THE EFFECTS OF ENDOPHYTIC *FUSARIUM VERTICILLIOIDES* ON THE INTERACTIONS OF MAIZE AND ITS FUNGAL PATHOGEN *USTILAGO MAYDIS*

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GOERGIANA MAY, Ph.D.
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Dedication

This work is dedicated to my wife, Sunmi Lee, who has supported me for years with her love, passion and patience.

Abstract

Diverse microbial organisms, including mycorrhizal fungi, endophytes and pathogens inhabit plants, interact with each other, and affect their fitness. Although theoretical studies suggest that the outcomes of multispecies interactions are often different from those of pairwise interactions, most empirical studies have focused on pairwise plant-pathogen interactions. Using endophytic isolates of *Fusarium verticillioides* (Sacc.) Nirenberg, the corn smut pathogen, *Ustilago maydis* DC (Corda) and maize, our studies suggest that endophytes could play important ecological roles for host defense and their impact needs to be appreciated when studying plant interactions with other organisms occurring in the same host. First, our results suggest that *F. verticillioides* likely interacts with *U. maydis* directly to reduce the host damage by pathogen infections, which we define here as 'aggressiveness'. Since the endophyte alone did not have detectable effects on plant growth, we inferred that *F. verticillioides* indirectly improves plant growth in the presence of the pathogen, *U. maydis*. Secondly, we found that *U. maydis* aggressiveness is constrained by the genetic association between traits governing aggressiveness and fitness, i.e., trade-off, and the endophyte, *F. verticillioides* enforces limits to *U. maydis* aggressiveness. Pathogen fitness decreases as the level of aggressiveness increases. Surprisingly, endophyte co-inoculation with the pathogen resulted in increased pathogen fitness, likely because the biotrophic pathogen, *U. maydis* depends on plant resources for its reproduction and plants in the endophyte co-inoculation treatments grow better than do plants in the pathogen only inoculation treatments. Lastly, we found strain-specific effects of the endophyte on the ecological and fitness outcomes of maize-*U. maydis*

interactions. The endophyte strain which produced least amount of fusaric acid had least impact on *U. maydis* aggressiveness, suggesting that the secreted secondary compound of the endophyte may play antagonistic role against the pathogen. Together, these results suggest that *F. verticillioides* endophytes play important defensive roles for host plants and that the evolution of plant-pathogen interactions is responsive to the microbial environment in which they occur.

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CHAPTER 1

Endophytic *Fusarium verticillioides* reduces disease severity caused by *Ustilago maydis* on maize

INTRODUCTION

Endophytic fungi form ubiquitous yet cryptic associations with many agricultural and natural plant hosts (Carroll, 1988; Harrison, 1999). However, their ecological and evolutionary impacts on host plants and co-occurring microbes are only now being explored for the vast majority of endophytes, those that are not host-specific (Arnold et al., 2003; Rodriguez et al., 2009). Here, we investigate interactions of endophytic isolates of *Fusarium verticillioides* with the corn smut pathogen, *Ustilago maydis*, to understand subsequent impacts on disease severity, plant mortality and plant growth.

The ascomycete *F. verticillioides* is one of the most commonly reported fungi infecting maize (Nelson et al., 1993; Leslie, 1996) and has two distinct life styles, that of an important pathogen (Marasas, 1996) and more commonly, as an endophyte (Yates et al., 2005). Previous studies have shown that *F. verticillioides* has negative effects on the incidence of pathogenic fungi, such as *F. graminearum* and *Stenocarpella maydis* (Berk.) Sutton (Rheeder et al., 1990) and other microbes (Blaney et al., 1986; Van Wyk et al., 1988), suggesting that *F. verticillioides* may protect hosts by suppressing more devastating pathogens. In contrast, some endophytic *F. verticillioides* apparently facilitates growth of other fungi by degrading maize antimicrobial compounds (Saunders & Kohn, 2008). Most of the interest in *F. verticillioides* has been in disease control and limiting mycotoxin contamination (Rheeder et al., 1990; Bacon et al., 2001), leaving basic questions about the nature of *F. verticillioides* interactions with the host and other symbionts largely unanswered.

The basidiomycete *U. maydis* provides an excellent model system with which to study interactions between endophytic and pathogenic fungi, as it is an obligate, biotrophic parasite of maize (*Zea mays* L. ssp. m*ays)* and the teosintes (e.g. *Zea mays* L. ssp. *parviglumis*) (Kämper et al., 2006). Like many grass smut pathogens, *U. maydis* is host specific and likely shares a long evolutionary history with *Zea* hosts predating maize domestication (Munkacsi et al., 2008). Haploid cells of *U. maydis* mate to form an infectious dikaryotic hypha, which penetrates the plant epidermis and proliferates to form large galls filled with a mass of diploid teliospores (Banuett & Herskowitz, 1996). The unique tumor development distinguishes smut disease symptoms from those caused by other pathogens, and the distinct stages of disease progression provide a tool to estimate the disease severity (Gold et al., 1997).

In this study, we investigated the effects of endophytic *F. verticillioides* on smut disease severity, plant mortality caused by smut disease, and on plant growth. Specifically, we asked: 1) does endophytic *F. verticillioides* reduce smut disease severity and plant mortality, 2) does endophytic *F. verticillioides* enhance plant growth, and 3) how do the genotypes of interacting endophytic *F. verticillioides* and *U. maydis* affect *U. maydis* disease severity, plant mortality and plant growth?

MATERIALS AND METHODS

Fungal cultures and inoculation

Two strains of the endophytic fungus *F. verticillioides*, FV1 and FV2, were isolated from asymptomatic field grown maize in St. Paul, MN, USA in a previous study (Pan et al.,

2008) and are deposited at the University of Minnesota Culture Collection (Culture ID: FV1, 49 56796-8 S; FV2, 20 57001-7 E). *F. verticillioides* cultures were grown at 28° on Potato Dextrose Agar (PDA; 24 g Potato Dextrose Broth (PDB; Difco); 1 L deionized water, 15 g of agar (Difco), amended with 25 mg/L penicillin and 25 mg/L streptomycin. Three haploid isolates of *U. maydis* were used; C7 (a1 b12) from northern Ohio, USA, A3 (a2 b3) from St. Paul, MN USA, and E11 (a2 b11) from Owatanna, MN USA. The strain C7 was used as a common parent with either A3 or E11 to generate two different *U. maydis* diploid genotypes, UM1 (A3 x C7) and UM2 (E11 x C7).

For plant inoculations, cultures of haploid *U. maydis* sporidia were grown in liquid media to a density of 2-3 x 10^8 cells/mL and harvested by centrifugation at 4,000 rpm for 10 min. Microconidia of *F. verticillioides* were collected by scraping the surface of 10 – 15 day PDA cultures and flooding these with sterile water (Yates et al., 1997). Spores of both fungi were washed twice with sterile water and re-suspended in sterile water to a final concentration of 10^7 cells/mL for *F. verticillioides* and 10^8 cells/mL for *U. maydis*. To inoculate plants, 0.2 mL of the *F. verticillioides* spore suspension (2 x 10^6 spores) or 0.2 mL of *U. maydis* sporidia suspension (2 x 10^7 sporidia) were pipetted into the leaf whorl with minimal damage to the plant. Inoculations of *U. maydis* were equal mixtures of the two mating compatible haploid cultures as described above. Mock controls were "inoculated" with 0.2 mL sterile water.

The sweet corn cultivar Jubilee (*Zea mays* var. *rugosa*; Jordan seeds, Inc. Woodbury, MN) is susceptible to both *F. verticillioides* (Huang et al., 1997) and to *U. maydis* (Pataky & Chandler, 2003). Seeds had been pre-treated with four fungicides

(Fludioxonil; Carboxin; Difenoconazole; Metalaxyl) and no *F. verticillioides* or other fungi were observed growing from 100 seeds germinating on PDA or from surface-sterilized maize tissues. Six seeds were planted in each 8-inch pot filled with Sunshine Professional Growing Mix (Sun Gro Horticulture Canada Ltd.). Greenhouse conditions were: 24 - 30 °C, 15h/9h light/dark cycle with light intensities at 120 - 200 µE m^{-2}, at the University of Minnesota plant growth facilities, St. Paul.

Experimental design

Our goal was to determine the impacts of *F. verticillioides* on smut disease severity, plant mortality due to smut disease, and on plant growth. We hypothesized that *F. verticillioides* would be more effective in limiting smut disease development if it was inoculated before *U. maydis* than if it was inoculated at the same time or after *U. maydis.* We reasoned that prior establishment of the endophyte would interfere with pathogen establishment or growth due to inhibitory compounds or physical exclusion. To generate differently timed treatments, *U. maydis* was inoculated at nine days after planting (DAP 9) while *F. verticillioides* was inoculated at one of the three different times; two days before *U. maydis* at DAP 7 (F > U), at the same time as *U.* maydis at DAP 9 (F = U), or two days after *U. maydis* at DAP 11 (U > F). To evaluate the overall effects of *U. maydis* on plant growth, we inoculated with *U. maydis* at DAP 9 but did not inoculate with *F. verticillioides* at any time (No FV treatment). To make a more robust study of interactions between *U. maydis* and the endophyte, two *U. maydis* diploid genotypes (UM1 and UM2) and two *F. verticillioides* isolates (FV1 and FV2) were included in a

full factorial design with the above inoculation treatments. Control treatments included mock inoculation (neither fungal species), and *F. verticillioides* only control treatments at three times, DAP 7, DAP 9 and DAP 11 (FV1 or FV2, no *U. maydis*). Each pot with six plants received only one treatment and constituted a replicate. We deployed 15 replicate pots per treatment in a randomized complete block design where three greenhouse benches were blocks.

Measurements of disease severity, plant mortality, and plant growth

We evaluated disease severity at DAP 19 because few plants had died and disease ratings could be made on all plants. We developed a quantitative measure of disease severity as the proportion of severely diseased plants by first rating smut disease symptoms on individual plants at DAP 19 as described previously (Gold et al., 1997); 0, no symptoms; 1, anthocyanin production and/or chlorosis; 2, small leaf galls; 3, small stem galls; 4, large stem galls; and 5, plant death. Individual plants were rated as severely diseased when the disease rating was ≥ 2 (leaf galls or stem galls). We then calculated the proportion of severely diseased plants as the number of plants in a pot with severe smut disease divided by n, the number of plants per pot. The proportions 0 and 1 were adjusted to 1/4n and (n – 1/4)/n, respectively as suggested by (Bartlett, 1947) in order to improve equality of variance. After adjustment, the disease severity values ranged between 0.04 (no smut galls) and 0.96 (smut galls on every plant). Because proportion variables tend to violate the assumptions of ANOVA for a normal distribution of variance, the adjusted proportion was then arc-sine transformed. Plant mortality due to smut infection was

analyzed as the proportion of dead plants at DAP 40, with the Bartlett correction applied for disease severity and the data arc-sine transformed, as above. Back-transformed proportions are presented in all figures. Plant height was measured as the length from the soil surface to the tip of the longest leaf, at DAP 17, 24 and 31.

Statistical analyses of smut disease severity and plant mortality

For smut disease severity and plant mortality data, we ran three-way ANOVA including a block effect using a weighted generalized linear model (GLM) (SPSS Inc., Chicago, IL) on a full-factorial design with four *F. verticillioides* inoculation treatments (No FV, F>U, F=U, U>F), two *U. maydis* genotypes (UM1, UM2), and two *F. verticillioides* strains (FV1, FV2) and determined significance of main treatment and treatment interaction effects. Because some pots had less than six plants due to plant death, we minimized the impact of variable numbers of plants per pot on results by using a weighting factor; the number of plants in a pot divided by the average number of plants per pot across the entire experiment.

Following the three-way ANOVAs, and for only those treatment factors having significant effects, we used Tukey's HSD (Honestly Significant Difference) test to determine significant differences among response means due to specific treatments. Differences were considered significant if two-tailed *P* values were < 0.05.

Statistical analyses of plant growth data

We measured plant height at three dates, DAP 17, 24 and 31, and these data were separately subjected to three-way ANOVA including a block effect using weighted GLMs, as above. Treatment factors were *F. verticillioides* treatment (No FV, F > U, F = U, U > F), *U. maydis* genotype (UM1 or UM2), and *F. verticillioides* strain (FV1 or FV2), as above. Because the repeated measurements at three dates on the same set of plants are not independent of each other, a Bonferroni correction was applied as □ = 0.05/3 to give an adjusted significance level at $P < 0.017$. In addition, since many smut diseased plants had died by DAP 31 and the number of plants remaining in the pot affected plant growth, plant height at DAP 31 was analyzed using the number of remaining plants in a pot at DAP 31 as a covariate. Tukey's HSD test was employed following the ANOVA results, as above.

Linear regression models and Pearson correlations were computed to investigate the relationship between disease severity at DAP 19 and the plant mortality at DAP 40, and between disease severity at DAP 19 and plant height at DAP 17.

RESULTS

Inoculation by *F. verticillioides* and *U. maydis* was effective in establishing infection. We randomly sampled leaves from 20 plants in the *F. verticillioides* only treatment at 24 hrs after inoculation, and recovered *F. verticillioides* from 19 out of 20 surface-disinfected leaves. Neither of the endophytic *F. verticillioides* isolates, FV1 or FV2, caused disease symptoms such as leaf blight or wilting, nor did *F. verticillioides* inoculation alone affect plant height. The level of infection by *U. maydis* was high, 60% overall, and similar to

that observed in field studies (Baumgarten et al., 2007) and in our other greenhouse studies. Lesions due to *U. maydis* infection were visible as early as two days after inoculation as small leaf tumors, followed by stem tumors later in disease development. Although some plants developed stem tumors without showing earlier leaf disease symptoms, the development of smut disease symptoms and progression of disease through time was even across plants; 82.4% of *U. maydis* infected plants formed small stem galls by DAP 19. Most infected plants died between DAP 25 and DAP 30. Plants without *U. maydis* inoculation did not exhibit smut disease symptoms.

Smut disease severity and plant mortality

We determined the effects of the timing of *F. verticillioides* inoculation, *U. maydis* genotypes and *F. verticillioides* strains on the response variables of smut disease severity at DAP19 and plant mortality at DAP40. Results of the three-way ANOVA showed significant main effects of *F. verticillioides* inoculation treatment and of *U. maydis* diploid genotype but not of *F. verticillioides* strain (Table 1-1). In addition, bench, the block factor, affected plant mortality but not disease severity. No interaction effects were significant. Evaluating the proportion of severely diseased plants provided an accurate gauge of damage to the plant as linear regression revealed a strong correlation between the disease severity at DAP 19 and the plant mortality at DAP 40 (Fig. 1-1, $r^2 = 0.89$, $P < 0.001$).

We had predicted that pre-inoculation by *F. verticillioides* would provide the greatest protection from *U. maydis* infection. However, we found that only simultaneous

co-inoculation treatments (F = U) significantly decreased disease severity compared to the other *F. verticillioides* treatments: No FV (*U. maydis* only), pre-inoculation (F > U) and post-inoculation (U > F) (Table 1-2). Pre-inoculation of *F. verticillioides* treatments were associated with only slightly lower smut disease severity than the No FV treatment, and surprisingly, treatments with post-inoculation by *F. verticillioides* (U > F) were associated with somewhat higher smut disease severity (Fig. 1-2). Although not statistically significant, the latter observation is consistent with results in preliminary experiments (data not shown) and with our finding that plant mortality increased and plant growth was reduced in these treatments, as described below.

Results for plant mortality were similar to those for disease severity. The ANOVA results showed significant effects of *F. verticillioides* inoculation treatment and of *U. maydis* genotype but not of *F. verticillioides* strain (Table 1-1). Results of the Tukey's HSD test showed that the plant mortality in the simultaneous co-inoculation treatments (F = U) was significantly lower than that of the other *F. verticillioides* treatments (No FV, F>U, U>F) (Table 1-2).

The *U. maydis* genotype had a significant effect on smut disease severity and plant mortality (Table 1-1). Over all treatments, the smut disease severity, measured as the proportion of severely diseased plants, in UM1 inoculated treatments averaged 0.45, while that of UM2 inoculated treatments was higher and averaged 0.72. The plant mortality at DAP 40 of UM1 inoculated treatments averaged 0.57 and that of UM2 inoculated treatments averaged 0.76. Thus, the UM2 genotype is more aggressive

towards the host plant than is the UM1 genotype and that difference should be due to differing contributions of the non-common parents; E11 in UM2 and A3 in UM1.

Plant growth

We next determined whether *F. verticillioides* treatments affected plant growth directly or indirectly through impacts on smut disease severity. Control experiments demonstrated that *F. verticillioides*, by itself, did not cause disease symptoms and did not have measurable effects on plant height (data not shown). Results of the three-way ANOVA showed that plant height was most strongly affected by *F. verticillioides* inoculation treatment, and *U. maydis* genotype, but not by *F. verticillioides* strain (Table 1-1). There was also a significant block effect, which was due to the environmental variation across benches in the greenhouse.

Results of the ANOVA analyses followed by Tukey's HSD test showed that the effects of *F. verticillioides* treatments on plant growth depended on the timing of *F. verticillioides* inoculation relative to *U. maydis* inoculation (Table 1-2). At DAP 17 and at DAP 24, co-inoculated (F=U) plants were significantly taller than were plants in the No FV treatment (*U. maydis* only), pre-inoculation (F>U) and post-inoculation (U>F) treatments (Table 1-2, Fig. 2-4). We noted that plants in post-inoculation (U > F) treatments at DAP17 and DAP24 were smaller than in other treatments, consistent with observations of slightly greater disease severity in this treatment. At DAP 31, plants in simultaneous co-inoculation treatments (F=U) were significantly taller than plants in No FV treatment (*U. maydis* only), but were not significantly taller than plants in pre-

inoculation (F>U) and post-inoculation (U>F) treatments. This was because many smut-diseased plants had died by DAP31 and only those surviving were measured for height. Linear regression analysis showed a strong negative correlation between plant height at DAP 17 and the smut disease severity at DAP 19 (r^2 = 0.51, P < 0.001, Fig. 2-3). These results together demonstrate that *F. verticillioides* treatments indirectly led to increased plant growth by reducing smut disease severity.

Plant height was significantly affected by *U. maydis* genotype (Table 1-1) with UM2 having a greater negative impact on plant growth than did UM1 at DAP 24 (39.1 cm *vs* 44.6 cm) and at DAP31 (67.0 cm *vs* 77.5 cm). Disease progress was likely not great enough to distinguish the differing effects of the two genotypes at DAP 17.

DISCUSSION

Contrary to our expectations, endophytic *F. verticillioides* significantly reduced disease severity only when it is co-inoculated with the pathogen *U. maydis*, and not when pre-inoculated. We infer that the endophyte competes with, or indirectly interferes with the early infection process of pathogen and slows disease development. Because horizontal transfer is important mechanism of dissemination for *F. verticillioides* (Munkvold et al., 1997) and the only mechanism of *U. maydis* infection, timing of infection will be important in their interaction. The impact of *F. verticillioides* on plant growth varied with *U. maydis* genotype as the more aggressive *U. maydis* genotype was apparently less sensitive to interference by *F. verticillioides* and caused more severe disease than did the less aggressive *U. maydis* genotype.

Pre-inoculation of *F. verticillioides* two days ahead of *U. maydis* inoculation had no significant impact on smut disease severity or plant mortality. Instead, co-inoculation of the two fungi was required to obtain a significant decrease in smut disease severity and plant mortality. These results suggest that the protection provided by endophytic *F. verticillioides* against the pathogen is not mediated by induced resistance such as systemic acquired resistance (SAR) (Durrant & Dong, 2004), as might be important for protection by host-specific endophytes of grasses (Clarke et al., 2006). Instead, this generalist endophyte likely interferes with *U. maydis* more directly, via fungal-fungal interactions (Arnold et al., 2003; Herre et al., 2007). The mechanism underlying these competitive fungal interactions are not clearly understood, however, *in vitro* experiments suggest that secreted secondary metabolites such as fusaric acid (FA) which accumulates during intercellular growth in plants (Bacon et al., 2006), has antagonistic effects against endophytic bacteria (Bacon et al., 2004; Bacon et al., 2006) and the biocontrol agent *Trichorderma harzianum* (El-Hasan et al., 2008). We found that addition of FA to liquid cultures of *U. maydis* decreases cell density by up to 99.8% compared to no FA controls (K. Lee, unpublished data). Our results suggest that *F. verticillioides* inhibits *U. maydis* infection of maize at early stages of smut disease development. Since both fungi commonly inhabit corn, their interactions are important for ameliorating smut disease in the crop host.

In post-inoculation treatments, we saw evidence that the presence of *F. verticillioides* actually increased disease severity and decreased growth. Interestingly, we sometimes observed necrotic lesions and *F. verticillioides* sporulation on *U. maydis*

infected plants but not on plants without smut disease. Further, differences between *U. maydis* genotypes in aggressiveness toward the plant were apparently associated with differing sensitivity to interference by *F. verticillioides*. These results together suggest that *F. verticillioides* and *U. maydis* may sometimes act synergistically (May et al., 2009) to affect disease severity.

The aim of our study was to elucidate ecological roles of endophytic *F. verticillioides* in the microbial community associated with a plant, maize. Our study clearly shows that the presence of endophytic *F. verticillioides* alters the intensity of maize-*U.maydis* interactions, and such results suggest that all three interacting species are important biotic factors affecting each other's fitnesses (Strauss & Irwin, 2004). The work contributes to our understanding how endophytes affect the dynamic microbial community *in planta* and provides empirical procedures that can be applied to other studies, such as evaluating biological control agents. Further studies will utilize the maize- *U. maydis* - *F. verticillioides* system to dissect the genetic determinants and molecular mechanisms underlying multispecies interactions.

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TABLES

Table 1-1. ANOVA of smut disease severity, plant mortality and plant height, with greenhouse bench, *F. verticillioides* inoculation treatment, *U. maydis* genotypes (UM), and *F. verticillioides* strains (FV), as treatment factors.

		F [a]				
Source of variation	df [b]	Disease severity at DAP 19	Plant mortality at DAP 40	Plant height at DAP 17	Plant height at DAP 24	Plant height at DAP 31[c]
Bench	2	2.62	**5.05***	**13.28*****	**7.51****	1.31
Inoculation	2	**21.46*****	**26.58*****	**20.34*****	**24.18*****	**4.48***
UM	1	**46.23*****	**20.82*****	0.62	**9.22*****	**21.17*****
FV	1	0.32	0.13	0.28	1.06	0.36
Inoculation x UM	2	0.59	0.53	1.66	0.06	0.79
Inoculation x FV	2	2.73	1.33	1.72	0.97	0.30
UM x FV	1	0.01	0.78	0.10	0.01	0.14
Inoc. x UM x FV	2	1.21	1.78	0.28	0.08	1.07

[a] Significant difference at $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) are shown in bold.

[b] Degrees of freedom for error was 166 except for plant height at DAP 31 (160).

[c] Maize growth at DAP 31 was analyzed using the number of remaining plants at DAP 31 as a covariate.

Table 1-2. Multiple comparisons using Tukey's HSD test[a]

	No FV	U > F	F > U	F = U
Disease severity at DAP 19	0.67 (a)	0.70 (a)	0.63 (a)	0.42 (b)
Plant mortality at DAP 40	0.71 (a)	0.77 (a)	0.67 (a)	0.45 (b)
Plant growth at DAP 17[b]	24.1 (ab)	22.0 (a)	24.8(b)	28.0 (c)
Plant growth at DAP 24	40.1 (a)	35.3 (a)	40.3 (a)	50.5 (b)
Plant growth at DAP 31[b]	64.6 (a)	69.0 (ab)	72.4 (ab)	75.8 (b)

[a]Means underlined are not significantly different from each other according to Tukey's HSD test at $P < 0.05$.
[b]Means with same letters in the parentheses are not significantly different according to Tukey's HSD test at $P < 0.05$.

FIGURES

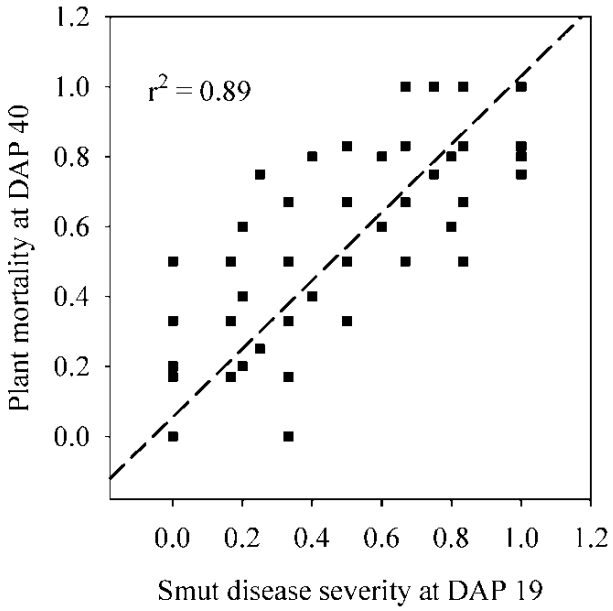

Fig. 1-1. **Correlation between disease severity at DAP 19 and plant mortality at DAP 40.** Linear regression showed a positive correlation between smut disease severity at DAP 19 and plant mortality at DAP 40 (r^2 = 0.89).

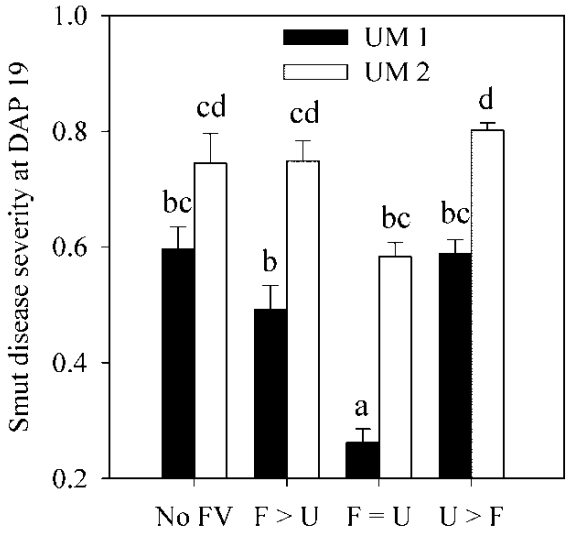

Fig. 1-2. **Effect of *F. verticillioides* inoculation on smut disease severity at DAP 19.** Smut disease severity was significantly lower when *F. verticillioides* was simultaneously co-inoculated with *U. maydis* but not when *F. verticillioides* was inoculated two days before (F > U) or two days after (U > F) *U. maydis* inoculation (Tukey's HSD test, $P < 0.05$). No FV represents treatments with *U. maydis* only. Results for the two *F. verticillioides* strains were combined. Values are means ± SE. Letters designate values that not significantly different at $P < 0.05$ (Tukey's HSD test).

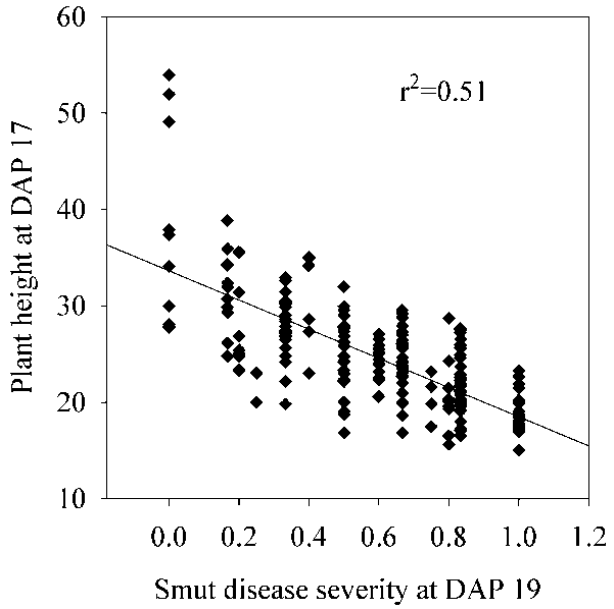

Fig. 1-3. **Correlation of disease severity and plant height.** Linear regression showed a negative correlation between smut disease severity at DAP 19 and plant height at DAP 17 (r^2 = 0.51, P < 0.0001).

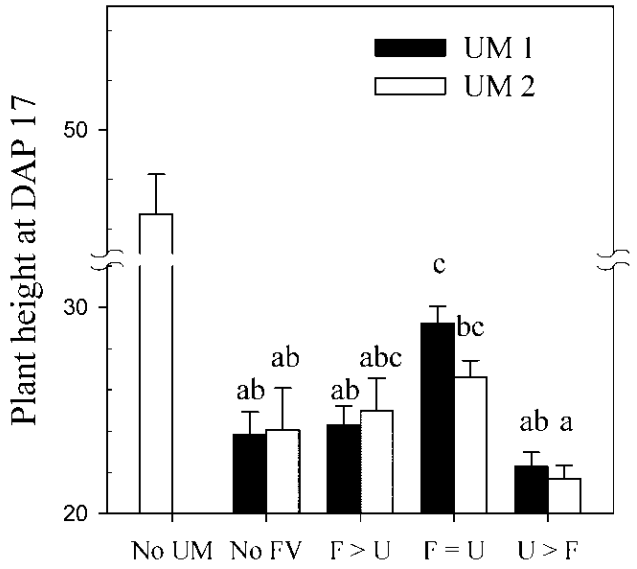

Fig. 1-4. **Effect of *F. verticillioides* inoculation treatments on plant growth.** Plant height was significantly greater only when *F. verticillioides* was simultaneously co-inoculated with *U. maydis* (F = U) but not when inoculated two days before (F > U) or two days after (U > F) *U. maydis* inoculation (Tukey's HSD test, $P < 0.05$). No UM represents treatments with *F. verticillioides* only and these plants grew to the same height as control plants with no fungal treatment. No FV represents treatments with *U. maydis* only. Results for the two *F. verticillioides* strains were combined. Values are means ± SE. Letters designate values that not significantly different at $P < 0.05$ (Tukey's HSD test).

CHAPTER 2

A microbial competitor enforces limits on pathogen aggressiveness

INTRODUCTION

Understanding why and how pathogens harm their hosts is arguably among the most important questions in the evolution of host-pathogen interactions, and will contribute toward better management of devastating diseases in human, agricultural and wild populations (Antia et al., 2003; de Roode et al., 2005; Dieckmann et al., 2002; Gandon et al., 2001; Thrall and Burdon, 2003). Most theoretical and empirical work has focused on interactions between two species (e.g., Alizon et al., 2009; Bremermann and Pickering, 1983; Frank, 1996; Gomulkiewicz et al., 2003; Mackinnon and Read, 1999a; May and Anderson, 1983; Sacristán and García-Arenal, 2008; Salvaudon et al., 2005), despite knowledge that multispecies interactions are ubiquitous and most hosts harbor many symbionts (Billick and Case, 1994; Juenger and Bergelson, 1998; Polis and Strong, 1996; Thompson, 2009). Very few studies have demonstrated fitness outcomes for species in pairwise interactions dependent on community context (Agrawal et al., 2006; Miller and Travis, 1996; Pilson, 1996; Strauss, 1991; Thompson, 1999), and consequently, we have limited understanding how a pathogen's aggressiveness toward its host is influenced by co-occurring community members.

In this study, we consider two models for the evolution of pathogen aggressiveness towards its host: the trade-off model (Ewald, 1983; May and Anderson, 1983) and a diffuse coevolutionary model (Janzen, 1980). Here, we define 'aggressiveness' in the sense that it is used in plant pathology; the degree of damage to a host due to a pathogen infection and reproduction (Jarosz and Davelos, 1995; Pariaud et al., 2009; Van der plank, 1963), and distinguish it from 'virulence' which denotes the qualitative ability of a pathogen to infect specific genotypes of a host in gene-for-gene

interactions (Flor, 1955, 1956, 1971). The trade-off model states that aggressiveness is an unavoidable consequence of the pathogen's growth within the host and predicts that a pathogen will evolve to an intermediate level of aggressiveness (Anderson and May, 1982; Ewald, 1983). Selection for greater pathogen aggressiveness will result if greater growth within the host translates into greater reproduction. However, selection for lower aggressiveness levels will result if a high level of aggressiveness damages the host or shortens its life span and reduces resources for pathogen reproduction. Indeed, some studies provide empirical support for the trade-off hypothesis: maximized fitness at an intermediate level of aggressiveness (Jensen et al., 2006) and a positive correlation of aggressiveness and transmission (Agnew and Koella, 1997; Cooper et al., 2002; Ferguson et al., 2003; Mackinnon et al., 2008; Mackinnon and Read, 1999a, b, 2003; Paul et al., 2004; Wickham et al., 2007). However, despite of considerable theoretical effort and the intuitive appeal of the trade-off model, there remains lack of empirical evidence for the trade-off model in many host-pathogen systems (Alizon et al., 2009; de Roode et al., 2008; Ebert and Bull, 2003). Here, we first show evidence for a trade-off between pathogen aggressiveness and reproduction in a plant-pathogen system, and then show the impact of a third interacting microbe on fitness outcomes for pathogen and host plant in the same system.

Although ecological interactions in nature often involve multiple species (e.g., Billick and Case, 1994; Juenger and Bergelson, 1998; Polis and Strong, 1996), few have considered the fitness consequences of community context; the impacts of a third party interactor on host-pathogen evolution. In contrast to strict pairwise coevolution, a diffuse coevolutionary model accounts for effects of a third party interactor on the evolutionary

dynamics of two species' interactions. Several authors have proposed a set of criteria to evaluate evidence for diffuse or pairwise coevolutionary process: (1) traits that are important to interactions with one species are independent of traits governing interactions with another species, (2) a third species does not affect the likelihood or intensity of interactions between other two species, and (3) the presence of a third species does not alter the impact of one species on the fitness of another interacting species (Hougen-Eitzman and Rausher, 1994; Iwao and Rausher, 1997; Stinchcombe and Rausher, 2002; Strauss and Irwin, 2004). If any of the three conditions are not satisfied, then there is potential for a diffuse evolutionary process.

In this study, we determine if the fungal endophyte *F. verticillioides* strongly alters fitness outcomes of the interaction between maize and the fungal pathogen *U. maydis*. Plants are host to diverse microorganisms, with none more prevalent than endophytes–the microbial associates living inside of plants without causing disease (Arnold et al., 2003; Carroll, 1988; Danielsen and Jensen, 1999; Wicklow et al., 2005). Thus it is important to understand how such diverse and ubiquitous endophytes influence the evolution of host-pathogen interactions. However, except for a few examples, the ecological impacts of endophytes on host-pathogen interactions have not been much appreciated (Herre et al., 2007; Rodriguez et al., 2009). Nonetheless, recent studies have shown that direct and indirect interactions among diverse microbial symbionts play important role in shaping the species composition and structure of the fungal community associated with plants (Pan et al., 2008; Pan and May, 2009; Saunders and Kohn, 2009).

Maize and its biotrophic pathogen, *U. maydis* comprise a tractable model system with which to study host-pathogen interactions (Banuett and Herskowitz, 1996). Maize

has quantitative resistance against *U. maydis* (Baumgarten et al., 2007; Ding et al., 2008) and the host and pathogen have long-shared coevolutionary history (Munkacsi et al., 2008). Moreover, *U. maydis* induces conspicuous tumors in the aerial tissues of the host plant such that disease progression and severity can be assessed visually without destructive sampling (Gold et al., 1997). The ascomycete *F. verticillioides* is among the most commonly reported fungi associated with maize (Marasas, 1996; Pan et al., 2008; Pan and May, 2009). Interestingly, *F. verticillioides* isolates vary in their interactions with maize, from disease-causing pathogens to asymptomatic endophytes (Bacon and Hinton, 1996). Previously, we have shown that endophytic *F. verticillioides* decreases smut disease severity caused by *U. maydis* and indirectly increases plant growth (Lee et al., 2009).

In this study, we utilize the maize, *F. verticillioides* and *U. maydis* system to investigate the ecological and genetic constraints on the evolution of aggressiveness by *U. maydis* towards maize. We ask first for evidence of a trade-off between pathogen aggressiveness and fitness with regard to interactions between the pathogen and plant host. Secondly, we ask whether the presence of a 'third party', the endophyte, alters the fitness landscape of the pathogen in its interactions with its plant host. Lastly, we investigate mechanisms of interaction as the endophyte might directly affect pathogen fitness if it interferes with smut infection and disease development (Lee et al., 2009) or it might indirectly affect pathogen fitness if it affects plant growth and resource allocation to the pathogen.

MATERIALS AND METHODS

Experimental design

Our overall goal was to determine the genetic and ecological factors that affect aggressiveness and fitness of the pathogen *U. maydis* in its interactions with maize. First, we generated 24 different diploid *U. maydis* genotypes with varying levels of aggressiveness towards the plant to determine evidence for a trade-off between pathogen aggressiveness and fitness. Second, we used these same *U. maydis* diploid genotypes in all pairwise interactions with two strains of endophytic *F. verticillioides* to determine the effect of the endophyte on the pathogen's reproduction and interaction with its host.

Fungal strains and plant cultivar

Two haploid strains, C7 (a1 b12; northern Ohio, USA) and E11 (a2 b11; Owatanna, MN USA), were mated on the plant to generate the parental diploid genotype (UM2-P; C7 x E11). Previous experiments had shown that UM2-P exhibits a more aggressive phenotype than many other genotypes (Lee et al., 2009 and K. Lee unpublished results). We germinated UM2-P teliospores on water agar (WA) plates where they undergo meiosis and isolated 240 haploid sporidia. We determined mating types by crossing with lab standards, and randomly chose 23 haploids that were mating compatible (a2 b11) with the parental strain C7 (a1 b12). The parental *U. maydis* diploid genotype, UM2-P (E11 x C7) was generated by co-inoculating strains E11 and C7. The 23 F1 progenies, UM2-1 to UM2-23, were generated by co-inoculating each of the 23 haploids above with the parental strain C7. Thus, each diploid genotype differs from others by a haploid genome. We used two strains of endophytic *F. verticillioides*, FV1 and FV2, which are deposited at the University of Minnesota Culture Collection (FV1: 49 56796-8 S; FV2: 20 57001-7

E). The sweet corn variety Jubilee (*Zea mays* var. *rugosa*; Jordan seeds, Inc. Woodbury, MN) used in these experiments is susceptible to *U. maydis*.

Plant inoculation

Plant inoculations were conducted as described previously (Lee et al., 2009). Briefly, cultures of haploid *U. maydis* cells (sporidia) were grown as yeast in Potato Dextrose Broth (PDB) to a density of 2-3 x 10^8 cells/mL, harvested by centrifugation, washed twice with sterile water and re-suspended in sterile water to a final concentration of 10^8 cells/mL. Microconidia of *F. verticillioides* were collected from 10 – 15 day-old cultures on Potato Dextrose Agar (PDA), washed twice with sterile water, and re-suspended in sterile water to a concentration of 10^7 cells/mL. An equal number of mating compatible *U. maydis* haploid sporidia (10^7 sporidia each) were mixed immediately before inoculation. To inoculate plants, 2 x 10^6 spores of *F. verticillioides* in 0.2 mL water or 2 x 10^7 sporidia of *U. maydis* sporidia in 0.2 mL water were pipetted onto the leaf whorl of seedlings at 10 days after planting (DAP 10) with minimal damage to the plant.

We inoculated each of the 24 *U. maydis* diploid genotypes by itself (UM only treatments) or simultaneously with one of the two *F. verticillioides* strains, FV1 and FV2. Control treatments were *F. verticillioides* only (FV1 or FV2, no *U. maydis*) and mock inoculation (sterile water). The complete factorial design yielded 24 UM treatment levels, three FV treatment levels (No FV, UM + FV1, and UM + FV2), and a total of 72 treatment combinations. Six plants in the same pot received the same treatment and the pot was treated as the unit of replication. We deployed eight replicate pots per treatment in a randomized complete block design where two greenhouse benches were blocks.

Eight-inch pots were filled with Sunshine Professional Growing Mix (Sun Gro Horticulture Canada Ltd.) and six seeds were planted in each pot. Greenhouse conditions were: 20 - 28 °C, 14h/10h light/dark cycle with light intensities at 120 - 200 μE m^{-2}, at the University of Minnesota plant growth facilities, St. Paul, MN.

Measurement of *U. maydis* aggressiveness and fitness

The aggressiveness of *U. maydis* diploid genotypes was measured as the proportion of severely diseased plants (Lee et al., 2009). We evaluated disease severity at 10 days after inoculation, (20 days after planting; DAP 20) as described by Gold et al. (1997). Individual plants were rated for smut disease symptoms at 0, no symptoms; 1, anthocyanin production and/or chlorosis; 2, small leaf galls; 3, small stem galls; 4, large stem galls; and 5, plant death. Second, we qualified a disease rating ≥ 2 (leaf galls or stem galls) as severely diseased because most plants with this disease rating at DAP 20, later died due to smut infection. The proportion of severely diseased plants was calculated as the number of plants in a pot with severe smut disease symptoms divided by n, the number of plants per pot. The proportion 0 was adjusted to 1/4n and 1 was adjusted to (n – 1/4)/n as suggested by Bartlett (1947) in order to improve equality of variance. The proportion values ranged between 0.04 (no smut galls) and 0.96 (smut galls on every plant). Indeed, the variance across all treatments was decreased from 0.05 to 0.02 after the adjustment. We further defined inherent aggressiveness as the proportion of severely diseased plants in UM only control treatments, and defined realized aggressiveness as the proportion of severely diseased plants in UM + FV treatments (UM + FV1, UM + FV2).

Fitness of *U. maydis* was measured as the total dry weight stem gall per plant because galls are primarily composed of teliospores covered by a thin layer of plant tissue. Smut galls were collected when mature at 3-4 weeks after inoculation (DAP 31 – 38). Galls from an individual plant were combined together, dried to a constant dry weight at 60°C and weighed. Plant height was measured at DAP 19.

Statistical Analyses

We performed analysis of variance (ANOVA) for *U. maydis* aggressiveness, *U. maydis* fitness and plant height using SPSS (SPSS Inc., Chicago, IL) to evaluate the significance of UM treatments (24 diploid genotypes), FV treatments (two strains of the endophytic *F. verticillioides*, FV1 and FV2, and no endophyte inoculation), as well as greenhouse bench. We ran Tukey's HSD (Honestly Significant Difference) test as a *post hoc* procedure to determine significant differences among treatment means. Differences were considered significant if two-tailed *P* values were < 0.05.

To determine if UM and FV treatments affect *U. maydis* aggressiveness, we ran a fixed effect ANOVA on *U. maydis* aggressiveness (proportion of severely diseased plants at DAP 20) using a generalized linear model (GLM) with SPSS. UM treatments, FV treatments and greenhouse benches were treated as fixed effects because we measured every replicate unit in the experimental population. The adjusted proportion of severely diseased plants was arc-sine transformed for ANOVA and back-transformed numbers are presented in all Figures. We determined the effects of FV treatments on *U. maydis* aggressiveness by evaluating the linear correlation (Pearson's product-moment correlation test) between inherent (no FV) and realized aggressiveness (in the presence of

FV) of *U. maydis*. We then determined if the slope of the regression line was significantly different from 1.0, the expected slope if inherent aggressiveness is the same as realized aggressiveness, using Students' t-test.

To determine if UM and FV treatments affect pathogen fitness, we ran a fixed effect ANOVA on dry gall weight per diseased plant using the same method as above. In order to improve equality of variance, dry weight stem gall per diseased plant was log transformed before the analysis. Since it is difficult to harvest small leaf galls without including a large and variable amount of leaf tissue, we ran ANOVA using only plants with stem galls (87% of all plants) and excluded data for plants with leaf galls only.

We determined the effect of *F. verticillioides* on the relationship between pathogen aggressiveness and fitness using an analysis of covariance (ANCOVA) with a GLM. Factors included were FV treatment (no FV, UM + FV1, and UM + FV2) as an independent factor and inherent aggressiveness as a covariate.

We examined the relationship between aggressiveness and fitness using linear regression models in SPSS. *U. maydis* fitness as dry weight stem gall per diseased plant was regressed on inherent aggressiveness separately for UM only, UM + FV1 and for UM + FV2 treatments. To determine whether the endophyte directly improves plant growth, we compared the plant height data for three control treatments without *U. maydis*, mock (water), FV1 only and FV2 only, using an ANOVA with two independent factors, greenhouse bench and FV treatment. To determine if *F. verticillioides* has indirect effects on plant growth via effects on *U. maydis* aggressiveness, the height for plants with stem galls were subjected to an ANOVA with a GLM. The independent factors were

greenhouse bench, UM genotypes (24 genotypes), and 3 FV treatments (UM only, UM + FV1, UM + FV2).

We ran a linear regression between inherent aggressiveness of *U. maydis* and mean height of diseased plants (plants with stem galls) at DAP 19 using SPSS to determine the relationship between pathogen aggressiveness and plant growth, in the presence and absence of the endophyte. We ran a linear regression analysis between *U. maydis* fitness (dry weight stem gall per diseased plant) and height of diseased plants at DAP 19 to determine the relationship of plant growth and *U. maydis* fitness.

RESULTS

We used 24 *U. maydis* genotypes with varying level of aggressiveness toward maize and two strains of *F. verticillioides* to examine the relationship between *U. maydis* aggressiveness and fitness, and the impacts of *F. verticillioides* on that relationship. The *U. maydis* inoculations were effective because 45% of inoculated plants exhibited smut disease symptoms, typical of experiments in which the plant is not damaged at inoculation (Baumgarten et al., 2007; Lee et al., 2009). *F. verticillioides* inoculations were effective because in >90% of inoculated plants, we could observe the presence of *F. verticillioides* as spores produced on the plant or smut gall surfaces, or as re-isolation of *F. verticillioides* from plant tissues.

Continuous variation is demonstrated in *U. maydis* aggressiveness

The 24 *U. maydis* diploid genotypes demonstrated continuous variation in inherent aggressiveness and the parental genotype exhibited an intermediate level (Fig. 2-1).

Strain UM2-3 exhibited the highest level of aggressiveness at 22% above the parent and strain UM2-23 exhibited the lowest level of aggressiveness as it did not cause visible galls on any of 141 plants. It was surprising to find a non-aggressive genotype for a biotrophic pathogen, even though we used a susceptible plant.

ANOVA demonstrated that the aggressiveness of *U. maydis*, as measured by the proportion of severely diseased plants, is significantly affected by greenhouse bench, *U. maydis* diploid genotype and FV treatments as well as FV x UM interaction effects (Table 2-1; Fig. 2-1). Significant and continuous variation in inherent aggressiveness provides evidence that several loci affect inherent aggressiveness. Differing aggressiveness among the 23 F1 diploid genotypes is attributable to the haploid genome derived from segregation of the parental diploid genotype, UM2-P.

F. verticillioides reduces *U. maydis* aggressiveness

The presence of the endophyte has negative impacts on pathogen aggressiveness across the 24 *U. maydis* genotypes. Realized aggressiveness in the presence of the endophyte averaged 41%, while inherent aggressiveness averaged 53% ($F_{1, 527} = 47.8$, $P < 0.001$). The FV x UM interaction term results from varying effects of *F. verticillioides* co-inoculation across *U. maydis* genotypes; for example, most *U. maydis* genotypes demonstrated reduced aggressiveness in the presence of *F. verticillioides* while a few genotypes demonstrate increased aggressiveness, U2-1 and U2-19 (Fig. 2-1), suggesting the endophyte may facilitate infection by some *U. maydis* genotypes. We then used linear regression to determine the relationship between inherent and realized aggressiveness of *U. maydis.* If *F. verticillioides* has no effect on *U. maydis* aggressiveness, the realized

aggressiveness will be equal to the inherent aggressiveness and the slope should be 1.0 (Fig. 2-2). Realized aggressiveness is linearly correlated with the inherent aggressiveness (UM + FV1, y = 0.84x – 0.05, r^2 = 0.41, P < 0.01; UM + FV2, y = 0.88x – 0.03, r^2 = 0.52, P < 0.01; y = realized aggressiveness, x = inherent aggressiveness). Slopes are not significantly different from 1.0, although they appear somewhat shallower. (Student's t-test, two-tailed; UM + FV1, t = 0.73, P = 0.47; UM + FV2, t = 0.65, P = 0.52). The similarity of slopes indicates that the effects of *F. verticillioides* on *U. maydis* aggressiveness are not independent of the level of inherent aggressiveness and that greater aggressiveness is associated with proportionally greater competitiveness towards *F. verticillioides.* Nonetheless, most *U. maydis* genotypes demonstrate reduced aggressiveness in FV treatments, consistent with our previous study (Lee et al., 2009).

U. maydis fitness is negatively correlated with the level of aggressiveness toward maize

We estimated *U. maydis* fitness directly as dry weight stem gall per diseased plant because there is a strong positive correlation between dry gall weight and number of teliospores produced per plant (Chapter 3; r^2 = 0.57). The fitness data for the non-aggressive genotype were excluded from the analysis because it produced no stem galls. The results of ANOVA revealed that *U. maydis* fitness, as measured by dry weight stem gall, is strongly affected by greenhouse bench, *U. maydis* genotypes and FV treatments (Table 2-2). The *U. maydis* genotypes differ two-fold in fitness, ranging from 111.2 ± 10.3 (mean ± SE) mg dry weight stem gall (UM2-1) to 209.7 ± 20.1 mg dry weight stem gall (UM2-18) per plant. Surprisingly, the presence of the endophyte leads to increased *U.*

maydis fitness; mean pathogen fitness in the presence of the endophyte was 164.8 ± 6.0 with FV1 and 160.0 ± 6.9 with FV2 but 145.9 ± 4.7 mg spores/diseased plant in UM only treatments without *F. verticillioides*.

There were no significant UM x FV interaction effects, perhaps surprising given significance of UM x FV interaction effects for aggressiveness. There were two reasons: 1) the *U. maydis* genotypes that had higher aggressiveness in the presence of the endophyte did not have dramatically lower fitness in the presence than in the absence of the endophyte, and 2) *U. maydis* fitness data only included diseased plants with stem galls, while *U. maydis* aggressiveness data included both uninfected plants and diseased plants with stem galls.

We next determined whether the relationship between pathogen aggressiveness and fitness demonstrated a trade-off and if so, whether the nature of that tradeoff is affected by the endophyte. In the absence of *F. verticillioides*, a trade-off is demonstrated by the negative linear relationship between inherent aggressiveness and *U. maydis* fitness as dry weight stem gall (solid line, y = -137x + 222; y = *U. maydis* fitness, x = inherent aggressiveness; $r^2 = 0.40$, $P < 0.01$; Fig. 2-3). In the presence of *F. verticillioides*, the relationship of fitness and aggressiveness also demonstrated a negative linear relationship (UM + FV1, dashed line, y = -192x + 276, $r^2 = 0.62$, $P < 0.01$; UM + FV2, dotted line, y = -182x + 268, $r^2 = 0.46$, $P < 0.01$; y = *U. maydis* fitness, x = inherent aggressiveness; Fig. 2-3). Interestingly, the slopes were not significantly different for UM treatments with and without FV (Student's t-test, one tailed; UM + FV1, t = -1.2, $P = 0.12$; UM + FV2, t = -0.9, $P = 0.18$). Most importantly, treatments including the endophyte resulted in greater *U. maydis* fitness; especially, the y-intercept in UM + FV1 treatments was

significantly greater than that for UM only treatments (Student's t-test, one tailed; t = 2.05, $P < 0.05$), although the y-intercept in UM + FV2 treatments was not significantly greater compared to UM only treatments (t = 1.56, $P = 0.06$).

F. verticillioides does not alter fitness-aggressiveness trade-off in *U. maydis*

The above results show that the pathogen's fitness negatively correlated with its aggressiveness level towards the plant, and that the level of fitness achieved at any level of aggressiveness greatly depends on the presence or absence of the endophyte. We ran ANCOVA to determine if *U. maydis* fitness significantly covaries with aggressiveness and to provide a better fit to models for the effects of *U. maydis* genotype and FV treatments on *U. maydis* fitness. There was significant covariance between inherent aggressiveness (Aggressiveness) and *U. maydis* fitness (Table 2-3), confirming that pathogen's fitness depends on its aggressiveness to its host plant. We found no significant effects of FV treatments (UM only, UM + FV1, UM + FV2) on *U. maydis* fitness, or of an interaction term for FV treatments x Aggressiveness.

The optimal level of aggressiveness at which pathogen fitness is greatest, is estimated to ~0.2, the lowest level represented in our experiments. Moreover, the presence of the endophyte does not greatly alter the apparent optimum aggressiveness, which is also estimated to ~0.2. Together, the ANCOVA, regression results, and optimality analyses show that treatments including the endophyte, *F. verticillioides* do not affect the fundamental relationship between the pathogen's aggressiveness towards the host and the fitness that it achieves.

We then asked questions regarding the mechanisms underlying these effects. Does *F. verticillioides* directly enhance plant growth and through increased plant resources indirectly benefit *U. maydis* fitness? Or does *F. verticillioides* indirectly increase plant resources to the pathogen by limiting *U. maydis* aggressiveness? ANOVA results for three control treatments, mock (No FV, no UM), FV1 only or FV2 only, showed that *F. verticillioides* alone does not directly improve plant growth ($F_{2, 133} = 0.6$, $P = 0.56$). Then, we used ANOVA on height of diseased plants with stem galls to show significant effects of greenhouse bench, *U. maydis* diploid genotypes and FV treatment (Table 2-4).

Given the ANOVA results, we subsequently determined the nature of the endophyte's effects by running linear regression between inherent aggressiveness and plant growth in the presence and absence of *F.* verticillioides. There was a strong negative correlation between inherent aggressiveness and plant height in treatments with or without the endophyte (Fig. 2-4; UM only, black circles, $y = -14.9x + 31.8$, $r^2 = 0.45$, $P < 0.001$; UM + FV1, white circles, $y = -13.2x + 34.4$, $r^2 = 0.29$, $P < 0.01$; UM + FV2, grey circles, $y = -18.2x + 36.1$, $r^2 = 0.53$, $P < 0.001$; y = mean height of diseased plants, x = inherent aggressiveness). There was no significant difference between the regression lines of UM only treatments and those for UM + FV treatments with regard to slope (Student's t-test, one-tailed; UM + FV1, $t = 0.45$, $P = 0.33$; UM + FV2, $t = 0.89$, $P = 0.19$). However, the y-intercept for UM + FV2 treatments was significantly greater than that for UM only treatments (Student's t-test, one-tailed; $t = 2.0$, $P < 0.05$) while the y-intercept for UM + FV1 treatments was not significantly greater than that for UM only treatments (Student's t-test, one-tailed; $t = 1.16$, $P = 0.13$). Across aggressiveness levels,

severely diseased plants with stem galls grew significantly better in the presence of the endophyte (UM + FV1, 26.1 ± 0.4 cm; UM + FV2, 24.5 ± 0.4 cm) than in the absence of the endophyte (UM only, 22.8 ± 0.3 cm; one way ANOVA, $F_{2,1277} = 22.6$, $P < 0.001$; Tukey's HSD test, $P < 0.05$).

F. verticillioides indirectly benefits *U. maydis* fitness

Lastly, we considered the effects of the endophyte jointly on *U. maydis* fitness and plant growth using linear regression between height of smut diseased plants and dry stem gall weight per plant. The results show a positive correlation; larger plants are associated with greater *U. maydis* reproductive output (Fig. 2-5; linear regression: UM only, solid line, y = 8.7x -58.5, $r^2 = 0.79$, $P < 0.001$; UM + FV1, dashed line, y = 6.4x – 11.2, $r^2 = 0.50$, $P < 0.001$; UM + FV2, dotted line, y = 8.8x - 64, $r^2 = 0.76$, $P < 0.001$). Interestingly, these analyses also show that fitness values in UM only treatments (black circles) were skewed toward the lower values of plant growth, while fitness values in UM + FV treatments (FV1, white circles; FV2, grey circles) were skewed toward the higher values of plant growth. It is perhaps not surprising to find that positive correlation since growth and reproduction of a biotrophic pathogen such as *U. maydis* depends on a living host's resources. Given that *F. verticillioides* itself does not directly enhance plant growth, and that FV treatments significantly reduce aggressiveness, we conclude that *F. verticillioides* affects plant growth indirectly by limiting the negative impacts of *U. maydis* aggressiveness on host plants, plants grow larger, and *U. maydis* fitness increases.

DISCUSSION

Using all results, we infer that *F. verticillioides* slows disease progression and decreases the negative impact of *U. maydis* disease on plant growth. *U. maydis* fitness benefits from the presence of the endophyte indirectly, because as plants grow larger, *U. maydis* fitness increases.

Our study demonstrated that the evolution of aggressiveness in *U. maydis* should be constrained by a trade-off between pathogen aggressiveness and within-host reproduction. While there is some empirical support for the trade-off model in animal systems (e.g., de Roode et al., 2008; Mackinnon and Read, 1999a,b), trade-offs have not been often demonstrated directly in plant-pathogen systems (Jarosz and Davelos, 1995; Thrall and Burdon, 2003) and fitness consequences are rarely reported (Salvaudon et al., 2005). The lack of support for the trade-off model in plant-pathogen systems may be attributable to a lack of experiments. Indeed, gene-for-gene systems have dominated studies of antagonistic plant-pathogen coevolution (Dodds et al., 2006; Ellis et al., 1999; Scofield et al., 1996; Thrall and Burdon, 2003), and consequently, research has focused on elucidating the molecular, genetic basis of the compatibility mechanism between plant hosts and their pathogens, leaving the quantitative aspects of host-pathogen interactions and their evolution relatively understudied. Interestingly, however, a trade-off is suggested in the gene-for-gene flax-flax rust system (Thrall and Burdon, 2003) as the number of spores produced per pustule apparently declines with increasing numbers of virulence genes.

The lack of empirical support for the trade-off model might also result if the measures of pathogen aggressiveness, infection efficiency (Clifford and Clothier, 1974; Knott and Mundt, 1991), lesion size (Kolmer and Leonard, 1986; Mundt et al., 2002), or

sporulation rate (Clifford and Clothier, 1974; Sache, 1997), are difficult to precisely estimate (Pariaud et al., 2009), or are simply not correlated with reductions in host fitness (Salvaudon et al., 2005). In our study, we measured the *U. maydis* aggressiveness as the proportion of severely diseased plants, which counts both the qualitative aspects, i.e. tumor induction (severe disease), and the quantitative outcome, i.e., proportion of severely diseased plants (Lee et al., 2009). Here, we demonstrate correlation between host fitness and pathogen aggressiveness and show that developing tools to measure pathogen aggressiveness and fitness accurately is an important step to demonstrating trade-offs.

The trade-off demonstrated in this study suggests a possible mechanism contributing to the long duration of maize resistance to *U. maydis* (Neuhauser et al., 2003). Often planted in large acreages, plant resistance to a pathogen is expected to be effective only for a short period of time (Mundt et al., 2002), but modern maize populations in North America have shown durable resistance to *U. maydis* for decades (Christensen, 1963; Shurtleff, 1980). Neuhauser et al. (2003) have proposed that quantitative resistance in maize and the obligately sexual reproductive system of *U. maydis* are the two important factors. Quantitative plant resistances are assumed to be more durable than the qualitative resistances (Lindhout, 2002) and recent studies have identified multiple QTLs contributing maize resistance to *U. maydis* (Baumgarten et al., 2007; Ding et al., 2008). In addition to these two factors, our study shows possible costs for a high level of aggressiveness suggesting that the genetic associations between traits governing aggressiveness and fitness may contribute to the remarkably durable maize resistance to *U. maydis*.

We infer that direct fungal-fungal interactions between the endophyte and the pathogen lead to reduced damage of host plants and thus, result in greater resource availability to the pathogen. Previous studies have demonstrated that *U. maydis* directly penetrates plant surface and suppresses the expression of host defense-related genes at 24 hours after inoculation. The early down-regulation of host immune system is a prerequisite for successful establishment of the biotrophic association (Doehlemann et al., 2008). The endophyte may reduce the number of viable *U. maydis* cells, inhibit *U. maydis* mating or interfere with repression of early host defensive genes, reducing the number of effective biotrophic infections within a host plant. Further studies could reveal whether the endophyte alters the gene expression of the pathogen at the early stage of the biotrophic phase.

In sum, our study not only provides evidence for a trade-off between aggressiveness and fitness but also underscores the importance of ecological interactions within communities to the fitness outcomes. This study suggests that endophytes may play important defensive roles for host plants against pathogens and that the ecological roles of non-host-specific endophytes can be better understood in the community context.

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TABLES

Table 2-1. ANOVA on *U. maydis* aggressiveness[a] with greenhouse bench, *F. verticillioides* treatment and *U. maydis* diploid genotypes as treatment factors.

Source	df[b]	SS[c]	MS[d]	*F*	*P*
Greenhouse bench	1	9.97	9.97	36.27	< **0.001**
U. maydis genotype (UM)	23	143.91	6.26	22.76	< **0.001**
F. verticillioides treatment[e] (FV)	2	13.58	6.79	24.69	< **0.001**
UM X FV	46	21.75	0.47	1.72	**0.003**
Error	503	138.28	0.27		

[a]Aggressiveness: proportion of severely diseased plants and arc-sine transformed before the analysis.
[b]degrees of freedom; [c]type III sum of squares; [d]mean square
[e]three levels: No FV (UM only), FV1 (UM + FV1), FV2 (UM + FV2)

Table 2-2. ANOVA on *U. maydis* fitness[a] with greenhouse bench, *F. verticillioides* treatment and *U. maydis* diploid genotypes as treatment factors.

Source	df[b]	SS[c]	MS[d]	*F*	*P*
Greenhouse bench	1	3.72	3.72	49.56	< **0.001**
U. maydis genotype (UM)	22	7.13	0.32	4.32	< **0.001**
F. verticillioides treatment[e] (FV)	2	0.85	0.43	5.69	**0.003**
UM X FV	43	2.57	0.06	0.80	0.825
Error	1211	90.79	0.07		

[a]*U. maydis* fitness was measured as dry weight stem gall per diseased plant
[a]degrees of freedom; [b]type III sum of squares; [c]mean square
[d]three levels: No FV (UM only), FV1 (UM + FV1), FV2 (UM + FV2)

Table 2-3. ANCOVA of *U. maydis* fitness[a] with *F. verticillioides* treatment as a treatment factor and inherent aggressiveness (Aggressiveness) as a covariate.

Source	df[b]	SS[c]	MS[d]	*F*	*P*
F. verticillioides treatment[e] (FV)	2	0.10	0.05	0.60	0.550
Aggressiveness[f]	1	3.37	3.37	42.43	**< 0.001**
FV x Aggressiveness	2	0.04	0.02	0.27	0.764
Error	1274	101.13	0.08		

[a]*U. maydis* fitness was measured as dry weight stem gall per diseased plant
[b]degrees of freedom; [c]type III sum of squares; [d]mean square
[e]three levels: No FV (UM only), FV1 (UM + FV1), FV2 (UM + FV2)
[f]proportion of severely diseased plants in UM only treatment (= inherent aggressiveness)

Table 2-4. ANOVA on plant height[a] with greenhouse bench, *F. verticillioides* treatment and *U. maydis* diploid genotypes as treatment factors.

Source	df[b]	SS[c]	MS[d]	*F*	*P*
Greenhouse bench	1	202.80	202.80	4.57	**0.033**
U. maydis genotype (UM)	22	9284.09	422.00	9.52	**<0.001**
F. verticillioides treatment[e] (FV)	2	2259.47	1129.74	25.48	**<0.001**
UM X FV	43	1658.83	38.58	0.87	0.709
Error	1217	53962.43	44.34		

[a]data for plants with stem galls were included for this analysis
[b]degrees of freedom; [c]type III sum of squares; [d]mean square
[e]three levels: No FV (UM only), FV1 (UM + FV1), FV2 (UM + FV2)

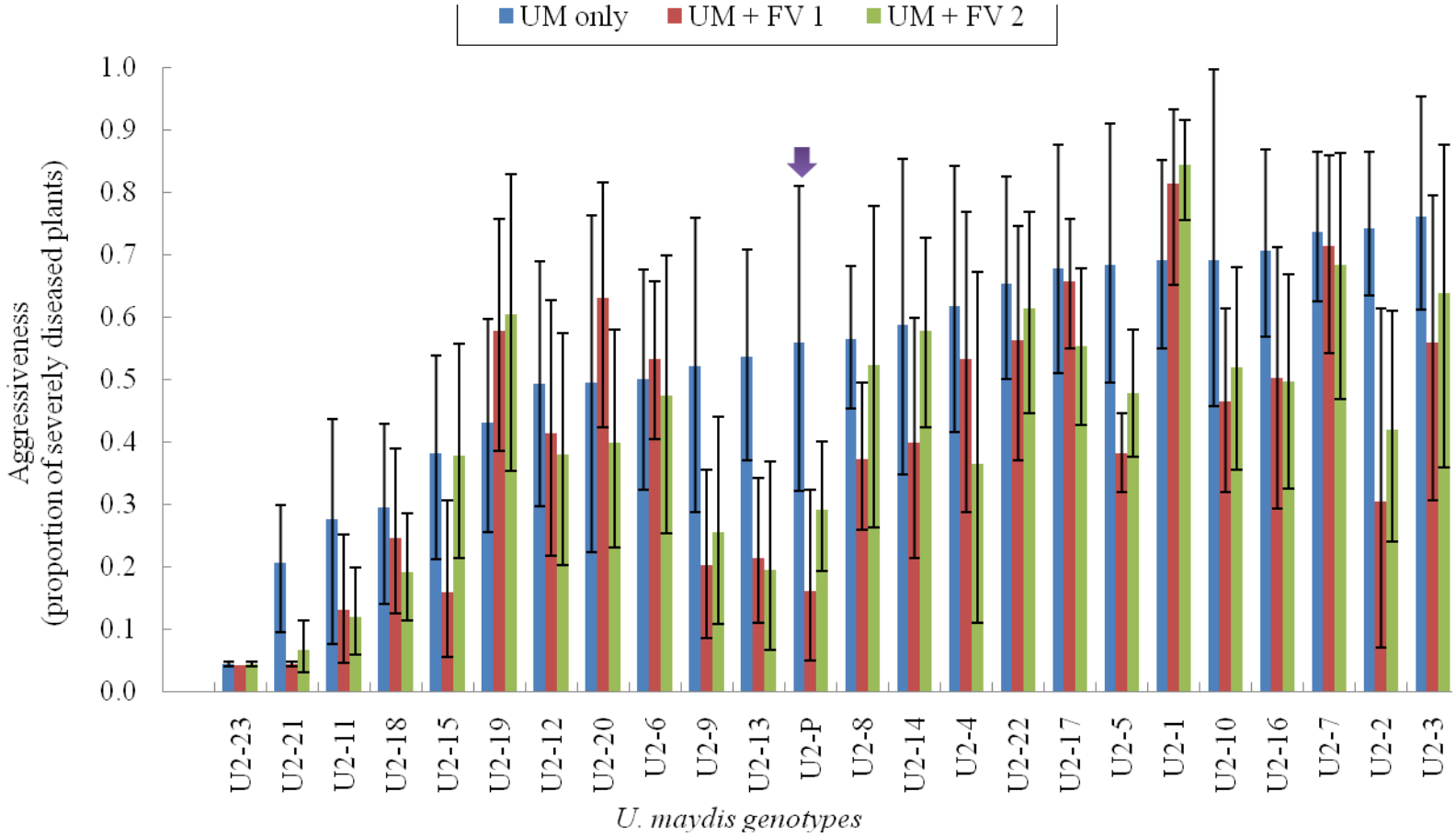
UM only
UM + FV 1
UM + FV 2
Aggressiveness
(proportion of severely diseased plants)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
U2-23
U2-21
U2-11
U2-18
U2-15
U2-19
U2-12
U2-20
U2-6
U2-9
U2-13
U2-P
U2-8
U2-14
U2-4
U2-22
U2-17
U2-5
U2-1
U2-10
U2-16
U2-7
U2-2
U2-3
U. maydis genotypes

Fig. 2-1. Variation in aggressiveness of *U. maydis* diploid genotypes.

The 24 *U. maydis* diploid genotypes are arranged with an increasing order of aggressiveness to illustrate continuous variation. Inherent aggressiveness was the proportion of severely diseased plants in UM only treatments, without the endophyte present (blue bars). Realized aggressiveness was measured as the proportion of severely diseased plants in UM + FV treatments (red bars, UM + FV1; green bars, UM + FV2). For most *U. maydis* genotypes, aggressiveness was reduced in the presence of the endophyte, while for a few genotypes (UM2-1 and UM2-19) aggressiveness was increased in the presence of the endophyte. The parental genotype (U2-P) is indicated by an arrow. Error bars represent 95% confidence intervals (CIs).

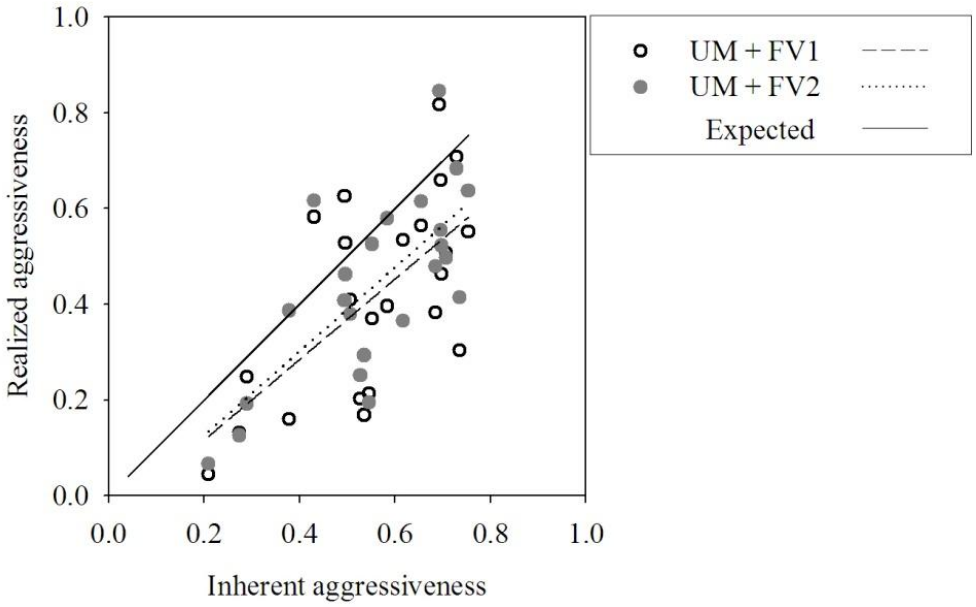

Fig. 2-2. Effect of *F. verticillioides* on *U. maydis* aggressiveness.

Realized aggressiveness, the proportion of severely diseased plants in the presence of the endophyte, was plotted against inherent aggressiveness, the proportion of severely diseased plants in *U. maydis* only treatments. The solid line represents the expected outcome if the *U. maydis* aggressiveness is not affected by the presence of the endophyte. Inherent and realized aggressiveness are positively correlated and slopes for UM + FV1 (dashed line; y = 0.84x – 0.05, r^2 = 0.41, $P < 0.01$; y = realized aggressiveness and x = inherent aggressiveness) and UM + FV2 (dotted line; y = 0.88x – 0.05, r^2 = 0.52, $P < 0.001$; y = realized aggressiveness and x = inherent aggressiveness) are not significantly different from 1.0 (Student's t-test, two-tailed; UM + FV1, t = 0.73, P = 0.47; UM + FV2, t = 0.65, P = 0.52). Realized aggressiveness is consistently lower than inherent

aggressiveness although y-intercepts for UM+FV1 and UM+FV2 are not significant different than 0.

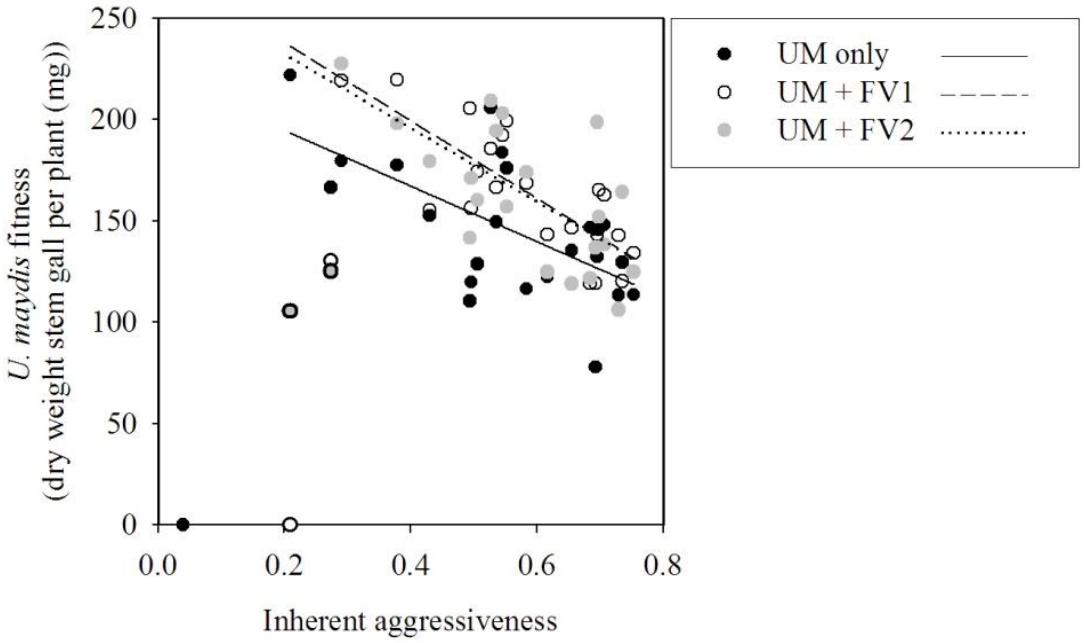

Fig. 2-3. *U. maydis* fitness decreases with increasing inherent aggressiveness

U. maydis fitness was measured as dry weight stem gall per diseased plant and plotted over inherent aggressiveness. Pathogen fitness was greater in the presence of the endophyte (UM+FV1, open circles, UM+FV2 grey circles) than in the absence of the endophyte (UM only, black circles). Linear regressions: UM only, solid line, y = -137x + 222, r^2 = 0.40, $P < 0.01$; UM + FV1, dashed line, y = -192x + 276, r^2 = 0.62, $P < 0.01$; UM + FV2, dotted line, y = -182x + 268, r^2 = 0.46, $P < 0.01$; y = *U. maydis* fitness and x = inherent aggressiveness. Slopes are not significantly different but y-intercept in UM + FV1 treatments are significantly greater than that for UM only treatments (Student's t-test, one tailed; t = 2.05, $P < 0.05$). The y-intercept in UM + FV2 treatments was not significantly greater than that for UM only treatment (Student's t-test, one tailed; t = 1.56,

P = 0.06). Data points with bold edge including the non-aggressive genotype (aggressiveness = 0.04) were treated as outliers because those *U. maydis* diploid genotypes either did not produce stem galls (aggressiveness = 0.2 and fitness = 0) or they have a substantial effect on the overall fit of the linear regression according to Cook's distance ($D > 1.0$) (Cook, 1979).

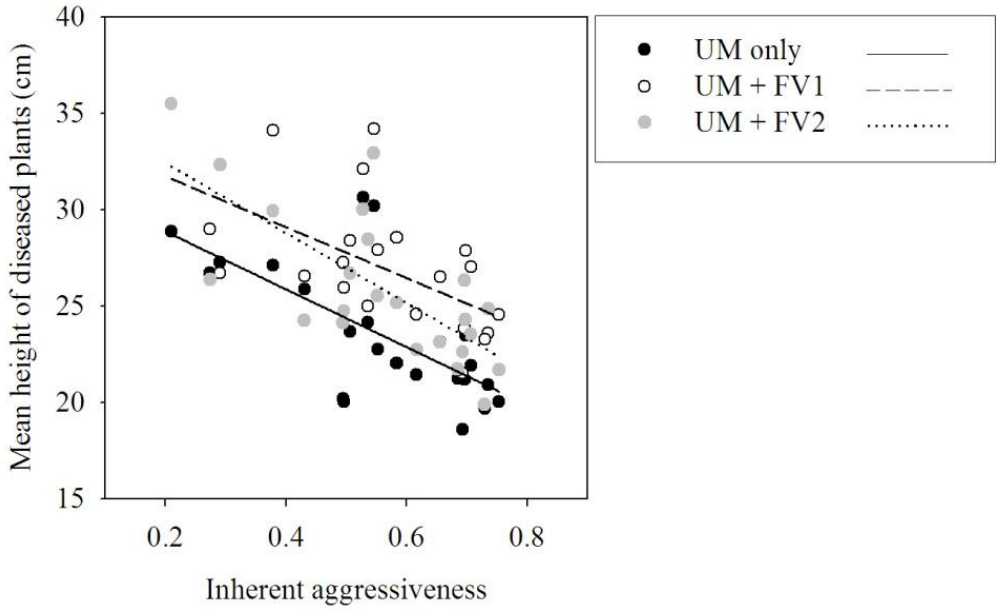

Fig. 2-4. Plant growth declines with increasing *U. maydis* aggressiveness.

Growth of smut diseased plants was negatively correlated with the level of *U. maydis* aggressiveness, both with and without *F. verticillioides* inoculation. However, greater plant growth was obtained with endophyte co-inoculation than without endophyte co-inoculation, across differing levels of inherent aggressiveness. Linear regression: UM only, solid line, y = -14.9x + 31.8, r^2 = 0.45, P < 0.001; UM + FV1, dashed line, y = -13.2x + 34.4, r^2 = 0.29, P < 0.01; UM + FV2, dotted line, y = -18.2x + 36.1, r^2 = 0.52, P < 0.001; y = mean height of diseased plants (cm) and x = inherent aggressiveness.

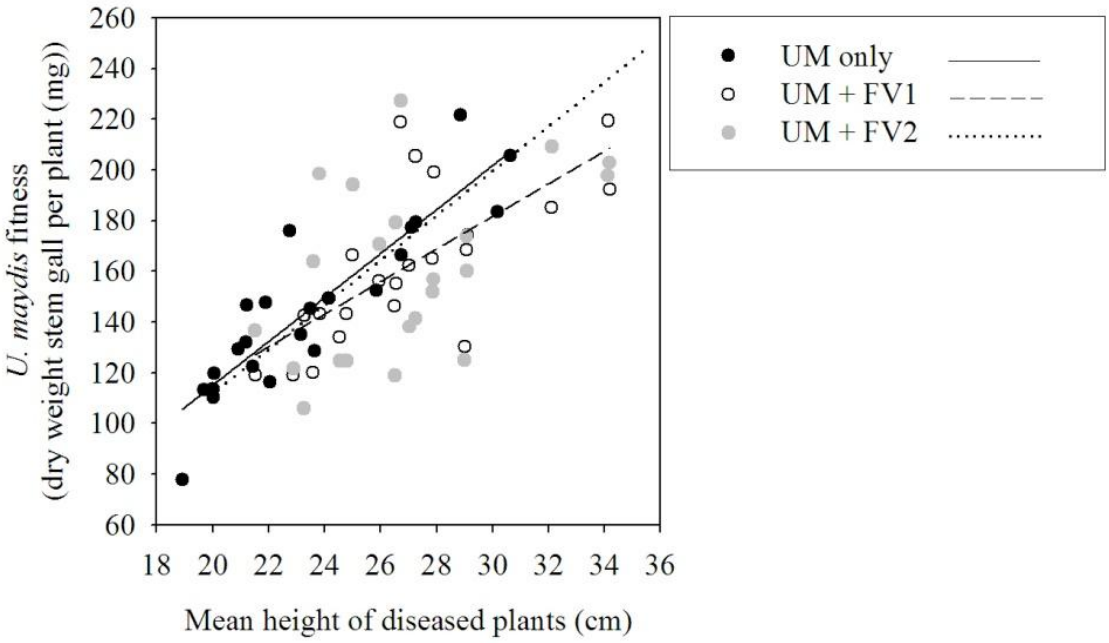

Fig. 2-5. *U. maydis* fitness increases with increasing plant height

There was a strong positive correlation between dry weight stem gall and mean height of diseased plants with stem galls. Linear regressions: UM only, black circles, y = 8.7x - 58.5, $r^2 = 0.79$, $P < 0.0001$; UM + FV1, white circles, y = 6.4x – 11.2, $r^2 = 0.50$, $P < 0.001$; UM + FV2, gray circles, y = 8.8x – 64.0, $r^2 = 0.76$, $P < 0.0001$; y = *U. maydis* fitness and x = mean height of diseased plants (cm). Neither slopes nor y-intercepts are significantly different between UM only treatments and UM + FV treatments.

CHAPTER 3

Fitness outcomes in interactions of *Ustilago maydis*, maize, and an endophyte depend on genotype

INTRODUCTION

Diverse microbial organisms, including mycorrhizal fungi, endophytes and pathogens, directly or indirectly interact with each other and with the host plant. Although theoretical studies suggest that fitness outcomes of multispecies interactions cannot always be explained as the sum of the component pairwise interactions (Hougen-Eitzman and Rausher, 1994; Iwao and Rausher, 1997; Strauss, 1991), most empirical studies have primarily focused on the direct interactions between a plant host and a pathogen (see Pariaud *et al.* 2009 and references therein). Thus, we have only a limited understanding how the interactions among the multiple community members affect fitness and evolution of a pathogen and its host. In this study, we determine effects of variation in a third interacting species on the fitness outcomes for a plant pathogen and its host plant.

Plants, endophytes and pathogens comprise excellent model systems with which to study the effects of co-occurring organisms on host-pathogen interactions. First, almost every plant species examined thus far is associated with endophytic fungi, which inhabit healthy plant tissues without causing disease symptoms (Petrini, 1991). Second, sessile plants encounter numerous pathogens and endophytes throughout their life span and these microbial species directly or indirectly interact with each other (Pan and May, 2009). Third, fungal endophytes produce diverse biologically active molecules (Schulz et al., 2002; Tan and Zou, 2001) and can trigger host defensive traits (e.g., Vu et al., 2006; Waller et al., 2005). Fourth, some endophytes can degrade host-synthesized antimicrobial molecules, thus can facilitate host infection by other microbial species which are susceptible to those host antimicrobial compounds (Saunders and Kohn, 2009). Therefore,

studying how endophytes affect plant-pathogen interactions not only helps us to better understand plant-pathogen coevolution in a multispecies context but also provides insight into the use of endophytes for biological control agents (Cavaglieri et al., 2004; Mejia et al., 2008).

Among the best-studied endophytes are the host-specific symbionts of grasses, which belong to the family Clavicipitaceae. These obligate intercellular symbionts are vertically transmitted and grow systemically throughout the above ground tissues in many grass species in the subfamily Pooideae (Schardl et al., 1997). Benefits of endophyte infections to host plants include growth enhancement, increased drought tolerance, systemic pathogen resistance and reduced herbivory (Clay, 1988; Clay and Schardl, 2002; Malinowski and Belesky, 1999; Saikkonen et al., 1998). While these grass endophytes are often considered as important evidence for plant-fungal mutualism, they account for a relatively small number of endophyte species (Schardl et al., 1997). In contrast, generalist endophytes occupying numerous hosts and include highly diverse taxa. These endophytes are usually horizontally transmitted and often grow locally within host tissues. Most studies of generalist endophytes have focused on the biodiversity and distribution of the microbial community in different plant species (Arnold *et al.*, 2000; Arnold *et al.*, 2001; Carroll, 1995; Crozier *et al.*, 2006; Joshee *et al.*, 2009), leaving an understanding of their ecological functions less well explored. Similar to host-specific endophytes, generalist endophytes may also play important defensive roles by limiting pathogen damage to the host plants in which they reside (Arnold et al., 2003; Campanile

et al., 2007; Danielsen and Jensen, 1999; Lee et al., 2009; Vu et al., 2006; Waller et al., 2005).

The underlying mechanisms of endophyte antagonism to pathogens are not well understood, but limited evidence suggests that some endophytes indirectly affect pathogens by inducing host immune system (e.g., Vu et al., 2006; Waller et al., 2005) or directly suppress pathogen growth in the host plants through the fungal-fungal interactions (e.g., Arnold et al., 2003; Mejia et al., 2008; Lee et al., 2009). While the details of direct antagonistic interactions between endophytes and pathogens need further investigation, recent studies suggest that secreted secondary metabolites might play important roles (e.g., Bacon et al., 2004).

In this work, we examine how a fungal endophyte, *F. verticillioides,* affects the outcomes of interactions between maize and its pathogen, corn smut *U. maydis*. Both *U. maydis* and *F. verticillioides* inhabit the above ground tissues of maize. The Basidiomycete fungus, *U. maydis* is a biotrophic pathogen of maize and has tracked the host expansion since the domestication of maize in southern Mexico (Munkacsi et al., 2007, 2008). Previously, we demonstrated that the endophyte *F. verticillioides* reduces *U. maydis* aggressiveness, consequently improves plant growth (Lee et al., 2009), and that these indirect effects lead to increased pathogen fitness (Chapter 2). In this study, we first ask if different strains of the endophyte, *F. verticillioides,* have varying effects on *U. maydis* aggressiveness, *U. maydis* fitness and host plant growth. Specifically, we ask if the varying levels of FA produced by different *F. verticillioides* strains *in vitro* are correlated with the effects on *U. maydis* aggressiveness during *in planta* interactions.

Secondly, we ask if *U. maydis* competitiveness towards *F. verticillioides* is correlated with *U. maydis* fitness in interactions of *U. maydis*, maize and *F. verticillioides*.

MATERIALS AND METHODS

Design of the plant inoculation experiments

The sweet corn variety Jubilee (*Zea mays* var. *rugosa*; Jordan seeds, Inc. Woodbury, MN) was used as a host because it is susceptible to *U. maydis.* We used five *F. verticillioides* (FV) treatments: controls without *F. verticillioides* (No FV) and inoculations with four different *F. verticillioides* genotypes (FV1 ~ FV4). *U. maydis* (UM) treatments consisted of seven different diploid genotypes. We chose four strains of endophytic *F. verticillioides* that demonstrated varying effects on *U. maydis* growth *in vitro* and produced varying amounts of FA during *in vitro* culture (Culture IDs at the University of Minnesota Culture Collection: FV1, 49 56796-8 S; FV2, 20 57001-7 E; FV3, 44 56796-6 E; FV4, 24 56796-6 E). These four *F. verticillioides* strains were individually inoculated or co-inoculated with each of the seven *U. maydis* genotypes that demonstrated varying aggressiveness toward maize in the previous experiments. The diploid genotype UM1 was generated by co-inoculating haploid strains A3 (a2 b3; St. Paul, MN USA) and C7 (a1 b12; northern Ohio, USA). Diploid genotype UM2 (= UM2-P) was generated by co-inoculating haploid strains C7 (a1 b12) and E11 (a2 b11; Owatonna, MN USA). The other five diploid genotypes were generated by co-inoculating the parental strain C7 (a1 b12) with each of five F-1 progenies (a2 b11) of UM2-P: UM2-1 + C7, UM2-2 + C7, UM2-7 + C7, UM2-13 + C7, UM2-19 + C7 (Chapter 2).

Control treatments included mock inoculation (sterile water) and FV only treatments (No UM). Each pot was a replicate unit and plants in the same pot received a single treatment. There were 12 replicate pots per treatment with a randomized complete block design (RCB) where two greenhouse benches were blocks.

Fungal cultures and plant inoculation

Fungal inocula for plant inoculation were prepared as previously described (Lee et al., 2009). Briefly, microconidia of *F. verticillioides* inoculum were scraped from 10-15 day-old cultures grown on Potato Dextrose Agar (PDA) (Yates et al., 1997), rinsed twice and resuspended in sterile water to a final concentration of 10^7 cells/mL. Haploid sporidial cultures of *U. maydis* were grown in Potato Dextrose Broth (PDB, Difco) for three days to a density of 2-3 x 10^8 cells/mL, rinsed twice and resuspended in sterile water to a final concentration of 10^8 cells/mL. The *U. maydis* inoculum was a mixture of an equal number of mating compatible haploid sporidia (10^7 sporidia each). Ten-day-old maize seedlings were inoculated with 2 x 10^6 *F. verticillioides* microconidia in 0.2 mL water or 2 x 10^7 *U. maydis* sporidia in 0.2 mL water. The mock treatments for either fungus were 0.2 mL sterile water.

Plant inoculation experiments were conducted in a 500 square-foot greenhouse room at the University of Minnesota plant growth facilities, St. Paul, MN. Six seeds were planted in each of the eight-inch pot filled with Sunshine Professional Growing Mix (Sun Gro Horticulture Canada Ltd.) as potting medium. Greenhouse conditions were optimal

for growing maize at 20 - 28 °C, 14h/10h light/dark cycle with 120 - 200 µE m^{-2} full spectrum light intensity.

Measurements

We measured *U. maydis* aggressiveness, fitness, and plant growth as outcomes of the interactions of pathogen, host plant, and endophyte. We evaluated disease severity at 20 days after planting (DAP 20) using the qualitative scale of Gold et al. (1997) and as previously described (Lee et al., 2009). Briefly, we considered severely diseased plants as those with stem or leaf galls, and calculated the proportion of severely diseased plants by dividing the number of severely diseased plants per pot by n, the total number of plants in the pot. To improve equality of variance, the proportion 0 was adjusted to 1/4n and the proportion 1 was adjusted to (n – 1/4)/n as suggested by Bartlett (1947). The adjusted proportion of severely diseased plants was then arcsine transformed for the analysis of variance (ANOVA) and back-transformed numbers are presented in all Figures. We defined inherent aggressiveness of *U. maydis* diploid genotypes as the proportion of severely diseased plants in *U. maydis* only treatments, and defined realized aggressiveness as the proportion of severely diseased plants caused by *U. maydis* in the presence of the endophyte (UM + FV treatments). We defined competitiveness of *U. maydis* towards *F. verticillioides* as the ratio of realized aggressiveness (RA) to inherent aggressiveness (IA), that is, competitiveness = RA/IA.

We determined if *U. maydis* fitness could be measured as total dry weight stem gall per plant. Smut galls were collected 3-4 weeks after inoculation (DAP 31 – 38) when

mature, combined together from each plant and dried at room temperature until the gall weight became constant. Results for plants with leaf galls only (12% of the total samples) were removed from the dataset because it was not possible to collect small leaf galls without including a variable amount of plant material. Dry weight stem gall per individual plant (mg spores) was log-transformed before ANOVA and back-transformed values presented in Figures. We then determined the correlation between total dry weight of stem galls per plant and *U. maydis* fitness, as number of teliospores. Dry galls were ground to release spores from plant tissues, and about 10 mg of the ground gall was weighed, and then suspended in 1mL of smut wash solution (sterile water amended with 50 µg/mL streptomycin and 50 µg/mL penicillin). Five µL of the spore suspension was pipetted onto a hemacytometer and spores per uL were counted. A total of 291 samples, with at least three samples per treatment, were counted and the number of total teliospores produced per plant was calculated. The correlation of dry weight stem gall per diseased plant (log-transformed) and the calculated number of teliospores (log-transformed) was determined using Pearson's product-moment test. Plant growth was measured as the number of leaves per plant at DAP 20.

In vitro production of fusaric acid (FA) by *F. verticillioides*

We measured amounts of FA produced by the four *F. verticillioides* strains described above during growth in Czapek-Dox medium as described previously (El-Hasan et al., 2008). The concentration of FA produced was estimated from the standard curve generated using authentic FA (≥ 99.0%, TLC; Sigma-Aldrich, Inc.), prepared in methanol

to the concentrations of 0.1, 0.3, 0.4, 0.5 and 0.8 μg/μL, and analyzed as were the biological samples described below.

The FA produced per gram mycelium was estimated for each of two replicates per strain using cultures grown at 25 °C for 30 days at 100 rpm in 25 mL of Czapek-Dox medium in 100 mL flasks. Cultures were filtered through the pre-weighed Whatman filter paper (No.1) and the mycelium was dried in a 60 °C oven over night, and the dry weight recorded. The culture filtrate was transferred to a 50 mL conical tube and adjusted to pH 4.0 with 1 N HCl. Sterile water was added to adjust the volume to 15 mL, an equal volume of ethyl acetate was added, and the tubes were shaken for 1 hr at 250 rpm. Extracts were then centrifuged at 4,000 rpm for 20 min, and 10 mL of the 15 mL of the upper organic phase was transferred to a new 50 mL conical tube. Ethyl acetate was evaporated completely using a vacuum dryer and the remaining pellet was then dissolved in 1 mL of methanol. We used an Ultra Performance Liquid Chromatography-Tandem Mass Spectrometric (UPLC-MS TOF; Waters, Inc.) (Mensch et al., 2007) at the Center for Mass Spectrometry and Proteomics at the University of Minnesota. The mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Four μL of each sample was injected and the corresponding peak area attributed to FA (mass/charge = 180.100) was calculated. Amounts of FA were estimated by comparing peak areas obtained from experimental samples to a regression of peak area for known FA concentrations against FA concentration (square-root transformed) to give FA concentration = $(x/10^4 + 0.0145)^2$, where x = peak area ($r^2 = 0.99$, $P < 0.001$).

Statistical analysis

We determined if variation across strains of the endophyte and the pathogen had significant effects on *U. maydis* aggressiveness, fitness, and plant growth using a fixed effect ANOVA with a generalized linear model (GLM) in SPSS (SPSS Inc., Chicago, IL) with dependent variables transformed before analysis as described above. The significance of treatment factors Block (greenhouse benches), *F. verticillioides* (FV) treatments and *U. maydis* (UM) genotypes, as well as interaction effects of FV treatments and UM genotypes were determined, treating these factors as fixed effects, as we measured every individual in the experimental population. We ran Tukey's HSD (Honestly Significant Difference) test as a *post hoc* procedure to determine significant differences among treatment means. Differences were considered significant at two-tailed P values < 0.05.

To determine whether *F. verticillioides* inoculation alone affects plant growth, we used one-way ANOVA (SPSS) to compare plant growth as the number of leaves per plant across control treatments lacking *U. maydis* inoculation: water control (mock), and each of the four endophyte strains (FV1 – FV4). Differences were considered significant at $P < 0.05$.

We hypothesized that the FA produced by the endophyte is an antagonistic molecule against the pathogen's growth in the host plant. Thus, we expected that increasing amounts of FA produced by different *F. verticillioides* strains would be correlated with greater reduction in *U. maydis* aggressiveness. We evaluated the degree to which aggressiveness was reduced in the presence of the endophyte as competitiveness

defined above as RA/IA. The relationship between FA production and the competitiveness of *U. maydis* towards *F. verticillioides* was determined using a linear correlation test as Pearson's product-moment test and a nonlinear correlation test as Spearman's rank test. Both tests were conducted with SPSS. Lastly, we determined the correlation of variation in *U. maydis* competitiveness towards *F. verticillioides* and *U. maydis* fitness using Pearson's product-moment correlation test (SPSS).

RESULTS

Inoculation of *U. maydis* onto maize plants was effective and resulted in inherent aggressiveness levels ranging from 0.77 to 0.91 (proportion of severely diseased plants). These values were higher than were those obtained in the previous study; values ranged from 0.43 to 0.74 for the same seven *U. maydis* genotypes (Chapter 2). Plants that were not inoculated with *U. maydis* did not show smut disease symptoms indicating that contamination was negligible. *F. verticillioides* inoculation was effective as we recovered *F. verticillioides* from 18 of 20 randomly sampled surface-sterilized maize leaves 2 days after inoculation. We also observed *F. verticillioides* growing on *U. maydis* diseased tissues in most UM + FV treatments. Plants inoculated with the endophyte alone did not develop any signs of disease symptoms, such as leaf blight and root or stem rots. The results of one-way ANOVA on plant growth in control treatments, mock (No UM, no FV) and each of the *F. verticillioides* strains, FV1 – FV4 (No UM), showed no significant difference among control treatments ($F_{4,335}$ = 1.1, P = 0.33), confirming that the endophyte alone does not have detectable effects on plant growth.

Endophyte inoculation had a dramatic effects on *U. maydis* disease development; about 85% of plants in UM-only inoculated treatments produced smut galls, while only 20% of plants produced galls in UM + FV inoculated treatments. Consistent with the results of reduced aggressiveness, plants in UM + FV treatments grew larger on average at 4.5 ± 0.1 (SE) leaves than did plants in UM only treatments at a mean of 2.8 ± 0.1 (SE) leaves. The four *F. verticillioides* strains produced the following amounts of FA: FV1, 1569 ± 114; FV2, 4171 ± 325; FV3, 2857 ± 308; FV4, 2454 ± 273 as mean μg FA/g mycelium ± SE.

Factors affecting *U. maydis* aggressiveness

We determined the factors affecting pathogen's aggressiveness toward its host using ANOVA on the full factorial design. Results show that pathogen aggressiveness, measured as the proportion of severely diseased plants, is significantly affected by *U. maydis* genotypes, FV treatments (No FV, FV1-FV4) and an interaction effect of *U. maydis* genotypes and FV treatments (Table 3-1). The presence of *F. verticillioides* resulted in dramatic reduction in *U. maydis* aggressiveness in all 28 combinations of UM x FV; *U. maydis* aggressiveness was consistently reduced by 31 ~ 91% relative to inherent aggressiveness (Fig. 3-1). Strains FV2, FV3 and FV4 had significantly greater negative effects on *U. maydis* aggressiveness than did FV1 although there were no significant differences among the three strains, FV2-FV4 (Tukey's HSD test, $P > 0.05$). The significant interaction effects (UM x FV) resulted because FV1 had stronger negative effects on less aggressive *U. maydis* genotypes (0.77 – 0.86) than did FV1 on more

aggressive *U. maydis* genotypes (0.87 – 0.90), while FV2-FV4 reduced aggressiveness greatly in all seven *U. maydis* genotypes (Fig. 3-1). Strain FV1 produced the least amount of FA during *in vitro* culture, suggesting that lower FA production is associated with lesser impacts on *U. maydis* aggressiveness. Taken together, these results provide evidence that *U. maydis* aggressiveness is affected by genotypes of interacting pathogen and endophyte.

Factors affecting *U. maydis* fitness

We found a strong positive correlation between dry weight stem gall and the number of teliospores produced per plant (r^2 = 0.57; Fig. 3-2). Consequently, we measured *U. maydis* fitness as dry weight stem gall per plant. The results of ANOVA showed significant effects of FV treatments on *U. maydis* fitness, but not of UM treatments (genotypes) or interactions of UM x FV (Table 3-2). A negative correlation between inherent aggressiveness and fitness was not found (Fig. 3-3, dark-blue circles) as in previous results (Chapter 2), likely because we obtained a very narrow range of high values for inherent aggressiveness, 0.77 – 0.90, in these experiments.

Consistent with the previous study (Chapter 2), *U. maydis* obtained greater fitness in the presence than in the absence of the endophyte. In the absence of the endophyte, dry weight stem gall per diseased plant averaged 89.7 ± 3.8 mg (mean ± SE), ranging from 77.7 ± 7.1 to 99.3 ± 10.1 mg across the seven genotypes. In the presence of the endophyte, *U. maydis* fitness averaged 159.6 ± 7.5 mg per diseased plant, ranging from 79.0 ± 7.2 to 376.7 ± 192.5 mg, across the seven genotypes. Tukey's HSD test demonstrated that *U.*

maydis fitness in FV2-FV4 co-inoculation treatments was significantly greater relative to UM only treatments ($P < 0.05$), while *U. maydis* fitness in FV1 co-inoculation treatments was not significantly different from that of the *U. maydis* only treatment (Tukey's HSD test, $P > 0.05$). Thus, these results show that pathogen fitness is affected by the endophyte strain as well as the presence or absence of the endophyte *F. verticillioides*.

Factors affecting plant growth

As shown above, *F. verticillioides* only inoculation has no significant effects on plant growth, suggesting the endophyte may indirectly affect host plant growth in the presence of the pathogen, *U. maydis*. The ANOVA results show that plant growth (leaf number per plant) was significantly affected by Block (greenhouse bench), *U. maydis* genotypes and FV treatments (Table 3-3). UM x FV interaction effects were not significant. The Block effects reflect the impacts of environmental variation across the greenhouse room on plant growth. Maize plants inoculated with the less aggressive genotypes (inherent aggressiveness, 0.77 ~ 0.86) grew significantly greater than did plants inoculated with the more aggressive genotypes (inherent aggressiveness, 0.88 – 0.90) (Tukey's HSD test, $P < 0.05$). Co-inoculation of *F. verticillioides* with *U. maydis* had a strong positive impact on plant growth; plants in UM + FV treatments had 3.5 ± 0.2 – 4.8 ± 0.1leaves per plant (mean ± SE), while plants in UM only treatments had only 2.5 ± 0.1 – 3.0 ± 0.1 leaves per plant (Fig. 3-4). Given that *F. verticillioides* alone did not have detectable effects on plant growth, these results suggest that plant growth increases in the presence of the

endophyte because the endophyte reduces the negative impacts of *U. maydis* towards the host plants.

Correlation between *U. maydis* fitness and plant size

To determine whether the greater *U. maydis* fitness is attributable to the increased plant size, we ran a linear correlation test for *U. maydis* fitness, measured as dry weight stem gall per plant, and the size of diseased plants, measured as the number of leaves per plant (Fig. 3-5). There was a positive correlation between plant size and *U. maydis* fitness (linear regression, y = 94.3x -106.3, where y = *U. maydis* fitness, x = plant size; $r^2 = 0.47$, $P < 0.0001$), demonstrating that *U. maydis* fitness increases as infected host plants grow larger. Importantly, the size of diseased plants is greater in the presence (2.93 ± 0.05 leaves per plant; mean ± SE) than in the absence (2.32 ± 0.03 leaves per plant) of the endophyte, *F. verticillioides*, showing that the beneficial effects of the endophyte on *U. maydis* fitness is via greater plant growth.

Correlation between plant size and *U. maydis* aggressiveness

As we found the indirect positive effects of the endophyte *F. verticillioides* on *U. maydis* fitness above via improved plant growth, we then asked if plant growth is strongly affected by the level of *U. maydis* aggressiveness. Consistent with our previous studies (Lee et al., 2009; Chapter 2), we found that the size of diseased plants was strongly, negatively correlated with *U. maydis* realized aggressiveness that achieved when endophyte was co-inoculated with the pathogen ($r = -0.97$, $P < 0.0001$; Fig. 3-6).

Together with the dependence of *U. maydis* fitness on plant growth as shown above, these results suggest that *U. maydis* fitness is dependent on the level of aggressiveness towards its host plant, which is strongly affected by co-inoculation of the pathogen with different strains of *F. verticillioides*. Thus, *F. verticillioides* indirectly benefits *U. maydis* fitness by slowing disease development and, consequently increasing plant growth.

Correlation between varying amounts of FA production and *U. maydis* aggressiveness

With the strain-specific effects of *F. verticillioides* on *U. maydis* aggressiveness and fitness shown above, we next determined if varying amount of FA produced by these strains *in vitro* was correlated with effects on *U. maydis* aggressiveness as measured by competitiveness (RA/IA). Both linear and non-linear correlation tests revealed significant correlations between FA level and *U. maydis* competitiveness towards *F. verticillioides* (Pearson's product-moment test, $r^2 = 0.46$, $P < 0.001$; Spearman's rank correlation test, $\rho = 0.62$, $P < 0.001$; Fig. 3-7). Yet, the significant correlation was obtained largely due to varying level of *U. maydis* genotype's competitiveness towards the endophyte strain producing the least FA, FV1. In contrast, these *U. maydis* genotypes demonstrated much lower competitiveness towards the strains FV2-FV4 (Tukey's HSD test, $P < 0.05$). Specifically, the average competitiveness across *U. maydis* genotypes towards FV1 was 0.47 ± 0.06 (mean ± SE), while the average competitiveness across the same *U. maydis* genotypes towards the other endophyte strains were 0.15 ± 0.02 (FV2), 0.14 ± 0.02 (FV3) and 0.15 ± 0.02 (FV4), respectively. Thus, these results suggest that if FA has an

antagonistic role against the pathogen, *U. maydis*, there is a threshold effect wherein strains producing the equivalent of 2454 μg FA/g mycelium or greater *in vitro* have the similar effects on *U. maydis* aggressiveness *in planta*.

Correlation between *U. maydis* competitiveness towards *F. verticillioides* and *U. maydis* fitness

Above results and those of the previous study (Chapter 2) suggest that *F. verticillioides* reduces *U. maydis* aggressiveness and as a result, *U. maydis* fitness increases. We determined if *U. maydis* genotypes that compete better against *F. verticillioides* have greater fitness using a linear correlation test (SPSS). There was a negative correlation between the competitiveness of *U. maydis* towards *F. verticillioides* and *U. maydis* fitness, suggesting that the pathogen might gain fitness if they are less competitive towards *F. verticillioides* ($r^2 = 0.35$, $P < 0.001$; Fig. 3-8). Most of the correlation of fitness with competitiveness is derived from the varying response of *U. maydis* genotypes to strain FV1. Interestingly, there was considerable variation in *U. maydis* fitness at the low range of competitiveness, likely due to the combined effects of variable abiotic environment and small sample sizes (3-5 replicates) for some data points represented. Taken together, these results show that fitness of both the pathogen and of the host are affected by the presence and the strain of *F. verticillioides*, effects mediated through the endophyte limiting negative impacts of pathogen aggressiveness and consequently improving plant growth and resources available for pathogen reproduction. The negative correlation between *U. maydis* competitiveness towards *F. verticillioides* and *U. maydis*

fitness further suggests that *U. maydis* fitness is not independent from its aggressiveness towards the host, thus antagonistic endophytes occurring in the same host plants may strongly influence the evolution of plant-pathogen interactions.

DISCUSSION

The results of our study show that co-occurring endophytes have the ecological potential to influence coevolutionary host-pathogen interactions. In the pairwise interactions, pathogen aggressiveness, pathogen fitness and plant growth are affected only by the genotypes of the pathogen and host. In contrast, in multispecies interactions, an endophyte significantly alters the fitness outcomes for the pathogen and its plant host. The presence of the endophyte limits pathogen aggressiveness, allowing increased plant growth, with which greater pathogen fitness results. Thus, *in planta* interactions among co-occurring microbial symbionts have the potential to affect the evolution of host-pathogen interactions.

Our results showed that *U. maydis* aggressiveness was significantly affected by the presence and strains of the endophyte *F. verticillioides*. The endophyte strain producing the least amount of FA (FV1) reduced *U. maydis* aggressiveness by 31 – 72% relative to inherent aggressiveness; while the other three *F. verticillioides* strains producing greater amounts of FA (FV2-FV4) decreased *U. maydis* aggressiveness by 75 – 90% relative to the inherent aggressiveness. Interestingly, *F. verticillioides* strains producing equal to or greater than 2454 μg FA/g mycelium had similar effects on *U. maydis* aggressiveness, demonstrating a threshold effect, which was also observed during

our preliminary experiments. During the *in vitro* culture, the growth of *U. maydis* haploid (strain E11) sporodia was inhibited by 4.6 – 17.2% relative to no FA control at 10 – 30 μg/mL FA, while that was inhibited by 96.4 – 99.8% at 50 – 100 μg/mL FA (unpublished data by K. Lee), suggesting that FA plays as an antagonistic role against *U. maydis*. Taken together, our study suggests that pathogen aggressiveness towards its host plant is not just determined by pathogen traits but also affected by interaction traits of community members.

We obtained much higher inherent aggressiveness over a narrower range in this study, 0.77 – 0.90, than did we in the previous study, 0.43 – 0.74, using the same *U. maydis* genotypes (Chapter 2). The differing outcomes between experiments reveal a strong impact of abiotic environment on disease development (McNew, 1960; Scholthof, 2007). Consistent with our results, Baumgarten et al. (2007) showed that abiotic environment significantly influences maize resistance to *U. maydis*. Although we did not manipulate specific abiotic factors in these experiments, results of inoculation experiments under controlled conditions showed that high light intensity (~ 200 μE m^{-2}) with natural quality and relative humidity of 60-80% favor greater *U. maydis* infections, while similar levels of *U. maydis* aggressiveness were obtained over a wide range of temperatures (18 – 28 °C; K. Lee unpublished data). Thus, our study suggests that both co-occurring symbionts, such as endophytes, and abiotic environments (Scholthof, 2007) are important factors in determining the outcomes of plant-pathogen interactions.

The trade-off model assumes a direct relationship between pathogen aggressiveness and fitness (Ewald, 1983; May and Anderson, 1983). In the Chapter 2

study, we have demonstrated a trade-off between *U. maydis* aggressiveness and fitness. Here, we provide further evidence for a fitness-aggressiveness trade-off; the dependence of *U. maydis* fitness on the level of its aggressiveness towards host plants was consistent between experiments, even though the levels of inherent aggressiveness considerably varied between experiments. For instance, for the same *U. maydis* genotypes, *U. maydis* fitness slightly increased (dry weight stem gall per plant) from 135.3 ± 5.0 mg to 145.9 ± 6.9 mg per plant by *F. verticillioides* co-inoculation in the Chapter 2 study, about 5% increase. In contrast, *U. maydis* fitness increased from 89.8 ± 3.7 mg to 156.0 ± 7.8 mg per plant by endophyte co-inoculation in this study, about 73% increase. The greater positive effects of the endophyte on the pathogen fitness in this study than were they in the Chapter 2 study is attributable to the greater reduction in the pathogen aggressiveness in this study than was in the previous study; co-inoculation of the endophyte reduced *U. maydis* aggressiveness from 0.85 to 0.20 in this study (0.65 reduction) but it only slightly reduced *U. maydis* aggressiveness from 0.62 to 0.49 in the Chapter 2 study (0.13 reduction). In addition, in the absence of *F. verticillioides, U. maydis* genotypes had much lower fitness in this study (89.8 ± 3.7: mean ± SE) than did they in the Chapter 2 study (135.3 ± 5.0; mean ± SE) due to the higher level of inherent aggressiveness, which averaged 0.85 in this study but averaged 0.62 in the Chapter 2 study. Likewise, in the presence of *F. verticillioides,* the same *U. maydis* genotypes had higher fitness in this study (156.0 ± 7.8) than did they in the Chapter 2 study (145.9 ± 6.9) due to the lower level of realized aggressiveness), which averaged 0.20 in this study but averaged 0.49 in the Chapter 2 study. Therefore, these results further suggest that the evolution of *U.*

maydis aggressiveness should be constrained by a trade-off between the level of aggressiveness towards its host plant and within-host reproduction.

To our knowledge, this is the first report to show the quantitative effect of interspecific interactions on pathogen fitness with the surprising result that the pathogen's fitness increases. Most previous studies on endophyte-pathogen interactions primarily aimed to determine whether endophytes confer disease resistance to host plants (Arnold et al., 2003; Bonos et al., 2005; Campanile et al., 2007; Clarke et al., 2006; Clay et al., 1989; Danielsen and Jensen, 1999; Narisawa et al., 2002). These studies have shown that many fungal endophytes provide host plants protection against fungal pathogens to some extent, but none of these studies examined how endophytes affect pathogen's fitness. Our study demonstrates that *F. verticillioides* not only affects the outcomes of ecological plant-pathogen interactions, i.e. aggressiveness, but also alters the fitness of co-occurring pathogen, suggesting that evolution of *U. maydis* aggressiveness traits might become associated with traits important for interactions with other co-occurring symbionts, such as *F. verticillioides*.

Generalist endophytes are both diverse and ubiquitous organisms occurring in associations with many plant hosts (Bacon and White, 2000; Petrini, 1991). Although these microbial species are less studied than pathogens, accumulating evidence shows that these organisms can have beneficial effects on host plants (Arnold et al., 2003; Lee et al., 2009; Mejia et al., 2008; Rodriguez et al., 2008). Further studies using plant, endophyte and pathogen systems can investigate the underlying molecular and genetic mechanism of endophyte-pathogen interactions. In addition, because endophytic fungi

produce diverse biologically active secondary metabolites, such as novel antibiotics (Castillo et al., 2003; Castillo et al., 2006), anti-oomycete compound (Strobel et al., 1999a), and antimycotics (Strobel et al., 1999b), studies using these systems will not only help us to better understand how interacting species pairs evolve in a complex community but also provide tools to utilize them as biological control agents for important pests in agriculture (Schulz et al., 2002; Tan and Zou, 2001).

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TABLES

Table 3-1. ANOVA on *U. maydis* aggressiveness with greenhouse bench, *F. verticillioides* strains and *U. maydis* diploid genotypes as treatment factors.

Source	df[a]	SS[b]	MS[c]	*F*	*P*
Block	1	0.02	0.02	0.77	0.381
U. maydis (UM)	6	1.75	0.29	9.77	< **0.001**
F. verticillioides (FV)	4	41.88	10.47	350.94	< **0.001**
UM X FV	24	1.18	0.05	1.65	**0.030**
Error	383	11.43	0.03		

[a]degrees of freedom; [b]type III sum of squares; [c]mean squares
[d]five levels: No FV (UM only) and FV1 – FV4

Table 3-2. ANOVA on *U. maydis* fitness (dry weight stem gall per plant) with greenhouse bench, *F. verticillioides* strains and *U. maydis* diploid genotypes as treatment factors.

Source	**df**[a]	**SS**[b]	**MS**[c]	***F***	***P***
Block	1	0.05	0.05	0.62	0.431
U. maydis (UM)	6	0.63	0.10	1.29	0.258
F. verticillioides (FV)	4	12.31	3.08	37.98	**< 0.001**
UM X FV	24	2.71	0.11	1.39	0.100
Error	666	53.98	0.08		

[a]degrees of freedom; [b]type III sum of squares; [c]mean squares
[d]five levels: No FV (UM only) and FV1 – FV4

Table 3-3. ANOVA on plant growth (average leaf number per plant) with greenhouse bench, *F. verticillioides* strains and *U. maydis* diploid genotypes as treatment factors.

Source	**df**[a]	**SS**[b]	**MS**[c]	***F***	***P***
Block	1	26.19	26.19	25.80	< **0.001**
U. maydis (UM)	6	45.26	7.54	7.43	< **0.001**
F. verticillioides (FV)	4	1295.82	323.96	319.07	< **0.001**
UM X FV	24	34.00	1.42	1.40	0.104
Error	383	388.86	1.02		

[a]degrees of freedom; [b]type III sum of squares; [c]mean squares
[d]five levels: No FV (UM only) and FV1 – FV4

FIGURES

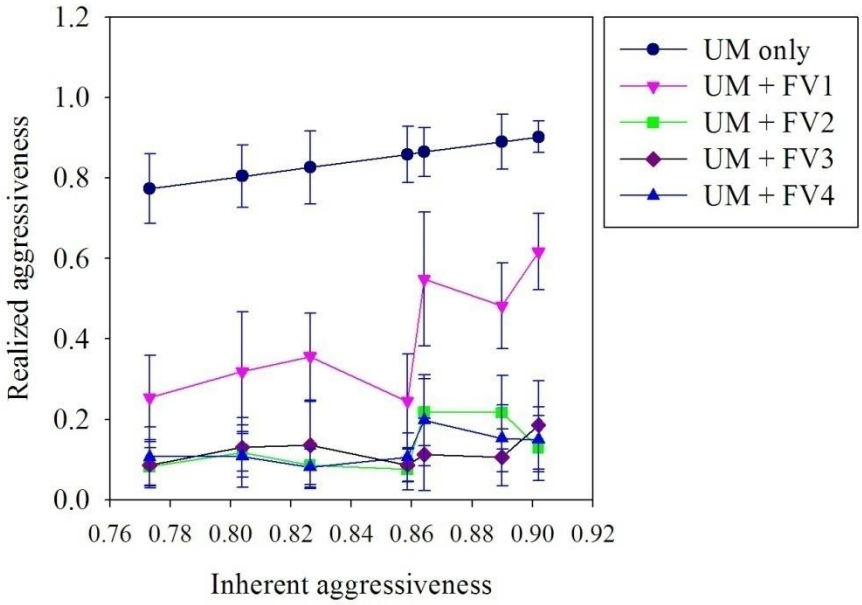

Fig. 3-1. Relationship of inherent and realized aggressiveness of *U. maydis*. *U. maydis* aggressiveness (as proportion of severely diseased plants) towards the plant in the presence of each of the four *F. verticillioides* strains and by itself, is presented. Co-inoculation of *F. verticillioides* resulted in significant reduction of *U. maydis* aggressiveness across all combinations of *U. maydis* genotypes and *F. verticillioides* strains. Results for UM only treatments represent inherent aggressiveness and are shown for reference (blue circle). Error bars represent 95% confidence intervals.

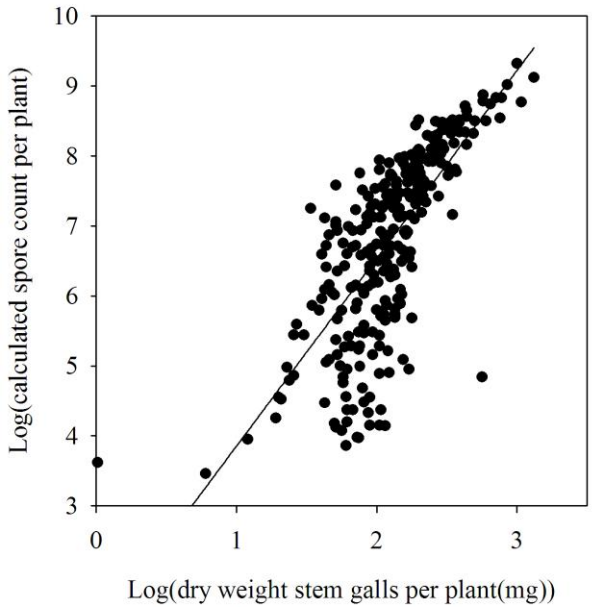

Fig. 3-2. Correlation of dry weight stem gall per plant and numbers of teliospores produced per plant. Total dry weight stem gall per diseased plant provides a measure of *U. maydis* fitness as calculated total teliospore produced per plant (r^2 = 0.57).

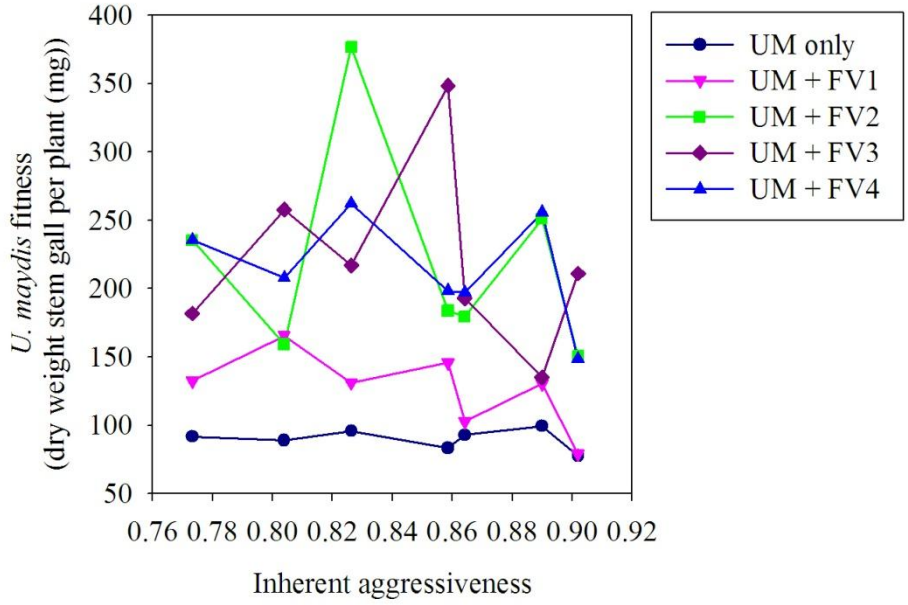

Fig. 3-3. Positive impact of *F. verticillioides* on *U. maydis* fitness. *U. maydis* had greater fitness when co-inoculated with *F. verticillioides* compared to UM only treatments, over the range of inherent aggressiveness (proportion of severely diseased plants).

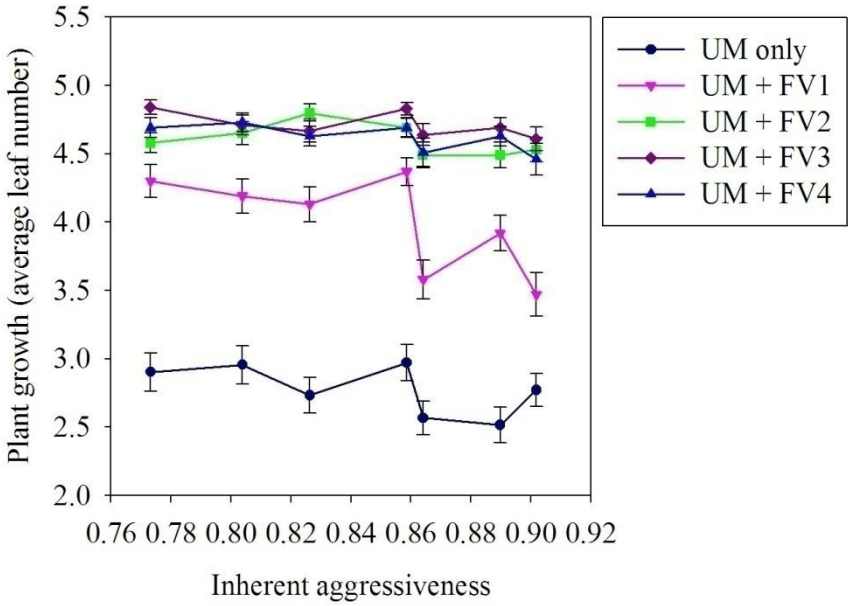

Fig. 3-4. Indirect effects of *F. verticillioides* on plant growth. Co-inoculation of *F. verticillioides* with *U. maydis* had a strong, positive impact on plant growth across the range of inherent aggressiveness (proportion of severely diseased plants). Strain FV1 had significantly less impact on plant growth in the presence of *U. maydis* than did other FV strains (FV2 – FV4) (Tukey's HSD test, $P < 0.05$). Error bars represent ±SE.

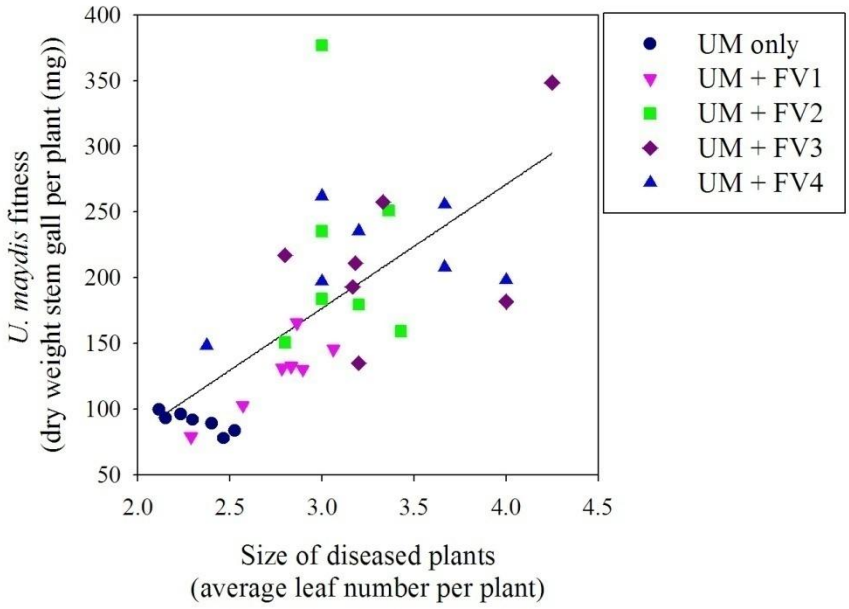

Fig. 3-5. *U. maydis* fitness is positively correlated with plant size. Diseased plants grew better in the presence (2.93 ± 0.05; mean ± SE) than in the absence (2.32 ± 0.03; mean ± SE) of *F. verticillioides* and were associated with greater *U. maydis* fitness (Student's t-test one-tailed: t = -11.5, $P < 0.0001$; linear regression, y = 94.3x -106.3, where y = *U. maydis* fitness, x = plant size; $r^2 = 0.47$, $P < 0.001$).

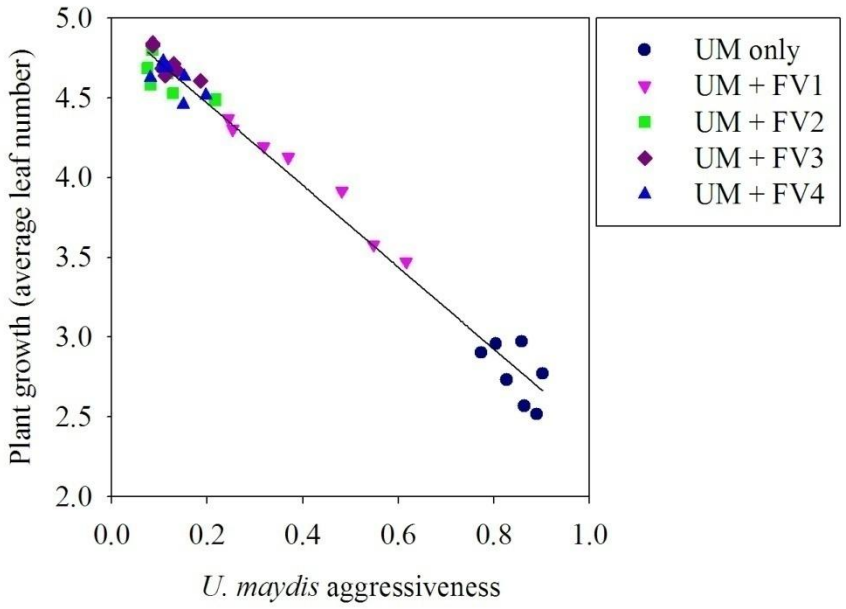

Fig. 3-6. Plant growth was negatively correlated with increased realized aggressiveness. Co-inoculation of *F. verticillioides* with *U. maydis* significantly reduced *U. maydis* aggressiveness, measured as proportion of severely diseased plants, and plants grew better, compared to UM only treatments (r = -0.97, $P < 0.001$).

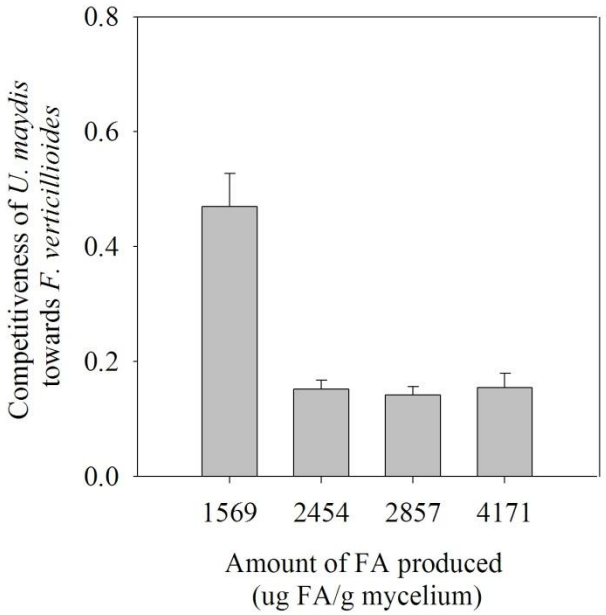

Fig. 3-7. Correlation between the amounts of FA and the *U. maydis* competitiveness. The competitiveness of *U. maydis* towards *F. verticillioides* (RA/IA) was negatively correlated with increasing FA production by different strains of *F. verticillioides* (Pearson's product-moment test, $r^2 = 0.46$, $P < 0.001$; Spearman's rank correlation test, $\rho = 0.62$, $P < 0.001$).

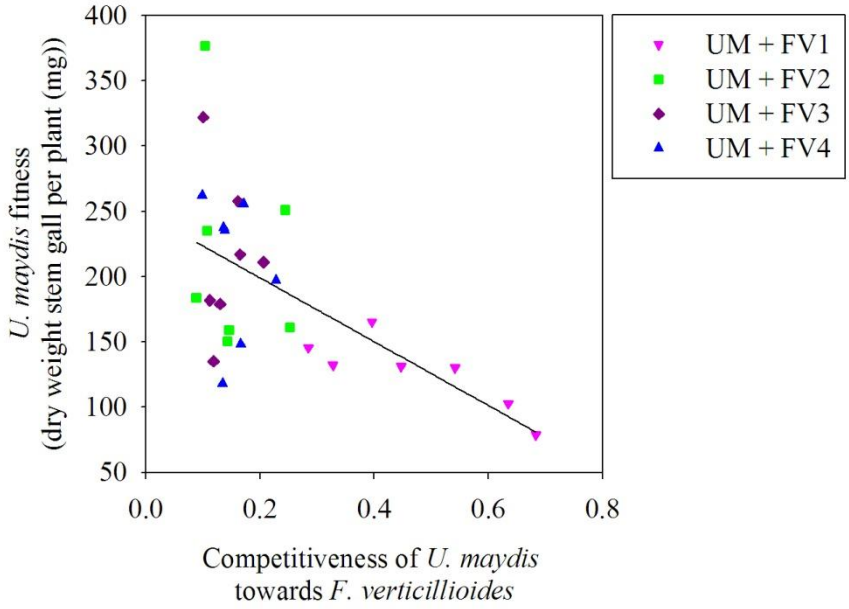

Fig. 3-8. *U. maydis* fitness is negatively correlated with competitiveness. Increased competitiveness as RA/IA, was associated with decreased fitness, especially for interactions involving FV1 (linear regression, y = -244x + 248, where y = *U. maydis* fitness, x = competitiveness of *U. maydis* towards *F. verticillioides*; $r^2 = 0.35$, $P < 0.001$). However, *U. maydis* fitness varied greatly at lower levels of competitiveness demonstrated in interactions with strains FV2 - FV4.

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