Polish Journal of Microbiology
2006, Vol. 55, No 3, 227–235

Characterization of Selected Groups of Microorganisms Occurring in Soil Rhizosphere and Phyllosphere of Oats

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Received 22 June 2006, revised 17 July 2006, accepted 18 July 2006

A b s t r a c t

Studies were carried out on the microflora of phyllosphere and soil rhizosphere of hulled (Chwat variety) and naked (Akt variety) oats. The material taken for study embraced samples of leaves and soil rhizosphere taken from cultivations differing in extent of nitrogen fertilization. The studies involved determination of total number of aerobic heterotrophic bacteria belonging to the genus *Pseudomonas* and microscopic hyphal fungi. Qualitative determinations focused on bacteria belonging to the genera *Azotobacter* and *Azospirillum* were also made.

Our results point to differences in number of microscopic hyphal fungi in the phyllosphere of both varieties of oats, depending on nitrogen fertilization dose. However, there were no significant differences in the number of bacteria of the different genera determined in the phyllosphere and rhizosphere. Strains of oligonitrophilic and diazotrophic bacteria were isolated from samples of the phyllosphere of oats and their N_2-fixing activity was determined by the acetylene reduction method using gas chromatography.

K e y w o r d s: microflora, nitrogenase, oats, phyllosphere, rhizosphere

Introduction

The surfaces of the above-ground parts of plants are inhabited by various groups of microorganisms (Hirano and Upper, 2000). Only some of these occur in this environments as transients, being deposited on the surface of flowers or leaves with precipitation or carried there by wind or insects. Most of the phyllosphere microorganisms are able to grow and multiply in these conditions. This group is defined as epiphytic microorganisms. The most numerous among them are bacteria, for which the phrase phyllobacteria has been coined (Beattie and Lindow, 1999). The qualitative composition of epiphytic microorganisms depends on many environmental factors, especially at the beginning of spring, in the stage of leaf development. Of equal importance are the conditions that the plant itself ensures (Hirano and Upper 2000; Mercier and Lindow, 2000; Chmiel, 2004).

The role of epiphytic microorganisms has not been fully elucidated. It is known that this group includes both plant pathogens and microorgansims that provide a protective barrier against them. Several species of phyllobacteria have also been found to synthesize plant hormones and have been suggested to play a role in stimulating plant growth (Lindow *et al.*, 1998; Beattie and Lindow, 1999).

The current study focuses on the effect of differentiated nitrogen fertilization on select groups of epiphytic microorganisms as well as those occurring in soil under oats cultivation.

Experimental

Materials and Methods

The studies embraced the microbiological analysis of leaf samples and rhizosphere soil taken from under oats cultivation. The material for the determinations was taken from field experiment set up at the Experimental Station of the Warsaw Agricultural University in Jaktorowo. In this experiment two types of oats were used: hulled Chwat and naked Akt. In each case, differentiated

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mineral nitrogen fertilization was employed: 0, 30, 60, 90 and 120 kg N/ha. The material for the studies was collected in May 2004, two months after sowing the plants.

Quantitative determinations. Total number of heterotrophic bacteria was determined using the method and medium described by Bunt and Rovira (1955). The number of microscopic hyphal fungi was estimated using Martin's medium (Martin**,** 1950) and the total number of bacteria using King B medium (King *et al.*, 1954). Bacteria belonging to the genera A*zotobacter* and *Azospirillum* were enumerated by routine MPN method. In the case of *Azotobacter* cultures were set up in liquid nitrigen-free medium and the presence of the bacteria was determined based on macroscopic observations of individual cultures, followed by *in situ* observations in optical microscope for characteristic shape of the cells, as described by Döbereiner *et al*., (1976). To determine the MPN of bacteria for bacteria belonging to the genus *Azospirillum* a semi-liquid nitrogen-free medium, supplemented with malate (Ebeltagy *et al*., 2001), was used. Initial test for the presence of these bacteria was based on the presence in the culture of a delicate film or layer just under the surface of the medium, such growth being characteristic of microaerophiles, followed by *in situ* observation of preparations from the culture using phase-contrast microscopy for vibroid cells filled with numerous granules of β-hydroxybutyric acid, moving by spiral rotations.

In order to enumerate epiphytic microorganisms oats leaves were placed in 100 ml sterile water and shaken for about 20 minutes. The obtained suspension was diluted in series and plated on selective media. The obtained results were calculated as colony forming units (cfu) per 1 g air dried leaves. To determine the number of microorganisms in rhizosphere soil oats roots were first delicately shaken and then placed in 100 ml sterile water and shaken for about 20 minutes. Consecutive dilutions were plated out on selective media. To estimate the mass of rhizosphere soil the original suspensions were transferred to evaporation vessels and after evaporation of water the soil was dried at 105°C and then weighed. The obtained results were calculated per 1 g dry weight of rhizosphere soil.

Qualitative determinations

Occurrence of bacteria belonging to the genera *Azospirillum* and *Azotobacter* in phyllosphere and rhizosphere soil of oats. Fragments of oats leaves and samples of soil rhizosphere were introduced in semi-liquid nitrogen-free medium with malate (NFb) as described by Döbereiner *et al*., (1976), and liquid azotobacter medium according to Girard and Rougieux (1967) supplemented with glucose. The routine procedures followed were basically as described above**.**

Characteristics of other groups of diazotrophic bacteria in the phyllosphere of oats. The enriched cultures obtained from phyllosphere of oats served as a source for the isolation pf pure cultures of bacteria. Each of them was again checked for ability to grow in the absence of nitrogen compounds. Selected strains of oligotrophic bacteria were examined in detail for their systematic position. The determinations included biochemical traits as described in detail elsewhere (Becking, 1974; Winslow *et al*., 1974; Holmes *et al*., 1987). The obtained data were further corroborated with the use of the biochemical test kits API: 20 E and NE and APILAB computer software for interpretation of the results and consequent identification of bacteria (bioMerieux).

Identification of nitrogenase activity of strains of diazotrophic bacteria. A culture enriched in bacteria from oats phyllosphere in nitrogen-free medium was used as a source of bacteria that were isolated to homogeneity and the obtained strains were tested for their ability to fix N_2. The nitrogenase activity of each of the bacterial strains was determined in cultures in semi-liquid media**:** azotobacter medium with glucose; NFb medium supplemented with maleic acid; modified NFb medium, in which maleic acid was replaced by glucose, mannitol or sucrose.

The method used in the studies was acetylene reduction, expressed as Acetylene Reduction Activity (ARA) (Hardy *et al.*, 1968). Measurements were made with UNICAM gas chromatograph – using flame ionization detector (FID) (temperature: doser 150°C, detector 200°C, column 60°C). The carrier gas was helium. Cultures of bacteria were set up in calibrated bottles containing 4 cm^3 semi-liquid medium and incubated for 2 hours in an atmosphere of 10% acetylene at 28°C (volume of gas phase 7 cm^3). The obtained results were calculated as number of nmoles C_2H_4 formed by a culture in one hour. All experiments were carried out in triplicate.

Statistical elaborations. The results of studies aimed at determining the number in soil and phyllosphere were subjected to statistical analysis using multifactor variance analysis. To compare the means from the studied experimental combinations, Tukey's multiple comparison test, with significance level $\alpha = 0.05$, was used (Blackman and Tukey, 1958). Uniform groups, that is means from experimental combinations between which there are no significant differences, are indicated on the individual figures using the same letters. On the contrary, means with different weights have been designated using different letters. Calculations were made using SAS 9.1 package (SAS Institute Inc., 2004).

Results and Discussion

Microorganisms colonizing the above-ground parts of plants usually occur in high numbers. A 1 cm^2 surface of a leaf may contain 10^5 to 10^7 bacterial cells (O'Brien and Lindow 1989; Hirano and Upper, 2000; Mercier and Lindow, 2000; Lindow and Leveau, 2002; Lindow and Brandl, 2003). These values can also be expressed as the number of bacteria per 1 gram fresh or dry weight of leaves (Brighigna *et al*., 2000). In such cases the number of bacteria per 1 gram fresh mass of leaves ranges from 10^5 to 10^8 colony forming units (cfu). Studies by Yang *et al*. (2001) indicate that generally microbial community structures are similar on different individuals of the same plant species, but unique on different plant species

In our studies on the microflora of oats a relatively low number of phyllobacteria 3.8×10^4 to 4.6×10^5 cfu per 1 g dry weight was found (Figs. 1 and 2). The number of moulds was also low and ranged from 2.1×10 to 6.3×10^2 cfu (Fig. 3). It seems that this may be caused by conditions determined by the plant itself. According to many authors the occurrence of microorganisms on the above-ground parts of plants depends on a number of factors, of which a critical role may be played by the genus and even species of the host plant (Mercier and Lindow, 2000; Lindow and Brandl, 2003; Chmiel, 2004). This is related to the availabil-

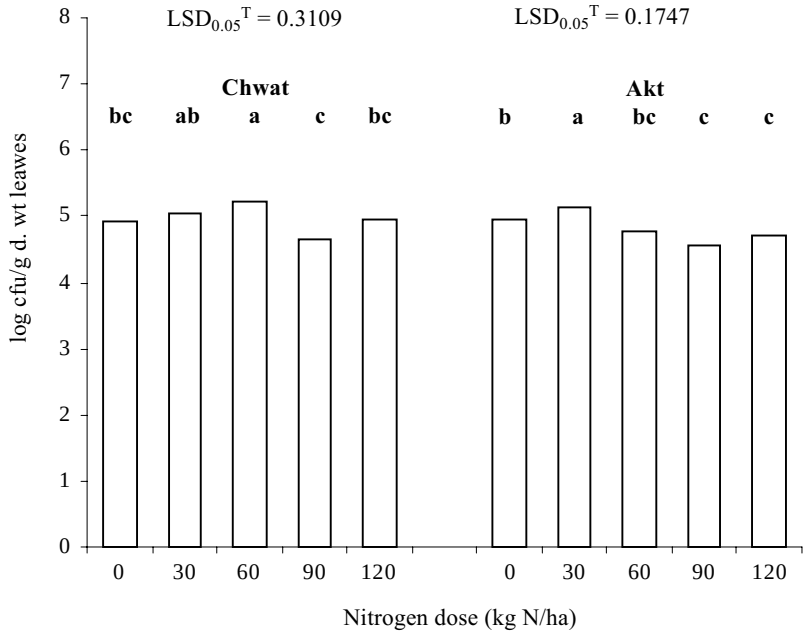

Fig. 1. Effect of nitrogen fertilization on number of heterotrophic bacteria in the phyllosphere of Akt and Chwat varieties of oats, determined in Bunt and Rovira medium

Explanations: LSD – least significant difference. The letters above the bars indicate significantly different means in the various experimental combinations employed

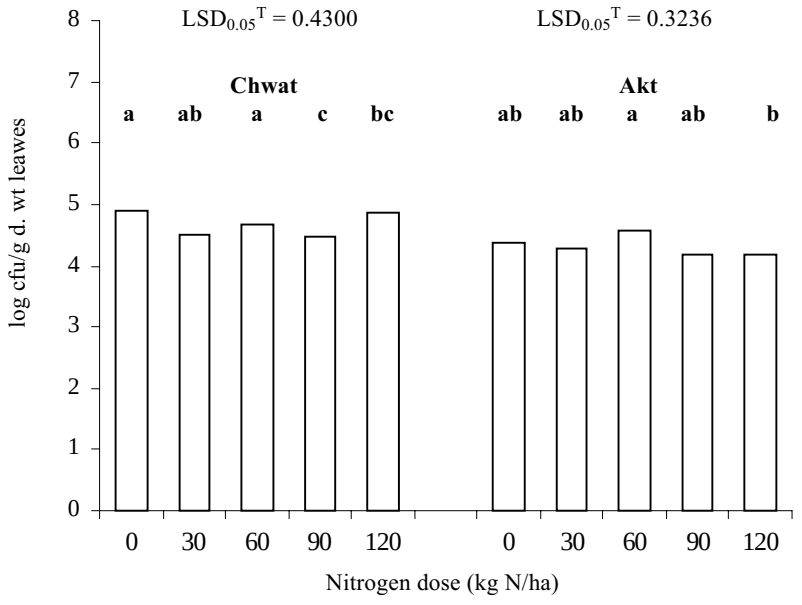

Fig. 2. Effect of nitrogen fertilization on number of bacteria in the phyllosphere of Akt and Chwat varieties of oats, determined in King B medium.

Explanations as to Fig. 1

ity of nutrients, above all saccharides (Wilson and Lindow, 1994a; 1994b; Wilson *et al*., 1995; Mercier and Lindow, 2000). Their low content in peas and cereals may strongly restrict the occurrence of epiphytic microorganisms on the surface of these plants.

It is known that the specificity of various plants as a habitat is also related to their secretions. Chmiel's work (2004) suggests that the presence of certain compounds may directly affect the differentiated numerical force of moulds in the phyllosphere of certain plants. In studies of several years the author found a significantly lower number of microscopic hyphal fungi in the case of cole, oats and corn compared to white and red clover and bulb and root plants. In her opinion this phenomenon is caused not only by limited

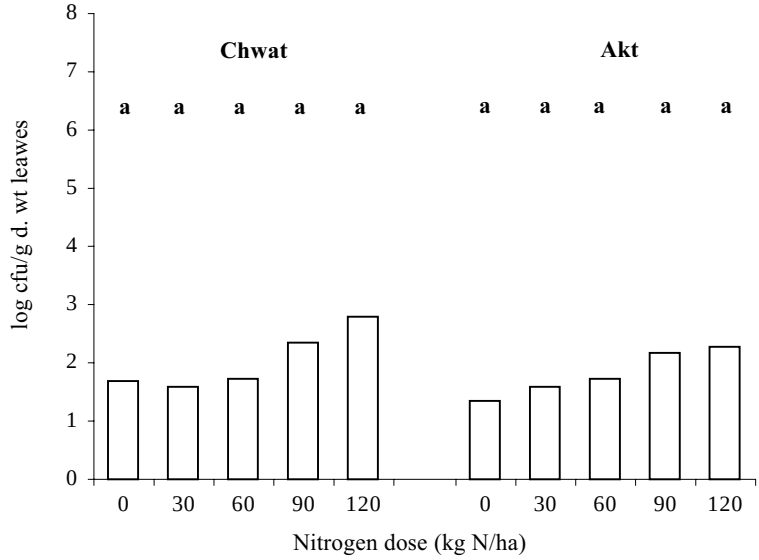

Fig. 3. Effect of nitrogen fertilization on number of microscopic hyphal fungi in the phyllosphere of Akt and Chwat varieties of oats
Explanations as to Fig. 1

nutrient resources in the phylloplane of these plants but also by organic compounds secreted by these plants. These include glycosides of pentacycylic triterpenoids, represented by avenacin A and alkylresorcinols with antifungal activity, which are synthesized by cereals.

In the view of many researchers the inhabiting of the phyllosphere of plants by microorganisms is strongly affected by environmental factors, such as humidity, temperature and chemical pollutants of the environment (*e.g.* Brighigna *et al*., 2000). This has been proven in the case of *Tilandsia caput medusae* and *Tillandsia schiedeana* in quantitative studies of phyllosphere depending on contamination of the environment with heavy metals. The sensitive group of organisms were epiphytic yeasts. Contamination of the environment with heavy metals resulted in their elimination from the phyllosphere of both species of the studied plants.

Surprisingly, there is practically no information in the literature regarding the effect of agrotechnical measures on the epiphytic microflora of cultivated plants. Consequently, this prompted our interest in determining the effect of mineral nitrogen fertilization on the number of microorganisms belonging to select groups inhabiting the phyllosphere of oats and the soil it is grown in. The studies embraced two varieties of oats – Chwat and Akt. Comparative analysis embraced the enumeration of moulds and heterotrophic bacteria, including bacteria belonging to the genus *Pseudomonas*. The results obtained in our studies suggest that the use of high N doses in mineral fertilization may stimulate the colonization of leaves by moulds and at the same time decrease the population size of heterotrophic bacteria. In the case of the Akt variety, an increase in N dose favoured the colonization of the surface of leaves by fungi (Fig. 1). In the case of the Chwat variety, such tendencies were observed in particular with fertilization of 90 and 120 kg N/ha. Statistical analysis did not reveal, however, any significant differences in the population sizes of moulds in the studied experimental combinations. However, it should be pointed out that the number of fungi in the phyllosphere of hulled oats was in general higher than in the case of naked oats. It therefore seems that not only the species but also the variety of a particular plants species can affect the qualitative composition of the population colonizing the leaves. The number of heterotrophic phyllobacteria in the studied oats' combinations showed slight differentiation, but in some cases the differences were statistically significant (Fig. 2). For both oats varieties the lowest number of bacteria was found on leaves from cultivations fertilized with 90 kg N/ha, and in the case of the Akt variety, also at 120 kg N/ha. This indicates that the use of high N doses in mineral fertilization may to a certain degree inhibit the colonization of oats leaves by heterotrophic bacteria.

Determinations for bacteria of the genus *Pseudomonas* in the phyllosphere of both varieties of oats did not demonstrate any significant differences in population size in the studied experimental combinations (Fig. 3). In the case of the variety Chwat a statistically significantly lower number of bacteria were found at fertilization level 90 kg N/ha, and in the case of the variety Akt at doses of both 90 and 120 kg N/ha.

The population size of moulds in the rhizosphere of Akt and Chwat oats was similar in the studied experimental combinations (Fig. 4). This was confirmed by statistical analysis. A slight effect of fertiliza-

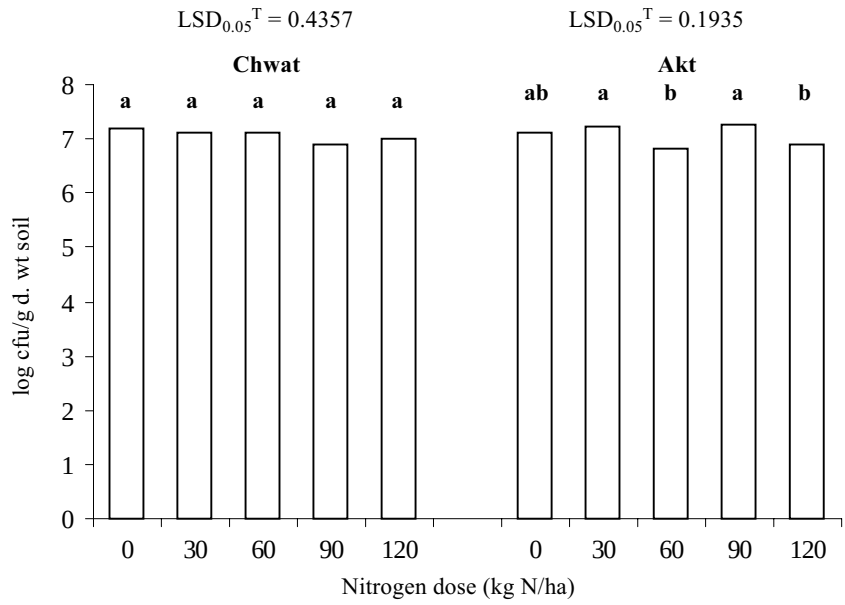

Fig. 4. Effect of nitrogen fertilization on number of heterotrophic bacteria in the rhizosphere of Akt and Chwat varieties of oats, determined in Bunt and Rovira medium

Explanations as to Fig. 1

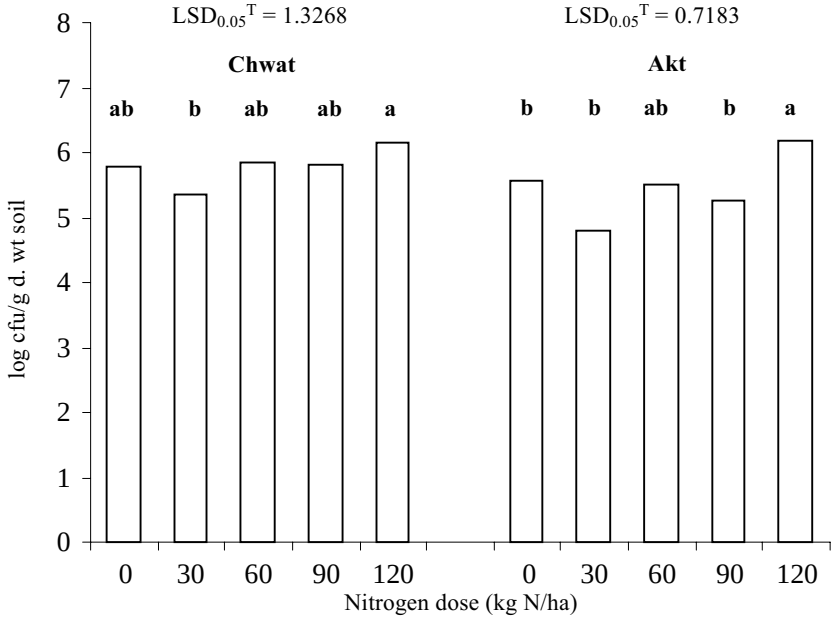

Fig. 5. Effect of nitrogen fertilization on number of bacteria in the rhizosphere of Akt and Chwat varieties of oats, determined in King B medium

Explanations as to Fig. 1

tion with mineral N was observed in the case of the population sizes of heterotrophic bacteria in the rhizosphere of the variety Akt (Fig. 5) and bacteria of the genus *Pseudomonas* (Fig. 6) in the case of both varieties. However, the obtained results do not allow any valid conclusions.

Quantitative determinations for N_2-fixing bacteria of the genera *Azotobacter* and *Azospirillum* were not successful. Plating out the suspension and its dilutions obtained after placing the leaves in sterile water gave negative results. The presence of these bacteria was determined only in the case of some samples when the media were directly inoculated with fragments of leaves. It seems that the number of bacteria of the genera *Azotobacter* and *Azospirillum* in the phyllosphere of oats is very low and it is possible that their presence could be determined only using highly sophisticated techniques, such as rRNA gene analysis, described by

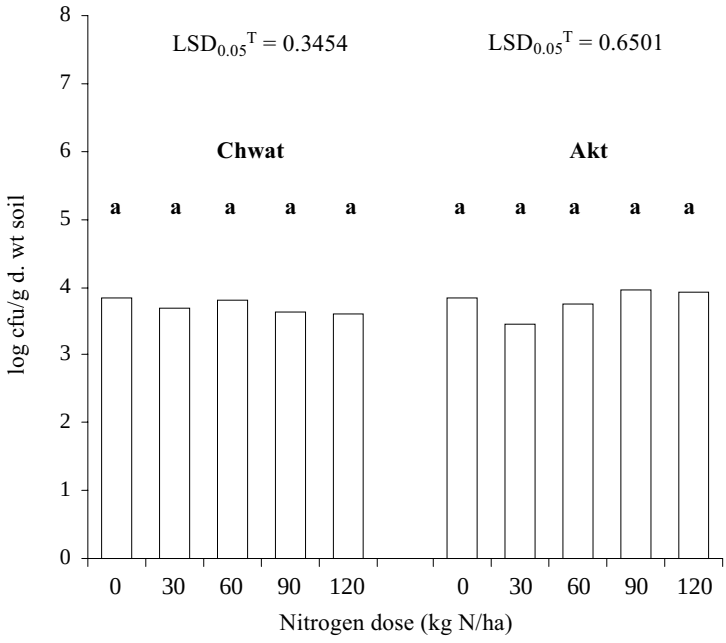

Fig. 6. Effect of nitrogen fertilization on number of microscopic hyphal fungi in the rhizosphere of Akt and Chwat varieties of oats

Explanations as to Fig. 1

Yang *et al*. (2001). The specific location of these bacteria on the surface of the leaves as well as the possibility of their presence in internal leaf tissues can also be considered since some microorganisms are known to colonize not only the surface of a plant but also to penetrate into its interior (Reinhold and Hurek, 1988;

Table I
Occurrence of bacteria belonging to the genera *Azospirillum* and *Azotobacter* in phyllosphere and rhizosphere soil of hulled Chwat variety and naked Akt variety of oats

Oats variety	N dose in mineral fertilization [kg N/ha]	Sample No	Phyllosphere		Rhizosphere	
			Azospirillum sp.	*Azotobacter* sp.	*Azospirillum* sp.	*Azotobacter* sp.
Chwat	0	947	–	–	+	+
	0	928	+	–	–	–
	30	930	+	–	+	+
	30	949	–	–	+	–
	60	927	–	–	+	+
	60	948	+	–	+	+
	90	929	–	–	+	–
	90	950	+	–	+	–
	120	926	–	–	–	+
	120	946	+	–	–	–
Akt	0	936	+	+	–	–
	0	968	–	–	+	–
	30	939	+	+	–	–
	30	969	+	+	–	+
	60	940	–	–	–	+
	60	966	–	–	–	–
	90	937	+	–	+	+
	90	970	+	+	–	+
	120	938	+	+	–	–
	120	967	+	+	–	+

Table II
Nitrogenase activity of strains of bacteria isolated from phyllosphere of oats varieties Chwat and Akt, expressed as acetylene reduction activity (ARA)

Oats variety	Identification of bacterial strain	Concentration of ethylene in cultures of the studied strains (nmol C_2H_4/culture/h)				
		Substrate used in semi-liquid medium				
		Malate	Glucose*	Sucrose	Mannitol	Glucose**
Chwat	927/1 *Agrobacterium radiobacter*	8.6	1.9	7.9	0.0	0.2
Chwat	927/2 *Agrobacterium radiobacter*	0.0	5.8	2.4	0.0	4.6
Chwat	930/1 *Agrobacterium radiobacter*	4.2	3.8	1.3	1.5	0.0
Chwat	950/c *Chryseomonas luteola*	0.0	0.0	19.4	3.8	0.0
Akt	939 *Azotobacter* sp.	20.5	nd	0	30.8	31.2
Akt	940/1 unidentified strain	8.01	3.1	2.2	2.8	10.0
Akt	940/2 *Chryseomonas luteola*	4.47	0	0	0	9.7
Akt	966 *Chryseomonas luteola*	6.2	0	7.4	0	0
Akt	967 *Pseudomonas fluorescens*	5.9	0	0	0	0
Akt	967/5 *Chryseomonas luteola*	15.7	13.1	11.6	11.6	0
Akt	967/12 *Chryseomonas luteola*	32.0	0.0	2.5	11.2	6.5
Akt	968 *Agrobacterium radiobacter*	6.7	2.2	2.5	0.0	3.0
Akt	970 unidentified strain	9.8	3.1	2.9	8.3	2.9
Akt	925 *Agrobacterium radiobacter*	13.2	8.3	5.3	0.0	0.0

* – determination made for culture in semi-liquid NFb medium
** – determination made for culture in semi-liquid azotobacter medium
nd – not determined

Beattie and Lindow, 1999). In general bacteria of the genus *Azospirillum* are associated with the roots of plants and are the most frequent in the rhizosphere and rhizoplane of cereals and grasses (Kulińska, 1983; Jaśkowska, 1995; 1994; Kirchhof *et al*., 1997). In combination with the plant, they form an association N_2-fixing system (Jaśkowska, 1989). Qualitative studies in the direction of N_2-fixing bacteria in the phyllosphere of oats revealed the presence of *Azotobacter* sp. only in the variety Akt (Tab. I). However, most of the studied of leaves of this variety (70 %) were colonized by bacteria belonging to the genus *Azospirillum*. In the case of the variety Chwat the percentage of positive identifications was 50 % of the samples.

The frequency of the occurrence of *Azotobacter* cells in the rhizosphere of oats was relatively low. For both varieties its presence was found in 5 out of 10 examined samples. On the other hand, bacteria of the genus *Azospirillum* were found in 7 samples of rhizosphere from hulled oats, whereas in the case of the variety Akt only two samples were positive for this species.

Enriched cultures set up from the phyllosphere of oats in nitrogen-free medium were used to isolated pure clones of bacteria. A comparison of the population of diazotrophic phyllobacteria on both varieties of oats indicated greater species differentiation in the case of naked oats (Tables I and II). In the case of leaves of the variety Chwat only 4 strains of diazotrophic bacteria were isolated, of which 3 were classified as the species *Agrobacterium radiobacter*, and one as *Chryseomonas luteola*. Ten strains of bacteria were isolated from the surface of leaves of the Akt variety of oats, of which 8 were positively identified. The dominating species among the isolates was *Chryseomonas luteola* (Tab. II). The remaining isolates were species of bacteria occurring on the surface of various plants: *Agrobacterium radiobacter*, *Pseudomonas fluorescens* and *Azotobacter* sp. (Jacques and Morris, 1995; Oehrie *et al*., 2000; Othman *et al*., 2003).

All the species o phyllobacteria isolated from the leaves of oats were shown to be able to fix N_2, but their activity was relatively low (Tab. II) as indicated from a comparison with the results obtained by other authors. Jaśkowska (1995) studied the nitrogenase activity of strains of *Azospirillum lipoferum* and *A. brasilense* isolated from the rhizoplane of barley, wheat and corn, and determined the reduction of acetylene to ethylene in the range from 6.0 to 860 nmoles C_2H_4/culture/h. According to Perzyński and Król (2001) active strains reduce in excess of 100 nmoles acetylene. In studies on the epiphytic microflora of oats the highest nitrogenase activity was found in *Chryseomonas luteola* culture in NFb medium supplemented with maleic acid (32 nmoles C_2H_4/culture/h). The remaining strains of the species demonstrated considerably

lower ARA activity (3.78–19.36 nmoles C_2H_4/culture/h). The ability of *C. luteola* to fix atmospheric nitrogen has been described earlier by Hai-Lian *et al*. (1999). In this case the strain was an endophytic one, isolated from rice plant.

In *Azotobacter* cultures with glucose and mannitol the activity of nitrogenase was similar, being 31.2 and 30.8 nmoles C_2H_4/culture/h, respectively. The same strain in NFb with maleic acic reduced 20.5 nmoles C_2H_4/culture/h. It can be seen that the studied strains of diazotrophic bacteria showed varied nitrogenase activity, depending on the carbon source introduced into the medium. An unidentified strain designated (940/1), isolated from Akt oats leaves reduced acetylene in cultures with each of the substrates employed. It showed highest activity in azotobacter medium with glucose (10.0 nmoles C_2H_4/culture/h). *Pseudomonas fluorescens*, on the other hand, demonstrated activity only in NFb medium with malate.

Overall, our results point to differences in number of microscopic hyphal fungi in the phyllosphere of both varieties of oats, depending on nitrogen fertilization dose. However, we were not able to detect any significant differences in the number of bacteria of the different genera determined in the phyllosphere and rhizosphere.

Acknowledgements. The authors wish to thank prof. Zdzisław Wyszyński for kindly providing study material from an experiment conducted by the Department of Agronomy at the Field Station of the Warsaw Agricultural Academy in Jaktorowo and Dariusz Mańkowski for assisting with the statistical elaboration of the results.

Literature

Beattie G.A. and S.E. Lindow. 1999. Bacterial colonization of leaves: a spectrum of strategies. *Phytopathology* **89**: 353–359.

Becking J.H. 1974. Family I. *Azotobacteriaceae*, p. 254. In: R.E Buchanan and N.E Gibbons (eds) Bergey's Manual of Systematic Bacteriology, 8th ed. The Williams and Wilkins Co, Baltimore.

Blackman R.B. and J.W. Tukey. 1958. The Measurement of Power Spectra From the Point of View of Communication Engineering, 190 pp., Dover Publications, New York.

Brighigna L., A. Gori, S. Gonnelli and F. Favilli. 2000. The influence of air pollution on the phyllosphere microflora composition of *Tillandsia leaves* (*Bromeliaceae*). *Rev. Biol. Trop*. **48**: 511–517.

Bunt J.S. and A.D. Rovira. 1955. Microbiological studies of some subantarctic soils. *J. Soil Sci.* **6**: 119–128.

Chmiel M.J. 2004. Saprophytic fungi in the phyllosphere of cultivated plants – a qualitative analysis. (in Polish) *Acta Agraria et Silvestria* **42**: 40–48.

Döbereiner J., J.E. Marriel and M. Nery. 1976. Ecological distribution of *Spirillum lipoferum* Beijerinck. *Can. J. Microbiol*. **22**: 1464 –1473.

Elbeltagy A.K., Nishioka, T. Sato, H. Suzuki, B. Ye, T. Hamada, T. Isawa, H. Mitsui and T. Minamisawa. 2001. Endophytic colonization and in planta nitrogen fixation by a *Herbaspirillum* sp. isolated from wild rice species. *Appl. Environ. Microbiol*. **67**: 5285–5293.

Girard H. and R. Rougieux. 1967. Techniques in Agricultural Microbiology. Dunod, Paris.

Hai-Lian Y., S. Xiao-Lu, S. Wei, W. Yun-Shan and M. Miao-Ying. 1999. Screening, identification and distribution of endophytic associative diazotrophs isolated from rice plant. *Acta Bot. Sin.* **41**: 927–931.

Hardy R.W.F., R.D. Holsten, E.K. Jackson and R.C. Burns. 1968. The acetylene-ethylene assay for N_2 fixation: laboratory and field evaluation. *Plant Physiol.* **43**: 1185–1207.

Hirano S.S. and C.D. Upper. 2000. Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae* – a pathogen, ice nucleus, and epiphyte. *Microbiol. Mol. Biol. Rev*. **64**: 624–653.

Holmes B., A.G. Steigerwalt, R.E. Weaver and D.J. Brenner. 1987. *Chryseomonas luteola* comb. nov. *Flavimonas oryzihabitans* gen. nov. comb. nov. *Pseudomonas*-like species from human clinical specimens and formerly know, respectively, as groups Ve-1 and Ve-2. *Int. J. Syst. Bacteriol*. **37**: 245–250.

Jacques M.A. and C.E. Morris. 1995. A review of issues related to the quantification of bacteria from the phyllosphere. *FEMS Microbiol. Ecol.* **18**: 1–14.

Jaśkowska H. 1989. *Azospirillum* associative N_2-fixing bacteria (in Polish). *Post. Mikrobiol*. **28**: 77–91.

Jaśkowska H. 1994. Occurrence of bacteria of the genus *Azospirillum* in rhizosphere of different cereal varieties. *Ann. of Warsaw Agricultural Univ*. **27**: 61–65.

Jaśkowska H. 1995. Occurrence and characteristic of *Azospirillum* spp. in the rhizosphere of cereals. *Acta Microbiol. Pol.* **44**: 69–77.

King E.O., M.K. Ward and D.E. Raney. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. *J. Lab. Clin. Med.* **44**: 301–307.

Kirchhof G., V.M. Reis, J.I. Baldani, B. Eckert, J. Dőbereiner and A. Hartmann. 1997. Occurrence, physiological and molecular analysis of entophytic diazotrophic bacteria in gramineous energy plants. *Plant Soil* **194**: 45–55.

Kulińska D. 1983. Occurrence of *Azospirillum* in Polish soils. *Acta Microbiol. Pol.* **44**: 69–77.

Lindow S.E. and M.T. Brandl. 2003. Microbiology of the phyllosphere. *Appl. Environ. Microbiol*. **69**: 1875–1883.

Lindow S.E. and J.H. Leveau. 2002. Phyllosphere microbiology. *Curr. Opin. Biotechnol*. **13**: 238–243.

Lindow S.E., C. Desurmont, R. Elkins, G. McGourty, E. Clark and M.T. Brandl. 1998. Occurrence of indole-3-acetic acid-producing bacteria on pear trees and their association with fruit russet. *Phytopathology* **88**: 1149–1157.

Martin J.P. 1950. Use of acid, rose bengal and streptomycin in the plane methods for estimating soil fungi. *Soil Sci.* **69**: 215–32.
Mercier J. and S.E. Lindow. 2000. Role of leaf surface sugars in colonization of plants by bacterial epiphytes. *Appl. Environ. Microbiol*. **66**: 369–374.
O'Brien R.D. and S.E. Lindow. 1989. Effect of plant species and environmental conditions on epiphytic population sizes of *Pseudomonas syringae* and other bacteria. *Phytopathology* **79**: 619–627.
Oehrie N.W., D.B. Karr, R.J. Kremer and D.W. Emerich. 2000. Enhanced attachment of *Bradyrhizobium japonicum* to soybean through reduced root colonization of internally seedborne microorganisms. *Can. J. Microbiol*. **46**: 600–606.
Othman A.A., W.A. Amer, M. Fayez, M. Monib and N.A. Hegazi. 2003. Biodiversity of diazotrophs associated to the plant cover of north Sinai desert. *Arch. Agron. Soil Sci*. **49**: 683–705.
Perzyński A. and M. Król. 2001. Influence of different carbon on nitrogenase activity of *Azospirillum* spp. strains. In: H. Dahm, A. Pokojska-Budziej (ed.). The Microorganisms of Soil Habitat – Physiological, Biochemical and Genetic Aspects. (in Polish). Marszałek,Toruń: p. 223–228.
Reinhold B. and T. Hurek. 1988. Location of diazotrophs in the root interior with special attention to the kallar grass association. *Plant Soil* **110**: 259–268.
SAS Institute Inc., 2004. SAS 9.1 Companion for windows SAS Publishing, SAS Institute INC, Cary, North Carolina, USA.
Wilson M., M.A. Savka, I. Hwang, S.K. Farrand and S.E. Lindow. 1995. Altered epiphytic colonization of mannityl opine-producing transgenic tobacco plants by a mannityl opine-catabolizing strain of *Pseudomonas syringae*. *Appl. Environ. Microbiol*. **61**: 2151–2158.
Wilson M. and S.E. Lindow. 1994a. Coexistence among epiphytic bacterial populations mediated through nutritional resource partitioning. *Appl. Environ. Microbiol*. **60**: 4468–4477.
Wilson M. and S.E. Lindow. 1994b. Ecological similarity and coexistence of epiphytic ice-nucleating (Ice) *Pseudomonas syringae* strains and a non-ice-nycleotin (Ice2) biological control agent. *Appl. Environ. Microbiol*. **60**: 3128–3137.
Winslow C., J. Broadhurst, R.E. Buchanan, Ch. Krumwiede, L.A. Rogers and G. Smith. 1974. Family I. *Pseudomonadaceae*, p. 217. In: R.E Buchanan and N.E Gibbons (eds) Bergey's Manual of Systematic Bacteriology, 8th ed. The Williams and Wilkins Co, Baltimore.
Yang C.-H., D.E. Crowley, J. Borneman and N. Keen. 2001. Microbial phyllosphere populations are more complex than previously realized. *Proc. Natl. Acad. Sci. USA* **98**: 3889–3894.