

***Daucus carota* L. - seed germination and natural infection by *Fusarium* spp. affected by *Pseudomonas* spp.**

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SUMMARY

The inhibitory effect of indigenous *Pseudomonas* strains on *Fusarium* spp. isolated from seeds of a new variety of carrot - 'Vizija' and their effects on seed germination were observed. *Pseudomonas* spp. strains were applied as bacterial suspensions (culture, 10⁶ CFU mL⁻¹) and cell-free supernatant (CFSa -10⁶ and CFSb -10⁸ CFU mL⁻¹) fractions by sowing seeds during 7 (I) and 14 (II) days of incubation. The germination of control 'Vizija' seeds was 19% after the first and 40% after the second incubation period. *Pseudomonas chlororaphis* Q16 strain exhibited statistically significant increases in seed germination with all applied fractions and incubation periods, showing values of 43–62% and 55.5–91%, i.e. increments of 24–43% and 15.5–51% compared to the control, after 7 and 14 days of incubation, respectively. *P. chlororaphis* K35 showed lower but significantly different seed germination values (38–67%) for all variants, except for 7 days old culture. *Pseudomonas* sp. Ek1 had weaker seed germination potential, showing statistically significant increment only for CFSa,b (I) and CFSb (II) fractions. All tested *Pseudomonas* strains inhibited the growth of three *Fusarium* species isolated from 'Vizija' seeds: *F. solani*, *F. oxysporum* and *F. subglutinans*. Natural infection was observed in 20% (I) and 54% (II) of 'Vizija' seeds. *P. chlororaphis* K35 was the most efficient antifungal strain, reducing seed infection 97.5–100%, followed by Q16 with 95–100%, showing no statistically significant mutual difference. *Pseudomonas* sp. Ek1 showed a weaker antifungal activity and reduced seed infection by 85–96.75%. The application of *P. chlororaphis* Q16 and K35 as strains effective in improvement of carrot seed germination and growth inhibition of the seed pathogens *F. solani*, *F. oxysporum* and *F. subglutinans*, can be further tested in carrot production for more beneficial effects.

Keywords: Carrots; Seed germination; *Fusarium*; *Pseudomonas*; Antifungal activity

INTRODUCTION

Over the last few decades, there has been a growing need for chemicals that effectively prevent plant diseases. Due to potential risks to human health, it is necessary to find a biological alternative to chemical control products for plant pests and diseases. Pests and chemicals do not always have long-term effects on plants, even when applied in early stages of plant growth. A lot of attention has been given to bacterial strains as biocontrol agents that are able to reduce disease incidence caused by pathogenic fungi and also to provide a long-term antifungal protection. Microbiological fungal control is important for some agro-economically important crops such as carrots.

Carrot (*Daucus carota* L.) is one of the most important vegetables, widely used for human consumption due to its high concentration of nutritional ingredients such as β -carotene. β -carotene is known for its beneficial effects on human immune system as a precursor of vitamin A. Recently, β -carotene has been examined for its potential anti-cancer properties. Carrots are also rich in other vitamins, proteins, fibers, energy, micro and macro elements, minerals, as well as antioxidants. Carrots have high Fe^{2+} , Cr^{2+} , K^+ and Ca^{2+} contents, and contain 40 calories per 100 g of dry weight, which is significantly higher in comparison with other vegetables used for human consumption (Hanif et al., 2006). Due to beneficial effects on human health, carrots are widely consumed as a fresh vegetable, as well as its juice, pickles and preserved carrots and candies (Sharma et al., 2011).

Besides the conventional orange carrot, a purple variety of this vegetable is also used for human consumption. The roots of purple carrots are characteristic for their high sugar content. It has been shown that purple carrots have higher concentrations of some important phenolic compounds, such as anthocyanins, in comparison with conventional orange carrots (Zadernowski et al., 2010). In this research, a new variety of purple carrot named 'Vizija' was used as a test-plant. This variety is characterized by a dark purple root exceeding 25 cm in length. 'Vizija' is characterized by a great yield potential, even under unfavorable cultivation conditions.

Carrots are very susceptible to fungal infection caused by *Fusarium* spp. The genus *Fusarium* consists of a variety of fungal species that can commonly be found in soil and water. These fungi are known to be plant-pathogenic for a wide array of economically and agriculturally important plants. Some of *Fusarium* species can produce a wide range of mycotoxins which are harmful for plants, as well as for humans and animals (Moretti, 2009). *Fusarium oxysporum* can be found

in various types of soil worldwide. These fungi have a great economic importance as they can induce root rot diseases, as well as vascular wilts in various crops. During the last few decades, *F. oxysporum* has attracted a lot of attention of researchers worldwide due to its disease inducing potential in a variety of plant hosts. Zhang et al. (2014) reported the first case of carrot disease caused by *F. solani* and *F. oxysporum* in China with disease incidence of up to 80%, causing symptoms such as dry rot lesions and brown cankers. In Serbia, Stanković et al. (2015) observed a carrot disease caused by *F. avenaceum* inducing similar symptoms, such as crown and root rots. *F. subglutinans* is another species, commonly causing pitch canker. This species is associated with a wide range of hosts, such as carrot, maize, pineapple, pine, sorghum and mango (Nirenberg, 1976).

Over the last few decades, some of the bacteria in the genus *Pseudomonas* have been used as microbiological fungal control agents against a wide array of fungal species, including *Fusarium*. Some of *Pseudomonas* bacteria have already shown a great potential when used as biocontrol agents against *Alternaria solani* (El-Sayed et al., 2008). *Pseudomonas chlororaphis* is most commonly used as a fungal antagonist due to its wide-spectrum of antifungal activity against soil-borne plant pathogens. Some of the most significant antifungal substances produced by *P. chlororaphis* are hydrophobic compounds, phenazine-1-carboxamine (PCN), chitinases, proteases and hydrogen cyanide (Chin-A-Woeng et al., 2000, 2005). *P. chlororaphis* is widely used for plant growth-promoting in the form of inoculants for biofertilization, biocontrol and phytostimulation (Bloembergen & Lugtenberg, 2001).

In this study, the potential of *P. chlororaphis* and *Pseudomonas* sp. strains for stimulating seed germination, and their antifungal activity against *Fusarium* spp. isolated from 'Vizija' seeds was tested.

MATERIAL AND METHODS

The samples of 'Vizija' seeds used in this study originated from the Institute for Vegetable Crops, Smederevska Palanka, Serbia (Figure 1). *Pseudomonas* sp. (Ek1) and two *P. chlororaphis* strains (Q16 and K35) used in this research are part of Project III46007 collection of the Institute of Soil Science, Belgrade, Serbia.

Pseudomonas strains were tested in the forms of bacterial suspension (culture) and cell-free supernatant (CFS) for fungal growth inhibition and improvement of seed germination. CFS fractions were obtained by centrifuging bacterial suspension at 13000 rpm for 5 min.

Supernatant was then re-centrifuged in centrifugal filter tubes with 0.22 μm microporous membrane (Millipore). Culture (10^6 CFU mL⁻¹) and CFS (CFSa - 10^6 and CFSb - 10^8 CFU mL⁻¹) of each tested strain were applied to seeds by soaking for 30 min. Each fraction of bacterial strains was applied to 100 seeds (25 seeds per each of 4 Petri dishes) in two independent experiments and the results were presented as mean values. Germinability and overall seed health status were examined on filter paper, using the paper towel method. The ratio of seed germination was counted after 7 (I) and 14 (II) days of incubation in the dark at 25°C by counting all germinated and ungerminated seeds.

Three *Fusarium* species were isolated from 'Vizija' seeds as natural infection agents, and were characterized based on their macroscopic and microscopic morphology as *F. solani*, *F. oxysporum* and *F. subglutinans*. The effect of *Pseudomonas* strains on *Fusarium* spp. was determined by counting naturally infected seeds. The

results showing the inhibitory effect of *Pseudomonas* strains were expressed as percentages of reduced infection incidence compared with the untreated control. The data were statistically processed using the Statistica 7 software, one-way ANOVA and Duncan's multiple range test at the significance level of $P < 0.05$.

RESULTS

Stimulation of carrot seed germination by *Pseudomonas* strains and difference between seed germination induced by each strain and the control sample is shown in Figure 2 and Table 1. *P. chlororaphis* Q16 induced a statistically significant increment in seed germination for all applied fractions and in both incubation periods. The weakest seed germination potential was observed in *Pseudomonas* sp. Ek1, showing the same or lower values than control samples when culture or CFSa (II) were applied.



Figure 1. *Daucus carota* cv. 'Vizija'

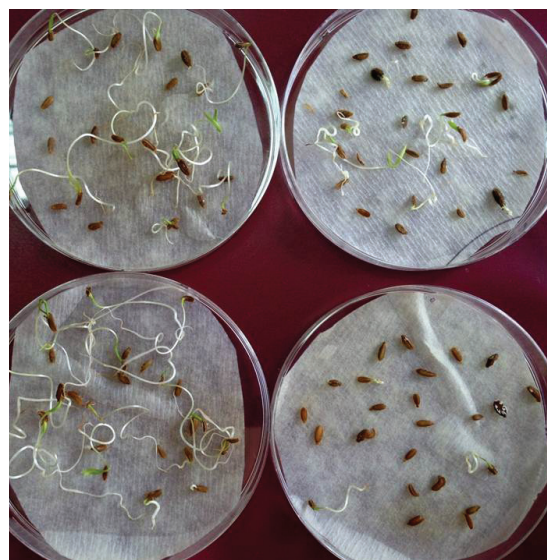


Figure 2. Seed germination of *Daucus carota* cv. 'Vizija' induced by *Pseudomonas* spp. strains K35, Ek1 and Q16

Table 1. Effects of *Pseudomonas* spp. strains on carrot seed germination (%)

Incubation period	7 days			14 days		
<i>Pseudomonas</i>	Culture*	CFSa	CFSb	Culture	CFSa	CFSb
Ek1	22b**	30c	30c	35 d	44c	48c
Q16	43a	48a	62a	55.5a	86a	91a
K35	27b	38b	41.5b	49b	52b	67b
Control	19b	19d	19d	40c	40c	40d

* bacterial suspension – culture; cell-free supernatant – CFSa (10^6 CFU mL⁻¹) and CFSb (10^8 CFU mL⁻¹)

**Dissimilar letters in each column mark statistically significant difference ($p \leq 0.05$) at the level of 5%, using Duncan's test

The CFSb of all strains showed the greatest seed germination potential during both experimental periods, while cultures showed the weakest potential. The seed germination potential of all tested strains gradually increased over time, and germinated seeds ranged from 40% in the control to 91% in the CFSb fraction of Q16 strain.

The applied *Pseudomonas* strains exhibited high antifungal potential against *Fusarium* spp. The results of seed infection and reduction in infection incidence for all tested strains and application forms are shown in Table 2 and Figure 3. All tested strains showed the best antifungal activity when applied as bacterial suspension; Q16 and K35 strains inhibited infection 100% during the first week. The inhibition of fungal growth was slightly lower during the next seven days for K35 and Q16, although without statistical significance, while Ek1 showed slightly weaker antagonism, and significantly lower values for the two CFS fractions.

DISCUSSION

P. chlororaphis Q16 and K35 were the most efficient strains which significantly reduced natural infection of the carrot variety 'Vizija' with three *Fusarium* species - *F. solani*, *F. oxysporum* and *F. subglutinans*. The highest seed germination potential was observed in *P. chlororaphis* Q16, followed by K35, and both significantly stimulated seed germination over time in all applied fractions. *Pseudomonas* sp. Ek1 was less effective than the two *P. chlororaphis* strains, showing lower values of seed germination and higher percent of seed infection, but still significantly beneficial compared with the control.

An improvement in germination rate of carrot seeds and efficacy of *Pseudomonas* spp. as biocontrol agent in control of the pathogen *Alternaria radicina* using pretreatment with hot water was reported by Lopez-Reyes et al. (2016). The results obtained in our study are consistent with other research reports showing that

Table 2. Effects of *Pseudomonas* spp. strains on carrot seeds naturally infected with *Fusarium* spp. (%)

Incubation period		7 days		14 days		
<i>Pseudomonas</i>	Culture*	CFSa	CFSb	Culture	CFSa	CFSb
Ek1	1a**	3b	3a	1.75a	4a	8b
Q16	0a	0.75a	1a	1.5a	2.5a	2.5a
K35	0a	0.75a	0.5a	1a	1a	1.25a
Control	20b	20c	20b	54b	54b	54c

* bacterial suspension – culture; cell-free supernatant – CFSa (10^6 CFU mL⁻¹) and CFSb (10^8 CFU mL⁻¹)

** dissimilar letters in each column mark statistically significant difference ($p \leq 0.05$) at the level of 5%, using Duncan's test

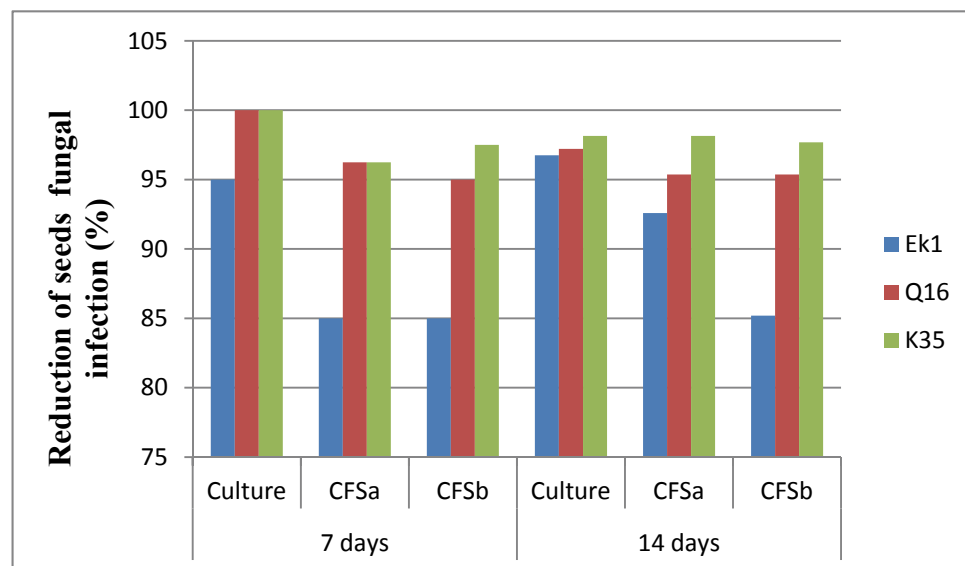


Figure 3. Effects of *Pseudomonas* spp. strains in reducing carrot seed infection with *Fusarium* spp. (%)

P. chlororaphis is a very strong antifungal agent. Mezaache-Aichour et al. (2016) tested the antifungal effects of *P. chlororaphis* against *F. solani* in dual culture, and it showed strong antifungal activity and inhibited fungal growth by approximately 65%. Ghosh et al. (2014) examined the antifungal effects of *Pseudomonas* spp. against *F. subglutinans* and found that fungal growth was inhibited up to 95% by *Pseudomonas* spp. in dual culture.

P. chlororaphis decreased the incidence of disease induced by *F. solani* by almost 50% in cotton plants cultivated in an extremely saline soil under gnotobiotic conditions (Egamberdieva et al., 2015). *P. chlororaphis* is proved to be one of the most efficient bacterial antagonists against *F. solani* in controlling tomato root rot in greenhouses (Postma et al., 2013). Chin-A-Woeng et al. (2000) reported that root colonization by *P. chlororaphis* under gnotobiotic sand conditions is one of the most important steps in bacterial biocontrol of *F. oxysporum*. Tziros et al. (2007) studied the antagonistic effects of some bacterial strains on *F. oxysporum* in control of watermelon wilt disease, and found that *P. chlororaphis* reduced disease severity by 41%, especially during the early stages of disease development, under gnotobiotic conditions. A combined application of *P. fluorescens* and *P. chlororaphis* showed a strong effect against the same pathogenic fungus. The authors suggested that *P. chlororaphis* could be used as a potentially strong agent against *F. oxysporum* infections.

P. chlororaphis Q16 reduced the disease incidence caused by *Alternaria tenuissima* up to 86.5% on basil (*Ocimum basilicum* L., Lamiaceae) under gnotobiotic conditions (Jošić et al., 2012a). Under the same conditions, this strain exhibited a strong antifungal effect on *A. tenuissima* and reduced disease incidence on cardoon (*Cynara cardunculus* L., Asteraceae) by 93%. In addition to their PCR detection of a gene for phenazine-1-carboxylic acid (PCA), the authors measured significant amounts of PCA and 2-hydroxy-phenazine-1-carboxylic acid (2-OH-PCA) (Jošić et al., 2012b). The harbouring genes for phenazine antibiotics and their production are among the properties responsible for the strong antifungal effects of *P. chlororaphis* Q16 strain.

CONCLUSION

P. chlororaphis Q16 and K35 strains tested in this study improved seed germination (19-51%, depending on the applied culture/fraction) and inhibited by 95-100% the fungal growth of carrot seed pathogens, such as *F. solani*, *F. oxysporum* and *F. subglutinans*.

Pseudomonas sp. Ek1 showed 85-96.75% inhibition of fungal pathogen incidence. In addition to the effects of *P. chlororaphis* strains as antifungal agents and seed germination inducers, field trials are necessary to confirm all their beneficial effects and biocontrol potential in carrot production.

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Daucus carota L. – klijavost semena i prirodna infekcija semena *Fusarium* spp. pod uticajem *Pseudomonas* spp.

REZIME

Ispitivan je inhibitorski efekat autohtonih *Pseudomonas* sojeva na gljive *Fusarium* spp. izolovane sa semena šargarepe sorte 'Vizija' i njihov uticaj na klijavost semena. *Pseudomonas* spp. sojevi su primenjeni u vidu bakterijske suspenzije (kulture, 10^6 CFU mL⁻¹) i supernatanta (CFSa - 10^6 i CFSb - 10^8 CFU mL⁻¹) potapanjem semena, a potom inkubirani tokom 7 (I) i 14 (II) dana. Klijavost semena 'Vizije' u kontroli iznosila je 19% posle prvog i 40% posle drugog perioda inkubacije. *P. chlororaphis* Q16 soj je uzrokovao statistički značajno povećanje klijavosti semena pri primeni svih frakcija i tokom oba inkubaciona perioda, dostižući 43-62% i 55.5-91% klijavosti, što je povećanje od 24-43% i 15.5-51% u poređenju sa kontrolom, posle 7 i 14 dana inkubacije. *P. chlororaphis* K35 je uslovio manje, ali statistički značajne vrednosti klijavosti semena (38-67%) za sve varijante, osim za kulturu posle 7 dana. *Pseudomonas* sp. Ek1 je pokazao slabiji potencijal klijavosti, sa statistički značajnim povećanjem samo za CFSa,b (I) i CFSb (II) frakcije. Svi ispitani *Pseudomonas* sojevi su inhibirali tri vrste gljiva iz roda *Fusarium*

koje su izolovane sa semena 'Vizije': *F. solani*, *F. oxysporum* i *F. subglutinans*. Prirodna infekcija semena je uočena kod 20% (I) i 54% (II) semena 'Vizije' u kontrolnoj varijanti. *P. chlororaphis* K35 je ispoljio najače antifungalno dejstvo, sa smanjenjem infekcije semena 97.5-100%, a zatim soj Q16 sa 95-100%, bez statističke značajnosti među njima. *Pseudomonas* sp. Ek1 je ispoljio slabiju antifungalnu aktivnost i redukovao infekciju semena 85-96.75%. Primena *P. chlororaphis* Q16 i K35, kao sojeva koji efikasno povećavaju klijavost semena šargarepe i inhibiraju rast patogenih semena *F. solani*, *F. oxysporum* i *F. subglutinans*, biće dalje testirana u proizvodnji šargarepe kako bi se procenili ostali pozitivni efekti ovih sojeva.

Ključne reči: Šargarepa; Klijanje semena; *Fusarium*; *Pseudomonas*; Antifungalno delovanje