

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

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Abstract

Endophytic fungi represent diverse taxa that inhabit plant hosts without causing disease symptoms. We used endophytic isolates of *Fusarium verticillioides* (Sacc.) Nirenberg to understand how endophytic fungi interact with pathogens, in this case, the corn smut pathogen, *Ustilago maydis* DC (Corda). Endophytic *F. verticillioides* strains were inoculated onto maize seedlings before, simultaneously, or after inoculation with *U. maydis*, and the effects on smut disease severity and on plant growth were assessed. When *F. verticillioides* is simultaneously coinoculated with *U. maydis*, smut disease severity is significantly decreased and plant growth is increased, compared with other treatments. Controls show that *F. verticillioides* by itself does not have measurable effects on plant growth. Together, our results suggest that a commonly occurring fungal endophyte on maize, *F. verticillioides*, ameliorates the effects of a host-specific pathogen, *U. maydis*, by interfering with the early infection process and limiting disease development, resulting in increased plant growth.

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 microorganisms are being explored only now 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 *Fusarium graminearum* and *Stenocarpella maydis* (Berk.) Sutton (Rheeder *et al.*, 1990) and other microorganisms (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. *mays*) and the teosintes (e.g. *Z. 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 (Munkacsy *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 plant growth. Specifically, we asked (1) whether endophytic *F. verticillioides* reduces smut disease severity and plant mortality, (2) whether endophytic *F. verticillioides* enhances 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, 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). *Fusarium verticillioides* cultures were grown at 28 °C on potato dextrose agar [PDA; 24 g potato dextrose broth (Difco); 1 L deionized water, and 15 g of agar (Difco)], amended with 25 mg L⁻¹ penicillin, and 25 mg L⁻¹ streptomycin. Three haploid isolates of *U. maydis* were used: C7 (a1 b12) from northern Ohio, A3 (a2 b3) from St Paul, MN, and E11 (a2 b11) from Owatanna, MN. The strain C7 was used as a common parent with either A3 or E11 to generate two different *U. maydis* diploid genotypes, UM1 (A3 × C7) and UM2 (E11 × C7).

For plant inoculations, cultures of haploid *U. maydis* sporidia were grown in liquid media to a density of 2–3 × 10⁸ cells mL⁻¹ and harvested by centrifugation at 1800 g for 10 min. Microconidia of *F. verticillioides* were collected by scraping the surface of 10–15-day-old PDA cultures and flooding these with sterile water (Yates *et al.*, 1997). Spores of both fungi were washed twice with sterile water and resuspended in sterile water to a final concentration of 10⁷ cells mL⁻¹ for *F. verticillioides* and 10⁸ cells mL⁻¹ for *U. maydis*. To inoculate plants, 0.2 mL of the *F. verticillioides* spore suspension (2 × 10⁶ spores) or 0.2 mL of *U. maydis* sporidia suspension (2 × 10⁷ 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 (*Z. 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 pretreated with four fungicides (fludioxonil, carboxin, difenoconazole, and 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, 15-h/9-h 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 9 days after planting (DAP 9) while *F. verticillioides* was inoculated at one of the three different times: 2 days before *U. maydis* at DAP 7 (F > U), at the same time as *U. maydis* at DAP 9 (F = U), or 2 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 time points: DAP 7, 9, and 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 arc-sine transformed data, 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, $F > U$, $F = U$, and $U > F$), two *U. maydis* genotypes (UM1 and UM2), and two *F. verticillioides* strains (FV1 and FV2) and determined the significance of the main treatment and the 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 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 honestly significant difference (HSD) 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 DAP 17, 24, and 31, and these data were separately subjected to a three-way ANOVA, including a block effect using weighted GLMs, as above. Treatment factors were *F. verticillioides* treatment (no FV, $F > U$, $F = U$, and $U > F$), *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 $\alpha = 0.05/3$ to give an adjusted significance level at $P < 0.017$. In addition, because 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 used following the ANOVA results, as above.

Linear regression models and Pearson's 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 h 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 2 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 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 DAP 19 and plant mortality at DAP 40. 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). 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 ($r^2 = 0.89$, $P < 0.001$, Fig. 1).

We had predicted that preinoculation by *F. verticillioides* would provide the greatest protection from *U. maydis* infection. However, we found that only simultaneous coinoculation treatments ($F = U$) significantly decreased disease severity compared with the other *F. verticillioides* treatments: no FV (*U. maydis* only), preinoculation ($F > U$), and

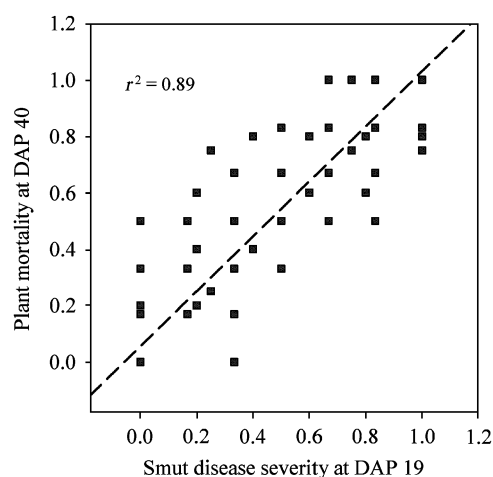
Table 1. ANOVA of smut disease severity, plant mortality, and plant height, with greenhouse bench, *Fusarium verticillioides* inoculation treatment, *Ustilago maydis* genotypes (UM), and *F. verticillioides* strains (FV), as treatment factors

Source of variation	d.f. [‡]	F				
		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 [§]
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 × UM	2	0.59	0.53	1.66	0.06	0.79
Inoculation × FV	2	2.73	1.33	1.72	0.97	0.30
UM × FV	1	0.01	0.78	0.10	0.01	0.14
Inoculation × UM × V	2	1.21	1.78	0.28	0.08	1.07

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

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

Significant difference at * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

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

postinoculation ($U > F$) (Table 2). Preinoculation of *F. verticillioides* treatments were associated with only slightly lower smut disease severity than the no FV treatment, and surprisingly, treatments with postinoculation by *F. verticillioides* ($U > F$) were associated with somewhat higher smut disease severity (Fig. 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). Results of the Tukey's HSD test showed that the plant mortality in

Table 2. Multiple comparisons using Tukey's HSD test

	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	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	64.6 (a)	69.0 (ab)	72.4 (ab)	75.8 (b)

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

the simultaneous coinoculation treatments ($F = U$) was significantly lower than that of the other *F. verticillioides* treatments (no FV, $F > U$, and $U > F$) (Table 2).

The *U. maydis* genotype had a significant effect on smut disease severity and plant mortality (Table 1). Overall 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 toward the host plant than is the UM1 genotype and that difference should be due to differing contributions of the noncommon 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

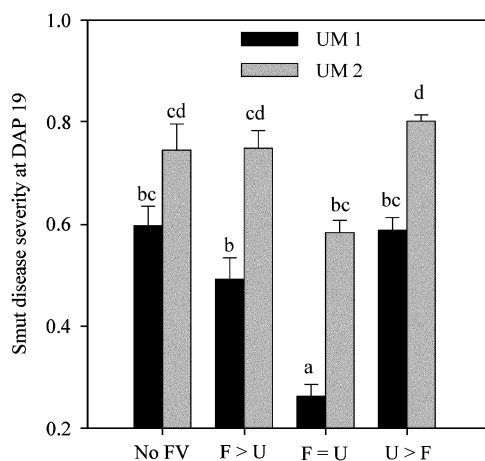


Fig. 2. Effect of *Fusarium verticillioides* inoculation on smut disease severity at DAP 19. Smut disease severity was significantly lower when *F. verticillioides* was simultaneously coinoculated with *Ustilago maydis*, but not when *F. verticillioides* was inoculated 2 days before (F > U) or 2 days 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 not different and were combined. Values are means \pm SE. Letters designate values that are not significantly different at $P < 0.05$ (Tukey's HSD test).

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). 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 2). At DAP 17 and 24, coinoculated (F = U) plants were significantly taller than the plants in the no FV treatment (*U. maydis* only), preinoculation (F > U), and postinoculation (U > F) treatments (Table 2, Fig. 3). We noted that plants in postinoculation (U > F) treatments at DAP 17 and 24 were smaller than in other treatments, consistent with observations of slightly greater disease severity in this treatment. At DAP 31, plants in simultaneous coinoculation treatments (F = U) were significantly taller than plants in no FV treatment (*U. maydis* only), but were not significantly taller than plants in preinoculation (F > U) and postinoculation (U > F) treatments. This was because many smut-diseased plants had died by DAP 31 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. 4). These results together show that

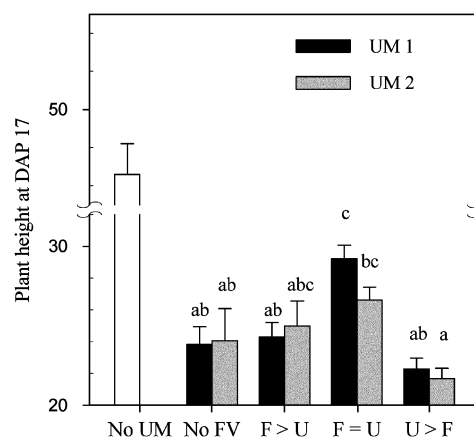


Fig. 3. Effect of *Fusarium verticillioides* inoculation treatments on plant growth. Plant height was significantly greater only when *F. verticillioides* was simultaneously coinoculated with *Ustilago maydis* (F = U), but not when inoculated 2 days before (F > U) or 2 days after (U > F) *U. maydis* 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 \pm SE. Letters designate values that are not significantly different at $P < 0.05$ (Tukey's HSD test).

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) with UM2 having a greater negative impact on plant growth than did UM1 at DAP 24 (39.1 vs. 44.6 cm) and at DAP 31 (67.0 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 coinoculated with the pathogen *U. maydis*, and not when preinoculated. 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 an 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.

Preinoculation of *F. verticillioides* 2 days ahead of *U. maydis* inoculation had no significant impact on smut

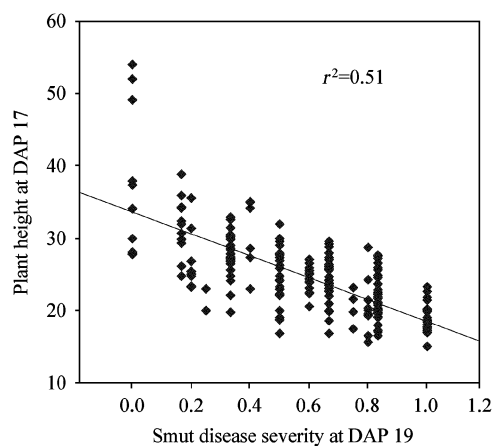


Fig. 4. Correlation of disease severity and plant height. Linear regression showed a negative correlation between smut disease severity at DAP 19 and plant height at DAP 17 ($r^2 = 0.51$, $P < 0.0001$).

disease severity or plant mortality. Instead, coinoculation 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 (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 observed in other systems (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.*, 1996, 2006), has antagonistic effects against endophytic bacteria (Bacon *et al.*, 2004, 2006) and the biocontrol agent *Trichoderma 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 with 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. Because both fungi commonly inhabit corn, their interactions are important for ameliorating smut disease in the crop host.

In postinoculation 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). This work contributes to our understanding of 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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