Bacillus spp. AS POTENTIAL BIOCONTROL AGENTS OF BACTERIAL SPOT ON PEPPER CAUSED BY *Xanthomonas euvesicatoria*

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Abstract

The objective of this research was to identify bacterial spot-causing pathogen and bacterial antagonists for management of the disease using biocontrol agents as environmentally friendly alternatives. Isolates of *Bacillus* spp. were obtained from the soil samples collected at different localities in Serbia. Antibacterial activity of natural antagonists toward the pathogen isolated from infected pepper leaves was examined using a modified well-diffusion assay and standard germination test. Our results confirmed the presence of *Xanthomonas euvesicatoria* as the causal agent of bacterial spot of pepper. Screening of 32 *Bacillus* spp. isolates for antibacterial activity showed that 8 isolates inhibit growth of examined *X. euvesicatoria* isolates. Four isolates identified as *Bacillus subtilis* exhibited the highest antibacterial activity by *in vitro* test (from 5 to 14 mm inhibition zone of bacterial growth). The isolates positively influenced germination of pepper seeds, causing up to 16% and 70% increase in germination and germination viability compared to control seeds infected with pathogen. The most effective isolates of *Bacillus subtilis* could be used as potential biocontrol agents of bacterial spot of pepper.

Introduction

Bacterial spot of pepper (*Capsicum annuum* L.) caused by *Xanthomonas* is one of the most damaging diseases resulting in direct economic loss [1]. The symptoms of bacterial spot infection are characterized by smaller lesions with yellow haloes which coalesce into larger ones and occur on leaves, stems, and fruits. Infection leads to germination decrease, leaf necrosis, complete defoliation and destruction of the crop. Bacterial spot-causing xanthomonads were reclassified into 4 species: *X. euvesicatoria*, *X. vesicatoria*, *X. perforans*, and *X. gardneri* [2]. The most common causative pathogen of bacterial spot on pepper plants listed in Serbia is *X. euvesicatoria*, while intensity of the infection depends on environmental conditions [3].

Chemical measures applied in order to prevent the recurrence of bacterial infection do not often provide satisfactory results in maintaining a low level of bacterial flora. Intensive application of synthetic pesticides became an increasing concern, primarily due to toxic chemical residues in food products, frequent environmental pollution and the development of resistant strains of pathogens [4]. Environmentally-friendly alternatives to pesticide such as application of beneficial microorganisms which inhibit the growth of pathogens and prevent the disease they cause, have been developed as a new strategy for biological control of soil-borne diseases [5].

Bacillus spp. are frequently found in soils and exert antagonistic activities against several fungal and bacterial pathogens [6]. The ability of forming endospores allows them to survive in different environmental conditions and facilitates the formulation of biocontrol preparatons [7]. *Bacillus*-based biopesticides contain strains which have the ability to colonize rhizosphere, promote plant growth and inhibit pathogen growth through various mechanisms [8]. Among these, *Bacillus subtilis, B. licheniformis, B. pumilus* and *B. amyloliquefaciens* are the most efective species in controlling plant diseases.
The objective of this research was to identify antagonistic *Bacillus* spp. strains for management of the bacterial spot of pepper.

Experimental

Isolation of antagonist

Different soil samples were randomly collected from various parts of Serbia. Soil sample collection included the rhizosphere of plants, agricultural and non-agricultural soils. Isolation of bacteria from the soil was done using the serial dilution and streak-plate techniques. Soil dilutions were prepared with 1 g of each soil sample suspended in 9 mL of 0.85% NaCl in sterile test tubes. A 0.1 ml aliquot of each dilution (10^{-3}-10^{-6}) was spread aseptically on nutrient agar (NA) (HiMedia Lab. Pvt. Ltd., Mumbai, India) and incubated at 30°C for 24 h. Round colonies, with entire or wavy margins, cream to light yellow and medium size, were recultivated five times to obtain pure cultures of *Bacillus* spp.

Isolation of pathogen

Isolation of the pathogen was made from infected pepper leaves showing typical bacterial spot lesions. Symptomatic leaves were rinsed with sterile water three times and then dried on a sterile filter paper under aseptic conditions. Small pieces of infected leaf tissues were macerated in sterile water, and the resulting suspension was streak-plated on nutrient agar (NA). After incubation at 27°C for 3–5 days, round, small, yellow and slimy colonies, with entire margins were purified by subculturing. For the evaluation of pathogenicity of isolates, bacterial suspensions (10^6/ml) were sprayed on pepper seedlings and bacterial spot symptoms were observed 3 weeks post-inoculation.

Molecular species identification

Bacillus and *Xanthomonas* isolates for DNA extraction were grown on NA plates for 24 h. DNA was extracted using a DNeasy Mini Kit (QIAGEN Inc., Hilden, Germany), according to the manufacturer's recommendations. For the amplification of 16S rDNA gene fragments of *Bacillus* isolates, universal primers fD1 (27F) (AGAGTTTGATCMTGGCTCAG) and rP3 (1492R) (TACGGYTACCTTGTTACGACTT) were used [9]. The target DNA gene fragments of *Xanthomonas* isolates, were amplified using primers xeF (CATGAAGAACTCGGCGTATCG) and xeR (GTCGGACATAGTGGACACATAC) [10]. The polymerase chain reaction (PCR) was done in 25-µl aliquots using S-thermal cycler (Eppendorf, Germany). Amplicons were electrophoresed in 1.5% agarose gel (Invitrogen) with ethidium bromide. Purification and sequencing of the PCR-amplified DNA fragments was done in the company MACROGEN, Seoul, South Korea (http://dna.macrogen.com). FinchTV Version 1.4.0. was used for sequence analysis, and nucleotide sequences were compared with the GenBank Database at the National Center for Biotechnology Information (NCBI).

Antibacterial activity assay

Antibacterial activity of *Bacillus* spp. against *Xanthomonas* was determined by modified well-diffusion assay [11]. *Bacillus* and *Xanthomonas* isolates were cultured for 24 h in nutrient broth (NB), at optimal temperature of 28°C. Petri dishes with NA solid medium were poured with 6 ml of soft NA_7 medium (the same medium, but with half amount of agar), previously inoculated with 60 μL of the pathogen culture (10^6/ml). The wells (R = 5 mm) were made in the medium using sterile bottom parts of the 200 μL pipette tips. Well-diffusion assay was completed by adding tested culture of *Bacillus* spp. (10^8/ml) into the well in the volume of 50 μL. Assay was done in three repetitions for each bacterial isolate. The plates were incubated overnight at 28°C. Antibacterial activity was detected by measuring the zone of inhibition from the edge of the well and expressed in mm.

Germination test

The effect of antagonistic bacteria *Bacillus* spp. on germination of pepper seeds infected by *Xanthomonas* was examined using a standard germination test [12]. Seed of pepper cultivar "Amfora" developed at the Institute of Field and Vegetable Crops in Novi Sad was used for test. Seeds were surface disinfested in 2% sodium hypochlorite (NaOCl, Sigma) for 2 to 3 min, rinsed with sterile distilled water four times, and then dried on sterile filter paper under aseptic conditions. Inoculation of seeds was performed with 5mL of pathogen suspension (10^6/ml) and 5 mL of antagonist suspension (10^8/ml). Control seeds were inoculated with 10 mL of pathogen suspension. Treated seeds were placed in Petri dishes (R = 140 mm), while moisturized filter paper was used as the medium. Germination was tested in a germination chamber at alternating temperature of 20- 30°C. Four replicates × 100 seeds were tested. Seed germination and germination viability were determined after 7 and 14 days.

Statistical analysis

Data was subjected to analysis of variance (ANOVA) using software STATISTICA 12.6 (Statsoft, Tulsa, Oklahoma, USA). Means were separated using Tukey's HSD (honest significant difference) test at the $P < 0.05$ level.

Results and discussion

This study confirmed that 8 out of the 32 isolates of *Bacillus* spp. from the soil were found positive for antibacterial activity against two isolates of *Xanthomonas* (X6, X18) by *in vitro* test (Table 1).

Table 1. Antibacterial activity of *Bacillus* spp. against *Xanthomonas*

Pathogen vs. Antagonist	X6	X18
	Inhibition (mm)	
B2	1.67 e	2.33 ef
B3	2.33 de	3.00 e
B5	11.33 a	14.33 a
B7	9.00 ab	10.67 b
B11	0.00 e	0.67 f
B13	5.00 cd	6.00 d
B23	2.00 e	4.33 de
B32	7.67 bc	8.33 c
Average	4.87 B	6.21 A

Values with different lowercase/capital letters within the same column/row differ significantly ($P < 0.05$). Values are the means of 3 replicates.

On average, *Bacillus* spp. isolates exhibited higher antibacterial activity against X18. The highest antagonistic activity was exhibited by isolates B5 and B7, followed by isolates B13 and B32. Isolates B2, B3, B11 and B23 had the least antagonistic effect on tested pathogens. Four of the best performing isolates (B5, B7, B13, B32) were selected for further assessment of their effect on germination of pepper seeds infected with pathogen (Table 2). *Bacillus* isolates positively influenced the germination of pepper seeds treated with *Xanthomonas*, and the effect varied depending on the examined isolate of antagonist and pathogen. The highest antibacterial activity against the pathogen X6 was observed using B5 isolate (16% and 34% increase in germination and germination viability), while B32 exhibited the highest antagonistic effect on X18 (14% and 70% increase in germination and germination viability). Isolate B7 also had good antagonistic potential, while the least effect was obtained by B13. Higher antagonistic effect on germination parameters was exhibited by pepper seeds treated with Xe18. These results are in agreement with *in vitro* testing of antibacterial activity.

Table 2**.** Effect of *Bacillus* on germination of pepper seeds infected with *Xanthomonas*

Pathogen vs. Antagonist	X6	X18	X6	X18
	Germination viability (%)		Germination (%)	
Control (Pathogen)	39.00 c	35.25 c	79.00 d	83.25 d
B5	52.25 a	58.00 a	91.25 a	93.00 b
B7	49.50 a	59.25 a	89.25 b	92.00 b
B13	43.50 b	49.00 b	88.00 bc	87.25 c
B32	45.75 b	59.75 a	87.00 c	95.25 a
Average	47.75 B	56.50 A	88.87 B	91.87 A

Values with different lowercase/capital letters within the same column/row differ significantly ($P < 0.05$). Values are the means of 4 replicates.

By comparing the sequences with the Genbank Database at NCBI, antagonistic isolates were identified as *Bacillus safensis* (B2), *Bacillus pumilus* (B3, B11, B23), and *Bacillus subtilis* (B5, B7, B13, B32) (Table 3), while pathogen isolates were identified as *Xanthomonas euvesicatoria* (X6, X18) (Table 4).

Table 3. Isolates of *Bacillus* spp. from soil

Isolate code	Isolation source	*Bacillus* species	NCBI
B2	Non-agricultural soil	*Bacillus safensis*	KU953932
B3	Rhizosphere (wheat)	*Bacillus pumilus*	KU953923
B5	Rhizosphere (sunflower)	*Bacillus subtilis*	KU953925
B7	Rhizosphere (maize)	*Bacillus subtilis*	KU953927
B11	Non-agricultural soil	*Bacillus pumilus*	KU953931
B13	Rhizosphere (maize)	*Bacillus subtilis*	KX444639
B23	Rhizosphere (wheat)	*Bacillus pumilus*	KX444649
B32	Non-agricultural soil	*Bacillus subtilis*	KX766373

Table 4. Isolates of *Xanthomonas euvesicatoria* from infected pepper leaves

Isolate code	Isolation source	*Xanthomonas* species	NCBI
X6	Pepper cv. Slonovo uvo	*Xanthomonas euvesicatoria*	KX512832
X18	Pepper cv. Palanačka bela	*Xanthomonas euvesicatoria*	KX512834

In general, the highest antagonistic activity toward this important bacterial plant pathogen was observed in *Bacillus subtilis* isolates, while B5, B7 and B32 were the best natural antagonists among them. The mechanisms of this antibacterial effect are uncertain, although it is known that *B. subtilis* can produce a variety of antimicrobial agents, including a broad spectrum of lipopeptides, such as surfactins, iturins and fengycins [13]. Similarly, Dimkić et al. [14] showed very strong inhibition of *Xanthomonas arboricola* by *Bacillus*, while Berić et al. [15] found that 104 out of 203 *Bacillus* isolates exhibit an antagonistic effect on *Xanthomonas oryzae* pv. *oryzae*. Since there is insufficient experimental confirmation on using *Bacillus* in biocontrol of *Xanthomonas*, especially against *X. euvesicatoria*, further research will be of great importance.

Conclusion

Based on our results, indigenous *Bacillus subtilis* isolates from soil generally had good antagonistic potential for biological control of bacterial spot of pepper caused by *Xanthomonas euvesicatoria*. Further selection of these isolates through *in planta* antagonistic testing will be necessary in order to determine their efficacy in inhibition of pathogen growth and suppression of disease under greenhouse and field conditions.

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