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SELECTION OF ANTAGONISTS FOR BIOCONTROL OF *Xanthomonas euvesicatoria*

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Xanthomonas euvesicatoria is a worldwide causer of pepper bacterial spot, a bacterial plant disease responsible for massive losses of fresh pepper fruits. Considering the current problems in management of bacterial plant diseases, biological control using antagonistic microbial strains with high potential for plant pathogens suppression emerges as a possible solution. The aim of this study was to select suitable antagonists for suppression of X. euvesicatoria among the bacteria, yeast and fungi from the genera Pseudomonas, Lactobacillus, Saccharomyces and Trichoderma, based on in vitro antimicrobial activity testing using the diffusion disc method. The results of this study have revealed that cultivation broth samples of the antagonists Lactobacillus MK3 and Trichoderma reseii QM 9414, as well as supernatant samples of the antagonist Pseudomonas aeruginosa I128, have showed significant potential to be applied in biological control of X. euvesicatoria. Further research would be required to formulate suitable cultivation medium and optimize bioprocess conditions for production of the proposed pepper bacterial spot biocontrol agents.

Keywords: *Pseudomonas* spp., *Lactobacillus* spp., *Saccharomyces cerevisiae*, *Trichoderma reseii*, antimicrobial activity

INTRODUCTION

Bacterial plant diseases represent a worldwide problem for sustainable food production due to difficulties and insufficient efficacy of existing agricultural practices in plant disease management. Furthermore, the lack of efficient disease suppression agents and heavy usage of copper-based chemicals and antibiotics have led to emergence of resistant bacterial pathogenic strains (1). Bacteria of the genus *Xanthomonas* are among the important plant pathogens, having a wide spectrum of plant hosts (2). The species *Xanthomonas euvesicatoria* is a causer of tomato and pepper bacterial spot, a plant disease responsible for massive fresh fruit losses, resulting in their degraded quality, lower market value and insufficient amount for industrial processing (3). Since usual bacterial spot disease management practices, such as crop rotation, usage of healthy planting material and copper bactericides (2) haven't given satisfying results in previous decades, biological control using microbial biocontrol agents emerges as a possible solution.

Bacteria of the genus *Pseudomonas* have been largely employed as biocontrol agents due to their several beneficial abilities: to colonize and multiply in the rhizosphere, to

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colonize plants endophytically, to aggressively compete with other microorganisms and to adapt to environmental stress (4). Furthermore, these bacteria produce wide range of different biocontrol metabolites: antibiotics, siderophores, volatiles and plant growth promoters (5). Biocontrol traits of *Lactobacillus* spp. rely on production of bioactive metabolites such as organic acids and bacteriocins (6). An additional advantage for their application as biocontrol agents is GRAS (Generally Regarded As Safe) and QPS (Qualified Presumption of Safety) status of several *Lactobacillus* strains (7). *Saccharomyces cerevisiae* is also a promising biocontrol agent with several biocontrol traits: competition and production of hydrolytic enzymes and volatiles (8). *Trichoderma* spp. are well-known as biocontrol agents exhibiting several indirect or direct biocontrol mechanisms, including competition for nutrients and space, modifying the environmental conditions, plant growth promotion, antibiosis, mycoparasitism and activation of plant defense mechanisms (9). Fungi of the genus *Trichoderma* produce wide range of compounds inducing localized or systemic resistance responses, and also contribute to substantial changes to plant proteome and metabolism, simultaneously promoting root growth and development, uptake and use of nutrients and crop productivity (10). Some of these compounds include plant growth factors, antibiotics, siderophores and enzymes (9).

The aim of this study was to select suitable antagonists for suppression of *X. euvesicatoria* pathogenic strains, isolated from pepper plants with symptoms of bacterial spot, among the isolates from the genera *Pseudomonas, Lactobacillus, Saccharomyces and Trichoderma*. The main indicator of antagonistic activity was inhibition zone diameter, obtained as a result of antimicrobial activity testing using the diffusion disc method.

EXPERIMENTAL

Antagonists and pathogens

In this study several antagonists were investigated: *Pseudomonas aeruginosa* ATCC 27853 (A1), *Pseudomonas aeruginosa* I128 (A2) isolated from water, *Pseudomonas putida* I315 (A3) isolated from water, three *Lactobacillus* strains isolated from cheese – *Lactobacillus* I14 (A4), *Lactobacillus* I19 (A5) and *Lactobacillus* MK3 (A6), *Saccharomyces cerevisiae* P31 (A7) and *Trichoderma reseii* QM 9414 (A8). Three phytopathogenic *Xanthomonas euvesicatoria* strains (X1, X2 and X3) were isolated from leaves of pepper plants with symptoms of bacterial spot in 2015 at the cadastral municipality Pivnice, Serbia.

Cultivation of antagonists

Inocula of the antagonistic strains were prepared using the following media: nutrient broth (HiMedia, India) for *Pseudomonas* spp. (A1, A2, A3), MHB (Mueller-Hinton broth – HiMedia, India) for *Lactobacillus* spp. (A4, A5, A6), SMB (Sabouraud maltose broth – HiMedia, India) for *Trichoderma reseii* QM 9414 (A8) and semi-synthetic medium for *Saccharomyces cerevisiae* P31 (A7) (11). Inocula were prepared on a laboratory shaker at 30 °C, with mixing (150 rpm) and spontaneous aeration during 48 h. Cultivation of antagonists was performed using the similar media as for the inocula preparation, under the similar conditions, except bioprocess duration was 96 h.

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Testing of antimicrobial activity

Three suspensions of pathogenic *X. euvesicatoria* strains (X1, X2 and X3) were prepared using a sterile saline to achieve 10^8 CFU/mL. These suspensions were used to inoculate the melted and tempered (50±1°C) test media – YMA (yeast maltose agar) (12). Samples for antimicrobial activity testing were cultivation broth samples, obtained after the end of the cultivation of selected antagonists, as well as their supernatants obtained by centrifugation of cultivation broth samples at 13000 g for 10 min (Rotina 380R, Hettich, Germany) which were afterwards filtrated through nylon syringe filters (0.22 μm pore diameter, Agilent Technologies, Germany) to completely remove biomass of antagonists. Commercial streptomycin disks containing 30 μg of streptomycin (Torlak, Serbia) were used as positive control, while sterile distilled water was used as negative control. Antimicrobial activity testing was performed in triplicates using the diffusion disc method (13) with 10 μL of sample per each disk. After incubation at 26 °C for 72 h, inhibition zone diameters were measured.

Experimental data analysis

The obtained data regarding inhibition zone diameters were processed using several statistical methods (Levene's test, ANOVA – analysis of variance, Duncan's multiple range test) using the software Statistica 13.5 (Tibco Software Inc., USA). All statistical analyses were performed at significance level of 95%. Mean values and standard deviations of inhibition zone diameters were calculated using Microsoft® Excel 2010 software (Microsoft Corporation, USA).

RESULTS AND DISCUSSION

After cultivation of eight selected antagonists, cultivation broth samples and biomass-free supernatants were tested for antimicrobial activity against three *X. euvesicatoria* phytopathogenic strains in order to select the most suitable antagonist and also to determine a suitable biocontrol agent (cultivation broth containing biomass of antagonists, or extracellular metabolites produced by the antagonists contained in biomass-free supernatants). The experimental data regarding the obtained inhibition zone diameters were hence analyzed separately for cultivation broth and supernatant samples. Levene's test was performed for both datasets and it confirmed hypothesis of variance homogeneity in both cases. Furthermore, one-way ANOVA was applied to determine statistical significance of the antagonists' and pathogens' effect to inhibition zone diameter. Finally, Duncan's multiple range test was performed to establish homogenous groups of antagonists and pathogens with the same level of statistical significance when it comes to their effect on antimicrobial activity of the tested cultivation broth and supernatant samples against *X. euvesicatoria*.

The results of one-way ANOVA for antimicrobial activity of cultivation broth samples of the selected antagonists against *X. euvesicatoria* are given in Table 1. As it could be observed, these results have revealed statistically significant effect of the tested antagonistic strains to the obtained inhibition zone diameters at the significance level of 99%, since the obtained *p*-value is less than 0.0001.

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Table 1. One-way ANOVA of inhibition zone diameters obtained as a result of antimicrobial activity testing of cultivation broth samples of the selected antagonists against *X. euvesicatoria*

Effect	SS	MS	DF	F-value	*p*-value
Intercept	41525.38	41525.38	1	32795.18	**<0.0001**
Antagonist	3843.95	480.49	8	379.48	**<0.0001**
Error	91.17	1.27	72	-	-

SS – sum of squares, MS – mean squares, DF –degree of freedom

Furthermore, homogenous groups of antagonists established by the Duncan's multiple range test when it comes to testing of antimicrobial activity of the cultivation broth samples are given in Table 2.

Table 2. Results of Duncan's multiple range test for inhibition zone diameters (mean values, standard deviations and significance levels) obtained as a result of antimicrobial activity testing of cultivation broth samples of the selected antagonists against *X. euvesicatoria*

Antagonist	Inhibition zone diameter (mm)
A7	15.72±0.97[a]
A1	16.83±1.41[b]
A5	17.28±0.83[b]
A3	17.89±1.05[b]
A2	20.00±0.87[c]
A4	23.89±1.05[d]
A8	26.50±1.12[e]
A6	27.22±1.39[e]
S	38.44±1.26[f]

A1 - *Pseudomonas aeruginosa* ATCC 27853, A2 – *Pseudomonas aeruginosa* I128, A3 – *Pseudomonas putida* I315, A4 - *Lactobacillus* I14, A5 - *Lactobacillus* I19, A6 - *Lactobacillus* MK3, A7 – *Saccharomyces cerevisiae* P31, A8 – *Trichoderma reseii* QM 9414, S – streptomycin

The lowest values of inhibition zone diameters were obtained using the cultivation broth sample of the antagonist A7 (*S. cerevisiae* P31), while the highest values of inhibition zone diameters in range 25.5-28.5 mm were obtained by the cultivation broth samples of *Lactobacillus* I14 (A4) and *T. reseii* QM 9414 (A8). These two antagonists are also at the same level of statistical significance, indicating that each of them could be successfully applied for suppression of *X. euvesicatoria*. In the study published by Daranas et al. (7) *Lactobacillus* spp. were successfully applied as biocontrol agents for suppression of *Xanthomonas* spp., where the most dominant *in vitro* inhibitory effect was lowering of the pH value due to production of lactic acid. Shrestha et al. (14) have also observed significant potential of *Lactobacillus* spp. to be used as biocontrol agents of pepper bacterial

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spot. *Trichoderma* spp. have provided systemic protection against *X. euvesicatoria* in the range 24.13-95.94% (15). *Trichoderma* strains in mixed culture with other antagonistic microorganisms, such as *Bacillus* strains, could also provide satisfying plant protection from bacterial spot (16).

When it comes to testing of antimicrobial activity of biomass-free supernatants obtained by centrifugation of cultivation broth samples of the selected antagonists, one-way ANOVA results (Table 3) have revealed statistically significant effect of the antagonists to inhibition zone diameters against *X. euvesicatoria* at the significance level of 99%.

Table 3. One-way ANOVA of inhibition zone diameters obtained as a result of antimicrobial activity testing of supernatant samples of the selected antagonists against *X. euvesicatoria*

Effect	SS	MS	DF	F-value	*p*-value
Intercept	13046.72	13046.72	1	22395.42	**<0.0001**
Antagonist	11327.34	1415.92	8	2430.50	**<0.0001**
Error	41.94	0.58	72	-	-

SS – sum of squares, MS – mean squares, DF –degree of freedom

The results of the Duncan's multiple range test have showed that biomass-free supernatant samples of three tested antagonists (A1 – *P. aeruginosa* ATCC 27853, A5 – *Lactobacillus* I19 and A7 – *S. cerevisiae* P31) hadn't showed any antimicrobial activity against *X. euvesicatoria* phytopathogenic strains, indicating that these antagonist don't have an ability to synthesize antimicrobial compounds in the form of extracellular metabolites effective against the tested bacterial phytopathogens. Each other antagonist has showed an ability to suppress growth of *X. euvesicatoria* by the mechanism of antimicrobial activity which includes synthesis of extracellular antibacterial compounds. The highest level of *X. euvesicatoria* suppression was achieved by the extracellular antibacterial compounds produced by *P. aeruginosa* I128 (A2), an antagonistic strain isolated from water. Since isolate from water has showed stronger antimicrobial activity against *X. euvesicatoria* in both cases of cultivation broth and biomass-free supernatant testing compared to referent strain A1 (*P. aeruginosa* ATCC 27853), these results have also confirmed the thesis that wild strains isolated from the environment usually express higher level of antimicrobial activity compared to referent strains, due to their better adaptation abilities in various ecosystems (17). Similarly, Spago et al. (18) have showed the ability of *P. aeruginosa* strain to produce secondary metabolites which have biological activity against different plant pathogenic *Xanthomonas* species. Production of extracellular compounds with antibiotic activities against *Xanthomonas* strains by *Pseudomonas* sp. has also been reported by Oliveira et al. (19). *Pseudomonas* spp. have also been successfully applied as a foliar treatment in biological control of bacterial spot (20). Bacteriocins, as the secondary metabolites produced by *Pseudomonas* spp., have also been investigated for suppression of *X. euvesicatoria* (21).

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Table 4. Results of Duncan's multiple range test for inhibition zone diameters (mean values, standard deviations and significance levels) obtained as a result of antimicrobial activity testing of supernatant samples of the selected antagonists against *X. euvesicatoria*

Antagonist	Inhibition zone diameter (mm)
A1	0.00 ± 0.00^{a}
A5	0.00 ± 0.00^{a}
A7	0.00 ± 0.00^{a}
A4	9.78 ± 0.71^{b}
A6	13.61 ± 1.11^{c}
A8	14.11 ± 0.78^{c}
A3	16.17 ± 0.79^{d}
A2	22.11 ± 0.82^{e}
S	38.44 ± 1.26^{f}

A1 - *Pseudomonas aeruginosa* ATCC 27853, A2 – *Pseudomonas aeruginosa* I128, A3 – *Pseudomonas putida* I315, A4 - *Lactobacillus* I14, A5 - *Lactobacillus* I19, A6 - *Lactobacillus* MK3, A7 – *Saccharomyces cerevisiae* P31, A8 – *Trichoderma reseii* QM 9414, S – streptomycin

Furthermore, one-way ANOVA was performed in order to estimate statistical significance of the tested *X. euvesicatoria* pathogenic strains to the obtained inhibition zone diameter during antimicrobial activity testing (Table 5). As it could be seen, the effect of pathogens to inhibition zone diameter wasn't statistically significant at the significance level of 95%, both in cases of cultivation broth and supernatant samples.

The fact that the effect of pathogens isn't statistically significant has further been confirmed by the Duncan's multiple range test (Table 6), which was performed in order to establish homogenous groups of pathogens according to their sensitivity towards the tested cultivation broth and supernatant samples of the selected antagonists.

Table 5. One-way ANOVA of inhibition zone diameters obtained as a result of antimicrobial activity testing against different *X. euvesicatoria* strains

Effect	SS	MS	DF	F-value	*p*-value
Intercept	41525.38^{CB} 13046.72^{S}	41525.38^{CB} 13046.72^{S}	1^{CB} 1^{S}	824.65^{CB} 89.53^{S}	**$<0.0001^{CB}$** **$<0.0001^{S}$**
Pathogen	7.41^{CB} 2.82^{S}	3.71^{CB} 1.41^{S}	2^{CB} 2^{S}	0.07^{CB} 0.01^{S}	0.9291^{CB} 0.9904^{S}
Error	3927.70^{CB} 11366.46^{S}	50.36^{CB} 145.72^{S}	78^{CB} 78^{S}	-	-

SS – sum of squares, MS – mean squares, DF –degree of freedom
^{CB} – samples of cultivation broth, ^{S} – samples of supernatant

The results given in Table 6 show that all pathogenic isolates (X1, X2 and X3) were at the same level of statistical significance, both in cases of antimicrobial activity testing using cultivation broth and supernatant samples. These results indicate that there are no statistically significant differences between the tested pathogenic strains, i.e. all of them

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are equally sensitive to antimicrobial action of the tested cultivation broth and supernatant samples of the selected antagonists, meaning that the selected antagonistic strains could be equally successfully applied against all tested phytopathogens.

Table 6. Results of Duncan's multiple range test for inhibition zone diameters (mean values, standard deviations and significance levels) obtained as a result of antimicrobial activity testing against different *X. euvesicatoria* strains

Pathogen	Inhibition zone diameter – samples of cultivation broth (mm)	Inhibition zone diameter – samples of supernatant (mm)
X1	22.28±6.86[a]	12.50±11.77[a]
X3	22.63±6.84[a]	12.63±11.96[a]
X2	23.02±7.57[a]	12.94±12.74[a]

CONCLUSION

The results of this study have revealed significant *in vitro* potential of cultivation broths containing biomass of the antagonists *Lactobacillus* MK3 and *T. reseii* QM 9414, as well as antibacterial compounds produced by the antagonist *P. aeruginosa* I128, to be successfully applied as biocontrol agents against *X. euvesicatoria*, causing pepper bacterial spot, which was also confirmed by the similar sensitivity of the tested pathogenic strains towards the investigated biocontrol agents. Identification and characterization of extracellular antibacterial compounds produced by the antagonist *P. aeruginosa* I128 would make a significant step towards the understanding of the mechanisms involved in biological control of *X. euvesicatoria*. Further research in this field should include optimization of bioprocess parameters, as well as cultivation medium, to produce sufficient amount of highly-efficient biocontrol agents through a cost-effective biotechnological process.

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