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

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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

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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.