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# Interactions between fungal endophytes and pathogens isolated from the invasive plant kudzu (Pueraria montana var. lobata)

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**ABSTRACT**

In this study, we examined the *in vitro* interactions between fungal endophytes and pathogens isolated from the invasive plant kudzu (*Pueraria montana* var. *lobata*) and test if endophytes might facilitate pathogen growth. This represents a required initial step towards the development of candidate fungal inocula that can aid in the suppression of kudzu. While most tested endophyte-pathogen assays suggest antagonism and/or competitive exclusion, we identified several pathogen/endophyte combinations that suggest pathogen facilitation. Additional work is needed to test if these may have *in planta* effects on phytopathogenicity. The present study accentuates on the potential of fungal endophytes as an effective ecological approach to manage invasive plants *via* pathogens facilitation.

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## Introduction

Invasive plants can pose serious threats to the environment by altering local biodiversity, hydrology, fire regimes (Brooks et al. 2004), as well as soil nutrient cycling (Jurskis 2012). These ecosystem modifications can facilitate additional invasions (Arim et al. 2006; Qi et al. 2014) and can mediate shifts in soil properties by modulating biogeochemical cycling dynamics (Rout et al. 2013) and *via* allelopathy (Callaway et al. 2008). These combined effects can further facilitate plant establishment and expansion (Gibbons et al. 2017).

A major invasive plant of concern in the United States is kudzu (*Pueraria montana* var. *lobata*), which is fast growing and its twining

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vines undergo rapid elongation (as fast as 0.3 m per day and 18 m per year) (Gulizia and Downs 2019). It is highly invasive in the United States and has a widespread ranged across the eastern United States with the heaviest infestations in the states of Alabama, Georgia and Mississippi (Miller 1996; Harrington et al. 2003; Gulizia and Downs 2019). Kudzu is a major contributor to local pollution through emission of large quantities of both nitric oxide and isoprene, which contributes to ground-level ozone pollution (Hickman et al. 2010). Further, kudzu is considered a major reservoir of leguminous pathogens (Jordan et al. 2010). For example, Asian Soybean rust, a major disease of concern for United States soybean producers, is caused by the fungal pathogen *Phakopsora pachyrhizi* and was first observed on kudzu in the United States in 2005 (Hershman et al. 2006). Kudzu may have been instrumental in the rapid spread of Asian soybean rust across the United States by acting as an alternative host. The combined cost of kudzu infestations to the United States forestry, railroads, agriculture and power line industries likely exceeds USD $1 billion per year (Lindgren et al. 2013).

Because of the rapid growth and regrowth of kudzu and the fact that it has deep tap roots with high starch levels, eradicating kudzu is extremely difficult. Kudzu can be treated with varied success with persistent (multi-year) herbicide treatments, livestock grazing, prescribed burning and disk harrowing (Harrington et al. 2003). However, control through chemical means can take more than 10 years of repetitive herbicide applications to induce mortality (Boyette et al. 2002). Even for the most extensive control programs, absolute kudzu mortality is difficult to achieve (Weaver et al. 2016). In addition, there are often concerns from landowners and the public, even if largely unfounded, about the safety and residency time of herbicides and how this persistent use may impact soil, environmental conditions, or human health outcomes (Berisford et al. 2006; Vincent and Davidson 2015; Van Bruggen et al. 2018; James et al. 2022).

Consequently, there has been an increased interest in biological control of invasive plants (Pedras et al. 2003; Boyette et al. 2011; Boyette and Hoagland 2015; Shahrtash and Brown 2020; 2021). Successful biological control agents may be able to provide long term control while reducing dependence on herbicides (Harding and Raizada 2015). Biological control of kudzu using phytopathogens has been actively studied with the fungal foliar pathogen *Albifimbria verrucaria* (Ascomycota, Stachybotryaceae; formally *Myrothecium verrucaria* (Lombard et al. 2016)), namely *A. verrucaria* strain CABI-IMI 368023 (Weaver et al. 2021, 2022), being the most promising to date. *A. verrucaria* has been evaluated for use as a stand-alone treatment as well as a part of integrated control programs

(Boyette et al. 2002; Weaver et al. 2009). However, infection of *A. verrucaria* on kudzu is not always successful. While little research has been done on kudzu as a pathosystem, a survey in its native range identified several fungal pathogens and diseases on kudzu (Sun et al. 2006) including *Synchytrium puerariae;* the causative agent of imitation rust, *Pseudocercospora pueraricola*, the causative agent of angular leafspot; *Cercospora puerario-thomsona*, the causative agent of brown spot; *Phomopsis* spp., *Colletotrichum lindemuthianum* and *Colletotrichum* spp., causal agents of anthracnose (Sun et al. 2006).

Fungal competition within a plant is rampant (Rodriguez et al. 2009) and the outcome of these interactions are achieved using numerous strategies including competition for space and nutrients, secretion of secondary metabolites and/or mycoparasitism (Zabalgogeazcoa 2008; Spadaro and Droby 2016; Barge et al. 2022). This competitive framework has been examined mainly to identify taxa that may suppress or modulate diseases causative pathogens (Padder and Sharma 2011; Singh 2014; Busby et al. 2016; Wisniewski et al. 2016; Deketelaere et al. 2017; Zhang et al. 2017), but emerging research has also pointed to the exacerbation of pathogenicity (Zelezniak et al. 2015; Busby et al. 2016; Parratt and Laine 2016; Shahrtash and Brown 2021). Further, there has been an increased interest in examining the role fungal endophytes play within plant pathosystems. Fungal endophytes are taxa that are present asymptomatically either within cells, in intercellular spaces, or in the vascular system within plant tissue (Stone et al. 2000; Kandel et al. 2017). Fungal pathogens are a well-documented tool to control invasive plants. There are several fungal genera that have been demonstrated to aid in the suppression of invasive plants including the necrotrophic genera *Penicillium* (Ismaiel and Papenbrock 2015), *Alternaria*, *Botrytis*, *Fusarium*, *Helminthosporium* and *Phoma* (Xu et al. 2021), and those with phytotoxic metabolites including *Sclerotinia*, *Alternaria*, *Chondrostereum* and *Puccinia*, among others (Harding and Raizada 2015; Xu et al. 2021). By incorporating a taxa interaction framework to facilitate increased pathogenicity, new control options for invasive plant management can be identified (Bailey 2014; Boyette and Hoagland 2015; Shahrtash and Brown 2021). While endophytic and bioherbicide inoculations for the control of invasive plants are promising as a sustainable management tool, the development and utilization is not without challenges. In addition to environmental safety and concerns about the longevity and bioherbicide efficacy (Triolet et al. 2020), maintaining host specificity of these bioherbicides is a matter of concern (Stastny and Sargent 2017).

Towards the goal of developing a pathogen-endophyte system to aid in the control of kudzu, this study takes the first step. We developed the first fungal culture library isolated from invasive kudzu (to our knowledge) and tested if interspecific interactions between kudzu endophytes and putative pathogenic agents are neutral, antagonistic or facilitatory. We tested these in both liquid and solid media to (1) identify and quantify the effects of interspecific interactions on fungal growth, and (2) to identify the directionality of these interactions. We predicted that some endophytes would facilitate growth of pathogens *in vitro*, which would lead to the identification of several good candidates for additional studies.

## Materials and methods

### *Sample collection and endophyte/pathogen culturing*

In September 2019, we collected healthy and disease symptomatic kudzu leaves (90 leaves) from across the greater Memphis, TN, USA metropolitan area (Figure 1, Supplementary material, Table S1). Mature leaves at leaf plastochron index (LPI) position 5 were excised from kudzu vines to avoid the most apical leaves and placed into clean individually labeled plastic bags, placed immediately on ice. Fungal isolation work began the same day as collected. Following (Shahrtash and Brown 2020), leaves were first rinsed under running RO water to remove debris and manually agitated in a series of washes: in sterile distilled $H_2O$ for 1 min, 1% Triton-X 100 (*w:v*) for 1 min, 3% NaClO (bleach) for 1 min, 70% ethanol for 1 min. These leaves were then agitated in three successive separate sterile distilled $H_2O$ containers for 1 min each. Excess moisture was wicked off on UV and microwave sterilized filter paper. Six randomly selected leaf disks were excised from each leaf (three on either side of the midrib)

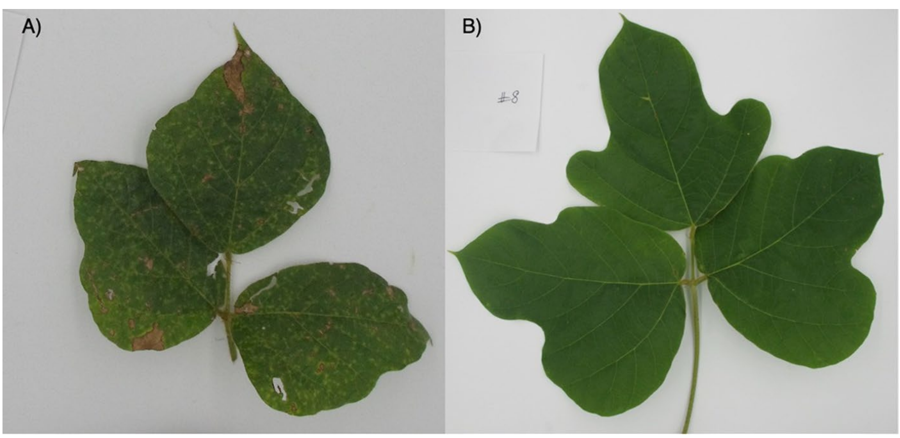


**Figure 1.** Representative examples of symptomatic (A) and asymptomatic (B) kudzu leaves used for culture library establishment across the Memphis TN metropolitan area (Shelby County TN, Fayette County TN, Hardeman County TN, Lauderdale County TN, and Haywood County TN).

using a sterilized handheld punch. Each leaf disk was placed in the center of a petri dish with either Potato Dextrose Agar (PDA) or Malt Extract Agar (MEA), both amended with antibiotics (0.1 mL of 10,000 U/mL Pen-Strep (Thermo Fisher Scientific, Waltham, MA, USA)). Plates were incubated at room temperature in the dark for 2 weeks until there was visible growth emerging from the leaf tissue. To obtain pure cultures, we conducted a series of sub-culturing events. Plugs of agar were drawn from the margins of growing colonies and sub-cultured onto fresh PDA plates until single fungal isolates were recovered. After initial culturing efforts, all isolates were maintained on PDA. In all, we obtained 82 pure fungal isolates with unique morphological characters (Morphotypes).

### Molecular identification of morphotypes

Using a sterile razor, hyphae and/or conidia from each fungus (~0.5 mg) were scraped from a 7 to 15-day-old pure fungal culture plate, and DNA was extracted from using IBI Soil Extraction Kits (IBI Scientific, Dubuque, IA, USA) followed by diluting to a concentration of 25 ng/µL. To identify the morphotypes, we amplified the complete ITS and partial LSU regions of the rRNA operon using the fungal specific primer pair ITS1F and LR3 (White et al. 1990; Gardes and Bruns 1993). PCRs were conducted in 25 µL reactions using Pure-Taq Ready-To-Go PCR Beads (Marlborough, MA, USA) with following concentrations: 2 µL DNA template (50 ng), 0.5 µM (2.5 µL) of each forward and reverse primer and 18 µL molecular grade $H_2O$. PCR parameters were 94 °C for 4 min, 30 cycles of 94 °C for 30 s (denaturing stage), 50 °C annealing temperature for 30 s (annealing stage) and 72 °C for 30 s (extension stage), followed by a final extension at 72 °C for 10 min; all ramp rates were 1 °C/s (SimpliAmp Thermal Cycler, Applied Biosystems, Foster City, CA, USA). Following verification of PCR amplification using gel electrophoresis, PCR products were cleaned to remove residual primers, nucleotides and enzymes, using ExoSap-IT (Thermo Fisher Scientific, Waltham, MA, USA). Samples were bi-directionally Sanger sequenced (using the sequencing primers ITS1F and LR3) at the Molecular Resources Center at University of Tennessee Health Science Center (UTHSC). Obtained chromatograms were corrected and assembled manually using the program Geneious Prime v.11.1.5 (Biomatters, Inc. Auckland, New Zealand) and full length ITS and partial LSU sequences were generated. We used BLASTn (nr/nt) to identify each obtained Sanger sequence where possible and fungal functional groups for these cultures were identified using the FungalTraits database (Põlme et al. 2020) to identify fungi that are putative pathogens and endophytes (Supplementary material, Table S2). From these initial fungal cultures, we identified those that (1) have been previously documented as pathogens on leguminous plants, or (2) are endophytes that have been previously documented to interact with pathogens in other systems. This information was used to design our pathogen-endophyte competition assays. In all, we tested (6) putative leguminous pathogens, and (4) putative endophytes. The isolates that were tested include the putative pathogenic isolates *Phoma* sp. – isolate A2, *Colletotrichum gloeosporioides* – isolate A3, *Fusarium* sp. isolate C7, *Microsphaeropsis olivacea* – isolate D12, *Nigrospora oryza* – isolate E11, *Alternaria* sp. – isolate D11; these were tested against the following putative endophytic isolates *Cladosporium uredinicola* – isolate E4, *Cochliobolus kusanoi* – isolate A7, *Epicoccum* sp. – isolate B12, and an unknown Sordariomycetes sp. – isolate A6.

### *Competition assays in liquid and solid media*

To examine the interaction types between two fungal species, we grew isolates together (co-culture) and compared them with fungal growth alone (monoculture). Agar plugs were taken from the margin of each isolate grown on PDA and placed into autoclaved and sterile Potato Dextrose broth (PDB) amended with 0.2% (v/v) Pen-Strep (as above). For each trial, 50 mL PDB was used in 250 mL flasks. Monocultures were replicated four times and co-cultures were replicated six times. Cultures were grown in the dark in a MaxQ$^{TM}$ 6000 incubator (Thermo Fisher Scientific, Waltham, MA, USA) at 25 °C and shaken at 75 rpm for 5 days. After incubation, the fungal mycelial mats were vacuum filtered onto a pre-weighed filter paper, oven dried at 80 °C (3700K Oven, Precision, Thermo Fisher Scientific, Waltham, MA, USA) for 48 h and biomass was determined (Accuris, Edison, NJ, United States). These biomass data were compared (see below) to determine modes of interactions (antagonistic, neutral and faciliatory).

To determine the interaction type between two fungal species on solid media, as well as to determine the directionality of observed interactions, we grew isolates in monoculture and co-culture and measured the area of fungal growth. We placed 5 mm diameter agar plugs collected from the margins of 7–15-day old cultures into the center of each compartment in four compartment culture dishes (12 replicates of each of monoculture and co-cultures) using Pen-Strep amended PDA (as above). For co-cultures, we placed competing agar plugs 1 cm apart. Cultures were grown at room temperature in the dark for 5 days. High resolution photographs were taken using a photography light box. The area of each isolate on PDA was measured and these values were used to determine the mode of interaction and directionality of this relationship (see below). All images were measured using the software Image J (v.1.15; Schneider et al. 2012).

### *Determination of interaction types and directionality*

Determination of interaction type was modified from a framework previously used (Li et al. 2019). We compared growth for each monoculture and co-culture with the assumption that if these isolates have a neutral interaction, then the growth in co-culture should equal the sum of the individual monocultures (*similar to* Foster and Bell 2012). We determined the average growth (biomass or area) of isolate *i* (indicated as $M_i$) and isolate *j* ($M_j$) and the growth (biomass or area) of the co-culture ($C_{i+j}$). Further we determined the 95% confidence interval (CI) of the co-culture growth. Interactions were determined to be neutral if $M_i + M_j$ falls within the 95% CI of $C_{i+j}$, antagonistic if $M_i + M_j >$ 95% CI of $C_{i+j}$, and faciliatory if $M_i + M_{j\,j} <$ 95% CI of $C_{i+j}$.

To determine directionality of this interaction in solid media, we quantified the ratio of interaction ($R_I$) using Equation (1).

$$R_I = \left| \frac{\bar{x}_{mi} - \bar{x}_{ci}}{\bar{x}_{mj} - \bar{x}_{ci}} \right| \tag{1}$$

where $\bar{x}_{mi}$ is the average area of isolate *i* in monoculture, $\bar{x}_{ci}$ is the average area of isolate *i* in co-culture, $\bar{x}_{mj}$ is the average area of isolate *j* in monoculture, $\bar{x}_{cj}$ is the average area of isolate *j* in co-culture. Where $R_I>1$, then isolate *i* is the dominate isolate and where $R_I<1$, then isolate *j* is the dominate isolate and the value of $R_I$ provides an indication of the strength of this interaction.

## Results

In this study we examined the antagonistic and facilitatory pairwise interactions between fungal endophytes and pathogens of kudzu in both liquid and solid media. After morphotype isolation and Sanger sequencing, we identified 52 isolates at the genus level: *Phoma* (8 isolates), *Colletotrichum* (4), *Cochliobolus* (6), *Fusarium* (7), *Epicoccum* (5), *Alternaria* (3), *Microsphaeropsis* (1), *Nigrospora* (1), *Cladosporium* (2), *Daldinia* (1), *Pestalotiopsis* (1), Fungal endophyte strain SV1888 (2), Fungal endophyte strain SV1874 (1). Additionally, there were several isolates that we were unable to place below Class using the ITS sequences (Sordariomycetes sp. (9) and Dothideomycetes (1)). Among these fungi at genus level, six genera were identified as putatively pathogens and nine genera were endophytes (Table 1). All full-length and partial LSU Sanger sequences are accessioned in GenBank at NCBI (accession numbers OP510067–OP510138).

**Table 1.** List of isolate taxonomic placement and functional roles based on the database FungalTraits, along with BLASTn identity values and NCBI GenBank accession of closest match.

| Isolate ID | BLAST result | Lifestyle | Percent identity | Accession |
|---|---|---|---|---|
| A1 | *Cochliobolus kusanoi* | Pathogen | 99.90% | JN943393.1 |
| A2 | *Phoma* sp. | Pathogen | 99.71% | KT462714.1 |
| A3 | *Colletotrichum gloeosporioides* | Pathogen | 100.00% | AJ301988.1 |
| A4 | *Colletotrichum gloeosporioides* | Pathogen | 100.00% | AJ301988.1 |
| A6 | *Sordariomycetes* sp. | Endophyte | 100.00% | JQ761153.1 |
| A7 | *Cochliobolus kusanoi* | Endophyte | 99.90% | JN943393.1 |
| A8 | *Phoma* sp. | Endophyte | 99.81% | KT462714.1 |
| A9 | *Fusarium* sp. | Pathogen | 100.00% | MN626675.1 |
| A11 | *Colletotrichum gloeosporioides* | Pathogen | 100.00% | AJ301988.1 |
| A12 | *Fusarium graminearum* | Pathogen | 100.00% | LT222056.1 |
| B1 | *Phoma* sp. | Pathogen | 99.90% | KT462714.1 |
| B3 | Fungal endophyte strain | Endophyte | 100.00% | KY765293.1 |
| B4 | *Cochliobolus kusanoi* | Endophyte | 99.90% | JN943393.1 |
| B7 | *Phoma aliena* | Pathogen | 99.62% | KC311486.1 |
| B8 | *Fusarium* sp. | Pathogen | 100.00% | MH582469.1 |
| B9 | *Fusarium* sp. | Pathogen | 100.00% | GQ505745.1 |
| B11 | *Cladosporium cladosporioides* | Endophyte | 99.26% | KJ596320.1 |
| B12 | *Epicoccum* sp. | Endophyte | 100.00% | MN153960.1 |
| C2 | *Phoma aliena* | Pathogen | 99.61% | KC311486.1 |
| C3 | *Phoma aliena* | Pathogen | 99.61% | KC311486.1 |
| C4 | *Fusarium* sp. | Pathogen | 100.00% | GQ505745.1 |
| C5 | Fungal endophyte strain SV1874 | Endophyte | 99.81% | KY765281.1 |
| C6 | *Epicoccum* sp. | Endophyte | 100.00% | MN153960.1 |
| C7 | *Fusarium* sp. | Pathogen | 100.00% | MH582464.1 |
| C8 | *Phoma* sp. | Pathogen | 99.90% | KF675768.1 |
| C9 | *Cochliobolus kusanoi* | Endophyte | 99.90% | JN943393.1 |
| C10 | Sordariomycetes sp. | Endophyte | 100.00% | KY413704.1 |
| C11 | *Microsphaeropsis olivacea* | Endophyte | 100.00% | MH855867.1 |
| C12 | *Epicoccum nigrum* | Endophyte | 100.00% | MT035964.1 |
| D1 | *Cochliobolus geniculatus* | Endophyte | 99.91% | JN943417.1 |
| D2 | Sordariomycetes sp. | Endophyte | 100.00% | JQ761100.1 |
| D4 | *Daldinia loculata* | Endophyte | 100.00% | JQ758841.1 |
| D5 | Sordariomycetes sp. | Endophyte | 100.00% | JQ761100.1 |
| D6 | *Pestalotiopsis trachicarpicola* | Endophyte | 100.00% | MN427963.1 |
| D7 | Sordariomycetes sp. | Endophyte | 100.00% | JQ761100.1 |
| D8 | Dothideomycetes sp. | Endophyte | 100.00% | GQ153080.1 |
| D9 | Sordariomycetes sp. | Endophyte | 100.00% | JQ761100.1 |
| D10 | *Cochliobolus kusanoi* | Endophyte | 99.90% | JN943393.1 |
| D11 | *Alternaria* sp. | Pathogen | 100.00% | MN153951.1 |
| E1 | Sordariomycetes sp. | Endophyte | 100.00% | JQ758841.1 |
| E2 | Epicoccum nigrum | Endophyte | 100.00% | JQ761100.1 |
| E3 | Fungal endophyte strain SV1888 | Endophyte | 100.00% | MN427963.1 |
| E4 | *Cladosporium uredinicola* | Endophyte | 100.00% | JQ761100.1 |
| E5 | *Fusarium tricinctum* | Endophyte | 100.00% | GQ153080.1 |
| E6 | *Alternaria* sp. | Endophyte | 100.00% | JQ761100.1 |
| E7 | *Epicoccum nigrum* | Endophyte | 99.90% | JN943393.1 |
| E8 | *Alternaria* sp. | Pathogen | 100.00% | MN153951.1 |
| E9 | *Sordariomycetes* sp. | Endophyte | 100.00% | JQ758841.1 |
| E10 | *Phoma aliena* | Endophyte | 100.00% | JQ761100.1 |
| E11 | *Nigrospora Oryzae* | Endophyte | 100.00% | MN427963.1 |
| E12 | Sordariomycetes sp. | Endophyte | 100.00% | JQ761100.1 |
| F1 | *Colletotrichum gloeosporioides* | Endophyte | 100.00% | GQ153080.1 |

From these obtained identities, we identified taxa that have either (1) been previously documented to cause diseases on other leguminous plants, as kudzu pathogens are not well described, and (2) are presumed to be endophytes, or at least have documented endophytic capability. Among the genera used for these additional analyses, *Colletotrichum* (Sun et al. 2006; Cannon et al. 2012; Chakraborty et al. 2019), *Fusarium* (Sampaio et al. 2020), *Phoma* (Kim and Chen 2019; Deb et al. 2020), *Alternaria* (Bankina et al. 2021), *Nigrospora* (Deepika et al. 2021) and *Microsphaeropsis* (Shabana et al. 2010), are well documented to cause diseases on other legumes, thus may also cause disease in kudzu. Further, *Colletotrichum* spp. have been demonstrated to cause anthracnose disease on kudzu in its native range (Sun et al. 2006). The isolates that were used as endophytes include representatives of the genera *Cladosporium*, *Epicoccum*, and *Cochliobolus* and the class Sordariomycetes. All other isolates were not used in competition assays as we could not have confidence in their ecological role within kudzu. Tested endophytes that have been previously reported as plant beneficial microbes include *Cladosporium* (Paul and Yu 2008; Barge et al. 2022) and *Epicoccum* (Schulz and Boyle 2005). It is important to note that some of our endophytic genera have been documented to be putative pathogens, but not on plants within Fabaceae. Co-culture experiments were performed across all pairwise interactions between all putative endophytes and putative kudzu pathogen combinations.

All pairwise interactions between fungal isolates in liquid media showed antagonistic interactions except two combinations, which were faciliatory. *Cochliobolus-Phoma* and *Epicoccum-Phoma* (*Phoma* sp. – isolate A2, *Cochliobolus kusanoi* – isolate A7, *Epicoccum* sp. – isolate B12) interactions facilitated increased biomass when grown together than compared to the sum of monocultures (Table 2). However, our liquid assays do not permit the elucidation of directionality of this facilitatory effect, that is we cannot determine if both isolates exhibit increased growth or does a single isolate dominate.

Consistent with liquid media assay, fungal competition assays in solid media indicated similar results, and most interactions indicated either antagonistic or neutral activities (Table 3). For this, the following isolates were presumed to be pathogens (*Phoma* sp. – isolate A2, *Colletotrichum gloeosporioides* – isolate A3, *Fusarium* sp. – isolate C7, *Microsphaeropsis olivacea* – isolate D12, *Nigrospora oryza* – isolate E11, *Alternaria* sp. – isolate D11) and the following were presumed to be endophytes (*Cladosporium uredinicola* – isolate E4, *Cochliobolus kusanoi* – isolate A7, *Epicoccum* sp. – isolate B12, Sordariomycetes sp. – isolate A6).

Facilitation was observed between *Cladosporium uredinicola* and *Microsphaeropsis olivacea* and between and *Cladosporium uredinicola* and

**Table 2.** Results of pairwise interactions in liquid media between putative pathogens and endophytes (isolate label is presented parenthetically for all taxa used under the column for Isolate *i*).

| Isolate *i* ID | Isolate *j* ID | $C_{i+j}$ (g) | 95% CI (g) | $M_i$ + $M_j$(g) | Interaction type |
|---|---|---|---|---|---|
| *Phoma* (A2) | *Nigrospora* | 0.293 | 0.255–0.331 | 0.569 | Antagonistic |
| *Epicoccum* (B12) | *Nigrospora* | 0.335 | 0.307–0.363 | 0.382 | Antagonistic |
| *Fusarium* (C7) | *Colletotrichum* | 0.542 | 0.504–0.58 | 0.747 | Antagonistic |
| *Fusarium*(C7) | *Alternaria* | 0.357 | 0.331–0.383 | 0.563 | Antagonistic |
| *Colletotrichum* (A3) | *Alternaria* | 0.439 | 0.397–0.48 | 0.668 | Antagonistic |
| *Alternaria* (D11) | *Sordariomycetes* | 0.107 | 0.098–0.116 | 0.461 | Antagonistic |
| *Sordariomycetes* (E1) | *Fusarium* | 0.351 | 0.309–0.392 | 0.539 | Antagonistic |
| *Epicoccum* (B12) | *Cladosporium* | 0.288 | 0.264–0.312 | 0.400 | Antagonistic |
| ***Epicoccum*** **(B12)** | ***Phoma*** | **0.304** | **0.274–0.335** | **0.193** | **Faciliatory** |
| *Phoma* (A2) | *Cladosporium* | 0.257 | 0.253–0.261 | 0.403 | Antagonistic |
| *Fusarium*(C7) | *Cladosporium* | 0.159 | 0.116–0.201 | 0.671 | Antagonistic |
| ***Phoma*** **(A2)** | ***Cochliobolus*** | **0.295** | **0.221–0.368** | **0.239** | **Faciliatory** |
| *Microsphaeropsis* (D12) | *Alternaria* | 0.010 | 0.245–0.266 | 0.543 | Antagonistic |
| *Microsphaeropsis* (D12) | *Sordariomycetes* | 0.266 | 0.195–0.337 | 0.520 | Antagonistic |
| *Phoma* (A2) | *Microsphaeropsis* | 0.097 | 0.062–0.131 | 0.362 | Antagonistic |
| *Alternaria* (D11) | *Cochliobolus* | 0.215 | 0.114–0.317 | 0.428 | Antagonistic |
| *Nigrospora* (E11) | *Alternaria* | 0.133 | 0.114–0.152 | 0.497 | Antagonistic |
| *Sordariomycetes* | *Cochliobolus* | 0.199 | 0.156–0.242 | 0.405 | Antagonistic |
| *Nigrospora* (E11) | *Cochliobolus* | 0.081 | 0.066–0.095 | 0.452 | Antagonistic |
| *Alternaria* (D11) | *Sordariomycetes* | 0.143 | 0.088–0.198 | 0.473 | Antagonistic |
| *Fusarium* (C7) | *Nigrospora* | 0.080 | 0.06–0.099 | 0.687 | Antagonistic |

Faciliatory interactions are in bold. Presented are the average mass (in grams) of co-cultures ($C_{i+j}$), the sum of each monoculture growth ($M_i$ + $M_j$), and the 95% confidence intervals (CI), and interaction type.

*Cochliobolus kusanoi* (Table 3) and in both instances, *C. uredinicola* facilitated the growth of *M. olivaceae* and *C. kusanoi*.

## Discussion

Experimental studies evaluating the efficacy of microbial consortia inoculations for invasive plant management are scarce and little is known about the precise contributions of how fungal endophytes might interact with pathogens. Nor is it well understood how these interactions may impact plant fitness and health. In this study, we explored the interactions between fungal endophytes and pathogens of kudzu *in vitro* and identify interactions that are faciliatory in nature. These endophyte-pathogen combinations are great candidates for future studies of *in planta* utility. These experiments are a crucial first step towards generating and optimizing microbial consortia to manage kudzu plants in its invaded range.

In this study, most tested isolates indicated antagonistic or neutral interactions and this was true for both liquid and solid assays. These types of interaction between microorganisms can be achieved through direct physical or chemical interactions (Schroeckh et al. 2009; Straight and Kolter 2009) or by exchanging signaling molecules and secretion

**Table 3.** Results of pairwise interactions in solid media for both sides of the interaction.

| Tested isolate | Isolate *i* ID | Isolate *j* ID | $C_{i+j}$ (cm$^2$) | 95% CI of $M_i$ OR $M_j$ (cm$^2$) | Interaction type | $R_I$ | Dominant fungi (Directionality) |
|---|---|---|---|---|---|---|---|
| *Alternaria* | *Alternaria* | *Colletotrichum* | 4.67 | 17.607–20.228 | Antagonistic | 2.977 | *Alternaria* |
| *Colletotrichum* | *Alternaria* | *Colletotrichum* | 14.562 | 14.089–24.740 | Neutral | | |
| *Alternaria* | *Alternaria* | *Epicoccum* | 7.156 | 17.607–20.228 | Antagonistic | 2.968 | *Alternaria* |
| *Epicoccum* | *Alternaria* | *Epicoccum* | 3.034 | 5.345–8.779 | Antagonistic | | |
| *Cladosporium* | *Cladosporium* | *Fusarium* | 2.473 | 3.405–6.147 | Antagonistic | 0.287 | *Fusarium* |
| *Fusarium* | *Cladosporium* | *Fusarium* | 7.091 | 13.735–16.507 | Antagonistic | | |
| *Cladosporium* | *Nigrospora* | *Cladosporium* | 5.332 | 3.405–6.147 | Neutral | 0.084 | *Nigrospora* |
| *Nigrospora* | *Nigrospora* | *Cladosporium* | 13.001 | 6.052–6.776 | Antagonistic | | |
| *Alternaria* | *Alternaria* | *Cladosporium* | 6.340 | 17.607–20.228 | Antagonistic | 18.176 | *Alternaria* |
| *Cladosporium* | *Alternaria* | *Cladosporium* | 5.4793 | 3.405–6.147 | Neutral | | |
| *Cladosporium* | *Cladosporium* | Sordariomycetes | 0.4305 | 3.405–6.147 | Antagonistic | 8.184 | *Cladosporium* |
| Sordariomycetes | *Cladosporium* | Sordariomycetes | 19.597 | 18.910–19.222 | Antagonistic | | |
| *Cladosporium* | *Cladosporium* | *Cochliobolus* | 2.546 | 3.405–6.147 | Antagonistic | 0.254 | *Cochliobolus* |
| *Cochliobolus* | *Cladosporium* | *Cochliobolus* | 13.566 | 11.899–12.462 | Faciliatory | | |
| *Cladosporium* | *Cladosporium* | *Microsphaeropsis* | 1.678 | 3.405–6.147 | Antagonistic | 0.663 | *Microsphaeropsis* |
| *Microsphaeropsis* | *Cladosporium* | *Microsphaeropsis* | 13.399 | 6.523–10.924 | Faciliatory | | |
| *Phoma* | *Phoma* | *Cladosporium* | 10.065 | 19.924–20.769 | Antagonistic | 25.241 | *Phoma* |
| *Cladosporium* | *Phoma* | *Cladosporium* | 5.183 | 3.405–6.147 | Neutral | | |
| *Cochliobolus* | *Cochliobolus* | *Alternaria* | 9.751 | 11.899–12.462 | Antagonistic | 0.345 | *Alternaria* |
| *Alternaria* | *Cochliobolus* | *Alternaria* | 4.678 | 17.607–20.228 | Antagonistic | | |
| *Cochliobolus* | *Cochliobolus* | *Colletotrichum* | 7.181 | 11.899–12.462 | Antagonistic | 0.248 | *Colletotrichum* |
| *Colletotrichum* | *Cochliobolus* | *Colletotrichum* | 9.737 | 14.089–24.740 | Antagonistic | | |
| *Cochliobolus* | *Cochliobolus* | *Epicoccum* | 9.079 | 11.899–12.462 | Antagonistic | 5.227 | *Cochliobolus* |
| *Epicoccum* | *Cochliobolus* | *Epicoccum* | 7.885 | 5.345–8.779 | Neutral | | |
| *Cochliobolus* | *Cochliobolus* | *Microsphaeropsis* | 10.434 | 11.899–12.462 | Antagonistic | 1.297 | *Cochliobolus* |
| *Microsphaeropsis* | *Cochliobolus* | *Microsphaeropsis* | 4.3603 | 6.523–10.924 | Antagonistic | | |
| *Cochliobolus* | *Cochliobolus* | *Nigrospora* | 11.216 | 11.899–12.462 | Antagonistic | 2.452 | *Cochliobolus* |
| *Nigrospora* | *Cochliobolus* | *Nigrospora* | 3.787 | 6.052–6.776 | Antagonistic | | |
| *Colletotrichum* | *Colletotrichum* | *Alternaria* | 18.381 | 14.089–24.740 | Neutral | 0.063 | *Alternaria* |
| *Alternaria* | *Colletotrichum* | *Alternaria* | 2.590 | 17.607–20.228 | Antagonistic | | |
| *Colletotrichum* | *Colletotrichum* | *Cladosporium* | 16.516 | 14.089–24.740 | Neutral | 0.904 | *Cladosporium* |
| *Cladosporium* | *Colletotrichum* | *Cladosporium* | 1.571 | 3.405–6.147 | Antagonistic | | |
| *Colletotrichum* | *Colletotrichum* | *Phoma* | 13.174 | 14.089–24.740 | Antagonistic | 0.425 | *Phoma* |
| *Phoma* | *Colletotrichum* | *Phoma* | 5.679 | 19.924–20.769 | Antagonistic | | |
| *Epicoccum* | *Epicoccum* | *Cladosporium* | 2.650 | 5.345–8.779 | Antagonistic | 2.99 | *Epicoccum* |
| *Cladosporium* | *Epicoccum* | *Cladosporium* | 3.301 | 3.405–6.147 | Antagonistic | | |
| *Epicoccum* | *Epicoccum* | *Colletotrichum* | 0.654 | 5.345–8.779 | Antagonistic | 14.042 | *Epicoccum* |
| *Colletotrichum* | *Epicoccum* | *Colletotrichum* | 19.87 | 14.089–24.740 | Neutral | | |
| *Epicoccum* | *Epicoccum* | *Nigrospora* | 4.379 | 17.607–20.228 | Antagonistic | 0.299 | *Nigrospora* |
| *Nigrospora* | *Epicoccum* | *Nigrospora* | 15.401 | 6.052–6.776 | Antagonistic | | |

Faciliatory interactions are in bold. Presented are the average area (in cm$^2$) of co-cultures ($C_{i+j}$), the 95% CI of average growth for the tested isolate ($M_i$ or $M_j$), the ratio of interaction ($R_I$), the interaction type and directionality (dominant fungi).

of secondary metabolites which leads to suppressed growth (Ola et al. 2013). Neutral interactions in wild systems occur when neither fungal species affects the growth of the other, but the chemical basis of neutral interactions of co-occurring species remains unclear. In antagonistic interactions, one fungus overgrows, suppresses, and/or excludes another usually *via* the release of secondary metabolites like antibiotics, mycotoxins, or pigments (Keller et al. 2005; Ujor et al. 2012; Boddy and Hiscox 2016; Chatterjee et al. 2016), or mycoparasitism (Benítez et al. 2004; Atanasova et al. 2013). For instance, comparative transcriptomics has revealed different strategies of *Trichoderma* antagonistic activity against *Rhizoctonia solani* (Atanasova et al. 2013), ranging from mycoparasitism, antibiotic production, and competitive exclusion (Sharma et al. 2017). In an *in vitro* bioassay study, it was demonstrated that *Streptomyces corchorusii* had a wide spectrum antagonistic activity against soil borne pathogens such as *Fusarium oxysporum* f. sp. *niveum* by damaging cell walls through secondary metabolite production (Yang et al. 2019). Additionally, antagonistic interactions likely involve competition for nutrients and space in culture media (Blumenstein et al. 2015; Ceci et al. 2019), or metabolite production (Heydari and Pessarakli 2010), which can lead to exclusion or suppression. For these reasons, it is not surprising that most of our tested interactions were antagonistic *in vitro* but the determination of the chemical basis for these antagonisms was beyond the scope of this current work. It remains to be seen if these observed *in vitro* interactions will be seen in *in planta*.

In our agar-based competition assays, we found that *Cladosporium uredinicola* facilitated the growth of *Microsphaeropsis olivacea* and *Cochliobolus kusanoi. Cladosporium* is a diverse genus which have diminutive conidia, which facilitates widespread dispersal (Park et al. 2004). *Cladosporium* spp. are often found as endophytes in plants (El-Morsy 1999) but also are commonly found on food, in soil and other organic matter. *Cladosporium* can be pathogenic (Batson et al. 2022), but they are often only pathogenic on fungi by acting as mycoparasites (Heuchert and Braun 2006). In partial support of these results, several studies have found antagonistic interactions between *Cladosporium* and other fungi. They can have widespread mycoparasitic effects on some common fungal pathogens' activity *via* direct and chemical interactions (Barge et al. 2022). It has also been reported that *Cladosporium cladosporoides* stunts the development of *Puccinia horiana*, the causal agent of white rust among *Chrysanthemum* species (Torres et al. 2017) and some *Cladosporium* species have been found to suppress the germination and mycelial growth of several plant pathogenic fungi including, *Alternaria alternata*, *Botrytis cinerea* and *Rhizoctonia solani* (Follstad 1966). Given that many *Cladosporium* are known plant pathogen antagonists, it is a bit surprising that we found that *Cladosporium* increases the growth of *Microsphaeropsis* and *Cochliobolus*, both of which are pathogenic. This reinforces the complex nature of fungal-fungal interactions which may limit the utility of “one size fits all” solutions. It also demonstrates the need for more research into the mechanisms of these interactions. Additionally, *Cladosporium* has been used as a biostimulant in agricultural systems and it has been shown that it has the potential to increase plant tolerances to biotic and abiotic stress and improve plant growth (Li et al. 2019; Răut et al. 2021).

*Cladosporium* increased the growth of *Microsphaeropsis* and *Cochliobolus* in this study. The genus *Cochliobolus* belongs to the class Dothideomycetes (Ohm et al. 2012) and has not been reported as a major causative agent of diseases of legumes, however it can be pathogenic in some Poaceae crops and grasses (Sindhu et al. 2008; Kang et al. 2018; Völz et al. 2020). *Microsphaeropsis* (family Didymosphaeriaceae) is a parasite and saprobe on several terrestrial plants (Pendle et al. 2004). To our knowledge, there has not been any previous reports of faciliatory interactions between *Cladosporium* and other fungal taxa before this study. The mechanisms behind this novel result are unknown and future work should investigate *Cladosporium* secondary chemistry and confirm these results *in planta*, but this is a promising result and suggests that some *Cladosporium* isolates may serve as a synergistic agent in a microbial consortium aimed toward invasive plant management.

In our liquid assays, we see that interactions between *Cochliobolus* and *Phoma* and between *Epicoccum* and *Phoma* suggest faciliatory growth *in vitro*. *Phoma* belongs to the class Dothideomycetes, and the genus is hyperdiverse with more than 3000 infrageneric taxa described with various host specificity (Montel et al. 1991; de Gruyter et al. 2009; Deb et al. 2020). *Phoma* is considered a saprophyte, and a weak pathogen (Sutton 1980; Kumar et al. 2015), mostly causing leaf and stem spots (Lin et al. 2014). *Phoma glomerata* has also been reported as a mycoparasite that suppress powdery mildew in sycamore trees (Sullivan and White 2000). There are other reports indicating that some *Phoma* species can have antagonistic activity against some plant pathogens. For example, the antagonistic activity of *Phoma etheridgei* against the aspen decay pathogen, *Phellinus tremulae* has been reported (Hutchison et al. 1994). Another study suggested *Phoma herbarum* as a significant biocontrol agent for controlling the pathogen *Colletotrichum gloeosporioides* (causative agent of Anthracnose) (Gupta et al. 2016). *Epicoccum* sp., belongs to the Didymellaceae, and contains numerous species (Hyde et al. 2017), most are plant endophytes (Schulz and Boyle 2005) and some have been tested for use as a biocontrol (Frari et al. 2019). There are several reports indicating the microparasitic activity of some *Epicoccum* spp. *via* the production of secondary metabolites on several plant pathogens including *Sclerotinia sclerotiorum* (Pieckenstain et al. 2001), *Claviceps africana* (Bhuiyan et al. 2003), *Rhizoctonia solani* (Lahlali and Hijri 2010) and *Pythium* spp. (Hashem and Ali 2004).

To our knowledge, this is the first report of *in vitro* interactions between these fungi. While the facilitation activities were characterized based on the increase in fungal biomass in co-cultures, the directionality of these interactions in this study are unknown. However, *Epicoccum* and *Phoma* have been found to be dominant genera found co-occurring on monument and rock surfaces (Sterflinger and Prillinger 2001). Another report indicated that *Fusarium*, *Epicoccum*, and *Phoma* readily co-occur and are highly abundant on maize leaves (Szilagyi-Zecchin et al. 2016). Our previous work in kudzu (Shahrtash and Brown 2020) also found these taxa have similar co-occurrence patterns in the foliar kudzu mycobiome. Due to these novel documented interactions, additional investigations into how these taxa interact molecularly and physiologically are needed before these systems can be tested for pathogen facilitation *in planta* but these are exciting targets for potential use.

It is not clear why the results from liquid and solid assays do not completely overlap. While most interacting isolates in both media types were seen to be antagonist or neutral, those that facilitated growth were not the same. In liquid media, we see that the *Phoma* isolate facilitated growth of both *Epicoccum* and *Cochilobolus*, while in solid media we see that *Cladosporium uredinicola* facilitated growth of both *Cochilobolus kusanoi* and *Microsphaeropsis olivacea*. In both assay types, *Cochilobolus* spp. growth was enhanced by different endophytes. The reason for this discrepancy remains hidden but fungal physiological differences often results if differential growth patterns or nutrient utilization in liquid and solid media (Walker and White 2017). This can be seen in wide range of situations including wood degrading Agaricomycetes (Mali et al. 2017), and in pathogens including *Macrophominia phaseolina* (Burton et al. 1995). Likely, this difference is mainly due to differential nutrient utilization of isolates in liquid and solid media and how the constitutive components of each isolate's Spitzenkörper directs hyphal elongation (Reynaga-Peña et al. 1997; Harris 2009), which directly impacts hyphal exploration types in solid media (Steinberg 2007). Whatever the reason for these differences, we identify here novel interactions between endophytes and pathogens of kudzu that direct additional investigations.

Until recently, the potential of using microbial consortia to manage plant disease, pests, invasive plants, and weeds have not received much study. The present work gives novel insights into the relationship between culturable endophytes of kudzu and their potential to be used as a component alongside biocontrol agents. Further investigation is needed to clarify and quantify the molecular signals and the secondary metabolites involved in the fungi-fungi and fungi-fungi-plant interactions (Ravikrishnan et al. 2020; Shahrtash and Brown 2021). Overall, our findings highlight the potential of using endophytes as an additional partner for the biological control of invasive plants. Combining compatible species with complementary synergistic effects on phytopathogens and investigating under which environmental conditions these results are valid may lead to the development of targeted biocontrol products with higher efficacy. Our findings particularly hint toward the importance of *Cladosporium uredinicola* and *Phoma* spp. as research targets for *in planta* experiments to confirm facilitation efficacy.

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## Declaration of interests

The authors declare no conflicts of interest.

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