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DR. TANJA BERIĆ (Orcid ID : 0000-0002-4860-2225)

PROF. SLAVIŠA STANKOVIĆ (Orcid ID : 0000-0003-0527-8741)

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Biological control of *Pseudomonas syringae* pv. *aptata* on sugar beet with *Bacillus pumilus* SS-10.7 and *Bacillus amyloliquefaciens* (SS-12.6 and SS-38.4) strains

Ivan Nikolić[1], Tanja Berić[1*], Ivica Dimkic[1], Tatjana Popović[2], Jelena Lozo[1], Djordje Fira[1] and Slaviša Stanković[1]

[1]Faculty of Biology, University of Belgrade, Studentski trg 16, 11000 Belgrade, Serbia;

[2]Institute for Plant Protection and Environment, Teodora Drajzera 9, 11040 Belgrade, Serbia

Abbreviated running headline: Biocontrol of *P. syringae* pv. *aptata* with *Bacillus*

Corresponding author:

Tanja Berić

Tel. number: 00381112637264

E-mail address: tanjab@bio.bg.ac.rs

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Abstract

Aim: Assessment of biological control of *P. syringae* pv. *aptata* using crude lipopeptide extracts (CLEs) of two *Bacillus amyloliquefaciens* strains (SS-12.6 and SS-38.4) and one *Bacillus pumilus* strain (SS-10.7).

Methods and Results: The minimum inhibitory concentration (MIC) of CLEs and their combinations against the pathogen and potential interaction between the extracts were determined *in vitro*. The most effective antibacterial activity was achieved with the CLE from *B. amyloliquefaciens* SS-12.6, with an MIC value of 0.63 mg ml^{-1}. Interactions between CLE combinations were mostly indifferent. The biocontrol potential of CLEs, mixtures of CLEs, and cell culture of *B. amyloliquefaciens* SS-12.6 was tested on sugar beet plants inoculated with *P. syringae* pv. *aptata* P53. The best result in inhibiting the appearance of tissue necrosis (up to 92%) was achieved with *B. amyloliquefaciens* SS-12.6 cell culture.

Conclusion: This work demonstrated significant biocontrol potential of the CLE and cell culture of *B. amyloliquefaciens* SS-12.6 which successfully suppress leaf spot disease severity on sugar beet plants.

Significance and Impact of the Study: The findings of biocontrol of sugar beet emerging pathogen will contribute to growers in terms of alternative disease control management. This study represent first assessment of biological control of *P. syringae* pv. *aptata.*

Keywords: *Pseudomonas syringae* pv. *aptata*, *Bacillus amyloliquefaciens*, biological control, sugar beet, lipopeptides.

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Introduction

Pseudomonas syringae represents a ubiquitous and economically very important plant pathogen which affects a number of hosts, including fruit trees, field crops, vegetables, and ornamental plants. Disease symptoms can be very diverse, including flower blast, necrotic leaf spots, discolored and blackened leaf veins and petioles, spots and blisters on fruit, and stem canker (Morris et al., 2000; Kennelly et al., 2007; Ferrante and Scortichini, 2010;). Detection and identification of leaf spot disease caused by *P. syringae* pv. *aptata* on sugar beet in Serbia, as well as genetic and pathogenic characteristics of collected strains, were reported by Stojšin et al. (2015) and Nikolić et al. (2018). Although large decreases of sugar beet yields have not been detected so far in Serbia, it is very important to make proper and environmentally friendly preparation for it if an outbreak happens. Recent frequent reports of sugar beet disease caused by *P. syringae* pv. *aptata* worldwide emphasize the importance of this pathogen (Dutta et al., 2014; Stiojšin et al., 2015; Arabiat et al., 2016).

Microbiological biopesticides, in particular products based on *Bacillus* strains, promote and protect the health of plants through many mechanisms and thereby draw attention to themselves as possible candidates for commercial application (Shafi et al., 2017). The activity of beneficial organisms, such as production of secondary metabolites (especially lipopeptides) with antimicrobial activity and the ability to promote plant growth, makes it possible to control diseases caused by bacterial pathogens (Butt et al., 1999; Fuentes-Ramirez and Caballero-Mellado, 2005; Ongena and Jacques, 2008). Trends in control management of all diseases caused by plant pathogens include the use of more and more alternative treatments that have certain advantages in terms of low toxicity, high resistance, good biodegradability, and ecological acceptability in comparison with chemical pesticides (Yang et al., 2006). Also, biopesticides usually have a mode of action different from that of conventional pesticides, which enables them to suppress already resistant pests and avoid development of further

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resistance (Cawoy et al., 2011). A number of *Bacillus* species, especially *B. subtilis*, *B. amyloliquefaciens*, and *B. pumilus* possess antimicrobial activity and their lipopeptides being mostly responsible for suppression of necrotizing plant pathogens (Dimkić et al., 2013; Chowdhury et al., 2015; Agarwal et al., 2017; Dimkić et al., 2017). Also, many *Bacillus* strains produce several lipopeptides from the surfactin, iturin, and fengycin families (Ongena and Jacques, 2008).

Lipopeptides can act synergistically and thereby increase their antimicrobial activity, a fact which has some ecological advantages and encourages the use of producer strains in biological control of plant pathogens (Falardeau et al., 2013; Liu et al., 2014). Biological control of *P. syringae* pathovars by *Bacillus* isolates was investigated in several recent studies (Sabir et al., 2017; Wicaksono et al., 2018). However, biological control of *P. syringae* pv. *aptata* has so far not been conducted, and the present paper represents the first report about biocontrol of this bacterial pathogen of sugar beet. Reports about biological control of pathogens causing diseases of sugar beet have mostly dealt with fungal pathogens, especially *Cercospora beticola*, *Sclerotium rolfsii*, and *Rhizoctonia solani* (Collins and Jacobsen, 2003; Errakhi et al., 2007; Zachow et al., 2010).

In order to test the biocontrol approach to management of leaf spot disease caused by *P. syringae* pv. *aptata*, we investigated the antibacterial activity of crude lipopeptide extracts (CLEs) of *B. amyloliquefaciens* SS-12.6, *B. amyloliquefaciens* SS-38.4, *B. pumilus* SS-10.7, and various combinations thereof *in vitro*; and that of *B. amyloliquefaciens* SS-12.6 cell culture *in planta*.

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Material and Methods

Bacterial strains and growth conditions

The strains used in this study are part of previously established and characterized collections (Stanković et al., 2012; Nikolić et al., 2018). From the *Pseudomonas syringae* collection, we used strain P12, strain P16, strain P53, and reference strain CFBP2473, which have been identified as *Pseudomonas syringae* pv. *aptata*. From the *Bacillus* collection, we used *Bacillus amyloliquefaciens* SS-12.6 and SS-38.4 strains and *Bacillus pumilus* SS-10.7 (Dimkić et al. 2017). The *Pseudomonas syringae* strains were grown on nutrient agar supplemented with 5% sucrose (NSA) and incubated for 2 days at 26°C, while the *Bacillus* strains were grown on Luria-Bertani (LB) medium and incubated for 24 h at 30°C with aeration. For antimicrobial assays, isolates of *P. syringae* were cultured in LB medium for 24 h at 30°C and suspensions were adjusted to McFarland standard turbidity (0.5) (BioMérieux, Marcy-l'Étoile, France), which corresponds to 10^8 CFU ml^{-1}.

Ethyl-acetate extraction of lipopeptides

Ethyl acetate extraction method, previously reported (Kuiper et al. 2004), was used for crude lipopetide extraction. All of the tested *Bacillus* strains were grown in 1000 ml of LB for 24 h at 30°C with aeration. Cell-free supernatant from cultures was obtained by centrifugation (5,000 × g, 20 min) at 4°C, and used for extraction of lipopeptides with ethyl-acetate. The culture supernatant and ethyl-acetate were mixed with the addition of NaCl (30 g l^{-1}) in a 1:1:1 volumetric ratio (*v/v/v*) and that suspension was mixed for 2 h on a magnetic stirrer. Rotary evaporator (Büchi Rotavapor R-215, Switzerland) was used for completely drying of ethyl acetate fraction. The dried CLEs were resuspended in DMSO (dimethyl sulphoxide) and filtered through a 0.45 µm DuraporeTM filter (Millipore, Billerica, USA).

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MIC assay

A broth microdilution method previously described elsewhere (Dimkić et al., 2016) was used for determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the tested CLEs of SS-10.7, SS-12.6, and SS-38.4, individually and in combinations. The combinations consisted of two and three CLEs. The combination referred to as MIX A consisted of CLEs of the SS-10.7 and SS-12.6 strains, MIX B consisted of CLEs of the SS-10.7 and SS-38.4 strains, while MIX C consisted of CLEs of the SS-12.6 and SS-38.4 strains. Combinations with CLEs of three strains were labelled as Mix 1 and Mix 2. Mix 1 represents a CLE obtained from co-culture of all three tested strains (which grew simultaneously), while Mix 2 represents a mixture of CLEs obtained from individual strains and then mixed. Two-fold serial dilutions of the samples with LB medium in 96-well microtitre plates were performed in the concentration range of from 2.5 to 0.04 mg ml^{-1}. DMSO was used as a solvent at final concentration 0.5%. Alongside of CLEs, a solvent control, sterility control, negative control and antibiotics as positive controls were tested. Streptomycin, gentamycin, vancomycin, and cefepime (Sigma-Aldrich, USA) were tested in the concentration range of 0.5 - 0.0004 mg ml^{-1}, while ampicillin was tested in the concentration range of 1 - 0.016 mg ml^{-1}. In addition, analytical-grade purity iturin A and surfactin standards (Sigma-Aldrich, USA) were used in the concentration range of 0.5 - 0.008 mg ml^{-1}as a positive control. Each well was inoculated with 20 µL of bacterial suspensions (1 $\times$ 10^{8} CFU ml^{-1}) of *P. syringae* pv. *aptata* (P12, P16, P53, and CFBP2473), reaching a final volume of 200 µL per well. In order to detect bacterial growth, 22 µL of resazurin (0.675 mg ml^{-1} final concentration, TCI, Belgium) was added to each well. All samples were tested in triplicate and the microtitre plates were put for incubation period of 24 h at 30°C. The lowest diluted concentration (mg ml^{-1}) which showed no change in color was defined as the MIC. Determination of MBC was performed by sub-culturing material from each well without color

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change on agar plates and incubating it for 18-24 h. The lowest diluted concentration (mg ml^{-1}) that showed no bacterial growth was marked as the MBC value.

Determination of effects of CLEs in different combinations

Determination of the type of interaction between crude lipopeptide extracts obtained from *B. amyloliquefaciens* and *B. pumilus* strains was done by the method presented above in description of the MIC assay. The concentration of tested uniform stocks of all CLEs dissolved in DMSO was 25 mg ml^{-1}. Double and triple mixtures of CLEs were made with individual CLEs present in ratios of 1:1 and 1:1:1, respectively. Serial dilution was carried out as in the MIC assay, and 20 μL of bacterial suspensions was added to each well, reaching a final volume of 200 μL. In order to detect bacterial growth, 22 μL of resazurin (0.675 mg ml^{-1} final concentration, TCI, Belgium) was added to each well. After 24 h of incubation at 30°C, the lowest concentration which showed no change in color was defined as the MIC value for certain combinations of CLEs. To establish the type of interactions of CLEs, the MICs of individual CLEs in different combinations were transformed into fractional inhibitory concentrations (FIC) according to following ratio:

$FIC_{(A)}$ = (MIC of compound A in the presence of compound B)/(MIC of compound A); and

$FIC_{(B)}$ = (MIC of compound B in the presence of compound A)/(MIC of compound B).

The fractional inhibitory concentration index (FICi) was calculated from FIC values for each CLE extract as follows: $FICi = FIC_{(A)} + FIC_{(B)}$, where A represents the first extract and B the second one in combinations. Calculations of the FIC indexes for triple mixtures were performed in the same manner. On the basis of FICi values, interaction type was presented as: synergistic effect when $FICi \leq 0.5$; additive effect when $FICi = 0.5 – 1$; indifferent effect when $FICi = 1 – 2$; and antagonistic effect when $FICi \geq 2$ (Dimkić et al., 2016).

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In planta biological control - treatment with crude lipopeptide extracts

Biological control of *P. syringae* pv. *aptata* P53 was tested on leaves of planted sugar beet (*B. vulgaris* L. cv. Marinela, KWS, Germany). Seeds were sterilized for 10 minutes in 0.3% (*v/v*) of sodium hypochlorite, washed four times in sterile distilled water, and stored at 4°C for 48 h. After 48 h seeds were put in plastic pots 10 cm in diameter and incubated in a climate chamber at 23°C with a photoperiod consisting of 12 h light and 12 h of darkness for 4 weeks prior to inoculation. Sugar beet plants at the age of 28 days were inoculated simultaneously with *P. syringae* pv. *aptata* P53 and CLEs (of individual strains and in various combinations), as previously described in the study of May et al. (1997). Individual CLEs were tested at a final concentration of 1 mg ml^{-1} dissolved in 0.66% DMSO. Mixtures of two and three individual CLEs in ratios of 1:1 and 1:1:1, respectively, were used, in addition to co-culture at 1 mg ml^{-1}. The suspension of the pathogenic bacterial strain was diluted up to 10^7 CFU ml^{-1}. Plants were inoculated using a sterile syringe needle to inject a mixture of CLEs and cell culture of strain P53 in a 4:1 ratio (*v/v*) in a total volume of 10 μL, as described previously (Montesinos and Bonaterra, 1996; Smith et al., 1997). Plants were also inoculated only with *P. syringae* pv. *aptata* P53 as a positive control, as well as only with a solvent as a negative control. The individual CLEs and CLE mixtures were used as a negative control. The inoculations were performed on 18 plants with three injection sites per each leaf, and the whole experiment was repeated twice. Following inoculation, plants were incubated for 7 days in a very humid (90%) climate chamber at 23°C with a constant photoperiod consisting of 12 h of light and 12 h darkness. After the incubation period, the presence or absence of symptom development was noted. For better visibility, the leaves were pulled off and then photographed (Fig. 1).

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***In planta* treatment with *B. amyloliquefaciens* SS-12.6 cell culture**

Biological control with SS-12.6 cell culture against two strains of *P. syringae* pv. *aptata* (P53 and CFBP2473) was tested on leaves of four cultivars of sugar beet, viz., Marinela, Serenada, and Jasmina (KWS, Germany); and Lara (NS, Serbia). Plants were prepared in the same manner as described above. Sugar beet plants at the age of 28 days were inoculated directly in petioles above the leaf plate with mixed cell cultures of *P. syringae* pv. *aptata* and *B. amyloliquefaciens* SS-12.6 in a 1:4 ratio (*v*/*v*). The initial concentrations of P53, CFBP2473, and SS-12.6 bacterial suspensions were 10^7 CFU ml^{-1}. Plants were inoculated only with the pathogen (P53 or CFBP2473) as a negative control. In order to assess the potential cause of necrosis of *B. amyloliquefaciens* SS-12.6, this strain was inoculated alone. Sixty plants were used for one experiment, where inoculations were performed in triplicate and the experiment was repeated twice. In each injection, 10 μL of the mixed suspension was added and the plants were incubated for 7 days in a climate chamber at 23°C with humidity 90% and a photoperiod consisting of 12 h of light and 12 h of darkness. After the incubation period, disease severity was rated on a scale of 1 to 6 (1 = no symptoms, 2 = 1–10%, 3 = 11–25%, 4 = 26–50%, 5 = 51–75%, and 6 = 76–100% leaf necrosis). Individual leaves disease severity were calculated according to Griesbach and Tyrach (1999).

Statistical analysis

Kolmogorov–Smirnov test for normality and Levene's test for homogeneity of variance was used for variance analysis. Data were subjected to analysis of variance (ANOVA) and means separation of disease severity *in planta* was performed using Tukey's HSD (honest significant difference) test. Statistically significant reduction of disease severity was taken at $P<0.05$. Statistical analyses were conducted by using STATISTICA v. 7 (StatSoft, Inc., USA) and IBM SPSS Statistics v. 20 (SPSS, Inc., USA) software.

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Results

In vitro biological control of *P. syringae* pv. *aptata*

The results of determining the MIC values of individual and combined CLEs against four different strains of *P. syringae* pv. *aptata* are shown in Table 1. The MIC value for the CLE of SS-12.6 was 0.63 mg ml^{-1} against all tested pathogen strains, except for CFBP2473, where the MIC value was 1.25 mg ml^{-1}. Otherwise, the MIC values for individual CLEs of SS-10.7 and SS-38.4 were 2.5 mg ml^{-1} against all tested strains. The MIX A (CLEs of SS-10.7 and SS-12.6) and MIX B (CLEs of SS-10.7 and SS-38.4) combinations of extracts had MIC values of 1.25 mg ml^{-1}, while MIX C (CLEs of SS-12.6 and SS-38.4) had MIC values of 0.63 mg ml^{-1}, against all tested strains. Gentamicin and streptomycin had the lowest MIC values, while the iturin A and surfactin standards had MIC values higher than 0.5 mg ml^{-1}. In order to determine the individual contribution of extracts and interaction between them in various combinations, FIC and the FICi were calculated (Table 1). An indifferent effect was determined for MIX A against strains P12 and P53, while antagonistic effect was determined for strains P16 and CFBP2473. Also, indifferent effect was determined for MIX C against all strains except CFBP2473, where an additive effect was scored. The MIX B combination of extracts had an additive effect on all *P. syringae* pv. *aptata* strains. The lipopeptide extracts of MIX 1 (CLE from co-cultivated SS-10.7, SS-12.6, and SS-38.4) showed MIC values of 0.83 mg ml^{-1}, while for MIX 2 (CLEs of SS-10.7, SS-12.6, and SS-38.4 extracted from individual strains and mixed together) the recorded MICs were in the range of 2.5-1.25 mg ml^{-1}. An antagonistic effect was observed only in the case of MIX 2 against P12, P16, and P53, as opposed to MIX 1, which exhibited an indifferent effect against all tested strains. The MBC values were very high or were not determined at all (data not shown).

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In planta biological control of *P. syringae* pv. *aptata* with crude lipopeptide extracts

Figure 1 shows the results of determining *in planta* antimicrobial activity of CLEs on sugar beet plants inoculated with *P. syringae* pv. *aptata* strain P53 after 7 days of incubation. Treatment only with CLEs and only with solvent did not cause necrotic lesions on leaf tissue. On the other hand, necrotic lesions of approximately 10 mm in diameter were observed on the leaves of sugar beet inoculated with only the pathogen. The leaves of plants inoculated with the pathogen and the CLE of SS-12.6, CLE of SS-38.4, and the combination of CLEs in MIX C did not show any symptoms of necrosis. The mixture of the pathogen and the combination of CLEs in MIX 1 did not cause necrosis, but rather just chlorosis, while the mixture of the pathogen and MIX 2 caused no chlorotic or necrotic lesions at all. Plants inoculated with the pathogen and the CLE of SS-10.7 developed necrotic lesions, as did ones inoculate with it in combination with MIX A and MIX B, but the lesions were smaller than in the control.

In planta biological control of *P. syringae* pv. *aptata* with *B. amyloliquefaciens* SS-12.6 cell culture

The experiment was performed by simultaneously inoculating leaf petioles of four cultivars of sugar beet with *B. amyloliquefaciens* SS-12.6 and *P. syringae* pv. *aptata* P53 and CFBP2473 pathogenic strains. The effect on potted plants after 7 days of incubation is shown in Fig. 2. The condition of plants treated only with pathogens was very poor, they lost turgor and the leaf blade was bent (Figs. 2a and 2b), while plants treated with SS-12.6 cell culture (Fig. 2c) and combinations of SS-12.6 cell culture and both pathogen strains (Figs. 2d and 2e) were evidently in good shape. As can be seen in Fig. 3, disease symptoms caused by P53 and CFBP2473 were ranged from 50 to 100% necrosis for all sugar beet cultivars. The highest disease severity (100%) for strains P53 and CFBP2473 was observed for the Serenada and Jasmina cultivars, respectively. The lowest disease severity for strain P53 (50%) was

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observed for the Lara cultivar. Strain CFBP2473 caused the lowest disease severity (65%) on the Serenada cultivar. On the other hand, plants treated with a mixture of SS-12.6 cell culture and pathogens developed necrosis in the range of from 8 to 40% (Fig. 3). The lowest disease severity on plants inoculated with SS-12.6 and P53 was 8% on the Serenada cultivar, while for SS-12.6 and CFBP2473 it was 9% on the Lara cultivar. Treatment with SS-12.6 cell culture and pathogens P53 and CFBP2473 completely inhibited necrotic lesions on the Jasmina and Lara cultivars, and no statistical differences were scored in comparison with the negative control. Not so high, but still statistically significant reduction of disease severity was observed for SS-12.6 cell culture and CFBP2473 on the Marinela and Jasmina cultivars. The weakest biocontrol effect of SS-12.6 on pathogen P53 was recorded for the Lara cultivar.

Discussion

Pseudomonas syringae is one of the most widespread of plant pathogens, an important environmental factor in the water cycle, and a very suitable model organism for exploration of plant-microbe interaction and microbial evolution in nature (Baltrus et al., 2017). Serious bacterial leaf spot disease was observed in 2013 on commercial cultivars of sugar beet (*Beta vulgaris subsp. vulgaris convar. vulgaris var. altissima*) in the Vojvodina Province, and *P. syringae* pv. *aptata* was confirmed as the causative agent (Nikolić et al., 2018).

Numerous studies have demonstrated the role played by lipopeptide compounds in exerting a direct effect on plant pathogens (Toure et al., 2004; Berić et al., 2012; Cao et al., 2012; Dimkić et al., 2013, 2015). A previous study of ours (Dimkić et al., 2017) assessed production and content of crude lipopeptide extracts of several *Bacillus* strains, including the isolates tested in the present study. It was determined that CLEs of SS-10.7, SS-12.6, and SS-38.4 are mostly composed of surfactin, fengycin A, and iturin A lipopeptides, of which iturin was responsible for inhibition of the growth of *P. syringae* pv. *aptata* P16. According to

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presented results in study Dimkić et al. (2017), we assessed biocontrol effect of CLEs and cell culture of already proven potent Bacillus strains *in vitro* and *in planta*, which could contribute to the potential application of these strains in the sugar beet control management.

Screening of potential antimicrobial substances against pathogens is commonly performed using the microdilution method to determine the minimal inhibitory concentration (Kadaikunnan et al., 2015; Balouiri et al., 2016). In this way, Bais et al. (2004) showed that surfactin produced by the *B. subtilis* 6051 strain has an MIC value of 25 mg ml^{-1}against *P. syringae* pv. *tomato* DC3000. In our study, the lowest MIC value of all crude lipopeptide extracts was recorded for the CLE of SS-12.6 (0.63 mg ml^{-1}). Also, all combinations which contained the CLE obtained from SS-12.6 had the lowest MIC value against tested strains of *P. syringae* pv. *aptata*. Crude extracts from co-cultivated *Bacillus* strains had low MIC values (0.83 mg ml^{-1}), although not as low as for the CLE of SS-12.6 alone, but considerably lower than that of a mixture of CLEs extracted from individual strains (2.5 mg ml^{-1}). The CLE from co-culture was used on the assumption that strains could have higher production of lipopeptides in co-culture growth. Zhi et al. (2017) showed that co-culture growth of two *B. amyloliquefaciens* strains led to 1.5-fold higher production of surfactin than in the case of individual strains. Results of other research (Bais et al., 2004: Arrebola et al., 2010) and also our inability to detection MBC values of tested CLEs points out that the primary mode of lipopeptide activity was bacteriostatic.

Bacillus strains often produce more than one antimicrobial lipopeptide, which can result in their higher activity due to interactions between the different compounds (Falardeau et al., 2013). Also, other types of antimicrobials that are bacilli capable of production, which we didn't follow in this work, could contribute to overall antimicrobial activity. Although many studies with different strain mixtures have been devoted to inhibition of fungal pathogens, studies on biological control of bacterial phytopathogens are scarce. Etchegaray et al. (2008)

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demonstrated that a mixture of surfactin and iturin lipopeptides successfully modifies and disintegrates the cell wall of *Xanthomonas campestris* pv. *campestris* and *Xanthomonas axonopodis* pv. *citri*, as causal agents of citrus canker. Also, Mora et al. (2015) confirmed a relationship between strong antibacterial activities of *Bacillus* isolates (*B. subtilis*/*amyloliquefaciens*) and the presence of surfactin and fengycin genes in action against *Ralstonia solanacearum, Erwinia amylovora, Pseudomonas syringae* pv. *syringae, Xanthomonas arboricola* pv. *fragariae,* and *Xanthomonas axonopodis* pv. *vesicatoria*. In order to investigate interactions between CLEs, we made mixtures of CLEs in different combinations. The combinations tested in the present study showed an indifferent or additive effect on pathogen strains P12, P16, P53, and CFBP2473. The combination of CLEs from strains SS-10.7 and SS-38.4 (MIX B) had an additive effect and a lower MIC value than CLEs of individual strains. In the conducted *in vitro* tests, we showed that the best antibacterial activity against *P. syringae pv. aptata* was exhibited by the CLE from strain SS-12.6, the combination of CLEs of SS-12.6 and SS-38.4 (MIX C), and the crude extract from co-cultivated strains (MIX 1). The simultaneous application of more than one active ingredient in a single product can be more effective and reliable under different conditions (Cawoy et al., 2011). Plant growth-promoting rhizobacteria strains INR7 (*B. pumilus*), GB03 (*B. subtilis*), and ME1 (*Curtobacterium flaccumfaciens*) were used in combinations for biological control of multiple cucumber pathogens, including *Pseudomonas syringae* pv. *lachrymans* (Raupach and Kloepper, 1998). The results showed more intensive promotion of plant growth and disease reduction in the mixture-treated plants. The use of agents with additive and synergistic effects in various combinations employing lower concentrations of individual extracts could be advantageous in combating bacterial pathogens.

All pathogenic strains of *P. syringae* pv. *aptata* tested *in vitro* had similar susceptibility to the CLEs of *Bacillus* strains, and we chose P53 for further investigation of biological control *in*

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planta. A study by Abdallah et al. (2015) showed antibacterial activity of a lipopeptide-enriched extract from *B. amyloliquefaciens* against *Agrobacterium tumefaciens* on carrot. Similarly, treatments of plants with the pathogen and CLEs of individual strains in our study were generally successful in inhibition of necrotic lesions, except in the case of CLE of SS-10.7 which did not inhibit necrotic lesions completely. Any combination of CLEs containing the CLE of strain SS-10.7 showed low antibacterial activity: lesions were detected on leaves, but they were smaller than on plants treated only with pathogen P53. These findings are in accordance with *in vitro* results, which indicated that the CLE of strain SS-10.7 had the highest MIC values. In contrast, MIX 2 which *in vitro* showed an antagonistic interaction, when used *in planta* did not cause any observable necrotic lesions. *In planta* testing of CLEs confirmed the potential of lipopeptide compounds in direct antagonism against plant pathogens. Also, the absence of leaf necrosis on sugar beet plants inoculated only with CLEs unquestionably recommends them for further research and safe use in biological control of *P. syringae* pv. *aptata*.

Numerous studies have been published on biological control of plant pathogens using pure bacterial culture of strains from the *B.subtilis* group (Zeriouh et al., 2011; Jiang et al., 2015; Huang et al., 2016). According to Lanna Filho et al. (2013), *B. amyloliquefaciens* and *B. pumilus* were successfully used for biocontrol of *P. syringae* pv. *tomato* on young tomato plants. They recorded reduction of the pathogen population by up to 84 and 97% on leaves pre-treated with *B. pumilus* and *B. amyloliquefaciens*, respectively. Fousia et al. (2016) showed that tomato plants treated with the pathogen *P. syringae* pv. *tomato* and after that with *B. subtilis* QST 713 had just 7% disease severity. Also, 60% of all plants treated with *B. subtilis* QST 713 were symptomless, while plants treated only with the pathogen and 70% of plants treated with copper hydroxide showed symptoms. Similarly, in our study we used pure culture of *B. amyloliquefaciens* SS-12.6 for inoculation of leaf petioles on sugar beet plants

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together with pathogenic strain P53, and it proved to be the best antagonist of all three tested strains. Necrosis spread slowly and only through the main nerve. Other parts of the leaf blade stayed intact and the general condition of the plants was good. The substantial level of disease suppression by strain SS-12.6, which was in the range from 60 to 92%, recommends this strain for application in biological control of *P. syringae* pv. *aptata*. Devastating disease symptoms with necrotic tissue on the main nerve and leaf blade appeared on plants treated only with the pathogen. The severity of *P. syringae* pv. *aptata* disease on sugar beet cultivars (Marinela, Jasmina, Serenada, and Lara) was assessed in a study of Nikolić et al. (2018), and the Jasmina and Marinela cultivars were found to be the most sensitive. In this study, suppression of disease symptoms was significant for all tested cultivars in comparison with plants treated only with the pathogen.

The present study represents the first report of potential biological control of *P. syringae* pv. *aptata* using *B. amyloliquefaciens* (SS-12.6 and SS-38.4) and *B. pumilus* (SS-10.7) strains. In it, the CLE of SS-12.6 had the lowest MIC values and successfully suppressed disease severity on sugar beet plants. *Bacillus amyloliquefaciens* SS-12.6 cell culture showed notable disease suppression *in planta*. These findings can serve as a stimulus for further research about pathogen-antagonist interactions and contribute to an alternative and effective strategy for disease control management. Serbia is one of the major sugar beet producers in Southeast Europe, which puts the results of our study in the focus of interest and emphasizes the need for additional research on potential applications in biological control. Future investigations will deal with testing of biopesticides based on *Bacillus* directly on sugar beet fields. Their implementation will represent the final step in research on biological control of this sugar beet plant pathogen.

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Conflict of Interest

The authors declare no conflict of interest.

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Table 1 The antibacterial activity and type of interaction of individual and different CLE combinations of *Bacillus* strains against *P. syringae* pv. *aptata* strains.

	***P. aptata* 12**	***P. aptata* 16**	***P. aptata* 53**	***P. aptata* CFBP2473**
	MIC	**MIC**	**MIC**	**MIC**
CLE of SS-10.7	2.50	2.50	2.50	2.50
CLE of SS-12.6	0.63	0.63	0.63	1.25
CLE of SS-38.4	2.50	2.50	2.50	2.50
MIX A	1.25	0.63	1.25	1.25
MIX B	1.25	1.25	1.25	1.25
MIX C	0.63	0.63	0.63	0.63
MIX 1	0.83	0.83	0.83	0.83
MIX 2	2.50	1.25	1.25	1.25
		$FIC_{MIX A}$		
$FIC_{10.7}$	0.50	0.25	0.50	0.50
$FIC_{12.6}$	2.00	1.00	2.00	1.00
FICi	2.50	1.25	2.50	1.50
Interaction	**An**	**I**	**An**	**I**
		$FIC_{MIX B}$		
$FIC_{10.7}$	0.50	0.50	0.50	0.50
$FIC_{38.4}$	0.50	0.50	0.50	0.50
FICi	1.00	1.00	1.00	1.00
Interaction	**Ad**	**Ad**	**Ad**	**Ad**
		$FIC_{MIX C}$		
$FIC_{12.6}$	1.00	1.00	1.00	0.50
$FIC_{38.4}$	0.25	0.25	0.25	0.25
FICi	1.25	1.25	1.25	0.75
Interaction	**I**	**I**	**I**	**Ad**
		$FIC_{MIX 1}$		
$FIC_{10.7}$	0.33	0.33	0.33	0.33
$FIC_{12.6}$	1.33	1.33	1.33	0.66

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$FIC_{38.4}$	0.33	0.33	0.33	0.33
FICi	1.99	1.99	1.99	1.33
Interaction	**I**	**I**	**I**	**I**
		$FIC_{MIX\ 2}$		
$FIC_{10.7}$	1.00	0.50	0.50	0.50
$FIC_{12.6}$	4.00	2.00	2.00	1.00
$FIC_{38.4}$	1.00	0.50	0.50	0.50
FICi	6.00	3.00	3.00	2.00
Interaction	**An**	**An**	**An**	**I**
		Standards and antibiotics		
Iturin A	>0.50	>0.50	>0.50	>0.50
Surfactin	>0.50	>0.50	>0.50	>0.50
Ampicillin	>1.00	>1.00	>1.00	>1.00
Cefepime	0.25	0.13	0.13	0.25
Gentamicin	0.0008	0.025	0.0006	0.0006
Streptomycin	0.0015	0.0125	0.0015	0.0015
Vancomycin	>0.50	>0.50	>0.50	>0.50

*__MIX A__ represents combination of CLEs from SS-10.7 and SS-12.6 strains; **MIX B** represents combination of CLEs from SS-10.7 and SS-38.4 strains; **MIX C** represents combination of CLEs from SS-12.6 and SS-38.4 strains; **MIX 1** represent mixture of CLEs from co-cultivated SS-10.7, SS-12.6 and SS-38.4 strains; **MIX 2** represents mixture of CLEs of SS-10.7, SS-12.6 and SS-38.4 extracted from individual strains.

Type of interaction: indifferent (I), additive (Ad), and antagonistic (An) effect. MIC values are expressed in mg ml^{-1}.

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FIGURE CAPTIONS

Figure 1 Biocontrol activity of crude lipopeptide extracts of SS-10.7, SS-12.6, and SS-38.4 strains alone and their combinations against *P. syringae* pv. *aptata* P53 on sugar beet after 7 days of incubation. Mix 1 represents CLEs from co-cultivated SS-10.7, SS-12.6, and SS-38.4 strains. Mix 2 represents a mixture of CLEs of SS-10.7, SS-12.6, and SS-38.4 obtained from individual strains. The solvent for CLEs was DMSO.

Figure 2 Biocontrol activity of *B. amyloliquefaciens* SS-12.6 cell culture against *P. syringae* pv. *aptata* P53 and reference strain CFBP2473 on four sugar beet cultivars after 7 days of incubation. Treatments were administered following: a) CFBP2473; b) P53; c) SS-12.6; d) SS-12.6 + CFBP2473; e) SS-12.6 + P53.

Figure 3 Estimation of disease severity 7 days after inoculation with *B. amyloliquefaciens* SS-12.6 and *P. syringae* pv. *aptata* P53 and CFBP2473 cultures (■CFBP2473; □P53; ▥SS-12.6 + CFBP2473; ■ SS-12.6 + P53; ▤ Control plant +SS-12.6). Differences between means within each histogram followed by the same letter are not significantly different (Tukey's HSD test, $P < 0.05$). The data represent average values of two independent experiments.

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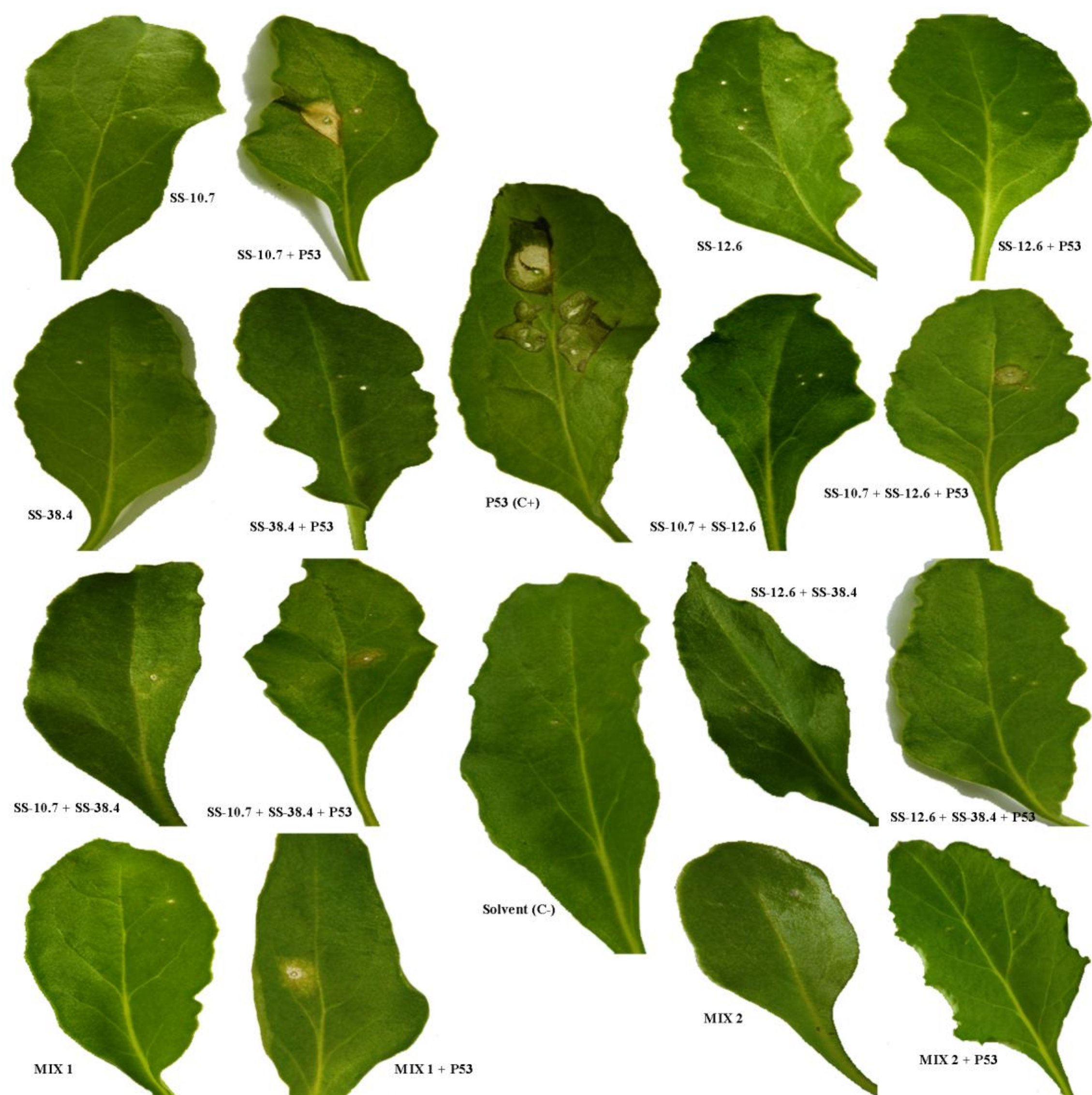

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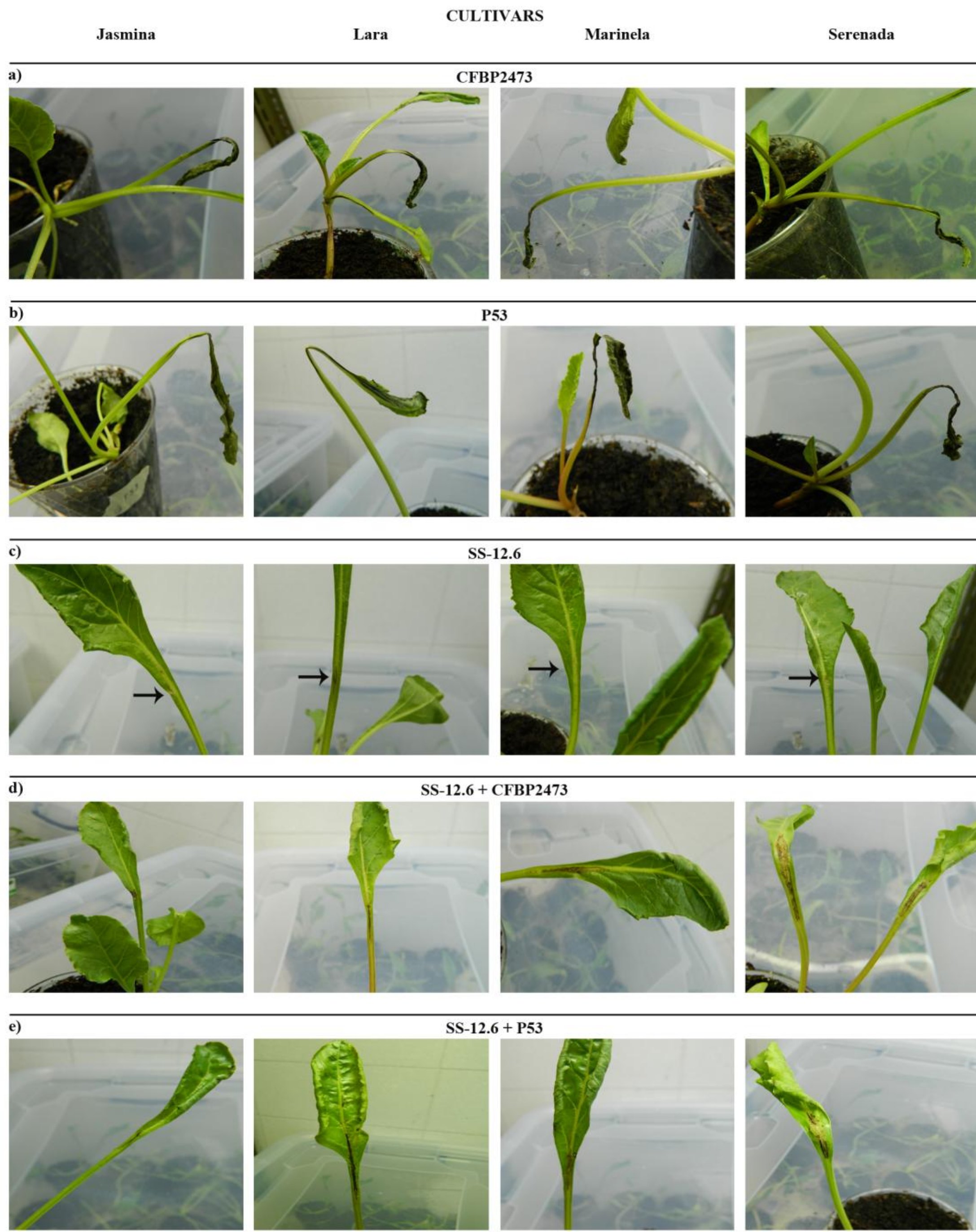

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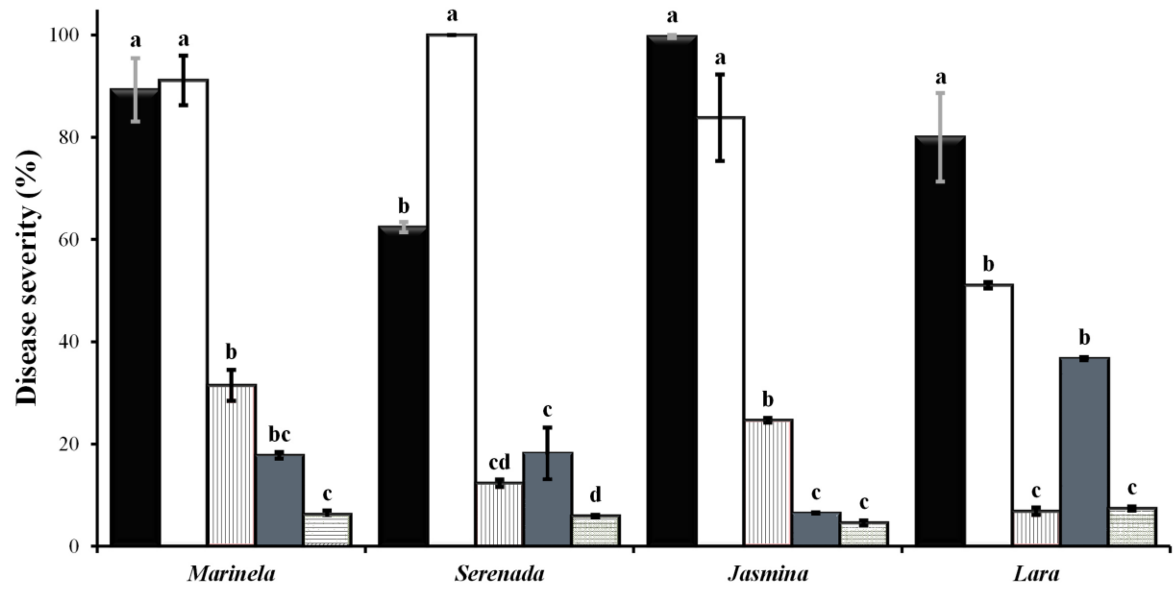

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