Summary of the PhD Dissertation

Towards Biocontrol of Tree Root Rot Pathogens from the Genus *Armillaria*

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1. INTRODUCTION

Armillaria species are among the most widely distributed fungal phytopathogens varying in pathogenecity and host range. They cause *Armillaria* root rot disease on a wide range of woody hosts and their impacts often result in destructive forest damages and huge economic losses. The serious ecological and economical damages resulting from pathogenic *Armillaria* species require effective control strategies. Concerning the environmental threats posed by fungicides and the undurable and unstable efficacy of silvicultural control practices, biological control measures seem to be promising alternatives. Biocontrol emphasizes on ecological sustainability and environmental protection usually by the exploitation and employment of beneficial microorganisms, including naturally occurring antagonistic fungi.

The use of free-living soil fungi from the genus *Trichoderma* has uncovered great potential to successfully eradicate the pathogenic activities of *Armillaria*. Particularly, native *Trichoderma* species isolated from soil or rhizosphere usually show a better adaptation and thus display more efficient control of diseases than introduced exotic microorganisms. The high efficacy by the employment of *Trichoderma* biocontrol agents to overcome the challenges caused by *Armillaria* has intrigued an increasing interest for unveiling their antagonistic strategies as well as their interaction mechanisms. Antagonistic *Trichoderma* species affected *Armillaria* species usually through competition for nutrients and space, by antibiosis reflected by the growth inhibition of *Armillaria*, or most importantly by direct mycoparasitic action. In our study, *Trichoderma* and *Armillaria* strains were isolated and identified from forest soils; the biocontrol efficiency of various *Trichoderma* isolates was examined, and their molecular interaction mechanisms were investigated through high throughput sequencing technology. Finally, the best biocontrol agents against *Armillaria* species were screened for further field applications.

2. OBJECTIVES

The frequent emergence of *Armillaria* root rot disease in the forests of the Northern Hemisphere and its severe economic consequences often led to the use of environmentally harmful, polluting fungicides. Above and beyond their commercial values, woody plants are essential components of wildlife habitats worldwide. Although *Armillaria* species are regular, natural components of the forests, under extreme biotic and abiotic conditions leading to loss of resistance of their woody host plants, *Armillaria* may become a dominant factor in the forests and cause severe diseases leading to compromised trees and seedlings. Although *Trichoderma* formulas have been applied broadly as important biocontrol agents for controlling a variety of plant pathogens, the

experimental investigation of the efficiency of *Trichoderma* species for the biological control of *Armillaria* species still has a long way to go.

Commercial products based on *Trichoderma* have been available on the market for plant protection. However, isolating and screening for antagonistic *Trichoderma* strains from diverse populations distributed at different geographic regions may be more helpful for developing efficient biocontrol agents against a broad range of pathogens from the genus *Armillaria*. Therefore, we focused on isolation and characterization of *Trichoderma* and *Armillaria* strains from forest soils, as well as on the examination of the biocontrol efficiency of various *Trichoderma* isolates. The best biocontrol agents could be applied for *Armillaria* biocontrol and further field applications.

The aims of this work were:

1) To isolate and identify *Trichoderma* as well as *Armillaria* strains from soil samples collected in both healthy and *Armillaria*-damaged forests
2) To screen for potential biocontrol candidates among the identified *Trichoderma* isolates using *in vitro* dual culture assays and assessing the antagonistic activities, as well as by detecting extracellular enzyme production and plant growth-promoting traits
3) To determine the biocontrol potential of selected *Trichdoderma* strains when confronted with both diploid and haploid isolates of *A. ostoyae*
4) To capture the relevant points of time for adequately assessing the characteristic interaction stages between a selected *Trichoderma* strain and *A. ostoyae* and to investigate the dual RNA-Seq profiles, assess the molecular background of metabolite-level and mycoparasitic (physical) interactions, and dissect the molecular interaction dynamics by time-course transcriptome analyses.
5) To analyze the mycoparasitism-related genes in the examined *Trichoderma* strain for the identification of biocontrol factors
6) To analyze the possible defense mechanisms of *A. ostoyae* for the identification of defense factors
7) To determine the potential of selected biocontrol candidate *Trichoderma* strains to control *Armillaria* in the field

3. METHODS

Collection of soil samples that contained native soil fungi

- Samples of bulk soil (soil outside the rhizosphere), upper rhizospheric soil, *Armillaria* rhizomorphs and their surrounding soil, as well as *Armillaria* fruiting bodies were collected from a heavily *Armillaria*-damaged oak stand (Keszthely Hills, Hungary) and healthy native spruce forests (Rosalia, Austria).

Isolation and identification of *Armillaria* and *Trichoderma* isolates

- *Armillaria* and *Trichoderma* strains were isolated from collected forest soil samples.
- For the identification of isolated strains, PCR-based molecular tools were applied.
- Fungal isolates were deposited in the Szeged Microbiology Collection (SZMC, www.szmc.hu), Szeged, Hungary.

Antagonistic activity assessment *in vitro* by dual culture assay

- *In vitro* dual-culture confrontation test was used for antagonistic activity assessment.
- Biocontrol Index (BCI) values were calculated.

Extracellular enzyme activity measurements

- β-Glucosidase, cellobiohydrolase, β-xylosidase and phosphatase enzyme activities were measured with the chromogenic substrates p-nitrophenyl-β-D-glucopyranoside, p-nitrophenyl-β-D-cellobioside, p-nitrophenyl-β-D-xylopyranoside and p-nitrophenyl-phosphate, respectively.

Quantitative analysis of indole-3-acetic acid production

- Indole-3-acetic acid (IAA) production of *Trichoderma* isolates was analyzed by colorimetric analysis using Salkowsky's reagent.
- The IAA concentration was determined.

Siderophore production

- Siderophore production of *Trichoderma* isolates was determined by using a modified chrome azurol S (CAS) agar test.

Transcriptome analysis of the interaction mechanisms between *Armillaria ostoyae* and *Trichoderma atroviride*

- Strains of *A. ostoyae* and *T. atroviride* were selected based on *in vitro* dual-culture confrontation tests for antagonistic activity assessment.
- Experimental design was set up based on time points including the time point before physical contact and the time point as physical contact happened.
- Mycelium samples were collected.
- Total RNA was extracted.
- Sequencing libraries were prepared for the transcriptome samples using the TruSeq RNA Library Prep Kit v2 (Illumina).
- Paired-end fragment reads were generated on an Illumina NextSeq sequencer using TG NextSeq® 500/550 High Output Kit v2 (300 cycles).
- Quantitative real-time reverse transcription PCR (qRT-PCR) were performed
- Time course analysis was performed.
- Secretory proteins were predicted.
- Functional characterization of proteins was performed by GO annotation and

InterproScan.

- CAZy annotation was performed.
- Proteases were identified.
- Secondary metabolite-related genes were predicted.

Field study in the Keszthely Hills

- A field study was set up in the Keszthely Hills in a forest clearing surrounded by a 2-meter-high fence, located in the central part of a heavily *Armillaria*-damaged Turkey oak (*Quercus cerris*) stand.
- Two-year-old, bare rooted seedlings of *Q. cerris* were planted. Before planting, the roots of seedlings were soaked in tap water (control group), whereas the roots of the other seedlings were soaked in tap water containing conidia of *T. virens* SZMC 24205 and *T. atrobrunneum* SZMC 24206 as treatment group.

4. RESULTS AND DISCUSSION

Screening for biocontrol *Trichoderma* strains against *Armillaria* species

Diversity of the genera *Armillaria* and *Trichoderma* in healthy and Armillaria-damaged forests

Four *Armillaria* species were identified by the sequence analysis of a fragment of the *tef1*α gene: the conifer-specific species *A. cepistipes* and *A. ostoyae* were abundant in the Rosalia spruce forest stands (Austria), whereas the presence of *A. mellea* and *A. gallica* was revealed in the Keszthely oak stand (Hungary). A total of 64 *Trichoderma* isolates were also isolated. Based on the sequence of a *tef1*α gene fragment, the isolates proved to represent 14 *Trichoderma* species. Population structure of *Trichoderma* species varied geographically between Keszthely and Rosalia in our study case; this variation might be closely related to the infested condition of the forests, as well as their associated *Armillaria* species.

In vitro antagonism of the isolated *Trichoderma* strains towards *Armillaria* species

Excellent biocontrol candidates: Antagonistic *Trichoderma* isolates were able to overgrow *Armillaria* colonies and intensely produce conidia on their surface, thereby potentially restricting *Armillaria* growth. Isolates such as *T. virens*, *T. atroviride*, *T. atrobrunneum* and *T. simmonsii* showed high *in vitro* antagonistic abilities indicated by BCI values. Most of the antagonistic isolates came from the oak stand in Keszthely. More antagonistic *Trichoderma* species dominated the severely *Armillaria*-infected soil, suggesting their great potential to be selected as native biocontrol agent.

Poor biocontrol candidates: Isolates belonging to *Trichoderma* species such as *T. koningii*, *T. asperellum*, *T. paraviridescens* and *T. longipile* had lower BCI values against almost all of the tested *Armillaria* isolates. Most of them were isolated from the spruce forest in Rosalia. Previously, species such as *T. koningii* and *T. asperellum*

showed excellent antagonistic activities during the application against other plant pathogens such as *Rhizoctonia solani*; however, in our study, they showed weak biocontrol ability when confronted with *Armillaria* species.

Extracellular enzyme production of the *Trichoderma* isolates

The 11 *T. koningii* isolates along with two *T. asperellum* and one *T. paraviridescens* showed good β-glucosidase and β-xylosidase activities. The examined *T. virens*, *T. atrobrunneum*, *T. simmonsii* and *T. atroviride* isolates showed lower activity levels for all enzymes tested, except for *T. atroviride* SZMC 26780 which had a very high β-xylosidase activity. Interestingly, the isolates of species with the best *in vitro* antagonistic abilities against *Armillaria* (*T. virens*, *T. atrobrunneum*, *T. simmonsii* and *T. atroviride*) were among the worst producers of these extracellular enzymes and *vice versa*, suggesting that the main antagonistic mechanism of these *Trichoderma* species against *Armillaria* may be mycoparasitism of hyphae and rhizomorphs rather than competition for polysaccharides or increasing phosphorous availability to the tree roots.

Potential plant growth-promoting traits of the isolated *Trichoderma* strains

From the forest-derived *Trichoderma* isolates, 40 were able to produce IAA. Characterization and detection of IAA production may be an important parameter to screen biocontrol candidates since IAA is important for plant growth. Most of the *Trichoderma* isolates tested were able to produce siderophores. The competition for iron may contribute to the anti-*Armillaria* activity of the examined *Trichoderma* isolates, as the production of siderophores proved to be a general feature among them.

Molecular dynamics of the biocontrol interaction between *T. atroviride* and *A. ostoyae*

Antagonistic effect of *T. atroviride* SZMC 24276 against *A. ostoyae* strains

During the 5 day co-incubation of dual cultures, *T. atroviride* SZMC 24276 showed a significant antagonistic effect against diverse diploid and haploid *A. ostoyae* strains. *T. atroviride* grew fast toward the colony of *A. ostoyae* and gradually invaded the growth area of *A. ostoyae* strains. Obviously, on the 5th day, haploid *A. ostoyae* derivatives were easier overgrown by *T. atroviride* and covered by abundant green conidia on the surface of the haploid *Armillaria* colony compared to that on diploid strains. Therefore, one of the haploid derivatives of *A. ostoyae* (AO), strain SZMC 23085 was selected for transcriptome analysis. The *T. atroviride* (TA) SZMC 24276 strain was selected as a biocontrol agent for our transcriptomic study.

Validation of differentially expressed genes using qRT-PCR

To confirm the reliability of the RNA-Seq data, the transcriptional level of 10 unigenes was examined by qRT-PCR. Taken together, all of these unigenes were upregulated in comparison with the control, consistent with the RNA-Seq data, indicating that our experimental results were valid

Time course analysis to understand the interaction dynamics between TA-AO

The dual co-culture method was employed to study the interaction between TA and AO. Time points of 53 and 62 hours after the inoculation of TA representing metabolite interaction before mycelial contact and mycoparasitic stage at physical contact, respectively, were analyzed. We performed time course analysis of the transcriptome data and generated 3 significant clusters for TA and AO. From the clusters, we identified the genes which showed the highest expression at the metabolite and mycoparasitic stages; and we also identified those genes showing continuous downtrend pattern in AO and TA.

Downtrend genes in TA and AO

We observed 768 and 747 downtrend genes in AO and TA respectively. Gene ontology (GO) enrichment analysis of downtrend genes in AO showed enrichment of cell cycle control such as the enrichment of DNA repair, mitotic cell cycle and microtubule-based process, etc. Genes related to the function of supramolecular structures were also found in the downtrend cluster. The downtrend gene cluster related to cell cycle and supramolecular structure seemed to indicate AO growth regression when the mycelia of TA gradually approached the AO colony.

Metabolite and mycoparasitic interaction stages

Defense reactions were induced in *A. ostoyae*: The overall transcriptional response of AO to the approaching TA revealed a defense reaction, such as oxidation-reduction and defense processes and metabolism of toxic compounds (high counts of genes responsible for glutathione peroxidase before contact, as well as DSBA-like thioredoxin domain and NADH:flavin oxidoreductase/NADH oxidase during physical contact were detected in AO), and transcriptional regulation (the transcriptional regulator NmrA-like domain protein was upregulated in AO at the mycoparasitic interaction stage). Upregulated defense-related genes included the genes encoding SnoaL-like domain and condensation domain for the biosynthesis of polyketides (PKs) and non-ribosomal peptides (NRPs) in AO before contact. Furthermore, other genes possibly protecting AO from the biocontrol agent at the mycoparasitic stage were upregulated, such as a malic acid transport protein and voltage-dependent anion channel for the efficient production of malic acid. Indoleamine 2,3-dioxygenase (IDO) was highly expressed in AO at the metabolite interaction stage before physical contact with TA; it is a tryptophan-degrading enzyme supplying nicotinamide adenine dinucleotide (NAD(+)) via the kynurenine pathway in fungi. Correspondingly, upregulation of the kynurenine pathway in AO probably leads to the production of an intermediate, the quinolinic acid (QA) at the metabolite stage. The antifungal properties of QA such as inhibition of fungal mycelia and fungal cell wall alterations indicated that AO still struggled to survive and deployed several defense strategies against TA.

Metabolite interactions were significantly induced: Metabolic activation in TA was

highlighted by genes implicated in the upregulation of condensation domain, short-chain dehydrogenase/reductase SDR and NAD-dependent epimerase/dehydratase that are involved in fundamental metabolic processes and the production of extracellular enzymes. The biological processes like cellular alcohol metabolic process were enriched in TA at the metabolite level interaction stage. The genes predicted to be involved in secondary metabolite biosynthesis (NRPS, PKS-like, PKS, NRPS-PKS hybrid and NRPS-like) were highly expressed in TA at the metabolite interaction stage, suggesting that TA actively antagonized AO through the production of antimicrobial compounds. Fungal ATP binding cassette (ABC) transporters which were well-characterized transmembrane proteins functioning in cellular detoxification were also expressed in the TA transcriptome during the mycoparasitic stage. In conclusion, genes encoding toxic secondary metabolites and ABC transporters possibly implicated in the production of antifungal components and toxic biocontrol molecules were highly expressed by TA when its mycelia gradually reached and physically contacted with AO. Peroxisome-related processes were activated in TA at the mycoparasitic stage. The peroxisome process also represents a type of defense systems that aims to protect the survival of the multicellular organism. It seems that peroxisomes play an essential role in the TA survival and growth process at physical contact with AO.

CAZymes play an important role in the biocontrol process: In the transcriptomes of TA, differential expression of CAZymes was found, including auxiliary activities (AAs) of redox enzymes, carbohydrate esterases (CEs), as well as glycoside hydrolases (GHs). The family GH containing the highest number of enzymes involved in fungal cell wall degradation is strongly expressed in TA at the metabolite interaction stage, which resulted in weaking AO. However, the expression of GHs in TA decreased significantly at the mycoparasitic stage, probably due to the saturation of these enzymes in the media. In the AO transcriptome, lower diversity and abundance of CAZymes were found before physical contact with TA; the expression of these CAZymes did not show significant change, except for the AAs of redox enzymes that are significantly upregulated at the mycoparasitic stage, which might be essential for AO survival and substrate usage under the competitive stress of TA.

Peptidase dynamics is a crucial defense response of AO: More abundance and more variation of peptidases induced in TA than in AO was found during the initial interaction stage before mycelial contact. As the incubation time was prolonged, genes related to the peptidase activities still dominated in the transcriptome of TA, possibly for further mycoparasitic interaction. However, considerable variations were found in the set of peptidases in TA expressed between the metabolite and mycoparasitic stages. This variation in TA seems to be induced by the defense reaction from AO. The results suggested that the involvement of peptidases and proteases in cell wall degradation seems to be necessary for *Trichoderma* mycoparasitism. On the other hand, AO showed

a dramatical reaction with a larger variety and more significant production of peptidases, suggesting that the biocontrol agent TA activates a typical defense process in AO. Peptidase and protease function in the detoxification of toxic molecules might play a crucial role in AO. The detoxification function of peptidases seems to be fully activated and put into effect in AO, as when the mycelia of TA extended towards interaction with AO.

Field experiment in a heavily *Armillaria*-damaged forest in the Keszthely Hills

Trichoderma virens SZMC 24205 and *T. atrobrunneum* SZMC 24206 were selected for a field experiment. Both strains were isolated from a Keszthely soil sample associated with decaying *Armillaria* rhizomorphs; furthermore, both exerted very good *in vitro* antagonistic abilities towards the tested *Armillaria* isolates and were able to produce hydroxamate-type siderophores and IAA. The isolates were applied to Turkey oak seedlings before planting as a root treatment in the form of a conidial suspension. The total survival rates calculated after 6 months for 120 treated and 115 control trees were 84.3% and 54.7%, respectively, indicating that the applied treatment had a beneficial effect on the survival of oak seedlings planted into the soil of an *Armillaria*-infested forest area.

SUMMARY

- Four *Armillaria* species and a total of 64 *Trichoderma* isolates representing 14 *Trichoderma* species were isolated from forest soils. The population structure of *Armillaria* and *Trichoderma* species varied geographically between Keszthely and Rosalia.
- Antagonistic *Trichoderma* isolates were screened based on the Biocontrol Index value (BCI) through *in vitro* dual culture confrontation assays. Antagonistic *Trichoderma* species dominated the severely *Armillaria*-infected soil, suggesting their great potential to be selected as native biocontrol agents.
- The isolates of *Trichoderma* species with the best *in vitro* antagonistic abilities against *Armillaria* were among the worst producers of the cellulolytic and xylanolytic enzymes as well as acidic phosphatase and *vice versa*, suggesting that the main antagonistic mechanism of these *Trichoderma* species against *Armillaria* may be mycoparasitism of hyphae and rhizomorphs rather than competition for polysaccharides or increasing phosphorous availability to the tree roots.
- 40 *Trichoderma* isolates were able to produce IAA. Most of the *Trichoderma* isolates tested were able to produce siderophores. IAA production is important for plant growth. The competition for iron may contribute to the

anti-*Armillaria* activity of the examined *Trichoderma* isolates.

- One of the haploid derivatives of *A. ostoyae* (AO) SZMC 23085 and the *T. atroviride* (TA) SZMC 24276 strain were selected for our transcriptomic study based on *in vitro* dual culture confrontation assays.
- To analyze major gene expression trends, we considered TA-AO genes showing continuous downtrend patterns or genes exhibiting the highest upregulation at the metabolite level before physical contact or mycoparasitic stages during physical contact for further analysis.
- The downtrend gene cluster related to cell cycle and supramolecular structure indicated AO growth regression when the mycelia of TA gradually grew towards the AO colony.
- The overall transcriptional response of AO to the approaching TA revealed a defense reaction, such as oxidation-reduction and defense processes, metabolism of toxic compounds and transcriptional regulation. Defense-related genes and other genes possibly protecting AO from the biocontrol agent were upregulated, such as indoleamine 2,3-dioxygenase (IDO) and kynurenine pathway, which in AO probably leads to the production of quinolinic acid (QA). All these transcriptional changes indicated that AO struggled to survive and deployed several defense strategies against TA.
- Genes required for the metabolic process, genes encoding toxic secondary metabolites and ABC transporters possibly implicated in the production of antifungal components and toxic biocontrol molecules were significantly upregulated in TA when its mycelia gradually reached and physically contacted with AO.
- Differential expression of CAZymes was found in TA. The family GH containing the highest number of enzymes involved in fungal cell wall degradation is strongly expressed in TA.
- Differential expression of peptidases was induced in TA affected by AO. The results indicated that the involvement of peptidases and proteases in cell wall degradation seems to be necessary for *Trichoderma* mycoparasitism. Whereas, AO showed a dramatical reaction with a larger variety and more significant production of peptidases, suggesting peptidase and protease function in the detoxification of toxic molecules might play a crucial role in AO.
- The higher survival rates of Turkey oak seedlings treated with the biocontrol candidates *T. virens* SZMC 24205 and *T. atrobrunneum* SZMC 24206 indicated that the applied *Trichoderma* treatment had a beneficial effect on the oak seedlings growth in the soil of an *Armillaria*-infested forest area.

LIST OF PUBLICATIONS

MTMT Author ID: 10057722

1. JOURNAL ARTICLES USED FOR ATTAINING THE PHD DEGREE

Kedves O, Shahab D, Champramary S, **Chen L**, Indic B, Bóka B, Nagy VD, Vágvölgyi C, Kredics L, Sipos G. Epidemiology, biotic interactions and biological control of Armillarioids in the Northern Hemisphere. PATHOGENS, 2021, 10(1):76. https://doi.org/10.3390/pathogens10010076 (IF2021: 3.492)

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Cumulative impact factor of the publications directly related to the thesis: 5.713

2. FURTHER PUBLICATIONS RELATED WITH THE TOPIC OF THE DISSERTATION

Conference paper:

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3. OTHER PUBLICATIONS

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Cumulative impact factor of publications not directly related to the thesis: 17.098

Total impact factor: 22.811

Scientific journal articles: 7

Conference paper: 1

Conference abstracts: 12

DECLARATION

I declare that the contribution of Liqiong Chen was significant in the below listed publications and the doctoral process is based on the publications listed. The results reported in the PhD dissertation were not used to acquire any PhD degree in the past and will not be used in the future either.

Chen, L.; Bóka, B.; Kedves, O.; Nagy, V.D.; Szűcs, A.; Champramary, S.; Roszik, R.; Patocskai, Z.; Münsterkötter, M.; Huynh, T.; Indic, B.; Vágvölgyi, C.; Sipos, G.; Kredics, L. Towards the Biological Control of Devastating Forest Pathogens from the Genus *Armillaria*. Forests 2019, 10, 1013. https://doi.org/10.3390/f10111013. IF_{2019}: 2.221

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Szeged, October 9, 2021

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Dr. László Kredics

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Prof. Dr. Csaba Vágvölgyi