IMPACT OF INFECTIONS WITH *Trichoderma pleurotum* AND *Trichoderma pleuroticola* ISOLATES ON YIELDING OF WILD STRAINS OF *Pleurotus ostreatus* (Fr.) Kumm. OBTAINED FROM NATURAL SITES

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Abstract. Green moulds of *Trichoderma* genus cause high losses in oyster mushroom cultivations in many countries. Recently two new species i.e. *T. pleurotum* and *T. pleuroticola* have been identified. In the experiment yielding levels of *Pleurotus ostreatus* growing on substrates infected with *Trichoderma* isolates were investigated. The following *Trichoderma* isolates were used: *T. pleurotum* designated as KWK/17 and *T. pleuroticola* designated as KMS/21 as well as three wild strains and one cultivated strain of *P. ostreatus*. It was found that the substrate infections with *T. pleurotum* and *T. pleuroticola* isolates caused significant yield reductions of the examined strains of oyster mushrooms. The infection with the *T. pleuroticola* KMS/21 isolate caused a significantly higher yield drops than the infection with the *T. pleurotum* KWK/17 isolate in comparison with yields recorded from uninfected substrates.

Key words: green moulds, molecular identification, substrate, oyster mushroom, yield reduction

INTRODUCTION

Occurrence of green moulds in cultivations of oyster mushrooms was reported in many different countries, among others in North America [Sharma and Vijay 1996], in South Korea [Park et al. 2004, 2006], in Italy [Woo et al. 2004], in Hungary [Hatvani et al. 2007] and in Romania [Kredics et al. 2006]. Recent experiments confirmed that oyster mushroom cultivations are infected by two genetically closely related, although phenotypically different, *Trichoderma* species, namely: *T. pleuroticola* and *T. pleuro-*

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tum [Komon-Zelazowska et al. 2007]. Both of the above-mentioned species were found in plantations and substrates in Europe, Iran and South Korea. The *T. pleuroticola* species was also isolated from soil and wood in Canada, USA, Europe, Iran and New Zealand [Park et al. 2004 a, 2004b, 2004c, 2006; Szekeres et al. 2005; Komon-Zelazowska et al. 2007]. It is not clear yet if the above species also occur in the natural environment in which *Pleurotus ostreatus* can be found. The above-mentioned species occurring in natural environment are likely to occupy various ecological and trophic niches [Komon-Zelazowska et al. 2007]. A number of *Trichoderma* species were found to occur in the neighbourhood of the *P. ostreatus* species. The most frequent species was that of *T. pleuroticola* but other species such as *T. harzianum*, *T. longibrachiatum* and *T. atroviride* were also identified [Kredics et al. 2009].

In recent years a PCR marker has been developed which can be employed for rapid identification of the two discussed *Trichoderma* species most frequently found in oyster mushroom cultivations, i.e. *T. pleuroticola* and *T. pleurotum* [Park et al. 2006, Kredics et al. 2009].

The aim of the performed experiments was to determine the impact of the infection with *T. pleuroticola* and *T. pleurotum* isolates on yields of wild strains and one cultivated strainof *Pleurotus ostreatus* (Fr.)Kumm obtained from natural sites.

MATERIAL AND METHODS

Table 1 provides information about places from which *Trichoderma* isolates used in the described experiments were obtained.

Table 1. List of isolates of *Trichoderma* used in the trial
Tabela 1. Wykaz izolatów *Trichoderma* użytych w doświadczeniu

Isolate – Izolat	Date of collection Data zbioru	Place of collection Miejsce zbioru	Identification Identyfikacja
T. pleuroticola KMS/21	2009	oyster mushroom farm boczniakarnia Łobez	Institute of Genetics Instytut Genetyki Poznań
T. pleurotum KWK/17	2009	oyster mushroom farm boczniakarnia Kościan	Institute of Genetics Instytut Genetyki Poznań

Trichoderma isolates were identified on the molecular level with the assistance of the analysis of ITS1 and ITS2 rDNA regional sequence and on the basis of the analysis of species-specific PCR marker.

Trichoderma isolates were grown in 50 ml Czapek-Dox broth (Sigma) with Yeast Extract (Oxoid) and streptomycin sulphate (50 mg/L$^{-1,}$ AppliChem), for 6 days at 25ºC on a rotary shaker (100 rpm). Mycelia were collected on filter paper in a Buchner funnel, washed with sterile water, frozen at -20ºC and freeze-dried. Total DNA was extracted using the CTAB method [Doohan et al. 1998].

The PCR for amplification of the internal transcribed spacers 1 and 2 of the rRNA gene cluster (ITS1 and 2 including the 5.8S RNA gene) was performed in 25 μl reaction mixture containing: 1 μl 50 ng/μl of DNA, 2.5 μl 10 × PCR buffer (50 mM KCl, 1.5 mM MgCl2, 10 mM Tris-HCl, pH 8.8, 0.1% Triton X-100), 1.5 μl 10 mM dNTP (GH Healtcare), 0.2 μl 100 mM of each primers, 19.35 μl MQ H2O, 0.25 μl (2 U/μl) DyNAzymeTM II DNA Polymerase (Finnzymes). Amplifications were performed in either a PTC-200 or PTC-100 thermocycler (MJ-Research, USA) under the following program conditions: 5 min at $94^{o}C$ – initial denaturation, 35 cycles of 45 s at $94^{o}C$, 45 s at $58°C$, 1 min at $72°C$, with the final extension of 10 min at $72°C$. The primers used for amplification and sequencing ITS were ITS4 and ITS 5.

PCR amplification of DNA marker for *T. pleurotum* and *T. pleuroticola* identification was performed according to Kredics et al. [2009]. The primers used for amplification of this marker were FPforw1, FPrev1 and PSrev1 [Kredics et al. 2009].

Amplification products were separated on a 1.5% agarose gel (Invitrogen) in 1 × TBE buffer (0.178 M Tris-borate, 0.178 M boric acid, 0.004 M EDTA) and stained with ethidium bromide. The 10 μl PCR products were combined with 2 μl of loading buffer (0.25% bromophenol blue, 30% glycerol). A 100 bp DNA Ladder Plus (Fermentas) was used as a size standard. PCR products were electrophoresed at 3 V/ cm for about 2 h, visualized under UV light and photographed (Syngen UV visualiser).

For sequencing of ITS1 and ITS2 rDNA fragment, the 3 μl PCR products were purified with exonuclease I and shrimp alkaline phosphatase according to Chełkowski et al. [2003]. Sequencing reactions were prepared using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit in 5 μl volume (AppliedBiosystems, Switzerland). DNA sequencing was performed on ABI PRISM 310 Genetic Analyzer (USA). Sequences were edited and assembled using Chromas v. 1.43 [Applied Biosystems]. ClustalW [Thompson et al. 1994] were used to align the sequences. Species were identified with use of BLAST [Altschul et al. 1990] and *Tricho*Key, *Tricho*BLAST [www.isth.info; Druzhinina et al. 2005].

Oyster mushroom strains used in experiments derived from different regions of Western Poland. Table 2 collates sites from which samples of oyster mushroom employed in these investigations were collected. The control was a P80 strain of oyster mushroom which derived from the collection of cultivated and medicinal mushrooms of the Department of Vegetable Crops of Poznań University of Life Sciences.

Maternal mycelia of wild strains and of the cultivated strain were prepared in the biological laboratory of the Department of Vegetable Crops of Poznań University of Life Sciences and later proliferated at the Cultivated Mushroom Plant in Łobez near Jarocin. The material used for substrate inoculation was grain mycelium prepared according to the formulation developed by Lemke [1971].

The substrate used in growing trials was straw cut into chaff of 3–5 cm length and soaked in water for 24 hours. Substrate pH value was fixed at 6.4–6.6 achieved by the addition of calcium carbonate $CaCO_3$ in the form of fertilizer chalk. The required addition of chalk was established on the basis of neutralisation curves. The prepared chaff of 70% moisture content was pasteurised at the temperature of $60^{o}C$ for the period of 48 h. After pasteurisation, the cut straw was cooled to the temperature of about $25^{o}C$, inoculated with mycelium and placed in perforated plastic bags. Each bag was filled with

12 kg substrate. Substrate incubation was conducted in the dark in a cultivation facility in which the temperature was maintained at the level of 20–21°C and humidity – at 80–85%.

Table 2.   List of *P. ostreatus* strains used in the experiment
Tabela 2. Wykaz ras *P. ostreatus* użytych w doświadczeniu

Strain Rasa	Date of harvest Termin zbioru	Place of harvest Miejsce zbioru	Site Stanowisko
KW.B110/5	X 2008	RDLP* Piła, Forest District Trzcianka Forest-range Jędrzejewo	on beech na buku
KW.F.14/7	IX 2009	RDLP Szczecinek Forest District Miastko Forest-range Biały Bór	on beech na buku
KW.A.19/5	X 2008	RDLP Poznań Forest District Piaski Forest-range Mchy	on oaks na dębach
P80	strain from the mushroom collection of the Department of Vegetable Crops of Poznań University of Life Sciences odmiana z kolekcji grzybów Katedry Warzywnictwa UP w Poznaniu		

*RDLP-Regional Directorate of State Forest/ Regionalna Dyrekcja Lasów Państwowych
Forest District/ Nadleśnictwo, Forest-range / Leśnictwo

Mycelium of *T. pleuroticola* and *T. pleurotum* isolates, similarly to the mycelium of oyster mushroom, was also prepared on wheat grain. The infection of the cultivation substrates with the grain mycelium of the above-mentioned *Trichoderma* species was carried out on the 12th day of incubation. The cultivation substrate of 12 kg weight was inoculated with the mycelium of the examined *Trichoderma* species using a tube of 3 cm diameter equipped in a small piston which allowed introduction of approximately 5 g of mycelium to the depth of 9–10 cm. The grain mycelium of *T. pleuroticola* and *T. pleurotum* isolates was injected into 5 precisely defined places on each side of the cultivation block in such a way as to achieve a uniform distribution of the inoculum. Each block was inoculated with 50 g of the grain mycelium of the above-mentioned *Trichoderma* isolates. Substrate inoculation after infection with *Trichoderma* isolates took place in darkness in the cultivation facility at the temperature of 20–21°C and humidity – at 80–85%.

In the described experiments, only carpophore yields of the first flush were harvested because, due to the infections with *Trichoderma* isolates, fruiting bodies of the second flush did not appear. Harvest was carried out when cap edges of carpophores turned up earlier, were coming to horizontal position. Single carpophores and their whole clusters were broken off from the substrate. In cases when several carpophores were developing from one base, they were harvested at once when majority of mushrooms in the cluster were opened up. The harvesting period in this trial lasted 16 days. Two cultivation cycles were carried out. Research results were analysed on the basis of

mean values from two cultivation cycles applying the analysis of variance for factorial experiments at α = 0.05 using Newman-Keuls test.

RESULTS AND DISCUSSION

Following the amplification reaction in the ITS1 and ITS2 region of rDNA carried out for the two *Trichoderma* isolates: KMS/21 and KWK/17, PCR products of 570 bp were obtained. The obtained amplification products were sequenced. On the basis of comparative analysis of ITS1 and ITS2 sequences obtained for KMS/21 and KWK/17 isolates with sequences stored in NCBI (www.ncbi.nlm.nih.gov) and ISTH (www.isth.info) databases, the isolates were identified as *T. pleuroticola* (KMS/21) and *T. pleurotum* (KWK/17). The comparative analysis of ITS sequences obtained in this study and ITS sequences of T1295 *T. pleuroticola* and CPK 21 17 *T. pleurotum* strains available in the GenBank NCBI database is presented in fig. 1.

As the result of the PCR carried out using Fpforw1 and Fprev1 starters [Kredics et al. 2009], a DNA fragment approximately 447 bp long was obtained both for KMS/21 and KWK/17 isolates. Following the PCR performed employing Fpforw1 and Psrev1 starters [Kredics et al. 2009], a 218 bp long amplification product was obtained only for the KWK/17 isolate. The presence of this PCR product indicated that the 7 isolate belonged to the *T. pleurotum* species. The obtained result is in keeping with the result obtained on the basis of the analysis of the ITS1 and ITS2 region of rDNA of the examined isolates. Moreover, it also corroborates the specificity of the PCR marker developed by Kredics et al. [2009] for *T. pleurotum* and *T. pleuroticola* species.

The highest yield on the non-infected substrate was recorded for the P80 strain (182 g·kg^{-1} fresh matter of substrate). The examined wild oyster mushroom strains gave much lower yields. The highest yield from the investigated strains was recorded for the KW.F.14/7 (123 g·kg^{-1} fresh matter of substrate) followed by the KW.B.110/5 and KW.A.19/5 strains. The cultivation of the above-mentioned wild strains and the cultivated strain on the substrate infected with *T. pleurotum* KWK/17 isolate caused a very significant yield reduction. The highest yield on the infected substrate was recorded for the KW.F.14/7 strain followed by the cultivated strain P80, while the lowest yields were observed in the case of the KW.A.19/5 strain (24 g·kg^{-1} fresh matter of substrate) (fig.2).

When analysing the percentage yield drop on the substrate infected with the above-mentioned isolate *T. pleurotum* KWK/17, it is worth stressing that it varied widely (tab. 3). The strongest yield decline was registered for the P80 strain (70.9%) and the wild strain KW.A.19/5 (68.8%), while the lowest yield decrease was recorded in the case of the wild strain KW.F.14/7 (44.7%). This relatively small yield drop in the course of cultivation on the substrate infected with the *T. pleurotum* KWK/17 isolate was responsible for the highest yield obtained when this strain was cultivated on the infected substrate (68 g·kg^{-1} fresh matter of substrate).

Analysing yields of the examined oyster mushroom strains on substrates infected with *Trichoderma pleurotum* KWK/21 isolate, it can be said that it caused a severe yield reduction. Yields obtained on infected substrates fluctuated from 10 to 35 g·kg^{-1} fresh
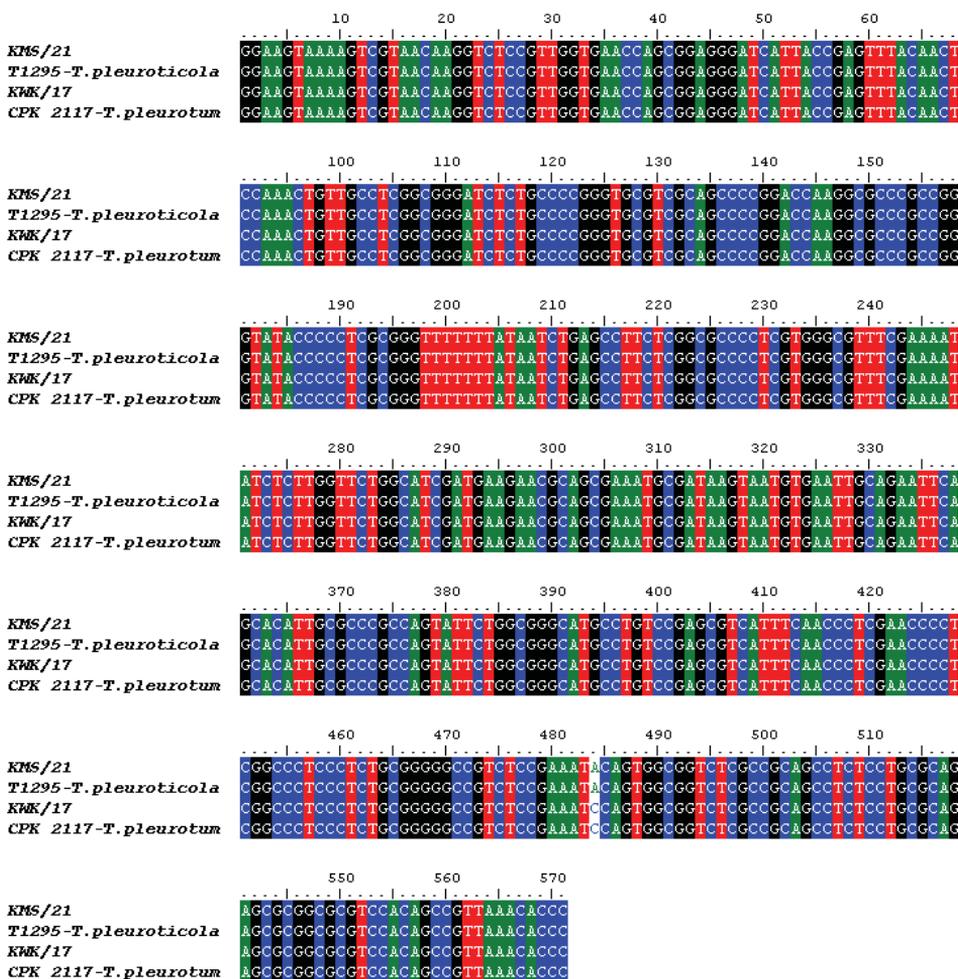
Fig. 1. Comparative analysis of ITS1 and ITS2 rDNA sequences obtained for M141 and T127 isolates and ITS1 and ITS2 rDNA sequences of T1295 *T. pleuroticola* and CPK 21 17 *T. pleurotum* strains available in the GenBank NCBI database

Rys. 1. Analiza porównawcza sekwencji ITS1 i ITS2 rDNA uzyskanych dla izolatów M141 i T127 oraz sekwencji ITS1 i ITS2 rDNA szczepów T1295 *T. pleuroticola* i CPK 21 17 *T. pleurotum,* dostępnych w bazie danych GenBank NCBI

matter of substrate, so it was considerably smaller in comparison with yields recorded on substrates infected with *T. pleurotum* KWK/17 isolate (fig. 3). The highest yield on the infected substrate was found in the case of the P80 strain (35 g·kg^{-1} fresh matter of substrate). Similar yields were recorded for the wild KW.F.14/7 (31 g·kg^{-1} fresh matter of substrate) and KW.F.110/5 (23 g·kg^{-1} fresh matter of substrate) strains. The lowest yield was found for the KW.A.19/5 strain (10 g·kg^{-1} fresh matter of substrate).
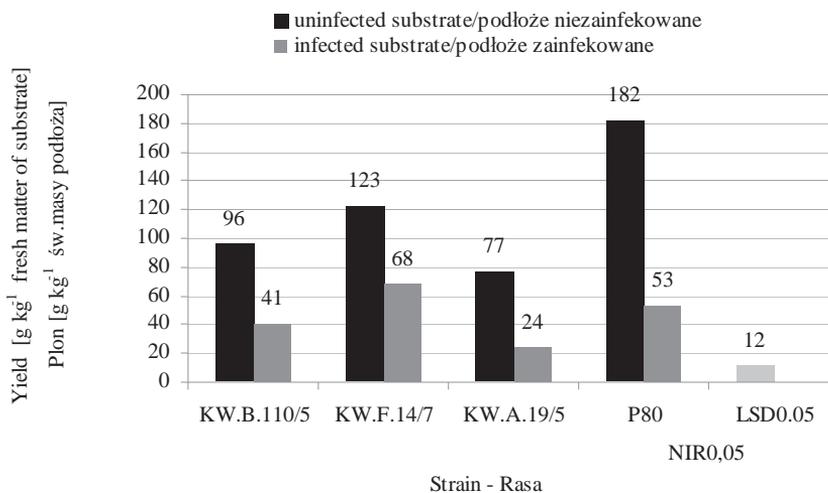
Fig. 2. Yielding of wild strains and a cultivated strain of *P. ostreatus* on substrate infected with *T. pleurotum* KWK/17 isolate

Rys. 2. Plonowanie ras dzikich i odmiany uprawnej *P. ostreatus* na podłożu zainfekowanym izolatem *T. pleurotum* KWK/17

The analysis of percentage yield losses in oyster mushroom growing on the substrate infected with *T. pleuroticola* KMS/21 isolate revealed that this reduction was similar in all combinations and ranged from 76.7% in the KW.F.14/7 strain to 81.9% in the cultivated P80 strain (tab. 4). Comparing the impact of the two investigated *Trichoderma* isolates on yields of three wild strains and the cultivated strain of the oyster mushroom, it can be concluded that the *T. pleurotum* KWK/17 isolate caused a relatively smaller yield loss than the *T. pleuroticola* KMS/21 isolate. One of the examined wild *P. ostreatus* strains – KW.F.14/7 – exhibited a relatively small yield reduction (44.7%) in relation to the cultivation on the uninfected substrate. On the other hand, in the case of infection with the *T. pleuroticola* KMS/21 isolate, none of the examined strains exhibited a distinctly higher yield loss.

Table 3. Percentage yield reduction of wild strains and a cultivated strain of *P. ostreatus* on substrate infected with *T. pleurotum* KWK/17 isolate

Tabela 3. Procentowa obniżka plonu ras dzikich i odmiany uprawnej *P. ostreatus* na podłożu zainfekowanym izolat *T. pleurotum* KWK/17

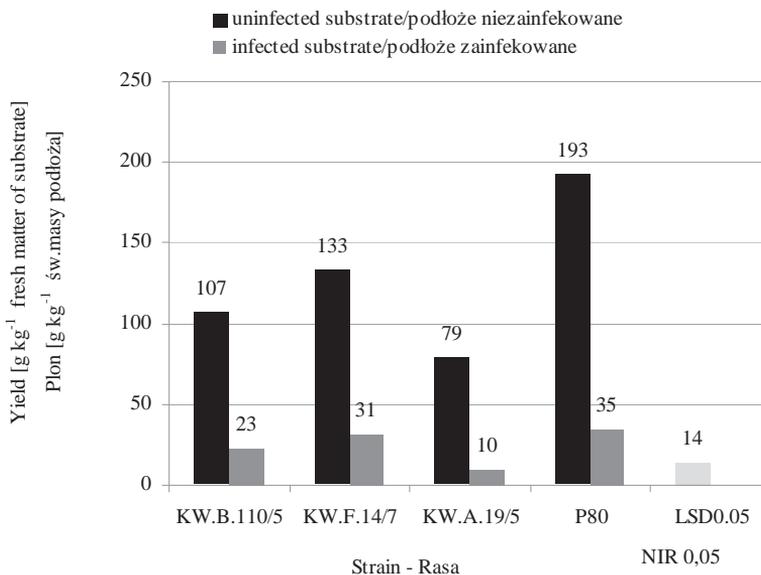| *P. ostreatus* strain + *T. pleurotum* isolate Rasa boczniaka + izolat *T. pleurotum*	Percentage yield reduction Procentowa obniżka plonu
KW.B.110/5 + *T. pleurotum* KWK/17	57.3
KW.F.14/7 + *T. pleurotum* KWK/17	44.7
W.A.19/5 + *T. pleurotum* KWK/17	68.8
P80 + *T. pleurotum* KWK/17	70.9

Fig. 3. Yielding of wild strains and a cultivated strain of *P. ostreatus* on substrate infected with *T. pleuroticola* KMS/21 isolate

Rys. 3. Plonowanie ras dzikich i odmiany uprawnej *P. ostreatus* na podłożu zainfekowanym izolatem *T. pleuroticola* KMS/21

Table 4. Percentage yield reduction of wild strains and a cultivated strain of *P. ostreatus* on substrate infected with *T. pleuroticola* KMS/21 isolate

Tabela 4. Procentowa obniżka plonu ras dzikich i odmiany uprawnej *P. ostreatus* na podłożu zainfekowanych izolatem *T. pleuroticola* KMS/21

P.ostreatus strain + *T. pleuroticola* isolate Rasa *P. ostreatus* + izolat *T. pleuroticola*	Percentage yield reduction Procentowa obniżka plonu
KW.B.110/5 + *T. pleurticola* KMS/21	78.5
KW.F.14/7 + *T. pleuroticola* KMS/21	76.7
W.A.19/5 + *T. pleuroticola* KMS/21	77.3
P80 + *T. pleuroticola* KMS/21	81.9

There is no data in the literature dealing with the effect of the examined *T. pleurotum* and *T. pleuroticola* isolates on yields of wild *P. ostreatus* strains derived from natural sites. Earlier investigations made by the authors indicated that fungi of *Trichoderma* genus, in particular the *T. aggessivum* f. *europaeum* species, can cause considerable yield reductions of the cultivated strain and wild strains of *Agaricus bitorquis* [Sobieralski et al. 2010a] as well as *Pleurotus eryngii* [Sobieralski et al. 2010b]. Other experiments carried out by the authors revealed that different strains of the *A. bisporus* genus, the so called white and brown strains, exhibited varying sensitivity to infection by fungi

of the *Trichoderma* genus. Brown varieties exhibited significantly lower sensitivity than white ones [Sobieralski et al. 2009]. Earlier studies of the identical nature conducted by the same researchers concerning the impact of different *Trichoderma* strains on the development of *Coprinus comatus* mycelium showed that the inhibition of mycelium growth by *T. longibrachiatum* and *T. atroviride* was significantly smaller in comparison with the growth inhibition caused by *T. aggressivum* [Frużyńska-Jóźwiak et al. 2010].

The performed investigations fully corroborated earlier results regarding high yield losses which fungi of the *Trichoderma* genus may cause in cultivations of edible mushrooms. The experiments showed that *T. pleuroticola* species caused considerably higher yield losses of *P. ostreatus*. The obtained results also corroborate, to a certain extent, earlier observations regarding the speed of growth in *in vitro* conditions of *T. pleurotum* and *T. pleuroticola* mycelium. In contrast to the *P. pleurotum*, *T. pleuroticola* did not cause any visible antagonistic reaction from the *P. ostreatus* culture and was able to overgrow the *Pleurotus* colony within 4 to 6 days [Komon-Zelazowska et al. 2007)].

CONCLUSIONS

1. All the examined wild *P. ostreatus* strains cultivated on the non-infected substrate gave much lower yields than P80 strain. Yield of one wild strain (KW.F.14/7) on substrate infected with *T. pleurotum* KWK/17 isolate was higher than in the case of P80 strain.

2. Substrate infection with *T. pleurotum* KWK/17 and *T. pleuroticola* KMS/21 strains caused significant yield reductions of the examined *P. ostreatus* strains.

3. Substrate infection with *T. pleurotum* KWK/21 isolate caused significantly higher yield losses than infection with *T. pleurotum* KWK/17 isolate in comparison with the yield recorded on the non-infected substrate.

4. Infection of the substrate with *T. pleurotum* KWK/17 isolate caused varying percentage yield losses of *P. ostreatus*. One of the examined wild strains (KW.F.14/7) exhibited a relatively low yield drop and differed considerably in this regard from the remaining wild strains as well as the cultivated strain.

5. All the examined *P. ostreatus* strains exhibited very high and similar yield losses when cultivated on the substrate infected with the studied *T. pleuroticola* KMS/21 isolate.

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**WPŁYW INFEKCJI IZOLATAMI *Trichoderma pleurotum*
I *Trichoderma pleuroticola* NA PLONOWANIE RAS DZIKICH
Pleurotus ostreatus (Fr.) Kumm POZYSKANYCH ZE STANOWISK
NATURALNYCH**

Streszczenie. W wielu krajach straty w uprawach boczniaka powodowane są przez zielone pleśnie rodzaju *Trichoderma*. W ostatnich latach zidentyfikowano dwa nowe gatunki *T. pleurotum* i *T. pleuroticola*. W doświadczeniu badano wielkość plonu *P. ostreatus* na podłożu zainfekowanym izolatami *Trichoderma*. Użyto izolatów *T. pleurotum* KWK/17 i *T. pleuroticola* KMS/21. Wykorzystano trzy rasy dzikie pochodzące ze stanowisk naturalnych oraz rasę uprawną *P. ostreatus*. Stwierdzono, że infekcja podłoża izolatami *T. pleurotum* oraz *T. pleuroticola* powodowała znaczną obniżkę plonu badanych ras dzikich oraz odmiany uprawnej *P. ostreatus*. Infekcja izolatem *T. pleuroticola* KMS/21 powodowała znacznie większą obniżkę plonu w stosunku do plonu uzyskanego z podłoża niezainfekowanego niż infekcja izolatem *T. pleurotum* KWK/17. Infekcja izolatem *T. pleurotum* KWK/17 powodowała bardzo różną procentową obniżkę plonu badanych ras *P. ostreatus*. Rasa KW.F.14/7 wykazała stosunkowo małą obniżkę plonu, wyraźnie różniąc się pod tym względem od pozostałych ras dzikich i odmiany uprawnej.

Słowa kluczowe: zielone pleśnie*,* identyfikacja molekularna, podłoże, boczniak ostrygowaty, obniżka plonu