

Genomic and Transcriptomic Analyses Reveal Mechanisms Associated with Pathogenesis of *Fusarium annulatum* and *F. commune* in Nursery-Grown Conifer Seedlings

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Abstract

In this study, we used coniferous and non-coniferous hosts to assess the pathogenicity of a *Fusarium annulatum* isolate derived from southwestern white pine (*Pinus strobiformis*), along with *F. commune* isolates derived from coniferous and herbaceous, non-conifer hosts. All isolates of both *Fusarium* spp. were found to be pathogenic to conifer hosts. For the tested non-coniferous hosts, *F. commune* isolates were found to be pathogenic to both rice and tomato, whereas *F. annulatum* was pathogenic to rice and potentially less aggressive on tomato. To investigate the molecular basis of pathogenicity, we identified differentially expressed pathogenicity-/virulence-associated genes by inoculating loblolly pine (*P. taeda*) seedlings with isolates of conifer-derived *F. commune* (collected from ponderosa pine [*P. ponderosa*]) and *F. annulatum* (collected from southwestern white pine), which were all previously confirmed to be pathogenic to loblolly pine in our assays. Seedlings were harvested at 12, 24, and 48 h postinoculation for transcriptomic analyses to identify pathogen genes

associated with early infection of the host. Among the upregulated in planta (UIP) genes, we identified putative pathogenicity-/virulence-associated genes, including secreted effectors, secondary metabolite gene clusters involved in mycotoxin biosynthesis, and carbohydrate-active enzymes. To identify the putative conifer pathogenicity profiles of these potential conifer pathogens, we compared these UIP genes with the predicted proteomes of 17 conifer-associated *Fusarium* spp. isolates. These putative pathogenicity profiles did not definitively correspond with pathogenicity on coniferous versus herbaceous hosts but rather aligned with *Fusarium* species complexes. A subset of these shared UIP genes may aid in the development of detection methods for conifer-specific pathogens based on *Fusarium* species complexes.

Keywords: conifer seedlings, *Fusarium annulatum*, *Fusarium commune*, pathogenicity, species complexes, transcriptomics, virulence factors

Warming climates, increased droughts, and more intense wildfires have created a growing demand for large-scale reforestation, which relies heavily on nursery-grown seedlings (Fargione et al. 2021). Conifer nurseries play an essential role in forest restoration and reforestation, supporting timber production, green-energy projects, and other ecological services. In North America, the Southeast, Southwest, and Pacific Northwest are key regions for conifer seedling production, with loblolly pine (*Pinus taeda*), ponderosa pine (*P. ponderosa*), and Douglas-fir (*Pseudotsuga menziesii*) as the predominant conifer seedling species, respectively. Due to limited seed availability, maximizing seedling yield under nursery settings is critical. However, seedling production is frequently hampered by damping-off and root disease pathogens, such as *Cylindrocarpon* spp. (syn. *Ilyonectria* and *Neonectria*),

Pythium spp., *Phytophthora* spp., and, most importantly, *Fusarium* spp. (Dumroese and James 2005; Dumroese et al. 2000; Weiland 2021; Weiland et al. 2013). A previous survey of federal, state, and private forest tree nurseries reported that losses caused by *Fusarium* pathogens can reach up to 40% in some years, which is also associated with high management costs (Dobbs et al. 2023). Because damping-off is most detrimental during the first 4 to 6 weeks following seed germination of conifer seedling growth (Cram 2003), it is critical to investigate early infection processes to identify pathogenicity/virulence genes involved in host specialization and disease development (Ma et al. 2013; Thatcher et al. 2016; Tu et al. 2023).

Many phytopathogenic *Fusarium* spp., such as *F. oxysporum* and *F. graminearum*, have been extensively studied to elucidate the mechanisms underlying disease development on agriculturally and/or economically important host species (Gordon 2017). However, understudied *Fusarium* spp. also pose a major threat to agricultural systems because their host ranges and virulence are not well understood. Recent studies have focused on understudied *Fusarium* spp., such as *F. commune* and *F. annulatum*, that were isolated from both conifers and non-conifers (Bustamante et al. 2022; Dobbs et al. 2023; Hamini-Kadar et al. 2010; Husna et al. 2021; Parra et al. 2022; Stewart et al. 2012). Although pathogenicity assays on limited hosts have shown that these understudied *Fusarium* spp. can cause damping-off and root disease (Hamini-Kadar et al. 2010; Husna et al. 2021; Mirghasempour et al. 2022; Stewart et al. 2012), effective control measures remain underdeveloped. Investigating mechanisms of pathogenicity for understudied *Fusarium* spp. is essential for developing effective management strategies, diagnostic tools for rapid detection/identification of pathogens, and resistance breeding approaches in agricultural and nursery settings.

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Multiple *Fusarium* spp. causing damping-off and root disease have been documented in conifer nurseries (Dobbs et al. 2023), yet the evolutionary history and specific mechanisms underlying conifer pathogenesis remain largely unexplored. Previous studies have determined that species within *Fusarium* species complexes share suites of potential pathogenicity genes (Dobbs et al. 2023, 2024a). In some *Fusarium* spp., such as *F. oxysporum*, entire chromosomes and even single genes within accessory chromosomes have the potential to be shared among other *F. oxysporum* strains and *Fusarium* spp. to confer pathogenicity (Bertazzoni et al. 2018; Li et al. 2020a, b). A better understanding of these pathogenicity genes and accessory chromosomes among conifer-pathogenic *Fusarium* spp. could inform proactive strategies to manage *Fusarium* pathogens before disease outbreaks occur (Burkhardt et al. 2019; Niehaus et al. 2016).

Globalization and changing environmental conditions are accelerating the emergence of both known and novel plant-pathogenic fungi (Kim et al. 2021; Kuang et al. 2022; Santini et al. 2018). Comparative genomics and transcriptomics offer powerful tools to develop detection and mitigation strategies, especially where effective management is currently lacking (Karunarathna et al. 2021; Yilmaz et al. 2021). For example, identifying genes involved in pathogenic lifestyles enhances our understanding of pathogen biology and supports the development of early detection tools (Ibarra Caballero et al. 2020; Ye et al. 2018). A previous study identified putative conifer-specific pathogenicity/virulence factors, including predicted secreted proteins and effectors, virulence-associated proteins, and secondary metabolites (e.g., mycotoxins, such as fumonisins and enniatin), in isolates of *Fusarium* spp. from conifer hosts (Dobbs et al. 2023, 2024a). These findings suggest that these predicted conifer-pathogenic *Fusarium* spp. have the capacity to cause disease based on their repertoire of pathogenicity/virulence genes, but it has not been verified whether these genes are expressed during host infection.

We hypothesize that conifer-pathogenic *Fusarium* spp. exhibit expression of shared pathogenicity/virulence genes that can be used to predict and rapidly identify *Fusarium* pathogens of conifers. Our overarching goal was to elucidate a genetic profile associated with conifer virulence in *Fusarium* spp. that can be used to

classify *Fusarium* isolates and distinguish conifer pathogens from non-pathogens. The specific objectives of this study were to (i) evaluate the pathogenicity/virulence of conifer- and non-conifer-collected *F. commune* isolates and conifer-collected *F. annulatum* on both coniferous (Douglas-fir, ponderosa pine, and loblolly pine) and herbaceous (rice [*Oryza sativa*] and tomato [*Solanum lycopersicum*]) hosts; (ii) analyze differentially expressed genes in representative conifer-collected *F. commune* (from ponderosa pine) and *F. annulatum* (from southwestern white pine [*P. strobiformis*]) isolates to identify genes involved in conifer pathogenicity/virulence; and (iii) use predicted proteomes of conifer-associated *Fusarium* spp. to identify upregulated genes in *F. commune* and *F. annulatum* during colonization of conifer hosts.

Materials and Methods

Fusarium spp. isolates and pathogenicity assays

To confirm conifer pathogenicity, five *F. commune* isolates (F10.2.2, F13.4.1, JICPIPO1, B62, and MiAE120) and one *F. annulatum* isolate (NASWWP3) were tested on three coniferous (Douglas-fir, ponderosa pine, and loblolly pine) and two herbaceous (rice and tomato) hosts (Table 1) (Dobbs et al. 2024a). The *F. annulatum* isolate (NASWWP3) was collected in a previous study (Dobbs et al. 2023) from a symptomatic southwestern white pine seedling, whereas the *F. commune* isolates were collected from both conifer (Dobbs et al. 2023) and non-conifer (Dobbs et al. 2024a) hosts (Table 1). Pathogenicity assays were conducted following Lai et al. (2020) in which seedling's roots were clipped and dipped in a spore suspension to ensure fungal colonization of host roots.

The inoculum for each isolate was produced in Erlenmeyer flasks containing 250 ml of potato dextrose broth (PDB) shaken at 90 rpm at room temperature. After 10 days in the dark, the mycelium was removed from the broth by straining through two layers of sterile cheesecloth. Spores (conidia) from the filtrate were pelleted through cold centrifugation at $5,000 \times g$ for 5 min at 10°C, and the final volume was adjusted with sterile water to obtain a concentration of 1×10^6 spores/ml as determined by a hemocytometer. Pathogenic-

TABLE 1. Isolates of *Fusarium* spp. used in this study (modified from Dobbs et al. 2024a)

<i>Fusarium</i> spp.	Isolate	GenBank accession number	<i>Fusarium</i> species complex ^a	Host
<i>F. tricinctum</i>	MES5	JAVTNN000000000	<i>F. tricinctum</i> (FTSC)	Ponderosa pine (<i>Pinus ponderosa</i>)
<i>F. avenaceum</i>	NC74	JAVTNV000000000	FTSC	Loblolly pine (<i>P. taeda</i>)
<i>F. flocciferum</i>	31MAPSF17A	JAVTNF000000000	FTSC	Ponderosa pine
<i>F. torulosum</i>	DCN062.2.3H	JAVTNI000000000	FTSC	Douglas-fir (<i>Pseudotsuga menziesii</i>)
<i>F. fujikuroi</i>	SC5	JAVTNY000000000	<i>F. fujikuroi</i> (FFSC)	Loblolly pine
<i>F. annulatum</i>	NASWWP3 ^{b,c}	JAVTNU000000000	FFSC	Southwestern white pine (<i>P. strobiformis</i>)
<i>F. fredkrugeri</i>	SC7	JAVTNZ000000000	FFSC	Loblolly pine
<i>F. lactis</i>	MES8	JAVTNP000000000	FFSC	Ponderosa pine
<i>F. verticillioides</i>	NAGrPIPO5	JAVTNR000000000	FFSC	Ponderosa pine
<i>F. clavum</i>	NAGrPIST2	JAVTNS000000000	<i>F. incarnatum-equiseti</i> (FIESC)	Southwestern white pine
<i>F. ipomoeae</i>	NC8	JAVTNW000000000	FIESC	Loblolly pine
<i>F. luffae</i>	CC	JAVTNH000000000	FIESC	Douglas-fir
<i>F. commune</i>	F10.2.2 ^b	JAVTNJ000000000	<i>F. nisikadoi</i> (FNSC)	Douglas-fir
<i>F. commune</i>	F13.4.1 ^b	JAVTNK000000000	FNSC	Douglas-fir
<i>F. commune</i>	JICPIPO1 ^{b,c}	JAVTNL000000000	FNSC	Ponderosa pine
<i>F. commune</i>	B62 ^b	—	FNSC	Rice (<i>Oryza sativa</i>)
<i>F. commune</i>	MiAE120 ^b	—	FNSC	Tomato (<i>Lycopersicon esculentum</i>)
<i>F. solani</i>	NAPSME6.1	JAVTNT000000000	<i>F. solani</i> (FSSC)	Douglas-fir
<i>F. falciforme</i>	SC35	JAVTNX000000000	FSSC	Loblolly pine
<i>F. inflexum</i>	JTHABCO3.1	JAVTNM000000000	<i>F. oxysporum</i> (FOSC)	White fir (<i>Abies concolor</i>)
<i>F. oxysporum</i>	MES6	JAVTNO000000000	FOSC	Ponderosa pine

^a Six *Fusarium* species complexes: FTSC (*F. tricinctum* species complex) including *F. acuminatum*, *F. avenaceum*, *F. flocciferum*, and *F. torulosum*; FFSC (*F. fujikuroi* species complex) including *F. annulatum*, *F. fredkrugeri*, *F. fujikuroi*, *F. lactis*, and *F. verticillioides*; FIESC (*F. incarnatum-equiseti* species complex) including *F. clavum*, *F. ipomoeae*, and *F. luffae*; FNSC (*F. nisikadoi* species complex) including *F. commune*; FSSC (*F. solani* species complex) including *F. falciforme* and *F. solani*; and FOSC (*F. oxysporum* species complex) including *F. inflexum* and *F. oxysporum*.

^b Used for pathogenicity assays.

^c Used for transcriptomic analyses.

ity assays consisted of two experiments, each with 15 replicates (seedlings) per isolate ($n = 30$). Seeds were surface disinfested using 10% commercial bleach (0.75% NaOCl) for 5 min in a beaker and agitated with a stir bar to ensure adequate coverage of all seeds. Bleach-disinfested seeds were germinated on sterile, moist filter paper within a Petri dish wrapped with Parafilm to prevent evaporation while allowing for gas exchange. At 7 days post-germination, seedlings were removed from the Petri dish and root tips were severed with a sterile scalpel to provide an infection court for the fungal spores (Lai et al. 2020). Germinants were placed in either a spore suspension containing one of the fungal isolates or sterile, distilled water (control) for 5 min. Subsequently, each treated germinant was transferred to a 50-ml conical tube filled with sterile, soilless potting mix and vermiculite (1:1). Sterile water was added as needed to maintain adequate moisture, and tubes were incubated in growth chambers under fluorescent, diurnal light (12-h day/night cycle) at 25°C. Seedlings were harvested after 14 days and examined for mortality and above-/belowground symptoms, then *Fusarium* was re-isolated onto potato dextrose agar (PDA) for confirmation of infection. To assess the virulence of each isolate on each host, disease was scored based on a 0 to 3 scale, where 0 indicated a non-symptomatic or healthy seedlings and 3 indicated disease-caused seedling mortality. Seedlings were rated for both above- and belowground symptoms (Fig. 1). Above- and belowground scores were combined for a total 0 to 6 scale. A disease severity index (DSI) was calculated ($DSI(\%) = [\text{sum}(\text{class frequency} \times \text{score of rating class})] / [(\text{total number of plants}) \times (\text{maximal disease index})] \times 100$) for each host separately and analyzed by the comparison with the control to show the overall disease caused by each pathogen on each host. All statistical analyses were conducted in RStudio (Posit Team 2023) using R v.4.3.2 (R Core Team 2023). An aligned rank transform (ART) two-way analysis of variance (ANOVA) was performed in RStudio using the ‘ARTool’ package (Wobbrock et al. 2011) on the ordinal ranked data, analyzing the total disease scores, to test the differences between the fixed effects of the isolates. A post-hoc analysis was performed using emmeans with a Holm

adjustment after the ART ANOVA to conduct pairwise comparisons of means among isolates.

Sample collection for transcriptomics

To identify virulence-associated genes during early infection of conifer seedlings, 3-week-old loblolly pine seedlings were inoculated with a 1×10^6 conidial suspension of either one *F. commune* isolate (JICPIPO1) collected from a symptomatic ponderosa pine seedling or one *F. annulatum* isolate (NASWWP3) collected from a symptomatic southwestern white pine seedling (Table 1) or sterile, distilled water, which served as a control. For the preparation of conidial suspensions, each isolate was first grown on 1/4-strength PDA at 25°C for 3 days. Hyphal tips were transferred to 500 ml of PDB and shaken at 90 rpm for 10 days at room temperature. Spores were separated from the mycelium by filtration through two layers of sterile cheesecloth. Spores were pelleted from the PDB in 50-ml, conical centrifuge tubes with $5,000 \times g$ centrifugation at 10°C for 5 min. The supernatant was removed, leaving the spore pellet, which was resuspended in sterile, distilled water. Solutions were diluted to 1×10^6 spores/ml using a hemocytometer for calibrations. Each loblolly pine seedling was inoculated by cutting the root tips with a sterile scalpel to provide an infection court and soaking the seedling roots in the spore solutions for 5 min before transferring each inoculated or mock-inoculated seedling to a conical cone-tainer (3.8×13.97 ; Stuewe and Sons) filled with soilless potting mix (PRO-MIX BX) (Lai et al. 2020). Seedlings were grown in a growth chamber at 25°C with a 12-h day/night cycle. A time-series approach was used to assess any early changes in gene expression during pathogenesis, with samples collected at 12, 24, and 48 h postinoculation intervals (hpi). For each *Fusarium* isolate and control, three seedlings were grown and harvested at each time point with three replicates ($n = 9$ per time point; 27 seedlings per treatment). Roots and a small portion of the stem above the root collar were harvested from each seedling. Samples from the three seedlings collected at each time point were pooled together in 2-ml screw-cap tubes to obtain approximately 0.1 g of wet weight and flash frozen using

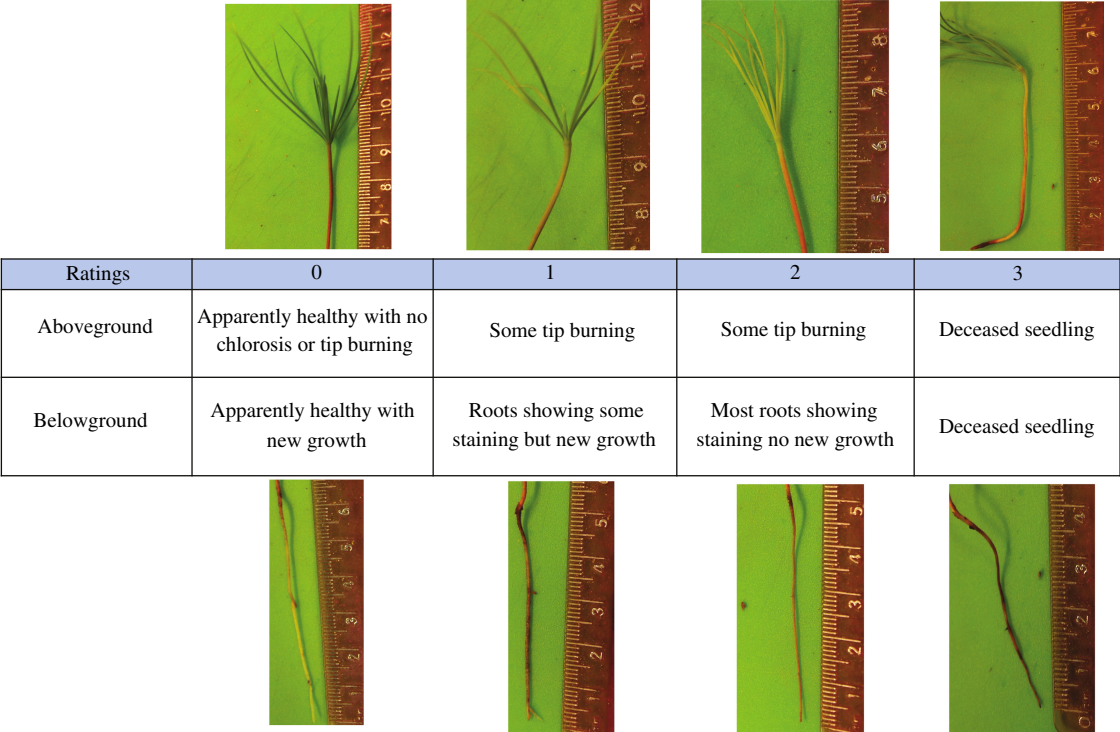


Fig. 1. Rating system used to calculate the disease severity of *Fusarium commune* and *F. annulatum* pathogenicity assays on coniferous (ponderosa pine [*Pinus ponderosa*], loblolly pine [*P. taeda*], and Douglas-fir [*Pseudotsuga menziesii*]) and herbaceous (rice [*Oryza sativa*] and tomato [*Solanum lycopersicum*]) seedlings.

liquid N₂. Root segments were stored at −80°C for subsequent RNA extraction.

RNA extraction

Total RNA was extracted using methods similar to those of Ata et al. (2022). Mycelial tip cultures from actively growing cultures on PDA of *F. commune* and *F. annulatum* isolates were grown in PDB for 3 days prior to RNA extraction. Wet mycelial samples were separated from the PDB using two layers of sterile cheesecloth and flash frozen in liquid N₂. Frozen mycelium and frozen root segments were each ground to a fine powder with a cold mortar and pestle, and approximately 0.1 g of ground mycelium/tissue was transferred to 2-ml microcentrifuge tubes. A 1-ml aliquot of warm RNA extraction buffer (OPS Synergy CTAB extraction buffer) with 2% polyvinyl pyrrolidone and 1% β-mercaptoethanol was added to each sample. Samples were homogenized in a FastPrep-24 (M.P. Biomedicals LLC) at 5.5× speed for 1 min, then incubated for 20 min at 65°C. An equal volume of chloroform/isoamyl alcohol (24:1) was mixed with the extraction buffer through inversion. Samples were centrifuged at 10,000 × g for 15 min, and the upper (aqueous) phase (approximately 400 µl) was transferred to new 2-ml microcentrifuge tubes. A quarter volume (approximately 100 µl) of 10 M lithium chloride was added to each sample and mixed by inversion. Total RNA was precipitated overnight at 4°C, and samples were centrifuged at 10,000 × g for 30 min at 4°C. The supernatant was discarded, and the RNA pellet was air dried before it was dissolved in 400 µl of SSTE buffer (1 M NaCl, 0.5% SDS, 10 mM Tris-HCl [pH 8.0], and 1 mM EDTA [pH 8.0] in nuclease-free water) preheated to 65°C. An equal volume of chloroform/isoamyl alcohol (24:1) was mixed with the extraction buffer through inversion. Samples were centrifuged for 15 min at 10,000 × g, and the aqueous phase was transferred to new 2-ml microcentrifuge tubes. A 3× volume of 100% ethanol was added to samples and mixed by inversion. Samples were left to precipitate overnight at −80°C before centrifugation at 10,000 × g at 4°C for 30 min. The supernatant was removed, and the pellet was dried. The RNA pellet was dissolved in molecular-grade H₂O and stored at −80°C until it was sent for sequencing. RNA samples were sent to Novogene Corporation for library preparation and sequencing. Raw reads were deposited to the NCBI SRA database (BioProject ID PRJNA1020577).

Genome-guided transcriptome assembly and analysis

Transcriptome assemblies were performed using methods similar to those of Dobbs et al. (2024b). HiSAT2 (v.2.2.1; Kim et al. 2019) and the STAR two-pass protocol (v.2.7.10b; Dobin and Gingeras 2015) were used to align reads to their respective *F. commune* or *F. annulatum* genomes and generate coordinate-sorted BAM files. BAM files were then used in Tigran (v.1.1; Zhao and Yu 2022) to integrate the mappings from two genome-guided aligners for in-depth analysis of microbial activity in inoculated loblolly pine seedlings. The resulting transcripts were clustered into isogroups using cd-hit-est (v.4.8.1; Fu et al. 2012) and a 0.99 cutoff value. RSEM (v.1.3.3; Li and Dewey 2011) was used to calculate expression after representation of reads was assessed using bowtie2 (v.2.5.0; Langmead and Salzberg 2012), in which reads from individual samples were mapped back to the isogroups of the combined transcriptome. Quantitative assessment of the transcriptome completeness, in terms of expected gene content, was assessed using BUSCO against ascomycota_odb10 (*n* = 1,706 genes), sordariomycetes_odb10 (*n* = 3,817 genes), and hypocreales_odb10 (*n* = 4,494 genes) (v.5.5.0; Manni et al. 2021). Counts of transcript abundance were analyzed after a variance stabilizing transformation of the data by producing a principal component plot using the plotpca function in the DESeq2 package v.1.42.0 (Love et al. 2014) in RStudio to assess sample correlations across conditions. Differentially expressed transcripts were identified using DESeq2 to compare expressed transcripts of the mycelium in inoculated seedlings collected at 12, 24, and 48 hpi (in planta) versus expressed transcripts of the mycelium

grown in broth (in vitro) at the analogous time points. Significant differentially expressed transcripts were determined as those with log₂ fold change > 1 and an adjusted *P* < 0.05 (Benjamini–Hochberg adjustment).

Orfipy v.0.0.3 (Singh and Wurtele 2021) was used to identify the longest open reading frames of each transcript that were subsequently converted to amino acids using the peptides flag (--pep). These longest open reading frames were used for predicted function analyses. Predicted functions of proteins encoded in the transcripts were determined using six databases: OrthoVenn3 for putative non-orthologous proteins (Xu et al. 2019), antiSMASH fungal database for secondary metabolite gene clusters discovery (Blin et al. 2023), InterProScan v.5.64–96.0 for protein family domains (Quevillon et al. 2005), Kyoto Encyclopedia of Genes and Genomes (KEGG) for metabolic pathways (Kanehisa and Goto 2000), dbCAN3 for putative carbohydrate-active enzymes (CAZymes) (Zhang et al. 2018), and PHI-base v.4.15 for putative pathogenicity/virulence-associated proteins (Urban et al. 2017). OrthoVenn3, antiSMASH, InterProScan, KEGG/KOfam, and dbCAN3 databases' online servers were used to analyze the predicted genes under default settings. NCBI BLAST+ was used for the PHI-base database. Selection of putative functions of proteins was based on an e-value < 1e-5, percent identity ≥ 65%, and coverage ≥ 50%.

Putative pathogenesis genes in *Fusarium* spp. genomes

Significantly upregulated transcripts in planta (UIP) expressed by *F. commune* and *F. annulatum* at any of the three time points were compared for their presence within genomes of *Fusarium* spp. isolates analyzed in Dobbs et al. (2024a) using OrthoFinder. Each *Fusarium* species' UIP transcriptome profiles were analyzed both separately and combined. Only transcriptome orthogroups that were also present in at least one genome were used for the analysis. Profile dissimilarity was contrasted by *Fusarium* species complex using Bray–Curtis distances with a permutational multivariate ANOVA using the 'vegan' v.2.6-4 package (Oksanen et al. 2020). Profile comparisons were visualized using principal coordinates analyses, and sets of UIP transcripts were visualized using an UpSet plot using the 'ComplexUpset' v.1.3.5 package (Lex et al. 2014). To further examine putative pathogenicity/virulence-associated genes shared among the *Fusarium* spp., the secretome and genes described as putative pathogenicity/virulence factors of each transcriptome were also compared with the 17 *Fusarium* spp. representing six *Fusarium* species complexes (Table 1) using the same criteria as above. The six *Fusarium* species complexes used in these comparisons are (i) FTSC (*F. tricinctum* species complex) including *F. acuminatum*, *F. avenaceum*, *F. flocciferum*, and *F. torulosum* (Laraba et al. 2022); (ii) FFSC (*F. fujikuroi* species complex) including *F. annulatum*, *F. fredkrugeri*, *F. fujikuroi*, *F. lactis*, and *F. verticillioides* (Yilmaz et al. 2021); (iii) FIESC (*F. incarnatum-equiseti* species complex) including *F. clavum*, *F. ipomoeae*, and *F. luffae* (Xia et al. 2019); (iv) FNSC (*F. nisikadoi* species complex) including *F. commune* (Han et al. 2023); (v) FSSC (*F. solani* species complex) including *F. falci-forme* and *F. solani* (Coleman 2016); and (vi) FOSC (*F. oxysporum* species complex) including *F. inflexum* and *F. oxysporum* (Bugingo et al. 2025).

Results

F. commune and *F. annulatum* pathogenicity assays

All *F. commune* isolates exhibited pathogenicity on all conifer and non-conifer hosts that were tested (Fig. 2). A statistically significant difference (*P* < 0.05) was found between each isolate compared with the control as assessed using the emmeans post-hoc test after the ART ANOVA. Using the post hoc test, the *F. annulatum* isolate was pathogenic on all hosts (Fig. 2); however, based on the ART ANOVA results, *F. annulatum* was not significantly different from the control on the tomato host (*P* = 0.09).

Transcriptome assembly

Illumina RNA sequencing generated an average of 59,665,694 reads per sample (Supplementary Table S1). Alignment of reads mapping to the *F. annulatum* reference genome resulted in 50 to 51% of reads from in vitro samples, 0.32 to 0.42% from 12-hpi samples, 0.53 to 0.93% from 24-hpi samples, and 0.93 to 1.59% from 48-hpi samples. Alignment of reads mapping to the *F. commune* reference resulted in 49 to 51% of reads from in vitro samples, 0.35 to 0.72% from 12-hpi samples, 0.36 to 0.69% from 24-hpi samples, and 0.80 to 4.11% from 48-hpi samples. The combined transcriptome assemblies generated 10,156 transcripts (mean length = 1,346 bases) for *F. annulatum* and 10,179 transcripts

(mean length = 1,265 bases) for *F. commune*. The transcriptomes had high BUSCO completeness scores: 91.5% (*F. annulatum*) and 88.9% (*F. commune*) with ascomycota_odb10; 89.9% (*F. annulatum*) and 88.3% (*F. commune*) with sordariomycetes_odb10; 88.7% (*F. annulatum*) and 86.5% (*F. commune*) with hypocreales_odb10. Principal component analysis was used to determine intergroup and intragroup variability of biological replicates from in vitro and in planta conditions (Fig. 3). Principal component 1 described the most variation (82% in *F. annulatum* and 81% in *F. commune*). The three replicates in mycelium and in planta were found to be more similar within groups than they were between groups (Fig. 3).

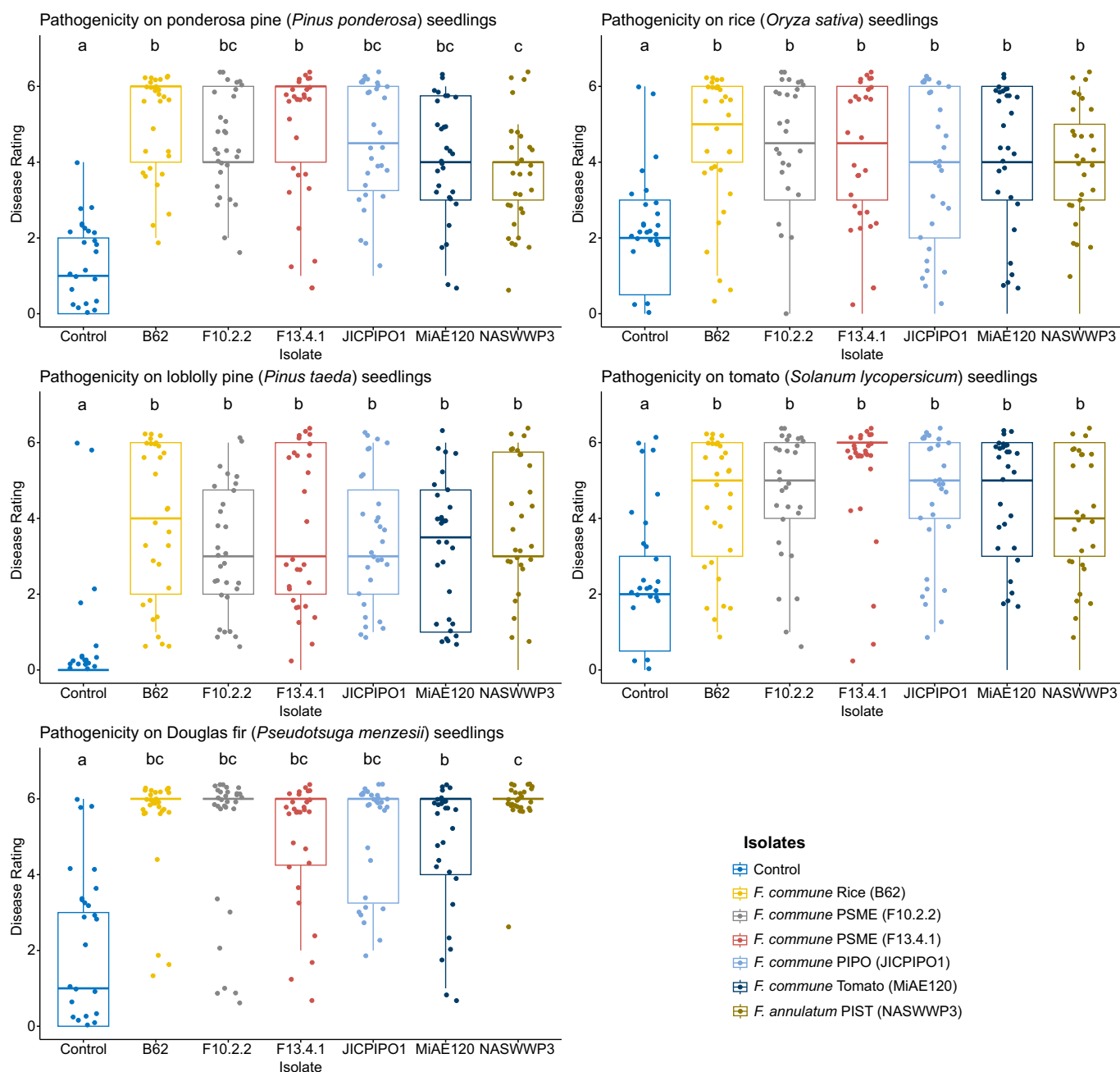


Fig. 2. Pathogenicity results based on the emmeans post hoc test of *Fusarium commune* isolates collected from Douglas-fir (*Pseudotsuga menziesii*, PSME; isolates F10.2.2 and F13.4.1), ponderosa pine (*Pinus ponderosa*, PIPO; isolate JICPIPO1), rice (*Oryza sativa*; isolate B62), and tomato (*Solanum lycopersicum*; isolate MIAE120) seedlings and one *F. annulatum* isolate (NASWWP3) collected from southwestern white pine (*P. strobiformis*; PIST). Each isolate was assessed for pathogenicity based on disease rating for above- and belowground disease symptoms observed on coniferous (ponderosa pine, loblolly pine [*P. taeda*], and Douglas-fir) and herbaceous (rice and tomato) hosts. All *F. commune* and *F. annulatum* isolates were found to cause significant disease compared with the mock-inoculated control. More detailed isolate information is available in Table 1. Isolates sharing the same post hoc letter are not significantly different, while those with different letters are.

Differential gene expression and putative function

Our analyses focused on differential expression in planta to identify pathogenicity-related genes associated with conifer pathogenesis. Differential expression analyses between in planta and in vitro samples generated 2,834 UIP transcripts in *F. annulatum* (Dobbs 2024a) and 2,414 in *F. commune* (Dobbs 2024b) isolates, with most upregulated genes expressed at 48 hpi (Fig. 4; Table 2). Putative functions were described for an average of 71.5% transcripts in the *F. annulatum* transcriptome and 72.5% transcripts in the *F. commune* transcriptome (Dobbs 2024a, b). Pfam and KEGG/KOfam described the functions of most UIP genes in both *Fusarium* species (Fig. 5). Among the putative pathogenicity-/virulence-associated genes of the two *Fusarium* species, functions were described as transporters, signaling pathways, effectors, transcription, chitin synthases, CAZymes, and genes involved in secondary metabolism pathways. Notably, genes involved in the jasmonic acid pathway (PHI: 4909) were expressed in *F. annulatum* at all three time points.

Transcripts encoding CAZymes were more abundant in in planta ($n = 191$ in *F. annulatum* and $n = 186$ in *F. commune*) than in in vitro samples ($n = 105$ in *F. annulatum* and $n = 114$ in *F. commune*) (Dobbs 2024a, b). Most of the extracellular CAZymes were described as involved in the degradation of cellulose, lignin, pectin, and other plant cell wall-forming carbohydrates (Dobbs 2024a, b; Fig. 6). Among the UIP transcripts, SWISS-PROT-described genes were involved in sesquiterpene biosynthesis, pigment biosynthesis, aurofusarin biosynthesis, and aromatic compound metabolism (Dobbs 2024a, b). Genes involved in secondary metabolism biosyn-

thesis differed between species. In *F. annulatum*, these genes were involved in the biosynthesis pathways of aspyridones and bikaverin, whereas five genes involved in the biosynthesis pathways of fusaoc-taxin A were described in *F. commune* (Dobbs 2024a, b). Gene clusters described by antiSMASH as involved in the biosynthesis of prolipyrone B/gibepyrone D were identified in the UIP transcripts in *F. annulatum*. In contrast, gene clusters involved in the biosynthesis of secondary metabolites were not identified in the UIP genes of *F. commune*. However, downregulated genes involved in the biosynthesis of enniatin were identified in both *Fusarium* species. In *F. annulatum*, genes not significantly regulated up or down in planta were identified as involved in the biosynthesis of fusarin C, choline, and ACT-Toxin II. Similar to the other databases, KEGG enrichment of UIP genes was found for genes involved in metabolic processes and biosynthesis of secondary metabolites (Fig. 7).

Orthologous protein clusters and Gene Ontology (GO) term enrichment

When comparing orthologous proteins among time points and species, *F. annulatum* had two unique orthogroups at 12 hpi, one at 24 hpi, and four at 48 hpi (Fig. 8). No GO enrichment was identified at 12 and 48 hpi; however, GO enrichment involved in mitochondrial electron transport (ubiquinol to cytochrome c) was detected at 24 hpi. *F. commune* did not produce any described unique orthogroups at 12 hpi, but one was produced at 24 hpi, and four were produced at 48 hpi (Fig. 8). Similar to *F. annulatum*, no GO terms were enriched at 12 and 48 hpi, but enriched

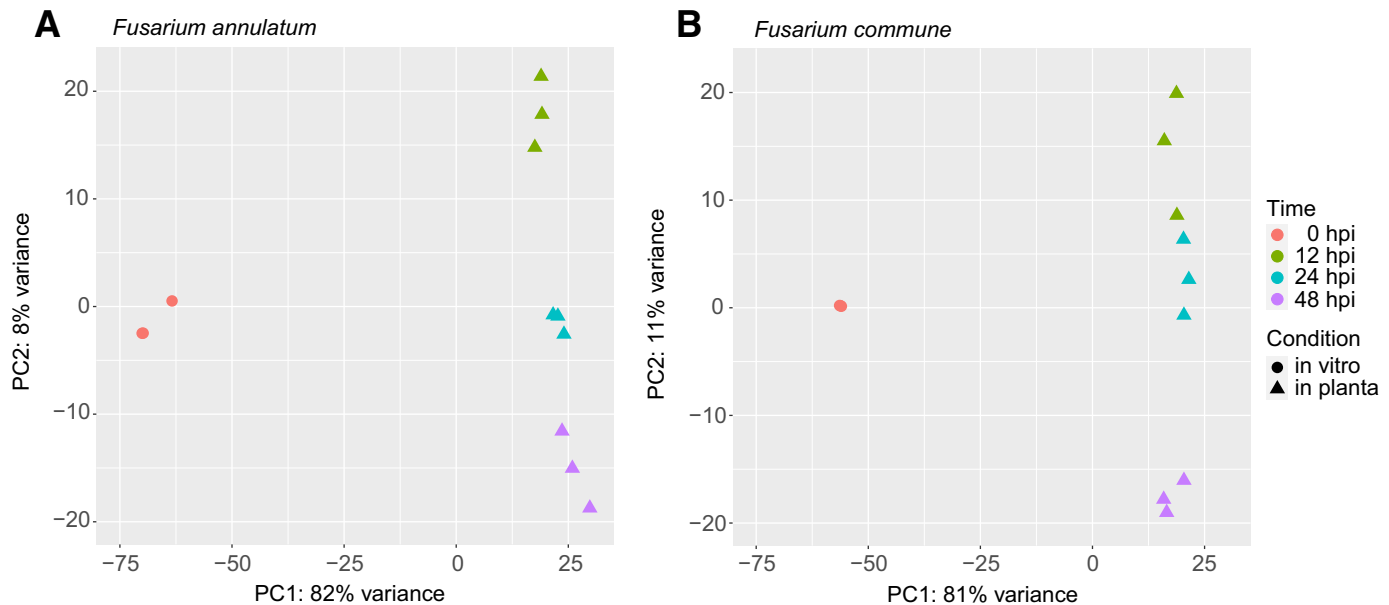


Fig. 3. Principal component (PC) analyses of three replicate samples of **A**, *Fusarium annulatum* and **B**, *F. commune* inoculated on loblolly pine (*Pinus taeda*) plants (in planta) compared with the mycelium of each isolate grown in potato dextrose broth (in vitro). Isolate RNA were collected at three time points postinoculation: 12 h (green), 24 h (blue), and 48 h (purple) postinoculation (hpi).

TABLE 2. Transcripts from plants inoculated with either *Fusarium annulatum* or *F. commune* isolates at three time points compared with 28,171 or 24,470 genes, respectively, extracted from the mycelium of cultures grown in potato dextrose broth to identify differentially expressed (DE) genes upregulated in planta (UIP) or downregulated in planta (DIP)

Isolate	<i>Fusarium</i> sp.	Time	Total DE	UIP	DIP	UIP described	UIP not described	Percent described	DIP described	DIP not described	Percent described
NASWWP3	<i>F. annulatum</i>	12 h	4,113	1,649	2,464	803	846	48.70%	945	1,519	38.35%
NASWWP3	<i>F. annulatum</i>	24 h	4,625	1,832	2,793	947	885	51.69%	1,020	1,773	36.52%
NASWWP3	<i>F. annulatum</i>	48 h	5,066	2,228	2,838	1,082	1,146	48.56%	1,062	1,776	37.42%
JICPIPO1	<i>F. commune</i>	12 h	3,753	1,320	2,433	663	657	50.23%	816	1,617	33.54%
JICPIPO1	<i>F. commune</i>	24 h	3,767	1,399	2,368	717	682	51.25%	836	1,532	35.30%
JICPIPO1	<i>F. commune</i>	48 h	4,222	1,865	2,357	957	908	51.31%	772	1,585	32.75%

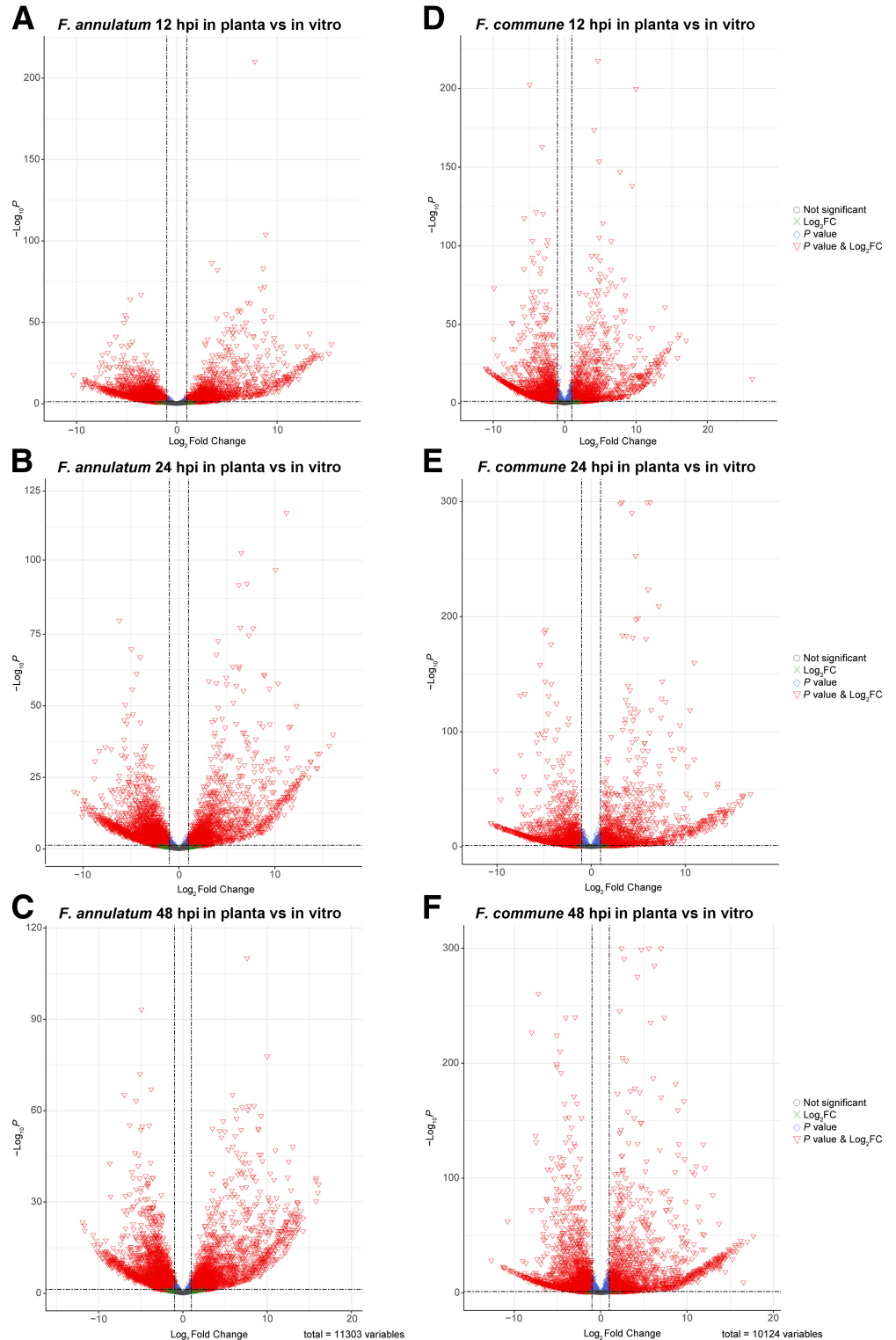
GO terms were identified at 24 hpi. Notably, GO terms described as involved in oxidoreductase activity and transmembrane transport were detected at 48 hpi. Of the unique orthogroup protein clusters that were identified, 413 were found in *F. annulatum* and 224 were found in *F. commune*. Among the *F. annulatum* unique orthogroups, enriched GO terms were associated with transport, oxidoreductase activity, aromatic compound catabolic process, terpenoid biosynthesis process, signal transduction, sterigmatocystin biosynthesis, and melanin biosynthesis process. In *F. commune*, enriched GO terms were associated with transport, transcription,

oxidoreductase activity, and sporulation. Shared among both *Fusarium* species, enriched GO terms were associated with transport, including toxin transport, quinate metabolic process, pathogenesis, sporulation, defense response, and signaling.

Secreted UIP genes

Among the differentially expressed transcripts of *F. annulatum* in planta, we found 317 secreted extracellular proteins, including 57 apoplastic effectors, 14 apoplastic/cytoplasmic effectors, and 30 cytoplasmic effectors (Dobbs 2024a). Most of the secreted

Fig. 4. Volcano plots of **A to C**, *Fusarium annulatum* and **D to F**, *F. commune* inoculated on loblolly pine (*Pinus taeda*) plants (in planta) compared with the mycelium of each isolate grown in potato dextrose broth (in vitro). Significantly differentially expressed genes in red were based on a $\log_2$ fold change > 1 and $P < 0.05$. Isolates were compared at three time points postinoculation (hours postinoculation [hpi]): A and D, 12 hpi; B and E, 24 hpi; C and F, and 48 hpi. Upregulated transcripts in planta are shown on the right of each plot and downregulated on the left.



proteins ($n = 286$) and effectors ($n = 93$) were expressed at 48 hpi (Dobbs 2024a). We found 321 transcripts encoding predicted secreted extracellular proteins, including 70 apoplastic effectors, 19 apoplastic/cytoplasmic effectors, and 22 cytoplasmic effectors in *F. commune* (Dobbs 2024b). Similar to *F. annulatum*, most of the secreted proteins ($n = 291$) and effectors ($n = 102$) were expressed

at 48 hpi (Dobbs 2024b). CAZymes described as involved in the degradation of pectin, hemicellulose, and cellulose were the most secreted effectors in both species (*F. annulatum* $n = 128$; *F. commune* $n = 134$). When comparing secreted proteins across time points, predicted effectors, pathogenicity-/virulence-related genes, and CAZymes increased from 12 to 48 hpi in both species (Fig. 6).

Fig. 5. Described upregulated transcripts in planta (UIP) of *Fusarium annulatum* (top) and *F. commune* (bottom) inoculated on loblolly pine (*Pinus taeda*) plants compared with the mycelium of each isolate grown in potato dextrose broth. Isolates were collected at 12, 24, and 48 h postinoculation (hpi). Counts are based on the number of genes described by each database.

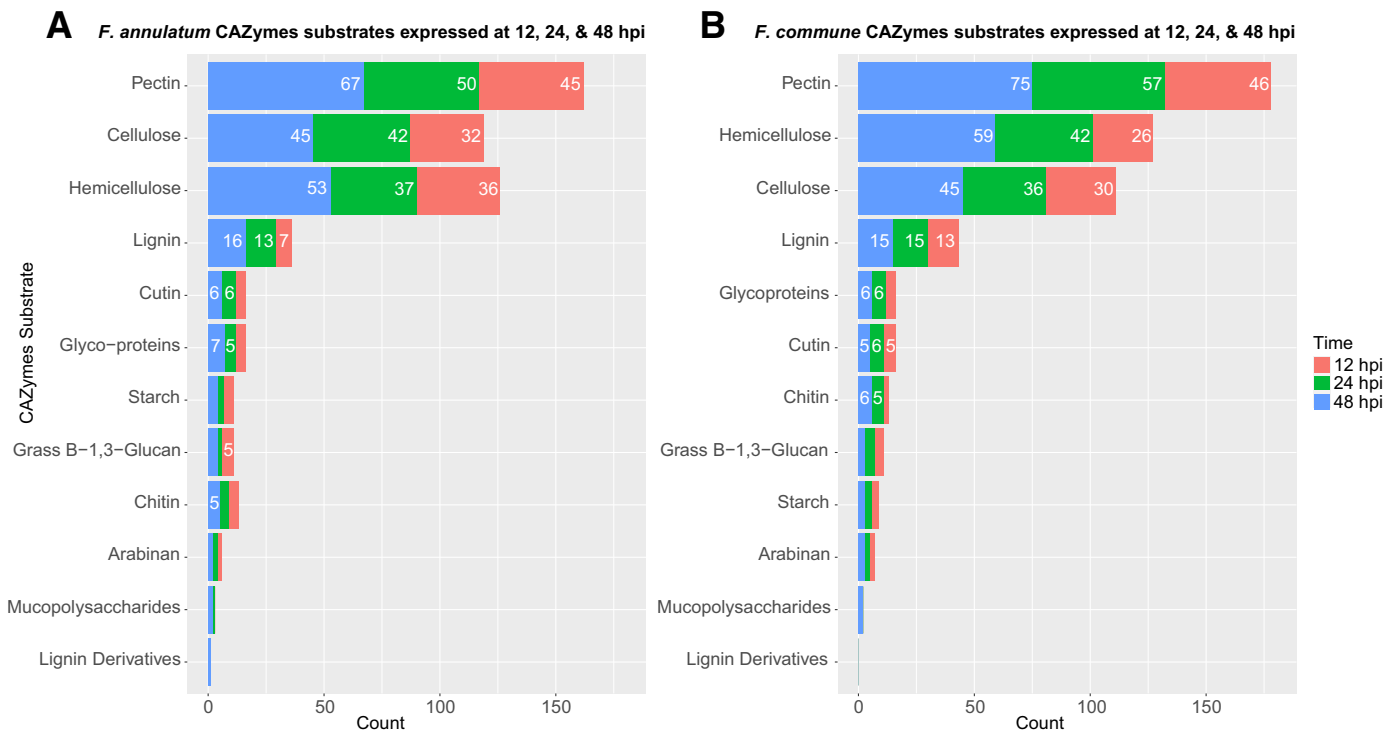
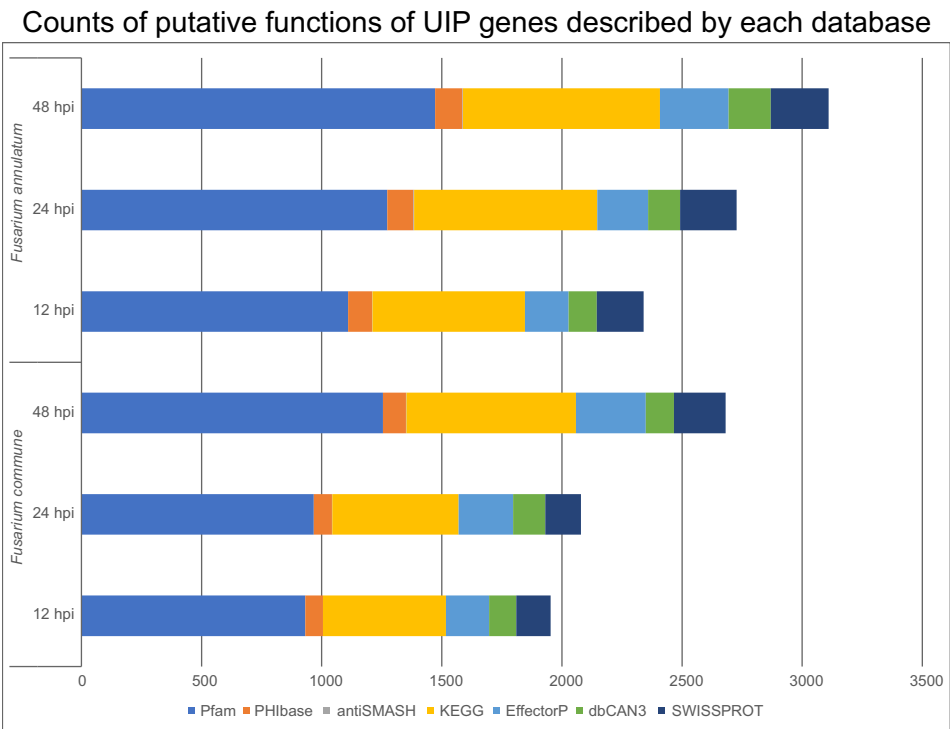


Fig. 6. Described carbohydrate-active enzymes in upregulated in planta (UIP) transcripts of **A**, *Fusarium annulatum* and **B**, *F. commune* inoculated on loblolly pine (*Pinus taeda*) plants compared with the mycelium of each isolate grown in potato dextrose broth. Isolates were collected at 12, 24, and 48 h postinoculation (hpi). Counts are based on the number of genes described at each time point. Only counts ≥ 5 genes were numbered on the graph.

Interestingly, in *F. annulatum*, one predicted secreted protein associated with secondary metabolism was described as part of the aurofusarin biosynthesis cluster and involved in pigment biosynthesis, and another was found to be involved in necrosis and ethylene induction. Similarly in *F. commune*, one secreted protein involved in secondary metabolism was described as part of the aurofusarin biosynthesis cluster and involved in pigment biosynthesis, and another was described as a hydrophobin-producing protein.

Putative pathogenesis genes in *Fusarium* spp. genomes

When comparing all the genes significantly UIP shared in both *F. annulatum* ($n = 1,010$ genes in 552 orthogroups) and *F. commune* ($n = 883$ genes in 529 orthogroups) with the 17 conifer-derived *Fusarium* spp. genomes from a previous survey on conifer seedlings (Dobbs et al. 2023, 2024a; Table 1), the UIP profiles were similar among *Fusarium* species complexes (Fig. 9A). Even

when comparing only secreted proteins (Fig. 9B) and pathogenicity-/virulence-associated genes (Fig. 9C), shared profiles were found within *Fusarium* species complexes. The closely related species complexes ordinated closer together, particularly the FNSC, FO SC, and FFSC (Fig. 9).

When investigating the putative function of the UIP gene orthogroup profiles in *F. annulatum* and *F. commune*, 101 and 93 orthologous genes, respectively, were found to be shared among *Fusarium* species complexes. Most UIP genes identified in these profiles were expressed at 48 hpi. Among six *Fusarium* species complexes (FTSC, FFSC, FIESC, FNSC, FSSC, and FO SC) (Table 1), the FFSC had the most orthologous genes shared among members with both the *F. annulatum* ($n = 657$) and *F. commune* ($n = 539$) transcriptome profiles (Table 3). Interestingly, all the *Fusarium* species complexes contained orthogroups found in the *F. annulatum* transcriptome profile that were involved in biosynthesis of

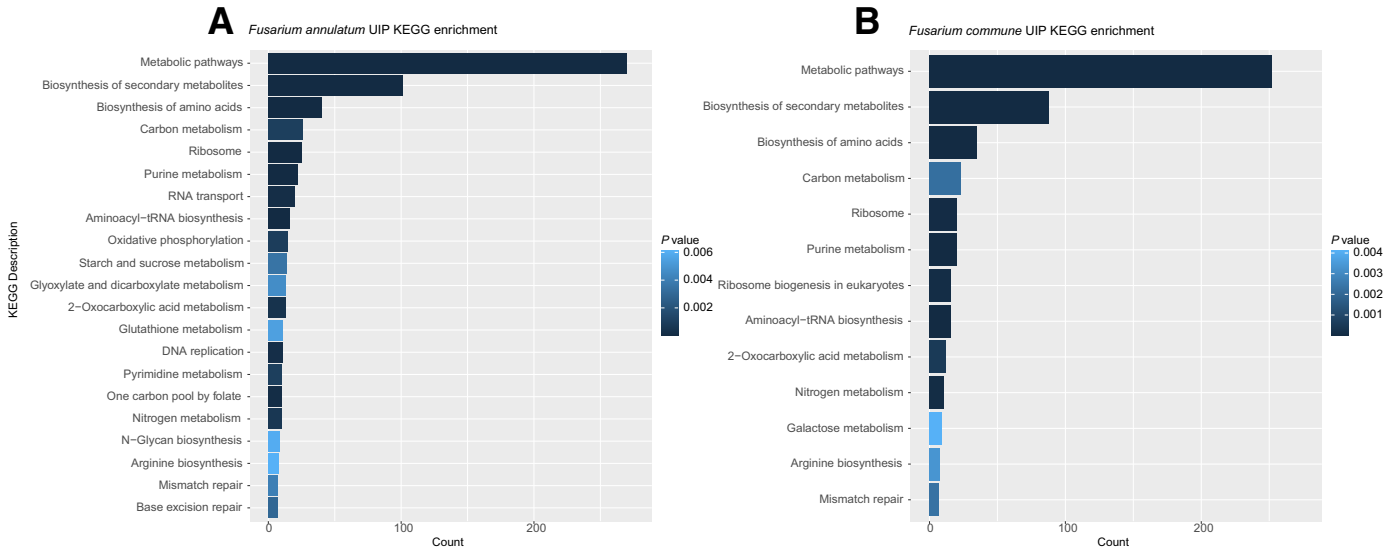
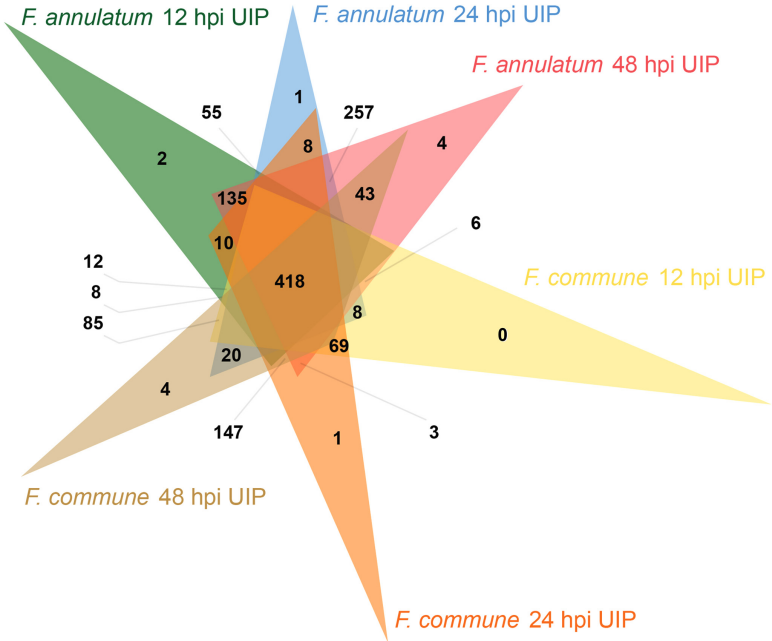


Fig. 7. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment of significantly upregulated in planta (UIP) genes of **A**, *Fusarium annulatum* and **B**, *F. commune* inoculated on loblolly pine (*Pinus taeda*) plants compared with the mycelium of each isolate grown in potato dextrose broth. In both species, significantly UIP genes were involved in metabolic pathways and biosynthesis of secondary metabolites.

Fig. 8. Venn diagram of shared and unique orthologous protein clusters of largest open reading frames of upregulated in planta (UIP) transcripts of *Fusarium annulatum* (top) and *F. commune* (bottom) inoculated on loblolly pine (*Pinus taeda*) plants compared with the mycelium of each isolate grown in potato dextrose broth. Isolates were collected at 12, 24, and 48 h postinoculation (hpi).



prolipyron B/gibepyrone D. No gene clusters from the anti-SMASH database involved in secondary metabolite biosynthesis were identified in the *F. commune* transcriptome profile. Though more orthogroups were found to be secreted in the FFSC and FNCS profiles based on the *F. annulatum* transcriptome profile, more effectors were identified in the *F. commune* transcriptome profile (Table 3).

Putative pathogenicity/virulence gene profiles unique to *Fusarium* species complexes

When comparing putative pathogenicity/virulence genes identified in the *F. annulatum* and *F. commune* transcriptome profiles with the predicted genes among 17 *Fusarium* isolates representing six *Fusarium* species complexes, unique putative pathogenicity/virulence genes were identified within species complexes that

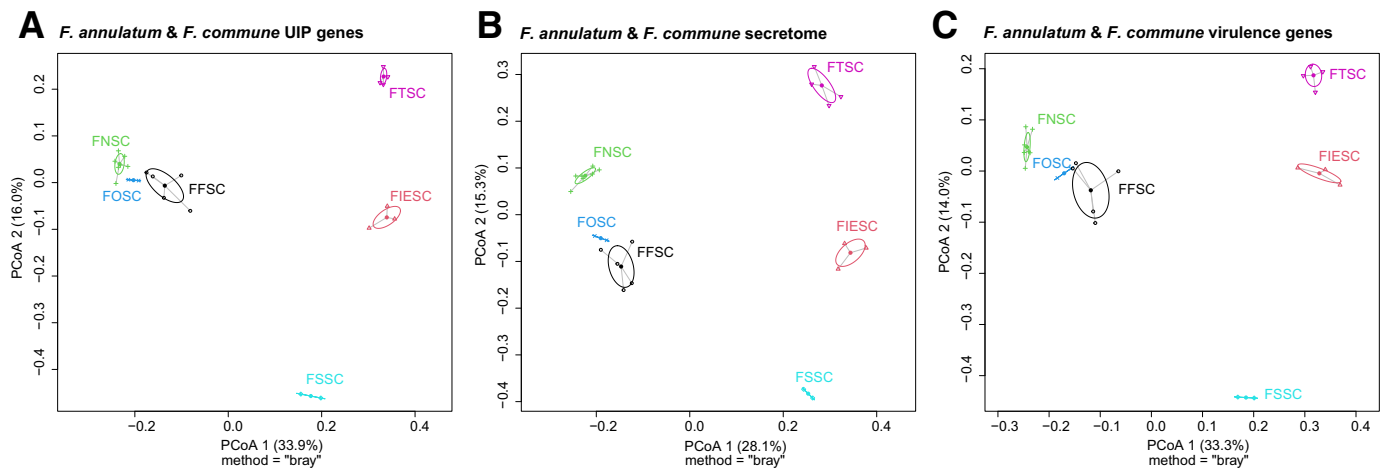


Fig. 9. Distribution of significantly upregulated in planta (UIP) genes identified in *Fusarium annulatum* and *F. commune* inoculated on loblolly pine (*Pinus taeda*) plants among *Fusarium* spp. isolates collected from conifer hosts. Principal coordinates analysis (PCoA) of UIP genes show similarity of **A**, UIP gene; **B**, secretome; and **C**, virulence gene profiles among *Fusarium* species complexes. Table 1 describes six *Fusarium* species complexes: FTSC (*F. tricinctum* species complex); FFSC (*F. fujikuroi* species complex); FIESC (*F. incarnatum-equiseti* species complex); FNCS (*F. nisikadoi* species complex); FSSC (*F. solani* species complex); and FOSC (*F. oxysporum* species complex).

TABLE 3. Profiles of upregulated in planta genes among species complexes based on either the *Fusarium annulatum* or *F. commune* transcriptomes at different hours postinoculation (hpi) described by databases of fungal virulence genes (PHI), secondary metabolite gene clusters (antiSMASH), and protein families (KEGG/KOfam, SWISS-PROT, and Pfam)^a

Condition, database, gene family	Fusarium species complexes						
	All	FNCS	FOSC	FFSC	FTSC	FIESC	FSSC
<i>F. annulatum</i> transcriptome	101	446	399	657	461	437	466
12 hpi	61	295	262	411	280	268	279
24 hpi	67	302	267	435	312	300	304
48 hpi	79	271	341	545	381	368	387
CAZymes	12	46	52	64	56	49	44
Extracellular	12	54	58	76	65	41	43
Apoplastic effector	5	5	7	10	10	6	4
Cytoplasmic effector	0	2	5	1	4	0	0
Apoplastic/cytoplasmic effector	0	0	0	0	0	0	0
PHI	3	11	12	30	12	15	21
antiSMASH	1	2	2	1	3	3	2
Secondary metabolites	Prolipyron B/ gibepyrone D	Prolipyron B/ gibepyrone D	Prolipyron B/ gibepyrone D	Prolipyron B/ gibepyrone D	Prolipyron B/ gibepyrone D	Prolipyron B/ gibepyrone D	Prolipyron B/ gibepyrone D
KEGG/KOfam	53	199	176	283	192	207	183
SWISS-PROT	20	46	39	67	44	50	51
Pfam	94	388	343	577	388	402	420
<i>F. commune</i> transcriptome	93	381	326	539	391	360	421
12 hpi	57	210	180	303	228	212	251
24 hpi	60	217	186	318	228	214	272
48 hpi	73	289	258	426	303	291	317
CAZymes	9	30	25	39	29	24	28
Extracellular	8	46	40	70	43	42	52
Apoplastic effector	1	7	4	13	7	11	6
Cytoplasmic effector	0	1	3	2	3	2	5
Apoplastic/cytoplasmic effector	0	4	3	6	2	4	2
PHI	8	24	20	29	26	23	20
antiSMASH	0	0	0	0	0	0	0
Secondary metabolites	0	0	0	0	0	0	0
KEGG/KOfam	41	146	116	195	145	141	168
SWISS-PROT	10	43	35	51	44	47	38
Pfam	66	250	210	351	256	243	276

^a FNCS (*F. nisikadoi* species complex), FOSC (*F. oxysporum* species complex), FFSC (*F. fujikuroi* species complex), FTSC (*F. tricinctum* species complex), FIESC (*F. incarnatum-equiseti* species complex), and FSSC (*F. solani* species complex).

had similar described functions across species complexes, including CAZymes, effectors, major facilitator supergroup (MFS) transporters, and secondary metabolite biosynthesis genes. However, differences among members of each species complex were also observed.

Orthologous genes in the *F. annulatum* transcriptome profiles that were found in the FNSC predicted genes included chitin synthases, transcription factors, transporters, and CAZymes, which are primarily involved in the degradation of pectin, cellulose, and hemicellulose. The *F. commune* isolate (JICPIPO1) collected from ponderosa pine had more MFS transporter genes than the other *F. commune* isolates. Interestingly, within the *F. commune* transcriptome profile, genes involved in transcription factors, glycoside hydrolases, and aurofusarin biosynthesis were also identified among the predicted genes in the FNSC. However, genes encoding cytochrome P450, transcription factors, and fungal trichothecene efflux pumps were present in all *F. commune* isolates.

The *F. annulatum* transcriptome profile shared a predicted MFS transporter with the genomes of all the FFSC isolates. However, a predicted necrosis-inducing protein and a transcription factor were found only in the *F. annulatum* genome. In contrast, a pathogenicity/virulence-related effector and a transcription factor were only found in the *F. verticillioides* genome, and another transcription factor was only found in the *F. fredkrugeri* genome. The *F. commune* transcriptome profile included a transcription factor that was shared among all FFSC isolates. Of further note, a transcription factor was only found in the *F. lactis* genome, and a TAG lipase was only identified in the *F. fujikuroi* genome. In addition, a glycoside hydrolase and an exopolysaccharuronase were only found in the *F. fredkrugeri* genome, and the aurofusarin biosynthesis gene was only found in the *F. lactis* and *F. verticillioides* genomes.

In the FOSC predicted genes, an MFS, a transcription factor, and a chitin synthase were identified in both FOSC species (*F. inflexum* and *F. oxysporum*) that were shared in the *F. annulatum* transcriptome profile. Aurofusarin biosynthesis and exopolysaccharuronase genes were also found in the genomes of both FOSC species based on the *F. commune* transcriptome profile. Of further note, a myosin and TAG lipase genes were only identified in the genome of *F. inflexum*.

In the FTSC predicted genes, a chitin synthase, a necrosis-inducing gene, and a ubiquitin fusion gene were identified in the transcriptome profile of *F. annulatum* and are shared in the genomes of *F. avenaceum* and *F. tricinctum*. The MFS transporter gene was found in the genomes of all examined FTSC genomes except *F. tricinctum*, and a TAG lipase gene was found only in the genome of *F. avenaceum*. In the *F. commune* transcriptome profile, transcription factors and exopolysaccharuronase genes were identified in the genomes of all FTSC isolates. Also, an aurofusarin biosynthesis gene was found in the genomes of *F. torulosum* and *F. avenaceum*. One unique transcription factor was found in the *F. tricinctum* genome, and one was found shared between the *F. flocciferum* and *F. torulosum* genomes.

The FIESC predicted genes included genes involved in MFS transporters that were present in genomes of all FIESC isolates and in the *F. annulatum* transcriptome profile. In contrast, a chitin synthase and leucine biosynthesis genes were found only in the genome of *F. ipomoeae*, and a transcription factor was found unique to the *F. luffae* genome. Based on the *F. commune* transcriptome profile, genes associated with intermediate steps in the aurofusarin biosynthesis pathway, along with a transcription factor, were identified in all FIESC isolates. In addition, a fusaoctaxin A (cytochrome P450) gene was only found in the genome of *F. clavum*. Furthermore, a transcription factor gene was found in the genomes of *F. clavum* and *F. ipomoeae*, and an exopolysaccharuronase gene was found only in the *F. luffae* genome.

The FSSC predicted genes included genes associated with MFS transporters, effectors, triacetylfulvarine C/siderophore biosynthesis, transcription factors, and two copies of an exopolysaccharuronase

that were found in all FSSC isolates and in the transcriptome profile of *F. annulatum*. A gene encoding chitinase was detected only in the *F. solani* genome. The *F. commune* transcriptome profile contained a gene encoding chitinase, which was only found in the *F. solani* genome, and genes encoding chitin synthase, transcription factor, and TAG lipase genes were present in the genomes of all FSSC isolates. An exopolysaccharuronase gene and a described extracellular protein from the PHI database were only found in the *F. solani* genome, whereas a glycoside hydrolase was identified only in the *F. falciforme* genome.

Discussion

In forest tree nurseries, managers are increasingly concerned about numerous fungal pathogens that can cause devastating losses to conifer seedlings. *Fusarium* spp., especially newly emerging plant-pathogenic species, are of major concern because of their high prevalence.

Furthermore, many of these conifer-associated *Fusarium* species remain understudied, with unknown virulence and poorly defined host ranges (Karunaratna et al. 2021; Stewart et al. 2019). Genomics and transcriptomics approaches offer valuable tools for elucidating host-pathogen interactions during pathogenesis. In this study, we generated reference-guided transcriptomes of two understudied *Fusarium* spp., *F. annulatum* and *F. commune*, both of which we confirmed as pathogens of conifer and non-conifer hosts. We initially hypothesized that *F. commune* isolates would exhibit host specificity corresponding to their original host of isolation, along with associated host-specific pathogenicity/virulence factors. Similarly, we also expected the *F. annulatum* isolate to exhibit specificity for conifer hosts. However, all tested isolates were found to be pathogenic to both conifer and non-conifer hosts in our assays compared with controls. However, we identified some unique pathogenicity/virulence pathways utilized by the *F. commune* isolate, compared with *F. annulatum*, suggesting that candidate genes encoding predicted secreted proteins within the transcriptomes may play a role in conifer pathogenesis. Of further note, we identified genes associated with mycotoxin biosynthesis pathways involved in the production of prolipyrone B/gibberpyrone D in the *F. annulatum* transcriptome. These genes in mycotoxin biosynthesis pathways were among the upregulated genes in planta, which further accentuates the importance of secondary metabolites in conifer pathogenesis. Subsequently, we surveyed for secreted pathogenicity/virulence genes among the predicted proteomes of conifer-associated *Fusarium* spp. and found that these profiles were associated with *Fusarium* species complexes rather than being affiliated with conifer versus non-conifer pathogens. For this reason, the conifer pathogenicity genes that we identified are likely not all required for conifer pathogenesis.

In our previous survey, *F. commune* was one of the most commonly isolated *Fusarium* spp. from conifer seedlings across the contiguous United States (Dobbs et al. 2023). Our current findings suggest that *F. commune* may function as a generalist pathogen, capable of infecting both conifer and non-conifer hosts—an insight with significant implications for ecosystem restoration efforts. Because restoration projects do not rely on single hosts, generalist pathogens pose a distinct threat because they can potentially infect a variety of hosts and can be transported on seedlings to planting sites. Even if the target restoration species are not susceptible to a *Fusarium* pathogen, some *Fusarium* pathogens can potentially persist as endophytes in nontarget hosts until a preferred host appears in out-planting sites (Montoya-Martínez et al. 2022). Our pathogenicity/virulence assays using the root clipping method in the *Pinaceae*, *Poaceae*, and *Solanaceae* plants demonstrated that *Fusarium* strains pathogenic to hosts in all three families may have devastating impacts on many common restoration and crop species within these families, such as those that are produced in tree/horticultural nurseries in the southwestern United States (Dreesen et al. 2002). The

root clipping method, which results in root wounding, may have provided an infection court allowing the pathogen to cause infections in plants that may not be common hosts for these pathogens.

Nonetheless, *F. commune* has already been reported as a pathogen to hosts within these families (Hamini-Kadar et al. 2010; Husna et al. 2021; Stewart et al. 2006, 2012), and it seems reasonable that strains of these pathogens, regardless of host origin, are a potential threat to conifer and forest restoration nurseries. A wide host range has even greater implications because many *Fusarium* spp., primarily in the FSSC and FO SC, have been reported as trans-kingdom pathogens, capable of infecting plants, animals, and humans (van Diepeningen and De Hoog 2016). Furthermore, switching between endophytic and pathogenic lifestyles may be transient in *Fusarium* with shared genetic features that support both lifestyles (Hill et al. 2022). As a consequence that warrants serious consideration for management, a pathogenic lineage may potentially persist within reservoir hosts as endophytes until favorable conditions or susceptible hosts are available to support a switch to a pathogenic lifestyle.

Similar to the *F. commune* isolates, the *F. annulatum* isolate caused significant disease compared with the controls on all conifer and non-conifer hosts tested. This result was expected, as *F. annulatum* has been recently reported as a pathogen on non-conifer hosts, such as wine/table grapes (*Vitis vinifera*) and cantaloupe (*Cucumis melo* var. *cantalupensis*) (Bustamante et al. 2022; Parra et al. 2022). Another consideration is that *F. annulatum* was also recently taxonomically separated from *F. proliferatum*, which was previously documented as a conifer pathogen (Stewart et al. 2016). Because our results demonstrate that *F. annulatum* was pathogenic to all tested conifer and rice hosts, this species should be further studied for pathogenicity to additional hosts, and managers should be aware of the potential damage this species can cause in their nurseries.

Due to the importance of secreted proteins and compounds involved in pathogenicity/virulence during host–pathogen interactions, this study focused on the predicted secretome, effectome, and pathogenicity/virulence-associated genes of *F. commune* and *F. annulatum* (Bradshaw et al. 2021). Many secreted proteins were described as glycoside hydrolases, including one (PHI_11176) identified in both transcriptomes of both *F. commune* and *F. annulatum* that was found to be involved in pathogenicity/virulence. This feature is not unexpected, as glycoside hydrolases have been found to be important for pathogenesis in other plant pathogens (Zhang et al. 2021). Both *Fusarium* species shared an aurofusarin biosynthesis gene in their secretome. Interestingly, aurofusarin has been found to protect *Fusarium* from predation by springtails, woodlice, and mealworms (Xu et al. 2019). Because many species within this study are soilborne and root pathogens, this aurofusarin pigment is likely not involved in pathogenicity/virulence but rather defense against soil-dwelling invertebrates. An apoplastic effector described as a hydrophobin gene, which has been described as involved in conidiation, pathogenicity, and secondary metabolism, was identified in the *F. commune* secretome (Shin et al. 2022). Additionally, a previous study found that hydrophobin genes in *F. graminearum* had multiple redundant copies, which is an indication of the biological importance of this pathogenicity gene (Shin et al. 2022). Interestingly, the described necrosis and ethylene inducing (NEP1-like) gene in the *F. annulatum* transcriptome has also been characterized as an apoplastic effector. This cytotoxic fungal NEP1-like protein was described in *Verticillium dahliae* as causing cell death after pathogen colonization within host tissue, and because it was expressed by the *F. annulatum* isolate at 24 and 48 hpi, it appears that *F. annulatum* was able to colonize the host within 48 hpi (Santhanam et al. 2013).

Effectors have various functions in both pathogenesis and evasion of host detection/defenses (Lo Presti et al. 2015). The most secreted effectors were observed at 48 hpi in both *F. commune* and *F. annulatum*. Secreted effectors, such as secreted in xylem (SIX) effectors, have been well studied in *F. oxysporum* (Jangir et al.

2021). The SIX2 gene was found in the predicted proteome of the *F. annulatum* isolate, though no transcription was detected under the tested conditions of this study. Although the significance of the SIX2 gene in pathogenesis of *F. annulatum* is yet to be determined, it has been previously found as unnecessary for pathogenicity/virulence in other *Fusarium* species (Gawehns et al. 2015). Another fungal effector identified in *Botrytis cinerea* (BcCLA4), which was previously found to mediate morphogenesis, growth, and cell cycle regulating processes (Minz-Dub and Sharon 2017), was also found in both the *F. annulatum* and *F. commune* transcriptomes but was only significantly UIP in *F. annulatum*. The incremental increase in effectors from 12 to 48 hpi suggests that effectors are important for early infection of conifer seedlings by *Fusarium*.

The effectome profiles of both *F. annulatum* and *F. commune* were dominated by apoplastic effectors, suggesting that during early infection, the pathogens were likely avoiding host detection while neutralizing host defenses and/or competing with other microbes in the apoplastic space (Rocafort et al. 2020). The identified cytoplasmic effectors have been described as important for inducing virulence functions within the targeted cell compartment (Rocafort et al. 2020). The putative effectors and the other secreted proteins described in this study represent promising targets for further investigations on identifying, detecting, and characterizing *Fusarium* pathogens.

The *F. annulatum* and *F. commune* transcriptomes contained approximately 6% CAZymes, particularly cellulose-, hemicellulose-, and pectin-degrading enzymes that were significantly UIP, with the highest expression occurring at 48 hpi. CAZymes, including cell wall-degrading enzymes, have been found to be necessary for continued infection and colonization of host tissues (Roy et al. 2020). This finding suggests that these secreted CAZymes are necessary for continued infection processes, which warrants further investigation. Similar studies in *Fusarium* spp. have observed significant expression of effectors and CAZymes at 4 days postinoculation (Kuang et al. 2022). However, in our study, we also observed a significant number of UIP genes involved in secondary metabolism.

Signal regulation is essential for fungal infection processes and avoiding host detection during pathogenesis (Hernandez-Chavez et al. 2017; Ma et al. 2013). In this study, many of the UIP genes in *F. annulatum* and *F. commune* were described as transcription factors and protein kinases involved in cell-signaling pathways. For example, a gene involved in the jasmonic acid pathway was identified in the UIP genes of *F. annulatum*. When jasmonates are produced by the host plant, this pathway can act as an essential host defense against necrotrophic fungi (Macioszek et al. 2023). However, when jasmonates are produced by the fungal pathogen, especially during late infections, it has been described as necessary for inducing cell death and facilitating continued infection (Chini et al. 2018).

The significant UIP genes within the *F. commune* and *F. annulatum* transcriptomes, including secreted proteins and described pathogenicity/virulence-associated genes, showed similarity to genome-encoded genes among *Fusarium* species complexes. This finding reflects what was described in a previous study using predicted proteomes to predict conifer pathogenesis gene profiles (Dobbs et al. 2024a), suggesting that conifer-specific pathogenicity/virulence may not be conferred by a shared accessory chromosome, as previously hypothesized (Stewart et al. 2012). Perhaps only a single gene or a few genes are necessary for conifer pathogenesis, as found among *F. oxysporum* strains pathogenic to melons (Li et al. 2020a); however, additional research is needed to determine the specific gene(s) responsible for conifer pathogenesis. Furthermore, *Fusarium* species complexes could be evolving independently and could utilize different pathways to cause damping-off and root disease on conifer seedlings (Abdullah et al. 2017; Summerell 2019). This evolutionary divergence could potentially be devastating to a host, given that multiple pathogens utilizing different pathogenicity/virulence pathways could develop pathogenicity to a single host,

especially if these pathogens, even weak pathogens, work in concert to overwhelm host defenses (Abdullah et al. 2017).

The clustering of the *Fusarium* species complexes based on the principal coordinates analyses analysis showed that closely related species and species complexes share similar pathogenicity/virulence gene profiles. These relationships concur with the phylogenetic analyses of both conserved genes and predicted proteomes found in other studies (Crous et al. 2021; Dobbs et al. 2024a; Geiser et al. 2021; Summerell 2019). We observed that some of the shared genes of the *F. annulatum* and *F. commune* transcriptomes were also shared among the profiles described for each *Fusarium* species complex. Pathogenicity/virulence genes described in the *F. annulatum* transcriptome included genes encoding MFS transporters, exopolysaccharuronases, transcription factors, and prolipyrone B/gibberpyrone D biosynthesis that were also shared among the profiles of *Fusarium* species complexes. The prolipyrone B/gibberpyrone D biosynthesis orthogroup is primarily contained in members of the FFSC (6) but is also contained in isolates from the FNSC (2), FOSC (1), and FTSC (3). Because expression of this gene has only been reported in members of the FFSC and another *Fusarium* species complex, the *F. graminearum* species complex (Adpressa et al. 2019), these encoded orthologous proteins are perhaps nonfunctional in pathogenicity of *F. annulatum*, or perhaps these proteins could produce a similar class of secondary metabolites. These broadly shared genes in *F. annulatum* and *F. commune* may be virulence related or broadly used for host colonization, because studied members within these species complexes (FFSC and FNSC) were also all collected from conifer hosts (Dobbs et al. 2024a). However, this uncertainty accentuates the need for further study to validate the function of these genes.

Similarly, genes encoding exopolysaccharuronases, transcription factors, and aurofusarin biosynthesis were found in the *F. commune* transcriptome profiles. However, expected differences among member profiles were observed within *Fusarium* species complexes. All these *Fusarium* species, except *F. fredkrugeri*, have been described as plant pathogens but on a variety of host species (Dobbs et al. 2024a). This finding suggests that perhaps these *Fusarium* species share virulence genes that may contribute to conifer pathogenesis, but the non-shared genes among the *Fusarium* profiles may confer host-specific pathogenicity (van der Does and Rep 2007).

Among the *Fusarium* species complex clades discussed in Dobbs et al. (2024a), SC1, consisting of the FFSC, FOSC, and FNSC, had 24 and 20 unique orthologous genes that were shared with the *F. annulatum* and *F. commune* transcriptome profiles, respectively. These consisted of transcription factors, inositol and MFS transporters, and glycoside hydrolases shared with the *F. annulatum* transcriptome, and transcription factors were shared with the *F. commune* transcriptome. SC2, consisting of the FTSC and FI-ESC, had 17 and 18 unique orthologous genes that were shared in the *F. annulatum* and *F. commune* transcriptome profiles, respectively. The *F. annulatum* transcriptome profile contained genes encoding beta-glucosidase, MFS transporters, and chitin synthases, and the *F. commune* transcriptome profile contained genes encoding aurofusarin biosynthesis, transcription factors, and a phosphoenolpyruvate carboxykinase. Both species complex clades had unique genes involved in transport, transcription, CAZymes, and secondary metabolism, all of which have been found to be associated with virulence in fungi (Guo et al. 2014; Ma et al. 2013; Roy et al. 2020). These unique genes indicate that these *Fusarium* species complexes can likely use unique pathways for disease development on their hosts. Such unique pathogenicity pathways should be considered when developing early detection tools to identify multiple *Fusarium* pathogens within nurseries for management to mitigate disease. However, because we did not conduct pathogenicity assays on all isolates in this study, functional assessments, such as proteomics or metabolomics, should be conducted to validate the repertoire of effectors and secondary metabolites nec-

essary for conifer pathogenesis among each species complex clade (Hooshmand et al. 2020; Zhao et al. 2020).

Conclusions

Our findings demonstrate that all *F. annulatum* and *F. commune* isolates used in this study were plant pathogens, regardless of their host origins, and further suggest that these isolates did not exhibit host specificity under the test conditions. Profiles of pathogenicity/virulence genes within the transcriptomes were identified as unique among *Fusarium* species complexes, but these profiles were not unique among conifer pathogens. Additionally, our results show that UIP genes in both *F. annulatum* and *F. commune* were involved in the production of important pathogenicity/virulence-associated, secreted proteins and secondary metabolites, which were primarily produced at 48 hpi. Although these putative pathogenicity profiles did not definitively correspond with pathogenicity on coniferous versus herbaceous hosts, they were associated with *Fusarium* species complexes. For this reason, a subset of these pathogenicity gene profiles could potentially aid in the development of detection methods and help characterize the pathogenicity mechanisms utilized by *Fusaria* in conifers.

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