Mycorrhiza (2008) 18:269–275
DOI 10.1007/s00572-008-0176-3

SHORT NOTE

Reducing airborne ectomycorrhizal fungi and growing non-mycorrhizal loblolly pine (*Pinus taeda* L.) seedlings in a greenhouse

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Received: 6 September 2007 / Accepted: 9 April 2008 / Published online: 15 May 2008

Abstract Atmospheric spores of ectomycorrhizal (ECM) fungi are a potential source of contamination when mycorrhizal studies are performed in the greenhouse, and techniques for minimizing such contamination have rarely been tested. We grew loblolly pine (*Pinus taeda* L.) from seed in a greenhouse and inside a high-efficiency particulate air-filtered chamber (HFC) constructed within the same greenhouse. Seedlings were germinated in seven different sand- or soil-based and artificially based growth media. Seedlings grown in the HFC had fewer mycorrhizal short roots than those grown in the open greenhouse atmosphere. Furthermore, the proportion of seedlings from the HFC that were completely non-mycorrhizal was higher than that of seedlings from the greenhouse atmosphere. Seedlings grown in sterilized, artificially based growth media (>50% peat moss, vermiculite, and/or perlite by volume) had fewer mycorrhizal short roots than those grown in sand- or soil-based media. The HFC described here can minimize undesirable ECM colonization of host seedlings in greenhouse bioassays. In addition, the number of non-mycorrhizal seedlings can be maximized when the HFC is used in combination with artificially based growth media.

Keywords Ectomycorrhizas · Soil inoculum potential · Bioassays · Greenhouse

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Introduction

Mycorrhizal studies that are performed in greenhouses include bioassays of soil inoculum potential (Herr et al. 1994; Teste et al. 2006), assessments of mycorrhizal community structure (Jones et al. 2003; Pilz and Perry 1984), and experimental inoculation with specific ectomycorrhizal (ECM) fungal symbionts (Beckjord and McIntosh 1983; Branzanti et al. 1999; Brundrett et al. 1996; Marx and Bryan 1969). There are two primary methods of assessing soil inoculum potential or mycorrhizal community structure. The first involves obtaining field soil, diluting it with different concentrations of sterile soil or a soilless medium ('most probable number' assay—see Brundrett et al. 1996) or obtaining intact soil cores and planting ECM host seed in the soil (Boerner et al. 1996). The second method involves producing non-mycorrhizal seedlings of an ECM host species and planting the seedlings in diluted soil (Brundrett et al. 1996) or intact soil cores (Smith et al. 1995), or outplanting the seedlings in undisturbed field soil (Tainter and Walstad 1977). In both cases, the 'trap plants' are left to grow for a period of time, then excavated and assessed for mycorrhizal colonization. However, aerial ECM fungal spores are a potential source of contamination when these tasks are performed in

greenhouses (Marx and Bryan 1969). Contamination by *Thelephora terrestris* Ehrh. (an ECM fungus) in greenhouses is particularly vexing due to its worldwide distribution and broad host range (Smith and Read 2002). Measures are often taken to reduce and test for fungal contamination, and it remains unclear whether an air-filtered growth environment is necessary to maintain soil bioassays free of contamination or for the growth of non-mycorrhizal seedlings.

Some groups have produced large numbers of non-mycorrhizal seedlings using only standard greenhouse space with different types of sterilized growth media (Boerner et al. 1996; Smith et al. 1995; Tainter and Walstad 1977). Others have used growth rooms with air filtration systems to remove airborne spores of mycorrhizal fungi from the atmosphere (Marx 1973; Ruehle 1982). However, Marx and Bryan (1969) indicated that non-mycorrhizal pine seedlings of at least 11 different species (including the focus of the current study, loblolly pine (*Pinus taeda* L.)) cannot be grown in field or greenhouse culture due to the pervasiveness of ectomycorrhizal propagules. Consequently, an electronically air-filtered, air-conditioned, growth room was used to produce non-mycorrhizal seedlings (Marx and Bryan 1969) and to keep greenhouse contaminants from colonizing seedlings inoculated with specific ECM fungal symbionts (Marx 1973). Limited information suggests that non-conifers (e.g., *Quercus* spp.) may be less susceptible to airborne contamination than conifers during greenhouse bioassays (Dickie et al. 2004) for unknown reasons.

Conventional growth chambers and laminar flow cabinets are costly, space-limited, and/or may not provide the level of air filtration necessary to reduce contamination of ECM bioassays. High-efficiency particulate air (HEPA) filtering technology may maintain lower levels of aerial contamination by ECM spores. Although HEPA filtering has not previously been used in a greenhouse setting, it has been successfully used in mushroom cultivation to prevent contamination from undesirable fungal propagules (Tisdale et al. 2006).

Various sterile growth media have been used in combination with filtered and unfiltered growth environments to grow non-mycorrhizal seedlings with variable success; these include sand, soil, vermiculite, and perlite (Boerner et al. 1996; Brundrett et al. 1996; Pilz and Perry 1984; Smith et al. 1995; Tainter and Walstad 1977). Whether specific growth media are structurally and/or nutritionally less conducive to mycorrhiza formation remains unclear.

The objectives of our study were to (1) test whether a HEPA-filtered chamber (HFC) was successful in reducing ECM colonization through reduction in aerial contamination and (2) determine whether sand- or soil-based and artificially based growth media promote different amounts of ECM colonization in pine seedlings.

Materials and methods

HEPA-filtered chamber

The study was conducted at the Clemson University Greenhouse Complex, Clemson, SC, USA (lat. 34° 40′ 8″; long. 82° 50′ 40″) which was built in 2002. The HFC was built inside the greenhouse to reduce the level of ECM spores in the growth atmosphere. The frame of the HFC was constructed of 5.1 cm×10.2 cm wood studs that enclosed a 152 cm×244 cm greenhouse bench (Fig. 1). The top and four sides of the HFC were lined with clear 2.65 mil, 7UVM Mylar film, and all seams were sealed with clear polyethylene tape. HEPA filtering of the HFC atmosphere was accomplished using a MAC 10® Fan Filter Unit (Envirco Corp.). The spores of ECM fungi range in diameter from 4 to 30 μm (Brundrett et al. 1996). The fan filter unit was rated to be 99.99% efficient at removing particles with a mean diameter of 0.3 μm and for circulating air at 1,105 m^3 h^{-1}. All air entered the HFC at one end through the fan filter unit and exited through the open bottom of the HFC. The HFC was thoroughly cleaned using a 10% household bleach solution (Izzo et al. 2006) and was purged with the fan filter unit running for 2 weeks prior to its use. A positive pressure, HEPA-filtered environment was maintained inside the HFC for the duration of the study.

Atmospheric measurements

Particle counts of total atmospheric particulate were performed using an electronic particle counter (Royco) at five positions distributed evenly in the HFC and in the greenhouse atmosphere 22 days prior to the start of the study. Additionally, five Petri dishes containing potato-dextrose agar (PDA; Difco; Cerrato et al. 1975) were placed at five different positions on the bench inside the HFC and on the bench immediately adjacent to the HFC in the greenhouse to capture atmospheric particulate (Marx and Bryan 1969) 20 days prior to commencing the study. The Petri dish traps were intended to confirm that fungal spores were present in the greenhouse atmosphere (Cerrato et al. 1975). Petri dishes were covered and cultured in the dark for 41 days at 22°C after 24 h of exposure. Fungal colonies were then counted on each Petri dish.

Measurements of photosynthetic photon-flux density (PPFD), ambient temperature, and relative humidity were obtained during the growth period both inside and outside of the HFC. PPFD was measured between 12:00 and 16:00 on four different days at six positions distributed evenly across the benches in the HFC and greenhouse environments using an AccuPAR LP-80 ceptometer (Decagon Devices, Inc.). PPFD was measured under different amounts of cloud cover and with and without 50% shade cloth draped across the growth area on four separate days.

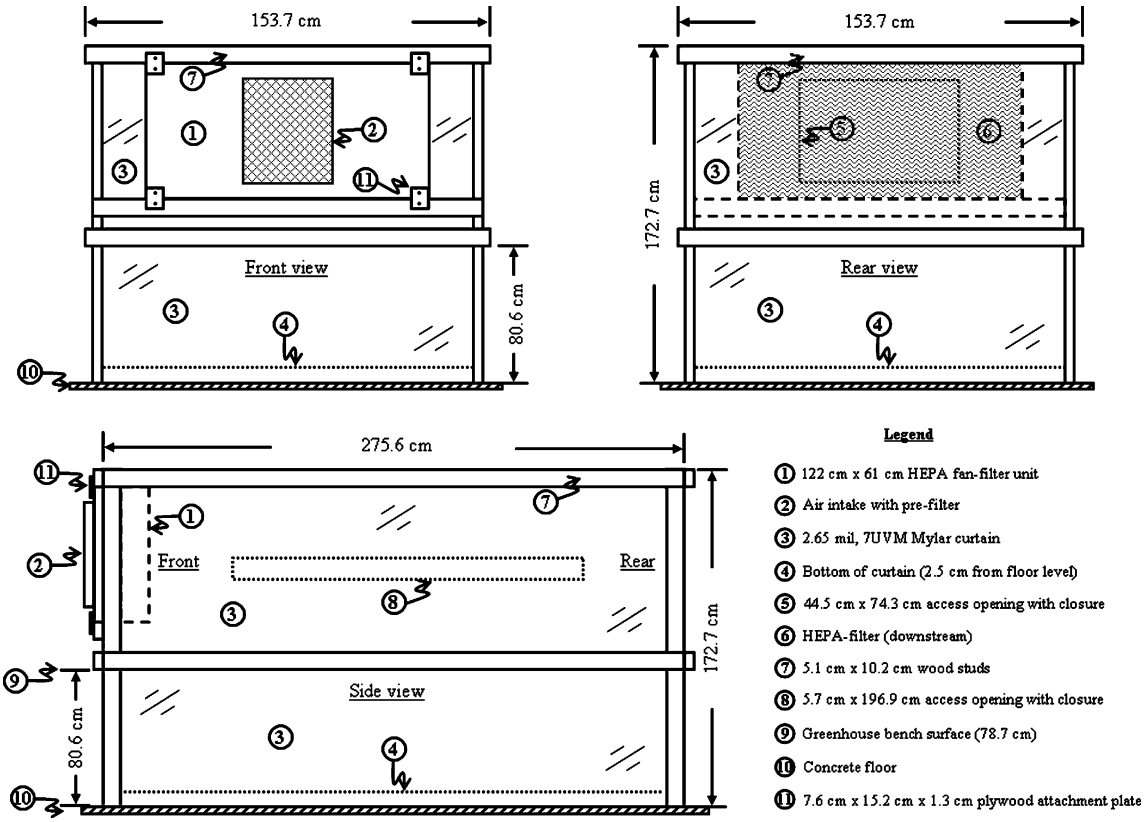

Fig. 1 Schematic diagram of a HEPA-filtered chamber that was built inside a greenhouse to reduce contamination by ectomycorrhizal fungal spores in the growth atmosphere

Ambient temperature and relative humidity were measured simultaneously both inside and outside of the HFC continuously for seven consecutive days using hygrothermographs (WEATHERtronics Corp.).

Sand- or soil-based and artificially based growth media

Ten 90 ml cavities of IPL® Rigi-Pots™ (Stuewe and Sons, Inc.) tube trays were filled with seven types of growth media, five of which have been used in previous greenhouse mycorrhizal studies (Boerner et al. 1996; Brundrett et al. 1996; Smith et al. 1995; Pilz and Perry 1984; Tainter and Walstad 1977). Growth media were classified as either sand- or soil-based or artificially based depending on the amount of peat moss, vermiculite, and/or perlite in the mixture. The four artificially based media contained >50% peat moss (Lambert), vermiculite (Palmetto), and/or perlite (Milolite) by volume and included a commercial mix (Fafard), 1:1 (*v*/*v*) vermiculite/perlite (Smith et al. 1995), 1:1:1 (*v*/*v*/*v*) peat moss/river sand/vermiculite (Tainter and Walstad 1977), and pure vermiculite. The river sand was collected in Oconee County, SC, USA. The commercial mix contained 5:3:1:1 (*v*/*v*/*v*/*v*) peat moss/processed pine bark/perlite/vermiculite and a packaged starter fertilizer (15:11:32).

The three soil-based media were river sand (Brundrett et al. 1996), 1:1 (*v*/*v*) river sand/peat moss (Boerner et al. 1996), and a forest soil (Pilz and Perry 1984). The forest soil was an ultisol (suborder Udult) collected from a loblolly pine stand in Clemson, SC, USA and included mineral A and B horizons to 10 cm in depth. Two tube trays filled with each type of growth media were autoclaved for 15 min at 122°C under 0.14 MPa (Fergus 1969) while inside a 64 cm×76 cm polypropylene autoclave bag which was closed with a wire tie. Trays remained inside the autoclave bags until the time of planting.

The study was a randomized complete block design with the HFC and greenhouse representing blocks and the two media categories representing treatments. One tube tray containing each media type was randomly distributed and positioned on the bench inside the HFC and on a bench immediately adjacent to the HFC in the greenhouse. The positions of the tube trays were not re-randomized during the experiment.

Ectomycorrhizal bioassays

Thirty-day stratified loblolly pine seeds (mixed lot, Telfair County, GA, USA) were surface-sterilized in 10% household bleach/water solution for 5 min (Dickie et al. 2001).

Two sterilized seeds were planted in each tray cavity to a consistent depth using a plastic dowel marked at 0.25 in. (0.64 cm). Germinates were thinned to one per cell after 14 days and allowed to grow under natural light for 14 additional weeks (16 weeks total) during which no fertilizer was applied. Seedlings were watered to field capacity two to three times per week with tap water. For each watering event, an irrigation hose and wand entered the HFC through an access opening (Fig. 1) during which positive pressure was maintained. Tap water samples were collected 20 days prior to commencing the study. The water was cultured on PDA media in Petri dishes for 41 days at 22°C to confirm that the water source did not contain fungal spores. The bioassay seedlings were harvested at 16 weeks, and excess growth medium was carefully removed from the root systems under running tap water.

Pine root systems are heterorhizic with distinct short roots and long (lateral) roots from which short roots subtend (Brundrett et al. 1996). Three lateral roots ≥6 cm in length were randomly selected from each root system. Each short root was tallied and classified as mycorrhizal or non-mycorrhizal using a dissecting microscope (Brundrett et al. 1996). Non-mycorrhizal short roots were slender and elongated, possessed root hairs and root caps, and lacked fungal mantles. Mycorrhizal short roots were bifurcate or monopodial, possessed fungal mantles, and lacked root hairs and root caps. No attempts were made to identify the mycorrhizas in any way. Percent ECM colonization was calculated by dividing the number of mycorrhizal short roots by the total number of short roots. In addition, the bioassay seedlings were dried (40°C for 24 h) and weighed to obtain measurements of root and shoot dry weight and root/shoot ratio.

A sub-sample of four seedlings in each of the seven growth media (28 seedlings total) were randomly selected and used to evaluate the methods used for recognizing mycorrhizas. For each of these seedlings, short roots were tallied and classified as mycorrhizal or non-mycorrhizal. The same roots were subsequently cleared with 10% KOH (6 to 12 h at 75°C), stained in trypan blue (6 h at 22°C), and de-stained in 50% glycerol (Brundrett et al. 1996). Each short root was classified as mycorrhizal or non-mycorrhizal based on the presence of Hartig net hyphae as observed under ×110 magnification using a compound microscope. Percentage colonization values were calculated as described previously.

Although we used only one HFC and one greenhouse environment, the 70 seedlings in each environment (HFC and greenhouse) were grown in individual 90 ml cavities of tube trays containing growth media. These 70 seedlings were treated as experimental units. However, we acknowledge that the seedlings grown in the same environment were not completely independent.

Statistical analysis

ECM colonization percentages were arcsin-transformed prior to statistical analyses. Analysis of variance was performed using PROC GLM (SAS Institute, Cary, North Carolina) to determine if there was a significant effect on ECM colonization of seedlings due to the category of growth media (sand- or soil-based or artificially based) that the seedlings were grown in.

Mean ECM colonization, shoot and root dry weight, and root/shoot ratio of seedlings were calculated for the two media categories in each environment. Additionally, the proportions of non-mycorrhizal seedlings produced in the two environments and in the two media categories were calculated. Chi-square tests were performed using PROC FREQ to compare the proportion of non-mycorrhizal seedlings produced in the two media categories. Due to the lack of replication of the environment factor, means were calculated, but no statistical comparisons were made between the HFC and greenhouse environments with respect to percentage ECM colonization, seedling growth, or proportion of non-mycorrhizal seedlings.

The sub-sample of 28 seedlings was used to evaluate how closely associated the percentage ECM colonization values determined using morphological characteristics and dissecting microscopy were to those determined using staining and compound microscopy. Percentage colonization values determined using different methodologies were compared to one another using correlation analysis (PROC CORR) with each seedling representing one observation. All data were expressed as the mean ± standard error of the mean.

Results

Electronic particle counts revealed that the mean number of particles ≥0.5 μm in diameter per m^3 air differed significantly between the air of the greenhouse environment ($1.34\times10^6\pm$ 66,800 particles) and that of the HFC (30±23 particles; $P<$ 0.0001). The average number of fungal colonies on Petri dish traps exposed to the greenhouse atmosphere (27±2.6) was also significantly higher than that on traps exposed to the HFC atmosphere (0.2±2.9; $P<0.0001$).

Measurements of PPFD and relative humidity revealed that the climatic conditions of the HFC and greenhouse environments were similar. Mean PPFD in the HFC (249.4± 29.4 mmol m^{-2} s^{-1}) was not significantly different than that in the greenhouse (293.8±48.1 mmol m^{-2} s^{-1}; $P=0.4610$). The difference in mean daily temperature between the HFC (25.7±0.24°C) and the greenhouse environment (24.7± 0.27°C) was statistically significant ($P=0.0104$) probably because the motor of the fan filter unit warmed the HFC

environment slightly. Mean daily relative humidity in the HFC (60.4±2.9%) was not significantly different than that of the greenhouse (60.4±2.6%; P=0.9954). There were no differences in PPFD, ambient temperature, or relative humidity, due to bench position, neither in the HFC nor in the greenhouse (data not shown). Culturing of tap water revealed that no fungal spores were present in the water source 20 days prior to commencing the study.

Analysis of variance revealed a significant blocking effect due to environment (P=0.0026). When all seven media were considered together, the mean percent ECM colonization of seedlings grown in the HFC (10.8±2.3%) was lower than that of greenhouse seedlings (23.1±3.0%). In addition, the proportion of seedlings that were non-mycorrhizal from the HFC was higher (by approximately 30%) than that of seedlings that were non-mycorrhizal from the greenhouse.

The mean percent ECM colonization of seedlings grown in artificially based media (10.6±2.1%) was significantly lower than that of seedlings grown in sand- or soil-based media (25.3±3.2%; P =0.0407). Mean percent colonization of seedlings, root and shoot dry weight, and root/shoot ratio are also given for individual growth media for the reader's information (Table 1). The category of media did not influence the proportion of non-mycorrhizal to mycorrhizal seedlings (P=0.5532).

Percentage colonization values determined for 28 seedlings using morphological characteristics and dissecting microscopy were strongly correlated with those determined for the same seedlings using the staining procedure and compound microscopy (r=0.7910, P<0.0001). This result provided verification that a dissecting microscope provided a sufficient level of magnification for the lab worker to effectively distinguish between mycorrhizal and non-mycorrhizal short roots based on morphological characteristics.

Mean seedling root dry weight was not significantly different between the two media categories (P=0.2069). Shoot dry weight of seedlings grown in artificially based media (0.30±0.02 g) was significantly higher than that of seedlings grown in sand- or soil-based media (0.22±0.01 g; P<0.0001). The root/shoot ratio of seedlings grown in artificially based media (0.26±0.02%) was significantly lower than that of seedlings grown in sand- or soil-based media (0.31±0.02%; P=0.0053).

Discussion

The results of this study strongly suggest that maintaining a reduced level of atmospheric fungal contaminants substantially reduces the amount of undesirable ECM colonization and increases the production of non-mycorrhizal seedlings. Furthermore, artificially based growth media discourage the formation of mycorrhizas compared to sand- or soil-based media.

Electronic particle counts and Petri dish traps exposed for 24 h indicated that fungal spores were abundant in the greenhouse atmosphere and essentially absent from the HFC atmosphere 22 days prior to the start of the study. Marx and Bryan (1969) found similar amounts of atmospheric fungal contamination in their filtered growth room

Table 1 Mean percent of ectomycorrhizal (ECM) colonization, root and shoot dry weight, and root/shoot ratio of loblolly pine (*Pinus taeda* L.) seedlings that were germinated in sand- or soil-based and artificially based growth media

Growth media	Greenhouse (n=70)				HEPA-filtered chamber (n=70)			
	% ECM colonization	Root dry weight (g)	Shoot dry weight (g)	Root/shoot ratio	% ECM colonization	Root dry weight (g)	Shoot dry weight (g)	Root/shoot ratio
River sand	31.0 (6.8)	0.07 (0.01)	0.18 (0.01)	0.40 (0.05)	12.3 (8.2)	0.07 (0.01)	0.17 (0.01)	0.34 (0.03)
1:1 (*v*/*v*) sand/peat	20.5 (8.6)	0.08 (0.01)	0.26 (0.02)	0.26 (0.01)	11.8 (7.9)	0.08 (0.01)	0.27 (0.01)	0.30 (0.05)
Forest soil[a]	41.4 (9.7)	0.06 (0.00)	0.26 (0.01)	0.24 (0.03)	13.5 (7.6)	0.07 (0.01)	0.20 (0.02)	0.34 (0.04)
Commercial mix[b]	19.1 (7.8)	0.14 (0.01)	0.48 (0.03)	0.26 (0.01)	11.4 (6.5)	0.15 (0.01)	0.67 (0.04)	0.21 (0.03)
1:1 vermiculite/perlite	9.3 (5.5)	0.05 (0.00)	0.18 (0.01)	0.22 (0.01)	7.4 (5.0)	0.06 (0.00)	0.20 (0.01)	0.32 (0.02)
1:1:1 peat/river sand/vermiculite	21.4 (5.6)	0.08 (0.01)	0.24 (0.01)	0.35 (0.03)	14.6 (5.2)	0.05 (0.00)	0.24 (0.01)	0.26 (0.01)
Vermiculite	18.6 (6.3)	0.06 (0.00)	0.18 (0.01)	0.28 (0.03)	4.8 (3.2)	0.04 (0.00)	0.20 (0.00)	0.16 (0.01)

Sand- or soil-based media included: river sand; 1:1 (*v*/*v*) sand peat; and forest soil. Artificially based media included: a commercial potting mix; 1:1 vermiculite/perlite; 1:1:1 peat/river sand/vermiculite; and 100% vermiculite. Two trays of each media type were sterilized and placed into a greenhouse and a HEPA-filtered chamber that was intended to reduce ECM fungal spore contamination. Standard errors of the means are in parentheses.

[a] The forest soil was an ultisol (suborder Udult) collected from a loblolly pine stand in Clemson, SC, USA and included mineral A and B horizons to 10 cm in depth.

[b] The commercial mix contained 5:3:1:1 (*v*/*v*/*v*/*v*) peat moss/processed pine bark/perlite/vermiculite and a packaged starter fertilizer (15:11:32).

after exposing malt extract agar, which is also non-selective and grows a variety of fungi (Atlas 2005), for only 30 min.

Some contamination may have been carried into the HFC on the surface of irrigation equipment, instruments for measuring the atmospheric environment, or clothing thus accounting for some of the colonization observed on the seedlings in the HFC. A sub-irrigating watering system would eliminate physical entry to the HFC through the access openings during weekly watering events and may further reduce contamination.

Many of the climatic conditions known to influence mycorrhiza formation (e.g., water, light, temperature, and relative humidity) were either controlled or measured with differences between the HFC and greenhouse atmospheres found to be not statistically significant. Although the environment factor lacked true replication, these results strongly suggest that the reduced level of ECM colonization exhibited by seedlings and the increased production of non-mycorrhizal seedlings was due to reduced airborne ecto-mycorrhizal contamination provided by the HFC.

Little difference in seedling root and shoot development suggests that mycorrhizal colonization was influenced by structural and/or nutritional differences between the media categories. Soil characteristics including nutrient content, pH, water-holding capacity, and porosity are known to influence the activity of mycorrhizal fungi (Marks and Kozlowski 1973; Schüepp et al. 1987). Schüepp et al. (1987) found that growth of vesicular–arbuscular mycorrhizal hyphae occurred at lower rates in calcined clay, peat moss, and chopped hay than in various sand- and soil-based media. They suggested that the effects of nutrition and/or pH on fungal growth, ease of hyphal penetration, texture, or moisture content may have caused differences in the rate of hyphal growth through the different media. Therefore, we suspect that peat moss, vermiculite, perlite, and pine bark may have had similar effects on fungal activity and mycorrhizal colonization in the present study.

Trap plants were used in some greenhouse studies to test for atmospheric contamination. Boerner et al. (1996) found that control seedlings growing in a sterilized sand/peat moss mixture were free of ECM colonization after 9 weeks in a greenhouse study of soil inoculum potential. In another study, non-mycorrhizal loblolly pine seedlings were propagated in an unfiltered greenhouse using a sterilized peat moss moss/vermiculite/sand mixture (Tainter and Walstad 1977). These results suggest that contaminants were not present in their greenhouse or were present but did not colonize control seedlings because the media mixture discouraged the formation of mycorrhizas with the seedlings. Results of the present study demonstrate that trap plants grown in control media that are dissimilar from the experimental soil or media mixture may misrepresent the amount of atmospheric contamination. However, such problems can be avoided by growing trap plants in the same type of medium that is under investigation.

The combination of sterilized artificially based growth media and a reduced level of atmospheric propagules should minimize the amount of undesirable colonization of seedlings during greenhouse studies involving ectomycorrhizas. Furthermore, the HFC is relatively inexpensive to build (<$1,000 USD) and appears to provide an atmosphere of reduced contamination that is needed for bioassays of soil ECM inoculum potential, studies of mycorrhizal community structure, or those that involve the isolation of specific fungal symbionts.

Acknowledgments We thank Richard Layton, Andy Nuffer, and Will Faulkner for assisting with greenhouse and laboratory work, Jim Harriss for performing electronic particle counts, and two anonymous reviewers for useful comments. Logistical support provided by Envirco Corporation, GBC Corporation, and the South Carolina Forestry Commission is greatly appreciated. Funding for this study was provided by the Interagency Joint Fire Science Program grant 04-2-1-33.

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