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TRICHODERMA spp. – THE CAUSE OF GREEN MOLD ON POLISH MUSHROOM FARMS

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Summary

The first reports concerning green mold on Polish mushroom farms are from 2002. In Europe and the United States *Trichoderma harzianum* was initially described as a causative agent of the disease. However, soon two biotypes of *Trichoderma,* Th2 in Europe and Th4 in America, were recognised as responsible for commercial losses and were designated as a new species named *T. aggressivum*. Until now it has not been clear which species of *Trichoderma* and which form of the species was a causative agent of the disease outbreak in Poland and what was the main source of infection. Therefore, studies were conducted on the composition of the *Trichoderma* species in Polish mushroom farms and in compost samples. The isolates were identified by phenotypic and microscopic studies. The classification of the strains recognised as *T. aggressivum* was then confirmed by PCR methods. The aggressiveness of *Trichoderma* spp., especially *T. aggressivum,* was estimated in *in vitro* studies. The most frequently isolated *Trichoderma* spp. were: *T. harzianum, T. aggressivum, T. atroviride* and *T. longibrachiatum.* Using the technique of PCR, multiplex PCR and PCR-RAPD it was determined that 24 isolates from Polish mushroom farms belonged to *T. aggressivum* f. *europeanum* (Th2). However, a large variation between isolates indicated the possibility that Polish biotypes of *T. aggressivum* were different from those, which were found in western Europe. All isolates of *T. aggressivum* were highly pathogenic towards *Agaricus bisporus*.

key words: *Trichoderma aggressivum*, PCR identification, epidemiology

INTRODUCTION

For at least two decades, commercial production of mushrooms has been seriously affected by green mold epidemics. The causative fungus was able to colonise compost, and resulted in crop losses of 30-100% (Seaby 1998). The first reported epidemics were in Ireland and United Kingdom (Seaby

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1989). Four distinct biotypes of *Trichoderma*, which were initially described as *T. harzianum*, have been associated with the disease (Morris *et al*. 1995, Seaby 1996). However, only two biotypes designated as Th2 for European isolates and Th4 for American, were responsible for the losses in commercial mushroom production (Seaby 1998, Samuels *et al*. 2002). These two forms were distinguished from other *Trichoderma* species by morphological traits, cultural characteristics and molecular identification by PCR methods (Muthumeenakshi *et al*. 1994, Castle *et al*. 1998, Chen *et al*. 1999 a,b). It was found that they were strongly separated from *T. harzianum* in sequences of the ITS-1 region of nuclear rDNA and an approximately 689 bp fragment of the protein coding translation elongation factor gene (Samuels *et al*. 2002). Finally, a new species named *T. aggressivum* was described, and two forms *T. aggressivum* f. *europeanum* (former Th2) and *T. aggressivum* f. *aggressivum* (former Th4) were separated (Samuels *et al*. 2002).

In Poland a serious epidemic of green mold in mushroom production was first observed in 2002 (Sakson 2004, Maszkiewicz 2006). However, until now it has not been clear which species of *Trichoderma* and which form of the species is a causative agent of the Polish disease outbreak and what was the main source of infection. Since 2004, studies on the composition of the species of the genus *Trichoderma* on Polish mushroom farms and compost samples have been performed. The isolates collected in the Research Institute of Vegetable Crops in Skierniewice were identified by phenotypic and microscopic studies. The classification of strains recognised as *T. aggressivum* was confirmed by molecular analyses. The aggressiveness of *Trichoderma* spp., especially *T. aggressivum,* was estimated in *in vitro* studies.

MATERIALS AND METHODS

Morphological identification of *Trichoderma* spp.

Studies commenced in 2004. Compost, casing material, floors, walls, dust of mushroom farms, trays, pallets, machinery and cloth stores were the sources of *Trichoderma* spp. isolates. The samples were obtained by dilution and plating of sampled material on rose bengal agar, by pressing plates with malt extract agar (MEA) to the studied surfaces or by dust sedimentation on the rose bengal medium. The hyphae of growing *Trichoderma* colonies were transferred on potato dextrose agar (PDA) amended with streptomycin sulfate (50 $mg \cdot L^{-1}$) and riphampicin (10 $mg \cdot L^{-1}$). Representative isolates from the samples were deposited for further studies.

For identification, fungal colonies were established on PDA. After two days of incubation at 27ºC, 5 mm diameter plugs of actively growing mycelium were transferred onto fresh 2% MEA and PDA. The plugs were placed mycelium-side-down approximately 5 mm from the edge of the plate. The plates were incubated in darkness at 27ºC. After 48 h the plates were briefly removed from the incubator and held up to the light. This was repeated at 24 h intervals. Identification was performed by observation of phenotypic characters of the

colonies and by microscopic studies of the conidiophores and conidia. The studies were supported by identification keys provided by Bissett (1984, 1991a,b), Gams & Bissett (1998) and http://nt.ars-grin.gov/taxadescriptions/keys/TrichodermaIndex.cfm. Among *Trichoderma* species the isolates of *T. aggressivum* were selected using additionally keys by Seaby (1996) and Samuels *et al*. (2002).

Molecular identification

Twenty four representative strains from Polish mushroom farms determined on the basis of morfological characters as *T. aggressivum* as well reference strains of *Trichoderma* obtained from the CBS collection (The Netherlands) were used for molecular analyses (Table 1).

Table 1. List of isolates used in this study

Isolate code	Locality and origin of samples
CBS 466.94 - *T. harzianum* Th1 CBS 693.94 - *T. atroviride* Th 3 CBS 450.95 - *T. aggressivum* f. *aggressivum* Th 4 CBS 689.96 - *T. aggressivum* f. *europeanum* Th 2	CBS* collection
SP.22.07	Maków (compost)
11.02	Częstochowa (compost)
28.05	Orzeł (compost)
7.06	Not known locality - (compost)
11.03	Mogielnica (casing)
16.11.2A	Lipinki (compost)
27.12.2	Lipinki (casing)
19.09.E	Szreniawa (casing)
11.08.C	Żelki (casing)
14.12.2C	Wojnowo (dust from cloth store)
T4/3.30.03	Skierniewice (peat)
13.02.B	Trzcianna (compost)
2.02.A	Maków (casing)
5/3.30.03	Skierniewice (dust collected from walls)
29.05.1B	Krzepielów (compost)
11.04	Polesie (compost)
3-9.08-4	Ignanie Nowe A (dust collected from walls)
5-0P-7.07-4	Kolonia Bolimowska (air sample)
5-0-7.07-9	Kolonia Bolimowska (casing)
2-0P-9.08-1	Ignanie Nowe B (dust collected from walls)
4-0-9.08-1	Skórzec (casing)
1-0P-9.08-3	Ignanie Nowe C (dust collected from walls)
PGU12	Skierniewice (compost)

CBS* - Centraalbureau voor Schimmelcultures, The Netherlands

DNA was extracted from mycelium grown on MEA according to the method by Aljanabi and Martinez (1997). DNA concentration and purity was determined spectrophotometrically and by visualization on 0.8% agarose gel. To identify of the pathogenic biotypes Th-2 and Th-4, a polymerase chain reaction (PCR) with specific primers Th-F and Th-R (Chen *et al*. 1999 a), PCR multiplex with Th-F/Th-R, β-tub-F/ β-tub-R primers and RAPD method with primer 232 (Chen *et al*. 1999 b) were used. The primer sequences are listed in Table 2.

Table 2. Primer sequences used in this study

Primers	Sequence 5' → 3'	Length of PCR products (bp)	Reference
Th-F Th-R	CGGTGACATCTGAAAGTCGTG TGTCACCCGTTCGGATCATCCG	444	Chen *et al*. (1999a)
β-tub-F β-tub-R	GTTGGTTCTGCCTTCTGG AACAGCTGGCCAAAGGGG	369	Chen *et al*. (1999b)
232	CGGTGACATC	1150	Chen *et al*. (1999b)

PCR and PCR multiplex reaction was performed in 20 µl volume containing 1 x PCR buffer (20 mM Tris-HCL pH 8.4, 50mM KCl), 2mM $MgCl_2$, 0.1 mM of each deoxynucleotide, 0.5 µM of each primer Th-F and Th-R and 0,25 µM of each β-tub-F/ β-tub-R primers, 1 U Taq DNA Polymerase (Invitrogen), 20 ng of DNA. The amplification reaction was carried out in GeneAmp 9700 (Applied Biosystems) thermal cycler. The cycle parameters were: 94ºC for 1 min followed by 40 cycles of 15 s at 93ºC, 20 s at 60ºC, 1 min at 72ºC, and the final extension time of 5 min at 72ºC.

Random amplified polymorphic DNA (RAPD) analysis with primer 232 was performed in 10 mM Tris HCl pH 8.3, 50 mM KCl, 0.1 mM of each dNTP, 2mM $MgCl_2$, 1 U Taq DNA polymerase (Sigma Aldrich), 0.001% gelatin, 0.3 µM of primer, 20 ng of genomic DNA in final volume of 20 µl. RAPD conditions were: 94ºC for 1 min followed by 45 cycles of 15 s at 92ºC, 25 s at 36ºC, 74 s at 72ºC, and the final extension time of 5 min at 72ºC. Amplification products were analysed by electrophoresis in 1.4% agarose gel in 1 x TBE buffer after staining with ethidium bromide.

Aggressiveness of *Trichoderma* spp. isolates

The aggressiveness of compost colonization by various *Trichoderma* spp. isolated from mushroom farms was studied. Twenty four isolates representing ten *Trichoderma* species were tested: *T. aggressivum* (8 isolates), *T. atroviride* (6), *T. viride* (2), *T. citrinoviride* (2), *T. koningii* (1), *T. virens* (1), *T. harzianum* (1), *T. ovalisporum* (1), *T. longibrachiatum* (1), *T. inhamatum* (1). Petri plates (17 cm diam.) containing 125 g of pasteurized compost colonised or not colonised with mycelium of *Agaricus bisporus,* were inoculated in the centre with the mycelial plug (5 mm) of tested *Trichoderma* fungus. The plates were sealed with parafilm and incubated in darkness at 24ºC for 24 days. Four plates were

prepared for each treatment, and the experiment was repeated three times. The radius of the area with distinct development of *Trichoderma* colony was measured after 8, 14, 19 and 24 days. To evaluate the significance of differences between treatments one-factorial analysis of variance was used. The means were compared with Newman-Keuls test for P=0.05.

RESULTS AND DISCUSSION

In the years 2004-2007, the most frequently isolated *Trichoderma* spp. were: *T. harzianum, T. aggressivum, T. atroviride*, *T. longibrachiatum* and *T. virens*. *T. aggressivum* was mostly isolated from compost and casing material. Usually, green mold on these substrates was already distinctly visible. It was probably a reason of such high contribution of this species in the total population of *Trichoderma* used for identification. When the colonies of the strains designed as *T. aggressivum* were observed on PDA and MEA, there were differences in the growth rate and colonies appearance. The studied isolates also slightly differed from reference strains CBS 689.94 and CBS 450.95 (Fig. 1). It was found that characters of the colony were especially distinct on MEA. Therefore, this medium seems to be useful for phenotypic characterisation and identification of this fungus (Seaby 1996). On this medium reference strain CBS 689.94 produced radially striate colonies with a smooth edge. Conidial production began in the middle near the inoculum plug, and then it was restricted to concentric rings (Fig. 1B). The colony was green. This appearance of the colony is consistent with the description of *T. aggressivum* f. *europeanum* presented by Seaby (1996) and Samuels *et al*. (2002). Conversly, the studied strains created radially striate colonies but with wavy edge, and conidial production was mostly concentrated in the middle of the colony forming characteristic dark green aggregations with white bands (Fig. 1C, D). Concentric rings of sporulation were only slightly marked near the margin.

The morphological identification of *T. aggressivum* strains was confirmed by PCR. With the use of Th-F and Th-R primers it was possible to observe a specific product of amplification at the 450 bp size for both reference strains of *T. aggressivum* CBS 689.94 (Th2) and CBS 450.95 (Th4) and for all tested isolates (Fig. 2A, line 3-18). This fragment was not identified in *T. harzianum* and *T. atroviride,* reference strains CBS 466.94 and CBS 693. 94 respectively (Fig 2A, line 1-2). As a result of DNA amplification with the use of the multiplex PCR technique, two products of amplification at the 370 and 450 bp size were obtained for the CBS 689.94 and CBS 450.95 strains and for all the tested Polish *T. aggressivum* isolates (Fig. 2B, line 3-18). However, in the profiles obtained for *T. harzianum* and *T. atroviride* only one DNA fragment 370 bp was visible (Fig. 2B, line 1-2). When the 232 RAPD primer was used, the specific DNA fragment 1150 bp was observed for *T. aggressivum* f. *aggressivum* CBS 450.95 (Fig. 3, line 3). This band was not detected in all tested Polish isolates or in reference strain CBS 689.94 (Fig. 3, line 4-16). This study may suggest that Polish isolates belonged to a biotype of *T. aggressivum* f. *europeanum* (Th2).

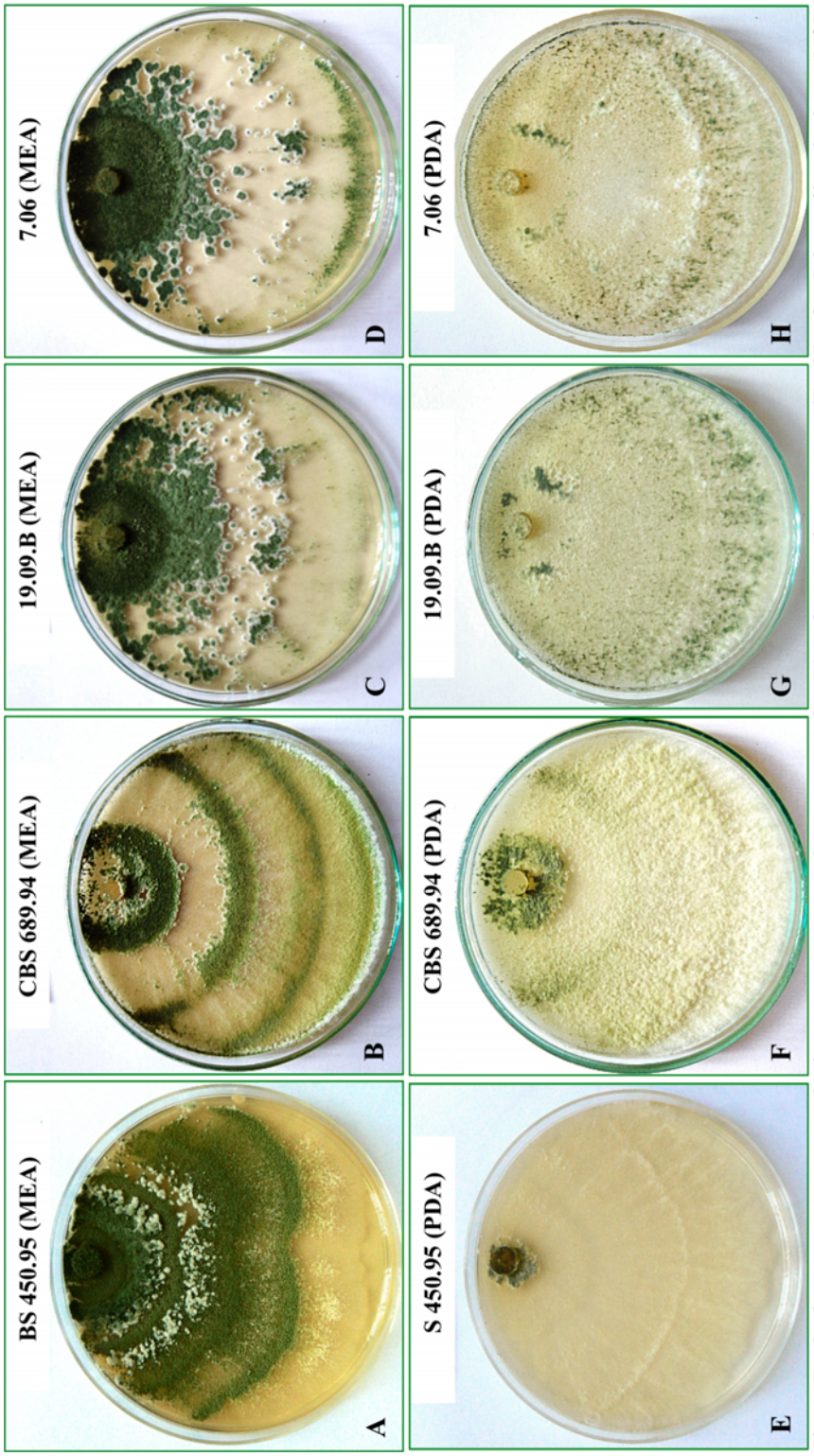


Fig. 1. *Trichoderma aggressivum* colonies appearance on malt extract agar (A - D) and potato dextorse agar (E – H) after 7 days of incubation at 27°C: A, E – reference strain *T. aggressivum* f. *aggressivum*, B, F – reference strain *T. aggressivum* f. *europeanum*, C, G and D, H – Polish strains of *T. aggressivum*

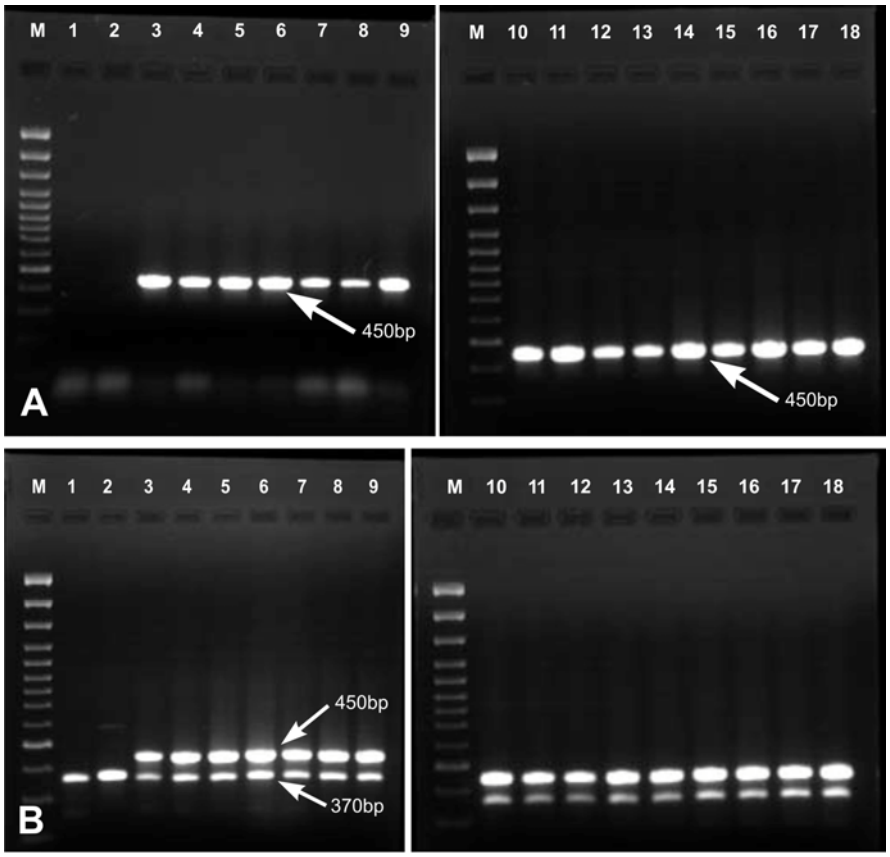


Fig.2. PCR amplification of genomic DNA using primer Th-F/Th-R (A) and multiplex PCR with primersTh-F/Th-R-and β tub-F/β-tubR(B).
Lanes:**1-4**,isolates obtained from CBS collection:**1**-*T. harzianum*,Th-1; **2**-*T. atroviride* ,Th-3; **3**-*T.agrgressivum* f*aggressivum* ,Th-4; **4**-T.*aggressivum* f.*europeanum* **,**Th-2; lines **5-18,**isolates from polish mushroom farms (presented inTable1); M–100 bp DNAladder

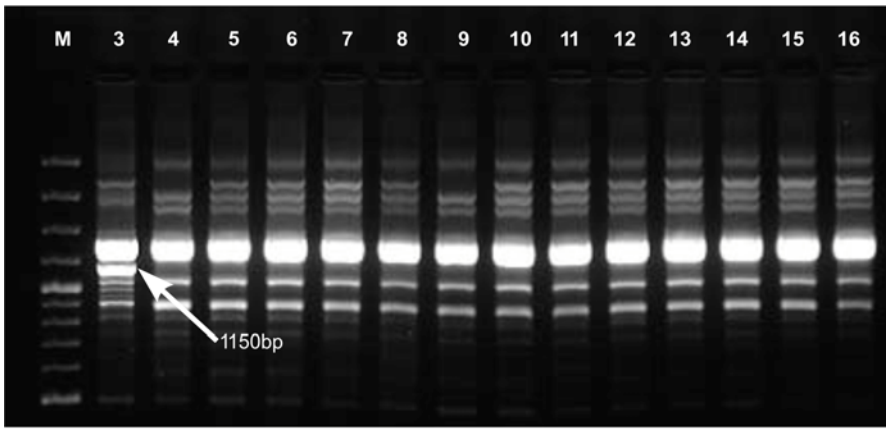


Fig 3. RAPD amplification of genomic DNA using primer 232
Lanes: **3 - 4**, isolates obtained from CBS collection: **3**- *T. agrgressivum* f.*aggressivum* , Th-4; **4**- *T. aggressivum* **f.** *europeanum* **,** Th-2**; 5-16,** isolates from polish mushroom farms (presented in Table 1) ; M – 100 bp DNA ladder
← specific RAPD fragment for reference isolate Th-4

The test on the aggressiveness of *Trichoderma* spp. has shown that all the strains designated as *T. aggressivum* grew intensively on compost colonised with *A. bisporus* (Table 3). However, in the absence of *A. bisporus* mycelium fungal development in the compost was not observed, even after 24 days of incubation. It has been found that metabolites of *A. bisporus* stimulated the growth of *T. aggressivum* f. *europeanum* (Mumpuni *et al*. 1998). In the case of other *Trichoderma* species used in the tests, neither of the isolates colonised the composts (data not presented). It suggests that only *T. aggressivum* is able to colonise aggressively the growing media for mushroom production and cause the green mold. The other *Trichoderma* species, even when present in the compost, casing material or in the growing houses, are not the disease agents.

Table 3. The growth of *T. aggressivum* isolates in the compost precolonised with *A. bisporus*

Isolate	Radius (mm) of *T. aggressivum* colony	
	after 8 days	after 14 days
SP.22.07	62	83
7.06	58	83
14.12.2C	58	83
11.02	60	83
16.11.2A	62	83
19.09.E	53	83
2.02.A	59	83
11.08.C	59	83

There were not significant differences between treatments at P=0.05

CONCLUSIONS

1. Morphological and molecular studies have shown that the Polish isolates of *Trichoderma* causing green mold belonged to the species T. *aggressivum* f. *europeanum*.
2. A variation between Polish isolates and reference strains of *T. aggressivum* f. *europeanum* indicated the possibility that Polish biotypes of *T. aggressivum* were different from those, which were found in western Europe.
3. The strains of *T. aggressivum* grew intensively on compost colonised with *A. bisporus*, but were not able to grow in the absence of *A. bisporus* mycelium.

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TRICHODERMA – SPRAWCA ZIELONEJ PLEŚNI W UPRAWIE PIECZAREK W POLSCE

Streszczenie

Pierwsze doniesienia na temat występowania zielonej pleśni w uprawie pieczarek w Polsce pochodzą z 2002 r. Początkowo, w Europie i Stanach Zjednoczonych jako

sprawcę zielonej pleśni wskazywano *Trichoderma harzianum*. Wkrótce jednak wyodrębniono dwa biotypy, które uznano za czynnik wywołujący straty. Oba agresywne biotypy oznaczono jako nowy gatunek *Trichoderma aggressivum*, przy czym biotyp Th2 określono jako *T. aggressivum* f. *europeanum* natomiast Th4 jako *T. aggressivum* f. *aggressivum*. Do tej pory nie ma jednak doniesień na temat, która z form *T. aggressivum* jest sprawcą choroby w pieczarkarniach w Polsce i co jest głównym źródłem infekcji. W związku z tym rozpoczęto ocenę składu gatunkowego grzybów z rodzaju *Trichoderma* występujących w pieczarkarniach, ze szczególnym uwzględnieniem form agresywnych. Uzyskane izolaty identyfikowano na podstawie cech morfologicznych. Wstępną klasyfikację form agresywnych *Trichoderma* potwierdzano przy użyciu metod opartych na łańcuchowej reakcji polimerazy (PCR). Badano epidemiologię *T. aggressivum*, a także stopień patogeniczności zebranych izolatów w porównaniu do szczepów wzorcowych pochodzących z innych krajów.

Najczęściej izolowanymi gatunkami *Trichoderma* w zakładach związanych z produkcją pieczarki były *T. harzianum, T. atroviride*, *T. aggressivum* oraz *T. longibrachiatum*. Przy wykorzystaniu techniki PCR , multiplex PCR i RAPD stwierdzono, że badane 24 izolaty pochodzące z polskich pieczarkarni należą do biotypu *T. aggressivum* f. *europeanum* (Th2). Stwierdzono dużą zmienność morfologiczną między izolatami sklasyfikowanymi jako *T. aggressivum,* wskazującą na możliwość wytworzenia biotypów odmiennych od występujących na terenie Europy Zachodniej. Mimo to wszystkie izolaty wykazywały bardzo silną patogeniczność wobec grzybni pieczarki (*Agaricus bisporus*).