STERILIZATION AND GERMINATION PROCESSES FOR IMPROVING MICROPROPAGATION EFFICIENCY OF THREE SOUTHERN PINES

A.M. Diner[1]

Abstract. In *vitro* adventitious bud initiation from newly germinated seedlings of loblolly, slash, and longleaf pine was studied to compare the effects of varying decontamination procedures and germination media. Seeds of loblolly and slash pines were either cold-stratified 1 month in moist peat moss, or immersed several days in 1% hydrogen peroxide at 29°C with seed coat micropyles perforated. Stratified, rinsed seeds were decontaminated in 30% hydrogen peroxide, then germinated on water-agar. Longleaf pine seed was germinated either by several days' seed immersion in 1% hydrogen peroxide, or by incubation of 30% hydrogen peroxide-sterilized seed on water-agar. Seedlings were germinated on agar at 20°, 25°, and 29°C. All aseptic seedlings of the three species were incubated intact on adventitious bud initiation medium for 2 days. Seedlings were then cut to cotyledons and hypocotyls for 12 days' additional incubation on initiation medium.

Decontamination procedures were almost completely effective. Growth of endogenous microbial contaminants was infrequent. Hydrogen peroxide-germinated seedlings were small, chlorotic and poorly developed. Micropropagation efficiency of each species was significantly higher using seedlings germinated on water-agar than in hydrogen peroxide.

Keywords: *Pinus taeda*, *Pinus elliotti*, *Pinus palustris*, tissue culture, in *vitro*

INTRODUCTION

Efficient in *vitro* micropropagation of conifers is hampered by several factors, possibly the most common of which is failure or inability to decontaminate the starting tissues completely. Numerous chemicals and processes have been employed for surface-sterilization of seed coats or decoated seedlings at least partially enclosed by gametophyte. Most of these processes include use of aqueous hypochlorite at solute concentrations ranging from 7.5% weight per volume (w/v) (von Arnold and Eriksson 1985) to 0.3% (Cheng and Voqui 1977). Though micropropagation success has been reported for numerous conifer species (Newton et al. 1989), decontamination efficiency relative to either pregerminated or germinated seeds has not been addressed, in spite of its pertinence to the costly, labor-intensive sequence of steps necessary to micropropagation, or to the limited availability of select seed lots.

[1] Plant physiologist, USDA Forest Service, Southern Forest Experiment Station, Alabama A&M University, Normal, AL 35762.

Seed dormancy in some species imposes an additional obstacle to immediate and direct use of seeds for micropropagation. While dormancy may be overcome by stratification, the use of a hydrogen peroxide seed soak has come into frequent use for rapid, high-frequency, relatively synchronous germination (Mott and Amerson 1981, Diner and Mott 1982, Newton et al. 1989). However, in preliminary tests at the same temperatures, hydrogen peroxide-germinated seedlings of loblolly (Pinus taeda L.), and slash (P. elliotti Engelm. var. elliotti) pines were chlorotic, smaller, and less well developed than those that had been first stratified, then germinated aseptically on water-agar.

The objectives of this study were (1) to develop protocols for decontamination of loblolly, slash, and longleaf (P. palustris L.) seed and germinated seedlings, and (2) to compare the micropropagation efficiency (ramets/genotype) of tissues from seedlings either germinated in hydrogen peroxide or stratified and then germinated on agar.

METHODS

Seed used in this study was obtained from International Forest Seed Co., Odenville, Alabama, and included loblolly pine seedlot RS22032 from Livingston Parish, Louisiana, slash pine seedlot IF BP-5 from south Georgia, and longleaf pine seedlot BC 12003 from Bay County, Florida. For germination of cold-stratified loblolly and slash pine, seeds were first bagged in cheesecloth and immersed in 4°C water for 24 hr. The water was then replaced with water-saturated peat moss, in which the bagged seeds were maintained at 4°C for 1 mo. Rinsed, stratified seeds were immersed 30 min in 30% aqueous hydrogen peroxide, rinsed three times in sterile distilled water, and placed on 1.6% w/v water-agar in petri dishes. Dishes with seeds were sealed with Parafilm and incubated under laboratory ambient illumination (50uE /m^2/sec) at 20°, 25°, or 29°C until germination terminated. Germinated seeds were decoated. Seedlings enclosed in gametophytes were agitated 15 to 20 min in approximately 100 ml 50% aqueous household bleach (5.25% hypochlorite) with a drop of Tween 20, then rinsed three times in sterile distilled water.

For germination of loblolly and slash pine in hydrogen peroxide, seed micropyles were perforated and seeds were immersed in 1% aqueous hydrogen peroxide at 29°C. The solution was replaced daily for 5 to 6 days. Germinated seeds were decoated and surface-sterilized in dilute bleach as described above.

Longleaf pine seeds were germinated either by immersion in 1% hydrogen peroxide as described above, or by placing decontaminated seeds on water-agar for several days. Decontamination was effected either by two successive 30-min soaks in 30% hydrogen peroxide separated by 5 to 6 hrs at room temperature, or by 45 to 60 min agitation in 50 to 100% household bleach to which 1 drop of Tween 20 per 100 ml disinfectant had been added. Rinsed seeds were then germinated at 20°, 25°, or 29°C on 1.6% water-agar in petri dishes as described above for loblolly and slash pines. Adventitious bud frequency differences by seed germination treatment and by species were tested using Chi-square.

In preliminary experiments, the asynchronous germination of all three species invariably led to seedlings of different ages in the germination medium, regardless of the method used. However, newly germinated seedlings of longleaf pine showed very early reduction in sensitivity to cytokinin, similar to that reported for P. radiata D. Don (Aitken-Christie et al. 1985). Therefore,

seedlings with roots extruded no more than 3 mm for all three species were used in this study. Seedlings were aseptically extracted from gametophytes of the three species and applied flat to 0.8% agar-solidified Brown and Lawrence medium (Brown and Lawrence 1968) modified to contain 1.46 gram per liter (g/l) glutamine as the sole source of amino nitrogen (BLG medium) and supplemented with 10 milligrams per liter (mg/1) 6-benzyladenine (BA) in petri dishes. Dishes sealed with Parafilm were incubated 2 days at 25°C under ambient laboratory illumination for growth and detection of any microbial contamination. Cotyledons and hypocotyls of aseptic seedlings were separated, and placed with hypocotyl apices inverted on medium of the same composition for an additional 12 days. Green, swollen tissues were then transferred to a Gresshoff and Doy half-strength (GD/2) medium (Mehra-Palta et al. 1978) with 10 g/1 each of sucrose and activated charcoal added. Cotyledons and hypocotyls of any genotype showing collective failure to enlarge on the BA-supplemented medium, as well as those developing an overall reddening or browning, were discarded. Subsequent biweekly tissue transfers were to Litvay's medium (Litvay et al. 1981) with 1.46 g/1 glutamine (LMG) and 10 g/1 sucrose. The pH of all media was adjusted to 5.5 before autoclaving. Water-agar and all solid media were gelled with 8.0 g/1 agar (Sigma Chemical Co., St. Louis, MO). Standard incubation conditions were 20°C, and 16-hr days of 250 uE/m^2/sec cool-white illumination for all stages of in vitro micropropagation following the above-described initial 2 days' treatment of intact seedlings at 25°C. Adventitious bud numbers were determined between 8 and 12 weeks from seed excision. Genotypes discarded prior to transfer to GD/2 medium were not included in the data.

RESULTS AND DISCUSSION

Seed of all three species germinated faster (3 to 5 days) and more synchronously at 29°C either in hydrogen peroxide or on water-agar than they did on water-agar at the lower temperatures of 25°C (4 to 9 days) or 20°C (5 to 14 days). Although endogenous contaminant growth was infrequent to negligible, the large amounts of condensation that accumulated on the inner suface of petri dish lids at 29°C tended to drip and spread any incidental contaminant growth. The moderate amount of condensation that accumulated at 25°C did not preclude selection of that temperature for further studies.

It should be mentioned that preliminary experiments to decontaminate seedlings had followed the suggestions of other workers (Crump et al. 1989), that seedlings extracted from their gametophytes be immersed for periods as short as 1 min in very dilute disinfectants. These included 0.01% household bleach or 0.01% aqueous sodium-o-phenylphenate tetrahydrate with 1 drop of Tween 20 per 100 ml disinfectant, or 0.001% Physan 20 (Maryl Products, Tustin, CA). These treatments produced no immediately noticeable tissue damage, but resulted in inevitable necrosis of at least the abaxial surface of cotyledons. Thus, cotyledons intact to their respective hypocotyls began to reflex rearward within 2 days on BLG medium. Cotyledons so affected commonly became tightly coiled during subsequent days when individually placed on BLG medium. Apparently, tissues at other surfaces of the cotyledons, protected in the closed needle cluster during disinfection, remained viable and thus responsive to the cell-stimulatory effects of BA, resulting in expansion of only those tissues.

Seeds of all three species germinated in 1% hydrogen peroxide were consistently chlorotic and less well developed than those germinated on water-agar at the same temperature and for similar periods of time. Tissues were soft and spongy and difficult to manipulate without damage manifested later by **necrotic zones.** Longleaf pine seedlings expressed these characteristics to a greater degree than did seedlings of either loblolly or slash pine. Longleaf pine hypocotyls showed little elongation in 1% hydrogen peroxide; roots remained extremely short though seeds were allowed to remain in the solution for several days under germination conditions. Germination of the three species on water-agar ultimately resulted in an overall average of 28.1 adventitious buds, compared with 11.5 for germination in hydrogen peroxide. These differences were highly significant ($p<0.01$) with a Chi-square value of 7.5. The average number of ramets per clone for each species was 21.0 for loblolly, 14.1 for slash, and 20.5 for longleaf pine. The species differences were not significant ($p>0.05$). Germination on water-agar more than doubled the final clone sizes in all three species relative to germination in hydrogen peroxide, as shown in Table 1. Maximum adventitious bud frequencies from hydrogen peroxide-germinated loblolly and slash pine were 111 and 46, respectively; those from stratified seedlings were 132 and 71, respectively. Hydrogen peroxide-germinated longleaf pine generated a maximum of 28 buds, while tissues of seedlings germinated on water-agar showed 102.

Table 1. Micropropagation efficiency in loblolly, slash and longleaf pine seedlings germinated in 1% hydrogen peroxide versus an agar substrate.

	Micropropagation Efficiency		
	Species		
	loblolly[1]	slash	longleaf
	x /n[2] Max.	x/n Max.	x/n Max.
Germination Medium:			
1% H_2O_2	13.6/67 111	9.0/35 46	11.0/40 28
Water-Agar	30.0/55 132	21.0/35 63	33.0/31 102

[1] Average ramets/genotype
[2] Number of genotypes tested

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