SUMMARY OF THE Ph.D. DISSERTATION

Mushroom pathogenic *Trichoderma* species: occurrence, biodiversity, diagnosis and extracellular enzyme production

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1. INTRODUCTION

World-wide mushroom cultivation is dominated by the production of *Agaricus bisporus* (champignon), *Lentinula edodes* (shiitake), and *Pleurotus ostreatus* (oyster mushroom). *Trichoderma* green mould infection in edible basidiomycetes has been known to be able to cause a drastical decrease in production or even entire crops can be wiped out. Pathogenic green moulds may colonize the substrate or grow on the surface of the emerging mushrooms, which thus can become severely spotted and often distorted, but in serious outbreaks no fruit bodies are produced. For *A. bisporus*, the association of *Trichoderma* species with the respective compost has been known for a long time to limit commercial production but it has become a severe problem following the occurrence of green mould epidemic - or epimycotic - in Northern Ireland in 1985, which was quickly succeeded by subsequent outbreaks in Ireland (1986), England and Scotland (1987), the Netherlands (1994), France (1997), Spain (1998) and Hungary (2004). In the early 1990s, a similar disease appeared in mushroom crops in the United States and Canada. The causative agents of the disease were described on the basis of morphological characteristics and the phylogenetic analyses of ITS 1 and the translation elongation factor 1-alpha (*tef1*) gene as the new species of *Trichoderma*, *T. aggressivum* f. *europaeum* (Europe) and *T. aggressivum* f. *aggressivum* (North America). Many pests and diseases can cause yield losses in *P. ostreatus*, and the association of *Trichoderma* species with the cultivation substratum has long been known to limit production. The first significant crop losses of cultivated *P. ostreatus* caused by green mould disease were reported in South Korea (2002), then severe infections were detected in Italy (2004), Hungary (2004) and Romania (2004). The causative agents of the oyster mushroom green mould have been reported to differ from *T. aggressivum* – the species responsible for *Agaricus* green mould disease – based on morphological characteristics, as well as DNA sequences, and therefore they have recently been introduced as the two species *T. pleurotum* and *T. pleuroticola.*

2. AIMS

Hungary is one of the leading European countries for mushroom production and export. Recently, Hungarian producers also noticed the occurrence of a *Trichoderma* green mould problem in their mushroom farms both for *A. bisporus* and for *P. ostreatus*, suggesting the simultaneous emergence of aggressive *Trichoderma* strains, resulting in a significant reduction in mushroom production. To the best of our knowledge no studies were carried out

aimed at *Trichoderma* species associated with cultivated mushrooms in Hungary, therefore we addressed the following questions arising from this situation:

- Is the green mould disease of *A. bisporus* due to *T. aggressivum* f. *europaeum*, i.e., has the Western European epidemic now spread to Central Europe?
- Which *Trichoderma* species are responsible for the green mould disease of *P. ostreatus* in Hungary?
- Are *T. aggressivum* and the *Pleurotus*-pathogenic green mould species specific for their substrates?

Our further aims were:

- To develop a PCR-based technique for the rapid and specific detection of the causal agents of the recently emerged green mould disease of oyster mushroom.
- To study the biodiversity of *Trichoderma* in the natural environment of *P. ostreatus.*

3. METHODS

3.1. Strain isolation

Trichoderma strains were isolated from samples of compost and substrate used for *A. bisporus* and *P. ostreatus* cultivation from three Hungarian mushroom farms (referred to further as A, B, and C) as well as from two facilities in Romania. Farm A produces *P. ostreatus*, farm B produces *A. bisporus* while farm C produces both mushrooms on the corresponding substrates. *Trichoderma* strains were isolated by placing from samples of compost and substrate on plates containing solid yeast extract-glucose medium (YEGM: glucose 5 g l^{-1}, yeast extract 1 g l^{-1}, KH_2PO_4 5 l^{-1} and agar 20 g l^{-1} in distilled water supplemented with streptomycin 0.1 g l^{-1} and chloramphenicol 0.1 g l^{-1}).

Fruiting bodies, as well as the growing substrate of *P. ostreatus* were collected in Hungarian forests, and inside the city of Szeged. *Trichoderma* strains were isolated as described above.

3.2. Polymerase chain reaction-based identification of *T. aggressivum*

A diagnostic polymerase chain reaction (PCR) test for *T. aggressivum* was carried out with primers Th-F and Th-R (Chen *et al.* 1999a).

3.3. Amplification and sequencing of the ITS1 and 2 regions, *tef1* and *chi18-5*

A region of nuclear rDNA, containing the ITS (internal transcribed spacer) 1 and 2 and the 5.8S rRNA gene, was amplifed by PCR using the primer combinations SR6R and LR1 (Gams and Meyer 1998). The amplification of a 0.4 kb fragment of endochitinase *chi18-5* (formerly named *ech42*) was carried out with the primer pair Chit42-1a and Chit42-2a, and an approximately 1-kb portion - including the fourth and fifth intron and a portion of the last large exon - of the *tef1* (translation elongation factor 1-alpha) gene was amplified and sequenced using primers EF1 and EF2 (O'Donnell *et al.* 1998) or with the primer pair EF1728F and TEF1LLErev (Jaklitsch *et al.* 2005). Purified PCR products for ITS1 and ITS2, *tef1*, and *chi18-5* were subjected to automatic sequencing. Sequences were aligned by the aid of the softwares ClustalX and GeneDoc, then they were deposited in NCBI GenBank and www.isth.info.

3.4. Identification of *Trichoderma* at the species level

Sequence analysis of ITS1, ITS2 and *tef1* was performed with the aid of the programs *TrichO*Key 1.0 (Druzhinina *et al.* 2005) and 2.0 (Druzhinina *et al.* 2006b) as well as *Tricho*BLAST (Kopchinskiy *et al.* 2005) available online at the home page of the International Subcommission on *Trichoderma* and *Hypocrea* Taxonomy (www.isth.info).

3.5. Phylogenetic analysis

For the phylogenetic analysis, DNA sequences were aligned using ClustalX and visually edited in Genedoc, version 2.6 (Nicholas *et al.* 1997). The interleaved NEXUS file was formatted using PAUP*, version 4.0b10 (Swofford 1998), and manually formatted for the MrBayes program, version 3.0B4. The Bayesian phylogenetic reconstructions have been performed as described in Jaklitsch *et al.* (2006). According to the protocol of Leache and Reeder (2002), posterior probability values lower than 0.95 were not considered significant while values below 0.9 were not shown on the consensus phylogram.

3.6. Restriction fragment length polymorphism analysis of mitochondrial DNA

Restriction fragment length polymorphisms (RFLPs) of mitochondrial DNA (mtDNA) were generated as described by Antal *et al.* (2006). For mtDNA characterization, the fast typing method of Varga *et al.* (1993) was used. Total DNA samples were isolated from lyophilized mycelia by the method of Leach *et al.* (1986) and digested with Hin6I (G/CGC) restriction enzyme. DNA fragments were separated by agarose gel electrophoresis and the

sizes of the mtDNA fragments were determined by using GelBase/GelBlot Pro Gel Analysis software (UltraViolet Products). The mtDNA profiles were converted to a similarity matrix and distance values were calculated with the PhylTools software (Buntjer 1997) to create dendrograms by the unweighted pair group method with arithmetic means using the NEIGHBOR program of PHYLIP version 3.57 (Felsenstein 1995).

3.7. Designing PCR primers specific to *T. pleurotum* and *T. pleuroticola*

Primers FPforw1 (5'- CAC ATT CAA TTG TGC CCG ACG A -3'), PSrev1 (5'- GCG ACA CAG AGC ACG TTG AAT C -3') and FPrev1 (5'- ACC TGT TAG CAC CAG CTC GC -3') were designed manually based on the translation elongation factor 1-alpha (*tef1*) sequence of *T. pleurotum* following an alignment containing the corresponding sequences of a series of *T. pleurotum*, *T. pleuroticola, T. harzianum, T. aggressivum* f. *europaeum* and f. *aggressivum* isolates, performed by the use of the software ClustalX. FPforw1 and FPrew1 were expected to amplify a 447 bp fragment from both species, while FPforw1 and PSrev1 were supposed to form a 218 bp PCR product only in the case of *T. pleurotum*.

3.8. PCR detection of *T. pleurotum* and *T. pleuroticola*

PCR was carried out in a final volume of 21 µl containing 95 mM 5X Green GoTaq™ Reaction Buffer, 0.38 mM dNTP Mix, 3.57 mM $MgCl_2$, 0.8 U GoTaq™ DNA polymerase (all from Promega Corporation), 190, 71 and 190 nM of primers FPforw1, FPrev1 and PSrev1, respectively, 0.5 µl bidistilled water, and 2 µl template DNA. Each experiment contained a negative control, where 2 µl bidistilled water was substituted for the template DNA. Amplification was performed in a Bio-Rad iCycler as follows: 1 cycle at 94 ^{o}C for 2 min, 35 cycles at 94 ^{o}C for 10 s and 68 ^{o}C for 20 s, and 1 cycle at 72 ^{o}C for 30 s.

3.9. Mutagenesis of *T. pleurotum* C15

From the sporulated colony of *T. pleurotum* C15 grown on solid YEGM for 5 days at 28 ^{o}C conidial suspension was prepared by washing the plate with 14 ml sterile distilled water. As a control to be used for the determination of the percentage of survival 1 ml of the suspension was taken and stored overnight at 10 ^{o}C. The rest of the suspension was exposed to UV irradiation by a Philips TUV 30 W germicide lamp at a wavelength of 254 nm from a distance of 30 cm for 5 minutes. After each minute of UV treatment 1 ml of the suspension was taken and stored overnight at 10 ^{o}C. A seven-step tenfold dilution of the suspension was prepared and 100 µl from each step was plated on solid YEGM supplemented with 0.1 %

Triton X-100 in order to obtain compact growth of the surviving colonies, which were counted following the incubation of the plates for 2-3 days at 28 ^{o}C. We attempted to isolate surviving colonies above 95 % of mortality but the treatment resulted in a lower degree of it, therefore the procedure was repeated with 7-12 minutes of irradiation.

3.10. Enzyme plate assays

Plate assays were performed in order to test the alterations in the function of pectinase, amylase, cellulase, lipase, protease, chitinase and glucanase enzyme system on solid media containing the corresponding substrates: pectin, starch, carboxymethyl-cellulose (CMC), Tween 20, gelatin, colloidal chitin and laminarin, respectively. Depending on the growth rate on the certain media, the diaterer of the colonies and the degradation zones were measured following incubation for 2-7 days at 28 ^{o}C.

3.11. Dual plate assays

The antagonistic ability of the mutants of *T. pleurotum* C15 showing altered enzyme production was tested against *P. ostreatus* in comparison with the parental strain on 3 different types of media: WAM (20 g l^{-1}agar in distilled water), YEGM (2 g l^{-1} glucose, 0.5 g l^{-1} yeast extract, 20 g l^{-1}agar in distilled water) and YEXM (2 g l^{-1} xylose, 0.5 g l^{-1} yeast extract, 20 g l^{-1} agar in distilled water) (Tokimoto 1982, Kitamoto *et al.* 1984). Mycelial disks of the colony *P. ostreatus* were inoculated onto the plates and after reaching a colony radius of approximately 1 cm, wild-type and mutant derivatives with altered production of certain enzyme systems of *T. pleurotum* C15 were inoculated next to them at a distance of 3 cm in the same way. After incubating the plates at room temperature for 5-7 days the overgrowth and conidiation of the *Trichoderma* strains was observed.

4. RESULTS

4.1. Identification of *Trichoderma* strains from *Agaricus* compost and *Pleurotus* substrate

Published in: Hatvani, L., Antal, Z., Manczinger, L., Szekeres, A., Druzhinina, I. S., Kubicek, C. P., Nagy, A., Nagy, E., Vágvölgyi, C. and Kredics, L. 2007. Green Mold Diseases of *Agaricus* and *Pleurotus* spp. Are Caused by Related but Phylogenetically Different *Trichoderma* Species. Phytopathology 97: 532-537.

A total of 66 *Trichoderma* strains were isolated from compost and substrate samples from three different Hungarian mushroom growing companies: 26 from farm A (producing *P. ostreatus*), 32 from farm B (producing *A. bisporus*), and 8 from farm C (producing both of them). In order to obtain a quick balance of how many of them were *T. aggressivum*, a diagnostic PCR amplification test was performed with the specific primers Th-F and Th-R. This resulted in the tentative identification of 16 isolates as *T. aggressivum*. However, several other isolates produced faint, yet detectable amplicons, rendering the result of this method uncertain. Therefore, fragments spanning the ITS1 and ITS2 sequences of the rRNA cluster were amplified, sequenced, and subjected to identification by the aid of *TrichO*Key. This strategy identified 17 isolates as *T. aggressivum*, thereby confirming all of the identifications reported above and additionally including one isolate that gave only a faint band in the diagnostic assay. The other 49 *Trichoderma* strains were also identified by means of *TrichO*Key: they included *T. harzianum*, *T. atroviride*, *T. asperellum*, and *T. ghanense* ((3, 9, 4 and 1 isolate, respectively). Four isolates belonged to the duplet of species *T. longibrachiatum*/*Hypocrea orientalis*, which exhibits identical ITS1 and ITS2 sequences and cannot be differentiated further by *TrichO*Key. Similarity searches were therefore performed with their *tef1* sequences (and *Tricho*BLAST), which revealed that they belong to the clade of *T. longibrachiatum*. The remaining 27 strains were identified as a yet unnamed phylogenetic species of *Trichoderma*, *Trichoderma* sp. DAOM 175924.

4.2. Distribution of *Trichoderma* species between *Agaricus* compost and *Pleurotus* substrate

Published in: Hatvani, L., Antal, Z., Manczinger, L., Szekeres, A., Druzhinina, I. S., Kubicek, C. P., Nagy, A., Nagy, E., Vágvölgyi, C. and Kredics, L. 2007. Green Mold Diseases of *Agaricus* and *Pleurotus* spp. Are Caused by Related but Phylogenetically Different *Trichoderma* Species. Phytopathology 97: 532-537.

T. aggressivum was exclusively isolated from *A. bisporus* compost, whereas *Trichoderma* sp. DAOM 175924 was exclusively found in the substrate for growth of *P. ostreatus*. Other species were found only in minor proportions, although occurring at higher frequency on *Agaricus* compost. The compost for production of *Agaricus* at location C - a farm with no significant *A. bisporus* losses due to *Trichoderma* sp. - was only contaminated by *T. harzianum*. These findings show that on mushroom farms there is a strict specificity of *T. aggressivum* and *Trichoderma* sp. DAOM 175924 for *A. bisporus* compost and *P.*

ostreatus substrate, respectively, which is still maintained when the two basidiomycetes are cultivated in close geographic vicinity. Based on differences in morphology and DNA sequences (ITS1 and 2, *tef1* and *chi18-5*) In parallel with our studies *Trichoderma* sp. DAOM 175924 was described as two distinct species, *T. pleurotum* and *T. pleuroticola* (Park *et al.* 2006). According to these findings the majority of the Hungarian isolates proved to belong to *T. pleurotum*, with a single isolate of *T. pleuroticola.*

4.3. *Trichoderma* sp. DAOM 175924 is co-specific with the *Pleurotus*-pathogenic *Trichoderma* isolates from Korea

Published in: Hatvani, L., Antal, Z., Manczinger, L., Szekeres, A., Druzhinina, I. S., Kubicek, C. P., Nagy, A., Nagy, E., Vágvölgyi, C. and Kredics, L. 2007. Green Mold Diseases of *Agaricus* and *Pleurotus* spp. Are Caused by Related but Phylogenetically Different *Trichoderma* Species. Phytopathology 97: 532-537.

Having demonstrated that the occurrence of green mould in *P. ostreatus* substrate is due to a different *Trichoderma* species than that in *Agaricus* compost (*T. aggressivum*), we wondered whether *Trichoderma* sp. DAOM 175924 would be the same as one of the two species which have recently been reported to be the agent of *Pleurotus* disease in Korea (Park *et al.* 2004a). To this end, we retrieved the available ITS1 and ITS2 sequences for *T. koreana* and *T. pleuroti* (accession numbers. DQ164405 to DQ164410) and submitted them to identification by *TrichO*Key and *Tricho*BLAST. All of these strains were identified as *Trichoderma* sp. DAOM 175924, and their sequence variation (1 nucleotide in ITS2) fell into the variation seen with other isolates of *Trichoderma* sp. DAOM 175924. We therefore conclude that the *Pleurotus* green mould isolates from Korea represent the same species as those found in the present study in Hungary.

4.4. Hungarian *T. aggressivum* isolates display the same mtDNA RFLP pattern as the early UK isolates

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In order to learn whether the green mould outbreak observed in Hungary indeed results from the spreading of the Western European *Agaricus* green mould epidemic, we compared the mtDNA RFLPs of the strains from this study with those of the *T. aggressivum* f. *europaeum* isolates collected during the epidemic onset in Ireland and England in the late 1980s (Seaby 1998). This comparison showed that both populations share exactly the same, uniform RFLP pattern, which was clearly different from that of *T. aggressivum* f. *aggressivum* (North-America).

4.5. *Trichoderma* sp. DAOM 175924 displays mtDNA RFLP heterogeneity

Published in: Hatvani, L., Antal, Z., Manczinger, L., Szekeres, A., Druzhinina, I. S., Kubicek, C. P., Nagy, A., Nagy, E., Vágvölgyi, C. and Kredics, L. 2007. Green Mold Diseases of *Agaricus* and *Pleurotus* spp. Are Caused by Related but Phylogenetically Different *Trichoderma* Species. Phytopathology 97: 532-537.

In contrast to *T. aggressivum* f. *europaeum*, isolates of *Trichoderma* sp. DAOM 175924 could be divided into three main distinct groups based on RFLPs of mtDNA. Samples from location A contained two of these groups (later identified as *T. pleurotum* and *T. pleuroticola*, respectively), and C, the third.

4.6. The *P. ostreatus*-associated strains comprise two phylogenetic *Trichoderma* species

Published in: Komoń-Zelazowska, M. Bissett, J., Zafari, D., Hatvani, L., Manczinger, L., Woo, S., Lorito, M., Kredics, L., Kubicek, C. P. and Druzhinina, I. S. 2007. Genetically closely related but phenotypically divergent *Trichoderma* species cause world-wide green mould disease in oyster mushroom farms. Appl. Environ. Microbiol. 73(22): 7416-7426.

Strains from oyster mushroom-producing farms having severe green mould infections from Hungary, Romania, Italy and South Korea exhibited the same or highly similar ITS1 and ITS2 sequences as the previously recognized putative new species "*Trichoderma* cf. *aureoviride* DAOM 175924" (NCBI GenBank accession no. AY605726) (Kullnig-Gradinger *et al.* 2002) and the recently described *Pleurotus* green mould agent from Korea, *T. pleuroticola* CNUMH 601 (NCBI GenBank DQ164409) (Park *et al.* 2006). We amplified and sequenced fragments from three different phylogenetic markers, i.e., a fragment spanning the ITS1-5.8S rRNA-ITS2 region of the rRNA gene cluster, a fragment covering the fourth and fifth introns and the last long exon of the translation elongation factor 1-alpha (*tef1*) gene, and a fragment including a portion of the fifth exon of the *chi18-5* gene encoding a family 18 chitinase. In order to compose the sample set for phylogenetic analysis, the resulting sequences were subjected to the sequence similarity search tool implemented in *Tricho*BLAST (Kopchinskiy *et al.* 2005; also www.ISTH.info). No identical hits except "*Trichoderma* sp. strain DAOM 175924" were detected. On the *chi18-5* tree, however, the hypothetical taxonomic unit node of *Pleurotus*-associated isolates and allied strains further bifurcated into two significantly supported clades. A similar divergence also takes place on the *tef1* tree: one clade comprises strain DAOM 175924, most of the Italian, one of the Hungarian, and two Romanian *Pleurotus* green mould strains, as well as environmental isolates from Iran, North America, and New Zealand (*T. pleuroticola*); the second clade includes most of the Hungarian and two Romanian green mould strains, together with one strain isolated from soft rot of wood in Germany. A single Italian isolate, CPK 1532, from a *Pleurotus* farm (*T. pleurotum*) occupies a basal position to this clade. All strains from *Pleurotus* farms were segregated into two ITS2 alleles which differed from each other by one single nucleotide polymorphism This divergence strictly corresponds to the two significant clades in the analyses of *tef1* and *chi18-5*. The concordant divergence of three loci showing two clades of *Pleurotus* green mould strains indicates the presence of two phylogenetic species. Because of the identity of the ITS1 and ITS2 sequences of strains DAOM 175924 and CNUMH 601 and of strains CPK 1532 and CNUMH 501 (NCBI GenBank DQ164405)

and based on the similar ecological characterizations, we assume that the detected new species correspond to *T. pleuroticola* type culture CNUMH 601 and *T. pleurotum* type culture CNUMH 501, which have been formally described by Park *et al.* (2006), in parallel with our studies.

4.7. Biogeography of *T. pleuroticola* and *T. pleurotum*

Published in: Komoń-Zelazowska, M. Bissett, J., Zafari, D., Hatvani, L., Manczinger, L., Woo, S., Lorito, M., Kredics, L., Kubicek, C. P. and Druzhinina, I. S. 2007. Genetically closely related but phenotypically divergent *Trichoderma* species cause world-wide green mould disease in oyster mushroom farms. Appl. Environ. Microbiol. 73(22): 7416-7426.

We performed a detailed analysis of the *tef1* alleles of *T. pleuroticola* and *T. pleurotum* to investigate possible biogeographic traits in the distribution of the isolates associated with *Pleurotus*. The scheme was constructed from one of 100 saved most parsimonious trees obtained using a heuristic search implemented in PAUP*, version 4b10. Six Hungarian and two Romanian strains of *T. pleurotum* showed almost no intraspecific variability since two groups of *tef1* sequences (four isolates each) were separated by only one A-G transition and one indel in one of several 5'-A*n*T*n*-3' spans of the intron. In contrast, two major alleles of *T. pleuroticola* were distinguished based on five diagnostic transitions. Six tested Italian strains of *T. pleuroticola* isolated from cultivated *Pleurotus* substratum were found to be polymorphic; one strain has the *tef1* allele identical to strain GJS 04-01 known to be a biocontrol agent from Montana used against *Cercospora* in sugar beet; three strains share the same allele with strain DZ56 isolated from *Agaricus* compost in Iran, and the two remaining strains have the *tef1* allele identical to that of reference strain DAOM 175924 isolated from *Acer* sp. in Canada. GJS 95-81, isolated from *Pleurotus* spawn in The Netherlands has one position that differs from the type allele. The only Hungarian isolate of *T. pleuroticola* (CPK 2104) belongs to the first major allele.

4.8. PCR-based detection of *T. pleurotum* and *T. pleuroticola*

In order to have a quick tool for the identification of the two *Pleurotus*-pathogenic *Trichoderma* spp. in hand, we screened an alignment of the sequences of ITS1 and 2, *tef1* and *chi18-5* for *T. pleurotum*, *T. pleuroticola,* and various isolates of *T. harzianum, T. aggressivum* f. *europaeum* and *T. aggressivum* f. *aggressivum* (cf. Komoń-Zelazowska *et al.* 2007) in order to find hallmark sequences appropriate for the development of specific primers

for *T. pleurotum* and *T. pleuroticola*. Thereby, three areas were found in *tef1* that allowed the specific setting of primers (FPforw1, FPrew1 and Psrev1, respectively), which were expected to amplify a 447 bp fragment from both species, and a 218 bp PCR product only in the case of *T. pleurotum*. In order to verify the applicability of these primers, a total of 13 *T. pleurotum* and 17 *T. pleuroticola* isolates were tested. This test resulted in two major bands in the case of all isolates of *T. pleurotum*, while only the larger fragment was produced with all DNA samples of *T. pleuroticola*. In order to assess that these primers are indeed specific and do not exhibit any cross reaction with other *Trichoderma* spp. or any other fungi, DNA samples of 28 other *Trichoderma* species (*T. harzianum, T. aggressivum* f. *europaeum, T. aggressivum* f. *aggressivum, T. minutisporum, T. crassum, T. oblongisporum, T. tomentosum, T. rossicum, T. fertile, T. cerinum, T. velutinum, T. polysporum, T. helicum, T. spirale, T. virens, T. brevicompactum, T. hamatum, T. atroviride, T. asperellum, T. viride, T. koningii, T. viridescens, T. gamsii, T. koningiopsis, T. aureoviride, T. longibrachiatum, T. citrinoviride* and *T. ghanense*), as well as 12 other fungal species (*Penicillium expansum, Aspergillus* sp., *A. niger, Mortierella* sp., *Thermomyces* sp., *Mucor circinelloides, Fusarium graminearum, F. oxysporum, F. culmorum, F. poae, F. sporotrichioides* and *Pleurotus ostreatus*) were tested. We should like to note that among these, *P. expansum, Aspergillus* sp., *Mortierella* sp., and *Thermomyces* sp. strains were isolated from wheat straw samples used for *Pleurotus* cultivation, while *P. ostreatus* derived from the company from whose infected substrate samples several *T. pleurotum* and *T. pleuroticola* strains had been isolated previously (Hatvani *et al.* 2007). In none of these cases were any PCR fragments produced, while controls with *T. pleurotum* and *T. pleuroticola* were always positive. We therefore conclude that our multiplex PCR method is appropriate for specific detection of the *Pleurotus*-pathogenic *Trichoderma* spp.

4.9. Identification of *T. pleuroticola* and *T. pleurotum* directly from *Pleurotus* substrate

Given the specificity of the primers developed in this study, we reasoned that they would also enable the direct detection of the mushroom pathogenic species without the necessity of subcultivation. To this end, we tested several substrata from three different stages of *Pleurotus* cultivation: straw; healthy substrate colonized by *Pleurotus*; and infected substrate. DNA was isolated from them and primers FPforw1, FPrev1 and PSrev1 used to amplify the *tef1* fragment(s) from potentially occurring *T. pleuroticola* and *T. pleurotum*. No *Trichoderma* amplicons were obtained from straw or from healthy substrate. However, the primers clearly formed the two bands characteristic to *T. pleurotum* in all cases of infected

straw, suggesting the presence of this species in the substratum, which was confirmed by subsequent ITS sequence analysis.

4.10. Identification of *Trichoderma* spp. growing on the natural substrate and on basidiocarps of *P. ostreatus*

With the aid of this multiplex PCR we tested whether any of these two species would occur in the immediate vicinity of wild-grown *P. ostreatus*. To this end, samples (stumps of *Populus alba*, *P. canadiensis* and *Tilia* sp. respectively) were taken from five areas located in the center of Hungary (Kisújszállás, Tőserdő, two from Kecskemét and Szeged region). The results were strongly different: whereas in sample 1, a forest dominated by *Convallario-Quercetum roboris,* all the 28 isolates were indeed identified as *T. pleuroticola.* No *T. pleuroticola* was detected in sample 2, where the dominant forest is *Fraxino pannonicae-Ulmetum*, as well as in sample 5, which is located in the city of Szeged, whereas in sample 4 it made up 5 of 19 isolates, and in sample 3/B a single isolate (of 11) was found. The lack of *T. pleurotum* was shown in all the five samples. We randomly tested the validity of our multiplex PCR identification, and obtained confirmation in all cases.

We also used sequence analysis of ITS1 and 2, and occasionally *tef1* (if needed) to identify the other *Trichoderma* spp. occuring in these samples. They were shown to consist of *T. harzianum*, *T. longibrachiatum* and *T. atroviride* exclusively. *T. harzianum* was the exclusive species found in sample 2, it also dominated sample 4 (14 out of 19 isolates) and made up for 50 % of the isolates (10 out of 20) in sample 5/B. *T. longibrachiatum* was present only in sample 3/B, where it appeared to be the dominant species (9 out of 11 isolates). *T. atroviride* was only found in sample 3/B (1 out of 11 isolates) and 5, where it accounted for the other 50 % of isolates (10 out of 20).

We also investigated whether *T. pleuroticola* or *T. pleurotum* are present on the basidiocarps of *P. ostreatus* found in samples 3 and 5. No *Trichoderma* spp. were found on *P. ostreatus* from sample 5. However, *T. pleuroticola* was the dominant species in sample 3/B (14 out of 20 isolates, respectively), and the remaining 6 isolates were all identified as *T. longibrachiatum*.

4.11. Survival of *T. pleurotum* C15 following UV-treatment

In order to induce mutations in genes involved in producing certain extracellular lytic enzymes, the parental isolate of *T. pleurotum* C15 was subjected to random mutagenesis by UV-irradiation. Reaching approximately 95% of mortality, 163 surviving colonies were

isolated from the samples having been exposed to 7, 8, 9 and 10 minutes of UV-treatment, and tested for their growth and sporulation. All surviving strains were involved in the enzymatic studies, but only the ones showing growth rate and sporulation similar to those of the parental strain were considered as putative mutants in enzyme production.

4.12. Enzyme plate assays

After the incubation period the transparency of the degradation halos were observed - only in the case of pectinase test - and the diameters of the colonies and the halos were measured in all cases. In order to relate polymer degradation with mycelial growth, halo/colony values were determined in at least 3 independent experiments. Based on the results, strain R3 was considered as a pectinase overproducer, while E6, F1, F5, O15, P13, T10, U3 and V5 as deficient mutants in pectinase production. Strains C4, D1 and D2 were considered as cellulase overproducers, while H2 and T14 as deficient mutants in the production of cellulases, being unable to degrade CMC. Strains T3 and U4 were supposed to be amylase-overproducers, while E5 and T2 deficient mutants in amylase production. S17, T8 and U25 seem to be lipase-overproducers, while P2, T13 and U17 show decreased ability of producing lipases. No protease-overproducers were found, while strains F7, R4, S10, S13, S16, U8 and V5 were considered as deficient mutants in protease production, with S13 and V5 forming no degradation zone. Strain P16 was identified as a chitinase-overproducer, while R2, R3, R4 and T14 were found to be deficient mutants in chitinase production. Based on these results strain C3, D1, H1 and M1 were considered as glucanase-overproducers, while F1, R2, R3, R4 and T3 as deficient mutants in the production of glucanases.

4.13. Dual plate assays

The most spectacular and unequivocal differences between the wild-type and some mutant strains appeared on YEX medium. The parental strain could overgrow and conidiate on the colony of *P. ostreatus* on all types of media examined. We could not observe any strains with higher mycoparasitic ability towards *Pleurotus* than the wild-type strain but in the case of several mutants the mycoparasitic potential appeared to be significantly lowered. Most of these mutants showed reduced mycoparasitic activity on the other media as well.

The mutants that showed normal growth but significantly lower mycoparasitic ability than the wild-type strain on YEX medium were:

H2 (amylase -, cellulase --, pectinase -, protease -), confirmed on YEGM and WAM

O15 (lipase 0-, pectinase -, protease 0-), confirmed on WAM

P2 (amylase 0-, lipase -)

P13 (pectinase -, protease 0-)

P16 (cellulase 0+, chitinase +, lipase +), confirmed on YEGM

R2 (amylase +, chitinase -, glucanase -, lipase -, protease -), confirmed on WAM

R3 (chitinase -, glucanase -, pectinase +, protease 0-), confirmed on YEGM and WAM

R4 (chitinase -, glucanase -, pectinase +, protease -), confirmed on YEGM

S10 (protease -), confirmed on YEGM

S13 (amylase ++, cellulase -, chitinase -, protease --), confirmed on YEGM

S16 (amylase +, glucanase 0-, protease -), confirmed on YEGM and WAM

T8 (cellulase -, lipase 0+, protease -)

T14 (cellulase --, chitinase -), confirmed on YEGM and WAM

According to these results we conclude that among the enzyme systems tested, proteases, lipases, chitinases and glucanases plaz role in the mycoparasitic activity of *T. pleurotum* towards *P. ostreatus*.

5. SUMMARY OF THE RESULTS

- We have demonstrated that the green mould disease of *A. bisporus* in Hungary is due to *T. aggressivum* f. *europaeum*, i.e., the Western European epidemic has now spread to Central Europe.
- We have shown that the causal agents of *Pleurotus* green mould disease in Hungary are *T. pleurotum* and *T. pleuroticola*, the same species as in other countries, which might indicate a world-wide epidemic. Furthermore, we have provided a more detailed characterization of the newly described species, and thereby confirmed their identity.
- *T. aggressivum* and the *Pleurotus*-pathogenic green mould species have proven to be specific for their substrates.
- We have developed a PCR-based technique for the rapid and specific detection of the causal agents of the recently emerged green mould disease of oyster mushroom.
- We have observed that one of the newly described pathogen of oyster mushroom, *T. pleuroticola* can not only be found but highly accumulated in the natural environment of *P. ostreatus.*
- Our results have shown that protease, lipase, chitinase and glucanase enzyme systems may act as important virulence factors of *T. pleurotum* against *P. ostreatus*.

List of publications related to the dissertation

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