

**Studies on 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.) – nutritional requirements
and physiological properties**

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■ **Abstract**

Studies were carried out on the nutritional and physiological properties of the fast and slow growing bacteria from the root-free soil, the rhizosphere and the mycorrhizosphere of seedlings and old trees of Scots pine (*Pinus sylvestris* L.). Among the fast and slow growing bacteria different nutritional groups were predominating (B, AG, Y, A). The differences in frequency of occurrence (depending on the source of isolation (type of rapidity of growth) of bacterial strains having the following physiological properties: hydrolytic activity, acidification of glucose, reduction of methylene blue, nitrate to nitrite reduction and ammonification were observed.

■ Key words

Scots pine, fast-growing bacteria, slow-growing bacteria, nutritional groups, physiological properties

■ Introduction

It was found in our previous studies (Gierasimiuk et al. 2001) that slow-growing bacteria were more frequent in the mycorrhizosphere than in the root-free soil and the rhizosphere of Scots pine (*Pinus sylvestris* L.). Also the generic composition of fast- and slow-growing bacteria depended both on the ecosystem type (nursery and 70-year old pine forest) and source of isolation (soil, rhizosphere and mycorrhizosphere) (Gierasimiuk et al. 2001). However physiological properties of the slow and the fast-growing soil bacteria of the Scots pine ecosystem still remain almost unknown. Therefore in this paper we present some results on physiological properties of the fast and the slow-growing bacteria of the root-free soil, the rhizosphere and the mycorrhizosphere of Scots pine (*Pinus sylvestris* L.) seedlings (1-year old) and 70-year old trees.

■ 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 Dobrzejewice Forest Inspectorate (near Toruń, Poland) from two sites:

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

Basic soil properties at site a) were: $\text{pH}_{\text{H}_2\text{O}} = 5.8$, $\text{pH}_{\text{KCl}} = 4.8$, $\text{C}_{\text{total}} = 2.4\%$, $\text{N}_{\text{total}} = 0.6\%$, $\text{C/N} = 15$ (loamy sand).

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

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

Enumeration and isolation

Bacteria were enumerated and isolated from the root-free soil, from the rhizosphere and the mycorrhizosphere of seedlings and trees. For enumeration and isolation of bacteria, plate dilution method was used (10^{-1} - 10^{-7} in sterile distilled water; for counting and isolation dilutions: 10^{-4} and 10^{-5} were used), the method and the results were described by Gierasimiuk et al. (2001).

Nutritional requirements of bacteria

Nutritional requirements were studied using the modified method of Lochhead and Chase (1943), where the liquid media were replaced with the solidified agar ones.

Bacteria were multiplied on the rich agar medium according to Hagedorn and Holt (1975) of the following composition ($\text{g}\times\text{l}^{-1}$): Tryptic Soy Agar (Difco) 4, Yeast Extract (Difco) 2, Bacto Agar (Difco) 15, distilled water 1000 cm^3 (pH 6.8-7.2; 26°C, 5 days). Subsequently the grown bacteria were replicated (using 25-pin multipoint inoculator) onto the following 7 agar media: medium "B" – basal medium (mineral salts + glucose), medium "A" – basal medium + amino acids, medium "G" – basal medium + growth factors, medium "AG" – basal medium + amino acids + growth factors, medium "Y" – basal medium + yeast extract, medium "S" – basal medium + soil extract, medium "YS" – basal medium + yeast extract + soil extract. After 6 days of incubation at 26°C, the growth response of each isolate was determined by assigning a value of 4 to the heaviest growth intensity (colony size) and rating the others relatively. A difference of not less than 2 points was considered significant.

Physiological properties of bacteria

12 physiological tests were carried out: six of them were performed in the liquid media (growth in N-free and C-free media, ammonification, reduction of nitrate to nitrite, reduction of methylene blue, acidification of glucose), and six of them were made with the use of solidified media [with 1.5% (w/v) of Agar (BTL, Łódź)]. Reduction of methylene blue test was used to detect dehydrogenase activity (general metabolic activity) of bacteria. Hydrolysis of carboxymethylcellulose (CMC) was tested using the Woods (1980) method. Hydrolysis of pectin was studied by the plate method described by Strzelczyk and Szpołański (1989). Starch hydrolysis was examined in the same basal medium as CMC and pectin hydrolysis, but containing 0.5% of soluble starch as substrate, and Lugol's solution as developing reagent. Gelatine and casein hydrolysis was tested, using the plate methods described by Strzelczyk et al. (1990). Lipolysis was examined in medium containing 0.5% of tributyrin as a substrate.

Ammonification, reduction of nitrate to nitrite, reduction of methylene blue and acidification of glucose were tested in liquid media given by Allen (1951). Bacteria for most of physiological studies were grown at 26°C for 7 days (except of CMC hydrolysis test – 14 days).

Ability of bacteria to grow oligotrophically was examined in the liquid basal "B" medium (Lochhead and Chase 1943), without the C or N source. After 14 days of culturing at 26°C, the turbidity of the cultures was checked visually.

Statistical evaluation of results

To calculate the per cent of microorganisms with a tested physiological property, their percentage to appropriate type of rapidity of growth / source of isolation was estimated and the obtained values were rescaled to 100%, corresponding to all the strains studied (total percentage for the sum of all groups : 1200% was assumed to be 100%, and other values – x%).

Frequency of occurrence of the strains having a given feature in sources of isolation / type of growth rapidity were tested for departures from the random distribution, using χ^2 tests. All statistical calculations were performed using Statistica for Windows, release 5.1 (1996, StatSoft, Tulsa, Oklahoma, USA).

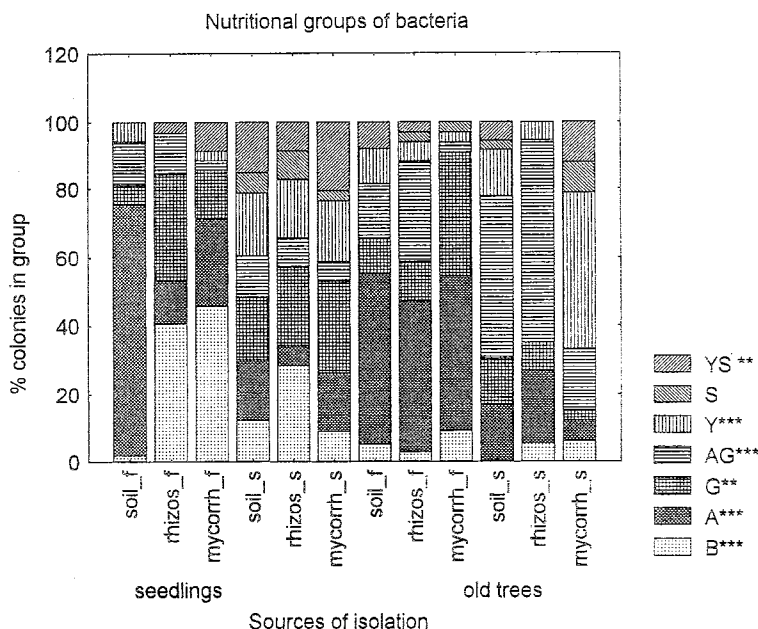
■ Results

Figure 1 shows nutritional groups of the fast and the slow growing bacteria of the root-free soil, the rhizosphere and mycorrhizosphere of 1-year old seedlings and 70-year old trees of Scots pine. Frequency of occurrence of the bacteria belonging to all of nutritional groups (except group S – requiring soil extract) were significantly different (χ^2 , $df = 11$, $p \leq 0.01$) depending on the three combined experimental factors (type of growth rate * age of pines * source of isolation). Among the fast growing microorganisms of the root-free soil both in seedlings and in the old trees, bacteria requiring amino acids (group A) were predominating (significantly more frequent for fast than for slow growers, $p < 0.0001$, and for the soil than the root zone, $p < 0.01$). In the rhizospheres and the mycorrhizospheres the fast growing bacteria of seedlings of pine, group B (showing good growth in the mineral medium according to Lochhead and Chase (1943) was the most frequent one [significantly higher frequency for the seedlings as compared to the mature trees ($p < 0.001$) and in the root zone than in the soil ($p < 0.01$)]. The slow growing bacteria of the root-free soil and the root zone of seedlings utilized in a similar degree the media: the mineral one (group B), with amino acids (group A), with growth factors (G), with yeast extract (Y), with soil and yeast extract (YS). In general, bacteria of more complex nutritional requirements (belonging to groups AG, Y, S and YS) were significantly more numerous ($p \leq 0.05$) among the slow than fast growers. The frequency of group AG was considerably higher ($p < 0.001$) in the mature forest than in the nursery. The fast growing microorganisms of the rhizosphere and the mycorrhizosphere of 70-years old trees belonged mostly to the group A, and to AG and G, respectively. Among the slow growing bacteria of the soil and the rhizosphere, microflora of the group AG was predominating (significantly more numerous than in the mycorrhizosphere) and in the case of the mycorrhizosphere, the group Y was dominating.

Some physiological properties of the fast and the slow growing bacteria from the root-free soil, the rhizosphere and the mycorrhizosphere of 1-year old and 70-years old trees are shown on Figure 2 and 3. Among the bacteria studied the most numerous were bacteria with the capability of oligonitrophilic ($\approx 80\%$) and of oligocarbophilic ($\approx 78\%$) growth (all groups of type of growth rate / sources of isolation), and the least were bacteria reducing nitrate to nitrite ($\approx 20\%$). Some of the bacteria of the respective type of growth rate / source of the isolates did not show some activities: e.g. the slow growing bacteria of mycorrhizosphere seedlings and old trees did not reduce the methylene blue. Results of χ^2 test ($df = 11$) indicate that frequency of the occurrence of bacteria having particular physiological properties (except the oligocarbophiles and oligonitrophiles) were significantly different depending on the all three experimental factors (type of growth rate * age of pines * source of isolation) (Fig. 2A).

The most active ammonifying organisms, reducing nitrate, reducing methylene blue, acidifying glucose, growing without the source of N or C were among the fast growing bacteria of the root-free soil of seedlings and old trees and among the fast growing bacteria of the rhizosphere of old trees ($\approx 32\%$ for all). The least active were the slow growing microorganisms of the mycorrhizosphere of seedlings and of the root-free soil of the old trees ($\approx 18\%$) (Fig. 2 B).

As indicated by χ^2 test, except of ammonifiers, bacteria revealing other physiological activities were significantly ($p < 0.05$) more frequent among the fast than the slow growers. No significant differences depending on the age of pines were stated. Organisms reducing



Values of χ^2 statistic and significance of χ^2 test for the separate experimental factors: * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$. (+) Group more frequent for rapid growers, and seedlings than for slow growers and trees, respectively (minus - means the reverse). For sources of isolation - marks in parentheses correspond to: soil, rhizosphere and mycorrhizosphere, respectively (+ more, - less frequent than in other sources of isolation).

Source of variation	Nutritional groups						
	B	A	G	AG	Y	S	YS
Type of growth rapidity (df = 1)	2.8	32.8	0.1	9.8	19.0	6.0	7.0
		***		**	***	*	**
		(+)		(-)	(-)	(-)	(-)
Age of pines (df = 1)	22.0	0.1	1.4	22.6	1.4	< 0.1	2.3
	***			***			
	(+)			(-)			
Source of isolation (df = 2)	15.0	15.3	4.0	15.9	5.7	0.9	4.8
	***	***		***			
	(+++)	(+-)		(++-)			

FIG. 1. Nutritional requirements of fast (f) and slow (s) growing bacteria isolated from soil, rhizosphere and mycorrhizosphere of 1-year old nursery seedlings and 70-year old trees of Scots pine (full name of the media are given in "Materials and methods"). Explanation: asterisks indicate significant differences in the occurrence of a given nutritional (physiological) group in 12 sources of isolation (χ^2 (chi square), df (degrees of freedom) = 11. * - $p < 0.01$, ** - $p < 0.05$, *** - $p < 0.001$]; soil - root-free soil, rhizos - rhizosphere, mycorr - mycorrhizosphere

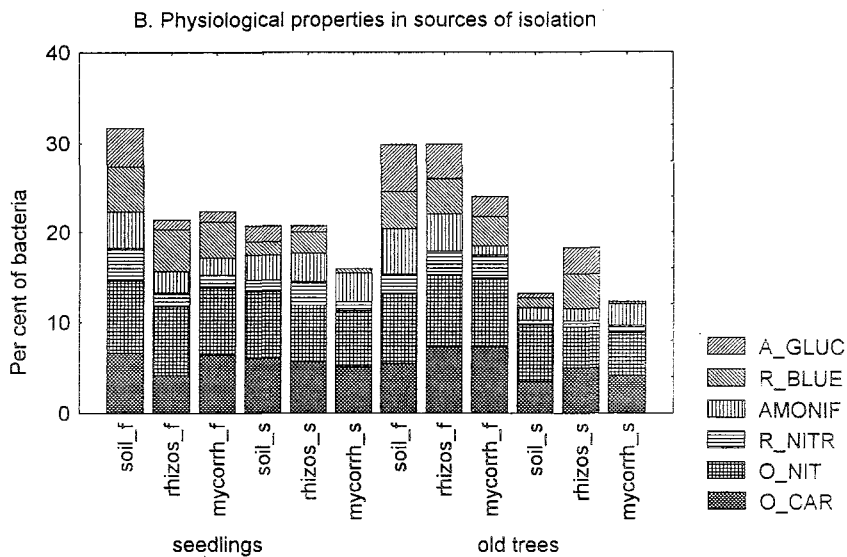
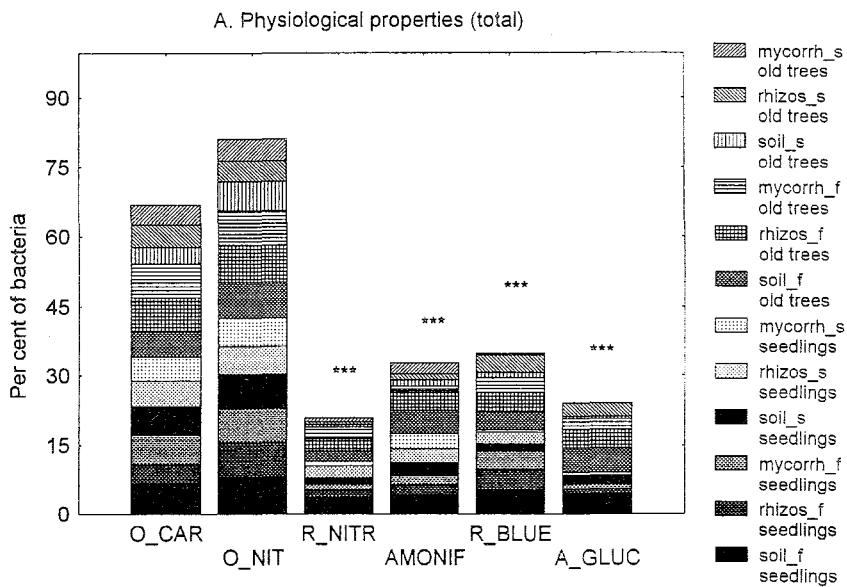


FIG. 2. Some physiological properties of fast (f) and slow (s) growing bacteria isolated from soil, rhizosphere and mycorrhizosphere of 1-year old nursery seedlings and 70-year old trees of Scots pine. Explanation: as in Fig. 1, O_CAR – oligocarbophilic bacteria, O_NIT – oligonitrophilic bacteria, R_NITR – nitrate reduction, AMONIF – ammonification, R_BLUE – reduction of methylene blue, A_GLUC – acidification of glucose

TABLE

Values of χ^2 (chi square) statistic and significance of χ^2 tests for the separate experimental factors: * – $p < 0.05$; ** – $p < 0.01$; *** – $p < 0.001$. (+) – Higher frequency for rapid growers and seedlings, than for slow growers and trees, respectively (minus – means the reverse); for sources of isolation, marks in parentheses correspond to: soil, rhizosphere and mycorrhizosphere, respectively (+ more, – less frequent than in the other sources of isolation)

A.

Source of variation	Selected physiological properties					
	Oligo-carbophiles	Oligo-nitrophiles	Reduction of NO_3^-	Ammonification	Red. of methylene blue	Acid from glucose
Type of growth rapidity (df=1)	4.18	6.62	12.38	3.64	29.26	28.68
	*	*	***		***	***
	(+)	(+)	(+)		(+)	(+)
Age of pines (df=1)	0.27	1.04	1.91	0.83	0.60	3.64
Source of isolation (df=2)	0.12	1.54	2.10	5.53	7.70	21.75
					*	***
					(++-)	(+++)

B.

Source of variation	Selected hydrolytic properties					
	Lipolytic	Proteolytic (casein)	Amylolytic	Pectolytic	Proteolytic (gelatine)	Cellulolytic (CMC)
Type of growth rapidity (df=1)	26.11	23.79	33.95	0.66	44.19	8.60
	***	***	***		***	**
	(+)	(+)	(+)		(+)	(+)
Age of pines (df=1)	2.17	6.85	7.69	9.42	0.05	18.67
		**	**	**		***
		(+)	(-)	(-)		(-)
Source of isolation (df=2)	20.10	55.46	43.59	13.57	30.39	5.41
	***	***	***	**	***	
	(+-)	(+-)	(+-)	(+++)	(+-)	

methylene blue and producing acid from glucose were significantly more frequent in the mycorrhizosphere than in the soil and in the rhizosphere ($p < 0.05$ and $p < 0.001$, respectively) (Tab. 1A).

Gelatine was the most frequently hydrolysed high molecular compound ($\approx 55\%$ of bacteria), and the least frequently hydrolysed compound was pectin ($\approx 27\%$) (Fig. 3A).

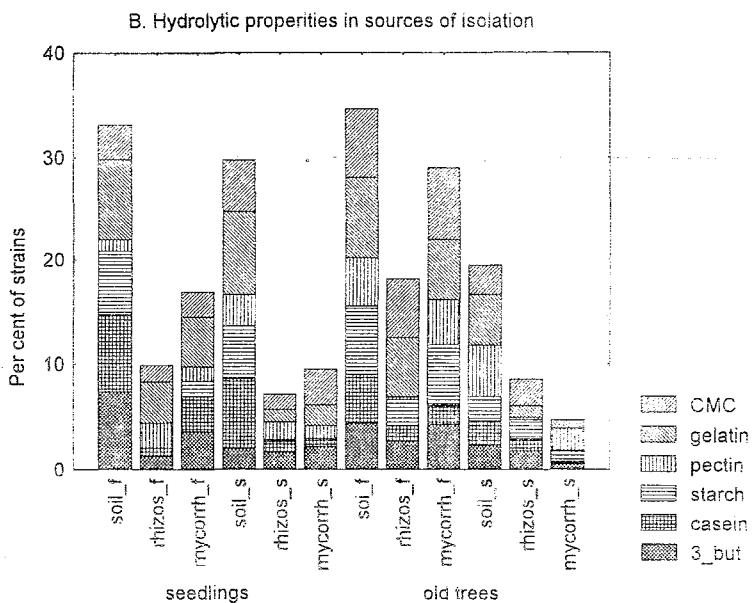
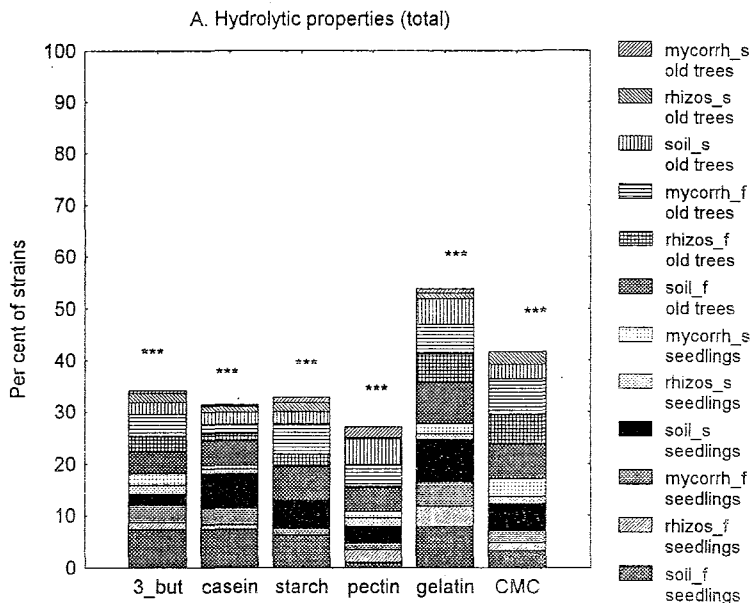


FIG. 3. Hydrolytic properties of fast (f) and slow (s) growing bacteria isolated from soil, rhizosphere and mycorrhizosphere of 1-year old nursery seedlings and 70-year old trees of Scots pine. Explanation: as in Fig. 1. 3_but – tributyrin, gelatin – gelatine

Among polysaccharides (CMC, starch, pectin), carboxymethylcellulose (CMC) was the most frequently hydrolysed ($\approx 40\%$) compound. Among proteins (gelatine, casein) the bacteria preferred gelatine. Tributyrin was hydrolysed by 35% of bacteria, similarly to starch, but better than casein and pectin (Fig. 3A).

The microorganisms of the root-free soil and the root zone of 70-years old trees of pine were more active in hydrolysis of high-molecular compounds than the bacteria from the nursery ecosystem (Fig. 3B). The highest hydrolytic activity was shown by the fast growing bacteria of the root-free soil of seedlings and 70-years old trees. The lowest biodegradative activity towards macromolecules was shown in the case of the slow-growing bacteria of the root zone of both tree ages. The fast growing microbes of old trees were more active than the slow growing ones. No clear difference in hydrolytic activity between fast and slow growing microflora associated with seedlings was found (Fig. 3B).

In all the tests, hydrolytic properties significantly departed from the equal distribution of frequency of occurrence of strains (χ^2 , $df=11$, $p<0.05$), i. e. there were significant differences in the occurrence of strains having hydrolytic properties, depending on the type of growth rate / source of isolation. Organisms hydrolysing organic compounds (except pectin) were significantly more frequent ($p<0.01$) among fast than slow growing bacteria. Relatively more ($p<0.01$) amylolytic, pectolytic and cellulolytic bacteria occurred in the mature forest than in the nursery. Isolates with hydrolytic properties were significantly more frequent ($p<0.01$) in the soil than in the root zone (Tab. 1B).

■ Discussion

In this paper the metabolic activities of fast and slow growing bacteria from the root-free soil, from the rhizosphere and mycorrhizosphere of seedlings and 70-years old trees of Scots pine are reported.

Our results indicate, that among bacteria the nutritional group A (seedlings: fast growing bacteria of the soil; old trees: fast growing bacteria – all sources of isolation), B (seedlings: fast and slow growing bacteria of the rhizosphere and fast growing ones of the mycorrhizosphere), AG (old trees: slow growing bacteria of the soil and the rhizosphere) and Y (old trees: slow growing bacteria of the mycorrhizosphere; seedlings: slow growing ones of the soil and the mycorrhizosphere) predominated. The above mentioned data are different from the results presented in the earlier works on bacteria of root-free soil and the root zone. The bacteria studied by Różycki et al. (1986) gave the best growth in the mineral medium (group B) and in the medium with amino acids (group A), however according to Dahm (1988) the optimal growth occurred – only in the presence of the amino acids. In the case of the soil and the root zone of grey and black alder, predominance of the group AG was noted (Różycki et al. 1998)

Bacteria with the ability to oligocarbophilic and oligonitrophilic growth were abundant in the soils studied here. Numerous occurrence of bacteria capable of growing in the media without source of carbon or nitrogen reported Dahm (1984) in her work on the microflora from the soil and the root zone of 60-years old Scots pines. Different results were noted by Dahm and Redlak (1998) – infrequent occurrence of oligocarbophilic bacteria and variable occurrence of oligonitrophilic bacteria (high frequency – in the mycorrhizosphere, low –

in the root-free soil and the rhizosphere of the trees of Scots pine). Quite common occurrence of oligotrophic bacteria in various soils was pointed out by Hattori (1985).

Ammonifying bacteria were rather numerous among the bacteria studied, which is in agreement with the results presented by Dahm and Redlak (1998).

The high physiological activity of bacteria from the root-free soil of the seedlings and the old trees of pine, observed by us, is in agreement with the results of Dahm and Redlak (1998). The high physiological activity (reduction of nitrate to nitrite, growth without C and N source) of bacteria from the root-free soil and the rhizosphere of grey and black alders seedlings was also noted by Różycki et al. (1998).

The results of our work have shown that tributyrin, casein, gelatine, starch, pectin and CMC were hydrolysed more frequently by the fast growing bacteria of the root-free soil of seedlings and old pine trees than by the bacteria from other sources of isolation / types of growth rate. This observation is in agreement with earlier studies on the bacteria from the root-free soil and the root zone of pine (Dahm et al. 1986, Różycki et al. 1986, Różycki 1987).

The higher hydrolytic activity of the bacteria from the root-free soil, the rhizosphere and the mycorrhizosphere of 70-years old trees than in the case of seedlings of Scots pine noted in our work, could be explained by the fact that the number of the bacteria of the root zone, capable of hydrolysing complex C compounds may increase during plant growth (Dumweld et al. 1998).

■ Conclusions

- Among the nutritional groups of bacteria the following predominated: B (fast and slow growing bacteria of the rhizosphere of seedlings and fast growing ones of the mycorrhizosphere of seedlings), A (fast growing bacteria of the soil and fast growing bacteria of all sources of isolation of old trees), AG (slow growing bacteria of the soil and the rhizosphere of 70-years old trees) and Y (slow growing bacteria from the mycorrhizosphere of old trees, and slow growing ones of the soil and the rhizosphere of the seedlings).
- The most active ammonifying organisms, reducing nitrate, reducing methylene blue, acidifying glucose, growing without the source of N or C were found among the fast growing bacteria of the root-free soil of seedlings and old trees and the fast growing bacteria of the rhizosphere of old trees ($\approx 32\%$ for all).
- The microorganisms of the root-free soil and the root zone of 70-years old trees of pine were more active in hydrolysis of high-molecular compounds than the bacteria of the soil, of the rhizosphere and mycorrhizosphere of seedlings.

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■ Streszczenie (Summary)

Badania nad bakteriami szybko i powoli rosnącymi, występującymi w glebie, ryzosferze i mikoryzosferze siewek szkółkarskich i siedemdziesięcioletnich drzew sosny zwyczajnej (*Pinus sylvestris* L.) – potrzeby pokarmowe i właściwości fizjologiczne

W pracy przedstawiono wyniki badań nad właściwościami fizjologicznymi i wymaganiami pokarmowymi bakterii szybko i powoli rosnących, wyizolowanych z gleby pozakorzeniowej, ryzosfery i mikoryzosfery siewek i starodrzewu sosny zwyczajnej. Wśród grup pokarmowych bakterii dominowały: B (bakterie szybko i powoli rosnące ryzosfery siewek oraz bakterie szybko rosnące mikoryzosfery siewek), A (bakterie szybko rosnące gleby pozakorzeniowej i wszystkie bakterie szybko rosnące starodrzewu), AG (powoli rosnące bakterie gleby i ryzosfery drzew siedemdziesięcioletnich) i Y (powoli rosnące bakterie mikoryzosfery drzew siedemdziesięcioletnich oraz powoli rosnące bakterie gleby i ryzosfery siewek).

Notowano również różnice w występowaniu szczepów bakteryjnych o określonych właściwościach fizjologicznych (hydroliza związków wielkocząsteczkowych, zakwaszenie glukozy, redukcja błękitu metylenowego, redukcja azotanów do azotynów, amonifikacja) w zależności od źródła izolacji i typu szybkości wzrostu.

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