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Interactions between *Fusarium verticillioides*, *Ustilago maydis*, and *Zea mays:* An endophyte, a pathogen, and their shared plant host

Alma E. Rodriguez Estrada [a,1], Wilfried Jonkers [b], H. Corby Kistler [c], Georgiana May [a,*]

[a] Department of Ecology, Evolution and Behavior, University of Minnesota, St. Paul, MN 55108, USA
[b] Department of Plant Pathology, University of Minnesota, St. Paul, MN 55108, USA
[c] USDA ARS Cereal Disease Laboratory, St. Paul, MN 55108, USA

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ABSTRACT

Highly diverse communities of microbial symbionts occupy eukaryotic organisms, including plants. While many well-studied symbionts may be characterized as either parasites or as mutualists, the prevalent but cryptic endophytic fungi are less easily qualified because they do not cause observable symptoms of their presence within their host. Here, we investigate the interactions of an endophytic fungus, *Fusarium verticillioides* with a pathogen, *Ustilago maydis,* as they occur within maize (*Zea mays*). We used experimental inoculations to evaluate metabolic mechanisms by which these three organisms might interact. We assessed the impacts of fungal–fungal interactions on endophyte and pathogen growth within the plant, and on plant growth. We find that *F. verticillioides* modulates the growth of *U. maydis* and thus decreases the pathogen's aggressiveness toward the plant. With co-inoculation of the endophyte with the pathogen, plant growth is similar to that which would be gained without the pathogen present. However, the endophyte may also break down plant compounds that limit *U. maydis* growth, and obtains a growth benefit from the presence of the pathogen. Thus, an endophyte such as *F. verticillioides* may function as both a defensive mutualist and a parasite, and express nutritional modes that depend on ecological context.

1. Introduction

An extraordinary diversity of endosymbiotic organisms occupies most eukaryotic hosts (e.g. Arnold et al., 2009; Moran et al., 2008), but the ecological and evolutionary processes determining species composition and function of these communities are not well understood (Johnson et al., 2006; Pan and May, 2009; Saunders et al., 2010). While some endosymbionts have apparent importance to host health, or add adaptive functionalities to their host, species within these endosymbiotic communities can be characterized as spanning the spectrum of mutualist to pathogenic nutritional modes (Harman et al., 2004; Rodriguez et al., 2009). Here, we investigate mechanisms of interaction between a fungal endophyte and a fungal pathogen within their shared plant host, and the outcomes of those interactions for fungal and plant growth.

Endophytic fungi colonize above and below ground plant organs, live inside the host without causing perceptible symptoms of infection (Wilson, 1995) and encompass a diverse array of fungal species, primarily Ascomycetes but including other fungal phyla (Rodriguez et al., 2009). In contrast to the host-specific endophytes related to *Epichloë* spp. that are primarily associated with cool season grasses (Schardl et al., 2004), the more diverse "generalist" endophytic fungal species associate with a broad range of plant and lichen hosts (U'Ren et al., 2010). At the phylogenetic level, transitions among the nutritional modes of parasite, mutualist, and saprophyte, and among associations with higher plants and lichens occur frequently over the evolutionary history of plant-associated fungi (Arnold et al., 2009), suggesting that functions of these organisms are not easily classified *a priori*. For example, the results of Lee et al. (2009) suggest that the endophytic *Fusarium verticillioides* may facilitate growth of *Ustilago maydis* in the plant, but also slows disease progress allowing greater plant growth. In field studies, Saunders and Kohn (2009) demonstrated that breakdown of the maize benzoxazolinones 6-methoxy-2-benzoxazolinone (MBOA) and 2-benzoxazolinone (BOA) by *F. verticillioides* facilitates colonization by fungal species less tolerant to these plant defense compounds. Together, these relatively few functional studies suggest that understanding the mechanisms of interaction among co-occurring symbionts of plants will improve prediction of ecological and evolutionary outcomes, and provide information for endophyte's potential use in biological control (Backman and Sikora, 2008; Meijía et al., 2008).

Here, we exploit an experimentally tractable system of maize (*Zea mays*), an endophytic fungus (*F. verticillioides*) and a common

* Corresponding author.
E-mail address: gmay@umn.edu (G. May).
[1] Present address: Department of Natural and Applied Sciences. University of Wisconsin Green Bay, Green Bay, WI 54311, USA.

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pathogen (*U. maydis*) of maize to investigate mechanisms of interactions between endophyte and pathogen within the host, as well as the effects of symbiont interactions on fungal and plant growth. Endophytic *F. verticillioides* and the pathogen *U. maydis* often co-occur in the same maize plant and the same tissue (Pan et al., 2008) and thus may have evolved mechanisms of interaction. Interestingly, *F. verticillioides* is commonly regarded as a pathogen of maize causing rots of the seed kernel, root, and stalk (Kommedahl and Windels, 1981) and is found in association with a wide range of plant hosts (Kuldau and Yates, 2000; Moretti et al., 2004). However, this Ascomycete species can be also isolated from symptomless plants (e.g., Leslie et al., 1990; Kuldau and Yates, 2000) and such isolates behave as endophytes when re-inoculated into plants (Pan et al., 2008; Saunders and Kohn, 2009) as do the isolates deployed in this study. The basidiomycete *U. maydis* is a smut pathogen with a long evolutionary history with cultivated maize (*Z. mays* var. *mays*) and its wild ancestor, teosinte (Munkacsi et al., 2008). Corn smut is characterized by the formation of hypertrophies (galls) that are filled with sooty black teliospores, allowing disease progress to be visually assessed (Gold et al., 1997; Banuett and Herskowitz, 1996). The availability of well-characterized genome sequences for both fungal symbionts (Kämper et al., 2006; Ma et al., 2010) and for maize (Schnable et al., 2009), provide a model system to study functional interactions among these species.

The specific mechanisms of interaction among co-occurring symbionts within hosts will strongly affect the ecological and evolutionary outcomes of those interactions (Buckling and Brockhurst, 2008). As we have learned from studies of biocontrol agents, the products involved in microbial interactions within plants may be as diverse as the organisms that produce them. The hallmarks of direct, parasitic interactions are chitinases and other cell-wall modifying enzymes (Chet and Inbar, 1994; Seidl et al., 2005; Harman, 2006). Chitinase gene families are amplified in the genomes of some fungal species with parasitic nutritional modes (Duo-Chuan, 2006; Karlsson and Stenlid, 2008). In contrast, indirect antagonistic interactions are more often mediated by secondary compounds (antibiosis) or by competition for nutrient resources via products such as iron siderophores (Nicoletti et al., 2004; Mathivanan et al., 2008; Vinale et al., 2008). In the system we use here, previous studies have shown that *F. verticillioides* and *U. maydis* interact through several of these mechanisms; cell wall degrading enzymes, key secondary metabolites, and competition for nutrients (Rodriguez Estrada et al., 2011, Jonkers et al., 2012). Interestingly, *F. verticillioides* breaks down the plant defense benzoxazolinone compounds that are also active against *U. maydis* (Basse, 2005), providing a potential mechanism by which *F. verticillioides* might facilitate *U. maydis* infection, as it does other maize-associated fungi (Saunders and Kohn, 2008, 2009). Specifically, among the wide range of secondary metabolites produced by *Fusarium* species (Bacon et al., 1996; Desjardins et al., 1993; Duffy et al., 2004; Mirocha et al., 1976), genes for the production of fusaric acid, fumonosins, and chitinases by *F. verticillioides* are upregulated in the presence of *U. maydis* (Jonkers et al., 2012). Although growth of *U. maydis* is slowed in co-culture with *F. verticillioides*, it is not defenseless. *U. maydis* produces a wide array of secondary metabolites (Bolker et al., 2008; Hewald et al., 2005; Teichmann et al., 2007; Rodriguez Estrada et al., 2011) and genes for the production of ustilagic acid, iron siderophores, and uncharacterized secreted proteins are upregulated in the presence of *F. verticillioides* (Jonkers et al., 2012).

In this study, we sought to understand the mechanisms of interaction between *F. verticillioides* and *U. maydis* as they occur in the plant maize, and the impact of those interactions on fungal and host plant growth. Because results of previous work suggest that *F. verticillioides* acts as a defensive mutualist against *U. maydis* in maize (Lee et al., 2009), we asked whether *F. verticillioides* gains a growth benefit during *in vivo* interactions as it apparently does during *in vitro* interactions with *U. maydis* (Rodriguez Estrada et al., 2011). We use defensive mutualist to describe a symbiont that limits pathogen damage, and thus confers a benefit, to the host. Using a maize variety conducive to infection by both *U. maydis and F. verticillioides*, we determined changes in each fungal species' biomass and secondary metabolite production when both fungal species were simultaneously inoculated on maize, compared to fungal biomass and secondary metabolite production in plants inoculated with a single fungal species. To understand the impacts of fungal interactions on pathogen aggressiveness towards the plant host, we compared plant growth in treatments using inoculations of single fungal species with plant growth in treatments with both fungi co-inoculated.

2. Materials and methods

2.1. Fungal strains and inocula preparation

We used two haploid genotypes of *F. verticillioides* (NR and F89) and two dikaryon genotypes of *U. maydis* (UM2, UM18 described below) that had previously been characterized for fungal interactions *in vitro* (Rodriguez Estrada et al., 2011) and for interactions *in planta* (Lee et al., 2009). In previous studies, the dikaryons UM2 and UM18 differed in aggressiveness towards maize (Lee, 2010). The dikaryon stage of *U. maydis* can only be generated by mating two compatible haploid sporidia on the plant. The dikaryon UM2 was generated by mating of the haploid strains U2 (a_2b_{11}) and C7 (a_1b_{12}) and the dikaryon UM18 was generated by mating U18 (a_2b_{11}) and C7 (a_1b_{12}). Inocula were prepared following the protocols of Lee et al. (2009) and Rodriguez Estrada et al. (2011). Briefly, the *F. verticillioides* and *U. maydis* strains were each separately grown in 50 mL of potato dextrose broth in 250 mL Erlenmeyer flasks for 3 days at 27 °C in a shaker incubator (100 rpm). The *F. verticillioides* cultures were subsequently filtered with sterile miracloth to remove mycelia and recover conidia. The *U. maydis* sporidia cultures and the filtered *F. verticillioides* conidia each were placed in 50 mL Falcon tubes and centrifuged at 4000 rpm for 6 min to pellet cells. Cells were washed and centrifuged three times with sterile, distilled water to remove remaining culture media, and after suspending in small amounts of sterile distilled water, were counted under the light microscope using a hemocytometer. The concentration of *F. verticillioides* conidia was adjusted with sterile water to yield 10^7 spores in 50 μL of water, inoculation volume. Since mating between two compatible haploid strains of *U. maydis* is needed for plant infection, the haploid sporidia concentration was adjusted to 5×10^6 sporidia in 25 μL water to give 10^7 cells per 50 μL inoculum. Compatible strains were mixed just before plant inoculation.

2.2. Experimental design

A full factorial block design was used with *F. verticillioides* (FV) and *U. maydis* (UM) as different treatment factors, each with three levels: genotype 1, genotype 2 and no fungus (control, same volume of water). Nine treatment combinations were generated: UM2, UM18, F89, NR, UM2 + F89, UM2 + NR, UM18 + F89, UM18 + NR and mock inoculated plants (negative control). Twenty replicate pots (19 cm in diameter and 14 cm in depth) per treatment were seeded with six kernels (see below). All seedlings per pot were given the same treatment. The experimental treatments were conducted in two groups within which all nine treatments were applied to half of the replicates 2 days apart and placed on different greenhouse benches (Block) within the same greenhouse. Pots representing treatments and pots were randomized across each bench.

2.3. Plant inoculation

The *Z. mays* cultivar Jubilee (sweet corn; Jordan seeds, Inc. Woodbury, MN) was used because it is highly susceptible to corn smut and develops disease symptoms under greenhouse conditions (Lee et al., 2009). Six corn seeds (one in the center and the rest distributed around the pot's perimeter) were planted in each pot containing All Purpose Potting Mix (LC8 Mix, Sun Gro). Seven day old seedlings were inoculated by pipetting the inocula onto the leaf whorls with minimum damage to the plant tissues. Control plants were inoculated with 100 μL of water while single treatment seedlings were inoculated with 50 μL of fungal inocula and then with the same volume of distilled sterile water. Co-inoculated treatments were inoculated by pipetting 50 μL of the *F. verticillioides* conidial suspension and 50 μL of *U. maydis* sporidia suspension (25 μL each of the two compatible haploid strains). The region where the inoculum made contact with the plant was marked using a waterproof marker (Fig. 1, bracketed region 'b').

2.4. Pathogen aggressiveness and disease severity

Aggressiveness of the pathogen *U. maydis* was evaluated as damage to plant growth and quantified by measuring plant height (Lee et al., 2009). Plant height was measured from the soil surface to the tip of the tallest extended leaf. In addition, we monitored disease progress through a qualitative score as follows: 0 = no disease symptoms, 1 = chlorosis and/or necrosis, 2 = small leaf galls, 3 = small stem galls, 4 = large stem galls, and 5 = plant death (Gold et al., 1997). Plant height and disease severity symptoms were recorded for all the plants in the experiment at 6 and 20 days post inoculation (dpi), which are 13 and 27 days after seedling emergence, respectively.

2.5. Tissue sample collection

Tissue from 12 plants per treatment (six plants per block) was sampled at 7 dpi because disease symptoms can be unequivocally scored at this time. While differences in plant height due to disease are already apparent, similar amounts of plant material can be harvested from all treatments. We sampled plants with the most common disease severity score for the treatment. The plant in the center of the pot was preferred in order to obtain the most even effects of experimental variables. All plants were measured for height but tissue samples were obtained for fewer plants because no more than 12 plants per treatment (108 total) per day could be processed for metabolite analyses.

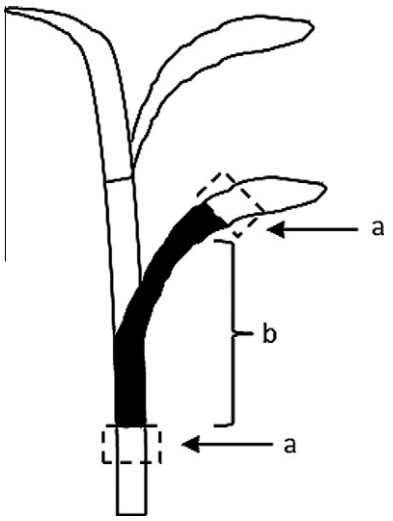

Fig. 1. Plant samples were collected at 7 days post-inoculation (dpi) for DNA and metabolite analyses. Regions of the leaf and stem where fungal inoculum made contact with the plant tissue are bracketed by (b). Two, 1 cm^2 fragments (a) were removed and used for fungal back-isolation. The remaining plant tissue of (b) (shaded) was frozen in liquid nitrogen and finely ground for DNA and metabolite extraction.

To estimate frequencies at which inoculated fungi became established in the plants, and to ascertain that additional fungal species had not infected the plants, we back-isolated fungi from two segments of tissue; a 1 cm^2 piece from the top (leaf) and the bottom (stem) of the marked region of the plant (Fig. 1, points a). For DNA and metabolite extraction, the remaining tissue from the inoculated region of each plant (Fig. 1, region b) was placed in 50 mL polypropylene tubes and immediately submerged in liquid nitrogen. Frozen plant tissue was ground to a fine powder with a mortar and pestle kept frozen with liquid nitrogen constantly poured in the mortar. Approximately 60 mg of tissue powder were immediately placed in frozen microcentrifuge tubes (2 mL) for DNA extraction and stored at −20 °C until processed. For metabolite extraction, approximately the same volume of frozen powdered tissue was transferred to 15 mL polypropylene tubes, weighed, and immediately processed.

2.6. Biomass quantification

Our goal was to compare each fungal species' biomass achieved in single inoculated plants with that achieved in co-inoculated plants. For each plant sample, we evaluated biomass for the two fungal species, and for the plant, as genome equivalents using a quantitative real-time polymerase chain reaction (qPCR) approach. Total DNA was extracted from plant samples using the OmniPrep™ kit for fungi (GBiosciences) following the manufacturer instructions with minor modifications. In short, cells were chemically lysed and total DNA was separated from organic components by chloroform extraction and centrifugation. DNA in the aqueous phase was precipitated with isopropanol, pelleted through centrifugation and washed with ethanol (70%). The DNA pellet was air dried to evaporate ethanol and re-suspended in RNAse in TE buffer for 37 °C for 1 h. DNA samples were stored at −80 °C until analyzed further.

The number of genome equivalents was estimated for each of the two fungal species using species-specific primers and labeled probes (TaqMan®) as described in Rodriguez Estrada et al. (2011). For *F. verticillioides*, primers FusqPCR_F (5′-TCGCTCTAGGCCAGATTACCA-3′), FusqPCR_R (5′-GAACCAGGAAAGTCGATGGTG-3′) and the probe Fusca (5′-VIC-CGCTCTCTCGGCCAGA-MGB-3′) were used. For *U. maydis*, primers Emt1qPCR_F (5′-GTCACTGGTTGCCGAA TGACT-3′), Emt1qPCR_R (5′-TGGGTCAAACAGGCTCTTACG-3′) and the probe UMemt1 (5′-6FAM-CATGGATGTTCACCGTTC-MGB-3′) were used. Real time PCR reactions were carried out in 384 well plates in an ABI Prism® 7900HT instrument (BioMedical Genomics Center, University of Minnesota, St. Paul). Concentrations of primers (400 nM of each primer) amplifying each fungal region and of the probes (250 nM for Fusca, 150 nM for UMemt1), components of the PCR reactions, and cycle parameters were the same as Rodriguez Estrada et al. (2011). Real-time PCR reactions for the two fungal species were conducted in multiplex to amplifying both fungal targets using the TaqMan® Environmental Master Mix (Applied Biosystems).

For analyses of plant genome equivalents as a measure of biomass, labeled primers and probes for *Z. mays* were designed (Primer Express, Applied Biosystems) based on the RuBisCo small subunit gene sequence (GenBank accession number: Y09214; Hahnen et al., 2003). Primers RubqPCR_F (5′-GATACCCTGCCTCGAG TTCA-3′) and RubqPCR_R (5′-CAGCTTCCACATGGTCCAGTA-3′) amplify a 100 bp region. The probe RubiscoNED is 5′-NED-CAAGCTCG GGTTCGTGT-MGB-3′. Real-time PCR reactions for the RuBisCo gene

were run separately from fungal samples but under the same conditions as those for the fungal qPCR. Primer and probe concentrations for the RuBisCo gene were optimized at 100 nM for the forward primer, 900 nM for the reverse and 250 nM for the probe in 15 μL reaction volumes.

Standard curves were created from a serial dilution of DNA extracted from pure fungal cultures or non-inoculated plants. Details regarding mass calculations of each fungal genome are as in Rodriguez Estrada et al. (2011). The mass (m) as gm per bp for the *Z. mays* diploid genome was calculated by the formula, $m = 2n$ ($1.096e^{-21}$ g/bp), where n (haploid genome size) is 2.5×10^9 bp (Applied Biosystems, 2003). DNA concentrations were measured using a fluorometer (Qubit®, Invitrogen) with the Quant-iT™ reagents (dsDNA Broad Range). DNA concentration for *F. verticillioides, U. maydis* and *Z. mays* were adjusted to 9.15, 4.3 and 54.8 ng μL^{-1}, respectively, in order to yield 3×10^5 genome equivalents at the highest concentration. Five 10-fold serial dilutions were done for each template so that the lowest dilution contained 30 genome equivalents. Data were acquired and processed with the Sequence Detection System Software (Enterprise edition). The amplification plots were visually assessed, threshold values manually positioned in the exponential phase. The regression equations for the relationship between genome equivalents and gene copies were then used to estimate the number of genome equivalents for each species in each sample. For each fungal species, genome equivalents in each sample were then normalized to the number of *Z. mays* genome equivalents estimated for the same sample. To standardize the growth responses, we report the fold-changes in each fungal biomass as the biomass in co-inoculated plants relative to the biomass in single fungal inoculated plants.

2.7. Fungal back-isolation frequencies

We back-isolated fungi from single- and co-inoculated plants in order to determine the presence of each fungus in the inoculated plants, further evaluate how the two fungal species might affect each other's establishment in the plant, and to determine whether the inoculated fungi were the only fungi present in the plants. Two 1 cm^2 pieces (at points at Fig. 1a) from each sampled plant were surface sterilized by serial rinses of sterile/deionized water, 70% ethanol (1 min), 40% bleach (2 min), 70% ethanol (1 min), and sterile/deionized water. Samples were then transferred to PDA medium in 90 mm Petri dishes and incubated at 27 °C in darkness. Samples were visually assessed for fungal growth at 3, 6 and 9 days after plating. Isolation frequencies from leaves and stems were calculated as IF = $Pi/n \times 100$, where Pi corresponds to the number of plants from which fungi were isolated and n the total number of plants sampled. We conducted a binomial test (Sokal and Rolf, 1981) to determine if *F. verticillioides* was back-isolated more frequently when co-inoculated with *U. maydis* than when inoculated alone.

2.8. Metabolite extraction and analysis

Metabolites were extracted from frozen, ground plant tissue and thus include compounds of both fungal and plant origin. Four mL of acidified methanol (99% methanol + 1% formic acid) were added to the plant tissue (avg. 545 ± 150 mg) in 15 mL polypropylene tubes (BD Falcon™). Samples were vortexed 5 s and then placed in ultrasonic bath for 1 h (Mettler Electronics). Samples were centrifuged at 4000 rpm for 10 m and 1.5 mL of the extract were placed in 2 mL microcentrifuge tubes and dried (SpeedVac, Savant, SC100) for 6 h at ambient temperatures. The pellet was resuspended in 250 μL of acidified methanol (99.9% methanol + 0.1% formic acid) using an ultrasonic bath for 10 m. Non-dissolved particles were removed through centrifugation (14,000 rpm for 10 min). Then, 100 μL of the supernatant was transferred to a 1.5 mL clear glass vial (Chrom Tech, Inc.) for analysis.

Metabolite analyses were conducted using an Ultra Performance Liquid Chromatography/Time of Flight/Mass Spectrometer (UPLC/TOF/MS) instrument (Acquity™ chromatograph coupled to a LCT Premier XE Micromass® spectrometer, Waters, Milford, MA) following Rodriguez Estrada et al. (2011) specifications. Chromatographic profiles were visually assessed (MassLynx software) in order to detect the chromatographic peaks characteristic of each treatment. Peaks were manually extracted using the monoisotopic mass of the most intense ion as reference (Rodriguez Estrada et al., 2011). Peak areas are estimates of each metabolite concentration and were automatically calculated using the QuanLynx software. ANOVA tests were performed to evaluate treatment effects on each individual compound's peak area as described below.

2.9. Statistical analyses

For plant height, we determined statistical significance of treatment effects (inoculation and fungal genotype) on plant height using full factorial, two-way ANOVA with Block and treatment interaction effects (aov function; R 2.10.1; The R Foundation for Statistical Computing 2009). Distribution of the residuals was visually assed for normality using normal quartile plots and height data were transformed by the Box–Cox method (Weisberg, 2005). The significance of differences among all pairwise comparisons of treatment means was subsequently determined using the Tukey–Kramer test that corrects for multiple, unplanned comparisons and is not overly conservative (Hayter, 1984). Tukey–Kramer tests were programmed in Excel following Sokal and Rolf (1981, pp. 246–252).

To evaluate treatment effects on each fungal species' biomass (as genome equivalents), we excluded controls where the fungal species is not present, and conducted two-way ANOVA to evaluate significance of the treatment factors of fungal genotype and co-inoculation in R 2.10.1 (aov function; The R Foundation for Statistical Computing 2009). For example, to evaluate *U. maydis* biomass as a response variable, treatment factors were UM genotypes (UMgenotype: UM2 or UM18), and *F. verticillioides* inoculation (FV: no endophyte, and co-inoculation with F89 or NR genotypes). Treatment interactions and Block were included in the model.

Similarly, for fungal metabolites, two-way ANOVA tested for the effect of the metabolite producing genotype. For example, treatments for metabolites identified as originating from *U. maydis* are UMgenotype (UM2 or UM18), *F. verticillioides* inoculation (FV: no endophyte, and co-inoculation with either F89 or NR), Block, and their interactions. As for fungal biomass, mock-inoculated controls where the fungus was not present were excluded from the analyses. For plant metabolites, the two-way ANOVA model included all of the mock controls and evaluated the effects of *F. verticillioides* inoculation (FV: no FV, F89 and NR), *U. maydis* inoculation (UM: no UM, UM2 and UM18), Block, and treatment interactions. Data were assessed for normality and transformed by the Box–Cox method (Weisberg, 2005).

3. Results

3.1. maydis aggressiveness is affected by interactions with F. verticillioides

We evaluated plant height as a measure of *U. maydis* disease aggressiveness. The full-factorial ANOVA for plant height at 6 dpi and 20 dpi demonstrated significant effects of UM, FV and Block (Table 1). Subsequent Tukey–Kramer tests for significant differences of means among all pairwise comparisons, show that the

effects of UM and FV treatments were primarily due to the presence or absence of these fungi, rather than the specific genotype inoculated. At 6 dpi, plants from UM-only treatments were significantly smaller than were plants in the mock-inoculated control, FV and FV + UM treatments (Fig. 2). On average, at 6 dpi, plants infected with *U. maydis* only measured 17.7 cm while the plants in other treatments measured more than 30.0 cm in height. At 20 dpi the surviving plants inoculated with *U. maydis* grew to an average of 37.7 cm while the plants in other treatments measured more than 59.0 cm in height (Fig. 2). Thus, plants co-inoculated with *F. verticillioides* and *U. maydis* grew as tall, on average, as did mock-inoculated plants and plants inoculated with *F. verticillioides* alone. The factor Block (bench effect) affected plant height differently at 6 and 20 dpi. In general, plants in Block one were taller at 6 dpi whereas plants in Block two were taller at 20 dpi (data not shown). We infer that strains of *F. verticillioides* we used here behave as endophytes because they cause no disease symptoms and do not damage plant growth.

3.2. Fungal genotype did not strongly affect U. maydis aggressiveness

Results for ANOVA (Table 1) and subsequent post hoc Tukey–Kramer tests demonstrate few significant differences in mean plant growth due specifically to the genotype of *U. maydis* or *F. verticillioides*. Often, the results suggest environmental sensitivity rather than differences directly attributed to genotype. For example at 6 dpi, plants co-inoculated with F89 and either *U. maydis* genotype were slightly taller than were plants co-inoculated with NR and *U. maydis*, but this result was observed in Block 1 and not Block 2. At 20 dpi, plants co-inoculated with F89 and *U. maydis* were slightly taller than plants co-inoculated with NR and *U. maydis* in Block 2 but not Block 1 (Fig. 3; data for Block not shown). Significant differences in plant growth due to the two *U. maydis* genotypes were also not observed (Fig. 4). Consequently, although we do observe significant fungal treatment (UM or FV) by Block interaction effects (Table 1), we concluded that apparent genotype effects could be due to the position of replicates in the greenhouse. In summary, plants co-inoculated with the endophyte *F. verticillioides* and the pathogen *U. maydis* gain as much growth as plants in controls and as those plants inoculated with the endophyte alone. In this way, both genotypes of *F. verticillioides* behave as defensive mutualists against smut disease in maize.

3.3. Fungal biomass is strongly affected by co-inoculation treatment

We estimated the fungal biomass within the plant as genome equivalents using qPCR, a method that estimates the number of specific gene sequences for each species in a sample. For *U. maydis*,

Table 1
Summary of ANOVA results for plant height at 6 days and 20 days post-inoculation (dpi). The effects of *U. maydis* (UM) and *F. verticillioides* (FV) inoculation treatments, greenhouse bench (Block), and their statistical interaction terms were estimated.

	6 dpi			20 dpi		
	df	*F*-stat	Pr (>*F*)[d]	*df*	*F*-stat	Pr (>*F*)
Block[a]	1	38.3157	***	1	45.137	***
UM[b]	2	118.598	***	2	118.7134	***
FV[c]	2	229.9783	***	2	28.4908	***
Block:UM	1	0.0644		1	85.7271	***
Block:FV	1	9.664	**	1	1.8203	
UM:FV	1	81.6258	***	1	8.9136	**
Block:UM:FV	1	1.8462		1	14.1358	***

[a] Block: two greenhouse benches.
[b] UM: *U. maydis* inoculation treatment: no UM, UM2 or UM18 genotypes.
[c] FV: *F. verticillioides* inoculation treatment: no FV, F89 or NR genotypes.
[d] Significance codes: *** <0.0001, ** <0.001, * <0.01.

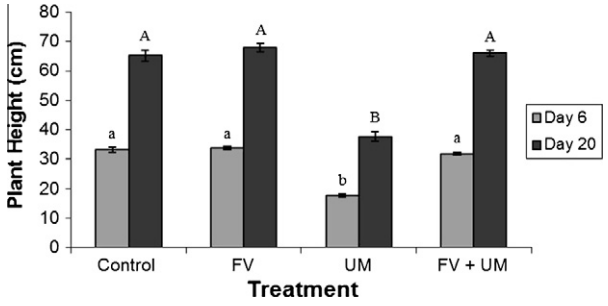

Fig. 2. Mean plant height at 6 and 20 days post-inoculation (dpi) for plants mock inoculated with water (Control), single-inoculated with either *F. verticillioides* (FV) or *U. maydis* (UM), or co-inoculated with both fungi (FV + UM). Different lower case letters indicate significant differences among means at 6 dpi and different upper case letters indicate significant differences among means at 20 dpi ($p < 0.05$; Tukey–Kramer test). Error bars indicate standard error of the mean.

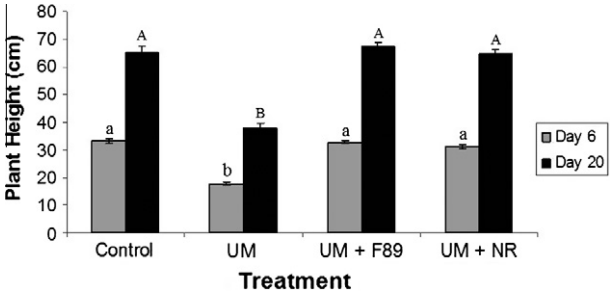

Fig. 3. Mean plant height at 6 and 20 days post-inoculation (dpi) for plants mock inoculated with water (Control), single-inoculated with *U. maydis* (UM, either genotype), or co-inoculated with *U. maydis* and *F. verticillioides* genotype F89 (UM + F89) or genotype NR (UM + NR). Different lower case letters indicate significant differences among means at 6 dpi and different upper case letters indicate significant differences among means at 20 dpi ($p < 0.05$; Tukey–Kramer test). Error bars indicate standard error of the mean.

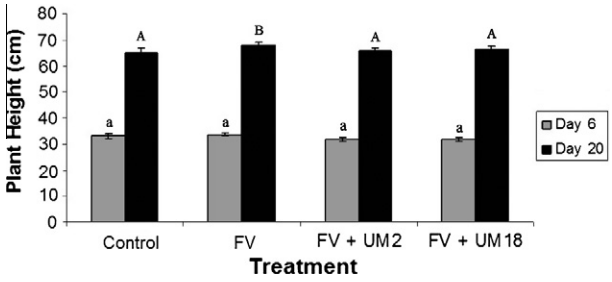

Fig. 4. Mean plant height at 6 and 20 days post-inoculation (dpi) for plants mock inoculated with water (Control), single-inoculated with *F. verticillioides* (FV, either genotype), or co-inoculated with *F. verticillioides* and *U. maydis* genotype UM2 (FV + UM2) or genotype UM18 (FV + UM18). FV single inoculated plants were slightly and significantly taller on average at 20 dpi, likely due to greater growth of F89 single inoculated plants in Block 2. Different lower case letters indicate significant differences among means at 6 dpi and different upper case letters indicate significant differences among means at 20 dpi ($p < 0.05$; Tukey–Kramer test). Error bars indicate standard error of the mean.

ANOVA results demonstrate that the endophyte inoculation treatment significantly affected the number of *U. maydis* genome equivalents present in the plant samples. The *U. maydis* genotype (UM2 or UM18) did not affect results, nor did Block (Table 2). Subsequent one-way ANOVA with the FV treatment factor only, and post hoc TukeyHSD analysis for differences among means in R 2.10.1 statistical package (functions aov and TukeyHSD; The R Foundation for Statistical Computing 2009), demonstrate that *U. maydis* biomass is significantly lower in co-inoculation treatments with either

F. verticillioides genotype (F89 or NR) than it is when *F. verticillioides* is not inoculated. However, the genotype of *F. verticillioides* co-inoculated into plants with *U. maydis* did not significantly affect *U. maydis* biomass.

For *F. verticillioides* biomass, ANOVA results demonstrate that UM inoculation treatment significantly affected the number of *F. verticillioides* genome equivalents present in the plant sample. The *F. verticillioides* genotype (F89 or NR) did not significantly affect mean endophyte biomass, nor did Block (Table 2). Subsequent one-way ANOVA with the UM inoculation treatment and post hoc TukeyHSD analysis for differences among means (functions aov and TukeyHSD; The R Foundation for Statistical Computing 2009) demonstrated that *F. verticillioides* biomass is significantly greater in co-inoculation treatments with either UM2 or UM18 than when *U. maydis* was not inoculated and *F. verticillioides* grew alone in the plant. However, the genotype of *U. maydis* co-inoculated into plants with *F. verticillioides* did not significantly affect *F. verticillioides* biomass.

We illustrate the changes in biomass for each fungal species when co-inoculated compared to the biomass achieved by each fungus growing alone in the plant in Fig. 5. We report this measure rather than absolute values because the two fungal genomes did not extract with equal efficiency from the plant material (data not shown). In summary, *U. maydis* biomass is 20- to 60-fold lower when co-inoculated with *F. verticillioides* than when inoculated alone. In contrast, the endophyte *F. verticillioides* gains a measurable, 10- to 20-fold growth benefit in the plant when co-inoculated with *U. maydis*, compared to growth of the endophyte alone in the plant (Fig. 5).

3.4. Fungal back-isolation frequencies

Back-isolation frequencies for both fungi was determined using 1 cm^2, surface sterilized plant segments from the leaf and stem of each sampled plant at 6 dpi. Neither of the inoculated fungal species was isolated from mock-inoculated plants nor were contaminating fungi found in plants from any treatment. Isolation frequencies for *U. maydis* could only be assessed in single inoculated plants because the fast growth of *F. verticillioides* mycelium prevented emergence of *U. maydis* colonies in samples of co-inoculated plants. The back-isolation frequency of *U. maydis* from single-inoculated plants was 91.7% on average, demonstrating that *U. maydis* successfully established infections in these plants. The observation of *U. maydis* sporidial colonies growing from the plant was unexpected because the dikaryon is considered an obligate pathogen on the plant. We determined that both *U. maydis* mating types were present (data not shown) and concluded that the infectious dikaryon breaks down to the haploid phase when grown on agar.

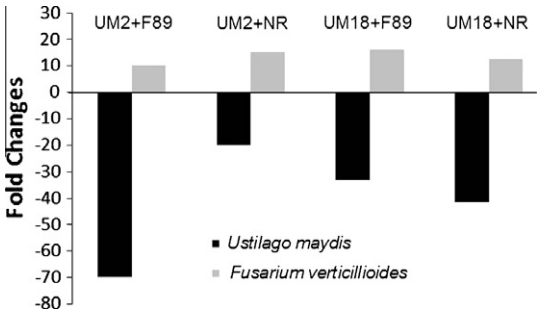

Fig. 5. Fold-changes in *Fusarium verticillioides* and *Ustilago maydis* biomass due to co-inoculation. Values show the ratio of biomass achieved in single- versus co-inoculated plants, for each species. Biomass was evaluated as genome equivalents using qPCR. *U. maydis* biomass is 20- to 60-fold lower in co-inoculated treatments compared to its biomass when growing alone in plants. *F. verticillioides* biomass is 10- to 20-fold greater in co-inoculated treatments compared to its biomass when growing alone in plants. Differences in each species' mean biomass due to genotype were not significant but are shown here to illustrate variation across experimental variables.

The average back-isolation frequency for *F. verticillioides* from co-inoculated plants was 64.7%, a higher frequency than the back-isolation frequency of 41.7% observed for plants inoculated with *F. verticillioides* only. While this observation is consistent with the growth advantage that *F. verticillioides* apparently gains from the presence of *U. maydis*, further analysis using a binomial test against the expectation for equal frequencies in single and co-inoculated treatments (Sokal and Rolf, 1981) revealed that the apparent increased back-isolation frequency of *F. verticillioides* from co-inoculated plants was somewhat dependent on genotype and was environmentally sensitive; back-isolation frequencies for *F. verticillioides* were higher in Block 1 than in Block 2 (data not shown). Overall, back-isolation frequencies are not an adequate indicator of the presence of these fungi because qPCR results above demonstrate that *F. verticillioides* was present in all plants inoculated with this species. However, they do serve as a good check that the inoculated fungi are the only fungi present in the plant.

3.5. Metabolite profiling identified compounds produced by the plant and U. maydis

A total of nine chromatographic peaks were characterized from the complex metabolite chromatograms obtained from plant extracts across all experimental treatments (Table 3). Metabolites

Table 2
Summary of ANOVA results for *U. maydis* and *F. verticillioides* biomass estimated at 7 dpi using qPCR. For *U. maydis* biomass, the effects of *U. maydis* genotype (UMgenotype) and *F. verticillioides* inoculation treatments (FV) were estimated. For *F. verticillioides* biomass, the effects of *F. verticillioides* genotype (FVgenotype) and *U. maydis* inoculation treatments (UM) were estimated.

	U. maydis biomass				*F. verticillioides* biomass		
	df	*F*-stat	Pr (>*F*)[f]		df	*F*-stat	Pr (>*F*)[f]
Block[a]	1	2.1087		Block[a]	1	0.3626	
UMgenotype[b]	1	0.1969		UM[d]	2	9.2777	***
FV[c]	2	52.2515	***	FV genotype[e]	1	3.43	
UMgenotype:FV	1	0.1193		UM:FVgenotype	1	0.517	

[a] Block: two greenhouse benches.
[b] UMgenotype: inoculation with either UM2 or UM18 genotype.
[c] FV: *F. verticillioides* inoculation treatment: no FV, F89 or NR genotype.
[d] UM is *U. maydis* inoculation treatment: no UM, UM2 or UM18 genotypes.
[e] FV genotype represents plants inoculated with either the F89 or NR genotype.
[f] Significance codes: *** <0.0001, ** <0.001, * <0.01.

Table 3
Characteristics of metabolites determined from extracts of plants inoculated with *U. maydis*, *F. verticillioides*, and co-inoculated in all combinations.

ID	*m*/*z*[a]	RT[b]	Putative origin[c]
1	731.189	2.14	*U. maydis*
2	853.204	3.05	*U. maydis*
3	284.125	3.48	*U. maydis*
4	649.246	3.69	*U. maydis*
5	647.236	4.11	*U. maydis*
6	421.096	1.73	*Z. mays*
7	769.192	2.33	*Z. mays*
8	797.228	2.71	*Z. mays*
9	851.392	6.97 and 7.11	*Z. mays*

[a] *m*/*z*: Mass to charge ratio for the most intense ion in the spectrum.
[b] RT: retention time.
[c] Metabolites were inferred to originate from *U. maydis* because they were detected in *U. maydis* single inoculated and co-inoculated plants. Metabolites were inferred to originate from *Zea mays* because they were detected across all treatments.

Table 4
Summary of ANOVA results for chromatographic peak areas of compounds attributed to *U. maydis*. The effects of *U. maydis* genotype (UMgenotype), *F. verticillioides* inoculation treatment (FV), the interaction term, and greenhouse bench (Block) were estimated.

Metabolite treatment	*df*[a]	1		2		3		4		5	
		F-stat	*P*[d]	*F*-stat	*P*	*F*-stat	*P*	*F*-stat	*P*	*F*-stat	*P*
UMgenotype[b]	1	4.59	0.0364	1.29	0.2594	0.57	0.4528	3.32	0.0741	3.12	0.083
FV[c]	2	17.27	<0.0001	9.88	0.0002	37.15	<0.0001	49.90	<0.0001	37.53	<0.0001
UMgenotype * FV	2	1.28	0.2855	0.86	0.4256	0.36	0.6967	3.27	0.0455	1.94	0.1527
Block	1	12.87	0.0007	1.63	0.2060	8.07	0.0064	2.55	0.1157	2.51	0.1186

[a] *df*: Degrees of freedom.
[b] UMgenotype: *U. maydis* genotype UM2 or UM18.
[c] FV: no endophyte, or coinoculation with F89 or NR.
[d] Pr (>*F*).

Table 5
Summary of ANOVA results for chromatographic peak areas of compounds attributed to *Zea mays*. The effects of *U. maydis* inoculation treatment (UM) and *F. verticillioides* inoculation treatment (FV), the interaction term, and Block were estimated.

Metabolites treatment	*df*[a]	6		7		8		9	
		F-stat	*P*[d]	*F*-stat	*P*	*F*-stat	*P*	*F*-stat	*P*
UM[b]	2	2.10	0.1276	1.38	0.256	45.98	<0.0001	0.12	0.8817
FV[c]	2	5.35	0.0064	2.14	0.1225	13.29	<0.0001	1.13	0.3267
UM * FV	4	0.10	0.9809	1.41	0.2358	4.62	0.002	1.66	0.1655
Block	1	37.28	<0.0001	8.11	0.0054	0.40	0.5254	1.69	0.1957

[a] Degrees of freedom.
[b] UM: inoculation with UM2, UM18, or no *U. maydis*.
[c] FV: inoculation with F89, NR, or no *F. verticillioides*.
[d] Pr (>*F*).

identified in fungal-inoculated plants that were not found in chromatograms obtained from mock-inoculated plants were assigned to be of fungal origin. Subsequently, a metabolite identified as being of fungal origin was then assigned to a species if the compound was observed in extracts of plants inoculated with that species but not in control plants that were not inoculated with that species. For example, we assumed that a chromatographic peak observed in UM and UM + FV treatments but not in FV treatments was produced by *U. maydis*. Five peaks (1–5) were inferred to be *U. maydis* metabolites because they were detected in chromatographic profiles in samples from plants inoculated with *U. maydis* or co-inoculated with *U. maydis* and *F. verticillioides*. The *m/z* values for metabolite 1 suggest a possible oligosaccharide. Four chromatographic peaks (6–9) were detected across all treatments, including control plants with no fungal inoculum, and we inferred these to be plant metabolites. No chromatographic peaks were detected in chromatograms corresponding to *F. verticillioides* single inoculated plants and we assumed that *F. verticillioides* metabolites were present below the instrument's detection limit or absent. The metabolite mass-charge ratio (*m/z*), retention time and putative species origin are shown in Table 3.

Judging by *m/z* ratio and retention time, none of the five metabolites putatively assigned to *U. maydis* correspond to those previously detected with *in vitro* experiments (Rodriguez Estrada et al., 2011; Jonkers et al., 2012). The following compounds have *m/z* values consistent with those we measured: metabolite 1, novel compounds produced by other pathogenic fungi (Bergstrom et al., 1993); metabolite 2, chitotetraose, a potential product of chitin catabolism (Price, 2006); and, metabolite 3, a pigment similar to the indole PyA (Zuther et al., 2008). For the compounds of putative plant origin, the *m/z* of metabolite 8 is consistent with xylan plant cell wall compounds (Brown et al., 2009).

3.6. Changes in metabolite concentrations resulted from co-inoculation

For each metabolite, we used chromatogram peak area normalized to plant tissue weight as a measure of metabolite concentrations in the plant tissue samples. ANOVA tests demonstrated significant differences in metabolite concentration due to treatments for all five peaks attributed to *U. maydis* (Table 4) and for two of the putative *Z. mays* metabolites (Table 5). Subsequent TukeyHSD tests ($p < 0.05$) show that mean concentrations of all putative *U. maydis* metabolites were lower in co-inoculation treatments with *F. verticillioides* (UM + F89 or UM + NR) compared to

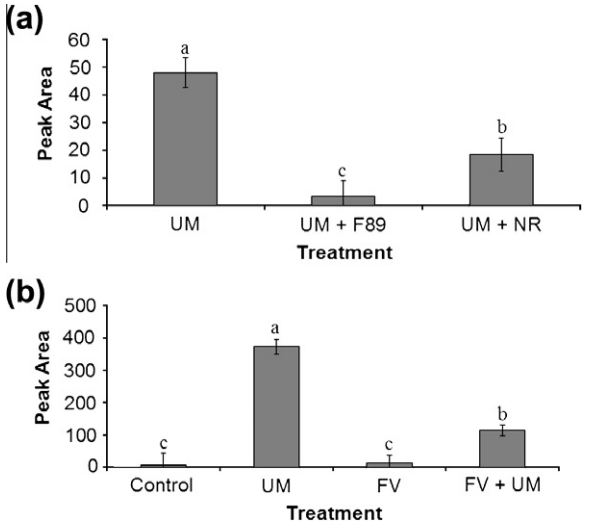

Fig. 6. The concentration of metabolites differed across inoculation treatments. (a) Metabolite 3 attributed to *U. maydis*. The mean concentration (peak area) of metabolite 3 was greatest in *U. maydis* single-inoculated plants (UM), significantly lower in plants co-inoculated with *F. verticillioides* genotype NR (UM + NR) and lower still in plants co-inoculated with *U. maydis* and *F. verticillioides* genotype F89 (UM + F89). (b) Metabolite 8 attributed to *Zea mays*. The mean concentration of metabolite 8 was significantly lower in mock-inoculate (Control) and *F. verticillioides* single-inoculated (FV) plants than in *U. maydis* single-inoculated (UM) or *F. verticillioides* and *U. maydis* co-inoculated (FV + UM) plants. Mean values that are statistically different at $p < 0.05$ are indicated by different letters (TukeyHSD test). Error bars indicate standard error of the mean.

inoculation of *U. maydis* alone (UM only). Concentrations of metabolites assigned to *U. maydis* were not significantly affected by *U. maydis* genotype, except for metabolite 1 where the concentration was higher in plants inoculated with UM2, than in plants inoculated with UM18. For putative *U. maydis* metabolite 3, mean concentrations (peak areas) were greatest in UM single inoculation treatments and were lower in F89 co-inoculation treatments than in NR co-inoculation treatments (Fig. 6a). Difference in the concentrations of other *U. maydis* metabolites were not observed due to the two *F. verticillioides* genotypes.

Of the putative plant metabolites (6–9) metabolites 6, 7 and 9 were not significantly affected by the presence of *U. maydis* whereas the peak area of metabolite 8 was. The most intense ion in the spectrum of the chromatographic peak corresponded to an *m/z* of 797.228. The concentration of compound 8 was significantly greater in single *U. maydis* inoculated plants than in any other treatment. The lowest concentration of this metabolite was observed in control plants and plants inoculated solely with *F. verticillioides*, perhaps because *U. maydis* was not present (Fig. 6b). Within the spectrum of compound 8, a fragment of *m/z* = 226.071 was detected which has a molecular weight consistent with N-(3-methoxy-2-hydroxyphenyl)-malonamic acid or HMPMA. HMPMA is a breakdown product of benzoxazolinones, plant defense compounds such as MBOA and BOA (Guillamon et al., 2004). The MBOA and BOA plant defense compounds are broken down by *F. verticillioides* (Glenn et al., 2002, 2003). Like other attributes of this system, metabolite concentrations were sensitive to environmental conditions. Concentrations of metabolites 1, 3, 6, 7 were significantly affected by Block and overall, metabolite concentrations for plants in Block 1 were higher than in Block 2.

4. Discussion

In this research, we addressed mechanisms and outcomes of interactions between a fungal endophyte of maize, *F. verticillioides*, a fungal pathogen, *U. maydis* and their host plant. The results show that pathogen aggressiveness towards the plant is lower when the endophyte is present than when the endophyte is absent. Previous studies had shown that disease development and plant mortality is influenced by pathogen and endophyte genotypes and by environmental conditions (Baumgarten et al., 2007; Lee et al., 2009). In the present study, uneven greenhouse conditions lead to variation in levels of disease severity and limited observation of differences due to *U. maydis* genotype. Nonetheless, the results are consistent with a growing body of research showing beneficial effects of endophytes in limiting pathogen and pest aggressiveness towards a plant host (Arnold et al., 2003; Lee et al., 2009; Matthews and Clay, 2001; Stein et al., 2008; Vu et al., 2006; Waller et al., 2005). Here, for the first time, we demonstrate a direct benefit to the endophyte of increased growth when growing with the pathogen present in the plant.

The results for fungal growth within the plant show that *U. maydis* achieves significantly less biomass when the endophyte is present, than when *U. maydis* is growing in the plant alone. This result is perhaps not surprising because *F. verticillioides* acts as an antagonist towards *U. maydis in vitro* (Rodriguez Estrada et al., 2011) and slows disease progress in maize (Lee et al., 2009). The results do show that *F. verticillioides* gains a significant 14-fold growth benefit in exchange for its role in sharply limiting pathogen growth in the plant. From these and microscopic observations (K. Lee, unpublished), we infer that *U. maydis* establishes the biotrophic phase of nutrition even in the presence of *F. verticillioides*.

Using our results and those of published work, we pose the following model for the exchange of fixed carbon among the pathogen, endophyte and host plant. The endophyte might "farm" *U. maydis* by harvesting sugars and other nutrients that *U. maydis* pulls from the plant (Wahl et al., 2010) because *U. maydis* reprograms young maize leaves to continue functioning as photosynthate sinks rather than becoming sources (Doehlemann et al., 2008a; Horst et al., 2009). In this model, the plant is trading goods (fixed carbon) for services (defense). However, it is not necessarily the case that the pathogen loses fitness. Lee (2010) showed that in the presence of *F. verticillioides*, smut disease development is slower, these diseased plants grow larger, and moderately aggressive *U. maydis* genotypes produce greater spore biomass. Together, these and previous results suggest that quantitative PCR estimates of fungal biomass can best be thought of as the standing biomass of actively growing fungal mycelia and thus, provide insight into the dynamics of fungal interactions within a host. Quantitative PCR should not be used as a proxy for fitness from such results alone and inferences for the long-term evolution should be restrained. In future studies, the fitness of the interacting fungi will be assessed by spore production over the life history of each species.

We found a complex array of secondary metabolite compounds in the sampled plant tissues, not surprising given the metabolic activities of all three organisms and their interactions. We attributed five of these metabolites to *U. maydis* because they were only observed in treatments with *U. maydis* present. All five decreased in concentration when *U. maydis* was co-inoculated with *F. verticillioides*, a result that could be attributed to any combination of less growth or lower production by *U. maydis*, or greater degradation in the presence of the endophyte. Among the putative *U. maydis* metabolites, the concentration of metabolite 3 declined significantly in plants co-inoculated with either F89 or NR, but declined more in the presence of F89. If metabolite 3 is an indole pigment with pathogenicity function (Zuther et al., 2008), the apparent greater ability of *F. verticillioides* genotype F89 to break this compound down may have contributed to the slightly greater growth observed for plants co-inoculated with *U. maydis* and F89. We were not able to detect metabolites produced by *F. verticillioides* such as fusaric acid that are apparently important during *in vitro* interactions between *F. verticillioides* and *U. maydis* (Rodriguez Estrada et al., 2011). However, we do show that the concentration of plant metabolite 8 is significantly greater in plants inoculated with *U. maydis* alone than in control plants, and that co-inoculation of *U. maydis* with *F. verticillioides* results in a still lower concentration. If compound 8 is a plant xylan wall compound, the results suggest that *U. maydis* produces xylanases (Cano-Canchola et al., 2000; Nadal et al., 2010). Maize likely produces xylanase inhibitors as do other Gramineae (Bellën et al., 2006). Whether cell wall degrading enzymes of *U. maydis* are important for pathogenicity, or for biotrophic growth, remains an open question (Doehlemann et al., 2008b).

The results show that the endophyte *F. verticillioides* behaves as a defensive mutualist, slowing disease progress, but it might at the same time, facilitate *U. maydis* growth in the plant. Results from *in planta* (Lee, 2010) and *in vitro* (Rodriguez Estrada et al., 2011; Jonkers et al., 2012) experiments suggest a role for fusaric acid in limiting *U. maydis* growth although we did not directly observe fusaric acid production here. We also observed decreased levels of an indol compound attributed to *U. maydis* in the presence of *F. verticillioides*. At the same time, *F. verticillioides* could modulate the growth of *U. maydis* in the plant by breaking down plant defense compounds such as BOA that are active against *U. maydis* (Basse, 2005; Niemeyer, 1988). Here, we identified the fragment (*m/z* = 226.071), which suggests the presence of HMPMA, a breakdown of BOA (Guillamon et al., 2004; Glenn et al., 2003). Infection by *U. maydis* induces DIMBOA production in maize, but *U. maydis* is insensitive to DIMBOA and may break it down (Basse, 2005). *F. verticillioides* encodes at least two genes active in the decomposition of BOA compounds in maize and different strains vary in their

sensitivity to and ability to break down DIMBOA, MBOA, and BOA (Glenn et al., 2002, 2003; Richardson and Bacon, 1995). Thus, we speculate that these two fungi may be "titrating" each other's growth in maize via the BOA-related compounds, with each species' catabolic activities producing compounds that either allow or restrict the growth of their antagonist. We do not know that such traits have resulted from a specific co-evolutionary interaction between *U. maydis* and *F. verticillioides* or in response to a diverse microbial community. The three-way interaction we study here is not all unusual, for example, the mycorrhizal *Piriformosporica indica* might limit the production of anti-oxidants by the root parasitic *F. verticillioides*, anti-oxidants that may combat the effects of the plant's defense system (Kumar et al., 2009). Given that either the presence of *F. verticillioides* (Saunders and Kohn, 2009) or the presence of *U. maydis* (Pan and May, 2009; Pan et al., 2008) strongly affects the distribution of other fungal species in maize, it is likely that both species-specific and general mechanisms of competition and defense have evolved in these complex communities.

5. Conclusions

The pathogen *U. maydis* and the endophyte, *F. verticillioides*, have long associated with their host plant maize and co-occur frequently in current maize culture. The results of this study shows that the endophyte ameliorates the severity of smut disease symptoms, likely as a result of antagonistic interactions between the endophyte and the pathogen. While the quantitative level of antagonism is sensitive to environmental conditions, the reduction in the rate of pathogen growth by the endophyte is consistently observed. Moreover, the endophyte gains a distinct growth benefit in the plant from interactions with *U. maydis*, and may facilitate *U. maydis* infection or growth during disease development. Consequently, plant symbiotic fungi such as *F. verticillioides* are not neatly categorized as mutualist or parasite; rather, their ecological roles depend on biotic and abiotic context.

Acknowledgments

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