The effect of psychrotrophic bacteria isolated from the root zone of winter wheat on selected biotic and abiotic factors

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Abstract: The roots of winter wheat plants, cv. Mikon, grown in 45-year monoculture, were analysed in the study. Twenty-two bacterial isolates obtained from the rhizosphere, rhizoplane, and endorhizosphere that were capable of growth at 8°C and at 28°C, were selected for further analysis. The isolated psychrotrophs accounted for 25% of all bacteria present in the wheat rhizosphere and capable of growth at 8°C. Psychrotrophic bacteria were analysed at a temperature of 10°C and 28°C to determine their ability to inhibit the growth of pathogenic fungi, solubilise mineral phosphates, and to determine their ability to degrade chitin and cellulose. Similarity between the isolates was determined by Enterobacterial Repetitive Intergenic Consensus – Polymerase Chain Reaction (ERIC-PCR) and Random Amplification of Polymorphic DNA – Polymerase Chain Reaction (RAPD-PCR). The majority of isolated psychrotrophs inhibited the growth of pathogenic fungi and solubilised mineral phosphates at both incubation temperatures. Psychrotrophic bacteria exerted a two-fold stronger inhibitory effect on mycelial growth at 10°C than at 28°C. The growth of *Fusarium culmorum* and *F. oxysporum* was inhibited to the highest extent at 10°C and at 28°C, respectively. Phosphate solubilisation rates were higher at 28°C, particularly in the rhizosphere. Regardless of temperature, the bacteria exhibited low chitin-degrading potential, and none of the isolates was capable of degrading cellulose. A high similarity between the selected psychrotrophs was revealed by ERIC-PCR and RAPD--PCR analyses. Based on RAPD-PCR, the analysed population was divided into a group of isolates obtained from the rhizosphere, and two groups comprising representatives of both the rhizoplane and the endorhizosphere. Due to their ability to grow over a wide temperature range and increase phosphorus availability to plants, and their antagonism against pathogens, psychrotrophic bacteria can be used to improve the growth and yield of cereal crops.

Key words: abiotic factors, antagonism, monoculture, psychrotrophic bacteria, winter wheat

Introduction

Psychrotrophic bacteria can grow in a wide range of temperatures. They can survive and thrive in a cold environment, at temperatures close to 0°C, and withstand mild temperatures of 15–30°C (optimum temperature is 15–20°C). Therefore, psychrotrops are the predominant spoilage bacteria in refrigerated foods. They have even been found in Antarctic ecosystems, in water, soil and plants (Druce and Thomas 1970; Lo Giudice *et al.* 2007; Altunatmaz *et al*. 2012). The beneficial influence of psychrotrophic bacteria on the natural environment, including biodegradation of undesirable substances and antagonistic activities against potential pathogens, has been widely documented (Lo Giudice *et al*. 2007; Pini *et al*. 2007).

Rhizosphere is the narrow zone of soil surrounding the roots of plants. This micro-ecosystem plays a key role in plant development as it affects the quality and quantity of soil nutrients available to plants. Numerous physical and chemical processes occur in the rhizosphere, which harbors many root-associated microorganisms. Biotic and abiotic factors, including the abundance and species composition of microbial communities, the structure and physicochemical parameters of soil, and partial pressures of O_2 and CO_2 in soil, are interrelated and determine nutrient and water availability to plants (Hinsinger *et al*. 2009).

Root-colonising bacteria that enhance plant growth are referred to as Plant Growth-Promoting Rhizobacteria (PGPR). The PGPRs exert their beneficial effects by suppressing the growth of pathogens, making nutrients available to plants, producing growth-promoting substances, enhancing abiotic stress tolerance mechanisms, and inducing systemic defense responses in plants. Rhizobacteria can colonise the rhizosphere, rhizoplane (the root surface) and the endorhizosphere (the interior of the roots) (Czaban *et al*. 2007; Cummings 2009; Yang *et al*. 2009; Beneduzi *et al*. 2012; Sallam *et al*. 2013).

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Among numerous taxa of bacteria and Archaea, there are also the Proteobacteria, Firmicutes, and Actinobacteria which can contribute significantly to plant protection against pathogens. The growth of root-infecting fungi can be effectively suppressed by γ-Proteobacteria (Mendes *et al*. 2012). The antagonistic effects of rhizosphere microorganisms are associated with their ability to produce non-ribosomal peptide synthetases, siderophores, hydrogen cyanide, chitinases, glucanases, antibiotics, and enzymes (Mendes *et al*. 2012; Noori and Saud 2012; Ashwini and Srividya 2013).

Free-living bacteria present in the soil and in the root zone are capable of fixing nitrogen, ammonification and solubilisation of mineral phosphate compounds. These compounds are mostly $Ca_3(PO_4)_2$ salts that are not available for uptake by plant roots. Crop yield and quality are affected by the abundance of beneficial soil microorganisms that participate in the conversion of important biogenic elements, and in the synthesis of phytohormones and substances that stimulate defense responses in plants (Rodríguez and Fraga 1999; Cummings 2009; Yang *et al*. 2009).

In the fall and early spring, low temperatures promote the growth of plant pathogens. As the temperature drops, the growth of antagonistic bacteria and microorganisms increases and nutrient availability slows down. Crop plants may become infected by pathogenic fungi. An insufficient supply of biogenic elements may reduce the plants' disease resistance, lodging resistance, and cold tolerance. Further research is needed to investigate the roles and functions of psychrophilic and psychrotrophic microorganisms in the rhizosphere of grain crops.

This paper describes one of the few studies that explore the structure of selected groups of psychrotrophic bacteria residing in the rhizosphere of winter wheat grown in long-term monoculture. There is a scarcity of published research on the potential use of rhizosphere bacteria in crop production. Thus, the current findings provide new insights into the applications of beneficial psychrotrophs for improving cereal crop productivity.

The first objective of this study was to determine the effect of psychrotrophic bacteria on biotic and abiotic factors in the rhizosphere of winter wheat grown in long-term monoculture. The second objective was to estimate the similarity between bacterial isolates.

Materials and Methods

Isolation of bacteria

The experimental materials comprised entire root systems collected with the surrounding soil (rhizosphere) from winter wheat plants grown in 45-year monoculture at the Agricultural Experiment Station in Bałcyny (Region of Warmia and Mazury, NE Poland), in October 2012. The experiment was established in 1967 by the Department of Agricultural Systems, University of Warmia and Mazury in Olsztyn, Poland. Whole plants were transported to the laboratory in sterile polyethylene bags. Bacteria were isolated and cultures were prepared on the sample collection day.

From the rhizosphere – 10 g samples of soil immediately surrounding the root system were placed in 250 ml flasks containing 90 ml of sterile saline solution (0.9% NaCl). As for the rhizoplane – the roots were cut off and rinsed with sterile demineralised water. Root segments were placed in 100 ml flasks containing sterile desalinated gravel and 50 ml of sterile demineralised water. The flasks were shaken for 30 min at 180 rpm. As for the endorhizosphere – the bacteria were isolated from roots which had been surface-sterilised (Czaban *et al*. 2007). Samples were rinsed with 5% NaClO followed by a rinsing in sterile water.

Following serial dilutions, 0.1 ml bacterial suspensions were poured into Petri dishes. Spore-forming bacteria were cultured by incubation at 80°C for 10 min. The following culture media were inoculated with aliquots of each successive dilution: N-free medium for the isolation of *Azotobacter* spp. (K_2HPO_4 – 1 g, $MgSO_4 \times 7H_2O$ – 0.2 g, $CaCO_3$ – 1 g, NaCl – 0.2 g, $FeSO_4 \times 7H_2O$ – 0.005 g, glucose – 10 g, agar – 15 g per liter of demineralised water, pH 7.0), Tryptone Soya Agar (TSA) for the isolation of spore-forming bacteria, Hagedorn and Holt medium (1975) for the isolation of *Arthrobacter*, Pseudomonas Selective Agar (PSA) for the isolation of *Pseudomonas* spp. Bacterial cultures were incubated at 8°C for 1–5 days. Bacterial colonies were stored at –80°C. To isolate psychrotrophic bacterial populations, the growth media were inoculated again with the isolates, and were incubated for up to 72 h at 28°C. Isolates capable of growth at 8°C and 28°C were selected for further analysis.

Antagonistic activity of psychrotrophic bacteria against pathogenic fungi

The inhibition of fungal growth was assessed on Potato Dextrose Agar (PDA) (dual culture analysis). The density of bacterial suspensions, measured photometrically, reached 5×10^8 CFU/ml. Samples of bacterial suspensions (10 µl) were streaked on the opposite edges of the PDA plate (3 cm from the center). At the center of the plate, a 5 mm mycelial disk was placed. Sterile distilled water served as the control. The plates were incubated at 28°C for 5 days, and at 10°C for 7 days. The antagonistic potential of bacteria against *Fusarium culmorum*, *F. oxysporum*, and *Monographella nivalis* was evaluated.

Phosphate-solubilising capability and chitin- and cellulose-degrading potential of psychrotrophic bacteria – a well test on solid media

The phosphate-solubilising capability of psychrotrophic bacteria was tested on NBRIP (National Botanical Research Institute's phosphate) growth medium (Nautiyal 1999). The chitin-degrading potential of psychrotrophic bacteria was assessed on agar medium containing colloidal chitin, prepared as described by Hsu and Lockwood (1975) with later modifications proposed by Ashwini and Srividya (2013). The cellulose-degrading potential of psychrotrophic bacteria was assessed on mineral growth medium (KH_2PO_4 – 1.0 g, $MgSO_4 \times 7H_2O$ – 0.5 g, NaCl – 0.5 g, $FeSO_4 \times 7H_2O$ – 0.01 g, $MnSO_4 \times H_2O$ – 0.01 g, NH_4NO_3

– 0.3 g, carboxymethyl cellulose – 2 g, agar – 15 g, in liter deionized water, pH 7.0). After incubation, the plates were flooded with 1% Congo-red solution for 15 min. Ten µl samples of bacterial suspensions with the density of 5×10^8 CFU/ml were used in each test. The plates were incubated at 28°C and at 10°C for 72 h, and the diameters of the zones around each well were measured.

Calculations and statistical analyses

The antagonistic activity of isolates against pathogens was determined based on the following formula (Ashwini and Srividya 2013):

$$\% \text{ Inhibition} = [1 - (\text{fungal growth in the presence of bacteria/control growth})] \times 100.$$

The phosphate solubilisation potential and chitin-degrading potential of psychrotrophic bacteria were estimated based on the diameters of zones around each well. The following scale was used:

– no potential,
• weak potential (< 1 mm),
•• moderate potential (1–3 mm),
••• strong potential (> 3 mm).

The results for each of the three analysed zones were averaged and expressed on a scale of 0.0 (no effect) to 3.0 (strong effect). Data were processed statistically by ANOVA and the significance of differences was estimated by Duncan's test at p = 0.05. All calculations were performed in the Statistica 10 application (StatSoft Inc. 2011).

Isolation of genomic DNA

The overnight culture on TSA (28°C) was centrifuged at 6,000 rpm for 10 min, and the supernatant was removed. Isolation was carried out according to the CTAB (cetyltrimethylammoniumbromide) protocol described by Chen and Ronald (1999). DNA was suspended in sterile demineralised water and stored for further analysis.

RAPD-PCR and ERIC-PCR analyses

Random Amplification of Polymorphic DNA – Polymerase Chain Reaction (RAPD-PCR) was performed as described by Williams *et al*. (1990) modified by Indira Devi *et al*. (2011), using the following oligonucleotides: OPB11, OPD07, OPG14, OPG17, and OPH05. Enterobacterial Repetitive Intergenic Consensus – Polymerase Chain Reaction (ERIC-PCR) was performed as described by Cirvilleri *et al*. (2005). ERIC 1R (5′-TGTAAGCTCCTGGGGATTCAC-3′) and ERIC 2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) primers were used. Electrophoresis was carried out in 1.2% agarose gel for 1 h 20 min at 100 V. The bands were analysed and the Jaccard index was calculated using MEGA 4.0 and DendroUPGMA software to construct dendrograms.

Results

Isolation of bacteria

Twenty-two isolates of psychrotrophic bacteria were selected from among 112 isolates of psychrophiles incubated at 8°C. *Pseudomonas* was the predominant genus in the analysed group. Psychrotrophs were most abundant in the rhizoplane (22.6%), and in the endorhizosphere and rhizosphere they accounted for 20.0% and 17.0% of psychrotrophic and psychrophilic bacteria isolated from all the zones, respectively. The above values were determined by the counts of psychrophiles, which were highest in the rhizosphere (39 isolates). *Azotobacter* spp. were isolated only from the rhizosphere, but they were incapable of growth at 28°C. *Arthrobacter* spp. had a low share of the psychrotroph population, but they were subdominant (together with *Pseudomonas* spp.) in the psychrophile population. Spore-forming bacteria capable of growth at both temperatures had a high proportion relative to all psychrophiles, which resulted from their low share of the entire population. Only two isolates of spore-forming psychrophiles, unable to grow at 28°C, were obtained from the endorhizosphere (Table 1).

Table 1. Ratio of psychrophiles to psychrotrophs

Sampling site	Bacteria	Psychrophile counts	Psychrotroph counts	Total	Share of psychrotrophs [%]	
Rhizosphere	*Azotobacter* spp.	3	0	3	0.0	17.0
	Pseudomonas spp.	19	4	23	17.4	
	Spore-forming	7	2	9	28.6	
	Arthrobacter spp.	10	2	12	16.7	
Rhizoplane	*Azotobacter* spp.	–	–	–	–	22.6
	Pseudomonas spp.	9	5	14	37.7	
	Spore-forming	4	1	5	20.0	
	Arthrobacter spp.	11	1	12	8.3	
Endorhizosphere	*Azotobacter* spp.	–	–	–	–	20.0
	Pseudomonas spp.	15	6	21	29.0	
	Spore-forming	2	0	2	0	
	Arthrobacter spp.	11	1	12	8.3	

"–" – not found in a given zone

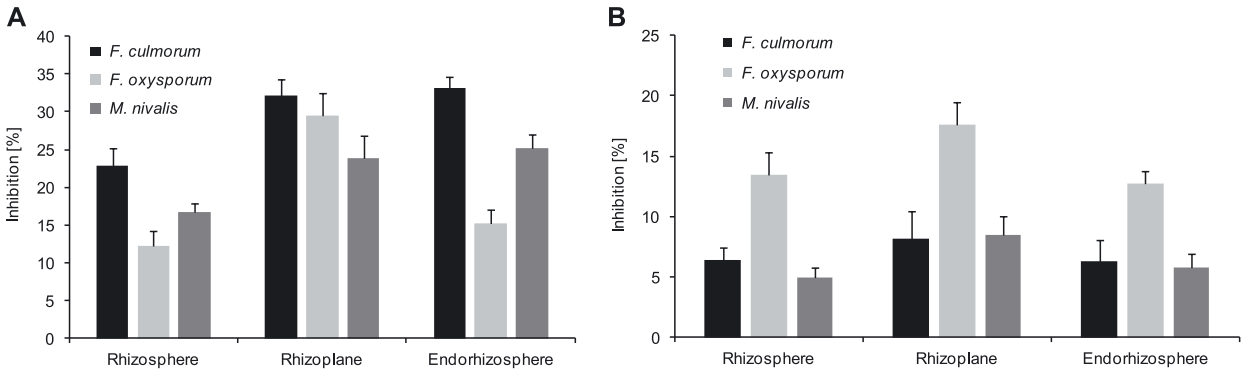

Fig. 1. Inhibition of mycelial growth by psychrotrophic bacteria at 10°C (A) and at 28°C (B)

Table 2. Antagonism of psychrotrophic bacteria against pathogens and phosphate solubilization rates

Origin	Isolate number	Inhibition of phytopathogen growth [%]						Phosphate solubilisation		Chitin degradation	
		F. culmorum		*F. oxysporum*		*M. nivalis*					
		10°C	28°C	10°C	28°C	10°C	28°C	10°C	28°C	10°C	28°C
Rhizosphere	RS-9	0.0 y	11.0 i–m	0.0 y	27.1 b–d	7.4 v–x	3.4 q–s	–	•••	•	•
	RS-13	32.9 e–h	10.3 i–o	13.0 r–v	11.1 i–l	14.8 p–t	6.0 l–q	•••	•••	•	•
	RS-23	23.6 j–n	2.0 q–s	18.0 m–r	7.3 j–q	14.8 p–t	14.2 g–i	••	•••	–	–
	RS-25	41.4 b,c	1.8 q–s	2.5 x,y	14.0 g–i	22.2 k–o	7.3 j–q	••	•••	–	•
	RS-33	14.3 p–t	0.0 s	20.0 l–q	1.8 q–s	24.1 j–m	3.2 q–s	–	••	–	–
	RS-34	32.9 e–h	4.6 q–s	2.0 x,y	5.3 o–s	7.4 v–x	5.3 n–s	–	••	–	•
	RS-43	20.7 l–p	7.0 j–q	34.0 d–h	11.7 i–k	20.4 l–q	0.0 s	••	•••	•	•
	RS-46	17.1 n–s	15.0 g–i	8.0 u–x	29.7 b,c	22.2 k–o	0.0 s	•	••	–	•
Rhizoplane	RP-2	32.1 f–i	0.0 s	6.0 w,x	10.8 i–m	14.8 p–t	3.9 q–s	••	••	–	–
	RP-3	34.8 d–g	0.0 s	32.0 f–i	13.3 h,j	20.4 l–q	7.1 j–q	••	•	–	–
	RP-4	12.1 r–w	20.0 e,f	60.0 a	35.6 a	60.2 a	19.1 e–g	•••	•	–	•
	RP-7	32.6 e–h	0.0 s	28.0 h–k	14.5 g–i	14.8 p–t	20.0 e,f	••	••	–	•
	RP-8	45.5 b	31.0 b	30.0 g–j	6.5 k–q	22.2 k–o	7.1 j–q	••	•••	–	•
	RP-25	25.0 j–l	3.2 q–s	26.0 i–l	14.2 g–i	23.1 k–n	1.8 q–s	–	••	–	•
	RP-27	42.9 b,c	3.3 q–s	25.0 j–l	28.0 b–d	11.1 s–w	0.0 s	•	•	–	–
Endorhizosphere	EN-33	24.2 j–m	25.0 c,d	7.0 v–x	10.2 i–p	7.4 v–x	5.5 n–r	••	•••	–	–
	EN-37	37.9 c–f	4.8 p–s	34.0 d–h	10.7 i–n	31.5 f–i	0.0 s	–	•••	•	•
	EN-38	38.6 c–e	0.0 s	8.0 u–x	12.1 i,j	25.0 j–l	0.0 s	••	••	•	•
	EN-39	37.9 c–f	0.0 s	14.0 q–u	23.7 d,e	25.9 i–l	5.7 m–q	•	–	–	•
	EN-40	39.4 c,d	0.0 s	18.0 m–r	14.4 g–i	25.9 i–l	5.1 o–s	••	••	–	–
	EN-41	22.9 k–n	14.2 g–i	16.0 o–t	5.9 l–q	35.2 d–g	7.3 j–q	•••	•••	•	•
	EN-60	31.4 f–i	0.0 s	10.0 t–w	12.3 h–j	25.9 i–l	17.5 f–h	••	••	–	•

*values marked with different letter(s) in the columns are statistically different according to Duncan's test ($p \leq 0.05$)
– no potential; • weak potential (< 1 mm); •• moderate potential (1–3 mm); ••• strong potential (> 3 mm)

Antagonistic activity of psychrotrophic bacteria against pathogenic fungi

Bacteria exerted a stronger inhibitory effect on mycelial growth at 10°C than at 28°C (23.2% *vs*. 9.3%, on average). At 10°C, the development of pathogenic fungi was suppressed by 28.5%, 24.6%, and 17.2% by bacteria isolated from the rhizoplane, endorhizosphere, and rhizosphere. At 28°C, the inhibition of pathogen growth reached 11.4% in the rhizoplane, and 8.3% in the endorhizosphere and rhizosphere (Fig. 1, Table 2).

At 10°C, the highest level of growth inhibition (over 30%) was noted in *F. culmorum* interacting with endorhizosphere and rhizoplane bacteria, and the lowest (12%) – in *F. oxysporum* interacting with rhizosphere bacteria. At 28°C, an over 10% (13–18%) growth inhibition was

observed in *F. oxysporum* interacting with psychrotrophs isolated from all analysed root zones. Rhizosphere bacteria exerted an insignificant antagonistic effect on *M. nivalis* (Fig. 1, Table 2).

In the majority of cases, the suppressive effect of the tested isolates on mycelial growth varied depending on temperature. At 10°C, only the RS-9 isolate did not inhibit the growth of *Fusarium* species. The number of isolates that did not exhibit antagonistic activity was much higher at 28°C. The highest number of isolates unable to suppress the growth of at least one pathogenic fungus were obtained from the endorhizosphere. All isolates inhibited the development of *F. oxysporum* (Table 2).

It was the RP-4 isolate that had the strongest inhibitory effect on the tested fungal pathogens. At 10°C, the RP-4 isolate suppressed the growth of *F. oxysporum* and *M. nivalis* by approximately 60%, and *F. culmorum* by 12.1%. At 28°C, RP-4 exhibited the strongest antagonistic activity against *F. oxysporum* (35.6%), followed by *F. culmorum*, and *M. nivalis* (ca. 20%).

Phosphate-solubilising capability of psychrotrophic bacteria

At 28°C, 21 isolates exhibited a phosphate-solubilising capability. Rhizosphere bacteria were characterised by the highest phosphate-solubilising capability (2.6 points), followed by isolates obtained from the endorhizosphere and rhizoplane (2.1 and 1.7 points, respectively). Phosphate solubilisation rates were lower at 10°C. The clear zone was observed in 17 isolates. The phosphate-solubilising potential was determined at 1.7 points in bacteria isolated from the rhizoplane and endorhizosphere, and 1.3 points in rhizosphere bacteria.

Chitin- and cellulose-degrading potential of psychrotrophic bacteria

The chitin-degrading potential of psychrotrophs was very low at both 10°C and 28°C. However, small clear zones on the growth medium were encountered more frequently after incubation at 28°C. An analysis of cellulose-degrading potential revealed that psychrotrophic bacteria were not able to break down cellulose, irrespective of the temperature.

Bacterial community structure

The RAPD-PCR analysis supported a more accurate grouping of isolates obtained from different root zones, compared with ERIC-PCR (Fig. 2). The plotted dendrogram, based on the combined results of RAPD-PCR, had two major clades. The larger clade had two branches. The first group was comprised of seven out of eight rhizosphere bacteria, two representatives of the rhizoplane and no representatives of the endorhizosphere. The isolates were characterised by a high degree of similarity (> 70%). The other group consisted of five endorhizosphere bacteria and two rhizoplane bacteria. The smaller clade included four isolates, with no predominant microbial groups. The remaining two branches contained single, less closely related RP-3 and RS23 isolates. Both RAPD and ERIC revealed a high degree of similarity between the isolated psychrotrophs, which was equal to or higher than 0.7 for approximately 70% of the community.

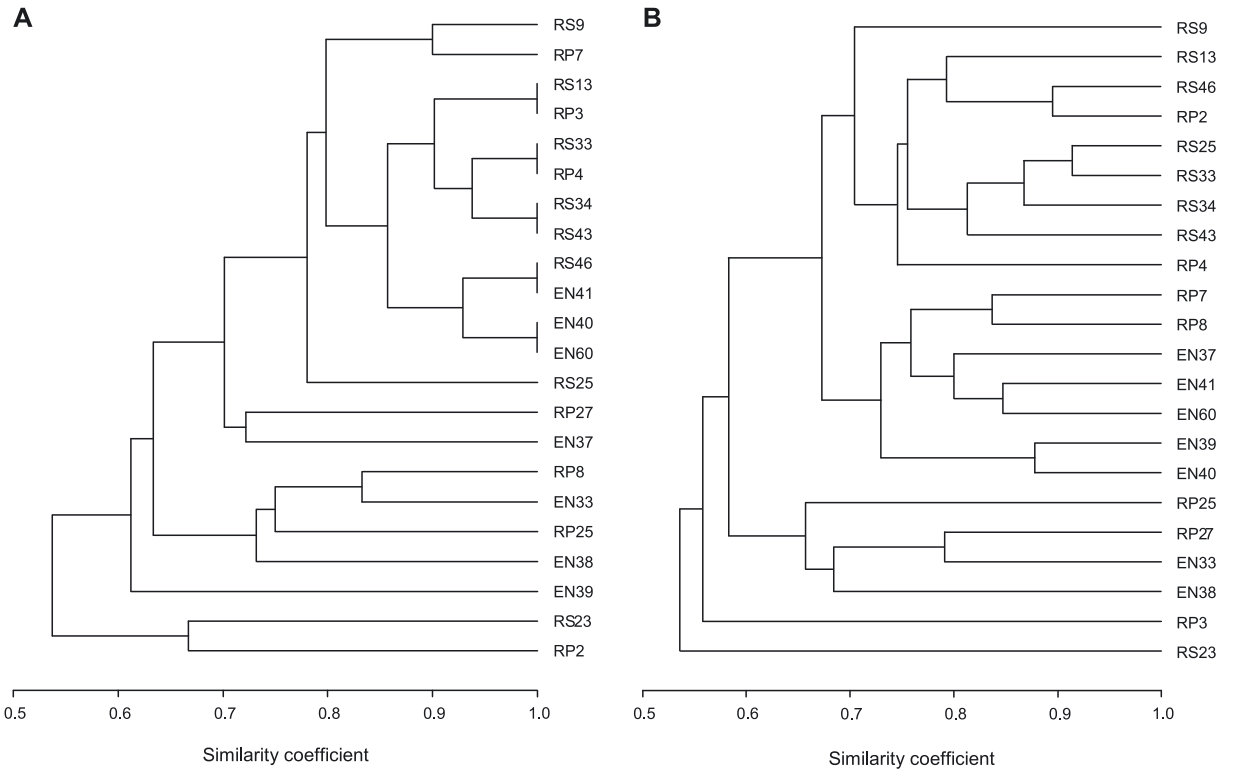

Fig. 2. Dendrograms illustrating similarity between the tested isolates, constructed based on the UPGMA algorithm: A – ERIC-PCR; B – RAPD-PCR

Discussion

Studies investigating bacteria capable of growing over a wide range of temperatures help find bacterial isolates that could be used as biocontrol agents and plant growth promoters. Such bacterial isolates could be used regardless of the ambient temperature, particularly in periods of unstable weather. Most laboratory tests are performed under optimum temperatures for microbial growth, which does not allow for determining the survival of bacteria at low temperatures.

The RP-4 isolate was characterised by the highest antagonistic activity against the tested pathogenic fungi (*F. culmorum*, *F. oxysporum*, and *M. nivalis*). The RP-4 isolate seems to be perfectly suited for biological wheat protection. Each of the isolated bacteria inhibited the growth of at least one pathogen species. According to Johansson (2003), PGPRs can effectively reduce the incidence of plant diseases caused by fungi of the genus *Fusarium*. The highest number of bacterial isolates with antagonistic potential, were obtained from plants of the family Brassicaceae, and from plants analysed in the spring.

Isolates RS-13 and EN-41 exhibit high phosphate-solubilising capability irrespective of the temperature. Bacteria of the genus *Pseudomonas* are able to solubilise phosphates over a wide temperature range, and show the maximum phosphate solubilisation potential at 21°C (Pandey *et al*. 2002). Das *et al.* (2003) examined *Pseudomonas fluorescens* strains and their cold-tolerant mutants for phosphate-solubilising activity at 25°C and 10°C. They found that in nearly all cases, the cold-tolerant mutants were characterised by higher phosphate solubilsation potential at both temperatures. The maximum phosphate solubilisation activity was noted after 6–7 days of incubation in the cold-tolerant mutants, and after 2–4 days in their wild-type counterparts.

The antagonistic activity of the tested isolates varied depending on temperature. In some cases, antagonistic effects were not observed at 10°C or 28°C. Similar trends were noted in respect to phosphate solubilisation potential. Psychrotrophs display high adaptability and respond to changes in their environment through changes in metabolic pathways. Some proteins (enzymes) are inactive at low temperatures, whereas cold-tolerant bacteria are able to maintain metabolism below their temperature optimum to survive through and prevent freezing damage to cells. As demonstrated in the present study and reported by other authors, considerable temperature variations in the environments inhabited by cold-tolerant bacteria lead to changes in their metabolic activity. Manifestation can be seen in the presence or absence of phenotypic traits such as antagonistic potential against pathogens and production of organic acids (Das *et al*. 2003; Chattopadhyay 2006; Lorv *et al*. 2014).

As shown by RAPD-PCR, the root zone microecosystem harbors closely related bacteria. Rhizosphere-dwelling psychrotrophs form a relatively homogeneous group that differs from other microbial groups. Bacteria isolated from the rhizoplane and endorhizosphere do not form specific communities. Such an indication means that they can easily migrate from the root surface to the root interior. RAPD-PCR can be a valuable tool for differentiating psychrotrophic PGPRs, but the authors hope to develop specific molecular markers that will enable the identification of bacteria isolated from the rhizosphere and the roots, and to determine the migration patterns of bacteria between those two zones. Studies that involve molecular markers confirm the suitability of random primers for analysis of soil bacteria. The method is both cost- and time-effective, and supports genomic DNA analysis without the need for microorganism identification (Bisht *et al*. 2013; Rincon-Florez *et al*. 2013). In the present study, the ERIC--PCR technique, which is suitable for the differentiation of Enterobacteriaceae, revealed that most bacterial isolates were similar regardless of the sampling site. Different results were delivered by RAPD-PCR, where the majority of rhizosphere bacteria belonged to the same branch. The above difference results from the low number of amplification products generated by ERIC-PCR, which indicates that this technique is not suitable for the differentiations of root-associated psychrotrophs. A high degree of similarity between cold-tolerant bacteria of the genus *Pseudomonas*, determined by rep-PCR (BOX, ERIC, GTG) and RAPD--PCR, was also reported by Bisht *et al*. (2013).

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