Ph.D. thesis

Comparative transcriptomic studies of plant - *Armillaria* interactions

by

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Under the supervision of:

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in

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INTRODUCTION

Armillaria fungi are notorious plant pathogens responsible for significant economic losses in forestry and agriculture. They infect living trees and possess a formidable ability to decompose wood, contributing to forest decline. Understanding the mechanisms employed by *Armillaria* species for both plant infection and wood decay is crucial for developing effective management strategies.

By investigating the enzymatic profiles of two *Armillaria* species with contrasting lifestyles, the highly virulent pathogen *Armillaria ostoyae* and the mostly saprophytic and secondary pathogen *Armillaria borealis*, this study addresses this gap in knowledge. Employing a two-pronged approach of transcriptome analysis and homology searches against known virulence factors, we aimed to identify key players involved in their pathogenic strategies and wood degradation capabilities. This research also explored the mycoremediation potentials of these fungal species.

This study seeks to uncover the intricate relationship between these fungi and plants, offering valuable knowledge on their molecular interactions and serving as a foundation for innovative strategies to manage Armillaria spread and combat other plant diseases. Ultimately, this research holds the potential to contribute significantly to protecting the health and resilience of forests.

OBJECTIVES

With this study we aimed to:

1. Identify and functionally characterise the virulence factors employed by *A. ostoyae* and *A. borealis.*
2. Compare the wood-degrading enzyme profiles of *A. ostoyae* and *A. borealis*.
3. Assess the mycoremediation potential of *A. ostoyae* and *A. borealis*.

METHODS

Experimental Setup:

- Freshly cut Norway spruce stems were divided into two groups: 'fresh' (F) and 'autoclaved' (A). The 'fresh' group represented live tree conditions, while the 'autoclaved' group represented dead wood, eliminating fungal growth and plant defense responses.
- Small 'windows' were cut in the bark to monitor fungal growth.
- Stems were incubated in controlled conditions (24°C, dark) on RSTO medium, a nutrient-rich substrate, inoculated with high and low virulent isolates of *A. ostoyae* and *A. borealis*.
- Mycelial fans formed under the bark were collected for RNA extraction.
- Control mycelia were grown on RSTO medium without stem segments.

RNA Extraction and Sequencing:

- Mycelial samples were grounded in liquid nitrogen.
- RNA was extracted using the RNeasy Plant Midi Kit (Qiagen).
- RNA quality and quantity were assessed using Qubit."
- RNA-seq libraries were prepared using the NEBNext Ultra II Directional RNA Library Prep Kit.
- Libraries were sequenced on the Illumina NovaSeq 6000 platform.

Transcriptome Analysis:

- Raw reads were quality-filtered using FastQC and Trimmomatic.
- Reads were aligned to the reference genomes using STAR.
- Gene expression was estimated using RSEM.
- Data was normalized using TMM method in edgeR.
- Differentially expressed genes (DEGs) were identified using Limma-Voom (log2 fold change ≥ 2, FDR ≤ 0.05).
- Functional annotation was performed using InterProScan, PANZERR, KofamScan, and dbCAN2.
- CAZyme analysis was performed using dbCAN2, focusing on plant cell wall degrading enzymes.

Virulence and Wood Degradation Gene Selection:

- Comparative transcriptome analysis was conducted to identify DEGs related to virulence (fresh stems) and wood degradation (autoclaved stems).

- SignalP, TMHMM, WoLF PSORT, DeepLoc, and OutCyte were used to predict protein localization and secretion.
- PHI database comparison was used to identify genes associated with pathogen-host interactions.
- Extracellularly targeted, upregulated genes were selected as candidate virulence and wood degradation factors.

Bioremediation Gene Identification:

- Comparative genomics analysis was performed using *Armillaria* and other fungal species.
- KEGG orthology IDs were assigned using KofamScan.
- Biocatalysis/Biodegradation Database and KEGG were used to identify xenobiotic degradation enzymes.
- Phylogenetic PCA and phylogenetic tree analysis were performed using R.
- Motif analysis was performed using MEME Suite.

RESULTS AND DISCUSSION

Focusing on highly virulent isolates, we identified DEGs with potential roles in virulence, with CAZymes emerging as a prominent group. Notably, *A. ostoyae* exhibited a significantly higher abundance of PCWDEs compared to both its less-virulent counterpart and *A. borealis* isolates, suggesting a more aggressive strategy for breaching the host cell wall. While some of the CAZyme repertoire is shared between the fungi, species-specific differences were also observed, highlighting unique enzymatic profiles.

The high abundance of PCWDEs observed in *A. ostoyae* suggests a well-developed enzymatic arsenal for dismantling plant cell walls. Key players include endopolygalacturonase, responsible for breaking down cell wall components, and cellobiohydrolases, adept at breaching plant defences. Peroxidases mitigate oxidative stress during infection, while xylanases facilitate fungal spread within the host. Pectate lyase further contributes by dismantling pectin, while multicopper oxidases secure vital iron required by the fungus. The presence of a glucosidase suggests a potential role in early infection stages, possibly influencing protein folding. The function of the CBM5 domain remains to be elucidated but may be linked to virulence.

A. ostoyae also possesses a broader array of virulence factor candidates beyond CAZymes. FAD-binding domain proteins may function in detoxifying host defences, while cytochrome P450 enzymes could be involved in either detoxification or virulence factor biosynthesis. An alkaline phosphatase homolog suggests the fungus might target the host's phosphate resources, and a palmitoyl protein thioesterase Ppt1 homolog hints at potential involvement in secondary metabolite biosynthesis, which could contribute to virulence. Finally, a FvScp1 homolog might be part of a complex regulating virulence factors.

While *A. borealis* shares some CAZymes with *A. ostoyae*, such as endopolygalacturonase and pectate lyases, it also possesses a distinct set of virulence factors. A GH28 protein homolog might contribute to pectin degradation. Similar to *A. ostoyae,* specific FAD-binding domain proteins and a

cytochrome P450 enzyme homolog are present, suggesting potential roles in detoxification. Cerato-platanin family homologs may manipulate the host's defences, while a peptidase S8/S53 domain homolog might target and degrade specific host proteins, suppressing their defence mechanisms. Finally, a thioredoxin domain homolog highlights its potential importance for virulence by ensuring proper protein folding.

Armillaria fungi are not just equipped to compromise host defences, they possess an arsenal of wood-degrading enzymes to break down and utilise the complex polysaccharides within the plant cell wall. The analysis of wood-degrading enzyme profiles in *A. ostoyae* and *A. borealis* reveals an interplay between shared and unique enzymatic capabilities. This tailored enzyme machinery allows both species to efficiently deconstruct the complex plant cell wall, but *A. ostoyae* appears to possess a potentially stronger lignin and pectin degradation arsenal.

Both *A. ostoyae* and *A. borealis* employ a diverse array of cellulases (GH families 1, 3, 6, 7, 12) for a coordinated attack with each family likely contributing to a specific enzymatic activity. This synergy is essential for efficient degradation. Endoglucanases create access points, while cellobiohydrolases steadily break down cellulose from the ends. While both species share this core set, A. ostoyae possesses the AA16 enzyme, potentially disrupting cellulose structure for other cellulases to access. A. borealis, on the other hand, has a unique β-1,4-endoglucanase (GH5_5) and inducible LPMOs (AA9) enzymes, suggesting a potentially distinct or adaptable strategy.

Analysis reveals a diverse array of hemicellulases (CE16, GH10, GH31, GH35, GH51) present in both species, targeting various hemicellulose components like xylan, arabinoxylan, and glucuronoxylan. This shared repertoire suggests a well-coordinated strategy for depolymerizing hemicellulose polymers, a crucial step in unlocking the nutrients they encapsulate. However, *A. ostoyae* possesses α-fucosidases (GH29), suggesting a unique enzymatic capability, while *A. borealis* has a unique endo-α-1,5-arabinanase (GH93), highlighting potential variations in their degradation strategies.

The identification of lignin-modifying enzymes (AA1_1, AA2, AA3_2) in both fungal species aligns with previous findings. However, *A. ostoyae* exhibits a higher abundance of laccases (AA1_1) and aryl-alcohol oxidases (AA3_2), enzymes that break down lignin components. This suggests a potentially stronger capacity for lignin breakdown, providing *A. ostoyae* with a selective advantage for faster cell wall degradation. Furthermore, *A. ostoyae* displays constitutive expression of the glyoxal oxidase AA5_1 enzyme, another H2O2-generating enzyme known to facilitate lignin breakdown by peroxidases.

Both species upregulate pectin-targeting enzymes (CE8, CE12, GH28, GH78, GH88), highlighting their ability to soften the cell wall and access nutrients. However, *A. ostoyae* displays a wider range of constitutively expressed pectinases compared to *A. borealis*, suggesting a potentially stronger and more efficient pectin degradation capability.

While CAZymes are the core wood-breakdown machinery, non-CAZyme secretory candidates reveal an intriguing dimension. This study identified a suite of non-CAZyme secretory candidates with diverse predicted functions, potentially acting synergistically with CAZymes to deconstruct wood components.

These candidates include enzymes like alpha/beta hydrolases (potentially aiding in hemicellulose and pectin breakdown), endonucleases/exonucleases/phosphatases (for various polysaccharide degradation), and fungal lipase-like domains (potentially involved in plant cell wall deacylation in *A. ostoyae*). Notably, the presence of intradiol ring-cleavage dioxygenases suggests a ligninolytic strategy, with *A. ostoyae,* the necrotroph, exhibiting more genes compared to the milder pathogen *A. borealis*.

Furthermore, enzymes like carboxylesterases (potentially modifying lignin structure in *A. ostoyae*) and cytochrome P450s (with multifaceted roles in detoxification and wood component modification) contribute to the overall degradation process. Additionally, calcineurin-like phosphoesterase domains and lysine-specific metallo-endopeptidases likely play supporting roles in nutrient acquisition and protein degradation, respectively.

These non-CAZyme candidates highlight the complexity of the wood-degrading arsenal in *Armillaria* species. Their diverse functionalities likely act together with CAZymes to achieve efficient wood deconstruction and nutrient acquisition during fungal colonisation.

This study also investigated the mycoremediation potential of *A. ostoyae* and *A. borealis* through a comparative genomic analysis. The results revealed promising capabilities for degrading various monocyclic aromatic compounds, while suggesting limitations for PAH degradation compared to other basidiomycetes.

Both *A. ostoyae* and *A. borealis* possess a richer genetic repertoire for degrading various monocyclic aromatic hydrocarbons compared to other fungal counterparts. This suggests a potentially enhanced capacity for these *Armillaria* species to tackle a wider range of aromatic substrates in contaminated environments. While genes associated with PAH degradation were identified, their overall abundance was lower compared to other basidiomycetes. Future research should focus on validating these findings in field studies, optimising environmental conditions for mycoremediation, exploring enzyme applications, and developing strategies for large-scale mycoremediation applications.

SUMMARY

This research provides valuable insights into the variations in pathogenic strategies of *Armillaria* species, laying the foundation for developing targeted approaches to control these destructive pathogens. Additionally, the study highlights the potential of *A. ostoyae* and *A. borealis* for bioremediation, particularly for monocyclic aromatic compounds. Further research efforts are crucial to translate these promising findings into practical applications for environmental cleanup. Understanding these mechanisms paves the way for a more comprehensive approach to managing *Armillaria* species in both agricultural and environmental contexts.

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