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Original scientific paper

Antagonistic potential of *Bacillus* spp. isolates against bacterial pathogens of tomato and fungal pathogen of pepper

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SUMMARY

In vitro antagonistic potential of eleven isolates of *Bacillus* spp. against two phytopathogenic bacteria and one fungus was tested in order to identify potential biocontrol agents in vegetable crops. The *Bacillus* spp. isolates demonstrated different levels of antagonistic effect against the tested pathogenic microorganisms. Data in the study proved *Xanthomonas vesicatoria* to be more sensitive to *Bacillus* spp. strains than *Clavibacter michiganensis* subsp. *michiganensis*. Ten *B. subtilis* strains induced growth inhibition of *X. vesicatoria*, while a strain of *B. pumilus* did not affect the growth of that bacterium. The largest inhibition zones against *X. vesicatoria* were induced by strains B-319, B-325 and B-358. The pathogenic strain *C. michiganensis* subsp. *michiganensis* was most inhibited by two *B. subtilis* strains (B-338 and B-348) with mean inhibition zone diameters of up to 20 mm. *B. subtilis* strain B-319 which was the best in inhibiting *X. vesicatoria,* showed the lowest inhibitory effect on *C. michiganensis* subsp. *michiganensis*. The largest growth inhibition percentage of *Verticillium* sp. (PGI approximately 70%) was induced by *B. subtilis* strains B-310 and B-322. The other *B. subtilis* strains showed PGI values ranging from 45% to 68%, while *B. pumilus* strain B-335 had the least antagonistic potential (PGI =34.43%) against the pathogen. This study identified at least one suitable biocontrol candidate, *B. subtilis* strain B-358, as effective *in vitro* against all three vegetable pathogens.

Keywords: Biological control; *Bacillus*; Bacterial pathogens; Fungal pathogen; Tomato; Pepper

INTRODUCTION

Bacterial and fungal pathogens associated with vegetables are causing substantial production losses worldwide. Being difficult to control, the most important are those pathogens that are seed transmitted and soilborne. Economically, the most damaging phytopathogenic bacteria affecting tomatoes are *Clavibacter michiganensis* subs. *michiganensis* (Smith, 1910; Davis et al., 1984), the causal agent of bacterial wilt and canker, and *Xanthomonas vesicatoria* (Doidge, 1920; Vauterin et al., 1995), the causal agent of bacterial spot.

These pathogens are on A2 lists both in Serbia and the EPPO region due to their devastating impact on tomato yield and quality (Pravilnik, 2015; EPPO, 2018). Among fungal pathogens, Verticillium wilt is one of the most important diseases of pepper, present in all commercial pepper growing areas. Its causal agents are fungi of the genus *Verticillium* (Pilar Santamarina & Roselló, 2006, Rekanović et al., 2007, 2010). Control of plant diseases incited by bacterial and fungal pathogens is regularly based on the use of conventional fungicides/bactericides and cultivation of unsusceptible crops. Control of bacterial diseases is additionally complicated by pathogen diversity, difficulties in finding durable resistance in host plants to the target pathogen, the ability of bacteria to reach high population densities in a short period of time and lack of effective chemical control (Jones et al., 2012). In contrast to a long list of products available for the control of fungal pathogens, there are very few bactericides with limited efficacy which are suitable for crop protection. Control of *Verticillium* spp. is considered complicated due to a long period of persistence of their resting structures in the field and a broad host range of some species. The pathogen is difficult to manage once it reaches the vascular plant tissue and fungicides appear to be ineffective. Moreover, chemical fumigants, which are able to reduce the primary inoculum of *Verticillium* in soil, are restricted because of their harmful effects on the environment. The fungicides commonly used to control Verticillium wilt of pepper are: thiophanate-methyl, difenoconazole, fluopyram, azoxystrobine, and prochloraz (Talboys, 1984; Tian et al., 1998; Rekanović et al., 2007; Mihajlović et al., 2015, 2017). Furthermore, disease control in vegetables is regularly provided by fungicide treatments of seeds, such as thiram and tebuconazole, as well as soil treatments with toxic compounds, such as methyl bromide, especially when soil-borne fungi are involved. The disease management approach that relies only on the use of chemicals has encountered problems with the occurrence of pathogens resistant to certain fungicides/bactericides (Bender & Cooksey, 1986; Stall et al., 1986; Bender et al., 1990; Manulis et al., 1998; Basim et al., 1999). Besides, restrictions on the use of chemical pesticides due to concerns for their impact on the environment and human health are increasing rapidly (Saha et al., 2012).

To achieve the goal of improving disease control, the use of microbial-based pesticides has been considered as a rational and safe alternative to chemical control (Milijašević-Marčić & Todorović, 2017). Many recent studies have focused on the use of microbial inoculants for biological control, utilizing their ability to antagonize the pathogen by multiple modes of action (Rekanović et al., 2007; Živković et al., 2010; Saha et al., 2012; Berić et al., 2012; Solanki et al., 2013; Dimkić et al., 2013; Gupta & Vakhlu, 2015; Stanojević et al., 2016; Milijašević-Marčić et al., 2017).

Bacillus sensu stricto represents a group of widely distributed Gram-positive, aerobic and facultative aerobic bacteria. These bacteria are catalase positive and able to produce resistant endospores which are metabolically active even under unfavorable environmental conditions (Logan et al., 2007). *Bacillus* species produce catalytic enzymes (proteases, chitinases and glucanases and peptide antibiotics (bacilizyn, fengimycin, bacitracin, bacilin, bacilomycin B, iturin) which are known as antifungal and antibacterial substances (Pal & McSpadden Gardener, 2006; Stein, 2005). The most studied *Bacillus* species used for biological control is *B. subtillis* (Asaka & Shoda, 1996; Krebs et al., 1998; Lin et al., 2001). In addition, members of the *B. subtilis* and *B. pumilus* group are considered to be harmless and have a status of "GRAS" organisms (Food and Drug Administration, 1999), which makes them good candidates for biocontrol agents.

The aim of this study was to determine *in vitro* antagonistic potentials of several natural isolates of *Bacillus* spp. against two phytopathogenic bacteria and one fungus, in order to identify potential biocontrol agents in vegetable crops.

MATERIALS AND METHODS

Test organisms and culture conditions

To determine antimicrobial activity of native *Bacillus* spp. strains, two bacterial and one fungal pathogens were used as indicator strains. The phytopathogenic bacteria used in the tests were: *C. michiganensis* subs. *michiganensis* P-5 (culture collection of the Institute of Pesticides and Environmental Protection, Belgrade, Serbia) and *X. vesicatoria* NCPPB 422 (National Collection of Plant Pathogenic Bacteria, UK) both originating from tomato plants. *C. michiganensis* subs. *michiganensis* strain P-5 was grown and maintained on nutrient agar (NA) plates, while *X. vesicatoria* NCPPB 422 was grown on yeast dextrose chalk agar (YDC). To prepare inoculum suspensions, bacteria were incubated on appropriate media at 26 °C for 48 h and resuspended in sterile distilled water to reach final concentrations of approximately 10^7 CFU/ml. The pathogenic fungus, *Verticillium* spp. isolate PS2V16 (culture collection of

the Institute of Pesticides and Environmental Protection, Belgrade, Serbia), was used as an indicator strain for testing bacterial antagonism. Stock culture of the fungal pathogen was maintained on potato dextrose agar (PDA) at 4 °C. Working culture was prepared by transferring stock agar plugs containing mycelium onto PDA plates and incubating them for 14 days at 22 °C.

Antagonistic bacteria

Natural isolates of *Bacillus* spp. from the culture collection of the Institute of Pesticides and Environmental Protection, used in the study, were isolated from different stages of mushroom growing substrate and manure obtained from the composting facility Učа & Co, Vranovo, Serbia (Table 1). Strains were previously identified based on colony morphology, Gram reaction, catalase reaction and a partial sequence of the hypervariant region of 16S rRNA gene (Gunjak, 2016).

Table 1. *Bacillus* spp. tested in the study

Strains	Source
B. subtilis B-308	Manure
B. subtilis B-309	Manure
B. subtilis B-310	Manure
B. subtilis B-313	Manure
B. subtilis B-319	Compost phase I, day 3.
B. subtilis B-322	Compost phase I, day 3.
B. subtilis B-325	Compost phase I, day 3.
B. pumilus B-335	Compost phase I, day 8.
B. subtilis B-338	Compost phase I, day 8.
B. subtilis B-348	Compost phase II
B. subtilis B-358	Compost phase III

In vitro antagonistic activity of Bacillus spp. against plant pathogenic bacteria

Eleven bacterial isolates characterized as members of the genus *Bacillus* and used in the screening were grown in nutrient broth (NB) at 30 °C in a rotary shaker (200 rpm) overnight. The culture broth was used in a well diffusion inhibition assay as described previously (Harris et al., 1989). Bacterial indicator strains were grown overnight according to their specific growth requirements and were added to molten NA cooled to 45 °C as a suspension, to reach a final concentration of approximately 10^6 CFU/ml. After solidification of NA medium, wells were made by sterile, glass borer and 50 µl of the culture broth of antagonistic bacteria were added to the wells in four replicates. Plates containing only indicator strains and sterile water in the wells were used as control. Zones of inhibition of sensitive indicator strains were measured after incubation of plates for three days at 26 °C.

In vitro antagonistic activity of Bacillus spp. *against pathogenic Verticillium sp.*

Preliminary screening of bacterial antagonism against the pathogenic isolate PS2V16 was carried out using the dual culture method, in triplicate (Fokkema, 1978). Agar discs (10 mm) of the tested pathogen were placed on one side of PDA plates and a loopful of antagonistic bacterial isolates from an overnight culture were streaked 3 cm away from the edge of the same plate. Plates inoculated only with the pathogen culture served as controls. In order to quantify the antagonistic potential of bacterial strains, the size of growth inhibition zones was measured after 14 days of incubation at 22 °C and the percent of growth inhibition (PGI) was calculated using the formula:

$$PGI\ (\%) = (KR - R1)/KR \times 100,$$

where KR represents the pathogen colony diameter in control plate, and R1 represents the colony diameter in treated plate (Korsten & De Jager, 1995).

Statistical analyses

Basic statistical parameters were calculated and the obtained data were presented in histograms as mean values of percent of mycelial growth inhibition for the tested fungal pathogen and mean values of inhibition zone diameter for the tested bacterial pathogens. The data were examined using the one-way analysis of variance (ANOVA). Tukey's HSD (honest significant difference) test was used to compare the inhibition of growth of the bacterial and fungal pathogens caused by the activity of 11 antagonistic bacterial strains. Significance was evaluated at $P<0.05$ for all tests. Statistical analyses were conducted by the general procedures of IBM SPSS Statistics v.19 (SPSS Inc.).

RESULTS

In vitro antagonistic activity of Bacillus spp. *against plant pathogenic bacteria*

The tested *Bacillus* spp. isolates demonstrated different levels of antagonistic effect against the pathogenic organisms. Of the two pathogenic bacteria tested in the study,

X. vesicatoria proved to be more sensitive to *Bacillus* spp. strains. Ten *B. subtilis* strains induced growth inhibition of *X. vesicatoria*, while a strain of *B. pumilus,* B-335, did not affect the growth of this bacterium. The largest inhibition zones against *X. vesicatoria* were induced by strains B-319, B-325 and B-358. Statistically significant differences were found between these three strains and all other tested strains (Fig. 1).

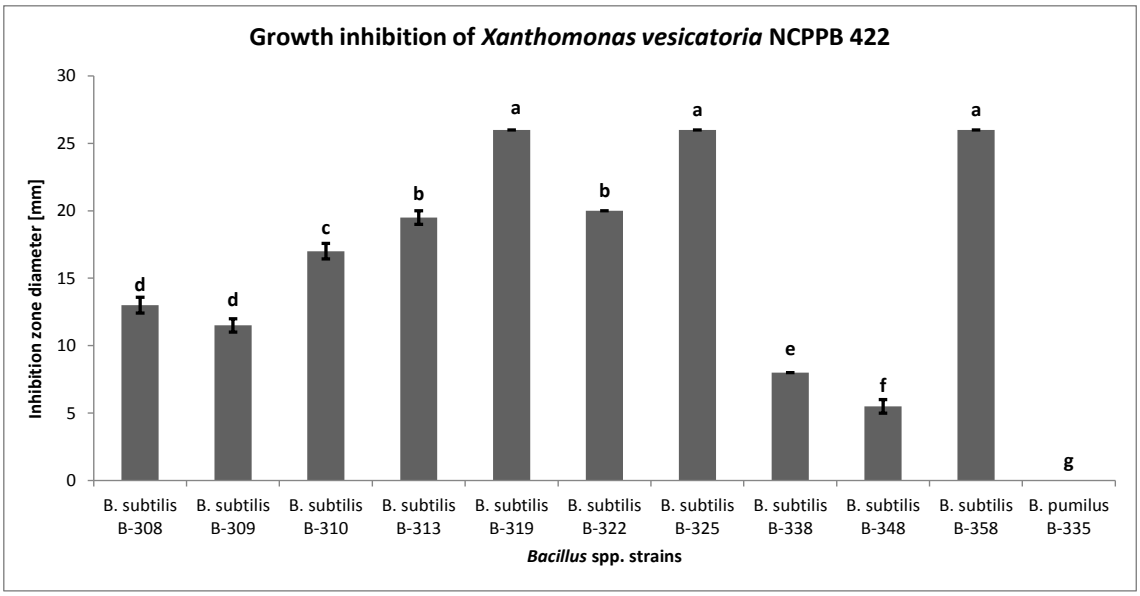

Figure 1. *In vitro* antibacterial activity of *Bacillus* spp. strains against *X. vesicatoria*. Mean values of inhibition zone diameter (n=4) with standard error for each antagonistic strain are shown. *Values marked by the same letter in columns for each strain are not significantly different (P<0.05), according to Tukey's HSD test.

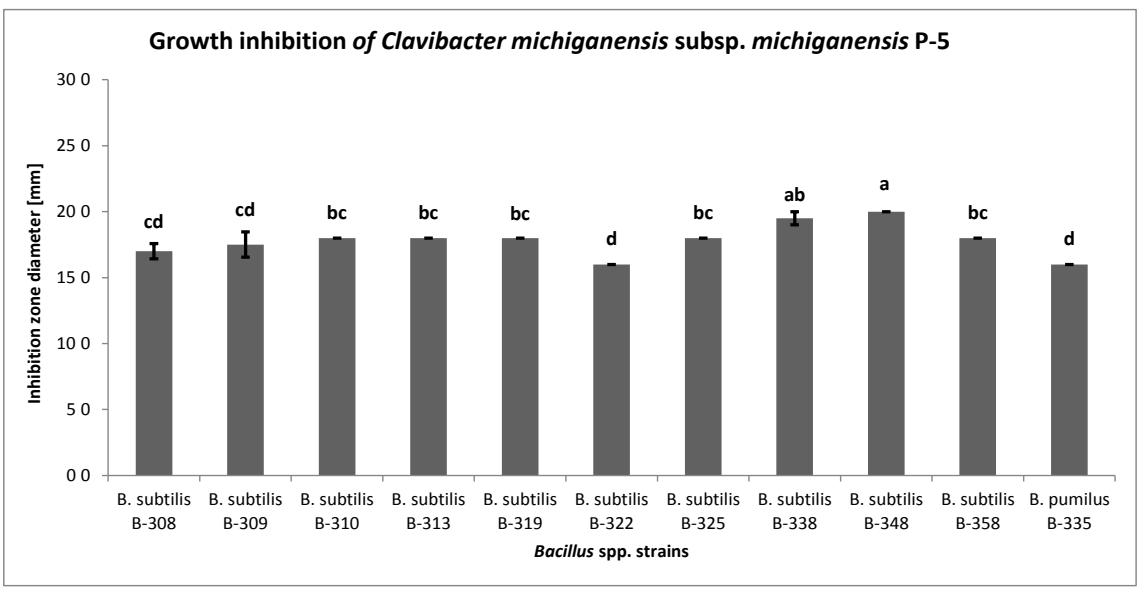

Figure 2. *In vitro* antibacterial activity of *Bacillus* spp. strains against *C. michiganensis* subsp. *michiganensis*. Mean values of inhibition zone diameter (n=4) with standard error for each antagonistic strain are shown. *Values marked by the same letter in columns for each strain are not significantly different (P<0.05), according to Tukey's HSD test.

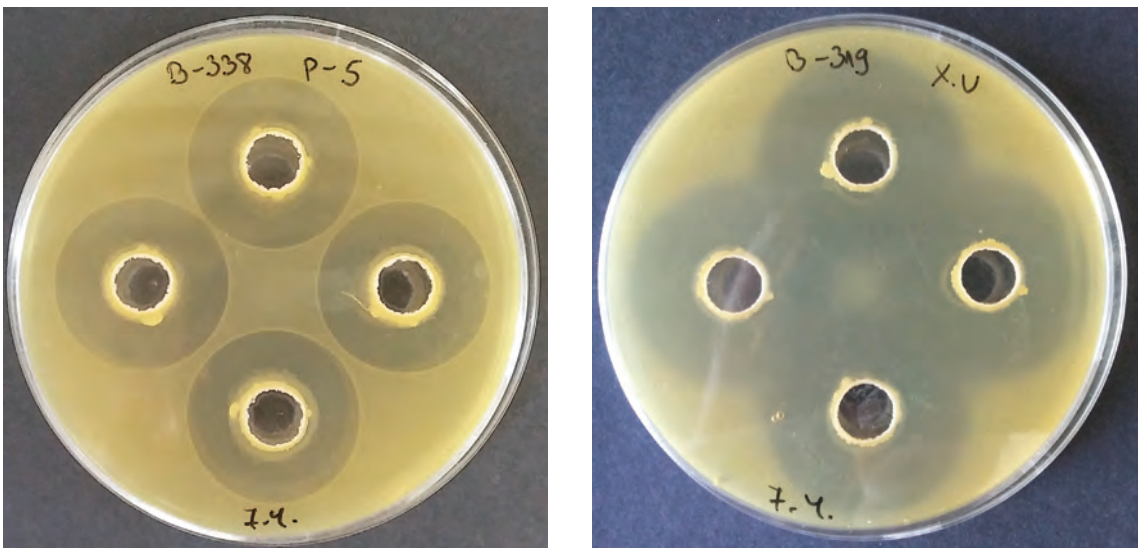

Figure 3. Well diffusion assay for determining the antagonistic potential of bacterial strains against bacterial pathogens. The activity of B-338 strain against *C. michiganensis* subsp. *michiganensis* – left; the activity of B-319 strain against *X. vesicatoria* – right.

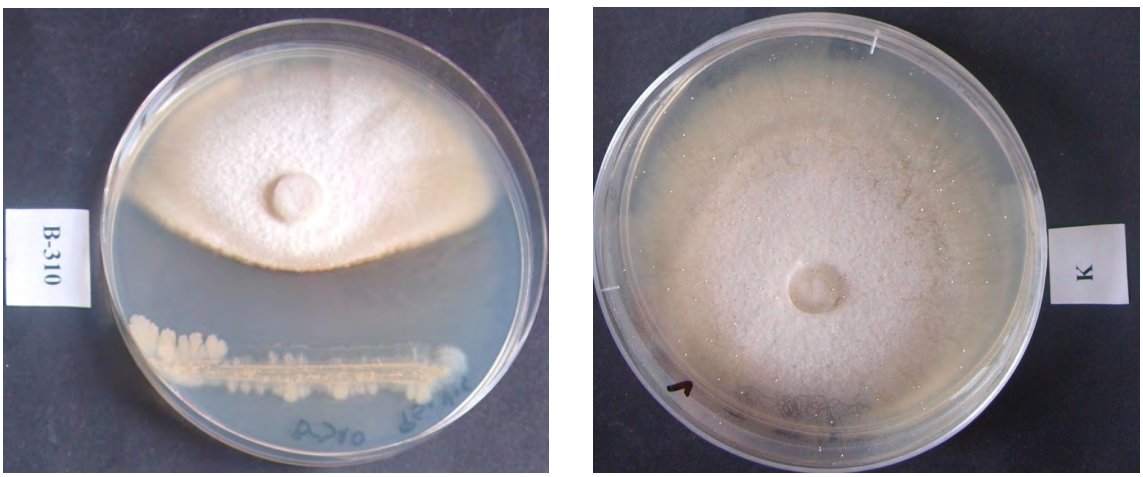

Figure 4. Dual culture method for determining the antagonistic potential of bacterial strains against fungal pathogen. The activity of B-310 strain against *Verticillium* sp. – left, control plate – right.

The pathogenic strain of *C. michiganensis* subsp. *michiganensis* was most inhibited by two *B. subtilis* strains (B–338 and B–348) with mean values of inhibition zone diameter of up to 20 mm (Figures 2 and 3).

In vitro antagonistic activity of Bacillus spp. *against pathogenic* Verticillium sp.

Antagonistic activity of native *Bacillus* spp. strains against the soil borne pepper pathogen (*Verticillium* sp. PS2V16) was evaluated in dual culture tests (Figure 4). The mean values of the growth inhibition percent (PGI) of the tested pathogen are shown in Figure 5.

The largest growth inhibition of *Verticillium* sp. (PGI value approximately 70%) was induced by the strains *B. subtilis* B-310 and B-322 (Figure 5). The other *B. subtilis* strains showed PGI values ranging from 45% to 68%, while *B. pumilus* strain B-335 had the least antagonistic potential (PGI =34.43%).

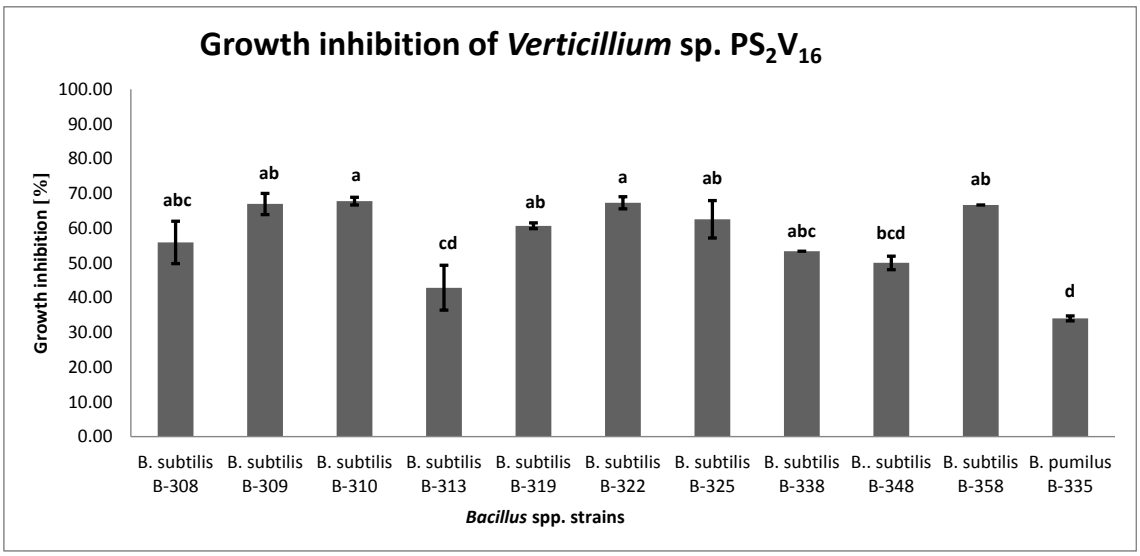

Figure 5. *In vitro* antifungal activity of *Bacillus* spp. strains against *Verticillium* sp. isolate. Mean values of percent of inhibition (n=3) of fungal growth with standard error for each antagonistic strain are shown. *Values marked by the same letter in columns for each isolate are not significantly different (P<0.05), according to Tukey's HSD test.

DISCUSSION

Some recent studies have shown potentials of several different genera of *Bacillus*, *Pseudomonas, Pantoea,* and *Streptomyces* for controlling bacterial pathogens (Obradovic et al., 2005; Stockwell & Stack, 2007; Stockwell et al., 2010; Berić et al., 2012; Dimkić et al., 2013; Milijašević-Marčić et al., 2016).

In this study, a collection of 11 native *Bacillus* strains from manure and composting material were used as a source for identification of strains with antimicrobial activity against several vegetable pathogens. The results of the screening performed with two phytopathogenic bacteria and one soil-borne fungus, used as indicator strains, showed that all *B. subtilis* strains (10) exhibited antagonistic activity against the three pathogens tested. However, the *B. pumilus* strain tested in this study did not show a significant level of inhibition of either fungal or bacterial pathogens. Considering bacterial indicators, *X. vesicatoria* proved to be more sensitive than *C. michiganensis* subsp. *michiganensis*. The tested strain of *X. vesicatoria* was most inhibited by strains B-319, B-325 and B-358, while the best inhibition of *C. michiganensis* subsp. *michiganensis* was achieved by strains B-338 and B-348. Interestingly, *B. subtilis* strain B-319, which was the best in inhibiting *X. vesicatoria,* showed the lowest inhibitory effect on *C. michiganensis* subsp. *michiganensis*. On the other hand, *B. subtilis* strain B-348 which showed the best inhibition of *C. michiganensis* subsp. *michiganensis*, had the lowest effect on *X. vesicatoria*. In a previous study of antagonistic activity of native *B. subtilis*, *B. amyloliquefaciens*, *B. pumilus* and *B. licheniformis* strains, Milijašević-Marčić et al., (2016) recorded significant inhibition of *C. michiganensis* subsp. *michiganensis*. The authors reported that three *B. amyloliquefaciens* showed the best inhibition of that pathogen; four *B. pumilus* strains had the lowest antagonistic activity, consistent with our present results, while one of the tested *B. pumilus* strains (B-217) produced inhibition zones of 14 mm. The study also reported that native *B. subtilis* strains produced inhibition zones of 5-9 mm, contrasting data in the present study that showed inhibition zones of 15-20 mm. In addition, Berić et al. (2012) reported a remarkable antimicrobial activity of broth culture and cell-free supernatants of natural *Bacillus* sp. isolates against a range of pathogenic bacteria: *Burkholderia glumae*, *B. cepacia*, *B. plantarii, Erwinia carotovora*, *Pseudomonas fuscovaginae*, *P. aeruginosa*, *Agrobacterium tumefaciens*, *Xanthomonas oryzae pv. oryzae*, and *Ralstonia solanacearum,* indicating potential uses of natural *Bacillus* strains as biocontrol agents. Dimkić et al. (2013) noted significant antibacterial activity of ethyl acetate extracts of cell-free supernatants of two *Bacillus* sp. strains against several postharvest fungal pathogens and two

phytopathogenic bacteria, *Xanthomonas arboricola* and *Pectobacterium carotovorum*.

This study proved well diffusion as an efficient method for preliminary screening of antagonistic activity among a large number of bacterial isolates, and enabled the selection of potential biocontrol candidates against two pathogenic bacteria in tomato crop. However, it deserves to be noted that the promising results *in vitro* sometimes do not correlate with experiments *in planta* and therefore further *in vivo* trials are needed.

As for the fungal pathogen tested, this study is considered to be the first step in identifying potential biocontrol agents for control of Verticillium wilt in pepper. In search for biologicals to control Verticillium wilt, candidates from the genus *Bacillus* have been well-explored. It is notable that over two thirds of the *Bacillus* strains tested belong to the species *Bacillus amyloliquefaciens* and *Bacillus subtilis* (Deketelaere et al., 2017). The results obtained in our study are promising, and suggest that bacterial antagonists are potential candidates for biological control of Verticillium wilt disease in pepper and its causal agent. Bacterial antagonists tested in the study, showed inhibition zones ranging from 10 to 20 mm against *Verticillium* sp. isolate. In a recent study of Mihajlović et al. (2017), mycelial growth of *Verticillium* sp. in the activity zone of *B. subtilis* was not completely inhibited, although a zone of partial inhibition of mycelial growth with a diameter of 16 mm was observed. However, when applied under greenhouse conditions, the product based on *B. subtilis* showed an efficacy of only 35.4% in Verticillium wilt control (Mihajlović et al., 2017). Therefore, the results of our study still have to be confirmed in *in vivo* trials. It is also important to note that although numerous studies have aimed at controlling Verticillium wilt using *Bacillus* species, only the strain *B. amyloliquefaciens* 5-127, isolated from tomato roots, was tested on different host plants (Deketelaere et al., 2017).

Disease suppressive soils are an interesting source of biological control agents with potentials against soil-borne diseases (Cook, 1985). Organic amendments have proved to be disease suppressive and therefore are also interesting reservoirs of potential biologicals. Several isolates controlling Verticillium wilt have been obtained from suppressive composts: two *Fusarium oxysporum* and two *Pseudomonas fluorescens* isolates originating from the rhizosphere of eggplants grown in soil amended with disease suppressive compost (Malandraki et al., 2008), while isolates belonging to *Arthrobacter* and *Blastobotrys* were obtained from disease suppressive olive mill compost (Papasotiriou et al., 2013). In our study, composting material proved to be a valuable source of bacterial isolates with antagonistic properties against vegetable pathogens. In addition, it is important to identify the antimicrobial spectrum of *Bacillus* spp. natural isolates in order to find the best candidates that could be used against a wide range of pathogens affecting vegetables. This study selected at least one suitable biocontrol candidate, *B. subtilis* strain B-358, effective *in vitro* against all three vegetable pathogens. The next step, aiming to confirm the colonization ability of the selected strain and testing its *in vivo* efficacy, will be the subject of a follow-up investigation.

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Antagonistički potencijal izolata *Bacillus* spp. protiv bakterija patogena paradajza i gljive patogena paprike

REZIME

Ispitivan je antagonistički potencijal jedanaest izolata *Bacillus* spp. protiv dve fitopatogene bakterije i jedne gljive u *in vitro* uslovima s ciljem identifikacije potencijalnih agensa biološke kontrole u povrtarskim usevima. Izolati *Bacillus* spp. ispoljili su različit stepen antagonističkog dejstva protiv testiranih patogenih mikroorganizama. Rezultati ispitivanja pokazali su veću osetljivost *X. vesicatoria* prema sojevima *Bacillus* spp. u poređenju sa *C. michiganensis* subsp. *michiganensis*. Deset sojeva *B. subtilis* izazvali su inhibiciju porasta *X. vesicatoria*, dok soj *B. pumilus* nije uticao na porast ove bakterije. Najveće zone inhibicije protiv *X. vesicatoria* izazvali su sojevi B-319, B-325 i B-358. Fitopatogenu bakteriju *C. michiganensis* subsp. *michiganensis* najviše su inhibirala dva soja *B. subtilis* (B–338 i B–348) sa prosečnim vrednostima zona inhibicije prečnika do 20 mm. Soj *B. subtilis* B-319 koji se pokazao najboljim u inhibiciji *X. vesicatoria,* ispoljio je najmanji inhibitorni efekat na *C. michiganensis* subsp. *michiganensis*. Najveći procenat inhibicije porasta (PGI) patogene gljive *Verticillium* sp. (PGI oko 70%), izazvali su sojevi *B. subtilis* B-310 i B-322. Ostali testirani sojevi *B. subtilis* imali su vrednosti PGI od 45% do 68%, dok je soj *B. pumilus* B–335 ispoljio najmanji antagonistički potencijal (PGI=34.43%) prema ovom patogenu. Rezultati ovog istraživanja identifikovali su najmanje jednog pogodnog kandidata za biološku kontrolu, i to soj *B. subtilis* B-358, koji se pokazao efikasnim *in vitro* protiv sva tri patogena povrća.

Ključne reči: Biološka kotrola; *Bacillus*; Patogene bakterije; Patogene gljive; Paradajz; Paprika