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ENZYMATIC AND ANTAGONISTIC POTENTIAL OBSERVED IN FLAX-ROOT-INFECTING FUNGI

Anna Ligocka, Zbigniew Paluszak, Stanisław Sadowski, Teresa Dziedzic
Department of Microbiology, University of Technology and Agriculture, Bydgoszcz, Poland

ABSTRACT

The quantitative and qualitative composition of the soil microorganisms depends, amongst others, on whether monoculture or crop rotation is applied. The main phytopathogen infecting flax (*Linum usitatissimum* L.) is the *Fusarium* genus as well as fungi of the *Alternaria, Phoma, Botrytis, Verticillium, Rhizoctonia* genera, however soil hyperparasites of the *Trichoderma* genus can control them successfully. Both types of microorganisms were isolated from the roots sampled both from monocuture and crop rotation and from plants in emergence and flowering stage. The enzymatic activity was researched for a variety of nutrient substrates (cellulose, pectin, starch, protein) as well as the capacity for dissolving triphosphates. There was also investigated the extent of *Trichoderma* fungal antagonistic activity towards pathogens. *Fusarium* spp. was most frequent over emergence in monoculture; the quantity was similar in monoculture and in crop rotation as well as over emergence and flowering. The greatest number of *Trichoderma* sp. isolates was obtained from crop rotation. Enzymatic tests show that all the pathogens are more active towards carbohydrates and protein, as compared with saprophytes. *Trichoderma* fungi, except one, turned very good antagonists towards pathogens. The isolates obtained from the monoculture inhibited the growth of parasites much less considerably than those from the crop rotation.

Key words: antagonism, *Fusarium, Trichoderma*, flax - *Linum usitatissimum* L., phytopathogenic fungi

INTRODUCTION

Arable land-soil phytosanitary status and the intensity of plant disease depend considerably on crop rotation. Monoculture can often affect the microflora development and the activity, hence frequent soil deterioration and decrease in yielding [8,12]. Flax cannot be sown more frequently than every 6-7 years in the same field, otherwise there can appear symptoms of soil exhaustion. The danger of disease decreases with a decrease in the

frequency of host plant cultivation on the same stand. All that is much affected by appropriate tolerant species selection [17] as well as the sequence in crop rotation [10], which makes the plants themselves as well as developing antagonistic microorganisms inhibit the pathogen development.

The greatest flax yield losses ranging from 10 to 20% are caused by fungal phytopathogens [24], including most frequent *Fusarium avenaceum, F. culmorum* and *F. oxysporum*, which inflict fusariosis with its wilting and plant decaying over flowering as well as water balance disturbances [11]. Similarly flax gets infested by *Rhizoctonia solani*, however its macroscopic symptoms are difficult to identify [23].

A special significance is attributed to soil microorganisms which prevent plant infection due to their competitive or hyperparsitic properties [21]. *Trichoderma* spp. is also quite frequent, participating in biocontrol and affecting other fungi directly [4,19,20] or indirectly producing numerous antibiotics [21].

The aim of the present paper was to compare the quantitative and qualitative composition of pathogenic and saprotrophic fungi in rhyzoplane of flax cultivated in 30-year monoculture and in 6-year crop rotation and to define the fungal enzymatic and antagonistic potential. Flax was addressed due its easily identifiable pathogen-inflicted symptoms, especially under long-established monoculture field experiment. The research hypothesis assumed a considerable quantitative and qualitative fungal composition both in monoculture and crop rotation; the latter enhances protection against pathogens, mainly due to a development of antagonistic microorganisms, which, in turn, helps the soil fungistatic properties.

MATERIAL AND METHODS

The research covered the rhizoplane of fibrous flax – *Linum usitatissimum* L., 'Artemida' cultivar, obtained from 6-year crop rotation (flax, rye, faba bean, winter triticale, potato, oats) and from 30-year monoculture, experimental plot of the Experiment Station at Bałcyny in the vicinity of Ostróda. The plants were sampled at emergence and flowering stages.

The material preparation included: 1 - rinsing plants under running water, drying; 2 - evaluating root health status with a 9-degree scale following Hillstrand and Auld [24]; 3 - preparing inocula (0.5 cm of main root) and rinsing under running water for 1h; 4 - surface-disinfecting of inocula with 75% alcohol and $HgCl_2$ (sublimate) and 3-times rinsing in sterile distilled water, 5- transferring of inocula onto the PDA medium (6 inocula per one Petri dish); 6 - incubating in thermostat over 4-5 days at 20°C; 7- establishing pure fungal cultures on PDA medium and their identification following mycological monographs [1,2,3,7].

1. Testing fungal enzymatic potential

The test covered the cellulose, amylase, pectin breakdown potential and proteolytic potential of the microorganisms isolated; the isolates were transferred onto media of different carbon sources: cellulose (CMC), starch, pectin and gelatine and incubated at 20°C over 7-14 days. Then there was investigated enzymatic potential as affected by the diameter of the nutrient substrate hydrolysis which was observed once the media were poured with a culture treated with reagents: medium with cellulose (CMC) was poured with the solution of Kongo red (15 min), which was followed by treatment with 1 M NaCl (15 min), starch medium – with iodine solution, pectin medium – with J in K, while gelatine medium – with Frazier reagent ($HgCl_2$ + HCl concentrated). There was also investigated an isolate capacity for transforming insoluble triphosphates into soluble monophosphates on the phosphate medium. The 3-week culture was tested for the occurrence of transparent zones around the microorganism growth. All the tests were carried out in three replications.

2. Evaluation of the antagonistic effect of *Trichoderma* genus on pathogens fungi

The experiment was carried out with the biotic series method which makes it possible to define the effects of the activity of antagonists in the environment [14,15,18,22]; it is often referred to as a two-species method where a single medium is used for simultaneous cultures of two fungi tested. A 6-degree scale was applied from 0 to 5 [18], where 0 stands for the fact that both fungal colonies tested (pathogen and hyperparasite) are tangible to each other along the straight line. The scale from +1 to +5 presents how the pathogenic fungus is gradually grown over by *Trichoderma* spp. (+5 - pathogen colony completely underdeveloped). The scale from –1 to –5 refers to a reverse situation where the development of *Trichoderma* spp. is limited by the pathogen.

A total of 18 fungal strains, including 8 *Trichoderma* sp. strains, were used to represent saprotrophic forms, and 5 strains of *Fusarium* sp. and one of each of the following species: *Verticilium* sp., *Phoma* sp., *Rhizoctonia solani*, *Botrytis cinerea*, *Alternaria alternata*, which represented phytopathogenic forms. A model experiment design included a two-species culture where a *Trichoderma* representative constituted one of the species and a pathogenic fungus – the other. The 96-hour fungal culture was used to cut out from the marginal mycelium 0.5 cm-in-diameter disks which were then transferred onto the test medium and placed 2 cm away from each other, with the mycelium facing the medium surface. Two disks were put onto one medium; one cut out from the saprotrophic fungus surface and the other one – from the pathogen culture. After 3 and 6 days of incubation, a stimulating or antagonistic effect observed between the fungi studied was defined following the grading scale.

RESULTS

1. Health status of fibrous flax roots

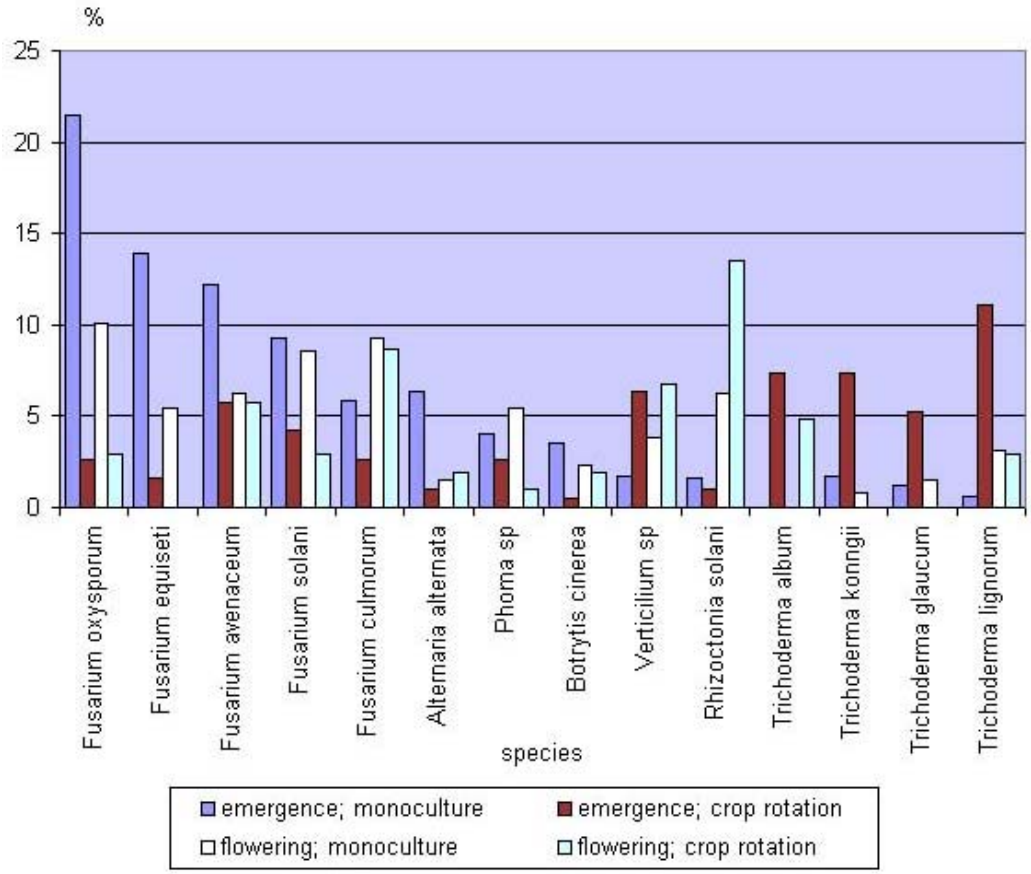
A 9-degree scale was used to evaluate the health status of 'Artemida' fibrous flax, including the development phase and the cultivation in monoculture and a 6-field crop rotation (Table 1). The flax infection index values for the cultivation in monoculture and a 6-field crop rotation show an unfavourable effect of monoculture on the fibrous flax growth, especially over emergence. One can also say that over emergence the crop root infection was greater than over flowering, both in monoculture and in crop rotation.

Table 1. Health status of 'Artemida' fibrous flax roots over emergence and flowering

Infection degree	Monoculture		Crop rotation	
	Emergence	Flowering	Emergence	Flowering
1	5.0	37.5	32.5	55.0
2	72.5	50.0	42.5	37.5
3	20.0	5.0	20.0	7.5
4	2.5	2.5	5.0	-
5	-	2.5	-	-
6	-	2.5	-	-
7	-	-	-	-
8	-	-	-	-
9	-	-	-	-
Infection index	24.42	19.42	21.93	17.6

There were obtained 605 fungal isolates out of which 14 representatives of both pathogens and saprotrophes were selected for further research (Fig. 1). *Fusarium* was the genus most often represented; its advantage over the other fungal species was considerable during emergence in monoculture (62.77%), while over flowering the percentage was much lower. The crop rotation showed a high decrease in the occurrence of these pathogens; 16.82% over emergence and 20.17% over flowering, respectively. The occurrence of other pathogenic fungi, including *Alternaria alternata*, *Botrytis cinerea*, *Rhizoctonia solani*, *Phoma* sp. *Verticilium* sp., is also of commercial importance. Over emergence in monoculture these fungi account for a total of 17.02% isolates, over flowering - 19.36% isolates, while in crop rotation – 11.56% and 24.99%, respectively, which shows that irrespective of crop rotation, a greater number of pathogens was recorded in summer – at full plant flowering. Similarly there is an interesting percentage of beneficial *Trichoderma* ssp. in the total number of other fungi. The greatest colonisation by these hyperparasites was observed in crop rotation, especially over emergence (31.03%). As for the comparison, their total percentage in the monoculture amounted to 8.9%. At flowering stage the share of these fungi was lower.

Fig. 1. Fungi population isolated from flax rhizoplane



2. Biochemical properties of fungi isolated from fibrous flax rhizoplane

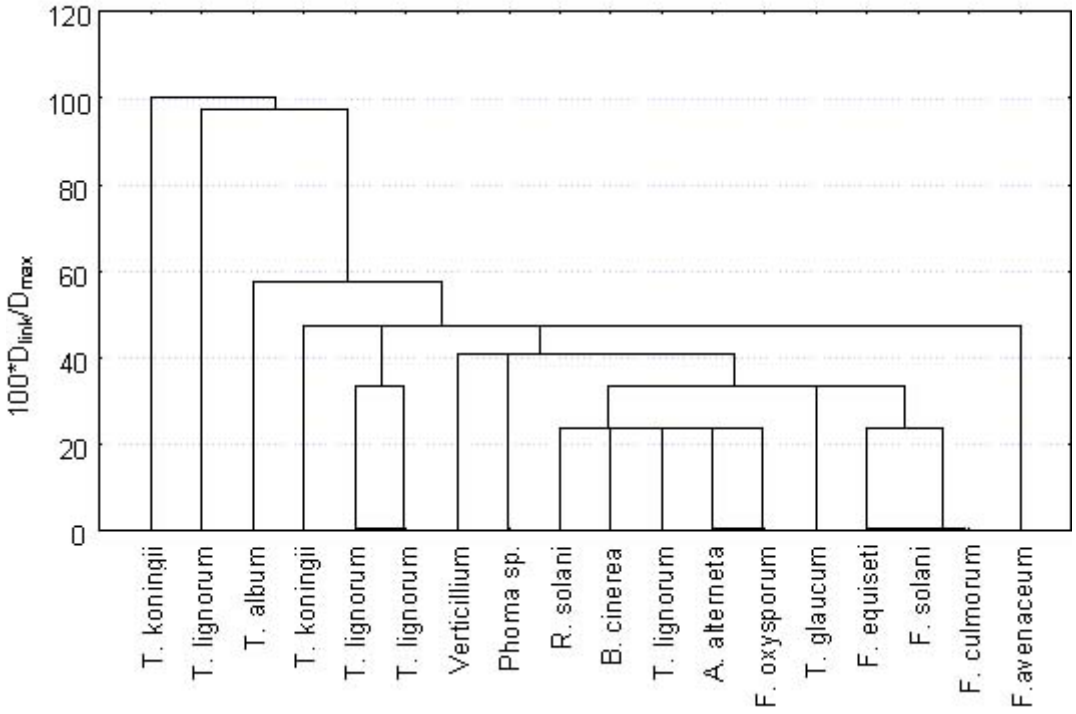
The properties were evaluated according to the strain capacity for the utilisation of nutrient substrates and transforming triphosphates into monophosphates. The results showing biochemical activity of pathogens and saprotrophes are presented in Table 2. All the *Fusarium* sp. analysed showed a high enzymatic potential towards the carbohydrates tested, especially pectin. *Fusarium solani*, *F. avenaceum* and *F. culmorum* hydrolysed pH 8 pectin better, while *F. oxysporum* and *F. equiseti* – pH 5 pectin, which shows some capacity for producing pectin lyases and polygalacturonases, respectively. Each of the fungi tested showed lower cellulose and amylase breakdown potentials. All the strains showed a high gelatine hydrolysis potential, however the most effective was *F. oxysporum* isolate. Similarly, it was the only species to show the potential for releasing phosphoric ions from mineral phosphorus forms. Other pathogens showed generally high potential for carbohydrate degradation, except for *Verticilium* sp., which showed their average hydrolysis potential. Besides the isolates of *B. cinerea, A. alternata* and *Phoma* sp., reacting with pectin showed higher potential for producing pectin lyase. The isolate which showed the lowest potential for proteolysis was *Verticilium* sp., while the highest one – *Rhizoctonia solani*. However, analysing the potential for releasing phosphate ions, it was noted that *R. solani* showed a high triphosphate dissolution activity, *A. alternata* – an average, while *Phoma* sp. – a low one. For *Verticilium* sp. and *Botrytis cinerea*, no such potential was observed. As for saprotrophic *Trichoderma* fungi, not all the isolates produced cellulases and pectinases. Some outstanding amylase breakdown properties were noted in *T. koningii* 544 and *T. lignorum* 264 isolates. However the pectin breakdown potential was noted in *T. lignorum* 574 and *T. koningii* 544 isolates. The proteolysis potential of these isolates was very high, unlike that of *T. album and T. glaucum*. *Trichoderma* spp. shows generally average and low potential for phosphate ion mobilisation; *T. lignorum* 264 isolate was the most active, while that of *T. album* showed no such activity. Irrespective of the crop rotation the isolates were obtained from, there were observed no significant differences in their biochemical potential.

Table 2. Variation in the biochemical activity of fungi against varied sources of C and non-organic phosphorus

Fungal strain	Pectin pH 8	Pectin pH 5	CMC	Starch	Protein	Phosphate
F. avenaceum Lm410	+++	++	++++	++++	++++	-
F. culmorum Lz869	++++	++++	++++	++++	++++	-
F. oxysporum Lm402	++++	++++	++++	++++	++++	++
F. solani Lm651	++++	++++	++++	++++	++++	-
F. equiseti Lm385	+++	++++	++++	++++	++++	-
Botrytis cinerea Lz132	++++	++++	++++	++++	++++	++++
Phoma sp. Lz162a	++++	+++	+++	++++	+++	+
A. alternata Lm369	++++	++++	++++	++++	+++	++
R. solani Lm664	++++	++++	++++	++++	++++	++++
Verticillum sp. Lz29a	+++	+++	+++	+++	++	-
T. koningii Lm544	++++	++++	-	++++	++++	++
T. koningii Lm339	-	-	-	+++	++++	+
T. lignorum Lz264	++++	++++	++++	++++	++++	+++
T. lignorum Lm574	++++	++++	++	++++	++++	++
T. lignorum Lz145a	++++	++++	++	+++	++++	+
T. lignorum Lz104	-	-	++++	++++	++++	++
T. glaucum Lm446	+++	++++	++++	++++	+++	+
T. album Lz51a	+++	++++	-	+++	++++	-

To group the flax root-infecting fungi, a cluster analysis was performed to test their enzymatic activity. The method of single linkage was used. The results obtained (Fig. 2) show a similar enzymatic activity of most of the fungi examined; *F. solani* and *F. culmorum* produced the same enzymes with similar activity. There is also a cluster that includes fungal strains with 75% enzymatic similarity and it consists of *R. solani*, *B. cinerea*, *T. lignorum* (a), *A. alternata*, *F. oxysporum*.

Fig. 2. Cluster analysis of flax-root infecting fungi and their enzymatic activity



3. Biotic effect of *Trichoderma* sp. on major pathogens isolated from fibrous flax rhizoplane

An evaluation of interaction of *Trichoderma* spp., with fungal pathogens has been based on the individual biotic effect [18]. The results confirm that there is a beneficial role of saprotrophic *Trichoderma* spp. in the control of plant pathogens. Seven out of eight saprothrophs limited a further growth of hyphae of all the pathogens evaluated (Table 3). Outstanding antagonistic potential was noted in *Trichoderma lignorum* 104 and 264, while moderate in *T. koningii* 339 and 544. Surprisingly low potential was observed in *T. glaucum*. Generally, *Trichoderma* spp. isolated from monoculture limited the growth of pathogens much less considerably than those from crop rotation.

Table 3. Effect of biotic activity of *Trichoderma* spp. on the *Fusarium* spp. isolated from the fibrous flax rhizoplane after 72- and after 120- hour incubation, black and red values, respectively

Fungi	*F. solani* Lm651	*F. avena-ceum* Lm410	*F. oxyspo-rum* Lm402	*F. culmorum* Lz869	*F. equiseti* Lm385	*Phoma* sp. Lz162a	*B. cinerea* Lz132	*Verticillum* sp. Lz29a	*R. solani* Lm664	*A. alternata* Lm369
Trichoderma lignorum Lz104	+4, +5	+5, +5	+4, +4	+4, +5	+4, +4	+4, +5	+4, +5	+4, +5	+4, +4	+4, +5
T. koningii Lm339	+4, +4	+4, +5	+3, +4	+3, +5	+3, +4	+5, +5	+4, +4	+4, +5	+4, +4	+4, +5
T. koningii Lm554	+4, +5	+5, +5	+4, +4	+3, +4	+3, +4	+5, +5	+4, +4	+3, +5	+4, +4	+4, +5
T. lignorum Lz145a	+3, +4	+3, +4	0, +1	+3, +4	+3, +3	+4, +5	+3, +3	+3, +5	+3, +3	+3, +5
T. lignorum Lm574	+3, +4	+4, +5	+3, +3	+3, +4	+2, +3	+3, +4	+3, +4	+3, +5	+3, +3	+3, +4
T. album Lz51a	+2, +3	+3, +3	0, 0	+2, +3	0, +3	0, +3	+2, +3	+3, +5	-3, -3	0, +3
T. lignorum Lz264	+4, +5	+5, +5	+4, +4	+5, +5	+3, +4	+4, +5	+4, +4	+5, +5	+4, +5	+4. +5
T. glaucum Lm446	-3, -3	0, 0	-3, -5	0, -1	-2, -4	0, 0	0, -4	0, 0	-4, -5	0, -1

DISCUSSION

The research which aimed at defining the flax root phytosanitary status definitely confirmed that plant cultivation in monoculture results in quantitative and qualitative changes in the population of fungi observed as a development of phytopathogens. The same is true for the development phase; with flax growth and development (flowering) there is observed a decrease in the number of some microorganisms infecting the roots, e.g. *Fusarium*, *Cephalosporium*, *Alternaria* or *Botrytis* genera. A similar decrease in the number of microorganisms with the plant age was noted by Lileroth and Baath [16] on spring barley roots as well as by Hagedorn et al. [9] on cotton roots. *Fusarium* spp. fungi were more often isolated in spring (over emergence) than over plant flowering; at the same time there was noted some decrease in the soil richness in saprotrophic species of *Trichoderma*. Similar observations were recorded by other authors. It is the monoculture which considerably disturbs the biological balance [6,24]. The monoculture poses a threat of pathogenic species accumulation in soil; hence toxins produced by some fungal species, including *Fusarium*, *Penicilium*, *Aspergillus* with their high bactericidal, phytotoxic and mutagenic activities [13,25]. The factor which can prevent or limit the negative pathogens impact on the soil condition and on the plant growth is an adequate crop-rotation which enhances the occurrence of beneficial microorganisms, e.g. *Trichoderma* fungi which take part in biological control.

The present research showed that the crop rotation is responsible for significant differences in the quantitative and qualitative composition of microorganisms population. Especially clear-cut changes were observed for both flax development phases in monoculture which was considerably responsible for the accumulation of pathogenic fungi, *Fusarium* mainly. A real threat of *Fusarium* spp. over emergence was greater than over flowering. As for other pathogenic fungi, it was noted that, irrespective of the crop rotation, their greater population was recorded over flowering.

The research covered also some biochemical microorganisms characteristics, including cellulose and pectin breakdown potential important both for pathogens and for hyperparasites. A high enzymatic potential was observed in pathogens towards all the carbon sources researched. *Fusarium*, *Phoma*, *Verticilium* and *Alternaria alternata*, *Botrytis cinerea* and *Rhizoctonia solani* used starch, CMC and pectin, unlike *Trichoderma* fungi, some isolates of which did not cause the hydrolysis of pectin and CMC, so they produced neither cellulases nor pectinases. One shall stress that the proteolytic potential was observed in all the fungi tested.

The research of the antagonistic interaction between *Trichoderma* hyperparasites and fungi pathogenic towards fibrous flax showed that *T. lignorum* and *T. koningii* exhibited a biotic potential against pathogenic fungi. The observations confirm the results recorded by Strzelec [26] who showed a positive role of *T. lignorum* in the control of one of the most dangerous flax pathogens – *Rhizoctonia solani.* The development of none of the pathogens was limited by *T. glaucum;* yet, according to Mańka [18], the fungus inhibited *Rhizoctonia solani.* Different results obtained in the present research could be attributed to individual characteristics of a given isolate.

Applying natural biological control in agriculture can modify the viability of pathogens. There is much hope in *Trichoderma* fungi, which is a parasite of *Rhizoctonia* sp. and which inhibits the development of other numerous fungi, including *Pythium* or *Fusarium*, which, in turn, inhibits the development of diseases caused by these pathogens [26]. The results reported confirm the key role of beneficial microorganisms, especially in the developing organic farming.

CONCLUSIONS

1. Flax cultivation in monoculture increases the intensity of diseases inflicted by *Fusarium* fungi which were most responsible for disease changes on roots of the emerging plants. *Fusarium oxysporum, F. avenaceum* and *F. equiseti* were most represented.
2. Other pathogenic fungi, namely *Alternaria alternata, Botrytis cinerea, Rhizoctonia solani, Phoma* sp. *and Verticilium* sp. attack plants cultivated both in crop rotation and monoculture with a similar intensity.
3. The greatest occurrence of hyperparasites of the *Trichoderma* genus was recorded in the crop rotation over plant emergence.
4. Populations of saprotrophic fungi of the *Trichoderma* genus differed in both development phases both for monoculture and crop rotation. *T. lignorum* and *T. koningii* showed an inhibitory effect on the pathogenic fungi development.
5. Most of the fungi researched showed very similar enzymatic activities.

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Anna Ligocka, Zbigniew Paluszak, Stanisław Sadowski, Teresa Dziedzic
Department of Microbiology,
University of Technology and Agriculture
Bernardyńska 6, 85-084 Bydgoszcz, Poland
E-mail: mikro@atr.bydgoszcz.pl
