Summary of Ph.D Dissertation

Characterization of plant growth-promoting activities of endophytic fungi isolated from Mongolian medicinal plants

Adiyadolgor Turbat

Supervisors:

Dr. András Szekeres, senior research fellow

Prof. Dr. Csaba Vágvölgyi

Doctoral School of Biology

Department of Microbiology

Faculty of Science and Informatics

University of Szeged

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1. INTRODUCTION

Endophytic fungi (EF) live in the tissues of plants without causing any symptoms of disease in their host. Their specific ecological niche with the continual metabolic interactions between the endophytic microorganism and the host plant seems to serve a remarkable strong evolutionary pressure and improves the possibilities of the synthesis of secondary metabolites of endophytes with novel properties. The diversity and population compositions of EF in plants are highly variable. EF are essential components of sustainable agriculture in the view of their ability to enrich plant growth, crop yield and enhance plant fitness through providing both biotic and abiotic stress tolerance. A considerable fact is that these fungi are agriculturally crucial as they can boost the growth of their host, have positive effects to increase nutrition and phytohormone production. The mineral solubilization is the indirect way of plant growth promotion via obtaining nutritional elements such as nitrogen and phosphorus or by mobilizing metals which are useful for plants. The direct way of plant growth promotion is to produce phytohormones, particularly gibberellins and indole-3-acetic acid (IAA), which are able to improve plant growth and reduce the adverse impacts of abiotic stress. A number of well-known plants have been investigated for the diversity and the production of secondary metabolites of their EF. The discovery of novel bioactive compounds gathered from many different types of endophytic microorganisms is crucial alternative to overcome the enriching levels of drug resistance to various pathogenic organisms.

In our study, investigation of EF isolated from Mongolian medicinal plants (*Convolvulus arvensis, Sphaerophysa salsula, Nitraria sibirica, Astragalus melilotoides, Thermopsis dahurica, Halerpestes salsuginosa, Oxytropis glabra, S. flavescens*) was undertaken. During the research work the EF were isolated from the plant parts and they were identified using molecular taxonomical tools. Then their direct and indirect plant promoting abilities were characterized and the bioactive potential of the strains were determined. Finally, biosynthetic pathways of the indoleacetic producer isolates were determined.

2. OBJECTIVES

Over the last few years, fungal endophytes have been reported as excellent candidates for producing various secondary metabolites and these compounds have significant impact on a field of medicine as well as agriculture. Whereas, it has been proved that endophytic fungi are able to enhance plant growth via producing plant growth regulating hormones and minerals. Thereby, we aimed to isolate, identify and investigate fungal endophytes harbored in Mongolian plants. Furthermore, to contribute significant insights into the plant hormon producing capabilities of fungal endophytes and reveal the antimicrobial activity of the secondary metabolites produced by the examined endophytic community.

For this purpose, our specific objectives were the followings:

- ❖ Isolation and identification of endophytic fungi from Mongolian medicinal plants.
- ❖ Invertigating of antibacterial and antifungal activity of endophytic fungi.
- ❖ Testing plant growth promoting activities of the isolates.
- ❖ Examination of IAA biosynthetic pathway of the IAA producer endophytic fungi.

3. METHODS

Collection of plant samples

- Fresh plants of the plants were collected from Mongolia

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

Bioactivities of the endophytic fungi

- Preparation of metabolite extracts from broth and mycelia
- Testing of antibacterial activities of the isolates
- Testing of antifungal activities of the isolates

***In vitro* plant growth promoting assays**

- Testing siderophore production of the isolates by agar plate assay
- Testing phosphate solubilizing activities of the isolates by agar plate assay
- Testing auxin production of the isolates by agar plate assay
- Confirming auxin production of the isolates by HPLC-MS

***In vivo* bioactivity assay of the extracts**

- Plant growth assay of the extracts on *Arabidopsis thaliana*

Determination of intermediates of auxin biosynthesis

- Detection of auxin related indole compounds by HPLC

4. RESULTS AND DISCUSSION

Isolation and identification of fungal endophytes: The plant samples were collected from 8 species of medicinal herbs randomly from two different locations in Mongolia. The leaf, stem, root and flower parts were separated, and these parts were examined for their EF content. Altogether, 62 endophytes were isolated in pure form from *Astragalus melilotoides* (7), *Convolvulus arvensis* (11), *Halerpestes salsuginosa* (4), *Nitraria sibirica* (4), *Oxytropis glabra* (9), *S. flavescens* (15), *Sphaerophysa salsula* (9), and *Thermopsis dahurica* (3), which harboured in the leaves (12), stems (18), flowers (10) and roots (22). Endophytic fungi were assigned into 22 taxa. Among 62 isolates, 44 isolates were identified at the species level, eighteen isolates at the genus level. The isolates belonged into 7 genera involving *Fusarium* (24), *Alternaria* (21), *Didymella* (9), *Penicillium* (3), *Phoma* (3), *Camarosporidiella* (1), and *Xylogone* (1). This study found some EF for the first time from the host plants such as, *Alternaria* species were detected for the first time from the host plants *S. flavescens, O. glabra, S. salsula* and *H. salsuginosa* and *Didymella* species from *S. flavescens, N. sibirica, S, salsula* and *C. arvensis.* In case of *Fusarium* species, they were isolated for the first time from *S. flavescens, S. salsula* and *H. salsuginosa* as well as *Xylogone* and *Camarosporidiella* species were firstly described as endophytes of *S. flavescens* and *A. melilotoides.* Furthermore, *Phoma* species were isolated from *A. melilotoides* and *S. salsula* as well as *Penicillium* species were isolated from *A. melilotoides* and *C. arvensis* first time in our work.

Bioactivities of the endophytic fungi

Antibacterial activity of the isolates: Altogether 372 extracts were tested against six microorganisms. In general, the ferment broth extracts of the isolates showed higher inhibitory activity on these bacteria than the mycelial extracts. *Staphylococcus aureus* and *Streptomyces albus* were the most sensitive against both mycelia and ferment broth extracts based on 287 and 260 extracts of the isolates with the ranges of 80% and 96.35%, respectively. Most of these isolates belong to *Fusarium* followed by *Alternaria* and *Didymella* species. Among three solvents (hexane, chloroform, ethyle-acetate) both ferment broth and mycelia extract of hexane caused the lowest inhibitory activity on the tested bacteria, while the application of ehyl-acetate extracts leaded to the highest antibacterial activities. Among all 62 isolates, *F. tricinctum* SZMC 27041 and *F. armeniacum* SZMC 26659 strains showed the highest antimicrobial activity on all six bacteria.

Antifungal activity of the isolates: During the antifungal activity tests, all 62 isolates were screened against the pathogenic *Candida albicans* and three filamentous fungi, *Aspergillus niger, F. culmorum* and *Rhizoctonia solani*. Based on these tests, *D. glomerata* (SZMC 26648, 26650, 26649), *Aspergillus* sp. (SZMC 26977), *Alternaria tenuissima* (SZMC 27025) and *F. redolens* (SZMC 26979) were found to be the most active against all tested fungi, meanwhile, *Fusarium* sp. (SZMC 26656, 26660), *F. tricinctum* (SZMC 26657, 26984), *A. alternata* (SZMC 26651) and *P. chrysogenum* (SZMC 26987) isolates were active against three out of the four tested fungi. In total, 64 extracts of the fungal isolates - most of them belong to *Fusarium* and *Alternaria* genera were found to show potential against *C. albicans*. It was observed that ethyl-acetate extracts have significant inhibition against *A. niger* and *R. solani* by involving 38 and 41 extracts of the isolates, respectively. On the contrary, only 21 extracts were active against *F. culmorum*. In general, chloroform-ferment broth extracts provided the most active solutions (44 extracts of the isolates) by giving significant potential against all tested fungi, while extracts of hexane-broth have the lowest inhibition against the 4 tested fungi. Based on these tests, ethyl-acetate extract of *Didymella glomerata* (SZMC 26649) showed the highest inhibition zone on *C. albicans*.

In vitro plant growth promoting assays

Siderophore production assay: During this assay, 34 endophytes caused orange/yellow zones around their colonies on CAS agar plates as a result of their siderophores sequestering and binding of iron from the medium. As a consequence, 9 *Fusarium* as well as to 9 *Alternaria* showed positive results. The largest zone (32 mm) appeared on the plate of the *F. tricinctum* (SZMC 26658) strain, but also high siderophore productions were detected in the case of *F. tricinctum* (SZMC 27019) (30 mm), *F. tricinctum* (SZMC 26984) (29 mm), *Didymella.* sp. (SZMC 26991) (29 mm) and *F.* sp. (SZMC 26656) (26 mm).

Phosphate solubilization assay: Regarding this assay, 31 isolates were capable to solubilize phosphate according to the observed zones on PKV agar medium. They were the member of the *Alternaria, Fusarium, Didymella, Xylogone, Camarosporidiella* and *Penicillium* genera with the highest activity of *F. tricinctum* SZMC 26657 (40 mm) followed by *F. armeniacum* SZMC 26981 (39 mm), *A. alternata* SZMC 26651 (39 mm), *Paraphoma chrysanthemicola* SZMC 27034 (37 mm) and *D. glomerata* SZMC 26648 (35 mm).

Auxin production assay: All isolates were proved to produce IAA either in the presence or in the absence of Trp or in both cultivation condition, from which 25 strains showed production only in the presence of Trp and 6 only in the absence of Trp as well as 25 in both cultivation conditions. This was detected after observing red ring formation in the membrane, which surrounded the colony after the treatment of Salkowski reagents. Most of the active isolates were *Fusarium* including 23 strains, followed by 21 strains belonging to the *Alternaria* genus. Two *Didymella* (SZMC 26648 and 26650), eight *Fusarium* (SZMC 26660, 26654, 26657, 26990, 27039, 27017, 27016 and 27001) and five *Alternaria* (SZMC 26652, 26985, 26982, 27004 and 27003) isolates were able to produce auxin on Trp supplemented media. Furthermore, two *Didymella* (SZMC 26647 and 26649), five *Alternaria* (SZMC 26651, 26653, 26977, 26996 and 26993) and two *Fusarium* (SZMC 26659, 27038) strains showed IAA production on both Trp and non-Trp containing media, meanwhile the two *Alternaria* (SZMC 26975 and 27002), the *F. proliferatum* (SZMC 26978), the *P. chrysogenum* (SZMC 26998), the *Phoma* sp.

(SZMC 27036) and the *Camarosporidiella moricola* (SZMC 27035) endophytes presented positive IAA results only on that media, which was not supplemented with Trp.

Confirming auxin production of the isolates by HPLC-MS: Similar to the results of the plate assays, each isolate showed remarkable IAA production either through the Trp-dependent or the Trp-independent pathway. In the case of mycelial extracts, two strains accumulated remarkable amount of IAA in PDB medium including *Alternaria* (SZMC 26993) and *Fusarium* (SZMC 26657) isolates. Meanwhile, 27 strains proved to be outstanding producers on PDB media supplemented with additional Trp source. These were five *D. glomerata* (SZMC 26647, 26648, 26649, 26650 and 27019), two *F. armeniacum* (SZMC 26980, 26659), a *F. tricinctum* (SZMC 26658), a *Didymella* sp. (SZMC 26991), a *F. incarnatum* (SZMC 27037), a *F. proliferatum* (SZMC 27038), two *F.* sp. (SZMC 26660, 26656) two *Alternaria* sp. (SZMC 26985 and 27004), two *A. tenuissima* (SZMC 26988, 26992), three *A. alternata* (SZMC 27024, 26652, 26653), a *F. oxysporum* (SZMC 26983), a *P. rubens* (SZMC 27006), a *C. moricola* (SZMC 27035), a *Phoma* sp. (SZMC 27005), two *F. verticillioides* (SZMC 27007 and 26990) and a *F. sporotrichioides* (SZMC 26654) isolates. Furthermore, mycelial extracts of *Alternaria* sp. (SZMC 26993, 26996), *A. alternata* (SZMC 26651), *F. tricinctum* (SZMC 26984), 26657, *F. proliferatum* (SZMC 26989), *D. glomerata* (SZMC 26655) and *Xylogone sphaerospora* (SZMC 26661) isolates were found to be higher IAA producers than their ferment broth extracts on PDB medium without Trp source. The ferment broth extracts of three isolates contained high IAA amounts on simple PDB medium, which were produced by *F. tricinctum* (SZMC 27041), *D. glomerata* (SZMC 26655) and *A. alternata* (SZMC 26651). On Trp supplemented PDB medium, the highest IAA production was detected for *D. glomerata* (SZMC 26648), which was the same as observed for mycelial extract. Significant productions were also measured in case of *Alternaria* sp. (SZMC 26975, 26976), *F. verticillioides* (SZMC 26990), *A. alternata* (SZMC 27024), *Didymella* sp. (SZMC 27021), *Phoma* sp. (SZMC 27036), *F. incarnatum* (SZMC 27037), *F. proliferatum* (SZMC 27038) and *F. oxysporum* (SZMC 27016).

In vivo bioactivity assay of the extracts:

The ferment broth extracts of six (SZMC 26648, 26652, 26658, 26660, 26651, and 26653) selected isolates were investigated for growth promoting activities in the model

plant *Arabidopsis thaliana*. The extract was diluted to 100, 10, 1, 0.1 and 0.01 µg/mL based on their original IAA contents and impact of these solutions was compared to the standard IAA solutions. At the higher concentration, the growth of primary root was inhibited (1 µg/mL, 10 µg/mL and 100 µg/mL), while it was stimulated at the lower levels of IAA (0.01 µg/mL and 0.1 µg/mL) compared to the untreated controls. However, the lengths of all *Arabidopsis* roots treated with fungal IAA were significantly higher than those of the IAA treatments at concentrations of 0.1 and 1 µg/mL at any of the days measured.

Comparing the biomass resulted in the plant assays, the IAA treatment led to remarkably higher level of biomass. Treatment with a fungal extract at 0.01 µg/mL IAA level resulted in a similar rate of production of photosynthetic pigments than with the IAA solution except for SZMC 26653 possessing increased amount of chlorophyll-a and total chlorophyll as well as carotenoids. The leaves of plants treated with the extracts of strains SZMC 26651, 26653 (at 0.1 µg/mL); SZMC 26660, 26653 (at 1 µg/mL); and SZMC 26660 (at 10 µg/mL and 100 µg/mL) contained higher chlorophyll-a, while SZMC 26648 (at 0.1 µg/mL); SZMC 26660 (at 1 µg/mL); SZMC 26648, 26652, 26658 and 26660 (at 10 µg/mL) as well as SZMC 26652, 26658 and 26660 (at 10 µg/mL) comprised increased quantities of chlorophyll-b pigment than the pure IAA treated plants. In the case of carotenoids, the extracts of SZMC 26660, 26651, 26653 (at 0.1 µg/mL); SZMC 26658, 26660, 26653 (at 1 µg/mL), SZMC 26660 (at 10 µg/mL) and SZMC 26652, 26660 (at 100 µg/mL) induced the productions in the plants.

Detection of auxin related indole compounds by HPLC-MS: to determine the background of the IAA production in the isolated endophytes, the possible members of the IAA biosynthetic pathway were measured and determined by HPLC techniques. These intermediates involved the TAM (2.3 min), IAM (7.9 min), IPyA (9.4 min), ILA (9.4 min), TOL (9.8 min), IAA (9.8 min), IAAld (9.8 min) and IAN (10.3 min). In our IAA producing isolates three type of Trp dependent IAA biosynthesis were identified including the IAM, IPyA and TAM pathways as well as the presence of TSO pathways could be also possible. The IAN was not detected at all in the samples. In general, it was the IPyA (26) pathway that most strains operated in their IAA biosynthesis, which was followed by IAM (16) pathway as well as certain strains have conducted two or more biosynthetic pathways for producing the IAA. Altogether, IAA production of 16

fungal endophytes functioned only one pathway including five isolates used IAM pathway, ten strains apply IPyA pathway and one fungus synthetized IAA via TSO pathway. In our work was established for the first time that *Didymella* and *Alternaria* species produce IAA via IAM pathway. It is important to point out that we have shown that IAM, IPyA, TAM pathways co-exist in *Fusarium* species (SZMC 26990, 27017 and 27037). Furthermore, fungal endophytes were capable to synthetize the IAA through 2 pathways including IPyA – TAM *Didymella* species (SZMC 26648 and 26991) and IPyA – IAM *Fusarium* species (26654 and 26979) and IAM – TAM *Fusarium* species (SZMC 26983) pathway pairs.

SUMMARY

- The investigation of the fungal endophytes isolated from Mongolian medicinal plants (*Convolvulus arvensis, Sphaerophysa salsula, Nitraria sibirica, Astragalus melilotoides, Thermopsis dahurica, Halerpestes salsuginosa, Oxytropis glabra, Sophora flavescens*) was undertaken.
- The isolates were identified at the species level, eighteen isolates at the genus level. The isolates belonged to 7 genera involving *Fusarium* (24), *Alternaria* (21), *Didymella* (9), *Penicillium* (3), *Phoma* (3), *Camarosporidiella* (1), and *Xylogone* (1). Based on the analysis of the ITS sequences of the strains, the members of *Alternaria* and *Fusarium* genus were found most frequently.
- 34 endophytes were positive for their siderophores sequestering and binding iron from the medium and the largest zone appeared as 32 mm on the plate of the *F. tricinctum* SZMC 26658 (isolated from the stem part of *S. flavescens*).
- 31 fungal endophytes were discovered as the solubilizing phosphate. The highest phosphate solubilization strain (SZMC 26657, 40.33 mm) was also the member of same species as the best siderophore producing strain (*F. tricinctum*) and both isolated from the stem of *S. flavescens*.
- IAA productions were generally improved due to the additional Trp in the ferment broth and mainly in the mycelia except for 7 strains. All isolates were able to produce higher amount of the IAA in cultivation media than

intracellularly.

- The six isolates with high IAA production were used to evaluate the effects of the endophytic IAA on the plants. The primary root lengths of *A. thaliana* were increased in several cases, however, the biomasses were significantly lower in the treated plants than in the control IAA treatment. The alteration of *A. thaliana* pigment contents did not show any specific trends.
- Fungal endophytes were able to produce IAA at least through 3 different pathways such as IAM, IPyA and TAM. Most of our fungal endophytes used IPyA pathway for their IAA biosynthesis followed by the IAM. Numerous isolates have conducted double or triple biosynthetic pathways for their IAA biosynthesis. We established for the first time that *Didymella* and *Alternaria* species produce IAA via IAM pathway.
- Regarding bioactivity, a total of 372 extracts were tested. For the antibacterial assay, the ferment broth extracts of the isolates showed higher inhibitory activity on the bacteria than the mycelial extracts. The highest number of the extracts were active on *Stap. aureus* and *Strep. albus* and most of those isolates belonged to the *Fusarium*, *Alternaria* and *Didymella* species.
- For the antifungal activity, 64 extracts were found to be the highest number to show potential against *C. albicans*. Furthermore, 38 and 41 extracts showed significant inhibition against *A. niger* and *R. solani*, respectively. Moreover, the lowest number of isolates were against *F. culmorum*. In addition, ethyle-acetate extract of SZMC 26649 (*D. glomerata*) ferment broth showed the highest inhibition zone in the whole test, which was detected against *C. albicans*.

5 LIST OF PUBLICATIONS RELATED TO THIS THESIS

Adiyadolgor Turbat, David Rakk, Aruna Vigneshwari, Sándor Kocsubé, Huynh Thu, Ágnes Szepesi, László Bakacsy, Biljana D. Škrbić, Enkh-Amgalan Jigjiddorj, Csaba Vágvölgyi and András Szekeres, 2020. Characterization of the plant growth-promoting activities of endophytic fungi isolated from *Sophora flavescens*. *Microorganisms,* 8, 683: doi:10.3390/microorganisms8050683.

Gábor Endre, Zsófia Hegedüs, **Adiyadolgor Turbat**, Biljana Škrbic, Csaba Vágvölgyi and András Szekeres, 2019. Separation and purification of aflatoxins by centrifugal partition chromatography. *Toxins,* 11, 309: doi:10.3390/toxins11060309.

Adiyadolgor Turbat, Gábor Endre, Dávid Rakk, Csaba Vágvölgyi, András Szekeres, 2021. Determination of indole-3-acetic acid biosynthetic pathways in fungal endophytes. ACTA MICROBIOLOGICA ET IMMUNOLOGICA HUNGARICA 68 : S1 p. 115 (2021)

Adiyadolgor Turbat, Enkh-Amgalan Jigjiddorj, Csaba Vágvölgyi, András Szekeres, 2018. Antimicrobial activity of endophytic fungi isolated from *Sophora flavescens*. In 16th Wellmann International Scientific Conference.

Adiyadolgor Turbat, David Rakk, Enkh-Amgalan Jigjid, Csaba Vágvölgyi and András Szekeres, 2017. Determination of plant-growth promoting compound in endophytes, isolated from *Sophora flavescens*. ACTA MICROBIOLOGICA ET IMMUNOLOGICA HUNGARICA 64 (Supplement 1): 183-184.

Adiyadolgor Turbat, Dávid Rakk, Enkh-Amgalan Jigjiddorj, Csaba Vágvölgyi, András Szekeres, 2017. Isolation of endophytes producing plant-growth promoting compounds. 5th Central European Forum for Microbiology.

Cumulative impact factor: 7.66

Scientific journal article: 2

Conference abstracts: 4

Number of independent citations: 16

DECLARATION

We, Dr. András Szekeres (Senior research fellow, Department of Microbiology, University of Szeged) and Prof. Dr. Csaba Vágvölgyi (Head of Department, Department of Microbiology, University of Szeged), the supervisors of the Ph.D. candidate Adiyadolgor Turbat, hereby certify, that we are familiar with the Ph.D. thesis entitled "Characterization of plant growth-promoting activities of endophytic fungi isolated from Mongolian medicinal plants". The thesis points of the dissertation are the results of the candidate. The candidate's contribution was significant on the below mentioned publications:

Adiyadolgor Turbat, David Rakk, Aruna Vigneshwari, Sándor Kocsubé, Huynh Thu, Ágnes Szepesi, László Bakacsy, Biljana D. Škrbić, Enkh-Amgalan Jigjiddorj, Csaba Vágvölgyi and András Szekeres, 2020. Characterization of the plant growth-promoting activities of endophytic fungi isolated from *Sophora flavescens*. *Microorganisms*, 8, 683: doi:10.3390/microorganisms8050683., IF: 3.531

Gábor Endre, Zsófia Hegedüs, **Adiyadolgor Turbat**, Biljana Škrbic, Csaba Vágvölgyi and András Szekeres, 2019. Separation and purification of aflatoxins by centrifugal partition chromatography. *Toxins*, 11, 309: doi:10.3390/toxins11060309., IF: 4.128

The results reported in the Ph.D. dissertation were not used to acquire any Ph.D. degree previously and will not be used in future either.

Szeged, January 25, 2022

Prof. Dr. Csaba Vágvölgyi Dr. András Szekeres