Symbiotic Interactions Between Indigenous Pacific Northwest Fungi and Wheat

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Authorization to Submit Thesis

This thesis of Shawna Lee Faulkner, submitted for the degree of Master of Science with a Major in Natural Resources and titled "**Symbiotic Interactions Between Indigenous Pacific Northwest Fungi and Wheat**," has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

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Abstract

Microbes that asymptomatically colonize the internal tissues and organs of plants are endophytes. Endophytes sometimes benefit their hosts by mediating responses to stress. Variation in endophyte interactions with hosts is dependent on environmental factors. Here, variation in endophyte interactions is examined in wheat. The functional roles of four *Penicillium* isolates were explored in wheat, cheatgrass, and cottonwood hosts. Demonstrated antagonists of Fusarium crown rot in wheat were taken to a field location to replicate antagonism; however, conditions were not conductive to disease development. Finally, Fusarium crown rot antagonists were applied to wheat challenged with Fusarium head blight. Some inoculants were facilitators and antagonists of disease severity. Tolerance was observed in previously classified susceptible and moderately resistant varieties. The disease facilitator and antagonists also contributed to tolerance as significant differences in disease levels were observed in inoculant treatments, but fecundity was not impacted. This may be the first report of endophytes as contributors to tolerance.

Acknowledgements

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Table of Contents

List of Tables

List of Figures

Chapter 1

Impact of four endophytic isolates of *Penicillium* on germination and seedling growth of three plant species

Abstract

Pathogens have negative effects on plants within their range, but no effects on plants outside that range. Endophytes also can have negative or no effects on plants, but they are distinguished from many pathogens in that positive effects are seen, at least in some plants. Here, the functionality of four isolates of *Penicillium* from asymptomatic cheatgrass and cottonwood vegetative materials is determined in the context of seed germination and seedling growth in wheat, cheatgrass, and cottonwood. Surface-sterilized seeds were imbibed in single *Penicillium* inoculant spore solutions, or sterile distilled water for controls. The inoculated seed then incubated for seven days under a diurnal light cycle of 15 daylight hours at room temperature. Ungerminated seeds, germinated seeds, seedlings with root hairs, and the number of roots and leaves were counted. Wheat germination was not impacted by any *Penicillium* inoculants, but cheatgrass and cottonwood germination were reduced compared to the control. In wheat, inoculants increased the proportion of seedlings with root hairs, a positive effect. In contrast, the percentages of cheatgrass and cottonwood seedlings with root hairs were reduced by inoculants, a clear negative effect. These *Penicillium* inoculants proved to be host-specific with cryptic, negative effects on two plant species and positive effects on the third.

Introduction

Fungi are classified based on their functional role in ecology (e.g., pathogens, saprotrophs, endophytes, mycoparasites, entomopathogens, mycorrhizae). Pathogens and endophytes are overlapping categories in that both groups can have negative effects on plants (Schulz and Boyle 2005; Saikkonen et al 1998). In the case of pathogens, negative effects tend to be obvious in the sense that pathogens cause disease with symptoms and sometimes signs (Gladieux et al 2011). Endophytes, on the other hand, can have negative effects, but these are cryptic in that no symptoms of disease are seen. Instead, seeds fail to germinate or emerge, or germinants fail to grow or develop, or seedlings fail to compete. These are negative effects that are inherently hidden or cryptic. While this feature can distinguish endophytes from many pathogens, root rot pathogens, like *Rhizoctonia solani*, have similarly cryptic negative effects on disease hosts (Grosch et al 2005). A distinguishing attribute of endophytes is that they can have positive effects in some plants whereas pathogens never do. Additionally, both pathogens and endophytes can be host-specific (Power and Mitchell 2004; Wisler and Norris 2005; Fraser et al 2017).

Endophytes that significantly benefit their hosts are functionally mutualists (Busby et al 2013; Comant et al 2005; Clay and Schardl 2002; Rodriguez and Redman 2008). Endophytes with no net effect on the host are considered functionally neutral (Carroll 1988; Stone et al 2004). Under the right environmental conditions, some endophytes can shift from neutrality to weak pathogens after a period of latency in an asymptomatic host; these are known as latent pathogens (Schulz and Boyle 2005; Sieber 2007; Malcolm et al 2013), a designation that again emphasizes the overlap between ‘pathogens’ and ‘endophytes’.

Pathogens can have unequal effects on competing plants, preferentially infecting the susceptible plant and thus favor the more tolerant plant (Beckstead 2010; Fraser et al 2017). Endophytes could do the same, although cryptically.

As an example, the fungus *Fusarium culmorum* is an important pathogen of some members of the Poaceae and thus host-specific to some extent. *Fusarium culmorum* also can be found asymptomatically as an endophyte in a number of host families, including the Salicaceae. Fraser et al (2017) demonstrated that two *F. culmorum* isolates from wheat and cottonwood were able to infect and significantly reduce the fitness of wheat while having no effect on cottonwood seedlings. By asymptomatically harboring a pathogen that can be transferred to a more susceptible competitor, cottonwood could potentially benefit. Classifying the function of *F. culmorum* in cottonwood as neutral seems inappropriate when the net effects are positive as community dynamics might be changed by these subtle interactions (Cobey and Lipsitch 2013; Orrock and Witter 2010; Newcombe et al 2009). Yet, since all host-specific pathogens could change plant community dynamics via unequal effects on different plants, it seems best to simply describe *F. culmorum* as a pathogen of some members of Poaceae that is otherwise endophytic outside its grass host range.

But, if pathogens have negative effects within their host ranges and no effects outside those ranges, what of endophytes? Endophytes could be like pathogens just with cryptic, negative effects within the host range and no effects outside. Or, they could differ in having either positive or negative effects depending on the plant that they affect. Here, the functional roles of four *Penicillium* endophytes are determined. *Penicillium* endophytes were isolated from asymptomatic cheatgrass and cottonwood vegetative materials. The effects of *Penicillium* inoculants on the germination and seedling growth of three Pacific Northwest

plants, including wheat, cheatgrass, and cottonwood, were measured to determine the functional roles of these isolates. Functional roles should be detectable as differences in seed germination and the proportion of seedlings with root hair development as surface area for water and nutrient absorption increases with root hair development, measured here in addition to the number of roots and leaves of seedlings. They could be pathogens, albeit with cryptic negative effects on some plants and no effects on others. Or, they could differ from pathogens as defined in this way.

Materials and Methods

Seed and Inocula Source

Wheat (*Triticum aestivum*) seed of the hard red winter wheat variety UI SRG were sourced from foundation seed. Cheatgrass (*Bromus tectorum*) and cottonwood (*Populus trichocarpa*) seed were harvested in Moscow, ID. Fungal isolations of *Penicillium* inoculants were made from asymptomatic cheatgrass and cottonwood vegetative materials (Table 1.1). Materials were plated onto potato dextrose agar. Once microbes emerged, they were further cultured onto potato dextrose agar. Multiple rounds of culturing were performed until single isolate cultures were obtained.

Inocula Preparation

Penicillium isolates were multiplied and grown to maturity on potato dextrose agar before processing. Petri dishes were washed with sterile distilled water and a surface-sterilized bent

glass rod was used to dislodge spores. Three drops of polysorbate 20 were mixed into each suspended spore solution to increase the dispersal of spores in solution by reducing hydrophobicity. A hemacytometer (Fisher Scientific, Asheville, NC) was used to calculate spore concentrations (Table 1.2).

Seed Inoculations, Germination, and Growth

Seeds were either imbibed in suspended spore solutions of single *Penicillium* inoculants or sterile distilled water for controls for four hours, removed, washed three times with sterile distilled water, and individually wrapped in pieces of sterilized paper towels dampened with sterile distilled water. Ten seeds were placed in a polyethylene bag for a total of 50 wheat and cheatgrass seeds per treatment and 50 to 56 cottonwood seeds per treatment combination of host and *Penicillium* inoculant. A total of 769 imbided seeds were incubated at room temperature, approximately 21^{o}C, and a diurnal light cycle of 15 daylight hours for one week before counts of ungerminated and germinated seeds, seedlings with root hairs, and the number of roots and leaves in seedlings were taken.

Data Analyses

R version 3.2.3 was used to analyze the data. A contingency table listing counts within each of two score categories was built using the stats package. Fisher's exact test for independence was used to analyze the contingency table data because some categories had counts less than or equal to five. Two Fisher's exact tests were performed to determine if

inoculants affected seed germination and seedling growth. Growing seedlings were defined as ones that developed root hairs whereas non-growers did not. Analyses of variance were used to determine if *Penicillium* inoculants impacted the observed number of leaves and roots in wheat and cheatgrass. If the *Penicillium* treatment effect was significant, a multiple comparison procedure was employed and a Dunnett test performed to determine the effects of individual *Penicillium* inoculants. Assumptions of normality and variance were verified using normal and residual plots. An alpha value of 0.05 was used as the threshold for significance.

Results

Wheat

Wheat seed germination was not significantly impacted by inoculation with *Penicillium* endophytes (Table 1.3). Significantly more wheat seedlings had root hairs in *Penicillium*-treated seed compared to the control. Seventy-four percent of control seedlings developed root hairs whereas there were 24, 18, 24, and 22% more seedlings with root hairs among seed inoculated with *Penicillium* sp. BT, *Penicillium* sp. POP1, *Penicillium* sp. POP2, and *Penicillium* sp. POP3*,* respectively (Table 1.4). The analyses of variance demonstrated a significant treatment effect of *Penicillium* inoculants on the number of observed roots (R^2 = 0.6973, $P < 2.2E^{-16}$) and leaves ($R^2 = 0.8298$, $P < 2.2E^{-16}$) in wheat. However, Dunnett tests from data treated with the multiple comparison procedure showed that while there was a significant treatments effect the numbers of leaves in wheat seedlings were not significantly impacted by inoculation with *Penicillium* when pair-wise comparisons were made. Yet, the

numbers of roots were significantly increased in all *Penicillium* treatments relative to the control ($P < 1E^{-4}$). On average, 60, 51, 71, and 69% more wheat seedlings had root hairs when inoculated with *Penicillium* sp. BT, *Penicillium* sp. POP1, *Penicillium* sp. POP2, and *Penicillium* sp. POP3 than control seeds.

Cheatgrass

Cheatgrass seed germination and seedling growth also were significantly reduced by inoculation with *Penicillium* endophytes. Inoculation of cheatgrass seed with *Penicillium* sp. BT, *Penicillium* sp. POP1, *Penicillium* sp. POP2, and *Penicillium* sp. POP3 reduced seed germination by 20, 32, 28, and 20% relative to the control (Table 1.3). Among control cheatgrass seedlings, 95.6% had root hair development; this was 61, 38, 28, and 20% greater than cheatgrass seed treated with *Penicillium* sp. BT, *Penicillium* sp. POP1, *Penicillium* sp. POP2, and *Penicillium* sp. POP3, respectively (Table 1.4). The numbers of leaves ($R^2 = 0.8345$, $P < 2.2E^{-16}$) and roots ($R^2 = 0.7677$, $P < 2.2E^{-16}$) were significantly impacted by *Penicillium* inoculation in cheatgrass as indicated by the analyses of variance (Figure 1.1). As in wheat, while there was an overall treatment effect of *Penicillium* inoculants, individual pair-wise comparisons in the Dunnett test on data treated with the multiple comparison procedure indicated that the numbers of leaves among cheatgrass seedlings were not significantly different. However, the numbers of roots were significantly reduced in cheatgrass seedlings treated with *Penicillium* inoculants when compared to the control in a Dunnett test. Seedlings treated with *Penicillium* sp. BT ($P = 0.02761$), *Penicillium* sp. POP1 ($P = 0.03089$), *Penicillium* sp. POP2 ($P = 0.00324$), and *Penicillium* sp. POP3 ($P = 0.01180$) had 35, 46, 39, and 30% fewer roots than the control treatment.

Cottonwood

Cottonwood seed germination and seedling growth were also significantly reduced by inoculation with *Penicillium* endophytes. Cottonwood seed germination was reduced 40, 55, 67, and 56% relative to the control when inoculated with *Penicillium* sp. BT, *Penicillium* sp. POP1, *Penicillium* sp. POP2, and *Penicillium* sp. POP3, respectively (Table 1.3). Seedlings with root hairs occurred at a 47% frequency among controls. When inoculated with *Penicillium* sp. BT, *Penicillium* sp. POP1, *Penicillium* sp. POP2, and *Penicillium* sp. POP3 the frequency was reduced by 34, 43, 45.2, and 41%, respectively (Table 1.4).

Discussion

Penicillium inoculants isolated from asymptomatic cheatgrass and cottonwood vegetative materials acted as pathogens of cheatgrass and cottonwood seeds and seedlings while wheat seed inoculation with these same isolates significantly benefited seedling growth. These results are contrary to those found by Fraser et al (2017) with *F. culmorum* in that neutral effects were not observed in the non-diseased host. Negative effects were observed in both cheatgrass and cottonwood and positive effects in wheat. It is possible that these *Penicillium* isolates are functionally pathogens only, but other factors contributed to the positive results seen in wheat. Wheat and cheatgrass are more similar in range and habit than either are to cottonwood; both are winter annual grasses native to Europe. Thus, neither of these factors appear to likely explain the results with *Penicillium* isolates. A potential explanation could be rates of maternal transmission, which refers to the ability of a parent plant to pass microbes called primary symbionts to its offspring. Cheatgrass and cottonwood seedlings generally

lack primary symbionts whereas wheat seedlings typically have them (Dr. George Newcombe, personal communications). In this scenario, primary symbionts could protect wheat seedlings from damage by *Penicillium* inoculants if wheat were a disease host whereas cheatgrass and cottonwood seedlings would be unprotected. Or, it is also possible these *Penicillium* inoculants are functionally pathogens of cheatgrass and cottonwood, but not wheat and that *Penicillium* inoculation in wheat stimulated primary symbionts that are found at a relatively high frequency and ultimately benefited the host when neutral effects would otherwise be observed. This study was not designed to address this aspect of the functional roles of these *Penicillium* isolates, yet other factors contributing to their functionally may be discussed.

As demonstrated in this study, the function of an endophyte is not only dependent on host species, but can also vary throughout the life cycle of the plant host. The fact that *Penicillium* inoculants filled multiple functional roles depending on host species and life cycle stage is worth consideration because it gives evidence to the need for additional categories to classify the functional roles of fungi. *Penicillium* inoculants should not be considered latent pathogens of cottonwood and cheatgrass because they did not first asymptomatically colonize seeds and seedlings. They might be considered weak pathogens of cottonwood and cheatgrass seeds and seedlings; yet, isolates came from asymptomatic plant material and the net effects in wheat were not neutral. Currently, the classification system for fungi lacks an appropriate term to adequately describe fungi that fill a diversity of functional roles in plant communities, like these *Penicillium* inoculants. The opposite reactions observed in wheat compared to cheatgrass and cottonwood demonstrates that our current understanding of microbial ecology also limits our ability to accurately describe

functional roles of fungi. Fungi play significant and complex roles in contributing to plant community dynamics, which require additional research to resolve the aforementioned problems with fungal classification.

Conclusions

New categories to classify fungi must be derived to accurately represent the functional diversity of fungi like these *Penicillium* species that have multiple ecological roles (i.e., pathogens and endophytes). These results are being incorporated into a manuscript with data on *F. culmorum* from Fraser et al (2016) and MySEQ data on the functional diversity of fungi found in cottonwood leaves (Dr. Posy Busby, personal communications) to further address the need for expansion of categories that describe the functional roles of fungi.

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Tables and Figures

Table 1.1. *Penicillium* endophytes selected for testing in wheat, cheatgrass, and cottonwood. *Penicillium* inoculants were collected from the native range of plant hosts and isolated from asymptomatic vegetative material of cheatgrass and cottonwood.

Endophyte	Source Host	Source Tissue	Recovery Site
Penicillium sp. BT	*Bromus tectorum*	stem	Ada County, ID, USA
Penicillium sp. POP1	*Populus trichocarpa*	leaf	Yakima River, WA, USA
Penicillium sp. POP2	*Populus trichocarpa*	leaf	Snohomish River, WA, USA
Penicillium sp. POP3	*Populus trichocarpa*	leaf	Snoqualmie River, WA, USA

Table 1.2. Concentrations of *Penicillium* isolates used to inoculate seed. Calculations were made with count data taken with a hemacytometer.

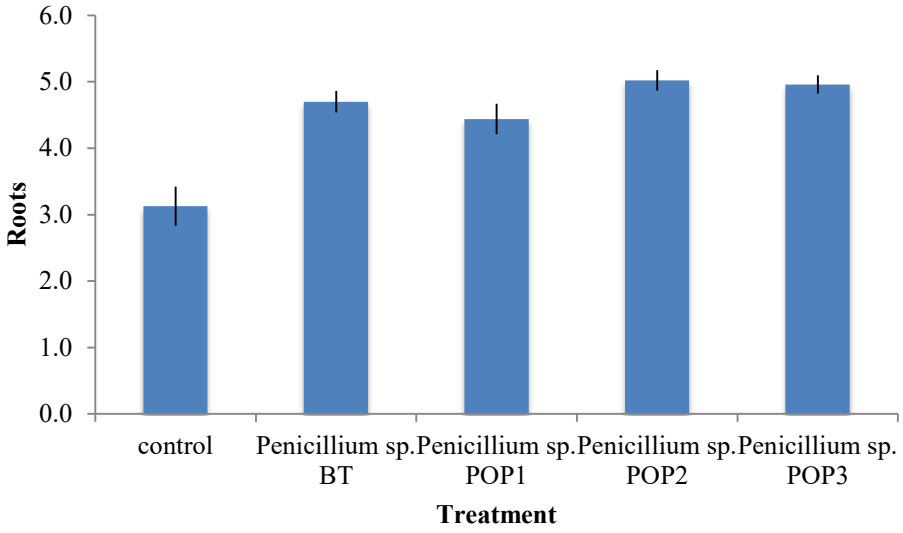
Inoculant	Inocula/mL
control	0
Penicillium sp. BT	$1.30E^{7}$
Penicillium sp. POP1	$1.37E^{7}$
Penicillium sp. POP2	$2.51E^{7}$
Penicillium sp. POP3	$1.23E^{7}$

Table 1.3. Germinated and ungerminated counts of seed in wheat, cheatgrass, and cottonwood after inoculation with *Penicillium* isolates.

Species	Inoculant	Germinated	Ungerminated	P-value
Triticum	*Penicillium* sp. BT	49	1	0.617
aestivum	*Penicillium* sp. POP1	46	4	1.000
	Penicillium sp. POP2	49	1	0.617
	Penicillium sp. POP3	49	1	0.617
	Control	47	3	
Bromus	*Penicillium* sp. BT	38	12	0.008
tectorum	*Penicillium* sp. POP1	32	18	0.000
	Penicillium sp. POP2	34	16	0.000
	Penicillium sp. POP3	38	12	0.008
	Control	47	2	
Populus	*Penicillium* sp. BT	16	39	0.000
trichocarpa	*Penicillium* sp. POP1	7	43	0.000
	Penicillium sp. POP2	1	55	0.000
	Penicillium sp. POP3	7	47	0.000
	Control	38	17	

Table 1.4. Counts of seedlings with and without root hairs in wheat, cheatgrass, and cottonwood after inoculation with *Penicillium* isolates.

Species	Inoculant	Root hairs	No hairs	P-value
Triticum	*Penicillium* sp. BT	49	1	0.001
aestivum	*Penicillium* sp. POP1	46	4	0.031
	Penicillium sp. POP2	49	1	0.001
	Penicillium sp. POP3	48	2	0.004
	Control	37	13	
Bromus	*Penicillium* sp. BT	35	15	0.001
tectorum	*Penicillium* sp. POP1	29	21	0.000
	Penicillium sp. POP2	34	16	0.000
	Penicillium sp. POP3	38	12	0.008
	Control	47	2	
Populus	*Penicillium* sp. BT	7	48	0.001
trichocarpa	*Penicillium* sp. POP1	2	48	0.000
	Penicillium sp. POP2	1	55	0.000
	Penicillium sp. POP3	3	51	0.000
	Control	24	31	



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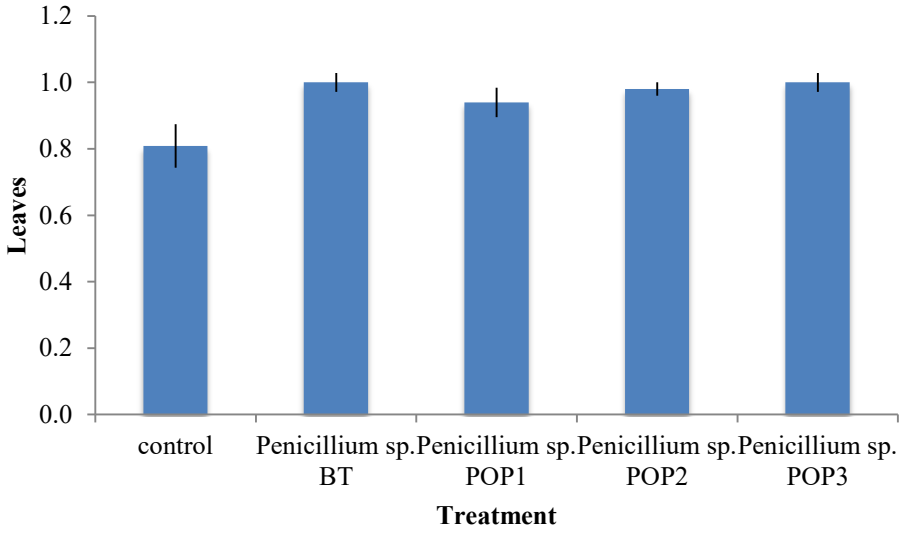
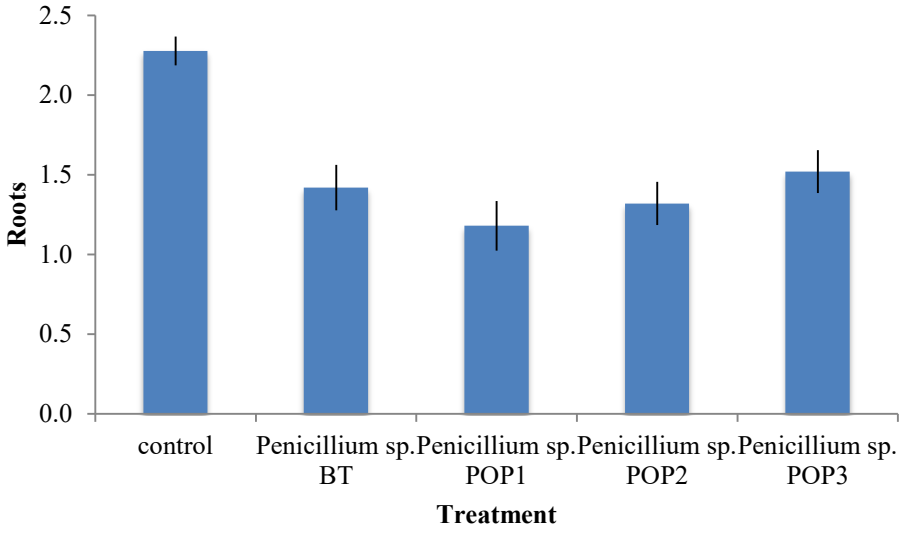
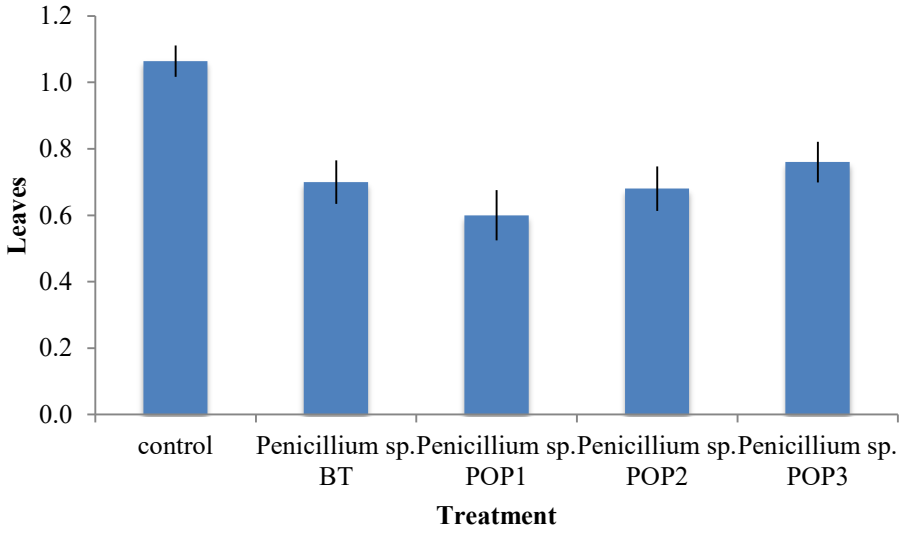


(b)

Figure 1.1. Mean number of roots (a) and leaves (b) observed in wheat seed treated with *Penicillium* inoculants and a control.



(a)



(b)

Figure 1.2. Mean number of roots (a) and leaves (b) in cheatgrass seed treated with *Penicillium* inoculants and a control.

Chapter 2

Field examination of demonstrated antagonists of Fusarium crown rot of wheat

Abstract

Pathogen antagonists reduce disease severity and thus benefit plants when disease would otherwise be favored. Antagonists have previously been shown to reduce Fusarium crown rot severity in wheat and increase seed yield, or fecundity, when disease was favored. Here, we apply these antagonists in the field to control Fusarium crown rot. Pathogen inoculum of the casual agent, *Fusarium culmorum*, was applied to some plots to supplement natural populations and increase disease severity; endophytes were applied to *Fusarium*-inoculated and control plots. The severity of Fusarium crown rot was then measured along with yield and the physiological variables test weight, plant height, infrared temperature, stomatal conductance, and foliar nutrient composition. Soil moisture was monitored as water stress favors Fusarium crown rot development. Inoculation with *F. culmorum* did not increase disease severity and high precipitation throughout June and July resulted in adequate soil moisture and thus conditions were not conductive to disease development. As expected, endophytes did not affect disease severity at uniformly low disease levels. Without endophyte reisolation frequency and maternal transmission data, interactions cannot be interpreted as there is no evidence infection occurred. A 2016-2017 field experiment has been planted to account for these factors so that the functional role of endophytes under field conditions can be determined.

Introduction

Antagonism of plant pathogens is a growing field of study as evidenced by the Phytobiome Initiative (Beattie and Leach 2014). There are several published records of endophytic antagonism in a variety of pathosystems where disease severity was significantly reduced when disease levels would otherwise be high (Busby et al 2016). Our understanding of antagonism comes primarily from agricultural studies. In one system, natural antagonists significantly reduced the severity of tan spot disease caused by *Pyrenophora tritici-repentis* in wheat (Istifadah and McGee 2006; Larran et al 2016). Dingle and McGee (2003) used antagonists to reduce disease severity and pustule size of the rust fungus *Puccinia recondita* f. sp. *tritici* in wheat. Antagonists are most often applied in regulated environmental conditions conductive to disease, such as in growth chamber and greenhouse experiments.

Antagonism of *Fusarium culmorum*, a casual agent of Fusarium crown rot, in wheat has been demonstrated under controlled conditions when disease was otherwise severe (Ridout and Newcombe 2016). In particular, yield was doubled in engineered drought conditions otherwise favorable to disease by the antagonist *Penicillium* sp. WPT. Additionally, inoculants mitigated abiotic stresses, like drought, that are associated with the development of Fusarium crown rot. Abiotic stress and other environmental factors can also modify the severity of Fusarium crown rot. Disease severity can be modified, depending less on inoculum loads and more on environmental conditions, including water stress late in the growing season (Cook 1980). In the Pacific Northwest on the Palouse, Fusarium crown rot severity increases with nitrogen application (Papendick and Cook 1980). Disease severity on the Palouse is also increased when early planting and direct seeding are practiced as

pathogen inoculum accumulates in the uppermost layer of soil (Paulitz et al 2002). To determine the efficacy of antagonists as agents for biological control of *F. culmorum* caused Fusarium crown rot, antagonism must be replicated in the field where conditions are not controlled and differences in environmental factors and cultural practices can result in variable disease levels.

Here, we take demonstrated *Fusarium culmorum* antagonists and physiologically beneficial endophytes (Ridout and Newcombe 2016) to a field location on the Palouse. Regionally, Fusarium crown rot severity varies based on weather meaning low disease levels are a risk for antagonism studies. Inoculum of *F. culmorum* was added to plots treated with demonstrated antagonists to ensure the pathogen would be consistently present and to try and increase disease. Pathogen inoculum was not added to plots treated with physiologically beneficial endophytes, but low levels were present in the soil. Disease severity was quantified using a scale and the number of internodes showing symptoms of disease recorded. Soil moisture data were taken as Fusarium crown rot severity is associated with this response variable. The effects of endophytes were then measured as yield, test weight, plant height, and plant stress. Additionally, stomatal conductance and foliar nutrient composition were measured among plots treated with physiologically beneficial endophytes. Foliar nutrient composition was monitored as previous studies indicate endophytes can influence the uptake of nutrients, in particular phosphorus (Jumponnen et al 1998; Haselwandter and Read 1982). Endophytes can impact water and nutrient uptake and thereby any reproductive, water, or nutrient stress can be quantified with these traits. The results of the field study are reported with recommendations for future studies.

Materials and Methods

Seed and Endophyte Inocula Source

Foundation seed of the hard red winter wheat variety UI SRG and soft white winter wheat variety Stephens were planted in this study. Endophyte inocula of *Clonostachys rosea, Morchella snyderi, Penicillium* sp. WPT*, Phialocephela* sp.*,* and *Pichia membranifaciens* were replicated on potato dextrose agar from archived cultures (Ridout and Newcombe 2016).

Study Site

Seed was planted on the University of Idaho Kambitsch Research Farm near Genesee, Idaho, USA in October 2015. This region is known as the Palouse and is characterized by rolling loess hills largely developed for agricultural production. Regionally, dryland wheat and legume production are possible because the soil has a high water holding capacity from its volcanic ash content. Each 5'x 20' plot was planted at a rate of 23 seeds/ft^2 into tilled ground using a seeder equipped with seven double-disc openers spaced 7 inches apart. Five foot buffer rows were planted with the awnless soft white winter wheat variety Brundage 96. A ripper-shooter was used to pre-plant incorporate a dry fertilizer containing NPS. A second fertilizer application using a dry formulation of urea (40-0-6, N-P-S), was flown on at a rate of 100 pounds per acre in early spring. The herbicides Huskie, Puma, and Harmony Extra XP were applied in early spring at rates of 12, 11, and 0.5 ounces per acre, respectively.

Experimental Design

Two levels of pathogen treatment included *F. culmorum*-inoculated plots and non-inoculated control plots with naturally low levels of *F. culmorum*. Demonstrated antagonists of *F. culmorum*, including *C. rosea, Penicillium* sp. WPT, and *P. membranifaciens* and a water control, were applied to pathogen treated and pathogen control plots. Physiologically beneficial endophytes including *M. snyderi, Penicillium* sp. WPT, and a *Phialocephela* sp. can benefit wheat in conditions otherwise conductive to Fusarium crown rot development (i.e., drought stress) and therefore may allow the host to cope with stress associated with the development of Fusarium crown rot. An endophyte control with low levels of *F. culmorum* inoculum was included. Treatment combinations were replicated once in each of five randomized complete blocks for a total of 5 plots of each cultivar per treatment combination and a total of 120 plots. Treatments were assigned to plot numbers with random permutations. Stephens plots were analyzed as a completely randomized design because the blocking factor was lost due to a planting error.

Inoculations

Two *F. culmorum* isolates, 97 and 126, obtained from Washington State University were grown on sterilized millet. Equal masses of the isolates were thoroughly mixed to homogenize pathogen inocula. Pathogen inoculum was added at a rate of 3.5 g/m linear row or 147 g/plot at the time of planting. Archived endophytes were replicated on potato dextrose agar and grown to maturity before processing. Spores of the species *Penicillium* sp. WPT, and *P. membranifaciens* were dislodged from mature cultures with a surface sterilized bent glass rod and sterile distilled water. Suspended filament and spore solutions of *C. rosea, M.*

snyderi, and *Phialocephela* sp. were made by blending mature cultures with sterile distilled water. Approximately 1 mL polysorbate 20 was added to each bulk solution to encourage inoculum dispersal by reducing spore hydrophobicity. A hemacytometer (Fisher Scientific, Asheville, NC) was used to calculate endophyte inocula concentrations (Table 2.1). Stephens plots were inoculated with endophytes once in November 2015 and again in the March 2016 to facilitate infection as antagonism in this variety has not been previously demonstrated. UI SRG plots were inoculated once in March 2016 as antagonism has been demonstrated in this variety. A stream of 1 mL endophyte inoculum/second, approximately 1 L of inoculant solution per plot, was applied to emerged seedlings with loaded backpack sprayers. Control plots were inoculated with water. Endophyte infection was facilitated by inoculating on afternoons where the relative humidity was high.

Severity of Disease

Fifteen random crowns were harvested from each plot for Fusarium crown rot disease scoring. Five crowns were harvested from the front, middle, and back locations of the plots. Soil and tillers were removed to allow evaluation of the main stem. A scale of 0 to 5 was created to score the severity of Fusarium crown rot disease with 0 indicating no disease and 5 representing high disease severity (Table 2.2). The number of internodes displaying Fusarium crown rot symptoms was recorded. Soil moisture measurements of volumetric water content were taken one day per week, for four consecutive weeks between June and July and recorded on an MPS-2 dielectric water potential sensor (Meter Group Pullman, WA) to determine whether conditions were conductive to the development of Fusarium crown rot and if soil moisture was homogenous across the study site.

Physiological Measurements

Yield (kg/ha), the measure for fecundity, was calculated in both *F. culmorum*-inoculated and control plots. Test weight (kg/hL), a standardized measure of the bulk density of seed, was calculated to determine if grain quality was affected in any treatment combinations. Plant height was recorded to determine if detectable differences in plant height occurred. Infrared thermometry was used as a metric to estimate plant stress (Hatfield 1990). Flag leaf surface temperatures were measured in each plot using a Model 8872 infrared thermometer (Spectrum Technologies, Paxinos, PA) one day per week, for six consecutive weeks at approximately the same time each day between June and July 2016. Data on stomatal conductance, temperature, and relative humidity of flag leaves were recorded on an SC-1 leaf porometer (Meter Group, Pullman, WA) one day a week, for four consecutive weeks in the early afternoon in June and July. Three random flag leaves were collected in the front, middle, and back of plots, respectively, stored in envelopes, and air-dried in an oven at 65^{o}C for 48 hours before processing. Dried leaves were ground to a fine powder using a F203 Grinder (KRUPS) and shipped to Midwest Laboratories for analysis of foliar N, NO_3N, P, K, Mg, Ca, S, Na, Fe, Mn, B, Cu, and Zn composition. Soil profile data were taken including nutrient composition and fertilizer recommendations to ensure the soil profile was homogenous in June during the growing season. After removing the top 4 cm of soil, twenty soil cores were taken in each of 5 and 4 blocks at a depth of 15 cm and stored at 15^{o}C prior to shipping. Nutrient composition measurements included percent organic matter, P_1, P_2, K, Mg, and Ca. Fertilizer recommendations were measured for Nox, N, NH_3, lime, and phosphate. Additional soil profile measurements included pH, buffer index, cation exchange

capacity, and percent saturation with K, Mg, Ca, and H. In September 2016, plots were individually harvested with a Wintersteiger plot combine.

Data Analyses

Data analyses for Fusarium crown rot severity and physiological measurements were completed using R version 3.2.3. Analyses of variance were used to determine if there were significant differences in disease severity as measured by yield, test weight, disease scores, and the number of internodes showing visible signs of disease. Analyses of variance were performed to determine if pathogen and endophyte treatments, blocks, and their two and three-way interactions were significantly different for the response variables plant height, infrared thermometry, stomatal conductance, and foliar nutrient composition. Analyses of variance were also used to test soil data and determine whether environmental conditions were conductive to Fusarium crown rot development and if the water and nutrient composition of the soil were homogenous across the study site. Residual and normal plots were used to verify that the data were normally distributed with equal variance. An alpha value of 0.05 was used as the threshold for significance.

Results

The pathogen and endophytes did not have any significant effects on the response variables measured in this study. Among those response variables representing the severity of disease, including disease scores and the number of internodes showing symptoms, there were not significant differences between pathogen and endophyte treatment combinations (Figures 2.1

and 2.2). Additionally, there were no detectable differences in soil moisture, the response variable positively correlated with Fusarium crown rot severity, as measured by volumetric water content (%) (Figure 2.3). There also were no differences in physiological response variables. Yield (kg/ha) was not affected by pathogen or endophyte inoculants at either low levels of disease as significant differences were not observed between treatment combinations in either variety (Figures 2.4 and 2.5). The quality of grain was not significantly impacted by pathogen or endophyte treatment combinations as test weights (kg/hL) were not significantly different (Figures 2.6 and 2.7). Plant height was not significantly different in any treatment combination (Figures 2.8 and 2.9) nor were differences in plant stress, as represented by infrared thermometry, biologically significant (Figure 2.10). Stomatal conductance was the same across treatment combinations (Figure 2.11). Foliar nutrient composition did not vary significantly among treatments (Table 2.3). Soil profile measurements showed there were no significant differences in the soil profile in nutrient composition, pH, buffer index, cation exchange capacity, or fertilizer recommendations (Table 2.4).

Discussion

The hypothesis was that antagonism of Fusarium crown rot observed under controlled environmental conditions could be replicated in the field. Endophytes did not demonstrate antagonism towards Fusarium crown rot likely because significant disease levels were not detected. Levels of Fusarium crown rot disease were uniformly low in both *F. culmorum*-inoculated and control plots as evidenced by yield, test weight, disease scores, and the

number of internodes showing symptoms of infection. Environmental conditions were not conductive to disease development as detected by soil moisture (Paulitz et al 2002). Despite attempts to increase disease severity by adding inoculum of *F. culmorum*, levels of crown rot disease were likely uniformly low because higher than normal precipitation fell in late June and early July resulting in little to no water stress and creating conditions that were not conductive to significant crown rot. The absence of significant differences in yield, test weight, and physiological measurements including plant height, IR temperature, water conductance, and foliar nutrient composition among *F. culmorum*-inoculated and control plots support this claim. Further, these results demonstrate that endophytes not only failed to be antagonistic, but also failed to physiologically impact hosts as has been demonstrated in some studies (Jumponnen et al 1998; Haselwandter and Read 1982; Ridout et al 2016). These results support the use of these endophytes as biological control agents of Fusarium crown rot because effects were not observed in low disease levels.

In some seasons, disease levels will inherently be low and the absence of effects of endophytes under these conditions is desired because it is impossible to forecast disease severity at the time endophytes must be applied. The potential impacts of biological control agents outside of the context in which they are being introduced are important to understand. Endophytes would not be viable options for control of Fusarium crown rot if effects such as significant reductions in yield were observed in low disease levels. Ultimately, antagonism must be demonstrated in the field, these results need to be replicated, and additional data need to be recorded to determine the efficacy of these endophytes in the control of Fusarium crown rot. These next steps must be taken to evaluate the potential applications of

antagonists of disease. Only then will we know if endophytes are generally viable options for biological control of Fusarium crown rot.

As demonstrated in this study, to be able to effectively test the suitability of endophytes, the factors that contribute to disease and the ecological interactions between host, pathogen, and endophyte must first be understood. A mixture of cultural practices was used in this experiment including split nitrogen applications and late planting. Differences in wheat production cultural practices significantly contribute to the development of Fusarium crown rot in wheat (Paulitz et al 2002). Increasing Fusarium crown rot severity has been linked with reduced tillage, direct seeding, high nitrogen applications, and early planting. It might be possible that different combinations of cultural practices result in higher disease levels. A defense hierarchy exists in the Fusarium crown rot pathosystem where effects of antagonists are contingent upon environmental factors that directly reduce disease. Low levels of disease in 2015-2016 winter wheat field plantings might be expected because soil moisture conditions were not conductive to disease. Late planting and above-average levels of precipitation in the spring and summer months, may have contributed to low levels of Fusarium crow rot in this study.

It is also possible that endophytes did not infect plants under field conditions. We do not know if endophytes infected plants because reisolation frequency data were not taken from vegetative material. Additionally, there was no evidence from seed grown in the field that suggested endophytes could be maternally transmitted, a process by which parent plants can pass on microbes to offspring. However, there was evidence of high, nearly 100%, maternal transmission of microbes other than our endophyte inoculants. A problem in pathogen antagonism studies is they are conducted under the assumption that significant

antagonism will be observed. If significant treatment effects of pathogens and endophytes are observed reisolation has not been considered necessary. But, as demonstrated in this study, when significant disease does not develop and reisolation data were not taken we do not know if endophytes infected plants. Inoculant interactions cannot be interpreted in these cases and their functional role remains unknown. Primary colonists from maternal transmission have also been ignored in antagonism studies, but matter given inoculated endophytes would interact with primary colonists. Again, without data on primary colonist isolation frequency, these interactions will remain unaddressed, but could significantly impact the observed plant-microbial interactions and the efficacy ranges of antagonists. It is prudent for endophyte reisolation data and maternal transmissions rates to always be measured in antagonism studies. Another option would be instead to work with primary colonists that demonstrate antagonism as they can be maternally transmitted. This study was not designed to address these factors, but a 2016-2017 field experiment has been planted that will do so.

Conclusions

For a number of reasons, conclusions should not be drawn regarding these endophyte inoculants. As environmental conditions were not conductive to disease, we do not know whether inoculants can be antagonistic in the field. Additionally, without information regarding rates of maternal transmission and inoculant reisolation frequency, the effects of inoculants cannot be interpreted. The field experiment planted this year addresses these factors.

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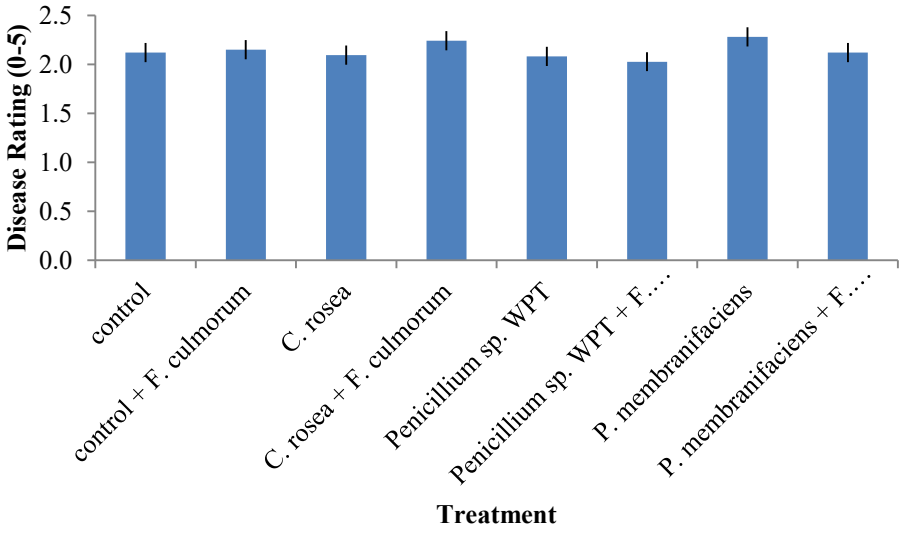
Tables and Figures

Table 2.1. Concentrations of antagonist field inocula in two different inoculations.

Inoculation Time	**Endophyte**	**Inocula/mL**
Fall 2015	control	0
	C. rosea	$1.08E^{7}$
	DSE	$4.94E^{5}$
	M. snyderi	$8.6E^{4}$
	P. sp. WPT	$1.825E^{7}$
	P. membranifaciens	$7.4E^{6}$
Spring 2016	control	0
	C. rosea	$5.95E^{6}$
	DSE	$1.4E^{6}$
	M. snyderi	$2.75E^{6}$
	P. WPT	$1.9E^{6}$
	P. membranifaciens	$7.3E^{6}$

Table 2.2. Descriptions of disease categories including the level of disease and signs of severity.

Score	Disease Level	Signs
1	low	some discoloration of the crown, none on the internodes
2	low-moderate	discoloration of the crown and some internodes
3	moderate	more discoloration of the crown and internodes
4	high	dark chocolate brown discoloration of crown and internodes, stalk may be deformed
5	severe	significant discoloration of crown and internodes, brittle crown and other tissues, stalk may be deformed



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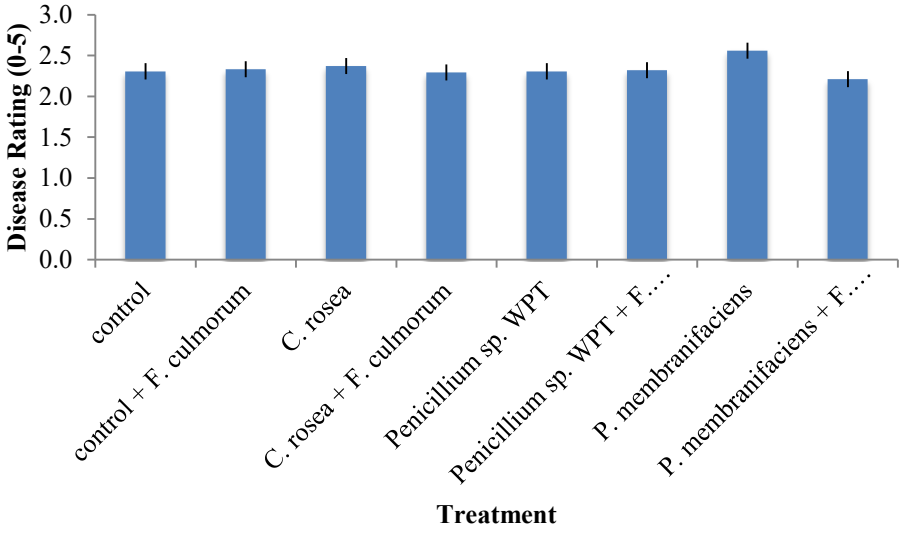
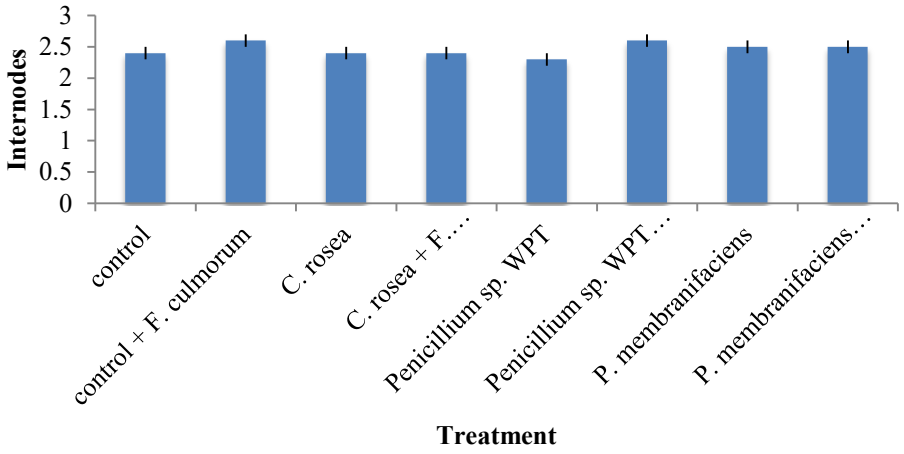


(b)

Figure 2.1. Mean scores of UI SRG (a) and Stephens (b) crowns with standard errors represented by black bars. Disease levels were uniformly low across treatments.



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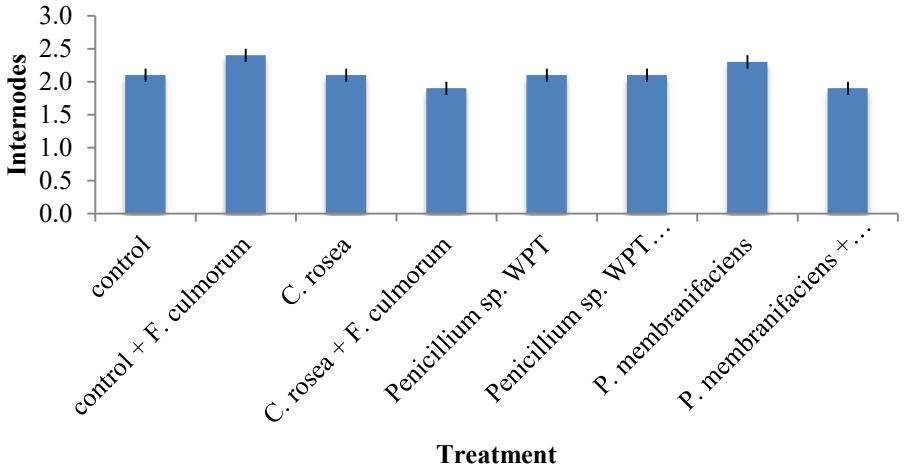


(b)

Figure 2.2. Mean number of internodes of UI SRG (a) and Stephens (b) plants showing crown rot symptoms with black bars to represent standard error. No significant differences in the number of internodes showing symptoms of disease were observed.

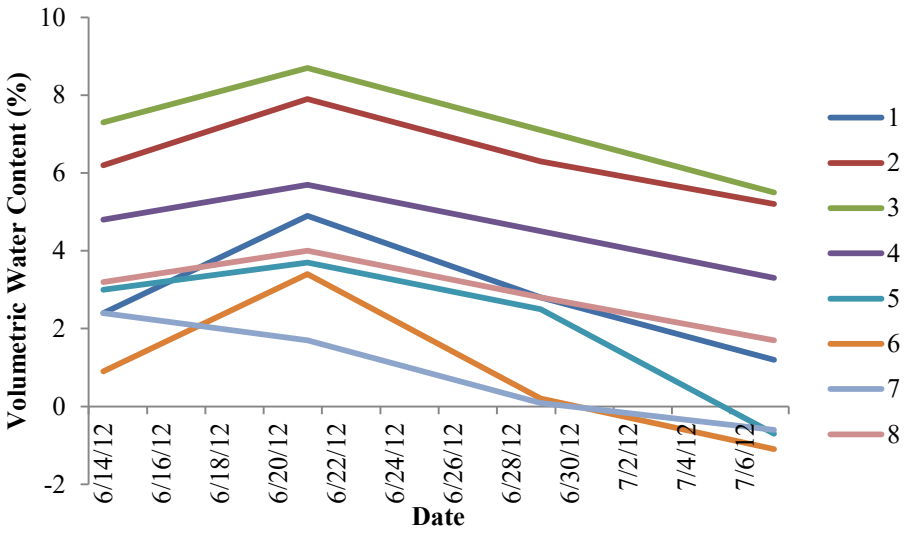
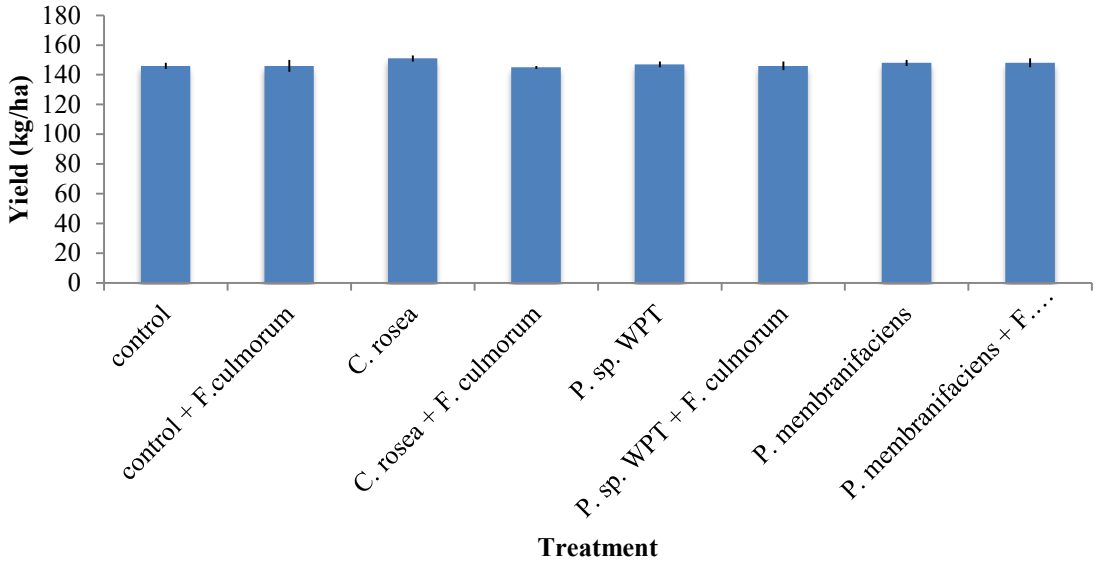


Figure 2.3. Soil moisture data from measurements taken during the growing season. Moisture is represented as volumetric water content (%) from 8 different probes, labeled 1 to 8, corresponding to geographic points measurements were taken at.



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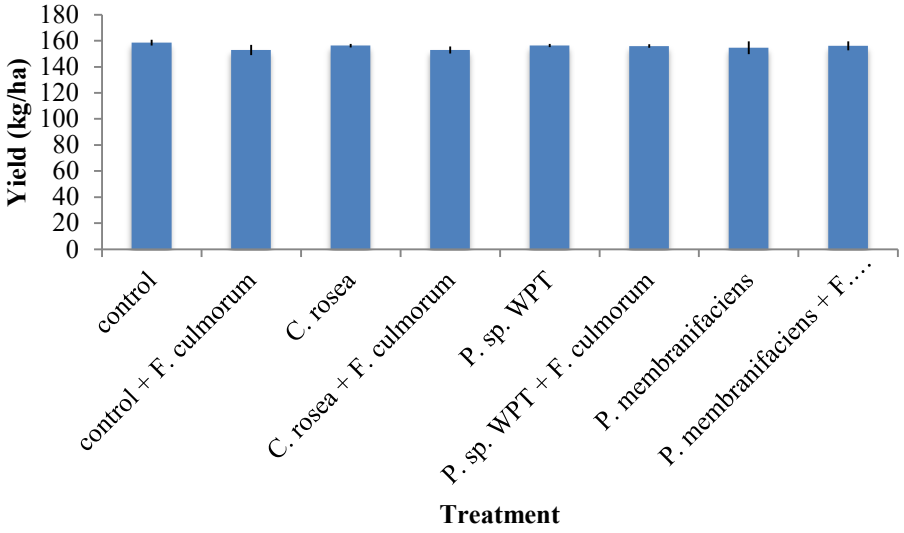
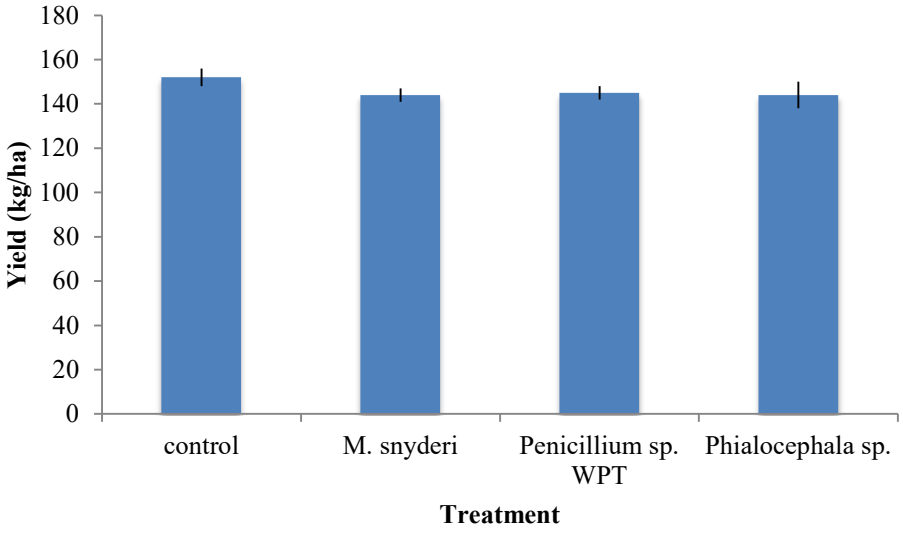


(b)

Figure 2.4. Mean yield (kg/ha) of UI SRG (a) and Stephens (b) pathogen and antagonist treatment combinations. Black bars represent standard errors of treatment combinations. No significant differences in yield were observed.



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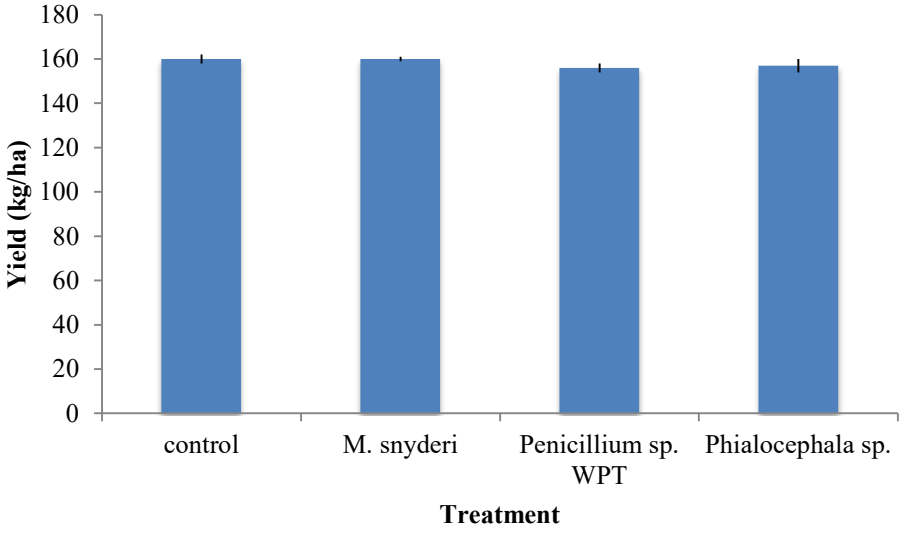
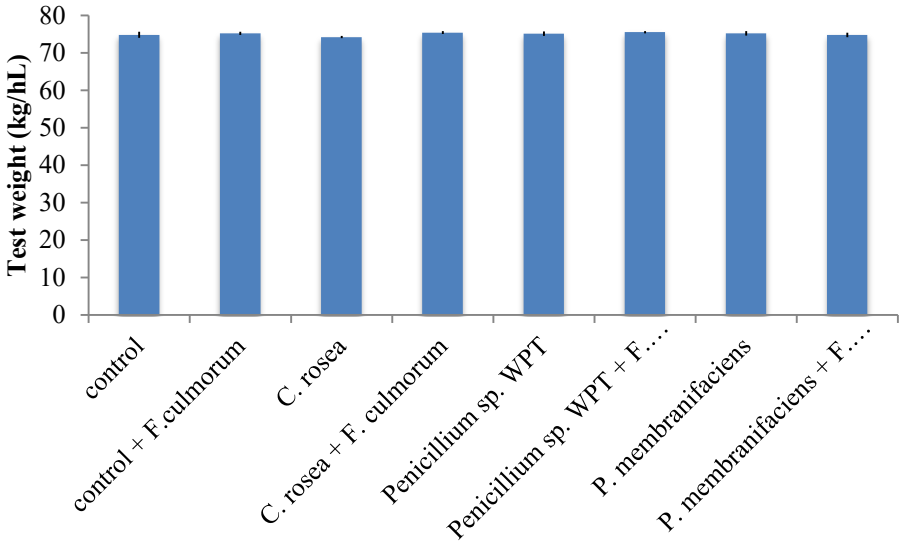


(b)

Figure 2.5. Mean yield (kg/ha) of UI SRG (a) and Stephens (b) plots treated with *M. snyderi, Penicillium* sp. WPT, *P. membranifaciens*, and a non-inoculated control. Black bars represent individual standard errors for each treatment. No significant differences in yield were observed.



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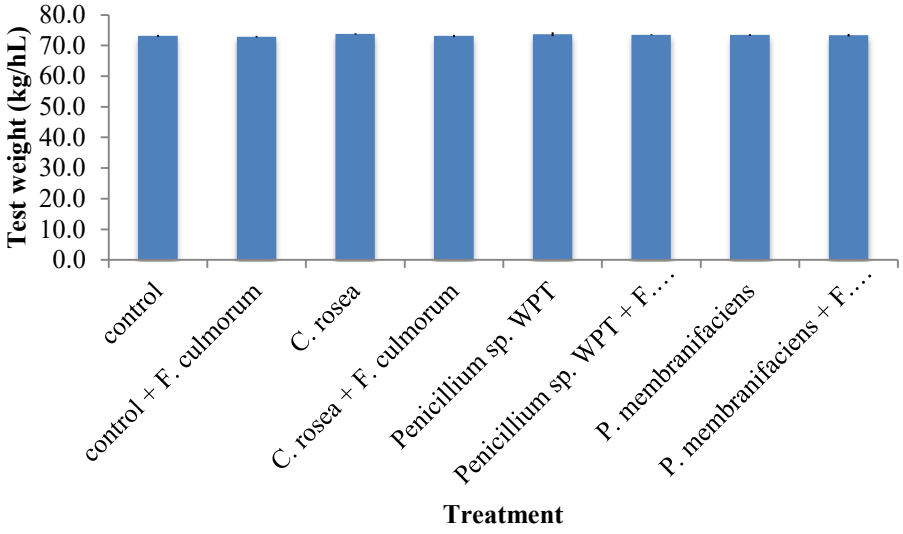
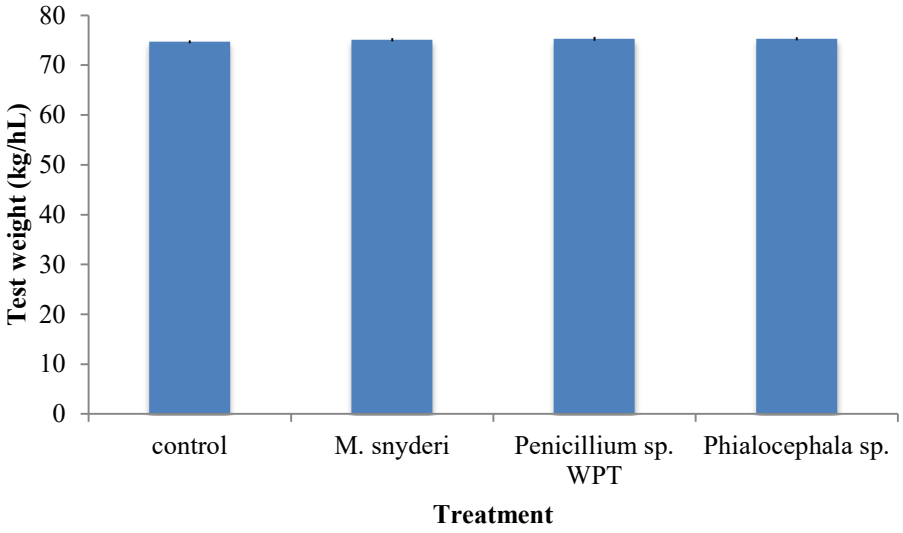


(b)

Figure 2.6. Test weight (kg/hL) for UI SRG (a) and Stephens (b) pathogen and antagonist treatment combinations. Standard errors for individual treatment combinations are represented by black bars. No significant differences in test weight were observed.



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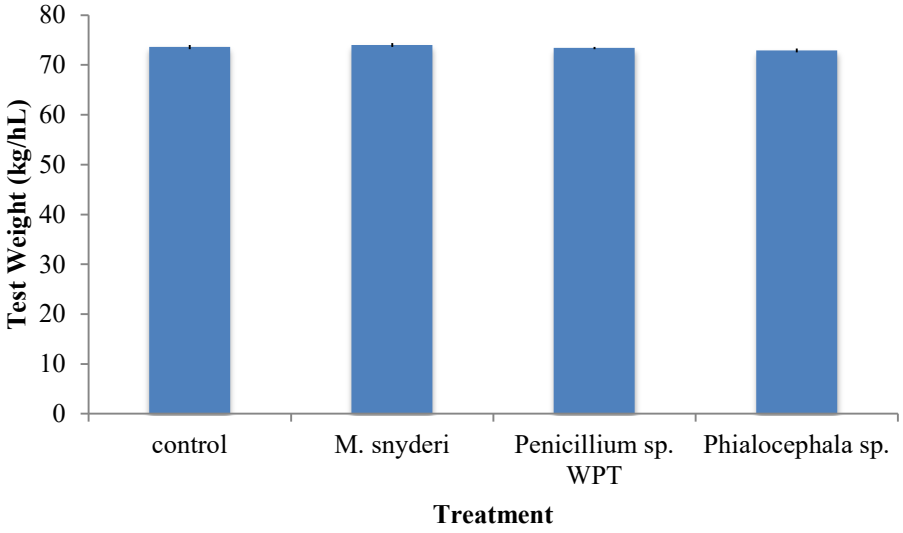
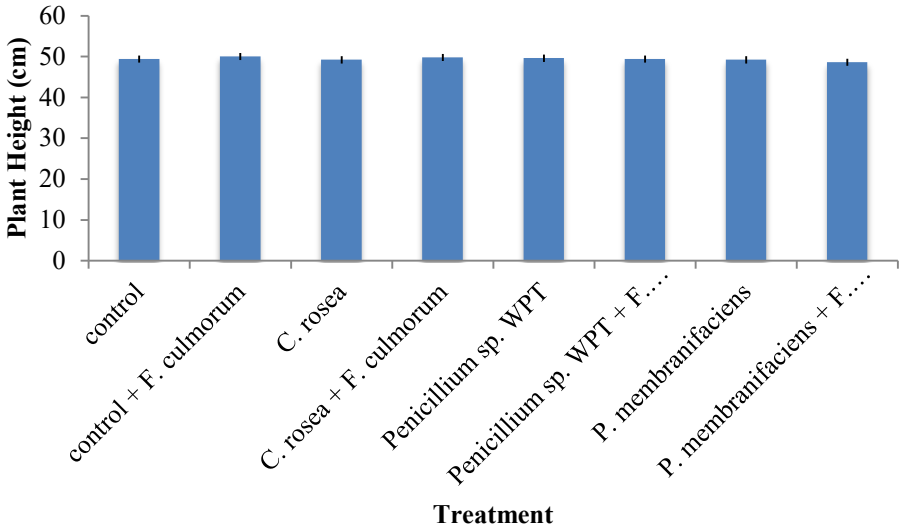


(b)

Figure 2.7. Test weights (kg/hL) for UI SRG (a) and Stephens (b) plots inoculated with physiologically beneficial endophytes. Standard errors of individual treatments are represented by black bars. No significant differences in test weight were observed.



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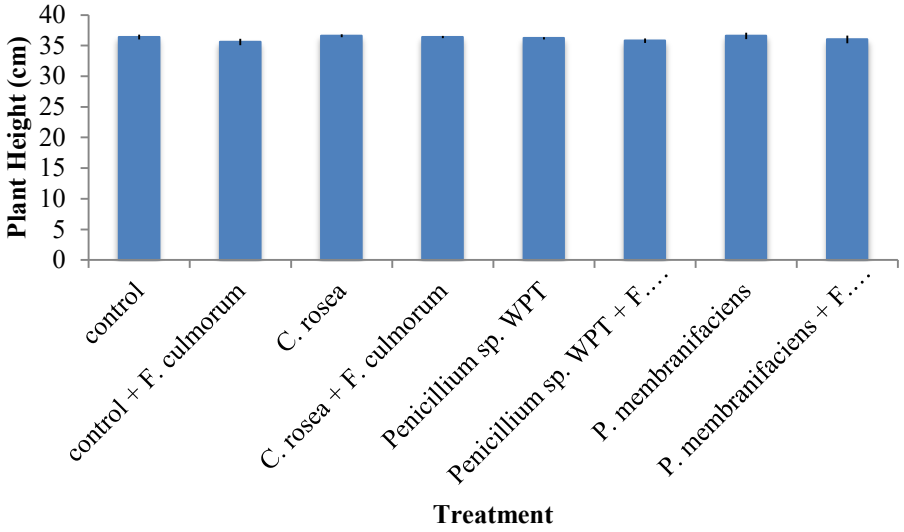
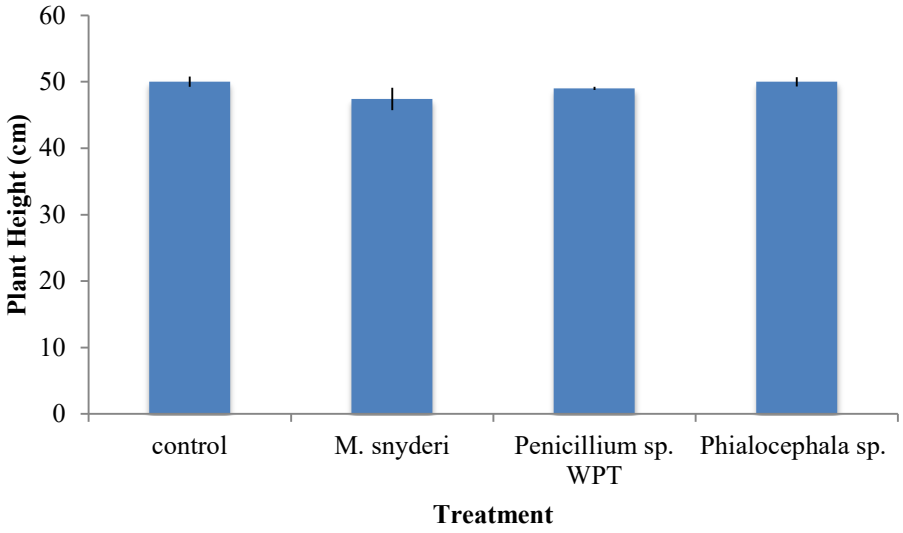


(b)

Figure 2.8. Mean plant height (cm) of UI SRG (a) and Stephens (b) plants among pathogen and antagonist treatment combinations. Black bars represent standard errors of individual treatment combinations. No significant differences in plant height were observed.



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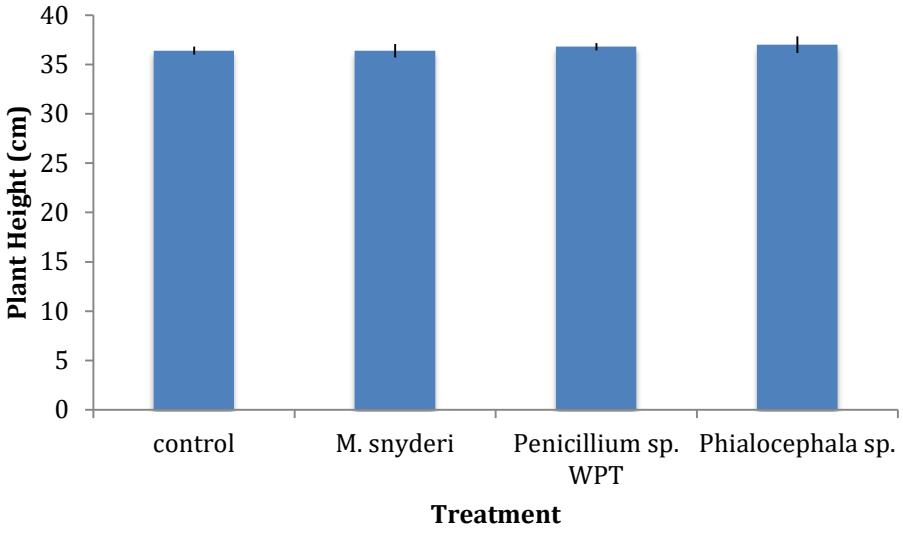
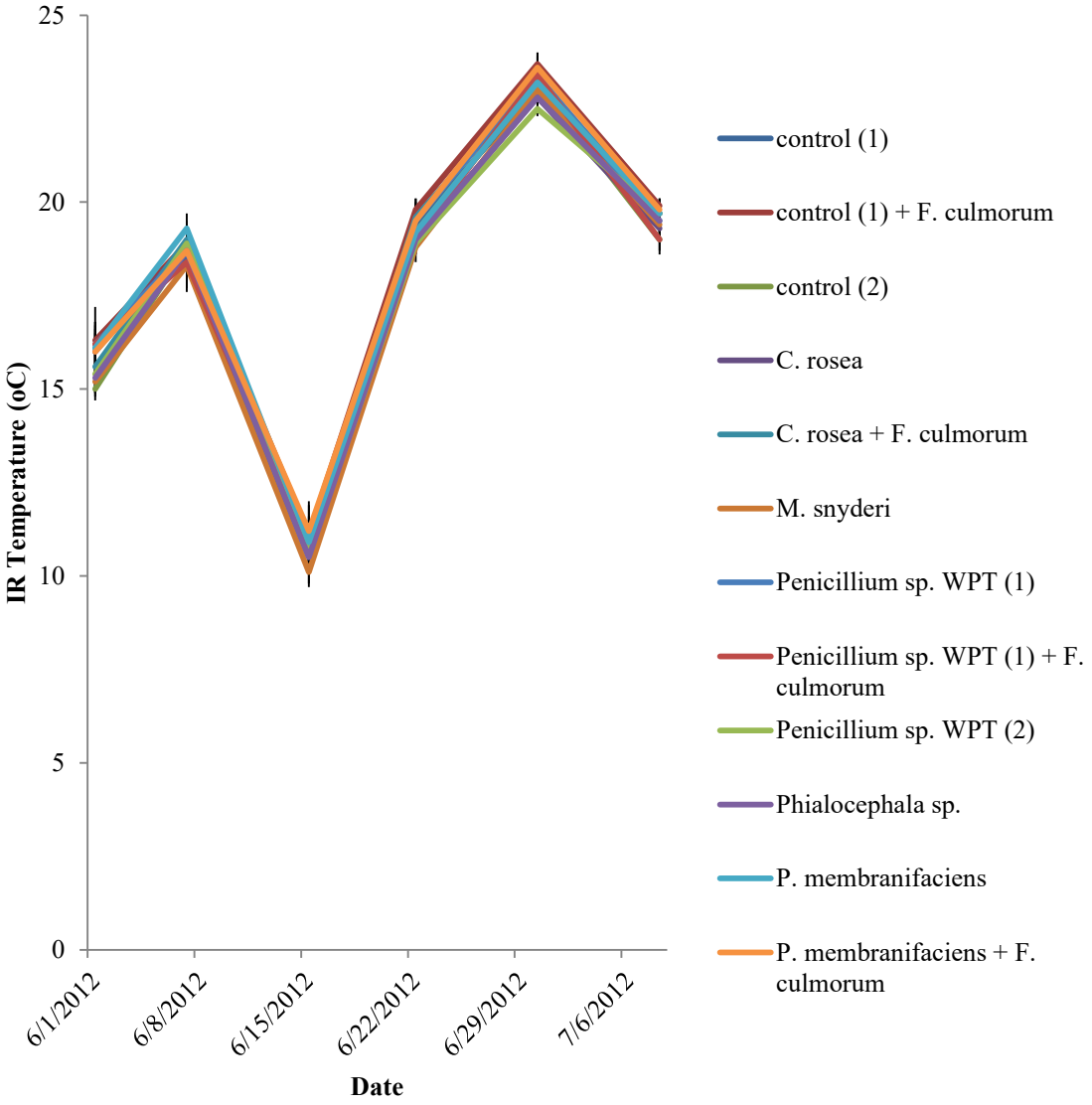


(b)

Figure 2.9. Mean plant height (cm) of UI SRG (a) and Stephens (b) plots treated with physiologically beneficial endophytes. Black bars represent standard errors for individual treatments. No significant differences in plant height were observed.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IR Temperature (oC)
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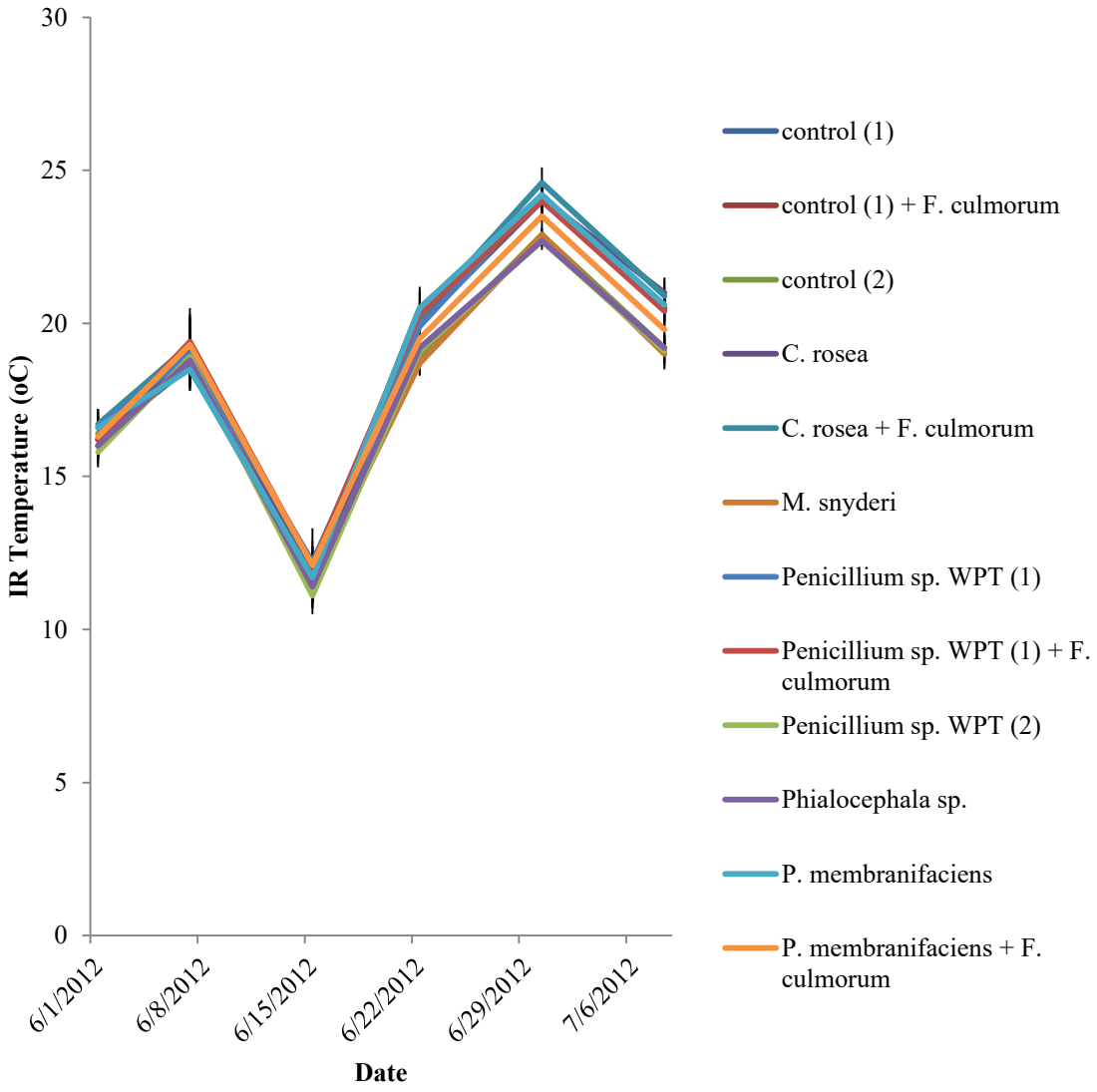
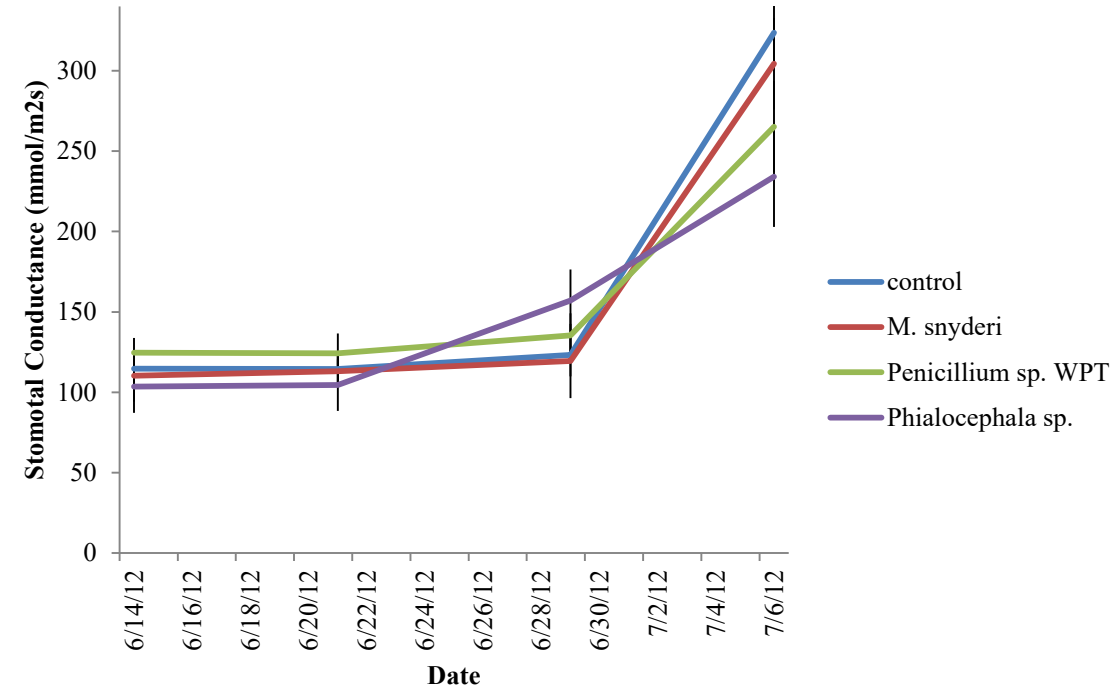
6/29/2012
7/6/2012
Date
control (1)
control (1) + F. culmorum
control (2)
C. rosea
C. rosea + F. culmorum
M. snyderi
Penicillium sp. WPT (1)
Penicillium sp. WPT (1) + F. culmorum
Penicillium sp. WPT (2)
Phialocephala sp.
P. membranifaciens
P. membranifaciens + F. culmorum

(a)

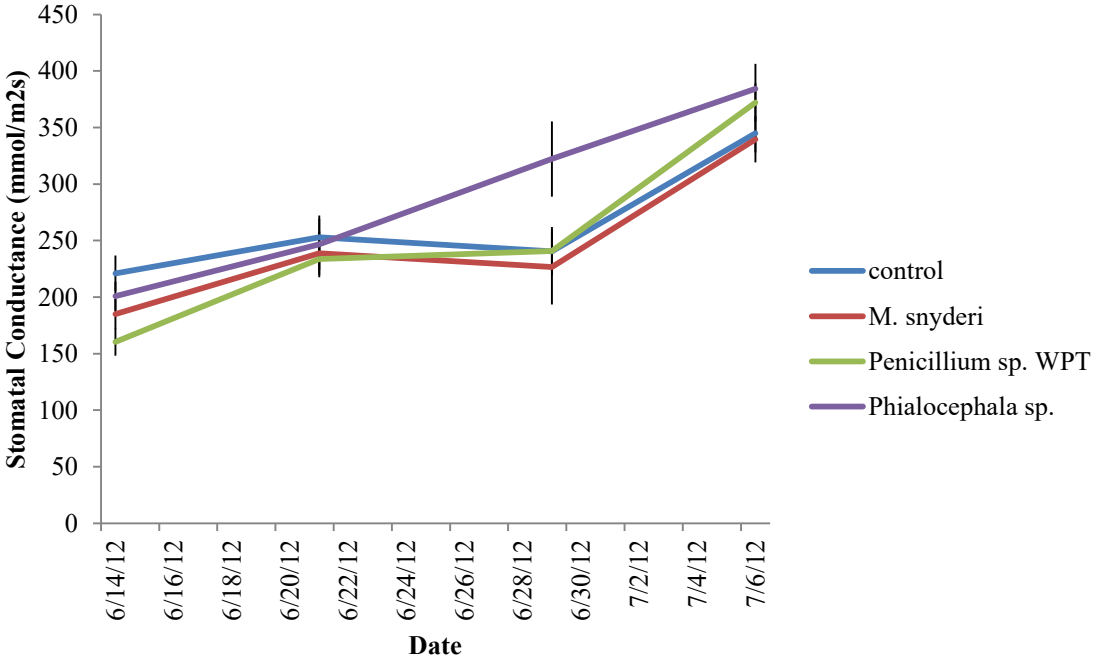


(b)

Figure 2.10. Mean infrared thermometry measurements for UI SRG (a) and Stephens (b) plots. Black bars represent standard errors of individual treatment combinations. No significant different in IR temperature were observed and thus plant stress was absent in this study. *Penicillium* sp. WPT (1) represents treatments from the study including pathogen inoculum while (2) represents the treatments in the study with physiologically beneficial endophytes.



(a)



(b)

Figure 2.11. Porometry data displaying mean stomatal conductance (mmol/m^2s) of UI SRG (a) and Stephens (b) plots treated with physiologically beneficial endophytes. Standard errors are shown as vertical black bars. No significant differences in conductance were seen.

Table 2.3. Foliar nutrient composition of N, P, K, Mg, Ca, S, and Na (%) and Fe, Mn, B, Cu, Zn, and NO_3N (ppm) among different antagonist treatments in low crown rot disease levels.

Variety	Nutrient	control	*M. snyderi*	*P.* sp. WPT	*P.* sp.
SRG	N (%)	3.92 ± 0.07	3.82 ± 0.05	3.85 ± 0.05	3.82 ± 0.06
	P (%)	0.25 ± 0.00	0.23 ± 0.00	0.24 ± 0.00	0.23 ± 0.01
	K (%)	2.12 ± 0.04	2.06 ± 0.052	2.04 ± 0.02	1.98 ± 0.07
	Mg (%)	0.15 ± 0.00	0.14 ± 0.00	0.15 ± 0.00	0.14 ± 0.01
	Ca (%)	0.35 ± 0.01	0.33 ± 0.01	0.34 ± 0.01	0.32 ± 0.07
	S (%)	0.29 ± 0.00	0.29 ± 0.01	0.29 ± 0.00	0.28 ± 0.01
	Na (%)	0.002 ± 0.000	0.002 ± 0.000	0.002 ± 0.000	0.002 ± 0.000
	Fe (ppm)	111 ± 9	95 ± 1	99 ± 2	96 ± 3
	Mn (ppm)	56 ± 2	51 ± 1	54 ± 2	51 ± 2
	B (ppm)	3 ± 0	3 ± 0	3 ± 0	3 ± 0
	Cu (ppm)	5 ± 0	4 ± 0	5 ± 0	5 ± 0
	Zn (ppm)	15 ± 0	15 ± 0	15 ± 0	14 ± 0
	NO_3N (ppm)	134 ± 25	109 ± 16	109 ± 18	135 ± 26
Stephens	N (%)	3.84 ± 0.04	3.88 ± 0.03	3.91 ± 0.05	3.94 ± 0.05
	P (%)	0.24 ± 0.00	0.23 ± 0.01	0.24 ± 0.00	0.24 ± 0.00
	K (%)	1.50 ± 0.03	1.47 ± 0.04	1.51 ± 0.04	1.53 ± 0.03
	Mg (%)	0.18 ± 0.02	0.18 ± 0.00	0.18 ± 0.00	0.17 ± 0.00
	Ca (%)	0.53 ± 0.02	0.52 ± 0.02	0.50 ± 0.02	0.51 ± 0.02
	S (%)	0.29 ± 0.00	0.28 ± 0.00	0.29 ± 0.01	0.29 ± 0.01
	Na (%)	0.002 ± 0.000	0.002 ±0.000	0.002 ± 0.000	0.002 ± 0.000
	Fe (ppm)	90 ± 2	89 ± 3	97 ± 5	89 ± 2

Mn (ppm)	95 ± 2	93 ± 2	93 ± 3	92 ± 3
B (ppm)	4 ± 0	4 ± 0	4 ± 0	4 ± 0
Cu (ppm)	5 ± 0	5 ± 0	5 ± 0	5 ± 0
Zn (ppm)	16 ± 0	16 ± 0	16 ± 0	16 ± 0
NO_3N (ppm)	42 ± 3	40 ± 5	51 ± 7	52 ± 10

Table 2.4. Mean soil nutrient composition and fertilizer recommendations for the field site.

	Mean ± SE
Organic Matter (%)	3.4 ± 0.0
P_1 (ppm)	38 ± 1
P_2 (ppm)	89 ± 2
K (ppm)	270 ± 8
Mg (ppm)	197 ± 5
Ca (ppm)	1569 ± 35
Soil pH	5.4 ± 0.0
Buffer index	6.4 ± 0.1
CEC (meg/100g)	14.4 ± 0.3
K Sat (%)	4.8 ±0.1
Mg Sat (%)	11.4 ±0.1
Ca Sat (%)	54.6 ± 0.5
H Sat (%)	29.2 ± 0.7
Nox (ppm)	3 ± 0
Nox (lbs/A)	6 ± 0
NH_3 (ppm)	4 ± 0
N (lbs/A)	80 ± 1
Lime	4725 ±580
Phosphate (lbs/A)	7 ± 1

Chapter 3

Endophytes contribute to tolerance of Fusarium head blight by modifying disease severity but not yield of wheat

Abstract

Microbes that impact plant disease are known as disease modifiers. Antagonists are disease modifiers that reduce disease severity whereas facilitators increase disease. We have assumed disease modifiers affect both growth and yield (fecundity) of their hosts by affecting disease severity. Here we employed endophytes expected to modify disease severity of Fusarium head blight of wheat. Plants were first inoculated with endophytes and then treated with either *F. culmorum* or *F. graminearum*, the primary causal agents of Fusarium head blight. Disease was scored using a Horsfall-Barratt scale. At maturity, heads were harvested and weighed. Each species of *Fusarium* did increase disease without affecting yield so both cultivars were to some extent tolerant. However, endophytes also significantly modified disease severity further without affecting yield. Two inoculants antagonized *Fusarium graminearum* by decreasing severity of head blight, but in so doing they decreased tolerance. On the other hand, a third inoculant increased tolerance by facilitating *Fusarium culmorum* and increasing disease severity without affecting yield. Overall, our findings indicate that disease tolerance may be more complex than previously believed because endophytes can modify it in both directions.

Introduction

Resistance and tolerance are two mechanisms plant hosts can use to modify disease levels that improve host fitness and are similar, but distinct. There are multiple definitions of resistance; here, resistance refers to traits that prevent or limit pathogen infection while tolerance refers to traits that do not affect infection, but reduce or limit fitness costs to the host (Roy and Kirchner 2000). By these definitions, if differences in both yield and disease severity are observed then resistance is impacted because infection levels are impacted and there should therefore be differences observed in both variables. Whereas, if differences in disease severity, but not yield, are observed then tolerance is impacted because infection is not affected, as reflected by differences in disease severity, yet costs to fecundity do not occur. Resistance and tolerance are also different in that resistance puts selective pressure on a pathogen to evolve while tolerance does not. Disease modifiers can also alter disease severity in plant hosts and have been recorded in many pathosystems (Busby et al 2016). Antagonism occurs when disease severity is reduced, while facilitation occurs when the severity of disease is increased. Whether disease modifiers affect resistance or tolerance has been largely unaddressed, yet we have assumed that impacts are on resistance. If yield and disease severity are affected by inoculant treatment, then inoculants impact resistance. If disease severity, but not yield, is affected by inoculant treatment, then inoculants impact tolerance as infection still occurs, but costs are limited as reflected in fecundity. Here, we use the Fusarium head blight-wheat pathosystem to address the potential impacts of disease modifiers on resistance and tolerance.

Fusarium head blight is the most significant disease of wheat in the United States (McMullen et al 1997). It can be caused by several species, but the two primary causal agents are *Fusarium graminearum* and *F. culmorum. Gibberella zeae,* the teleomorph of *F. graminearum,* also can cause disease; there has not been a teleomorph of *F. culmorum* observed (Obanor et al 2010). Infection of wheat heads occurs at anthesis and disease develops through kernel-filling (Osborne and Stein 2007). Severe disease tends to develop when conditions are humid and warm in the spring and summer months (Sutton 1982). Given the significant economic losses from Fusarium head blight and the concern for mycotoxin buildup in grain, antagonism of the disease has been of interest. Xue et al (2009) showed that a strain of *Clonostachys rosea* could significantly reduce the severity of Fusarium head blight caused by *Gibberella zeae* in wheat. In another study, antagonists belonging to the bacterial genus *Lactobacillus* significantly reduced the severity of Fusarium head blight caused by five different *Fusarium* species, including *F. graminearum* and *F. culmorum,* in durum wheat (Baffoni et al 2015). As neither of these studies reported yield, the effects of disease modifiers on resistance and tolerance are unknown.

Here, demonstrated antagonists of *Fusarium culmorum* causing Fusarium crown rot in wheat are applied in the context of Fusarium head blight. The hypothesis was that antagonists would have general interactions with the host, pathogen, and microhabitat making them affective controls for Fusarium head blight. In the first study, *F. culmorum* inoculum was used to induce Fusarium head blight in controlled greenhouse conditions; a second set of similar studies explored antagonism using *F. graminearum*. Antagonists previously shown to antagonize *F. culmorum,* in addition to a novel *Chaetomium* sp., were used as endophyte treatments. Factors including host variety, pathogen species, endophyte

application method, and environmental conditions were modified with experimentation to promote disease. The Fusarium head blight susceptible hard red winter wheat variety UI SRG was used in the first study and the moderately resistant soft white spring wheat variety UI Stone in the second set. Yield and disease severity were measured and can be used to determine the effects of disease modifiers on resistance and tolerance.

Materials and Methods

Seed and Inocula Source

Wheat (*Triticum aestivum*) seed of the hard red winter wheat variety UI SRG and soft white spring wheat variety UI Stone were sourced from certified foundation seed. Seed were planted in one-gallon pots with a soilless mix (Sungro, Agawam, MA). Each plant was fertilized every two weeks with 350 mL of 200 ppm N. Fungal isolates of *F. culmorum, F. graminearum, Chaetomium* sp.*, C. rosea*, *Penicillium* sp. WPT, and *P. membranifaciens* were replicated on potato dextrose agar from pure cultures (Ridout and Newcombe 2016).

Inocula Preparation

All fungal isolates were grown to maturity before being washed with sterile distilled water and a surface sterilized bent glass rod used to dislodge spores. Three drops of polysorbate 20 were mixed into each suspended spore solution to increase the dispersal of spores in solution by reducing hydrophobicity. A hemacytometer (Fisher Scientific, Asheville, NC) was used to calculate spore concentrations (Table 3.1).

Inoculations

UI SRG: *Chaetomium* sp., *Clonostachys rosea,* and *Pichia membranifaciens*

In the susceptible variety UI SRG, disease modifier inoculations were done at stage one of the Feekes scale when plants had two leaves or more (Large 1954). Equal volumes of suspended spore solutions were liberally applied to seedling crowns and incubated in polyethylene moisture chambers overnight. In tandem with anthesis, approximately 1 mL of suspended *F. culmorum* inoculum was applied to each pathogen treated head. Controls were inoculated with sterile distilled water. Heads were incubated overnight in polyethylene moisture chambers. After inoculations, plants were arranged with treatments replicated once each in 20 blocks. *F. culmorum* inoculations were done as wheat heads went to anthesis using these procedures. After endophyte and pathogen inoculation additional humid conditions, which are associated with significant Fusarium head blight development, such as mist irrigation systems were not employed.

UI Stone: *Penicillium* sp. WPT and *Clonostachys rosea*

In the moderately resistant variety UI Stone, approximately 1 mL of suspended antagonist spore solution was applied to individual heads at anthesis, or stage 10.5 on the Feekes scale (Large 1954). Heads were covered with polyethylene bags and incubated overnight. After 24 hours, approximately 1 mL of suspended *F. graminearum* inoculum was applied to each head. Controls were inoculated with equal volumes of sterile distilled water. Polyethylene bags were used to incubate heads overnight. Plants were completely randomized on greenhouse benches equipped with a mist irrigation system set to run four times a day every

6 hours for two minutes to mimic humid conditions conducive to Fusarium head blight development.

Disease Severity

Disease levels in heads were scored when symptoms ceased progressing, but prior to senescence. A Horsfall-Barratt scale was implemented to visually estimate the percent of infected kernels in each head (Stack and McMullen 2010). Heads were placed into one of 10 score categories based on the percent of infected wheat spikelets per spike- 0, 7, 14, 21, 33, 50, 66, 79, 90, and 100%. By measuring counts of heads in each category, severity of disease can be analyzed. At maturity, individual heads were harvested, cured, and processed. Yield per head (g) was measured to determine if there were any significant differences between pathogen and disease modifier treatment combinations. Significant differences in yield would reflect benefits or costs to overall plant fecundity and can thus be used a metric to determine differences in disease severity.

Data Analyses

R version 3.2.3 was used to analyze the data. A completely randomized block design was used for the UI SRG trial while UI Stone studies used a completely randomized experimental design because they were small and with fewer treatment combinations making blocking unnecessary. Analyses of variance were conducted to test for significant differences in yield per head between different treatment combinations. Residual and normal plots were used to confirm data were normally distributed with equal variance. The distribution of disease score data showed the data were right-skewed with many observations of zero, so the data were

analyzed using the non-parametric Kruskal-Wallis one-way analysis of variance. An alpha value of 0.05 was used as the threshold for significance.

Results

Yield was not impacted by pathogens or disease modifiers. The analyses of variance showed no significant treatment effects of disease modifiers, pathogens, or time of inoculation on fecundity as measured by yield per head in any study (Figures 3.1-3.3a). Count data were right-skewed with many observations having a disease severity of 0% meaning that the incidence of disease was relatively low (Figures 3.1-3.3c). Yet, a greater proportion of pathogen treated heads scored in higher non-zero score categories than in controls and some endophyte and pathogen treatment combinations resulted in differences in the proportions of scores in different score categories. Additionally, significant differences in disease severity were observed in all studies (Figures 3.1-3.3b). Among pathogen treated UI SRG heads the average percent coverage with Fusarium head blight was 14% while it was 2% in the pathogen control (Figure 3.1b). In the *Penicillium* sp. WPT study in UI Stone, heads treated with the pathogen averaged 36% coverage while it was 0% in the pathogen control (Figure 3.2b). In the *C. rosea* study in UI Stone, average coverage was 32% in pathogen treated heads and 2% in pathogen controls (Figure 3.3b). The Kruskal-Wallis test indicated significant differences in levels of disease were obtained between control and pathogen treated heads in the UI SRG study ($X^2 = 12.499$, $P = 4.071E^{-4}$). Significant differences in disease levels between pathogen treated and control heads also developed in the *Penicillium* sp. WPT and *C. rosea* UI Stone studies ($X^2 = 30.553$, $P = 3.249E^{-8}$; $X^2 = 81.245$, $P < 2.2E^{-16}$,

respectively). Among pathogen treated UI SRG heads, disease severity of *Chaetomium* sp. treated heads was significantly greater than endophyte controls treated with the pathogen (X^2 = 5.2974, P = 2.136E^{-2}). Specifically, endophyte controls averaged 14% disease severity, while *Chaetomium* sp. treated heads averaged 42% (Figure 3.1b). Treatment with *C. rosea* and *P. membranifaciens* in this study did not result in significant differences in disease severity. Among pathogen treated UI Stone heads, disease severity was significantly reduced by the endophytes *Penicillium* sp. WPT and *C. rosea* (X^2= 16.637, P = 4.527E^{-5}; X^2 = 40.803, P = 1.684E^{-10}, respectively). In the *Penicillium* sp. WPT study, endophyte control heads treated with the pathogen averaged 36% disease severity whereas pathogen treated heads also treated with *Penicillium* sp. WPT averaged 6% (Figure 3.2b). Similarly, pathogen treated heads in the *C. rosea* study in UI Stone averaged 32% while pathogen treated heads also treated with *C. rosea* showed less disease averaging 10% (Figure 3.3b). In summary, yield was not affected by any treatment combination, yet disease severity was increased by pathogen inoculation, relative to the control, and some disease modifiers either increased or decreased disease levels.

Discussion

It was hypothesized endophytes would modify disease of Fusarium head blight, measured by yield and disease severity, as some did in Fusarium crown rot. There were no significant treatment effects of pathogen, endophytes, or time of inoculation on fecundity as measured by yield. However, pathogen inoculation significantly increased disease severity in all studies. By definition, UI SRG and UI Stone are to some extent tolerant to Fusarium head

blight because infection occurred without adverse affects on fecundity (Roy and Kirschner 2000; Simms and Triplett 1994). This is interesting given UI SRG and UI Stone have been previously classified as susceptible and moderately resistant, respectively, to Fusarium head blight. To our knowledge this is the first demonstration of tolerance in either variety. Some pathogen and endophyte treatment combinations also resulted in variable disease severity including *Chaetomium* sp., *Penicillium* sp. WPT, and *C. rosea.* By definition, these disease modifiers can impact tolerance (Roy and Kirschner 2000; Simms and Triplett 1994). As many disease modification studies have only addressed disease severity and yield was not measured (Busby et al 2016), resistance and tolerance have been largely unaddressed. Among disease modification studies where yield and disease severity were measured, disease modifiers have only been shown to impact resistance as yield was improved and disease severity reduced when it was otherwise severe (Droby et al 2002; Ridout and Newcombe 2016). It also has been assumed disease modifiers either impact host resistance or directly interact with the pathogen.

Here, we demonstrate that disease modifiers can also modify disease by impacting host tolerance. *Chaetomium* sp. facilitated disease severity as significant increases were seen in pathogen and endophyte treated heads relative to the endophyte control treated with the pathogen. *Penicillium* sp. WPT and *C. rosea* antagonized disease severity as significant reductions in disease severity were observed in coinoculations of pathogen and endophyte treated heads relative to the endophyte control treated with the pathogen. Antagonism of the casual agent of Fusarium head blight by *C. rosea* demonstrated in the moderately resistant variety UI Stone is consistent with the findings of Xue et al (2009) where disease severity was reduced; however, yield was not measured in this study. All of these disease modifiers

impacted tolerance because changes in disease severity were observed, but not fecundity (Roy and Kirschner 2000; Simms and Triplett 1994). Disease modifiers impacted tolerance differently based on whether they facilitated or antagonized the pathogen.

The effect of disease modifiers on tolerance must be considered in light of both its impact on fecundity as well as disease severity. Facilitators increase tolerance because fecundity remains unaffected in higher disease levels. Antagonists decrease tolerance because lower disease levels are observed without differences in fecundity being observed. In the case of these studies, the facilitator *Chaetomium* sp. increased tolerance as fecundity was not impacted in higher disease levels. The antagonists *Penicillium* sp. WPT and *C. rosea* reduced tolerance because fecundity was not impacted in lower disease levels. These experiments were not designed to address why these patterns were observed and the literature is lacking in studies that do so.

Primarily, it is problematic that a number of variables were varied between experiments to promote disease development (Table 3.2). These included host variety, pathogen species, environmental conditions, and the point of endophyte inoculation on the Feekes scale. Based on the recommendations of the Fusarium head blight expert Dr. Juliet Marshall from the University of Idaho Extension Program, host variety was changed, endophyte inoculation time, and a mist irrigation system created to promote disease development. We switched pathogen species from *Fusarium culmorum* to *Fusarium graminearum* because the later produces mycotoxins that are of significant concern on regional, national, and global scales. Because of the exploratory nature of this research, conclusions outside of antagonism and facilitation, and resistance and tolerance cannot be made. Patterns of specificity between host, pathogen, endophyte, and microhabitat could be

explored in future studies by standardizing these variables. Future disease modification studies would benefit from measuring yield to determine whether disease modifiers impact resistance or tolerance.

Conclusions

Future disease modification studies would benefit from measuring fecundity so that conclusions may be drawn regarding inoculant impacts on resistance and tolerance. Future studies should also standardize host, pathogen, inoculant, and microhabitat factors so that we may begin to examine how disease modifiers can impact tolerance.

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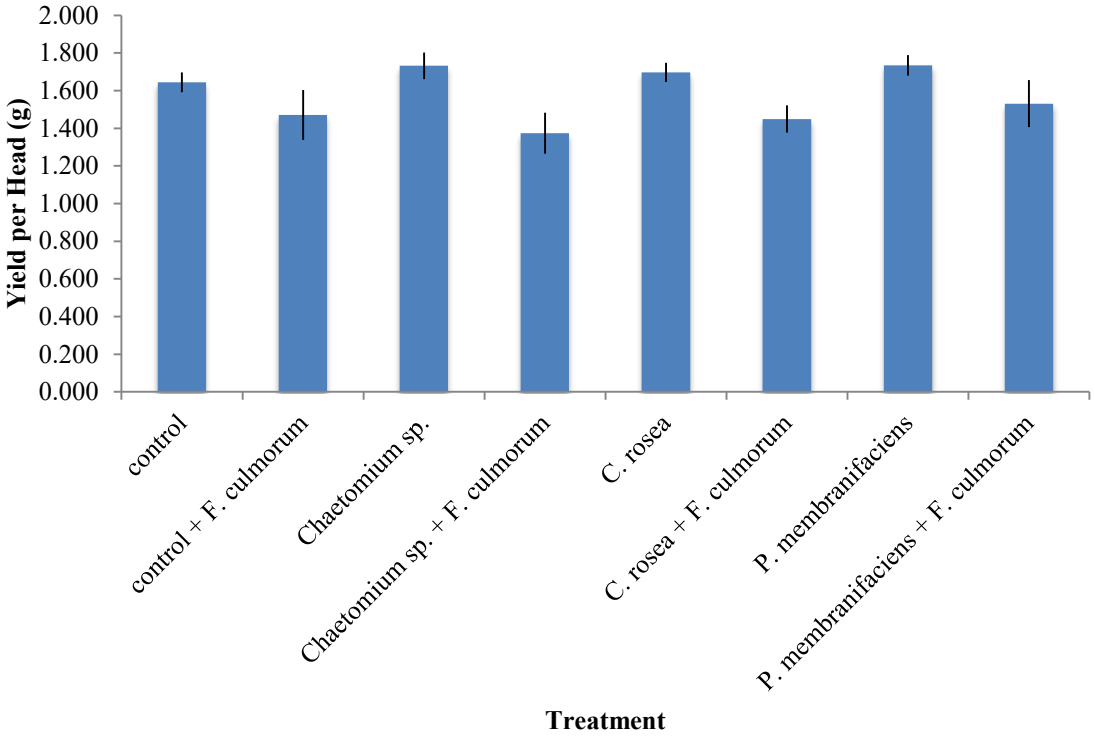
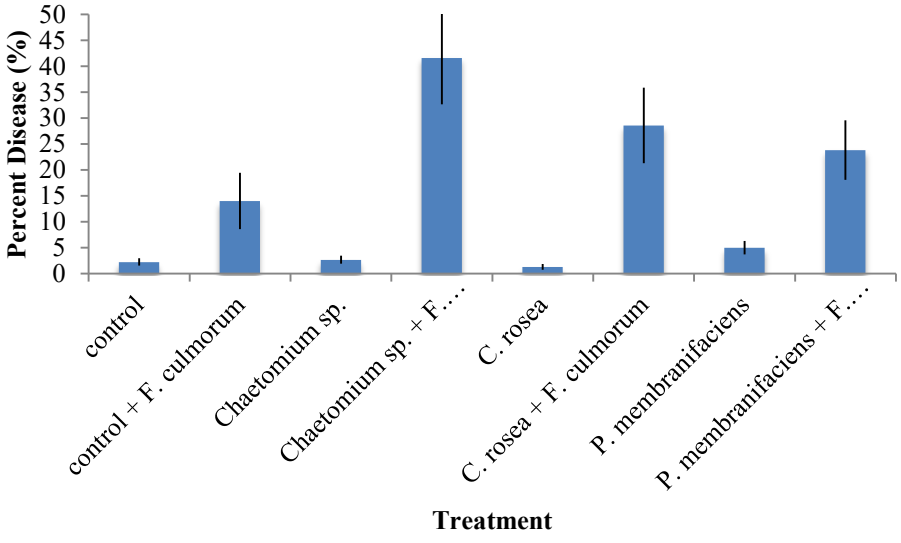
Tables and Figures

Table 3.1. Concentrations of antagonist spore solutions for each study and inoculation time.

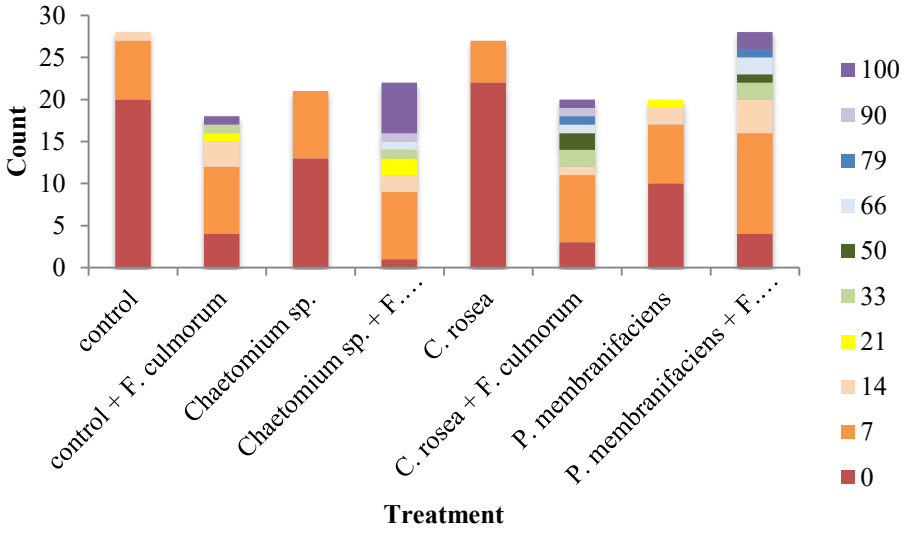
Experiment	Inoculant Solution	Treatment	Concentration
UI SRG	1	control	0
	1	*Chaetomium* sp.	$6.14E^{6}$
	1	*C. rosea*	$3.14E^{7}$
	1	*P. membranifaciens*	$2.27E^{7}$
	1	*F. culmorum*	$2.50E^{5}$
UI Stone-	1	control	0
Penicillium	1	*Penicillium* sp. WPT	$1.64E^{6}$
sp. WPT	1	*F. culmorum*	$4.20E^{5}$
	2	control	0
	2	*Penicillium* sp. WPT	$1.39E^{6}$
	2	*F. culmorum*	$3.90E^{5}$
UI Stone-	1	control	0
C. rosea	1	*C. rosea*	$7.70E^{6}$
	1	*F. culmorum*	$1.44E^{6}$


Yield per Head (g)
2.000
1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
control
control + F. culmorum
Chaetomium sp.
Chaetomium sp. + F. culmorum
C. rosea
C. rosea + F. culmorum
P. membranifaciens
P. membranifaciens + F. culmorum
Treatment

(a)

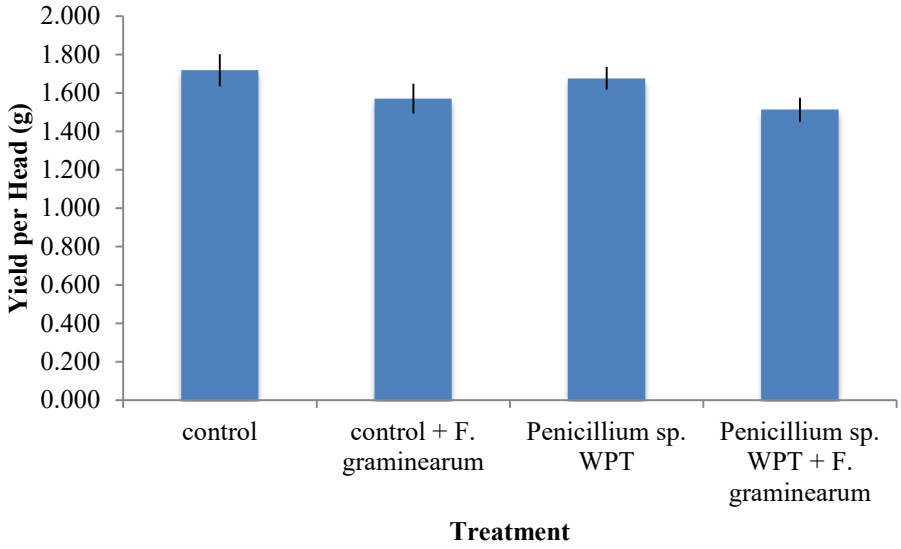

Percent Disease (%)
50
45
40
35
30
25
20
15
10
5
0
control
control + F. culmorum
Chaetomium sp.
Chaetomium sp. + F.…
C. rosea
C. rosea + F. culmorum
P. membranifaciens
P. membranifaciens + F.…
Treatment

(b)

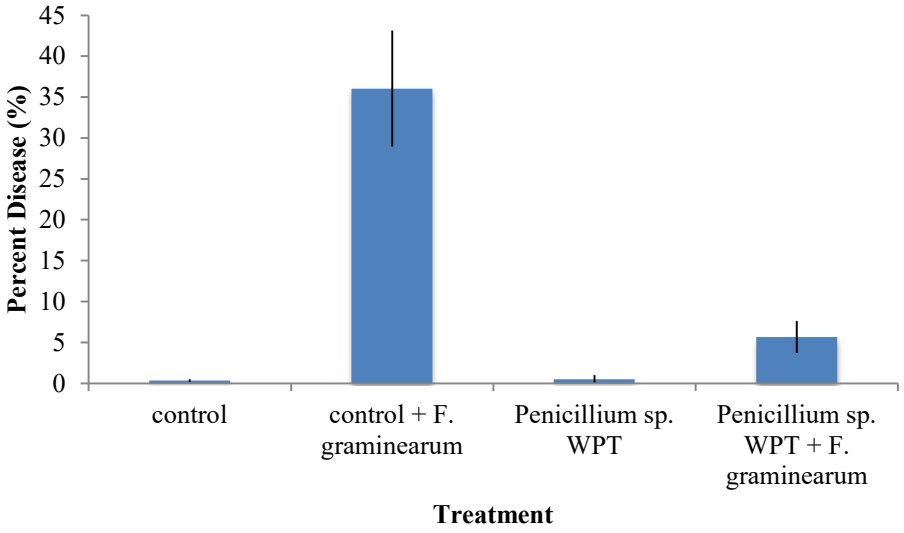


(c)

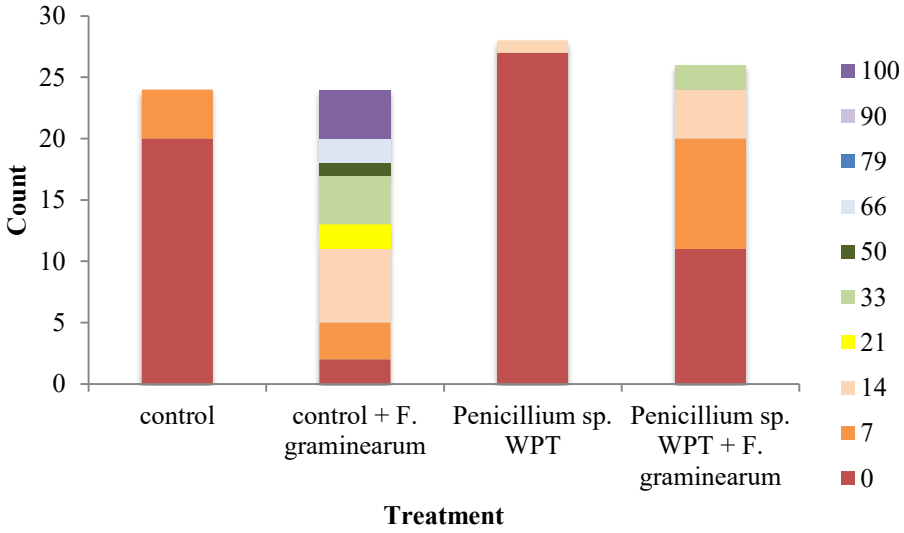
Figure 3.1. Mean yield per head (g) in the UI SRG study **(a),** mean percent infection of heads (%) with Fusarium head blight symptoms **(b)**, and count data for heads in 10 different score categories **(c)**. Black bars are standard errors for individual treatment combinations.



(a)

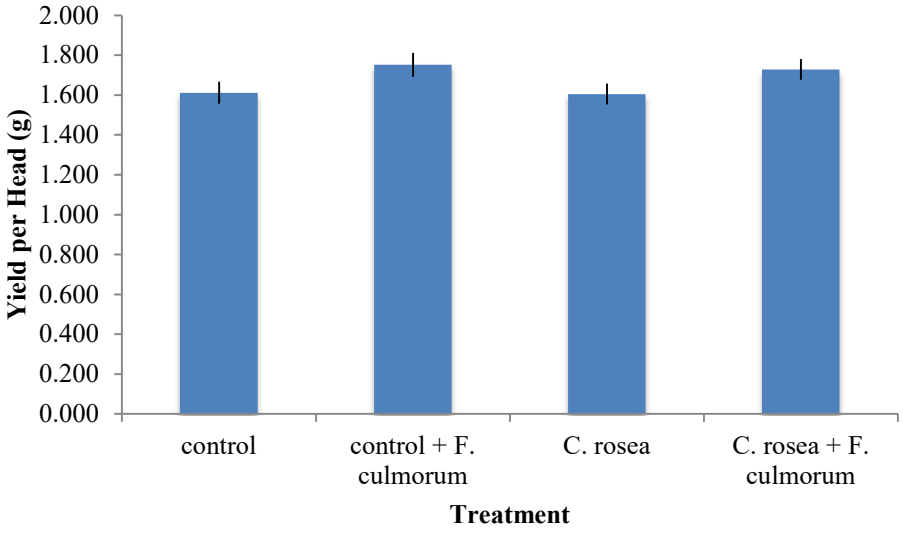


(b)

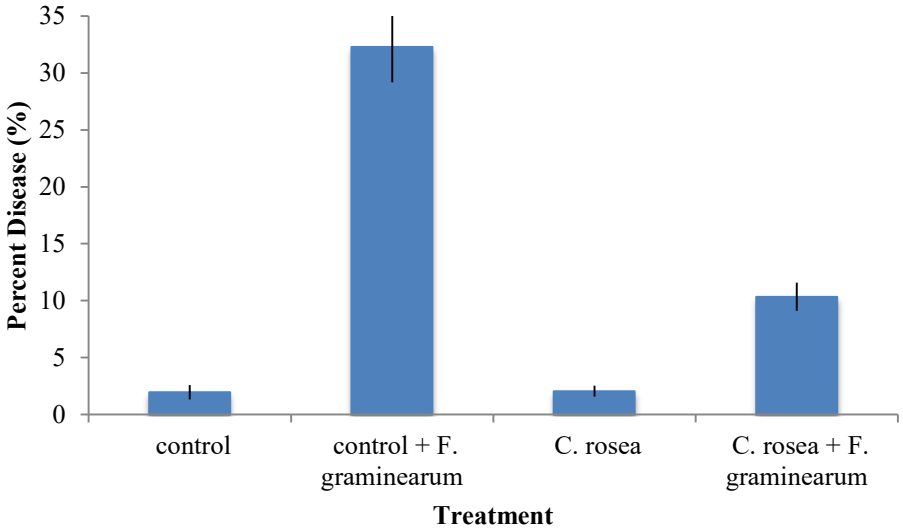


(c)

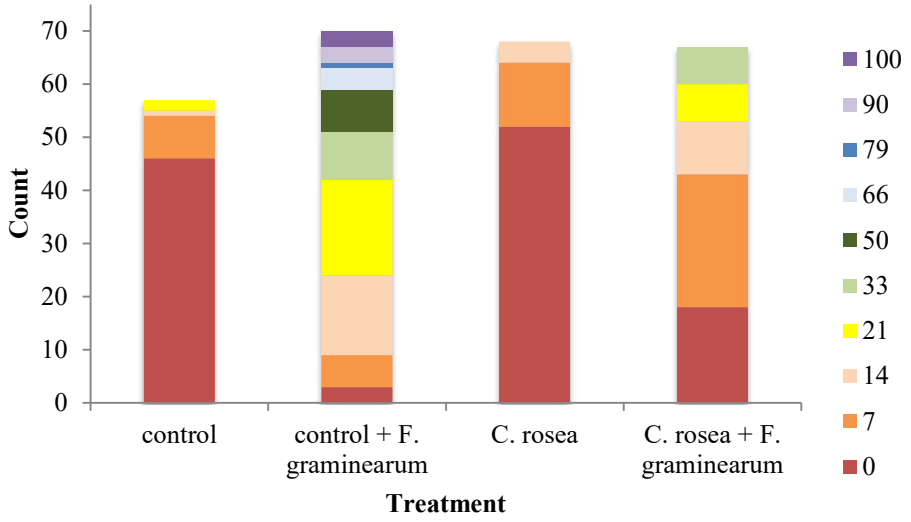
Figure 3.2. Mean yield per head (g) in the UI Stone-*P.* sp. WPT study **(a)** and mean percent infection of heads (%) with Fusarium head blight **(b)**, and count data for heads in 10 different score categories **(c)**. Black bars are standard errors for individual treatment combinations.


2.000
1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
Yield per Head (g)
control
control + F. culmorum
C. rosea
C. rosea + F. culmorum
Treatment

(a)


35
30
25
20
15
10
5
0
Percent Disease (%)
control
control + F. graminearum
C. rosea
C. rosea + F. graminearum
Treatment

(b)



(c)

Figure 3.3. Mean yield per head (g) in the UI Stone-*C. rosea* study **(a)** and mean percent cover of head (%) with Fusarium head blight **(b)**, and count data for heads in 10 different score categories **(c)**. Black bars are standard errors for individual treatment combinations.

Table 3.2. List of factors changed in different experiments to facilitate Fusarium head blight. The ways in which each factor was changed are briefly described.

Variety	Resistance	Endophyte	Endophyte	Pathogen	Irrigation
UI SRG	susceptible	*Chaetomium* sp.	crown	*F. culmorum*	NA
	susceptible	*C. rosea*	crown	*F. culmorum*	NA
	susceptible	*P. membranifaciens*	crown	*F. culmorum*	NA
UI Stone	moderate	*Penicillium* sp. WPT	head	*F. graminearum*	mist
	moderate	*C. rosea*	head	*F. graminearum*	mist