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Biological Control as an Alternative Method of Protecting Crops Against Fungal Pathogens

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1. Introduction

The intensive development of agriculture in the 20th century has led to a significant increase in food production through the cultivation of efficient crop varieties and the widespread use of fertilizers and chemical plant protection products. Intensive agricultural production has contributed significantly to environmental degradation and pollution. The great challenge for modern societies is to increase plant production in a sustainable, environmentally friendly way, assuming that global food production must keep pace with an increasingly urbanized and growing population. Another problem is the adverse effects of climate change. In order to feed the world, the existing approach to agriculture should be re-examined, which includes the use of artificial fertilizers, herbicides, fungicides and insecticides (Morrissey et al., 2004). Meeting the global challenges of climate change and population growth, and better understanding and control of soil processes will be one of the most important challenges of science in the next decade (McNear Jr. 2013). An increasing number of scientists dealing with increasing the efficiency of agricultural production can see the potential hidden in the roots and adjacent soil included the richness of microorganisms, called the rhizosphere. It is even believed that the roots and rhizosphere are the key to the further development of agriculture (Gewin 2010). In the rhizosphere there are many interactions between different groups of microorganisms and between microorganisms and plants. They are both positive and negative (Whipps 2001). Positive interactions between microorganisms and plants can be symbiotic or non-symbiotic and contribute to the stimulation of plant growth (Glick 2012, Kalitkiewicz & Kępczyńska 2008), whereas between microorganisms they can rely on the mutual use of metabolism products (Kołwzan et al. 2006). Negative interactions between microorganisms and plants are caused by plant pathogens and they lead to

disruption of basic physiological functions of plants, causing diseases and thus a decrease in productivity in agricultural production. Negative interactions between microorganisms can be used to biological control of plant pathogens. Importantly, the use of microorganisms to inhibit the development of plant diseases is an environmentally friendly solution (Bolwerk & Lugtenberg 2005, Lugtenberg & Kamilova 2009). Bacteria can limit the development of pathogens, including through competition for space on the root, reducing iron availability to pathogens by chelation to siderophores, synthesis of antifungal and antibacterial metabolites, including antibiotics and production of enzymes lysing fungal cell walls (Glick, 2012). Rhizobacteria that produce siderophores may limit the occurrence of certain diseases by hindering phytopathogens to obtain enough iron, thus limiting their ability to develop properly. They achieve this mainly due to the fact that the siderophores have a much greater affinity for iron binding compared to fungal pathogens (Dowling et al. 1996). Many bacteria also produce peptide antibiotics. These are oligopeptides that inhibit the synthesis of cell walls of pathogens (Maksimov et al 2011), including 2,4 - diacetylphloroglucinol (2,4-DAPG), hydrogen cyanide, oomycin A, phenazine, tensin and cyclic lipopeptides produced by bacteria from the genus *Pseudomonads*, as well as oligomycin A, zwittermycin and xantobacin produced by *Bacillus*, *Streptomyces* and *Stenotrophomonas* spp. (Compant et al. 2005). Extremely important is the ability of bacteria to produce enzymes lysing the cell walls of the fungal pathogens and thereby limit their spreading on the soil. These enzymes include chitinases, cellulases, β-1,3 glucanases, proteases and lipases. Chitinases and glucanases are key enzymes responsible for the hydrolysis of chitin and glucan, the main components of fungal cell walls (Kisiel & Kępczyńska 2017). It has been proven the effectiveness of antifungal activity of bacteria, including to *Streptomyces*, *Bacillus*, *Pseudomonas* or *Serratia* against such plant pathogens as *Fusarium*, *Botrytis*, *Rhizoctonia* and many others (Kisiel & Kępczyńska 2017).

The aim of this study was to determine the effectiveness of bacteria isolated from alfalfa (*Medicago sativa*) rhizosphere to reduce the growth of fungal pathogens, as well as to check the possible mechanisms used for this purpose by these bacteria.

2. Material and methods

A total of 16 strains of bacteria were isolated from the soil collected from the alfalfa (*Medicago sativa*) rhizosphere cultivated near Stargard (Zachodniopomorskie). Isolates were subjected to molecular identification based on the 16S rRNA gene fragment (Kisiel & Kępczyńska 2016). The present study used the following strains: from the family *Bacillaceae* (*Bacillus niacini* KK 1b, *B. megaterium* KK 11, *Lysinibacillus fusiformis* KK 2 i *L. fusiformis* KK 3, *Paenibacillus*

odorifer KK 1a, *P. borealis* KK 4, *P. amylolyticus* KK 9a), family *Pseudomonadaceae* (*Pseudomonas brassicacearum* KK 5, *P. corrugata* KK 7 and *P. corrugata* KK 12), family *Xantomonadaceae* (*Stenotrophomonas maltophilia* KK 8b and *S. maltophilia* KK 9b), family *Enterobacteriaceae* (*Citrobacter muliniae* KK 10, *Leclercia adecarboxylata* KK 6 and *Raoultella planticola* KK 8a) and family *Rhizobiaceae* (*Sinorhizobium meliloti* KK 13*)*.

Fungal pathogens used in this study *Phoma medicaginis* Malbr. strain Ph 33 and *Fusarium culmorum* strain Cul-3 were made available from the collection of the Institute of Plant Genetics, PAS in Poznań. A sporulating mycelium was used to make an inoculum. Using a hemocytometer (Bürker chamber), the density of the spore suspension was determined using the formula:

$$Ld = a \times b \times 2500 \times 1000$$

where:
Ld – number of spores in 1 ml,
a – average number of spores,
b – dilution used.

The studies used a suspension with a density of 10^6 spores per 1 ml of inoculum.

The effect of bacteria on fungal growth evaluated on solid and liquid media. In the first method, 7-day *Fusarium culmorum* or *Phoma medicaginis* culture and bacterial colonies were placed on opposite poles of the dish with PDA medium. After 7 days, the diameter of the mycelium was measured. To assess the growth of the fungus in liquid cultures, bacteria grown in liquid medium Czapek DOX were used. The culture supernatant was aseptically filtered through 0.45 μm sterile membrane filters. For the falcon tubes containing the Czapek DOX medium, in combination with the 5% bacterial culture filtrate, 100 μl of the inoculum was added. After 5 days, the cultures were placed on filter paper and dried, and weighed to determine the dry weight. The inhibition rate of fungal growth in both cases was expressed in relation to the control being pure fungal cultures.

The activity of β-1,3-glucanase in bacterial cells was determined by spectrophotometric method for Lim et al. (1991). Bacteria were grown in M9 or DF media with the addition of 0.02% laminarin (from *Laminaria digitata*). Measurements were made in a plate spectrophotometer at 540 nm. The calculations were made after determining the standard curve for glucose in the range of 0 - 10 mM. The unit of β-1,3-glucanase activity was defined as 1 μmol of released glucose/mg protein/min.

Chitinase activity was determined based on the ability of the strains to hydrolyze chitin in the medium. The medium was supplemented with a suspen-

sion of colloidal chitin, prepared according to the procedure described by Rodriguez-Kabana et al. (1983). The medium for determining the chitinase activity consisted of (g/l) $(NH_4)_2SO_4$ – 1.0; KH_2PO_4 – 1.34; yeast extract – 5.0; bacteriological agar – 20.0; 0.01 M Tris-HCl topped to 1L and 1% colloidal chitin. Bacterial colonies were applied to the plates and incubated at 28°C for 14 days. After this time the clearing zones around the colony were measured.

The production of siderophores was determined using CAS-Agar medium and assessing clearing zones around the bacterial colonies. The Chrome azurol S (CAS) culture medium was prepared according to the procedure according to Alexander and Zuberer (1991). HDTMA, a cationic detergent that stabilizes Fe-CAS and gives a blue color, which turns orange in the presence of siderophores, was added to the medium. Bacteria were inoculated into this prepared medium and incubated in a thermostat at 28°C for 5 days. After this time, the ability to produce siderophores was estimated based on the diameter of the orange zones visible after iron binding by the siderophores. To evaluate the effects of siderophores and chitinases used the 5-grade scale: „-" – 0-0,5 mm; „+/-" – 0,5-1 mm; „+" – 1-2 mm; „++" – 2- 3 mm; „+++" - > 3 mm.

3. Results and discussion

The pathogens that most often attack alfalfa crop (*Fusarium culmorum* Cul-3 oraz *Phoma medicaginis* Ph-33) was used to evaluate the potential use of bacteria for biological control against fungal pathogens. It was found that all bacterial strains inhibit the growth of *F. culmorum* Cul-3 on both solid and liquid media (Fig. 1A). The most inhibitory effect on this fungus on solid medium, as much as 42.2% showed *Raoultella planticola* KK 8a strain. A significant inhibition of fungal growth was also caused by *Pseudomonas corrugata* KK 12 strain (39.3%) and both strains *Stenotrophomonas maltophilia* KK 8b (36.4%) and KK 9b (31.1%). A higher degree of fungal growth inhibition was observed on the liquid medium in the presence of bacterial filtrates. Similarly to the solid medium, the most effective strains were *Raoultella planticola* KK 8a (64.5%) and *Pseudomonas corrugata* KK 12 (46.5%). Previously, antifungal activity of bacteria of the genus *Pseudomonas* and *Bacillus* has been demonstrated against pathogens such as *Fusarium oxysporum* and *Rhizoctonia solani* or *Sclerotinum rolfsii* (Wahyudi et al. 2011a and b). The strains of the genus *Pseudomonas* (Khan et al. 2006) and *Bacillus*, as well as *Stenotrophomonas* (Kamil et al. 2007) proved effective against *Fusarium culmorum*.

All isolated strains also inhibited the growth of *P. medicaginis* (`. 1B). The highest inhibition of this fungus on solid media was found for *Pseudomonas brassicacearum* KK 5, it was 35.5%. Two strains from the *Bacillaceae* family, *Lysinibacillus fusiformis* KK 3 and *Bacillus megaterium* KK 11 also significantly limited

the growth of this fungus by 25.1% and 22.6%, respectively. In liquid cultures of *P. medicaginis*, as in the case of *Fusarium*, most strains showed a higher ability to inhibit fungal growth compared to cultures on solid media. *Pseudomonas corrugata* KK 7 and *Leclercia adecarboxylata* KK 6 inhibited the growth of fungal pathogen in 46%, while strains from the *Enterobacteriaceae* family, *Raoultella planticola* KK 8a and *Citrobacter murliniae* KK 10, respectively in 35.9% and 33.5%. Efficacy of bacteria in limiting the development of *Phoma medicaginis*, one of the most dangerous fungal pathogens of alfalfa, has been previously observed for bacteria of the genus *Pseudomonas* (Guevara & Lukezic 2000), *Sinorhizobium* (Mrabet et al. 2011) or *Bacillus* (Slimene et al. 2015).

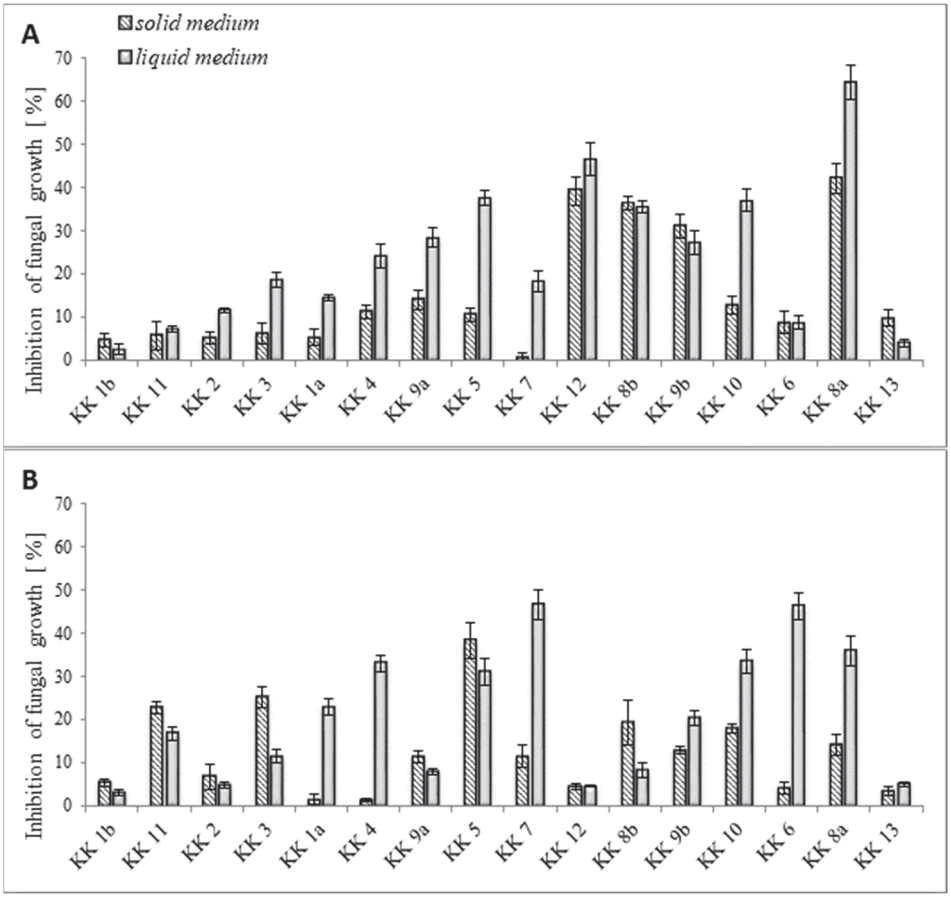

Fig. 1. Inhibition of growth *Fusarium culmorum* Cul-3 (A) and *Phoma medicaginis* Ph-33 (B) by bacteria

Various mechanisms were found as the core of the antifungal activity of rhizobacteria: the ability to produce antibiotics and metabolites (HCN, siderophores, 2,4-DAPG) and cell wall lysing enzymes (glucanases and chitinases) (Beneduzi et al. 2012). After confirming that all strains of isolated bacteria were more or less able to inhibit the growth of both fungal pathogens *F. culmorum* Cul-3 and *P. medicaginis* Ph-33, it was necessary to check whether this could be due to the production of enzymes that hydrolyse the fungal cell wall or in the case of *Pseudomonas* strains, the ability to produce the 2,4-DAPG antibiotic (Table 1).

Table 1. Bacterial features affecting antifungal activity

Strain of bacteria	β-1,3-glucanase activity (μM glucose/mg protein/min)	chitinase activity	siderophore production
KK 1b	62,7 ± 5,1	+	-
KK 11	13,3 ± 7,7	-	+++
KK 2	70 ± 0,2	-	-
KK 3	60 ± 6,8	-	+
KK 1a	51,7 ± 9,1	++	-
KK 4	72,5 ± 5,9	+	+
KK 9a	37,3 ± 11	-	+++
KK 5	12,8 ± 5,1	-	+++
KK 7	2,3 ± 0,1	-	+++
KK12	13,2 ± 7,7	-	++
KK 8b	9,2 ± 4,7	+++	+++
KK 9b	43,5 ± 11,9	+	++
KK 10	6 ± 2,4	-	++
KK 6	16,3 ± 3,3	-	+++
KK 8a	2,4 ± 0,2	-	+
KK 13	2,2 ± 0,4	-	+

All the bacterial strains isolated from *M. sativa* rhizosphere showed β-1,3-glucanase activity in the range of 2.3 to 72.5 μmol glucose/mg protein/min. The highest activity of this enzyme was found in strains belonging to the family *Bacilliaceae*, and in particular in *Paenibacillus borealis* KK 4 (72.5 μM), both strains belonging to *Lysinibacillus fusiformis* KK 2 (70 μM) and KK 3 (60 μM) and *Bacillus niacini* KK 1b (62.7). Of the other strains, fairly high activity of this enzyme was found in *S. maltophilia* KK 9b (43.5). The lowest activity (2.2) was observed for the *Sinorhizobium meliloti* KK 13. Of all the isolated bacterial strains, only five showed chitinolytic activity. The highest activity of chitinases was characterized by a strain of *Stenotrophomonas maltophilia* KK 8b, the

slightly lower *Paenibacillus odorifer* KK 1a, the lowest *Bacillus niacini* KK 1b, *Paenibacillus borealis* KK 4 and *S. maltophilia* KK 9b. In addition, thirteen out of the 16 analyzed strains of rhizobacteria were decomposing the colored complex present in the CAS medium (CAS-Fe(III)), was evidence of the production of iron chelating compounds. The most efficient siderophore producers were the following 6 strains: *Bacillus megaterium* KK 11, *Paenibacillus amylolyticus* KK 9a, *Pseudomonas brassicacearum* KK 5, *P. corrugata* KK 7, *Stenotrophomonas maltophilia* KK 8b, *Leclercia adecarboxylata* KK 6. Due to the ability to produce chelating compounds, whose primary function is to chelate the ferric iron, bacterial make it available for plant thereby while limiting the availability of the element for pathogens (Glick 2012). In earlier studies siderophores has been detected among others in bacteria of the genus *Pseudomonas* (Luján et al. 2015), *Bacillus* (Wahyudi et al. 2011b) or *Stenotrophomonas* (Kumar & Audipudi 2015).

Ahmad et al. (2008) showed a close relationship between the anti-fungal effect of bacteria and the production of HCN and siderophores. Similarly, as in the case of these studies, in other studies the production of bacterial chitinases did not correlated with the fungal growth inhibition (Wahyudi et al 2011a). In turn, Slimene et al. (2015) showed that in *Bacillus licheniformis* S213 rhizobacteria, the activity of chitinolytic enzymes can be increased by cultivating them on the medium with the addition of colloidal chitin, which provided high activity this bacteria in inhibiting the growth of fungi, such as *F culmorum* or *P. medicaginis*. The same bacteria cultured on medium without colloidal chitin limited the growth of these fungal pathogens to little or none at all. This may be due to the fact that bacterial chitinases activate with a significant delay, which is related with the long time needed for the degradation of high molecular weight chitin that is found in the cell walls of fungi. In addition, the production of chitinases by bacteria is closely related to the concentration of colloidal chitin, incubation time and nutrient composition or pH (Gomaa 2012).

It is known that not only fungal cell wall hydrolysing enzymes, i.e. glucanases and chitinases, are involved in inhibiting the growth of fungal pathogens, but also antibiotics and secondary metabolites produced by bacteria. This group of antimicrobial compounds includes the broad-spectrum antibiotic, 2,4-diacethylphloroglucinol (2,4-DAPG). *Pseudomonas* bacteria can produce this metabolite (Raaijmakers et al. 1997). Of all three isolates from the Pseudomonadaceae family analyzed in this work in *P. brassicacearum* KK 5 and *P. corrugata* KK 12, the presence of the *phlD* gene, responsible for the synthesis of monoacetylglucinol, the precursor 2,4-DAPG, was previously confirmed (Kisiel & Kępczyńska 2016). This gene has been detected in many strains belonging to *Pseudomonas*, including *P. brassicacearum* (Kwak & Weller 2013).

The observed inhibition of the growth of both pathogens *Medicago* spp. (*Phoma medicaginis* and *Fusarium culmorum*) by *P. brassicacearum* KK 5 and

P. corrugata KK 12 may be probably the resultant production of the metabolite 2,4-DAPG and β-glucanase activity. Participation in this process does not take endocitinase, in three *Pseudomonas* species no activity of these enzymes has been detected. It is difficult to determine which mechanisms are crucial for antifungal activity. The most effective bacteria that inhibit fungal growth included both, which at the same time were capable of high production of siderophores and were found to have chitinases and glucanases activity, such as *Stenotrophomonas maltophilia* KK 8b and those in which the production of siderophores was at a low level, as was the activity of enzymes that hydrolyzed fungal cell walls, such as *Stenotrophomonas maltophilia* KK 8a.

4. Conclusions

Biological control can be an alternative to chemical plant protection products like pesticides and help reduce them and the same contribute to protect the environment. This study confirms the potential of bacteria isolated from the rhizosphere to control the dangerous biological fungal pathogens that limit crops.

1. The results presented here confirm that bacteria isolated from the Medicago sativa rhizosphere were able to biological control of fungal pathogens such as Fusarium culmorum and Phoma medicaginis in vitro.
2. The observed inhibition of growth of these pathogens by the tested bacteria may be related to the production of enzymes that hydrolyse fungal cell walls, i.e. chitinases and glucanases, and in the case of bacteria of the *Pseudomonas* genus, the production of the secondary metabolite like 2,4-DAPG.
3. Potentially, in limiting the development of fungal pathogens may also be the useful ability of bacteria to produce siderophores that bind iron and thus prevent the fungus from taking this element.

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Abstract

Progressive degradation of the environment caused, among others, by the excessive use of chemical pesticides forces us to look for alternative methods of protecting crops against pathogens. Definitely beneficial for the environment, but also, as confirmed by numerous studies, the use of biological control mechanisms can be an effective solution. The bacteria can inhibit the growth of fungi through the production of enzymes that lyse their cell walls, such as chitinases and glucanases, but also by limiting the availability of microelements important to growth, such as iron, by chelating them to siderophores, and finally producing antibiotics and secondary metabolites, like 2,4-DAPG.

The present study examined 16 strains of bacteria isolated from *Medicago sativa* rhizosphere for their suitability for the control of fungal pathogens such as *Fusarium culmorum* and *Phoma medicaginis*. Among of bacteria were strains belonging to the family *Bacillaceae* (genus *Bacillus*, *Lysinibacillus* and *Paenibacillus*), the family *Pseudomonadaceae* (genus *Pseudomonas*), the family *Xantomonadaceae* (genus *Stenotrophomonas*) of the *Enterobacteriaceae* family (genus *Citrobacter*, *Leclercia* and *Raoultella*) and of the family *Rhizobiaceae* (genus *Sinorhizobium*).

In vitro, both on solid and liquid media, all bacterial strains were able to limit the growth of *Fusarium culmorum* and *Phoma medicaginis*. The effective inhibitors of *Fusarium culmorum* Cul-3 were *Roultella planticola* KK 8a, *Pseudomonas corrugata* KK

12, and both strains belonging to *Stenotrophomonas maltophilia* KK 8b and KK 9b. Limiting the development of the second *Medicago* pathogen, *Phoma medicaginis*, was the most effective after the use of *Pseudomonas corrugata* KK 7, *Leclercia adecarboxylata* KK 6 and *Pseudomonas brassicacearum* KK 5.

By studying the mechanisms that may be potential for bacteria to inhibit the growth of fungi was tested production of lytic enzymes the cell walls, and siderophores. All bacterial strains showed β-1,3-glucanase activity in the range of 2.3 to 72.5 μmol glucose /mg protein/min. Five strains showed chitinolytic activity, with *Stenotrophomonas maltophilia* KK 8b being the most active. Thirteen of the 16 analyzed strains of rhizobacteria were able to produce iron chelating compounds, siderophores. In addition, the presence of the *phlD* gene was confirmed in *Pseudomonas brassicacearum* KK 5 and *P. corrugata* KK 12, which may indicate the production of 2,4-DAPG.

The results obtained indicate that the bacteria isolated from the rhizosphere have the potential for biological control of fungal pathogens, which limit the plant cultivation, using various mechanisms. The use of rhizobacteria as biopesticides is an environmentally friendly alternative to chemical plant protection products.

Keywords:
fungal pathogens, biological control, rhizobacteria

Zwalczanie biologiczne jako alternatywna metoda ochrony upraw przed patogenami grzybowymi

Streszczenie

Postępująca degradacja środowiska spowodowana między innymi nadmiernym wykorzystywaniem chemicznych środków ochrony roślin zmusza nas do poszukiwania alternatywnych metod zabezpieczania upraw przed patogenami. Zdecydowanie korzystnym dla środowiska ale również, jak potwierdzają liczne badania, skutecznym rozwiązaniem może być wykorzystanie mechanizmów zwalczania biologicznego. Bakterie mogą ograniczyć rozwój patogenów grzybowych poprzez produkcję enzymów lizujących ich ściany komórkowe, takich jak chitynazy i glukanazy ale także poprzez ograniczenie dostępności ważnych dla ich wzrostu mikroelementów, jak żelazo, chelatując je do sideroforów, czy wreszcie produkując grzybobójcze antybiotyki i metabolity wtórne, jak 2,4-DAPG.

W niniejszych badaniach przeanalizowano 16 szczepów bakterii wyizolowanych z ryzosfery *Medicago sativa* pod kątem ich przydatności do zwalczania patogenów grzybowych takich jak *Fusarium culmorum* i *Phoma medicaginis*. Wśród bakterii były szczepy należące do rodziny *Bacillaceae* (rodzaju *Bacillus*, *Lysinibacillus* i *Paenibacillus*), z rodziny *Pseudomonadaceae* (rodzaju *Pseudomonas*), z rodziny *Xantomonadaceae* (rodzaju *Stenotrophomonas*), z rodziny *Enterobacteriaceae* (rodzaju *Citrobacter*, *Leclercia* i *Raoultella*) oraz z rodziny *Rhizobiaceae* (rodzaju *Sinorhizobium*).

W warunkach *in vitro* zarówno na pożywkach stałych jak i płynnych wszystkie analizowane szczepy bakterii były zdolne do ograniczenia wzrostu *Fusarium culmorum* oraz *Phoma medicaginis*. Do skutecznych inhibitorów rozwoju *Fusarium culmorum* Cul-

3 należy zaliczyć szczepy *Roultella planticola* KK 8a, *Pseudomonas corrugata* KK 12, oraz oba szczepy należące do *Stenotrophomonas maltophilia* KK 8b i KK 9b. Ograniczenie rozwoju drugiego patogena *Medicago*, tj. grzyba *Phoma medicaginis* było najskuteczniejsze po zastosowaniu szczepów *Pseudomonas corrugata* KK 7, *Leclercia adecarboxylata* KK 6 i *Pseudomonas brassicacearum* KK 5.

Badając mechanizmy, które mogą stać za potencjałem bakterii do hamowania wzrostu grzybów sprawdzono produkcję enzymów lizujących ściany komórkowe oraz sideroforów. Wszystkie szczepy bakterii wykazywały aktywność β-1,3-glukanazy w zakresie od 2,3 do 72,5 μmol glukozy/mg białka/min. Pięć szczepów wykazało aktywność chitynolityczną, największą aktywnością charakteryzował się szczep *Stenotrophomonas maltophilia* KK 8b. Trzynaście z 16 analizowanych szczepów ryzobakterii było zdolnych do produkcji związków chelatujących żelazo, sideroforów. Ponadto u *Pseudomonas brassicacearum* KK 5 i *P. corrugata* KK 12 potwierdzono obecnośc genu *phlD*, co może świadczyć o produkcji 2,4-DAPG.

Jak wynika z przeprowadzonych badań bakterie izolowane z ryzosfery posiadają potencjał do zwalczania patogenów grzybowych ograniczających uprawy, wykorzystując do tego szereg mechanizmów. Zastosowanie ryzobakterii jako biopestycydy jest przyjazną dla środowiska alternatywą dla chemicznych środków ochrony roślin.

Słowa kluczowe:
patogeny grzybowe, zwalczanie biologiczne, ryzobakterie