PhD THESIS

Echophysiological and molecular investigation of *Trichoderma* strains isolated from Hungarian soil samples

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INTRODUCTION

The efficient control of fungal plant pathogens causing substantial losses in agricultural production is an important issue in all plant cultivation systems. Species belonging to the genus *Trichoderma* are imperfect filamentous fungi of multiple importance. Members of this genus are well known as cellulase producers of biotechnological relevance. Certain *Trichoderma* species are on the growing list of potential fungal pathogens in immunocompromised humans, while others are harmful in mushroom cultivation as the causative agents of green mold epidemics.

Furthermore, the genus involves promising biocontrol candidates with excellent antagonistic abilities against a series of plant pathogenic fungi including *Fusarium*, *Pythium*, *Rhizoctonia*, *Botrytis* and *Sclerotinia* species. Several modes of action, including antibiosis by the production of antifungal metabolites, competition for space and nutrients, plant growth promotion, induction of the defense responses in plants and mycoparasitism have been proposed to play roles in biocontrol capabilities. These processes are supposed to act synergistically. For the study of this complex synergistic system, it is crucial to clear the relative importance of the individual mechanisms involved in the antagonistic process. Both competition by colonizing the ecological niche favoured by the pathogen and mycoparasitism by penetration of the host hyphae require hydrolitic enzyme systems, that are playing important roles in the digestion of the

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available natural substrates in the soil and the polymers constituting the cell-wall and cytoplasm of the target fungi. Based on the involvement in the biocontrol process, the extracellular enzymes secreted by *Trichoderma* species can be separated into the two major classes of mycoparasitic and competitive enzymes. Certain enzyme systems take part in both mechanisms. The secretion of extracellular enzymes may occur constitutively or inductively. From the aspect of effective biological control it is favorable if the biocontrol strains produce large amounts of enzymes either constitutively, or as an early response to induction.

APPLIED METHODS

In our taxonomical works, *Trichoderma* strains were isolated from roots of winter wheat grown in agricultural fields of southern Hungary, and the identity of species was examined based on morphological, biochemical as well as molecular characters. Morphological data were collected by measuring the structure and shape of conidiophores, phialides and conidia. The different electrophoretic types revealed by cellulose-acetate electrophoresis mediated isoenzyme analysis were used as biochemical markers. For the investigation of molecular diversity, sequence analysis of the internal transcribed spacer region was performed.

The ecophysiological properties of the strains were described based on the examinations of the production of extracellular enzymes and antibiotics as well as the measurement of the biocontrol activity.

In the extracellular enzyme assays, the production of β-xylosidase, α-glucosidase, β-glucosidase, β-galactosidase, cellobiohydrolase, trypsin-, chymotrypsin- and chymoelastase-like proteases and *N*-acetyl-β-glucosaminidase were investigated. These enzymes are important for the progress of competition and mycoparasitism. The secretion of enzymes was measured by means of specific chromogenic *p*-nitroanilide and *p*-nitrophenyl substrates. The secretion of the antibiotics was detected using biological tests. The biocontrol properties were tested *in vitro* by direct confrontation assays and modified aggressiveness tests. The direct confrontation assay was applied for recording the inhibition effect, which was expressed as the value of biocontrol index (BCI) calculated from the image analysis of ratio of the occupied area of fungal colonies. Natural-like conditions were reached with the application of the modified aggressiveness tests.

UV-irradiation mediated mutagenesis method was carried out for the improvement of the selected *Trichoderma* strains for higher antagonistic capacity.. Mutant strains were selected for *p*-fluorophenyl-alanine resistance or altered colony morphology.

NEW SCIENTIFIC RESULTS

1. One hundred and sixteen *Trichoderma* strains were isolated at 18 different test holes from roots of winter wheat grown in 5 agricultural fields of southern Hungary. The isolates were purified and deposited in the Microbiological Collection of the University of Szeged. [2, 15, 17, 18, 21]
2. We identified 89 *Trichoderma* isolates at the species level by morphological methods. These strains belong to *T. harzianum* (44), *T. virens* (31), *T. atroviride* (9) and *T. longibrachiatum* (5). [2, 15, 17, 18, 21]
3. A method useful in taxonomical point of view, based on cellulose-acetate electrophoresis mediated isoenzyme analysis was worked out to determine the taxonomical position of the *Trichoderma* isolates using previously identified clinical strains deriving from culture collections. The reproducibility and reliability of the method as well as its applicability in clinical diagnostic were proved. [6, 9, 10, 17, 19, 22]
4. The Hungarian *Trichoderma* isolates were classified by the cellulose-acetate electrophoresis mediated isoenzyme analysis, and the phylogenetic relationships between them were described based on their biochemical properties. [2, 15, 17, 18, 21, 25]
5. The molecular taxonomical examination of Hungarian *Trichoderma* isolates was undertaken based on the DNA sequences of the ITS 1 and ITS 2 regions. Nine of sixteen isolates were identified at the species level and 18 at the clade level with the oligonucleotid barcode method. The comparison of the CAE- and barcode results enabled the identification of further 15 strains at the species level. [15, 17, 18, 25]
6. In the phylogenetic analysis of the DNA sequences, 4 novel genotypes of *T. harzianum* and 3 of *T. virens* were determined. Furthermore, we suggested the description of five new *Trichoderma* species based on their high phylogenetic distance from all known species. [15, 17, 25]
7. In the collective phylogenetic analysis of the isoenzyme patterns and the ITS 1 and ITS 2 sequences we proposed the phylogenetic positions of the *Lutea* and *Semiorbis* clades as well as the „*Lone lineages*" group. [17, 25]
8. We have determined the Shannon diversity indices at certain sample holes of the 13 identified *Trichoderma* species (8 already described: *T. harzianum*, *T. virens*, *T. atroviride*, *T. longibrachiatum*, *T. brevicompactum*, *T. spirale*, *T. rossicum* and *T. oblongisporum*; 5 presumably new species). Furthermore, our study is the first to demonstrate the occurrence of *T. virens*, *T. rossicum*, *T. spirale*, *T. brevicompactum* and *T. oblongisporum* in Hungary. [17, 25]
9. We estimated the secretion of 10 extracellular enzymes and the antibiotic production of the isolated strains, their antibiotics were partially purified. [3, 4, 11, 12, 13, 17, 24]
10. In our examinations, significant correlations were usually not detected between the secretion of the extracellular enzymes. Howewer, on

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minimal medium, relatively high correlations were between β-1,4-*N*-acetyl-glucosaminidase and β-glucosidase, between certain proteases and Leu-aminopeptidase, while on yeast extract medium between β-galactosidase and α-glucosidase, β-xylosidase and β-galactosidase, cellobiohydrolase and β-galactosidase, and between certain proteases . Nevertheless, no correlation could be found between the taxonomical position and the enzyme production of the strains in our investigation system. [17, 24]

11. Among the isolates, 17 proved to be antibiotic producers under the conditions applied. The produced compounds showed antimicrobial activity against the tested Gram-negative bacteria. The detection of antibiotics was in correlation with the taxonomical position of the isolates: except for a single strain (*T. harzianum*), all of the producers proved to belong to *T. virens*. [4, 11, 13, 17]
12. We developed an *in vitro* method for the determination of the biocontrol activity based on image analysis, which has the following advantages: it takes the most important mechanisms of antagonism, namely competition for space and nutrients, production of antifungal metabolites and mycoparasitism into consideration by enabling their development during the incubation time of ten days. Furthermore, isolates with biocontrol potential examined in different laboratories around the world are comparable using the BCIs calculated under the defined experimental parameters. Finally, the described method is

simple to carry out and provides accurate quantitative values for the evaluation of *in vitro* antagonism, which may help the characterization of biocontrol abilities of fungal antagonists. [5, 14, 16, 17, 23]

13. Trying to simulate natural conditions, a new *in vitro* biocontrol model, the modified aggressiveness test was developed for the robust measurement of the biocontrol activity of *Trichoderma* strains. [17, 24]
14. We examined the statistical relationships between the values of antagonism and extracellular enzyme activity, and proved that the enzymes playing a role both in the competition and mycoparasitism had an influence on the *in vitro* antagonism. [17, 24]
15. To improve the fungal antagonistic capacity of the selected *Trichoderma* strains, a mutagenetic program was undertaken for the construction of protease overproducing derivates. The mutant strains were obtained by means of UV-irradiation and were selected for *p*-fluorophenyl-alanine resistance or altered colony morphology. Both trypsin-like and chymotrypsin-like protease secretion was elevated in most of the mutant strains. The profiles of isoenzymes were different between the mutants and the wild-type strain, when examined by gel filtration chromatography. Certain mutants proved to be better antagonists against plant pathogens in *in vitro* antagonism experiments. These results suggest the possibility of using mutants with improved constitutive extracellular protease secretion against plant pathogenic fungi. [1, 3, 7, 8, 12, 17, 19, 20]

LIST OF PUBLICATIONS

1. Publications related to the PhD study

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1. **Szekeres, A.,** Kredics, L., Antal, Z., Manczinger, L., and Kevei, F. (2004): Isolation and characterization of protease overproducing mutants of *Trichoderma harzianum. FEMS Microbiol. Lett.* 233, 215-222. **(IF: 2,057)**
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