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The Tripartite Interaction Between Arbuscular Mycorrhizal Fungi, Rice, and Insects

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THE TRIPARTITE INTERACTION BETWEEN ARBUSCULAR MYCORRHIZAL FUNGI, RICE, AND INSECTS

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This dissertation is dedicated to my
loved and inspiring parents, Flor Alvarado and
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Abbreviations

AM	Arbuscular mycorrhiza
AMF	Arbuscular mycorrhiza fungi
cm	Centimeter
DAF	Days after flooding
FAW	Fall armyworm
g	Gram
IPM	Integrated pest management
N	Nitrogen
P	Phosphorus
RDW	Root dry weight
RWW	Rice water weevil
SDW	Shoot dry weight
ShB	Sheath blight of rice

Abstract

Losses caused by pests remain an important limitation to achieving high rice yields in the United States. Arbuscular mycorrhizal fungi (AM fungi) are able to modify plant physiology by increasing plant growth or inducing defense responses against insect herbivores. However, studies of the role of AM fungi in agroecological factors, including natural occurrence, plant resistance, soil dependency, and plant tolerance, with specific regards to pests that feed on rice plants have not been conducted before.

A three-year study revealed natural occurring colonization by AM fungi on rice roots sampled in four rice-producing areas in the southern United States. Overall, rice-AM fungi associations were greatest in Arkansas followed by Mississippi, Texas, and then Louisiana.

In the plant resistance study, larval performance and pathogen infection of different pests on rice cultivars inoculated with AM fungi in Louisiana were investigated. Results from this study revealed that densities of rice water weevil (RWW) larvae, weight gains of fall armyworm (FAW) larvae, and susceptibility to sheath blight infection were higher on rice plants treated with AM fungi inoculum.

In the soil-dependent study, the susceptibility to RWW and FAW was increased in AM fungi-treated rice plants, but this effect was soil dependent. The enhanced effect on plant biomass was also soil dependent, but the inoculation of AM fungi had no effect on N or P concentrations nor on rice yields in both soil types.

In the tolerance study, AM fungi seed treatment did not reduce RWW densities, but NipsIt INSIDE seed treatments reduced RWW densities. In addition, plant biomass and yields were higher in AM fungi-treated plants compared to untreated plants. This study provided strong support that the effects of AM fungi seed treatments can be more effective to increase rice biomass and yields.

Taken together, findings from this work reveal that rice plants inoculated with AM fungi may provide an effective method for herbivore control (especially for the RWW) for

increasing plant biomass and yields, but also highlight the complicated nature of the various factors governing rice-AM fungi-pest interactions. The broader implications of this study are important due to the potential impact that AM fungi may have on IPM and future studies. Thus, gaining a better understanding of the underlying mechanisms of AM fungi on rice-pest interactions will contribute to the development of more effective and sustainable strategies to control or reduce pest damage in rice.

Chapter 1
General Introduction

1.1 The Model System

Globally, rice (*Oryza sativa* L.,) is the second most important cereal crop following corn with about 161 million hectares of rice planted, producing more than 480 million metric tons in 2017 (FAO & USDA, 2018). Rice is the staple food of an estimated 3.5 billion people worldwide, providing half of the daily calories consumed by humans (Goff et al., 2002). Worldwide rice production in 2017, based on area harvested, was led by India and China combining to a total of half of all the rice produced globally (FAO, 2018).

In the United States rice is produced on approximatively 1.3 million hectares, which represents less than 1% of the total rice production (USDA, 2018). This crop is grown in two distinct regions, California and the southern states of Arkansas, Louisiana, Texas, Mississippi, and Missouri. Louisiana is the third leading rice producing state with 161 thousand hectares of rice planted and 1.5 million metric tons of rice produced in 2017 (USDA & NASS, 2018).

Rice in Louisiana is grown annually under flooded conditions on natural flatlands. This type of land allows for mechanization and more efficient crop management. However, rice as a monocrop creates a vulnerable environment, which is exposed to biotic and abiotic stresses that may reduce the yield and value of the rice grain. Therefore, to maintain the stability of rice production or, more importantly, to increase its production in Louisiana, it is necessary to control the threats from the various rice pests that are involved in rice production.

The model system investigated is presented in Figure 1.1. Among the rice pests, insect herbivores are an economic problem that attack rice fields during the entire planting season. In the southern United States, the rice water weevil (RWW), *Lissorhoptrus*

oryzophilus (Kuschel), is the most important early season insect pest and the most destructive insect pest of rice. Both adults and larvae of this species feed on rice, but feeding by adults generally does not result in economic injury. However, root pruning by larvae can severely reduce both growth and yield of rice (Way, 1990; Zou et al., 2004a). In Louisiana, larval infestations can reduce yields up to 25% in untreated plots, or even more under heavy pressure (Stout et al., 2000; Zou et al., 2004b).

The fall armyworm (FAW), *Spodoptera frugiperda* (J. E. Smith), is one of several occasional rice lepidopteran pests in Louisiana. It can attack rice throughout the growing season, but in Louisiana, it most frequently attacks early season rice before flooding. This pest can occur in high densities and can quickly defoliate young rice plants (Pantoja et al., 1986). Cultural and chemical control tactics are commonly used for controlling armyworms. Cultural control consists of flooding rice fields to kill armyworm larvae, but, as with rice water weevil control, chemical application has always been the preferred tactic for managing this pest.

The sugarcane borer, *Diatraea saccharalis* (F.), the Mexican rice borer, *Eoreuma loftini* (Dyar), and the rice stalk borer, *Chilo plejadellus* (Zincken), constitute the group of mid- to late season Lepidopteran stem borers that attack rice fields in the United States (Way, 2003). Of these stem-boring species, the Mexican rice borer has the potential for significant economic damage (Reay-Jones et al., 2008). According to Reay-Jones et al. (2008), this pest will invade the Louisiana rice and sugarcane industry by 2035 with potential annual losses of up to $200 million. Currently, insecticide control is the most commonly used management tactic but it is not very cost-effective; in addition, insecticide applications have adverse environmental effects on non-target organisms.

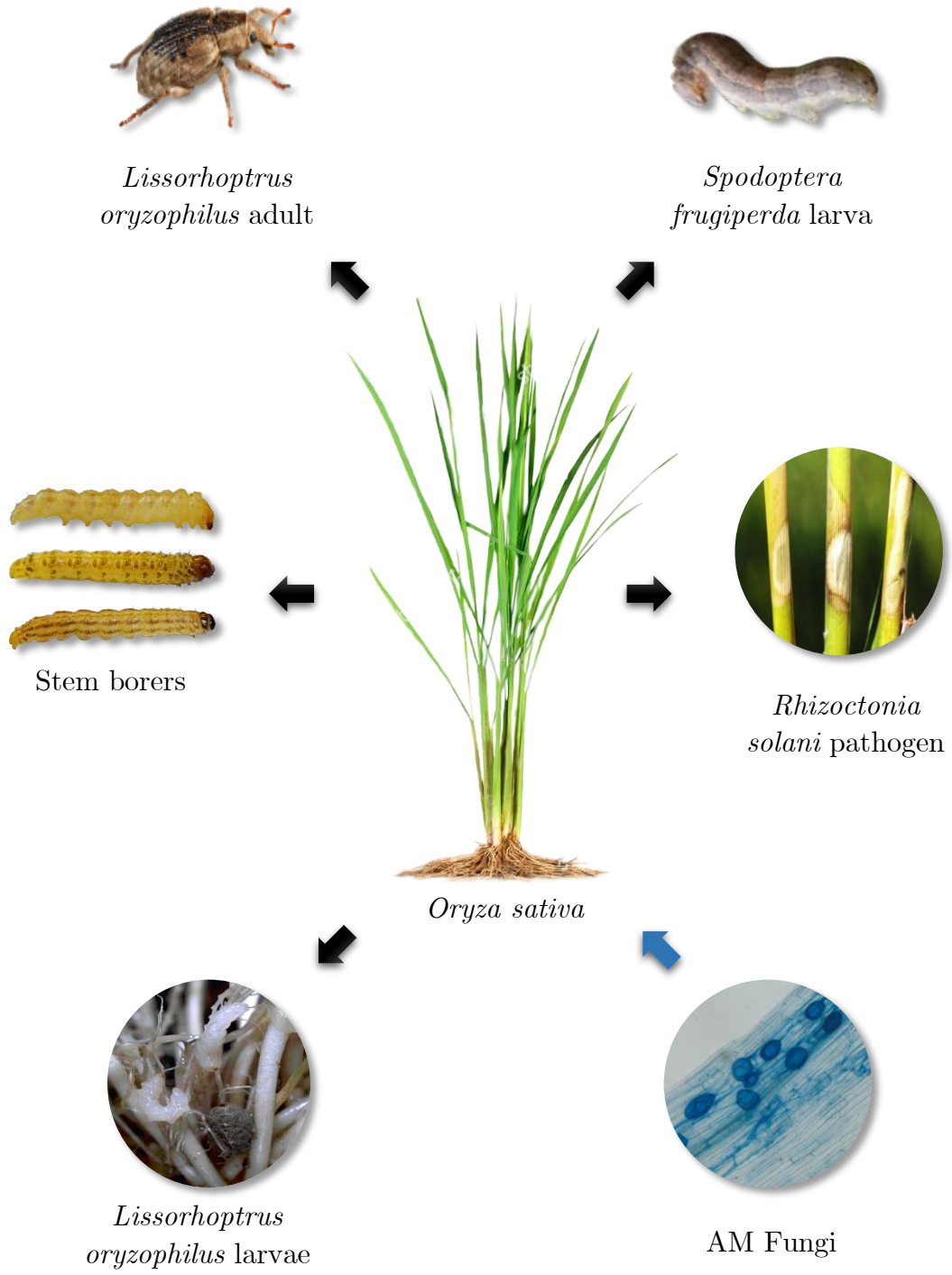

Figure 1.1. Model system used in this thesis consists of organisms from above- and below-ground.

The sheath blight (ShB), *Rhizoctonia solani* (Kuhn), is currently one of the most damaging diseases to rice crops in Louisiana since the early 1970s. Cultural practices and fungicides are the two most effective options today to control the pathogen. Reducing plant density of rice and the use of fertilizer, especially nitrogen, can help reduce amount of inoculum in the soil (Blanche et al., 2009).

According to Hokkanen (2015), pest management programs based on the use of pesticides will eventually become unstable and unsustainable. Host plant resistance instead, is considered a sustainable and effective tactic against insect pests that can be incorporated into integrated pest management (IPM) to reduce the indiscriminate use of chemical pesticides (Stenberg, 2017). In Louisiana, rice varieties exhibit various resistance levels to arthropod pests (Mohamad Saad et al., 2018), providing an alternative to the use of insecticides. Additionally, the level of resistance expressed by a host plant depends on the interactions of the plant with microorganisms in its environment, especially microbes that inhabit the rhizosphere (Mariotte et al., 2018; Pineda et al., 2010; Wardle et al., 2004). Interaction of the plant with belowground microbes can positively or negatively alter plant resistance to herbivores, a particular topic of study that is of increasing importance, yet still not well studied in Louisiana agriculture.

Arbuscular mycorrhizal (AM) fungi are one of the most important and common types of fungal components in terrestrial ecosystems. They can be found in both natural and agricultural sites forming symbiotic associations with a wide variety of host plants (Smith & Read, 2008). AM fungi have been classified in the phylum Glomeromycota based on DNA sequences (Schüβler et al., 2001). It appears that, through their roles in nutrient uptake, AM fungi were probably important in the colonization of land by plants and remain a major determinant of plant interactions in ecosystems today (Smith & Read, 2008).

AM fungi assist with plant growth and nutrition, which can benefit or harm the host plant. It is already known that AM fungi can establish in different environments; however,

input-intensive agriculture based on tillage, fertilization, or chemical application can degrade crop soils thereby potentially reducing or eliminating indigenous AM species (Barber et al., 2013). It may be possible to reintroduce AM fungi into agricultural fields to support plant growth and improve pest management programs. However, relatively little is known in the United States about the role of AM fungi in rice and the effect of colonization by AM fungi on rice pests. Success in controlling rice pests in Louisiana is variable, perhaps because the role of AM fungi has not been fully considered as part of pest management programs.

1.2 Research objectives and outline of the thesis

The presence of soil organisms that often pass ignored, have demonstrated to play a major role not only structuring aboveground plant-insect interactions but also belowground communities by affecting the survival, growth and development of foliar- and root-feeding insects, respectively (Van der Putten et al., 2001). The main goal of this PhD study is to improve our understanding on the basic mechanisms that mediate the tripartite interactions between AM fungi, rice plants and its pests.

I approached this topic with basic questions about the natural occurrence of AM fungi in rice producing areas, and I applied questions about the response of rice plants inoculated with AM fungi and their combined interactions in resistance or tolerance to rice pests. This was conducted using field, greenhouse, and laboratory experiments that investigated the effects of colonization by AM fungi of rice plants on rice water weevils, fall armyworms, and stem borers, as well as the pathogen sheath blight. To achieve this goal I had four research objectives that comprise chapters 3, 4, 5, and 6.

In Chapter 2, I present the state of the art that focus on the tripartite interactions between rice, AM fungi, insects and/or pathogens. Belowground organisms and aboveground insects can interact influencing each other via plant-mediated mechanisms.

This chapter reviews and summarizes the literature on how a symbiotic fungi influences plant interactions with different pests.

In Chapter 3, the objective was to determine the extent of natural colonization by AM fungi of non-flooded rice plants grown under conditions typical of commercial fields in the southern United States. Rice plant samples were collected from areas across Texas, Mississippi, Arkansas, and two research stations in Louisiana. I quantified the occurrence of AM fungi colonization in insecticide-free rice roots over three consecutive years (2014–2016). The results revealed natural colonization of AM fungi in all rice producing areas. In all the three years of survey, rice-AM fungi associations were the greatest in Arkansas, followed by Mississippi, Texas, and finally Louisiana.

In Chapter 4, the objective was to investigate the influence of AM fungi on rice resistance against pests. I inoculated rice plants with a commercial, granular formulation of AM fungi in several field and greenhouse experiments to test whether the interaction of AM fungi with rice roots changes the resistance of rice against two chewing insects, the rice water weevil and the fall armyworm, and one pathogenic microorganism, sheath blight. Both in field and greenhouse experiments, the performance of insects and the pathogen on rice was enhanced when plants were inoculated with AM fungi. In the field, inoculating rice plants with AM fungi resulted in higher numbers of RWW larvae on rice roots. In the greenhouse, more RWW first instars emerged from AM fungi-colonized rice plants than from non-colonized control plants. Weight gains of FAW larvae were higher on rice plants treated with AM fungi inoculum. Lesion lengths and susceptibility to ShB infection were higher in rice plants colonized by AM fungi. Although AM fungi inoculation enhanced the growth of rice plants, nutritional analyses of root and shoot tissues indicated no major increases in the concentrations of nutrients in rice plants colonized by AM fungi. The large effects on rice susceptibility to pests in the absence of large effects on plant nutrition suggest that AM fungi colonization influences other mechanisms of susceptibility (e.g., defense signaling processes). Given the widespread occurrence of AM fungi, our

findings provide a different perspective on the causal bases of rice resistance/susceptibility to insects and pathogens.

In Chapter 5, the objective was to investigate whether commercial inoculation with AM fungi can successfully establish in different soil types and enhance plant growth and resistance to rice pests. In rice, more attention has been given to investigations of the direct effects of AM fungi on root colonization, plant growth, and crop production. Here, I conducted a broad study to investigate the effects of AM fungi inoculation on rice plants with two different unsterilized field soils under field and greenhouse conditions in two consecutive seasons in the United States. I tested whether inoculation with AM fungi boosted plant biomass, nutrient uptake, resistance to pests, and yields. Our results showed that commercial inoculation increased root colonization by AM fungi in all soils, regardless of soil phosphorus (P) availability. Inoculation with AM fungi increased susceptibility to two insect pests, rice water weevil and fall armyworm, but this effect was soil dependent. Inoculation with AM fungi had no effect on either nitrogen (N), phosphorus concentrations, or rice yields. The enhanced effect on plant biomass was also soil dependent. Our study provides evidence that commercial inoculation by AM fungi results in successful colonization of the roots of rice plants, but effects on the rice susceptibility to pests and plant biomass appear to be soil dependent. Moreover, I provide further evidence that of AM fungi-inoculated rice, nutrient status, based on N and P concentrations, is not the reason for the increased susceptibility. I highlight the importance of considering soil feedbacks in sustainable agriculture and the role of AM fungi species.

Subsequently, in Chapter 6 I investigated the effects of AM fungi on rice yields and tolerance to rice water weevil injury. In particular, I hypothesized that rice growth would be greater and yield losses from RWWs would be smaller in the presence of AM fungi than in the absence of. I also hypothesized that the inoculation with AM fungi would increase plant biomass and yields in rice. I used a 2x2 factorial experimental design, using two levels of insecticide (root injury) and two levels of AM fungi symbiosis (AM fungi-

inoculated or not inoculated) with 10 replications each in field experiments over three years. The insecticide used, NipsIt INSIDE, is a neonicotinoid seed treatment. Results showed significant effects on plant density depending on the interaction between AM fungi treatments and insecticide treatments. In all experiments, mycorrhizal seed treatments showed the highest AM fungi colonization. As in previous experiments, mycorrhizal treatments increased population densities of RWW relative to untreated controls and insecticidal seed treatment significantly reduced weevil densities. AM fungi increased rice biomass and a clear significant increase in yield was observed. AM fungi may mediate plant interaction by influencing plant biomass, and rice inoculated with AM fungi may provide an effective method for increasing rice yields.

The ultimate goal of this dissertation is to provide a more comprehensive understanding of the use of AM fungi into rice pest management programs. General conclusions of the results are presented in Chapter 7. Here, I also suggest directions for future research to continue in the topical theme of understanding interactions between AM fungi, rice and their favorite pests.

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Chapter 2
Literature Review

2.1 Rice

Rice is a member of the *Oryza sativa* Poaceae family. The life cycle of rice begins with the germination of seed and ends with the formation of grain. During this period, the rice plant exhibits a series of continuous changes in its growth and development, which can be divided into two phases: vegetative and reproductive (Blanche et al., 2009). The vegetative phase starts from seed germination (emergence), and progresses through seedling development, tillering, and internode elongation. The reproductive stage includes prebooting, heading, grain filling, and maturity (Blanche et al., 2009). Rice is a diploid plant with 24 (n = 12) chromosomes and was the first sequenced crop genome with a small genome size of ~430 Mbp (Goff et al., 2002).

All plants are hosts for, and interact with, below- and above-ground organisms. In the past two decades, interactions of plant roots with below-ground soil organisms has received increased attention because of their implications in plant fitness.

2.2 Biology of target insects of rice in the southern United States

Insect pests are a major threat worldwide rice production. The rice water weevil, *Lissorhoptrus oryzophilus,* and the rice stink bug, *Oebalus pugnax*, are the two major insect pests of rice in Louisiana. In addition, a group of sporadic pests such as fall armyworm, *Spodoptera frugiperda*, a complex of rice stem borers, and the South American rice miner can be serious pests of rice under heavy infestation levels (Blanche et al., 2009).

2.2.1 Rice water weevil

The rice water weevil (RWW), *Lissorhoptrus oryzophilus* Kuschel (Coleoptera: Curculionidae) is the most important early season insect pest, not only in Louisiana but also in other southern rice producing states (Blanche et al., 2009). Native to North America (Saito et al., 2005), this insect has been associated with rice since the crop was introduced into the United States (Bowling, 1957). The life cycle begins in the presence of standing water, typically peak oviposition occurs one to two weeks after a permanent flood has been established in a rice field (Shang et al., 2004). The sheaths of young rice plant leaves are preferred for oviposition of the eggs of RWW. Upon hatching, larvae move to rots and begin feeding upon the underwater root system. Dense infestations can prune rice roots considerably, effecting a reduction of tillers, above-ground biomass, and yield. Larvae range in size from initially 1/32-inch long to almost a quarter inch by their fourth and final larval instar. Pupation occurs in the roots of rice plants and pupae appear as small, brown balls.

The RWW require approximately one month, depending on temperature, to pass through all instar and pupal stages to reach adulthood. Thus, one or two generations of RWW may be supported in a single growing season of southern Louisiana (Shang et al., 2004), where warm temperatures favor rapid RWW development. The 1/8-inch long adults feed on leaves, producing longitudinal scars; however, this type of injury is not considered economically important and so contrasts greatly with the damage that root-feeding larvae can inflict. Also, since RWW prefer to feed on younger rice plants, they tend to more frequently infest new fields rather than stay in one place where plants have already had a chance to mature. Furthermore, they overwinter in grasses, debris, and wooded areas neighboring rice fields.

2.2.2 Fall armyworm

The fall armyworm (FAW), *Spodoptera frugiperda* J. E. Smith (Lepidoptera: Noctuidae), does not specifically target rice or maize, but is still a severe threat to rice. It is also polyphagous and tends to feed on younger cereal crops as well as grasses found in and around rice fields; defoliation can ravage seedlings if infestations are particularly advanced. Female FAW may oviposit groups of 50 or more eggs on leaves of rice and other host plants. Since temperature influences development rate for FAW, larvae may eclose from eggs within two to ten days. They usually transition through six larval instars and grow to nearly one inch, but do so in three weeks or less. Larvae can vary in color, from light tan to green to nearly black, with stripes running the length of the body. Mature larvae can be distinguished from other members of the family by the presence of an inverted "Y" on the front of the head capsule (Blanche et al., 2009). The chance of successfully pupating and emerging as an adult decreases in flooded rice fields, because the mature larvae will attempt to pupate in soil inside of a cocoon that is not watertight (Blanche et al., 2009). Adults measuring about one inch in length emerge after ten to 15 days. The relatively shorter lifecycle usually allows the FAW to produce four generations per growing season in southern Louisiana.

2.2.3 Stem Borers

The rice stalk borer, *Chilo plejedellus* Zink (Crambidae), the Mexican rice borer, *Eoreuma loftini* Dyar (Crambidae), and the sugarcane borer, *Diatraea saccharalis* F. (Crambidae) share both a similar life cycle and a propensity for attacking rice crops. Stem borers overwinter as larvae or pupae, usually inside a rice stalk, sugar cane, in stubble, or other appropriately structured crops and weeds. Pupation requires seven to ten days in the spring. The adult moths measure ¾-inch to 1-inch in length and visit various host plants while mating.

Sugarcane borer eggs can be found on either the top or bottom of rice leaves and in groups of 100 or as few as ten or less. It will take three to five days before the larvae can emerge, crawl down the leaf, and begin boring into the host plant's stem (Blanche et al., 2009). Once feeding concludes between 15 and 20 days after entering the stem, the larvae will chew an exit hole and in the stem wall and begin pupating. The slightly larger rice stalk borer, measuring 1 inch in length, feeds slightly longer at 24 to 30 days (Blanche et al., 2009). Unlike the sugarcane borer, the rice stalk borer constructs a silken web in which to pupate (Blanche et al., 2009). This stage will also last slightly longer with the insect needing seven to ten days before emerging. Mexican rice borers pose a serious threat as they can overwinter in almost any grass large enough to afford the size of the larvae (Beuzelin et al., 2016). On rice plants, they feed within leaf sheaths for about a week before boring into the culm. Exit holes, covered by a layer or two of plant material, are created before pupation as well (Blanche et al., 2009). Because these three pests bore into the stalk of the host plant, foliar insecticides are ineffective during most of the larval stage.

Symptoms of attack from the borers are commonly referred to as whiteheads and deadhearts. Both conditions arise from the hollowing of the stem, which can no longer properly transport resources to some parts of the rice plant. Younger leaves withering and dying off in the host plant's vegetative stage is a distinction of deadheart. Whitehead occurs when borers attack the rice stems supporting panicles, resulting in white, lightweight, upright panicles containing no grains.

2.2.4 Sheath Blight

The fungus *Rhizoctonia solani* (Basidiomycota) causes sheath blight (ShB), one of the most damaging diseases of rice in Louisiana. Warm temperatures and high humidity allow this pathogen to thrive in densely planted rice plants. To establish itself, *R. solani* forms either hyphae (thread-like structures) in plant debris or sclerotia, masses of mycelium

wrapped in a hydrophobic secretion, on the stem of a host plant. Either form can ride the surface of irrigation waters to propagate to other plants. First signs of infection by sheath blight are noted by the appearance of oval-shaped discolorations on leaf sheaths. When the rice plant begins tillering, 0.5 cm^2 to 3 cm^2 sized lesions begin forming just above the waterline of the rice culms. Mycelia grow up the host plant's sheath, spreading infection and forming new lesions. Once the rice plant passes out of its vegetative stage and panicles emerge, the infection can sometimes spread rapidly to the flag leaf. The life cycle continues without the release of spores, but by infecting other tillers, spreading to other plants by physical contact, or by floating inoculum at the water line. Host plants infected with sheath blight disease have weakened culms and may therefore lodge or collapse (Blanche et al., 2009).

2.3 The rhizosphere

The narrow zone of soil that surrounds and is influenced by plant roots is called rhizosphere. This interface is home to an overwhelming number of microorganisms and invertebrates and is considered to be one of the most dynamic zones on earth (Philippot et al., 2013). These organisms include nematodes, fungi, bacteria, and arthropod herbivores, which alone or in combination, may interact with the host plant (van Dam & Bouwmeester, 2016) (Figure 2.1). Soil microbial community influences important ecosystem services such as plant productivity, carbon storage, nutrient cycling, and water pollution among others (Köhl et al., 2014). Therefore, soil microbes directly and/or indirectly can have important consequences on food security.

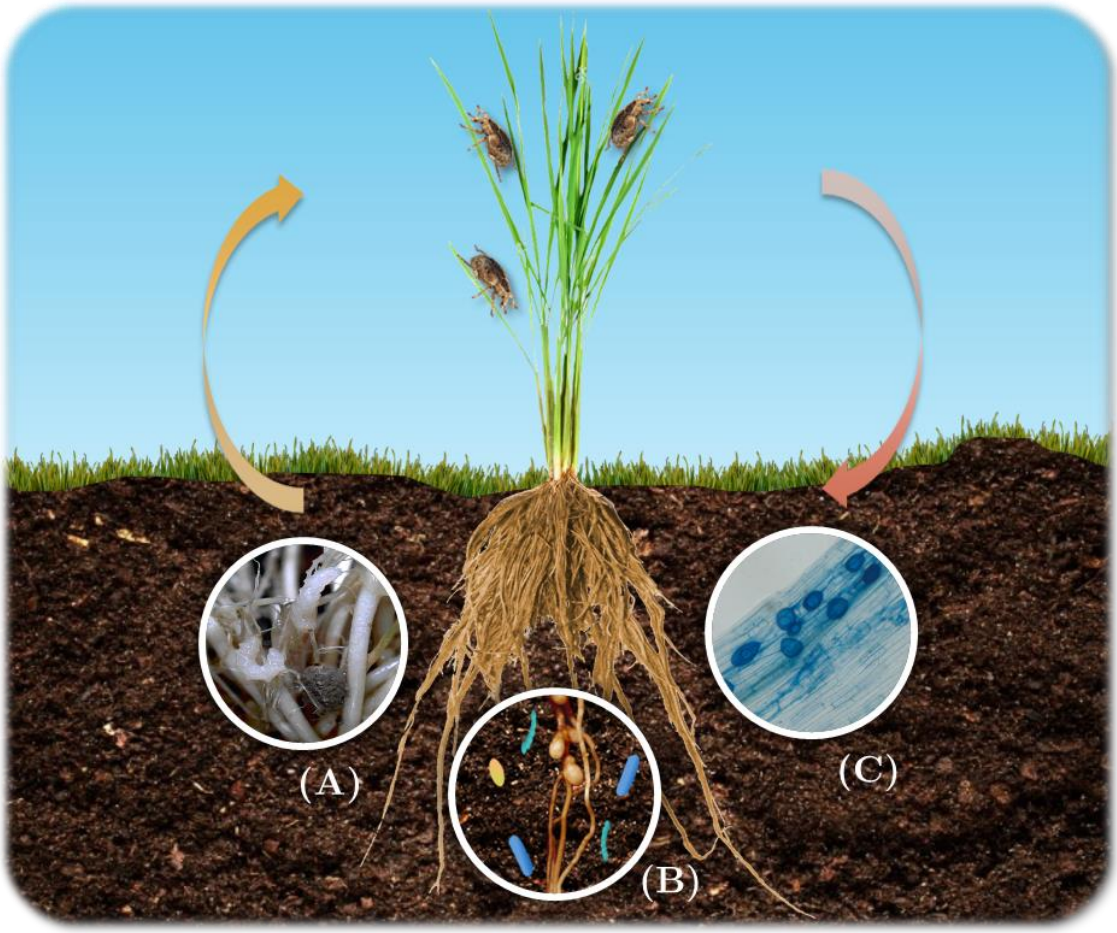

Figure 2.1. The rhizosphere showing the different organisms surrounding the root of the rice plant. (A) herbivores, (B) pathogens, and (C) symbionts.

2.4 Origins of mycorrhizal symbiosis

The symbiosis formed between terrestrial plants and mycorrhizal fungi is as old as land plants themselves (Humphreys et al., 2010). A mycorrhiza represent the most ancestral and unique type of mycorrhizal interaction between two eukaryotes: the obligate biotrophic soil-inhabiting fungus (called mycorrhizal fungi) and roots of its host plant, leading to an improvement of the fitness of the interacting partners (Smith & Read, 2008). The oldest fossils of arbuscules date to the Devonian protracheophyte *Aglaophyton major* (400 million year ago) and evidence of AM fungal spores and hyphae exists from the Ordovician (460 million year old). These indications suggested that association with AM

fungi was necessary for plants to colonize dry lands and most ecosystems by higher plants (Pirozynski & Malloch, 1975), a hypothesis supported by paleobotanical data (Berbee & Taylor, 2007; Brundrett, 2002), and phylogenetic analyses based on DNA sequences (James et al., 2006).

Various types of mycorrhizal associations have been described, based on the place where the fungus has been found in the root surface: ectomycorrhiza (with only intercellular colonization) and endomycorrhiza or arbuscular mycorrhizal (AM) (with both intracellular and intercellular colonization) (Smith & Read, 2008). In this dissertation, I will focus only in AM fungi.

2.4.1 AM fungi

AM fungi are obligate biotrophs, which have never been grown axenically (Hart et al., 2001). The name 'arbuscular' is derived from structures characteristic of AM fungus, the arbuscules (Figure 2.2). These are highly branched hyphal structures that develop within the cortical cells of many plant roots colonized by AM fungi and are responsible of the release of nutrients to the plants (Smith & Read, 2008). The 'vesicles' are the storage structures located within or between the cells. These structures have been considered an important diagnostic for identifying colonization by AM fungi (Figure 2.2). The fungi also form extensive hyphal networks in the soil, which can extend farther than plant roots into the rhizosphere and provide more access to nutrients and water (Jansa et al., 2008). AM fungi form symbiotic associations with more than 95% of plant species (Smith & Read, 2008). This mutualistic relationship allows AM fungi to exchange carbohydrates in the form of sugars and lipids (Luginbuehl et al., 2017) necessary for completing their life cycle, and in return, plants get the nutrients in the form of nitrogen (N) and phosphorus (P) important for the proper plant growth (Smith & Read, 2008).

The cycle of AM fungi starts with germination of the spores and initiation of extraradical hyphae. Once a host has been recognized, hyphae can specialize as appressoria, which flatten against and enter the host with turgor pressure capable of penetrating cortical root cells (Parniske, 2008). At this point, AM fungi begin to form their namesake arbuscules within the host plant for nutrient exchange, with special emphasis on phosphorus, carbon, nitrogen, and other micronutrients (Parniske, 2008).

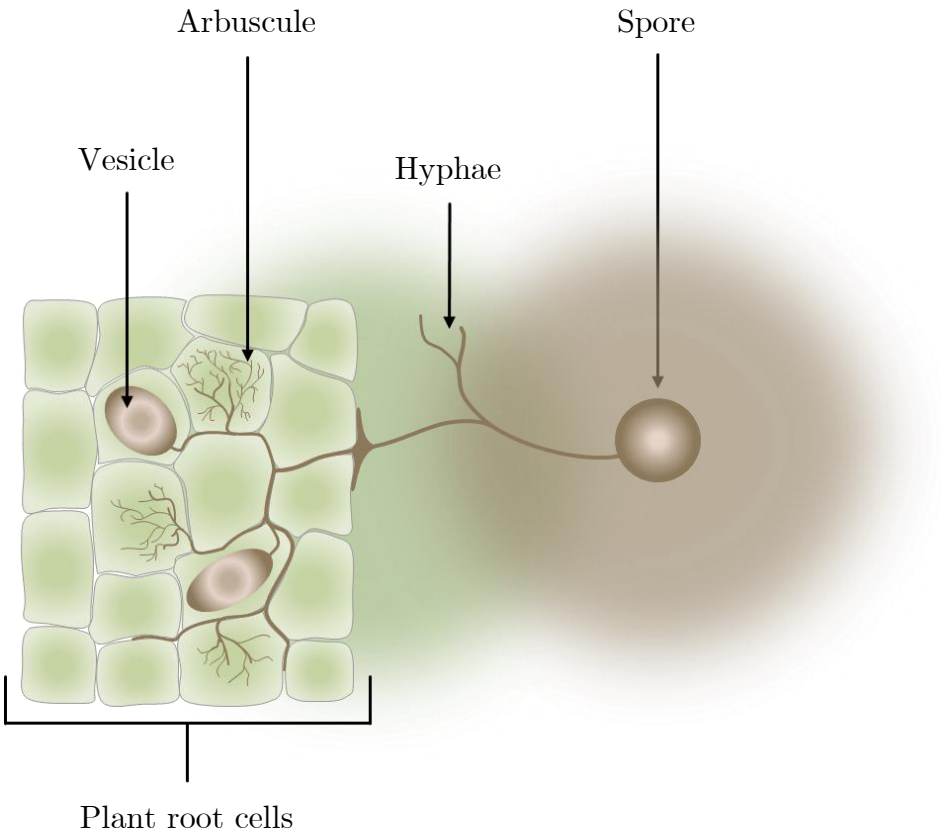

Figure 2.2. Diagrammatic representation of AM fungal structures within the root cell.

2.4.2 Key roles of AM fungi in ecosystems

AM fungi are ubiquitous soil organisms that exist in almost all types of soil ecosystems (Jansa et al., 2009). AM fungi can influence interactions with plants in more ways than their role in plant nutrition. For instance, the presence of AM fungi can increase the

movement of water through plants and provide protection under periods of drought stress (Ruiz-Sanchez et al., 2010). AM fungi can also improve growth, nutrient uptake and tolerance of plants exposed to salt stress (Chen et al., 2017; Wang et al., 2018). Under pathogen infection, colonization of roots by AM fungi can result in suppression of fungal and nematode plant pathogens (Borowicz, 2001; Veresoglou & Rillig, 2012; Wehner et al., 2010). AM fungi can alter the growth responses of plants to insect herbivores (Bennett & Bever, 2007; Cosme et al., 2011; Gange & West, 1994).

Furthermore, AM fungi can play important roles on the productivity and diversity of plant communities (van der Heijden et al., 1998). The transfer of fixed carbon from plants to AM fungi can result in a substantial sink of carbon to the soil (Olsson & Johnson, 2005), and influencing nutrient cycling. Also, the presence of AM fungi hyphal networks in soils influences the microbial communities for soil structure through biochemical and biological processes (Rillig & Mummey, 2006).

2.4.3 Effects of AM fungi on plant resistance to pests

Plant-mediated interactions between above- and below-ground organisms include more participants than just herbivores. AM fungi also interact with plants and herbivores via multiple mechanisms (Bennett et al., 2006; Gehring & Bennett, 2009). They can positively influence above-ground insect herbivores by improving plant vigor and foliar nutrient concentrations (Borowicz, 1997), but they also negatively influence above-ground herbivores with changes in constitutive and inducible defenses against herbivory (Bennett et al., 2006). On one hand, Barber et al. (2013) demonstrated that different farming practices influenced root colonization of AM fungi in cucumber plants. Also, these farming practices, such as organic versus conventional fertilization, differed significantly in their typical mineral content, concluding that these nutrients and AM fungi may have altered plant traits in ways that could have altered the response to insect herbivores. On the

other hand, AM fungi root colonization can significantly increase the production of plant signaling hormones, such as jasmonic acid, that can reduce performance and growth of some above-ground herbivores (Jung et al., 2012). Other studies have suggested that root colonization by AM fungi can also enhance the production of volatile organic compounds that are attractive to aphids (Babikova et al., 2014). Furthermore, the association with AM fungi can indirectly influence above-ground herbivores by mediating plant attraction of natural enemies of herbivores (Gehring & Bennett, 2009; Hoffmann et al., 2011).

While studies on the effects of AM fungi on plant-insect interactions have increased substantially, the responses of these tripartite interactions are complex and will vary depending of the host plant, AM fungi, and herbivore involve. For instance, Koricheva et al. (2009) reported that colonization by AM fungi had a negative effect on the performance of generalist chewing insects, and a positive effect on the performance of generalist sucking insects and specialists (Koricheva et al., 2009). The negative effect on the performance of chewing insects is thought to be due to the priming of plants by AM fungi for jasmonic acid (JA)-related defense compounds (Pozo & Azcon-Aguilar, 2007), whereas the positive effect on sap-sucking insects is deemed to result from the suppression of AM fungi for salicylic-acid (SA)-related defenses due to the negative crosstalk between JA and SA signaling pathways (Jung et al., 2012; Pozo & Azcon-Aguilar, 2007).

2.5 AM fungi and rice

During the last two decades, different aspects of the mutualistic symbiosis between AM fungi and rice plants have been studied extensively in other parts of the world under different agricultural conditions (Campos-Soriano et al., 2010; Sawers et al., 2008). However, in the southern United States very little or almost no attention has been paid on the study of AM fungi in rice producing areas.

In recent years, the application of commercial inoculum of AM fungi has increased its significance in the field of agriculture. Application of AM fungi inoculum has demonstrated to increase soil nutrients and root colonization in rice plants (Bhattacharjee & Sharma, 2011; Lumini et al., 2011; Vallino et al., 2009). Other studies have shown that AM fungi induced significant changes in plant host architecture (Gutjahr et al., 2009), and harvest index in rice under lab conditions (Li et al., 2012). However, AM fungi colonization and plant responsiveness depend on plant and fungus combinations as well as environmental conditions (Davison et al., 2015; Rodríguez-Echeverría et al., 2017; Rúa et al., 2016).

2.6 The tripartite interaction between rice, AM fungi, and pests

Very little is known about the tripartite interactions between AM fungi, rice, and pests. On the one hand, Campos-Soriano et al. (2011) reported that root colonization by the AM fungus is accompanied by the systemic induction of genes that play a regulatory role in the host defense response in rice leaves of mycorrhizal plants in the absence of pathogen infection, which confer enhanced resistance to infection by the rice blast fungus. On the other hand, Cosme et al. (2011) showed that root colonization of rice plants by AM fungi enhanced aboveground oviposition of the rice water weevil and increased nitrogen (N) and phosphorus (P) concentrations in plant tissues. They suggested that rice water weevil females are able to discriminate plants for oviposition based on AM fungi-mediated changes in plant quality. Even though plant resistance due to AM fungi association is well known in some plant species (Babikova et al., 2013; Fritz et al., 2006; Jung et al., 2012; Pozo & Azcon-Aguilar, 2007), the findings of Cosme et al. (2011) in rice, necessitate further investigation given the variable response of AM fungi to rice pests. For instance, single or commercial formulations of fungi show different benefits to the same plant under the same environmental conditions; also, the same commercial

formulations show differential benefits to the same plant under different environmental conditions.

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Chapter 3
Natural Colonization of Rice by Arbuscular Mycorrhizal Fungi in Different Production Areas*

3.1 Introduction

Arbuscular Mycorrhizal Fungi (AMF, Phylum Glomeromycota) are important components of the soil microbial communities. AMF form mutualistic associations with roots of most terrestrial plants, including many agricultural crops. In many agricultural plants, these mutualistic associations have shown the potential to increase crop productivity, thereby playing a key role in the functioning and sustainability of agroecosystems (Gianinazzi et al., 2010). The most important function of these symbiotic associations involves the transfer of nutrients such as organic carbon (C), in the form of sugars and lipids (Jiang et al., 2017; Luginbuehl et al., 2017), to the fungi by the plants and the transfer of phosphorus (P) and nitrogen (N) to the plants by the fungi (Smith & Read, 2008). AMF-mediated improvement in mineral uptake may lead to increased growth and development of plants, and may confer resistance to abiotic and biotic stresses (Gianinazzi et al., 2010; Liu et al., 2007; Smith & Read, 2008). In addition to these benefits to plants, AMF may improve soil structure, ameliorate drought and salinity stress, and affect the diversity of plant communities (Rillig & Mummey, 2006; Smith et al., 2010; van der Heijden, 2010; van der Heijden et al., 1998). The benefits provided by AMF may be critical to increasing agricultural yields and productivity in a low-input manner.

AMF share a long history of coevolution with plants in various ecosystems, resulting in their adaptation to specific areas (Gosling et al., 2006). The majority of research on AMF associations involve laboratory or greenhouse experiments, in which plants are

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cultivated in sterilized soil, with particular AMF species. They ignore indigenous AMF species that could alter plant responses or compete with the AMF inoculant (Munkvold et al., 2004). In addition, these studies ignore the complexity of soil biological communities that could influence the establishment of the AMF symbiosis and its impact on plant fitness (Lekberg & Koide, 2005).

During the last two decades, different aspects of the association of crop plants with AMF have been studied extensively in different geographical regions and under different agricultural conditions (Gianinazzi et al., 2010; Srivastava et al., 1996). Those studies have shown variable effects of AMF on crop plants, ranging from mutualistic to parasitic. The effects of AMF can depend on soil moisture, the inorganic nutrients available in the soil, pH, species of AMF, and host plant species. Along with these factors, a number of agricultural management practices affect the soil environment, and therefore, mycorrhizal abundance and activity.

Rice (*Oryza sativa* L.) is one of the world's most important cereal crops. In the United States of America, it is cultivated in two distinct regions, California and several southern states, including Arkansas, Louisiana, Mississippi, Missouri and Texas. In the southern region, the majority of rice acreage is grown under a delayed-flood cultural system in which rice is drill-seeded and surface-irrigated as necessary to establish a stand (Hamm et al., 2010). Timing of the permanent flood in this system varies, but flooding is generally delayed until rice begins to tiller, four to five weeks after planting. The period from seeding to flooding favors the colonization of root systems by AMF (Dhillion, 1992; Secilia & Bagyaraj, 1994).

Colonization by indigenous or native AMF species in cereal crops in general and rice in particular has been reported earlier (Campos-Soriano et al., 2010; Cosme et al., 2011; Maiti et al., 1995; Sawers et al., 2008). Despite this, in USA, almost no attention has been paid to AMF associations in rice. In another study, we showed that the performance of insects and a pathogen on rice was enhanced when plants are colonized by AMF, and

AMF colonization can be manipulated by inoculating plots with a commercial AMF product (unpublished data). It will be necessary to evaluate the natural association of AMF with rice plants, particularly in regions where rice is produced, to facilitate agricultural exploitation of the symbiosis.

Given the paucity of information on the natural association of AMF with rice in different production areas of the United States, our goal was to survey rice fields from several locations in the southern United States to determine the extent of AMF colonization associated with commercial varieties before flooding. We tested the hypotheses that AMF establish natural association with rice roots, and that the AMF colonization would differ among locations. Unlike previous studies of natural AMF colonization in rice (Dhillion, 1992; Dhillion & Ampornpan, 1992), this study was carried out in most of the rice-producing areas of the southern United States and demonstrated natural colonization of AMF in rice fields, may have practical implications for increasing rice production and sustainability.

3.2 Materials and methods

3.2.1 Sampling sites

Sampling to determine the extent of natural AMF colonization was conducted over three production seasons from 2014 to 2016. Four (2014 and 2015) or five (2016) collection sites were included in each year to represent a range of production environments in the southern United States (Table 3.1). The climate in the rice-producing regions of the southern United States belongs to the humid subtropical type, with average annual rainfall of 1000 to 1600 mm. In these areas, the summers are warm and humid, and the daily maximum temperatures usually range from 32^{o}C to 37^{o}C during the growing season. Average temperatures in late spring are about 21^{o}C, while 28^{o}C in summer and about 25^{o}C in early fall (US Climate Data, 2018).

Table 3.1. Arbuscular mycorrhizal fungi (AMF) colonization percentage (presence of hyphae, arbuscular and vesicles) in fields during 2014–2016.

Rice field	County, State	Coordinate	Soil type	Variety	AMF colonization percentage (%)		
					2014	2015	2016
H. Rouse Caffey Rice Research Station, Crowley (CR)	Acadia, Louisiana	30°14'23.4"N 92°20'46.1"W	Silt loam	Cocodrie	3.8 ± 0.4	19.0 ± 2.1	59.3 ± 4.1
				Jupiter	3.8 ± 0.9	N.S	N.S
				Lemont	1.8 ± 0.4	N.S	N.S
				Mermentau	N.S	N.S	22.0 ± 4.1
				Cheniere	N.S	N.S	58.0 ± 2.2
Macon Ridge Research Station, Winnsboro (WB)	Franklin, Louisiana	32°08'33.0"N 91°42'23.6"W	Sharkey clay	CL151	N.S	N.S	16.0 ± 0.6
Delta Research & Extension Center, Stoneville (SV)	Washington, Mississippi	33°25'24.1"N 90°54'39.1"W	Sharkey clay	Cocodrie	16.7 ± 2.6	N.S	N.S
				Wells	N.S	29.8 ± 2.8	27 ± 0.8
Texas A&M AgriLife Research & Extension Center, Beaumont (BM)	Jefferson, Texas	30°04'19.8"N 94°17'58.1"W	League clay	Antonio	5.8 ± 0.8	25.8 ± 1.3	18.0 ± 1.8
Rice Research & Extension Center, Stuttgart (ST)	Stuttgart, Arkansas	34°28'31.9"N 91°25'05.6"W	Dewitt silt loam	Wells	11.8 ± 1.1	61.4 ± 6.3	61.2 ± 4.6

NS, Not sampled. Values are Mean ± SE (n = 7 to 10).

Environmental conditions and cultural practices varied from year to year and site to site, but in all cases were typical of rice fields in the southern USA. In all these environments, rice was grown as a single crop per year, drill-seeded and irrigated. Plot sizes at all sites were at least 1.5 m x 6 m. At the Winnsboro (WB) site, rice was grown in experimental plots in fields that had been under a continuous rice cultivation system for several years; for the Crowley (CR) and Beaumont (BM) sites, rice was grown in fields that had been in a rice-fallow rotation for the past 50 years; for the Stuttgart (ST) and Stoneville (SV) sites, rice was grown in rotation with soybeans. Planting dates were within the range of normal planting dates for each site, ranging from March or April at the CR and BM sites to May at the WB, ST and SV sites. Fertilization practices at each site were performed based on soil test results (Blanche et al., 2009). Only in CR and WB sites, all nitrogen was applied pre-flood, whereas split applications were employed at the other sites. As is typical for a survey spanning a large region, each site has a different soil type (Table 3.1). Soil types were silt loam at CR site, League at BM site, DeWitt silt loam at ST site, and sharkey clay at WB and SV sites. The rice cultivars collected from each site location over three years of survey were: Cocodrie, Jupiter, Lemont, Mermentau and Cheniere at CR; CL151 at WB; Antonio at BM; Cocodrie and Wells at SV; and Wells at ST.

3.2.2 Collection of samples

Rice samples, consisting of leaves, roots, and soil, were collected from each site four to five weeks after seeding but before application of permanent flood. Seven to ten samples of rice plants at the early tillering stage were collected by pulling out plants from soil by hand or using a metal core sampler measuring 9.2 cm (diameter) x 7.6 cm (depth) and attached to a metal handle. Each sample contained three to four whole plants. The roots of plants were washed under pressure over a sieve to remove soil. The roots of each sample

were immediately wrapped in moist paper towels, and entire plant was loosely wrapped in newspaper for shipping. Each sample was placed in plastic bags and shipped overnight to Louisiana State University, Baton Rouge. Samples were processed in the lab as described below.

3.2.3 Quantification of mycorrhizal colonization

The percentages of roots exhibiting signs of AMF colonization at each site were determined using the trypan blue staining method of Koske and Gemma (1989) with minor modifications. Color and texture were used to distinguish live roots from dead roots (dead roots were darker than live ones, and the great majority of roots survived the sampling and shipping process with little damage). Roots from each collected sample were cut into ca. 2-cm-long segments and placed in tissue processing cassettes (Ted Pella, Redding, CA). Approximately 200 of these small root pieces per sample were cleared in 10% KOH at 90°C for 30 minutes in a water bath. Cleared pieces of roots were rinsed five times with tap water to remove KOH, and roots were immersed in 2% HCl at room temperature for 15-20 min to ensure the roots were adequately acidified for staining. Cassettes containing roots were immediately stained with 0.05% trypan blue (Sigma-Aldrich, St. Louis, USA) by incubation overnight and then transferred to vials containing lactoglycerol at 4°C to allow excess stain to leach out of the roots. Stained root samples were stored in lactoglycerol solution for 48 hours before being mounted in the same solution on a microscopic slide.

Mycorrhizal colonization by AMF structures was determined by assessing five slides with ten segments per slide from each sample and scoring the amount of colonization using the magnified intersections method of McGonigle et al. (1990) with minor modifications. A total of 50 stained root segments per sample were examined with a compound microscope (Olympus CH2, Tokyo, Japan) at 40X to 100X magnification for confirmation

of mycorrhizal colonization of rice plants. Root pieces showing presence of blue-stained mycorrhizal structures including arbuscules, hyphae, or vesicles were scored as positive for AMF. All microscopic examinations were carried out by the same individual. Photos of mycorrhizal structures on colonized roots were taken using a microscope-mounted 5.0 megapixel digital camera (Leica DFC480, Cambridge, UK). Root colonization percentage was averaged for the seven to ten samples at each site and calculated by the following formula:

$$Root\ colonization = \left(\frac{Number\ of\ segments\ colonized\ with\ AMF}{Total\ number\ of\ segments\ observed}\right) x\ 100\%$$

3.3 Results

All the rice samples collected from multiple locations over multiple years were colonized by AMF, with root colonization percentage ranging from 1.8 to 61.4% (Table 3.1). AMF structures typical of plant-AMF symbioses such as hyphae, vesicles, and arbuscules were present in all screened rice roots at each location (Figure 3.1). The most common structures were hyphae, which appeared in all samples. Few arbuscules were observed in our survey because these structures tend to degrade quickly (Parniske, 2008); vesicles were found in greater number.

SV and ST sites had the highest colonization percentage in all years (Table 3.1). AMF colonization in 'Cocodrie' increased substantially in CR from 2014 to 2016. Texas, Mississippi, and Arkansas samples showed consistent AMF colonization, with slight fluctuations between years. In 2014, the highest mycorrhizal colonization was recorded in SV (16.7%) followed by ST (11.8%), and the lowest colonization was found in CR in 'Lemont' cultivar (1.8%) (Table 3.1). In 2015, the highest colonization percentage was found in ST (61.4%) and the lowest colonization was found again in CR (19%). In 2016, CR and ST had the highest colonization percentages (59.3 and 61.3%, respectively)

followed by 'Cheniere' (58.0%) in CR, and the lowest colonization was in WB (16.0%) (Table 3.1).

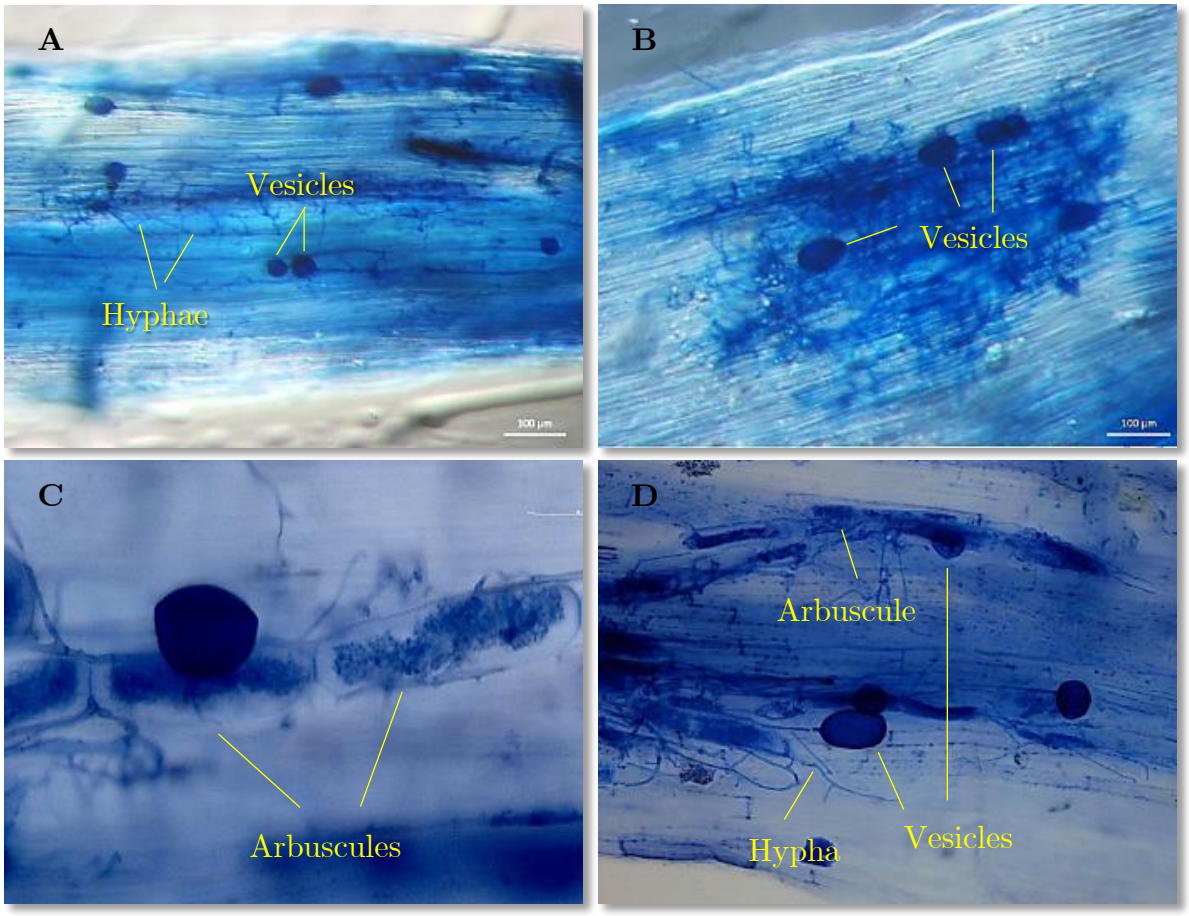

Figure 3.1. Examples of arbuscular mycorrhizal fungal structures used as indicators of rice root colonization collected from Mississippi (A), Arkansas (B), Texas (C), and Louisiana (D, Crowley) rice fields. The roots were stained with trypan blue. Bars correspond to 50 (C) and 100 (A, B and D) µm.

3.4 Discussion

The presence and importance of AMF in rice production systems have received some recent attention (Vallino et al., 2014; Wang et al., 2015; Zhang et al., 2015), but no study has addressed the questions of whether AMF naturally colonize rice in commercial farming systems and to what extent natural colonization by AMF differs among conventional rice farming regions in the southern United States. The present survey was carried out over three years at five sites representative of rice production areas in the southern United States. Natural AMF colonization was found in all sampling sites, confirming our hypothesis that natural AMF colonization is widespread in unflooded rice across the southern USA.

Our results are in agreement with Watanarojanaporn et al. (2013) and Wang et al. (2015), who showed that AMF are commonly present in rice roots from conventional rice fields (paddy wetlands) in Thailand and China at early growth stages and before flooding is established. However, our results differ from those of Lumini et al. (2011) and Vallino et al. (2009), who showed that AMF colonization was absent or lower, respectively, in rice roots grown under a conventional cultivation system in Italy. Rice roots in southern agricultural fields exhibited higher levels of colonization by native AMF than other crops in a different environment such as winter wheat in southern Switzerland (Mäder et al., 2000). Because environmental conditions and cultural practices were not manipulated over years and at the different locations in our survey, the factors responsible for variation in levels of AMF colonization in our study cannot be unequivocally determined. However, likely contributors to this variation include rice variety, AMF species, and soil fertility.

In this study, the extent of AMF colonization was relatively stable over the three years at each location. Some sites showed consistently higher AMF colonization than the others. For example, colonization at the ST site was consistently higher than those at the others. The exception to this seemed to be the CR site, where colonization increased over the years. Differences in rice varieties could be among the factors that contributed to the

differences among sites. Alternatively, differences among sites may have been due to differences in abundance and geographic distribution of AMF species, which in turn may have resulted from differences among sites in soil fertility, soil type, environmental conditions such as temperature and precipitation (soil moisture), past use of pesticides (fungicides), and crop rotational history. Environmental factors and agricultural management practices in rice fields such as fertilizer input and water management have been shown to influence both symbiosis and diversity of AMF communities (Barber et al., 2013; Gosling et al., 2006; Lumini et al., 2011).

One environmental factor in particular that is known to have a negative impact on AMF symbiosis is P availability. At lower soil P concentrations, when plants are P-limited, they tend to allocate a higher fraction of available carbon to AMF, thus stimulating AMF colonization (Johnson, 2010). At higher soil P concentrations, less carbon is allocated to AMF from the host plant and AMF can become carbon-limited. As a result, low colonization is expected at high P concentrations (Treseder & Allen, 2002). We hypothesize that the high rates of AMF colonization at the ST and CR field sites were due to the low-medium levels of P in the soil. In contrast, low rates of root colonization in some rice fields of our study may be due to higher levels of P in the soil due to addition of P fertilizer. However, we did not have soil nutrition analyses from the different field sites. Therefore, we recommend more studies to develop a better understanding of the relationship between soil fertility and AMF colonization.

At present, there are only few studies that provide information of the effects of AMF colonization on rice growth and physiology, and there is still no clear picture of the potential direct benefit of this association on crop yield in rice. Van Der Heijden et al. (2006) showed that growth responses of plants to different species of AMF were temporally variable and plant-species dependent, where *Lotus* and *Trifolium* performed better with one AMF species in the first growing season, but grow best with a mixture of several AMF in the second season. Future work will be needed to understand the composition of the

microbial communities on rice roots by identifying colonizing AMF species present in rice fields of the southern United States. Currently, we are assessing the benefits of AMF colonization on rice growth and yields in a field trial. Preliminary data reveal that colonization by AMF influences plant performance by increasing shoot biomass. Yields of field grown rice were up to 13% higher in rice plants inoculated with AMF than non-inoculated plants (unpublished data). This information will give a better idea of the beneficial effects of AMF in rice-producing areas.

This study demonstrates for the first time the natural association of AMF in rice in commercial fields in the southern U.S. Fungi living in intimate relationship with rice plants may have effects on their host ranging from beneficial to detrimental, depending on the partners involved and other biotic and abiotic factors in a highly context-dependent manner. This work builds on an earlier study, in which we showed that inoculation of rice plots with a commercial AMF inoculum influences plant-herbivore and plant-pathogen interactions as well as rice growth (Bernaola et al., 2018). The information gathered from this study can be used to further investigate the impact of the symbiotic relationship between AMF and rice, which is becoming increasingly relevant for sustainable agriculture, where soil organisms may be useful for plant production (Berruti et al., 2016).

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Chapter 4

Belowground Inoculation with Arbuscular Mycorrhizal Fungi Increases Local and Systemic Susceptibility of Rice Plants to Different Pest Organisms*

4.1 Introduction

Plants are active organisms capable of adapting to fluctuating environmental conditions; accordingly, they exhibit a high degree of phenotypic plasticity (Pozo et al., 2015). As an important example, plants respond to diverse biotic threats from above- and belowground herbivores and pathogens using a variety of direct and indirect defense mechanisms (Kessler & Baldwin, 2002; Robert-Seilaniantz et al., 2011). Because plant responses to herbivores and pathogens are both local and systemic, above- and belowground organisms may influence each other's fitness through changes in the shared host plant (Ali et al., 2013; Bezemer & van Dam, 2005; Soler et al., 2007; Soler et al., 2009). The presence of soilborne microbes in the rhizosphere plays a considerable role in ecosystem functioning by changing nutrient uptake by plants (thereby influencing quality of the host plant for herbivores), promoting plant growth, and altering plant defense pathways independently of plant nutrition (Pozo & Azcon-Aguilar, 2007; Smith & Read, 2008; van der Heijden et al., 1998). The interplay of these various changes controls the final impact of soilborne microbes on the structure of communities associated with plants.

Arbuscular mycorrhizal fungi (AMF) are well-known, essential components of soil biota within natural and agricultural ecosystems (Smith & Read, 2008). AMF form associations with the root systems of more than 85% of vascular plant species, including many important crops (Smith & Read, 2008). The symbiosis between AMF and plants

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results in a continuum of effects on plant growth and fitness, from highly mutualistic to antagonistic (Barber et al., 2013a; Currie et al., 2011; Johnson et al., 1997; Smith & Read, 2008). Most often, however, associations with AMF facilitate the acquisition by plants of essential nutrients such as nitrogen, phosphate, and water from the soil (Smith & Read, 2008). In exchange, the fungal partner receives photosynthetically fixed carbon, which is used to grow more mycelial networks that allow the root system to expand in the soil and absorb more nutrients (Bonfante & Genre, 2010; Parniske, 2008; Smith & Read, 2008). Although in agricultural ecosystems the association of plants with AMF often results in plant yield increases (Gosling et al., 2006), the effects of AMF can also vary markedly along a parasitism-mutualism continuum (Fesel & Zuccaro, 2016; Johnson et al., 1997; Paszkowski, 2006). Because AM fungi are important components of soil microbial communities and are a central part of agro-ecosystems, they can potentially provide benefits but also costs to farmers.

Colonization of plant roots by AMF has been shown to alter plant quality for both above- and below-ground insect herbivores and pathogens (Currie et al., 2011; Gange, 2001; Goverde et al., 2000; Koricheva et al., 2009) and AMF can contribute to improved resistance or tolerance against abiotic (Maya & Matsubara, 2013; Ruiz-Sanchez et al., 2010) and biotic stresses, such as those caused by root and shoot herbivores and pathogens (Campos-Soriano et al., 2011; Gange, 2001; Pozo & Azcon-Aguilar, 2007; Smith & Read, 2008; Vannette & Hunter, 2011). However, the effects of mycorrhizal colonization on insect fitness or pathogen infection vary depending on the identity of both AMF and host plant, the insect or pathogen involved, and environmental factors (Bennett et al., 2006; Borowicz, 2009; Campos-Soriano et al., 2011; Currie et al., 2011; Gange, 2001, 2007; Gange et al., 2002; Gange & West, 1994; Gehring & Bennett, 2009; Koricheva et al., 2009; Pineda et al., 2010; Vannette & Hunter, 2011). It has been proposed that generalist herbivores and necrotrophic pathogens are usually negatively affected by the presence of AMF, whereas specialist herbivores and biotrophic pathogens are usually positively affected,

performing better on mycorrhizal plants (Borowicz, 2013; Currie et al., 2011; Gange et al., 2002; Hartley & Gange, 2009; Koricheva et al., 2009). A meta-analysis of 34 studies showed that AMF predominantly have negative effects on the performance of generalist chewing herbivores, but positive effects on specialist chewing insects (Koricheva et al., 2009).

The mechanisms by which mycorrhizal colonization alters plant resistance, and the effects of agricultural practices on the presence and effectiveness of AMF symbiosis in crop plants, are not fully understood. Increases in plant growth and improvements in nutrient uptake resulting from mycorrhizal colonization might make plants more attractive or susceptible to herbivores and pathogens (Roger et al., 2013). Alternatively, evidence from tomato plants showed that mycorrhizal colonization may change plant resistance by altering plant defense such as the jasmonic acid pathways (Jung et al., 2012). A large body of evidence also shows that insect herbivores and plant pathogens frequently induce plant defense responses, but the indirect effects of AMF on these induced responses are not thoroughly understood. Importantly, agricultural practices often reduce the presence and effectiveness of AMF symbiosis in the soil (Barber et al., 2013a), which may reduce or delay colonization of the crop by AMF relative to herbivore infestation or pathogen attack. A better understanding of the changes in crop plants in response to root colonization by AMF in agricultural settings, principally in major crops, and how these changes affect plant-herbivore or plant-pathogen relationships, is urgently needed to more effectively utilize mycorrhizae in agriculture.

Cereal crops are an important group of plants that establish symbiotic associations with AMF (Campos-Soriano et al., 2011; Gutjahr et al., 2009; Gutjahr et al., 2015b; Sawers et al., 2008; Vallino et al., 2009). Rice (*Oryza sativa* L) is a staple for more than half the globe's population and represents a promising model system for studies of AMF interactions in general and plant-AMF-herbivore interactions in particular. The presence of AMF associations in rice roots has received increased attention in recent years

(Campos-Soriano et al., 2011; Edwards et al., 2015; Gutjahr et al., 2009). In a recent study, a detailed characterization of the root-associated microbiomes of the rice plant revealed dynamic changes in these microbial communities as a function of geographical location, soil source, host genotype, and cultivation practices (Edwards et al., 2015). However, only a few studies have investigated the interacting effects of AMF symbiosis in rice plants and the implications of these interactions for insect herbivores or pathogens (Campos-Soriano et al., 2011; Cosme et al., 2011). For instance, mycorrhizal rice plants showed enhanced resistance to the rice blast fungus, *Magnaporthe oryzae* and this resistance appeared to rely on both the systemic activation of defense regulatory genes in the absence of pathogen challenge and priming for stronger expression of defense genes during pathogen infection (Campos-Soriano et al., 2011).

The aim of the current study was to understand how AMF inoculation influences rice-herbivore and rice-pathogen interactions. We used as model organisms three important pests of rice in the southern U.S.: larvae of the rice water weevil (RWW; *Lissorhoptrus oryzophilus* Kuschel; Coleoptera: Curculionidae), larvae of the fall armyworm (FAW, *Spodoptera frugiperda* J.E. Smith; Lepidoptera: Noctuidae), and sclerotia of sheath blight (ShB, *Rhizoctonia solani;* Basidiomycete). Of these three study organisms, only the effects of AMF on rice water weevils have been previously investigated. Cosme et al. (2011) found, in a greenhouse experiment, that females of the grass-specialist RWW laid double the amount of eggs in AMF-inoculated rice plants, an effect they speculated was caused by AMF-mediated increases in plant nutrient concentrations. In light of these prior results with RWW, we explored the hypothesis that colonization of roots by AMF would reduce the resistance of rice to the RWW in the field and greenhouse experiments. Then, in light of new results, we addressed a second hypothesis that AMF colonization might reduce the resistance of rice to other pest organisms such as FAW and ShB under greenhouse conditions. We asked the following questions: (1) Does AMF inoculation reduce rice resistance against a root- and foliar-feeding herbivore in the field and greenhouse? (2)

Does AMF inoculation affect resistance to a fungal pathogen? (3) Does AMF inoculation increase plant biomass? (4) Does AMF inoculation influence the nutritional status of rice plants? To answer these questions, we carried out a series of field and greenhouse experiments in rice by manipulating the availability of AMF (inoculated and non-inoculated plants) using a commercial inoculum containing six AMF species from the Glomeraceae family. We found that the performance of insects and the pathogen on rice was enhanced when plants were colonized by AMF, which was consistent with results from Cosme et al. (2011); however, this susceptibility was not correlated with changes in plant nutritional status.

4.2 Materials and methods

4.2.1 Study system: plants, fungi, and insects

To study plant-AMF-herbivore and plant-AMF-pathogen interactions, we used two commercial varieties of rice as the host plant. 'Lemont' and 'Cocodrie' are high-yielding, early-maturing, conventional varieties developed at the Texas A&M University Agricultural Research and Extension Center (Beaumont, TX, USA) and the Louisiana State University Agricultural Center (LSU AgCenter) H. Rouse Caffey Rice Research Station (Crowley, Acadia, LA, USA), respectively (Bollich et al., 1985; Linscombe et al., 2000). 'Cocodrie' is a susceptible variety grown widely in the southern U.S. 'Lemont' is not widely grown currently but was chosen because it had been used in previous studies of rice-AMF interactions (Dhillion, 1992). Seeds of rice were kindly provided by the breeding and foundation seed program at the LSU AgCenter H. Rouse Caffey Rice Research Station. 'Lemont' was used for experiments in 2012 and 'Cocodrie' for experiments in 2013.

A commercial inoculum prepared *in vivo* to contain only AMF propagules (ECOVAM^{TM} VAM Endo Granular, Horticultural Alliance Inc., Sarasota, FL, USA) was

used to promote and establish symbiosis with the host plants in the field and greenhouse experiments. The inoculum contained six species of AMF (*Rhizophagus irregularis*, *Funneliformis mosseae*, *Glomus deserticola*, *Rhizophagus fasciculatum*, *Sclerocystis dussii*, and *Glomus microaggregatum*) and consisted of spores, hyphae and colonized root fragments. All AMF species were originally obtained from the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM, West Virginia University, USA). The AMF propagules were carried in an inert-like material consisting of a uniform mixture of zeolite, pumice, vermiculite, perlite and attapulgite. According to the supplier, quantification of the number of spores per gram of inert material was accomplished by the wet sieving and decanting method of Gerdemann and Nicolson (1963) followed by sucrose gradient centrifugation according to the modification proposed by Schenck (1982). For the extraction of spores, 20 g of inert material was blended for ten seconds in one liter of tap water. Counting was carried out under an optical microscope using a counting slide of 1 mL. The formulated material contained an average of 132 spores of AMF (all species) per gram, in addition to hyphae and colonized root fragments.

The RWW is the most destructive insect pest of rice in the United States (Hamm et al., 2010; Stout et al., 2002; Tindall & Stout, 2003). RWW adults feed on young rice leaves, producing longitudinal scars. However, this form of injury is not economically important; rather, the larvae have a strong impact on plant yields when they feed on roots of flooded rice (Cosme et al., 2011). Adult rice water weevils were collected from rice fields at the H. Rouse Caffey Rice Research Station 24 h prior to conducting greenhouse experiments. Field experiments relied on natural infestations of RWWs, which are abundant at the field site. Weevils were maintained in glass jars with freshly cut rice leaves and water until use. Before starting the experiment, weevils were captured in copula or sexed under a dissecting microscope in order to ensure equal numbers of males and females.

The FAW is a sporadic pest of rice that causes harm by consuming aboveground portions of rice with its chewing mouthparts. Adult female armyworms oviposit a large number of eggs on leaves, which give rise to larvae that begin to feed on leaves (Stout et al., 2009). Larvae of the FAW used in these experiments were obtained from a colony maintained continuously on meridic diet in a laboratory. The colony originated from larvae collected in rice fields near Crowley, LA, in 2011. Genetic variability and vigor of the colony were maintained annually with field-collected larvae. The diet used for rearing of larvae was a commercial formulation designed specifically for this species (Southland Products Incorporated, Lake Village, AR, USA). Pupae were placed in buckets containing vermiculite, wax paper as a substrate for oviposition, and two dental rolls soaked in a mixture of honey and beer (150ml honey-150ml beer- 300ml water-12g ascorbic acid) and covered with cheesecloth. After emergence, adults mated and females oviposited eggs onto the cheesecloth, which were collected daily and placed in 8-cell trays (Bio-Serv, Frenchtown, NJ, USA) with a moistened cotton ball and sealed with lids. When neonates began to emerge, they were placed in cups supplied with artificial diet. Larvae were maintained on meridic diet until use for feeding assays. The colony was maintained under controlled environmental conditions (L14: D10, 28 ± 2°C, 38 ± 2% R.H).

Rhizoctonia solani (Basidiomycete), the causal agent of ShB of rice, is a soilborne pathogen with a wide host range. The disease caused by this organism in rice usually develops after the tillering stage of rice growth, and initial infection appears on the stem near the water line as oval lesions, which dry and turn tan (Lee & Rush, 1983). The fungal isolate LR172 of the ShB pathogen used in this study was originally isolated in 1972 from a naturally infected rice plant (cv. 'Lebonnet') in Louisiana. LR172 was generously provided by D. Groth (LSU AgCenter H. Rouse Caffey Rice Research Station) and maintained on potato dextrose agar (PDA). Mycelial growth and sclerotia production were typical of *R. solani*. The isolate of *R. solani* was examined for mycelial growth with a compound microscope (Olympus CH2, Pittsburgh, PA). A verified isolate of *R. solani*

was subcultured by placing sclerotia in the center of a 9-cm-diameter petri dish filled with PDA medium to produce active mycelia and grown at room temperature (22 to 25°C) under continuous light. These cultures were used to prepare agar blocks of 5-day-old cultures inoculation.

4.2.2 Experimental design

4.2.2.1 Evaluating effects of AMF on RWW performance (field study)

To evaluate whether inoculation of rice plants with AMF affects the resistance of rice plants to *L. oryzophilus*, three small-plot field experiments were conducted during the 2012 and 2013 growing seasons at the LSU AgCenter H. Rouse Caffey Rice Research Station (Crowley, Acadia Parish, LA). In 2012, one experiment, referred to as Experiment-1 (Exp-1) was conducted; in 2013, two experiments, Experiment-2 (Exp-2) and Experiment-3 (Exp-3) (Table 4.1), were conducted. Each experiment comprised three treatments. For the first treatment (F, fungicide) rice seeds were treated with a mixture of the fungicides Maxim 4FS (fludioxonil, 4.16 mg a.i. 300 g^{-1} of seeds; Syngenta Crop Protection, Greensboro, NC, USA), Apron XL 3LS (mefenoxam, 26.33 mg a.i. 300 g^{-1} of seeds; Syngenta Crop Protection, Greensboro, NC, USA) and Dynasty (azoxystrobin, 20.79 mg a.i. 300 g^{-1} of seeds; Syngenta Crop Protection, Greensboro, NC, USA) and planted in soil with sterilized AMF inoculum. Rice seeds were treated with a mixture of fungicides before planting to eliminate the presence of any fungi from experimental plots. For the second treatment (NM, nonmycorrhizal), rice seeds were sown in soil with sterilized AMF inoculum. The sterilized inoculum was used in nonmycorrhizal plots to control for the possibility that inert ingredients in the commercial inoculum altered soil properties. For the F and NM treatments, commercial inoculum was sterilized by autoclaving for 60 min at 120°C to destroy living AMF inoculum. For the third treatment (M, mycorrhizal), rice seeds were planted in soil inoculated with live AMF. For all three

experimental treatments, rice plants were grown from seeds in the field; thus the soil was not sterilized and likely contained native AMF. Sterilized mock or live AMF inoculum was applied on the surface of the soil and gently raked in to incorporate the live or mock inoculum into the upper 2.5 cm of the soil. Experiments were laid out in a randomized complete block design (RCBD; in Exp-1) or in a completely randomized design (CRD; in Exp-2 and 3) with a total of eight and ten blocks (replications) per treatment per experiment for 2012 and 2013, respectively.

Table 4.1. Planting and sampling dates for three field experiments conducted in 2012 and 2013 for evaluating the effects of arbuscular mycorrhizal fungi on the performance of rice water weevil in rice plants.

Year	Trial	Planting date	Flooding date	Larval sampling dates (cores)
2012	Experiment-1	17th April	30th May	15th June & 20th June
2013	Experiment-2	4th April	30th May	19th, 24th June & 2nd July
	Experiment-3	6th June	24th June	15th, 22th & 29th July

Rice was hand-seeded on the dates specified in Table 4.1 at a rate of 10 g of seeds per plot. Plots measured 0.762 m x 0.762 m. A soil sample was collected from the plots before seeding in 2013 and sent for analysis to the LSU AgCenter Soil Testing & Plant Analysis Laboratory (STPAL, LSU, Baton Rouge, LA). The principal chemical properties of the soil are reported in Supplementary Table A1. Each plot was inoculated with 1.5 kg (2012) or 2 kg (2013) of sterilized AMF inoculum (F and NM) or live inoculum (M). The inoculum amounts used in 2012 and 2013 corresponded to approximately 200 and 260 thousand AMF spores per plot, respectively. To avoid the spread of AMF inoculum from plot to plot during irrigation, plots were surrounded by an enclosure constructed of metal roofing

flashing 20 cm high and held in place by pushing into the soil before planting. Plots were flushed with well water as necessary for the first month after seeding to establish stands of rice. We did not incorporate small filtrate aliquots of AMF inoculum into plots because we assumed that the large volumes of flooding water were sufficient to allow some homogenization among treatments in terms of water-soluble microflora, whereas the loose AMF spores, which are denser than water, were expected to remain precipitated. After allowing the plants to grow for approximately one month, a permanent flood was applied on the dates specified in Table 4.1. Plants possessed 4-5 leaves (early tillering) at permanent flooding. Metal flashing was removed after flooding. Plots in these experiments were not fertilized.

After natural infestation, densities of RWW larvae and pupae were determined by taking root/soil core samples from each plot (Stout et al., 2001). The core sampler was a metal cylinder with a diameter of 9.2 cm and a depth of 7.6 cm attached to a metal handle (Figure A1). Core sampling was conducted twice for all experiments between three and five weeks after permanent flood. Dates of core samplings are shown in Table 4.1. For each sampling date, two (2012) or three (2013) core samples were taken from each plot. Core samples were placed into a 40-mesh screen sieve bucket to wash the soil and larvae from roots, buckets were placed into basins of salt water, and larvae and pupae were counted as they floated to the water surface (N'Guessan et al., 1994). RWW counts from two to three core samples per plot per sampling date were averaged to obtain an average number of larvae/pupae per core sample.

In order to confirm if the inoculum enhanced the abundance of AMF living in rice roots in Exp-2 and 3, the percentage of the root system containing AMF colonization was determined by observation of sub-sampled root fragments as described below. For Exp-2, the percentage of root fragments colonized by AMF was evaluated two times during plant development, before and after flood. For Exp-3, this parameter was evaluated one time after the flood was established. On May 15th (41 dai) and June 7th (64 dai), 12 root

samples from Exp-2 were randomly collected and analyzed from four plots of each treatment group per sampling date. The same number of root samples from Exp-3 were collected and analyzed from four plots of each treatment group on July 8th (32 dai). Sampling in Exp-2 and 3 was conducted by taking 9.2 cm diameter soil-root cores adjacent to plants. Each soil-root core (two to four plants) was placed in plastic bags (one core per bag) and taken to the laboratory to be processed as described below for root staining. For the purpose of this study, one core represented one plant sample. A list of the experiments conducted in 2012 and 2013 are summarized in Supplementary Table A2.

4.2.2.2 Evaluating effects of AMF on plant resistance to RWW (Greenhouse study)

To further evaluate whether AMF inoculation alters the resistance of rice to *L. oryzophilus*, two choice experiments (RWW1 and RWW2) were conducted in the summer of 2013 in a greenhouse on the campus of Louisiana State University, Baton Rouge, LA. For each experiment, two treatments were employed, namely mycorrhizal (M) and nonmycorrhizal plants (NM; control). All plants were grown in 2 liter round (15 cm diameter) plastic pots (Hummert International, Earth City, MO) filled with a sterilized soil mix (2:1:1, soil: peat moss: sand), to which 50 g of AMF inoculum (corresponding to approximately 6500 AMF spores) or 50 g sterilized inoculum were added. For all greenhouse experiments, the soil substrate was sterilized by autoclaving for 60 min at 120 ^{o}C to eradicate the indigenous AMF. The AMF inoculum was mixed with the soil, and rice seeds were sown directly into pots. Plants were maintained under greenhouse conditions with temperatures ranging from 25^{o}C to 35^{o}C and ambient lighting. Plants were maintained in large wooden basins lined with heavy black plastic pond liner to hold flood waters when necessary as indicated in Stout and Riggio (2002). As for the field study, we assumed that flooding waters were suffice to allow some homogenization of water-soluble microflora. Approximately 10 days after planting, seedlings were thinned to

a density of two or three plants per pot (RWW1 and RWW2, respectively). Experiments were conducted using two-week-old plants (3-leaf stage). Because these experiments were conducted with rice at an early stage of growth, additional fertilizer was not necessary for adequate plant growth.

To initiate the choice experiments, two pots of each treatment were placed into each of seven (RWW1) or six (RWW2) infestation cages (Table A2, Figure A2). Cages were set in the greenhouse basins and basins were flooded to a depth of ~20 cm. Infestation cages were cylindrical wire frames (46 cm diameter x 61 cm tall) covered with a mesh fabric screening. After flooding, weevils were released into cages at a density of three weevils per plant (24 and 36 weevils per cage in RWW1 and RWW2, respectively) and allowed to feed, mate, and oviposit on plants of both treatments for 5 days. After that, pots were removed from cages and weevils were discarded.

The resistance of M and NM plants to *L. oryzophilus* was evaluated by counting first instars as they emerged from eggs laid in leaf sheaths of plants. Procedures for estimating larval densities were adapted from Stout and Riggio (2002). Briefly, after the 5-day adult infestation, plants for each pot were removed from the soil, washed free of soil, and placed individually in water in clean test tubes. Test tubes were labeled, arranged in a test tube rack, and placed in a growth chamber (30°C, 14:10 L:D). Using this method, weevils that infest plants hatch from eggs, emerge from leaf sheaths and settle on the bottom of the test tubes (Heinrichs et al., 1985). Larvae were removed by shaking roots free of larvae and then pouring water from test tubes into a petri dish for counting. After that, plants were placed back into the test tubes, and tubes were refilled with fresh water. Larva counts were started 3 days after placing plants in the tubes, and larvae were counted daily until no additional larvae were found for two consecutive days.

The percentage of root fragments colonized by AMF was measured in RWW2. Root samples from 5 plants of each mycorrhizal treatment were sampled on Jul 18th, 31 dai. A total of 10 plant samples were collected from this experiment.

4.2.2.3 Evaluating effects of AMF on plant resistance to FAW (Laboratory study)

To assess whether AMF inoculation influences resistance of rice to *S. frugiperda*, three laboratory feeding assays were conducted in 2012 (FAW1) and 2013 (FAW2 and FAW3). To this end, we cut leaf material from greenhouse-grown plants with or without AMF inoculum to determine *S. frugiperda* larval growth. 'Lemont' and 'Cocodrie' rice plants were grown under two treatments, namely M and NM. Plants were grown in the greenhouse as previously described. Six rice seeds were planted in each pot and thinned to three plants immediately before starting feeding assays for FAW1, FAW2 and FAW3 (Table A2). Plants from which leaf material was taken were 3 weeks old and possessed three or four leaves. Because these experiments were conducted with rice at an early stage of growth, additional fertilizer was not necessary for adequate plant growth.

To initiate the assays, larvae of 4 to 5 days in age were selected from meridic diet and stage-synchronized at head capsule slippage. Synchronized larvae were starved for three hours to ensure that their guts were voided before their masses were determined using an analytical balance (model XS105, Mettler-Toledo LLC, Columbus, OH, USA). Larvae with similar masses were used in these experiments. Feeding assays were conducted in 9 cm plastic petri dishes lined with moistened cotton batting to maintain turgor in excised tissues (Figure A3). Youngest fully-expanded leaves were removed from plants of each treatment group using scissors, transported on ice to the laboratory, cut into ca. 2 cm pieces and placed in petri dishes. Weighed larvae were placed together in petri dishes with foliage and allowed to feed on excised leaf material for 4 days (FAW1), 7 days (FAW2) or 10 days (FAW3). Larvae were observed daily to ensure they were not food-limited and leaves were changed every other day, but in later larval stage the leaves were changed daily. After ending the feeding assay, larvae were starved for three hours to ensure that the larval gut was emptied before final mass was determined and recorded. For each experiment, 15 larvae (replicates) were used for each treatment for a total of 28, 30, and

30 observations for FAW1, FAW2, and FAW3, respectively (insects that died during feeding assays were excluded).

The percentage of root fragments colonized by AMF was measured in FAW2. To this end, root samples from 5 plants of each treatment were sampled on May 24th, 35 dai in 2013, and processed as described below. For the experiment FAW3 described here, RWW1 described above, and ShB1 described below, only one assessment of AMF colonization was conducted as these three experiments were planted at the same time and the inoculation success had been previously confirmed. From a total of 100 pots planted (50 M and 50 NM) in these three experiments, five M and five NM plants were sampled on Jun 27th, 36 dai in 2013. A total of 20 plant samples were collected from the four experiments.

4.2.2.4 Evaluating effects of AMF on plant resistance to rice sheath blight (Greenhouse study)

To investigate whether AMF inoculation influences susceptibility of rice to infection by the fungus *R. solani*, two experiments (ShB1 and ShB2) were conducted in the summer of 2013. To obtain uniform disease development, rice plants at late tillering growth stage (approximately 8-weeks-old) were used for inoculation with *R. solani*. As in previous experiments, M and NM treatment plants were set up in the greenhouse filled with sterilized soil mix. Six rice seeds were planted in each pot and thinned to five and three plants immediately before pathogen inoculation for ShB1 and ShB2, respectively (Table A2). Plants in each pot were collectively considered an experimental unit (replication). Fifteen pots of each treatment group were used for each experiment and arranged in a completely randomized design in greenhouse basins. Because these experiments were conducted with rice at late stage of growth, additional fertilizer was necessary for adequate plant growth. Urea (46% N) was applied at 0.5 g (134 kg N/ha) per pot in all pots (ShB1 and ShB2). Fertilizer was applied twice at 20 days and 40 days after planting.

Agar blocks (0.5 cm squares) of a 5-day-old culture of LR172 were cut from the outer growing area of culture plate using a pipette tip. Using forceps, one tiller of each plant, i.e. five or three tillers in each pot, was inoculated with *R. solani* by placing the mycelial agar block beneath the leaf sheath, ensuring that mycelia were in contact with the plant. The leaf sheath and agar block were covered immediately with aluminum foil as described by Park et al. (2008). Inoculated plants were maintained in the greenhouse, where relative humidity was favorable for the growth of ShB. When typical lesions started to appear 3 days after inoculation (dai), the aluminum foil was removed to allow for disease development (Figure A4). Susceptibility of rice plants to ShB was evaluated 7 dai for each tiller by counting the number of lesions and measuring the lesion length of each inoculated plant. For each plant, measurements of lesion length were used to derive the maximum lesion length and the mean lesion length.

4.2.2.5 Processing and quantification of mycorrhizal colonization

The trypan blue method of Koske and Gemma (1989) for root staining was used for quantification of mycorrhizal colonization with some modifications. Clearing and staining procedures require root samples to be washed from soil to remove all soil particles and then separating root and shoot tissues. For subsampling, roots of each plant were cut into 2-cm-long segments and placed in tissue processing cassettes (Ted Pella, Redding, CA). At least 200 small root pieces per root sample were cleared in 10% KOH at 90°C for 20 min in a water bath. Clear pieces of roots were rinsed 5X with tap water to remove KOH, and roots were immersed in 2% HCl at room temperature for 10-15 min to ensure the roots were adequately acidified for staining. Cassettes containing roots were immediately stained with 0.05% trypan blue (Sigma-Aldrich, St. Louis, MO, USA) by incubation overnight and then transferred to vials containing lactoglycerol at 4°C to allow excess

stain to leach out of the roots. Stained root samples were stored in destaining lactoglycerol solution for 48 h before being mounted in the same solution on a microscopic slide.

In order to quantify the abundance of AMF living in rice roots, the 2-cm-long root fragments were mounted after staining on microscopic slides as previously described (McGonigle et al. (1990). Five microscope slides, each containing ten stained randomly selected root fragments, were prepared from each plant sample. The random selection of root fragments is representative for the whole root system as it was often not possible to disentangle the root types. A total of 50 stained root segments per sample were examined with a compound microscope (Olympus CH2, Tokyo, Japan) at 40X magnification in order to confirm the levels of AMF colonization. Root fragments that contained blue-stained AMF structures such as intraradical aseptate hyphae linked to either fungal arbuscules or vesicles/spores were scored as colonized by AMF (Figure A5) (DeMars & Boerner, 1996). Percent of root fragments with AMF colonization was averaged per treatment for the analyzed experiments. Photos of AMF structures on mycorrhizal colonized roots were taken using a microscope-mounted 5.0-megapixel digital camera (Leica DFC480, Cambridge, UK).

4.2.2.6 Evaluating effects of AMF on plant biomass

To determine the effect of AMF on plant biomass, rice samples were collected from Exp-2 and from a separate greenhouse experiment (PB1) conducted in 2013 using previously sterilized field soil from the LSU AgCenter H. Rouse Caffey Rice Research Station. For PB1, NM and M treatments were established with 12 replications for each treatment as described previously (Table A2). Entire plants were collected on June 18th from Exp-2 and on Sep 24th for PB1 at 75 and 30 dai, respectively. Pots for PB1 were not fertilized. Soil was washed from roots, and the shoots and roots were separated and blotted dry with a paper towel. Fresh weights of shoots and roots were recorded, and plant

material was dried in an oven (60°C for 1 week) and reweighed (shoot and root dry weight) to calculate plant dry biomass as well as the ratio of root dry weight (RDW)/shoot dry weight (SDW).

4.2.2.7 Evaluating effects of AMF on plant nutritional status

To evaluate whether AMF inoculation affected the concentrations of nutrients in leaves and roots of rice, above- and belowground plant tissue samples from each of the treatments in Exp-1, Exp-2 and PB1 were collected on May 30th, June 18th, and September 24th at 43, 75 and 28 dai, respectively. Plant material was washed and transported to the laboratory. Samples were dried in an oven at 60°C for 1 week, ground in a Wiley mill (Thomas Wiley® Mini-Mill, Mexico) and submitted to the LSU AgCenter's Soil Testing & Plant Analysis Laboratory (STPAL, LSU, Baton Rouge, LA) to determine nutrient concentrations in shoot and root tissues. The STPAL determined N and C concentrations by dry combustion using a LECO TruSpec™ CN analyzer (LECO Corp., St. Joseph, MI, USA), while the concentrations of the remaining nutrients (Ca, Mg, S, P, K, Al, B, Cu, Fe, Mn, Na, and Zn) were determined by inductively coupled plasma (ICP) analysis.

4.2.2.8 Statistical Analyses

Data were analyzed using SAS 9.4 (SAS Institute, 2014). The effects of AMF inoculation on rice plant responses for each experiment were analyzed separately by one-way analysis of variance (ANOVA) using PROC MIXED. For the RWW field experiments, effects of AMF inoculation on average number of larvae/pupae per core sample were analyzed as appropriate for a RCBD with treatment (F, NM or M) as a fixed effect and block (replication) as a random effect for Exp-1 or CRD with treatment (F, NM or M) as fixed effect for Exp-2 and Exp-3. For the RWW choice experiments, data were analyzed with treatment as a fixed effect and infestation cages (replication) as a

random effect. For the FAW experiments, weight gain (final weight – initial weight) was the response variable, treatment was a fixed effect, and experiment was a random effect. For ShB experiments, disease ratings (lesion length and numbers of lesions) from five and three individual plants in each pot, respectively, were averaged as a single replication. The two experiments were analyzed independently with lesion length and number of lesions as dependent variables with treatment considered as a fixed effect. The data on AMF colonization were analyzed based on the percentage of root fragments colonized (see above) for Exp-2, Exp-3, RWW2, FAW2, and FAW3/RWW1/ShB1 experiments. Data for SDW and RDW were analyzed with the two treatments (M and NM) as fixed effects. For nutritional analyses, data for each nutrient (N, P, K, and C) were analyzed separately. Means were separated using the least significant difference (LSD) test in each of the experiments when there was a significant difference between treatments.

4.3 Results

4.3.1 Root colonization by AMF

The microscopic analyses of root fragments collected from M, NM or F treated rice plant samples in experiments Exp-2, Exp-3, RWW2, FAW2 and in a random sampling of FAW3, RWW1 and ShB1 combined (see Materials and Methods above) confirmed that AMF inoculation significantly enhanced the percentage of root fragments colonized by AMF in relation to the non-inoculated controls. This was observed in greenhouse grown plants and in field grown plants (Table 4.2, Figure A5); except in Exp-2 prior flooding at 41 dai, in which the enhanced percentage of root fragments colonized by AMF was only apparent in M plants compared with the non-inoculated plants. For both field experiments (Exp-2 and Exp-3), we detected a small percentage of fragments colonized by AMF in the non-inoculated plants or in the plants treated with fungicide (Table 4.2), probably due to native AMF already present in soil. Overall, although the percentages of root fragments

colonized by AMF in rice were generally low, our data confirm that inoculation with AMF enriched the abundance of AMF living in rice roots grown under greenhouse and field conditions.

Table 4.2. Percentage (%) of root fragments colonized by arbuscular mycorrhizal fungi (AMF) in rice plants. The percentage of colonized root fragments was determined from two field experiments (Experiment-2, Experiment-3), and from five greenhouse experiments (FAW2, RWW2, and from the combined experiments FAW3/RWW1/ShB1). Means ± standard errors are shown (n = 4 or 5 for field and greenhouse, respectively). Different letters indicate significant differences between mycorrhizal levels within each mycorrhizal treatments according to Least Significant Difference mean comparisons ($P < 0.05$; LSD). The F, NM, and M refer to AMF treatments of F: rice seeds + fungicides + sterilized AMF, NM: rice seeds + sterilized AMF, and M: rice seeds + live AMF.

Treatments	Root fragments colonized by AMF (%)		
Field 2013 (Mean of 4 samples each)	Exp-2 (41 dai[1]) Mean ± SE	Exp-2 (64 dai) Mean ± SE	Exp-3 (32 dai) Mean ± SE
Fungicide (F)	1.5 ± 0.95b	0.5 ± 0.50b	0.5 ± 0.50b
Nonmycorrhizal (NM)	4 ± 1.83ab	1.5 ± 0.95b	3 ± 1.29b
Mycorrhizal (M)	9 ± 2.08b	6 ± 2.16a	7 ± 1.29a
$F_{2,9}$	5.10	4.41	9.00
$P\text{-}value$	0.033	0.046	0.007
Greenhouse 2013 (Mean of 5 samples each)	RWW2 (31 dai) Mean ± SE	FAW2 Mean ± SE	FAW3/RWW1/ShB1 (36 dai) Mean ± SE
Nonmycorrhizal (NM)	0.8 ± 0.49b	0.4 ± 0.40b	0 ± 0b
Mycorrhizal (M)	8.4 ± 2.48a	11.6 ± 1.72a	13.6 ± 1.72a
$F_{1,8}$	9.03	40.20	62.49
$P\text{-}value$	0.017	0.0002	< 0.0001

[1]dai, days after inoculation

4.3.2 Effects of AMF inoculation on RWW performance in the field

Under field conditions, the susceptibility of AMF-inoculated rice plants to RWW was measured by the densities of RWW larvae and pupae compared with that of rice plants treated with sterilized inoculum or with fungicides and sterilized inoculum (Figure 4.1). For Exp-1, we observed a significant positive impact of AMF inoculation on rice susceptibility to RWW larvae and pupae on both core sampling dates (June 15: $F_{2,14} = 7.45$, $P = 0.0063$; June 20: $F_{2,14} = 21.06$, $P < 0.0001$) (Figure 4.1). The highest immature densities were found in plots of plants inoculated with AMF on both sampling dates, whereas densities were lowest, at nearly equal numbers, in plots inoculated with sterilized inoculum or with fungicide and sterilized inoculum. Also, densities increased over time: weevil densities were lowest at 15 (core 1) days after permanent flood and highest at 20 (core 2) days after permanent flood. Increases in RWW densities in plots of AMF-inoculated plants ranged from 91.4% in core 1 (2.94 ± 1.01 to 0.25 ± 0.13, mean ± SE) to 94.3% in core 2 (7.75 ± 1.13 to 0.44 ± 0.19, mean ± SE) when compared to NM plants. For Exp-2, the AMF-mediated susceptibility of rice to RWW larvae and pupae was only significant in the first core sampling, while in the second and third core samplings the enhanced susceptibility was not apparent (June 19: $F_{2,18} = 4.15$, $P = 0.0331$; June 24: $F_{2,18} = 2.64$, $P < 0.0990$; July 2: $F_{2,18} = 1.26$, $P = 0.3074$). As in Exp-1, weevil densities in Exp-2 increased with sampling date, being lowest at 19 (core 1) days after permanent flood, intermediate at 24 (core 2) days, and highest at 32 (core 3) days after permanent flood (Figure 4.1). The increase in weevil densities in plots of AMF-inoculated plants in core 1 was 37% (5.70 ± 0.92 to 3.60 ± 0.52, mean ± SE) when compared to NM control plants. In second and third core samplings, increases were not meaningful with 24.2% (11.95 ± 1.72 to 9.05 ± 1.09, mean ± SE) and 12.3% (12.20 ± 1.60 to 10.70 ± 1.02, mean ± SE), respectively. In Exp-3, densities of RWW were significantly higher in AMF-inoculated plants in the first and third core samplings (July 15: $F_{2,18} = 4.32$, $P = 0.0293$; July 29: $F_{2,18} = 6.20$, $P = 0.0090$) but not in the second core sampling (July 22: $F_{2,18} =$

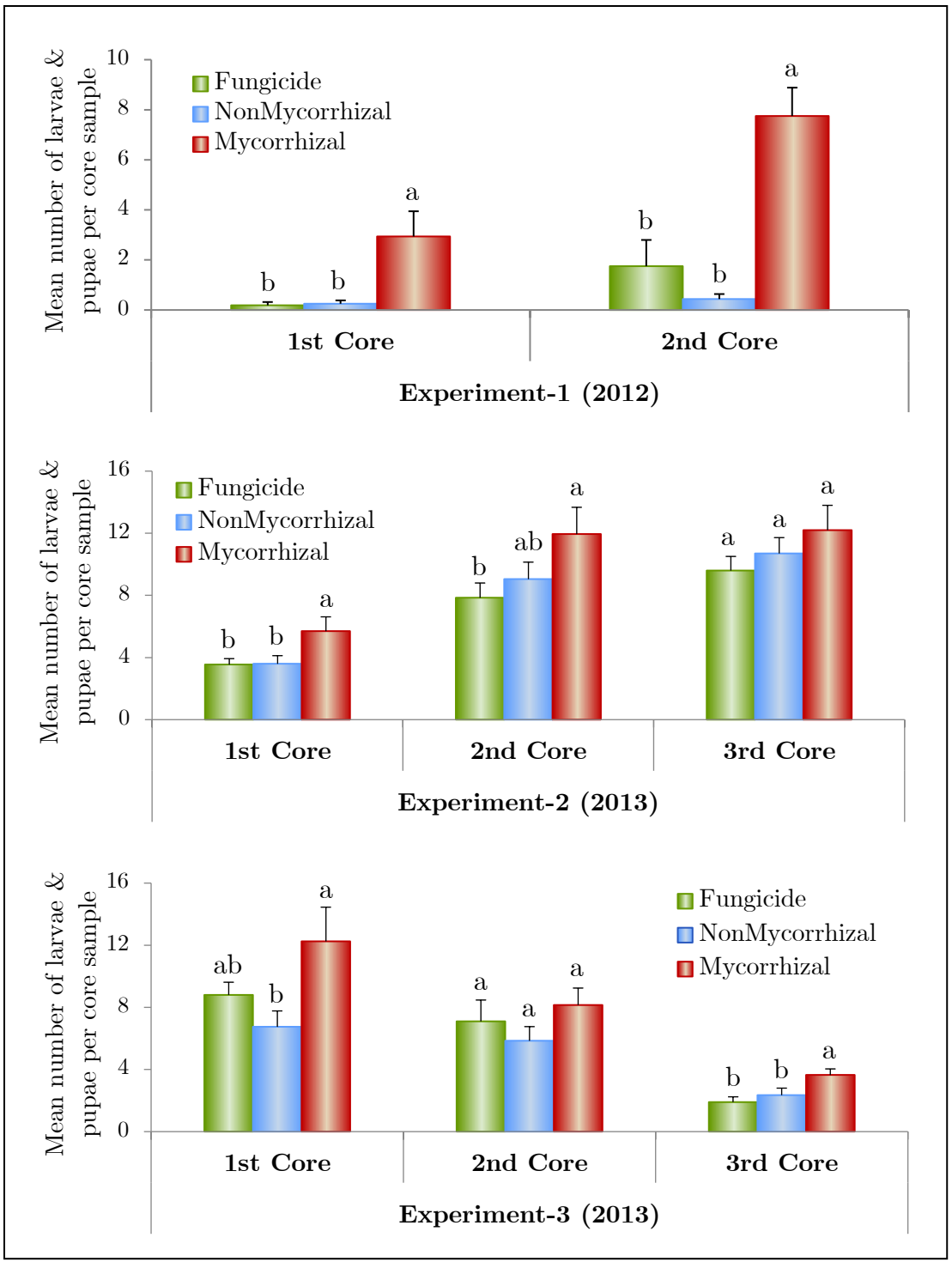

Figure 4.1. Effects of arbuscular mycorrhizae fungi treatments on the densities (larvae and pupae per core sample) of *Lissorhoptrus oryzophilus* (± SE) in three field experiments (Experiment-1, Experiment-2, and Experiment-3) during 2012 and 2013. Fungicide: rice seeds + fungicides + sterilized AMF, NonMycorrhizal: rice seeds + sterilized AMF, Mycorrhizal: rice seeds + live AMF. Bars and lower case letters at the column head indicate that means differ significantly (LSD, $P \leq 0.05$).

1.11, $P < 0.3497$), compared with both non-inoculated control treatments. Unlike previous experiments, weevil densities in Exp-3 decreased with sampling date: weevil densities were highest at 21 (core 1), intermediate at 28 days (core 2), and lowest at 35 (core 3) days after permanent flood. Increases in RWW densities in plots of AMF-inoculated plants ranged from 45% in core 1 (12.25 ± 2.20 to 6.75 ± 1.02, mean ± SE) to 36% in core 3 (3.65 ± 0.39 to 2.35 ± 0.45, mean ± SE) when compared to NM control plants. Overall, the inoculation of rice plants with AMF enhanced the susceptibility of rice to RWW in all three field experiments (Experiment-1: $F_{2,14} = 26.44$, $P < 0.0001$; Experiment-2: $F_{2,18} = 5.59$, $P = 0.013$; Experiment-3: $F_{2,18} = 7.00$, $P = 0.0056$).

4.3.3 Effects of AMF inoculation on plant resistance to RWW in the greenhouse

AMF colonization can increase rice susceptibility to oviposition by RWW females (Cosme et al., 2011), but it was yet unclear whether this affects subsequent developmental stages. In order to address this question, we assessed the number of RWW first instars emerging from rice plants subjected to oviposition under controlled conditions. In two independent experiments (RWW1 and RWW2) inoculation with AMF of rice roots significantly increased the numbers of RWW first instars emerging from M treated rice plants (Figure 4.2; RWW1: $F_{1,48} = 6.99$, $P = 0.0110$; RWW2: $F_{1,65} = 13.66$, $P = 0.0005$). Numbers of RWW first instars emerging from M rice plants were 34% and 47% greater in RWW1 (12.39 ± 1.43 to 8.21 ± 0.95, mean ± SE) and in RWW2 (10.19 ± 1.11 to 5.44 ± 0.95, mean ± SE), respectively, compared to NM control plants. Therefore, AMF inoculation also has a positive impact on the performance of early stages of RWW.

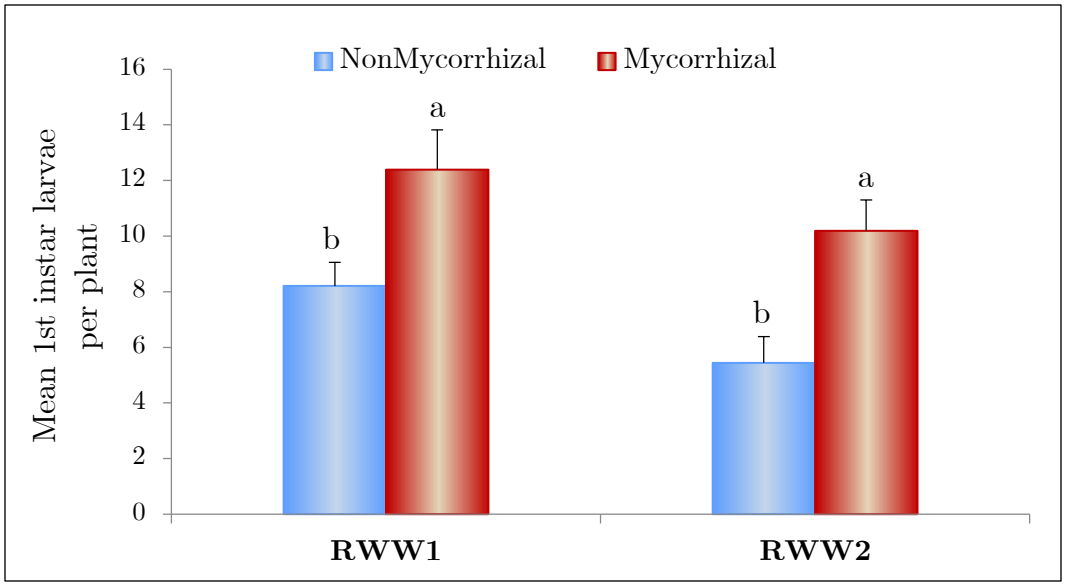

Figure 4.2. Mean number of *Lissorhoptrus oryzophilus* larvae per plant (± SE) in a greenhouse experiment using mycorrhizal (M) and nonmycorrhizal (NM) rice plants of the variety 'Cocodrie'. Plants were infested with pairs of rice water weevil adults to feed on each plant for 5 days. NonMycorrhizal: rice seeds + sterilized AMF, Mycorrhizal: rice seeds + live AMF. Bars and lower case letters at the column head indicate that means differ significantly (LSD, $P \leq 0.05$).

4.3.4 Effects of AMF inoculation on FAW growth

To understand whether the increase in susceptibility of rice plants colonized by AMF is specific to RWW, we assessed the impact of inoculation with AMF on growth of FAW larvae. For all three FAW experiments, FAW larvae gained more weight when fed leaf material from plants inoculated with AMF compared with larvae fed leaf material from NM plants (FAW1: $F_{1,26} = 6.72$, $P = 0.015$; FAW2: $F_{1,28} = 16.82$, $P = 0.0003$; FAW3: $F_{1,28} = 159.24$, $P < 0.0001$) (Figure 4.3). Increases in larval growth on M rice plants ranged from 30.2% in FAW1 (0.053 ± 0.004 to 0.037 ± 0.003, mean ± SE), 31.4% in FAW2 (0.118 ± 0.004 to 0.014 ± 0.007, mean ± SE) to 75% in FAW3 (0.056 ± 0.003 to 0.014 ± 0.002, mean ± SE) compared with the NM control plants. These results show that the impact of AMF on rice susceptibility to herbivores affects aboveground herbivores as well as root feeding herbivores.

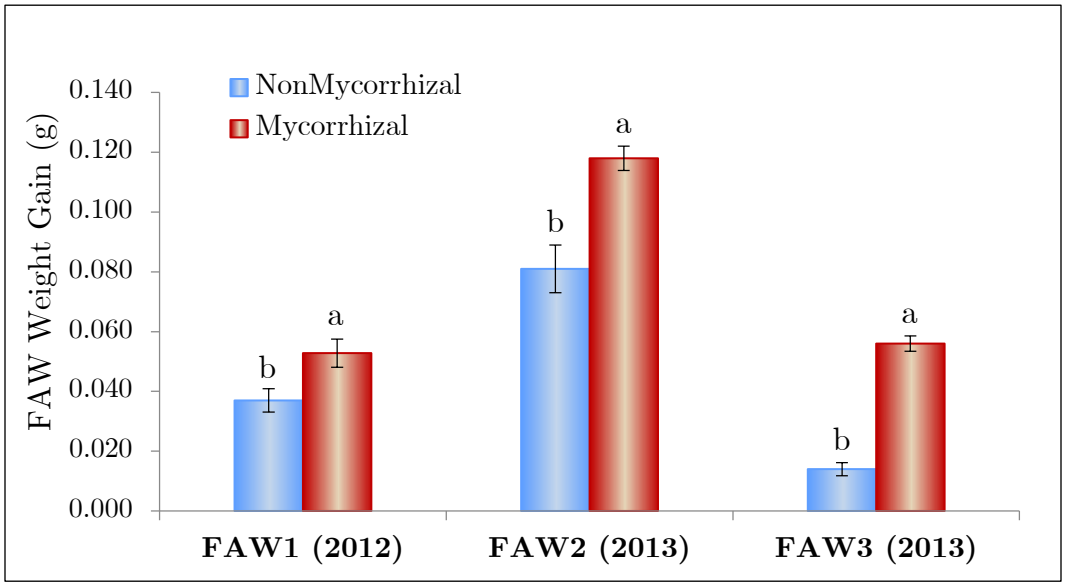

Figure 4.3. Weight gain (g ± SE) of *Spodoptera frugiperda* larvae fed on rice leaves from nonmycorrhizal (NM) and mycorrhizal (M) plants in lab studies during 2012 and 2013. Feeding assays were performed for 4, 7 and 10 days with larvae of 4 to 5 days old. NonMycorrhizal: rice seeds + sterilized AMF, Mycorrhizal: rice seeds + live AMF. Bars and lower case letters at the column head indicate that means differ significantly (LSD, $P \leq 0.05$).

4.3.5 Effects of AMF inoculation on plant resistance to sheath blight

In order to determine whether AMF-induced rice susceptibility also extends to pathogenic microorganisms, we analyzed the infection levels by ShB in rice stems. In two independent experiments, inoculation of rice roots with AMF significantly increased both measures of damage caused by ShB, i.e. lesion length (ShB1: $F_{1,28} = 11.83$, $P = 0.0018$; ShB2: $F_{1,28} = 31.80$, $P < 0.0001$) and numbers of lesions (ShB1: $F_{1,28} = 17.06$, $P = 0.0003$; ShB2: $F_{1,28} = 34.27$, $P < 0.0001$). Lesion length in M rice plants was 38% and 40% greater in ShB1 (3.86 ± 0.38 cm to 2.40 ± 0.20 cm, mean ± SE, n = 15) and ShB2 (10.85 ± 0.56 to 6.53 ± 0.52 cm, mean ± SE, n = 15), respectively, compared with lesion length in NM control plants. Similarly, the numbers of lesions in the two experiments were greater on M rice plants as compared to the NM plants (37% greater in ShB1: 3.67 ± 0.30 to 2.31 ± 0.14, mean ± SE, n = 15 and 38% greater in ShB2: 8.29 ± 0.39 to 5.16 ± 0.36, mean ±

SE, n = 15). Leaves from M plants developed clear symptoms of infection at 3 days post-inoculation. At this time, only small necrotic spots were evident on NM plants. Lesions advanced aggressively on the leaves of mycorrhizal plants, and after 7 days post-inoculation these leaves were severely damaged (Figure A4). Overall, these results show that AMF-induced rice susceptibility is also observed with an aboveground fungal pathogen (Figure 4.4).

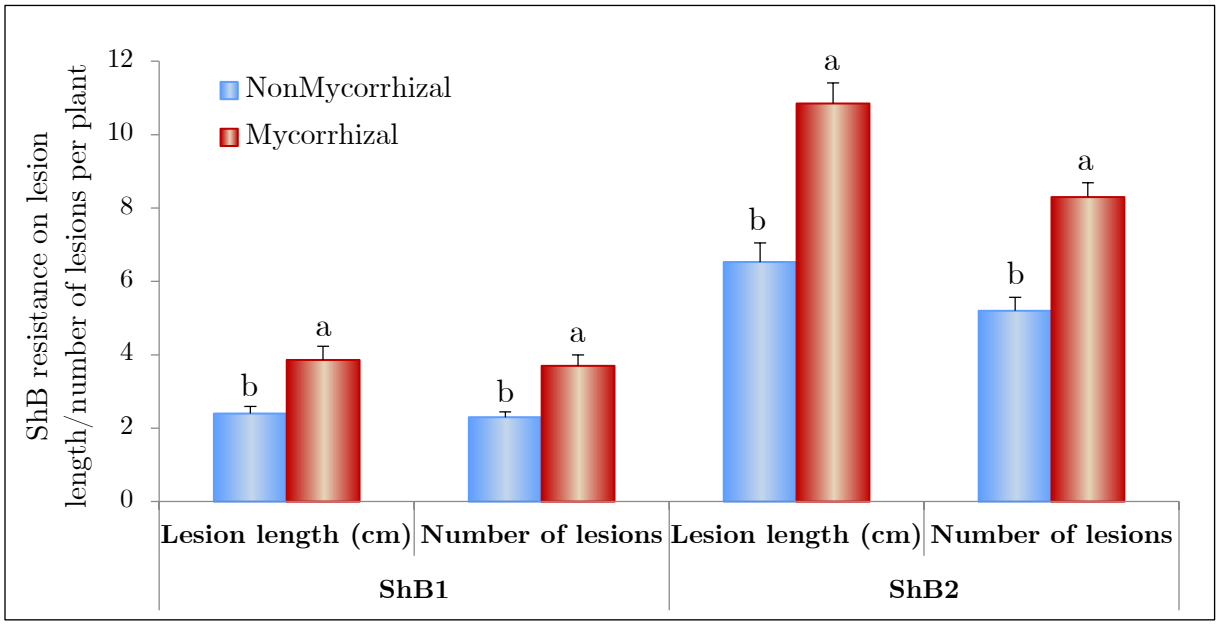

Figure 4.4. Rice sheath blight disease variables (lesion length and number of lesions) measured after inoculation with isolate LR172 of *Rhizoctonia solani* in mycorrhizal and nonmycorrhizal rice plants in greenhouse experiments in the summer 2013. NonMycorrhizal: rice seeds + sterilized AMF, Mycorrhizal: rice seeds + live AMF. Bars and lower case letters at the column head indicate that means differ significantly (LSD, $P \leq 0.05$).

4.3.6 Effects of AMF inoculation on plant biomass

In Exp-2, the shoot biomass of M rice plants differed significantly from the shoot biomass of rice plants treated with sterilized inoculum (NM) or with fungicides and sterilized inoculum (F) ($F_{2,6} = 12.15$, $P = 0.008$), ranging from 2.17 to 3.94 g (Table 4.3).

The effect of AMF inoculation on root biomass and root-to-shoot ratio was not significant (Table 4.3). In 75-day-old rice plants, the SDW of M rice plants was 32.7% higher than the SDW of NM plants. In the PB1 experiment, M rice plants exhibited significantly higher shoot biomass than NM plants ($F_{1,11} = 6.53$, $P = 0.027$) (Table 4.3), ranging from 0.88 to 1.09 g (Table 4.3). As in Exp-2, neither root biomass nor root-to-shoot ratio of rice plants differed among the different AMF treatments (Table 4.3). The SDW of the 30-day-old rice plants was 19.3% higher in M plants as compared to NM plants (Table 4.3).

4.3.7 Effects of AMF inoculation on plant nutritional status

No effects of AMF inoculation on concentrations of plant nutrients were found in either the field experiment, Exp-2, which showed low levels of AMF colonization in the non-inoculated controls, or in the greenhouse experiment (PB1), which had a nonmycorrhizal control without AMF (Supplementary Table A3). Therefore, the increases in shoot biomass and susceptibility to pests in AMF-inoculated plants were not accompanied by increases in concentrations of N, P, K or C (Supplementary Table A3).

Table 4.3. Results from one-way ANOVA on the effect of arbuscular mycorrhizal fungi (AMF) on the shoot and root dry weight biomass and root: shoot ratio of 75 and 30 day-old rice plants from a field (Exp-2) and a greenhouse experiment (PB1) in 2013. DW = Dry Weight. Mean values followed by different letters within columns indicate a significant difference among treatments by Least Significant Difference mean comparisons ($P < 0.05$; LSD). The F, NM, and M refer to AMF treatments of F: rice seeds + fungicides + sterilized AMF, NM: rice seeds + sterilized AMF, and M: rice seeds + live AMF. *The relative change (%) in root, shoot and ratio was calculated by dividing the difference of AMF and non-AMF by AMF treatment.

Treatments	Shoot DW (g)	Root DW (g)	Root DW / Shoot DW
Field 2013 (Exp-2)	Mean ± SE	Mean ± SE	Mean ± SE
Fungicide (F)	2.17 ± 0.38b	1.02 ± 0.08b	0.50 ± 0.07a
Nonmycorrhizal (NM)	2.65 ± 0.48b	1.19 ± 0.27a	0.45 ± 0.04a
Mycorrhizal (M)	3.94 ± 0.36a	1.25 ± 0.21a	0.34 ± 0.08a
	(32.7%)*	(4.8%)*	(-32.4%)*
$F_{2,6}$	12.15	0.38	2.15
P-value	0.008	0.699	0.198
GH 2013 (PB1)	Mean ± SE	Mean ± SE	Mean ± SE
Nonmycorrhizal (NM)	0.88 ± 0.05b	0.51 ± 0.05a	0.57 ± 0.05a
Mycorrhizal (M)	1.09 ± 0.06a	0.60 ± 0.04a	0.56 ± 0.04a
	(19.3%)*	(15.0%)*	(-1.8%)*
$F_{1,11}$	6.53	2.46	0.02
P-value	0.027	0.145	0.901

4.4 Discussion

Interactions among AMF and plants can alter the suitability of plants for herbivores and pathogens. These effects have been investigated in a number of systems (Currie et al., 2011; Gange & West, 1994; Pineda et al., 2010) but have not been extensively investigated in rice, one of the most important crops not only in the United States but also worldwide (Campos-Soriano et al., 2011; Cosme et al., 2011). In this study, we used a commercial formulation of AMF containing multiple species from the Glomeraceae family to investigate the effects of inoculation with AMF on rice resistance against two important herbivores and one important pathogen. These biotic interactions were investigated in a wetland rice system. It is widely recognized for wetland systems that, although AMF can live through the year and occur in all plant developmental stages, flooding strongly suppresses levels of AMF colonization of roots (Miller & Bever, 1999; Miller & Sharitz, 2000; Purakayastha & Chhonkar, 2001; Solaiman & Hirata, 1995, 1996, 1997). Previously observed colonization levels in wetland rice under flooded conditions have ranged from 4% at 14 dai (Cosme et al., 2011), 5% at 30 dai (Campos-Soriano et al., 2010), 2-12% at 60 dai (Solaiman & Hirata, 1995), 14-29% at 40 dai (Purakayastha & Chhonkar, 2001), and >30% at 75 dai (Solaiman & Hirata, 1997). Such low levels of colonization by AMF in wetland rice have nonetheless been associated with significant impacts on plant growth and nutrition (Purakayastha & Chhonkar, 2001; Solaiman & Hirata, 1995, 1996, 1997). In addition to the suppressive effects of flooding on AMF colonization, not all tissues of rice roots are susceptible to AMF colonization. Previous studies have shown that only large lateral roots of rice are substantially susceptible to AMF colonization, whereas crown roots are generally poorly colonized and fine lateral roots are never colonized (Gutjahr et al., 2009; Gutjahr et al., 2015a). Such specialization in colonization dilutes the levels of colonization in the whole root system. Thus, the low levels of colonization of rice roots by AMF observed using the sampling and staining techniques described in this study were not surprising. Despite the low levels of

colonization in our experiments, we detected significant impacts of AMF on susceptibility of rice to both below- and above-ground pest organisms. We found that AMF inoculation caused a strong positive effect on the performance of the leaf-feeding insect FAW and the root-feeding RWW, as well as on the severity of disease caused by a fungal pathogen. The increased susceptibility of rice to herbivores and a pathogen in AMF-inoculated plants was not associated with changes in plant nutrient concentrations but was associated with an increase in shoot biomass. Taken together, these results show that the interactions of rice roots with AMF caused a broad-spectrum reduction in resistance to pests of rice, perhaps by altering defense-related pathways.

The increases in susceptibility to RWW in AMF-inoculated field plots, particularly in Exp-1, were greater than the differences in RWW densities typically observed among resistant and susceptible varieties of rice (N'Guessan et al., 1994; Stout et al., 2001), suggesting that the symbiotic status of rice plants might be a crucial component of susceptibility to RWW in the field. There was, however, some variability in the response of rice to AMF inoculation. In the second and third core samplings of Exp-2, and again in the second core sampling of Exp-3, densities of immature RWW did not differ between the M and NM treatments. The reasons for this variability in response to AMF inoculation are not known. One possible reason is that sample and plot sizes might not have been sufficiently large to detect a weak effect of AMF inoculation among treatments, and it is interesting to note that all means in all core samplings trended in the direction of higher weevil densities in AMF-inoculated plants. Furthermore, experiments in 2012, when effects of AMF inoculation were large, and experiments in 2013, when effects were smaller, utilized different rice varieties ('Lemont' in 2012 and 'Cocodrie' in 2013), and were subject to different environmental conditions because they were conducted in different fields. With respect to the effect of rice variety, plant responses to AMF inoculation are known to vary among varieties within a plant species (Sawers et al., 2010).

The effectiveness of our experimental treatments in establishing AMF symbiosis was verified by quantifying AMF colonization in root samples in seven of our experiments. Although AMF colonization was not verified in all individual experiments, the substantial and statistically significant increases in colonization in response to commercial inoculants in the seven experiments in which colonization was assessed supports the assumption that addition of inoculum led to increased colonization in experiments in which mycorrhizal colonization was not quantified. An unresolved question in our experiments is whether actual colonization of rice roots differed among the six species of fungi in our inoculum, as we did not examine changes in colonization by individual fungal species. Different species and combinations of AMF are known to have different effects on plant resistance to herbivores (Gange, 2001; Roger et al., 2013).

The effects of AMF colonization on plant-herbivore and plant-pathogen interactions have been variable in previous studies (Barber et al., 2013b; Bennett & Bever, 2007; Currie et al., 2011; Gange, 2001; Hartley & Gange, 2009; Jung et al., 2012; Koricheva et al., 2009). The effects of AMF colonization on herbivores and pathogenic microorganisms depend on numerous factors, including host plant species, AMF species, herbivores or pathogens involved, and environmental conditions (Pineda et al., 2010). Our study contributes to a growing body of evidence that the effects of AMF in plants do not always lead to priming of plant tissues for a more efficient activation of defense mechanisms (Pozo & Azcon-Aguilar, 2007). This study also extends a previous report of positive effects of AMF inoculation on RWW oviposition (Cosme et al., 2011) and shows that the positive effects of AMF inoculation on RWW are observed in a different developmental stage of RWW. Furthermore, the oviposition preference of RWW for mycorrhizal over nonmycorrhizal plants (Cosme et al., 2011) coupled with the higher performance of RWW larvae on mycorrhizal plants (this study) provides support for the preference-performance hypothesis for belowground herbivores, which predicts that when insect herbivores have

offspring with limited mobility, there will be strong selection pressure for adults to oviposit on plants that maximize offspring performance (Johnson et al., 2006).

As noted above, several previous studies have, like this one, found positive effects of AMF inoculation on herbivore performance. Currie et al. (2011) found colonization of clover plants by AMF increased on survival of larvae of the specialist clover root weevil (*Sitona lepidus*). Likewise, Goverde et al. (2000) reported that survival and larval weights of the common blue butterfly (*Polyommatus icarus*) were greater in larvae that fed on *Lotus corniculatus* plants colonized by AMF. Gange et al. (2002) demonstrated that AMF colonization increased the larval growth of the specialists lace border (*Scopula ornata*), mint moth (*Pyrausta aurata*), and redcurrant aphid (*Cryptomyzus ribis*) on plants in the Lamiaceae family. The stronger performance of RWW, an oligophagous insect that specializes on grasses, on AMF-inoculated rice is consistent with results of a meta-analysis (Koricheva et al., 2009) that noted a general pattern in which most specialist chewing insects, but not most generalist insects, perform better on plants colonized by AMF than on non-colonized plants. However, our results with the generalist FAW, which showed higher larval growth on AMF-inoculated rice plants, contradicts this general pattern. Gange et al. (2002), similarly found that AMF colonization had a positive effect on the growth of the generalist aphid (*Myzus persicae*), and Hoffmann et al. (2009) showed that females of the generalist two-spotted spider mite (*Tetranychus urticae*) preferentially resided and oviposited at a higher rate on common bean plants colonized by AMF.

The effects of AMF colonization on aboveground pathogenic microorganisms have also been investigated in several prior studies. In rice in particular, Campos-Soriano et al. (2011) found that AMF confers enhanced rice resistance against infection by the rice blast fungus. In our experiments with ShB, we found that mycorrhizal rice plants were more susceptible to infection by *R. solani* than nonmycorrhizal plants. Because flooded rice plants were used in our study, and non-flooded plants in the study by Campos-Soriano et al. (2011), it is possible that water regime might affect the impact of AMF on rice

resistance to ShB, although other experimental differences may also have contributed to these contrasting results. Altogether, our results underscore the variability of the effects of AMF colonization in plant-insect and plant-pathogen interactions.

There are three major hypotheses to explain the increases in rice susceptibility when colonized by AMF in this study. First, the interaction of AMF with rice might increase susceptibility to pests by increasing plant quantity (biomass) with no change in plant quality. Bennett et al. (2006) refer to this hypothesis as the "nutritional quantity hypothesis". Second, AMF colonization might increase the quality of plant tissues for herbivores by improving plant nutrient status, which is referred by Bennett et al. (2006) as the "nutritional quality hypothesis". In our experiments, we found no support for the nutritional quality hypothesis; no significant differences in concentrations of P, N, K and C, the nutrients that are most frequently studied in plant-AMF experiments, were found among AMF-inoculated plants and non-inoculated controls. In a previous study using the same rice-RWW system, however, (Cosme et al., 2011) found that increased oviposition preference of RWW adults on mycorrhizal rice plants was associated with increased N and P concentrations. The effects of AMF on plant nutritional status have been widely studied in other systems, particularly effects of AMF on P, where P deficiency in soil promotes mycorrhizal formation (Babikova et al., 2014b; Cosme & Wurst, 2013; Secilia & Bagyaraj, 1994). In contrast to the results for nutrient status, we observed that AMF inoculation increased shoot biomass of rice plants in field and greenhouse studies (Table 4.3), which is in agreement with previous studies (Campos-Soriano et al., 2010). This result is consistent with the nutritional quantity hypothesis for RWW first instars, FAW and ShB, which live on above ground plant tissues. However, the relatively moderate increases in shoot biomass observed are unlikely to fully account for the substantial increases in susceptibility to pests found in greenhouse experiments. This is particularly true for the increase in FAW susceptibility, as the FAW assay used excised leaf tissue and insects were never food-limited.

A third major hypothesis to explain increases in rice susceptibility in this study involves AMF-mediated changes in the expression of plant defenses via modulation of phytohormone signaling and consequent reprogramming of defense-related gene expression and other processes (Gutjahr, 2014; Jung et al., 2012; Pozo et al., 2015). There is evidence that AMF colonization can prime or otherwise affect jasmonic acid (JA)- and salicylic acid (SA)-dependent pathways (Herrera-Medina et al., 2008; Jung et al., 2012; Koricheva et al., 2009; Pozo & Azcon-Aguilar, 2007), and that these changes in plant signaling can lead to enhanced or decreased plant resistance against herbivores or pathogens (Campos-Soriano et al., 2011; Jung et al., 2012). Fontana et al. (2009) demonstrated that mycorrhizal symbiosis induced qualitative and quantitative changes in the production of volatile compounds of *Plantago lanceolata* plants when they were infested by caterpillars of *Spodoptera* spp. In another study, Jung et al. (2012) reported that AMF plants were usually more resistant to necrotrophs and chewing insects, which are affected by JA-dependent defense responses, and more susceptible to biotrophs (Jung et al., 2012). Thus, the evolution of plant-AMF interactions has apparently resulted in a repertoire of responses to AMF colonization that influence interactions with insects and pathogens (Babikova et al., 2014a; Babikova et al., 2014b; Gehring & Bennett, 2009; Gilbert & Johnson, 2015; Gutjahr & Paszkowski, 2009; Jung et al., 2012; Kiers et al., 2010; Pozo et al., 2015). However, the impact of AMF on plant defense hormone levels and gene transcription vary depending on the genotypes of the partners and other factors (Fernández et al., 2014).

In rice in particular, inoculation of unflooded roots with AMF induces a complex transcriptomic reprogramming, leading to enrichment of transcripts associated with phytohormones and secondary metabolism (Fiorilli et al., 2015; Gutjahr et al., 2015a). In our study, the fact that large effects of AMF inoculation on plant resistance were observed despite low levels of AMF colonization suggest that inoculation with AMF induced a systemic reprogramming of defense-related processes. However, the exact AMF-induced

changes in JA and SA signaling and consequent changes in gene expression that influence the systemic susceptibility of wetland rice remain to be elucidated. Work is in progress to investigate expression levels of genes involved in the JA and SA signaling pathways of leaf tissues following AMF inoculation and FAW feeding using an RNA-seq and real time -PCR.

In summary, this study demonstrates that inoculation of rice plants with AMF rendered the plants more susceptible to pests without causing dramatic changes in plant nutrient concentrations. Our study highlights that AMF can compromise plant resistance and suggests that caution should be used when considering large scale applications of commercial AMF inoculant. However, despite the negative effects on plant resistance observed in this study, it would be premature to conclude that AMF does not have practical benefits for rice production. The higher shoot biomass of AMF-inoculated plants observed in two experiments in this study suggests that AMF inoculation may positively impact rice growth and perhaps yields under some circumstances. Moreover, the negative impact of AMF on plant resistance may not occur in all soil environments. Barber et al. (2013b), for example, found that the effects of AMF on plant nutrition vary with soil source and therefore soil characteristics may influence the effects of AMF colonization on herbivores. Although the effects of AMF on rice susceptibility were consistent in our study, the strength of these effects appeared to vary under the different conditions present in different experiments. Work is in progress to investigate whether different soil attributes, (e.g., soil P concentrations), alter the effects of AMF inoculation on the performance and growth of RWW and FAW in rice. Moreover, experiments are also being conducted to characterize the impacts of AMF inoculation on rice growth and yield when insects are not present. Responses to AMF provide a unique window for studying the traits or characteristics that make rice plants more susceptible or tolerant to insect and pathogen attack. A better understanding of the interactions of rice and other crops with AMF in the rhizosphere and with the different organisms they encounter both above and below

ground may be a key to increasing plant productivity and improving pest management with less input of harmful chemicals.

4.5 References

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Chapter 5
Effects of Arbuscular Mycorrhizal Fungi on Rice-Herbivore Interactions are Soil-Dependent

5.1 Introduction

Arbuscular mycorrhizal fungi (AM fungi) belong to the phylum Glomeromycota and are obligate symbionts that form mutualistic associations with the roots of ca. 90% of terrestrial plants (Smith & Read, 2008). AM fungi are found in almost all soils (Bernaola et al., 2018a; Jansa et al., 2009) and share a long history of coevolution with plants in various ecosystems, resulting in adaptation to specific geographic areas (Gosling et al., 2006). The most important function of these symbiotic associations involves the transfer of nutrients such as phosphorus (P) and nitrogen (N) by the fungus to the host plant in exchange for carbon (C), in the form of sugars and lipids (Luginbuehl et al., 2017; Smith & Read, 2008), to the fungi by the plants. Colonization by AM fungi alters plant growth and also influences the interactions of plants with insect herbivores (Barber et al., 2013b), although the mechanisms remain to be elucidated. The effects of colonization by AM fungi on plant-herbivore interactions are variable; colonization by AM fungi can have beneficial, detrimental, or no effects on herbivore fitness (Gehring & Bennett, 2009; Hartley & Gange, 2009; Koricheva et al., 2009). For example, a detrimental effect was reported for black wine weevil feeding on AM fungi-inoculated strawberry plants (Gange, 2001), beneficial effects were reported for rice water weevil feeding on AM fungi-colonized rice (Bernaola et al., 2018b; Cosme et al., 2011) and clover root weevil feeding on AM fungi-colonized clover plants (Currie et al., 2011), and no effect was seen for *Junonia* coenia feeding on *Plantago lanceolata* (Bennett & Bever, 2007). The net effect of colonization by AM fungi on herbivores may depend on the balance of the positive effects resulting from increases in concentrations of plant nutrients and the negative effects resulting from increases in

plant defenses against herbivores (Bennett et al., 2006; Currie et al., 2011; Vannette & Hunter, 2011).

Inoculation of soil with commercial AM fungi has been proposed as an alternative production practice that may contribute to more efficient nutrient use in crops (Kohl et al., 2016). Despite extensive research on the effects of AM fungi on their host plants, the impacts of agricultural practices such as fertilization, tillage, and monoculture that can affect the soil environment and, therefore, AM fungi colonization are insufficiently known (Gosling et al., 2006; Köhl et al., 2014; Lekberg & Koide, 2005; Verbruggen et al., 2010). For instance, Barber et al. (Barber et al., 2013a) reported that intensive conventional agriculture may select for inferior mutualists such as AM fungi. Furthermore, it has been demonstrated that high concentrations of P in the soil negatively influence AM fungi colonization in different crop plants (Gosling et al., 2013). Inoculation of soil with commercial AM fungi has been proposed as an alternative production practice that may contribute to more efficient nutrient use in crops (Kohl et al., 2016). However, the effectiveness of soil inoculation with AM fungi varies their response to the same AM fungi species mix (Berruti et al., 2016). The disadvantages of soil inoculation with commercial formulations of AM fungi in agricultural fields include high application costs, the lack of positive effects of AM fungi under conditions of high nutrient (especially P) availability, and lack of effect on plant growth in some plants in some environments (Ryan & Graham, 2002). Despite these challenges, a meta-analysis conducted by Berruti et al. (2016) revealed that soil inoculation with AM fungi increased root colonization rates, and increased root colonization rates led in turn to increased root and shoot biomass, improved plant nutrition, and higher crop yields under diverse experimental conditions. Because the effects of inoculation with AM fungi on plant nutrition and other plant traits vary with soil source, soil characteristics will likely influence the effects of AM fungi colonization on herbivores (Barber et al., 2013b).

Rice (*Oryza sativa* L.) is one of the world's most important cereal crops and is also important crop in the southern United States. In the southern U.S., including Louisiana, the majority of rice is grown under a delayed-flood cultural system in which rice is drill-seeded into dry soil, surface-irrigated as necessary to establish a stand, and flooded approximately four weeks after seeding (Hamm et al., 2010). Rice is very susceptible to different insect pests, which are one of the major problems during the growing season. The rice water weevil (*Lissorhoptrus oryzophilus*, RWW) and fall armyworm (*Spodoptera frugiperda*, FAW) are two chewing pests that can cause significant economic losses in rice production (Hamm et al., 2010; Stout et al., 2009). Current management practices to control these pests rely on the use of insecticides, but insecticides are expensive and also can cause environmental harm. Only a few studies have explored how AM fungi colonization influences the resistance of rice plants to herbivore feeding or pathogen infection and their consequences for rice fitness, with contrasting results (Campos-Soriano et al., 2011; Cosme et al., 2011). Campos Soriano et al. (2011) reported that inoculation with AM fungi enhanced resistance to the foliar pathogen *Magnaporthe oryzae*, while Cosme et al. (2011) found that females of the root-feeding RWW laid more eggs in rice plants inoculated with AM fungi, an effect that may have been caused by AM fungi-mediated increases in plant nutrient concentrations. Recently, Bernaola et al. (2018b) demonstrated that AM fungi inoculation increases local and systemic susceptibility of rice plants to different pest organisms, including RWW and FAW in field and greenhouse conditions. It is still not clear how soil characteristics influence colonization by AM fungi or the effects of colonization by AM fungi on the interaction between rice and its insect herbivores. In particular, whether AM fungi colonization reduces rice resistance in all soil environments is still not known.

In this study, we investigated how soil type altered the effects of inoculation of rice plants with a commercial formulation of AM fungi on plant growth and plant-herbivore interactions. We conducted field and greenhouse experiments with two soil types differing

in nutrient concentration levels. A commercial formulation of AM fungi containing six species of *Glomus* was used, and effects of inoculation with AM fungi on performance of two insects were assessed. This study represents the first study to demonstrate the soil dependency of the effects of AM fungi inoculation on plant-herbivore interactions in rice. Here, two hypotheses were tested:

(H1) The effects of inoculation with AM fungi on rice-herbivore interactions differ in soils that have different properties such as concentrations of P and/or N.

(H2) The effects of inoculation with AM fungi on plant growth, plant nutrient concentrations and yield differ in soils that have different properties.

These data will facilitate the agricultural exploitation of AM fungi-crop symbiosis.

5.2 Materials and methods

Experiments were conducted under both field and greenhouse conditions. Field experiments were conducted at two locations with different soil properties to compare effects of inoculation with AM fungi on rice growth and RWW population densities in soils with different properties. Greenhouse experiments were conducted using soil collected from the two field locations to compare effects of inoculation with AM fungi on FAW growth rates in different soil types.

5.2.1 Plants, fungi, insects, and soil sources

Two commercial varieties of rice (*Oryza sativa* L.) were used in our experiments. 'Cocodrie' and 'CL111' are both long-grain, high-yielding, early-maturing conventional varieties developed at the Louisiana State University Agricultural Center (LSU AgCenter) H. Rouse Caffey Rice Research Station (Crowley, Acadia, LA, USA). 'Cocodrie' is susceptible to RWW and grown widely in the southern U.S., and was chosen for this study

because it had been used in previous studies of rice-mycorrhizal-herbivore interactions (Bernaola et al., 2018b). 'CL111' is an herbicide-tolerant variety chosen because it was the most widely grown rice variety in Louisiana in 2014-2015. Seeds of rice were kindly provided by the breeding and foundation seed program at the LSU AgCenter H. Rouse Caffey Rice Research Station.

A commercial inoculum of AM fungi containing only AM fungal propagules (ECOVAM^{TM} VAM Endo Granular, Horticultural Alliance Inc., Sarasota, FL, USA) was selected to establish and promote symbiosis with rice plants in both field and greenhouse experiments. The inoculum consisted of spores, hyphae and colonized root fragments of six species of AM fungi as described in Bernaola et al. (2018b). All AM fungi species were originally obtained from the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM, West Virginia University, USA). The AM fungi propagules were carried in inert material consisting of a uniform mixture of zeolite, pumice, vermiculite, perlite and attapulgite. The formulated material contained an average of 132 spores of AM fungi (all species) per gram, in addition to hyphae and colonized root fragments.

The rice water weevil (RWW; *Lissorhoptrus oryzophilus* Kuschel; Coleoptera: Curculionidae) is the most destructive insect pest of rice in the United States (Hamm et al., 2010; Stout et al., 2002; Tindall & Stout, 2003). Field experiments relied on natural infestations of RWWs, which are abundant at the field sites (Hamm et al., 2010). Adult RWWs feed on young rice leaves, producing longitudinal scars, and females lay eggs primarily in leaf sheaths of flooded rice plants. Larval RWW have a strong impact on rice yields by feeding on roots of flooded rice (Cosme et al., 2011).

Larvae of the fall armyworm (FAW, *Spodoptera frugiperda* J.E. Smith; Lepidoptera: Noctuidae) were obtained from a colony maintained continuously on meridic diet in a laboratory. The colony originated from larvae collected in rice fields near Crowley, LA, in 2013. Adult female armyworms oviposit eggs on leaf blades and other substrates, giving

rise to larvae that feed on leaves (Stout et al., 2009). The diet used for rearing of larvae was Fall Armyworm Diet (Southland Products Incorporated, Lake Village, AR, USA). The colony was maintained under controlled environmental conditions (L14: D10, 28 ± 2°C, 38 ± 2% R.H).

Field experiments were conducted at, and soils were sourced from, two locations in southwest Louisiana. The first location was the LSU AgCenter H. Rouse Caffey Rice Research Station (Crowley, Acadia Parish, 30°14'22" N, 92°20'46" W), while the second location was in a farmer's field in Mamou, Louisiana (Evangeline Parish, 30°38'28" N, 92°27'33" W). The physicochemical properties of soils from the two sites were analyzed by the LSU AgCenter Soil Testing & Plant Analysis Laboratory (STPAL, LSU, Baton Rouge, LA). The soils varied in their properties as shown in Table 5.1. Notably, soil P and K were at least four and three times higher in the Crowley soil than in the Mamou soil, respectively. The Mamou soil was more acidic (pH 5.1) than the Crowley soil (pH 7.4).

For greenhouse experiments, soils were collected from the top 6 inches of topsoil at each of the field sites described, in early summer in 2014. Before used in greenhouse experiments, soil was sterilized at 121°C for 60 min. After sterilization, Crowley and Mamou soils had a pH of 7.7 and 4.7, a total P content of 31.5 and 10.9 mg/kg, and a total K content of 132.4 and 44.5 mg/kg, respectively.

Table 5.1. Properties of soils collected from two different locations for experiments conducted in 2014 and 2015. Average values for soils collected over two years are shown (means ± SE, n = 2).

Rice field	Soil name	Soil type	Location	pH	N %	P mg/kg	K mg/kg
H. Rouse Caffey Rice Research Station	Crowley	Silt loam	Acadia Parish, Louisiana	7.4 ± 0.2	0.097 ± 0.0	33.3 ± 0.5	117.6 ± 101
Kenneth LaHaye Farm	Mamou	Mowata silt loam	Evangeline Parish, Louisiana	5.1 ± 0.0	0.099 ± 0.0	8.6 ± 0.8	36.5 ± 6.5

5.2.2 Field experiments

Previous small-plot experiments conducted at the Crowley location established that inoculation with a commercial formulation of AM fungi often increased the susceptibility of rice to RWW (Bernaola et al., 2018b). For the current study, four small-plot field experiments (one in 2014 and three in 2015) were carried out to evaluate the effects of soil type on the susceptibility of RWW to AM fungi inoculation. Experiments were designated as: Rice Water Weevil Mamou 1 (RWW-M1), Rice Water Weevil Mamou 2 (RWW-M2), Rice Water Weevil Crowley 1 (RWW-C1) and Rice Water Weevil Crowley 2 (RWW-C2) (Table 5.2).

All experiments were laid out in a completely randomized design (CRD) and each experiment included two treatments, one in which plots were inoculated with AM fungi and one in which plots were inoculated with a nonmycorrhizal control. Each of the two treatments was replicated five times, resulting in 10 plots per experiment. For the nonmycorrhizal control, plots were seeded into soils treated with a mock inoculum containing all the inert ingredients of the AM fungi inoculum but without the AM fungi. For the mycorrhizal treatment, rice seeds were sown in soil inoculated with live AM fungi.

Mock or live inoculum was applied to the surface of the soil after planting and gently raked in to incorporate the live or mock inoculum into the upper 2.5 cm of the soil. Because rice was grown in the field, soil was not sterilized and likely contained native AM fungi.

Rice was drill-seeded on the dates specified in Table 5.2 at a rate of 85 g (68 kg/ha) of seeds per plot. Plots measured 1.4 m x 4.9 m. Each plot was inoculated with 17 kg of mock inoculum or live inoculum. The inoculum amounts used in both years corresponded to approximately 2.2 million AM fungi spores per plot. Plots were flushed with well water as necessary for the first month after seeding to establish stands of rice. After allowing the plants to grow without a flood for approximately one month, permanent floods were applied on the dates specified in Table 5.2. Plants possessed 4-5 leaves (early tillering) at permanent flooding.

Densities of RWW larvae and pupae were determined after permanent flooding by taking root/soil core samples from each plot (Stout et al., 2001). The core sampler was a metal cylinder with a diameter of 9.2 cm and a depth of 7.6 cm attached to a metal handle. Core sampling was conducted twice at the Mamou site and three times at the Crowley site for all experiments. All core sampling was conducted between three and five weeks after permanent flood. Dates of core samplings are shown in Table 5.2. For each core sampling, two or three (2014) and three or four (2015) core samples were taken from each plot. Core samples were transported in plastic bags to a processing facility, where each sample was placed into a 40-mesh screen sieve bucket to wash the soil and larvae from roots. Buckets with rinsed samples were placed into basins of salt water, and larvae and pupae were counted as they floated to the water surface (N'Guessan et al., 1994). RWW counts from two to four core samples from each plot per sampling date were averaged to obtain mean densities of immature weevils (larvae and pupae) per core sample.

5.2.3 Greenhouse experiments

Additional experiments were conducted in the greenhouse to further test the hypothesis that differential effects of inoculation with AM fungi on susceptibility to insects were attributable to differences in the properties of soil at the two field sites. Two laboratory feeding assays were conducted in 2014 using cut leaf material to determine whether mycorrhizal inoculation affected growth of FAW larvae. Experiments were designated as Fall Armyworm 1 (FAW-1) and Fall Armyworm 2 (FAW-2) (see Table 5.2). 'Cocodrie' rice plants were grown under two treatments, namely mycorrhizal and nonmycorrhizal.

All plants were grown in 2 liter round (15 cm diameter) plastic pots (Hummert International, Earth City, MO) filled with sterilized soil from one of the two field sites to which 50 g of mycorrhizal inoculum or 50 g mock inoculum were added. The inoculum was thoroughly mixed with the soil before filling pots. Four rice seeds were sown per pot and a total of 25 pots per treatment were set up. Plants were maintained under greenhouse conditions with temperatures ranging from 25°C to 35°C and ambient lighting. Rice seedlings were thinned to two plants per pot two weeks after planting. Leaves for FAW feeding assays were taken from plants that were three weeks old; plants possessed three or four leaves at the time experiments were initiated. Because these experiments were conducted with rice at an early stage of growth, additional fertilizer was not necessary for satisfactory plant growth.

Neonate FAW that had eclosed within 24 hours were used for feeding assays. Feeding assays were conducted in 9 cm plastic petri dishes lined with moistened cotton batting to maintain turgor in excised tissues. Youngest fully-expanded leaves were removed from plants of each treatment group using scissors, transported on ice to the laboratory, cut into ca. 7 cm pieces, and placed in petri dishes. Three neonates were placed together in each petri dish with foliage and allowed to feed on excised leaf material for 10 days in

Table 5.2. Planting and insect sampling dates for field and greenhouse experiments conducted over the 2014 and 2015 growing seasons to evaluate the effects of inoculation with AM fungi on the performance of rice water weevil and the growth of fall armyworm on rice plants

Year	Experiment	Planting date	Flooding date	AM fungi sampling date	RWW core sampling dates
Field					
2014	RWW-M1	21st April	23rd May	20th May & 6th June	12th & 18th June
2015	RWW-M2	31st March	15th May	5th May	5th & 9th June
	RWW-C1	25th March	15th May	8th May	9th, 16th & 23rd June
	RWW-C2	4th May	10th June	5th June	30th June, 6th & 13th July
Year	Experiment	Planting date	AM fungi sampling date		FAW final weight measurements
Greenhouse					
2014	FAW-1	1st Jul	30th July		11th August
	FAW-2	26th Aug	-		17th October

each experiment. Larvae were observed daily to ensure they were not food-limited and leaves were changed every other day (every day for larvae in later stages). After ending the feeding assay, larvae were starved for three hours to ensure that the larval gut was emptied before final masses were determined. The mean mass of the remaining larvae in each petri dish was calculated. Weight gain (final weight) was recorded as the response variable and initial weight of neonates was considered to be zero. For each experiment, 20 petri dishes (replicates) were used for each treatment for a total of 80 observations for each of the FAW experiments. Insects that died during feeding assays were excluded.

5.2.4 Quantification of mycorrhizal colonization

In order to verify the effectiveness of AM fungi inoculations, the extent of AM fungi colonization was measured in each experiment. Root colonization by AM fungi was evaluated twice during plant development in RWW-M1, before and after flood establishment. Root colonization was evaluated once (before flooding) in the other field (RWW-M2, RWW-C1 and RWW-C2) and greenhouse (FAW-1) experiments. Sampling was conducted by taking 9.2 cm diameter soil-root cores from field plots, or washing the roots from greenhouse pots containing entire rice plants. For the purpose of this study, one soil-root core (field experiments) or pot (greenhouse experiments) represented one plant sample. Ten root samples from each experiment were randomly collected from five plots or pots of each treatment group per sampling date (Table 5.2). Each soil-root core or pot, containing two to four plants, was placed in plastic bags (one core per bag) and taken to the laboratory to be processed for root staining.

The trypan blue method of Koske and Gemma (1989) was used with minor modifications for root staining of AM fungi colonization. Clearing and staining procedures require root samples to be washed from soil to remove all soil particles and then separating root and shoot tissues. For subsampling, roots from each soil-root core or pot were cut

into 2-cm-long segments and placed in tissue processing cassettes (Ted Pella, Redding, CA). At least 250 small root pieces per root sample (either soil-root core or pot) were cleared in 10% KOH in a water bath at 90°C for 20 min. Clear pieces of roots were rinsed five times with tap water to remove KOH, and roots were immersed in 2% HCl at room temperature for 10-15 min to ensure the roots were effectively acidified for staining. Cassettes containing roots were immediately stained with 0.05% trypan blue (Sigma-Aldrich, St. Louis, MO, USA) by incubation overnight and then transferred to vials containing lactoglycerol at 4°C to allow excess stain to leach out of the roots. Stained root samples were stored in destaining lactoglycerol solution for 48 h before being mounted in the same solution on a microscope slide.

The method of McGonigle et al. (1990) was used with modification for quantifying the abundance of AM fungi colonization. Five microscope slides for each root sample, each containing ten 2-cm-long root fragments, were mounted after staining on microscopic slides. Root fragments were randomly selected from each root sample and are representative of the whole root system as it was not possible to separate root types. A total of 50 root samples were collected from four field experiments and 20 root samples from one greenhouse experiment. For each root sample, 50 stained root fragments (250 stained root fragments per treatment) were examined with a compound microscope (Olympus CH2, Tokyo, Japan) at 40X magnification in order to confirm the levels of AM fungi colonization. The presence of blue-stained mycorrhizal structures in the root fragments including intraradical aseptate hyphae linked to either arbuscules or vesicles/spores were scored as colonized by AM fungi (Figure 5.1) (DeMars & Boerner, 1996). Photos of AM fungi structures on mycorrhizal colonized roots were taken using a microscope-mounted 5.0-megapixel digital camera (Leica DFC480, Cambridge, UK). Percent of root fragments with AM fungi colonization was averaged per treatment for the analyzed experiments.

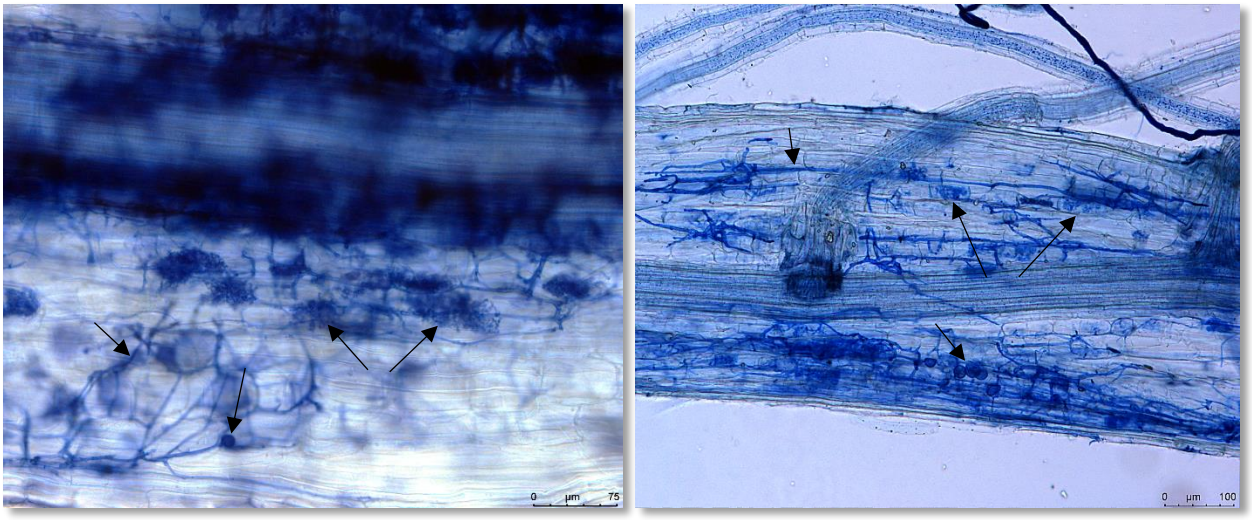


Figure 5.1. Root fragments stained with trypan blue showing arbuscular mycorrhizal fungi structures in rice plants. Light micrographs of mycorrhizal inoculated-root fragments from some experiments conducted in 2015 show: (A) Hyphae (h), arbuscule (a), and spore (v). (B) Hyphae, arbuscule, and spore (s).

5.2.5 Effects of AM fungi on rice growth and nutrient concentrations

To determine the effect of inoculation with AM fungi on plant biomass, entire plants were collected from AM fungi-inoculated and control plots. Four to five weeks after planting, entire plants were harvested from field plots by taking one soil-root core per plot. Entire plants were also collected from pots in greenhouse experiments (see above). Soil was washed from roots, and the shoot (leaf + stem), and root portions of plants were separated and blotted dry with a paper towel. Plant material was dried in an oven (60^{o}C for 1 week) and shoot dry weight (SDW) and root dry weight (RDW) were measured for each plant.

To evaluate whether AM fungi inoculation affected nutrient concentrations in leaves and roots of rice plants, the same plant tissue samples collected for plant biomass were used for plant analysis. After the samples were dried and weighed, portions of plants were submitted to the LSU AgCenter Soil Testing & Plant Analysis Laboratory (STPAL, LSU, Baton Rouge, LA) to determine nutrient concentrations in shoot and root tissues. N and

C content were determined by dry combustion using a LECO TruSpec^{TM} CN analyzer (LECO Corp., St. Joseph, MI, USA), while concentrations of the remaining nutrients (Ca, Mg, S, P, K, Al, B, Cu, Fe, Mn, Na and Zn) were determined by inductively coupled plasma (ICP) analysis.

To assess the effect of the AM fungi inoculation on plant growth (field experiments only), mycorrhizal growth responses (MGR) were calculated as effect sizes using the individual biomass dry weights of the AM fungi-inoculated plants and mean biomass dry weight values of mock-inoculated control plants (average of five plots per treatment).

$$\%MGR = \frac{Dry\ weight\ (\text{AM fungi}-\text{inoculated}) - mean\ dry\ weight\ (\text{mock}-\text{inoculated})}{mean\ dry\ weight\ (\text{mock}-\text{inoculated})} \times 100$$

Yield data were obtained only for field experiments. Four rice rows in the center of each plot were harvested at maturity by a mechanical combine and grain yield (expressed at 12% moisture) was calculated.

5.2.6 Statistical analyses

Prior to analysis, data were analyzed to verify that they met assumptions of normality. Statistical analyses were conducted using SAS 9.4 (SAS Institute 2014). For field experiments, the effect of AM fungi inoculation on root colonization rates, RWW larval densities, plant biomass, nutrient concentrations, and grain yields were analyzed separately with analysis of variance (ANOVA) in PROC MIXED (SAS., 2013). Data for RWW larval densities were analyzed independently each year by repeated measures ANOVA. Inoculation treatment was used as fixed effect and block as a random effect.

For greenhouse experiments, the effect of AM fungi inoculation on root colonization rates and FAW weight gain were analyzed by two-way ANOVAs with 'soil type' (Crowley and Mamou), 'Inoculation treatment', and their interaction as fixed effects, with

replication as a random effect. Means were separated using the least significant difference (LSD) test.

5.3 Results

5.3.1 Field experiments

AM fungi root colonization rates

Colonization of roots of field-grown plants by AM fungi was higher in plots inoculated with commercial AM fungal inoculant than in control plots (Figure 5.2A). The effect of inoculation with AM fungi was significant in RWW-M1 (29 dai, $F_{1,8} = 23.04$, $P = 0.001$), RWW-M2 (40 dai, $F_{1,8} = 140.31$, $P < .0001$), and RWW-C1 (44 dai, $F_{1,8} = 25.57$, $P = 0.001$) (Table 5.3). For RWW-M1, in which colonization was assessed before and after flooding, 29-day-old rice plants inoculated with AM fungi exhibited a colonization rate of 13% before flooding. This colonization rate decreased after 13 days of flooding; colonization rates of 45-day-old (RWW-M1) rice plants inoculated with AM fungi decreased from 13 to 4% (Figure 5.2A) after flooding. The largest values detected for AM fungi colonization in the field experiments were for mycorrhizal plants in RWW-C1 and RWW-M2 with 68.0% and 68.8%, respectively. Overall, our data confirmed that the AM fungi inoculation increased the abundance of AM fungi living in rice roots grown under field conditions even in soils with different P availability.

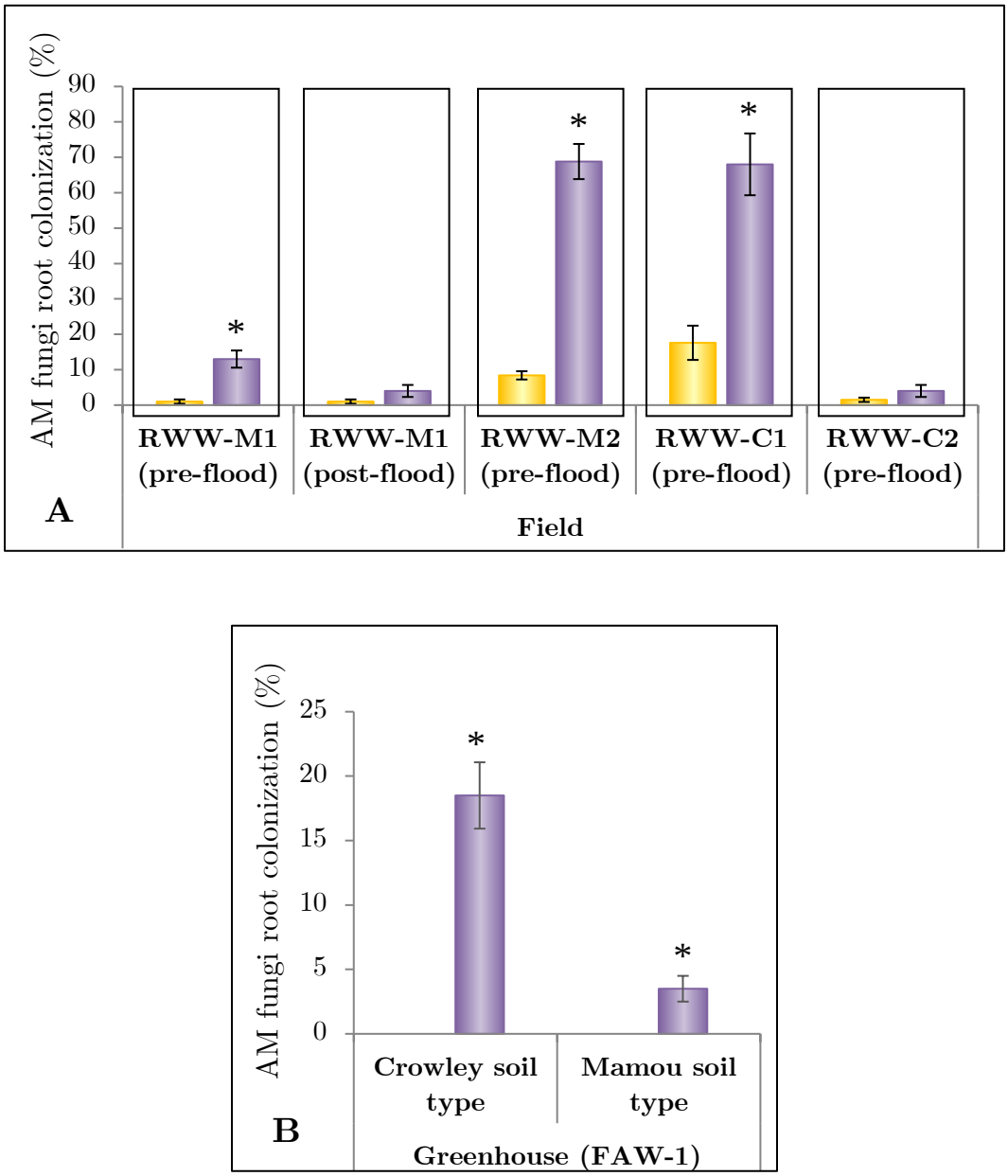


Figure 5.2. Effects of inoculation with a commercial formulation of arbuscular mycorrhizal fungi (AM fungi) on percent colonization by AM fungi in rice plants grown in field (A) and greenhouse (B) conditions in two types of soil (Crowley and Mamou). Soils were either treated with mycorrhizal inoculum (orange bars) or with nonmycorrhizal inoculum (yellow bars). Quantification of colonization was carried out for field and greenhouse experiments in 2014-2015. Percentages are means ± SE, n=5. Asterisks at the column heads indicate that means differed significantly (LSD, $P \leq 0.05$).

Table 5.3. Results for the mixed models assessing effects of inoculation treatment (Mycorrhizal and Nonmycorrhizal) on colonization by AM fungi, infestation by RWW, root and shoot dry weights, and nutrient concentrations of rice plants in the experiments conducted in the field in 2014 and 2015. Values are means of 10 replicates.

Source of variation	RWW-M1			RWW-M2		
	d.f.	*F*	*P*	*d.f.*	*F*	*P*
AMF % colonization	1, 8	23.04	0.001	1, 8	140.3	<.0001
RWW density (core)	1, 16	0.92	0.35	1, 16	0.36	0.56
Shoot dry weight (g)	1, 8	14.34	0.02	1, 8	1.99	0.19
Root dry weight (g)	1, 8	9.01	0.04	1, 8	3.57	0.13
Shoot N concentration				1, 8	0.01	0.91
Shoot P concentration				1, 8	0.00	0.97
Root N concentration				1, 8	0.01	0.93
Root P concentration				1, 8	0.07	0.79
Adjusted yield (lb/ha)	1, 8	0.05	0.83	1, 8	1.08	0.33
	RWW-C1			RWW-C2		
	d.f.	*F*	*P*	*d.f.*	*F*	*P*
AMF % colonization	1, 8	25.57	0.001	1, 8	1.92	0.20
RWW density (core)	1, 24	11.20	0.003	1, 18	3.85	0.07
Shoot dry weight (g)	1, 8	1.71	0.23	1, 6	7.73	0.03
Root dry weight (g)	1, 8	6.30	0.03	1, 6	6.62	0.04
Shoot N concentration	1, 8	0.18	0.68	1, 6	0.01	0.93
Shoot P concentration	1, 8	14.65	0.01	1, 6	2.47	0.17
Root N concentration	1, 8	2.83	0.13	1, 6	1.48	0.27
Root P concentration	1, 8	1.40	0.27	1, 6	1.37	0.29
Adjusted yield (lb/ha)	1, 8	0.00	0.96	1, 6	1.10	0.33

Insect performance in response to AM fungi inoculation in two soil types

In experiments conducted at the Mamou field location (RWW-M1 & RWW-M2), densities of RWW larvae and pupae in core samples collected-three and four weeks after flooding did not differ among AM fungi treatments (Figure 5.3, Table 5.3). In the experiments conducted at the Crowley location, in contrast, larval densities were significantly higher in plots inoculated with AM fungi than in control plots in RWW-C1 ($F_{1,24} = 11.20$, $P = 0.003$). In addition, a marginally significant increase in larval densities in AM fungi-inoculated plots was observed in RWW-C2 ($F_{1,18} = 3.85$, $P = 0.06$). Increases in RWW densities in AM fungi-inoculated plots ranged from 35% in RWW-C1 to 24% in RWW-C2 (Figure 5.3). Thus, the effect of inoculation with AM fungi on insect densities showed a soil dependency under field conditions

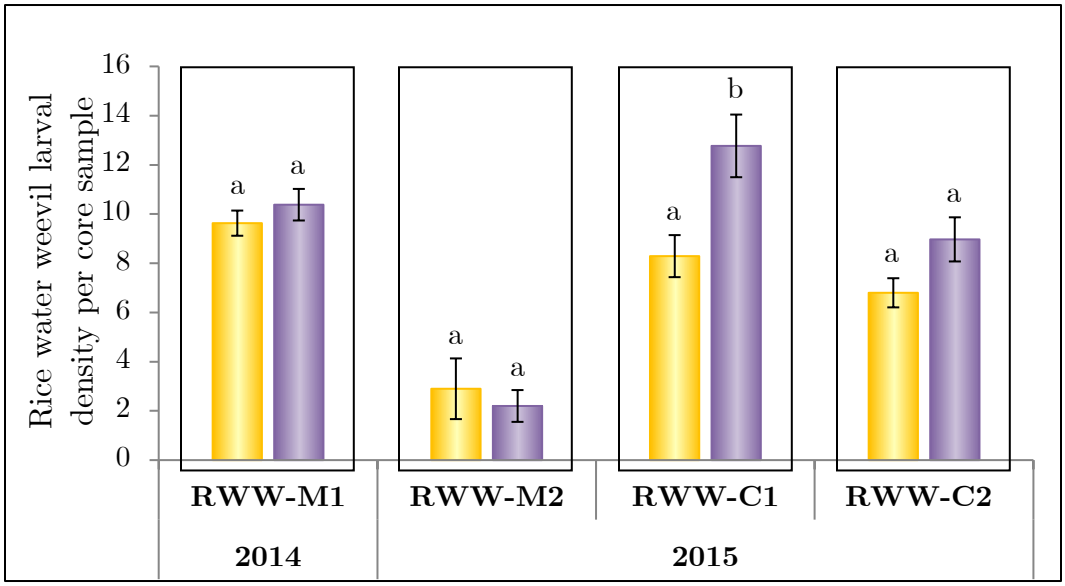


Figure 5.3. Effects of inoculation of rice with arbuscular mycorrhizal fungi on the densities of rice water weevils (larvae and pupae per core sample ± SE) on rice plants grown in four field experiments with either mycorrhizal (grey bars) or mock inoculum (nonmycorrhizal, open bars). Experiments were conducted in two locations with different soil types: Crowley (RWWW-C1, RWW-C2) or Mamou (RWW-M1, RWW-M2) during the 2014 and 2015 growing seasons. Values are means ± SE, n=5. Different letters accompanying bars indicate means that differ significantly (LSD, P ≤ 0.05).

Plant growth responses to AM fungi inoculation in two soil types

The shoot (leaf + stem) dry weights (SDW) of plants varied with AM fungi inoculation (Figure 5.4). At the Mamou location, analysis of the SDW data revealed a significant increase with AM fungi inoculation in RWW-M1 ($F_{1,8} = 14.34$; $P = 0.02$). As with SDW, root dry weights (RDW) of mycorrhizal plants were greater than that of the nonmycorrhizal plants in RWW-M1, as indicated by a significant main effect of inoculation with AM fungi (Table 5.3; $F_{1,8} = 9.01$; $P = 0.04$). Inoculation with AM fungi did not increase SDW or RDW in RWW-M2 (Figure 5.4; Table 5.3), but a trend toward higher weights in mycorrhizal plants was observed. At the Crowley location, an increase in SDW ($F_{1,6} = 6.62$; $P = 0.04$) was observed in RWW-C2, but no significant effect of AM fungi inoculation on SDW was observed in RWW-C1 ($F_{1,8} = 1.71$; $P = 0.23$) (Figure 5.4). A significant increase in RDW with AM fungi inoculation was observed in both experiments (RWW-C1: $F_{1,8} = 6.30$; $P = 0.03$; RWW-C2: $F_{1,6} = 6.62$; $P = 0.04$) (Figure 5.4; Table 5.3). Overall, the highest shoot biomass increase was observed in RWW-C2 (26.0%) and RWW-C1 showed the highest increase in root biomass (27.0%).

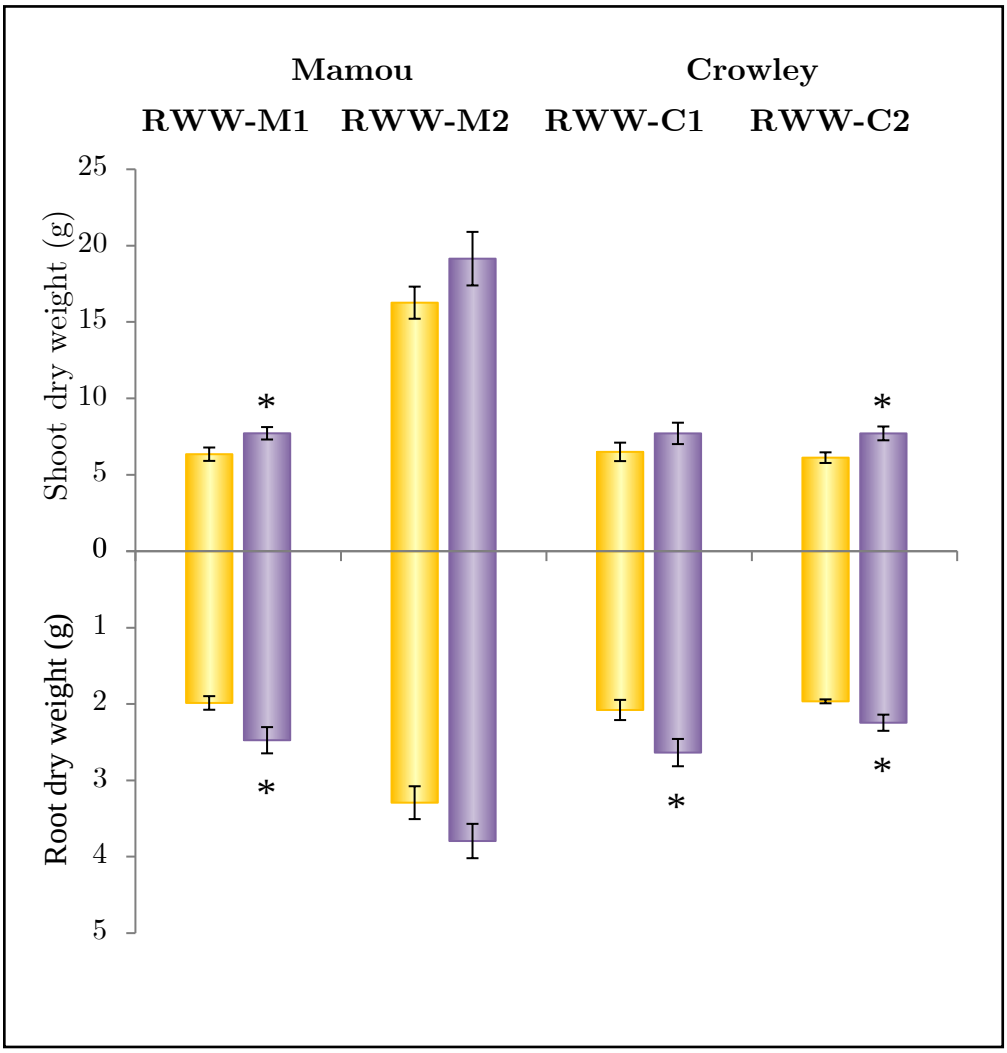


Figure 5.4. Mean shoot (above x-axis) and root (below x-axis) dry weights (grams ± S.E.) for rice plants grown in two different soils (Crowley and Mamou) in four field experiments. Rice plants were inoculated with AMF (orange columns) or with mock inoculum (nonmycorrhizal, yellow columns). Values are means ± SE, n=5 (field experiments). Asterisks represent significant differences in shoot or root dry biomass between mycorrhizal and nonmycorrhizal treatments for each experiment.

Plant nutrient responses to AM fungi inoculation in two soil types

Nutrient (N and P) concentrations in plant tissues were largely unaffected by inoculation with AM fungi (Figure 5.5A and B; Table 5.3). The concentration of P in shoot tissues was affected by AM fungi inoculation only in RWW-C1, with significantly higher concentrations encountered in the nonmycorrhizal control as compared to mycorrhizal plants ($F_{1,8} = 14.65$; $P = 0.01$; Figure 5.5B).

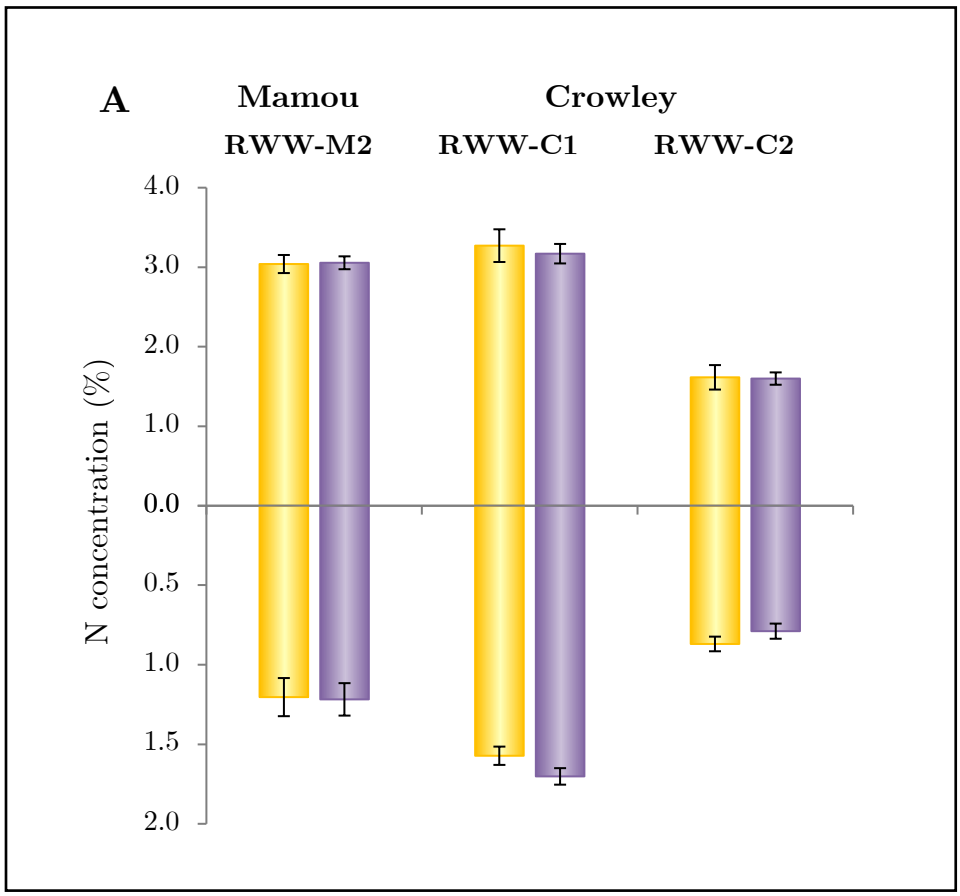
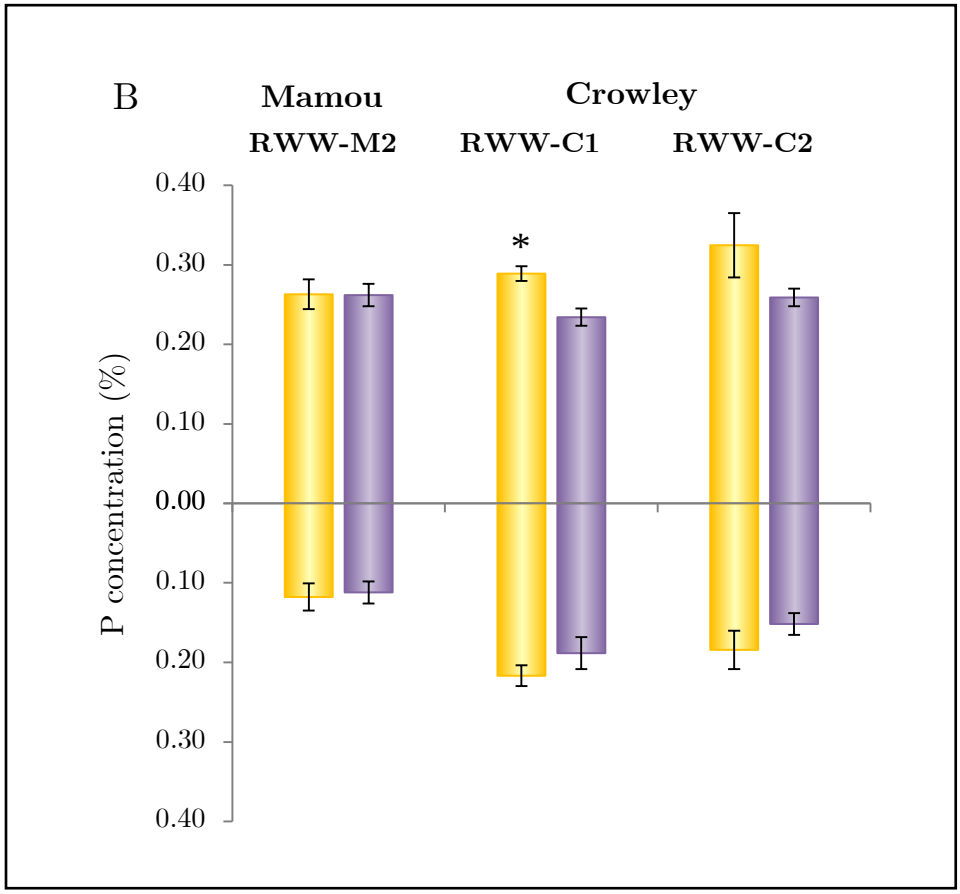


Figure 5.5. Effects of inoculation with AM fungi on concentrations of N (A) and P (B) in shoots (above x-axis) and roots (below x-axis) in two field soils (Crowley and Mamou) for three field experiments conducted at two locations, Crowley and Mamou. Rice plants were inoculated with AM fungi (orange bars) or with mock inoculum (yellow bars). Values are mean ± SE, n=5 (field experiments). Asterisks represent significant differences in shoot or root concentrations between mycorrhizal and nonmycorrhizal plants for each experiment.
(Figure cont'd.)



Grain yields to AM fungi inoculation and two soil types

Grain yields were not affected by inoculation with AM fungi in any of the field experiments (Figure B1; Table 5.3).

5.3.2 Greenhouse experiments

AM fungi root colonization

In the greenhouse, sterilization of the soil prevented colonization by AM fungi in the roots of nonmycorrhizal plants independently of soil type in FAW-1 (Figure 5.2B; root

colonization was not evaluated in FAW-2). Inoculation with AM fungi significantly enhanced the percentage of root fragments colonized by AM fungi in both soil types, with inoculation leading to higher colonization in Crowley soil (19 ± 2.6%) than in Mamou soil (3.5 ± 1.0%) (Figure 5.2B, Table 5.4). The effects of inoculation on the percentage of root colonized by AM fungi depended on soil type as shown by a highly significant 'soil type' x 'AM fungi inoculation' interaction ($F_{1,\ 12} = 34.39$, $P < .0001$, Table 5.4).

Effects of AM fungi inoculation on FAW growth in two soil types

Two-way ANOVA evaluating the effects of inoculation with AM fungi and soil type on growth of FAW larvae showed a soil dependency in effects of inoculation with AM fungi on larval growth. Weight gains of larvae were significantly affected by inoculation with AM fungi in both experiments (FAW-1: $F_{1,76} = 14.18$; $P = 0.0003$ and FAW-2: $F_{1,76} = 8.95$; $P = 0.004$) (Table 5.4). Weight gains of FAW larvae were also affected by 'soil type' in both experiments (FAW-1: $F_{1,76} = 15.90$; $P = 0.0002$ and FAW-2: $F_{1,76} = 16.43$; $P = 0.0002$) (Table 5.4), but the interaction of 'soil type' and 'inoculation' was significant only in FAW-1 ($F_{1,76} = 10.00$; $P = 0.002$) (Figure 5.6). In both experiments, the increase in FAW growth on plants inoculated with AM fungi was seen for insects reared on plants grown in the Crowley soil but not the Mamou soil. Increases in larval growth on mycorrhizal plants in Crowley soil averaged about 46% over both experiments (FAW-1: 0.039 ± 0.003 to 0.021 ± 0.002, mean ± SE; and FAW-2: 0.013 ± 0.001 to 0.007 ± 0.001, mean ± SE) when compared to the nonmycorrhizal control plants.

Table 5.4. Results of two-way ANOVAs assessing effects of soil source (Crowley and Mamou), inoculation treatment (Mycorrhizal and Nonmycorrhizal), and their interaction on % AMF colonization and fall armyworm growth on rice plants grown in the greenhouse in 2014.

Parameter	Factor	FAW-1			FAW-2		
		d.f.	*F*	*P*	*d.f.*	*F*	*P*
Total % AMF Colonization	Soil type	1, 12	34.39	<.0001			
	Inoculation	1, 12	73.99	<.0001			
	Soil x Inoculation	1, 12	34.39	<.0001			
FAW Weight gain (g)	Soil type	1, 76	15.90	0.0002	1, 57	16.43	0.0002
	Inoculation	1, 76	14.18	0.0003	1, 57	8.95	0.004
	Soil x Inoculation	1, 76	10.00	0.002	1, 57	0.09	0.7715

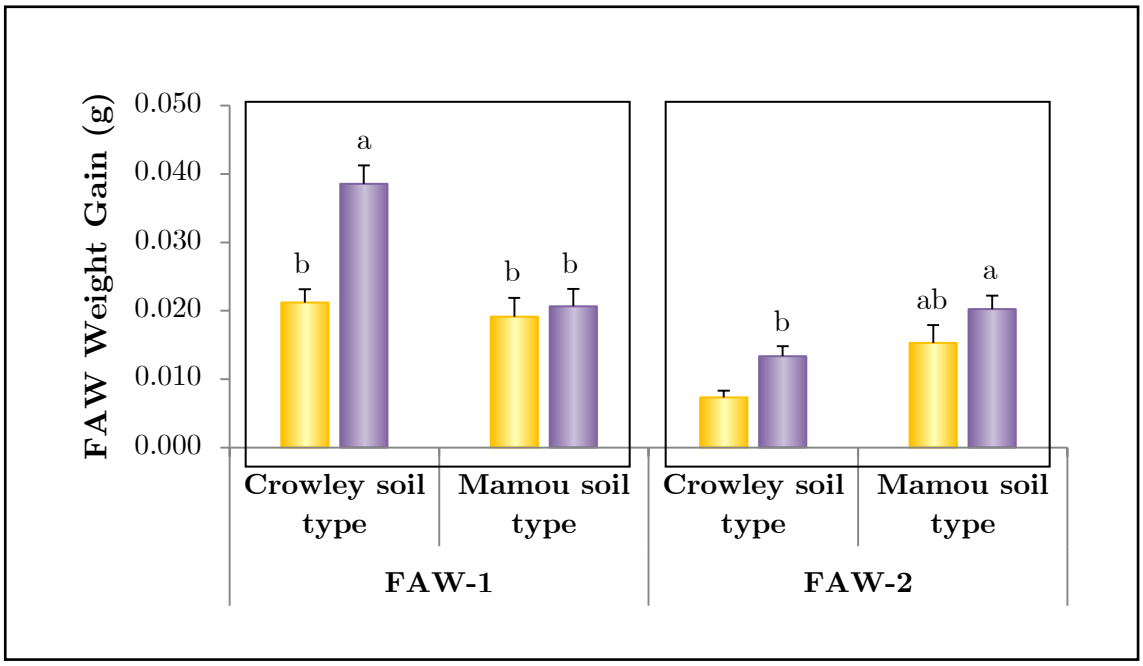


Figure 5.6. Effects of inoculation of rice plants with AM fungi on weight gains of fall armyworm larvae in two experiments using two different soil sources (Crowley and Mamou). Two inoculation treatments, either with mycorrhizal (orange bars) or with mock inoculum (nonmycorrhizal, yellow bars) are shown. Values are means ± SE, n=20. Bars accompanied by different letters indicate that means differ significantly (LSD, $P \leq 0.05$).

5.4 Discussion

In agricultural ecosystems, crop plants often interact simultaneously with herbivores and with AM fungi, and AM fungi and herbivores may interact indirectly through changes in their shared host plant. These tripartite interactions may be influenced by environmental factors. Building on past studies that have focused on the effects of inoculation with AM fungi on rice growth and resistance to pests (Bernaola et al., 2018b; Cosme et al., 2011), our study investigated the effects of soil type on AM fungi-rice-herbivore interactions in two different soil types under controlled and field conditions over two years. Our results highlight the context-dependency of the effects of inoculation with AM fungi on rice growth and the interaction of rice with its herbivores.

AM fungi are known to have widespread geographical distributions (Savary et al., 2017) and to be well-adapted to agricultural ecosystems (Barber et al., 2013b). Verbruggen et al. (2013) reported that compatibility with the environment is an important factor determining successful establishment of AM fungal inoculants in agricultural soils. In this study, colonization by AM fungi was successfully established using a granular commercial formulation of AM fungi over multiple years and locations. Increased root colonization levels after inoculation with AM fungi in rice fields indicated that AM fungi are compatible with different soil conditions as shown by colonization in soils with variation in pH (5.1 to 7.4), P availability (8.6 to 33.3 mg/kg), K availability (36.5 to 117.6 mg/kg), and organic matter content (0.96% to 2.25%) (Table 5.1), and is consistent with other studies showing that inoculation with AM fungi usually enhances root colonization by AM fungi in other plant species (Janoušková et al., 2013; Kohl et al., 2016; Robinson Boyer et al., 2016). While these other studies focused in crop systems such as clover, alfalfa, and strawberry in different parts of the world, the results from our study support the hypothesis that inoculation with AM fungi increases root colonization in rice plants in different locations in Louisiana, and therefore perhaps, other rice-producing areas of the world as well.

In addition to soil type, other factors may have been important in determining levels of root colonization. Since only two rice cultivars were used in the experiments, data from this study are insufficient to clearly indicate whether rice variety influenced root colonization. As seen in Figure 5.2, there was no evident correlation between colonization and rice variety, but future studies should include this aspect in their experimental design, because root colonization after inoculation with AM fungi inoculation has been shown to vary among varieties within a plant species (Sawers et al., 2010). Another aspect to consider when interpreting the results of these experiments is whether colonization rates differed among the six AM fungi species in the commercial inoculum. Quantification of colonization by AM fungi in this study focused on colonization by all fungal structures, regardless of fungal species identity. Different species of AM fungi are known to vary not only in their ability to provide nutrients to plants (Smith & Read, 2008) but also in their effects on plant resistance to herbivores (Roger et al., 2013). Irrespective of these two factors, data from this study demonstrates that AM fungi were able to influence plant biomass and yield under field experiments.

Insect performance on rice was either positively affected or not affected by inoculation with AM fungi, depending on the soil in which the plants were grown: inoculation increased densities of a root-feeding herbivore (RWW larvae) and growth of a leaf-feeding herbivore (FAW larvae) in the Crowley soil type but not the Mamou soil type. Bernaola et al. (2018b) had previously shown that inoculation of rice plants with AM fungi increased susceptibility to RWW and FAW and a rice pathogen (sheath blight) in experiments conducted in the Crowley soil. Our results are consistent with these findings and extend them to demonstrate that this AM fungi-induced susceptibility is soil dependent. Currie et al. (2011) and Koricheva et al. (2009) have also shown root and chewing insects benefited from colonization by AM fungi, but Yang et al. (2014) and Gange (2001) found that colonization by AM fungi inhibited the growth of root-feeding insects. Koricheva et al. (2009) suggested that specialist herbivores perform better on AM fungi inoculated

plants, whereas generalists do worse. However, in this study, we demonstrated that both specialist root-feeding and generalist shoot-feeding chewing insects were positively affected by AM fungi inoculation. To our knowledge, this is the first direct demonstration of soil dependence in the effect of AM fungi on rice-insect interactions. However, there are a few other studies have shown soil dependence in AM fungi-insect interactions in different crop systems (Barber et al., 2013a; Barber et al., 2013b).

Increased susceptibility of rice inoculated with AM fungi to herbivores was not associated with significant effects of AM fungi on plant nutrient concentrations. In particular, inoculation with AM fungi did not affect concentrations of P or N, the nutrients most commonly studied in plant-AM fungi interactions. Similarly, Barber et al. (2013b) found that commercial AM fungi inoculum did not change leaf nutrient content. As plant nutrient status does not explain the positive effects of AM fungi on rice-herbivore interactions in this study, changes in other plant traits such as plant defenses might have been responsible for observed effects. Future efforts could also focus on effects of colonization by AM fungi on less-studied macro- or micronutrients such as K, Na, or Zn. It has been shown that the presence of these nutrients in plant tissues can influence the performance of insect herbivores (Barber et al., 2013b; Behmer & Joern, 2012; Joern et al., 2012).

It has been previously hypothesized that effects of AM fungi inoculation on plant growth are context-dependent. In particular, it has been found that inoculation with AM fungi increases the growth of plants under P limitation (Smith & Smith, 2011), but not under conditions of P abundance. In this study, AM fungi inoculation stimulated plant growth in all field experiments and effects of plant growth were not influenced by the nutrient (N and P) status of the plant. Unlike Bernaola et al. (2018b), who found that AM fungi inoculation increased only shoot biomass of rice plants in field and greenhouse studies, this study showed that AM fungi inoculation increased both shoot and root biomass in field experiments at the Mamou location. In general, AM fungi inoculation is

known to have positive effects on plant biomass, but it also possible that other parameters are involved, such as concentrations of other soil nutrients in agricultural fields, climatic conditions, soil microflora, P application rates, since these interactions are not fully understood yet and require future study.

Previous studies on the effect of inoculation with AM fungi inoculation on rice grain yields have been contradictory, some reporting higher yields (Li et al., 2011; Secilia & Bagyaraj, 1994; Zhang et al., 2015), lower yields, or unchanged yields as a result of inoculation with AM fungi (Solaiman & Hirata, 1998). In this study, grain yields did not differ between AM fungi treatments at either the Crowley or Mamou sites. However, the lack of an effect on grain yield may need further study, as yield components that might be affected by inoculation with AM fungi were not studied.

Our study reports for the first time that effects of inoculation of rice with AM fungi on plant growth and rice-herbivore interactions are context dependent and differ in different soil types. Future work will include identification of soil characteristics responsible for this context dependency to facilitate an understanding of how production practices mediate the potential benefits of AM fungi in rice plants. In addition, selecting more soil locations with varying properties, not only in Louisiana but also other rice-producing areas, will be necessary to determine the effect of inoculation with AM fungi in those areas. Understanding how AM fungi inoculation interacts with the rice plant and how inoculation with AM fungi changes plant responses to biotic stresses is important in order to improve rice production and to promote effective and sustainable management of rice pests in ecological and agronomic contexts.

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Chapter 6
The Effect of Mycorrhizal Seed Treatments on Rice Growth, Yield, and Tolerance to Rice Water Weevil Injury

6.1. Introduction

The below-ground herbivores, pathogens and symbionts associated with a host plant can affect above-ground portions of a plant, and vice versa (Soler et al., 2012), which ultimately affects yield of plants. Arbuscular mycorrhizal (AM) fungi, members of the phylum Glomeromycota, are ecologically essential components of soil communities (Borowicz, 2001) and form obligate mutualistic associations with the roots of many plants (Smith & Read, 2008). AM fungi provide their host plants with nutrients (such as N and P) and water; in return, fungi receive sugars and lipids (Luginbuehl et al., 2017; Smith & Read, 2008). Herbivores affect plants by removing their biomass and reducing photosynthetic area (Agrawal et al., 2012). Both AM fungi and insect herbivores interact in complex and multifaceted ways with their host plants, and can interact with each other via changes they induce in their shared host plant (Gehring & Bennett, 2009).

In the context of crop protection, resistance and tolerance are two major strategies that plants employ to reduce the impact of herbivore attack (Mitchell et al., 2016). Resistance comprises plant traits that limit herbivore injury to the plant, while tolerance involves plant traits or physiological processes that reduce amount of damage (yield loss) per unit herbivore injury (Stout, 2013). Whereas there is extensive information on plant resistance to insects, tolerance is less studied, and the traits responsible are not well understood (Peterson et al., 2017). Previous studies have demonstrated that AM fungi can modify the pairwise interactions between plants and herbivores (Barber et al., 2013; Bennett et al., 2006; Kempel et al., 2010; Koricheva et al., 2009; Yang et al., 2014) through these two strategies. First, association with AM fungi has been shown to both increase and decrease resistance to herbivores in different crop systems by inducing plant defense

responses or by improving plant quality to herbivores (Cosme et al., 2011; Currie et al., 2011; Gange, 2001; Gehring & Bennett, 2009; Koricheva et al., 2009). Second, AM fungi may indirectly enhance plant tolerance by changing plant nutrient status or plant growth (Bennett & Bever, 2009).

Modern cereal crops have retained the ancient capacity to interact with AM fungi (Sawers et al., 2008). Rice (Oryza sativa L.) is an important cereal crop in the economy of the United States. Rice is produced in the six states of Arkansas, California, Louisiana, Mississippi, Missouri and Texas. Despite the high-input agricultural practices used in U.S. rice, rice plants in U.S. production areas are colonized by AM fungi (Bernaola et al., 2018a). At the same time, throughout their development, rice plants interact with a diverse complex of above- and below-ground insect herbivores (Lu et al., 2015; Stout et al., 2009). The two major early and late season insect pests of rice in the U.S. are the rice water weevil (*Lissorhoptrus oryzophilus*) and the rice stink bug (*Oebalus pugnax*), respectively. In addition, fall armyworms (*Spodoptera frugiperda*), rice stem borers (*Eoreuma loftini, Diatraea saccharalis*), leafminers (*Hydrellia wirthi*), and aphids can be economically important rice pests when they infest at high levels (Blanche et al., 2009).

In rice, it has been shown that the associations with AM fungi result in changes in plant competitive ability (Roger et al., 2013), ecotype-specificity (Diedhiou et al., 2016), functional diversity (Li et al., 2011), nutrient acquisition, and growth and gene transcription (Angelard et al., 2010; Colard et al., 2011). Insect herbivores cause significant transient effects in metabolism that may increase the defense or tolerance of the host plant (Johnson et al., 2016; Schultz et al., 2013). Although rice-AM fungi and rice-herbivore interactions have been studied mostly separately, a few studies have investigated tripartite interactions among AM fungi, herbivores, and rice plants. In particular, a few studies have focused on the effects of AM fungi on the resistance or susceptibility of rice plants to herbivores and pathogens. For instance, Cosme et al. (2011) showed that the oligophagous herbivore rice water weevil oviposited at higher rates on

rice plants colonized by the AM fungus Rhizophagus intraradices. The positive effect of the AM symbiosis on the herbivore was attributed to changes in plant nutrition caused by colonization by AM fungi. In a recent study, Bernaola et al. (2018b) also showed that inoculation of rice with a commercial AM fungi inoculant increased the susceptibility of rice plants to three antagonists. Increases in plant susceptibility to those pests caused by AM fungi was not associated with changes in the concentrations of essential nutrients in rice plants. The authors suggested that AM fungi colonization influenced defense signaling processes in rice plants, and thereby influenced susceptibility to the antagonists. In contrast, Campos-Soriano et al. (2011) reported enhanced resistance to the pathogenic fungus rice blast, *Magnaporthe oryzae*, in rice plants colonized by AM fungi. The negative effects of symbiosis on the pathogen appeared to arise from both the systemic activation of defense regulatory genes in the absence of pathogen attack and priming for stronger expression of defense genes during pathogen infection. However, studies are lacking to characterize the impact of AM fungi on the tolerance of rice to insect herbivores.

The purpose of this study was to investigate whether colonization by AM fungi increases the growth, yield, and tolerance of rice plants to root injury by the rice water weevil. To investigate these questions, we used a factorial experimental design with two levels of root injury and two levels of AM symbiosis. Root injury was manipulated by treating or not treating rice seeds with an insecticide (neonicotinoid), and symbiosis by AM fungi was manipulated by inoculating or not inoculating rice seeds with AM fungi. Using this factorial design we addressed two questions:

1. Does inoculation with AM fungi increase plant biomass, nutrition, colonization or yield in rice?

2. Does inoculation with AM fungi increase tolerance to root herbivory? If AM fungi increases plant tolerance, then the difference in plant biomass or yield between insecticide-treated and -untreated plots would be smaller in mycorrhizal plots than nonmycorrhizal plots.

6.2. Material and Methods

6.2.1. Study system

Seeds of O. sativa cultivar 'CL111' were used as the host plant in all three years of the study. 'CL111' is a long-grain, high-yielding, early-maturing conventional rice variety. Seeds of 'CL111' were provided by the breeding and foundation seed program of the Louisiana State University Agricultural Center (LSU AgCenter) H. Rouse Caffey Rice Research Station (Crowley, Acadia, LA, USA).

A commercially available mixture of AM fungi (Valent® USA, Walnut Creek, CA, USA) was used in all experiments. This mixture consisted of four endomycorrhizal fungi species (*Rhizophagus irregularis*, *Glomus aggregatum*, *Funneliformis mosseae*, and *Claroideoglomus etunicatum*) containing spores, hyphae and colonized root fragments (see methodology for more details).

The rice water weevil is the most important insect pest of rice in the United States (Hamm et al., 2010). Adult rice water weevils feed on leaves resulting in longitudinal scars parallel to the leaf veins of rice plants. After flooding of rice fields, females lay eggs in leaf sheaths below the water surface. Neonate larvae migrate down to the plant roots, where they feed on flooded roots and pass through four larval instars and a pupal stage (Zou et al., 2004). Feeding on rice roots reduces rice growth and yields (Zou et al., 2004).

The rice stem borer complex that attacks rice fields in the United States comprises the Mexican rice borer (*Eoreuma loftini*; Lepidoptera: Crambidae), sugarcane borer (*Diatraea saccharalis*; Lepidoptera: Crambidae), and rice stalk borer (*Chilo plejadellus*; Lepidoptera: Crambidae) (Way, 2003). Stem borer larvae injury occurs during rice vegetative or reproductive stages producing two different symptoms known as deadheart and whitehead, respectively (Way, 2003). Stem borer activity varies each year; however, an increase in their occurrence in recent years has been noted (Way et al., 2006).

6.2.2. Experimental design

To evaluate whether inoculation with AM fungi influences rice productivity and tolerance to rice water weevil, four field experiments were conducted during the 2016, 2017, and 2018 growing seasons at the LSU AgCenter H. Rouse Caffey Rice Research Station (Acadia Parish, 30o14'22" N, 92o20'46" W) on Crowley silt-loam soils. Experiments were referred to as Experiment-1 (Exp-1) in 2016, Experiment-2 (Exp-2) in 2017, and Experiment-3 (Exp-3) and Experiment-4 (Exp-4) in 2018. All experiments utilized randomized complete block designs incorporating factorial combinations of AM fungi and insecticide treatments as described below. Management practices during those years followed the recommendations of the LSU AgCenter for drill-seeded rice (Blanche et al., 2009).

Each experiment consisted of four treatments with 10 replicates of each treatment. Each block consisted of four plots assigned to factorial combinations of two levels of AM fungi seed treatment (+AMF and -AMF) and two levels of insecticide seed treatment (+NsI and –NsI) giving a total of 40 plots. The insecticide formulation used to treat seeds was NipsIt INSIDE (clothianidin 47.8%, Valent® USA Corporation, Walnut Creek, CA). The AM fungi formulation used was MycoApply® EndoMaxx (6.6%). Seeds were treated by the manufacturer in each year (Valent® USA Corporation). Seed treatment rates in all years were 17 µg AI/seed for clothianidin and 14 g AI/ha for MycoApply EndoMaxx. Rice plants were grown from seeds in the field; thus, the soil was not sterilized and likely contained native AM fungi. NipsIt INSIDE seed treatments have shown to reduce densities of RWW larvae and pupae in field experiments (Hummel et al., 2014; Hummel & Stout, 2009).

Rice seeds were drilled-seeded on the dates specified in Table 6.1 at a rate of 50 g of seeds per plot (67 kg/ha) in all experiments. Field plots measured 5.4 m x 1.8 m. A soil sample was collected from the rice field before seeding in each year and sent for analysis to the LSU AgCenter Soil Testing & Plant Analysis Laboratory (STPAL, LSU, Baton

Rouge, LA, USA). Soil physical and chemical properties are reported in supporting information Table C1. Approximately three weeks after planting, fields were surface-irrigated for 24 h to facilitate plant stand establishment. Permanent flood was applied at the 4-5 leaf (early tillering) stage of rice on the dates specified in Table 6.1. Nitrogen was applied in all years with a single application, one day before permanent flood was established, in the form of Urea (46% N) at 134 kg N/ha, for adequate plant growth; fields were not fertilized with P and K.

For all experiments, densities of rice seedlings in plots were evaluated when plants attained the two- to three-leaf stage (approximately two weeks after rice emergence, Table 6.1) to assess the effect of treatments on seedling densities (Hamm et al., 2014). Densities of plant stands were assessed by counting the number of seedlings present in three or two randomly selected quadrats of 0.09 m2 per plot. Mean stand counts for each plot were used for analysis.

Extent of root colonization by AM fungi was evaluated the day that permanent flood was established (Table 6.1) in all experiments. A core sample containing at least one plant was taken from the center of each plot. Roots were rinsed completely free of soil with running tap water. Samples were transported to the laboratory. Roots from each sample were cut into 2 cm pieces and placed into tissue cassettes. Subsequently, roots were cleared by boiling for 30 min in 10% KOH, washing with tap water 5 X, then for 20 min in 2% HCl, and stained overnight in 0.05% blue stain solution. Percentage of AM fungi colonization was estimated according to the modified method of McGonigle et al. (1990) at 40X magnification to score AM fungal structures, including hyphae, arbuscules, vesicles and spores per root sample (Figure 6.1).

Table 6.1. Activities for four experiments conducted in the field over three growing seasons (2016-2018).

Activity	Year			
	2016	2017	2018-I	2018-II
Trial	Exp-1	Exp-2	Exp-3	Exp-4
Planting date	16th May	15th March	27th March	3rd May
Stand count	27th May	25th April	29th April	31st May
Flood date	15th June	26th April	9th May	5th June
AMF sampling	15th June	27th April	10th May	7th June
First core sampling	7th July	18th May	31st May	28th June
Second core sampling	14th July	24th May	7th June	5th July
Third core sampling	20th July	30th May	14th June	12th July
Plant Biomass & Nutrient analysis (Before flooding)	15th June	27th April	11th May	14th June
Plant Biomass & Nutrient analysis (After flooding)	20th July	5th June	7th June	28th June
Whiteheads count	1st & 8th August	29th June & 6th July	28th June, 5th, 12th, 19th, & 26th July	26th July, 2nd, 9th, 16th, & 23rd August
Heading percent			28th June & 5th July	26th July & 2nd August
Maturity percent			12th & 19th July	9th & 16th August
Plant yield	8th September	28th July	17th August	20th September

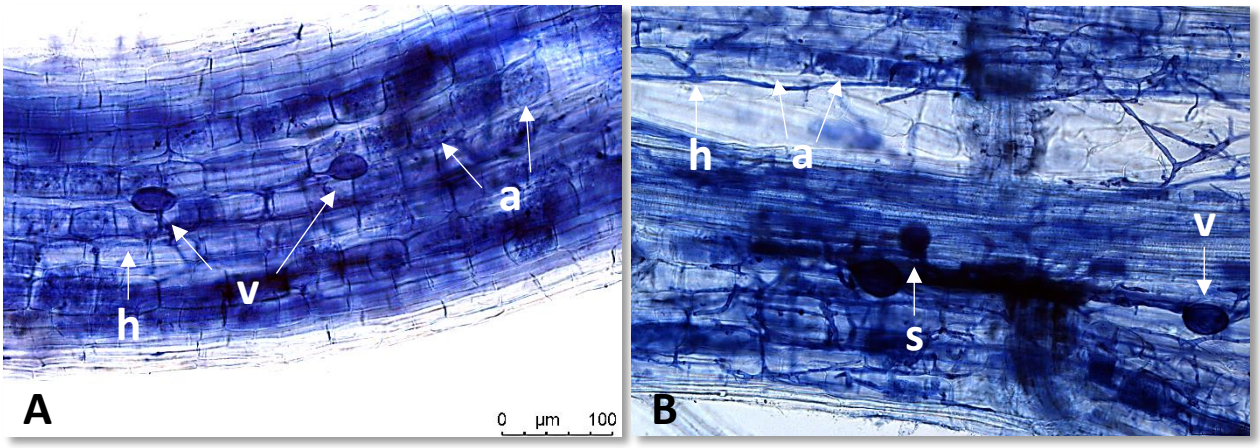

Figure 6.1. Root fragments stained with trypan blue showing arbuscular mycorrhizal fungi structures in rice plants. Light micrographs of mycorrhizal inoculated root fragments from some experiments conducted in 2018 show: (A) Hyphae (h), arbuscule (a), and vesicle (v). (B) Hyphae, arbuscule (a), spore (s) and vesicle.

Densities of rice water weevil immatures (larvae and pupae) associated with roots of rice plants were determined on three dates after flooding by taking root-soil core samples from each treatment. Field experiments relied on natural infestations of rice water weevils, which are abundant at the field site. Core sampling was conducted between three and five weeks after flooding (Table 6.1). Three core samples were taken from each plot in 2016 and 2017, and two core samples were taken from each plot in 2018. Roots of rice plants from core samples were washed free of soil under medium pressure in a sieve bucket (40-mesh screen). Buckets were then placed into basins of salt water, which caused larvae to float to the surface of the salt solution, where they were counted. Pupae were counted as they settled in the bottom of sieve buckets (Figure C1) (N'Guessan et al., 1994). Average numbers of immature weevils found in the two or three soil cores from each plot were calculated and used for analysis.

Incidence of whiteheads resulting from stem borer infestations on rice in reproductive-stage rice was determined by counting the total number of whiteheads in each plot weekly on two (Exp-1 and 2) or five different dates (Exp-3 and 4) (Table 6.1). Plants showing

whiteheads symptoms were collected and all tillers with whiteheads were dissected by opening longitudinally with a knife to identify the stem borer species. The numbers of whiteheads from two or five weeks was summed to obtain a total number of whiteheads in each plot.

Biomass of plants (roots and above-ground portions) were assessed twice in each experiment, before and after flood was established (Table 6.1). Entire plants were pulled by hand from soil. Soil was washed from roots with tap water, and roots were separated from above-ground material and blotted dry with a paper towel. Plant material was stored in a paper bag and placed in a drying oven at 60^{o}C for 1 week. Shoot (stem + leaves) dry weight (SDW) and root dry weight (RDW) were recorded for each plant. Dried plant biomass was submitted to the LSU AgCenter's Soil Testing & Plant Analysis Laboratory to determine nutrient concentrations in root and shoot tissues. The STPAL determined N and C content by dry combustion using a LECO TruSpecTM CN analyzer (LECO Corp., St. Joseph, MI, USA), while concentrations of the remaining nutrients (Ca, Mg, S, P, K, Al, B, Cu, Fe, Mn, Na and Zn) were determined by inductively coupled plasma (ICP) analysis.

The average of two visual ratings of panicle heading and maturity were determined for each treatment in Exp-3 and Exp-4 in two consecutive weeks (Table 6.1), and expressed in percentages for each plot of days after planting (DAP). A plot was considered to have started heading if at least 30% of panicles were emerged from the leaf sheath. Maturity was defined as the time at which 80% of all spikelets were ripe (i.e., when grain had lost green color). Entire plots were harvested at grain maturity using a harvester/thresher machine and grain yields (expressed at 12% moisture) were recorded.

6.2.3. Statistical analyses

All statistical analyses were carried out using SAS 9.4 (SAS Institute 2016). Plant stands, numbers of whiteheads, plant biomass, nutrient concentrations, heading, maturity, and yields were analyzed as factorial RCBD experiments with AM fungi, insecticide, and their interaction as fixed effects and block as a random effect in PROC MIXED. Percentages of root fragments colonized by AM fungi were arcsin square root-transformed to meet the assumptions of normality and were analyzed using ANCOVA in PROC GLM. Root biomass was included as the covariate in the analysis of root colonization to control for variation in root biomass that could influence colonization. Immature weevil densities over three weeks were analyzed separately for each experiment by repeated-measures ANOVA.

Percent yield losses from rice water weevil were determined in the following manner. For each block, the difference between yields from insecticide-treated plots and yields from insecticide-untreated plots were calculated separately for plots inoculated with AM fungi and plots not inoculated with AM fungi, dividing this difference by the yield of the appropriate insecticide-treated plot, and multiplying by 100. These numbers were analyzed using a one –way ANOVA with the factor treatment in PROC MIXED. In addition, a meta-analysis using the means from yield and percent of yield losses from the four experiments was used to reduce sampling error. Means were separated using LSD test.

6.3. Results

6.3.1. Effect of seed treatments on AM fungi root colonization

Seed treatment with AM fungi successfully increased colonization of roots by AM fungi in all four experiments (Table 6.2; Figure 6.1). NipsIt INSIDE seed treatment had a significant negative effect on percentages of root colonization by AM fungi only in Exp-1

(Table 6.2; Figure 6.2). AM fungi root colonization was significantly affected by the interaction between AM fungi and insecticide seed treatment only in Exp-4 (Table 6.2; Figure 6.2). However, in Exp-1 and Exp-3, even though the interaction between AM fungi and insecticide seed treatments did not affect significantly root colonization by AM fungi, colonization was lower in the combination of AMF and NipsIt than AMF alone (Figure 6.2). This interaction suggests that AMF inoculation increases colonization in absence of NipsIt but not in presence of NipsIt. Furthermore, there was no effect of root biomass (covariate) on percentages of root colonization by AM fungi in any of the experiments (Table 6.2).

Table 6.2. ANCOVA results for the effects of inoculation with AM fungi and insecticide seed treatments as well as their interaction on arcsin square root transformed values of the percentage of rice roots colonized by AM fungi of four experiments conducted in the field over three years (2016-2018).

Factor	Exp-1			Exp-2			Exp-3			Exp-4		
	d.f	*F*	*P*	*d.f*	*F*	*P*	*d.f*	*F*	*P*	*d.f*	*F*	*P*
Block	9	1.81	0.115	9	0.96	0.495	9	1.14	0.37	4	0.80	0.551
AM fungi	1	51.6	$<.0001$	1	56.6	$<.0001$	1	6.38	0.018	1	19.1	0.001
Insecticide	1	25.8	$<.0001$	1	0.08	0.782	1	3.25	0.083	1	3.82	0.076
AM fungi x Insecticide	1	3.72	0.065	1	3.55	0.071	1	3.04	0.093	1	14.6	0.003
Root biomass	1	1.05	0.315	1	0.87	0.361	1	0.39	0.539	1	0.00	0.994
Error	26			26			26			11		

Root biomass values were included as a covariate in order to control for variation in biomass that could influence quantitative measures of percentage of colonization by AM fungi. Bold numbers indicate significant effects.

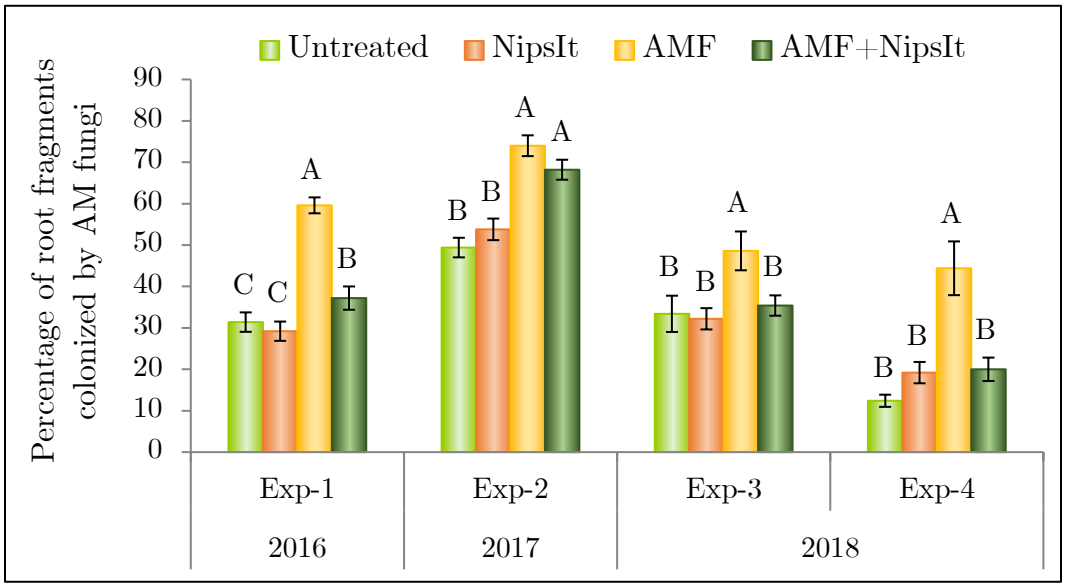

Figure 6.2. Effects of inoculation with AM fungi and treatment of seeds with insecticide as well as their interaction on the percent of root fragments colonized by AM fungi in rice plants of four experiments conducted in the field over three years (2016-2018). Untreated (-AMF/-NsI, light green columns), insecticide only (-AMF/+NsI, orange columns), mycorrhizal only (+AMF/-NsI, yellow columns), or combination of mycorrhizal and insecticide (+AMF/+NsI, dark green columns). The percentages are the means of 10 replications per treatments. Letters at the column head indicate that means differ significantly (LSD, $P \leq 0.05$).

6.3.2. Effect of seed treatments on plant densities

AM fungi seed treatment significantly decreased stand counts of rice plants per 0.09 m2 in Exp-1 (Table 6.3; Figure 6.3) but not in Exp-2, Exp-3, or Exp-4. Clothianidin seed treatment did not affect densities of rice seeding in any of the four experiments (Table 6.3). Stand counts were significantly affected by the interaction between AM fungi treatment and insecticide treatment in Exp-1, Exp-3, and Exp-4 (Table 6.3), however, there was not a consistent pattern on the effect of these interactions on plant densities (Figure 6.3).

Table 6.3. Results of ANOVA for the effects of inoculation with AM fungi (+AMF and –AMF) and insecticide seed treatments (+NsI and –NsI) as well as their interaction on stands of rice plants grown in four experiments conducted in the field over three years (2016-2018).

Factor	Exp-1			Exp-2			Exp-3			Exp-4		
	d.f.	*F*	*P*	*d.f.*	*F*	*P*	*d.f.*	*F*	*P*	*d.f.*	*F*	*P*
AMF	1, 36	5.72	0.022	1, 27	0.81	0.375	1, 27	0.17	0.679	1, 27	0.00	0.986
Insecticide	1, 36	0.00	0.992	1, 27	0.90	0.351	1, 27	0.60	0.445	1, 27	1.71	0.202
AMF x Insecticide	1, 36	4.42	0.043	1, 27	0.01	0.930	1, 27	4.87	0.036	1, 27	6.58	0.016

Bold numbers indicate significant effects.

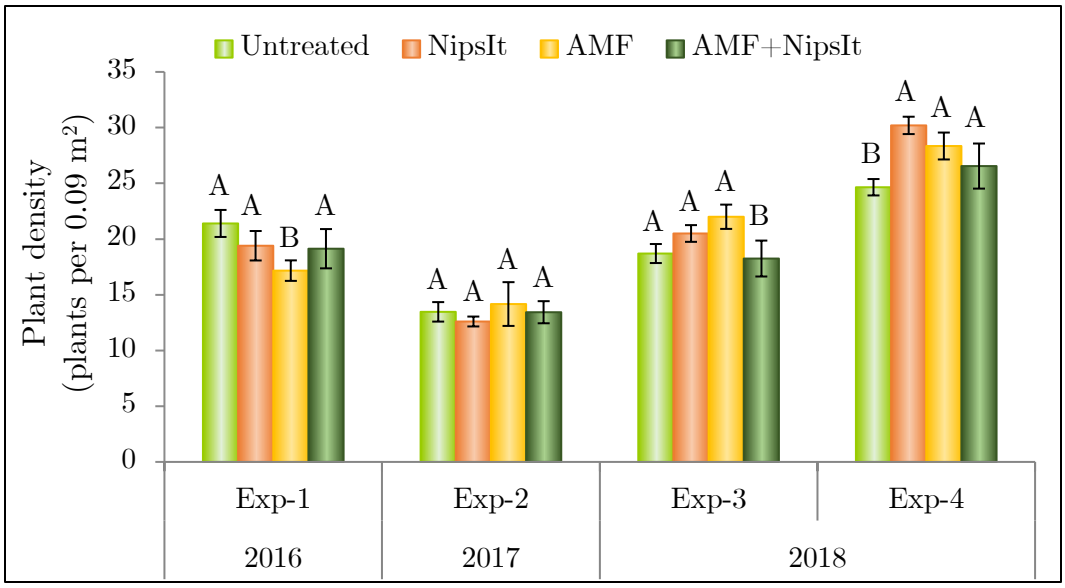

Figure 6.3. Effects of inoculation with AM fungi and insecticide seed treatment as well as their interaction on densities of rice seedlings (plants per 0.09 m2 ± S.E.) of four experiments conducted in the field over three years (2016-2018). Untreated (-AMF/-NsI, light green columns), insecticide only (-AMF/+NsI, orange columns), mycorrhizal only (+AMF/-NsI, yellow columns), or combination of mycorrhizal and insecticide (+AMF/+NsI, dark green columns). The numbers are the means of two stand counts. Letters at the column head indicate that means differ significantly (LSD, $P \leq 0.05$).

6.3.3. Effect of seed treatments on RWW larval densities

Densities of rice water weevil larvae and pupae were significantly higher in plots with AM fungi-treated rice plants in Exp-1, Exp-2, and Exp-3 than in plots not inoculated with AM fungi (Table 6.4; Figure 6.4A). Increases in weevil densities in AM fungi-treated plots ranged from 10.3% in Exp-1, 21.2% in Exp-2, 22.7% in Exp-3 to 9% in Exp-4 when compared to non-AM fungi treated plots (Figure 6.4A). Treatment of seeds with NipsIt INSIDE significantly reduced population densities of immature rice water weevils in all four experiments (Table 6.4; Figure 6.4B). Reductions in weevil densities in insecticide-treated plots ranged from 56.6% in Exp-1, 48.4% in Exp-2, 76.7% in Exp-3 to 61.0% in Exp-4. The interaction of AM fungi and insecticide seed treatment was not significant in

any experiment (Table 6.4). In addition, weevil densities were significantly affected by time (core sampling) in all four experiments (Table 6.4; Figure C2). Weevils were lowest at the third week after permanent flood (WAPF), highest at the fourth WAPF and started to decrease at the fifth WAPF (Figure C2). Densities of immature weevils were also significantly affected by the interaction of time and AM fungi in Exp-1 (Table 6.4). Insect densities were higher in AM fungi-treated plots than in control plots in the core samplings taken three and four weeks after flooding, but not in the core sampling taken the fifth week. Weevil densities were also significantly affected by the interaction of time and insecticide in Exp-1, Exp-3, and Exp-4 (Table 6.4; Figure C3). The interaction suggests that when rice plots are left untreated with Clothianidin, insect numbers tend to be higher over time and started to decrease at the last week of sampling compared to the steady effect of insecticide-treated plants (Figure C3).

Table 6.4. Repeated measures ANOVA of the effects of time (core sampling date), inoculation with AM fungi (+AMF and –AMF), treatment of seeds with insecticide (+NsI and –NsI) as well as their interaction on densities of larvae and pupae of rice water weevil in experiments conducted in the field over three years (2016-2018).

Factor	Exp-1			Exp-2			Exp-3			Exp-4		
	d.f.	*F*	*P*	*d.f.*	*F*	*P*	*d.f.*	*F*	*P*	*d.f.*	*F*	*P*
AM fungi	1, 99	5.44	0.027	1, 99	10.0	0.002	1, 99	6.45	0.013	1, 99	2.46	0.120
Insecticide	1, 99	269	<.0001	1, 99	110	<.0001	1, 99	240	<.0001	1, 99	276	<.0001
AM fungi* Insecticide	1, 99	2.52	0.124	1, 99	2.25	0.137	1, 99	2.28	0.134	1, 99	1.35	0.247
Time (core)	2, 99	15.3	<.0001	2, 99	79.4	<.0001	2, 99	8.76	0.0003	2, 99	14.9	<.0001
Time*AM fungi	2, 99	4.58	0.013	2, 99	1.09	0.342	2, 99	0.30	0.740	2, 99	0.35	0.708
Time*Insecticide	2, 99	6.35	0.003	2, 99	1.61	0.206	2, 99	16.9	<.0001	2, 99	6.06	0.003
Time*AM fungi*Insecticide	2, 99	0.42	0.656	2, 99	0.51	0.600	2, 99	1.25	0.289	2, 99	0.43	0.654

Bold numbers indicate significant effects.

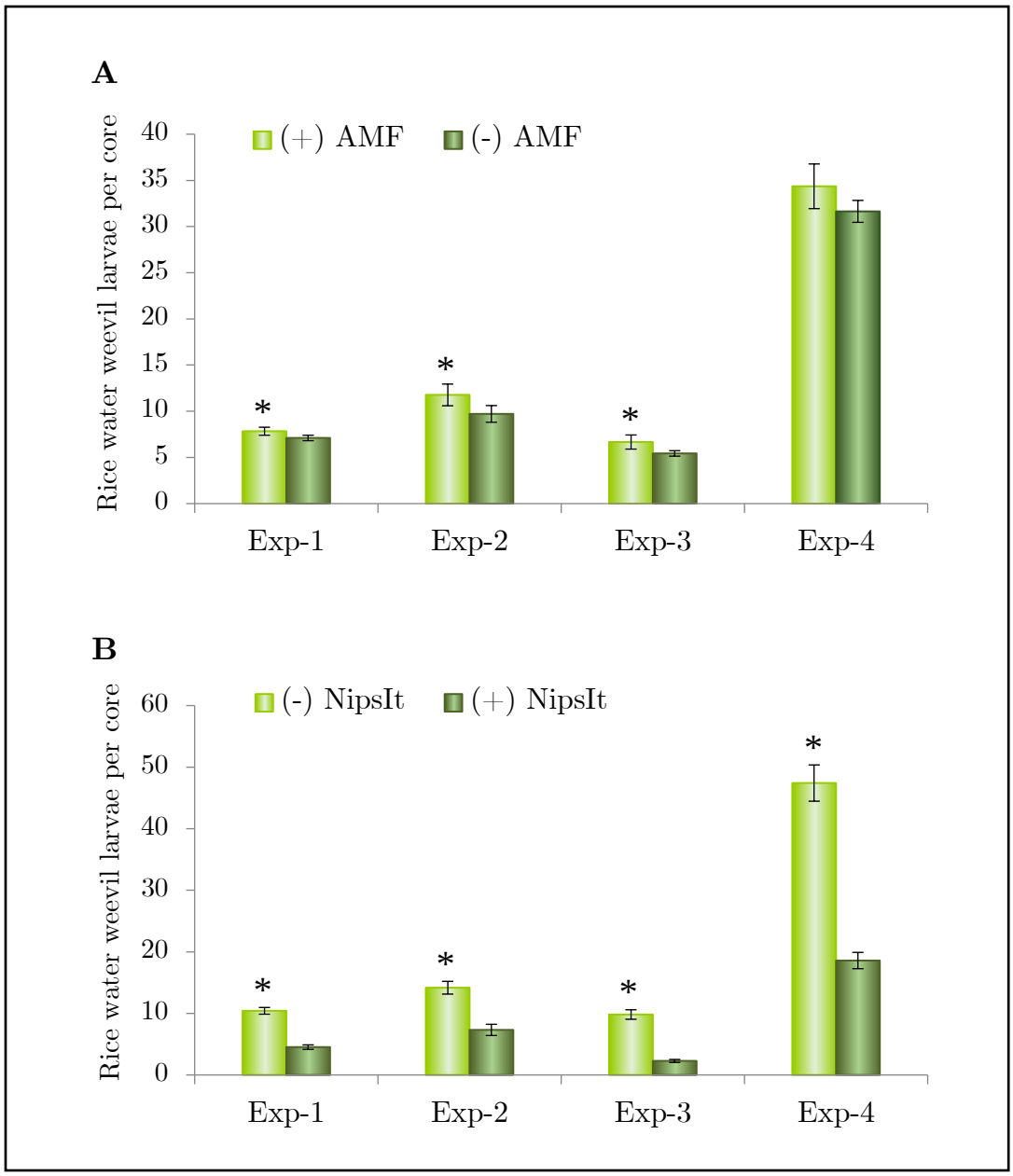

Figure 6.4. Main effects of inoculation with AM fungi and treatment of seeds with insecticide on densities of rice water weevil (larvae and pupae per core sample ± S.E.) in rice plots of four experiments conducted in the field over three years (2016-2018). (A) main effect of AM fungi treatment (+AMF and –AMF); (B) main effect of insecticide (+NsI and –NsI) treatment. The numbers are the means of 10 replications. Asterisk at the column head indicate that means differ significantly between treatments (LSD, $P \leq 0.05$).

6.3.4. Effect of seed treatments on whitehead numbers

Numbers of whiteheads per plot were used as a measure of stem borer infestation in all four experiments. About 70% of stem borer larvae collected after dissecting rice stems were found to be Mexican rice borer, with the remaining larvae a mix of sugarcane borer and rice stalk borer. AM fungi seed treatment significantly increased whitehead numbers compared to non-AM fungi rice plots in Exp-1 and Exp-4 (Table C2). Increases in number of whiteheads in AM fungi-treated plots ranged from 29.0% in Exp-3 and 4 to 80.3% in Exp-1 and 82.9% in Exp-2 (Figure C4A). NipsIt INSIDE seed treatment significantly reduced whitehead numbers in treated plots compared to untreated rice plots only in Exp-4 (Table C2). Reductions in whitehead densities ranged from 11.2% in Exp-1, 27.2% in Exp-3, 35.0% in Exp-2 to 54.5% in Exp-4 (Figure C4B). There were no significant interactions between AM fungi and insecticide treatments in any years.

6.3.5. Effect of seed treatments on plant biomass

Before flooding, inoculation of rice with AM fungi had a greater influence on root biomass than on shoot biomass (Figure 6.5A). RDW was greater in AM fungi treatments in Exp-1, Exp-2 and Exp-3 (Table 6.5) and TDW was greater in +AMF treatments in Exp-2 and Exp-3 (Table 6.5; Figure C5A). SDW on the other hand, was increased by inoculation with AM fungi only in Exp-2 (Figure 6.5A). There were no main effects of insecticide treatment on plant biomass in any of the pre-flood samplings. In addition, the interaction between AM fungi and insecticide treatments significantly influenced the TDW, SDW, or RDW in Exp-1; TDW and RDW in Exp-3; and SDW in Exp-4 (Table C3). These interactions suggest that AM fungi treatments did not impact plant biomass significantly in the presence of NipsIt INSIDE treatments; however, AM fungi have a positive impact on biomass in NipsIt INSIDE-untreated plots.

In contrast to the results observed before flooding, inoculation with AM fungi had a greater effect on shoot weights than root weights after flooding (Figure 6.5B). After flooding, AM fungi treatments significantly increased the TDW (Figure C5B) or SDW in Exp-2, the SDW in Exp-3, and the TDW and SDW in Exp-4 (Table 6.5; Figure 6.5B). NipsIt INSIDE seed treatment positively influenced the SDW or RDW in Exp-3, and TDW, SDW or RDW in Exp-4 (Table 6.5; Figure 6.5B). Moreover, the interaction between AM fungi and insecticide treatments significantly influenced the TDW, SDW, or RDW in Exp-3, and TDW or RDW in Exp-4 (Table C4). These interactions suggest that, after flooding, AM fungi treatments still influence plant growth significantly in the absence or presence of insecticide treatments towards shoot tissues.

Table 6.5. ANOVA results for the mixed effects of inoculation with AM fungi, treatment of seeds with insecticide as well as their interactions on the dry weight in total (TDW), shoot (SDW) and root (RDW) biomass collected twice, before (B.F.) and after (A.F.) flooding were established, in experiments conducted in the field over three years (2016-2018).

Trial	Fixed effect	TDW		SDW		RDW	
		$F_{1,\,27}$	P	$F_{1,\,27}$	P	$F_{1,\,27}$	P
Exp-1	AM fungi						
	B.F.	0.67	0.42	0.23	0.63	4.06	0.05
	A.F.	0.19	0.66	0.54	0.47	0.62	0.44
	Insecticide						
	B.F.	0.19	0.67	0.05	0.83	1.52	0.23
	A.F.	0.07	0.79	0.03	0.87	0.41	0.53
	AM fungi*Insecticide						
	B.F.	10.20	0.004	4.67	0.039	44.64	$<.0001$
	A.F.	0.26	0.62	0.32	0.57	0.04	0.83
Exp-2	AM fungi						
	B.F.	8.47	0.007	6.86	0.01	8.67	0.007
	A.F.	7.57	0.01	8.91	0.006	2.23	0.15
	Insecticide						
	B.F.	2.07	0.16	2.86	0.10	0.72	0.40
	A.F.	1.76	0.20	1.37	0.25	2.19	0.15
	AM fungi*Insecticide						
	B.F.	1.81	0.19	2.83	0.10	0.41	0.53
	A.F.	1.53	0.23	1.21	0.28	1.85	0.19
Exp-3	AM fungi						
	B.F.	4.57	0.04	2.74	0.11	8.73	0.006
	A.F.	3.26	0.08	4.46	0.044	1.18	0.29
	Insecticide						
	B.F.	0.05	0.82	0.13	0.72	0.04	0.85
	A.F.	8.23	0.008	5.89	0.02	6.81	0.01
	AM fungi*Insecticide						
	B.F.	4.27	0.049	2.55	0.12	8.19	0.008
	A.F.	12.54	0.002	9.49	0.005	9.86	0.003
Exp-4	AM fungi						
	B.F.	0.53	0.47	0.43	0.52	0.78	0.39
	A.F.	16.26	0.0004	18.18	0.0002	2.00	0.17
	Insecticide						
	B.F.	1.29	0.27	1.17	0.29	1.39	0.25
	A.F.	8.93	0.006	6.91	0.01	4.66	0.04
	AM fungi*Insecticide						
	B.F.	3.72	0.06	3.91	0.05	2.41	0.13
	A.F.	5.92	0.02	2.99	0.09	6.84	0.01

Bold numbers indicate significant effects.

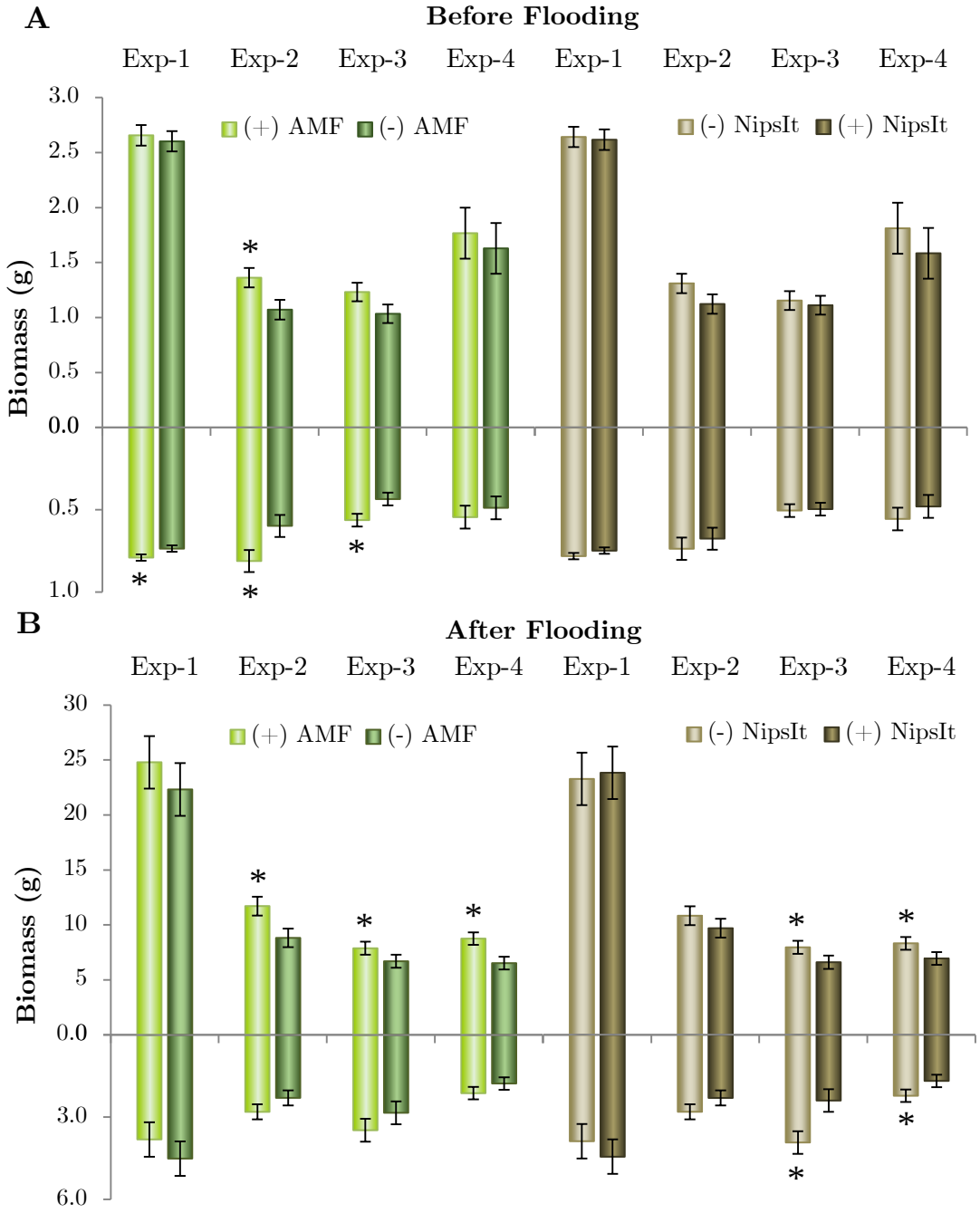

Figure 6.5. Main effects of inoculation with AM fungi (+AMF and –AMF) and treatment of seeds with insecticide (+NsI and –NsI) on shoot (above x-axis) and root (below x-axis) dry weights of rice plants sampled from plots twice: (A) before and (B) after flooding, of four experiments conducted in the field over three years (2016-2018). AM fungi-treated plants (light green) and non-AM fungi (dark green). Values are the means of 10 replications. Asterisk at the column head indicate that means differ significantly (LSD, $P \leq 0.05$).

6.3.6. Effect of seed treatments on concentrations of plant nutrients

The effects of AM fungi and insecticide seed treatments on concentrations of nutrients in rice shoots and roots were inconsistent, and only a handful of significant responses were observed. Before flooding, treatment with AM fungi significantly increased shoot N concentrations in Exp-1 and root N concentrations in Exp-3 (Table C5). Also, inoculation with AM fungi significantly decreased shoot N concentrations in Exp-2 and Exp-3 and root N concentrations in Exp-1 (Figure C6A). Insecticide seed treatment significantly decreased shoot N and increased root N concentrations in Exp-3 (Figure C6A). AM fungi treatment significantly decreased shoot P concentrations in Exp-2 and decreased root P concentrations in Exp-3 (Table C5; Figure C6B). Treatment with AM fungi significantly increased shoot C concentrations in Exp-1 and decreased shoot C concentrations in Exp-2 (Table C5; Figure C6C). NipsIt INSIDE seed treatment significantly decreased shoot C concentrations in Exp-2 and increased root C concentrations in Exp-3 (Table C5; Figure C6C). Additionally, the interaction between inoculation with AM fungi and insecticide seed treatments significantly influenced shoot and root P concentrations in Exp-2, as well as N and C concentrations of shoots and roots in Exp-3 (Table C6). The interactions between seed treatments suggest that AM fungi treatments did not influence significantly shoot and root nutrient concentrations in Exp-2 and Exp-3 in the absence of insecticide treatments, but these interactions influenced plant nutrient concentrations in insecticide-treated plots.

The effects of treatments on nutrient concentrations after flooding were also inconsistent. Treatment with AM fungi significantly increased shoot N concentrations in Exp-4 (Table C5; Figure C6D). NipsIt INSIDE seed treatment significantly increased root N concentrations in Exp-1 and -3 (Figure C6D). Insecticide treatment significantly increased shoot P concentrations in Exp-4, and root P concentrations in Exp-1 and -2 (Table C5; Figure C6E). Treatment with AM fungi significantly decreased shoot C concentrations in Exp-3 and increased shoot C concentrations in Exp-4 (Table C5; Figure

C6F). Insecticide seed treatment significantly decreased shoot C concentrations in Exp-2 and -4, and increased root C concentrations in Exp-1 and -3 (Figure C6F). Also, the interaction between AM fungi and insecticide treatment significantly influenced root P concentrations in Exp-1, shoot N concentrations and root N, P and C concentrations in Exp-3, and shoot N and C concentrations in Exp-4 (Table C7). Interactions between AM fungi and insecticide treatments show that AM fungi treatment did not affect significantly shoot and root nutrient concentrations mostly in Exp-1, Exp-3 and Exp-4 without insecticide treatments.

6.3.7. Effect of seed treatments on yields and tolerance

Data for percentages of heading and maturity were not taken in Exp-1 and Exp-2. AM fungi seed treatment and the interaction of AM fungi and insecticide seed treatments did not accelerate heading or maturity of rice plants in Exp-3 and Exp-4 (Table 6.6; Figure 6.6A). In contrast, insecticide seed treatment significantly accelerated percentages of heading at 87.5 DAP and maturity at 101.5 DAP of NipsIt INSIDE-treated plots in Exp-4 (Table 6.6). At 87.5 DAP, panicle heading in plots of insecticide-treated plants was 32.8% earlier in Exp-4 when compared to untreated plots (Figure 6.6B). At 101.5 DAP, panicle maturity in NipsIt INSIDE-treated plots hastened 10.0% in Exp-4 when compared to untreated plots (Figure 6.6B).

Table 6.6. Results of ANOVA for effects of inoculation with AM fungi (+AMF and -AMF), treatment of seeds with insecticide (+NsI and –NsI) as well as their interaction on panicle heading and maturity percentages of rice plots of the two experiments conducted in the field in 2018.

Factor	Heading (%)						Maturity (%)					
	Exp-3			Exp-4			Exp-3			Exp-4		
	d.f.	*F*	*P*	*d.f.*	*F*	*P*	*d.f.*	*F*	*P*	*d.f.*	*F*	*P*
AMF	1, 27	3.05	0.09	1, 27	1.82	0.19	1, 27	0.12	0.74	1, 27	0.01	0.93
Insecticide	1, 27	0.01	0.98	1, 27	30.1	$<.0001$	1, 27	1.49	0.23	1, 27	6.63	0.02
AMF*Insecticide	1, 27	2.57	0.12	1, 27	2.34	0.14	1, 27	0.52	0.47	1, 27	0.20	0.66

Bold numbers indicate significant effects.

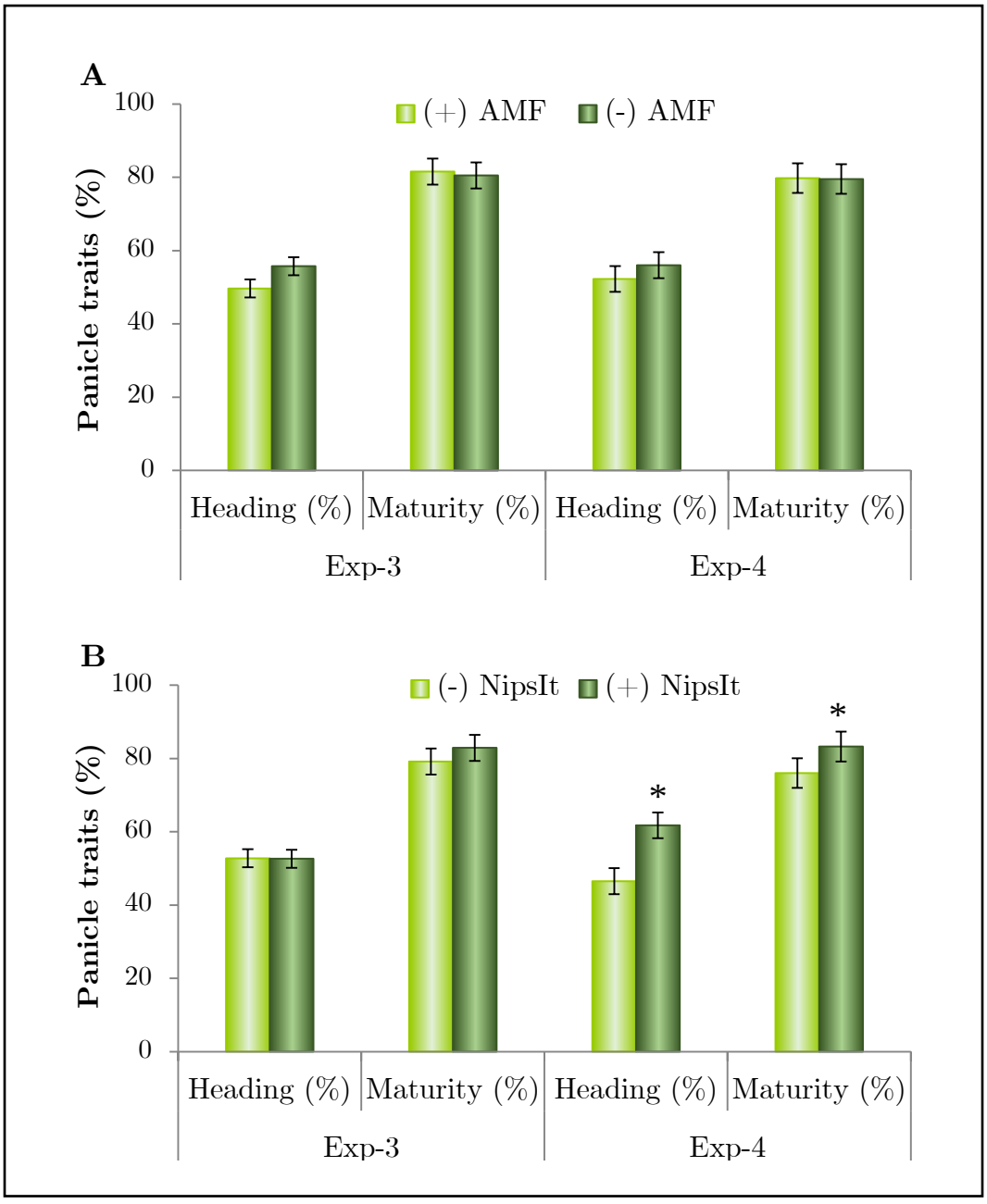

Figure 6.6. Main effects of inoculation with AM fungi and treatment of seeds with insecticide on percentages of panicle heading and maturity (% Mean ± S.E.) in rice plots of two experiments conducted in the field in 2018. (A) main effect of AM fungi treatment (+AMF and –AMF); (B) main effect of insecticide (+NsI and –NsI) treatment. The numbers are the means of 10 replications. Asterisk at the column head indicate that means differ significantly between treatments (LSD, $P \leq 0.05$).

Yields from rice seeds inoculated with AM fungi were significantly higher than from plots not treated with AM fungi in Exp-1, Exp-2 and Exp-4 (Table 6.7). Yields from AM fungi plots were higher by 410 kg/ha in Exp-1, 632 kg/ha in Exp-2, and 1151 kg/ha in Exp-4 (Figure 6.7A). No effect of AM fungi treatment on yield was observed in Exp-3. NipsIt INSIDE seed treatment significantly affected yields in Exp-3 and Exp-4 (Table 6.7). Yields from insecticide-treated plots were higher by 346 kg/ha and 1338 kg/ha in these experiment (Figure 6.7B). Interaction between AM fungi and insecticide did not affect rice yields in any of the experiments (Table 6.7). A meta-analysis of yield from all four experiments showed that AM fungi-treated plots had significantly higher yields compared to non-AM fungi plots ($F_{1, 148}=16.90$, $P<0.0001$), and that NipsIt INSIDE-treated plots had significantly higher yields compared to untreated plots ($F_{1, 148}=14.73$, $P=0.0002$).

Table 6.7. Results of ANOVA for effects of inoculation with AM fungi (+AMF and -AMF), treatment of seeds with insecticide (+NsI and –NsI) as well as their interaction on yields of rice plots of four experiments conducted in the field over three consecutive years (2016-2018). Yields were adjusted to 12% moisture.
Bold numbers indicate significant effects.

Factor	Exp-1			Exp-2			Exp-3			Exp-4		
	d.f.	*F*	*P*	*d.f.*	*F*	*P*	*d.f.*	*F*	*P*	*d.f.*	*F*	*P*
AMF	1, 36	6.49	0.02	1, 24	7.66	0.01	1, 36	0.52	0.48	1, 36	7.71	0.009
Insecticide	1, 36	0.63	0.43	1, 24	2.29	0.14	1, 36	7.19	0.01	1, 36	10.4	0.003
AMF*Insecticide	1, 36	0.18	0.68	1, 24	0.31	0.58	1, 36	0.11	0.74	1, 36	1.15	0.29

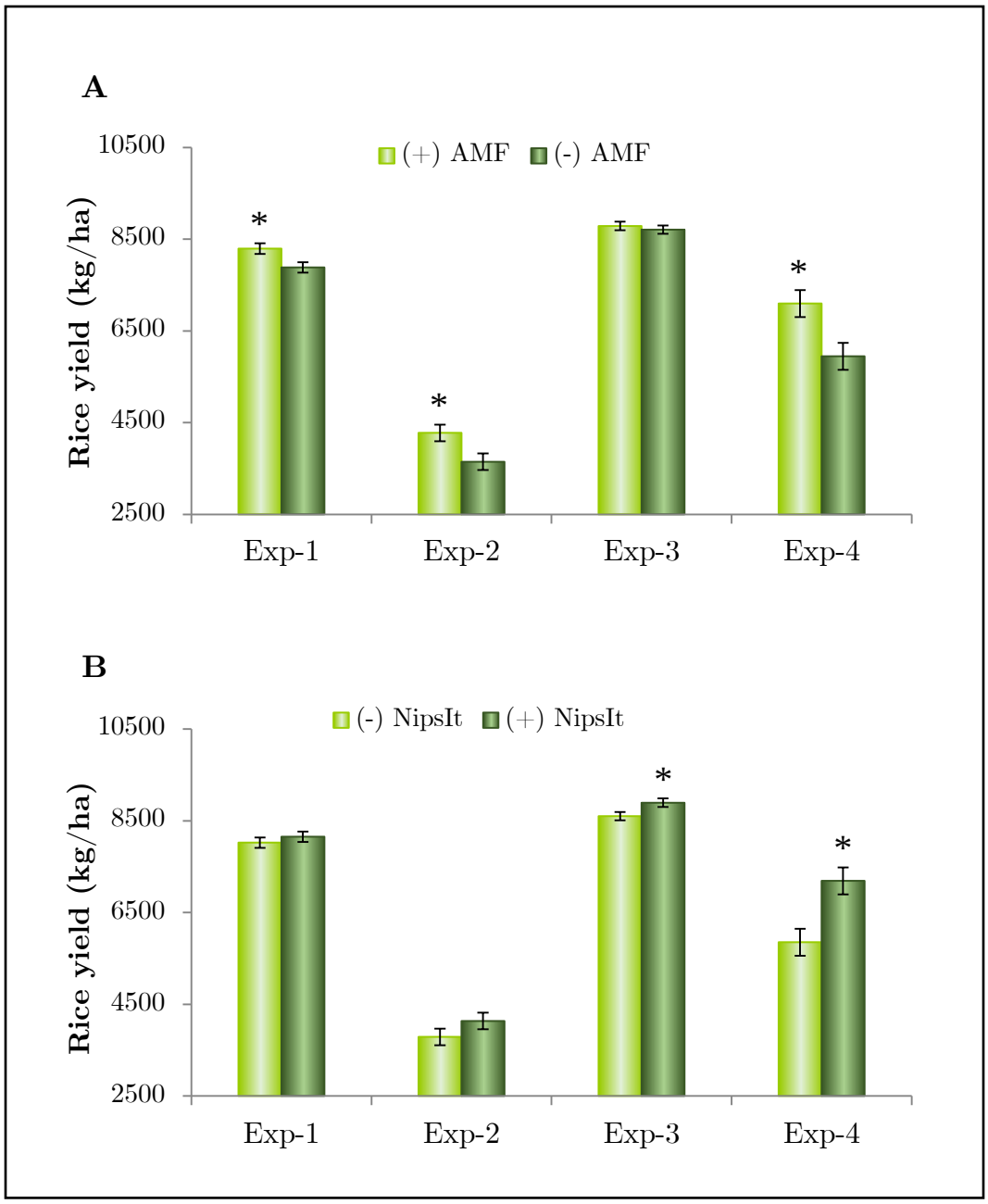

Figure 6.7. Main effects of inoculation with AM fungi (+AMF and –AMF) and treatment of seeds with insecticide (+NsI and –NsI) seed treatments on yields (kg/ha) of rice plots of four experiments conducted in the field over three consecutive years (2016-2018). AM fungi-treated plants (light green) and non-AM fungi (dark green). Yields were adjusted to 12% moisture. Values are the means of 10 replications. Asterisk at the column head indicate that means differ significantly (LSD, $P \leq 0.05$).

Differences in yield loss (kg/ha) from weevils in presence of AM fungi with or without insecticide and not inoculated with AM fungi are not significant in any of the four experiments (Table C8). However, AM fungi plots had a trend towards higher yield losses in all four experiments than those with or without insecticide only (Table C8). Yield losses from weevils in presence of AM fungi were higher by 134 kg/ha in Exp-1, 255 kg/ha in Exp-2, 87 kg/ha in Exp-3, and 889 kg/ha in Exp-4 than yield losses in presence of insecticide. A meta-analysis to compare yield loss in AM fungi plots with yield loss in not inoculated with AM fungi plots from all four experiments also showed no significance difference in yield loss ($F_{1, 72.1}=1.04$, $P=0.312$). Yield losses from weevils in presence of AM fungi ranged from 2 to 19% depending on the experiment, while yield losses from weevils in presence of insecticide ranged from 1 to 12% (Figure C7).

6.4. Discussion

Louisiana is largely an agricultural state. Rice is the second most important crop of the state, but the continuous use of pesticides and chemical fertilizers keeps contaminating our ecosystem which, in turn, affects agricultural outputs negatively. AM fungi inoculation has become a sustainable approach to overcome the reduction in plant yield produced by insects by altering the physiological and biochemical properties of the host plant. The result of the symbiosis between AM fungi and their host plants is variable, with the environmental factors influencing the quantity, effectiveness, and nature of resource exchange (Bever, 2015; Johnson et al., 2010; Wardle et al., 2004). On one hand, the positive effects of AM fungi on plant growth, nutrient concentrations (Solaiman & Hirata, 1996, 1997), defense against pathogens (Campos-Soriano et al., 2011), and photosynthetic rates (Black et al., 2000) have been shown in several studies. On the other hand, negative effects on resistance to herbivore have also been well documented (Bernaola et al., 2018b;

Cosme et al., 2011), but their impact on tolerance to root-herbivore injury and yield loss is poorly understood in crop systems.

In the current study, the plants inoculated with AM fungi resulted in greatly increased AM fungi root colonization, which in turn, modulated the belowground dualistic interaction of its host plant, *O. sativa*, and the root herbivore RWW, to the fitness benefit of the host plant. Overall, AM fungi plants overcompensated for herbivore injury (Strauss & Agrawal, 1999). AM fungi increased RWW densities, and the associated root damage caused on the rice plants was compensated by the marginal increase of rice yields. Even though the presence of AM fungi increased rice biomass and yield, shoot and root nutrient concentrations were not consistent in this study.

One method to measure plant tolerance, which is defined as the ability to maintain plant fitness (biomass or yield) after herbivore injury, is by estimating fitness differences between damaged and undamaged plants (Garrido et al., 2010). In Garrido et al. (2010), no increases in plant biomass were shown in a greenhouse experiment when using a commercial AM fungi and manual defoliation. In contrast, Bernaola et al. (2018b) showed that AM fungi inoculation had a positive effect on plant biomass, in the field, when high densities of immature RWWs were present. Similar to this, we found evidence that AM fungi colonization provided an advantage to root-herbivore injured plants. In fact, AM fungi had a stronger effect on plant biomass in the presence of root injury, suggesting that the presence of RWW larvae does not limit the plant's ability to benefit from AM fungi. In contrast to the manual defoliation in the work of Garrido et al. (2010), the natural infestation of RWW in our studies with commercial AM fungi, in four different field experiments across three years, show that AM fungi-treated plots exhibited increased plant biomass. The consistency of our field experiments suggest that commercial AM fungi has the potential to compensate for herbivory injury. Furthermore, Dhillion (1992) showed that indigenous AM fungi species, collected from the field in Louisiana, significantly increased plant growth among different rice cultivars. Combined with our results, this

suggests that rice plants can achieve similar performance when growing with either commercial inoculum or natural inoculum.

Consistent among all AM fungi treatments, inoculation with AM fungi increased immature weevil densities. This study found that AM fungi positive effects on herbivores depended on feeding sites, which aligns with the results of Currie et al. (2011), Bernaola et al. (2018b), as well as Koricheva et al. (2009). Currie et al. (2011) found that AM fungi increased larval survival of root-feeding insects; Bernaola et al. (2018b) reported that inoculation with AM fungi increased densities of root-feeding rice water weevil; Koricheva et al. (2009) addressed that chewer insects benefited from AM fungi. On the other hand, our results are opposite to Gange (2001), who demonstrated that AM fungi significantly reduced the larval survival and biomass of root-feeding black vine weevil. Therefore, these differences might be caused by diet breadth, where AM fungi probable had more positive effects on specialist insect herbivores than on generalist ones (Hartley & Gange, 2009; Koricheva et al., 2009). Positive effects of AM fungi on root-feeding herbivores might be mediated by increasing delivery of nutrient (N or P) concentrations, which in turn, make plants nutritionally superior and attractive for herbivores (Cosme et al., 2011; Currie et al., 2011; Vannette et al., 2013). In our study, AM fungi do not always increase plant nutrient concentrations in all experiments, but AM fungi clearly modulated the concentration of nutrients before flooding was established.

Despite the lack of negative effects of AM fungi on rice water weevil, yields were affected by AM fungi. AM fungi seed treatment improved rice yields in Exp-1 and Exp-2 by 5% and 14%, respectively, when compared to non-AM fungi plots. The high levels of AM fungi colonization in all experiments of AM fungi-treated plants were correlated with a clear increase of rice yields, root dry weight (before flooding), and shoot dry weight (after flooding) in field experiments. Beneficial effects of AM fungi on rice yield have also been previously reported. Two field experiments conducted in Japan showed that one of three varieties, ARC5955, exhibited a strong tendency toward increase (up to 42%) in rice

yield in AM fungi-treated plots (Sisaphaithong et al., 2017). Inoculation with AM fungi also increased grain yield by 14-21% higher than not inoculated plots under field conditions but AM fungi colonization did not influence grain yield under greenhouse conditions (Solaiman & Hirata, 1997). Similarly, Diedhiou et al. (2016) showed that upland rice varieties treated with AM fungi had higher grain yield (up to 52%) when compared to non-inoculated plants under field conditions. In addition, the increases in yield of AM fungi-treated plots (5-14%) were higher than the increases of NipsIt INSIDE (2.0-8.4%) over untreated plots.

This study revealed that AM fungi inoculation had beneficial effects on plant growth and yields. The fact that AM fungi seed treatment increased plant growth and yield of rice plants inoculated with AM fungi and after herbivory injury, opens an alternative to use commercial formulations of AM fungi as a potential part of pest management programs in the southern United States. Despite the positive effect of AM fungi on the performance of insect pests in this study, AM fungi still have an important role in rice production. However, further studies should be conducted using different formulations of AM fungi, including native species from the field of study, as well as more rice varieties in order to determine a suitable AM fungi-rice combination, which can enhance more yield in inoculated plants versus non-inoculated ones.

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Chapter 7
Conclusions and Future Directions

With the global population increasing rapidly for many years, the challenges to meet the demands of food security are still a major concern. Insect herbivores are a constant threat to plants, which are the primary producers in terrestrial ecosystems. In addition to the detrimental organisms that interact with plants, plants host a diversity of beneficial microorganisms (root-associated microbes) in the rhizosphere as well. These interactions between plants and mutualistic microbes such as AM fungi can also affect plant interactions with insect herbivores and plant pathogens. Within the soil microbiome, more attention has been given to pathogenic effects of fungal and bacterial diseases on rice (Melanson et al., 2017; Nalley et al., 2016; Shrestha et al., 2016), but the role of mutualistic organisms in insect management and control has been largely ignored in Louisiana. The main goal of the present dissertation research was to explore and improve our understanding of the novel effects of AM fungi, a soil-borne microbe, on rice resistance to different pests. AM fungi are known to play various key ecological roles in nature and might as well have a role in rice-insect interactions. This study represents the first study conducted in the United States in rice showing the effects of AM fungi in rice-insect interactions.

7.1. Conclusions

Laboratory, greenhouse and field experiments were carried out, and in the light of key findings from the observations reported in the foregoing chapters, it can be concluded that:

AM fungi establish natural associations with the roots of commercial rice varieties in different rice production areas in the southern United States. This information can be used in future rice research to facilitate the agricultural exploitation of the symbiosis.

AM fungi increase rice susceptibility to insects and a pathogenic fungi that specialize on different plant tissues. This information provides a different perspective on the causal bases of rice resistance to insects and pathogens.

The effects of AM fungi on rice-insect interactions is soil dependent. This information highlight the importance of considering soil feedback in sustainable agriculture and the role of AM fungi species.

The effect of AM fungi seed treatments in rice tolerance after root herbivore feeding seem to be more effective for increasing plant growth and yields than AM fungi granular formulation applied to the soil.

It would certainly be premature, given the results of this study, to view AM fungi as the ultimate solution to pest control in rice. However, one thing is clear is that this mutualistic symbiosis has a potential role to play as far as yield increase is concerned after herbivore injury. However, more insight is still needed to increase our understanding of the tripartite interaction of AM fungi, insects and rice plants before any recommendation can be made. It is my hope that future projects on above-belowground interactions in rice will benefit from the research findings gained here.

7.2. Future directions

AM fungi are a fundamental part of agroecosystems, with potential to provide both benefits and costs to farmers. The management decisions of farmers drive evolutionary selection in these diverse soil organisms (Verbruggen & Kiers, 2010). More work on the host-mycorrhizal-insect interactions may allow us to provide specific management

recommendations that can increase yield and meet the demand of an increasingly world population.

The data obtained from this study should be further deepened and extended. One major goal would be to focus on the fundamental basis of induce resistance using mutants. An expansion for Chapter 3, the identification of the native or indigenous species of AM fungi should be conducted with the aim to gather more information about the common or other species of AM fungi present in different rice producing areas of the Unite States.

An expansion for Chapter 4, in addition to inoculation with a commercial formulation of AM fungi, the use of native species of AM fungi in the field of study should be necessary to determine the effectiveness of both inoculation systems in rice. Native species are also important components present in the soil community of agricultural fields and there is a lot of controversy in the use of native versus commercial formulations of AM fungi.

An expansion for Chapter 5, identification of soil characteristics responsible for this context dependency to facilitate an understanding of how production practices influence the potential benefits of AM fungi in rice plants and its interaction with rice pests. In addition, selecting more soils locations with varying properties, not only in Louisiana but also other rice-producing areas, will be necessary to determine the effect of inoculation with AM fungi in those areas.

An expansion for Chapter 6, additional rice varieties should be included and screened with the aim to test the effects of AM fungi on rice tolerance after root herbivore injury not only against the RWW but also other rice pests since it has been shown that rice varieties respond differently to RWW feeding and using a single cultivar may reduce the variability of tolerance to RWW.

7.3. References

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Appendix A: Supplementary information for Chapter 4

Table A1. Properties of soil collected from the Crowley site for experiments conducted in 2012 and 2013

Soil Properties	2012		2013	
	Amount	Rating*	Amount	Rating*
Texture	Silt loam		silt loam	
pH (in water)	5.57	OP	5.97	OP
% organic matter	2.33		1.77	
CEC	9		12.54	
P (ppm)	11	L	13	L
K (ppm)	110	H	74	M
Ca (ppm)	1,341	M	1202	VH
Mg (ppm)	459	VH	254	VH
Na (ppm)	138	OP	54	VL
S (ppm)	11.6	L	4.23	L
Cu (ppm)	1.8	H	1.39	H
Zn (ppm)	4.3	H	8.1	H

*According to recommendation sheet: http://www.stpal.lsu.edu\recsheets\C-150.RTF
CEC = cation exchange capacity; OP = optimal; L = low; M = medium; VH = very high; VL = very low; H = high.

Table A2. Summary of field and greenhouse experiments conducted in 2012 and 2013.

Year	Trial	Treatments	N° of reps/ treatment	N° plants/ treatment	Root colonization assessed
2012	Experiment-1	F, NM & M	8	> 400	No
	FAW1	NM & M	14	42	No
2013	Experiment-2	F, NM & M	10	> 400	Yes
	Experiment-3	F, NM & M	10	> 400	Yes
	RWW1	NM & M	14	28	Yes
	RWW2	NM & M	12	36	Yes
	FAW2	NM & M	15	45	Yes
	FAW3	NM & M	15	45	Yes
	ShB1	NM & M	15	75	Yes
	ShB2	NM & M	15	45	No
	PB1	NM & M	12	48	No

The F, NM and M refer to AMF treatments of F: rice seeds + fungicides + sterilized AMF, NM: rice seeds + sterilized AMF, and M: rice seeds + live AMF. The Experiment-1, 2 and 3 are field experiments conducted against the rice water weevil. The RWW1, RWW2, FAW1, FAW2, FAW3, ShB1 and ShB2 experiments were conducted against the rice water weevil, fall armyworm and sheath blight of rice. The PB1 refers to plant biomass greenhouse experiment using field soil.

Table A3. Results of ANOVA (Proc Mixed) of arbuscular mycorrhizal fungi (AMF) treatment effects on plant (root and shoot tissue) nutrient concentration of 30-day-old rice plants taken from field and greenhouse experiments in 2012 and 2013.

Root tissue	N (%)	P (%)	K (%)	C (%)
Field 2012 (Exp-1)	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
Fungicide (F)	1.80 ± 0.08	0.12 ± 0.01	1.15 ± 0.02	37.08 ± 1.18
Nonmycorrhizal (NM)	1.71 ± 0.02	0.11 ± 0.01	1.11 ± 0.07	36.45 ± 1.18
Mycorrhizal (M)	1.86 ± 0.02	0.13 ± 0.01	1.19 ± 0.07	38.28 ± 0.19
$F_{2,6}$	3.59	3.59	0.95	0.91
$P\text{-}value$	0.095	0.094	0.437	0.451
Field 2013 (Exp-2)	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
Fungicide (F)	0.78 ± 0.03	0.13 ± 0.01	0.89 ± 0.07	35.55 ± 2.36
Nonmycorrhizal (NM)	0.89 ± 0.04	0.14 ± 0.01	1.06 ± 0.05	36.55 ± 1.93
Mycorrhizal (M)	0.87 ± 0.03	0.15 ± 0.01	0.98 ± 0.05	36.73 ± 0.83
$F_{2,6}$	2.67	0.68	2.09	0.12
$P\text{-}value$	0.148	0.543	0.204	0.889
GH 2013 (PB1)	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
Nonmycorrhizal (NM)	1.41 ± 0.05	0.19 ± 0.01	1.26 ± 0.06	39.23 ± 0.38
Mycorrhizal (M)	1.41 ± 0.06	0.18 ± 0.01	1.26 ± 0.04	38.75 ± 0.25
$F_{1,5}$	0.00	1.00	0.01	1.14
$P\text{-}value$	0.980	0.364	0.911	0.335

The F, NM and M refer to AMF treatments of F: rice seeds + fungicides + sterilized AMF, NM: rice seeds + sterilized AMF, and M: rice seeds + live AMF. Concentrations of four elements did not differ significantly among treatments.

(Table cont'd.)

Shoot tissue	N (%)	P (%)	K (%)	C (%)
Field 2012 (Exp-1)	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
Fungicide (F)	3.15 ± 0.04	0.16 ± 0.01	2.56 ± 0.06	40.45 ± 0.26
Nonmycorrhizal (NM)	3.05 ± 0.14	0.14 ± 0.01	2.44 ± 0.07	39.75 ± 0.10
Mycorrhizal (M)	3.25 ± 0.05	0.16 ± 0.01	2.45 ± 0.03	40.28 ± 0.24
$F_{2,6}$	2.01	0.65	1.62	3.80
$P\text{-}value$	0.214	0.554	0.275	0.086
Field 2013 (Exp-2)	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
Fungicide (F)	1.45 ± 0.12	0.27 ± 0.01	2.09 ± 0.12	39.48 ± 0.32
Nonmycorrhizal (NM)	1.70 ± 0.09	0.29 ± 0.01	2.19 ± 0.12	39.85 ± 0.17
Mycorrhizal (M)	1.45 ± 0.12	0.29 ± 0.01	2.09 ± 0.04	39.45 ± 0.21
$F_{2,6}$	3.50	2.75	0.73	0.87
$P\text{-}value$	0.098	0.142	0.518	0.467
GH 2013 (PB1)	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
Nonmycorrhizal (NM)	1.95 ± 0.06	0.23 ± 0.01	3.42 ± 0.05	38.11 ± 0.09
Mycorrhizal (M)	2.13 ± 0.13	0.22 ± 0.01	3.28 ± 0.05	38.18 ± 0.13
$F_{1,11}$	2.26	1.54	3.89	0.18
$P\text{-}value$	0.161	0.241	0.074	0.679

The F, NM and M refer to AMF treatments of F: rice seeds + fungicides + sterilized AMF, NM: rice seeds + sterilized AMF, and M: rice seeds + live AMF. Concentrations of four elements did not differ significantly among treatments.

Figure A1. Photographic representation of rice water weevil injury. A: rice field under flooded conditions triggers rice water weevil infestations; B: core sampler used to collect plants from rice plots to determine weevil densities; and C: red arrows pointing larvae of rice water weevil feeding in rice roots.

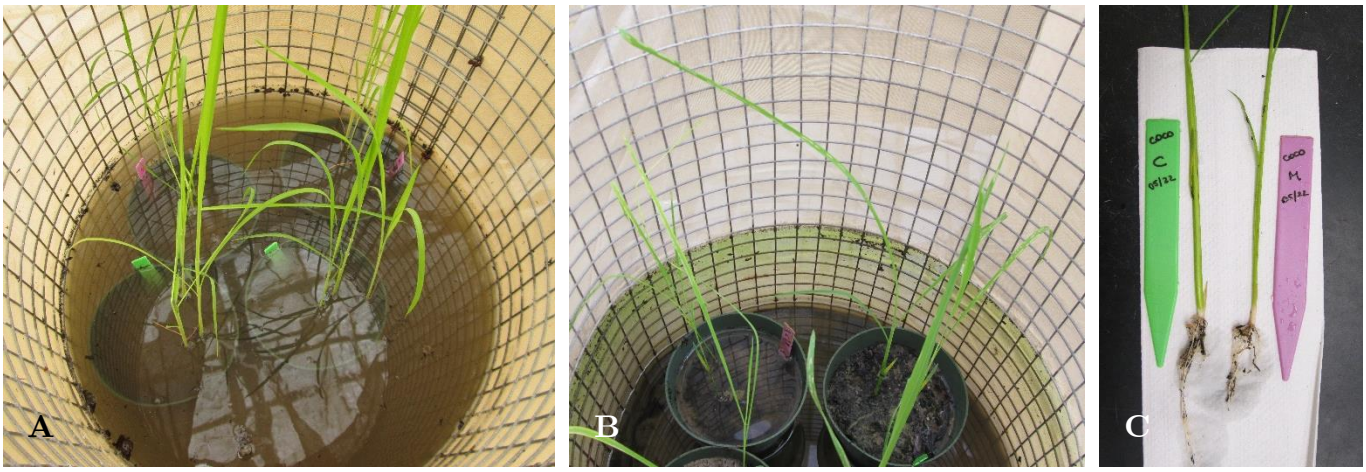


Figure A2. Photographic representation of rice water weevil choice experiments. A: mycorrhizal and nonmycorrhizal pots placed in a cage under flooded conditions before weevil infestation; B: mycorrhizal and nonmycorrhizal pots showing leaf injury (white scars) after weevil infestation; and C: mycorrhizal and nonmycorrhizal plants showing differences in the root system after weevil feeding.

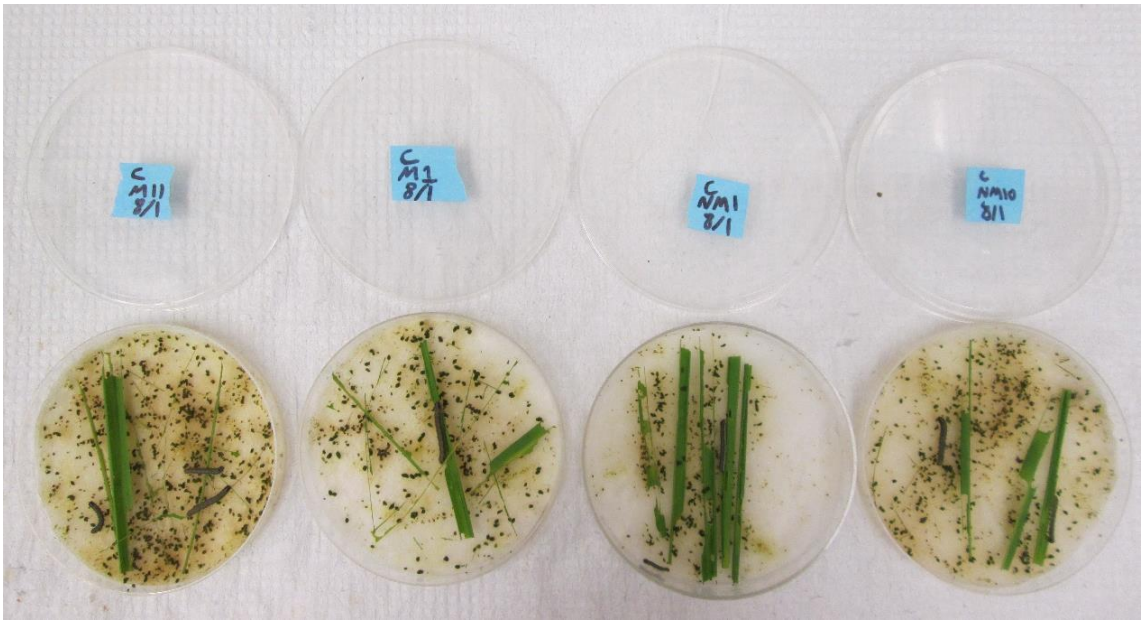


Figure A3. Photographic representation of typical fall armyworm feeding assays. Feeding assays were conducted in petri dishes lined with moistened cotton batting to maintain turgor in freshly cut leaf tissues. This picture shows difference among treatments (mycorrhizal and nonmycorrhizal tissues) at the end of the fall armyworm feeding experiment.

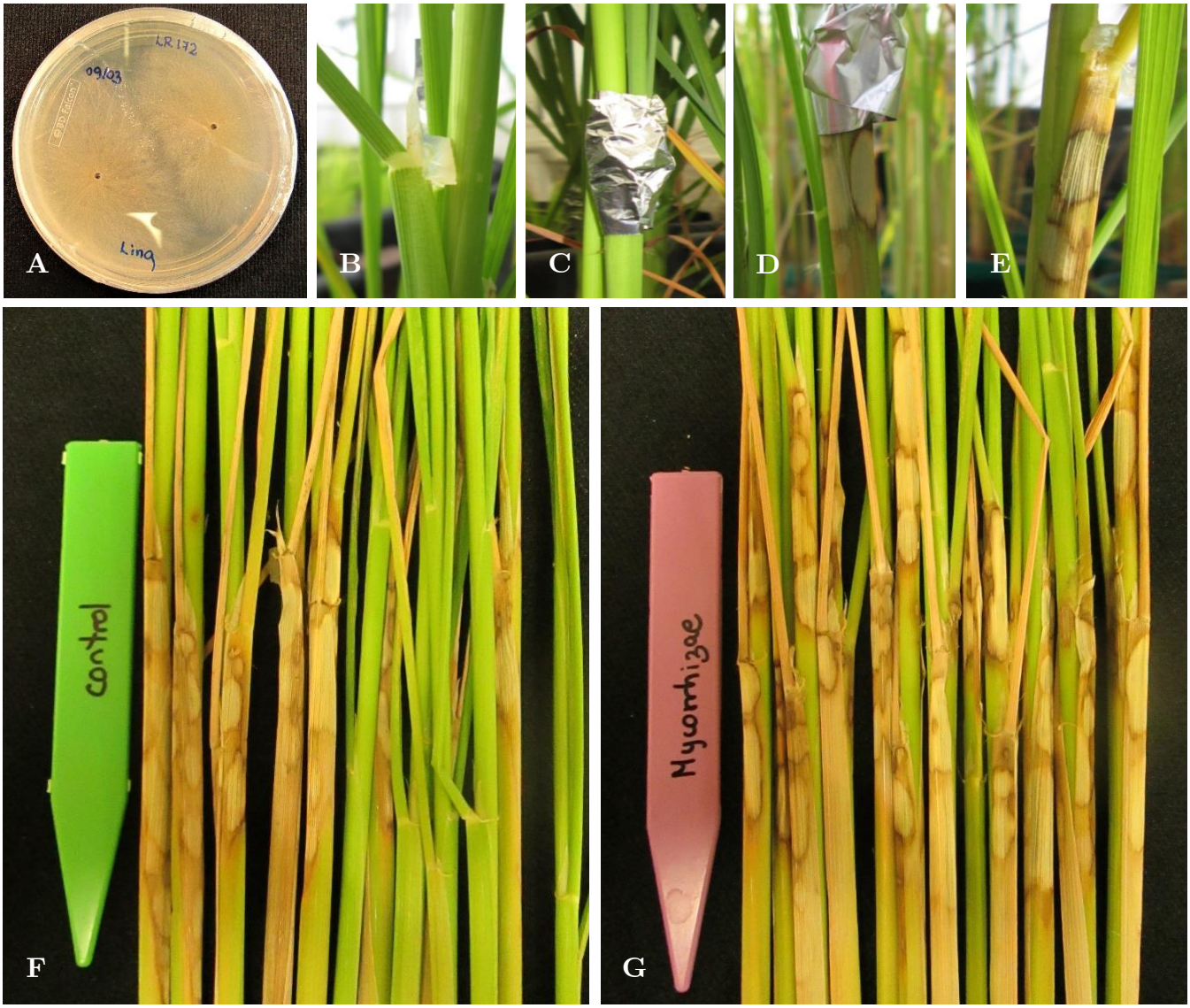


Figure A4. Photographic representation of sheath blight inoculation. A: sclerotia of *Rhizoctonia solani* on potato dextrose agar; B: inoculation of mycelia ball beneath leaf sheath; C: inoculated sheath covered with aluminum foil; D: appearance of lesions (symptoms) 3 days after inoculation; E: removal of aluminum foil 7 days after inoculation; F and G: level of infection in nonmycorrhizal and mycorrhizal rice plants, respectively.

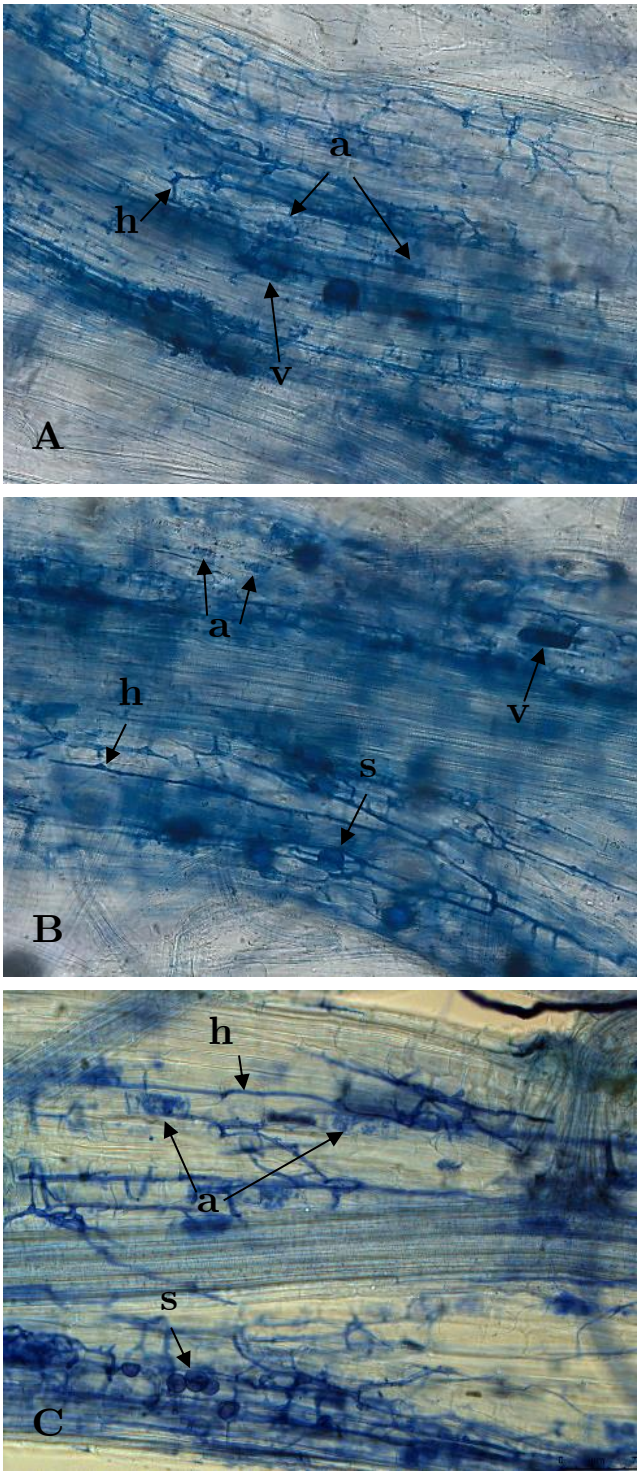


Figure A5. Root fragments stained with trypan blue showing arbuscular mycorrhizal fungi structures in rice plants. Light micrographs of mycorrhizal inoculated root fragments from some experiments conducted in 2013 show: (A) Hyphae (h), arbuscule (a), and vesicle (v). (B) Hyphae, arbuscule, spore (s) and vesicle. (C) Hyphae, arbuscule, and spore.

Appendix B: Supplementary information for Chapter 5

Table B1. t-values of one simple t-test with the effect size of mycorrhizal parameters (MGR) for each of the two soil types (Crowley and Mamou) of field experiments. *MGR* mycorrhizal growth response

Experiment	Parameter	Soil type					
		Mamou			Crowley		
RWW-1		d.f.	t	P	d.f.	t	P
	Shoot dry weight (g)	4	3.37	0.03	4	1.94	0.12
	Root dry weight (g)	4	3.13	0.04	4	3.54	0.02
RWW-2							
	Shoot dry weight (g)	4	1.68	0.17	4	4.41	0.02
	Root dry weight (g)	4	2.36	0.08	4	3.02	0.05

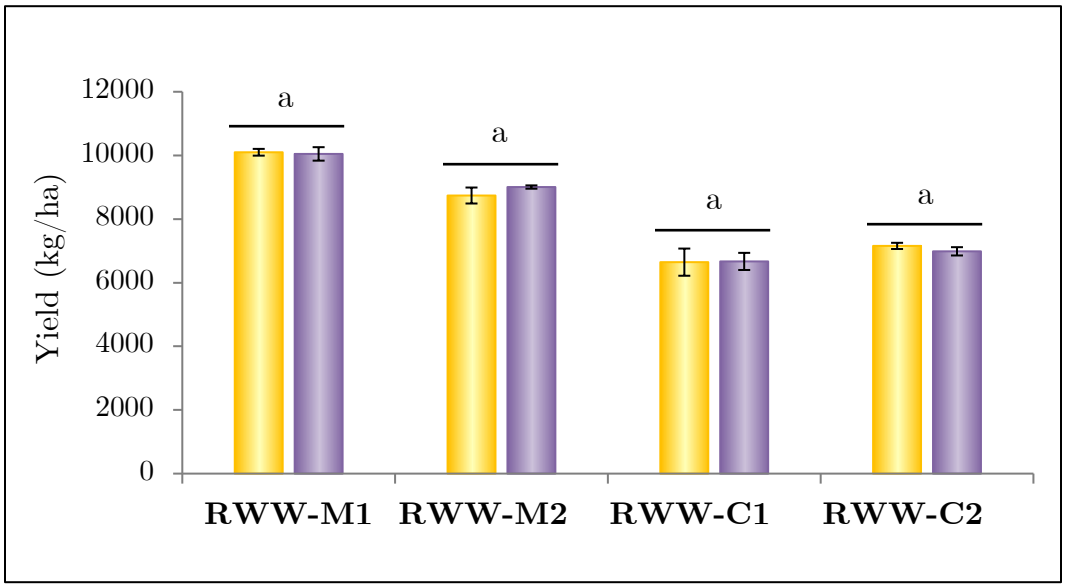


Figure B1. Influence of arbuscular mycorrhizal fungi inoculation on yields from field experiments in two different locations during 2014-2015. Yields were adjusted to 12% moisture. Rice plants were inoculated with AMF (orange bars) or with NM control inoculum (orange bars). Bars represent means of five ± SE. Bars and upper case letters at the column head indicate that means differ significantly (LSD, $P \leq 0.05$).

Appendix C: Supplementary information for Chapter 6

Table C1. Properties of soil collected from the Crowley site for seed treatment experiments conducted in 2016, 2017 and 2018. Numbers show the average of both years (mean ± SE, n = 2).

Soil Properties	2016		2017		2018	
	Amount	Rating*	Amount	Rating*	Amount	Rating*
Texture	Silty clay loam		Silt loam		Silt loam	
pH (in water)	7.5 ± 0.2	H	7.9 ± 0.2	VH	7.12	H
% organic matter	1.1 ± 0.1		1.2 ± 0.1		1.4 ± 0.1	
CEC	17.2 ± 0.1		11.5 ± 0.1		11.8 ± 0.1	
P (ppm)	13.0 ± 0.2	L	15.0 ± 0.2	L	20 ± 0.2	L
K (ppm)	87.0 ± 0.1	M	122 ± 0.1	H	108 ± 0.2	H
Ca (ppm)	1,870	VH	1,665	VH	1,627	VH
Mg (ppm)	266 ± 0.2	VH	344 ± 0.2	VH	237 ± 0.3	VH
Na (ppm)	131 ± 0.1	OP	96.0 ± 0.1	OP	67 ± 0.1	OP
S (ppm)	7.0 ± 0.1	L	1.7 ± 0.1	L	7.1 ± 0.1	L
Cu (ppm)	2.1 ± 0.2	H	2.7 ± 0.2	H	2.0 ± 0.2	H
Zn (ppm)	20.0 ± 0.1	H	11.0 ± 0.1	H	6.4 ± 0.2	H

*According to recommendation sheet: http://www.stpal.lsu.edu\recsheets\C-150.RTF
CEC = cation exchange capacity; OP = optimal; L = low; M = medium; VH = very high; H = high.

Figure C1. Photographic representation of densities of rice water weevil immatures (larvae and pupae). (Left) Larvae are counted as they float to the surface of salt water. (Right) Pupae are counted as they settled in the bottom of sieve buckets and are highlighted on red circle.

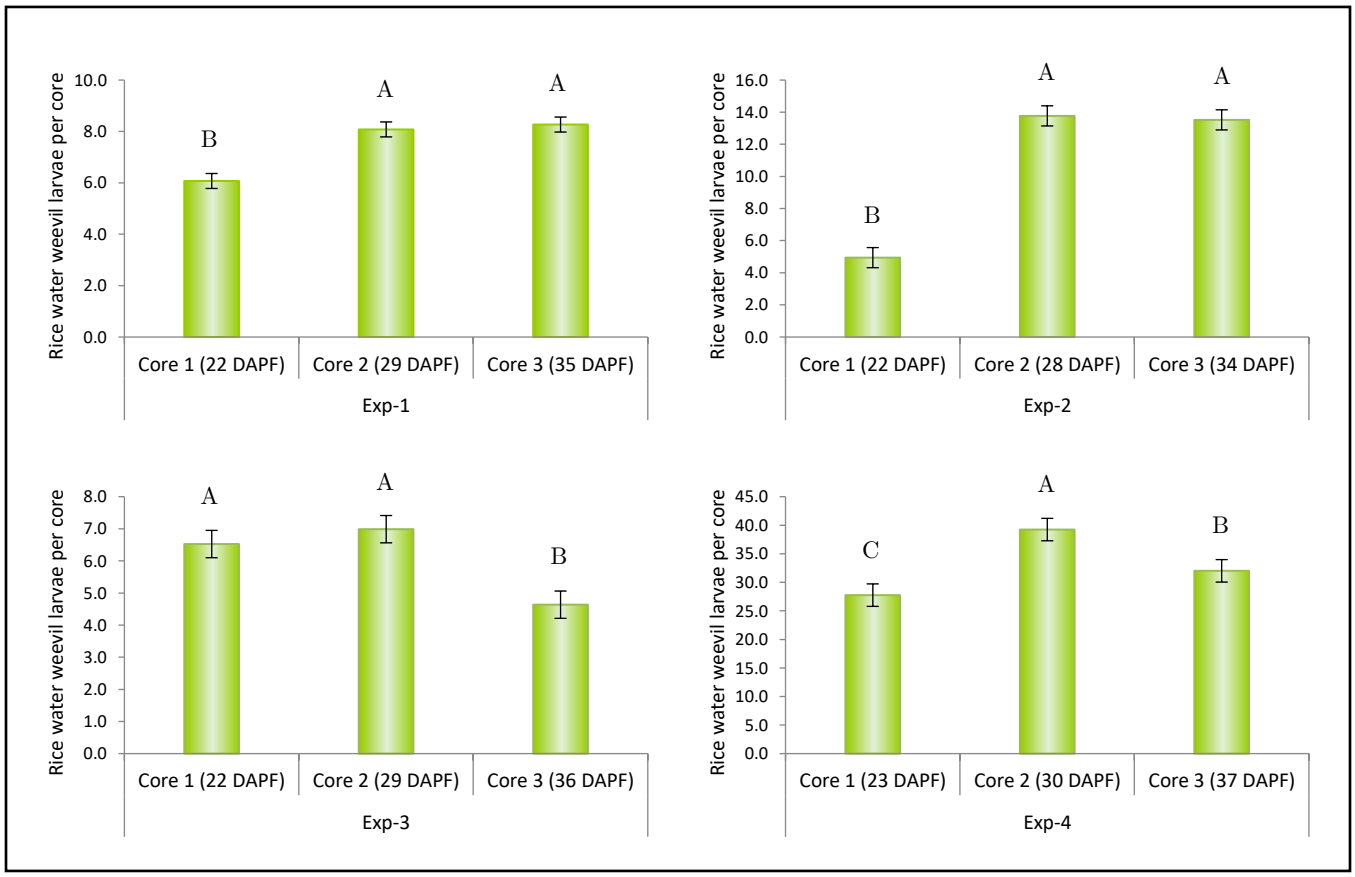


Figure C2. Influence of time (core sampling date) on densities of rice water weevil (larvae and pupae per core sample ± S.E.) in rice plots of four experiments conducted in the field over three years (2016-2018). (Top left) Exp-1, (Top right) Exp-2, (Bottom left) Exp-3, and (Bottom right) Exp-4. The numbers are the means of 10 replications. Different letters at the column head indicate that means differ significantly between treatments (LSD, P $\leq$ 0.05).

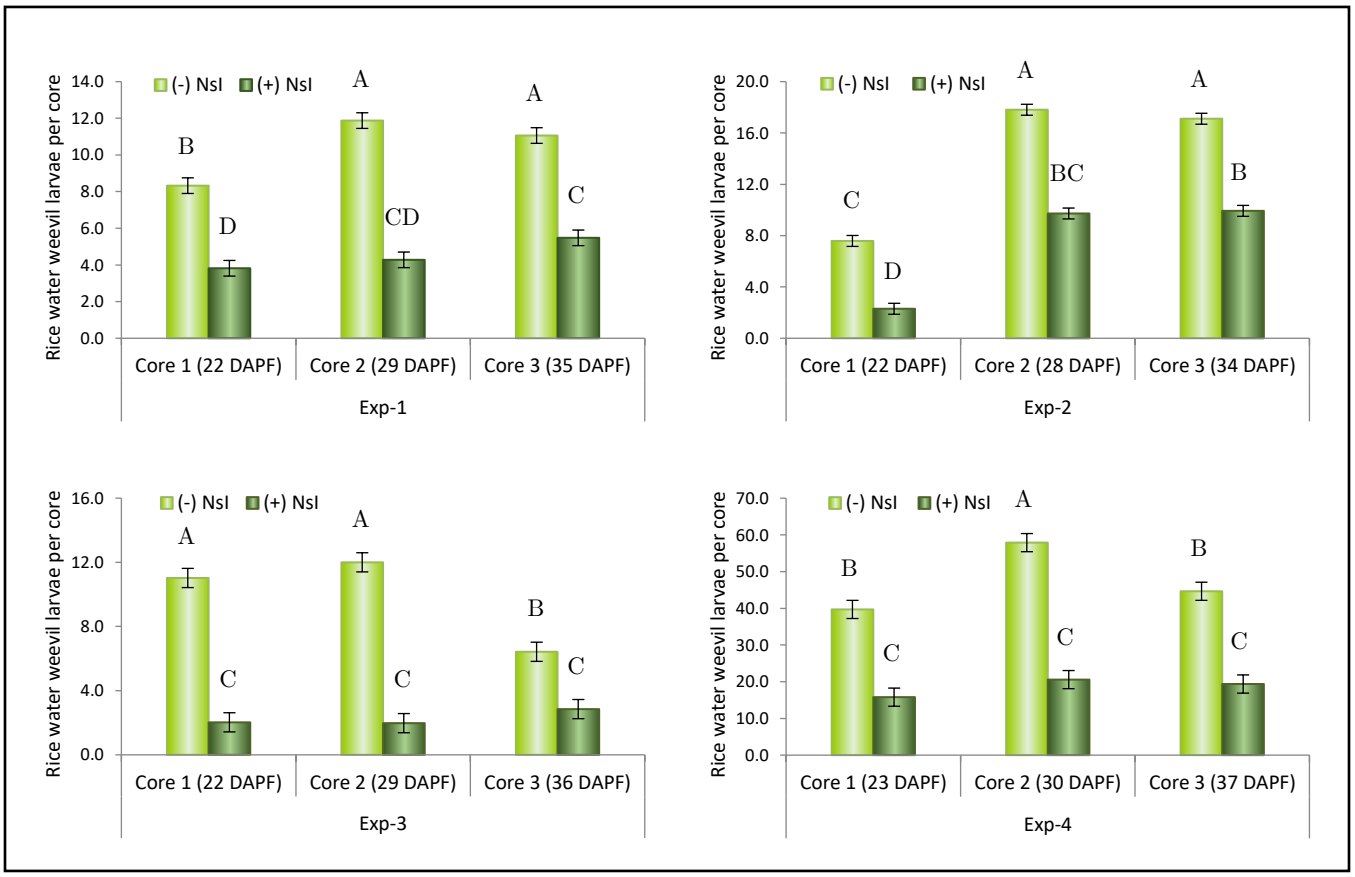


Figure C3. Influence of time (core sampling date) and treatment of seeds with insecticide (-NsI and +NsI) on densities of rice water weevil (larvae and pupae per core sample ± S.E.) in rice plots of four experiments conducted in the field over three years (2016-2018). (Top left) Exp-1, (Top right) Exp-2, (Bottom left) Exp-3, and (Bottom right) Exp-4. The numbers are the means of 10 replications. Different letters at the column head indicate that means differ significantly between treatments (LSD, $P \leq 0.05$).

Table C2. Results of ANOVA for effects of inoculation with AM fungi (+AMF and -AMF) and treatment of seeds with insecticide (+NsI and –NsI) as well as their interaction on numbers of whiteheads produced by stem borers in experiments conducted in the field over three years (2016-2018).

Factor	Exp-1			Exp-2			Exp-3			Exp-4		
	d.f.	*F*	*P*	*d.f.*	*F*	*P*	*d.f.*	*F*	*P*	*d.f.*	*F*	*P*
AMF	1, 76	5.54	**0.02**	1, 76	2.93	0.09	1, 196	1.55	0.21	1, 196	4.23	**0.04**
Insecticide	1, 76	0.24	0.63	1, 76	1.53	0.22	1, 196	2.41	0.12	1, 196	36.5	**<.0001**
AMF*Insecticide	1, 76	0.24	0.63	1, 76	0.42	0.52	1, 196	1.28	0.26	1, 196	1.70	0.19

Bold numbers indicate significant effects.

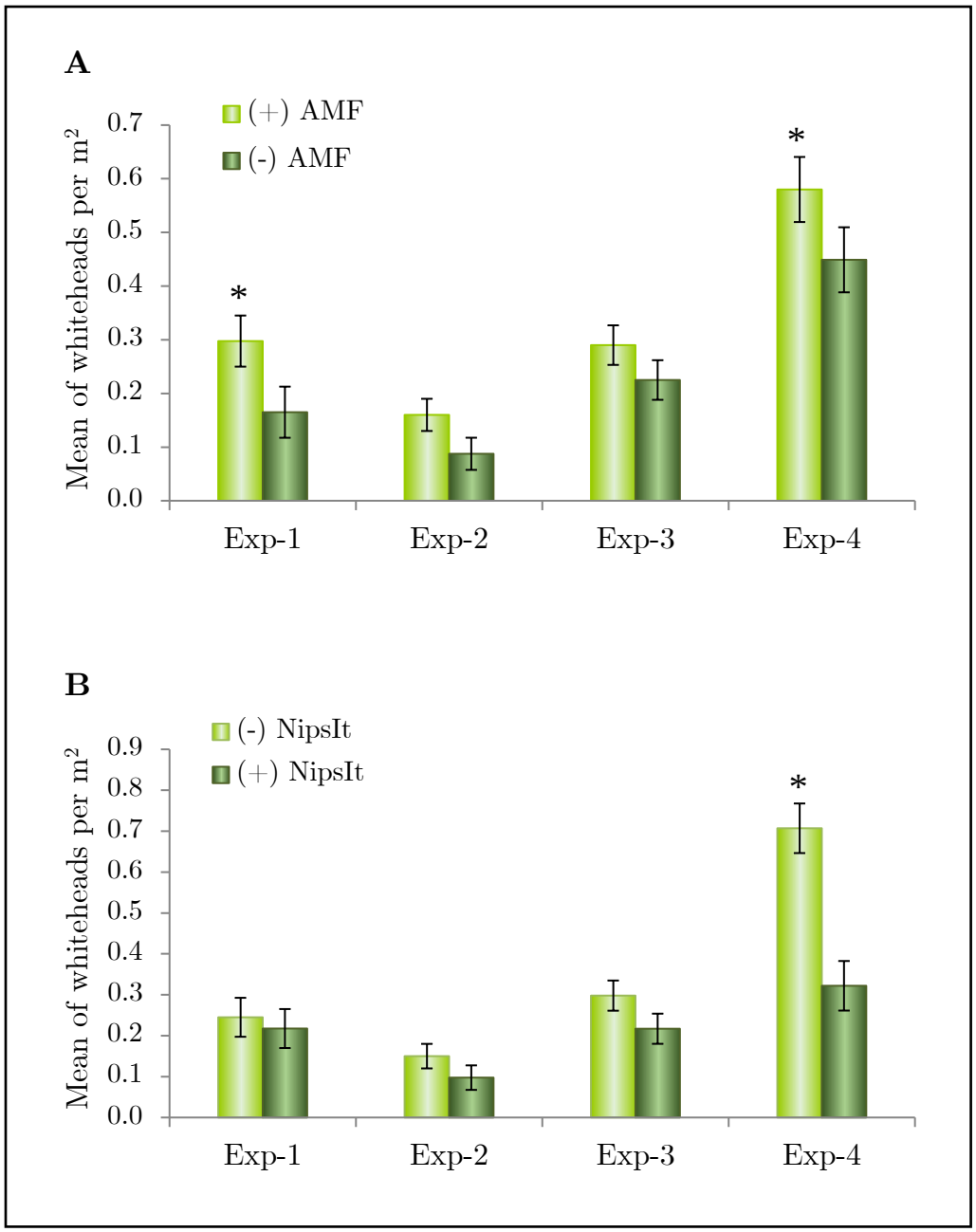


Figure C4. Main effects of inoculation with AM fungi and treatment of seeds with insecticide on numbers of whiteheads produced by stem borers in experiments conducted in the field over three years (2016-2018). (A) main effect of AM fungi treatment (+AMF and –AMF); (B) main effect of insecticide (+NsI and –NsI) treatment. The numbers are the means of 10 replications. Asterisk at the column head indicate that means differ significantly (LSD, $P \leq 0.05$).

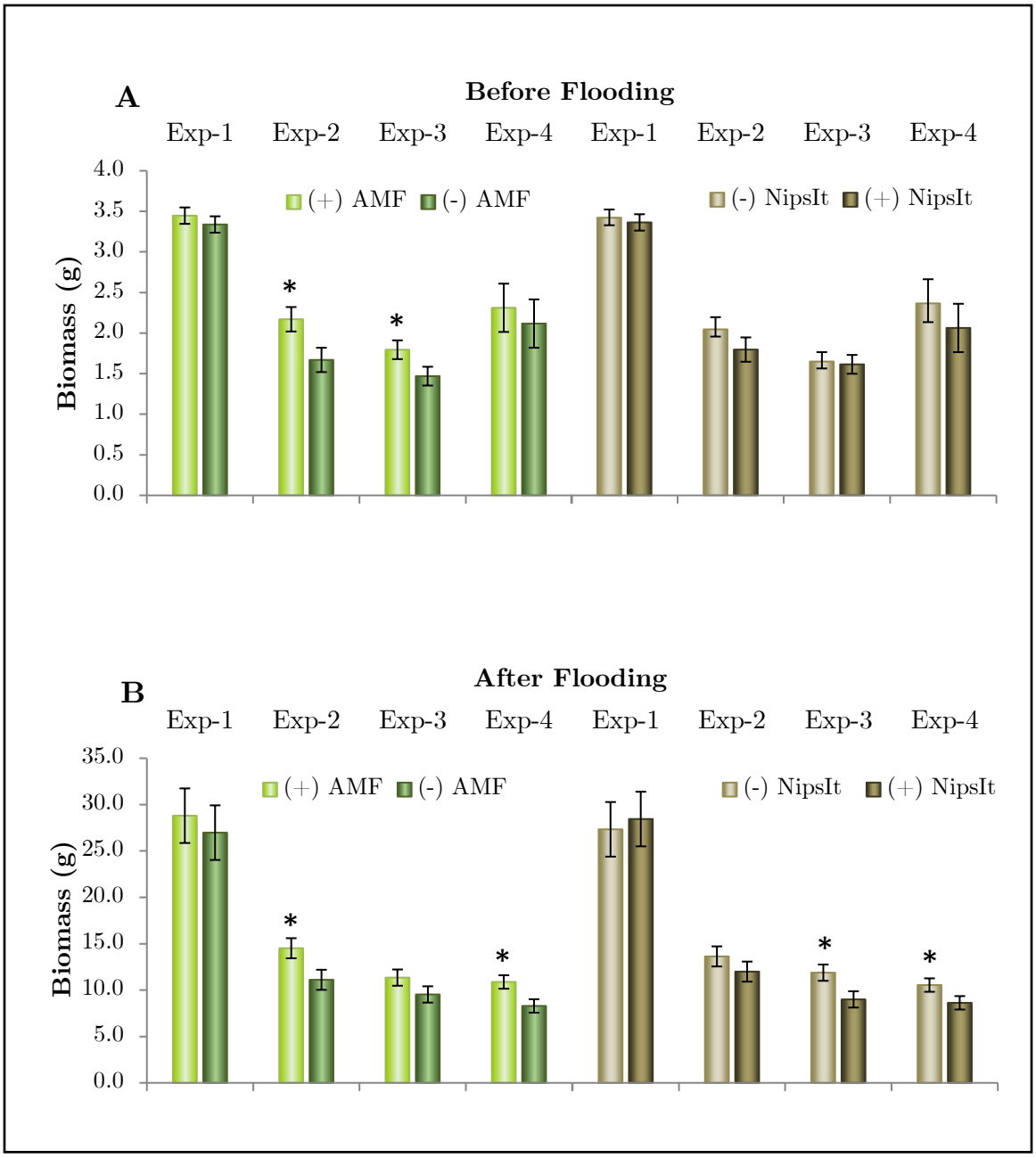


Figure C5. Main effects of inoculation with AM fungi (+AMF and –AMF) and treatment of seeds with insecticide (+NsI and –NsI) on total dry weights of rice plants sampled from plots twice: (A) before and (B) after flooding, of four experiments conducted in the field over three years (2016-2018). AM fungi-treated plants (light green) and non-AM fungi (dark green). The numbers are the means of 10 replications. Asterisk at the column head indicate that means differ significantly (LSD, $P \leq 0.05$).

Table C3. Effects of the interaction of inoculation with AM fungi and insecticide seed treatment on total (TDW), shoot (SDW), and root (RDW) dry weights of rice plants sampled before permanent flooding of four experiments conducted in the field over three years (2016-2018). The numbers are the means of 10 replications. Asterisk at the column head indicate that means differ significantly (LSD, $P \leq 0.05$).

Treatment combination	TDW (g, mean ± S.E.)			
	Exp-1	Exp-2	Exp-3	Exp-4
-AMF/-NsI	3.15 ± 0.14 b	1.68 ± 0.15 b	1.33 ± 0.14 b	0.48 ± 0.08 ab
-AMF/+NsI	3.52 ± 0.18 ab	1.66 ± 0.15 b	1.61 ± 0.18 ab	2.22 ± 0.46 ab
+AMF/-NsI	3.69 ± 0.07 a	2.41 ± 0.26 a	1.97 ± 0.16 a	2.72 ± 0.31 a
+AMF/+NsI	3.21 ± 0.14 b	1.93 ± 0.20 ab	1.62 ± 0.15 ab	1.90 ± 0.34 b

Treatment combination	SDW (g, mean ± S.E.)			
	Exp-1	Exp-2	Exp-3	Exp-4
-AMF/-NsI	2.49 ± 0.13 a	1.07 ± 0.11 b	0.96 ± 0.12 b	1.54 ± 0.20 ab
-AMF/+NsI	2.71 ± 0.15 a	1.07 ± 0.09 b	1.11 ± 0.12 ab	1.72 ± 0.36 ab
+AMF/-NsI	2.79 ± 0.07 a	1.55 ± 0.17 a	1.35 ± 0.13 a	2.09 ± 0.24 a
+AMF/+NsI	2.52 ± 0.13 a	1.17 ± 0.10 b	1.12 ± 0.10 ab	1.45 ± 0.27 b

Treatment combination	RDW (g, mean ± S.E.)			
	Exp-1	Exp-2	Exp-3	Exp-4
-AMF/-NsI	0.66 ± 0.02 c	0.61 ± 0.05 b	0.37 ± 0.03 b	0.48 ± 0.08 a
-AMF/+NsI	0.81 ± 0.04 b	0.59 ± 0.07 b	0.50 ± 0.05 a	0.50 ± 0.10 a
+AMF/-NsI	0.90 ± 0.02 a	0.87 ± 0.10 a	0.62 ± 0.06 a	0.63 ± 0.07 a
+AMF/+NsI	0.68 ± 0.02 c	0.76 ± 0.11 ab	0.51 ± 0.05 a	0.46 ± 0.08 a

Table C4. Effects of the interaction of inoculation with AM fungi and insecticide seed treatment on total (TDW), shoot (SDW), and root (RDW) dry weights of rice plants sampled after permanent flooding of four experiments conducted in the field over three years (2016-2018). The numbers are the means of 10 replications. Asterisk at the column head indicate that means differ significantly (LSD, $P \leq 0.05$).

Treatment combination	TDW (g, mean ± S.E.)			
	Exp-1	Exp-2	Exp-3	Exp-4
-AMF/-NsI	25.37 ± 3.53 a	12.69 ± 1.24 ab	9.20 ± 1.17 b	8.47 ± 0.66 b
-AMF/+NsI	28.59 ± 4.04 a	9.53 ± 0.62 b	9.87 ± 1.12 b	8.12 ± 0.80 b
+AMF/-NsI	29.30 ± 5.44 a	14. 57 ± 1.02 a	14.57 ± 1.36 a	12.62 ± 0.99 a
+AMF/+NsI	28.31 ± 3.30 a	14.46 ± 2.19 a	8.13 ± 0.79 b	9.14 ± 0.93 b

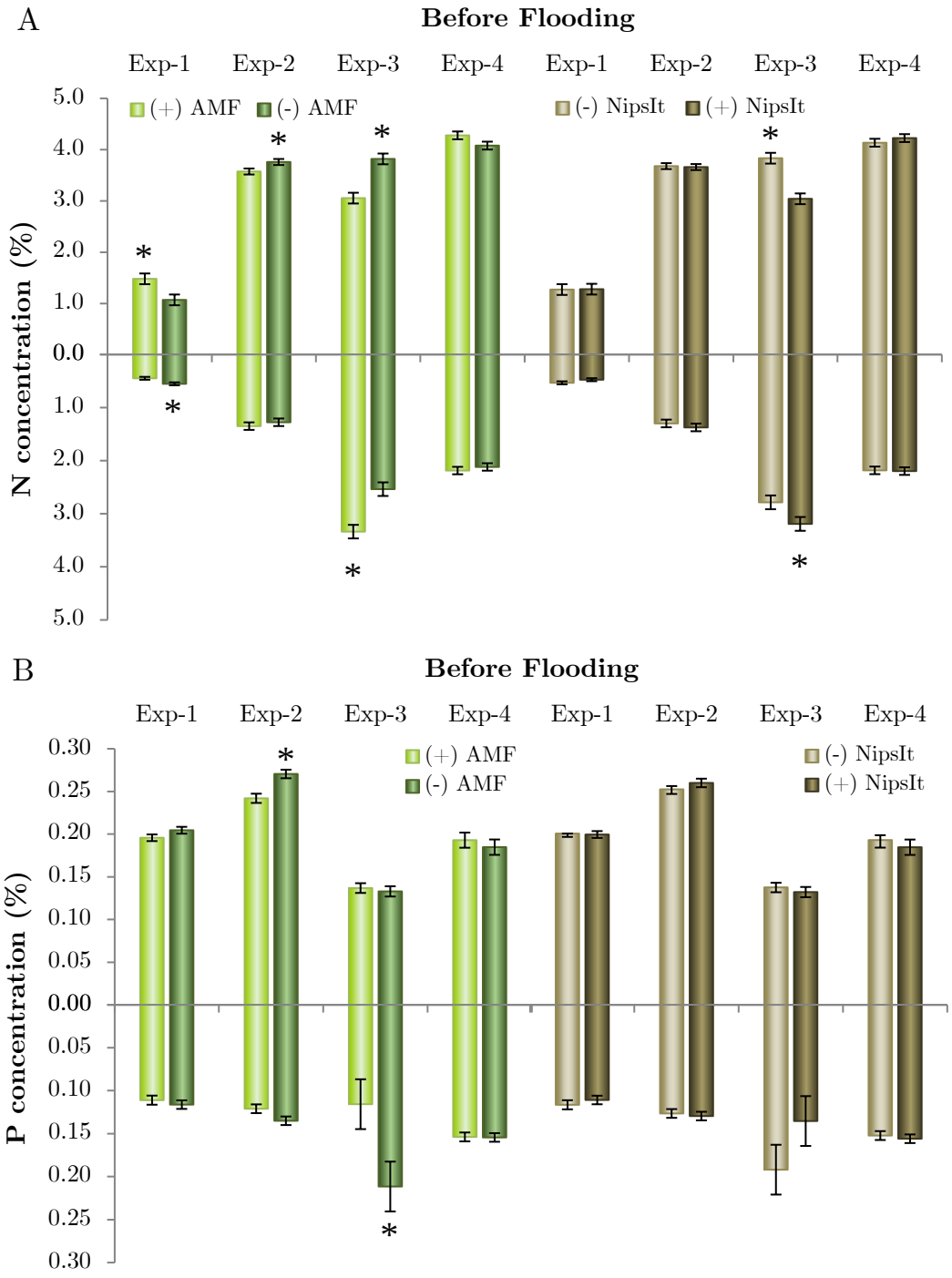
Treatment combination	SDW (g, mean ± S.E.)			
	Exp-1	Exp-2	Exp-3	Exp-4
-AMF/-NsI	21.08 ± 2.58 a	9.92 ± 0.97 ab	6.51 ± 0.72 b	6.76 ± 0.55 b
-AMF/+NsI	23.56 ± 3.15 a	7.72 ± 0.55 b	6.88 ± 0.65 b	6.29 ± 0.56 b
+AMF/-NsI	25.47 ± 4.60 a	11.75 ± 0.75 a	9.41 ± 0.79 a	9.89 ± 0.86 a
+AMF/+NsI	24.11 ± 2.81 a	11.68 ± 1.73 a	6.33 ± 0.68 b	7.61 ± 0.73 b

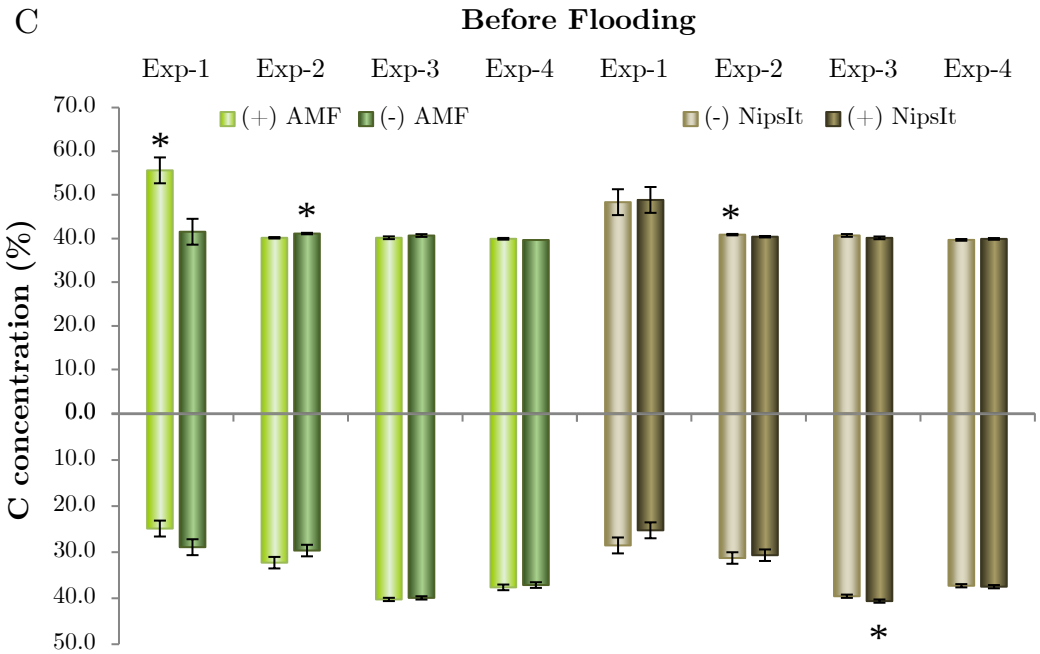
Treatment combination	RDW (g, mean ± S.E.)			
	Exp-1	Exp-2	Exp-3	Exp-4
-AMF/-NsI	4.13 ± 0.99 a	2.78 ± 0.45 ab	2.69 ± 0.54 b	1.72 ± 0.19 b
-AMF/+NsI	4.88 ± 1.06 a	1.81 ± 0.14 b	3.00 ± 0.81 b	1.83 ± 0.32 b
+AMF/-NsI	3.62 ± 0.82 a	2.83 ± 0.31 a	5.16 ± 0.63 a	2.72 ± 0.37 a
+AMF/+NsI	3.99 ± 0.60 a	2.79 ± 0.47 ab	1.79 ± 0.18 b	1.53 ± 0.25 b

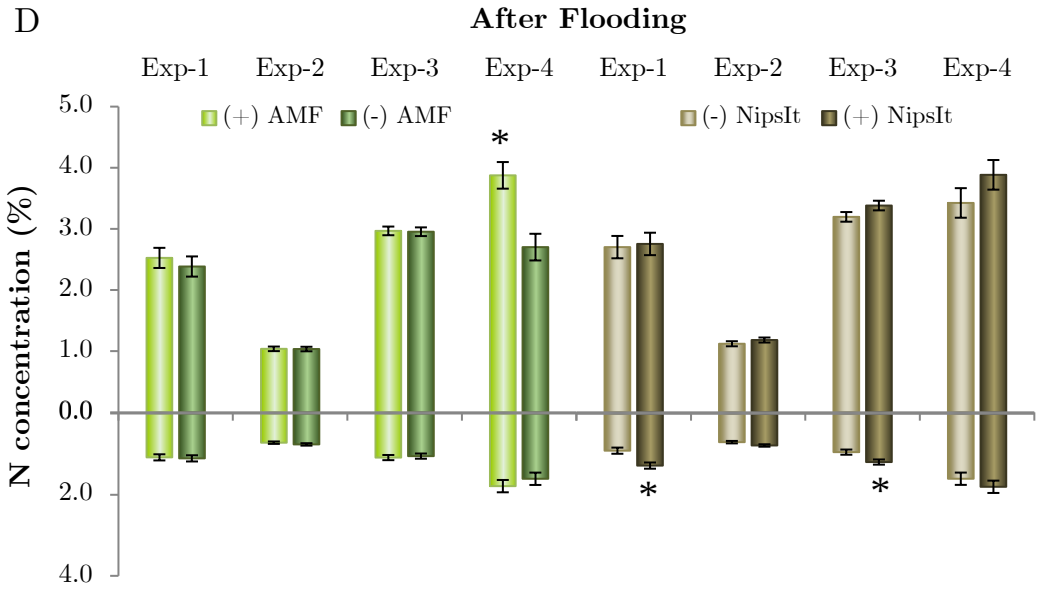
Table C5. ANOVA results for effects of AM fungi, insecticide seed treatment, and their interactions on the concentration of N, P, and C in shoot (SNC, SPC, and SCC) or root (RNC, RPC, and RCC) biomass of rice plants sampled before permanent flooding of four experiments conducted in the field over three years (2016-2018). Bold numbers indicate significant effects.

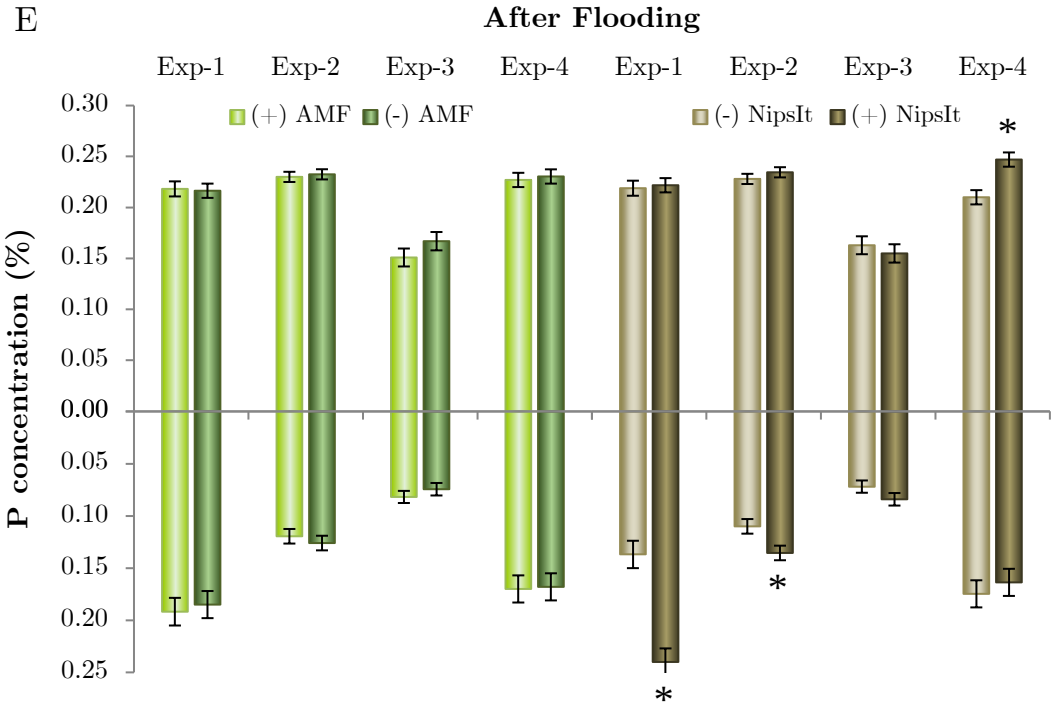
Trial	Fixed effect	SNC		SPC		SCC		RNC		RPC		RCC	
		$F_{1, 36}$	P	$F_{1, 36}$	P	$F_{1, 36}$	P	$F_{1, 36}$	P	$F_{1, 36}$	P	$F_{1, 36}$	P
Exp-1	AMF												
	B.F.	8.88	**0.006**	3.60	0.07	11.7	**0.002**	7.18	**0.011**	0.49	0.49	2.99	0.10
	A.F.	0.42	0.53	0.03	0.86	0.02	0.89	0.05	0.82	0.13	0.72	0.08	0.78
	Insecticide												
	B.F.	0.00	0.96	0.06	0.81	0.02	0.90	3.04	0.09	0.55	0.46	1.97	0.17
	A.F.	0.05	0.83	0.76	0.40	0.08	0.79	11.8	**0.003**	30.3	**<.0001**	11.3	**0.004**
	AMF*Insecticide												
	B.F.	0.37	0.55	0.27	0.61	0.69	0.41	0.03	0.87	0.86	0.36	0.21	0.65
	A.F.	0.01	0.91	0.76	0.40	2.64	0.13	1.67	0.21	6.77	**0.019**	3.42	0.08
Exp-2	AMF												
	B.F.	5.02	**0.03**	13.9	**0.01**	17.5	**0.01**	0.54	0.47	3.69	0.06	2.27	0.14
	A.F.	0.00	0.95	0.11	0.74	0.11	0.74	1.01	0.32	0.45	0.51	0.52	0.48
	Insecticide												
	B.F.	0.02	0.88	0.96	0.33	4.10	**0.05**	0.54	0.47	0.17	0.68	0.12	0.73
	A.F.	1.50	0.23	0.76	0.39	4.58	**0.04**	3.59	0.07	6.89	**0.01**	2.63	0.11
	AMF*Insecticide												
	B.F.	0.48	0.49	4.11	**0.05**	0.49	0.49	0.08	0.78	5.45	**0.03**	0.06	0.81
	A.F.	0.02	0.89	0.04	0.84	0.00	0.99	0.63	0.43	0.45	0.51	0.28	0.60
Exp-3	AMF												
	B.F.	34.9	**<.0001**	0.40	0.53	1.33	0.26	19.7	**<.0001**	8.11	**0.01**	0.45	0.51
	A.F.	0.02	0.89	2.90	0.10	4.56	**0.04**	0.16	0.69	0.79	0.38	0.07	0.80
	Insecticide												
	B.F.	37.4	**<.0001**	0.81	0.38	1.60	0.21	4.88	**0.03**	2.84	0.10	4.43	**0.04**
	A.F.	2.73	0.11	0.74	0.40	1.17	0.29	7.47	**0.009**	2.06	0.16	4.18	**0.05**
	AMF*Insecticide												
	B.F.	21.0	**<.0001**	0.81	0.38	4.45	**0.04**	23.4	**<.0001**	1.48	0.24	9.30	**0.01**
	A.F.	8.36	**0.007**	1.55	0.22	0.91	0.35	14.7	**0.0005**	15.9	**0.01**	7.37	**0.01**
Exp-4	AMF												
	B.F.	3.29	0.08	0.43	0.51	1.00	0.32	0.53	0.47	0.01	0.92	0.34	0.56
	A.F.	21.3	**<.0001**	0.16	0.69	5.40	**0.03**	0.72	0.40	0.02	0.89	1.06	0.31
	Insecticide												
	B.F.	0.70	0.41	0.45	0.50	0.67	0.42	0.02	0.89	0.34	0.57	0.06	0.81
	A.F.	2.64	0.12	21.5	**<.0001**	5.83	**0.02**	0.86	0.36	0.58	0.45	1.67	0.20
	AMF*Insecticide												
	B.F.	0.02	0.88	0.28	0.60	0.55	0.46	2.15	0.15	1.18	0.29	0.02	0.89
	A.F.	5.48	**0.027**	0.62	0.44	5.75	**0.02**	0.22	0.64	0.46	0.50	0.11	0.75

Figure C6. Main effects of inoculation with AM fungi (+AMF and –AMF) and insecticide (+NsI and –NsI) seed treatments on shoot (above x-axis) and root (below x-axis) tissue N concentrations (A and D), P concentrations (B and E), and C concentrations (C and F) of rice plants sampled before and after flooding. The numbers are the means of 10 replications. Asterisk at the column head indicate that means differ significantly (LSD, $P \leq 0.05$).




C
Before Flooding
Exp-1
Exp-2
Exp-3
Exp-4
Exp-1
Exp-2
Exp-3
Exp-4
(+) AMF
(-) AMF
(-) NipsIt
(+) NipsIt
C concentration (%)
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
10.0
20.0
30.0
40.0
50.0


D
After Flooding
Exp-1
Exp-2
Exp-3
Exp-4
Exp-1
Exp-2
Exp-3
Exp-4
(+) AMF
(-) AMF
(-) NipsIt
(+) NipsIt
N concentration (%)
5.0
4.0
3.0
2.0
1.0
0.0
2.0
4.0


E
After Flooding
Exp-1
Exp-2
Exp-3
Exp-4
Exp-1
Exp-2
Exp-3
Exp-4
(+) AMF
(-) AMF
(-) NipsIt
(+) NipsIt
P concentration (%)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.05
0.10
0.15
0.20
0.25
*
*
*

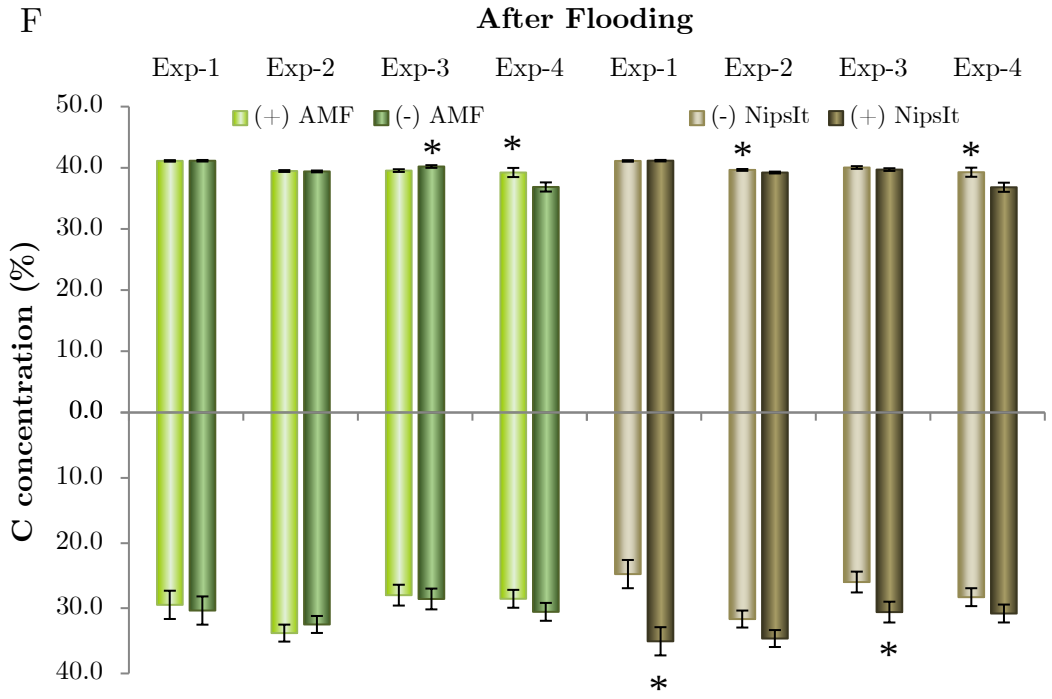

F
After Flooding
Exp-1
Exp-2
Exp-3
Exp-4
Exp-1
Exp-2
Exp-3
Exp-4
(+) AMF
(-) AMF
(-) NipsIt
(+) NipsIt
C concentration (%)
50.0
40.0
30.0
20.0
10.0
0.0
10.0
20.0
30.0
40.0
*
*
*
*
*
*

Table C6. Effects of the interaction of inoculation with AM fungi and insecticide seed treatment on the concentration of N, P, and C in shoot (SNC, SPC, and SCC) or root (RNC, RPC, and RCC) biomass of rice plants sampled before permanent flooding of four experiments conducted in the field over three years (2016-2018). The numbers are the means of 10 replications.

Treatment combination	SNC (% mean ± S.E.)				RNC (% mean ± S.E.)			
	Exp-1	Exp-2	Exp-3	Exp-4	Exp-1	Exp-2	Exp-3	Exp-4
-AMF/-NsI	1.02 ± 0.03	3.80 ± 0.13	3.92 ± 0.07	4.05 ± 0.09	0.59 ± 0.04	1.26 ± 0.08	2.77 ± 0.26	2.04 ± 0.11
-AMF/+NsI	1.11 ± 0.09	3.73 ± 0.04	3.72 ± 0.15	4.12 ± 0.09	0.52 ± 0.04	1.30 ± 0.06	2.30 ± 0.09	2.19 ± 0.08
+AMF/-NsI	1.52 ± 0.17	3.55 ± 0.05	3.75 ± 0.19	4.23 ± 0.13	0.48 ± 0.04	1.30 ± 0.08	2.70 ± 0.22	2.25 ± 0.10
+AMF/+NsI	1.44 ± 0.20	3.60 ± 0.08	2.36 ± 0.12	4.33 ± 0.12	0.42 ± 0.04	1.40 ± 0.16	3.97 ± 0.08	2.13 ± 0.09

Treatment combination	SPC (% mean ± S.E.)				RPC (% mean ± S.E.)			
	Exp-1	Exp-2	Exp-3	Exp-4	Exp-1	Exp-2	Exp-3	Exp-4
-AMF/-NsI	0.20 ± 0.01	0.26 ± 0.01	0.13 ± 0.01	0.19 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	0.26 ± 0.07	0.15 ± 0.01
-AMF/+NsI	0.21 ± 0.01	0.28 ± 0.01	0.13 ± 0.01	0.18 ± 0.02	0.12 ± 0.01	0.15 ± 0.01	0.16 ± 0.03	0.16 ± 0.01
+AMF/-NsI	0.20 ± 0.01	0.25 ± 0.01	0.14 ± 0.01	0.19 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	0.12 ± 0.01	0.16 ± 0.01
+AMF/+NsI	0.19 ± 0.00	0.24 ± 0.01	0.13 ± 0.01	0.19 ± 0.01	0.10 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.15 ± 0.01

Treatment combination	SCC (% mean ± S.E.)				RCC (% mean ± S.E.)			
	Exp-1	Exp-2	Exp-3	Exp-4	Exp-1	Exp-2	Exp-3	Exp-4
-AMF/-NsI	39.6 ± 0.38	41.3 ± 0.15	40.5 ± 0.27	39.5 ± 0.25	30.0 ± 2.56	29.7 ± 1.62	40.2 ± 0.26	37.1 ± 1.26
-AMF/+NsI	43.5 ± 2.74	40.9 ± 0.15	40.9 ± 0.21	39.9 ± 0.11	27.8 ± 2.65	29.6 ± 1.60	39.7 ± 0.19	37.2 ± 0.65
+AMF/-NsI	57.1 ± 5.19	40.5 ± 0.20	40.9 ± 0.27	39.9 ± 0.27	27.0 ± 1.64	32.8 ± 1.61	40.0 ± 0.94	37.5 ± 0.74
+AMF/+NsI	54.2 ± 5.87	39.9 ± 0.36	39.4 ± 0.77	40.0 ± 0.40	22.7 ± 2.59	31.8 ± 2.14	41.6 ± 0.17	37.8 ± 0.67

Table C7. Effects of the interaction of inoculation with AM fungi and insecticide seed treatment on the concentration of N, P, and C in shoot (SNC, SPC, and SCC) or root (RNC, RPC, and RCC) biomass of rice plants sampled after permanent flooding of four experiments conducted in the field over three years (2016-2018). The numbers are the means of 10 replications.

Treatment combination	SNC (% mean ± S.E.)				RNC (% mean ± S.E.)			
	Exp-1	Exp-2	Exp-3	Exp-4	Exp-1	Exp-2	Exp-3	Exp-4
-AMF/-NsI	2.37 ± 0.12	1.01 ± 0.06	3.02 ± 0.09	2.79 ± 0.18	0.99 ± 0.07	0.71 ± 0.04	1.10 ± 0.07	1.56 ± 0.19
-AMF/+NsI	2.40 ± 0.25	1.06 ± 0.05	2.89 ± 0.12	2.61 ± 0.33	1.22 ± 0.13	0.82 ± 0.06	1.00 ± 0.09	1.66 ± 0.23
+AMF/-NsI	2.49 ± 0.19	1.01 ± 0.05	2.74 ± 0.11	3.37 ± 0.36	0.83 ± 0.12	0.70 ± 0.04	0.79 ± 0.08	1.64 ± 0.15
+AMF/+NsI	2.56 ± 0.30	1.07 ± 0.03	3.20 ± 0.08	4.38 ± 0.23	1.33 ± 0.09	0.74 ± 0.02	1.38 ± 0.12	1.94 ± 0.27

Treatment combination	SPC (% mean ± S.E.)				RPC (% mean ± S.E.)			
	Exp-1	Exp-2	Exp-3	Exp-4	Exp-1	Exp-2	Exp-3	Exp-4
-AMF/-NsI	0.22 ± 0.01	0.23 ± 0.01	0.18 ± 0.02	0.22 ± 0.01	0.16 ± 0.02	0.11 ± 0.01	0.09 ± 0.01	0.18 ± 0.01
-AMF/+NsI	0.22 ± 0.01	0.24 ± 0.01	0.16 ± 0.01	0.25 ± 0.01	0.21 ± 0.01	0.14 ± 0.01	0.06 ± 0.01	0.16 ± 0.02
+AMF/-NsI	0.21 ± 0.01	0.23 ± 0.01	0.15 ± 0.01	0.21 ± 0.01	0.12 ± 0.01	0.11 ± 0.01	0.06 ± 0.01	0.17 ± 0.01
+AMF/+NsI	0.23 ± 0.01	0.23 ± 0.01	0.15 ± 0.01	0.25 ± 0.01	0.27 ± 0.02	0.13 ± 0.01	0.10 ± 0.01	0.17 ± 0.02

Treatment combination	SCC (% mean ± S.E.)				RCC (% mean ± S.E.)			
	Exp-1	Exp-2	Exp-3	Exp-4	Exp-1	Exp-2	Exp-3	Exp-4
-AMF/-NsI	41.3 ± 0.24	39.6 ± 0.18	40.2 ± 0.12	39.3 ± 0.28	28.0 ± 3.38	30.5 ± 1.93	29.3 ± 2.68	29.6 ± 2.05
-AMF/+NsI	41.0 ± 0.06	39.2 ± 0.12	40.2 ± 0.11	34.4 ± 2.02	32.7 ± 1.50	34.5 ± 1.56	27.8 ± 2.10	31.5 ± 1.65
+AMF/-NsI	41.0 ± 0.18	39.7 ± 0.15	39.9 ± 0.53	39.2 ± 0.22	21.5 ± 4.84	32.8 ± 2.33	22.6 ± 2.43	27.0 ± 1.33
+AMF/+NsI	41.3 ± 0.17	39.3 ± 0.28	39.2 ± 0.30	39.2 ± 0.30	37.5 ± 0.67	34.8 ± 1.40	33.4 ± 1.70	30.1 ± 2.53

Table C8. Yield loss (kg/ha) attributed to rice water weevil root damage of four experiments conducted in the field over three years (2016-2018). The numbers are the means of 10 replications.

Treatment	Yield loss (kg/ha ± S.E.)			
	Exp-1	Exp-2	Exp-3	Exp-4
(+)AMF[a]	194.34 ± 198.97 a	473.00 ± 334.54 a	342.03 ± 174.46 a	1782.73 ± 775.43 a
(-)AMF[b]	60.57 ± 334.07 a	218.20 ± 333.22 a	255.17 ± 167.24 a	893.57 ± 513.32 a
F[c]	0.14	0.29	0.13	0.91
$P > F$	0.720	0.597	0.722	0.352

[a] Difference between yields from AM fungi-inoculated and insecticide-treated plots versus plots only treated with AM fungi.
[b] Difference between yields from insecticide-treated plots versus untreated plots.
[c] Exp-1: df = 1, 9; Exp-2: 1, 16; Exp-3: 1, 9; and Exp-4: 1, 18.

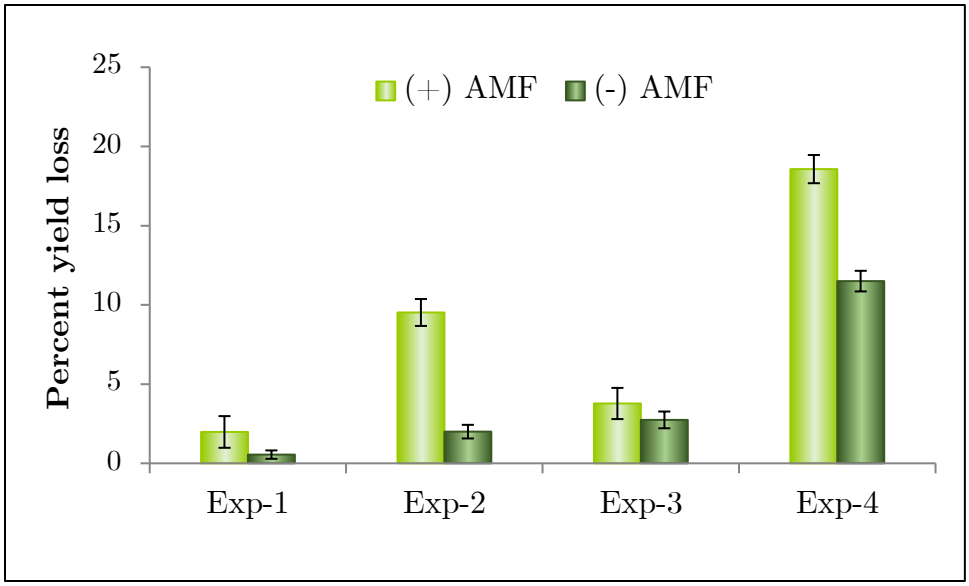


Figure C7. Yield loss percentage (%) attributed to rice water weevil root damage of four experiments conducted in the field over three years (2016-2018). (+)AMF: difference between yields from AM fungi-inoculated and insecticide-treated plots versus plots only treated with AM fungi. (-)AMF: difference between yields from insecticide-treated plots versus untreated plots.

Appendix D: Letter of Permission for Chapter 3

Title: Natural Colonization of Rice by Arbuscular Mycorrhizal Fungi in Different Production Areas

Author: Lina Bernaola,Grace Cange,Michael O. Way,Jeffrey Gore,Jarrod Hardke,Michael Stout

Publication: Rice Science

Publisher: Elsevier

Date: May 2018

Appendix E: Letter of Permission for Chapter 4

From: Frontiers in Plant Science <plantscience@frontiersin.org>
Subject: RE: Permission request/letter for using my previously published work
Date: Thu 8/23, 4:26 AM
To: Bernaola, Lina

AMF Research

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Vita

Lina Bernaola-Alvarado was born and raised in the city of Magdalena del Mar, in Lima, Peru, as the first daughter of electrical engineer David Bernaola and accountant Flor Alvarado. At age 17, she was accepted to San Marcos National University in Peru where she studied Biology with a minor in Genetics and Molecular Biology. During her senior year, her passion for the world of plants began to bloom when she was selected as an undergraduate scholar to conduct a bachelor thesis at the International Potato Center (CIP) under the direction of Dr. Marc Ghislain. After completing and successfully defending her thesis, Lina was hired at the National Institute of Agricultural Research (INIA) as a research assistant to work on genetically modified organisms in maize for the department of molecular biology and plant genetic resource conservation. It is after her experience at CIP and INIA where she further developed an interest in reducing crop losses due to different plant pests.

Her motivation and determination led her to attend graduate school at Louisiana State University. In 2010, she joined Dr. Baisakh's laboratory to pursue an M.Sc. in Agronomy and Crop Sciences. She had the opportunity to learn new molecular techniques for her research, which also made her realize she wanted to develop more skills working with applied research in the field to deepen her knowledge on plant-pathogen-insect interactions.

In 2012, Lina became a Ph.D. student of Dr. Michael Stout in the rice laboratory of the Department of Entomology to study the tripartite interaction between mycorrhizal fungi, rice, and insects. Upon completion of her Ph.D.'s degree, Lina will continue her passion for research and career in plant-insect interactions.

Lina and her husband James got married in April 2017 while attending graduate school.