Acta Microbiologica Polonica
1992, Vol. 41, No. 3/4, 193–201

Effect of Different Carbon and Nitrogen Sources and Vitamins on Growth of *Azospirillum* spp. Isolated from Coniferous Ectomycorrhizae and Sporocarps of Ectomycorrhizal Fungi.

HENRYK RÓŻYCKI[1], EDMUND STRZELCZYK[1], EWA RACZKOWSKA[1]
and CHING-YAN LI[2]

[1] Laboratory of Microbiology, Institute of Biology, Nicolaus Copernicus University,
87-100 Toruń, Gagarina 9, Poland
[2] United States Department of Agriculture – Forest Service,
Pacific Northwest Research Station, Forestry Sciences Laboratory,
3200 S.W. Jefferson Way, Corvallis, Oregon 97331, U.S.A.

Received 15 July, 1992

Abstract

Effects of different substrates (sources of C and N, vitamins, soil – and pine root extracts, root exudates of pine seedlings, extracts from mycelia of ectomycorrhizal fungi) on the growth of three isolates of *Azospirillum* spp. associated with ectomycorrhizal fungi (*Rhizopogon vinicolor, Laccaria laccata*, and *Hebeloma crustuliniforme*) were studied.
Physiological differences between different bacterial strains were observed. These differences involved: growth kinetics, abilities for utilization of different carbon sources, and the reaction to the presence of natural exudates and/or extracts in the medium.

Introduction

The microbiological studies of a few recent years have confirmed the hypothesis that N_2-fixing bacteria are associated with micorrhizal fungi of forest trees (Li and Castellano, 1985, 1987; Li and Hung, 1987; Tilak *et al.*, 1988; Pachlewski *et al.*, 1922; Li *et al.*, 1992). The trials of simultaneous inoculation of crop plants with *Azospirillum* and with endomycorrhizal fungi of the genus *Glomus* have given positive (synergistic) results – crop increase (Barea *et al.*, 1983; Rao, *et al.*, 1985).

The purpose of the present studies was to characterize the physiology of carbon and nitrogen sources utilization by three strains of *Azospirillum* spp. associated with ectomycorrhizal fungi.

Experimental

Materials and Methods

Bacterial strains. In our studies 3 strains of *Azospirillum* spp. were used. They were isolated from within ectomycorrhizae of Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] formed by *Rhizopogon vinicolor* (strain No. 1) from within sporocarp of *Laccaria laccata* (strain no 2) and from within sporocarp of *Hebeloma crustuliniforme* (strain No. 3) – by Li and Castellano, 1987.

Culture medium. For culturing' storage and for growth experiments with *Azospirillum* modified Döbereiner's (1980) basal medium was used. Composition: K_2HPO_4 – 0.4 g, KH_2PO_4 – 0.1 g, $MgSO_4 \times 7H_2O$ – 0.2 g, NaCl – 0.1 g, $CaCl_2$ – 0.02 g, solution of microelements (containing: FeNa EDTA – 1 g/l, $MnSO_4 \times 2H_2O$ – 1 g/l, $Na_2MoO_4 \times 2H_2O$ – 0,2 g/l) – 10 ml, distilled H_2O – 1000 ml. As a basal carbon source (medium for culturing/storage and a positive control in growth experiments) disodium malate (5g/liter) was used. Similarly as a basal nitrogen source ammonium chloride (0.2 g/liter) was used. Medium for culturing/storage (semisolid – containing 4 g of agar/liter) did not contain NH_4Cl, but was supplemented with trace amount (20 mg/liter) of yeast extract.

Bacterial growth under aerobic conditions. The growth kinetics of *Azospirillum* spp. was studied under aerobic conditions in the presence of the following carbon sources: a) sodium salts of organic acids: succinate, propionate, fumarate, pyruvate, α-ketoglutarate; and b) sugars: glucose, fructose, galactose, xylose, arabinose, sucrose, as well as – in the presence of different nitrogen sources (with sodium malate as a constant carbon source): $NaNO_3$, alanine, glutamic acid, lysine, Casamino Acids (Difco), Yeast Extract (Difco).The above mentioned compounds were added to the basal medium in amount corresponding to: 1.225 g of carbon contained in 5 g of disodium malate (positive control) in case of carbon sources and 52.4 mg of nitrogen contained in 200 mg of NH_4Cl (positive control) in case of nitrogen sources. 20 ml portions of media in 100 ml Erlenmeyer flasks (with side arm) after autoclaving were inoculated with 0.2 ml of young (36–48 hour-old) liquid culture of a given bacterium (2 replicates per combination). Cultures were incubated at 28°C under static conditions, but with occasional shaking on Vortex mixer (immediately before reading optical density).

Optical density of the bacterial cultures was estimated on Klett-Summerson Photoelectric Colorimeter Model 800-3 (with a blue filter No. 42) after 0, 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, 24, 25, 26, 27, 28, 30, 32, 34, 36, 38, 48, 54, 60 and 72 hours of culturing. On the basis of resulting growth curve the following growth parameters were estimated and/or calculated: duration of the lag phase, growth yield after 36 hours (in optical density units, *i.e.* in Klett units), absolute growth yield (maximum of growth, growth rate during the exponential phase – *i.e.* the reciprocal of the generation time or the number of cell divisions per time unit (1 hour). Growth rate was calculated according to the following formulea:

$$\mu = \frac{\ln N - \ln N_o}{t - t_o}$$

where: N – number of cells in a culture at the time t; N_0 – number of cells in the culture at time t_o, *i.e.* immediately after inoculation.

Instead of absolute cell numbers we used the corresponding values of optical density (OD – expressed in Klett units) – assuming that OD is directly proportional to N.

Bacterial growth under microaerophilic conditions. Under microaerophilic conditions we studied the ability of utilization of the same carbon sources by *Azospirillum* spp. as in the case of aerobic conditions, but the medium did not contain 200 mg NH_4Cl/l; it contained 20 mg of yeast extract/l instead (trace nitrogen). We also tested the same nitrogen sources as in the case of aerobic conditions, but in trace amounts (30 mg/l) – in media containing 5 g of disodium malate per liter. Similarly the effect of vitamins (added separately and in combinations): a) thiamine (100 μg/l), b) biotin (100 μg/l), c) pantothenic acid (100 μg/l) on microaerophilic growth of *Azospirillum* was also estimated. Additionally the effects of soil – pine root extracts as well as – those of root exudates of pine seedlings and extracts of the mycelia of mycorrhizal fungi was tested.

Soil extract was prepared according to Lochhead and Chase (1943) and was added to the basal medium (with malate, but without nitrogen) in concentration 10%(v/v).

Pine root extract was prepared as follows: to 6 g of air-dried roots (of diameter up to 2mm) 100 ml of basal mineral medium was added and left for 24 hours at 4°C. Subsequently roots in extraction medium were heated in boiling water bath for 10 min. After filtering, adding the carbon source (5g of malate/l) and tubing – the medium was sterillized by repeated heating in boiling water bath for 10 min. at 24-hour intervals.

In order to obtain the root exudates – pine seeds were surface sterilized (30% H_2O_2) and put onto 0.1% peptone water agar (1%). Subsequently germinated, uncontaminated seeds were put onto cheese cloth covering the glass cylinders placed in 750 ml (wide neck) Erlenmeyer flasks containing 100 ml of mineral basal medium (hydroponic cultures). Seedlings were cultured for 30 days in plant growth chamber at temperature 21°C with 16-hour photoperiod (light intensity: 4500 lux). Post-culture liquids of pine seedlings after adding malate and tubing were sterillized by tyndalization.

To prepare mycelial extracts *Laccaria laccata* and *Hebeloma crustuliniforme* were cultured on cellophane squares put onto Lamb's (1974) agar medium; *Rhizopogon vinicolor* was cultured in liquid medium. After 26 days of culturing mycelia were harvested (ca.1 g, 0.4 g, 1 g for *Laccaria laccata, Rhizopogon vinicolor* and *Hebeloma crustuliniforme*, respectively), homogenized in 120 ml of mineral basal medium and filtered through Whatman GF/D glass fibre filter. To resulting extracts malate was added and after tubing they were sterilized by tyndalization.

In all experimental combinations to obtain microaerophilic conditions media were distributed in 5 ml quantities into small (140 × 14 mm) culture tubes (3 replicates per combination). They were inoculated with 0.05 ml of young liquid bacterial culture (36–48 hour-old, grown under aerobic conditions in malate-NH_4Cl medium) and grown at 28°C for 72 hours. As a control served basal medium containing disodium malate (5 g/l), but no nitrogen. Growth kinetics was not followed due to scarce bacterial growth. A growth yield after 72 hours was estimated turbidimetrically at 560 nm using the spectrophotometer S-1/K (EMCO, Płock, Poland).

Statistical evaluation of the results. All the data obtained (aerobic conditions: growth rate and 36-hour growth yield; microaerophilic conditions: 72-hour growth yield) were evaluated by the method of 1-factor analysis of variance and Newman-Keuls multiple range test ($p/<0.05$). Statistical calculations were performed using CSS: STATISTICA package for IBM PC and compatibles (release 3E, 1991, StatSoft, Tulsa, Oklahoma, U.S.A.).

Results

The results obtained in the present work are presented in Tables I–IV. The growth kinetics parameters of *Azospirillum* spp. in the presence of different substrates under aerobic conditions are shown in Tables I–III. Among sodium salts of organic acids the best substrate for all three strains was pyruvate; the worst – propionate (Table I). Strain No. 1 grew best in the medium with glucose; strain No. 3 – with arabinose and strain No. 2 did not utilize sugars at all (Table II). In general the best nitrogen source was glutamic acid and the worst – lysine, but there were differences depending on the analysed growth parameter (growth rate or growth yield) (Table III).

Table IV presents the results of studies performed under microaerophilic conditions. Among salts of organic acids – pyruvate and α-ketoglutarate were best utilized by strain No 1.; for strains Nos. 2 and 3 pyruvate but not α-ketoglutrate was the best carbon source. Glucose and fructose were the best utilized sugars by strain No. 1; for strains Nos. 2 and 3 fructose, but not glucose was the optimal carbon among sugars. In general under microaerophilic conditions (similarly as under aerobic conditions) salts of organic acids were better carbon sources for azospirilla than sugars. Trace amounts (20 mg/l) of nitrogen sources stimulated microaerophilic growth of bacteria studied – particularly alanine and $NaNO_3$ (Table IV).

Table I

Growth kinetics parameters of *Azospirillum* spp. in the presence of sodium salts of organic acids as carbon sources (aerobic conditions, N-NH_4Cl, 28°C). Mean values of 2 replications. Values marked with the same letters do not differ significantly ($p \leqslant 0.05$)

Strain No.	Sodium salts of organic acids	Time (hours) of*		Growth rate ($hour^{-1}$)**		Growth (O. D.) after 36 hours of culturing***	
		lag phase duration	reaching the maximum of growth	Value	%	Value	%
1	Malate (Control)	3	26	0.340 c	100	151.5 c	100
	Succinate	4	30	0.207 b	61	194.0 d	128
	Propionate	16	> 60	0.059 a	17	8.5 a	6
	Fumarate	3	30	0.259 bc	76	167.0 c	110
	Pyruvate	2	32	0.417 cd	122	172.5 cd	114
	α-Ketoglutarate	2	26	0.203 b	60	128.0 b	84
2	Malate (Ctrl)	1	54	0.189 a	100	165.0 c	100
	Succinate	1	38	0.238 a	126	165.5 c	100
	Propionate	16	> 72	0.057 a	30	23.5 a	14
	Fumarate	1	> 72	0.248 a	131	125.7 b	76
	Pyruvate	0	> 72	0.207 a	109	225.0 d	136
	α-Ketoglutarate	2	> 72	0.163 a	86	143.5 bc	87
3	Malate (Ctrl)	1	36	0.343 a	100	184.5 c	100
	Succinate	3	34	0.238 a	69	151.5 b	82
	Propionate	14	> 72	0.084 a	0.2	23.5 a	13
	Fumarate	2	38	0.246 a	72	141.0 b	76
	Pyruvate	2	34	0.178 a	52	199.0 c	108
	α-Ketoglutarate	2	38	0.170 a	50	176.0 c	95

* aproximate values
** a reciprocal of generation time during the exponential growth phase (μ)
*** growth yield

Table II

Growth kinetics parameters of *Azospirillum* spp. in the presence of sugars as carbon sources (aerobic conditions, N-NH_4Cl, 28°C). Mean values of 2 replications. Values marked with the same letters do not differ significantly ($p \leqslant 0.05$)

Strain No.	Sugars	Time (hours) of*		Growth rate ($hour^{-1}$)**		Growth (O. D.) after 36 hours of culturing***	
		lag phase duration	reaching the maximum of growth	Value	%	Value	%
1	Malate (Ctrl)	1	38	0.412 b	100	183.3 cd	100
	Glucose	5	48	0.210 ab	51	196.0 d	107
	Fructose	2	> 72	0.176 a	43	107.5 b	59
	Galactose	4	> 72	0.185 ab	45	158.0 c	86
	Xylose	5	60	0.136 a	33	114.5 b	62
	Arabinose	3	60	0.152 a	37	159.0 c	87
	Sucrose	0	> 72	0.011 a	3	2.5 a	1

c.d. tab. II

1	2	3	4	5	6	7	8
2	Malate (Ctrl)	0	> 72	0.291 b	100	121.0 b	100
	Glucose	1	> 72	0.006 a	2	2.0 a	2
	Fructose	1	> 72	0.014 a	5	10.5 a	9
	Galactose	1	> 72	0.000 a	0	3.5 a	3
	Xylose	0	> 72	0.011 a	4	8.0 a	7
	Arabinose	2	> 72	0.000 a	0	3.0 a	2
	Sucrose	2	> 72	0.009 a	3	4.5 a	4
3	Malate (Ctrl)	1	38	0.437 b	100	159.5 c	100
	Glucose	0	> 72	0.146 ab	33	172.5 c	108
	Fructose	2	> 72	0.137 ab	31	108.5 b	68
	Galactose	.0	> 72	0.150 ab	34	162.0 c	102
	Xylose	1	> 72	0.118 ab	27	132.5 ab	63
	Arabinose	0	> 72	0.211 ab	48	180.0 c	113
	Sucrose	0	> 72	0.013 a	3	6.5 a	4

Explanations – see Table I

Table III

Growth kinetics parameters of *Azospirillum* spp. in the presence of different nitrogen sources (aerobic conditions, C – malate, 28°C). Mean values of 2 replications. Values marked with the same letters do not differ significantly ($p \leqslant 0.05$)

Strain No.	Nitrogen sources	Time (hours) of*		Growth rate ($hour^{-1}$)**		Growth (O. D.) after 36 hours of culturing***	
		lag phase duration	reaching the maximum of growth	Value	%	Value	%
1	NH_4Cl (Ctrl)	2	38	0.559 d	100	213.5 d	100
	$NaNO_3$	3	60	0.220 b	39	155.0 c	73
	Casamino Ac.	3	38	0.195 b	35	68.0 b	32
	Yeast Extract	1	> 72	0.284 b	51	68.0 b	31
	Alanine	4	60	0.135 ab	24	132.5 c	62
	Glutamic acid	3	38	0.339 bc	61	213.5 d	100
	Lysine	30	> 72	0.032 a	6	7.0 a	3
2	NH_4Cl (Ctrl)	2	38	0.313 b	100	131.5 c	100
	$NaNO_3$	4	60	0.123 ab	39	142.5 c	108
	Casamino Ac.	2	> 72	0.343 b	109	60.0 ab	46
	Yeast Extract	3	> 72	0.191 ab	61	92.0 b	70
	Alanine	0	> 72	0.123 ab	39	104.0 bc	79
	Glutamic acid	3	60	0.266 b	85	200.0 d	152
	Lysine	5	> 72	0.036 a	11	42.0 a	32
3	NH_4Cl (Ctrl)	2	38	0.304 b	100	188.0 c	100
	$NaNO_3$	4	> 72	0.279 ab	92	170.5 bc	91
	Casamino Ac.	3	38	0.217 ab	72	66.5 ab	35
	Yeast Extract	2	38	0.199 ab	65	93.0 b	49
	Alanine	3	48	0.301 b	99	138.5 bc	74
	Glutamic acid	2	38	0.319 b	105	212.5 cd	113
	Lysine	36	> 72	0.040 a	13	2.5 a	1

Explanations – see Table I

Table IV

Growth of *Azospirillum* spp. in the presence of different substrates under microaerofilic conditions (mean values from 5 replications)

Growth substrate	Final growth yield – optical density (560 nm) after 72 hoursof culturing (% of control)					
	Strain No. 1		Strain No. 2		Strain No. 3	
Organic acid						
Control	0.0404 b	100%	0.1708 cd	100%	0.0416 ab	100%
Succinate	0.0390 b	96%	0.1444 c	84%	0.0398 ab	96%
Propionate	0.0278 a	69%	0.0672 a	39%	0.0376 ab	90%
Fumarate	0.0330 ab	82%	0.1468 c	86%	0.0282 a	68%
Pyruvate	0.0466 b	115%	0.2054 de	120%	0.0466 b	112%
α-ketoglutarate	0.0500 bc	124%	0.1100 b	64%	0.0374 ab	90%
Sugars						
Control	0.0248 b	100%	0.0526 c	100%	0.0292 ab	100%
Glucose	0.0698 d	281%	0.0298 a	57%	0.0260 ab	89%
Fructose	0.0466 c	188%	0.0514 c	98%	0.0342 b	117%
Galactose	0.0392 c	158%	0.0348 ab	66%	0.0262 ab	90%
Xylose	0.0372 c	150%	0.0308 a	55%	0.0248 ab	85%
Arabinose	0.0410 c	165%	0.0390 ab	74%	0.0268 ab	92%
Sucrose	0.0112 a	45%	0.0418 b	79%	0.0204 a	70%
Nitrogen sources						
Control	0.0056 a	100%	0.1282 b	100%	0.0110 a	100%
NH_4Cl	0.0708 c	1264%	0.1024 a	80%	0.0590 c	536%
$NaNO_3$	0.1086 d	1939%	0.1358 bc	106%	0.0932 d	847%
Casamino acids	0.0318 ab	568%	0.1086 ab	85%	0.0108 a	98%
Yeast extract	0.0186 ab	332%	0.1072 ab	84%	0.0168 a	153%
Alanine	0.0368 b	657%	0.1710 d	133%	0.0488 c	444%
Glutamic acid	0.0268 ab	479%	0.1524 c	119%	0.0340 b	309%
Lysine	0.0128 ab	229%	0.1378 bc	107%	0.0240 ab	218%
Vitamins						
Control	0.0086 a	100%	0.1028 ab	100%	0.0174 a	100%
Thiamine (T)	0.0204 b	237%	0.1146 b	111%	0.0124 a	71%
Biotin (B)	0.0144 a	167%	0.1214 bc	118%	0.0162 a	93%
Pantothenate (P)	0.0120 a	139%	0.0978 ab	95%	0.0174 a	100%
T + B	0.0108 a	126%	0.0882 a	86%	0.0106 a	61%
B + P	0.0084 a	98%	0.0914 a	89%	0.0128 a	74%
T + P	0.0084 a	98%	0.0898 a	87%	0.0100 a	57%
T + B + P	0.0860 a	100%	0.0972 ab	94%	0.0094 a	54%
Extracts/exudates.						
Control	0.0056 a	100%	0.1282 a	100%	0.0110 a	100%
Soil extract	0.0820 b	1464%	0.1680 a	131%	0.0760 a	691%
Pine root extr.	0.5058 c	9032%	0.3280 b	256%	0.4424 b	4022%
Root exudates of pine seedlings	0.0416 c	732%	0.1120 a	87%	0.4860 a	422%
Myc. ex. of *Rh. v.*	0.0618 ab	1104%	0.1240 a	97%	0.0860 a	782%
Myc. ex. of *Lac. l.*	0.0170 a	304%	0.1128 a	88%	0.0308 a	280%
Myc. ex. of *Heb. c.*	0.0610 ab	1089%	0.1016 a	79%	0.0870 a	791%

Explanations: Control – basal mineral medium containing 5 g malate/l without bound nitrogen. Values in a given substrate group marked with the same letter do not differ significantly ($p \leq 0.05$)

Studies on the effects of vitamins on the microaerophilic growth of azospirilla have shown that growth of strain No. 1 was most strongly stimulated by thiamine. In case of strain No. 2 the most stimulatory was biotin; in general this organism at the presence of vitamins gave severalfold better growth than the two remaining ones. Vitamins inhibited growth of strain No. 3, except pantothenic acid (Table IV).

Addition to the medium of natural extracts and/or exudates stimulated the growth of strains Nos. 1 and 3 – most strongly at the presence of the pine root extract; in case of the mycelial extracts the most effective were extracts from *Rhizopogon vinicolor* and *Hebeloma crustuliniforme* mycelia. Growth of strain No. 2, although stimulated only by soil- and pine root extracts – in all experimental combinations was significantly stronger than in two remaining strains (TableIV).

Discussion

The present studies have shown that in *Azospirillum* spp. derived from within sporocarps of ectomycorrhizal fungi, as well as from within the ectomycorrhizae of Douglas-fir, growth kinetics under aerobic conditions in the presence of different growth substrates was different depending on the strain studied. The differences in the "carbon/nitrogen source utilization order", depending on the growth parameter analysed (growth rate during the exponential phase [μ] or growth yield), *e.g.* high μ and low yield in given experimental combination can be explained by high availability of a given substrate with simultaneous fast production of self-inhibitory and/or self-toxic metabolites.

We have observed that sodium salts of organic acids were better carbon sources for azospirilla than sugars. It was also stated by other researchers (Okon *et al.* 1976 a, b; Das and Mishra, 1983). High availability of organic acids in *Azospirillum* spp. may be of importance under natural conditions, considering the positive chemotactic reaction of these bacteria towards the ions of organic acids contained in root exudates (Heinrich and Hess, 1985; Reinhold *et al.*, 1985; Zhulin *et al.* 1988).

In the second part of our studies, performed under microaerophilic (N_2-fixation inducing) conditions – the strains studied grew very weakly. It could be caused by the actual level of pO_2 in "microaerophilic" tube cultures – not exactly corresponding to the optimal pO_2 for N_2-fixation by azospirilla; optimal pO_2 ranges between 0.005–0.007 atm (Okon *et al.*, 1977). However we have shown a stimulatory effect of bound nitrogen traces (20 mg of N compound per liter) – mainly of $NaNO_3$ and alanine. Other workers recommended addition to the medium 0.002% (=20 mg/l) of yeast extract (Döbereiner, 1980) or NH_4Cl (Hartmann *et al.*, 1988; Tilak *et al.*, 1988) acting as "growth starters" and facilitating N_2-fixation.

In the present work relatively weak and/or inconsistent effects of vitamins on microaerophilic growth of azospirilla were determined. This could be ascribed (at least partly) to weak growth of these organisms at low pO_2 – and to the resulting high experimental error. Further studies on this problem are needed, involving the use of more accurate methods.

On the other hand the observed strong stimulatory effect of the extract from pine roots on microaerophillic growth of *Azospirillum* spp. can be described both to the trophic action of its organic constituents and to the presence of some specific growth factors. Also more detailed studies are needed on the action of natural exucates/extracts (involving their chemical analyses) on N_2-fixing bacteria.

The phenomenon of growth stimulation in N_2-fixing bacteria by extracts from sporocarps of ectomycorrhizal fungi, which bacteria were isolated from – was described by Li and Castellano (1985). We did not find any specific action of extracts made from the host-fungus mycelia on the appropriate associated bacteria (*Azospirillum* spp.); there non-specific stimulation of bacterial growth occured.

It is noteworthy that strain No. 2 of *Azospirillum* (deriving from *Laccaria laccata* sporocarp) was considerably different than the two remaining strains. For instance, unlike to strains Nos. 1 and 3, it was uncapable of utilizing sugars under aerobic conditions. Besides, strain No. 2 grew better under microaerophilic conditions than the two remaining ones (independently of the carbon source in the medium). Due to its generally better growth we could not notice any distinct stimulatory effect of mycelial extracts and pine root exudates in the case of this strain. Tilak *et al.* (1988) studying the same strains did not detect any significant differences between them in the results of qualitative diagnostic tests. On the basis of these results they suggested that these 3 strains belong to one species of *Azospirillum* – most probably to: *A. brasilense.* However – considering the results of our (more quantitative) studies it can be supposed that they belong to different ecotypes.

Summarizing, it can be said that to get better knowledge of N_2-fixing bacteria associated with mycorrhizal fungi and/or with mycorrhizae further studies are needed on: a) their isolation (*e.g.* from sporocarps) and identification; b) physiological properties – especially physiology of growth and on the activity on nitrogenase under different environmental conditions. As a continuation (extension) of this kind of studies there could be trials of their practical use in forestry as components of mycorrhizal inocula in conjunction with the fungi they are associated with – to inoculate seedlings of forest trees in nurseries.

Acknowledgements. This research was carried out under a joint program performed in cooperation with the United States Department of Agriculture and the Polish Ministry of Agriculture and Food Production under the Second M. Skłodowska-Curie Fund (Project No. PL-AES-139).

Literature

Barea J.M., Bonis A.F., Olivares J., 1983: Interactions between *Azospirillum* and VA mycorrhiza and their effects on growth and nutrition of maize and ryegrass. *Soil Biol. Biochem.* **15**: 705–709.

Das A., Mishra A.K., 1983: Utilization of fructose by *Azospirillum brasilense. Can. J. Microbiol.* **29**: 1213–1217.

Döbereiner J., 1980: Forage grasses and grain crops p. 535–555. In: Methods for evaluating biological nitrogen fixation (Bergersen F. J., Ed.). John Wiley and Sons, Inc., New York.

Hartmann A., Fu H., Burris R.H., 1988: Influence of amino acids on nitrogen fixation ability and growth of *Azospirillum spp. Appl. Environ. Microbiol.* **54** (1): 87–93.

Heinrich D., Hess D., 1985: Chemotactic attraction of *Azospirillum lipoferum* by wheat roots and characterization of some attractants. *Can. J. Microbiol.* **31**: 26–31.

Lamb R.J., 1974: Effect of D-glucose on utilization of single carbon sources by ectomycorrhizal fungi. *Trans. Brit. Mycol. Soc.* **63** (2): 295–306.

Li C.Y., Castellano M.A., 1985: Nitrogen fixing bacteria isolated from within sporocarps of three ectomycorrhizal fungi, p. 264. Proceedings of 6th North American Conference on Mycorrhiza(Molina R., Ed.) Oregon State University, Forestry Research Laboratory, Corvallis.

Li C.Y., Castellano M.A., 1987: *Azospirillum* isolated from within sporocarps of the mycorrhizal fungi *Hebeloma crustuliniforme, Laccaria laccata* and *Rhizopogon vinicolor.Trans. Br. Mycol. Soc.* **88** (4): 563–565.

Li C.Y., Hung L.L., 1987: Nitrogen-fixing (acetylene reducing) bacteria associated with ectomycorrhizae of Douglas-fir. *Plant* and *Soil* **98**: 425–428.

Li C.Y., Massicote H.B., Moore L.V.H., 1992: Nitrogen-fixing *Bacillus* sp. associated with Douglas-fir tuberculate ectomycorrhizae. *Plant* and *Soil* **140**: 35–40.

Lochhead A.G., Chase F.E., 1943: Qualitative studies of soil microorganisms. IV. Nutritional requirements of the predominant bacterial flora. *Soil Sci.* **55**: 185–195.

Okon Y., Albrecht S.L., Burris R.H., 1976 a: Factors affecting growth and nitrogen fixation of *Spirillum lipoferum. J. Bacteriol.* **127** (3): 1248–1254.

Okon Y., Albrecht S.L., Burris R.H., 1976 b: Carbon and ammonia metabolism of *Spirillum lipoferum. J. Bacteriol.* **128** (2): 592–597.

Okon Y., Houchins J.P., Albrecht S.L., Burris R.H., 1977: Growth of *Spirillum lipoferum* at constant partial pressures of oxygen, and the properties of its nitrogenase in cell-free extracts. *J. Gen. Microbiol.* **98**: 87–93.

Pachlewski R., Kermen J., Chruściak E., Trzcińska E., 1992: Studies of pine ectendomycorrhizas in nurseries. *Acta Mycologica*, in press.

Rao S.N.S., Tilak K.V.B.R., Singh C.S., 1985: Synergistic effect of vesicular-arbuscular mycorrhizas and *Azospirillum brasilense* on the growth of barley in pots. *Soil Biol. Biochem.* **17** (1): 119–121.

Reinhold B., Hurek T., Fendrik I., 1985: Strain specific chemotaxis of *Azospirillum* spp. *J. Bacteriol.* **162** (1): 190–195.

Tilak K.V.B.R., Li C.Y., Trappe J.M., 1988: Characterization of nitrogen-fixing *Azospirillum* isolated from within sporocarps of ectomycorrhizal fungi associated with Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco]. *Indian J. Microbiol.* **28** (4): 315–319.

Zhulin I.B., Tretyakova S.E., Ignatov V.V., 1988: Chemotaxis of *Azospirillum brasilense* towards compounds typical of plant root exudates. *Folia Microbiol.* **33** (4): 277–280.