ISSN 1644-0692
www.acta.media.pl

Acta Sci. Pol. Hortorum Cultus, 15(6) 2016, 3-18

ANTAGONISTIC ACTIVITY OF PLANT-ASSOCIATED MICROORGANISMS AGAINST *Phytophthora infestans*

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Abstract. *Phytophthora infestans* is a pathogen threatening potato and tomato tillages. Environmentally safe biological methods are searched for the prevention against this pathogen. Many biocontrolling agents occur in plants or on their surfaces. Our studies were aimed at searching for effective antagonists against *P. infestans* among the isolates of endophyte and epiphyte bacteria. 37 endophyte and 26 epiphyte bacterial strains isolated from *Equisetum arvense, Elymus repens* and *Chenopodium album* L. were studied. The bacterial strains were screened for their antagonistic activity against *Phytophthora infestans*. The inhibitory effect of the bacteria was estimated based on the calculation of the per cent of relative growth. All tested isolates showed antagonistic properties against *P. infestans*. The strongest activity was observed for the *B. subtilis* strain. Plant species and the tested parts of the plants had an important influence on the antagonistic activity of bacterial strains isolated from these plants. The endophyte growth rate inhibition of *P. infestans* was higher than for the epiphyte strains and was over 40% for most isolates. Bacterial biocontrolling agents should be searched among endophytes of the studied plants and not among bacteria colonizing their phyllosphere.

Key words: endophytes, epiphytes, bacterial biocontrolling agents, blight of potato and tomato

INTRODUCTION

Microbes accompany plants from the first stages of their lives. They include microorganisms promoting plant growth and phytopatogens. The first group, i.e. plant-associated bacteria *sensu lato* colonize the plant rhizosphere, phyllosphere and en-

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dosphere [Pini et al. 2011]. The rhizosphere comprises soil attached to roots, and the bacteria and fungi present there play fundamental environmental roles. A completely different zone, due to the prevailing conditions, is the phyllosphere, that is the zone encompassing the surface of the above-ground portions of plants: leaves, stems, flowers and fruits. It is inhabited by epiphytic organisms, dominated by bacteria, and accompanied by yeasts and archaea [Yang et al. 2000, Whipps et al. 2008]. Microorganisms can also penetrate into plants becoming endophytes, that is microorganisms, which by inhabiting the internal portions of roots, stems, leaves or seeds do not have a harmful influence on the plant host [Hallmann et al. 1997]. All these zones are niches with high agricultural and environmental significance. Microbes that are present in each of these zones can stimulate plant growth by giving access to nutrients, producing phytohormones, and increasing plant resistance to environmental stress [Nongkhlaw and Joshi 2014]. They may also impede plant colonization by pathogens, due to which they become a valuable source of microbes used in biocontrol. This paper presents the results of studies on the antagonistic activity of endophytes and epiphytes in three plants: common horsetail, couch grass and white goosefoot against *Phytophthora infestans*.

Microorganisms from the genus *Phytophthora* belong to the class Oomycetes, phylum Stramenopiles, encompassing species which attack agricultural and garden plants as well as forest stands [Hardham 2005, Jung and Burgess 2009, Beakes et al. 2012]. An important species is *P. infestans*, which causes potato disease. It can also infect tomatoes and a few related plants from the Solanaceae family. In some cases, *P. infestans* also infects peppers (*Capsicum* L.) and eggplants (*Solanum melongena*) [Griffith et al. 1992, Gisi and Cohen 1996]. This pathogen is common worldwide, in particular in cool and humid conditions [Nelson 2008]. Attacked portions of the plant include leaves, stems, fruits and tubers. Disease development is influenced by temperature and humidity. The sporangia are formed under the leaves, and sporulation takes place at 3–26°C. Spores germinate at 21–26°C, whereas below 18°C the sporangia produce 6 to 8 zoospores, which require water for transportation. Each zoospore may initiate the disease, which explains why the disease is troublesome in cool and humid conditions. Zoospores contain a transcript for several enzymes important in the pathogenesis: cutinases, polygalacturonases, pectate lyases, β-1,4-glucanases, hemicellulases (xylanases and others), glucanase inhibitor proteins, and protease inhibitors [Judelson and Blanco 2005]. Potato disease caused by *P. infestans* belongs to the most common potato disease, resulting in great hazard to this crop and extremely high economical loss [Schlenzig et al. 1999, Sedláková et al. 2011]; in Poland these losses reach 20–25%. Currently, crop protection is linked with the application of fungicides combined with environmental monitoring [Nelson 2008, Sedláková et al. 2011]. Despite these measures, the protection is often ineffective due to the appearance of pathogen resistance to phenyloamides and metalaxyl contained in the fungicides [Cooke and Lees 2004, Sedláková et al. 2011]. At present, attempts are undertaken to find biological protective agents against this pathogen with the application of plant extracts [Stephan et al. 2005, Moushib et al. 2013] and microorganisms acting as successful antagonists [Daayf et al. 2003, Son et al. 2008, Chandrakala et al. 2012, Maksimov et al. 2014, Puopolo et al. 2014].

The aim of our study was to search for effective endophytic and epiphytic antagonists against *P. infestans* WD40, isolated from three plants: couch grass (*Agropyron*

repens), white goosefoot (*Chenopodium album* L.) and common horsetail (*Equisetum arvense*).

MATERIALS AND METHODS

Bacterial antagonists against *Phytophthora infestans*. The analysed material included epiphytic and endophytic bacteria isolated from the above-ground portions of three plant species: couch grass (*Agropyron repens*), white goosefoot (*Chenopodium album L.) and common horsetail (Equisetum arvense*). Isolation of epi- and endophytes was conducted according to the procedure described by Rekosz-Burlaga et al. [2014]. The diversity of microbes settled in the above-ground portions of the analysed plants is the topic of a separate paper currently in preparation.

Oomycete strain. Bacterial isolates obtained from the analysed plants were assessed with regard to their antagonistic activity against the oomycete *Phytophthora infestans* WD40. This was accomplished using the procedure described by Daayf et al. [2003]. A total of 63 bacterial strains was assessed. The test strain of *Phytophthora infestans* WD40 used in the analyses was from the collection of the Forest Research Institute in Sękocin near Warszawa.

Antagonist test. Determinations were made on 24-h cultures of bacteria on a nutrient agar medium and several-day cultures of *P. infestans* WD40 on a potato dextrose agar (PDA) medium. Dense cell suspensions in saline solutions were prepared from the bacterial cultures. Later, the medium with the developed oomycetes was sub-divided with a scalpel into 5 × 5 × 5 mm cubes.

The antagonist test on the cultures was carried out on Petri dishes with a diameter of 90 mm on a potato dextrose agar medium. 5 µl of the bacterial suspension were inserted in four, equidistant points of the dish (1, 2, 3, 4). This means that each bacterial isolate occurred in four repetitions on a single dish. For each isolate the test was established on two Petri dishes, resulting in eight repetitions. The dishes were incubated for 24 h at 28 (±2)°C. Next, an earlier prepared cube of a medium with *P. infestans* WD40 was inserted in the central point of the dish. The control batch included dishes with a fragment of *P. infestans* WD40 without bacteria. Further incubation was conducted at room temperature to the moment when *P. infestans* in the control batch grew to the margins of the Petri dish, i.e. reached a diameter of 85 mm. On this day the radius of oomycete growth was measured in the cultures with each of the bacterial strains. This measurement was the base to assess the bacterial antagonistic properties. Based on the obtained data, the coefficient of growth inhibition of *P. infestans* WD 40 was calculated using the formula presented by Daayf et al. [2003]. The percent inhibition provided by each bacterium was calculated as follows:

$$\% \text{ inhibition} = 100 - [(BP/WP) \times 100],$$

where BP and WP represent the infected area (mm) on leaves that were pretreated with a bacterium (BP) or with sterile, distilled (WP) water, respectively, and then inoculated with *P. infestans*.

Statistical analysis. The results of antagonism against *P. infestans* WD 40 were subject to two-factor variance analysis at significance level $p < 0.05$ using the ANOVA statistic model (coefficient of growth inhibition ~ plant + strain bacteria nested plant). The analyzed factors included the plant, from which the bacteria were isolated and the nested factor, which included the strain of bacteria, taking into account the plant from which it was isolated.

Identification of bacterial isolates. Isolates with a strong antagonistic activity were further studied to evaluate their taxonomic affiliation. The identification was performed based on morphological observations, biochemical properties, 16S rRNA gene sequence analysis and randomly amplified polymorphic DNA analysis (RAPD-PCR).

The biochemical characteristics of the selected isolates was tested, including their ability to ferment and utilize different substrates and to produce different metabolites. The obtained results were analyzed according to Bergey's Manual of Systematic Bacteriology [Logan and De Vos 2009]. API 50CHB Biomerieux tests were conducted to confirm the obtained results.

Genomic DNA was isolated using the Genomic Mini AX Bacteria kit (A&A Biotechnology) following the manufacturer's instructions and resuspended in sterile water. Amplification of 16S rRNA gene was performed using F27 (5'-AGAGTTTGATCATGGCTCAG-3') and R1492 (5'-TACGGCTACCTTGTTACGACTT-3') primers [Lagace et al. 2004]. PCR reaction mixtures contained: 4 µl of dNTPs (2.5 mM), 5 µl of each primer (5 µM), 10 × Taq buffer (100 mM Tris-HCl, 500 mM KCl, 0.8% (v/v) Nonidet P40),) 2 µl of $MgCl_2$ (50 mM), 1 µl of DNA, and 2 µl of Taq Nova-RED polymerase. PCR was performed on Bio-Rad T100 Thermal Cycler and the annealing temperature was 56°C. The PCR products were analyzed on 1% agarose gel, purified using a commercial kit (Clean up; A&A Biotechnology) and then sequenced using an automated DNA sequencer (454 GS FLX Titanium, Roche). The 16S rRNA gene sequences were compared with 16S ribosomal RNA sequences (Bacteria and Archaea) in the NCBI database (www.ncbi.nlm.nihgov/blast) using Standard Nucleotide BLAST.

To confirm the species identification, RAPD-PCR was performed in Bio-Rad T-100 Thermal Cycler using 1 µg of isolated DNA as a template, 10 pmol primer S30 (5'-GTGATCGCAG-3') and 1U Run Polymerase (A&A Biotechnology) [Kwon et al. 2009]. Amplification conditions included an initial denaturation step at 94°C for 5 min, 40 cycles each consisting of 94°C for 15 s, 35.5°C for 15 s, 72°C for 2 min and final extension at 72°C for 4 min. RAPD-PCR products were electrophoresed on 2% agarose gel (Bio-Rad apparatus).

RESULTS

Thirty seven endophytic and twenty six epiphytic strains of bacteria were isolated from the analyzed plants. Among them, forty nine strains refer to bacteria capable to develop spores, whereas the remaining strains are bacilli that do not develop spores. Twenty six bacterial strains were isolated from couch grass, of which sixteen represent endophytes and ten – epiphytes (tab. 1). Thirteen endophytes and eight epiphytes have

been isolated from white goosefoot. The lowest number of bacterial strains was isolated from common horsetail – eight endophytes and eight from the phyllosphere. The growth inhibition coefficient *P. infestans* WD40 was determined for each isolate.

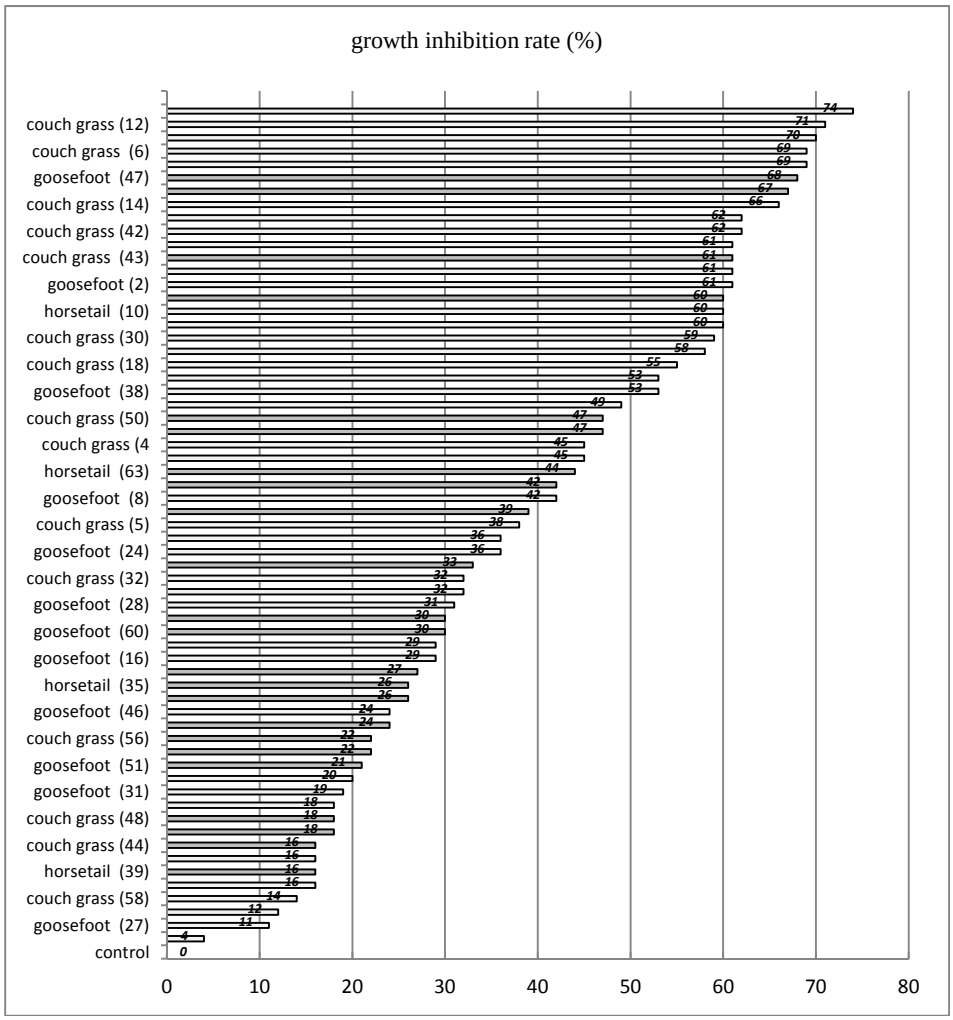

Fig. 1. Growth inhibition of *Phytophthora infestans* WD40 by bacterial strains (%) (in vitro assay). *light bars – endophyte, dark bars – epiphyte

Figure 1 presents the comparison between the coefficient of *P. infestans* WD40 growth inhibition determined for each bacterial isolate: endophytes (light bars) and epiphytes (dark bars). All analysed bacterial strains inhibited the growth of the tested oomycetes and their activity was very variable. The highest value of the coefficient was determined for spore bacilli nos. 22, 12 and 40. The determined coefficient of growth inhibition was 74, 71 and 70%, respectively. All these strains are endophytes, among

which isolates nos. 12 and 40 were from couch grass and isolate no. 22 – from white goosefoot. Additionally, the remaining most active strains, for which the coefficient of *P. infestans* growth inhibition exceeded 60%, also represent spore bacilli. They include endophytes of couch grass: nos. 6 (fig. 1), 14, 11, 42, 34, 43; endophytes of white goosefoot: nos. 23 and 2, as well as epiphytes of white goosefoot: nos. 47 and 45. In this group there is only one isolate from common horsetail, i.e. endophytic strain no. 9.

Table 1. Percentage and number of bacterial isolates inhibiting the growth of *P. infestans*

Plant	Endophyte epiphyte	Number of isolates tested	Number (n) and percentage (%) of bacterial isolates inhibiting the growth of *P. infestans* WD40							
			≥ 40%		40–49%		50–59%		≥ 60%	
			n	%	n	%	n	%	n	%
Couch grass	endophyte	16	11	68.8	2	12.5	2	12.5	7	43.8
	epiphyte	10	4	40.0	2	20.0	0	0.0	2	20.0
	sum	26	15	57.7	4	15.4	2	7.7	9	34.6
Common horse-tail	endophyte	8	4	50	1	12.5	1	12.5	2	25.0
	epiphyte	8	1	12.5	1	12.5	0	0.0	0	0.0
	sum	16	5	31.3	2	12.5	1	6.3	2	12.5
White goosefoot	endophyte	13	7	53.9	1	7.7	2	15.4	4	30.8
	epiphyte	8	3	37.5	1	12.5	0	0.0	2	25.0
	sum	21	10	47.6	2	9.5	2	9.0	6	28.6

Comparison of the antagonistic activity of strains with their origin suggests the presence of some relationships. Among 16 isolates from common horsetail, only five revealed antagonism to *P. infestans* reflected by the coefficient of growth inhibition equal to or higher than 40% (tab. 1). This number represents only 31.3% of all isolates from this plant. From this group, only in the case of two isolates the coefficient of growth inhibition exceeded 60%, whereas only for one isolate it exceeded 50%. In both cases the isolates represented endophytes.

The frequency of antagonism occurrence (growth inhibition coefficient exceeding 40%) in isolates from the two remaining plants was much higher: 47.6% for white goosefoot and 57.7% for couch grass. Worth noting is the fact that the growth inhibition coefficient above 60% was determined in 28.6% isolates from white goosefoot and in 34.6% isolates from couch grass. In twenty six strains from couch grass, only eleven had the coefficient of *P. infestans* WD40 growth inhibition below 40%. Although the frequency of strain occurrence with high antagonistic activity was higher for bacteria isolated from couch grass, the most active strains were isolated from tissues of white goosefoot.

The obtained results were verified using bi-factor analysis of variance at significance level of $p < 0.05$. The analysis shows that plant species is a factor significantly influencing the antagonistic activity of bacterial strains isolated from these plants at $p < 0.0004285$. It also evidenced that the antagonistic properties of bacteria depend on the strain. In this case two nested variables were accepted as factors in the analysis of

variance: the strain and the plant species. Nested factors were used because the obtained strain was strictly linked with the plant species of its origin (tab. 2).

Table 2. Analysis of the variance factor inhibiting the growth of *Phytophthora infestans*

	Degrees of freedom (Df)	Sum of squares (SS)	Mean squares (MS)	Value statistics F (F value)	Significance level (Pr > F)
Plant	2	5338	2669	7.8772	0.0004285 ***
Plant: strain	3	20326	6775	19.9970	2.978e-12 ***
Results	498	168731	339		

Table 3. Comparison of the antagonist activity of endophytes and epiphytes isolated from the tested plants

Endophyte epiphyte	Number of isolates tested	Number of isolates with a coefficient of growth inhibition at different levels							
		40–49%		50–59%		≥ 60%		≥ 40%	
		n*	%*	n	%	n	%	n	%
Endophyte	37	4	11	5	14	13	35	22	59.5
Epiphyte	26	4	15	0	0	4	15	8	30.8

* – % the percentage of isolates exhibiting antagonist activity of the isolates tested in this group, n – indicates the number of isolates having an antagonist activity

Table 4. Comparison of 16S rRNA gene of tested isolates with the most similar sequences submitted to NCBI database

Strain ID	Closest species based on 16S rRNA sequence similarities (%)	GenBank accession number	Sequence similarities (%)
12	*Bacillus subtilis*	CPO 10053.1 CPO10052.1	99
22	*Bacillus subtilis* *B. subtilis* sub. *subtilis*	CPO0053.1 CP01052.1	99
40	*Bacillus* sp.	KM289136.1	99
		KM289135.1	99
	Bacillus subtilis	KJ496376.1	99
		KM823958.1	99
		KM492825.1	99
		KM492823.1	99
		KM492822.1	99
		KM492820.1	99
6I	*Bacillus subtilis*	KJ496376.1	99
		KM823958.1	99
		KM492825.1	99
		KM492823.1	99
		KM492822.1	99
	Bacillus vallismortis	KM084863.1	99
		KM084861.1	99
	Bacillus tequilensis	KJ870196.1	99

Furthermore, analysis of the relationship between the antagonism and the origin of bacterial isolates included calculation and comparison of the percentage contribution of endophytes and epiphytes (tab. 3) between the antagonists. As was noted, 59.5% of the studied endophytes showed antagonistic activity reflected in the growth inhibition coefficient of the oomycete at equal to or higher than 40%. Among the epiphytes, such activity was noted in only 30.8% of the isolates. These results were not verified statistically due to the lack of a specially designed statistical model.

Identification of the most active antagonists of *Phytophthora infestans* WD40. Analysis of the morphological and biochemical properties of the isolates with the strongest antagonistic activity (nos. 6, 12, 22 and 40), enabled to identify them as *Bacillus* species. Analysis of the results supported by Biomerieux APILAB software enable to identify isolates nos. 40, 12, 6 as *Bacillus subtilis* species (with a probability of 96, 89, and 94%, respectively). Isolate no. 22 was identified as *B. licheniformis* with a probability of 93% (tab. 5).

Table 5. Characteristics of bacterial isolates

Characteristic	6	12	22	40
Pigment colonies	–	–	–	–
Yellow-pink-red				
Dark brown/black	–	–	–	–
Spore formation	+	+	+	+
Ellipsoidal				
Swell sporangia	–	–	–	–
motility	–	?	nt*	nt
Parasopral cristals	–	–	–	–
catalase	+	+	+	+
Aerobic grow	+	+	+	+
Anaerobic grow	+	+	?	?
Voges-Proskauer	+	+	+	?
Hydrolysis of starch	+	–	+	?
Nitrate reduction	+	+	+	?
Grow in NaCl 6.5%	+	+	+	+
Grow at 55°C	+	+	+	+
Gas from gulcose	–	–	–	nt
Acid from:				
L-Arabinose	+	+	+	+
D-Glucose	+	+	+	+
Glycogene	+	+	?	–
Mannitol	+	+	+	+
Salicine	–	–	+	+
Glycerol	+	+	+	–
Erthritol	–	–	–	–
D-Arabinose	–	–	–	–
D-Mannose	+	+	+	+

Ribose	+	+	–	+
DXylose	+	+	+	+
LXylose	–	–	–	–
Adonitol	–	–	–	–
Methyl-xyloside	–	–	–	–
Galactose	–	–	–	–
D-Fructose	+	+	+	+
L-Sorbose	–	–	–	–
Rhamnose	–	–	–	–
Dulcitol	–	–	–	–
Inositol	+	+	+	+
Sorbitol	+	+	+	+
Methyl-D-mannoside	–	–	–	–
Methyl-D-glucoside	+	+	+	+
N Acetyl glucosamine	–	–	–	–
Amygdaline	+	+	+	+
Arbutine	+	+	+	—
Esculine	+	+	+	+
Celiobiose	+	+	+	+
Maltose	+	+	+	+
Lactose	–	–	–	–
Meliobiose	+	+	+	+
Saccharose	+	+	+	+
Trehalose	+	+	+	+
Inuline	–	–	–	–
Melezitose	–	–	–	–
D-Raffinose	+	+	+	+
Amidon	–	–	–	–
Xylito	–	–	–	–
Gentiobiose	–	–	–	–
D-Turanose	+	+	+	+
D-Lyxose	–	nt	–	–
D-Tagatose	–	nt	–	–
D-Fucose	–	nt	–	–
L-Fucose	–	nt	–	–
D-Arabitol	–	nt	–	–
L-Arabitol	–	nt	–	–
Gluconate	–	nt	–	–
2 ceto-gluconate	–	nt	–	–
5 ceto-gluconate	–	nt	–	–

nt – no tested

16S rRNA analysis allow to confirm that isolates nos. 12 and 40 are closely related to *Bacillus subtilis*, isolate no. 22 – to *B. subtilis* subsp. *subtilis*, whereas isolate no. 6 was related to three species: *B. subtilis*, *B. vallismortis* and *B. tequilensis* (tab. 4).

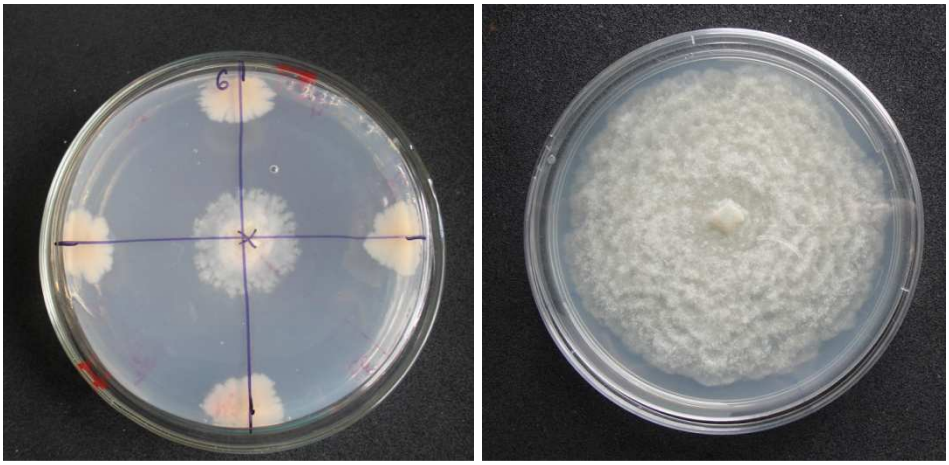

Fig. 2. Antagonistic activity of a bacterial strain (isolate no. 6) against *Phytophthora infestans* WD40 (to the left) and growth of the *P. infestans* control strain (to the right)

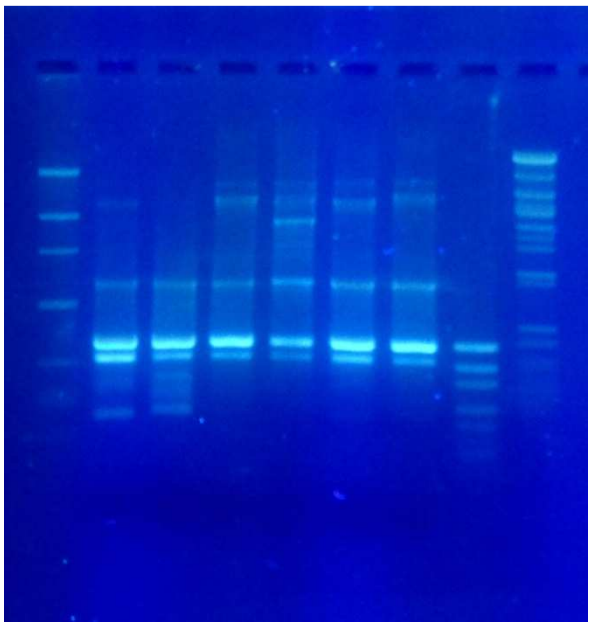

Fig. 3. RAPD-PCR profiles of six *Bacillus* sp. isolates. Line 1, DNA Dramix size ladder (A&A Biotechnology); line 2, *Bacillus* sp. 6; line 3, *Bacillus* sp.12; line 4, *Bacillus* sp. 22; line 5, *Bacillus* sp. 40; line 6, *Bacillus* sp. 6; line 7, *Bacillus* sp. 12; line 8, pUC/MspI size ladder (A&A Biotechnology); line 9, DNA Lambda/AvaII size ladder (A&A Biotechnology)

The RAPD-PCR profiles of the tested isolates (fig. 3) were compared with the profiles of *Bacillus* sp. strains presented by Kwon et al. [2009]. All tested isolates produced common bands of 0.5 and 0.88 kb in size, which were also observed in the *B. subtilis* reference strains. There were no bands of 1.25 or 1.70 kb presented in the *B. licheniformis* reference strains, or 1.1 and 1.5 kb characteristic of *B. amyloliquefaciens* reference strains [Kwon et al. 2009].

DISCUSSION

In plant production arge focus is placed on biological methods, being alternatives for pesticides or aiding in restricting their application [Bacon et al. 2001, Raaijmakers et al. 2002, Sunaina and Ajay 2007, Tran et al. 2007, Cawoy et al. 2011, Chandrakala et al. 2012, Patel et al. 2015]. These methods based on the potential of organisms naturally occurring in the environment and demonstrate antagonistic activity in relation to pathogens or they can increase plant resistance to pathogens. A valuable source of biological agents for biocontrol are internal plant tissues or the phyllosphere, inhabited by microbe populations with specific properties and activities.

In the conducted research, 63 bacterial isolates have been isolated from the phyllo- and endosphere of the above-ground portions of three plants. These bacterial isolates were evaluated with regard to their antagonistic activity against the plant pathogen *P. infestans* WD40. The obtained results show that in the presence of each of the bacterial isolates, *P. infestans* WD40 growth inhibition took place. The largest number of active antagonists was noted in couch grass. For 9 isolates in this group, the studied coefficient was equal to or higher than 60%, which refers to 34.6% of bacteria from this plant. As generally known, couch grass is a very common and oppressive weed. The tip of the runner terminal bud grows through the roots, bulbs and tubers of other plants, and even tree roots. In potato tillages it causes tuber damage. The antagonistic activity of endophytes and epiphytes from this plant against *P. infestans* suggests that the presence of couch grass in tillages may be positive and decrease the susceptibility of potatoes to potato disease. This hypothesis requires further confirmation by field studies. According to the investigations of Daayf et al. [2003], assessment of antagonistic properties depends on the applied method. Moreover, experiments conducted in laboratory conditions do not always find confirmation in field experiments. This fact has been evidenced by research on the activity of *Bacillus subtilis* and *B. pumilus* as antagonists of *Cerospora beticola* (a pathogen of sugar beet) in field conditions. The effective operation of both species was lower by about 20% compared to laboratory conditions [Esh et al. 2011].

The lowest activity in relation to oomycetes had endophytes and epiphytes of common horsetail. Only in the case of two isolates, the coefficient of growth inhibition for *P. infestans* exceeded 60%. This suggests a question on the existence of a relationship between the plant species and the antagonistic activity of bacteria linked with this plant. This hypothesis was confirmed by verifying the results using analysis of variance, which evidenced that the plant species is a significant factor influencing the antagonistic activity of bacteria.

Another interesting result is linked with the comparison of the antagonistic activity of epiphytes and endophytes from the three analysed plants. Among the active antagonists against *P. infestans* WD40, there are almost twice more endophytes than bacteria from the phyllosphere.

The largest activity had bacteria from the genus *Bacillus*. Based on biochemical investigations, three of the isolates were classified to the species *Bacillus subtilis*, and the forth isolate was classified as the species *B. licheniformis*. Analysis of the 16S rRNA sequence has confirmed the result for the first thee isolates; isolate no. 22 was classified to the subspecies *B. subtilis* subsp. *subtilis*. The latter determination was not possible on the basis of biochemical tests, because *B. subtilis* subsp. *subtilis* is phenotypically similar to *B. antrophus* and distinguishable from that species only by pigmentation. Moreover, it is not distinguishable from *Bacillus mojavensis*, *B. subtilis* subsp. *spizixenii* and *B. vallismortis* by phenotypic tests. 16S rRNA sequencing did not give an explicit result for isolate no. 6; in this case 99% homology to three species was obtained. Therefore, further molecular tests – RAPD-PCR were conducted. Isolates nos. 6, 12, 22, and 40, with very strong antagonistic activity, produced bands of 0.5 kb and 0.88 kb, which is in accordance with the results obtained by Kwon et al. [2009] and Jiyeon et al. [2011] for *Bacillus subtilis* reference strains. Kwon et al. [2009] compared 6 tested isolates with 14 reference *Bacillus subtilis* strains and 5 reference *B. licheniformis* strains. This team confirmed that by using the S30 primer it is possible to generate species-specific bands for *Bacillus* sp. strains. *B. subtilis* reference strains produced common bands of 0.5 and 0.88 kb, whereas *B. licheniformis* reference strains produced bands of 1.25, 1.70 and 1.90 kb. For one reference strain, *B. licheniformis* ATCC 14580, 0.5 and 0.88 kb bands were also present. Similar results were obtained by Jiyeon et al. [2011]. The outcomes obtained by Kwon et al. [2009] suggest that the *B. subtilis* species-specific pattern obtained by RAPD-PCR with the S30 primer consists of 0.5 and 0.88 kb bands and there are no bands between 1.2 and 1.9 kb, whereas the *B. licheniformis* species-specific pattern consists of 1.25, 1.70 and 1.90 kb bands. Patterns obtained for our isolates (nos. 6, 12, 22, 40) consisted of 0.5 and 0.88 kb bands but there were no bands between 1.20 and 1.90 kb, which confirms the identification to the species *Bacillus subtilis*.

Available reports confirm the antagonistic properties of *B. subtilis* against many plant pathogens. It has been evidenced that fungal growth was inhibited by *F. maniliforme* [Bacon et al. 2001], *B. cinerea* [Walker et al. 2002] and *C. beticola* [Altahli 2009], as well as *P. capsici* [Khabbaz et al. 2015] and *P. infestans*. In the experiments presented by Daayf et al. [2003], the coefficient of growth inhibition of *P. infestans* for the strains of *B. subtilis* was: 41, 60 and 68% for strains J1, B3 and B1, respectively. Experiments conducted by our team showed a higher activity of bacteria from the *B. subtilis* group. For instance, for strain no. 40, the coefficient of growth inhibition for *P. infestans* WD40 was 70%. The most active antagonist was *B. subtilis* sub. *subtilis* isolated from white goosefoot. The coefficient of growth inhibition for this isolate was 74%. Scientific literature supplies data on the application of *Bacillus* bacteria in biopesticides [Cawoy et al. 2011]. The BD170 (Biopro®) preparation with *B. subtilis* is applied in the biocontrol against fire blight on pears and apples. The product hampers the spreading of *E. amylovora* bacteria, of serious concern in pear and apple orchards [Broggini et al. 2005]. Application of the bacterial isolates, obtained during this re-

search, in practical plant protection requires further studies on the assessment of their abilities to colonize plants, survivability in new conditions and assessment of the antagonist properties in field conditions.

CONCLUSION

1. Antagonistic activity of endophytes and epiphytes depends on host-plant species.
2. Aboveground parts of the white goosefoot (*Chenopodium album* L.) and common horsetail (*Equisetum arvense*) are inhabited by bacteria with antagonistic activity against *P. infestans.*
3. Bacterial biocontrolling agents should be searched among endophytes of the studied plants and not among bacteria controlling their phyllosphere.
4. RAPD-PCR with the S30 primer enable to identify tested strains as *Bacillus subtilis* species.
5. The feasibility of the application of the most active antagonists in plant protection requires further tests in field conditions.

REFERENCES

Altalhi, A. (2009). Genotypic characterization of *Bacillus subtilis* strains colonizing pomegranate phyllosphere and their antagonistic activities against *Cercospora beticola*. Arch. Phytopathol. Plant Prot., 42(6), 524–532.

Bacon, C.W., Yates, I.E., Hinton, D.M., Meredith, F. (2001). Biological control of Fusarium moniliforme in Maize. Environ Health Persp., 109(2), 325–332.

Beakes, G.W., Glockling, S.L., Sekimoto, S., 2012. The evolutionary phylogeny of the oomycete "fungi". Protoplasma, 249(1), 3–19. doi: 10.1007/s00709-011-0269-2

Broggini, G.A.L., Duffy, B., Holliger, E., Schärer, H., Gessler, C., Patocchi, A. (2005). Detection of the fire blight biocontrol agent *Bacillus subtilis* BD170 (Biopro) in a Swiss apple orchard. Eur. J. Plant Pathol., 111(2), 93–100.

Cawoy, H., Bettiol, W., Fickers, P., Ongena, M. (2011). *Bacillus* – based biological control of plant diseases. In: Pesticides in the modern world – pesticides use and management, Stoytcheva, M. (ed.). Pub. InTech, 273–302.

Chandrakala, A., Chandrashekar, S.C., Jyothi, G., Ravikumar, B.M. (2012). Effect of cell-free culture filtrates of bio-control agents on the spore germination and infection by *Phytophthora infestans* causing late blight of potato. Glob. J. Biol. Agricult. Health Sci., 1(2), 40–45.

Cooke, D.E.L., Lees, A.K. (2004). Markers, old and new, for examining *Phytophthora infestans* diversity. Plant Pathol., 53, 692–704.

Daayf, F., Adam, L., Fernando, W.G.D. (2003). Comparative screening of bacteria for biological control of potato late blight (strain US-8), using in vitro, detached-leaves, and whole-plant testing systems. Can. J. Plant Pathol., 25, 276–284.

Esh, A.M., Atia, M.M.M., Tohamy, M.R.A., Taghian, S. (2011). Systemic resistance in sugar beet elucidated by non-pathogenic, phyllosphere-colonizing *Bacillus pumilus* and B. *subtilus* against the pathogen *Cercospora beticola* Sacc. J. Plant Protect. Pathol., 2(1), 67–83.

Gisi, U., Cohen, Y. (1996). Resistance to phenylamide fungicides: a case study with *Phytophthora infestans* involving mating type and race structure. Ann. Rev. Phytopath., 34, 549–572.

Griffith, J.M., Davis, A.J., Grant, B.R. (1992). Target sites of fungicides to control Oomtcetes. In: Target sites of fungicide action, Koller, W. (ed). London, CRC Press, 69–100.

Hallmann, J., Quadt-Hallmann, A., Mahaffee, W.F., Kloepper, J.W. (1997). Bacterial endophytes in agricultural crops. Can. J. Microbiol., 43(10), 895–914.

Hardham, A.R. (2005). Pathogen profile *Phytophthora cinnamomi*. Mol. Plant Pathol., 6(6), 589–604.

Jiyeon, L., Kwon, G.-H., Park, J.-Y., Park, C.-S., Kwon, D.Y., Lim, J., Kim, J.S., Kim, J.H. (2011). A RAPD-PCR Method for the rapid detection of *Bacillus cereus*. J. Microbiol. Biotechnol., 21(3), 274–276. doi: 10.4014/jmb.1008.08031

Judelson, H.S., Blanco, F.A. (2005). The spores of *Phytophthora*: weapons of the plant destroyer. Nat. Rev. Microbiol. 3, 4 7–58.

Jung, T., Burgess, T.I. (2009). Re-evaluation of *Phytophthora citricola* isolates from multiple woody hosts in Europe and North America reveals a new species, *Phytophthora plurivora* sp. nov. Persoonia, 22, 95–110.

Kwon, G.H., Lee, H.A., Park, J.Y., Kim, J.S., Lim, J. (2009). Development of a RAPD-PCR method for identification of *Bacillus* species isolated from Cheonggukjang. Int. J. Food Microbiol., 129, 282–287.

Khabbaz, S.E., Zhang, L., Cáceres, L.A., Sumarah, M., Wang, A., Abbasi, P.A. (2015). Characterisation of antagonistic *Bacillus* and *Pseudomonas* strains for biocontrol potential and suppression of damping-off and root rot diseases. Ann. Appl. Biol., 1–16. doi: 10.1111/aab.12196

Lagace, L., Pitre, M., Jacques, M., Roy, D. (2004). Identification of the bacterial community of maple sap by using amplified ribosomal DNA (rDNA) restriction analysis and rDNA sequencing. Appl. Environ. Microbiol., 70(4), 2052–2060. doi: 10.1128/AEM.70.4.2052-2060

Logan, N.A., De Vos, P. (2009). Genus I. *Bacillus* Cohn 1872, 174AL. In: Bergey's manual of systematic bacteriology, De Vos, P., Garrity, G.M., Jones, D., Krieg, N.R., Ludwig, W., Rainey, F.A., Schleifer, K.H., Whitman, W.B. (eds). 2nd Edition, Springer, New York, 3, 21–128.

Maksimov, I.V., Abizgildina, P.P., Sorokan', A.V., Burkhanova, G.F. (2014). Regulation of peroxidase activity under the influence of signaling molecules and *Bacillus* subtilis 26D in potato plants infected with *Phytophthora infestans*. Appl. Biochem. Microbiol., 50(2), 197–202.

Moushib, L., Wtzell, J., Lenman, M., Liljeroth, E., Andreasson, E. (2013). Sugar beet extract induces defense against *Phytophthora infestans* in potato plants. Eur. J. Plant Pathol. 136(2), 261–271. doi: 10.1007/s10658-012-0160-9

Nelson, S.C. (2008). Late blight of tomato (*Phytophthora infestans*). Plant Disease, PD-45, 1–10.

Nongkhlaw, F.M.W., Joshi, S. R. (2014). Epiphytic and endophytic bacteria that promote growth of ethnomedicinal plants in the subtropical forests of Meghalaya, India. Home, 62(4). http://revistas.ucr.ac.cr/index.php/rbt/article/view/12138/15463

Patel, R.R., Thakkar, V.R., Subramanian, B.R. (2015). A *Pseudomonas guariconensis* strain capable of promoting growth and controlling collar rot disease in *Arachis hypogaea* L. Plant Soil, 390 (1), 369–381. doi 10.1007/s11104-015-2436-2

Pini, F., Galardini, M., Bazzicalupo, M., Mengoni, A. (2011). Plant-bacteria association and symbiosis: Are there common genomic traits in *Alphaproteobacteria*? Genes, 2, 1017–1032. doi:10.3390/genes2041017

Puopolo, G., Cimmino, A., Palmieri, M.C., Giovannini, O., Evidente, A., Pertot, I. (2014). *Lysobacter capsici* AZ78 produces cyclo(L-Pro-L-Tyr), a 2,5-diketopiperazine with toxic activity against sporangia of *Phytophthora infestans* and *Plasmopara viticola*. J. Appl. Microbiol., 117, 1168–1180.

Raaijmakers, J.M., Vlami, M., de Souza, J.T. (2002). Antibiotic production by bacterial biocontrol agents. Antonie van Leeuwenhoek, 81, 537–547.

Rekosz-Burlaga, H., Borys, M., Goryluk-Salmonowicz, A. (2014). Cultivable microorganisms inhabiting the aerial parts of *Hypericum perforatum*. Acta Sci. Pol. Hortorum Cultus, 13(5), 117–129.

Schlenzig, A., Habermayer, J., Zinkernagel, V. (1999). Serological detection of latent infection with *Phytophthora infestans* in potato stems. Zeitschrift für Pflanzenkrankheiten und Pfl, 105(1), 22–33.

Sedláková, V., Dejmalová, J., Hausvater, E., Sedlák, P., Doležal, P., Mazáková, J. (2011). Effect of *Phytophthora infestans* on potato yield in dependence on variety characteristics and fungicide control. Plant Soil Environ., 57(10), 486–491.

Son, S.W., Kim, H.Y., Choi, G.J., Lim, H.K., Jang, K.S., Lee, S.O., Lee, S., Sung, N.D. (2008). Bikaverin and fusaric acid from *Fusarium oxysporum* show antioomycete activity against *Phytophthora infestans*. J. Appl. Microbiol., 104, 692–698.

Stephan, D., Schmitt, A., Martins Carvalho, S., Seddon, B., Koch, E. (2005). Evaluation of biocontrol preparations and plant extracts for the control of *Phytophthora infestans* on potato leaves. Eur. J. Plant Pathol., 112, 235–246.

Sunaina, V., Ajay, S. (2007). Evaluation of biocontrol agent *Bacillus subtilis* b5 for control of *Phytophthora infestans* on potato leaves. Potato J., 34(1–2), 79–80.

Tran, H., Ficke, A., Asiimwe, T., Höfte, M., Raaijmakers, J.M. (2007). Role of the cyclic
lipopeptide massetolide a in biological control of *Phytophthora infestans* and in colonization of tomato plants by *Pseudomonas fluorescens*. New Phytolog., 175(4), 731–742.

Yang, Ch., Crowley, D.E., Borneman, J., Keen, N.T. (2000). Microbial phyllosphere populations are more complex than previously realized. PNAS, 98(7), 3889–3894.

Walker, R., Ferguson, C.M.J., Booth, N.A., Allan, J. (2002). The symbiosis of *Bacillus subtilis* L-forms with Chinese cabbage seedlings inhibits conidial germination of *Botrytis cinerea.* Lett. Appl. Microbiol. 34(1), 42–45.

Whipps, J.M., Hand, P., Pink, D., Bending, G.D. (2008). Phyllosphere microbiology with special reference to diversity and plant genotype. J. Appl. Microbiol., 105(6), 1744–1755.

AKTYWNOŚĆ ANTAGONISTYCZNA MIKROORGANIZMÓW ŻYJĄCYCH W ASOCJACJI Z ROŚLINAMI WOBEC *Phytophthora infestans*

Streszczenie. *Phytophthora infestans* jest patogenem zagrażającym uprawie ziemniaków I pomidorów. Aby chronić rośliny przed tym patogenem, poszukuje się metod biologiczne bezpiecznych dla środowiska. W roślinach i na ich powierzchni jest obecnych wiele naturalnych czynników kontroli. Badania miały na celu poszukiwanie skutecznych antagonistów *P. infestans* wśród bakterii pozyskanych z trzech roślin: *Equisetum arvense*, *Elymus repens* oraz *Chenopodium album* L. Badaniom poddano 37 izolatów bakterii endofitycznych oraz 26 izolatów bakterii epifitycznych. Hamujący wpływ bakterii na wzrost *P. infestans* został oszacowany na podstawie współczynnika relatywnego wzrostu. Wszystkie testowane izolaty bakterii ograniczały wzrost *P. infestans*. Największą ak-

tywność stwierdzono dla gatunku *Bacillus subtilis*. Aktywność antagonistyczna bakterii była istotnie zróżnicowana w zależności od gatunku rośliny, z której zostały wyizolowane oraz od miejsca ich występowania (endosfera, fyllosfera). Endofity hamowały wzrost *P. infestans* znacznie skuteczniej niż epifity (o ponad 40% dla większości izolatów). Wnioskuje się, że bakteryjnych czynników biokontroli należy szukać wśród endofitów, a nie wśród epifitów badanych roślin.

Słowa kluczowe: endofity, epifity, bakteryjne czynniki biokontroli, zaraza ziemniaczana

Accepted for print: 26.04.2016

For citation: Rekosz-Burlaga, H., Tokarczyk, E., Szczepaniak, J., Goryluk-Salmonowicz, A. (2016). Antagonistic activity of plant-associated microorganisms against *Phytophthora infestans*. Acta Sci. Pol. Hortorum Cultus, 15(6), 3–18.