Acta Agrophysica, 2001, 52, 151-160

N_2 - FIXING BACTERIA ISOLATED FROM THE ROOTS OF CROPS AND GRASSES

M.J. Król, A. Perzyński, J. Kobus

Department of Agricultural Microbiology, Institute of Soil Science and Plant Cultivation
Królewska 1, 24-100 Puławy, Poland

A b s t r a c t. Free-living bacteria fixing-N_2 (from genera of *Azospirillum* and *Acinetobacter*-like) occurred in the rhizosphere of barley, cultivated on different soils and also in the rhizosphere of maize and grasses grown on medium alluvial soil. In the rhizosphere of barley occurrence of *Azospirillum* sp. strains was found in 84%, and in the rhizosphere of maize and grasses in the amount of 100% and 94%, respectively. Bacteria of the genus *Azospirillum* were not found in the rhizosphere of *Lolium perenne, Phleum pratense* and *Poa palustris*.

In the rhizosphere of barley cultivated on rendzinas and black earth developed from slightly loamy sand and in the rhizosphere of grasses, were found of halophylic strains of *Azospirillum* sp. (growth in medium with 3% NaCl) and strains, which preliminary determined as *Acinetobacter*-like (growth in medium with 6% NaCl).

The most probable number (MPN) of bacteria from the genus *Azospirillum* counted in the rhizosphere of barley, maize and grasses ranged from 0 to 2450 cells g^{-1} of soil dry matter. No correlation between the frequency of their occurrence and content of available components, humus and exchangeable cations in the soil sampling date was observed. The nitrogenase activity of *Azospirillum* spp. strains isolated from the barley, maize and grasses rhizosphere ranged from 1.6 to 228.5 nM C_2H_4 h^{-1} cm^{-3} of gas phase. The nitrogenase activity of the isolated *Acinetobacter*-like strains ranged from 8,4 to 59,4 nM C_2H_4 $h^{-1}cm^{-3}$ of the gas phase.

K e y w o r d s: cereals, rhizosphere, *Azospirillum*, *Acinetobacter*-like, acetylene reduction.

INTRODUCTION

Bacteria of the *Azospirillum* genus are nitrogen-fixing organisms that live in a close association with plants in the rhizosphere. They commonly occur in the soil and plant rhizosphere in the tropical and temperate zones [1,4,5,14,20,22,24-26].

In the beginning, *Azospirillum* was considered a typical bacterium of tropical climates [2,3,5,28]. Later, is was isolated as bacteria from the soil and rhizosphere of temperate regions and a cold climate of Finland [8].

Occurrence of *Azospirillum* bacteria in the rhizosphere of various cereals cultivated on Polish soils was examined by Jaśkowska [12].

In literature, there are reports on the *Azospirillum* isolation from the soils, but to the best of our knowledge, a specific analysis of the rhizosphere/soil ratio has not been performed [10,11]. Little is known about the occurrence of *Azospirillum* bacteria in the Polish soils and in the crop plants rhizosphere [12,13,15,19].

MATERIALS AND METHODS

Samples were taken from the rhizosphere of barley grown on different soils, the rhizosphere of maize and grasses grown on the alluvial soil (*Elymus arenarius* - sand of the Baltic sea). In order to record *Azospirillum* occurrence in the rhizoplane, roots were rinsed under tap water, sterilised on the surface with sodium dichloroisocyanurate and cut into 5 mm pieces. Several pieces of roots of the above plant variety were put into each of the ten test tubes with 5 cm^3 of semi-solid nitrogen-free medium Nfb [5]. The cultures were incubated for 72 hours at 30 ^oC. The presence of *Azospirillum* was macro- and microscopically tested. Strains of bacteria were studied for growth in the presence of d-glucose, d-mannitol, sucrose and in medium with 3% and 5% NaCl [27]. Acidification of peptone-base glucose and requirement for biotin were examined. The most probable number (MPN) of *Azospirillum* was counted according to the method of Hegazi *et al.* [9] with the use of Mc Crady's statistical tables [7]. Nitrogenase activity of *Azospirillum* was examined, and C_2H_2 reduction was estimated by closing the vials of the semi-solid Nfb medium with rubber stoppers and incubation in the air with 10% C_2H_2 for one hour. Concentration of C_2H_4 was estimated using a Pay Unicam - 204 gas chromatograph fitted with a hydrogen flame ionisation detector.

RESULTS AND DISCUSSION

The number of free-living, fixing-N_2 bacteria that occurred in the rhizosphere of barley grown in 15 different soils and also in the rhizosphere of maize and grasses grown on medium alluvial soil (Table 1) was determined. These soils were developed from clayey sand, clay, loess, silt loam, peat and the soils developed from limestone. The structure of the analysed soils was differentiated. Among the examined 15 of the soils only 5 (loamy sand, podzolic soil developed from loess, heavy alluvial soil, black earth developed from slightly loamy sand, black earth developed from sandy loam) were acidic and lightly acidic, the remaining soils

Table 1. Granulometric composition of soil and their reaction

No. of sample	Soil	Origin of sample	Percentage of mechanical fractions (mm)				pH H_2O
			0.002	1.0-0.1	0.1-0.02	<0.02	
1	Silty closely caly sand	Kłoda/PołaniecVoi. Tarnobrzeskie	3	49	33	18	5.4
2	Silty closely clay sand	Osiny/Iłża Voi. Radomskie	3	47	34	19	6.8
3	Silty closely caly sand	Gnojno/Chmielnik Voi. Kieleckie	7	56	44	20	7.9
4	Silty sandy clay	Kończyce/Chełmża Voi. Toruńskie	4	35	37	21	7.4
5	Podzolic soil developed from clay	Młynary/Braniewo Voi. Elbląskie	16	17	62	46	6.8
6	Podzolic soil developed from loess	Kiełczany/Bogucin Voi. Kieleckie	5	11	62	27	5.5
7	Brown-gray soil developed from loess	Las Stocki/Wąwolnica, Voi. Lubelskie	6	1	42	37	6.4
8	Chernozem developed from loess	Kazimierza Wielka Voi. Kielckie	21	7	47	51	7.3
9	Chernozem developed from loess	Hrubieszów Voi. Zamojskie	18	2	25	51	6.8
10	Alluvial soil heavy	Nowy Dwór Gdański, Voi. Gdańskie	25	11	22	64	5.9
11	Rendzina developed from limestone	Stara Wieś/Frampol Voi. Zamojskie	3	67	5	11	6.2
12	Rendzina developed from limestone	Busko Zdrój Voi. Kieleckie	16	71	7	24	7.0
13	Black earth developed from loam and clay	Rudniki/Połaniec Voi. Tarnobrzeskie	2	86	36	7	4.6
14	Black earth developed from light sand	Ostaszewo/Toruń Voi. Toruńskie	5	40	-	24	5.0
15	Peat-mucky slimpy soil	Nakło/Noteć Voi. Bydgoskie	-	-		-	6.8

were neutral and alkaline soil. The results of investigation on the content of available components and humus in the studied soils are shown in Tables 2 and 3. The humus content ranged from 0.89 to 3.80%. Most the humus was found in the chernozem developed from loess, heavy alluvial soil, black earth developed from slightly loamy sand and podzolic soil developed from loam. Low content of humus was observed in the rendzina from limestone and loamy sand. Content of other available components such as P_2O_5, K_2O and Mg was determined for various pH soil levels. In the investigated soils, the content of available phosphorus ranged from 2.5 to 46.8 mg P_2O_5 in 100 g of soil. Most of the available phosphorus was found in the rendzinas from limestone and silty light loam, respectively: 46.8 and 39.0 mg P_2O_5 in 100 g of soil. Low amount of available phosphorus was found in the soils developed from loamy sand and loess.

Table 2. Content of available nutrients and humus in the soils

No. of soil sample*	Available nutrients (mg dm^{-3} soil)			Humus (%)
	P_2O_5	K_2O	Mg^{2+}	
1	2.5	4.6	6.0	2.43
2	4.6	13.3	1.8	1.10
3	18.9	12.7	2.8	1.64
4	39.0	23.5	4.8	1.48
5	15.4	13.3	22.8	2.59
6	5.6	3.1	6.6	1.84
7	12.6	4.9	10.7	2.13
8	5.8	8.6	20.0	3.80
9	14.5	5.2	17.4	2.90
10	6.5	7.7	22.4	3.65
11	7.0	8.6	3.3	0.89
12	46.8	18.2	4.2	2.00
13	5.0	11.7	4.4	2.85
14	7.2	13.3	6.2	1.95
15	15.9	3.4	17.4	-

*see Table 1.

The content of available potassium ranged from 3.1 to 23.5 mg K_2O in 100 g of soil. Low amount of available potassium was determined in the podzolic soil developed from loess and peat-muck soil, and the highest amount in the silty light loam and rendzinas developed from limestone.

The content of available magnesium ranged from 1.8 to 22.8 mg Mg^{2+} in 100 g of soil. High amount of available magnesium was observed in the podzolic soil developed from loam, heavy alluvial soil and in chernozem.

Chemical properties of the soils were determined on the basis of exchangeable cations structure in the soil samples Ca^{2+}, Mg^{2+}, $K^{+}$and Na^{+} (Table 3).

Table 3. Content of exchangeable cations in the soils

No. of soil sample*	Exchangeable cations m.e./100 g soil			
	Ca^{2+}	Mg^{2+}	K^{+}	Na^{+}
1	4.24	0.61	2.21	0.06
2	14.72	0.36	0.42	0.10
3	2.50	0.23	0.30	0.03
4	1.62	0.31	0.34	0.03
5	15.34	2.55	0.61	0.13
6	3.37	0.13	0.38	0.03
7	7.86	0.39	0.72	0.06
8	3.49	0.53	0.19	0.06
9	18.71	2.14	0.40	0.19
10	39.42	2.63	0.72	0.29
11	5.49	0.50	0.50	0.19
12	3.12	0.38	0.38	0.06
13	24.20	2.55	0.55	0.22
14	20.83	2.55	0.50	0.28
15	23.15	0.59	0.83	0.13

*see Table 1.

The most probable number of bacteria from the genus *Azospirillum* counted in the rhizospheres of barley, maize and grasses ranged from 0 to 2450 cells g^{-1} of soil dry matter (Tables 4 and 5). Most of the bacteria (MPN) were found in the soil developed from loamy sand and in the rendzina, and the least in heavy alluvial soil [7]. Among the 14 grasses, bacteria of *Azospirillum* spp. were not found in the rhizosphere of *Lolium perenne, Phleum pratense* and *Poa palustris* (Table 5).

Similar results on the amount of azospirilla in the samples of rhizosphere soil was obtained by other authors [4,13,21]. Studies on the occurrence of azospirilla in relation to the soil pH showed that the studied strains were present in about 34% of the samples of acidic soil, in 67% of the samples of lightly acidic soil, in 100% of the neutral samples and in 86% of the alkaline soil (Table 6). For instance, a great number of azospirilla appeared in the black earth developed from slightly loamy sand (pH=4.6, MPN-2240) and in grey-brown podzolic soil developed from loess (pH=6.4, MPN-0). However, it was not found in the rhizosphere of *Phleum pratense* (pH=6.0; MPN-0) and *Poa palustris* (pH=7.0; MPN-0). Other authors isolated azospirilla from the soil pH in the range from 4.0 to 8.0, mostly in the light acidic and alkaline soil.

Table 4. Occurrence of *Azospirillum* spp. in rhizosphere of barley grown on different soils (in 1 g of sample d.m.)

No. of soil sample*	pH_{H_2O} **	MPN***
1	5.4	2350
2	6.8	2450
3	7.6	2380
4	7.4	2350
5	6.8	2220
6	5.5	0
7	7.7	0
8	7.3	2150
9	6.8	2320
10	5.9	10
11	6.2	0
12	7.0	2450
13	4.6	2240
14	5.0	0
15	6.8	120

*see Table 1; ** pH of rhizosphere soil; ***most probable number after method of Hegazi *et al.* [9].

Table 5. Occurrence of *Azospirillum* spp. in rhizosphere of different species of grasses grown on alluvial soil (in 1 g of sample d.m.)

No.*	Grass species	pH_{H_2O} **	MPN***
1	*Agropyron sibiricum*	7.2	235
2	*Agrostis vulgaris*	6.4	1888
3	*Arrhenatherum elatius*	7.0	1813
4	*Bromus inermis*	6.3	2242
5	*Dactylis glomerata*	7.2	2357
6	*Elymus glaucus*	6.5	2260
7	*Festuca rubra*	7.2	33
8	*Hierochloe australis*	6.9	2352
9	*Lolium perenne*	6.8	0
10	*Miscanthus purpurescens*	7.0	2220
11	*Phalaris arundinacea*	7.5	33
12	*Phleum pratense*	6.0	0
13	*Poa angustifolia*	7.0	2306
14	*Poa palustris*	7.0	0

*see Table 1; **pH of rhizosphere soil; ***most probable number after method of Hegazi *et al.* [9].

In literature, data on the abundance of azospirilla in the soil samples is scarce [5,6] but a specific analysis of the rhizosphere/soil ratio has not been performed [10,11]. Our examination on the presence of azospirilla in the rhizospheres suggested that the amount of bacteria is higher than in the soil.

Table 6. The occurrence of *Azospirillum* spp. in relation to pH of soil in barley rhizosphere

pH_{H_2O} of rhizosphere soil	Number of test tube	Number of positive results	Percentage of positive results
4.6-5.5	3	1	34
5.6-6.5	3	2	67
6.6-7.2	2	2	100
>7.2	7	6	86
Sum	15	11	

De Coninck *et al.* [4], analysed the presence of azospirilla in a field-inoculation experiment and she found from 10 to 100 times more azospirilla in rhizosphere samples than in the soil samples. There was no correlation between the frequency of their occurrence and clay and silt content of the soil. Jaśkowska [12,13] examined the occurrence of the *Azospirillum* bacteria in the rhizospheres of 30 different cereal varieties. The amount of *Azospirillum* in the rhizosphere was lower, usually amounting to some cells in 1 g of dry matter of soil. Higher numbers, i.e. from 10^4 to 10^7 cells in 1 g of soil dry matter was found in the soil and rhizosphere of plants in the tropical climate [1,2,5]. This amount was not well correlated with the frequency of their occurrence and the content of mechanical fractions, and carbon and nitrogen in the soil. When soil conditions and agrotechnical soil treatments were alike, the above may be assumed as a criterion for the selection of bacteria in plant rhizosphere.

Among the bacteria isolated for the genus of *Azospirillum*, the most numerous group were the bacteria that belonged to *Azospirillum brasilense*.

In the rhizosphere of barley occurrence of strains of *Azospirillum* sp. was found in 84% and in the rhizosphere of maize and grasses in amount of 100% and 94%, respectively (Table 7). In the rhizosphere of maize, *Azospirillum lipoferum*, *Azospirillum brasilense* and *Azospirillum* sp. in amount of 15%, 62% and 23%, respectively was found. In the rhizosphere of grass taken from Sobieszewo, the presence of *Azospirillum lipoferum*, *Azospirillum brasilense* and *Azospirillum* sp. in amount of 6%, 76% and 4% was determined. The level of *Azospirillum* in the rhizosphere of grass taken from Świnoujście was, respectively, 6%, 76% and 4%.

Halophylic bacteria of the genus *Azospirillum* occurred in about 22% of all samples (Table 7). In the rhizosphere of barley halophylic strains of *Azospirillum* sp. were found in 84% and *Acinetobacter*-like in 16% of all the isolates. In the rhizosphere of maize, the presence of *Azospirillum* sp. and *Acinetobacter*-like was found in 23% and 0%, respectively. The rhizosphere of *Elymus arenarius* taken

Table 7. Arrangement of nitrogen-free fixing bacteria isolated from the monocotyledone plants rhizoplane

Plant	Total number of isolates	*Azospirillum*						*Acinetobacter*-like**	
		lipoferum		*brasilense*		sp*			
		Total	%	Total	%	Total	%	Total	%
Barley	25	0	0	0	0	21	84	4	16
Maize	68	10	15	42	62	16	23	0	0
Elymus arenarius[a)]	32	2	6	24	76	4	12	2	6
Elymus arenarius[b)]	21	2	14	7	33	1	5	10	48
Sum	146	15		73		42		16	

*Growth in medium with 3% NaCl; **Growth in medium with 6% NaCl; according to metods Tarrand *et al.* [27] and tests API, S.A.; [a)] Grass from Sobieszewo; [b)] Grass from Świnoujście.

from Sobieszewo contained the *A.*sp. and *A.*-like in amount of 12 and 6%, respectively. *A.*sp. (5%) and *A.*-like (48%) were found in the rhizosphere of gramineum taken from Świnoujście. Levels of *Azospirillum* in the rhizosphere of the examined plants was low. These bacteria were not found in all the replications. Their amount was usually some cells in 1 g of rhizosphere soil dry matter, only occasionally coming to several thousand.

In the rhizosphere of barley cultivated on rendzinas and black earth developed from slightly loamy sand and in the rhizosphere of grass *Elymus arenarius* - taken from sand of the Baltic sea, halophylic strains of *Azospirillum* sp. were characteristic. They grew on the medium with 3% NaCl, together with the strains which were preliminary determined as *Acinetobacter*-like [18]. They grew and fixed N_2 in a semi-solid medium with 3% NaCl (147 nM C_2H_4 h^{-1} cm^{-3} of gas phase). It also grew in the medium with 6% NaCl. These strains were related to the genus *Pseudomonas stutzeri* [17].

Characteristic bacteria capable for pectin disintegration of the genus *Azospirillum* was found in the rhizosphere of barley cultivated on various soils, and also in the rhizosphere of grasses and maize [16]. The association isolated included 57 bacteria of the genus of *Azospirillum* that grew in the nitrogen-free, semi-solid medium with pectin and produces pectinolitic enzymes. Pectinolitic bacteria of the genus *Azospirillum* occurred in all of the samples, i.e. in the rhizosphere of barley (*Hordeum sativum*) taken from rendzinas developed from limestone and black earth developed from loam and in the rhizosphere of grasses (*Elymus arenarius*) taken from the sandy soil and in the rhizosphere of maize (*Zea mays*) taken from alluvial soil [16]. Probably, the strains of these bacteria were related to the genus *Azospirillum irakense*.

The nitrogenase activity of *Azospirillum* spp. strains isolated from the barley, maize and grasses rhizosphere ranged from 1,6 to 228,5 nM C_2H_4 h^{-1} cm^{-3} of gas phase. The nitrogenase activity of isolated *Acinetobacter*-like strains ranged from 8,4 to 59,4 nM C_2H_4 $h^{-1}cm^{-3}$ of gas phase [15,18].

Comparing the results of acetylene reduction of *Azospirillum lipoferum* with *Azospirillum brasilense* isolated from the maize rhizosphere and *Elymus arenarius* grasses, we could observe that the strains isolated from the maize rhizosphere were several times more numerous. Most the nitrogenase active were found in the two strains of *Azospirillum brasilense* isolated from maize rhizospheres and of halophilic strains growing in the medium with 3% NaCl (respectively: 228.5 and 213 nM C_2H_4 h^{-1} cm^{-3} of gas phase), [18]. Also Jaśkowska [12] observed that highest nitrogenase activity among the bacteria of *Azospirillum brasilense* was from the rye rhizosphere. Most of the authors, however, deals with nitrogenase activity in the rhizosphere of genetically differentiated plants of the same species [10,11]. On the basis of the data obtained, it may not be assumed that there exists too high the nitrogenase activity in soil environment.

CONCLUSIONS

1. The number of bacteria from the genera of *Azospirillum* in the rhizosphere of the examined crop plants is low.
2. No correlation between the frequency of their occurrence and content of available components, humus and exchangeable cations in the soil samples was observed.

REFERENCES

1. **Baldani V.L.D., Döbereiner J.:** Host - plant specificity in infection of cereals with *Azospirillum* sp., Soil Biol. Biochem., 12, 433, 1980.
2. **Blow J.W.F., Döbereiner J.:** Potential for nitrogen fixation in maize genotypes in Brazil. Proc. Nat. Acad. Sci., 72, 2389, 1975.
3. **Day J. M., Döbereiner J.:** Pysiological aspects of N_2-fixation by *Spirillum* from *Diditaria* roots. Soil Biol. Biochem., 8, 45, 1976.
4. **De Coninck K., Horemans S., Randombage S., Vlassak K.:** Occurrence and survival of *Azospirillum* spp. in temperate regions. Plant Soil, 110, 213, 1988.
5. **Döbereiner J., Marriel J. E., Nery M.:** Ecological distribution of *Spirillum lipoferum* Beijerinck. Can. J. Microbiol., 22, 1464, 1976.
6. **Favilli F., Balloni W., Cresta E., Messini A.:** *Azospirillum* spp. ecology of Italian soils. [in] Advances in Nitrogen Fixation Research. (Eds) Veeger C., Newton W.E. Nijhoff and Junk, Pudoc, 339, 1984.
7. **Girard H., Rougieux R.:** Techniques de microbiologic agricole. Dound, Paris 1967.

8. **Haahtela K., Wartiovaara T., Sundman V., Skujins J.:** Root-associated N_2 fixation (acetylene reduction) by *Enterobacter* and *Azospirillum* strains in cold-climate spodosols. Appl. Environ. Microbiol., 41, 203, 1981.
9. **Hegazi N.A., Amer H.A., Monib M.:** Enumeration of N_2 - fixing spirilla, Soil Biol. Biochem., 1, 437, 1979.
10. **Jagnow G.:** Growth and survival of *Azospirillum lipoferum* in soil and rhizosfere as influenced by ecological stress conditions. [In:] *Azospirillum*. Genetics. Physilogy. Ecology, [Ed.] W. Klingmuller, Birkhauser Verlag, Basel-Boston-Stuttgart, Experientia Suppl., 42, 100, 1982.
11. **Jagnow G.:** Nitrogenase activity of sterile cereal sedlings inoculated with *Azospirillum brasilense*: differences between cultivars and possible significance. [In:] *Azospirillum* IV. Genetics, Physiology, Ecology, (Ed. W. Klingmuller) Springer Verlag, Berlin-Heidelberger-New York-Tokyo, 189, 1988.
12. **Jaśkowska H.:** Występowanie i charakterystyka bakterii z rodzaju *Azospirillum* w ryzosferze roślin zbożowych. Praca doktorska, SGGW, Warszawa, 1993.
13. **Jaśkowska H.:** Occurrence of bacteria of the genus *Azospirillum* in rhizosphere of different cereal varieties. Ann. Warsaw Agricul. University, Agriculture, 27, 61, 1994.
14. Kamas K.M., Ageron E., Grimont P.A.D., Kaiser P.: *Azospirillum irakense* sp. nov., a nitrogen-fixing bacterium associated with rice roots and rhizosphere soil, Res. Microbiol., 140, 679, 1989.
15. **Król M.:** Występowanie bakterii z rodzaju *Azospirillum* w ryzosferze traw. Zesz. Nauk. AR Szczecin, Rolnictwo, 68, 181, 133, 1997.
16. **Król M., Perzyński A., Kobus J.:** Hydroliza pektyn przez bakterie z rodzaju *Azospirillum*. Zesz. Nauk. AR Szczec. Rolnictwo, (w druku), 1998.
17. **Król M., Kobus J., Eckert B.:** Characterization biochemical of free-living N_2-fixing bacteria isolated from the endorhizosphere of barley - *Hordeum sativum* and *Elymus arenarius*. Third European Nitrogen Fixation Conference. Septembers 20-24, De Blije Werelt Lunteren. The Netherlands, 183, 1998.
18. **Król M.J., Kobus J.:** Bakterie halofilne wiążące azot wyizolowane z endoryzosfery roślin jednoliściennych. Drobnoustroje w środowisku. Akademia Rolnicza. W. Barabasz. Kraków, 291, 1997.
19. **Kulińska D.:** Occurrence of *Azospirillum* in Polish soils, Acta Microbiol. Pol., 32, 265, 1983.
20. **Magalhaes F.M.N., Baldani J. I., Souto S.M., Kuykenda J.R., Dbereiner J.:** A new acid tolerant *Azospirillum* species, Ann. Acad. Bras. Cien., 55, 417, 1983.
21. **Markus P., Kramer J.:** Importance of nonsymbiotic nitrogen-fixing bacterian organic farming systems. In: *Azospirillum*. Part IV. Genetics. Physiology. Ecology, ed.W. Klingmuller, Springer Verlag, Berlin-Heidelberg-New York Tokyo, 197, 1988.
22. **Michiels K., Vanderleyden J., Van Gool A.:** *Azospirillum* - plant root associations: A review, Biol. Fertil. Soils, 8, 356, 1989.
23. **New P.B., Kennedy I.R.:** Regional distribution and pH sensitivity of *Azospirillum* associated with wheat roots in Eastern Australia, Microb. Ecol., 17, 299, 1989.
24. **Okon Y.:** Advances in agronomy and ecology of the *Azospirillum* / plant association. Nitrogen Fixation: Fundamentals and applications, Kluwer Academic Publishers, Netherlands, s.635, 1995.
25. **Patriguin D.G.:** New developments in grass-bacteria association. In: Advances in agricultural microbiology, ed. N. S. Subba Rao, s.139, 1982.
26. **Reinhold B., Hurek T., Fendrik J., Pot B., Gillis M., Kersters K., Thielemans S., De Ley J.:** *Azospirillum halopraeferans* sp. nov. of Kallar grass (*Leptochloa fusca* L. Kunth), Int. J. Syst. Bacteriol., 37, 43, 1987.
27. **Tarrand J.J., Krieg N.R., Dbereiner J.:** A taxonomic study of the *Spirillum lipoferum* group, with descriptions of a new genus, *Azospirillum gen. nov.* and two species *Azospirillum lipoferum* (Beijerinck) *comb. nov.* and *Azospirillum brasilense* sp. *nov.*, Can. J. Microbiol., 24, 967, 1978.
28. **Tyler M.E., Milam J.R., Smith R.L., Schank S.C., and Zuberer D.A.:** Isolation of *Azospirillum* from diverse geographic regions. Can. J. Microbiol., 25, 693, 1979.