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Antagonistic effect of soil bacteria against fusarium wilt of pepper *in vitro*

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Abstract: The aim of this work was to isolate bacteria from the rhizosphere of tomato and pepper plants infected with *Fusarium oxysporum* and from soils where the fusarium wilt pathogen was noticed earlier, as well as to explore the possibility for control of *Fusarium oxysporum*, the causal agent of pepper wilt, using their antagonistic traits, *in vitro*. A total of 25 visually different bacteria were isolated from soil and 1 was isolated from melon leaf and included in the research as it showed a strong antagonistic effect against the isolated fungus. Eleven out of 26 isolates had a more or less antagonistic effect on this pathogen. Isolate Ab_{23} showed the strongest inhibition rate with PIRG value (percentage of inhibition of radial growth) of 70.98%. Isolates Ab_7 (61.24%), Ab_{17} (57.73%) and Ab_1 (56.56%) also produced a satisfactory effect. Interestingly, isolate Ab_9 even though not being from the soil showed a very high value of inhibition of radial growth of pathogen mycelia (59.74%). Inhibition rate of isolates Ab_{18}, Ab_{21}, Ab_{10}, Ab_{22}, Ab_5, and Ab_{20} was 37.67% - 28.99%. Given the above, there are bacteria in the rhizosphere of diseased plants of tomato and pepper or in the soil where fusarium wilt of tomato and pepper has been previously reported that have an antagonistic effect against the causal agent of pepper wilt, *Fusarium oxyporum*, and that can be successfully used in control of this pathogen, *in vitro*. Even the isolate of non-soil-borne bacteria can show antagonism against soil pathogen, *in vitro*.

Key words: *Fusarium oxysporum*, Pepper, Antagonist, Bacteria, Biocontrol

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Introduction

Sweet pepper (*Capsicum annuum* L.) is one of the most significant vegetable crops both worldwide and in Serbia. Due to its great importance it is necessary to preserve and protect its production. The greatest risk to pepper production are phytopathogenic fungi, soil ones in particular. In greenhouse and open field cultivation of pepper, several soil-borne plant pathogens can severely jeopardize its production, being present in diverse pepper growing areas. The most important among them seem to be Phytophthora capsici Leonian, Verticillium dahliae Kleb., Rhizoctonia solani Kuhn. (Cartia *et al.* 1989, Di Vito *et al*. 2000; Douira *et al.* 1995, Slusarski and Pietr 2009, Tsror (Lahkim) *et al.* 1998). In recent years, increasingly frequent occurrence of fusarium wilt of sweet pepper has been observed on open-field-grown peppers. Fusarium wilt is a disease caused by the soil pathogen *Fusarium oxysporum* which negatively affects crop yield, significantly decreasing the quantity and the quality of the crop (Sahi and Khalid 2007, Wongpia and Lomthaisong 2010).

Healthy plants can become infected by this pathogen if the soil used for their cultivation is contaminated with the fungus. The fungus can invade a plant with its sporangial germ tube or mycelium by invading plant roots, through wounds or directly through the root tip or at the formation point of lateral roots. Once inside the plant the mycelium grows through the root cortex intercellularly towards xylem vessels, into which it enters through xylem pits (Agrios 1988). Inside the xylem, due to mycelial growth, plant water supply is greatly affected, which leads to leaf stomata closure and occurrence of wilt symptoms. Eventually, the plant dies due to water deficiency.

It is only when death occurs that the pathogen invades the parenchymatous tissue, until it reaches the surface of the dead tissue, where it sporulates abundantly, thus providing inoculum for spreading (Agrios 1988).

Effective means of control of this pathogen, and *F. oxysporum* in general include soil disinfestation and use of resistant/tolerant plant material. Soil disinfestation using methyl-bromide has been very effective but banned by the Montreal protocol (Djordjevic *et al.* 2010a, Ivanovic and Ivanovic 2007, Mao *et al.* 1998). Methyl bromide being labeled as ozone depletory opened the door for new strategies and solutions for controlling *F. oxysporum* and other soil pathogens.

Pathogen biocontrol is a promising strategy for the replacement of chemical treatments (Compant *et al.* 2005, Dubey *et al.* 2007, Fravel *et al.* 1998). This strategy consists of several solutions. One of the solutions is implementation of microorganisms that have an antagonistic effect against *F. oxysporum*. This aspect of biocontrol involves association of microorganisms which have the capacity to restrict pathogen development or completely suppress it, in order to improve or preserve the health of plants and represent interaction of at least three

organisms (Handelsman and Stabb 1996). If we take this into consideration and add the fact that the use of microbial antagonists for the suppression of soil plant pathogens, such as *Fusarium oxysporum*, is taking place in a very dynamic environment, such as soil, then it is easy to conclude that this is a very complex interaction. Indeed, despite the great potential for applications in agriculture, this form of biocontrol is not well enough explained, because of the very complex mechanisms of bio-control (Bapat and Shah 2000, Handelsman and Stabb 1996).

The mechanism of bio-control of plant pathogens using antagonists may be through competition for space and food or by stimulating host plant by inducing tolerance or resistance to the pathogen, or antibiosis, ie. production of low-molecular fungitoxic compounds or enzymes (Matar *et al.* 2009). Antibiosis or the production of one or more antibiotics is a highly effective mechanism for prevention and control of pathogens in the rhizosphere (Handelsman and Stabb 1996). The mechanism of biological control and the antagonistic relationship of microorganisms to the plant pathogens in general was studied by several authors (Cavagliery *et al.* 2005, Dubey *et al.* 2007, Djordjevic *et al.* 2010a, Guo *et al.* 2004, Jiang *et al.* 2001, Larkin and Fravel 1998, Matar *et al.* 2009, Sahi and Khalid 2007).

Due to the fact that every organism has its enemy in nature, then, if the pathogen, fusarium wilt, of pepper is present in the soil it is very likely that there are antagonistic microorganisms of this pathogen present, as well. In view of the above, the objective of this research is to: a) isolate bacteria from the rhizosphere of diseased plants of pepper and tomato with symptoms of fusarium wilt and confirm presence of their antagonistic effect against *Fusarium oxysporum* pathogen of pepper, and b) form a collection of such bacteria for further research regarding identification and *in vivo* application.

Experiment

2.1 Isolation of pathogen

Pathogen *Fusarium oxysporum* was isolated from diseased pepper plants on open fields of Institute for vegetable crops, Smederevska Palanka, Serbia. Isolation and identification of pathogen was performed by using standard methods and isolating single conidia and growing it on PDA, as described by Levic (2008) and Booth (1971). After growing mycelia of pathogen from single conidia, isolates of pathogen were kept on PDA in refrigerator at 4°C, until further use.

2.2 Isolation of antagonists

Isolation of potential antagonists was carried out from the rhizosphere of tomato and pepper plants that have expressed symptoms of fusarium wilt

characteristic for them, and from the soil where this pathogen has been reported in previous growing seasons (Djordjevic *et al.* 2010). Isolation was done from the soil samples taken from the rizosphere of diseased plants and soil. Samples were first passed through a sieve to remove parts of plant roots and then 3g of every sample were measured and homogenized with 100 ml sterile distilled water in a magnetic shaker for 30 min at 150 r/min. Then, serial dilution (10^{-1} – 10^{-9}) was made and 1 ml of two highest dilutions was transferred by a micropipette on YDC and King B mediums (Djordjevic *et al.* 2010a, Larkin and Fravel 1998). After 24 or 48h, i.e. after development of bacterial colonies, visually different individual colonies of bacteria were transferred on YDC to obtain single colonies (Shaad *et al.* 2001). Single colonies were transferred and kept on YDC in refrigerator at 4°C until further use.

Apart from the strains of soil bacteria isolated as described above, one isolate was obtained by isolation from the melon leaf. When isolating the causal agent of the spots that appeared on the melon leaves, colonies of bacteria with signs of antagonistic action on fungal colonies that developed next to them (Fig. 1) developed as a by-product and were, therefore, included in this study. After obtaining individual colonies, it was kept in the same way as the above-mentioned bacteria.

Fig. 1. Antagonism of isolate Ab_9 isolated from melon leaf

2.3 *In vitro* testing of antagonistic effect of isolated bacteria against *F. oxysporum*

In order to determine whether the isolated bacteria demonstrated antagonism to the observed pathogen, the trial was set by the method described by Rosenzweig and Stotzky (1979). The method involved inoculation using a loop-full of 24 h old bacterial culture that was transferred to Petri dishes (R = 9 cm) close to the wall of the plate with as a strike line. Then, a mycelial plug (5 x 5 mm) of pathogen was transferred from 7 day old fungal culture of fungi to the opposite side of the Petri dish. Petri dishes were then kept in a thermostat for seven days in total darkness and at a temperature of 24 °C. After seven days, the

dishes were examined and the antagonism was evaluated (present/not present). If the fungi mycelium reached bacteria or crossed over it, the antagonism was not present.

Testing of the antagonistic relationship of isolates to *F.oxysporum* was carried out by the dual culture method (Matar *et al.* 2009, Rozenzweig and Stotzky 1979, Suparman *et al.* 2002). Mycelium plug (5x5 mm) was transferred from 7-day-old culture to Petri dishes, 9 cm in diameter with a PDA background in it, at the center of the box. At 2.5 cm away from the plug in the center of the box, a loop-full of 24 hour old bacterial culture was transferred. Then Petri dishes were kept in a thermostat for 7 days at a temperature of 24°C in complete darkness. After this period the growth of mycelium towards the bacteria was measured, as well as growth of mycelia opposite the bacteria. During this process, the percentage of inhibition of radial growth (PIRG) was calculated using the following formula:

$$PIRG = \left((r_c - r_i) / r_c \right) \times 100,$$

where r_c denotes mycelial growth on the opposite side of the bacteria and r_i indicates mycelial growth towards the observed bacteria.

The experiment was set up twice in a completely randomized design with five replications. The results were subjected to analysis of variance and the significance of the differences was checked by Duncan test ($P<0.05$). All data were analyzed using the MATLAB Ver. 7.0 mathematical program.

Results and Discussion

Twenty-five visually different bacteria were isolated from the rhizosphere of diseased plants of tomato and pepper (Table 1). One isolate obtained from melon leaf that showed strong inhibition of fungal mycelia was included in the research.

Of the 26 test isolates, 11 showed an antagonistic effect and inhibited the growth of pathogenic mycelia (Fig. 2). The strongest antagonism was exhibited by the isolate Ab_{23} with PIRG value of 70.98%. Isolates Ab_7 (61.24%), Ab_9 (59.74%), Ab_{17} (57.73%) and Ab_1 (56.56%) had a lower efficacy in inhibiting the radial growth of pathogenic mycelia. The lowest PIRG value was found in Ab_{20} (28.99%) (Fig. 3, Table 1). Isolates that showed a higher inhibiting effect on the mycelial growth of the pathogen showed the first signs of inhibition after three days. From day 3 to day 7 there was little or no mycelial growth of the pathogen towards antagonistic bacteria.

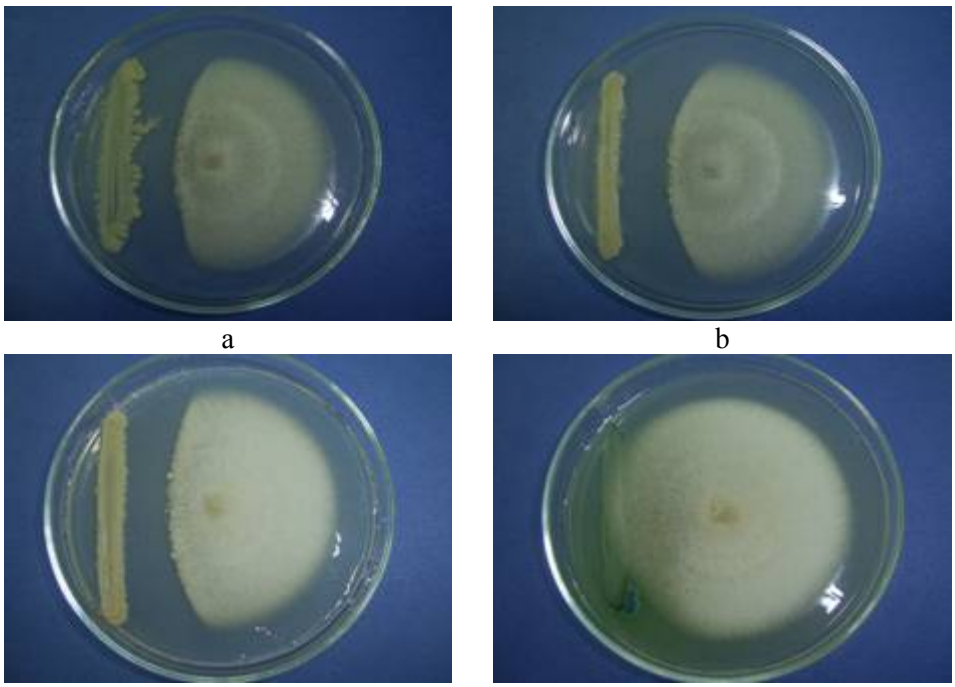

Fig. 2 Presence of the antagonistic effect of isolates a) Ab_{23}, b) Ab_9, c) Ab_7 a and d) absence of antagonism of Ab_6

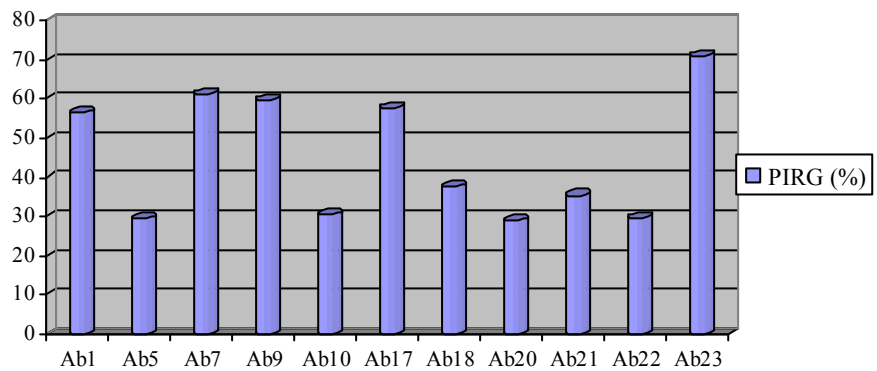

Fig. 3 Percentage of inhibition of radial growth (PIRG) of *F. oxysporum* due to influence of antagonistic bacteria

Pepper production is one of the largest vegetable production processes in Serbia and in the world. The occurrence of fusarium wilt of pepper caused by *Fusarium oxysporum* is a disease of pepper. There are as much as over 50% of

infected plants on some open fields. In order to protect this important production process and at the same time ensure environmentally friendly production, it is necessary to find a sustainable solution to control this disease. The use of biological measures could serve as a solution. This method implies the use of antagonistic bacteria (Djordjevic *et al.* 2010a, Dubey *et al.* 2007, Mazurier *et al.* 2009).

This paper explores the presence of bacteria that are antagonists to the causal agent of fusarium wilt of pepper, *Fusarium oxysporum* in the rhizosphere of pepper and tomato plants already infected by fusarium wilt pathogen (*Fusarium oxysporum* and *F. oxysporum* f. sp. *lycopersici*), as well as in the soil where the presence of these pathogens was previously noted. Also, the objective of this research was to evaluate the strength of the trait i.e., the strength of inhibition of mycelium growth by isolated bacteria *in vitro*.

The antagonistic effect against *F.oxysporum* was exhibited by 11 out of 26 isolates. The strongest inhibitory effect was produced by isolate Ab_{23} with PIRG value 70.98%. Isolates Ab_7, Ab_9, Ab_{17} and Ab_1 were very effective with PIRG values ranging from 61.24% to 56.56%. Isolates Ab_{18}, Ab_{21}, Ab_{10}, Ab_{22}, Ab_5 and Ab_{20} showed a significantly lower inhibitory effect as compared to the above isolates, with PIRG range being between 37.67% and 28.99%.

Biological control of *Fusarium oxysporum*, the causal agent of wilt of pepper, using antagonistic soil-borne bacteria, has not been much studied. Sahi and Khalid (2007) examined bio-control of *Fusarium oxysporum*, the causal agent of wilt of pepper, using different strains of antagonistic fungi of *Trichoderma* sp., *in vitro*. They observed considerable inhibition of the mycelial growth of pathogen as a response to the effect of *Trichoderma* sp. antagonism.

The test isolates of soil bacteria were used in another research where biological control of different pathogens was tested *in vitro*, as well. Djordjevic *et al.* (2010a, 2010b, 2010c, 2011) tested these isolates for antagonism against fusarium wilt of peas (*Fusarium oxysporum* f. sp. *pisi*), tomato (*F. oxysporum* f. sp. *lycopersici*) and eggplant (*F.oxysporum*), and grey mold of tomato (*Botrytis cinerea*).

Table 1. Origin of isolated bacteria tested for antagonism and their PIRG values

Bacteria	Locality	Isolated from	Presence of antagonism	PIRG (%)	Duncan test*
Ab_1	Vranjska Banja	soil	+	56.56	bcde
Ab_2	Vranjska Banja	soil	-	0	/
Ab_3	Vranjska Banja	soil	-	0	/
Ab_4	Vranjska Banja	soil	-	0	/
Ab_5	Vranjska Banja	soil	+	29.37	h
Ab_6	Smederevska Palanka	rhizosphere of tomato	-	0	/
Ab_7	Smederevska Palanka	rhizosphere of tomato	+	61.24	b
Ab_8	Vranovo	rhizosphere of tomato	-	0	/
Ab_9	Vranovo	melon leaves	+	59.74	bc
Ab_{10}	Vranovo	rhizosphere of tomato	+	30.54	gh
Ab_{11}	Smederevska Palanka	rhizosphere of tomato	-	0	/
Ab_{12}	Smederevska Palanka	rhizosphere of tomato	-	0	/
Ab_{13}	Smederevska Palanka	soil	-	0	/
Ab_{14}	Smederevska Palanka	soil	-	0	/
Ab_{15}	Smederevska Palanka	soil	-	0	/
Ab_{16}	Smederevska Palanka	soil	-	0	/
Ab_{17}	Desimirovac	rhizosphere of pepper	+	57.73	bcd
Ab_{18}	Desimirovac	rhizosphere of pepper	+	37.67	f
Ab_{19}	Vranovo	soil	-	0	/
Ab_{20}	Vranovo	soil	+	28.99	h
Ab_{21}	Pancevo	soil	+	35.37	fg
Ab_{22}	Pancevo	rhizosphere of tomato	+	29.71	h
Ab_{23}	Banatski Brestovac	soil	+	70.98	a
Ab_{24}	Banatski Brestovac	soil	-	0	/
Ab_{25}	Jabuka	soil	-	0	/
Ab_{26}	Jabuka	soil	-	0	/

* values with the same letter are not significantly different ($P<0.05$)

Isolate Ab_{23} that showed the highest value of PIRG in this research was the most effective in the growth inhibition of mycelium of *Botrytis cinerea* (82.20%) and *F.oxysporum* f. sp. *lycopersici* (73.33%) (Djordjevic *et al.* 2010b, 2011). The PIRG value of isolate Ab_{23} when inhibiting fusarium wilt of pea was 58.26%, and

fusarium wilt of eggplant 66.61% (Djordjevic *et al.* 2010a, 2010c). For inhibition of growth of fusarium wilt of pea the most effective were isolates Ab_{17} (80.26%), Ab_1 (72.37%), Ab_{10} (71.48%) and Ab_6 (58.7%) (Djordjevic *et al.* 2010a). Testing of the antagonistic effect of these isolates against the causal agent fusarium wilt of tomato showed that the highest values of PIRG were exhibited by Ab_{23}, as mentioned above, followed by Ab_1 (69.19%) and Ab_{10} (64.44%) (Djordjevic *et al.* 2010b). Djordjevic *et al.* (2010c) determined the highest PIRG values of these isolates in Ab_1 (69.49%), Ab_{23} (66.61%) and Ab_{10} (63.47%). The antagonistic effect of the test isolates against *Botrytis cinerea* was evaluated by Djordjevic *et al.* (2011) who found that isolate Ab_{23} had the highest PIRG value (82.22%) followed by Ab_3 (80%), Ab_{12} and Ab_{18} (76.89%), Ab_{20} (76%) and Ab_9 (75.11%).

Isolate Ab_6 exhibited antagonism only against the fusarium wilt of pea but not against the fusarium wilt of tomato, eggplant and pepper. Isolate Ab_3 was antagonistic only against *Botrytis cinerea* despite it being a soil microorganism.

Interestingly, isolate Ab_9, although originating from the soil, showed a certain degree of antagonism to all these pathogens, both soil and *Botritys cinerea*. The PIRG value of this isolate in this research was 59.74%, while in other experiments it varied from 48.69% for fusarium wilt of eggplant, 54.13% for fusarium wilt of tomato, 59.09% for fusarium wilt of pea to 75.11% for grey mold (Djordjevic *et al.* 2010a, 2010b, 2010c, 2011). This suggests that this isolate can be used as an antagonist to a wide range of plant pathogens.

Other scientists in their research showed that using antagonistic bacteria as a tool for control of soil-borne and leaf pathogens can be a solution for control of these pathogens (Anis *et al.* 2010, Dubey *et al.* 2007, Gheorghe *et al.* 2008, Husen 2003, Mazurier *et al.* 2009, Matar *et al.* 2009, Suparman *et al.* 2002, Validov *et al.* 2006).

Conclusions

The growth of the world population and the lack of food, which is a very frequent occurrence and will be even more so in the future, have resulted in an increase in food prices. Fungicides are one of major inputs in agricultural production used to protect from disease-causing agents. The excessive use of fungicides and inadequate substitution also lead to an increase in production costs and, hence, agricultural products. One of the solutions to protect food production, reduce costs and increase quality is the use of easily accessible natural resources. Due to the fact that plant rhizosphere and soil itself have a wide range of diverse populations of microorganisms, the population of *F. oxysporum* pepper pathogen being one of them, some of these populations necessarily act as its natural enemies.

This paper justifies the use of soil-borne microorganisms in controlling plant diseases and highlights the related tendency of the modern plant protection

profession. Our further work should focus on determination of the mechanism of antagonism of these bacteria towards *F. oxysporum* and investigation of this relationship *in vivo*.

It would be especially interesting to perceive *in vivo* the antagonistic effect of bacteria isolated from melon leaf and establish their reaction in the rhizosphere, given the prevailing completely different ecological conditions and microbiological relations.

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ANTAGONISTIČKI EFEKAT ZEMLJIŠNIH BAKTERIJA PREMA PROUZROKOVAČU FUZARIOZNOG UVENUĆA PAPRIKA *IN VITRO*

- originalni naučni rad -

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Rezime

Cilj istraživanja je bio da se izoluju bakterije iz rizosfere biljaka paradajza i paprike zaraženih patogenom *Fusarium oxysporum* ali i iz zemljišta gde je ovaj patogen prisutan od ranije, kao i da se ispita mogućnost kontrole patogena prouzrokovača fuzarioznog uvenuća paprike, korišćenjem njihovih antagonističkih osobina, *in vitro*. U istraživanju je korišćeno ukupno 25 vizuelno različitih bakterija izolovanih iz zemljišta kao i jedna bakterija izolovana sa lista dinje koja je uključena u istraživanje zbog zapaženih jakih antagonističkih svojstava prema gljivama. Jedanaest izolata od 26 je pokazalo manje ili jače izražen antagonistički efekat prema ovom patogenu. Izolat Ab_{23} je pokazao najjaču inhibiciju porasta sa vrednosti PIRG-a (procenat inhibicije radijalnog porasta) 70,98%. Izolati Ab_7 (61,24%), Ab_{17} (57,73%) i Ab_1 (56,56%) su takođe imali zadovoljavajući efekat. Interesantno je da je izolat Ab_9, iako nije poreklom zemljišni, imao vrlo visok nivo inhibicije radijalnog porasta micelije patogena (59,74%). Procenat inhibicije izolata Ab_{18}, Ab_{21}, Ab_{10}, Ab_{22}, Ab_5, i Ab_{20} se kretao između 37,67% i 28,99%. Uzimajući sve u obzir možemo zaključiti da postoje bakterije u rizosferi obolelih biljaka paradajza i paprika ili u zemljištu gde je patogen fuzarioznog uvenuća ovih kultura ranije zabeležen, koje imaju antagonističkog efekta prema prouzrokovaču fuzarioznog uvenuća paprike *Fusasrium oxysporum*, ida se one mogu sa uspehom primeniti u kontroli ovog patogena, *in vitro*. Čak i izolat bakterije koja nije zemljišna može imati antagonistički efekat prema zemljišnom patogenu, *in vitro*.