

**Comparative -omics analyses to understand  
wood decay strategies and evolution of  
pathogenicity in *Armillaria* spp.**

**Thesis Booklet**

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## Introduction

*Armillaria* spp. are devastating pathogens that cause root-rot disease in trees resulting in severe losses in the economy, health, and long-term productivity of forest ecosystems. This group comprises about 70 known species, which exhibit their most prominent ecological lifestyle as a necrotrophic pathogen. Depending on environmental conditions and host availability, *Armillaria* species are reported to attack a wide range of plant hosts, including oak, pine, and agronomic crops, in both the Northern and Southern hemispheres and a range of climates. Whilst being a pathogen, they are also essential in forest ecosystems as saprotrophs thus making them suitable for studying mechanisms of pathogenicity and wood-decay systems in fungi. During the saprotrophic phase, they decay the woody plant biomass by producing a wide arsenal of PCWDEs, thereby playing an important role in carbon and nutrient cycling in forests.

*Armillaria* species have evolved a range of unique features, which conceivably emerged in the most recent common ancestor (MRCA). These include a very low mutation rate, extreme longevity, and size of colonies (>2,500 years, >900 hectares), diploidy, bioluminescence, specialized underground structures known as rhizomorphs, and, economically most importantly, the ability to infect and kill live trees. Once *Armillaria* species infect the root system, they colonize and kill the cambium, ultimately causing plant death. Although the cause and spread of *Armillaria* root disease are well documented, the molecular mechanisms

underlying the infection process remain unclear. Therefore, understanding the biology and molecular aspects of *Armillaria* infection is essential for the development of effective disease control strategies. Recent genome studies have identified that *Armillaria* genomes have an expanded repertoire of protein-coding genes enriched in PCWDEs, chitin-binding proteins, secondary metabolites, and putative pathogenicity genes. Ongoing research explores the mechanisms of pathogenicity in *Armillaria* species and their potential impact on ecosystems and global climate change. Genomic and transcriptomic studies of *Armillaria*, as well as studies of soil microbial communities, have also provided new insights into *Armillaria* biology and potential disease control strategies. However, more research is needed to fully understand the complex interactions between *Armillaria* spp. and their hosts and to develop effective strategies to control diseases spread by *Armillaria* in forest stands.

## Objectives

The genus *Armillaria* includes mushroom-forming fungi that are among the largest and oldest terrestrial organism on Earth. These species efficiently degrade lignocellulosic plant biomass and are also known to cause root-rot disease in woody ecosystems worldwide. Despite their huge impact on forest ecosystems, the biology of *Armillaria* species, especially the molecular level details of the interaction between *Armillaria* and host plants, as well as the strategies for wood-decay are not well explored. A major reason for this is the lack of genetic manipulation tools available for these non-model fungi.

In my doctoral research, we used a combination of gene-expression studies and phylogenomic comparisons to explore the evolutionary routes that contributed in making *Armillaria* species such devastating forest pathogens and efficient wood-decaying saprotrophs. The objectives of this thesis were to answer the following questions:

- What are the wood decay strategies employed by pathogenic and saprobic *Armillaria*?
- Do gene repertoire variations drive the functional diversity in *Armillaria*?
- How distinct wood-decay traits and pathogenicity evolve in *Armillaria*?
- What are the molecular mechanisms underlying the infection process?

# Methods

## **Wood-decay omics in two *Armillaria* species**

- Experimental setup
- Sample preparation and sequencing
- Analysis of multi-omics data (transcriptomic and proteomic analyses of wood decay by *A. ostoyae* and *A. cepistipes*)
- Clustering and functional annotations

## **Comparative phylogenomics with new *Armillaria* genomes**

- Sample preparation for nucleic acid sequencing (8 new *Armillaria* genomes)
- Taxon sampling and dataset preparation
- Gene family evolution in *Armillaria*
- Analyses of CAZymes in *Armillaria* spp.
- Analyses of horizontally transferred genes
- Analysis of new expression data (in-planta and stem-invasion assays)

## Summary of Results

### **Overview of wood-decay omics data in two *Armillaria* species**

To explore the wood-decay patterns employed by *Armillaria* species we investigated the colonization of sterilized spruce roots by *A. ostoyae* and *A. cepistipes* using morphological observations and transcriptomic and proteomic techniques. Our results showed that spruce roots were colonized primarily by mycelium, which had a higher number of differentially expressed genes (DEGs) and differentially expressed proteins (DAPs) compared to rhizomorphs in both species. The upregulation of transporters in the rhizomorphs of both species indicated their possible role in nutrient transfer. In addition, the saprotrophic species *A. cepistipes* showed a higher number of DEG/DAP PCWDEs than the pathogenic (necrotroph) *A. ostoyae*, reflecting a general difference in wood decay strategies between saprotrophic and pathogenic species. We also found a shared gene expression response to spruce root colonization, with a total of 779 co-orthologous genes showing similar DEG patterns in the invasive mycelium of both species. These included gene families such as oxoglutarate/iron-dependent dioxygenases, galactose-binding-like domain superfamily proteins, and intradiol ring cleavage dioxygenases, as well as several families involved in plant cell wall degradation, among which pectinases were the most abundant. Surprisingly, the two *Armillaria* species were found to exhibit a wood-decay profile that, unlike that of white rot (WR) fungi, largely bypassed the

expression of lignin-degrading genes and was characterized by the upregulation of other PCWDEs such as pectinases and cellulases. These expression patterns are more suggestive of soft rot, a known rot type in Ascomycota. Along with *Armillaria*, this appears to be a common wood decay approach among early-diverging Agaricomycetes that contain lignin-degrading genes in their genome, but still produce soft rot (SR) like traits or decay patterns that do not necessarily fit into the traditional WR and brown-rot (BR) dichotomy. We hypothesize that WR fungi evolved in an early ancestor of Agaricomycetes that inherited soft-rot machinery from its common ancestors with the Ascomycota, leading to the combination of WR and SR toolkits in *Armillaria* and other unusual WR fungi. The evolution of such a rot apparatus may allow these fungi to selectively employ either strategy for wood rot. To put our findings in an evolutionary perspective and understand the evolution of the wood-decay apparatus in *Armillaria* species, we further used a comparative phylogenomic approach with new and previously published *Armillaria* genomes.

### **Phylogenomic comparisons using new *Armillaria* genomes**

We examined genomic and gene repertoire variations in *Armillaria* spp. using phylogenomic comparisons with other Agaricales from different ecological lifestyles. Our findings showed that *Armillaria* spp. have an expanded repertoire of protein-coding genes and several novel gene families, including a plethora of lignocellulose degrading PCWDEs. We also

highlighted an over-representation of putative pathogenesis-related gene families in *Armillaria* genomes, including genes related to pectin-degradation, cellulose binding, and other putative extracellular and aromatic compound breakdown processes.

With the new genomes, we were able to make prediction whether variations in the plant cell wall degrading gene repertoire are responsible for the functional diversity of wood-decay strategies in *Armillaria*. Phylogenetic PCAs suggested that variations in the plant cell wall degrading gene repertoire might be responsible for the observed differences in wood-decay strategies between *Armillaria* and other WR fungi. Gene copy numbers of PCWDEs suggested that both gene gains and losses have played a role in the evolution of trophic modes in these fungi and that the strategy of wood decay in *Armillaria* may hint towards soft rot in Ascomycota. Additionally, we identified CAZy orthogroups that were significantly co-enriched and overrepresented in both *Armillaria* and Ascomycota, suggesting co-expansion or horizontal gene transfer (HGT) events.

Using a two-step method to systematically, we identified 101 strongly supported HGT events corresponding to a total of 775 genes in *Armillaria* genomes. Most of these events had Ascomycota donors, predominantly from the Sordariomycetes and Dothideomycetes. Functional annotation of these horizontally transferred genes revealed genes involved in pathogenicity and PCWDEs. Of these a number of



PCWDEs were responsible for the co-enrichment signal observed between Ascomycota and *Armillaria*. These findings suggest that HGT events have played a crucial role in shaping the evolution of pathogenicity and plant cell wall degrading attributes of *Armillaria* species.

Further, to understand the molecular mechanisms underlying the infection process, we analyzed different transcriptomic datasets including our wood-decay omics, previously published developmental data and new RNA-Seq experiments from our collaborators (stem-invasion and root-colonization). Our comparative analysis revealed distinctive enrichment patterns of different gene groups in wood decay, stem colonization, *in-planta* assay, and fruiting body/rhizomorph growth. We found that PCWDE genes associated with cellulose, hemicellulose, pectin, and lignin displayed a distinct enrichment pattern in stem invasion and wood-decay experiments. However, *in-planta* time series experiments showed a downregulation of PCWDEs, and an upregulation of other genes involved in virulence such as cerato-platanins, CAP domains, catalases, and genes related to oxidative stress during host infection. Our analysis also revealed that species and strains may respond differently to substrates/hosts, suggesting that variations in lifestyle or pathogenicity may affect species-specific enrichment patterns.

Overall, based on our results, we find that the evolution of *Armillaria* genomes was marked with multiple types of genetic innovations, including horizontal gene

transfer from Ascomycota. The findings of this work, in combination with existing and emerging pathosystems, could improve research into the molecular mechanisms of plant colonization.

## List of publications (MTMT ID: 10071410)

### **Mandatory peer-reviewed publications for the fulfillment of the doctoral process:**

1. **Sahu, N.**, Indic, B., Wong-Bajracharya, J., Merényi, Z., Ke, H.-M., Ahrendt, S., Monk, T.-L., Kocsubé, S., Drula, E., Lipzen, A., Bálint, B., Henrissat, B., Andreopoulos, B., Martin, F.M., Harder, C.B., Rigling, D., Ford, K.L., Foster, G.D., Pangilinan, J., Papanicolaou, A., Barry, K., LaButti, K., Virágh, M., Koriabine, M., Yan, M., Riley, R., Champramary, S., Plett, K.L., Grigoriev, I.V., Tsai, I.J., Slot, J., Sipos, G., Plett, J., Nagy, L.G., 2022. Genomic innovation and horizontal gene transfer shaped plant colonization and biomass degradation strategies of a globally prevalent fungal pathogen (preprint). <https://doi.org/10.1101/2022.11.10.515791> (IF: NA)
2. **Sahu, N.**, Merényi, Z., Bálint, B., Kiss, B., Sipos, G., Owens, R.A., Nagy, L.G., 2021. Hallmarks of Basidiomycete soft- and white-rot in wood-decay -omics data of two *Armillaria* species. *Microorganisms* 9, 149. <https://doi.org/10.3390/microorganisms9010149> (IF<sub>2021</sub>: 4.926)

### **Complete list of publications (Cumulative impact factor: 71.64)**

1. Nagy, L.G., Vonk, P.J., Künzler, M., Földi, C., Virágh, M., Ohm, R.A., Hennicke, F., Bálint, B., Csernetics, Á., Hegedüs, B., Hou, Z., Liu, X.B., Nan, S., Pareek, M., **Sahu, N.**, Szathmári, B., Varga, T., Wu, H., Yang, X., Merényi, Z., 2023. Lessons on fruiting body

morphogenesis from genomes and transcriptomes of Agaricomycetes. Stud. Mycol.

<https://doi.org/10.3114/sim.2022.104.01> (IF<sub>2021</sub>: 25.56)

2. Merenyi, Z., Krizsan, K., **Sahu, N.**, Liu, X.-B., Balint, B., Stajich, J., Spatafora, J.W., Nagy, L.G., 2022. Taxonomic vs genomic fungi: contrasting evolutionary loss of protistan genomic heritage and emergence of fungal novelties (preprint).

<https://doi.org/10.1101/2022.11.15.516418>

3. **Sahu, N.**, Indic, B., Wong-Bajracharya, J., Merényi, Z., Ke, H.-M., Ahrendt, S., Monk, T.-L., Kocsubé, S., Drula, E., Lipzen, A., Bálint, B., Henrissat, B., Andreopoulos, B., Martin, F.M., Harder, C.B., Rigling, D., Ford, K.L., Foster, G.D., Pangilinan, J., Papanicolaou, A., Barry, K., LaButti, K., Virágh, M., Koriabine, M., Yan, M., Riley, R., Champramary, S., Plett, K.L., Grigoriev, I.V., Tsai, I.J., Slot, J., Sipos, G., Plett, J., Nagy, L.G., 2022. Genomic innovation and horizontal gene transfer shaped plant colonization and biomass degradation strategies of a globally prevalent fungal pathogen (preprint). <https://doi.org/10.1101/2022.11.10.515791>

4. Chen, L., Champramary, S., **Sahu, N.**, Indic, B., Szűcs, A., Nagy, G., Maróti, G., Pap, B., Languar, O., Vágvölgyi, C., Nagy, L.G., Kredics, L., Sipos, G., 2022. Dual RNA-Seq profiling unveils mycoparasitic activities of *Trichoderma atroviride* against haploid *Armillaria ostoyae* in antagonistic interaction assays (preprint).

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5. Pareek, M., Hegedüs, B., Hou, Z., Csernetics, Á., Wu, H., Virágh, M., **Sahu, N.**, Liu, X.-B., Nagy, L., 2022. Preassembled Cas9 ribonucleoprotein-mediated gene

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<https://doi.org/10.1128/aem.00940-22> (IF<sub>2021</sub>: 5.005)

6. Virágh, M., Merényi, Z., Csernetics, Á., Földi, C., **Sahu, N.**, Liu, X.-B., Hibbett, D.S., Nagy, L.G., 2022. Evolutionary morphogenesis of sexual fruiting bodies in Basidiomycota: Toward a new evo-devo synthesis. Microbiol. Mol. Biol. Rev. 86, e00019-21. <https://doi.org/10.1128/MMBR.00019-21> (IF<sub>2021</sub>: 13.044)

7. **Sahu, N.**, Merényi, Z., Bálint, B., Kiss, B., Sipos, G., Owens, R.A., Nagy, L.G., 2021. Hallmarks of Basidiomycete soft- and white-rot in wood-decay -omics data of two *Armillaria* species. Microorganisms 9, 149. <https://doi.org/10.3390/microorganisms9010149> (IF<sub>2021</sub>: 4.926)

8. Almási, É\*, **Sahu, N\***, Krizsán, K., Bálint, B., Kovács, G.M., Kiss, B., Cseklye, J., Drula, E., Henrissat, B., Nagy, I., Chovatia, M., Adam, C., LaButti, K., Lipzen, A., Riley, R., Grigoriev, I.V., Nagy, L.G., 2019. Comparative genomics reveals unique wood-decay strategies and fruiting body development in the Schizophyllaceae. New Phytol. 224, 902–915. <https://doi.org/10.1111/nph.16032> (\* Shared first author) (IF<sub>2021</sub>: 10.323)

9. Krizsán, K., Almási, É., Merényi, Z., **Sahu, N.**, Virágh, M., Kószó, T., Mondo, S., Kiss, B., Bálint, B., Kües, U., Barry, K., Cseklye, J., Hegedüs, B., Henrissat, B., Johnson, J., Lipzen, A., Ohm, R.A., Nagy, I., Pangilinan, J., Yan, J., Xiong, Y., Grigoriev, I.V., Hibbett, D.S., Nagy, L.G., 2019. Transcriptomic atlas of

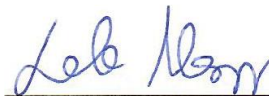
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## Co-author waiver form

As the corresponding and/or contributing author of the below mentioned peer reviewed publication I declare that the authors have no conflict of interest related to this study. I also declare that the PhD candidate Neha Sahu worked under my supervision and her contribution was prominent in obtaining the results, and these publications were not used for PhD defense by anyone of the co-authors.

**1** Sahu, N., Indic, B., Wong-Bajracharya, J., Merényi, Z., Ke, H.-M., Ahrendt, S., Monk, T.-L., Kocsubé, S., Drula, E., Lipzen, A., Bálint, B., Henrissat, B., Andreopoulos, B., Martin, F.M., Harder, C.B., Rigling, D., Ford, K.L., Foster, G.D., Pangilinan, J., Papanicolaou, A., Barry, K., LaButti, K., Virágh, M., Koriabine, M., Yan, M., Riley, R., Champramary, S., Plett, K.L., Grigoriev, I.V., Tsai, I.J., Slot, J., Sipos, G., Plett, J., Nagy, L.G., 2022. Genomic innovation and horizontal gene transfer shaped plant colonization and biomass degradation strategies of a globally prevalent fungal pathogen (preprint).

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