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

Ph.D. dissertation

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2008

Szeged

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2. ABBREVIATIONS

chi18-5: endochytinase 18-5 (previously named ech42)

CMC: carboxymethyl-cellulose

CPK: the culture collection of the Research Area Gene Technology and Applied Biochemistry, Institute of Chemical Engineering, Vienna University of Technology, Vienna, Austria

IPM: integrated pest management

ITS: internal transcribed spacer

MEA: malt extract – agar medium

MIC: minimal inhibitory concentration

mtDNA: mitochondrial DNA

RFLP: restriction fragment length polymorphism

rpb2: RNA polymerase II

SMC: substrate moisture content

SzMC: Szeged Microbiological Collection, the culture collection of the Department of Microbiology, Faculty of Science and Informatics, University of Szeged, Szeged, Hungary

tef1: translation elongation factor 1-alpha

WAM: water-agar medium

YEGM: yeast extract-glucose medium

YEXM: yeast extract-xylose medium

3. INTRODUCTION

3.1. The genus *Trichoderma*

Trichoderma species are asexual, soil-inhabiting filamentous fungi with teleomorphs belonging to the genus *Hypocrea* (Ascomycota, Pyrenomycetes, Hypocreales, Hypocreaceae). Besides the industrial importance of the genus (Kubicek and Penttilä 1998), certain *Trichoderma* species are well known to have the ability of antagonizing a series of plant pathogenic fungi (Papavizas 1985). Proposed mechanisms of antagonism include mycoparasitism by the action of cell-wall degrading enzymes, antibiosis by the production of antibiotics, competition for space and nutrients through rhizosphere competence, facilitation of seed germination and growth of the plants via releasing important minerals and trace elements from soil and induction of the defense responses in plants (Herrera-Estrella and Chet 2003; Howell 2003; Benítez *et al.* 2004). During the last decades, representatives of the genus have been reported to be harmful as emerging opportunistic pathogens of humans (Kredics *et al.* 2003, Antal *et al.* 2005) and as the causative agents of green mould, a disorder that results in substantial losses in the production of cultivated mushrooms, including champignon (*Agaricus bisporus)* and oyster mushroom (*Pleurotus ostreatus)*.

3.2. Mushroom green mould disease

World-wide mushroom cultivation is dominated by the production of *Agaricus bisporus* (champignon), *Lentinula edodes* (shiitake), and *Pleurotus ostreatus* (oyster mushroom) (Chang 1999). *Trichoderma* green mould infection in edible basidiomycetes has a long history (Sinden and Hauser 1953). Chen and Moy (2004) have stated that parameters of mushroom cultivation, such as the sources of carbon and nitrogen, high relative humidity, warm temperatures, a fluctuation of these factors, and the absence of light during spawn run are ideal environmental conditions for moulds as well, which can easily lead to a contamination. Among these preferred conditions moulds exhibit fast growth, therefore they can compete for space and nutrients more effectively than the mushrooms, furthermore, they are able to produce toxic secondary compounds, extracellular enzymes, as well as volatile organic compounds (Mumpuni *et al.* 1998, Williams *et al.* 2003), which can result in 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. *Trichoderma* spp. produce whitish mycelia indistinguishable from those of the mushrooms during spawn run, therefore it is difficult to recognize the infection at this stage (Won 2000, Largeteau-Mamoun *et al.* 2002).

3.2.1. *Agaricus bisporus*

For *A. bisporus*, the association of *Trichoderma* species with the respective compost has been known for plenty of time to limit commercial production (Sinden and Hauser 1953). Until the 80's it was considered only a minor problem that is associated mainly with the low-quality of the compost or poor hygiene (Geels *et al.* 1988). This point of view changed basically after the first 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), and Spain (1998) (Hermosa *et al.* 1999, Mamoun *et al.* 2000, Muthumeenakshi *et al.* 1994, 1998). In the early 1990s, a similar disease appeared in mushroom crops in the United States and Canada (Castle *et al.* 1998, Ospina-Giraldo *et al.* 1998, 1999). The escalation of green mold evoked extensive research efforts to identify and study the causative agent. Although a number of *Trichoderma* spp. have been isolated from mushroom compost, aggressive colonization resulting in epidemic outbreaks has been originally suggested to be restricted to *T. harzianum* (Seaby 1987, 1989, Doyle 1991).

Compost isolates from the British Isles identified as *T. harzianum* have been differentiated into three biological forms, Th1, Th2 and Th3, that differ in their growth rate, sporulation pattern and aggressiveness in compost colonization, with Th2 being the predominant agent responsible for green mould epidemics (Seaby 1987). This initial grouping was later confirmed with a series of molecular techniques, including restriction fragment length polymorphism (RFLP) of ribosomal and mitochondrial DNA, random amplified polymorphic DNA (RAPD) and sequence analysis of the internal transcribed spacer 1 (ITS 1) (Muthumeenakshi *et al.* 1994). These techniques were used later for the molecular characterization of *Trichoderma* strains isolated from American mushroom farms, which revealed that the aggressive isolates in North America (group Th4) were very similar to, but clearly distinct from those belonging to Th2 (Castle *et al.* 1998, Muthumeenakshi *et al.* 1998). As *T. harzianum* is a species often used for biological control of fungal plant pathogens, concerns have emerged regarding the possible

involvement of biocontrol strains in the development of mushroom green mold. However, molecular phylogenetic studies based on RAPD analysis (Ospina-Giraldo *et al.* 1999) as well as sequence analysis of the ITS 1-5.8S rDNA-ITS 2 region revealed that biocontrol and green mould isolates can be clearly distinguished. Supporting these molecular data, pathogenicity trials indicated that commercial biocontrol *T. harzianum* strains and related ones from the Th1 biotype were not pathogenic on *A. bisporus*, in contrast to Th4 isolates (Romaine *et al*. 2001). A primer pair for the specific identification of the aggressive biotypes Th2 and Th4 was also developed for screening of biocontrol candidates for potential pathogenicity (Chen *et al.* 1999a). This PCR-based test is useful in disease management programmes as well. Furthermore, it has been applied for the comparison of *Trichoderma* strains sampled in the United States during and prior to the outbreak of the green mould epidemic (Chen *et al*. 1999b), which revealed no evidence for the preepidemic existence of Th4, suggesting the recent emergence of a highly virulent genotype. Based on the molecular differences between the biotypes Th1-3, Muthumeenakshi *et al*. (1994) already suggested that they may represent three different species. Molecular evidences indicated later, that biotype Th3 is actually *T. atroviride* (Castle *et al.*. 1998, Ospina-Giraldo *et al.* 1998), while Th1 was recognized as *T. harzianum* sensu stricto (Gams and Meyer 1998). More recently, the two aggressive biotypes, Th2 and Th4 were redescribed on the basis of morphological characteristics and the phylogenetic analyses of ITS 1 and the translation elongation factor 1-alpha (*tef1*) gene as *T. aggressivum* f. *europaeum* and *T. aggressivum* f. *aggressivum*, respectively (Samuels *et al.* 2002).

3.2.2. *Pleurotus ostreatus*

Apart from being the third most commercially important edible mushroom world-wide (Chang 1996, Chang 1999), *P. ostreatus* – commonly known as oyster mushroom - is used for the bioconversion of agricultural and industrial lignocellulose debris (Ballero *et al.* 1990, Puniya *et al.* 1996) and as a source of enzymes and other metabolites for industrial and medical applications (Gunde-Cimerman 1999, Marzullo *et al.* 1995). *P. ostreatus* can be grown on a wide range of agricultural by-products and industrial wastes (Pani *et al.* 1997), although pasteurized straw is most commonly used. 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 (Anonymous 2005). Sharma and Vijay (1996) reported green mould of oyster mushroom caused by *T.*

viride in North America, but the first significant crop losses of cultivated *P. ostreatus* caused by green mould disease were reported in South Korea by Yu (2002). According to these results the cultural and morphological characteristics of more than one hundred *Trichoderma* strains isolated from oyster mushroom substrate were examined and based on the results the following species were identified: *T. viride* (13.6%), *T. harzianum* (8.2%), *T. koningii* (5.5%), and the majority of the isolates (65.5%) belonged to an unidentified species of *Trichoderma.* According to DNA band pattern, internal transcribed spacer 1 and 2 (ITS1 and 2) sequences, random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) analysis, these isolates were supposed to belong to *T. harzianum* but considered as a new variety because they formed different phylogenetic clades. Two species of *Hypocrea* were also found on mushroom beds, one of them forming brown (*Hypocrea* sp. 1), while the other one (*Hypocrea* sp. 2) possessing white stroma. *Hypocrea* sp. 1 produced an anamorph with gliocladium-like morphology, but *Hypocrea* sp. 2 did not form the asexual stage on mushroom beds. The *in situ* and *in vitro* interactions between *P. ostreatus* and the green mould strains were examined, and *Trichoderma* isolates occurred to dominate *Pleurotus* on both potato dextrose agar (PDA) medium and mushroom beds (Yu 2002). Mesumbe (2006) reported about the green mould disease of *Lentinula edodes* and *Pleurotus* spp. caused by *T. viride* in Cameroon. Woo *et al.* (2004) reported serious green mould infections of *P. ostreatus* in Italy, which have led to a crisis situation in *Pleurotus* cultivation in the country. Preliminary results obtained from the morphological and genetic characterization of the infective agents have suggested that the isolates belong to the species *T. harzianum.* Serious cases of green mould diseases of *P. ostreatus* in commercial operations were detected recently also in South Korea (Park *et al.* 2004a) and Romania (Kredics *et al.* 2006). In the latest years the green mould disease of cultivated *P. ostreatus* occurred in Hungary as well, and several *Trichoderma* strains were isolated from green mould affected *P. ostreatus* substrate samples (Hatvani *et al.* 2007).

The causative agent of the oyster mushroom green mould has been reported to be morphologically and culturally distinct from *T. aggressivum* (Park *et al.* 2004a,b, Woo *et al.* 2004). Park *et al.* (2004a,b) examined South Korean green mould isolates in details. Among the 26 *Trichoderma* strains isolated from Korean oyster mushroom farms they could distinguish and identify four groups based on cultural and morphological characteristics: *Trichoderma* sp. K1-type (K1), *Trichoderma* sp. K2-type (K2), *T. harzianum* and *T. atroviride.* The predominant species was K2, followed by K1 and *T.*

atroviride. According to colony morphology, growth rate, phialide and conidial morphology, K1 and K2 could be distinguished from *T. harzianum* and *T. atroviride*, as well as from each other. The phylogenetic analysis of the ITS region of the isolates was carried out in comparison with *Trichoderma aggressivum* f. *europaeum* and f. *aggressivum*, the causative agents of *A. bisporus* green mould disease. Based on the results, K1 and K2 could be clearly separated from *T. harzianum*, *T. atroviride*, *T. aggressivum* f. *europaeum* and *T. aggressivum* f. *aggressivum*. Furthermore, they proved to be different from each other as well according to a single A/C transversion: K1 had "A", while K2 had "C" at the position 447 of the ITS2 region. Their sequences were identical at all other positions. K1 and K2 could be differentiated from *T. harzianum*, *T. atroviride*, *T. aggressivum* f. *europaeum* and f. *aggressivum* and from each other based on the phylogenetic analysis of the fourth intron of *tef1* and motif six and seven of RNA polymerase II (*rpb2*) gene as well (Park *et al.* 2004a,b). Unfortunately only the ITS1 and 2 from sequences of six isolates from these studies have been deposited in public databases. According to the above findings, the authors proposed *Trichoderma* sp. K1-type and *Trichoderma* sp. K2-type to be described as new species under the names *T. koreana* and *T. pleuroti*, but they did not provide nomenclaturally valid species descriptions (Park *et al.* 2004a). Finally the two species were introduced as *Trichoderma pleurotum* S. H. Yu & M. S. Park, sp. nov. and *Trichoderma pleuroticola* S. H. Yu & M. S. Park sp. nov. (Park *et al.* 2006), however, the ex-type cultures of these species were not deposited in publicly accessible culture collections, and for the molecular characterization the authors only referred to articles that are not easily accessible (Park *et al.* 2004c; Park *et al.* 2005). However, these findings were strongly supported by Komoń-Zelazowska *et al.* (2007), therefore the identity of the two species were confirmed from several points of view. In this study an integrated approach was used for the comprehensive characterization of several *T. pleurotum* strains from Hungary, Italy and Romania, as well as *T. pleuroticola* isolates from Canada, the USA, Italy, Hungary, Romania, Iran, the Netherlands, Germany and New Zealand. According to the results of this study, similarly to *T. aggressivum* - the causal agent of *Agaricus* green mould disease - both species were shown to belong to the Harzianum Clade of *Hypocrea/Trichoderma*. Morphological studies have revealed that *T. pleuroticola* shows pachybasium-like properties, which is characteristic in the Harzianum Clade, while *T. pleurotum* possesses gliocladium-like conidiophore morphology. Biolog Phenotype Microarrays were used to determine the carbon source utilization profile of the isolates. The results have shown unequivocal differences between the two species, namely

the growth of *T. pleurotum* was slower or impaired on the majority of the carbon sources tested as compared to *T. pleuroticola*, which showed similar growth to that of *T. aggressivum*, indicating a closer relationship. The results suggest that the evolution of *T. pleurotum* was accompanied with the loss of the utilization ability of certain carbon sources. The phylogenetic analysis of a fragment including 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 *tef1*; and a fragment including a portion of the fifth exon of the endochitinase *chi18-5* (previously named *ech42*) gene encoding a family 18 chitinase confirmed the responsibility of the two distinct species for causing the green mould disease of oyster mushroom world-wide. A DNA BarCode for identification of these species based on ITS1 and 2 sequences was also provided and integrated in the main database for *Hypocrea/Trichoderma* (www.ISTH.info). *In vitro* antagonistic tests revealed that both species are able to antagonize *A. bisporus* in a similar degree like *T. aggressivum*, but *T. pleuroticola* showed stronger inhibitory effect on *P. ostreatus* than *T. pleurotum.* Furthermore, while *T. pleurotum* has so far been found only in association with cultivated *P. ostreatus*, *T. pleuroticola* can be isolated from environmental samples, such as soil and wood world-wide. These findings suggest that *T. pleuroticola* means the most serious threat to oyster mushroom cultivation. Green mould infection of *P. ostreatus* is supposed to be transmitted via the substratum for mushroom cultivation, and differences in species distribution may be due to the use of certain substrata which depending on the manufacturer may consist of cereal straw, sawdust, bagasse, or waste cotton. In this study *T. pleuroticola* is reported to be dominant in samples from Italian *Pleurotus* farms, while the majority of the isolates from Hungary belong to the species *T. pleurotum* (Komoń-Zelazowska *et al.* 2007).

Yu (2002) and Woo *et al.* (2004) have observed that *Trichoderma* species are present at the initial phase of substrate preparation, then they disappear with the pasteurization, but they can be found again in the substrate after spawning (inoculation with *Pleurotus*), during spawn run (incubation phase) and the harvesting cycles. Yu (2002) studied the efficacy of pasteurization on the development of green mould infection caused by *Trichoderma* and *Hypocrea* at different temperatures, durations and substrate moisture content (SMC) values. As for *Trichoderma*, the results have shown that mycelial growth was completely inhibited by pasteurization at 60 ^{o}C for 10 hours or longer at both 50 and 70 % SMC, while *Hypocrea* cannot survive following heat treatment at 50 ^{o}C for 5 hours. However, when pasteurization time is determined, heat conduction rate of substrate

(depending on the kind of material, substrate volume and moisture content) must be taken into consideration, because substrate moisture content can become too low, which might let the substrate harbor the pathogenic fungi alive in it. The effect of substrate moisture content on the growth of *Pleurotus* and *Trichoderma* was also examined in this study. The optimum of oyster mushroom fell into the range of 60-70 %, and the growth of it was inhibited at 80 %. In contrast to this, the mycelial growth of green mould isolates occurred to be proportional to SMC, reaching its maximum at 80 %. This is important to know, since a mushroom growing room has a varying SMC according to the height of the shelf. Although the temperature ideal for the growth of oyster mushroom varies among strains, room temperatures of approximately 25 ^{o}C, 13-15 ^{o}C and 18 ^{o}C are needed for spawn run, induction of the development of fruit bodies and fruiting, respectively. The substrate is exposed to green mould infection mostly during spawn run, when the substrate temperature is elevated up to 30 ^{o}C due to the generation of metabolic heat by mushroom mycelia. The study of the influence of different temperatures on the mycelial growth of *Trichoderma* revealed that the growth of *Trichoderma* was favored by higher temperatures, reaching a maximum at 30 ^{o}C, while no growth was observed at 15 ^{o}C. This finding suggests that the temperature of growing room should be maintained between 15 and 18 ^{o}C after spawn run in order to minimize the possibility of the development and spreading of green mould infection. Woo *et al.* (2004) examined the effect of temperature and pH on the mycelial growth of green mould isolates and different varieties of *P. ostreatus*. The temperature optimum for the growth of *Pleurotus* was 28 ^{o}C, while *Trichoderma* could grow well at a wider range (20-28 ^{o}C), and exceeded the growth rate of *Pleurotus* by three times at 25 ^{o}C. The pH optimum for the growth of *Pleurotus* was alkaline (pH 8-9) whereas *Trichoderma* preferred acidic-neutral conditions (pH 5-7). This finding suggests that adjusting the pH of the substrate to 8-9 might slow down the growth of *Trichoderma*, resulting in the decrease in the spreading of the infection. The inhibitory effect of several fungicides commonly used in agriculture (prochloraz, thiabendazole, dichloran, benomyl, propiconazole, thiofanatomethyl) was also tested in this study, and both prochloraz and thiabendazole, the pesticides allowed in edible mushroom production, were found to inhibit the growth of the aggressive *Trichoderma* isolates without having a negative effect on *Pleurotus*. In order to prevent the contamination from spreading, Won (2000) suggested the application of calcium hydroxide onto the affected area, and the use of fungicides benomyl, thiabendazole and prochloraz was also reported to be effective. Yu (2002, 2003) has examined the means of disease control in details as well. The effect of

various fungicides on spore germination and the mycelial growth of *Trichoderma* isolates aggressive to *Pleurotus* was tested. Unfortunately several *Trichoderma* strains have been found to exhibit resistance to the the fungicides involved in the study. However, prochloraz was shown to be the most effective fungicide for inhibition of the mycelial growth of the green moulds, because the amount of resistant *Trichoderma* isolates was the lowest in the case of this fungicide. Prochloraz, benomyl and probineb were found to inhibit spore germination of benomyl-susceptible isolates in a proper way, while chlorothalonil was effective for that of benomyl-resistant strains. Other 7 fungicides including captan inhibited mycelial growth of *P. ostreatus* rather than that of *Trichoderma* sp., which means that these chemicals cannot be applied in disease control. Low toxic chemicals, such as wood vinegar and vinegar had no inhibitory effect on the mycelial growth of the green moulds, but surface activator inhibited spore germination. Administering chemicals to the substrate (waste cotton) during water adjustment or substrate preparation proved to be the most effective chemical treatment for the prevention and control of green mould disease. By using fungicide treatment before substrate sterilization, infection of *Hypocrea* sp. was effectively prevented during the whole cultivation process of oyster mushroom, but fungicide treatment after spawning might threaten with chemical toxicity. Control value of prochloraz and thiabendazole on the mushroom bed contaminated with green mould was 78.5 % and 70.9 %, respectively. However, benomyl treatment had no inhibitory effect on *Trichoderma*. Although prochloraz showed harmful effect on the mycelial growth and fruit body development of *Pleurotus* sp. in high concentrations, it was selected as the most effective fungicide for the control of green mould disease on mushroom beds. The treatment of waste cotton substrate with prochloraz at 250 ppm has lead to shorten the time for fruit body initiation and increase the yields. Fungicide residues in the fruit bodies of oyster mushroom harvested from chemical treated substrates and mushroom beds were analyzed. Prochloraz, thiabendazole and benomyl residues in the fruit bodies were below 0.095, 0.431-0.495 and 0.33-0.58 ppm, respectively, which are much lower than tolerance for fungicide residue in the mushroom. The bacterial strain CNU LI-1 has been found to inhibit the mycelial growth of *Trichoderma* spp. and *Hypocrea* sp., therefore it might be a potential candidate to be used in the biological control of green mould disease. The inoculation of the strain into the pre-sterile substrate was effective for preventing the occurrence of *Trichoderma* spp. and *Hypocrea* sp. on mushroom beds. However, the treatment of mushroom beds already colonized by green mould had no effect of control.

3.2.3. Our research background

Both *T. pleuroticola* and *T. pleurotum* have been found on cultivated *Pleurotus* and its substratum in Europe, Iran and South Korea, but *T. pleuroticola* has also been isolated from soil and wood in Canada, the United States, Europe, Iran, New Zealand (Park *et al.* 2004a, Park *et al.* 2006, Komoń-Zelazowska *et al.* 2007) and Hungary (Szekeres *et al.* 2005). It is not known yet whether any of these species occur also in association with wild-grown *P. ostreatus*. Our purpose was therefore to test the occurence of *T. pleuroticola* and *T. pleurotum* on wood colonized by *P. ostreatus*, as well as on the surface of the fruit bodies. In order to facilitate identification of these two *Trichoderma* spp., we have developed three oligonucleotide primers (based on sequence regions within the 4^{th} and 5^{th} introns in the gene encoding translation elongation factor 1-α (*tef1*), which identify them in a multiplex PCR assay. We have also shown that these primers can be used to detect the presence of *T. pleurotum* and/or *T. pleuroticola* directly in the growing substrate of oyster mushroom, without the necessity of cultivation. Furthermore, this method may provide a useful tool for studies aimed at the identification of the possible sources and potential vectors of *Trichoderma* green mould infections in *Pleurotus* producing facilities.

4. AIMS

Hungary is one of the leading European countries for mushroom production and export (38,000 and 16,000 tons in the year 2000, respectively; source: Hungarian Vegetable and Fruit Board). 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.*

5. MATERIALS AND METHODS

5.1. Strain isolation and maintenance

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* on wheat straw as substrate, while farm B produces *A. bisporus* on compost containing wheat straw, chicken manure, horse manure, and gypsum, supplemented with soy meal as an additive. Farm C produces both mushrooms on the corresponding substrates described above. Samples were put 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}). After the occurrence of conidiation, conidial suspensions were prepared, diluted, and plated on solid YEG medium. Agar plugs cut from separately growing young *Trichoderma* colonies were transferred to new plates with the same medium. The isolates were deposited in the culture collection of the Department of Microbiology, Faculty of Science and Informatics, University of Szeged, Szeged, Hungary (Szeged Microbiological Collection, SzMC). The strains and their origins are listed in Table 4 (RESULTS).

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 and maintained as described above, they were then deposited in the culture collection of the Research Area Gene Technology and Applied Biochemistry, Institute of Chemical Engineering, Vienna University of Technology, Vienna, Austria (CPK) and in SzMC. *Trichoderma* isolates derived from the substrate as well as the surface of wild-grown *P. ostreatus*, and their origins are listed in Table 1, while Fig. 1 shows the geographical location of the sampling sites.

Table 1. Origin of samples and *Trichoderma* strains isolated from the substrate and the fruiting body of wild-grown *P. ostreatus*

Strain number
Sample 1: *Populus alba* stump, *Convallario-Quercetum roboris*, Kisújszállás, Hungary
CPK 3193, 3194, 3195, 3196, 3197, 3198, 3199, 3200, 3201, 3202, 3203, 3204, 3205, 3206, 3207, 3208, 3209, 3210, 3211, 3212, 3213, 3214, 3215, 3216, 3217, 3218, 3219, 3220
Sample 2: *Populus alba* stump, *Fraxino pannonicae-Ulmetum*, Tőserdő, Hungary
CPK 3247, 3248, 3249, 3250, 3251, 3252, 3253, 3254, 3255, 3256, 3257, 3258
Sample 3: *Populus alba* stump, *Populetum cult*., Kecskemét, Nyomási erdő, Hungary
3/A: surface of fruit body
CPK 2884, 2885, 2886, 2887, 2888, 2889, 2890, 2891, 2892, 2893, 2894, 2895, 2896, 2897, 2898, 2899, 2900, 2901, 2902, 2903
3/B: substrate
CPK 3271, 3272, 3273, 3274, 3275, 3276, 3277, 3278, 3279, 3280, 3281
Sample 4: *Populus canadensis* stump, along the road, Kecskemét, Hunyadiváros, Hungary
CPK 3259, 3260, 3261, 3262, 3263, 3264, 3265, 3266, 3267, 3268, 3269, 3270, 3271, 3272, 3273, 3274, 3275, 3276, 3277, 3278, 3279, 3280, 3281, 3282, 3283, 3284, 3285, 3286, 3287, 3288
Sample 5: *Tilia sp.* stump, inside the city of Szeged, Hungary
5/A: surface of fruit body
No *Trichoderma* was found.
5/B: substrate
CPK 3375, 3376, 3377, 3378, 3379, 3380, 3381, 3382, 3383, 3384, 3385, 3386, 3387, 3388, 3389, 3390, 3391, 3392, 3393, 3394

Figure 1. Geographical location of the origin of wild-grown *P. ostreatus* samples

5.2. Fungal strains applied in the development of the method for the specific detection of *T. pleurotum* and *T. pleuroticola*

All fungal strains used in this study were derived from culture collections SzMC or CPK. The strains of *Trichoderma* and of other ascomycetes that have been employed in testing the method are given in Table 2.

Table 2. List of fungal isolates have been involved in testing the primers specific for *T. pleurotum* and *T. pleuroticola*

Species	Strain number	Origin
T. pleurotum	A1, CPK 2095	substrate of cultivated *P. ostreatus*, Hungary
	A8, CPK 2096	substrate of cultivated *P. ostreatus*, Hungary
	A11, CPK 2097	substrate of cultivated *P. ostreatus*, Hungary
	A16, CPK 2098	substrate of cultivated *P. ostreatus*, Hungary
	A25, CPK 2100	substrate of cultivated *P. ostreatus*, Hungary
	A28, CPK 2103	substrate of cultivated *P. ostreatus*, Hungary
	C4, CPK 2109	substrate of cultivated *P. ostreatus*, Hungary
	C5, CPK 2110	substrate of cultivated *P. ostreatus*, Hungary
	C14, CPK 2112	substrate of cultivated *P. ostreatus*, Hungary
	C15, CPK 2113	substrate of cultivated *P. ostreatus*, Hungary
	C21, CPK 2114	substrate of cultivated *P. ostreatus*, Hungary
	C25, CPK 2116	substrate of cultivated *P. ostreatus*, Hungary
	C27, CPK 2117	substrate of cultivated *P. ostreatus*, Hungary
T. pleuroticola	A37, CPK 2104	substrate of cultivated *P. ostreatus*, Hungary
	CPK 230, DAOM 175924, CBS 121144	Canada
	CPK 882, CBS 121146	Iran
	CPK 1401, DAOM 175924, CBS 628.77	The Netherlands
	CPK 1540, CBS 121217	From *Pleurotus*, Italy
	CPK 1541	From *Pleurotus*, Italy
	CPK 1542	From *Pleurotus*, Italy
	CPK 1543	From *Pleurotus*, Italy
	CPK 1544	From *Pleurotus*, Italy
	CPK 1545	From *Pleurotus*, Italy
	CPK 1546	From *Pleurotus*, Italy
	CPK 1547	From *Pleurotus*, Italy
	CPK 1548	From *Pleurotus*, Italy
	CPK 1549	From *Pleurotus*, Italy
	CPK 1550	From *Pleurotus*, Italy
	CPK 1551	From *Pleurotus*, Italy
	CPK 1715, G.J.S. 04-01	Biocontrol agent of *Cercospora* in sugar beet, USA, Montana
T. harzianum	CPK 7, CBS 960.68	Unknown

T. aggressivum f. *europaeum*	CPK 361, IMI 359824	United Kingdom
T. aggressivum f. *aggressivum*	CPK 366, CBS 435.95	Mushroom compost, Canada, British Columbia
T. minutisporum	CPK 22, G.J.S. 95-216	Unknown
T. crassum	CPK 63, CBS 336.93, DAOM 164916	Soil under *Picea excelsa*, Canada, Quebec
T. oblongisporum	CPK 93, CBS 343.93	Wood of *Thuja plicata*, Canada, British Columbia
T. tomentosum	CPK 97, CBS 349.93	Material under bark of *Ulmus* sp.Canada, Ontario
T. rossicum	CPK 223, DAOM 230008	Cultivated soil, Russia, Krasnoyarsk region, Siberia
T. fertile	CPK 232, DAOM 167161	Unknown
T. cerinum	CPK 293, DAOM 230012, TUB F-778	Soil, Nepal, Annapurna Himal
T. velutinum	CPK 298, DAOM 230013, TUB F-784	Forest soil, Nepal, Annapurna Himal
T. polysporum	CPK 462, G.J.S. 99-159	Bark, Australia , New South Wales
T. helicum	CPK 414, DAOM 230016	Soil near seashore, Malaysia
T. spirale	CPK 679, TUB F-825	Brazil, Rio de Janeiro
T. virens	CPK 2141, CNRA 146	Rhizosphere of *Theobroma cacao*, plantation, Ivory Coast
T. brevicompactum	CPK 1580	Iran
T. hamatum	CPK 16	Soil, USA, Florida
T. atroviride	CPK 626, TUB F-337	Soil near seashore, Jamaica
T. asperellum	CPK 674, TUB F-756	Brazil
T. viride	CPK 625, TUB F-371	Soil, castle park Japan, Osaka
T. koningii	CPK 1370, G.J.S. 90-18	USA, Wisconsin
T. viridescens	CPK 2069, UNISS 3-76 STS	Soil, Italy, Sardinia
T. gamsii	CPK 2070, UNISS, 4-102	Soil, Italy, Sardinia
T. koningiopsis	CPK 1813, PPRC J7	Soil, Ethiopia, Jimma
T. aureoviride	CPK 10, ICMP 3090	Unknown
T. longibrachiatum	CPK 45, IMI 297702	Unknown
T. citrinoviride	CPK 343, TUB F-706	Soil, USA, Boston
T. ghanense	CPK 1255, NRRL 3091	Unknown
Fusarium poae	CPK 2786, CBS 115696	From *Triticum aestivum*, Poland, Zulawy region
F. graminearum	CPK 1117, RM 40	Unknown
F. oxysporum	CPK 1842, PPRC H6	Soil, Ethiopia, Harerga
F. culmorum	CPK 2747	From wheat, Austria
F. sporotrichioides	CPK 2787, CBS 115700	From *Fagopyrum esculentum*, Poland, Warmia region
Penicillium expansum	FAM-1	Straw used for preparing *Pleurotus* substrate, Hungary

Penicillium expansum	FAM-3	Straw used for preparing *Pleurotus* substrate, Hungary
Aspergillus sp.	FDM-5	Straw used for preparing *Pleurotus* substrate, Hungary
Mortierella sp.	FDM-7	Straw used for preparing *Pleurotus* substrate, Hungary
Thermomyces sp.	FAT-1	Straw used for preparing *Pleurotus* substrate, Hungary
Pleurotus ostreatus	B7	Mushroom farm, Hungary
A. niger	SzMC 608	Brazil
Mucor circinelloides	SzMC 12028	Unknown

5.3. DNA extraction from fungal cultures, straw and *Pleurotus* substratum

Cultures were grown on solid MEA medium (30 g l^{-1} malt extract and 20 g l^{-1} agar in distilled water) covered with a cellophane membrane for 1-2 days, then 70-80 mg fresh mycelium was harvested and disrupted in Eppendorf tubes containing glass pearls by a TissueLyser, RETSCH MM 301 (20/s, 1 min). The disruption procedure was performed twice. DNA extraction was carried out using Quiagen DNeasy Plant Mini Kit or Sigma GenElute^{TM} Plant Genomic DNA Miniprep Kit according to the protocol provided by the manufacturers. Straw, as well as healthy and *Trichoderma*-affected *Pleurotus* substrata were disrupted with a pestle in a mortar filled with liquid nitrogen, which was followed by DNA extraction as described above. DNA extracts derived from fungal cultures were diluted to 1:100, while those from straw and *Pleurotus* substrata to 1:10 with bidistilled water for PCR amplification.

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

The diagnostic polymerase chain reaction (PCR) test for *T. aggressivum* developed by Chen *et al.* (1999a) was carried out with primers Th-F (5′-CGGTGACATCTGAAAAGTCGTG-3′) and Th-R (5′-TGTCACCCGTTCGGATCATCCG-3′) in a T3 thermocycler (Biometra, Gottinger, Germany) with 1 cycle at 94°C for 2 min, 35 cycles at 94°C for 15 s, 62°C for 30 s, and 74°C for 30 s, and a final elongation step at 72°C for 7 min.

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

A region of nuclear rDNA, containing the ITS1 and 2 and the 5.8S rRNA gene, was amplifed by PCR using the primer combinations SR6R (5'-AAG TAT AAG TCG TAA CAA GG-3') and LR1 (5'-GGT TGG TTT CTT TTC CT-3') (Gams and Meyer 1998) in 50 µl volumes using the following parameters: 1 min initial denaturation at 94 °C, followed by 30 cycles of 1 min denaturation at 94 °C, 1 min primer annealing at 50 °C, 90 s extension at 74 °C, and a final extension period of 7 min at 74 °C. The amplification of a 0.4 kb fragment of endochitinase *chi18-5* (formerly named *ech42*) was carried out with the primer pair Chit42-1a (5'-GCTYTCCATCGGTGGCTGGAC-3') and Chit42-2a (5'-GGAGTTGGGGTAGCTCAGC-3'), and the following amplification protocol: 1 min initial denaturation at 94 °C, 30 cycles each of 1 min at 94 °C, 1 min at 62 °C, and 1 min at 74 °C, and a final extension period of 7 min at 74 °C. An approximately 1-kb portion - including the fourth and fifth intron and a portion of the last large exon - of the *tef1* gene was amplified and sequenced using primers EF1 [5'-ATGGGTAAGGA(A/G)GACAAGAC-3'] and EF2 [GGA(G/A)GTACCAGT(G/C)ATCATGTT-3'] (O'Donnell *et al.* 1998). or with the primer pair EF1728F and TEF1LLErev (Jaklitsch *et al.* 2005). The parameters of amplification were: 1 min initial denaturation at 94 °C, followed by 30 cycles of 1 min denaturation at 94 °C, 1 min primer annealing at 59 °C, 50 s extension at 74 °C, and a final extension period of 7 min at 74 °C. All amplifications were performed in a Bio-Rad iCycler. Purified PCR products for ITS1 and ITS2, *tef1*, and *chi18-5* were subjected to automatic sequencing at MWG (Martinsried, Germany). Sequences were aligned by the aid of softwares ClustalX and GeneDoc, then they were deposited in NCBI GenBank and www.ISTH.info.

5.6. 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).

5.7. 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. Haplotype networks were constructed manually based on detected shared polymorphic sites and confirmed using statistical parsimony analysis as implemented in TCS, version 2.11 (Clement *et al.* 2000), and maximum parsimony analysis using PAUP*, version 4.0b10 (Swofford 1998).

5.8. 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 enzymes. DNA fragments were separated by agarose gel electrophoresis and visualized under UV light. The sizes of the mtDNA fragments were determined by using GelBase/GelBlot Pro Gel Analysis software (UltraViolet Products), using l–pUC mix (Fermentas) as the molecular mass marker. 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).

5.9. 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*. The data of the primers are shown in Table 3, while the binding sites are indicated on Figure 2 (A,B).

Table 3. Data of primers specific to *T. pleurotum* and *T. pleuroticola*

Primer	Specificity	Sequence	Lentgh	Tm	Amplicon
FPforw1	*T. pleurotum* and *T. pleuroticola*	5'- CACATTCAATTGTGCCCGACGA -3'	22	58.22 °C	
FPrev1	*T. pleurotum* and *T. pleuroticola*	5'- ACCTGTTAGCACCAGCTCGC -3'	20	59.21 °C	447 bp
PSrev1	*T. pleurotum*	5'- GCGACACAGAGCACGTTGAATC -3'	22	58.89 °C	218 bp

Figure 2A. Binding sites of primers FPforw1, FPrev1 and PSrev1, used in the multiplex PCR for the detection of *Pleurotus* pathogenic *Trichoderma* spp. Schematic presentation of the *tef1* fragment with the indication of the primer binding sites. Numbers indicate nucleotide positions within the introns.

Figure 2B. Binding sites of primers FPforw1, FPrev1 and PSrev1, used in the multiplex PCR for the detection of *Pleurotus* pathogenic *Trichoderma* spp. Alignment of sequence areas within the 4th and 5th intron of the *tef1* gene that were used for primer design. Positions that are variable between *T. pleurotum, T. pleuroticola* and related *Trichoderma* species are indicated in red within the sequences corresponding to the specific primers. The aligned sequences derived from the GenBank (accession numbers: EF601679, AY605769, AY605798, AF348095 and AF348099).

```
position within 4th intron:                      29↓                                                              91↓
                                              FPforw1: 5'...CACATTCAATTGTGCCCGACGA...3'
T. pleurotum CPK 2814:......................5'...CTCCCTCCACATTCAATTGTGCCCGACGATTCTGCAGAGAATTTTCGTGT-CGACAATTGAT-AT...3'
T. pleuroticola DAOM 175924:................5'...CTCCCTCCACATTCAATTGTGCCCGACGATTCTGCAGAGAATTTTCGTGT-CGACAATTTTTCAT...3'
T. aggressivum f. aggressivum DAOM 222154:..5'...CTCCCTCCACATTCAATTGTGCTCGATCATTCTGAAGAGAATT-----GT-CGACAATTTTTCAT...3'
T. aggressivum f. europaeum CBS 100525:.....5'...CTCCCTCCACATCCAATTGTGCTCGATCATTCTGAAGAGAATT-----GT-CGACAATTTTTCAT...3'
T. harzianum CBS 273.78:....................5'...CTCCCTCTACATTCAATTGAACCCGACAATTCTGAAGAGAATTTTCGTGTTCGACAATTTTTCAT...3'

position within 4th intron:                     196↓                                                             256↓
                                                                    PSrev1: 3'...CTAAGTTGCACGAGACACAGCG...5'
T. pleurotum CPK 2814:......................5'...TTTTTTCTGCTTCACTCCCCCCACTGGCCCAGTCATGATTCAACGTGCTCTGTGTCGC----CAT...3'
T. pleuroticola DAOM 175924:................5'...TTT---CTGCTTCACTCTCCC-ACTG-CCCAGTCATCATTCAACGTGCTCTGTGTCTCC---CAT...3'
T. aggressivum f. aggressivum DAOM 222154:..5'...TTTTT-GTGCTTCACTATCACTA----CCCAGCCGTCGTTCAACGTGCTCTGTCTCTC---TCAT...3'
T. aggressivum f. europaeum CBS 100525:.....5'...TTTTTTGTGCTTCACTATCACTA----CCCAGCCGTCGTTCAACGTGCTCTGTCTCTC--GTCAT...3'
T. harzianum CBS 273.78:....................5'...TTTTCT--GCTTCAC--TCACTT----CCCAGCCATCATTCAGCGTGTTCTGTGTCCTTGGTCAT...3'

position within 5th intron:                       1↓                                                              65↓
                                                                    FPrev1: 3'...CGCTCGACCACGATTGTCCA...5'
T. pleurotum CPK 2814:......................5'...GTATGTCTGCTGCTCCATCACCTCCATGCAGGAATGGCGAGCTGGTGCTAACAGGTCATGCGCAG...3'
T. pleuroticola DAOM 175924:................5'...GTATGTCTGCT---CCATCATCTTGATGCAGGAATTGCGAGCTGGTGCTAACAGGTAATTCGCAG...3'
T. aggressivum f. aggressivum DAOM 222154:..5'...GTATGTCTCCT--TC-ATCACCCCGATGCAGCAATTACAAGCCAGTGCTAACAGGCAATTCACAG...3'
T. aggressivum f. europaeum CBS 100525:.....5'...GTATGTCTCCT--TC-ATCACCCCGATGCAGCAATTACAAGCCAGTGCTAACAGGCAATTCACAG...3'
T. harzianum CBS 273.78:....................5'...GTATGTCTTCT--TC-ATTAACTTCATGCTTCAATTGCAAGTCAGTGCTAACAGGCAATTCACAG...3'
```

5.10. 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 (see Table 4), 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 °C for 2 min, 35 cycles at 94 °C for 10 s and 68 °C for 20 s, and 1 cycle at 72 °C for 30 s. PCR products were subjected to electrophoresis at 80 V for 30 min in 1.5 % agarose gel prepared in TAE buffer (4.84 g l^{-1} Tris base, 1.142 ml l^{-1} glacial acetic acid, 2 ml l^{-1} 0.5 M EDTA, pH 8.0 in distilled water, pH adjusted to 8.5) containing 200 ng/ml ethidium-bromide. Electrophoresis buffer was the same TAE as specified above. GeneRuler™ 1kb DNA Ladder (Fermentas) was used as a standard. DNA was visualized by UV illumination and photographed by a Bio-Rad Gel Doc 2000 device.

5.11. Mutagenesis of *T. pleurotum* C15

From the sporulated colony of *T. pleurotum* C15 grown on solid yeast extract-glucose medium (YEGM: glucose 5 g l^{-1}, yeast extract 1 g l^{-1}, KH_2PO_4 5 g l^{-1} and agar 20 g l^{-1} in distilled water) for 5 days at 28 °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 °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 °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 °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. Reaching approximately 95% of mortality, 163

surviving colonies were isolated and maintained on solid YEGM. Those showing growth and sporulation similar to that of the wild-type strains were chosen for further enzymatic tests.

5.12. 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 as follows:

Pectinase test:

Component A of the medium contained 5 ml l^{-1} mineral solution ($(NH_4)_2SO_4$ 1 g l^{-1}, KH_2PO_4 2 g l^{-1}, Na_2HPO_4 3 g l^{-1}, $FeSO_4$ x 7 H_2O 0.1 g l^{-1}, $CaCl_2$ 0.5 g l^{-1}, H_3BO_3 0.005 mg l^{-1}, $MnSO_4$ 0.005 mg l^{-1}, $ZnSO_4$ 0.035 mg l^{-1}, $CuSO_4$ 0.025 mg l^{-1}, MoO_3 0.005 mg l^{-1} in distilled water), yeast extract 2 g l^{-1} and pectin10 g l^{-1}, while component B consisted of agar 30 g l^{-1} in distilled water. The pH of both components was adjusted to 5 by adding 37 % HCl or 1 M NaOH, and equal amounts them were mixed following sterilization at 116 ^oC for 15 minutes. From the actively growing edge of the colonies mycelial disks with the diameter of 8 mm were inoculated on the medium and pectinase activity was observed after 2 days of incubation at 28 ^oC by flooding the plates with 5 ml 1 % (10 gl^{-1} in distilled water) hexadecyltrimethylammonium bromide (C-TAB), previously sterilized at 120 ^oC for 20 minutes. After 2 hours of incubation the reagent was discarded and the diameter of the colony and the degradation halo was measured (Hankin and Anagnostakis 1975).

Cellulase test:

The test medium consisted of mineral solution ($(NH_4)_2SO_4$ 1 g l^{-1}, urea 0.3 g l^{-1}, KH_2PO_4 2 g l^{-1}, $CaCl_2$ 0.3 g l^{-1}, $MgCl_2$ x 6 H_2O 0.3 g l^{-1}, $FeSO_4$ x 7 H_2O 0.005 g l^{-1}, $MnSO_4$ x H_2O 0.014 g l^{-1}, $CaCl_2$ x 2 H_2O 0.002 g l^{-1},) 20 ml l^{-1}, carboxymethyl-cellulose (CMC) 10 g l^{-1}, agar 15 g l^{-1} and 0.1% Triton X-100 1ml l^{-1} in distilled water. pH was adjusted to 5 as described above and the medium was sterilized at 116 ^oC for 15 minutes. Inoculation was carried out as described above, and cellulase activity was visualized after 7 days of

incubation at 28 ^{o}C by flooding the plates with 5 ml aqueous solution of Congo red (1 g l^{-1} in distilled water). The plates were shaken at 50 rpm for 15 minutes on a rotary shaker, then the reagent was discarded and the diameter of the colony and the degradation halo was measured (Sazci *et al.* 1986).

Amylase test:

The medium contained nutrient agar 23 g l^{-1} and starch 2 g l^{-1} in distilled water. pH was adjusted to 6 and the medium was sterilized at 120 ^{o}C for 20 minutes. Inoculation was performed as described above and amylase activity was checked after 3-4 days of incubation at 28 ^{o}C by flooding the plates with Iodine/Potassium iodide solution (Fluka). After 10 min of incubation the reagent was discarded and the diameter of the colony and the degradation zone was measured (Hankin and Anagnostakis 1975).

Lipase test:

The medium consisted of peptone (Difco) 10 g l^{-1}, NaCl 5 g l^{-1}, $CaCl_2$ x 2 H_2O 0.1 g l^{-1}, agar 20 g l^{-1} in distilled water. pH was adjusted to 6 and the medium was sterilized at 120 ^{o}C for 20 minutes, then 10 ml l^{-1} Tween 20 was added, sterilized previously at 120 ^{o}C for 15 minutes. Inoculation was carried out as described and following incubation at 28 ^{o}C 3 days the diameter of the colony and the degradation zone was measured (Hankin and Anagnostakis 1975).

Protease test:

Test medium was prepared by adding nutrient agar (OXOID) 28 g l^{-1} in distilled water. After adjusting the pH to 6 the medium was sterilized at 120 ^{o}C for 20 minutes, then 50 ml gelatin solution (80 g l^{-1}, sterilized at 120 ^{o}C for 20 minutes) was added. A cellophane membrane (sterilized twice at 120 ^{o}C for 40 minutes) was placed on the solidified medium and inoculation was carried out as described after the membrane has become dry. Plates were incubated at 28 ^{o}C for 3-4 days then colony diameters were measured and cellophane membranes containing the colonies were removed. If the degradation halos were not visible, plates were flooded with saturated (0.67 g l^{-1}) $(NH_4)_2SO_4$ and halos were measured after the removal of the reagent (Hankin and Anagnostakis 1975).

Chitinase test:

20 g agar was sterilized in 1 l of distilled water at 116 ^{o}C for 15 minutes, then 1 g l^{-1} colloidal chitin, prepared according to the method of Hsu and Lockwood (1975), was added to the medium. Inoculation was performed as described above and colony and degradation zone diameters were measured following incubation at 28 ^{o}C for 2-3 days.

Glucanase test:

The medium contained agar 20 g l^{-1} and laminarin 0.5 g l^{-1}in distilled water, and sterilization followed at 120^{o}C for 20 minutes. Inoculation was performed as described above and the plates were incubated at 28 ^{o}C for 2 days. Plates were flooded with 3 ml aqueous solution of 0.1 % Methyl Blue (Fluka) (Cote *et al.* 1989) and shaken at 100 rpm for 30 minutes on a rotary shaker, then the reagent was discarded and degradation zones were visualized by UV illumination using a UVP white/UV transilluminator (TMW-20, 220/240 W, 50 Hz, 0.6 Amps, San Gabriel CA 91778 USA).

5.13. 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 with the diameter of 5 mm from the actively growing edge 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.

6. RESULTS

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

A total of 66 *Trichoderma* isolates were isolated from compost and substrate samples from three different Hungarian mushroom growing companies (Fig. 3A and B): 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* (Fig. 4). 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 and *Tricho*BLAST. 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 (Fig. 4, B34). The other 49 *Trichoderma* strains were also identified by means of *TrichO*Key: they included *T. harzianum* (allele of the ex-type culture, three isolates), *T. atroviride* (nine isolates), *T. asperellum* (four isolates), and *T. ghanense* (one isolate). 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, 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. This preliminary denomination is based on a strain with the same ITS type isolated from decayed *Acer* stump in Ontario, Canada (Kullnig-Gradinger *et al.* 2002). The results of strain identification and the accession numbers of some selected sequences submitted to NCBI GenBank from this study are indicated in Table 4.

Figure 3A. Examples for isolating *Trichoderma* strains from Hungarian *Pleurotus* substrate (1) and *Agaricus* compost (2) samples

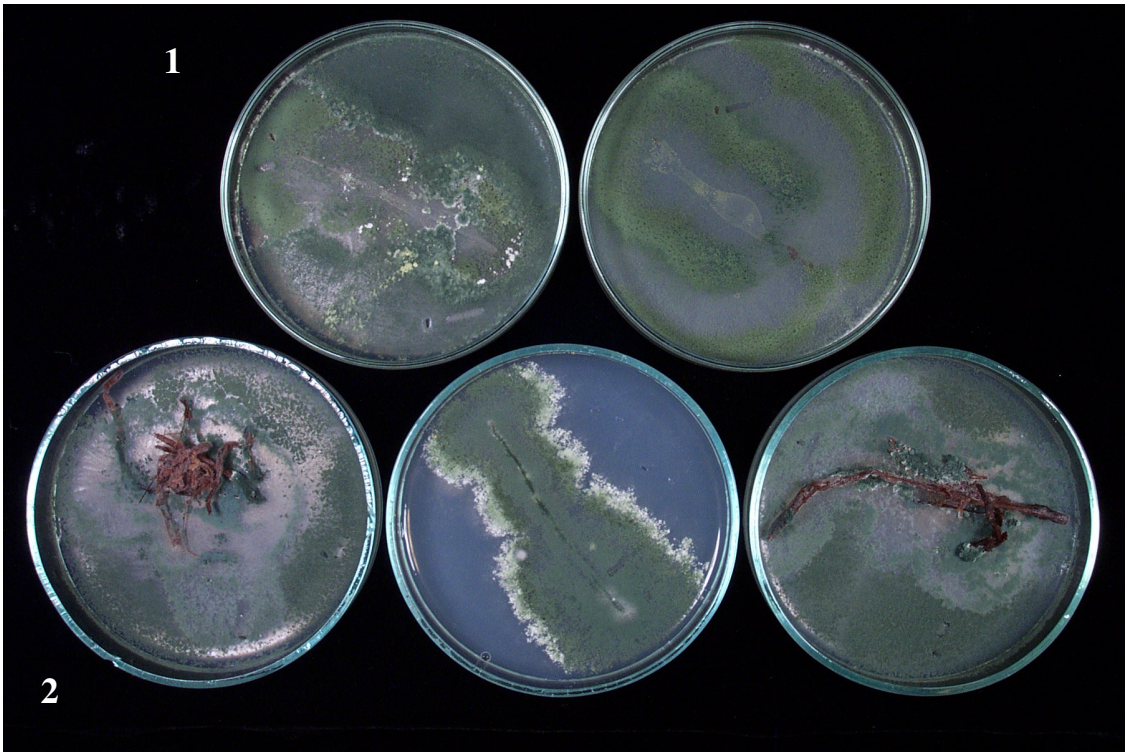


Figure 3B. Examples of *Trichoderma* strains isolated from Hungarian *Pleurotus* substrate (1) and *Agaricus* compost (2) samples

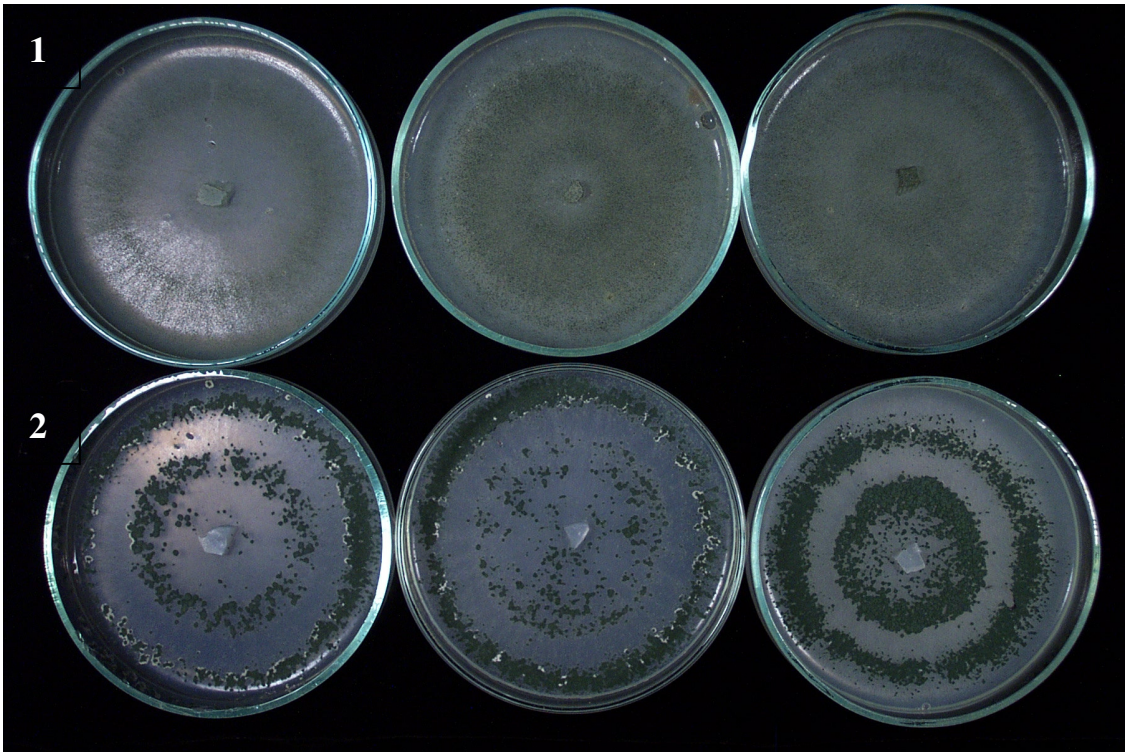


Figure 4. Screening of Hungarian *Trichoderma* isolates from mushroom compost and substrate for *T. aggressivum* by diagnostic PCR. +: positive control (*T. aggressivum* f. *europaeum* CBS 100526), –: negative control (with bidistilled water substituted for DNA)

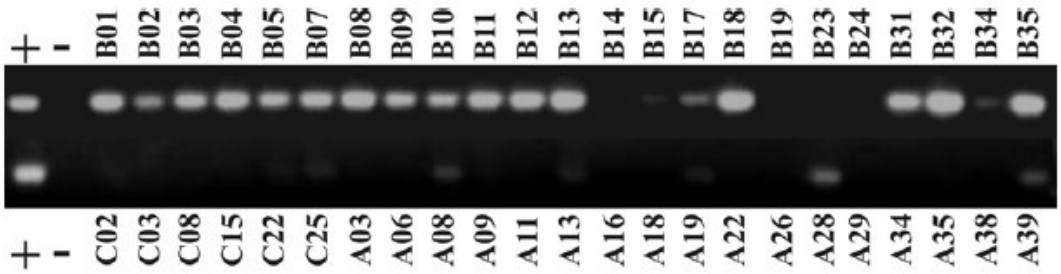


Table 4. Identification of *Trichoderma* strains isolated from *Agaricus* compost and *Pleurotus* substrate samples, sources of isolation and GenBank accession numbers of ITS1, ITS2 and *tef1* sequences

Strain	Source of isolation	Taxon	ITS1	ITS2	*tef*1
CBS 450.95, CBS100527, CBS100528	*Agaricus* compost, North America	*T. aggressivum* f. *aggressivum*	DQ328880	DQ328904	
CBS 100525, CBS 100526, CBS433.95, CBS689.94	*Agaricus* compost, United Kingdom	*T. aggressivum* f. *europaeum*	DQ328879	DQ328903	
A03	*Pleurotus* substrate, Farm A, Hungary	*T.* sp. DAOM 175924	DQ328882[a]	DQ328912	
A06	*Pleurotus* substrate, Farm A, Hungary	*T.* sp. DAOM 175924	DQ328882[a]	DQ328911	
A08	*Pleurotus* substrate, Farm A, Hungary	*T.* sp. DAOM 175924	DQ328897	DQ328939	
A09, A13, A33	*Pleurotus* substrate, Farm A, Hungary	*T.* sp. DAOM 175924	DQ328882	DQ328907	
A11	*Pleurotus* substrate, Farm A, Hungary	*T.* sp. DAOM 175924	DQ328898	DQ328939	
A14	*Pleurotus* substrate, Farm A, Hungary	*T.* sp. DAOM 175924	DQ328882	DQ328913	
A15, A20, A22, A23, A27, A30, A34	*Pleurotus* substrate, Farm A, Hungary	*T.* sp. DAOM 175924	DQ328882	DQ328915	
A16	*Pleurotus* substrate, Farm A, Hungary	*T.* sp. DAOM 175924	DQ328882	DQ328906	
A18	*Pleurotus* substrate, Farm A, Hungary	*T.* sp. DAOM 175924	DQ328882	DQ328914	
A19	*Pleurotus* substrate, Farm A, Hungary	*T.* sp. DAOM 175924	DQ328883	DQ328906	
A25	*Pleurotus* substrate, Farm A, Hungary	*T.* sp. DAOM 175924	DQ328898	DQ328939	
A26	*Pleurotus* substrate, Farm A, Hungary	*T.* sp. DAOM 175924	DQ328888	DQ328907	
A28	*Pleurotus* substrate, Farm A, Hungary	*T.* sp. DAOM 175924	DQ328884	DQ328907	
A29	*Pleurotus* substrate, Farm A, Hungary	*T.* sp. DAOM 175924	DQ328882	DQ328916	
A35	*Pleurotus* substrate, Farm A, Hungary	*T. longibrachiatum*	DQ328902	DQ328943	DQ364636
A37	*Pleurotus* substrate, Farm A, Hungary	*T.* sp. DAOM 175924	DQ328898	DQ328940	
A38	*Pleurotus* substrate, Farm A, Hungary	*T. asperellum*	DQ328889	DQ328917	
A39	*Pleurotus* substrate, Farm A, Hungary	*T. atroviride*	DQ328890	DQ328918	
B01	*Agaricus* compost, Farm B, Hungary	*T. aggressivum* f. *europaeum*	DQ328892	DQ328903	
B02	*Agaricus* compost, Farm B, Hungary	*T. aggressivum* f. *europaeum*	DQ328899	DQ328903	
B03, B04, B07, B12, B18, B31, B35	*Agaricus* compost, Farm B, Hungary	*T. aggressivum* f. *europaeum*	DQ328879	DQ328903	
B05	*Agaricus* compost, Farm B, Hungary	*T. aggressivum* f. *europaeum*	DQ328879	DQ328921	
B08	*Agaricus* compost, Farm B, Hungary	*T. aggressivum* f. *europaeum*	DQ328900	DQ328903	
B09	*Agaricus* compost, Farm B, Hungary	*T. aggressivum* f. *europaeum*	DQ328879	DQ328922	
B10	*Agaricus* compost, Farm B, Hungary	*T. aggressivum* f. *europaeum*	DQ328879	DQ328923	
B11	*Agaricus* compost, Farm B, Hungary	*T. aggressivum* f. *europaeum*	DQ328893	DQ328903	
B13	*Agaricus* compost, Farm B, Hungary	*T. aggressivum* f. *europaeum*	DQ328879	DQ328925	
B14	*Agaricus* compost, Farm B, Hungary	*T. ghanense*	DQ328894	DQ328924	
B15	*Agaricus* compost, Farm B, Hungary	*T. atroviride*	DQ328885	DQ328908	
B17	*Agaricus* compost, Farm B, Hungary	*T. longibrachiatum*	DQ328886	DQ328909	DQ364633
B19	*Agaricus* compost, Farm B, Hungary	*T. longibrachiatum*	DQ328902	DQ328942	DQ364634
B20	*Agaricus* compost, Farm B, Hungary	*T. atroviride*	DQ328890	DQ328926	
B21	*Agaricus* compost, Farm B, Hungary	*T. atroviride*	DQ328890	DQ328927	
B22	*Agaricus* compost, Farm B, Hungary	*T. atroviride*	DQ328890	DQ328929	
B23	*Agaricus* compost, Farm B, Hungary	*T. asperellum*	DQ328889	DQ328928	
B24	*Agaricus* compost, Farm B, Hungary	*T. atroviride*	DQ328895	DQ328930	
B26	*Agaricus* compost, Farm B, Hungary	*T. asperellum*	DQ328889	DQ328936	

B27	*Agaricus* compost, Farm B, Hungary	*T. atroviride*	DQ328890	DQ328931	
B28	*Agaricus* compost, Farm B, Hungary	*T. atroviride*	DQ328890	DQ328932	
B29	*Agaricus* compost, Farm B, Hungary	*T. longibrachiatum*	DQ328887	DQ328910	DQ364635
B30	*Agaricus* compost, Farm B, Hungary	*T. atroviride*	DQ328890	DQ328933	
B32	*Agaricus* compost, Farm B, Hungary	*T. aggressivum* f. *europaeum*	DQ328879	DQ328934	
B34	*Agaricus* compost, Farm B, Hungary	*T. aggressivum* f. *europaeum*	DQ328879	DQ328935	
B37	*Agaricus* compost, Farm B, Hungary	*T. asperellum*	DQ328889	DQ328937	
C01	*Agaricus* compost, Farm C, Hungary	*T. harzianum*	DQ328891	DQ328919	
C02	*Pleurotus* substrate, Farm C, Hungary	*T.* sp. DAOM 175924	DQ328882	DQ328920	
C03	*Pleurotus* substrate, Farm C, Hungary	*T.* sp. DAOM 175924	DQ328896	DQ328938	
C08	*Agaricus* compost, Farm C, Hungary	*T. harzianum*	DQ328901	DQ328941	
C15	*Pleurotus* substrate, Farm C, Hungary	*T.* sp. DAOM 175924	DQ328898	DQ328939	
C22	*Agaricus* compost, Farm C, Hungary	*T. harzianum*	DQ328881	DQ328905	
C25, C27	*Pleurotus* substrate, Farm C, Hungary	*T.* sp. DAOM 175924	DQ328897	DQ328939	

[a]Identical GenBank accession numbers indicate that the sequences of the corresponding isolates are identical.

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

The distribution of the identified species within the samples derived from cultivation of *A. bisporus* and *P. ostreatus* revealed an interesting pattern. *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* (Table 5A and B). 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.

Table 5A. Distribution of *Trichoderma* species in Hungarian *Agaricus* compost and *Pleurotus* substrate samples

Species	Sample				Total
	A (*Pleurotus* substrate)	B (*Agaricus* compost)	C/1 (*Pleurotus* substrate)	C/2 (*Agaricus* compost)	
T. harzianum	0	0	0	3	3
T. sp. DAOM 175924*	23	0	5	0	28
T. aggressivum	0	17	0	0	17
T. atroviride	1	8	0	0	9
T. asperellum	1	3	0	0	4
T. longibrachiatum	1	3	0	0	4
T. ghanense	0	1	0	0	1
Total	26	32	5	3	66

**T.* sp. DAOM 175924 was later described as two distinct species, *T. pleurotum* and *T. pleuroticola* (Park *et al.* 2006). Their distribution in the samples are shown in Table 5B.

Table 5B. Distribution of *T. pleurotum* and *T. pleuroticola* isolates in Hungarian *Agaricus* compost and *Pleurotus* substrate samples

Species	Sample				Total
	A (*Pleurotus* substrate)	B (*Agaricus* compost)	C/1 (*Pleurotus* substrate)	C/2 (*Agaricus* compost)	
T. pleurotum	22	0	5	0	27
T. pleuroticola.	1	0	0	0	1

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

Having demonstrated that the occurrence of green mold 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 mold isolates from Korea represent the same species as those found in the present study in Hungary.

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

In order to learn whether the green mold outbreak observed in Hungary indeed results from spreading of the Western European *Agaricus* green mold 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 RFLP pattern (Fig 5A and C), which was clearly different from that of *T. aggressivum* f. *aggressivum.* For the latter, a common pattern could be observed apart from the small variance resulting from the presence or absence of a 2.2-kb band (Fig. 5A), the derivative of a DNase sensitive, S1 nuclease and RNase resistant plasmid of approximately 5.0 kb, which was detected in the undigested DNA Fig. 6 A and B).

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

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 (Fig. 5B and C). Samples from location A contained two of these groups (later identified as *T. pleurotum* and *T. pleuroticola*, respectively), and C, the third. In the course of this investigation, we also observed the presence of a plasmid-derived 2.2-kb band in some but not all isolates of *T. pleurotum* and in *T. pleuroticola* (Fig. 5B).

Figure 5. Mitochondrial DNA polymorphism of *T. aggressivum* and *T.* sp. DAOM 175924 strains revealed by RFLP analysis with the restriction enzyme *Hin*6I. A: 1. *T. aggressivum* f. *europaeum* B1, 2. *T. aggressivum* f. *europaeum* B7, 3. *T. aggressivum* f. *europaeum* B8, 4. *T. aggressivum* f. *europaeum* CBS 433.95, 5. *T. aggressivum* f. *europaeum* CBS 689.94, 6. *T. aggressivum* f. *aggressivum* CBS 450.95, 7. *T. aggressivum* f. *aggressivum* CBS 100528, 8. *T. aggressivum* f. *aggressivum* CBS 100527, M: Lambda DNA/*Hin*dIII marker; B: M: Lambda DNA/*Hin*dIII marker, 1. *T.* sp. DAOM 175924 (*T. pleurotum*) C15, 2. *T.* sp. DAOM 175924 (*T. pleurotum*) A8, 3. *T.* sp. DAOM 175924 (*T. pleurotum*) A16, 4. *T.* sp. DAOM 175924 (*T. pleurotum*) A28, 5. *T.* sp. DAOM 175924 (*T. pleuroticola*) A37; C: Dendrogram of *T. aggressivum* and *T.* sp. DAOM 175924 strains based on their mtDNA profiles. The scale bar represents genetic distance.

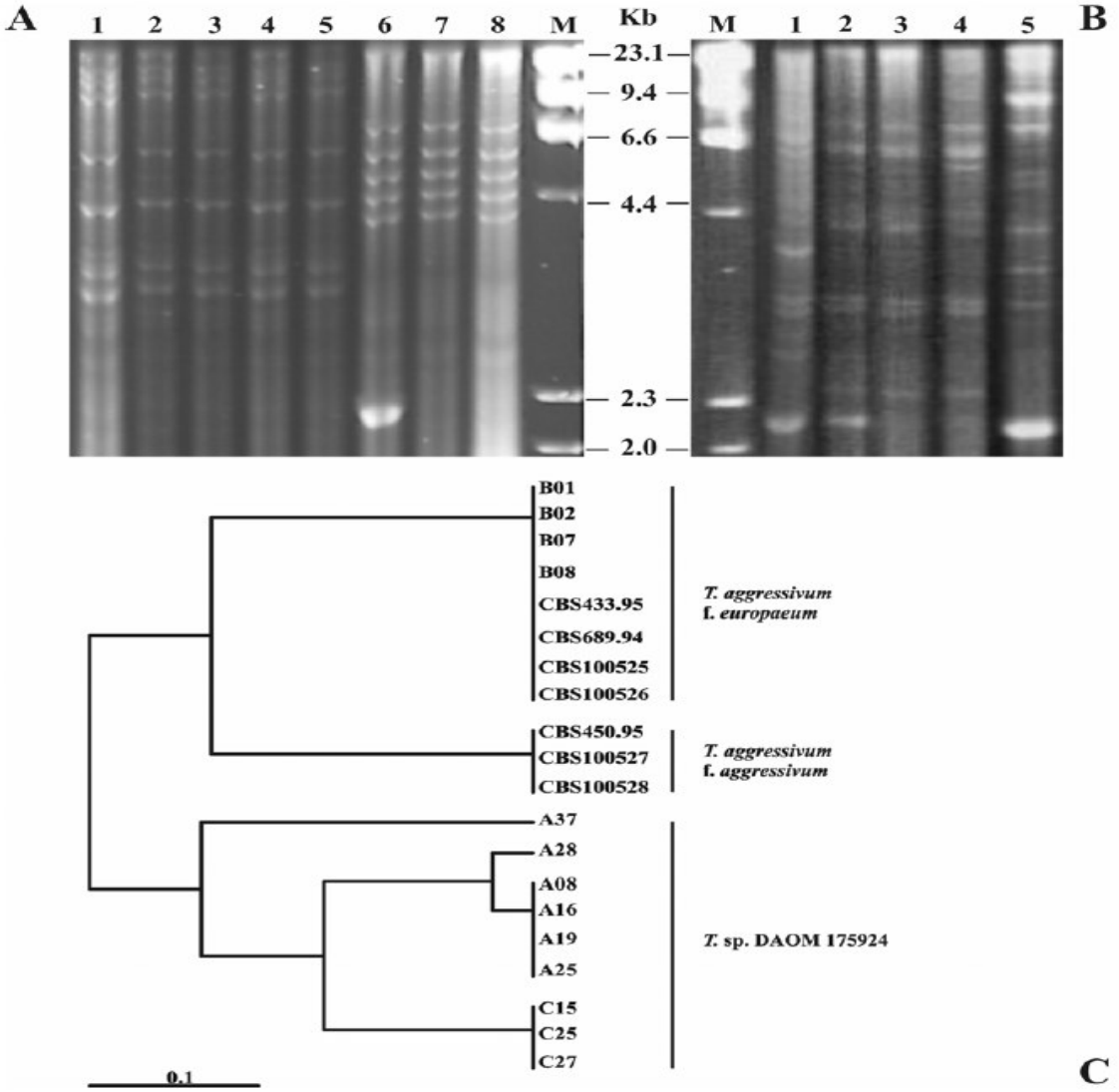
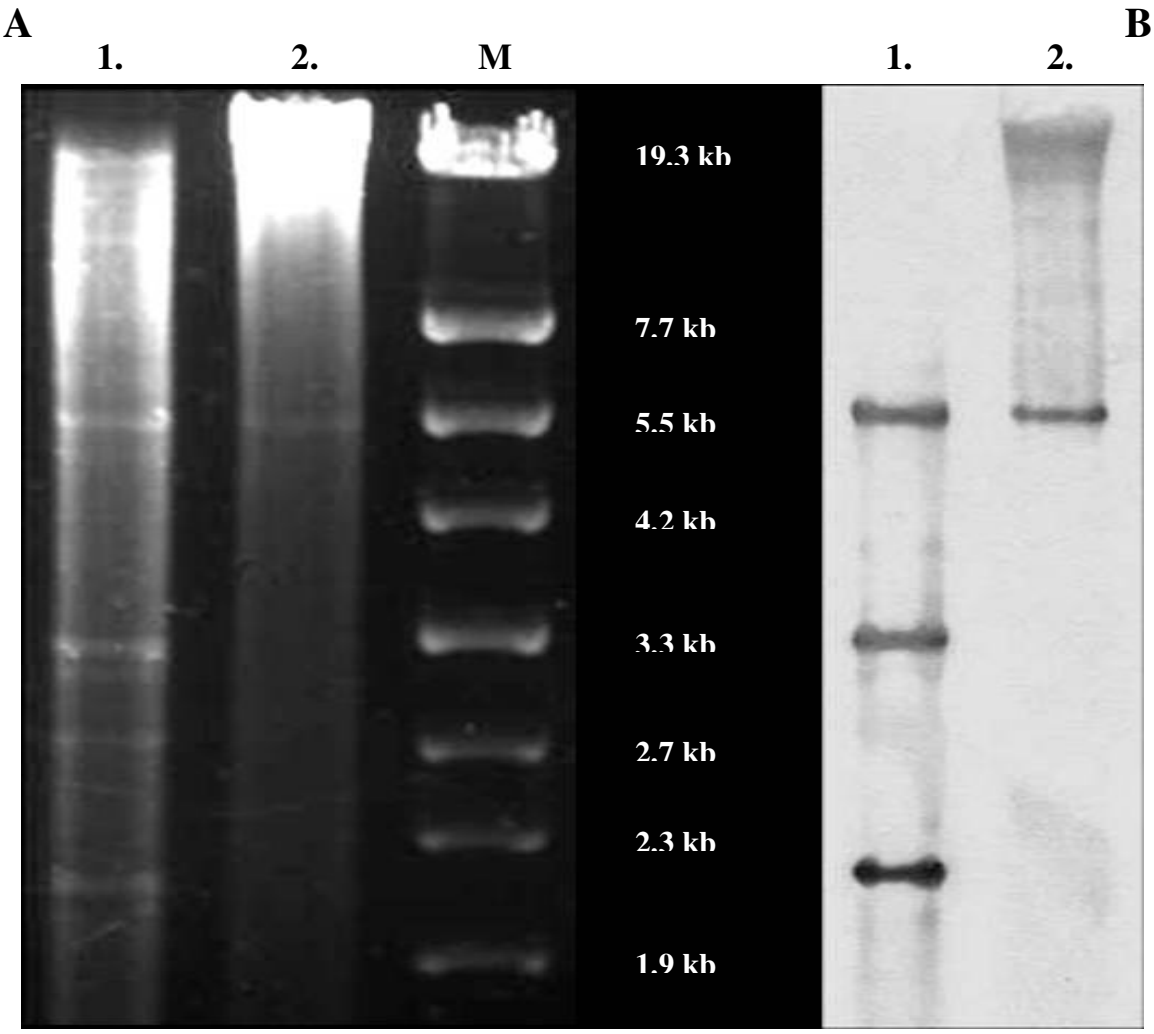


Figure 6. A gel (A) and its blot (B) hybridized with the 5.0 kb fragment as probe. 1. The total DNA of *T. aggressivum* f. *aggressivum* strain CBS 450.95 digested with *Eco*RI, 2.Undigested total DNA of strain CBS 450.95, M: pUC-mix molecular size marker



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

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). The strain DAOM 175924 was previously reported to form a separate phylogenetic branch in the vicinity of *T. harzianum* and *T. aggressivum* in the Harzianum clade (Kullnig-Gradinger *et al.* 2002, Lieckfeldt *et al.* 2001). We applied two criteria of Dettman *et al.* (2003) employing multilocus genealogies to determine if the various isolates associated with green mould on *Pleurotus* represent a single species or more than one distinct species. The criterion of genealogical concordance requires that the clade must be present in the majority of single-locus trees. The genealogical nondiscordance criterion recognizes a clade as an independent evolutionary lineage when it is reliably supported by at least one single-locus genealogy and if it is not contradicted by any other single gene tree determined by the same methods. To do this, 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, but the highest sequence homology was shown for species from the Harzianum clade (Druzhinina *et al.* 2006a). The phylogenies obtained from independent analyses of *chi18-5* (Fig. 7A) and *tef1* (Fig. 7B) markers placed the *Pleurotus* green mould isolates and cospecific strains in one supported hypothetical taxonomic unit close to *H. lixii*/*T. harzianum*. On the *chi18-5* tree 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.

Figure 7A. Phylogenetic analysis of *T. pleurotum* and *T. pleuroticola* isolates based on *chi18-5* (Komoń-Zelazowska *et al.* 2007)

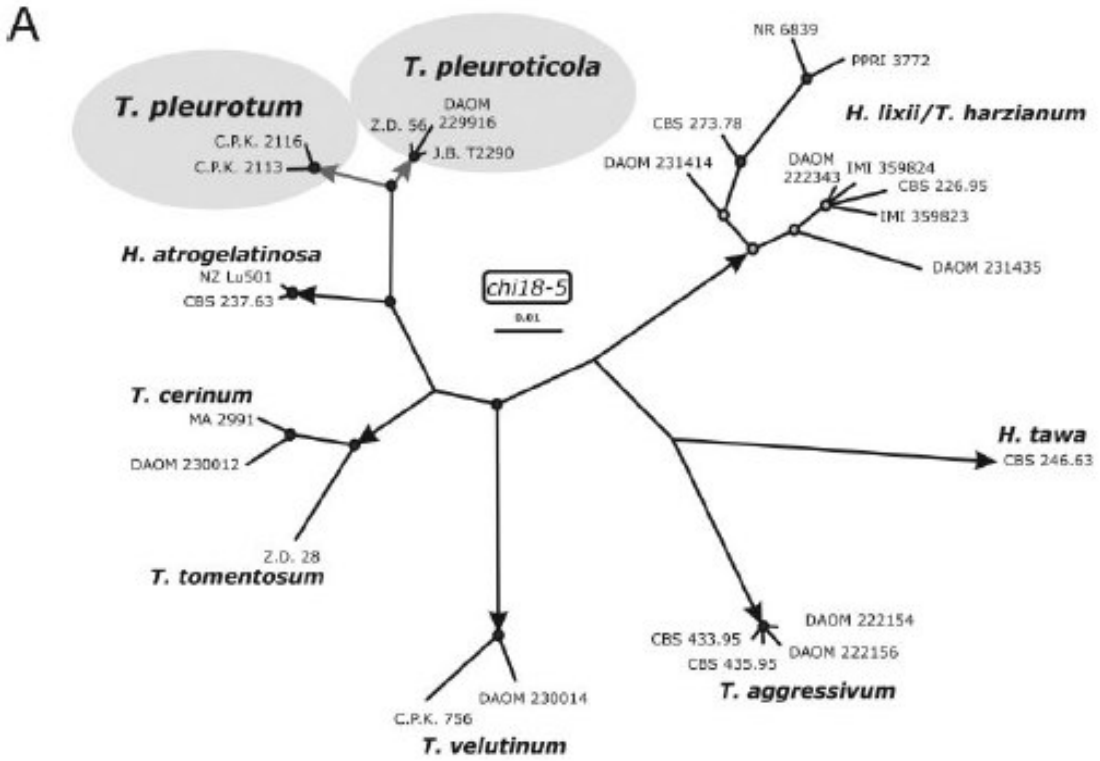
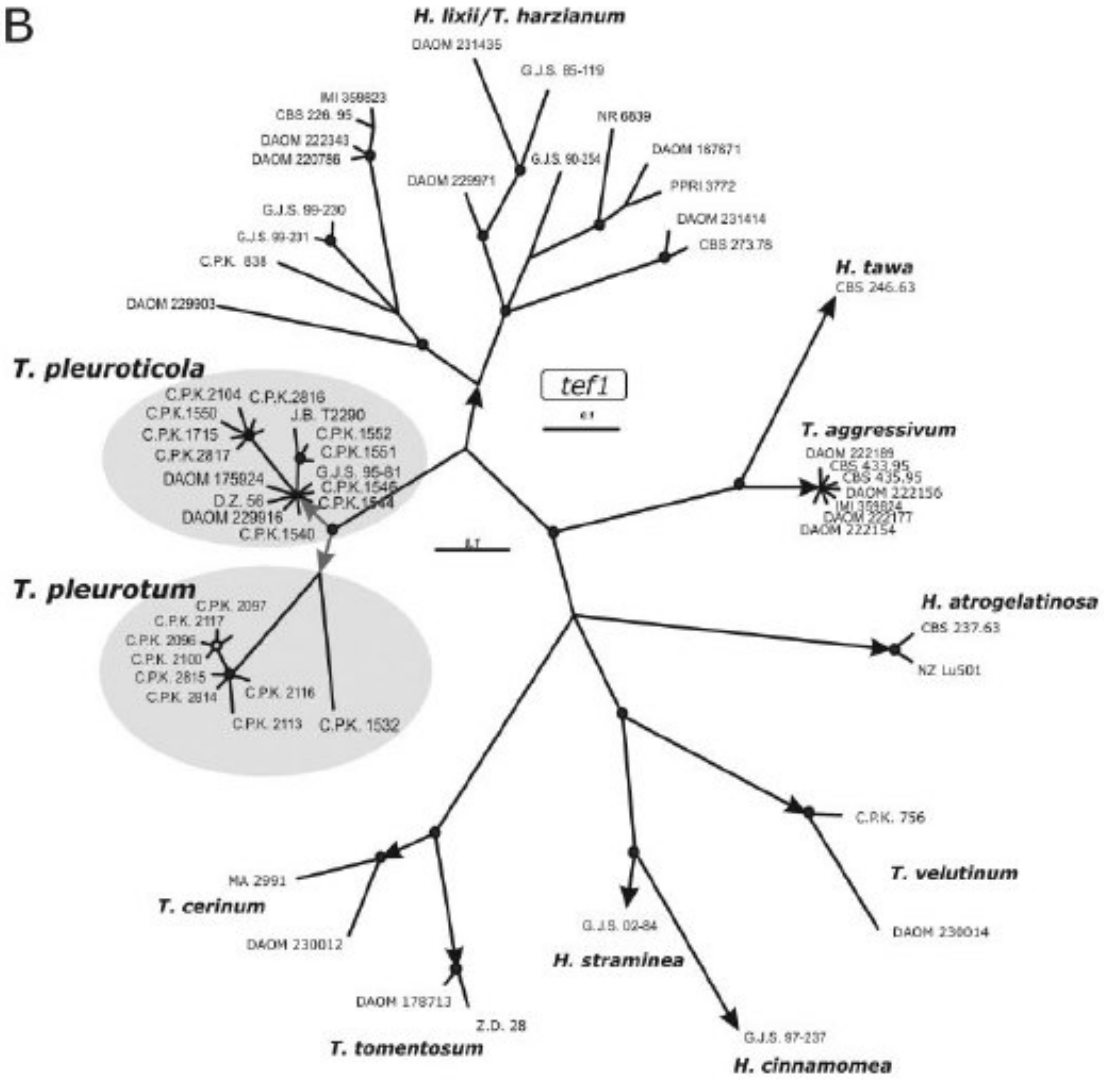
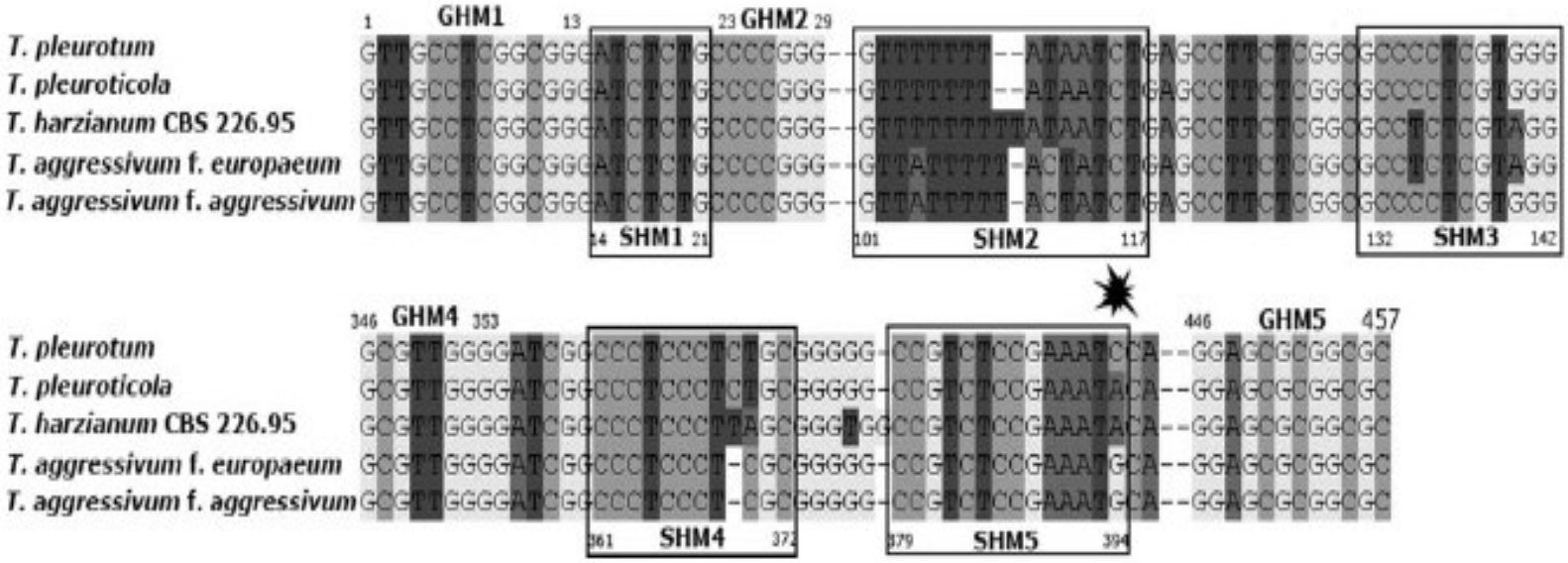


Figure 7B. Phylogenetic analysis of *T. pleurotum* and *T. pleuroticola* isolates based on *tef1* sequences (Komoń-Zelazowska *et al.* 2007)



Visual analysis of ITS1 and ITS2 sequences (Fig. 8) support the relationship of the studied strains to the Harzianum clade. All strains from *Pleurotus* farms were segregated into two ITS2 alleles which differed from each other by one single nucleotide polymorphism (position 394 from the first nucleotide of the first genus-specific hallmark) (Druzhinina *et al.* 2005). This divergence strictly corresponds to the two significant clades in the analyses of *tef1* and *chi18-5*. Both of these alleles were 5 to 6 nucleotides different from the type allele of *T. harzianum* (CBS 226.95) and from the two known alleles of *T. aggressivum*. 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). Since ex-type cultures of these species were not deposited in publicly accessible culture collections and therefore were not available for this study, we refer to strains DAOM 175924 and CPK 1532 as reference strains for *T. pleuroticola* and *T. pleurotum*, respectively. For rapid molecular identification, oligonucleotide BarCodes based on differences in ITS1 and ITS2 sequences among *T. pleurotum*, *T. pleuroticola*, and related species have been implemented in the database for the *Hypocrea/Trichoderma* DNA oligonucleotide BarCode program *TrichO*KEY (Druzhinina *et al.* 2005, Druzhinina *et al.* 2006b; also www.ISTH.info).

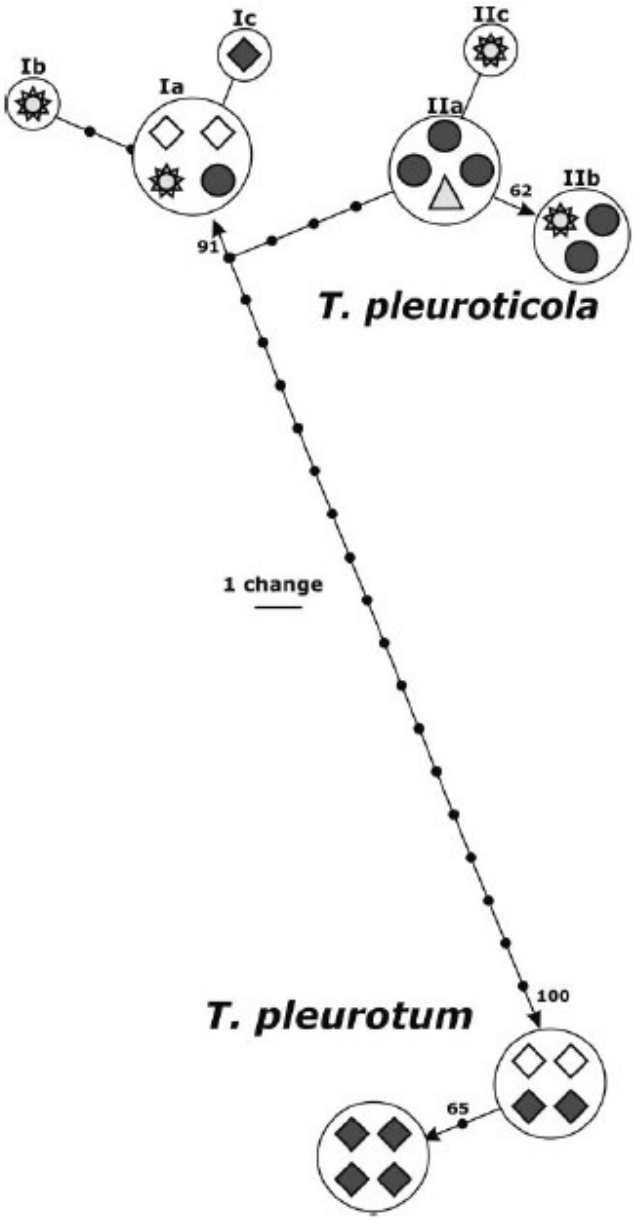
Figure 8. ITS-based oligonucleotide BarCode for identification of mushroom green mould species. GHM1 to GMH5 and SHM1 to SHM5 indicate positions of genus- and species-specific hallmarks as indicated in Druzhinina *et al.* (2005). The star shows the position of the diagnostic substitution inside SHM5 for *T. pleurotum*, *T. pleuroticola*, *T. aggressivum*, and *H. lixii/T. harzianum*. Type sequences were retrieved using accession numbers given in the study of Druzhinina *et al.* (2005) (Komoń-Zelazowska *et al.* 2007).



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

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*. Figure 9 shows the distribution of individual *tef1* alleles among isolates from different locations. 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* (Fig. 9., I and II) 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 Ia 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 (IIa) with strain DZ56 isolated from *Agaricus* compost in Iran, and the two remaining strains have the *tef1* allele (IIb) 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.

Figure 9. Distribution of individual *tef1* alleles among isolates from different locations. The scheme was manually constructed based on one of the 100 saved most parsimonious trees obtained using a heuristic search implemented in PAUP*, version 4b10 (Swofford 1998). ♦: isolates from Hungary; ◊: isolates from Romania; ●: isolates from Italy; ☼: isolates from North America; Δ: single isolate from Iran. Arabic numbers correspond to bootstrap coefficients; roman numbers show main *tef1* alleles (Komoń-Zelazowska *et al.* 2007).

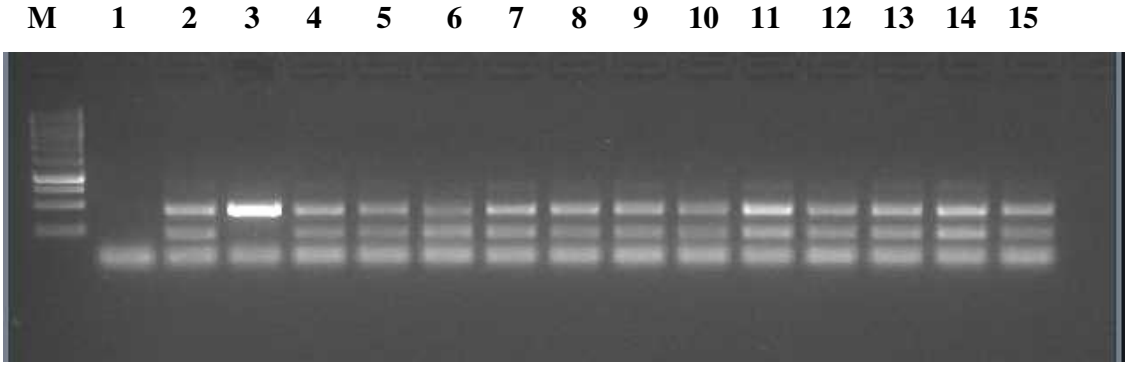


6.8. Design of PCR-primers selectively identifying *T. pleurotum* and *T. pleuroticola*

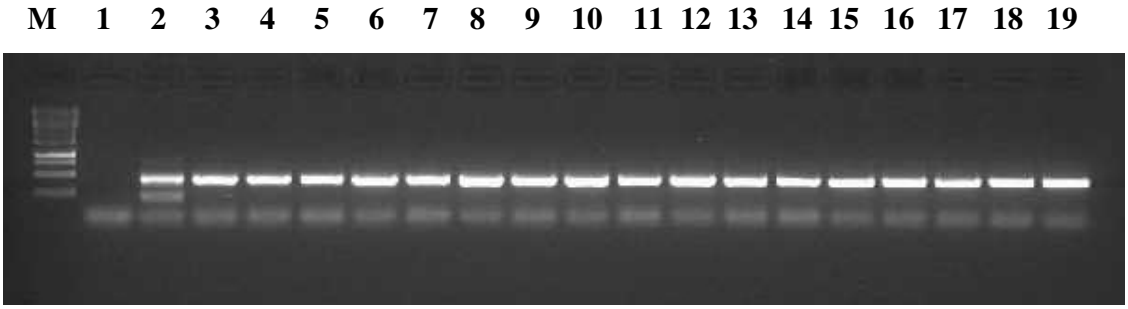
In order to have a quick tool for the identification of the two *Pleurotus*-pathogenic *Trichoderma* spp. in hand, we screened an alignment of the sequences of ITS1 and 2, *tef1* and *chi18-5* for *T. pleurotum*, *T. pleuroticola,* and various isolates of *T. harzianum, T. aggressivum* f. *europaeum* and *T. aggressivum* f. *aggressivum* (cf. Komon-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* (see Fig. 2A) that allowed the specific setting of primers (FPforw1, FPrew1 and Psrev1, respectively; see Table 3), 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* (see Fig 2B). In order to verify the applicability of these primers, a total of 13 *T. pleurotum* and 17 *T. pleuroticola* isolates were tested (Table 2, Fig. 10A and B). 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*.

Figure 10. Fragments amplified in the reaction using primers specific for *T. pleurotum* and *T. pleuroticola* in the case of 13 *T. pleurotum* isolates (A) M: GeneRuler^{TM} 1 kb DNA Ladder (Fermentas), 1. negative control, 2. *T. pleurotum* A8, 3. *T. pleuroticola* A37 (positive controls), *T. pleurotum* strains: 4. A1, 5. A11, 6. A16, 7. A25, 8. A28, 9. C4, 10. C5, 11. C14, 12. C15, 13. C21, 14. C25, 15. C27; and with 17 *T. pleuroticola* isolates (B) M. GeneRuler^{TM} 1 kb DNA Ladder (Fermentas), 1. negative control, 2. *T. pleurotum* A8, 3. *T. pleuroticola* A37 as positive controls, *T. pleuroticola* strains: 4. CPK 230, 5. CPK 882, 6. CPK 1401, 7. CPK 1540, 8. CPK 1541, 9. CPK 1542, 10. CPK 1543, 11. CPK 1544, 12. CPK 1545, 13. CPK 1546, 14. CPK 1547, 15. CPK 1548, 16. CPK 1549, 17. CPK 1550, 18. CPK 1551, 19. CPK 1715

A



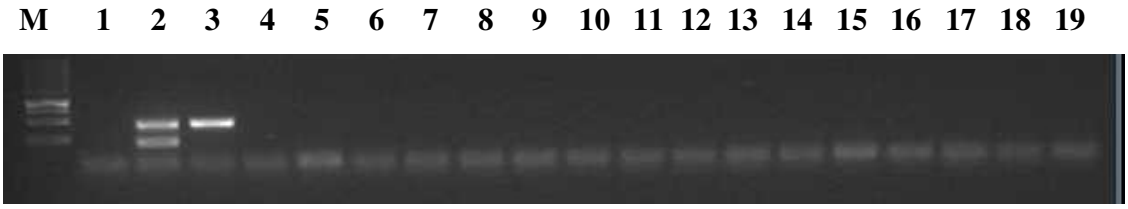
B



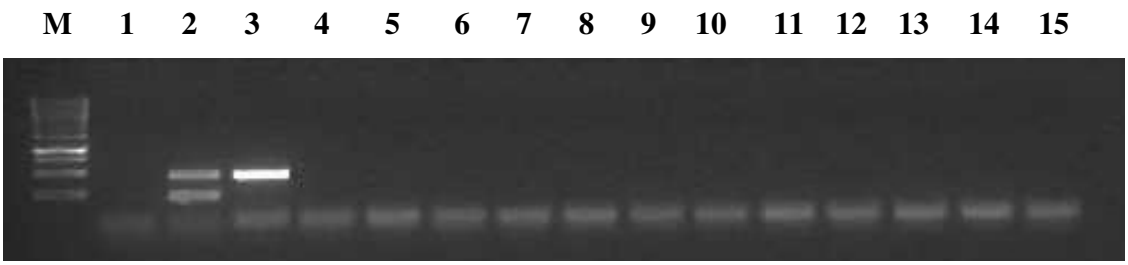
In order to assess that these primers are indeed specific and do not exhibit any cross reaction with other *Trichoderma* spp. or any other fungi, DNA samples of 28 other *Trichoderma* species (*T. harzianum, T. aggressivum* f. *europaeum, T. aggressivum* f. *aggressivum, T. minutisporum, T. crassum, 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* strains had been isolated previously (Hatvani *et al.* 2007). In none of these cases, however, were any PCR fragments produced, while controls with *T. pleurotum* and *T. pleuroticola* were always positive (a small number of examples are given in Fig. 11A and B). We therefore conclude that our multiplex PCR method is appropriate for specific detection of the *Pleurotus*-pathogenic *Trichoderma* spp.

Figure 11. PCR products formed in the reaction using primers specific for *T. pleurotum* and *T. pleuroticola* in the case of 16 different *Trichoderma* isolates from other species (A) M: GeneRuler™ 1 kb DNA Ladder (Fermentas), 1. negative control,2. *T. pleurotum* A8, 3. *T. pleuroticola* A37 as positive controls, 4. *T. harzianum,* 5. *T. aggressivum* f. *europaeum,* 6. *T. aggressivum* f. *aggressivum,* 7. *T. oblongisporum,* 8. *T. tomentosum,* 9. *T. rossicum,* 10. *T. helicum,* 11. *T. spirale,* 12. *T. virens,* 13. *T. hamatum,* 14. *T. atroviride,* 15. *T. asperellum,* 16. *T. viride,* 17. *T. koningii,* 18. *T. aureoviride,* 19. *T. longibrachiatum*; and with 12 different fungal species from other genera (B) M: GeneRuler™ 1 kb DNA Ladder (Fermentas), 1. negative control, 2. *T. pleurotum* A8, 3. *T. pleuroticola* A37 as positive controls, 4. *Penicillium expansum,* 5. *Aspergillus* sp., 6. *A. niger,* 7. *Mortierella* sp., 8. *Thermomyces* sp., 9. *Mucor circinelloides,* 10. *Fusarium graminearum,* 11. *F. oxysporum,* 12. *F. culmorum,* 13. *F. poae,* 14. *F. sporotrichioides,* 15. *Pleurotus ostreatus*

A



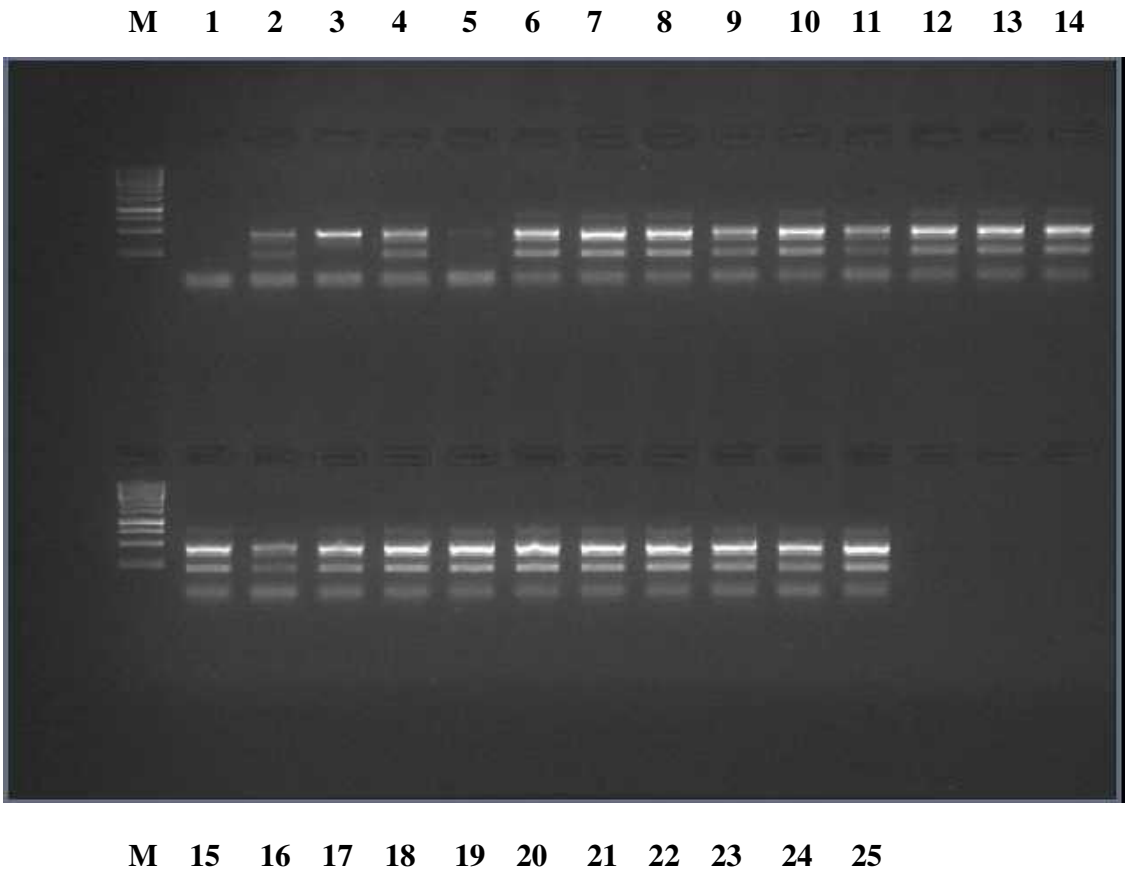
B



6.9. 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*. The results are given in Fig. 12. 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 (data not shown).

Figure 12. Amplicons gained using primers specific for *T. pleurotum* and *T. pleuroticola* in the reaction with DNA extracted directly from infected substrata and from *Trichoderma* strains isolated from them. M: GeneRuler™ 1 kb DNA Ladder (Fermentas), 1. negative control, 2. *T. pleurotum* A8, 3. *T. pleuroticola* A37 (positive controls), 4. DNA from infected substrate PSAII/3, 5-15. DNA from monospore cultures isolated from substrate PSAII/3, 16. DNA from infected substrate PSAII/4, 17-25. DNA from monospore cultures isolated from substrate PSAII/4



6.10. 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; see Fig. 1.). The results were strongly different (Table 6): 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 (data not shown).

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*.

Table 6. Origin of samples and identification of *Trichoderma* strains isolated from the substrate and the fruit body of wild-grown *Pleurotus ostreatus*

Strain number	**Specific PCR**	**ITS sequence analysis**	***tef1* sequence analysis**
Sample 1: *Populus alba* stump, *Convallario-Quercetum roboris*, Kisújszállás, Hungary			
CPK 3193	*T. pleuroticola*	Confirmed * EU918140	Confirmed * EU918160
CPK 3194	*T. pleuroticola*	Confirmed * EU918140	Confirmed * EU918160
CPK 3195	*T. pleuroticola*	Confirmed * EU918140	Confirmed * EU918160
CPK 3196	*T. pleuroticola*	Confirmed * EU918140	Confirmed * EU918160
CPK 3197	*T. pleuroticola*	Confirmed * EU918140	Confirmed * EU918160
CPK 3198	*T. pleuroticola*	N. d.	N. d.
CPK 3199	*T. pleuroticola*	N. d.	N. d.
CPK 3200	*T. pleuroticola*	N. d.	N. d.
CPK 3201	*T. pleuroticola*	N. d.	N. d.
CPK 3202	*T. pleuroticola*	N. d.	N. d.
CPK 3203	*T. pleuroticola*	N. d.	N. d.
CPK 3204	*T. pleuroticola*	N. d.	N. d.
CPK 3205	*T. pleuroticola*	N. d.	N. d.
CPK 3206	*T. pleuroticola*	N. d.	N. d.
CPK 3207	*T. pleuroticola*	N. d.	N. d.
CPK 3208	*T. pleuroticola*	N. d.	N. d.
CPK 3209	*T. pleuroticola*	N. d.	N. d.
CPK 3210	*T. pleuroticola*	N. d.	N. d.
CPK 3211	*T. pleuroticola*	N. d.	N. d.
CPK 3212	*T. pleuroticola*	N. d.	N. d.
CPK 3213	*T. pleuroticola*	N. d.	N. d.
CPK 3214	*T. pleuroticola*	N. d.	N. d.
CPK 3215	*T. pleuroticola*	N. d.	N. d.
CPK 3216	*T. pleuroticola*	N. d.	N. d.
CPK 3217	*T. pleuroticola*	N. d.	N. d.

CPK 3218	*T. pleuroticola*	N. d.	N. d.
CPK 3219	*T. pleuroticola*	N. d.	N. d.
CPK 3220	*T. pleuroticola*	N. d.	N. d.
Sample 2: *Populus alba* stump, *Fraxino pannonicae-Ulmetum*, Tőserdő, Hungary			
CPK 3247	Negative	*T. harzianum/H. lixii* * EU918151	N. d.
CPK 3248	Negative	*T. harzianum/H. lixii* * EU918151	N. d.
CPK 3249	Negative	*T. harzianum/H. lixii* * EU918151	N. d.
CPK 3250	Negative	*T. harzianum/H. lixii* * EU918149	*T. harzianum* * EU918162
CPK 3251	Negative	*T. harzianum/H. lixii* * EU918151	N. d.
CPK 3252	Negative	*T. harzianum/H. lixii* * EU918151	N. d.
CPK 3253	Negative	*T. harzianum/H. lixii* * EU918151	N. d.
CPK 3254	Negative	*T. harzianum/H. lixii* * EU918151	*T. harzianum* * EU918163
CPK 3255	Negative	*T. harzianum/H. lixii** EU918151	*T. harzianum* * EU918163
CPK 3256	Negative	*T. harzianum/H. lixii* * EU918151	*T. harzianum* * EU918164
CPK 3257	Negative	*T. harzianum/H. lixii* * EU918149	*T. harzianum* * EU918162
CPK 3258	Negative	*T. harzianum/H. lixii* * EU918151	N. d.
Sample 3: *Populus alba* stump, *Populetum cult.*, Kecskemét, Nyomási erdő, Hungary			
3/A: surface of fruit body			
CPK 2884	Negative	*T. longibrachiatum/H. orientalis*, * EU918139	*T. longibrachiatum* * EU918159
CPK 2885	*T. pleuroticola*	Confirmed * EU918141	Confirmed * EU918161
CPK 2886	*T. pleuroticola*	Confirmed * EU918142	N. d.

CPK 2887	*T. pleuroticola*	Confirmed * EU918148	N. d.
CPK 2888	Negative	*T. longibrachiatum/H. orientalis*, * EU918139	*T. longibrachiatum* * EU918159
CPK 2889	Negative	*T. longibrachiatum/H. orientalis*, * EU918139	*T. longibrachiatum* * EU918159
CPK 2890	Negative	*T. longibrachiatum/H. orientalis*, * EU918139	*T. longibrachiatum* * EU918159
CPK 2891	*T. pleuroticola*	Confirmed * EU918143	Confirmed * EU918160
CPK 2892	*T. pleuroticola*	N. d.	N. d.
CPK 2893	*T. pleuroticola*	N. d.	N. d.
CPK 2894	*T. pleuroticola*	Confirmed * EU918144	N. d.
CPK 2895	*T. pleuroticola*	Confirmed * EU918145	N. d.
CPK 2896	*T. pleuroticola*	Confirmed * EU918146	N. d.
CPK 2897	*T. pleuroticola*	Confirmed * EU918148	Confirmed * EU918160
CPK 2898	Negative	*T. longibrachiatum/H. orientalis*, * EU918139	*T. longibrachiatum* * EU918159
CPK 2899	*T. pleuroticola*	Confirmed * EU918147	Confirmed * EU918160
CPK 2900	*T. pleuroticola*	Confirmed * EU918148	N. d.
CPK 2901	*T. pleuroticola*	Confirmed * EU918148	Confirmed * EU918160
CPK 2902	*T. pleuroticola*	Confirmed * EU918148	N. d.
CPK 2903	Negative	N. d.	*T. longibrachiatum* * EU918159
3/B: substrate			
CPK 3271	Negative	*T. longibrachiatum/H. orientalis*, * EU918139	*T. longibrachiatum* * EU918159
CPK 3272	*T. pleuroticola*	Confirmed	Confirmed

		* EU918148	* EU918160
CPK 3273	Negative	*T. longibrachiatum/H. orientalis*, * EU918139	*T. longibrachiatum* * EU918159
CPK 3274	Negative	*T. longibrachiatum/H. orientalis*, * EU918139	*T. longibrachiatum* * EU918159
CPK 3275	Negative	*T. longibrachiatum/H. orientalis*, * EU918138	N. d.
CPK 3276	Negative	*T. longibrachiatum/H. orientalis*, * EU918139	*T. longibrachiatum* * EU918159
CPK 3277	Negative	*T. atroviride/H. atroviridis* * EU918133	*T. atroviride* * EU918154
CPK 3278	Negative	*T. longibrachiatum/H. orientalis*, * EU918139	*T. longibrachiatum* * EU918159
CPK 3279	Negative	*T. longibrachiatum/H. orientalis*, * EU918139	*T. longibrachiatum* * EU918159
CPK 3280	Negative	*T. longibrachiatum/H. orientalis*, * EU918139	*T. longibrachiatum* * EU918159
CPK 3281	Negative	*T. longibrachiatum/H. orientalis*, * EU918139	*T. longibrachiatum* * EU918159
Sample 4: *Populus canadensis* stump, along the road, Kecskemét, Hunyadiváros, Hungary			
CPK 3259	Negative	*T. harzianum/H. lixii* * EU918150	*T. harzianum* * EU918165
CPK 3260	Negative	*T. harzianum/H. lixii* * EU918150	N. d.
CPK 3261	*T. pleuroticola*	Confirmed * EU918148	Confirmed * EU918160
CPK 3262	Negative	*T. harzianum/H. lixii* * EU918150	N. d.
CPK 3263	Negative	*T. harzianum/H. lixii* * EU918152	*T. harzianum* * EU918166
CPK 3264	Negative	*T. harzianum/H. lixii* * EU918150	*T. harzianum* * EU918165
CPK 3265	Negative	*T. harzianum/H. lixii* * EU918152	N. d.
CPK 3266	*T. pleuroticola*	Confirmed * EU918148	Confirmed * EU918160

CPK 3267	Negative	*T. harzianum/H. lixii* * EU918152	N. d.
CPK 3268	Negative	*T. harzianum/H. lixii* * EU918152	N. d.
CPK 3269	Negative	*T. harzianum/H. lixii* * EU918150	*T. harzianum* * EU918165
CPK 3270	*T. pleuroticola*	Confirmed * EU918148	Confirmed * EU918160
CPK 3282	Negative	*T. harzianum/H. lixii* * EU918150	N. d.
CPK 3283	*T. pleuroticola*	Confirmed * EU918148	Confirmed * EU918160
CPK 3284	*T. pleuroticola*	Confirmed * EU918148	Confirmed * EU918160
CPK 3285	Negative	*T. harzianum/H. lixii* * EU918153	N. d.
CPK 3286	Negative	*T. harzianum/H. lixii* * EU918152	N. d.
CPK 3287	Negative	*T. harzianum/H. lixii* * EU918152	N. d.
CPK 3288	Negative	*T. harzianum/H. lixii* * EU918152	*T. harzianum* * EU918166
Sample 5: *Tilia sp.* stump, inside the city of Szeged, Hungary			
5/A: surface of fruit body			
No *Trichoderma* was found.			
5/B: substrate			
CPK 3375	Negative	N. d.	*T. harzianum* * EU918166
CPK 3376	Negative	N. d.	*T. harzianum* * EU918167
CPK 3377	Negative	*T. atroviride/H. atroviridis* * EU918134	*T. atroviride* * EU918155
CPK 3378	Negative	N. d.	*T. harzianum* * EU918168
CPK 3379	Negative	N. d.	*T. harzianum* * EU918168

CPK 3380	Negative	*T. atroviride/H. atroviridis* * EU918135	*T. atroviride* * EU918156
CPK 3381	Negative	N. d.	*T. harzianum* * EU918165
CPK 3382	Negative	*T. atroviride/H. atroviridis* * EU918135	*T. atroviride* * EU918155
CPK 3383	Negative	*T. atroviride/H. atroviridis* * EU918135	*T. atroviride* * EU918155
CPK 3384	Negative	*T. atroviride/H. atroviridis* * EU918136	*T. atroviride* * EU918157
CPK 3385	Negative	*T. atroviride/H. atroviridis* * EU918135	*T. atroviride* * EU918155
CPK 3386	Negative	*T. atroviride/H. atroviridis* * EU918135	*T. atroviride* * EU918155
CPK 3387	Negative	N. d.	*T. harzianum* * EU918168
CPK 3388	Negative	*T. atroviride/H. atroviridis* * EU918137	*T. atroviride* * EU918158
CPK 3389	Negative	*T. atroviride/H. atroviridis* * EU918135	*T. atroviride* * EU918155
CPK 3390	Negative	*T. atroviride/H. atroviridis* * EU918135	*T. atroviride* * EU918155
CPK 3391	Negative	N. d.	*T. harzianum* * EU918168
CPK 3392	Negative	N. d.	*T. harzianum* * EU918168
CPK 3393	Negative	N. d.	*T. harzianum* * EU918169
CPK 3394	Negative	N. d.	*T. harzianum* * EU918168

* GenBank accession number, identical numbers refer to identical sequences.

N. d.: Not determined

6.11. 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. The number of the surviving colonies resulting from the certain lengths of UV-treatment was counted and expressed as the percentage of the control samples 6 days after the treatment. Table 7 shows the percentage of survival in comparison with the untreated control.

Table 7. The percentage of surviving colonies of *T. pleurotum* in comparison with the untreated control

Duration of UV-treatment	***Survival (%)***
1 min	Uncountable
2 min	Uncountable
3 min	Uncountable
4 min	Uncountable
5 min	Uncountable
7 min	**3.8 %**
8 min	**0.88 %**
9 min	**0.43 %**
10 min	**0.08 %**
11 min	0 %
12 min	0 %

Based on these results, 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.

6.12. Growth and sporulation of the isolated surviving colonies

Growth and sporulation of the surviving colonies was observed and compared to those of the parental strains after 5 days of inoculation, data are shown in Table 8. (in APPENDIX). 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.

6.13. 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. Results of the parental strain and its derivatives with altered pectinase, cellulase, amylase, lipase, protease, chitinase and glucanase production are shown in Table 9, 10, 11, 12, 13, 14 and 15, respectively..Data are given as means of 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 (Table 9). 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 (Table 10). Fig. 13 shows the cellulase assay of the wild-type and a cellulase-deficient derivative of *T. pleurotum* C15. Strains T3 and U4 were supposed to be amylase-overproducers, while E5 and T2 deficient mutants in amylase production (Table 11). Fig. 14 shows the amylase assay of the wild-type and an amylase-deficient mutant of *T. pleurotum* C15. S17, T8 and U25 seem to be lipase-overproducers, while P2, T13 and U17 show decreased ability of producing lipases. (Table 12). 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 (Table 13). Strain P16 was identified as a chitinase-overproducer, while R2, R3, R4 and T14 were found to be deficient mutants in chitinase production (Table 14). 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 (Table 15).

Table 9. Pectinase production of the wild-type and mutant strains of *T. pleurotum* C15

0: transparency of the degradation halo produced by the wild-type strain

-: degradation halo with less transparency

--: degradation halo with far less transparency

Strain number	***Colony diam.***	***Halo diam.***	***Halo/Colony***	***Halo transp.***
Wild-type	**52**	**40**	**0.77**	**0**
E6	45	30	0.67	-
F1	44	22	0.50	--
F5	44	27	0.61	0
O15	40	21	0.53	--
P13	51	25	0.49	--
R3	52	48	0.92	0
T10	45	27	0.60	-
U3	47	26	0.55	--
V5	28	16	0.57	-

Table 10. Cellulase production of the wild-type and mutant strains of *T. pleurotum* C15

Strain number	***Colony diam.***	***Halo diam.***	***Halo/Colony***
Wild-type	**31**	**41**	**1.32**
C4	23	34	1.48
D1	26	38	1.46
D2	27	40	1.48
H2	31	0	0
T14	25	0	0

Figure 13. Degradation of CMC by the wild-type isolate (A) and the mutant strain T14 (B) of *T. pleurotum* C15

A **B**

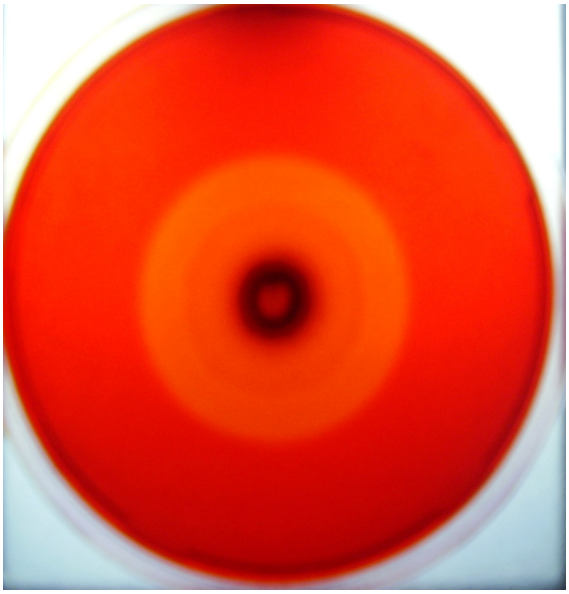


Table 11. Amylase production of the wild-type and mutant strains of *T. pleurotum* C15

Strain number	***Colony diam.***	***Halo diam.***	***Halo/Colony***
Wild-type	**76**	**28**	**0.37**
E5	72	18	0.25
T2	76	19	0.25
T3	68	42	0.62
U4	70	35	0.50

Figure 14. Degradation of starch by the wild-type isolate (A) and the mutant strain E5 (B) of *T. pleurotum* C15

A **B**

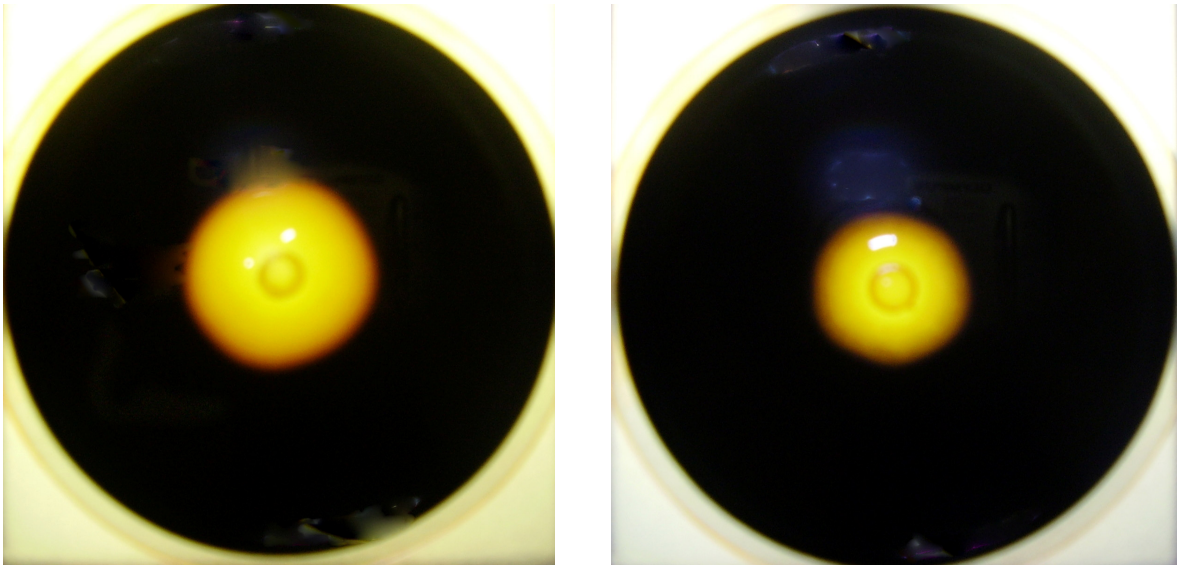


Table 12. Lipase production of the wild-type and mutant strains of *T. pleurotum* C15

Strain number	***Colony diam.***	***Halo diam.***	***Halo/Colony***
Wild-type	**48**	**32**	**0.67**
P2	43	26	0.60
S17	39	31	0.79
T8	35	30	0.86
T13	48	26	0.54
U17	42	24	0.57
U25	45	34	0.76

Table 13. Protease production of the wild-type and mutant strains of *T. pleurotum* C15

Strain number	***Colony diam.***	***Halo diam.***	***Halo/Colony***
Wild-type	**33**	**39**	**1.18**
F7	30	28	0.93
R4	40	30	0.75
S10	30	21	0.70
S13	23	0	0
S16	37	33	0.89
U8	25	22	0.88
V5	24	0	0

Table 14. Chitinase production of the wild-type and mutant strains of *T. pleurotum* C15

Strain number	***Colony diam.***	***Halo diam.***	***Halo/Colony***
Wild-type	**53**	**41**	**0.77**
P16	30	27	0.9
R2	37	22	0.59
R3	57	40	0.70
R4	45	27	0.60
T14	23	13	0.56

Table 15. Glucanase production of the wild-type and mutant strains of *T. pleurotum* C15

0: halo produced by the wild-type strain

+: larger halo as compared to that of the wild-type strain

-: smaller halo as compared to that of the wild-type strain

Strain number	**Colony diameter**	***Halo size as compared to the wild-type***
Wild-type	**49**	**0**
C3	14	+
D1	42	+
F1	48	-
H1	36	+
M1	37	+
R2	42	-
R3	48	-
R4	45	-
T3	43	-

6.14. Dual plate assays

The most spectacular and unequivocal differences between the wild-type and some mutant strains appeared on YEX medium (Fig. 15). 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

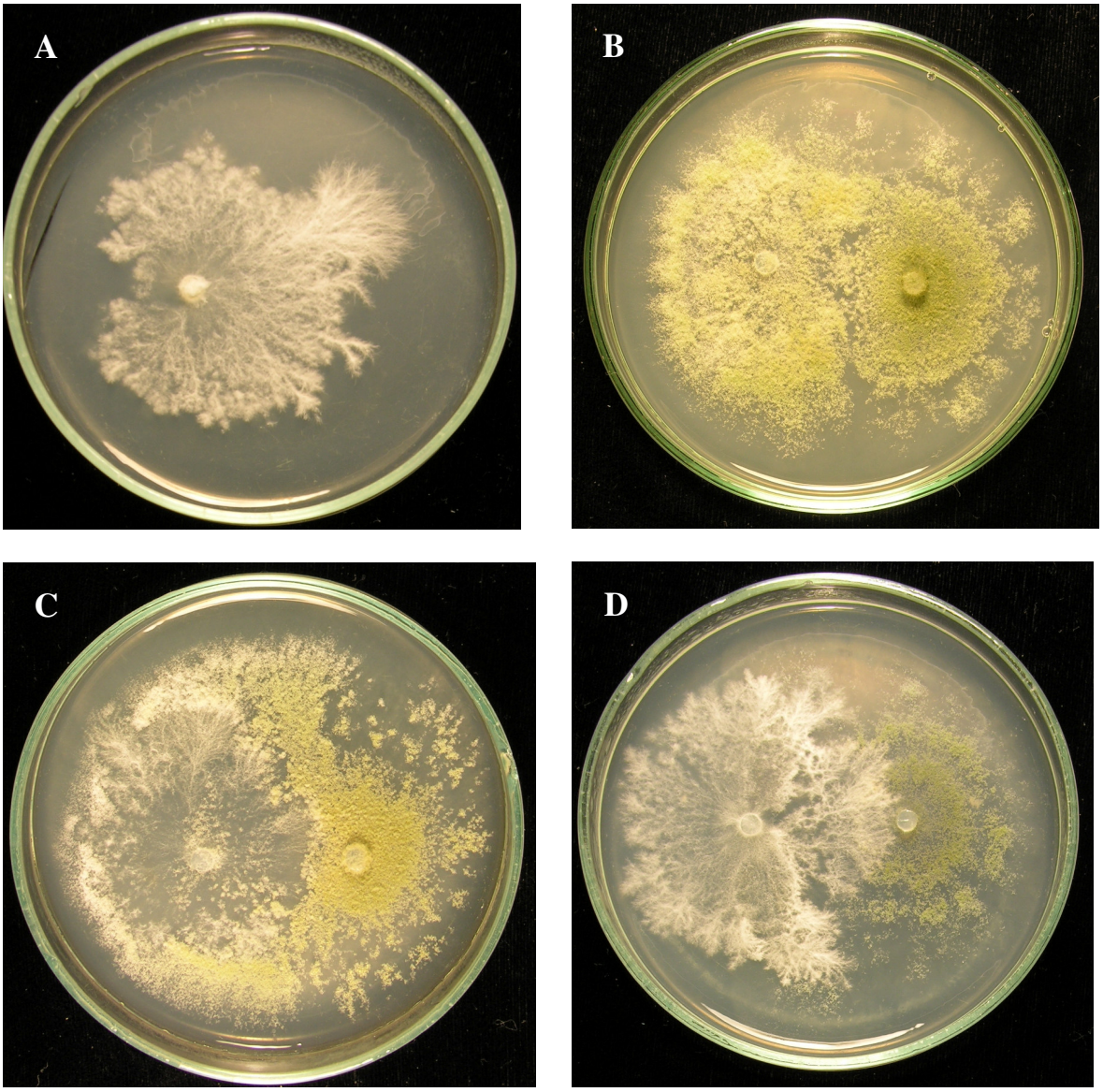
S13 (amylase ++, cellulase -, chitinase -, protease --), confirmed on YEGM

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

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

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

Figure 15. *In vitro* confrontation assays between *P. ostreatus* and the wild-type, as well as mutant derivatives of *T. pleurotum* C15 on YEXM. A: *P. ostreatus*, B: *P. ostreatus* + *T. pleurotum* wild-type, C: *P. ostreatus* + *T. pleurotum* R2 (amylase+, chitinase-, glucanase-, lipase-, protease-), D: *P. ostreatus* + *T. pleurotum* T14 (cellulase--, chitinase-)



According to these results we conclude that among the enzyme systems tested, deficiencies in the protease, lipase, chitinase and glucanase systems reduce the mycoparasitic potential of *T. pleurotum* towards *P. ostreatus* mostly.

7. DISCUSSION

We performed a survey of *Trichoderma* spp. present in the substrates for commercial production of *Agaricus bisporus* and *Pleurotus ostreatus* in Hungary. According to the currently accepted taxonomy of *Trichoderma,* the genus can be divided into three sections: *Longibrachiatum*, *Trichoderma* and *Pachybasium* B (Druzhinina and Kubicek 2005). Representatives of all three sections could be found among the *Trichoderma* strains isolated from Hungarian *Agaricus* compost and *Pleurotus* substrate samples: *T. longibrachiatum* and *T. ghanense* from section *Longibrachiatum*, *T. atroviride* (formerly known as *T. harzianum* Th3) from clade *Rufa* and *T. asperellum* from clade *Pachybasium* A of section *Trichoderma,* as well as *T. harzianum*, *T.* sp. DAOM 175924 and *T. aggressivum* f. *europaeum* (previously known as *T. harzianum* Th2) from clade *Lixii/catoptron* of section *Pachybasium* B. ITS sequence analysis by *TrichO*key 1.0 proved to be appropriate for the accurate identification of strains belonging to 6 out of the 7 *Trichoderma* species detected. Although an ITS-based identification was not possible for 4 isolates belonging to the duplet of species *T. longibrachiatum/Hypocrea orientalis*, sequence analysis of *tef1* carried out by TrichoBlast enabled the accurate identification of these strains as *T. longibrachiatum.* The data obtained for *A. bisporus* are in agreement with those of studies in Western Europe and the United States, i.e., that *T. aggressivum* is the major contaminant of *Agaricus* compost. The ITS 1 and ITS 2 sequences of most *T. aggressivum* f. *europaeum* isolates proved to be identical. In the case of both forms of *T. aggressivum,* low levels of variability or complete identity of ITS 1 sequences were reported for the sets of strains examined previously, while a five-base-pair difference exists between *T. aggressivum* f. *europaeum* and f. *aggressivum* (Muthumeenakshi *et al.* 1994, 1998; Samuels *et al.* 2002). Some further species such as *T. atroviride*, *T. asperellum*, *T. harzianum*, and *T. longibrachiatum* were also found in significant proportions in *Agaricus* compost (Castle *et al.* 1998). In contrast wit this situation, and in agreement with the findings of Park *et al.* (2004a), the substrate for oyster mushroom cultivation contained almost exclusively a new species of *Trichoderma*, the unnamed phylogenetic species *Trichoderma* sp. DAOM 175924, which was later found to comprise two distintct species of *Trichoderma*,

identical with *T. pleurotum* and *T. pleuroticola* (Park *et al.* 2006). This indicates differences in the biology of the *Trichoderma* pathogens of the two basidiomycetes. The *Pleurotus*-pathogenic species apparently seem to be a strong competitors of other *Trichoderma* spp. In fact, the *Pleurotus* green mould is known to be due to a heavy colonization and sporulation of *Trichoderma* spp. on the substrate, which ultimately inhibits growth of *P. ostreatus*. There are further significant differences between the *Trichoderma* pathogens of *A. bisporus* and *P. ostreatus. T. aggressivum* was so far isolated only from compost for production of *A. bisporus*, it was never observed in any field study. Seaby (1998) speculated that red pepper mites may act as the vector for *T. aggressivum*. Such a mechanism of distribution would be consistent with the slow but steady migration of this infection within Europe and also within the United States, and with the fact that f. *aggressivum* and f. *europaeum* still maintain their allopatric distribution, and renders the compost and the origin of its constituents a major source for the infection. Similarly, *T. pleurotum* has never been found in nature but in association with cultivated *P. ostreatus.* In contrast, isolates of *T. pleuroticola* have widely been found in soil or on decaying wood in the United States, Canada, New Zealand, Europe, India (I. S. Druzhinina and C. P. Kubicek, *unpublished data*), Iran (D. Zafari, *personal communication*), and China (Zhang *et al*. 2005). The widespread occurrence of this new species raises the questions as to why infections by it have just only recently been observed and what makes this species specific for antagonizing just *P. ostreatus.* Interestingly, one of the *Trichoderma* sp. DAOM 175924 isolates (originally identified as "*T. harzianum*" HEND) is used in New Zealand as a biocontrol agent against *Armillaria novae-zealandiae* and *Armillaria limonea* on kiwifruit and pine, respectively (Dodd *et al.* 2000). *Trichoderma* sp. DAOM 175924 was originally misidentified as *T. aureoviride* (Lieckfeldt *et al.* 2001), but molecular analyses proved it to be a new phylogenetic entity that forms a sister group to *T. harzianum* and *T. aggressivum* (Kullnig-Gradinger *et al.* 2002). Hatvani *et al.* (2007) reported that the Hungarian oyster mushroom green mould species has the same internal transcribed spacer (ITS) 1 and 2 sequences as the *Trichoderma* sp. strain DAOM 175924 (Kullnig-Gradinger *et al.* 2002) and that its ITS1 and ITS2 sequences were also identical with those deposited for four *Trichoderma* pathogens of *P. ostreatus* from South Korea, which were described as *Trichoderma*

pleurotum S. H. Yu & M. S. Park, sp. nov. and *Trichoderma pleuroticola* S. H. Yu & M. S. Park sp. nov. (Park *et al.* 2006). But unfortunately the ex-type cultures of these species were not deposited in publicly accessible culture collections, and concerning molecular characterization the authors only referred to articles hardly accessible (Park *et al.* 2004c; Park *et al.* 2005). Coincidentally, Komoń-Zelazowska *et al.* (2007) used an integrated approach for the comprehensive characterization of several *T. pleurotum* strains from Hungary, Italy and Romania, as well as *T. pleuroticola* isolates from Canada, the USA, Italy, Hungary, Romania, Iran, the Netherlands, Germany and New Zealand. Similarly to *T. aggressivum* - the causal agent of *Agaricus* green mould disease - both species belong to the Harzianum Clade of *Hypocrea/Trichoderma*. Morphological studies have revealed that *T. pleuroticola* shows pachybasium-like properties, which is characteristic in the Harzianum Clade, while *T. pleurotum* possesses gliocladium-like conidiophore morphology. Biolog Phenotype Microarrays were used to determine the carbon source utilization profile of the isolates. The results have shown unequivocal differences between the two species, namely the growth of *T. pleurotum* was slower or impaired on the majority of the carbon sources tested as compared to *T. pleuroticola*, which showed similar growth to that of *T. aggressivum*, indicating a closer relationship. The results suggest that the evolution of *T. pleurotum* was accompanied with the loss of the utilization ability of certain carbon sources. The phylogenetic analysis of a fragment including 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 *tef1*; and a fragment including a portion of the fifth exon of the endochytinase *chi18-5* (previously named *ech42*) gene encoding a family 18 chitinase confirmed the responsibility of the two distinct species for causing the green mould disease of oyster mushroom world-wide. A DNA BarCode for identification of these species based on ITS1 and 2 sequences was also provided and integrated in the main database for *Hypocrea/Trichoderma* (www.ISTH.info). According to the results of *in vitro* antagonistic tests both species are capable of antagonizing *A. bisporus* in a degree similar to that of *T. aggressivum*, but *T. pleurotum* was found to be less inhibitory on *P. ostreatus* than *T. pleuroticola.* Furthermore, since *T. pleuroticola* can be found in environmental samples, such as soil and wood world-wide, while *T. pleurotum* has so far been isolated only from association

with cultivated *P. ostreatus*, *T. pleuroticola* is suggested to mean the most serious threat to oyster mushroom cultivation. In this study *T. pleurotum* was shown to be dominant in samples from Hungarian *Pleurotus* farms, while most of the isolates from Italy were found to belong to the species *T. pleuroticola* (Komoń-Zelazowska *et al.* 2007). The findings of this study provided a detailed characterization of the two newly described *Pleurotus*-pathogenic *Trichoderma* species and confirmed their identity from several different aspects.

The analysis of mtDNA, due to its relatively small size and higher mutation rate in comparison with chromosomal DNA, is a powerful tool for differentiation of fungal isolates below the species level. mtDNA RFLP was used earlier to distinguish *Trichoderma* strains isolated from mushroom compost (Muthumeenakshi *et al.* 1994, 1998). Here we applied this method to compare the mtDNA RFLP patterns of Hungarian *T. aggressivum* isolates with those of strains from the British Isles and North America, in order to assess whether the Hungarian isolates of *T. aggressivum* f. *europaeum* belong to the same population as the first isolates from the British Islands, which started the epidemic. The results documented that there are no essential differences between the Hungarian isolates and the strains from Northern Ireland (CBS 433.95) and England (CBS 100525 and CBS 100526). In a previous study, only minor differences in mtDNA distinguishing Irish isolates from those in Great Britain could be found with the restriction enzyme *Hin*dIII (Muthumeenakshi *et al.* 1994). RFLP patterns of *T. aggressivum* f. *europaeum* strains proved to be clearly different from *T. aggressivum* f. *aggressivum* isolates. Similar results were obtained with other restriction enzymes by Muthumeenakshi *et al*. (1998). Our results indicate that *T. aggressivum* f. *europaeum* has an essential clonal structure, and that the Hungarian strains likely have been derived from the Western European epidemic lineage. This report therefore indicates that the green mould epidemic of *A. bisporus* also has spread eastward to reach Central Europe. In contrast to *T. aggressivum*, mtDNA RFLP of the isolates of *Trichoderma* sp. DAOM 175924 revealed at least four different RFLP patterns, three of which were even present in strains isolated from the same *Pleurotus* substrate (sample A), indicating that this species has a much higher genetic variability than *T. aggressivum*. It would be interesting to study whether there is a correlation between mtDNA RFLPs and the vigor of infection

of *Pleurotus* substrates. Interestingly, plasmid molecules could be detected both in undigested and digested DNA samples of certain Hungarian *T. harzianum*, *T.* sp. DAOM 175924 and North American *T. aggressivum* f. *aggressivum* isolates. The presence in the same sample of both plasmidless and plasmid-harbouring strains belonging to the DAOM2a mtDNA haplotype suggests the possibility of horizontal transmission. The occurrence of mitochondrial plasmids in *Trichoderma* was described first by Meyer (1993). Since then, plasmid molecules have been reported from North American mushroom compost isolates belonging to biotypes Th1 (*T. harzianum*) and Th4 (*T. aggressivum* f. *aggressivum*) (Castle *et al.* 1998) as well as from the biocontrol isolate *T. harzianum* T95 (Antal *et al.* 2002). In *Fusarium oxysporum* f. *conglutinans*, mitochondrial plasmids have been identified as one of the factors determining the host specificity (Kistler and Leong 1986). Further studies are necessary to determine whether the plasmids of *Trichoderma* sp. DAOM 175924 have any effect on its virulence to *P. ostreatus*.

Our results have confirmed that the causal agent for the oyster mushroom green mould disease, which recently started to spread in Europe and Asia, is actually two different although genetically closely related species of *Trichoderma* which correspond to the recently described taxa *T. pleurotum* and *T. pleuroticola* (Park *et al.* 2006). Both species are also closely related to the *H. lixii*/*T. harzianum* species aggregate and to *T. aggressivum*. These findings are in accordance with those reported by Park *et al.* (2004a), who used parsimony analysis of ITS1 and ITS2, *rpb2*, and *tef1* sequences to separate the *Pleurotus* pathogenic strains into two clusters which they called "*Trichoderma* sp. strain K1" and "strain K2." Unfortunately, sequences from their study, except for ITS1 and ITS2 from six isolates, have not been deposited in public databases and thus could not be included in our analysis. The available ITS1 and ITS2 sequences indicate that their six strains of *Trichoderma* sp. strain K1 isolated from *Pleurotus* substrata (rice straw, cotton waste, and sawdust) from four locations in South Korea correspond to *T. pleuroticola* (reference GenBank accession numbers DQ164409 and DQ164410 for strains CNU601 and CNU646, respectively) subsequently described by the same authors (Park *et al.* 2006). Similarly, strain K2 represents *T. pleurotum*, 14 strains of which were isolated from the same substrata in six locations (reference GenBank accession numbers

DQ164405, DQ164406, DQ164407, and DQ164408 for strains CNU501, CNU523, CNU538 and CNU571, respectively). According to Park *et al.* (2004a, 2006) both species coexist in South Korean *Pleurotus* farms with no clear dominance of one or the other species. There are several indications that the infection is introduced to farms via the substratum for mushroom cultivation, and differences in species distribution may be due to the use of certain substrata which, depending on the manufacturer, may consist of cereal straw, sawdust, bagasse, or waste cotton. *T. pleuroticola* dominates in samples from Italian *Pleurotus* farms, while *T. pleurotum* is abundant among Hungarian isolates (Komoń-Zelazowska *et al.* 2007). Although wheat straw is used as a major component for the *Pleurotus* substratum in both countries, the difference in species composition may be due to the addition of pulverized "tufo" in Italian farms, which is a natural calcareous rock of volcanic origin that raises the substratum pH to around 8. To the best of our knowledge, there is no such technological stage in Hungarian farms, where wheat straw is moisturized in the open air before use as *Pleurotus* substratum. The hypothesis of a possible reduction of *T. pleurotum* infection by the alkalization of the substratum may be further supported by the fact that *Pleurotus* green mold is not reported to be a severe problem in the United States, where the addition of lime to increase pH to 7.5 is widely practiced (http: //mushroomspawn.cas.psu.edu/). However, this treatment seems to be ineffective against *T. pleuroticola*. There may be another explanation for the occurrence of the two *Pleurotus*-associated green mould species in mushroom farms. *T. pleuroticola* is also frequently isolated from soil and plant debris, and environmental strains are known from Canada, the United States, Europe, and New Zealand. It seems to have a global occurrence, although possibly favoring a temperate climate. *T. pleuroticola* infections may therefore have multiple origins and even be due to introductions from the surrounding environment (Komoń-Zelazowska *et al.* 2007). In contrast, *T. pleurotum*, just like *T. aggressivum*, has so far never been isolated from areas outside of mushroom farms. Seaby (1989) reported evidence that *T. aggressivum* could be carried by red pepper mites into *Agaricus* mushroom farms. Another possibility would be that *T. pleurotum* could be an endophyte of plants used for preparation of the mushroom substratum (possibly wheat, rice, and cotton). The consistent co-occurrence of these two species in mushroom farms in Romania, Italy, Hungary, and South Korea is interesting.

Yet recent metagenomic studies on the occurrence of *Trichoderma* in Austrian soils frequently reveals the presence of *T. pleuroticola* but never that of *T. pleurotum* (M. A. Friedl and I. S. Druzhinina, unpublished data), suggesting that these two species occupy different ecological and trophic niches in nature. The large phenetic divergence of *T. pleuroticola* and *T. pleurotum*, morphologically and metabolically, in spite of the very close phylogenetic relationship, is a unique finding, as fungi are believed to develop phenotypic differences only after accumulation of some genetic distance, which gives rise to "cryptic" species which can hardly be phenotypically distinguished. The fact that *T. pleurotum* occupies a more terminal position than *T. pleuroticola* in all gene trees and that the latter exhibits similar morphological and metabolic characteristics as its phylogenetically close members in the Harzianum clade of *Hypocrea/Trichoderma* (*T. harzianum* and *T. aggressivum*) suggest that this change in morphology is due to a loss rather than a gain of gene function. Kullnig-Gradinger *et al.* (2002), comparing the morphotypes and phylogeny of *Trichoderma* species have speculated that the switch from fungicolous to saprophytic habitats was accompanied by the expression of the pachybasium-like conidiophore morphology. In this sense, the return to gliocladium-like morphology may be advantageous under the conditions of the natural niche of *T. pleurotum*. The gliocladium-like morphology is rare in the Harzianum clade although it is known for the anamorph of *Hypocrea tawa*.

Our study also places some caveats on the use of some *Trichoderma* isolates as biocontrol agents. One of the *T. pleuroticola* isolates we studied had been obtained from other researchers as biofungicides against soil-borne diseases (Table 1). In view of the present identification of *T. pleuroticola* as a causative agent of oyster mushroom green mould, this application could be problematic. However, the fact that infections by *T. pleuroticola* and *T. pleurotum* - although probably common for decades (see reference Sinden and Hauser1953) - only recently increased dramatically suggests there must be a special trigger for the infections, which may involve the source of the substrate used for cultivation, its preparation, or other conditions of mushroom cultivation. This, in turn, implies that if this trigger can be determined, the risk of infection can be managed.

Because of the emerging cases of fungal pathogenesis on mushrom plants, there is an increasinging need of rapid methods for their detection in order to be able to

efficiently control the diseases caused by them. Zijlstra *et al.* (2008) used a TaqMan PCR test for timely detection of *Verticillium fungicola* var. *aleophilum* and var. *fungicola*, the causal agents of dry bubble disease of *A. bisporus* in North America and Europe, respectively. Chen *et al.* (1999a) described a polymerase chain reaction-based test for the identification of the so-called *T. harzianum* biotypes 2 and 4 – which were later recognized not to be *T. harzianum* and described as *T. aggressivum* f. *europaeum* and *aggressivum*, respectively (Samuels *et al.* 2002) – responsible for the world-wide green mould epidemic in cultivated *A. bisporus*. This PCR-based test has been applied for the comparison of *Trichoderma* strains sampled in the United States during and prior to the outbreak of the green mould epidemic (Chen *et al.* 1999b) which revealed no evidence for the preepidemic existence of Th4, suggesting the recent emergence of a highly virulent genotype. The method was also successfully adapted in our study for the identification of the causal agent of green mould disease of cultivated *A. bisporus* in Hungary as *T. aggressivum* f. *europaeum*.

In order to facilitate identification of the two *Pleurotus*-pathogenic *Trichoderma* spp. (*T. pleuroticola* and *T. pleurotum*), we have developed three oligonucleotide primers which identify them in a multiplex PCR assay. Based on intron sequences of the *tef1* gene, we designed the primers FPforw1, FPrew1 and Psrev1 which were expected to amplify a 447 bp fragment from both species, and a 218 bp PCR product in the case of *T. pleurotum* only. The primers were found to amplify the expected DNA fragments in the case of all *T. pleurotum* and *T. pleuroticola* isolates tested, while no PCR products were formed with the DNA samples of 28 other *Trichoderma* species and 12 species from other fungal genera. Thus our results demonstrate that *T. pleurotum* and *T. pleuroticola* can be distinguished unequivocally from each other, as well as from other fungal species by the application of this three-primer set. The reaction was tested with DNA extracted directly from wheat straw, as well as healthy and *Trichoderma*-affected substrata of cultivated oyster mushroom. No amplicons were formed in the case of straw and healthy substrata, while with DNA from the infected ones the two bands characteristic to *T. pleurotum* (or *T. pleurotum* and *T. pleuroticola*) were detected, suggesting the presence of this species in the sample. We must note that the two bands could be due to the presence of either *T. pleurotum* only, or due to the presence of *T. pleuroticola* and *T.*

pleurotum together, and we therefore cannot decide if the substratum contains only *T. pleurotum* or both species. Therefore, in order to support this finding we isolated *Trichoderma* strains from the infected samples, and besides the multiplex PCR reaction, their identity as *T. pleurotum* was confirmed also by subsequent ITS1 and 2 sequence analysis. These data clearly show that the primers designed in this study can detect *Pleurotus*-pathogenic *Trichoderma* spp. both among laboratory conditions and in mushroom farms, even without preceding cultivation. Our finding may help to recognize the green mould disease of *Pleurotus ostreatus* caused by *T. pleurotum* and *T. pleuroticola* in its early phase, and therefore allow the application of appropriate disease control strategies.

The presence and harm of the newly described species of *Trichoderma*, *T. pleurotum* and *T. pleuroticola* (Park *et al.* 2006) in the cultivation of *P. ostreatus* is well-known world-wide (Yu 2002, Park *et al.* 2004a,b,c, Woo *et al.* 2004, Park *et al.* 2005, Kredics *et al.* 2006, Hatvani *et al.* 2007, Komoń-Zelazowska *et al.* 2007, Hatvani *et al.* 2008), however, to the best of our knowledge, their association with wild-grown oyster mushroom has not yet been reported. Therefore we tested the occurence of these species on wood colonized by *P. ostreatus*, as well as on the surface of its fruit bodies. The above discussed specific detection method revealed that *T. pleuroticola* can be present in both types of habitats.

The accumulation of *T. pleuroticola* in the substratum and on the fruit body of wild-grown oyster mushroom suggests that these might be potential sources of infections observed in mushroom farms. In contrast, *T. pleurotum* could not be detected in any of the samples. Similarly to *T. aggressivum*, *T. pleurotum* has never been found in environmental sources. Recent metagenomic studies on the occurrence of *Trichoderma* in Austrian soils frequently reveal the presence of *T. pleuroticola* but never that of *T. pleurotum* (Friedl, M. A. and Druzhinina, I. S., unpublished), suggesting that these two species may occupy different ecological and trophic niches in nature (Komoń-Zelazowska *et al.* 2007).

We also studied the biodiversity of *Trichoderma* species in the vicinity of wild-grown *P. ostreatus*. Besides *T. pleuroticola*, the other species occurring in these habitats were *T. harzianum*, *T. longibrachiatum* and *T. atroviride*. It is interesting to compare

these results with the biodiversity of *Trichoderma* observed in the cultivation of *P. ostreatus*. Hatvani *et al.* (2007) examined *Trichoderma* strains isolated from the growing substratum of cultivated oyster mushroom in Hungary. Among 31 isolates, *T. pleurotum* proved to be most frequent (27 isolates). In addition, single isolates of *T. pleuroticola*, *T. atroviride*, *T. asperellum* and *T. longibrachiatum* were also found, however, in contrast with the samples of wild-grown *P. ostreatus*, no *T. harzianum* was detected. To the best of our knowledge, this is the first study dedicated to the examination of the biodiversity of *Trichoderma* in association with wild-grown oyster mushroom, applying the first PCR-based method developed for the rapid and specific detection of the two recently described *Pleurotus* pathogenic *Trichoderma* species, *T. pleurotum* and *T. pleuroticola*.

A strain of *T. pleurotum* – isolated from a green mould-affected *Pleurotus* farm in Hungary - was subjected to UV-mutagenesis. 163 surviving colonies were isolated and tested for their ability of producing pectinases, cellulases, amylases, proteases, lipases, chitinases and glucanases in plate assays. Mutants with altered ability of producing certain enzymes were tested for their antagonistic potential towards *P. ostreatus* in dual plate assays on three different types of media: water-agar (WAM), yeast extract-glucose (YEGM) and yeast extract-xylose (YEXM) medium in comparison with the parental strain. The most spectacular and unequivocal differences between the antagonistic ability of the wild-type and some mutant strains appeared on YEXM. The parental strain could overgrow and conidiate on the colony of *P. ostreatus* on all kinds of media tested. 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. Extracellular enzymes have been proven to act as important components of mycoparasitic activity of several biocontrol *Trichoderma* strains. The complex mycoparasitic process was extensively reviewed by Herrera-Estrella and Chet (1998), and Viterbo *et al.* (2002) presented an overview of the data available on lytic enzymes from the mycoparasitic fungus *Trichoderma*, while the data available about the biocontrol mechanisms of the genus were summarized by Benítez *et al.* (2004). Among chitinases, the expression of N-acetylglucosaminidase 1 (*nag1*) was found to be induced in *T. atroviride* by pathogen cell walls or chitin

degradation products but was not triggered prior to contact by the fungus with *Rhizoctonia solani* (Mach *et al.* 1999). The NAG1 protein was purified from *T. atroviride* P1 and synergistic activity with a ß-glucosidase in the inhibition of *Botrytis cinerea* spore germination has been reported (Lorito *et al.* 1994). Two GlcNAcases, CHIT73 and CHIT102, were detected in the growth medium of *Trichoderma* isolates T-Y and TM (Haran *et al.* 1995, 1996). CHIT102 was the first chitinase to be produced upon contact with *Sclerotium rolfsii*. Therefore, it has been speculated that the 102 kDa chitinase plays an important role in inducing the expression of other chitinolytic enzymes (Haran *et al.* 1996). The 42 kDa endochitinase is also supposed to play a key role in the mycoparasitic interaction. Contradictory data have been reported on the biocontrol activity of knockout or overexpressor mutants, however, it has been shown that manipulation of this gene can have positive as well as negative effects (Carsolio *et al.* 1999; Woo *et al.* 1999). The *ech42* gene was found to be up-regulated during the pre-contact stage of the confrontation between *T. atroviride* and *R. solani* (Kulling *et al.* 2000). *Trichoderma* strains overexpressing another endochitinase, CHIT33, were found to possess increased antifungal activity towards *R. solani* (Limon *et al.* 1999). Confrontation experiments with *R. solani* as the host have revealed that *chit33* is expressed only during, and not earlier than the stage of overgrowth on the pathogen. These data show that *chit33* is indeed involved in mycoparasitism (Dana *et al.* 2001). Another endochitinase, named CHIT36 was isolated from *T. harzianum* isolate TM (Viterbo *et al.* 2001). Data demonstrate a strong inhibitory effect of this enzyme on *B. cinerea* spore germination and *in vitro* growth of *S. rolfsii* and *Fusarium oxysporum* f. sp. *melonis*. Another 37 kDa endochitinase has been isolated from *T. harzianum* 109 with lytic activity on cell walls of *Crinipellis perniciosa*, the causal agent of witches' broom disease of cocoa (De Marco *et al.* 2000). Glucanases are another group of key enzymes involved in the mycoparasitism of *Trichoderma* spp. ß-1,3-glucan (laminarin) and 1,4- ß-D-glucan (cellulose) are the major components of fungal cell walls along with chitin. Laminarin is hydrolyzed mainly by ß-1,3-glucanases, which are further classified into endo- and exo-ß-glucanases, releasing oligosaccharides and monosaccharides, respectively. *Trichoderma* species also produce ß-1,6-glucanases under specific conditions. These enzymes hydrolyze ß-1,6-glucans, the minor structural polymers of the fungal cell wall (Viterbo *et al.* 2002). Direct

evidence for the involvement of glucanases in mycoparasitism has been demonstrated by Lorito *et al.* (1994); using an *in vitro* bioassay in which a purified 78 kDA endo-ß-1,3-glucanase inhibited *B. cinerea* spore germination in synergistic cooperation with *N-acetylglucosaminidases.* An endo ß-1,3-glucanase of similar molecular weight has also been isolated from strain T-24 (El-Katatny *et al.* 2001). This enzyme has been shown to inhibit the growth of *S. rolfsii* in synergism with a T-24 43 kDa endochitinase. Glucanases appear to be involved in the antagonism of plant-pathogenic oomycetes, such as *Pythium* by *Trichoderma. Pythium* is exceptional in that its cell walls contain ß-(1,3)-(1,6)-glucans and cellulose instead of chitin as their major structural components (Viterbo *et al.* 2002). Transformants overexpressing BGN13.1 have been reported to inhibit the growth of *B. cinerea*, *R. solani* and *Phytophthora citrophthora*. Transformant T28, which had the highest BGN13.1 glucanase activity under both repressing and inducing conditions, showed the highest inhibition of pathogens. Antagonism was higher against *P. citrophthora* - oomycete with cellulose and glucans as the main cell wall components (Benítez *et al.* 1998) - than against *Botrytis* or *Rhizoctonia* (A. M. Rincón, PhD thesis). Transformants of *T. longibrachiatum* with extra copies of the ß-1,4 endoglucanase (*egl1*) gene were found to exhibit higher biocontrol activity than the parental strain against *P. ultimum* on cucumber seedlings (Migheli *et al.* 1998). *T. harzianum* isolate T3 also appeared to be very effective in the control of this pathogenic fungus (Thrane *et al.* 2000), suggesting the involvement of this enzyme in the mycoparasitic process of *Trichoderma*. BGN16.2 – a ß-1,6- glucanase – was shown to exhibit antifungal properties alone or in combination with chitinases (Benítez *et al.* 1998) and reduced the growth of *B. cinerea* and *Gibberella fujikuroi* (De la Cruz and Llobell 1999). Transformants producing BGN16.2 controlled the growth of *R. solani* and *B. cinerea* (Benítez *et al.* 1998). Djonovic *et al.* (2006) studied the role of Tv*bgn3*, another ß-1,6- glucanase in *T. virens* through the comparison of wild-type and transformant strains, in which Tv*bgn3* was disrupted (GKO) or constitutively overexpressed (GOE). Gene expression analysis revealed induction of Tv*bgn3* in the presence of host fungal cell walls, indicating regulation during mycoparasitism. While deletion or overexpression of Tv*bgn3* had no evident effect on growth and development, GOE and GKO strains showed an enhanced or reduced ability, respectively, to inhibit the growth of the plant pathogen *Pythium*

ultimum compared to results with those of the wild-type. The relevance of this activity in the biocontrol ability of *T. virens* was confirmed in plant bioassays. Deletion of the gene resulted in levels of disease protection that were significantly reduced as compared to levels observed in the case of the wild-type, while GOE strains showed a significantly increased biocontrol capability. These results demonstrate the involvement of ß-1,6-glucanase in mycoparasitism and its relevance in the biocontrol activity of *T. virens*. Other hydrolases, such as α-1,3-glucanases, have been purified from *T. harzianum* strain 2413, and their genes have been isolated and overexpressed, which resulted in increased biocontrol activity of the transformant strains (Ait-Lahsen *et al.* 2001). The review of extracellular proteases of *Trichoderma* species was prepared by Kredics *et al.* (2005). Mischke (1996) reported that the specific activity of proteases produced by *Trichoderma* strains do not correlate with their known biocontrol ability, however, other studies do not support this statement (Kredics *et al.* 2005). Transformation systems were developed for increasing the copy number of the *T. atroviride prb1* gene (Flores *et al.* 1997, Goldman and Goldman 1998). Transformants exhibited increased control of *R. solani*, suggesting that *prb1* is a mycoparasitism-related gene (Flores *et al.* 1997),. Overexpression of *tvsp1* in *T. virens* also resulted in an increased biocontrol activity against *R. solani.* Results of these studies suggest that the overexpression of protease encoding genes is a powerful tool for strain improvement. The method of UV-mutagenesis with the selection for *p*-fluorophenyl-alanine resistant or colony morphology mutants was used by Szekeres *et al.* (2004) for the isolation of protease overproducing strains from *T. harzianum*. Certain mutants appeared to be better producers of extracellular trypsin- and chymotrypsin-like proteases with manifold levels of the activities of the wild type strain. The increase in the proteolytic activities of these mutants was low when compared to transformants overexpressing the proteinase gene *prb1* (Flores *et al.* 1997), but they proved to be much better antagonists of plant pathogens than the parental strain. Benhamou and Chet (1993) suggested the involvement of lipases in the of mycoparasitic activity of *Trichoderma* spp. Calistru *et al.* (1997) detected lipolitic activity in *T. viride* and *T. harzianum*, and their culture filtrates were found to have inhibitory effect on *Fusarium moniliforme* and *Aspergillus flavus*. The aggressive behaviour demonstrated by *Trichoderma* spp. may be partly explained by the liberation of extracellular enzymes by these fungi. Van Tilburg

and Thomas (1993) observed high activity of lipases in *Gliocladium virens* when the fungus was grown on medium containing washed cell walls of *R. solani*. Sivan and Chet (1989) have speculated that a co-ordinated action of polysaccharidases, lipases and proteases is important in antibiosis exhibited by *Trichoderma* spp..

The lysis of the mycelium of shiitake *(Lentinus edodes)* caused by mycolytic enzymes of *T. harzianum* was examined by Tokimoto (1982). The two fungi were grown in an antagonistic state, and crude enzyme prepared from culture filtrates of *T. harzianum* or from *L. edodes* invaded by *T. harzianum* was found to be able to dissolve *L. edodes* mycelium, and had high activities of ß-1,3-glucanase and chitinase. The activities of these mycolytic enzymes appeared to correlate positively with the degree of *L. edodes* damage caused by *T. harzianum.* Kitamoto *et al.* (1984) studied the production of lytic enzymes against cell walls of basidiomycetes by *T. harzianum.* A strain of *T. harzianum*, which had been isolated from shiitake logs was induced to produce ß-1,3-glucanase and chitinase and showed a remarkable cell wall lytic activity when the fungus was cultured in a medium containing the cell walls or the powder of mycelia and fruiting bodies of basidiomycetes as carbon source. The above findings - correlating with our observations - suggest that these enzymes might play role in the mycoparasitic activity of *Trichoderma.* Williams *et al.* (2003) investigated the mycoparasitic and saprotrophic behavior of isolates representing groups of *Trichoderma* to establish a mechanism for the aggressiveness towards *A. bisporus* in infested commercial compost. Mycoparasitic structures were infrequently observed in interaction zones on various media, including compost, with cryoscanning electron microscopy. *T. harzianum* grew prolifically in compost in the absence or presence of *A. bisporus*, *T. aggressivum* f. *europaeum* and f. *aggressivum* isolates produced significantly higher biomasses (6.8- and 7.5-fold, respectively) in compost than did non-aggressive - group Th1 - isolates. All groups secreted depolymerases that could attack the cell walls of *A. bisporus* and of wheat straw, and some were linked to aggressiveness. Growth on mushroom cell walls *in vitro* resulted in rapid production of chymoelastase and trypsin-like proteases by only *T. aggressivum* f. *europaeum* and *aggressivum* isolates. These strains also produced a dominant protease isoform (pI 6.22) and additional chitinase isoforms. On wheat straw, *T. aggressivum* f. *aggressivum* produced distinct isoforms of cellulase and laminarinase, but there was no

consistent association between levels or isoforms of depolymerases and aggressiveness. Th3's distinctive profiles confirmed its reclassification as *Trichoderma atroviride.* Proteases and glucanases were detected for the first time in sterilized compost colonized by *Trichoderma*. Xylanase dominated, and some isoforms were unique to compost, as were some laminarinases. The authors therefore hypothesize that aggressiveness results from competition, antagonism or parasitism but only as a component of, or following extensive saprotrophic growth involving degradation of wheat straw cell walls. Kredics *et al.* (2008) examined the production of extracellular proteases by several isolates belonging to *T. pleurotum* and *T. pleuroticola* under non-inductive conditions and upon induction by *Pleurotus* powder, wheat straw powder, or both. The capability of the strains to produce extracellular trypsin-like, chymotrypsin-like and chymoelastase-like protease activites was studied and compared with those of *T. aggressivum* f. *aggressivum*, *T. aggressivum* f. *europaeum* and *T. harzianum*. Significant differences could be observed between the two *Pleurotus* pathogenic species in the production of proteolytic activities. *T. pleuroticola* isolates produced 3-8 times higher amounts of chymotrypsin-like protease activities on *Pleurotus* powder than *T. pleurotum* isolates. In contrast, *T. pleurotum* isolates produced significantly higher amounts of both trypsin-, chymotrypsin- and chymoelastase-like protease activities on wheat straw powder than *T. pleuroticola* isolates. Thus the proteolytic system of *T. pleurotum* seems to be highly inducible by the wheat straw substrate used for *Pleurotus* cultivation, while the proteases of *T. pleuroticola* – especially chymotrypsin-like activities – seem to be induced by the presence of *P. ostreatus* and not by the substrate (Kredics *et al.* 2008). These results suggest that the two closely related *Pleurotus* pathogenic *Trichoderma* species may use different enzymatic strategies for the adaptation to the circumstances existing during *Pleurotus* cultivation.

Based on our results we conclude that among the enzyme systems tested, deficiencies in protease, lipase, chitinase and glucanase system reduce the mycoparasitic potential of *T. pleurotum* towards *P. ostreatus* mostly. Some mutant derivatives of *T. pleurotum* with lower ability of producing cellulases and pectinases were also found to possess decreased mycoparasitic potential towards oyster mushroom in our study. However, we suppose that these enzyme systems do not have direct importance in the

mycoparasitic activity of *T. pleurotum* towards *P. ostreatus* but they might be in regulatory connection with some other enzyme systems, which were not examined in these experiments.

8. FUTURE PLANS

In order to combat the green mould problem successfully, research efforts will be made to the further examination of the physiology of isolates belonging to *T. pleurotum*, *T. pleuroticola* and *T. aggressivum* f. *europaeum*, as well as to reveal the origin of contamination at mushroom farms, which could help the design of efficient control strategies and preventive measures. Our further purposes are:

- To examine green mould samples from other countries
- To study the occurrence and circulation of mushroom pathogenic *Trichoderma* species in air samples of cultivation facilities using aeromicrobiological techniques
- To evaluate the role of the insect fauna in the propagation of *Trichoderma* species responsible for green mould
- To investigate the biodiversity of *Trichoderma* in the natural environment of *Agaricus* spp., with special attention to finding *T. aggressivum*
- To identify the certain enzymes involved in the mycoparasitic activity of *T. pleurotum* towards *P. ostreatus* in the mutants by the use of specific (chromogenic and fluorogenic) substrates

9. ACKNOWLEDGEMENTS

Herein I would like to express my gratitude to:
My supervisors, Dr. László Manczinger and Dr. László Kredics,
The head of my home Department, Dr. Csaba Vágvölgyi,
Prof. Christian P. Kubicek,
Prof. Giovanni Vannacci,
Dr. Irina S. Druzhinina,
Dr. Levente Karaffa,
Dr. Sabrina Sarrocco,
Dr. Zsuzsanna Antal,
Dr. András Szekeres,
Monika Komoń-Zelazowska,
Mária Lele,
Melinda Deákné Kulcsár,
Sándor Kocsubé,
Maurizio Forti,
Valentina Catalano,
Nicola Leoni,
László Nagy,
Anikó Pósa
And all my colleagues at the Department of Microbiology, University of Szeged, who have helped my work in any way.

The work was supported by:

- the grant OTKA F68381 of the Hungarian Scientific Research Fund,
- the Hungarian Government with research grant NKFP OM-00083/2004,
- the grant of the Marie Curie Foundation (2005),
- the grant of the Hungarian Scholarship Board (2006),
- the János Bolyai Research Scholarship of the Hungarian Academy of Sciences (2006),
- the grant of Stiftung Aktion Österreich-Ungarn (2007),
- Austrian Science Foundation grants FWF P-12748-MOB, FWF P-16601 and P-17895-B06,
- NKTH (National Office for Research and Technology, Hungary) and ÖAD (Österreichischer Austauschdienst, Austria) under the bilateral project TéT A02/2006 - WTZ HU 03/2007.

10. REFERENCES

Ait-Lahsen H, Soler A, Rey M, de la Cruz J, Monte, E. and Llobell, A. 2001. An antifungal exo-α-1,3-glucanase (AGN13.1) from the biocontrol fungus *Trichoderma harzianum*. Appl. Environ. Microbiol. 67(12): 5833-5839.

Anonymous. 2005. Consensus document on the biology of *Pleurotus* spp. (oyster mushroom). Series on harmonisation of regulatory oversight in biotechnology, no. 34 Organisation for Economic Co-operation and Development, Environment Directorate, Paris, France.

Antal, Z., Kredics, L., Pakarinen, J., Dóczi, I., Andersson, M., Salkinoja-Salonen, M., Manczinger, L., Szekeres, A., Hatvani, L., Vágvölgyi, C. and Nagy, E. 2005. Comparative study of potential virulence factors in human pathogenic and saprophytic *Trichoderma longibrachiatum* strains. Acta Microbiol. Immunol. Hung. 52: 341-350.

Antal, Z., Manczinger, L., Kredics, L., Kevei, F. and Nagy, E. 2002. Complete DNA Sequence and Analysis of a Mitochondrial Plasmid in the Mycoparasitic *Trichoderma harzianum* Strain T95. Plasmid 47: 148–152.

Antal, Z., Varga, J., Kredics, L., Szekeres, A., Hatvani, L., Manczinger, L., Vágvölgyi, C., and Nagy, E. 2006. Intraspecific mitochondrial DNA polymorphism within the emerging filamentous fungal pathogen *Trichoderma longibrachiatum*. J. Med. Microbiol. 55: 31-35.

Ballero, M., Mascia, E., Rescigno, A. and Teulada, E. S. D. 1990. Use of *Pleurotus* for transformation of polyphenols in waste waters from olive presses into proteins. Micol. Ital. 19: 39–41.

Benhamou, N. and Chet, I. 1993. Hyphal interaction between *Trichoderma harzianum* and *Rhizoctonia solani*: ultrastructure and gold chemistry of the mycoparasitic process. Phytopathology 83: 1062–71.

Benítez T., Delgado-Jarana J., Rincón A. M., Rey M. and Limón M. C. 1998. Biofungicides: *Trichoderma* as a biocontrol agent against phytopathogenic fungi.

In: Pandalai SG (ed) Recent research developments in microbiology, vol. 2. Research Signpost, Trivandrum, 129-150.

Benítez T., Rincón A. M., Limón M. C. and Codón A. C. 2004. Biocontrol mechanisms of *Trichoderma* strains. Int. Microbiol. 7: 249–260.

Buntjer, J. B. 1997. Phylogenetic computer tools (PhylTools). Version 1.32 for Windows. Laboratory of Plant Breeding, Wageningen University, Wageningen, the Netherlands.

Carsolio, C., Benhamou, N., Haran, S., Cortes, C., Gutierrez, A., Chet, I. and Herrera-Estrella, A. 1999. Role of the *T. harzianum* endochitinase gene, ech42, in mycoparasitism. Appl. Environ. Microbiol. 65: 929–935.

Calistru, C., McLean, M. and Berjak, P. 1997. *In vitro* studies on the potential for biological control of *Aspergillus flavus* and *Fusarium moniliforme* by *Trichoderma* species. *A study of the production of extracellular metabolites by* Trichoderma *species.* Mycopathologia 137: 115–124.

Castle, A., Speranzini, D., Rghei, N., Alm, G., Rinker, D. and Bissett, J. 1998. Morphological and molecular identification of *Trichoderma* isolates on North American mushroom farms. Appl. Environ. Microbiol. 64: 133-137.

Chang, R. 1996. Functional properties of edible mushrooms. Nutr. Rev. 54: 91–93.

Chang, S. T. 1999. World production of cultivated and medicinal mushrooms in 1997 with emphasis on *Lentinus edodes* (Berk.) Sing. In China. Int. J. Med. Mushrooms. 1: 291-300.

Chen, A.W. and Moy, M. 2004. Mushroom cultivation: building mold contamination. At: <http://www.mushworld.com> Accessed on 28. October 2004.

Chen, X., Romaine, C. P., Ospina-Giraldo, M. D. and Royse, D. J. 1999a. A polymerase chain reaction-based test for the identification of *Trichoderma harzianum* biotypes 2 and 4, responsible for the worldwide green mold epidemic in cultivated *Agaricus bisporus*. Appl. Microbiol. Biotechnol. 52: 246-250.

Chen, X., Romaine, C. P., Tan, Q., Schlagnhaufer, B., Ospina-Giraldo, M. D., Royse, D. J. and Huff, D. R. 1999b. PCR-based genotyping of epidemic and preepidemic *Trichoderma* isolates associated with green mold of *Agaricus bisporus*. Appl. Environ. Microbiol. 65: 2674-2678.

Clement, M., Posada, D. and Crandall K. A. 2000. TCS: a computer program to estimate gene genealogies. Mol. Ecol. 9: 1657–1659.

Cote, F., Letarte, J., Grenier, J., Trudel, J. and Asselin, A. 1989. Detection of beta-1,3-glucanase activity after native polyacrylamide gel electrophoresis: application to tobacco pathogenesis-related proteins. Electrophoresis 10(7): 527-529.

Dana, M. M., Limón, M. C., Mejias, R., Mach, R. L., Benítez, T., Pintor-Toro, J. A. and Kubicek, C. P. 2001. Regulation of chitinase 33 (chit33) gene expression in *Trichoderma harzianum*. Curr. Genet. 38: 335–342.

De la Cruz, J. and Llobell, A. 1999. Purification and properties of a basic endo-β-1,6-glucanase (BGN16.1) from the antagonistic fungus *Trichoderma harzianum*. Eur J Biochem 265: 145-151.

De Marco, J. L., Lima, L. H. C., valle de Sousa, M. and Felix, C. R. 2000. A *Trichoderma harzianum* chitinase destroys the cell wall of the phytopathogen *Crinipellis perniciosa*, the casual agent of witches' broom disease of cocoa. World J. Microbiol. Biotechnol. 16: 383–386.

Dettman, J. R., Jacobson, D. J. and Taylor J. W. 2003. A multilocus genealogical approach to phylogenetic species recognition in the model eukaryote *Neurospora*. Evolution 57: 2703–2720.

Djonovic, S., Pozo, M. J. and Kenerley, C. M. 2006. Tvbgn3, a ß-1,6-Glucanase from the Biocontrol Fungus *Trichoderma virens*, Is Involved in Mycoparasitism and Control of *Pythium ultimum*. Appl. Environ. Microbiol. 72(12): 7661-7670.

Dodd, S. L., Crowhurst, R. N., Rodrigo, A. G., Samuels, G. J., Hills, R. A. and Stewart, A. 2000. Examination of *Trichoderma* phylogenies derived from ribosomal DNA sequence data. Mycol. Res. 104: 23-34.

Doyle, O. 1991. *Trichoderma* green mould – update. Irish Mushroom Rev. 3: 13-17.

Druzhinina, I. S., Kopchinskiy, A.G., Komon, M., Bissett, J., Szakács, G. and Kubicek, C. P. 2005. An oligonucleotide barcode for species identification in *Trichoderma* and *Hypocrea*. Fungal Genet. Biol. 42: 813-828.

Druzhinina, I. S., Kopchinskiy, A. G. and Kubicek, C. P. 2006a. The first one hundred of *Trichoderma* species is characterized by molecular data. Mycoscience 47: 55–64.

Druzhinina, I. S. and Kopchinskiy, A. G. 2006b. *TrichO*KEY v. 2 - a DNA oligonucleotide BarCode program for the identification of multiple sequences of *Hypocrea* and *Trichoderma*, p. 53–59. *In* W. Meyer and C. Pearce (ed.), Proceedings of the 8th International Mycological Congress, Cairns, Australia. Medimond-Monduzzi, Bologna, Italy.

Druzhinina, I. S. and Kubicek, C.P. 2005. Species concepts and biodiversity in *Trichoderma* and *Hypocrea*: from aggregate species to species clusters? J. Zhejiang Univ. Sci. 6B, 100-112.

El-Katatny, M. H., Gudelj, M., Robra, K. H., Elnaghy, M. A. and Gübitz, G. M. 2001. Characterization of a chitinase and an endo-ß-1,3-glucanase from*T. harzianum* Rifai T-24 involved in control of the phytopathogen *Sclerotium rolfsii*. Appl. Microbiol. Biot. 56: 137–143.

Felsenstein, J. 1995. PHYLIP (Phylogeny Inference Package). Version 3.57c. Department of Genetics, University of Washington, Seattle.

Flores, A., Chet, I. and Herrera-Estrella, A.1997. Improved biocontrol activity of *Trichoderma harzianum* by over-expression of the proteinase-encoding gene *prb1*. Curr. Genet. 31: 30–37.

Gams, W. and Meyer, W. 1998. What exactly is *Trichoderma harzianum*? Mycologia 90: 904-915.

Geels, F., van den Geijin, J. and Rutjens, A. 1988. Pests and diseases. In: (van Griensven L.J.L.D. Ed): The cultivation of mushrooms. Interlingua, East Grinstead, Sussex, England, 361-422.

Goldman, M. H. S., Goldman, G. H. 1998. *Trichoderma harzianum* transformant has high extracellular alkaline proteinase expression during specific mycoparasitic interactions. Gen. Mol. Biol. 21: 329–333.

Gunde-Cimerman, N. 1999. Medicinal value of the genus *Pleurotus* (Fr.) P. Karst. (*Agaricales* s.l. Basidiomycetes). Int. J. Med. Mushrooms 1: 69–80.

Hankin, L. and Anagnostakis, S. L. 1975. The use of solid media for detection of enzyme production by fungi. Mycologia 67: 597-607.

Haran, S., Schickler, H., Oppenheim, A. B and Chet, I. 1995. New components of the chitinolytic system of *Trichoderma harzianum*. Mycol. Res. 99: 441–446.

Haran, S., Schickler, H., Oppenheim, A. B and Chet, I. 1996. Differential expression of *T. harzianum* chitinases during mycoparasitism. Phytopathology 86: 980–985.

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.

Hatvani, L., Manczinger, L., Kocsubé, S., Antal, Z., Szekeres, A., Druzhinina, I. S., Kubicek, C. P., Nagy, A., Vágvölgyi, C. and Kredics, L. 2008. The green mould disease global threat towards the cultivation of oyster mushroom (*Pleurotus ostreatus*): a review. Pp. 485–495 in 'Science and cultivation of edible and medicinal fungi: Mushroom Science XVII', ed. by M. van Gruening. Proceedings of the 17th Congress of the International Society for Mushroom Science, Cape Town, South Africa, 20–24 May 2008. South African Mushroom Farmers Association, Pretoria, South Africa (CD-ROM).

Hermosa, M. R., Grondona, I., and Monte, E. 1999. Isolation of *Trichoderma harzianum* Th2 from commercial mushroom compost in Spain. Plant Disease 83: 591.

Herrera-Estrella, A. and Chet, I. 1998. Biocontrol of bacteria and phytopathogenic fungi. In: Altman A (Ed). Agricultural Biotechnology 263–282. Marcel Dekker Inc. New York.

Herrera-Estrella, A. and Chet, I. 2003. The biological control agent *Trichoderma* from fundamentals to applications. In: Arora D. K., Bridge P. D. and Bhatnagar D. (eds), Fungal Biotechnology in Agricultural, Food, and Environmental Applications, Marcel Dekker, New York, 147-156.

Howell, C. R. 2003. Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: the history and evolution of current concepts. Plant Dis. 87: 4–10.

Hsu, S. C., and Lockwood, J. L. 1975. Powdered Chitin Agar as a Selective Medium for Enumeration of Actinomycetes in Water and Soil, Appl. Microbiol. 422-426.

Jaklitsch, W. M., Komon, M., Kubicek, C. P. and Druzhinina, I. S. 2006. *Hypocrea crystalligena* sp. nov., a common European species with a whitespored *Trichoderma* anamorph. Mycologia 98: 500–514.

Jaklitsch W. M., Komon, M., Kubicek, C. P. and Druzhinina, I. S. 2005. *Hypocrea voglmayrii* sp. nov. from the Austrian Alps represents a new phylogenetic clade in *Hypocrea/Trichoderma*. Mycologia 97: 1391–1404.

Kistler, H. C. and Leong, S. A. 1986. Linear plasmidlike DNA in the plant pathogenic fungus *Fusarium oxysporum* f. *conglutinans.* J. Bacteriol. 167: 587-593.

Kitamoto Y., Kono R., Tokimoto K., Mori N. and Ichikawa Y. 1984. Production of lytic enzymes against cell walls of basidiomycetes from *Trichoderma harzianum.* Trans. Mycol. Soc.]apan 26: 69-79.

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.

Kopchinskiy, A. G., Komon, M., Kubicek, C. P. and Druzhinina, I. S. 2005. *Tricho*BLAST: a multilocus database for *Trichoderma* and *Hypocrea* identifications. Mycol. Res. 109: 657–660.

Kredics, L., Antal, Z., Dóczi, I., Manczinger, L., Kevei, F. and Nagy, E. 2003. Clinical importance of the genus *Trichoderma*. Acta Microbiol. Immunol. Hung. 50: 105-117.

Kredics L., Antal, Z., Szekeres, A., Hatvani, L., Manczinger, L., Vágvölgyi, C. and Nagy, E. 2005. Extracellular proteases of *Trichoderma* species. A review. Acta Microbiol Immunol Hung. 52(2): 169-84.

Kredics, L., Cseh, T., Körmöczi, P., Hatvani, L., Manczinger, L. and Vágvölgyi, C. 2008. Extracellular enzyme production of the two causative agents of oyster mushroom green mould under inductive and non-inductive conditions. Abstracts of the 6th International Conference on Mushroom Biology and Mushroom Products. Bonn, Germany, 29. September – 3. October 2008.

Kredics, L., Hatvani, L., Antal, Z., Manczinger, L., Druzhinina, I. S., Kubicek, C. P., Szekeres, A., Nagy, a., Vágvölgyi, C. and Nagy E. 2006. Green mould disease of oyster mushroom in Hungary and Transylvania. Acta Microbiol. Immunol. Hung. 53: 306–307.

Kubicek, C. P. and Penttilä, M.E. 1998. Regulation of production of plant polysaccharide degrading enzymes by *Trichoderma*. In: Harman G.E. and Kubicek C.P. (eds), *Trichoderma* and *Gliocladium*, Vol. 2: Enzymes, Biological Control and Commercial Applications, Taylor and Francis Ltd., London, 49-71.

Kulling, C., Mach, R. L., Lorito, M. and Kubicek, C. P. 2000. Enzyme diffusion from *T. atroviride* (=*T. harzianum* P1) to *R. solani* is a prerequisite for triggering of *Trichoderma* ech42 gene expression before mycoparasitic contact. Appl. Environ. Microbiol. 66: 2232–2234.

Kullnig-Gradinger, C. M., Szakács, G. and Kubicek, C. P. 2002. Phylogeny and evolution of the fungal genus *Trichoderma* - A multigene approach. Mycol. Res. 106: 757-767.

Largeteau-Mamoun, M. L., Mata, G. and Savoie, J. M. 2002. Green mold disease: Adaptation of *Trichoderma harzianum* Th2 to mushroom compost. Pp.179-187. in Mushroom Biology and Mushroom Products. Proceedings of The 4th International Conference on Mushroom Biology and Mushroom Products, Cuernavaca, Mexico, 20-22. February 2002. ed. by Sanchez *et al*.

Leach, J., Finkelstein, D. B. and Rambosek, J. A. 1986. Rapid miniprep of DNA from filamentous fungi. Fungal Genet Newslett 33: 32–33.

Leache, A. D. and Reeder T. W. 2002. Molecular systematics of the eastern fence lizard (*Sceloporus undulatus*): a comparison of parsimony, likelihood and Bayesian approaches. Syst. Biol. 51: 44–68.

Lieckfeldt, E., Kullnig, C. M., Kubicek, C. P., Samuels, G. J. and Börner, T. 2001. *Trichoderma aureoviride*: phylogenetic position and characterization. Mycol. Res. 105: 313–322.

Limón, M. C., Pintor-Toro, J. A. and Benítez, T. 1999. Increased antifungal activity of *T. harzianum* transformants that overexpress a 33-kDa chitinase. Biol. Control 89: 254–261.

Lorito, M., Hayes, C. K., Di Pietro, A., Woo, S. L. and Harman, G. E. 1994. Purification, characterization and synergistic activity of a glucan 1,3-β-glucosidase and an *N*-acetylglucosaminidase from *T. harzianum*. Phytopathology 84: 398–405.

Mach, R. L., Peterbauer, C. K., Payer, K., Jaksits, S., Woo, S. L., Zeilinger, S., Kullnig, C. M., Lorito, M. and Kubicek, C. P. 1999. Expression of two major chitinase genes of *T. atroviride* (*T. harzianum* P1) is triggered by different regulatory signals. Appl. Environ. Microbiol. 65: 1858–1863.

Mamoun, M. L., Savoie, J. M. and Olivier, J. M. 2000. Interactions between the pathogen *Trichoderma harzianum* Th2 and *Agaricus bisporus* in mushroom compost. Mycologia 92: 233-240.

Marzullo, L., Cannio, R., Giardina, P., Santini, M. T. and Sannia, G. 1995. Veratryl alcohol oxidase from *Pleurotus ostreatus* participates in lignin biodegradation and prevents polymerization of laccase-oxidized substrates. J. Biol. Chem. 270: 3823–3827.

Mesumbe, B. N. 2006. Mushroom Industry in Cameroon. At: <http://www.mushworld.com> Accessed on 28. December 2006.

Meyer, R., J. 1991. Mitochondrial DNAs and plasmids as taxonomic characteristics in *Trichoderma viride*. Appl. Environ. Microbiol. 57: 2269-2276.

Migheli, Q., Gonzales-Candelas, L., Dealessi, L., Camponogara , A. and Ramon-Vidal, D. 1998. Transformants of *T. longibrachiatum* overexpressing the β-1,4 endoglucanase gene egl1 show enhanced biocontrol of *Pythium ultimum* on cucumber. Phytopathology 88: 673–677.

Mischke, S. 1996 Evaluation of chromogenic substrates for measurement of protease production by biocontrol strains of *Trichoderma*. Microbios. 87: 175–183.

Mumpuni, A., Sharma, H. S. S. and Brown, A. E. 1998. Effect of Metabolites Produced by *Trichoderma harzianum* Biotypes and *Agaricus bisporus* on Their Respective Growth Radii in Culture. Appl. Environ. Microbiol. 64(12): 5053-5056.

Muthumeenakshi, S., Brown, A. E. and Mills, P. R. 1998. Genetic comparison of the aggressive weed mould strains of *Trichoderma harzianum* from mushroom compost in North America and the British Isles. Mycological Research 102: 385-390.

Muthumeenakshi, S., Mills, P. R., Brown-Averil, E., and Seaby, D. A. 1994. Intraspecific molecular variation among *Trichoderma harzianum* isolates colonizing mushroom compost in the British Isles. Microbiology (UK) 140: 769-777.

Nicholas, K. B., and Nicholas, H. B. Jr. 1997 Genedoc: a tool for editing and annotating multiple sequence alignments. http://www.psc.edu/biomed/genedoc.

O'Donnell, K., Cigelnik, E. and Nirenberg, H. L. 1998. Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. Mycologia 90: 465–493.

Ospina-Giraldo, M. D., Royse, D. J., Chen, X. and Romaine, C. P. 1999. Molecular phylogenetic analyses of biological control strains of *Trichoderma harzianum* and other biotypes of *Trichoderma* spp. associated with mushroom green mold. Phytopathology 89: 308-313.

Ospina-Giraldo, M. D., Royse, D .J., Thon, M. R., Chen, X. and Romaine, C. P. 1998. Phylogenetic relationships of *Trichoderma harzianum* causing mushroom green mold in Europe and North America to other species of *Trichoderma* from world-wide sources. Mycologia 90: 76-81.

Pani, B. K., Panda, S. N. and Das, S. R. 1997. Utilization of some agricultural bioproducts and other wastes for sporophore production of oyster mushroom. Orissa J. Hortic. 25: 36–39.

Papavizas, G. C. 1985. *Trichoderma* and *Gliocladium*: biology, ecology, and potential for biocontrol. Annu. Rev. Phytopathol. 23: 23–54.

Park, M. S., Bae, K. S. and Yu, S. H. 2004a. Molecular and morphological analysis of *Trichoderma* isolates associated with green mold epidemic of oyster mushroom in Korea. J. Huazhong Agric. Univ. 23: 157-164.

Park, M. S., Bae, K. S. and Yu, S. H. 2004b. Morphological and molecular analysis of *Trichoderma* species associated with green mold epidemic of oyster mushroom in Korea. At: <http://www.mushworld.com> Accessed on 11. October 2004.

Park, M. S., Bae, K. S. and Yu, S. H. 2004c. Morphological and molecular analysis of Trichoderma species associated with green mold epidemic of oyster mushroom in Korea. Pp. 143-158. in New Challenges in Mushroom Science. Proceedings of the 3rd Meeting of Far East Asia for Collaboration on Edible Fungi Research, Suwon, Korea 2004.

Park, M. S., Bae, K. S. and Yu, S. H. 2006. Two new species of *Trichoderma* associated with green mold of oyster mushroom cultivation in Korea. Mycobiology 34: 111-113.

Park, M. S., Seo, G. S., Lee, K. H., Bae, K. S. and Yu, S. H. 2005. Characterization of *Trichoderma* spp. associated with green mold of oyster mushroom by PCR-RFLP and sequence analysis of ITS regions of rDNA. Plant Pathology Journal 21: 229-236.

Puniya, A. K., Shah, K. G., Hire, S. A., Ahire, R. N., Rathod, M. P. and Mali, R. S. 1996. Bioreactor for solid-state fermentation of agro-industrial wastes. Indian J. Microbiol. 36: 177–178.

Romaine, C. P., Chen, X., Ospina-Giraldo, M. D. and Royse, D. J. 2001. Molecular genetics and pathogenicity of biocontrol and mushroom *Trichoderma*. IOBC wprs Bull. 24(3): 333-336.

Samuels, G. J., Dodd, S. L., Gams, W., Castlebury, L. A. and Petrini, O. 2002. *Trichoderma* species associated with the green mold epidemic of commercially grown *Agaricus bisporus*. Mycologia 94: 146-170.

Sazci A., Radford A. and Erenler K. 1986. Detection of cellulolytic fungi by using Congo red as an indicator: a comparative study with the dinitrosalicylic acid reagent method. Journal of Applied Bacteriology 61: 559-562.

Seaby, D.A. 1989. Further observation on *Trichoderma*. Mushroom J. 197: 147-151.

Seaby, D.A. 1987. Infection of mushroom compost by *Trichoderma* species. Mushroom J. 179: 355-361.

Seaby, D. 1998. *Trichoderma* as a weed mould or pathogen in mushroom cultivation. Pages 267-287 in: *Trichoderma* and *Gliocladium*. Vol. 2. Enzymes, Biological Control and Commercial Applications. Kubicek, C. P. and Harman, G. E. eds. Taylor and Francis, London.

Sharma, S. R. and Vijay B. 1996. Yield loss in *Pleurotus ostreatus* spp. caused by *Trichoderma viride*. Mushroom Research 5: 19–22.

Sinden, J. and E. Hauser. 1953. Nature and control of three mildew diseases of mushrooms in America. Mushroom Sci. 2:177–180.

Sivan, A. and Chet, I. Degradation of fungal cell wall by lytic enzymes of *Trichoderma harzianum*. J. Gen. Microbiol. 1989; 135: 675-82.

Swofford, D. L. 1998. PAUP*: phylogenetic analysis using parsimony (* and other methods), version 4. Sinauer, Sunderland, MA.

Szekeres, A., Kredics, L., Antal, Z., Hatvani, L., Manczinger, L. and Vágvölgyi, C. 2005. Genetic diversity of *Trichoderma* strains isolated from winter wheat rhizosphere in Hungary. Acta Microbiol. Immunol. Hung. 52(S): 156.

Szekeres, A., Kredics, L., Antal, Z., Kevei, F., Manczinger, L. 2004. Isolation and characterization of protease overproducing mutants of *Trichoderma harzianum*. FEMS Microbiol. Lett. 233: 215–222

Thrane, C., Jensen, D. F. and Tromso, A. 2000. Substrate colonization, strain competition, enzyme production *in vitro*, and biocontrol of *Pythium ultimum* by *Trichoderma* spp. isolates P1 and T3. Eur. J. Plant Pathol. 106: 215–225.

Tokimoto, K. 1982. Lysis of the mycelium of *Lentinus edodes* caused by mycolytic enzymes of *Trichoderma harzianum* when the two fungi were in an antagonistic state. Trans. Mycol. Soc.]apan 23:13-20.

Van Tilburg, A.-U. B. and Thomas, M. D. 1993. Production of Extracellular Proteins by the Biocontrol Fungus *Gliocladium virens.* Appl. Environ. Microbiol. 59(1): 236-242

Varga, J., Kevei, F., Fekete, C., Coenen, A. F., Kozakiewicz, Z. and Croft, J. H. 1993. Restriction fragment length polymorphisms in the mitochondrial DNAs of the *Aspergillus niger* aggregate. Mycol. Res. 97: 1207–1212.

Viterbo, A., Haran, S., Friesem, D., Ramot, O. and Chet, I. 2001. Antifungal activity of a novel endochitinase gene (chit36) from *T. harzianum* Rifai TM. FEMS Microbiol. Lett. 200: 169–174.

Viterbo, A., Ramot, O., Chernin, L. and Chet, I. 2002. Significance of lytic enzymes from *Trichoderma* spp. in the biocontrol of fungal plant pathogens. Ant. Leeuw. 81: 549–556.

White, T. J., Bruns, T., Lee, S. and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH,

Sninsky JJ, White TJ, eds. PCR protocols: a guide to methods and applications. San Diego, California: Academic Press. 315–322.

Williams, J., Clarkson, J. M., Mills, P. R. and Cooper, R. M. 2003. Saprotrophic and Mycoparasitic Components of Aggressiveness of *Trichoderma harzianum* Groups toward the Commercial Mushroom *Agaricus bisporus.* Appl. Environ. Microbiol. 69(7): 4192-4199.

Won, Y. 2000. Oyster Mushroom Disease: Green Mould. At: <http://www.mushworld.com> Accessed on 1. November 2000.

Woo, S. L., Di Benedetto, P., Senatore, M., Abadi, K., Gigante, S., Soriente, I., Ferraioli, S., Scala, F. and Lorito, M. 2004. Identification and characterization of *Trichoderma* species aggressive to *Pleurotus* in Italy. Journal of Zhejiang University, Agriculture and Life Sciences 30: 469-470.

Woo S. L., Donzelli, B., Scala, F., Mach, R., Harman, G. E., Kubicek, C. P., del Sorbo, G, and Lorito, M. 1999. Disruption of the *ech42* (endochitinase-encoding) gene affects biocontrol activity in *T. harzianum* P1. Mol. Plant Microbe In. 12: 419–429.

Yu, S. H. 2002. Integrated Control of Oyster Mushroom Green Mould. At: <http://www.mushworld.com> Accessed on 1. May 2002.

Yu, S. H. 2002. Integrated Control of Oyster Mushroom Green Mould (2). At: <http://www.mushworld.com> Accessed on 1. October 2002.

Yu, S. H. and Seo, G. S. 2002. Integrated Control of Oyster Mushroom Green Mould (1). At: <http://www.mushworld.com> Accessed on 1. August 2002.

Yu, S. H. and Seo, G. S. 2002. Integrated Control of Oyster Mushroom Green Mould (3). At: <http://www.mushworld.com> Accessed on 1. November 2002.

Yu, S. H. and Seo, G. S. 2002. Integrated Control of Oyster Mushroom Green Mould (4). At: <http://www.mushworld.com> Accessed on 1. December 2002.

Yu, S. H. and Seo, G. S. 2003. Integrated Control of Oyster Mushroom Green Mould (5). At: <http://www.mushworld.com> Accessed 1. January 2003.

Zhang, C. L., Druzhinina, I. S., Kubicek, C. P. and Xu, T. 2005. Biodiversity of *Trichoderma* in China: Evidence for a North to South difference of species distribution in East Asia. FEMS Microbiol. Lett. 251: 251-257.

Zijlstra, C., de Weerdt, M., Baar, J. and Baars, J. 2008. A TaqMan PCR test for timely detection of the causal agents of dry bubble disease. Abstracts of the 17th Congress of the International Society for Mushroom Science, Cape Town, South Africa, 20–24 May 2008.

11. SUMMARY

World-wide commercial mushroom production is currently threatened by massive attacks of green mould disease. Producers of champignon (*Agaricus bisporus*) and oyster mushroom (*Pleurotus ostreatus*) are facing recent incidents of green mould epidemics in Hungary as well. We isolated 66 *Trichoderma* strains from *Agaricus* compost and *Pleurotus* substrate samples from three Hungarian mushroom producing companies, and examined them by a PCR-based diagnostic test for *T. aggressivum*, sequence analysis of the internal transcribed spacer region (ITS) 1 and 2 and (selectively) of the fourth and fifth intron of translation elongation factor 1-α (*tef1*), as well as by restriction fragment length polymorphism (RFLP) analysis of the mitochondrial DNA. Eight *Trichoderma* species were identified: *T. aggressivum* f. *europaeum*, *T. harzianum*, *T. longibrachiatum*, *T. ghanense*, *T. asperellum*, *T. atroviride*, and the recently described phylogenetic species, *T. pleuroticola* and *T. pleurotum* (17, 3, 4, 1, 4, 9, 1 and 27 isolates, respectively). *T. aggressivum* f. *europaeum* was exclusively derived from *A. bisporus* compost, whereas *T. pleurotum* and *T. pleuroticola* exclusively occurred in the substrate for *Pleurotus* cultivation. Sequences of the latter strains were co-specific with those for *Trichoderma* pathogens of *P. ostreatus* in Korea. The widespread occurrence of these new species raises questions as to why infections by them have just only recently been observed. Our initial data documented that the green mould disease caused by *T. aggressivum* f. *europaeum* has geographically expanded to Central Europe; the green mould disease of *P. ostreatus* in Hungary is due to the same *Trichoderma* species as in Korea and the world-wide distribution of the new species indicates the possibility of spreading epidemics; and on mushroom farms, the species are specialized on their different substrates.

Having applied the genealogical concordance of multiple phylogenetic markers (ITS1 and ITS2 sequences; partial sequences of *tef1* and *chi18-5*), we have determined that the causal agents of *Pleurotus* green mould disease are two genetically closely related, but phenotypically strongly different species of *Trichoderma*, which have been recently described as *T. pleurotum* and *T. pleuroticola.* They belong to the Harzianum

clade of *Hypocrea/Trichoderma* which also includes *T. aggressivum*, the causative agent of green mould disease of *Agaricus*. Both *Pleurotus*-pathogenic species have been found on cultivated oyster mushroom and its substratum in Europe, Iran, and South Korea, but *T. pleuroticola* has also been isolated from soil and wood in Canada, the United States, Europe, Iran, and New Zealand. A DNA BarCode for identification of these species based on ITS1 and ITS2 sequences has been provided and integrated in the main database for *Hypocrea/Trichoderma* (www.ISTH.info).

We present a polymerase chain reaction (PCR)-based technique for the rapid and specific detection of *T. pleurotum* and *T. pleuroticola*, the two newly described pathogens of *P. ostreatus*. Based on sequence regions within the 4th and 5th introns in the gene encoding translation elongation factor 1-α (*tef1*) we designed three oligonucleotide primers which identify these species in a multiplex PCR assay. We have also found that these primers can be used to detect the presence of *T. pleurotum* and/or *T. pleuroticola* directly in the growing substrate of oyster mushroom, without the necessity of cultivation and ITS sequence analysis, which makes identification significantly faster. We used this method to examine the presence of the new species in the natural environment of *P. ostreatus* and we have found that *T. pleuroticola* is often accumulated in the growing substrate, as well as on the surface of the fruit body of the mushroom. Besides *T. pleuroticola* exclusively *T. harzianum*, *T. longibrachiatum* and *T. atroviride* were detected in these habitats, and *T. pleurotum* was still not found in any of the samples.

We intended to examine enzyme systems potentially involved in the mycoparasitic activity of *T. pleurotum*. From a wild-type isolate - that had previously shown high aggressivity towards *P. ostreatus* - we developed mutants with altered ability of producing several extracellular enzymes (pectinases, cellulases, amylases, proteases, lipases, chitinases and glucanases) by the use of UV-mutagenesis. The mycoparasitic potential of the parental strain and its mutant derivatives towards *P. ostreatus* was tested in dual plate assays. Based on our results we conclude that among the enzyme systems tested, deficiencies in protease, lipase, chitinase and glucanase system reduce the mycoparasitic potential of *T. pleurotum* towards *Pleurotus ostreatus* mostly.

12. ÖSSZEFOGLALÓ

A gombatermesztést az utóbbi évtizedekben egyre inkább fenyegeti a zöldpenész betegség világszerte, így a magyarországi csiperke- és laskatermesztőknek is jelentős károkkal kell számolniuk. Munkánk első részében 66 *Trichoderma* törzset izoláltunk magyarországi gombatermesztő üzemekből származó csiperkekomposztból és laskaszubsztrátból. Alávetettük őket egy *T. aggressivum*-specifikus, polimeráz láncreakció (PCR)-alapú diagnosztikus vizsgálatnak, elvégeztük az ITS (internal transcribed spacer region) 1 és 2 régiók és a *tef1* (translation elongation factor 1-α) gén 4. és 5. intronjának szekvenciaanalízisét, valamint tanulmányoztuk az izolátumok mitokondriális DNS-ét RFLP (restriction fragment length polymorphism) módszerrel. Eredményeink alapján a következő nyolc *Trichoderma* fajt azonosítottuk: *T. aggressivum* f. *europaeum*, *T. harzianum*, *T. longibrachiatum*, *T. ghanense*, *T. asperellum*, *T. atroviride*, és a nemrégiben leírt fajok, *T. pleurotum* és *T. pleuroticola* (17, 3, 4, 1, 4, 9, 27, illetve 1 izolátum). A *T. aggressivum* f. *europaeum* fajt kizárólag csiperkekomposztból tudtuk izolálni, míg a *T. pleurotum* és a *T. pleuroticola* cask a laskatermesztésben alkalmazott szubsztrátban fordult elő. Az utóbbi fajok DNS-szekvenciái kospecifikusnak bizonyultak a Dél-Koreában izolált laskapatogén *Trichoderma* törzsekéivel. Ezen új fajok széleskörű elterjedése felveti a kérdést, hogy az általuk okozott fertőzés miért csak az utóbbi időben jelentkezett. Eredményeink azt mutatják, hogy a *T. aggressivum* f. *europaeum* által okozott csiperke-zöldpenész terjedt át Közép-Európába, a laskagomba zöldpenész betegségét pedig Magyarországon is a Dél-Koreában leírt fajok okozzák. A patogén fajok világméretű elterjedése a járvány terjedését vetítik elő, és az egyes fajok specializálódtak a különböző gazdaszervezetekre, illetve termesztési körülményekre.

Számos filogenetikai marker (ITS1 és 2 szekvenciák, a *tef1* és a *chi18-5* gén részleges szekvenciái) együttes vizsgálatával megállapítottuk, hogy a laska-zöldpenész kórokozói két genetikailag közeli rokon, de fenotípus alapján erősen különböző *Trichoderma* faj, melyeket nemrég *T. pleurotum* és *T. pleuroticola* néven új fajokként írtak le. Mindkettő a *Hypocrea/Trichoderma* nemzetségek Harzianum kládjába tartozik,

ami magában foglalja a *Trichoderma aggressivum* fajt, a csiperke-zöldpenész kórokozóját is. Mindkét laskapatogén *Trichoderma* faj megtalálható a termesztett laskagombán és annak szubsztrátján Európaszerte, Dél-Koreában és Iránban, de a *T. pleuroticola* környezeti mintákból (talaj, fa) is gyakran izolálható (Kanada, Egyesült Államok, Európa, Irán és Új-Zéland).

Kifejlesztettünk egy polimeráz láncreakció (PCR)-alapú módszert a két újonnan leírt laskapatogén *Trichoderma* faj, a *T. pleurotum* és a *T. pleuroticola* gyors és specifikus kimutatására. A *tef1* (translation elongation factor 1-α) gén szekvenciái alapján három primert terveztünk, melyek segítségével egy együttes reakcióban azonosítható a két faj. Kimutattuk, hogy ezen primerek használatával a *T. pleurotum*, illetve a *T. pleuroticola* kimutatható tenyésztés és az ITS szekvenciák analízise nélkül is, közvetlenül a laskagomba szubsztrátjából, ami jelentősen meggyorsítja az azonosítást.

A leírt módszerrel vizsgáltuk a két új faj jelenlétét a laskagomba természetes környezetében, és azt találtuk, hogy a *T. pleuroticola* jelentősen feldúsulhat mind a szubsztrátban, mind a gomba termőtestjén. A *T. pleuroticola* mellett kizárólag *T. harzianum*, *T. longibrachiatum* és *T. atroviride* volt kimutatható ezeken az élőhelyeken, de a *T. pleurotum* faj egyik mintában sem volt jelen.

Vizsgálni szándékoztuk a *T. pleurotum* laskagombával szembeni mikoparazitizmusában feltételezhetően szerepet játszó extracelluláris enzimrendszereket. Ehhez egy szülői izolátumot, amely korábbi kísérleteinben nagy aggresszivitást mutatott a laskával szemben, UV-kezelésnek vetettük alá, és ezt követően számos mutáns törzset izoláltunk, melyek a vad izolátumtól különböző mértékben voltak képesek különböző extracelluláris enzimrendszerek (pektinázok, cellulázok, amilázok, proteázok, lipázok, kitinázok és glülanázok) termelésére. A szülői törzs és mutáns származékei laskával szembeni mikoparazita képességét *in vitro* konfrontációs kísérletekben hasonlítottuk össze, és eredményeink alapján arra következtettünk, hogy a *T. pleurotum* laskával szembeni antagonizmusában az általunk vizsgált enzimrendszerek közül a proteázok, lipázok, kitinázok és a glülanázok játszanak leginkább szerepet.

13. APPENDIX

Table 8. Growth and sporulation of the surviving colonies of *T. pleurotum* C15 following UV-treatment

+++: growth and sporulation similar to those of the wild-type strain

++: reduced growth or/and sporulation

+: highly reduced growth or/and sporulation

Strain number	***Duration of UV-treatment***	***Growth***	***Sporulation***
Wild-type		+++	+++
A1	7 min	+++	+
B1	8 min	+++	+
B2	8 min	+++	+++
C1	8 min	+++	+++
C2	8 min	+++	+++
C3	8 min	++	+++
C4	8 min	++	+++
D1	8 min	++	+++
D2	8 min	++	+++
D3	8 min	+	+++
E1	9 min	+++	+++
E2	9 min	+++	+++
E3	9 min	++	+++
E4	9 min	+	+++
E5	9 min	++	+++
E6	9 min	++	+++
F1	9 min	+++	+++
F2	9 min	+++	+++
F3	9 min	+++	+++
F4	9 min	+++	+++
F5	9 min	+++	+++
F6	9 min	+++	+++
F7	9 min	+++	+++
F8	9 min	+++	+++

F9	9 min	+	+++
G1	9 min	++	+++
G2	9 min	+++	+++
G3	9 min	+++	+++
H1	9 min	++	+++
H2	9 min	+++	+++
I1	9 min	+++	+++
I2	9 min	+++	+++
I3	9 min	+	+++
L1	10 min	+++	+
L2	10 min	+++	+++
L3	10 min	+++	+++
L4	10 min	+	+++
L5	10 min	+	+++
M1	10 min	++	+++
M2	10 min	++	+++
M3	10 min	++	+
N1	10 min	+++	+++
O1	7 min	++	+++
O2	7 min	+++	+++
O3	7 min	+++	+++
O4	7 min	+++	+++
O5	7 min	+++	+++
O6	7 min	+++	+++
O7	7 min	+++	+++
O8	7 min	+++	+++
O9	7 min	+++	+++
O10	7 min	+	+++
O11	7 min	++	+++
O12	7 min	++	+++
O13	7 min	+	+++
O14	7 min	+	+++
O15	7 min	+++	+++
O16	7 min	+	+++
P1	7 min	+++	+++
P2	7 min	+++	+++

P3	7 min	+++	+++
P4	7 min	+++	+++
P5	7 min	+++	+++
P6	7 min	+++	+++
P7	7 min	+++	+++
P8	7 min	+++	+++
P9	7 min	+++	+++
P10	7 min	+++	+++
P11	7 min	+++	+++
P12	7 min	+++	+++
P13	7 min	+++	+++
P14	7 min	+	+++
P15	7 min	+++	+++
P16	7 min	+	+++
P17	7 min	+++	+++
P18	7 min	+	+++
P19	7 min	+++	+++
P20	7 min	+	+++
R1	7 min	+++	+++
R2	7 min	+++	+++
R3	7 min	+++	+++
R4	7 min	+++	+++
R5	7 min	+++	+++
R6	7 min	+++	+++
R7	7 min	+++	+++
R8	7 min	+++	+++
R9	7 min	+	+++
R10	7 min	++	+++
R11	7 min	+	+++
R12	7 min	+	+++
S1	8 min	+++	+++
S2	8 min	+++	+++
S3	8 min	+	+++
S4	8 min	+++	+++
S5	8 min	++	+++
S6	8 min	+++	+++

S7	8 min	+++	+++
S8	8 min	+++	+++
S9	8 min	+++	+++
S10	8 min	+++	+++
S11	8 min	+++	+++
S12	8 min	+++	+++
S13	8 min	+++	+++
S14	8 min	+++	+++
S15	8 min	+	+++
S16	8 min	++	+++
S17	8 min	++	+++
S18	8 min	+++	+++
S19	8 min	++	+++
T1	8 min	++	+++
T2	8 min	++	+++
T3	8 min	++	+++
T4	8 min	+++	+++
T5	8 min	+++	+++
T6	8 min	+++	+++
T7	8 min	++	+++
T8	8 min	++	+++
T9	8 min	+++	+++
T10	8 min	++	+++
T11	8 min	++	+++
T12	8 min	+++	+++
T13	8 min	+++	+++
T14	8 min	+++	+++
T15	8 min	++	+++
T16	8 min	++	+++
T17	8 min	+	+++
T18	8 min	+++	+++
T19	8 min	+++	+++
T20	8 min	+++	+++
T21	8 min	++	+++
U1	8 min	+++	++
U2	8 min	+++	+++

U3	8 min	+++	+++
U4	8 min	+++	+++
U5	8 min	+++	+++
U6	8 min	++	+++
U7	8 min	+++	+++
U8	8 min	+++	+++
U9	8 min	+++	+++
U10	8 min	+	++
U11	8 min	++	+++
U12	8 min	+	++
U13	8 min	+++	+++
U14	8 min	+	+
U15	8 min	+++	+++
U16	8 min	++	+++
U17	8 min	+++	+++
U18	8 min	+++	+++
U19	8 min	+	++
U20	8 min	+	+++
U21	8 min	++	+++
U22	8 min	+	++
U23	8 min	++	+++
U24	8 min	++	+++
U25	8 min	+++	+++
V1	9 min	++	+++
V2	9 min	++	+++
V3	9 min	++	+++
V4	9 min	++	No
V5	9 min	++	+++
V6	9 min	+++	+++
V7	9 min	++	+++
V8	9 min	+	++