

Development of *Trichoderma*-based bioeffectors for biocontrol and plant growth promotion

Ph.D. THESIS

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2016

1. INTRODUCTION

Members of the genus *Trichoderma* are cosmopolitan fungi, widespread throughout the world as permanent members of different ecosystems. They can utilize wide scale of available nutrients and possess high reproductive capacity and competitive properties. A number of *Trichoderma* species has a significant impact on human welfare and numerous opportunities for application. They are the most commonly used biofungicides and plant growth promoters and they are also good resources of extracellular enzymes that can be used by several industries. *Trichoderma* species produce wide range of different secondary metabolites. Some species of the genus have clinical importance while others play roles in soil bioremediation. Despite the success of modern agriculture, there are still a lot of practices (e.g. excessive use of inorganic fertilizers) that increase the risk of diseases affecting plants. Moreover, plant pathogenic fungi present in the soils may raise further problems. The damages caused by *Pythium*, *Phytophthora*, *Botrytis*, *Rhizoctonia* and *Fusarium* species are increasing from year to year. The application of different chemicals (chemical control) is predominant to control their growth, however, these chemicals have negative environmental impacts and the emergence of resistant strains is also a serious problem. Nowadays there is a growing interest towards the alternative agricultural techniques, as they can provide e. g. a good opportunity for biological control. A number of bacterial- and fungal-based biocontrol products became available and a high incidence of *Trichoderma* strains can be observed among them. Within the genus *Trichoderma*, the species *T. virens*, *T. viride* and the THSC species complex are the most frequently used biocontrol agents. The success of *Trichoderma* species as biocontrol agents is based on their numerous positive properties such as the high reproductive capacity and a high degree of tolerance of adverse condition. They have high efficiency to utilize various nutrients as different carbon sources and the capability to colonize the

rhizosphere. These mostly soilborne fungi have been marketed successfully in order to increase the crop yield by their plant growth promoting effects and biocontrol abilities against plant pathogenic microorganisms (bacteria, fungi, oomycetes).

2. AIMS

The aims of our work were:

1. to isolate *Trichoderma* strains from Hungarian vegetable rhizosphere samples and to identify them by molecular tools,
2. to determine the *in vitro* antagonistic abilities of the isolates against different plant pathogenic fungi in dual confrontation tests,
3. to investigate the effects of various environmental factors (temperature, pH, water activity) on their mycelial growth,
4. to examine the cellulose-degrading and phosphate-mobilizing capabilities, as well as the examination of their laccase production,
5. to determine their sensitivity to different fungicides,
6. to study the plant growth promoting effect of the most promising strain, and
7. to select bioeffector *Trichoderma* strains with biocontrol and plant growth promoting capabilities for the purposes of practical application.

3. METHODS

- ◆ Isolation of *Trichoderma* strains from vegetable rhizosphere samples
- ◆ Sequence-based identification of the isolated strains
- ◆ Determination of the *in vitro* antagonistic abilities in dual confrontation tests
 - 14 *Trichoderma* strains
 - 9 plant pathogenic fungi
 - Image analysis

- Determination of the Biocontrol Index (BCI) values
- ◆Determination of the effects of various environmental factors
 - Temperature: 5-40 °C
 - pH: 2.2-8
 - Water activity (a_w): 0.977-0.922
- ◆Investigation of the cellulose-degrading and phosphate-mobilizing capabilities
 - cellulose-degrading capabilities with paranitrophenyl- β -D-cellobioside and paranitrophenyl- β -D-glucopyranoside substrate
 - phosphate-mobilizing capabilities with paranitrophenyl-phosphate substrate
- ◆Laccase enzyme production of the strains
 - qualitative analysis of the laccase enzymes on indicator media
 - quantitative analysis of the laccase production in liquid cultures with ABTS substrate
 - investigation of the pH dependence of the laccase activities
- ◆Determination of the Minimal Inhibitory Concentration (MIC) values of different fungicides towards *Trichoderma* isolates
 - Microdilution method
 - 14 fungicides included
- ◆Experiments on tomato plants
 - determination of the biomass production
 - determination of stomatal conductance and CO₂ assimilation
 - determination of chlorophyll *a* fluorescence induction parameters
 - investigation of photosynthetic pigment contents
 - investigation of total sugar content

4. SUMMARY OF RESULTS

We isolated 45 *Trichoderma* strains from 16 vegetable rhizosphere samples collected in Hungary. Based on ITS-sequence analysis we could identify 10 different *Trichoderma* species. The most abundant taxon among the isolates was the *Trichoderma harzianum* species complex (THSC 55.6%). The species *T. pleuroticola* - known as one of the causal agents of oyster mushroom green mould disease - was the second most common species. We also identified representatives of the *T. longibrachiatum*/*T. orientale* and *T. citrinoviride* species. These strains are potential opportunistic human pathogens and may cause infections and mycoses in immunocompromised patients. In addition to the above mentioned species, we also isolated *T. koningiopsis*/*T. ovalisporum*, *T. hamatum*, *T. virens* and *T. gamsii* strains from tomato, carrot, salad, and paprika rhizosphere samples.

In order to describe the *in vitro* antagonistic abilities of the *Trichoderma* strains, we performed dual confrontation assays and determined the biocontrol index (BCI) values. The highest BCI values against the tested plant pathogenic fungi were determined in the case of the two *T. asperellum* strains. Both of these strains proved to be the most effective against 3-3 plant pathogenic fungi: *T. asperellum* SZMC 20866 was the most effective against FSSC SZMC 11057F, SZMC 11064F and *Phoma cucurbitacearum*, while *T. asperellum* SZMC 20786 was the most effective against the other two FSSC isolates (SZMC 11067F and SZMC 11070F) and *Alternaria alternata* SZMC 16085.

We studied the effects of various environmental factors (temperature, pH, water activity) on the mycelial growth in the case of 14 *Trichoderma* strains selected for detailed characterisation. None of the examined *Trichoderma* strains could grow at 5 °C and only 2 strains (*T. hamatum* SZMC 20784, *T. longibrachiatum* SZMC 20788) were able to grow at 10 °C. The strains showed slight growth at 15 °C, while the optimal growth temperatures were between 20

and 30 °C. The mycelial growth of the strains decreased gradually by rising the temperature to 35 and 40 °C. We should treat *T. citrinoviride* and *T. longibrachiatum* strains as potential human pathogens, as they are able to grow between 35 and 40 °C. The *Trichoderma* strains showed mycelial growth in a wide range of pH values. All strains had their pH optima at 3 or 5. Decreased mycelial growth was observed above pH 4. In the case of water activity, more than half of the examined strains showed optimal growth at the a_w value of 0.997, whereas the growth optimum was at 0.991 in the case of the remaining strains. When the water activity was decreased, the diameters of the colonies decreased as well. Only 3 strains showed growth at a_w 0.945, while none of them could grow at 0.922.

The cellulose degrading and phosphate mobilizing capabilities of the selected *Trichoderma* strains were examined on liquid minimal and corn stem powder containing media. The mycelium-free ferment broths were used for the enzyme activity measurements. In liquid minimal medium, strains THSC SZMC 20761 and *T. hamatum* SZMC 20784 had the highest cellobiohydrolase and β -glucosidase activities, however, the enzyme productivity of these strains decreased in corn stem powder containing liquid medium. The enzyme production of THSC SZMC 20869 increased dramatically in corn stem powder containing liquid medium, but in minimal liquid medium its cellobiohydrolase and β -glucosidase activities were lower than that of the previously mentioned two strains. None of the examined *Trichoderma* strains showed phosphatase activities in minimal liquid medium. In corn stem powder containing liquid medium, THSC SZMC 20869 had the highest phosphatase activity.

The laccase production capability of *Trichoderma* strains derived from rhizosphere samples were studied on Petri plates containing a medium supplemented with ABTS or guaiacol as substrate. Three laccase-producing *Trichoderma* strains (*T. asperellum* SZMC 20786, SZMC 20688 and *T.*

atroviride SZMC 20780) could be identified. The relative laccase activities of these strains were the highest on the second day of the fermentation. *T. atroviride* SZMC 20780 showed 3 fold higher laccase activities than the *T. asperellum* strains. We used ferment broths derived from the 2nd and 3rd day of fermentation for the determination of the pH optimum of the laccase enzymes. The optimum pH for the laccases produced by the above mentioned *Trichoderma* strains were pH 3.5 and 4. We could measure a decrease in laccase activities above pH 6.

We determined the MIC values of 14 fungicides towards the selected *Trichoderma* strains. Our results showed that these rhizosphere-derived *Trichoderma* strains possess natural tolerance against the tested fungicides. Only 6 fungicides (cyproconazol, imazalil, penconazol, spiroxamine, thiophanate-methyl, thiram) showed inhibitory effects towards the examined *Trichoderma* strains.

We studied the plant growth promoting effect of *T. asperellum* SZMC 20786 strain on tomato plants. Significant growth in the length of shoots and roots could be detected. This positive effect was present in the case of plant fresh weight. After the treatment with *Trichoderma*, the photosynthetic efficacy of the plants was examined. We also collected data about the total sugar, chlorophyll *a+b* and carotenoid contents of the tomato plants. The increased stomatal conductance and CO₂ assimilation, as well as the higher total sugar content suggest that the photosynthesis was induced in the tomato plants after the *Trichoderma* treatment.

Based on our results, 2 *Trichoderma* strains were selected as the components of a composite bioeffector product. The first one was *T. asperellum* SZMC 20786, a strain possessing excellent *in vitro* antagonistic and laccase-producing capabilities as well as tolerance towards a wide range of fungicides and plant growth promoting effects in tomato, while the second one was THSC

SZMC 20869, a strain with the ability to produce high amounts of cellulase and phosphatase enzymes and polyresistance to the tested fungicides. The planned bioeffector product has been amended with a *Streptomyces albus* strain with excellent peroxidase-producing abilities as a humus-producing component, and an *Azotobacter vinelandii* strain with the capability to grow on nitrogen source-free medium, thus having the potential to provide excess nitrogen for crops. The previously mentioned strains were provided to the BioeGO Ltd. After the licencing of the product, it was named as “BioeGO soil organizer” and introduced into the market in 2015. Since then the product was recognised with the Southern Plain’s Innovation Award.

5. PUBLICATION LIST

Papers

Körmöczi P, Danilovic G, Manczinger L, Jovanovic L, Pankovic D, Vágvölgyi C and Kredics L (2013). Species composition of *Trichoderma* isolates from the rhizosphere of vegetables grown in Hungarian soils. *Fresen Environ Bull* 22:(6) pp. 1736-1741. **IF: 0,527**

Körmöczi P, Marik T, Manczinger L, Sajben-Nagy E, Vágvölgyi Cs and Kredics L (2014). *Trichoderma* isolates from vegetable rhizosphere samples: potential for the biological control of *Botrytis* species. *Review on Agriculture and Rural Development* 3:(1 CD Supplement) pp. 324-330.

Körmöczi P, Manczinger L, Sajben-Nagy E, Vágvölgyi C, Danilović G, Panković D, Jovanović L, Pucarević M and Kredics L (2013). Screening of *Trichoderma* strains isolated from rhizosphere samples for laccase production. *Review On Agriculture And Rural Development* 2:(1) pp. 325-330.

Book chapters:

Kredics L, Hatvani L, Naeimi S, **Körmöczi P**, Manczinger L, Vágvölgyi C and Druzhinina I (2014). Biodiversity of the Genus *Hypocrea/Trichoderma* in Different Habitats. In: Gupta VK; Schmoll M; Herrera-Estrella A; Upadhyay RS; Druzhinina I; Tuohy M (szerk.) *Biotechnology and Biology of Trichoderma*. Amsterdam: Elsevier Science B.V., 2014. pp. 3-24.

Körmöczi P, Danilovic G, Ljubinko J, Manczinger L, Pankovic D, Vágvölgyi C and Kredics L (2013). Magyarországon termesztett zöldségek rizoszférájából származó *Trichoderma* izolátumok *in vitro* antagonista képességeinek vizsgálata. In: Bacskainé Bódi É, Fekete I, Kovács B (szerk.) *Fiatalkutatók az egészséges ételmiszerért: Tudományos ülés*. pp. 135-140.

Abstracts published in journals:

Körmöczi P, Szekeres A, Leitgeb B, Danilović G, Panković D, Manczinger L, Vágvölgyi C and Kredics L (2013). Application of the image analysis-based biocontrol index calculation method for the assessment of *in vitro* antagonistic abilities of *Trichoderma* isolates from vegetable rhizosphere samples. *Acta Microbiologica Et Immunologica Hungarica* 60:(S) pp. 40-41.

Körmöczi P, Oláh S, Marik T, Terhes D, Danilovic G, Pankovic D, Manczinger L, Vágvölgyi C and Kredics L (2012). A *Trichoderma* nemzetség biodiverzitása magyarországi zöldségrhizoszféra mintákban *Mikol Közlekedés* 51:(1) pp. 140-141.

Kredics L, Marik T, Oláh S, Terhes D, Danilovic G, Pankovic D, Manczinger L, Vágvölgyi C and **Körmöczi P** (2012). Species composition of *Trichoderma* communities in Hungarian soils used for vegetable cultivation. *Review on agriculture and rural development* 1:(1) p. 483.

Körmöczi P, Kredics L, Danilovic G, Jovanovic L, Manczinger L, Pankovic D and Vágvölgyi Cs (2013). Possibilities of bioremediation, biocontrol and plant growth promotion with *Trichoderma* strains isolated from vegetable rhizosphere samples. *Acta Microbiologica Et Immunologica Hungarica* 60:(S) pp. 163-164.

Abstracts published in conference proceedings:

Körmöczi P, Danilovic G, Ljubinko J, Manczinger L, Pankovic D, Vágvölgyi C and Kredics L (2013). Magyarországon termesztett zöldségek rizoszférájából származó *Trichoderma* izolátumok *in vitro* antagonista képességeinek vizsgálata. Fiatal kutatók az egészséges ételmisszerért: Tudományos ülés.

Körmöczi P, Kredics L, Danilovic G, Jovanovic L, Manczinger L, Pankovic D and Vágvölgyi Cs (2013). Possibilities of bioremediation, biocontrol and plant growth promotion with *Trichoderma* strains isolated from vegetable rhizosphere samples. Acta Microbiologica Et Immunologica Hungarica 60:(S) pp. 163-164. 4th Central European Forum for Microbiology.

Körmöczi P, Bóka B, Szabó S, Tarnai G, Manczinger L, Chandrasekaran M, Shine K, Naiyf A, Vágvölgyi C and Kredics L (2015). Combined application of *Trichoderma*, *Streptomyces* and *Azotobacter* strains in the soil conditioner BioeGO. 17th Danube-Kris-Mures-Tisa (DKMT) Euroregional Conference on Environment and Health: Program and Abstracts. 78 p.

Kredics L, **Körmöczi P**, Bóka B, Szabó S, Tarnai G, Chandrasekaran M, Manczinger L and Vágvölgyi C (2015). BioeGO: development of a fungal-bacterial consortium based soil inoculant for plant growth promotion and biocontrol. 6th Congress of European Microbiologists, Abstract P 1308.

Kredics L, Bóka B, **Körmöczi P**, Szabó S, Tarnai G, Manczinger L, Marik T, Szekeres A and Vágvölgyi C (2015). BioeGO: a new soil inoculant based on the combined application of beneficial bacteria and fungi. miCROPE International Symposium: Microbe-assisted crop production - opportunities, challenges & needs. p. 219.

Danilović G, **Körmöczi P**, Vágvölgyi C, Kredics L, Jovanovic L and Panković D (2014). Species composition of *Trichoderma* isolates from vegetable rhizosphere in conventional and organic farming. In: XVI. International Congress on Plant-Microbe Interactions. P129.

Danilović G, **Körmöczi P**, Kredics L, Panković D and Nešić L (2014). Variability and antagonism properties of *Trichoderma* strains isolated from the rhizosphere of plants grown in different soil types. In: XVI. International Congress on Plant-Microbe Interactions. P135.

Danilovic G, **Körmöczi P**, Kredics L, Pankovic D and Jovanovic L (2014). *Trichoderma* as biocontrol agent - benefits and risks. EU Project Collaborations: Challenges for Research Improvements in Agriculture. P53.

Körmöczi P, Marik T, Sajben-Nagy E, Manczinger L, Vágvölgyi Cs and Kredics L (2014). Biocontrol potential of *Trichoderma* isolates from pepper and lettuce rhizosphere against plant pathogenic *Botrytis cinerea* and *B. pseudocinerea* strains. 16th Danube-Kris-Mures-Tisa (DKMT) Euroregion Conference on Environment and Health: Book of Abstracts. p 40.

Kredics L, **Körmöczi P**, Bóka B, Tarnai G, Szabó S, Manczinger L and Vágvölgyi C (2014). Development of a four-component liquid soil biofertilizer: BioeGO. A Magyar Mikrobiológiai Társaság 2014. évi Nagygyűlése and EU FP7 PROMISE Regional Meeting: Absztraktfüzet. 82 p.

Körmöczi P, Marik T, Manczinger L, Sajben-Nagy E, Vágvölgyi C, Danilovic G, Pankovic D, Jovanovic L, Pucarevic M and Kredics L (2013). Laccase production of *Trichoderma* strains from vegetable rhizosphere. 15th Danube-Kris-Mures-Tisa (DKMT) Euroregion Conference on Environment and Health with satellite event LACREMED Conference "Sustainable agricultural production: restoration of agricultural soil quality by remediation"

Danilović G, **Körmöczi P**, Kredics L, Vágvölgyi C, Jovanović L, Vrić MM and Panković D (2012). Genetic variability of *Trichoderma* isolates from the rizosphere of vegetables in agricultural soils in Serbia. International Conference NEWENVIRO - New approaches for assessment and improvement of environmental status in Balkan region: interactions between organisms and environment.

Körmöczi P, Sajben E, Manczinger L, Danilovic G, Pankovic D, Leitgeb B, Szekeres A, Vágvölgyi C and Kredics L (2012). Screening of *Trichoderma* isolates from vegetable rhizosphere for *in vitro* antagonistic potential against plant pathogenic fungi and extracellular laccase production. 14th DKMT Euroregional Conference on Environment and Health.

Kredics L, Marik T, Oláh S, Terhes D, Danilovic G, Pankovic D, Manczinger L, Vágvölgyi C and **Körmöczi P** (2012). *Trichoderma* species occurring in the rhizosphere of vegetables in different regions of Hungary. 14th DKMT Euroregional Conference on Environment and Health.

Further Papers

Hassan AS, Al-Hatmi AMS, Shobana CS, van Diepeningen AD, Kredics L, Vágvölgyi C, Homa M, Meis JF, de Hoog GS, Narendran V, Manikandan P, Varga J, Galgóczy L, Kocsubé S, **Körmöczi P**, Papp T, Baranyi N, Szekeres A, Revathi R, Anita R, Gomathi P, Mythili A, Punitha T, Rajendran R, Selvam KP, Chandrasekaran M, Dóczi I, Leitgeb B, IHFK Working Group (2016). Antifungal susceptibility and phylogeny of opportunistic members of the genus *Fusarium* causing human keratomycosis in South India. *Medical Mycology* 54:(3) pp. 287-294. **IF: 2,164**

Kredics L, Narendran V, Shobana CS, Vágvölgyi C, Manikandan P, Varga J, Galgóczy L, Kocsubé S, Németh TM, Papp T, Homa M, Baranyi N, Szekeres A, **Körmöczi P**, Krizsán K, Anita R, Revathi R, Gomathi P, Hassan AS, Singh YRB, Mythili A, Selvam KP, Dóczi I and Leitgeb B (2015). Filamentous fungal infections of the cornea: A global overview of epidemiology and drug sensitivity. *Mycoses* 58:(4) pp. 243-260. **IF: 2,332**

Kredics L, Ládai M, **Körmöczi P**, Manczinger L, Rákhely G, Vágvölgyi C and Szekeres A (2011). Genetic and biochemical diversity among *Trichoderma* isolates in soil samples from winter wheat fields of the Pannonian Plain. *Acta Biol Szeged*.

Kredics L, Ládai M, **Körmöczi P**, Manczinger L, Rákhely G, Vágvölgyi C and Szekeres A (2011). *Trichoderma* communities of the winter wheat rhizosphere. *Agrár- és Vidékfejlesztési szemle* 6: S413-S418.

Magyar D, Eszéki ER, Oros G, Szécsi Á, Kredics L, Hatvani L and **Körmöczi P** (2011). The air spora of an orchid greenhouse. *Aerobiologia* 27: 121-134. **IF: 1,173**

Kredics L, Hatvani L, **Körmöczi P**, Manczinger L and Vágvölgyi C (2010). A termesztett gombák zöldpenészes fertőzése. *Zöldség és gyümölcs piac* 2010: p. 13.

Kredics L, **Körmöczi P**, Cseh T, Hatvani L, Manczinger L, Nagy A and Vágvölgyi C (2009). Green mould disease of oyster mushroom in Hungary and Romania: ecophysiology of the causative agent. Annals of Faculty of Engineering Hunedoara-International Journal of Engineering 7: 195-198.

Kredics L, Cseh T, **Körmöczi P**, Nagy A, Kocsubé S, Manczinger L, Vágvölgyi C and Hatvani L (2009). A termesztett laskagomba zöldpenészes fertőzése. Mikol Közl Clusiana 48: 81-92.

Cumulative impact factor: 6.196