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PHENAZINES PRODUCING *PSEUDOMONAS* ISOLATES DECREASE *ALTERNARIA TENUISSIMA* GROWTH, PATHOGENICITY AND DISEASE INCIDENCE ON CARDOON

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Abstract – Phenazines, secondary metabolites of fluorescent *Pseudomonas*, represent a group of heterocyclic nitrogen-containing compounds showing a broad spectrum of antibiotic properties. Phenazines producing fluorescent *Pseudomonas* species are studied extensively for their application in plant disease management. In this study, we examined the antifungal activity of different indigenous *Pseudomonas* isolates (Q16, B25 and PS2) against the phytopathogenic fungus *Alternaria tenuissima*, which had infected cardoon (*Cynara cardunculus* L., Asteraceae). An *in vitro* experiment demonstrated the antifungal activity of selected indigenous isolates. In addition, an *in vivo* experiment under gnotobiotic conditions showed suppression of *C. cardunculus* disease caused by *A. tenuissima*. The quantification of phenazines revealed significant amounts of phenazine-1-carboxylic acid (PCA) and 2-hydroxy-phenazine-1-carboxylic acid (2-OH-PCA). PCR analysis confirmed the presence of PCA genes in all examined indigenous *Pseudomonas* isolates. Based on our results, we assume that these *Pseudomonas* isolates have potential in controlling plant diseases caused by *A. tenuissima*.

Key words: Pseudomonas, Cynara cardunculus, Alternaria tenuissima, phenazines, plant disease management

INTRODUCTION

Plant diseases represent one of the biggest problems of modern agriculture. Phytopathogenic fungi, the most common plant pathogens, are capable of infecting different types of plant tissues. Among the main aims in agriculture is finding adequate strategies for their suppression. One of these strategies is biological control (biocontrol) of plant diseases that relies on the use of natural antagonists of phytopathogenic fungi (Heydari and Pessarakli, 2010).

A special place among the natural antagonists of phytopathogenic fungi belongs to rhizobacteria that show beneficial effects on plant growth. They are referred to as plant growth promoting rhizobacteria – PGPR (Zehnder et al., 2001). These bacteria use various mechanisms for their action: production of plant hormones, asymbiotic fixation of N_2, antagonism towards phytopathogenic microorganisms and the ability to solubilize mineral phosphates and other nutrients (Cattelan et al., 1999). Different isolates of fluorescent *Pseudomonas* species take prominent place in this respect. Consequently, these isolates have been intensively studied. Fluorescent *Pseudomonas* species are present in temperate and tropical soils, often dominant among rhizobacteria (Ayyadurai et al., 2007). They belong

to PGPR because of the ability to colonize the roots of plants and stimulate growth by decreasing the frequency of diseases. Suppression of diseases includes the inhibition of pathogens by competition and/or by antagonism (Couillerot et al., 2009). The prominent feature of fluorescent *Pseudomonas* species is the production of antibiotics as inhibitory compounds that play a role in the suppression of diseases caused by phytopathogenic fungi (Haas and Défago, 2005).

One of the best-studied antibiotics of fluorescent *Pseudomonas* species are phenazines, nitrogen-containing heterocyclic compounds (Fernando et al., 2005). The only known natural producers of phenazines are bacteria (Pierson and Pierson, 2010). The most common phenazine derivatives produced by *Pseudomonas* sp. are phenazine-1-carboxylic acid (PCA), 2-hydroxy-phenazine-1-carboxylic acid (2-OH-PCA), phenazine-1-carboxamide (PCN) and hydroxy-phenazines (PHZ-OH) (Fernando et al., 2005). Phenazines such as PCA and 2-OH-PCA exhibit a wide range of activities against various fungi (Mavrodi et al., 1998).

Fluorescent *Pseudomonas* species are capable of inhibiting the phytopathogenic fungus *Alternaria tenuissima*. This common plant pathogen in several regions of the world (USA, South Africa, Europe) infects various crops such as cereals, vegetables (carrot, cauliflower, potato, etc.) and fruits (lemon, apple, etc.) (Gannibal et al., 2007). A significant characteristic of this phytopathogenic fungus is causing secondary infections and producing spores in conditions of favorable moisture (rains, large amounts of dew) and temperature (19-23°C). Symptoms of infection are represented by dark necrotic spots on leaves, which often wilt and fall off (Blodgett and Swart, 2002).

One of the hosts of *A. tenuissima* is cardoon (*Cynara cardunculus* L., Asteraceae). On the leaves of plants infected with *A. tenuissima*, disease is manifested by the appearance of necrotic spots (Yang et al., 1988). *C. cardunculus* is a perennial herbaceous plant that grows in many regions of the world (Europe, North Africa, South America), often in harsh conditions (high temperatures and water stress in summer) on unproductive and stony soils (Quilhó et al., 2004). Apart from its use in nutrition, *C. cardunculus* is also used in medicine because of its antioxidant properties (Ceccarelli et al., 2010).

A. tenuissima is a very aggressive plant pathogen of large numbers of medicinal plants. *A. tenuissima* isolated from *C. cardunculus* was the most aggressive compared with those isolated from other medicinal plants in our earlier investigations. The aim of this study was to examine the antifungal activity of different indigenous phenazines producing *Pseudomonas* isolates (Q16, B25 and PS2) against the phytopathogenic fungus *Alternaria tenuissima* which had infected cardoon (*Cynara cardunculus* L., *Asteraceae*).

MATERIALS AND METHODS

Determining the effect of different indigenous Pseudomonas isolates on the germination of conidia of Alternaria tenuissima isolated from cardoon (Cynara cardunculus)

The effects of indigenous *Pseudomonas* isolates Q16, B25 and PS2 on *A. tenuissima* conidial germination were examined *in vitro* by the McCallan method. Conidial germination of *A. tenuissima* was determined under the influence of different concentrations of *Pseudomonas* Q16, B25 and PS2 (10^7, 10^5 and 10^3 cfu/ml), prepared from a liquid overnight culture in King B medium based on OD_{600} measuring. The test fungus, *A. tenuissima*, was grown on sterile seeds of *C. cardunculus* on potato dextrose agar (PDA) at 23°C. Every dilution of *Pseudomonas* isolates was mixed on microscopic slides with a conidial suspension of *A. tenuissima* in ratio the 1:1. Three control variants were included in the experiment: the mixture of the conidial suspension of *A. tenuissima* with sterile distilled (1) water, (2) sterile saline solution and (3) fungicide DACOFLO 0.2%. After incubation for 24 h at 25°C, the conidial germination of *A. tenuissima* was determined microscopically (Matijević and Gavran, 1993).

Determination of the antagonistic action of different indigenous Pseudomonas isolates toward Alternaria tenuissima isolated from cardoon (Cynara cardunculus)

Determination of the antagonistic actions of the examined *Pseudomonas* isolates Q16, B25 and PS2 toward *A. tenuissima* were conducted on two nutrient media – King B and Waksman agar plates. Overnight cultures of *Pseudomonas* isolates Q16, B25 and PS2, optimized to $1 \cdot 10^7$ cfu/ml were used: a) to examine the influence of extracellular metabolites on cells (1 ml of cultures was centrifuged at 13000 rpm for 10 min and resuspended in the same volume of sterile saline solution); b) thermostable extracellular metabolites – heat stable antifungal factors (HSAF) influence (1 ml of cultures was centrifuged at 13000 rpm for 10 min, supernatant was filtered and filtrate was incubated at 70°C for 30 min).

The assay on every examined isolate was conducted in four variants: cell free without extracellular metabolites in (1) King B and (2) Waksman agar plates, suspension of thermostable extracellular metabolites (HSAF) added to (3) King B and (4) Waksman agar plates. Sowing 10 µl of these two agents was done near the edges of Petri dishes and mycelia of *A. tenuissima* were placed in the centre. Control variants contained only mycelia of *A. tenuissima* on King B and Waksman agar plates. Observation and the measuring of zones of growth inhibition of mycelia around bacterial colonies were performed after seven and fourteen days of incubation at 25°C (Nair and Anith, 2009). The percentage of growth inhibition of *A. tenuissima* mycelia was calculated by the formula: % Inhibition = [(Control - Treatment)/Control] x 100 (Ogbebor and Adekunle, 2005).

Experiment under gnotobiotic conditions

In vivo screening for suppression of *C. cardunculus* disease (caused by *A. tenuissima*) with indigenous *Pseudomonas* isolates Q16, B25 and PS2 was performed under gnotobiotic conditions. Uninfected seedlings of *C. cardunculus* and seedlings infected with *A. tenuissima* were used in the experiment. Infected seedlings were planted fifteen days after infection. The controls included (1) plants developed from uninfected seedlings, (2) infected seedlings that had not been inoculated with the examined *Pseudomonas* isolates (Q16, B25 and PS2), and (3) infected seedlings treated with fungicide DACOFLO 0.2%. Every variant of the experiment was set in eight repetitions. Inoculation with 1 $x \cdot 10^9$cfu of *Pseudomonas* isolates Q16, B25 and PS2 was performed at the base of the plant two times during the experiment (the 4th and the 14th day). Based on the developed symptoms forty days after planting, the percentage of *C. cardunculus* leaves infected by *A. tenuissima* was calculated using the formula: % of leaf incidence = (no. of leaves infected with *A. tenuissima* / no. of leaves observed) x 100 (Portisanos et al., 2006); the efficiency was calculated using the formula: Efficiency (%) = [(Control – Variant) / Control] x 100 (Gado, 2007).

Quantification of phenazine-1-carboxylic acid and 2-hydroxy-phenazine-1-carboxylic acid

Extraction of total phenazines was performed from 5 ml of Q16, B25 and PS2 overnight cultures on nutrient broth adapted to 1 $x \cdot 10^9$ cfu/ml. After 15 min of centrifugation at 3000 rpm, the supernatants were acidified with concentrated HCl (pH<2) and total phenazines were extracted by benzene (v/v). After benzene evaporation, the phenazines were resuspended in 0.1N NaOH and quantified spectrophotometrically at OD_{367} (PCA) and OD_{468} (2-OH-PCA) (Chancey et al., 1999). The concentration of PCA and 2-OH-PCA was calculated using known molar extinction coefficients: $\varepsilon_{PCA\ (367nm)}$ of 3019 $M^{-1}cm^{-1}$ and $\varepsilon_{2\text{-}OH\text{-}PCA\ (468nm)}$ of 7943 $M^{-1}cm^{-1}$ (Maddula et al., 2008).

PCR detection of the gene for phenazine-1-carboxylic acid

DNA samples were isolated from the overnight cultures of the examined indigenous *Pseudomonas* isolates Q16, B25 i PS2. Two hundred µl of the overnight cultures of all the examined isolates were resuspended in 500 µl of sterile distilled water and incubated for 10 min at 95°C. The samples were then

incubated for 5 min at -20°C and centrifuged (13000 rpm, 5 min). The supernatant was stored at -20°C. Dream Taq Green PCR Master Mix and PCA2a/PCA3b primers were used for PCR reaction according to Raaijmakers et al. (1997). The temperature profile of PCR amplification of initial denaturation of DNA at 94°C (2 min), 30 cycles (denaturation at 94°C (60 s), annealing at 67°C (45 s), extension at 72°C (60 s)) and final extension at 72°C (8 min), was applied. PCR products were separated on 1% agarose gel in 1xTBE buffer, stained with ethidium bromide, visualized under the UV light of a transilluminator and photographed (Raaijmakers et al., 1997).

Statistical analyses were performed by the Duncan multiple test.

RESULTS AND DISCUSSION

Determining the effect of different indigenous Pseudomonas isolates on the germination of conidia of Alternaria tenuissima isolated from cardoon (Cynara cardunculus)

The toxicity and slope of the regression lines were calculated from the obtained results of the number of germinated conidia of *A. tenuissima* under the influence of different concentrations of the examined *Pseudomonas* isolates (Q16, B25 and PS2). These values are shown in Table 1.

Based on these results it is evident that the *Pseudomonas* isolate Q16 showed the highest toxicity on the conidial germination of *A. tenuissima*, i.e. $5.219 \cdot 10^4$ cfu/ml. The indicator of the activity of *Pseudomonas* isolates Q16, B25 and PS2 in the inhibition of conidial germination is the slope of the regression lines. The lowest value of 0.29 for *Pseudomonas* isolate B25 shows that this isolate has a delayed action on the conidial germination of *A. tenuissima* compared with isolate PS2, whose regression line slope was the highest (0.33). The impact of different concentrations of *Pseudomonas* isolates Q16, B25 and PS2 is shown in Fig. 1. Also, the treatment with fungicide DACOFLO 0.2% caused total inhibition of *A. tenuissima* conidial germination of (Fig. 2).

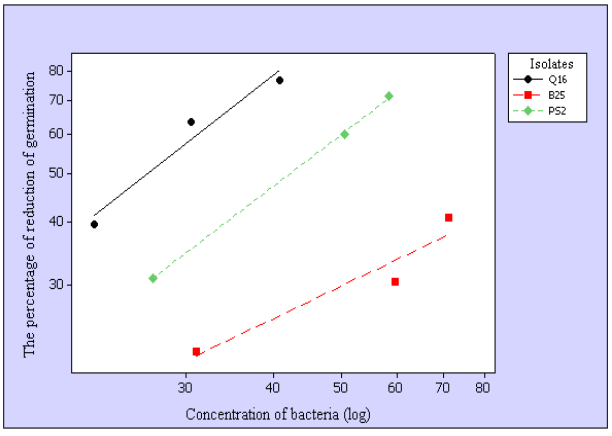

Fig. 1. Reduction of conidial germination of *A. tenuissima* by indigenous *Pseudomonas* strains.

The results are consistent with the study of Mishra et al. (2011) that showed that *P. fluorescens* isolate MA-4 inhibits the spore germination of the phytopathogenic fungus *Alternaria alternata* (in the range 40.3-82.7%). Kumaresan et al. (2005) showed that *P. chlororaphis* isolate PA23 inhibits spore germination of *A. solani* as a consequence of phenazine action (phenazine caused a high percentage of inhibition of spore germination *in vitro*, while germination was not inhibited in untreated controls). Different concentrations of suspensions of *P. fluorescens* isolates A-5, C-03, CRM-3, L-5 and Pf4-1 exhibited antifungal activity against *A. cajani* (by inhibition of sporulation) in the study of Srivastava and Shalini (2008), where all five isolates showed different levels of inhibition of spore germination: the highest concentration of suspension of these isolates exhibited the highest percentage of inhibition.

Determination of the antagonistic action of different indigenous Pseudomonas isolates toward Alternaria tenuissima isolated from cardoon (Cynara cardunculus)

The impact of cells free of extracellular metabolites and heat stable antifungal factors (HSAF) of *Pseudomonas* isolates Q16, B25 and PS2 on growth inhibition of *A. tenuissima* isolates from *C. cardunculus* is shown in Table 2.

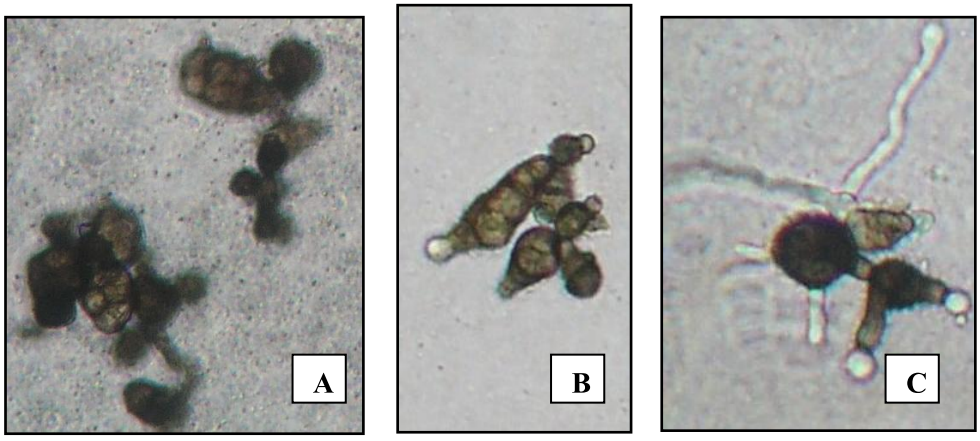

Fig. 2. (A) Impact of fungicide DACOFLO 0.2%, (B) *Pseudomonas* isolate Q16 and (C) B25 (concentration 10^5) on germination of conidia of *A. tenuissima*.

In the case of cells devoid of extracellular metabolites, the highest percentage of growth inhibition of *A. tenuissima* on Waksman agar plates was caused by *Pseudomonas* isolate PS2 (51.5%), whereas the lowest percentage of inhibition was that of *Pseudomonas* isolate Q16 (43.7%). On King B agar plates, the highest inhibitory effect was caused by *Pseudomonas* isolate B25 (80.0%) and the lowest by *Pseudomonas* isolate Q16 (58.7%). All values of percentages of inhibition on the Waksman agar plates were statistically significant, but on the King B agar plates the only statistically significant value was obtained in the case of isolate B25. The HSAF of isolates Q16 and PS2 showed a stronger effect on the growth inhibition of *A. tenuissima* (53.7%) compared with isolate B25 (37.7%). Fungal growth inhibition of 80% caused by B25 cells and the low inhibition caused by HSAF suggest a high enzymatic activity of the B25 isolate growing in the KB medium.

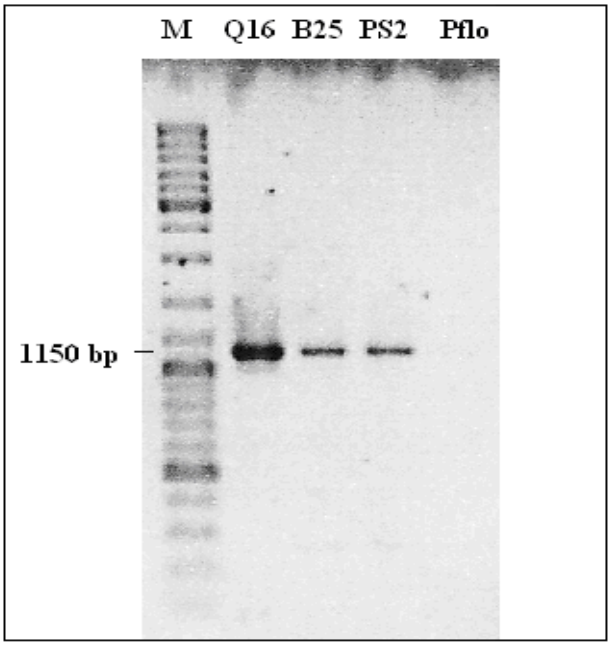

Fig. 3. PCA gene amplicons (1150 bp) from *Pseudomonas* sp. Q16, B25, PS2 and *P. fluorescens* as negative control.

The examined *Pseudomonas* isolates inhibited mycelial growth of *A. tenuissima* in the range of 34.0-80.0%, which is consistent with the results of Seetha et al. (2010), where *P. fluorescens* inhibits the mycelial growth of *A. tenuissima* by 83.3%. Mishra et al. (2011) showed the effect of 3 and 4 day-old culture filtrates of *P. fluorescens* MA-4 on the growth of the phytopathogenic fungus *Alternaria alternata*. Fluorescent *Pseudomonas aeruginosa* BFPB9, *P. plecoglossicida* FP12 and *P. mosselli* FP13 show a wide spectrum of antifungal activity against different phytopathogenic fungi (*Rhizoctonia solani*, *Macrophomina phaseolina*, *Fusarium oxysporum* f. sp. *cubense*, *F. oxysporum* f. sp. *vasinfectum*, etc. (Jha et al., 2009). Similar to our results, Goud and Muralikrishnan (2009) revealed

Table 1. Toxicity of bacterial isolates on germination of conidia *A. tenuissima* (cfu/ml).

Pseudomonas isolate	Toxicity (cfu/ml)	Slope (b)
Q16	$5.219 \cdot 10^4$	0.3031
PS2	$5.430 \cdot 10^4$	0.3291
B25	$6.236 \cdot 10^4$	0.2918

Table 2. Inhibition of *A. tenuissima* depending on the type of inhibitory agent.

Inhibitory agent	Isolate of *Pseudomonas* sp.					
	Q16		B25		PS2	
	Inhibition (%)		Inhibition (%)		Inhibition (%)	
	WA	KB	WA	KB	WA	KB
cells	43.7 a	58.7 b	46.5 a	80.0 a	51.5 a	65.8 b
HSAF	34.0 a	53.7 a	36.9 a	37.7 b	41.0 a	53.7 a

Table 3. The degree of ilness in *C. cardunculus* infected with *A. tenuissima* and disease suppression efficiency by treatment with *Pseudomonas* sp.

	Variants		Diseased leaves (%)	Efficiency (%)
Seed infected with *A. tenuissima*	Inoculation with strain	Q16	37.40 a	43.33 b
		B25	37.40 a	42.78 b
		PS2	52.30 a	18.99 c
	Treatment with fungicide DACOFLO 0.2%		4.1 d	93.75 a
	Positive control		65.8 a	/
Control – Uninfected seed			0.00 d	

Table 4. Production of PCA and 2-OH-PCA by isolates of *Pseudomonas* sp.

Isolate of *Pseudomonas* sp.	c_{PCA} μg/ml	$c_{2\text{-OH-PCA}}$ μg/ml
Q16	124	15
B25	57	12
PS2	27	8

that *P. fluorescens* has significant effect (50-80%) on growth inhibition of different phytopathogenic fungi (*Pythium ultimum*, *Macrophomina phaseolina* and *Pyricularia oryzae*).

Experiment under gnotobiotic conditions

The infection percentages by *A. tenuissima* of *C. cardunculus* plants are presented in Table 3. Inoculation with *Pseudomonas* isolates Q16 and B25 showed a higher percentage of reduction of symptoms caused by *A. tenuissima* compared with isolate PS2. The lowest efficiency, with the lowest statistical significance, was that of PS2 (18.99%). Ramjegathesh et al. (2011) reported that *P. fluorescens* reduces leaf blight disease of onion caused by *Alternaria alternata* by 34.90%. The results of Tabarraei et al. (2011) show that *P. putida, P. aeruginosa* and *P. fluorescens* have poten-

tial for decreasing diseases caused by *Phytophthora drechsleri*. The treatment of soil with bacterial suspensions of different isolates of these bacteria reduces frequency of disease (30.0-83.7%) compared with the control, and treatment of the seeds with bacterial suspensions reduces disease incidence in the range 36.0-85.0%. The obtained results are significant since there is little known data about the biocontrol of *A. tenuissima* by fluorescent *Pseudomonas* in gnotobiotic and field conditions.

The research of Portisanos et al. (2006) on the *Pseudomonas chlororaphis* isolate PA23 showed that this isolate provides significant protection against leaf infection by the fungus *Sclerotinia sclerotiorum*, highly reducing the intensity of the disease. In the untreated control, there were no infected leaves, in plants treated with isolate PA23 about 10% and in the positive control about 75% of diseased leaves. The study of Selin et al. (2010) of *P. chlororaphis* PA23 also showed biocontrol activity of this isolate towards *S. sclerotium,* because this bacterium produces phenazine, which inhibits the growth of *S. sclerotiorum*: isolate PA23-63, with deficiency in the production of this antibiotic, is equivalent to a wild-type isolate in its ability to control fungal infection and reduce disease intensity. Compared with the positive control, there was no difference in leaf incidence. This indicates that phenazines can, to a certain degree, inhibit phytopathogenic fungi, even though they have a minor role in disease suppression, which is consistent with the results obtained in this study.

Quantification of phenazine-1-carboxylic acid and 2-hydroxy-phenazine-1-carboxylic acid

The established concentrations of PCA and 2-OH-PCA (Table 4) indicate that all examined isolates produced both types of phenazine derivatives, but isolate Q16 proved to be the best producer of PCA and 2-OH-PCA. These results are consistent with the study of Mavrodi et al. (1998) which found that PCA can be accumulated in media up to a concentration of 1 g/l. Furthermore, Timms-Wilson et al. (2000) identified PCA as an inhibitory agent for *P. ultimum*. Maddula's et al. (2008) quantification of PCA and 2-OH-PCA in their study of the *P. chlororaphis* isolate 30-84 revealed a production of 28.5 μg/ml of PCA and 6.7 μg/ml of 2-OH-PCA. This study showed that isolates with no phenazine production do not inhibit the growth of fungus – the loss of 2-OH-PCA production results in a significant reduction of inhibition of the fungal pathogen *Gaeumannomyces graminis* var. tritici.

PCR detection of the gene for phenazine-1-carboxylic acid

Electrophoresis on agarose gel confirmed the amplification of the gene for phenazine antibiotic PCA of all three examined indigenous *Pseudomonas* isolate. Q16, B25 and PS2. This result is consistent with the study of Raaijmakers et al. (1997), where the primers PCA2a and PCA3b amplified the predicted DNA fragment of *P. fluorescens* isolate 2-79. In addition, their study confirmed the amplification of the same sized fragment from the DNA of all the other examined phenazine-producing isolates (*P. aureofaciens* 30-84, PGS12, AP9, 13985, TAMOak81, *Pseudomonas* spp. BS1391, BS1393).

The study of Djurić et al. (2011) shows that the indigenous *Pseudomonas* isolate PS2 is an efficient antifungal agent due to production of extracellular enzymes (chitinases and lytic enzymes) and siderophores. Because of this, isolate PS2 could be further investigated for its application as a biocontrol agent. In our study, indigenous *Pseudomonas* isolates Q16, B25 and PS2 showed similar properties, indicating that such investigations should be carried out on isolates Q16 and B25. Apart from the detected antibiotics (PCA and 2-OH-PCA) from the phenazine group, the ability of indigenous *Pseudomonas* isolates Q16, B25 and PS2 to produce other antibiotics (whose role in biocontrol has been proved to be very significant) could be further examined.

CONCLUSIONS

Biological control of *A. tenuissima,* the most aggressive isolate from medicinal plants in Serbia, isolated from *C. cardunculus*, is an ecological method of plant

protection. Our investigation confirmed the antifungal activity of indigenous *Pseudomonas* isolates Q16, B25 and PS2. They had an inhibitory effect on the conidial germination and mycelial growth of *A. tenuissima* and decreased the symptoms of the disease caused by this fungus. PCR analysis confirmed the presence of the gene for PCA in all three isolates. Quantification of PCA and 2-OH-PCA showed that the isolates Q16, B25 and PS2 are good producers, with Q16 as the best of them. Phenazine productions classify our isolates as a promising group of PGPR. Further analyses of other antifungal metabolites are needed to support their role in the biocontrol of *C. cardunculus* disease caused by *A. tenuissima*.

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