

ARMILLARIA ROOT ROT OF PEACH:
BIOCHEMICAL CHARACTERIZATION, DETECTION OF RESIDUAL INOCULUM, AND
INTERSPECIES COMPETITION

by

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(Under the Direction of Harald Schemm)

ABSTRACT

In the southeastern United States, the practice of replanting of peach trees on the same orchard site and expansion of production into cleared forest lands have resulted in an increased prevalence of *Armillaria* root and crown rot, which develops in these situations due to contact between the roots of newly planted trees and infected residual root pieces in the soil. The limited success in managing *Armillaria* root disease is in part due to a lack of knowledge regarding the biology of fungi in the genus *Armillaria* in orchard ecosystems. A series of three studies was carried out to clarify selected aspects related to establishment, spread, and persistence of *Armillaria* in peach orchards. Specifically, these studies provided basic information on the biochemical characterization of *Armillaria* species, the extent of potential inoculum in the form of residual root pieces in orchard replant situations, and the potential for restricting colonization and persistence of *Armillaria* on peach roots with saprophytic antagonists. Using fatty acid methyl ester (FAME) analysis, it was determined that thallus type (mycelium, sclerotial crust, or rhizomorphs) did not affect overall cellular fatty acid composition of *Armillaria*, and that FAME profiles could be used to identify *Armillaria* isolates to species. Ground-penetrating radar was

used to detect residual peach roots in the field, quantify residual root mass following orchard clearing, and document that residual root fragments are of a size favoring *Armillaria* survival and infection. In an investigation of interactions between several species of saprophytic lignicolous fungi and *Armillaria*, such fungi induced hyphal and mycelial interference reactions against *Armillaria* and reduced growth of the pathogen when paired with it on peach roots, indicating the potential for restricting *Armillaria* colonization of dead or dying root tissue in the field. Knowledge gained from these studies contributes to a better understanding of and the potential for improved management of *Armillaria* root rot on peach and other stone fruits in the southeastern United States.

INDEX WORDS: *Armillaria* root and crown rot, Peach, *Prunus persica*, *Armillaria mellea*, *Armillaria. tabescens*, fatty acid methyl ester, FAME, ground-penetrating radar, lignicolous fungi, inoculum, hyphal interference, mycelial interference, antagonism, residual roots

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DEDICATION

I dedicate this dissertation to Dr. Harald Scherm for making a scientist out of me.

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CHAPTER 1

INTRODUCTION

Peach (*Prunus persica* (L.) Batsch) is an important fruit crop in the United States, planted on ca. 60,772 ha and having an annual value of \$467 million in 2003 (USDA National Agricultural Statistics Service 2004). Depending on the year, Georgia ranks from second to third in the United States in total production. In 2003, statewide peach production in Georgia covered >5,463 harvested ha and was valued at \$32.5 million (USDA National Agricultural Statistics Service 2004).

Armillaria root rot, a disease caused by several species of the basidiomycete *Armillaria* (Fr.:Fr) Staude, occurs worldwide on a wide variety of hardwood and softwood plants including many stone fruit species (Cooley 1943; Savage *et al.* 1953; Raabe 1962; Hood *et al.* 1991). In peach orchards in the southeastern United States, the disease has been problematic since the early 1950s (Savage *et al.* 1953; Rhoads 1954) and continues to cause extensive tree mortality during replanting and expansion of orchards (Cox *et al.* 2004), resulting in considerable economic losses to producers (Cooley 1943; Savage *et al.* 1953; Rhoads 1954; Steiner 1976). For example, in South Carolina peaches from 1980 to 1992, *Armillaria* root rot is estimated to have caused \$3.86 million in direct losses annually (Miller 1994). In addition to these direct losses, persistence of the pathogen in the soil on infested residual root pieces prevents the establishment of productive orchards in previously infested sites, thereby causing additional loss of income (Rhoads 1954; Doepel 1962; Chandler and Daniel 1982).

Root and crown tissues infected by *Armillaria* appear water-soaked, becoming white to yellow in color, spongy, and gelatinous. Removing the bark at the crown and roots often reveals

the presence of white to pale yellow fan-like sheets of mycelium, indicative of the pathogen. Although rarely seen on stone fruits in the Southeast, dark, shoestring-like rhizomorphs (produced only by the species *A. mellea* (Vahl.:Fr.) P. Kumm.) may originate from infected tissues. As the disease progresses and the cambium in the roots and crown is rotted, a rapid yellowing and defoliation of the tree occurs, followed by the death of individual limbs above diseased roots (Shaw and Kile 1991). Eventually, the entire tree is killed. These above-ground symptoms may not appear until severe damage to the root systems already has occurred, and young trees may die within the same season that symptoms manifest (Savage *et al.* 1953; Shaw and Kile 1991). Given proper temperature and moisture conditions, clusters of the yellow to brown basidiocarps can be found at the base of infected trees.

In southeastern peach orchards, the disease is caused primarily by *A. tabescens* (Scop.) Dennis *et al.* (= *Clitocybe tabescens* (Scop.) Bres.) and *A. mellea*. Although the two species are morphologically very similar, there are several features that distinguish them. The basidiocarps of *A. tabescens* lack an annulus just below the cap and can be a darker shade of brown than those of *A. mellea*. By contrast, an annulus is present on the basidiocarps of *A. mellea*, and in some cases rhizomorphs may be produced from host tissues infected by this species. Although the two species are distinguished easily by their basidiocarps, the latter rarely are produced in southeastern orchards, making it difficult to determine which species is more prevalent. *Armillaria mellea* is more aggressive than *A. tabescens* (Rhoads 1945), but little is documented regarding the prevalence of the two species and their roles in *Armillaria* root disease in peach production systems in the southeastern United States.

Armillaria root rot frequently develops in orchards planted on cleared forest stands or on replanted former orchard sites harboring endemic *Armillaria* populations. These populations are

capable of surviving for decades on infected root pieces that remain in the soil after tree removal (Reaves *et al.* 1993; Roth *et al.* 2000). Contact between live peach roots and infected root pieces in the soil is thought to initiate the disease in the orchard, whereas spores produced from basidiocarps in nearby forests are unlikely to contribute to the establishment of disease (Savage *et al.* 1953; Rishbeth 1964). Once this contact is made, the fungus grows throughout the roots to the crown, rotting cambial tissues. In the southeastern United States, rhizomorphs (produced only by *A. mellea*) are rare, hence spread of the disease throughout orchards occurs primarily by root-to-root contact. This root-to-root infection results in the outward expansion of clusters of diseased trees as neighboring trees subsequently become infected (Termorshuizen 2000). New sites of infection also can become established in non-infested areas of the orchard by moving infected root pieces through cultivation, erosion gullies, and careless tree removal practices (Steiner 1976).

Management options for *Armillaria* root rot are extremely limited (Doepel 1962; Steiner 1976; Guillaumin 1977; Hagle and Shaw 1991). Since *Armillaria* can survive in infected root pieces for decades, crop rotation has very little effect in reducing inoculum potential. Resistant rootstocks with acceptable horticultural features are not available at present, although breeding programs are addressing this problem (Proffer *et al.* 1988; Guillaumin *et al.* 1991; Beckman *et al.* 1998; Beckman and Pusey 2001; Beckman and Lang 2003). Thorough removal of residual root pieces throughout the entire rooting zone, which would be required to control the disease effectively in replant situations (Roth *et al.* 2000), is expensive and labor-intensive. Pre-plant fumigation with commonly used fumigants, although effective in some trials (Bliss 1951; Chandler 1969; Savage *et al.* 1974; Sherman and Beckman 1999), is not cost-effective and faces a very uncertain regulatory future. Post-plant management strategies that have been shown to be

somewhat effective are either too laborious (e.g., digging trenches between infected and healthy trees to avoid spread by root contact; Homer 1991) or unlikely to be labeled for commercial use (e.g., trunk injection of fungicides; Adaskaveg *et al.* 1999).

The limited potential for management of *Armillaria* root rot on peach is in part due to a lack of knowledge regarding several key aspects of the biology of *Armillaria* in the orchard ecosystem. Particularly, little is known about the relative prevalence of the two *Armillaria* species in southeastern United States and how to distinguish them in the absence of basidiocarps or rhizomorphs. There is also little information regarding the amount of potential inoculum remaining in the field after standard pre-plant and replant tree removal practices, or to what extent the remaining root fragments are of a size that would favor survival of the pathogen. In addition, there are numerous saprophytic lignicolous fungi present on dead or dying peach trees in the Southeast but very limited information regarding the interactions between them and *Armillaria*; such information may be useful for developing biocontrol tactics for competitive exclusion of *Armillaria* from peach roots in the orchard.

The overall goal of this research is to provide basic information on *Armillaria* species characterization, orchard inoculum, and interactions with other saprophytic fungi present in orchards. Specific objectives are to 1) characterize *Armillaria* species in southeastern peach orchards using fatty acid methyl ester profiles; 2) detect and quantify the extent of potential root inoculum in orchard replant situations using ground-penetrating radar; and 3) investigate the interactions between saprophytic fungi and *Armillaria* in controlled environments to determine the potential for competitive exclusion of *Armillaria* in the field.

Characterizing *Armillaria* spp. from southeastern peach orchards using fatty acid methyl ester profiling

Little is known about the cellular biology of *Armillaria*, including the composition of its fatty acids and the extent to which they could be used to answer questions about the ecology of *Armillaria*. Indeed, the cellular fatty acid composition of basidiomycetes in general, particularly those in the Agaricales, largely has been undocumented until recently (Müller *et al.* 1995; Stahl and Klug 1996; Dimou *et al.* 2002; Brondz *et al.* 2004). Despite the influences of age and the environment on fungal fatty acid profiles, there is also little information on how thallus or propagule type affects the profile. Different fungal thallus types (e.g., mycelium, sclerotial crust, and rhizomorphs) have different physiological functions (e.g., survival, dissemination, and infection), and each could thus have different fatty acid profiles.

In southeastern peach orchards, *A. tabescens* and *A. mellea* have been implicated as etiological agents of Armillaria root disease, but it is unclear which species is more important in causing the disease. Unlike *A. tabescens* (Rhoads 1945), *A. mellea* is capable of producing rhizomorphs, which allows it to potentially spread more efficiently. There is also evidence that *A. mellea* is more aggressive on peach than *A. tabescens* (Rhoads 1945; Raabe 1967). Potential differences in aggressiveness and rate of spread between the two species mandate the need to determine their prevalence and geographical distribution accurately.

Identification of *Armillaria* species based on morphology can be difficult because of variability in the distinguishing features (e.g., rhizomorphs and basidiocarps) and the critical dependence of their formation on environmental conditions (Rishbeth 1978; Guillaumin *et al.* 1991). Indeed, basidiocarps or rhizomorphs are rare enough in southeastern peach orchards to render them all but useless in species identification. Identification based on cultural techniques

such as mating assays (Korhonen 1978) is also often inconclusive because results can be variable and their interpretation subjective. Based on these considerations, molecular identification tools have been developed for distinguishing among species of *Armillaria* worldwide (Harrington and Wingfield 1995; Guillaumin *et al.* 1996; Schulze *et al.* 1997; Chillali *et al.* 1998; White *et al.* 1998; Perez-Sierra *et al.* 1999). Despite the success of the molecular techniques, there has been considerable difficulty using them with *Armillaria* isolates from peach in Georgia and South Carolina. Indeed, significant ITS and IGS heterogeneity has been revealed in these isolates in addition to the existence of inter-species ITS hybrids (Bryson *et al.* 2003; Kelly 2004). Based on these considerations, complementary identification tools are needed to determine to which species *Armillaria* isolates from peach in the Southeast are physiologically or biologically most similar. Given its successful application in other basidiomycetes such as *Rhizoctonia*, *Pleurotus*, and *Heterobasidion* (Stevens Johnk and Jones 1992; Müller *et al.* 1995; Baird *et al.* 2000; Dimou *et al.* 2002; Priyatmojo *et al.* 2002), fatty acid profiling may be useful as such a complementary tool.

The overall goal of this project was to provide a comprehensive characterization of the cellular fatty acids of the basidiomycete *Armillaria*. Specifically, I used fatty acid methyl ester (FAME) analysis to characterize the influence of thallus type on the fatty acid composition of *Armillaria*; determined whether *Armillaria* species known to occur in the southeastern United States can be distinguished from one another and from other saprophytic fungi typically occurring on peach using FAME analysis; and applied FAME profiling in an attempt to classify unknowns from naturally infected peach tissues and to validate results obtained with molecular classification tools. This could provide a better understanding of *Armillaria* species on peach in the southeastern United States than currently provided by molecular techniques alone.

Ground-penetrating radar to detect and quantify residual root fragments following peach orchard clearing

Peach orchards commonly are replanted when orchard profitability begins to decline, which may be as early as 4 to 6 years after initial establishment (Steiner and Lockwood 2004). *Armillaria* root rot is becoming more prevalent as producers are increasingly forced to plant into recently cleared forest lands or old orchard sites with endemic *Armillaria* populations. These fungal populations are capable of surviving for decades on infected root pieces that remain in the soil after tree removal (Reaves *et al.* 1993; Roth *et al.* 2000), and contact between growing roots of replanted trees and infected residual root pieces in the soil is thought to initiate the disease (Savage *et al.* 1953; Rishbeth 1964). Residual roots with diameters as small as 0.7 cm can support survival of *Armillaria*, while those of larger diameters are more important for establishing infection based on their greater inoculum potential (Bliss 1951; Patton and Riker 1959; Chandler and Daniel 1982). Thus, thorough removal of large root pieces after orchard clearing is an important strategy for reducing the disease in replant situations (Steiner 1976; Shaw and Kile 1991; Cox *et al.* 2004).

There is little information regarding the quantity of residual roots remaining in peach orchards after commercial tree removal and land clearing practices, or the extent to which these root fragments are of a size that would support survival of *Armillaria*. These questions could be addressed using ground-penetrating radar (GPR), a radar system that allows detection of changes in the soil profile including the presence of underground objects such as roots. GPR has been used successfully to map tree root systems in forest stands and in urban environments under concrete and asphalt (Hruska *et al.* 1999; Cremak *et al.* 2000; Butnor *et al.* 2001) and may have the potential for detection and quantification of residual roots in cleared peach orchards. The

purpose of this objective was to provide proof of concept for GPR detection of residual peach root fragments in the soil following orchard clearing. Specifically, I wished to 1) characterize GPR reflector signals from root pieces in a controlled burial experiment and 2) quantify the amount of residual roots remaining after orchard clearing typical of commercial practice. Information derived from this study could shed light on the potential amount of *Armillaria* inoculum remaining in peach orchards after commercial tree removal and replant practices. The study could also help clarify the extent to which residual root fragments are of a size favoring survival of *Armillaria*.

Interaction dynamics between saprophytic lignicolous fungi and *Armillaria* in controlled environments: Investigating the potential for competitive exclusion of *Armillaria*

Because of the limited effectiveness and/or feasibility of conventional management tactics, biological control of *Armillaria* root disease has been pursued for a number of years. In particular, species of the mycoparasite *Trichoderma* have been closely studied in this context. However, the inability to maintain adequate *Trichoderma* populations needed for control has limited the potential for *Armillaria* management in the field (Shaw and Roth 1978). Furthermore, *Armillaria*'s occupation of recalcitrant woody tissue and its ability to spread quickly to cambial tissues, noted by Rishbeth (1976), present inherent difficulties for a biocontrol agent such as *Trichoderma* that does not colonize these ecological niches.

Alternatively, saprophytic lignicolous fungi may be promising antagonists of *Armillaria*. Such fungi are non-pathogenic and soil-inhabiting, unlike *Armillaria*, and can be more aggressive colonizers of dead or decaying woody tissues. Moreover, these fungi, especially cord-forming basidiomycetes, have adopted combative strategies involving hyphal interference, gross

mycelial contact, and mycoparasitism to replace or confine other wood-rotting and pathogenic fungi (Boddy 1993, 2000). Still other saprophytes such as Xylariaceous ascomycetes are successful at early colonization of woody tissue, and once established can “hold their territory” against other wood-decay organisms (Boddy 2000).

In peach, the first tree deaths caused by *Armillaria* can occur a few years after orchard establishment (Savage *et al.* 1953; Shaw and Kile 1991). In subsequent years, the roots of infected trees become entirely colonized by the fungus, serving as an inoculum source for adjacent trees (via root-to-root contact) and providing massive inoculum levels in the case of replanting. If the root systems of dead or dying trees could be colonized by an effective competitor of *Armillaria* before tree removal, the extent of root colonization by *Armillaria* could be reduced, thereby decreasing the threat to adjacent trees and/or subsequent plantings. Such competitors may be found among saprophytic, lignicolous fungi which can be aggressive colonizers of dead or dying roots and are often observed on peach trees when *Armillaria* is absent (Adaskaveg *et al.*, 1993). These include several cord-forming basidiomycetes that are present in the southeastern United States (Nakasone 1993) and possess the ability to exclude pathogenic fungi such as *A. ostoyae* or *Heterobasidion annosum* in forest systems (Pearce 1990; Pearce *et al.* 1995; Varese *et al.* 1999, 2003; Chapman and Xiao 2000).

Based on the above considerations, saprophytic lignicolous fungi would be excellent candidates for competitive exclusion of *Armillaria* from peach root systems. However, there is little information regarding interactions between *Armillaria* and such potential antagonists in peach orchards. A detailed investigation of fungal interactions in controlled conditions could provide the necessary proof of concept for exclusion of *Armillaria*. Because of the very long-term nature (up to 10 years) of field experiments on managing *Armillaria* root disease, it is

critical that detailed micro- and mesocosm experiments on the ecology of and interactions between competitive saprophytic fungi and *Armillaria* precede field-scale evaluation of this approach.

The purpose of this objective was to examine the interactions between five species of saprophytic lignicolous fungi (*Ganoderma lucidum*, *Hypholoma fasciculare*, *Phanerochaete velutina*, *Schizophyllum commune*, and *Xylaria hypoxylon*) and two *Armillaria* species (*A. tabescens* and *A. mellea*) to determine the potential for competitive exclusion of *Armillaria* from peach roots. Specifically, I wished to determine the nature of interactions between the potential antagonists and *Armillaria* 1) at the microscopic or hyphal level, 2) at the colony level on woody tissue, and 3) at the microcosm level on peach root segments focusing on competitive colonization and fungal displacement.

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CHAPTER 2

LITERATURE REVIEW: ARMILLARIA ROOT ROT OF STONE FRUITS

Geographical prevalence and early reports

Armillaria root rot, a disease caused by the basidiomycete fungus *Armillaria* (Fr.:Fr) Staude, occurs worldwide on a wide range of hardwood and softwood plants including many stone fruit (*Prunus*) species (Cooley 1943; Savage *et al.* 1953; Raabe 1962; Hood *et al.* 1991). The disease causes severe tree mortality in orchards in Europe, North America, Africa, Asia, and Australia (Doepel 1962; Adaskaveg and Ogawa 1990; Hood *et al.* 1991). In Europe, *Armillaria* root rot causes considerable economic losses to peach, apricot, cherry, and almond orchards in the main region of stone fruit production in southern France and in Mediterranean regions of Spain and Italy (Guillaumin *et al.* 1991). In North America, the disease was first reported in California on peach in 1901 and then in prune, cherry, plum, almond, and apricot orchards soon thereafter (Gardner and Raabe 1963; Wilson and Ogawa 1979). During the 1930s, the disease was reported from peach orchards in Oklahoma, Arkansas, Missouri, and North Carolina, while it was rarely observed in Maryland, Pennsylvania, and Virginia (Cooley 1943; Rhoads 1954). In Georgia and South Carolina, *Armillaria* root rot was first documented in Spartanburg, South Carolina, and in several peach orchards at the Georgia Experiment Station during a series of surveys in the early 1950s (Savage *et al.* 1953; Rhoads 1954).

Since the early reports of *Armillaria* root rot in North American orchards, the disease has become more prevalent, primarily due to the expansion of stone fruit production into cleared forest lands (Adaskaveg and Ogawa 1990) and replanting into previous orchard sites. Since

1945, worldwide demand for fruit and nuts has increased, which resulted in an intensification of stone fruit production in the Sacramento and San Joaquin valleys of California (Adaskaveg and Ogawa 1990; Adaskaveg *et al.* 1993; 1999). Similarly, the disease is becoming more prevalent in Georgia and South Carolina as producers are increasingly forced to plant into forest lands or old orchard sites with endemic *Armillaria* populations (Savage *et al.* 1953; Rhoads 1954; Miller 1994).

Armillaria root rot continues to cause considerable economic losses in nearly all of the major stone fruit-producing states. California stone fruit orchards have a high incidence of root and crown rots; from 1986 to 1988, the incidence of wood decay, including that caused by *Armillaria*, ranged from 21 to 92% in the Sacramento and San Joaquin Valleys (Adaskaveg and Ogawa 1990). *Armillaria* root rot also causes substantial tree mortality in stone fruit orchards of Washington, Oregon, and Michigan (Proffer *et al.* 1988; Shaw and Kile 1991). In South Carolina between 1987 and 1992, the disease was estimated to have caused \$3.86 million in annual losses (Miller 1994), and in Georgia from 2000 to 2002 combined, *Armillaria* root rot, including control costs, resulted in >\$1.5 million in losses to the peach industry (Williams-Woodward, 2001, 2002, 2003). In addition to direct losses, persistence of the pathogen in the soil on infested residual root pieces prevents the establishment of productive orchards in previously infested sites, thereby causing additional loss of income (Rhoads 1954; Doepel 1962; Chandler and Daniel 1982).

Historically in the southeastern United States, *Armillaria* has been the second leading contributor of premature peach tree mortality after peach tree short life (PTSL) (Rhoads 1954; Miller 1994; Beckman and Pusey 2001). In recent years, however, premature death of trees caused by PTSL has decreased due to the widespread adoption of the PTSL-tolerant 'Guardian'

rootstock (Okie *et al.* 1994). By contrast, tree deaths due to *Armillaria* will likely increase as a greater proportion of trees on PSTL-tolerant rootstocks reach age classes susceptible to *Armillaria*.

***Armillaria* taxonomy and species identification**

Since the adoption of *Armillaria* as a tribe of *Agaricus* by Fries in 1821, the taxonomy of *Armillaria* has been in a constant state of change and controversy. In 1857, Staude raised Fries' *Armillaria* tribe designation to a genus. A question over authority for this action generated considerable controversy and was debated until the latter half of the 20th century. From this controversy the genus name *Armillariella* was adopted for several decades, which is now an obligate synonym for the genus *Armillaria*. In addition, a proposal for the incorporation of members of the genus *Armillaria* into the genus *Clitocybe* (Fr.) Staude by Ricken in 1915 added to the confusion. However, this ultimately resulted in incorporating *Clitocybe tabescens* (Scop.) Bres. into the genus *Armillaria* because of similarities in basidiome development, basidiospore wall structure, and bifactorial heterothallism (Shaw and Kile 1991).

Soon after the generic description of the fungus had been established, debate about the species within the genus arose. In the early 1960s, *A. mellea* (Vahl:Fr) P. Kumm. was known as a single species of variable morphology and pathogenicity. A decade later, Hintikka (1973) developed a compatibility test involving the pairing of single-basidiospore haploid isolates that demonstrated the existence of different species within the *A. mellea* complex, referred to as *A. mellea sensu lato*. These new species were subsequently referred to as "biological species" since they were biologically incompatible. Using this method, it was determined that there were five biological species of *Armillaria* in Europe and ten biological species in North America

(Korhonen 1978; Anderson and Ulrich 1979). Currently, biological species designations rarely are used, and most of the North American and European biological species have been described and named (Marxmüller 1992; Burdsall and Volk 1993).

Historically, >200 species have been attributed to the genus *Armillaria*, but most of these can now be placed in a number of modern genera to which they are more related (Burdsall and Volk 1993). More recently, 42 species of *Armillaria* were recognized (Fox 2000). Of these, only a few cause disease in stone fruits. *Armillaria mellea* is the primary species that causes root rot in stone fruits worldwide, although other species can be present or even predominant in a given region (Shaw and Kile 1991; Fox 2000). In Europe, particularly in France, England, and Italy, *A. mellea* is the predominant the species attacking stone fruits. However, *A. borealis* and *A. cepistipes* are known to attack wild cherry in France, and *A. gallica*, which is weakly pathogenic, also attacks stone fruit in France, England, and Italy (Guillaumin *et al.* 1991, 1993). In Australia, *A. luteobubalina* is the most common and most pathogenic species of *Armillaria* causing root rot in orchard crops including peach and nectarine (Kile and Watling 1988). By contrast, the species responsible for the disease in North American stone fruit orchards is variable depending on the region. In the western United States, *A. mellea* primarily causes the disease, particularly in California, Washington, and Oregon (Adaskaveg and Ogawa 1990). In Michigan cherry orchards, *Armillaria* root rot is predominantly caused by *A. ostoyae* followed by *A. mellea* and *A. calvescens* (Proffer *et al.* 1988). In the southeastern and midwestern states, the disease is believed to be caused primarily by *A. tabescens* and to a lesser extent by *A. mellea*. Indeed, *A. tabescens* attacks stone fruits in Oklahoma, Arkansas, and Missouri (Rhoads 1954); in Georgia and South Carolina, peach orchards are reportedly attacked almost exclusively by *A. tabescens* (Rhoads 1954; Cox *et al.* 2004).

Numerous methods have been developed to distinguish among species of *Armillaria*. Identification of *Armillaria* species based on morphology is possible but can be difficult because of variability in the distinguishing features (e.g., rhizomorphs and basidiocarps) and the critical dependence of their formation on environmental conditions (Rishbeth 1978; Guillaumin *et al.* 1991). Indeed, basidiocarps or rhizomorphs are rare enough in southeastern peach orchards to render them all but useless in species identification (Cox *et al.* 2004). Mating tests have been used extensively as a means of identifying *Armillaria* species. These tests are based on the same principles as used to establish the biological species concept in the 1970s (Hintikka, 1973; Korhonen 1978). The technique takes advantage of *Armillaria*'s heterothallic bifactorial sexual incompatibility system to distinguish species by the occurrence of a change in colony morphology resulting from a successful mating event. The mating itself involves the pairing of an unknown isolate against a series of haploid tester strains (single-basidiospore isolates) from all other known species. When a compatible mating reaction occurs on solid media, the typical white fluffy mycelium of haploid colonies becomes a stable diploid colony appearing reddish brown, flat, and crustose. Mating tests have been used to determine the occurrence and distribution of pathogenic species of *Armillaria*. Using mating tests, it was demonstrated that *Armillaria* root rot in Michigan cherry orchards was caused by three different *Armillaria* species localized in different production regions (Proffer *et al.* 1987, 1988). This subsequently allowed Michigan cherry growers to orient their rootstock breeding program toward the species present locally. Despite their relative simplicity, mating tests have the disadvantages of taking 6 to 8 weeks to complete, difficulty in interpreting the culture condition, and a constant need for fresh tester strains given that haploid cultures tend to lose their mating ability over time (Schulze *et al.* 1997).

Given the difficulties associated with morphological and cultural assays, a variety of biochemical and nucleic acid-based methods have been developed which allow rapid identification of inter- and intra-specific differences in *Armillaria*. Isozyme analysis, which differentiates multiple molecular forms of the same enzyme, can differentiate species as well as genets within species of *Armillaria* (Bergmann *et al.* 1996; Bragaloni *et al.* 1997; Robene-Soustrade and Lung-Escarmant 1997). For example, Bragaloni *et al.* (1997) were able to distinguish all European *Armillaria* species using a combination of esterase, glutamic-oxalacetic transaminase, alcohol dehydrogenase, phospho-gluco-mutase, and polygalaturonase isozyme patterns.

Nucleic acid-based identification tools are currently the most widely used and most reliable means of *Armillaria* species differentiation (Harrington and Wingfield 1995; Guillaumin *et al.* 1996; Schulze *et al.* 1997; Chillali *et al.* 1998; White *et al.* 1998; Perez-Sierra *et al.* 1999). These tools involve PCR amplification, often followed by restriction digestion, of conserved regions of rDNA, particularly the internal transcribed spacer (ITS) and intergenic spacer (IGS) regions. Indeed, North American, European, and Japanese species of *Armillaria* may be distinguished by IGS restriction enzyme patterns or by sequencing the amplified IGS region (Harrington and Wingfield 1995; Terashima *et al.* 1998; Perez-Sierra *et al.* 1999). In addition, PCR of the IGS region of *Armillaria* from dried basidiomes and infected wood scrapings has been demonstrated (Harrington and Wingfield 1995). However, despite the success of the molecular techniques, there has been considerable difficulty using them with *Armillaria* isolates from Georgia and South Carolina from peach. Indeed, significant ITS and IGS heterogeneity has been revealed in these isolates in addition to the existence of inter-species ITS hybrids (Bryson *et al.* 2003; Kelly 2004).

Symptoms, development, and spread

Armillaria infects root and crown tissues which results in the development of below- and above-ground symptoms. Below-ground the decay of woody tissue appears water-soaked, becoming white to yellow in color, spongy, and gelatinous (Doepel 1962). Removing the bark at the crown and roots reveals the presence of white to pale yellow fan-like sheets of mycelium, indicative of *Armillaria*. These mycelial sheets are usually 1 to 3 mm thick and often are marked with varying degrees of perforation (Rhoads 1945; Ross 1970; Shaw and Kile 1991). Although very rarely seen in the Southeast, dark, shoestring-like rhizomorphs (produced only by *A. mellea*) may originate from infected tissues. Given proper temperature and moisture conditions, clusters of the yellow to brown basidiocarps can be found at the base of infected trees. For *A. tabescens* the basidiocarps lack an annulus and are a darker shade of brown than those of *A. mellea*, which have an annulus and are a lighter honey color (Fox 2000).

Above-ground symptoms include chlorotic and stunted leaves with little terminal growth of shoots. A distinctive symptom in stone fruits is the curling of leaves along the midrib (sometimes accompanied by a bronzing of the foliage and stems) followed by wilting (Cox *et al.* 2004). As the disease progresses, a rapid yellowing and defoliation occurs, followed by the death of individual limbs above diseased roots (Steiner 1976; Guillaumin 1977; Shaw and Kile 1991). Eventually, the entire tree is killed. Gum produced from the cambium may exude from cracks in the bark after the infection has reached the root collar. These above-ground symptoms may not appear until severe damage to the root systems has already occurred, and young trees may die within the same season that symptoms manifest (Savage *et al.* 1953; Shaw and Kile 1991).

Armillaria root rot frequently develops in orchards planted on cleared forest stands or due to replanting into old orchard sites with endemic *Armillaria* populations. These populations are

capable of surviving for decades on infected root pieces that remain in the soil after tree removal and land clearing (Savage *et al.* 1953; Rhoads 1954; Reaves *et al.* 1993; Roth *et al.* 2000).

Contact between live peach roots and infected root pieces in the soil is thought to initiate the disease in the orchard, whereas basidiospores produced from basidiocarps in nearby forests are unlikely to contribute to the establishment of disease (Savage *et al.* 1953; Rishbeth 1964). Once this contact is made, the fungus grows throughout the roots to the crown, rotting the cambial tissues. In the southeastern United States, rhizomorphs are rare, hence spread of the disease throughout stone fruit orchards occurs primarily by root-to-root contact (Marsh 1952; Kable 1974). This root-to-root infection results in the outward expansion of clusters of diseased trees as neighboring trees subsequently become infected (Termorshuizen 2000). The expansion of these “infection centers” proceeds more rapidly in the direction of irrigation (Kable 1974), in mature orchards, and in higher-density plantings where the number of root-to-root contacts is greatest (Steiner 1976). Moreover, new sites of infection can also become established in non-infested areas of the orchard by moving infected root pieces through cultivation, erosion gullies, and careless tree removal practices (Steiner 1976).

Management of Armillaria root rot

Various cultural, biological, and chemical management strategies have been employed in attempts to control or reduce the incidence of Armillaria root rot, but the success of current management options has been limited (Doepel 1962; Steiner 1976; Guillaumin 1977; Hagle and Shaw 1991). Cultural methods of control focusing on the avoidance and reduction of *Armillaria* inoculum have met with little success or have been too cost-prohibitive. In a 20-year study in a severely diseased pine stand, Roth *et al.* (2000) evaluated several inoculum reduction strategies

and demonstrated that multiple mechanical ripping of the soil and successive hand removal of large roots were needed after stump removal to achieve any benefit in disease reduction. In kiwi orchards, *Armillaria* root rot is often initiated by spread from willow shelterbelts, followed by subsequent within-orchard spread by root contacts; this spread can be minimized and in some cases stopped completely by digging 1 to 2-m-deep trenches between infected and healthy vines, lining the trench insides with plastic tarp, and refilling with removed soil (Homer 1991). However, inoculum reduction strategies involving hand removal of root pieces or digging trenches are too expensive and labor-intensive to be practical. In addition, crop rotation has very little effect in reducing inoculum potential as *Armillaria* can survive in infected root pieces for decades. Resistant rootstocks with acceptable horticultural features are not available at present, although active breeding programs are addressing this problem (Proffer *et al.* 1988; Guillaumin *et al.* 1991; Beckman *et al.* 1998; Beckman and Pusey 2001; Beckman and Lang 2003). In general, plum rootstocks have good tolerance to *Armillaria* compared with those of peach, apricot, and sour cherry, which are highly susceptible (Guillaumin *et al.* 1991, Beckman *et al.* 1998). Indeed, in field trials involving natural and artificial inoculation, plum lines have shown better survival when compared with peach, almond, and peach × almond hybrids, although no lines are completely resistant (Guillaumin *et al.* 1991, Beckman *et al.* 1998). Several more recently developed, complex plum and plum × peach hybrids root stocks proved to be more susceptible than currently available standards (Beckman and Pusey 2001).

Chemical control of *Armillaria* root rot has been attempted, but because of the protected nature of *Armillaria* inoculum (i.e., being encased underneath the bark of roots and surrounded by soil), chemical management has met with limited success. Much of the research on fumigation, soil drenches, and tree injection with chemicals has been inconclusive or carried out

without sufficient field testing (Pawsey and Rahman 1976). However, there have been a few demonstrated successes with chemical control. Bliss (1951) found that carbon disulfide (CS₂) reduced the viability of *Armillaria* on buried citrus root pieces and enhanced the activity of resident antagonist *Trichoderma* populations which, in turn, led to the reduction of *Armillaria* in citrus soils. Similarly, in almonds grown on peach rootstock, pre-plant applications of sodium tetrathiocarbonate, a liquid that evolves CS₂ gas, was found to reduce the recovery of *Armillaria* from infected root segments and decrease disease severity in infected trees, but had no effect on tree mortality (Adaskaveg *et al.* 1999). The fumigant methyl bromide also prevented *Armillaria* root rot in peach replants for 3 to 5 years when applied after pre-plant inoculum reduction in dry, sandy soils (Sherman and Beckman 1999). Overall, however, pre-plant fumigation has not been cost-effective and faces an uncertain regulatory future. Post-plant passive injections of propiconazole into 7 to 8-year old infected almond trees grown on peach rootstocks reduced *Armillaria*-induced mortality compared with controls over a 2-year period (Adaskaveg *et al.* 1999). However, this tactic was only partly effective and is unlikely to be labeled for commercial use.

Because of the limited effectiveness and/or feasibility of conventional management tactics, biological control of *Armillaria* root disease has been pursued for a number of years. Species of the mycoparasite *Trichoderma*, in particular, have been studied closely in this context. The antagonism of *Armillaria* by *Trichoderma* species has been demonstrated *in vitro* and is believed to be a major factor in the destruction of *Armillaria* in studies involving sublethal fumigation or sublethal heating. Indeed, *Trichoderma* can replace *Armillaria* from buried woody inoculum (Garrett 1956, 1957; Munnecke *et al.* 1973, 1976; Otieno *et al.* 2003) and has been shown to suppress root disease caused by the pathogen in citrus orchards when used in

combination with sublethal fumigation (Bliss 1951). *Armillaria* is also much less tolerant to heating than *Trichoderma*, hence *Armillaria* in infested peach roots was more easily parasitized by *Trichoderma* after being weakened by heating and drying (Munnecke *et al.* 1976). Despite the aforementioned successes, the inability to maintain adequate *Trichoderma* populations needed for control has limited the potential for *Armillaria* management in the field (Shaw and Roth 1978). Furthermore, *Armillaria*'s occupation of recalcitrant woody tissue and its ability to spread quickly to cambial tissues, noted by Rishbeth (1976), present inherent difficulties for a biocontrol agent such as *Trichoderma* which does not colonize these ecological niches.

Alternatively, saprophytic lignicolous fungi may be promising antagonists of *Armillaria*. Such fungi are non-pathogenic soil-inhabitants and can be more aggressive colonizers of dead or decaying root and crown tissue than *Armillaria*. Moreover, these fungi, especially cord-forming basidiomycetes, have adopted combative strategies involving hyphal interference, gross mycelial contact, and mycoparasitism to replace or confine other wood-rotting and pathogenic fungi (Boddy 1993, 2000). Indeed, several cord-forming species have the documented ability to exclude pathogenic fungi such as *A. ostoyae* or *Heterobasidion annosum* in forest systems (Pearce 1990; Pearce *et al.* 1995; Varese *et al.* 1999, 2003; Chapman and Xiao 2000). For example, Pearce *et al.* (1995) found that species of *Hypholoma* and *Phanerochaete* in combination with ammonium sulfamate were able to reduce colonization, and in some cases completely exclude, *A. luteobubalina* from eucalypt stumps. Similarly, *H. fasciculare* also readily colonized the root systems of pine stumps in sites severely infested with *A. ostoyae*, excluding it in some cases (Chapman and Xiao 2000). Furthermore, many of these cord-forming basidiomycetes, aside from being avid colonizers of woody tissue, colonize in a subcortical manner similar to that of *Armillaria* (Redfern 1968).

Knowledge gaps

In the past 50 years, there has been a considerable amount of research on *Armillaria* root rot of various woody hosts. In particular, progress has been made in the identification and molecular characterization of *Armillaria* species involved in causing the disease. However, the current molecular methods have not been completely successful in differentiating and identifying *Armillaria* isolates from certain regions, such as those present in peach orchards of Georgia and South Carolina. Furthermore, while establishment and spread of the disease in the orchard is well understood and the mechanisms leading to outward expansion of infection centers are known, little is known about the amount of residual root inoculum remaining in orchard replant situations or the survival and inoculum potential of this root inoculum. Unfortunately, progress has been most limited in the area of management of *Armillaria* root rot. Given legislative restrictions and high costs associated with agricultural fumigants such as methyl bromide, genetic methods of control would be ideal; however, progress in breeding efforts for *Armillaria*-tolerant plum and plum-hybrid rootstocks has been limited to date. As for biological control, much is known about antagonistic *Trichoderma* species from laboratory and greenhouse-based studies, but little has been developed in terms of field-scale application. Perhaps the best avenue for biocontrol of *Armillaria* is with certain species of saprophytic lignicolous fungi, particularly species of cord-forming fungi such as *Hypholoma* and *Phanerochaete* which can quickly colonize the crown and root systems of trees and have been documented to reduce *Armillaria* colonization in forest systems. In orchard settings, there is only anecdotal information regarding the interactions between such antagonists and *Armillaria*, which points to the need for future investigations to explore their biocontrol potential.

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CHAPTER 3

**CHARACTERIZING *ARMILLARIA* SPP. FROM SOUTHEASTERN PEACH
ORCHARDS USING FATTY ACID METHYL ESTER (FAME) PROFILING¹**

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Characterization of *Armillaria* spp. from southeastern peach orchards using fatty acid methyl ester (FAME) profiling

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ABSTRACT

There is little information regarding the composition of cellular fatty acids in *Armillaria* or the extent to which fatty acid profiles can be used to characterize species in this basidiomycete genus. Fatty acid methyl ester (FAME) profiles generated from cultures of *A. tabescens*, *A. mellea*, and *A. gallica* consisted of 15 to 26 fatty acids ranging from 12 to 24 carbons in length, with 9-*cis*,12-*cis*-octadecadienoic acid (9,12-18:2), hexadecanoic acid (16:0), and other 18-carbon fatty acids being the most abundant. FAME profiles generated from different thallus morphologies (mycelium, sclerotial crust, or rhizomorphs) displayed by cultures of *A. gallica* showed that thallus type had no significant effect on the cellular fatty acid composition, suggesting that FAME profiling would be robust enough for species differentiation despite potential differences in thallus morphology among species. The three *Armillaria* species included in this study could be distinguished from other lignicolous basidiomycete species commonly occurring on peach (*Schizophyllum commune*, *Ganoderma lucidum*, *Stereum hirsutum*, and *Trametes versicolor*) on the basis of FAME profiles using stepwise discriminant analysis (average squared canonical correlation = 0.967), with hexadecanoic acid (16:0), 12-*cis*-hexadecenoic acid (C16:1), and tricosanoic acid (23:0) being the three strongest contributors to

distinguish between *Armillaria* and the outgroup species. In a separate stepwise discriminant analysis, *A. tabescens*, *A. mellea*, and *A. gallica* also could be separated from one another on the basis of their fatty acid profiles (average squared canonical correlation = 0.963), with tetradecanoic acid (14:0), 8-*cis*-octadecenoic acid (C18:1), and iso-tetracosanoic acid (iso-24:0) being the strongest contributors. When fatty acids were extracted directly from mycelium dissected from naturally infected peach tissue, the FAME-based discriminant functions developed in the preceding experiments classified 14 out of 16 samples as *A. tabescens* and the remaining two as *A. mellea*; when applied to cultures isolated from the same naturally infected samples, all 16 unknowns were classified as *A. tabescens*. Thus, FAME species classification of *Armillaria* unknowns directly from infected tissues may yield heterogeneous results. Species designation of unknown *Armillaria* cultures by FAME analysis was identical to that indicated by IGS-RFLP classification with *Alu* I, suggesting that this method can complement existing molecular tools for characterizing *Armillaria*.

INTRODUCTION

Armillaria root disease, caused by species of the basidiomycete *Armillaria* (Fr.:Fr) Staude, occurs worldwide on a variety of hardwood and softwood plants, including many stone fruit species (Savage *et al.* 1953; Raabe 1962; Shaw and Kile 1991). The disease can cause extensive tree mortality in peach (*Prunus persica* L. Batsch) production regions worldwide (Childs and Zeller 1929; Doepel 1962; Gardner and Raabe 1963; Hood *et al.* 1991), including orchards in Georgia and South Carolina (Savage *et al.* 1953; Rhoads 1954; Miller 1994). In South Carolina, *Armillaria* root rot was estimated to have caused \$3.86 million in losses annually during the period from 1987 to 1992 (Miller 1994). In Georgia in 2000, *Armillaria* root rot,

including control costs, caused an estimated \$660,000 loss to the peach industry (Williams-Woodward 2001).

Despite the potential for substantial losses, several key aspects of the biology and ecology of *Armillaria* in southeastern peach orchards are not well understood. Indeed, most of the information currently available on the prevalence of different *Armillaria* species, their population ecology, and their mode of infection and spread is based primarily on observational or anecdotal data (Cox *et al.* 2004).

Little is known about the cellular biology of *Armillaria*, including its fatty acid composition and the extent to which fatty acids could answer questions about the ecology of the pathogen. Indeed, the cellular fatty acid composition of basidiomycetes in general, particularly those in the Agaricales, has only been documented recently (Müller *et al.* 1995; Stahl and Klug 1996; Dimou *et al.* 2002; Brondz *et al.* 2004). Of the basidiomycetes, only *Thanatephorus* (*Rhizoctonia*), *Pleurotus*, and *Heterobasidion* are well characterized in terms of cellular fatty acid composition (Stevens Johnk and Jones 1992; Müller *et al.* 1995; Baird *et al.* 2000; Dimou *et al.* 2002; Priyatmojo *et al.* 2002a).

Fungal fatty acid profiles consist of cellular fatty acids derived from neutral lipids found in storage vesicles and those from phospholipids of membrane-bound organelles. These profiles are typically composed of 11 to 18 fatty acids ranging in length from 12 to 24 carbons, with linoleic acid (9-*cis*,12-*cis*-octadecadienoic acid) and palmitic acid (hexadecanoic acid) being the most abundant (Müller *et al.* 1995; Stahl and Klug 1996; Dimou *et al.* 2002; Larkin and Groves 2003; Brondz *et al.* 2004). Fungal fatty acid profiles are influenced by physiological age and environmental factors such as temperature and growth substrate (Graham *et al.* 1995; Müller *et*

al. 1995; Larkin and Groves 2003); because of these influences, fatty acid profiles may represent different physiological states of the fungus.

Despite the influences of age and the environment on fungal fatty acid profiles, there is little information on how thallus or propagule type affects the profile. Fatty acid profiles of fungi typically are obtained by extracting fatty acids from either mycelium or spores; however, the contribution of these or other fungal structures such as mycelial aggregates (e.g., stromata, sclerotia, or rhizomorphs) to the fatty acid profile is largely undocumented. Different fungal thallus types have different physiological functions (e.g., survival, dissemination, or infection), and therefore each could have different fatty acid profiles. *Armillaria* is an ideal candidate in which to study the contribution of thallus type to the cellular fatty acid composition as it readily produces several different thallus morphologies in culture..

Aside from providing an assessment of physiological or cellular chemical composition, analysis of cellular fatty acids is a useful taxonomic and diagnostic tool. It can identify and classify bacteria and yeasts (Moss 1981; Nichols *et al.* 1986; Augustyn *et al.* 1989, 1990; Veys *et al.* 1989). Similarly, analyzing fungal fatty acids have been used to identify and classify *Rhizoctonia* and various endomycorrhizal fungi (Stevens Johnk and Jones 1992; Graham *et al.* 1995; Bentivenga and Morton 1996; Baird *et al.* 2000). In *Pleurotus* and *Heterobasidion*, fatty acid and sterol content (FAST) profiles can distinguish among species and biological species (intersterility groups), respectively (Müller *et al.* 1995; Dimou *et al.* 2002). Species differentiation on the basis of fatty acid profiles may also provide a better means of identifying *Armillaria* species in the southeastern United States.

In southeastern stone fruit orchards, *Armillaria tabescens* (Scop.) Dennis *et al.* (= *Clitocybe tabescens* (Scop.) Bres.) and *A. mellea* (Vahl.:Fr.) P. Kumm. are the etiological

agents of *Armillaria* root disease, but it is unclear which species is more important in causing the disease. Unlike *A. tabescens* (Rhoads 1945), *A. mellea* is capable of producing rhizomorphs, which allows it to potentially spread more efficiently. There is also evidence that *A. mellea* is more aggressive on peach than *A. tabescens* (Rhoads 1945; Raabe 1967). Potential differences in aggressiveness and rate of spread between the two species suggests the need to determine their prevalence and geographical distribution accurately.

Identification of *Armillaria* species based on morphology is difficult because of variability in the distinguishing features (e.g., rhizomorphs and basidiocarps) and the critical dependence of their formation on environmental conditions (Rishbeth 1978; Guillaumin *et al.* 1991). Indeed, basidiocarps or rhizomorphs are rare enough in southeastern peach orchards as to render them all but useless in species identification. Identification based on cultural techniques, such as mating assays (Korhonen 1978), also is often inconclusive because results can be variable and their interpretation subjective. Based on these considerations, molecular identification tools have been developed for distinguishing among species of *Armillaria* worldwide (Harrington and Wingfield 1995; Guillaumin *et al.* 1996; Schulze *et al.* 1997; Chillali *et al.* 1998; White *et al.* 1998; Perez-Sierra *et al.* 1999). These tools involve PCR amplification, often followed by restriction digestion, of conserved regions of rDNA, particularly the internal transcribed spacer (ITS) or intergenic spacer (IGS) regions. Despite the success of molecular techniques, there has been considerable difficulty using them with *Armillaria* isolates obtained from Georgia and South Carolina peach orchards. Indeed, significant ITS and IGS heterogeneity in these isolates suggests that inter-species ITS hybrids exist (Bryson *et al.* 2003; Kelly and Taylor, *unpublished*). Based on these considerations, complementary identification tools are needed to determine to which species *Armillaria* isolates from peach in the southeastern United

States are physiologically or biologically most similar. Given its successful application in other basidiomycetes such as *Rhizoctonia*, *Pleurotus*, and *Heterobasidion*, fatty acid profiling may be useful as such a complementary tool.

The overall goal of this study was to provide a comprehensive characterization of the cellular fatty acids of the basidiomycete *Armillaria*. Specifically, we 1) used fatty acid methyl ester (FAME) analysis to characterize the influence of thallus type on the fatty acid composition of *Armillaria*; 2) determined whether *Armillaria* species that occur in the southeastern United States can be distinguished from one another and from other saprophytic fungi typically occurring on peach using FAME analysis; and 3) applied FAME profiling in an attempt to classify unknowns from naturally infected peach tissues and to validate results obtained with molecular classification tools. This could provide a better understanding of *Armillaria* species on peach in the southeastern United States than currently provided by molecular techniques alone.

MATERIALS AND METHODS

FAME profiles of different thallus types. The experiment was carried out with *A. gallica* isolate C6/C (Table 3.1) which readily produced undifferentiated mycelium, sclerotial crusts, and rhizomorphs *in vitro* (Fig. 3.1). A single isolate was used in order to minimize confounding by species or isolate effects. Cultures were grown on malt extract agar (30g/L) containing peptone (5g/L) in the dark at room temperature (ca. 23°C) until the desired thallus type had formed (ca. 1 week for mycelium and 3 to 4 weeks for sclerotial crusts and rhizomorphs). Samples of each thallus type (and agar as a control) were dissected from each culture and freeze-dried in a VirTis Series 2K freeze-dryer (VirTis, Gardiner, NY). FAMES were obtained using a direct trans-esterification protocol (Matsumoto *et al.* 1997; Sönnichsen and

Müller 1999; Lewis *et al.* 2000; Riley *et al.* 2000) with a glass bead-shredding step to improve tissue disruption. Briefly, 50 mg of freeze-dried tissue from each sample was added to 1.5 mL 3M methanolic hydrochloric acid and shredded for 3 min at 300 rpm using a Mini-BeadBeater (BioSpec Products Inc., Bartlesville, OK). Methyl-esterification of fatty acids was completed by transferring the organic phase to Pyrex test tube with 0.5 mL hexane (to improve lipid extraction) and incubating for 45 min at 100°C with vortexing for 15 sec every 15 min. FAMES were extracted by adding another 0.5 mL hexane and 1.0 mL methyl tert-butyl ether followed by rotary mixing for 10 min. The organic phase was transferred to a new Pyrex tube and washed by adding 3 mL HPLC-grade water followed by 5 min of rotary mixing. The organic layer was transferred to a chromatography vial, evaporated under N₂ gas, and resuspended in 0.5 mL of an equal-part hexane:methyl tert-butyl ether mixture. FAMES were analyzed on a Hewlett-Packard 5890 gas chromatograph (GC) (Agilent Technologies, Wilmington, DE) equipped with a flame ionization detector and an autosampler coupled to a Microbial Identification System (MIS Version 1.06; MIDI, Newark, DE). The FASTWO method (MIDI) and its corresponding calibration standards were used to define GC run conditions. Fatty acids were identified on the basis of their retention times relative to the calibration standard. For each of the three thallus types, FAME profiles were developed from four independent samples in each of three experimental runs (replicates).

Samples were analyzed based on the amount of detected fatty acids (expressed as a percentage of the total fatty acids for each sample). The SAS General Linear Model procedure (version 8.02; SAS Institute Inc., Cary, NC) was used to determine if there were significant ($P \leq 0.05$) differences among the three thallus types in the amount of each of the fatty acids detected.

FAME profiles of *Armillaria* species. To determine if southeastern *Armillaria* species can be distinguished from one another and from other wood-decaying basidiomycetes (outgroup species) that commonly occur on peach (Adaskaveg *et al.* 1993), FAME profiles were developed for four isolates each of *A. mellea*, *A. tabescens*, and *A. gallica*; and two isolates each of *Ganoderma lucidum*, *Schizophyllum commune*, *Stereum hirsutum*, and *Trametes versicolor* (Tables 3.1 and 3.2). Species identity of *Armillaria* isolates was confirmed by IGS-restriction fragment length polymorphism (RFLP) analysis with *Alu* I according to Harrington and Wingfield (1995) (Fig. 3.2).

All fungi were grown on poplar wood blocks ($6.3 \times 3.1 \times 0.5$ cm) that had been autoclaved in deionized water for 1 hour and placed on water agar dishes to prevent drying. The wood blocks were inoculated from 30-day-old agar cultures growing on malt agar with peptone. When the fungal hyphae had covered the blocks (ranging from ca. 2 weeks for *G. lucidum* to 4 weeks for the *Armillaria* spp.), they were scraped from the block, freeze-dried, and FAMEs extracted as described in the previous section. For each isolate, FAME profiles were developed from two independent samples in each of three experimental runs, with both wood block and agar controls included along with a reagent blank. FAME profiles were analyzed based on the amount of detected fatty acids (expressed as a percentage of the total fatty acids for each isolate) using discriminant analyses (SAS). FAME profiles were subjected to two separate stepwise discriminant analyses to determine which of the fatty acids was most significantly ($\alpha = 0.15$) separating 1) between *Armillaria* and the outgroup species and 2) among the three *Armillaria* species. To illustrate the differences among isolates graphically, canonical discriminant analyses were performed based on the fatty acids selected by stepwise discriminant analysis. Finally, discriminant function analyses were conducted on the fatty acids selected by the stepwise

procedures to parameterize discriminant functions for subsequent classification of unknowns; separate discriminant functions were developed to distinguish between *Armillaria* and the outgroup fungi and among the *Armillaria* species.

Classification of unknowns from naturally infected tissue. Infected root and crown tissues were collected from seven peach trees and one plum tree in four orchards in Georgia and two in South Carolina (Table 3.3). In the laboratory, mycelial sheets or fans resembling those of *Armillaria* were dissected from underneath the bark and stored at -80°C . At the same time, isolations were made from the infected tissue and the isolates cultured on poplar wood blocks as described above. Once blocks were completely colonized, the mycelium was scraped off and freeze-dried; mycelium obtained directly from the naturally infected tissue samples was freeze-dried at the same time. FAMES were analyzed in two experimental runs with appropriate agar, wood block, root, and reagent controls; each sample originated from different roots in the cases of naturally infected tissue and from two independent cultures in the case of isolates. The resulting FAME profiles (based on the percent contribution of each fatty acid) were entered into the discriminant functions developed in the previous section for classification of genera and species. To compare FAME classification with molecular classification, genomic DNA was extracted (DNeasy Plant Mini Kit; Qiagen Inc., Valencia, CA) from the unknown *Armillaria* isolates growing in culture, and IGS-RFLP analysis using *Alu* I was performed according to Harrington and Wingfield (1995).

RESULTS

FAME profiles of different thallus types. FAME profiles for the three thallus types consisted of 19 to 24 fatty acids ranging from 12 to 24 carbons in length. Only 11 of these fatty

acids were consistently present in their respective thallus type for all three experimental runs and contributed >0.5% of the total fatty acid content (Table 3.4); the remaining fatty acids were not considered in subsequent analyses. All 11 of the primary fatty acids were present in all three thallus types, with 9-*cis*,12-*cis*-octadecadienoic acid (9,12-18:2), hexadecanoic acid (16:0), and heneicosanoic acid (21:0) being the most abundant. There was variability in the amount of several fatty acids among the three thallus types, particularly for docosanoic acid (22:0) which was highest in mycelium ($1.6 \pm 0.9\%$) and near absent in rhizomorphs. However, there were no statistically significant differences in percent abundance for any of the fatty acids among the three thallus types.

FAME profiles of *Armillaria* and outgroup basidiomycete species. Across the seven fungal species, 48 fatty acids were detected which ranged from 12 to 24 carbons in length, with 9-*cis*,12-*cis*-octadecadienoic acid (9,12-18:2) and hexadecanoic acid (16:0) being most abundant. Five fatty acids were unique to the three *Armillaria* spp. and were not present in the other basidiomycetes tested. Conversely, 22 fatty acids were only found in the outgroup basidiomycetes. However, several of these were present in trace amounts only (<0.5%) and most were not named by the FASTWO method.

Armillaria species were distinguished from the other basidiomycete species on the basis of their FAME profile using discriminant analysis. Thirteen fatty acids were selected into the stepwise discriminant analysis (average squared canonical correlation = 0.967), with hexadecanoic acid (16:0), 12-*cis*-hexadecenoic acid (C16:1), and tricosanoic acid (23:0) being the three strongest contributors to the distinction between *Armillaria* and the outgroup species (Table 3.5). Canonical discriminant analysis, performed using only the fatty acids selected by the stepwise discriminant analysis, readily separated *Armillaria* species from outgroup species by the

first canonical axis (Fig. 3.3). Discriminant functions to distinguish the two groups are given in Appendix 1.

Discriminant analysis distinguished *A. tabescens*, *A. mellea*, and *A. gallica* from one another on the basis of their fatty acid profiles. Eleven fatty acids were selected into the stepwise discriminant analysis (average squared canonical correlation = 0.963) with tetradecanoic acid (14:0), 8-*cis*-octadecenoic acid (C18:1), and iso-tetracosanoic acid (iso-24:0) being the three strongest contributors to the distinction among the three *Armillaria* species (Table 3.6).

Canonical discriminant analysis performed using only the fatty acids selected by the stepwise discriminant analysis revealed that *A. mellea* is distinguished from *A. tabescens* and *A. gallica* by the first canonical axis, while *A. gallica* and *A. tabescens*, more similar in FAME profiles, are separated by canonical axis 2 (Fig. 3.4). Discriminant functions to distinguish among *Armillaria* species on the basis of FAME profile are given in Appendix 2.

Classification of unknowns from naturally infected tissue. FAME profiles of the samples from both the infected tissues and the corresponding isolates were similar to those obtained for *A. tabescens*, *A. mellea*, and *A. gallica* above in that they consisted of 15 primary fatty acids (>0.5% abundance) ranging from 12 to 24 carbons in length with 9-*cis*,12-*cis*-octadecadienoic acid (9,12-18:2), hexadecanoic acid (16:0), and 8-*cis*-octadecenoic acid (C18:1) being the most abundant. Fatty acid profiles obtained directly from infected host tissues were similar to those of the corresponding cultures, except that heneicosanoic acid (21:0) and 2-hydroxy-hexadecanoic acid (OH-16:0) were higher in samples from cultures, while tricosanoic acid (23:0) was consistently higher in samples from infected tissue.

All of the unknowns were classified as *Armillaria* using the discriminant function described above for distinguishing *Armillaria* from the outgroup basidiomycetes. The

discriminant function for separating the three *Armillaria* species classified 14 out of 16 samples obtained from host tissues as *A. tabescens* (Table 3.3); however, at two orchard locations, one of the two host tissue samples was classified as *A. tabescens*, while the second sample was classified as *A. mellea* (Table 3.3). By contrast, all samples obtained from fungal cultures isolated from the same roots were classified as *A. tabescens* (Table 3.3). Furthermore, IGS-RFLP analysis of these fungal cultures resulted only in banding patterns indicative of *A. tabescens* (Table 3.3, Fig. 3.5).

DISCUSSION

This is the first in-depth study quantifying the cellular fatty acid composition of *Armillaria* and the influence of thallus type (mycelium, sclerotial crusts, or rhizomorphs) on the fatty acid profile in this genus. Using *A. gallica* as a model, we showed that thallus type had no significant effect on the cellular fatty acid composition. When combined with discriminant analysis, FAME profiling distinguished *Armillaria* species from one another and from other lignicolous basidiomycetes commonly found on peach. The potential for classification of unknowns from naturally infected host tissue and agreement with DNA-based classification suggest that FAME profiling could be of practical use in determining the identity of *Armillaria* species occurring on peach in the southeastern United States.

The fatty acids detected for *Armillaria* in this study, similar to those reported for other basidiomycetes (Müller *et al.* 1995; Stahl and Klug 1996; Dimou *et al.* 2002; Brondz *et al.* 2004), ranged from 12 to 24 carbons in length with 9-*cis*,12-*cis*-octadecadienoic acid (9,12-18:2), hexadecanoic acid (16:0), and other 18-carbon fatty acids being the most abundant. However, the profiles from *Armillaria* contained more fatty acids than reported in previous

studies with other basidiomycetes: FAME profiles for *Armillaria* contained 15 to 26 fatty acids, while between four (*Hypholoma* and *Cortinarius*) and 15 (*Heterobasidion*) have been reported in other basidiomycetes (Müller *et al.* 1995; Stahl and Klug 1996; Dimou *et al.* 2002; Brondz *et al.* 2004). Brondz *et al.* 2004 found only seven fatty acids in basidiospores of *A. borealis*, which is less than half the number of fatty acids reported for any of the mycelial profiles in the current study. Since most of the aforementioned studies used similar direct trans-esterification protocols, the higher number of fatty acids in our profiles may be due in part to the incorporation of a bead-shredding step to improved tissue homogenization. Indeed, Riley *et al.* (2000) reported that fatty acid extraction and consistency in the ascomycete *Ceratocystis* was enhanced by vortexing with glass beads, as compared with standard methods. The difference in fatty acid numbers between our profiles and those reported for *A. borealis* (Brondz *et al.* 2004) may also be due to species differences or differences in fungal structures examined. All of the thallus types included in the present study consisted of secondary mycelium (diploid), while the basidiospores analyzed by Brondz *et al.* (2004) are haploid and may only contain the most essential fatty acids necessary for undergoing germination and hyphal fusion. Discrepancies in fatty acid composition between secondary mycelium and basidiospores may help explain the physiological infection success of the former compared with the latter.

We found no significant differences in fatty acid composition among the thallus types important for infection and survival (mycelium, sclerotial crusts, and rhizomorphs) in *A. gallica*. This is somewhat surprising given that the three thallus types in our study were primarily the result of different physiological ages of cultures and that culture age has been shown previously to influence fungal fatty acid profiles (Stahl and Klug 1996; Larkin and Groves 2003). In endomycorrhizal zygomycetes, certain fatty acids are found exclusively or in higher

concentrations in spore tissue than infected roots (Graham *et al.* 1995); however, the fastidious nature of endomycorrhizal fungi prevents meaningful in-depth comparisons between spore and mycelial fatty acid profiles. In the current study, the lack of differences in fatty acid composition between mycelium, sclerotial crusts, and rhizomorphs could indicate either that fatty acids are not important for the physiology of these structures or that such differences are not expressed in laboratory conditions.

Armillaria tabescens, *A. mellea*, and *A. gallica* were readily distinguished from other basidiomycete genera on the basis of fatty acid composition. Indeed, when comparing *Armillaria* FAME profiles with those of the other genera, there were differences in both the presence/absence of individual fatty acids as well as in the amounts of fatty acids common to all genera examined. Similarly, Stahl and Klug (1996) found that only four of ten fatty acids were common among five basidiomycete genera, and Brondz *et al.* (2004) reported that only four of eleven fatty acids were common among species in five genera of Agaricales and in one genus of Russulales. Based on the combined evidence from the current study and previous reports, it is therefore reasonable to conclude that basidiomycete genera have little commonality in fatty acid composition.

When combining FAME profiles with discriminant analysis, it was possible to differentiate among the three *Armillaria* species present in the southeastern United States, i.e., *A. tabescens*, *A. mellea*, and *A. gallica*. There have been similar reports of basidiomycete differentiation to the species and biological species (sterility group) level based on fatty acid analysis. Indeed, ten species of *Pleurotus* were differentiated using three fatty acid ratios, while three intersterility groups of *Heterobasidion annosum* were distinguished using discriminant analysis of FAST profiles (Müller *et al.* 1995; Dimou *et al.* 2002). Furthermore, several studies

have demonstrated that species, and in some cases isolates, of *Rhizoctonia* are distinguishable on the basis of fatty acid profiles (Matsumoto *et al.* 1997; Priyatmojo *et al.* 2002a, b). In the context of the present study, differentiation beyond the species level seems unwarranted until a better understanding of the southeastern *Armillaria* species complex is reached.

Identification of the unknowns using fatty acid analysis combined with the discriminant functions revealed that the unknowns were all *Armillaria* species, primarily *A. tabescens*. This is in agreement with field observations regarding the prevalence of *A. tabescens* basidioma in southeastern peach orchards (Rhoads 1954; Steiner 1976; Cox *et al.* 2004). To the best of our knowledge, this is the first study to use fatty acid profiles derived from reference isolates to classify unknown fungal samples collected at a later date. Furthermore, this appears to be the first study to attempt fungal classification from natural sources (i.e., infected root and crown tissues). In endomycorrhizal zygomycetes, fatty acid profiles from artificially infected roots have been used to distinguish species (Bentivenga and Morton 1994, 1996; Graham *et al.* 1995), but none of these studies attempted classification of unknowns.

Examination of the unknowns by fatty acid analysis indicated some discrepancies between samples extracted directly from infected host tissues compared with those from the corresponding fungal cultures. At two of the orchard sites, mycelium dissected from one of the two host tissue samples was classified as *A. tabescens* while the second sample was classified as *A. mellea*; the corresponding cultures isolated from these tissues were all classified as *A. tabescens*. Each of the naturally infected samples came from host tissues located in close proximity, which could have been infected by different *Armillaria* species. Presence of multiple *Armillaria* species within the same orchard has been observed (*unpublished*) but is thought to be uncommon. It is more likely that fatty acid classification from naturally infected samples is

affected by variations in substrate condition, e.g., the level of tissue decay. Indeed, Müller *et al.* (1995) observed differences in the FAST profiles of *H. annosum* when different isolates were grown on different media. They also found that Euclidean distances between average FAST profiles for different intersterility groups of *H. annosum* changed depending on the culture medium.

Another possible explanation for discrepancies between samples from infected host tissues compared with the corresponding cultures could be that fatty acids from a contaminating organism influenced sample classification from host tissues. Pending further validation, species classification of *Armillaria* unknowns directly from infected tissues may be possible, but multiple samples from the source may be needed to achieve reliable results.

When applied to *Armillaria* cultures grown *in vitro*, the species designation by FAME profiling was identical to that indicated by IGS-RFLP analysis, suggesting that FAME profiling could complement existing molecular tools for identification of *Armillaria*. In endomycorrhizal fungi, Bentivenga and Morton (1994) demonstrated stability and heritability of FAME profiles, indicating that some level of evolutionary conservation occurs in fungal fatty acid composition. Furthermore, studies involving bacteria and bacterial community differentiation indicated a good agreement between FAME profiling and DNA-based methodologies (Ka *et al.* 1994; Ritchie *et al.* 2000). FAME profiling would be especially useful as a physiological indicator of species in situations where ribosomal DNA heterogeneity and hybrids are reported to occur (Bryson *et al.* 2003; Hanna *et al.* 2003; Kelly 2004).

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Table 3.1. *Armillaria* isolates used in the present study

Isolate	<i>Armillaria</i> species	Origin	Host	Collector ^a	FAME ^b	IGS-RFLP ^c
OZZ 522	<i>A. mellea</i>	Ozark Forest, MO	<i>Quercus alba</i>	JB	AM	490, 180
CLY40A	<i>A. mellea</i>	West Chesnee, SC	<i>Prunus persica</i>	TB	AM	490, 180
49-8	<i>A. mellea</i>	Provincetown, MA	<i>Acer rubrum</i>	JA	AM	490, 180
97-1	<i>A. mellea</i>	Provincetown, MA	<i>Acer rubrum</i>	JA	AM	490, 180
M1	<i>A. tabescens</i>	Columbia, MO	n/d ^d	JB	AT	430, 240
M12-4	<i>A. tabescens</i>	Ozark Forest, MO	<i>Quercus rubra</i>	JB	AT	430, 240
94I-71	<i>A. tabescens</i>	Byron, GA	<i>Prunus persica</i>	TB	AT	430, 240
96I-88	<i>A. tabescens</i>	Byron, GA	<i>Prunus persica</i>	TB	AT	430, 240
C6/C	<i>A. gallica</i>	Missouri	n/d	JB	AG	582, 240
90-4	<i>A. gallica</i>	Burlington, VT	<i>Fraxinus</i>	JA	AG	582, 240
90-10	<i>A. gallica</i>	Burlington, VT	<i>Fraxinus</i>	JA	AG	582, 240
137-1	<i>A. gallica</i>	Ann Arbor, MI	n/d	JA	AG	582, 240

^a JB: Johann Bruhn, TB: Tom Beckman, JA: James Anderson (isolates obtained via Alan Jones).

^b Indicates to which species the isolate was classified by fatty acid methyl ester (FAME) profiling; AM: *Armillaria mellea*, AT: *Armillaria tabescens*, AG: *Armillaria gallica*.

^c Band sizes (in base pairs) resulting from *Alu* I restriction digest of the intergenic spacer (IGS) region of rDNA (Fig. 3.2).

^d Not determined.

Table 3.2. Isolates of outgroup basidiomycete species used in the present study

Isolate ^a	Species	Origin	Host
FP-1011971	<i>Schizophyllum commune</i>	Davis, CA	<i>Paulownia</i>
TjV-93-5-Sp	<i>Schizophyllum commune</i>	Blackhawk Ridge, WI	<i>Toxicodendron</i>
CS-70-11A	<i>Ganoderma lucidum</i>	Murphysboro, IL	<i>Quercus palustris</i>
JHG-211-Sp	<i>Ganoderma lucidum</i>	Warrensburg, NY	n/d ^b
FP-135626	<i>Stereum hirsutum</i>	Liberty, NY	<i>Acer</i>
TJV-93-161	<i>Stereum hirsutum</i>	Olympic Natl. Park, WA	<i>Alnus rubra</i>
FP-105578-Sp	<i>Trametes versicolor</i>	Elma, WA	<i>Prunus sp.</i>
FP-1250572-T	<i>Trametes versicolor</i>	Gilford, NH	<i>Prunus sp.</i>

^a Isolates provided by Rita Rentmeester, USDA Forest Service, Forest Products Laboratory, Madison, WI.

^b Not determined.

Table 3.3. Samples from naturally infected host tissues used in the classification of *Armillaria* unknowns by fatty acid methyl ester (FAME) profiling

Sample	Origin	Host	FAME1 ^a	FAME2 ^b	IGS-RFLP ^c
Eldn1	Clemson, SC	<i>Prunus persica</i>	AT ^d	AT	430, 240
Eldn2	Clemson, SC	<i>P. domestica</i>	AT	AT	430, 240
Lwsn	Quitman, GA	<i>P. persica</i>	AT	AT	430, 240
Ln	Fort Valley, GA	<i>P. persica</i>	AT	AT	430, 240
Mssr1	Seneca, SC	<i>P. persica</i>	AT	AT	430, 240
Mssr2	Seneca, SC	<i>P. persica</i>	AT	AT	430, 240
Msll	Musella, GA	<i>P. persica</i>	AT, AM ^e	AT	430, 240
Byrn	Byron, GA	<i>P. persica</i>	AM, AT ^e	AT	430, 240

^a Fatty acid methyl ester (FAME)-based species classification of mycelium dissected directly from infected host tissue.

^b FAME-based species classification of *Armillaria* cultures isolated from infected host tissue.

^c Band sizes (in base pairs) resulting from *Alu* I restriction digest of the intergenic spacer (IGS) region of rDNA (Fig. 3.5).

^d Indicates to which species the sample was classified by FAME profiling; AM: *Armillaria mellea*, AT: *Armillaria tabescens*.

^e One sample identified as *A. tabescens*, the other as *A. mellea*.

Table 3.4. Fatty acid methyl ester (FAME) profiles of three thallus morphologies of *Armillaria gallica* isolate C6/C

Fatty acid		Thallus morphology ^a		
Abbreviation	Name	Mycelium	Sclerotial crust	Rhizomorphs
12:0	Dodecanoic acid	1.0 ± 0.5	0.3 ± 0.2	0.3 ± 0.3
15:0	Pentadecanoic acid	2.1 ± 0.2	2.2 ± 0.0	2.1 ± 0.3
16:0	Hexadecanoic acid	18.4 ± 2.5	20.8 ± 0.4	22.7 ± 1.4
OH-16:0	2-Hydroxy-hexadecanoic acid	4.0 ± 0.6	3.6 ± 0.5	2.9 ± 0.2
9,12-18:2	9- <i>cis</i> ,12- <i>cis</i> -octadecadienoic acid	44.6 ± 7.6	51.9 ± 6.2	50.6 ± 4.6
18:0	Octadecanoic acid	3.6 ± 0.1	2.9 ± 0.5	4.8 ± 0.8
OH-18:0	2-Hydroxy-octadecanoic acid	1.5 ± 0.6	1.0 ± 0.1	0.2 ± 0.2
21:0	Heneicosanoic acid	8.1 ± 5.3	5.1 ± 2.8	3.6 ± 2.2
22:0	Docosanoic acid	1.6 ± 0.9	0.8 ± 0.6	Trace
OH-22:0	2-Hydroxy-docosanoic acid	7.5 ± 2.5	5.1 ± 0.6	4.6 ± 0.2
24:0	Tetracosanoic acid	1.4 ± 0.5	1.0 ± 0.2	0.1 ± 0.1

^a Values represent means and standard errors ($n = 3$) of the percentage contribution of each fatty acid to the total cellular fatty acid content for a given thallus type.

Table 3.5. Results of a stepwise discriminant analysis to distinguish *Armillaria* species (*A. mellea*, *A. tabescens*, and *A. gallica*) from outgroup basidiomycete species (*Schizophyllum commune*, *Ganoderma lucidum*, *Stereum hirsutum*, and *Trametes versicolor*) by fatty acid methyl ester (FAME) profiling^a

Fatty acid		Partial R^2	F -value	Pr> F	Avg. Squared Canonical Correlation
Abbreviation	Name				
16:0	Hexadecanoic acid	0.442	93.4	<0.0001	0.443
10-16:1	10- <i>cis</i> -hexadecanoic acid	0.353	60.4	<0.0001	0.914
23:0	Tricosanoic acid	0.335	54.8	<0.0001	0.863
10-18:1	10- <i>cis</i> -octadecenoic acid	0.244	37.8	<0.0001	0.582
13-18:1	13- <i>cis</i> -octadecenoic acid	0.223	33.3	<0.0001	0.796
21:0	Heneicosanoic acid	0.213	30.3	<0.0001	0.937
8-18:1	8- <i>cis</i> -octadecenoic acid	0.222	33.1	<0.0001	0.735
CH ₃ -18:0	8-methyl- octadecenoic acid	0.182	26.2	<0.0001	0.656
ECL 21.6	n/d ^b	0.164	20.9	<0.0001	0.957
9,12-18:2	9- <i>cis</i> ,12- <i>cis</i> - octadecadienoic acid	0.122	14.4	0.0002	0.964
OH-16:0	2-Hydroxy-Hexadecanoic acid	0.063	7.1	0.0091	0.967
OH-22:0	2-Hydroxy-docosanoic acid	0.062	6.3	0.0133	0.966
Iso-24:0	Iso-tetracosanoic acid	0.063	6.3	0.0139	0.966

^a Fungal species used in the analysis are given in Tables 3.1 and 3.2.

^b Fatty acid not named by the FASTWO method.

Table 3.6. Results of a stepwise discriminant analysis to distinguish among *Armillaria tabescens*, *A. mellea*, and *A. gallica* by fatty acid methyl ester (FAME) profiling^a

Fatty acid		Partial R^2	F -value	Pr> F	Avg. Squared Canonical Correlation
Abbreviation	Name				
14:0	Tetradecanoic acid	0.431	25.9	<0.0001	0.668
23:0	Tricosanoic acid	0.332	16.9	<0.0001	0.735
18:0	Octadecanoic acid	0.316	14.7	<0.0001	0.782
8-18:1	8- <i>cis</i> -octadecenoic acid	0.449	25.7	<0.0001	0.862
21:0	Heneicosanoic acid	0.386	19.9	<0.0001	0.894
Iso-24:0	Iso-tetracosanoic acid	0.494	30.2	<0.0001	0.915
17-23:1	17- <i>cis</i> -tricosacenoic acid	0.266	10.9	<0.0001	0.924
12-16:1	12- <i>cis</i> -hexadecenoic acid	0.279	11.2	<0.0001	0.943
16:0	Hexadecanoic acid	0.373	18.1	<0.0001	0.951
OH-18:0	2-Hydroxy-octadecanoic acid	0.252	9.8	0.0002	0.962
ECL 21.6	n/d ^b	0.173	6.2	0.0036	0.963

^a Fungal isolates used in the analysis are given in Table 3.1.

^b Fatty acid not named by the FASTWO method.

Fig. 3.1. Different thallus morphologies of *Armillaria gallica* isolate C6/C from which fatty acids were extracted. **A**, **B** and **C** represent examples of mycelium, sclerotial crust, and rhizomorphs (arrows), respectively. Scale bar = 1 cm.

Fig. 3.1.

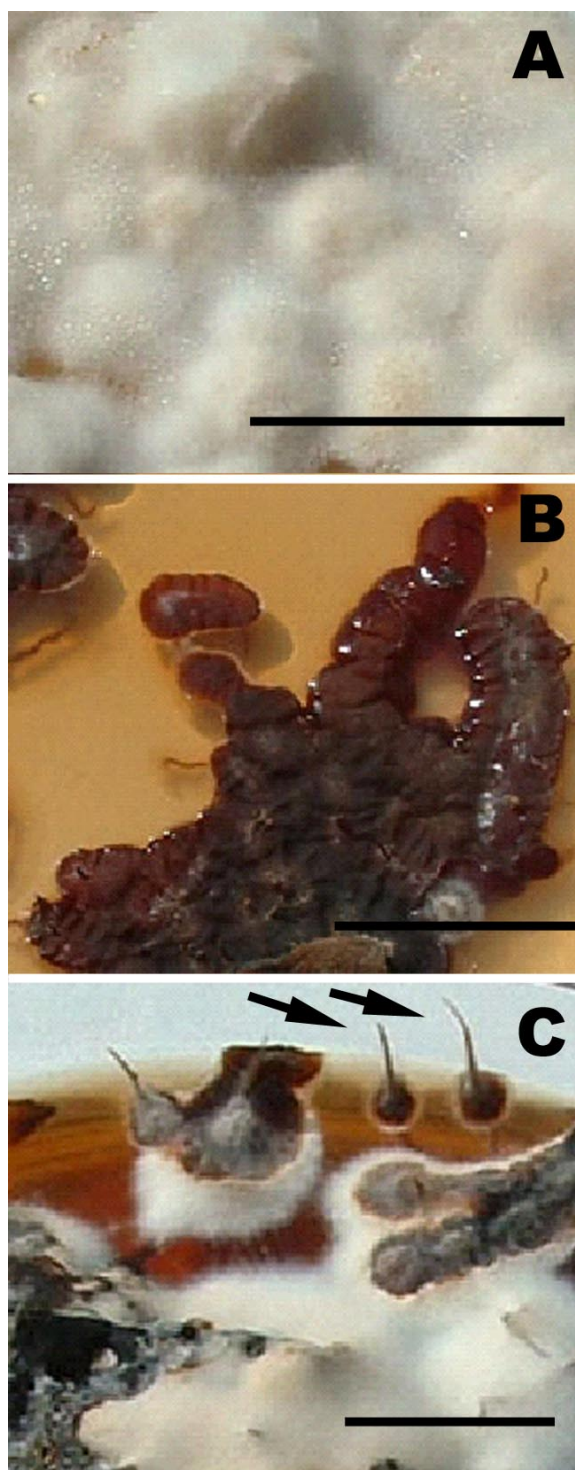


Fig. 3.2. Agarose gel (1%) image showing restriction fragments resulting from *Alu* I digest of the rDNA intergenic spacer (IGS) region of the *Armillaria* isolates used in the present study. Isolates depicted in lanes 1 through 12 are: M1, OZZ 522, C6/C, M12-4, CLY40A, 90-4, 49-8, 94I-71, 90-10, 97-1, 96I-88, and 137-1 (Table 3.1). The presence of 490 + 180, 430 + 240, and 582 + 240 bp bands indicates *A. mellea*, *A. tabescens*, and *A. gallica*, respectively (Harrington and Wingfield 1995). Size marker: 100 bp ladder, with arrows indicating the 500 and 200 bp bands.

Fig. 3.2.

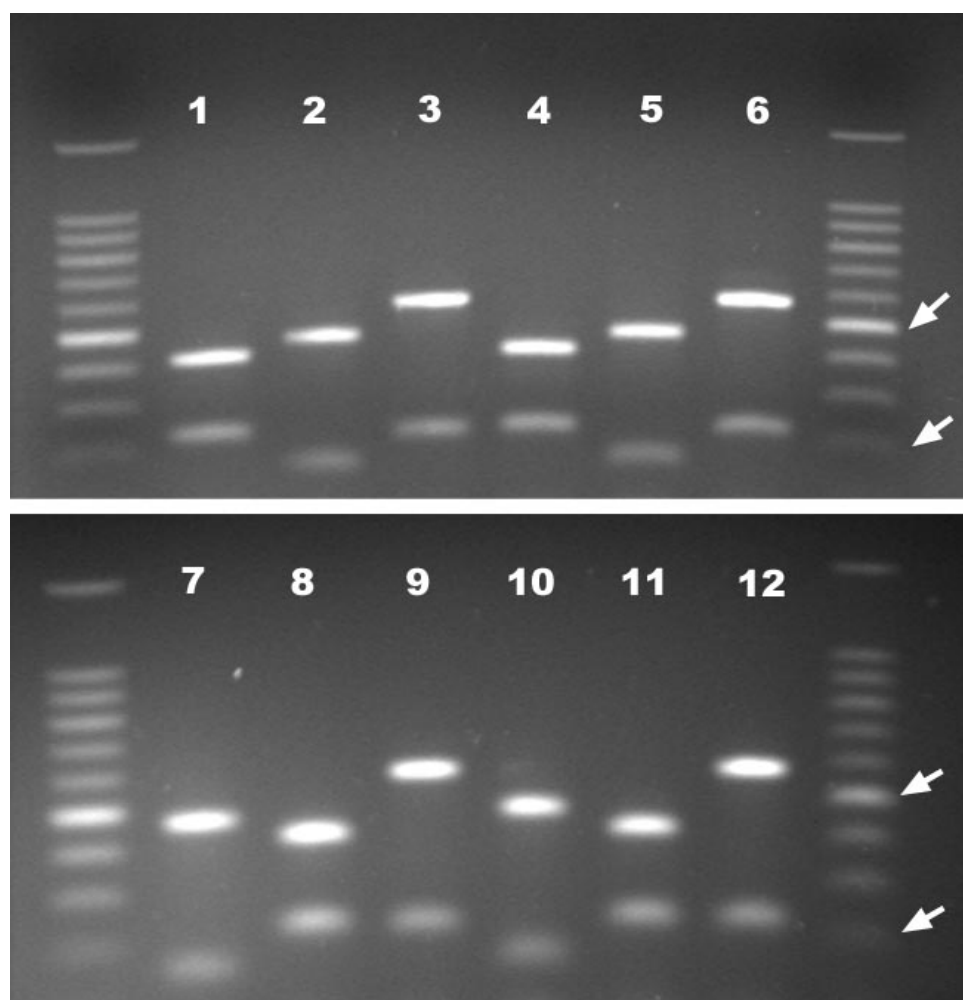


Fig. 3.3. Box plot illustrating the separation of *Armillaria* species (*A. tabescens*, *A. mellea*, and *A. gallica*) from outgroup basidiomycete species (*Schizophyllum commune*, *Ganoderma lucidum*, *Stereum hirsutum*, and *Trametes versicolor*) by the first canonical axis obtained from canonical discriminant analysis conducted on fatty acid methyl ester (FAME) profiles. Isolates used in the analysis are given in Tables 3.1 and 3.2.

Fig. 3.3.

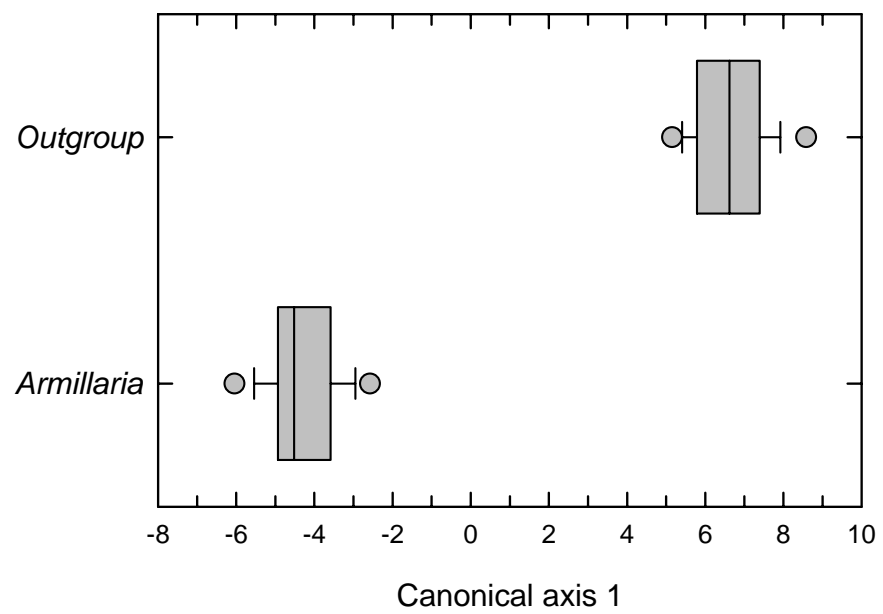


Fig. 3.4. Scatter plot illustrating the separation among *Armillaria tabescens*, *A. mellea*, and *A. gallica* by the first and second canonical axes obtained from canonical discriminant analysis conducted on fatty acid methyl ester (FAME) profiles. Isolates used in the analysis are given in Table 3.1. Different symbols within species indicate different isolates.

Fig. 3.4.

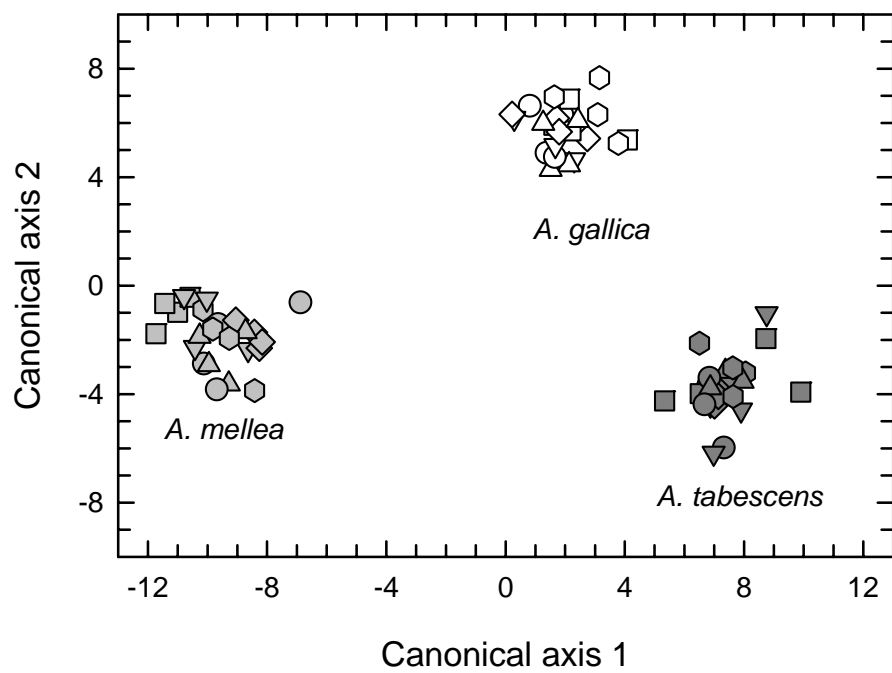
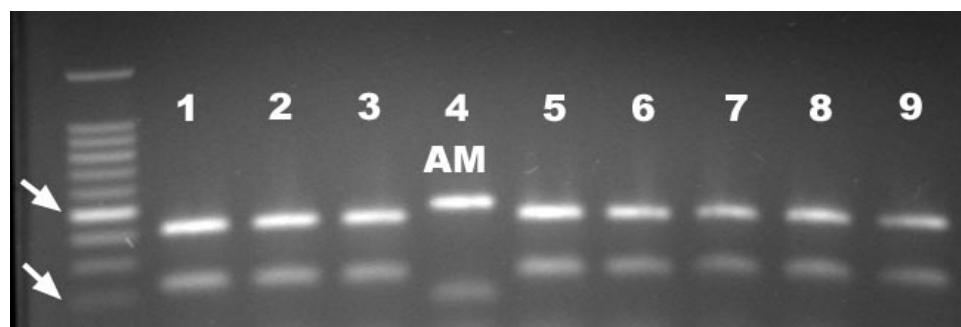


Fig. 3.5. Agarose gel (1%) image showing restriction fragments resulting from *Alu* I digest of the rDNA intergenic spacer (IGS) region of the *Armillaria* unknowns isolated from naturally infected host tissues. Isolates depicted in lanes 1 through 9 are: Byrn, Msl1, Mssr2, OZZ 522 (*A. mellea* control), Mssr1, Ln, Lwsn, Eldn2, and Eldn1 (Table 3.3). The presence of 490 + 180 and 430 + 240 bp bands indicates *A. mellea* and *A. tabescens*, respectively (Harrington and Wingfield 1995). Size marker: 100 bp ladder, with arrows indicating the 500 and 200 bp bands.

Fig. 3.5.



APPENDIX 1

Discriminant function to distinguish *Armillaria* species (*A. tabescens*, *A. mellea*, and *A. gallica*) from the outgroup basidiomycete species *Schizophyllum commune*, *Ganoderma lucidum*, *Stereum hirsutum*, and *Trametes versicolor* based on stepwise discriminant analysis of fatty acid methyl ester (FAME) profiles from cultures of the fungal species. The percent contribution of each fatty acid to the total fatty acid content is entered into the equation to compute a DF_v value. DF_v values for unknown samples >83.06 (dividing point) are classified as *Armillaria*, otherwise they are classified as an outgroup basidiomycete.

$$\begin{aligned}
 DF_v = & (16:0 \times 4.22) + (13-18:1 \times -10.22) + (CH_3-18:0 \times -2.83) + (8-18:1 \times -7.43) \\
 & + (10-18:1 \times 27.43) + (23:0 \times 6.94) + (10-16:1 \times -24.93) + (21:0 \times -2.08) \\
 & + (ECL\ 21.6 \times -19.46) + (9,12-18:2 \times 0.57) + (Iso-24:0 \times 5.8) \\
 & + (OH-22:0 \times -4.72) + (OH-16:0 \times 4.7)
 \end{aligned}$$

Variable	Fatty acid	Variable	Fatty acid
16:0	Hexadecanoic acid	CH ₃ -18:0	8-methyl- octadecenoic acid
10-16:1	10- <i>cis</i> -hexadecanoic acid	ECL 21.6	n/d
23:0	Tricosanoic acid	9,12-18:2	9- <i>cis</i> ,12- <i>cis</i> -octadecadienoic acid
10-18:1	10- <i>cis</i> -octadecenoic acid	OH-16:0	2-Hydroxy-hexadecanoic acid
13-18:1	13- <i>cis</i> -octadecenoic acid	OH-22:0	2-Hydroxy-docosanoic acid
21:0	Heneicosanoic acid	Iso-24:0	Iso-tetracosanoic acid
8-18:1	8- <i>cis</i> -octadecenoic acid		

APPENDIX 2

Discriminant functions to distinguish among *Armillaria tabescens*, *A. mellea*, and *A. gallica* based on stepwise discriminant analysis of fatty acid methyl ester (FAME) profiles from cultures of the species. The percent contribution of each fatty acid to the total fatty acid content is entered into each of the three equations. An unknown sample is classified as the *Armillaria* species that has the highest value for the equation.

$$A. \textit{tabescens} = (14:0 \times 401.39) + (23:0 \times -20.17) + (18:0 \times 17.73) + (8-18:1 \times 26.17) + (21:0 \times 7.47) + (\text{Iso-24:0} \times -11.65) + (17-23:1 \times -197.52) + (\text{ECL } 21.6 \times 100.94) + (16:0 \times 19.42) + (\text{OH-18:0} \times -10.49) + (\text{ECL } 21.6 \times -52.25) - 311.98$$

$$A. \textit{gallica} = (14:0 \times 433.94) + (23:0 \times -24.8) + (18:0 \times 79.56) + (8-18:1 \times 10.37) + (21:0 \times 0.611) + (\text{Iso-24:0} \times 1.79) + (17-23:1 \times -263.46) + (12-16:1 \times 119.50) + (16:0 \times 23.17) + (\text{OH-18:0} \times -0.9) + (\text{ECL } 21.6 \times -58.41) - 476.94$$

$$A. \textit{mellea} = (14:0 \times 192.43) + (23:0 \times -11.23) + (18:0 \times 97.10) + (8-18:1 \times -14.36) + (21:0 \times -9.12) + (\text{Iso-24:0} \times 15.18) + (17-23:1 \times -187.71) + (12-16:1 \times 61.96) + (16:0 \times 15.22) + (\text{OH-18:0} \times 12.13) + (\text{ECL } 21.6 \times -32.61) - 261.24$$

Variable	Fatty acid	Variable	Fatty acid
14:0	Tetradecanoic acid	17-23:1	17- <i>cis</i> -tricosanoic acid
23:0	Tricosanoic acid	12-16:1	12- <i>cis</i> -hexadecanoic acid
18:0	Octadecanoic acid	16:0	Hexadecanoic acid
8-18:1	8- <i>cis</i> -octadecenoic acid	OH-18:0	2-Hydroxy-octadecanoic acid
21:0	Heneicosanoic acid	ECL 21.6	n/d
Iso-24:0	Iso-tetracosanoic acid		

CHAPTER 4

**GROUND-PENETRATING RADAR TO DETECT AND QUANTIFY RESIDUAL ROOT
FRAGMENTS FOLLOWING PEACH ORCHARD CLEARING¹**

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Ground-Penetrating Radar to Detect and Quantify Residual Root Fragments Following Peach Orchard Clearing

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ABSTRACT

Consecutive replanting of peach trees on the same orchard site can result in various replant problems and diseases. One such disease is *Armillaria* root and crown rot, which develops in replant situations due to contact between the roots of newly planted trees and infested residual root pieces in the soil. There is little information regarding the quantity of roots remaining in stone fruit orchards following tree removal and land clearing. We investigated the utility of ground-penetrating radar (GPR) to characterize reflector signals from peach root fragments in a controlled burial experiment and to quantify the amount of residual roots remaining after typical, commercial orchard clearing. In the former experiment, roots ranging from 2.5 to 8.2 cm in diameter and buried at depths of 11 to 114 cm produced characteristic parabolic reflector signals in radar profiles. Image analysis of high-amplitude reflector area indicated significant linear relationships between signal strength (mean pixel intensity) and root diameter ($r = -0.517$, $P = 0.0097$, $n = 24$) or the combined effects of root diameter and burial depth, expressed through a depth \times diameter term ($r = -0.680$, $P = 0.0003$, $n = 24$). In a peach orchard in which trees and roots had been removed following standard commercial practice (i.e., trees were pushed over, burned, and tree rows were subsoiled), a GPR survey of six 4-m \times 8-m plots revealed that the majority of reflector signals indicative of root fragments were located in the top 30 to 40 cm of

soil. Based on ground-truth excavation of 100 selected sites within plots, reflectors showing a strong parabolic curvature in the radar profiles corresponded to residual root fragments with 100% accuracy, while those displaying a high amplitude area represented roots in 86.1% of the cases. By contrast, reflectors with both poor curvature and low amplitude yielded roots for <10% of the excavated sites, while randomly selected sites lacking reflector signals were devoid of any roots or other subsurface objects. A high level of variability in the number of residual roots was inferred from the radar profiles of the six plots, suggesting an aggregated distribution of root fragments throughout the field. The data further indicated that at least one residual root fragment would be present per m³ of soil, and that many of these fragments have diameters corresponding to good to excellent inoculum potential for *Armillaria* root disease.

Additional index words. *Prunus persica*, georadar

INTRODUCTION

Peach (*Prunus persica* L. Batsch) orchards commonly are replanted when orchard profitability begins to decline, which may be as early as 4 to 6 years after initial establishment (Steiner and Lockwood 2004). Replanting on the same orchard site can result in various replant problems and diseases as soil conditions deteriorate and soilborne pathogen populations increase (Fehrmann 1988). One such replant disease is *Armillaria* root and crown rot, which is caused by several species of the basidiomycete *Armillaria* (Fr.:Fr) Staude. *Armillaria* species occur worldwide, and they attack a wide variety of hardwood and softwood plants, including many stone fruit species (Cooley 1943; Raabe 1962; Shaw and Kile 1991). This disease can cause extensive tree mortality in Georgia and South Carolina peach orchards (Savage *et al.* 1953;

Rhoads 1954; Miller 1994) and is becoming more prevalent as producers increasingly are forced to plant into forest lands or old orchard sites with endemic *Armillaria* populations. These fungal populations are capable of surviving for decades on infected root pieces that remain in the soil after tree removal (Reaves *et al.* 1993; Roth *et al.* 2000), and contact between growing roots of replanted trees and infested residual root pieces in the soil is thought to initiate the disease (Savage *et al.* 1953; Rishbeth 1964). Residual roots with diameters as small as 0.7 cm can support survival of *Armillaria*, while those of larger diameters are more important for establishing infection based on their greater inoculum potential (Bliss 1951; Patton and Riker 1959; Chandler and Daniel 1982). Thus, thorough removal of large root fragments after orchard clearing is an important strategy for reducing disease incidence in replant situations (Steiner 1976; Shaw and Kile 1991; Cox *et al.* 2004).

There is little information regarding the quantity of residual roots remaining in peach orchards after commercial tree removal and land clearing practices, or the extent to which these root fragments are of a size that would support survival of *Armillaria*. Various excavation techniques, ranging from manual digging to pressurized air or water excavation, have been employed to study the root systems of trees *in situ* before or after tree removal (Jenik 1978; Bohm 1979; Carlson *et al.* 1988; Nicoll and Armstrong 1998; Pareek *et al.* 1993; Rizzo and Gross 2000; Stokes *et al.* 2002), but these techniques are often too invasive and/or too laborious to survey large areas. Similarly, indirect techniques, such as measuring sap flow or the use of radioactive tracers to study root systems (Woods 1969; Cremák *et al.* 1980; Cremák and Kucera 1990), are uninformative of root structure and unsuitable for quantifying residual root fragments. By contrast, ground-penetrating radar (georadar, GPR) is a nondestructive geophysical technique that can both detect tree roots and characterize their distribution. GPR is a pulse radar system in

which pulses of electromagnetic energy are transmitted into the soil from an antenna. Electromagnetic waves are partially reflected back to the antenna off of subsurface features of varying electromagnetic properties, and these were subsequently converted into a digitized image of waveforms (Morey 1974; Ulriksen 1982; Daniels 1996; Butnor *et al.* 2001). Due to the change in travel time of the electromagnetic waves as the antenna passes over them (Barker and Doolittle 1992), roots and other discrete subsurface features produce hyperbolic reflector signals in the radar profile, and the shape and intensity of these characteristic reflectors can be used to identify roots in radar profiles (Butnor *et al.* 2001). GPR has been applied successfully to detect, characterize, and map tree root systems in the subsurface of forest stands and urban environments under concrete and asphalt (Hruska *et al.* 1999; Cremák *et al.* 2000; Butnor *et al.* 2001; Stokes *et al.* 2002). However, the technique has not been used previously to detect and quantify residual root pieces beneath the soil surface following orchard clearing.

The purpose of this study was to provide proof of concept for GPR detection of residual peach root fragments in the soil following orchard clearing. Specifically, we 1) characterized reflector signals from root pieces in a controlled burial experiment, and 2) quantified the amount of residual roots remaining after orchard clearing typical of commercial practice. This study could help determine the potential amount of *Armillaria inoculum* remaining in peach orchards after commercial tree removal and replant practices; it could further help clarify the extent to which residual root fragments are of a size favoring survival of *Armillaria*.

MATERIALS AND METHODS

Controlled burial experiment. The purpose of this experiment was to characterize the GPR reflector signals of peach roots in nearly ideal conditions. It was carried out on a fallow

Pelham loamy sand (92.5% sand, 7.2% silt, 0.5% clay) at the Bowen Farm near Tifton, GA. A trench 20 m long, 1.5 m wide, and 1.2 m deep was dug with a backhoe, and peach root segments or PVC pipes (used as standards) were inserted into the trench face at different depths and in different orientations as described below. The root segments (2.5 to 8.2 cm in diameter and >50 cm in length) had been collected at the USDA-ARS Southeastern Fruit and Tree Nut Research Laboratory, Byron, GA, from mature peach trees on 'Lovell' rootstock which had been pushed over using a front-end loader. Roots were stored in a freezer between collection and use to avoid desiccation of the tissue. The PVC pipes were 3.1 to 6.0 cm in diameter and cut to 70 cm length to ensure complete coverage across the signal footprint when the antenna was pulled across the soil surface next to the trench (see below).

The controlled burial experiment was designed to determine the influence of root diameter, depth of burial, and orientation of the root segment relative to the ground plane (Table 4.1). Root diameters were within a range most relevant for *Armillaria* survival (Bliss 1951; Chandler and Daniel 1982). With the aid of a rubber mallet, root segments and pipe standards were pushed into the trench face at four depth classes (ca. 25, 45, 60, and 80 cm) that encompass the range of typical stone fruit tree rooting depths (Glen and Welker 1993; Godara *et al.* 2000). The direction of insertion was either horizontal, angled upward, or angled downward to simulate varying root orientations. There were a total of 12 root segments and 12 PVC pipes, with individual objects spaced ca. 1 m apart to avoid overlap in GPR reflector signals. Most insertions were made in the eastern face of the trench as the deposition of excavated soil on the western trench shelf allowed only surveying up to 6 m on that side.

Two days following insertion of the objects into the trench face, both shelves of the trench were surveyed using a ground-coupled 900-MHz antenna (3101D; Geophysical Survey

Systems, Inc., North Salem, NH), each in a series of five transects 25 cm apart starting at the edge of the trench face. Multiple transects were done to ensure complete coverage of the roots and pipes. The antenna was calibrated for gain and soil dielectric constant estimated on site. Radar profiles were collected in 8-bit files with a range of 30 ns. The radar profiles were viewed in color table 17 (grayscale) with linear color transform, and processing was limited to marker editing and horizontal scaling (distance normalization) using RADAN for Windows NT v. 3.1 (Geophysical Survey Systems, Inc.). An example profile from the western trench face is shown in Fig. 4.1. Further processing with RADAN using Finite Impulse Response (FIR) filters to remove background noise and Kirchoff migration to collapse hyperbolic diffractions was performed to determine if clarity of reflector signals could be improved.

Following color table customization and horizontal scaling, the qualitative radar profiles were converted to numerical data using Scion Image software (Scion Corporation, Frederick, MD) to quantify the signal strength of root and pipe reflectors. The high-amplitude area of each reflector was selected and analyzed for mean intensity on an 8-bit scale, giving intensity values from 0 to 255, with high- and low-amplitude areas appearing lighter (lower values) and darker (higher values), respectively. The resulting mean pixel intensity for each object was used as a measure of relative signal strength in subsequent linear regression analyses to investigate the dependence of pixel intensity on depth and diameter of roots and pipes (SigmaPlot v. 7.0; SPSS Inc., Chicago, IL).

GPR survey of peach root fragments following orchard clearing in commercial conditions. The experiment was conducted in a commercial peach orchard near Byron, GA. The soil was a Faceville fine sandy loam (85.4% sand, 10.3% silt, 4.3% clay) with a hardpan containing higher percentages of clay and silt at a depth of 30 to 40 cm. The orchard had been

cleared by the producer in July of 2003, which involved pushing the trees over, piling and burning them, and subsoiling the tree rows. Apart from mechanical weed control with a harrow, the cleared area lay fallow throughout the subsequent fall and winter. In January of 2004, just prior to replanting, six 4×8 -m plots were established randomly across the fallow orchard. Multiple small plots were used to increase the likelihood of including areas with varying residual root levels, and the size of plots was chosen to encompass the area typically allocated to an individual tree under standard spacing practices. Each plot was surveyed with a ground-coupled 900-MHz antenna (3101D, Geophysical Survey Systems, Inc.) in a series of nine 8-m transects (*y*-direction) with 50 cm spacing between transects to ensure minimal signal overlap (Fig. 4.2). Each plot also was surveyed in the *x*-direction to produce 4-m transects, but only the *y*-transects were used for analysis. The antenna was calibrated for gain on site, and radar profiles (range 30 ns) were collected and processed as described above for the controlled burial experiment.

The individual radar profiles were examined for characteristic parabolic reflectors indicative of roots, the position of these reflectors was marked, and signal characteristics (parabolic curvature and amplitude) were recorded. Signals potentially corresponding to roots were categorized into four classes: 1) having contrasting bands of high amplitude and a well-defined parabolic shape; 2) having poor amplitude contrast, but a well-defined parabolic shape; 3) having contrasting bands of high amplitude, but only slight curvature; and 4) having poor amplitude contrast and slight curvature (Fig. 4.3). Across all six plots, 100 validation sites (each of ca. 25 cm radius) were selected for ground-truth excavation to a depth of ca. 60 cm, with 75 selected from areas containing potential root signals (i.e., having characteristics of signal classes 1 through 4 above) and 25 selected from areas lacking any reflector signals in the radar profiles. The number of excavation sites that yielded root fragments was tallied for each of the four signal

classes and used as a measure of accuracy for predicting the presence of root fragments based on the characteristics of that signal class.

For each signal class, mean root characteristics (diameter, length, and dry weight) were calculated across all roots excavated as part of that class. Then, each radar profile was inspected for the total number of reflectors in each signal class, and the density of root fragments per m³ of soil was calculated by tallying the numbers of reflectors from each of the signal classes with the highest ground-truth accuracy (i.e., classes 1 through 3; see below) and dividing this number by the volume of soil ($8 \times 4 \times 0.6 \text{ m}^3$) considered in the survey of each plot. Similarly, the biomass contribution of each signal class was calculated by multiplying the mean dry weight of roots in that class by the number of reflectors belonging to the signal class. Root biomass was then estimated for each plot by summing the biomass contributions of each class within the plot and dividing by the volume of soil considered in the survey.

RESULTS AND DISCUSSION

Controlled burial experiment. With minimal downstream signal processing, all root segments and PVC pipes produced discernible parabolic reflector signals in the radar profiles (Fig. 4.1). Additional processing with FIR filters and Kirchoff migration to remove background noise and collapse hyperbolic diffractions, respectively, did not improve clarity or position of reflector signals further (*data not shown*). The reflectors varied in the clarity of shape and in signal strength, but there were no obvious differences in signals between roots and pipes of similar diameter, burial depth, and orientation (Fig. 4.1). The reflector shapes and intensities produced by the roots in our study were similar to those described in previous reports (Truman *et al.* 1988; Barker and Doolittle 1992; Hruska *et al.* 1999; Butnor *et al.* 2001).

With the GPR system all roots included in the controlled burial experiment were detected, regardless of diameter (2.5 to 8.2 cm) and burial depth (11 to 114 cm) (Table 4.1). By contrast, Hruska *et al.* (1999), using a 400-MHz antenna, were only able to resolve oak roots of 3 to 4 cm diameter with a 1- to 2-cm margin of error. The antenna used in their study has a lower resolution but a greater depth of penetration (up to 2 m) compared with the 900-MHz antenna used here. Moreover, the presence of multiple, overlapping roots in their survey of intact root systems *in situ* further compromised the potential to resolve roots of diameters <3 cm accurately. However, Butnor *et al.* (2001) were able to clearly distinguish pine roots as small as 0.5 cm in diameter using a 1.5-GHz antenna and larger roots (>3.7 cm diameter) using a 400-MHz antenna *in situ*. The 1.5-GHz antenna provided sufficient resolution to distinguish small-diameter roots at depths of 35 to 60 cm depending on soil type, while the 400-MHz antenna allowed for detection of larger roots at depths >1 m.

Relative to assessing potential *Armillaria* inoculum on peach roots, root pieces <2.5 cm in diameter are less conducive to survival and weaker in inoculum potential than fragments with larger diameters (Bliss 1951; Garrett 1956, 1957; Chandler and Daniel 1982). Moreover, most coarse peach roots are found in the top 50 cm of soil (Glen and Welker 1993; Godara *et al.* 2000), and only few roots are observed below 60 cm in southeastern peach orchards (*unpublished data*). The use of a 900-MHz antenna in our study therefore represents a sound compromise in terms of resolution, depth of penetration, and biological relevance of the resulting information.

Apart from merely detecting the presence of roots, there were relationships between reflector signal strength (indicated by reflector pixel intensity following image analysis) and depth and diameter of roots. A significant negative correlation existed between signal strength

and the diameter of root and pipes ($r = -0.517$, $P = 0.0097$, $n = 24$) (Fig. 4.4A), indicating that larger roots produced higher-amplitude signals (which correspond to lower values of pixel intensity on the 0 to 255 scale). By contrast, the relationship between signal strength and burial depth was significant only at $\alpha = 0.10$ ($r = -0.384$, $P = 0.0643$, $n = 24$) (Fig. 4.4B). Interestingly, the combined effect of depth and diameter, accounted for through use of a depth \times diameter term in the regression analysis, resulted in a stronger correlation with signal strength ($r = -0.680$, $P = 0.0003$, $n = 24$) (Fig. 4.4C). Butnor *et al.* (2001) explored similar relationships with root diameter and biomass using the high-amplitude area of cottonwood and loblolly pine root reflector signals in sandy soils. They reported significant correlations between pine root diameter and high-amplitude area at depths of 15 and 30 cm. Interestingly, the strength of the relationship declined with increasing depth, and there was no significant association between cottonwood root diameter and high-amplitude area (Butnor *et al.* 2001). In the current study, after accounting for depth, a combined depth-diameter term better explained reflector signal strength.

GPR survey of peach root fragments following orchard clearing in commercial conditions. All six plots contained radar profiles with parabolic reflector signals suggestive of root fragments. The majority of these reflectors were located in the top 30 to 40 cm of soil (Table 4.2) above a hardpan in which few reflectors were present. Excavation at the 25 randomly selected validation sites which lacked reflector signals revealed no roots or other subsurface objects. Of the 75 reflectors selected for excavation and validation, 21 were classified into signal class 1 (high amplitude and a well-defined parabolic shape), six into class 2 (low amplitude, but well-defined parabolic shape), 15 into class 3 (high amplitude, but only slight curvature) and 33 into class 4 (low amplitude with slight curvature) (Table 4.2). In signal classes 1 and 2, which represented reflectors with well-defined parabolic shape, all validation sites yielded root

fragments. On average, these root fragments had the largest diameters and the greatest biomass of all roots recovered from validation sites. While the majority (15 out of 25) of reflectors in signal class 3 corresponded to root fragments, few in class 4 yielded any roots. These root fragments had the smallest average diameters and biomass on average (Table 4.2). Overall, reflectors showing a strong parabolic curvature in the radar profiles corresponded to residual root fragments with 100% accuracy, while those displaying a high amplitude area represented roots in 86.1% of the cases. By contrast, reflectors with both poor curvature and low amplitude yielded roots for <10% of the excavated sites and most likely represented background noise. It is unlikely that these faint reflectors represent a significant proportion of the residual roots remaining in the soil after orchard removal.

Based on the high level of accuracy for root fragment detection using signal classes 1 through 3, total root counts were obtained for each plot based on the number of reflectors corresponding to these three classes in the radar profiles (Table 4.3). Plots 2 through 5 had the highest numbers of reflectors in signal classes that consistently corresponded to root fragments. These plots also had the highest predicted root density and biomass. Variability in predicted root density among plots may be due to an aggregated distribution of root fragments throughout the field. This is consistent with commercially used orchard clearing practices whereby trees are typically pushed over with a bulldozer or front-end loader, which often causes trees to break off at the crown, leaving the majority of the roots in the soil. The subsequent tractor raking, typical of the land-clearing process may fail to remove all of the remaining roots in tree rows, resulting in alternating higher and lower areas of residual root density. Although the planting pattern of the cleared orchard was not known when our survey plots were established, it is possible that plots 2

through 5 were located near or above the old tree rows, hence the higher root biomass in these plots.

Armillaria root disease is characterized by patchy distribution in both forests and orchards (Kable 1974; Steiner 1976; van der Kamp 1995; Baumgartner and Rizzo 2002). Indeed, it is common to observe patches of dead trees, killed by *Armillaria*, surrounded by apparently healthy trees. This pattern is thought to be due to clumping of residual root inoculum resulting from inefficient tree removal or land-clearing, which allows for contact between roots of newly planted trees and infested residual root fragments from the previous planting (Savage *et al.* 1953; Steiner 1976). Baumgartner and Rizzo (2002) found that the patchy distribution of *Armillaria* root disease in a California vineyard was due to a concomitant distribution of decaying residual roots. In the current study, the patchy residual root distribution among plots is consistent with the patchy incidence of *Armillaria* root disease commonly observed in peach orchards in the southeastern United States.

Following peach orchard removal, our study indicates the presence of numerous residual root fragments that may serve as potential inoculum for *Armillaria* root disease. Patton and Riker (1959) demonstrated that pine root segments with diameters from 0.7 to 5 cm supported survival of *Armillaria* for 10 to 35 months; they also concluded that the size of inoculum was not important for fungal survival. However, other studies in peach and citrus asserted that *Armillaria* root inoculum of larger diameters favors longer-term survival (Bliss 1951; Chandler and Daniel 1982). Furthermore, large-diameter (≥ 1.75 cm) roots are thought to be more important for *Armillaria* infection, as they have greater inoculum potential than those of smaller diameters. In experiments in which potato tubers were colonized from root inoculum of different sizes, Garrett (1956, 1957) concluded that larger *Armillaria* inoculum provides greater energy for infection,

and conversely, the proportion of successful infections decreases with decreasing inoculum size, due to a decrease in inoculum potential. In the current study, numerous root fragments with diameters ≥ 1.75 cm (a size that is both conducive to survival of *Armillaria* and provides a good to excellent inoculum potential *sensu* Garrett) were detected with GPR. Indeed reflector signal classes 1 to 3 represent root pieces with diameters ≥ 1.7 cm on average and account for the majority of the roots detected in our survey (Table 4.2). With the exception of plot 1, it was predicted that at least one residual root fragment would be present in every m^3 of soil, and many of these fragments correspond to signal classes representing diameters with good inoculum potential (Tables 4.2 and 4.3).

In the GPR survey of the commercial replant orchard, the predicted residual root biomass per plot ranged from 16.6 to 77.5 g m^{-3} of soil (Table 4.3); this could pose a substantial infection threat to newly planted trees, as only 4 g of root inoculum can provide enough inoculum potential for successful infection by *Armillaria* (Garrett 1956). To address the practical relevance of residual root inoculum, Roth *et al.* (2000) conducted a 20-year study using different land-clearing strategies for *Armillaria* inoculum reduction in a ponderosa pine stand. For the land-clearing treatment most akin to that used in peach production (trees pushed over and maximum root removal by machine), the authors reported a residual dry-weight root biomass of 14.72 kg m^{-3} of soil, which is almost 20 times higher than the highest biomass inferred in our survey. The pine trees removed in their study were both much taller (site index of 32 m at 100 years) and growing at a higher density (tree spacing < 1.9 m) than is the case for trees in a peach orchard. Thus, the observed difference in the magnitude of residual root biomass between the two studies with similar land-clearing practices is likely due to differences in root biomass input between pine stands and peach orchards. Interestingly, Roth *et al.* (2000) reported that maximum root

removal by machine offered no reduction in *Armillaria* root disease incidence on replanted pine trees in their study. Similar long-term disease monitoring in peach orchards will be needed to determine to what extent the much lower levels of root biomass remaining in the soil in the current study pose a threat to replanted trees in the form of potential *Armillaria* inoculum.

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Table 4.1. Aspects of the controlled burial experiment to characterize reflector signals of peach root fragments or PVC pipe standards by ground-penetrating radar

Peach root fragments				PVC Pipes ^a	
Diameter (cm)	Depth (cm) ^b	Length (cm)	Orientation ^c	Diameter (cm)	Depth (cm)
3.1	44	60	horizontal	3.1	63
3.2	27, 18	51	upward	4.0	82
2.5	41, 11	90	upward	6.0	45
2.7	28	62	horizontal	3.1	35
6.1	58, 52	60	upward	6.0	23
3.5	78, 70	52	upward	6.0	57
5.2	52, 77	70	downward	4.0	25
8.2	86	70	horizontal	6.0	86
2.5	38, 52	51	downward	4.0	44
7.3	64	60	horizontal	3.1	82
2.5	85, 113	55	downward	4.1	66
3.3	88, 114	70	downward	3.1	29

^a All pipes were 70 cm long and inserted into the trench face horizontally.

^b Numbers correspond to the insertion depth of the root fragment into the trench face and the depth at the endpoint for roots oriented ‘upward’ or ‘downward’.

^c Orientations ‘upward’ and ‘downward’ indicate insertions at an angle up or down into the face of the trench.

Table 4.2. Classification of ground-penetrating radar reflector signals and associated root fragment characteristics determined by independent ground-truth excavation in a peach orchard following orchard clearing according to commercial practice

Signal class ^a	Reflector characteristics ^a			n_1 ^f	n_2 ^g	Root characteristics ^b			
	High amplitude ^c	Strong parabola ^d	Weak parabola ^e			Diameter (cm)	Depth (cm)	Length (cm)	Dry weight (g)
1	+	+	-	21	21	3.2	26.2	25.1	82.4
2	-	+	-	6	6	2.2	28.3	22.8	51.7
3	+	-	+	15	10	1.7	30.5	19.4	18.0
4	-	-	+	33	3	1.2	16.7	13.7	10.0
5	-	-	-	25	0	-	-	-	-

^a Reflector characteristics and corresponding signal classes are illustrated in Fig. 4.3.

^b Based on means across all roots in the signal class.

^c Indicated by the presence of strong contrasting bands of high amplitude in the signal.

^d Possessing a well-defined parabolic shape.

^e Faint in contrast or slight in curvature.

^f Number of excavations in the signal class.

^g Number of excavations that yielded root fragments.

Table 4.3. Predicted root density and biomass based on ground-penetrating radar reflector signal classes in a peach orchard following orchard clearing according to commercial practice

Plot ^b	Number of roots ^a			Predicted root density (m ⁻³)	Predicted biomass (g m ⁻³) ^c
	Signal class 1	Signal class 2	Signal class 3		
1	1	0	3	0.31	16.6
2	6	3	14	1.80	76.8
3	3	6	5	1.09	59.9
4	9	3	2	1.09	77.5
5	7	3	6	2.03	67.8
6	2	3	8	1.02	45.6

^a Indicates number of reflector signals possessing the characteristics of the classes defined in Table 4.2 and Fig. 4.3.

^b Each plot was 4 m × 8 m in area.

^c Calculated from number of roots per signal class per plot and mean dry weight of excavated roots in each signal class (Table 4.2).

Fig. 4.1. Representative radar profile from the west trench face in the controlled burial experiment to characterize reflector signals of peach root fragments or PVC pipe standards by ground-penetrating radar. Parabolic shapes indicated by long and short arrows correspond to roots and pipes, respectively.

Fig. 4.1.

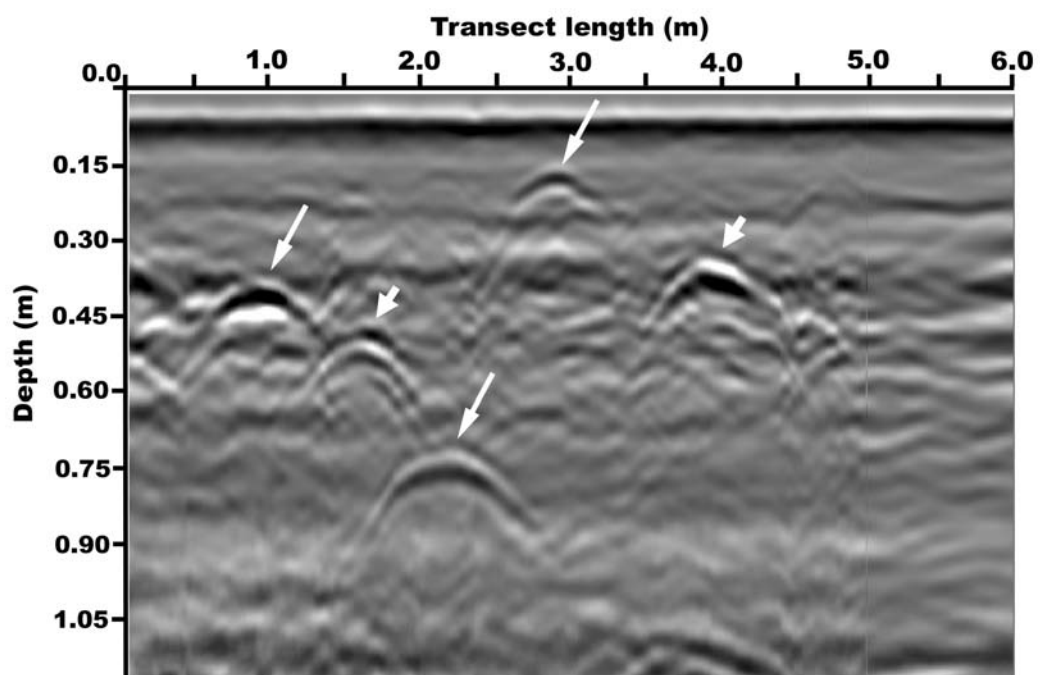


Fig. 4.2. Schematic representation of one of the plots surveyed to detect and quantify residual peach root fragments by ground-penetrating radar following orchard clearing according to commercial practice. Individual survey transects, indicated by arrows, were spaced 50 cm apart. Each plot was surveyed in two directions as indicated by the directionality of transects.

Fig. 4.2.

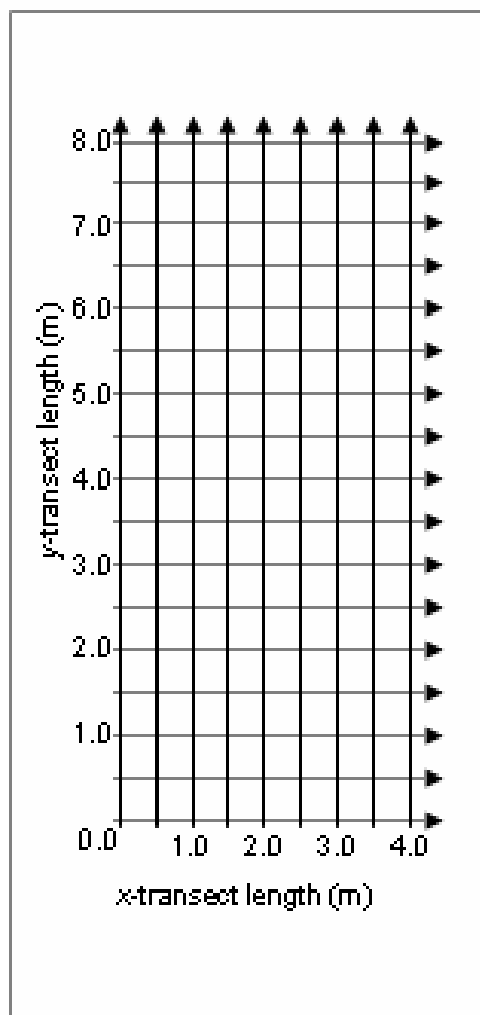


Fig. 4.3. Ground-penetrating radar reflector signals representing different combinations of signal characteristics in a peach orchard cleared according to commercial practice. Signals potentially corresponding to root fragments were categorized into four classes: having contrasting bands of high amplitude and a well-defined parabolic shape (**A**); having poor amplitude contrast, but a well-defined parabolic shape (**B**); having contrasting bands of high amplitude, but only slight curvature (**C**); and having poor amplitude contrast and slight curvature (**D**). Scale bar = 50 cm.

Fig. 4.3.

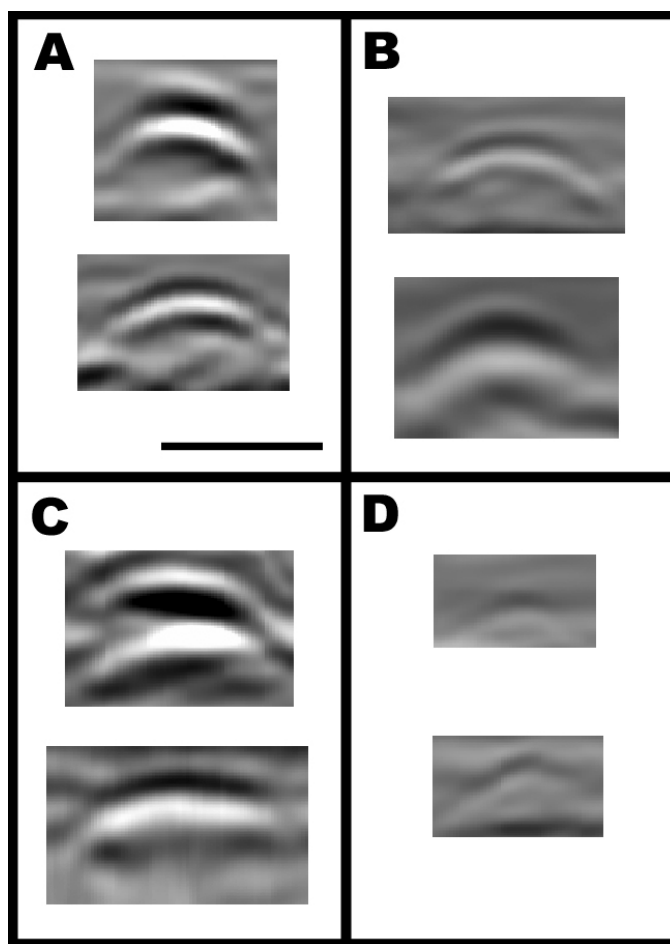
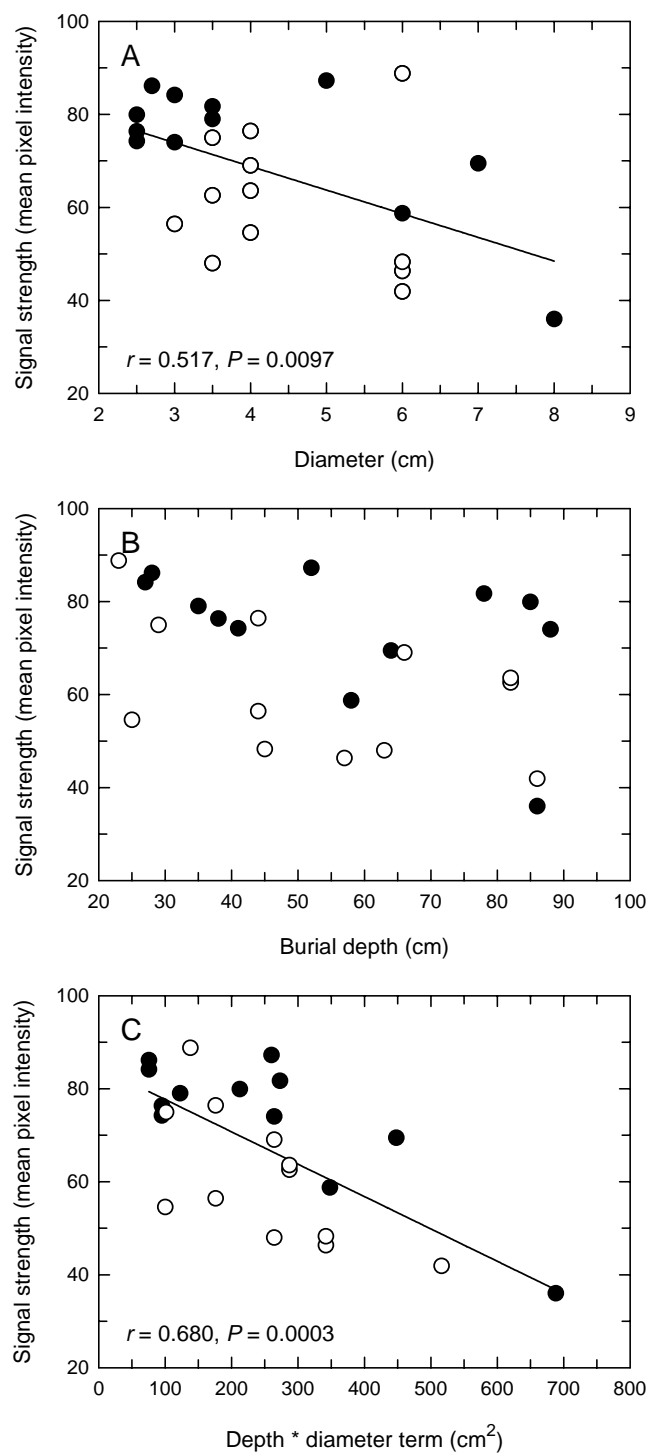


Fig. 4.4. Relationships between signal strength and root characteristics derived from the controlled burial experiment to characterize reflector signals of peach root fragments (●) or PVC pipe standards (○) by ground-penetrating radar. Signal strength is expressed as the mean pixel intensity (range 0 to 255) for the high-amplitude area of reflector signals, with lower values indicative of stronger intensity. The depth × diameter term in **C** corresponds to the product of burial depth and root diameter. Lines in **A** and **C** indicate significant ($P \leq 0.05$) linear regression relationships for the combined root-pipe data.

Fig. 4.4.



CHAPTER 5

**INTERACTION DYNAMICS BETWEEN SAPROPHYTIC LIGNICOLOUS FUNGI
AND *ARMILLARIA* IN CONTROLLED ENVIRONMENTS: INVESTIGATING THE
POTENTIAL FOR COMPETITIVE EXCLUSION OF *ARMILLARIA*¹**

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Interaction Dynamics Between Saprophytic Lignicolous Fungi and *Armillaria* in Controlled Environments: Investigating the Potential for Competitive Exclusion of *Armillaria*

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ABSTRACT

Armillaria root disease, caused by *Armillaria tabescens* and *A. mellea*, is a major contributor to premature tree death in peach orchards in the southeastern United States. The root systems of infected trees can become entirely colonized by *Armillaria*, serving as an inoculum source for adjacent trees and providing massive inoculum levels in replant situations. If dead or dying trees could be colonized by an effective competitor of *Armillaria* before their removal, the extent of root colonization by the pathogen could be reduced, thus decreasing the threat to adjacent trees and/or subsequent plantings. An investigation of interactions between five species of saprophytic lignicolous fungi (*Ganoderma lucidum*, *Hypholoma fasciculare*, *Phanerochaete velutina*, *Schizophyllum commune*, and *Xylaria hypoxylon*) and the two *Armillaria* species was carried out in controlled conditions to provide proof of concept for competitive exclusion of *Armillaria* from peach roots. On agar-coated glass slides, all five potential antagonists induced detrimental reactions in >58% of the *Armillaria* hyphae observed, with the majority of the interactions resulting in hyphal swelling or granulation. On poplar wood blocks, all antagonists consistently either overgrew *Armillaria* colonies or – in the case of *S. commune* – engaged in deadlock reactions; in all cases, the viability of *Armillaria* colonies was reduced to <30% of that of unchallenged controls. When inoculated simultaneously onto opposite ends of peach root

segments, all antagonists consistently reduced growth and viability of *Armillaria* on and underneath the bark, whereby reduction of pathogen growth underneath the bark, the primary ecological niche of *Armillaria*, was most pronounced for *G. lucidum*, *S. commune*, and *X. hypoxylon*. When root segments were allowed to be colonized entirely by *Armillaria* before being inoculated with the antagonists, the latter were able to overgrow the pathogen on the root surface but unable to pre-empt it from underneath the bark. In summary, *G. lucidum*, *S. commune*, and *X. hypoxylon* caused strong hyphal and mycelial interference reactions and the most pronounced reductions in growth of *Armillaria* above and below the bark, indicating that they would be the most promising candidates for field-scale evaluations to restrict colonization of dead or dying peach trees by *Armillaria* in the orchard.

INTRODUCTION

Armillaria root disease, which can be caused by several members of the basidiomycete genus *Armillaria* (Fr.:Fr) Staude, occurs worldwide on a wide variety of hardwood and softwood plants including many stone fruit species such as peach (*Prunus persica* L. Batsch) (Raabe 1962; Savage *et al.* 1953; Hood *et al.* 1991). In southeastern peach orchards, where the disease is caused primarily by *A. tabescens* (Scop.) Dennis *et al.* (= *Clitocybe tabescens* (Scop.) Bres.) and *A. mellea* (Vahl.:Fr.) P. Kumm., Armillaria root rot has been a problem since the early 1950s (Savage *et al.* 1953; Rhoads 1954). The disease causes extensive tree mortality during replanting and expansion of orchards in Georgia and South Carolina (Miller 1994; Cox *et al.* 2004), resulting in considerable economic losses to producers (Cooley 1943; Savage *et al.* 1953; Rhoads 1954; Steiner 1976). In addition to these direct losses, pathogen persistence in the soil on infested residual root pieces prevents the establishment of productive orchards in previously

infested sites, thereby causing additional loss of income (Rhoads 1954; Doepel 1962; Chandler and Daniel 1982).

Current management options for *Armillaria* root rot are limited (Doepel 1962; Steiner 1976; Guillaumin 1977; Hagle and Shaw 1991). Since *Armillaria* can survive in infected root pieces for decades, crop rotation has little effect on reducing inoculum potential. Resistant rootstocks with acceptable horticultural features are not available, although active breeding programs are addressing this problem (Proffer *et al.* 1988; Guillaumin *et al.* 1991; Beckman *et al.* 1998; Beckman and Pusey 2001; Beckman and Lang 2003). Thorough removal of root pieces throughout the entire rooting zone, which would be required to control the disease effectively in replant situations (Roth *et al.* 2000), is expensive and labor-intensive. Pre-plant fumigation, although effective in some trials (Bliss 1951; Chandler 1969; Savage *et al.* 1974; Sherman and Beckman 1999), is not cost-effective and faces an uncertain regulatory future. Post-plant management strategies are either too laborious (e.g., digging trenches between infected and healthy trees to avoid spread by root contact; Homer 1991) or unlikely to be labeled for commercial use (e.g., trunk injection of fungicides; Adaskaveg *et al.* 1999).

Because of the limited effectiveness of conventional management tactics, biological control of *Armillaria* root disease is another alternative. Species of the mycoparasite *Trichoderma*, in particular, have been studied closely in this context. *Trichoderma* can replace *Armillaria* from buried woody inoculum (Garrett 1956, 1957; Munnecke *et al.* 1973, 1976; Otieno 2003) and suppresses root disease caused by the pathogen in citrus orchards when used in combination with sublethal fumigation (Bliss 1951). However, maintaining adequate *Trichoderma* populations needed for control of *Armillaria* in the field is difficult (Shaw and Roth 1978). Furthermore, *Armillaria*'s presence in woody tissue and its ability to spread quickly to

cambial tissues, noted by Rishbeth (1976), present inherent difficulties for biocontrol agent such as *Trichoderma* which does not colonize these ecological niches.

Alternatively, saprophytic lignicolous fungi may be promising antagonists of *Armillaria*. Such fungi are non-pathogenic soil-inhabitants and can be more aggressive colonizers of dead or decaying root and crown tissue than *Armillaria*. Moreover, these fungi, especially cord-forming basidiomycetes, have adopted combative strategies involving hyphal interference, gross mycelial contact, and mycoparasitism to replace or confine other wood-rotting and pathogenic fungi (Boddy 1993, 2000). Still other saprophytes, such as Xylariaceous ascomycetes, are successful at early colonization of woody tissue, and once established can “hold their territory” against other wood-decay organisms (Boddy 2000).

In peach, the first tree deaths caused by *Armillaria* can occur within the first few years after orchard establishment (Savage *et al.* 1953; Shaw and Kile 1991). In subsequent years, the roots of infected trees become entirely colonized by the fungus, serving as an inoculum source for adjacent trees and providing massive inoculum levels in replant situations. If dead or dying trees could be colonized by an effective competitor of *Armillaria* before their removal, the extent of root colonization by *Armillaria* could be reduced, thereby decreasing the threat to adjacent trees and subsequent plantings. Such competitors may be found among saprophytic, lignicolous fungi which can be aggressive colonizers of dead or dying roots and are commonly observed on peach trees when *Armillaria* is absent (Adaskaveg *et al.* 1993). These include several cord-forming basidiomycetes that are present in the southeastern United States (Nakasone 1993) and possess the ability to exclude pathogenic fungi such as *A. ostoyae* or *Heterobasidion annosum* in forest systems (Pearce 1990; Pearce *et al.* 1995; Varese *et al.* 1999, 2003; Chapman and Xiao 2000).

Based on the above considerations, saprophytic lignicolous fungi would be excellent candidates for competitive exclusion of *Armillaria* from peach root systems. However, little information exists regarding interactions between *Armillaria* and such potential antagonists in peach orchards. A detailed investigation of fungal interactions in controlled conditions could provide the necessary proof of concept for exclusion of *Armillaria*. Because of the long-term nature (up to 10 years) of field experiments on managing *Armillaria* root disease, it is critical that detailed micro- and mesocosm experiments on the ecology of and interactions between competitive saprophytic fungi and *Armillaria* precede field-scale evaluation of this approach.

The purpose of this study was to examine the interactions between five species of saprophytic lignicolous fungi (*Ganoderma lucidum*, *Hypholoma fasciculare*, *Phanerochaete velutina*, *Schizophyllum commune*, and *Xylaria hypoxylon*), which either occur on peach or have documented combative ability and occur in the southeastern United States, and two *Armillaria* species (*A. tabescens* and *A. mellea*) to determine their potential for competitive exclusion of *Armillaria* from peach roots. Specifically, we determined the nature of interference interactions between the potential antagonists and *Armillaria* 1) at the microscopic or hyphal level, 2) at the colony level on woody tissue, and 3) at the microcosm level on peach root segments focusing on competitive colonization and fungal displacement.

MATERIALS AND METHODS

Fungal isolates are listed in Table 5.1. The fungi were cultured on malt (30g/L) peptone (5g/L) agar medium at 25°C in the dark. In the experiments described below, the five potential antagonists were paired individually with those of the two *Armillaria* species in all possible combinations; unchallenged controls consisted of each fungal species growing alone.

Hyphal interactions on glass slides. To elucidate the nature of the interactions between hyphae of the potential antagonists and those of *Armillaria*, colony interfaces between paired fungal isolates were examined using light microscopy. Agar plugs ($0.5 \times 0.5 \times 0.5 \text{ cm}^3$) from each fungus to be paired were placed 5 cm apart on a glass slide covered with a thin layer of malt-peptone agar. Inoculated slides were incubated on a Petri dish of water agar at 25°C in the dark and examined daily. At the first sign of contact between colonies, the slide was removed from the agar dish and the colony interface was examined for characteristic hyphal interaction events using a compound microscope with Nomarski optics (400×). The frequency of hyphal bursting, granulation, tip bursting, and swelling (vacuolation), (Skidmore and Dickson 1976; Huub *et al.* 1990) in four microscope fields per slide was recorded for growing fronts of both *Armillaria* and the antagonist in the colony interface (Fig. 5.1); frequencies were converted to percentages based on the total number of hyphae in each microscope field. Each fungal pairing was examined on four replicate slides in three experimental runs.

Colony-level interactions on wood blocks. Mycelial interactions between colonies of *Armillaria* and those of the potential antagonists were examined on woody tissue. Wood blocks ($2.5 \times 7.5 \times 0.5 \text{ cm}^3$), cut from ChoiceWood poplar boards (Weyerhaeuser, Federal Way, WA) were autoclaved twice for 1 hour in deionized water and placed in sterile Magenta boxes. Agar plugs from each of the paired fungi were placed 7 cm apart on the surface of the moist wood block. The boxes were sealed and incubated in the dark at two temperatures (15 and 25°C) to simulate cool and warm-season conditions and favor growth of the antagonists and *Armillaria*, respectively (Rishbeth 1978; Dowson *et al.* 1989). The fungal pairings were monitored every 3 days, and the nature of the interaction between colonies was assessed when the mycelium of the antagonist fungus, inoculated singly as a control, had reached the distal end of the wood block.

Interactions were scored using the key developed by Porter (1924), which consists of five types of colony interference: 1) mutual intermingling of hyphae without observable reaction between fungi; 2) hyphal overgrowth of one fungus by another whereby the overgrown fungus is inhibited in colony growth; 3) inhibition of hyphal growth after colonies are almost in contact with one another (1 to 2 mm apart); 4) hyphal growth of one fungus around the colony of another fungus without reaction (“deadlock”); and 5) mutual inhibition of colony growth of both fungi at a distance >2 mm. The percentage of blocks with these modes of colony interaction was calculated based on observations from 16 wood blocks per pairing at each temperature in each of three experimental runs. Wood blocks were arranged in a split-split-plot design with temperature as the main-plot, antagonist fungal species as the sub-plot, and *Armillaria* species as the sub-sub-plot; the three experimental runs were considered replicates (blocks) in the analysis of variance (ANOVA) (SAS version 8.02; SAS Institute Inc., Cary, NC). Main effects and interactions were considered statistically significant at $\alpha = 0.05$.

For each fungal pairing, the viability of *Armillaria* was determined by removing five fragments of the *Armillaria* mycelium from the colony, placing them onto poplar wood blocks resting on a thin layer of malt-peptone agar in Petri dishes, and incubating them for 2 weeks at 25°C in the dark. Percent viability of *Armillaria* was determined for each fungal pairing based on five isolations taken from each of five wood blocks in each of the three experimental runs.

Competitive colonization and fungal displacement on peach root segments. Roots (2.6 to 3.2 cm in diameter) of peach cv. Lovell were collected at the USDA-ARS Southeastern Fruit and Tree Nut Research Laboratory, Byron, GA; trimmed to 25 cm length; and stored at -20°C until use. Prior to inoculation, root segments were autoclaved twice for 1 hour and placed

on a 2-cm layer of autoclaved moist vermiculite in autoclaved 1.9-liter Mason jars (lying on their sides).

Simultaneous inoculations -- Agar plugs of the paired fungi were attached to opposite ends of the root segments with sterile pins, and jars were sealed and incubated in the dark at 20°C, a temperature supporting growth of both *Armillaria* and the potential antagonists (Rishbeth 1978; Dowson *et al.* 1989). After 40 days, root segments were dissected and the distance (in mm) of external (on the root surface) and internal (underneath the bark) colonization by *Armillaria* was measured based on the presence of characteristic mycelial sheets. Growth distances were converted into growth rates (mm/day), and percent reductions in external and internal growth rates were calculated relative to unchallenged *Armillaria* controls. Each fungal pair was examined on duplicate root segments in each of four experimental runs. Data were analyzed by ANOVA for a factorial design (SAS) with *Armillaria* species and antagonist species as the two treatment factors. For each fungal pairing, the viability of *Armillaria* was determined as described above for the wood block experiments.

Consecutive inoculations -- The potential for fungal displacement was investigated on autoclaved peach root segments pre-colonized by first inoculating with *Armillaria* and then challenging with one of the antagonists species or vice versa. Root segments were incubated in Mason jars as described above at a temperature favorable for the pre-colonizing fungus (25°C for *Armillaria*, 15°C for the potential antagonists) until they were completely colonized on their surface. The segments were inoculated with the challenging fungus on the opposite end and incubated for 120 days to allow for potential long-term displacement. In root segments pre-colonized by *Armillaria*, the distance of external and internal growth of the invading saprophyte was measured and vice versa. Each of the fungal pairings was examined on duplicate root

segments in each of four experimental runs. Colonization distance (in mm) by the challenging fungus was analyzed by ANOVA for a split-plot design (SAS) with incubation temperature as the main-plot and a factorial arrangement of *Armillaria* species and antagonist species in the subplot. The analysis was carried out only for root segments pre-colonized with *Armillaria* as there was no growth of *Armillaria* onto roots pre-colonized by any of the potential antagonists. The viability of *Armillaria* was verified by isolation on autoclaved wood blocks as described above.

RESULTS

Hyphal interactions on glass slides. All five antagonists induced detrimental reactions in >58% of the *Armillaria* hyphae observed on agar-coated glass slides (Table 5.2). *Xylaria hypoxylon*, *S. commune*, and *P. velutina*, in that order, consistently induced higher numbers of detrimental hyphal reactions in both species of *Armillaria* than *G. lucidum* and *H. fasciculare*. Across all antagonists, the majority of the interactions resulted in swelling or granulation of *Armillaria* hyphae (Table 5.2). Hyphal bursting of *Armillaria* was observed exclusively in encounters with *P. velutina*, with a greater percentage occurring in *A. tabescens* (22.2%) than in *A. mellea* (4.0%). Only *X. hypoxylon* consistently induced hyphal tip bursting in *Armillaria*. With the exception of hyphal bursting induced by *P. velutina*, both *Armillaria* species responded similarly to the presence of the antagonists.

Colony-level interactions on wood blocks. All antagonists consistently either overgrew *Armillaria* colonies or engaged in a deadlock reaction with the pathogen, preventing further growth of the latter (Fig. 5.2). In addition to overgrowing the *Armillaria* colonies, the two cord-forming antagonists (*H. fasciculare* and *P. velutina*) and *X. hypoxylon* also formed nutrient-foraging cords to the *Armillaria* colonies (Fig. 5.2A). In deadlock reactions, the *Armillaria*

colony was usually <1.5 cm and walled off by the antagonist, which overtook the rest of the block (Fig. 5.2B).

ANOVA revealed no significant effect of incubation temperature and *Armillaria* species on overgrowth and deadlock reactions (Table 5.3). However, there were highly significant ($P < 0.0001$) differences among antagonist species, with *S. commune* having a significantly lower frequency of overgrowth reactions and a significantly higher frequency of deadlock reactions than the other four antagonists (Figs. 5.3A, B).

In addition to inhibiting *Armillaria* through overgrowth or deadlock reactions, all antagonists reduced the viability of *Armillaria* colonies. Indeed, <30% of the isolations from the *Armillaria* colonies overgrown by the antagonists produced viable colonies (Fig. 5.3C), compared with a viability of almost 100% for unchallenged controls (wood blocks singly inoculated with *Armillaria*).

Competitive colonization and fungal displacement on peach root segments.

Simultaneous inoculations -- All antagonists consistently reduced both the external and internal growth rate of *Armillaria* by between 32.6 and 100% relative to that of unchallenged controls (Fig. 5.4). Both *G. lucidum* and *X. hypoxylon* caused a significantly greater reduction in external growth of *Armillaria* compared with *H. fasciculare* and *P. velutina* (Fig. 5.4A; Table 5.4). For these two antagonist species, the reduction in external growth rate was >65% compared with unchallenged controls for both *Armillaria* species. Overall, there was no significant difference between *A. tabescens* and *A. mellea* in how they responded to the presence of the antagonists ($P = 0.1461$ and 0.4776 for external and internal growth, respectively). However, there was a significant *Armillaria* species \times antagonist interaction for internal growth; the greatest inhibition

was obtained with *S. commune* for *A. tabescens* (75%) and with *G. lucidum* for *A. mellea* (100%) (Fig. 5.4B).

Ganoderma lucidum and *X. hypoxylon* often coated the entire root segment with a rubbery mat of basidiome-like mycelium and a stromatized crust, respectively (Figs. 5.5A, B). By contrast, *H. fasciculare* and *P. velutina* consistently colonized the rhizomorph initials of both *Armillaria* species on the root surface (Fig. 5.5C).

In addition to reducing external and internal growth, all antagonists reduced the viability of *Armillaria* underneath the bark of root segments. Less than 60% of the isolations from mycelial sheets produced viable *Armillaria* colonies compared with a viability of almost 100% for unchallenged controls (root segments singly inoculated with *Armillaria*) (Fig. 5.4C).

Consecutive inoculations -- On pre-colonized root segments, all antagonists were able to colonize the surface of the roots pre-colonized by *Armillaria* to some extent (Fig. 5.6). However, there were no significant ($P > 0.08$) effects of temperature, *Armillaria* species, and antagonist species relative to external colonization of *Armillaria*-infested roots by the antagonists (*data not shown*). In addition, none of the antagonists was able to internally colonize root segments pre-colonized by *Armillaria*. Similarly, *Armillaria* was unable to displace any of the antagonists from underneath the bark (*data not shown*).

DISCUSSION

The hyphal interactions between the saprophytic, lignicolous antagonists and *Armillaria* observed in the present study were indicative of hyphal interference, a form of antagonism whereby cessation of growth and subsequent vacuolation (swelling), granulation, and lysis of hyphae occur as species come in close proximity (Ikediugwu and Webster 1970; Skidmore and

Dickson 1976; Shankar *et al.* 1994). These interactions are mediated by non-enzymatic, diffusible metabolites (antibiotics) that alter the permeability of the plasma membranes of the antagonized hyphae leading to plasmolysis and hyphal death (Ikediugwu and Webster 1970; Skidmore and Dickson 1976; Boddy 2000). Such hyphal interference reactions are well documented among basidiomycetes, and are particularly important in the succession of fungi during wood and dung decomposition (Ikediugwu and Webster 1970; Boddy 2000). Based on these considerations, the hyphal interference observed *in vitro* in the current study may be a reflection of the natural succession of *A. tabescens* and *A. mellea* by other lignicolous fungi in the root decay process.

Observations of overgrowth of the *Armillaria* colony and of deadlock reactions between *Armillaria* and the antagonists on wood blocks and peach root segments suggest a larger-scale interaction mechanism termed mycelial interference. Mycelial interference is the result of gross mycelial contact between fungi that ultimately results in overgrowth (also referred to as “replacement” in the literature) or deadlock depending on the resource and microclimate (Boddy 2000). The occurrence of mycelial interference between the five antagonists and *Armillaria* on the peach root segments in the current study may indicate that the hyphal interference reactions observed on glass slides occur on a large enough scale to have notable effects on resource competition. This has important implications relative to the potential for competitive exclusion of *Armillaria* from root fragments, the main source of primary inoculum of the pathogen on peach in the southeastern United States (Savage *et al.* 1953; Steiner 1976; Cox *et al.* 2004). Species of *Ganoderma*, *Hypholoma*, *Phanerochaete*, and *Xylaria* have been reported previously to deadlock and/or overgrow other fungi including *Armillaria* in woody tissue and to reduce *Armillaria* rhizomorph growth on agar (Pearce 1990; Nicolotti *et al.* 1999; Boddy 2000), but

there has been no comprehensive investigation of the antagonistic potential of these fungi at different spatial scales.

Although most of the antagonists readily overgrew *Armillaria* on wood blocks and on root surfaces, successful overgrowth of the pathogen underneath the bark of peach roots was not observed. *Armillaria* spreads as mycelial sheets throughout the root and crown between the bark and cambium (Shaw and Kile 1991), and colonization of this region by an antagonist would be most important for biocontrol of the pathogen. The lack of internal (below the bark) overgrowth of *Armillaria* by antagonists may be in part related to resource and inoculum characteristics. Indeed, the quality of inoculum and the level of resource decay affects the potential for fungal replacement (Boddy 2000). For example, Holmer and Stenlid (1993) showed that lignicolous fungi inhabiting larger woody inoculum were more combative when challenged by other fungal species. In the current study, the use of small agar-plug inoculum on non-decayed (fresh) roots may not have supplied the resources necessary for overgrowth by the antagonists during the period of time investigated. Aside from inoculum potential, microclimate, particularly water content and to a lesser degree temperature, affects the outcome of mycelial interference in interactions between lignicolous fungi (Boddy 2000). *Armillaria* favors moist wood conditions, while decay saprophytes such as *Phanerochaete* and *Xylaria* favor drier wood as they are unable to penetrate the surface of felled branches due to high moisture content (Chapela and Boddy 1988; Chapela *et al.* 1988). Although temperature had no significant effect on overgrowth of *Armillaria* on wood blocks or root segments in the present study, the high moisture content of roots (due to sterilization in water and incubation on moist vermiculite to prevent desiccation of agar inoculum) may have favored colonization by *Armillaria* and compromised growth and combative ability of the antagonist underneath the bark. Successful use of these antagonists to

colonize and competitively exclude *Armillaria* from dead or dying peach trees in the field may depend on soil moisture and the level of root decay.

Despite the lack of replacement reactions underneath the bark, all antagonists used in this study did reduce the internal growth of *Armillaria* on roots considerably, which indicates promise for delaying or stopping colonization by *Armillaria* on infected root systems and for preempting the pathogen from uncolonized resources. Reduction of growth of *Armillaria* underneath the bark was most pronounced for *G. lucidum*, *S. commune*, and *X. hypoxylon*. The latter fungus also induced the strongest hyphal interference reactions of any the antagonists in this study, was the only one to stimulate hyphal bursting to an appreciable extent, and consistently overgrew *Armillaria* on wood blocks. Although this species has not been used as a biological control agent previously, it has been shown to replace *Armillaria*, to reduce rhizomorph growth on agar medium (Pearce 1990; Boddy 2000), and to be resistant to replacement by other fungi in woody tissue (Chapela *et al.* 1988). The formation of a stromatized crust on the peach root surface further attests to the ability of *X. hypoxylon* to secure “territory” and resist replacement by other fungi.

Similar to *X. hypoxylon*, *G. lucidum* and *S. commune* caused appreciable reductions in growth rates of *Armillaria* on and in root tissue. *Ganoderma lucidum* consistently colonized root and wood block surfaces with a rubbery basidiome-like mat, which would further assist in the exclusion of *Armillaria* from roots if this reaction occurred in the field. By contrast, gross mycelial contact between *S. commune* and *Armillaria* resulted in a considerable proportion of deadlock reactions where the pathogen was completely walled off, especially on wood blocks. The literature regarding combative interactions between species of *Ganoderma* or *Schizophyllum* and other lignicolous fungi is scarce. However, Pearce (1990) showed that *G. applanatum* was

capable of overgrowing *A. luteobubalina* on agar and of reducing its rhizomorph growth. To the best of our knowledge, species of neither *Ganoderma* nor of *Schizophyllum* have been used as biocontrol agents for soilborne diseases, and although undocumented, there is some concern that *G. lucidum* is mildly pathogenic on peach in the southeastern United States as it is observed fruiting on seemingly healthy trees.

The two cord-forming basidiomycetes *H. fasciculare* and *P. velutina*, while less effective in reducing internal colonization of root segments by *Armillaria*, induced swelling and eventual bursting (lysis) of hyphae of *Armillaria* and consistently overgrew the pathogen on wood blocks. Species of *Hypholoma* and *Phanerochaete* are highly combative saprophytes (Boddy 1993; Boddy and Abdalla 1998; Boddy 2000), and the formation of mycelial cords by *H. fasciculare* and *P. velutina* toward *Armillaria* colonies on wood blocks in our study is similar to foraging behavior and resource colonization documented for these species (Dowson *et al.* 1986; Hughes and Boddy 1996; Boddy and Abdalla 1998); this may indicate that *Armillaria* was being used as a nutrient source.

In summary, although only species of the cord-forming fungi *Hypholoma* and *Phanerochaete* have been used previously to reduce colonization of dead or dying roots and crowns by *Armillaria* in the field (Pearce *et al.* 1995; Chapman and Xiao 2000), *G. lucidum*, *S. commune*, and *X. hypoxylon* resulted in similar hyphal and mycelial interference reactions and more pronounced reductions in *Armillaria* growth underneath the bark of inoculated root segments in the present study. Based on these results, and given potential pathogenicity concerns with *G. lucidum*, *S. commune* and *X. hypoxylon* would be the most promising candidates for field-scale evaluations to restrict colonization of dead or dying peach trees by *Armillaria* in the orchard.

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Table 5.1. Isolates of *Armillaria* and saprophytic lignicolous fungi used in the present study

Isolate ^a	Fungal species	Origin	Host
CLY40A	<i>Armillaria mellea</i>	West Chesnee, SC	<i>Prunus persica</i>
94I-71	<i>Armillaria tabescens</i>	Byron, GA	<i>Prunus persica</i>
CS-70-11A	<i>Ganoderma lucidum</i>	Murphysboro, IL	<i>Quercus palustris</i>
OKM-7107-Sp	<i>Hypholoma fasciculare</i>	Laurel, MD	<i>Vaccinium</i>
HHB-4369-Sp	<i>Phanerochaete velutina</i>	Laurel Falls, TN	<i>Pinus</i>
TjV-93-5-Sp	<i>Schizophyllum commune</i>	Blackhawk Ridge, WI	<i>Toxicodendron</i>
RTH790	<i>Xylaria hypoxylon</i>	Athens, GA	<i>Liquidambar styraciflua</i>

^a Isolates of *Armillaria*, *X. hypoxylon*, and the basidiomycete fungi were provided by Tom Beckman (USDA-ARS, Southeastern Fruit and Tree Nut Research Laboratory), Richard Hanlin (University of Georgia), and Rita Rentmeester (USDA Forest Service, Forest Products Laboratory), respectively.

Table 5.2. Percentage of interference reactions^a in hyphae of *Armillaria tabescens* and *A. mellea* induced by saprophytic lignicolous fungi on agar-coated glass slides

Hyphal reaction ^b	<i>Ganoderma lucidum</i>	<i>Hypholoma fasciculare</i>	<i>Phanerochaete velutina</i>	<i>Schizophyllum commune</i>	<i>Xylaria hypoxylon</i>
<i>A. tabescens</i>					
Swelling	34.3 ± 6.5	47.0 ± 7.0	15.9 ± 6.0	50.4 ± 16.1	35.5 ± 18.2
Bursting	0	0	22.2 ± 3.8	0	0
Tip bursting	0	9.3 ± 7.3	0	3.5 ± 3.0	18.8 ± 7.8
Granulation	29.4 ± 1.5	17.3 ± 0.9	41.9 ± 4.9	19.7 ± 14.9	24.8 ± 12.4
Total detrimental reactions	63.7 ± 5.9	73.6 ± 8.6	80.0 ± 4.1	73.6 ± 13.3	79.1 ± 13.6
<i>A. mellea</i>					
Swelling	17.4 ± 5.4	35.1 ± 5.0	3.9 ± 2.2	57.2 ± 2.9	36.0 ± 7.7
Bursting	0	0	4.0 ± 2.5	0	0
Tip bursting	10.1 ± 10.1	10.1 ± 9.8	0	0	23.2 ± 4.5
Granulation	41.7 ± 2.8	13.2 ± 4.8	63.9 ± 11.9	10.9 ± 9.3	38.0 ± 10.6
Total detrimental reactions	69.2 ± 4.6	58.4 ± 9.1	71.8 ± 8.3	68.1 ± 6.1	97.2 ± 2.6

^a Percentages based on frequency of hyphal reactions observed in four microscope fields (400×) per slide. Values are means and standard errors across three experiments.

^b Interference reactions illustrated in Fig. 5.1.

Table 5.3. Results of a split-split-plot analysis of variance to determine the effects of temperature, *Armillaria* species, antagonist species, and their interactions on *Armillaria* overgrowth and deadlock reactions in fungal pairings on poplar wood blocks^a

Source	df	Overgrowth			Deadlock		
		Mean Square	<i>F</i>	<i>P</i> > <i>F</i>	Mean Square	<i>F</i>	<i>P</i> > <i>F</i>
Replication (block)	2	0.107	6.34	0.0074	0.100	6.06	0.0088
Temperature (main-plot)	1	0.000	0.02	0.8988	0.002	0.11	0.7482
Main-plot error	2	0.023	---	---	0.022	---	---
Antagonist species (sub-plot)	4	0.802	47.7	<0.0001	0.758	45.7	<0.0001
Temperature × Antagonist	4	0.010	0.59	0.6762	0.011	0.69	0.6068
Sub-plot error	16	0.044	---	---	0.041	---	---
<i>Armillaria</i> species (sub-sub-plot)	1	0.011	0.66	0.4273	0.013	0.77	0.3896
Temperature × <i>Armillaria</i> species	1	0.054	3.22	0.0877	0.058	3.50	0.0760
Antagonist species × <i>Armillaria</i> species	4	0.017	1.01	0.4239	0.019	1.15	0.3625
Temperature × Antagonist species × <i>Armillaria</i> species	4	0.034	2.05	0.1262	0.037	2.21	0.1049
Sub-sub-plot error	20	0.017	---	---	0.017	---	---

^a Analysis based on arcsine-square root-transformed percentages. Levels of the three factors are: temperature, 15 and 25°C; *Armillaria* species, *A. tabescens* and *A. mellea*; antagonist species, *Ganoderma lucidum*, *Hypholoma fasciculare*, *Phanerochaete velutina*, *Schizophyllum commune*, and *Xylaria hypoxylon*.

Table 5.4. Results of a factorial analysis of variance to determine the effects of *Armillaria* species, antagonist species, and their interactions on reduction of *Armillaria* growth in fungal pairings on peach root segments ^a

Source	df	External growth			Internal growth		
		Mean Square	<i>F</i>	<i>P</i> > <i>F</i>	Mean Square	<i>F</i>	<i>P</i> > <i>F</i>
Replication (block)	3	0.402	12.98	<0.0001	0.572	6.34	0.0021
Antagonist species	4	0.376	12.12	<0.0001	0.641	7.11	0.0005
<i>Armillaria</i> species	1	0.069	2.24	0.1461	0.047	0.52	0.4776
Antagonist species × <i>Armillaria</i> species	4	0.035	1.13	0.3614	0.291	3.22	0.0276
Error	27	0.031	---	---	0.090	---	---

^a Analysis based on arcsine-square root-transformed percentages. Levels of the two factors are: *Armillaria* species, *A. tabescens* and *A. mellea*; antagonist species, *Ganoderma lucidum*, *Hypholoma fasciculare*, *Phanerochaete velutina*, *Schizophyllum commune*, and *Xylaria hypoxylon*.

Fig. 5.1. Interference reactions in hyphae of *Armillaria* induced by saprophytic lignicolous fungi on agar-coated glass slides. Bursting of *A. tabescens* induced by *Phanerochaete velutina* (**A**); tip-bursting of *A. mellea* induced by *Xylaria hypoxylon* (**B**), swelling (vacuolation) of *A. tabescens* induced by *Schizophyllum commune* (**C**), and granulation of *A. mellea* hyphae induced by *Xylaria hypoxylon*. Arrows indicate hyphae or hyphal fragments (in **A** only) of *Armillaria*. Scale bar = 40 μm .

Fig 5.1.

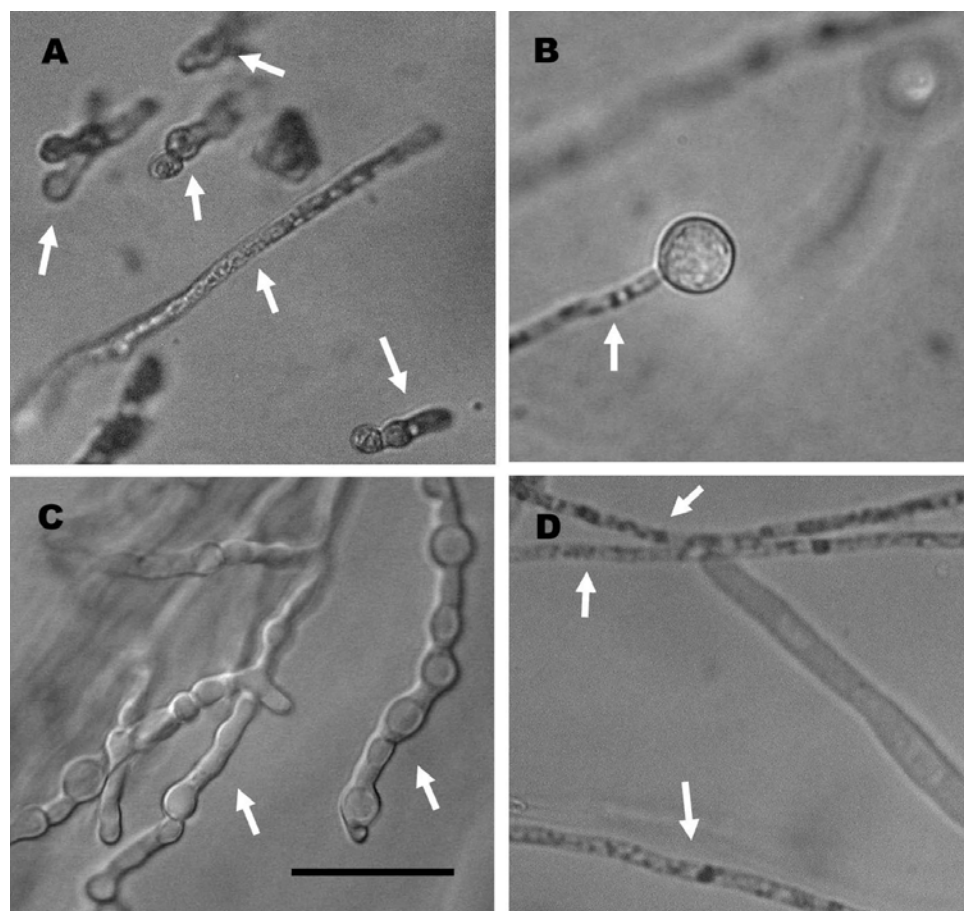


Fig. 5.2. Examples of overgrowth and deadlock reactions between *Armillaria* and saprophytic lignicolous fungi on poplar wood blocks: **A)** Nutrient-foraging cords of *Hypholoma fasciculare* covering and connected to a colony of *A. tabescens* (AT); **B)** typical deadlock reaction whereby *Schizophyllum commune* surrounds a colony of *A. mellea* (AM). Scale bar = 1.0 cm.

Fig. 5.2.

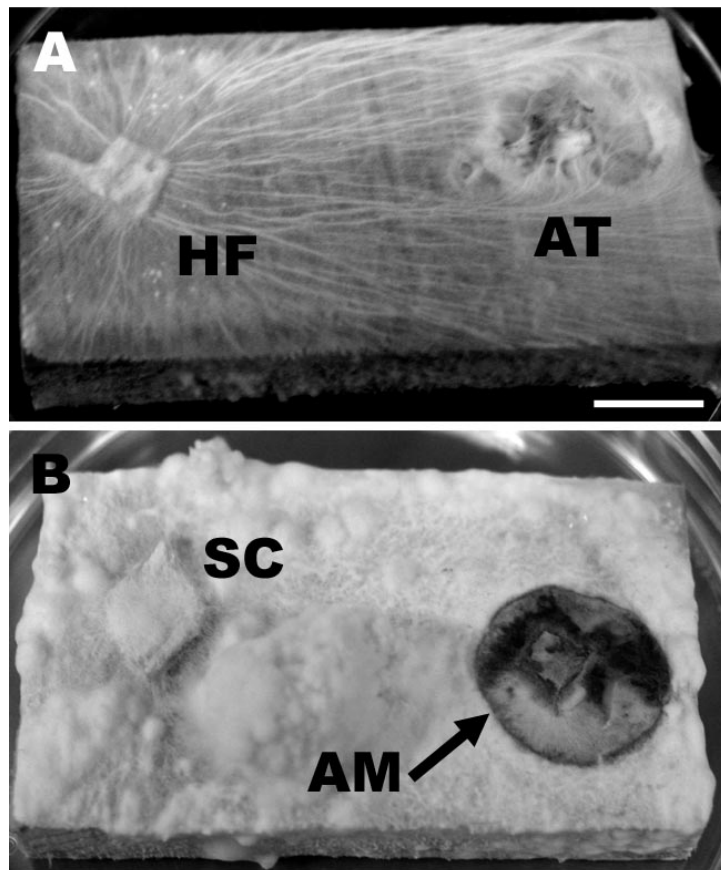


Fig. 5.3. Frequency of overgrowth (**A**) and deadlock (**B**) reactions between *Armillaria* and saprophytic lignicolous fungi on poplar wood blocks, and percent viability of *Armillaria* mycelium isolated from the blocks (**C**). Values are means and standard errors of three experimental runs averaged across two temperatures (15 and 25°C). In **A** and **B**, antagonist species followed by the same letter are not significantly different according to Fisher's protected LSD test (analysis of main effects at $\alpha = 0.05$, arcsine square root-transformed values). GL = *Ganoderma lucidum*, HF = *Hypholoma fasciculare*, PV = *Phanerochaete velutina*, SC = *Schizophyllum commune*, and XY = *Xylaria hypoxylon*.

Fig. 5.3.

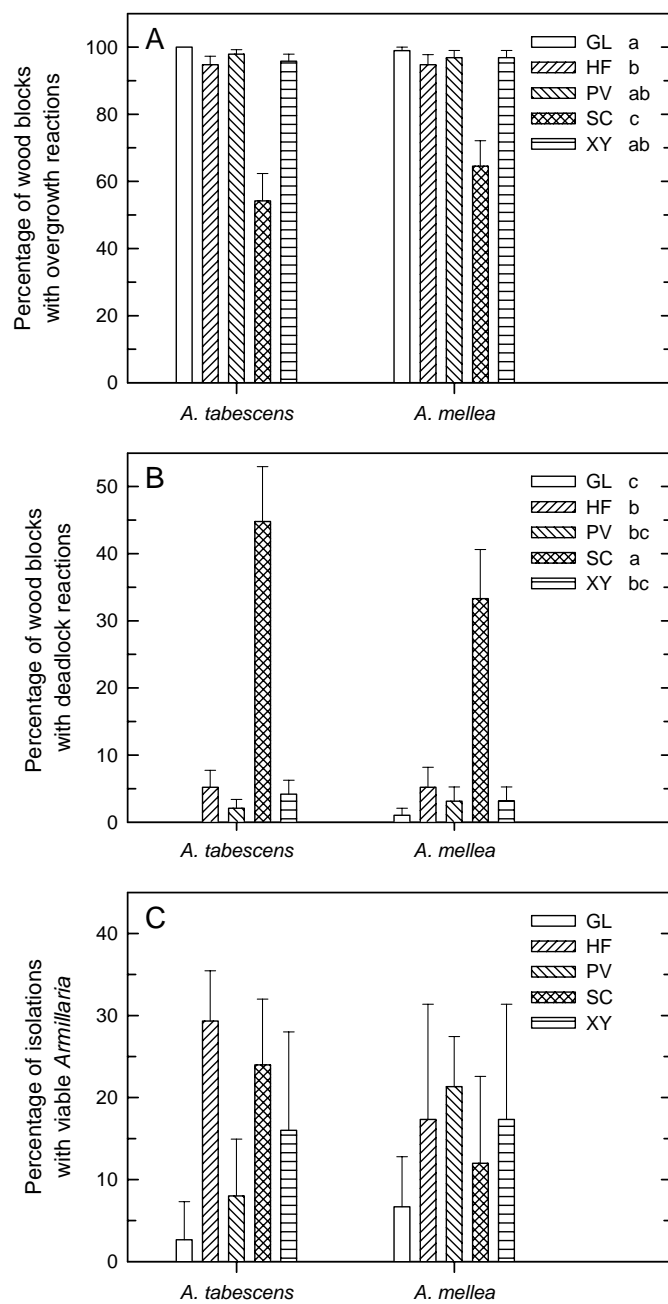


Fig. 5.4. Percent reduction of external (**A**) and internal (**B**) growth of *Armillaria* on peach root segments in the presence of saprophytic lignicolous fungi, and percent viability of *Armillaria* mycelium isolated from the roots (**C**). Root segments were inoculated simultaneously with *Armillaria* and the antagonist on opposite ends of the root. Values are means and standard errors of four experimental runs. In **A** and **B**, antagonist species followed by the same letter are not significantly different according to Fisher's protected LSD test ($\alpha = 0.05$, arcsine square root-transformed values). In **B**, means separation was carried out separately for *A. tabescens* and *A. mellea* because of a significant antagonist species \times *Armillaria* species interaction ($P = 0.0276$). GL = *Ganoderma lucidum*, HF = *Hypholoma fasciculare*, PV = *Phanerochaete velutina*, SC = *Schizophyllum commune*, and XY = *Xylaria hypoxylon*.

Fig.5.4.

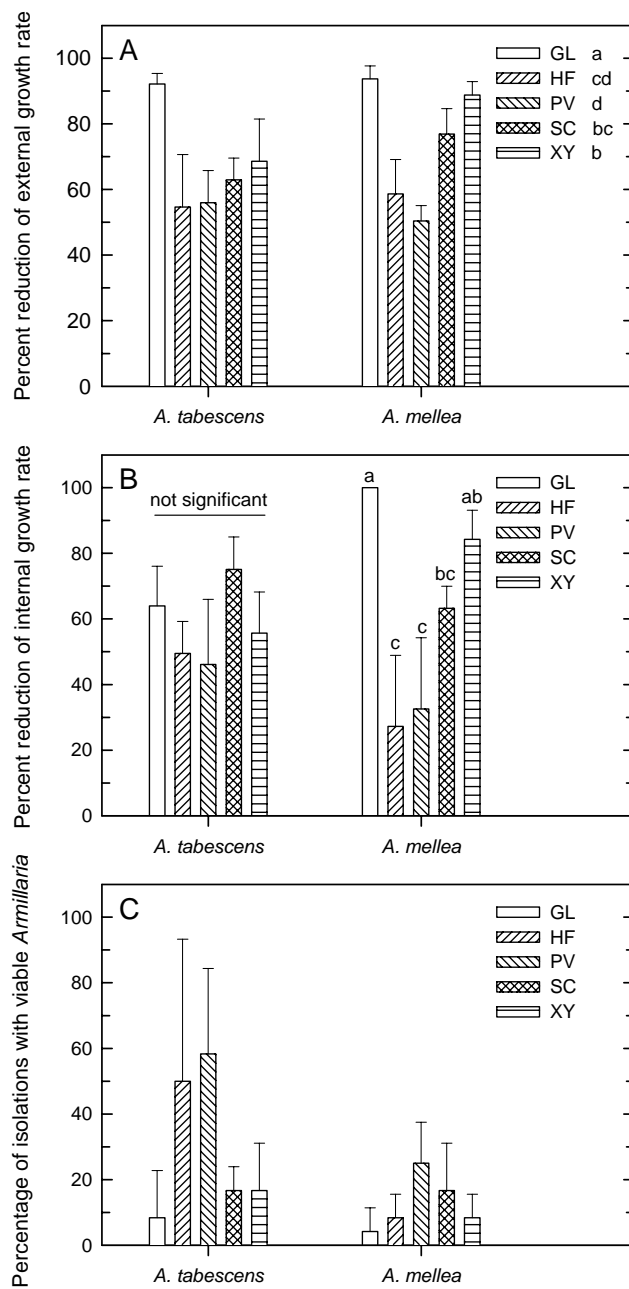


Fig. 5.5. Examples of interactions between *Armillaria* and saprophytic lignicolous fungi on peach root segments. Rubbery mats of basidiome-like mycelium produced by *Ganoderma lucidum* (GL) covering the root surface (**A**); hard, stromatized crust produced by *Xylaria hypoxylon* covers the surface of a root (left) compared with a control root (right) (**B**); rhizomorph initials of *A. mellea* (AM) colonized by *Phanerochaete velutina* (PV) (**C**). Scale bar = 1.5 cm in **A** and **B**, and 0.5 cm in **C**.

Fig. 5.5.

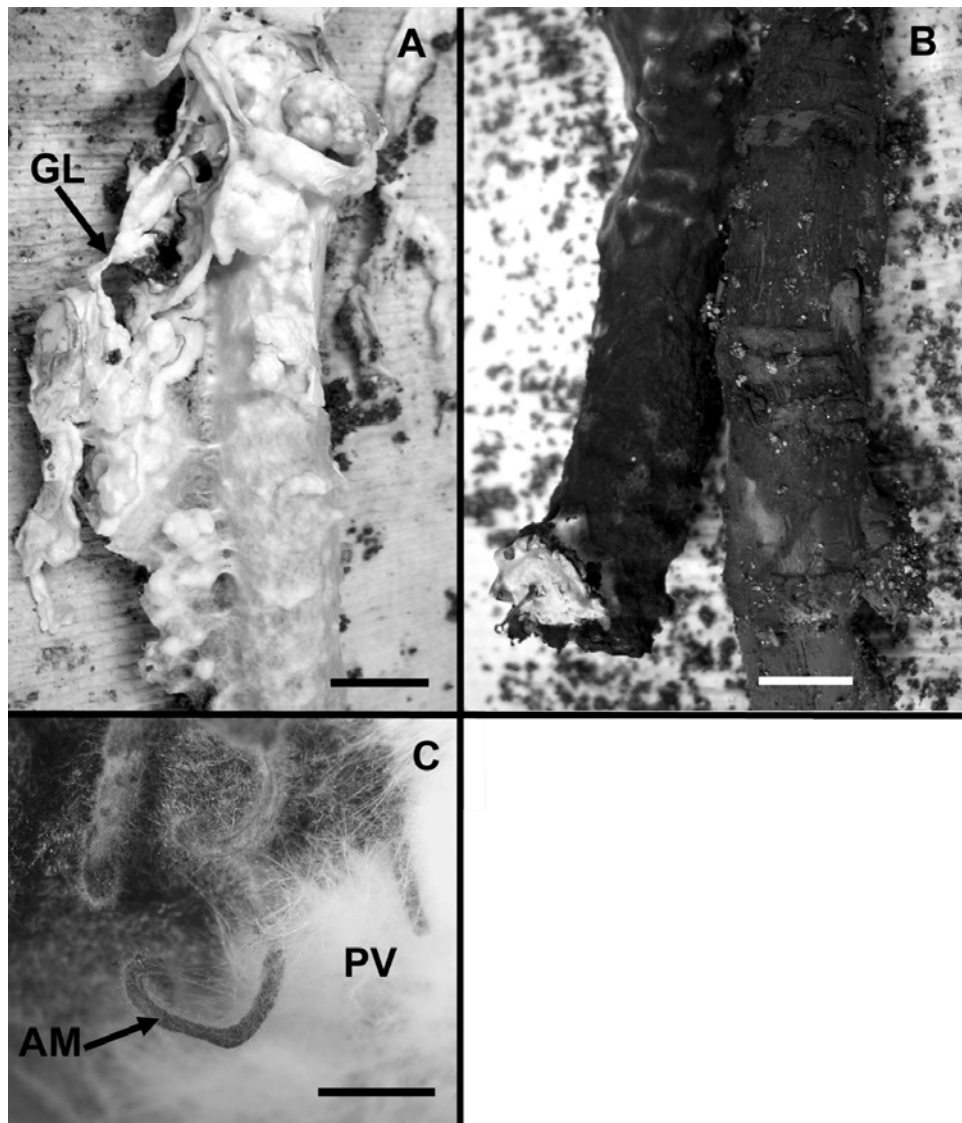
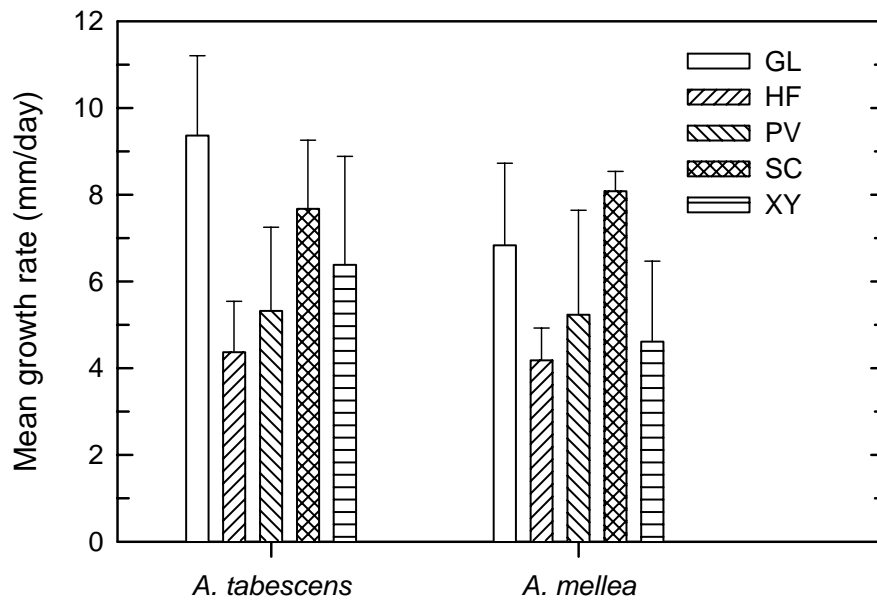


Fig. 5.6. External growth of saprophytic lignicolous fungi on peach root segments that had been pre-colonized by *Armillaria*. Values are means and standard errors of four experimental runs averaged across two temperatures (15 and 25°C). GL = *Ganoderma lucidum*, HF = *Hypholoma fasciculare*, PV = *Phanerochaete velutina*, SC = *Schizophyllum commune*, and XY = *Xylaria hypoxylon*.

Fig. 5.6.



CHAPTER 6

CONCLUSIONS

In southeastern peach orchards, *Armillaria* root rot, caused by *A. tabescens* (Scop.) Dennis *et al.* (= *Clitocybe tabescens* (Scop.) Bres.) and *A. mellea* has been problematic since the early 1950s (Savage *et al.* 1953; Rhoads 1954) and continues to cause extensive tree mortality during replanting and expansion of orchards (Cox *et al.* 2004), resulting in considerable economic losses to producers (Cooley 1943; Savage *et al.* 1953; Rhoads 1954; Steiner 1976; Miller 1994). Current management options for the disease are limited (Doepel 1962; Steiner 1976; Guillaumin 1977; Hagle and Shaw 1991). Since *Armillaria* can survive in infected root pieces for decades, crop rotation has very little effect in reducing inoculum potential. Resistant rootstocks with acceptable horticultural features are not available at present (Beckman *et al.* 1998; Beckman and Pusey 2001; Beckman and Lang 2003). Thorough removal of root pieces throughout the entire rooting zone, which would be required to control the disease effectively in replant situations (Roth *et al.* 2000), is expensive and labor-intensive. Pre-plant fumigation, although effective in some trials (Bliss 1951; Chandler 1969; Savage *et al.* 1974; Sherman and Beckman 1999), is not cost-effective and faces an uncertain regulatory future. Post-plant management strategies that have been shown to be somewhat effective are either too laborious (e.g., digging trenches between infected and healthy trees to avoid spread by root contact; Homer 1991) or very unlikely to be labeled for commercial use (e.g., trunk injection of fungicides; Adaskaveg *et al.* 1999).

The limited success in managing *Armillaria* root rot of peach is in part due to a lack of knowledge regarding several key aspects of the biology of *Armillaria* in the orchard ecosystem.

In particular, little is known about the relative prevalence of the two *Armillaria* species in the southeastern United States and how to distinguish between them and other lignicolous fungi in the absence of basidiocarps. There is also little information regarding the amount of potential inoculum remaining in the field after standard pre-plant and replant tree removal practices, or to what extent the remaining root pieces are of a size that would favor survival of the pathogen. In addition, there are numerous saprophytic lignicolous fungi present on dead or dying peach trees but very limited information regarding the interactions between them and *Armillaria*; such information may be useful for developing biocontrol tactics for competitive exclusion of *Armillaria* from peach roots in the orchard.

The research carried out as part of this dissertation addressed selected aspects related to establishment, spread, and persistence of *Armillaria* root rot in peach orchards in a series of three studies. The first of the studies focused on different species of the pathogen and their characterization and discrimination using fatty acid methyl ester (FAME) analysis. The second study addressed detection and quantification of residual root fragments, the source of primary inoculum of *Armillaria* root rot in replant situations. In the third study, interactions between *Armillaria* and lignicolous fungal antagonists were investigated in controlled conditions to determine the potential for restricting colonization and persistence of *Armillaria* on root tissue in the field.

When *Armillaria* species were characterized using fatty acid analysis, FAME profiles generated from cultures of *A. tabescens*, *A. mellea*, and *A. gallica* consisted of 15 to 26 fatty acids ranging from 12 to 24 carbons in length, with 9-*cis*,12-*cis*-octadecadienoic acid (9,12-18:2), hexadecanoic acid (16:0), and other 18-carbon fatty acids being the most abundant. FAME profiles from different thallus morphologies (mycelium, sclerotial crust, or rhizomorphs)

displayed by cultures of *A. gallica* showed that thallus type had no significant effect on the cellular fatty acid composition. The three *Armillaria* species included in this study could be distinguished from other lignicolous basidiomycete species commonly occurring on peach (*Ganoderma lucidum*, *Schizophyllum commune*, *Stereum hirsutum*, and *Trametes versicolor*) on the basis of their FAME profiles using stepwise discriminant analysis, with hexadecanoic acid (16:0), 12-*cis*-hexadecenoic acid (C16:1), and tricosanoic acid (23:0) being the three strongest contributors to the distinction between *Armillaria* and the “outgroup” species. In a separate stepwise discriminant analysis, *A. tabescens*, *A. mellea*, and *A. gallica* could also be separated from one another on the basis of their fatty acid profiles, whereby tetradecanoic acid (14:0), 8-*cis*-octadecenoic acid (C18:1), and iso-tetracosanoic acid (iso-24:0) made the strongest contributors to the distinction among the three species. When fatty acids were extracted directly from mycelium dissected from naturally infected peach tissue, the FAME-based discriminant functions developed in the preceding experiments classified 14 out of 16 samples as *A. tabescens* and the remaining two as *A. mellea*; when applied to cultures obtained from the same naturally infected samples, all 16 unknowns were classified as *A. tabescens*. Thus, FAME species classification of *Armillaria* unknowns directly from infected tissues may yield heterogeneous results. Species designation of unknown *Armillaria* cultures by FAME analysis was identical to that indicated by IGS-RFLP classification with *Alu* I, suggesting that this method can be used to complement existing molecular tools for characterization of *Armillaria*.

In the second objective, I investigated the utility of ground-penetrating radar (GPR) to characterize reflector signals from peach root fragments in a controlled burial experiment and to quantify the amount of residual roots remaining after orchard clearing typical of commercial practice. In the former experiment, roots ranging from 2.5 to 8.2 cm in diameter and buried at

depths of 11 to 114 cm produced characteristic parabolic reflector signals in radar profiles. Image analysis of high-amplitude reflector area indicated significant linear relationships between signal strength (mean pixel intensity) and root diameter ($r = -0.517$, $P = 0.0097$, $n = 24$) or the combined effects of root diameter and burial depth, expressed through a depth \times diameter term ($r = -0.680$, $P = 0.0003$, $n = 24$). In a peach orchard in which trees and roots had been removed following commercial practice, a GPR survey of six 4-m \times 8-m plots revealed that the majority of reflector signals indicative of roots were located in the top 30 to 40 cm of soil. Based on ground-truth excavation of selected sites within plots, reflectors showing a strong parabolic curvature in the radar profiles corresponded to residual root fragments with 100% accuracy, while those displaying a high amplitude area represented roots in 86.1% of the cases. By contrast, reflectors with both poor curvature and low amplitude yielded roots for <10% of the excavated sites, while randomly selected sites lacking reflector signals were devoid of roots or other subsurface objects. A high level of variability in the number of residual roots was inferred from the radar profiles of the six plots, suggesting an aggregated distribution of root fragments throughout the field. The data further indicated that at least one residual root fragment would be present per m³ of soil, and that many of these fragments have diameters corresponding to good to excellent inoculum potential for Armillaria root disease. Further GPR surveys involving different levels of land clearing, combined with long-term monitoring of Armillaria disease incidence in replanted trees, will be necessary to ascertain the disease threat posed by the levels of residual root biomass observed in this study.

Given the importance of residual root fragments as primary inoculum for inciting Armillaria root disease, an investigation of interactions between five species of saprophytic lignicolous fungi (*Ganoderma lucidum*, *Hypholoma fasciculare*, *Phanerochaete velutina*,

Schizophyllum commune, and *Xylaria hypoxylon*) and the two *Armillaria* species was carried out in controlled conditions to provide proof of concept for competitive exclusion of *Armillaria* from peach roots. On agar-coated glass slides, all five potential antagonists induced detrimental reactions in >58% of the *Armillaria* hyphae observed, with the majority of the interactions resulting in hyphal swelling or granulation. On poplar wood blocks, all antagonists consistently either overgrew *Armillaria* colonies or – in the case of *S. commune* – engaged in deadlock reactions; in all cases, the viability of *Armillaria* colonies was reduced to <30% of that of unchallenged controls. When inoculated simultaneously onto opposite ends of peach root segments, all antagonists consistently reduced growth and viability of *Armillaria* on and underneath the bark, whereby reduction of pathogen growth underneath the bark in its primary ecological niche was most pronounced for *G. lucidum*, *S. commune*, and *X. hypoxylon*. When root segments were allowed to be colonized entirely by *Armillaria* before being inoculated with the antagonists, the latter were able to overgrow the pathogen on the root surface but unable to pre-empt it from underneath the bark. Overall, *G. lucidum*, *S. commune*, and *X. hypoxylon* caused strong hyphal and mycelial interference reactions and the most pronounced reductions in growth of *Armillaria* above and below the bark, indicating that they would be the most promising candidates for field-scale evaluations to restrict colonization of dead or dying peach trees by *Armillaria* in the orchard.

FINAL REMARKS

These studies provided basic information on establishment, spread, and persistence of *Armillaria* root rot in peach orchards, specifically on the biochemical characterization of the *Armillaria* species involved, on the extent of potential inoculum in the form of residual root

pieces in replant situations, and on the potential for restricting colonization and persistence of *Armillaria* on peach root tissue. Using fatty acid analysis, I determined that thallus type did not affect overall cellular fatty acid composition of *Armillaria*, and that FAME analysis could be used to identify *Armillaria* isolates to species, while corroborating molecular identification. I also established that GPR could be used to detect peach roots in the field, quantify potential root inoculum following orchard removal, and document that residual roots are of a size favoring *Armillaria* survival and infection. In the investigation of interactions between several species of saprophytic lignicolous fungi and *Armillaria*, I established that such potential antagonists can induce hyphal and mycelial interference reactions against *Armillaria* and reduce the external and internal growth of the pathogen when paired with it on peach roots, indicating the potential for restricting *Armillaria* colonization of dead or dying root tissue in the field. Knowledge gained from these studies contributes to a better understanding of and the possibility for improved management of *Armillaria* root rot occurring on peach in the southeastern United States.

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