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Antagonistic effect of fungi from Scots pine stump roots against *Heterobasidion annosum* and *Armillaria ostoyae*

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The study presents quantitative and qualitative aspects of fungal colonization of the 2-year-old stump roots of the 30- and 49-year-old Scots pines, and biotic relations between fungi inhabiting the stump roots and major agents of butt and root rot in Poland, i.e.: *H. annosum* nad *A. ostoyae*. Compared to the live roots, the increase in density of fungi communities as well as the frequency of the fungi antagonistic towards *H. annosum* and *A. ostoyae*, particularly of *Trichoderma* species, in pine stump roots resulted in the increase of the suppressive effect of these communities towards both pathogens, studied *in vitro*. This finding may suggest a stronger resistance of pine stump roots to *H. annosum* and *A. ostoyae* what under forest conditions may be the example of natural control of both pathogens.

Key words: antagonistic fungi, *Armillaria ostoyae*, *Heterobasidion annosum*, Scots pine roots, *Trichoderma*.

INTRODUCTION

In forest the infection of trees by *Heterobasidion annosum* (Fr.) Bref. and *Armillaria ostoyae* (Romagn.) Herink can be accomplished through the contact of diseased and healthy roots. As far as this "route" of infection is considered, so far, it has been generally assumed that the infection of stumps does not differ from the infection of live trees. Recently, however, it has been proved that *A. ostoyae* becomes established in stumps by infecting only live trees or colonizing fresh stumps immediately after thinning operations (S h a w and K i l e 1991). In North America *A. ostoyae* is considered incapable of colonizing stumps that have not been already infected as living trees (F i l i p 1989). The reasons for this apparent inability are not clear. They may reflect the species limited capacity for spreading by rhizomorphs as well as its deficiency as a saprotrophic competitor.

The paper discusses the earlier colonization of Scots pine stump roots by successfully competing saprotrophic organisms, mostly *T. viride* what may reduce the infection by *A. ostoyae* and *H. annosum*.

MATERIAL AND METHODS

Root material was collected from 2-year-old stumps of the 30 and 49-year-old Scots pines in the Huta Pusta Forest District (western Poland, 17°10′ E, 52°50′ N), division 37d and 37h, in September 1993. Both divisions were situated on the former forest ground. The 49-year-old stand was infected by *H. annosum* in 30%. In every stand, samples were taken from two locations situated 200 m apart. Stump root complexes of approx. 30 cm length, lying 120° apart from each other, were excavated from B-horizon (30-50 cm) under each of 5 stumps, in each location. In laboratory 3 to 5 randomly selected 2 cm long segments of fine (0.5-1 mm diam.) and one segment of thicker (5 mm diam.) roots were taken from each root complex.

The segments were serially washed 10 times for 3 min. in flasks. The first 8 and the 10th flask contained 100 ml of distilled sterile water, the 9th contained 70 ml of distilled sterile water and 30 g of sterile quarz sand. Roots were dried in sterile filter paper. The fine roots were divided into 5 mm long subsegments and the thick ones into 1 mm thick discs which were put onto 2% PDA agar with chlorotetracycline (0.004 g l^{-1} agar). Each location and each kind of roots were represented by 180 inocula placed on 30 Petri plates (6 per 1 dish). The plates were incubated at 20-22°C. After 10 days the fungal colonies were transferred onto PDA slants. Fungi were identified according to their morphology on SNA, PDA, Czapek-Dox and 2% Malt – extract-agar. The percentage of occurrence was defined as the frequency in the whole recovered community.

Fungi were tested for their effect on the growth of *H. annosum* and *A. ostoyae* according to the biotic series method by Mańka (1974). The *H. annosum* – type P, heterocaryotic culture, with clamp connections (Korhonen 1978) was isolated from the fruit body found on a young Scots pine tree in the Huta Pusta Forest District. The Diploid *A. ostoyae* culture, characterized by the crustose colony, was isolated from the basidiome found on a *Quercus* stump in the Huta Pusta Forest District. The test was performed on 2% PDA (pH 5.6) for *H. annosum* and 2% Malt agar (pH 5.5) for *A. ostoyae*, using 15 most frequently occurring species of fungi (if the whole community comprised more than 15 separate species) or all species (if the community comprised 15 or less species). Individual biotic effect (IBE), after 10 days with *H. annosum* and 20 days with *A. ostoyae*, was estimated and then general (GBE) and summary biotic effect (SBE) were calculated.

Differences in fungal community structure in fine and thick roots, at two locations in one stand, and differences among individual fungal species populations were examined by multifactory analysis of variance.

RESULTS

The fine and thicker roots of 2-year-old stumps created after felling of the 30- and 49-year-old Scots pines were inhabited by 71 fungal species (Table 1).

In two locations of the 30-year-old Scots pine stumps **the fine roots** were colonized by 395 and 489 isolates represented by 26 species. In two locations of the 49-year-old stumps **the fine roots** were colonized by 274 and 224 isolates represented by 36 and 20 species, respectively. The fungal communities were strongly quantitatively and qualitatively differentiated. Only 18 species occurred in both stands. 19 and 24 separate species occurred in the 30- and 49-year-old stand, respectively. Among the most abundant species *T. viride*, *M. gracilis*, *M. r. atrovirens*, *P. daleae*, *M. vinacea*, *Z. moelleri* and *S. schenckii* occurred in both locations of both stands. *T. virens* did not occur in I location of older stand. *Cylindrocarpon destructans*, *M. acuminata*, *M. humilis*, *M. macrocystis*, *M. zonata* were present only in the 30-year-old stand and *H. annosum* was isolated sporadically only from the 49-year-old pine stumps. Only 15 and 14 species occurred in both locations of the 30- and 49-year-old stand respectively. The number of species occurring only in one location of the 30- and 49-year-old stands was respectively 22 and 28.

In two locations of the 30-year-old Scots pine stumps **the thick roots** were colonized by 344 and 183 isolates represented by 14 and 19 species. In two locations of the 49-year-old pine stumps **the thick roots** were colonized by 146 and 201 isolates represented by 15 and 8 species. 13 species occurred in both stands but among the most abundant only *T. viride*, *S. schenckii*, *M. vinacea* and *Z. moelleri* occurred in both locations of both stands. *Cylindrocarpon destructans* and *M. r. atrovirens* were absent in II location and *T. virens* in I location of the 49-year-old stand. Only 10 and 4 separate species occurred in both locations of the 30- and 49-year-old stand, respectively. The number of species occurring only in one location of 30- and 49-year-old stand was respectively 13 and 15.

There were significant differences ($P < 0.01$) between numbers of isolates from fine and thick roots in each location as well as among individual fungal species populations in each community.

Mycelium r. atrovirens, *Mucorales* and *Penicillia* occurred more often in fine roots, compared to thicker ones. The differences in the frequency of *Mucorales* and *Penicillia* were particularly high in the 49-year-old stand.

Table 1

Frequency of fungi in 2-year-old stump roots of 30- and 49-year-old Scots pine and their biotic effect on *Heterobasidion annosum* and *Armillaria ostoyae* growth

Species of fungi	30-year-old stand		49-year-old stand		30-year-old stand		49-year-old stand		Individ. biot. effect IBE	
	fine roots I	fine roots II	fine roots I	fine roots II	thick roots I	thick roots II	thick roots I	thick roots II	*H. annosum*	*A. ostoyae*
1	2	3	4	5	6	7	8	9	10	11
Absidia coerulea Bainier			2						+1	+4
Absidia cylindrospora Hagem		10	3		1	1	2		+1	+4
Alternaria alternata (Fr.) Keissler			1							
Aspergillus kanagawaensis Nehira		1								
Basidiomycotina sp.	2				1				−2	+4
Cladosporium cladosporioides (Fres) de Vries	1									
Cladosporium herbarum Link ex Fr.	3	1	1	1					−5	0 to +3
Cladosporium sphaerospermum Penz.					1					
Clonostachys sp.						3			−5	0
Cylindrocarpon destructans (Zinssm.) Scholten	74	105			20	35	1		+1	+5 to +6
Exophiala sp.			2							
Fusarium sp.			1						−5	1
Geotrichum candidum Link ex Lehman						1		8	+6	+7

Gliocladium roseum Bainier				1					−5	+1
Heterobasidion annosum (Fr.) Bref.			1	2			2		0	+7
Mariannaea elegans (Corda) Samson				1					−1	+3
Melanospora fimicola Hansen				1					−3	+1
Mortierella acuminata Linn.	2	9							0	+3
Mortierella alpina Peyronel				1					0	+4
Mortierella gracilis Linn.	68	51	65	37		7			0 to +1	+4
Mortierella humilis Linn.	5	4							+1	+4
Mortierella hygrophila var. *minuta* Linn.			1							
Mortierella isabellina Oud. et Koning					2				−7	+4
Mortierella macrocystis W. Gams	3	4							−6	+4
Mortierella minutissima van Tieghem	14		3						+1	+4
Mortierella nana Linn.		1								
Mortierella pulchella Linn.			1							
Mortierella spinosa Linn.		1								
Mortierella turficola Ling-Young	1									
Mortierella vinacea Dixon-Stewart	37	23	15	9	22	32	1	3	−4 to −5	+4
Mortierella zonata Linn.	11	16							+3 to +4	+4
Mortierella sp.			3							
Mucor ambiguus Vuill.			2							

cont. Table 1

1	2	3	4	5	6	7	8	9	10	11
Mucor fragilis Bainier						1				
Mycelium radicis atrovirens Melin	68	95	31	1	1	18	10		−6 to −7	+2 to +4
Oidiodendron griseum Robak		2								
Penicillium cf. *aculeatum* Raper et Fennell	3									
Penicillium adametzii Zaleski			2		1	2			+20to +22	+2
Penicillium brevicompactum Dierckx	1									
Penicillium commune Thom			1	1						
Penicillium daleae Zaleski	30	49	10	15	10	7	1		+1 to +2	+2 to +3
Penicillium glabrum (Wehmer) Westling			3	1					−5	+4 to +5
Penicillium janczewskii Zaleski		1	3			4	2		+8 to +10	0
Penicillium lividum Westl.			2							
Penicillium raistrickii Smith		1	1							
Penicillium roquefortii Thom			1							
Penicillium spinulosum Thom			8	1					−5	+5
Penicillium steckii Zaleski		1	4	5		1	3		0 to −3	0
Penicillium variabile Sopp.			1							
Pythium cf. *rostratum* E. Butler			2							
Sesquicillium candelabrum (Bon.) W. Gams		1	1	1					−5	+3

Sporothrix schenckii Hectoen et Perkins	3	8	5	3	113	29	3	1	−5	+2 to +3
Thysanophora penicillioides (Roum.) Kendr.		1								
Torulomyces lagena Delitsch			1							
Trichoderma fertile Bissett	1									
Trichoderma harzianum Rifai							3		+7	+7
Trichoderma koningii Oudemans		2	3			8	2		+8	+7
Trichoderma longipilis Bissett	20		2						+7	+7
Trichoderma polysporum (Link ex Pers.) Rifai	2	9	2			2	5		+8	+6
Trichoderma pubescens Bissett			6					4	+7 to +8	+7
Trichoderma strictipilis Bissett	3								+7	+7
Trichoderma strigosum Bissett	12					4			+8	+7
Trichoderma virens (Mill. Gidd. et Fost.) von Arx	2	23		2	13	3		71	+8	+7
Trichoderma viride Pers. ex Fr.	14	46	68	126	152	7	102	109	+8	+7
Trichoderma sp.								4	+8	+7
Zygorhynchus moelleri Vuill.	15	24	16	12	6	18	1	1	+8	+4
Sterile mycelium Kc S II 11				3					0	+4
Sterile mycelium Kg S I 13					1				−8	0
Sterile mycelium Kg S II 7							8		−6	0
Number of isolates	395	489	274	224	344	183	146	201		
Number of species	26	26	36	20	14	19	15	8		

The frequency of *Trichoderma* increased in thick roots, particularly in the 49-year-old stand (Table 2). The most common species in *Trichoderma* group were *T. viride* and *T. virens*.

Table 2

Frequency (%) of the most common taxa in fine and thick stump roots of 30- and 49-year-old Scots pine

	30-year-old pine				49-year-old pine			
	fine roots		thick roots		fine roots		thick roots	
	location				location			
	I	II	I	II	I	II	I	II
Mycelium radicis atrovirens	17.2	19.4	0.3	9.8	11.3	0.4	6.8	0
Mucorales	39.5	29.2	9.0	32.2	40.5	26.3	2.7	2.0
Penicillia	8.6	10.6	3.2	7.6	13.1	10.3	4.1	0
Trichoderma	13.7	16.4	48.0	13.1	29.6	57.1	76.7	93.5
T. virens + *T. viride*	4.1	14.1	48.0	5.5	24.8	57.1	69.9	89.6

Table 3

The increase in number of isolates, in %, in roots of 2-year-old stumps of Scots pine, compared to roots of living trees

	30-year-old pine		49-year-old pine	
	location			
	I	II	I	II
Fine roots	52.5	96.3	7.4	8.2
Thick roots	66.9	−10.7	11.4	47.7

Table 4

Changes in frequency (%) of the most common taxa in stump roots, compared to living roots

	30-year-old pine				49-year-old pine			
	fine roots		thick roots		fine roots		thick roots	
	location				location			
	I	II	I	II	I	II	I	II
Cylindrocarpon spp.	+4.9	+5.4	+5.8	+19.1	0	0	−7.0	0
Mycelium radicis atrovirens	+12.6	+1.3	−84.1	−45.3	−6.0	−18.0	−19.9	−49.3
Mucorales	+5.2	−4.7	+6.1	+31.7	−20.7	−15.7	+0.4	+2.0
Penicillia	−26.9	+3.8	+1.7	+7.1	−1.4	−7.1	−42.5	−31.6
Trichoderma	+6.8	−2.1	+37.8	+13.1	+22.9	+40.7	+75.9	+93.5

Table 5

The number and frequency (%) of fungal isolates* from Scots pine roots antagonistic** to *Heterobasidion annosum*

	30-year-old pine				49-year-old pine			
	location							
	I		II		I		II	
	absolute	%	absolute	%	absolute	%	absolute	%
Fine roots	266	71	337	71	177	73	155	71
Thick roots	203	59	97	54	119	82	197	98

* – numbers include 15 the most common species or all species in cases of smaller fungi communities

** – individual biotic effect (IBE) > +1

Table 6

Comparison of the summary biotic effect of fungi communities from Scots pine roots and Scots pine stump roots in relation to *Heterobasidion annosum* and *Armillaria ostoyae* (Kwaśna 1997)

	H. annosum		*A. ostoyae*	
	fine roots	thick roots	fine roots	thick roots
30-year Scots pine				
location I	+128	−1068	+909	+346
location II	+9	−1215	+1109	+484
49-year Scots pine				
location I	+366	−447	+898	+442
location II	−22	−642	+792	+451
30-year Scots pine stumps				
location I	+86	+741	+1770	+1747
location II	+399	+73	+2178	+728
49-year Scots pine stumps				
location I	+533	+789	+1096	+856
location II	+1075	+1540	+1220	+1391

Compared to living roots the number of isolates from stump roots increased over 52% and 96% as well as 7% and 8% in fine roots and 66% as well as 11% and 47% in thick roots (Table 3). The differences were significant ($P < 0.01$). The changes in frequency of the most common taxa in stump roots compared to live ones (Kwaśna 1997) are given in table 4. The fungi communities from fine roots comprised 71–73% isolates antagonistic to *H. annosum*. In thick roots their frequency was 54–98% (Table 5). The summary biotic effects (SBE) of fungi communities from the Scots pine stumps roots in relation to *H. annosum* and *A. ostoyae*, compared to SBEs from the living tree roots are given in table 6.

DISCUSSION

The fungal communities from 2-year-old stump roots were more numerous compared to the communities from live roots (K w a ś n a 1997). The increase in the frequency of fungi was particularly conspicuous in the 30-year-old stand. There was also a bigger diversity of communities from stump roots compared to live roots. The fungi communities in stump roots of the 49-year-old pines were smaller as compared to the 30 year-old pines. Each community was dominated by *Mucorales* nad *Trichoderma* spp., though, compared to living roots, the frequency of *Mucorales* increased in thick roots and usually decreased in fine roots (Table 4). The most frequent were *M. gracilis* and *M. vinacea*. *Penicillia* were more frequent in fine roots of stumps. The frequency of *Penicillia* decreased in majority of cases compared to live roots. The combination of *P. daleae* + *P. adametzii* + *P. janczewskii* common in live fine roots was replaced by *P. daleae* alone or with *P. spinulosum* and *P. glabrum* or *P. steckii* in stump roots. The last species are characteristic for decaying vegetation. *Trichoderma* frequency increased from almost 7% to over 93% in stump roots, compared to live ones (Table 4). The slight decrease of *Trichoderma* frequency in fine stump roots of the 30-year-old stand (II location) is not correlated with the decrease in its density. The most common species were *T. viride* and *T. virens*. Both species are common in coniferous stump roots (M a ń k a and G i e r c z a k 1961; N e l s o n et al. 1987; P r z e z b ó r s k i 1989).

The frequency of *M. r. atrovirens* increased slightly in fine stump roots of the 30-year-old stand. In most cases, however, if the fungus was not totally eliminated from the stump roots, its population decreased. *Cylindrocarpon destructans* is considered often as a pathogen with preferences for the living root tissues. The fungus occurred in fine roots of 30-year-old pines (K w a ś n a 1997) and succesfully survived in fine and thick stump roots where often became the most frequent member of community. Its frequency in stump roots, compared to the live ones, increased from over 4% to 19% (Table 4). The fungus did not occur either in roots of 49-year-old pines or in roots of their stumps (with the single exception). The presence of *M. r. atrovirens* and *C. destructans* suggests the high vitality of fine stump roots in the 30-year-old stand. The increase of *Trichoderma* population was small in fine stump roots what might support the supposition on the lower preferences of *Trichoderma* for the living tissues (P r z e z b ó r s k i 1989). *T. viride* favours the decaying organic matter (G o l d f a r b et al. 1989) and even participates actively in wood decomposition (D o m s c h et al. 1980). Analysis of the fungi communities structure suggests that the quantitative and qualitative changes of the fungi communities colonizing stump roots do not result from the age of trees but rather from the degree of root

decomposition which in the case of stumps from the 49-year-old pines might be much more advanced compared to stumps from the 30-year-old pines.

The changes in structure of communities caused the increase in number of isolates antagonistic to both pathogens in stump roots, compared to living roots. In the case of *H. annosum* they were higher in number about 12%–27% in fine and 48%–95% in thick stump roots (K w a ś n a 1997). This resulted in the increase of the SBE value in relation to *H. annosum* and *A. ostoyae* (Table 6). The increase of the SBE suggests the higher suppressiveness of the fungi communities from 2-year-old stumps towards *H. annosum* and *A. ostoyae*. These findings partly agree with the earlier studies by P r z e z b ó r s k i (1989); the increase of *Trichoderma*, mostly *T. viride* population, resulted in the continuous increase of suppressive effect of fungi communities from pine stump roots on *H. annosum* growth *in vitro*.

Inability to maintain effective populations of organisms antagonistic to *Armillaria* under field conditions has been, however, the main factor limiting successful biological control (S h a w and R o t h 1980). Fortunately, *Trichoderma* species are common and ubiguitous soil inhabitants (D o m s c h et al. 1980) what might suggest that applying *Trichoderma* inoculum is generally unnecessary but the favourable, conditions for its development should be created. Often, beneficial consequences of the increase of particular fungi population are achieved after certain operations. Thinning is considered as an important treatment affecting the development and condition of the stand. Its beneficial effect on the biotic relations in the soil environment of the pine stands was pointed out many times (M a ń k a 1986; M a ń k a et al. 1993). The profound increase in *T. viride* density also in the soil beneath stumps (K w a ś n a 1995) and in very intensively thinned stand (M a ń k a et al. 1993) suggests that thinning favours the increase of *Trichoderma* fungi population in the soil habitat.

T. viride combines properties of very good competitor, i.e. fast growth rate, heavy sporulation, common occurrence in temperature forests, antagonism toward many fungi due to the production of fungitoxic substances (mostly peptide antibiotics), complex hydrolase production, cellulolytic capabilities, good growth on many substrates, easy utilization of available organic and inorganic compounds and ability of parasitism. It seems that *T. virens* – the second species with similar properties, producing gliotoxin and viridin can act antagonistically as efficiently as *T. viride* but in the natural habitat the fungus occurs more rarely. *Trichoderma* species colonize the stump roots gradually and systematically. *T. viride* accounted for 11% and 22% of the total number of isolates in fine stump roots 4 and 10 months after thinning (P r z e z b ó r s k i 1989). The present results suggest that the thicker roots, where after 2 years even 90% increase in fungus population was observed, may be colonized even more efficiently.

CONCLUSION

The fungi communities in Scots pine stump roots had more suppressive effect on the growth of *H. annosum* and *A. ostoyae*, *in vitro*, compared to communities in live roots. The big increase of the SBE values was strongly corellated with the increase of *Trichoderma* population. This might increase the stump resistance to the infection and be the example of natural control of both pathogens accomplished particularly in acid forest soils favouring the growth of *Trichoderma* species.

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Efekt antagonistyczny grzybów z korzeni pniaków sosnowych w stosunku do *Heterobasidion annosum* i *Armillaria ostoyae*

S t r e s z c z e n i e

Korzenie cienkie i grube 2-letnich pniaków powstałych po ścięciu 30- i 49 letnich sosen zasiedlone były przez 71 gatunków grzybów. Najliczniej występowały: *Mycelium radicis atrovirens*, *Mucorales*, *Penicillia* i *Trichoderma*. W porównaniu z korzeniami sosen żywych, populacja *M. r. atrovirens* wzrosła w korzeniach cienkich w drzewostanie 30-letnim, *Mucorales* zmalała w korzeniach cienkich, a wzrosła w grubszych, *Penicillium* wzrosła w drzewostanie 30-letnim, a zmalała w 49-letnim, grzybów z rodzaju *Trichoderma* wzrosła w korzeniach cienkich i grubszych.

Wartości sumarycznego efektu biotycznego grzybów z korzeni pniaków były wyższe zarówno w stosunku do *H. annosum* jak i *A. ostoyae*, w porównaniu z podobnymi wartościami ustalonymi dla grzybów z korzeni sosen żywych. Silniejsze działanie antagonistyczne zbiorowisk grzybowych z korzeni pniaków było wynikiem wzrostu populacji grzybów jak również wzrostu populacji gatunków antagonistycznych w stosunku do *H. annosum* i *A. ostoyae*, zwłaszcza *Trichoderma* spp.