THE BIOLOGY AND ECOLOGY OF *TUBER AESTIVUM* MYCORRHIZAE ESTABLISHMENT IN THE GREENHOUSE AND THE FIELD

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By

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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

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THE BIOLOGY AND ECOLOGY OF *TUBER AESTIVUM* MYCORRHIZAE ESTABLISHMENT IN THE GREENHOUSE AND THE FIELD

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ABSTRACT

Truffles, of the ectomycorrhizal genus *Tuber*, are cultivated in plantations to offset declining wild production. Although methods for truffle plantation management have been published, methods for the production of truffle-colonized seedlings are rarely published. In a greenhouse study I tested the effect of lime type, inoculation technique, and truffle source on oak growth and mycorrhizal colonization. I found that the type of lime used to raise potting mix pH can affect the growth rate of root systems inoculated with Burgundy truffle (*Tuber aestivum*). I also report on the effects of two greenhouse seedling production systems on growth and colonization of oak seedlings. I found that book-type containers and a peat-based medium produced smaller yet better-colonized seedlings than seedlings produced in RPM® containers with ground bark-based RPM medium. Finally, I identified and monitored the ectomycorrhizal community on inoculated seedlings for three years and found that both *T. aestivum* colonization levels and native species richness increased in the field, indicating that native species, in the short term, did not displace *T. aestivum*.

Chapter 1 - INTRODUCTION AND RESEARCH QUESTIONS

Truffle cultivation – Perspectives for the USA

Due to the high market value of European black truffles, entrepreneurs and researchers alike are establishing truffle orchards (truffière) worldwide. As with any developing agricultural crop, questions about the truffle species' tolerance of different climates and environments abound. Folk knowledge and historical truffle cultivation research provide incomplete guidance concerning truffle growth and production in exotic environments, because most research has been conducted within the truffle's natural range (Hall *et al.* 2001). In the 1980's, Ian Hall from New Zealand Crop and Food Research Ltd challenged the traditional truffle cultivation paradigm by producing truffles in climates and soils quite different from natural ranges. This sparked a movement to establish truffières worldwide (Hall *et al.* 2001). As of 2007, artificial truffières in Europe (Chevalier & Frochot 1989; Chevalier 1999), New Zealand (Hall & Yun 2001), Australia (Malaczuk), Sweden (Wedén 200X), and the United States (Garland 2001; Michaels 2007) are producing truffles. Orchards are planned for Chile (Slice of Heaven Truffiére, www.sohtruffiere.com, Talca, Chile), Canada (Berch *et al.* 2005), and Finland (Salem Shamekh) . From experiences in Europe and New Zealand, it appears that the most important geographic factors to consider when establishing a producing truffière are climate, vegetation history, soil pH,

and other soil characteristics (Chevalier *et al.* 2001; Hall *et al.* 2001). Additionally, prospective growers should reflect on what species of host plant to use, what species and strain of truffle fungus to use, and finally how to prepare and maintain the truffière. Here I will address what is known about truffle biology and ecology including the geographic range limitations, the host species associations, and the different cultivation methods available to grow both the Perigord Black truffle (*Tuber melanosporum* Vitt.) and the Burgundy truffle (*T. aestivum* Vitt., syn. *T. uncinatum* Chatin). I will then discuss greenhouse production techniques of Burgundy truffle-colonized oaks as they relate to my research questions. The subsequent chapters provide detailed descriptions of my research questions, results, and conclusions.

Truffle biology and ecology

Root symbionts including ectomycorrhizal fungi play an integral role in controlling ecosystem function in dominant temperate forests worldwide (Smith & Read 1997). They provide their host plants with trace minerals and protection from pathogens, pests, and drought (Garbaye 2000; Landeweert *et al.* 2001; Allen *et al.* 2003; Govindarajulu *et al.* 2005), and in return they receive organic carbon from their hosts (Smith & Read 1997). The mushrooms produced by ectomycorrhizal fungi are important sources of food for forest animals (Carey *et*

al. 2002) and of medicine and sustenance for humans in many societies (de Román *et al.* 2006).

Ectomycorrhizal fungi in the genus *Tuber*, produce subterranean fruit bodies that emit volatile sulphuric hydrocarbons (Bellesia *et al.* 1998; Bellesia *et al.* 2001) which attract vectors that disperse the fungal spores (Bellina-Agostinone *et al.* 1987). Due to the aromatic volatiles, truffles have long been prized for their gastronomic characteristics (Hall *et al.* 2001). Currently, the Perigord black truffle (*Tuber melanosporum* Vitt.) is the second most valuable truffle in the world selling for 250-1200 US$/kg (wholesale) in 2000 (Hall *et al.* 2003).

Over the last 100 years there has been a dramatic decline in forest mushroom production and subsequent harvest (Cherfas 1991). This decline is attributed to water and air pollution, changes in forest structure including deforestation, and loss of local harvesting knowledge because of world wars (Hall *et al.* 2003). In the Netherlands, the number of mushroom species collected per foray declined from 72 in 1912 to 38 by 1982 (Eef Arnold in Cherfas 1991). Additionally, in central Europe, Johannes Schmitt found that chanterelles for sale in 1975 were fifty times smaller than in those in 1958 (in Cherfas 1991). Additionally, the volume of Perigord black truffles (*T. melanosporum*) sold in France declined from 2000 tons per year at beginning of 20th century to less than 150 tons by the beginning of the 21st century (Hall *et al.* 2003).

To address the deficit of truffles collected from naturally infected trees, scientists and landowners in the 1970s began developing methods for the

cultivation of truffle fungi in manmade orchards (Fontana & Bonfante 1971; Chevalier & Grente 1979). Orchards currently produce approximately half of the truffles harvested world wide (Hall *et al.* 2003). The majority of orchards occur within the natural geographic range of the truffle fungi.

Truffle natural geographic range

Geographically, *T. melanosporum* is more restricted than *T. aestivum* but also brings higher prices in world markets (Chevalier *et al.* 2001). It is found in France, Italy, Spain, Bulgaria, Croatia, and Portugal (Hall *et al.* 2001). In its native range, *T. melanosporum* competes and fruits best in rocky, warm, open forests with moderate winters and regular rainfall during the summer (Sourzat 2002). Production of fruiting bodies is most abundant on slopes protected from cold or dry wind (Sourzat 2002). Climatically, average rainfall of 600-1500 mm, average summer temperatures of 17.5-22 °C, and average winter temperatures of 1-8 °C are optimum for *T. melanosporum* (Demas 1983; Zambonelli & Di Munno 1992). Areas experiencing consistently frozen soil during the winter are not appropriate for *T. melanosporum* production because the fungus fruits from December to March and fruit bodies spoil once frozen. In New Zealand, truffières planted in colder and wetter coastal regions have not produced truffles (Hall *et al.* 2001).

Potential USA range

The most appropriate areas in the US to grow *T. melanosporum* based on the climate restrictions outlined above include areas where the ocean buffers extreme temperature variation throughout the year, but where fog and rain are limited during the summer so the truffière floor receives solar radiation that warms and dries the soil. This would include areas along the Pacific coast that receive moderate, but not extreme quantities of rain (central Washington, western and central Oregon, and northern and central California (National Climatic Data Center 2002). Additionally, areas along the Gulf of Mexico and on the Atlantic coast where extreme temperature variations are moderated may be appropriate (National Climatic Data Center 2002). Regions that experience frozen soil in the winter should be excluded.

With respect to rainfall, areas from central Oklahoma east to the Atlantic ocean and parts of the Pacific coast all receive sufficient precipitation to support *T. melanosporum* production (National Climatic Data Center 2002). Previous studies of truffle production reinforce the importance of regular, but not high levels of precipitation or irrigation during the summer (Chevalier 1999; Chevalier *et al.* 2001; Sourzat 2002). As a general rule, orchards should be irrigated after 20 days without rain (Chevalier 1999). Many areas with suitable temperature (WA, OR, CA, MO) receive little to no summer rainfall, so prospective growers should ensure access to irrigation during the summer. Truffle production is a long-term investment, and in many areas of the United States demand for water

is predicted to increase (Gleick *et al.* 2005). Producers should establish orchards in areas where water is inexpensive and projected to be available for agricultural irrigation in the future. Consequently, much of southern California, Arizona, Colorado, Nevada, New Mexico, and Texas should be excluded from consideration for truffle production. In summary, geographic limitations based on climate and rainfall trends will impose regional restrictions on *T. melanosporum* production.

The reduced market price of *Tuber aestivum* when compared to *T. melanosporum* is offset by *T. aestivum's* tolerance of higher planting densities and the subsequent increase in truffle yield per hectare. Additionally, *T. aestivum* tolerates more variable climates, thus there is a wider potential geographic range in the USA. *Tuber aestivum* is found from Northern Africa to Sweden and from Ireland to Russia (Chevalier & Frochot 1997; Wedén 2004) in oceanic settings with little seasonal change and evenly distributed rainfall, mountainous settings with warm dry summers and long cold winters, and continental settings with hot dry summers and cold rainy winters (Chevalier *et al.* 2001). In Italy, it is native to areas with heavy spring and fall rain (Zambonelli & Di Munno 1992). Because of the variability of its native range, and the fact that it fruits earlier in the autumn and winter, *T. aestivum* is appropriate for slightly colder areas with more extreme weather than *T. melanosporum*. Regardless, *T. aestivum* should not be grown in areas where soil freezes before December. Unlike *T. melanosporum* which flourishes in warm, relatively dry sites, *T. aestivum* requires higher quantities of

water during the summer so availability of irrigation is an important consideration in truffière establishment.

Climate provides guidance on the appropriate region for truffle growth, but within-site characteristics such as soil properties and site history should be evaluated before selecting the specific orchard site. In Europe, *T. melanosporum* orchards that produce consistent yields occur in iron-rich, free draining limestone soils that have organic matter levels less than 8%, high levels of Ca and Mg, moderate levels of P, low levels of Na, and are well aerated and granular in texture (Poitou 1986; Poitou 1990; Hall & Yun 2001). Additionally, and of critical importance, the soil must have a minimum pH of 7.5 (optimum pH 7.9) (Hall & Yun 2001). Soil can be limed to increase the pH to this level but if the soil is artificially limed, the pH level needs to be maintained in the top 15 cm of the soil where the fungus is found. In fact it may be preferable to select a truffle site with a pH range between 6.0 and 7.0 and use lime to artificially increase the pH. This strategy will reduce colonization of trees by native mycorrhizal competitors that do not tolerate the higher pH.

Tuber aestivum has more flexible soil requirements than *T. melanosporum*. It flourishes under conditions of high C/N ratios (9-12 to 20) (Lulli *et al.* 1999) and roots shaded by a litter layer or mulch (Chevalier & Frochot 1997; Wedén *et al.* 2004a; Zambonelli *et al.* 2005). It tolerates higher levels of nitrogen, and more clay and silt, but less fine sand in the soil than *T. melanosporum* (Chevalier *et al.* 2001). The minimum pH requirement for *T. aestivum* fruiting is 7.0, with the optimum around 7.5, but it is found in nature in

soils with pH below 6.9 and above 8.0 (Chevalier *et al.* 2001; Wedén *et al.* 2004a).

Truffière management

When choosing a planting location, site history is critically important. Orchards should be planted at least 75 m away from forested areas containing native, competitive mycorrhizae (Hall & Yun 2001). Good choices for sites include old agriculture fields that will have low ectomycorrhizal spore banks, but hypogeous ectomycorrhizal sporebanks may nevertheless be pre-established by mycophagous animals (Ashkannejhad & Horton 2006). Areas to be avoided include clearcut forests, areas that are very wet or have poor drainage, and locations with pH less than 5.5 (Hall & Yun 2001).

In combination with site selection, proper host plant choice will help ensure a successful truffière (Sourzat 2002). Effective host plants need to form mycorrhizal relationships with the truffle fungus, be well-adapted to the local environment, be drought, disease, and pest resistant, and have a growth pattern that is most appropriate to the colonizing truffle fungus (columnar or vase-shaped canopy for *T. melanosporum* to allow sunlight penetration; spreading canopy for *T. aestivum* to ensure shade and a litter layer). Also, hosts should be long-lived to maximize the productivity of the orchard. Successful plant/fungus pairs from other continents can be used as a guide when selecting appropriate host plants native to a target region. For example, in France, successful hosts for *T.*

melanosporum are pubescent oak (*Q. pubescens*), holm oak (*Q. ilex*), and Byzantium hazel (*Corylus colurna*) while less successful hosts include penduculate oak (*Q. robur*) and common hazel (*C. avellana*) (Chevalier 1999). In New Zealand and North America, common hazel (*C. avellana*) are quite successful (Garland 2001; Hall *et al.* 2001). Productive hosts for *T. aestivum* include common hazel (*C. avellana*), white hornbeam (*Ostrya carpinifolia*), black pine (*Pinus nigra*), and peduculate oak (*Q. robur*) (Chevalier *et al.* 2001). North American species closely related to the European hosts, such as American hazel (*Corylus americana*) and native white oaks tolerant of high pH soils would be good choices for evaluation as plant hosts in the US.

Once the orchard location, the host species, and the truffle species have been selected, then any soil imbalances at the planting site must be addressed prior to putting the truffle colonized seedlings in the ground. Land can be limed, fertilized, or have micro- or macronutrient imbalances corrected as necessary (Sourzat 2002). Soil amendments should be incorporated to a depth of 10-20 cm, and soils should be retested after six months to ensure that amendments were effective. Once the soil is properly adjusted, the seedlings can be planted. The best time for planting trees is in the late fall or early spring once the soil is workable, without danger of compaction.

Current truffière management practices range from planting the trees and walking away to yearly tilling and pruning (Hall *et al.* 2001; Sourzat 2002). The management method chosen depends on how much time and money the producer wants to spend maintaining the truffière. The Tanguy method which

consists of mowing the orchard as needed can produce good yields, but the onset of production is late (10+ year) (Chevalier 1999). The Pallier model, based on fruit orchard management, includes soil tilling, irrigation (as needed), and tree pruning. It is expensive, but can produce good results earlier than the Tanguy method (Chevalier 1999). As a rule, regardless of management intensity, excessive irrigation and fertilization should be avoided (Chevalier 1999). Fertilization should be based on sound soil test results (Chevalier 1999). Tilling the soil too deeply should also be avoided to reduce damage to colonized roots (Chevalier 1999).

While the production practices discussed above are considered successful in Europe, there is little is known about their success in the USA, because most truffières are less than five years old and no controlled studies have been reported.

Greenhouse-based seedling production

From an orchard owner's point of view, the functional details of seedling production are less relevant than the knowledge that the seedlings purchased are well colonized by the truffle fungus. Researchers and nursery owners, on the other hand, have a vested interest in optimizing the greenhouse based seedling inoculation and colonization processes to ensure consistent production of high quality seedlings. Currently, greenhouse based truffle tree production consists of germinating host seeds in trays on media or vermiculite, transplanting the

germinated seeds to larger containers filled with truffle-spore inoculated soil, then allowing the seedling to grow in the greenhouse for one to two years to ensure good colonization of the root system by the fungus (Chevalier & Grente 1979). Although there are many published accounts of effective cultural practices for truffle orchard establishment and management (Chevalier & Frochot 1997; Callot 1999; Olivier 2000; Hall *et al.* 2001; Olivier *et al.* 2002; Sourzat 2002) published recommendations for the process of producing truffle-colonized seedlings are few and generally vague. In order to ensure high yielding truffle orchards it is critical to plant seedlings well colonized by the truffle fungus. Currently, most publications on truffle cultivation treat the production of truffle-colonized seedlings as a trade secret, simply suggesting that landowners purchase seedlings that are certified by an impartial authority to be well-colonized by the advertised truffle species. In some countries, notably France, Italy, Spain, and New Zealand, this is possible (Fischer & Colinas 1996). Unfortunately, this is not yet the case in the USA, and the number of nurseries that reportedly produce consistently well-colonized seedlings is quite low. To the extent that effective production methods are kept as trade secrets, the industry is hampered in its efforts to improve upon the status quo. Truffles have the potential to be a lucrative agroforestry crop in many areas of the world, but the lack of public knowledge about greenhouse-based methods of producing well-colonized seedlings limits the industry (Hall *et al.* 2003).

Hall states that, “poor seedling quality remains a serious problem in *Tuber*-inoculated plants (Hall *et al.* 2003),” and that “factors related to inoculum

processing and seedling growth and care remain trade secrets (Hall *et al.* 2003)." Consequently, there is a need to establish and publish production methods that ensure that nurseries produce and sell well colonized seedlings. My research focuses on the combination of lime type, truffle source, inoculation technique, and production method which best produce well colonized and healthy seedlings. I also investigated the diversity of native mycorrhizal species which may become competitors of the truffle fungus in the greenhouse and in the field.

Research questions and experiments

Chapter 2: Does lime type, truffle source, or inoculation technique affect *Tuber aestivum* colonization of *Quercus robur* host roots?

European black truffles can be profitable agroforestry crops outside their native ranges. Truffle fungi grow symbiotically as ectomycorrhizae on the roots of host trees, notably hazels and oaks. Conditions in the central USA appear conducive to cultivation of the Burgundy truffle (*Tuber aestivum* Vitt. syn. *T. uncinatum* Chatin), but research is needed to determine effects of management practices on truffle establishment and fruiting. In a greenhouse study we tested the effect of lime type, inoculation technique, and two truffle sources on Pedunculate oak (*Quercus robur* L.) growth and mycorrhizal colonization. We found that the type of lime used to raise potting mix pH can differentially affect the growth rate of root systems inoculated with different selections of Burgundy truffle inoculum. Seedlings inoculated with one selection of the truffle and grown

in potting mixes amended with natural crushed dolomitic limestone developed larger root systems with more truffle mycorrhizae compared with potting mix amended with high-calcium pelletized quick-release lime. Seedlings inoculated with a second truffle selection were not affected by lime source and developed root systems as large as those developed with the first truffle source grown with natural lime. Supplemental root dip inoculation did not improve levels of colonization beyond those accomplished by potting mix infestation with truffle ascospores. Use of a hygroscopic polymer to maintain ascospore suspension in the inoculum slurry used to infest the potting mix had no effect on root system development or mycorrhiza formation.

Chapter 3: Which seedling production method, 1) standard methods and media or 2) RPM methods and media produce seedlings with greater *Tuber* colonization and larger size?

Ectomycorrhizal fungi play integral roles in many forest ecosystems. Anthropogenic factors have caused precipitous declines in fruiting by some ectomycorrhizal mushroom species. Truffles, the hypogeous ascocarps of the ectomycorrhizal genus *Tuber,* are currently cultivated in orchards to offset declining wild production. Although methods for truffle orchard establishment and management have been published, there are not yet published methods for the production of truffle-colonized seedlings. Here we address a series of four questions related to greenhouse production of oak seedlings well colonized by

the Burgundy truffle fungus (*Tuber aestivum*). In Experiments 1 & 2, we compared the effects of two seedling production systems on growth of *Quercus bicolor x Quercus robur* seedlings inoculated with *T. aestivum*. We found that a peat-based medium produced smaller better-colonized seedlings. In experiment 3, we modified the bark-based medium in an attempt to favor the fungus, but did not achieve acceptable colonization levels. In the final experiment, we assessed the effect of pH on *T. aestivum* colonization. We conclude that the peat-based medium is most appropriate for growing seedlings well colonized by *T. aestivum*. We are currently investigating the effects of watering and fertilizer regimes on *T. aestivum* colonization of *Q. bicolor* x *Q. robur* seedlings.

Chapter 4: Competitive native mycorrhiza

Ectomycorrhizal fungi play integral roles in many forest ecosystems. Anthropogenic factors have caused precipitous declines in fruiting by some ectomycorrhizal mushroom species. Truffles, the hypogeous ascocarps of the ectomycorrhizal genus *Tuber,* are currently cultivated in orchards to offset declining wild production. Truffle cultivation begins by germinating host seeds, inoculating the host seedlings with truffle spores, growing the seedlings in greenhouses until the mycorrhizal relationship is well established, and then outplanting the seedlings. Little is known in the USA about the effect of native ectomycorrhizal species on colonization of host trees by the European Burgundy truffle fungus. Here we identify the fungal community composition in the

greenhouse in three types of potting media, and then track fungal community composition for two years after outplanting. We found that the infection rates of fungal species commonly present in the greenhouse decline to low levels in the field. We also found that after two years of field growth, both *Tuber* colonization levels and native ectomycorhizal species richness and abundance increased,

indicating that native species, in the short term, do not displace the introduced *Tuber* spp.

Final thoughts

Producing truffles outside of their native range is a growing industry. There is still much to be learned about the optimum climate, soil conditions, and management regimes. Because there are still lots of lessons to be learned in this field, we can expect many false starts. The challenge will be for researchers to learn as much as possible early on, to ensure that citizens are provided with proper guidance when making biologically based business decisions.

Chapter 2 - COLONIZATION OF PEDUNCULATE OAK BY THE BURGUNDY TRUFFLE FUNGUS IS GREATER WITH NATURAL THAN WITH PELLETIZED LIME

Introduction

The Périgord and Burgundy black truffles of Europe (*Tuber melanosporum* Vitt., and *T. aestivum* Vitt. syn. *T. uncinatum* Chatin, respectively) are among the most valuable gourmet mushrooms in the world (Hall & Yun 2001; Hall *et al.* 2003). These fungi function in nature as symbiotic ectomycorrhizal fungi (Smith & Read 1997), aiding their host trees in acquisition of minerals and water from the soil. In Europe, these fungi occur in both extensively and intensively managed truffières (truffle-producing forests and plantations) on suitable sites (Riousset *et al.* 2001). Even in Europe, the influences of site factors and management practices on successful truffière establishment, maintenance, and productivity are incompletely understood and under vigorous study. Nevertheless, both of these truffle species have been successfully cultivated in their native Europe, and the Périgord black truffle has also been cultivated in New Zealand (Hall *et al.* 2001), Australia (James Trappe, pers. comm.), and the USA (Garland 2001). We expect additional successes using these and other truffle species.

Several sets of interdependent decisions need to be made in the process of establishing truffières. First, an appropriate site must be found, based on land use history, landform position, soil properties, and local climate (e.g., Chevalier *et*

al. 2001; Hall *et al.* 2001). Local climate will be a determining factor in selecting which truffle and host tree species to consider. Though the soils on many otherwise appropriate sites are too acid to support truffle production, this deficiency can be overcome with large-scale applications of lime to the topsoil prior to planting (Garland 2001; Hall *et al.* 2001; Hall & Zambonelli 2005). The Burgundy truffle fruits best in the range of pH 7.1—8.0 (Chevalier 1978b; Wedén *et al.* 2004a), whereas soils in the central USA generally range in pH 5.0—6.5 (NRCS on going). Natural dolomitic lime is readily available throughout the central USA, but varies greatly in chemistry, including the Ca/Mg ratio. More highly refined forms of agricultural lime (pelletized, with very high Ca/Mg) are also available from fewer sources, and therefore at greater expense in general due to shipping costs. Our study addresses the effectiveness of these two classes of lime in supporting Burgundy truffle mycorrhiza formation with Pedunculate oak (*Quercus robur* L.) in the seedling production phase.

The second set of decisions in truffière establishment involves production of seedlings well-colonized by the chosen truffle species. Successful truffle mycorrhiza development requires efficient root contact with truffle propagules (ascospores or mycelial fragments) in a potting mix that is conducive to both seedling root growth and truffle mycelial growth. Host seed is often germinated in advance of inoculation, to permit early selection of seedlings based on initial growth performance. Once germlings of the selected host tree species are planted in the infested potting mix, the greenhouse environment must be maintained within limits that favor efficient truffle mycorrhiza formation. In

addition to evaluating the effects of lime type on root system growth and Burgundy truffle mycorrhiza formation, our study addressed the effectiveness of supplementary root dip inoculation in addition to potting mix infestation, and the effect of including Stockosorb, a hygroscopic gel, in the inoculum suspension. Our hypothesis was that Stockosorb would enhance mycorrhiza formation by maintaining more uniform levels of propagules in the potting mix.

The final set of decisions involves the management of truffières once the seedlings are outplanted. While a great deal has been learned about truffière management in recent decades (Chevalier & Frochot 1997; Chevalier *et al.* 2001; Hall *et al.* 2001; Sourzat 2002; Ricard 2003), this is the subject of intensive on-going research. Much of this research is inherently long-term, because truffières generally require four or more years for adequate establishment prior to first fruiting (Hall *et al.* 2001). Seedling root system size is a determinant of seedling survival (Dey & Parker 1997), and may influence the extent to which root systems colonize truffière sites. Our study reports differential effects of lime type on overall root system development using two strains of the Burgundy truffle.

Materials and methods

Experimental design

This experiment compared the effects on root system development of two types of lime, three root dip inoculation techniques, two soil infestation methods,

and two sources of Burgundy truffle inoculum, resulting in 24 treatment combinations. The experiment was arranged in the greenhouse as a randomized complete block experiment with 10 replicates for a total of 240 seedlings.

Potting mix, host plants, and fungal inoculum

The potting mix consisted of a 2:1:1 mixture of air-dried field soil, vermiculite, and perlite (v:v:v). The field soil was loamy silt topsoil excavated at the University of Missouri Horticulture and Agroforestry Research Center, New Franklin, MO, USA from an old alfalfa field that had been cleared of trees for over 100 years. The soil was hand crumbled, and then sufficient lime was incorporated to raise pH to ca. 7.5, well within the range known to support Burgundy truffle fruiting (Wedén *et al.* 2004a; Wedén *et al.* 2004b). Half the substrate was amended with MFA Pel-Lime® (Missouri Farmers Association, Columbia MO, USA); the other half with a natural dolomitic lime with higher magnesium (Mg) content. MFA Pel-Lime® had a $CaCO_3$ equivalent (CCE) of 90.5, an effective neutralizing material (ENM) value of 660, and a Mg content of 3.6% (AOAC 1984). The natural dolomitic lime with substantial magnesium had a CCE of 101.4, an ENM of 639, and a Mg content of 12.6% (AOAC 1984).

We selected *Quercus robur* as our experimental host because it is one of the prominent host species for the Burgundy truffle in Europe. The Burgundy truffle is found as far north as Gotland, Sweden (Wedén *et al.* 2004a) and south to Spain and Italy (Chevalier *et al.* 2001). *Quercus robur* acorns from a cold

hardy planting in Indiana were acquired from Vallonia State Nursery, Vallonia, IN, USA.

Burgundy truffle ascocarps (truffles) representing one French and one Swedish population of *Tuber aestivum* Vitt. were obtained from Dr. Gérard Chevalier (INRA, Clermont-Ferrand, France) and Dr. Eric Danell (Uppsala University, Sweden), respectively. Both sources were harvested October 2001. The Swedish truffles were shipped fresh by air from Gotland, Sweden, and frozen on arrival. The French truffles were sliced and lightly-dried, and then transported to the U.S.A. in February 2002. Ascospores from lightly-dried truffles are known to function well as inoculum (Chevalier pers. comm.). Ascospore inoculum was produced by removing the outer “rind” (peridium) of each truffle, suspending the fragmented fertile interior (gleba) in tap water, and blending the material to produce a suspension of nearly uniform particle size. Inocula produced from the dried French truffles and from the fresh-frozen Swedish truffles are referred to as Ta-1 and Ta-2, respectively.

Inoculation and seedling production

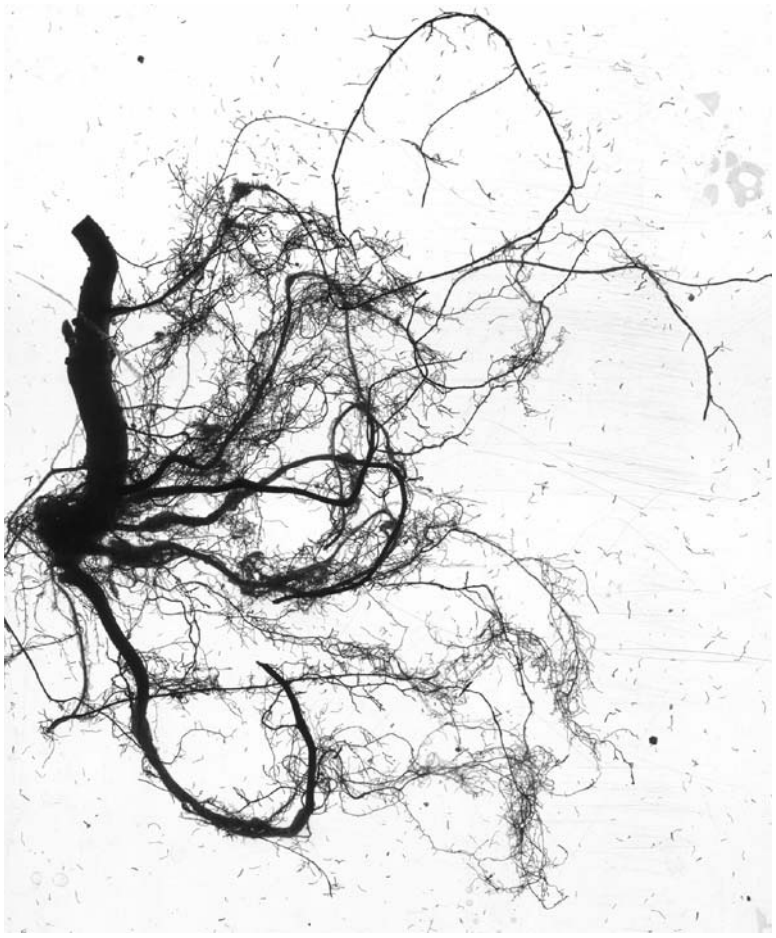
Acorns were germinated in the greenhouse in Promix (Premier Horticulture Ltd, Quebec, Canada) for approximately 2 months during June-July 2002. In August 2002, the seedlings were removed from their pots, the Promix was washed from their roots, and they were randomly assigned to treatments.

Inoculation involved a potting mix infestation treatment and a root dip treatment with either Ta-1 or Ta-2, so that each tree had spores from one source incorporated into its potting mix and also placed directly onto its roots. The two potting mix infestation treatments both involved mixing an aqueous slurry of

ascospores (W + Ta) into the potting mix at the concentration of 0.7 x 10^6 ascospores L^{-1} potting mix (Figure 2-1). In one of the two treatments (W + Ta + S), the ascospore slurry was amended with 0.18 mL L^{-1} Stockosorb® (Stockhausen, Greensboro, NC, USA). Stockosorb® is a gel-based compound that when hydrated helps to maintain moisture in potting mixes. We used Stockosorb® to help keep the spores suspended during inoculation treatments.

The three root dip treatments were: a) roots dipped in water with no spores (W); b) roots dipped in an aqueous spore slurry (W + Ta); or c) roots dipped in an aqueous spore slurry containing Stockosorb® (W + Ta + S). The root dip slurries contained 2 x 10^7 ascospores L^{-1}, sufficient to provide 10^6 ascospores per plant to the extent that the spores could be distributed equally and completely. In the W + Ta+ S root dip treatment, 0.18 mL L^{-1} Stockosorb® was added to the ascospore slurry. After root-dipping, seedlings were planted singly in waxed cardboard containers provided with drainage (10 cm x 10 cm x 26 cm deep) and filled to 20 cm with ca. 1.85 L of appropriately limed and inoculated potting mix. Seedlings were maintained in a greenhouse and watered approximately 3 times per week.

Figure 2-1: A typical image produced by scanning a root system with an Epson EU22 flat-bed scanner. Such images were analyzed using WinRhizo software to estimate total root system volume, length, and number of root tips.



Data collection

After 11 months in treatment, the seedlings were removed from their pots and their roots were gently washed free of potting mix. Each root system was then spread on a flat surface and scanned with an Epson EU22 scanner (Epson America Inc., Long Beach, CA, USA) (Figure 2-1). The scanned image of each root system was analyzed with WinRhizo image analysis software (Régent Instruments, Montreal, Quebec, Canada) to estimate three growth metrics: total root system volume, total root system length, and total number of root tips.

The extent of fungal colonization was also assessed for each root system. Three hundred root tips from each seedling were randomly sampled by spreading the root system onto a numbered grid and harvesting a root segment containing 30 root tips from each of 10 randomly selected locations. These root samples were frozen (-20 C) in 15% glycerol for later examination of fungal colonization. Upon thawing, each root tip was examined by light microscopy (100X and 400X) for the presence of an ectomycorrhizal fungal mantle (Peterson *et al.* 2004). Ectomycorrhizal root tips were identified microscopically as Burgundy truffle-colonized or "other" (Müller *et al.* 1996; Rauscher *et al.* 1996). The ectomycorrhizal condition of each seedling was characterized by multiplying the total number of root tips scanned by the percentages of sampled root tips which were ectomycorrhizal or, more specifically, Burgundy truffle-colonized. Broken tips were excluded from analysis. The number of seedlings in each treatment that died during the experiment was also recorded.

Data Analysis

Root system metrics (total root system volume, total root system length, and total number of root tips) and fungal colonization metrics (total number of ectomycorrhizal root tips and total number of Burgundy truffle-colonized root tips) for each seedling were analyzed by linear regression (PROC GLM, $\alpha = 0.05$; SAS/STAT System Release 7.1, Inst. Inc., Cary, NC) (Table 4-1). Least significant means were used to determine differences among treatments.

The effects of the three root dip inoculation techniques and inoculum source on seedling mortality were analyzed separately for the two inoculum sources (Ta-1 and Ta-2) using contingency table analysis and the χ^2 statistic (Sokal & Rohlf 1995) to compare expected values with observed mortality. All expected values were > 3.0, thus providing a robust analysis (Steel & Torrie 1980).

Results

Truffle inoculum source

After 11 months in treatment, seedling root systems inoculated with the Ta-2 truffle source had grown larger than those inoculated with the Ta-1 source (Table 2-1 and Figure 2-2). Inoculation with Ta-2 resulted in 31% greater root volume ($P < 0.001$, Figure 2-2a), 29% greater total root length ($P < 0.001$, Figure 2-2b),

and 25% more total root tips ($P < 0.001$, Figure 2-2c) than did inoculation with Ta-1. Root systems inoculated with Ta-2 also developed 22% more ectomycorrhizal root tips ($P < 0.001$), including more root tips identified as Burgundy truffle-colonized (Table 2-1 and Figures 2-2d and 2-2e, respectively). Nevertheless, the numbers of ectomycorrhizal root tips per cm^{-1} of root length and the numbers of truffle-colonized root tips cm^{-1} of root length were very similar for root systems inoculated with either Ta-1 or Ta-2. Seedlings inoculated with Ta-1 and Ta-2 developed 2.47 and 2.43 mycorrhizal root tips cm^{-1} of root length and 1.55 and 1.56 truffle-colonized root tips cm^{-1} of root length, respectively. Seedlings inoculated with Ta-1 experienced greater mortality than seedlings inoculated with Ta-2 (26% vs. 9%, respectively, $\chi^2 = 12.5$, df = 1, $P < 0.001$).

Figure 2-2: Characteristics of root systems inoculated with Ta-1 or Ta-2 sources of Burgundy truffle ascospores. a) Root system volume. b) Root system length. c) Root tips per plant. d) Number of ectomycorrhizal root tips. e) Number of ectomycorrhizal tips colonized by Burgundy truffle. Within each panel, histogram bars with different letters represent significantly different means (α = 0.05).

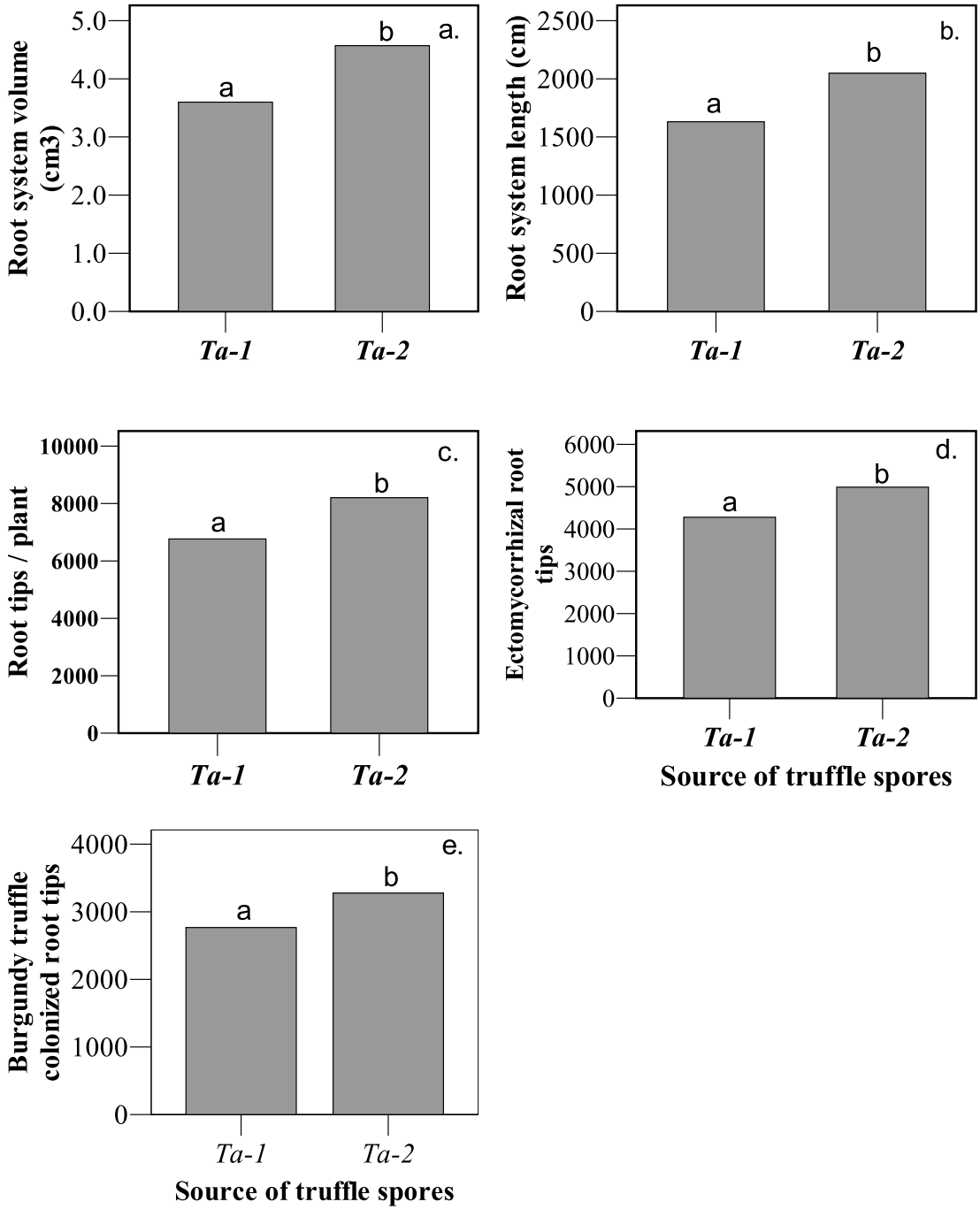

a.
Root system volume (cm3)
5.0
4.0
3.0
2.0
1.0
0.0
a
b
Ta-1
Ta-2
b.
Root system length (cm)
2500
2000
1500
1000
500
0
a
b
Ta-1
Ta-2
c.
Root tips / plant
10000
8000
6000
4000
2000
0
a
b
Ta-1
Ta-2
d.
Ectomycorrhizal root tips
6000
5000
4000
3000
2000
1000
0
a
b
Ta-1
Ta-2
Source of truffle spores
e.
Burgundy truffle colonized root tips
4000
3000
2000
1000
0
a
b
Ta-1
Ta-2
Source of truffle spores

Table 2-1: ANOVA table. Results of analysis of variance to identify effects of lime type, inoculation methods, and truffle inoculum source on *Quercus robur* root system growth and mycorrhization in the greenhouse.

Table 2-1: ANOVA table. Results of analysis of variance to identify effects of lime type, inoculation methods, and truffle inoculum source on *Quercus robur* root system growth and mycorrhization in the greenhouse.

	Response variables[b]									
	Total root volume		Total root length		Total no. root tips		Total no. mycorr. root tips		Total truffle-colonized root tips	
Source of variation[a]	df	F	df	F	df	F	df	F	df	F
Model	32	2.07**	32	2.34***	32	2.54***	32	3.01***	32	2.27***
Block	9	1.33	9	1.56	9	2.99**	9	4.74***	9	2.78**
Lime	1	1.91	1	4.73*	1	8.06**	1	3.08	1	6.2*
Root Dip	2	10.71***	2	7.57***	2	6.55**	2	10.63***	2	9.02***
Potting Mix Infest.	1	0.37	1	0.17	1	0.10	1	0.41	1	0.38
Source	1	17.53***	1	19.47***	1	15.34***	1	13.38***	1	10.59**
Lime x Root Dip	2	3.10*	2	3.52*	2	2.10	2	3.69*	2	3.55*
Lime x Potting Mix Infest.	1	0.31	1	1.13	1	0.07	1	1.50	1	0.53
Lime x Source	1	1.77	1	10.93**	1	10.17**	1	6.95**	1	1.81
Root Dip x Potting Mix Infest.	2	0.07	2	0.62	2	0.44	2	0.69	2	0.69
Source x Root Dip	2	1.41	2	3.70*	2	5.63	2	2.11	2	1.07
Source x Potting Mix Infest.	1	3.34	1	0.81	1	0.82	1	1.17	1	1.89
Lime x Source x Root Dip x Potting Mix Infest.	9	0.48	9	0.74	9	0.82	9	1.38	9	0.91
Within Groups	170	—	170	—	170	—	156	—	156	—
Total	202	—	202	—	202	—	188	—	188	—

[a] sources of variation: Block, 10 randomized complete blocks; Lime, Pel-lime® or natural dolomitic lime; Root Dip, water (W) or water + *Tuber aestivum* spores (W + *Ta*) or water + *T. aestivum* spores + Stockosorb® (W + *Ta* + S); Potting Mix Infest., potting mix mixed with water and *T. aestivum spores* (W + *Ta*) or water + *T. aestivum* + Stockosorb® (W + *Ta* + S); Source, *T. aestivum* ascospores from *Ta*-1 or *Ta*-2 inoculum source.

[b] df, degrees of freedom; *, **, *** indicate F-value significance at $P < 0.05$, 0.01, and 0.001, respectively.

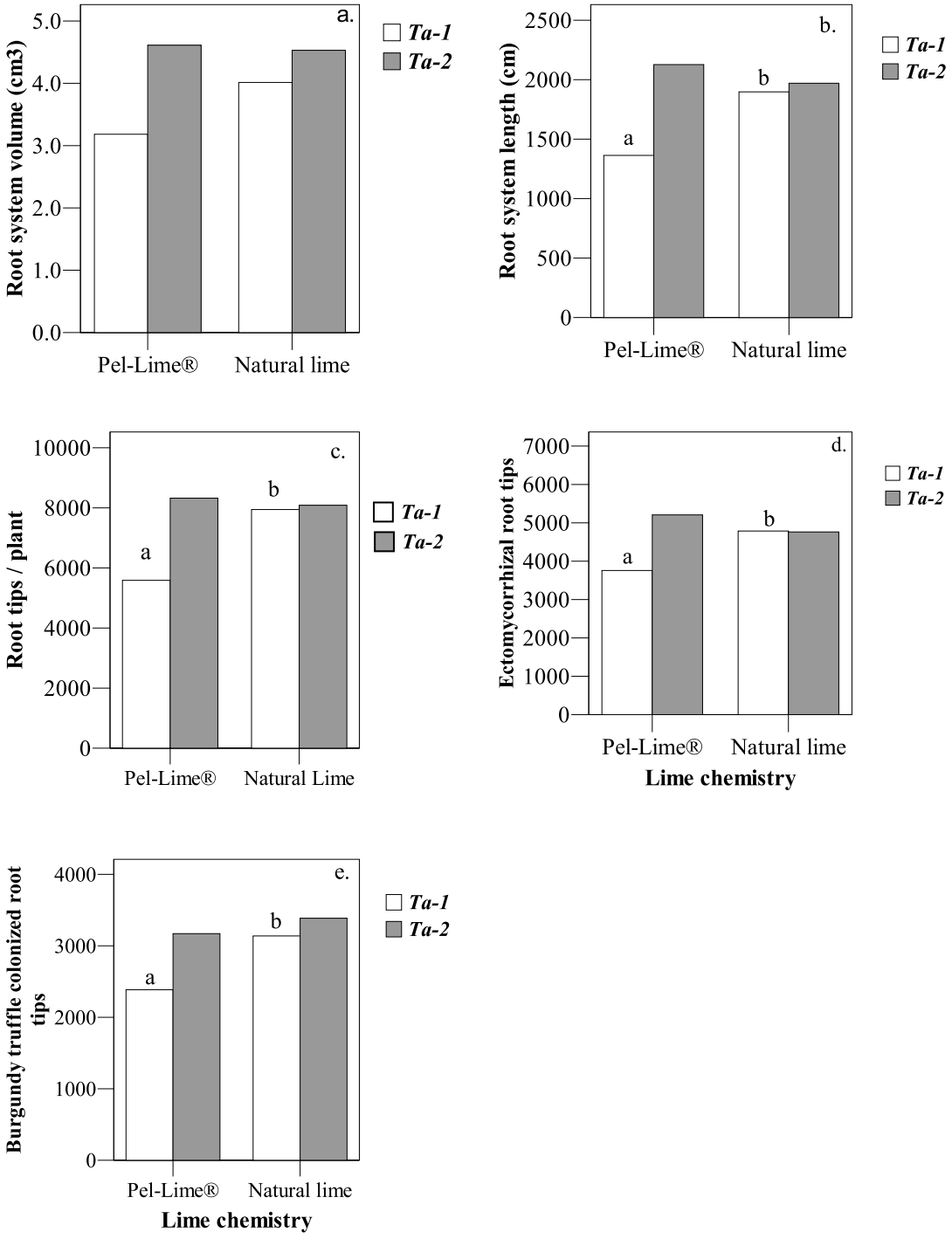
Lime type

Root systems grown in the potting mix amended with natural dolomitic lime developed greater total root length ($P < 0.05$), more total root tips ($P < 0.01$), and more identifiable Burgundy truffle-colonized root tips ($P < 0.05$) than did root systems grown in potting mix amended with Pel-Lime® (Table 2-1). There was also a clear statistical interaction between inoculum source and the type of lime used (Table 2-1). Root systems inoculated with Ta-1 and grown in potting mix amended with Pel-Lime® were 28% shorter ($P < 0.01$) and produced 30% fewer root tips ($P < 0.01$) of which 22% fewer were ectomycorrhizal ($P < 0.01$), as compared with seedlings grown in potting mix amended with the natural dolomitic lime (Figures 2-3b, 2-3c, and 2-3d, respectively). Root systems inoculated with Ta-2 performed equally well in potting mix amended with either type of lime (Figure 2-3). There was no apparent effect of lime type on mortality of seedlings inoculated with either Ta-1 or Ta-2.

Potting mix infestation

Addition of Stockosorb® to the aqueous spore suspension used to infest the potting mix with Burgundy truffle ascospores had no effect on root system growth or mycorrhization (Table 2-1). In addition, no interactions were detected between potting mix infestation treatment and type of lime used, inoculum source, or root dip inoculation treatment (Table 2-1).

Figure 2-3: Characteristics of root systems grown in potting mix amended with Pel-Lime® or natural dolomitic lime and inoculated with Ta-1 or Ta-2 sources of Burgundy truffle ascospores. a) Root system volume. b) Root system length. c) Root tips per plant. d) Number of ectomycorrhizal root tips. e) Number of ectomycorrhizal tips identified as Burgundy truffle. Mean response values were compared separately for each inoculum source. Within each panel, histogram bars with different letters represent significantly different means (α = 0.05). There were no significant differences in any response variable with respect to lime source using the Ta-2 inoculum source.

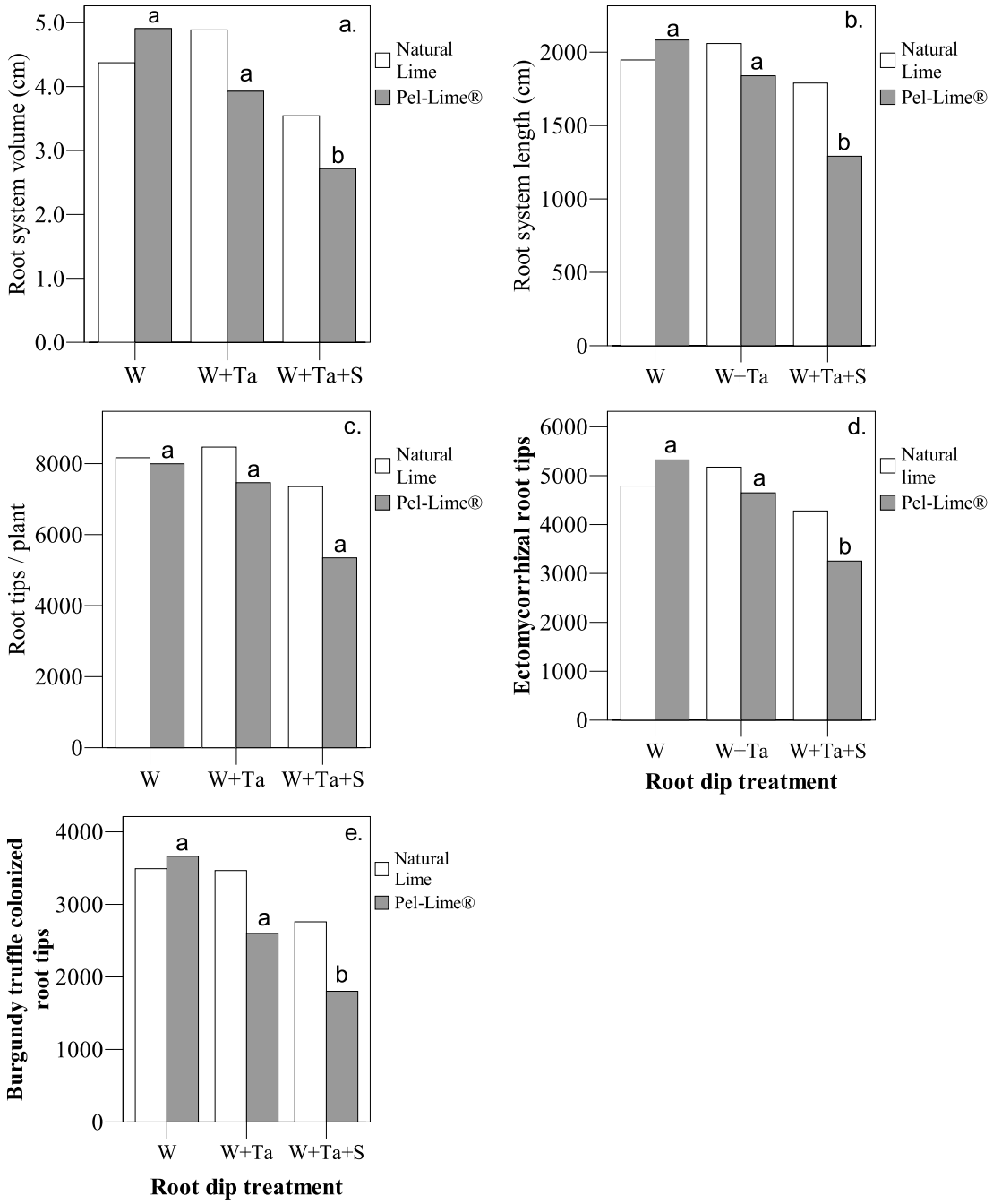

a.
Root system volume (cm3)
5.0
4.0
3.0
2.0
1.0
0.0
Pel-Lime®
Natural lime
Ta-1
Ta-2
b.
Root system length (cm)
2500
2000
1500
1000
500
0
a
b
Pel-Lime®
Natural lime
Ta-1
Ta-2
c.
Root tips / plant
10000
8000
6000
4000
2000
0
a
b
Pel-Lime®
Natural Lime
Ta-1
Ta-2
d.
Ectomycorrhizal root tips
7000
6000
5000
4000
3000
2000
1000
0
a
b
Pel-Lime®
Natural lime
Lime chemistry
Ta-1
Ta-2
e.
Burgundy truffle colonized root tips
4000
3000
2000
1000
0
a
b
Pel-Lime®
Natural lime
Lime chemistry
Ta-1
Ta-2

Root dip inoculations

Neither of the root dip inoculation treatments increased root system size or ectomycorrhizal development beyond the effect of potting mix infestation (Figure 2-4). Adding Stockosorb® to the aqueous spore suspension (W + Ta + S) resulted in smaller and shorter root systems containing fewer ectomycorrhizal root tips ($P < 0.001$) and fewer truffle-colonized root tips ($P < 0.001$) as compared to root systems dipped in an aqueous spore suspension (W + Ta) or the water-only root dip (W) (Table 2-1). The effect of Stockosorb® was greater in the Pel-Lime® treatments than in the natural dolomitic lime treatments (Figure 2-4). Seedlings dipped in the Stockosorb® amended spore slurry (W + Ta + S) experienced 31% mortality compared to 14% mortality for the aqueous spore suspension (W + Ta), and 9% for the water-only root dip (W) ($\chi^2 = 15.2$, $df = 2$, $P < 0.001$).

A significant block effect developed near the end of the study (Table 2-1), because it took eight weeks to process the seedlings. Blocks were processed sequentially, to protect treatment differences. However, it was apparent that mycorrhizal development continued during processing.

Figure 2-4: Characteristics of root systems which experienced different root dip techniques and grown in potting mix amended with Pel-Lime® or natural dolomitic lime. a) Root system volume. b) Root system length. c) Root tips per plant. d) Number of ectomycorrhizal root tips. e) Number of ectomycorrhizal tips identified as Burgundy truffle. Root dip treatments consisted of: water (W); and aqueous suspension of Ta-1 or Ta-2 ascospores (W + Ta); an aqueous suspension of Ta-1 or Ta-2 ascospores and Stockosorb® (W + Ta + S). Mean response values were compared separately for each lime type. Within each panel, histogram bars with different letters represent significantly different means (α = 0.05). There were no significant differences in response variables among root dip treatments where natural.


a.
Root system volume (cm)
5.0
4.0
3.0
2.0
1.0
0.0
a
a
b
W
W+Ta
W+Ta+S
Natural Lime
Pel-Lime®
b.
Root system length (cm)
2000
1500
1000
500
0
a
a
b
W
W+Ta
W+Ta+S
Natural Lime
Pel-Lime®
c.
Root tips / plant
8000
6000
4000
2000
0
a
a
a
W
W+Ta
W+Ta+S
Natural Lime
Pel-Lime®
d.
Ectomycorrhizal root tips
6000
5000
4000
3000
2000
1000
0
a
a
b
W
W+Ta
W+Ta+S
Root dip treatment
Natural lime
Pel-Lime®
e.
Burgundy truffle colonized root tips
4000
3000
2000
1000
0
a
a
b
W
W+Ta
W+Ta+S
Root dip treatment
Natural Lime
Pel-Lime®

Discussion

Seedlings inoculated with Ta-1, the French truffle source, produced smaller root systems in potting mix amended with Pel-Lime® than did seedlings inoculated with Ta-2, the truffle source from Sweden. However, the number of root tips and of Ta-colonized root tips specifically cm^{-1} root length were very similar for both truffle sources regardless of the form of lime used. This result indicates a differential effect of our two forms of lime on structural root growth as mediated by the two Burgundy truffle sources used as mycorrhizal inoculum.

The basis for the sensitivity of the Ta-1 biotype to the pelletized, quick-release Pel-Lime® is uncertain and merits further study. High levels of calcium in European soils (commonly associated with soil pH > 7.0) are widely thought to be important for the growth and/or fruiting of many Tuber species (Chevalier *et al.* 2001; Hall *et al.* 2001; Riousset *et al.* 2001; Sourzat 2002). García-Montero *et al.* (2005) observed greater truffle production in Portuguese soils with higher active carbonate content and suggested that high soil carbonate activity specifically is more closely related to truffle fruiting than is the total quantity of calcium in the soil (García-Montero *et al.* 2005). Although we did not measure the carbonate activity of the differently limed potting mixes in our study, the CCE (calcium carbonate equivalent) of the dolomitic lime was 11% higher than the CCE of the Pel-Lime®. Nevertheless, the calcite Pel-lime® contained negligible magnesium compared to our dolomitic lime source. Because calcium and magnesium compete for cation exchange sites on soil particles, we suspect that our Ta-1

truffle source is more susceptible to the exceptionally high Ca:Mg ratio established using calcite Pel-lime® compared with dolomitic lime. We certainly can not draw any geographic conclusions from our two T. aestivum sources. Wedén *et al.* (2004b) pointed out that productive truffle sites in both France and Sweden vary widely in their soil Ca:Mg ratios. Rather, our results demonstrate that sensitivity to exceptionally high Ca:Mg ratios may be common among strains of *T. aestivum*.

All seedlings were planted into potting mix infested with ascospores of one or the other truffle source. The spore suspension used to infest half of the potting mix with each truffle source contained Stockosorb®. Addition of Stockosorb® to the inoculum suspension used to infest the potting mix had no influence on root system size or infection.

We anticipated that a supplemental root-dip inoculation with an aqueous spore suspension would increase at least root infection level beyond that achieved by potting mix infestation, but this was not the case. In fact, addition of Stockosorb® to the root dip suspension significantly reduced root system size and Ta infection level in potting mix amended with Pel-Lime®. Other studies have indicated that gel-based polymers enhance, suppress, or act neutrally towards plant growth depending on the system (Austin & Bondari 1992; Volkmar & Chang 1995). In our case, however, addition of Stockosorb®-amended root dip treatment was counter productive in potting mix amended with Pel-Lime®. The Pel-Lime® component of this effect may again be explained by the exceptionally high Ca:Mg ratio of calcitic Pel-Lime®. Additionally, the Stockosorb® particles may

have physically separated some spores from fine roots and/or maintained a moisture content unfavorable for Ta infection. Because spores were more uniformly distributed throughout Stockosorb® suspensions compared to simple aqueous suspension, spores may have become largely distributed along structural root sections (where infection is less likely) instead of flowing to more susceptible root tips. The basis for the interaction between Stockosorb® root-dip inoculation and Pel-Lime® is uncertain.

While our source of Q. robur was selected for it's cold-hardiness, it was also uniformly susceptible to powdery mildew. Powdery mildew infection levels were uniform across all treatments throughout the experiment, resembling levels observed in Sweden (Johansson 2001). We did not attempt to treat the powdery mildew, as the potential effects of fungicides on truffle ecology are uncertain. We suspect that the greater mortality experienced among seedlings inoculated with Ta-1 was associated with the smaller root systems of these seedlings in general. A host more resistant to powdery mildew might have fared better.

Our seedlings experienced low levels of colonization by other ectomycorrhizal fungi. No effort was made to identify these species. Most likely these competitors were introduced both during the greenhouse growth period and in the field live soil which formed the basis for our potting mix. Although the soil excavation site has lacked ectomycorrhizal hosts for perhaps 100 years, various mycophagous mammals (e.g. deer and burrowing rodents) have undoubtedly deposited fecal pellets rich in ectomycorrhizal inoculum over this period (Ashkannejhad & Horton 2006). We intentionally used live field soil from

our eventual outplanting site in order not to exclude the effects of indigenous microbiota, including competing ectomycorrhizal fungi, from consideration.

Conclusions

This study was conceived as a direct response to concerns expressed by nurserymen eager both to grow planting stock well-infected by Burgundy truffle and to know the importance of cation balance for truffle infection and ultimately fruiting in limed soils. The critical issue is the cost of transporting the large volumes of lime needed to raise soil pH to favorable levels at plantation sites. Many potential growers are closer to sources of natural dolomitic lime than they are to manufacturers of more highly refined lime products with the highest Ca-levels (e.g., calcite Pel-Lime®). We conclude from this study that calcite Pel-Lime® is not superior to our test source of natural dolomitic lime in supporting Burgundy truffle mycorrhiza formation with Pedunculate oak. In fact, Pel-Lime® appears capable of suppressing structural root development in partnership with some sources of Burgundy truffle. We can not recommend that growers undertake extra expense in order to use a refined high-Ca liming product.

Neither Stockosorb® nor root-dip inoculation improved Burgundy truffle infection levels beyond those accomplished by potting mix infestation using an aqueous suspension of truffle ascospores. (Jimenez-Aguilar & Uceda-Marfil 2001) had similar findings in Spain.

We have shown that different sources of Burgundy truffle responded differently to calcite Pel-Lime® in partnership with our source of Pedunculate oak. No difference was detected between truffle sources in response to our source of dolomitic lime. From this we also conclude that the gentle drying technique used to prepare our Ta-1 source for transportation did not adversely affect inoculum viability. Researchers use various ways to prepare inoculum (Hall *et al.* 2003). Our findings indicate that there is not a significant difference between freezing and drying.

Chapter 3 - GREENHOUSE PRODUCTION OF BURGUNDY TRUFFLE MYCORRHIZAE ON OAK ROOTS

Introduction

Root symbionts including ectomycorrhizal fungi play integral roles controlling ecosystem function in temperate forests worldwide (Smith & Read 1997). They provide their host plants with phosphorus and trace minerals and protection from pathogens and pests, and in return receive organic carbon from their hosts. The mushrooms produced by ectomycorrhizal fungi are important sources of food for forest animals (Carey *et al.* 2002) and of medicine and sustenance for humans in many societies (de Román *et al.* 2006). Over the last 100 years there has been a dramatic decline in forest mushroom production, and consequently in harvest as well (Cherfas 1991). This decline is attributed to drought and air pollution, changes in forest composition and structure including both neglect and deforestation, and loss of local harvesting knowledge because of world wars (Hall *et al.* 2003). In the Netherlands, Arnolds found that the number of mushroom species collected per foray declined from 72 in 1912 to 38 by 1982 (Cherfas 1991). In central Europe, Schmitt found that chanterelles for sale in 1975 were fifty times smaller than those in 1958 (Cherfas 1991).

Declining mushroom production is most easily quantified by focusing on species with high commercial value such as truffles, for which extensive and long-term trade records exist. Truffles, the subterranean fruit bodies of

ectomycorrhizal fungi in the genus *Tuber,* have long been prized for their gastronomic characteristics (Hall *et al.* 2001). Records suggest that *Tuber melanosporum* Vitt. (the Perigord black truffle) sales in France declined from 2000 tons per year at the beginning of the 20th century to less than 150 tons by the beginning of the 21st century (Hall *et al.* 2003). Currently, *T. melanosporum* is the second most valuable truffle in the world, selling wholesale for 250-1200 US$/kg in 2000 (Hall *et al.* 2003).

To offset the declining wild truffle harvest, scientists and landowners in the 1970s began developing methods for the cultivation of truffle fungi in orchards. Orchards currently produce approximately half of the truffles harvested worldwide (Hall *et al.* 2003). Although there are many published accounts discussing cultural practices for truffle orchard establishment and management (Chevalier & Frochot 1997; Callot 1999; Olivier 2000; Hall *et al.* 2001; Riousset *et al.* 2001; Olivier *et al.* 2002; Sourzat 2002; Ricard 2003), most publications on truffle cultivation treat the production of truffle-colonized seedlings as a trade secret, simply suggesting that landowners purchase seedlings that are certified by an impartial authority to be well-colonized by the advertised truffle species. Certified seedlings are available in some countries: notably France, Italy, Spain, and New Zealand (Fischer & Colinas 1996). Unfortunately, this is not yet the case in the USA, where very few nurseries produce consistently well-colonized seedlings. Published processes for production of truffle-colonized seedlings often lack detail or are not designed for commercial use. In order to grow this industry and ensure

high-yielding truffle orchards, it is critical to have open discussions of methods for producing seedlings well-colonized by the target truffle species.

Truffles have the potential to be a lucrative agroforestry crop in many areas of the world, but the lack of public knowledge about greenhouse-based methods of producing well-colonized seedlings limits development of both the industry (Hall *et al.* 2003) and the underlying science.

Truffle seedling growers use a variety of potting media with varying levels of colonization success. Forrest Keeling Nursery (Elsberry, Missouri, USA) has developed a plant production system called Root Production Method (RPM®, patent pending) that air-prunes vertically-oriented roots while stimulating lateral root development high on the taproot, thus generating vigorous plants with many root tips and little mortality when field planted (Lovelace 1998). The RPM method involves planting stratified seeds in February, culling, transplanting, and inoculating the plants in March, transplanting again in May, and outplanting the following spring. The bark-based, high porosity RPM potting medium differs substantially from more typical peat-based potting media used in Europe to grow truffle-colonized seedlings. RPM thus presents an interesting alternative to more typical truffle seedling production methods which involve a single potting event. Seedlings with more numerous and intensively-branched lateral root systems might more efficiently colonize a planting site. However, this would only prove useful if their root systems could be well-colonized by the truffle fungus in the greenhouse.

Although *T. melanosporum* is the most valuable black truffle, Perigord black truffles mature during the winter and are destroyed by prolonged freezing (Riousset *et al.* 2001). *Tuber aestivum* Vitt. syn. *T. uncinatum* Chatin (the Burgundy truffle) commands approximately half the price of the Perigord truffle but fruits during the late autumn (Riousset *et al.* 2001; Wedén *et al.* 2004a). *Tuber aestivum* also fruits more densely than *T. melanosporum,* owing to its preference for denser spacing of host trees (Chevalier *et al.* 2001). For these reasons, we have chosen to study cultivation of *T. aestivum* in Missouri, USA. Further, we have selected *T. aestivum* truffles from Sweden for our experiments (Wedén *et al.* 2004a) because the climate and soils on the Swedish island of Gotland are more similar to those found in the midwestern USA than those found in the mainland European range of *T. aestivum* (Wedén *et al.* 2004a). On Gotland, *T. aestivum* grows wild in association with *Quercus robur* L. (pedunculate oak) and *Corylus avellana* L. (European hazel) in soils with pH values ranging from 6.8 to 7.9 (Wedén *et al.* 2004a). Previous results showed that *Q. robur* seedlings inoculated with Swedish *T. aestivum* performed well in a Missouri soil (Pruett *et al.* 2008).

We used a *Quercus bicolor* Willd. x *Quercus robur* L. hybrid (swamp white oak x pedunculate oak) as the host for *T. aestivum* in our studies. This hybrid was used because *Q. robur* is a natural *T. aestivum* host in Europe (Riousset *et al.* 2001; Wedén *et al.* 2004a), and because its hybrid with *Q. bicolor* tolerates high soil pH and is more resistant to powdery mildew and native insect pests than are its component species (Wayne Lovelace, pers. comm.).

We have addressed a series of four questions related to greenhouse production of *T. aestivum*-colonized seedlings. 1) Does a typical European-like production method utilizing small containers and peat-based medium produce seedlings more or less well-colonized by *T. aestivum* than an RPM-based method that utilizes larger containers and a bark-based medium? Our evaluations spanned one year in the greenhouse and two years in the field. 2) Does the medium or the container play a more influential role in development of a well-colonized root system? 3) Do modifications of the bark-based RPM medium designed to increase pH improve truffle colonization? 4) Does the pH of the root medium influence the level of root system colonization by the truffle fungus?

Materials and methods

Experiment 1: RPM vs. a Typical seedling production system

Potting media, host plants, and fungal inoculum

We compared the effects of two seedling production systems on growth of *Q. bicolor* x *Q. robur* seedlings inoculated with *T. aestivum.* We grew seedlings according to the RPM method, using RPM containers and RPM media with and without manure, and according to a more typical production method in book-type containers (book planters) using a peat-based medium.

The potting media tested are referred to as: RPM, RPM + Manure, and Typical. The RPM potting medium consisted of 5:3:2 (v:v:v) ground pine bark, rice hulls, and sand, amended with the manufacturer recommended quantities of Osmocote fertilizer (*19-5-8*, The Scotts Company, Marysville, OH) and Terra-sorb, an anhydrous-based gel used to prevent dehydration (Plant Health Care, Inc., Pittsburg, PA). The RPM + Manure medium consisted of 5:3:1:1 (v:v:v:v) ground pine bark, rice hulls, sand, and cattle manure (plus Osmocote and anhydrous gel as in the RPM medium). Forrest Keeling Nurseries (Elsberry, Missouri, USA) provided the premixed RPM and RPM + Manure media. The Typical medium, based on a generalized recipe provided by a European nursery growing certified truffle-colonized seedlings, consisted of 10:8:1:1 (v:v:v:v) peat, sand, perlite, and vermiculite plus Osmocote (*14-14-14*) at one-half the recommended rate. The Typical medium was prepared in the University of Missouri – Columbia, Ashland Gravel Road Greenhouse (Columbia, Missouri, USA). Agriculture lime (Mississippi Lime Company, Genevieve, Missouri) was incorporated into all three media at a rate of 25 g L^{-1} using a Big Cat cement mixer (Red Lion, Canada). The RPM and RPM + Manure media were pasteurized at 65 C for at least 2 h on two consecutive days using a Bouldin and Lawson model # 02884 steam cart (McMiniville, TN). The Typical medium was pasteurized in a Samsung microwave oven (model MW1150WA) at 65 C for 30 min on two consecutive days (Ferris 1984).

Acorns from a *Q. bicolor* x *Q. robur* hybrid were cold-stratified at 3 C for 90 days prior to germination (U.S. Forest Service 1974). The seedlings used in the

RPM and RPM + Manure treatments were grown according to the RPM system, which consists of two repotting events using three different types of containers (Lovelace 1998). In February 2004, acorns were placed in 6.5 cm x 36 cm x 46.5 cm germination trays (Anderson Tool & Dye, Portland, OR) with a lattice "floor" containing RPM or RPM + Manure potting medium. The acorns were laid on their sides and gently pressed into the potting medium so that half of their diameter was below the soil surface. Once their first set of leaves had formed, they were transplanted to 6 cm x 6 cm x 6 cm square band-pots with a lattice "floor" (Anderson Tool & Dye, Portland, OR) containing RPM or RPM + Manure potting medium inoculated with *T. aestivum* spores. Once the second set of leaves formed, seedlings were transplanted from the band-pots to 8 L round pots (Nursery Supply, Inc. Chambersburg, PA, USA) containing *T. aestivum*-infested potting medium, and allowed to grow in the greenhouse until May 2005.

Seedlings in the Typical medium treatment were grown in 183 cm^3 Hillons book planters (Hummert International, Earth City, MO), supported in plastic frames (model # 170 - 4). Each frame supported 10 books, and each book contained four 3.8 cm x 3.8 cm x 12.7 cm seedling compartments. Acorns were germinated in February 2004 in book planters containing truffle-infested Typical medium, and were allowed to grow in the greenhouse until May 2005.

Seedlings in all three treatments were grown in the same greenhouse environment, with temperatures ranging from 16 to 33 C and natural sunlight with natural photoperiod. Seedlings were watered every three days or less to avoid overwatering.

Truffle inoculum was obtained through Dr. Eric Danell (Uppsala University, Sweden), shipped fresh by air from Gotland, Sweden, and frozen on arrival in Missouri. Ascospore inoculum was produced by removing the peridium (outer "rind") of each truffle, coarsely chopping and suspending the gleba (fertile interior) in tap water, and blending the gleba to produce a suspension of nearly uniform particle size. The aqueous spore suspension was incorporated thoroughly by hand into the potting medium at a rate of 2 g of truffle gleba / seedling (ca 5 x 10^5 spores L^{-1} potting medium) in the RPM and RPM + Manure potting medium treatments and 1 g of truffle / seedling (1 x 10^7 spores L^{-1} potting medium) in the Typical treatment.

Each treatment was replicated 30 times for a total of 90 seedlings. A random sample of 15 seedlings from each treatment were destructively harvested after one year in the greenhouse to provide baseline metrics on seedling root systems (including fungal colonization levels) prior to outplanting.

In May 2005, fifteen experimental trees representing each treatment were planted with the root collar at soil-line in Howard County, Missouri, USA, at the University of Missouri Horticulture and Agroforestry Research Center (GPS 39 °N, 93 °W) in an old alfalfa field. The planting soil is a well-drained silt loam on a loess ridge. Trees were planted in a completely randomized design on a 3 m grid and surrounded by a 1.8 m^2 square sheet of black water-permeable weed barrier fabric (DeWitt Co., Sikeston, MO) to control competing vegetation. Seedlings were watered as needed for the first growing season using Rainbird overhead sprinklers (Glendora, CA, USA). In October 2005, after the first

growing season, the weed barrier fabric was removed and the seedlings were no longer watered.

Data collection

Fifteen trees representing each potting medium treatment were destructively sampled in May 2005 after 14 months in the greenhouse. A potting medium sample was collected from each container to measure medium pH (w) (Soil and Plant Analysis Council 1980). The potting medium was gently rinsed from each root system using tap water, and the aerial plant parts were separated from the root system at the root collar. Tissue volumes for the aerial and root portions of each seedling were assessed using a water displacement method (Harrington *et al.* 1994). Each root system was spread on a clear plastic tray and scanned with an Epson EU22 scanner (Epson America Inc., Long Beach, CA, USA) modified to illuminate the root sample both from above and below. Root system architecture was analyzed using WinRhizo image analysis software (version 4.1c, Régent Instruments, Montreal, Quebec, Canada) to estimate the total root system length and total number of root tips for each seedling.

Approximately 1,000 tips were removed arbitrarily from the root system of each destructively harvested seedling. From those 1,000 tips, we randomly selected 300 root tips to estimate the percentage of root tips colonized by ectomycorrhizal species in general, and by *T. aestivum* specifically (Fischer & Colinas 1996). *Tuber aestivum* ectomycorrhizae were recognized on the basis of

their characteristic anatomy and morphology (Agerer 1996) using dissecting and compound microscopy. All ectomycorrhizal root tips were morphotyped (Gardes & Bruns 1996a) and stored at 3 C for subsequent DNA sequence-based identification. For each seedling, the total number of root tips was applied to the percent colonization data to estimate the total number of root tips colonized by *T. aestivum* and other ectomycorrhizal fungi (Pruett *et al.* 2008).

For the remaining outplanted trees, data were collected annually in May 2005 (prior to planting), 2006, and 2007. Tree growth was characterized by annual measurements of seedling height and stem diameter at 5 cm above the root collar. In 2005, just before outplanting, fungal colonization of each seedling was monitored by arbitrarily collecting a pooled sample of roots from the potting medium, representing 3 depths and 3 distances from the stem. In 2006 and 2007, fungal colonization and soil pH (w) were monitored from two soil cores taken near each seedling (one upslope and one downslope) to a depth of 20 cm using a 2.5 cm dia soil corer. Because we wanted to measure new root growth beyond the pot edge, seedlings in the Typical medium treatment were sampled 5 cm from the stem and seedlings in the two RPM treatments were sampled 10 cm from the stem. For each seedling, the two cores were pooled into one sample for which we measured pH (w). Each composite sample was then soaked in tap water and sieved using a #20 soil sieve (850 µm aperture) to recover the roots for analysis.

Mycorrhizal species identification

Mycorrhizal tips grouped by morphotype were stored in tap water for no more than 3 days prior to DNA extraction. DNA was extracted from three to five root tips per morphotype per tree using DNEasy Plant Mini Kits (Qiagen Inc., Valencia, CA, USA) following the manufacturer's protocol. Extracted DNA was stored in a – 80 C freezer until PCR processing. Polymerase chain reactions (PCR) were conducted to amplify the ITS region using primers ITS1F (5' CTTGGTCATTTAGAGGAAGTAA 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) according to Gardes and Bruns (1996b). Each PCR reaction consisted of 2 mM dNTPs, 2.5 mM $MgCl_2$, 0.5 mM of each primer, 2.5 mM 10X PCR Buffer (Promega, Madison, WI, USA), 0.2 units of *Taq* DNA Polymerase (Promega), 1-5 ng DNA, and sterile water to 25 µL. The PCR product was sequenced by the University of Missouri – Columbia DNA Core Facility using ITS1F and ITS4 as primers. Sequencher version 4.5 for Macintosh (Gene Codes Corporation, Ann Arbor, MI) was used to generate the consensus sequences. DNA sequences were BLASTed against GenBank (http://www.ncbi.nlm.nih.gov/) and UNITE (http://unite.ut.ee/) databases in an effort to determine their identity.

Experiment 2: Potting media vs. container system effects

In this experiment, we compared *T. aestivum* colonization of *Q. bicolor* x *Q. robur* oak seedlings grown under all four combinations of our two potting media (RPM and Typical) and our two container system potting processes (RPM and Typical).

Potting media, host plants, and fungal inoculum

RPM and Typical potting media were prepared on site according to the recipes described for Experiment 1, except that slow-release Osmocote (*14-14-14*) was applied at one-half the recommended rate and the anhydrous gel material was excluded from the RPM medium. Previous experiments showed that fertilizer and anhydrous gels can suppress fungal colonization (Smith & Read 1997; Pruett *et al.* 2008). Hybrid *Q. bicolor* x *Q. robur* acorns and Swedish *T. aestivum* inoculum were obtained and processed according to the methods described for Experiment 1.

Seedlings receiving the RPM potting process treatment were grown according to the RPM potting process described above for Experiment 1. In February 2006, cold-stratified acorns were placed in germination trays containing either RPM or Typical potting medium. Seedlings were transplanted at the first leaf stage into the appropriate substrate infested with truffle inoculum as described for Experiment 1, and allowed to grow until January 2007.

The seedlings receiving the Typical potting process were grown in Hillons book planters containing either RPM or Typical substrate. Acorns were

germinated in February 2006 in book planters containing truffle-infested potting medium and allowed to grow in the greenhouse until January 2007, when seedling growth was measured.

Seedlings in all four treatments were grown simultaneously in a uniform greenhouse environment as in Experiment 1. The seedlings were arranged in a randomized complete block with 20 replicate blocks of each of the four treatments.

Data collection

In January 2007, after 12 months in the greenhouse, we measured potting medium pH (w), stem diameter at 5 cm above the root collar, seedling height, and root system volume by displacement. We used WinRhizo software to characterize root system growth as described for Experiment 1. Root system colonization by ectomycorrhizal fungi in general, and *T. aestivum* specifically, was again estimated by the same methods used in Experiment 1.

Experiments 3A and 3B: RPM potting medium modification

Potting media, host plants, and fungal inoculum

Few potting media have pH values in the range favorable for *T. aestivum* (6.9 - 7.9 (Wedén *et al.* 2004a). In Experiment 3A, we compared the growth responses of *Q. bicolor* x *Q. robur* seedlings grown in six RPM-like media designed to provide higher pH while maintaining RPM substrate structure (water holding capacity and percent air space) (Hayden 2005) with seedlings grown in the premixed RPM medium provided by Forrest-Keeling Nursery. The modified RPM treatments comprised 4:4:2 (v:v:v) composted rice hulls, ground bark, and a mixture of sand and dolomitic limestone. We tested two bark types (substituting hardwood bark for pine bark in half of the treatments) and three proportions of sand:limestone (2:0, 1:1, 0:2), for a total of six modifications (see Results). The premixed RPM potting medium consisted of 5:3:2 (v:v:v) ground pine bark, rice hulls, and sand, and was amended with Osmocote fertilizer, Terra-sorb anhydrous-based gel, and 25 g / L^{-1} of agricultural lime as in Experiment 1. Acorns were germinated in April 2005, randomly assigned to the seven treatments, and then grown for 12 weeks in the greenhouse in 160 cm^3 cylindrical plastic containers (5 cm diameter x 8 cm height) containing potting media infested with Swedish *T. aestivum* inoculum at a rate of 1 x 10^5 spores L^{-1} of potting mix (2 g truffle pot^{-1}). The seedlings were watered approximately every three days as necessary. Each treatment was replicated 10 times.

In Experiment 3B, we evaluated the colonization of *Q. bicolor* x *Q. robur* seedlings by *T. aestivum* in three of the seven potting media tested in Experiment 3A (see Results). These three potting media were selected for having pH values within an acceptable range for *T. aestivum* while also

producing robust seedlings. Acorns were germinated in January 2006, randomly assigned to the three treatments, inoculated as described for Experiment 3A, and allowed to grow for 12 months in the greenhouse. Each treatment was replicated 15 times.

Data collection

In July 2005, the seedlings in Experiment 3A were destructively sampled. Total porosity, aeration porosity, and water holding porosity (Landis *et al.* 1990), and pH (w) of the substrate were measured. Seedling height and stem diameter were measured and WinRhizo software was used to calculate the root volume and number of root tips. In January 2007, the seedlings in Experiment 3B were destructively sampled and similar data collected, except that the number of ectomycorrhizal tips and colonization of the root system by *T. aestivum* were also estimated in Experiment 3B.

Experiment 4: Effect of lime level on *T. aestivum* colonization of typical medium.

In this experiment, we evaluated plant growth and fungal colonization of seedlings grown in Typical peat-based potting media modified to provide a range of pH values. The Typical potting medium was amended with agriculture lime at rates of 25, 30, 35, 40, and 45 g L^{-1}, intended to generate pH (w) values ranging 6.9–8.9. Limed potting media were then infested with 1 x 10^5 spores L^{-1} potting

mix (2 g pot^{-1}), as in Experiment 1. In January 2006, 25 *Q. bicolor* x *Q. robur* acorns were germinated, randomly assigned to the pH treatments, and then grown for 12 months in the greenhouse in the same 160 cm^3 plastic cylindrical containers as in Exp. 3.

Data collection

In January 2007, the seedlings were destructively sampled and potting medium pH (w) and seedling height were measured. Then WinRhizo Software was used to calculate root system volume and number of root tips, and 300 root tips per seedling were evaluated for percent colonization by *T. aestivum.*

Data analysis

In Experiment 1, seedling growth, fungal colonization, and pH data for the destructively sampled greenhouse seedlings were analyzed by Analysis of Variance using the GLM procedure of the SAS 9.1 statistical software (SAS Institute Inc., Cary, NC). For the remaining trees, tree growth, fungal colonization, and pH (w) data were analyzed by repeated measures Analysis of Variance using PROC MIXED in SAS 9.1 statistical software. Least significant means were used to determine differences among treatments (Sokal & Rohlf 1995). Percent colonization data were $\sqrt{\arcsin}$ transformed prior to data analysis (Gotelli & Ellison 2004).

For Experiments 2, 3A, and 3B, data were analyzed by linear regression (PROC GLM, $\alpha = 0.05$; SAS/STAT System Release 9.1, Inst. Inc., Cary, NC). Least significant means were used to differentiate among treatments. For Experiment 4, data were analyzed by polynomial regression analysis in Excel (Microsoft Corp., Redmond, WA).

Results

Experiment 1: RPM vs. a Typical seedling production system

After 14 months in the greenhouse, seedlings grown in Typical peat-based potting medium in book planters were significantly shorter ($P < 0.001$) and smaller in diameter ($P < 0.001$), with less root tissue volume ($P < 0.001$) and total root length ($P < 0.001$) (Table 3-1, Figure 3-1), resulting in 65% fewer root tips per seedling when compared to the RPM + Manure or RPM treatments (Figure 3-1). However, seedlings in the Typical treatment had 208 root tips cm^{-3} potting medium volume while RPM and RPM + Manure had 10.5 and 11.2, respectively. Additionally, seedlings in the Typical treatment had the same number of root tips cm^{-1} of root length, but 7 and 190 times ($P < 0.001$) more *T. aestivum*-colonized root tips on average than seedlings grown in the RPM + Manure or RPM treatments, respectively (Figure 3-1). This increase corresponds to an average of 13.4, 0.92, and 0.02 *Tuber aestivum*-colonized root tips cm^{-1} of root length and

62.87, 0.20, and 0.01 *Tuber aestivum*-colonized root tips cm^{-3} potting medium volume in the Typical treatment, RPM + Manure, and RPM treatments, respectively. Further, the root systems of seedlings in the RPM + Manure treatment had greater percentages of root tips colonized by other ectomycorrhizal fungi than those in the Typical or RPM treatments ($\alpha = 0.05$, Figure 3-1). Potting media pH (w) differed among the three treatments, and was highest in the Typical system ($\alpha = 0.05$, Figure 3-1).

After one year of growth in the field, seedlings grown with RPM, RPM + Manure, and Typical treatments increased 2.0 %, 44.5 %, and 67.3 % in height, and 52 %, 84.5 % and 78.1 % in stem diameter, respectively. By the end of their second year in the field, RPM, RPM + Manure, and Typical seedlings had increased 25%, 120%, and 170% in height and 197%, 245%, and 253% in stem diameter, respectively, since planting. Yet after two years in the field, RPM seedlings had the greatest height and stem diameter while the Typical seedlings where still the smallest ($\alpha = 0.05$, Figure 3-2).

Seedling colonization both by *T. aestivum* and by competing ectomycorrhizal fungi increased during the first year in the field. *Tuber aestivum* levels after one year were 1.8 %, 13 %, and 42 %, respectively, for RPM, RPM + Manure and Typical seedlings (Figure 3-2). During their second year in the field, the RPM + Manure and Typical seedlings experienced a decline in *T. aestivum* colonization levels ($\alpha = 0.05$, Figure 3-2). Nevertheless, at each time of measurement, Typical seedlings had the greatest number of *T. aestivum* mycorrhizae.

Table 3-1: Results of ANOVA to identify effects of three potting treatments (RPM, RPM + Manure, Typical) on tree growth and fungal ectomycorrhizal colonization of root systems over three years of growth

Table 3-1: Results of ANOVA to identify effects of three potting treatments (RPM, RPM + Manure, Typical) on tree growth and fungal ectomycorrhizal colonization of root systems over three years of growth

Source of variation[a]	Response variables[b]									
	Height (cm)		Stem diameter (mm)		Soil pH(w)		% *Tuber*		% Competitive mycorrhizae	
	df	F	df	F	df	F	df	F	df	F
Treatment	2	72.20***	2	33.39***	2	1.64	2	40.38***	2	8.22***
Year	2	100.27**	2	252.23***	2	2.04	2	16.83***	2	126.28***
Treatment x Year	4	4.70**	4	7.71***	4	0.29	4	3.16*	4	1.7
Total	8	—	8	—	8	—	8	—	8	—

[a] sources of variation: Treatment - RPM, RPM + Manure, and TYPICAL potting processes; Year - after 1 year in greenhouse, after 1 year in the field, after 2 years in the field

[b] Response variables: height–seedling height (cm); diameter–seedling diameter (mm), measured 5 cm above the root crown; soil pH(w)–measured by the water method; % Tuber and % competitive mycorrhizae–% of total number of mycorrhizal root tips identified either as *Tuber aestivum* or not; df, degrees of freedom; *, **, *** indicate F-value significance at $P < 0.05, 0.01$, and 0.001, respectively

Figure 3-1: Characteristics of *Tuber aestivum*-inoculated *Quercus bicolor* x *Q. robur* seedlings grown according to three different systems: RPM, RPM + Manure, or Typical. Potting media pH (w). Height (cm). Stem diameter (mm). Root system volume (cm^3). Root system length (cm). Total root tips (Total # of root tips per root system). Total *Tuber* mycorrhizae (Total # of *Tuber* colonized root tips per root system). Total competitor mycorrhizae (Total # of competitive mycorrhizal root tips per root system). Seedlings were destructively sampled after 14 months in the greenhouse. Within each response variable, histogram bars with different letters represent significantly different means as determined by ANOVA (α = 0.05).

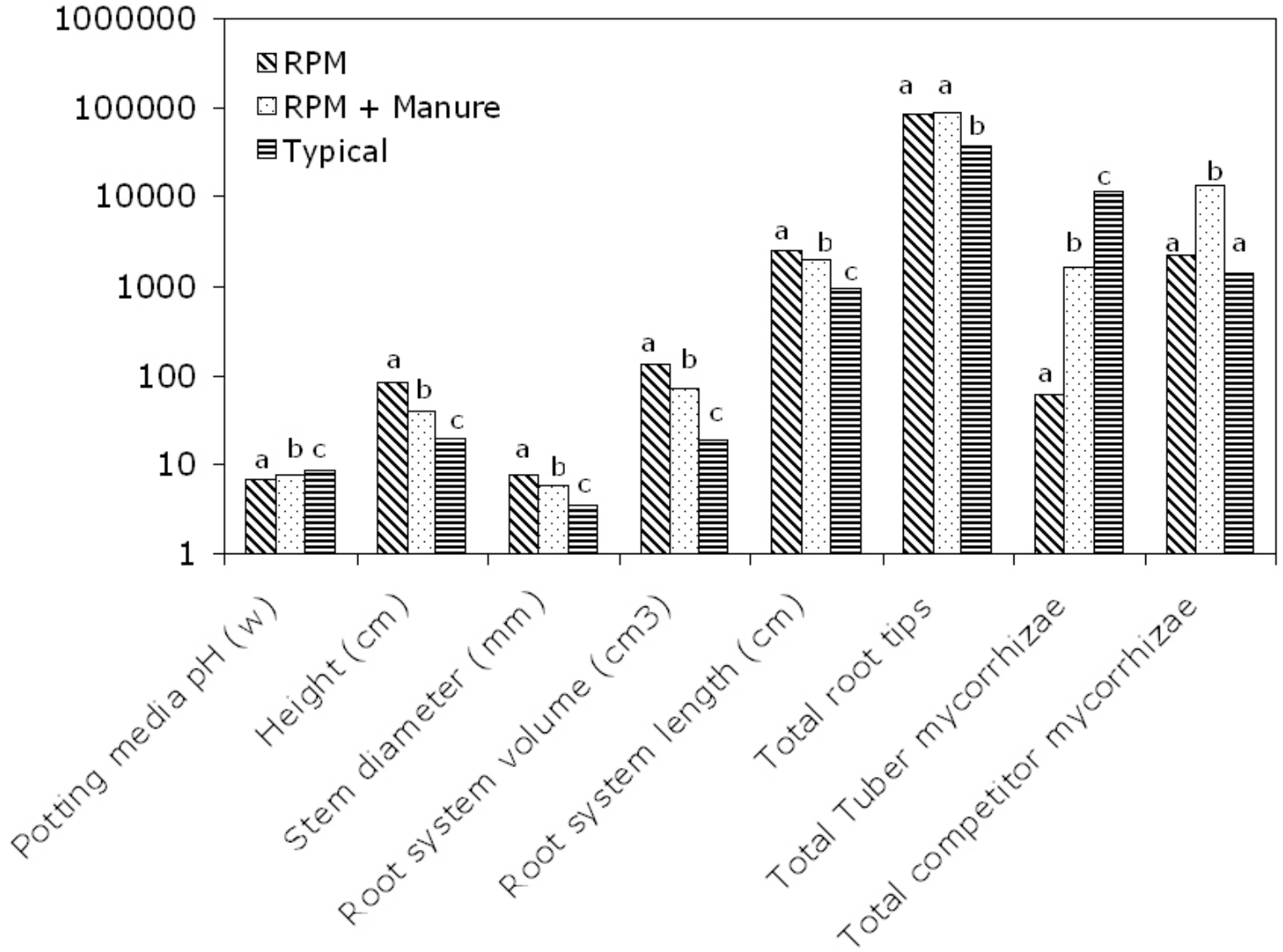
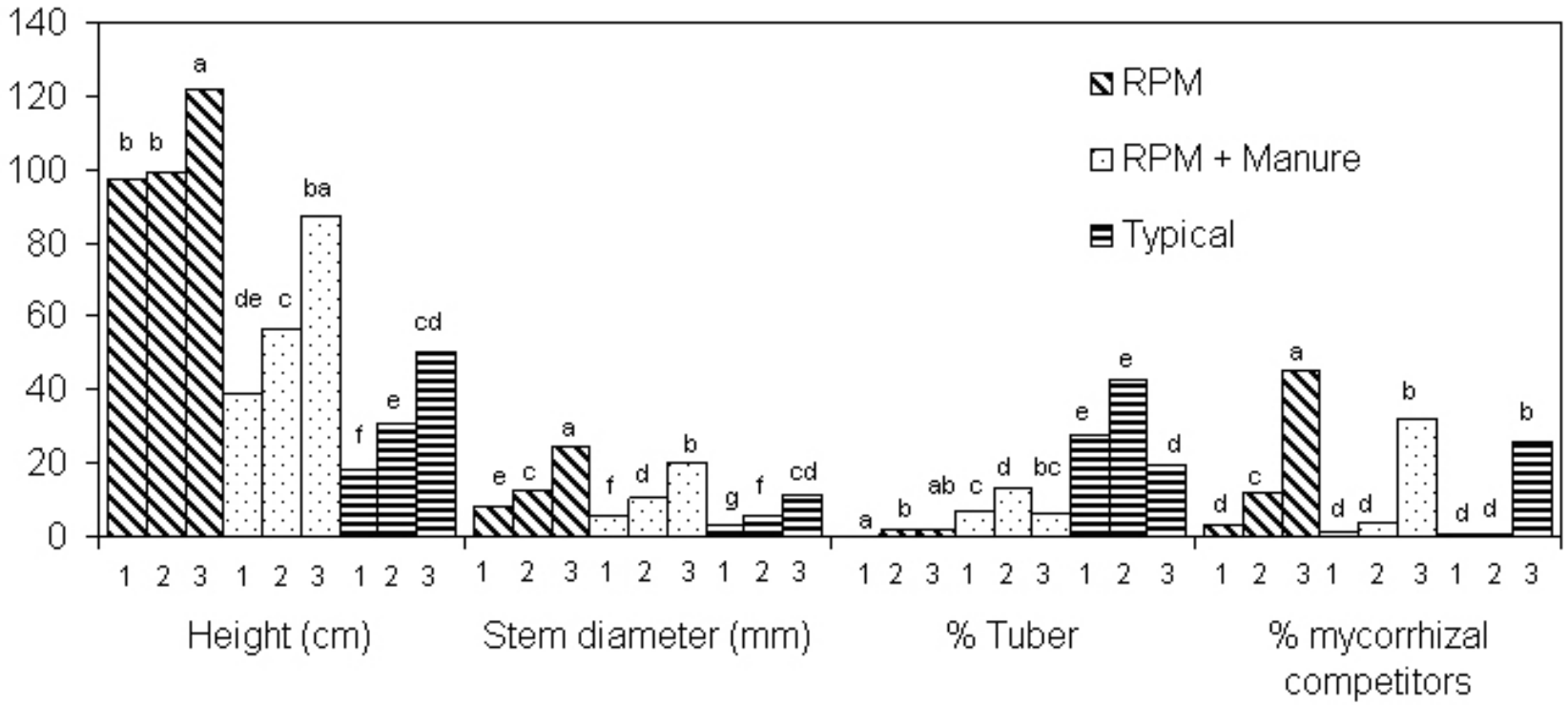

1000000
100000
10000
1000
100
10
1
RPM
RPM + Manure
Typical
a b c
a b c
a b c
a b c
a b c
a a b
a b c
a b c
Potting media pH (w)
Height (cm)
Stem diameter (mm)
Root system volume (cm3)
Root system length (cm)
Total root tips
Total Tuber mycorrhizae
Total competitor mycorrhizae

Figure 3-2: Characteristics of *Tuber aestivum*-inoculated *Quercus bicolor* x *Q. robur* seedlings grown according to RPM, RPM + Manure, or Typical systems at 1, 2, and 3 years after germination. Height (cm). Stem diameter (mm), 5 cm above the root collar. *Tuber aestivum* mycorrhizae and other competing ectomycorrhizae (% of total root tips). During the first year, the seedlings were grown in containers in the greenhouse. During the subsequent two years, seedlings were grown in the field. Within each response variable, histogram bars with different letters represent significantly different means (α = 0.05).


140
120
100
80
60
40
20
0
RPM
RPM + Manure
Typical
b b a de c ba f e cd
e c a f d b g f cd
a b ab c d bc e e d
d c a d d b d d b
1 2 3 1 2 3 1 2 3
Height (cm)
Stem diameter (mm)
% Tuber
% mycorrhizal competitors

All treatments had higher levels of competitors after outplanting, but the RPM seedlings experienced the fastest gain (2.8-fold increase) and the highest level of colonization by competing ectomycorrhizal fungi in the first year (12 % vs 3.7 % and 0.92 % for RPM + Manure and Typical, respectively) ($\alpha = 0.05$, Figure 3-2). After the second year in the field, RPM seedlings still had the highest level of competing ectomycorrhizae ($\alpha = 0.05$, Figure 3-2).

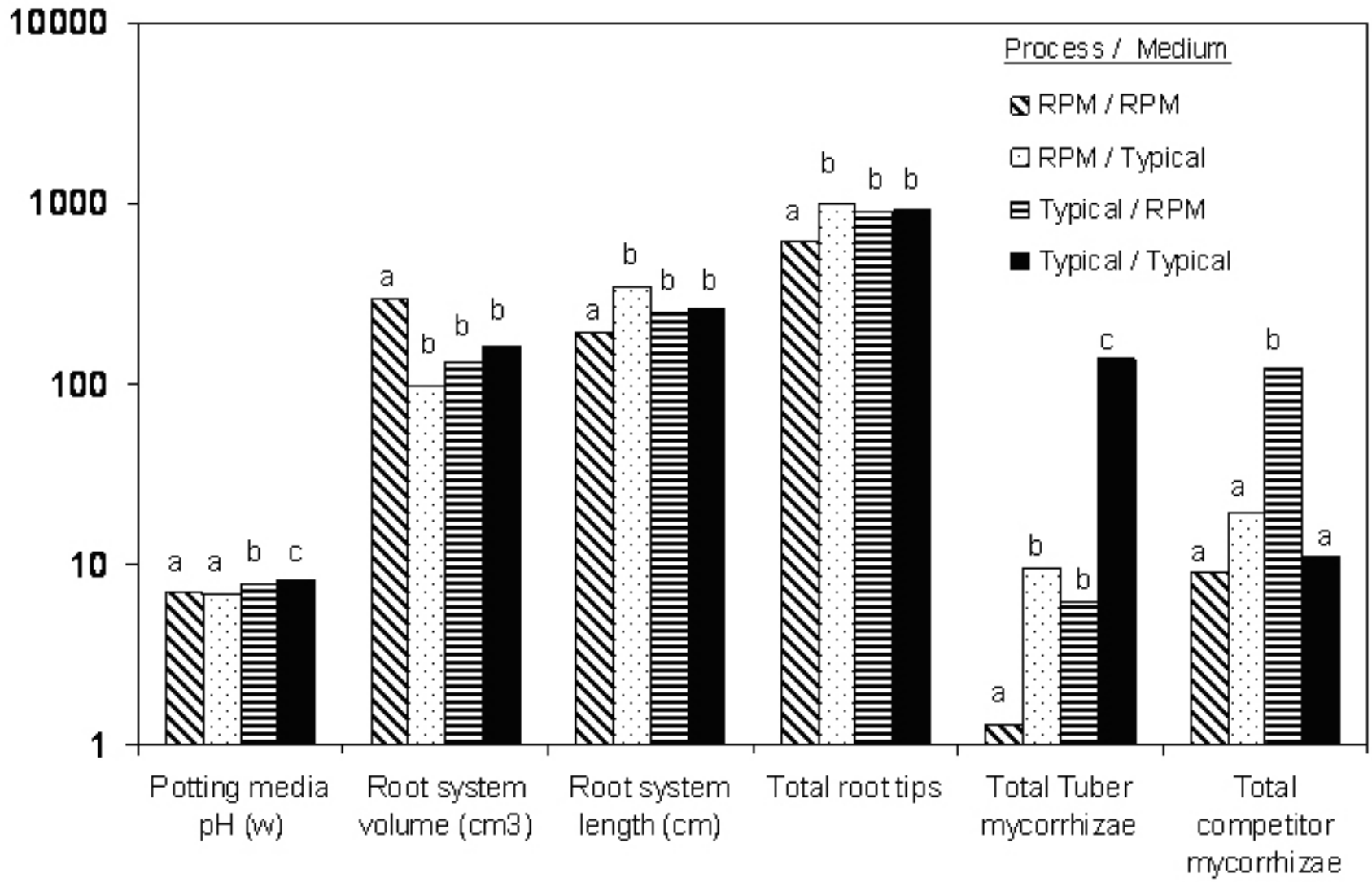
Experiment 2: Potting media vs. container system effects

Seedlings grown in book planters experienced higher soil pH than those grown in RPM containers ($P < 0.01$, Figure 3-3). Although potting medium pH was similar for all seedlings grown in the RPM containers (mean pH = 6.9), the peat-based Typical medium developed a higher mean pH (8.3) in the Typical book planters ($P < 0.001$) than did the bark-based RPM medium (mean pH = 7.8) (Figure 3-3).

Seedlings grown in peat-based Typical medium were on average 23 % taller ($P < 0.01$) and 59 % larger in stem diameter ($P < 0.01$) regardless of the container system to which they were assigned (data not shown). Root system characteristics were influenced by the interaction between container system and potting medium. Seedlings grown in bark-based RPM medium with neither fertilizer nor anhydrous-based gel amendments in RPM containers had root system volumes twice as large as the other treatments ($\alpha = 0.05$), but with 21%fewer root tips per seedling ($P < 0.05$, Figure 3-3). Additionally, seedlings

grown in RPM containers had less than one root tip cm^{-3} potting medium volume while seedlings grown in book planters had greater than four root tips cm^{-3} potting medium volume. Seedlings grown in peat-based Typical medium in book planters had 14 times more *T. aestivum*-colonized root tips than seedlings grown in the other treatments, with root systems colonization levels of 15% for the peat-based Typical medium / Typical book planter treatment and less than 1% for all other treatments ($P < 0.001$, Figure 3-3). This corresponds to an average of 0.5 and < 0.05 *T. aestivum*-colonized root tips cm^{-1} of root length in the peat-based Typical medium / Typical book planter treatment vs. all others, respectively. Seedlings grown in peat-based Typical medium in the RPM containers also had 5.3 times more competitor ectomycorrhizae than the other treatments ($\alpha = 0.05$, Figure 3-3).

Figure 3-3: Characteristics of *Tuber aestivum*-inoculated *Quercus bicolor* x *Q. robur* seedlings grown according to two different potting processes (RPM and Typical) in two different potting media (RPM and Typical). Potting media pH(w). Root system volume (cm^3). Root system length (cm). Total root tips (Total # of root tips per root system). Total *Tuber* mycorrhizae (Total # of *Tuber* colonized root tips per root system). Total competitor mycorrhizae (Total # of competitive mycorrhizal root tips per root system). Seedlings were destructively sampled after 11 months in the greenhouse. For each response variable, histogram bars with different letters represent significantly different means (α = 0.05).


10000
1000
100
10
1
Process / Medium
RPM / RPM
RPM / Typical
Typical / RPM
Typical / Typical
a a b c
a b b b
a b b b
a b b b
a b b c
a a b b
Potting media pH (w)
Root system volume (cm3)
Root system length (cm)
Total root tips
Total Tuber mycorrhizae
Total competitor mycorrhizae

Experiments 3A and 3B: RPM potting medium modification

After 12 weeks in the greenhouse, there were no significant differences in total porosity (mean = 45% vol. pore space / total vol. medium) or water holding porosity (mean = 41%) among the seven RPM-based potting media tested, but the original RPM potting medium had higher aeration porosity (mean = 10%) than the other treatments (mean = 3%) (data not shown, df = 6, F = 10.53, $\alpha < 0.05$). The modified RPM-based media had pH values averaging 0.3-0.8 units higher than the proprietary RPM medium (df = 6, F = 24.39, $P < 0.05$, Table 3-2). Seedling growth differed among treatments, with seedlings grown in potting media without lime or with low levels of lime (Treatments 1, 4, and RPM) exhibiting among the greatest mean heights and tissue volumes (Table 3-2). Bark type (hardwood vs. pine) did not affect seedling growth, but hardwood bark did increase the potting medium pH ($\alpha < 0.05$). Of the six modified RPM-based media, only treatments 1, 2, and 4 had pH values ≤ 8.4 and also displayed good seedling growth (Table 3-2).

In Experiment 3B, seedlings were grown for one year in three of the potting media tested in Experiment 3A (Treatments 1, 2, and 4) infested with *T. aestivum* spores. The average potting medium pH for all treatments in Experiment 3B at the time seedlings were planted was within 0.5 pH units of the final values observed in Experiment 3A (Table 3-2), but after 12 months was significantly lower for all treatments (Table 3-3).

Table 3-2: Components of modified RPM potting media, final potting media pH (w), and resultant 4-month old seedling characteristics as compared with seedlings grown in the premixed RPM medium amended with agricultural lime provided by Forrest-Keeling Nursery.

Table 3-2: Components of modified RPM potting media, final potting media pH(w), and resultant 4-month old seedling characteristics as compared with seedlings grown in the premixed RPM medium amended with agricultural lime provided by Forrest-Keeling Nursery

Treatment	Ground bark type	% sand (vol)	% Ag lime (vol)	Response variables [a, b]			
				Final pH	Height (cm)	Diameter (mm)	Tissue volume (cm^3)
1	pine	20	0	7.99a	12.4a	2.6a	8.5ab
2	pine	10	10	8.23ab	9.1abc	1.9ab	7.5ab
3	pine	0	20	8.26b	3.4c	0.9b	4.1b
4	hardwood	20	0	8.40bc	10.7ab	1.9ab	7.7ab
5	hardwood	10	10	8.43bcd	4.9bc	1.4ab	5.4b
6	hardwood	0	20	8.50cd	9.4abc	1.7a	4.9b
RPM (premixed)	pine	20	(25 g/L)	7.69e	12a	2.8a	13.8a

[a] For each response variable, different letters represent statistically different means (Least Square Mean analysis, $\alpha = 0.05$)
[b] Response variables: final pH–potting media pH(w); height–seedling height (cm); diameter–seedling diameter (mm), measured 5 cm above the root crown; volume–total seedling volume (cm^3)

Table 3-3: Components of modified RPM potting media, and resultant 12-month old seedling characteristics, including fungal colonization after 12 months of growth. Treatments 1, 2, and 4 in Experiment 3B were selected from the preliminary Experiment 3A for having pH values within an acceptable range for *T. aestivum,* while also producing robust seedlings.

Table 3-3: Components of modified RPM potting media, and resultant 12-month old seedling characteristics, including fungal colonization after 12 months of growth. Treatments 1, 2, and 4 in Experiment 3B were selected from the preliminary Experiment 3A for having pH values within an acceptable range for *T. aestivum,* while also producing robust seedlings.

Treatment	Ground bark type	% Sand (vol)	% Ag lime (vol)	Response variables [a, b]						
				Initial pH	Final pH	Height (cm)	Stem diameter (mm)	Root tips	% *Tuber*	% Competitor
1	pine	20	0	7.8a	5.8a	15.9a	2.7a	1399b	2.3a	2.4a
2	pine	10	10	8.0a	6.5b	12.8b	2.3a	1066a	0.6a	0.8a
4	hardwood	20	0	8.1a	6.7b	15.2a	2.6a	1684c	1.6a	1.0a

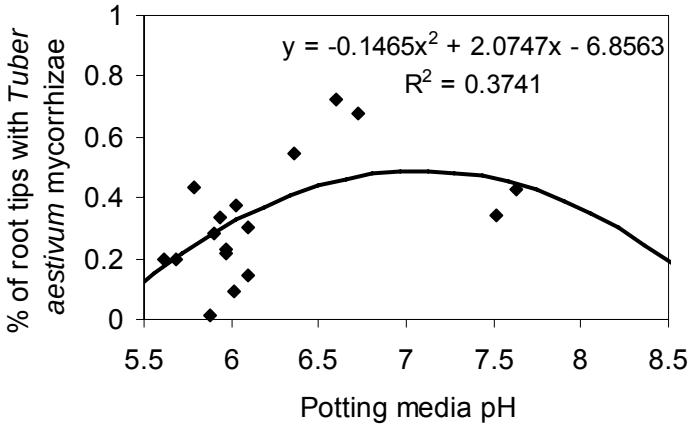
[a] For each response variable, different letters represent statistically different means (Least Square Mean analysis, $\alpha = 0.05$)
[b] Response variables: initial pH and final pH–potting media pH (w); height–seedling height (cm); diameter–seedling diameter (mm), measured 5 cm above the root crown; root tips–total count; % Tuber and % competitor–percentage of root tips colonized by *T. aestivum* or by other ectomycorrhizal fungi

Treatment 1 had a significantly lower final mean pH than Treatments 2 and 4 ($\alpha < 0.05$, Table 3-3). Adding lime to the pine bark-based RPM medium (Treatment 2) reduced seedling height and number of root tips ($\alpha < 0.05$), but did not increase potting medium pH or percent colonization by *T. aestivum* above the levels of the other treatments. All three treatments produced poor levels of mycorrhizal colonization in general, with levels of *Tuber* mycorrhizae less than three percent of total number of root tips (Table 3-3).

Experiment 4: Effect of lime level on *T. aestivum* colonization of typical medium

Although lime was added at levels expected to generate potting medium pH values ranging from 6.9 to 8.9, the potting medium pH values achieved ranged from 5.3 - 7.6 after 12 months of seedling growth. The polynomial regression analysis of 12–month final pH values with *T. aestivum* colonization levels suggests that maximum colonization levels will result with potting medium pH in the range of 6.5 – 7.5 ($\alpha < 0.05$, $R^2 = 0.374$, Figure 3-4). Of the original 25 trees, only 18 survived the entire 12 months.

Figure 3-4: Polynomial regression of percent of *Q. bicolor* x *Q. robur* seedling root tips displaying identifiable *T. aestivum* mycorrhizae across a pH gradient with seedlings grown in Typical media.


y = -0.1465x2 + 2.0747x - 6.8563
R2 = 0.3741
% of root tips with Tuber aestivum mycorrhizae
Potting media pH
1
0.8
0.6
0.4
0.2
0
5.5
6
6.5
7
7.5
8
8.5

Discussion

We presume that the productivity of a *T. aestivum* “plantation truffière” is directly related to the uniformity and density with which *T. aestivum* mycorrhizae are distributed throughout the orchard. Seedling production systems that stimulate prolific lateral root initiation in the greenhouse might result in more uniform and intensive colonization of a plantation site by *T. aestivum* mycorrhizae. Nevertheless, it does not necessarily follow that a more highly developed lateral root system in the greenhouse will be better colonized by *T. aestivum*, even in the presence of abundant inoculum. In our Experiment 1, the relative density of *T. aestivum* mycorrhizae on seedling root systems leaving the greenhouse appeared to determine the effectiveness with which *T. aestivum* proliferated in competition with indigenous mycorrhizal species.

The RPM seedling production system (Lovelace 1998) is a very effective method for the rapid production of very large tree seedlings with large and prolifically branched lateral root systems. The RPM system differs in two fundamental ways from the more typical and much less expensive production of seedlings in book type containers. The RPM potting medium is designed to maximize porosity, and the RPM container system promotes lateral root proliferation and growth while suppressing tap root development (Lovelace 1998). RPM seedlings are extensively used for rapid establishment of diverse tree species in challenging environments (Grossman *et al.* 2003). We set out to

determine whether or not the RPM system could be adapted to the production of high quality *T. aestivum*-infected seedlings.

In our first experiment, we found that seedlings grown according to a typical method in peat-based medium and book planters were significantly smaller yet much more densely colonized by *T. aestivum* than were seedlings grown in either the RPM or RPM + Manure medium in the RPM container system. After two years in the field, the Typical seedlings were still smaller than the RPM or RPM + Manure seedlings, but they had experienced the greatest percent increase in height and stem diameter, especially compared to seedlings produced in the RPM medium. Lighter grazing pressure by deer may have contributed to the large comparative growth increase for the smaller Typical seedlings. The seedlings produced by the Typical process had also gained the greatest percentage of *T. aestivum* mycorrhizae after 1 year in the field. The most common and effective method of infection of uncolonized root tips is by contagious hyphal tip-to-tip infection (Smith & Read 1997). Consequently, in a conducive plantation environment, seedlings well-colonized by *T. aestivum* in the greenhouse would have the best chance of developing well-colonized root systems in the field. The larger number of root tips produced per unit volume of potting medium in our Typical treatment, coupled with the associated greater inoculum density, probably explains the higher *T. aestivum* colonization levels for seedlings in our Typical treatment both in the greenhouse and in the field.

The *T. aestivum* colonization levels of the Typical and RPM + Manure seedlings declined during their second year in the field. There are at least two

possible explanations for this downturn in *T. aestivum* colonization levels. First, after a warm March, the central USA experienced a severe early April freeze in 2007, after most oaks had broken dormancy and prior to our third mycorrhizal sampling. We look forward to determining whether or not *T. aestivum* levels on these seedlings rebound in the coming years. Second, we may have influenced *T. aestivum* levels in the plantation by removing the weed barrier fabric from our seedlings after their first year in the field. Mulching techniques have been shown to differentially influence *T. aestivum* mycorrhizal levels in an experimental plantation truffière (Zambonelli *et al.* 2005). Clearly, there is much to learn about the environmental sensitivity of *T. aestivum* mycorrhizae and their formation.

Seedlings grown in RPM medium with the RPM container system developed the greatest percentage of indigenous competitor mycorrhizae after both one and two years in the plantation. We attribute this development to the greater total number of root tips available for mycorrhizal colonization and very low level of mycorrhizal infection associated with RPM seedlings leaving the greenhouse.

Our second experiment distinguished the effects of the bark-based RPM vs. peat-based Typical media from the effects of the RPM vs. Typical container systems. Seedlings grown in the peat-based medium in book planters developed far more *T. aestivum* mycorrhizae and no more competitor mycorrhizae than did seedlings in the other three treatments. Additionally, we note that total mycorrhizal counts (*T. aestivum* plus competitors) were greater in the Typical book planters regardless of the medium used, with *T. aestivum* dominating in the

peat-based Typical medium and competitors dominating in the bark-based RPM medium. We believe the increased level of mycorrhizal colonization in the Typical book planters is due to the greater number of root tips per unit volume of potting medium, as compared to the RPM container system.

Potting medium structure plays a critical role in seedling growth and mycorrhizal colonization (Tinus & McDonald 1979). The peat-based Typical medium had higher water holding capacity and lower porosity than the pine-bark based RPM medium. Porosity can affect seedling size by increasing air exchange and decreasing ambient CO_2 levels within the medium, thus increasing metabolism and growth (Landis *et al.* 1990). Based on the results of our first experiment, we hypothesized that the lower porosity of the Typical medium would reduce seedling growth. Unexpectedly, in this second experiment, seedlings grown in the Typical medium had the greatest height and stem diameter as well as root system length when grown in similar containers. The robust growth of the RPM trees in our first experiment was likely due to the presence of fertilizer and hygroscopic gel, which were reduced and removed (respectively) from the RPM medium in our second experiment. Seedlings grown in bark-based media require higher levels of supplemental N than seedlings grown in more easily decomposed media, to offset the nitrogen demands of composting microorganisms (Tinus & McDonald 1979). Bark-based media also have reduced cation exchange capacity (CEC), compared to peat-based mixes, further reducing the availability of plant nutrients (Tinus & McDonald 1979) and increasing seedling dependence on supplemental fertilizers.

In contrast to Experiment 1, seedlings grown in the RPM medium in RPM containers in Experiment 2 had the greatest root system volumes but significantly lower root system length and fewer root tips. Water and nutrient stress in field and pot experiments can lead to higher allocation of plant biomass to root systems and a reduction of root tips due to continuous damage to fine tissues (Gregory 2006). The reduction in seedling height between Experiments 1 and 2 and the relative increase in root system volume further indicate the need for supplemental fertilizer in the RPM media to generate large seedlings. Incorporation of slow release fertilizer and hygroscopic gels (for water retention) into the RPM and RPM + Manure would further reduce mycorrhizal colonization because both of these amendments have been shown to suppress mycorrhizal species in general (Wang & Gregg 1990; Austin & Bondari 1992; Volkmar & Chang 1995), and *T. aestivum* specifically (Pruett *et al.* 2008). Removing these amendments in our second experiment did not improve *T. aestivum* colonization levels, reduced seedling size and increased the root tissue volume.

In our third set of experiments we endeavored to determine if modifications to the RPM medium designed to raise medium pH would favor both *T. aestivum* colonization and seedling root system development. Even those modifications of the RPM medium which produced pH values expected to favor *T. aestivum* while supporting vigorous seedling growth, failed to support adequate levels of *T. aestivum* infection. Potting medium composition and structure appear to have the greatest influence on *T. aestivum*-colonization levels of seedling root systems. Very large pore space in the RPM and RPM + Manure

media may have led to high quantities of inoculum washing out of the media during watering or to poor contact between seedling root tips and *T. aestivum* inoculum. This effect appears to be species specific, because native mycorrhizal colonization in the greenhouse was highest on seedling root systems grown in the RPM medium amended with manure.

In our fourth experiment we sought to determine the best potting medium pH for maximizing root system colonization levels. The peat-based Typical medium had a higher buffering capacity than expected, resulting in lower than expected pH values based on the quantity of lime added. Nevertheless, we detected a downturn in *T. aestivum* mycorrhiza formation in Typical medium modified to pH values above 7.5. Root system colonization increased with pH values up to approximately 6.7, and might be expected to peak at approximately 7.1 based on our analysis.

Management implications

Many successful techniques have been developed for the production of healthy tree seedlings. The plethora of available container designs and potting medium components, amendments, and recipes can be bewildering. When selecting a seedling production system, if the objective is solely to grow a healthy tree seedling, then at least the target organism can be easily visualized and evaluated.

The situation becomes much more complex when the goal is to produce healthy seedlings well-colonized by a specific ectomycorrhizal fungus (*e.g.*, *T.*

aestivum). In such situations, the potting medium and greenhouse environment need to provide conditions conducive to fine root proliferation and physiological vulnerability to infection, while simultaneously providing conditions favorable for *T. aestivum* spore germination, seedling infection, and aggressive vegetative spread throughout the developing seedling root system. In addition, evaluation of success (or failure) requires microscopic observation of seedling root systems to determine the abundance of *T. aestivum* ectomycorrhizae. Our research is helping to evaluate our Typical and novel seedling production techniques for their potential to facilitate production of high-quality well-infected seedlings for establishment of more successful plantations truffières.

The RPM seedling production system quickly produces large tree seedlings with large and well ramified lateral root systems that demonstrate excellent survival in the field. Commercially available mycorrhizal inocula, in conjunction with naturally occuring inocula of contaminant mycorrhizal species in the greenhouse, generally result in prolific ectomycorrhiza development on RPM seedlings. However, we were not able to devise a modification of the RPM medium that favored colonization by *T. aestivum* (the Burgundy truffle). In contrast, our Typical peat-based potting medium permitted excellent root system colonization by *T. aestivum*.

Both the RPM potting process and the RPM potting medium depressed the extent of root system colonization by *T. aestivum*, as compared with the effects of book planters and our peat-based medium. Further, though RPM

seedlings are growing well in the field, *T. aestivum* has not proliferated on their root systems to the extent experienced by seedlings in our Typical treatment.

Having increased significantly in density during the first year following outplanting, *T. aestivum* mycorrhizal density declined significantly during the second year in the field, as estimated in late spring 2007. This decline in *T. aestivum* abundance corresponded with a significant increase in abundance of ectomycorrhizae of competing fungus species. This shift may be associated with removal of the water-permeable weed barrier fabric following the first field season. We have found that this fabric conserves soil moisture while moderating soil temperature. We removed this protection following seedling establishment at the end of the first field season because it is widely accepted that *T. aestivum* does not thrive in wet soil. Nevertheless, *T. aestivum* does thrive under closed forest canopies and beneath a shallow litter layer. The observed shift in mycorrhizal abundance may also have been caused by the unusually warm March followed by several days of freezing temperatures during April 2007 prior to May sampling.

Chapter 4 - TEMPORAL DYNAMICS OF ECTOMYCORRHIZAL COMMUNITY COMPOSITION ON ROOT SYSTEMS OF OAK SEEDLINGS INFECTED WITH BURGUNDY TRUFFLE

Introduction

Ectomycorrhizal fungi (EMF) play important roles in forest ecosystems through their symbiotic relationships with the roots of host plants. EMF influence host plant growth, drought tolerance, and nutrient accessibility (Smith & Read 1997). Additionally, their reproductive fruit bodies are important food sources for animals (Carey *et al.* 2002) and humans (de Román *et al.* 2006). Fungi in the genus Tuber form ectomycorrhizal relationships with a great variety of host genera including oak (*Quercus* spp.) and hazel (*Corylus* spp.) (Chevalier 1978a; Riousset *et al.* 2001). These fungi produce subterranean ascocarps called "truffles", mature specimens of several species being highly-esteemed for their superb flavor. Records of human consumption of truffles date as far back as the Greek empire (Hall *et al.* 2001).

Over the last century, truffle production in Europe has declined precipitously, from more than 1,000 tons per year to less than 100 tons per year in France alone (Hall *et al.* 2003). The decline is attributed to multiple factors including pollution and deforestation (Hall *et al.* 2003). To supplement the declining natural truffle production, cultivation of truffle fungi on the root systems of host plants was initiated in the early to mid-1970s, with the first truffles collected from an intentionally-established orchard in the late 1970s (Chevalier &

Grente 1979). Many successful orchards have been established in the subsequent years, and currently one-half of the truffles sold in Europe are harvested from such orchards (Hall *et al.* 2003). Unfortunately, not all orchards are successful and not all trees within a producing orchard generate truffles. The causes of orchard failure are poorly understood, but it has been hypothesized that many orchards fail due to poor site selection and/or poorly colonized planting stock (Mamoun & Olivier 1993a; Mamoun & Olivier 1993b; Hall *et al.* 2003), under competition from native EMF (Baciarelli-Falini *et al.* 2006) .

While progress has been made in identifying EMF community composition below ground (Dahlberg 2001; Horton & Bruns 2001), little is known about the factors, both biotic and abiotic, that influence the development of a given community structure. Additionally, the role that interspecific competition plays in determining EMF community composition is, as yet, poorly understood. Several studies have provided evidence for the role of interspecific competition in ectomycorrhizal community development. Ectomycorrhizal species in a pine forest in Pennsylvania, USA, have lower than expected levels of co-occurrence, indicating negative relationships among species (Koide *et al.* 2004). *Tuber borchii* competed successfully with *Laccaria bicolor* and *Hebeloma sinapizans* on seedlings of *Pinus pinea* both in the greenhouse and after outplanting (Zambonelli *et al.* 2000). *Rhizopogon occidentalis* out competed *R. salebrosus* when grown on the root systems of *Pinus muricata* in microcosms (Kennedy & Bruns 2005) and in the field (Kennedy *et al.* 2007). *Rhizopogon occidentalis* also colonizes root systems more quickly than *Tomentella sublilacina*, but *R.*

occidentalis colonization levels peak and then decline, while the number of *T. sublilacina*-colonized root tips increased steadily over time (Lilleskov & Bruns 2003). Most of the recent work on EMF competition involves pine systems or simplified systems with only two or three competing EMF. Little is known of the interactions among EMF in more complex communities.

If competition between EMF and intentionally introduced Tuber spp. is truly a factor influencing truffle orchard failure, the mechanisms of that competition may be direct or indirect (Kennedy & Bruns 2005). Direct competition between EMF species may take the form of allelopathy, sporophagy or mycelial overgrowth in order for fungal species to acquire higher levels of carbon from the host. Competition for carbon may also be mediated indirectly through the host in the form of carbon rationing by the host to EMF root colonizers (Kennedy & Bruns 2005). The plant may be more supportive of mycorrhizal formation with a fungus that demands lower levels of carbon. Additionally, EMF strains adapted to local climates may have a competitive advantage against diseases and predators, which may influence EMF composition. If certain indigenous EMF species are able to out compete *T. aestivum*, either directly or indirectly in the field, early detection of these species could inform land management decision-making.

Here, we report on a greenhouse and field study in which we monitored EMF community development annually for three years on oak seedling root systems inoculated in the greenhouse with the Burgundy truffle fungus (*T. aestivum*), beginning at outplanting in May 2005. We wished to determine the

effects of selected potting media on the colonization of oak seedlings in the greenhouse by *T. aestivum* and other EMF, and the ability of *T. aestivum* to compete with indigenous EMF following outplanting.

Materials and methods

Experimental design

We monitored EMF community composition on the root systems of *Q. bicolor* X *Q. robur* seedlings for three years following inoculation with *T. aestivum*. We grew seedlings in the greenhouse using three approaches: (1 and 2) the RPM method (Lovelace 1998) in RPM media with or without manure, and (3) according to a "Typical" method (Pruett *et al.* 200X) in a medium containing less organic matter. The RPM media consisted of ground pine bark, rice hulls, and sand (supplemented or not with cow manure), and was provided by Forrest-Keeling Nurseries (Elsberry, Missouri, USA). The Typical medium consisted of peat, sand, and vermiculite and was prepared at the University of Missouri – Columbia, according to Pruett *et al.* (200X). Acorns from a *Q. bicolor* x *Q. robur* hybrid were cold stratified for 90 days, moved to the greenhouse in Feb 2004, planted and managed as in Pruett *et al.* (200X), and allowed to grow in the greenhouse until May 2005.

Fresh truffle inoculum from Sweden was transported by air and frozen on arrival in December 2004 for experiments beginning in February 2005. Ascospore inoculum was prepared according to Pruett *et al.* (200X). Prior to

planting, an aqueous spore suspension was incorporated into the potting medium at a rate of 2 g truffle gleba per seedling (ca 5 x 10^5 spores L^{-1}) in the RPM and RPM + Manure potting media and 1 g truffle per seedling (1 x 10^7 spores L^{-1}) in the Typical potting medium. Each potting medium treatment was replicated 20 times in the case of the RPM and RPM + Manure treatments, and 15 times in the case of the Typical treatment, for a total of 55 seedlings. In May 2005, the trees were planted in the field (Pruett *et al.* 200X) in a completely randomized design in a 3 m grid. Each seedling was surrounded by a 1.8 m^2 sheet of black water-permeable mulching fabric to moderate soil temperature and moisture during the first field season. The fabric was removed in October, 2005 (Pruett *et al.* 200X).

The field site is a long-abandoned alfalfa (*Medicago sativa*) field at the University of Missouri Horticulture and Agroforestry Research Center (HARC), located in New Franklin, Missouri. The plantation was established on a loess ridge, with forested drainages approximately 20-30 m away on each side (Pruett *et al.* 200X). The site was limed at a rate of 5 tons $hectare^{-1}$ with agricultural crushed limestone (Mississippi Lime Company, Genevieve, Missouri) in December 2004 prior to planting. The limestone was lightly incorporated into the soil using a tractor disk to a depth of approximately 10 cm.

Data Collection

Annually in May of 2005, 2006, and 2007, we measured the pH (w) (Soil and Plant Analysis Council 1980) of soil or potting media samples from which

ectomycorrhizae were extracted. In May 2007, we also collected one-litre samples of soil from ten uniformly distributed locations across the plot. These samples were individually analyzed by the University of Missouri Extension Soil Analysis Lab (Columbia, MO, USA) to determine soil texture by particle size determination, pH (w), exchangeable calcium (Ca), assimilable phosphorus (P_2O_5), exchangeable magnesium (Mg), exchangeable potassium (K), Ca:Mg, K:Mg, organic matter, organic carbon, organic nitrogen, and C:N ratio. The results from the 10 samples were then used to generate mean, standard deviation, and range for comparison with soil values from sites within the native *T. aestivum* range. Air and soil temperature were collected hourly from the truffle plantation site using a Cambell Scientific Micrologger (Cambell Scientific Inc., Logan, Utah). Daily precipitation was monitored by HARC staff approximately 800 m from the plantation.

EMF colonization of seedling root systems was evaluated by randomly removing approximately 300 root tips from each seedling to determine the percentage of root tips colonized by EMF (Fischer & Colinas 1996) based on root tip morphology (Agerer 1996) and molecular confirmation (Pruett *et al.* 200X). Mycorrhizal root tips were morphotyped (Gardes & Bruns 1996a) and DNA was extracted from three to five root tips per morphotype per tree using the DNEasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's protocol. The ITS region of the DNA was amplified with primers ITS1F (5' CTTGGTCATTTAGAGGAAGTAA 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') in PCR reactions (Gardes & Bruns 1996b)

conducted in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). Each PCR reaction consisted of 200 µM dNTPs, 2.5 mM Mg^{2+}, 0.5 µM of each primer, 0.25 units of Taq DNA Polymerase (Promega, Madison, WI, USA), 1-5 ng DNA, and PCR water to 25 µL. PCR products were submitted to the University of Missouri – Columbia DNA Core Facility for sequencing. We then used BLAST searches in GenBank (http://www.ncbi.nlm.nih.gov/) and UNITE (http://unite.ut.ee/) in combination with the development of phylogenetic, neighbor-joining trees (Geneious Pro 3.0.6, Biomatters Ltd, Auckland, NZ) to group our unknowns into clades. We incorporated sequences representing confirmed species identities from UNITE into our trees to add validity to the clade taxonomic classification of the sequenced morphotypes. The UNITE organization only allows EMF sequences into their database that have been verified as accurately identified.

Data Analysis

Richness (S = the number of species present) and the Shannon Diversity index (*H*, derived from the abundance of the species present and the evenness of their representation) (Shannon & Weaver 1949) were generated annually for each seedling. Data were grouped by potting medium treatment (i.e., RPM, RPM + Manure, Typical) and year, for analysis using repeated measures Analysis of Variance (PROC MIXED, SAS 9.1, SAS Institute Inc., Cary, NC). To compare EMF community composition between seedling root systems we used the

nonmetric multidimensional scaling ordination technique (NMDS) (Minchin 1987), in combination with the Gower dissimilarity coefficient (Gower & Hand 1996), to produce cluster diagrams. The Gower methodology incorporates both presence/absence and abundance information when generating dissimilarity coefficients for communities. In the ordination plots, the Euclidean distance between plotted points varies linearly with the similarity of the fungal communities. Thus, points closer to each other represent seedlings with more similar EMF communities compared with points at a greater absolute distance.

Results

EMF community composition

At the time of outplanting, all seedlings had low EMF species diversity (*H*) and richness (S) (Figures 4-1 and 4-2). However, few seedlings grown in the RPM medium had become colonized by *T. aestivum* (Table 4-1). By the end of their first year in the field, the EMF communities of seedlings grown in the RPM medium were more diverse than those which developed in the Typical medium ($\alpha = 0.05$, Figure 4-1). By the end of the second year in the field, seedlings grown in the RPM medium had also acquired higher numbers of native EMF species ($\alpha = 0.05$, Figure 4-2). Both S and *H* increased during the two years following outplanting ($\alpha = 0.05$, Figure 4-1 and 4-2). Richness nearly quadrupled for the seedlings grown in RPM medium.

Figure 4-1: Ectomycorrhizal species diversity (*H* = Shannon Diversity Index) for *Tuber aestivum*-inoculated *Quercus bicolor* x *Q. robur* seedlings grown in RPM, RPM + Manure, or Typical potting medium. Seedlings were evaluated one year after germination (just prior to outplanting) (Year 1), and after one and two years in the field (Year 2, 3). Histogram bars with different letters represent significantly different means (α = 0.05).

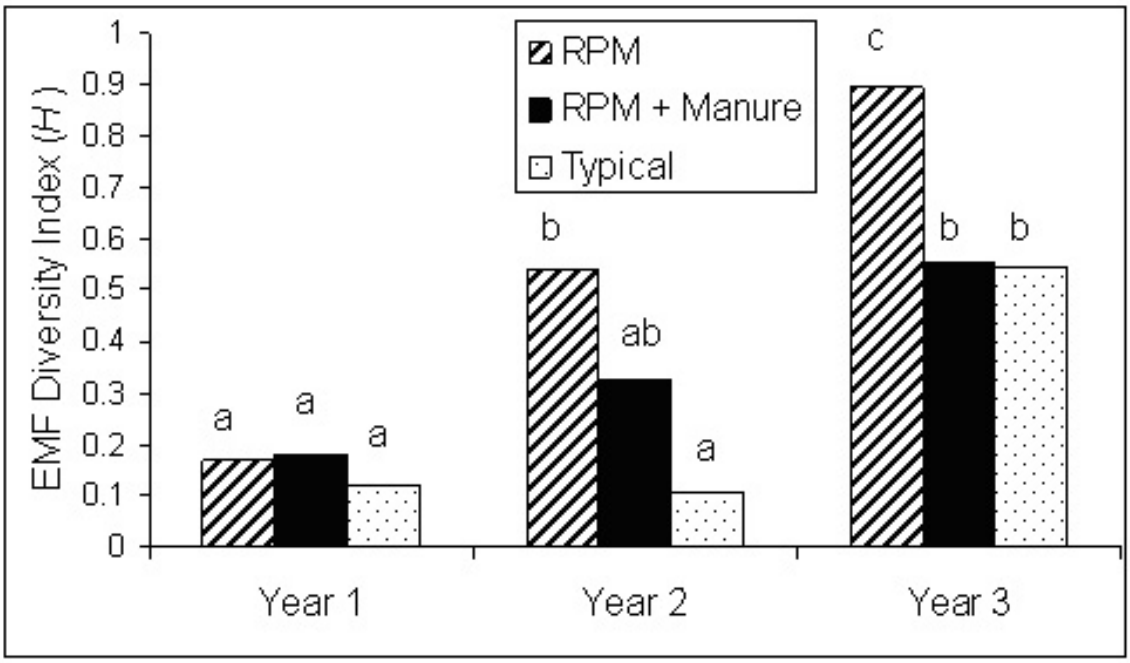
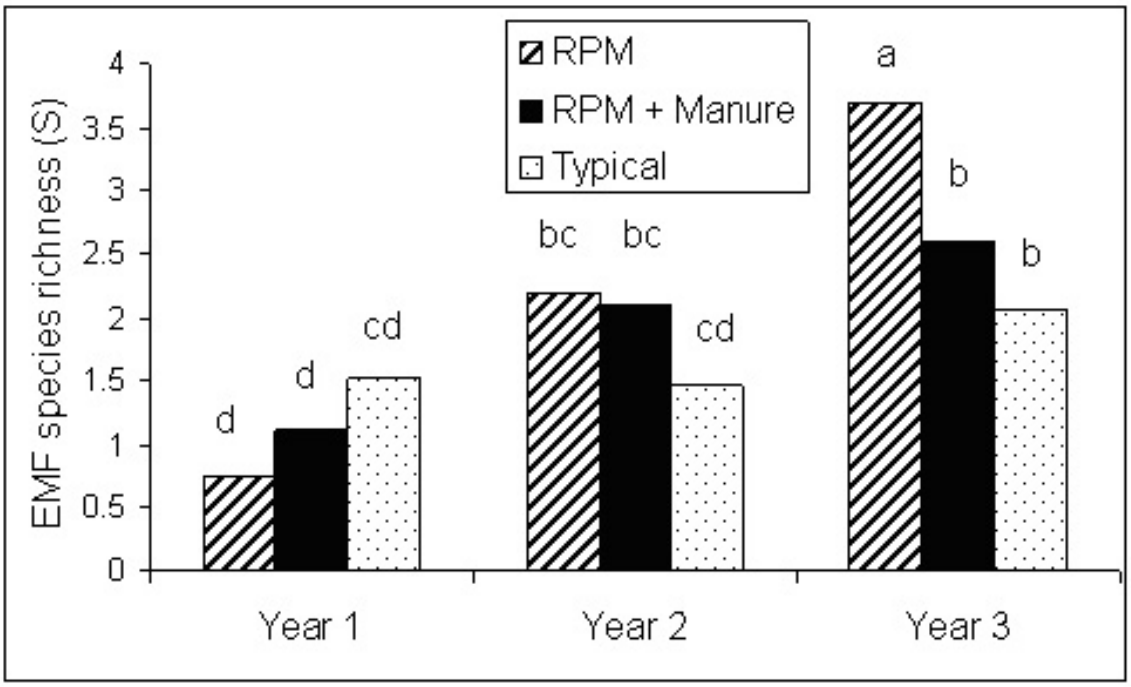

EMF Diversity Index (H')
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
RPM
RPM + Manure
Typical
a
a
a
b
ab
a
c
b
b
Year 1
Year 2
Year 3

Figure 4-2: Ectomycorrhizal species richness (S = number of species present) for *Tuber aestivum*-inoculated *Quercus bicolor* x *Q. robur* seedlings grown in RPM, RPM + Manure, or Typical potting medium. Seedlings were evaluated one year after germination (just prior to outplanting) (Year 1), and after one and two years in the field (Year 2, 3). Histogram bars with different letters represent significantly different means (α = 0.05).


RPM
RPM + Manure
Typical
EMF species richness (S)
4
3.5
3
2.5
2
1.5
1
0.5
0
d
d
cd
bc
bc
cd
a
b
b
Year 1
Year 2
Year 3

We detected a total of 34, 28, and 15 EMF species on seedlings grown in the RPM, RPM + Manure, and Typical media, respectively, during the three years of the study (Table 4-1). After one year in the greenhouse, seedling EMF communities comprised at most three species including: the introduced *T. aestivum*, *Tomentella ellisii*, and an as yet unidentified species (Table 4-1). After one year in the field, the total EMF community in the plantation also included one *Hebeloma* sp., three *Scleroderma* spp., a second (unidentified) *Tomentella* sp., two *Tuber* spp. with affinities to *T. maculatum* and *T. whetstonense*, and six unknowns (Table 4-1). Of the three EMF detected in the greenhouse, only *T. aestivum* and *T. ellisii* were still detected two years after outplanting. *Tuber aestivum* was least frequently detected on RPM seedlings and *T. ellisii* was least frequently detected on Typical seedlings. Two years after outplanting, the number of detected *Hebeloma*, *Tomentella* and *Tuber* species had increased, and six species in the Thelephorales were detected for the first time (Table 4-1). *Hebeloma* spp. only occurred on seedlings in the RPM treatment, and *Scleroderma* spp. occurred most often on RPM seedlings (Table 4-1). *Tomentella* spp. (except for *T. ellisii*) were rarely found on seedlings produced in the Typical medium (Table 4-1). All native *Tuber* spp. were much less frequently found on Typical seedlings than on RPM seedlings, whereas *T. aestivum* was least frequently found on RPM seedlings (Table 4-1). Eight of the 15 EMF species detected after one year in the field were not detected a year later (Table 4-1), while 28 new EMF spp. were detected after two years in the field. Only nine of

these 28 species were detected on Typical seedlings, whereas 21 of these species were detected on RPM seedlings.

NMDS ordination of seedling EMF communities using the Gower dissimilarity index highlighted both the temporal changes in EMF communities and the underlying treatment effects (Figure 4-3, a-f). The EMF communities on seedlings produced in all three media declined in similarity over time, but especially during their second year in the field (Figure 4-3, a-c). Qualitative shifts from year to year in EMF communities are also evident from the shifting positions of points representing different years in each treatment (Figure 4-3, a-e).

Direct year by year comparison of treatment effects on EMF community development (Figure 4-3, d-f) shows the relatively greater similarity of EMF communities developed in RPM and RPM + Manure media as compared with the EMF communities that developed in the Typical medium after 1 year in the greenhouse (Figure 4-3d). After one year in the field, EMF communities that developed in Typical medium remained similar as compared with EMF communities developing in the RPM medium (Figure 4-3e). However, after two years in the field, the EMF communities on seedlings produced in the Typical medium began to display reduced similarity, though they remained more similar than seedlings produced in the RPM medium (Figure 4-3f).

Table 4-1: Ectomycorrhizal fungus species detected in the greenhouse and the field on seedlings produced in three potting medium treatments.

Table 4-1: Ectomycorrhizal fungus species detected in the greenhouse and the field on seedlings produced in three potting medium treatments.

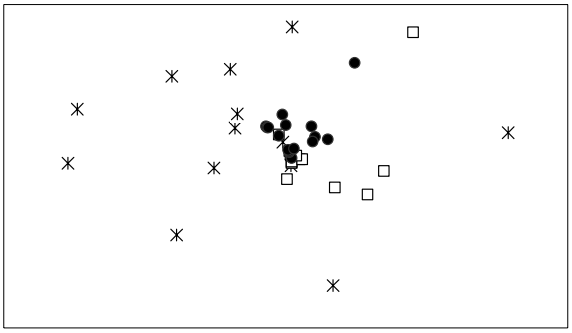
	Year[a]			Detections (N)[b]			
Identifier	1	2	3	RPM	RPM+ Manure	Typical	GenBank accession no.[c]
Hebeloma sp1	-	-	+	2	0	0	EU202687
Hebeloma sp2	-	-	+	2	0	0	EU202688
Hebeloma sp3	-	+	+	3	0	0	EU202689
Scleroderma sp1	-	-	+	0	0	1	EU202690
Scleroderma sp2	-	+	+	7	2	3	EU202691
Scleroderma sp3	-	+	-	3	0	0	EU202692
Scleroderma verrucosum clade1	-	+	-	1	0	0	EU202693
Thelephoraceae1	-	-	+	1	0	0	EU202694
Thelephoraceae2	-	-	+	0	0	1	EU202695
Thelephoraceae3	-	-	+	1	1	0	EU202696
Thelephorales sp1	-	-	+	1	1	0	--
Thelephorales sp2	-	-	+	1	0	0	--
Thelephorales sp3	-	-	+	2	5	3	--
Tomentella ellisii	+	+	+	32	26	12	EU202697
Tomentella sp1	-	-	+	2	3	1	EU202698
Tomentella sp2	-	+	-	1	1	0	EU202699
Tomentella sp3	-	-	+	0	1	0	EU202700
Tomentella sp4	-	-	+	0	1	0	EU202701
Tomentella sp5	-	-	+	0	2	0	EU202702
Tomentella sp6	-	-	+	1	2	0	EU202703
Tuber aestivum	+	+	+	17	37	39	EU202704
Tuber lyonii	-	-	+	1	2	1	EU202705
Tuber maculatum clade1	-	-	+	8	8	3	EU202706
Tuber maculatum clade2	-	-	+	3	0	1	EU202707
Tuber maculatum clade3	-	+	+	4	1	0	EU202708
Tuber rufum clade1	-	-	+	4	4	0	EU202709
Tuber rufum clade2	-	-	+	1	0	0	EU202710
Tuber whetstonense clade1	-	-	+	4	2	2	EU202711
Tuber whetstonense clade2	-	-	+	11	4	6	EU202712
Tuber whetstonense clade3	-	+	+	5	4	1	EU202713
Tuber whetstonense clade4	-	-	+	2	1	0	EU202714
Unknown Agaricales	-	-	+	0	1	0	--
Unknown Ascomycete	-	+	-	0	1	0	EU202715
Zalerion varium	-	+	-	0	1	1	EU202716
Unknown Pezizales	-	+	-	2	2	0	EU202717
Unknown1	+	+	-	3	1	1	--
Unknown2	-	+	+	1	2	0	--
Unknown3	-	-	+	4	1	0	--
Unknown4	-	-	+	0	1	1	--
Unknown5	-	-	+	1	0	0	--
Unknown6	-	+	-	1	0	0	--
Unknown7	-	-	+	2	0	0	--

Unknown8	-	-	+	1	0	0	--

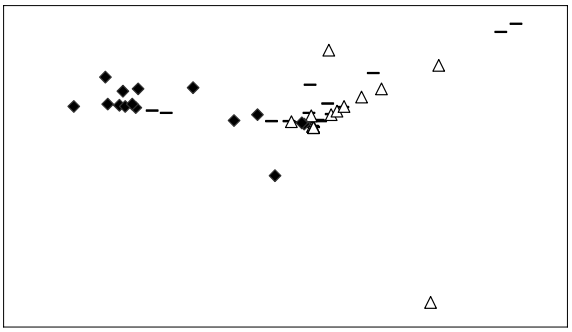
[a]Year, the year(s) in which a species was detected: 1=2005; 2=2006; 3=2007.
[b]Detections (N), the number of seedlings in each treatment on which a given ectomycorrhizal fungus species was detected over the three years of the study. RPM and RPM+Manure treatments were replicated 20 times and the Typical treatment was replicated 15 times. Each fungus could be detected each of three years for a total of 60 possible detections in the RPM and RPM+Manure treatments and 45 possible detections in the Typical treatment.
[c]Genbank accession number, entries with -- did not have adequate sequence quality for submission.

Figure 4-3: Scatterplots of ectomycorrhizal fungus community composition of seedling root systems using nonmetric multidimensional scaling (NMDS) and the Gower dissimilarity index. Each panel represents an independent analysis of community similarity. Panels a-c divide the data by treatment medium, thus presenting the change in similarity over the three years of study on seedlings produced in each experimental medium. Panels d-f divide the data by year, thus presenting the similarity among seedlings produced in the three media. As the community similarity decreases, the distance between points increases.

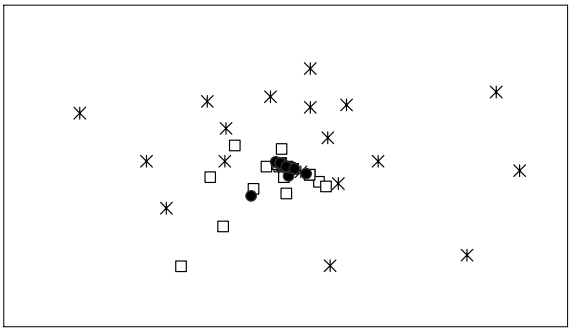
a. Typical medium – All years



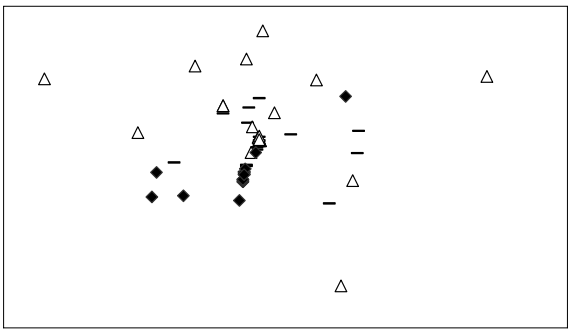
d. Year 1 – All treatments



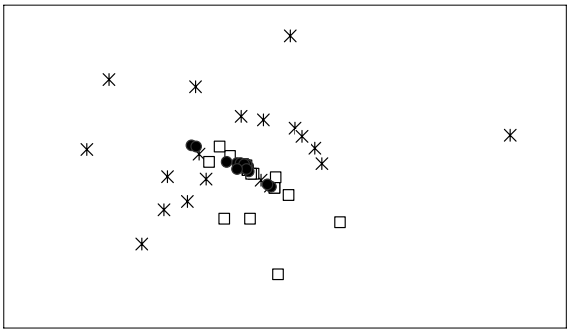
b. RPM medium – All years



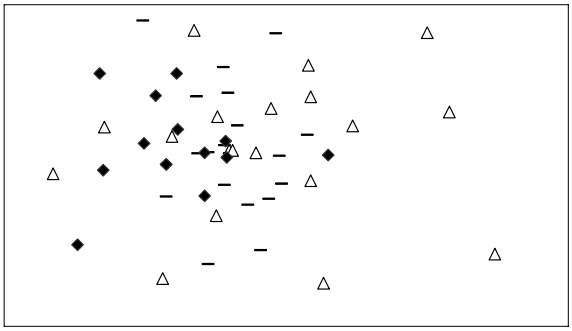
e. Year 2 – All treatments



c. RPM + Manure medium – All years



f. Year 3 – All treatments



● = Year 1 □ = Year 2 * = Year ♦ = Typical Δ = RPM ▬ = RPM + Manure

Soil analysis and climate data

Soil and climate characteristics of our plantation site are compared with those of Swedish (Gotland) and French (Burgundy) *T. aestivum* habitats in Tables 4-2 and 4-3, respectively. Considering the wide range of soil conditions conducive to *T. aestivum* growth in Europe (Chevalier & Frochot 1997; Wedén 2004) our Missouri soil falls well within the range of particle size distribution, pH (w), Mg, K, Ca:Mg, K:Mg, and organic matter encountered in Europe (Table 4-2). Missouri, Sweden, and France receive similar levels of precipitation in the fall and winter, but Missouri experiences more rain during the spring and summer months (April – Aug.) than do Gotland or Burgundy (Table 4-3). Missouri also has colder winters than Burgundy (similar to Gotland) and warmer summers than either Burgundy or Gotland (Table 4-3).

Table 4-2: Soil comparison of natural Swedish and French *T. aestivum* sites, and our Missouri *T. aestivum* plantation.

Table 4-2: Soil comparison of natural Swedish and French *T. aestivum* sites, and our Missouri *T. aestivum* plantation.

	Data range		
Measured parameter	USA (Missouri)	Sweden (Gotland)[a]	France[b]
Clay <2 µm %	10.0 – 15.0*	10.4 – 32.6	13.6 – 52.8
Silt 2-50 µm %	65.0 – 70.0	9.8 – 64.7	17.3 – 67.4
Sand 50-2000 µm %	17.5 – 22.5*	12.9 – 79.8	2.8 – 69.1
Water pH	7.7 – 8.0*	6.8 – 7.9	7.1 – 8.0
Exchangeable Ca (ppm)	1,200 – 2,200	3,600 – 10,700	2,800 – 7,900
Assimilable phosphorus (P2O5) (ppm)	10 – 23	20 – 1,200	20 – 820
Exchangeable magnesium (Mg) (ppm)	80 – 110*	90 – 450	50 – 410
Exchangeable potassium (K) (ppm)	130 – 180*	80 – 630	250 – 1,040
Ca/Mg	15.0 – 22.7*	12.4 – 67.7	19.5 – 116.5
K/Mg	1.5 – 2.3*	0.3 – 4.0	1.3 – 8.1
Organic matter (ppm)	25,000 – 36,000*	60,000 – 210,000	4,400 – 211,000
Organic carbon (ppm)	14,600 – 21,000	35,000 – 123,000	26,000 – 123,000
Organic nitrogen (ppm)	2,000 – 2,600	3,000 – 11,000	3,000 – 8,000
C/N Ratio	6.3 – 9.5	9.7 – 18.2	8.9 – 20.4

[a]data from Weden (2004).

[b]data from Chevalier & Frochot (1997).

*soil characteristics that are within the range occurring in Sweden and/or France.

Table 4-3: Monthly mean precipitation and temperature in Sweden (Gotland), France (Burgundy), and USA (Missouri).

Table 4-3: Monthly mean precipitation and temperature in Sweden (Gotland), France (Burgundy), and USA (Missouri).

	Monthly mean precipitation (mm)		
Month	Sweden (Gotland)[a]	France (Burgundy)[a]	USA (Missouri) ± SD[a]
January	43.3	73.6	65.4 ± 41.9
February	32.6	69.3	54.0 ± 42.0
March	34.6	69.3	58.0 ± 28.4
April	29.3	60.4	75.1 ± 34.6
May	28.0	81.9	131.6 ± 62.2
June	39.5	75.9	136.7 ± 52.7
July	51.6	65.4	98.8 ± 52.6
August	48.5	66.4	108.6 ± 82.9
September	56.8	72.0	60.9 ± 74.1
October	50.5	80.0	69.4 ± 47.9
November	57.5	83.2	56.2 ± 61.2
December	55.5	87.1	32.0 ± 31.7

	Monthly mean temperature (°C)		
Month	Sweden (Gotland)	France (Burgundy)	USA (Missouri) ± SD
January	-1.1	2.7	-0.2 ± 2.4
February	-1.8	3.6	0.9 ± 2.1
March	0.1	6.5	7.7 ± 2.5
April	4.0	9.0	14.5 ± 1.6
May	9.6	13.2	18.7 ± 1.5
June	14.3	16.0	23.2 ± 1.2
July	16.3	18.7	26.3 ± 0.4
August	15.9	18.6	25.8 ± 0.6
September	12.1	15.1	20.0 ± 1.6
October	8.1	10.9	13.4 ± 1.4
November	3.9	5.9	7.3 ± 2.4
December	0.7	3.8	1.2 ± 3.4

[a]Swedish data from Weden *et al.* (2004); French data from Chevalier & Frochot (1997); Missouri data from our research plantation at the University of Missouri Horticulture and Agroforestry Research Center, New Franklin, Missouri, USA.

Discussion

The diversity and richness of the EMF communities in each experimental treatment increased over the three years of our study. Seedlings in the RPM treatment had the lowest levels of colonization by *T. aestivum* and the greatest number of uncolonized root tips at outplanting (Pruett *et al.* 200X). These factors undoubtedly predisposed them to greater colonization by indigenous EMF in the field. Indeed, seedlings grown in the RPM medium developed the most diverse and species rich EMF communities by the end of the study. Duñabeitia *et al.* (2004) found that seedlings grown in uninoculated soil had higher colonization rates of native EMF in the field than seedlings inoculated with EMF in the greenhouse or nursery.

After one year in the greenhouse, the total greenhouse EMF community was limited to the introduced *T. aestivum* and two contaminant species, *Tomentella ellisii* and an unidentified species. During the subsequent two years in the field, levels of *T. ellisii* declined substantially, and the unidentified greenhouse morphotype disappeared entirely. In Alberta, Canada, pine seedlings inoculated with six EMF species in the nursery retained only one of these species after five years in the field (Gagné *et al.* 2006). The occurrence of EMF species under greenhouse or nursery conditions can be poorly correlated with their survival in the field. Competitive ability both in the seedling production system and in the field seem to be necessary for successful establishment of an EMF species in the field. In our study, the two greenhouse contaminant EMF species

colonized only a few percent of seedling root tips in any of the three potting media tested. Likewise, *T. aestivum* colonized very few root tips on seedlings grown in the RPM medium. However, *T. aestivum* colonized almost 10 percent of the root tips on seedlings grown in the RPM + Manure medium, and over 25 percent of root tips on seedlings grown in the Typical medium. Subsequent survival of *T. aestivum* in the field demonstrates the advantage of high levels of root tip colonization at outplanting on an EMF species' ability to compete with indigenous species.

Indigenous EMF species detected on the roots of seedlings after one year in the field included one *Hebeloma* sp., three *Scleroderma* spp., two *Tomentella* spp. (including *T. ellisii*), three *Tuber* spp. (including *T. aestivum*), and six unidentified morphotypes. Our field site is an old unmanaged alfalfa field on a loess ridge that has not supported EMF hosts for many decades. Consequently, the indigenous EMF species that infected our seedlings are most likely abundant in the neighboring forests (approximately 30 m distant from our research plantation). Some of these EMF species have probably developed spore banks in the soil at our study site. *Hebeloma cylindrosporum* has been shown to re-colonize sites annually via airborne basidiospores instead of mycelial colonization (Guidot *et al.* 2003). *Scleroderma* spp. are generally considered early colonizers of disturbed sites (Duñabeitia *et al.* 2004) and can thrive in dry conditions due to their long-distance exploration lifestyle defined by the use of hyphal-cords in foraging (Bakker *et al.* 2006). *Tuber* species are commonly vectored to new areas by forest animals that depend upon them for food (Carey

et al. 2002; Ashkannejhad & Horton 2006). The deer and rodents that frequent both our site and the neighboring forest patches may be a primary inoculum source for *Tuber* on our seedling root systems.

After two years in the field, we detected several additional species in the genera already known to be represented, as well as species in the Thelephorales. The EMF assemblage was still depauperate (32 taxa), compared to native EMF communities in California tanoak groves which can support 119 EMF species (Bergemann & Garbelotto 2006), pinyon pines in northern Arizona with 55 RFLP types (Gehring 1998), and Norway spruce forests in Sweden with more than 50 EMF taxa (Dahlberg *et al.* 1997). Our site, due to its disturbed nature, is potentially more similar to a clear-cut site in Alberta where only 11-13 EMF were recognized on the roots of the experimental pine seedlings after 5 years of growth (Gagné *et al.* 2006).

The role of a greenhouse potting medium is to help facilitate the establishment of seedling root systems supporting robust populations of the desired EMF species. It is apparent that successful establishment in the field of a new EMF species depends on adequate seedling colonization by that EMF species in the greenhouse (Pruett *et al.* 200X; Fischer & Colinas 1996). Field soil characteristics must take on a larger role long-term in shaping EMF community composition than the potting media treatment of seedlings in the greenhouse. The C:N ratio and soil pH are thought to play central roles in determining the belowground microbial community, including EMF species (Högberg *et al.* 2007). Agricultural sites generally have C:N ratios of less than 10 because of past N

fertilization and because they have a reduced litter layer compared to undisturbed forest soils (Eriksson *et al.* 1997). Low C:N may select against many forest-dwelling EMF species that depend on a litter layer (Högberg *et al.* 2007). In the Alps, species of *Tomentella*, *Tuber*, and *Hebeloma* were all most abundant in the mineral A-horizon of the soil. This horizon was derived from limestone and noted for relatively high soil pH, Ca, and K content as well as low C:N ratios (Baier *et al.* 2006). It is interesting that the competitive indigenous EMF species colonizers on our research site are those that specialize in limestone-derived mineral soil and tolerate increased pH and low C:N ratios. Our research site was specifically amended with crushed limestone to increase soil pH to favor *T. aestivum*. The edaphic characteristics of the site may be acting as a filter affecting colonization by local EMF species. The low C:N ratio and high pH of the soil at our research site appear to limit the diversity of colonizing EMF species and may suppress competition against *T. aestivum*.

The suppression of competitors by the environment in a truffle orchard is only important if *T. aestivum* is able to survive and produce truffles under the full range of conditions present. Our study site soil has a high enough pH (w) and low enough phosphorus level to support fruiting by *T. aestivum* (Wedén *et al.* 2004a). Though our soil is generally more silty than soils associated with fruiting in the Burgundy truffle's native range, *T. aestivum* has been shown to fruit over a wide range of soil textures (Wedén *et al.* 2004a). The soil Ca:Mg ratio at our study site is within the range of values associated with fruiting in Europe. Many truffle species (including *T. aestivum*) are thought to require high levels of soil Ca

for growth and/or fruiting. Both Ca and Mg compete for soil cation exchange sites, so high levels of Mg may reduce the supply of Ca to the fungus (Wedén *et al.* 2004a). Soil organic matter at our study site is within the range reported for *T. aestivum* fruiting, but the C:N ratio is near the low end of reported values, perhaps because our Missouri site is an old alfalfa field while the Swedish and French sites are natural forests (Compton & Boone 2000). As the trees in our study plantation mature, a litter layer will form that should increase the C:N ratio (Compton & Boone 2000).

Missouri winters are no colder on average than those on Gotland, but experience more fluctuation in monthly temperatures from year to year. This might influence the quality of the truffle harvest from year to year, but more so for winter-fruiting truffle species such as *T. melanosporum* than for late-autumn fruiting species such as *T. aestivum*. Summers are generally warmer and wetter in Missouri than in France or Sweden. Truffle primoridia (young fruit bodies) are thought to be very sensitive to drought in spring and summer (Sourzat 2002), so the higher levels of precipitation in Missouri during this period may favor truffle fruiting. It is unknown how the warmer temperatures in combination with the increased rainfall will influence truffle development in mid- to late- summer in Missouri.

During their first year in the field, the EMF communities on our Typical seedlings maintained low diversity and species richness, and correspondingly tight clustering in our NMDS scatter plots of the Gower Dissimilarity Index, all reflecting the dominance of *T. aestivum* on these seedlings' root systems (Pruett

et al. 200X). However, sampling after two years in the field showed a significant rise in both diversity and richness along with looser clustering of Gower dissimilarity values for Typical seedlings. This abrupt change in EMF community structure for Typical seedlings corresponded with two environmental events of potential significance. First, removal of the mulching fabric in October 2005 may have influenced seedling EMF community structure through effects on soil temperature and moisture. Water-permeable mulch fabric can favor *T. aestivum* colonization while suppressing competing EMF species (Zambonelli *et al.* 2005). Perhaps the mulch fabric provides an environmental effect analogous to canopy closure and/or development of a litter layer on the forest floor, both of which are factors associated with natural *T. aestivum* sites. The second notable environmental event involved an unusually warm month of March 2007, followed by a severe freeze that lasted several days at the beginning of April. It seems possible that this unusual weather pattern might have influenced the EMF communities we sampled in May 2007. In any case, we have replaced the mulch fabric in our plantation, and will continue to monitor the EMF communities.

Edaphic characteristics of our truffle orchard site appear to limit and filter the local native EMF colonizers to those that can tolerate high pH, low C:N ratios, and a limited litter layer. As the litter layer builds and the canopy closes over the life of the orchard, it will be interesting to see which species are added to the community cohort. There is slight, but not strong evidence for the greenhouse potting media predisposing seedlings to one community composition or another, but the predisposition may be due more to the different levels of mycorrhizal

colonization of the seedlings at outplanting and less to the media itself. The selected orchard site appears to have acceptable edaphic and climatic characteristics to support *Tuber aestivum* growth over the life of the truffle orchard.

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