4.0	2.3 \pm 2.6
3% Sucrose			
0 mM glutamine	0	0	0
15 mM glutamine	1.3 \pm 1.2	0.3 \pm 0.5	0.3 \pm 0.3
50 mM glutamine	1.3 \pm 1.2	5.7 \pm 3.1	10.3 \pm 7.4
6% Sucrose			
0 mM glutamine	0	0	0
15 mM glutamine	1.0 \pm 1.4	0.7 \pm 0.9	1.7 \pm 2.4
50 mM glutamine	4.7 \pm 1.7	10.3 \pm 3.8	23.3 \pm 4.2
12% Sucrose			
0 mM glutamine	0	0	0
15 mM glutamine	1.0 \pm 1.4	4.0 \pm 2.2	6.3 \pm 8.3
50 mM glutamine	1.5 \pm 0.5	4.3 \pm 4.0	13.3 \pm 5.4

In treatments where embryo development was not observed, proliferation of embryogenic callus was usually apparent. Media containing 12% sucrose and 0 or 19 μ M ABA at all glutamine levels were the exceptions. On these media, the embryogenic tissue turned bright yellow but growth was minimal. Growth and development were also retarded on media containing 150 mM glutamine (data not shown). The best medium for embryo development to stage 2 contained 50 mM glutamine, 6% sucrose, and 38 μ M ABA.

Although the effects of glutamine on conifer somatic embryo development have not been previously quantified, glutamine, at lower concentrations, has been used successfully for development of sugar pine (~1.4 mM) (Gupta and Durzan, 1986) and loblolly pine (~3.1 mM) (Gupta and Durzan, 1987) somatic embryos. Higher levels of glutamine have also been reported to be beneficial for somatic embryo development in alfalfa (30 mM) (Stewart and Strickland, 1984) and cotton (15 mM) (Finer, 1988).

Abscisic acid at 38 μ M promoted embryo development to stage 2 in eastern white pine. Enhancement of conifer somatic embryo development by ABA addition has previously been reported for white spruce (Hakman and von Arnold, 1988), Norway spruce (Becwar *et al.*, 1987; Boulay *et al.*, 1988; von Arnold and Hakman, 1988) and Douglas-fir (Durzan and Gupta, 1987). In eastern white pine, this promotion was enhanced by raising the sucrose concentration from 3% to 6% (Table III). Hakman and von Arnold (1988) also reported interactions between ABA and sucrose in white spruce embryo development and subsequent germination from embryogenic suspension culture material. Lu and Thorpe (1987) showed by sorbitol substitution that the sucrose enhancement of embryo development in their white spruce system was an osmotic effect. They reported no improvement of somatic embryo development by addition of ABA.

This paper reports on the efficient production of embryogenic callus and suspension cultures of eastern white pine (*P. strobus*). Embryos obtained using the procedure described in this paper have recently developed to stage 3 following transfer to a growth regulator-free medium. Efforts are underway to induce these embryos to germinate and form whole plants.

Acknowledgements. The authors wish to acknowledge Raymond Saxton and Judith Smith for their technical assistance.

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