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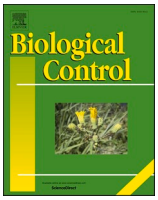

Biological control of major pathogenic bacteria of potato by *Bacillus amyloliquefaciens* strains SS-12.6 and SS-38.4

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HIGHLIGHTS

- Two *B. amyloliquefaciens* strains inhibited the growth of potato pathogens *in vitro*.
- Antagonistic *B. amyloliquefaciens* strains produced antibacterial lipopeptides.
- *Bacillus* spp. strains efficiently suppressed blackleg/soft rot on potato tubers.
- Antagonistic strains reduced the bacterial wilt symptoms *in planta*.

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ABSTRACT

Potato is ranked as one of the most important food crops. Bacterial wilt caused by *Ralstonia solanacearum* and blackleg and soft rot caused by different species from genera *Pectobacterium* and *Dickeya* are considered two of the most important diseases of the potato. Biological control is the optimal strategy for controlling pathogens in crops generally, including bacteria. The present study aimed to evaluate the antagonistic effects of two *Bacillus amyloliquefaciens* strains, SS-12.6 and SS-38.4, against bacterial pathogens isolated from the potato in Serbia, such as *R. solanacearum*, *Pectobacterium carotovorum*, *Pectobacterium brasiliense*, and *Dickeya dianthicola*. The diameter of the inhibition zones formed by ethyl-acetate extracts of SS-12.6 and SS-38.4 strains show much higher values than the inhibition zones of supernatants, which implies the main power of these antagonists' potential lies in lipopeptides. The effectiveness of the treatment (19.7–44.5%), based on the difference in weight of potato tubers on the fifth and 15th day after treatment (DAT), showed that the antagonistic strains were almost equally effective in the suppression of *P. carotovorum*, *P. brasiliense*, and *D. dianthicola* strains. Strains SS-12.6 and SS-38.4 exhibited the efficacy in the suppression of *R. solanacearum* wilt from 28.64 to 60.22%. The analysis of the area under the disease progress (AUDPC) confirmed differences among pathogen control treatments and biocontrol treatments with *B. amyloliquefaciens* strains in all trials. This study shows that the two *B. amyloliquefaciens* strains, SS-12.6 and SS-38.4, can potentially be used as biocontrol agents against potato pathogens.

1. Introduction

Potato (*Solanum tuberosum* L.) is one of the most globally important crops, next to maize, wheat, and rice (Devaux et al., 2020). Its yield is affected by various diseases, but those caused by bacterial pathogens could lead to a loss of even up to 80% (Mousa et al., 2022). According to Charkowski et al. (2020), the potato's two most significant bacterial diseases are bacterial wilt caused by *Ralstonia solanacearum* (designated as a quarantine organism in the EU zone), and blackleg and/or soft rot that a wide range of bacteria could cause. There are twelve species from the *Pectobacterium* genus (*P. aroidearum, P. atrosepticum, P. brasiliense, P. carotovorum, P. odoriferum, P. parmentieri, P. parvum, P. peruviense, P.*

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Table 1
Plant pathogenic bacteria used in this study.

Bacteria	Strains	Potato cultivars	Year of isolation	Field source of isolation	Reference
R. solanacearum	Rs0115	Crisps4all	2015	Srpski Miletić	Marković et al., 2021a
	Rs6616	Lady Claire	2016	Sombor	
	Rs8118	Rudolph	2018	Zmajevo	
P. carotovorum	Pcc324	Lady Claire	2016	Kulpin	Unpublished
P. brasiliense	Pcb133	Crisps4all	2018	Maglić	Unpublished
	Pcb62	Lady Claire	2018	Obrovac	Marković et al., 2021b
	Pcb2544	Lady Claire	2019	Maglić	
	Pcb2811	Lady Claire	2019	Kulpin	
	Pcb2842	Lady Claire	2019	Kulpin	
D. dianthicola	Dd31	Lady Claire	2018	Obrovac	Marković et al., 2021b

polaris, P. punjabense, P. versatile, and *P. wasabiae*) and five species/subspecies from *Dickeya* genus (*D. chrysanthemi, D. dadantii* subsp. *dadantii, D. dianthicola, D. solani,* and *D. zeae*) that cause blackleg and/or soft rot of potato.

Based on scientific and economic importance, *R. solanacearum*, *P. carotovorum,* and *P. atrosepticum* are listed in the top ten most important bacterial plant pathogens (Mansfield et al., 2012). *R. solanacearum* race 3 biovar 2 infects several high-value crops, causing brown rot of potato, tomato, and eggplant plants (Kheirandish & Harighi, 2015). Also, once *R. solanacearum* is established in the field, it is very difficult to eradicate it because it spreads via contaminated plant debris, soil, and water. Bacteria from the genera *Pectobacterium* and *Dickeya* have an extensive host range. Accordingly, *Pectobacterium* species have been identified as pathogens of at least 20 dicots and 12 monocot families (Ma et al., 2007; Czajkowski et al., 2015). The *Dickeya* has a narrower host range and can infect plant species from at least 12 dicots and 10 monocot families (Samson et al., 2005; Ma et al., 2007). Potato is the most economically important host for *Dickeya* spp. Some *Pectobacterium* and *Dickeya* species can also be spread by soil and water, as well as on weed plants (Charkowski et al., 2020).

The frequency of certain diseases is increasing with the continual growth of crop productivity and international trade, thus requiring more frequent use of pesticides (Lahlali et al., 2022). Ensuring potato production sustainability could be found in integrated pest management as the optimal strategy in plant protection to guarantee healthy crop production with minimal environmental consequences (Berlin et al., 2018; Helepciuc & Todor, 2022). Studies indicate that potato is one of the crops most treated with chemical pesticides (Vincent et al., 2013). Nowadays, chemical bactericides and antibiotics like streptomycin and its derivatives are commonly used to control potato' bacterial pathogens (Gracia-Garza et al., 2002; Yuliar and Toyota, 2015). However, their excessive use is undesirable due to the negative impact on the environment and human health and the risk of the emergence of resistant strains of bacteria (Jess et al., 2014; La Torre et al., 2018; Sundin & Wang, 2018; Mann et al., 2021).

Biological treatments with antagonistic microorganisms are emerging as a promising alternative to chemical treatments and can significantly reduce their negative environmental impact (Lahlali et al., 2022). For these reasons, some countries are reducing the use of chemical pesticides by 50% in their plant protective plans (Macfadyen et al., 2014) and introducing biological treatments as their replacement. Biopesticides imply the use of beneficial microorganisms or products of their metabolism in disease control (Ongena & Jacques, 2008; Jiang et al., 2017; Etminani & Harighi, 2018). Among biological control agents, bacteria dominate with 90% (Yuliar and Toyota, 2015), with strains from the genera *Bacillus, Agrobacterium*, and *Pseudomonas* (Fravel, 2005) being the most exploited.

The great interest in the *Bacillus* spp. lies in their ability to produce a wide range of active molecules that act potentially inhibitory on the growth of plant pathogens and exhibit different mechanisms of action (Ongena & Jacques, 2008; Cawoy et al., 2011; Fira et al., 2018; Helepciuc & Todor, 2022). Lipopeptides, secondary metabolites with antibiotic features, are particularly interesting for biocontrol (Ongena & Jacques, 2008). Besides the direct effect on cell viability, lipopeptides can inhibit biofilm formation and even disrupt formed biofilms of phytopathogens due to their biosurfactant properties (Meena & Kanwar, 2015; Crouzet et al., 2020; Malviya et al., 2020).

In recent years, the commercialization of products based on *Bacillus* spp. has expanded significantly (Helepciuc & Todor, 2022). Numerous studies have shown the great potential of *B. amyloliquefaciens* in suppressing *R. solanacearum*, as well as bacteria from the genus *Pectobacterium* (Chen et al., 2019; Osei et al., 2021). In this study, two *B. amyloliquefaciens* strains, SS-12.6 and SS-38.4, with previously proven antagonistic potential against several pathogens, were examined for antimicrobial activity against pathogenic bacteria isolated from the potato in Serbia using *in vitro*, *in situ*, and *in planta* tests to find effective and environmentally friendly control agents against destructive potato diseases.

2. Material and methods

2.1. Bacterial strains

Previously identified and described antagonistic *B. amyloliquefaciens* SS-12.6 and SS-38.4 strains (Dimkić et al., 2017) were used to determine antimicrobial activity against Serbian *R. solanacearum* race 3 biovar 2, *P. carotovorum*, *P. brasiliense*, and *D. dianthicola* strains originally isolated from potato (Table 1). Antagonistic *Bacillus* spp. strains were isolated from soil at different locations in Serbia and belong to the bacterial strain collection of the Laboratory for Microbiology (University of Belgrade – Faculty of Biology). Phytopathogenic strains used in this study belong to the collection of the Laboratory for Phytopathology of the Institute for Plant Protection and the Environment in Serbia. Antagonistic strains were maintained on Luria-Bertani Agar (LA) [tryptone 10 g L^{-1}, yeast extract 5 g L^{-1}, NaCl 5 g L^{-1}, agar 15 g L^{-1}] at 30 °C. Pathogenic strains were maintained on Sucrose Peptone Agar (SPA) [sucrose 20 g L^{-1}, bacto-peptone 5 g L^{-1}, K_2HPO_4 0.5 g L^{-1}, $MgSO_4{\cdot}7H_2O$ 0.25 g L^{-1}, bacto-agar 15 g L^{-1}] for *R. solanacearum* strains and Nutrient Agar (NA) (Torlak, Serbia) for *Pectobacterium* and *Dickeya* strains and incubated at 26 °C for 24–48 h. Until the moment of manipulation, both antagonist and phytopathogenic strains were kept at −20 °C, in a Luria-Bertani (LB) [tryptone 10 g L^{-1}, yeast extract 5 g L^{-1}, NaCl 5 g L^{-1}] medium supplemented with 20% (v/v) of sterile glycerol (Zorka Pharma-Hemija, Serbia).

2.2. In vitro assay of antagonistic activity of B. amyloliquefaciens strains SS-12.6 and SS-38.4

2.2.1. Preparation of pathogenic and B. amyloliquefaciens strains for in vitro tests

For *in vitro* tests, all pathogenic strains were grown on an appropriate medium under the same conditions (26 °C for 24–48 h). After the incubation period, a single colony of each pathogenic strain was transferred to LB and incubated overnight at 26 °C while shaking (180 rpm).

The whole liquid cultures were then centrifuged (10 min at 2000 *g*), and the obtained supernatant was discarded while the pellet was dissolved in sterile 10 mM $MgSO_4$ buffer (in final concentrations 10^{6-8} CFU mL^{-1}).

Bacillus strains SS-12.6 and SS-38.4 were maintained on LA medium at 30 °C for 24 h. A single colony of each strain was inoculated in LB medium and incubated at 30 °C overnight while shaking (180 rpm). Thereafter, the whole liquid cultures were centrifuged (10 min at 2000 *g*) and the pellets were dissolved in sterile 10 mM $MgSO_4$ to the final concentration of 10^8 CFU mL^{-1} to obtain cell suspensions (CS). Supernatants (SN) were collected after centrifugation and filtered (0.22 μm Durapore™ Millipore, Billerica, USA) to remove residual cells and endospores. So prepared CSs and cell-free SNs were used for testing the *Bacillus* spp. antagonistic activity *in vitro*.

Ethyl acetate extracts (EAEs) of *B. amyloliquefaciens* strains SS-12.6 and SS-38.4 were obtained according to the extraction method reported by Kuiper et al. (2004). One mL of a whole culture of the two tested antagonistic strains was added to 1000 mL of fresh LB medium and incubated overnight at 30 °C in an orbital shaker (180 rpm). Cells were discarded after centrifugation (20 min at 5000 *g*). Ethyl-acetate (Zorka Pharma-Hemija, Serbia) in a 1:1 volumetric ratio (*v/v*) and NaCl (30 g L^{-1}) were added to each supernatant and mixed on a magnetic stirrer for 2 h. A rotary evaporator (Rotavapor R-215; Büchi, Switzerland) was used for complete drying. The precipitates were dissolved with methanol (Zorka Pharma-Hemija, Serbia) to the final concentration of 20 mg mL^{-1} for both strains, purified through a filter (0.45 μm Durapore™ Millipore, Billerica, USA), and used for further *in vitro* tests.

2.2.2. Testing of B. amyloliquefaciens CS, SN, and EAE

The antagonistic activity of *B. amyloliquefaciens* SS-12.6 and SS-38.4 CS was tested by applying droplets (5 μl) to the surface of the LA soft medium (5 mL) [tryptone 10 g L^{-1}, yeast extract 5 g L^{-1}, NaCl 5 g L^{-1}, agar 7 g L^{-1}], previously inoculated with 50 μl of a suspension of each pathogenic strain (10^8 CFU mL^{-1}) and poured over LA medium. The antagonistic activity of strains SS-12.6 and SS-38.4 was evaluated 48 h after incubation at 30 °C by measuring the diameter of inhibition zones of pathogenic strains' growth, formed around applied droplets of whole bacterial cultures. The experiment was performed in triplicate and the diametres of inhibition zones were expressed in millimeters.

The activity of SN and EAE of *B. amyloliquefaciens* strains SS-12.6 and SS-38.4 was tested according to the modified well-diffusion method from Harris et al. (1989). For this purpose, wells were made in LA soft medium (7 mL) mixed with 140 μl of suspension of pathogenic strain (10^8 CFU mL^{-1}). Exactly 50 μl of SNs or EAEs (20 mg mL^{-1}) was added to wells. After incubation at 30 °C for 48 h, inhibition zone diameters were measured and expressed in mm. The experiment was carried out in triplicate.

2.2.3. Determination of minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of B. amyloliquefaciens EAEs

MIC of EAE of *B. amyloliquefaciens* was determined in 96-well round-bottom microtiter plates (Sarstedt, Germany) according to the method from Dimkić et al. (2016). Suspensions of pathogenic strains (10^8 CFU mL^{-1}) and EAE of antagonistic strains (20 mg mL^{-1}) were prepared as previously described. Serial dilutions of each extract in the concentration range from 2 to 0.03125 mg mL^{-1} were prepared in an LB medium. For each microtiter plate, pure LB medium was used as a negative control (medium sterility), methanol was used as a solvent control (10 to 0.16%), and streptomycin (Sigma-Aldrich, USA) in the range of 0.4 to 0.00625 mg mL^{-1} was used as a positive control of pathogen suppression. Suspensions of pathogenic bacteria were added to wells to obtain the final concentration of 10^6 CFU mL^{-1}. The final volume in each well was 200 μl. Resazurin (SERVA Electrophoresis GmbH, Germany) in a concentration of 0.675 mg mL^{-1} was used to visualize the presence/abscence of bacterial growth. The plates were incubated at 30 °C for 24 h. The lowest concentration with no change in color was designated as the MIC. To determine the MBC, 5 μl from each well without color change was inoculated on LA medium. After incubation (24 h, 30 °C), the growth of bacteria was observed, and the lowest concentration which showed no growth was selected as MBC. The experiment was performed in triplicate.

2.2.4. Inhibition of pathogenic bacteria biofilm formation by EAE of B. amyloliquefaciens

The ability of pathogenic strains to produce biofilm and the inhibition of biofilm formation by EAEs was tested in the 96-well flat-bottom microtiter plates (Sarstedt, Germany) according to Stepanović et al. (2003), with some modifications. In each well of plates, 180 μl of LB medium and 20 μl of pathogenic strains suspensions (final concentration 10^6 CFU mL^{-1}) were added, and plates were incubated for 48 h at 30 °C. Wells filled only with LB medium served as a negative control. All pathogens were tested in triplicate. After incubation, the contents of the microtiter plates (medium and planktonic cells) were discarded carefully by pipetting, and the plates were washed three times with 300 μl of sterile distilled water (SDW). After drying the plates overnight in the upside-down position at room temperature, 200 μl of methanol was added to the wells to fix the adherent cells, and the plates were incubated for 20 min at room temperature. Methanol was poured off by hand and the plates were dried at room temperature for 30 min. After drying, 200 μl of 0.1% crystal violet (Lach-Ner, Czech Republic) was added to the wells to stain the biofilms. The plates were incubated for 15 min at room temperature and then washed thoroughly with tap water to remove any unbound stain, and the plates were air-dried upside-down. Crystal violet bound to adherent cells was dissolved by adding 200 μl of absolute ethanol (Zorka Pharma-Hemija, Serbia). After 10 min, the optical density (OD) at 570 nm was measured (Multiskan FC microplate reader, Thermo Scientific, China). The cut-off OD (ODc) values (three standard deviations (SD) above the mean OD value of the negative control) were defined for each strain based on their OD values. The following scale was used to determine the category of biofilm production: 0 – no biofilm formation ($OD \leq ODc$), 1 – weak ability to form a biofilm ($ODc < OD \leq 2 \times ODc$), 2 – moderate ability to form a biofilm ($2 \times ODc < OD \leq 4 \times ODc$), 3 – pronounced ability to form a biofilm ($4 \times ODc < OD$).

The influence of *B. amyloliquefaciens* EAE on the formation of biofilms was performed as described, with some differences. A suspension of pathogenic strains in LB medium (10^6 CFU mL^{-1}) was added to each well of the 96-well flat-bottom microtiter plates in a total volume of 200 μl. After incubation for 24 h at 30 °C, the contents of the wells were discarded by pipetting, and the plates were washed with SDW three times. A fresh LB medium with EAE of the antagonistic strains SS-12.6 and SS-38.4 was added to the wells in four concentrations (0.25, 0.125, 0.063 and 0.032 mg mL^{-1}) in a final volume of 200 μl. As the solvent control, methanol was added to wells in the range of 1.25 to 0.16%. The experiment was performed in triplicate. The following steps (washing with SDW, fixation by methanol, staining with crystal violet) were the same as described. The OD at 570 nm was measured and the percentage of inhibition of biofilm formation compared to the control was calculated according to the following formula:

$$\text{Inhibition of biofilm formation (IBF\%)} = [(OD_{control} - OD_{sample})/OD_{control}] \times 100$$

2.3. In situ assay of antagonistic activity of B. amyloliquefaciens strains SS-12.6 and SS-38.4 against soft rotting bacteria

The antagonistic potential of *B. amyloliquefaciens* strains SS-12.6 and SS-38.4 was tested *in situ* on potato tubers inoculated with strains of three different pathogenic bacteria: *P. carotovorum* (Pcc324), *P. brasiliense* (Pcb133, Pcb62, Pcb2544, Pcb2811, Pcb2842), and *D. dianthicola* (Dd31), according to the modified method by Hadizadeh et al. (2019). The tubers were washed with tap water and surface-

Table 2
The inhibition zones (in mm, mean ± SD, n = 3) of tested phytopathogenic strains induced by cell suspensions (CS), cell-free supernatants (SN), and ethyl-acetate extracts (EAE) of *B. amyloliquefaciens* strains SS-12.6 and SS-38.4 *in vitro*. Values with the same letter are not significantly different according to Tukey's Honestly Significant Difference test ($p < 0.05$).

Pathogenic strains		*B. amyloliquefaciens* strains					
		CS		SN		EAE	
		SS-12.6	SS-38.4	SS-12.6	SS-38.4	SS-12.6	SS-38.4
R. solanacearum	Rs0115	4.67 ± 0.58 *a **a ***C	7.33 ± 0.58 a a AB	16.67 ± 1.53 a ab A	15.33 ± 1.53 a ab AB	33.67 ± 2.08 a ab A	26.00 ± 1.00 a ab B
	Rs6616	5.67 ± 0.58 a a BC	8.33 ± 0.58 a a A	15.00 ± 1.73 a abc AB	12.33 ± 2.51 a abc B	36.67 ± 2.52 a a A	27.67 ± 1.16 a a B
	Rs8118	5.00 ± 1.00 a a C	7.33 ± 0.58 a a AB	17.33 ± 0.58 a a A	15.67 ± 0.58 a a AB	35.00 ± 2.00 a a A	26.67 ± 0.58 a ab B
P. carotovorum	Pcc324	0	0	15.67 ± 1.15 abc A	13.67 ± 0.58 abc A	19.00 ± 1.00 c A	17.33 ± 1.53 e A
P. brasiliense	Pcb133	0	0	14.00 ± 1.00 ab bcde AB	12.00 ± 1.00 a abc ABC	21.67 ± 0.58 c c D	21.67 ± 0.58 c d D
	Pcb62	0	0	14.67 ± 0.58 a abcd A	11.67 ± 0.58 a bc BC	34.67 ± 1.15 a a A	28.67 ± 0.58 a a B
	Pcb2544	0	0	11.67 ± 0.58 b de BC	11.00 ± 1.00 a c C	29.67 ± 1.52 b b B	22.67 ± 1.15 bc cd CD
	Pcb2811	0	0	12.67 ± 1.53 ab cde ABC	11.00 ± 1.00 a c C	33.00 ± 1.00 a ab A	24.67 ± 0.58 b bc C
	Pcb2842	0	0	13.33 ± 0.58 ab cde ABC	11.67 ± 1.53 a bc BC	32.67 ± 0.58 a ab A	24.67 ± 1.53 b bc C
D. dianthicola	Dd31	1.67 ± 0.58 b A	2.67 ± 0.58 b A	11.00 ± 1.00 e A	10.33 ± 1.15 c A	17.67 ± 0.58 c A	15.67 ± 1.53 e A

*lowercase letters in the same column indicate differences between *R. solanacearum* strains or *P. brasiliense* strains for each antagonistic strain.
**underlined lowercase letters in the same column show differences between all phytopathogenic strains for each antagonistic strain.
***capital letters in the same column show differences in the action of both antagonistic strains SS-12.6 and SS-38.4 on each phytopathogen.

sterilized with 70% ethanol. After drying, 60 punctures (about 5 mm deep) were made on the tuber's surfaces using a sterile needle. Prepared tubers were immersed in suspensions of *B. amyloliquefaciens* strains SS-12.6 and SS-38.4 (2×10^8 CFU mL^{-1}) for 30 min. After drying, the tubers were inoculated with a suspension of the tested pathogenic strains (10^8 CFU mL^{-1}) using a hand sprayer.

Tubers immersed in SDW and then inoculated with a suspension of pathogenic strains served as a positive control, while tubers immersed only in SDW served as a negative control. To eliminate the influence of antagonistic strains SS-12.6 and SS-38.4 on soft rot, another set of tubers was primarily immersed in SDW and then in a suspension of these strains. In the last step, the weight of each tuber was measured, and the tubers were placed in plastic boxes with wet filter paper and stored at room temperature. For each treatment, the experiment was performed on five tubers in three independent replicates.

The evaluation of the experiment was carried out on the first, fifth, and 15th day after the treatment (DAT) (i) visually and (ii) by measuring the weight of each tuber. Visual assessment was based on the rating of each tuber according to a scale of 0–5 (0-no wet rot and 5-complete rot) defined by Colyer & Mount (1984). Visually assessed disease intensity (DIv%) was calculated according to the formula:

$$\mathrm{DIv}\,(\%) = \frac{\sum (f \times v)}{\mathrm{N} \times \mathrm{X}} \times 100$$

Wherein: f - infection class frequencies, v - number of tubers of each class, N - the total of observed tubers, and X - the highest value of the evaluation scale (McKinney, 1923).

The disease intensity (DIw%) evaluation based on the difference in tuber weight was carried out by measuring tuber weight before and after treatment (the macerated tissue was removed under tap water before measuring). The intensity of the disease was estimated and calculated according to the formula given by Hadizadeh et al. (2019):

$$\mathrm{DIw}\,(\%) = \frac{\text{Tuber weight before treatment (g)} - \text{Tuber weight after treatment (g)}}{\text{Tuber weight before treatment (g)}} \times 100$$

The antagonistic effect of *B. amyloliquefaciens* strains SS-12.6 and SS-38.4, i.e., their influence on the reduction of disease intensity (treatment efficiency, TE%), was calculated according to the formula given by Hadizadeh et al. (2019):

$$\mathrm{TE}\,(\%) = \frac{[\text{Weight loss control (g)} - \text{Weight loss treatment (g)}]}{\text{Weight loss control (g)}} \times 100$$

The area under the disease progress curve (AUDPC) was calculated using the mean values of tuber's weights rated 1, 5, and 15 DAT according to the formula given by Iličić et al. (2023).

2.4. *In planta* assay of antagonistic activity of *B. amyloliquefaciens* SS-12.6 and SS-38.4 strains against bacterial wilt

The antagonistic potential of *B. amyloliquefaciens* strains SS-12.6 and SS-38.4 was tested *in planta* against *R. solanacearum* (Rs0115, Rs6616, and Rs8118) according to the modified method by Kheirandish & Harighi (2015). Plastic pots were sterilized with 70% ethanol and filled with 2 kg of Klasmann's plant-growing medium. Potato sprouts were cut (cone-shape), surface sterilized (70% ethanol), and immersed in a suspension of antagonistic strains (2×10^8 CFU mL^{-1}) for 1 h. Inoculated potato sprouts were dried on filter paper and planted in pots. After 24 h, a suspension of pathogenic strains (10^8 CFU mL^{-1}) was made and added into pots (10 mL). Sprouts immersed in SDW served as a negative

Table 3
Minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) of *B. amyloliquefaciens* SS-12.6 and SS-38.4 ethyl-acetate extracts (EAE) on the tested phytopathogenic bacteria expressed in mg mL^{-1}.

Pathogenic strains		*B. amyloliquefaciens* strains			
		SS-12.6 EAE		SS-38.4 EAE	
		MIC	MBC	MIC	MBC
R. solanacearum	Rs0115	0.063	0.125	0.125	0.25
	Rs6616	0.063	0.125	0.125	0.25
	Rs8118	0.063	0.125	0.125	0.25
P. carotovorum	Pcc324	0.25	0.5	0.25	0.5
P. brasiliense	Pcb133	0.125	0.25	0.25	0.5
	Pcb62	0.063	0.125	0.25	0.5
	Pcb2544	0.125	0.25	0.25	0.5
	Pcb2811	0.125	0.25	0.25	0.5
	Pcb2842	0.125	0.25	0.25	0.5
D. dianthicola	Dd31	0.25	0.5	0.25	0.5

control, while those treated only with a suspension of tested pathogenic strains served as positive controls. Sprouts treated only with suspensions of strains SS-12.6 and SS-38.4 were used to examine their effect on plant growth. The pots were placed in a growth chamber at a temperature of 25 °C and a light regime of 16 h day and 8 h night. All plants were watered regularly. Five plants were used for each treatment, as well as the control. The evaluation of the experiment was carried out 52, 56 and 60 days after the day treatment (DAT). The plants were evaluated visually by determining the number of total and wilted leaves per plant. The fresh weight of plants was measured at the 60th day. Thereafter, plants were dried at a temperature of 75 °C for 48 h, and their weight was measured again. The experiment was performed three times independently.

Disease intensity (DI%) and treatment efficiency (TE%) of *B. amyloliquefaciens* strains SS-12.6 and SS-38.4 were calculated using the following formulas (Kheirandish & Harighi, 2015):

$$\mathrm{DI}\,(\%) = \frac{\text{Number of wilted leaves per treatment}}{\text{Total number of leaves per treatment}} \times 100$$

$$\mathrm{TE}\,(\%) = \frac{(\text{DI in control pots} - \text{DI in treatment pots})}{\text{DI in control pots}} \times 100$$

The AUDPC was calculated using the mean values of percentages of wilted leaves rated 52, 56, and 60 DAT.

2.5. Statistical analysis

For the statistical analysis of the results obtained *in vitro*, *in situ,* and *in planta*, Minitab v 19. was used. The obtained data were subjected to analysis of variance (ANOVA) using the *post hoc* Tukey's HSD test. When the p-value was < 0.05, the results were considered statistically significant.

3. Results

3.1. In vitro antagonistic activity of B. amyloliquefaciens strains SS-12.6 and SS-38.4

R. solanacearum strains Rs0115, Rs6616, and Rs8118 were sensitive to CS of the *B. amyloliquefaciens* strains SS-12.6 and SS-38.4 without statistically significant difference (Table 2; Supplementary Fig. 1a). The SS-38.4 exhibited statistically significantly stronger antagonistic activity for all *R. solanacearum* strains. Pathogenic strains of *P. carotovorum* Pcc324 and *P. brasiliense* Pcb133, Pcb62, Pcb2544, Pcb2811, and Pcb2842 were not susceptible to CS of *B. amyloliquefaciens* strains SS-12.6 and SS-38.4 while on *D. dianthicola* strain Dd31 they exhibited weak antagonistic activity. There were no statistically significant differences between the two antagonistic strains (Table 2; Supplementary Fig. 1b). Significant differences were determined between *R. solanacearum* and *D. dianthicola* strains for CS of SS-12.6 and SS-38.4 strains. *D. dianthicola* strain showed higher resistance to both CSs of antagonistic strains than the *R. solanacearum* strains.

All *R. solanacearum* strains were equally susceptible to the antagonistic activity of the SN of both *B. amyloliquefaciens* strains (Table 2; Supplementary Fig. 2a). The strains of *Pectobacterium* and *Dickeya* were also sensitive to the SN of SS-12.6 and SS-38.4 (Table 2; Supplementary Fig. 2 b-d). For *P. brasiliense* Pcb61, SN of SS-12.6 showed significantly higher inhibition.

EAE of SS-12.6 and SS-38.4 showed antimicrobial activity against all *R. solanacearum* strains (Table 2). The largest inhibition zones were determined for Rs6616, while the smallest inhibition zones were recorded for Rs0115 (Supplementary Fig. 3a). The differences among *R. solanacearum* strains were not significant for each antagonistic strain. Significant differences were recorded for the two antagonistic strains for all *R. solanacearum* strains, and the EAE of SS-12.6 showed stronger antagonistic activity than SS-38.4. EAE of SS-12.6 and SS-38.4 also were active against all *Pectobacterium* and *Dickeya* spp. strains (Supplementary Fig. 3b-d). The statistically significant differences among

Table 4
Inhibition of biofilm formation (mean ± SD, n = 3) of pathogenic strains after treatment with four different concentrations of *B. amyloliquefaciens* SS-12.6 and SS-38.4 ethyl-acetate extracts (EAE). The different letters in the same row indicate the significant difference between the grades according to Tukey's Honestly Significant Difference test ($p < 0.05$).

Pathogenic strains	Inhibition of biofilm formation [%]							
	SS-12.6 EAE				SS-38.4 EAE			
	0.25 mg mL^{-1}	0.125 mg mL^{-1}	0.063 mg mL^{-1}	0.032 mg mL^{-1}	0.25 mg mL^{-1}	0.125 mg mL^{-1}	0.063 mg mL^{-1}	0.032 mg mL^{-1}
R. solanacearum								
Rs0115	92.21 ± 2.58^{ab}	78.02 ± 2.44^{c}	39.09 ± 2.23^{e}	27.19 ± 1.76^{f}	87.79 ± 2.45^{ab}	76.32 ± 2.16^{cd}	27.25 ± 3.19^{f}	17.38 ± 3.06^{gh}
Rs6616	98.33 ± 3.15^{a}	84.67 ± 2.26^{bc}	52.95 ± 2.55^{d}	26.01 ± 2.13^{f}	95.74 ± 2.58^{a}	71.53 ± 2.22^{d}	41.14 ± 2.35^{e}	14.46 ± 2.67^{h}
Rs8118	97.5 ± 2.47^{a}	89.94 ± 2.61^{ab}	54.43 ± 1.52^{d}	23.78 ± 0.97^{f}	89.37 ± 3.11^{ab}	84.43 ± 3.24^{bc}	23.39 ± 3.14^{fg}	22.40 ± 2.27^{fgh}
P. carotovorum								
Pcc324	93.08 ± 1.89^{a}	74.15 ± 1.80^{b}	38.80 ± 2.73^{c}	*n.e.	84.99 ± 3.15^{a}	78.44 ± 3.09^{a}	61.68 ± 1.25^{b}	36.92 ± 2.67^{c}
P. brasiliense								
Pcb133	100.00 ± 0.00^{a}	89.61 ± 2.49^{bc}	69.53 ± 3.23^{de}	28.04 ± 3.53^{gh}	87.13 ± 2.20^{ab}	58.79 ± 2.14^{c}	33.93 ± 0.82^{fg}	11.68 ± 3.07^{j}
Pcb62	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	97.42 ± 3.26^{a}	87.75 ± 2.61^{c}	95.82 ± 2.28^{a}	84.00 ± 3.63^{b}	29.93 ± 1.34^{gh}	13.35 ± 2.18^{ij}
Pcb2544	100.00 ± 0.00^{a}	86.55 ± 2.61^{c}	63.42 ± 2.88^{e}	34.15 ± 2.47^{g}	84.07 ± 2.53^{b}	58.79 ± 3.14^{c}	47.12 ± 1.14^{de}	14.75 ± 1.77^{ij}
Pcb2811	100.00 ± 0.00^{a}	95.70 ± 3.24^{ab}	55.97 ± 2.00^{f}	28.81 ± 2.26^{gh}	84.82 ± 1.09^{b}	45.77 ± 2.82^{de}	21.31 ± 1.53^{hi}	11.54 ± 3.03^{j}
Pcb2842	100.00 ± 0.00^{a}	71.28 ± 3.39^{d}	54.25 ± 1.79^{f}	21.94 ± 2.89^{h}	77.96 ± 2.32^{b}	52.68 ± 1.53^{cd}	41.01 ± 2.08^{ef}	18.65 ± 1.99^{ij}
D. dianthicola								
Dd31	92.21 ± 2.26^{a}	72.15 ± 2.52^{b}	46.23 ± 1.88^{c}	13.47 ± 2.44^{d}	72.65 ± 3.84^{a}	61.55 ± 2.72^{b}	41.45 ± 2.71^{c}	11.06 ± 1.52^{d}

*n.e. - not established.

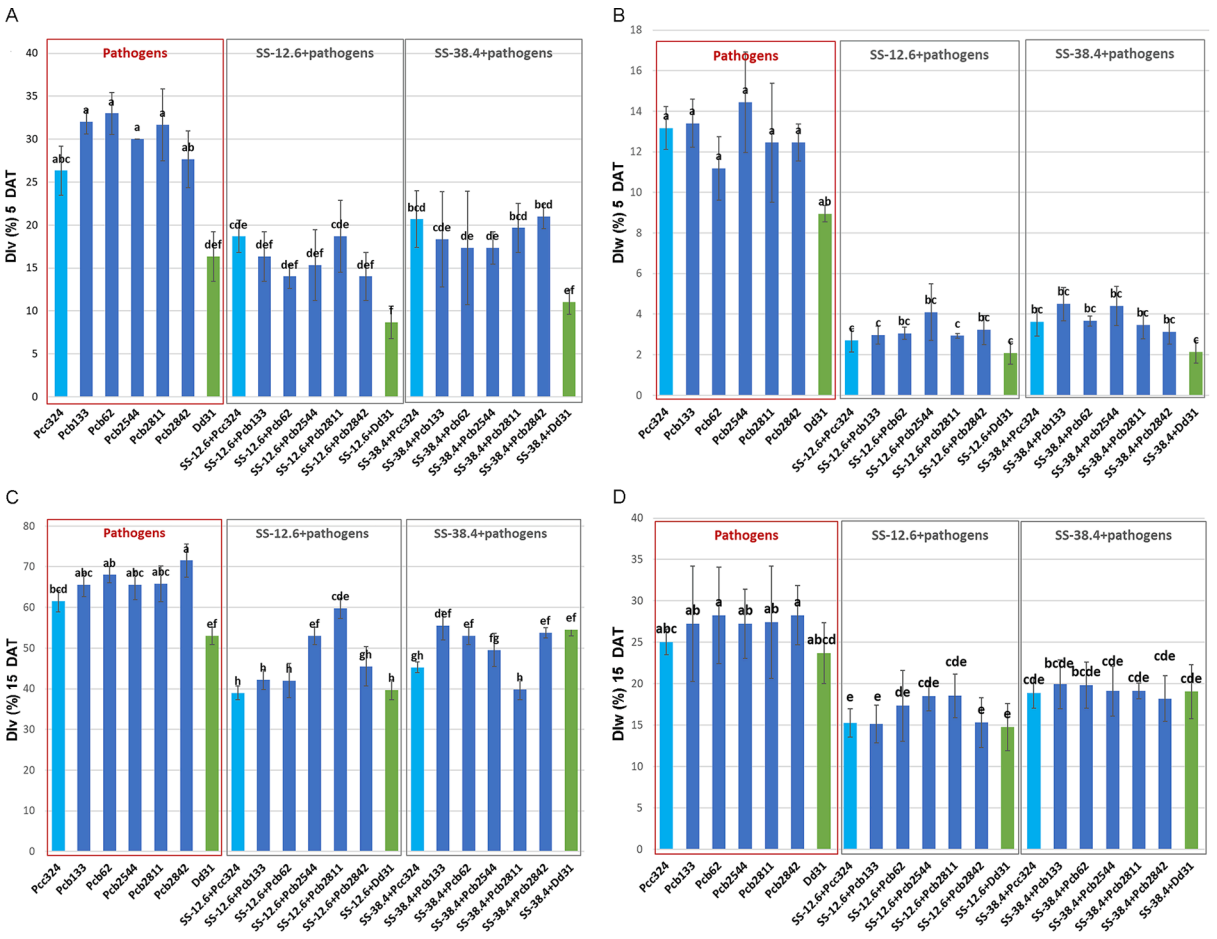

Fig. 1. Soft rot disease intensity (%) of potato tubers determined: A) visually five days after treatment (DAT), B) five DAT based on weight loss, C) visually 15 DAT and D) 15 DAT based on weight loss. Potato tubers inoculated with pathogenic strains alone are marked as „Pathogens" while those treated with antagonistic *Bacillus amyloliquefaciens* SS-12.6 or SS-38.4 strains and then inoculated with pathogens are marked as "SS-12.6 + pathogens" and "SS-38.4 + pathogens". Pathogenic strains used were: *Pectobacterium carotovorum* (Pcc324), *Pectobacterium brasiliense* (Pcb133, Pcb62, Pcb2544, Pcb2811, Pcb2842) and *Dickeya dianthicola* (Dd31). Data are presented as mean values (n = 15) ± standard errors (SE). Values with the same letter are not significantly different according to Tukey's Honestly Significant Difference test ($p < 0.05$).

P. brasiliense strains were found for Pcb2544 and Pcb133 (SS-12.6), and Pcb62 (SS-38.4). For strains of *Pectobacterium* and *Dickeya*, statistical differences for antagonistic bacteria were observed for Pcb62, Pcb2544, Pcb2811, and Pcb2842, where SS-12.6 showed stronger inhibition activity.

For *R. solanacearum* strains, Rs0115, Rs6616, and Rs8118, the same MIC and MBC were obtained for each EAE (Table 3). EAE of SS-12.6 exhibited a stronger antagonistic effect on *R. solanacearum* strains than the EAE of SS-38.4. Among *Pectobacterium* strains, the strongest effect was noticed on *P. brasiliense* Pcb62 with EAE of SS-12.6, with MIC and MBC 0.63 and 1.25 mg mL^{-1}, respectively. The same was noted in all *P. brasiliense* strains. Strain *P. carotovorum* Pcc324 showed higher MIC and MBC than all *P. brasiliense* strains. *D. dianthicola* Dd31 exhibited the same sensitivity (MIC 0.25 mg mL^{-1} and MBC 0.5 mg mL^{-1}) to EAE of both antagonistic strains.

All tested *R. solanacearum* strains (Rs0115 $OD_{570} = 0.214$; Rs6616 $OD_{570} = 0.164$; Rs8118 $OD_{570} = 0.157$) produced a similar quantity of biofilm and were classified in category 1 (weak biofilm formation ability). The same was determined for *Pectobacterium* spp. strains (Pcc324 $OD_{570} = 0.229$; Pcb133 $OD_{570} = 0.201$; Pcb62 $OD_{570} = 0.219$; Pcb2544 $OD_{570} = 0.232$; Pcb2811 $OD_{570} = 0.218$ and Pcb2842 $OD_{570} = 0.237$). *D. dianthicola* Dd31 ($OD_{570} = 0.397$) showed a moderate ability to form biofilm and was classified as category 2.

The inhibition of biofilm formation for all tested pathogenic species by EAE of SS-12.6 and SS-38.4 depended on the concentration. Statistically significant differences in the inhibitory activity of the EAE of both antagonistic strains were established for all tested phytopathogens (Table 4).

3.2. *In situ* antagonistic activity of *B. amyloliquefaciens* strains SS-12.6 and SS-38.4 against soft rotting bacteria

Soft rot symptoms were not visible on tubers one day after treatment (DAT) with pathogens. Also, treatment with antagonistic strains and/or pathogens did not affect the weight of tubers.

On the fifth DAT, soft rot symptoms were visually observed on tubers that were a positive control and the tubers in the treatments (Supplementary Fig. 4). The tubers inoculated with *Pectobacterium* and *Dickeya* strains (positive controls) showed symptoms of soft rot to a greater extent than those from the treatment (Fig. 1a). Between positive controls and treatments, statistically significant differences were observed for strains Pcb133, Pcb62, Pcb2544, and Pcb2811. Treatments with CS of SS-12.6 and SS-38.4 exhibited similar efficiency in reducing disease intensity (DIv).

After the visual evaluation of tubers, their weight was measured five DAT, and the disease incidence (DIw) was calculated (Fig. 1b). In tubers treated with *Pectobacterium* and *Dickeya* strains (positive controls), the weight loss was significantly higher than those treated with the

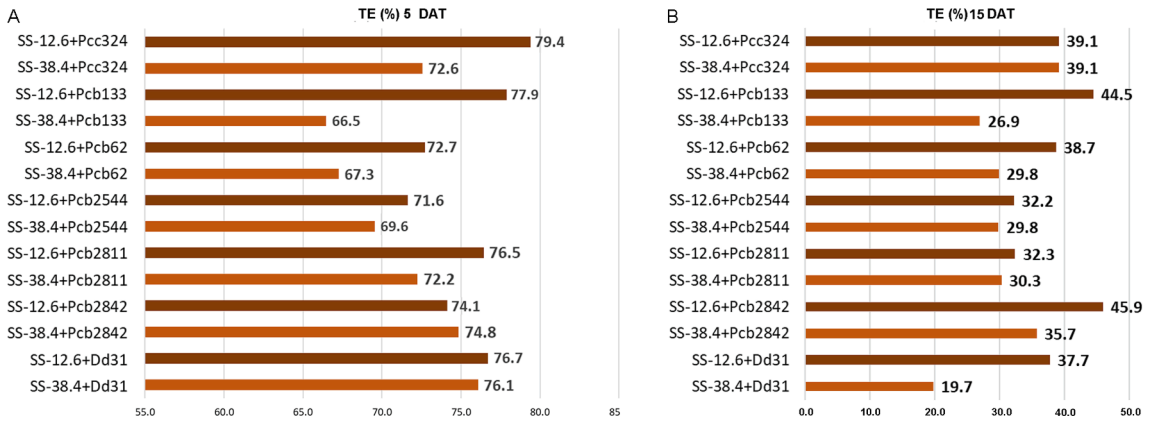

Fig. 2. Treatment efficiency – TE (%) of *Bacillus amyloliquefaciens* SS-12.6 and SS-38.4 in reducing potato soft rot caused by *Pectobacterium carotovorum* (Pcc324), *Pectobacterium brasiliense* (Pcb133, Pcb62, Pcb2544, Pcb2811, Pcb2842) and *Dickeya dianthicola* (Dd31) strains based on potato tuber weight loss: A) five days after treatment (DAT) and B) 15 DAT. Data are presented as mean values (n = 15).

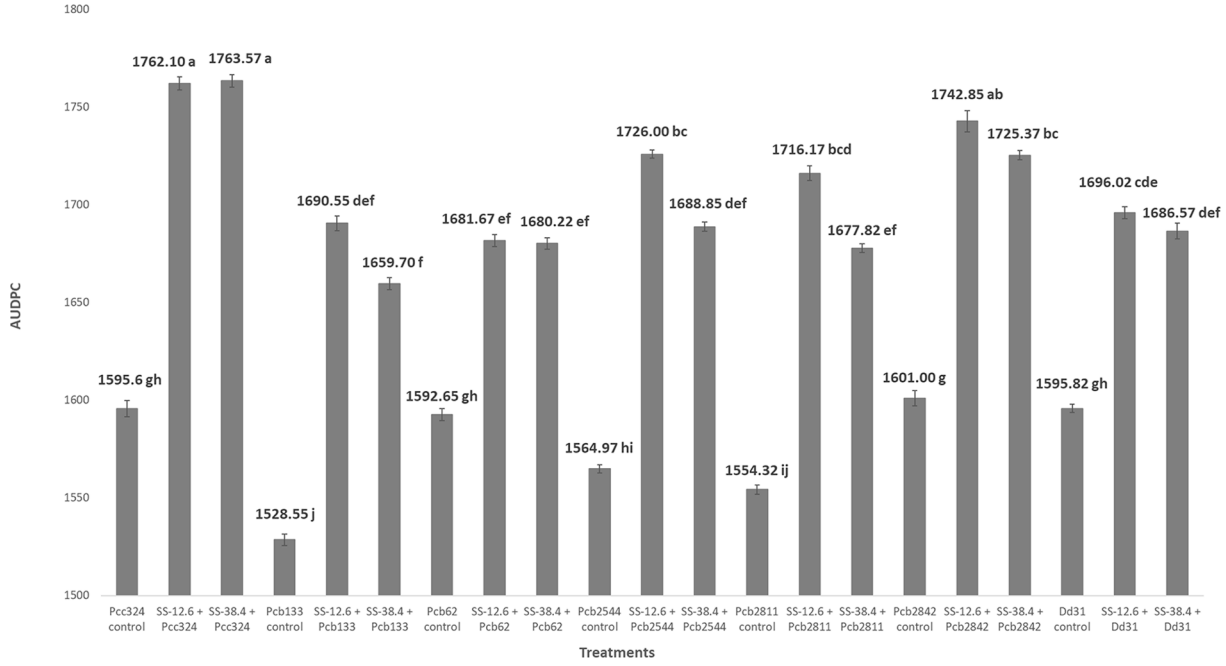

Fig. 3. Disease progression, as the area under the disease progress curve (AUDPC), rated from potato soft rotted tubers inoculated by *Pectobacterium carotovorum* (Pcc324), *Pectobacterium brasiliense* (Pcb133, Pcb62, Pcb2544, Pcb2811, Pcb2842) and *Dickeya dianthicola* (Dd31) alone (control) and tubers treated with *Bacillus amyloliquefaciens* strains SS-12.6 and SS-38.4. The AUDPC was calculated based on tuber's weights rated 1, 5, and 15 days after treatment. Data are presented as mean values (n = 15) ± standard errors (SE). Different letters represent significant statistical differences.

pathogen and antagonistic strains SS-12.6 and SS-38.4 (treatments). No differences were observed between the treatments with SS-12.6 and SS-38.4. Potato tubers that were negative control, treated only with *B. amyloliquefaciens* SS-12.6 and SS-38.4, did not show soft rot symptoms.

Visually observed, fifteen DAT tubers inoculated with pathogenic bacteria showed soft rot symptoms to a greater extent than the treatments.

Differences in disease intensity (%) determined visually between the tubers inoculated with *Pectobacterium* and *Dickeya* strains and those treated with *B. amyloliquefaciens* SS-12.6 and SS-38.4 were statistically significant for Pcb324, Pcb133, Pcb62, Pcb2544, Pcb2842 (Fig. 1c). *B. amyloliquefaciens* SS-12.6 and SS-38.4 showed similar efficacy in reducing disease intensity without statistically significant differences.

After the visual evaluation of the tubers, their weight was measured, and the disease intensity (%) was calculated (Fig. 1d). Significant differences were found between the tubers treated with *Pectobacterium* and *Dickeya* strains and all treatments with the antagonistic strain SS-12.6. For Pcb62, Pcb2544, Pcb2811, and Pcb2842, significant differences were detected in treatment with SS-38.4. *B. amyloliquefaciens* SS-12.6 and SS-38.4 showed similar efficacy in reducing disease intensity without statistically significant differences.

Treatment efficiency (%) based on the difference in tuber weight, measured by five DAT, shows that both *B. amyloliquefaciens* SS-12.6 and SS-38.4 were effective (66.5–79.4%) in suppressing potato tuber soft rot (Fig. 2a). Tubers that were negative control and tubers treated with antagonistic *B. amyloliquefaciens* strains SS-12.6 and SS-38.4 did not show soft rot symptoms.

The treatment efficacy (%) based on the differences in tuber weight 15 DAT showed that both SS-12.6 and SS-38.4 were effective (19.7–44.5%) in suppressing potato tuber soft rot (Fig. 2b). For *D. dianthicola* strain Dd31, a difference in the efficiency of SS-12.6

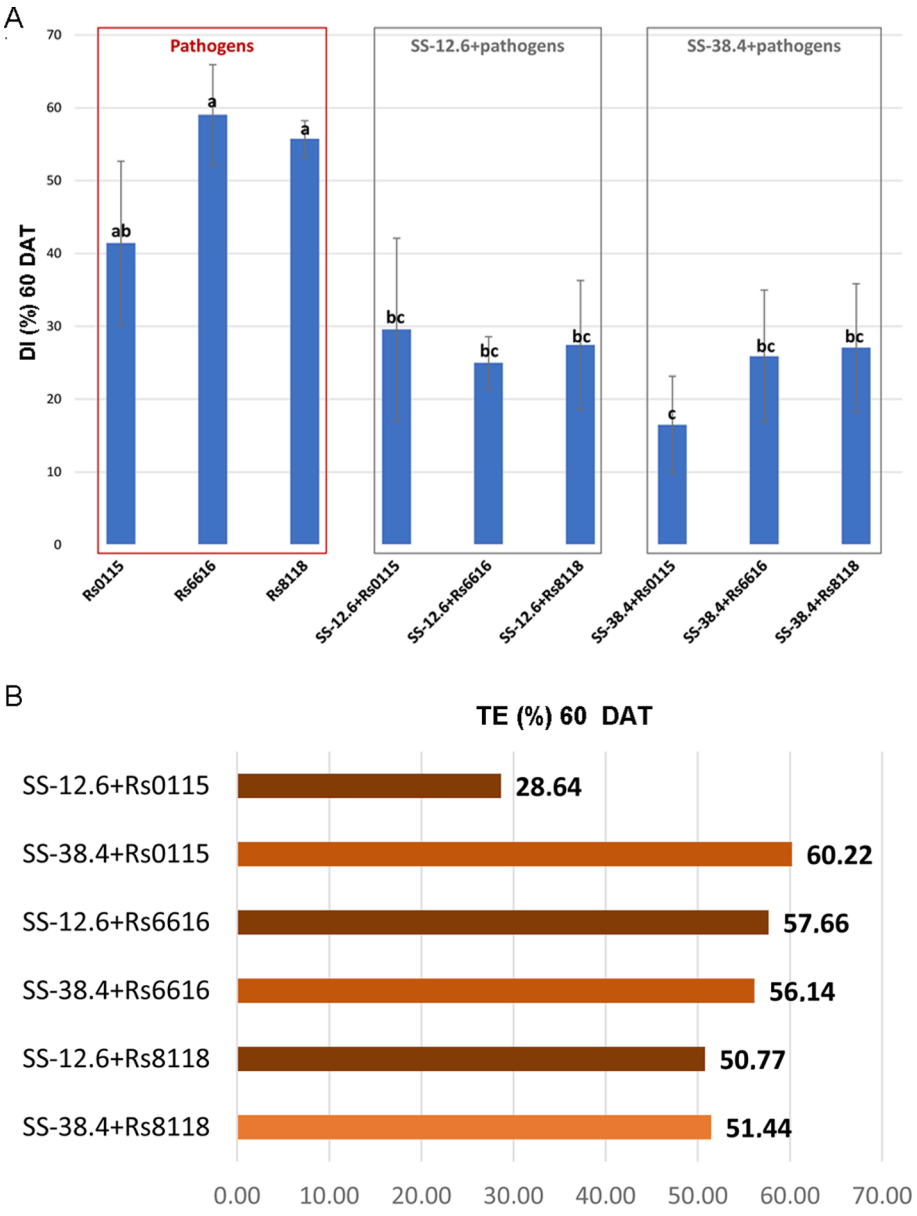

Fig. 4. Bacterial wilt disease intensity – DI (%) of potato plants inoculated with *Ralstonia solanacearum* strains (Rs0115, Rs6616 and Rs8118) alone ("Pathogens") and treated with *Bacillus amyloliquefaciens* SS-12.6 ("SS-12.6 + pathogens") and SS-38.4 ("SS-38.4 + pathogens") strains (A) and treatment efficiency – TE (%) of *Bacillus* spp. strains in suppressing potato wilt (B), determined visually by the number of wilted leaves 60 days after treatment (DAT). Data are presented as mean values (n = 15) ± standard errors (SE). Values with the same letter are not significantly different according to Tukey's Honestly Significant Difference test ($p < 0.05$).

(37.7%) and SS-38.4 (19.7%) was noted.

The AUDPC values ranged from 1528.55 for the Pcb133 control treatment to 1763.57 for the biocontrol treatment with *B. amyloliquefaciens* strain SS-38.4 vs. Pcc324 (Fig. 3). All control treatments were statistically at the same level (AUDPC 1528.55–1601), and they differed from treatments with biocontrol strains which were all at another statistical level (AUDPC 1659.7–1763.57).

3.3. *In planta antagonistic activity of B. amyloliquefaciens strains SS-12.6 and SS-38.4 against bacterial wilt*

By visual assessment of plants infected with *R. solanacearum* strains Rs0115, Rs6616, and Rs8118 and treated with *B. amyloliquefaciens* SS-12.6 and SS-38.4, we determined that antagonistic strains reduced disease intensity (Fig. 4a) 60 DAT. Significant differences in disease intensity were determined between plants treated with Rs6616 and Rs8118 and appropriate controls. No differences were observed between treatments with strains SS-12.6 and SS-38.4. Plants that served as negative control and plants treated with antagonistic strains SS-12.6 and SS-38. 4 did not show wilting symptoms.

The effectiveness of the treatment, calculated based on the visual assessment of the number of leaves with wilting symptoms, indicated that both *B. amyloliquefaciens* strains SS-38.4 and SS-12.6 reduced the disease in the range of 28.64–60.22% (Fig. 4b; Fig. 5; Supplementary Fig. 5). The lowest treatment efficiency was recorded for the pathogenic strain Rs0115 after treatment with antagonistic strain SS-12.6 (28.64%).

The calculated AUDPC ranged from 74.64 for biocontrol treatment with *B. amyloliquefaciens* SS-38.4 against *R. solanacearum* Rs0115 to 235.84 for the *R. solanacearum* Rs6616 control treatment (Fig. 6). There was a statistically significant difference for AUDPC among all controls (AUDPC were 181.64, 209.88 and 235.84 for *R. solanacearum* strains Rs0115, Rs8118 and Rs6616, respectively). Biocontrol trials with *B. amyloliquefaciens* SS-12.6 vs. strains Rs0115 and Rs8118 and *B. amyloliquefaciens* SS-38.4 against strains Rs6116 and Rs8118 were grouped together. Treatments with *B. amyloliquefaciens* SS-38.4 against *R. solanacearum* Rs0115 and SS12.6 against Rs6616 were mutually different and separated from the previous group (Fig. 6).

The fresh and dry weights of plants were measured, and the mean value was calculated (Fig. 7). Statistically significant differences in plants' fresh weight were found between plants that served as positive

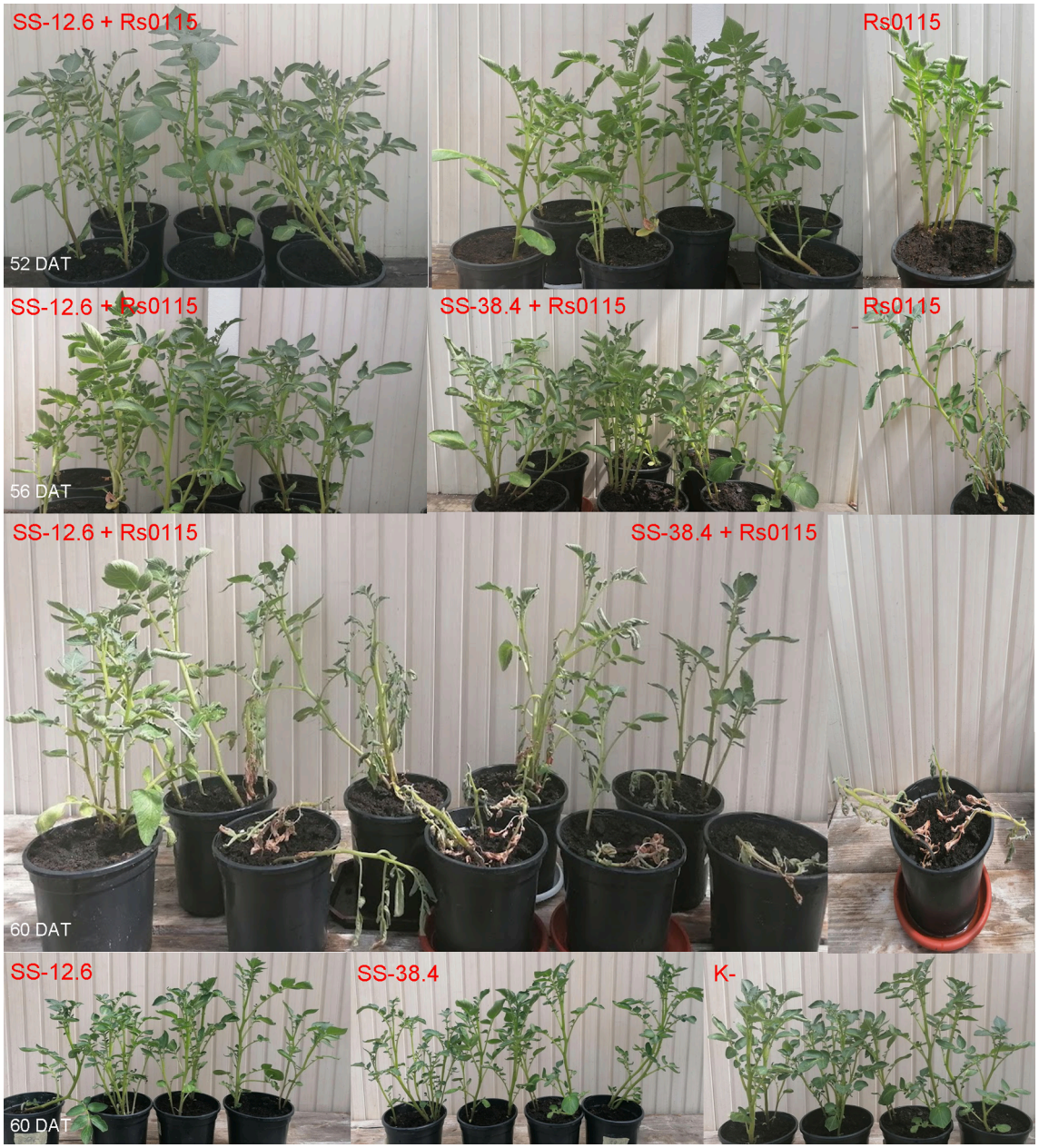

Fig. 5. Evaluation of the *in planta* efficacy of *Bacillus amyloliquefaciens* SS-12.6 and SS-38.4 strains in the supression of bacterial wilt of potato caused by *Ralstonia solanacearum* Rs0115, 52, 56 and 60 days after treatment (DAT).

control and plants treated with *B. amyloliquefaciens* SS-12.6 and SS-38.4 (Fig. 7a). There was no significant difference in plants' fresh weight between treatments. After drying and re-measuring plants' weight, similar results were obtained (Fig. 7b). A significant difference was recorded between the positive controls and the treatment with antagonistic strains, and a significant difference in the plants' dry weight was not observed between treatments.

4. Discussion

Bacterial phytopathogens such as *R. solanacearum, P. carotovorum, P. brasiliense, P. punjabense, P. versatile*, and *D. dianthicola* were isolated from potato plants and tubers in Serbia during the past decade (Milijašević-Marčić et al., 2013; Marković et al., 2021a; Marković et al., 2021b; Loc et al., 2022; Marković et al., 2022). Due to their wide host range, spreading potential, and environmental persistence, controlling these pathogens is difficult and mainly relies on preventive measures and monitoring (Charkowski et al., 2020; Ho et al., 2020). An efficient, safe, and eco-friendly means of disease control would significantly contribute to potatoes' pre- and postharvest protection. The objective of the present study was to evaluate the antagonistic effects and biocontrol efficacy of *B. amyloliquefaciens* strains SS-12.6 and SS-38.4 against *R. solanacearum, P. carotovorum, P. brasiliense,* and *D. dianthicola* isolated from potatoes in Serbia. The two *B. amyloliquefaciens* strains used in this study previously showed a broad spectrum of antagonistic activity towards bacterial and fungal pathogens *in vitro* and *in planta*, due to their ability to produce lipopeptides from iturin, surfactin, fengycin, and kurstakin families (Dimkić et al., 2017; Nikolić et al., 2019).

This study's results suggest that lipopeptide compounds alone were responsible for the pronounced antimicrobial activity of the antagonistic strains SS-12.6 and SS-38.4 against the sensitive pathogenic strains *in vitro*. Overall, the diameter of the inhibition zones induced by the EAE of these strains was greater than with SN. The CSs of SS-12.6 and SS-38.4 did not inhibit the growth of *P. carotovorum* and *P. brasiliense* strains.

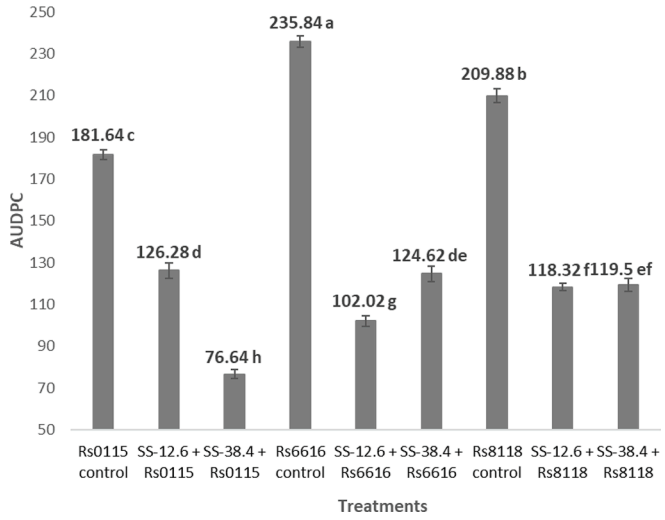

Fig. 6. Disease progression, as the area under the disease progress curve (AUDPC) of potato plants infected with *Ralstonia solanacearum* (Rs0115, Rs6616 and Rs8118) alone (control) and infected plants treated with *Bacillus amyloliquefaciens* strains SS-12.6 and SS-38.4 The AUDPC was calculated based on the percentages of wilted leaves rated 52, 56, and 60 days after treatment. Data are presented as mean values (n = 15) ± standard errors (SE). Different letters represent significant statistical differences.

D. dianthicola showed the highest resistance, *P. carotovorum* and *P. brasiliense* average, and *R. solanacearum* had the lowest resistance, confirmed by minimal inhibitory and minimal bactericidal concentrations of EAE of *B. amyloliquefaciens* SS-12.6 and SS-38.4 (Table 3). EAE of SS-12.6 previously showed inhibitory activity towards two *P. carotovorum* strains isolated from cabbage (Dimkić et al., 2013). Gerayeli et al. (2018) noted that whole cultures of *B. subtilis* and *B. pumilus* strains harboring lipopeptide genes (for surfactins, iturins, fengycins and bacillomycin L) inhibited the growth of *P. carotovorum* subsp. *carotovorum in vitro* (10 mm diameter of the inhibition zone). *Bacillus amyloliquefaciens* S917 with the potential for production of various antimicrobial compounds showed antagonistic acitivity against *P. carotovorum* subsp. *brasiliense* by reducing the soft rot lesion diameters in chilli peppers (Li et al., 2023). Also, the inhibition of *R. solanacearum* FJAT-91 induced by the crude lipopeptides produced by *B. amyloliquefaciens* FJAT-2349 was shown by Chen et al. (2019) and Villegas-Escobar et al. (2018) for the lipopeptides of *Bacillus* sp. EA-CB0959 against *R. solanacearum* and *Pectobacterium* spp. Chen et al. (2020) also showed that *B. velezensis* FJAT-46737 had strong antibacterial activity towards *R. solanacearum* due to the secretion of lipopeptides, especially fengycins. In the said study, crude lipopeptide extract induced the inhibition of *R. solanacearum* from tomato and peanut by 18.52 and 14.57 mm, respectively, at 10 mg mL^{-1}. In our study, the inhibition zone diamaters of all three *R. solanacearum* strains induced by tested EAEs were larger (26.00–36.67 mm), but the concentration of the EAEs was twice as high. Additional experiments are necessary to reveal the exact compound responsible for the inhibitory effect of *B. amyloliquefaciens* SS-12.6 and SS-38.4 towards the examined phytopathogens of the potato.

In our study, *D. dianthicola* showed a moderate ability to form a biofilm, while *P. carotovorum*, *P. brasiliense*, and *R. solanacearum* strains showed a weak biofilm formation ability. The most susceptible to the anti-biofilm activity of EAE of *B. amyloliquefaciens* SS-12.6 and SS-38.4 were *P. brasiliense* followed by *R. solanacearum* strains. Also, SS-12.6 was more efficient in inhibiting the formation of biofilms of most tested pathogenic bacteria. That could be attributed to the differences in lipopeptide composition of the two EAE (Dimkić et al., 2017). Since we tested the EAE of *Bacillus* strains, we hypothesized that lipopeptides from EAE are responsible for inhibiting biofilm formation. Earlier was shown that SN of *Bacillus* spp. inhibited *R. solanacearum* biofilm formation (Almoneafy et al., 2014), and SN of surfactin-, iturin- and fengycin-producing *B. amyloliquefaciens* and *B. velezensis* strains demonstrated anti-biofilm activity towards *Dickeya dadantii* (Hossain et al., 2020). Since biofilms represent important virulence factors in pathogens (Kang et al., 2002; Ham et al., 2004; Mori et al., 2016; Tanui et al., 2017), biocontrol agents targeting biofilms could offer efficient phytopathogen suppression with lower risk for development of resistance to antimicrobial compounds.

Suppression of soft rot symptoms by *B. amyloliquefaciens* SS-12.6 and SS-38.4 was evaluated on whole tubers artificially infected with *Pectobacterium* spp. and *Dickeya* spp. strains. Visual examination of potato tubers on the fifth and 15th DAT revealed that disease intensity was two to three times higher than disease intensity obtained by measuring the weight of the tubers. These observations imply that soft rot spreads on the surface. Therefore, the method of determining the intensity of the disease by measuring the weight was proved far more accurate. Differences in disease intensity determined by weight loss 15 DAT between the inoculated control and the treatments with *B. amyloliquefaciens* SS-12.6 were statistically significant for all phytopathogenic strains and with SS-38.4 for some strains (Fig. 1d). The calculated AUDPC values also indicated a statistical difference between the control treatments (soft rot bacteria) and treatments using biocontrol strains. A study by Azaiez et al. (2018) showed that pretreatment of tubers with *B. amyloliquefaciens* Ar10 strain reduced *P. carotovorum* infection after 10 days of incubation, but the reduction depended on the duration of pretreatment. The lowest weight loss reduction (1.43%) was observed after treatment for one hour, while the highest protection was noted after pretreatment for 72 h (85%). The weight loss reduction (treatment efficiency) in the current study, five DAT with strains SS-12.6 and SS-38.4 (that lasted 30 min), was 71.6–79.4% and 66.5–74.8%, and 15 days after treatment, it was 32.2–45.9% and 26.9–39.1%. Another study showed that two-hour pretreatment of tubers by *Bacillus pumilus* SN could protect *P. carotovorum* subsp. *carotovorum* for 12 weeks under storage conditions (Abd-El-Khair et al., 2021).

Reports on biological control of *D. dianthicola* are scarce in contrast to *D. solani*, which proved to be more aggressive on tubers, causing more severe symptoms of the disease (Blin et al., 2021). Compared to the study where *D. solani* strain Ds0432-1 caused a disease intensity of 9.4% (Hadizadeh et al., 2019), *D. dianthicola* strain Dd31 in our study showed similar disease intensity (8.95%) five days after inoculation. We also found that on the 15th day after the inoculation, the disease intensity for *D. solani* and *D. dianthicola* was the same (23.3 and 23.71%, respectively). In the same study, antagonistic strain *B. subtilis* P48 achieved protection of tubers by 65.1% 30 days after treatment, which was notably higher than TE of *B. amyloliquefaciens* SS-12.6 and SS-38.4 that reduced soft rot by 37.7 and 19.7% fifteen DAT, respectively. Overall, it would be useful to examine if prolonged pretreatment of tubers with antagonistic strains could improve their efficacy in supression of soft rot.

Since the control of *R. solanacearum* is challenging to achieve once it has been established in the field (Abd El-Rahman and Shaheen, 2016), numerous studies were conducted in search of biocontrol agents for bacterial wilt suppression (Yuliar and Toyota, 2015). The potential of *B. amyloliquefaciens* SS-12.6 and SS-38.4 for biocontrol of three *R. solanacearum* strains was evaluated *in planta* to confirm results obtained *in vitro*. The treatment efficacy evaluated 52, 56 and 60 days after planting showed that *B. amyloliquefaciens* SS-12.6 and SS-38.4 reduced the bacterial wilt symptoms in potato plants by 28.64–57.66% and 51.44–60.22%, respectively. Aliye et al. (2008) reported a more pronounced wilt reduction (82.7%) by a *B. subtilis* strain 65 days after the treatment. However, contrary to our findings, the *B. subtilis* strain had a plant growth promotion effect, whereas plants treated with *B. amyloliquefaciens* SS-12.6 and SS-38.4 alone had the same fresh and dry weight as in the negative control. Another study showed that the endophytic *Bacillus licheniformis* Bl17 strain reduced the potato wilt by 41.31% (Bahmani et al., 2021). Elazouni et al. (2019) showed that

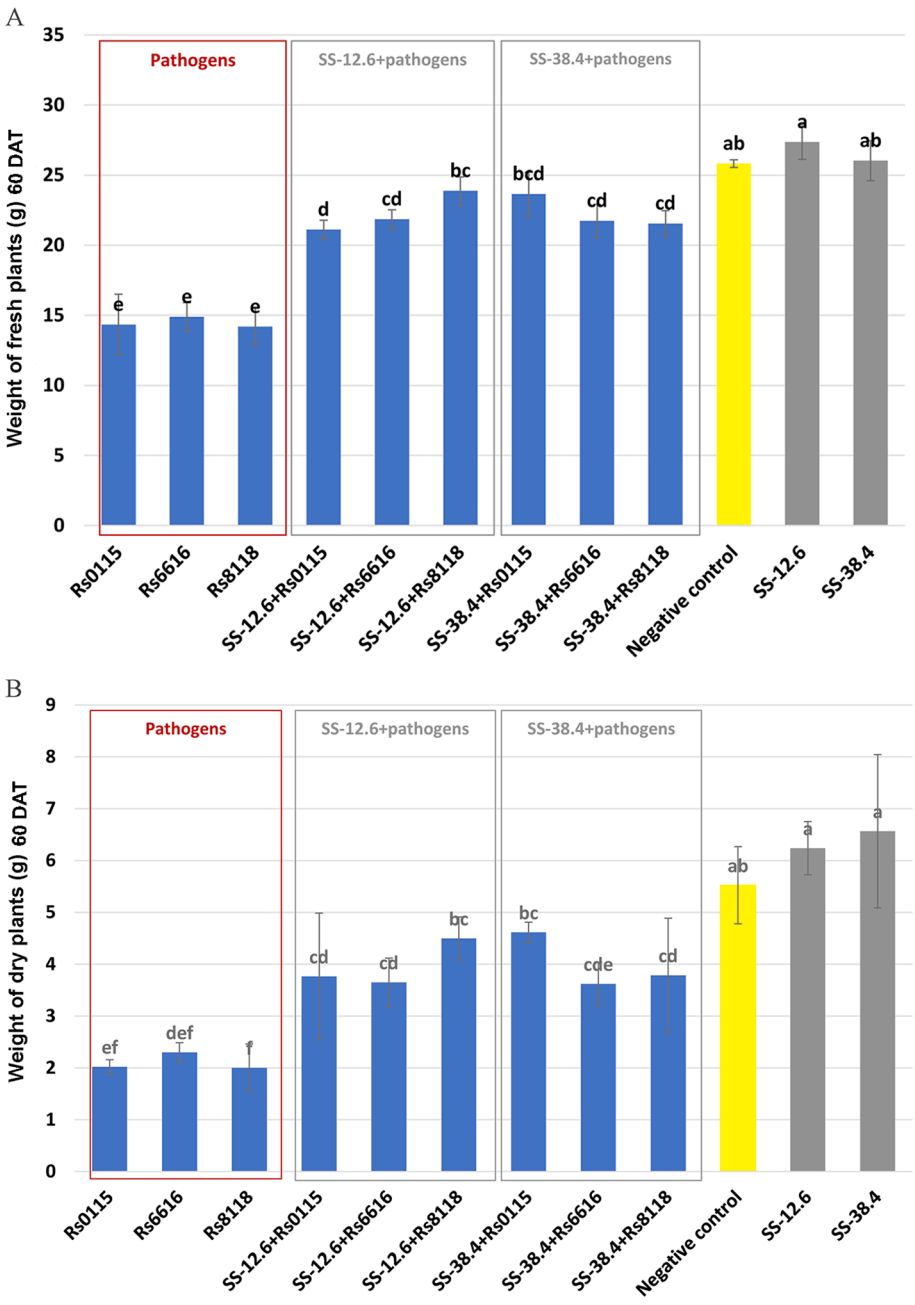

Fig. 7. Fresh (A) and dry (B) weight [g] of potato plants inoculated with *Ralstonia solanacearum* (Rs0115, Rs6616 and Rs8118) only ("Pathogens") and those treated with *Bacillus amyloliquefaciens* SS-12.6 ("SS-12.6 + pathogens") and SS-38.4 ("SS-38.4 + pathogens"). Potato sprouts treated with antagonistic *Bacillus* spp. strains alone marked as "SS-12.6" and "SS-38.4" served to exclude any harmful effect on the host plants, while "Negative control" represented potato sprouts immersed in sterile distilled water. The weight of potato plants was evaluated 60 days after treatment (DAT). Data are presented as mean values (n = 15) ± standard errors (SE). Values with the same letter are not significantly different according to Tukey's Honestly Significant Difference test ($p < 0.05$).

treatment of different potato cultivars by several potential biocontrol agents reduced the disease by 19.5–79.37%, with *P. fluorescens* and *B. subtilis* being the most efficient. Considering the tomato wilt, Singh et al. (2016) reported two *B. amyloliquefaciens* strains with biocontrol efficacy (63 and 68%) in supression of tomato wilt. Also, *B. amyloliquefaciens* strains were shown to exhibit biocontrol efficacy in supression of tomato wilt of almost 80% when both tomato seedlings and soil were treated with biocontrol strains (Tan et al., 2013). This indicates that the two-fold treatment with antagonists should be further examined, along with varying times and number of treatments, to identify the optimal strategy for suppresion of bacterial wilt of potato.

In conclusion, results given in this study pointed out that strains of *B. amyloliquefaciens* SS-12.6 and SS-38.4 showed high *in vitro*, *in situ*, and *in planta* antagonistic activity against *R. solanacearum*, *P. carotovorum*, *P. brasiliense*, and *D. dianthicola*. These *B. amyloliquefaciens* strains could therefore be exploited as a broad-spectrum biocontrol preparation for suppressing bacterial wilt and soft rot and/or potato blackleg. Future work should focus on determining precise modes of action of biocontrol strains, examining their efficacy in tuber protection and persistence under actual storage conditions for extended periods, and verifying their effectiveness under field conditions.

CRediT authorship contribution statement

S. Stanković: Conceptualization, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2023.105238.

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