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ANTIMICROBIAL ACTIVITY OF ANTARCTIC STREPTOMYCETES AGAINST PEPPER BACTERIAL SPOT CAUSING AGENTS

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Abstract: Streptomycetes are microorganisms that synthesize various biologically active substances, most of which with antimicrobial activity. In this study three *Streptomyces* strains isolated from Antarctic soils were screened for *in vitro* and *in vivo* antibacterial activity towards causative agents of bacterial spot of pepper – *Xanthomonas vesicatoria, Xanthomonas euvesicatoria* and *Xanthomonas gardneri*. Cell-free filtrates of cultural media and mycelium extracts obtained in 120 h of the cultivation of the streptomycetes were used. The *in vitro* antimicrobial activity was determined by the agar diffusion method. All streptomycetes showed antibacterial effect which varied for different test-bacteria and the producer strains. The extracts from the mycelia of the strains were more active. The potential of the streptomycetes for *in vivo* control of phytopathogenes was investigated by treatment of pepper seeds infected with *X. vesicatoria, X. euvesicatoria* and *X. gardneri* using streptomycetes cell-free filtrates and mycelium extracts. The cell-free extracts did not show activity, whereas the extracts manifested high decontamination activity (between 94 and 100%) decreasing slightly seed germination (between 8 and 30%).

INTRODUCTION

Phytopathogenic bacteria are a serious problem in the agricultural practice. They cause diseases in many plant species and each year farmers suffer huge losses worldwide. The bacteria of the genus *Xanthomonas* are typical phytopathogenic

bacteria that attack over 200 species of plants, including peppers and tomatoes traditionally grown in Bulgaria and Macedonia. These bacteria cause bacterial spot disease, which results in the appearance of necrotic spots on leaves, fruit, and leaf stalks, leading to significant yield reductions (Campbell et al., 2006). Traditional methods to control these phytopathogens include different physical approaches that require more time and are not always sufficiently sensitive. Continuous use of chemical agents affects biocenotic relationships in agroecosystems and lead to the development of resistance among bacterial populations of pathogens. This causes an increase in the doses of the drugs, which in turn leads to environmental pollution and problems related to the health of consumers. The attention of many researchers is directed to the development of an alternative means to replace the chemical products. Attention is paid to the use of natural antagonists of pathogens or products of their metabolism. Some actinomycetes have potential as biological control agents against phytopathogens, which is best expressed by representatives of the genus *Streptomyces* (Manian and Sowndhararajan, 2010; Dhanasekaran et al., 2012).

Several investigators have described *in vitro* and *in vivo* antimicrobial activity of many actinomycete species (Moncheva et al., 2002; Oskay et al., 2004; Nedialkova and Naidenova, 2005; Badzhinerov et al., 2005, 2008, 2009). Their mode of action involves a parasitism on fungal hyphae (El-Tarabily and Sivasithamparam, 2006), sclerotia or oospori (Crawford et al., 1993), competition with pathogens (Kunoh, 2002), the synthesis of antibiotics (Igarashi, 2004), a siderophore (Khamna et al., 2009) and enzymes such as cellulases, hemicellulases, chitinases, glucanases, and amylases (Yuan and Crawford, 1995). *S. hygroscopicus* 155 synthesized complex of antibiotics, which inhibit the growth of tomato bacterial disease agents (Bogatzevska et al., 1989). *Streptomyces lydicus* WYEC108 inhibit *Pythium ultimum* and *Rhizoctonia solani in vitro* by the production of antifungal metabolites (Yuan and Crowford, 1995).

The aim of this study was in vitro and in vivo screening of streptomycete strains isolated from Antarctic soils against the causative agents of pepper bacterial spot – *Xanthomonas vesicatoria, Xanthomonas euvesicatoria* and *Xanthomonas gardneri*.

MATERIALS AND METHODS

Microorganisms

Three *Streptomyces* strains – *Streptomyces* sp. 2M, *Streptomyces* sp. 30-1 and *Streptomyces* sp. 54N isolated from Antarctic soils (Badzhinerov et al., 2009; Encheva et al., 2013) were screened in this study for production of antibacterial substances. The following phytopathogenic bacterial species were used as test-microorganisms: *Xanthomonas vesicatoria* 8b (isolate from Bulgaria, PT pathotype) and 44M (isolate from Macedonia, P pathotype), *Xanthomonas*

euvesicatoria 41b (isolate from Bulgaria, P pathotype) and 37M (isolate from Macedonia, PT pathotype) and *Xanthomonas gardneri* 62t (isolate from Bulgaria, PT pathotype) (Kizheva et al., 2011, 2013).

Cultivation conditions

The streptomycetes were maintained on Gauze Mineral I medium and the phytopathogenic bacteria on potato dextrose agar (PDA). For determination of the antibacterial activity the streptomycetes were cultivated in the following liquid medium (g/l): mannitol, 10; corn meal, 8; soy groats, 12; distilled water, up to 1 l) on orbital shaker at 240 rpm, 28°C for 120 hours. The cell-free supernatants were obtained by centrifugation of the culture liquids at 5000 rpm for 10 min and then filtered through membrane filter (0.22 μm). The extracts were prepared by stirring of the streptomycete mycelia with methanol for 30 min followed by centrifugation (5000 rpm, 10 min).

***In vitro* antibacterial activity**

The *in vitro* antibacterial activity of streptomycetes was determined by well-diffusion method using cell-free supernatants and methanol extracts on PDA previously inoculated by the cell suspensions of the test-bacteria (0.5 McFarland). The antimicrobial activity was measured by the diameters of the inhibition zones formed around the wells after an incubation period of 24 - 48 hours at 28°C. The methanol was used as a control.

***In vivo* antibacterial activity**

The *in vivo* antibacterial activity was tested using pepper seeds ("Dzhulinska Shipka" with 90% of germination) previously inoculated by vacuum infiltration (Bogatzevska, 2002) with the phytopathogenic bacteria (10^8 cfu/ml). The experiment was performed in the following variants: seeds inoculated independently with the five strains of the used phytopathogenic bacteria and treated with the cell-free supernatants and the extracts of streptomycetes. Two types controls were used: seeds uncontaminated with the phytopathogens and treated by the supernatants and extracts, and contaminated and non-treated seeds. For every variant 100 seeds were used. One series of the seeds was placed on PDA medium in Petri dishes and incubated during 5-6 days at 28°C after that the average percentage (rate) of healthy seeds was calculated. The second series of seeds was put on moist filter paper, incubated for 5-6 days at room temperature and the rate of germination was determined. All the analyses were performed in triplicates.

RESULTS AND DISCUSSION

Three Streptomyces strains isolated from Antarctic soils were screened for *in vitro* antibacterial activity towards three phytopathogenic bacterial species - the causative agent of pepper bacterial spot and isolated from pepper plantations in Bulgaria and Macedonia – *X. vesicatoria, X. gardneri* and *X. euvesicatoria*. For

the screening cell-free filtrates from the culture media and methanol extracts from the mycelia were used. The filtrates and the extracts were obtained from 48 to 120 h of the cultivation of the streptomycetes.

It was shown that all the strains synthesized antibacterial substances at the beginning of the cultivation period, showing a different activity depending on the test bacteria (Tabl. 1). The highest activity was observed at the 120 hour of the cultivation of the streptomycetes. The extracts from the mycelia of all the strains were more active than the cell-free supernatants (Tabl. 1).

X. euvesicatoria was the most sensitive species to the antibacterial substances synthesized by the streptomycetes (Tabl. 1), whereas *X. gardneri* was the least affected one. The activity of the substances produced by *Streptomyces* sp. 54N and *Streptomyces* sp. 2M was higher than those from *Streptomyces* sp. 30-1. The differences in the antibacterial activity among the strains might be due to the nature of the produced substances. In a previous work we screened the potential of several actinomycete strains isolated from Antarctic soils to produce antibacterial substances active against Gram-positive and Gram-negative bacteria including phytopathogens (Moncheva et al., 2002; Badzhinerov et al., 2005, 2008, 2009). Naidenova and Nedialkova (2005) and Mavengere (2008) also reported the possibility of several Antarctic actinomycetes to synthesize antibacterial substances. The present study confirmed the broad potential of these microorganisms to produce bioactive compounds with activity towards phytopathogenic bacteria, especially those causing the bacterial spot disease of pepper.

Table 1. *In vitro* antibacterial activity of streptomycetes.

Test-bacteria	Streptomycete strain	Cell-free filtrates/ inhibition zone, mm				Extracts/ inhibition zone, mm			
		48 h	72 h	96 h	120 h	48 h	72 h	96 h	120 h
X. gardneri 62t	*Streptomyces* sp. 54N	-	18	20	22	14	17	17	24
	Streptomyces sp. 2M	12	13	15	17	11	14	16	18
	Streptomyces sp. 30-1	-	11	12	13	-	-	17	18
X. vesicatoria 8b	*Streptomyces* sp. 54N	18	20	23	26	25	30	32	33
	Streptomyces sp. 2M	23	24	27	29	23	30	33	37
	Streptomyces sp. 30-1	-	16	18	20	-	20	25	27
X. euvesicatoria 41b	*Streptomyces* sp. 54N	23	26	28	31	21	24	25	33
	Streptomyces sp. 2M	20	22	24	25	19	22	25	27
	Streptomyces sp. 30-1	-	20	23	24	-	19	21	24

In vivo experiments showed that the supernatants had no antibacterial effect (data not shown) towards the phytopathogens regardless of the *in vitro* tests where the supernatants showed such effect, whereas the extracts significantly

inhibited the bacteria and confirmed the efficacy determined for them in the *in vitro* experiments (Tabl. 2). It should be noted, however, that the extracts of the strains 30-1 and 2M caused a slight decrease of the germination. The percentage of the germinated contaminated and non-treated seeds varied between 83 and 90 (Tabl. 3). The different extracts possessed approximately the same antibacterial effect. This study confirmed the antibacterial activity of strain 54N established in a previous work of ours (Badzhinerov et al., 2009) and reported additional information about the spectrum of its activity.

The treatment of seeds should be seen as a valuable step in crop protection and the selection of specific antibacterial substances obtained from streptomycetes could be the key in limitation of the disease. There are insufficient data published for the effect of natural antibacterial substances against the phytopathogenic strains used by us and this study is giving initial information about the streptomycetes potential to help to obtain a sustainable reduction of harmful pathogens of pepper.

Table 2. *In vivo* antibacterial effect of the Antarctic streptomycetes.

No	Variant	Healthy seeds, % / Streptomyces strain			Germination, % / Streptomyces strain		
		30-1	2M	54N	30-1	2M	54N
1	Control (uninoculated with the pathogens seeds and treated with the extracts)	100	100	100	70	74	89
2	Seeds inoculated with *X. gardneri* and treated	95	96	96	70	70	79
3	Seeds inoculated with *X. euvesicatoria* 41b and treated	95	98	99	71	73	90
4	Seeds inoculated with *X. euvesicatoria* 37M and treated	100	100	100	72	76	89
5	Seeds inoculated with *X. vesicatoria* 8b and treated	94	96	98	72	74	88
6	Seeds inoculated with *X. vesicatoria* 44M and treated	100	100	98	71	72	89

Table 3. The germination of the inoculated and non-treated seeds.

Variant	Germination, %
Seeds inoculated with *X. gardneri*	83
Seeds inoculated with *X. euvesicatoria* 41b	84
Seeds inoculated with *X. euvesicatoria* 37M	84
Seeds inoculated with *X. vesicatoria* 8b	89
Seeds inoculated with *X. vesicatoria* 44M	90

In conclusion, the present study showed that the used streptomycetes possessed antibacterial effect against the causative agent of bacterial spot in pepper and had a good potential to be used as an alternative method for decontamination of pepper seeds.

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REFERENCES

1. Badzhinerov, N., Bogatzevska, N., Moncheva, P. 2005. Compounds from Streptomycetes with activity against plant pathogenic bacteria and their application in biological control. *Ecology and Future*, 7 (1): 26-31.
2. Badzhinerov, N., Atanasov, V., Dorkov P., Moncheva, P. 2008. Isolation and partially characterization of antimicrobial substance produced by an antarctic streptomycete strain. *Ecological Engineering and Environment Protection*, 2: 24-32.
3. Badzhinerov, N., Tishkov, S., Stoyanova, M., Bogatzevska, N., Moncheva, P. 2009. Activity of antarctic streptomycetes strains against phytopathogenic bacteria. *Ecologica*, 16: 307-311.
4. Bogatzevska, N. S. 2002. Phytopathogenic bacteria of the genus Pseudomonas goup syringae and genus Xanthomonas group vesicatoria and axonopodis – growth phases. [dissertation], Kostinbrod: Plant Protection Institute of the Agricultural Academy.
5. Bogatzevska, N. S., Tishkov, S.H., Moncheva, P. A. 1989. An antibacterial antibiotic preparation for the decontamination of the seeds of tomatoes from the agents of bacterioses. *Proc. of 7th International Conference of Plant Pathogenic Bacteria*, 237-242, Budapest, Hungary.
6. Crawford, D.L., Lynch, J.M, Whipps, J.M, Ousley, M.A. 1993. Isolation and characterization of actinomycete antagonists of a fungal root pathogen. *Appllied and Environmental Microbiology*, 59: 3899-3905.
7. Dhanasekaran, D., Thajuddin N., and Panneerselvam, A. 2012. Applications of Actinobacterial Fungicides in Agriculture and Medicine. In: D. Dhanasekaran, D., Thajuddin, N., and Panneerselvam, A. (Eds.). Fungicids for Plant and Animal Diseases, InTech, pp. 29-54.
8. El-Tarabily, K. A., Sivasithamparam, K. 2006. Potential of yeasts as biocontrol agents of soil-borne fungal plant pathogens and as plant growth promoters. *Mycoscience*, 47: 25-35.
9. Encheva, M., Zaharieva, N., Kenarova, A., Chipev, N., Chipeva, V., Hristova, P., Ivanova, I., Moncheva, P. 2013. Abundance and activity of soil actinomycetes from Livingston Island, Antarctica. *Bulgarian Journal of Agricultural Science*, 19 (2): 68-71.
10. Igarashi, Y. 2004. Screening of novel bioactive compounds from plant-associated actinobacteria. *Actinomycetologia*, 18: 63-66.
11. Ji, P., Campell, H. L., Kloepper J. W., Jones, J. B., Suslow, T. V., Wilson, M. 2006. Integrated biological control of bacterial speck and spot of tomato under field conditions using foliar biological control agents and plant growth-promoting rhizobacteria, *Biological control*, 36: 358-367.
12. Khamna, S., Yokota, A., Lumyong, S. 2009. Actinobacteria isolated from medicinal plant rhizosphere soils: diversity and screening of antifungal compounds, indole-3-acetic acid and siderophore production. *World Journal of Microbiology and Biotecnology*, 25: 649-655.
13. Kizheva, Y., Vancheva, T., Hristova, P., Stoyanova, M., Bogatzevska, N., Moncheva, P. 2011. Diversity of *Xanthomonas* Spp. causal agents of bacterial spot on pepper and

tomato plants in Bulgaria. *Biotechnology and Biotechnological Equipment*, 25 (4) suppl.: 98-104.
14. Kizheva, Y., Vancheva, T., Hristova, P., Stoyanova, M., Stojanovska, M., Moncheva, P. and Bogatzevska, N. 2013. Identification of Xanthomonas strains from tomato and pepper and their sensitivity to antibiotics and copper. *Bulgarian Journal of Agricultural Science*, 19 (2): 80–82.
15. Kunoh, H. 2002. Endophytic actinobacteria: attractive biocontrol agents. *Journal of General and Plant Pathology*, 68: 249-252.
16. Manian S., Sowndhararajan K. 2010. Microbial biopesticides. In: D. K. Maheshwari, R. C. Dubey, R. Saravanamurthu (Eds.). Industrial Exploatation of Microorrganism, I. K. International Pub. House, New Delhi, pp. 51-69.
17. Mavengere NR. Isolation, identification and characterization of novel actinomycetes from Antarctic soil samples [dissertation]. Cape Town: University of the Western Cape Academic.
18. Moncheva, P., Tishkov, S., Dimitrova, N., Chipeva, V., Antonova-Nikolova, S., Bogatzevska, N. 2002. Characteristics of soil Actinomycetes from Antarctica. *Journal of Culture Collections*, 3: 3-14.
19. Nedialkova, D., Naidenova, M. (2004-2005). Screening the antimicrobial activity of Actinomycetes strains isolated from Antarctica. *Journal of Culture Collections*, 4 (1): 29-35.
20. Oskay, M., Üzame, A. T., Azeri, C. 2004. Antibacterial activity of some actinomycetes isolated from farming soils in Turkey. *African Journal of Biotechnology*, 3(9): 441-446.
21. Yuan, W.M., and Crawford, D.L. 1995. Characterization of streptomyces lydicus WYEC108 as a potential biocontrol agent against fungal root and seed rots. *Appllied and Environmental Microbiology* 61: 3119-3128.