

Endophytic Fungi as a Promising Biocontrol Agent to Protect Wheat from *Fusarium graminearum* Head Blight

Zachary A. Noel,¹ Ludmilla V. Roze,¹ Mikaela Breunig,¹ and Frances Trail^{1,2,†}

¹ Department of Plant, Soil, and Microbial Sciences, Michigan State University, East Lansing, MI 48823

² Department of Plant Biology, Michigan State University, East Lansing, MI 48823

Abstract

The search for beneficial endophytes that can be part of a constructed microbial community has increased in recent years. We characterized three endophytic fungi previously isolated from wheat for their in vitro and in planta antagonism toward the *Fusarium* head blight pathogen, *Fusarium graminearum*. The endophytes were phylogenetically characterized and shown to be *Alternaria destruens*, *Fusarium commune*, and *Fusarium oxysporum*. Individual fungal endophytes significantly increased seed weight and lowered the accumulation of the mycotoxin deoxynivalenol compared with *F. graminearum*-infected wheat heads without endophyte pretreatment. Investigation into the mechanism of competition in vitro showed that endophytes competitively excluded

F. graminearum by preemptive colonization and possible inhibition over a distance. Investigations on the use of these endophytes in the field are in progress. Identification of these three endophytes highlights a common quandary in searching for beneficial microbes to use in agriculture: species definitions often do not separate individual isolates' lifestyles. A greater understanding of the risks in using intraspecific variants for biocontrol is needed and should be examined in the context of the ecology of the individuals being investigated.

Keywords: cereals and grains, cultural and biological practices, endophytes, *Fusarium graminearum*, head blight

Fusarium head blight (FHB) is one of the most devastating diseases of cereals worldwide. The disease is primarily caused by the fungus *Fusarium graminearum*. The pathogen overwinters on crop residue, and perithecia release ascospores that infect wheat heads during anthesis (Leonard and Bushnell 2003; Paul et al. 2019; Trail 2009). Infected spikelets bleach, and grain is rendered small, shriveled, and discolored (Cowger and Arellano 2013). *F. graminearum* infection can result in grain contamination with the mycotoxins deoxynivalenol (DON) and its derivatives 3-acetyldeoxynivalenol (3ADON) and 15-acetyldeoxynivalenol (15ADON), the estrogen-mimic zearalenone, aurofusarin (and monomer rubrofusarin), and several others (Brown et al. 2003; Frandsen et al. 2011; Gaffoor and Trail 2006; Gaffoor et al. 2005; Kim et al. 2005). Importantly, DON also acts as a virulence factor; thus reduction of DON production may also reduce disease (Harris et al. 1999; Proctor et al. 1995). DON and derivatives affect the digestive system and organ function of humans and livestock, resulting in acute emetic effects, as well as more serious health consequences, with chronic exposure (Pestka 2010). Alternatively, *F. graminearum* can cause root rot, plant stunting, and damping-off (Broders et al. 2007). Therefore, the management of this disease is essential to food security and human health.

†Corresponding author: F. Trail; trail@msu.edu

Z. A. Noel and L. V. Roze contributed equally to this work.

Current address for Z. A. Noel: Department of Entomology and Plant Pathology, Auburn University, Auburn, AL 36849.

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Management of FHB requires an integrated approach of planting varieties with moderate or partial resistance, timely fungicide applications, and cultural practices such as crop rotation and tillage (Llorens et al. 2019; McMullen et al. 2012; Ripa et al. 2019). However, because at this time there is no complete host resistance available for *F. graminearum* (Paul et al. 2019), fungicides remain an important tool to fight this disease. Widespread reliance on fungicide applications every year may lead to fungicide resistance because variations in sensitivity have been found among *F. graminearum* isolates (Anderson et al. 2020; Ishii and Hollomon 2015; Llorens et al. 2019; McMullen et al. 2012; Ripa et al. 2019; Spolti et al. 2012; Yin et al. 2009). Hence, integrated approaches, including biocontrol, are essential for sustainable management of FHB (Llorens et al. 2019; McMullen et al. 2012; Ripa et al. 2019).

Biocontrol applications have been successfully applied to several crops, but few have targeted mycotoxin contamination. Cotty and collaborators developed the use of wild strains of *Aspergillus flavus*, which did not produce the potent carcinogen and mycotoxin aflatoxin (Adhikari et al. 2016; Atehnkeng et al. 2016; Bandyopadhyay et al. 2016; Cotty 1994). These strains occupied soil and plant niches that would be colonized by toxigenic wild strains in their absence, and the strategy is now used worldwide with locally adapted strains (Probst et al. 2011). Recent work has mainly focused on the use of endophytes, which are microorganisms that live inside plants asymptotically and can provide protection to the plant against a variety of biotic and abiotic stresses. For example, biocontrol of fungal diseases from nonpathogenic endophytic strains of *Fusarium oxysporum* has been documented (de Lamo and Takken 2020; Probst et al. 2011). *Metarhizium anisopliae* has been reported to have potential as a biocontrol agent against FHB (Hao et al. 2021) with moderate effects on disease severity on sprayed heads in the field. *Sarocladium zeae*, a wheat endophyte, has also been reported to have reduced symptoms and DON content of wheat heads in greenhouse assays, in which the mechanism is an alteration in defense signaling response to the pathogen (Kemp et al. 2020). For biocontrol in wheat, bacteria and bacterial endophytes have been tested for activity against *F. graminearum* (Llorens et al. 2019). Serenade and Sonata are commercial products that contain bioactive *Bacillus* spp. effective against some foliar diseases in wheat but are not labeled for FHB management (Matarese et al. 2012; Schöneberg et al. 2015). Although fungal antagonists against *F. graminearum* have been studied, there are no commercial fungal biocontrol options for wheat.

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The prospect of using biocontrol agents, particularly in the form of constructed microbial communities, has stimulated interest in this area. Here, three previously isolated endophytic fungi from wheat (Gdanetz and Trail 2017) were tested for their antagonism to *F. graminearum*. The objectives of this study were to (i) phylogenetically characterize the endophytes using multiple loci, (ii) assess the in vitro competition against seven isolates of *F. graminearum*, and (iii) assess the ability of the endophytes to increase seed mass and decrease mycotoxins in the presence of *F. graminearum* when applied to developing wheat heads in the greenhouse. Our results demonstrate promising but varied activities for the three endophytes.

Materials and Methods

Fungal strains and growth conditions: Wheat and millet cultivars. *Fusarium* and *Alternaria* endophytes used in this study were isolated from wheat stems or heads in 2013 and previously identified to the genus level by sequencing the internal transcribed spacer region (Gdanetz and Trail 2017). *F. graminearum* isolates used in in vitro competition experiments were collected as indicated in Table 1. *F. graminearum* isolate PH-1 (FGSC 9075; NRRL 31084) (Trail and Common 2000) was the first *F. graminearum* strain to have its genome sequenced (Cuomo et al. 2007) and has been used worldwide to study head blight disease. PH-1 is only known to produce DON and 15-ADON forms of the trichothecene deoxynivalenol (Alexander et al. 2011). The other six isolates were chosen from a survey in Michigan to represent a diversity of locations (Breunig et al., unpublished data). These isolates were confirmed to be *F. graminearum* by sequencing of translation elongation factor 1- α (TEF1- α). Unless otherwise noted, all isolates were stored at -80°C as colonized malt extract agar (MEA) blocks in 35% glycerol and were commonly grown on 2% MEA medium. The spring wheat cultivar Wheaton is a susceptible variety that was used throughout the study. Winter wheat cultivar Ambassador, a scab-susceptible variety, was used to test the effects of the endophytes for protecting head infection of this type of wheat. Proso millet (*Panicum miliaceum*) was used for generating inoculum for plant inoculation (Gdanetz and Trail 2017).

Molecular identification of endophytes. Endophytes were grown on MEA for 5 days and then three plugs were transferred to Erlenmeyer flasks containing 100 ml of yeast extract peptone dextrose broth. Mycelium was harvested after 3 days of growth in yeast extract peptone dextrose broth, and 50 mg was ground with 0.08 ml of lysing matrix A (MP Biomedicals, Houston, TX) and a 4-mm diameter steel ball (SPEX SamplePrep, Metuchen, NJ) using a Fast-Prep FP120 (Thermo Fisher Scientific, Waltham, MA), and genomic DNA (gDNA) was extracted using the DNeasy plant Mini Kit (Qiagen Sciences Inc., Germantown, MD) following the manufacturers' instructions. The two endophytes in the *Fusarium* genus were identified to species level by amplifying and sequencing the TEF1- α along with the two subunits of the RNA polymerase II gene (RPB1 and RPB2). TEF1- α was amplified using the primers EF1 and EF2. RPB1 was amplified and sequenced in two overlapping segments using the primer pairs Fa with R8 and F7 with R9 (Hofstetter et al. 2007; O'Donnell et al. 2010). The first half of RPB2 (RPB2-1) was amplified and sequenced using the primer pair 5f2 and 7cr (Liu et al. 1999; Reeb et al. 2004). The second half of RPB2 (RPB2-2) was amplified and sequenced using the primer pair 7cf and 11ar. The

endophyte identified within the *Alternaria* genus was identified by amplifying the plasma membrane ATPase and calmodulin genes, as suggested by Lawrence et al. (2013). Primers for the ATPase were the ATPDF1 and ATPDR1. The calmodulin gene was amplified using the primer pairs CALDF1 and CALDR1. Primer sequences can be found in Supplementary Table S1. PCR consisted of a final concentration of 1 \times Phusion Green HotStart II High-Fidelity DNA Polymerase (Thermo Fisher Scientific) mix containing 0.5 μM forward and reverse primers, for each respective locus, and 10 to 40 ng of genomic DNA. Thermal cycling conditions for EF1- α , RPB2-1, and RPB2-2 were as follows: 98°C for 30 s, followed by 35 cycles at 98°C for 10 s, 59°C for 30 s, and 72°C for 1.5 min, followed by a final extension at 72°C for 7 min. RPB1 was amplified using the same thermal cycling conditions except for the annealing temperature at 57°C (Fa and R8) or 61°C (F7 and R9). Amplicons were separated by gel electrophoresis, and successfully amplified amplicons were purified via gel extraction with the Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI) or by adding 3 U of exonuclease I and 0.5 U of shrimp alkaline phosphatase (Thermo Fisher Scientific) and incubating at 37°C for 45 min followed by 85°C for 10 min to inactivate enzymes. Amplicons were Sanger-sequenced at the Michigan State University Genomics core facility (<https://rtsf.natsci.msu.edu/genomics/>). Forward and reverse sequences were trimmed and assembled with Codon Code Aligner version 4.2.7, and consensus sequences were compared against a curated set of *Fusarium* species using the CBS-KNAW Fungal Biodiversity Centre's *Fusarium* Multilocus Sequence Typing database (<https://fusarium.mycobank.org/>).

Fusarium RPB1 and RPB2 and *Alternaria* ATPase and calmodulin gene sequences were used for phylogenetic analyses. RPB1 and RPB2 sequences of *Fusarium* endophytes were aligned to sequences from *Fusarium* species within the Gibberella clade (O'Donnell et al. 2013) using MUSCLE version 3.8.31. *Alternaria* ATPase and calmodulin gene sequences were aligned to *Alternaria* species within sections *Alternaria*, *Sonchi*, and *Alternantherae* (Lawrence et al. 2013) using MUSCLE version 3.8.31. The Markov Chain Monte Carlo algorithm was implemented in MrBayes version 3.2.6 (Ronquist et al. 2012) to generate a Bayesian phylogeny using the combined RPB1 and RPB2 or ATPase and calmodulin alignments, treating each gene sequence as a separate partition. The Markov Chain Monte Carlo algorithm was run with a GTR+I+G molecular model of evolution, 5,000,000 generations, trees sampled every 1,000 generations, and a 25% burn-in.

Competition of endophytes in vitro. Colonies of the six *F. graminearum* isolates and endophytes were cultured separately on MEA in constant light. After 5 days, a 5-mm plug of each was placed mycelial side down 3 cm apart on MEA agar medium within 100-mm Petri dishes. Dual cultures were incubated in the dark at 25°C for 18 days. Photographs were taken of each Petri dish at 4, 11, 14, and 18 days post-inoculation (dpi). A 1-cm $^{-2}$ calibration card was included within each image to convert pixels to distances within the image analyzer Fiji (Schindelin et al. 2012). The area occupied by *F. graminearum* was estimated from the images by tracing the colony area. Petri dishes containing only *F. graminearum* (no endophyte) served as a negative control.

To test for the effect of potential endophyte volatiles on the growth of *F. graminearum*, the pathogen and endophytes were grown physically separated on MEA medium, as described above, and a 5-mm plug of each was placed mycelium side down on separate 60-mm Petri dishes containing MEA medium. Two Petri dishes containing agar medium with endophytes and one Petri dish containing agar medium with *F. graminearum* were placed within an empty 150-mm Petri dish. Fungi were incubated within the larger Petri dishes in the dark at 25°C for 6 days. Images were taken 6 dpi, and the colony area was estimated as described above.

Preparation of inoculum for soil infestation. The inoculum was prepared by growing *F. graminearum* PH-1 in spawn bags containing moistened seeds of proso millet. Briefly, white millet seeds (1 kg) were covered with sterile water and allowed to imbibe for 24 h at room temperature, after which they were distributed into two spawn bags (ECAB2430; Fungi perfecti, Olympia, WA),

Table 1. *Fusarium graminearum* isolates used in this study

Isolate ID	Year collected	Origin	Host	County
107M	2017	Wheat head	Wheat	Presque Isle
107H	2017	Wheat head	Wheat	Presque Isle
24A	2016	Wheat head	Wheat	Huron
76J	2017	Wheat head	Wheat	Lenawee
76O	2017	Wheat head	Wheat	Lenawee
93D	2017	Wheat head	Wheat	Sanilac
PH-1	1995	Corn stalk	Corn	Ingham

and sterilized for 45 min at 121°C in an autoclave. Millet was allowed to cool and stored at 4°C until use. *F. graminearum* conidia (10^4 conidia in 100 µl of water per plate) were spread onto Petri dishes (100 × 15 mm) containing potato dextrose agar (Difco; Becton, Dickinson and Co., Sparks, MD) with chloramphenicol (0.17 mg ml⁻¹) and erythromycin (0.25 mg ml⁻¹) and grown for 7 days at room temperature in constant fluorescent light. One Petri dish colonized with *F. graminearum* PH-1 culture was combined with one plate of fresh potato dextrose agar and 130 ml of distilled water then homogenized in a sterilized glass blender. The slurry (120 ml) was then poured into a spawn bag containing the sterilized millet. The bag was sealed, the contents were mixed, and it was left under continuous fluorescent light at room temperature for 10 days. After incubation, the contents of the bag were air-dried in a flow cabinet at room temperature for 3 days and then maintained at 4°C until use.

Pretreatment of wheat seeds with endophytes. Agar plugs colonized with a single endophyte were placed in the center of MEA Petri dishes (60 × 15 mm) and grown under continuous fluorescent light for 4 to 5 days. Seeds were surface-sterilized first by soaking in 95% ethanol for 10 s, rinsing with sterile water three times, soaking in 0.4% sodium hypochlorite containing 0.01% Tween-20 for 3 min, and finally rinsing three times with sterile water. Surface-sterilized wheat seeds were placed on the edges of the colonies and incubated for 3 days; control seeds were placed on MEA, which did not contain an endophyte. Germinated seeds were planted one seed per cone-tainer (50 ml; Steuwe and Sons, Inc., Tangent, OR) containing an equal mix of nonautoclaved potting soil (Suremix Perlite, Michigan GrowerProducts, Inc., Galesburg, MI) and field soil (from an agricultural field in Mason, MI) with or without 5% *F. graminearum* PH-1 inoculum.

Treatment of wheat heads with endophytes and *F. graminearum*. Wheat plants of the cultivar Ambassador were vernalized before planting in the greenhouse. To vernalize wheat seeds (400 counts) of cultivar Ambassador, seeds were surface-sterilized for 8 min in 2.5% sodium hypochlorite amended with 20% SDS and then washed with sterile distilled water five times. The sterilized seeds were spread on sterile filter paper soaked with sterile distilled water and placed on the bottom of sterilized plastic boxes and covered with a lid. The boxes were placed into a vernalization chamber (10°C, supplemental lighting) located in the AgFarm Complex, Michigan State University. After 9 weeks, the healthy seedlings were transferred to the greenhouse. Wheat plants of both cultivars (four per 9-inch pot) were grown in the greenhouse at 21 to 22°C with supplemental lighting and inoculated as previously described (Guenther and Trail 2005). Conidia were produced by growing each endophyte on MEA for 1 week and then rubbing off the conidia with a bent glass rod and rinsing with 0.05% Tween 20 in water that was collected from the plate and centrifuged to separate spores from solution. The supernatant was removed, and spores were suspended in 35% glycerol to 2.8×10^5 conidia ml⁻¹. Approximately 4 to 5 weeks after planting, wheat heads were at the beginning of anthesis (defined as 50% heads producing visible anthers) and were pretreated with endophytes by pipetting 30 µl of fresh conidia on the rachis, between florets, and on awns. The heads were covered with a glassine pollination/grain bag (Canvasback G27, Seedbuco Company, Des Plaines, IL) to keep humidity high. After 6 days, the heads were infected with *F. graminearum* PH-1 by pipetting 10 µl of conidia (1.5×10^5 conidia ml⁻¹ in 35% glycerol) between the lemma and palea of a single floret central per head. Wheat heads were similarly inoculated with 35% glycerol without fungi to serve as negative controls, and heads inoculated with *F. graminearum* PH-1 served as a positive control. Watering of plants was stopped after 4 weeks, when the seeds were filled, to allow the seed to dry. After 3 additional weeks, the mature seeds were harvested from each head, air-dried, and weighed. Seed weight (i.e., average seed weight per head) was analyzed with a linear mixed model with the treatment as a fixed effect and biological replicate as a random factor. Additionally, seeds were analyzed for mycotoxins.

Mycotoxin quantification in wheat seeds. Determination of DON and 15-ADON concentration in seeds from greenhouse trials was conducted by Dr. Yanhong Dong at the Mycotoxin Diagnostic

Laboratory and extracted in the Department of Plant Pathology, University of Minnesota, St. Paul, using gas chromatography-mass spectrometry as described by Mirocha et al. (1998). Mycotoxin data were analyzed with a Kruskal-Wallis test followed by pairwise comparisons using the Wilcoxon rank-sum test with a Benjamini-Hochberg *P* value adjustment to control the false discovery rate.

Statistical analysis and data availability. All statistical analysis was conducted in R version 3.5.2 using the packages 'lme4' (Bates et al. 2015) and 'emmeans' (Lenth 2016). All data and R code used in this manuscript are available on GitHub (<https://github.com/noelzach/EndophyteBiocontrol>) or upon request. TEF1- α , RPB1, RPB2, ATPase, and calmodulin gene sequences were deposited in GenBank under accession numbers MW917147-MW917154.

Results

Molecular identification of endophytes. Three endophytes isolated from wheat microbiomes and previously shown to have antagonistic effects toward *F. graminearum* were phylogenetically characterized. The ATPase gene (1,194 bp) from *Alternaria* endophyte #37 had a 99.83% match with 100% coverage to *Alternaria destruens* CBS 121454, which is the only sequence available for this species. The calmodulin gene (776 bp) had a 1 bp mismatch and 100% coverage to *A. destruens* CBS 121454 and *Alternaria lini* CBS 106.34. According to Woudenberg et al. (2015), both species have been synonymized with *Alternaria alternata*. However, in this manuscript, we refer to *Alternaria* endophyte #37 as *A. destruens*. This name is corroborated with the phylogenetic placement because *Alternaria* endophyte #37 is grouped in a well-supported (posterior probability 1.0) clade with *A. destruens* CBS 121454 (Fig. 1A). Bayesian phylogeny of *Alternaria* ATPase and calmodulin genes with *Alternaria* endophyte #37 are shown in Figure 1A.

Based on RPB1 and RPB2 sequences, *Fusarium* endophyte #70 grouped with *F. oxysporum* NRRL 34936 in a well-supported (posterior probability = 1.0) monophyletic group (Fig. 1B). In terms of sequence identity, *Fusarium* #70 RBP2 sequence (1,887 bp) had a 99.75% match with 97.35% overlap to *F. oxysporum* NRRL 1943. The RPB1 sequence (1,470 bp) had a 99.66% match with a 100% coverage to *F. oxysporum* NRRL 20433. Additionally, the EF1- α sequence (684 bp) had a 99.27% match and 99.56% overlap with *F. oxysporum* NRRL 1943.

For *Fusarium* endophyte #40, the RPB1 sequence exactly matched (100% identical, 100% coverage) *F. commune* NRRL 28387. The RPB2 sequence for #40 had a 99.76% match with 92.73% overlap with two *F. commune* isolates (NRRL 13816 and NRRL 28058). Additionally, the EF1- α sequence had an exact match (100% identical, 100% overlap) to two *F. commune* isolates (NRRL 28058 and NRRL 13816). The identification of #40 as *F. commune* was corroborated by phylogenetic placement because it grouped in a well-supported clade (posterior probability = 1.0) with *F. commune* NRRL 28387 (Fig. 1B). Hereafter, we refer to the endophytes in this study as *F. commune* #40, *F. oxysporum* #70, and *A. destruens* #37.

Interactions of endophytes with *F. graminearum*. In vitro competitions between the three wheat endophytes and six *F. graminearum* isolates were tested in one assay allowing physical contact and another assay without physical contact. The assay with physical contact was designed to test competition via preemption of unoccupied space, whereas the assay without physical contact tested reduction in the growth of *F. graminearum* via volatile production. The percentage of surface area in the culture occupied by *F. graminearum* was significantly lower ($P < 0.001$) in the presence of the endophytes than without them (Fig. 2A; Supplementary Table S2). The two *Fusarium* endophytes (*F. commune* #40 and *F. oxysporum* #70) restricted the area occupied by *F. graminearum* isolates by 36.5 to 42.6% after 14 dpi (Fig. 2A). The mean area occupied on the Petri dish of the six *F. graminearum* isolates tested in the presence of *F. commune* #40 or *F. oxysporum* #70 14 dpi was 12.7 ± 1.06 mm² and 12.1 ± 1.11 mm², respectively, compared with 41.1 ± 3.13 mm² for *F. graminearum* without endophytes present. *A. destruens* #37 significantly restricted the growth of *F. graminearum*, albeit to a lesser extent (18.2 ± 1.42 mm²), but similarly preemptively

occupied space on the Petri dish (Fig. 2A). However, growth restriction was not observed when *F. graminearum* and endophytes were physically separated, indicating that potential volatiles produced by the endophytes did not contribute to competition at a distance (data not shown). *F. commune* #40 and *A. destruens* #37

inhibited the growth of the initial colony of *F. graminearum* before the endophytes reached the colony edges. Furthermore, in the presence of *F. oxysporum* #70, growth of the *F. graminearum* colony resembled that of PH-1 without an endophyte (Fig. 2B). Continued growth restriction by the former two endophytes on all sides

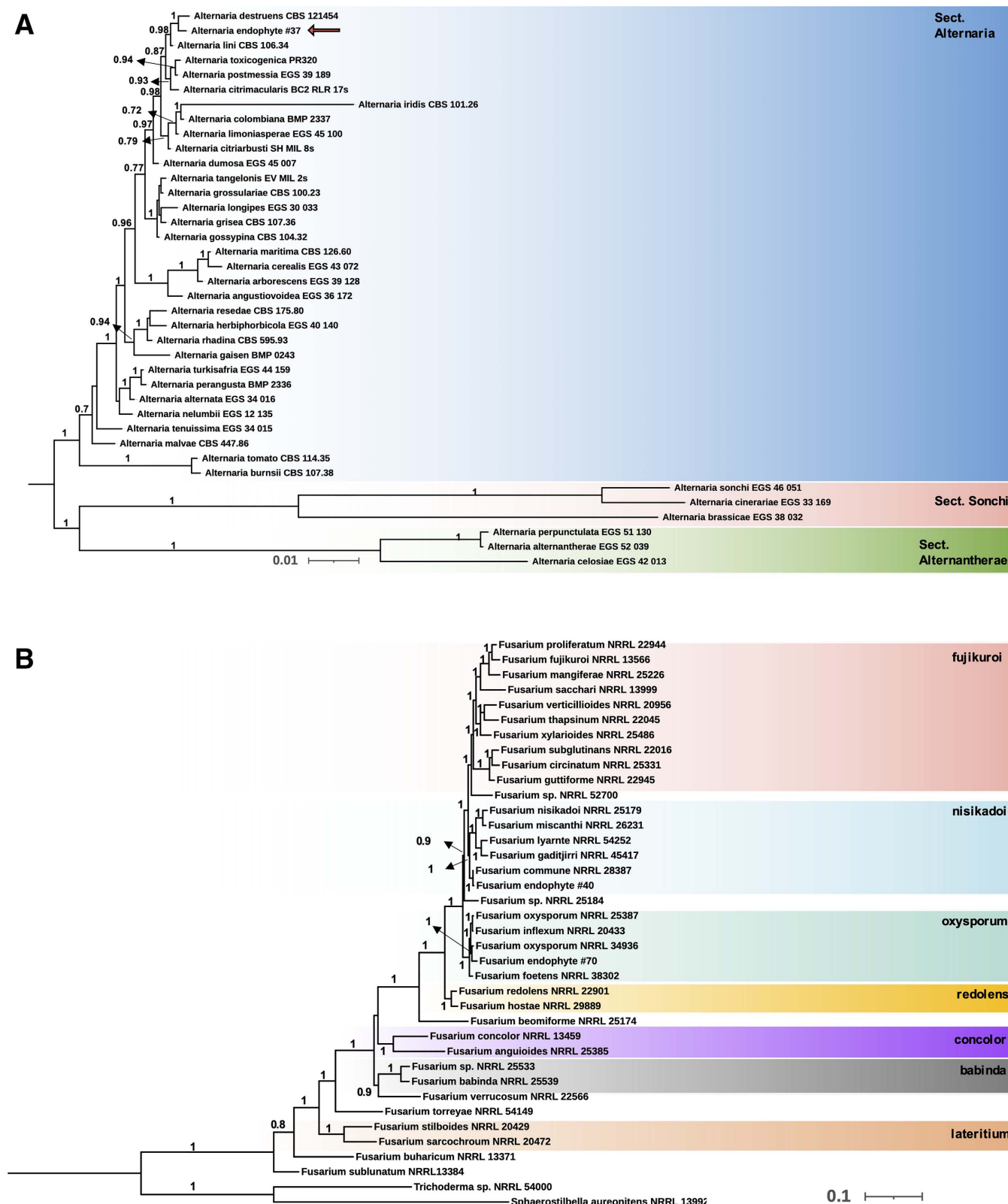


Fig. 1. Identification of wheat endophytes using phylogenetics. **A**, Bayesian phylogeny of *Alternaria* ATPase and calmodulin genes from Lawrence et al. (2013) with *Alternaria endophyte* #37 (*Alternaria destruens*) included (red arrow). **B**, Bayesian phylogeny of RPB1 and RPB2 genes from the *Fusarium* Gibberella clade obtained from O'Donnell et al. (2013) with *Fusarium endophyte* #40 (*Fusarium commune*) and *Fusarium endophyte* #70 (*Fusarium oxysporum*) (red arrows). Bifurcations with posterior probability <0.7 do not show the support values.

defines a smooth-edged PH-1 colony. Additionally, morphological observations on the interaction between *F. commune* #40 and *F. graminearum* indicated that upon physical contact, a proliferation of *F. commune* #40 hyphae was observed at the juncture along with an increase in the red pigment observed at 18 dpi compared with *F. commune* #40 grown without *F. graminearum* (Fig. 2B). Seven isolates were tested, and all seven *F. graminearum* isolates were restricted in growth similarly.

Effects of pretreatment of wheat heads and roots with endophytes.

Because the endophytes were able to preemptively occupy noncolonized portions of the medium and restrict further growth of all six *F. graminearum* isolates, we tested whether these endophytes could similarly restrict the growth of *F. graminearum* PH-1 and reduce disease severity and mycotoxin contamination on wheat heads treated during anthesis. Seed mass after infection from *F. graminearum*

PH-1 was significantly greater when *F. commune* #40 ($P < 0.001$) or *A. destruens* #37 ($P = 0.0023$) endophytes were applied to Wheaton but not Ambassador wheat heads before inoculation of the pathogen (Table 2). For Wheaton, precolonization with *F. commune* #40 resulted in near doubling of seed weight when compared with *F. graminearum* PH-1-inoculated heads without preinoculation of endophytes. However, the mean seed weight of *F. graminearum* PH-1-infected heads preinoculated with any of the endophytes was still significantly lower than control heads without inoculation (Table 2). All three endophytes significantly reduced DON and 15A-DON levels in the seeds of both wheat cultivars compared with the *F. graminearum* PH-1-infected heads (Table 2). There was no evidence that endophytes reduced disease severity on roots or increased plant biomass in Wheaton (Supplementary Fig. S1).

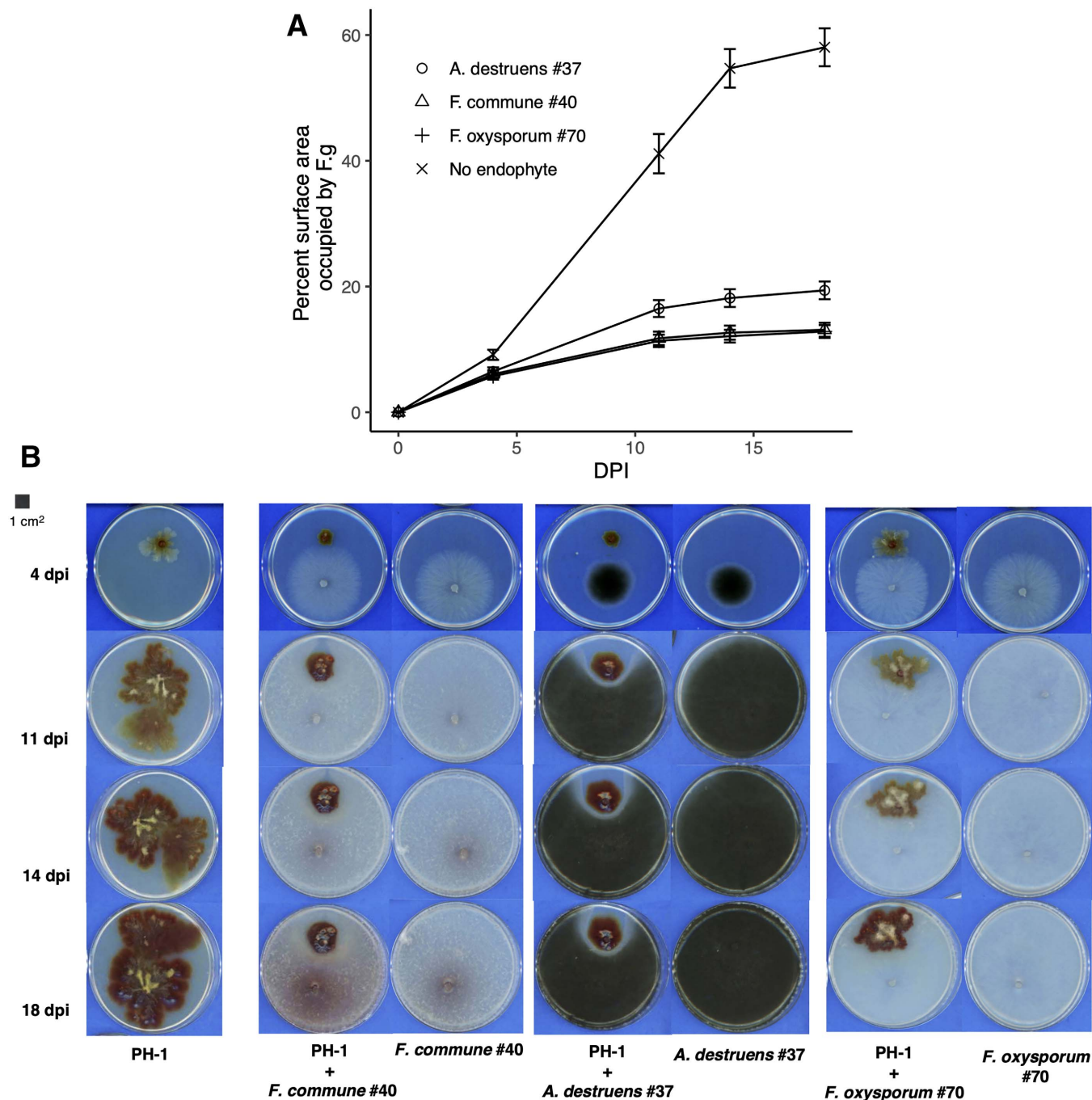


Fig. 2. Endophytes preemptively restrict the growth of *Fusarium graminearum* in vitro by occupying space and restricting spread over time. **A**, Mean growth restriction of six *F. graminearum* isolates occurs after physical contact via endophyte preemptive colonization ($n = 9$). **B**, Colonization restriction of *F. graminearum* PH-1 over time by the three endophyte isolates used in this study.

Table 2. Seed mass and mycotoxin concentration after infection with *Fusarium graminearum* and protection of endophytes

Treatment	Wheaton			Ambassador		
	Mean seed mass per seed (mg) \pm SE ^y	DON (mg/kg) ^z	15-ADON (mg/kg)	Mean seed mass per seed (mg) \pm SE	DON (mg/kg)	15-ADON (mg/kg)
Control	29.2 \pm 2.40 a	0 a	0 a	37.5 \pm 0.92 a	0 a	0 a
Fg	11.3 \pm 0.90 d	87.6 \pm 18.8 b	2.7 \pm 0.437 b	21.4 \pm 1.50 b	38.7 \pm 7.18 b	1.8 \pm 0.221 b
Fg + <i>Alternaria destruens</i> #37	18.1 \pm 1.44 bc	25.3 \pm 5.30 c	0.8 \pm 0.170 c	24.1 \pm 1.51 b	21.7 \pm 3.76 c	0.8 \pm 0.121 c
Fg + <i>Fusarium commune</i> #40	21.8 \pm 1.69 b	17.6 \pm 4.30 c	0.7 \pm 0.174 c	22.6 \pm 1.22 b	18.8 \pm 3.30 c	0.7 \pm 0.118 c
Fg + <i>Fusarium oxysporum</i> #70	16.1 \pm 1.10 cd	19.2 \pm 4.58 c	0.7 \pm 0.165 c	20.8 \pm 1.41 b	27.9 \pm 4.89 c	0.8 \pm 0.108 c

^y Means followed by the same letters within columns are not significantly different according to Tukey's honest significant difference ($\alpha = 0.05$). SE, standard error.

^z Means followed by the same letters within columns are not significantly different according to pairwise Wilcoxon ranked sum test with Bonferroni correction ($\alpha = 0.05$). DON, deoxynivalenol; 15-ADON, 15-acetyldeoxynivalenol.

Discussion

In wheat and barley, the development of robust control of FHB is a critical goal because plant resistance fails in the presence of highly conducive environmental conditions, and chemical control may not be a reliable long-term solution because of fungicide resistance. Here, three endophytes previously isolated from wheat (Gdanetz and Trail 2017) were characterized phylogenetically and for their abilities to attenuate FHB and accumulation of mycotoxins in greenhouse studies. Mycotoxin-reducing and antifungal (*F. graminearum*) effects of these endophytes were consistently observed. In addition, seed mass was significantly increased by two of the three endophytes when they were introduced to the developing wheat head before inoculation with the pathogen. The endophytes preemptively colonized unoccupied space and restricted the growth of *F. graminearum* in Petri dish assays, which correlated with the colonization of wheat heads in the greenhouse. Taken together, these results suggest that wheat endophytes are a promising biocontrol for FHB.

The endophytes identified in this study were associated with three species that include common pathogens and biologicals (*F. oxysporum*, *F. commune*, and *A. destruens/alternata*). *F. commune* was previously reported as an endophytic species for biocontrol because it is effective at protecting rice flowers from *F. fujikuroi* (Saito et al. 2021). *A. destruens* is registered as a biocontrol agent of parasitic plants, such as dodder (U.S. Environmental Protection Agency 2005), and is used as a spray and granular application to the soil. Nonpathogenic *F. oxysporum* strains have been identified and used to varying degrees as biocontrol agents (Constantin et al. 2019; de Lamo and Takken 2020), and there is some concern over the possible transfer of virulence genes between nonpathogenic and pathogenic strains when they are used in the field (de Lamo and Takken 2020). There is the possibility that these endophytic strains are latent pathogens under certain environmental conditions. However, we did not notice any pathogenic capabilities of these endophytes under repeated inoculations of wheat seeds, wheat heads, or wheat roots. Additionally, in the summer of 2020, we did attempt a field trial with two of these endophyte strains, but results were inconclusive because of lack of conditions favorable for FHB development in the field (data not shown). However, there was no obvious indication that these endophyte strains were pathogenic in field conditions. Future studies can address field efficacy of these endophytes.

Other strains of these endophyte species are known to produce mycotoxins, including beauvericin, bikaverin, enniatin (*F. oxysporum*) and beauvericin, bikaverin, and moniliformin (*F. commune*; O'Donnell et al. 2013), although no report was found for mycotoxin production by *A. destruens*. These results highlight a common quandary in the search for beneficial microbes to use in agriculture: species definitions often do not separate the lifestyles of individual isolates. Furthermore, confusion and alarm can be generated when these strains are used in the public sector. A greater understanding of the risks in using intra-species variants for biocontrol is needed, as is an increased understanding by users and public sectors.

Competition for space among fungi in plant tissue is an important part of biocontrol efficacy. Fungi compete for space by various mechanisms (Sridhar 2019). For example, fungi can produce volatile or diffusible compounds that inhibit the growth of competitors without physical contact. Fungi can restrict the growth of competitors by preemption of unoccupied space, thus impeding the competitor's growth over time, with the atoxigenic *Aspergillus* species mentioned previously as an example. Lastly, a parasitic relationship can result in a replacement of the competitor's mycelium after physical contact (Rodriguez et al. 2009). Growth of *F. graminearum* PH-1 in culture was inhibited in the presence of *F. commune* #40 and *A. destruens* #37 but not in the presence of *F. oxysporum* #70. Growth restriction was not observed when PH-1 was grown in the presence of endophytes with physical separation. These results indicate that soluble materials are likely to be inhibiting *F. graminearum* colony expansion but that potential volatiles produced by the endophytes did not contribute to competition at a distance.

The red pigment seen in the cultures produced by *F. graminearum* is likely to be aurofusarin/rubrafusarin (AUR), a monomer/dimer mycotoxin known to affect egg quality in chickens (Dvorska et al. 2001; Medentsev et al. 1993), but it also possesses some antimicrobial activity (Sondergaard et al. 2016). AUR is induced in some media, such as MEA (as seen in Fig. 2B) and often is seen in wheat and corn crop residues. In culture, aurofusarin has been shown to be negatively correlated with vegetative growth. When aurofusarin production is arrested with gene knockouts of the regulating transcription factor GIP2, mycelia grow faster. When GIP2 is overexpressed, aurofusarin is overproduced and mycelial growth is slower than wild-type (Kim et al. 2006). In knockouts of the polyketide synthase gene (FGSG02324; *pkS12*), reports of mycelial growth rate have shown an increase in strain 3639 (Kim et al. 2006) or similarity to PH-1 wild-type (Gaffoor et al. 2005). In these situations, a general antimicrobial agent would be beneficial to the fungus to protect fruiting bodies, overwintering hyphae in plant residue, and hyphae in colonized plant tissue. The use of MEA (Fig. 2) for in vitro competition illustrates the limits of certain media for these kinds of assays. Because PH-1 generates copious amounts of AUR and has uneven growth on this medium, it is difficult to speculate the reaction to the endophytes on V8 juice or carrot medium when AUR is not induced during initial colonization.

The prospect of using biocontrol agents, particularly in the form of constructed microbial communities, has stimulated interest in characterizing the endophytes used in this study. A corollary to this goal is the ability to use fungicides and protective microbial communities in the same crop. Using a combination of approaches requires ingenuity and careful management of both strategies, at least until microbial protection is robust enough to obviate the need for fungicides.

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