Summary of Ph.D. dissertation

Exploring the molecular machinery of Armillaria ostoyae: Advancing insights into pathogenicity, bioremediation and self-defences

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INTRODUCTION

Armillaria, a fungal genus widely known as the "honey fungus," belongs to the *Physalacriaceae* family and is renowned for its remarkable wood decay abilities. Some of the pathogenic species, such as *Armillaria ostoyae*, are known to cause root rot diseases in trees worldwide, leading to substantial economic losses. These pathogenic *Armillaria* species, or opportunistic pathogens, infect woody plants, employing various virulence- and pathogenicity-associated proteins and wood-degrading genes to achieve their parasitic objectives.

This dissertation delves into the intricate roles of secretory genes, long non-coding RNAs (lncRNAs), and associated microbiomes in the interaction between Armillaria fungi and plants, aiming to elucidate their influence on fungal virulence. Moreover, the thesis explores the potential mycoremediation capabilities of *Armillaria* species through in silico methods, along with an examination of genes involved in mycoparasitic interactions between *A. ostoyae* and *Trichoderma atroviride*.

OBJECTIVES

1. Conduct in vivo interaction studies between Norway spruce (*Picea abies*) and *A. ostoyae* to reveal the genetic factors involved in *A. ostoyae* virulence and pathogenicity.

2. Identify and characterize long non-coding RNAs (lncRNAs) within the *A. ostoyae* genome to elucidate their role in the pathogenicity and virulence of the fungus.

3. Investigate the microbial associations with *A. ostoyae* during the plant infection process and explore their potential functional roles in the infection mechanism.

4. Identify and analyze the expression of biocontrol-related genes during the interaction between haploid *A. ostoyae* and *T. atroviride*.

5. Explore the mycoremediation potential of the armillarioids by conducting a comparative analysis with other extensively used fungi in biodegradation processes.

METHODS

Inoculum preparation and infection of host plants

- Inoculum preparation followed the method of Rigling *et al*. (2003).
- Hazelnut stem segments were initially placed in a polypropylene dish with Norway spruce wood chips and double sterilized.
- After sterilization, *A. ostoyae* culture was added, incubated in the dark at 25°C for 4 months.
- Spruce seedlings were cultivated in a nursery and transferred to pots with Okohum GmbH soil and fertilizer.
- Plastic pipes served as a stand-in for *A. ostoyae* inoculum. Control pots without seedlings were prepared for *A. ostoyae* control.
- Plastic pipes were replaced with *A. ostoyae*-colonized hazelnut stem segments and covered with soil.
- Inoculated seedlings were kept in the forest nursery and watered as needed.

Assessment of fungus-inoculated seedlings and sample collection:

- Monthly monitoring for *Armillaria* root rot symptoms in seedlings.
- Sixteen months after inoculation, seedlings were categorized based on health status and symptoms they showed. The outcomes depicted by infected *P. abies* could be classified into two groups: sudden dieback (Necro) or Symptomatic (Symp).

- *A. ostoyae* rhizomorphs (Rhizo) harvested from pots having only soil were used as control and mycelium harvested from sudden dieback (Necro) and symptomatic (Symp) seedlings as test.

RNA-Sequencing and bioinformatics analysis:

- RNA from Rhizo of *A. ostoyae* and mycelium samples from Symp and Necro seedlings were randomly extracted.
- Library preparation, sequencing, and quality assessment were performed using standard protocols.
- Transcriptome analysis included read quality check, alignment, quantification, and differential expression analysis.
- Functional annotation, enrichment analysis, and identification of secretory proteins were carried out.
- Feature identification was conducted utilizing supervised learning algorithms, including Random Forest and XG-Boost to pinpoint specific features that might contribute to the white-rot lifestyle of *A. ostoyae.*
- Long non-coding RNAs (lncRNAs) were predicted using our own pipeline and differential expression analysis was performed.
- Target gene prediction and functional enrichment analysis of lncRNAs
- For metatranscriptome analysis, host and fungal reads were identified and removed.
- Bacterial community identification and metatranscriptome differential expression analysis.

Transcriptomic analysis of mycoparasitic interactions: A. ostoyae vs T. atroviride

- Dual-culture assay between *A. ostoyae* and *T. atroviride* was performed.
- Samples were collected at three different time points: 0^{th} (Control), 53^{rd} (Metabolite interaction stage) and 62^{nd} (Mycoparasite interaction stage) hours.
- Molecular mechanisms of mycoparasitic interaction were studied for both the fungi.

Comparing biodegradation prospects of the armillarioids

- A total of 36 fungal species were included in the comparative analysis.
- Phylogenetic PCA analysis was performed to understand how armillarioids grouped based on xenobiotic-degrading enzyme copy numbers.
- Motif analysis was conducted to identify conserved patterns or motifs within the sequences of interest.
- Gene expression profiles of xenobiotic degrading genes were verified in vitro stem invasion assays.

RESULTS

In vivo infection of spruce seedlings and investigation of secretory proteins:

- Two outcomes observed: curled leaves and wilting, sudden dieback with drying brown needles.
- Kofam enrichment analysis demonstrated that the Symp exhibited a significant enrichment of genes associated with detoxification, cellulose degradation, and MFS transporters whereas Necro exhibited dominant enrichment of genes related to carboxypeptidase, pectin, and lignin degradation.

Comparative study of secretory genes during in vivo and in vitro plant tissue invasions:

- Analysis compared secretory genes upregulated in Symp and Necro with those in wood-degrading and live stem invasion experiments.
- Unique gene expression patterns during *in vivo* invasion, especially in Necro.
- UpsetR analysis of protein families and CAZymes identified those uniquely upregulated in Symp and Necro.
- The signaling mucin MSB2 gene was present only in the *in vivo* experiment and was upregulated in both Symp and Necro.

CAZyme and secondary metabolite analysis:

- Differential expression of 114 CAZyme-related genes grouped into 74 families.

- CAZyme comparison highlighted unique groups in Symp and Necro, indicating involvement in live tissue interactions.
- Seventy-two secondary metabolite-related genes were upregulated, with distinct patterns in Rhizo, Symp, and Necro.
- Terpene-related genes were dominant in Rhizo, while NRPS-like genes were prevalent in Symp and Necro.
- NRPS- and siderophore-related cluster genes appeared specific to Necro.

Pathogenicity model for A. ostoyae infection strategies:

- Boruta and XGBoost identified 82 and 109 orthogroups as essential features to distinguish white rot fungi from soft rots, brown rots and mycorrhizal fungi.
- Machine learning algorithms (Boruta and XGBoost) identified essential features, including protein families like the major facilitator superfamily, fungal ligninase, zinc finger, GMC-oxidoreductases (AA3_2) and GH28.
- Strong correlation between captured features and fungal lifestyle, particularly in the context of white-rot invasion.

lncRNAs in the A. ostoyae genome:

- *A. ostoyae* 4116 lncRNAs were discovered using *A. ostoyae* genome.
- The majority of identified lncRNAs were intergenic (70.9%), followed by antisense (16.7%), novel (7.09%), intronic (2.6%), and sense (2.5%) lncRNAs.

- A significant proportion (83.01%) of *A. ostoyae* lncRNAs had three or fewer exons, contrasting with protein-coding transcripts (21.8%).

Differential gene expression analysis:

- 59, 34, and 38 lncRNAs were upregulated in Rhizo, Symp, and Necro, respectively.
- MSTRG.7390.2 and MSTRG.17337.1, exhibited the highest expression in Symp, while MSTRG.10210.4 and MSTRG.15406.1 in Necro.

lncRNAs as co-regulators of infection:

- Functional analysis of target transcripts regulated by differentially expressed lncRNAs identified enrichment in various biological processes related to cell cycle, chromatin regulation, metabolism, signaling, and stress response.
- The analysis revealed 2939 cis- and 2185 trans-regulated targets of the differentially expressed lncRNAs.
- Genes associated with response to chemical stimulus, gene silencing, signal transduction, and transport were overrepresented among the cis-regulated targets.
- Trans-regulated targets included genes involved in gene silencing, regulation of mitochondrion organization, and cyclase activity regulation.
- The study identified correlations between DE lncRNAs and DE genes involved in regulating gene expression, plant cell wall degradation, transport, degradation of aromatics, secondary metabolite synthesis, and signal transduction.

Microbiome analysis of A. ostoyae:

- CCMetagen classified reads into 55 phyla and 765 genera, with dominant bacteria including Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes.
- Genera like *Pseudomonas, Rhizobium, Streptomyces* constituted 26 percent of the identified genera.

Bacterial community patterns:

- Rhizo exhibited higher microbial richness and faith diversity compared to Symp and Necro.
- Symp showed dominance of particular species, while Necro demonstrated an even distribution of species compared to Symp.
- The PERMANOVA test indicated significant differences in community compositions among Rhizo, Symp, and Necro.
- Top 20 taxa contributing to the discrimination of Symp, Necro and Rhizo included *Sphingomonas, Rhizobium, Frankia, Acidovorax,* and others.

Differential abundance analysis:

- Significant differences ($p < 0.01$) in bacterial taxa between Rhizo vs. Symp and Rhizo vs. Necro.
- Top 5 significant genera in Rhizo included *Acidovorax, Rhodoferax, Bdellovibrio, Byssovorax*, and *Luteolibacter*.
- *Chroococcidiopsis, Geodermatophilus, Nevskia, Nakamurella,* and *Enterobacter* were found to be significantly occurring in Symp, while

Salinibacterium, Subtercola, Cryptosporangium, Methylobacterium, and *Aeromicrobium* were found to be significant in Necro
- Genera such as *Actinomycetospora, Friedmanniella, Hymenobacter, Microlunatus,* and *Sphingomonas* were significantly enriched in both Symp and Necro.

Differential metatranscriptome analysis:

- 42,374 transcripts analyzed, revealing 5,971 differentially expressed genes (DE) between samples.
- 3,147 genes upregulated in Symp, 3,116 in Necro, with 1,844 genes upregulated in both.

Functional analysis of the metatranscriptome:

- 8% of transcripts successfully annotated using InterPro database.
- GO enrichment analysis indicated biological processes related to membrane proteins and respiration in both Symp and Necro.
- 133 carbohydrate-active enzymes (CAZymes) upregulated in Symp, 114 in Necro, with GT2 type CAZyme most commonly upregulated in both conditions.

Interaction of A. ostoyae with T. atroviride:

- A total of 414 Small Secreted Proteins (SSPs) were identified in *A. ostoyae*, with 382 consistently expressed during the interaction. Notably, SSPs associated with lectin activity, hydrophobin activity, and chitin binding showed significant elevation.

- *T. atroviride* exhibited differential expression of 157 SSP-related genes during the 62nd hour, with cerato-platanin, cerato-ulmin hydrophobin, RlpA-like protein, and peptidase S51 activities being highly elevated.
- *A. ostoyae* displayed upregulation of 110 proteins with CAZyme annotation during the interaction, particularly genes associated with CBM50, mannanases, glucanases, and chitin-binding proteins.
- *T. atroviride* expressed 40 genes associated with FCWD, including mannanases, glucanases, chitin-binding proteins, and other cell wall-degrading enzymes, indicating its response to the interaction.
- Peptidase-related genes with differential expression were found in both fungi, with *A. ostoyae* showing upregulation of 151 genes, particularly aspartic peptidase A1A and serine peptidase S12 during the 62nd hour. *T. atroviride* profiles showed 52 differentially expressed secreted peptidases, with notable upregulation of aspartic peptidases (A01A) and serine peptidases (S01A, S08A, S09X, S12, and S54) during the 62^{nd} hour.
- The overexpression of A1 and G1 peptidases in *T. atroviride* during the mycoparasite stage suggests a potential role in pathogenicity and host adaptability towards *A. ostoyae*.

Genome-level comparative analysis identifies specialization of armillarioid fungi:

- Comparative analysis of 92 distinct enzymes related to biodegradation activities revealed a distinct

clustering of armillarioid species, setting them apart from other white-rotting fungi.

- Notably, genes encoding benzoate-4-monooxygenase and NADPH2 dehydrogenase with high copy numbers in armillarioid genomes contributed significantly to this distinctive clustering.

Checking the expression profiles of mycoremediation-related genes:

- Analysis of genes potentially involved in mycoremediation, using raw RNA-Seq data from *in vitro* stem invasion assays, revealed that a significant proportion of identified mycoremediation genes were active in both *A. ostoyae* and *A. borealis*.
- In particular, benzoate-4-monooxygenase genes exhibited interesting expression patterns in response to stem invasion conditions, with a notable presence of armillarioid-specific residues in certain binding sites.
- A significant proportion of benzoate-4-monooxygenase genes were upregulated in both *A. ostoyae* and *A. borealis* during stem invasion, suggesting their active role in the plant environment.
- One gene in *A. borealis* (Ambor|1721289) was identified as significantly overexpressed in the virulent isolate compared to the less virulent counterpart under conditions of plant invasion, indicating its potential contribution to virulence.

SUMMARY

The study examined the effects of an *A. ostoyae* inoculum on *P. abies* seedlings and found two different infection outcomes. *A. ostoyae* mycelia from symptomatic and sudden dieback plants were subjected to transcriptome studies, which revealed distinct gene expression patterns associated with the breakdown of cellulose, pectin, hemicellulose, and aromatics. Tissue-specific upregulation of secretory proteins during host invasion was observed by gsecretome profiling. Functional characterization linked several gene groups, including metallopeptidases and hydrophobins, to virulence. The orthogroup study revealed relationships between fungal tactics and orthogroup repertoires, allowing for the lifestyle-based differentiation of fungi. The discovery of long non-coding RNAs points to their potential regulatory function during infection. According to the microbial community analyses, *A. ostoyae* mycelia was associated with aromatics degrading bacteria. The study provides insights into potential applications for the treatment of fungal infections and advances our knowledge of the infection mechanisms of *A. ostoyae*.

In addition to studying the interactions between *A. ostoyae* and *T. atroviride*, the research explores gene expression differences during various stages of their interaction, revealing the upregulation of cerato-ulmin hydrophobin and G1 peptidase in *T. atroviride*. The study delves into the dynamics of carbohydrate-active enzymes (CAZymes) and proteases during the interaction, suggesting the involvement of these enzymes in mycoparasitism. The investigation also highlights the mycoremediation potential of

armillarioid species, emphasizing their expanded repertoires of benzoate 4-monooxygenase-degrading genes and NADPH2 dehydrogenases, making them suitable for handling specific environmental contaminants.

LIST OF PUBLICATIONS

MTMT Author ID: 10072893

Cumulative impact factor (IF) of the publications directly related to the thesis: 61.5

Mandatory peer-reviewed publications for the fulfilment of the doctoral process:

1. **Champramary, S***., Indic, B., Szűcs, A., Languar, O., Hasan, F. K., Szekeres, A., ... & Sipos, G. The mycoremediation potential of the Armillarioids: a comparative genomics analysis. Frontiers in Bioengineering and Biotechnology, 11, 1189640. (IF 2023: 6.1)

2. Chen, L*., **Champramary, S***., Sahu, N., Indic, B., Szűcs, A., Nagy, G., ... & Sipos, G. (2023). Dual RNA-Seq profiling unveils mycoparasitic activities of *Trichoderma atroviride* against haploid *Armillaria ostoyae* in antagonistic interaction assays. Microbiology Spectrum, e04626-22. (IF 2023: 9.0)

Other Publications:

Sahu, N., Indic, B., Wong-Bajracharya, J., Merényi, Z., Ke, H. M., Ahrendt,….., **Champramary S**.,…... & Nagy, L. G. (2023). Vertical and horizontal gene transfer shaped plant colonization and biomass degradation in the fungal genus *Armillaria*. Nature Microbiology, 1-14. (IF 2023 : 31.0)

Bhattacharya, A., **Champramary, S**., Tripathi, T., Thakur, D., Ioshikhes, I., Singh, S. K., & Nandi, S. (2021). Identification of the conserved long non-coding RNAs in myogenesis. BMC genomics, 22(1), 336. (IF 2021 : 4.6)

Kedves, O., Shahab, D., **Champramary, S**., Chen, L., Indic, B., Bóka, B., ... & Sipos, G. (2021). Epidemiology, Biotic

Interactions and Biological Control of Armillarioids in the Northern Hemisphere. Pathogens, 10(1), 76. (IF 2021: 3.5)

Chen, L., Bóka, B., Kedves, O., Nagy, V. D., Szűcs, A., **Champramary, S**., ... & Kredics, L. (2019). Towards the biological control of devastating forest pathogens from the genus *Armillaria*. Forests, 10(11), 1013. (IF 2019: 2.2)

Conference paper:

Simang, Champramary ; Boris, Indic ; László, Kredics ; György, Sipos. A comparison of the wood decay abilities of common white-rot fungi from the Carpathian Basin. 10TH HARDWOOD CONFERENCE PROCEEDINGS: University of Sopron Press (2022) 323 p. pp. 188-193.

Liqiong, Chen ; Danish, Shahab ; Orsolya, Kedves ; **Simang, Champramary**; Boris, Indic ; Viktor, Dávid Nagy ; Csaba, Vágvölgyi ; László, Kredics ; György, Sipos. Armillarioid root rot invasion: possibilities of silvicultural and chemical control. Hardwood Conference Proceedings 9TH HARDWOOD PROCEEDINGS: PART II pp. 90-97. , 8 p. (2021)

Conference abstracts:

Boris, Indic ; **Simang, Champramary** ; Liqiong, Chen ; Huynh, Thu ; Orsolya, Kedves ; Ferenc, Lakatos ; Csaba, Vágvölgyi ; László, Kredics ; György, Sipos. Phylogenetic analysis shows contrasting genetic diversity among various Armillarioid species in Pannonian forests. 10TH HARDWOOD CONFERENCE PROCEEDINGS: University of Sopron Press (2022) 323 p. p. 203

Chen L ; **Champramary S**; Sahu N; Indic B ; Csaba, Vágvölgyi ; László, Kredics ; György, Sipos et al., *In vitro* transcriptome level interactions between mycoparasitic

Trichoderma atroviride and haploid *Armillaria ostoyae* uncovered by dual RNA-Seq profiling: (2022) 54 p. p. 12 , 1 p.

Bencsik-Bóka, B ; Sahu, N ; Huynh, T ; Kedves, O ; Merényi, Z ; Kovács, G ; Chen, L ; **Champramary, S** ; Patocskai, Z ; Münsterkötter, M et al., Classical and 'omics' approaches towards the biological control of devastating forest pathogens from the genus *Armillaria*. Hungarian Society for Microbiology (2018) 70 p. p. 6

Champramary, S, Münsterkötter M, György, S. Rhizomorph formation in *Armillaria ostoyae*: the contribution of rhizomorph-specific genes (2019), Zürich Mycology Symposium 2019, 2019.01.18.,

Indjic, B ; **Champramary, S** ; Münsterkötter, M ; Kredics, L ; Sipos, G. The Genome Of The Pathogenic White Rot Fungus *Armillaria Ostoyae* Encodes A Distinctive Genetic Potential To Degrade Aromatic Compounds. HASAT INTERNATIONAL AGRICULTURE AND FOREST CONGRESS:Adnan Menderes University (2019) p. 267 , 1 p.

Other publication not related to thesis:

Hasan, K. F., **Champramary, S**., Al Hasan, K. N., Indic, B., Ahmed, T., Pervez, M. N., ... & Bejó, L. (2023). Eco-friendly production of cellulosic fibers from Scots pine wood and sustainable nanosilver modification: A path toward sustainability. Results in Engineering, 101244. (IF 2023: 5.1)

Declaration

I declare that the contribution of Simang Champramary was significant in the listed publications and the doctoral process is based on the publications listed. The results reported in the Ph.D. dissertation and the publications have not been used to acquire any PhD degree previously and will not be used in the future either.

Szeged, 09.01.2024

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Prof. Dr. Sipos György

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Dr. Nagy Gábor