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Original Research

Occurrence, Characteristics, and Genetic Diversity of *Azotobacter chroococcum* in Various Soils of Southern Poland

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Abstract

The presence of *Azotobacter* sp. in soils has beneficial effects on plants, but the abundance of these bacteria is related to many factors, especially soil pH and fertility. The presented study evaluated the abundance of *Azotobacter* sp. in various soils of southern Poland and confirmed the relationship between soil properties and the presence of these bacteria. Diagnostic tests indicate that all isolates belonged to *A. chroococcum* species. The studied bacteria were present in 43% of soil samples. Restriction analysis of the bacterial Internal Transcribed Spacer region indicate that the selected isolates were identical. Two fingerprinting methods, PCR Melting Profile and Random Analysis of Polymorphic DNA, revealed high population diversity. Four (PCR MP) and five (RAPD) congruent clusters were defined. No correlation was found between the sources of strain isolation and their positions in clusters. PCR MP and RAPD techniques appeared to be useful in intraspecies differentiation of *A. chroococcum*.

Keywords: *Azotobacter chroococcum*, soil, southern Poland, genetic diversity, fingerprinting

Introduction

It has been proved that inoculation with free-living aerobic bacteria *Azotobacter* spp. causes beneficial effects for the growth of many plant species [1-3]. It is mostly related to their nitrogen fixation ability as well as to production of plant growth promotion and fungicidal substances, vitamins, and siderophores. Therefore, *Azotobacter* spp. is often regarded as a member of "plant growth-promoting rhizobacteria (PGPR)" [4-6]. The genus *Azotobacter* includes 6 species, among which *A. chroococcum* is the most abundant in Polish soils [7]. The presence of these bacteria in soils is correlated with soil pH and fertility. *Azotobacter* spp. occurs most frequently in neutral and slightly alkaline soils, whereas in acidic soils they are absent or occur in very low numbers [7, 8].

One of the objectives of the present study was to evaluate the abundance of *Azotobacter* spp. in various soils of Southern Poland, including forests, urban lawns, parks, fallow lands, field crops, gardens, and industrial areas. Evaluation of the predominant species of isolated strains of the genus *Azotobacter* was done in accordance with Bergey's Manual of Systematic Bacteriology [8]. These analyses indicated that all isolates belonged to *A. chroococcum* species. Additionally, all strains were inoculated on *Azotobacter chroococcum* agar. Restriction analysis of the bacterial Internal Transcribed Spacer (ITS) region was used to confirm the systematic position of the isolates. An additional point of this study was to evaluate the significance of the relationship between *A. chroococcum* abundance in the

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analyzed soils and some soil properties, e.g. pH, total N, and organic C content, soil type, and soil use. Two fingerprinting methods – PCR Melting Profiles (PCR MP) [9] and Pandom Amplification of Polymorphic DNA (RAPD) [10] – were applied to assess the genetic diversity of the isolated strains. The latter two methods have strong discriminatory power [11-13] and can be applied at low diversity levels e.g. in epidemiological studies of hospital infections in a short period of time.

Experimental Procedures

Sampling Strategy and Analysis of Samples

This study included 7 types of soil use: forests, urban lawns, parks, fallow lands, field crops, gardens, and industrial areas. A total of 100 soil samples were collected in spring and autumn 2008 from Lesser Poland and Silesian Voivodeships in southern Poland. Sampling and further storage of samples was carried out according to international standards [14]: the collected soil samples (30 cm deep), weighing up to one kilogram were transported to the laboratory in sterile containers. Immediately after sampling, the soils were analyzed for *Azotobacter* spp. abundance and then stored in a refrigerator at 4ºC [15] for further physico-chemical analyses. Soil pH was determined in soil and water suspension in a 1:2 ratio [15], total nitrogen concentration using Kiejdahl's method [16] and organic carbon content – using Tiurin's method modified by Oleksynowa [17] were analyzed. Soil dry weight was determined by the weight loss of 10 g samples after drying in an oven at 105ºC for 24 h [18]. Locations of the sampling sites with soil type, soil use, pH, total nitrogen, and organic carbon content of all 100 samples are summarized in Table 1.

Determination of *Azotobacter chroococcum* Abundance and Isolation of Strains

The abundance of *Azotobacter* spp. bacteria in soil samples was determined by the serial soil dilutions technique [19] on plates containing Ashby's medium [20]. Ten grams of soil from each sample was drawn and serially diluted aseptically to 10^{-1} and 10^{-2}. Then 1 cm^3 of each sample was pipetted aseptically onto a sterile Petri dish containing 15-20 cm^3 of Ashby's medium [15]. This was repeated three times to give three replicates. After 72 h of incubation at 28ºC, numbers of colony forming units (CFU) were calculated. Numbers of CFU on the three replicate Petri dishes for each soil sample were then standardized to CFU per gram of soil.

Taxonomic Identification

Taxonomic identification was done according to Bergey's Manual of Systematic Bacteriology [8] based on macroscopic and microscopic (Gram-stained smears) observations. The obtained strains were characterized by their ability to use rhamnose, caproate, caprylate, meso-inositol, mannitol, starch, glutarate, and glycolate as carbon sources. Subsequently, all isolates were inoculated on *Azotobacter chroococcum* agar (agar, 20.0 g; $CaCO_3$, 20.0 g; glucose, 20.0 g; K_2HPO_4, 0.8 g; $MgSO_4 \times 7H_2O$, 0.5 g; KH_2PO_4, 0.2 g; $FeCl_3 \times 6H_2O$ 0.1 g; and $Na_2MoO_4 \times 2H_2O$, 0.05 g in 1000 cm^3 of distilled water) [21] to confirm their taxonomic identification.

DNA Isolation

Before isolation the bacteria were cultured in liquid LB (lysogeny broth) medium [21] for 24 h at 28ºC. After this time 0.5 cm^3 of each culture was centrifuged and rinsed with 100 µl Tris solution (10 mM Tris-HCl, pH 8.5). Bacterial DNA was isolated using an isolation kit for bacterial genomic DNA (Genomic Mini – A&A Biotechnology) according to manufacturer's instructions.

Restriction Analysis of the ITS Region

PCR amplification of the rDNA ITS region was performed according to the procedure proposed by Jensen et al. [22]. Two primers, G1 (5'-GAAGTCGTAACAAGG-3') and L1 (5'-CAAGGCATCCACCGT-3'), were used for amplification of the rDNA 16S-23S spacer. Each PCR reaction was carried out in 25 µl volume containing 1 µl (20 ng) of genomic DNA, 2.5 µl PCR buffer 10× (Fermentas), 25 mM dNTP mix, 1 µl of each primer (10 mM), 2 µl $MgCl_2$ (25 mM), and 0.5 µl *PwoHyp* polymerase [2 $U \times \mu l^{-1}$], with the following temperature profile: initial denaturation at 94ºC for 5 min, 25 amplification cycles of denaturation at 94ºC for 60s, annealing at 55ºC for 60s and elongation at 72ºC for 60s, and final elongation at 72ºC for 5 min. The PCR products (6 µl) were electrophorezed in 1% agarose gel (ethidium bromide stained up to 0.5 $mg \times cm^{-3}$ concentration) in 1×TAE buffer. After electrophoresis for 30 min at 7 $V \times cm^{-1}$ the gel was analyzed with UV illumination (VersaDoc Imaging System v. 1000) and Quantity One software (Bio-Rad Laboratories Inc.). The PCR products were analyzed by *HindIII* (Fermentas) digestion according to the endonuclease manufacturer's instructions. The RFLP profile was analyzed by electrophoresis through 1% agarose gel as described above.

PCR Melting Profiles

The PCR Melting Profiles (PCR MP) method was applied according to the modified procedure proposed by Krawczyk et al. [23]. The analysis consists of two steps. First, the initial temperature optimization experiment (with genomic DNA of 1 strain and 12 different denaturation temperatures) was performed to determine the denaturation temperature. After that, the analysis of all 43 strains was carried out using the denaturation temperature adjusted in step one.

Table 1. Characteristics of the sampling sites.

No.	Location	Coordinates	Soil use	pH in H_2O	Total N [g×kg^{-1}]	Soil type	Org. C [%]	CFU×g^{-1} (isolate No.)
1	Poręba	N50º28’36” E19º20’15”	forest	6.2	1.648	Rendzina	2.257	5 (1)
2	Łącko	N49º33’57” E20º26’32”	forest	7.5	3.239	Luvisol	3.931	38 (2)
3	Żakowiec	N49º59’57” E19º41’4”	forest	7.0	0.795	Podsol	3.978	0
4	Zagacie	N50º0’8” E19º41’33”	forest	5.8	0.276	Podsol	1.110	0
5	Dąbrowa Szlachecka	N50º0’5” E19º43’36”	forest	4.8	0.473	Podsol	3.209	0
6	Rodaki	N50º24’11” E19º32’24”	forest	6.3	0.057	Rendzina	0.601	0
7	Zawiercie	N50º30’13” E19º26’30”	forest	5.2	1.465	Podsol	0.866	0
8	Laskowa	N49º45’8” E20º26’17”	forest	6.0	1.686	Cambisol	1.508	0
9	Parkoszowice	N50º31’48” E19º28’25”	forest	6.1	1.570	Podsol	2.669	0
10	Morsko	N50º32’59” E19º30’30”	forest	5.2	0.615	Podsol	1.589	0
11	Rudniki	N50º31’8” E19º26’37”	forest	5.5	0.565	Rendzina	0.939	0
12	Czarny Potok	N49º34’30” E20º28’15”	forest	4.4	1.444	Cambisol	1.554	0
13	Wieniec	N49º54’46” E20º18’7”	forest	4.0	0.682	Luvisol	0.402	0
14	Wielmoża	N50º15’37” E19º49’58”	forest	4.4	1.428	Cambisol	1.390	0
15	Czernichów	N49º59’12” E19º41’17”	field crop	9.7	1.314	Fluvisol	2.705	7 (3)
16	Wielmoża	N50º15’40” E19º48’58”	field crop	7.5	2.616	Cambisol	5.166	47 (4)
17	Wołowice – corn	N49º59’12” E19º43’12”	field crop	8.1	0.339	Fluvisol	1.050	0
18	Wołowice – wheat	N49º59’6” E19º43’10”	field crop	7.1	0.231	Fluvisol	1.613	0
19	Rusocice	N49º59’2” E19º36’53”	field crop	6.5	0.483	Fluvisol	0.688	0
20	Łazy Biegonickie	N49º34’0” E20º40’54”	field crop	7.5	1.001	Luvisol	1.317	0
21	Wędzoce	N49º59’11” E19º43’20”	field crop	4.2	0.156	Fluvisol	0.784	0
22	Pichonówka	N49º59’17” E19º43’16”	field crop	4.4	0.913	Fluvisol	0.916	0
23	Korzkiew	N50º9’43” E19º52’41”	field crop	7.3	0.620	Cambisol	2.863	0
24	Park Krakowski	N50º4’0” E19º55’26”	park	6.5	1.268	Anthrosol	2.393	3 (5)
25	Park Jordana	N50º3’38” E19º55’4”	park	6.0	1.338	Anthrosol	2.111	6 (6)
26	Planty	N50º3’22” E19º56’6”	park	5.5	1.001	Anthrosol	1.266	7 (7)
27	Strzelecki Garden	N50º3’55” E19º56’59”	park	7.2	1.002	Anthrosol	2.273	14 (8)
28	Solvay Park	N50º1’3” E19º55’43”	park	6.2	0.493	Anthrosol	1.213	0
29	Park Bednarskiego	N50º2’28” E19º56’55”	park	6.9	0.268	Anthrosol	1.063	0
30	Bielany	N50º3’3” E19º52’56”	urban lawn	8.1	2.874	Luvisol	2.669	37 (9)
31	Kr – Mickiewicza Avenue	N50º3’54” E19º55’24”	urban lawn	8.0	2.511	Anthrosol	2.760	92 (10)
32	Kr – Oleandry Street	N50º3’36” E19º55’17”	urban lawn	8.2	3.591	Anthrosol	4.374	112 (11)
33	Kr – 29th November Street	N50º5’0” E19º57’8”	urban lawn	7.0	1.741	Anthrosol	2.273	44 (12)
34	Kr – Bujwida Street	N50º3’41” E19º57’5”	urban lawn	7.5	2.745	Anthrosol	2.909	61 (13)
35	Kr – Ingardena Street	N50º3’43” E19º55’13”	urban lawn	7.0	1.611	Anthrosol	2.305	11 (14)
36	Kr – Odrzańska Street	N50º1’36” E19º55’42”	urban lawn	6.0	1.729	Fluvisol	1.756	12 (15)
37	Kr – Reymonta Street	N50º4’3” E19º54’15”	urban lawn	6.8	1.481	Anthrosol	1.983	23 (16)
38	Kr – Piastowska Street	N50º3’48” E19º54’10”	urban lawn	6.8	1.433	Fluvisol	2.236	24 (17)
39	Kr – Balicka Street	N50º4’50” E19º51’58”	urban lawn	7.4	1.001	Fluvisol	1.082	75 (18)
40	Kr – Armii Krajowej Street	N50º4’36” E19º53’21”	urban lawn	6.6	0.203	Anthrosol	1.155	3 (19)
41	Kr – Wielicka Street	N50º1’40” E19º58’34”	urban lawn	6.8	1.369	Anthrosol	2.801	22 (20)

Table 1. Continued.

No.	Location	Coordinates	Soil use	pH in H_2O	Total N [g×kg^{-1}]	Soil type	Org. C [%]	CFU×g^{-1} (isolate No.)
42	Kr – Podgórska Street	N50º3’5” E19º57’19”	urban lawn	7.2	2.179	Anthrosol	3.454	21 (21)
43	Kr – Malborska Street	N50º1’33” E19º58’26”	urban lawn	7.0	1.053	Anthrosol	1.158	3 (22)
44	Kr – Ofiar Katynia Roundabout	N50º5’15” E19º53’33”	urban lawn	7.0	1.150	Cambisol	4.172	49 (23)
45	Kr – Lindego Street	N50º4’49” E19º52’13”	urban lawn	7.0	1.121	Anthrosol	1.210	4 (24)
46	Kr – Wiślicka Street	N50º5’16” E20º0’2”	urban lawn	6.4	0.770	Anthrosol	1.185	1 (25)
47	Kr – Stella-Sawickiego Street	N50º4’28” E20º0’15”	urban lawn	6.0	1.242	Anthrosol	1.726	2 (26)
48	Kr – Bociana Street	N50º5’42” E19º57’19”	urban lawn	6.9	0.826	Anthrosol	1.194	10 (27)
49	Kr – Mackiewicza Street	N50º5’24” E19º56’37”	urban lawn	6.7	0.843	Anthrosol	3.445	28 (28)
50	Kr – Kluczborska Street	N50º5’11” E19º56’16”	urban lawn	6.3	0.892	Anthrosol	1.711	2 (29)
51	Kr – 3rd May Street	N50º3’34” E19º55’21”	urban lawn	8.9	0.337	Fluvisol	0.997	0
52	Kr – Ugorek Street	N50º4’32” E19º58’48”	urban lawn	7.7	0.323	Anthrosol	0.725	0
53	Kr – Borsucza Street	N50º1’39” E19º55’40”	urban lawn	7.0	0.258	Fluvisol	1.497	0
54	Kr – Widok Street	N50º3’32” E19º58’52”	urban lawn	6.8	0.320	Anthrosol	2.072	0
55	Kr – Podwawelskie Estate	N50º2’29” E19º55’27”	urban lawn	7.0	0.535	Fluvisol	2.680	0
56	Kr – Kijowska Street	N50º4’6” E19º54’48”	urban lawn	6.5	0.701	Anthrosol	1.658	0
57	Kr – Podchorążych Street	N50º4’30” E19º54’23”	urban lawn	7.0	0.385	Anthrosol	3.577	0
58	Kr – Chełmońskiego Street	N50º5’17” E19º54’37”	urban lawn	7.7	0.702	Anthrosol	0.758	0
59	Kr – Halszki Street	N50º0’48” E19º57’0”	urban lawn	7.0	0.496	Anthrosol	1.302	0
60	Kr – Opolska Street	N50º5’27” E19º55’11”	urban lawn	5.5	0.094	Anthrosol	0.930	0
61	Kr – Ojcowska Street	N50º5’27” E19º52’54”	urban lawn	6.6	0.512	Cambisol	0.455	0
62	Kr – Mydlniki	N50º5’0” E19º50’41”	urban lawn	6.4	0.336	Anthrosol	1.831	0
63	Kr – Montelupich Street	N50º4’30” E19º56’21”	urban lawn	7.1	0.451	Anthrosol	0.973	0
64	Kr – Słowackiego Avenue	N50º4’24” E19º56’1”	urban lawn	5.0	0.307	Anthrosol	3.435	0
65	Czeladź	N50º19’9” E19º6’8”	urban lawn	6.0	0.129	Rendzina	1.422	0
66	Kr – Kadrówki Street	N50º3’37” E19º55’21”	urban lawn	7.9	1.469	Anthrosol	2.289	0
67	Kr – Fabryczna Street	N50º3’35” E19º58’15”	industrial area	7.3	1.677	Anthrosol	2.181	17 (30)
68	Kr – Czyżyńskie Roundabout	N50º4’21” E20º1’12”	industrial area	6.6	1.327	Anthrosol	1.932	8 (31)
69	Kr – Plac Centralny	N50º4’16” E20º2’12”	industrial area	7.1	1.940	Anthrosol	3.849	35 (32)
70	Kr – Makuszyńskiego Street	N50º5’18” E20º2’49”	industrial area	7.3	2.939	Anthrosol	4.318	83 (33)
71	Kr – Teatralne Estate	N50º4’44” E20º1’46”	industrial area	6.8	1.441	Anthrosol	2.217	22 (34)
72	Kr – Pleszów Estate	N50º4’23” E20º7’2”	industrial area	6.1	0.800	Anthrosol	1.119	19 (35)
73	Kr – Wzgórza Krzesławickie Estate	N50º5’56” E20º5’28”	industrial area	7.3	2.179	Anthrosol	2.516	28 (36)
74	Kr – Mittal Steel Nowa Huta	N50º4’46” E20º3’53”	industrial area	7.3	1.635	Anthrosol	1.751	8 (37)
75	Kr – Mogila Fort	N50º3’52” E20º4’54”	industrial area	6.4	0.369	Anthrosol	3.190	0
76	Kr – Igołomska Street	N50º4’3” E20º4’9”	industrial area	7.1	0.541	Anthrosol	1.975	0
77	Kr – Szkolne Estate	N50º4’30” E20º2’48”	industrial area	6.5	0.308	Anthrosol	2.733	0
78	Herby	N50º45’2” E18º53’11”	industrial area	6.2	1.428	Podsol	4.033	0
79	Kr – Borsucza Street	N50º1’47” E19º55’41”	garden	8.0	2.992	Fluvisol	4.528	19 (38)
80	Zielonki	N50º7’10” E19º55’9”	garden	8.9	1.274	Cambisol	2.668	73 (39)

Table 1. Continued.

No.	Location	Coordinates	Soil use	pH in H_2O	Total N [g×kg^{-1}]	Soil type	Org. C [%]	CFU×g^{-1} (isolate No.)
81	Olszanica	N50º4'3" E19º50'0"	garden	8.9	1.543	Luvisol	2.602	0
82	Czernichów	N49º59'17" E19º40'29"	garden	7.9	0.154	Fluvisol	1.659	0
83	Ptaszkowa	N49º36'3" E20º53'28"	garden	6.5	0.460	Cambisol	2.281	0
84	Kr – Blonia	N50º3'33" E19º55'0"	fallow land	7.0	2.129	Fluvisol	1.881	8 (40)
85	Kr – Chrobrego Street	N50º4'31" E19º57'40"	fallow land	7.2	3.352	Anthrosol	4.786	33 (41)
86	Kr – Nowosądecka Street	N50º1'26" E19º58'43"	fallow land	6.9	1.418	Rendzina	1.941	4 (42)
87	Siewierz	N50º27'52" E19º13'45"	fallow land	6.3	1.744	Podsol	1.405	3 (43)
88	Wieniec	N49º54'44" E20º17'51"	fallow land	4.4	0.029	Luvisol	1.498	0
89	Czernichów	N49º59'12" E19º40'26"	fallow land	8.1	0.288	Fluvisol	3.641	0
90	Kr – Bobrzyńskiego Street	N50º1'36" E19º54'18"	fallow land	6.0	0.446	Anthrosol	3.514	0
91	Kr – Bułhaka Street	N50º2'52" E19º55'50"	fallow land	6.9	0.726	Anthrosol	4.443	0
92	Łazy	N50º26'4" E19º24'16"	fallow land	7.4	0.455	Rendzina	2.873	0
93	Włodowice	N50º33'17" E19º26'54"	fallow land	7.0	0,567	Podsol	0.850	0
94	Parkoszowice	N50º32'7" E19º27'46"	fallow land	6.8	0.245	Podsol	1.014	0
95	Zawiercie	N50º29'44" E19º26'23"	fallow land	6.7	0.316	Phaeozem	1.730	0
96	Skałka	N50º31'36" E19º23'46"	fallow land	7.0	0.257	Podsol	1.012	0
97	Ptaszkowa	N49º36'11" E20º53'23"	fallow land	6.1	0.500	Cambisol	1.480	0
98	Czarny Potok	N49º34'22" E20º29'25:	fallow land	7.5	2.181	Cambisol	1.660	0
99	Łącko	N49º33'16" E20º26'43"	fallow land	4.0	0.540	Cambisol	1.285	0
100	Koziegłowy	N50o35'56" E19º9'3"	fallow land	6.6	0.611	Podsol	2.569	0

Kr – Kraków

Genomic DNA was *HindIII* (Fermentas) digested at 37ºC for 30 min. Subsequently, the following ligation mix was added: 2 µl of adaptor-forming oligonucleotides (5'-AGCTGTCGACGTTGG-3' and 5'-CTCACTCTCAC-CAACAACGTCGAC-3'; 20pM of each), 2.5 µl of T4 DNA Ligase buffer (10× – Fermentas), 0.5 µl of a 25 mM ATP solution, and 0.5 µl of T4 DNA Ligase buffer (10× – Fermentas). The restriction/ligation mixture was then incubated at 37ºC for 30 min., heated in a thermo-block at 70ºC for 10 min, and cooled at room temperature for 10 min. The subsequent PCR reaction was carried out in 20 µl volume containing 1 µl of restriction/ligation mixture, 2.5 µl *PwoHyp* 10×PCR buffer (DNA Gdańsk), 2.5 µl 20 mM $MgCl_2$ solution, 2.5 µl of dNTPs (2 mM each), 0.5 µl (1U) of *PwoHyp* DNA polymerase (DNA Gdańsk), and 0.25 µl of adaptor-complementary primer (5'-CTCACTCTCAC-CAACGTCGACAGCTT-3', 100 mM). The PCR reaction was performed in a Tgradient Engine thermal cycler (Biometra) with the following temperature profile: initial denaturation at 72ºC for 2 min to release unligated oligonucleotides and to fill in the single-stranded ends, followed by 22 cycles of denaturation at 88ºC for 30s, annealing and elongation at 72ºC for 90s, and final elongation at 72ºC for 2 min. 5 µl of PCR products were electrophoresed on 6% polyacrylamide gel ethidium bromide stained (up to 0.5 mg×cm^{-3} concentration) in 1×TBE buffer. After electrophoresis for 3h at 12 V×cm^{-1} the gel was analyzed with UV illumination by a VersaDoc Imaging System v. 1000 and Quantity One software.

Random Amplification of Polymorphic DNA (RAPD)

The Random Amplification of Polymorphic DNA (RAPD) analysis was carried out with two random primers (5'-AGTCAGCCAC-3' and 5'-AAGAGCCCGT3-3') in a single reaction. Amplification was performed in 25 µl volume containing 1 µl of genomic DNA (20 ng), 2.5 µl *PwoHyp* 10×PCR buffer (DNA Gdańsk), 3 µl of each primer (10 mM), 3 µl $MgCl_2$ solution (25 mM), 2.5 µl dNTP mix (25 mM), and 0.5 µl of *PwoHyp* DNA polymerase (DNA Gdańsk). The PCR reaction was performed with the following temperature profile: initial denaturation at 94ºC for 5 min, 25 amplification cycles of denaturation at 94ºC for 30s, annealing at 36ºC for 30s and elongation at 72ºC for 60s, followed by 5 min of final elongation at 72ºC. The PCR products (6 µl) were electrophoresed in 1% agarose gel ethidium bromide stained (up to 0.5 mg×cm^{-3} concentration) in 1×TAE buffer. After electrophoresis for 30 min at 7 V/cm the gel was analyzed with UV illumina-

Table 2. *A. chroococcum* isolations in samples of various soil use.

Occurrence of *A. chroococcum* in samples of various soil use								
Soil use	Forest	Fallow land	Garden	Field crop	Urban lawn	Industrial area	Park	Total
No. of samples	14	17	5	9	37	12	6	100
No. (%) of samples with successful isolation	2 (14.3)	4 (23.5)	2 (40.0)	2 (22.2)	21 (56.7)	8 (66.7)	4 (66.7)	43 (n/a)
No. (%) of CFU	43 (4.3)	48 (4.0)	92 (26.0)	54 (8.4)	636 (24.2)	220 (26.0)	30 (7.1)	1,123 (100)
Average no of CFU per sample	3.1	2.8	18.4	6.0	17.2	18.3	5.0	n/a

n/a – non applicable

tion by VersaDoc Imaging System v. 1000 and Quantity One software.

Statistical Analysis

Pearson's correlation coefficient (r) was used to compare the relationship between the CFU×g^{-1} and the soil properties. The statistical analysis was performed by Statistica v. 9 (StatSoft) software. Electrophoretic patterns obtained with PCR MP and RAPD methods were compared using Quantity One software. DNA relatedness was calculated by the band-based Dice coefficient. Band tolerance was 2%. Unweighted Pair Group Method with Arithmetic Mean Algorithm (UPGMA) was used to perform hierarchical cluster analysis and to construct dendrograms. A cut-off value of 90% similarity was determined.

Results and Discussion

Various populations of *Azotobacter* spp. were detected in 43 out of 100 studied soil samples (Table 1). When present, numbers of *A. chroococcum* CFU per 1 gram of soil varied from one to more than 100 (soil Nos. 46 and 32, respectively).

No macroscopic or microscopic differences were observed between isolated colonies from each site. Therefore, to simplify the tests further molecular analyses were performed on one strain from each location. All isolated strains turned dark brown after 5-7 days of incubation and, after identification according to Bergey's Manual of Systematic Bacteriology [8], all of them were inoculated on *A. chroococcum* agar to confirm their taxonomic identification. All isolates belonged to *A. chroococcum* species. The occurrence of *A. chroococcum* was different for samples of various soil use (Table 2). Gardens and industrial areas represented the highest numbers of CFU per sample – this may result from multiple factors such as total N or organic C contents or soil pH. Garden soils with successful *A. chroococcum* isolations were alkaline and had relatively high total N and organic C contents. Excluding sample no. 72, the situation is similar for industrial areas, where soils were alkaline and neutral. Industrial soils were sampled from the area of Kraków steelworks (ArcelorMittal Steel Kraków). But since

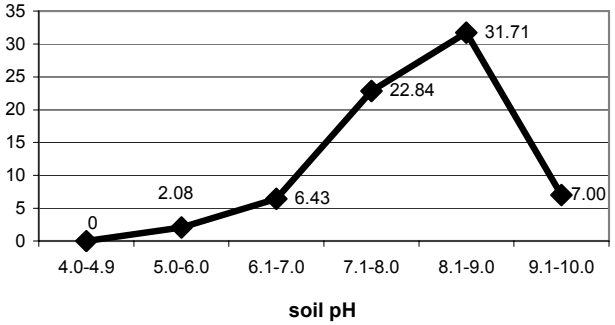

Fig. 1. Percentage of *A. chroococcum* CFU depending on soil pH.

these soils may be heavy metal-contaminated, they were not assessed in this study. Bacterial strains were present in only 22.2% of agricultural soil samples and only 8.4% of strains were derived from these soils. These results were much lower than reported by Martyniuk and Martyniuk (51.6%) [7]. Such differences may result from various sampling sites, the application of different mineral fertilizers, pesticides, or differences in soil properties. It has been documented that mineral N fertilizers may cause acidification of soil, particularly when such fertilizers are used in high doses without limitations [7, 24-26]. Some of the examined agricultural soils were slightly acidic and acidic (Nos. 19, 21, and 22, respectively) and *A. chroococcum* strains were absent in these samples.

It has been proved that *Azotobacter* spp. is sensitive to soil acidity [7, 27]. The results obtained for interactions between soil pH and *A. chroococcum* abundance in the analyzed soils confirm that these bacteria occur mostly in neutral and slightly alkaline soils (Fig. 1). The optimum pH for the isolated strains of *A. chroococcum* ranged from 7.1 to 9.0, which is consistent with results obtained in other studies [19, 28]. To verify interactions between the abundance

Table 3. Correlation coefficients between numbers of *A. chroococcum* CFU×g^{-1} and various soil properties. The coefficients are significant, with $p<0.05$.

Soil type	pH in H_2O	Total N [g×kg^{-1}]	Org. C [%]
0.180	0.368	0.664	0.455

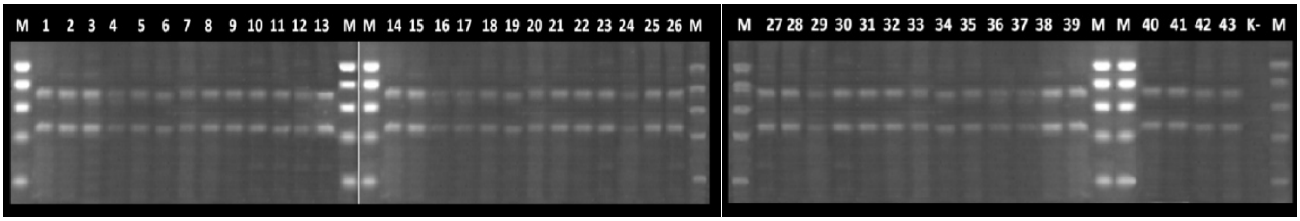

Fig. 2. Two fragments (~0.23 and 0.36 kb) obtained from *HindIII* digestion of the 16S-23S rDNA PCR product for 43 *A. chroococcum* strains.

of *A. chroococcum* in the studied soil samples, correlation coefficients were calculated (Table 3). Numbers of these bacteria were strongly correlated ($p<0.05$) with total N content and to a lesser extent with organic C content in the analyzed soils. There was also moderate correlation between *A. chroococcum* occurrence and soil pH. On the other hand, there was low correlation between soil type and *A. chroococcum* abundance. Many authors emphasize the strong influence of soil pH on *Azotobacter* spp. occurrence in soils [7, 19, 27], but significant correlation between *A. chroococcum* abundance and total N and organic C contents in the studied soils prove that soil fertility is another important factor influencing soil colonization by these bacteria [29, 30].

As a result of amplification of 16S-23S rDNA fragments one PCR product ~0.63 kb was obtained for all 43 examined strains. After *HindIII* digestion of the amplified fragment the same pattern was obtained for all isolates – two fragments, ~0.23 and 0.36 kb (Fig. 2). This result indicated a 100% similarity between the analyzed isolates. Restriction analysis of the ITS region may be used as a reliable method to identify *Azotobacter* spp. bacteria at the level of genus or species, but it is not useful for detecting intraspecific differentiation.

Using a PCR MP method, 43 different patterns were obtained. Fig. 3 shows the UPGMA dendrogram built from a distance matrix calculated from the PCR MP fingerprints. Four clusters (AI, AII, AIII, AIV) were defined using a 35% threshold value. Fig. 4 presents the UPGMA dendrogram built from a distance matrix calculated from the RAPD fingerprints. Five clusters (BI, BII, BIII, BIV, and BV) were defined using a 40% threshold value. High degree of congruence between PCR MP and RAPD clusters was found, but small differences were observed for both methods. There was no statistically significant relationship between the strains grouped in clusters and the sources of isolation or their properties, either for PCR MP or RAPD. Isolates of diverse origin were joined together in all clusters.

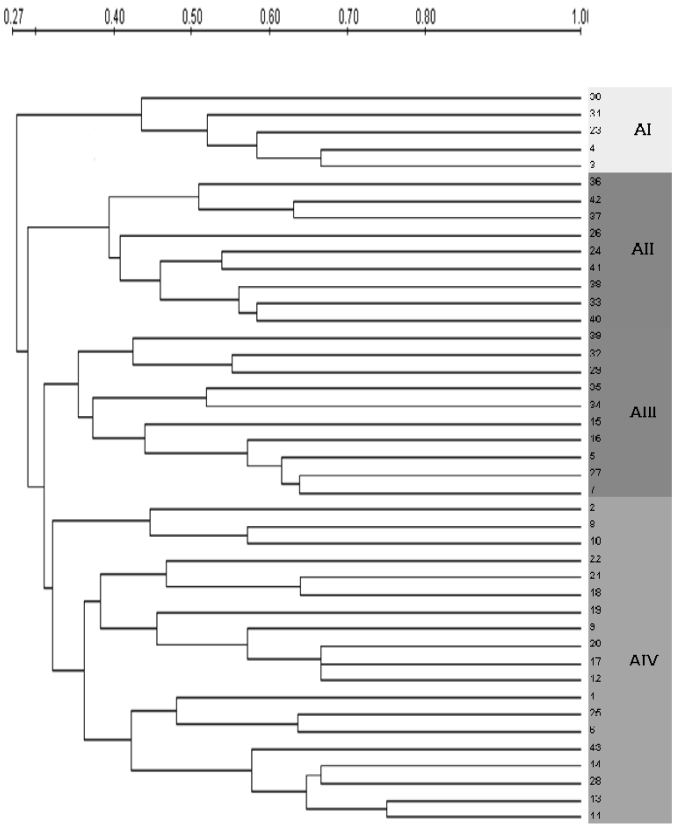

Fig. 3. UPGMA dendrogram of 43 *A. chroococcum* strains (PCR MP); 1-43: No. of isolates; AI-AIV: clusters.

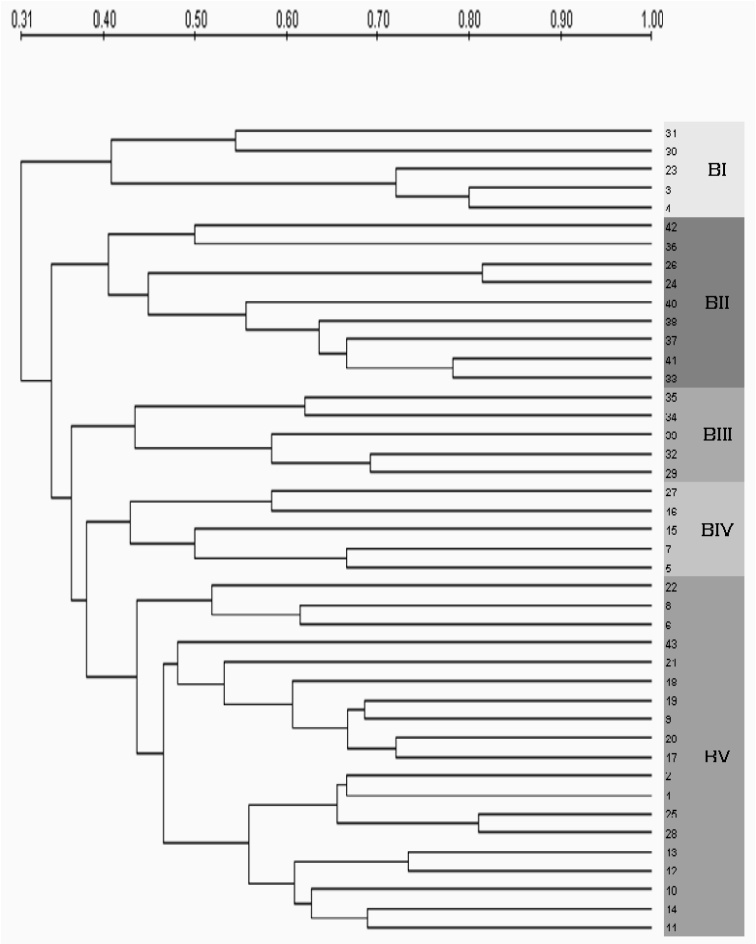

Fig. 4. UPGMA dendrogram of 43 *A. chroococcum* strains (RAPD); 1-43: No. of isolates; BI-BV: clusters.

Relatively close relationships were detected between strains 3 and 4 in cluster AI, strains 42 and 37 in cluster AII, strains 7 and 27 in cluster AIII, and strains 13 and 11 in cluster AIV for PCR MP. For RAPD a relatively close relationship was detected between strains: 3 and 4 in cluster BI, 24 and 26 in cluster BII, 29 and 32 in cluster BIII, 5 and 7 in cluster BIV, and 25 and 28 in cluster BV. But the location of these strains was very distant so that no correlation could be found between the sources of isolation and strain positions in clusters. Such heterogeneity most probably resulted from the fact that the bacteria were derived from various locations and from various soil types and soil use, as well as the fact that other soil properties were different. Other studies report high genetic diversity of bacteria independent of their origin [31]. Overall, genetic similarity between *A. chroococcum* isolates reached 27% for PCR MP technique and 31% for RAPD. Similarity between the strains within the clusters did not exceed 75% for PCR MP and 85% for RAPD. No genetically identical strains (over 90% similarity) were detected using both methods. Compared to other reports, the obtained results indicated high diversity of the isolated strains, but some bacterial species may present much higher diversity despite identical origins [32]. Nevertheless, genetic diversity of *A. chroococcum* species has not yet been widely studied, therefore it is difficult to estimate if the similarity level between strains was actually low. Diversity of various isolates of *Azotobacter* spp. in different cotton soils of India has been estimated based on restriction fragment length polymorphism (RFLP) analysis of *nif*H gene [33]. This analysis depicted a similarity of ≥80% between strains that originated from four various regions and even belonged to different species. On the other hand, the presented diversity results are based on genomic DNA analysis with a technique of much stronger discriminatory power, making the two studies impossible to compare.

It is necessary to continue studies on diversity of *A. chroococcum* in various regions as well as within individual locations to obtain a complete picture of variability in a bacterial population. Moreover, detailed genetic analyses may help to generate evidence of genome plasticity and evolution, leading to bacterial adaptation to various environmental conditions [34, 35].

Conclusions

Based on the performed diagnostic analyses, all isolated strains were defied as *Azotobacter chroococcum* – it can be stated that this species is the most abundant in Polish soils. The occurrence of *A. chroococcum* is affected by soil pH as well as by total N and organic C content – fertility of

soils is as important as pH. Restriction analysis of ITS region indicates that all isolates belonged to the same species and were 100% identical. Fingerprinting methods (PCR MP and RAPD) indicated high genetic diversity of all isolated strains, and the results obtained by these methods are comparable. The defined clusters joined together strains that in some cases originated from very distant locations. A comparison of RFLP of the ITS region and both PCR MP and RAPD techniques indicates that the latter two are much more useful for intraspecies differentiation of *A. chroococcum*, while the first technique can be applied for rapid identification at the species level.

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References

1. KHOSRAVI H., SAMAR S.M., FALLAHI E., DAVOODI H., SHAHABIAN M. Inoculation of 'Golden Delicious' apple trees on M9 rootstock with *Azotobacter* improves nutrient uptake and growth indices. J. Plant Nutr. **32**, 946, **2009**.
2. BIARI A., GHOLAMI A., RAHMANI H.A. Growth promotion and enhanced nutrient uptake of maize (*Zea mays* L.) by application of plant growth promoting rhizobacteria in arid region of Iran. J. Biol. Sci **8**, 1015, **2008**.
3. BEHL R.K., NARULA N., VASUDEVA M., SATO A., SHINANO T., OSAKI M. Harnessing wheat genotype x *Azotobacter* strain interactions for sustainable wheat production in semi arid tropics. Tropics **15**, 121, **2006**.
4. ORDOOKHANI K., KHAVAZI K., MOEZZI A., REJALI F. Influence of PGPR and AMF on antioxidant activity, lycopene and potassium contents in tomato. Afr. J. Agric. Res. **5**, 1108, **2010**.
5. RODELAS B., GONZÁLEZ-LÓPEZ J., MARTÍNEZ-TOLEDO M., POZO C., SALMERÓN V. Influence of *Rhizobium/Azotobacter* and *Rhizobium/Azospirillum* combined inoculation on mineral composition of faba bean (*Vicia faba* L.). Biol. Fertil. Soils **29**, 165, **1999**.
6. ABBASS Z., OKON Y. Plant growth promotion by *Azotobacter paspali* in the rizosphere. Soil Biol. Biochem. **25**, 1075, **1993**.
7. MARTYNIUK S., MARTYNIUK M. Occurrence of *Azotobacter* spp. in some Polish soils. Pol. J. Environ. Stud. **12**, 371, **2003**.
8. TCHAN Y.T., NEW P.B. *Azotobacteraceae*. [In:] HOLT J.G. (Ed.) Bergey's Manual of Systematic Bacteriology Vol 1. Williams & Wilkins: Baltimore, pp. 220-229, **1989**.
9. MASNY A., PŁUCIENNICZAK A. Ligation mediated PCR performed at low denaturation temperatures – PCR melting profiles. Nucl. Acids Res. **31**, 1, **2003**.
10. WILLIAMS J.G.K., KUBELIK A.R., LIVAK K.J., RAFALSKI J.A., TINGEY S.V. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl. Acids Res. **18**, 6531, **1990**.
11. KRAWCZYK B., LEIBNER-CISZAK J., MIELECH A., NOWAK M., KUR J. PCR Melting Profile (PCR MP) – a new tool for differentiation of *Candida albicans* strains. BMC Infect. Dis. **9**, 177, **2009**.
12. LARASSA J., GARCÍA-SÁNCHEZ A., AMBROSSE N., PARRA A., ALONSO J.M., REY J.M., HERMOSO-DE-MENDOZA M., HERMOSO-DE-MENDOZA J. Evaluation of randomly amplified polymorphic DNA and pulsed field gel electrophoresis techniques for molecular typing of *Dermatophilus congolensis*. FEMS Microbiol. Lett. **240**, 87, **2004**.
13. SMITH S., CANTET F., ANGELINI F., MARAIS A., MÉGRAUD F., BAYERDŐFFER E., MIEHLKE S. Discriminatory power of RAPD, PCR-RFLP and Southern Blot analyses of *ure*CD or *ure*A gene probes on *Helicobacter pylori* isolates. Z. Naturforsch. **57c**, 516, **2002**.
14. ISO 11465. Soil quality – Determination of dry matter and water content on a mass basis – Gravimetric method. International Organization for Standardization. Geneve, Switzerland, **1999**.
15. SHARMA S.D., KUMAR P., RAJ H., BHARDWAJ S.K. Isolation of arbuscular mycorrhizal fungi and *Azotobacter chroococcum* from local litchi orchards and evaluation of their activity in the air-layers system. Sci. Hort. **123**, 117, **2009**.
16. TROCHIMCZUK A.W., KOLARZ B.N., JERMAKOWICZ-BARTKOWIAK D. Metal ion uptake by ion + exchange-chelating resins modified with cyclohexene oxide and cyclohexene sulphide. Eur. Polym. J. **37**, 559, **2001**.
17. OLEKSYNOWA K., TOKAJ J., JAKUBIEC J., KOMORNICKI T. Guidebook to practices with soil science and geology. part 2. Agricultural Academy in Krakow **1991** [In Polish].
18. BARNES R.J.,BAXTER S.J., LARK R.M. Spatial covariation of *Azotobacter* abundance and soil properties: a case study using the wavelet transform. Soil Biol. Biochem. **39**, 295, **2007**.
19. AQUILANTI L., FAVILLI F., CLEMENTI F. Comparison of different strategies for isolation and preliminary identification of *Azotobacter* from soil samples. Soil Biol. Biochem. **36**, 1475, **2004**.
20. OLYUNINA L.N., MATSKOVA Y.A., GONCHAROVA T.A., GUSHINA Y.Y. Evaluation of thermal resistance of *Azotobacter chroococcum* 66 using atomic force microscopy. Appl. Biochem. Microbiol. **45**, 38, **2009**.
21. ATLAS R.M., PARKS L.C. Handbook of Microbiological Media, 2nd ed., CRC Press: Boca Raton, **126**, **1997**.
22. JENSEN M.A., WEBSTER J.A., STRAUS N. Rapid identification of bacteria on the basis of Polymerase Chain Reaction – amplified ribosomal DNA spacer polymorphisms. Appl. Environ. Microbiol. **59**, 945, **1993**.
23. KRAWCZYK B., SAMET A., LEIBNER J., ŚLEDZIŃSKA A., KUR J. Evaluation of a PCR Melting Profile technique for bacterial strain differentiation. J. Clin. Microbiol. **44**, 2327, **2006**.
24. FILIPEK T., KACZOR A. The dynamics of anthropogenic acidification pressure on agricultural production in Poland, [In:] KUKUŁA S. (Ed.) Protection and use of agricultural production in Poland. IUNG Puławy, Poland, pp. 35-43, **1997**.
25. MERCIK S. Most important soil properties and yielding on long-term static fertilising experiments in Skierniewice. Rocz. Gleboz. **44**, 71, **1994**.
26. MYŚKÓW W., WRÓBLEWSKA B., STACHYRA A., PIERZYŃSKI A. Effect of long-term organic and mineral fertilization on the biological fixation of atmospheric nitrogen and the productivity of light soils. Pam. Puł. **93**, 131, **1988**.

27. DŐBEREINER J. Isolation and identification of aerobic nitrogen-fixing bacteria from soil and plants. in: ALEF K., NANNIPIERI P. (Eds.) Methods in Applied Soil Microbiology and Biochemistry. Academic Press: London, pp. 134-141, **1995**.
28. LIMMER C., DRAKE H. L. Non-symbiotic N_2-fixation in acidic and pH-neutral forest soils: aerobic and anaerobic differentials. Soil Biol. Biochem. **28**, 177, **1996**.
29. SAFARI SINEGANI A.A., SHARIFI Z. Land use effect on the occurrence and distribution of *Azotobacter* in Hamadan soils, Iran. Proceeding of the Fourth International Iran and Russia Conference in Agriculture and Natural Resources. Sep. 8-10, Shahrekord, Iran, pp. 614-618, **2004**.
30. OCAMPO J.A., BAREA J.M., MONTOYA E. Interactions between *Azotobacter* and "phosphobacteria" and their establishment in the rhizosphere as affected by soil fertility. Can. J. Microbiol. **21**, 1160, **1975**.
31. VERMA V., RAJU S.C., KAPLEY A., KALIA V.C., DAGINAWALA H.F., PUROHIT H.J. Evaluation of genetic and functional diversity of *Stenotrophomonas* isolates from diverse effluent treatment plants. Biores. Technol. **101**, 7744, **2010**.
32. VALDEZATE S., VINDEL A., MARTÍN-DÁVILA P., SÁNCHEZ DEL SAZ B., BAQUERO F., CANTÓN R. High genetic diversity among *Stenotrophomonas maltophilia* strains despite their originating at a single hospital. J. Clin. Microbiol. **42**, 693, **2004**.
33. BHATIA R., RUPPEL S., NARULA N. *Nif*H-based studies on azotobacterial diversity in cotton soils of India. Arch. Microbiol. **191**, 807, **2009**.
34. BHATTACHARYA D., SARMA P.M., KRISHNAN S., MISHRA S., LAL B. Evaluation of genetic diversity among *Pseudomonas citronellosis* strains isolated from oily sludge-contaminated sites. Appl. Environ. Microbiol. **69**, 1435, **2003**.
35. RENDERS N., RŐMLING U., VERBRUGH H., VAN BELKUM A. Comparative typing of *Pseudomonas aeruginosa* by Random Amplification of Polymorpfic DNA or Pulsed-Field Gel Electrophoresis of DNA macrorestriction fragments. J. Clin. Microbiol. **34**, 3190, **1996**.