EFFECT OF INOCULATION OF *PSEUDOMONAS CHLORORAPHIS* 30-84 AND / OR *RHIZOPHAGUS IRREGULARIS* DAOM 197198 ON MAIZE ANTHRACNOSE LEAF BLIGHT AND TOMATO ZEBRA CHIP DISEASE

A Thesis

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ABSTRACT

Anthracnose leaf blight (ALB) and Zebra Chip disease (ZC) represent serious threats to maize and Solanaceous crop production respectively. ALB control relies on resistant lines, but high genetic plasticity in the fungal causative agent, *Colletotrichum graminicola,* resulting in the evolution of new pathotypes makes it challenging to breed for durable genetic resistance. ZC control is based on antibiotic treatment against the causative agent '*Candidatus* Liberibacter solanacearum' (CLso) and insecticide applications to control the psyllid vector, *Bactericera cockerelli*. However, this will lead to resistance emergence in the psyllids and CLso. A potential alternative is to boost host resistance by employing Plant Growth-Promoting Rhizobacteria (PGPR) and Arbuscular Mycorrhizal Fungi (AMF).

I found in vitro that the PGPR *Pseudomonas chlororaphis* 30-84 inhibited the growth of *C. graminicola* M1.001 via phenazine production. In vivo, the AMF *Rhizophagus irregularis* and *P. chlororaphis* 30-84 triggered effective systemic resistance to *C. graminicola*-induced foliar lesions on the susceptible B73 maize line. The level of disease control was similar to that of the resistant line W438. Both biocontrol agents (BCAs) reduced foliar fungal biomass. Coinoculation mirrored *R. irregularis* effect and revealed different and competitive mechanisms triggered by each BCA. Mutation of the ZMLOX12 gene enhanced disease severity on bacterized plants, whereas mycorrhized plants were still resistant. When introduced sequentially, I found no effect on the rate of root colonization by either organism on the other.

To investigate the role of mycorrhizae on Zebra Chip disease development and psyllid survival, I used a 'no-choice' assay, in which three couples of male and female *B*.

cockerelli harboring either CLso haplotype A or B (LsoA and LsoB respectively) were placed on a single leaf. Despite reports suggesting mycorrhization facilitates feeding by phloem-feeding insects, *R. irregularis* substantially delayed and reduced symptoms of ZC diseases on 'Moneymaker' tomato as compared to non-mycorrhized plants treated with psyllids. Mycorrhization also impaired the survival of psyllid larvae from parents harboring LsoA but increased slightly the survival of those from parents harboring LsoB. PCR with specific CLso primers revealed that mycorrhization did not prevent CLso transmission by the insect or translocation to newly formed leaves.

DEDICATION

To ***Jaine*** and ***Jarod***, my darling children, to my dear brothers ***Pacome*** and ***Jean-Jonathan***, my lovely sisters ***Mireille*** and ***Marie-José,*** for your indefectible support, I leave this poem from Bessie Anderson Stanley (born Caroline Elizabeth Anderson):

"Qu'est-ce que la réussite?

C'est rire beaucoup et souvent;

C'est gagner le respect des gens intelligents;

Tout autant que l'affection des enfants;

C'est mériter l'appréciation des gens honnêtes;

Et supporter la trahison de faux amis;

C'est apprécier la beauté des êtres;

C'est trouver en chacun le meilleur;

C'est apporter sa contribution, aussi modeste soit-elle:

Un enfant bien portant, un jardin en fleurs,

Une vie qu'on a rendue plus belle;

C'est savoir qu'on a facilité l'existence

De quelqu'un par notre simple presence;

Voilà ce qu'est la réussite.".

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NOMENCLATURE

ABA	Abscisic Acid
ALB	Anthracnose Leaf Blight
AM	Arbuscular Mycorrhizae
AMF	Arbuscular Mycorrhizal Fungus
ASR	Anthracnose Stalk Rot
BA	Biocontrol Agent
BXD	Benzoxazinones
CFU	Colony Forming Unit
CLso	*Candidatus* Liberibacter solanacearum
CSSP	Common Symbiosis Signaling Pathway
DAI	Days After Infestation
DAPG	2,4-diacetylphloroglucinol
DAS	Days After Sowing
DIMBOA	2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one
ET	Ethylene
ETI	Effector-Triggered Immunity
GA	Gibberellic acid
IAA	Indole-3-acetic acid
ISR	Induced Systemic Resistance
JA	Jasmonic Acid
LB	Lysogeny Broth Medium

LOX	Lipoxygenase
LPC	Lysophosphatidylcholine
LsoA	CLso haplotype A
LsoB	CLso haplotype B
MAMP	Microbial-Associated Molecular Pattern
MIR	Mycorrhiza-Induced Resistance
MTI	MAMP-Triggered Immunity
Myc-LCO	Mycorrhiza-specific Lipochitooligosaccharide
PGPF	Plant Growth-Promoting Fungi
PGPP	Plant Growth-Promoting Pseudomonas
PGPR	Plant Growth-Promoting Rhizobacteria
s/nsMyc-LCOs	sulfated and nonsulfated lipochitooligosaccharides
SA	Salicylic Acid
SAR	Systemic acquire Resistance
SNP	Single Nucleotide Polymorphism
VOCs	Volatile Organic Compounds
WAI	Weeks After Infestation
WAS	Weeks After Sowing
ZC	Zebra Chip disease

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

CHAPTER I

GENERAL INTRODUCTION

Plants host a very diversified microbiome (1). Plant Growth-Promoting Microbes (PGPM) comprise one of the main groups of microsymbionts of economic and scientific research importance. Among PGPM, Plant Growth-Promoting Rhizobacteria (PGPR) and Arbuscular Mycorrhizal Fungi (AMF) are well-recognized because they provide essential host functions along with ecosystem roles. For instance, AMF coevolved with plants and facilitated their land settlement in the Devonian period (2). PGPR are known to form biofilms on roots and to produce secondary metabolites that play substantial roles in plant health by affecting plant nutrition, photosynthesis, development, and health (3). Besides modulating root architecture, PGPM also enhance plant phenotypic plasticity in response to unstable environments (2).

The plant genotype actively selects among PGPM and determines the resulting nature of the interaction. For instance, among AMF, the outcome of plant association with *Rhizophagus irregularis* is clearly in favor of disease resistance compared to *Gigaspora* sp., which predominantly promotes plant growth. These findings suggest that plants manipulate PGPM and enhance interactions that help them cope with stressful environments (2). Thus, these results support the hypothesis by Smith and Gotham (1999) that manipulation of the genes involved in plant-microbe relations would advance understanding of these interactions (3).

PLANT GROWTH-PROMOTING *PSEUDOMONAS* (PGPP)

Although a few are well studied as plant disease causative agents, the *Pseudomonas* genus hosts rhizosphere-inhabiting species capable of colonizing a wide variety of wild and cultivated plant species, including maize and wheat, where they function as biofertilizers, photostimulators, and biocontrol agents. Species like *P. fluorescens*, *P. putida*, and *P. chlororaphis* can directly stimulate plant growth and development through alteration of plant traits including root branching for better soil exploration and shoot growth via production of auxins and cytokinins (2). They also act indirectly by priming systemic resistance in the host. The primed plant defense is regulated via the jasmonate signaling pathway. Moreover, PGPP produce many different secondary metabolites capable of antagonizing plant pathogens, including phenazines, 2,4-diacetylphloroglucinol (DAPG), pyrrolnitrin, pyocyanin, and hydrogen cyanide (4–16) as well as volatile organic compounds (VOCs) such as acetoin (17–19). For instance, *P. fluorescens* strains secrete DAPG, which inhibits the growth of a diversity of fungal plant pathogens including *Pythium ultimum, Phoma beta, Rhizopus stolonifera* and *Fusarium oxysporum* (20) and suppresses the fungal take-all disease on wheat caused by *Gaeumannomyces graminis* var. *tritici* (2). In addition to triggering Induced Systemic Resistance (ISR), PGPP also elicit Induced Systemic Tolerance (IST) towards water and moderate drought stresses (21). As Goh et al. (2013) explain, inducing systemic resistance involves alterations in the chemical plasticity of both the plant and bacterial partners since bacterial and plant VOCs impact photosynthesis as well as enable roots to explore efficiently nutrient-poor soils and acquire iron. In bacteria, plant growth promotion

or ISR often results from overproduction of virulence factors, antibiotics or iron-scavenging siderophores (2).

Phenazines are a class of antimicrobial compounds produced by *Pseudomonas* strains (8, 13). *P. chlororaphis* 30-84 produces three phenazine derivatives, phenazine-1-carboxylic acid (PCA), 2-hydroxy-phenazine-1-carboxylic acid (2OHPCA), and 2-hydroxy-phenazine (2OHPZ). These phenazines are important for suppression of take-all disease of wheat caused by *Gaeumannomyces graminis var. tritici* (13). Phenazines also are essential for rhizosphere survival and biofilm formation in *P. chlororaphis* 30-84 (5–7), regulating gene expression in *P. chlororaphis* and *P. aeruginosa* (12, 22), inducing plant defense pathways and functioning in electron shuttling and iron chelation (12, 23). Phenazine production is under the control of conserved regulatory systems, which include GacS/GacA two-component signal transduction (TCST), PhzR/PhzI quorum sensing (QS), and post-transcriptional control by small non-coding RNAs (8).

PGPP also use secreted effector molecules and phytohormones to suppress plant diseases after overcoming the Microbial Associated Molecular Patterns-Triggered Immunity (MTI) or Effector-Triggered Immunity (ETI) in the plant. For example, *P. fluorescens* Q8r1-96- root bacterization of wheat up-regulated Jasmonic Acid (JA) signaling and *P. fluorescens* GM30- Arabidopsis root colonization up-regulated abscisic acid (ABA) and ethylene (ET)-dependent pathways (24). In contrast, *P. fluorescens* SS101-Arabidopsis colonization up-regulated a salicylic acid (SA)-dependent pathway (24). Moreover, PGPP colonization of maize resulted in significant increases in important plant defense phytoalexins such as 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), Benzoxazinones (BXD), coumarins and flavonoids (24).

ARBUSCULAR MYCORRHIZAL FUNGI (AMF)

Arbuscular mycorrhizae (AM) is a 400 million -years-old symbiosis thought to be indispensable for early colonization of land by plants. AM involves beneficial and obligate symbiotic Glomeromycetes. These fungi supply more than 80 % of the terrestrial plant species with water and nutrients (mainly inorganic phosphorus, nitrogen). In return the extraradical mycelia store up to 20% of plant-fixed carbon in the form of lipid- and sugar -like molecules (25, 26). Arbuscules are the diagnostic fungal structures within plant cortical cells where the nutrient exchange occurs (27), even though the two symbionts stay delimited and separated by their plasma membranes (26).

AM formation is under the control of specific plant-fungal signaling. Plant roots exude strigolactones, which promote AMF metabolism and branching. AM fungi release the so-called 'Myc factors' that trigger symbiotic root reprogramming and activation of specific plant genes (27). The Myc factors are primarily sulfated and nonsulfated lipochitooligosaccharides (s/nsMyc-LCOs). They have been purified from *Rhizophagus irregularis* (formerly *Glomus intraradices*) exudates (28). Upon sensing of released strigolactones in the soil, AM fungal spores secrete the “Myc factors,” which causes plant transduction of a Ca^{2+}-signal mediated by numerous components of a common symbiosis signaling pathway (CSSP) also partially used during rhizobial nodulation. These plant responses lessen plant defense mechanisms against the AMF’s Microbe-Associated Molecular Patterns (MAMPs) and facilitate root colonization by AMF hyphae (26). Indeed, within minutes of strigolactones perception, AM fungal spores germinate hyphae that form a hyphopodium on the root surface to penetrate the epidermis after the plant creates a pre-

penetration passageway (29). The intraradical hyphae invade the cortex intercellularly and intracellularly where arbuscules, coiled hyphae, and vesicles are formed, whereas the extraradical hyphae produce spores (26). Late in the AM formation, lysophosphatidylcholine, which is a lipophilic signal, enables plant uptake of orthophosphate that is released from the AM fungus (26).

Other plant and AM fungal phytohormones such as auxins, ethylene (ET), jasmonates (JAs), salicylic acid (SA), strigolactones and gibberellins (GAs) play roles in the maintenance of the endosymbiosis (30, 31). Phytohormones regulate AM development differently. Early steps of AM development are positively regulated by strigolactones and auxin whereas ET negatively affects fungal root penetration as well as the first AM-specific gene expression (32, 33). Auxin signaling also controls strigolactone concentrations in pre-symbiosis stages but not arbuscule formation (32, 33). On the contrary, a GA-negative impact on arbuscule formation was demonstrated by severe reduced arbuscule numbers in *Medicago truncatula* and *Oryza sativa* in the absence of DELLA proteins which are negative regulators of GA signaling (30). Conjugated auxin accumulation improves AM fungi infection and growth (34). Salicylic acid (SA) accumulation is related to reduced root colonization at the debut of the interaction (34). Several studies reported a concentration-dependent effect of Jasmonic Acid (JA) on mycorrhizal plants. Indeed, low concentration favors AM infection, whereas high level impairs root colonization (34).

Later stages of AM development are positively regulated by JA via enhanced allocation of assimilates to the root system (31). *R. irregularis*-root colonization of rice is up-regulated by the JA-dependent pathway and down-regulated by pathogenesis-related (PR) gene expression both locally and systemically (35). In the very late phase of mycorrhization,

increased levels of cytokinins were detected in AM plants compared to non-mycorrhizal plants. This increased level is accompanied by the shutdown of two plant defense PR genes (34). Increased levels of GAs in leaves of AM plants are coupled with decreased levels of GA in the AM roots compared to the non-AM ones (34). An increased level of ET is associated with decreasing AM root colonization and abnormal, swollen and highly branched appressoria non-adapted for cortex cell penetration (36). Ethylene formation is especially increased in flax (*Linum usitatissimum*) roots colonized by *R. irregularis* (34). Contrasting data suggest that the interaction between the level of IAA and AM colonization is plant-genotype dependent as well as AM establishment-phase dependent.

AMF- PRIMED ISR AGAINST HERBIVORY INSECT AND DISEASE TRANSMITTED BY INSECT

Mycorrhized plants differ biochemically, physiologically and morphologically as hosts to insects as compared to nonmycorrhized plants (37, 38). In mycorrhized plant – insect interactions, the outcome depends on diverse factors including the response variable measured and the type of AMF and insect feeding specialization (39). For example, positive correlations have been observed between mycorrhization and oviposition in several types of chewing insects (38, 40). Additionally, because of their improved nutritional quality, it is noted that chewing insects feed preferentially on mycorrhizal plants. However, mycorrhizal plants are able to compensate for losses due to herbivory by enhanced growth (37). Moreover, it is thought that mycorrhization reduces chewing insect survival, especially for polyphagous insects compared to specialists, potentially due to the production of VOCs or

antifeeding compounds induced by mycorrhization (37). The mechanism of the effect could be the priming of the plant defenses where JA signaling plays a prominent role (39). In contrast, mycorrhization does not affect damage caused by phloem feeders (e.g. sucking insect feeding) (37, 41, 42). Despite the findings mentioned above, there is insufficient data on host plant -vectored pathogen - insect interactions in the literature to draw a formal conclusion on the effect of AMF towards sucking insects. Moreover, informations regarding the effects of mycorrhization on disease agents transmitted by different types of insect feeders are scarce (43).

SYNERGISTIC ROLE OF PGPP AND AMF IN STRESS TOLERANCE.

The rhizosphere offers a dynamic environment for plant–plant, root–microbe, and microbe–microbe interactions. The outcome of these interactions, synergistic or antagonistic, depends on the partner species or strains, but favorable manipulation of these interactions is a promising approach for sustainable agriculture (44).

In the mycorrhizosphere, AMF may interact with Mycorrhizal Helper Bacteria (MHB) such as *Bacillus* and *Pseudomonas* to synergistically promote plant growth and enhance each other's survival (44). Also, MHB such as *Paenibacillus brasilensis* can increase colonization by AMF such as *Funneliformis mosseae* (formerly *G. mosseae*) by releasing compounds that increase root cell permeability and exudation, which are known to stimulate hyphal growth. Nadeem et al. (2014) cited Linderman (1992) who reported a positive effect on nitrogen-fixing and phosphorus solubilizing bacteria by AM fungi in their activity to intensify plant growth (44).

Protection of plants from various plant pathogens or stresses is another example of synergistic interaction. For example, increased tomato growth or chrysanthemum resistance to phytoplasma infection has been reported following co-inoculation by *P. fluorescens, P. putida and G. mosseae* BEG12 (45, 46, 46, 47). Conclusive findings state that the disease interaction outcomes may be specific, e.g. PGPP may behave differently with different fungal species. For example, co-application of *R. irregularis* with *P. fluorescens* improved the dry weight of wheat infected by *Microdochium nivale* (a pathogenic fungus), but did not provide this protection when co-applied with *P. brasilensis*. Dual inoculation of PGPP and AMF also stimulated the growth of plant facing abiotic stress. For instance, co-inoculation improved lettuce growth compared to mycorrhization alone under drought stress (44).

My study is interested in two pathosystems discussed separately in the next chapters: anthracnose leaf blight (ALB) of maize and Zebra Chip disease (ZC) of Solanaceous crops, with tomato as the host of interest. Presently, ALB control relies on resistant lines, but high genetic plasticity in the fungal causative agent, *Colletotrichum graminicola,* resulting in the evolution of new pathotypes makes it challenging to breed for durable genetic resistance. ZC control is currently based on antibiotic treatment against the causative agent '*Candidatus* Liberibacter solanacearum' (CLso) and insecticide applications to control the psyllid vector, *Bactericera cockerelli*. However, this will lead to resistance emergence in the psyllids and CLso. A potential alternative is to boost host resistance by employing PGPP and AMF. I evaluated the effect of inoculation of the PGPP *P. chlororaphis* 30-84 and/or the AMF *R. irregularis* DAOM 197198 on Maize anthracnose leaf blight and Zebra Chip disease. My study consisted of these activities:

1. I assessed the ability of *R. irregularis* and/or *P. chlororaphis* 30-84 to induce systemic resistance (ISR) against fungal pathogen *C. graminicola,* the causative agent of maize leaf blight when applied onto the leaves of maize line B73 and its *lox12-1* mutant. In doing so, I observed the role of each beneficial agent in ISR;
2. Using a split root system that separated the root colonists, I determined the nature of the interaction between the symbionts *in planta*;
3. In a factorial experiment, I evaluated the mycorrhizal effect on ZC disease development in a susceptible tomato cultivar.
4. In a non-choice assay (e.g. where the insects are forced to feed on one leaf), I assessed the mycorrhizal effect on the psyllid oviposition and larval survival.

CHAPTER II

EFFECT OF INOCULATION OF *R. IRREGULARIS* DAOM 197198 AND *PSEUDOMONAS CHLORORAPHIS* STRAIN 30-84 ON MAIZE LEAF BLIGHT

INTRODUCTION

Colletotrichum species, the etiological agents of anthracnose, are among the top 10 fungal plant pathogens of economically important crops (Balmer et al., 2013; Vargas et al., 2012). *C. graminicola* causes severe anthracnose seedling and leaf blight (ALB) and stalk rot (ASR) diseases on maize and sorghum (48). Although the pathogen infects all parts of the plant at every growth stage (49, 50), leaf necrosis is the most damaging aspect as it results in 50% or more in yield reductions (51). Moreover, although leaf blight and stalk rot are the major symptoms, root infection by *C. graminicola* also may result in systemic infections (49). Stalk infection is facilitated by wounds caused by insects, yet the pathogen's narrow hyphae also slowly but directly penetrate the pith tissues through small openings in cell walls (48). *C. graminicola* is a well-studied model for hemibiotrophic pathogens (52, 53).

Genetic resistance stands as the most efficient control of ALB and ASR. In maize, one single dominant gene, CgL*,* seems to control the resistance to ASR at both the seedling and mature stages of development whereas two co-dominant genes controlled resistance to ALB (54). However, Bergstrom & Nicholson (1999), and da Costa et al., (2014) reported that polygenes might be involved in ALB resistance (54, 55). Thus, the inheritance of resistance in maize remains controversial. Also, the effectiveness and durability of maize resistance as a management approach against ALB relies on the variability of *C. graminicola* (54). Indeed*,*

the genus *Colletotrichum* contains considerable phenotypic plasticity, and rapid evolution of races or pathotypes has been reported (53, 54, 56–58). Therefore, it is challenging to diagnose disease accurately and to breed for durable genetic resistance (54, 59). Subsequently, according to Valèrio et al., (2005), long-lasting control of anthracnose relies on inoculum source reduction and management of both host resistance genes and the host-pathogen-environment (52). In this perspective, Arbuscular Mycorrhizal Fungi (AMF) and Plant Growth-Promoting Rhizobacteria (PGPR) are suitable to alter the host-pathogen-environment, mainly because of their ability to prime systemic resistance against both the biotrophic and necrotrophic lifestyles of *C. graminicola*. Mycorrhization due to AMF inoculation has been shown to both improve host plant growth and defenses (37, 60, 61). AMF colonization can prime the jasmonic acid-dependent responses of plants to wounds and resistance to pathogenic fungi (62, 63). PGPR can locally antagonize soil borne pathogens and induce systemic resistance against pathogens via the entire plant. Pathogen control *in plantae* and indirect promotion of plant growth have been related to the production of siderophores, antibiotics, and volatile organic compounds (64–66). It is hypothesized that co-inoculation with PGPR and AMF could positively interact to stimulate plant growth and control of fungal diseases since they both improve plant nutrient status, and thus significantly affect crop growth and fruit quality (67) and may share a common symbiosis signaling pathway (29, 68, 69). For example, maize plants inoculated with AMF and/or *P. fluorescens* Pf4 showed increased growth and yield compared to non-inoculated plants (67). Pf4 increased grain starch content, especially the digestible components, whereas AMF enriched storage compounds, especially zein content (67). Thus, plant inoculation with Pf4 and AMF resulted in additive effects on grain composition (67). There are numerous examples where

PGPR alone or in combination were shown to inhibit diseases on different hosts. For example, combinations of pseudomonads including *P. chlororaphis* 30-84 have been shown to suppress take-all disease caused by *Gaeumannomyces graminis* var. *tritici* (70, 71). Maize roots inoculated with *P. putida* KT2440 and later challenged by *C. graminicola* showed resistance to leaf necrosis, and fungal growth was significantly reduced compared to non-bacterized controls (24). *P. chlororaphis* PCL1391 controlled tomato foot and root rot caused by *Fusarium oxysporum* f. s. *radices-lycopersici* (72). AMF *R. irregularis* and *F. mosseae* showed antagonism towards *C. gloeosporioides* anthracnose on Cyclamen (73). Pre-mycorrhization by *R. irregularis* BEG110 reduced disease severity in cucumber plants caused by *C. orbiculare* (74). Cucumber plants pre-inoculated with *P. putida* and *Micrococcus luteus* or *Serratia marcescens* 90-166 and *P. fluorescens* 89B61 were resistant to anthracnose after inoculation with *C. orbiculare* (74–76).

Although numerous examples demonstrate that both AMF and PGPR can prime plant defenses, it is unclear how they act together. This is somewhat difficult to predict since the interactions can be species related, at the microbe-plant level and or the microbe-microbe level. For instance, Pivato et al., (2008) showed that *P. fluorescens* C7R12 promoted *Glomus mosseae* saprophytic growth and its mycorrhization of *Medicago truncatula* and *Lycopersicum esculentum* but not promote growth or mycorrhization by *Gigaspora rosea* (77). It was also shown that rhizobial nodulation systemically and negatively influenced AMF root colonization and vice versa in an alfalfa split-root system assay (78).

One area that still requires investigation is the plant signaling pathways that mediate interactions with beneficial microbes and pathogens and how established interactions with one type of beneficial microbe alters plant signaling to affect subsequent interactions with

other beneficial or pathogenic agents. It now is well established that lipoxygenases (LOX), key regulatory components of plant signaling pathways including JA signaling, affect interactions with important rhizosphere microorganisms. JA is one of the key regulators of plant stress responses and plant development. Interaction with microbes and wounding of leaves by insects activate a complex signaling pathway in which jasmonates are increasingly synthesized following a feedforward regulatory loop since the genes coding for JA biosynthetic enzymes are also activated (34). Plant defense genes that code for proteinase inhibitors, enzymes of phytoalexins synthesis, vegetative storage proteins, thionins, and defensins are also activated (34, 79). JA is synthesized from the octadecanoid pathway (79, 80). The biosynthesis starts by plastid membranes releasing α-linolenic acid (α-LeA). Oxygenation at the position 9 or 13 is catalyzed by 9- or 13-lipoxygenases respectively, resulting in the formation of (9 or 13-*S*)-hydroperoxy linolenic acid (9- or 13-HPOT). Although, 13-HPOT can be utilized by at least seven different pathways, only its conversion by dehydration into an unstable allene oxide (81) by an allene oxide synthase (AOS) leads to JA via the *cis*-(+)-enantiomer (9*S*,13*S*) of OPDA and cyclization by allene oxide cyclase (AOC) (34, 81). The AOC is of particular relevance in JA biosynthesis. The final steps in this biosynthesis include the reduction of OPDA by the OPDA reductase 3 (OPR3) and three cycles of β oxidation of the carboxylic acid side chain by acyl-CoA oxidase (ACX), multifunctional proteins (MFP) and 3-ketoacyl-CoA thiolase (KAT) (34). In collaboration with Dr. Michael Kolomiets (Department of Plant Pathology and Microbiology, Texas A&M University), I have access to a collection of maize *lox* mutants in the maize B73 background. Each mutant has the function of one of the approximately twelve LOX genes interrupted by insertions of Mutator transposable elements (82, 82–92). In my work, I will be focusing on

lox12-1, a 9- lipoxygenase involved in the synthesis of JA. Interestingly, different mutations in this JA signal pathway affect root architecture, root-root interactions (allelopathy), root interactions with plant growth promoting fungi like *Trichoderma*, and stress tolerance (to drought, disease, and herbivory) differently, producing different ecological phenotypes (92). Mutations can be responsible for susceptibility or resistance to stress. The effect of mutations in the LOX genes on AMF and PGPR colonization and disease control has yet to be investigated.

My study assessed the effect of co-inoculation by *R. irregularis* and *P. chlororaphis* 30-84 on ALB. I hypothesized that the establishment of a synergistic defense by *R. irregularis* and *P. chlororaphis* 30-84 against the maize ALB pathogen occurs via the Jasmonic acid pathway. I based my expectation on the above-mentioned findings of Pivato et al. (2009). My study is the first report on the nature of the interaction between *P. chlororaphis* 30-84 and *R. irregularis* DAOM 197198 and their effect on maize ALB in plantae. In this study, I first examined the ability of *R. irregularis* and/or *P. chlororaphis* 30-84 when preinoculated onto the roots of the susceptible maize inbred line B73 to induce resistance against *C. graminicola* applied to the leaves. Subsequently, I compared responses of B73 and *lox12-1* maize mutants when preinoculated with *R. irregularis, P. chlororaphis* 30-84 or both in a dual inoculation and challenged by the pathogen. I also used a split root system whereby I preinoculated half of the root system with one of the beneficial microbes three weeks before I inoculated the other half with the other beneficial microbe. This split pot experiment helped achieve the second objective, which was to determine the nature of the interaction between the symbionts *on plants*. Finally, I assessed whether the beneficial microbes synergistically act to control the ALB.

RESULTS

P. chlororaphis 30-84 inhibits *C. graminicola* M1.001 growth *in vitro*

I first examined whether *P. chlororaphis* 30-84 (hereafter 30-84) was directly inhibitory to the mycelial growth of *C. graminicola* strain M1.001 (hereafter *C. graminicola*). An in vitro dual culture assay was conducted on potato dextrose agar (PDA) plates. The fungal strain was initially grown on PDA for seven days, and 3-mm-diameter plugs were taken from the leading edge of the mycelial growth. These were transferred to the center of fresh PDA plates, to grow for two days before 30-84 was spotted to the plates. I included several 30-84 derivatives in the assay: 30-84 (wild-type), 30-84ZN (non-phenazine producer, *phzB::lacZ*, hereafter Phz$^-$ mutant) and 30-84GacA$^-$ (a spontaneous *gacA* mutant deficient in the production of several secondary metabolites essential for biological control, including phenazines hereafter GacA$^-$ mutant). Overnight bacterial cultures were adjusted to OD_{620} = 0.8, and five µl of each was spotted onto the plate 3 cm from the center where the fungus was inoculated. After ten days, the zone of inhibition was measured as the distance between the edge of the bacterial colony and the fungal mycelium (Figure 1).

There was a significant difference among bacterial strains in the size of the zone of inhibition, indicating differences in the production of diffusible compounds responsible for halting mycelial growth. The inhibition zone caused by 30-84 was 7.36 ± 0.07 times greater than that of the Phz$^-$ mutant. I concluded that diffusible compounds produced by 30-84 wild type but not the Phz$^-$ mutant inhibited *C. graminicola* growth in vitro, and this inhibition was likely due to phenazine production. The non-phenazine producer mutant was able to limit

fungal growth to some extent, although this effect was short-lived (e.g. the Phz$^-$ spots were fully overgrown by 12 days, data not shown), whereas the GacA$^-$ mutant was unable to inhibit mycelial growth. These data indicate that other secondary metabolites produced by 30-84 also play roles in fungal growth inhibition, but are not as effective or persistent as phenazines.

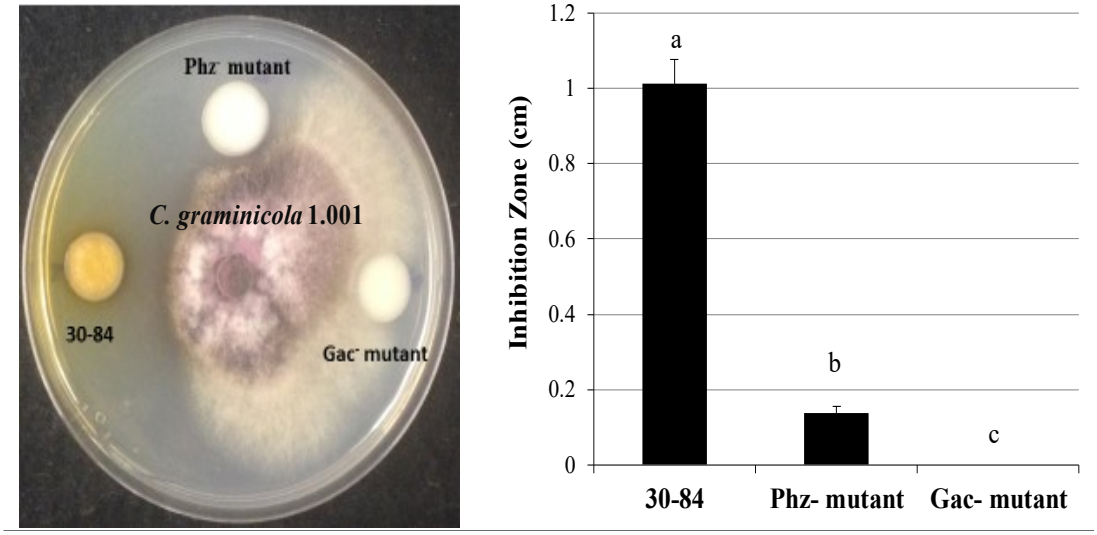


Figure 1: *C. graminicola* growth inhibition assay using 30-84 wild type and the Phz$^-$ and Gac$^-$ mutants

The fungal strain was applied to the center of a PDA plate. Bacterial overnight cultures of 30-84 wild-type, Phz- mutant (non-phenazine producer, phzB::lacZ) and Gac- mutant (spontaneous *gacA* mutant), were spotted 3 cm from the fungal plug two days later. After ten days, the zone of inhibition was measured. Bars represent means of eight replicates ±.S.E

P. chlororaphis 30-84 and *R. irregularis* suppress *C. graminicola*-induced leaf lesions

In a randomized complete block design, I tested the ability of *P. chlororaphis* 30-84 and *R. irregularis* to suppress *C. graminicola*-induced leaf lesions at V3 growth stage of maize seedlings of lines that were ALB susceptible (B73), ALB resistant (W438), or mutated

in ZmLOX12 (*lox12-1*). All lines were preinoculated with *R irregularis* (hereafter Ri) and/or strain 30-84 before being leaf-infected three weeks later with 1×10^6 conidia/ml of *C. graminicola* on the 3rd leaf. The control consisted of plants receiving no biocontrol inoculation, e.g. inoculated only with the pathogen. Three days after pathogen inoculation, infected leaves were removed from the plant, and lesion area scanned and estimated using ImageJ 1.50i software.

I first evaluated the levels of 30-84 and Ri root colonization during the assay. Previous studies suggest that populations of 10^5 or greater are required for biological control activity on wheat (70). Good colonization by Ri is considered to be 30-40% of the root length (93, 94). I was particularly interested in colonization rates in the dual treatment since 30-84 is known to inhibit the mycelial growth of many fungi. However, it was unknown whether 30-84 would inhibit AM formation or if AM formation would affect 30-84 colonization. Root colonization rates are shown in Figure 2.

I found that 30-84 achieved population sizes of 10^5 - 10^7 CFU/g of root and root length colonization by *R. irregularis* was 57 – 97%. 30-84 populations from single or dual inoculum differed by less than tenfold, whereas *R. irregularis* was somewhat more variable. These data indicate no consistent impact of one population on the other when applied simultaneously to the same root system. Since *R. irregularis* genome lacks toxin genes, it made sense that mycorrhization did not impair root bacterization by 30-84 (95). Previous study demonstrated the ability of *R. irregularis* to modulate and even downregulate fungal toxin gene expression in the plant pathogen *Fusarium sambucinum* (96). Thus, unaffected rate of mycorrhization in dual inoculation might be explained by the fact that *R. irregularis* might have impaired phenazine production in 30-84. Moreover, data demonstrated that

colonization rates were the same on all maize lines, indicating that mutation of ZmLOX12 did not affect colonization by either biocontrol agent.

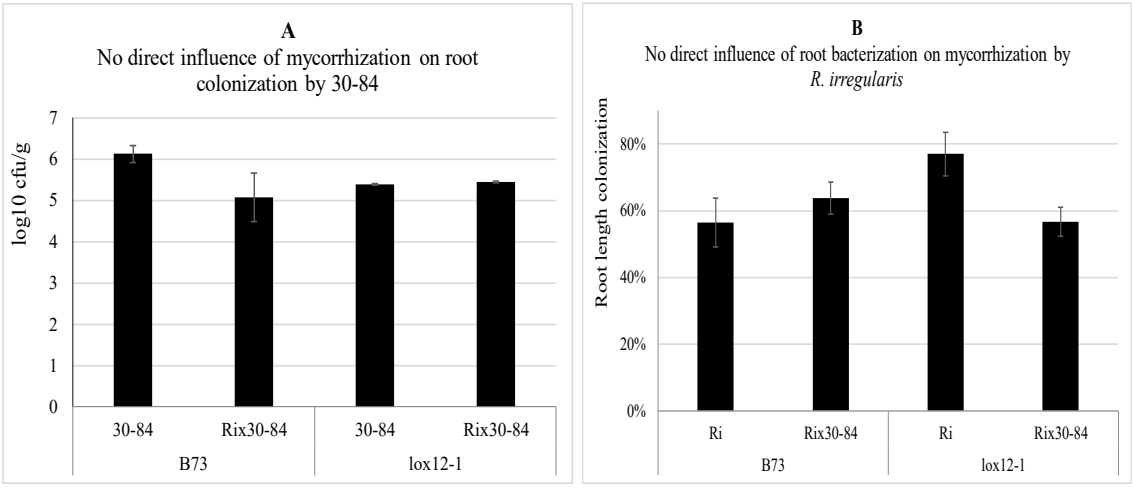


Figure 2: Level of *P. chlororaphis* 30-84 (A) and *R. irregularis* root colonization (B) during the antagonism and plant growth promoting assays
Ri = *R. irregularis* DAOM 197198. 30-84 = *P. chlororaphis* 30-84. Rix30-84 = Coinoculation (e.g. dual treatments applied together to the entire root system). Three weeks old plants were preinoculated with *R. irregularis* and/or 30-84 and colonization rates were checked at V3 growth stage. 30-84 colonization is expressed as the colony forming units/gram of root (log_{10}). Ri colonization is measured as the percentage of the root length colonized. Bars are means of at least 3 measures ± S.E. Experiments were repeated twice. There was no statistical difference.

Maize response to *C. graminicola* infection

After confirming that root colonization was sufficient, I measured the response of the plants to *C. graminicola* infection (Figure 3 & 4). Both beneficial agents reduced disease severity on susceptible B73. Pre-inoculation of B73 with Ri, Rix30-84 or 30-84 resulted in lesions areas that were 4.45±0.03, 2.76±0.03 and 23.59±0.02 times smaller respectively than the lesion areas produced on the unprotected control (Figure 3A) and this level of control is similar to that observed for the unprotected resistant line W438 (3B). These reductions were significantly different when compared to the control. Neither of the phenazine deficient

mutants (Phz$^-$ or Gac$^-$) elicited the same response as the wild type, indicating that phenazine production may be involved in systemic disease suppression.

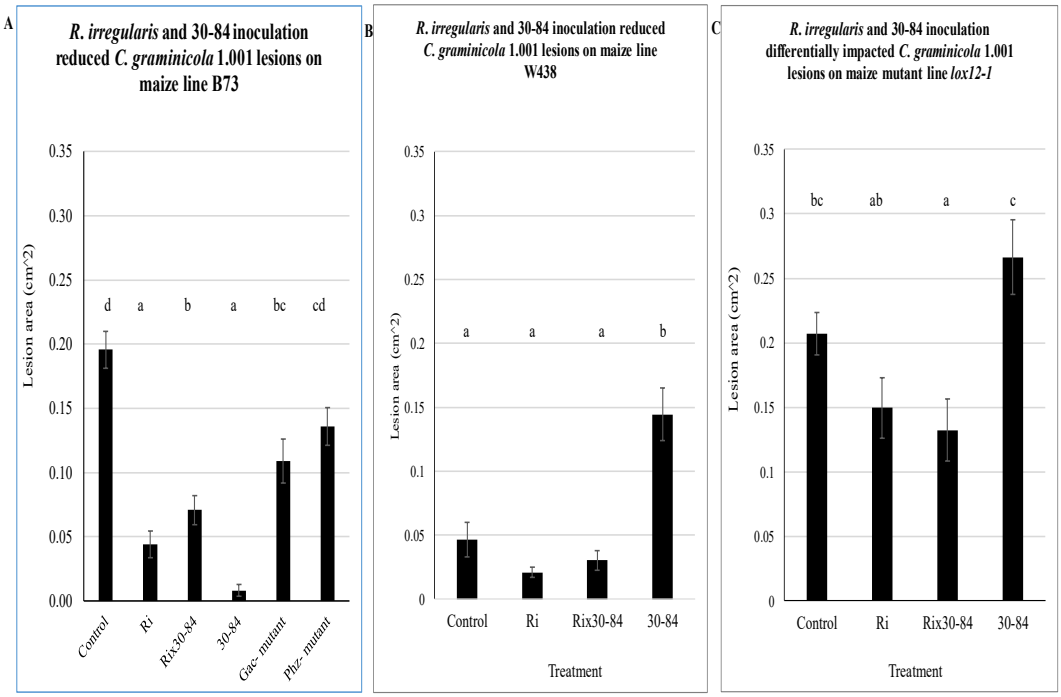


Figure 3: Lesion area observed on A) ALB susceptible B73, B) ALB resistant W438, and C) *lox12-1* on plants receiving no pre-treatment (control) or pretreatment with different beneficial agents' combinations.
Ri = *R. irregularis* DAOM 197198. 30-84 = wild type 30-84, Rix30-84 = co-inoculation, Phz- mutant = non-phenazine producing 30-84 mutant, Gac- mutant = non-phenazine producing Gac mutant. Three weeks old plants were preinoculated with *Ri* and/or 30-84 and later at V3 growth stage were leaf-infected with 10 days old of *C. graminicola* culture. Leaves were removed from the plant and scanned 3 days after pathogen application and lesion area were measured with ImageJ software. Bars are mean of 38 (B73), 13 (W438), or 20 (*lox12-1*) lesion measurements ± S.E. Experiments were repeated twice. Bars with the same letters are not significantly different.

Although the lesion areas observed on B73 plants treated with 30-84 and Ri were not statistically different, there was a qualitative difference in the lesion type according to the grading scale defined by da Costa et al., 2014 (54). The lesions on 30-84-inoculated plants were small chlorotic/necrotic punctuations without any sporulation or yellow chlorotic halo;

disease severity rated less than 1% (Table 1). This reaction correlated to a rating of Resistance/Hypersensitivity. Ri-inoculated plants had lesions that were circular to irregular, pale brown to brown, and often surrounded by a chlorotic or yellow halo, with an absence of sporulation; disease severity rated less than 5%. This reaction is rated as Resistant. Disease severity in Rix30-84 inoculated plants was less than 20%, but Rix30-84 treatment yielded to the same lesion type as the Ri treatment, so it also rated as Resistant. Control plant lesions had predominantly an oval shape, greenish gray color on both leaf surfaces, the presence of concentric growing halos, and sporulation. The disease severity was more than 21% indicating an accentuated susceptible reaction (Table 1, Figure 4).

Table 1: Assessment according to the descriptive scale as per da Costa et al., 2014

Corn line	Treatment	Note	Reaction	Lesion type
B73	Control	4	Susceptible	Lesions chiefly were oval, gray color on both leaf surfaces, and surrounding by growing chlorotic halos. Disease Severity: 21–40%.
	Ri	2	Resistant	Brown and circular to irregular shaped lesions which, often surrounded by a yellow halo. Disease Severity: less than 5%.
	Rix30-84	3	Resistant	Brown and circular to irregular shaped lesions which, often surrounded by a yellow halo. Maximum disease severity: 20%.
	30-84	1	Resistant / hypersensitivity	Small necrotic punctuations, absence of chlorotic halos and sporulation. Disease Severity: less than 1%

Table 1 Continued

Corn line	Treatment	Note	Reaction	Lesion type
W438	Control	2	Resistant	Brown and circular to irregular shaped lesions which, often surrounded by a yellow halo. Disease Severity: less than 5%.
	Ri	2	Resistant	Brown and circular to irregular shaped lesions which, often surrounded by a yellow halo. Disease Severity: less than 5%.
	Rix30-84	2	Resistant	Brown and circular to irregular shaped lesions which, often surrounded by a yellow halo. Disease Severity: less than 5%.
	30-84	3	Resistant	Brown and circular to irregular shaped lesions which, often surrounded by a yellow halo. Maximum disease severity: 20%.
lox12-1	Control	4	Susceptible	Lesions chiefly were oval, gray color on both leaf surfaces, and surrounding by growing chlorotic halos. Disease Severity: 21–40%.
	Ri	3	Resistant	Brown and circular to irregular shaped lesions which, often surrounded by a yellow halo. Maximum disease severity: 20%.
	Rix30-84	3	Resistant	Brown and circular to irregular shaped lesions which, often surrounded by a yellow halo. Maximum disease severity: 20%.
	30-84	4	Susceptible	Lesions chiefly were oval, gray color on both leaf surfaces, and surrounding by growing chlorotic halos. Disease Severity: 21–40%.

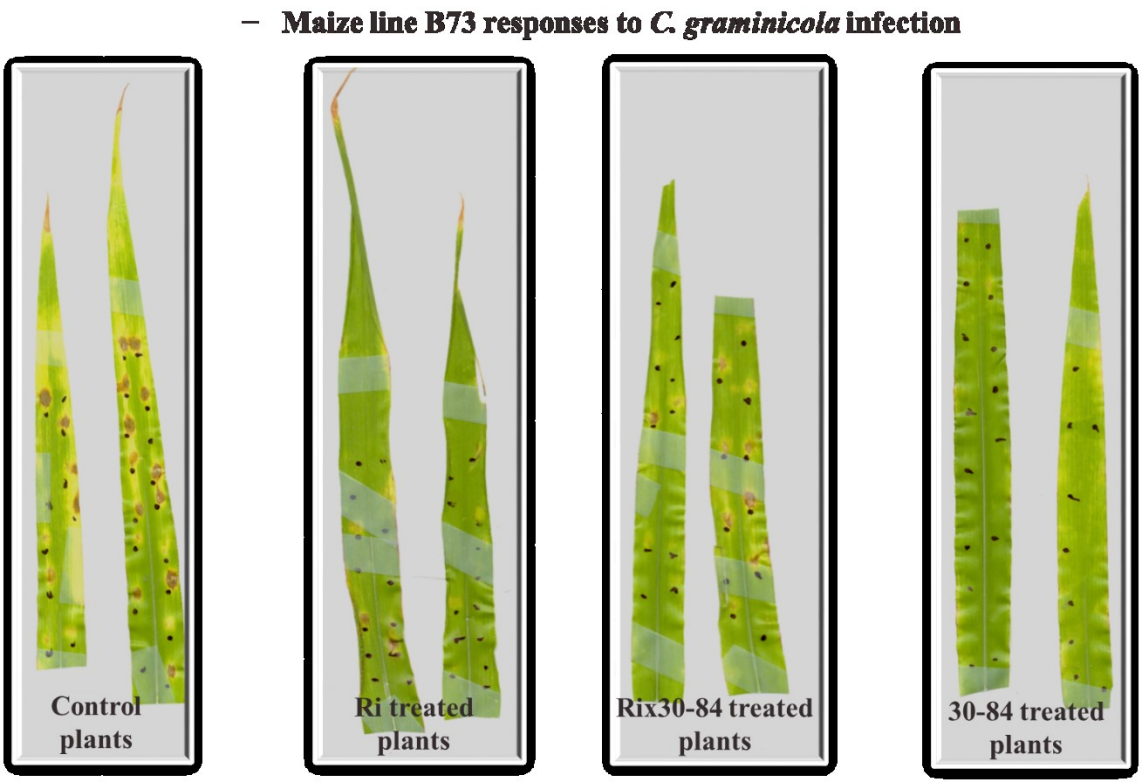
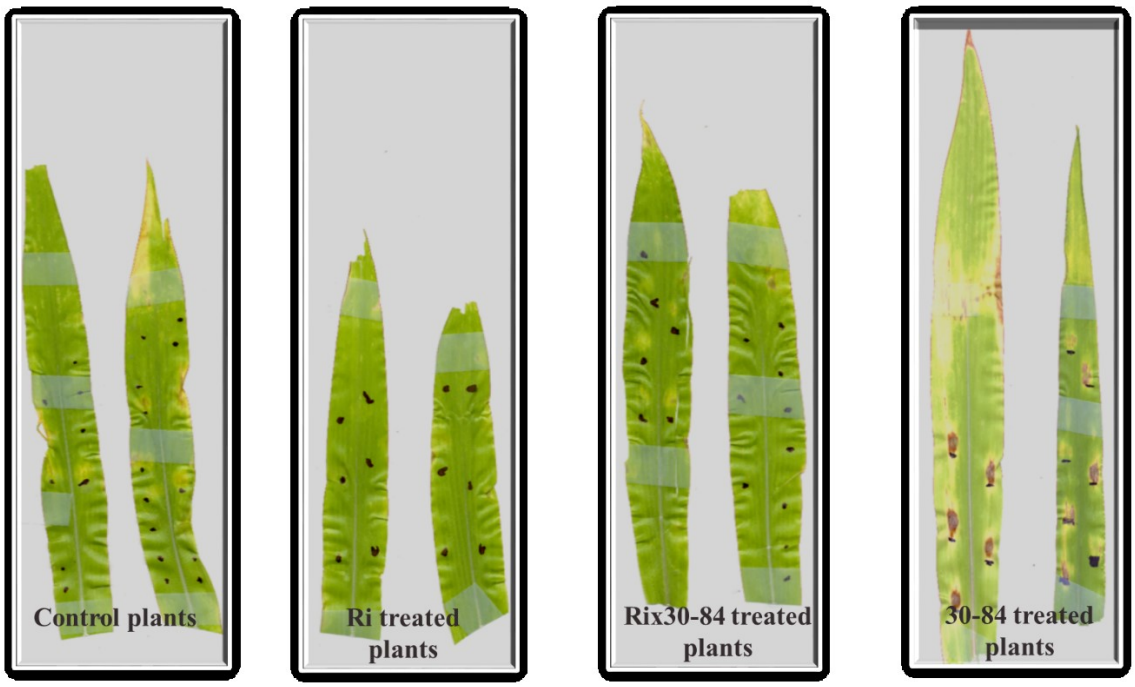


Figure 4: Effect of *R. irregularis* and 30-84 on *C. graminicola* lesions on maize lines
Ri = *R. irregularis* DAOM 197198. 30-84 = wild type 30-84, Rix30-84 = co-inoculation. Three weeks old plants were preinoculated with *Ri* and/or 30-84 and later at V3 growth stage were leaf-infected with 10 days old of *C. graminicola* culture. Leaves were removed from the plant and scanned 3 days after pathogen application and lesion area were measured with ImageJ software.

– **Maize line W438 responses to *C. graminicola* infection**



– **Maize *lox12-1* mutant responses to *C. graminicola* infection**

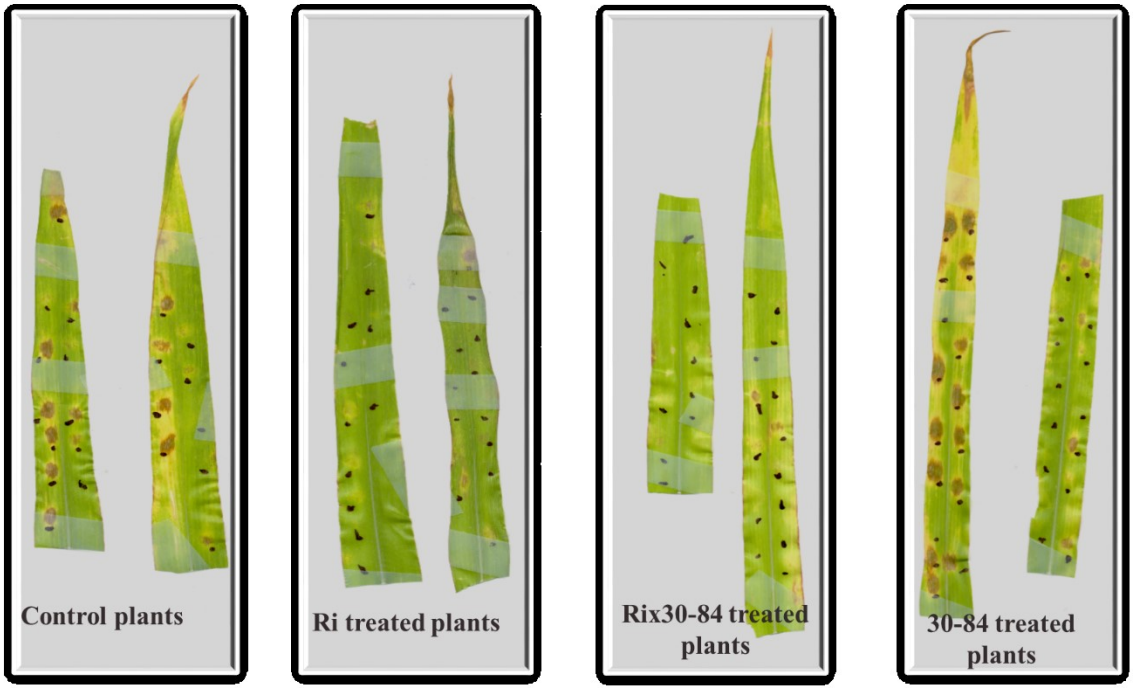


Figure 4 Continued

W438 is referenced as resistant to ALB (84), and yet the biocontrol agents (BCA) produced very different responses. Lesion areas were significantly *greater* in 30-84-inoculated plants compared to all the other treatments, which were statistically and qualitatively similar to each other (Figure 3B, Table 1, Figure 4). Thus, preinoculation with 30-84 impaired the level of the resistance present in W438.

Disease resistance measurements on the *lox12-1* mutant provided information on the role of this lipoxygenase in the interactions between the plant, pathogen, and biocontrol agents. Comparison of control plant responses revealed that B73 and *lox12-1* showed the same level of susceptibility (Figure 3A, C). Pretreatment of *lox12-1* with Ri and Rix30-84 resulted in leaf lesions areas that were 1.39± 0.04 and 1.57± 0.04 times smaller than those found on the unprotected control. Surprisingly, pretreatment with 30-84 resulted in lesions 1.29±0.05 times *larger* than those found on the unprotected plants, indicating that 30-84 inoculation enhanced *lox12-1* disease symptoms (Figure 3C). From a qualitative assessment view, control and 30-84-inoculated *lox12-1* plants were rated susceptible and highly susceptible, respectively, whereas Ri and Rix30-84 treatments provided a mild resistance in this Lox mutant (Table 1, Figure 4).

To determine whether lesion area was related to foliar colonization by the pathogen, I estimated foliar pathogen biomass by determining ergosterol content of lesions (97-99) (Figure 5).

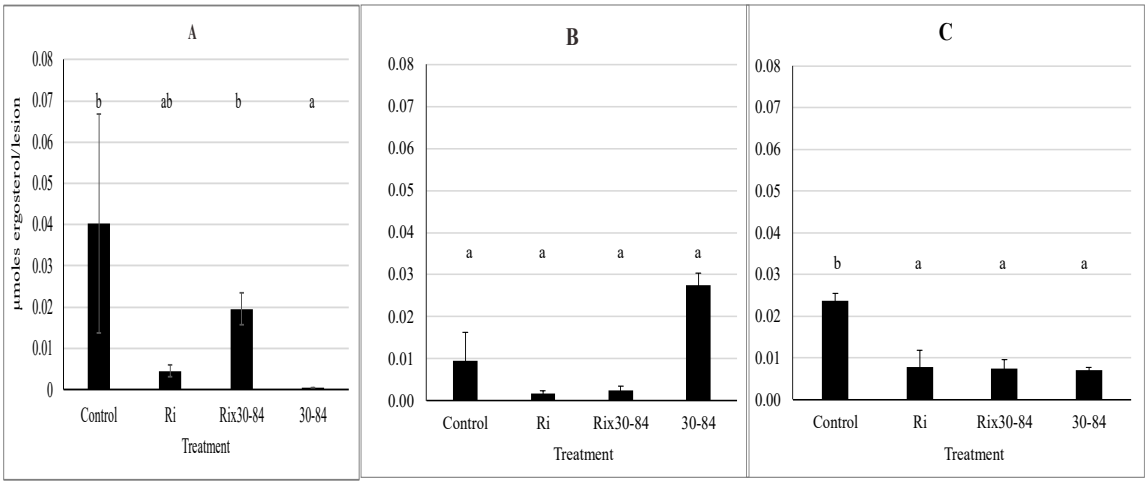


Figure 5: Measurement of ergosterol content by lesion as an indicator of *C. graminicola* growth (biomass) in leaves of A) B73, B) W438, and C) *lox12-1*.
Ri = *R. irregularis* DAOM 197198. 30-84 = *P. chlororaphis* 30-84. Rix30-84 = Coinoculation. Three weeks old plants were preinoculated with *R. irregularis* and/or 30-84 at V3 growth stage were leaf-infected with 10 days old of *C. graminicola* culture. Leaves were cut, treated in liquid nitrogen and kept in -80°C before ergosterol being measured by LC/MS. Bars are mean of at least 8 measures ± S.E. Experiments were repeated twice in growth chamber: cycle of dark (20°C, during 12h) and light (30°C, during 12h). Same letters on bars show non-significant difference.

Consistent with lesion area measurements, ergosterol content in B73 leaves treated with 30-84, Ri or dual inoculation was 111.78±0.03, 8.87±0.03 and 2.2±0.03 times lower than in the untreated control leaves, respectively (Fig. 5A). Ergosterol levels in W438 leaves also mirrored disease severity, although there were no significant differences in ergosterol content among the treatments (Fig. 5B). In *lox12-1* leaves, differences in ergosterol levels among all BCA treatments were not significant, but they were significantly reduced compared to the unprotected *lox12-1* plants. Surprisingly, despite the accentuated disease symptoms on 30-84 treated *lox12*-1 (Fig. 3C), ergosterol content per lesion was lower than untreated plants (Fig. 5C), indicating that the size of the lesion areas is not only related to pathogen growth but also to plant reaction.

To get a clearer understanding of the plant's response, I also measured disease incidence (e.g. the number of leaves that once inoculated developed symptoms). Disease incidence provides information on the number of leaves that escape infection whereas disease severity is a measure of symptom development once lesions are established. Disease incidence (DI %) was calculated per treatment according to this formula (21, 100–102). Lesions developed on all the maize lines but with different relative frequencies. On B73 leaves, the lowest disease incidence was observed on 30-84-inoculated plants where only 50% of these plants showed the symptoms. Mycorrhized plants presented the lowest disease incidence on W438 and *lox12-1* (Table 2).

Table 2: Disease incidence, mycorrhizal, bacterial and dual inoculation effect

Maize line	Treatment	DI	MIE	BIE	DIE
B73	Control	100%			
	Ri	80%	-77.51%		
	Rix30-84	100%			-63.76%
	30-84	50%		-95.76%	
W438	Control	40%			
	Ri	33%	-54.82%		
	Rix30-84	50%			-34.74%
	30-84	75%		210.57%	
lox12-1	Control	100%			
	Ri	75%	-27.87%		
	Rix30-84	60%			-36.17%
	30-84	100%		28.56%	

Mycorrhizal Inoculation Effect (MIE %) has been used to calculate the effect of mycorrhization on disease severity and is calculated using this formula (21, 100–102). Mycorrhizal Inoculation caused a reduction of pathogenic foliar lesions on all maize lines tested. The strongest reduction as shown by MIE was recorded on B73, which was 22.69%

and 49.64% greater than the value recorded on W438 and *lox12-1* respectively (Table 2). Similarly, Bacterial Inoculation Effect (BIE %) was calculated per maize line. Bacterial inoculation caused a reduction of pathogenic foliar lesions on B73 but not on W438 or *lox12-1*. Dual Inoculation Effect (DIE %) also was calculated. Dual inoculation caused a reduction of pathogenic foliar lesions on B73 which was 29.02% and 27.59% superior to that recorded on W438 and *lox12-1* respectively (Table 2).

Together these data indicated *R. irregularis* and/or *P. chlororaphis* 30-84 systemically suppress corn leaf blight due to *C. graminicola* strain M1.001 on B73. This response appears to be dependent on the production of phenazines since phenazine deficient mutants did not cause the same response. This reaction also appears to be JA pathway dependent for 30-84 since suppression is lost in the *zmlox12* mutant (*lox12-1*), which does not produce JA. Interestingly, although all the W438 treatments had low disease symptoms, the symptoms expressed on the 30-84-treated plants were enhanced, suggesting a potential conflict in the mechanisms of disease resistance provided by 30-84 and present in W438. For the most part, applying both Ri and 30-84 together induced systemic resistance, but in a manner that mirrored the Ri effect. On B73 there appeared to be a weak interaction between the two that resulted in less disease suppression than observed for either biocontrol agent. On the *lox12-1* mutant, this antagonism was not evident suggesting ZmLOX12 may mediate this weak interaction.

Neither BCA promoted maize growth under the experimental conditions

Both beneficial agents are capable of promoting plant growth. I used the number of green leaves after six weeks after sowing (WAS), height, and root and shoot fresh weight biomass as measures of B73 growth. However, *under my experimental conditions,* I found no significant differences among treatments or compared to the non-inoculated control in any of the growth parameters measured (Figure 6). Future studies will need to focus on changes in leaf and root area relative to biomass as an indicator of changes in biomass allocation due to colonization.

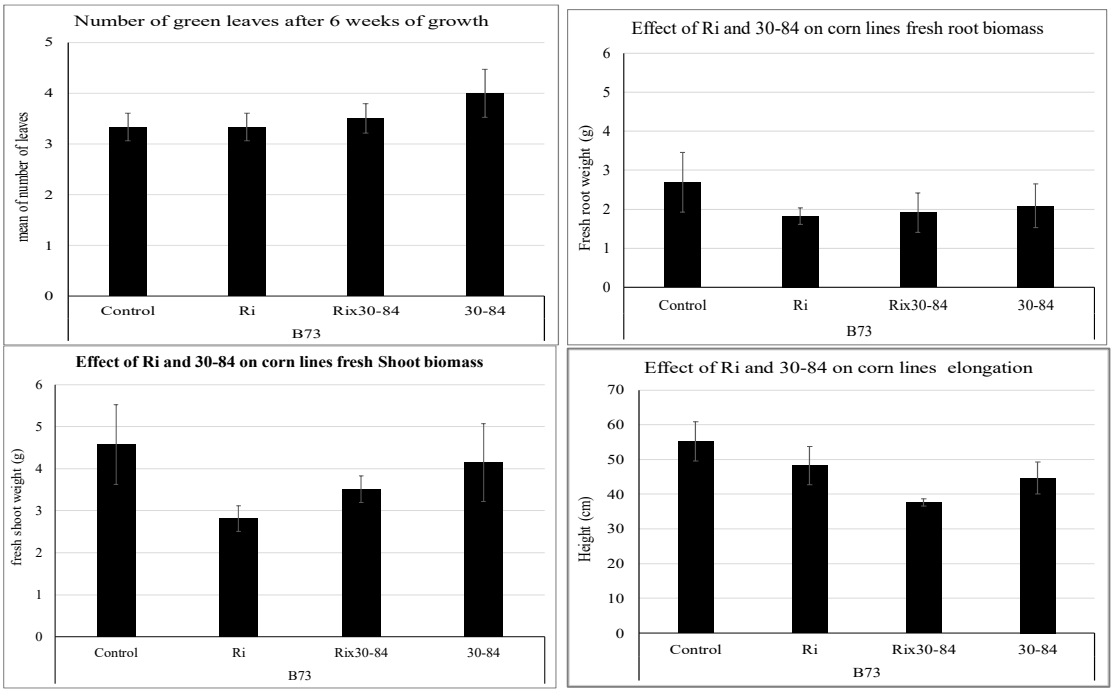


Figure 6: Effect of *R. irregularis* and 30-84 inoculation on B73 growth.
Three weeks old plants were preinoculated with R. irregularis and/or 30-84 and 42 DAS later, were leaf-infected at V3 growth stage with *C. graminicola*. Following analysis of root colonization and disease symptoms growth, parameters were measured. Bars are means of at least 4 measures ± S.E. Experiments were repeated twice.

Investigation of the effect of pre-colonization by one biocontrol agent on the ability of the second to colonize using a split pot assay

The results of my previous experiments indicated that both 30-84 and Ri were capable of inducing a systemic response to *C. graminicola* on B73 three weeks after pre-colonization by these agents. On *lox12-1* mutants, the BCAs differed in their effect on pathogen inhibition: 30-84 enhanced disease symptoms whereas Ri reduced them. I was interested in knowing whether pre-colonization by one of the beneficial agents would also induce a systemic response to colonization by the other beneficial agent and whether this interaction would differ between maize lines. I characterized the nature of the interaction between the two beneficial microbes using a split root/dual inoculation system in which one part of the root system was pre-inoculated with one of the two beneficial organisms, and the second part (in a separate pot) was inoculated three weeks later by the other one. In this way, inoculation by the two beneficial microbes was separated spatially and temporally. Colonization was measured three weeks after the second inoculation (5, 6, 103). Differences in colonization rates among treatments were analyzed via non-parametric tests and are presented in Figure 7.

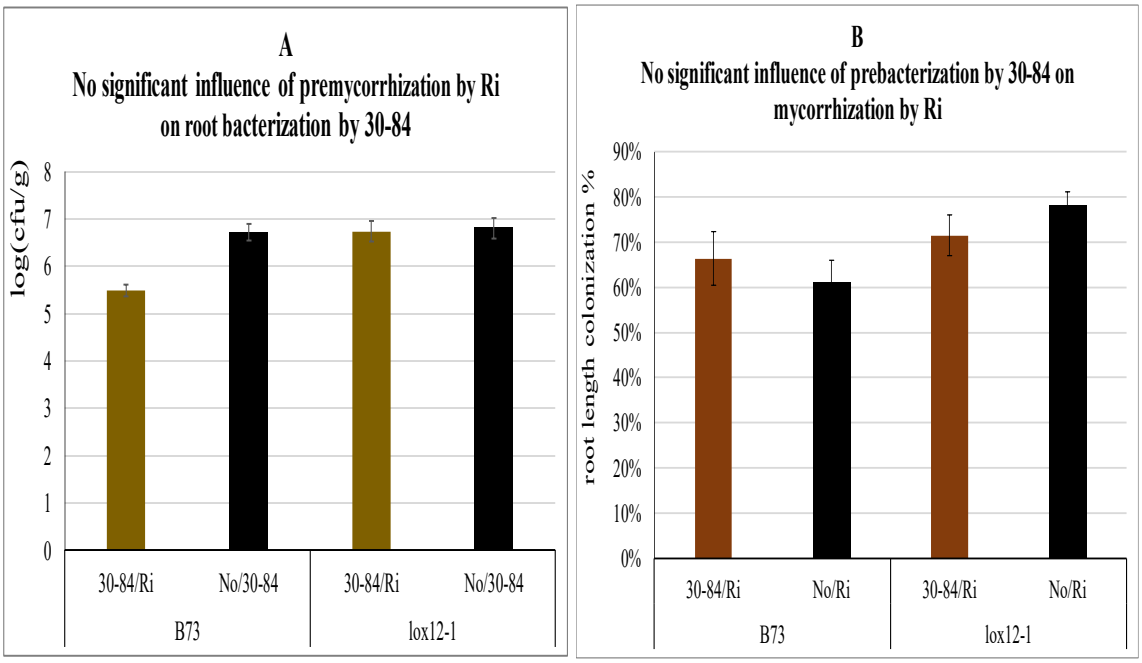


Figure 7: Effect of root pre-colonization by one beneficial on the colonization of the other when applied to B73 or *lox12-1*, and when split roots were pre-mycorrhized (A) or pre-bacterized (B)

The roots of two weeks old plants were split, and half the root system was preinoculated with one of the two beneficial organisms. The second part was inoculated three weeks later by the other one. Sequential treatments are symbolized by '/.' Three weeks after the second inoculation, root colonization by 30-84 was determined as colony forming units (CFU) /g (log_{10}) and colonization of Ri as root length colonized. Bars are means of at least 6 measures ± S.E. Experiments were repeated twice.

Similar to when the beneficial microbes were applied at the same time to the entire root system, in the split root assay 30-84 achieved population sizes of 10^6 - 10^7 CFU/g of root and *R. irregularis* colonized of 60 – 80% of the root length even though one beneficial was applied before the other. This indicates no consistent impact of one beneficial agent on the other. These results show that although pre-colonization by either of the beneficials on B73 results in induced resistance to the pathogen *C. graminicola* (Figure 8), plant response to pre-colonization does not interfere with colonization by the other beneficial.

Moreover, there was no effect of ZmLOX12 mutation on colonization rates (figure 7). Similar to when the dual treatment was applied to the entire root system, all single and dual treatments were equally effective in suppressing disease, regardless of the order of application (Figure 8).

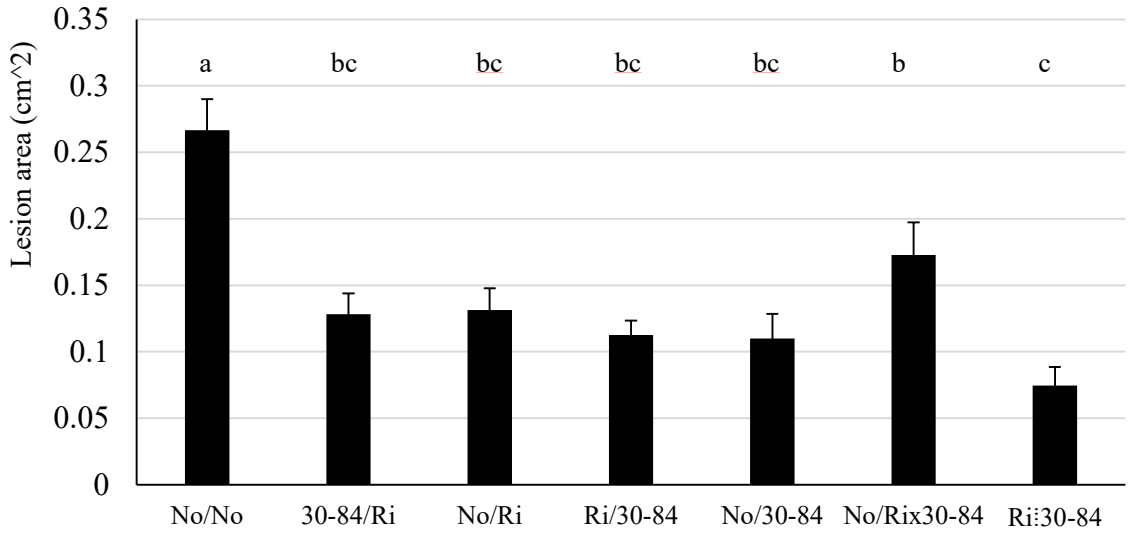


Figure 8: Lesion area observed on ALB susceptible B73 when root is pre-colonized by one beneficial and 3 weeks later or at the same time colonized by the other beneficial. The roots of two weeks old plants were split, and half the root system was preinoculated with one of the two beneficial organisms. The second part was inoculated three weeks later by the other one (symbolized by '/') or the same time (symbolized by '⁞'). Bars are means of at least 18 measures ± S.E. Experiments were repeated twice.

DISCUSSION

My data indicate *P. chlororaphis* 30-84 and *R. irregularis* considerably reduced *C. graminicola* M1.001 virulence on susceptible maize line B73. Interestingly, I demonstrated that 30-84 directly interfered with the mycelial growth of *C. graminicola,* primarily via the production of phenazines. Although the importance of this interaction needs to be tested in the field, this direct antagonism has noteworthy management implications since *C. graminicola* can infect plant roots and crowns leading to ASR (100). Both beneficial agents suppress foliar symptoms of ALB caused by *C. graminicola* infections on B73, however to a different extent. Pre-colonization of B73 by 30-84 resulted in lower disease severity, incidence, and fungal biomass whereas Ri pre-colonization led to reductions in severity and fungal growth but not necessarily incidence. Disease suppression by 30-84 also appeared to be dependent on phenazine production. Moreover, suppression of disease and fungal growth resulting from co-inoculation mirrored Ri inoculation on all maize lines. These results suggest that the two beneficial agents do not act in an additive manner to affect disease suppression and may suppress disease through different and potentially competing mechanisms. This hypothesis is supported by observations of increased disease severity on W438 and *lox12-1* pre-colonized by 30-84 as compared to pre-colonized with Ri. On W438, 30-84 may down-regulate the inherent mechanism(s) of disease suppression, likely through the modulation of plant hormones and/ or specific defense pathways. On *lox12-1*, loss of disease suppression in plants pre-colonized with 30-84 can be related directly to the absence of ZmLOX12. Similarly, pre-colonization of B73 with *Trichoderma virens* strain GV29-8 results in suppression of *C. graminicola,* which is lost in zmlox*3*-4 mutants (84). These

results demonstrate that different beneficial agents can induce resistance to *C. graminicola* through the action of various lipoxygenases in the JA-dependent pathway. In mycorrhized plants, loss of ZmLOX12 and JA (via the loss of ZmLOX12) does not affect disease suppression. One possibility is that salicylic acid and/or abscisic acid-dependent pathways contributing to Systemic Acquired Resistance, may be involved (49). This hypothesis is supported by previously published observations that Mycorrhiza-Induced Resistance (MIR) shares characteristics of both SAR and ISR (104–106). The next step is to analyze plant responses using the same factorial design (comparing untreated and 30-84 and Ri pre-inoculation across all three maize lines) and transcriptomic approaches to analyze gene expression patterns, in particular for the different lipoxygenase genes and defense genes regulated by the SA and JA pathways.

In nature, plants recruit and are colonized by a diversity of beneficial microorganisms. Thus, co-inoculation by beneficials is likely to be a ubiquitous occurrence. Overall, the results of this study indicate that application of 30-84 and *R. irregularis* have potential for boosting plant resistance to plant diseases such as *C. graminicola.* However, the plant response ***may be cultivar specific***. Moreover, the potential for negative interactions among the beneficials both regarding their effect on plant defense responses as well as on each other's establishment in the rhizosphere should be considered. This study is one of the first to examine the indirect interaction between beneficials using a split root system specifically the interaction between *P. chlororaphis* and *R. irregularis*. I found a neutral influence on the rate of root colonization by each organism on the other on all lines tested. The neutral interaction was somewhat surprising given that previous studies employing AMF and PGPP strains from the same genera or family led to the conclusion that the interactions

among beneficials may be very specific to the partners. For example, Pivato et al., (2008) revealed that dual inoculation of *P. fluorescens* C7R12 promoted *Glomus mosseae* saprophytic growth and its mycorrhization of *Medicago truncatula* and *Lycopersicum esculentum* but not *Gigaspora rosea* (77). In the 2008 study, the beneficials were introduced together (e.g. not in a split root experiment) so it is unclear if this outcome is the result of direct or indirect interactions. Interestingly nodulation by *Sinorhizobium meliloti* systemically and negatively influenced mycorrhization by *Glomus mosseae* and vice versa on alfalfa split-root system assay (78). Inhibition also was observed when Nod factors (lipo-chitooligosaccharides) purified from *S. meliloti* were applied prior to nodule formation and mycorrhization. Analysis of C^{14} allocation excluded competition for carbohydrates as the regulatory mechanism, indicating that systemic regulatory mechanisms were responsible for the negative interaction apparently through the shared symbiosis signaling pathway (78). In the case of 30-84 and Ri, systemic disease resistance appeared to be triggered by different signals, but it is unclear how each is able to circumvent host recognition and/or plant defense responses primed by the other to effectively colonize the plant host.

CONCLUSIONS

The effectiveness and durability of maize genetic resistance as a management strategy against anthracnose depends on knowledge of the pathogenic variability of *C. graminicola*. Since the genus *Colletotrichum* is characterized by a rapid evolution of races or pathotypes, it is challenging to diagnose disease accurately, and to breed for durable genetic resistance. However, Arbuscular Mycorrhizal Fungi and Plant Growth-Promoting Rhizobacteria are suitable to alter the host-pathogen-environment for the plant, mainly by priming systemic resistance. This study revealed the following:

- AMF *R. irregularis* DAOM 197198 and a PGPP *P. chlororaphis* 30-84 separately suppress disease symptoms caused *C. graminicola* on susceptible maize line B73. Phenazine production by 30-84 was responsible for both direct antagonisms of mycelial growth and systemic resistance. Dual inoculation also resulted in disease suppression, although not significantly better than the individual effects.
- Disease severity on resistant line W438 was significantly higher on 30-84 pre-colonized plants than untreated controls indicating that 30-84 may interfere with the disease resistance mechanisms present in W438. Moreover, these data indicate how interactions between “beneficials” and host plants may be cultivar specific.
- Disease suppression by 30-84 appears to be ZmLOX12 and JA signaling dependent as indicated by the loss of disease suppression on *lox12-1*. However, disease suppression by Ri is not altered on *lox12-1* indicating a different mechanism for disease suppression.

- Dual inoculation revealed effective systemic resistance induced against *C. graminicola* M1.001. However, the two agents do not negatively or positively interfere with each other's root colonization.

For a further understanding of the interaction between *R. irregularis* and *P. chlororaphis* 30-84 inoculated maize and the pathogen *C. graminicola*, local and systemic molecular and chemical defense responses of maize leaves and roots should be simultaneously investigated. I should also compare gene expression and hormonal alterations in both above- and below-ground organs. Metabolomics profiling could reveal significant differences in the composition of secondary metabolites in leaves and roots, indicating that my BCA organs employ distinct chemical defense systems. It is also important to see how the beneficial microbes contribute to maize productivity and plant defense in the field. Future studies focused on plant responses to rhizosphere colonization will help elucidate how PGPM avoid host defenses as well as similarities and differences in the way they interact with plant defense responses to promote disease resistance. Future studies focused on lipoxygenase signaling mutants may be key to unraveling critical signaling events and identifying QTLs for breeding better plant-PGPM interactions. Here below I outline two further activities of immediate interest.

1- Determination of newly synthesized molecules or host genes activated in inoculated B73 involved in the expression of resistance using metabolomic and transcriptomic analyses.
2- Assessment of AMF and 30-84 co-inoculation on plant yield and disease suppression under field conditions.

MATERIALS AND METHODS

Plant and soil material

Plant materials consisted of the ALB-susceptible maize inbred line B73, a near isogenic *lox12-1* mutant of B73, and the ALB resistant line W438.All seeds were obtained from Dr. Michael Kolomiets (Department of Plant Pathology and Microbiology, Texas A&M University). Seeds were surface sterilized in diluted commercial bleach (3%) for 30 min and rinsed twice with sterilized water. Surface sterilized seeds were pregerminated on water agar for 4-5 days. Pregerminated seeds were transferred to 1 gal pots filled with Metromix 900 and grown in a growth chamber. I maintained a dark cycle of 12 H at 20°C and the light cycle of 12h at 30°C. For the split root assays, one week, old plants were transferred to 164 ml conetainers filled with a turface and sand mix (1:1 v:v), which enabled the setup of a split-root system. One week old plants were used in the split root assays because the hypocotyl at this growth stage fits in the opening that sits on the two jointed conetainers and divides the root system. For all other experiments, two-week old plants were, transferred into 164ml conetainers that contain a turface and sand mix (1:1, v:v). The composition of turface used in these experiments is listed in Appendixes (Appendix 1). All plants were grown in the aforementioned growth chambers conditions.

Fungal growth inhibition in vitro assay

C. graminicola strain M1.001 was initially cultured on potato dextrose agar (PDA) for seven days, and a 3-mm-diameter plug was taken from the edge of the colony and transferred to the center of fresh PDA plate. After two days, 5 µl of 30-84 wild-type, Phz- mutant (non-phenazine producer, *phzB::lacZ*) and Gac- mutant (non-secondary metabolite producer, spontaneous *gacA* mutant deficient in the production of phenazines and other compounds essential for biological control) cultures, grown in 3 ml LB medium at 28°C for overnight with rapid shaking, were spotted onto 3 cm apart from the center of fungal plug. The distance between the edge of the mycelial and bacterial colony (zone of inhibition) was measured ten days after bacterial treatment.

***C. graminicola* foliar inoculation protocol**

C. graminicola strain M1.001 was routinely cultured for two weeks on PDA at room temperature with continuous illumination from a fluorescent light source (48) (107). Preparation of inoculum was performed as previously described (48). Briefly, conidia were collected by adding 10 ml sterile water to the plate and gently scraping the surface with a plate spreader. The conidial suspension was then filtered through sterile glass wool and washed with sterile water following two runs of centrifugation at 1741×g or 3000 rpm for three min. The concentration of conidia was then adjusted to 1×10^6 conidia per ml using a hemocytometer. For foliar inoculations, 0.01% Tween-20 was added to the spore suspensions. Aliquots of 10 µl of the conidial suspension were used for infection of the 3rd

leaf of each plant at V3 growth stage indicated by the total number of nodes that develop (3)(108). Prior to inoculation, these leaves were tapped on moist sterile paper to ensure high relative humidity. After inoculation, all plants were covered for 18 h and kept in the dark at 27±2°C (48). The leaf lesion areas were assessed 3 days after infection (54).

Inoculation of *R. irregularis* and *P. chlororaphis* 30-84.

The beneficial microbes utilized in this study were an AMF commercial product (*R. irregularis*) and a spontaneous rifampicin-resistant derivative of *P. chlororaphis* 30-84. For AMF inoculation, 100 µl of Myke® Pro Potato L (Premier Tech, Québec, Canada), which contains 10,500 viable spores/ml of *R. irregularis* was injected at a determined time according to each experimental conduct.

For the bacterial inoculation, a single colony of *P. chlororaphis* 30-84, which contains GFP expression plasmid pGT2-PsP*gfp* was grown in 25 ml of LB medium at 27°C with rapid agitation. After 12 h, cells were collect by centrifugation at 3095 × g (4000 rpm) for 15 min, and bacterial inoculum was normalized to OD_{620} of 0.4 (ca. 10^8 - 10^9 CFU/ml) with phosphate buffered saline (PBS, pH 7.4). Afterward, 2.5 ml of bacterial inoculum was applied directly to the plant growth medium.

Water and fertilizer regime

Plants were watered with 30 ml every other day. One gram of slow release rock phosphate [2-3% of hydroxyapatite (HA), $Ca_{10}(PO_4)_6(OH)_2$] was supplied to 100 g of the mixed substrate prior to plant. Rock phosphate formula was 0-3-0. N and K were provided by the ½ strength Hoagland solution 0.5N (Appendix 2).

Assessment of *R. irregularis* and *P. chlororaphis* 3084 effects on ALB

Experimental units consisted initially of 5 plants per treatments grown as previously described. Treatments consisted of plants with or without inoculation of *R. irregularis* DAOM 191798, *P. chlororaphis* 30-84 and *C. graminicola* strain M1.001 as outlined in Table 3. The control treatment consisted of plants only inoculated with the pathogen. Plants receiving the biocontrol agents were preinoculated at the age of 3 weeks (t_0) with at least one of the beneficial microbes and infected three weeks later (t_3 equivalent to V3 stage) with the pathogen.

Table 3: Treatments of the evaluation of Ri and 30-84 antagonism to *C. graminicola*

BCA inoculation	**Pathogen application 3 weeks later after BCA inoculation**
Control: No Inoculation	*C. graminicola* strain M1.001 applied on the 3rd leaf
Ri	
30-84 Strains (Gac^- and Phz^- mutant and the 30-84)	
Co-inoculation: Rix30-84	

Three days after *C. graminicola* strain M1.001 inoculation, inoculated leaves were removed, and lesion areas scanned and measured using the ImageJ 1.50i software (109). Statistical differences were inferred via non-parametric test since conditions of ANOVA were violated. Induced resistance and disease severity were also qualitatively scored using a modified version of da Costa et al., (2014) grading scale below (54). Immediately after lesion area scanning, all leaves were treated in liquid nitrogen and kept in -80℃ before ergosterol being measured by LC/MS as described in Appendix 3. Subsequently, I calculated Disease incidence (DI %) per maize line and treatment according to this formula:

$\mathrm{DI} = 100 \times \frac{\text{Number of necrotic leaves}}{\text{Total number of inoculated leaves}}$. Mycorrhizal Inoculation Effect (MIE %) was also calculated per maize line using this formula:

$$\mathrm{MIE} = 100 \times \frac{\text{Mean lesion area of Ri\_inoculated plants} - \text{Mean lesion area of control plants}}{\text{Mean lesion area of control plants}}$$

Bacterial Inoculation Effect (BIE %) was calculated per maize line using this formula:

$\mathrm{BIE} = 100 \times \frac{\text{Mean lesion area of 3084\_inoculated plants} - \text{Mean lesion area of control plants}}{\text{Mean lesion area of control plants}}$. Finally, Dual Inoculation Effect (DIE %) was calculated per maize line using this formula:

$$\mathrm{DIE} = 100 \times \frac{\text{Mean lesion area of Rix3084\_inoculated plants} - \text{Mean lesion area of control plants}}{\text{Mean lesion area of control plants}}$$

Assessment of plant growth promotion and root colonization

Growth measurements were made seven weeks after sowing and included fresh weights of shoot and root, dry weight of shoot, height, and counting of green leaves. Data were subjected to non-parametric tests. Root colonization was measured seven weeks after sowing.

R. irregularis root colonization was verified using following the staining protocol of Hayman and Phillip (1970) and the gridline intersect method (103, 110, 111). Briefly, roots were washed to remove adhering soil particles. Then, cortical cell cytoplasmic content was cleared by 10% KOH solution heated in a water bath at 90C during 2h. After rinsing to remove KOH solution, roots were stained in 0.05% Trypan solution during 30 - 60 min in water bath at 90°C. I then, performed the gridline intersect method (103, 111), in which roots were randomly distributed in an 8.5 cm diameter Petri plate with an 11/14 cm (approx. 1/2 inch) grid lines. I scanned along these grid lines with a compound light microscope to quantify intersections between grid lines and roots — which were designated as either colonized or non-mycorrhizal. Total count of intersection between a fragment and a grid line at which there was any mycorrhizal structure was divided by the total count of lines crossed by any root fragment. Data were subjected to nonparametric tests to compare mean values between treatments.

30-84 root colonization was assessed by determining the CFU/g of root. Briefly, after roots were gently washed to remove soil, they were left to dry briefly on a sterile paper towel and weighted. I then immersed them then in 40 ml of PBS (pH7.4) and removed bacteria from roots by alternating vortexing (10 seconds) and sonicating (10 seconds) three times. Finally, I spread 100µl of 10000-fold dilution on LB plates containing rifampicin (100 µg/ml) and cycloheximide (50 µg/ml). Plates were incubated at 27 °C. After two days, colony forming units (CFU) were counted and standardized per gram of fresh root (5, 6).

Interaction between *R. irregularis* & *P. chlororaphis* 30-84 in split root system

In this objective, I focused on the interaction between the biocontrol agents (BAs) in the absence of the *C. graminicola* strain M1.001. Experimental units consisted of 1 week-old seedlings in which the root system was divided for growing in two pots. In this split root system, one part of the split root was preinoculated with one of the two beneficial organisms and the second part (in a separate pot) was inoculated three weeks later or at the same time by the other one. Four plants were used for treatment outlined in Table 4 and in which I measured 30-84 CFU/g of root and *R. irregularis* root length colonization six weeks after sowing (103, 5-6). Differences in treatments effects were analyzed via non-parametric tests

Table 4: Treatments of the assessment of Ri and 30-84 interaction in split pot design

Treatment on each root side		Symbol of dual treatments
First Treatment on root side A (15 DAS)	Second Treatment on root side B (30 DAS)	
No Inoculation	No Inoculation	No/No
No Inoculation	*R. irregularis*	No/Ri
No Inoculation	*P. chlororaphis 30-84*	No/30-84
R. irregularis	*P. chlororaphis 30-84*	Ri/30-84
P. chlororaphis 30-84	*R. irregularis*	30-84/Ri
No Inoculation	*R. irregularis* & *P. chlororaphis 30-84* in same pots	No/Rix30-84

CHAPTER III

ARBUSCULAR MYCORRHIZAL INTERACTIONS WITH TOMATO AND EFFECTS ON ZEBRA CHIP DISEASE DEVELOPMENT AND PSYLLIDS FITNESS

INTRODUCTION

Zebra Chip disease (ZC) is a substantial economic threat to the potato and tomato industries in the US, Mexico, Guatemala, Honduras and New Zealand. In potato, it affects fresh market produce as well as that destined for specialty markets such as chip and fry production. The disease is caused by '*Candidatus* Liberibacter solanacearum' (CLso), a non-culturable Gram-negative bacteria belonging to the class α-proteobacteria and the family Phyllobacteriaceae. In plants, it is phloem-limited, has a rod-shaped morphology and is about 0.2 µm wide and 4 µm long. It is heat-sensitive to temperatures above 32°C. Temperatures at or below 17°C may significantly slow the infection in potato (112).

CLso is closely related to four other '*Candidatus* Liberibacter' species: '*Ca*. L. asiaticus' (CLas), '*Ca*. L. africanus' (CLaf) and '*Ca*. L. americanus' (CLam), which are responsible for citrus 'greening' Huanglongbing (HLB), and '*Ca*. L. europaeus' a non-pathogen in pears (113). CLso and CLas are among the most important emerging major plant pathogens in the United States. All of these pathogens are vectored and transmitted solely by insects from the Hemiptera - Triozidae clade (114–118).

The Hemiptera clade includes several important phloem-feeding pest insects, which host various endosymbionts on which they depend for essential amino acids. This dependency often results in co-speciation (115). Vectors of CLso include the potato/tomato

psyllid *Bactericera cockerelli* (Sulç), the carrot psyllid *Trioza apicalis* (Förster) and *Bactericera trigonica* (Hodkinson). *B. cockerelli* has at least three biotypes in North America that differ in mating and ecology (116).

CLso exists in at least five different haplotypes (LsoA to LsoE), which currently are delineated by the host and geographic ranges. Haplotypes are revealed by PCR using simple sequence repeat (SSR) markers (LSO- SSR- 1F and LSO- SSR- 1R primers). The haplotypes are defined according to SNPs (Single Nucleotide Polymorphism) on the 16s rRNA, 16s/23s ISR (intergenic spacer region) and 50s rplJ and rplL ribosomal protein genes. These SNPs are inherited together (119). LsoA and LsoB infect Solanaceous crops. They were detected in the Americas and New Zealand (119, 120). LsoA and LsoB have numerous genomic rearrangements (inversions and relocations) and numerous SNPs distinctions. There also are differences in phage-related regions, including the locations and sequences of various prophages. Moreover, bioinformatics analyses revealed 46 putative genes in LsoA that are lacking in LsoB (120). LsoC and LsoD were identified from carrots, but LsoC was discovered in Finland, Sweden and Norway whereas LsoD was from Spain and the Canary Islands. Both are harbored by the psyllid *B. trigonica* (119, 120). LsoE was identified in celery and carrot in Spain, after inspecting fractional 16S and 50S rRNA genes sequences (121).

Currently, the most widely used approach to manage ZC disease is early and repeated application of insecticides (116, 117, 122). Potential control of ZC also exists by using isolates of *Streptomyces* producing chitinases and antibiotics (123), streptomycin sulfate injections, SAR compounds, and nutrient supplements, but heavy insecticide application remains the preferred treatment (124–126). However, the consequences of the existing

methods are the development of insecticide resistance in psyllid populations (127) and antibiotic-resistance in CLso. To date, efforts to select natural resistant plant cultivars have not been successful, but have identified differences in susceptibility; e.g. cultivars with some degree of tolerance have been identified. (122).

A potential alternative control may be the use of beneficial microbes capable of promoting the plant's growth and natural resistance mechanisms for tolerating biotic and abiotic stress. Fungi capable of forming arbuscular mycorrhizas with 90% of vascular plants (128–130) are good candidates. AMF are known to improve plant biomass, nutrient content, alter the source-sink nutrient distribution pattern within plants, and modulate plant defenses, all of which may influence the suitability of foliar tissues to attackers (37, 60–63, 131, 132). Mycorrhization primes the jasmonic acid-dependent resistance responses of plants to herbivorous insects and pathogens. For example, mycorrhization by *Rhizophagus irregularis* (formerly *Glomus intraradices*) DAOM 197198 significantly reduced herbivory by chewing insect, whereas mycorrhization by *Glomus fasciculatum* and *Funneliformis mosseae* (*G. mosseae*) negatively affected chewing and sucking insects (60, 62, 133–135). However, little is known regarding the effects of AM on phloem-feeding insects on tomato and potato, especially psyllids harboring phloem-limited pathogenic bacteria such as CLso.

In this study, I used tomato as the host to examine the effects of *R. irregularis* (hereafter Ri) on this pathosystem. Although disease symptoms are less pronounced in tomato than potato, this host was used because it grows more rapidly than potato. The first aim of the study was to assess growth promotion by AMF *R. irregularis* DAOM 197198 on tomato variety Moneymaker, an open-pollinated, "heirloom" variety. I also examined the

effect of mycorrhization on aspects of tomato-psyllid-pathogen interactions, including ZC symptom development as well as psyllid oviposition and survival of larvae. Aboveground symptoms of ZC on tomato include distorted growth (especially shortened internodes or stunting), leaf symptoms (purpling of leaves or midveins, curling), wilt (yellowing, interveinal chlorosis and vein greening, mottled or chlorotic leaves and plant collapse), and in some cases death (116, 117). Fruit occasionally will be misshapen with a strawberry-like appearance. The leaf axil or stalk also may become long, and fruit development may be uneven (112). Oviposition is the process of laying or depositing eggs by the female insects. Oviposition and larval survival relate to insect fitness and reproduction and thus to disease epidemiology. I used a factorial design to compare tomato plants treated with or without *R. irregularis* and with or without psyllids harboring CLso. Experiments were replicated four times, twice with insects harboring LsoA and twice with insects harboring LsoB. I hypothesized that the formation of Arbuscular Mycorrhizae (AM) would promote plant growth (height and number of green leaves) and biomass (fresh and dry shoot weight), and delay the appearance of wilt symptoms, which in turn would reduce disease severity. Moreover, I hypothesized that compared to non-mycorrhizal plants, mycorrhization would delay the detection of CLso (via PCR-based methods) in newly formed leaves (typically observed around 6-8 weeks after infestation), a correlative measure of the bacteria's ability to reproduce and move in the plant. These expectations are based on observations that preinoculation of plants with *Funneliformis mosseae* BEG12 and *Pseudomonas putida* S1Pf1Rif led to a reduction in disease severity that was associated with a decrease in the titer of the Chrysanthemum yellows phytoplasma. This phloem-limited, obligate parasite is transmitted by the pestiferous leafhopper *Macrosteles quadripunctulatus* (Hemiptera:

Cicadellidae) (45). Furthermore, AM formation on grafted tomato and pear resulted in the reduction of disease symptoms caused by a phytoplasma (132). It was suggested that mycorrhization could elicit an ISR-type response capable of conferring resistance to this type of pathogens (45, 132). I also hypothesized that mycorrhization may decrease the number of eggs laid and the survival of larvae on leaves. I based this expectation on the fact that mycorrhizal tomatoes were shown previously to accumulate antifeedant volatiles accompanied by the transcriptional up-regulation of defense-related genes that impair herbivory directly (via larvae and adults feeding performance) and indirectly via oviposition (i.e. number of eggs laid on leaves) (132, 136).

RESULTS

Four combinations of Ri and psyllid treatments were used in this study: plants with and without Ri pre-inoculation and with and without psyllid infestation. Plants receiving *R. irregularis* (hereafter Ri) treatment were inoculated at approximately three weeks after sowing. Ri-treated and untreated plants receiving psyllids were infested six weeks after sowing (e.g. three weeks after mycorrhization). All psyllids harbored LsoA or LsoB, e.g. the experiment was repeated twice with psyllids harboring LsoA and twice with psyllids harboring B. Infestation included the application of 3 male and 3 female adult psyllids to a single leaf per plant, and these were removed two days after infestation (DAI). Development of ZC symptoms (especially wilt) on leaves was surveilled. Mycorrhization was evaluated at the end of the experiment.

The first step was to evaluate Ri root colonization (Figure 9). Previous studies suggest that good colonization by Ri is considered to be 30-40% of the root length (93, 94). I found that root length colonization by *R. irregularis* ranged from 69 to 74% in all experiments. In plants pre-treated with *R. irregularis*, there was no significant difference in the rate of mycorrhization between plants with or without infestation by CLso-infected psyllids. These data indicate ZC stress did not affect the rate of mycorrhization by *R. irregularis* on tomato cultivar Moneymaker.

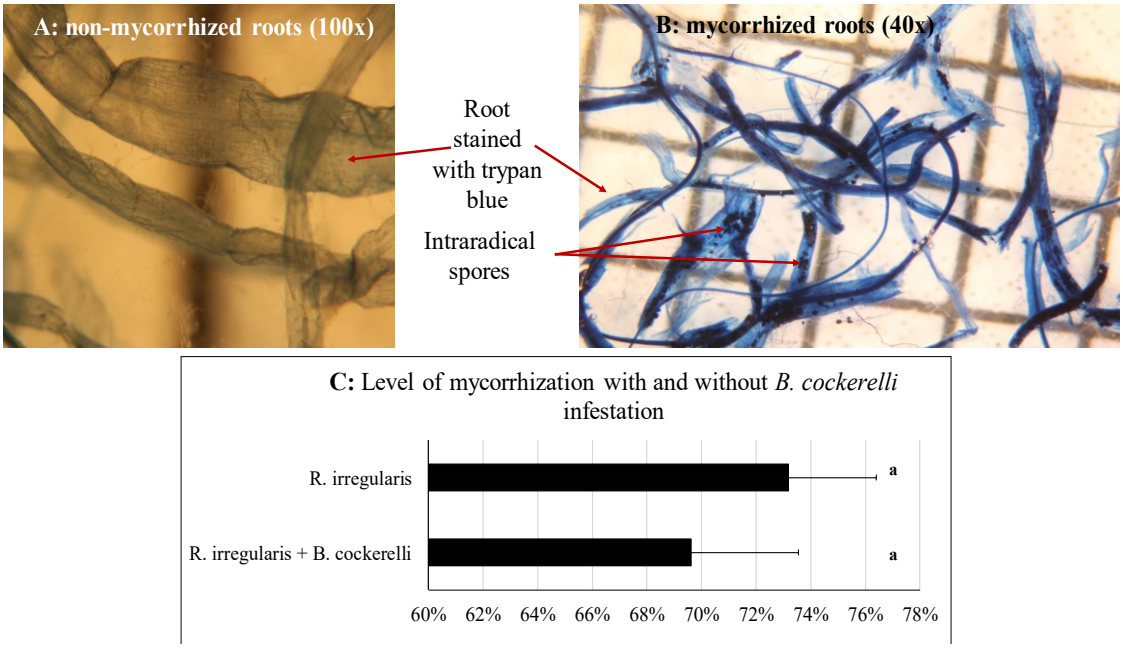


Figure 9: Moneymaker mycorrhization rate with and without *B. cockerelli* infestation
Two weeks old plants were inoculated with *R. irregularis* and three weeks later leaf-infested with three couples of adult male and female of *B. cockerelli* harboring CLso. The experiment was replicated twice with insects harboring each haplotype, and the pooled results from all experiments are presented. Root was collected and checked for mycorrhization eight weeks after infestation. Bars are mean of 10 measures ± S.E. A: non-mycorrhized roots, B: mycorrhized roots, C: Level of mycorrhization with and without *B. cockerelli* infestation.

R. irregularis delays ZC symptom development

I scored the severity of foliar symptom twice weekly following infestation according to a 0 to 4 scale, where a score of 1 indicated slight curling of the freshly emerged leaves and a score of 4 was given to a plant showing extreme stunting, wilting or yellowing of all leaves (Table 5). Disease symptoms were observed only on psyllids-infested plants. Regardless of CLso haplotype, distorted growth (especially shortened internodes or stunting) was visible on both mycorrhized and non-mycorrhized plants at six weeks after infestation (WAI). However, wilt symptoms were first observed on non-mycorrhized plants 6 WAI (Figure 10A,

Table 5). Some mycorrhized plants showed wilt symptoms after 7WAI (Figure 10B, Table 5). In general, mycorrhized plants received lower disease scores compared to non-mycorrhized plants, which achieved the highest scores on the scale during the 6-9 WAI observation period (Table 5). After 9WAI, the experiment was terminated because the cages limited further growth of the plants. These data indicate that mycorrhization delayed and reduced the development of symptoms of ZC and psyllid yellowing due to both CLso and *B. cockerelli* (Figure 10).

Table 5: Scoring of ZC symptoms on tomato cultivar Moneymaker

Treatments	Score at 6 -7 WAI	Symptom type	Score at 8-9 WAI	Symptom type
Control	0	No ZC symptom	0	No ZC symptom
R. irregularis	0	No ZC symptom	0	No ZC symptom
B. cockerelli	3.5	Accentuated stunting, yellowing, interveinal chlorosis. Presence of vein greening mottled or chlorotic leaves	4	Extreme stunting and extreme scorching, wilt, yellowing or interveinal chlorosis. Mottled or chlorotic leaves. Plant collapse and death of the plant.
R. irregularis + *B. cockerelli*	2	Mild stunting of plant, or purpling of leaves	2.5 - 3	Mild to Accentuated stunting, yellowing, interveinal chlorosis. Presence of vein greening mottled or chlorotic leaves

Plants were treated with or without *R. irregularis* and CLso-infected *B. cockerelli,* and symptoms scored at 6-7 weeks after infestation (WAI) and 8-9 WAI. Results are pooled across experiments using psyllids vectoring LsoA or LsoB.

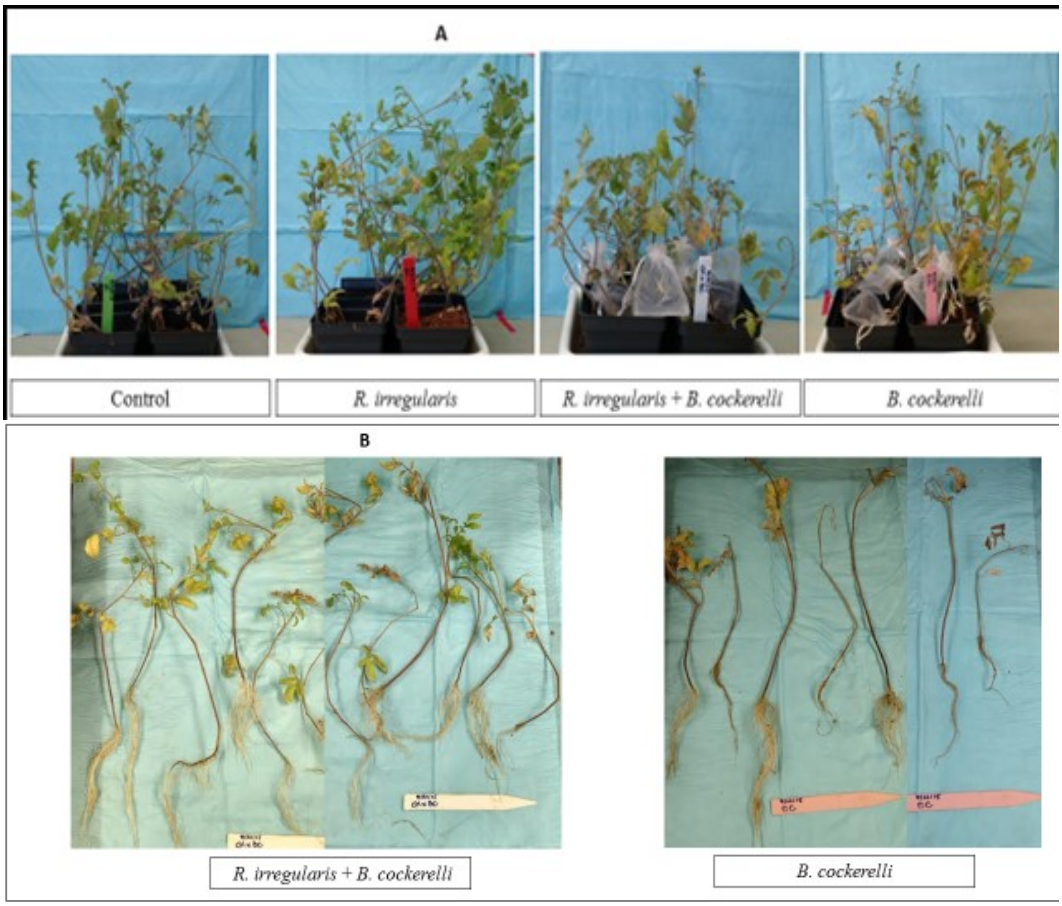


Figure 10: ZC symptom development in plants with and without *R. irregularis*
Two-week-old plants were inoculated with *R. irregularis* and three weeks later leaf-infested with three couples of adult male and female of *B. cockerelli* harboring CLso. The psyllids were removed two days later. A) 6 Weeks after Infestation (WAI) and B) 8 WAI. Data are from one representative experiment using psyllids harboring LsoB.

In addition to measuring the rate and severity of disease development, disease incidence was measured at 6 WAI. Disease incidence (DI %) was calculated according to this formula: $\mathrm{DI} = 100 \times \frac{\text{Number of diseased plants}}{\text{Total number of plants}}$. DI was 100% on plants without Ri pre-inoculation infested with psyllids and 46.15% in mycorrhized plants treated with psyllids. Mycorrhizal Inoculation Effect (MIE %) on ZC development was calculated at 6 WAI as: $\mathrm{MIE} = 100 \times ((\text{Nb of diseased plants treated with } R.\ irregularis \,\&\, B.\ cockerelli) -$

(Nb of diseased plants treated only with $B.\ cockerelli$))/

(Nb of diseased plants treated only with $B.\ cockerelli$).

Negative values of MIE indicate Ri mycorrhization reduced the occurrence of ZC wilt. Positive values of MIE show a putative promoting effect. Here, MIE = -33.33%. Mycorrhization caused a reduction in disease incidence compared to unprotected plants infested with *B. cockerelli*.

R. irregularis **mycorrhization does not prevent translocation of CLso**

Detection of CLso in leaves (other than the one treated with insects) is a useful measure of the ability of the pathogen to replicate and spread within the plant. Previous work demonstrated that newly formed leaves are good "sinks" for substances including pathogens translocated in the phloem (116). I was interested in whether Ri pre-treatment could reduce pathogen infection and spread in the plant. At intervals of three, five, six and seven weeks after psyllids application, newly formed leaves were collected for PCR-based pathogen detection. Conventional PCR using genomic DNA extracted from leaves and LsoTx16/23 primers were utilized for CLso detection. These analyses detected CLso in some new leaves 3 WAI in both Ri-treated and untreated plants and most Ri-treated and unprotected plants 6 WAI (Figure 11C, D). The ability to amplify tomato β-tubulin (data not shown) and elongation factor in leaf samples indicated that the extraction conditions for most samples were successful (Figure 11A). Analysis of the insects revealed nearly all insects harbored detectable amounts of CLso (Figure 11B).

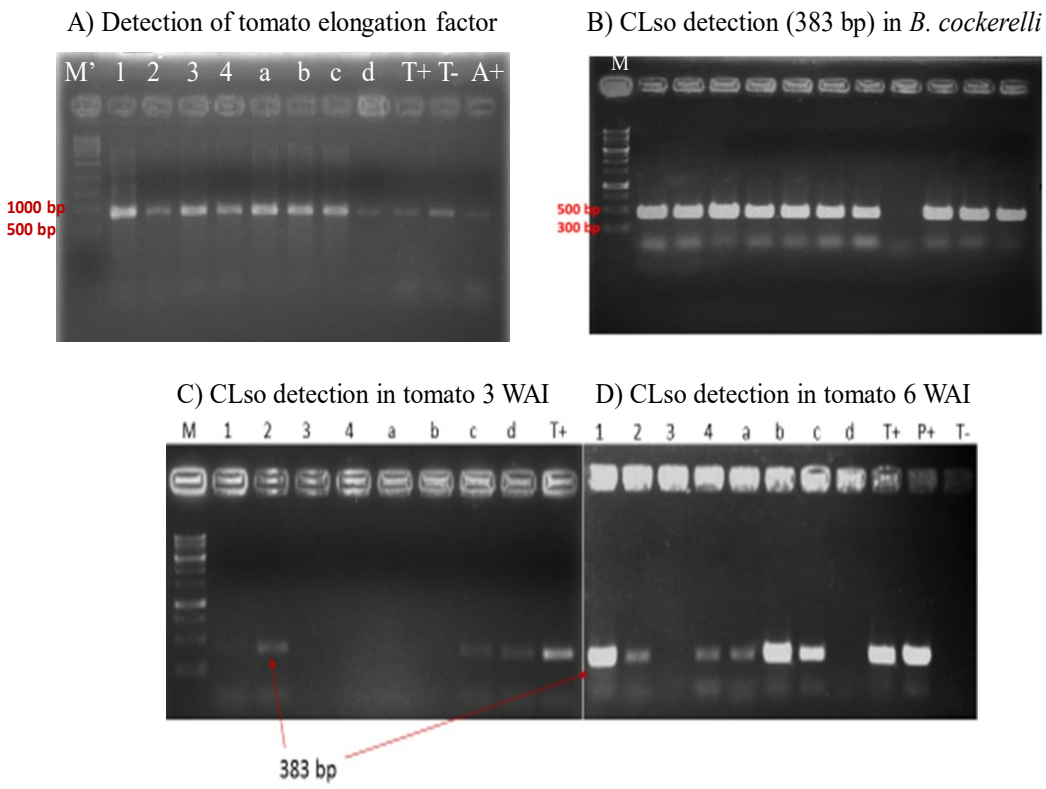


Figure 11: Detection of CLso in newly formed leaves of mycorrhized and non-mycorrhized tomato plants

A) Detection of tomato elongation factor 1α (900 bp) at 6 WAI. B) CLso detection (383 bp) in *B. cockerelli.* C) CLso detection in tomato 3 WAI, D) CLso detection in tomato 6 WAI. M and M'= 1kb ladder DNA marker (300 – 10 000 bp and 0.5 – 10 kb respectively, markers at 300, 500, 700 or 1000 are highlighted as references). 1 to 4 = DNA of mycorrhized plants, a to d = DNA of non-mycorrhized plants, T+=DNA of CLso infected tomato, T-=DNA of CLso tomato, Atl+ = DNA of CLso-infected Atlantic potato, W = Water (negative control). P+=DNA from CLso infected psyllid. WAI = Weeks After Infestation. Data are for four randomly selected plants from one experiment.

R. irregularis mycorrhization does not effect *B. cockerelli* ovipositioning

I also was interested in whether mycorrhization by Ri could reduce the ovipositioning of the CLso vector *B. cockerelli*. I compared the number of eggs laid by psyllids on leaves of plants treated with or without Ri. This assessment was repeated twice for each CLso haplotype. The findings consistently showed that Ri mycorrhization did not impair *B.*

cockerelli oviposition since there was no significant difference in the number of eggs laid on mycorrhized plants compared to non-mycorrhized plants (Figure 12).

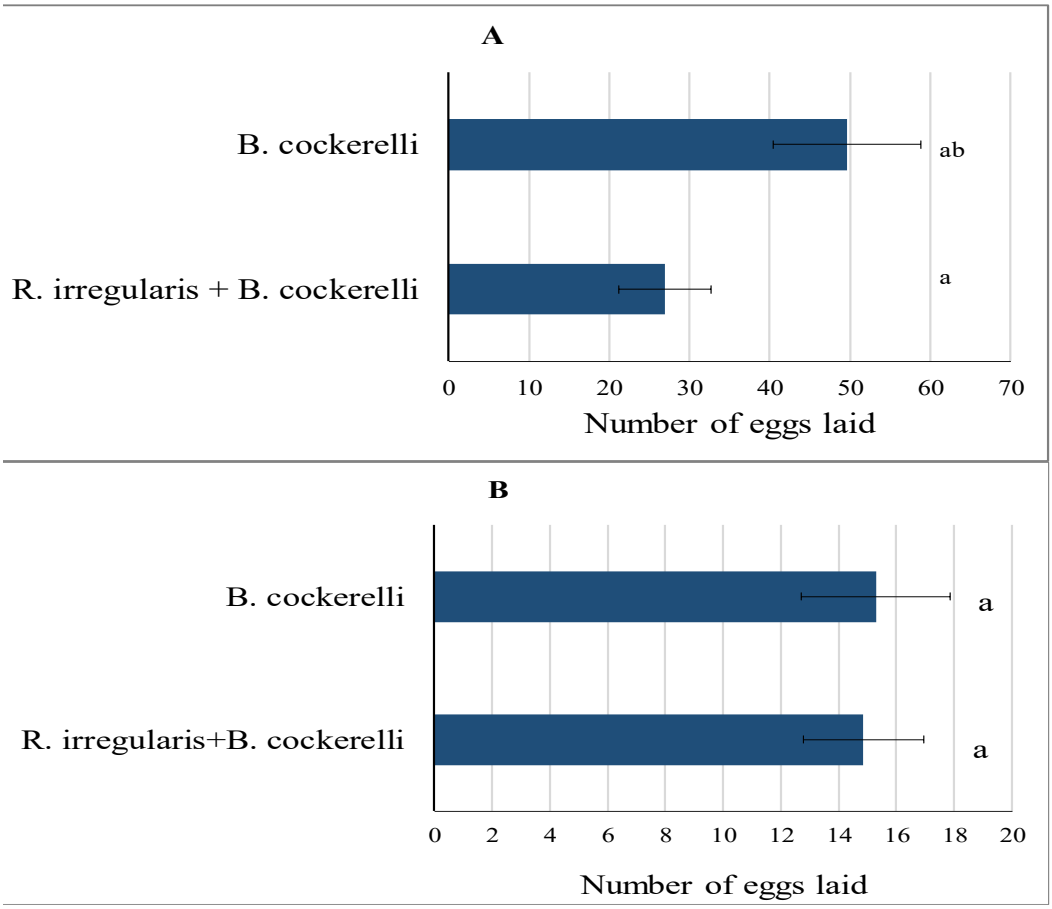


Figure 12: Influence of mycorrhization on *B. cockerelli* oviposition
A) Psyllids vectoring LsoA being vectored, B) Psyllids vectoring LsoB. Two-week old plants were inoculated with *R. irregularis* and three weeks later leaf-infested with three couples of adult male and female *B. cockerelli*. The psyllids were removed two days later, and number of eggs laid were counted. Bars are means of counts on six replicate leaves ± S.E in A and ten replicate leaves B. Levels with same letters are not statistically difference.

Effects of *R. irregularis* mycorrhization on larval survival of *B. cockerelli*

I also was interested in the effects of *R. irregularis* mycorrhization on larval survival, since larval feeding is an important mechanism for CLso acquisition and transmission as well as for causing symptoms of psyllid yellows. I found that mycorrhization by Ri reduced the survival of larvae from *B. cockerelli* parents harboring LsoA, but slightly promoted survival of larvae from *B. cockerelli* parents harboring LsoB. The number of living larvae was counted one week after removing the adult psyllids from the leaves and every two days thereafter. Compared to plants without AMF treatment, larval survival from LsoA vectoring parents was significantly less on mycorrhized plants (Figure 13A). The two-sample t-test gave a p-value of 0.002 at the threshold of significance $\alpha = 5\%$. Surprisingly, survival of larvae from parents harboring LsoB, was slightly greater on mycorrhized plants than non-mycorrhized plants (Figure 13B). The two-samples t-test gave a p-value of 0.03 at the threshold of significance $\alpha = 5\%$.

I also found consistently with Yao et al., (2016) on unprotected tomato plants (cv Moneymaker) that LsoB negatively impacted the numbers of eggs laid (Figure 12), the number of eggs hatched (data not shown), and larval survival (Figure 13) as compared to LsoA (137).

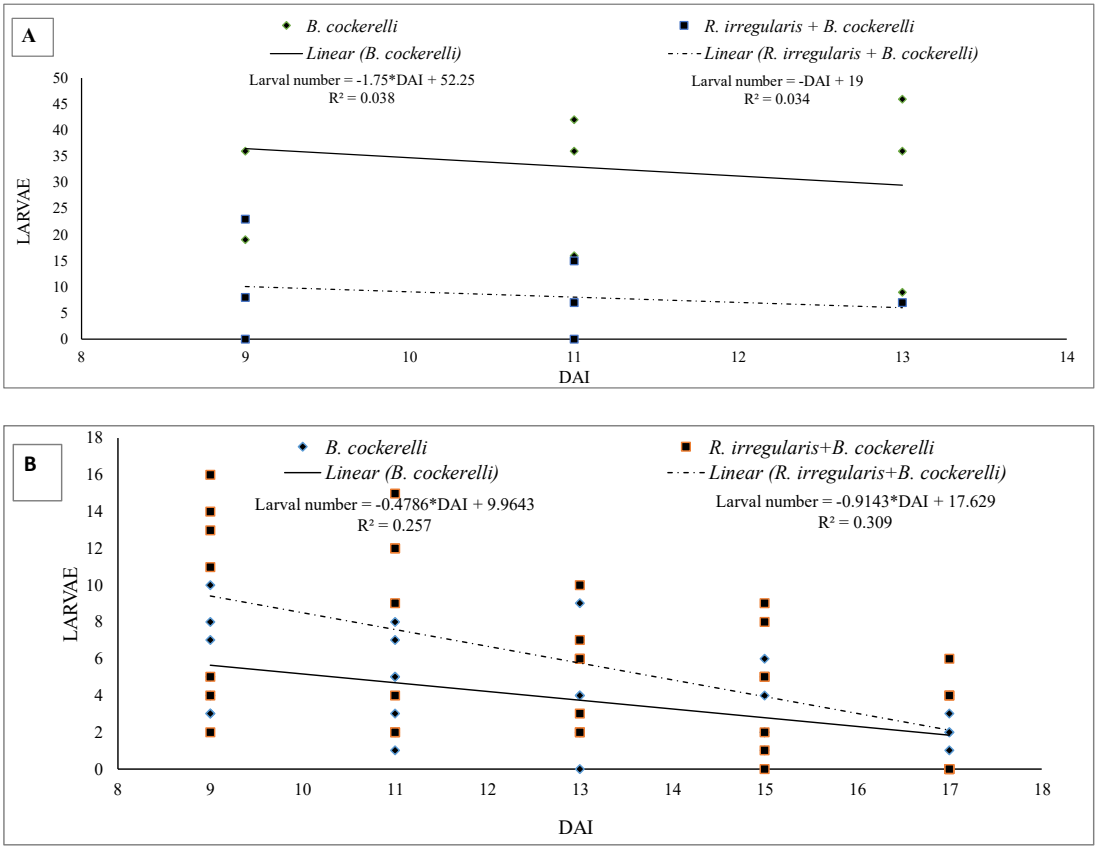


Figure 13: Survival of larvae harboring different CLso haplotype on plants with and without mycorrhization.
A) Larvae from parents Harboring LsoA, **B**) Larvae from parents vectoring LsoB. Two week old plants were inoculated with *R. irregularis* and three weeks later leaf-infested with three couples of adult male and female of *B. cockerelli*. The psyllids were removed two days later, and number of larvae were counted every two days from 9th DAI.

Effect of *R. irregularis* mycorrhization on tomato growth

R. irregularis mycorrhization is capable of promoting plant growth. I used the number of green leaves after eight WAI, height, and root and shoot biomass as measures growth. However, *under my experimental conditions,* I found no significant differences among Ri-inoculated and non-inoculated control plants in fresh root biomass, elongation, and number of green leaves compared to the control. (Figure 14A, C, D). Only fresh shoot

biomass was significantly increased in mycorrhized plants compared to the control (Figure 14B).

I also compared the effects of mycorrhization on plants that had been infected with CLso. Regardless of CLso haplotype, all growth parameters for mycorrhized and non-mycorrhized plants were not significantly different (Figure 14A, B, C, D), indicating that mycorrhization did not enhance the growth of CLso infected plants.

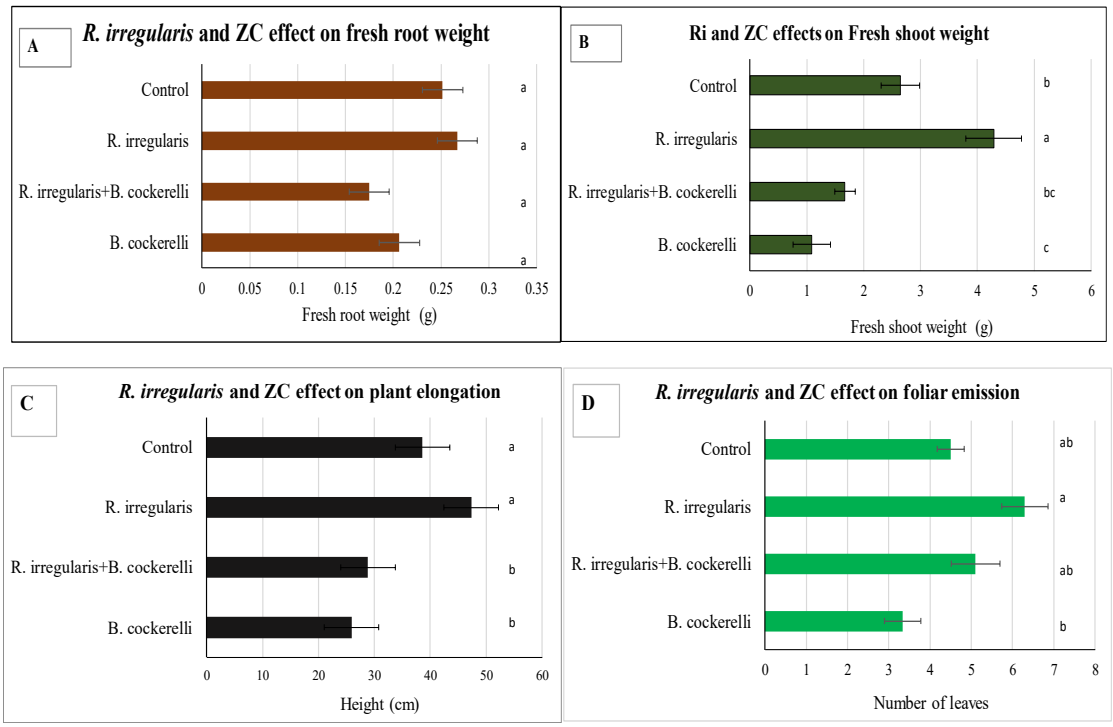


Figure 14: Effect of *R. irregularis* and CLso infection on tomato growth
A, B, C, D Effect on fresh root weight, fresh shoot weight, height, and number of leaves, respectively. Two weeks old plants were inoculated with *R. irregularis* and three weeks later leaf-infested with three couples of adult male and female of *B. cockerelli*. The psyllids were removed two days later, and final growth measures were taken after 8 WAI.

DISCUSSION

Mycorrhization distinctly delayed and reduced disease severity and incidence compared to non-mycorrhized plants. The measurements of plant growth parameters indicating symptoms of psyllid yellowing and ZC disease, the counting of eggs laid on leaves, the assessment of larval survival and the detection of CLso DNA in plants at 3 and 6 WAI served to discriminate the mycorrhizal effect on tomato under the attack of potato psyllids harboring CLso. Differences in symptoms, especially wilt, which is the main aboveground symptom of ZC on tomato, were clearly visible between 6 to 9 WAI. Moreover, mycorrhization was effective against both LsoA and LsoB haplotypes. It has been reported that LsoA-infected tomato displayed reduced size compared to control plants, whereas LsoB-infected plants displayed dramatic symptoms leading to plant death as time progressed (Tamborindeguy unpublished). Additionally, Lso density in LsoB-infected tomato plants increased, whereas Lso density decreased in LsoA-infected plants to undetectable levels by week 9 (Tamborindeguy unpublished). Both Lso haplotypes kill potato plants. However, milder ZC symptoms develop on LsoA infected plants (Tamborindeguy unpublished). In my study, I looked at the effect of mycorrhization on disease development due to infestation by psyllids harboring LsoA and LsoB in *separate experiments*, so I was not able to compare differences in disease severity due to haplotype directly. Moreover, I terminated my experiments at 8 weeks due to cage constraints, so I did not observe disease progression into flowering. However, the most consistent finding was the large difference in symptom development between mycorrhizal and non-mycorrhizal plants, regardless of CLso haplotype. The results suggest that mycorrhization by *R. irregularis* is triggering MIR against

ZC symptoms caused by CLso. However, my results suggest this resistance is not due to prevention of pathogen movement or replication as I was able to detect CLso in the newly developing leaves of both mycorrhized and non-mycorrhized plants at about the same time. Previous observations with susceptible and ZC tolerant potato varieties similarly showed no correlation between symptom development and pathogen movement or titer (116). As expected, mycorrhization promoted shoot biomass production in non-infested plants. That is clearly the primary impact of mycorrhization (128–130). However, there was no benefit to plant growth for plants that had been infected with CLso. This lack of growth promotion in infected plants may indicate a trade-off in benefits conferred by the mycorrhization between growth promotion and resistance to disease.

MIR seemed to be more efficient against CLso than the vector since mycorrhization did not significantly antagonize the vector oviposition behavior of insects harboring either LsoA or LsoB. I did not check the effect of mycorrhization on the psyllid feeding performance since numerous authors have already documented feeding is positively correlated with mycorrhization (37, 39, 60, 62, 132, 136). These findings are consistent with previous results that show that the JA signaling dependent defense, which is the main mechanism triggered by MIR against herbivores, is known to be ineffective in reducing psyllid oviposition behavior as well as damage caused by phloem feeding (37, 39, 49, 50, 62, 63, 132, 136). However, I found a negative impact of arbuscular mycorrhization on the survival of larvae from parents vectoring LsoA at least one WAI. These results suggest that larval feeding for as little as one week is sufficient to trigger MIR. In contrast, I found that mycorrhization improved slightly the survival of larvae from parents vectoring LsoB.

I do not know the mechanism underlining the effects of MIR against CLso or the survival of larvae from parents vectoring LsoA. I hypothesize that larvae might be directly affected by MIR induced production of VOCs, phytoalexins, DIMBOA or other defense related molecules produced by plants in response to insect herbivory (138). In the case of LsoB, I do not know what contributes to the slight improvement in larval resistance to MIR.

My results constitute the first report of MIR against ZC disease. My results reinforce the idea that mycorrhization confers resistance to phloem-limited pathogens (45, 132). Future experiments under greenhouse conditions and ultimately in the field will be required to determine whether mycorrhization can protect plants sufficiently to improve marketable yield and to dissect whether there are differences in the outcome of yield trials due to different Lso haplotypes.

CONCLUSIONS

This is the first report of mycorrhization antagonistic effect against ZC. Mycorrhizal interaction appeared as a potential approach for ZC management. MIR clearly induced delays and reductions in ZC symptom development, although the effects on the vector were less pronounced and dependent on insect life stage and CLso haplotype that was harbored by the insect. However, further investigations are needed to enlighten the mechanisms involved in MIR. Field experiments are required to confirm the MIR and potential for agricultural applications to control ZC on tomato and potato, with particular attention to effects on marketable yield.

Future work utilizing metabolomics profiling could be used to reveal differences between mycorrhizal and non-mycorrhizal plants regarding the composition of secondary metabolites and chemical defense systems elicited in response to *B. cockerelli* feeding and CLso infection. I plan to repeat these aforementioned works with 30-84 since my previous work demonstrated its ability to promote ISR. Moreover, like *P. putida,* a close relative shown to suppress disease symptoms caused phloem-limited phytoplasma, it produces auxin, aided in the recovery from phytoplasma infection (45, 139).

MATERIALS AND METHODS

Plant and soil material

I used a ZC susceptible tomato variety “moneymaker”. Seeds were surface sterilized in diluted commercial bleach (3%) for 30 min and rinsed twice with sterilized water. Surface sterilized seeds were pregerminated on water agar for 4-5 days. Pregerminated seeds were transferred to 4 inches pots filled with an autoclaved mix of turface and sand (1:1, v:v), and were placed on light shelves with dark-and-light cycles of 8h and 16 h at room temperature. To avoid cross infestation by psyllids, plants were always grown in mesh-cages .

Insect materials

B. cockerelli harboring LsoA or LsoB were obtained from Dr. Cecelia Tamborindeguy (Department of Entomology, Texas A&M University). To ensure that the psyllid colonies harbored CLso, I performed PCR as describe below (Figure 11). Psyllid DNA was extracted by grinding two psyllids per Eppendorf tube in 75 µl of sterile water. To validate the PCR, I amplified the 28S rDNA gene using *B. cockerelli* genomic DNA as a template and the primer set D2BC F/ D2BC R (GCGAGGACTCAGTTTCGTGT / AGAGCTCGACTCGGATTGTC) (140). For CLso detection, I used primer set to amplify 383 bp of the 16S-23S rDNA intergenic region (Lso TX 16/23 Forward 5'-AATTTTAGCAAGTTCTAAGGG -3’ and Lso TX 16/23 Reverse 5'-GGTACCTCCCATATCGC -3’) (Figure 11) (141–143). A 15 µl PCR mixture was prepared

with 7.5 µl of the GoTaq Master Mix (Promega Corp. Madison, WI), 1 µl of DNA extract, 1 µl of each primer (10 µM initial concentration), and 4.5 µl of sterilized, deionized water. The following cycling conditions were employed: initial denaturation 95°C for 3 min; 35 cycles of denaturation at 95°C for 40 s, annealing at 55°C for 40 s, extension at 72°C for 1 min; and a final extension for 10 min at 72°C. All PCR reactions were performed in an Eppendorf Thermocycler (Hamburg, Germany).Gel electrophoresis was used to differentiate amplicons. Sterile water and psyllid genomic DNA containing CLso DNA were as well used as negative and positive control respectively. Following confirmation that the colonies contained CLso, six adult insects comprising the same number of females and males were attached to a single leaf inside an "organza-bags" (116) in the middle tier of the shoot system, 3 weeks after AMF inoculation and removed two days after infestation

AMF material and inoculation protocol

The beneficial microbe utilized in this study was an AMF commercial product (*R. irregularis*). 100 µl of Myke® Pro Potato L (Premier Tech, Québec, Canada), which contains 10,500 viable spores/ml of *R. irregularis* was injected into the substrate supporting three weeks old plants growing in 4 inches pots. Plant roots were checked three weeks later to verify the establishment of the mycorrhization by the staining protocol of Hayman and Phillip (144, 145) and the gridline intersect method (110).

Water and fertilizer regime

Every other day, plants were watered with 30 ml. Only once, 100 g of the mixed substrate received 1g of slow-release rock phosphate [2-3% of hydroxyapatite (HA), $Ca_{10}(PO_4)_6(OH)_2$] prior to plant. Rock phosphate formula was 0-3-0. N and K were provided by the ½ strength Hoagland solution 0.5N (Appendix 2).

Treatments of the experiment

In this study, there were four treatments which are combination of Ri and psyllid applications regardless of CLso haplotype (Table 6):

Table 6: Treatments for the evaluation of *R. irregularis* antagonism to ZC disease

	Without *R. irregularis*	**With *R. irregularis***
Without *B. cockerelli*	Control	Treatment ♯1: *R. irregularis*
with CLso-infected *B. cockerelli*	Treatment ♯2: *B. cockerelli*	Treatment ♯3: *R. irregularis* + *B. cockerelli*

Treatment ♯1 and Control plants were preinoculated with and without Ri, respectively, and neither were infested with CLso-infected *B. cockerelli*. Treatment ♯2 consisted of plants receiving 3 couples of female and male adult psyllids six weeks after sowing. Treatment ♯3 was comprised of Ri-preinoculated plants that were infested with psyllids 6 weeks after sowing. Each treatment contained 3 and 6 plants for the first and second repetition of the same experiment.

Assessment of plant growth promotion and root colonization

Growth measurements were performed at 8 WAI and included fresh weights of shoot and root, dry weight of shoot, height, and number of green leaves. Data were subjected to non-parametric tests. Final root length colonization was performed at 8 WAI.

R. irregularis root colonization was checked by following the staining protocol of Hayman and Phillip (144, 145) and the gridline intersect method (103, 110, 111). Briefly, roots were first washed to remove adhering soil particles. Then, cortical cell cytoplasmic content was cleared by 10% KOH solution heated in a water bath at 90°C during 2h. After rinsing to remove KOH solution, roots are stained in 0.05% Trypan solution during 30-60 min in water bath at 90°C. I then, performed the gridline intersect method (103, 111), in which roots are randomly distributed in an 8.5 cm diameter Petri plate with an 11/14 cm (approx. 1/2 inch) grid lines. I scanned along these grid lines with a compound light microscope to quantify intersections between grid lines and roots which were designated as mycorrhized or not. Total count of intersections between a root fragment and a grid line at which there was any mycorrhizal structure was divided by the total count of lines crossed by any root fragment. Data were subjected to nonparametric tests.

Assessment of the *R. irregularis* effect on ZC development

After infestation, I surveiled the ZC symptom development on 2 days per week until the end of 9 WAI. The severity of foliar symptoms was scored twice a week based on the 0 to 4 scale described in Table 7.

Table 7: Grading scale of ZC development symptoms on Moneymaker

Score of foliar ZC symptom severity	Symptom type
0	No symptom
1	Slight curling and/or purpling of leaves
2	Mild stunting of plant, wilting and midveins purpling of leaves
3	Accentuated stunting, yellowing, interveinal chlorosis. Presence of vein greening mottled or chlorotic leaves
4	Extreme stunting and extreme scorching, wilt, yellowing or interveinal chlorosis. Mottled or chlorotic leaves. Plant collapse and death of the plant.

Disease incidence (DI %) was calculated per treatment according to this formula:

$\mathrm{DI} = 100 \times \frac{\text{Number of diseased plants}}{\text{Total number of plants}}$.

Mycorrhizal Inoculation Effect (MIE %) on ZC development was calculated using this formula: $\mathrm{MIE} = 100 \times$

((Number of diseased plants treated with $R. irregularis$ and $B. cockerelli$) −

(Number of diseased plants treated with only $B.$ cockerelli))/

(Number of diseased plants treated only with $B. cockerelli$).

Newly formed leaf tissues (mid-vein) have been being sampled at 3, 6 and 7 weeks after infestation (WAI) for CLso detection by PCR as described earlier (116). Tomato genomic DNA was extracted according to *Arabidopsis thaliana* DNA extraction protocol (Appendix 4). The positive control of the PCR was psyllid DNA that was previously authenticated as infected by CLso DNA (116) whereas the negative control was CLso free tomato genomic DNA. The conditions of PCR were the same as those described previously. I used the tomato elongation factor-1 gene (EF1) to validate the PCR conditions and to control for false negatives in plant samples. The primer set used for the control PCR reaction was EF1 F/EF1 R (AGATGGTCAGACCCGTGAAC/ GTCAAACCAGTAGGGCCAAA) (146).

Assessment of the effect on *B. cockerelli* oviposition and larval survival

Two days after infestation, the adult psyllids were removed, and all the eggs deposited on the leaves were counted. One week after the removal of the adults, I counted the living larvae every two days until of the appearance of young adults.

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APPENDIXES

Appendix 1: Turface MVP chemical composition

1.	Materials:	A calcined, non-swelling illite and silica clay
2.	Porosity:	Total 74%, with 39% Capillary and 35% Non Capillary
3.	pH range:	5.5 ± 1.0
4.	CEC:	33.6 mEq/100g
5.	Particle Stability:	Sulfate Soundness testing (ASTM C-88) and static degradation test not to exceed 4% loss over 20 years
6.	Bulk Density:	37 ± 2 lb./ft³
7.	Color Range:	Reddish/Tan
8.	Packaging:	50 pound valve bags, 2000 pound super sacks, bulk dump truck loads

MVP® SIEVE ANALYSIS:

	% Retained
6 MESH (3.36 mm)	11.5%
8 MESH (2.38 mm)	35.6%
12 MESH (1.68 mm)	29.0%
20 MESH (.841 mm)	23.1%
30 MESH (.595 mm)	0.6%
40 MESH (.420 mm)	0.1%
Pan	0.1%

PRODUCT DESCRIPTION: Must be an illite silica blend at 40% minimum and 60% minimum amorphous silica. Material must be processed in a rotary kiln operation at temperatures not less than 1200 degrees Fahrenheit. Product must then be screened and de-dusted.

TYPICAL CHEMICAL DESCRIPTION:
SiO_2 - 74%
Al_2O_3 - 11%
Fe_2O_3 - 5%
All other chemicals equal less than 5% and include : CaO, MgO, K_2O, Na_2O and TiO_2

Appendix 2: ½ Hoagland's stocks recipe

Dissolve in distilled water and autoclave these stocks

Stock I 400x, per liter:
236.2g Ca(NO3)2 4H2O (calcium nitrate)
101.11g KNO3 (potassium nitrate)

Stock II 1000x, per liter:
246.48g MgSO¬4 7H2O (magnesium sulfate)

Stock III 2000x, per liter:
36.7g NaFeEDTA (Ethylenediaminetetraacetic acid iron(III) sodium salt)
Wrap bottle in foil to keep out light.

Stock IIII 5000x per liter:
13.6g KH2PO4 (potassium phosphate monobasic)

Stock V 1000x, per liter:
618.4mg H3BO3 (boric acid)
48.4mg Na2MoO4 2H2O (sodium molybdate)
287.6mg ZnSO4 7H2O (zinc sulfate)
395.8mg MnCl2 4H2O (manganese chloride)

124.8mg CuSO4 5H2O (cupric sulfate)
47.6mg CoCl2 6H2O (cobalt chloride)
1.25ml 10M HCl or 1.033ml 12.1M HCl

Stock VI 1000x, per liter:
97.5g MES
****
For ½ Hoagland's 0.5xN of 10 liters:
25ml Stock I
10ml Stock II
5 ml Stock III
2ml Stock IV
10ml Stock V
10ml Stock VI
Adjust pH to 6.1.

Appendix 3: Ergosterol Protocol

1. Put one leaf of each sample in separate scintillation vials and chop it
2. Add 2-3 ml of 2:1 chloroform:MeOH solution
3. Mix overnight shaking dark, capped at RT
4. Syringe filter 1 ml of extract into amber vials for storage
5. Pipette 10µl from amber vial into autosampler vial and add 90µl C_{13} cholesterol.
6. Run on LC/MS

Appendix 4: Arabidopsis quick genomic DNA prep for PCR

Primary Author: Elliot Meyerowitz, CALTECH
Note: this protocol was found on the iprotocols website in 2002. As of September 2008, the URL has been deleted.
Reagents:
50ml extraction buffer (make fresh each time):

100mM Tris pH 8	5ml of 1M
500mM eDTA pH 8	5ml of 0.5M
500mM NaCl	5ml of 5M
10mM beta ME	34.7µl of 14.4M
Water, molecular grade, to 50ml	35ml

20% SDS

5M potassium acetate

Isopropyl alcohol

3M sodium acetate

Procedure (adapted for use without liquid N_2):

1. Set heat block for 65°C. Make appropriate solutions.
2. Clip ~1/2 of large Arabidopsis leaf, or 1 whole small leaf; place in labeled microtube.

Steps 3-9 are to be performed under a fume hood.

3. Add 500µl extraction buffer and homogenize tissue using a pestle in the tube; alternately,
   place leaf sample in mortar and grind with pestle outside of the tube, adding 500µl to wash back into the tube.
4. Add 35µl 20% SDS.
5. Incubate on 65 C heat block for 10 min.
6. Add 130µl 5M potassium acetate.
7. Incubate 5min on ice or put the sample in the -20C freezer.
8. Spin at 15,000g 10min.
9. Transfer supernatant to new labeled tube.
10. Add 640µl isopropyl alcohol.
11. Add 60µl 3M sodium acetate. Invert briefly.
12. Incubate in freezer 2 hours (or overnight) at -20°C).
13. Spin at 15,000g 10min. Discard supernatant.
14. Wash with 70% ethanol (EtOH) (add EtOH and invert 3 times).
15. Spin at 15,000g for 5min. Discard EtOH.
16. Re-suspend pellet in 40µl molecular grade water. Add RNAse (20µg/ml) to eliminate RNA that could interfere with PCR.
17. Use 2µl of re-suspension for PCR applications.