



Patrycja Golińska, Hanna Dahm

Antagonistic properties of *Streptomyces* isolated from forest soils against fungal pathogens of pine seedlings

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Abstract: Biological control of plant diseases has received worldwide attention in recent years mainly as a response to use of hazardous chemicals in the environment. Soil actinomycetes particularly *Streptomyces* spp. enhance soil fertility and have antagonistic activity against wide range of soil-borne plant pathogens. In study of antifungal activity of streptomycetes against plant fungal-pathogens *Fusarium oxysporum* (Schlecht.), *F. culmorum* (Wm. G. Sm.) Sacc. and *Rhizoctonia solani* (Kühn) causing damping-off of pine seedlings, *in vitro* and *in vivo* tests were carried out. Two types of *in vitro* tests in mixed cultures of actinomycetes and fungi were performed. In majority of the cases, an inhibitory effect of actinomycetes on fungi was noticed. Three isolates (SG9, SR2, SR4) were highly active against pathogenic fungi and were chosen for exopolysaccharides extraction and *in vivo* tests. The sensitivity to exopolysaccharide action was noted in fungi from the genus *Fusarium*. *Fusarium oxysporum* showed a higher sensitivity to the analyzed exopolysaccharides than *F. culmorum*. Biocontrol interactions both in sterile and non-sterile soil were found for the actinomycete *Streptomyces cyaneus* (strain SG9) isolated from root free soil of the pine. The latest experiment confirmed the strong pathogenicity of the fungus *Rhizoctonia solani*.

Antifungal properties of *Streptomyces cyaneus* (strain SG9) need further comprehensive studies.

Additional key words: *Streptomyces* sp.; antifungal activity, *Fusarium oxysporum*, *F. culmorum*, *Rhizoctonia solani*, biocontrol activity

Address: P. Golińska, H. Dahm, Department of Microbiology, Nicolaus Copernicus University, Faculty of Biology and Environmental Protection, Lwowska 1, 87-100 Toruń, Poland, e-mail: golinska@umk.pl

Introduction

Gram-positive bacteria from the genus *Streptomyces* are one of the basic components of soil microorganisms. Actinomycetes of cultivated soils are well known and are still being intensively investigated. Less attention has been paid to actinomycetes of forest soils as acid forest soils are not believed to favor the development of these microorganisms (Barabasz and Voříšek 2002; Marcinowska 2002; Zakalyukina et al. 2002; Aghighi et al. 2004; Zakalyukina et al. 2004).

Members of the genus *Streptomyces* are important in nature mainly because of their high enzymatic activity. They actively participate in the biodegradation of various soil polymers both of plant and animal origin in the form of polysaccharides (starch, pectin, cellulose, chitin), proteins, including keratin and elastin which are difficult to degrade, and lignocelluloses and aromatic compounds (McCarthy 1987; Crawford 1988; Lechevalier 1988; McCarthy and Williams 1992; Marcinowska 1993; Kämpfer 2006). Due to their hydrolytic abilities and high adaptive

capability they are an important link in the circulation of matter and energy (Dahm et al. 1986; Marcinowska 1993).

Over one-half of microorganisms isolated from soil show antagonistic properties. Among them actinomycetes are predominant, mainly from the genera *Streptomyces* and *Micromonospora* (Marcinowska 1993; Marcinowska 2002; Shirokikh et al. 2002). The production of bioactive metabolites which are secreted and accumulated in the cells and their effects on other microorganisms, mainly pathogenic ones, still arouses scientific and social interest (Marcinowska 2002; Shirokikh et al. 2002). One of the matters arousing an increasing interest among various scientific groups, especially plant pathologists, is biocontrol. New microorganisms are constantly being sought which could be used for combating fungal pathogens, the main causes of plant diseases (Aghighi et al. 2004).

Members of the genus *Streptomyces* show activity which can be used for the biocontrol of fungal pathogens of plants (Chattopadhyay and Nandi 1982; Fravel and Roberts 1991; Whipps 2001; de Boer et al. 2003; Dong et al. 2003; Kucuk and Kivanc 2003). This includes the production of various secondary metabolites (e.g. antibiotics), extracellular polysaccharides (EPS) and enzymes hydrolyzing fungal cell walls (Aghighi et al. 2004; Gohar et al. 2006).

In Central Europe the main causes of seedlings damping-off are fungi from the genus *Fusarium* (*F. oxysporum*, *F. culmorum*) and *Rhizoctonia solani*. Damping-off is a dangerous disease in forest nurseries and it is a burden in artificial renewal sowing and self-sowing. Fungi from the species *Rhizoctonia solani* are strong plant pathogens and cause damping-off of seedlings, especially in coniferous trees, including the Scots pine. *Fusarium oxysporum* also attacks pine seedling roots, mainly their root collar (Mańka 1992).

The purpose of the investigations was to show antagonistic properties of forest soil actinomycetes against dangerous fungal pathogens of pine seedlings and the selection of strains with potential biocontrol properties.

Materials and Methods

Bacterial and fungal strains

The microbial material analyzed were actinomycetes isolated from bulk soil (10 strains) and the rhizosphere (10 strains) of Scots pine (*Pinus sylvestris* L.) and pathogenic fungi causing damping-off of pine seedlings. The strains of actinomycetes were isolated from 87-year old Scots pine trees stand in the Forestry Bielawy near Toruń, Poland (Lat 53°01'46"; Long 18°42'24") and were identified up to species by Golińska and Dahm (2011b) according to the meth-

ods described by Shirling and Gottlieb (1966), Kutzner (1981) and Williams et al. (1983).

Strains used for the investigations:

- From the bulk soil: *Streptomyces griseoviridis* (SG1, SG6, SG8), *Streptomyces cyaneus* (SG2, SG4, SG9), *Streptomyces varsoviensis* (SG5), *Streptomyces exfoliatus* (SG7), *Streptomyces chromogenus* (SG10) and *Streptoverticillium olivovertici* (SG3).
- From the rhizosphere: *Streptomyces exfoliatus* (SR1, SR5, SR8, SR9, SR10), *S. xanthochromogenes* (SR2), *Streptomyces phaeochromogenes* (SR3), *Streptomyces anulatus* (SR4), *Streptomyces griseoviridis* (SR7) and *Streptoverticillium olivovertici* (SR6).
- Pathogenic fungi: *Fusarium oxysporum* (Schlecht.), *Fusarium culmorum* (Wm. G. Sm.) Sacc. and *Rhizoctonia solani* (Kühn) (strain no. 13, 15, 411 and 476). Because of the large strain differentiation of *Rhizoctonia solani* four strains of this species were used for investigations.

Antagonism of *Streptomyces* strains against pathogenic fungi – *in vitro* tests

These investigations were performed in mixed cultures of actinomycetes and fungi in Petri dishes with PDA medium (Difco) according to methods in Yuan and Crawford (1995) and Aghighi et al. (2004).

In Yuan and Crawford (1995) the results of mutual interactions between the actinomycetes and the fungus were observed after 5 days of incubation. The radius of the fungal colony in the fungal control culture and mixed cultures with actinomycetes were measured. The degree of antagonism of actinomycetes against fungi was expressed as an index of % inhibition which was calculated according to the formula:

% inhibition = $(1 - (\text{fungal radius in a mixed culture with actinomycetes} / \text{fungal radius in control colony})) \times 100$ (Coombs et al. 2004).

In Aghighi et al. (2004) the size of inhibition zones formed in fungal cultures [mm] was measured.

On the basis of the obtained results, three highly antagonistic actinomycete strains (SG9, SR2, SR4) were chosen for further investigations.

Antifungal activity of exopolysaccharides (EPS) isolated from selected *Streptomyces* strains

In order to select optimal culture conditions for actinomycete growth, microorganisms were grown in 3 liquid media with different compositions (M1, M2 and M3) according to Gohar et al. (2006).

Exopolysaccharides were extracted from supernatant of actinomycetes culture broth according to Gohar et al. (2006).

Investigations on the effects of EPS derived from actinomycetes against pathogenic fungi were performed on Petri dishes with PDA medium (Difco) us-

ing two-layer plates method modified from Aghighi et al. (2004). After inoculation of the basal layer of the PDA medium with a pathogenic fungus, filter paper discs (5 mm in diameter) were placed on the surface of the top agar layer. Each disc received 20 μ l of the aqueous EPS solution. Antagonistic activity of extracted EPS was examined in triplicates. The cultures were maintained at 26°C for 7 days after which fungal inhibition zones [mm] were measured.

Biocontrol activity of *Streptomyces* strains – *in vivo* tests

Pine seedlings were grown from certified seeds obtained from the forest nursery in Klosnowo near Chojnice, Poland.

The culture medium was soil from under Scots pine, taken from a stand from which actinomycetes had previously been isolated. Cultures of seedlings were performed in two variants – in non-sterile and sterile soil. Sterile soil was obtained by sterilizing 3 times in an autoclave at 121°C for 20 minutes.

The seedlings were grown in a culture chamber at 22 \pm 4°C in the light of sodium lamps with an illumination of 7 thousand lux (appr. 100 μ mol m⁻²s⁻¹ PAR – photosynthetically active radiation – 16 hours and 8 hours in the dark).

For seedling inoculation a suspension of actinomycete spores in peat with sand prepared according to the method of Yuan and Crawford (1995) was used.

Fungi (*Fusarium oxysporum*, *F. culmorum*, *Rhizoctonia solani*) were grown in liquid Czapek-Dox medium (Difco). After three days of culture with shaking a suspension for seedling inoculation was prepared. The culture was centrifuged in a centrifuge for 10 minutes at 3500·g. The mycelium was suspended in 300 ml sterile distilled H₂O and homogenized. Subsequently 1 ml inoculum was used to inoculate three-week old pine seedlings.

As controls seedlings not inoculated with any microorganism or inoculated only with the appropriate fungus or actinomycetes were used. The test seedlings were inoculated with two microorganisms – an appropriate fungus and an actinomycete. The experiments were performed in both the sterile and the non-sterile soil medium. Each variant of the experiment was performed in 10 replicates.

After 14 weeks duration of the experiment, seedling analysis was performed and the following measurements were taken: main root length (cm), fresh root mass (mg), dry root mass (mg), shoot length (cm), fresh shoot mass (mg), dry shoot mass (mg).

Results

Antagonistic activity of streptomyces strains isolated from the rhizosphere and bulk soil of Scots pine against pathogenic fungi – *in vitro* tests

Analysis of antagonistic interactions of actinomycetes against fungal pathogens of pine determined by the degree (%) of fungal growth inhibition in mixed cultures indicated that in the majority of cases, an inhibitory effect of actinomycetes was seen on fungi. However, in some cases a stimulation of fungal growth was observed, e.g. stimulation of *Fusarium oxysporum* by the *Streptomyces exfoliatus* (SR8) strain. Stimulation of the growth of this fungus was as high as 159% (Fig. 1B). For the same fungus a small stimulation of growth (1–9.8%) by *Streptomyces varsoviensis* (SG5), *S. exfoliatus* (SG7), *S. cyaneus* (SG9) and *S. griseoviridis* (SR7) was observed. Similar results were obtained for the culture of *Fusarium culmorum* and *Streptomyces cyaneus* (SG4) (Fig. 1A).

Actinomycetes showed the highest antagonism towards *Fusarium culmorum* and strains of *Rhizoctonia solani*. Antagonism was demonstrated both by soil actinomycetes [*Streptoverticillium olivovertici* (SG3), *Streptomyces varsoviensis* (SG5), *S. exfoliatus* (SG7), *S. griseoviridis* (SG8), *S. cyaneus* (SG9)], as well as rhizospheric ones [*S. xanthochromogenes* (SR2), *S. phaeochromogenes* (SR3), *S. anulatus* (SR4)]. The degree of fungal growth inhibition was 40–50%. The strongest antagonists of *Fusarium oxysporum* growth were strains belonging to the species *Streptomyces griseoviridis* (strains SG1 and SG6) and a strain of *Streptomyces exfoliatus* (strain SR5), which inhibited fungal growth by 40% (Fig. 1B, Phot. 1). The four strains of *Rhizoctonia solani* (13, 15, 411, 476) analyzed in this work differed in their sensitivity to interaction of actinomycetes (Fig. 1C–F). No effects on the analyzed strains of *R. solani* were found for two *Streptomyces cyaneus* strains (SG2 and SG4), *Streptomyces chromogenes* (SG10) and *Streptomyces exfoliatus* (SR8).

It was observed that actinomycete strains belonging to the same species differed in the degree of fungal inhibition and thus the fungal inhibition was actinomycete strain property. Among three strains of *Streptomyces cyaneus* (SG2, SG4, SG9) only one (SG9) showed a high antagonism against fungi. The remaining strains did not affect fungal growth or only had a small influence. Strains of *Streptomyces exfoliatus* isolated from the rhizosphere (SR1, SR5, SR8, SR9, SR10) were very weak antagonists, whereas strain (SG7), of the same species derived from root free soil inhibited pathogen growth by 40–50%.

Among actinomycetes isolated from the bulk soil, more strains antagonistic to fungi were observed than among strains isolated from the rhizosphere. *Fusarium*

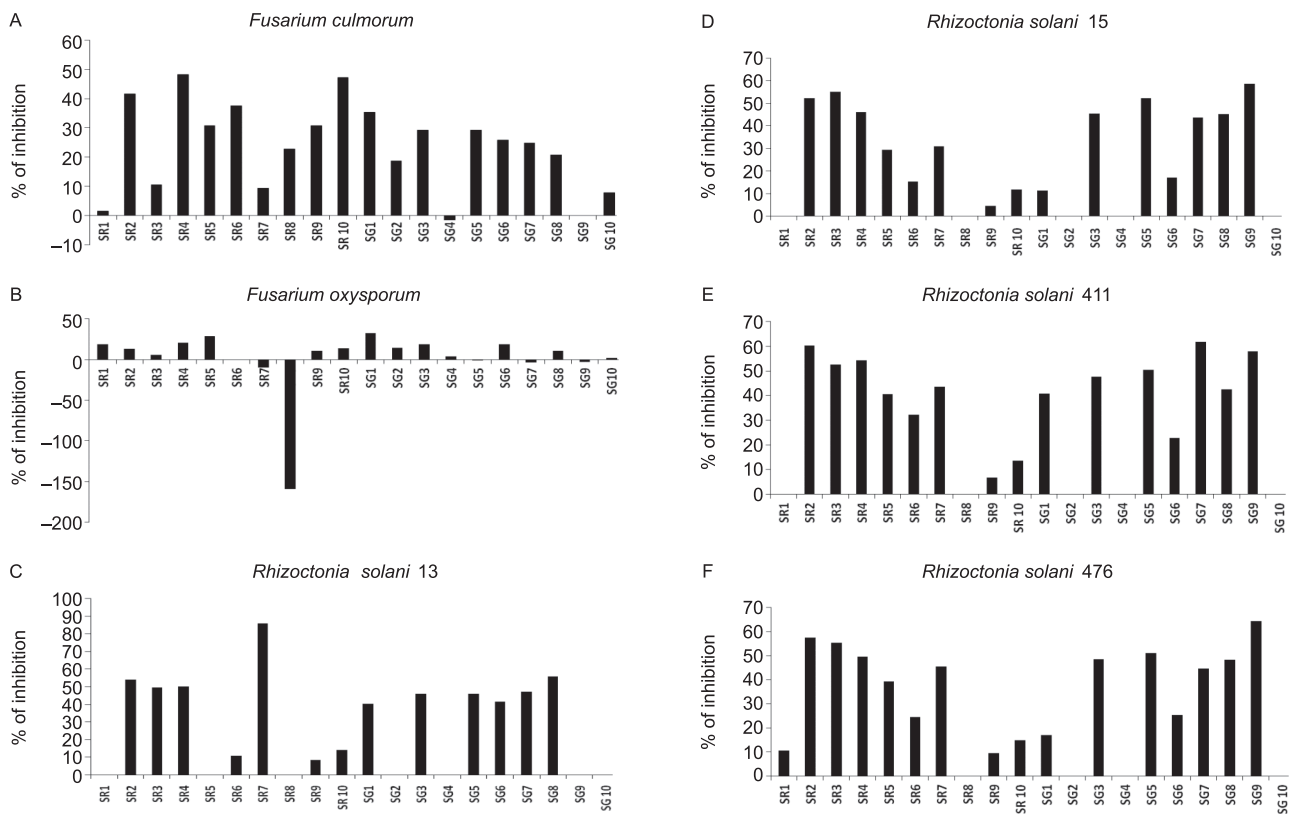


Fig. 1. Antagonism *Streptomyces* strains isolated from rhizosphere and bulk soil of Scots pine (*Pinus sylvestris* L.) against pathogenic fungi according to Yuan and Crawford method (1995) (fungi were inoculated five days after *Streptomyces*) SR1-SR10 – *Streptomyces* strains isolated from rhizosphere of Scots pine; SG1-SG10 – *Streptomyces* strains isolated from bulk soil of Scots pine

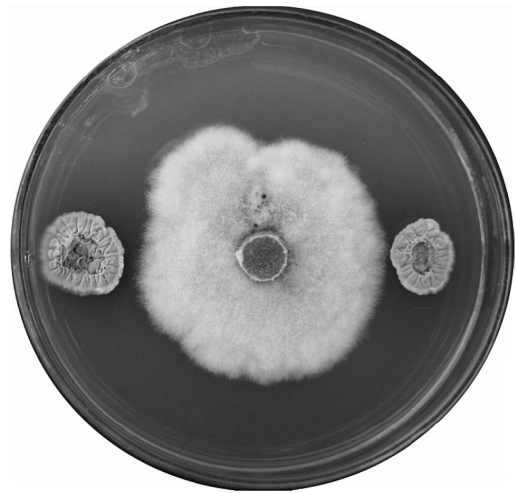
oxysporum showed low sensitivity to actinomycetes inhibition. Even though the inhibition of growth of this fungus was observed in the presence of almost all actinomycetes, the degree of this inhibition was lower than for the remaining fungi. The growth of another species from this genus, *Fusarium culmorum*, was inhibited by 18 of the 20 analyzed strains of actino-

mycetes. The degree of inhibition of the growth of this fungus was differentiated.

After using an alternative method of analysis according to Aghighi et al. (2004), conclusions as to inhibition of fungi were made on the basis of clear zones of growth inhibition or of zones (which were not clear) of poorer fungal growth around an actino-



Fusarium oxysporum (Control)



F. oxysporum + *S. griseoviridis* (SG1)

Fot. 1. Antagonistic activity of *Streptomyces* isolated from rhizosphere and bulk soil of Scots pine according to Yuan and Crawford (1996) method (an example)

SG1 – *Streptomyces griseoviridis* isolated from bulk soil of Scots pine

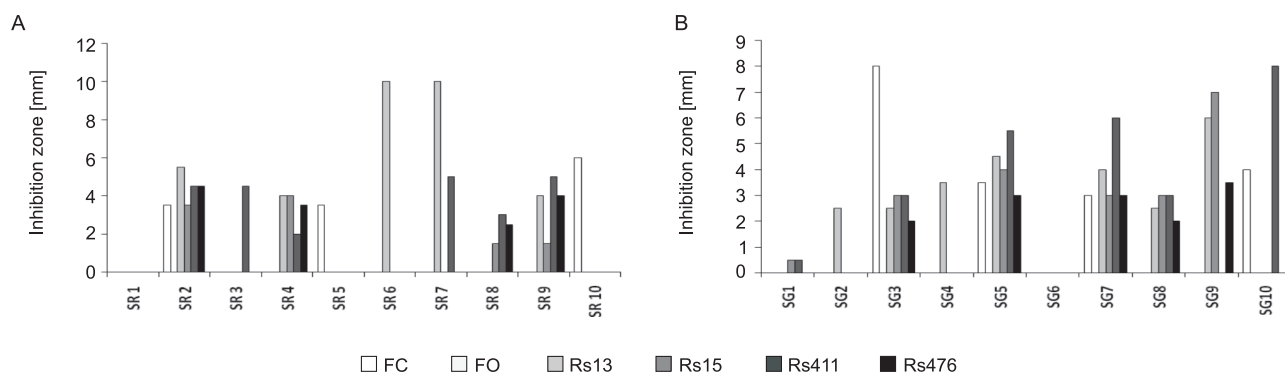


Fig. 2. Antagonism *Streptomyces* strains isolated from rhizosphere and bulk soil of Scots pine (*Pinus sylvestris* L.) against pathogenic fungi according to Aghighi et al. (2004) method (*Streptomyces* and fungi were inoculated simultaneously) SR1-SR10 – *Streptomyces* strains isolated from rhizosphere of Scots pine; SG1-SG10 – *Streptomyces* strains isolated from bulk soil of Scots pine; FC – *Fusarium culmorum*; FO – *Fusarium oxysporum*; Rs13 – *Rhizoctonia solani* 13; Rs15 – *Rhizoctonia solani*15; Rs 411 – *Rhizoctonia solani* 411; Rs 476 – *Rhizoctonia solani* 476

mycetes colony. Soil and rhizospheric actinomycetes inhibited the growth of *Rhizoctonia solani* (3 among 4 strains) most strongly with clear zones of inhibition. The strongest antagonists of pathogenic fungi were soil actinomycetes (*Streptoverticillium olivovertici* (SG3), *Streptomyces varsoviensis* (SG5), *S. exfoliatus* (SG7), *S. griseoviridis* (SG8)) and in lower numbers rhizosphere actinomycetes (*Streptomyces anulatus* (SR4), *Streptomyces exfoliatus* (SR9)) (Fig. 2A, 2B, Phot. 2).

Inhibitory properties of EPS (third experimental variant) were deduced on the basis of clear zones of growth inhibition or zones of decreased fungal growth around a filter paper disc containing suspension of the extracted exopolysaccharide. The sensitivity to exopolysaccharide activity was noted in fungi from the genus *Fusarium*. No effect of exopolysaccharides on the growth of *Rhizoctonia solani* strains was observed.

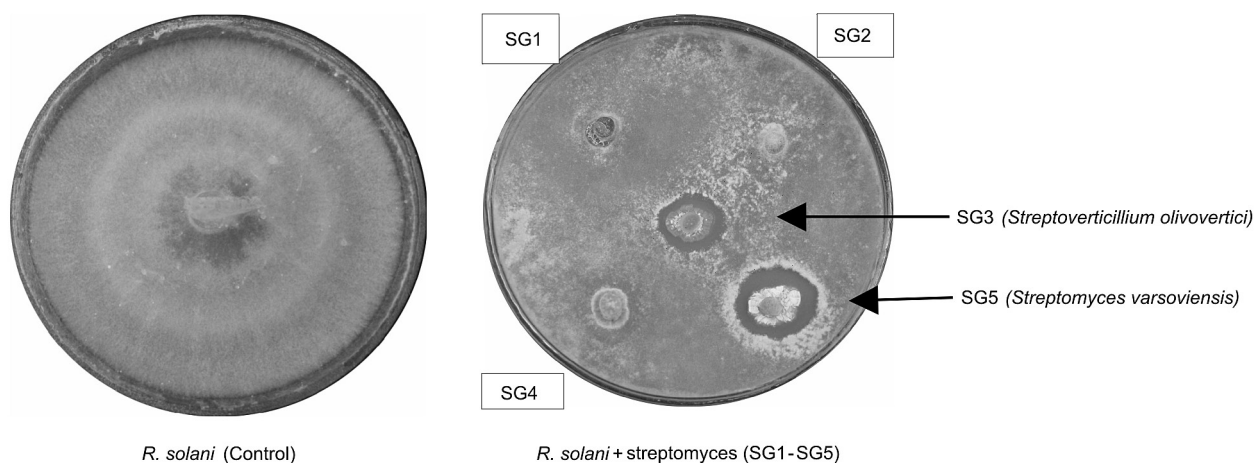
Fusarium oxysporum showed a higher sensitivity to the analyzed exopolysaccharides than *Fusarium*

Table 1. Antagonistic activity of exopolysaccharides (EPS) isolated from *Streptomyces* strains against pathogenic fungi (growth inhibition or fungistatic zone [mm])

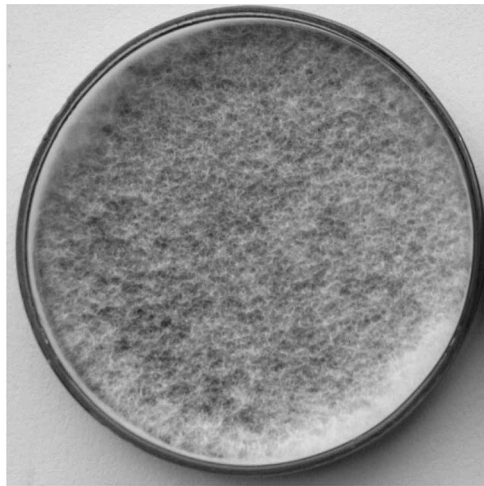
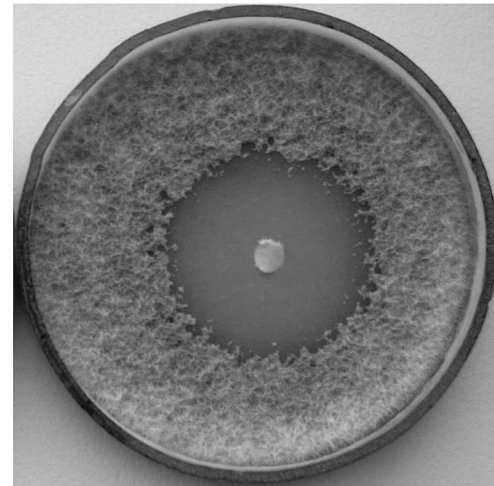
EPS source	<i>Fusarium culmorum</i>	<i>Fusarium oxysporum</i>	EPS g/ml
SR2	8.2*	17**	0.40
SR4	not inhibited	not inhibited	0.84
SG9	2.5**	7.8*	0.23

Rhizoctonia solani strains were not inhibited by studied exopolysaccharides

SR2 – *Streptomyces xanthochromogenes*; SR4 – *Streptomyces anulatus* isolated from rhizosphere of Scots pine; SG9 – *Streptomyces cyaneus* isolated from bulk soil of Scots pine; *Fungistatic activity (decreased fungal growth); **Inhibition zone



Phot. 2. Antagonistic activity of *Streptomyces* strains isolated from rhizosphere and bulk soil of Scot pine against pathogenic fungi according to Aghighi et al. (2004) method (an example) SG1-SG5 *Streptomyces* strains isolated from bulk soil of Scots pine

*Fusarium oxysporum* (Control)EPS (SR2) + *Fusarium oxysporum*

Fot. 3. Antagonistic activity of exopolysaccharides (EPS) isolated from *Streptomyces xanthochromogenes* (SR2) against *Fusarium oxysporum* (an example)

SR2 – *Streptomyces xanthochromogenes* isolated from rhizosphere of Scots pine

Table 2. Influence of *Streptomyces* strains on the growth of Scots pine seedlings after inoculation of *Rhizoctonia solani* [average values (n=10) ± standard deviations]

Experimental combinations	Number of dead seedlings	Sterile soil			
		Root		Shoot	
		Length of main root [cm]	Dry mass [mg]	Length [cm]	Dry mass [mg]
<i>Rhizoctonia solani</i> – control	7	6,38 ± 9,0	26,14 ± 39,0	3,36 ± 2,1	48,48 ± 69,7
<i>R. solani</i> + SR2	4	100% 13,15 ± 10,8	100% 45,70 ± 47,1	100% 3,37 ± 1,49	100% 46,16 ± 38,2
<i>R. solani</i> + SR4	7	206,11% 5,95 ± 7,5	174,82% 44,25 ± 71,2	100,29% 2,66 ± 2,1	95,21% 41,37 ± 63,0
<i>R. solani</i> + SG9	2	93,26% 14,21 ± 9,0*	169,28% 52,90 ± 39,0	79,16% 4,20 ± 2,1	85,33% 55,29 ± 69,7
		222,72%	202,37%	125%	114,04%
Experimental combinations	Number of dead seedlings	Non-sterile soil			
		Root		Shoot	
		Length of main root [cm]	Dry mass [mg]	Length [cm]	Dry mass [mg]
<i>Rhizoctonia solani</i> – control	7	7,14 ± 8,3	18,89 ± 29,3	3,35 ± 2,1	49,82 ± 73,8
<i>R. solani</i> + SR2	2	100% 16,26 ± 8,4*	100% 27,88 ± 22,8	100% 5,56 ± 2,1*	100% 112,09 ± 80,56
<i>R. solani</i> + SR4	3	227,73% 13,89 ± 8,5	147,59% 34,64 ± 26,1	165,97% 4,99 ± 2,6	224,99% 138,64 ± 102,6*
<i>R. solani</i> + SG9	1	194,53% 17,14 ± 6,1*	183,87% 50,77 ± 24,1*	148,95% 5,39 ± 2,4	278,28% 128,87 ± 60,2*
		240,05%	268,76%	160,89%	258,67%

SR2 – *Streptomyces xanthochromogenes*; SR4 – *Streptomyces anulatus* isolated from rhizosphere of Scots pine; SG9 – *Streptomyces cyaneus* isolated from bulk soil of Scots pine; * Significant differences as compared to control (t-test)

Table 3. Three-factor analysis of variance (ANOVA), comparing the kind of growth medium, streptomyces inoculation and pathogenic fungi inoculation on the growth parameters of Scots pine seedlings roots

Source of variation	<i>p</i> (significance level)			
	df	Length of root	Fresh mass of root	Dry mass of root
Medium	1	0,1021	0,0000	0,0000
Streptomyces	3	0,0071	0,1135	0,0309
Pathogenic fungi	2	0,0000	0,0000	0,0000
Newman-Keuls multiple range test ($p \leq 0,05$)				
Soil:	Length of root [cm]	Fresh mass of root [mg]	Dry mass of root [mg]	
sterile	16,0066 a	292,4792 b	sterylne: 80,0683 b	
non-sterile	17,1200 a	152,3508 a	niesterylne: 43,5266 a	
Streptomyces	Control: 14,8833 a	Control: 225,9333 a	Control: 57,8900 a	
	SR2: 17,5983 b	SR2: 208,0283 a	SR2: 55,9866 a	
	SR4: 15,9916 ab	SR4: 251,4617 a	SR4: 71,9766 b	
	SG9: 17,7800 b	SG9: 204,2367 a	SG9: 61,3366 ab	
	Control: 18,6700 b	Control: 272,1675 b	Control: 79,3162 c	
Pathogenic fungi	<i>F. oxysporum</i> : 19,2550 b	<i>F. oxysporum</i> : 261,5237 b	<i>F. oxysporum</i> : 68,4300 b	
	<i>R. solani</i> : 11,7650 a	<i>R. solani</i> : 133,5538 a	<i>R. solani</i> : 37,6462 a	

df – degrees of freedom; SR2 – *Streptomyces xanthochromogenes*; SR4 – *Streptomyces anulatus* isolated from rhizosphere of Scots pine; SG9 – *Streptomyces cyaneus* isolated from bulk soil of Scots pine; mean values marked with the same letter do not differ significantly ($p \leq 0,05$)

culmorum. The strongest action inhibiting the growth of *F. oxysporum* was noted for EPS obtained from strain *Streptomyces xanthochromogenes* (SR2) isolated from the rhizosphere of Scots pine (Phot. 3). The exopolysaccharide also inhibited growth of *Fusarium culmorum* causing the formation of non-clear zones of growth inhibition, but this action was weaker in comparison to that observed in *F. oxysporum*. EPS obtained from strain *Streptomyces anulatus* (SR4), even though it was tested at a concentration of two times higher

(0.84 g/ml) than the EPS from strain SR2 and four times higher than that from the strain SG9, did not inhibit the growth of any of the tested fungi (Table 1).

Biocontrol activity of streptomyces isolated from the rhizosphere and bulk soil of Scots pine against pathogenic fungi – *in vivo* tests

The experiment confirmed the strong pathogenicity of the fungus *Rhizoctonia solani*. Seedlings inocu-

Table 4. 3 Three-factor analysis of variance (ANOVA), comparing the kind of growth medium, streptomyces inoculation and pathogenic fungi inoculation on the growth parameters of seedlings of Scots pine shoots

Source of variation	<i>p</i> (significance level)			
	df	Length of shoot	Fresh mass of shoot	Dry mass of shoot
Medium	1	0,0000	0,0000	0,0000
Streptomyces	3	0,1109	0,1121	0,2089
Pathogenic fungi	2	0,0000	0,0000	0,0265
Newman-Keuls multiple range test ($p \leq 0,05$)				
Soil	Length of shoot [cm]	Fresh mass of shoot [mg]	Dry mass of shoot [mg]	
sterile	4,8058 a	299,3983 a	88,3288 a	
non-sterile	5,8358 b	540,4517 b	152,597 b	
Streptomyces	Control: 4,9500 a	Control: 391,4600 a	Control: 109,8900 a	
	SR2: 5,3430 a	SR2: 410,5083 a	SR2: 115,5000 a	
	SR4: 5,4500 a	SR4: 485,2100 b	SR4: 138,6583 a	
	SG9: 5,5200 a	SG9: 392,5217 a	SG9: 107,2317 a	
	Control: 5,8387 b	Control: 467,6537 b	Control: 128,4867 b	
Pathogenic fungi	<i>F. oxysporum</i> : 6,0137 b	<i>F. oxysporum</i> : 519,6550 b	<i>F. oxysporum</i> : 145,8333 b	
	<i>R. solani</i> : 4,1100 a	<i>R. solani</i> : 272,4662 a	<i>R. solani</i> : 87,0700 a	

df – degrees of freedom; SR2 – *Streptomyces xanthochromogenes*; SR4 – *Streptomyces anulatus* isolated from rhizosphere of Scots pine; SG9 – *Streptomyces cyaneus* isolated from bulk soil of Scots pine; mean values marked with the same letter do not differ significantly ($p \leq 0,05$)

lated with this fungus had significant inhibition of root and shoot growth in seedlings cultivated both in sterile and non-sterile soil (70% dead seedlings).

About 30–80% seedlings cultivated in sterile soil survived after inoculation with *R. solani* and different actinomycetes strains.

In non-sterile soil the positive effect of actinomycete protection was even more pronounced. The percentage of live seedlings was 70–90%. Among the analyzed actinomycetes, the most favorable biocontrol interactions both in sterile and non-sterile soil were found in *Streptomyces cyaneus* (strain SG9) isolated from root free soil of the pine (Table 2).

No pathogenic effects of *Fusarium oxysporum* was observed in pine seedlings. Seedling growth parameters (both in sterile and non-sterile soil) after inoculation with this fungus were similar to control.

Growth substrate sterility was important for the condition of the seedlings. Cultured seedlings in non-sterile soil were characterized by a statistically different elongation and mass of the shoot, whereas in sterile soil better development of the seedling root system was observed.

Inoculation of the seedlings with only actinomycetes had no negative effects on the growth of pine seedlings, while the positive effect was small. In general all actinomycetes elongated the root to a small extent, and one of the strains of *Streptomyces anulatus* (SR4) additionally increased the shoot mass (Table 3, 4).

Discussion

The ability of actinomycetes to produce antibiotics with a differentiated spectrum of activity and biological specificity is commonly known. Practical application of these secondary metabolites is not limited to medicine and veterinary medicine. These compounds are also used in phytopathology and in combat with plant pathogens, albeit uncommonly (Saadoun and Gharaibeh, 2003; Davelos et al. 2004; Oskay et al. 2004).

Metabolites released into the environment play an important role in shaping the interactions between microorganisms (Chmiel 1982; Dahm et al. 1986; Marcinowska 1993) and maintaining biological equilibrium in natural environments (Bressan 2003; Tian et al. 2004; Aghighi et al. 2004; Zaitlin et al. 2004).

Our investigations on the interactions between microorganisms, similar to those of other authors (Chmiel 1982; Shoda 2000) indicate that these interactions may be inhibitory, stimulating, synergistic or neutral. The inhibitory effect of actinomycetes on other microorganisms is mainly ascribed to antibiotics, while other aspects of actinomycetes metabolic activity also play an important role in these processes.

The stimulating effect of actinomycetes on other microorganisms in the environment may be linked to

the formation and secretion of metabolites such as amino acids, sugars, organic acids and vitamins into the environment.

According to some scientists the site of intense synthesis of antibiotic compounds is bulk soil, even though greater competition for space and nutrients occurs in the rhizosphere of plants (Shirokikh et al. 2002). Therefore other researchers believe that microorganisms find particularly appropriate conditions for antibiotic synthesis in the plant rhizosphere (Crawford et al. 1993; Marcinowska 1993; Sturz et al. 1997). Antibiotic production being highly regulated, is believed to be produced under starvation conditions when the colony is ready for sporulation and not in a nutrient-rich environment (Hopwood 2007). Aerial mycelium of *Streptomyces* colonies develops at the expense of nutrients obtained from the decomposition of the substrate mycelium. The released metabolites (sugars, amino acids and others) may be attractants for many microorganisms and the antibiotics produced by actinomycetes may act in this way at small distances (Hopwood 2007).

The results of our investigations are similar to the observations of Shirokikh et al. (2002) and indicate that the environment with the highest number of actinomycetes antagonistic to pathogenic fungi is root-free soil and that strains isolated from the rhizosphere are characterized by a weaker antagonism. However, the investigations of Crawford et al. (1993) have shown that actinomycetes isolated from bulk soil and the rhizosphere demonstrated a similar degree of inhibition of the pathogenic fungus *Pythium ultimum*.

Investigations *in vitro* on the antagonistic interactions of actinomycetes and pathogenic fungi performed by various research groups often differ in the type of culture medium used, the temperature of the culture or the method of inoculating the media (Crawford et al. 1993; Yuan and Crawford 1995; Trejo-Estrada et al. 1998; Getha and Vikineswary 2002). Such factors may affect the results obtained in these investigations.

In our investigations two methods were used to determine the mutual interactions of actinomycetes and fungi: according to Yuan and Crawford (1995) and according to Aghighi et al. (2004), differing in the time of inoculation of the fungus and the actinomycetes. Different or similar results were obtained depending on the analyzed actinomycetes strain. With simultaneous inoculation of the medium with the fungus and *Streptomyces varsoviensis* (SG5) a strong antagonism of the actinomycetes was observed, whereas when the fungus was inoculated into a 5-day actinomycete culture the interaction was much weaker. Some actinomycetes (*Streptoverticillium olivovertici* (SG3) and *Streptomyces griseoviridis* (SG8)) inhibited fungal growth to a similar extent regardless of the time of inoculation of the medium with the fungus.

A significant effect of the time of culture on the degree of actinomycete antagonism was also demonstrated by the investigations of Yuan and Crawford (1995). The antagonism of *Streptomyces lydicus* WYEC108 to different species of pathogenic fungi increased with the time of duration of the mixed cultures and was stronger on the 5th than on the 2nd day of mixed culture.

Few investigations have addressed the effect of exopolysaccharides produced by actinomycetes on fungi. In this work the inhibitory action of exopolysaccharides was only demonstrated against fungi from the genus *Fusarium*. Strains of *Rhizoctonia solani* were resistant to the action of these compounds.

In the antagonism of actinomycetes against fungi an important role is played by chitinases, enzymes which hydrolyze their cell wall. Many authors have shown a relationship between chitinolytic activity of actinomycetes and their antifungal activity (Mahadevan and Crawford 1997; de Boer et al. 1998; Hoster 2005). Comparison of the relationship between chitinolytic activity of actinomycetes analyzed by us (Golińska and Dahm 2011a) and the observed degree of inhibition of fungi in mixed culture has shown that these parameters were not always correlated, e.g. *Streptomyces cyaneus* (SG9) and *Streptomyces anulatus* (SR4) did not produce chitinases in the conditions of the experiments performed or showed a low activity of these enzymes, but in cultures with fungi strongly inhibited their growth. Experiments performed by de Boer et al. (1998) have also shown that the growth of fungi was inhibited both by chitinolytic microorganisms and by those which were incapable of the synthesis of such enzymes. However, in other analyzed strains there was a correlation between the degree of fungal inhibition and the chitinolytic activity of actinomycetes. A high chitinolytic activity and a strong antagonism against fungi were found in the actinomycetes *Streptomyces griseoviridis* (SG1), *S. varsoviensis* (SG5), *S. griseoviridis* (SG8). These observations correspond to the conclusions of Hoster et al. (2005), who demonstrated a high correlation between the chitinolytic activity of various strains of *Streptomyces* and the degree of their antagonism against pathogenic fungi. For the biological control of diseases caused by *Phytophthora infestans*, *Streptomyces violaceusniger* (strain YCED-9), was selected, a strong antagonist *in vitro* of this strong fungal pathogen. The antagonism of this strain was not linked with chitinolytic activity but with antibiotic synthesis. This one strain may produce several compounds with antibiotic character: AFA (with activity against *Fusarium*), a fungicidal polyene complex similar to guanidylfungin A or nigericin and geldanamycin, strongly inhibiting the growth of the fungi *Pythium* and *Phytophthora* spp.

The success of biocontrol depends to a large extent on the basic knowledge on the subject of the selected antagonist (Bressan 2003). In general, microorganisms as potential biocontrol factors are selected in *in vitro* tests analysing their antagonism against pathogens. After the analysis of our results we selected three strains of actinomycetes (*Streptomyces xanthochromogenes* (SR2), *S. anulatus* (SR4) and *S. cyaneus* (SG9)) showing a strong antagonism to the analysed pathogenic fungi and used them in investigations of respective interactions of actinomycetes and pathogenic fungi on the roots of pine seedlings in greenhouse experiments.

The control of plant pathogens using biological factors is still not applied on a large scale but many investigations have shown the large possibilities of using this method (Bressan 2003).

Most investigations on biocontrol concentrate on pathogens and microorganisms which combat those of them which are associated with cultivated plants (rice, maize, tomato, tobacco etc.) – this is understandable from the economic point of view. However, the search for biological factors as an alternative to chemical compounds which combat forest tree pathogens (especially in forest nurseries) is also important (Dumroese et al. 1998; Rudawska 2000).

The observations performed by us in greenhouse experiments have shown a role for actinomycetes in protecting pine seedlings against *Rhizoctonia solani*.

The best biocontrol organism was the strain *Streptomyces cyaneus* (SG9), isolated from free root soil. It protected seedlings grown in both sterile and non-sterile soil against infection by this pathogen. The rhizosphere actinomycetes *Streptomyces xanthochromogenes* and *Streptomyces anulatus* showed a lower biocontrol ability and only in non-sterile soil.

The hypothesis is probable that plant pathogens are effectively inhibited by mixed groups of antagonists. Different microorganism populations may indicate differentiated mechanisms alleviating or fully reducing the effects of plant diseases, as well as the possibility of their occurrence (Raupach and Kloepper 1998).

Fusarium oxysporum was found to be non-pathogenic for pine seedlings even though it was obtained from a collection of strong pathogens. Fungi may lose pathogenicity among others after successive passages (Mańka 1992). The growth of seedlings inoculated with *F. oxysporum* did not differ significantly from the growth of control seedlings, and moreover inoculation of the seedlings with an actinomycete (especially *Streptomyces anulatus* isolated from the rhizosphere) significantly stimulated their growth.

Berg et al. (2005) moreover found that non-pathogenic or weakly pathogenic strains of well-known species of pathogenic fungi (among others *Fusarium*

oxysporum) may be antagonists of other pathogenic fungi e.g. *Verticillium dahliae* Kleb.

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