Agraarteadus
2 • XXXII • 2021 303–306

Journal of Agricultural Science
2 • XXXII • 2021 303–306

CASE STUDY: INFLUENCE OF PROBIOTICS-BASED PRODUCTS ON PHYTOPATHOGENIC BACTERIA AND FUNGI IN AGROCENOSIS

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Saabunud: Received: 02.11.2021

Aktsepteeritud: Accepted: 22.12.2021

Avaldatud veebis: Published online: 23.12.2021

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Keywords: probiotics, agrophytocenosis, phytopathogenic bacteria, *Bacillus subtillis*.

DOI: 10.15159/jas.21.41

ABSTRACT. In recent years bacterial diseases of agricultural plants have reached a new level of epiphytoty and they appear as an essential factor that influences crop yield. It is important to use products, which provide the eradication of pathogenic agents and the prevention of their development and propagation. The objective of our study is to define the effect of probiotics, based on *Bacillus subtillis,* on bactericidal and fungicidal activities, and to develop the recommendations for probiotics application in a biological system of plant protection. The results show that studied solutions Sviteco-PPW, Sviteco-OPL and Sviteco-Agrobiotic-01 affect phytopathogenic bacteria. Sviteco-Agrobiotic-01 either is the most active product, in its native state or diluted. It has demonstrated high antagonizing activity against all studied phytopathogenic bacteria. Hence, Sviteco-Agrobiotic-01 has the best potential to be used in the biological protection system of grain crops, grain legumes, vegetables from the most dangerous and widely spread pathogenic bacteria. Research results don't show a significant effect of studied probiotics on phytopathogenic fungicidal activity.

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Introduction

Pathogenic organisms, invaders or weeds are the main factors that do not allow to obtain high crop yield. Ecological conditions of ecosystem development are changing significantly and that is why scientists are more likely to consider either plant resistance support or to take advantage of the natural potential of agroecosystems. On the other hand, the harmful impact of plant protection products on the agroecosystem and, more precisely on humans, stimulate the industry to use biological or microbiological methods of plant protection against pathogens. These approaches are ecologically safe for the ecosystems and human beings (Patyka *et al.* 2014a, Patyka *et al.* 2014b).

Microbiological techniques of plant protection against diseases and pathogens have an important role in ecologically sustainable agriculture. The demand for such drugs is growing in recent years. Biological methods of protection permit significantly reduce the costs of the production technology. Moreover, combined methods of protection prevent the uprise of resistances that are only observed upon the usage of chemical pesticides.

There is a sustainable market of different products based on probiotic cultures, which indicates the benefit of them. The scientific research proves the mechanisms of their action. The capacity of spore-forming bacteria to perform probiotic activity has led to the development of probiotic-based products, which are considered to be a new generation of antagonists (Green *et al.*, 1999). The unique feature of this bacterial group is the high G+C ratio (from 32% to 69%) (Nakano *et al.*, 1998).

Bacillus is a genus with about 77 species that create a large group of Gram-positive chemoorganotrophic rod-shaped aerobes or facultative anaerobes forming endospores (List of Prokaryotic names with Standing in Nomenclature). The representatives of this genus have a high and diverse spectrum of biological activity. Very often it is also characterized by antagonizing activity against pathogens.

The crucial features of probiotic strains are a high antagonism to pathogens and opportunistic microorganisms, the resistance to low pH of the environment, low adhesive activity, the absence of genes of antibiotic resistance and virulence.

As for today, we know about microbiological manure based on nitrogen-assimilating and phosphate-mobilizing bacteria. Many strains used in these fertilizers have an antagonizing effect on phytopathogenic flora. Therefore, a new and perspective way of disease prevention and crop yield increase is the application of probiotics.

The research aimed to determine the influence of microbiology products, based on *Bacillus subtilis* on bactericidal and fungicidal activity and their further usage in the biological system of agrophytocenosis protection.

Materials and Methods

Our experiment envisaged the study of three probiotic-based products: Sviteco-PPW, Sviteco-OPL, Sviteco-Agrobiotic-01 (Chrisal NV, Lommel, Belgium), based on *Bacillus subtillis*. Was made the determination of their antibacterial and fungicidal activity on phytopathogenic bacteria.

The bacterial sensibility was determined using a serial dilution method on a solid medium – potato dextrose agar. The product has been applied using a disc diffusion method (instead of a disc with an antibiotic we have used a drop of a probiotic drug). Petri dish with potato dextrose agar (PA) was inoculated with 0.1 ml of a bacterial suspension of studied bacteria (the concentration of suspension was 1×109 CFU ml^{-1}) and then we used a spreader to homogenize the pathogen culture. Afterwards have added 0.1 ml of a tested drug in different concentrations. After 24–48 hours in an incubator at 28 °C, we have made the accounting of inhibition zones. Every experiment has been repeated three times. The absence of inhibition zones indicated the resistance of pathogens to this concentration of a product. Zones with a diameter higher than 15 mm signify the low sensitivity to a product. Inhibition zones with a diameter of 15–25 mm are characteristic of sensible bacteria. Highly sensible bacteria show the inhibition zones with a diameter of more than 25 mm (Dankevych *et al.,* 2014). Bactericidal action of solutions was determined using test cultures: *Pseudomonas syringae* – UKM B-1027^{7} (IMB 8511); *Pseudomonas fluorescens, Pectobacterium carotovorum* – UKM B-1095^{T} (IMB 8982); *Xanthomonas campestris pv. campestris* – UKM B-1049 (IMB 8003); *Clavibacter michiganensis* subsp. *michiganensis* 10_2; *Agrobacterium tumefaciens* (*Rhizobium vitis*) 8628.

The fungicidal activity was determined using a wort agar (Merck, Germany). Phytopathogenic strains of *Fusarium oxysporum* and *Alternaria* sp. were used to determine the fungicidal action of the products. For this experiment, we have used products in a 100% concentration and diluted state (1:10, 1:100, 1:1000 and 1:10000). The conclusion about the biocide activity was made from inhibition zones on the third and fifth day of incubation.

Results

Identification of influence of probiotics on bactericidal activity

Obtained results indicate, that Sviteco-Agrobiotic-01 is the most effective among the three tested products. Both in native state and diluted it has shown high antibacterial activity against all tested phytopathogenic bacteria (Table 1).

Table 1. Effect of probiotics on phytopathogenic bacteria (diameter of the inhibition zones, mm)

Phytopathogenic bacteria	Native state	Concentration					
		$1:10^{-1}$	$1:10^{-2}$	$1:10^{-3}$	$1:10^{-4}$	$1:10^{-5}$	$1:10^{-6}$
			Sviteco-PPW				
P. syringae	20	15	0	0	0	0	0
P. fluorescens	0	0	0	0	0	0	0
P. carotovorum	0	0	0	0	0	0	0
X. campestris pv. campestris	Full BA	40	28	0	0	0	0
C. michiganensis	Full BA	35	22	5	0	0	0
A. tumefaciens	0	0	0	0	0	0	0
			Sviteco-OPL				
P. syringae	15	13	10	BsA-18	BsA-9	0	0
P. fluorescens	0	0	0	0	0	0	0
P. carotovorum	30	20	0	0	0	0	0
X. campestris pv. campestris	50	40	15	13	0	0	0
C. michiganensis	50	35	24	15	0	0	0
A. tumefaciens	20	13	0	0	0	0	0
			Sviteco-Agrobiotic-01				
P. syringae	50	30	25	25	10	weak BsA	0
P. fluorescens	30						
P. carotovorum	50	25	22	27	0	0	0
X. campestris pv. campestris	40	35	30	15	10	0	0
C. michiganensis	60	30	18	15	13	0	0
A. tumefaciens	50	35	15	5	0	0	0

BA – bactericidal action, BsA – bacteriostatic action

It was found, that bacterial species *Pseudomonas syringae*, *Xanthomonas campestris* and *Clavibacter michiganensis* is sensible even to a solution diluted 1:10000. Sviteco-Agrobiotic-01 diluted in 1000 times has shown high antibacterial activity against *Pseudomonas syringae* and *Pseudomonas fluorescens*; moderate antibacterial activity on *Xanthomonas campestris* and *Clavibacter michiganensis*; weak activity against *Agrobacterium tumefaciens*. To limit the spread of phytopathogenic bacteria *Pseudomonas syringae* and *Pectobacterium carotovorum* Sviteco-Agrobiotic-01, diluted 1:1000, can be used.

Sviteco-PPW and Sviteco-OPL have shown selective antibacterial activity against certain pathogens. Hence, Sviteco-PPW has antibacterial activity on *Pseudomonas syringae* only in a native (non-diluted) state and in a 1:10 dilution. *Xanthomonas campestris* and *Clavibacter michiganensis* were not sensible to a product neither in the native state nor in 1:10 or 1:100 dilutions. There was no toxic action observed against *Pseudomonas fluorescens* or *Agrobacterium tumefaciens*.

Sviteco-OPL has a selective activity to phytopathogenic bacteria mostly in a native state. The exception is the antibacterial activity on *Xanthomonas campestris* and *Clavibacter michiganensis*, which has been revealed even using a 1:1000 dilution.

Thus, 1:100 or 1:1000 dilutions of Sviteco-Agrobiotic-01 can be used to develop biological methods of plant protection against all tested phytopathogenic bacteria. Sviteco-OPL can be employed to protect plants only against *Xanthomonas campestris* and *Clavibacter michiganensis*.

Identification of influence of probiotics on fungicidal activity

The results of our study of fungicidal activity prove that probiotics have no toxic action against all tested phytopathogenic fungi (Table 2).

Table 2. Effect of probiotics on phytopathogenic bacteria (diameter of the inhibition zones, mm)

Micromycete	Native state	Concentration			
		$1:10^{-1}$	$1:10^{-2}$	$1:10^{-3}$	$1:10^{-4}$
Sviteco-PPW					
Fusarium oxysporum	0	0	0	0	0
Alternaria sp.	0	0	0	0	0
Sviteco-OPL					
Fusarium oxysporum	15	0	0	0	0
Alternaria sp.	15	0	0	0	0
Sviteco-Agrobiotic-01					
Fusarium oxysporum	10	0	0	0	0
Alternaria sp.	10	0	0	0	0

During the examination of Sviteco-PPW all the dilution and in the native state has no zones of growth inhibition. Products Sviteco-OPL and Sviteco-Agrobiotic-01 show fungicidal activity only in a native state. However, the diameter of inhibition zones was not bigger than 15 mm, which indicates the pathogen resistance to this probiotics. Hence, products, based on probiotics, do not affect to the development of *Fusarium oxysporum* and *Alternaria* sp.

Discussion

Scientific research (Vandenberghe *et al.*, 2017) showed probiotic microorganisms, as bioprotectants, bio-controllers, biofertilizers, or biostimulants, are beneficial microorganisms that offer a promising alternative and reduce health and environmental problems. These microorganisms are involved in either a symbiotic or free-living association with plants and act in different ways, sometimes with specific functions, to achieve satisfactory plant development. The use of probiotics as an alternative soil fertilization source in agriculture improves nutrient supply and conserves field management and causes no adverse effects.

Another research (Barriuso *et al.*, 2008; Zhang *et al.*, 2008) confessed salt tolerance in plants, a decrease in the disease index of 61.2%, and a reduction in mortality due to salt stress of 72.4% after application *Bacillus* sp. strain. Additionally, significant differences were found in the growth of plants and photosynthesis. Showed an increase in dry biomass, total soluble sugars and proline content in wheat crops (Upadhyay *et al.*, 2012).

The study of the potential of *Bacillus* sp. as a probiotic was made by Khadieva *et al.* (2018). The strains were resistant to a wide range of the ambient pH, characterized by antagonistic properties against phytopathogenic micromycetes, as well as against pathogenic and opportunistic enterobacteria. Therefore, were concluded to be promising strains for use as probiotics. *Bacillus subtilis* strain showed strong ability against many common plant fungal pathogens in vitro (Gong *et al.*, 2006).

The research results of Avdeeva *et al.* (2015) has defined high antagonizing activity of *B. Subtilis* against *Shigella flexnery, Proteus vulgaris, P. Vulgaris, Staphylococcus aureus* as well as a moderate level of antagonism on *Salmonella thyphimurium*, *Ecsherichia coli* and a low level of antagonism against *S. enteri3a, S. Derby, Pseudomonas aeruginosa*. Hrabova *et al.* (2015) have found out the antagonizing effect of 100 strains of genus *Bacillus* against ancient and actual strains of phytopathogenic bacteria and fungi. It was identified that the level of antagonizing activity against phytopathogenic bacteria has varied depending on the genus of pathogens. The average level of antagonism against fungi has been revealed among 30% of bacilli. The strain *Bacillus sp.* has been isolated. It has a high and moderate level of antagonism against bacterial and fungal phytopathogens. Hence, this strain is considered to have a lot of potential for biological drug development for plant growth.

High antimicrobial activity of *B. Subtilis* has been studied by Cutting (2011); Karlsson *et al.* (2017); Khadieva *et al.* (2018). They have characterized the perspective of these cultures to be used as probiotics. The resistance to a wide range of pH has been confirmed along with the identification of antagonizing effect on phytopathogenic micromicelles.

Thus, studies of scientists confirm our research of antagonizing effect of probiotic cultures. So, the application of these probiotic-based drugs to control phytopathogens is a perspective and innovative approach.

Prospects for future research is field investigation of probiotic products depending on the type of crops and method of application.

Conclusion

Research results show that studied probiotics affect phytopathogenic bacteria. Sviteco-Agrobiotic-01 has a high antibacterial activity on bacteria species *Pseudomonas syringae, Pseudomonas fluorescens, Pectobacterium carotovorum* subsp. *carotovorum* in a 1:1000 dilution; or on *Xanthomonas campestris* pv. *campestris and Clavibacter michiganensis subsp. michiganensis* in a 1:100 dilution. Sviteco-PPW and Sviteco-OPL have a selective antibacterial activity in a 1:100 dilution.

Studied products Sviteco-PPW, Sviteco-OPL and Sviteco-Agrobiotic-01 haven't fungicidal activity against fungal test cultures: *Fusarium oxysporum* and *Alternaria* sp.

Therefore, among studied products, Sviteco-Agrobiotic-01 has the best potential to be used in the biological protection system of grain crops, grain legumes, vegetables from the most dangerous and widely spread pathogenic bacteria.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Author contributions

PP, MSa – editing and approving the final manuscript.
AT – the corresponding author, writing of the manuscript.
YT, MSe – design/sampling/analysis.
All authors read and approved the final manuscript.

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