Fungal Ecology 68 (2024) 101327

Contents lists available at ScienceDirect

Fungal Ecology

journal homepage: www.elsevier.com/locate/funeco

Fungal endophytes of the invasive grass *Eragrostis lehmanniana* shift metabolic expression in response to native and invasive grasses

Taylor A. Portman [a,b], A. Elizabeth Arnold [b,c,d], Robin G. Bradley [b,e], Jeffrey S. Fehmi [e], Craig Rasmussen [a], Malak M. Tfaily [a,d,*]

[a] *Environmental Science, University of Arizona, Tucson, AZ, USA*
[b] *School of Plant Science, University of Arizona, Tucson, AZ, USA*
[c] *Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ, USA*
[d] *BIO5 Institute, University of Arizona, Tucson, AZ, USA*
[e] *School of Natural Resources and the Environment, University of Arizona, Tucson, AZ, USA*

ARTICLE INFO

Handling editor: Prof. Benjamin Wolfe

Keywords:
Invasion
Poaceae
Lehmann's lovegrass
Fusarium sp.
Pseudothielavia sp.
Pseudophialophora sp.
Metabolomics
Mass spectrometry

ABSTRACT

Plant-fungal interactions shape ecosystem dynamics and are increasingly recognized as important in the success of invasive plants. Although diverse fungal endophytes are known to inhabit plants, including grasses, the precise chemical mechanisms through which they influence their hosts remain inadequately understood. We used untargeted metabolomics to characterize substrate use and compound production of three fungal endophytes isolated from an invasive grass, *Eragrostis lehmanniana,* characterizing the metabolome of these fungal isolates grown alone (axenically) and in the presence of seeds from invasive *E. lehmanniana* and co-occurring native grasses (*E. intermedia, Bouteloua curtipendula,* and *Leptochloa dubia*). We found that each fungal isolate expressed a different metabolic profile in response to *Eragrostis* seeds, relative to seeds of non-*Eragrostis* native grasses. Coupled with results of germination trials, these findings suggest that plant-fungal interactions mediated by the fungal metabolome may play a key role in determining the success of a major invasive species.

1. Introduction

Plant-fungal interactions represent a range of complex and dynamic relationships that often are important for the well-being of plants (Rodriguez et al., 2009; Peay et al., 2016; Pozo et al., 2021; Verma et al., 2021). Fungal symbionts of plants that occur within plant tissues – endophytes – are ubiquitous among plants and have evolved with plants since colonization of land (Lutzoni et al., 2018). In certain cool-season grasses, vertically transmitted endophytes (specifically of the family *Clavicipitaceae*) provide their hosts with protection against natural enemies and abiotic stress (Clay, 1989; Llorens et al., 2019; Schardl et al., 2004). However, many plants, including warm-season grasses, also harbor diverse communities of horizontally transmitted endophytes (Higgins et al., 2011; Knapp et al., 2019). These fungi often establish in roots, leaves, and seeds from the surrounding environment, and some confer fitness benefits relevant to stress resilience and protection against disease and herbivores (Arnold et al., 2003; Rodriguez et al., 2009; Kivlin et al., 2013; Busby et al., 2016; Card et al., 2016; Rho et al., 2018; Verma et al., 2021). As they grow within the living tissues of plants, endophytes create avenues for optimizing primary metabolite nutrient exchange and secondary metabolite production, such as metabolites that may elicit diverse physiological responses from their host plants (e.g., Lu et al., 2021). Given the capacity for some horizontally transmitted fungal endophytes to benefit their hosts, it is plausible that they play a role in facilitating plant establishment under stressful conditions, including during biological invasions.

Biological invasions are a global phenomenon whereby organisms such as plants are introduced and become established outside their geographic range, with negative impacts on biodiversity, ecosystem services, or human sustainability (IUCN, 2021). Grassland biomes, which cover more than 40% of terrestrial lands, are threatened throughout the world by conversion to croplands, urbanization, woody plant encroachment, and invasive species (Petermann and Buzhdygan, 2021). Compounded by stress due to climate change, ongoing and new invasions are expected to increase in prevalence and impact in coming decades (e.g., Ali and Bucher, 2022; Overpeck and Udall, 2020).

In southwestern North America, land use over the past century has contributed to ecosystem disruption including invasions by diverse

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[*] Corresponding author. Environmental Science, University of Arizona, Tucson, AZ, USA.
E-mail address: tfaily@arizona.edu (M.M. Tfaily).

https://doi.org/10.1016/j.funeco.2023.101327
Received 1 August 2023; Received in revised form 29 November 2023; Accepted 6 December 2023

grasses (McClaran, 2003). In the early 1900s, land management practices often included the introduction of non-native plants to provide agricultural or ecosystem services, in some cases leading to the establishment and spread of invasive species and suppression of native species (e.g., Anable et al., 1992; Smith et al., 2012).

Eragrostis lehmanniana (Lehmann lovegrass), a warm-season perennial bunchgrass, was introduced to Arizona (USA) from Africa in 1932 to stabilize soils and provide forage for cattle (Anable et al., 1992). It rapidly became the predominant perennial grass in areas where it was initially established, becoming more common than all native grasses combined by a decade after introduction (McClaran, 2003). Now it is established across much of the American Southwest in a range that stretches from California to Texas (Burruss et al., 2022).

Factors that are often invoked as influencing the invasion success of *E. lehmanniana* include its rapid regeneration after fire, resistance to cattle grazing, prolific seed production, drought resistance, and tolerance of high temperatures (Anable, 1990; D'Antonio and Vitousek, 1992; Uchytil, 1992). Many of these factors may be influenced by microbes, which remain underexplored in this system (see Fehmi et al., 2021). Lehr (2018) established a culture library of endophytic fungi from roots and stems of *E. lehmanniana* in southern Arizona. Subsequently, Bradley et al. (2020, 2021) observed that a subset of these isolates enhanced seed germination of *E. lehmanniana* while inhibiting the germination of co-occurring native grasses. It was proposed that the metabolites produced by such fungi may play an important role in influencing seed germination and early growth responses. However, despite recent interest in the metabolic capacity of diverse endophytes, and longstanding interest in secondary metabolites of endophytes for applied purposes (e.g., Bascom-Slack et al., 2012), metabolomics has not yet been used widely in ecological studies of such plant-fungal interactions (see Gupta et al., 2022; Nagarajan et al., 2021). Thus, specific compounds or metabolites produced or consumed by these fungi remain unexplored, until now.

In this study, we employed untargeted metabolomics using liquid chromatography-tandem mass spectrometry to comprehensively and without bias detect and identify various small molecules within the metabolomes of representative endophytic fungi from *E. lehmanniana*. This analysis was conducted in the context of assessing how these fungi affect germination of seedlings from both native and invasive grass species. We asked: **Q1,** to what extent do fungal isolates with similar ecological niches differ in their metabolic capacity? **Q2,** are there specific secondary metabolites produced by these fungal endophytes that are consistently associated with increased or decreased seedling germination? With our data we test two focal hypotheses: **H1,** similarity in metabolic expression will reflect phylogenetic relatedness of the endophytes, and **H2,** endophytes have the ability to adjust their metabolic activity by upregulating specific metabolites in response to seeds of different plant taxa, ultimately enhancing or suppressing seed germination. This study contributes to understanding a potential functional role of endophytes associated with an important invasive grass, providing a baseline for characterizing the metabolic capacity of these fungi and their potential role in seedling establishment under natural conditions.

2. Materials and methods

Experimental system. Our study was conducted at the Santa Rita Experimental Range (SRER), situated approximately 80 km south of Tucson, AZ, USA. Encompassing a 21,000-ha expanse of mesquite-grass savanna and Sonoran Desert vegetation, the SRER boasts elevations ranging from approximately 900 m–1400 m. The region experiences an average rainfall of 213 mm in the summer and 158 mm in the winter, as reported by McClaran (2003). The SRER has a long history of research focusing on restoration of vegetation, including multiple attempts in the early 1900s to establish exotic grasses for erosion control and forage (McClaran, 2003). Lehmann lovegrass (*E. lehmanniana* Nees, ERLE) was introduced for these reasons. It is now the predominant grass species over most of the SRER, with an introduced range beyond the SRER of more than 140,000 ha across the American southwest. At SRER, *E. lehmanniana* co-occurs with native grasses such as *Eragrostis intermedia* Hitchc. (ERIN), *Bouteloua curtipendula* (Michx.) Torr. (BOCU), and *Leptochloa dubia* (Kunth.) Nees (syn. *Disakisperma dubium* (Kunth.) P. M. Peterson & N. Snow) (LEDU). We selected these grass species for our study because they represent the diverse genera of perennial grasses present in the region. *Eragrostis intermedia* represents a native grass of the same genus as the invasive *E. lehmanniana*, while *B. curtipendula* and *L. dubia* represent native species of differing genera to account for differences in seed morphology and other traits that vary among plant lineages.

Sample collection and processing. Fungal cultures used in this study were isolated from fresh roots collected in August 2013–March 2014 from healthy, mature *E. lehmanniana* occurring at high density in the mesquite savanna at SRER (Lehr, 2018). Briefly, roots were collected intact, rinsed in tap water, cut into 2 mm pieces, and surface sterilized by sequential washes in 95% ethanol (10 s), 10% Clorox bleach (0.53% NaOCl-; 2 min) and 70% ethanol (2 min) (Higgins et al., 2011; Massimo et al., 2015). Root pieces were placed on the surface of 2% malt extract agar (MEA), a standard growth medium, and cultivated at ca. 21 °C for up to one month under 12-h light-dark cycles (Lehr, 2018). Emergent fungi were isolated to axenic culture and vouchered as living cultures in sterile water at the Robert L. Gilbertson Mycological Herbarium at the University of Arizona. These vouchers suppress metabolic activity and allow fungi to be revived readily by transfer to new growth media. Each strain was characterized via Sanger sequencing of the fungal barcode locus (internal transcribed spacers and 5.8S region, ITSrDNA) and an adjacent portion of the nuclear ribosomal large subunit (partial LSUrDNA) following U'Ren and Arnold (2016). ITSrDNA-partial LSUrDNA sequences were edited manually and assembled into operational taxonomic units (OTUs) based on 95% sequence similarity (Arnold and Lutzoni, 2007; Lehr, 2018; U'Ren et al., 2009). All isolates used in this study belong to the subphylum Pezizomycotina (Ascomycota). Their phylogenetic placement was estimated in T-BAS v2.3 using the Pezizomycotina v2.1 reference set with the Evolutionary Placement Algorithm (Carbone et al., 2017, 2019) (Table 1).

We selected three taxa that were the most common associates of *E. lehmanniana* roots at SRER (Table 1), together representing nearly 25% all isolates obtained from roots. We focused on root endophytes because they complete their life cycles in soil, where they can come into contact with seeds of *E. lehmanniana* and native species (Bradley, 2023). Root endophytes of *E. lehmanniana* represent a subset of the endophyte diversity that occurs at SRER, with the focal taxa considered here occurring at low abundance in bulk soils and roots of native plants (Arnold, unpubl. data).

Experimental design. Three isolates were cultured on 2% MEA from sterile vouchers (Table 1). From each actively growing culture, we used a sterile borer to obtain a mycelial plug (6 mm in diameter), which we homogenized with a sterile micropestle in a sterile 1.5 mL microcentrifuge tube with 600 μl of sterile water (Bradley et al., 2020). Fifty microliters of homogenate were transferred to 60 mm Petri plates containing 2% MEA, and spread evenly with a sterile cell spreader. Fungal

Table 1
Names, abbreviations, and taxonomy for three fungal endophytes of *Eragrostis lehmanniana* isolated from roots of healthy, mature plants at the Santa Rita Experimental Range (SRER), southeastern Arizona, USA. All are members of the Ascomycota, subphylum Pezizomycotina, class Sordariomycetes (Zhang et al., 2006).

Isolate	Species	Order	Family
F1246	*Pseudothielavia* sp.	Sordariales	Chaetomiaceae
F1154	*Fusarium* sp.	Hypocreales	Nectriaceae
F1177	*Pseudophialophora* sp.	Magnaporthales	Magnaporthaceae

lawns were allowed to grow for 8 days at ca. 22 °C with 12-h light/dark cycles (Bradley et al., 2021). For each isolate fifteen fungal lawns were grown.

Seeds of each of four plant species (*E. lehmanniana*, *E. intermedia*, *B. curtipendula*, or *L. dubia*, obtained from Granite Seed, UT) were surface-sterilized and placed onto three replicate fungal lawns of each isolate. In addition, seeds of each plant were placed on three replicate plates of 2% MEA, composed of 20 g malt extract and 20 g of agar per liter, serving as negative controls. An additional three lawns of each isolate were grown for the same duration with no seeds present, serving as positive controls (Fig. 1).

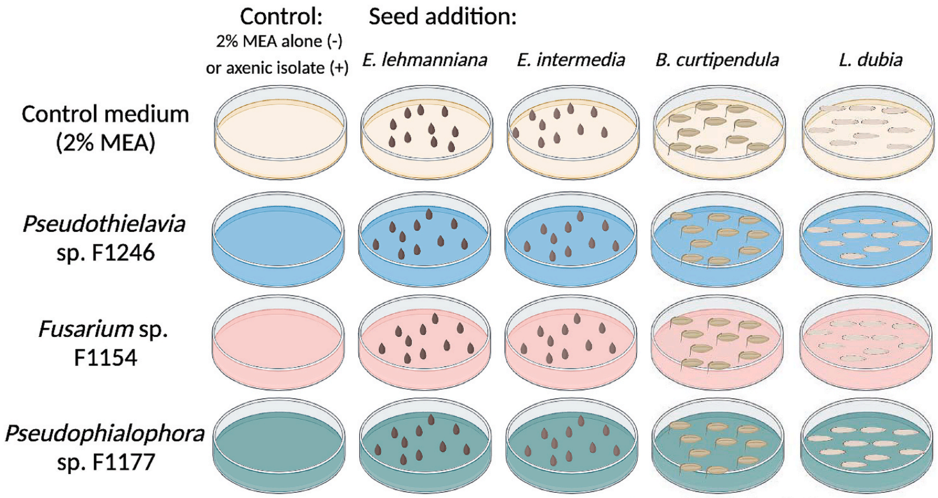
The number of seeds added ranged from 10 to 37 per plate. *Eragrostis lehmanniana* and *E. intermedia* seeds were too small to transfer using sterile forceps and were instead suspended in sterile water and pipetted onto fungal lawns, in some cases causing more than 10 seeds to be dispersed in the plate. Plates were incubated for 12 days at 22 °C with 12-h light/dark cycles, and germination was tracked daily.

Following seed germination, sterile transfer tubes (Spectrum Transfertube, 190195P) were used to remove five 6 mm fungal plugs from the site of interaction between fungal isolate on 2% MEA and germinating seeds, ensuring that each sample contained the same fungal biomass as measured by surface area. In samples that had fewer than five germinated seeds, samples were collected under non-germinated seeds. Fungal plugs were stored immediately in 1.5 mL microcentrifuge tubes at −80 °C, then lyophilized for 48 h (FreeZone 2.5 Plus, Labconco). Lyophilized samples were bead-beaten in 500 μL of sterile water with two 3.2 mm stainless steel beads (Mini-Beadbeater-96, BioSpec Products), dried in a SpeedVac (SPD11V, ThermoScientific), and stored at −80 °C. Controls (2% MEA media alone, and cultures of each fungal isolate with no seeds present) were handled as above, with plugs collected in a star pattern. We took care to isolate mycelium for this work such that we anticipate compounds from the fungus would be more prevalent than any exudates from the germinating seeds. Plugs were collected and processed together resulting in one technical replicate for each biological replicate.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS). Metabolites were extracted by suspending lyophilized and bead-beaten plugs in an 80:20 solution of MeOH:sterile MilliQ water (600 μl MeOH: 150 μl sterile MilliQ water). Samples were briefly vortexed, sonicated in a water bath for 30 min at 20–30 °C (FisherBrand CPX3800), and centrifuged for 10 min at 500 rpm (Eppendorf Centrifuge 5430) to pellet media and cellular debris. The supernatant (800 μl) was transferred to two 2 ml glass autosampler vials (400 μl each), dried in a vacuum centrifuge (Eppendorf Vacufuge plus), and stored at −80 °C. Samples were then reconstituted in 80:20 water: methanol (for reverse phase), and 50:50 water: acetonitrile (for hydrophilic interaction liquid chromatography), prior to injection into the instrument.

A ThermoScientific Vanquish Duo ultra-high performance liquid chromatography system (UHPLC) was used for liquid chromatography. Extracts were separated using a Waters ACQUITY HSS T3 C18 column for reverse phase (RP) chromatography separation and a Waters ACQUITY BEH amide column for hydrophilic interaction liquid chromatography (HILIC) separation. A pooled quality control (QC) sample was created by combining aliquots from the individual biological samples under investigation. Pooled QC-based area corrections were then employed to standardize ionization levels across the entire experiment and aid in the correction of various systematic biases inherent in large-scale metabolomics studies.

Samples were injected in a 1 μl volume column and eluted as follows: for RP, a gradient from 99% mobile phase A (0.1% formic acid in H_2O) to 95% mobile phase B (0.1% formic acid in methanol) over 16 min; for HILIC, a gradient from 99% mobile phase A (0.1% formic acid, 10 mM ammonium acetate, 90% acetonitrile, 10% H_2O) to 95% mobile phase B (0.1% formic acid, 10 mM ammonium acetate, 50% acetonitrile, 50% H_2O). Both columns were run at 45 °C with a flowrate of 300 μl/minute. A ThermoScientific Orbitrap Exploris 480 was used for spectral data collection with a spray voltage of 3500 V for positive mode (for RP) and 2500 V for negative mode (for HILIC). In both positive and negative ionization modes, samples were analyzed using higher-energy collision dissociation (HCD). The source parameters for the heated electrospray ionization (HESI) were set as follows: a spray voltage of 3.5 kV for positive mode and 2.5 kV for negative mode, capillary temperature at 350 °C, S lens RF level at 50 arbitrary units, and an auxiliary gas heater temperature of 350 °C. Full MS scan data were acquired at a resolving power of 120,000 FWHM at m/z 200, with a scanning range from m/z 65 to 975. The automatic gain control (AGC) target was set at 50%, with



Each plate depicted above represents one of three biological replicates.

Fig. 1. Three fungal endophytes isolated from roots of *Eragrostis lehmanniana*, shown on 2% malt extract agar (MEA), with a dendrogram showing their relative similarity in terms of metabolites (A). Metabolic characterization of each isolate is represented by the heatmap (B), where each column is an individual feature identified by LC-MS/MS and clustered by Pearson's correlation; colors indicate the relative abundance of each compound in the corresponding sample. Sample three of *Pseudophialophora* sp. F1177 is not included because the extraction efficiency was insufficient. One sample of *Fusarium* sp. F1154 is clustered separately due to sample variation. Grey samples indicate compound abundances for 2% MEA with no isolates present.

a maximum injection time of 100 ms. Data-dependent acquisition (DDA, dd-MS2) parameters used to obtain product ion spectra included a resolving power of 30,000 FWHM at m/z 200, AGC target of 50% ions with the maximum injection time set to auto, an isolation width of 1.2 m/z, and HCD collision energies of 20%, 40%, and 80%. The top ten intense features were selected per MS1 for the DDA experiment. The raw spectra are available as a MassIVE data set at ftp://massive.ucsd.edu/v02/MSV000093311.

LC-MS/MS spectra were analyzed in Compound Discoverer (ThermoScientific, version 3.3) using an adaptive curve alignment model, with a maximum retention time (RT) shift of 30 s and mass tolerance of 5 ppm. Features were picked at an intensity tolerance of 30% with a signal to noise threshold of 3. Features were then grouped with a mass tolerance of 5 ppm and a RT tolerance of 18 s using preferred ions of $[M-H]^{-1}$ for HILIC, and $[M+H]^{+1}$ for RP spectra. The features' presumed elemental compositions were determined by predicting them through the analysis of exact mass, isotopic pattern, fine isotopic pattern, and MS/MS data, with reference to the HighChem Fragmentation Library of reference fragmentation mechanisms. Subsequently, the MS/MS data were compared to the mzCloud database, based on both *m/z* values and fragment matching with a fragment mass tolerance of 10 ppm**.** Predicted compositions were assigned based on a mass tolerance of 5 ppm, a signal to noise threshold of 3, and a minimum spectral fit of 30% with fragment matching mass tolerance of 5 ppm. The ChemSpider database was then used to match features based on their *m/z* values with a mass tolerance of 5 ppm using the following databases: BioCyc, ChEBI, KEGG, NIST, PlantCyc, and the Yeast Metabolome Database. Both mzCloud and ChemSpider follow the level 2 identification criteria outlined by the Metabolomics Standards Initiative (MSI) to provide a putative annotation (Salek et al., 2013). Compounds with a putative annotation were then mapped to KEGG pathways (Kyoto Encyclopedia of Genes and Genomes; Kanehisa et al., 2023), and Metabolika pathways using predicted compositions and a mass tolerance of 5 ppm. Compounds were assigned their annotation based on the following priority order: mzCloud search, predicted composition, ChemSpider search, and Metabolika search. The complete workflow is summarized in the Supplementary Fig. S1.

Features that were given names and structures (i.e., were putatively annotated) were also compared against the mycotoxin database from Renaud and Sumarah (2018). Compounds were manually checked for duplication of features at multiple retention times and mis-assignments, and filtered for non-biologically relevant feature assignments. Non-gap-filled data were used to create Venn diagrams comparing feature presence/absence. Normalized gap-filled data were used for all other analysis to compare relative abundances across all samples.

A total of 2037 total features were picked following the criteria summarized above, including 550 features in the RP dataset and 1487 features in the HILIC dataset. The RP and HILIC data are available as supplementary data 1 and supplementary data 2, respectively. For RP chromatography 223 total compounds were annotated, including chemical names and structures for 117 features from mzCloud/ChemSpider and molecular formulas for 106 features based on predicted composition. For HILIC 401 total compounds were annotated, including chemical names and structures for 227 features from mzCloud/ChemSpider and molecular formulas for 174 features based on predicted composition. In the RP data set one sample (a control: *Pseudophialophora* sp. F1177 grown without seeds) was omitted due to poor extraction efficiency.

Statistical analyses. All statistical analyses were done in R (version 4.2.3). The proportion of seeds germinated per sample was calculated by dividing the number of germinated seeds by the initial number of seeds. Germination values were standardized to controls by subtracting the average number of seeds that germinated in the control plates for each plant species.

LC-MS/MS feature peak abundance values were normalized through cyclic loess normalization to account for systematic variability (Ejigu et al., 2013). Principal components were calculated from the normalized data and used for principal component analysis (PCA), and distance matrices were calculated via Bray-Curtis dissimilarity and used for permutational multivariate ANOVA (PERMANOVA) in the package vegan, with visualization in ggplot2 (Dixon, 2003; Villanueva and Chen, 2019).

To identify meaningful differences in compound expression between treatments, log2 fold change (L2FC) values were calculated for each identified feature comparing between (1) control samples containing only 2% MEA vs. fungal lawns of each isolate grown axenically; or (2) fungal lawns of each isolate grown axenically vs. fungal lawns of each isolate with seeds present. In addition, L2FC values were calculated by comparing seeds grown alone on 2% MEA against control media samples (2% MEA) to establish a baseline seed metabolome for each plant species. T-tests were performed according to the groups listed above to determine statistical significance. The Benjamini-Hochberg procedure was used to control the false discovery rate, which provided adjusted p-values (Padj.) to account for multiple testing (Benjamini and Hochberg, 1995; Hur et al., 2013). Values were adjusted for RP and HILIC data sets separately. Compounds were then filtered to select for those with an associated Padj. of <0.05, which we classified as upregulated or produced by the isolate when $L2FC > 0$, or downregulated or consumed by the isolate when $L2FC < 0$. Gibbs free energy (GFE), which represents the thermodynamic potential of a compound and thus provides insight into metabolic dynamics from an energy perspective, was calculated for all unique peaks assigned a molecular formula. Compound molecular formulas were used to calculate the nominal oxidation state of C (NOSC) using the following formula from LaRowe and Van Cappellen (2011):

$$NOSC = 4 - \frac{4c + h - 2o - 3n + 2s + 5p - z}{c}$$

Letters represent the stoichiometric numbers for the elements with the corresponding capital symbol (C, H, O, N, S, P), z is the charge of the molecule. The average NOSC was then used to calculate the GFE of a given compound using the following formula from LaRowe and Van Cappellen (2011):

$$\Delta G^{\circ}_{ox} = 60.3 - 28.5 * NOSC(kJper\ mol\ C)$$

Heatmaps indicating the relative abundance of each compound for a given sample were rendered in the R package pheatmap with rows and columns clustered by Pearson correlations (Kolde and Kolde, 2018). Of the total 624 compounds given a name/structure, 298 were found to be differentially expressed (Padj <0.05). We then extracted the international chemical identifier keys (InChIKeys), which are standardized, compacted text representations of chemical compounds, in order to classify the compounds into standard chemical taxonomy using ClassyFire (Djoumbou Feunang et al., 2016). We curated a list of compounds, 20 in total, based on their biological relevance and designated structure, comprising the 10 most positive and 10 most negative L2FC (with $P < 0.05$) found in isolates grown axenically. Additionally, we compiled a separate list of 10 compounds, featuring the 5 most positive and 5 most negative L2FC (with $P < 0.05$), identified in *Fusarium* sp. F1154 when in the presence of different seed types. By highlighting these specific compounds we aim to provide a concise and in-depth examination of the most critical metabolic changes relevant to this study.

3. Results

3.1. Fungal endophytes differed markedly in their metabolic capacity

In axenic culture, the metabolic capacity of fungal endophytes from roots of *E. lehmanniana* differed among isolates, but not in a manner consistent with their phylogenetic relatedness (see Zhang et al., 2006). *Fusarium* sp. F1154 and *Pseudophialophora* sp. F1177 had a more similar metabolic profile to each other than did either isolate to *Pseudothielavia* sp. F1246 (PERMANOVA; RP: $P < 0.001$, $R2 = 0.885$, $F = 17.894$; HILIC:

$P = 0.066$, $R2 = 0.453$, $F = 2.212$) (Figs. 2 and 3A, B).

All three fungal isolates shared 156 significantly upregulated features and 359 significantly downregulated features in comparison to respective media controls. However, significant differences were observed in terms of the substrates used for growth and those produced by the isolates (Fig. 3C). For example, *Fusarium* sp. F1154 consumed mostly organic oxygen compounds such as 1-O-acetyl-alpha-maltose. *Pseudophialophora* sp. F1177 consumed mostly organoheterocyclic compounds such as 4-methoxy-6-(prop-2-en-1-yl)-2H-1,3-benzodioxole, theophylline, and 1,5-anhydro-D-fructose. *Pseudothielavia* sp. F1246 consumed the most diverse range of compounds, including organoheterocyclic compounds, organic nitrogen compounds, and organic acids and derivatives such as xanthine and 2-oxobutyric acid (Fig. 3C). All isolates consumed compounds associated with primary metabolism such as hordenine, inosine-5′-monophosphate (IMP), 1,4-anhydro-1-(2,4-dioxo-1,2,3,4-tetrahydro-5-pyrimidinyl)-5-O-phosphonopentitol, beta-D-ethyl glucuronide, D-(+)-maltose, gluconic acid, and furaltadone (Table 2). All isolates consumed compounds of lower Gibbs free energy (GFE) and produced compounds of higher GFE (Fig. 3D).

The most abundant compound produced by *Fusarium* sp. F1154 was an organooxygen compound, 3′4′-dimethoxyacetophenone. Other highly abundant compounds included vanillic acid 4-sulfate, L-iditol, and 6-chloro-5-methyl-1H-1,2,3-benzotriazole (Fig. 3E, Table 2). *Pseudophialophora* sp. F1177 had the fewest total number of produced compounds. Its most abundant product was the primary metabolite L-iditol, followed by 6-chloro-5-methyl-1H-1,2,3-benzotriazole (Fig. 3E, Table 2). The most abundant compound produced by *Pseudothielavia* sp. F1246 was the organoheterocyclic compound 4-methoxy-6-(prop-2-en-1-yl)-2H-1,3-benzodioxole. *Pseudothielavia* sp. F1246 had a markedly distinct overall metabolic profile (Figs. 2B and 3A, B), with several compounds downregulated or present at low abundance in *Fusarium* sp. F1154 and *Pseudophialophora* sp. F1177 but upregulated in *Pseudothielavia* sp. F1246, including 3,4-dihydroxyphenylpropionic acid, 3,5-dihydroxybenzoic acid, trachelogenin, and 1-(dimethylamino)-2-propanyl acetate (Supplementary Table S1).

3.2. Fungi changed metabolite expression in response to seeds

Metabolic activity of the three isolates differed when grown with native grasses *B. curtipendula* and *L. dubia*, as compared to their metabolic profile when grown with *E. intermedia* and *E. lehmanniana*, or in the absence of any seeds (axenic control) (PERMANOVA; RP-1154 P <

Table 2

Twenty of the most abundant up- and downregulated compounds from three fungal isolates when grown axenically (no seeds present) as compared to control (2% MEA). Names were assigned according to Compound Discoverer, with biogeochemical class assigned by ClassyFire. Log 2 fold change (L2FC) values and adjusted P-values (Pval.adj) are reported for each corresponding isolate.

Feature ID	Name	Formula	Mass (Da)	Biogeochemical Class	GFE (kJ/mol)	L2FC			Pval.adj			RP/HILIC
						1154	1177	1246	1154	1177	1246	
feature0232	Hordenine	C10H15NO	174	Benzene and substituted derivatives	88.8	−10.5	−10.4	−1.5	0	0	0	RP
feature 0333	Vanillic acid 4-sulfate	C8H8O7S	270.1	Benzene and substituted derivatives	31.8	6.7	9.3	6.7	0.3	0.1	0	HILIC
feature 0843	Trachelogenin	C21H24O7	522.2	Furanoid lignans	73.9	0.2	2.4	6.3	0.1	0.4	0	HILIC
feature 0237	Inosine-5′-monophosphate (IMP)	C10H13N4O8P	414.1	Purine nucleotides	31.8	−10.5	−10.3	−8.4	0	0	0	RP
feature 0334	1-(Dimethylamino)-2-propanyl acetate	C7H15NO2	157.1	Carboxylic acids and derivatives	92.9	3	0.5	9	0.3	0.5	0	RP
feature 1358	Xanthine	C5H4N4O2	162.1	Carboxylic acids and derivatives	−8.1	−4.2	−3.3	−8.4	0.1	0.1	0	HILIC
feature 0007	2-Oxobutyric acid	C4H6O3	104	Keto acids and derivatives	60.3	−8.5	−8.4	−5.2	0.1	0.1	0	HILIC
feature 1479	1-O-acetyl-alpha-maltose	C14H24O12	497.9	Organooxygen compounds	60.3	−9.6	−8.1	−2.9	0.1	0	0	HILIC
feature 0037	1,4-Anhydro-1-(2,4-dioxo-1,2,3,4-tetrahydro-5-pyrimidinyl)-5-O-phosphonopentitol	C9H13N2O9P	381.9	Organooxygen compounds	41.3	−6.9	−3.6	−2.9	0	0	0	RP
feature 0413	beta-D-Ethyl glucuronide	C8H14O7	239.1	Organooxygen compounds	60.3	−10.9	−10.1	−4.9	0	0	0	HILIC
feature 1264	D-(+)-Maltose	C12H22O11	405.1	Organooxygen compounds	60.3	−11.5	−10.3	−4.8	0	0	0	HILIC
feature 0580	Gluconic acid	C6H12O7	205	Organooxygen compounds	50.8	−5.6	−4.4	−4.7	0	0	0	HILIC
feature 0380	L-Iditol	C6H14O6	191.1	Organooxygen compounds	69.8	6.5	6.3	3.5	0.2	0	0.1	HILIC
feature 0090	4-methoxy-6-(prop-2-en-1-yl)-2H-1,3-benzodioxole	C11H12O3	202	Benzodioxoles	75.8	−0.4	−0.2	10.6	0.3	0.5	0	RP
feature 1129	6-Chloro-5-methyl-1H-1,2,3-benzotriazole	C7H6ClN3	176.1	Benzotriazoles	49.6	5.2	5.7	3	0.3	0	0	HILIC
feature 0255	Furaltadone	C13H16N4O6	382.2	Furans	42.8	−4.6	−3.9	−5	0	0	0	HILIC
feature 1000	Theophylline	C7H8N4O2	188	Imidazopyrimidines	27.7	−4.4	−4	−6.8	0.1	0	0	HILIC
feature 1334	1,5-Anhydro-D-fructose	C6H10O5	166.1	Oxanes	60.3	−9.3	−8.8	−4.9	0	0	0	HILIC
feature 0063	3′4′- Dimethoxyacetophenone	C10H12O3	180.1	Phenylpropanoic acids	77.4	12.1	10	0.3	0	0.5	0.7	RP
feature 0453	3,4-Dihydroxyphenylpropionic acid	C9H10O4	172	Phenylpropanoic acids	66.6	−0.9	−1.3	7.3	0.1	0.1	0	HILIC

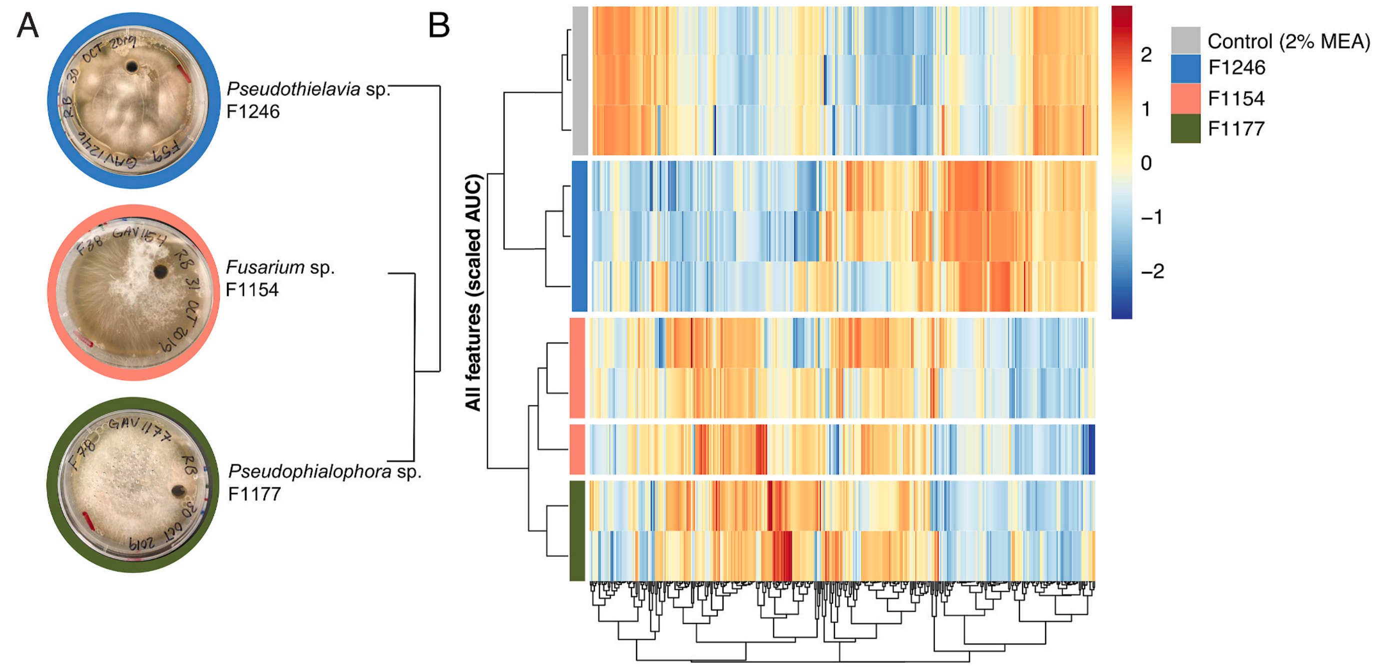


Fig. 2. Experimental design showing 2% MEA culture plates with each isolate grown axenically (column one), and in the presence of invasive and native seeds *Eragrostis lehmanniana, Eragrostis intermedia, Bouteloua curtipendula,* and *Leptochloa dubia.*

0.001, $R^2 = 0.435$, $F = 4.623$; RP-1177 $P = 0.017$, $R^2 = 0.327$, $F = 2.675$; RP-1246 $P = 0.004$, $R^2 = 0.446$, $F = 4.833$) (Fig. 4B–D). Our experimental design allowed us to relate these observations of metabolic activity to seed germination. The final percent germination for each grass-isolate combination is shown in Fig. 4A.

Some isolates had a more marked response to the presence of seeds than others. When comparing across isolates, *Fusarium* sp. F1154 had the most noticeable differences in expression due to the presence of seeds, with the other isolates not responding as strongly. Thus we will focus much of our attention in the results/discussion on *Fusarium* sp. F1154.

Fusarium sp. F1154 isolates decreased germination of most native seeds, while moderately increasing germination of the invasive grass (Fig. 4A). Upregulated metabolites present in *Fusarium* sp. F1154 with *E. lehmanniana* seeds present included the phenylpropanoid 3′,4′-dimethoxyacetophenone, and the organoheterocyclic compound 1-(2-furyl)-1,2-ethanediol. A fatty acyl, (±)9-HpODE, increased in the presence of both *Eragrostis* grasses. D-erythrose and anhydroecgonine decreased in the presence of both *Eragrostis* grasses but not in the presence of the other grass species. Phenactin and dipropylene glycol dimethyl ether were present in *Fusarium* sp. F1154 when it was grown in the presence of *B. curtipendula* and *L. dubia*.

In the presence of all grasses, *Fusarium* sp. F1154 utilized compounds associated with plant growth and primary metabolism. These compounds include IMP, γ-aminobutyric acid (GABA), and hordenine, which is involved in the plant defense response (Table 3 and its supplement). No common mycotoxins were characterized in *Fusarium* sp. F1154, but the mycotoxin beauvericin was detected in *Pseudophialophora* sp. F1177.

Fusarium sp. F1154 had higher relative abundance of caffeic acid when grown with the native grasses *B. curtipendula* and *L. dubia*, but a lower relative abundance in the presence of *Eragrostis* grasses (Supplementary Fig. S2A). Caffeic acid is involved in the phenylpropanoid biosynthesis pathway, secondary metabolite production, and lignin synthesis. To investigate whether caffeic acid influenced seed germination in isolation, we compared germination of seeds grown on fungal lawns of *Fusarium* sp. F1154 to those grown in the presence of caffeic acid. However, caffeic acid alone did not influence seed germination (Supplementary Fig. S2B).

Seed germination of all four grasses decreased relative to controls in the presence of *Pseudophialophora* sp. F1177 (Fig. 4A). Overall, the metabolite expression of this strain was similar to that of *Fusarium* sp. F1154 in the presence of seeds. *Pseudothielavia* sp. F1246 had mixed effects on germination of native grasses, increasing germination of *L. dubia* while decreasing germination of *B. curtipendula*. Of the identified up- and downregulated compounds for *Pseudothielavia* sp. F1246, most had relatively low log2 fold change rates compared to *Fusarium* sp. F1154 (Supplementary Table S1). Additional information for all compounds characterized in Tables 2 and 3 can be found in the supplementary information.

4. Discussion

Metabolomic characterization of three fungal endophytes revealed distinct metabolic capacities despite their shared ecological mode of residing within healthy root tissues of the same plant species. While certain metabolic traits persisted across isolates, likely owing to shared ancestral metabolism, our analyses delineated specific differences in each isolate when grown axenically on a standard medium, and in the presence of seeds of focal grasses during the germination phase.

All three isolates consumed nutrient-rich organic compounds and produced an array of complex secondary metabolites, including phenylpropanoids, benzenoids, and lipids. This pattern aligns with common observations from soils, where organisms favor compounds with lower GFE as energy sources. This preference reflects the general principle that organisms tend to break down or utilize compounds that can provide more energy for their metabolic needs (Fudyma et al., 2021; Wilson et al., 2021).

Interestingly, certain compounds present in plant-derived media, such as hordenine, inosine-5′-monophosphate, D-(+)-maltose, beta-D-ethyl glucuronide, and gluconic acid, were universally consumed by all isolates, indicating their shared utilization of these nutrient sources (Ramachandran et al., 2006; Moffatt and Ashihara, 2002). Conversely, the production of more complex secondary metabolites including the antimicrobial compound vanillic acid 4-sulfate, the secondary metabolite L-iditol, and the antioxidant 3′4′-dimethoxyacetophenone, emphasizes the metabolic versatility of these representative endophytes (see

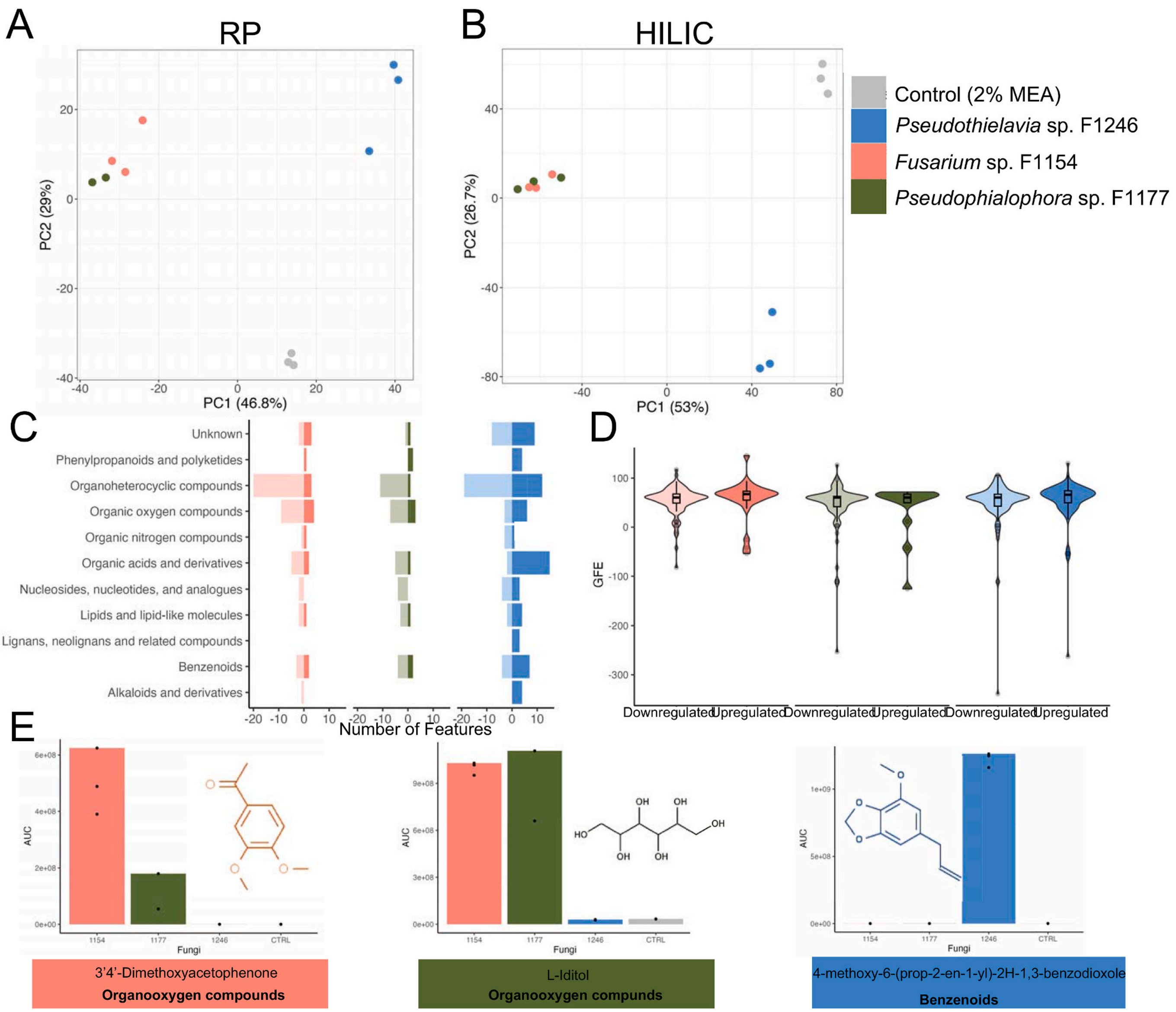


Fig. 3. Principal component analysis (PCA) representing LC-MS/MS features detected by reverse phase (RP) chromatography (A), and hydrophilic interaction liquid chromatography (HILIC) (B) showing differences in overall feature composition between each fungal isolate and 2% MEA negative control. Identified compounds which were upregulated (+) or downregulated (−) in each isolate compared to control were characterized by biogeochemical class and filtered based on Padj. <0.05 (C). Gibbs free energy (GFE) was calculated for up- and downregulated features given a chemical formula, represented by violin plots of each isolate for up- and downregulated features (D). Relative abundance was compared based on area under the curve (AUC) values for the most abundant compound reported for each isolate (E).

Koivistoinen et al., 2012; Rencoret et al., 2018; Barnhart-Dailey et al., 2019; Ibrahim et al., 2020 Singh et al., 2023).

Notable differences in substrate use and metabolite production among the strains provides a compelling lens through which to view the diversity of endophytes that can occur within plants. Despite sharing the same niche by growing within living plant tissues, endophytes may differ in their utilization of specific plant compounds, as suggested by our observation of differential use of some media components by the focal strains. In turn, their production of distinctive metabolites may speak to their defining distinct chemical phenotypes in the plant tissues they inhabit. For a detailed list of specific compounds that differed between isolates and relevant literature on these compounds, see the supplement to Table 2.

Our work was set against the backdrop of plant invasions, with a focus on evaluating whether endophytes associated with an invasive grass, *E. lehmanniana,* express metabolites that may inhibit germination of native grass species in the invaded range, and/or could promote germination of *E. lehmanniana* seeds. If so, it is plausible that endophytes of the invader may represent an important but cryptic element of the success of *E. lehmanniana* on the landscape. We observed a shift in overall metabolic expression of all three fungal endophytes when grown in the presence of *Eragrostis* seeds compared to native seeds. However, our analyses also showed that metabolites produced by the fungi generally were similar when exposed to native *E. intermedia* as well as invasive *E. lehmanniana*. The congruent metabolome profile generated from fungal associations with both the native and invasive *Eragrostis* species aligns with previous work in both invasive and natural ecosystems, where horizontally transferred fungi show preference for hosts of similar phylogenetic origin (see Gilbert and Webb, 2007; Higgins et al., 2007; Ricks and Koide, 2019; U'Ren et al., 2019; Wearn et al., 2012). We hypothesize that *E. lehmanniana* may have co-opted symbiotic partnerships with fungi already primed for *Eragrostis* due to the presence of *E.*

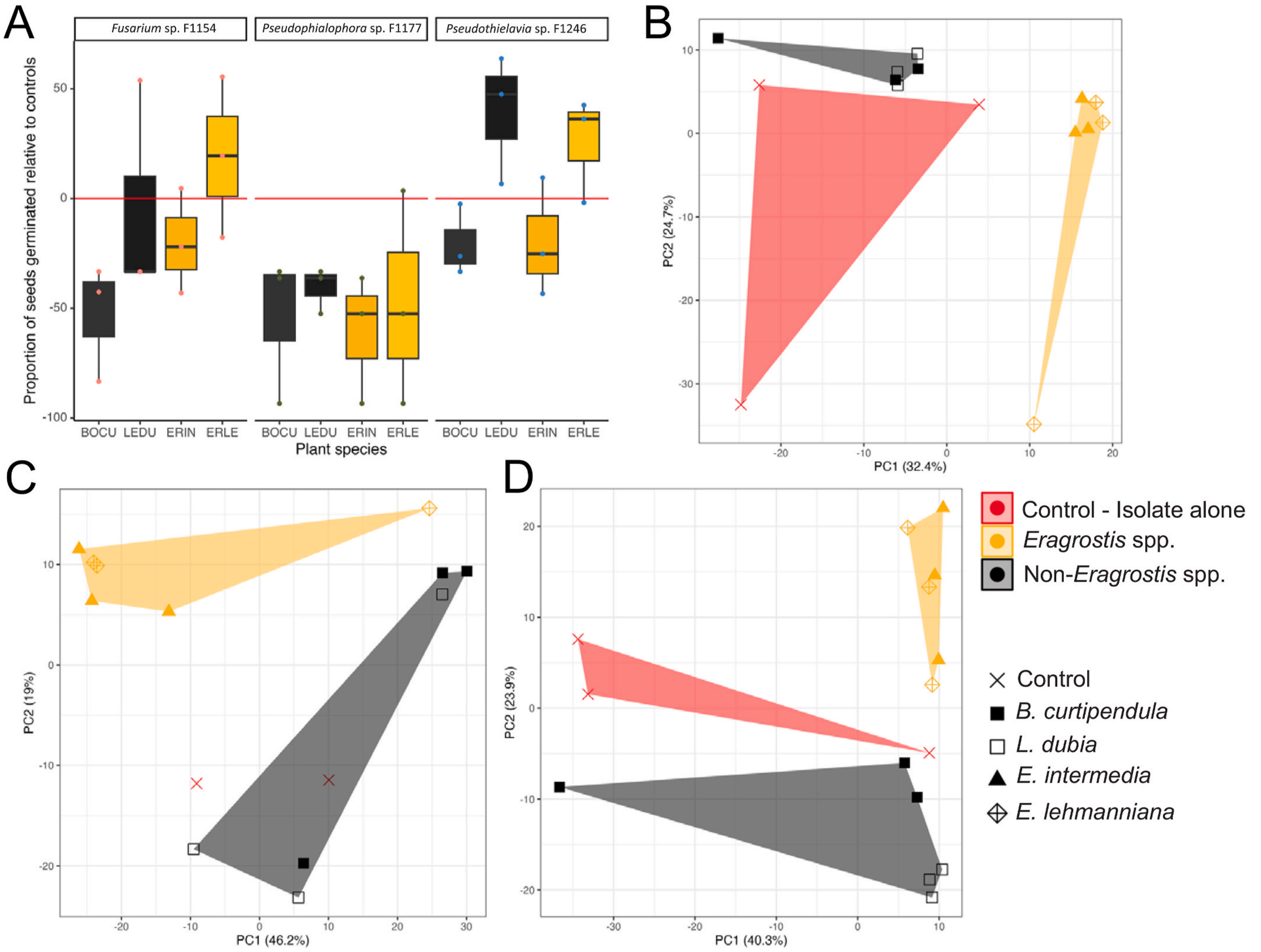


Fig. 4. Seedling germination for two *Eragrostis* grasses – *Eragrostis lehmanniana* (ERLE, invasive) and *E. intermedia* (ERIN, native) – as well as two additional native grasses (*Bouteloua curtipendula,* BOCU; *Leptochloa dubia,* LEDU) in the presence of each fungal isolate (A). Percent germination was scaled relative to the number of seeds that germinated in controls (2% MEA). Values > 0 indicate that the sample had greater germination in the presence of the associated isolate relative to control, and values < 0 indicate that the sample had decreased germination in the presence of the isolate relative to the control. PCA ordination plots of LC-MS/MS features from RP chromatography of *Fusarium* sp. F1154 (B), *Pseudophialophora* sp. F1177 (C), and *Pseudothielavia* sp. F1246 (D) in the presence of four seed types, including *B. curtipendula* and *L. dubia*; *Eragrostis* spp., including native *E. intermedia* and invasive *E. lehmanniana* (yellow); and axenically grown fungal isolates (red).

intermedia in the region, congruent with our observation that endophytes of *E. lehmanniana* appear to represent lineages of fungi from the American southwest, not from southern Africa (Arnold, in prep.). Further investigations are warranted to examine mechanisms underlying these symbiotic partnerships and their potential contributions to invasion dynamics. By investigating the metabolic profiles of endophytes associated with *E. lehmanniana* and their interactions with native and invasive grasses, this study contributes to our understanding of the metabolic strategies employed by endophytes in potentially promoting or altering the demography of plants.

The compounds identified in our assays with seeds and fungi are unlikely to be solely plant products. When compared to the metabolome of seeds alone, the focal compounds were low in abundance (L2FC $< \pm 0.05$). Thus, we interpret the upregulated compounds as products of the interaction between the fungi and the plant tissue. The specific compounds observed in the metabolomes of these fungi can foster new hypotheses regarding their ecological interactions.

For example, upregulated compounds of *Fusarium* sp. F1154 in the presence of *Eragrostis* seeds at the germination phase include the compound 9-HpODE, a hydroperoxy polyunsaturated fatty acid that is thought to play a role in plant defense and can alter the secondary metabolism of some fungi, including *Fusarium* spp. (Fischer and Keller, 2016). In studies of Fusarium wilt caused by *Fusarium oxysporum,* 9-HpODE has been identified as a potential mediator of plant-fungal signaling, altering secondary metabolism linked to defense responses (Beccaccioli et al., 2022; Fernandes and Ghag, 2022). While the grasses studied here do not suffer from Fusarium wilt, such defense response signals may benefit seeds during the germination stage, in part because *Fusarium* species are among the most prevalent seed-associated fungi in the area (Hamzazai, 2020).

In turn, downregulated compounds of *Fusarium* sp. F1154 in the presence of *Eragrostis* seeds include 3′4′-dimethoxyacetophenone, a byproduct of the breakdown of guaiacyl lignin, commonly associated with C4 grass lignin synthesis through the phenylpropanoid biosynthesis pathway (Kabuyah et al., 2012; Saiz-Jimenez and De Leeuw, 1986). In addition to lignin synthesis, phenylpropanoids can increase ultraviolet (UV) stress tolerance in plants (Singh et al., 2023), which may contribute to *E. lehmanniana*'s success in grasslands with little canopy cover. Additional information and relevant literature on compounds influencing *Fusarium* sp. F1154 in the presence of native and invasive grasses can be found in the supplement to Table 3. An upstream compound in the phenylpropanoid biosynthesis pathway is the

Table 3

Ten most up- and downregulated compounds expressed by isolate *Fusarium* sp. F1154 in the presence of grass seeds (invasive, *E. lehmanniana* (ERLE); native, *E. intermedia* (ERIN), *B. curtipendula* (BOCU), *L. dubia* (LEDU)) as compared to axenic growth (no seeds present). Compound names were assigned according to Compound Discoverer with biogeochemical class assigned by ClassyFire. Log 2 fold change (L2FC) values were calculated for *Fusarium* sp. F1154 in the presence of each seed type with associated adjusted P values (Pval.adj).

Feature ID	Name	Formula	Mass (Da)	Biogeochemical Class	GFE (kJ/mol)	L2FC				Pval.adj			LEDU	RP/HILIC
						F1154				F1154				
						ERLE	ERIN	BOCU	LEDU	ERLE	ERIN	BOCU		
feature 0090	Phenacetin	C10H13NO2	179.0946	Benzene and substituted derivatives	77.4	−0	−0.86	0.043	0.041	0.88	0.29	0.022	0.011	RP
feature 0195	Hordenine	C10H15NO	165.23	Benzene and substituted derivatives	88.8	−0.63	−3.61	−0.59	−0.66	6E-04	0.18	0.003	3E-04	RP
feature 0489	(−±)9-HpODE	C18H32O4	312.4	Fatty Acyls	98.3	2.142	2.22	0.684	1.095	0.21	0.04	0.55	0.427	HILIC
feature 0189	Inosine-5′-monophosphate (IMP)	C10H13N4Na2O9P	410.18	Purine nucleotides	31.8	−0.61	−0.41	−0.6	−0.6	7E-04	0.19	7E-04	7E-04	RP
feature 0001	γ-Aminobutyric acid (GABA)	C4H9NO2	103.0633	Carboxylic acids and derivatives	74.55	−0.08	−1.4	−0.04	−0.06	0.003	0.18	0.617	0.082	RP
feature 1381	D-Erythrose	C4H8O4	120.1	Organooxygen compounds	60.3	−6.05	−5.26	0.133	0.114	0.058	0.02	0.811	0.768	HILIC
feature 0215	Dipropylene glycol dimethyl ether	C8H18O3	162.1256	Organooxygen compounds	103.05	0.053	−4.52	0.026	0.029	0.104	0.18	0.014	0.006	RP
feature 0337	1-(2-Furyl)-1,2-ethanediol	C6H8O3	128.0473	Heteroaromatic compounds	69.8	0.355	−3.96	0.279	0.319	5E-05	0.18	0.067	1E-04	RP
feature 0256	Anhydroecgonine	C9H13NO2	167.0947	Pyrrolidines	79.3	−0.11	−2.48	0.039	0.077	0.039	0.34	0.552	0.028	RP
feature 0293	3′,4′-Dimethoxyacetophenone	C10H12O3	180.0786	Phenylpropanoic acids	77.4	0.61	−2.76	0.738	0.726	4E-04	0.18	0.015	0.001	RP

hydroxycinnamic acid, caffeic acid. We examined the effect of this compound on seed germination, but under *in vitro* conditions it did not influence germination rates. It is plausible that it is important under field conditions. It also may accompany or be less important than other signals, such as hormones or small secreted proteins, which could be the drivers of germination responses (see Castelluccio et al., 1995). Additional research is needed since subsequent experiments showed that *Fusarium* sp. F1154 still enhanced the germination rates of *E. lehmanniana*, Fig S2.

Overall, our study provides new information on the breadth and diversity of fungal metabolism while illuminating the spectrum of compounds that may be synthesized within fungi that occur in this common ecological niche. Importantly, untargeted metabolomics offers identification of key compounds and highlights their relative abundance, and in the case of the present study points to knowledge gaps in the study of fungal metabolites. For example, our literature review revealed that only 13 out of 624 compounds identified here had been cataloged previously in research focused on fungi (Birkinshaw et al., 1936; Guo et al., 2000; Nielsen et al., 2011; Hemtasin et al., 2016). Moreover, only seven of these compounds were documented in the Natural Product Atlas (NPA) of fungal and bacterial products. Thus, our study not only deepens our understanding of fungal metabolism and metabolic capacity by exploring the metabolomes of endophytes, but also sheds light on the remarkable diversity of compounds that may be important within this ecological framework. These findings underscore a cryptic role played by the fungal metabolome in shaping plant demography, and highlight how such interactions can significantly impact the success of invasive species. Under a changing climate, *E. lehmanniana* is projected to continue to spread outside of its current range (Schussman et al., 2006). Our study delves into potential mechanisms underlying interactions between endophytic fungi and native and invasive hosts, highlighting a cryptic element of plant invasions in ecosystems worldwide.

Author contributions

Conceived of study: MMT, AEA, TAP. Provided materials and support for research: MMT, AEA, JSF, CR. Gathered and analyzed data: TAP, RGB, MMT and AEA. Provided funding: MMT, AEA, TAP. Wrote manuscript: TAP, MMT, AEA, with input from all authors.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank Faith Mendoza and Megan Nickerson for laboratory assistance, Ming-Min Lee for logistics support, and Christian Ayala-Ortiz and Viviana Freire Zapata for programming and computational assistance. We thank Krishna Parsawar and Lawrence Walker of the University of Arizona Mass Spectrometry Core for their analytical chemistry expertise and data acquisition via LC-MS/MS. This study represents a portion of the thesis work of TAP, a Fellow supported by the National Science Foundation NRT-BRIDGES program in Ecosystem Genomics at the University of Arizona. Additional support was provided by United States Department of Agriculture - National Institute of Food and Agriculture, USDA NIFA) awards to AEA and colleagues for research regarding arid-land microbiomes (ARZT-1361340-H25-242) and to AEA for support of the Robert L. Gilbertson Mycological Herbarium (ARZT-1259370-S25-200). Additionally, this research was supported, in part, by the Department of Energy, Office of Science Biological and Environmental Research Grant DE-SC0021349, awarded to MMT. We thank the UArizona College of Agriculture, Life and Environmental Sciences, the Gilbertson Herbarium, the School of Plant Sciences, and the Ecosystem Genomics Graduate Interdisciplinary Program for additional support. This material is based upon work supported by the National Science Foundation under Grant No. DGE-2022055. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation.

Appendix Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funeco.2023.101327.

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