Biblid: 1821-4487 (2022) 26; 1; p 15-18
UDK: 621.785
DOI: 10.5937/jpea%v-36660

Original Scientific Paper
Originalni naučni rad

Bacillus velezensis - BIOCONTROL ACTIVITY OF CELLS AND EXTRACELLULAR COMPOUNDS AGAINST *Xanthomonas spp.*

Bacillus velezensis – BIOKONTROLNA AKTIVNOST ĆELIJA I EKSTRACELULARNIH JEDINJENJA PROTIV *Xanthomonas spp.*

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ABSTRACT

Bacillus velezensis is a novel biocontrol species exhibiting several mechanisms in biological control of plant pathogens, including antibiosis, production of other types of antimicrobials, such as volatile organic compounds, direct competition for nutrients and growth space, and induction of plant immunity towards pathogens. The aim of this study was to assess the potential of Bacillus velezensis IP22, cultivated on the optimized medium with glycerol as carbon source, for in vitro suppression of phytopathogenic bacteria - Xanthomonas campestris and Xanthomonas euvesicatoria. Different potential biocontrol agents were investigated: the overall cultivation broth, containing cells of Bacillus velezensis IP22 and produced extracellular metabolites, and cell-free supernatant obtained by centrifugation of the cultivation broth (10000 rpm, 10 min), which contained only the produced extracellular compounds. Furthermore, supernatant samples were subjected to heat treatment to assess whether there were thermosensitive extracellular compounds present in the supernatant. Vacuum evaporation was performed to concentrate supernatant samples in order to compare the effect of higher concentration of extracellular compounds to pathogens' growth. The results have indicated average inhibition zone diameters of 66.00 mm for the cultivation broth samples, 25.67 mm for the supernatants, 10.00 mm for the heat treated supernatants and 43.50 mm for the concentrated supernatants. Further research should include optimization of the production processes aimed at maximization of Bacillus velezensis IP22 growth and/or biosynthesis of the antimicrobial metabolites, as well as their precise identification and characterization to better understand the mechanism(s) of biocontrol activity against phytopathogenic Xanthomonas spp.

Key words*: antimicrobial activity, cultivation broth, supernatant, heat treatment, Xanthomonas campestris, Xanthomonas euvesicatoria.*

REZIME

Bacillus velezensis je nova biokontrolna vrsta koja ispoljava nekoliko mehanizama biološke kontrole biljnih patogena, uključujući antibiozu, proizvodnju drugih vrsta antimikrobnih jedinjenja, kao što su isparljiva organska jedinjenja, direktnu kompeticiju u pogledu hranljivih materija i prostora za rast, kao i indukciju imunog odgovora biljaka prema patogenima. Cilj ovog istraživanja bila je procena potencijala soja Bacillus velezensis IP22, kultivisanog na optimizovanoj podlozi sa glicerolom kao izvorom ugljenika, za in vitro suzbijanje fitopatogenih bakterija - Xanthomonas campestris i Xanthomonas euvesicatoria. Ispitani su različiti potencijalni biokontrolni agensi: kultivaciona tečnost, koji sadrži ćelije Bacillus velezensis IP22 i proizvedene ekstracelularne metabolite, i supernatant oslobođen ćelija dobijen centrifugiranjem kultivacione tečnosti (10000 rpm, 10 min), koji je sadržao samo proizvedena ekstracelularna jedinjenja. Pored toga, uzorci supernatanta su podvrgnuti termičkom tretmanu da bi se utvrdilo da li u supernatantu postoje termosenzitivna ekstracelularna jedinjenja. Vakuum uparavanje je izvedeno sa ciljem koncentrisanja uzoraka supernatanta kako bi se uporedio efekat veće koncentracije ekstracelularnih jedinjenja na rast patogena. Rezultati su pokazali prosečne prečnike zona inhibicije od 66,00 mm za uzorke kultivacione tečnosti, 25,67 mm za uzorke supernatanta, 10,00 mm za termički tretirane uzorke supernatanta i 43,50 mm za uzorke koncentrovanog supernatanta. Dalja istraživanja u ovoj oblasti treba da obuhvate optimizaciju proizvodnih procesa u cilju maksimizacije sadržaja biomase Bacillus velezensis IP22 i/ili biosinteze antimikrobnih metabolita, kao i njihovu preciznu identifikaciju i karakterizaciju radi boljeg razumevanja mehanizama biokontrolne aktivnosti protiv fitopatogena Xanthomonas spp.

Ključne reči*: antimikrobna aktivnost, kultivaciona tečnost, supernatant, termički tretman, Xanthomonas campestris, Xanthomonas euvesicatoria.*

INTRODUCTION

Modern agriculture assumes intensive farming systems aimed at obtaining as high yield as possible with a minimal utilization of the available resources. The inevitable consequence of heavy agrochemicals usage is serious degradation of the soil quality, as well as contamination of the natural ecosystems with residues of chemical pesticides and inorganic fertilizers, including underground water, water reservoirs and air (*Payá Pérez and Rodríguez Eugenio, 2018*). Due to high exposure of the food chain and environment to the agrochemicals' residues, they have also been present in the final food products, while the chronic effects of a long-term exposure to small amounts of pesticides have been showing a numerous serious negative effects to human health (*Jennings and Li, 2020*). The overuse of chemical pesticides to secure the harvest yield and pathogen-free food has resulted in the emergence of super-resistant microbial plant pathogens (*Raman et al., 2020*), which is especially important when it comes to plant pathogenic bacteria, due to very fast vertical transfer of the resistance genes across the generations, as well as the possibility of horizontal transfer of the resistance genes to the other bacterial species (*Chen et al., 2018*). Bacterial phytopathogens, for example, have become almost impossible to manage, hence the shift was made towards the good agricultural practice and healthy and certified seed and planting material as the prevention of bacterial infections occurrence (*Bihn and Reiners, 2018*). Bacteria of the genus *Xanthomonas* are among the most important bacterial plant pathogens in terms of infection severity, yield loss and the economic consequences for vegetable production (*Potnis et al., 2015*). Usual management practices when it comes to *Xanthomonas*-caused infections of vegetables include application of copper-based pesticides, as well as antibiotics, although their application is forbidden in the majority of countries across the globe (*Sundin and Wang, 2018*).

In a world faced with questionable sustainability of food supply in the future, mostly due to unsustainable agricultural practices and agrochemicals overuse, the shift must be made to the more sustainable alternatives when it comes to control of plant diseases. Microbial biocontrol agents in the form of microbial biopesticides appear as one of the alternatives which have already found their place on the global market of the agents used for plant pathogen management (*Köhl et al., 2019*). A very high number of commercially available biopesticides in based on usage of bacteria of the genus *Bacillus* due to their several favorable traits for biopesticide production and formulation, including sporulation ability resulting in higher resistance towards different environmental conditions and better survival rates, which is especially important for the formulation procedure, storage and application, as well as the possibility to produce the wide palette of compounds with antimicrobial activity against the wide range of bacterial and fungal plant pathogens (*Fira et al., 2018*). *Bacillus velezensis* is a relatively novel biocontrol species with remarkable antimicrobial activity against plant and human pathogens (*Ye et al., 2018; Baharudin et al., 2021*). Different strains of this species exhibit high potential for application in biological control of plant pathogens due to strong genetic basis for production of many compounds with antimicrobial activity, including lipopeptides, such as surfactin, iturin, fengycin (*Pajčin et al., 2020; Mácha et al., 2021*), volatile organic compounds and enzymes (*Alenezi et al., 2021*).

The aim of this study was to assess the potential of different biocontrol agents based on *Bacillus velezensis* IP22, including cultivation broth, biomass-free supernatant obtained by centrifugation, thermally treated supernatant and concentrated supernatant with increased concentrations of the produced antimicrobial compounds, for suppression of bacterial plant pathogens causing black rot of cruciferous crops (*Xanthomonas campestris*) and pepper bacterial spot (*Xanthomonas euvesicatoria*).

MATERIAL AND METHOD

Microorganisms

Biocontrol strain applied in this study was *Bacillus velezensis* IP22, isolated from fresh cheese and identified using 16S rRNA gene sequencing (*Pajčin et al., 2020*). This strain is being kept in glycerol solution (20%, v/v) at -20 °C and it was transferred to nutrient agar slant for incubation at 28 °C for 48 h prior to the cultivation. *Xanthomonas campestris* Mn 7-2, the black rot pathogen, was isolated from cabbage plants, while *Xanthomonas euvesicatoria* PL1 as the bacterial spot pathogen was isolated from pepper plants. The both pathogens were kept at YMA (yeast maltose agar) slant (*Grahovac et al., 2021*) and transferred to the same medium for incubation at 26 °C for 48 h prior to antimicrobial activity testing.

Cultivation

Inoculum preparation was performed in Erlenmayer flasks using nutrient broth (Himedia, India) at 28 °C for 48 h with agitation of 150 rpm on the orbital shaker. Cultivation was performed in the bioreactor (Biostat® Aplus, Sartorius, Germany) under the following conditions: working volume 2 L, inoculum volume 200 mL, temperature 28 °C, agitation rate 250 rpm, aeration rate 2 L/(L·min), duration 96 h. The composition of the applied medium based on glycerol was previously optimized (*Pajčin et al., 2020*) and it was sterilized at 121 °C and 2.1 bar for 20 min. The sample from the end of the *Bacillus velezensis* IP22 cultivation was used as a basis for all of the tested biocontrol agents, as explained further in the next section.

Preparation of samples and antimicrobial activity testing

The following samples were tested against *Xanthomonas campestris* Mn 7-2 and *Xanthomonas euvesicatoria* PL1 for their antimicrobial activity: the overall cultivation broth, containing viable cells of *Bacillus velezensis* IP22 and the produced extracellular metabolites, and biomass-free supernatant obtained by centrifugation of the cultivation broth (10000 rpm, 10 min, Rotina 1080R, Hettich, Germany), which contained only the produced extracellular compounds. Furthermore, supernatant samples were subjected to heat treatment (100 °C, 15 min) to assess whether there were thermosensitive extracellular compounds present in the supernatant. Vacuum evaporation at 40 °C was performed to concentrate supernatant samples, while the concentrates were resuspended in distilled water to achieve 25% of the initial sample volume in order to compare the effect of higher concentration of extracellular compounds to pathogens' growth. *In vitro* antimicrobial activity testing was performed using the diffusion disc method on YMA medium in triplicate tests under the following conditions: pathogen suspension concentration 10^8 CFU/mL, inoculation volume ratio 1:15 (suspension:medium), sample volume 15 µL, incubation temperature 26 °C, incubation time 72 h. Statistica software (v. 14.0.0.15, Dell Inc., USA) was applied for all statistical analyses of the obtained experimental results regarding inhibition zone diameters, performed at the significance level of 95%, including Levene's test, One-way ANOVA (analysis of variance) and Duncan's multiple range test.

RESULTS AND DISCUSSION

In order to assess the effects of the different biocontrol agents (cultivation broth, supernatant, thermally treated supernatant and concentrated supernatant) on antimicrobial activity against *Xanthomonas campestris* Mn 7-2 and *Xanthomonas euvesicatoria* PL1, One-way ANOVA was applied. Levene's test has confirmed homogeneity of variances with the p-value of 0.6504. The ANOVA results presented in Table 1 have suggested statistically significant effect at the significance level of 95% of biocontrol agent type/treatment to inhibition zone diameters obtained by antimicrobial activity testing against the *Xanthomonas* spp. phytopathogens, with p-value less than 0.0001.

Table 1. One-way ANOVA of inhibition zone diameters for different biocontrol agents tested against Xanthomonas campestris Mn 7-2 and Xanthomonas euvesicatoria PL1

Effect	SS	MS	DF	F-value	p-value
Intercept	31610.04	31610.04	1	61180.73	<0.0001
Biocontrol agent	10432.13	3477.38	3	6730.40	<0.0001
Error	10.33	0.52	20		
SS – sum of squares, MS – mean squares, DF –degree of freedom					

In order to assess the biocontrol agent with the highest potential for cruciferous black rot and pepper bacterial spot suppression, Duncan's multiple range test was applied to establish homogenous groups of biocontrol agents at the same level of statistical significance when it comes to inhibition zone diameters resulting from the antimicrobial activity testing. As presented in Figure 1, all of the tested biocontrol agents were at different levels of statistical significance. The lowest values of inhibition zone diameters (approximately 10 mm) were obtained in case when thermally treated supernatant was applied as biocontrol agent, suggesting the presence of thermally sensitive antimicrobial compounds as extracellular metabolic products of *Bacillus velezensis* IP22.

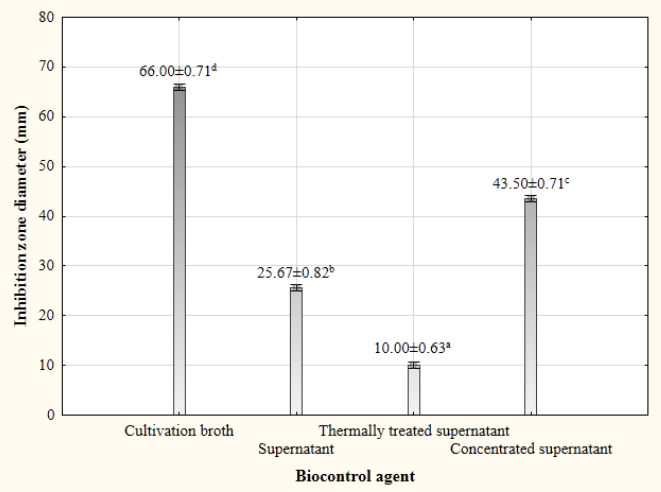

Figure 1. Antimicrobial activity of different biocontrol agents based on Bacillus velezensis IP22 against Xanthomonas campestris Mn 7-2 and Xanthomonas euvesicatoria PL1 – Duncan's multiple range test (different superscript letters designate the different levels of statistical significance)

The value of inhibition zone diameters obtained when the supernatant of the cultivation broth was applied as biocontrol agent was approximately 2.5 fold higher (25.67±0.82 mm) compared to thermally treated supernatant. *Baharudin et al. (2021)* have reported the opposite results for the 5 kDa peptide antimicrobial compound produced by the *Bacillus velezensis* PD9, where the culture supernatant was stable at different temperatures from 40 °C to 80 °C. The same was reported for the aflatoxin-biodegrading compounds from the cell-free supernatant of *Bacillus velezensis* DY3108, which were stable at the temperature range 20-90 °C (*Shu et al., 2018*). *Chen et al. (2020)* have shown solid thermal stability of the antimicrobial compounds present in the cell-free filtrate of *Bacillus velezensis* ZW-10 culture broth even at 121 °C. On the other hand, *Wang et al. (2020)* have shown the rapid decrease of antimicrobial activity of the thermally treated supernatant of *Bacillus velezensis* BM21 cultivation broth when increasing the temperature from 20 °C to 100 °C, with an assumption that antimicrobial compounds are of a lipopeptide origin. In order to assess whether the higher concentration of the antimicrobial compounds produced by *Bacillus velezensis* IP22 would significantly affect the antimicrobial activity against *Xanthomonas campestris* Mn 7-2 and *Xanthomonas euvesicatoria* PL1, the supernatant was concentrated four folds using the vacuum evaporation. Vacuum evaporation was selected as a mild technique in terms of low temperature required for the liquid evaporation and the overall sample volume reduction. The obtained average value of the inhibition zone diameter of 43.50 mm indicated almost doubled antimicrobial activity compared to the supernatant with the lower concentration of the produced extracellular compounds, indicating that other techniques for the supernatant volume decrease could be applied in order to achieve higher extent of biocontrol action, however, thermal sensitivity of the produced antimicrobial compounds must be taken into account when choosing the appropriate downstream technique. However, the highest values of inhibition zone diameters (approximately 66 mm) were achieved when cultivation broth was used as biocontrol agent against the pathogenic *Xanthomonas* spp. Cultivation broth contained biomass, i.e. living cells of *Bacillus velezensis* IP22, besides the produced extracellular antimicrobial compounds, which could compete with pathogens in terms of growth space and nutrients, as well as potentially produce antimicrobial compounds at the interaction site (*Mácha et al., 2021*). Hence, the synergistic effect of biomass and extracellular metabolites of *Bacillus velezensis* IP22 has resulted in the highest biocontrol potential for suppression of black rot and pepper spot causers.

CONCLUSION

The results of this study have indicated a significant potential of different biocontrol agents based on *Bacillus velezensis* IP22 to be used for management of black rot of cruciferous crops and pepper bacterial spot. The highest level of the antimicrobial activity was achieved in case of *Bacillus* cells application, indicating the possibility to formulate the cultivation broth itself as a source of commercial biocontrol agent without the necessity for downstream processing, thus avoiding the overall bioprocess cost increase due to expensive downstream operations. Furthermore, the extracellular metabolites produced by *Bacillus velezensis* IP22 could also be successfully applied as a separate biocontrol agent, with a higher antimicrobial activity achieved when their concentration had been increased. Furthermore, thermal sensitivity of the produced antimicrobial compounds must be taken into account when choosing the method for the supernatant volume reduction. Further research should include precise identification and characterization of the antimicrobial compounds produced by *Bacillus velezensis* IP22, as well as *in planta* testing of the most perspective biocontrol agents to choose the appropriate one for field management of plant bacterial infections caused by pathogenic *Xanthomonas* spp.

ACKNOWLEDGEMENT: This research was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia within the framework of the project 451-03-68/2022-14/200134 (2019-2022).

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Received: 25.02.2022. Accepted:14.03.2022.