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Occurrence of actinomycetes in forest soil

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Abstract: Actinomycetes from the genus *Streptomyces* are Gram-positive bacteria commonly and in large numbers isolated from the soil, which is their natural habitat. Due to their metabolic activity and high adaptative capabilities they are an important link in the circulation of matter and energy. They play an important role in the formation of bioactive metabolites, mainly antibiotic compounds.
In contrast to numerous elaborations concerning actinomycetes of cultivated soils, little is known about their occurrence in forest soils. The main factor limiting actinomycete development in forest soils is believed to be the low pH, as the development of most actinomycetes is facilitated by a neutral or alkaline soil reaction. However, these microorganisms have also been isolated from strongly acidic soils, which was confirmed in our studies.
The pH value of analysed soils was in the range 4.0–4.3 (4.0 for bulk soil of alder; 4.1 for bulk soil of Scots pine; 4.3 for bulk soil of birch).
Root soil of the analyzed trees contained more microorganisms than soil outside the range of the roots. This also concerned actinomycetes, eubacteria as well as saprophytic fungi. In our investigations the greatest number of eubacteria were connected with the alder (bulk soil, rhizosphere and rhizoplane), and the smallest number with the pine, whereas actinomycetes were the most numerous in the birch rhizosphere.
The analyzed actinomycete strains were identified to 18 taxa, with a dominant species *Streptomyces exfoliatus*.

Additional key words: actinomycetes; forest soils and trees; *Streptomyces* identification; biodiversity.

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Introduction

Actinomycetes (*Actinomycetales*) are important in nature mainly because of their ability to decompose many complex compounds, among others proteins, pectins, cellulose, hemicellulose, lignins and chitin (Dahm et al. 1986; Marcinowska 1993). They play an important role in the formation of bioactive metabolites, mainly antibiotic compounds (Marcinowska 2002; Mordarska and Paściak 2002; Oskay et al. 2004).

Among *Streptomyces* there are organisms which are plant pathogens (among others *S. scabies*, *S. ipomoea*, *S. parvulus*, *S. sparsogenes*, *S. flavovirens*) (Sutherland et al. 1979; Kennedy and Alcorn 1980; Lechevalier 1988; Natsume et al. 2001). A smaller *Streptomyces* group are animal and human pathogens (e.g. *S. somaliensis*).

In most soils there are 10^4 to 10^7 colony forming units (CFU) of actinomycetes in 1 gram soil which constitutes 1–20% of the total number of microorganisms (Dahm et al. 1986; Shirokikh et al. 2002). However, in some soils actinomycetes are the dominant group. Among them representatives of the *Streptomycetaceae* family, mainly the genus *Streptomyces*, make up 90% or even more of all actinomycetes.

Reports about the occurrence of acidophilic and acid-tolerant actinomycetes appeared for the first time in the scientific literature in the 1970s (Williams et al. 1971; Williams and Flowers 1978). Until that

time actinomycetes were considered to be neutrophilic organisms.

In contrast to many elaborations concerning actinomycetes of cultivated soils, the reports about their occurrence in forest soils are less numerous. The main factor limiting actinomycetes development in forest soils is believed to be the low soil pH, as the development of most actinomycetes is favoured by a neutral or slightly alkaline pH. However, these microorganisms have also been isolated from strongly acidic forest soils (Barabasz and Voøišek 2002; Marcinowska 2002; Zakalyukina et al. 2002, 2004). Williams et al. (1971) isolated from acidic soil of a pine forest actinomycetes developing both on a neutral substrate as well as acidophilic ones with an optimum growth pH of 4.5. Acidophiles differed from neutrophiles not only by different requirements in respect to pH but also by other physiological properties. Williams and Robinson (1981) state that acidophilic actinomycetes play an important role in chitin decomposition in acid forest soils. As a result of N-acetyloglucosamine decarboxylation they can increase the pH of the environment, thus enabling the growth of neutrophilic actinomycetes. The lesser numbers and smaller differentiation of actinomycetes in forest ecosystems is also linked to the properties of forest soils. Forests have the ability to regulate the water relation of the soil, have a specific effect on phytoclimate and soil climate and also ensure a distinct type of biological circulation of matter and energy flow (Puchalski and Prusinkiewicz 1990).

Assignment of *Streptomyces* to species creates considerable problems because of the large number of described and published taxa, most of which are based on the description of a single strain. Thus currently there is no one recommended method or even several methods in order to assign actinomycetes of the genus *Streptomyces* to species. Since a clear concept of a species within the genus *Streptomyces* still does not exist, careful assignment of taxa on the basis of results obtained using one of the known methods of identification is recommended (Atalan et al. 2000; Mellouli et al. 2003; Kämpfer 2006).

The aim of the present work was to analyze the number and to identify actinomycetes isolated from acid forest soils and from the root zone of forest trees in Poland.

Materials and methods

The analyzed material were actinomycetes (and in the preliminary investigations also eubacteria and saprophytic fungi, in order to obtain general microbiological characteristics of the environment) isolated from root-free soil and the root zone (rhizosphere – soil strongly associated with roots and rhizoplane – homogenate of surface-sterilized roots) of old pine (*Pinus sylvestris* L.), birch (*Betula pendula* Roth) and black alder (*Alnus glutinosa* L.) stands. The samples were collected in a mixed forest in the Dobrzejewice forest inspectorate, Bielawy forest district in Toruń.

The pH of forest soils in water solution was determined.

To isolate microorganisms the commonly used Koch dilution method was applied. The general obtained number of microorganisms (c.f.u.) which could be cultured was estimated per 1 g dry mass of soil or roots.

In order to isolate actinomycetes (and eubacteria) appropriately prepared material was plated on starch-casein medium (SCA – Starch Casein Agar) (according to Küster and Williams 1964) with nystatin and actidione (50 mg/dm^3 medium).

For isolation of fungi the fungal selective Martin medium (BTL) was used, supplemented with streptomycin (30 mg/dm^3 medium). Assignment of the actinomycete strains to species was performed using the computer program "Probabilistic Identification of Bacteria" using probability matrices (Bryant 1995). For this purpose a number of tests were performed determining the morphological and physiological properties of the analyzed actinomycetes.

Actinomycete morphology

In these investigations we have analyzed the colour of the aerial and substrate mycelium and the colour of the diffusing dye, and also the type of the formed conidial chains. The mycelium colour and the type of conidial chains was determined on ISP 4 medium (Inorganic Salts – Starch Agar, Difco), and the colour of the diffusing dye on ISP 5 medium (Glycerol –

Table 1. Research plot characteristics

Forest stand	Forest department	Largeness of department (ha)	Trees composition	The site of sample	Age of trees (in years)	pH of soil (in H_2O)
Scots pine	6 g	2.96	~100% Scots pine	53°01'46" N 18°42'24" E	87	4.1
Birch	10 g	4.03	10% birch; 90% Scots pine	53°01'27" N 18°42'28" E	58	4.3
Alder	3 m	0.72	100% alder	53°02'01" N 18°42'59" E	72	4.0

Asparagine Agar, Difco). The method proposed by Shirling and Gottlieb (1966) was used for the investigations.

The colour of the aerial and substrate mycelium and the colour of the diffusing dye was determined according to the Munsell atlas of colours. The morphology of conidial chains was determined by the microculture method (according to Kutzner 1981). The strains were classified into appropriate groups on the basis of the structure of the chains: spiral – S (Latin *Spira*), straight – RF (Latin *Rectus-Flexibilis*), incomplete spiral – RA (Latin *Retinaculum- Apertum*).

Differentiating physiological properties of actinomycetes according to Williams et al. (1983)

Physiological properties of actinomycetes were determined by defining their ability to decompose some organic compounds (xanthine, xylane, pectins, lectins, tributyrates, arbutins, urea, allantoins, hippurates), formation of hydrogen sulphide, melanin production, reduction of nitrates to nitrites, and also determining their ability to utilize different sources of carbon and nitrogen.

The ability of actinomycetes to grow in the presence of inhibitors (7% NaCl; 0.1% phenol; 0.01% sodium azide, 0.001% potassium telluride) and at 45°C were also tested. The antagonist properties of actinomycetes were tested in respect to the following test microorganisms: *Bacillus subtilis* ATTC 6633, *Micrococcus luteus* ATTC 10240, *Streptomyces murinus* PCM 2369, *Aspergillus niger* ATTC 16404, *Saccharomyces cerevisiae* ATTC 9763, *Candida albicans* ATTC 10231 and resistance to antibiotics in respect to penicillin, rifampicin and neomycin.

Molecular identification of actinomycetes

For the investigations 30 strains isolated from the soil and pine roots were selected as pine is the tree occurring the most frequently in Poland. For this purpose DNA was isolated and the 16S rDNA fragment was amplified and sequenced.

DNA isolation (Kirby mix procedure) (Kieser et al. 2000)

This procedure involves lysis with phenolic detergent mix and phenol/chloroform extraction.

The isolated DNA was stored at 4°C until further analysis or for longer storage at –20°C.

The obtained DNA was checked qualitatively and quantitatively by the spectrophotometric method performing measurements at wavelengths of 230, 260 and 280 nm using a Nano Drop ND 1000 spectrophotometer and ND 1000V 3.3.0 software.

Polymerase Chain Reaction (PCR)

The isolated DNA was amplified by PCR using a thermocycler (Authorized Thermal Cycler – Eppendorf AG 223331, Hamburg). For amplification PCR primers were used (Biomers.net GmbH Ulm, Germany): Forward (FW) 5'-AGA GTT TGA TCC TGG CTC AG-3'

Reverse (RW) 5' – AAG GAG GTG ATC CAG CCG CA-3'. Fragments 1600 bp in length were amplified.

The PCR reaction mixture was as follows: FW (100 pmol/μl) 10 μl; RW (100 pmol/μl) 10 μl; dNTP (10 mM) (Fermentas) 5 μl; 10×TagBuffer z $(NH_4)_2SO_4$ (Fermentas) 10 μl; $MgCl_2$ (25 mM) (Fermentas) 12 μl; DMSO (100%) (Sigma) 5 μl; Taq polymerase (500U) (Fermentas) 2 μl; isolated template DNA 1–2 ng/100 μl sample and ddH_2O (after taking into consideration the amount of DNA template the sample for the PCR reaction was supplemented with ddH_2O to a volume of 100 μl).

Conditions of the PCR reaction: step 1 – denaturation (95°C; 5 minutes); step 2 (30 cycles) – denaturation (95°C; 20 seconds); annealing (55°C; 30 seconds); synthesis (72°C; 90 seconds); step 3 – elongation (72°C; 5 minutes); step 4 – storage (4°C).

The amplified DNA was analyzed in a 1% agarose gel (Invitrogen). For gel analysis a UV transilluminator and Doc – It®LS Image Analysis Software from UVP, Inc. Upland, CA in Cambridge, UK were used.

The length of the amplified DNA fragment was determined on the basis of a molecular weight standard. In the investigations Lambda DNA/PstI Marker (λ/PstI, Fermentas) containing linear DNA fragments with a length of 210 bp (0.21 kb) to 11509 bp (11.5 kb) was used.

For purifying the proper PCR products the QIAquick Gel Extraction Kit (Qiagen) was used according to the producer's instructions.

Amplified and purified genetic material was sequenced using an ABI Prism™ Big Dye™ Terminator Cycle Sequencing Kit in the Laboratory of DNA Sequencing and Oligonucleotide Synthesis of the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences in Warsaw.

Results

The number of microorganisms (actinomycetes, bacteria and fungi) isolated from the root zone (rhizosphere, rhizoplane) and bulk soil of the analyzed trees

Root soil of the analyzed trees contained more microorganisms than soil outside the range of the roots. This also concerned actinomycetes, eubacteria as well as saprophytic fungi.

Most eubacteria were detected in the alder rhizosphere, whereas actinomycetes were the most numerous in the birch root zone. The tree with the lowest numbers of bacteria and actinomycetes was the pine. The number of fungi was the lowest in pine soil and the highest in alder soil (Fig. 1).

Identification of actinomycetes isolated from under pine, birch and alder by the probabilistic method using a probability matrix for actinomycetes (Bryant 1995)

Tables 2–4 contain the results of identification of actinomycetes isolated under pine, birch and alder taking into consideration the probability of identification of particular strains expressed by means of the Wilcox coefficient. Satisfactory identification was achieved for over 55% of the analyzed actinomycetes. The threshold value of the Wilcox coefficient for these strains was 0.98. 42.22% strains were similar to a given taxon. For them the threshold value of the Wilcox coefficient was in the range of 0.98–0.6. The remaining strains (2.22%) were identified with a low probability of <0.6 (Table 5).

The analyzed actinomycete strains were identified to 18 taxa, with a dominant species *Streptomyces exfoliatus*. No dominance of a specific species was observed in particular sources from which actinomycetes were isolated (root-free soil, rhizosphere, rhizoplane) but the species *S. exfoliatus* occurred the

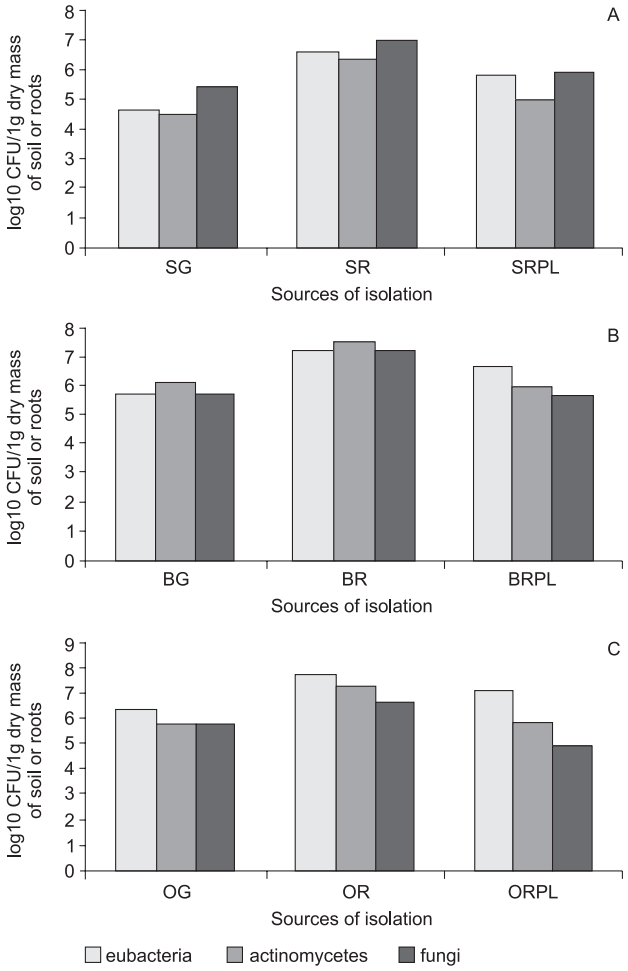

Fig. 1. The number of microorganisms (eubacteria, actinomycetes and fungi) in bulk soil, rhizosphere and rhizoplane of Scots pine (A), birch (B) and alder (C). SG – bulk soil of Scots pine; SR – rhizosphere of Scots pine; SRPL – rhizoplane of Scots pine; BG – bulk soil of birch; BR – rhizosphere of birch; BRPL – rhizoplane of birch; OG – bulk soil of alder; OR – rhizosphere of alder; ORPL – rhizoplane of alder

Table 2. Results of identification of actinomycetes isolated from bulk soil and root zone of Scots pine by the probabilistic method using a probability matrix for actinomycetes (Bryant 1995)

Isolated strain	Identified species	Probability of the identification (Wilcox Index)
SG1	*Streptomyces griseoviridis*	0.98
SG2	*Streptomyces cyaneus*	0.79
SG3	*Streptoverticillium olivovertici**	0.98
SG4	*Streptomyces cyaneus*	0.87
SG5	*Streptomyces varsoviensis*	0.98
SG6	*Streptomyces griseoviridis*	0.98
SG7	*Streptomyces exfoliatus*	0.92
SG8	*Streptomyces griseoviridis*	0.96
SG9	*Streptomyces cyaneus*	0.80
SG10	*Streptomyces chromogenus*	0.98
SR1	*Streptomyces exfoliatus*	0.88
SR2	*Streptomyces xanthochromogenes*	0.98
SR3	*Streptomyces phaeochromogenes*	0.85
SR4	*Streptomyces anulatus*	0.79
SR5	*Streptomyces exfoliatus*	0.98
SR6	*Streptoverticillium olivovertici**	0.98
SR7	*Streptomyces griseoviridis*	0.98
SR8	*Streptomyces exfoliatus*	0.98
SR9	*Streptomyces exfoliatus*	0.96
SR10	*Streptomyces exfoliatus*	0.96
SRPL1	*Streptomyces exfoliatus*	0.98
SRPL2	*Streptomyces xanthochromogenes*	0.98
SRPL3	*Streptomyces exfoliatus*	0.98
SRPL4	*Streptomyces olivaceoviridis*	0.95
SRPL5	*Streptomyces exfoliatus*	0.98
SRPL6	*Streptomyces olivaceoviridis*	0.98
SRPL7	*Streptomyces diastaticus*	0.98
SRPL8	*Streptomyces graminofaciens*	0.98
SRPL9	*Streptomyces olivaceoviridis*	0.79
SRPL10	*Streptomyces cyaneus*	0.87

SG – bulk soil of Scots pine; SR – rhizosphere of Scots pine; SRPL – rhizoplane of Scots pine

*the genus *Streptoverticillium* is currently included in the genus *Streptomyces*

Table 3. Results of identification of actinomycetes isolated from bulk soil and root zone of birch by the probabilistic method using a probability matrix for actinomycetes (Bryant 1995)

Isolated strain	Identified species	Probability of the identification (Wilcox Index)
BG1	*Streptomyces chromogenus*	0.98
BG2	*Streptomyces varsoviensis*	0.98
BG3	*Streptomyces cyaneus*	0.65
BG4	*Streptomyces chromofuscus*	0.41
BG5	*Streptomyces cellulosae*	0.98
BG6	*Streptomyces cyaneus*	0.73
BG7	*Streptomyces chromofuscus*	0.97
BG8	*Streptomyces exfoliatus*	0.98
BG9	*Streptomyces griseoluteus*	0.60
BG10	*Streptomyces diastaticus*	0.71
BR1	*Streptomyces fulvissimus*	0.95
BR2	*Streptoverticillium olivovertici**	0.98
BR3	*Streptomyces exfoliatus*	0.98
BR4	*Streptomyces griseoviridis*	0.61
BR5	*Streptomyces griseoviridis*	0.98
BR6	*Streptomyces anulatus*	0.70
BR7	*Streptomyces chromofuscus*	0.98
BR8	*Streptomyces cyaneus*	0.92
BR9	*Streptomyces exfoliatus*	0.98
BR10	*Streptomyces exfoliatus*	0.98
BRPL1	*Streptomyces exfoliatus*	0.98
BRPL2	*Streptomyces chromofuscus*	0.89
BRPL3	*Streptomyces exfoliatus*	0.98
BRPL4	*Streptomyces phaeochromogenes*	0.70
BRPL5	*Streptomyces chromofuscus*	0.93
BRPL6	*Streptomyces aurantiacus*	0.91
BRPL7	*Streptomyces exfoliatus*	0.73
BRPL8	*Streptomyces chromofuscus*	0.98
BRPL9	*Streptomyces exfoliatus*	0.98
BRPL10	*Streptomyces phaeochromogenes*	0.98
BG1	*Streptomyces chromogenus*	0.98
BG2	*Streptomyces varsoviensis*	0.98
BG3	*Streptomyces cyaneus*	0.65
BG4	*Streptomyces chromofuscus*	0.41
BG5	*Streptomyces cellulosae*	0.98
BG6	*Streptomyces cyaneus*	0.73
BG7	*Streptomyces chromofuscus*	0.97
BG8	*Streptomyces exfoliatus*	0.98
BG9	*Streptomyces griseoluteus*	0.60
BG10	*Streptomyces diastaticus*	0.71
BR1	*Streptomyces fulvissimus*	0.95
BR2	*Streptoverticillium olivovertici**	0.98
BR3	*Streptomyces exfoliatus*	0.98
BR4	*Streptomyces griseoviridis*	0.61
BR5	*Streptomyces griseoviridis*	0.98
BR6	*Streptomyces anulatus*	0.70
BR7	*Streptomyces chromofuscus*	0.98
BR8	*Streptomyces cyaneus*	0.92
BR9	*Streptomyces exfoliatus*	0.98
BR10	*Streptomyces exfoliatus*	0.98
BRPL1	*Streptomyces exfoliatus*	0.98
BRPL2	*Streptomyces chromofuscus*	0.89
BRPL3	*Streptomyces exfoliatus*	0.98
BRPL4	*Streptomyces phaeochromogenes*	0.70
BRPL5	*Streptomyces chromofuscus*	0.93
BRPL6	*Streptomyces aurantiacus*	0.91
BRPL7	*Streptomyces exfoliatus*	0.73
BRPL8	*Streptomyces chromofuscus*	0.98
BRPL9	*Streptomyces exfoliatus*	0.98
BRPL10	*Streptomyces phaeochromogenes*	0.98

BG – bulk soil of birch; BR – rhizosphere of birch; BRPL – rhizoplane of birch

* – the genus *Streptoverticillium* is currally included in the genus *Streptomyces*

Table 4. Results of identification of actinomycetes isolated from bulk soil and root zone of Scots pine by the probabilistic method using a probability matrix for actinomycetes (Bryant 1995)

Isolated strain	Identified species	Probability of the identification (Wilcox Index)
OG1	*Streptomyces diastaticus*	0.90
OG2	*Streptomyces exfoliatus*	0.98
OG3	*Streptomyces exfoliatus*	0.81
OG4	*Streptomyces exfoliatus*	0.98
OG5	*Streptomyces diastaticus*	0.97
OG6	*Streptomyces exfoliatus*	0.98
OG7	*Streptomyces chromogenus*	0.98
OG8	*Streptomyces exfoliatus*	0.98
OG9	*Streptomyces exfoliatus*	0.98
OG10	*Streptomyces exfoliatus*	0.98
OR1	*Streptomyces exfoliatus*	0.98
OR2	*Streptomyces exfoliatus*	0.97
OR3	*Streptomyces diastaticus*	0.60
OR4	*Streptomyces diastaticus*	0.98
OR5	*Streptomyces roseus*	0.98
OR6	*Streptomyces diastaticus*	0.98
OR7	*Streptomyces exfoliatus*	0.83
OR8	*Streptomyces chromofuscus*	0.60
OR9	*Streptomyces anulatus*	0.98
OR10	*Streptomyces chromofuscus*	0.95
ORPL1	*Streptomyces diastaticus*	0.96
ORPL2	*Streptomyces diastaticus*	0.93
ORPL3	*Streptomyces exfoliatus*	0.98
ORPL4	*Streptomyces diastaticus*	0.54
ORPL5	*Streptomyces diastaticus*	0.77
ORPL6	*Streptoverticillium olivovertici**	0.98
ORPL7	*Streptomyces diastaticus*	0.98
ORPL8	*Streptoverticillium olivovertici**	0.98
ORPL9	*Streptomyces exfoliatus*	0.98
ORPL10	*Streptomyces exfoliatus*	0.98

OG – bulk soil of alder; OR – rhizosphere of alder; ORPL – rhizoplane of alder

* – the genus *Streptoverticillium* is currally included in the genus *Streptomyces*

Table 5. Wilcox Index for strains isolated from under Scots pine, birch and alder

Wilcox Index	0.98	<0.98–0.6	<0.6
Number of studied strains	50	38	2
[%] all studied strains	55.55	42.22	2.22

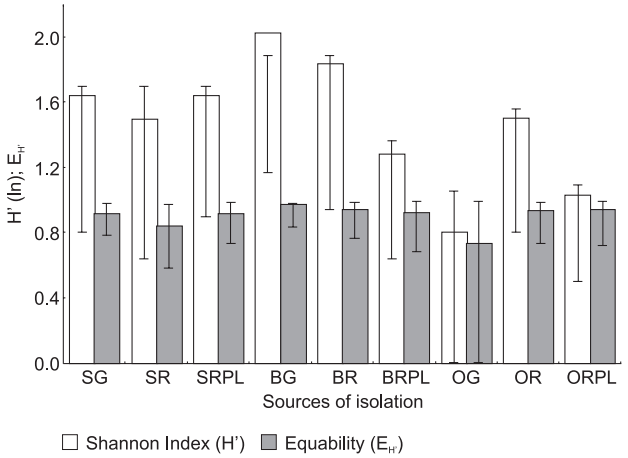

Fig. 2. Shannon index (H') and its equability ($E_{H'}$) for species composition of actinomycetes isolated from bulk soil and root zone (rhizosphere and rhizoplane) of Scots pine, birch and alder. SG – bulk soil of Scots pine; SR – rhizosphere of Scots pine; SRPL – rhizoplane of Scots pine; BG – bulk soil of birch; BR – rhizosphere of birch; BRPL – rhizoplane of birch; OG – bulk soil of alder; OR – rhizosphere of alder; ORPL – rhizoplane of alder (error bars are 95% confidence limits, completed using a bootstrap technique)

most frequently among actinomycetes isolated from under alder, especially among soil actinomycetes.

Among 90 identified strains 6 species which occur the most frequently observed were: *Streptomyces exfoliatus* (30 strains), *S. diastaticus* (12 strains), *S. chromofuscus* (8 strains), *S. cyaneus* (7 strains) and *Streptoverticillium olivovertici* (5 strains).

The most diverse strain composition was observed among actinomycetes isolated from under the birch (14 species) and pine (12 species). The habitat containing the lowest number of species was root-free soil and the root zone of the alder, where 7 different species of actinomycetes were found.

The Shannon calculated coefficient of species diversity (H') indicated that these were diverse habitats (from H' ≈ 0.8 to H' ≈ 2.0). The most diverse sources of isolation were bulk soil (H' ≈ 2.0) and the rhizosphere of birch (H' ≈ 1.8) and the least diverse habitat was root-free alder soil (H' ≈ 0.8) and the alder rhizoplane (H' ≈ 1.0). The remaining isolation sources were moderately diverse. The value of the Shannon coefficient was in the range from H' ≈ 1.3 to H' ≈ 1.6. The identified species occurred in general in a similarly homogeneous fashion in defined sources of isolation (Fig. 2).

It was also noted that some species of actinomycetes are associated exclusively with a given tree. *Streptomyces graminofaciens*, *S. olivaceoviridis* and *S. xanthochromogenes* occur only among actinomycetes

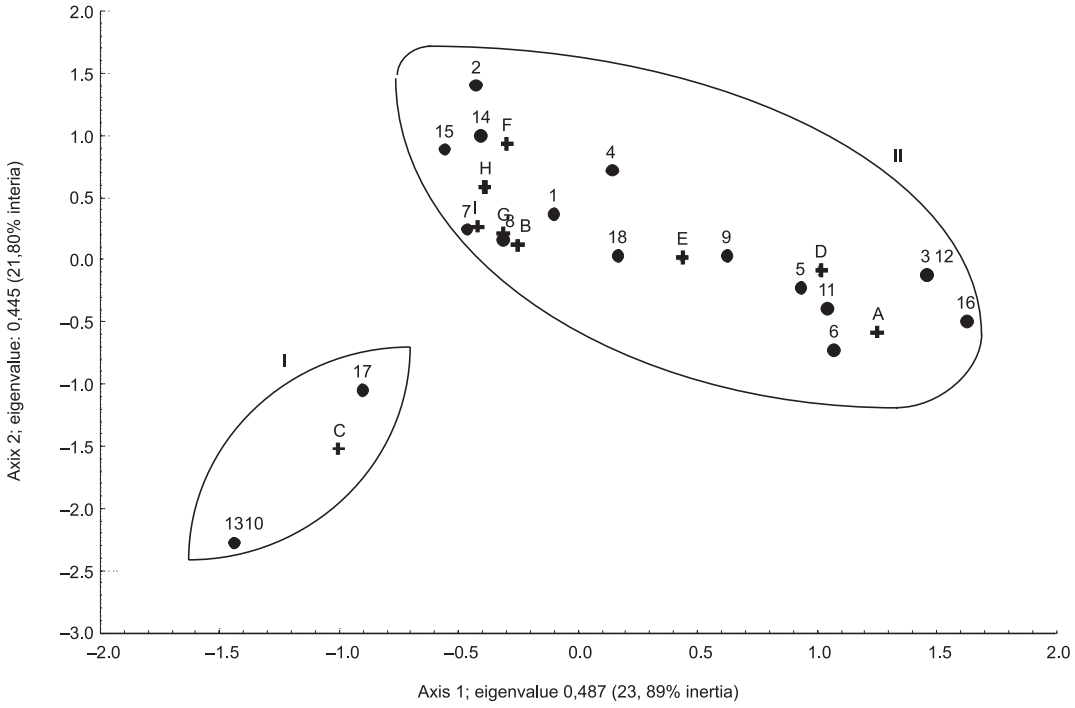

Fig. 3. Correspondence analysis (CA) of clusters of the sources of isolation and species of actinomycetes associated with them
Identified species of *Streptomyces*: 1. *S. anulatus;* 2. *S. aurantiacus;* 3. *S. cellulosae;* 4. *S. chromofuscus;* 5. *S. chromogenus;* 6. *S. cyaneus;* 7. *S. diastaticus;* 8. *S. exfoliates;* 9. *S. fulvissimus;* 10. *S. graminofaciens;* 11. *S. griseoviridis;* 12. *S. griseoluteus;* 13. *S. olivaceoviridis;* 14. *S. phaeochromogenes;* 15. *S. roseus;* 16. *S. varsoviensis;* 17. *S. xanthochromogenes;* 18. *St. olivovertici*
Sources of isolation: A – bulk soil of Scots pine; B – rhizosphere of Scots pine; C – rhizoplane of Scots pine; D – bulk soil of birch; E – rhizosphere of birch; F – rhizoplane of birch; G – bulk soil of alder; H – rhizosphere of alder; I – rhizoplane of alder

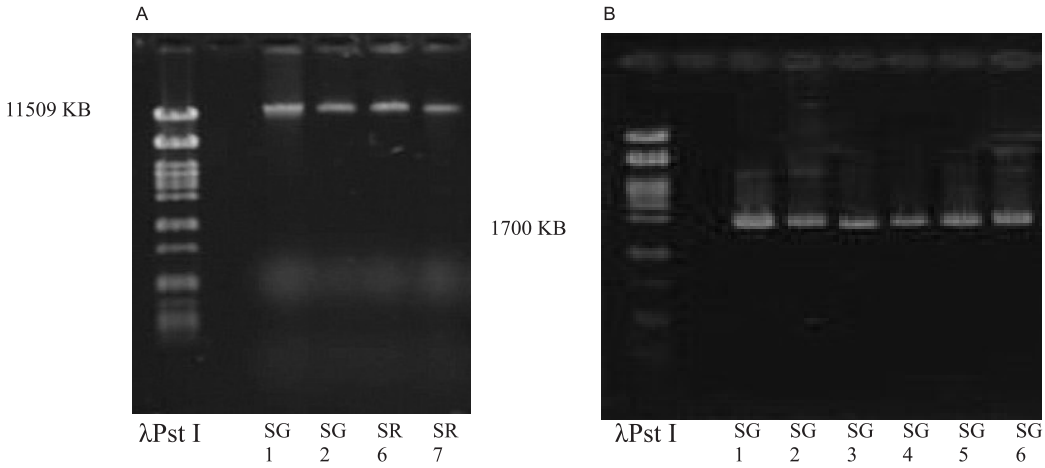

Fig. 4. Results of DNA isolation (A) and 16rDNA amplification (B) from actinomycetes isolated from under Scots pine (chosen strains). SG – strains isolated from bulk soil of Scots pine; SR – strains isolated from rhizosphere of Scots pine

isolated from under the pine, and *S. aurantiacus*, *S. fulvissimus*, *S. griseoluteus* and *S. cellulosae* among actinomycetes linked only to the birch. An actinomycete species only associated with the alder was *S. roseus*.

As a result of correspondence analysis (Fig. 3) two clusters of the sources of isolation and taxa of actinomycetes associated with them were found. The first cluster encompasses the pine rhizoplane and three taxa of actinomycetes associated with it: *S. graminofaciens*, *S. olivaceoviridis* and *S. xanthochromogenes*. The second cluster encompasses all remaining identified species and the sources of isolation associated with them (bulk soil and rhizosphere of the pine, birch, alder and the rhizoplane of the birch and alder). This cluster in considerably diverse – especially along the first axis of correspondence analysis (explaining the largest part of the variation among all axes).

Molecular identification of *Streptomyces*

Molecular assignment of actinomycetes to species on the basis of 16S rRNA sequence analysis was not possible because of the too small variation within this gene. This method made it only possible to assign the analyzed strains to the appropriate genera and in combination with other tests performed for identification allowed their correct classification (Fig. 4).

Discussion

The number of microorganisms in the soil and their diversity depend on many factors, both biotic and abiotic ones (Schlegel 2000; Saadoun and Gharaibeh 2003). Among biotic factors plants are of great importance. Their root system can liberate various chemical compounds into soil. Root exudates can be divided into two types: one of them is common in most plants, the an other is the special one which is produced by certain plant species or under certain conditions (Tu et al. 2000). It was shown that different species had similar components as well as different ones. Difference of components may occur in the same plant species that were in different age.

Plant root exudates are under influence of many factors such as plant genotype, age, nutrition status and microbial activity in the rhizosphere (Uselman et at. 1999; Tu et al. 2000; Sandnes et al. 2005).

Exudates from conifers (Douglas-fir, grand fir, noble fir, Pacific silver fir, ponderosa pine, lodgepole pine, Engelmann spruce) contained glucose, sucrose, fructose, rhamnose and ribose. Aspartic acid, glutamic acid and glutamine were the main amino acids in the exudates of all species with lesser amounts of glycine, serine, asparagine, arginine, leucine and alanine. The pH of the exudates ranged from 5.3 to 5.7 (Ketchie and Lopushinsky 1981). Those authors stated that except for the relatively high concentrations of sugars in the exudates from grand fir and noble fir, there were no species related differences in concentrations of the various chemical substances.

Sandnes et al. (2005) reported on low molecular weight organic acid in root exudates of Norway spruce and silver birch. Dominated monocarboxylic acids – fumaric acid was exclusive for spruce, while lactic, malonic, butyric and phthalic acids were only found in the birch exudates. In spruce oxalic, lactic, formic, butyric and phthalic acids were found and citric, adipic, propionic, succinic and acetic acids were observed in the rhizosphere of birch.

Tree species, developmental stage, root density, mycorrhizal status and growth conditions were important for composition and the amount of organic acids. However authors conclude that the rhizosphere of birch contains more organic acids and at higher concentrations than spruce.

In our investigations the greatest number of eubacteria were associated with the alder (bulk soil, rhizosphere and rhizoplane), and the smallest number with the pine, whereas actinomycetes were the most numerous in the birch rhizosphere.

Some scientists believe (Zakalyukina et al. 2002) that the composition of actinomycete genera is determined by the properties of the soil, mainly the pH, whereas the species of microorganisms are selected by the plant.

The considerable number of actinomycetes found by us in acid forest soils (pH 4.0–4.3) constituting 13.6–43.7% of the isolated bacteria and fungi (22.1–59.2% of the isolated bacteria) indicates the importance of these microorganisms in forest tree stands. Similarly other researchers believe that actinomycetes are, after eubacteria, the second largest group of soil microorganisms (Küster 1971; Kutzner 1981; Mordarska and Paściak 2002; Shirokikh et al. 2002).

Birch is considered as a pioneer tree settling easily into cuttings after a spruce forest and improving soil-forming and microbiological parameters of this habitat (Lettl and Hysek 1994). In our investigations we have noted the highest numbers of actinomycetes in the rhizosphere and the soil from under the birch.

Analyzing the occurrence of the actinorrhizal actinomycete *Frankia* in forest soils, Huss-Danell and Frej (1986) observed that the numbers of this microorganisms in the soil do not depend absolutely on the presence of actinorrhizal plants. Often larger numbers of *Frankia* were noted under non-actinorrhizal plants (e.g. *Betula* sp.) than under actinorrhizal ones (e.g. *Alnus* sp.). Smolander (1990) suggested that because of the phylogenetic similarity of birch and alder (*Betulaceae* family) the birch could similarly to alder be an actinorrhizal plant. However, so far no actinorrhizal symbiosis has been observed on birch roots.

Actinomycetes are an important group of soil microorganisms which successfully compete in this environment with other microorganisms (Mordarska and Paściak 2002).

Many of the investigations performed so far on actinomycetes concerned the antagonistic action of *Actinomycetes* on other microorganisms, and also their metabolic activity mainly the ability to produce enzymes and vitamins, especially in agricultural soils (Crawford et al. 1993; Pędziwilk 1995; Paul and Clark 2000).

The member of actinomycetes on which the most attention has been focused from the beginning of the 20th century is *Streptomyces coelicolor*. It is considered to be a model organism in physiological, genetic and molecular investigations (Hobbs et al. 1992; Hood et al. 1992; Chater and Losick 1996; Kieser et al. 2000; Bentley et al. 2002; Sheeler et al. 2005; Kois et al. 2009).

So far little attention has been focused on the identification of forest soil actinomycetes. The methods used so far are mainly based on analyzing a number of morphological and physiological properties of strains according to the method proposed by the International *Streptomyces* Project (ISP) (Shirling and Gottlieb 1966; Williams et al. 1983; Mehling et al. 1995; Hain et al. 1997; Saadoun and Gharaibeh 2008).

According to some researchers (Kämpfer 2006) identification of *Actinomycetes* by methods of the analysis of the sequence of 16S rRNA may be difficult because of the low variation in this gene.

Also our attempts to identify actinomycetes allowed us only to determine the genus. Species of *Streptomyces* were identified by a method also used by other researchers in agreement with ISP, described by Shirling and Gottlieb (1966).

On the basis of morphological and biochemical properties supported by successful analysis of 16S rRNA sequences, Srivibool et al. (2004) assigned two particularly biochemically active strains of thermotolerant soil actinomycetes as belonging to the species *Streptomyces tendae* and *S. maritimus*.

The results of identification of forest soil and tree root actinomycetes indicate their large species diversity. Knutson et al. (1980) among forest soil actinomycetes have considered *Streptomyces parvulus* as the dominant species, and in the mycorrhizosphere and inside mycorrhizas *Streptomyces collinus*, *S. longisporus*, *S. rochei*, *S. aureomonopodialis*, *S. atrolivaceus*, *S. griseobrunneus* and *S. resistomycificus* were dominant. Różycki (1984) in a pine wood forest considered as the dominant species *Streptomyces longispororuber*, and from the rhizosphere and mycorrhizosphere *Streptomyces venezeuelae*, *S. lavendulae*, *S. griseus*, *S. tendae* and *S. violaceoniger* were frequently isolated.

Our probabilistic assignment of members of the genus *Streptomyces* to species, based on several dozen easily reproducible diagnostic morphological and physiological properties made it possible to distinguish 18 taxa. The greatest number of *Streptomyces* strains were assigned to the species *S. exfoliatus* (33.3%). Fewer strains were assigned to *S. diastaticus* (13.3%), *S. chromofuscus* (8.9%), *S. cyaneus* (7.8%) and *S. olivovertici* (5.5%).

The analysis of the diversity of actinomycetes in the analyzed sources of isolation (different soils and rhizosphere of different trees) indicated a lack of significant differences between these microorganisms. The only sources of isolation which were relatively diverse were the root-free soil of birch (Shannon coefficient at the limit of 2.0) and the birch rhizosphere (H' index $\approx$ 1.8).

The lowest diversity of actinomycetes was found in bulk soil and the actinorrhizal rhizoplane of alder.

According to Burges and Raw (1971) groups of microorganisms which are the most diverse are those in stabilized, equilibrated biotopes. Such a view would indicate that the microorganisms of root-free soil should be the most differentiated in comparison with the rhizosphere which is subject to the effects of root secretions. The results of our investigations are not in

a full agreement with a view that natural habitats rich in nutrients contain poorly differentiated species because of the antagonistic interactions between microorganisms leading to the dominance of selected groups.

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