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Diazotrophic bacteria in root-free soil and in the root zone of pine (*Pinus sylvestris* L.) and oak (*Quercus robur* L.)

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Abstract

Studies on the occurrence, identity and potential nitrogenase activity of diazotrophic bacteria in soil and the root zone of Scots pine (*Pinus sylvestris* L.) and common oak (*Quercus robur* L.) were carried out. Diazotrophic bacteria were more numerous in soil and the root zone of oak than in those of pine. Most of the bacteria isolated from the roots of both trees belonged to the genera *Pseudomonas* and *Bacillus*. The majority of these bacteria had nitrogenase activity (tested by the acetylene reduction assay method) within the range from 4 to 20 nmoles C_2H_4 per culture per hour. No clear relationships between the taxonomic identity and/or source of isolation of the bacteria studied and their nitrogenase activity were found. Biotin, *p*-aminobenzoic acid (PABA) and yeast extract, applied both separately and in combination, significantly (at least several-fold) stimulated the activity of N_2 fixation in three selected (nitrogenase highly active) bacterial strains, as compared to a control without growth factors.

Keywords: Pine; Oak; Rhizosphere; Diazotrophic bacteria; Dinitrogen fixation

1. Introduction

N_2-fixation by free-living and associative bacteria, occurs in forest soils (Dawson, 1983; Limmer and Drake, 1996). According to Granhall and Lindberg (1978) it occurs mainly in forest litter and in the plant rhizosphere. Any deficiency of soil organic and inorganic nitrogen compounds, stimulates microbial N_2-fixation (Limmer and Drake, 1996).

It is difficult to assess the contribution of free-living diazotrophs (occurring in forest litter and in the rhizosphere of trees) to total N_2-fixation – as compared with the contribution of associative N_2-fixing bacteria, occurring within the fungal sporocarps and mycelia as well as within the mycorrhizal roots (Florence and Cook, 1984; Li and Castellano, 1987; Li and Hung, 1987). In situ N_2-fixation was generally not measured; only the presence of bacteria responsible for N_2-fixation was demonstrated. It can be assumed that the nitrogenase activity of associative bacteria may be very important, because of their direct impact on the mycorrhizal symbiont and the host plant (Garbaye, 1994).

N_2-fixation by microorganisms associated with ectomycorrhizal roots has been postulated and proved

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indirectly (Dawson, 1983; Li and Castellano, 1985). Nitrogenase activity (of bacterial origin) has also been detected in sporocarps of the ectomycorrhizal fungi *Laccaria laccata*, *Hebeloma crustuliniforme*, *Rhizopogon vinicolor* and *Thelephora* sp.: *Clostridium* spp. and N_2-fixing, associative *Azospirillum* spp. were isolated from within sporocarps of the above fungi (Li and Castellano, 1987; Li and Hung, 1987). Occurrence of N_2-fixing bacteria of the genus *Bacillus* within the tuberculate ectomycorrhizas of Douglas-fir (Li et al., 1992) and within Scots pine ectendomycorrhizas (Pachlewski et al., 1991) has been reported. N_2 fixation can occur also in decaying tree stumps (Griffiths et al., 1993; Crawford et al., 1997).

The potential beneficial effects of diazotrophic, free-living and associative bacteria on mycorrhizal fungi and/or on the host plant are not limited to providing both mycorrhizal partners with nitrogen in poor forest soils. They can be also due to production of B-vitamins, amino acids, plant growth hormones and other biologically active substances by the mycorrhizosphere bacteria, including diazotrophs (Kampert and Strzelczyk, 1984; Różycki and Strzelczyk, 1986; Garbaye, 1994; Strzelczyk et al., 1994a, b). Despite the potential importance of diazotrophic, free-living and associative bacteria in forest soils, studies on their physiology and ecology are rather scarce (Li et al., 1992; Bormann et al., 1993; Barkmann and Schwintzer, 1998). In particular, their occurrence in soil, rhizosphere/rhizoplane and mycorrhizosphere/mycorrhizoplane of forest trees (as well as within roots), taxonomic composition and potential nitrogenase activity (including factors affecting nitrogen fixation) remain largely unknown. Thus, the study was designed to assess and determine N_2-fixing microorganisms associated with Scots pine and common oak, growing on an undisturbed site (forest reservation).

2. Materials and methods

2.1. Origin of samples

The samples of soil and mycorrhizal (and/or feeder) and non-mycorrhizal (suberized) roots of pine (*Pinus sylvestris* L.) and oak (*Quercus robur* L.) were taken from the A_1 soil horizon (podzolized rusty soil, formed from windblown terase sands of the 'old' Vistula River valley) in the forest reservation 'Las Piwnicki' near (≈10 km north) Toruń (Poland) from PL. Mixed samples (300–500 g; depth: 5–10 cm) were taken from underneath 3–4 trees, chosen at random. The soil underneath Scots pine had pH_{H_2O} 4.6, and mass loss on ignition was 5.1%; the soil underneath common oak had pH_{H_2O} 5.9, and loss on ignition was 5.4% (A_1 horizon in both cases). The closest pine and oak trees were 7–15 m apart. This is a mixed conifer–hardwood forest (*Pino-Quercetum*) with a predominance of pine (Prusinkiewicz and Biały, 1976).

2.2. Enumeration of diazotrophs by MPN method

Mycorrhizal (and/or feeder) and non-mycorrhizal (suberized) roots of pine and oak were hand sorted out, and 10 g soil samples were shaken on a rotary shaker (120 rpm) for 30 min with 100 ml sterile distilled water, yielding 10^{-1} dilution. A series of 10-fold dilutions (10^{-2}–10^{-7}) was then made. Culture tubes with semisolid (5 g of agar per liter) Rennie (1981) medium for diazotrophic bacteria (supplemented with growth factors: biotin – 5 μg/l, *p*-aminobenzoic acid (PABA) – 10 μg/l and yeast extract – 25 mg/l) were inoculated with 0.1 ml of suspensions obtained in the above dilution procedure (five replicates per dilution). Inoculated tubes were incubated for 4 weeks at 30°C; then the nitrogenase activity in the mixed bacterial cultures obtained was estimated, using the acetylene reduction assay method (Section 2.3). On the basis of the number of inoculated culture tubes in which acetylene reduction was recorded, the most probable number (MPN) of diazotrophic bacteria and its 95% confidence limits were estimated, using the program developed by Bennet et al. (1988).

2.3. Assessment of nitrogenase activity

The roots, previously shaken on a rotary shaker in sterile distilled water as described in Section 2.2 were further washed by shaking for 30 min in a sterilized water solution of 0.1% Tween 80. Then 5 serial washings of the roots in sterile, distilled water were carried out, shaking them for 30 min each time ('mechanical rinsing'). Half-portions of mechanically rinsed roots were surface sterilized chemically with 30% hydrogen peroxide for ca. 1 min. After sterilization the roots were rinsed seven times with sterile

distilled water. The purpose of mechanical rinsing was to remove most of the rhizosphere soil bacteria and to allow only rhizoplane (root surface) bacteria to grow. The purpose of surface chemical sterilization was to remove all root surface bacteria, allowing only these 'purely associative' organisms, which live within root segments to grow. The roots (both cleaned mechanically and sterilized chemically) were cut aseptically into sections of length ca. 1 cm. Mycorrhizal/feeder and non-mycorrhizal/suberized sections of pine and oak roots were put aseptically into semisolid N-free Rennie (1981) medium (without growth factors) in 14 mm × 100 mm culture tubes (≈12 root sections of each kind; one root section per tube). The tubes with root segments were incubated at 30°C for 6 days. Bacterial growth in Rennie's (1981) medium indicated the presence of oligonitrophilic and/or diazotrophic bacteria within the roots. Nitrogenase activity in the mixed cultures obtained was estimated by the acetylene reduction assay method. The results provided a basis for selecting nitrogenase active cultures so that pure strains could be isolated from them. Nitrogenase activities ranged from 0 (or low detection limit) to relatively low values (data not shown), depending on the tree (pine or oak), kind of roots (suberized or feeder/mycorrhizal) and the treatment (mechanical rinsing or chemical sterilization).

Culture tubes with visible bacterial growth were plugged with sterile serum stoppers. Acetylene was injected into the tubes to constitute 10% of the total gas volume. After 24 h of incubation at 30°C, gaseous samples were removed from the tubes and analyzed for ethylene with a Schimadzu GC 14A gas chromatograph fitted with 2 m × 1.8 mm 80–100 mesh, Porapak R column with oven temperature of 70°C. Injector and flame-ionization detector temperatures were adjusted to 100°C and the flow rate of nitrogen carrier gas was 40 cm^3 per min. Data from the GC were transmitted to the IBM PC compatible computer through the data acquisition system (by Elektronika Jądrowa, Kraków, Poland).

Nitrogenase activity was expressed as nmoles of ethylene formed per culture per hour, and calculated according to modified Mãrtennson (1993) equation:

$A = (\%C_2H_4 \times (PV/RT) \times 10^{-7})/24$, where:

A – nitrogenase activity (nM C_2H_4 per culture per hour],

$\%C_2H_4$ – percentage of ethylene peak area,

P – atmospheric pressure in atmospheres (≈1.0),

V – volume of acetylene injected into the culture tube (cm^3),

R – gas constant = 82.054 cm^3-atm/mol °K,

T – temperature in Kelvins (= 273 + °C).

The mixed cultures in which nitrogenase activity was detected were kept at 4°C, and then used for isolation of pure cultures of diazotrophic bacteria.

2.4. Isolation, maintenance and characterization of diazotrophic bacteria

Pure cultures of diazotrophic bacteria were obtained from mixed cultures showing nitrogenase activity using the streak-plate technique on solid (1.5% agar) N-free Rennie's (1981) medium (two replicate plates for each active culture). One set of plates was incubated under aerobic and the second under microaerophilic conditions using an anaerostat with most of the oxygen bound by alkaline pyrogallol solution. In both cases, plates were incubated at 26°C for 10 days. Bacterial colonies differing in morphology were subcultured and stored in 'R2A/4' semisolid medium [modified R2A medium (Difco)] of the following composition (g l^{-1}): glucose 1.0, Yeast Extract (Difco) 0.5, Bacto R2A Agar (Difco) 4.55, soil extract 100 ml, distilled water 900 ml; pH 7.0–7.2. Bacterial strains were purified on R2A Agar (Difco) plates enriched with 10% soil extract, v/v. A total of 42 strains were isolated.

Nitrogenase activity in pure bacterial strains was tested using the acetylene reduction assay method after 3-day culturing at 30°C in semisolid N-free Rennie's medium.

In order to characterize and tentative identify the cultures, the following morphological/cultural/physiological/biochemical tests were performed:

a) Gram stain and cell morphology (after 1, 3 and 5 days of culturing at 26°C in the liquid 'YS' medium – according to Lochhead and Chase, 1943);

b) alternative tests for the Gram reaction [formation of slime with 3% KOH according to Powers (1995) and vancomycin sensitivity (Biolog, 1993b)];

c) motility;

d) survival of pasteurization [90°C for 30 min, according to Behrendt et al. (1997)];
e) cytochrome oxidase test (Collins, 1967);
f) catalase test (Fung and Hartman, 1975);
g) metabolism (oxidative, fermentative or alkalization) of glucose in Hugh and Leifson (1953) medium;
h) growth in the medium selective for Gram-negative bacteria (Bacto EMB Agar–Difco);
i) growth in the medium selective for *Arthrobacter* (Hagedorn and Holt, 1975);
j) growth in the medium selective for *Pseudomonas* (Cetrimide Agar – Merck, Germany);
k) growth and production of fluorescent pigments King's B agar – for *Pseudomonas* ssp. (Geels and Schipper, 1983);
l) growth in D1 agar – selective for *Agrobacterium* (Kado and Heskett, 1970);
m) growth in D3 agar – selective for *Erwinia* (Kado and Heskett, 1970).

Additionally, bacterial strains were differentiated and, wherever possible, identified, by studying the utilization of different carbon sources contained in BIOLOG test microplates for Gram-negative (GN) and Gram-positive (GP) bacteria, respectively (Biolog, 1993a, b, c).

2.5. Impact of growth factors on nitrogenase activity

The impact of growth factors contained in the full Rennie's (1981) medium formula [vitamins: biotin and *p*-aminobenzoic acid (PABA) and yeast extract (YE)] on nitrogenase activity in three selected, active strains (No. 15 – from mycorrhizal/feeder roots of pine and 26 and 27 – from non-mycorrhizal roots of oak) was studied. The strains were selected on the basis of consistently high nitrogenase activity, detected in 2–3 independent assays.

The following experimental variants (five replicates of each) of Rennie's (1981) medium were made:

a) control – without vitamins and yeast extract;
b) biotin (5 μg/l);
c) PABA (10 μg/l);
d) yeast extract (YE)–(25 mg/l);
e) biotin + PABA;
f) PABA + YE;
g) biotin + YE;
h) biotin + PABA + YE.

The three selected strains were inoculated into the full (with vitamins and YE), semisolid Rennie's medium and grown for 7 days at 30°C. Bacteria grown in this medium were used for loop-inoculation of culture tubes with different (a–h) experimental combinations of semisolid Rennie's medium. Cultures were grown for 7 days at 30°C, and the nitrogenase activity was quantified using the acetylene reduction assay method.

2.6. Statistical evaluation of the results

Results of bacteriological tests (20 unit, binary characters for 42 strains) described in Section 2.4 were subjected to cluster analysis (measure of dissimilarity – % disagreement, algorithm of amalgamation – Ward's method). The results are presented in the form of a dendrogram; major clusters being differentiated at 20% of the maximum distance level.

The results of studies on the impact of growth factors on nitrogenase activity were statistically evaluated using one- and two-factor analysis of variance (ANOVA) and Newman–Keuls multiple range test ($p \leq 0.05$; for comparison of averages). To evaluate the impact of growth factors on nitrogenase activity in single strains one-factor ANOVA was used; while two-factor ANOVA was used to compare the effects of strains and growth factors on nitrogenase activity. All statistical calculations were performed using Statistica for Windows, release 5.1 (1996; StatSoft, Tulsa, OK, USA).

3. Results

3.1. Occurrence of diazotrophic bacteria

Numbers of diazotrophic bacteria (as determined by MPN) in root-free soil and in the root zone of pine and oak were within the range 60–4353 thousands per g of dry mass and were in the following order: mycorrhizal/feeder roots of oak > oak soil > non-mycorrhizal roots of oak > non-mycorrhizal roots of pine > mycorrhizal/feeder roots of pine > pine soil. Most probable bacterial numbers in soil and mycorrhizal/feeder roots

Table 1
MPN (most probable numbers) of diazotrophic bacteria in soil and the root zone of pine and oak in Rennie's (1981) medium (MPN g^{-1} of dry mass, five replicate tubes per dilution, 95% confidence limits are given)

Source of isolation	MPN (in thousands)	MPN –95% confidence limit	MPN + 95% confidence limit
Pine root-free soil[a]	61	20	220
Non-mycorrhizal pine roots[ab]	197	60	651
Mycorrhizal/feeder pine roots[ab]	172	52	570
Oak root free soil[b]	739	224	2441
Non-mycorrhizal oak roots[ab]	385	117	1272
Mycorrhizal/feeder oak roots[b]	4353	1318	14373

Habitats marked with different letters have MPN estimations significantly different (based on ~ non-overlapping 95% confidence limits).

(but not in suberized roots) of oak can be considered as significantly higher than those in soil and the mycorrhizal/feeder root zone of pine (Table 1).

3.2. Characterization and identification of bacteria

Fig. 1 shows the results of cluster analysis of 42 strains of oligonitrophilic and/or diazotrophic bacteria isolated from the roots of pine and oak – in conjunction with the results of their identification using the computerized ID system developed by BIOLOG. Three major cluster were obtained (I–22 strains, II–9 strains, III–11 strains) – at the 20% of maximum distance level. Cluster I was joined with cluster II at about 35% of maximum distance level; the pair of clusters I + II was maximally distant from cluster III. Gram-negative rods and one coryneform strain were the only members of clusters I and II; spore forming bacilli and one coccus (i.e. Gram-positive bacteria) belonged exclusively to cluster III. This indicates very high dissimilarity between Gram-negative bacteria and Gram-positive bacteria, even on the basis of quite a low number (20) of characters. No clear relationship between the source of isolation of strains and their major cluster membership was found, although in cluster I Gram-negative rods from non-mycorrhizal roots of pine and oak predominated, and in cluster III – bacilli from mycorrhizal/feeder roots of both trees (Fig. 1).

Twenty-eight of the 42 strains (67%) were identified to the genus level and 19 of these were identified to the species level (similarity to the reference species $\geq$ 0.5). Among *Pseudomonas* spp., the most frequent were strains similar to or identical with species *P. glathei* (14 strains); four cultures of this genus, which produced a fluorescent pigment in King's B medium were similar to or identical with the species *P. putida*. The occurrence of bacterial genera was in the following order: *Pseudomonas* (55%) > *Bacillus* (24%) > *Xanthomonas* (10%) > *Burkholderia* (5%) > *Agrobacterium* (2%); coccus (unidentified; 2%); coryneform bacterium (2%) (Fig. 1).

3.3. Nitrogenase activity of pure bacterial strains

Most of the bacterial strains studied (35 of 42) had nitrogenase activity as measured by the acetylene reduction assay method (4–20 nmol C_2H_4 per culture per hour) (Table 2). Of these 35 strains, 23 had nitrogenase activity above 10 nmol per culture per hour and only one had activity above 20 nmol C_2H_4 per culture per hour. No clear relationship between the taxonomic identity and/or source of isolation of the bacteria studied – and their nitrogenase activity was noted (Table 2).

3.4. Impact of growth factors on nitrogenase activity

In the three selected, nitrogenase-active bacterial strains the growth factors (except PABA) increased nitrogenase activity – at least severalfold (Fig. 2). In strain No. 15 (*Pseudomonas glathei*) all the three growth factors used in combination (biotin + PABA + yeast extract) gave the strongest stimulation of nitrogen fixation. Nitrogenase activity in strain No. 26 (*Xanthomonas sp.*), was almost equally strongly stimulated by the combination of biotin and yeast extract as by biotin + PABA + yeast extract. In

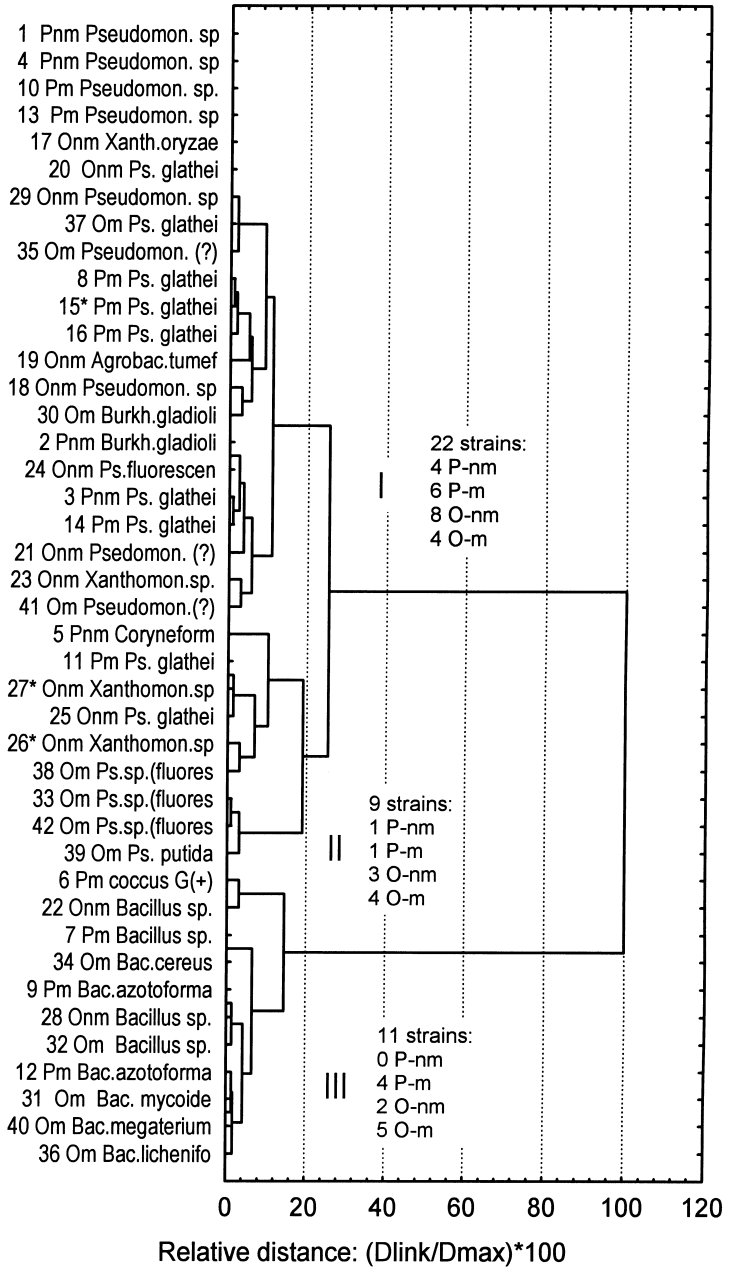

Fig. 1. Results of cluster analysis of the diazotrophic bacteria listed in Table 2 which were isolated from the roots of pine and oak. Measure of distance: % disagreement; amalgamation algorithm – Ward's method. Major clusters (I–III) were differentiated at the level of 20% of maximum distance. Source of isolation – given after the strain No.: P – pine, O – oak, m – mycorrhizal/feeder roots, nm – non-mycorrhizal (suberized) roots. Strains marked with asterisks were selected for further studies.

the case of strain No. 27 (*Xanthomonas sp.*), the stimulation of nitrogenase activity by the growth factors was in the following order: yeast extract $\leq$ biotin < biotin + PABA < PABA + yeast extract < biotin + yeast extract < biotin + PABA + yeast extract (Fig. 2).

Table 2
Nitrogenase activity of bacterial strains isolated from the roots of pine and oak, estimated using the acetylene reduction assay method

Strain No.	Strain symbol	Source of isolation[a]	Result of identification	Nitrogenase activity (nmol of C_2H_4 per culture per hour)
1	SnmN3	P-nm	*Pseudomonas* sp.	8
2	SnmN4	P-nm	*Burkholderia gladioli*	13
3	SnmN4	P-nm	*Pseudomonas glathei*	14
4	SnmN3I	P-nm	*Pseudomonas* sp.	11
5	SnmN3II	P-nm	coryneform [G(±)] rod	18
6	SmN5III	P-m	G(+), unidentified coccus	9
7	SmN4II	P-m	*Bacillus* sp.	8
8	SmN4I	P-m	*Pseudomonas glathei*	10
9	SmN5II	P-m	*Bacillus azotoformans*	0
10	SmN5I	P-m	*Pseudomonas* sp.	11
11	SmN2	P-m	*Pseudomonas glathei*	8
12	SmN2II	P-m	*Bacillus azotoformans*	12
13	SmN5II	P-m	*Pseudomonas* sp.	19
14	SmN4I	P-m	*Pseudomonas glathei*	11
15[b]	SmN2I	P-m	*Pseudomonas glathei*	16
16	SmN5I	P-m	*Pseudomonas glathei*	8
17	DnmSt12II	O-nm	*Xanthomonas oryzae* pv *oryzae* E	7
18	DnmN5I	O-nm	*Pseudomonas* sp.	9
19	DnmN5I	O-nm	*Agrobacterium tumefaciens* A	4
20	DnmN8I	O-nm	*Pseudomonas glathei*	0
21	DnmN8II	O-nm	similar to the genus *Pseudomonas*	20
22	DnmN8III	O-nm	*Bacillus* sp.	0
23	DnmSt12II	O-nm	*Xanthomonas* sp.	8
24	DnmN5II	O-nm	*Pseudomonas fluorescens* type G	17
25	DnmN8	O-nm	*Pseudomonas glathei*	9
26[b]	DnmSt12I	O-nm	*Xanthomonas* sp.	18
27[b]	DnmSt12I	O-nm	*Xanthomonas* sp.	15
28	DnmSt12I	O-nm	*Bacillus* sp.	15
29	DnmN5I	O-nm	*Pseudomonas* sp.	12
30	DmSt9I	O-m	*Burkholderia gladioli*	12
31	DmSt6	O-m	*Bacillus mycoides*	0
32	DmN7II	O-m	*Bacillus* sp.	11
33	DmN7II	O-m	*Pseudomonas* sp. (fluorescence)	11
34	DmSt9II	O-m	*Bacillus cereus / thuringiensis*	6
35	DmSt9II	O-m	similar to the genus *Pseudomonas*	
36	DmSt9II	O-m	*Bacillus licheniformis*	10
37	DmN7I	O-m	*Pseudomonas glathei*	0
38	DmN7IV	O-m	*Pseudomonas* sp. (fluorescence)	0
39	DmN7II	O-m	*Pseudomonas putida* type A1	0
40	DmSt6	O-m	*Bacillus megaterium*	13
41	DmSt9I	O-m	similar to the genus *Pseudomonas*	12
42	DmN7III	O-m	*Pseudomonas* sp. (fluorescence)	11

[a] P – pine, O – oak, m – mycorrhizal/feeder roots, nm – non-mycorrhizal (suberized) roots.
[b] Strains selected for further studies.

The results of two-factor analysis of variance (ANOVA), comparing the effects of strains and growth factors added to the Rennie's medium on nitrogenase activity show that growth factors had a much more important effect than strains (Table 3). No significant interaction between growth factors and strains occurred. The Newman–Keuls multiple range test ($p \leq 0.05$) showed significantly higher nitrogen fixa-

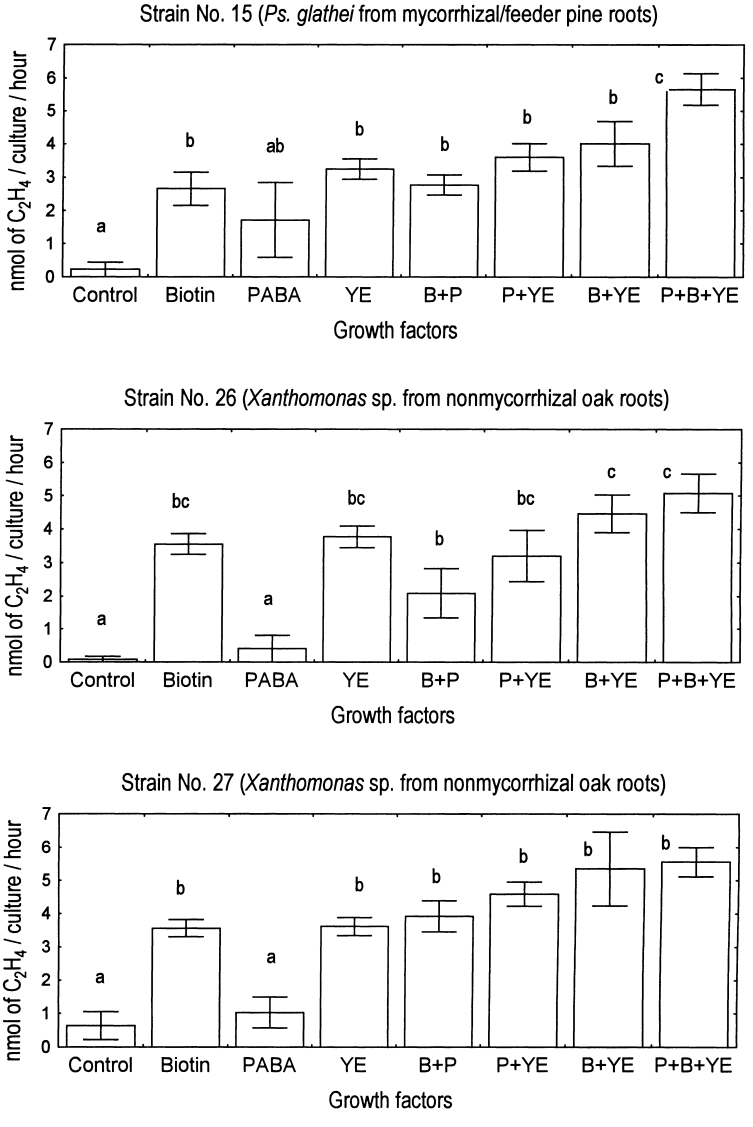

Fig. 2. Effect of growth factors on nitrogenase activity in three selected strains of bacteria (having consistently high nitrogenase activity in 2–3 repeated assays). Mean values ($n = 5$) ± standard error (marked with the same letter do not differ significantly, $p \leq 0.05$). Abbreviations in descriptions of combinations: B – biotin, P – PABA, YE – yeast extract.

tion activity by strain No. 35 than by strains No. 30 and 34. Averages for the growth factors were in the following order, with statistically homogenous groups of averages given in parentheses (Table 3): (biotin + PABA + yeast extract > biotin + yeast extract) > PABA + yeast extract > yeast extract > (biotin > biotin PABA) > (PABA > control).

4. Discussion

The results show that diazotrophic bacteria were more numerous in the root zone than in root-free soil under pine. This is in agreement with the data obtained by other researchers, as bacteria (including diazotrophs) are more frequent and metabolically active

Table 3
Two-factor analysis of variance (ANOVA), comparing the effects of strains and growth factors on nitrogenase activity

Source of variation		Variance	df[a]	*F* parameter	*p* (significance level)
Strains[b]		5.49	2	3.74	0.02728
Growth factors[b]		43.76	7	29.81	<0.000001
Interaction		1.22	14	0.83	0.6323
Error		1.47	96		
Strains					
No. 15	2.98 a				
No. 26	2.83 a				
No. 27	3.53 b				
Growth factors					
Control	0.31 a				
Biotin	3.26 b				
PABA	1.05 a				
Yeast extract (YE)	3.55 bc				
Biotin + PABA	2.92 b				
PABA + YE	3.79 bc				
Biotin + YE	4.60 c				
PABA + Biotin + YE	5.43 c				

[a] Degrees of freedom.
[b] Indicate the experimental factors, which affected significantly the nitrogenase activity.
[c] Mean values marked with the same letter do not differ significantly ($p \leq 0.05$).
Newman–Keuls multiple range test ($p < 0.05$).

in the root zone because of carbon inputs in the form of root exudates than in root-distant soil (Rambelli, 1973; Richards, 1973; Grayston et al., 1997).

Our results indicate that the numbers of diazotrophs were considerably higher in the soil and in the root zone of oak than in those of pine. Most probably the abundance of these bacteria was affected by soil pH which was lower for pine (4.6) than oak (5.9) soil. It is well known that heterotrophic bacteria including nitrogen fixers, are less numerous in acidic than in neutral forest soils (Jones and Bangs, 1985; Limmer and Drake, 1996). According to Jones and Bangs (1985), a pH close to 6.0 is optimal for nitrogen fixation by forest soil bacteria. It is also worthy of note that even though the closest pines and oaks were only 7–15 m apart the chemical and microbiological properties of the soil underneath them were much different. Differences in soil pH underneath pine and oak and differences in occurrence of diazotrophic bacteria can be ascribed to less acidic conditions and more easily decomposable litter of oak as compared with pine (Prusinkiewicz and Biały, 1976).

The absolute numbers of diazotrophs in soils and root zones of forest trees noted in our work were comparable to those reported by Jurgensen and Davey (1971).

The results of identification of bacteria obtained in this work, using the computer-aided system BIOLOG have shown that only about half of the strains could be identified satisfactorily. In most cases they belonged to the genera *Pseudomonas* and *Bacillus*. Limitations of the BIOLOG system for identification of soil bacteria were discussed in detail by Wünsche and Babel (1996). According to these authors, only about 50% of culturable soil bacteria can be identified successfully using the BIOLOG system, which is in an agreement with the results of our work. Wünsche and Babel (1996) stressed that such results should always be checked and/or confirmed by a determination of a few, fundamental, easily determinable, diagnostically important bacterial characters to avoid misleading results on a large scale. Such characters of morphological, cultural and physiological/biochemical nature (20 of them) were determined in this work and were used (a) to choose the correct BIOLOG test plates [for G(−) or G(+) bacteria]; (b) to make initial taxonomic grouping of the strains (genus level at best); and (c) for cluster analysis. Although 'con-

ventional' and BIOLOG testing were designed to be complementary, results obtained using both techniques are quite concordant (e.g. bacteria belonging to taxonomically close species/genera are also located close together on the dendrogram in Fig. 1).

It is worthy of note that the majority of bacteria studied in the present work were diazotrophic, as in our earlier studies (unpublished data) we frequently isolated oligonitrophilic bacteria unable to fix N_2 both on Rennie's and on other N-free media. It can be supposed that most soil bacteria may possess genetic information encoding the nitrogen fixation capability, but the expression of nitrogen fixation genes may be hindered by environmental conditions (Schlegel, 1996). Most of the diazotrophs studied belonged to the genus *Pseudomonas*. The predominance of this genus can be attributed to its very common occurrence both in the soil and in the root zone [according to Höflich (1989) it may comprise up to 60% of the rhizosphere bacteria], its low nutritional requirements, its capacity to utilize numerous complex organic substrates (including heterocyclic and aromatic compounds) (Stanier et al., 1966) and its tolerance to low pH (Dawson, 1983; Schlegel, 1996). The second most abundant genus among the diazotrophic bacteria studied in this work was *Bacillus*. The aerobic (and/or facultatively anaerobic) sporeforming bacilli are also very common in forest soils, especially those which are saccharolytic and have low nitrogen requirements, e. g. species like *Bacillus circulans* and *B. polymyxa* (Jurgensen and Davey, 1971; Buchanan and Gibbons, 1974; Różycki, 1987; Schlegel, 1996). Nitrogenase-active members of this genus were also isolated by other researchers, e.g. from within Scots pine ectendomycorrhiza (Pachlewski et al., 1991) and from within tuberculate ectomycorrhiza of Douglas-fir (Li et al., 1992).

It is difficult to compare the nitrogenase activity of bacterial strains studied in this work with the results obtained by others – mainly due to different methods used and different ways of expression the activity of nitrogen fixation.

The results of our studies on the effects of growth factors on nitrogenase activity are in agreement with the results of Li et al. (1992), who reported stimulatory effects of thiamine, biotin and yeast extract, but not PABA on nitrogenase activity of diazotrophic *Bacillus* sp. isolated from within tuberculate ectomycorrhiza of Douglas-fir. Stimulatory effects of trace amounts (20–25 mg/l) of yeast extract on the growth and nitrogenase activity of diazotrophic bacteria can be explained by its action as a source of 'starter nitrogen' – enabling the initiation of bacterial growth and nitrogen fixation under microaerophilic conditions (Döbereiner, 1980; Krieg and Döbereiner, 1984; Różycki et al., 1992). The results of the present work indicate that biotin (especially in combination with trace amounts of yeast extract) exerted a much more stimulatory effect on the growth and nitrogenase activity in the bacteria studied than PABA. Biotin is known to participate in heterotrophic CO_2 fixation and caboxylation and decarboxylation reactions (Oertli, 1987). According to Różycki et al. (1996) some vitamins (thiamine and pantothenate) but not biotin stimulated the microaerophilic growth of *Azospirillum*. However, the diazotrophic bacteria from the roots of pine and oak, studied in our work did not belong to the genus *Azospirillum* and they could react to the addition of vitamins to the medium (among them –also biotin) in a different manner. It is worthy of note, that Rennie's medium growth factors, which are known to have a stimulatory impact on the growth of diazotrophs also enhanced nitrogenase activity in the bacteria studied by us. The influence of vitamins on growth may not always be directly and positively correlated with their impact on nitrogenase activity (our unpublished data).

It is difficult to draw any conclusions about the ecological implications of nitrogen fixation in the bacterial strains studied in this work, as the results were obtained in pure cultures (in vitro) and it is almost impossible to extrapolate them to natural conditions. There is no agreement between researchers on the ecological role of nitrogen fixed in forest ecosystems. For example, Bormann et al. (1993) claimed that pine forest ecosystems can be nitrogen self-sufficient due to the non-symbiotic and associative N_2-fixation, while Barkmann and Schwintzer (1998) argued that N_2-fixation rates associated with pine are low and have little impact on the N budget of pine forest ecosystems. We believe that our study contributed to a better understanding of the biology of diazotrophic bacteria inhabiting the roots of pine and oak. In addition, these associative N_2 fixers may have ecological significance in enhancing mycorrhizal formation, as demonstrated by Garbaye (1994).

The results of our work have shown a quite common potential for nitrogen fixation as measured by nitrogenase activity among bacteria associated with the roots of pine and oak. Since associative N_2-fixation is targeted within the rhizosphere, we can assume that its significance to trees may be greater than indicated by simply calculating inputs on a kilogram per hectare basis. On N-limited sites, even small amounts of N added by bacteria living in association with mycorrhizae or roots could enhance the performance of individual plants. Our work indicates that both Scots pine and common oak provide not only mycorrhizae, but also associative N_2 fixers as well.

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