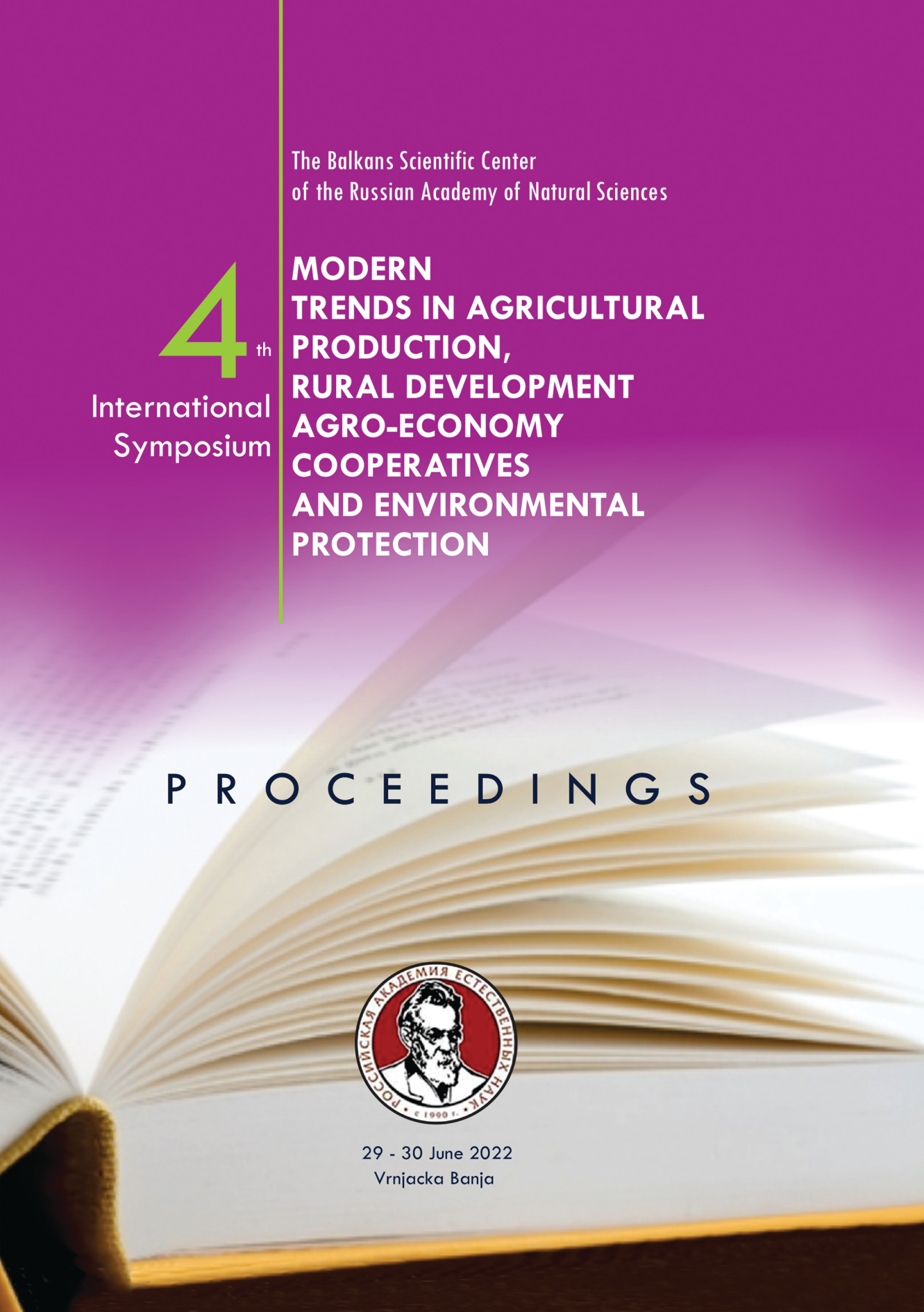
The Balkans Scientific Center
of the Russian Academy of Natural Sciences

4th International Symposium

MODERN TRENDS IN AGRICULTURAL PRODUCTION, RURAL DEVELOPMENT AGRO-ECONOMY COOPERATIVES AND ENVIRONMENTAL PROTECTION



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BIOCONTROL ABILITY OF *BACILLUS HALOTOLERANS* AGAINST STONE FRUIT PATHOGENS

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Abstract

The biocontrol ability of Bacillus halotolerans strain B33 against the most significant stone fruit bacterial and fungal pathogens was investigated under in vitro conditions. The results indicate that the inhibition zone toward bacterial pathogen Xanthomonas arboricola pv. pruni originating from peach and apricot (strains Xp219 and Xp320, respectively) ranged from 20 to 45 mm in diameter, depending of the used B. halotolerans concentration. B. halotolerans B33 did not inhibit the growth of bacteria Pseudomonas syringae pv. syringae (strains RE05 and RE3 originating from sweet cherry), P. s. pv. morsprunorum race 1 (strain Pm5 originating from sweet cherry and Pm26 from plum) and P. cerasi (strain RE10 originating from wild cherry). In the case of fungal pathogen Monilinia fructicola strain 116, 41.66−61.00% growth inhibition was observed when pure culture of B. halotolerans was used. *From the obtained results in this work, B. halotolerans strain B33 could be recommended to be potentially used as a suitable biocontrol agent for the control of Xanthomonads plant pathogenic bacteria and Monilinia spp. on stone fruit.*

Keywords: *fungi, bacteria, pathogen, stone fruit, biological control.*

Introduction

Fruit growing is an important part of agriculture in Serbia, with stone fruit orchards occupying 67% of the area designated for this purpose. Stone fruit (*Prunus* spp.) cultivation depends on a wide range of natural factors, including soil characteristics, climate, diseases and pests (Keserović et al., 2014). However, many fungal and bacterial pathogens undermine stone fruit quality and yield, resulting in significant economic losses. In general, fungal pathogens from the genus *Monilinia* (species *laxa, fructigena,* and *fructicola*) are considered the most destructive for all types of stone fruits, as they cause blossom and shoot blight, as well as brown rot in the fruit ripening stage (Hrustić et al., 2015). Pathogen *M. fructicola* is listed as an A2 quarantine organism in EU (EPPO, 2020). Ample evidence also indicates that the most significant bacterial pathogens of stone fruit species *Pseudomonas syringae* pathovars (*syringae* and *morsprunorum*), *P. cerasi* and *Xanthomonas arboricola* pv. *pruni* are responsible for dieback, as well as leaf and fruit bacterial spots (Iličić, 2016; Balaž et al., 2016; Iličić et al., 2018; Iličić & Popović, 2021; Iličić et al., 2022). Bacterium *X. arboricola* pv. *pruni* is listed as a quarantine organism in EU (EPPO, 2021).

Pathogen control has traditionally relied on the use of synthetic pesticides. However, increasing adoption to integrated pest management (IPM) has created an urgent need to find alternative measures that are less dependent on chemicals in order to satisfy the growing food demand while maintaining fruit protection and food safety. The most promising strategies are based on the use of biocontrol beneficial bacteria (Jiménez-Gómez et al., 2021) with zero residues and no withdrawal period.

Bacillus species are plant growth promoting, root-associated bacteria (PGPR) and are thus promising biocontrol agents. Plant growth promotion of *Bacillus* species bacteria is usually a result of combined action of two or more mechanisms, such as plant yield enhancement, soil biofertilization, abiotic stress and drought mitigation (Slama et al., 2019). One of the most promising activities of these bacteria is their ability to inhibit many plant pathogenic fungi and bacteria (Siddiqui et al., 2001; Jošić et al., 2011; Berić et al., 2012; Zdravković et al., 2015; Milijašević-Marčić et al., 2018; Nikolić et al., 2019; Marković et al., 2020; Jelušić et al., 2021). Bacterium *Bacillus halotolerans* is beneficial rhizobacteria which recently started receiving extensive attention due to its antagonistic activity, physiological traits, and other beneficial effects such is potential to promote plant growth and tolerance to drought and salinity stress (Zhang et al., 2018; Slama et al., 2019; Wang et al., 2021; Wu et al., 2021). Different strains of this bacterium were recently described as potent antagonistic agents against plant pathogenic

fungi (Sagredo-Beltrán et al., 2018; Slama et al., 2019) root-knot nematodes (Xia et al., 2019), which implies its perspective in wide use. *B. halotolerans* is listed on Biosafety level 1 Risk group (=biological agents which are unlikely to cause disease human disease) (https://bacdive.dsmz.de/pdf.php?id=1095&doi=10.13145/bacdive1095.20170829.2).

Guided by this evidence, the aim of this study was to determine the biocontrol potential of *B. halotolerans* strain B33 in the control of stone fruit pathogens, i.e. bacteria *P. syringae* pv. *syringae*, *P. s.* pv. *morsprunorum* race 1, *P. cerasi*, *X. arboricola* pv. *pruni* and fungi *Monilinia fructicola*, *in vitro* assays.

Materials and Methods

The strain of *B. halotolerans* coded as B33, obtained from the culture collection of the Institute for Plant Protection and Environment, was grown on Nutrient Broth (NB) for 24 h at 26 °C.

Its biological activity was tested against strains of bacteria *P. syringae* pv. *syringae* (strains RE05 and RE3 originating from sweet cherry), *P. s.* pv. *morsprunorum* (strains Pm5 and Pm26 originating from sweet cherry and plum, respectively), *P. cerasi* (strain RE10 originating from wild cherry), *Xanthomonas arboricola* pv. *pruni* (strains Xp219 and Xp320 originating from peach and apricot, respectively) and fungi *Monilinia fructicola* (strain 116 originating from peach). Bacterial strains were grown on Nutrient Agar (NA) for 48−72 h at 26 °C, while strain 116 was grown of Potato Dextrose Agar (PDA) for 10 days at 23 °C.

Inhibitory effect of B33 on the bacterial strains growth was evaluated by Agar-diffusion assay using holes in medium (Gojgić-Cvijović & Vrvić, 2003). Bacterial suspension of each tested strain was added to NA to obtain the final concentration of 10^8 cells mL^{-1} and was poured into sterilized Petri plates (ø 90 mm). As soon as the medium had solidified, the holes (ø 7 mm) were made on agar in Petri plates. To obtain the minimum inhibitory concentration (MIC), six bacterial concentrations of the strain B33 were prepared (10^4–10^9 cells mL^{-1}) and 10 µL of each of the tested concentrations was placed in the plate holes. Petri plates were then left to incubate at temperature 26 °C to promote bacterial development. The interaction between the tested bacterial pathogens and *B. halotolerans* strain B33 was expressed by the formation of the inhibition zone, the diameter (expressed in mm) of which was determined three days after incubation. Sterile distilled water served as a control treatment.

Antagonism of the strain B33 against the fungal pathogen *M. fructicola* (strain 116) was determined using the dual culture method. Agar discs (ø 7 mm)

of the tested fungi pathogen strain were placed in the center of PDA plates (ø 90 mm). Drops (8 µl in volume) of bacterial suspension of the strain *B. halotolerans* B33 adjusted to 10^8 cells mL^{-1} were placed on four sides near the edge of the Petri plate. Sterile distilled water served as a control treatment. In order to quantify the antagonistic potential of *B. halotolerans*, pathogen growth area was measured after 14 days of incubation at 23 °C, and the percent of inhibition was calculated using the formula given by Zarrin et al. (2009).

Three replicates were conducted for each of the tested bacterial or fungal strains, and for each treatment/ *B. halotolerans* B33 concentration. All experiments were performed twice.

The results were statistically analyzed by conducting analysis of variance (ANOVA), whereby Duncan's Multiple Range Test ($p < 0.05$) was performed for testing the differences between means related to different treatments.

Results and Discussion

Due to the increasing health and environmental risks posed by the pesticide use in agriculture, less harmful alternatives such as plant growth-promoting bacteria to suppress phytopathogens must be explored. At present, *Bacillus* species are considered the most promising group of bacteria for plant pathogen control, as they have the capacity to act as biopesticides, biostimulators and biofertilizators. Most important bioactive molecules from the genus *Bacillus* are synthesized peptides and lipopeptides, polyketide compounds, bacteriocins and siderophores. In general, they exhibit a broad spectrum of antagonistic activity against plant pathogenic bacteria, fungi and viruses (Romero et al., 2007; Fira et al., 2018).

The results obtained in the present study indicate that *B. halotolerans* strain B33 has the capacity to suppress the growth of most common bacterial and fungal pathogens of stone fruit. The results of assays including bacterial pathogens, presented in Table 1, indicate that B33 was effective in the suppression of *X. arboricola* pv. *pruni*. Both strains Xp219 (originating from diseased peach leaves) and Xp320 (originating from diseased apricot fruits) were suppressed in the applied concentrations 10^6–10^9 cells mL^{-1} of *B. halotolerans*, while lower concentrations (10^4 and 10^5 cells mL^{-1}) were ineffective. Thus, 10^6 was established as the MIC, resulting in an inhibitory zone of 20.00−20.33 mm diameter, which increased to 28.00−29.00 mm, 38.33−38.66 mm, and 45.00 mm as the concentration increased to 10^7, 10^8 and 10^9, respectively. However, in this study, no inhibitory effect was obtained when strains of *P. syringae* pv. *syringae*

(RE05 and RE3) isolated from diseased sweet cherry, as well as strains of *P. syringae* pv. *morsprunorum* race 1 (Pm5 and Pm26, isolated from sweet cherry and plum, respectively) and *P. cerasi* (RE10, isolated from wild cherry) were used, irrespective of the *B. halotolerans* B33 concentration.

The inhibitory activity of *B. halotolerans* strain B33 against fungal pathogen *M. fructicola* strain 116 was assessed using the dual culture method. Based on the obtained results, *B. halotolerans* achieved 41.66−61.00% inhibition of *M. fructicola* growth. Referring to the obtained results, it could be concluded that applied treatments with *B. halotolerans* were effective in pathogen control, indicating that this strain should be considered for use in organic agriculture, and especially in product storage.

If bacterium *B. halotolerans* is used as a biocontrol agent, it could effectively inhibit many plant pathogenic fungi such are *Fusarium* spp., *Alternaria alternata*, *Botrytis cinerea*, *Rhizoctonia bataticola* and *Phytophthora infestans* (Slama et al., 2019; Wang et al., 2021). According to Wang et al. (2021) treatment with *B. halotolerans* strain KLBC XJ-5 controlled mycelial growth as well as conidial germination of *B. cinerea* (grey mould) *in vitro*. The disease in strawberries inoculated with *B. halotolerans* KLBC XJ-5 was lower in comparison with that in the control fruit. Slama et al. (2019) found four bacterial isolates of *B. halotolerans*, designated BFOA1, BFOA2, BFOA3 and BFOA4 that proved very active against *Fusarium oxysporum* f. sp. *albedinis*; active against *Fusarium* isolates belonging to four species: *F. oxysporum*, *F. solani*, *F. acuminatum* and *F. chlamydosporum* and exhibited strong activities against another four major phytopathogens: *Botrytis cinerea*, *Alternaria alternata*, *Phytophthora infestans*, and *Rhizoctonia bataticola.* Authors also proved *in vivo* antifungal activity of strain BFOA4 against *F. oxysporum* f. sp. *radicis-lycopersici* infection on tomato fruits. *B. halotolerans* strain MS50-18 A can inhabit the growth of plant pathogens and is good for pepper plantlet roots (Zhang et al., 2018).

In this study for the first time we demonstrated antibacterial activity of *B. halotolerans* strain B33 against xanthomonads plant pathogenic bacteria. According to Jelušić et al. (2021) two bacteria from the genus *Bacillus*, *B. velezensis* and *B. megaterium* were found to be effective against crucifers pathogen *Xanthomonas campestris* pv. *campestris in vitro* and *in vivo* when applied as a whole-culture and as a cell-free supernatant. Rabbee et al. (2022) reported that *B. velezensis* Bv–21 can be used as an effective and eco-friendly biocontrol agent either by itself or as an active compound against the wild-type and streptomycin-resistant *Xanthomonas citri* subsp. *citri*, which causes bacterial canker disease in citrus fruit. Nikolić et al. (2019) demonstrated significant

biocontrol potential of the crude lipopeptide extracts and cell culture of *Bacillus amyloliquefaciens* SS-12.6 in suppression of leaf spot disease (*P. syringae* pv. *aptata*) severity on sugar beet plants. Some *Bacillus* species were reported as an antagonist of *P. s.* pv. *syringae* pathogen of the citrus blast in the study of Mougou and Boughalleb-M'hamdi (2018).

The evidence obtained in the present study confirms the importance of the developed *in vitro* antagonistic assays for screening different bacteria for their antagonistic activity. Finally, the *B. halotolerans* strain B33 constitutes a promising biocontrol agent which produces antibacterial compounds against *X. arboricola* pv. *pruni*, as well as exhibits antifungal activity against pathogen *M. fructicola.* Future studies will thus be performed *in planta* under different production conditions.

Table 1. *Inhibition zone caused by Bacillus halotolerans against different bacterial pathogens of stone fruits*

B. halotolerans strain B33 − Bacterial concentration	10^9	10^8	10^7	10^6	10^5	10^4
Plant pathogenic bacteria / Strains	Inhibitory zone (mm)					
X. arboricola pv. *pruni*, strain Xp320	45.00^e	38.33^d	29.00^c	20.33^b	0^a	0^a
X. arboricola pv. *pruni*, strain Xp219	45.00^e	38.66^d	28.00^c	20.00^b	0^a	0^a
P. syringae pv. *syringae*, strain RE05	0^a	0^a	0^a	0^a	0^a	0^a
P. syringae pv. *syringae*, strain RE3	0^a	0^a	0^a	0^a	0^a	0^a
P. syringae pv. *morsprunorum*, strain Pm5	0^a	0^a	0^a	0^a	0^a	0^a
P. syringae pv. *morsprunorum*, strain Pm26	0^a	0^a	0^a	0^a	0^a	0^a
P. cerasi, strain RE10	0^a	0^a	0^a	0^a	0^a	0^a

*Mean values of inhibition zone diameters are shown. Values followed by the same letter are not significantly different ($p < 0.05$) according to the Duncan's multiple range test results.

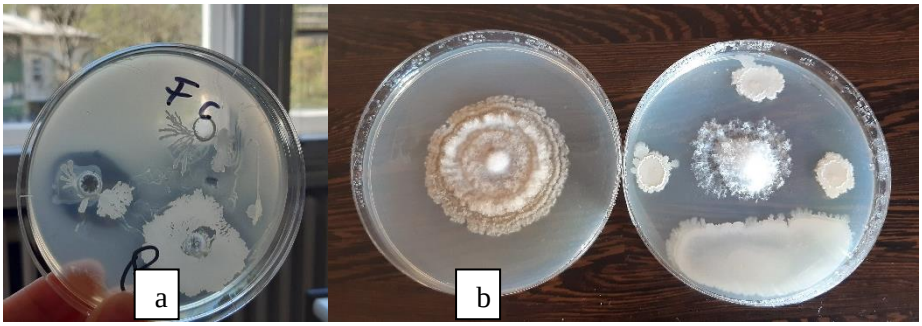

Figure 1. *Biocontrol ability of B. halotolerans strain B33 with respect to:*
(a) inhibition of X. arboricola pv. pruni bacterial strain Xp219; and
(b) M. fructicola strain 116 control treatment (left) and treatment with B33 (right).

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