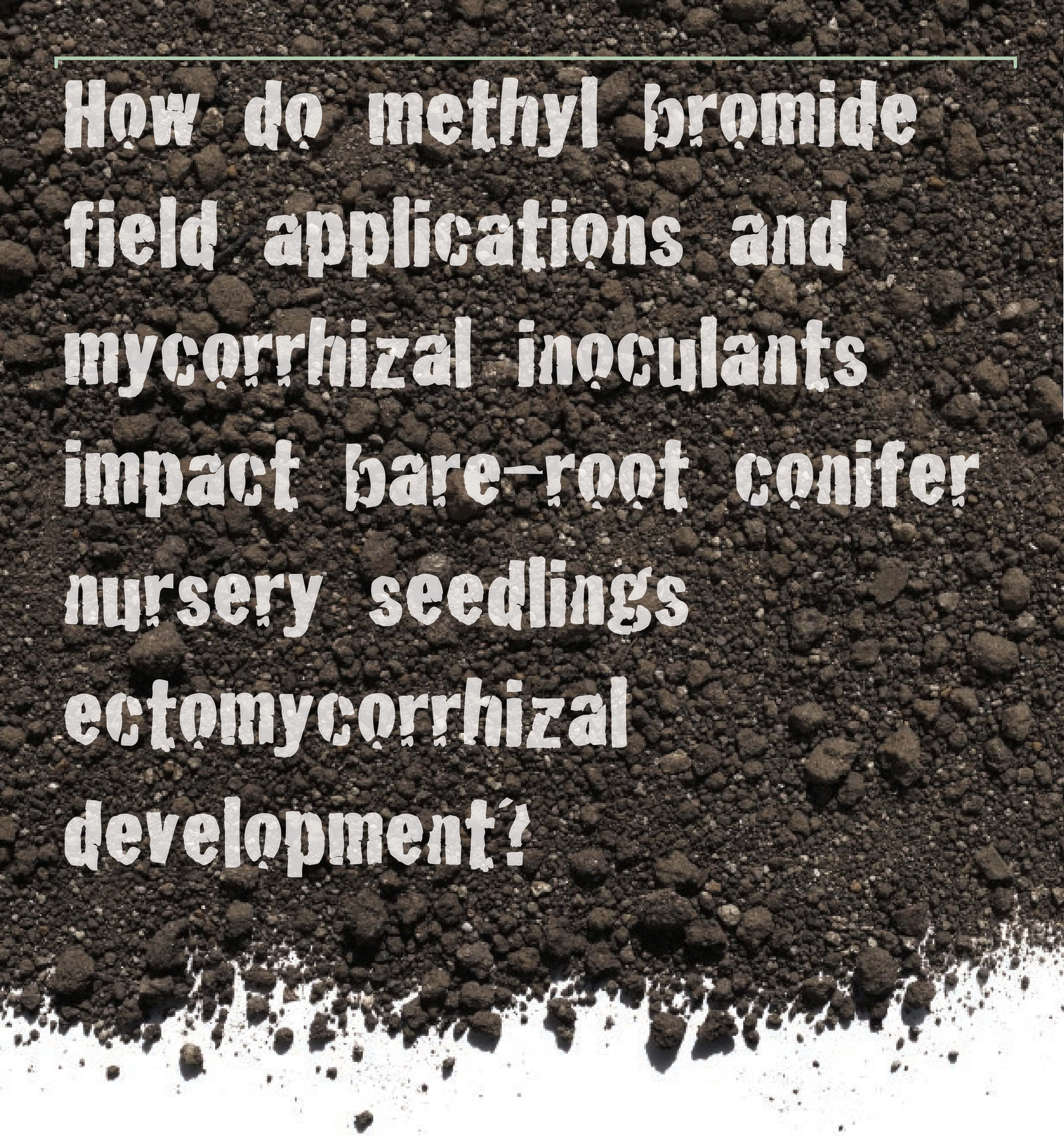
How do methyl bromide field applications and mycorrhizal inoculants impact bare-root conifer nursery seedlings ectomycorrhizal development?



BRYAN RENNICK, GIAN MARIA NICCOLÒ BENUCCI, GREGORY BONITO
DEPARTMENT OF PLANT SOIL AND MICROBIAL SCIENCES, MICHIGAN STATE UNIVERSITY

Soil-borne pathogens can cause pre- and post-emergence damping off in bare-root Christmas tree seedlings nurseries. Fungi (e.g. *Rhizoctonia* and *Fusarium*) and Oomycota (e.g. *Pythium*, *Phytophthora*) are the main culprits that can attack fir, spruce and pine seedlings. Once soil-borne pathogens establish in a seedling nursery they can cause significant seedling loss, depending on weather conditions, and they may spread between trees and other soils and sites when transplanted.

One strategy some bare-root seedling growers in Michigan have adopted to mitigate against soil-borne pathogens is the use of soil-fumigation prior to planting. While broad-spectrum chemistries including methyl bromide and chloropicrin are active against diverse target organisms such as oomycetes, fungi and nematodes, beneficial non-target organisms such as ectomycorrhizal root-symbionts of Christmas trees are also susceptible to these fumigants. Thus, Christmas tree nurseries have been interested to know whether the use of fumigants leads to the absence of beneficial ectomycorrhizal fungi on seedling roots post-fumigation, and if so, any potential impacts that this could have on seedling growth and health.

To investigate how soil fumigants impact the development of fungal communities on Christmas tree seedling roots in bare root nurseries, our research team at Michigan State University (MSU) teamed up with Jerry and Josh Peterson of Peterson's Riverview Nursery, LLC (Allegan, MI) and with Dan Wahmhoff of Wahmhoff Farms Nursery (Gobles, MI) for some on-farm research. Our goals in this partnership were to address the following questions:

(1) Are ectomycorrhizal fungi present on the roots of seedlings that have been germinated and grown in soils treated with methyl bromide fumigation?

(2) Can local, exotic or commercial ectomycorrhizal fungi inoculated into soils prior to seeding establish and persist on the roots of seedling roots grown in fumigated soils?

(3) How does plant host species and nursery site impact the diversity of ectomycorrhizal fungi that establish and persist on Christmas tree seedlings?

(4) Does the timing or approach of ectomycorrhizal inoculation impact the establishment and persistence of the inoculum?

To address these questions, two separate experiments were carried out. Both experiments focused on *Picea abies* (Norway spruce), *Pinus sylvestris* (Scotch pine), and *Pseudotsuga menziesii* (Douglas fir) since the tree species are important to Michigan Christmas tree industry and are expected to be most compatible with the ectomycorrhizal fungal inoculum that we used for this study. As part of standard site preparation, both farms fumigated with Tri Brom 80-20 (80.0% Methyl Bromide and 19.9% Chloropicrin) at a rate of 240 lbs per acre in the fall prior to planting. In the first experiment carried out in 2018, spores of three local ectomycorrhizal species (*Pisolithus arendrius, Laccaria bicolor, Scleroderma citrinum*) and those of three exotic commercial truffle species (*Tuber aestivum, T. borchii, T. indicum*) were applied after the soils were tilled but

Table 1. Inoculum Application Rates in Grams per Meter

A. Experiment 1

	Site A			Site B		
	Picea abies	*Pinus sylvestris*	*Pseudotsuga menziesii*	*Picea abies*	*Pinus sylvestris*	*Pseudotsuga menziesii*
Tuber aestivum	20	20	20	N/A	N/A	N/A
Tuber borchii	20	20	20	20	20	20
Tuber indicum	20	20	20	20	20	20
Pisolithus	10	N/A	10	N/A	N/A	N/A
Scleroderma	10	10	10	10	10	10
Laccaria	10	N/A	10	10	10	10
Negative Control	0	0	0	0	0	0

B. Experiment 2

	Site A		Site B
	Picea abies	*Pseudotsuga menziesii*	*Pinus sylvestris*
Tuber aestivum	20	20	20
Tuber indicum	20	20	20
Commercial inoculum	12	12	12
Negative Control	0	0	0

Table 1. Inoculum application rates (grams of fungal spores per square meter). A) Rates of application for site A and B during experiment 1. B) Rates of application for site A and B during experiment 2. Site A did not grow Scotch pine and site B did not grow either Norway spruce or Douglas fir during the second year for experiment 2.

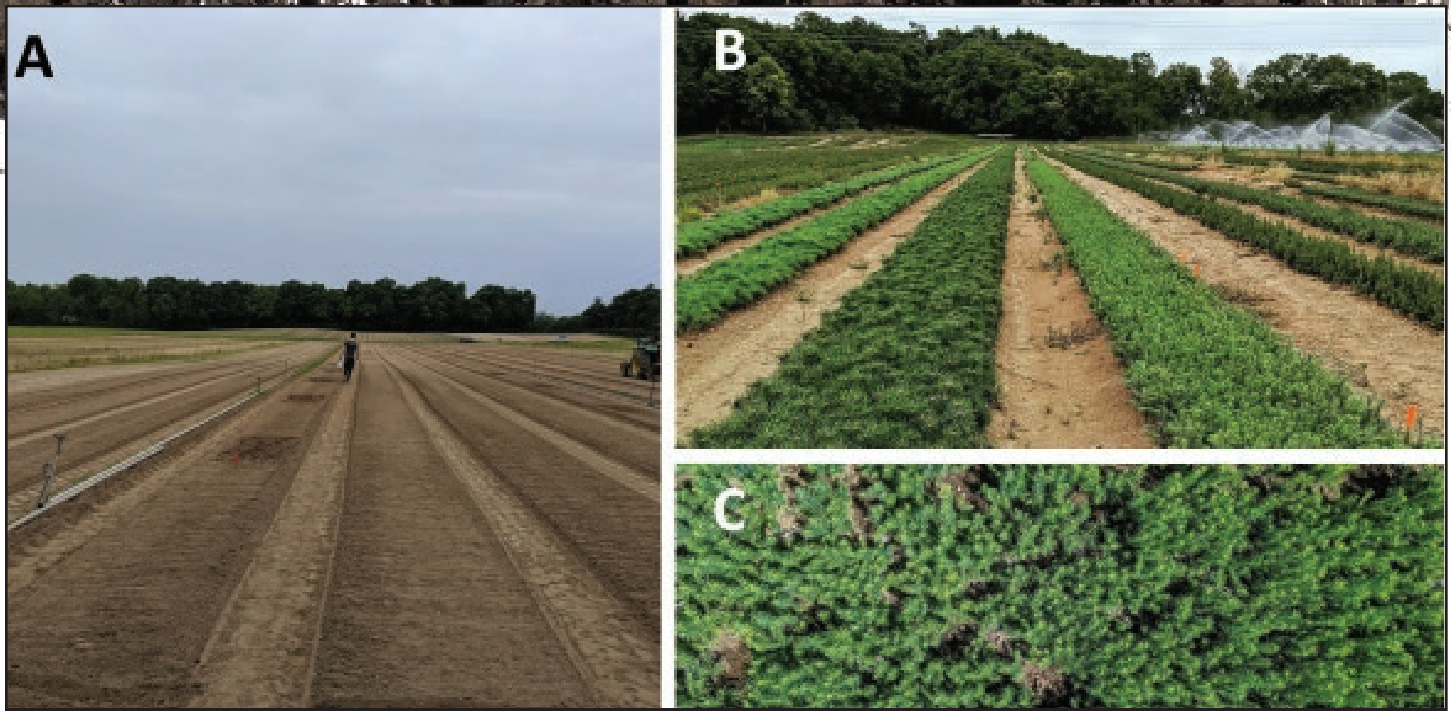


Figure 1. Inoculation (A) and experimental nursery seedlings (B & C) at harvest for Experiment 1.

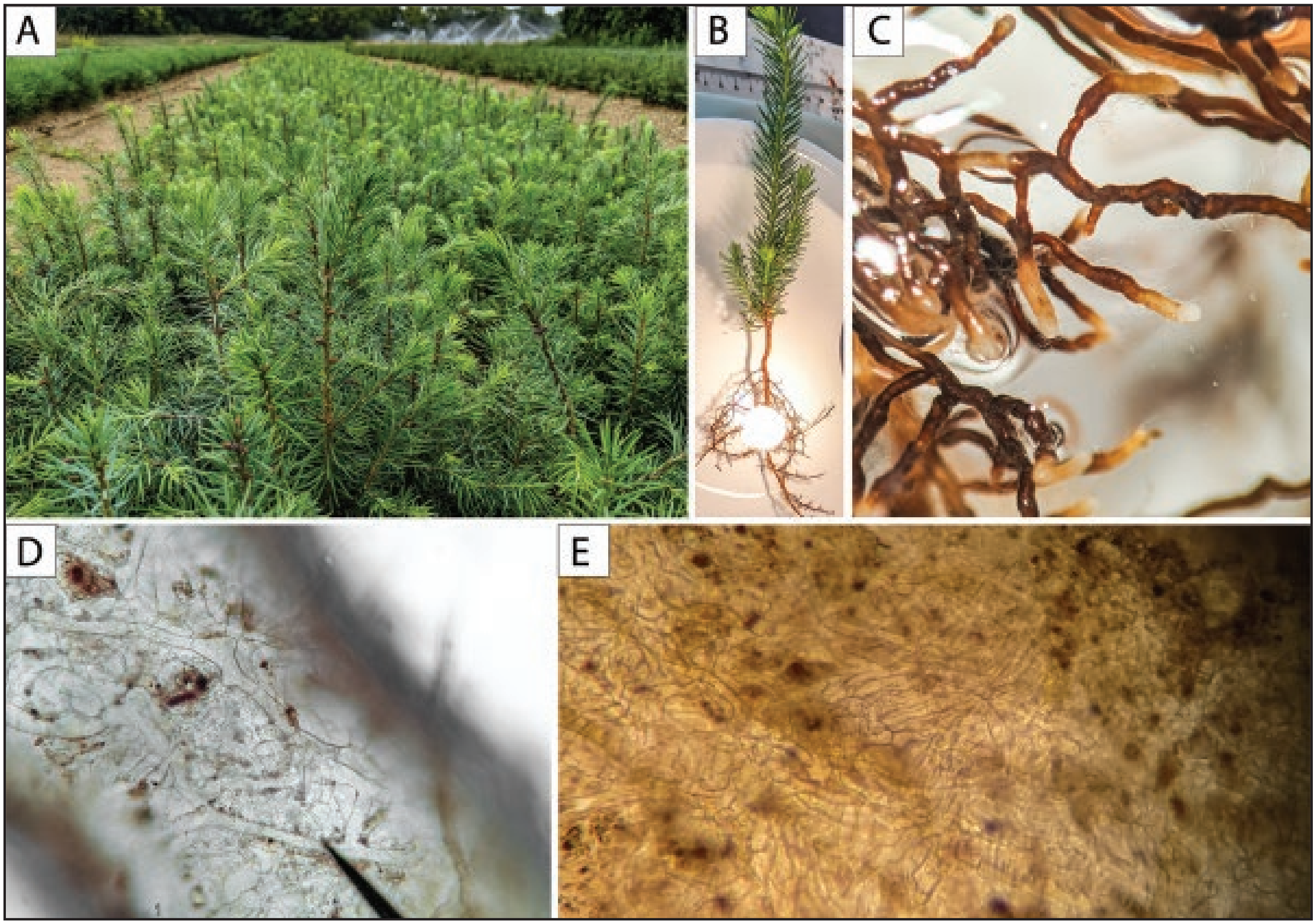


Figure 2. Seedling selection and processing. (A) Douglas fir seedlings growing in high density in inoculated soils. (B) Bare root seedling under a light microscope for assessment of root system. (C) Colonized ectomycorrhizal root tips exhibiting characteristic swollen appearance and extramatrical hyphae. (D) Root tip in early stages of colonization with needle pointing to an individual hyphae of the mantle covering the root surface. (E) A mature EMF mantle composed of densely packed fungal cells formed over a well colonized root tip.

before seeds were sown such that the seed would be pushed into the inoculant. In the second experiment carried out in 2019, spores of two truffle species and those of a commercial inoculum were applied right before soils were tilled and seeds sown such that the spores were homogenized through the upper ~5 inches of soil seed-bed where roots establish. The commercial inoculum was said to contain four species of Glomus, four species of *Rhizopogon, Pisolithus,* and two species of *Scleroderma*.

In both experiments, spore inoculants were spread across the rows and we maintained gaps between treatments as large as was possible to reduce cross contamination and edge effects. We had a total of seven treatments on three host species and two sites in experiment 1, and four treatments across two sites and a total of three host species in experiment 2 (see Table 1). Treatment blocks in experiment 1 were $1m^2$ and those in experiment 2 blocks were $5m^2$ each (see Figure 1). Each treatment was replicated 5 times at each farm, and also included sections where no inoculants were applied to act as a control. Application rates for each tree at each site is laid out in Table 1. After 1 year of growth, replicated sub-samples of seedlings from each treatment were brought back to MSU for analysis. Seedling height, stem girth and other health parameters were recorded. A subset of roots were washed to remove soil and were visualized with microscopy to assess whether mycorrhizas were present. To assess belowground fungal communities on seedlings, roots were cleansed of soil particles and fungal DNA was extracted, PCR amplified and sequenced. We then analyzed the sequences to assess the impacts of our treatments on the fungal community on seedling roots.

Visual inspection of seedlings, shown in Figure 2, indicated that ectomycorrhizas were present in all treatments, including control treatments where no exogenous ectomycorrhizal spores were applied. Notably, the morphology of the ectomycorrhizas observed through

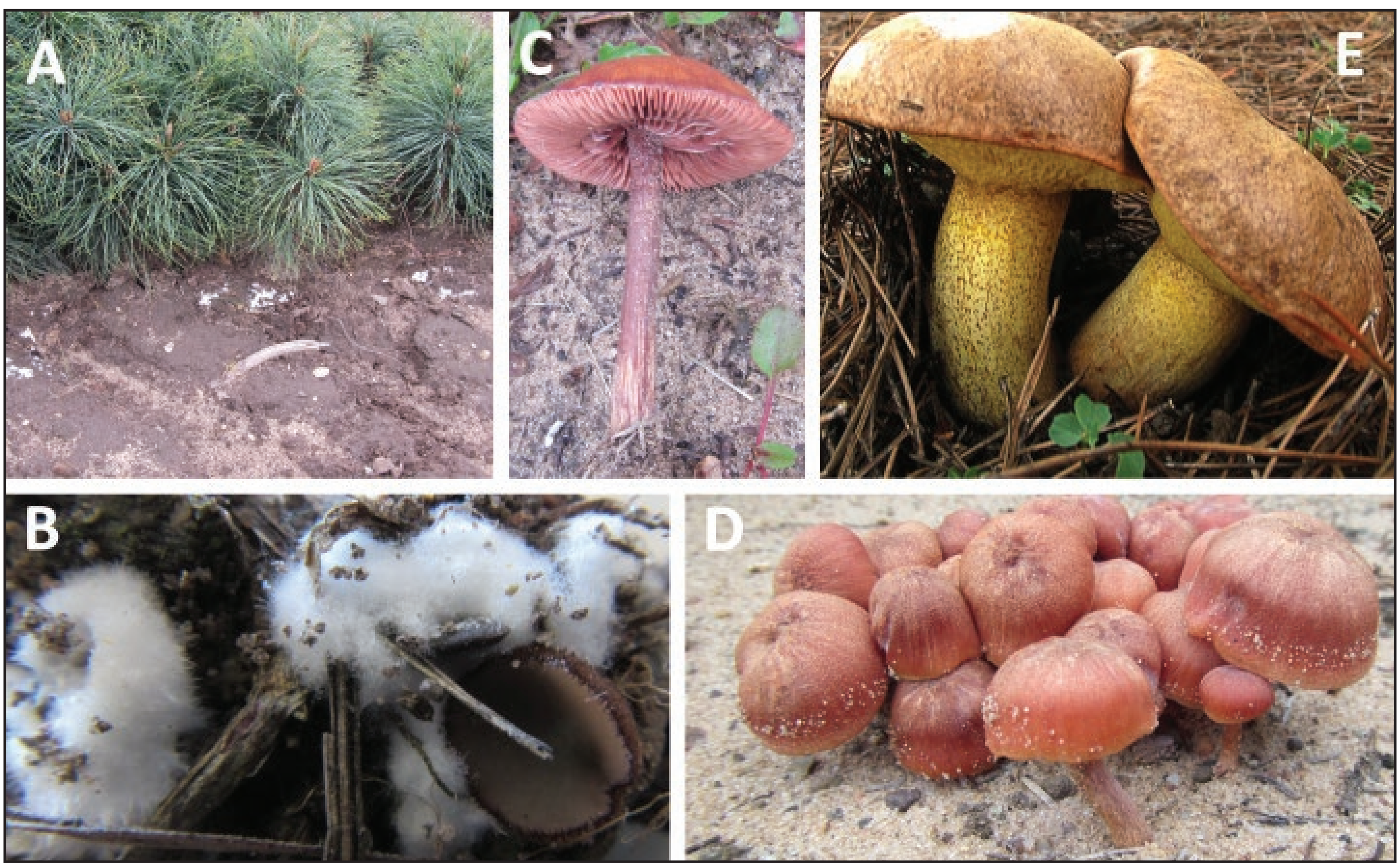


Figure 3. Visualization of ectomycorrhizal fruiting bodies of species detected on Christmas tree seedling roots. *Wilcoxia* produces white asexual spore mats (A & B) and small brownish colored cup-shaped fruiting bodies. *Laccaria* species produce small to medium size purplish mushroom fruiting bodies (C & D) and associates with many types of host seedlings, while *Suillus* produces larger fruiting bodies and is strictly a pine associate (E).

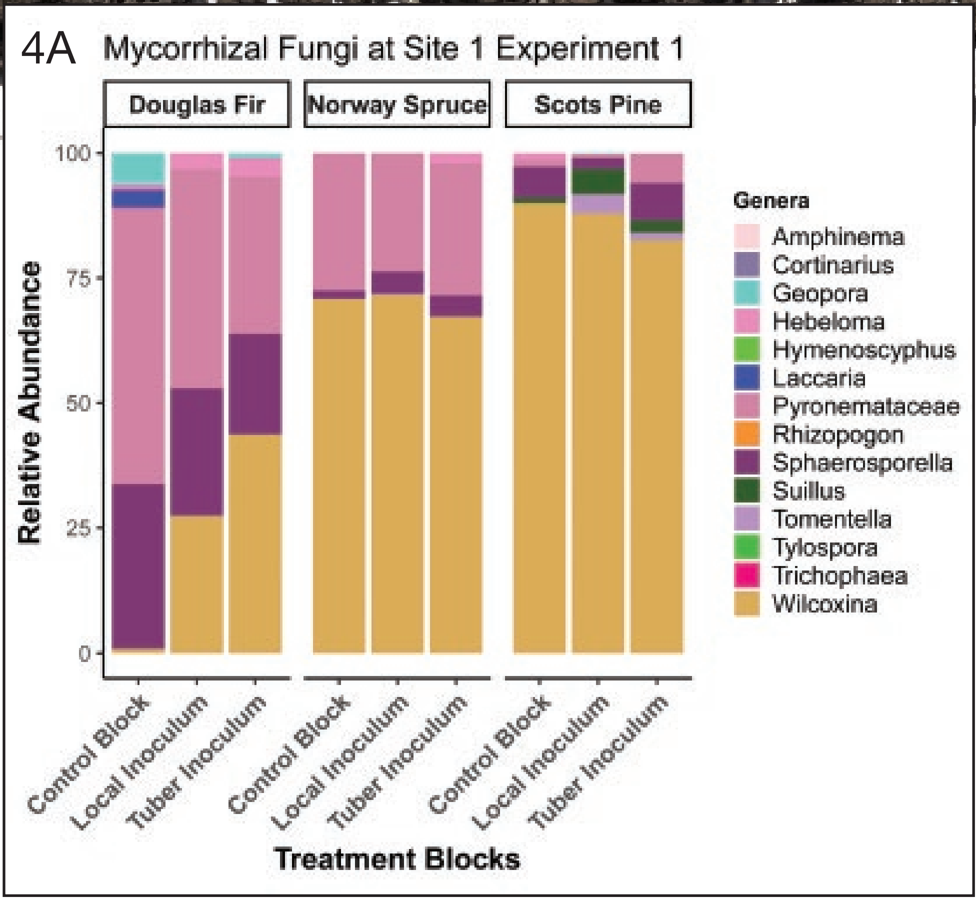
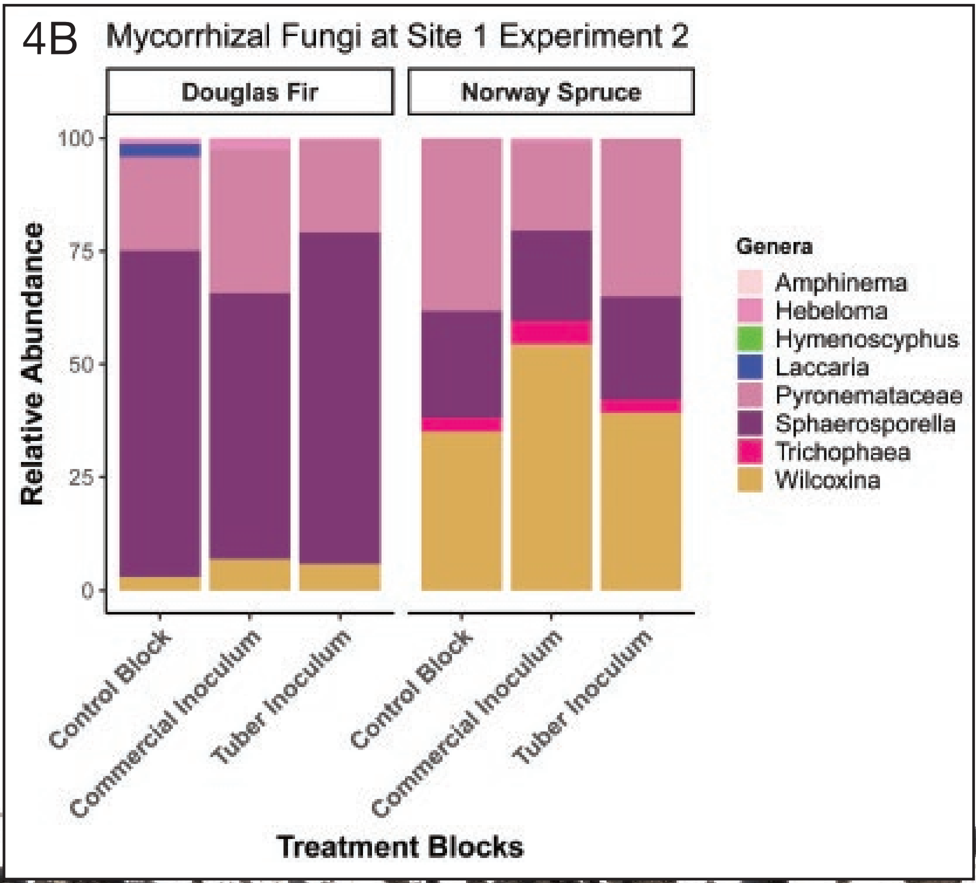


Figure 4. (A, B, C, D) Mycorrhizal community structure. Relative abundances of fungal taxa showing a dominant presence of *Wilcoxina, Sphaerosporella, Pyronemataceae* are seen with each tree at each site for both experiments. *Suillus* appears at both sites associated with Scotch pine, and Tuber can be seen in purple at site 2, especially on Douglas fir (C).

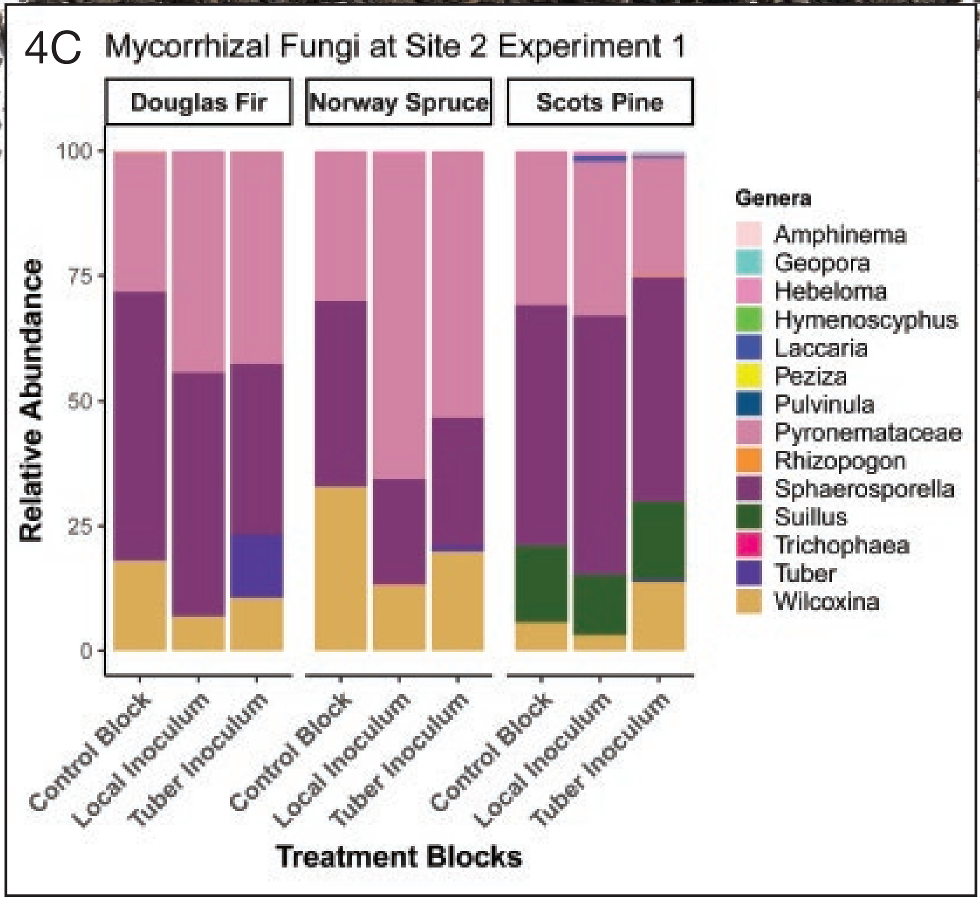


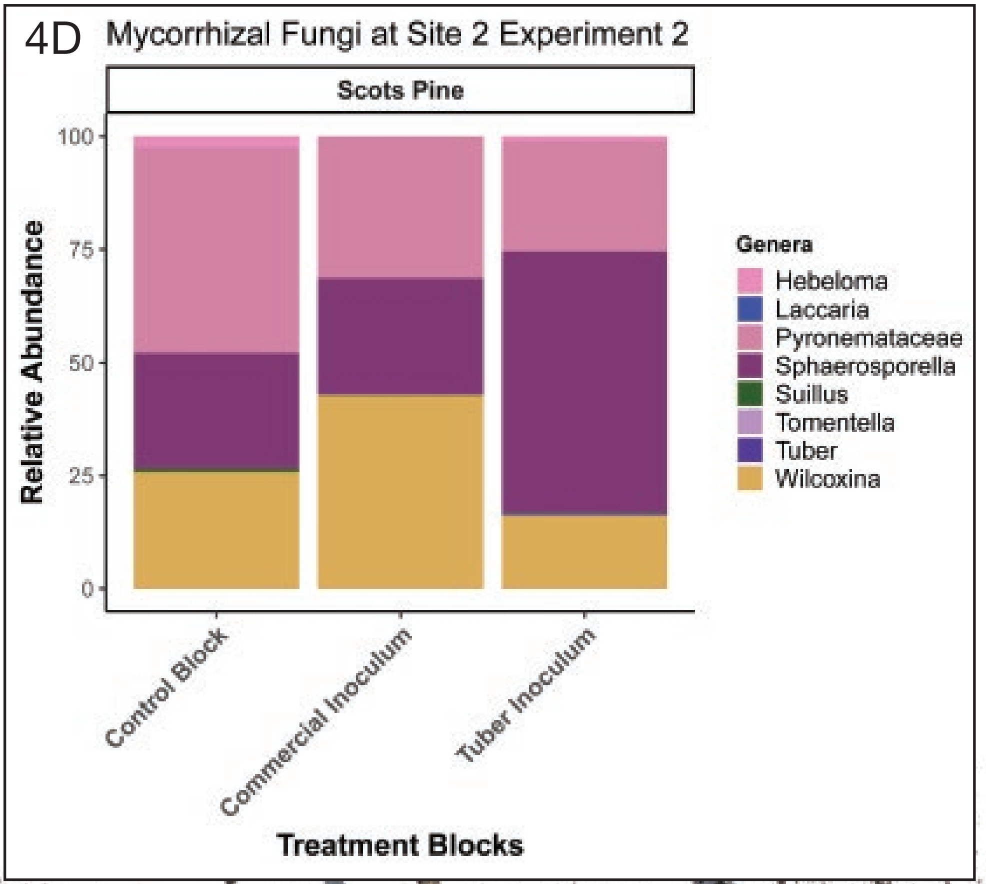
microscopy was different from what we would have expected for the species that we inoculated with. Additionally, there were no statistically relevant growth or health impacts correlating to any of the inoculation treatments.

Sequencing data confirmed that ectomycorrhizal fungi were abundant on seedling roots across all treatments, sites and seedling species. Ectomycorrhizal species diversity differed by sites and seedling hosts, yet ascomycete species belonging to *Wilcoxina, Sphaerosporella* and *Pyronemataceae* dominated across all plant hosts and treatments at both sites. As shown in Figure 3, these fungi can make white spore mats on the soil surface and also make small and somewhat inconspicuous fruiting bodies. There were site specific variations in the less abundant fungi, but the three aforementioned ascomycete taxa were the same. The ectomycorrhizal species that we detected to be dominant across the treatments are known to be well adapted to disturbed habitats and likely have large populations in the sampled area. They must produce spores in great enough numbers to arrive and establish across the Christmas tree nurseries studied. Perhaps unsurprisingly, the strict pine-associated Suillus was also a mainstay in the Scotch pine treatments (see Figure 3E & Figure 4A & C).

Our data demonstrate that some of the inoculated ectomycorrhizal species were able to establish and persist on seedling roots, as shown in Figure 4. Of the local ectomycorrhizal taxa used in this experiment, only *Laccaria bicolor* established and persisted on seedling hosts, but it was present in a very low abundance. Similarly, in experiment 2 the European truffle *Tuber borchii* persisted among Douglas fir and to a lesser extent Norway spruce and Scotch pine, but only at a low relative abundance (Figure 4). Notably, we were not able to detect species from the commercial ectomycorrhizal inoculum on seedling roots above background levels, and most of the taxa including *Glomus* and *Rhizopogon* species were not detected at all, indicating that these taxa likely did not establish or persist in these two sites. As presented in Figure 5, ectomycorrhizal taxa were dominant and accounted for roughly 80% of the sequences generated from Christmas tree roots. Some of the more common non-ectomycorrhizal fungal taxa that were detected were *Didymella, Fusarium, Rhizoctonia, Trichoderma,* and *Paraphoma*. Pathogens remain a concern for Christmas tree nurseries. Given that all treatments had formed ectomycorrhizas, including uninoculated control plots, we were therefore unable to assess how the absence of ectomycorrhizas impacts the susceptibility of seedlings to soil-borne pathogens. This is a worthwhile topic for future research.

In conclusion, Christmas tree nursery growers are often concerned that fumigants such as methyl bromide may kill beneficial fungi, including ectomycorrhizas, in the process of soil fumigation leaving the seedlings subject to reduced nutrient uptake and lower defense against soil-borne pathogens. However, based on our field results from two nurseries across two years, a lack of ectomycorrhizas in fumigated bare-root Christmas tree nurseries is not a problem. We tested whether ectomycorrhizal populations could be enhanced through spore inoculation approaches, but mycorrhizal spore inoculation did not appear to be necessary or particularly effective for establishing mycorrhizas after fumigation in bare-root nursery





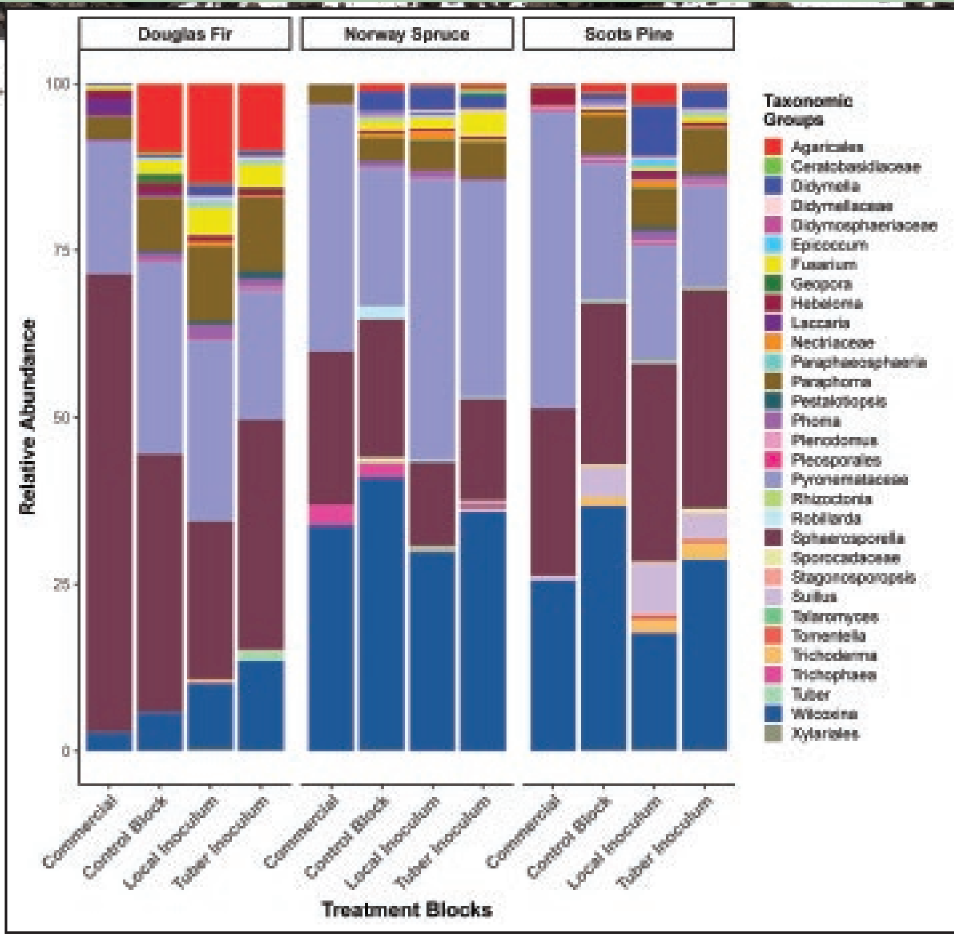


Figure 5. Diversity and relative abundance of all fungi detected on Douglas fir, Norway spruce and Scotch pine seedlings. *Wilcoxina, Sphaerosporella,* and *Pyronemataceae* accounted for the highest relative abundance of ectomycorrhizal taxa, while Paraphoma, Didymella, Fusarium, and Trichoderma were the most common non-mycorrhizal taxa detected on seedling roots.

environments. Rather, it appears that the spore load of ectomycorrhizal fungi that are dispersed naturally into the system post-fumigation is robust enough to support mycorrhizal establishment of Christmas tree seedlings at our experimental sites. This was evident by the high ectomycorrhizal occupancy of control plants that were not inoculated with ectomycorrhizal fungi, and the low persistence of inoculated fungi detects on seedlings after 1 year of growth. Thus, neither collecting and using spores from nearby mushrooms, nor purchasing and applying commercial mycorrhizal blends, can be recommended as a wise investment of resources or time.

ACKNOWLEDGEMENTS

We are grateful to those who have supported this research and made it possible, including Jerry and Josh Peterson of Peterson's Riverview Nursery, LLC (Allegan, MI) and with Dan Wahmhoff of Wahmhoff Farms Nursery (Gobles, MI), and support from the Michigan Christmas Tree Growers Association and Project GREEEN (GR18-076).