DISSERTATION

# COMMUNITY STRUCTURE AND PATHOGENOMICS OF *PINACEAE*-INFECTING *FUSARIUM* SPP.

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# ABSTRACT

## COMMUNITY STRUCTURE AND PATHOGENOMICS OF *PINACEAE*-INFECTING *FUSARIUM* SPP.

Due to a warming climate, the need for nursery grown conifer seedlings is continually increasing. However, several *Fusarium* spp. that cause pre- and post-emergent damping-off and root disease can hinder production of conifer seedlings. These soil or seed-borne *Fusarium* pathogens of conifers infect seedlings through the developing roots, and their similar effects on conifer hosts suggests that these pathogens may share a common evolutionary history. The shared ecological function of these *Fusarium* pathogens is likely associated with lineage-specific (LS) chromosomes or virulence gene(s) that are shared among these species. Identifying these potentially shared chromosomes or gene(s) and their functionality is best approached through the use of multiple ‘omics technologies. Taken together, genomics, transcriptomics, proteomics, and metabolomics provide a comprehensive overview of the plant-microbial interactions at the time of *Fusarium* infection. This research accentuates how a combination of these technologies, such as genomics and transcriptomics, can be used to elucidate the biology of *Fusarium* pathogens and identify the presence of virulence-associated LS chromosomes or virulence gene(s) that facilitate the development of tools to rapidly identify and track these important pathogens.

Chapter two, published in *Frontiers in Plant Science*, presents the observed regional effect on community structure of Fusarioid fungi collected from conifer seedlings among nurseries across the contiguous USA. The need for a global consensus to establish and maintain databases based on Fusarioid species type strains as references due to the continuing taxonomic

disputes about the appropriate classification of *Fusarium* spp. designations was also discussed. For this reason, phylogenetic placement of the isolates was used for species identification; however, it is recognized that more research, such as whole genome sequencing, is needed to further validate the taxonomic identify of the isolates used in this study. Chapter three presents the whole genome comparisons of 17 *Fusarium* spp. isolates collected from conifer seedlings. Based on phylogenetic analyses of 16 conserved loci and composition of predicted genes, species were shown similar within and among *Fusarium* species complexes. Putative profiles of pathogenicity/virulence genes, including secreted in xylem (SIX) genes 2, 3, 9, and 14, and secondary metabolites, including the mycotoxins fumonisin and deoxynivelanol, were identified among the species complexes, but validation of expression of these genes is needed to demonstrate their functionality. Chapter four explores the mechanisms of pathogenicity and/or virulence of two understudied *Fusarium* spp., *F. commune* and *F. annulatum*, on conifer and non-conifer hosts and the differential gene expression in a susceptible conifer species. Further, the putative secretome profiles of *Fusarium* spp. within species complexes were identified, containing secreted carbohydrate-active enzymes, major facilitator supergroup transporters, apoplastic effectors, and gene products involved in secondary metabolite biosynthesis such as prolipyrone B/gibepyrone D, aurofusarin, and deoxinivelanol. Results from this study showed *F. annulatum* and *F. commune* caused disease on young conifer and non-conifer seedlings and identified putative genes associated with broad pathogenicity, and possibly indicating age-related resistance within the conifer host to certain upregulated pathogenicity genes.

Due to the threat of spreading fungal pathogens from nurseries to field sites through latent infected seedlings and seed, this research highlights the need for robust early detection methods, while also providing insight into the biology of 17 *Fusarium* spp. that are potentially

pathogenic to conifer seedlings. This research will help further develop technologies that aid managers for controlling Fusarium damping-off and root disease and mitigating the spread of novel haplotypes across regions.

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# CHAPTER 1: MULTI-OMICS TOOLS FOR TRACKING *FUSARIUM* CONIFER NURSERY PATHOGENS FOR EARLY DETECTION AND TO PREVENT SPREAD

## Introduction

Fungal plant pathogens are globally problematic for agricultural plant producers including nurseries and forests. Movement of pathogens to new environments increases the risk of detrimental losses of hosts that were not adapted to these introduced pathogens. With an increased need for nursery grown conifer seedlings due to wildfires in the U.S.A., more seed and seedlings may need to be transferred among nurseries and field sites leading to more potential movement of novel pathogens to field plantings (Fargione et al. 2021). To mitigate these introductions, monitoring nursery plant movement and early detection of plant pathogens can minimize losses caused by these novel pathogen introductions. A plethora of techniques are currently used to detect and monitor plant pathogens, e.g., disease modeling, molecular environmental (air, water, plant, and soil) screening, and assessments of morphological and diagnostic features. To accurately characterize a plant pathogen requires confirmation that the isolate causes disease and an understanding of the pathogen's biology, including at the molecular level to elucidate mechanisms used for disease development. However, the diversity of fungal plant pathogens and the intricacy of their genomic mechanisms used in host pathogenicity makes it impractical to rely on an individual tool to detect and track pathogens. *Fusarium* is a cosmopolitan fungal genus that contains many plant pathogenic species. Many cause the similar symptoms on shared hosts, however the shared evolutionary history of these pathogens are not known. We hypothesize that this shared evolutionary history is likely due to the horizontal transfer of host-specific lineage-specific chromosomes and/or pathogenicity/virulence genes that

can be discerned using a combination of ‘omics technologies. Additionally, in recent years, the number of species and species identification methods in the genus has been in dispute highlighting the need for a standardized identification method. In this review, recommendations for the use of multi-omics developed tools as an early detection method for *Fusarium* pathogens in conifer nurseries is discussed.

**Fusaria**

*Fusarium* is a genus of diverse plant and human pathogens, innocuous soil saprophytes, and endophytes (Summerell et al. 2019). *Fusarium* spp. have been found to cause diseases on above- and belowground plant parts including cankers, root rots, damping-off, wilts, and blights on a wide range of hosts (Drenkhan et al. 2020; Rivera-Jimenez et al. 2018; Dobbs et al. 2020; Shude et al. 2020). Differentiating *Fusarium* spp., as well as pathogenic versus non-pathogenic strains, can be difficult because morphological characteristics can be plastic across species and unreliably unique (Gordon et al. 2020; O’Donnell et al. 2022; Stewart et al. 2012). Additionally, some pathogenic species have host-specific strains referred to as formae speciales (Edel-Hermann & Lecomte 2019; Lievens et al. 2008; van Dam et al. 2016). *Fusaria* produce many secondary metabolites and effectors to infect and feed on host tissues; the composition of which has been described as contributing to or responsible for this host-specificity (Li et al. 2016; Li et al. 2020a; van Dam et al. 2016). Conditionally dispensable, lineage-specific chromosomes (LS) house pathogenicity and virulence factors have been characterized on some *Fusarium* spp. that can be involved in host-specificity (Yang et al. 2020; Hatta et al. 2002; Li et al. 2016; Li et al. 2020b; Li et al. 2020c; Li et al. 2020d; Witte et al. 2021). These LS chromosomes are able to be gained or lost without affecting normal cellular function within a strain (Yang et al. 2020), and can also be horizontally transferred among strains and species through anastomoses that allow

previously saprophytic strains to become pathogenic (Vlaardingerbroek et al. 2016; van Dam et al. 2017). Therefore, molecular methods that identify these virulence-related LS chromosomes are necessary to differentiate pathogenic and non-pathogenic strains.

**Fusaria in conifer nurseries**

In conifer nurseries, numerous *Fusarium* spp. have been identified from conifer hosts including Douglas-fir (*Pseudotsuga menziesii*), pine (*Pinus* spp.), larch (*Larix* spp.), and spruce (*Picea* spp.) (Dobbs et al. 2023a; Drenkhan et al. 2020; James 2004; James 2005). However, pathogenicity has not been confirmed for all described species associations. Species that have been identified as pathogenic to conifers include *F. oxysporum*, *F. circinatum*, *F. avenaceum*, *F. acuminatum*, *F. commune*, and *F. proliferatum* (James et al. 1996; Stewart et al. 2012; Stewart et al. 2016). However, *F. circinatum* (causes pitch canker) in recent years has been the most prevalently studied due to its devastating impact on conifers [e.g., Monterey pine (*Pinus radiata*)] both in nurseries and in field sites, as well as its new classification as a quarantine species (Fernandes et al. 2023). Losses due to fungal diseases in conifer nurseries have been reported as high as 40% of sown seed and cost up to $40,000 annually for management (Dobbs et al. 2023a). This highlights the risk of unmitigated spread and difficulty of removing fungal pathogens in nurseries.

As best practices for identifying *Fusarium* isolates to species are currently in dispute (Crous et al. 2021; Geiser et al. 2021; O'Donnell et al. 2020; O'Donnell et al. 2022), an alternative method may be necessary to detect and determine pathogenic *Fusarium* lineages. Additionally, identifying a *Fusarium* species in a nursery may not necessitate management as pathogenicity or virulence are not associated with species but rather strains within species. *Fusarium* species within species complexes can utilize similar genetic pathways for disease development, e.g.,

toxin production or regulatory genes, and using these as targets may provide a robust method for determining *Fusarium* pathogens presence in nursery systems (Bakker et al. 2018; Niehaus et al. 2016; Perincherry et al. 2019; van Dam et al. 2016). Identifying *Fusarium* pathogens of conifer hosts may be possible using mass spectrometry or amplification of genes involved in the production of these mycotoxins or other virulence factors (Jerome Jeyakumar et al. 2018; Wigmann et al. 2019; van Dam et al. 2018).

Current management practices in conifer nurseries focus on preventative measures such as pre-planting soil fumigation to reduce the pathogen pressure on newly germinating seedlings (Gordon et al. 2020). Studies have shown that after using soil fumigation *Trichoderma* spp. were the initial colonizers of the treated soil and acted as an early deterrent for fungal pathogens (Cram et al. 2007). After preventative treatments, host symptom development informs managers when cultural and/or chemical practices are necessary to attempt to eradicate or mitigate further spread of fungal pathogens. However, once established in a nursery it is difficult to remove soil-borne pathogens, like Fusaria. Due to the hazards of methyl bromide use, alternatives are being encouraged, such as cultural methods (Hildebrand et al. 2004), solarization (Frankel et al. 2020), biological controls (Dumroese et al. 2012; Martin-Garcia et al. 2019; Pederson et al. 1999; Zhang et al. 2017), enzyme and nanoparticle treatments (Aleksandrowicz-Trzcińska et al. 2018), and resistance breeding programs (Martin-Garcia et al. 2019; Dudley et al. 2020). Early detection, before spread and symptom development, can mitigate the need for costly and lengthy management programs. Using multiple molecular detection methods that differentiate pathogens from saprophytes can inform managers when management is necessary. Utilizing a modified top-down data reduction approach to integrating multi-omics tools for systems biology investigations

can provide a targeted approach to developing robust molecular detection tools (Pinu et al. 2021; Figure 1.1).

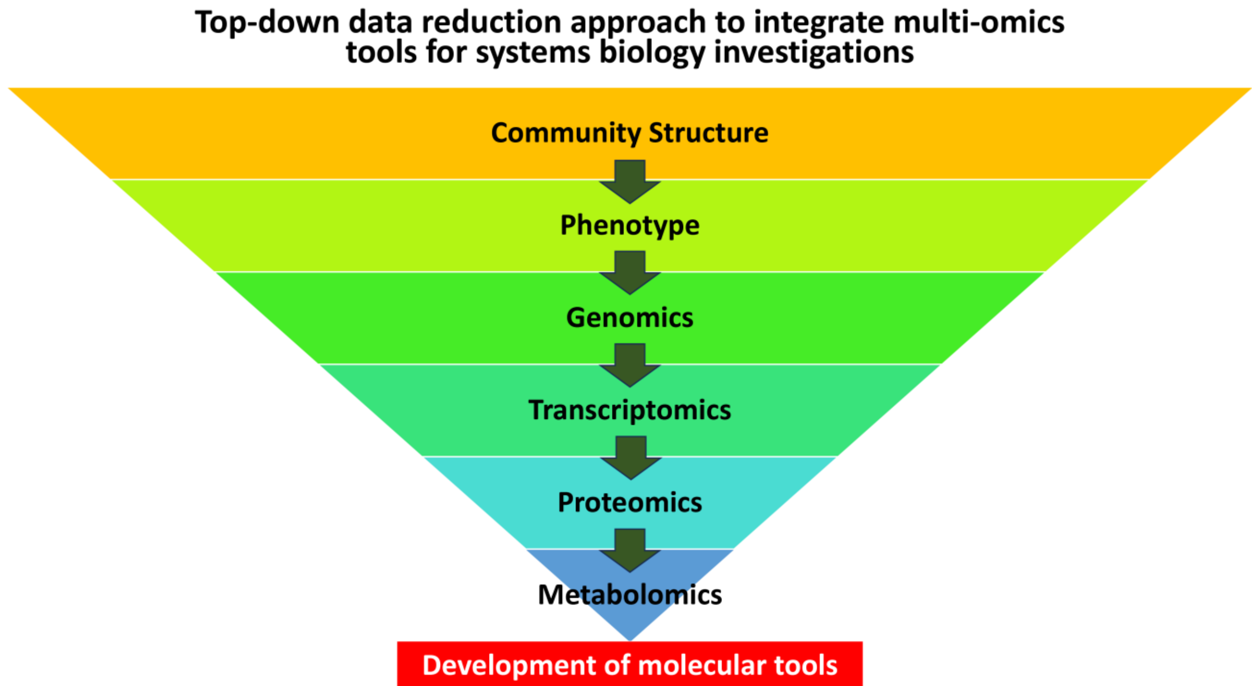


Figure 1.1 – Top-down data reduction approach in systems biology to develop molecular tools for rapid identification of pathogenic Fusaria. Step 1: assess the community structure of *Fusarium* spp. within a nursery; Step 2: confirm pathogenicity of those *Fusarium* isolates to conifer hosts; Step 3: conduct whole genome analyses to identify putative genes shared among potential pathogens; Step 4: conduct transcriptomics analyses to identify differentially expressed genes within the host produced by the pathogen; Step 5: conduct proteomics analyses to identify expressed secreted proteins and effectors; Step 6: conduct metabolomics analyses to identify downstream products including mycotoxins involved in conifer pathogenesis; Final Step: integrate the data to develop diagnostic molecular tools based on the identified pathogenicity/virulence pathways and genes.

**Community and population analyses for determination of pathogen pressure**

Identifying community structure and population analyses can help determine pathogen pressure on hosts. Some pathogenic fungi are considered weak pathogens but may work in concert with other pathogens to cause more significant disease (Abdullah et al. 2017). Identifying shifts in community structure through metabarcoding or shotgun sequencing requires detailed analyses of within nursery conditions for container and bareroot systems. These

communities may shift when seedlings are planted into field sites, however, more studies are needed to identify this shift. Microbial communities will need to be reassessed if continual monitoring in field sites is desired.

Previous studies have found that region has a significant effect on *Fusarium* community structure (Dobbs et al. 2023a; Yergeau et al. 2009). However, more surveys are needed to determine if host associations have a significant effect on *Fusarium* community structure within a region. Climate factors may be a driving force in the community structure observed in these studies (Timmusk et al. 2020; Yergeau et al. 2009). This may be important due to increasing temperatures have been reported in recent years. Increasing temperature for some pathogenic *Fusarium* spp. has been found to increase virulence in as few as 5°C (increase from 25° to 30°C) (Cram 2003; Dumroese & James 2005; Huang & Kuhlman 1991). More studies are needed to determine if a broad range of *Fusarium* spp. may increase virulence at increased temperatures and if cohabitating species may impact this increased virulence at higher temperatures. Moisture has also been determined to be a driver of disease (Cram 2003). Increased moisture makes conditions more favorable to pathogens and have resulted in recommendations of decreasing moisture when pathogen pressure is high (Dumroese et al. 2018). Understanding the environmental influences on community structure is important for understanding the pathogens biology but to accurately determine *Fusarium* communities and populations, accurate species identification is necessary.

**Characterizing Fusaria in conifer nurseries**

Traditional methods included collecting soil or plant samples and plating on selective media, e.g., Komada's agar or peptone-PCNB agar (PPA), to isolate *Fusarium* spp. (Leslie et al. 2006; Summerell et al. 2003). Then identifying the isolates to species using morphological features

produced on low nutrient agars, e.g., carnation leaf agar and SNA petri plates (Summerell et al. 2003). This method, while affordable, can be time consuming and may not detect a pathogen before it establishes in a nursery. Downstream analyses are also necessary to determine if isolates are indeed pathogenic through Koch's postulates. Identifying the microbial community of a nursery system can indicate a potential shift in the community when a pathogen is introduced (Ata et al. 2022a; Ata et al. 2022b; Ibarra Caballero et al. 2023; Stewart et al. 2021). Utilizing environmental screening techniques within a nursery would allow a nursery to identify this shift from a healthy microbial community to an inhospitable environment for the plant host. Collection of air, water, and/ or soil would allow for the extraction of eDNA, eRNA, and metabolites that could determine the microbial community health.

*Fusarium* species diversity within nurseries can be determined through high throughput sequencing methods such as metabarcoding and metagenomics, as they are powerful tools that can rapidly identify microbial communities. Metatranscriptomics, as well, is a powerful tool to identify genes involved during host-microbe interactions. Shotgun sequencing of eDNA allows for the construction of whole genomes of microbial organisms in the sample (Kestel et al. 2022; Piombo et al. 2021; Quince et al. 2017). Metagenomics also has the benefit of being able to identify unculturable microbes (Albertsen et al. 2013; Tringe & Rubin 2005; Lapidus & Korobeynikov 2021). This is similar but more informative than metabarcoding techniques that rely on gene loci to identify microbial communities, e.g., internal transcribed spacer (ITS) for fungal and oomycete identification (Edel-hermann et al. 2015; Tedersoo et al. 2022). This could rapidly identify *Fusarium* spp. composition and associated microbes to assess the overall health of the microbial communities to determine if pathogen pressure is high within the nursery. Metatranscriptomics could be utilized within these pathosystems to identify virulence genes

produced by pathogens in the rhizosphere soil and within the host tissue (Ata et al. 2022a; Guo et al. 2021; Kuske et al. 2015; Schneider et al. 2021; Wang et al. 2023). Pathogenicity and/or virulence factors expressed during host infection could be identified and used to inform greenhouse managers when a pathogen is present in a nursery and warrants management. Environmental and host tissue sampling are useful preventative methods to keep apprised on pathogen pressure.

**Pathogen biology informs molecular identification**

Molecular detection techniques are inexpensive methods to detect pathogens prior to outbreaks. However, to accurately design these tools, it is essential to characterize pathogen biology, lifestyle (Constantin et al. 2021; Dobbs et al. 2023a; Hill et al. 2022), and host range (Borah et al. 2018; Dewing et al. 2022; Graham-Taylor et al. 2020). Assessing molecular plant-pathogen interactions (Elhamouly et al. 2022; Guttman et al. 2014; Ma et al. 2013), and the environmental conditions for optimal growth and infection of the pathogen and host (Corredor-Moreno et al. 2020; Cruz et al. 2019; Gordon et al. 2019) can elucidate pathogen biology prior to designing molecular tools. These are essential first steps into any integrated pest management strategy that can develop robust recommendations for control measures of pathogens.

Identifying a previously reported pathogenic species does not necessarily identify a pathogen in another system, as lifestyles can vary greatly among strains as innocuous saprophytes, opportunistic pathogens, and primary pathogens. Using multiple ‘omics tools can aid in developing more robust pathogen-specific probes and detection methods (Niehaus et al. 2016). Individually each tool provides a degree of information about plant pathogens how disease development occurs, but taken together these tools provide greater understanding what differentiates pathogens from non-pathogens (Guttman et al. 2014; Pang et al. 2022). Elucidating

the underlying mechanisms involved in the complex molecular plant-microbe interactions through multiple ‘omics tools can be utilized to produce pathogen-specific probes. As sequencing and diagnostic tool costs have decreased, molecular tools have become more widely accessible to a broader community (Pinu et al. 2019).

Whole genome comparisons and transcriptomics analyses can be predictive tools in determining molecular targets for molecular identification (Dobbs et al. 2020; Ye et al. 2018). However, genomic analyses identify genes within a tool kit that a pathogen could potentially use, but the whole repertoire of genes may not be expressed during infection and disease development (Ibarra Caballero et al. 2019). To characterize a pathogen’s tool kit, a high quality genome is needed and can be generated either though combining long and short read technologies such as Illumina-based short reads and PacBIO or oxford nanopore technologies (Yun et al. 2019; Tai et al. 2023) or through structural annotation methods like Hi-C (Zaccaron et al. 2022). However, obtaining these high-quality genomes can increase costs and the extraction of high quality, high molecular weight DNA can be laborious and difficult for some organisms.

Transcriptomics, the sequencing of expressed RNA transcripts, is another valuable tool to identify genes being expressed, but also does not provide a precise depiction of what downstream products are produced and used by a pathogen during infection. Typically, however, this method provides more detail about the putative pathways utilized by a pathogen (Tu et al. 2023). This method can also become expensive as best practices to answer your biological question may require collecting multiple samples and replicates under various conditions to provide a more accurate depiction of genes involved in pathogenesis (Conesa et al. 2016; Upton et al. 2023). Combining methodologies such as RNAseq and tagseq can reduce costs per sample to make transcriptomics more affordable (Lohman et al. 2016). Genomics and transcriptomics

together (Ibarra Caballero et al. 2020; Dobbs et al. 2023b) can provide insight into the pathogens biology through potential molecular pathways utilized by the pathogen and predict best tools for validation through *in silico* methods such as primer development or functional methods such as proteomics and/or metabolomics.

Molecular identification methods including SNP based probes based on genes identified in the genomes and/or transcriptomes have been used to differentiate pathogens from non-pathogens (Dobbs et al. 2020; Luchi et al. 2020). Diagnostic primers including locus specific amplification, e.g., quantitative polymerase chain reaction (qPCR; Thomas et al. 2022), loop-mediated isothermal amplification (LAMP; Deng et al. 2022), recombinase polymerase amplification–lateral flow dipstick (RPA-LFD; Xu et al. 2023), and PCR based enzyme linked immunosorbent assay (ELISA; Omori et al. 2018), are useful tools as they can quantify pathogen pressure and techniques like LAMP don't require specialized tools to inform managers that the pathogen is present.

Systems biology investigations of pathogens aim to understand the complex pathogen-host interactions (Dix et al. 2016; Pinu et al. 2019). Using a top-down approach provides a holistic view of these interactions and uses genomics, transcriptomics, proteomics, and metabolomics techniques to elucidate the mechanisms and pathways used by pathogens to cause disease (Dix et al. 2016; Pinu et al. 2019). Integrating these technologies provides a detailed view of the mechanisms and pathways utilized by the pathogen to cause disease on a susceptible host (Dix et al. 2016; Pinu et al. 2019). Proteomics and metabolomics provide insight into the secreted proteins/effectors or secondary metabolites, respectively, used by the pathogen during its interaction with the host (Hooshmand et al. 2020; Zhao et al. 2020). Mass spectrometry is emerging as a powerful tool to identify pathogens within plant hosts and rhizosphere soil. Mass

spectrometry methods provide an accurate depiction of the detectable proteins that are expressed at the time for further investigation (He et al. 2023; Zhao et al. 2020), thus validating the genomic or transcriptomic predicted secreted proteins and effectors as well as the regulatory enzymes involved in determining the production of metabolites during pathogen infection (Rauwane et al. 2020; Zhao et al. 2020). Metabolomics similarly identifies the detectable metabolites including phytohormones and mycotoxins expressed during infection (Hooshmand et al. 2020; Pang et al. 2022). Mass spectrometry has been used to identify *Fusarium* mycotoxins within crops, e.g., zearalones, trichothecenes, and fumonisins (Ekwomadu et al. 2021; Fallahi et al. 2019). Liquid (LC-MS) and gas (GC-MS) chromatography mass spectrometry have been used to differentiate pathogens within host tissue through identifying pathogen-produced toxins, hormones, and metabolites (Hooshmand et al. 2020; Jeyakumar et al. 2018; Nordstrom et al. 2022). However, there are limitations to both technologies as LC-MS is more useful for dissolvable compounds and GC-MS is more useful for volatile compounds (Beale et al. 2018). Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF) is less informative compared to LC-MS and GC-MS but is useful in comparing pathogen fingerprints based on mass spectra within a sample. *Fusarium* spp. can be differentiated from each other based on subproteome mass spectra similarly to multi-locus DNA sequencing (Wigmann et al. 2019). In combination, these methods would inform the functional genes that would provide the best targets for pathogen identification. Using the predicted and functional genes combined informs about the profiles of genes used by closely related pathogens that may be combined to identify when a pathogen to the host is present rather than a pathogenic species is present within a system (Luchi et al. 2020; Nordstrom et al. 2022). This would be of greater use to managers as this informs a manager when sanitation methods are necessary rather than

potential of a pathogen needs further investigation. However, more detail about the diversity among communities and populations would provide insight to whether this is possible for inter- or intraspecies detection.

**Tracking and potential danger of introducing pathogens into new environments**

Introducing novel strains of pathogenic species can be problematic for crops. In other plant pathogens, transferring strains to field sites can cause severe damage to out-planted seedlings and neighboring trees (Simamora et al. 2018; Weiland 2021). *Fusarium circinatum* has been found to be problematic in nurseries and in out-planted field sites (Santana et al. 2015). It has been posited that nursery adapted strains are not well-suited to field sites (Dumroese et al. 2005), however introducing novel virulence genes may be horizontally transferred among strains that may affect pathogenicity and virulence. Developing tools that rapidly identify *Fusarium* pathogens can mitigate this spread.

Predictive modeling in combination with quarantine measures are useful to mitigate the spread of pathogens. Models can be based on many factors including climate variables changing across time (Ali et al. 2022; Alkhalifah et al. 2023) and sensing host response to pathogen secreted compounds (Cui et al. 2022). Integrating climate variables that are influential to the pathogen and the detection of the pathogen through secreted effectors, secondary metabolites, or virulence genes would provide a robust model to generate predictive models for use by regulatory agencies and nursery managers, in particular bareroot nurseries since they are not as capable of controlling environmental factors as container systems. This would require generating a comprehensive pathogen profile database consisting of diagnostic tools, including primers and mass spectral fingerprints, and virulence mechanisms produced by the pathogen identified through multi-omics tools. A compliment to the pathogen profile databases creation is that a

requirement should be implemented that federal agencies require purchasers of seed or seedlings that cross state or federal boarders be required to pay for pathogen testing to limit the chances of spreading pathogens to novel environments. However, enforcement by nurseries, seed companies, and/or APHIS would be necessary to ensure these recommendations are enacted appropriately. The database would aid in more accurate detection of pathogenic *Fusaria*.

**Conclusions**

Due to the complexity of *Fusarium* genomics, to accurately and efficiently identify conifer pathogens, a systems biology approach should be used to generate pathogen profiles for robust detection tools like qPCR probes, LAMP assays, and mass spectral fingerprints. Managers can have their nursery environments, e.g., soil, water, or air, and/or plants tested for the presence of *Fusarium* pathogens using tools targeted to *Fusarium* pathogens rather than *Fusarium* spp.

Combined, early detection methods provide a more accurate representation of the pathogen community present in a nursery. Environmental or another culture independent genomic or transcriptomic method should be used either through air, water, soil, or plant tissue to identify pathogenic species or species complexes present in the nursery. Mass spectrometry, MALDI-TOF or LC-MS/GC-MS of plant tissue should be used to identify the presence of pathogen secondary metabolites and mycotoxins. Prescreening of seeds and plant material would reduce the likelihood of introducing novel pathogenic strains to a nursery or field site.

More research is needed to determine the driving factors for conifer pathogenesis including temperature, moisture, and/ or community structure. Curated, diagnostic databases are needed that include virulence factors such as genes, genomes, transcripts, mass spectra, and metabolites for rapid identification of conifer pathogenic *Fusarium* species. These techniques and databases can be used and developed for other pathosystems.

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# CHAPTER 2: FUSARIOID COMMUNITY DIVERSITY ASSOCIATED WITH CONIFER SEEDLINGS IN FOREST NURSERIES ACROSS THE CONTIGUOUS USA[1]

**Summary**

**Introduction:** Fusarioid fungi that cause damping-off and root diseases can result in significant losses to conifer crops produced in forest nurseries across the USA. These nurseries are vital to reforestation and forest restoration efforts. Understanding the diversity of Fusarioid fungi associated with damping-off and root diseases of conifer seedlings can provide an approach for targeted management techniques to limit seedling losses and pathogen spread to novel landscapes.

**Methods:** This study identifies 26 *Fusarium* spp. (*F. acuminatum*, *F. annulatum*, *F. avenaceum*, *F. brachygibbosum*, *F. clavus*, *F. commune*, *F. cugenangense*, *F. diversisporum*, *F. elaeagni*, *F. elaeidis*, *F. flocciferum*, *F. fredkrugeri*, *F. fujikuroi*, *F. grosmichelii*, *F. ipomoeae*, *F. lactis*, *F. languescens*, *F. luffae*, *F. odoratissimum*, *F. oxysporum*, *F. queenslandicum*, *F. redolens*, *F. torulosum*, *F. triseptatum*, *F. vanleeuwenii*, & *F. verticillioides*), 15 potential species within *Fusarium* and *Neocosmospora* species complexes (two from *F. fujikuroi* species complex, nine from *F. oxysporum* species complex, three from *F. tricinctum* species complex, and one from *Neocosmospora* species complex), and four *Neocosmospora* spp. (*N. falciforme*, *N. metavorans*, *N. pisi*, & *N. solani*) and associated host information collected from conifer-producing nurseries across the contiguous USA.

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**Results:** Phylogenetic analyses identified Fusarioid fungi haplotypes that were associated with 1) host specificity, 2) localization to geographic regions, or 3) generalists found on multiple hosts across diverse geographic regions.

**Discussion:** The haplotypes and novel species identified on conifer seedlings should be considered for further analysis to determine pathogenicity, pathogen spread, and assess management practices.

## Introduction

Forest nurseries produce seedlings of diverse conifer and hardwood species for timber production, forest restoration, and reforestation. In the United States (USA), 1.2 billion conifer seedlings are produced annually through container and/or bareroot stock types, primarily in the southern, northwestern, and northcentral regions (Starkey et al., 2015a; Starkey et al., 2015b; Haase et al., 2020). However, nursery seedling production of both stock types for diverse purposes is frequently hampered by seedling diseases. Container nurseries tend to support higher seedling pathogen density, while bareroot nurseries tend to support greater pathogen species richness (Okorski et al., 2019). After transferring seedlings from nurseries to planting sites, seedling performance (i.e., survival and growth) is decreased when post-planting monitoring and treatments are lacking (Fargione et al., 2021). In addition, seedling survival after outplanting may be influenced by the introduction of nursery pathogens into the forest planting sites. For example, *Phytophthora* spp. can devastate hosts in natural stands after these pathogen species are carried on nursery stock and introduced into novel landscapes (Frankel et al., 2020). Once introduced, these pathogens are difficult to manage and can adversely impact tree planting programs and native vegetation surrounding planting sites.

Conifer seedlings are susceptible to a variety of soil- and seedborne pathogens that can hinder production or have costly management implications due to the diversity in pathogen genera and species. Members of the genera *Cylindrocarpon* (syn: *Ilyonectria* & *Neonectria*), *Cylindrocladium* (syn: *Calonectria*), *Fusarium*, *Pythium*, *Phytophthora*, and *Rhizoctonia* have been found to cause damping-off and root disease (Dumroese et al., 2000; Cram, 2003; Dumroese and James, 2005; Vujanovic et al., 2007). Recently, *F. solani* species complex was placed in the genus *Neocosmospora* (Crous et al., 2021). Damping-off is the most common disease resulting in seedling wilting and mortality in North American forest nurseries, which primarily affects conifer seedlings within the first 4 to 6 weeks after germination (Cram, 2003). Symptoms of damping-off can range from wilting to pre- and post-emergent mortality of seedlings (Cram, 2003). If left untreated, damping-off and root disease pathogens can also cause root rot, stunting, and wilt in older seedlings (James et al., 1989; James et al. 1990b). Of the pathogens that cause seedling diseases, *Fusarium* spp., which are the most commonly isolated fungal pathogens from symptomatic seedlings, cause major economic losses and severe damage to conifer seedling production (James, 2004; Stewart et al., 2012).

Fusarioid fungi are very diverse and contain diverse pathogenic species that affect many important plant species for agriculture, silviculture, and horticulture worldwide, including grains, timber trees, and orchard trees (Marek et al., 2013; Boutigny et al., 2019; Dobbs et al., 2021). Several Fusarioid fungi have been isolated and identified from conifer seedlings, but not every identified species has been tested for pathogenicity on conifer hosts (James et al., 1989; James et al., 1990a; Stewart et al., 2012; Stewart et al., 2016). The most common Fusarioid fungi identified on conifer roots and in surrounding rhizosphere soil are *F. commune*, *F. oxysporum*, *F. proliferatum*, and *Neocosmospora solani*. In subsequent pathogenicity assays, isolates of these

species have all been found to be pathogenic to the hosts from which they were isolated (James 2005; Stewart et al., 2012). Virulence across strains within a species can vary, however, from non-pathogenic to highly virulent (Stewart et al., 2006).

Because morphology alone is insufficient to distinguish among strains of Fusarioid fungi that differ in virulence, molecular characterization or extensive assays are required to assess strain virulence. Further, molecular characterization of Fusarioid fungi is required because distinguishing morphological characteristics are rare or unreliable across some species (Stewart et al., 2012). Some species, such as *F. oxysporum*, have been found to contain host-specific formae speciales, but the full host range and level of specificity remains undetermined for many of these pathogenic strains (EdelHermann & Lecomte, 2019). The diversity observed within Fusarioid fungi inherently complicates their management, and lacking identification methods for pathogenic strains increases the risk of introducing these strains into novel landscapes. *Fusarium circinatum* is an economically and ecologically important canker pathogen that can spread easily from nurseries to planting sites through latent infections that decrease seedling establishment success and cause damage to mature trees in landscapes (Gordon et al., 2015). Unmitigated spread of these pathogens through anthropogenic means may increase the likelihood of pathogen adaptation to new hosts and landscapes, which could result in costly losses of seedlings in forestry landscapes and loss to existing forests (Corredor-Moreno & Saunders, 2020; Fargione et al., 2021).

Our research aim was to assess the diversity of Fusarioid fungi associated with conifer-producing forest nurseries throughout the contiguous USA, based on DNA sequencing of two genes [translation elongation factor 1-a (tef1a) and RNA polymerase II second largest subunit (rpb2)]. We surveyed the nurseries to 1) identify patterns of geographic distribution and host

range across Fusarioid fungi, and 2) determine if Fusarioid fungi haplotypes were widespread or isolated within a region.

## Materials and Methods

### *Sample and isolate collection*

We made two collections of Fusarioid fungi. The first collection (from 2012 through 2016) was made across 11 states (representing federal, private, and state entities) throughout the western, midwestern, and southern USA (Stewart et al., 2020). For this collection, we visited container and bareroot forest nurseries and collected more than 300 isolates from soil, container substrates, and roots from asymptomatic and symptomatic conifer and non-conifer hosts; however, to increase the likelihood of identifying conifer pathogens, only 66 of those isolates that were collected from conifer host roots are reported in this study. Our second collection (from 2017 through 2020) targeted underrepresented conifer hosts and states identified after the first collection. Ten nurseries across eight states were surveyed about potential root disease problems. Based on their responses (Supplemental Table 1 https://www.frontiersin.org/articles/10.3389/fpls.2023.1104675/full#supplementary-material), five nurseries shipped conifer seedlings in 3.8-L, zip-lock-type bags on ice with the roots wrapped in damp paper towels to prevent desiccation. We visited three nurseries in 2021 and attempted to collect up to 20 seedlings (10 asymptomatic and 10 symptomatic) of each conifer species grown at each nursery. According to our survey agreements, specific-site information will remain confidential, and analyses were only conducted at the state level. The number of samples per site varied from 14 to 101 with 1 to 21 seedlings per host.

Fusarioid fungal isolates were obtained from plant samples by culturing root segments on Komada's agar (Komada, 1976). Five root segments per seedling were surface disinfested in

10% commercial bleach for 2 minutes, washed twice in sterile water, and plated. After at least 3 days, when initial mycelial growth from root segments was observed, hyphal tips were transferred to ¼-strength potato dextrose agar (PDA; BD Difco™ Dehydrated Culture Media, Franklin Lakes, New Jersey, USA) in Petri dishes. Cultures were grown at 25°C for 14 days in the dark. Cultures were stored in the Stewart collection (Colorado State University) in glycerol broth at -80°C and/or on sterile filter paper stored at -20°C in coin cases with a desiccant to keep isolates dry.

In total, we collected samples in 16 states (Figure 2.1) and obtained a total of 741 isolates from 27 host species. We selected 325 isolates collected from asymptomatic and symptomatic conifer hosts with associated tree species and state information for further analyses (these analyses excluded isolates from Michigan and Utah). A total of 66 isolates were obtained from the 2012-2016 collection and the remaining 259 isolates were from the 2017-2020 collection (Supplemental Table 2 https://www.frontiersin.org/articles/10.3389/fpls.2023.1104675/full#supplementary-material).

*DNA Sequencing of* Fusarium *isolates*

The 325 isolates were grown on ¼-strength PDA in Petri dishes and initially extracted using a Chelex100® resin extraction protocol similar to Dobbs et al. (2021). Mycelia were scraped from cultures using a sterile spatula and transferred to 0.2-ml, 8-strip PCR tubes, followed by thermolyzation using a thermal cycler program of 100°C for 35 minutes and held at 4°C until ready for use in polymerase chain reaction (PCR). If poor quality DNA sequencing was obtained in the forward direction or low-quality DNA was obtained from the isolate, a CTAB extraction protocol from Dobbs et al. (2021) was used as an alternate DNA extraction method. Isolates were sequenced at two loci, *tef1a* (Primers EF-1: 5'-ATGGGTAAGGARGACAAGAC-

3’ and EF-2: 5’-GGARGTACCAGTSATCATGTT-3’; O’Donnell et al., 1998) and *rpb2* (Primers RPB2-6F: 5’-TGGGGKWTGGTYTGYCCTGC-3’ and fRPB2-7cR: 5’-CCCATRGCTTGYTTRCCCAT-3’; Liu et al., 1999). Both loci were amplified using a PCR cycle program of 94°C for 2 min, 40 cycles of 94°C for 40s, 58°C for 40s, and 72°C for 30s, and 72°C for 5 min (Dobbs et al., 2020). Products were electrophoresed on a 1.5% agarose gel to visualize amplified PCR product using GelRed® (Biotium). Amplified products were sequenced in both directions using Sanger sequencing at Eurofins Genomics (https://eurofinsgenomics.com/en/ home/). As described in O’Donnell et al. (2021), sequences were checked visually for base-score quality using Geneious Prime v. 2022.0.1 (https://www.geneious.com/)andidentified to putative species through BLAST analysis in the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/), Fusarium-ID v. 3.0 (http://isolate.fusariumdb.org/blast.php), (O’Donnell et al. 2015; O’Donnell et al., 2021). Species were confirmed through phylogenetic analysis using DNA sequences from *Fusarium* spp. type strains obtained from GenBank links from the Fusarioid-ID database (Crous et al., 2021).

*Phylogenetic analysis*

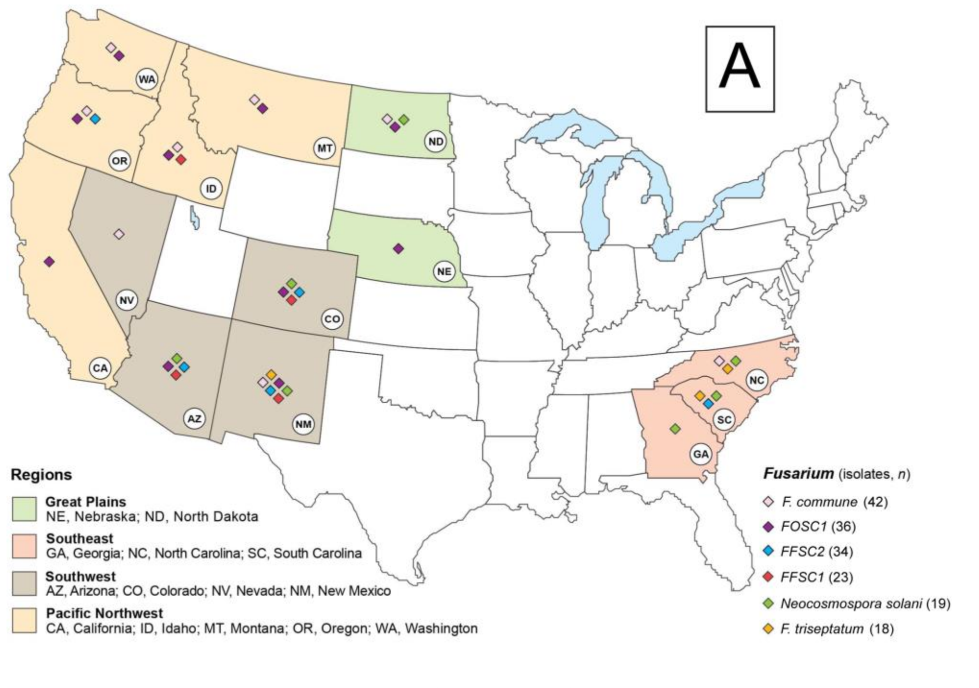
A Bayesian inference phylogeny was constructed from partitioned, concatenated tef1a and rpb2 sequences. Sequences from representative Fusarioid fungi type strains were aligned to survey isolate sequences with MUSCLE sequence aligner using default settings (Edgar, 2004). Haplotypes were generated using DNAsp 6 (Rozas et al., 2017). Iqtree2 v2.2.0 was used to generate the maximum likelihood bootstrap support values for the concatenated phylogeny from the haplotype file with 1000 pseudoreplicates, and Modelfinder was used to determine substitution model for the phylogeny (Minh et al., 2020). Substitution models for each locus

were determined independently by partitioning. Iqtree2 symmetry testing was conducted to ensure each partition did not reject the stationary and homogeneous sequence evolution assumptions (Naser-Khdour et al., 2019). Beauti2 was used to format the aligned sequences for subsequent use in BEAST2 to generate the Bayesian phylogeny (Bouckaert et al., 2019).

*Statistical analyses*

All statistical analyses were conducted in R v. 4.0.2 (R Core Team, 2022). To visualize the distribution of potentially pathogenic haplotypes within Fusarioid fungi, relative abundance heatmaps of Fusarioid fungi by host and by state were generated from 325 isolates using the "gplots" and "Bbmisc" packages (Bischl et al., 2017; Warnes et al., 2020).

To test the hypotheses that the composition of Fusarioid fungi communities differed among regions and between host genera, we conducted a permutational multivariate analysis of variance (PERMANOVA). Fusarioid fungi communities were grouped into four regions, 1) Pacific Northwest consisting of Idaho, Oregon, Washington, Montana, and California; 2) Southwest consisting of Colorado, New Mexico, Arizona, and Nevada; 3) Southeast consisting of Georgia, North Carolina, and South Carolina; and 4) Great Plains consisting of Nebraska and North Dakota (Figure 2.1). Community dissimilarity was contrasted by host genus and region using Bray-Curtis distances with a PERMANOVA using the 'vegan' package (Oksanen et al., 2020).



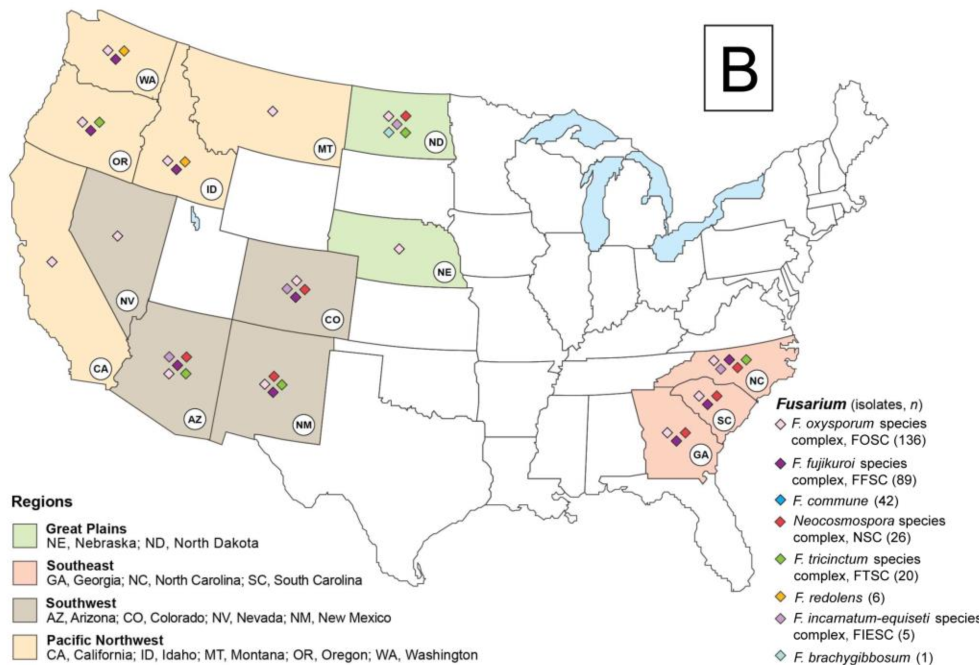




Figure 2.1 – Map of the United States (USA) indicating the six most abundant Fusarioid fungi (A) and the Fusarioid species complexes (B) identified through a damping-off and root disease survey of conifer seedlings in states within the Southwest, Southeast, Pacific Northwest, and Great Plains regions. The colors on the states indicate their regional designation and the colored diamonds over the states indicates the presence of one of the six Fusarioid fungi identified in that state.

## Results

*Phylogenetic analyses*

Each partition did not reject the stationary and homogeneous sequence evolution assumptions with p-values greater than 0.05 (tef1a: SymPval = 0.300, MarPval = 0.184, & IntPval = 0.495; rpb2: SymPval= 0.673, MarPval = 0.615, & IntPval =0.526). Phylogenies were constructed using sequences from the 325 isolates that included isolates that were sequenced at either or both the tef1a and rpb2 loci (Supplemental Table 2 https://www.frontiersin.org/articles/10.3389/fpls.2023.1104675/full#supplementary-material). Sequences for these isolates were aligned and sorted into 191 haplotypes with *Neonectria ditissima*, a sister taxon to *Fusarium* (Geiser et al., 2021), collected from GenBank (accession # tef1a: JF735783.1; rpb2: DQ789798.1) serving as the outgroup (Supplemental Table 2 https://www.frontiersin.org/articles/10.3389/fpls.2023.1104675/full#supplementary-material). Of the eight *Fusarium* species complexes [*F. fujikuroi* species complex (FFSC); *F. incarnatum-equiseti* species complex (FIESC); *F. nisikadoi* species complex (FNSC); *F. oxysporum* species complex (FOSC); *F. redolens* species complex (FRSC); *F. sambucinum* species complex (FSAMSC); *Neocosmospora* species complex (NSC); *F. tricinctum* species complex (FTSC)], most formed well-supported clades, with all with bootstrap values and posterior probabilities above 75 and 0.80 in the maximum likelihood and Bayesian phylogenies, respectively (Figure 2.2). The majority of the haplotypes were collected from single hosts (85%; 124/ 146) and states (83%; 121/146). The remaining 15% (22/146) and 17% (25/146) of haplotypes were derived from two to seven host genera and two to eight states, respectively (48 haplotypes were derived from GenBank references only).

*Fusarium oxysporum* species complex

*Fusarium fujikuroi* species complex

*Fusarium commune*

*Fusarium redolens*

*Fusarium tricinctum* species complex

*Fusarium incarnatum-equiseti* species complex

*Fusarium brachygibbosum*

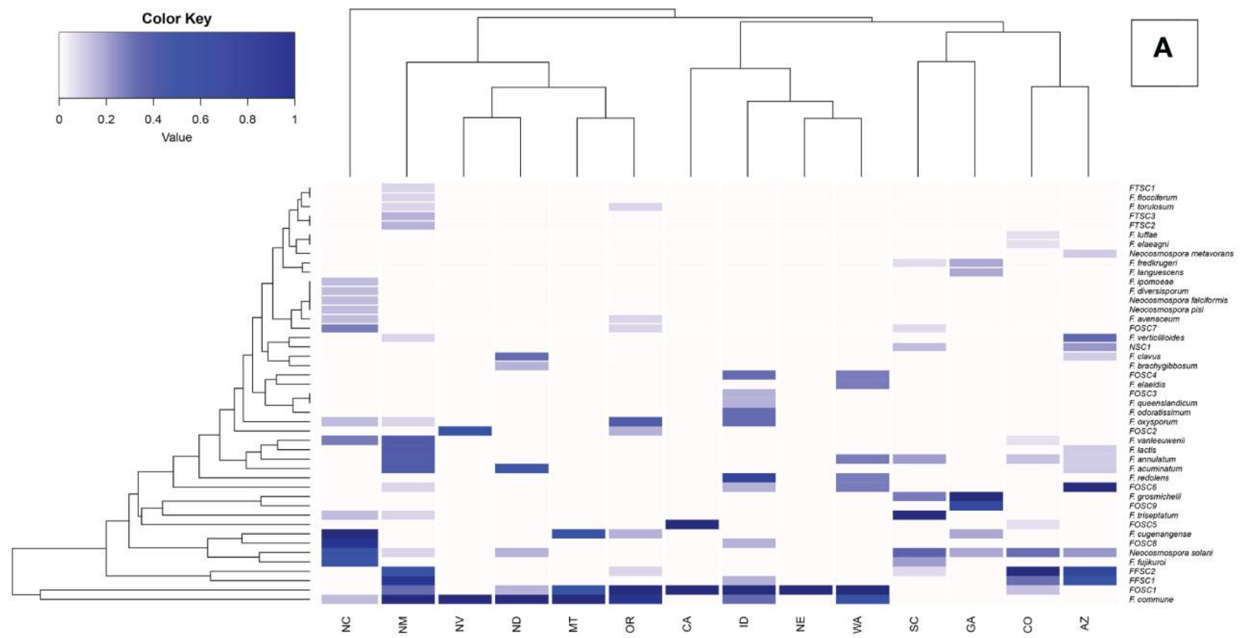
*Neocosmospora* species complex

0.03

Figure 2.2 – Bayesian posterior probability phylogeny inferred from concatenated *tef1α* and *rpb2* haplotypes from sequences of 325 Fusarioid fungi isolates collected from conifer hosts across the contiguous USA. Node values indicate maximum likelihood bootstrap support based on 1000 pseudo-replicates and Bayesian posterior probability values above 70%/0.75, respectively. The tree is rooted to *Neonectria ditissima* isolate sequences collected from GenBank (accession # tef1α: JF735783.1; rpb2: DQ789798.1). For each haplotype (denoted by H#), states are listed and colored circles indicate the host genera from which the isolates within that haplotype were collected. Fusarioid fungi and species complex haplotype clades are grouped together within colored boxes.

*Fusarioid species and relative abundance*

From 325 isolates with *tef1a* and/or *rpb2* sequences, a total of 26 *Fusarium* spp. were identified, and 15 potential *Fusarium* spp. were recognized that could not be assigned to the species level because only two loci (tef1a and rpb2) were sequenced. However, these 15 potential *Fusarium* spp. could all be assigned to four *Fusarium* species complexes [two from FFSC (FFSC1, FFSC2), nine from FOSC (FOSC1, FOSC2,...FOSC9), three from FTSC (FTSC1, FTSC2, FTSC3), and one from NSC (NSC1) members]. In addition, four *Neocosmospora* spp. were identified from the NSC (Supplemental Tables 2, 3 https://www.frontiersin.org/articles/10.3389/fpls.2023.1104675/full#supplementary-material). Relative abundances of Fusarioid fungi were analyzed by state (Figure 2.3A) and host (seedling species) (Figure 2.4A). The most commonly collected species, in order of relative abundance, were *F. commune*, FOSC1, FFSC2, FFSC1, *Neocosmospora solani* (syn. *F. solani*), and *F. triseptatum*. Members within the FOSC and FFSC were the most commonly collected species (Figures 2.3B, 2.4B).



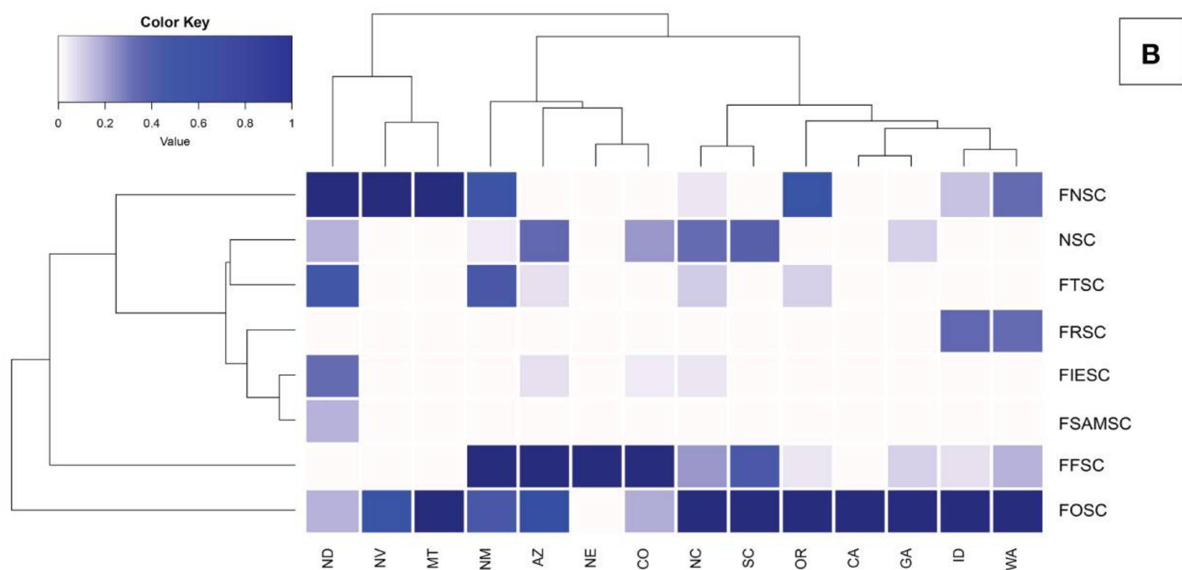
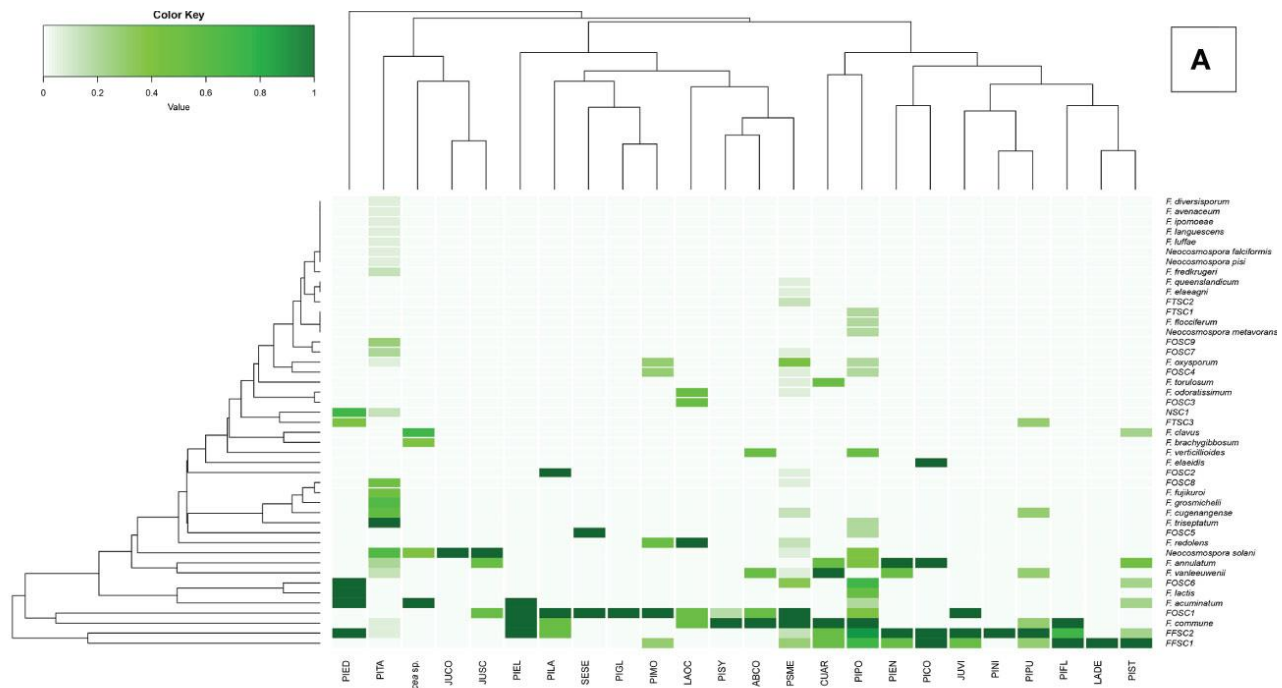




Figure 2.3 – Relative abundance of Fusarioid fungi isolates (A) and Fusarioid species complexes (B) collected from conifer seedling host species from 14 states surveyed across the contiguous USA. Abbreviations for species complexes include FNSC – *Fusarium nisikadoi* species complex; NSC – *Neocosmospora* species complex; FTSC – *Fusarium tricinctum* species complex; FRSC – *Fusarium redolens* species complex; FIESC – *Fusarium incarnatum-equiseti* species complex; FSAMSC – *Fusarium sambucinum* species complex; FFSC – *Fusarium fujikuroi* species complex FOSC – *Fusarium oxysporum* species complex. Data were normalized to indicate the proportion of Fusarioid fungi isolates collected from each conifer host with mint-cream to green signifying the range of proportion from 0 to 1, respectively. Dendrogram shows the hierarchical relationship between each Fusarioid fungi (y-axis) and host (x-axis).



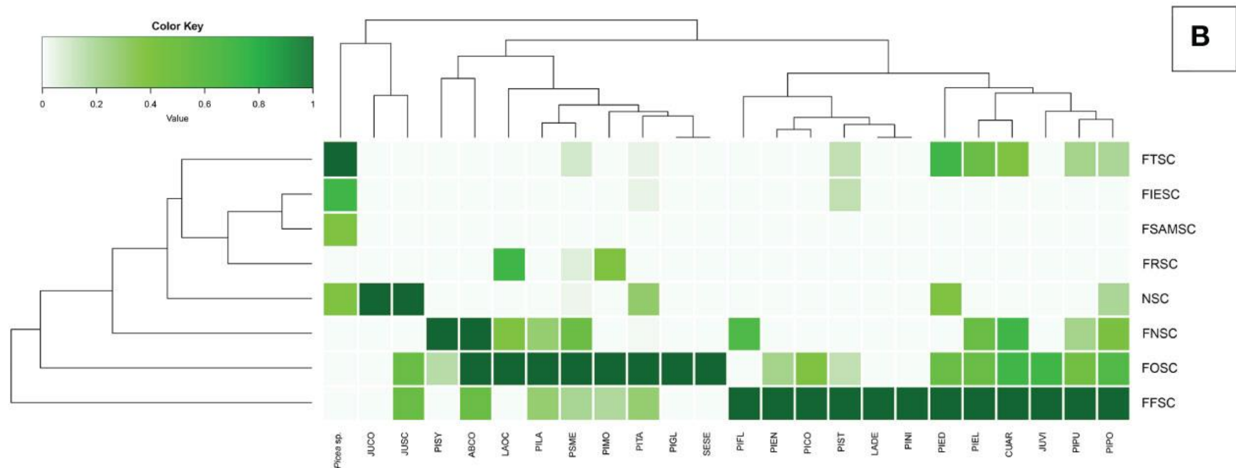




Figure 2.4 – *Fusarium* species relative abundance from isolates collected from conifer seedling host species from 14 states surveyed across the contiguous U.S.A. Data were normalized to indicate the proportion of *Fusarium* species isolates collected from each conifer host with mint-cream to green signifying the range of proportion from 0 to 1, respectively. Dendrogram shows the hierarchical relationship between each *Fusarium* sp. (y-axis) and hosts (x-axis). *Fusarium oxysporum* was the most often collected *Fusarium* species from the most hosts followed by *F. proliferatum* and *F. commune*. ABCO-*Abies concolor*; CUAR-*Cupressus arizonica*; JUCO-*Juniperus communis*; JUSC-*Juniperus scopulorum*; JUVI-*Juniperus virginiana*; LADE-*Larix decidua*; LAOC-*Larix occidentalis*; PICO-*Pinus contorta*; PIED-*Pinus edulis*; PIEL-*Pinus eldarica*; PIEN-*Picea engelmanii*; PIFL-*Pinus flexilis*; PIGL-*Picea glauca*; PILA-*Pinus lambertiana*; PIMO-*Pinus monticola*; PINI-*Pinus nigra*; PIPO-*Pinus ponderosa*; PIPU-*Picea pungens*; PIST-*Pinus strobiformis*; PISY-*Pinus sylvestris*; PITA-*Pinus taeda*; PSME-*Pseudotsuga menzesii*; SESE-*Sequoia sempervirens*; THOC-*Thuja occidentalis*; THPL-*Thuja plicata*; TSHE-*Tsuga heterophylla*

Of these six species, FOSC1 was collected from the most states, *F. triseptatum* from the least, with the other four species intermediate (Table 2.1). The number of plant species that were associated with each Fusarioid fungal species followed the same general pattern, although FFSC1 was associated with the highest number of plant species (Table 2.1).

Table 2.1 – The six most commonly collected *Fusarium* and *Neocosmospora* spp. in order of abundance (left to right), the number of USA states in which they were observed, and the number (and percentage) of host species surveyed (n = 27) and the conifer genera from which they were isolated.

| | *F. commune* | *FOSC1*[a] | *FFSC2*[b] | *FFSC1*[c] | *N. solani* (syn. *F. solani*) | *F. triseptatum* |
|---|---|---|---|---|---|---|
| States (n) | 8 | 9 | 5 | 4 | 7 | 2 |
| Associations (n; %) | 11; 40.7 | 12; 44.4 | 14; 51.8 | 11; 40.7 | 6; 22.2 | 2; 7.4 |
| Genera | | | | | | |
| *Abies* | X | X | | | | |
| *Cupressus* | X | | X | X | | |
| *Juniperus* | | X | X | X | X | |
| *Larix* | X | X | | X | | |
| *Picea* | X | X | X | X | X | |
| *Pinus* | X | X | X | X | X | X |
| *Pseudotsuga* | X | X | X | X | X | |
| *Thuja* | | X | | | X | |

[a]FOSC1, Fusarium oxysporum species complex 1.
[b]FFSC2, Fusarium fujikuroi species complex 2.
[c]FFSC1, Fusarium fujikuroi species complex 1.

*Fusarioid community variation*

Variation among Fusarioid community structure among geographic regions was best described along two principal axes which explained 44.6% and 9.8% of the variation, respectively (Figure 2.5A). Variation among Fusarioid community structure among host genera was also best described along two principal axes which explained 41.7% and 14.4% of the variation, respectively (Figure 2.5B). The PERMANOVA highlighted differences within the Fusarioid community that were associated with geographic region (R2 = 0.2197, F = 3.378, P = 0.003), but no distinct communities were found to be associated with the conifer host genus (R2

= 0.1853, F = 1.0236, P = 0.385). The Fusarioid communities identified in the Southeast and Great Plains regions were most similar (ordinated closer to one another) followed by the Southeast and Pacific Northwest (Figure 2.5A). The Southwest region was the most dissimilar (ordinated furthest away from other regions) region (Figure 2.5A). Fusarioid communities associated with *Pseudotsuga* and *Picea* were more similar to each other, while those associated with *Pinus* were the most distinct but still similar to the other host genera (Figure 2.5B).

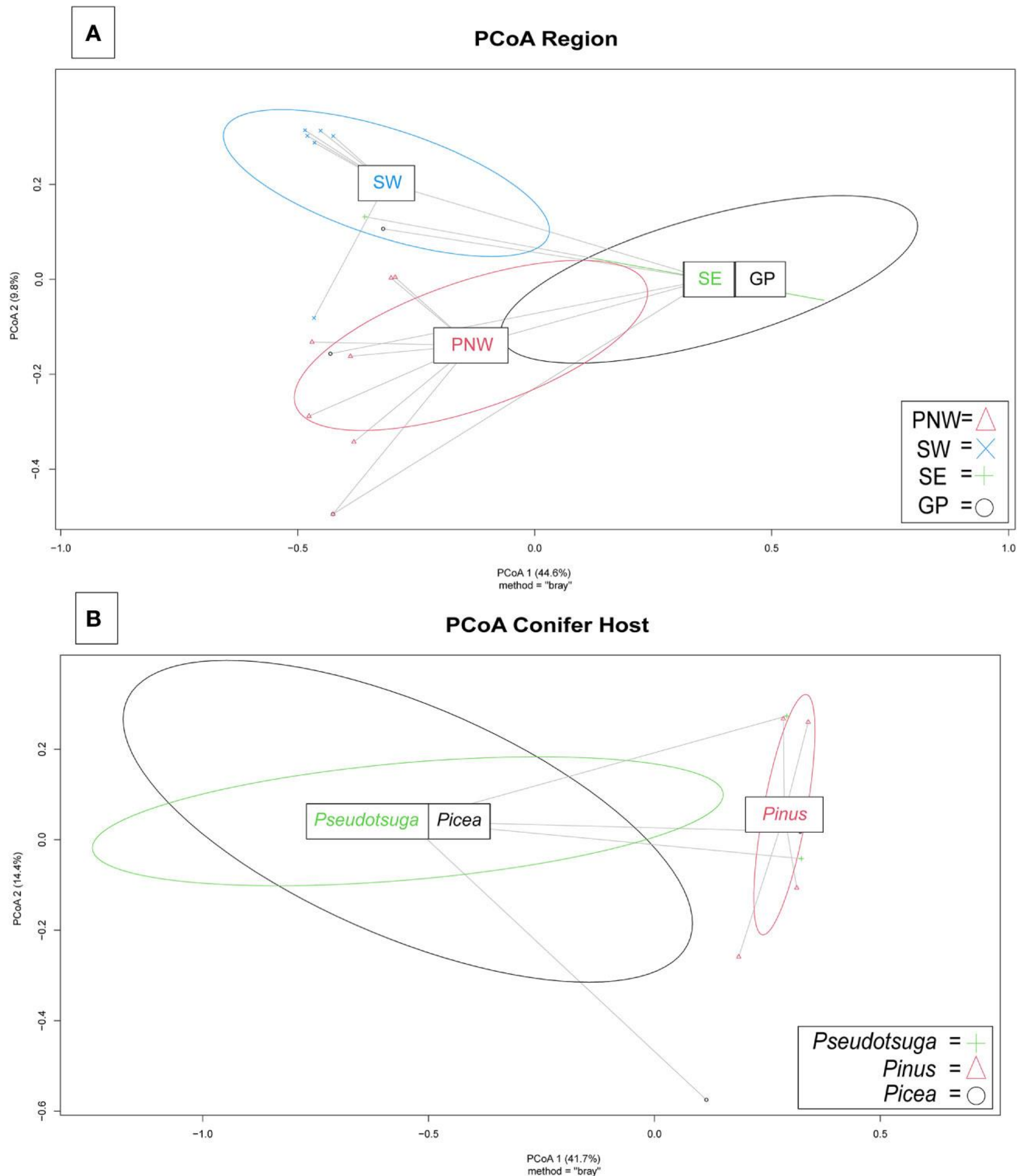



Figure 2.5 – Principal coordinates analysis (PcoA) plots of Fusarioid fungi analyzed by the four regions (A) [Pacific Northwest (PNW), Southwest (SW), Southeast (SE), and Great Plains (GP)] of the USA, where isolates were collected and the three most common host genera (B).

## Discussion

In this study, we surveyed the diversity of Fusarioid fungi and strains in conifer-producing forest nurseries in relation to geographic range and host species, which provides a baseline toward understanding disease threats and invasive potential associated with these Fusarioid fungi and genetic strains. We identified 26 *Fusarium* spp., 14 potential *Fusarium* spp., one potential *Neocosmospora* sp., and four *Neocosmospora* spp. associated with conifers across the USA. Of these, several species (e.g., *F. annulatum*, *F. clavus*, *F. cugenangense*, *F. diversisporum*, *F. elaeagni*, *F. elaideidis*, *F. flocciferum*, *F. fredkrugeri*, *F. grosmichelii*, *F. inflexum*, *F. ipomoeae*, *F. lactis*, *F. languescens*, *F. odoratissimum*, *F. queenslandicum*, *F. torulosum*, *F. triseptatum*, *F. vanleeuwenii*, *N. falciforme*, *N. metavorans*, and *N. pisi*) have not previously been identified in association with conifer seedlings in North America. Further, since Fusarioid fungi been the subject of considerable taxonomic revision in recent years, not much is currently known about several of the species that were identified in our study. Our study highlights the need for further characterization and understanding of the role of these species as pathogens in conifer nurseries.

The importance of accurate phylogenetic analysis and maintaining genomic databases was highlighted in this study. as many Fusarioid fungi have been reclassified (e.g., *Neocosmospora solani* syn. *Fusarium solani*) (Šišić et al., 2018; Lombard et al., 2019; Xia et al., 2019; Crous et al., 2021; Yilmaz et al., 2021). Curated databases are also necessary for accurate species identification, such as Fusarium-ID (http://isolate.fusariumdb.org/blast.php) or Fusarioid-ID (Crous et al., 2021). Because these databases sometimes resulted in multiple species identifications for our isolates, we relied on phylogenetic analyses with type specimens included to help accurately identify our isolates at the species level (Crous et al., 2021). However, we

found that molecular analyses reduce, but do not eliminate, the need for time-consuming morphological identification. Based on the two loci, *tef1a* and *rpb2*, we were not able to resolve all isolates at the species level. The use of diagnostic morphological features, although not always produced for all species (Stewart et al., 2006), may have further resolved some isolates to species.

Determining Fusarioid fungi composition and genetic variation of strains within species is an essential first step toward understanding the disease severity caused by Fusarioid fungi on conifer seedlings. Many Fusarioid fungi are morphologically similar and require molecular techniques to differentiate these cryptic species (Stewart et al., 2012). Variation in pathogenicity and virulence observed among cryptic species and other strains has management implications because some endophytic and saprophytic strains serve beneficial roles (e.g., Dumroese et al., 2012) that require no management, whereas others may be highly virulent pathogens to the plant of interest (Constantin et al., 2021). Furthermore, the characterization of Fusarioid fungi and genetically distinct strains is requisite for identifying potential movement of pathogenic strains between nurseries, states, and/or regions to prevent/limit further spread and reduce the impact of these strains on seedling production (Liebhold et al., 2012; Bate et al., 2016).

In this study, geographic region had a more significant influence on Fusarioid community compositions than the host genus. Even though seedling movement between nurseries was not tracked in this study, we did observe divergent community compositions of Fusarioid fungi that were differentiated by geographic and regional locations, suggesting that the movement of Fusarioid fungi and/or genotypes on seedlings and seed across regions may influence or shift Fusarioid fungi communities. In addition, the distinct Fusarioid fungi composition between the Pacific Northwest and Southwest regions, where we conducted site visits, could have been

attributed to differences in site conditions and cultural practices that we observed. Management practices likely alter population densities of Fusarioid fungi and the frequency and severity of the diseases they cause. Members of the FOSC were found most prominently in this study, representing 42% (137/325) of the total isolates. *Fusarium oxysporum* has been recently divided and reclassified into several different species based on the genetic distinctiveness of isolates that belonged to many formae speciales (Lombard et al., 2019). *Fusarium oxysporum* has historically been noted as a pathogen on conifers (James et al., 1989; James, 2004; Stewart et al., 2012). However, pathogenic species on conifers within the newly described FOSC species have not yet been characterized, and this deserves further investigation as we found several new species associated with conifer in our study. We did not, however, conduct virulence assays in this study, which would be necessary to delineate pathogenic species of conifers within this species complex. We also identified isolates that belonged to several species complexes outside of the FOSC, including *F. fujikuroi* (27%; 89/325), *F. nisikadoi* (that contains *F. commune*) (13%; 41/325), *Neocosmospora* (8%; 26/325), *F. tricinctum* (6%; 20/ 325), and *F. redolens* (2%; 6/325). Members within these species complexes (e.g., *F. proliferatum*, *F. commune*, *Neocosmospora solani*, and *F. acuminatum*) have been found to be pathogenic to conifer species (James, 2004; Stewart et al., 2012; Stewart et al., 2016), though pathogenicity assays on conifers should be completed on newly named species for confirmation.

Several of the species identified in our study have been characterized as pathogens that cause root rot diseases on non-coniferous hosts. These pathogens may pose a potential hazard to landscape plantings as they may be adapted to native, non-conifer hosts surrounding planting sites. For examples, *F. flocciferum* causes root rot of pea (*Pisum sativum*) and faba bean (*Vicia faba*) (Šišić et al., 2020), *F. lactis* causes internal fruit rot of sweet pepper (*Capsicum anuum*)

(Sekiguchi et al., 2021), and *F. torulosum* causes crown rot of wheat (*Triticum* sp.) (Karlsson et al., 2021). Previously, *F. inflexum* and *F. ipomoeae* were found as pathogens on non-coniferous hosts, causing wilt disease of beans (*Vicia faba*) (Schneider & Dalchow, 1975) and leaf spot disease of peanuts (*Arachis hypogaea*) (Xu et al., 2021), respectively. *Neocosmospora falciforme* was previously found in association with two *Pinus* spp., but it was not tested for pathogenicity (Herron et al., 2015). In addition, *F. fredkrugeri* is a newly described species that was isolated from rhizosphere soils of non-coniferous hosts, but this species has not been identified as a pathogen (Sandoval-Denis et al., 2018). Several Fusarioid fungi, *F. acuminatum*, *F. commune*, *F. oxysporum*, *F. proliferatum*, and *Neocosmospora solani* (syn. *F. solani*), are known to cause similar symptoms on commonly grown conifer species, including Douglas-fir (*Pseudotsuga menziesii*) and ponderosa pine (*Pinus ponderosa*) (Stewart et al., 2006; James & Dumroese, 2007). The shared evolutionary history of these damping-off and root rot pathogens has not been well studied and deserves further investigation. Although these Fusarioid fungi may work independently as pathogens, they may also function in concert with other Fusarioid fungi. Multiple weak pathogens may work in concert to overwhelm host defenses to cause the development of disease and associated symptoms (Abdullah et al., 2017). Microbial species compositions can influence the plant host's ability to defend against pathogens (Vujanovic et al., 2007). The influence of Fusarioid fungi composition on host range and disease severity is, however, not well studied, and remains unknown for several newly characterized species, including *F. annulatum*, which was recently separated from *F. proliferatum*. It remains undetermined how Fusarioid fungi that are identified as pathogens on non-coniferous hosts may influence, either directly or indirectly, disease severity on coniferous seedlings and vice versa.

Further investigations are needed to determine if shared virulence genes among these Fusarioid fungi contribute to pathogenicity on the same hosts.

Fusarioid fungi, such as *F. oxysporum*, and *Neocosmospora solani* (syn. *F. solani*), can transfer conditionally dispensable, lineage-specific chromosomes that may increase the competitiveness of receiving strains to cause disease on plant hosts (Dobbs et al., 2020; Ayukawa et al., 2021). Some *Fusarium* spp., such as *F. oxysporum* f. sp. *koae*, can cause death of older host trees if conditions become favorable for the pathogen and the host trees become stressed (Dobbs et al., 2020). With the increasing effects of climate change, higher temperatures may exacerbate tree stress and increase their susceptibility to opportunistic pathogens (Dumroese & James, 2005). The virulence of some weaker *Fusarium* spp. has been found to increase by raising temperatures from 20°C to 30°C (Huang & Kuhlman, 1991). The ever-increasing threat of more diverse pathogens on conifer seedlings amplifies the need for rapid identification of pathogenic *Fusarium* strains.

To accurately identify pathogenic Fusarioid fungi and strains, a better understanding of what determines and differentiates virulence among pathogenic species and strains within species is needed. Functional genomics analysis can provide a comprehensive understanding of damping-off/root rot pathogenesis of Fusaroid fungi. This genomics information can be further used to develop pathogen-specific primers for identification, detection, and monitoring. The haplotypes and novel species identified on conifers should be considered for further analysis to determine pathogen spread as it relates to management practices. The findings of this study emphasize the importance of mitigating spread of Fusarioid pathogens across regions and preventing introductions of genetically distinct pathogens into new landscapes.

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# CHAPTER 3: COMPARATIVE GENOMICS OF PINACEAE-ASSOCIATED *FUSARIUM* SPP.

**Summary**

Recent studies have shown numerous *Fusarium* spp. are associated with symptomatic conifer seedlings in both bareroot and container nursery systems. Some of these species have been found pathogenic to conifer seedlings (e.g., *F. avenaceum*, *F. commune*, *F. oxysporum*, *F. solani*, and *F. verticillioides*), but the mechanisms and shared evolutionary history of these conifer pathogenic species have not been well studied in these pathosystems. We compared whole genomes of 17 *Fusarium* spp. associated with conifer (specifically *Pinaceae*) seedlings to elucidate putative shared pathogenicity/virulence gene profiles presumably expressed for roles in causing damping-off and/or root disease on conifer seedlings. In addition, this work provides draft genomes of *Pinaceae*-associated *Fusarium* spp. and genomes not previously referenced in public databases (e.g., *F. lactis*, *F. fredkrugeri*, *F. ipomoeae*, and *F. flocciferum*). We identified pathogenicity/virulence genes associated with *Fusarium* spp. pathogens of conifers including effectors, the secreted in xylem (SIX) genes 2, 4, 9, & 14 and secondary metabolites, and the mycotoxins fumonisin and deoxynivalenol. We conclude that gene profiles are shared within *Fusarium* species complexes and among closely related *Fusarium* species complexes; however, these shared profiles are widely distributed across all *Fusarium* pathogens. These findings highlight potential targets for detecting and/or identifying *Fusarium* pathogens of conifers, but multiple methods and/are targets will be required depending on the species complexes and clades. More research is needed to determine the roles of expressed pathogenicity/virulence genes and the downstream metabolic products that result in pathogenesis to conifers.

## Introduction

*Fusarium* comprises cosmopolitan fungi that infect many agriculturally important plant species. Due to the ubiquitous nature of many *Fusarium* spp., global interest has arisen to better understand diverse species and strains of pathogenic *Fusarium*. Currently, the host ranges of many *Fusarium* species are not well documented, and it is unknown whether certain *Fusarium* species are limited to pathogenic, saprophytic, and/or endophytic lifestyles. As climate change and extreme weather events (e.g., drought) results in stressed plant hosts and conditions favoring plant disease, native and seemingly innocuous saprophytic fungi can emerge as "new" plant pathogens, which elevates the need to better understand the pathogenic potential of host-associated fungal species and strains (Herron et al. 2015). Of particular note, some *Fusarium* spp. were previously thought to only cause localized disease due to their soil-borne habits; however, recently these species have been reported as causing atypical disease on aboveground plant parts (Li et al. 2023). For example, one apparent shift in pathogenic behavior from soil to foliage environments is perhaps attributable to changes in climatic conditions that increase plant susceptibility to the pathogen or favor pathogen infection of foliage (e.g., high humidity facilitating infection via airborne conidia) (Rekah et al. 2007). Currently, the molecular mechanisms that drive such lifestyle changes are not well understood.

Conifer nurseries are vital for forest restoration, reforestation, and timber production. However, nursery production can be impeded by conifer seedling-infecting *Fusarium* pathogens that cause pre- and post-emergent damping-off and root diseases (Dobbs et al. 2023), a major problem for nursery managers. In addition, as changing climatic conditions occur, diseases caused by *Fusarium* pathogens may increase because of lifestyles shifting from saprophytic or opportunistic/low-virulence pathogens to high-virulence pathogens under the new climatic

conditions (Quesada et al. 2019). Reforestation of areas impacted by fire is an ever-increasing need that translates to a growing requirement for nursery-grown seedlings. Heightened nursery production can contribute to inadvertent introductions of new pathogens to a broader suite of host species that can lead to the development of pathogens with broader host ranges (Fargione et al. 2021). Since the geographic region of *Fusarium* spp. can significantly affect its community structure, the spread of these pathogens across regions into nurseries or field sites could result in devastating seedling losses (Dobbs et al. 2023). Furthermore, multiple *Fusarium* spp. have been documented to cause damping-off and root disease on the same host (Gordon et al. 2015). If multiple conifer-pathogenic *Fusarium* species can co-infect a susceptible host utilizing multiple virulence metabolic pathways to overcome host defenses, the likely result is devastating disease (Abdullah et al. 2017). Multiple *Fusarium* pathogens can each utilize unique virulence mechanisms to overwhelm their hosts' defenses. It has been posited that multiple pathogens, even weakly virulent pathogens, can work in concert to cause disease on a given host, and repeated introductions of a pathogen on a host can facilitate the development of host-specific, virulence mechanisms in the pathogen (Abdullah et al. 2017; Newman et al. 2020).

The host-range of many *Fusarium* spp. has not been well documented, especially in recently described or understudied species, such as *F. commune* and *F. annulatum* (Skovgaard et al. 2003; Yilmaz et al. 2021). For example, *F. commune* has been reported as a pathogen of both conifer and non-conifer hosts; however, it has not been characterized as a generalist pathogen and it is unknown if pathogenic strains display host specificity (Stewart et al. 2012; Hamini-Kadar et al. 2010; Kim et al. 2012). Species like *F. oxysporum* have been separated into strains by *formae speciales* designations that reflect host specificity (Czislowski et al. 2021). Generalist and host-specific pathogens have different management implications. As examples, treatments

are typically required once a generalist pathogen is identified in a nursery, whereas further investigation may be required before a treatment is deemed necessary for host-specific pathogens.

The current reclassification of *Fusarium* spp. coupled with the potential of multiple species causing a threat to conifer seedling production have generated considerable uncertainty about the appropriate disease management practices for nurseries and other agricultural systems. DNA sequence-based techniques, such as Sanger or whole-genome sequencing, are integral to pathogen identification, but these methods still rely on confirmation of species identification through databases (Torrez-Cruz et al. 2022), and the main databases for *Fusarium* species identification do not always agree (Geiser et al. 2021; Crous et al. 2021). For this reason, an alternative method for identifying *Fusarium* pathogens is needed that does not rely on the conventional conserved-locus BLAST search that is currently used by the main databases. For example, one alternative method could utilize genetic markers that target pathogenicity genes expressed by the pathogens during disease development. Such pathogenicity-based identification methods would be more useful for managers in determining the presence of pathogens and informing when pathogen management is necessary.

*Fusarium* contains species complexes that comprise members that share hosts and virulence mechanisms – features that would be useful for rapid identification (Ma et al. 2013). Furthermore, such similarities among species complexes may provide insights for developing robust disease-management methods. However, the evolutionary history and genetic similarities have not been characterized for multiple *Fusarium* spp. that cause root rot and damping-off diseases on conifer seedlings. Identification of shared genes among diverse *Fusarium* pathogens of conifers will facilitate the development of affordable and robust management strategies.

Pathogenomics is the first step in developing genomic and virulence-associated gene profiles that can be used for developing these management strategies (Rampersad 2020).

It has been hypothesized that *Fusarium* pathogens of conifers may share similarities in virulence factors, possibly due to horizontal gene or chromosome transfer (Stewart et al. 2012). To further investigate this hypothesis, our goal was to determine if predicted virulence genes are shared among *Pinaceae*-pathogenic *Fusarium* strains. Toward this goal, our research was directed toward the following objectives: 1) investigate genomic similarities among *Fusarium* species collected from *Pinaceae* seedlings; 2) identify isolates collected from conifers for further analyses, based on their genomic similarities among their respective *Fusarium* species complexes; and 3) identify putative conifer-pathogenicity/virulence genes used by *Fusarium* pathogens of conifer.

**Materials and methods**

Fusarium *spp. isolates*

DNA extraction was conducted on 21 *Fusarium* spp. isolates, 19 collected from symptomatic conifer (*Pinaceae*) seedlings from the southwestern, Pacific northwestern, and southeastern USA, which were previously described in Dobbs et al. (2023), and two from non-conifer hosts (Table 3.1). In preparation for DNA extraction, the isolates were grown on ¼-strength potato dextrose agar (PDA) at 25°C for 3 days. These *Fusarium* isolates represent 17 *Fusarium* spp.[1–17] that are contained within six *Fusarium* species complexes [*F. fujikuroi* species complex (FFSC) including *F. annulatum*[1], *F. fredkrugeri*[2], *F. fujikuroi*[3], *F. lactis*[4], and *F. verticillioides*[5]; *F. nisikadoi* species complex (FNSC) including *F. commune*[6]; *F. oxysporum* species complex (FOSC) including *F. inflexum*[7] and *F. oxysporum*[8]; *F. incarnatum-equiseti* species complex (FIESC) including *F. clavum*[9], *F. ipomoeae*[10], and *F. luffae*[11]; *F. tricinctum*

species complex (FTSC) including *F. acuminatum*[12], *F. avenaceum*[13], *F. flocciferum*[14], and *F. torulosum*[15]; and *F. solani* species complex (FSSC) including *F. falciforme*[16] and *F. solani*[17]] (Table 3.1).

Table 3.1 - List of reference genomes available in GenBank and the isolates used in this study, including those without an available reference genome.

| ***Fusarium* species** | **Isolate** | **Accession #** | **Species complex*** | **Host: Common name** | **Host: Scientific name** | **Reference** |
|---|---|---|---|---|---|---|
| *F. acuminatum* | CHS3 | JAPTGE000000000 | FTSC | | *Bupleurum scorzonerifolium* | |
| *F. acuminatum* | MES5 | JAVTNN000000000 | FTSC | Ponderosa pine | *Pinus ponderosa* | This study |
| *F. ambrosium* | NRRL62606 | NKCL00000000 | FSSC | Beetles | | Gesier et al. 2021 |
| *F. annulatum* | F8_4S_2B | JAHAPR000000000 | FFSC | ISS environmental surface | | |
| *F. annulatum* | NASWWP3 | JAVTNU000000000 | FFSC | Southwest white pine | *Pinus strobiformis* | This study |
| *F. avenaceum* | NRRL54939 | JPYM00000000 | FTSC | Barley | *Hordeum vulgare* | Gesier et al. 2021 |
| *F. avenaceum* | NC74 | JAVTNV000000000 | FTSC | Loblolly pine | *Pinus taeda* | This study |
| *F. brachygibbosum* | Fb-HN-1 | JAEKIX000000000 | *sambucinum* | Maize | *Zea mays* | |
| *F. circinatum* | NRRL25331 | JAAQPE000000000 | FFSC | Monterey pine | *Pinus radiata* | Gesier et al. 2021 |
| *F. clavum* | NRRL66337 | QGEC00000000 | FIESC | Oats | *Avena sativa* | |
| *F. clavum* | NAGrPIST2 | JAVTNS000000000 | FIESC | Southwest white pine | *Pinus strobiformis* | This study |
| *F. commune* | NRRL28387 | JABFES000000000 | FNSC | Tomato | *Solanum lycopersicum* | Gesier et al. 2021 |
| *F. commune* | B62 | JAVTNG000000000 | FNSC | Rice | *Oryza sativa* | This study |
| *F. commune* | F10.2.2 | JAVTNJ000000000 | FNSC | Douglas-fir | *Pseudotsuga menziesii* | This study |
| *F. commune* | F13.4.1 | JAVTNK000000000 | FNSC | Douglas-fir | *Pseudotsuga menziesii* | This study |
| *F. commune* | JCM11502 | BCHB00000000 | FNSC | Soil | | This study |
| *F. commune* | JICPIPO1 | JAVTNL000000000 | FNSC | Ponderosa pine | *Pinus ponderosa* | This study |
| *F. commune* | MiAE120 | JAVTNQ000000000 | FNSC | Tomato | *Solanum lycopersicum* | This study |
| *F. equiseti* | NRRL66338 | QGEB00000000 | FIESC | Oats | *Avena sativa* | Gesier et al. 2021 |
| *F. falciforme* | NRRL43529 | JABEEK000000000 | FSSC | Human | *Homo sapiens* | Gesier et al. 2021 |
| *F. falciforme* | SC35 | JAVTNX000000000 | FSSC | Loblolly pine | *Pinus taeda* | This study |
| *F. flocciferum* | 31MAPSF17A | JAVTNF000000000 | FTSC | Ponderosa pine | *Pinus ponderosa* | This study |
| *F. foetens* | NRRL38302 | JABFMM000000000 | FOSC | Monterey pine | *Pinus radiata* | Gesier et al. 2021 |
| *F. fredkrugeri* | SC7 | JAVTNZ000000000 | FFSC | Loblolly pine | *Pinus taeda* | This study |
| *F. fujikuroi* | NRRL5538 | GCF_900079805.1 | FFSC | Rice | *Oryza sativa* | Gesier et al. 2021 |
| *F. fujikuroi* | SC5 | JAVTNY000000000 | FFSC | Loblolly pine | *Pinus taeda* | This study |

| *F. gaditjirrii* | NRRL45417 | JABFAI000000000 | FNSC | | | Gesier et al. 2021 |
|---|---|---|---|---|---|---|
| *F. guttiforme* | NRRL22945 | JAAQRL000000000 | FFSC | Pineapple | *Ananas comosus* | Gesier et al. 2021 |
| *F. hainanense* | NRRL66475 | JABFEW000000000 | FIESC | | | Gesier et al. 2021 |
| *F. hostae* | NRRL29888 | JABCJX000000000 | *redolens* | Hostas | *Hosta* sp. | Gesier et al. 2021 |
| *F. illudens* | NRRL22090 | JABFEX000000000 | FSSC | Tawa | *Beilschmiedia tawa* | Gesier et al. 2021 |
| *F. incarnatum* | NRRL66325 | GCA_004367075.1 | FIESC | Pointed gourd | *Trichosanthe dioica* | |
| *F. inflexum* | JTHABCO3.1 | JAVTNM000000000 | FOSC | White fir | *Abies concolor* | This study |
| *F. ipomoeae* | NC8 | JAVTNW000000000 | FIESC | Loblolly pine | *Pinus taeda* | This study |
| *F. irregulare* | NRRL31160 | QGEA00000000 | FIESC | Human | *Homo sapiens* | Gesier et al. 2021 |
| *F. lactis* | MES8 | JAVTNP000000000 | FFSC | Ponderosa pine | *Pinus ponderosa* | This study |
| *F. luffae* | NRRL66473 | GCA_013184325.1 | FIESC | | | |
| *F. luffae* | CC | JAVTNH000000000 | FIESC | Douglas-fir | *Pseudotsuga menziesii* | This study |
| *F. lyarnte* | NRRL54252 | JAAVUB000000000 | FNSC | | | Gesier et al. 2021 |
| *F. mangiferae* | NRRL25226 | FCQH00000000 | FFSC | Mango | *Mangifera indica* | Gesier et al. 2021 |
| *F. metavorans* | FSSC_6 | LWBZ00000000 | FSSC | Asian longhorned beetle | *Anoplophora glabripennis* | |
| *F. miscanthi* | NRRL26231 | JAAVUA000000000 | FNSC | Silver grass | *Miscanthus* sp. | Gesier et al. 2021 |
| *F. neocosporiellum* | NRRL22166 | SSHR00000000 | FSSC | | *Heterodera glycines* | Gesier et al. 2021 |
| *F. nisikadai* | NRRL25179 | JABFFB000000000 | FNSC | | *Phyllostachys nigra* var. *henonis* | Gesier et al. 2021 |
| *F. oxysporum* | NRRL34936 | AAXH00000000 | FOSC | Tomato | *Solanum lycopersicum* | Gesier et al. 2021 |
| *F. oxysporum* | NRRL32931 | AFML00000000 | FOSC | | *Homo sapiens* | Gesier et al. 2021 |
| *F. oxysporum* | MES6 | JAVTNO000000000 | FOSC | Ponderosa pine | *Pinus ponderosa* | This study |
| *F. proliferatum* | ET1 | FJOF00000000 | FFSC | Orchid | | |
| *F. redolens* | NRRL22901 | JAAVUJ000000000 | *redolens* | Douglas-fir | *Pseudotsuga menziesii* | Gesier et al. 2021 |
| *F. sacchari* | NRRL66326 | JABSTH000000000 | FFSC | | | Gesier et al. 2021 |
| *F. scirpi* | NRRL66328 | QHHJ00000000 | FIESC | Debris from soil | | Gesier et al. 2021 |
| *F. solani* | NAPSME6.1 | JAVTNT000000000 | FSSC | Douglas-fir | *Pseudotsuga menziesii* | This study |
| *F. subglutinans* | NRRL66333 | JAAOAV000000000 | FFSC | Acoma blue maize | *Zea mays* | Gesier et al. 2021 |
| *F. thapsinum* | NRRL22049 | JAAOAX000000000 | FFSC | Sorghum | *Sorghum bicolor* | Gesier et al. 2021 |

| | | | | | | |
|---|---|---|---|---|---|---|
| *F. torulosum* | NRRL22747 | JABFMN000000000 | FTSC | | | Gesier et al. 2021 |
| *F. torulosum* | DCN062.2H | JAVTNI000000000 | FTSC | Douglas-fir | *Pseudotsuga menziesii* | This study |
| *F. tricinctum* | NRRL25481 | JAALXJ000000000 | FTSC | Winter wheat | *Triticum aestivum* | Gesier et al. 2021 |
| *F. vanettenii* | NRRL45880 | ACJF00000000 | FSSC | | | Gesier et al. 2021 |
| *F. verticillioides* | NRRL20956 | AAIM00000000 | FFSC | Maize | *Zea mays* | Gesier et al. 2021 |
| *F. verticillioides* | NAGrPIPO5 | JAVTNR000000000 | FFSC | Ponderosa pine | *Pinus ponderosa* | This study |
| *F. virguliforme* | NRRL31041 | JABEEP000000000 | FSSC | Soybean | *Glycine max* | Gesier et al. 2021 |
| *F. xylarioides* | NRRL25486 | JABFFK000000000 | FFSC | Coffee | *Coffea* sp. | Gesier et al. 2021 |

**F. fujikuroi* (FFSC), *F. nisikadoi* (FNSC), *F. oxysporum* (FOSC), *F. incarnatum-equiseti* (FIESC), *F. tricinctum* (FTSC), and *F. solani* (FSSC) species complexes

*Whole genome sequencing and assembly*

To examine host-specificity in an understudied species reported as pathogenic to both conifer and non-conifer hosts, DNA was also extracted from five *F. commune* isolates [Representative isolates were collected from symptomatic hosts including rice (*Oryza sativa*), tomato ‘Montfavet’ (*Solanum lycopersicum*), ponderosa pine (*Pinus ponderosa*), and Douglas-fir (*Pseudotsuga menziesii*)] (Table 3.1). Hyphal tips were transferred to 200 ml of potato dextrose broth and shaken at 90 rpm for 7 days at room temperature. Mycelium was collected using a Buchner funnel and filtered through a Whatman® No. 1 filter paper under vacuum. Mycelium was placed in 2-ml cryotubes and frozen at -75°C for 24 hours prior to extraction. Total genomic DNA was extracted using a cetyl trimethyl ammonium bromide (CTAB) extraction method similar to Dobbs et al. (2020). Tissue preparation was modified in that frozen samples were ground to a fine powder using liquid $N_2$ in a flame-sterilized mortar and pestle. Tissue was homogenized after adding 750 µl of CTAB extraction buffer (1% w/v CTAB; 1 M NaC1; 100 mM Tris; 20 mM EDTA; 1% w/v polyvinylpolypyrrolidone) to each sample using a FastPrep-24 (M.P. Biomedicals LLC, Santa Ana, CA, USA) at 5.5x speed for two 60-second runs. Samples were incubated for 30 min at 70°C in a heat block. One volume of chloroform:isoamyl alcohol (24:1) was added to each sample and mixed on a shaker for 20 min then centrifuged at 10,000 x g for 5 min. The upper, aqueous phase (600 µl) was transferred to a new 2-ml tube. Two volumes (1200 µl) of precipitation buffer were added to each sample, mixed in a shaker for 5 min, and then centrifuged at 13,000 x g for 15 min. The supernatant was removed and re-suspended in 350 µl 1.5 M NaCl. To each sample, 20 µl of 10 mg/ml RNase was added, followed by incubation at 37°C for 30 min. One volume of chloroform:isoamyl alcohol (24:1) was added to each sample and mixed on a shaker for 20 min, then centrifuged at 10,000 x g for 5 min. The upper phase was

transferred to a new 1.5-ml tube and 0.6 volume of ice-cold isopropanol was added to each sample. Samples were mixed by inversion and incubated at 20°C for 1 hour. After incubation, samples were centrifuged at 14,000 x g for 5 min at room temperature. The supernatant was discarded and 1 ml 70% ethanol was added to each sample. After 1-min incubation at room temperature, samples were centrifuged at 14,000 x g for 3 min at room temperature. Each pellet was dried and resuspended in 80 µl TE buffer (10 mM TrisHCl, 1 mM EDTA). Extracted DNA was accessed for quality using a Nanodrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA) and gel electrophoresed in a 0.8% agarose gel. DNA was quantified using a Qubit™ fluorometer (Invitrogen, Carlsbad, CA, USA). Extracted DNA was sent to Novogene (U.C. Davis DNA Sequencing Center, Davis, CA, USA) for TruSeq Illumina shotgun sequencing with paired-end reads of 2 x 151 bp. Paired-end reads were trimmed using BBDuk (Sourceforge.net/projects/bbmap/) in Geneious Prime v2022.0.1 (https://www.geneious.com/).

*Whole genome assembly*

The trimmed reads were used in *de novo* genome assemblies that were constructed using SPAdes v3.15.4 (Bankevich et al. 2012). The SPAdes genome assemblies were assessed for quality using QUAST (Gurevich et al. 2013). Contigs were checked for similarity with other *Fusarium* spp. sequences using a local BLAST of the National Center for Biotechnology Information (NCBI) nucleotide (nt) database displaying the top five results per hit (query sequence with similarity to the nucleotide database). Contigs were screened for presence of bacterial DNA, and sequences that had three or more results containing similarity to bacterial DNA were removed.

*Comparative genome analysis*

Reference *Fusarium* spp. genomes and the genome of a closely related sister species (*Neonectria ditissima*), for use as an outgroup, were retrieved from GenBank (Table 3.1). Genomes of conifer-associated *Fusarium* spp. isolates were compared for size and quality assessment using QUAST (Gurevich et al. 2013) and for quantitative assessment of genome completeness using BUSCO v5.4.4 against the ascomycota_od10 database (Manni et al. 2021). A maximum likelihood (ML) phylogeny was inferred from 16 concatenated loci (*acl1, cal1, dpa1, dpe1, fas1, fas2, lcb2, mcm7, pgk1, rpb1, rpb2, tef1, top1, tsr1, tub1*, and *tub2*) previously used to speciate *Fusaria* (Geiser et al. 2021) with 1000 pseudoreplicates using IQtree2 and Modelfinder to determine substitution model for the phylogeny (Minh et al. 2020). Abbreviations for loci names are described in Table 3.2. Loci substitution models were determined independently from one another through partitioning of each locus. Beauti2 was used to format the aligned sequences for subsequent use in BEAST2 to generate the Bayesian phylogeny (Bouckaert et al. 2019). Bayesian posterior probability branch support and the maximum likelihood bootstrap values were added to the ML phylogeny. DNA sequences were aligned with MUSCLE sequence aligner using default settings (Edgar 2004).

Table 3.2 - Phylogenetic data summary of 16 genes analyzed in this study.

| **Locus*** | **Identity** | **Model** |
|---|---|---|
| *acl1* | ATP citrate lyase large subunit | TN+F+I+G4 |
| *cal1* | Calmodulin | TIM2+F+I+G4 |
| *dpa1* | DNA polymerase alpha subunit | TNe+I+G4 |
| *dpe1* | DNA polymerase epsilon subunit | TN+F+I+G4 |
| *fas1* | Fatty acid synthase alpha subunit | TIM2+F+I+G4 |
| *fas2* | Fatty acid synthase beta subunit | TN+F+I+G4 |
| *lcb2* | Sphinganine palmitoyl transferase subunit | TIM+F+I+G4 |
| *mcm7* | DNA replication licensing factor | TIM2+F+I+G4 |
| *pgk1* | Phosphoglycerate kinase | TN+F+I+G4 |
| *rpb1* | RNA polymerase largest subunit | TN+F+I+G4 |
| *rpb2* | RNA polymerase second largest subunit | TIM2+F+I+G4 |

| | | |
|---|---|---|
| *tef1* | Translation elongation factor 1-alpha | GTR+F+I+G4 |
| *top1* | Topoisomerase | TIM2+F+I+G4 |
| *tsr1* | Ribosomal biogenesis protein | TN+F+I+G4 |
| *tub1* | Tubulin alpha subunit | TIM2e+I+G4 |
| *tub2* | Tubulin beta subunit | SYM+I+G4 |
| *Selected based on Geiser et al. 2021 | | |

*Genome annotation*

Genomes were annotated in a manner similar that of Dobbs et al. (2020). The *de novo* assembled genomes were annotated using MAKER v.3.01.03 annotation pipeline (Cantarel et al. 2007) with RepeatMasker v.4.1.2 (Smit et al. 2013-2015). A genome-specific repeat library was constructed using RepeatModeler v.2.0.3 (Smit et al. 2015) to mask interspersed repeats and low complexity DNA sequences. Three gene predictors, GeneMark-ES (Ter-Hovhannisyan et al. 2008), SNAP (Zaharia et al. 2011), and AUGUSTUS (Keller et al. 2011), were used in the pipeline, with *F. graminearum* serving as the species model for AUGUSTUS. To identify tRNA genes, tRNAscan-SE v2.0.9 (Lowe et al. 1997) was used with default settings. Predicted transcripts (≥150 bp) and proteins (≥50 amino acids) were analyzed for putative function. Putative functions of genes (i.e., virulence-associated genes, secreted proteins, effectors, carbohydrate degrading enzymes (CAZymes), secondary metabolites, and orthologous gene clusters) were predicted using the following databases: PHI-base 4.14 (Urban et al. 2019), deeploc2 (Thumuluri et al. 2022), EffectorP 3.0 (Sperschneider & Dodds 2022), dbCAN2 (Zhang et al. 2018), antiSMASH (Blin et al. 2019), and OrthoFinder (Emms & Kelly 2019), respectively. Online servers antiSMASH and dbCAN2 were used to analyze the predicted genes at default settings. Only proteins with dbCAN2 hits in at least two of the three databases (HMMER, DIAMOND, and e-CAMI) were considered. OrthoFinder with multiple sequence alignment, deeploc2, and EffectorP were ran locally with default settings and a local BLAST was

used for the PHIbase database. Selection of putative proteins from BLASTx results was based on criteria of e-value < 1e-5, percent identity ≥ 65%, and a coverage of ≥ 50%. Additionally, a local BLAST of Secreted In Xylem (SIX) genes collected from GenBank was used to predict the presence of SIX genes in the genomes used in this study (Table 3.3). Comparisons of gene contents were analyzed for each database using R studio.

To further investigate the similarity among *Fusarium* taxa and the *F. commune* isolates from conifer and non-conifer hosts, including two reference genomes of *F. commune* [GCA 001599515.1 collected from soil; NRRL28387 collected from tomato (*Lycopersicon esculentum*)], principal coordinates analyses (PCoA) were conducted using Bray-Curtis dissimilarities based on abundance of predicted genes. An UpSet plot and a phylogenetic principal component analysis were generated based on orthologous proteins among *Fusarium* spp. using ComplexUpset (Lex et al. 2014) in R studio. Conifer-unique orthogroups were identified based on species previously identified as pathogens of conifers and analyzed based on putative function including virulence-associated proteins, secreted proteins and effectors, and CAZymes.

Table 3.3 - SIX gene references

| SIX Gene | GenBank Accession# |
|---|---|
| SIX1 | KC296735.1 |
| SIX2 | KX435003.1 |
| SIX3 | MT846906.1 |
| SIX4 | KX435037.1 |
| SIX5 | MN745210.1 |
| SIX6 | KR855770.1 |
| SIX7 | KM893940.1 |
| SIX8 | FJ755837.1 |
| SIX9 | KC701447.1 |
| SIX10 | LC648777.1 |
| SIX11 | KC701449.1 |
| SIX12 | KC701450.1 |
| SIX13 | KP964997.1 |

| SIX14 | LC648782.1 |
|---|---|
| SIX15 | MW160901.1 |

**Results**

*Whole genome assembly and annotation*

Assembled genomes in this study were deposited into NCBI GenBank databank (BioProjectID: PRJNA1020449; Table 3.1). Genomes ranged from ca. 39-56 Mb with *F. ipomoeae* having the smallest genome and *F. falciforme* having the largest, both collected from loblolly pine (*P. taeda*) (Table 3.3). All assembled genomes had a completeness of >97% based on 1,706 genes of the ascomycota_db10 (Table 3.4). GC content averaged 48.38%, which is expected for *Fusarium* spp. (Table 3.4). Genome size was incongruent with the number of predicted genes (Table 3.5). As examples, *F. facliforme* has the largest genome size (56.13 Mb) in this study with a modest number of predicted genes (17,518), whereas *F. lactis* (genome size 54.97 Mb) has the highest (22,049) number of predicted genes (Table 3.5).

Table 3.4 - Comparison of genome size, completeness and GC content of the conifer (*Pinaceae*) collected *Fusarium* spp. genomes used in this study.

| Isolate | *Fusarium* Species | Host Collected From | Genome Size (Mb) | # Contigs | BUSCO completeness (%) | GC% |
|---|---|---|---|---|---|---|
| NASWWP3 | *F. annulatum* | *Pinus strobiformis* | 45.96 | 400 | 98.6 | 48.3 |
| NC74 | *F. avenaceum* | *P. taeda* | 42.5 | 275 | 98.7 | 47.99 |
| NAGrPIST2 | *F. clavum* | *Pinus strobiformis* | 47.15 | 2456 | 98.8 | 47.93 |
| B62 | *F. commune* | *Oryza sativa* | 45.96 | 410 | 98.6 | 47.63 |
| F10.2.2 | *F. commune* | *Pseudotsuga menziesii* | 48.85 | 648 | 98.7 | 47.74 |
| F13.4.1 | *F. commune* | *Pseudotsuga menziesii* | 49.96 | 1441 | 98.7 | 47.68 |
| JCM11502* | *F. commune* | Soil | 46.18 | 19 | 99.1 | 47.62 |
| JICPIPO1 | *F. commune* | *P. ponderosa* | 49.43 | 1555 | 98.4 | 48.62 |
| MiAE120 | *F. commune* | *Solanum lycopersicum* | 45.71 | 640 | 98.9 | 47.8 |
| NRRL28387* | *F. commune* | *Solanum lycopersicum* | 48.32 | 1926 | 98.5 | 48.11 |
| SC35 | *F. falciforme* | *P. taeda* | 56.13 | 1247 | 98.9 | 49.49 |
| 31MAPSF17A | *F. flocciferum* | *P. ponderosa* | 40.77 | 197 | 99 | 47.57 |
| SC7 | *F. fredkrugeri* | *P. taeda* | 46.23 | 334 | 98.6 | 47.24 |
| SC5 | *F. fujikuroi* | *P. taeda* | 43.8 | 171 | 98.4 | 48.23 |
| JTHABCO3.1 | *F. inflexum* | *Abies concolor* | 52.61 | 1637 | 98.8 | 47.74 |
| NC8 | *F. ipomoeae* | *P. teada* | 38.79 | 56 | 98.6 | 48.09 |
| MES8 | *F. lactis* | *P. ponderosa* | 54.97 | 1164 | 98.5 | 50.58 |
| CC | *F. luffae* | *Pseudotsuga menziesii* | 50.75 | 217 | 97.8 | 52.45 |
| MES6 | *F. oxysporum* | *P. ponderosa* | 49.71 | 805 | 98.6 | 47.6 |
| NAPSME6.1 | *F. solani* | *Pseudotsuga menziesii* | 51.79 | 601 | 98.7 | 50.82 |
| DCN062.2H | *F. torulosum* | *Pseudotsuga menziesii* | 43.12 | 499 | 98.9 | 47.55 |
| MES5 | *F. tricinctum* | *P. ponderosa* | 44.45 | 253 | 99 | 47.71 |
| NAGrPIPO5 | *F. verticillioides* | *P. ponderosa* | 42.84 | 467 | 98.4 | 48.25 |

Table 3.5 - Comparison of gene content within the *Fusarium* genomes used in this study.

| Isolate | *Fusarium* Species | Predicted Genes | Secretome | Effectors | PHIbase | CAZymes | Secondary Metabolite Genes | Predicted Metabolites |
|---|---|---|---|---|---|---|---|---|
| NASWWP3 | *F. annulatum* | 15320 | 1680 | 583 | 552 | 675 | 65 | 20 |
| NC74 | *F. avenaceum* | 13485 | 1555 | 563 | 540 | 699 | 63 | 20 |
| NAGrPIST2 | *F. clavum* | 17440 | 2207 | 897 | 545 | 1024 | 45 | 11 |
| B62 | *F. commune* | 14958 | 1682 | 621 | 557 | 697 | 60 | 17 |
| F10.2.2 | *F. commune* | 15624 | 1727 | 614 | 573 | 705 | 58 | 19 |
| F13.4.1 | *F. commune* | 15969 | 1795 | 657 | 598 | 770 | 61 | 18 |
| JCM11502* | *F. commune* | 14567 | 1632 | 589 | 559 | 704 | 59 | 18 |
| JICPIPO1 | *F. commune* | 17105 | 2029 | 742 | 567 | 740 | 60 | 20 |
| MiAE120 | *F. commune* | 14573 | 1522 | 569 | 560 | 629 | 53 | 18 |
| NRRL28387* | *F. commune* | 14960 | 1689 | 600 | 577 | 701 | 60 | 19 |
| SC35 | *F. falciforme* | 17518 | 1921 | 596 | 561 | 826 | 50 | 13 |
| 31MAPSF17A | *F. flocciferum* | 12561 | 1429 | 540 | 532 | 640 | 56 | 16 |
| SC7 | *F. fredkrugeri* | 14850 | 1540 | 541 | 553 | 704 | 58 | 19 |
| SC5 | *F. fujikuroi* | 14692 | 1621 | 566 | 550 | 746 | 60 | 18 |
| JTHABCO3.1 | *F. inflexum* | 16624 | 1894 | 674 | 583 | 853 | 55 | 15 |
| NC8 | *F. ipomoeae* | 12721 | 1431 | 511 | 535 | 636 | 49 | 10 |
| MES8 | *F. lactis* | 22049 | 3136 | 1110 | 547 | 923 | 65 | 20 |
| CC | *F. luffae* | 21632 | 2969 | 1097 | 536 | 803 | 57 | 12 |
| MES6 | *F. oxysporum* | 16150 | 1767 | 618 | 566 | 834 | 56 | 13 |
| NAPSME6.1 | *F. solani* | 16955 | 1817 | 571 | 560 | 776 | 45 | 11 |
| DCN062.2H | *F. torulosum* | 13374 | 1478 | 557 | 541 | 657 | 58 | 21 |
| MES5 | *F. tricinctum* | 14046 | 4623 | 604 | 549 | 706 | 62 | 21 |
| NAGrPIPO5 | *F. verticillioides* | 14078 | 1552 | 522 | 540 | 701 | 57 | 16 |

*Phylogenetics and phylogenomics of Fusarium spp.*

The maximum likelihood phylogeny using 16 loci resolved isolates into six *Fusarium* species complexes [FFSC, FNSC, FOSC, FIESC, FTSC, and FSSC) (Figure 3.1A). Because reference genomes were not available for five *Fusarium* spp. (*F. flocciferum*, *F. fredkrugeri*, *F. inflexum*, *F. ipomoeae*, and *F. lactis*), the identity of these *Fusarium* spp. and phylogenetic relationships of their 16 conserved loci with closely related species was confirmed using the BLAST similarity algorithm to match the translation elongation factor 1-alpha (*tef1*) and the RNA polymerase II second largest subunit (*rpb2*) loci to the GenBank (https://www.ncbi.nlm.nih.gov/) and Fusarioid-ID databases (Crous et al. 2021). These five genomes without associated reference genomes were placed within the expected species complex clades (SC1–SC3, please see details below) based on their respective BLAST sequence similarities (Figure 3.1A). Clades of closely related species complexes were shown to be well supported among the FFSC, FNSC, and FOSC hereafter referred to as species complex clade 1 (SC1); FIESC and FTSC were found to be similar (SC2); and the FSSC was unique (SC3). Species complex clades (S1–SC3) were further confirmed based on the 5,677 orthologous proteins shared among the assembled *Fusarium* spp. isolates identified through the OrthoFinder analysis (Figure 3.1B).

A

F. verticillioides NRRL20956
NAGrPIPO5
F. thapsinum NRRL22049
MES8
F. xylarioides NRRL25486
F. circinatum NRRL25331
F. subglutinans NRRL66333
F. guttiforme NRRL22945
F. annulatum F8.4S.2B
NASWWP3
F. proliferatum ET1
F. fujikuroi NRRL5538
SC5
F. mangiferae NRRL25226
F. sacchari NRRL66326
SC7
B62
JICPIPO1
F. commune JCM11502
F10
F13
F. commune NRRL28387
MiAE120
F. miscanthi NRRL26231
F. nisikadoi NRRL25179
F. gaditjirrii NRRL45417
F. lyrante NRRL54252
F. oxysporum NRRL34936
MES6
F. oxysporum NRRL32931
JTHABCO3.1
F. foetens NRRL38302
F. hostae NRRL29888
F. redolens NRRL22901
F. clavum NRRL66337
NAGrPIST2
NC8
F. scirpi NRRL66328
F. equiseti NRRL66338
MES7
CC
F. luffae NRRL66473
F. irregulare NRRL31160
F. incarnatum NRRL6632
F. hainanense NRRL664
F. acuminatum F829
F. brachygibbosum HN1
F. tricinctum NRRL25481
MES5
F. avenaceum NRRL54939
NC74
DCN
F. torulosum NRRL22747
31MAPSF17A
F. falciforme NRRL43529
SC35
NAPSME6.1
F. solani FSSC5
F. vanettenii NRRL45880
F. metavorans FSSC6
F. ambrosium NRRL62606
F. neocosporiellum NRRL22166
F. virguliforme NRRL31041
Neonectria ditissima NRRL20485
0.05

SC1
SC2
SC3

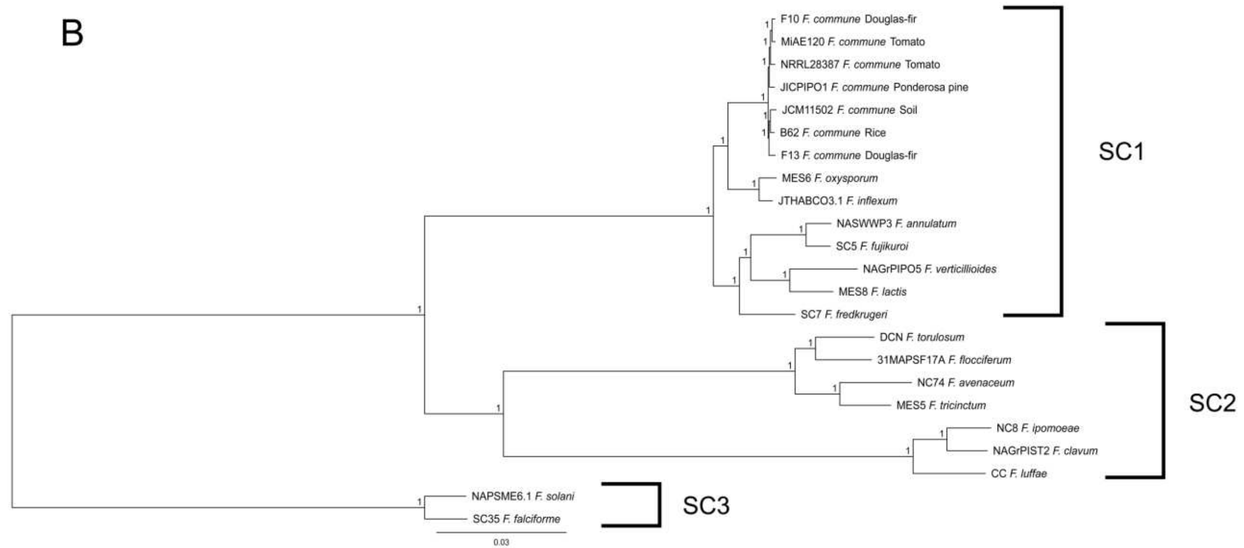



Figure 3.1 – A) Maximum likelihood phylogeny inferred from 16 loci (*acl1, cal1, dpa1, dpe1, fas1, fas2, lcb2, mcm7, pgk1, rpb1, rpb2, tef1, top1, tsr1, tub1*, and *tub2*) with bootstrap values >78 and Bayesian posterior probability values >0.99 showing good support for *Fusarium* species

complex clades (SC1: species complex clade 1 including *F. fujikuroi*, *F. nisikadoi*, and *F. oxysporum* species complexes; SC2: species complex clade 2 including *F. incarnatum-equiseti* and *F. tricinctum* species complexes; SC3: species complex clade 3 including *F. solani* species complex). The phylogeny resolved isolates analyzed in this study to species that had available reference genomes. Those isolates (*F. flocciferum*, *F. fredkrugeri*, *F. inflexum*, *F. ipomoeae*, and *F. lactis*) without available reference genomes were confirmed based on species complex and BLAST analysis. B) Species tree inferred from 5,677 orthologous proteins identified by OrthoFinder of 17 *Fusarium* species used in this study. Species complex clades (SC1–3) were confirmed compared to the 16 conserved loci. Abbreviations for loci names are described in Table 3.2.

*Comparative gene profiles*

To compare gene profiles among species and species complexes, PCoA were generated based on total orthologous proteins (Figure 3.2) and pathogenicity-related genes (Figure 3.3). Species within the SC1 (e.g., FNSC, FFSC, and FOSC) ordinated most closely together, suggesting they share similar gene profiles likely involved in pathogenicity and secondary metabolite production (Figure 3.3A and 3.3B). The SC2 (e.g., FTSC and FIESC) also ordinated closely together when comparing virulence-associated genes (Figure 3.3A) but are less similar when comparing secondary metabolite genes (Figure 3.3B). The SC3 and the species comprised within were the most dissimilar suggesting their gene profiles were uniquely distinct from the other *Fusarium* spp. This pattern was corroborated when comparing the profiles of virulence-associated genes and secondary metabolite genes (Figure 3.3A and 3.3B). However, the gene profiles were more similar when comparing CAZymes (Figure 3.3C). Virulence-associated genes unique to species complexes (FFSC, FNSC, FOSC, FIESC, FTSC, and FSSC) were identified (Supplemental Table S1). Interestingly, the histone deacetylase inhibitor (DEP1-5) mitogen activated protein kinase (MAPK) signaling pathway was shared between the FFSC, FNSC, and FOSC, which are clustered within SC1 (Figure 3.1). The BLAST search of SIX genes that was conducted across genomes found that SIX2, SIX4, SIX9, and SIX14 were present in species

collected from conifer seedlings in the FFSC, FNSC, and *F. redolens* species complexes (Supplemental Table S2). These species complexes also possessed some unique predicted secondary metabolites including some well characterized species complex-specific toxins, such as fujikurin and fumonisin, that are only found in the FFSC (Supplemental Table S3). However, when comparing CAZyme profiles, no significant groupings were observed that indicated similarities or differences among the species complexes (Figure 3.3C).

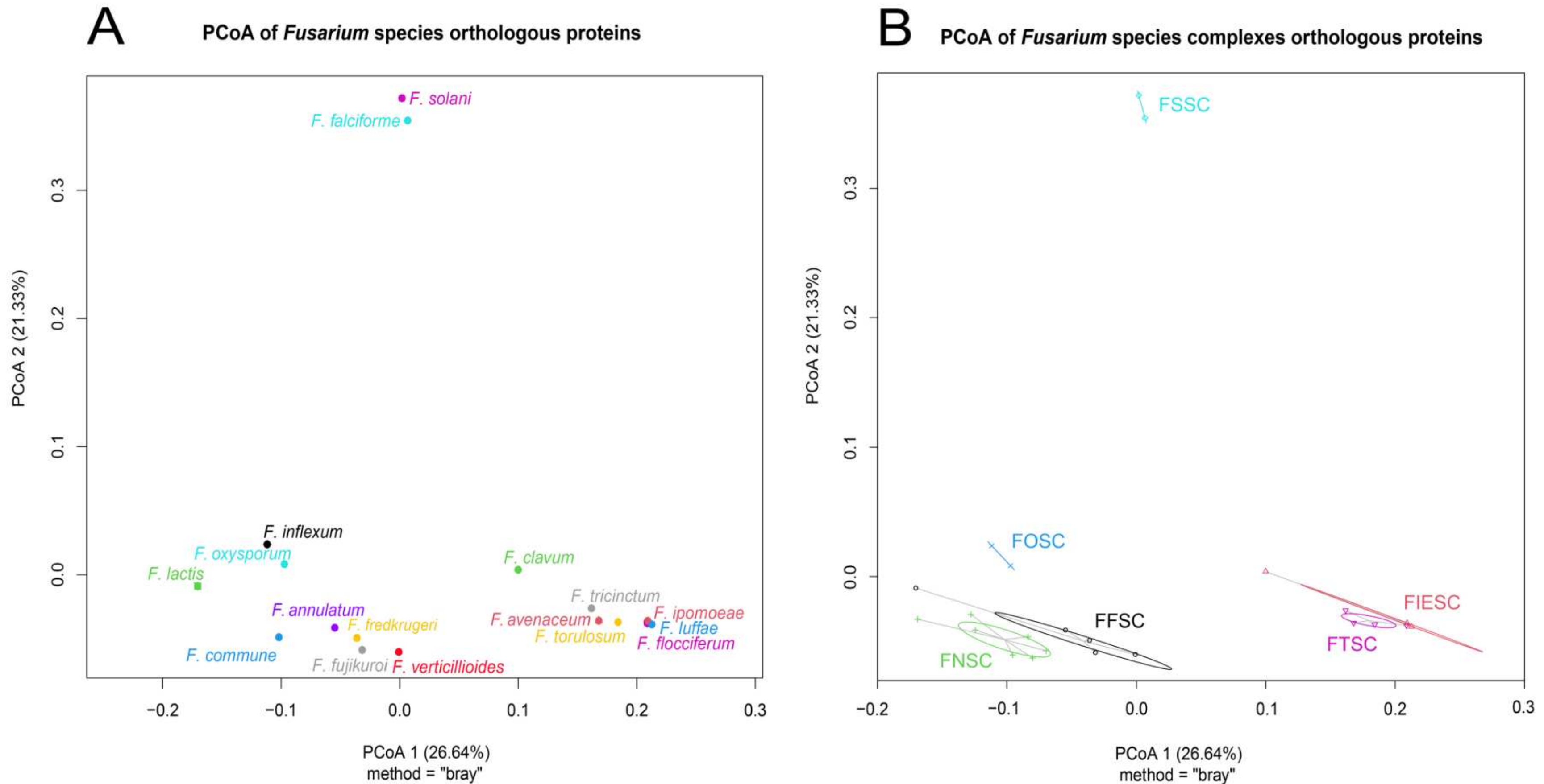



Figure 3.2 – A) A principal coordinates analysis (PCoA) of orthologous proteins shared among and between 17 *Fusarium* species. B) Of the six identified *Fusarium* species complexes, the *F. solani* species complex (FSSC) was the most dissimilar. The *F. fujikuroi* (FFSC), *F. oxysporum* (FOSC), and *F. nisikadoi* (FNSC) species complexes ordinated closely and likewise, the *F. tricinctum* (FTSC) and *F. incarnatum-equiseti* (FIESC) species complexes ordinated close together.

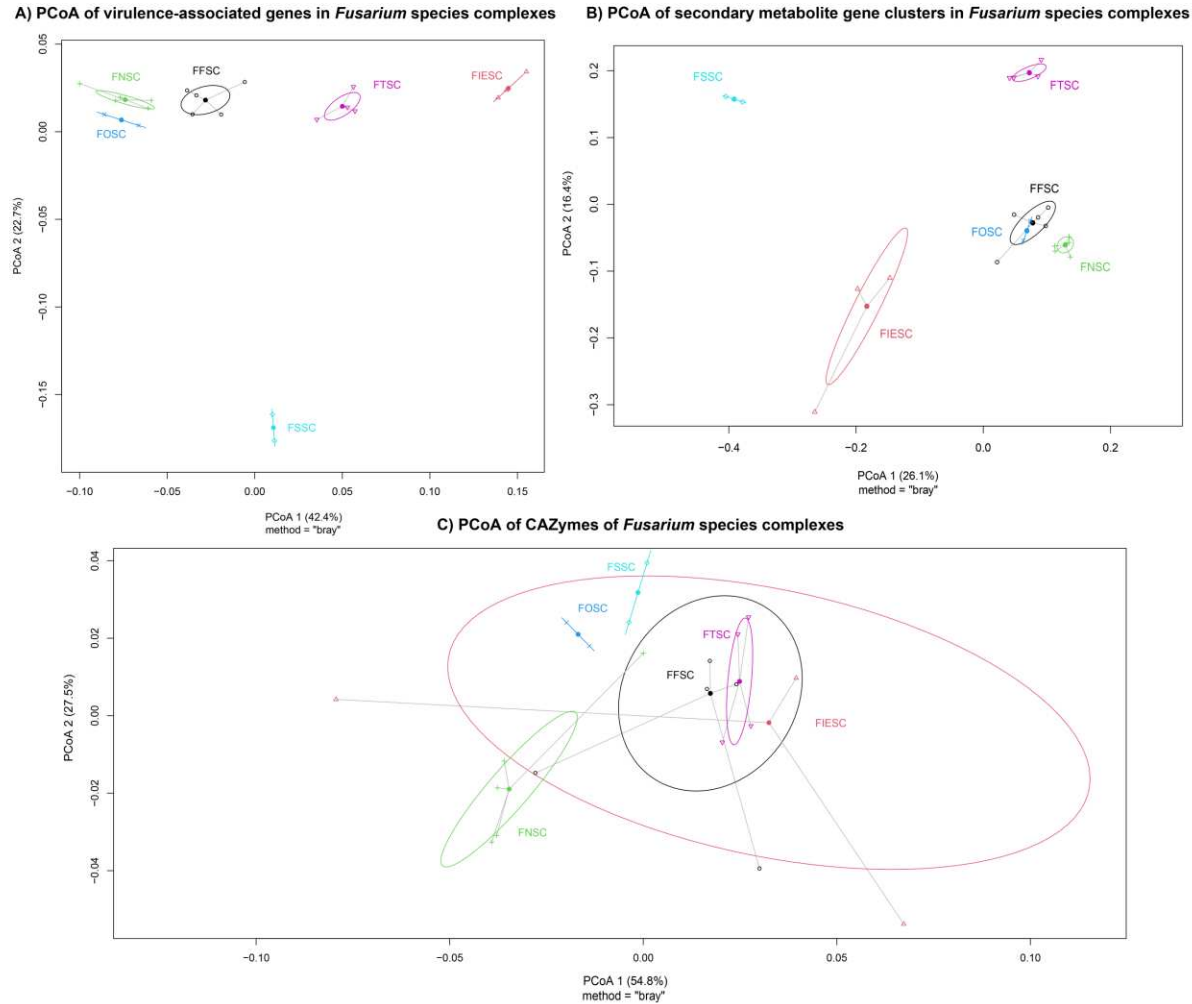



Figure 3.3 – Principal coordinates analysis (PCoA) of putative gene functions shows clustering of *Fusarium* species complexes when comparing virulence-associated genes (A) and secondary metabolites (B). *F. nisikadoi* (FNSC), *F. fujikuroi* (FFSC), and *F. oxysporum* (FOSC)ordinated closely together and may indicate that they may contain similar virulence pathways and secondary metabolites. Analysis of CAZymes (C) did not show the same clustering of *Fusarium* species complexes. Note: *F. incarnatum-equiseti* (FIESC), *F. tricinctum* (FTSC), and *F. solani* (FSSC) species complexes.

*Similarity among conifer pathogens*

Of the 8,928 conifer accessory gene orthogroups identified in this study, 730 were found unique among species previously reported as pathogens to conifers (*F. avenaceum*, *F. commune*, *F. oxysporum*, *F. solani*, and *F. verticillioides*) (Figure 3.4). Among the 730 conifer-unique orthogroups, 473 orthogroups had a predicted function including transcription, transport, carbohydrate and lipid metabolism, and mycotoxin biosynthesis (Supplemental Table S4). Within these orthogroups, nine were identified as CAZymes (e.g., CMB21, AA3_2, GH43, GH3, and GT1) (Supplemental Table S4). The 19 orthogroups identified as putative virulence-associated proteins were primarily found to be involved in secondary metabolite production and detoxification and among other conifer-unique orthrogroups, 173 were found to contain signal peptides associated with extracellular localization (e.g., secreted proteins), 61.8 % of which are predicted as effectors that are further divided into 55.3% cytoplasmic effectors and 6.6% apoplastic effectors. When examining putative function, five virulence-related genes were identified: FUG1 (PHI:6262) associated with pathogenicity and fumonisin biosynthesis; FDB2 (PHI:4602) N-malonyltransferase encoding gene; FTF1 (PHI:5483) *Fusarium* transcription factor; FgPEX1_(FGSG_07104) (PHI:9222) peroxin involved in deoxynivalenol (DON) biosynthesis; and FgABC1_Fg10995_Fgm5_(FGSG_10995) (PHI:3924, 8224, 9036) ATP-binding cassette (ABC) transporter involved in pleiotropic drug resistance (Supplemental Table S4).

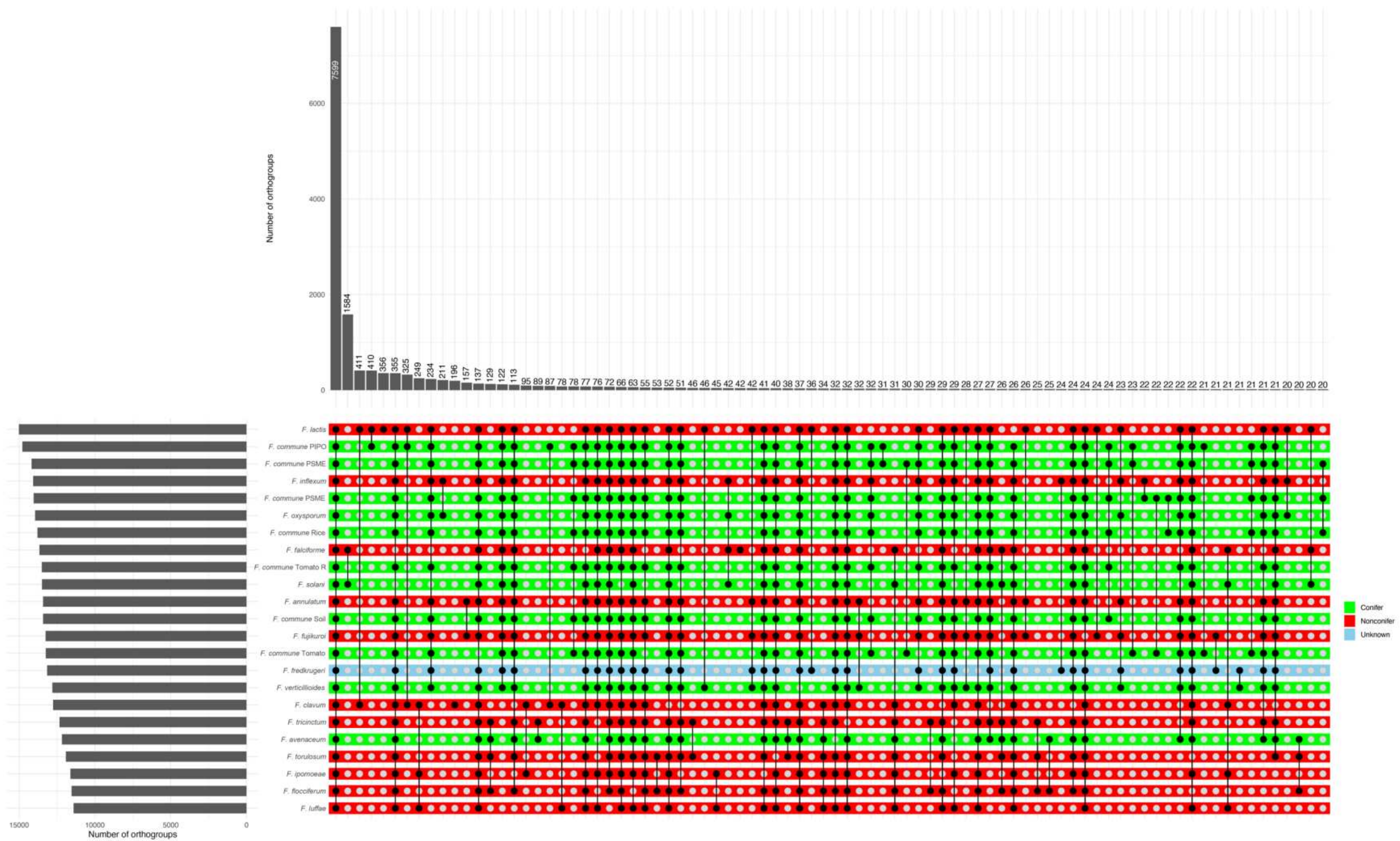


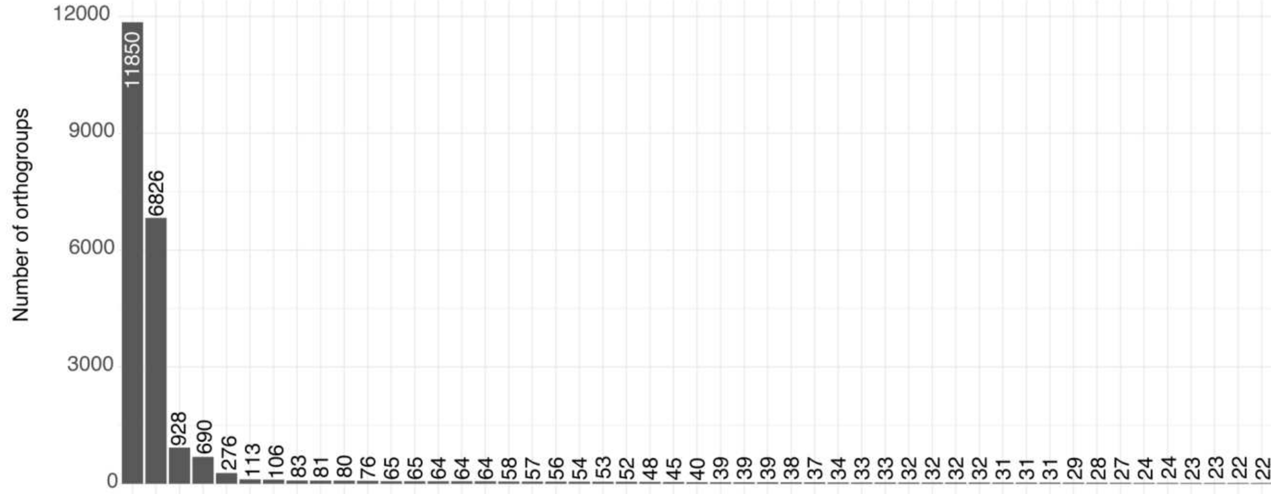

Figure 3.4 – An UpSet plot of orthologous proteins from 23 *Fusarium* spp. isolates (17 *Fusarium* spp.) collected from *Pinaceae*-associated hosts. Orthogroups of species identified as pathogenic to conifers (in green), non-conifers (in red), or unknown (in light blue) were highlighted.

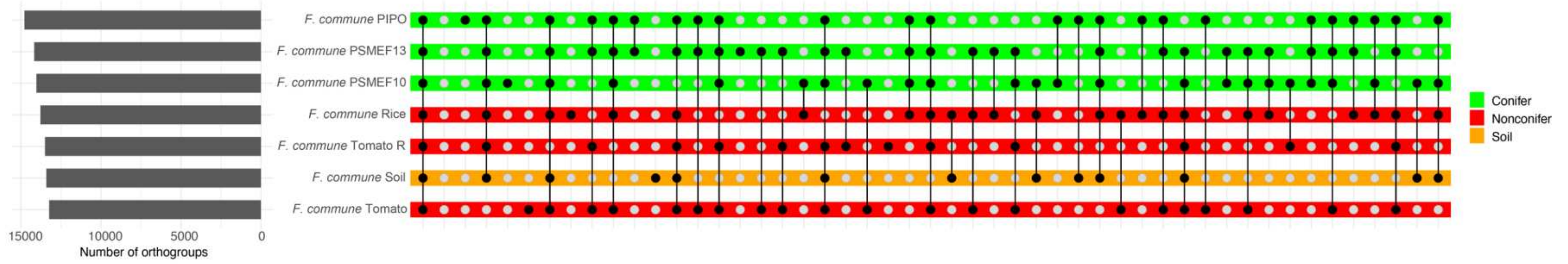
Analyses of total genes and their pathogenicity gene profiles for *F. commune* isolates collected conifer hosts (e.g., Douglas-fir, ponderosa pine), non-conifer hosts (e.g., rice, tomato), and soil revealed that the orthologous protein profiles were dissimilar among isolates originating from a conifer host, non-conifer host, and soil (Figure 3.5). In contrast, comparisons of virulence-associated, secondary metabolite, and CAZyme gene profiles did not reveal significant separation among isolates derived from conifer hosts, non-conifer hosts, and soil (Figure 3.6). For this reason, genes conveying host specificity could not be clearly attributed to specific functions. However, some virulence-associated genes including VFGLU1 (PHI:323 Beta-1,6-glucanase), Mocapn9 (PHI:6612 Calpain), HOG1 (PHI:149 MAP kinase), and BcSAK1 (PHI:1031 stress activated MAPK) were found only in *Pinaceae*-associated isolates F13.4.1 (collected from Douglas-fir) and JICPIPO1 (collected from ponderosa pine). In contrast, PCK1 (PHI:424 Phosphoenolpyruvate carboxykinase), Mopsr1 (PHI:11582 Serine/threonine-protein phosphatase dullard), and Annexin_A7 (PHI:2115 Unknown) were only found in non-conifer host-associated isolates (Dobbs 2024a).

Although significant genomic separation was not found among *F. commune* isolates based on their hosts or substrate, origin-associated variation was found for some aspects of the predicted metabolome. For example, differences were observed in secondary metabolite gene clusters and predicted metabolites, including the following: 1) the RiPP-like genes, lucilactaene, ilicolin H, and butyrolactone were uniquely identified in the ponderosa pine-associated isolate; 2) lijiquinone, fusatrixin, fungal RiPP-like genes, fosfonochlorin, enniatin, choline, and AbT1 were identified in both the *Pinaceae*- and non-conifer-associated isolates, but were not found in the soil-derived isolate; 3) more copies of fungal RiPP-like genes were found in *Pinaceae*-associated isolates, with higher numbers in isolates JICPIPO1 and F13.4.1; 4) fusatrixin was

found in both conifer- and rice-associated isolates; 5) more copies of T1PKs and terpenes were found in non-conifer-associated and soil-derived isolates; and 6) subglutinol A & B were only found in the rice-associated isolate (Dobbs 2024a).

A)



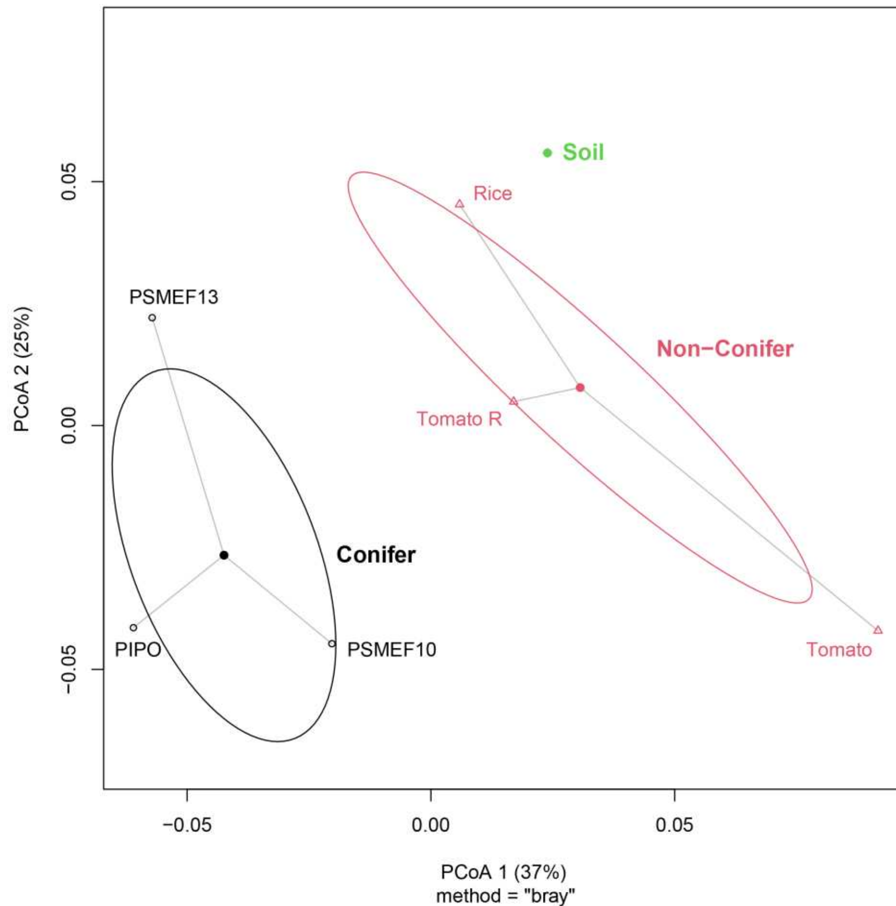



Figure 3.5 – A) An UpSet plot of orthologous proteins from *Fusarium commune* isolates collected from conifer [ponderosa pine (*Pinus ponderosa*; PIPO), and Douglas-fir (*Pseudotsuga menziesii*; PSME)], non-conifer hosts [rice (*Oryza sativa*) and tomato (*Solanum lycopersicum*)], and soil. Some orthogroups were found exclusive among conifer collected isolates. B) A principal coordinates analysis (PCoA) of *Fusarium commune* isolates collected from conifer (in green), non-conifer (in red), and soil (in orange) showed clustering based on host origins. When conducting a PCoA, the isolates separate based on host origins.

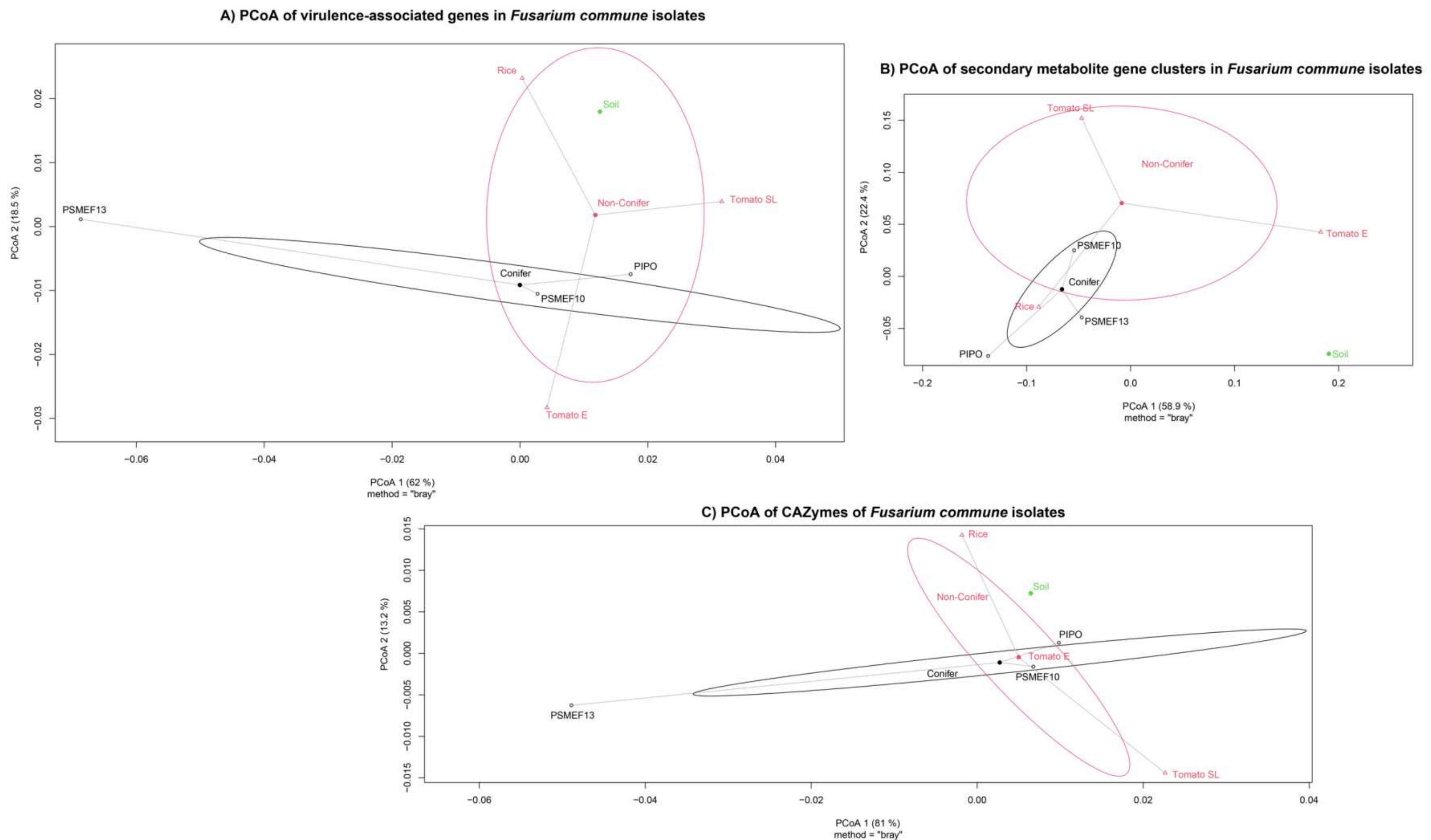



Figure 3.6 – Principal coordinates analysis (PCoA) of putative gene functions shows no significant separation ($p < 0.05$) of *Fusarium commune* isolates collected from conifer [ponderosa pine (*Pinus ponderosa*; PIPO), and Douglas-fir (*Pseudotsuga menziesii*; PSME)], non-conifer [rice (*Oryza sativa*) and tomato (*Solanum lycopersicum*)], and soil when comparing virulence-associated genes (A), secondary metabolites (B), and CAZymes (C)

**Discussion**

In this work, we used whole-genome sequencing and annotation to compare the genomes of 17 *Fusarium* spp. collected from symptomatic conifer (*Pinaceae*) seedlings, of which seven (*F. lactis*, *F. torulosum*, *F. flocciferum*, *F. ipomoeae*, *F. clavum*, *F. luffae*, and *F. falciforme*) were only recently reported on conifers (Dobbs et al. 2023). We found orthologous proteins that were shared among *Fusarium* species complexes, which suggests a shared evolutionary history among virulence-associated proteins and secondary metabolite production within six *Fusarium* species complexes (FFSC, FNSC, FOSC, FIESC, FTSC, and FSSC) and larger species complex clades (SC1 including FFSC, FNSC, and FOSC; SC2 including FIESC and FTSC; and SC3 including FSSC). We identified unique genes shared among putative *Fusarium* pathogens of conifers, suggesting that *Pinaceae*-associated isolates may contain host-specific genes involved in host colonization, pathogenicity, or virulence. These genes could serve as targets for identification/detection of conifer pathogens. Although we identified genes that were unique to *Pinaceae*-associated isolates, we further showed that it may not be possible to identify a single gene across *Fusarium* species and species complexes that determines pathogenicity to conifer seedlings. In contrast, we provide evidence that it may be reasonable and necessary to identify multiple genes or downstream products that play roles in multiple pathways used by *Fusarium* lineages for pathogenicity to conifer hosts. For this reason, further study is necessary to better understand the roles of virulence- and secondary metabolite-associated gene expression in the pathogenicity and host-specificity of *Fusarium* species and lineages.

In this study, we investigated *Pinaceae*-associated *Fusarium* species contained within six *Fusarium* species complexes: *Fusarium fujikuroi* (FFSC; SC1), *F. nisikadoi* (FNSC; SC1), *F. oxysporum* (FOSC; SC1), *F. incarnatum-equiseti* (FIESC; SC2), *F. tricinctum* (FTSC; SC2), and

*F. solani* (FSSC; SC3) species complexes. Based on the phylogenetic and orthologous putative protein analyses, we observed a correlation between species complex clades (i.e., SC1, SC2, and SC3) and similarities among the gene profiles of closely related species complexes (Figure 3.1A and 3.1B), which parallels similar results that have been found in previous phylogenetic and phylogenomic studies where multi-gene phylogenies were constructed for *Fusarium* (Geiser et al. 2021; Tobarti et al. 2018; Crous et al. 2021).

Since most of the *Fusarium* species that were examined in this study have been reported as plant pathogens, we expected to find both unique and shared virulence-associated genes among these species/species complexes and their species complex clades (SC1–SC3). For example, the SGE1 gene, which has been identified as important for virulence as a regulator of effectors and secondary metabolite production (Brown et al. 2014), was found in the SC1 and SC2, but interestingly, was lacking in the SC3. Many of the unique virulence-associated proteins in this study were described as associated with secondary metabolite production. In *Fusarium* spp., secondary metabolites are compounds produced by the fungus that are non-essential, except for roles in pathogenesis and competition with other microbes (Avalos et al. 2022). In genomics analyses, gene clusters have been identified in the production of these secondary metabolites (Blin et al. 2023), and combinations of these secondary metabolites have been linked to host-specificity (Niehaus et al. 2016; Witte et al. 2021). Interestingly, in the *Fusarium* genomes of the three species complex clades (SC1–SC3) analyzed in this study, SC1 uniquely possessed the depudecin gene cluster (dep1-5), which was previously identified in the virulence-associated genes and secondary metabolite gene through comparative genomics (Wight et al. 2009). Depudecin is a polyketide mycotoxin that acts a histone deacetylase inhibitor (Wight et al. 2009). Fusaric acid was also identified as unique to SC1, as was expected since it has been previously

reported in species within the FFSC and FOSC (Liu et al. 2020). Enniatin, which is an important mycotoxin, was shared among FNSC and FOSC, and it has been previously found in the FFSC and FOSC (Gautier et al. 2020). A primary finding of our study is that combinations of secondary metabolite genes, unique and common across species, may be necessary for conifer pathogenesis, but further research is needed to better understand the pathogenic roles of secondary metabolite gene combinations.

*Similarities among hosts and virulence pathways within* Fusarium *species complex clades (SC1–SC3)*

Fusarium *species complex clade 1 (SC1 including FFSC, FNSC, and FOSC species complexes)*

Many agriculturally important *Fusarium* species have been reported as belonging to the *F. fujikuroi* species complex (FFSC). In this study, we analyzed the genomes of five *Pinaceae*-associated *Fusarium* spp. in the FFSC, including *F. annulatum*, *F. fujikuroi*, *F. fredkrugeri*, *F. lactis*, and *F. verticillioides*. *Fusarium annulatum*, *F. fujikuroi*, and *F. lactis* have all been previously reported as pathogens to non-conifer hosts including grapevine (*Vitis* sp.), millet (*Panicum* sp.), melon (*Cucumis* sp.), wheat (*Triticum aestivum*), corn (*Zea mays*), rice (*Oryza sativa*), and sweet pepper (*Capsicum annuum*) (Sekiguchi et al., 2021; Choi et al. 2021; Wiemann et al. 2013; Medeiros Araujo et al. 2021; Niehaus et al. 2016; Brown et al. 2014). *Fusarium verticillioides* has been reported as pathogenic to conifer hosts (Maciel et al. 2017); however, *F. fredkrugeri* has not been reported as a pathogen (Sandoval-Denis et al. 2018). In the FFSC, virulence-associated genes, such as SGE1, have been found to be vital to virulence as regulators of the production of many effectors and the production of secondary metabolites (e.g., fumonisins and fujikurins) (Brown et al. 2014; Atanasoff-Kardjalieff et al. 2022). In this study, the SGE1 gene was found in the isolates of *F. avenaceum*, *F. commune*, *F. inflexum*, *F.*

*oxysporum*, *F. torulosum*, and *F. verticillioides*, suggesting that these isolates are likely pathogenic. The SGE1 gene was not found in the isolate of *F. fredkrugeri*, a potential indication that this isolate is not a plant pathogen.

*Fusarium inflexum* is a member of the *F. oxysporum* species complex (FOSC) that is not well studied or well characterized. It was originally described as a pathogen on beans (*Vicia faba*) (Schneider et al. 1975), but recent investigations of this species have focused on its potential use as a biocontrol agent against nematodes (Lira et al. 2020). In contrast, *F. oxysporum* is a very well-characterized species with high-quality genomes available and descriptions of its various formae speciales (Edel-Hermann et al. 2019). Effector profiles have been well characterized for several *Fusarium* species, including *F. oxysporum*. In particular, the SIX effectors have been used to determine host-specificity and a single gene has been identified as necessary for host specialization (Czislowski et al. 2021; Li et al. 2020a). Based on a BLAST search of SIX genes present in the *Fusarium* spp. genomes studied here, SIX2, SIX4, SIX9, and SIX14 genes were identified in *Pinaceae*-associated *Fusarium* spp. SIX2 was found in *F. annulatum* and *F. circinatum*, which was expected since it has previously been found to be shared among other members of the FFSC – a potential example of horizontal gene transfer of SIX genes from *F. oxysporum* to other species (van Dam et al. 2017). SIX4 has been identified as necessary for high virulence on plant hosts due to its role in repressing host defense response (Jangir et al. 2021; Jenkins et al. 2021). SIX4 was found in *F. commune* and *F. verticillioides*, which have both been described as pathogens of conifer seedlings, suggesting a potential role in of SIX4 in virulence on conifers. SIX9, found in *F. redolens,* and SIX14, found in *F. lactis* and *F. verticillioides*, have both been described as unnecessary for pathogenicity; however, these genes could be related to virulence in specific hosts (Jenkins et al. 2021).

Although SIX genes are important for pathogenicity and/or virulence, their expression and that of SGE1 are regulated by two transcription factors, *Fusarium* transcription factors (FTF) 1 and 2 (Jangir et al. 2021). FTF1 appears to be lineage-specific and housed on accessory chromosomes (Jangir et al. 2021). Because FTF1 was only found in isolates of two species in this study, *F. commune* and *F. oxysporum*, it could be inferred that these isolates can express these SIX genes or they may share a lineage-specific chromosome necessary for SIX gene expression (Jangir et al. 2021). However, FTF1 and the SIX4 gene were found in two separate isolates of *F. commune*, which could be interpreted as SIX genes are not necessary for *Fusarium* pathogenicity and/or virulence on conifers. Alternatively, if these two isolates interacted, they could potentially transfer the FTF1-containing accessory chromosome to the SIX gene-containing strain, and potentially affecting pathogenicity/virulence on conifer seedling hosts. The *F. oxysporum* isolate in this study did not contain any identified SIX genes, which may indicate that it is a non-pathogenic strain or that SIX genes are not necessary for pathogenicity to conifer seedlings. However, FOW2 (PHI:734), a Zn finger transcription factor that has also been described as important for virulence in FOSC members, was found in all the *Fusarium* isolates surveyed in this study. FOW2 was identified within the *F. oxysporum* genome as being necessary for invasion and colonization of plant hosts (Jangir et al. 2021). This perhaps indicates that all the studied isolates are potential plant pathogens, but it likely does not indicate that all of these isolates are conifer pathogens.

*Fusarium commune* is the only member of the *F. nisikadoi* species complex (FNSC) that we investigated since it was the only species in this complex that we recovered from conifer seedlings in our previous survey (Dobbs et al. 2023). Recently, *F. commune* has been described as a pathogen on both coniferous and non-coniferous hosts including rice, tomato, lotus

(*Nelumbo nucifera*), and Douglas-fir (Stewart et al. 2012; Hamini-Kadar et al. 2010; Kuang et al. 2022; Husna et al. 2020). A transcriptome analysis of *F. commune* pathogen of lotus root showed that genes involved in carbon metabolism and effectors were important for pathogenesis (Kuang et al. 2022). In this study, we found that the isolates collected from symptomatic ponderosa pine and Douglas-fir seedlings (JICPIPO1 & F13, respectively) had the most predicted effectors and CAZymes compared to the other *F. commune* isolates, which may indicate that these isolates are highly virulent isolates to conifer seedlings. However, virulence assays are necessary to confirm the connections among effectors and CAZymes with pathogenicity/virulence to coniferous hosts. Based on their genome content, all studied isolates have the potential to produce secondary metabolites, such as enniatin, depudecin, gibberellin, fusaric acid, alterpyranone, and subglutinol A/B, while infecting their hosts. Metabolomic and/or transcriptomic analyses would allow for the identification of pathogenicity/virulence genes involved in metabolism and effector production that are expressed during pathogenesis on conifer seedlings.

Fusarium *species complex clade 2 (SC2 including FIESC and FTSC species complexes)*

We analyzed three members of the *F. incarnatum-equiseti* species complex (FIESC), *F. ipomoeae*, *F. luffae*, and *F. clavum,* all of which have been described as pathogens of non-coniferous hosts including peanut (*Arachis hypogaea*), maize, and tomato (Gilardi et al. 2021; Parime et al. 2022; Xu et al., 2021). None of these species have been tested for pathogenicity on conifers. Secondary metabolite gene clusters and products, such as enniatin, fusarin, and zearalone, have been found to vary among members of the FIESC, likely due to horizontal gene transfers that effect functionality of the metabolic pathways (Villani et al. 2019). Zearalone was identified in *F. clavum* and *F. luffae*, while deoxynivalenol (DON) was identified in *F. ipomoeae* and *F. luffae*. However, enniatin and fusarin were not identified in any of these three species (*F.*

*clavum*, *F. luffae*, and *F. luffae*), suggesting isolates studied herein may be pathogens but the lack some mycotoxin-associated genes that may affect pathogenicity or virulence on conifer hosts.

The *F. tricinctum* species complex (FTSC) members are well-known cereal pathogens that cause head and seedling blight on wheat and barley (*Hordeum* sp.) and crown rot on wheat, but FTSC members have also been described as pathogens of pea (*Pisum sativum*) and faba bean (*Vicia faba*) (Šišić et al. 2020; Inbaia et al. 2023; Karlsson et al. 2021; Wang et al. 2022). In addition, FTSC members are known for producing mycotoxins, such as enniatins and moniliformin, that can contaminate grain (Wang et al. 2022). The genomes of *F. tricinctum*, *F. avenaceum*, *F. flocciferum*, and *F. torulosum* were examined in this study, of which, only *F. avenaceum* has been previously reported as a pathogen of conifer seedlings (Morgan 1983; James et al. 1989). It has been posited that *F. tricinctum* is not a pathogen of conifer seedlings, even though it is commonly isolated from conifer seedlings (James 2004). In this study, isolates of *F. tricinctum* and *F. torulosum* did not share secondary metabolites that were identified in *F. avenaceum*, further suggesting these two species likely are not conifer pathogens (Supplemental Table S2). The *F. avenaceum* isolate had unique predicted secondary metabolites and shared secondary metabolites with SC2. Enniatin, which has been found to be important for pathogenicity on plant hosts (Inbaia et al. 2023), was not found in the *F. avenaceum* isolate; however, apicidin gene clusters were identified in the *F. avenaceum* isolate. The apicidin gene cluster was recently found on accessory chromosomes in a closely related species, *F. poae* (Witte et al. 2021), suggesting that this secondary metabolite may be important for conifer pathogenesis and that an accessory chromosome may be present in this *F. avenaceum* isolate.

Fusarium *species complex clade 3 (SC3 including FSSC species complex)*

Taxonomists are currently disputing the taxonomic placement of the *F. solani* species complex (FSSC), and changing of the genus from *Fusarium* to *Neocosmospora* has been recently proposed (Crous et al. 2021). A recently described species, *F. falciforme* (syn. *N. falciforme*) (Herron et al. 2015) has been reported as a pathogen of melons (Medeiros Araujo et al. 2021). Another member of FSSC, *F. solani* (syn. *N. solani*), has been described as a pathogen of both conifer and non-conifer hosts (James et al. 2000; Šišić et al. 2018). Pectase lyase genes, which were identified in the FSSC species, have been described as important for pathogenicity of *F. solani* (Covey et al. 2014).

*Unique genes for conifer pathogens*

Identification of core and accessory genes is a prerequisite toward focusing on gene targets for host-specific pathogenicity factors across genomes. In our study, we identified 730 conifer pathogen-exclusive, accessory genes. Most of these genes were characterized as putative effectors (61.8%). The putative pathogenicity/virulence-associated genes were found to be involved in transcription, transport, and secondary metabolism based on database matches; all of which have been found to be important for host-specific pathogenicity/virulence (Inoue et al. 2022; Li et al. 2020b). In addition, effectors, such as the previously described SIX genes, play vital roles in host-specific pathogenicity/virulence and infection (Inoue et al. 2022). Further investigations of these effectors and their functions may elucidate conifer pathogen-specific, secreted proteins.

In previous studies of fungi, CAZymes have been found to be enriched in fungi with saprophytic life stages (Steindorff et al. 2020). Even though CAZymes were among the conifer pathogen-unique genes, in this study, we did not observe CAZymes as significantly different among species, species complexes, or among host origins of *F. commune* isolates. This finding may

indicate that these CAZymes may not be necessary for host-specific pathogenicity/virulence, but they may contribute to host penetration or disease progression within the host. In contrast, it has been previously reported that CAZymes are important for pathogenicity in *Fusarium* and other fungi, and CAZymes have been found important for pathogen lifestyle adaptation (Ma et al. 2019; Rampersad 2020). Secreted lipases were among the conifer pathogen-unique CAZymes, and secreted lipases have also been reported as import for pathogenicity/virulence in *F. graminearum* (Rampersad 2020).

Of the putative pathogenicity/virulence-associated proteins, most were identified as involved in secondary metabolite production or detoxification. Fumonisin- and DON-production proteins were identified among the conifer pathogen-unique orthogroups, and these proteins are important for plant host infection (Perincherry et al. 2019). Gene clusters associated with the production of these mycotoxins were predicted among members of the FFSC (*F. annulatum*, *F. fujikuroi*, and *F. verticillioides*) and FIESC (*F. ipomoeae* and *F. luffae*). Identification of these mycotoxins could signify that secondary metabolites may play an important role in pathogenicity to conifer seedling. Genes associated with secondary metabolites also includes genes associated with detoxification of host-produced metabolites, such as the FDB2 (PHI:4602) protein, which has been found to be involved in overcoming host-produced toxins and may be important for the pathogen to survive within the conifer seedling host (Karagianni et al. 2023). Such genes that are associated with secondary metabolite production could serve as targets for the development of conifer pathogen-specific probes. Transcriptomic and metabolomic analyses could be used to validate expression of these genes when the pathogen is infecting conifer seedling hosts.

*Detection techniques*

Methods to rapidly identify pathogenic members within the *Fusarium* species complexes through genetic, genomic, proteomic, and metabolomic analyses are necessary to develop robust management strategies to detect conifer pathogens, mitigate pathogen spread, and manage disease outbreaks. Conserved gene loci and comparative genomics have been previously used to generate PCR-based detection methods for *Fusarium* spp. (Dobbs et al. 2020; Pramunadipta et al. 2022). Soil and plant material samples serve as substrates for detecting/identifying *Fusarium* spp. pathogens within a growing system by metabarcoding of environmental DNA (eDNA) (Cobo-Diaz et al. 2019). Loop-mediated isothermal amplification (LAMP) is also another useful detection tool that targets pathogen-specific DNA sequences, and use of this tool is becoming more common, as LAMP is a rapid, accurate, and cost-effective detection method (Deng et al. 2022). Effector gene profiles and protein mass spectral fingerprinting have also been used to identify pathogenic *Fusarium* spp. (Wigmann et al. 2019; Czislowski et al. 2021). However, secondary metabolites and the genes associated with their production can also be used to effectively and robustly differentiate pathogenic isolates of *Fusarium* spp. (Niehaus et al. 2016; Wiemann et al. 2013). The gene profiles and the putatively produced secondary metabolites identified in this study may prove useful for the development of rapid detection tools to identify conifer pathogens within *Fusarium*.

**Conclusions**

In this study, we used whole-genome sequencing and predicted genes to analyze the relationships among *Fusarium* isolates and found clustering among closely related species complexes. This work provides draft genomes for *Pinaceae*-associated *Fusarium* spp. and other genomes that were not previously referenced in public databases (e.g., *F. lactis*, *F. fredkrugeri*, *F. ipomoeae*, and *F. flocciferum*). We also took a closer look at a single species, *F. commune*, to

determine which genes associated with host-specialization were present among isolates derived from conifer and non-conifer hosts. Also in this study, we predicted *Fusarium* pathogenicity-related genes for conifers through genome comparisons; however, phenotypic validation through pathogenicity/virulence assays, transcriptomic and metabolomic analysis are necessary to confirm the pathogenicity/virulence of the 17 *Fusarium* spp. isolates used in this study, confirm expression of putative pathogenicity-related genes for conifers, and confirm the presence of secondary metabolites that contribute to pathogenicity and/or virulence. Based on our previous survey, we found associations of *Fusarium* species complexes with multiple conifer hosts including *Picea* spp. (FFSC, FOSC, FNSC, FIESC, FTSC, and FSSC), *Pinus* spp. (FFSC, FOSC, FNSC, FIESC, FTSC, and FSSC), *Pseudotsuga menziesii* (FFSC, FOSC, FNSC, FTSC, & FSSC), *Larix* spp. (FFSC), *Cupressus* spp. (FFSC, FOSC, FNSC, & FTSC), *Juniperus* spp. (FFSC, FOSC, & FSSC), *Sequoia* spp. (FOSC), and *Abies* spp. (FFSC, FOSC, & FNSC). These wide host associations suggest that directed pathogenicity assays of *Fusarium* spp. on these host genera would prove useful to determine conifer host specialists and generalists.

Mycotoxins were shown to be specific to species complexes of the *Pinaceae*-associated *Fusarium* spp. isolates used in this study. This finding is noteworthy as it determines that potential targets for detecting/identifying conifer pathogens within *Fusarium* will likely require multiple methods, depending on the species complex clades. Our results also support the importance of mass spectrometry as an alternative method for pathogen detection method. In addition, host response to these mycotoxins is perhaps another useful approach that warrants further investigation for rapid detection of infected hosts through air sampling within nurseries to detect pathogens and disease.

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# CHAPTER 4: PATHOGENOMICS OF *PINACEAE*-ASSOCIATED PATHOGENIC *FUSARIUM ANNULATUM* AND *F. COMMUNE*

**Summary**

In this study, we assessed pathogenicity and/or virulence associated genes in *Fusarium annulatum* and *F. commune* pathogenic to loblolly seedlings and compared these transcriptomes to the genomes of *Fusarium* spp. to assess the pathogenomics of *Fusarium* spp. associated with conifers (*Pinaceae*). In the pathogenicity tests, we tested isolates from both species on both conifer and non-conifer seedlings. We discovered that all tested isolates were pathogenic to all hosts, but that the virulence among hosts varied but did not correlate with host origins. We identified differentially expressed pathogenicity/virulence genes by comparing broth grown mycelium cultures against conifer pathogenic and conifer-derived *F. commune* (collected from ponderosa pine) and *F. annulatum* (collected from southwest white pine) inoculated loblolly pine seedlings harvested at 12-, 24-, and 48-hours post-inoculation. These isolates were selected because of their high virulence on loblolly pines in our pathogenicity assays. Among the upregulated genes *in planta* (UIP), we identified pathogenicity/virulence-associated genes including secreted effectors, genes involved in biosynthesis of mycotoxins, carbohydrate-active enzymes, etc. We then compared these UIP genes with the predicted proteomes of 17 *Fusarium* spp. isolates collected from conifer hosts and identified putative pathogenicity profiles of these potential pathogens. We assessed the presence of secreted pathogenicity/virulence genes among the predicted proteomes of *Pinaceae*-associated *Fusarium* spp. and found that profiles were associated with *Fusarium* species complexes rather than conifer versus non-conifer pathogens.

However, these profiles may aid in the development of conifer pathogen-specific detection methods based on *Fusarium* species complexes.

**Introduction**

As warming climates, increased droughts, and more intense fires, reforestation efforts are relying on nursery grown seedlings (Fargione et al. 2021). Conifer nurseries are vital resources for forest restoration and reforestation and for timber production for green energy projects and lumber. The pacific northwest, southeast, and southwest are important conifer seedlings producing areas where Douglas-fir (*Pseudotsuga menziesii*), loblolly pine (*Pinus taeda*), and ponderosa pine (*Pinus ponderosa*) are the predominant species produced, respectively. However, due to increased seed shortages, it is imperative that nurseries can get the most seedlings from the limited seed they have. This can be hampered by damping-off and root disease pathogens including, *Cylindrocarpon* spp. (syn. *Ilyonectria* & *Neonectria*), *Pythium* spp., *Phytophthora* spp., and most importantly, *Fusarium* spp. (Dumroese et al. 2000; Dumroese & James 2005; Weiland et al. 2013; Weiland 2021). In a previous survey, it was reported that losses caused by *Fusarium* pathogens can be as high as 40% and management costs up to $45,000 annually (Dobbs et al. 2023a). Since damping-off is primarily detrimental during the first 4-6 weeks in conifer seedlings (Cram 2003), investigations into initial and early infection is necessary to identify pathogenicity/virulence genes in host selection and disease development (Ma et al. 2013; Thatcher et al. 2016; Tu et al. 2023).

Understudied *Fusarium* spp. also pose a threat to agricultural systems as the virulence and host range of many of these species has not been well documented. Emerging investigations of the understudied *Fusarium* spp., such as *Fusarium annulatum* and *F. commune*, are ongoing within the literature as these pathogens are collected from a variety of hosts including conifers

and non-conifers (Bustamante et al. 2022; Dobbs et al. 2023a; Hamini-kadar et al. 2010; Husna et al. 2021; Parra et al. 2022; Stewart et al. 2012). Recent studies have found these species to cause damping-off and root disease on the limited hosts through pathogenicity assays (Hamini-kadar et al. 2010; Husna et al. 2021; Mirghasempour et al. 2022; Stewart et al. 2012). However, limited described control measures have been documented. Investigating the pathogenicity mechanisms utilized by these pathogens would aid in providing pathogen mitigation recommendations and the development of tools for rapid identification and breeding for agricultural and nursery managers.

Multiple *Fusarium* spp. that cause damping-off and root disease in conifer nurseries (Dobbs et al. 2023a). However, the evolutionary history and the mechanisms involved in conifer pathogenesis have not been determined for many of these conifer pathogenic species. Previous studies have determined that *Fusarium* spp. within species complexes share profiles of potential pathogenicity genes (Chapter 3). In some *Fusarium* spp., like *F. oxysporum*, entire chromosomes and even single genes among accessory chromosomes have been shown to be able to be shared among other *F. oxysporum* strains and *Fusarium* spp. to confer pathogenicity (Bertazzoni et al. 2018; Li et al. 2020a; Li et al. 2020b). Investigations into these pathogenicity genes and accessory chromosomes among conifer pathogenic *Fusarium* spp. would aid in developing strategies to mitigate pathogens before outbreaks occur (Niehaus et al. 2016; Burkhardt et al. 2019).

New and emerging fungal pathogens have been increasing because of globalization and changing environmental conditions. Comparative genomics and transcriptomics can be useful in developing mitigation and detection tools, especially for fungal pathogens where there is a lack of detection and control methods that also hinders understanding of pathogen biology

(Karunaratha et al. 2021; Yilmaz et al. 2021). Identifying genes involved in pathogenic lifestyles is useful for understanding pathogen biology and developing early detection tools based on pathogenicity/virulence genes (Ibarra Caballero et al. 2020; Ye et al. 2018). Putative conifer-specific pathogenicity/virulence factors including, predicted secreted proteins and effectors, virulence-associated proteins, and secondary metabolites (e.g., mycotoxins such as fumonisins, deoxynivelanol, and enniatin) were identified in a previous survey (Chapter 3). This suggests 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 confirmed if these genes are expressed during the infection of a susceptible host.

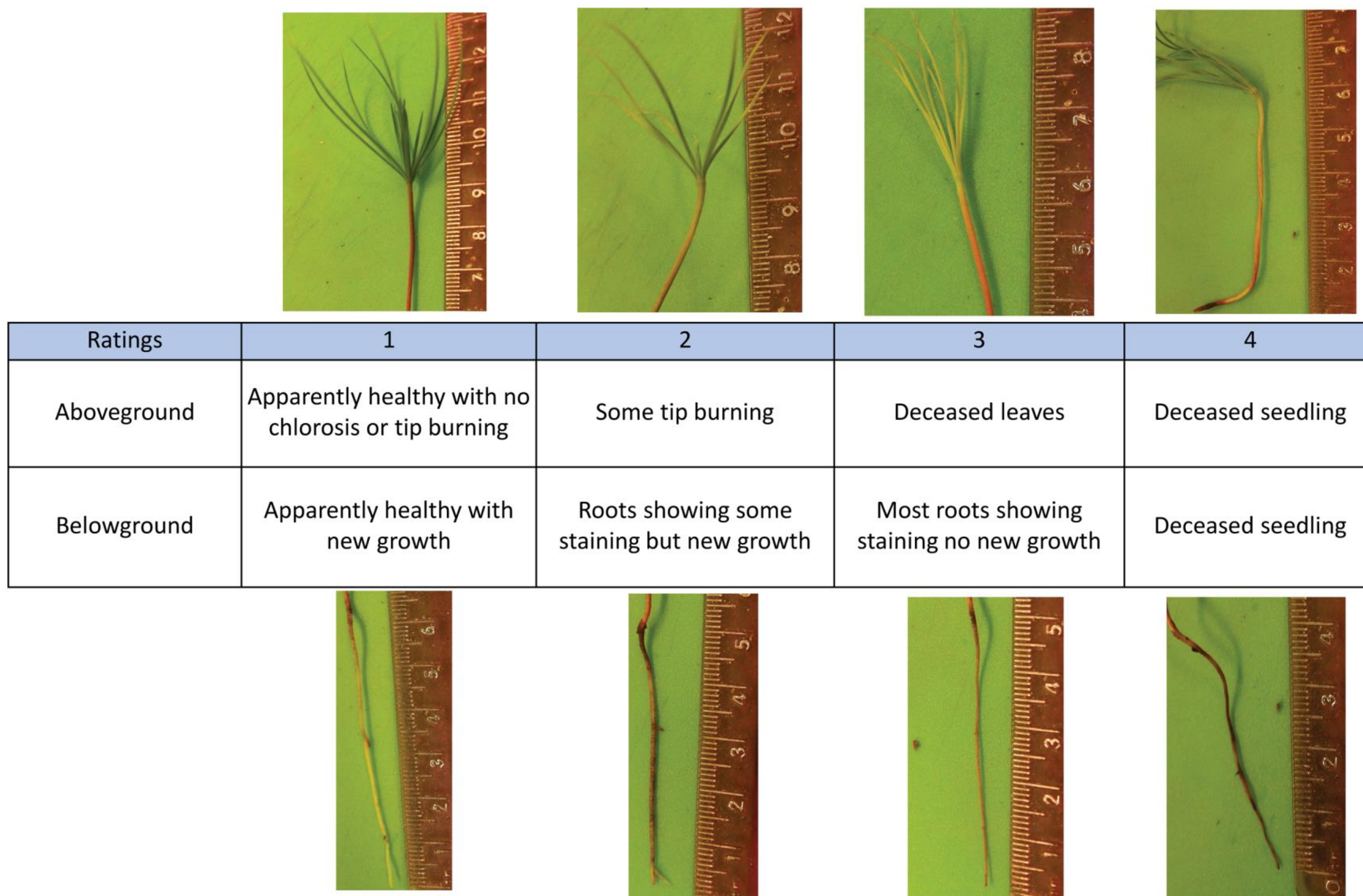
We hypothesized that conifer (*Pinaceae*) pathogenic *Fusarium* spp. have similar expressed pathogenicity/virulence genes that can be used to predict and rapidly identify *Fusarium* pathogenic to conifers. Our overarching goal was to elucidate a *Fusarium* spp. conifer pathogen virulence profile and use this to classify and distinguish conifer pathogenic *Fusarium* isolates from non-pathogens. The specific objectives were to: 1) evaluate the pathogenicity/virulence of conifer and non-conifer collected *Fusarium commune* isolates on both conifer and non-conifer hosts; 2) evaluate the virulence of conifer collected isolates on three conifer hosts; 3) investigate the differentially expressed genes of representative conifer collected *F. annulatum* and *F. commune* to determine conifer pathogenicity/virulence genes; and 4) identify the presence of *F. annulatum* and *F. commune* upregulated genes in host plants within *Pinaceae*-associated *Fusarium* spp. predicted proteomes.

## Materials and methods

### *Pathogenicity assays*

To confirm conifer pathogenicity of *Fusarium annulatum*, a representative isolate collected from a symptomatic southwest white pine seedling (*Pinus strombiformis*) was assessed for virulence on three conifer hosts [ponderosa pine (*Pinus ponderosa*), loblolly pine (*Pinus taeda*), and Douglas-fir (*Pseudotsuga menziesii*)]. *F. commune* isolates collected from conifer and non-conifer hosts were tested for virulence on three conifer (Douglas-fir, loblolly pine, and ponderosa pine) and two non-conifer hosts [rice (*Oryza sativa*) and tomato (*Solanum lycopersicum*)]. Inoculum for each isolate was produced in Erlenmyer flasks of 250ml potato dextrose broth medium. After seven days in the dark, mycelium was removed from the broth through 2 layers of cheese cloth. Spores from the filtrate were condensed through cold centrifugation. A $1x10^6$ spores/ml concentration was obtained using a hemocytometer. Pathogenicity assays included 15 replicates in two experiments (n=30) for each isolate. Bleach disinfested seeds were germinated on filter paper. Seven days post-germination, each germinant had their root tips severed with a sterile scalpel and placed in either the spore solution or sterile water for 5 min. Each 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, and tubes were incubated in growth chambers under fluorescent, diurnal light (12/12) at either 25°C. Seedlings were harvested after 14 days and examined for mortality and above/belowground symptoms, then re-isolated onto PDA. A disease severity index was used to assess isolate virulence based on a 0 (indicating a non-symptomatic or healthy seedlings) to 3 (indicating a deceased seedling) rating for both above- and below-ground symptoms (Figure 4.1). A mixed effect model was used to test for significance of the random effect between the two separate experiments. Statistical

significance of disease severity was assessed using an analysis of variance (ANOVA) and Tukey ad-hoc test in R.



| Ratings | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Aboveground | Apparently healthy with no chlorosis or tip burning | Some tip burning | Deceased leaves | Deceased seedling |
| Belowground | Apparently healthy with new growth | Roots showing some staining but new growth | Most roots showing staining no new growth | Deceased seedling |

Figure 4.1 – Rating system used to calculate disease severity of *Fusarium annulatum* and *F. commune* pathogenicity assays.

*Sample collection for transcriptomics*

To identify virulence-associated genes produced during early infection of conifer seedlings, three-week-old loblolly pine (*Pinus taeda*) seedlings were inoculated with a $1x10^6$ conidia suspension of either one *F. commune* isolate collected from a symptomatic ponderosa pine (*Pinus ponderosa*) seedling and one *F. annulatum* isolate collected from a symptomatic southwest white pine (*Pinus strombiformis*). Sterile distilled water was used as a control. In preparation of spore solutions, each isolate was first grown on ¼-strength potato dextrose agar (PDA) at 25°C for 3 days. Hyphal tips were transferred to 500 ml of potato dextrose broth (PDB) and shaken at 90 rpm for 10 days at room temperature. Spores were separated from mycelium through two layers of cheesecloth. Spores were separated from PDB through cold centrifuging at 10°C and then was strained in 50 ml conical centrifuge tubes at 5,000 x g for 5 min. Supernatant was removed leaving the spore pellet that was resuspended in sterile distilled water. Solutions were calibrated to $1x10^6$ using a hemocytometer. Each seedling was inoculated by severing the root tips with a sterile scalpel to provide an infection court and soaking the samples in the spore solutions for 5 min. before transferring to forestry tubes filled with soil-less potting mix (PRO-MIX BX, Quebec, CA). Seedlings were grown in a growth chamber kept at 25°C with a 12-hour day-night cycle. A time series approach was used, at 12-, 24-, and 48-hours post-inoculation intervals, to determine if there were early changes from early infection to pathogenesis. Three seedlings were grown and harvested at each time point with two replicates (9 total seedlings per time point; 27 seedlings per treatment). Roots and a small portion of the stem above the root collar were harvested from each seedling. The three seedlings at each time point were pooled together in 2 ml screw cap tubes to 0.1 g wet weight and quenched using liquid nitrogen. Root segments were stored at -80°C for later RNA extraction.

*RNA extraction*

Total RNA was extracted using methods similar to Ata et al. (2022). Mycelial tip cultures from active growing cultures on PDA of both *F. commune* and *F. annulatum* isolates were grown in PDB for three days prior to RNA extraction. Wet mycelium tissue samples were extracted from broth using two layers of cheesecloth and flash frozen in liquid N. Frozen mycelium and frozen root segments were ground to a fine powder with a cold mortar and pestle and transferred 0.1 g of ground tissue to 2 ml microcentrifuge tubes. Warm extraction buffer with 2% polyvinyl pyrrolidine (PVP) and 10µl β-mercaptoethanol (500 µl) was added to the samples. Samples were homogenized in a FastPrep-24Tm (M.P. Biomedicals LLC, Santa Ana, CA, USA) at 5.5x speed for 1 minute 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 10,000g for 15 min. and the upper aqueous phase (ca. 400 µl) was transferred to new 2 ml microcentrifuge tubes. A quarter volume (ca. 100 µl) of 10 M lithium chloride (LiCl) was added to each sample and mixed by inversion. Total RNA was precipitated overnight at 4°C. Samples were centrifuged at 10,000g for 30 min. at 4°C. Supernatant was removed and the RNA pellet was left to air dry. Pellet was dissolved in 400 µl of SSTE buffer (preheated at 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 10,000g. Aqueous phase was transferred to new 2 ml microcentrifuge tubes. Three times volume of 100% ethanol was added to sample and mixed by inversion. Samples were left to precipitate overnight at -80°C. Samples were centrifuged at 10,000g at 4°C for 30 min. Supernatant was removed and pellet was left to dry. Pellet was dissolved in molecular grade water and stored at -80°C until sent for sequencing.

RNA samples were sent to Novogene Corporation, Inc. for library preparation and sequencing. Raw reads were deposited to NCBI SRA database (BioProjectID PRJNA1020577).

*Genome-guided transcriptome assembly and analysis*

Genomes of each isolate were retrieved from GenBank BioProjectID PRJNA1020449: *Fusarium commune* collected from ponderosa pine (*Pinus ponderosa*) (AccessionID: JAVTNL000000000) and *F. annulatum* collected from southwest white pine (*Pinus strobiformis*) (AccesionID: JAVTNU000000000). Quality of sequences was visually inspected using FastQC (Andrews 2010). No reads in any sample were trimmed due to high quality Phred scores (>30). Transcriptome assemblies were performed using methods similar to Dobbs et al. (2023b). HiSAT2 (v2.2.1; Kim et al. 2019) and STAR two-pass protocol (v2.7.10b; Dobin et al. 2013) 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 Tiglon (v1.1; Zhao & Yu 2022) to integrate the two alignments and utilize the integrated analysis of mappings from two genome-guided aligners for in-depth analysis of microbial activity in inoculated loblolly pine seedlings. Resulting transcripts were clustered into isogroups using cd-hit-est (v4.8.1; Fu et al. 2012) and a 0.99 cutoff value. RSEM (v1.3.3; Li et al. 2011) was used to calculate expression after representation of reads was assessed using bowtie2 (v2.5.0; Langmead & Salzberg 2012) where 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 the ascomycota_odb10 (n=1706 genes) and sordariomycetes_odb10 (n=3817 genes) (v5.5.0; Manni et al. 2021). Counts of transcript abundance was analyzed after a variance stabilizing transformation (vsd) of the data by producing a principal component (PCA) plot using the plotpca function in the DESeq2 package

v1.42.0 (Love et al. 2014) in R v4.3.2 (R Core Team 2023) to assess sample correlation across conditions. Differentially expressed (DE) transcripts were identified using DESeq2 to compare mycelium or inoculated seedlings collected at 12-, 24-, and 48-hours post inoculation (hpi) (*in planta*) vs mycelium grown in broth (*in vitro*) and hours post-inoculation. Significant DE transcripts were determined as those with $\log_2$ fold change > 1 and an adjusted p-value < 0.05 (BH adjustment).

Orfipy v0.0.3 (Singh et al. 2021) was used to identify the longest open reading frames (orfs) of each transcript and were additionally converted to amino acids using the peptides flag (--pep). These longest orfs were used for predicted function analyses. Predicted functions of proteins coded by the transcripts were determined using seven databases: OrthoVenn3 for putative non-orthologous proteins (Xu et al. 2019), antiSMASH fungal database for secondary metabolite gene clusters discovery (Blin et al. 2017), InterProScan v5.64-96.0 for protein family domains (Quevillon et al. 2005), KEGG: Kyoto Encyclopedia of Genes and Genomes for metabolic pathways (Kanehisa et al. 2000), dbCAN3 for putative carbohydrate-active enzymes (Zhang et al. 2018), PHI-base v4.15 for putative pathogenicity/virulence-associated proteins (Urban et al. 2017), and SWISS-PROT for similarity to proteins in the curated database (Bairoch et al. 2000). OrthoVenn3, antiSMASH, KEGG KOfam, and dbCAN3 databases' online servers were used to analyze the predicted genes at default settings. Mmseqs2 (Mirdita et al. 2021) was used to identify sequence similarity of proteins in the SWISS-PROT, Pfam for protein family domains (release 36.0), and PHI-base database. Selection of putative functions of proteins was based on a e-value < 1e-5, percent identity ≥ 65% and a coverage ≥ 50%. UniProt's ID mapping tool based on SWISS-PROT identities was used to generate equivalent GO terms and Pathway identification.

*Putative pathogenesis genes in* Fusarium *spp. genomes*

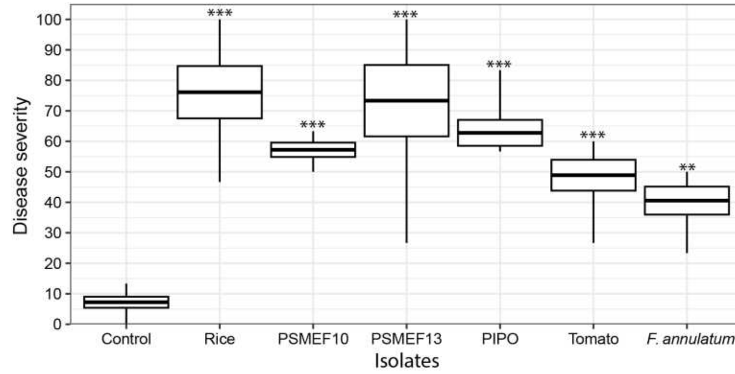
Significantly upregulated transcripts *in planta* (UIP) expressed by *F. commune* and *F. annulatum* at any of the three time points were compared for presence within genomes of *Fusarium* spp. isolates used in Chapter 3 using OrthoFinder. Each *Fusarium* species' UIP transcriptome profiles were analyzed both separately and combined. Only transcriptome orthogroups that were also present in at least one genome were kept for the analysis. Profile dissimilarity was contrasted by *Fusarium* species complex using Bray-Curtis distances with a PERMANOVA using the 'vegan' v2.6-4 package (Oksanen et al. 2020). Profile comparisons were visualized using principal coordinates analyses (PCoA) and sets of UIP transcripts were visualized using an UpSet plot using the 'ComplexUpset' v1.3.5 package (Lex et at. 2014). A phylogenetic principal components analysis (PhylPCA) was conducted using a concatenated maximum likelihood phylogeny of 16 conserved loci (Supplemental Figure S1) and the UIP profiles used for the PCoA analysis using the 'phytools' v2.0-3, 'ape' v5.7-1, and 'geiger' v2.0.11 packages (Revell & Harmon 2022). 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 to the 23 *Fusarium* spp. using the same criteria as above.

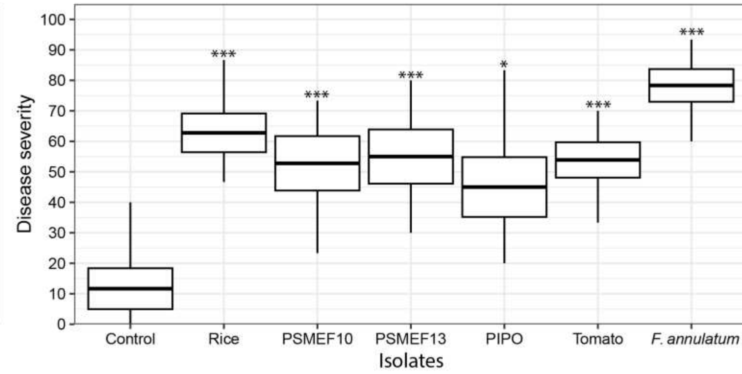
**Results**

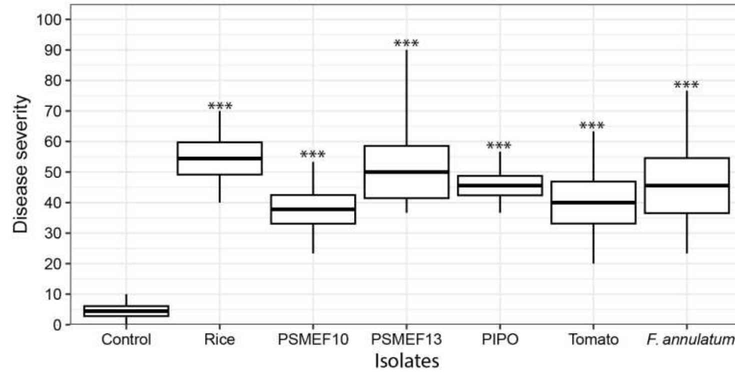
Fusarium annulatum *and* F. commune *pathogenicity assays*

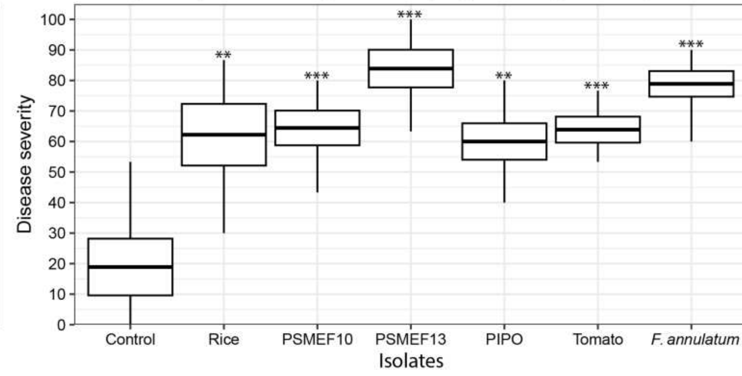
All the tested *Fusarium commune* and *F. annulatum* isolates were found to be pathogenic across the hosts that were surveyed. Based on the mixed effect model, the random effect of the two separate experiments was not significant for the ponderosa pine (p=0.821), loblolly pine (p=0.118), and tomato (p=0.226) seedlings. However, the random effect of the two experiments

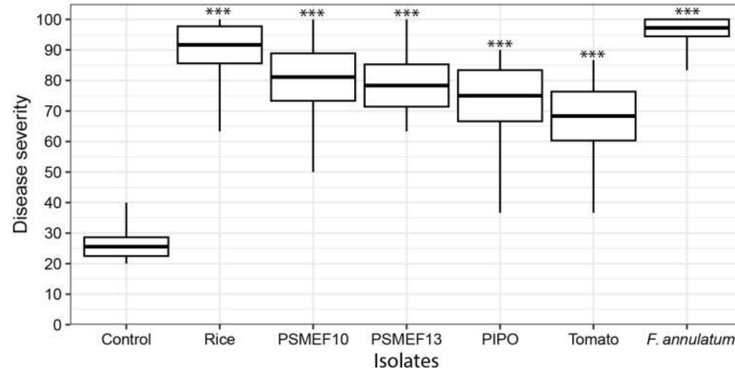
was significant for the rice (p=0.009) and Douglas-fir (p=0.002) seedlings. This explained the observed variability in disease severity of isolates across experiments in the rice collected isolate (B62) and one of the Douglas-fir collected isolates (F13) (Figure 4.2). All isolates caused significantly more disease compared to the uninoculated controls. When comparing isolates to each other, the *F. commune* collected from Douglas-fir (F13) and rice caused significantly more disease compared to the *F. annulatum* isolate on ponderosa pine seedlings. On Douglas-fir seedlings, the *F. commune* collected from tomato caused significantly less disease compared to the *F. annulatum* isolate. The *F. commune* from ponderosa pine caused significantly less disease on rice seedlings compared to the *F. annulatum* isolate.




<u>Significance compared to control</u>

*** p< 0.001
** p< 0.01
* p< 0.05

Figure 4.2 – Pathogenicity results of *Fusarium commune* isolates collected from Douglas-fir (*Pseudotsuga menziesii*; PSME), ponderosa pine (*Pinus ponderosa*; PIPO), rice (*Oryza sativa*), and tomato (*Solanum lycopersium*) seedlings and one *F. annulatum* isolate collected from southwest white pine (*Pinus strobiformis*). Each isolate was assessed for virulence based on disease severity on conifer (A: ponderosa pine, B: loblolly pine, & C: Douglas-fir) and non-conifer (D: rice & E: tomato) hosts. All isolates were found to cause significant disease compared to the uninoculated control.

*Transcriptome assembly*

Illumina sequencing of RNA samples produced an average of 59,665,694 reads (Table 4.1A). Alignment of reads mapping to the *F. annulatum* reference genome resulted in 50-51% of reads in *in vitro* samples, 0.31-0.40% in 12 hpi samples, 0.50-0.88% in 24 hpi samples, and 0.89-1.51% in 48 hpi samples. Alignment of reads mapping to the *F. commune* reference resulted in 50-52% of reads in *in vitro* samples, 0.34-0.71% in 12 hpi samples, 0.36-0.69% in 24 hpi samples, and 0.79-4.07% in 48 hpi samples. The combined *F. annulatum* transcriptome assemblies generated 11,303 transcripts with a mean length of 2,629 bases. The *Fusarium commune* transcriptome assembly generated 10,124 transcripts with a mean length of 2,340. The transcriptomes were of good quality based on high BUSCO completeness scores of the ascomycota_odb10 (92.4% in *F. annulatum* and 88.7% in *F. commune*) and sordariomycetes_odb10 (90.6% in *F. annulatum* and 88.3% in *F. commune*). A principal components analysis (PCA) was used to determine inter- and intragroup variability of biological replicates from *in vitro* and *in planta* conditions (Figure 4.3). 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.

Table 4.1A - Illumina raw reads and read quality at sequencing of RNA extracted from plants inoculated with either *Fusarium annulatum* or *F. commune* isolates and from mycelium of cultures grown in potato dextrose broth and the total assembled transcripts from the three replicates of each isolate and condition.

| Sample | *Fusarium* sp. | Replicate | HPI | Tissue | Raw reads | Effective (%) | Error (%) | Q20 (%) | Q30 (%) | GC (%) | Reads alignment (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| NR112H | *F. annulatum* | 1 | 12 | *in planta* | 60843106 | 98.28 | 0.03 | 96.00 | 89.56 | 44.57 | 0.40 |
| NR212H | *F. annulatum* | 2 | 12 | *in planta* | 59929554 | 98.22 | 0.03 | 96.20 | 89.90 | 44.67 | 0.36 |
| NR312H | *F. annulatum* | 3 | 12 | *in planta* | 57165924 | 97.82 | 0.03 | 96.54 | 90.56 | 44.52 | 0.31 |
| NR124H | *F. annulatum* | 1 | 24 | *in planta* | 59488296 | 96.79 | 0.03 | 96.47 | 90.42 | 44.43 | 0.64 |
| NR224H | *F. annulatum* | 2 | 24 | *in planta* | 63728398 | 96.60 | 0.03 | 96.42 | 90.43 | 44.44 | 0.50 |
| NR324H | *F. annulatum* | 3 | 24 | *in planta* | 55579268 | 97.58 | 0.03 | 96.17 | 89.83 | 44.35 | 0.88 |
| NR148H | *F. annulatum* | 1 | 48 | *in planta* | 59205094 | 97.71 | 0.03 | 96.49 | 90.53 | 44.66 | 0.99 |
| NR248H | *F. annulatum* | 2 | 48 | *in planta* | 59511462 | 97.28 | 0.03 | 95.76 | 89.05 | 44.60 | 1.51 |
| NR348H | *F. annulatum* | 3 | 48 | *in planta* | 59405932 | 97.84 | 0.03 | 96.41 | 90.42 | 44.33 | 0.89 |
| NAUR1M | *F. annulatum* | 1 | NA | *in vivo* | 63111046 | 97.35 | 0.03 | 96.55 | 90.98 | 52.25 | 50.62 |
| NAUR2M | *F. annulatum* | 2 | NA | *in vivo* | 59226918 | 97.52 | 0.03 | 96.30 | 90.30 | 51.62 | 50.63 |
| NAUR3M | *F. annulatum* | 3 | NA | *in vivo* | 62681926 | 97.94 | 0.03 | 96.30 | 90.21 | 51.70 | 51.64 |
| JR112H | *F. commune* | 1 | 12 | *in planta* | 68425386 | 94.53 | 0.03 | 96.86 | 91.34 | 43.35 | 0.45 |
| JR212H | *F. commune* | 2 | 12 | *in planta* | 56661490 | 98.18 | 0.03 | 96.05 | 89.62 | 44.37 | 0.34 |
| JR312H | *F. commune* | 3 | 12 | *in planta* | 59429582 | 98.32 | 0.03 | 95.28 | 88.11 | 44.45 | 0.71 |
| JR124H | *F. commune* | 1 | 24 | *in planta* | 57740852 | 98.44 | 0.03 | 95.97 | 89.43 | 44.67 | 0.36 |
| JR224H | *F. commune* | 2 | 24 | *in planta* | 58578480 | 97.84 | 0.03 | 95.96 | 89.45 | 44.36 | 0.54 |
| JR324H | *F. commune* | 3 | 24 | *in planta* | 64899002 | 97.08 | 0.03 | 96.74 | 91.05 | 44.44 | 0.69 |
| JR148H | *F. commune* | 1 | 48 | *in planta* | 58991634 | 98.05 | 0.03 | 96.15 | 89.75 | 44.57 | 0.84 |
| JR248H | *F. commune* | 2 | 48 | *in planta* | 55446480 | 97.43 | 0.03 | 96.35 | 90.17 | 44.76 | 0.79 |
| JR348H | *F. commune* | 3 | 48 | *in planta* | 53251758 | 97.60 | 0.03 | 96.06 | 89.56 | 45.10 | 4.07 |
| JicR1M | *F. commune* | 1 | NA | *in vivo* | 62130642 | 98.28 | 0.03 | 96.75 | 91.16 | 50.96 | 50.88 |
| JicR2M | *F. commune* | 2 | NA | *in vivo* | 52745324 | 96.79 | 0.03 | 95.89 | 89.55 | 51.13 | 52.92 |
| JicR3M | *F. commune* | 3 | NA | *in vivo* | 63799120 | 97.50 | 0.03 | 96.77 | 91.36 | 51.04 | 52.28 |

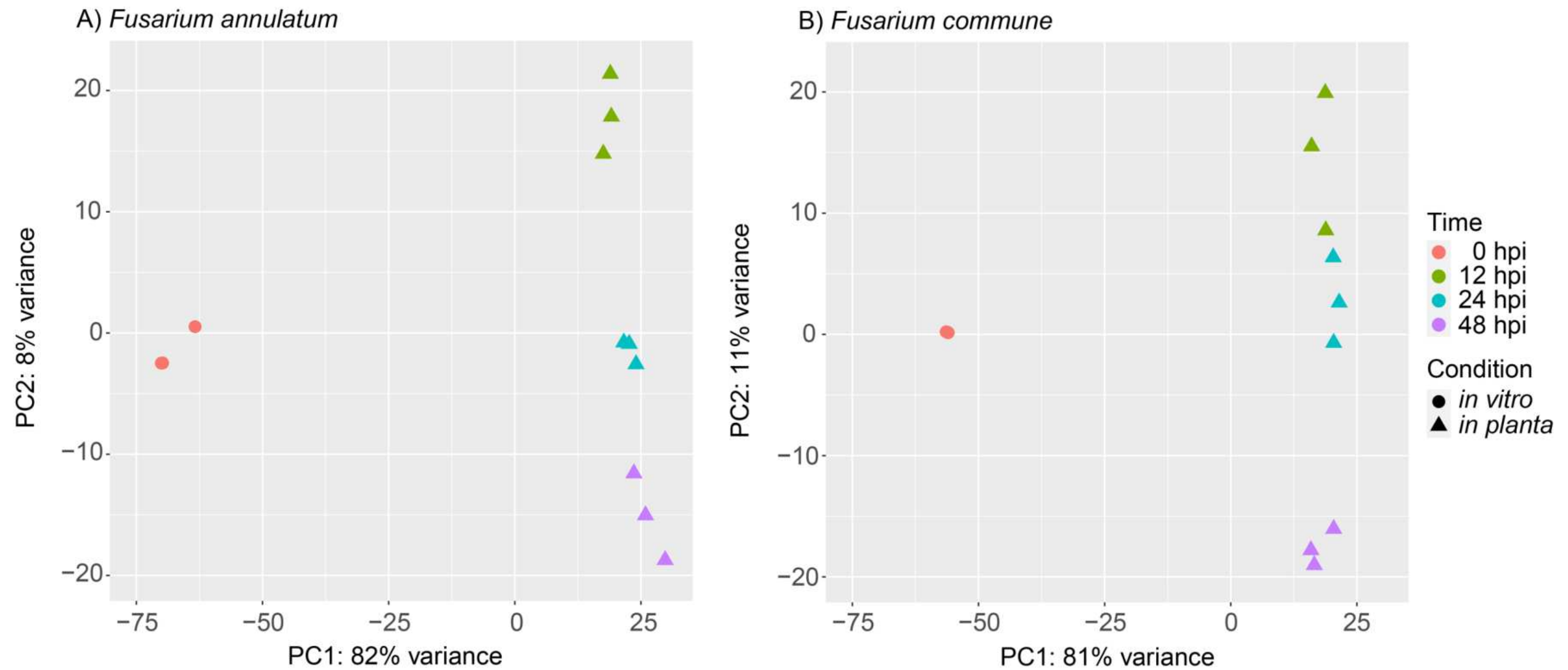


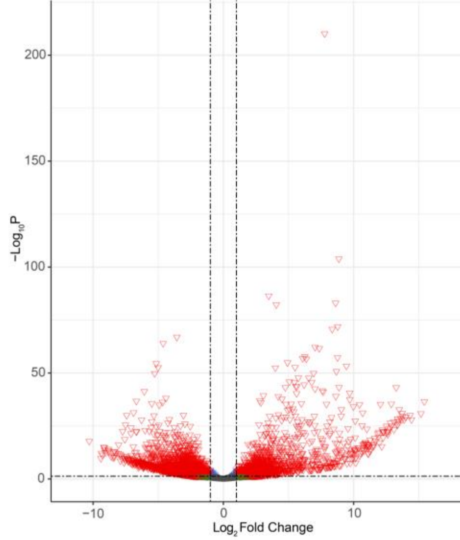

Figure 4.3 – Principal components analyses of three replicate samples of *Fusarium annulatum* (A) and *F. commune* (B) inoculated loblolly pine (*Pinus taeda*) plants (*in planta*) compared to mycelium of each isolate grown in potato dextrose broth (*in vitro*). Isolates were collected at three time points post-inoculation collected at 12 hours (green), 24 hours (blue), and 48 hours (purple).

*Differential gene expression and putative function*

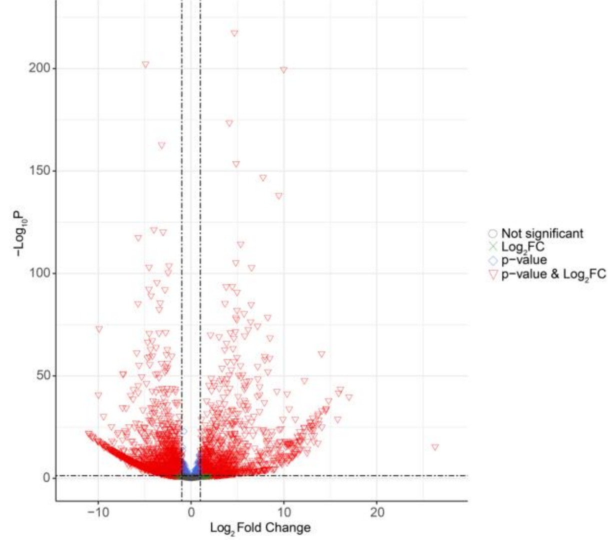
Our analyses focused on differential expression (DE) *in planta* to identify pathogenicity/virulence related genes associated with conifer pathogenesis. DE analyses between *in planta* and *in vitro* samples generated 2,835 and 2,415 upregulated transcripts *in planta* (UIP) in the *F. annulatum* and *F. commune* isolates respectively, with most upregulated genes expressed at 48 hours post inoculation (hpi) (Figure 4.4, Table 4.1B). Putative functions were described for an average of 71.5% transcripts in the *F. annulatum* transcriptome and 72.5% in the *F. commune* transcriptome (Dobbs 2024b; Dobbs 2024c). Pfam and KEGG Kofam described the functions of most of the UIP genes in both species (Figure 4.5). Among the putative pathogenicity/virulence associated genes of both species, functions were described as transporters, signaling pathways, effectors, transcription, chitin synthases, CAZymes, and genes involved in secondary metabolism including genes involved in tricothecene and deoxynivelanol biosynthesis. Interestingly, genes involved in jasmonic-acid pathway (PHI: 4909) were expressed in *F. annulatum* at all three time points.

Table 4.1B - Transcripts from plants inoculated with either *Fusarium annulatum* or *F. commune* isolates compared with 28,171 or 24,470 genes, respectively, extracted from mycelium of cultures grown in potato dextrose broth to identify differentially expressed (DE) genes upregulated *in planta* (UIP) or downregulated *in planta* (DIP).

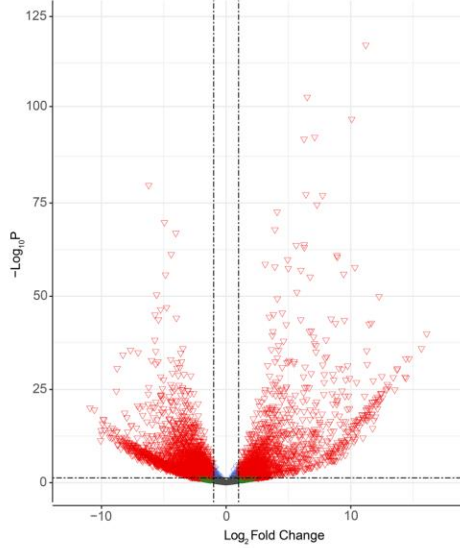
| Isolate | *Fusarium* sp. | Time | Total DE | UIP | DIP | UIP Described | UIP Not Described | Percent Described | DIP Described | DIP Not Described | Percent Described |
|---|---|---|---|---|---|---|---|---|---|---|---|
| NASWWP3 | *F. annulatum* | 12H | 4113 | 1649 | 2464 | 803 | 846 | 48.70% | 945 | 1519 | 38.35% |
| NASWWP3 | *F. annulatum* | 24H | 4625 | 1832 | 2793 | 947 | 885 | 51.69% | 1020 | 1773 | 36.52% |
| NASWWP3 | *F. annulatum* | 48H | 5066 | 2228 | 2838 | 1082 | 1146 | 48.56% | 1062 | 1776 | 37.42% |
| JICPIPO1 | *F. commune* | 12H | 3753 | 1320 | 2433 | 663 | 657 | 50.23% | 816 | 1617 | 33.54% |
| JICPIPO1 | *F. commune* | 24H | 3767 | 1399 | 2368 | 717 | 682 | 51.25% | 836 | 1532 | 35.30% |
| JICPIPO1 | *F. commune* | 48H | 4222 | 1865 | 2357 | 957 | 908 | 51.31% | 772 | 1585 | 32.75% |



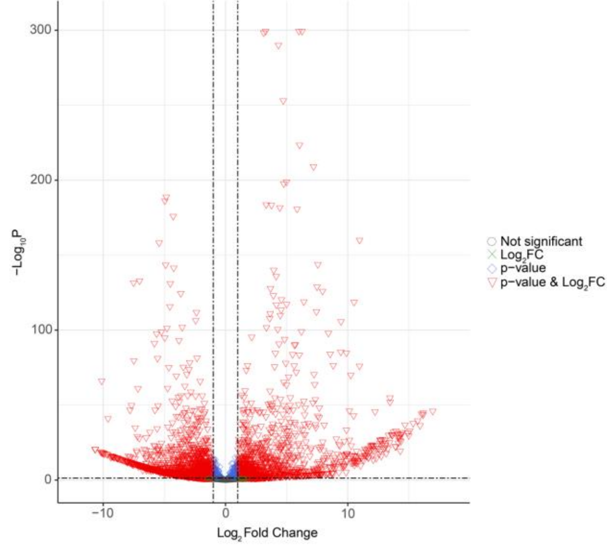
A) F. annulatum 12 hpi in planta vs in vitro
$-Log_{10}P$
$Log_2$ Fold Change

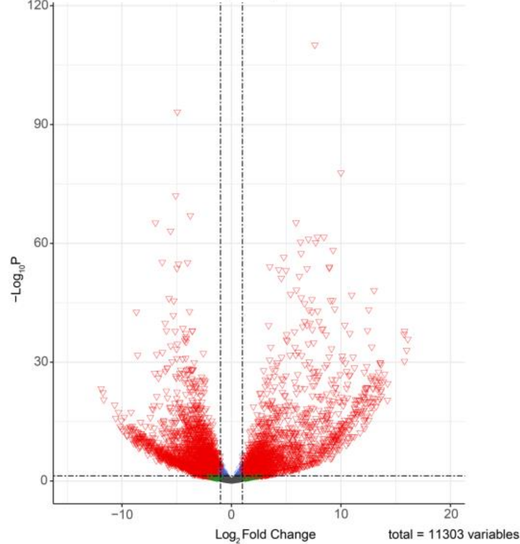
D) F. commune 12 hpi in planta vs in vitro
$-Log_{10}P$
$Log_2$ Fold Change
Not significant
$Log_2FC$
p−value
p−value & $Log_2FC$

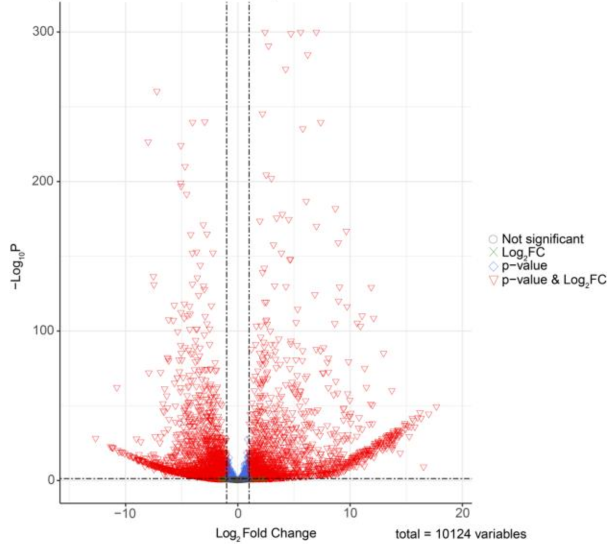
B) F. annulatum 24 hpi in planta vs in vitro
$-Log_{10}P$
$Log_2$ Fold Change

E) F. commune 24 hpi in planta vs in vitro
$-Log_{10}P$
$Log_2$ Fold Change
Not significant
$Log_2FC$
p−value
p−value & $Log_2FC$

C) F. annulatum 48 hpi in planta vs in vitro
$-Log_{10}P$
$Log_2$ Fold Change
total = 11303 variables

F) F. commune 48 hpi in planta vs in vitro
$-Log_{10}P$
$Log_2$ Fold Change
total = 10124 variables
Not significant
$Log_2FC$
p−value
p−value & $Log_2FC$

Figure 4.4 – Volcano plots of *Fusarium annulatum* (A, B, & C) and *F. commune* (D, E, & F) inoculated loblolly pine (*Pinus taeda*) plants (*in planta*) compared to 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 a p-value < 0.05. Isolates were compared at three time points post-inoculation (hpi) collected at 12 hours (A & D), 24 hours (B & E), and 48 hours (C & F). Upregulated transcripts in plant are shown on the right of each plot and downregulated on the left.

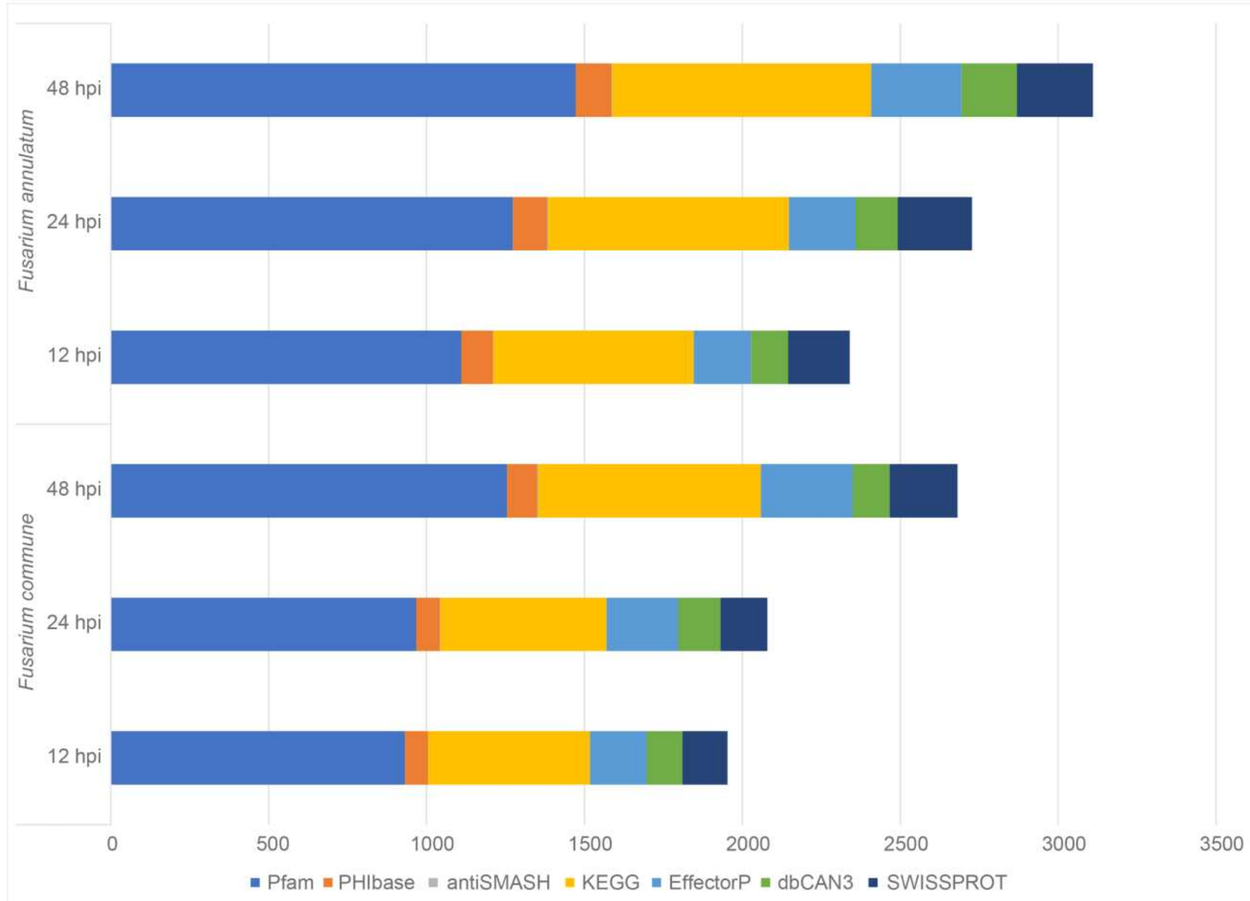



Figure 4.5 – Described upregulated transcripts of *Fusarium annulatum* (top) and *F. commune* (bottom) inoculated loblolly pine (*Pinus taeda*) plants (UIP) compared to mycelium of each isolate grown in potato dextrose broth. Isolates were collected at 12 hours, 24 hours, and 48 hours post-inoculation (hpi). Counts are based on number of genes described by each database.

Transcripts coding for CAZymes were more abundant *in planta* (n=191 in *F. annulatum* and n=186 in *F. commune*) when compared to *in vitro* samples (n=105 in *F. annulatum* and n=114 in *F. commune*) (Dobbs 2024b; Dobbs 2024c). Most of the extracellular CAZymes were described as involved in the degradation of cellulose, lignin, pectin, and other plant cell wall forming carbohydrates (Dobbs 2024b; Dobbs 2024c; Figure 4.6).

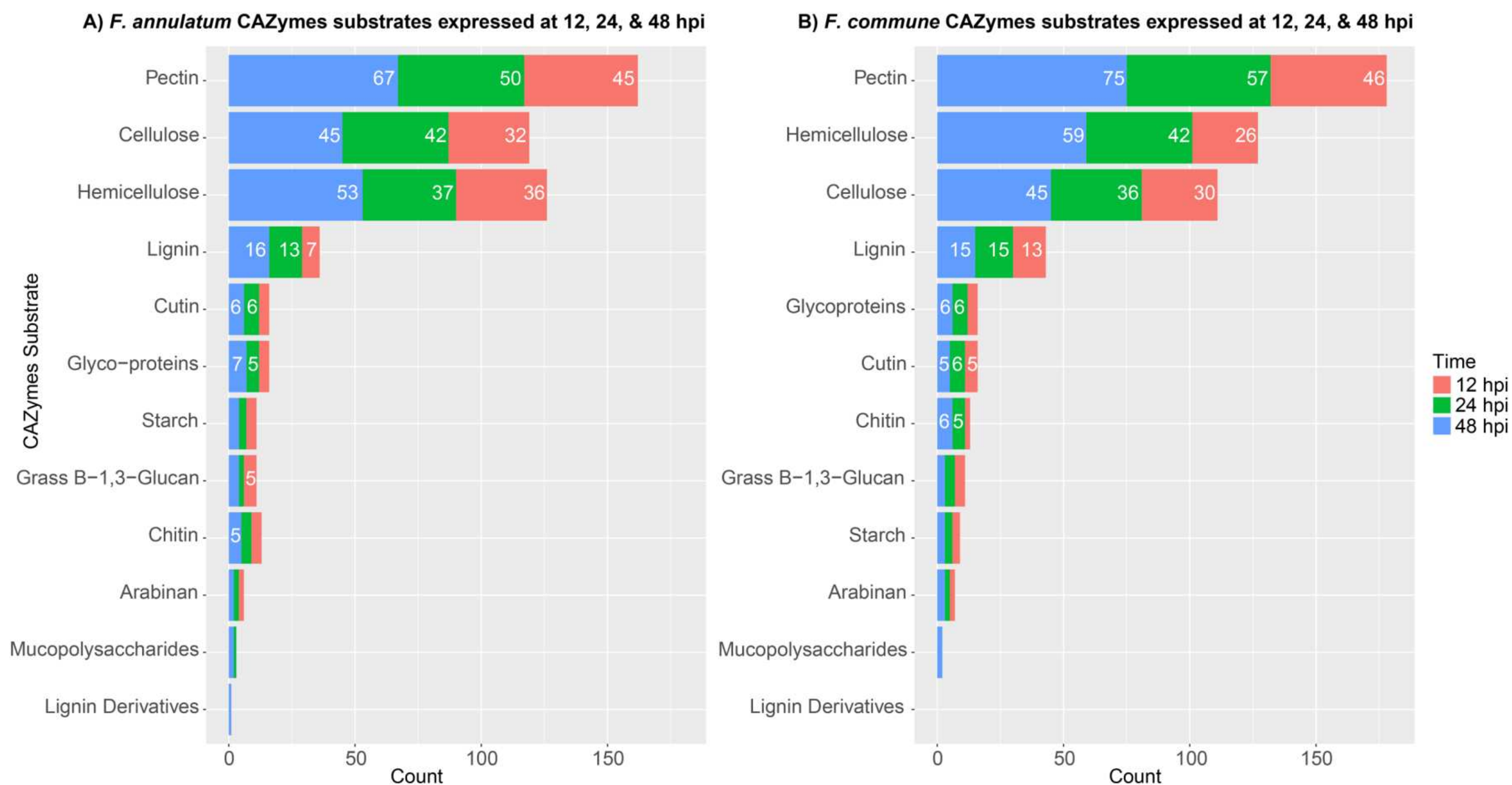



Figure 4.6 – Described carbohydrate-active enzymes in upregulated transcripts of *Fusarium annulatum* (A) and *F. commune* (B) inoculated loblolly pine (*Pinus taeda*) plants (UIP) compared to mycelium of each isolate grown in potato dextrose broth. Isolates were collected at 12 hours, 24 hours, and 48 hours post-inoculation (hpi). Counts are based on number of genes described at each time point. Only counts $\geq$ 5 genes were numbered on the graph.

Among the UIP transcripts, SWISS-PROT-described genes were involved in sesquiterpene biosynthesis, aurofusarin pigment biosynthesis, and aromatic compound metabolism (Dobbs 2024b; Dobbs 2024c). Genes involved in secondary metabolism biosynthesis differed between species. In *F. annulatum*, these included biosynthesis of aspyridones and bikaverin while five genes involved in biosynthesis of fusaoctaxin A were described in *F. commune* (Dobbs 2024b; Dobbs 2024c). Gene clusters described by antiSMASH involved in the biosynthesis of prolipyrone B/gibepyrone D were identified in the UIP transcripts in *F. annulatum*. In *F. commune*, gene clusters involved in biosynthesis of secondary metabolites were not identified in the UIP genes. However, downregulated genes involved in the biosynthesis of enniatin were identified in both species. In *F. annulatum*, genes not significantly regulated up or down in plant 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 were found to be involved in metabolic processes and biosynthesis of secondary metabolites (Figure 4.7).

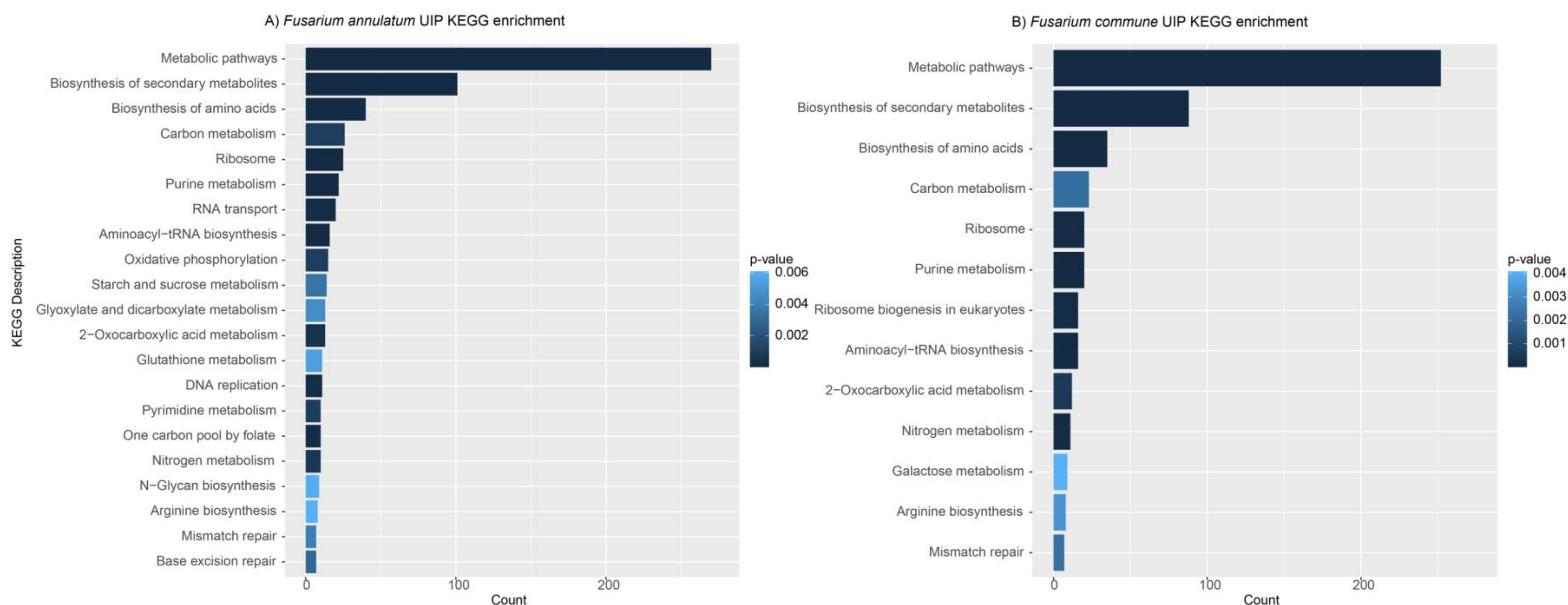



Figure 4.7 – KEGG enrichment of significantly upregulated genes of *Fusarium annulatum* (A) and *F. commune* (B) inoculated loblolly pine (*Pinus taeda*) plants (UIP) compared to mycelium of each isolate grown in potato dextrose broth. In both species, significantly UIP genes were involved in metabolic pathways and biosynthesis of secondary metabolites.

*Orthologous protein clusters and GOterm 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 (Figure 4.8). No GO enrichment was identified at 12 hpi. At 24 hpi, GO enrichment was found to be involved in mitochondrial electron transport, ubiquinol to cytochrome c. At 48 hpi, GOterms described as involved in carbohydrate metabolic process were described. *F. commune* did not have any described unique othrogroups at 12, 24, or 48 hpi (Figure 4.8). Unique orthogroup protein clusters were identified as 481 and 462 in *F. annulatum* and *F. commune*, respectively. Among the *F. annulatum* unique orthogroups, GOterms were enriched in transport, oxidoreductase activity, aromatic compound catabolic process, terpenoid biosynthesis process, signal transduction, sterigmatocystin biosynthesis, and melanin biosynthesis process. In *F. commune*, GOterms enriched in transport, transcription, oxidoreductase activity, and sporulation were described. Shared among both species, GOterms were enriched in transport including toxin transport, quinate metabolic process, pathogenesis, sporulation, defense response, and signaling.

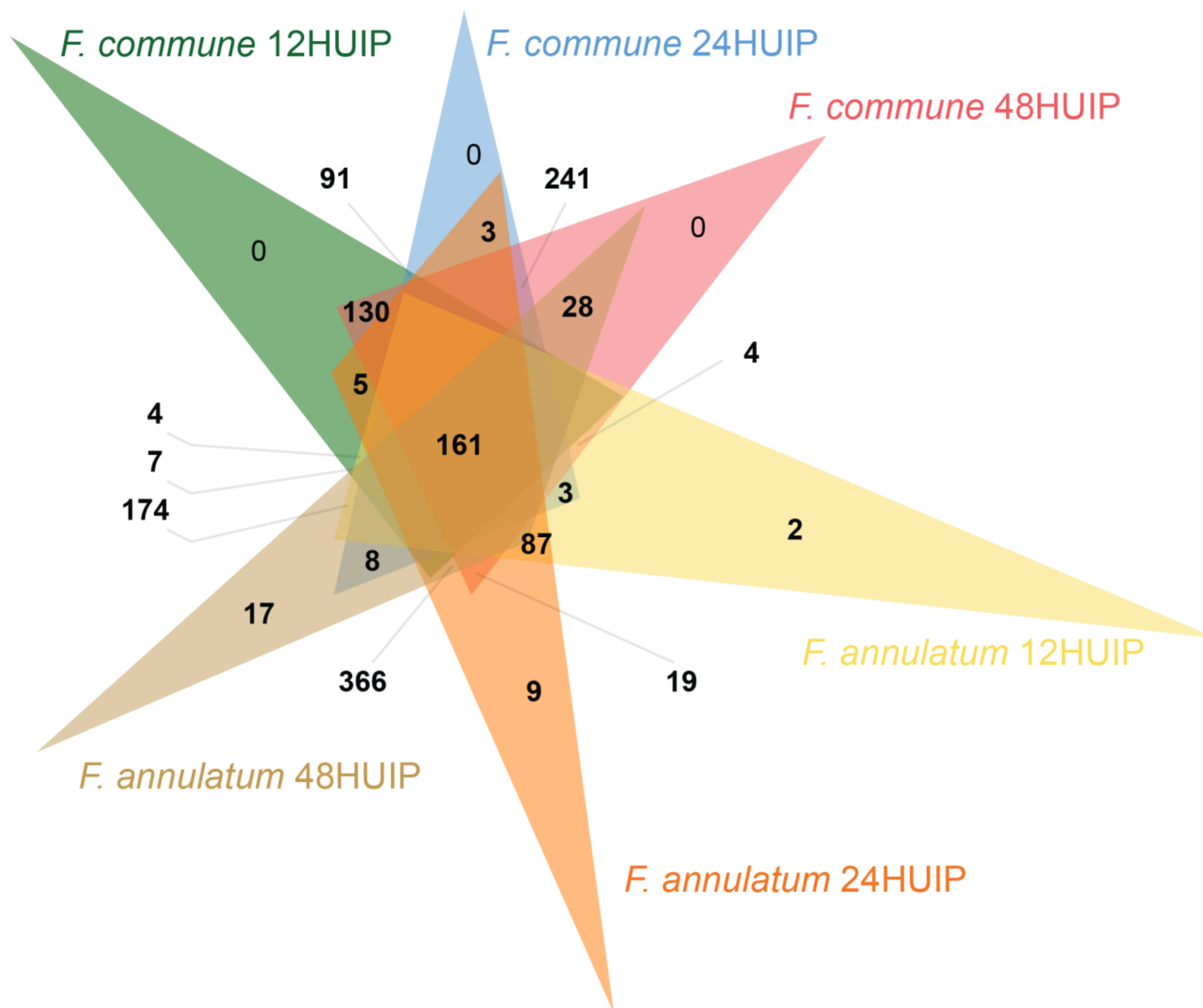



Figure 4.8 – Venn diagram of shared and unique orthologous protein clusters of largest open reading frames of upregulated transcripts of *Fusarium annulatum* (top) and *F. commune* (bottom) inoculated loblolly pine (*Pinus taeda*) plants (UIP) compared to mycelium of each isolate grown in potato dextrose broth. Isolates were collected at 12-, 24-, and 48-hours post-inoculation (hpi).

*Secreted UIP genes*

Among the differentially expressed transcripts *in planta*, in *F. annulatum*, we found 317 secreted extracellular proteins consisting of 57 apoplastic effectors, 14 apoplastic/cytoplasmic effectors, and 30 cytoplasmic effectors (Dobbs 2024b). Most of the secreted proteins (n=286) and effectors (n=93) were expressed at 48 hpi (Dobbs 2024b). We found 321 transcripts coding for secreted predicted extracellular proteins, including 70 apoplastic effectors, 19 apoplastic/cytoplasmic effectors, and 22 cytoplasmic effectors in *F. commune* (Dobbs 2024c). Similar to *F. annulatum*, most of the secreted proteins (n=291) and effectors (n=102) were expressed at 48 hpi (Dobbs 2024c). 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 hpi to 48 hpi in both species (Figure 4.6). Interestingly in *F. annulatum*, one secreted protein involved in secondary metabolism was described as part of the aurofusarin biosynthesis cluster and involved in pigment biosynthesis and another was found 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 significantly upregulated genes *in planta* shared in both *F. annulatum* (n=1010 genes in 552 orthogroups) and *F. commune* (n=883 genes in 529 orthogroups) with the 19 conifer-derived and four non-conifer-derived *Fusarium* spp. genomes from a previous survey on conifer seedlings (Dobbs et al 2023a; Chapter 3), the UIP profiles

were similar among *Fusarium* species complexes (Figure 4.9A). Even when comparing only secreted proteins (Figure 4.9B) and pathogenicity/virulence-associated genes (Figure 4.9C), shared profiles were found within *Fusarium* species complexes. The closely related species complexes ordinated closer together, and in particular, the *Fusarium nisikadoi*, *F. oxysporum*, and *F. fujikuroi* species complexes.

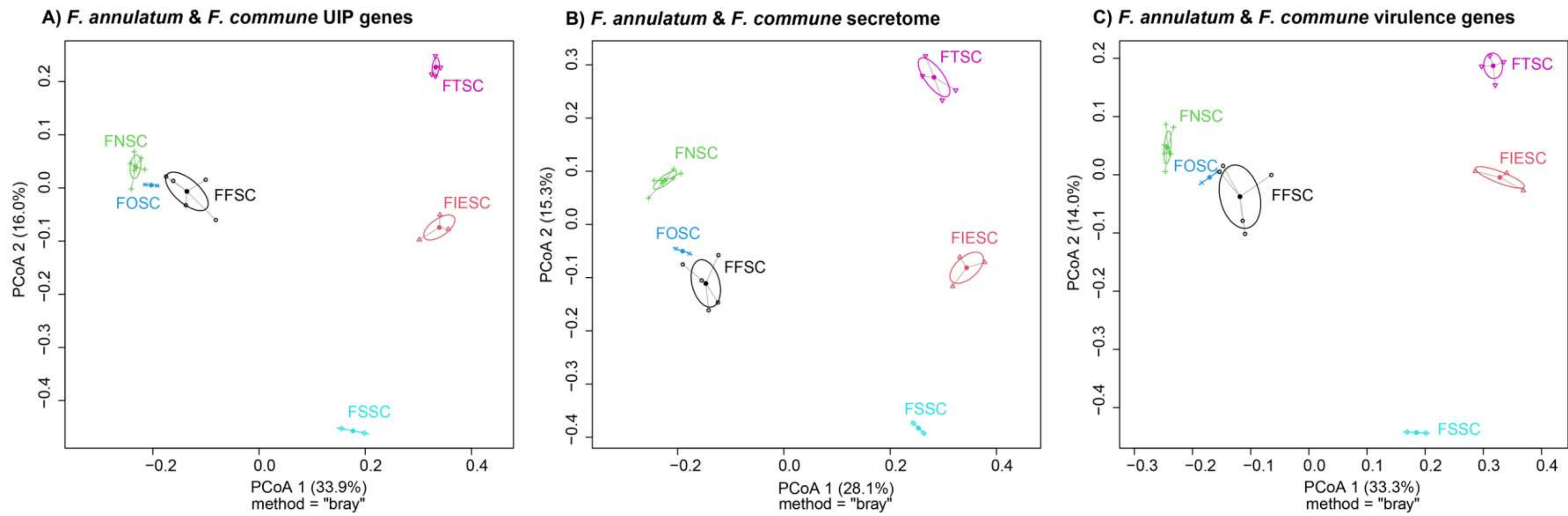
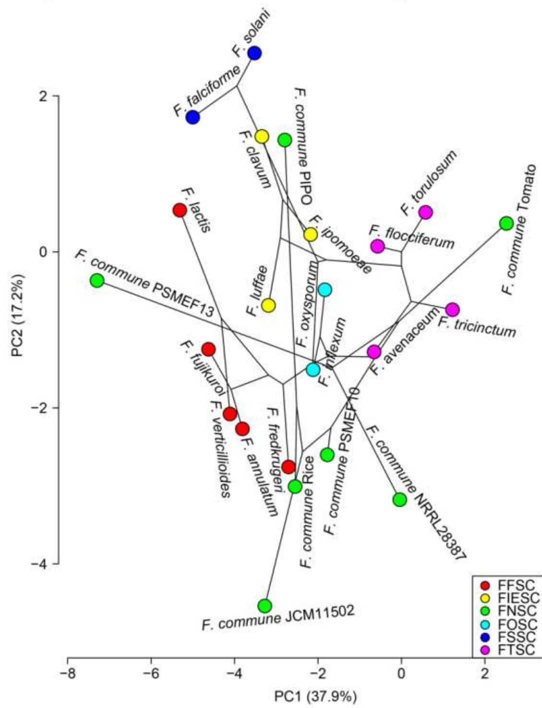
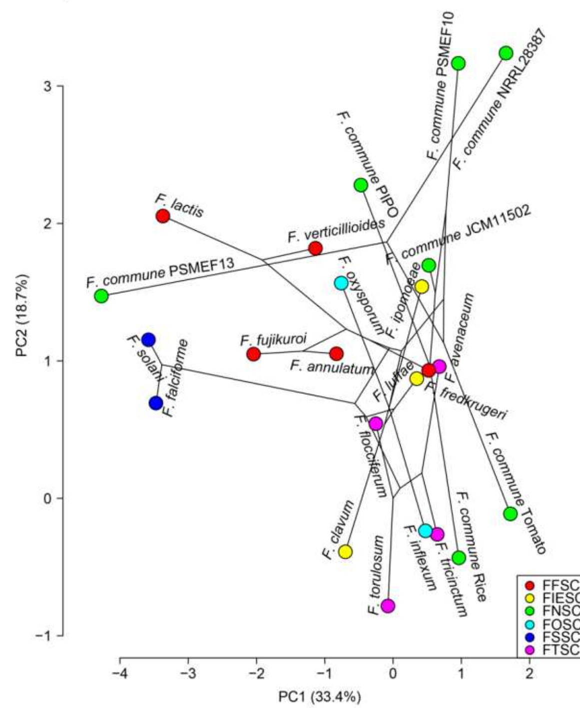



Figure 4.9 – Distribution of significantly upregulated genes identified in *Fusarium annulatum* and *F. commune* inoculated loblolly pine (*Pinus taeda*) plants (UIP) among *Fusarium* spp. isolates collected from conifer hosts. Principal coordinates analysis of UIP show similarity of UIP (A), secretome (B), and virulence gene (C) profiles among species complexes.

The trends observed in the PCoA analyses were similar with our UIP PhylPCA analyses, whereby *Fusarium* species complexes mostly clustered together (Figure 4.10). However, the *F. commune* isolates did not cluster as well as the other species complexes. The *F. commune* isolate collected from ponderosa pine and one of the isolates collected from Douglas-fir were consistently different from the other *F. commune* isolates in total UIP (Figure 4.10A), secretome (Figure 4.10B), and pathogenicity/virulence gene (Figure 4.10C) profiles.



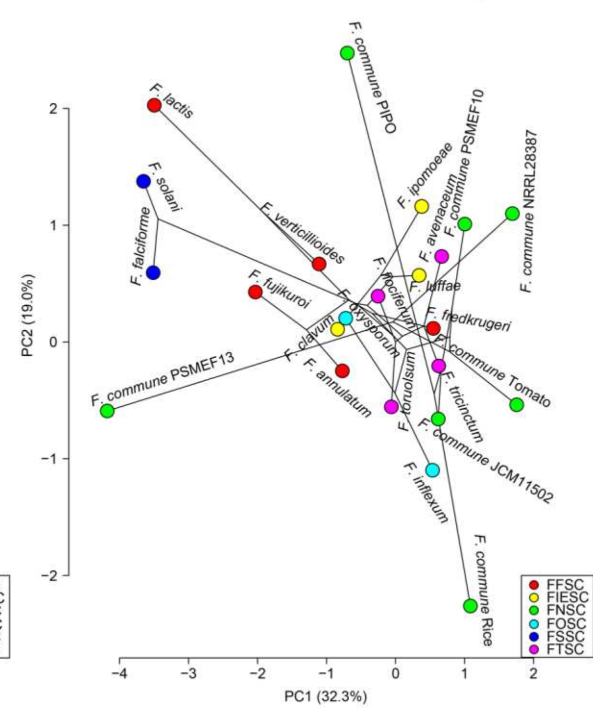




Figure 4.10 – Distribution of secreted upregulated transcripts identified in *Fusarium annulatum* (A) and *F. commune* (B) inoculated loblolly pine (*Pinus taeda*) plants (UIP) among *Fusarium* spp. isolates collected from conifer hosts. Phylogenetic principal components analysis of UIP (A), secreted proteins (B), and virulence genes (C) show similarity of *Fusarium* species complexes profiles but *F. commune* isolates (green) did not cluster as well as other species complexes.

When investigating the putative function of the *F. annulatum* and *F. commune* UIP gene orthogroup profiles, 101 and 93 orthologous genes, respectively, were found shared among species complexes. Most genes identified in the profiles were expressed at 48 hpi. FFSC had the most orthologous genes shared among members with both the *F. annulatum* (n=657) and *F. commune* (n=539) transcriptome profiles (Table 4.2). Interestingly, all the species complexes contained orthogroups found in the *F. annulatum* transcriptome profile that were involved in biosynthesis of prolipyrone B/gibepyrone D, whereas orthogroups involved in patulin biosynthesis were found in the *F. commune* transcriptome profile. No gene clusters from the antiSMASH 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 FNSC profiles based on the *F. annulatum* transcriptome profile, more effectors were identified in the *F. commune* transcriptome profile. Though many genes were found shared within species complexes, differences among members of each species complex were observed.

Within the FNSC, putative gene functions included chitin synthases, transcription factors, transporters, deoxynivelanol (DON) biosynthesis, aurofusarin biosynthesis, patulin biosynthesis, cytochrome P450, fungal trichothecene efflux pumps, and CAZymes involved in mostly pectin, cellulose, and hemicellulose degradation. These genes were identified in all of the *F. commune* isolates. However, the ponderosa pine *F. commune* isolate had more MFS transporter genes (sugar transporters) than all the other isolates and the DON biosynthesis was only found in the Douglas-fir isolate (F10) and the two tomato isolates.

In the FFSC, putative gene functions included transcription factors, patulin biosynthesis, and MFS transporters among all isolates. However, a necrosis inducing protein and a transcription factor were found only in *F. annulatum*. While a pathogenicity/virulence-related

effector was only in *F. verticillioides*. A transcription factor was only found in *F. fredkrugeri*. A transcription factor and DON biosynthesis gene were only found in *F. lactis*, while a TAG lipase was only identified in *F. fujikuroi*. A glycoside hydrolase and exopolygalacturonase were only found in *F. fredkrugeri*, while the aurofusarin biosynthesis gene was only found in *F. lactis* and *F. verticillioides*.

In the FOSC, putative gene functions included MFS transporters, transcription factors, aurofusarin biosynthesis, exopolygalacturonase, and chitin synthases were identified in both species. A myosin and TAG lipase genes were only identified in *F. inflexum*.

In the FTSC, putative gene functions included transcription factors, exopolygalacturonase, and patulin biosynthesis genes identified in all isolates, while chitin synthase, necrosis inducing, and ubiquitin fusion genes were only identified in *F. avenaceum* and *F. tricinctum*. A MFS transporter gene was found in all isolates but *F. tricinctum*, while a TAG lipase gene was found only in *F. avenaceum*. DON biosynthesis genes were found in all but *F. flocciferum*. An aurofusarin biosynthesis gene was found in *F. torulosum* and *F. avenaceum*. One unique transcription factor was found in *F. tricinctum* and one transcription factor was found in *F. flocciferum* and *F. torulosum*.

In the FIESC profiles, putative gene functions included aurofusarin biosynthesis, patulin biosynthesis, transcription factor, DON biosynthesis and MFS transporters in all isolates, while a chitin synthase and leucine biosynthesis genes were found only in *F. ipomoeae*. A transcription factor was found unique to *F. luffae*. A fusaoctaxin A (cytochrome P450) gene was only found in *F. clavum*. A transcription factor and DON biosynthesis genes were found in *F. clavum* and *F. ipomoeae*. While an exopolygalacturonase was found only in *F. luffae*.

In the FSSC profiles, putative gene functions included MFS transporters, effectors, triacetylfusarine C/sideropore biosynthesis, transcription factors, chitin synthase, patulin biosynthesis, TAG lipase, and exopolygalacturonases in all isolates. A chitosanase was only found in *F. solani*. An exopolygalacturonase gene and a described extracellular protein from the PHI database were only found in *F. solani*, while a glycoside hydrolase was only identified in *F. falciforme*.

Table 4.2 - profiles of up-regulated *in planta* genes among species complexes based on either the *Fusarium annulatum* or *F. commune* transcriptomes described by databases of fungal virulence genes (PHI), secondary metabolite gene clusters (antiSMASH), and protein families (Kofam, SWISS-Prot, & Pfam).

| | All | FNSC | FOSC | FFSC | FTSC | FIESC | FSSC |
|---|---|---|---|---|---|---|---|
| *F. annulatum* 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 | Prolipyrone B/ Gibepyrone D | Prolipyrone B/ Gibepyrone D | Prolipyrone B/ Gibepyrone D | Prolipyrone B/ Gibepyrone D | Prolipyrone B/ Gibepyrone D | Prolipyrone B/ Gibepyrone D | Prolipyrone B/ Gibepyrone D |
| 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 |
| *F. commune* 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 |
| 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 |

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

Among the *Fusarium* species complexes, putative pathogenicity/virulence genes were shown to be unique among species complexes but they had similar described functions including CAZymes, effectors, major facilitator supergroup (MFS) transporters, and secondary metabolite biosynthesis genes.

The *F. annulatum* transcriptome profile identified 21 (FNSC), 16 (FOSC), 88 (FFSC), 54 (FTSC), 82 (FIESC), and 80 (FSSC) unique genes within species complexes. All species complexes had unique secreted proteins mostly described as CAZymes. CAZymes in the FOSC were found to be involved in the degradation of cellulose, lignin, and pectin. CAZymes in the FFSC were found to be involved in the degradation of cellulose, grass B-1,3 glucan, hemicellulose, pectin, and starch. CAZymes in the FTSC were found to be involved in the degradation of lignin and pectin. CAZymes in the FIESC were found to be involved in the degradation of cellulose, pectin, lignin, glycoproteins, chitin, and mucopolysaccharides. CAZymes in the FSSC were found to be involved in the degradation of pectin, hemicellulose, and lignin derivates. Unique apoplastic effectors were identified in FFSC, FTSC, and FSSC, while unique cytoplasmic effectors were identified in the FNSC and FSSC. Secondary metabolism like cytochrome P450s, oxidoreductases, and monooxygenases were found among the species complexes. Unique mycotoxin biosynthesis genes were found in the FOSC (sesquiterpene biosynthesis), FTSC (choline biosynthesis), and FIESC (DON and aflatoxin biosynthesis). Most of the PHIbase described genes were identified in the FFSC (n=8), followed by the FSSC (n=7), FIESC (n=6), FTSC (n=2), FOSC (n=1), and FNSC (n=0), respectively. The *F. commune* transcriptome profile identified 30 (FNSC), 14 (FOSC), 81 (FFSC), 57 (FTSC), 56 (FIESC), and 69 (FSSC) unique genes within species complexes. All species complexes had

unique secreted proteins mostly described as CAZymes. CAZymes in the FNSC were found to be involved in the degradation of pectin. CAZymes in the FFSC were found to be involved in the degradation of cellulose, glycoproteins, hemicellulose, and pectin. CAZymes in the FTSC were found to be involved in the degradation of lignin and cellulose. CAZymes in the FIESC were found to be involved in the degradation of cutin, hemicellulose, and pectin. Unique apoplastic effectors were identified in FNSC, FFSC, FTSC, FIESC, and FSSC, while unique cytoplasmic effectors were identified in the FOSC, FTSC, FIESC, and FSSC. Though all the species complexes had unique transporters and/or transcription factors, most of the species complexes did not have identified unique genes involved in secondary metabolism. However, unique cytochrome P450s and monooxygenases were found in the FIESC and FSSC. Unique mycotoxin biosynthesis genes were only found in the FIESC (fusaoctoxin biosynthesis). Most of the PHIbase described genes were identified in the FTSC (n=5), followed by the FIESC (n=4), FFSC (n=3), FSSC (n=1), FOSC (n=0), and FNSC (n=0), respectively.

**Discussion**

In forest tree nurseries there are numerous fungal pathogens of concern to managers that can cause devastating losses to their conifer seedlings. *Fusarium* spp., especially new and emerging plant pathogenic species, are a major concern because they are frequently isolated from symptomatic conifer seedlings and many of these *Pinaceae*-associated species are understudied and/or have unknown virulence and host range (Karunnarathna et al. 2021; Stewart et al. 2019). Genomics and transcriptomics are valuable tools to understand the host-pathogen interactions during host pathogenesis. In this study, we generated reference-guided transcriptomes of two understudied *Fusarium* spp., *F. annulatum* and *F. commune*, that we confirmed as pathogens to both conifer and non-conifer hosts. We expected to see host-specificity among *F. commune*

isolates based on the hosts they were collected from and therefore host-specific pathogenicity/virulence factors. Similarly, we expected to see conifer host-specificity of the *F. annulatum* isolate. However, we found that all isolates tested were pathogenic to the hosts assayed. We did find some unique pathogenicity/virulence pathways utilized by the *F. commune* isolate when compared to *F. annulatum*, thereby identifying some potential gene targets necessary for conifer pathogenesis among the transcriptomes predicted secreted proteins. We also identified mycotoxin biosynthesis genes involved in the production of prolipyrone B/gibepyrone D in the *F. annulatum* transcriptome and patulin in the *F. commune* transcriptome that were among the upregulated genes *in planta* that highlights the importance of secondary metabolites in conifer pathogenesis. We then assessed the presence of these secreted pathogenicity/virulence genes among the predicted proteomes of *Pinaceae*-associated *Fusarium* spp. and found that these profiles were found to be associated with *Fusarium* species complexes rather than highlighting conifer versus non-conifer pathogens. Therefore, we identified conifer pathogenicity genes, however they are likely not all required for conifer pathogenesis.

*Host-specialization vs generalism*

In our previous survey *F. commune* was one of the most common *Fusarium* spp. collected from conifer seedlings across the contiguous USA (Dobbs et al. 2023a). Our findings that *F. commune* may be a generalist pathogen to seedlings of both conifer and non-conifer hosts has management implications for restoration. As restoration projects do not rely on single hosts like many timber projects or crops, generalist pathogens may pose a greater threat as they can infect the variety of hosts that may cause disease or follow seedlings into planting sites. However, even if the target restoration species are not susceptible to a *Fusarium* pathogen, it has been reported that pathogens can persist as endophytes in non-target hosts until a preferred host

is present in out-plantings (Montoya-Martinez et al. 2022). As we tested pathogenicity/virulence on Pineaceae, Poaceae, and Solonaceae plants, we showed that strains pathogenic to hosts in all three families may cause devastating impacts on many common restoration and crop species within these families that are produced in tree nurseries in the southwest (Dreesen et al. 2002). Since *F. commune* has already been reported as a pathogen to hosts within these families, it is easy to posit that strains of these pathogens, regardless of host origin, are a threat to conifer and forest restoration nurseries. This has even broader implications as many *Fusarium* spp., primarily in the *Fusarium solani* and *F. oxysporum* species complexes, have been reported as being pathogens across trans-kingdom and can be human and animal pathogens as well as plant pathogens (van Diepingen et al. 2016). It has also been reported that lifestyle changes in *Fusarium* can be transient and endophytes and phytopathogens have shared genetic compliments (Hill et al. 2022). A pathogenic lineage may be able to remain within reservoir hosts as they can persist as endophytes until conditions are favorable to switch to a pathogenic lifestyle and warrants serious consideration for management.

Like the *F. commune* isolates, the *F. annulatum* isolate caused significant disease compared to the controls on all hosts surveyed. This was expected as *F. annulatum* has been recently reported as a pathogen on non-conifer hosts, wine and table grapes (*Vitis vinifera*) and cantaloupe (*Cucumis melo* var. *cantalupensis*) (Bustamante et al. 2022; Parra et al. 2022). *F. annulatum* was also recently separated from *F. proliferatum*, which has been documented as a conifer pathogen (Stewart et al. 2016). Due to the pathogenicity shown in this study of *F. annulatum* across the hosts surveyed, this species should be further studied, and managers should be aware of the potential damage this species can cause in their nurseries.

The *F. commune* isolates caused significant disease on all hosts tested, suggesting that *F. commune* isolates may be pathogenic to hosts regardless of host they were collected from. However, due to the observed effect of gene profiles related to host origin observed in Chapter 3, the root clipping method may have allowed the pathogen an advantage that may not normally exist. By providing an infection court through wounding the roots, isolates from non-conifer hosts may have lacked genes necessary for conifer host penetration. Though the clip-n-dip method is commonly used, to gain a better understanding of an isolate's "true" virulence, a method like corn meal agar incorporated into soil, soaking roots in a $1x10^6$ spore solution without clipping them, or drenching seedlings with a $1x10^6$ spore solution should be used (Stewart et al. 2012; Lai et al. 2020).

*Conifer pathogenesis genes expressed during initial and early infection*

An emphasis in this study was made on the predicted secretome, effectome, and pathogenicity/virulence-associated genes due to the importance of secreted proteins and compounds involved in pathogenicity/virulence during host-pathogen interactions (Bradshaw et al. 2021). Many secreted proteins were described as glycoside hydrolases including one identified in both transcriptomes that was found to be involved in pathogenicity/virulence (PHI:11176). This was to be expected as glycoside hydrolases have been found important for pathogenesis in other plant pathogens (Zhang et al. 2021). Both 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). Since many species within this study are soilborne and root pathogens, this aurofusarin pigment is likely not involved in pathogenicity/virulence but rather defense from soil dwelling invertebrates. An apoplastic effector described as a hydrophobin gene was identified in the *F. commune* secretome

which has been described as involved in conidiation, pathogenicity, and secondary metabolism (Shin et al. 2022). Additionally, a previous study found that hydrophobins in *F. graminearum* had multiple redundant copies which highlights 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 142oloni* as causing cell death after colonization within a host suggesting that since it was found expressed at 24 and 48 hpi, the *F. annulatum* isolate was able to colonize the host within 48 hpi (Santhanam et al. 2013).

More secreted effectors were observed at 48 hpi in both species. Secreted effectors, e.g., secreted in xylem (SIX) effectors, have been well studied in *Fusarium oxysporum* (Jangir et al. 2021). The SIX2 gene was found in the *F. annulatum* isolate predicted proteome but was not identified as expressed under the conditions of this study. Though the significance of this SIX gene has not been elucidated in *F. annulatum* pathogenesis, it has been previously found not necessary for pathogenicity/virulence in other *Fusarium* species (Gawehns et al. 2015). However, a fungal effector identified in *Botrytis cinerea* (BcCLA4) was found in both the *F. annulatum* and *F. commune* transcriptomes but was only significantly upregulated *in planta* in *F. annulatum* and was previously found to mediate morphogenesis, growth, and cell cycle regulating processes (Minz-Dub et al. 2017). Effectors have various functions in both pathogenesis as well as host evasion (Lo Presti et al. 2015). The incremental increase in effectors from 12 hpi to 48 hpi suggests that effectors are important for early infection in conifer seedlings. The *F. annulatum* and *F. commune* effectomes were characterized with mostly apoplastic effectors suggesting that during early infection, they were still primarily avoiding host detection, neutralizing host defenses, or competing with other microbes in the apoplastic space

(Rockafort et al. 2020). The identified cytoplasmic effectors have been described as important for virulence as they enter the host cells and induce their virulence function within the targeted cell compartment (Rockafort et al. 2020). The putative effectors and the other secreted proteins described in this study may be of interest for further investigation.

Both the *F. annulatum* and *F. commune* transcriptomes contained ca. 6% carbohydrate-active enzymes, particularly cellulose, hemicellulose, and pectin degrading enzymes. CAZymes include cell wall degrading enzymes and have been found necessary for continual infection and colonization of host tissues (Ma et al. 2019; Roy et al. 2020). The significantly upregulated *in planta* genes described as CAZymes suggest that *F. commune* and *F. annulatum* were still actively infecting the loblolly seedlings; emphasized by more significantly upregulated genes described as CAZymes than down regulated *in planta* (Achari et al. 2021). This suggests that these secreted CAZymes are necessary for continual infection and warrants further investigation. Similar studies in *Fusarium* spp. have observed significant expression of effectors and CAZymes at 4 days post inoculation (Kuang et al. 2022). However, in our study, we observed a significant number of genes involved in secondary metabolism.

The importance of secondary metabolite biosynthesis as an essential component of plant host pathogenesis has been highlighted in many *Fusarium* spp. (Perincherry et al. 2019; Li et al 2020c). Some genes described in both *F. annulatum* and *F. commune* that contribute to these secondary metabolite biosynthesis pathways were described as polyketide synthases, transporters, and cytochrome P450 genes. Transporters are important for both host pathogenesis and for detoxification of the host to allow the pathogen to persist within the host and continue to infect (Guo et al. 2014). Pathways involved in the production of deoxynivelanol (DON), trichothecenes, sesquiterpenes, prolipyrone B/gibepyrone D, fusaric acid, choline, and ACT-

Toxin II were all found in *F. annulatum* in this study; all pathogenicity important *Fusarium* produced mycotoxins and secondary metabolites (Ayada et al. 2022; Engle et al. 2007; Li et al. 2020c). However, only DON, trichothecenes, sesquiterpenes, and prolipyrone B/gibepyrone D genes were found upregulated *in planta* in *F. annulatum*. The identified polyketide synthase involved in the biosynthesis of prolipyrone B/gibepyrone D was previously described as solely necessary for biosynthesis of this mycotoxin (Westphal et al. 2018). This mycotoxin may be necessary for *F. annulatum* to cause phytotoxicity in conifers and further study involving metabolomics, diagnostic primers, or gene knockouts should be conducted to confirm this. The down-regulated expression of gene clusters involved in enniatin, fusaric acid, choline, and ACT-Toxin II biosynthesis suggests that these mycotoxins may play a role in pathogenicity/virulence during later disease progression and were not significantly upregulated *in planta* at initial or early infection. In the *F. commune* transcriptome, DON, patulin, fusaoctaxin A, trichothecenes, sesquiterpenes, and enniatin were found in this study. All the *F. commune* described genes involved in secondary metabolite biosynthesis were described as significantly upregulated *in planta* except for enniatin. Patulin and fusaoctaxin A are also important fungal and *Fusarium* mycotoxins that aid in disease development (Jia et al. 2019; Tannous et al. 2020). Interestingly, gene clusters involved in the biosynthesis of the mycotoxins enniatin were found downregulated in both *F. annulatum* and *F. commune*. This suggests that enniatin may not be a necessary mycotoxin for conifer pathogenesis as the host may be able to detoxify this mycotoxin and render it inert (Westrick et al. 2021).

Signal regulation is important in pathogenesis of fungi for fungal infection and for avoiding detection within the host (Ma et al. 2013; Hernandez-Chavez et al. 2017). Many of the UIP genes were described as transcription factors and protein kinases involved in cell signaling

pathway in this study. A gene involved in the jasmonic acid pathway was found in the UIP genes in *F. annulatum*. When jasmonates are produced by the host plant, this pathway is essential for host response to 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 facilitate continual infection (Chini et al. 2018).

*Conifer pathogenesis genes among conifer-derived* Fusarium *spp. isolates*

As discussed previously, the *F. annulatum* and *F. commune* transcriptomes were found to comprise genes involved in secondary metabolite biosynthesis, cell signaling, and CAZymes in this study. The significantly upregulated *in planta* genes (UIP) including secreted proteins and described pathogenicity/virulence-associated genes showed similarity among species complexes. This concurs with what has been described in a previous study using predicted proteomes to predict conifer pathogenesis gene profiles (Chapter 3). This suggests that there may not be a shared accessory chromosome that confers conifer-specific pathogenicity. This likely means that there may be only a few or a single gene necessary for conifer pathogenesis like among *F. oxysporum* strains pathogenic to melons (Li et al. 2020a) and further investigation is needed to tease out the specific gene(s) for conifer pathogenesis. This may also indicate that *Fusarium* species complexes may be evolving independently and utilizing different pathways to cause damping-off and root disease on conifer seedlings (Abdullah et al. 2017; Summerell et al. 2019). This could be devastating to a host if multiple 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 PCoA and phylogenetic PCA analyses showed that closely related species and species complexes share similar pathogenicity/virulence gene profiles. This concurs with the phylogenetic analyses of both

conserved genes and predicted proteomes found in other studies (Chapter 3; Crous et al. 2021; Geisier et al. 2021; Summerell et al. 2019). Among the secreted profiles described by each species complex, based on shared genes among *F. annulatum* and *F. commune* transcriptomes, there were some genes shared among all species complexes suggesting that these genes are virulence related or more broadly used for host colonization since members within the species complexes were all collected from conifer hosts (Chapter 3). Pathogenicity/virulence genes described in the *F. annulatum* transcriptome including major facilitator supergroup (MFS) transporters, exopolygalacteronases, transcription factors, and prolipyrone B/gibpyrone D biosynthesis genes were shared among species complex profiles. The prolipyrone B/gibepyrone D biosynthesis orthogroup alignment showed that the orthologous proteins among the species complexes shared ca. 28% pairwise identity with the gene described in the *F. annulatum* transcriptome. This meets the 20% threshold used to describe proteins with orthologous functions (Pearson 2013). In the phylogeny, however, the highlighted clade that contained the prolipyrone B/gibepyrone D gene from the *F. annulatum* transcriptome shared ca. 32% pairwise identity with the other 11 species (Supplemental Figure S1). These species were comprised mostly members of the FFSC (6) but also contained FNSC (2), FOSC (1), and FTSC (3) species. Since this gene has only been described as expressed in members of the FFSC and *F. graminearum* species complex (Adpressa et al. 2019), this may indicate that these orthologous proteins are either not functional or may indicate that they produce a similar class of secondary metabolites emphasizing the need to validate the function of these genes in further studies. Similarly, in the *F. commune* transcriptome profiles, exopolygalacturonases, transcription factors, and aurofusarin and patulin biosynthesis genes were identified. All of these species, except *F. fredkrugeri*, have been described as plant pathogens but on a variety of host species

(Chapter 3). This could mean that they share virulence genes that may contribute to conifer pathogenesis, but the non-shared genes among profiles are likely host-specific (van der Does & Rep 2007). For example, DON has been described as important to full virulence in *Fusarium* especially members within the *F. tricinctum* (FTSC) and *F. incarnatum-equiseti* (FIESC) species complexes and was identified in all species in the FIESC and FTSC except *F. flocciferum* (Bai et al. 2002; O'Mara et al. 2023; Villani et al. 2019). In our study, DON was only identified in *F. commune* (FNSC) isolates collected from Douglas-fir and tomato and *F. lactis* (FFSC). However, based on the pathogenicity assays, the other *F. commune* isolates also caused disease on hosts, suggesting that at least in seedlings, DON may not be a necessary pathogenicity pathway in *F. commune*. The DON biosynthesis may aid in disease development in older plants since head blight causing *Fusarium* spp. infect their hosts at flowering (Alisaac et al. 2023). Metabolomics analyses would aid in determining the expression of DON in conifers.

In the phylogenetic PCA analysis, the *F. commune* collected from Douglas-fir (F13) and the ponderosa pine (PIPO) isolate were consistently different. When investigating the profile genes, F13 had a unique NRPS-like and leucine biosynthesis gene, whereas the PIPO isolate had more MFS transporters, specifically sugar transporters compared to the other *F. commune* isolates. The PIPO isolate also had an amino acid synthesis pathway and a lysine tRNA synthetase protein not present in the other isolates. These unique genes likely explain the distinctiveness in the phylogenetic PCA and were likely involved in the observed higher virulence of F13 than the PIPO isolate on all hosts assayed (not significantly). The leucine biosynthetic pathway has been described as crucial for virulence in *Aspergillus fumigatus* and may be necessary in *F. commune* for high virulence on conifer seedlings (Orasch et al. 2019).

Functional assessments like CRISPR/CAS9 gene knockouts of this gene on the virulence on conifer seedlings would be needed to test its effects on virulence (Shinkado et al. 2022).

Among the species complex clades discussed in Chapter 3, SC1 consisting of the FFSC, FOSC, and FNSC had 24 and 20 unique orthologous genes in the *F. annulatum* and *F. commune* transcriptome profiles, respectively. Both species complex clades had unique genes involved in transport, transcription, carbohydrate-active enzymes, and secondary metabolism all of which have been found associated with virulence in fungi (Guo et al. 2014; Ma et al. 2013; Roy et al. 2020). These unique genes may signify that these species complexes use unique pathways for disease development on their hosts. This should be considered when developing early detection tools to identify multiple pathogens within a nursery to mitigate disease. However, since in this study we did not conduct pathogenicity assays on all isolates, functional assessments such as proteomics or metabolomics should be conducted to validate the repertoire of effectors and secondary metabolites that are necessary for conifer pathogenesis among each species complex clade (Hooshmand et al. 2020; Zhao et al. 2020).

**Conclusions**

Our findings show that *Fusarium annulatum* and *F. commune* isolates, investigated in this study, were plant pathogens regardless of host origins suggesting that these isolates did not exhibit host-specificity. Profiles of pathogenicity/virulence genes within the transcriptomes were identified as unique among *Fusarium* species complexes, not between conifer pathogens. However, this study was an initial investigation, and the mechanisms involved in plant host virulence across more diverse hosts should be investigated. 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 and were

primarily produced at 48 hpi. The profiles identified in this study can further be used to conduct targeted analyses of the products of the genes involved in transport, transcription, carbohydrate-active enzymes, and secondary metabolism among species complex clades that can be used to identify pathogenic members within these species complexes. Additionally, investigations of the host response to the pathogen would aid in understanding the systems biology and develop detection techniques like air sampling detection methods that can rapidly identify an infected host plant. A systems biology investigation utilizing proteomics and metabolomics analyses of the proteins and secondary metabolites produced during conifer pathogenesis may identify conifer-specific effectors and mycotoxins that can be used in the development of early detection and pathogen mitigation techniques. However, additional studies may also determine that these identified pathogenicity/virulence genes may be broad host pathogenicity genes as we did not identify host-specific or non-pathogenic strains in this study. Our pathogenicity assays indicate that young conifer seedlings may be susceptible to a broader range of pathogenic *Fusarium* spp. until they become more mature and develop age-related resistance to non-host-specific strains. However, since there were many important downregulated mycotoxins identified in this study, e.g., fusaric acid, ACT-toxin II, and enniatin, the genes involved in the biosynthesis of these toxins and their expression in more mature seedlings should be explored.

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# CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

## Conclusions

*Fusarium* spp. pathogens are becoming an increasing concern to forest tree nurseries especially now that there is a growing need for nursery grown seedlings due to climate change (Fargione et al. 2021). However, there is a lack of knowledge of the evolutionary history of the multiple *Fusarium* spp. that cause Fusarium damping-off and root disease to young conifer seedlings. Our limited understanding of the pathogenicity/virulence genes, including secreted proteins, effectors, and secondary metabolites, used by conifer pathogenic *Fusarium* spp. leaves nurseries unprepared to prevent outbreaks and limit spread of pathogens among nurseries and field sites. Understanding the systems biology of the molecular interaction of/among conifer pathogenic *Fusarium* spp. with their hosts will provide the basis needed to develop early detection and monitoring tools to inform managers when control and spread mitigation methods are necessary (Dix et al. 2016; Yeung et al. 2019). Due to the current reclassification of *Fusarium* spp., alternative methods are necessary to identify conifer pathogens and utilizing pathogenicity/virulence pathways may be a viable alternative to genetic identification methods (Crous et al. 2021; Geiser et al. 2021; O'Donnell et al. 2022). To improve our knowledge of the pathogenicity/virulence genes necessary for *Fusarium* conifer pathogenicity, I used: 1) genetics to identify the community structure of *Pinaceae*-associated *Fusarium* spp.; 2) genomics comparisons to identify putative pathogenicity/virulence gene profiles among putative pathogenic *Fusarium* spp.; and 3) transcriptomics of confirmed pathogenic *Fusarium* spp. at initial and early infection time points in a susceptible host to identify upregulated pathogenicity/virulence genes. This research identified conifer pathogenicity/virulence genes,

however these genes are likely not all required for all conifer pathogens. The identified pathogenicity/virulence genes in this study may be broad host pathogenicity/virulence factors that young conifer seedlings are susceptible to and that age-related resistance may render these genes inert as the seedling ages (Hu et al. 2019). Furthermore, due to the pathogenicity/virulence gene profiles showing a difference in evolutionary history based on closely related *Fusarium* species complexes, this likely means there is not a singular lineage-specific chromosome responsible for conifer pathogenesis and likely a single or multiple gene(s) necessary for conifer pathogenesis.

This dissertation focuses on the initial components of a top-down data reduction approach to integrate multi-omics tools for systems biology investigations (Ch1:Figure1; Pinu et al. 2019). In Chapter 2, the spatial distribution and host relationships of Fusarioid fungi were investigated to assess community structure of potential pathogenic *Fusarium* spp., determining that geographic region had a significant effect on Fusarioid fungi community structure. Understanding the community or population structure is a vital first step in determining the overall health of system by identifying the *Fusarium* spp. associated with different hosts and regions to identify shifts in communities from healthy to diseased (Ibarra Caballero et al. 2023; Yergeau et al. 2010). This regional effect on Fusarioid fungi communities highlights the importance of limiting the spread of new pathogens to regions where hosts are not adapted to the introduced pathogens. As the climate warms, *Fusarium* spp. and strains adapted to warmer climates may increase their habitable range to regions where the hosts are not adapted to the increased temperature or the mechanisms used by these pathogens to cause disease leading to more outbreaks from emerging or introduced pathogens (Corredor-Moreno et al. 2019).

In Chapter 3, 17 *Fusarium* spp. genomes were compared and determined that predicted pathogenicity/virulence gene profiles were shared among closely related species complexes. The predicted pathogenicity/virulence genes such as the effectors and mycotoxins identified in this study provide potential targets for detecting *Fusarium* conifer pathogens. The profiles identified in the potential host-specialization in *F. commune* isolates derived from conifer and non-conifer hosts and soil, showed that there were significantly different profiles associated with isolates collected from conifer, non-conifer, or soil. This highlighted the possibility of host-specificity among the pathogens (Li et al. 2020). This chapter also highlighted the current need for high-throughput sequencing to properly place *Fusarium* spp. As the debate continues about the classification of *Fusarium* spp., type strains and phylogenetic placement are necessary to properly identify *Fusaria* to species (Crous et al. 2021; O'Donnell et al. 2022).

In Chapter 4, conifer pathogenicity of two *Fusarium* spp., *F. annulatum* and *F. commune*, was confirmed and the differentially expressed genes within a host at three time points 12-, 24-, and 48-hours post inoculation (hpi) were identified on one of the susceptible hosts, loblolly pine (*Pinus taeda*). Pathogenicity/virulence genes were predominantly expressed during 48-hpi in both species likely indicating that host colonization had been successful by this time. Species complex pathogenicity/virulence gene profiles were developed based on the *F. annulatum* and *F. commune* upregulated genes in the host plant and shown to be similar among closely related *Fusarium* species complexes. Though unique genes and pathways were shown among members within a species complex, this emphasized the multiple pathogenicity/virulence pathways that seedlings are encountering, possibly concurrently, overwhelming their underdeveloped defenses (Abdullah et al. 2017). Interestingly, when revisiting the host-specificity of *F. commune* isolates derived from conifer and non-conifer hosts, we did not observe host-specificity. All isolates

caused disease across the five hosts surveyed, further emphasizing that seedling age may play a major role in the defense against *Fusarium* pathogens. This also highlighted the need for multi-omics analyses to investigate the virulence genes expressed during infection of each host. As the previous chapter identified differences in pathogenicity/virulence gene profiles based on host origins, the same trend was not observed based on the transcriptome-based profiles.

Results from this research provides insight into the pathways and pathogenicity/virulence genes used by conifer pathogenic *Fusarium* spp. and the generated profiles provides a focus for future targeted analyses. The recommended modified top-down data reduction approach [1) assess the community structure; 2) determine pathogenicity on hosts; 3) conduct whole genome comparisons to identify shared and unique pathogenicity/virulence genes/pathways; 4) identify upregulated genes and pathways involved in pathogenicity/virulence; 5) conduct proteomics analyses to validate expression of secreted proteins and effectors; and 6) conduct metabolomics analyses to identify mycotoxins and secondary metabolites produced by the pathogen during disease progression] and results from this research provide a framework for future studies of un- or under-studied pathosystems in designing targeted investigations for the development of rapid diagnostic tools.

**Future directions**

These studies introduce new research questions that should be investigated. Since a regional effect on Fusarioid fungi community structure was observed in this research, what are the main drivers of this effect? Are they climactic or possibly culturable practices that differ in regions that contribute to this regional effect. Studies simulating the changes in climate should also be conducted to identify traits involved in projected warmer, drier conditions (Ghosh et al. 2022). Since there was an observed regional effect on community structure, there is likely

already temperature and moisture influences on *Fusarium* conifer pathogens. Increased temperatures have shown to increase virulence of isolates and species and strains (Cram 2003; Dumroese & James 2005; Huang & Kuhlman 1991). Furthermore, surveys should be conducted to identify potential host associations among Fusarioid fungi communities within regions. This would allow for the identification of potentially host-specific virulence factors used by conifer pathogenic *Fusarium* spp.

What is the best method for identifying *Fusarium* spp.? Since current debate on proper placement of *Fusarium* spp. is relying on anywhere from eight to 19 recommended conserved loci, it is becoming more economical and feasible to conduct metagenomics to identify communities and should be considered for future research (Crous et al. 2021; Geiser et al. 2021; O'Donnell et al. 2022). Understanding the lifestyle of a pathogen is vital to create robust management methodologies. To gain a better understanding of conifer pathogens, a direct comparison of species or strains with pathogenic lifestyles with host-specific and non-pathogenic lifestyle species or strains should be conducted. Comparing the different lifestyles allows for the identification of pathogenicity/virulence factors that may not be highlighted in a direct comparison of shared lifestyle isolates. However, a variety of isolates of varying lifestyles should be used to prevent identification of genomic or pathogenicity/virulence genes only identifiable in certain lineages, e.g., only highly virulent lineages (Dobbs et al. 2020).

How does environment influence the molecular host-pathogen interactions in conifer nurseries? As temperature has been seen to influence pathogen virulence (Cram 2003; Dumroese & James 2005; Huang & Kuhlman 1991), studies should be conducted to identify the temperature range of this virulence and utilize multi-omics tools to identify the molecular mechanisms involved in these changes. Additionally, since all isolates used in this study were

shown to be broad pathogens on young seedlings, how does seedling age influence the molecular host-pathogen interactions in conifer nurseries? The above should be conducted on a range of seedling ages as some species can be grown for years in a nursery before out-planting (Duryea 1984). Integrating transcriptomics with proteomics and/or metabolomics would provide the best comparisons to identify the different functional genes and pathways necessary for conifer pathogenesis and under the above conditions (Pinu et al. 2019).

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APPENDICES

Supplemental Table S1 - Shared and unique predicted virulence-related genes based on conifer seedling-collected isolates of *Fusarium* spp. in six *Fusarium* species complexes, *F. fujikuroi* (FFSC), *F. nisikadoi* (FNSC), *F. oxysporum* (FOSC), *F. incarnatum-equiseti* (FIESC), *F. tricinctum* (FTSC), and *F. solani* (FSSC) species complexes.

| | **Species Complex Clade 1 (SC1)** | | | | | | | | **Species Complex Clade 2 (SC2)** | | | | | | | **Species Complex Clade 3 (SC3)** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **FFSC*** | | | | | **FNSC** | **FOSC** | | **FIESC** | | | **FTSC** | | | | **FSSC** | |
| | ***1*** | ***2*** | ***3*** | ***4*** | ***5*** | ***6*** | ***7*** | ***8*** | ***9*** | ***10*** | ***11*** | ***12*** | ***13*** | ***14*** | ***15*** | ***16*** | ***17*** |
| FAM1 | X | X | X | X | X | X | X | X | | | | | | | | | |
| FoSep1 | X | X | X | X | X | X | X | X | | | | | | | | | |
| FvCP02 | X | X | X | X | X | X | X | X | | | | | | | | | |
| FvHAP2 | X | X | X | X | X | X | X | X | | | | | | | | | |
| FvHAP3 | X | X | X | X | X | X | X | X | | | | | | | | | |
| GIV1 | X | X | X | X | X | X | X | X | | | | | | | | | |
| THIOL | X | X | X | X | X | X | X | X | | | | | | | | | |
| Fub1 | X | X | X | | | X | X | X | | | | | | | | | |
| DEP2 | X | X | | X | X | X | X | | | | | | | | | | |
| FgLDHL1_(FGSG_00145) | X | | X | X | | X | X | X | | | | | | | | | |
| FUM19 | X | | X | | X | | | | | | | | | | | | |
| VFGLU1 | X | | X | | | X | X | | | | | | | | | | |
| FUM21 | X | | X | | | X | | | | | | | | | | | |
| MgAtr7 | X | | X | | | X | | | | | | | | | | | |
| SIX2 | X | | | | | | | | | | | | | | | | |
| DEP5 | | X | | | X | X | X | X | | | | | | | | | |
| DEP1 | | X | | | X | X | X | | | | | | | | | | |
| DEP4 | | X | | | X | X | X | | | | | | | | | | |
| Fgm4_(FGSG_10994) | | X | | | | | | | | | | | | | | | |
| MoPLC3 | | | X | | X | X | | | | | | | | | | | |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Apa2 | | | | X | | | | | | | | | | | | | |
| BbbqrA | | | | | X | | | | | | | | | | | | |
| MoCDIP4 | | | | | X | | | | | | | | | | | | |
| FTF1 | | | | | | X | | X | | | | | | | | | |
| BcSAK1 | | | | | | X | | | | | | | | | | | |
| Fghyd3_(FGSG_09066) | | | | | | X | | | | | | | | | | | |
| Fghyd4_(FGSG_03960) | | | | | | X | | | | | | | | | | | |
| Mocapn9 | | | | | | X | | | | | | | | | | | |
| SsFdh1_(SS1G_10135) | | | | | | X | | | | | | | | | | | |
| ABC3 | | | | | | | X | | | | | | | | | | |
| VdSge1 | | | | | | | X | | | | | | | | | | |
| MoPEX7 | | | | | | | | X | | | | | | | | | |
| FgNoxR | | | | | | | | | X | X | X | X | X | X | X | | |
| MoLys2 | | | | | | | | | X | X | X | X | X | X | | | |
| Ss-odc2 | | | | | | | | | X | X | X | X | X | X | | | |
| FgFSR1 | | | | | | | | | X | X | X | X | | | | | |
| FgAP2s_(FGSG_02015) | | | | | | | | | X | X | X | | X | | | | |
| Dcl1 | | | | | | | | | X | X | X | | | | | | |
| Dcl2 | | | | | | | | | X | X | X | | | | | | |
| FgCrpA_(FGRAMPH1_01G10037) | | | | | | | | | X | X | X | | | | | | |
| FgFRP1 | | | | | | | | | X | X | X | | | | | | |
| FgLAI12 | | | | | | | | | X | X | X | | | | | | |
| FgMed1 | | | | | | | | | X | X | X | | | | | | |
| FgMkk1 | | | | | | | | | X | X | X | | | | | | |
| FGSG_02077 | | | | | | | | | X | X | X | | | | | | |
| FgVe1_(FgVeA) | | | | | | | | | X | X | X | | | | | | |
| GzC2H007 | | | | | | | | | X | X | X | | | | | | |
| MGV1 | | | | | | | | | X | X | X | | | | | | |
| Moatg6 | | | | | | | | | X | X | X | | | | | | |
| Mofzo1_(MGG_05209) | | | | | | | | | X | X | X | | | | | | |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| VPS9_(FGRAMPH1_01G01477) | | | | | | | | | X | X | X | | | | | | |
| TRI10 | | | | | | | | | X | X | | | | | | | |
| TRI5__Tri5 | | | | | | | | | X | X | | | | | | | |
| GzGPA2 | | | | | | | | | X | | X | X | X | X | X | | |
| FGSG_05586 | | | | | | | | | X | | | | | | | | |
| KRE5 | | | | | | | | | X | | | | | | | | |
| (Sc_Tpk2)__CPK1 | | | | | | | | | | X | | | | X | | | |
| MLT1 | | | | | | | | | | | X | | | | | | |
| MoBRE1 | | | | | | | | | | | X | | | | | | |
| Siz1 | | | | | | | | | | | X | | | | | | |
| TRI6 | | | | | | | | | | | X | | | | | | |
| MoAbp1_(MGG_06358) | | | | | | | | | | | | X | X | | | | |
| FpAda1_(FPSE_04421) | | | | | | | | | | | | X | | | | | |
| TOXF | | | | | | | | | | | | X | | | | | |
| VdTHI20 | | | | | | | | | | | | | X | | | | |
| CPKA__CpkA | | | | | | | | | | | | | | X | | | |
| Pes1 | | | | | | | | | | | | | | X | | | |
| AbcB__AbcG1 | | | | | | | | | | | | | | | X | | |
| BcSOD1__Bcsod1 | | | | | | | | | | | | | | | X | | |
| MGG_01707 | | | | | | | | | | | | | | | X | | |
| Leu4_(MGG_13485) | | | | | | | | | | | | | | | | X | X |
| MoDUO1 | | | | | | | | | | | | | | | | X | X |
| MoJMJ1 | | | | | | | | | | | | | | | | X | X |
| MoVPR | | | | | | | | | | | | | | | | X | X |
| PmcC | | | | | | | | | | | | | | | | X | X |
| VdThit | | | | | | | | | | | | | | | | X | X |
| VEDA_05199 | | | | | | | | | | | | | | | | X | X |
| SCD1 | | | | | | | | | | | | | | | | X | |
| Vph1 | | | | | | | | | | | | | | | | X | |
| ARP1_(orf19.2641) | | | | | | | | | | | | | | | | | X |

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| FGSG_07409__GzHMG024 | | | | | | | | | | | | | | | | | X |
| MET6 | | | | | | | | | | | | | | | | | X |
| MGG_07259 | | | | | | | | | | | | | | | | | X |
| VEDA_05196 | | | | | | | | | | | | | | | | | X |

**F. fujikuroi* (FFSC), *F. nisikadoi* (FNSC), *F. oxysporum* (FOSC), *F. incarnatum-equiseti* (FIESC), *F. tricinctum* (FTSC), and *F. solani* (FSSC) species complexes
1-*F.annulatum*,2-*F.Fredkrugeri*,3-*F.fujikuroi*,4-*F.lactis*,5-*F.verticillioides*,6-*F.commune*,7-*F.inflexum*,8-*F.oxysporum*,9-*F.clavum*,10-*F.ipomoeae*,11-*F.luffae*,12-F.avenaceum,13-F.flocciferum,14-F.torulosum,15-F.tricinctum,16-F.falciforme,17-F.solani

Supplemental Table S2 - Secreted in xylem (SIX) genes identified in species used in this study.

| Isolate | *Fusarium* spp. | Species complex* | Host Collected From | SIX 1 | SIX 2 | SIX 3 | SIX 4 | SIX 5 | SIX 6 | SIX 7 | SIX 8 | SIX 9 | SIX 10 | SIX 11 | SIX 12 | SIX 13 | SIX 14 | SIX 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MES5 | *F. tricinctum* | FTSC | Conifer | | | | | | | | | | | | | | | |
| NASWWP3 | *F. annulatum* | FFSC | Conifer | | X | | | | | | | | | | | | | |
| NC74 | *F. avenaceum* | FTSC | Conifer | | | | | | | | | | | | | | | |
| NRRL25331 | *F. circinatum* | FFSC | Conifer | | X | | | | | | | | | | | | | |
| NAGrPIST2 | *F. clavum* | FIESC | Conifer | | | | | | | | | | | | | | | |
| F10.2.2 | *F. commune* | FNSC | Conifer | | | | | | | | | | | | | | | |
| F13.4.1 | *F. commune* | FNSC | Conifer | | | | | | | | | | | | | | | |
| JICPIPO1 | *F. commune* | FNSC | Conifer | | | | X | | | | | | | | | | | |
| SC35 | *F. falciforme* | FSSC | Conifer | | | | | | | | | | | | | | | |
| 31MAPSF17A | *F. flocciferum* | FTSC | Conifer | | | | | | | | | | | | | | | |
| NRRL38302 | *F. foetens* | FOSC | Conifer | | | | | | | | | | | | | | | |
| SC7 | *F. fredkrugeri* | FFSC | Conifer | | | | | | | | | | | | | | | |
| SC5 | *F. fujikuroi* | FFSC | Conifer | | | | | | | | | | | | | | | |
| JTHABCO3.1 | *F. inflexum* | FOSC | Conifer | | | | | | | | | | | | | | | |
| NC8 | *F. ipomoeae* | FIESC | Conifer | | | | | | | | | | | | | | | |
| MES8 | *F. lactis* | FFSC | Conifer | | | | | | | | | | | | | | X | |
| CC | *F. luffae* | FIESC | Conifer | | | | | | | | | | | | | | | |
| MES6 | *F. oxysporum* | FOSC | Conifer | | | | | | | | | | | | | | | |
| NRRL22901 | *F. redolens* | *redolens* | Conifer | | | | | | | | | X | | | | | | |

| NAPSME6.1 | *F. solani* | FSSC | Conifer | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| DCN062.2H | *F. torulosum* | FTSC | Conifer | | | | | | | | | | | | | | | |
| NAGrPIPO5 | *F. verticillioides* | FFSC | Conifer | | | | X | | | | | | | | | | X | |
| F8_4S_2B | *F. annulatum* | FFSC | Environmental | | X | | | | | | | | | | | | | |
| JCM11502 | *F. commune* | FNSC | Environmental | | | | X | | | | | | | | | | | |
| NRRL66328 | *F. scirpi* | FIESC | Environmental | | | | | | | | | | | | | | | |
| NRRL43529 | *F. falciforme* | FSSC | Human | | | | | | | | | | | | | | | |
| NRRL31160 | *F. irregulare* | FIESC | Human | | | | | | | | | | | | | | | |
| NRRL32931 | *F. oxysporum* | FOSC | Human | | | | | | | | X | | | | | | | |
| NRRL62606 | *F. ambrosium* | FSSC | Insect | | | | | | | | | | | | | | | |
| FSSC_6 | *F. metavorans* | FSSC | Insect | | | | | | | | | | | | | | | |
| CHS3 | *F. acuminatum* | FTSC | Non-conifer | | | | | | | | | | | | | | | |
| NRRL54939 | *F. avenaceum* | FTSC | Non-conifer | | | | | | | | | | | | | | | |
| Fb-HN-1 | *F. brachygibbosum* | *sambucinum* | Non-conifer | | | | | | | | | | | | | | | |
| NRRL66337 | *F. clavum* | FIESC | Non-conifer | | | | | | | | | | | | | | | |
| NRRL28387 | *F. commune* | FNSC | Non-conifer | | | | | | | | | | | | | | | |
| B62 | *F. commune* | FNSC | Non-conifer | | | | | | | | | | | | | | | |
| MiAE120 | *F. commune* | FNSC | Non-conifer | | | | | | | | | | | | | | | |
| NRRL66338 | *F. equiseti* | FIESC | Non-conifer | | | | | | | | | | | | | | | |
| NRRL5538 | *F. fujikuroi* | FFSC | Non-conifer | | X | | | | | | | | | | | | | |
| NRRL22945 | *F. guttiforme* | FFSC | Non-conifer | | | | | | | | | | | | | | X | |
| NRRL29888 | *F. hostae* | *redolens* | Non-conifer | | | | | | | | | | | | | X | X | |
| NRRL22090 | *F. illudens* | FSSC | Non-conifer | | | | | | | | | | | | | | | |
| NRRL66325 | *F. incarnatum* | FIESC | Non-conifer | | | | | | | | | | | | | | | |
| NRRL25226 | *F. mangiferae* | FFSC | Non-conifer | | X | | | | | | | | | | | | | |
| NRRL26231 | *F. mIscanthi* | FNSC | Non-conifer | | X | | | | | | | | | | | | | |
| NRRL22166 | *F. neocosporiellum* | FSSC | Non-conifer | | | | | | | | | | | | | | | |
| NRRL25179 | *F. nisikadoi* | FNSC | Non-conifer | | | | | | | | | | | | | | | |
| NRRL34936 | *F. oxysporum* | FOSC | Non-conifer | X | X | X | | X | X | X | X | X | X | X | X | X | X | X |

| | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ET1 | *F. proliferatum* | FFSC | Non-conifer | | | | | | | | | | | | | | | |
| NRRL66333 | *F. subglutinans* | FFSC | Non-conifer | | X | | | | | | | | | | | | X | |
| NRRL22049 | *F. thapsinum* | FFSC | Non-conifer | | X | | | | | | | | | | | | X | |
| NRRL25481 | *F. tricinctum* | FTSC | Non-conifer | | | | | | | | | | | | | | | |
| NRRL20956 | *F. verticillioides* | FFSC | Non-conifer | | X | | | | | | | | | | | | X | |
| NRRL31041 | *F. virguliforme* | FSSC | Non-conifer | | | | | | | | | | | | | | | |
| NRRL25486 | *F. xylarioides* | FFSC | Non-conifer | | | | | | | X | | | X | | X | | | |
| NRRL45417 | *F. gaditjirrii* | FNSC | | | X | | | | | | | | | | | | | |
| NRRL66475 | *F. hainanense* | FIESC | | | | | | | | | | | | | | | | |
| NRRL66473 | *F. luffae* | FIESC | | | | | | | | | | | | | | | | |
| NRRL54252 | *F. lyarnte* | FNSC | | | X | | | | | | | | | | | | | |
| NRRL66326 | *F. sacchari* | FFSC | | | X | | | | | | | | | | | | | |
| NRRL22747 | *F. torulosum* | FTSC | | | | | | | | | | | | | | | | |
| NRRL45880 | *F. vanettenii* | FSSC | | | | | | | | | | | | | | | | |

**F. fujikuroi* (FFSC), *F. nisikadoi* (FNSC), *F. oxysporum* (FOSC), *F. incarnatum-equiseti* (FIESC), *F. tricinctum* (FTSC), and *F. solani* (FSSC) species complexes

Supplemental Table S3 - Unique predicted secondary metabolites based on *Fusarium* spp. in six *Fusarium* species complexes, *F. fujikuroi* (FFSC), *F. nisikadoi* (FNSC), *F. oxysporum* (FOSC), *F. incarnatum-equiseti* (FIESC), *F. tricinctum* (FTSC), and *F. solani* (FSSC) species complexes.

| | **Species Complex Clade 1 (SC1)** | | | | | | | | **Species Complex Clade 2 (SC2)** | | | | | | | **Species Complex Clade 3 (SC3)** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **FFSC*** | | | | | **FNSC** | **FOSC** | | **FIESC** | | | **FTSC** | | | | **FSSC** | |
| | ***1*** | ***2*** | ***3*** | ***4*** | ***5*** | ***6*** | ***7*** | ***8*** | ***9*** | ***10*** | ***11*** | ***12*** | ***13*** | ***14*** | ***15*** | ***16*** | ***17*** |
| Fusaric acid | X | X | X | X | X | X | X | X | | | | | | | | | |
| Fujikurin A/B/C/D | X | X | X | X | | | | | | | | | | | | | |
| Trichoxide | X | X | | | | | | | | | | | | | | | |

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fumonisin | X |  | X |  | X |  |  |  |  |  |  |  |  |  |  |  |  |
| Gibberellin | X |  | X |  |  | X |  |  |  |  |  |  |  |  |  |  |  |
| Acetylaranotin | X |  | X |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Arylpoyene | X |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Solanapyrone D | X |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Depudecin |  | X |  |  | X | X | X |  |  |  |  |  |  |  |  |  |  |
| Alternapyrone |  | X |  |  |  | X |  |  |  |  |  |  |  |  |  |  |  |
| Subglutinol A/B |  | X |  |  |  | X |  |  |  |  |  |  |  |  |  |  |  |
| Falvoglaucin |  |  | X | X | X |  |  |  |  |  |  |  |  |  |  |  |  |
| APE Vf |  |  |  | X |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Crochelin A |  |  |  | X |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Kijanimicin |  |  |  | X |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Phomopsin A/B/E |  |  |  | X |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Plantaribactin |  |  |  | X |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Gregatin A |  |  |  |  | X |  |  |  |  |  |  |  |  |  |  |  |  |
| Enniatin |  |  |  |  |  | X | X |  |  |  |  |  |  |  |  |  |  |
| Butyrolactone |  |  |  |  |  | X |  |  |  |  |  |  |  |  |  |  |  |
| Huperzine A |  |  |  |  |  |  |  |  | X | X | X | X |  |  |  |  |  |
| Chrysogine |  |  |  |  |  |  |  |  | X | X |  | X |  |  |  |  |  |
| Zearalenone |  |  |  |  |  |  |  |  | X |  | X |  |  |  |  |  |  |
| UNIIYC2Q1094PT |  |  |  |  |  |  |  |  | X |  |  |  |  |  |  |  |  |
| NI-siderophore |  |  |  |  |  |  |  |  |  | X | X |  | X |  |  |  |  |
| Deoxynivalenol |  |  |  |  |  |  |  |  |  | X | X |  |  |  |  |  |  |
| Apicidin |  |  |  |  |  |  |  |  |  | X |  | X |  |  |  |  |  |
| Alcaligin |  |  |  |  |  |  |  |  |  |  | X |  |  |  |  |  |  |
| Ectoine |  |  |  |  |  |  |  |  |  |  | X |  |  |  |  |  |  |
| PR-toxin |  |  |  |  |  |  |  |  |  |  | X |  |  |  |  |  |  |
| Aurofusarin |  |  |  |  |  |  |  |  |  |  |  | X |  |  |  |  |  |
| Culmorin |  |  |  |  |  |  |  |  |  |  |  | X |  |  |  |  |  |
| Fusarielin H |  |  |  |  |  |  |  |  |  |  |  | X |  |  |  |  |  |

| Fusaristatin A | | | | | | | | | | | | X | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Orcinol/orsellinic acid | | | | | | | | | | | | X | | | | | |
| Peramine | | | | | | | | | | | | X | | | | | |
| Pneumocandin A0/B0 | | | | | | | | | | | | X | | | | | |
| W493A/B | | | | | | | | | | | | X | | | | | |
| Duclauxin | | | | | | | | | | | | | | | | X | X |
| Fusarubin | | | | | | | | | | | | | | | | X | X |
| Metachelin C | | | | | | | | | | | | | | | | X | X |
| Sansalvamide | | | | | | | | | | | | | | | | X | X |
| Clavaric acid | | | | | | | | | | | | | | | | X | |
| Monacolin K | | | | | | | | | | | | | | | | | X |
| Ochratoxin A | | | | | | | | | | | | | | | | | X |

**F. fujikuroi* (FFSC), *F. nisikadoi* (FNSC), *F. oxysporum* (FOSC), *F. incarnatum-equiseti* (FIESC), *F. tricinctum* (FTSC), and *F. solani* (FSSC) species complexes
1-*F.annulatum*,2-*F.Fredkrugeri*,3-*F.fujikuroi*,4-*F.lactis*,5-*F.verticillioides*,6-*F.commune*,7-*F.inflexum*,8-*F.oxysporum*,9-*F.clavum*,10-*F.ipomoeae*,11-*F.luffae*,12-F.avenaceum,13-F.flocciferum,14-F.torulosum,15-F.tricinctum,16-F.falciforme,17-F.solani

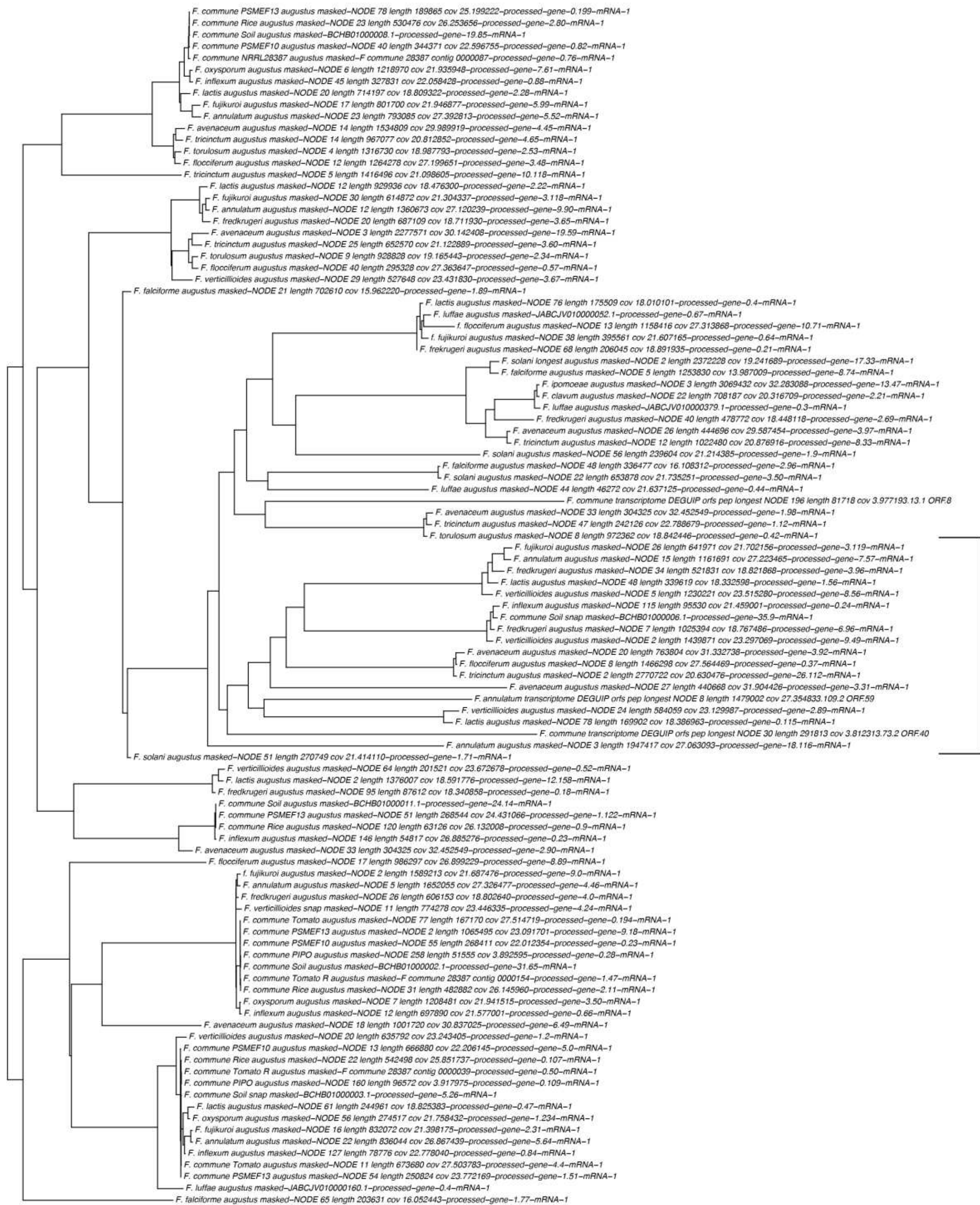
Supplemental Table S4 - Unique genes shared among putative conifer pathogenic *Fusarium* spp.

| Conifer pathogenic species unique genes | | |
|---|---|---|
| Total orthogroups | 730 | containing 2133 proteins |
| Secreted proteins | 173 | |
| Effectors | 1319 | 61.80% |
| Cytoplasmic effectors | 1179 | 55.30% |
| Apoplastic effectors | 140 | 6.60% |
| Interproscan | | |
| 2EXR domain | IPR045518 | 2 |
| ABC transporter type 1, transmembrane domain | IPR011527 | 5 |
| Alcohol dehydrogenase-like, C-terminal | IPR013149 | 8 |
| Alcohol dehydrogenase-like, N-terminal | IPR013154 | 9 |
| Aldehyde dehydrogenase domain | IPR015590 | 9 |
| Alpha-L-rhamnosidase, six-hairpin glycosidase domain | IPR035396 | 4 |
| Amidase signature domain | IPR023631 | 7 |
| Amidohydrolase 3 | IPR013108 | 1 |
| Amine oxidase | IPR002937 | 2 |
| Amino acid transporter, transmembrane domain | IPR013057 | 2 |

| | | |
|---|---|---|
| Aminoacyl-tRNA synthetase, class II (D/K/N) | IPR004364 | 6 |
| Aminotransferase class-III | IPR005814 | 4 |
| Aminotransferase, class I/classII | IPR004839 | 4 |
| Ankyrin repeat | IPR002110 | 40 |
| ATPase, AAA-type, core | IPR003959 | 7 |
| B-box-type zinc finger | IPR000315 | 1 |
| Bile acid:sodium symporter/arsenical resistance protein Acr3 | IPR002657 | 1 |
| Carotenoid oxygenase | IPR004294 | 6 |
| Cation-transporting P-type ATPase, C-terminal | IPR006068 | 4 |
| CBM21 (carbohydrate binding type-21) domain | IPR005036 | 3 |
| Ccc1 family | IPR008217 | 2 |
| CDP-alcohol phosphatidyltransferase | IPR000462 | 1 |
| Cellulose-binding domain, fungal | IPR000254 | 2 |
| Copper amine oxidase, catalytic domain | IPR015798 | 4 |
| Copper amine oxidase, N2-terminal | IPR015800 | 4 |
| Copper amine oxidase, N3-terminal | IPR015802 | 4 |
| Cupin 2, conserved barrel | IPR013096 | 3 |
| Cytochrome b5-like heme/steroid binding domain | IPR001199 | 3 |
| Cytochrome P450 | IPR001128 | 8 |
| Domain of unknown function DUF1996 | IPR018535 | 4 |
| Domain of unknown function DUF676, lipase-like | IPR007751 | 1 |
| EamA domain | IPR000620 | 3 |
| Endonuclease/exonuclease/phosphatase | IPR005135 | 1 |
| FAD linked oxidase, N-terminal | IPR006094 | 2 |
| FAD-binding domain | IPR002938 | 4 |
| Fatty acid desaturase, N-terminal | IPR021863 | 1 |
| F-box domain | IPR001810 | 1 |
| FMN-dependent dehydrogenase | IPR000262 | 6 |
| Fumarylacetoacetase, N-terminal | IPR015377 | 1 |
| Fumarylacetoacetase-like, C-terminal | IPR011234 | 3 |
| Fungal lipase-like domain | IPR002921 | 5 |
| Fungal transcription factor | IPR021858 | 7 |
| GLEYA adhesin domain | IPR018871 | 1 |
| Glucose-methanol-choline oxidoreductase, C-terminal | IPR007867 | 3 |
| Glucose-methanol-choline oxidoreductase, N-terminal | IPR000172 | 4 |
| Glutathione S-transferase, C-terminal | IPR004046 | 3 |
| Glycoside hydrolase family 1 | IPR001360 | 2 |
| Glycoside hydrolase, family 3, N-terminal | IPR001764 | 2 |
| Glycoside hydrolase, family 43 | IPR006710 | 4 |
| Glycosyl hydrolase family 32, N-terminal | IPR013148 | 3 |

| | | |
|---|---|---|
| Glycosyltransferase family 28, N-terminal domain | IPR004276 | 4 |
| Glyoxalase/fosfomycin resistance/dioxygenase domain | IPR004360 | 2 |
| GNAT domain | IPR000182 | 5 |
| HAT, C-terminal dimerisation domain | IPR008906 | 2 |
| Heavy metal-associated domain, HMA | IPR006121 | 2 |
| HECT domain | IPR000569 | 2 |
| Helicase, C-terminal | IPR001650 | 1 |
| Heterokaryon incompatibility | IPR010730 | 10 |
| HUWE1/REV1, ubiquitin-binding motif | IPR025527 | 6 |
| Integrase zinc-binding domain | IPR041588 | 1 |
| Kinetochore-associated protein Dsn1/Mis13 | IPR013218 | 2 |
| Kynurenine formamidase/cyclase-like | IPR007325 | 2 |
| Lipase, secreted | IPR005152 | 1 |
| Luciferase-like domain | IPR011251 | 3 |
| Major facilitator superfamily | IPR011701 | 7 |
| Major facilitator, sugar transporter-like | IPR005828 | 18 |
| Metallo-beta-lactamase | IPR001279 | 4 |
| Multicopper oxidase, C-terminal | IPR011706 | 4 |
| Multicopper oxidase-like, N-terminal | IPR011707 | 3 |
| Mycotoxin biosynthesis protein UstYa-like | IPR021765 | 1 |
| NACHT nucleoside triphosphatase | IPR007111 | 4 |
| NAD(P)-binding domain | IPR016040 | 2 |
| NAD-dependent epimerase/dehydratase | IPR001509 | 4 |
| NADPH-dependent FMN reductase-like | IPR005025 | 9 |
| Nitroreductase | IPR029479 | 3 |
| NWD NACHT-NTPase, N-terminal | IPR031359 | 1 |
| Peptidase M3A/M3B catalytic domain | IPR001567 | 4 |
| Plasmid encoded RepA protein | IPR006881 | 1 |
| Prion-inhibition and propagation, HeLo domain | IPR029498 | 2 |
| Protein kinase domain | IPR000719 | 8 |
| Protein of unknown function DUF3292 | IPR021709 | 3 |
| Protein of unknown function DUF3295 | IPR021711 | 2 |
| Protein of unknown function DUF3659 | IPR022124 | 41 |
| Protein of unknown function DUF4748 | IPR031833 | 2 |
| RadC-like JAB domain | IPR025657 | 2 |
| Reverse transcriptase domain | IPR000477 | 6 |
| Reverse transcriptase, RNase H-like domain | IPR041373 | 2 |
| Reverse transcriptase/retrotransposon-derived protein, RNase H-like domain | IPR041577 | 1 |
| Rhodopsin domain, fungi | IPR049326 | 11 |
| Sel1-like repeat | IPR006597 | 1 |

| | | | |
|---|---|---|---|
| Septin-type guanine nucleotide-binding (G) domain | IPR030379 | 2 | |
| Short-chain dehydrogenase/reductase SDR | IPR002347 | 6 | |
| SPRY domain | IPR003877 | 1 | |
| SPRY-associated | IPR006574 | 2 | |
| SsuA/THI5-like | IPR015168 | 2 | |
| Staphylococcal nuclease (SNase-like), OB-fold | IPR016071 | 1 | |
| Starter unit:ACP transacylase | IPR032088 | 1 | |
| Sulfatase, N-terminal | IPR000917 | 2 | |
| Tetratricopeptide TPR-3 | IPR011716 | 4 | |
| Transcription factor domain, fungi | IPR007219 | 16 | |
| Translocator protein BipB-like, C-terminal domain | IPR006972 | 1 | |
| tRNA-guanine(15) transglycosylase-like | IPR002616 | 3 | |
| UPF0758, N-terminal | IPR046778 | 1 | |
| v-SNARE, coiled-coil homology domain | IPR042855 | 1 | |
| WD40 repeat | IPR001680 | 4 | |
| Xaa-Pro dipeptidyl-peptidase, C-terminal | IPR013736 | 7 | |
| Xaa-Pro dipeptidyl-peptidase-like domain | IPR000383 | 2 | |
| YjgF/YER057c/UK114 family | IPR006175 | 2 | |
| Zinc finger, CCHC-type | IPR001878 | 1 | |
| Zinc finger, RING-type, eukaryotic | IPR027370 | 1 | |
| Zn(2)Cys(6) fungal-type DNA-binding domain | IPR001138 | 7 | |
| CAZymes | | | |
| CMB21 | 1 | | |
| AA3_2 | 4 | | |
| GH43 | 1 | | |
| GH3 | 2 | | |
| GT1 | 1 | | |
| Virulence-Associated | | | |
| FUG1 | PHI:6262 | 2 | Role in pathogenicity and fumonisin biosynthesis |
| FDB2 | PHI:4602 | 7 | N-malonyltransferase encoding gene |
| FTF1 | PHI:5483 | 2 | *Fusarium* transcription factor |
| FgPEX1_(FGSG_07104) | PHI:9222 | 5 | Peroxin involved in Deoxynivalenol (DON) biosynthesis |
| FgABC1_Fg10995_Fgm5_(FGSG_10995) | PHI:3924_8224_9036 | 3 | ATP-binding cassette (ABC) transporter |




Supplemental Figure S1 – OrthoFinder phylogeny of Orthogroup 0000000 containing the *Fusarium annulatum* prolipyrone B/gibepyrone D biosynthesis gene and the orthologous proteins of 11 *Fusarium* spp.