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In vitro interactions between *Fusarium verticillioides* and *Ustilago maydis* through real-time PCR and metabolic profiling

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ABSTRACT

The goal of this research was to determine mechanisms of interaction between endophytic strains of *Fusarium verticillioides* (Sacc.) Nirenberg and the pathogen, *Ustilago maydis* (DC) (Corda). Endophytic strains of the fungus *F. verticillioides* are commonly found in association with maize (*Zea mays*) and when co-inoculated with *U. maydis*, often lead to decreased disease severity caused by the pathogen. Here, we developed methods (liquid chromatography–mass spectrometry) to evaluate changes in relative concentration of metabolites produced during *in vitro* interactions between the endophyte and pathogen. Fungi were grown on two different media, in single and in confronted cultures. We used real-time PCR (qPCR) assays to measure relative changes in fungal biomass, that occurred in confronted cultures compared to single cultures. The results showed that most secondary metabolites are constitutively produced by each species. Metabolite profiles are complex for *U. maydis* (twenty chromatographic peaks detected) while relatively fewer compounds were detected for *F. verticillioides* (six chromatographic peaks). In confronted cultures, metabolite ratio (metabolite concentration/biomass) generally increases for *U. maydis* metabolites while no significant changes were observed for most *F. verticillioides* metabolites. The results show that *F. verticillioides* is a strong antagonist of *U. maydis* as its presence leads to large reductions in *U. maydis* biomass. We infer that few *U. maydis* metabolites likely serve antibiotic functions against *F. verticillioides*. The methods described here are sufficiently sensitive to detect small changes in biomass and metabolite concentration associated with differing genotypes of the interacting species.

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1. Introduction

Plants are hosts to diverse microorganisms that cause a range of detrimental to beneficial effects on the host's growth and survival. Of particular interest are the endophytic fungi or bacteria that live inside a plant without causing apparent disease symptoms (Rodriguez et al., 2009). While the functional roles of endophytes are understudied, they may act as defensive mutualists by conferring increased tolerance to pathogens (Arnold et al., 2003; Lee et al., 2009) or conferring competitive abilities to the host (Clay and Holah, 1999; Wilson, 1995). While effects on the host have been explored to some extent, mechanisms of interaction among plant-associated microbes have only been studied recently (Saunders and Kohn, 2008). Here, we develop methods to evaluate mechanisms and outcomes of interactions between two fungi commonly found in maize; the endophyte *Fusarium verticillioides* and the pathogen *Ustilago maydis*. We consider the potential role of metabolites during interactions of these two species and infer those which may function as antibiotics limiting the growth of their competitor.

Interactions among microbes, including the endophytes, encompass antagonistic or competitive, facilitative, and apparently neutral outcomes (Mazurier et al., 2009; Saunders and Kohn, 2008; Pan and May, 2009). Interacting fungi may limit each other's growth through antibiosis, where secondary compounds limit mycelial growth, spore production or spore germination of the opposing fungi (Altomare et al., 2000; Goodman and Burpee, 1991; Nicoletti et al., 2004). Fungi also may compete for nutrients if they use the same nutritional resource (Janisiewicz et al., 2000). Less well studied, fungi may facilitate each other's growth if one provides a compound that the other metabolizes or if one species detoxifies a given environment, making it more suitable for the growth of other fungi (Saunders and Kohn, 2008). In this research, we evaluated the modes of interaction between endophytic *F. verticillioides* and the pathogen *U. maydis*.

The ascomycete *F. verticillioides* (syn *F. monilliformes* Sheldon, teleomorph *Gibberella moniliformis* Wineland) is found in

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association with a wide range of plant hosts (Kuldau and Yates, 2000; Moretti et al., 2004). In maize, *F. verticillioides* can cause rot in ears, seeds, roots or stalks (Kommedahl and Windels, 1981), but also can be found as endophyte (Leslie et al., 1990; Pan et al., 2008; Shepherd et al., 1996). Lee et al. (2009) demonstrated that endophytic *F. verticillioides* may decrease disease severity caused by *U. maydis*. The genus *Fusarium* is well known to produce a wide range of secondary metabolites including mycotoxins, organic acids and pigments (Bacon et al., 1996; Chelkowski, 1989; Steyn et al., 1979). Evidence that those compounds directly affect the plant–pathogen interaction and disease development is ambiguous, leading to the speculation that they play ecological roles in competition against other microorganisms (Bacon et al., 2004; Duffy et al., 2004; Keyser et al., 1999; Toyoda et al., 1988). The basidiomycete *U. maydis* and closely related smut fungi produce a variety of secondary compounds such as ustilagic acid and mannosylerythrol glycolipids but their role as virulence factors or during ecological interactions with other microbes is unclear (Hewald et al., 2005; Lemieux, 1953; Teichmann et al., 2007).

We report molecular and biochemical techniques to study relative changes in fungal biomass and secondary metabolite concentrations during *in vitro* interactions between *F. verticillioides* and *U. maydis*. Our immediate goal was to distinguish among antagonistic, facilitative or neutral effects of these species on each other's biomass, and to determine the roles of secondary metabolites in those interactions. We utilized a metabolic profiling approach to evaluate metabolites produced by these fungi when grown in single culture, and determined changes in metabolites concentration occurring in confronted cultures. Quantitative PCR (qPCR) assays were used to monitor relative changes in fungal biomass due to interactions. Different isolates of each interacting species were included in this work to determine if these techniques were sufficiently sensitive to detect variation across genotypes. The methods developed and tested in this work can be used to study fungal interactions *in planta*.

2. Methods

2.1. Fungal cultures and culture media

Two strains of *F. verticillioides* (NR and F17) were included in this study because they varied in color and fusaric acid production (Keunsub Lee, personal communication, Iowa State University). Both F17 and NR strains behave as endophytes in maize under greenhouse conditions. The F17 strain was isolated from symptomless plants in a previous study (Pan et al., 2008) and species identification was verified by sequencing of the translation elongation factor (TEF; Keunsub Lee, personal communication). The NR strain (NRRL 20956) was obtained from the Agricultural Research Service Culture Collection and has been fully sequenced (Ma et al., 2010). *U. maydis* is a dimorphic fungus wherein the infectious, dikaryotic filamentous stage results from a compatible mating of two haploid yeasts. We utilized three different *U. maydis* haploids: C7 (mating type a_1b_{12}), U2 and U18 genotypes (both mating type a_2b_{11}) which were paired to form genotypes UM2 (U2 × C7) and UM18 (U18 × C7). Strain F17 is kept in liquid nitrogen at the University of Minnesota Culture Collection (Culture ID: 49 56796-8 S). NR is stored at −80 °C in 15% glycerol and *U. maydis* haploids are stored in silica gel at −20 °C in the May laboratory. All strains are available on request.

Secondary metabolite production is influenced by the composition of the growing media. Therefore, we used standardized, commercially available media; Czapek-Dox Agar (CDA, Difco Scientific; Sucrose 30 g, $NaNO_3$ 3 g, K_2PO_4 1 g, $MgSO_4$ 0.5 g, KCl 0.5 g, $FeSO_4$ 0.01 g, Agar 15 g per Liter) and Potato Dextrose Agar (PDA, Difco Scientific; Potato starch 4 g, Dextrose 20 g, Agar 15 g per Liter).

2.2. Experimental design

Two experiments, on Potato Dextrose Agar (PDA) and on Czapek-Dox Agar (CDA) media (see below), were conducted independently to evaluate fungal biomass and metabolite production in single and confronted cultures. Within each experiment, a full factorial design was employed with the fungal species, *U. maydis* and *F. verticillioides*, as treatment factors. Each factor had three levels. *U. maydis* levels were: UM2, UM18, and no *U. maydis*. *F. verticillioides* levels were: NR, F17, and no *F. verticillioides*. This gave nine treatment combinations (UM2 only, UM18 only, NR only, F17 only, UM2 + F17, UM2 + NR, UM18 + F17, UM18 + NR and media inoculated with water). For each treatment combination, fungal biomass, metabolite concentrations and metabolite ratios (metabolite concentration/biomass) were evaluated. The water control was used as a negative control for biomass determination and to determine compounds attributed to the agar media. Five replicate plates were evaluated per treatment combination.

2.3. Inoculum preparation and culture sampling

For *F. verticillioides*, spore inoculum was prepared by harvesting spores from cultures grown on PDA medium at 28 °C for 10 days. Seven milliliter of sterile deionized water were added to the culture, and the mycelium was gently scraped with a glass rod to loosen the spores. The *U. maydis* haploids (C7, U18 and U2) were each grown separately in 50 mL of potato dextrose broth (PDB) in Erlenmeyer flasks (125 mL) for 3 days at 28 °C in a shaker incubator (100 rpm). After harvest, the *U. maydis* sporidia and *F. verticillioides* spores were each centrifuged at 4000 rpm for 6 min. The sporidia or spores pellets were re-suspended in 15 mL of sterile deionized water. This washing process was repeated two additional times. Inoculum of *F. verticillioides* was adjusted to 2×10^4 spores μL^{-1} and 50 μL (10^6 spores) were inoculated onto plates. Inoculum of *U. maydis* was adjusted to 1×10^4 sporidia μL^{-1}, 40 μL of each compatible mating type were mixed, and 80 μL (10^6 spores total) were inoculated onto the medium. The number of spores and sporidia inoculated was thus the same for both fungi. Preliminary experiments determined that the somewhat larger volume for *U. maydis* inoculum allowed the colony to grow before the arrival of the *F. verticillioides* mycelium.

Petri dishes (90 mm) containing 20 mL of PDA or CDA nutrient media were inoculated (five replicates per treatment combination). For confronted cultures (UM2 + F17; UM2 + NR; UM18 + F17 and UM18 + NR), fungi were inoculated 2.5 cm apart from each other. Single cultures were inoculated alone in the center of the Petri dish and mock treatments were inoculated with sterile deionized water. Petri dishes were incubated for 10 days at 28 °C in darkness.

We removed two agar plugs (7 mm in diameter × 5 mm in thickness) to sample each plate culture for biomass and two plugs for metabolite analysis. In confronted cultures, these plugs were cut from the interaction zone between the *F. verticillioides* and *U. maydis* colonies (see Fig. 1). For the single cultures, agar plugs were removed at approximately the same distance as in confronted cultures; from the edge of the *U. maydis* colony and at 3 cm from the center of the *F. verticillioides* colony.

2.4. Metabolite extraction

Metabolites were extracted following a modified version of the micro-scale extraction procedure published by Smedsgaard (1997). As described above (Section 2.3), for confronted cultures, two agar plugs were cut from each of the five replicate plates from the interaction zone between *F. verticillioides* and *U. maydis* colonies (see Fig. 1). For the single cultures, two agar plugs were removed from each of the five replicate plates.

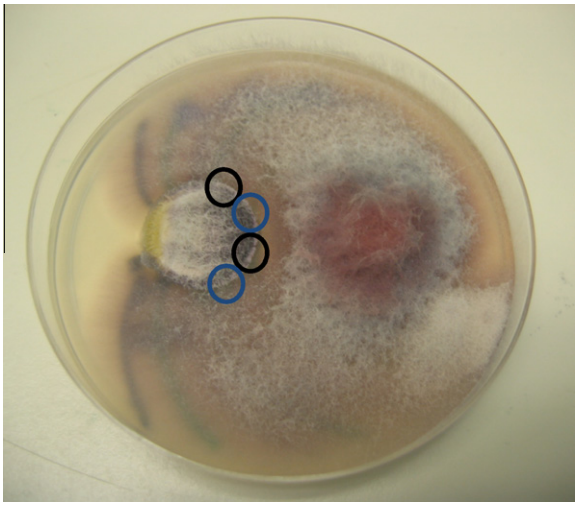

Fig. 1. *Ustilago maydis* (UM18) and *Fusarium verticillioides* (F17) interactions in PDA at 10 days after inoculation. The aerial mycelium of *F. verticillioides* is reduced in the interaction zone before it overgrows *U. maydis*, then mycelium heavily accumulates over the *U. maydis* colony. Black circles indicate plugs taken for metabolites extraction. Blue circles indicate plugs taken for biomass quantification. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

To extract metabolites from cultures grown on PDA medium, a mixture of toluene/methanol/formic acid (1:1:0.01 v/v/v) was used (1.5 mL of solvent per two agar plugs). Toluene (benzene derivative) was expected to have more affinity for the fungal cyclic pigments expected in this medium. Extractions were conducted in an ultrasonic bath (Mettler Electronics) for 1 h at room temperature. To extract metabolites from cultures grown on CDA medium, 1.5 mL of methanol (⩾99.9% Sigma–Aldrich, spectrophotometric grade) and formic acid (1:0.01 v/v) were added to the agar plugs. Extraction was done for 12 h at 4 °C as recommended by Peiris et al. (2008). The extracts were placed in clean 2 mL tubes and dried in a speed vacuum (SpeedVac, Savant, SC100) for 6 h. The resulting pellet was stored at 4 °C for no more than 1 week before analysis.

2.5. Metabolite analysis

Dried pellets were dissolved in 500 μL of acidified methanol (⩾99.9% Sigma–Aldrich) and formic acid (1:0.001 v/v) with ultrasonication for 10 min and subjected to centrifugation at 14,000 rpm for 10 min. One hundred microliters of the supernatant from each sample was transferred to clear glass vials (1.5 mL, Chrom Tech, Inc.) and diluted 1:3 volume sample to volume acidified methanol. Samples from five biological replicates per treatment (2 μL per injection) were analyzed in random order 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). Fungal metabolites were separated using an Acquity UPLC® BEH C_{18} reverse phase column (2.1 × 100 mm; 1.7 μm particle size) and a Van Guard™ pre-column (2.1 × 5 mm). The mobile phase was constituted from mixtures of two components: water/formic acid (A, 1:0.001 v/v) and acetonitrile/formic acid (B, 1:0.001, v/v), and delivered at a constant flow rate of 0.5 mL/min. Compound elution was accomplished using a linear gradient of 2–20% of B over 1 min, 20–100% B for 11 min. Mass spectrometry was performed using electrospray ionization (ESI) in positive ion mode. Accurate mass measurements (8 ppm mass error) were obtained by monitoring the first isotopic peak of the calibrant, leucine enkephalin (m/z = 557.280, solution is 50 ng mL^{-1}, in 50% methanol, delivered at 10 μL/min). The calibrant signal was monitored 5% of the time at regular intervals and was used to automatically correct fluctuations in the instrument calibration from scan to scan. The MassLynx™ software (v 4.1, Waters) was utilized to operate the instrument, process and visualize the data.

2.6. LC–MS data processing

Data retrieved from the UPLC/TOF/MS instrument includes three components: (1) retention time (elution time from the column), (2) monoisotopic mass (mass to charge ratio m/z, mostly ($M+H^+$) and ($M+Na^+$)), and (3) ion intensity (signal in counts per second). We used these three components to manually extract each chromatographic peak and automatically estimate peak areas (see Fig. 2). Peaks found in mock treatments (media inoculated with water) were identified and excluded from further analysis. Compound identification was facilitated by comparing highly accurate (±8 ppm mass error) monoisotopic mass measurements with those values calculated from structures of secondary compounds previously described for *F. verticillioides* (Balan et al., 1970; Chelkowski, 1989; Gelderblom et al., 1988; Mayser et al., 2007) and for *U. maydis* (Bölker et al., 2008; Kulakovskaya et al., 2005; Teichmann et al., 2007; Zuther et al., 2008).

2.7. Tryptophan internal standard

Tryptophan (m/z = 205.097 $[M+H]^+$) was added to each sample at a final concentration of 0.033 mg μL^{-1} to serve as an internal standard. Peak area of the tryptophan was obtained for each analysis and used to normalize all peaks in the chromatogram by dividing peak area of each metabolite by peak area of the internal standard (Peiris et al., 2008).

2.8. Fusaric acid quantification

We sought to measure differences in concentrations of all compounds produced by *F. verticillioides* and *U. maydis* under different treatment conditions, including compounds which had not been previously identified or purified. To validate the use of peak areas to estimate metabolite concentration, we constructed two standard curves (0.01–0.1 μg μL^{-1} and 0.1–0.6 μg μL^{-1}) for fusaric acid (FA; Sigma–Aldrich). Peak areas were plotted against known concentration for each range of dilutions. A regression line was fitted and the resulting equations were used to calculate FA concentration (Microsoft Excel 2007). The FA serial dilutions were run along with the samples from the CDA experiment since preliminary results showed that FA is produced by *F. verticillioides* on CDA medium and not on PDA medium.

2.9. Biomass quantification

We estimated fungal biomass in agar plugs removed from single and confronted cultures as described above (Section 2.3). We used real-time PCR (absolute quantification method, qPCR) to calculate the copy number (genome equivalents) of target genes for each species. Species-specific primers and labeled probes (TaqMan® MGB) were designed based on gene sequences of the calmodulin (*F. verticillioides*) and glycosyl transferase gene (*U. maydis*) which we assumed were single-copy genes in these strains as in the fully sequenced strains (Ma et al., 2010; Kämper et al., 2006). Primers VER1 (5′-CTTCCTGCGATGTTTCTCC-3′) and VER2 (5′-AATTGG CCATTGGTATTATATATCTA-3′) amplify a 578 bp region of the calmodulin gene in *F. verticillioides* (Mule et al., 2004). Primers emt1_F (5_-GCAGCCACAATGGTCCGCG-3) and emt1_R (5_-CGAGTCTGTG

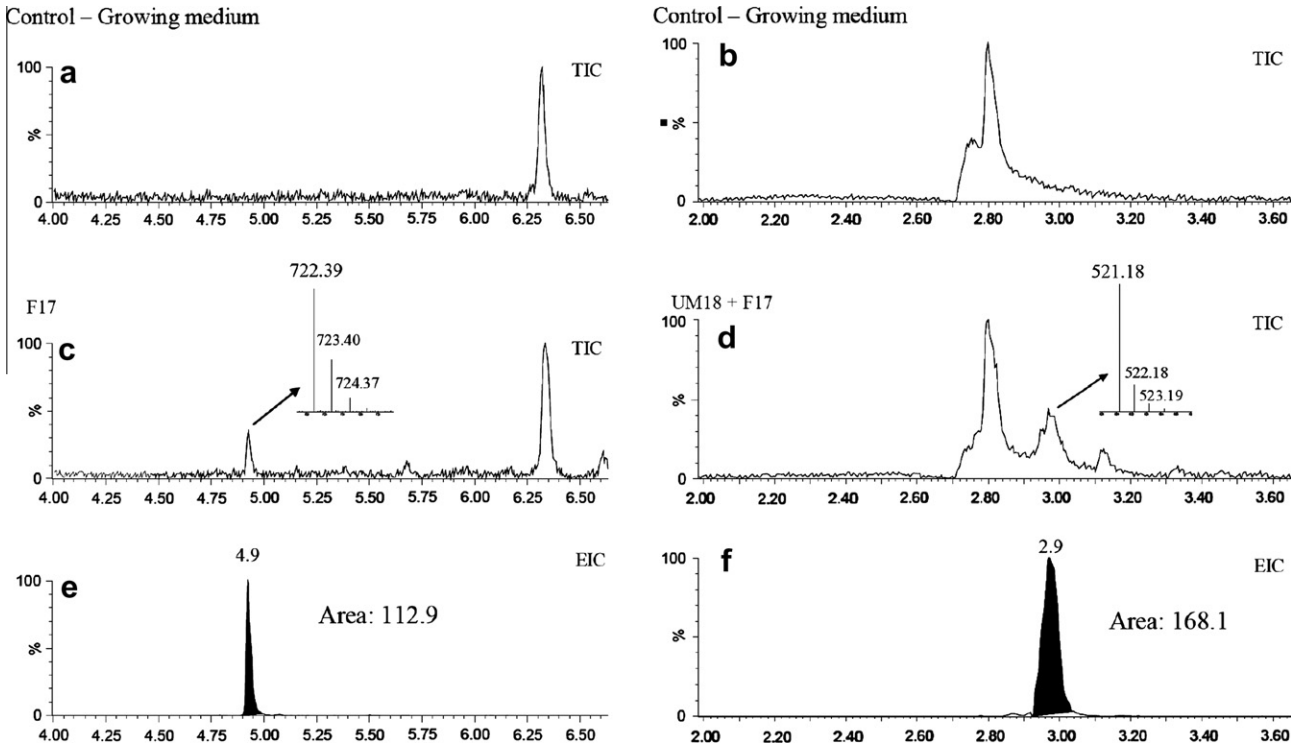

Fig. 2. Peak extraction and integration from chromatograms. (a and b) Chromatogram region corresponding to PDA medium (mock treatment). (c) Fumonosins B1 (m/z = 722.398 $[M+H]^+$) from *Fusarium verticillioides* (peak and spectrum). (d) Metabolite U (m/z = 521.183) from *Ustilago maydis* (peak and spectrum). (e) Integrated peak corresponding to fumonosins B1 (retention time and peak area). (f) Integrated peak corresponding to unidentified metabolite (retention time and peak area). The monoisotopic peaks from the mass spectra for each metabolite were used for peak extraction and integration. TIC = total ion chromatogram, EIC = extracted ion chromatogram.

AGGCCAGGCGTG-3) amplify a 456 bp region of the glycosyl transferase gene in *U. maydis* (Hewald et al., 2005). After the sequences were obtained, PRIMER 3 (http://frodo.wi.mit.edu/primer3/) and Primer Express (Applied Biosystems) software were used to design primers and labeled probes (UMemt1 and Fusca) for species specific real-time PCR assays (Table 1).

To conduct the qPCR, total genomic DNA was extracted from two agar plugs (7 mm diameter × 5 mm thick) colonized with fungi taken from the same plate and directly adjacent to plugs used for metabolite extraction (see Fig. 1). Plugs were dried under a vacuum for 4 h and total DNA was extracted with a modified version of the phenol chloroform method (Beckerman et al., 2001). Glass beads (~324 mg), TENTS buffer (0.5 mL; 10 Mm Tris/HCl pH 7.5, 1 mM EDTA, 100 mM NaCl, 2% Triton X-100, 1% SDS) and phenol:chloroform (0.5 mL, 1:1) were added to each sample. Cell walls were mechanically disrupted by vigorously vortexing and agitating the samples with a cell disruptor (Vortex-Genie®-2) for 4.5 min. The aqueous and organic layers were separated by centrifugation at 14,000 rpm for 10 min at room temperature. DNA was precipitated from the aqueous phase with 50 μL of ammonium acetate (7.5 M) and 1.0 mL of isopropanol, pelleted by centrifugation (14,000 rpm for 15 min) and then rinsed with 100 μL of 70% cold ethanol. After, ethanol was evaporated in a speed vacuum for 5 min. The DNA pellet was re-suspended in 50 μL of elution buffer (10 mM Tris–Cl, Qiagen)/RNase A (6.25 μg/mL) and incubated at 37 °C for 3 h.

Table 1
Ustilago maydis and *Fusarium verticillioides* primers and labeled probes for real-time PCR.

Species (amplicon length)	Primer or probe name[a]	Sequence 5′ → 3′
Ustilago maydis (93)	Emt1qPCR_F	GTCACTGGTTGCCGAATGACT
	Emt1qPCR_R	TGGGTCAAACAGGCTCTTACG
	UMemt1	6FAM-CATGGATG TTCACCGTTC-MGB
Fusarium verticillioides (137)	FusqPCR_F	TCGCTCTAGGCCAGATTACCA
	FusqPCR_R	GAACCAGGAAAGTCGATGGTG
	Fusca	VIC-CGCTCTCTCGGC CAGA-MGB

[a] Primers were purchased from the BioMedical Genomics Center at the University of Minnesota. Probes were purchased from Applied Biosystems.

Standard curves for each species were created from serial dilutions of total DNA extracted from single cultures (Applied Biosystems, 2003). Mass of each fungal genome was calculated with the formula $m = (n)\ (1.096e^{-21}$ g/bp) where n is the haploid genome size (41.78 Mb for *F. verticillioides* and 19.68 Mb for *U. maydis*). Initial concentrations of total DNA were measured with a fluorometer (Qubit®, Invitrogen) using Quant-iT™ dsDNA Broad Range reagents. DNA templates of *F. verticillioides* and *U. maydis* were adjusted to 9.15 and 4.3 ng μL^{-1}, respectively. Then, five 10-fold serial dilutions were made to yield a calculated range of 30–3 × 10^5 genome equivalents of each single copy gene added to the real-time PCR reactions as described below.

Quantitative PCRs were conducted in multiplex with primer and probe sets included for both species (StepOnePlus™, Applied Biosystems). In preliminary experiments, amplification efficiencies of multiplex and singleplex reactions were compared to demonstrate that no cross reactivity occurred among primers and probes for the two fungal species (data not shown). Real-time PCR amplifications were done with the TaqMan® Environmental Master Mix (Applied Biosystems) in 15 μL reactions arrayed in 96 well plates. Primer concentration in the reactions was 400 nM each, and probe concentrations were 150 and 250 nM for the UMemt1 and Fusca probes, respectively. DNA extracted from agar plugs was diluted fivefold and from these dilutions, 1.5 μL were added to the PCR reaction. Cycling conditions were: 10 min at 95 °C, 45 cycles of

15 s at 95 °C and 60 s at 60 °C. Two 96 well plates were used per experiment. For each plate, one multiplex standard curve was included and each dilution was replicated three times (technical replicate). Data were recovered and processed with the StepOne™ Software (V 2.0, Applied Biosystems). Threshold values were set in the exponential phase of the amplification plot. Regression equations for each standard curves were automatically calculated by the StepOnePlus™ software. After data processing, the number of genome equivalents was expressed as number of single-gene copies $\times 10^3$. These values were used to estimate relative changes in fungal biomass under different treatments.

2.10. Metabolite ratio (metabolite concentration/biomass)

Metabolites and DNA were extracted from closely adjacent agar plugs on the same agar plate (see Fig. 1). We normalized metabolite concentration (peak area) to its corresponding biomass (genome equivalents) giving the metabolite ratio values (metabolite concentration/biomass).

2.11. Statistical analyses

The JMP® software (V. 7.0.1) was used to analyze the data generated. Experiments for each growth medium (CDA and PDA) were conducted at different times and thus results for each experiment were evaluated separately. Data sets comprise values of the following response variables: (1) metabolites concentration (peak areas), (2) biomass (number of genome equivalents $\times 10^3$), and (3) metabolite ratios (metabolite concentration/biomass). The effects of genotype, condition (single and confronted cultures with both confronting genotypes) and interaction terms (genotype × condition) were evaluated for each response using two-way ANOVA. Since that analysis included the presence or absence of a confronting species, we next determined effects of genotype on response variables using two-way ANOVA with the genotype factor defined by the genotype of the species for which metabolites or biomass were being evaluated (*U. maydis* or *F. verticillioides* genotypes), and condition defined by the genotype of the confronting species. For example, for *U. maydis* metabolite and biomass in confronted cultures, genotype is UM2 or UM18, and condition is confrontation with either strain F17 or NR. Normality was visually assessed through normal quantile plots and statistically by the Shapiro–Wilk *W* test (Shapiro and Wilk, 1965). Values that were not normally distributed were transformed by the Box–Cox method as recommended by Weisberg (2005). Significant differences among means were determined with the Tukey's Honestly Significant Difference (HSD) test (Hsu, 1996). Fold-changes in metabolite concentration, biomass, or metabolite ratios were expressed as increases or decreases in confronted cultures relative to non-confronted cultures.

3. Results

3.1. Cultures

F. verticillioides mycelia reached *U. maydis* colonies at 5–6 days after inoculation. No obvious inhibition zone was observed although the aerial mycelium of *F. verticillioides* within the interaction zone was not as dense as in the rest of the fungal colony. Once *F. verticillioides* overgrew *U. maydis*, its mycelia accumulated conspicuously over the *U. maydis* colony (Fig. 1).

3.2. LC–MS analysis

Chromatographic peaks were detected and extracted from the metabolic profiles corresponding to single and confronted cultures in each medium (Fig. 2). Observed mass to charge ratio (*m/z*), peak identifier label, putative identification and presence or absence in each medium are shown in Table 2. For *U. maydis*, the metabolic profiles (number of peaks and *m/z*) were very similar for both media and both *U. maydis* genotypes (UM2 and UM18). On PDA medium, 19 peaks (putative metabolites) and on CDA medium, 18 peaks were detected for both *U. maydis* genotypes grown in single cultures (Table 2a). Metabolite T was only detected on PDA whereas metabolites G and S were only detected on CDA. All *U. maydis* metabolites were detected in both single and confronted cultures except for the unidentified metabolite U (*m/z* = 521.183) (Table 2b). Metabolite U was only found in trace amounts (peak area <5) in single *U. maydis* cultures grown on CDA but it was found in considerably greater quantities when *U. maydis* was

Table 2
Ustilago maydis and *Fusarium verticillioides* chromatographic peaks (metabolites) detected (+) or absent (−) in single and confronted cultures grown in Czapek-Dox agar (CDA) and potato dextrose agar (PDA) media.

Peak	Metabolite[a]		Media[b]	
ID	*m/z*	ID	PDA	CDA
(a) Ustilago maydis				
A	461.160	Unidentified	+	+
B	489.191	Unidentified	+	+
C[c,d]	693.330	MEL D	+	+
D	327.211	Unidentified	+	+
E[c,e]	807.419	UA B	+	+
F	805.424	Unidentified	+	+
G	777.395	Unidentified	+	–
H[c,g]	791.406	UA A	+	+
I[c,h]	835.432	UA B	+	+
J[c,f]	819.439	UA A	+	+
K	775.411	Unidentified	+	+
L	803.442	Unidentified	+	+
M	743.418	Unidentified	+	+
N	643.330	Unidentified	+	+
O	669.346	Unidentified	+	+
P	695.360	Unidentified	+	+
Q	671.363	Unidentified	+	+
R	697.376	Unidentified	+	+
S	685.415	Unidentified	+	–
T	677.335	Unidentified	–	+
Total			19	18
(b) Ustilago maydis + Fusarium verticillioides				
U	521.183	Unidentified	+	+
Total			1	1
(c) Fusarium verticillioides				
I	722.398	Fumonosins B1	+	–
II	706.401	Fumonosins B2	+	–
III	383.074	Bikaverin	+	–
IV	690.405	Unidentified	+	–
V	180.100	Fusaric acid	–	+
VI	495.207	Unidentified	–	+
Total			4	2

[a] Metabolite: *m/z* is the mass to charge ratio of the most intense ion in the spectrum. ID is a putative identification based on mass to charge ratio of the most intense ion in the spectrum.
[b] Growing media: CDA (Czapek-Dox agar); PDA (potato dextrose agar).
[c] Mass to charge ratio of the most intense ion within the spectrum. The most intense ion might be an abduct or fragment of the parental ion.
[d] MEL D: mannosylerythritol lipid, $C_{24}H_{44}O_{11}$ $[+1]^+ = 509.296$.
[e] UA B $[M+Na]^+$: ustilagic acid B (R1 = OH; R2 = OH; *n* = 2), $C_{36}H_{64}O_{18}$ $[23+]^+ = 807.419$.
[f] UA A $[M+Na]^+$: ustilagic acid A (R1 = H; R2 = OH; *n* = 4), $C_{38}H_{68}O_{17}$ $[23+]^+ = 819.439$.
[g] UA A $[M+Na]^+$: ustilagic acid A (R1 = H; R2 = OH; *n* = 2), $C_{36}H_{64}O_{17}$ $[23+]^+ = 791.406$.
[h] UA B $[M+Na]^+$: ustilagic acid B (R1 = OH; R2 = OH; *n* = 4), $C_{38}H_{68}O_{18}$ $[23+]^+ = 835.432$.

grown in confrontation with *F. verticillioides*. Therefore, we infer that metabolite U is a compound synthesized by *U. maydis*.

We observed simpler metabolic profiles for *F. verticillioides* than for *U. maydis* (Table 2c). On PDA medium, four peaks were detected (compounds I–IV). On CDA medium, only two peaks were detected (compounds V and VI). PDA medium has low nitrogen availability and includes a complex carbohydrate source (potato starch) whereas CDA medium contains inorganic nitrate as the nitrogen source and sucrose as the carbon source. The strongly differing results for *F. verticilloides* on the two media likely reflect effects of differing nitrogen and carbon sources (Yu and Keller, 2005) but also may reflect differences in extraction methods. Overall, genotype and growing media did not strongly affect *U. maydis* metabolites production, but these factors were more influential for *F. verticillioides*.

The methods we employed were sufficient to isolate and identify previously described compounds for both *U. maydis* and *F. verticillioides*. For *U. maydis*, mannosylerythritol lipid (MEL D; compound C), different forms of ustilagic acid (UA), and a possible breakdown product of ustilagic acids (compound D) were detected. Different forms of ustilagic acid were detected in masses consistent with ustilagic acid A (compounds H, J) and B (compounds E, I) and several isomeric forms. Isomers are likely the result of glycosyl moiety heterogeneity and were observed as peaks with identical masses but slightly different retention times. For example, a mass consistent with ustilagic acid B was observed with chromatographic features eluting at 5.7, 5.9 and 6.1 min. For the purposes of this study, peaks with identical mass to charge ratios (*m/z*) were treated as the same metabolite and peak areas were added together for statistical analyses. For *F. verticillioides*, four of the six detected compounds were identified based on the monoisotopic mass of the most intense ion, consistent with previously described *F. verticillioides* metabolites (Table 2c). The polyketide fumonisins, B1 (compound I) and B2 (compound II) as well as the pigment bikaverin (compound III), were detected on PDA medium but not on CDA medium. The pigment bikaverin was detected in F17 but not in NR profiles. Lastly, fusaric acid (compound V) was produced by both *F. verticillioides* genotypes grown on CDA medium, but not on PDA medium.

3.3. Tryptophan internal standard

Peak areas of the internal standard tryptophan were compared across samples in order to detect variation due to technical artifacts. The ANOVA tests did not show significant differences in peak areas for tryptophan across replicates, or among treatments ($p > 0.05$) (data not shown). To further validate our technique, we obtained response ratios dividing peak area of each chromatographic peak by the peak area of tryptophan. ANOVA tests conducted with the response ratio were identical to direct analysis of peak areas across treatments. Therefore, the results of analyses reported here are not normalized to the internal standard tryptophan.

3.4. Fusaric acid quantification

Because FA is available in purified form, we further validated the use of peak areas as measures of metabolite concentration by correlating known fusaric acid quantities with peak areas. The regression results for FA quantities at 0.01–0.1 μg μL^{-1} ($y = 2397.2x + 2.1216$; $R^2 = 0.99$) and 0.1–0.6 μg μL^{-1} ($y = 685.7x + 184.5$; $R^2 = 0.99$) demonstrated strong correlations between FA concentration and peak area. ANOVA tests confirmed very similar results with significant effects of genotype, condition and interactions ($p < 0.05$) for FA concentration when calculated values (based on the standard curve) or direct comparisons of peak areas were used (e.g. Fig. 3a). Because the resulting regression model demonstrates a strong linear relationship between peak area and metabolite concentration, we concluded that peak area is an accurate representation of metabolite concentration for FA and other compounds.

3.5. Changes in metabolite concentration due to interactions

ANOVA tests were conducted for metabolite concentrations (peak area) for each metabolite in response to treatment variables

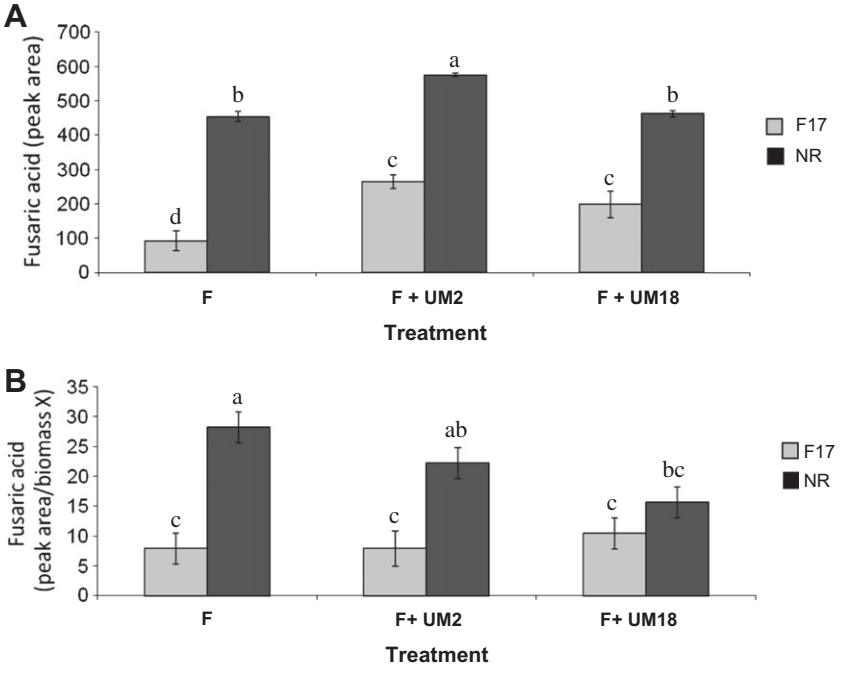

Fig. 3. Fusaric acid produced by *Fusarium verticillioides* in single and confronted cultures on Czapek-Dox medium. (A) Peak areas, (B) metabolite ratio (peak area/biomass, transformed data X). *Fusarium verticillioides* isolates F17 and NR were confronted with two genotypes of *Ustilago maydis* (UM2 and UM18). Mean values that are statistically different at $p < 0.05$ are indicated by different letters (Tukey's HSD test). Error bars indicate standard error of the mean.

(data not shown). Changes in metabolic concentration expressed as the fold-changes in confronted cultures compared to single cultures for *U. maydis* (UM2) and *F. verticillioides* metabolites (F17 and NR) on PDA medium are shown in Fig. 4a. Differing results due to *U. maydis* genotype or growth on CDA medium are explained in text below. As a general trend, most *U. maydis* metabolites significantly decreased in concentration when cultures were grown in confrontation with *F. verticillioides* (14 of the total 19 metabolites detected on PDA, 12 of the 18 total *U. maydis* metabolites detected on CDA). On both media, confrontation with *F. verticillioides* strain NR generally led to greater decreases in *U. maydis* metabolite concentrations than did strain F17 (Figs. 4a and 5a). In contrast to most metabolites, compounds D, M and U increased in concentration when *U. maydis* was grown in confrontation with *F. verticillioides* on either medium. The spectrum of metabolite D (m/z = 327.211) contains a fragment of m/z = 305.435 corresponding to one hypothetical product of flocculosin degradation ($M+H^+$) (Mimee et al., 2009). Since flocculosin is a glycolipid of nearly identical structure to ustilagic acid, we believe that compound D is a breakdown product of ustilagic acid. A significant increase in metabolite D during interactions suggests catabolism of ustilagic acid by *U. maydis* or *F. verticillioides*.

In contrast to results for *U. maydis*, all *F. verticillioides* metabolites significantly increased in concentration when grown in confronted cultures on PDA (Fig. 4a) or on CDA medium (Fig. 3a, for fusaric acid), compared to concentrations when grown alone. We next explain the effects of confronted cultures on the two species' biomass and then integrate results for changes in metabolite concentration and biomass as metabolite ratios (concentration per biomass).

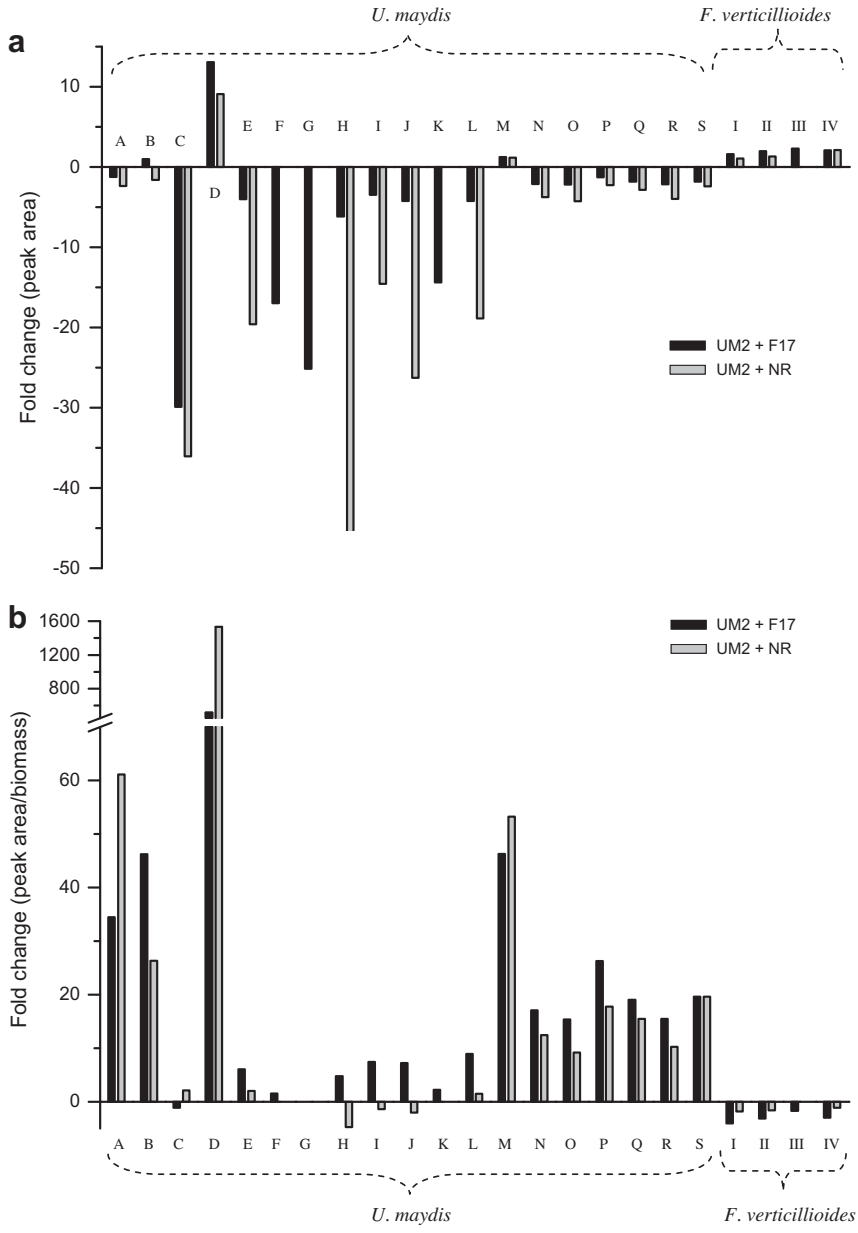

Fig. 4. Average fold changes (increase or decrease) of *Ustilago maydis* (A–S) and *Fusarium verticillioides* (I–IV) metabolites in the interaction zone (PDA medium). (a) Changes in metabolite concentrations. (b) Changes in metabolite ratios (peak area/biomass). UM2 genotype confronted with both *F. verticillioides* genotypes is shown. Metabolites F, G and K were not detected in UM2 + NR confrontations and compound III was not detected in strain NR under any growth condition.

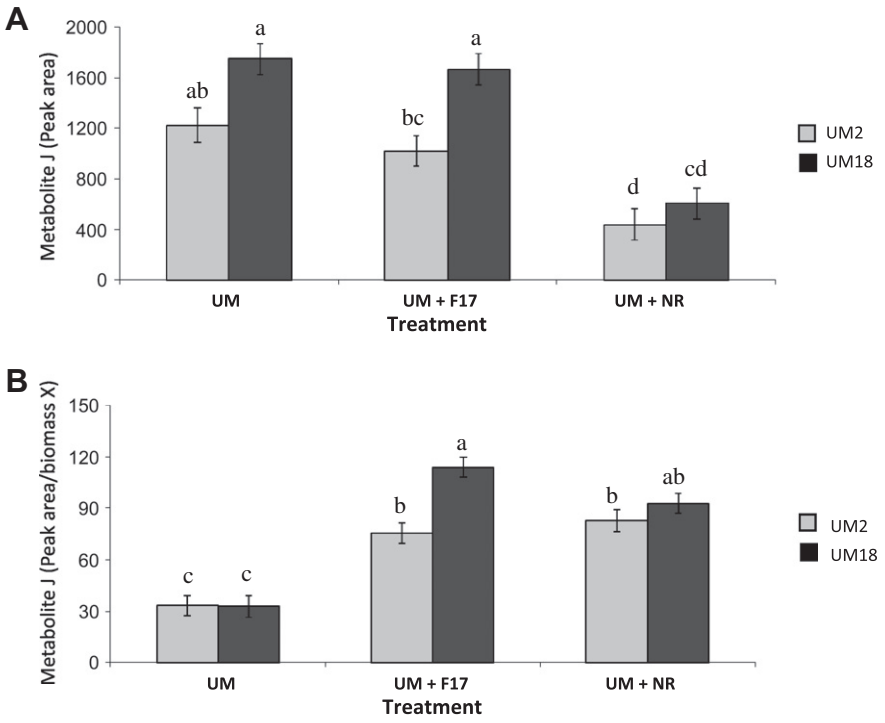

Fig. 5. Ustilagic acid A (metabolite J) produced by *Ustilago maydis* in single and confronted cultures on Czapek-Dox agar medium. (A) Peak area, (B) metabolite ratio (peak area/biomass, transformed data). Means that are statistically different ($p < 0.05$) are indicated by different letters. Error bars indicate standard error of the mean.

3.6. Biomass quantification

Standard curves were automatically calculated by the StepOnePlus™ software, and resulting amplification efficiencies, R^2 values, slopes and Y-intercepts were similar between species and among plates (Table S1).

The ANOVA results for *U. maydis* biomass (number of genome equivalents $\times 10^3$) on PDA medium showed significant effects of UM genotype, condition, and interaction of genotype and condition. On CDA medium, the condition and interaction term had significant effects on *U. maydis* biomass but not UM genotype (Table 3). *U. maydis* biomass was dramatically lower by 9- to 400-fold in confronted cultures compared to single cultures, while *F. verticillioides* biomass showed much smaller, but significant, gains of two- to fourfold in confronted cultures compared to single cultures, in both media and with either *U. maydis* genotype (Table 4). In general, *U. maydis* biomass decreased more in confronted cultures on PDA medium than on CDA medium. On either medium, UM18 apparently incurred greater losses of biomass in confronted cultures than did UM2. The *F. verticillioides* genotype NR caused greater biomass loss in *U. maydis* than did F17 (Table 4). Growth of the two *F. verticillioides* strains was similar in single culture, and both strains showed similar gains of two- to fourfold greater biomass in confronted cultures, although NR was typically slightly higher. The greater loss of *U. maydis* biomass in confronted cultures on PDA, compared to CDA, is paralleled by slightly greater growth gains by *F. verticillioides* on PDA than on CDA.

Table 3
P-values obtained from ANOVA for effects of genotype, condition and their interactions, on biomass (genome equivalents $\times 10^3$) in *Ustilago maydis* and *Fusarium verticillioides*.

Source of variation	df	PDA	CDA
Ustilago maydis			
Genotype (UM2 and UM18)	1	0.0490*	0.7258
Condition (*U. maydis* only, +F17 and +NR)[a]	2	<0.0001*	<0.0001*
Genotype × condition	2	0.0409*	0.0031*
Fusarium verticillioides			
Genotype (F17 and NR)	1	0.5510	0.8908
Condition (*F. verticillioides* only, +UM2 and +UM18)[b]	2	0.0112*	0.0156*
Genotype × condition	2	0.1930	0.1911

[a] *Ustilago maydis* only is a non-confronted culture, +F17 and +NR are *U. maydis* cultures confronted with *F. verticillioides* isolates F17 or NR.
[b] *Fusarium verticillioides* only is a non-confronted culture, +UM2 and +UM18 are *F. verticillioides* cultures confronted with UM2 or UM18.

3.7. Metabolite ratio (metabolite concentration/biomass)

For confronted cultures, we observed above that metabolite concentrations of *U. maydis* largely decreased and *F. verticillioides* metabolite concentrations increased, while *U. maydis* lost biomass and *F. verticillioides* gained biomass. To discern changes in metabolite concentrations relative to biomass, we conducted ANOVA tests for metabolite ratios, here defined as metabolite concentration/biomass. Data obtained for metabolite ratios were not normally distributed and therefore transformed through the Box–Cox transformation method (Weisberg, 2005). The ANOVA showed significant differences among treatments for all *U. maydis* metabolite ratios and effects of the genotype, condition and interactions were also significant for most metabolite ratios in either media (Tables 5 and S2). Metabolite ratios for single cultures of *U. maydis* strains UM2 and UM18 were not different from each other on PDA or on CDA media. However, in confronted cultures, metabolite ratios were significantly different between the two *U. maydis* genotypes for 12 metabolites in PDA media (data not shown) and for 11 metabolites on CDA (Fig. 5b shows ustilagic acid A as an example). We note that metabolite ratios more often obtained greater values in UM18 than in UM2 perhaps because UM2 lost less biomass than UM18 in confronted cultures.

For *U. maydis* metabolites A, B, and M through S, changes in metabolite concentration were small in confronted cultures (Fig. 4a), and thus with biomass loss, metabolite ratios (peak

Table 4
Fold changes in *Fusarium verticillioides* and *Ustilago maydis* biomass (copy genes × 10^3) in confronted cultures as compared to single culture, in PDA and CDA media.

Confronted cultures	PDA[a] biomass fold change		CDA[a] biomass fold change	
	Fusarium verticillioides	*Ustilago maydis*	*Fusarium verticillioides*	*Ustilago maydis*
F17 + UM2	+2.3	−23.9	+1.2	−8.9
F17 + UM18	+1.3	−29.2	+1.5	−35.2
NR + UM2	+2.6	−148.6	+2.1	−26.6
NR + UM18	+4.1	−404.8	+2.9	−50.9

[a] Media: PDA (Potato dextrose agar); CDA (Czapek-Dox agar).

area/biomass) increased in confronted culture (Fig. 4b).Concentrations of metabolites C, and E through L, were significantly lower in confronted cultures than in single cultures (Fig. 4a) while the metabolite ratios for these compounds show little response to confronted cultures (Fig. 4b), suggesting that their production is closely tied to the number of living *U. maydis* cells. Compounds F, G, and K were not detected in *U. maydis* cultures confronted with *F. verticillioides* strain NR indicating that NR might catabolize these more than does strain F17. Compound D increased 518 and 1533-fold during F17 and NR confrontations, respectively. These results imply that compound D is a breakdown product of ustilagic acid as discussed above, and that it accumulates in confronted cultures.

Compounds U and S, produced by *U. maydis*, might be considered as having antibiotic activity against *F. verticillioides*. Compound U is barely detected in single cultures but increases in confronted cultures, suggesting that this metabolite is "up-regulated" in response to confrontation between the two species. Interestingly, compound U increases more in the presence of F17 than with NR. For example, in PDA, concentration of compound U in UM + F17 was 11 times higher than in UM + NR. Both S and U are produced in greater amounts by UM2 than by UM18 (data not shown).

F. verticillioides metabolite production is strongly responsive to the culture medium as well as to confrontations with *U. maydis*. The four metabolites (I–IV) detected on PDA increase in concentration and decrease in metabolite ratio in confronted cultures (Fig. 4). Similarly, the two metabolites (V, VI) produced on CDA increase in concentration and decrease in metabolite ratio in confronted cultures compared to single cultures. For example, the concentration of metabolite V (fusaric acid) produced by NR is greater than that produced by strain F17, and both increase in confrontation with *U. maydis* (Fig. 3a). Metabolite ratios for fusaric acid stay the same or decrease in confrontations with *U. maydis*, likely as a reflection of greater biomass accumulation by *F. verticillioides* (Fig. 3b).

4. Discussion

Changes in secondary metabolites concentration and biomass during interactions between *U. maydis* and *F. verticillioides* were monitored using biochemical and molecular approaches developed in this work. We demonstrated that the metabolic profiling and qPCR techniques are sensitive enough to detect variation due to genotype. With one exception, *U. maydis* metabolite U, metabolites evaluated here were constitutively produced and changed in concentration, rather than having been induced by fungal confrontations, results differing from the induction of some metabolites observed for other *in vitro* fungal–fungal interactions (Haynes et al., 2007; Peiris et al., 2008).

Given the highly variable biological community that any pathogen or endophyte might encounter within a plant, it is perhaps not surprising that most metabolites produced by *U. maydis* are expressed constitutively. However, since *F. verticillioides* is common in maize, it was unexpected to find that few *U. maydis* metabolites are likely to serve in defense against *F. verticillioides*. We would expect that antibiotic defense compounds should be produced in greater amounts by strain UM2 than by strain UM18 since UM2 incurred less biomass loss when confronted with *F. verticillioides* than did UM18. Only metabolite S was produced in much greater amounts by UM2 than by UM18 in single cultures. Furthermore, concentration of compound U strongly increases in confronted cultures and it increases more in confrontation with F17 than NR. *F. verticillioides* F17 had less effect on the growth of *U. maydis*. Together, the results for compounds S and U most clearly fulfill expectations for compounds functioning as antibiotics against *F. verticillioides*.

Our results also show that previously described metabolites for *U. maydis*, the ustilagic acids (metabolites E, H, I, J), and Mel D (metabolite C) likely have functions other than antibiosis. For example, the metabolite ratio for ustilagic acid A (metabolite J) was about the same for the two *U. maydis* genotypes in single culture, and for both, increases in confronted cultures. The metabolite ratio for ustilagic acid A (compound J) was significantly higher when UM18 was confronted with F17, than when UM2 was confronted with F17. Many *U. maydis* metabolites, for example the ustilagic acids, MEL D and unidentified K, L, and T compounds, were produced in greater concentrations by UM18, the strain experiencing greater biomass loss, than by UM2. The ustilagic acids are thought to primarily function in mating recognition and not in inter-specific interactions (Hewald et al., 2005; Teichmann et al., 2007). We infer that the ustilagic acids, Mel D, and unknown compounds, K, L, and T, serve functions other than defense, at least against *F. verticillioides*. Lastly, the results for *U. maydis* metabolite D, suggest that compound metabolite D is a breakdown product of ustilagic acids. Mimee and colleagues (2009) showed that *Pseudozyma flocculosa*, a close relative of *U. maydis*, was able to catabolize flocculosin during conditions of nutrient limitation to a breakdown product very similar to compound D.

While *U. maydis* metabolites were constitutively expressed on both media, *F. verticillioides* metabolite production responded strongly to the growth medium. We expected that the more aggressive *F. verticillioides* strain, NR, would produce greater quantities of any metabolite involved in antagonism toward *U. maydis*, than would the less aggressive strain F17. On PDA, Fumonisins B1 and B2 were produced and may have limited *U. maydis* growth, as previously shown for fumonosins' effects on other fungi (Keyser et al., 1999). On CDA, fusaric acid and compound VI are also produced in greater amounts by NR than by F17 and thus may have contributed to the reduction of *U. maydis* growth during interactions on this medium.

While *F. verticillioides* is a strong antagonist of *U. maydis*, leading to decreased *U. maydis* biomass and possibly catabolizing some *U. maydis* compounds, it gains a relatively small growth advantage of only 2–4-fold. *F. verticillioides* clearly limits the growth, perhaps killing and feeding on *U. maydis* cells, and might be considered a mycoparasite. But, if so, it is a relatively inefficient parasite and

Table 5
Statistical significance obtained by ANOVA on data corresponding to *Ustilago maydis* and *Fusarium verticillioides* metabolite ratios (peak area/biomass).

	Metabolites[a]																		
	A	B	C	D	E	F[d]	G[d]	H	I	J	K[d]	L	M	N	O	P	Q	R	S	T
Ustilago maydis																				
PDA																				
Model[b,c]	***	***	***	***	***	*	**	***	***	***	***	***	***	***	***	***	***	***	***	n/d[f]
G	–	***	*	*	**	**	***	***	***	***	***	***	*	–	–	–	–	–	–	
C	***	***	***	***	***	*	–	***	***	***	***	***	***	***	***	***	***	***	***	
G * C	–	*	***	*	–	–	–	–	*	*	–	**	–	***	**	**	**	**	**	
CDA																				
Model[b,c]	***	***	***	***	***	***		***	***	***	***	***	***	***	***	***	***	***		**
G	–	**	***	–	***	***	n/d[f]	***	***	**	***	***	**	–	–	–	–	–	n/d[f]	**
C	***	***	***	–	***	–		**	**	*	**	**	–	*	**	**	*	**		*
G * C																				

	Metabolites[a]						
	I	II	III[e]	IV	V	VI	
Fusarium verticillioides							
PDA							
Model[b,c]	*	–	–	–	n/d[f]	n/d[f]	
G	*	–	–	–			
C	*	–	–	–			
G * C	–	–	–	–			
CDA							
Model[b,c]	n/d[f]	n/d[f]	n/d[f]	n/d[f]	***	–	
G					***	*	
C					–	–	
G * C					–	–	

	Metabolites[a]
	U
U. maydis + F. verticillioides	
PDA	
Model[b,c]	**
G	**
C	–
G * C	–
CDA	
Model[b,c]	*
G	**
C	–
G * C	–

[a] Metabolites correspond to those identified in Table 2.
[b] G = genotype effect (UM2 or UM18 for *U. maydis*; F17 or NR for *F. verticillioides*); C = Condition effect (*Ustilago maydis* only, +F17 or +NR for *U. maydis*; *F. verticillioides* only, +UM2 or + UM18 for *F. verticillioides*).
[c] Statistical significance: $p < 0.05$ *, $p < 0.01$**, $p < 0.001$***, – not statistically significant.
[d] *Ustilago maydis* metabolites not detected in NR interactions.
[e] *Fusarium verticillioides* metabolites detected only in F17.
[f] n/d = not detected.

specialized structures for attack of *U. maydis* have not been observed in these species' interactions. At least the endophytic forms of *F. verticillioides* apparently compete by killing and limiting the growth of *U. maydis* through production of secondary compounds, an interference mechanism that could leave more resources available to the endophyte *in planta*.

U. maydis and *F. verticillioides* commonly co-occur and affect each other's growth in maize (Lee et al., 2009). In this research, we developed biochemical and molecular techniques to detect changes in secondary compounds and biomass during fungal confrontation *in vitro*, and demonstrate that specific metabolites might be important to these interactions. Further studies will monitor secondary metabolites, biomass and gene expression of both fungi as they interact in the host.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2011.06.006.

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