In Vitro Colonization of Micropropagated *Pieris floribunda* by Ericoid Mycorrhizae. II. Effects on Acclimatization and Growth

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Abstract. Inoculation of microshoots of *Pieris floribunda* (Pursh ex Sims) Benth. and Hook. (mountain andromeda) with isolates of *Hymenoscyphus ericae* (Read) Korf and Kernan ericoid mycorrhizal fungi stimulated growth during 1 month in vitro. However, no benefits were apparent after 3 months in a greenhouse. Acclimatization of plantlets of *P. floribunda* to greenhouse conditions following in vitro inoculation improved survival (42% vs. 16% for controls). The protocol reported herein is similar to procedures utilized currently for micropropagation of various ericaceous species and has potential to improve plantlet survival during acclimatization.

Pieris floribunda is an ericaceous, ever-green shrub native to the southeastern United States and has several desirable landscape and cultural characteristics. Unfortunately, supplies are often limited because conventional propagation by sexual means is unreliable and vegetative propagation by stem cuttings results in poor rooting (Dirr and Heuser, 1987). A protocol was developed for successful micropropagation of *P. floribunda*, but greenhouse acclimatization of rooted

microshoots was <38% (Starrett et al., 1993).

Often the greatest losses during micropropagation occur during plantlet acclimatization to greenhouse conditions (Preece and Sutter, 1991). Losses of 20% to 40% have been reported for plantlets of *Rhododendron* L. (rhododendron) during acclimatization (Anderson, 1978). Typically, 10% of micropropagated plants in the Ericaceae either die or do not attain market standards during acclimatization, causing significant commercial losses (Lemoine et al., 1992). Plantlets produced in vitro also lack mycorrhizal associations and often experience transplant problems when exposed to the natural environment (Ravolanirina et al., 1988). In vitro inoculation with an arbuscular mycorrhizal fungus reduced losses during acclimatization of micropropagated plants of *Persea americana* Mill. (avocado) (Vidal et al., 1992). Unfortunately, inoculation in vitro with ericoid mycorrhizal fungi does not always improve acclimatization of ericaceous species.

Plantlet survival and subsequent root growth of rooted microcuttings of *Rhododendron minus* var. *chapmanii* (A. Gray) Duncan & Pullen [syn. *Rhododendron chapmanii* A. Gray (Chapman's rhododendron)] were improved after ex vitro inoculation with *Hymenoscyphus ericae* (Read) Korf and Kernan [syn. *Peizella ericae* (Read)] (Barnes and Johnson, 1986). Inoculation with isolates of *H. ericae* of seedlings of *Vaccinium corymbosum* L. (highbush blueberry) (Starrett et al., 1995) and *Calluna vulgaris* (L.) Hull (heather) (Berta and Gianinazzi-Pearson,

1986), increased growth when plants were cultured in vitro. In contrast, growth was not stimulated by ex vitro inoculation of plantlets of *Vaccinium angustifolium* Ait. [lowbush blueberry, (Smagula and Litten, 1989)] with *H. ericae*. Similarly, no positive effects were noted following in vitro inoculation of micropropagated shoots of *V. corymbosum* with ericoid mycorrhizal fungi (Lareau, 1985).

There are no published reports on the influence of in vitro inoculation with ericoid mycorrhizae during micropropagation of *P. floribunda* and subsequent evaluation of plant growth ex vitro. Therefore, the objective of this study was to determine the effects of selected ericoid mycorrhizae on ex vitro survival, acclimatization, and subsequent growth of micropropagated plantlets of *P. floribunda*.

Materials and Methods

On 30 Dec. 1995, 249 flat-bottomed glass shell vials [95 × 25-mm Kimble™ opticlear® (Kimble Glass, Toledo, Ohio)] were prepared with 10 mL of a mixture of 1 steam-pasteurized, sifted peat : 1 fine vermiculite (v/v) medium moistened with 5 mL liquid Woody Plant Medium [WPM (Lloyd and McCown, 1980)] with 200 mg·L⁻¹ NaH₂PO₄, 80 mg·L⁻¹ adenine hemisulfate, and 5 g·L⁻¹ sucrose. The pH was adjusted to 5.2 with 1 N KOH prior to placement in the vials. Vials were covered with 25-mm Magenta 2-way caps (Magenta Corp., Chicago), autoclaved for 15 min at 121 °C, and allowed to cool overnight.

On 1 Jan. 1996, hyphae of ericoid mycorrhizal fungi were transferred aseptically to vials containing the cooled peat : vermiculite medium. Wefts of hyphae ≈ 3 mm² growing actively on the surface of 1-month-old malt agar cultures of isolate HE [= *H. ericae* (ATCC #32985) American Type Culture Collection, Rockville, Md.] and of isolate DA [= isolate A, a putative isolate of *H. ericae* (Lemoine et al., 1992)] were transferred to 83 vials each. A third set of 83 vials remained noninoculated (controls). Vials were recapped and sealed with Parafilm® "M" (American National Can, Greenwich, Conn.). Cultures were placed in a controlled-environment chamber maintained at 23 ± 1 °C with a 16-h photoperiod provided by two cool-white fluorescent lamps suspended 20 cm above the tops of the vials. Lamps provided a photosynthetic photon flux [PPF (400–700 nm)] of ≈ 68 μmol·m⁻²·s⁻¹ as measured at the tops of the vials. These and all other light measurements were recorded with a LI-COR LI-185A quantum/radiometer/photometer (LI-COR, Lincoln, Nebr.).

On 21 Jan. 1996, five unbranched microshoots of *P. floribunda* 2.5 cm in length were selected from cultures started on 10 July 1993 (Starrett et al., 2001). Microshoots were placed vertically to a depth of 1 cm in each of 60 small jars (vol. = 140 mL). Each jar contained 30 mL WPM solidified with 0.8% TC agar (JRH Biosciences, Lenexa, Kans.), with 200 mg·L⁻¹ NaH₂PO₄, 80 mg·L⁻¹ adenine hemisulfate, 20 g·L⁻¹ sucrose, and 62.2 μM of the potassium (K) salt of 1H-indole-3-butyric acid (K-IBA). This medium was adjusted to pH 5.2

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with 1 N KOH prior to placement in the jars. Cultures were capped with Magenta B-caps (Magenta Corp.), sealed with Parafilm®, and maintained at $23 \pm 1^\circ\text{C}$ under a 16-h photoperiod provided by two cool-white fluorescent power groove tubes (GE model F48PG17W; GE Lighting, Cleveland) and two, 25-W, soft-white incandescent bulbs suspended 60 cm above the tops of the jars. Tubes and bulbs provided a PPF of $\approx 33 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at the tops of the jars.

After 10 d on this medium, microshoots with visible root initials were transferred to the vials previously inoculated with ericoid mycorrhizal fungi and to control vials. Shoots were inserted to a depth of 1 cm (one shoot per vial). Cultures were maintained at $23 \pm 1^\circ\text{C}$ under a 16-h photoperiod provided by four, cool-white fluorescent tubes suspended 12 cm above the tops of the vials ($\text{PPF} \approx 116 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$).

After 1 month, data were recorded for total shoot length (main shoot + lateral shoots), branch number, and leaf number. After evaluation, Parafilm® was removed from the vials, and caps were loosened to reduce humidity and to begin acclimatization of the plantlets. The following day, plantlets were transferred to styrofoam cups (7.5 cm diameter, vol. = 225 mL) containing a steam-pasteurized, pine bark medium that had been sifted through a 0.6-mm mesh sieve. Prior to transfer of plantlets, holes were made in the base of each cup for drainage. A Spoonula™ lab-spoon (Fisher Scientific, Pittsburgh) was used to transfer plantlets and all the medium from vials to cups. Plantlets were misted immediately with deionized water. Cups containing plantlets inoculated with the same fungus or those that were noninoculated were grouped on racks (nine cups per rack).

Racks were placed on carts, three racks per cart (one rack each: noninoculated, inoculated with isolate HE, and inoculated with isolate DA), for a total of five carts. Individual racks were separated by clear, plexiglass partitions to prevent cross-contamination. Carts were placed in a glass greenhouse at the Southeastern Plant Environment Laboratory (Phytotron). The greenhouse was maintained at 9-h days/15-h nights of $22/18^\circ\text{C}$. Plants were subjected to long-day conditions by daily interruption of the dark period from 2300 to 0200 HR with $\approx 11\text{--}12 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ from incandescent lamps (Downs and Thomas, 1991). Tops and sides of carts were covered with three layers of white cotton gauze suspended 73 cm over the racks, which provided $\approx 70\%$ reduction of ambient irradiance ($\approx 500\text{--}600 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) as measured at solar noon on a clear day at onset of placement of carts in the Phytotron. In addition, upper portions of carts were wrapped with clear plastic along the sides from shelf level (77 cm above floor level) to the top of the gauze (150 cm above floor level). Air movement around racks was maintained through the cart shelves, which were open mesh, and the gauze shade cloth.

Plantlets on carts were misted by deflection-type mist nozzles suspended 50 cm above cups and were fertilized daily with standard Phytotron nutrient solution (Downs and Thomas,

1991). For the first 3 d, plantlets were misted daily with deionized water every 30 min for 15 s. The following 4 d, mist frequency was maintained at 30-min intervals during daylight (9 h) but was reduced to 15 s every hour during the night (15 h). Mist frequency was reduced over time as follows: week 2 = mist every hour (daylight), every 2 h (night); week 3 = mist every 2 h (daylight), every 4 h (night); week 4 = mist every 4 h (daylight), off (night). Plants were not misted after week 4. Thereafter, plantlets were watered once daily with Phytotron nutrient solution. Irradiance was increased to 40% of ambient after 1 month and increased to 50% of ambient after an additional month by removal of cotton gauze layers from carts.

The study was terminated 3 months after placement of plantlets in the Phytotron. Plants were harvested and divided into leaves, stems, and roots. Total leaf area and shoot length were measured with a Monochrome AgVision Root and Leaf Analysis System (Decagon Devices, Pullman, Wash.). Leaves were counted and roots and shoots were dried at 70°C for 48 h and weighed. Prior to drying, 50 mg (fresh weight) of each root system was removed to evaluate mycorrhizal colonization. Root subsamples were selected from the same region on each root system, proximal to the base of the shoot. Roots were cleared and stained using a protocol adapted from Brundrett et al. (1984) and examined by bright field microscopy. To quantify root colonization, individual roots were examined for the presence of infected cortical cells. Any root with at least one infected cell was classified as "colonized." Intensity of colonization was determined by counting the number of colonized cortical cells at $\times 400$ magnification using light microscopy ($\approx 25\text{--}30$ cells per observation, four observations per subsample).

The experiment was conducted four times, and each experiment (run) was considered a replicate in a randomized complete-block design (RCBD) with treatments in a nested factorial. An experimental unit consisted of one rack (average of nine cups per rack). Because of high plantlet mortality, averages per rack were calculated based on values from living plants only. GLM and LSMEANS were used for statistical analysis (SAS Inst., Cary, N.C., 1990).

The methodology reported herein can be integrated into a micropropagation protocol developed for *Rhododendron* sp. (Anderson, 1978). The difference, however, between the present study and the protocol of Anderson (1978), is the addition of ericoid mycorrhizal fungi to the medium during in vitro rooting. Time spent under mist in the Phytotron was similar to that used for acclimatization of micropropagated plantlets of *Rhododendron* sp. (Anderson, 1978). Unfortunately, *Pieris* requires an additional root initiation phase that is not necessary for *Rhododendron* (Pennell, 1990). This extra step to induce rooting was incorporated into the micropropagation protocol developed for *P. floribunda* (Starrett et al., 1993). The time at which plantlets of *P. floribunda* grown in the present study were evaluated for initial growth and development (3 months) is the average time when greenhouse-

grown plantlets of *Rhododendron* are transplanted to larger pots (Anderson, 1978).

Results and Discussion

Prior to placement of plantlets in the Phytotron, effects of mycorrhizal inoculation on shoot length, branch number, and leaf number were evaluated. After incubation for 1 month, in vitro inoculation with isolates HE or DA had significantly increased total shoot length, and both branch and leaf number (Table 1).

Inoculation with isolates HE or DA increased shoot growth of *P. floribunda* (Table 1). This contrasts with the data of Lareau (1985) who found no positive effects on shoot growth of *V. corymbosum* produced and inoculated in vitro with *H. ericae*. Pons et al. (1982) reported an increase in plant size and vigor in seedlings of *Vaccinium myrtillus* L. (whortleberry) and *C. vulgaris* following in vitro inoculation with *H. ericae*. Response to inoculation was rapid, and differences between inoculated and noninoculated seedlings were observed within 15 d (Pons et al., 1982). Shoot growth of seedlings of *Vaccinium macrocarpon* Ait. (cranberry) was stimulated by in vitro inoculation with *H. ericae* (Stribley and Read, 1974; Stribley et al., 1975). Shoot length was increased when seedlings of *V. corymbosum* were inoculated in vitro with isolates of *H. ericae* (Starrett et al., 1995). In addition, shoot length, branch number, and leaf number were increased by inoculation of plantlets of *P. floribunda* in vitro with isolates of *H. ericae* (Starrett et al., 1996).

Inoculation with either fungus significantly increased survival (Table 1). Survival of micropropagated plantlets of *R. minus* var. *chapmanii* was also improved by inoculation with *H. ericae*; however, the response was dependent on the pH of the medium (Barnes and Johnson, 1986). Inoculation during rooting increased survival of transplants of the ericaceous plant *Arctostaphylos uva-ursi* (L.) Spreng. (bearberry) (Holden, 1978). Similarly, inoculation with peat that contained ericoid mycorrhizal fungi increased survival of cuttings of *V. corymbosum*, although survival of cuttings inoculated with pure cultures of *H. ericae* was less than that of noninoculated cuttings or those inoculated with mycorrhizal-peat (Powell and Bagyaraj, 1984).

The presence of isolates of *H. ericae* increases root development on cuttings, seedlings, and microcuttings of ericaceous plants (Berta and Gianinazzi-Pearson, 1986; Pons et al., 1982; Starrett et al., 1995, 1996). Although root development was not quantified prior to placement of plantlets in the Phytotron, root initials were present on all microshoots placed in vials containing the peat : vermiculite medium, with or without fungal inoculum. Furthermore, roots were well-developed on microshoots during transfer of plantlets from vials to cups for placement in the Phytotron. Previously, recommendations for acclimatization were to transplant microshoots just after initiation of roots but prior to root elongation, as roots may be damaged or killed during transfer (Conner and Thomas, 1981; Debergh and Maene, 1981).

Table 1. Influence of in vitro mycorrhizal inoculation on total shoot length, branch number, and leaf number of rooted microshoots of *Pieris floribunda* after 1 month of in vitro culture and on survival after 3 months of acclimatization in a greenhouse.

Fungal inoculum	Total shoot length (cm)	Branch no.	Leaf no.	Survival (%)
Noninoculated control	2.4 a ²	0.3 a	10 a	16 a
Isolate HE ²	3.1 b	0.6 b	11 b	42 b
Isolate DA ³	3.2 b	0.6 b	12 b	42 b

²Mean separation within columns by Fisher's protected LSD at $P \leq 0.01$.

³HE = *Hymenoscyphus ericae* [(ATCC #32985) American Type Culture Collection, Rockville, Md.].

⁴DA = Putative isolate of *H. ericae* from Dijon, France.

Inoculation with mycorrhizal fungi did not affect total shoot length, branch number, leaf number, leaf area, top dry weight (shoots and leaves), or root dry weight (data not shown) during 3 months of growth in the greenhouse. Lack of initial response may be attributed to the constant availability of nutrients provided by daily fertigation with nutrient solution. Limited supplies of readily available nutrients, especially N, are present in typical heathland soils (Read, 1991) or the soils typical of the habitat where *P. floribunda* is native, and inoculation with ericoid mycorrhizae is beneficial only when N is limiting (Stribley and Read, 1974, 1976; Stribley et al., 1975). Ericaceous plants use ammonium as a source of N (Townsend, 1970). The amount of available N from ammonium alone in the Phytotron nutrient solution is 14 mg·L⁻¹, which is adequate for plant growth when supplied daily. A constant quantity of ammonium N from daily fertilization might have obscured growth benefits from inoculation.

Extent of root colonization was significantly greater for plantlets inoculated with DA than with HE. Following inoculation with HE, only 48% of the root system was colonized, whereas 68% was colonized after inoculation with DA. Similarly, intensity of colonization of root cortical cells was slightly, but not significantly, greater for DA (63%) than for HE (57%). Noninoculated controls were not infected.

Root colonization levels in the present study were comparable with those reported for inoculated microshoots of *R. minus* var. *chapmanii* (Barnes and Johnson, 1986) and *V. corymbosum* (Powell, 1982). In another study, however, lower levels of colonization (4.5% to 9.0%) occurred in roots of micropropagated plantlets of *V. angustifolium* after a period of growth similar to that used in our study (Litten et al., 1992). Low amounts of root colonization by *H. ericae* are also reported for rooted cuttings of *V. corymbosum* [(5% to 30%) (Powell and Bagyaraj, 1984)]. Surveys of *V. corymbosum* in North Carolina revealed only 1% to 3% mycorrhizal infection in commercial plantings, with levels up to 85% found in native populations (Boyer et al., 1982). Thus, the extent of root colonization of ericaceous plants by ericoid mycorrhizal fungi appears to be variable.

The protocol reported herein is similar to procedures utilized currently for micropropagation of various ericaceous species, and thus has potential to improve survival of plantlets during acclimatization. The protocol also provides a means of inoculating plantlets with mycorrhizal fungi that might aid in later field establishment and growth. Microshoots of *P. floribunda* inoculated with isolate HE or DA

grew more than did noninoculated microshoots during 1 month in vitro. However, mycorrhizal inoculation did not stimulate root or shoot development of plantlets provided with adequate nutrition during 3 months growth in a greenhouse. Acclimatization of plantlets of *P. floribunda* to greenhouse conditions following in vitro inoculation with ericoid mycorrhizal fungi improved survival (42%) over noninoculated plantlets (16%). However, additional research is warranted to further improve survival.

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