Summary of Ph.D Dissertation

Bioprospecting and biodiversity investigations of endophytic fungi isolated from medicinal plants and mosses

Ramasamy Aruna Vigneshwari

Supervisor:

Dr. András Szekeres, senior research fellow

Doctoral School of Biology

Department of Microbiology

Faculty of Science and Informatics

University of Szeged

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1. INTRODUCTION

Endophytic fungi are a group of highly diverse microorganisms that live in the interior tissues of plants. They possess a complex relationship with their hosts ranges from symbiotic, commensals to latent pathogens. Furthermore, endophytic fungi of medicinal plants are generally considered as a significant source of bioactive metabolites. In certain cases, it has been observed that the plant endophytes produce the bioactive metabolite similar to the host. Bioprospecting of endophytic fungi for host associated metabolites would not only reduce the need to harvest slow growing and possibly rare plants, but also can preserve the world's ever-diminishing biodiversity. Scientific efforts have also been made to utilize endophytes to meet the requirement of new antimicrobial agents.

In our study 4 plants were selected to investigate endophytic fungi producing novel bioactive metabolite, which were collected from the southern Hungarian areas. The *Hypericum perforatum* is known for its traditional medicinal value containing biologically active compounds hypericin and emodin, which are an antidepressant and anticancer compounds, respectively. Previously an endophytic fungi *Thelavia subthermophila* isolated from *H. perforatum* and identified as a producer of these metabolites. The *Juniperus communis* is a medicinal plant, which contains many bioactive compounds like deoxypodophyllotoxin, podophyllotoxin and the extracts of the plants acts as anti-diarrheal, anti-inflammatory, and antimicrobial agents. *Artemisia asiatica* is widely used for its medicinal value possessing the bioactive compounds eupatilin and jaceosidin, which reported as anticancer, antimicrobial, anti-inflammatory and antioxidative agents. Despite their pharmaceutical importance, the endophytic fungal community of this plant was never investigated. The mosses are non-vascular plants belons to the division Bryophyta. They are abundant in vast variety of habitats from arctic cold to hot desserts. Previously reported studies showed that the endophytic bacterial community from mosses play an important role in plant growth promotion. Due to the limited investigations regarding the endophytic fungi of mosses, these plants were also selected for our study**.**

2. OBJECTIVES

The aim of this work was to isolate, identify as well as evaluate and compare the bioprospects of fungal endophytes harbored in Hungarian plants. Furthermore, to provide fundamental insights into the host metabolite producing abilities of endophytic fungi and reveal the antimicrobial activity of the secondary metabolites produced by the examined endophytic community.

The main objectives are,

1. Screening for the host metabolite producing endophytic fungi from the *H. perforatum.*

2. In the case of finding host metabolite producer strains, detailed investigation of the host metabolite producing endophytic fungi regarding the taxonomy, yield of the metabolites and dependence of the production on certain cultivation conditions.

3. Isolation and identification of the endophytic fungi from *J. communis*, *A. asiatica* and several mosses and evaluate their biodiversity.

4. Determination of antimicrobial activities of metabolites extracted with different organic solvents from the ferment broth and mycelia of isolated endophytic fungi.

3. METHODS

Collection of plant samples

- Fresh plants of *H. perforatum* and *J. communis* were collected from southern region of Hungary
- Plant samples of *A. asiatica* and mosses were provided by Department of Pharmacognosy, University of Szeged

Isolation and identification of fungal endophytes

- Fungal endophytes were isolated from surface sterilized fresh sterile segments of selected plants
- For the identification of isolated strains PCR based molecular tools were applied

Targeted screening of host metabolite production of *H. perforatum* isolates

- Preparation of metabolite extracts from broth and mycelia
- Screening for hypericin and emodin production by HPLC-UV measurements
- Confirmation of hypericin and emodin production using HPLC-HRMS and HPLC-HRMS/MS
- Testing of antibacterial activities of hypericin and emodin

Test the effects of different cultivation parameters on the production of hypericin and emodin

- Cultivation in dark and light condition
- Fermentation in different cultivation media n
- Sub-cultivation studies on producer strains
- Testing the effect of different elicitors on the attenuated strains
- Following the effects of the treatments on the hypericin and emodin production by HPLC-MS/MS analysis

Biodiversity mapping of isolates from *J. communis*, *A. asiatica* and mosses.

- Calculation of isolation rate, biodiversity parameters as well as host and tissue specificity of fungal endophytes of *J. communis, A. asiatica* and mosses, based on the isolation and identification data

Screening of bioactive metabolite producing isolates from J. communis, A. asiatica and mosses.

- Preparation of metabolite extracts from ferment broth and mycelia using solvents with different polarity
- Antibacterial activity testing of prepared extracts against four test bacteria including *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* by microdilution method
- **Antifungal activity testing of prepared extracts against two yeasts**, ***Candida albicans* and *C. krusei* as well as** two filamentous fungi, *Rhizoctonia solani* and *Fusarium culmorum* by agar diffusion method

4. RESULTS AND DISCUSSION

Screening for host metabolite producing endophytic fungi from *H. perforatum*

From 48 parts of different tissues of *H. perforatum* eight endophytic fungi were isolated and identified as *Epicoccum nigrum* (1), *Alternaria* sp. (6) and *Trichoderma harzianum* (1). Among the 32 prepared metabolite extracts, the presence of plant host metabolites, hypericin and emodin were determined in the mycelial extract of *E. nigrum* as well as emodin was detected in the mycelial extracts of two *Alternaria* species via HPLC-UV analyses. Then the comprehensive confirmation of both metabolites was done by HPLC/HRMS and HPLC-HRMS/MS analysis by comparing the retention of times of authentic standards of hypericin and emodin.

The yield of emodin in *E. nigrum* (320 ng/ml) was approximately three quarters of hypericin (428 ng/ml) produced also by this strain and emodin was 20 times higher than other two *Alternaria* (~20 ng/ml of each) strains

Effect of different cultivation parameters on host metabolite production

Effect of Dark and Light condition: During the cultivation of producer strains in dark and light conditions, it was observed that the production of host metabolites and the biomass accumulation of both strains were higher in light than in dark. The results suggest that light has significant impact both on the growth of the strain and on the production of hypericin and emodin.

Effect of different cultivation media: The production of hypericin and emodin was measured in four different fermentation media, potato dextrose broth (PDB), malt extract broth (MEB), Czapek-Dox broth (CDB) and defined media (DM). It was observed that the production of both host metabolites was highest in PDB followed by MEB and was significantly lower in the synthetic media. Thus, it was concluded that PDB proved to be suitable media to produce hypericin and emodin in both the strains.

Sub-cultivation studies on producer strains: The sub-cultivation of both *E. nigrum* and an *Alternaria* species was resulted the attenuation of hypericin and emodin production after certain generations in all four applied media. In the case of *E. nigrum* the production of both emodin and hypericin ceased in the tenth generation in both PDB and MEB, while amount of emodin reduced completely also at the tenth generation in the case of *Alternaria* sp. In both cases, the production of the metabolites

was stopped two generations earlier in the two synthetic media.

Testing the effect of elicitors on attenuated strains: To restore the production of hypericin and emodin, different types of elicitors were added to the ferment broth of attenuated strains of *E. nigrum* then the presence of the metabolites were analyzed in the mycelia of the strain sub-cultivated again in the PDB. Our results revealed that the addition of the ferment broth of the producer strains did not have any positive impact on the production of both hypericin and emodin in the attenuated strains. The effect of tea solution of *H. perforatum* were also tested in two concentrations, where the applied higher tea amount could restore the production of hypericin in low amount, but no traces of emodin were observed. Furthermore, when emodin was added in two different concentrations or the fermentation medium broth was supplemented with the fresh tissues of *H. perforatum* as an elicitor, hypericin was also detected in the mycelial extracts of the sub-cultivated strain.

Biodiversity mapping of isolates from *J. communis, A. asiatica* and *mosses.*

Altogether 240 segment of *J. communis*, 126 segments of *A. asiatica* and 40 different specimens of mosses were used for endophytic isolation and altogether 254 strains were isolated, from which 197 strains were used for further investigations. The isolated strains belonged to 3 orders, Sordariomycetes, Dothideomycetes and Eurotiomycetes.

Calculation of isolation rate, biodiversity parameters and specificity of fungal endophytes of *J. communis:* The isolation rate was 0.54 in *J. communis.* The stems of *J. communis* harbored 11 unique fungi, whereas 4 and 2 were found in leaf and cone showing that some species seem to be tissue specific. *Xylaria* species were found only in cone, while *Pestalitiopsis* and *Bipolaris* were found only in leaf, whereas *Curvularia*, *Aspergillus, Didymella* and *Purpureocillium* species were specifically found in stems. The biodiversity parameters revealed higher diversity of endophytic fungal species in the stem compared to that of the other parts in *J. communis.*

Calculation of isolation rate, biodiversity parameters and specificity of fungal endophytes of *A. asiatica:* For *A. asiatica,* the isolation rate was recorded as 0.75 and the fungal richness of fungi was 17. The number of fungi in the stem was higher than

leaves, but this difference was comparatively low. *Curvularia, Phomopsis* and *Simplicillium* species were found only in leaves, while *Aspergillus, Trichoderma* and *Stemphylium* species were isolated only from the stem.

Calculation of isolation rate, biodiversity parameters and specificity of fungal endophytes of mosses: Totally, 40 endophytic fungi were isolated from 126 plant segments of 42 different mosses at an isolation rate of 0.37. *Trichoderma* and *Alternaria* genera were dominantly found, which were followed by *Aspergillus* and *Fusarium.* The presence of *Dothiorella gregaria* as moss endophytes were only observed in our study. Comparing to other two plants the endophytic fungal community is less diverse in mosses.

Screening of bioactive metabolite producing endophytic isolates

Antimicrobial effects of fungal extracts of *J. communis* endophytes: Most of the tested extracts were active against Gram positive than Gram negative bacteria. More number of the ethyl acetate extracts (55) were active against *B. subtilis* with respect to taxa, *Fusarium, Pestalotiopsis* (SZMC 27205*), A. fumigatus* (SZMC 27164*)* and *Purp. lilacinum* (SZMC 27031) showed significantly high bioactivity against the tested strains. Altogether, 27 extracts showed high inhibitory effects (90%) against yeasts. Mycelial extracts of *Trichoderma* and *Purp. lilacinum* were found to be active against the tested filamentous fungi.

Antimicrobial effects of fungal extracts of *A. asiatica* endophytes: Altogether, 328 extracts were tested against the test strains. Low percentage of extracts were active against Gram negative bacteria including *E. coli* (31%) and *P. aeruginosa* (28%). It should highlight that *Didymella glomerata* (SZMC 27102) isolate exhibited remarkable high activity against all of the tested microbes. Most of the extracts of *Fusarium* species exhibited significant antimicrobial activities against yeasts, but none of them were active against the two filamentous fungi. Taxa wise, *Aspergillus, Penicillium* and *Fusarium* species were found to have metabolites with effective antifungal activity.

Antimicrobial effects of fungal extracts of *moss* endophytes: Totally, 85% of the extracts of moss endophytes showed inhibitory activity against at least one test

bacterium. 54 % of the EtOAc extracts and 52% of mycelial extracts showed inhibition against Gram negative bacteria. The *Trichoderma* isolates were found to be active against most of the tested pathogens. It is important to highlight that the mycelial extracts of 20 isolates significantly inhibited the growth of the plant pathogen *R. solani.*

Altogether several extracts containing the endophytic metabolites proved to be active against the tested pathogens. These isolates will be examined in detail in the future and the chemical nature of the active metabolites will be determined that may possibly lead to novel compounds for the pharmaceutical applications.

SUMMARY

- In the targeted approach, *E. nigrum* were identified as the alternative source for both hypericin and emodin, while two *Alternaria* sp. isolates were described as the producer of emodin alone.

- The strains were cultivated in different media including PDB, MEB, DM and CDB. The results revealed that PDB medium was found to be superior, when compared to other ones for both biomass accumulation and for host metabolite production in the case of both *E. nigrum* and *Alternaria* sp.

- Our results on sub-cultivation of the producer strains clearly indicated that both of them lost their ability to produce host metabolites during subsequent generations. The usage of high amount of tea solution, pure emodin and fresh plant parts of *H. perforatum* in the fermentation medium as an elicitor re-induced the synthesis of hypericin in an attenuated strain *E. nigrum*. However, further studies should be carried out on elucidating biosynthetic pathways, and employing metabolic reprogramming, reversal of epigenetic silencing to tackle the attenuation problem.

- In our work, altogether 254 strains from 406 plant fragments of *A. asiatica* (95), *J. communis* (132) and mosses (44) were isolated and finally 82, 75 and 40 fungi were chosen investigation. Where the *Trichoderma, Fusarium*

and *Alternaria* species were found to be dominant in all the three plants

- Totally 788 extracts were tested for their bioactivity against 4 bacterial pathogens, 2 yeast and 2 plant pathogenic fungi. 61% of the extracts were found to be effective against at least one test microorganism including 367 against *B. subtilis*, 341 against *S. aureus,* 221 against *E. coli* and 262 against *P. aeruginosa*. In the case of yeast, *C. albicans* were susceptible to 92 extracts and 63 were showing inhibitory against *C. krusei.*

- The *Pestalotiopsis sp.* (SZMC 27205)*, D. glomerata* (SZMC 27102*),* and *Purp. lilacinum* (SZMC 27031) were highly active against all tested bacterial strains

- *Purp. lilacinum* (SZMC 27031) and *Trichoderma atroviride* (SZMC 27261) of mosses were inhibiting the growth of tested plant pathogens.

- Endophytic fungi produce plethora of bioactive secondary metabolites and our results also revealed that the isolated fungal endophytes in our study are excellent reservoirs of bioactive compounds.

5. LIST OF PUBLICATIONS RELATED TO THIS THESIS

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6. OTHER PUBLICATIONS

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