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STUDIES ON THE FAST- AND SLOW GROWING BACTERIA
OCCURRING IN THE ROOT-FREE SOIL, RHIZOSPHERE AND
MYCORRHIZOSPHERE OF NURSERY SEEDLINGS AND 70-YEAR
OLD TREES OF SCOTS PINE (*Pinus sylvestris* L.). ENUMERATION
AND IDENTIFICATION

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Abstract. The numbers of bacteria were higher in the mycorrhizosphere of both the seedlings and old trees than in other sources of isolation. The numbers of bacteria forming fast and slow-growing colonies were different depending on their origin. In the same sources of isolation dominated the same groups/taxons for old trees and nursery seedlings.

Soil micro-organisms are integral components of forest soil ecosystems playing an important role in the nutrient cycle, maintenance of soil structure and regulation of plant growth [14, 18].

The influence of soil micro-organisms on other ecosystem components may depend on their potential growth rate, which can be expressed as a time of colony formation [9, 13]. Colonies appearing on a given day can be considered as a rough equivalence of species; hence it is possible to calculate diversity indices of soil bacteria [9]. Interpretation of such calculation results was based on the theory of K- and r-selection. K-strategists dominate in the densely inhabited biotopes, and r-strategists in the uninhabited ones that are rich in nutrients. R-strategists form colonies in the first two days after inoculation; those, which form colonies later, are K-strategists [9].

Up-to-date studies on the occurrence of the slow- and the fast-growing soil micro-organisms in the soil and rhizosphere of some crop plants have been

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performed [9, 21]. Practically nothing is known about these two groups of microbes occurring in the root-free soil and in the root zone of forest trees. For that reason this study was undertaken.

MATERIALS AND METHODS

Origin of samples and sampling procedure. Samples of root free soil, mycorrhizal and non-mycorrhizal (suberized) roots of pine (*Pinus sylvestris* L.) were taken on October 14, 1998 in the Dobrzejewice Forest Inspectorate (near Toruń, Poland) from two sites:

- a) Scots pine nursery with 1-year old seedlings;
- b) 70-year old Scots pine forest.

Basic soil properties of the site a) were: $\text{pH}_{\text{H}_2\text{O}} = 5.8$, $\text{pH}_{\text{KCl}} = 4.8$, $C_{\text{total}} = 2.4$ %, $N_{\text{total}} = 0.16$ %, $C/N = 15$ (loamy sand).

Basic soil properties of the site b) were: $\text{pH}_{\text{H}_2\text{O}} = 4.5$, $\text{pH}_{\text{KCl}} = 3.8$, $C_{\text{total}} = 1.0$ %, $N_{\text{total}} = 0.04$ %, $C/N = 25$ (podzol formed from loose sand). Habitat type at the site b) was: sub-continental pine forest (*Peucedano-Pinetum*).

Mixed samples (300-500 g; depth 5-10 cm) were taken from the underneath of 10-12 seedlings and 4-5 trees chosen at random.

Enumeration and isolation. Bacteria were enumerated and isolated from the root-free soil, rhizosphere and mycorrhizosphere of the nursery seedlings and 70-year old trees of Scots pine. Soil Extract Agar according to Lochhead and Chase [16] was used as the plating medium (7 replicates). To evaluate the time-course of the bacterial colony development and to differentiate between fast-growing (appearing after 1-2 days) and slow-growing (appearing after 6-10 days) colonies, the colonies were counted after 1, 2, 3, 4, 5, 6 and 10 days of incubation at a temperature of 26°C [9]. The bacteria were sub-cultured and stored in R2A/4 semisolid medium [modified R2A medium (Difco) – its composition was given by Różycki *et al.* [20]]. 30–50 strains were obtained from each source of isolation. As some strains were lost during examination, their final number was 432 (222 – for the seedlings, 210 – for the old trees of Scots pine).

Identification of bacteria. Bacteria were identified using the scheme given by Acero *et al.* [1]. The following results were then analysed: results of morphological characterisation of bacterial cells [G(-) rods, G(+) cocci, G(+) sporulating bacilli and pleomorphic forms] and results of the following tests: survival after pasteurisation [2], production of catalase [10], cytochrome oxidase [4], glucose metabolism in Hugh and Leifson [16] medium and growth in selective media: King B [11], for *Arthrobacter* [12].

Statistical evaluation of the results. The results of determination of the total number of bacteria were statistically evaluated, using 1-way analysis of variance (ANOVA) – after previous log-transformation of the c.f.u. (colony forming units)

data [$y = \log_{10}(x + 1)$]. A 2-way ANOVA was used to compare the effect of tree age (1; nursery seedlings and 70-years old trees) and sources of isolation (2; soil, rhizosphere and mycorrhizosphere) on the bacterial numbers. The colony development index (CDI) was calculated to evaluate bacteria growth rate. The CDI was a sum of ratios of bacterial colonies appearing on the days of determination which were divided by the number of days when given colonies appeared and expressed in per cent; its values could range from 10 to 100% [21]. To evaluate diversity of types of bacterial colonies growth rate, Eco-Physiological Index (EPI) was calculated. It corresponds to the Shannon's H' index which considers types of bacterial colonies growth rate (the sum of proportions of respective types of colonies formation rate multiplied by their logarithms) [9]. A 2-way analysis of variance was used to compare the effect of tree age and sources of isolation on the growth rapidity and diversity. Differences among average values for sources of isolation / type of growth rate were tested for significance using Newman-Keuls multiple range test ($p \leq 0.05$). All statistical calculations were performed using Statistica for Windows, version 5.1 (1996, StatSoft, Tulsa, Oklahoma, USA) software.

RESULTS

The number of bacteria and actinomycete in the root – free soil, rhizosphere and mycorrhizosphere of seedlings and 70-year old trees of Scots pine are shown in Fig. 1 – after log-transformation of the c.f.u. data. The number of bacteria for all sources of isolation increased in the following order: root-free soil (old forest) < rhizosphere (old forest) < root-free soil (nursery) < rhizosphere (nursery) < mycorrhizosphere (old forest) < mycorrhizosphere (nursery), ranging from $\log_{10} \approx 6.7$ to $\log_{10} \approx 8.5$ (Fig. 1).

Figure 2 points out to the proportion of fast and slow-growing bacteria colonies in the soil and the root zone of seedlings and old trees. The highest percentage of fast-growing bacteria, noted for the soil and rhizosphere of Scots pine seedlings ($\approx 55\%$) was significantly ($p \leq 0.05$) higher than in all other sources of isolation (25-32%). The slow-growing bacteria were most frequent in the mycorrhizosphere of the Scots pine seedlings ($\approx 58\%$; other sources of isolation: 15-30%) (Fig. 2).

The 2-way ANOVA indicates that both tree age (1) and sources of isolation (2) significantly affected [$p \ll 10^{-6}$; effect of (2) stronger than the effect of (1)] bacterial numbers. Bacteria were significantly more numerous in the ecosystem of the nursery than in the old forest, and the bacterial number increased significantly ($p \leq 0.05$) in the following order: soil < rhizosphere < mycorrhizosphere (Table 1).

Only the sources of isolation significantly affected the values of EPI and CDI. Both indices were significantly higher ($p \leq 0.05$) for the root-free soil and rhizosphere than for the mycorrhizosphere (Table 1).

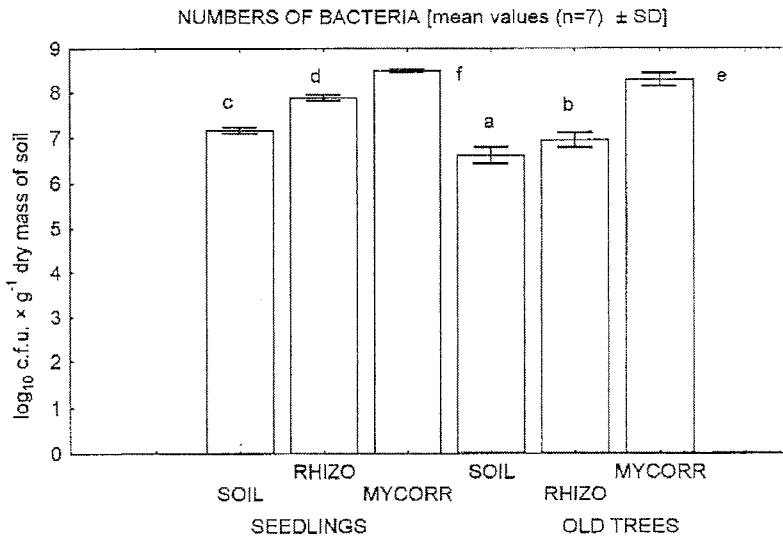


Fig. 1. Plate count of bacteria and actinomycetes (mean values of \log_{10} c.f.u., $n = 7$) on soil extract agar (c.f.u. – colony forming units) for soil, rhizosphere and mycorrhizosphere of nursery seedlings and 70-year old trees of Scots pine (*Pinus sylvestris* L.). Explanations: soil – root-free soil, rhizo – rhizosphere, mycorr – mycorrhizosphere; SD – standard deviation. Mean values indicated with the same letter do not differ significantly ($p \leq 0.05$).

Figure 3 shows calculation results for colony development index (CDI) and the eco-physiological diversity index (EPI). The CDI indicates that the colonies of bacteria from the root-free soil and the rhizosphere in the case of both the seedlings and old trees grew significantly faster than those from the mycorrhizosphere.

The Shannon H' index shows a significantly lower type diversity in time in the case of bacterial colonies in the mycorrhizosphere of the seedlings and 70-year old trees than in the case of the soil and the rhizosphere (Fig. 3).

Table 2 shows identification results of the fast and slow-growing bacteria of seedlings and old trees. Most of the identified bacterial taxons were common both in the nursery seedlings and in the old forest. Among the taxons *Bacillus*, *Arthrobacter*, coryneforms other than *Arthrobacter*, *Pseudomonas* - group I (non-pigmented colonies, oxidative catabolism of glucose) dominated both for the seedlings and old trees. The most numerous genus / group of soil bacteria (fast-growing; both for the seedlings and old trees) was *Bacillus*, of the rhizosphere organisms *Moraxella* (fast-growing; seedlings), *Pseudomonas* (slow-growing; seedlings and old trees), coryneforms other than *Arthrobacter* (fast-growing; old trees), of the mycorrhizosphere isolates coryneforms other than *Arthrobacter* (both types of growth rapidity for the ecosystems of the seedlings and old trees, respectively). The dominating taxons were similar for the seedlings and 70-year old trees when the same sources of isolation / types of growth rate were considered (Table 2).

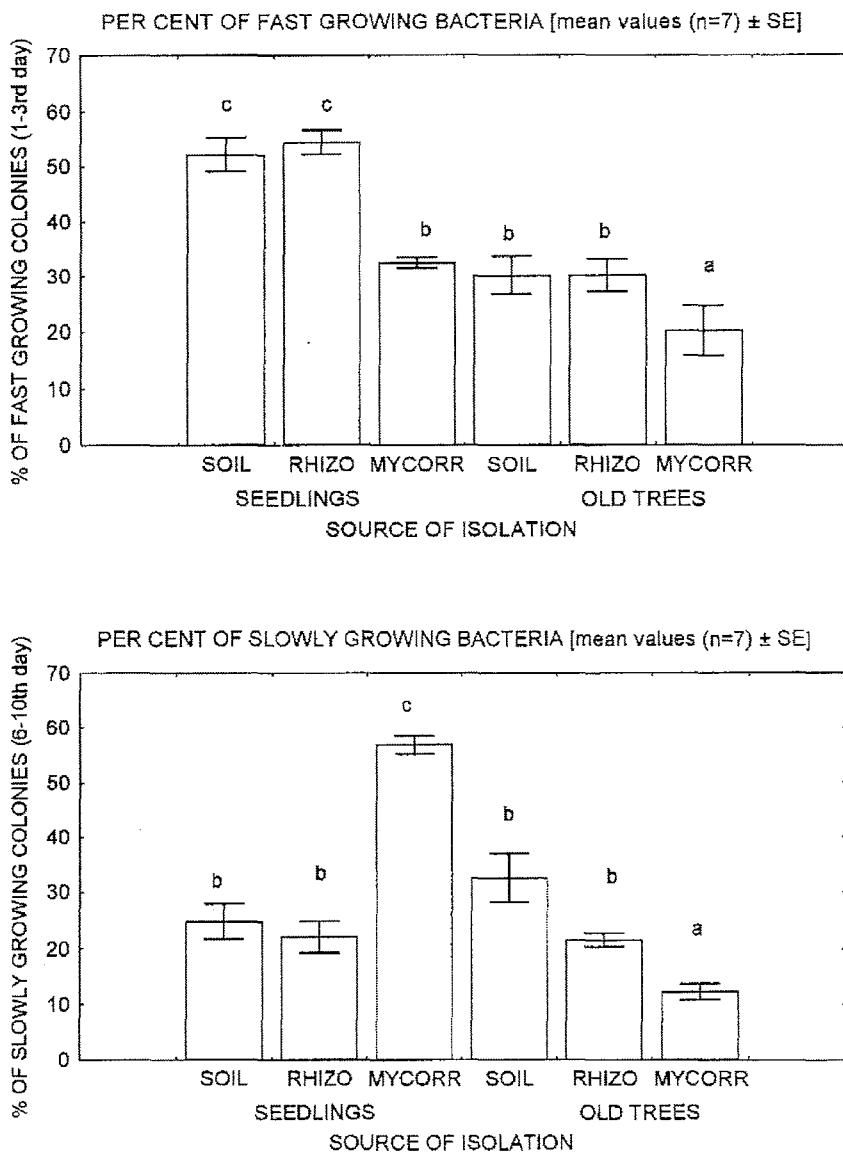


Fig. 2. Per cent occurrence of fast (colonies appearing after 1–3 days) and slow growing bacteria (colonies appearing after 6–10 days), grown on soil extract agar, among organisms isolated from soil, rhizosphere and mycorrhizosphere of nursery seedlings and old trees of Scots pine. SE – standard error; other explanations – see Fig 1.

TABLE 1. TWO-WAY ANALYSIS OF VARIANCE (ANOVA), COMPARING THE EFFECT OF THE AGE OF TREES (1; NURSERY SEEDLINGS AND 70- YEAR OLD TREES) AND SOURCES OF ISOLATION (2; SOIL, RHIZOSPHERE AND MYCORRHIZOSPHERE) ON THE NUMBER OF BACTERIA (A), ON EPI (B), AND ON CDI (C)

A. Bacterial numbers (\log_{10} c.f.u.)				
Source of variation	Variance	df @	F	p (significance level)
Age of trees	3,34	1	213,99	$<< 10^{-6}$
Source of isolation	8,11	2	520,24	$<< 10^{-6}$
Interaction	0,46	2	29,50	$< 10^{-6}$
Error	0,02	36		

Newman-Keuls multiple range test: @@

I. Age	a) seedlings (nursery)	7,84	a
	b) trees (70 years)	7,28	a
II. Source of isolation	a) Soil	6,88	a
	b) Rhizosphere	7,41	b
	c) Mycorrhizosphere	8,38	c

B. Eco-Physiological Index (EPI)				
Source of variation	Variance	df @	F	p (significance level)
Age of trees	0,0011	1	0,53	0,472
Source of isolation	0,0721	2	36,32	$< 10^{-6}$
Interaction	0,0025	2	1,24	0,302
Error	0,0020	36		

Newman-Keuls multiple range test: @@

I. Age	a) seedlings (nursery)	0,69	a
	b) trees (70 years)	0,70	a
II. Source of isolation	a) Soil	0,74	b
	b) Rhizosphere	0,73	b
	c) Mycorrhizosphere	0,61	a

C. Colony Development Index (CDI)				
Source of variation	Variance	df @	F	p (significance level)
Age of trees	1,10	1	0,05	0,833
Source of isolation	203,92	2	8,36	0,001
Interaction	25,85	2	1,06	0,357
Error	24,38	36		

Newman-Keuls multiple range test: @@

I. Age	a) seedlings (nursery)	29,31	a
	b) trees (70 years)	29,64	a
II. Source of isolation	a) Soil	31,39	b
	b) Rhizosphere	31,96	b
	c) Mycorrhizosphere	25,08	a

Explanations: @ - degrees of freedom (df), @@ mean values indicated with the same letter do not differ significantly ($p < 0.05$).

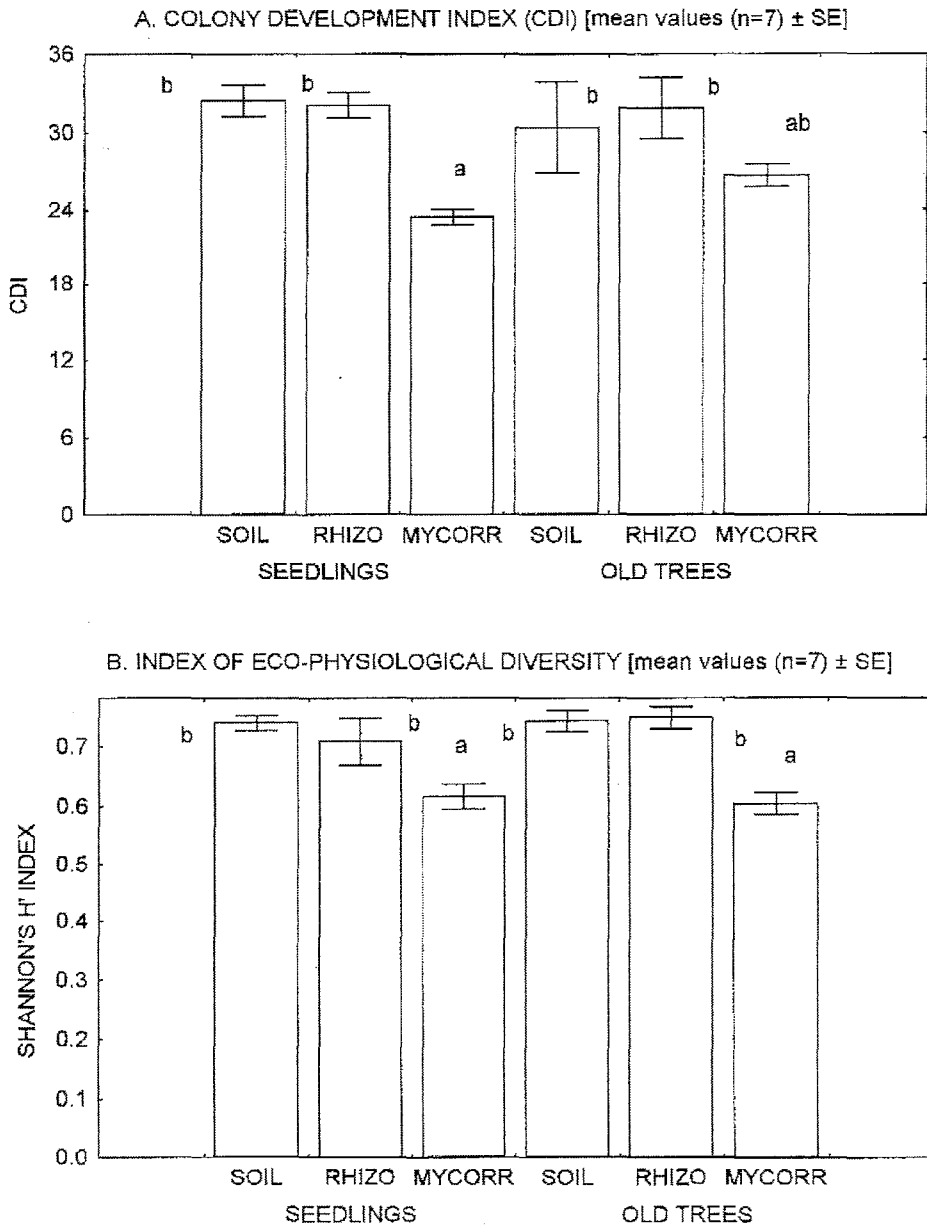


Fig 3. Colony Development Index (CDI) for plates inoculated with the suspension (dilution: 10^{-5}) of root-free soil, rhizosphere and mycorrhizosphere of Scots pine (A) and index of eco-physiological diversity of the same colonies (B). Explanations: - see Figs 1 and 2.

TABLE 2. RESULTS OF TENTATIVE IDENTIFICATION OF BACTERIA FROM SOIL AND ROOT-ZONE OF SEEDLINGS AND 70-YEAR OLD TREES OF SCOTS PINE (*PINUS SYLVESTRIS*L.) TO GENUS/GROUP, ACCORDING TO THE SCHEME BY ACERO *et al.*, [1] (% OF STRAINS). CATALASE-NEGATIVE STRAINS WERE CONSIDERED AS THE UNIDENTIFIED ONES

No.	Names of genus/ group	% of strains of seedling similar to genus						Sum	% of strains of old trees similar to genus						Sum
		fast growing bacteria			slow growing bacteria				fast growing bacteria			fast growing bacteria			
		soil	rhizo- sphere	mycor- rhozo- sphere	soil	rhizo- sphere	mycor- rhozo- sphere		soil	rhizo- sphere	mycor- rhozo- sphere	soil	rhizo- sphere	mycor- rhozo- sphere	
%															
1	<i>Acinetobacter</i>		9		6	6	6	4	5			3	3	13	4
2	<i>Agrobacterium</i>				6	6	12	4	3	9	3		3	3	3
3	<i>Alcaligenes</i>	2										3			
4	<i>Enterobacteriaceae</i>		3		18	3	9	5					11		2
5	<i>Erwinia</i>	2	6	9	3	9	6	5	3				8	6	3
6	<i>Erwinia-like</i>		6					1							
7	<i>Flavobacterium</i>		3	6	3	6		3							
8	<i>Xanthomonas</i>		3	6				1				3			0
9*	<i>Pseudomonas I</i>	6	6	6	6	26	12	9		9	6	31	24	13	14
10**	<i>Pseudomonas II</i>	6		6		3	3	2	5		9	3	3		3
11***	<i>Pseudomonas III</i>								5		3			3	2
12	<i>Moraxella</i>		16	3			6	4				3			0
13	unidentified [Gram(-), Catalase(-)]		13	9	3		6	6	3		9	8	3	6	5
14	<i>Bacillus</i>	66	9	11	21	3		7	45	21	12	11	11	9	19
15	<i>Micrococcus</i>			9				1						3	0
16	<i>Arthrobacter</i>	6	13	11	18	6	15	9	18	24		3	14	3	10
17	coryneforms other than <i>Arthrobacter</i>	8	13	20	6	17	18	11	11	32	36	22	14	38	25
18	unidentified [Gram(+) Catalase(-)]	6		6	9	6	9	5	3	6	21	11	8	3	9

Explanations: * *Pseudomonas*, group I: non-pigmented colonies, oxidative catabolism of glucose; ** *Pseudomonas*, group II: pigmented colonies, oxidative catabolism of glucose; *** *Pseudomonas*, group III: non-pigmented colonies, no acidification of glucose.

DISCUSSION

The results presented in our paper indicate that the numbers of bacteria were different and related to the source of isolation and the tree age. Bacteria were more numerous in the root-free soil and root zone of seedlings than in the old trees. This is in an agreement with the results of Dahm and Redlak [8] obtained in their studies on the soil micro-flora of Scots pine. In our study, the most numerous micro-organisms were observed in the mycorrhizosphere of seedlings, slightly lower numbers were found in the mycorrhizosphere of the 70-year old trees as compared with other sources of isolation. Similar results were noted by Strzelczyk *et al.* [22], Dahm *et al.* [6], Różycki [19], Dahm and Redlak [8]. This may be explained by a strong stimulating action of the plant root and fungal exudates on the development of micro-flora of the root zone [5, 19].

The time-course of bacterial colony formation indicated that the fast-growing organisms dominated in the root-free soil and the rhizosphere of the Scots pine seedlings, and the slow-growing ones dominated in the mycorrhizosphere of the seedlings. Among bacteria of the old tree ecosystem, no clear-cut differentiation of the two types of growth rate was observed. These results were similar to those noted by Różycki *et al.* [20] for black and grey alder seedlings. In our study, only the results for the seedlings were in agreement with the ecological theory of r- and K-selection. According to this theory, the organisms of the root-free soil and the seedling rhizosphere of Scots pine were r-strategists, and those of the seedling mycorrhizosphere belonged to K-strategists. Presumably the mycorrhizosphere of the seedlings was a densely populated zone in the state of ecological equilibrium.

The Colony Development Index calculated in the present study showed a faster growth of the colonies from the root-free soil and the rhizosphere of seedlings and 10-year old trees than from the mycorrhizosphere. The CDI data given by Parathchandra *et al.* [21] were significantly lower for the rhizosphere soil than for the rhizoplane of *Trifolium repens*. The type diversity of bacterial colonies growth rate in the mycorrhizosphere of the pine seedlings and old trees was lower than in the remaining sources of isolation as indicated by H' Shannon's index. Parathchandra *et al.* [21] showed that the values of EPI did not differ significantly among different sources of isolation.

In the present work, 18 bacterial groups / taxons were found. In the root-free soil of both the seedlings and old trees, *Bacillus* was predominant when both types of bacterial colonies growth rate was considered - [except for the *Pseudomonas* group I (non-pigmented colonies, oxidative catabolism of glucose) - in the case of the slow-growing bacteria of old trees], in the rhizosphere - *Pseudomonas* group I and coryneforms other than *Arthrobacter*, and in the mycorrhizosphere - coryneforms other than *Arthrobacter*, were predominant. The above results differed from those by Różycki [22], who observed mainly *Arthrobacter* in the

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root-free soil, rhizosphere and mycorrhizosphere of pine. Taxonomic diversity of the micro-organisms studied by the present authors was higher in the rhizosphere and mycorrhizosphere than in the root-free soil. It could be a result of the *Bacillus* predominance in the soil which could inhibit the growth of other bacteria due to competition for space and nutrients [3]. The present results are in agreement with the observations by Nurmiäho-Lassila *et al.* [17]. The above authors stated that the root surface of *Pinus sylvestris* is inhabited by a large number of morphologically different cells.

CONCLUSIONS

1. Bacteria were most numerous in the mycorrhizosphere of both the seedlings and 70-year old trees.

2. Bacteria forming slowly growing colonies dominated only in the mycorrhizosphere of the seedlings which resulted in the occurrence fast-growing colonies in the root-free soil and rhizosphere. Bacteria isolated from the soil and the root zone of the old trees did not differ in the percentage occurrence of slow and fast-growing colonies.

3. Results of the ANOVA programme pointed out that the effect of the isolation source on the bacterial number was stronger than the effect of tree age.

4. *Bacillus* was predominant in the root-free soil (except for the *Pseudomonas* group I in the slow-growing colonies of the old trees); *Moraxella* and coryneforms other than *Arthrobacter* were predominant in the rhizosphere, and coryneforms other than *Arthrobacter* - in the mycorrhizosphere.

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BADANIA NAD BAKTERIAMI SZYBKO I POWOLI ROSNĄCYMI,
WYSTĘPUJĄCYMI W GLEBIE POZAKORZENIOWEJ, RYZOSFERZE
I MIKORYZOSFERZE SIEWEK SZKÓŁKARSKICH I 70-LETNICH DRZEW SOSNY
ZWYCZAJNEJ (*Pinus sylvestris* L.). OZNACZANIE LICZEBNOŚCI
I IDENTYFIKACJA

W pracy przedstawiono wyniki badań nad liczebnością i identyfikacją bakterii szybko i powoli rosnących wyizolowanych z gleby pozakorzeniowej i strefy korzeniowej siewek szkółkarskich i drzew 70-cio letnich sosny zwyczajnej (*Pinus sylvestris*).

Bakterie były bardziej liczne w mikoryzosferze zarówno siewek, jak i drzew starych. Powoli rosnące bakterie dominowały w mikoryzosferze, zaś szybko rosnące w glebie pozakorzeniowej i ryzosferze. Bakterie wyizolowane z gleby i strefy korzeniowej drzew starych nie różniły się liczbą bakterii szybko i powoli rosnących. ANOVA (analiza wariancji) wykazała silniejszy wpływ źródeł izolacji niż wieku drzew na liczebność bakterii. W glebie pozakorzeniowej dominowały bakterie z rodzaju *Bacillus* [wyjątek stanowiły *Pseudomonas* grupy I (bezbarwne kolonie, oksydatywny katabolizm glukozy) - dla bakterii powoli rosnących starodrzewu], w ryzosferze - *Moraxella* i pleomorfy inne niż *Arthrobacter*, zaś w mikoryzosferze - pleomorfy inne niż *Arthrobacter*.