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ORIGINAL PAPER

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EFFICACY OF BIOCHEMICAL PREPARATIONS AND EXTRACT FROM *Hypericum perforatum* AGAINST BACTERIAL DISEASES

Małgorzata Schollenberger, Sylwia Pudło, Elżbieta Paduch-Cichal✉, Ewa Mirzwa-Mróz

Department of Plant Pathology, Faculty of Horticulture, Biotechnology and Landscape Architecture, Warsaw University of Life Sciences, WULS-SGGW, Poland

ABSTRACT

The biotechnical preparations: Biosept Active (based on a grapefruit extract) and BioZell (based on thyme oil) as well as *Hypericum perforatum* extract, streptomycin solution and fungicide Champion 50WP (active ingredient substance – e.i. 50% copper hydroxide) were investigated for antimicrobial effects against plant pathogenic bacteria: *Agrobacterium tumefaciens*, *Pseudomonas syringae* pv. *syringae* and *Xanthomonas arboricola* pv. *corylina*. The screening was carried out *in vitro* on three media: Nutrient Agar (NA Difco), Pseudomonas Agar F (Merck) – analogue of King B and 523. In the experiments, the agar plate method was applied. There were no statistically significant differences in the effect of streptomycin and Champion 50WP on the growth inhibition of three bacteria strains for medium 523 and Nutrient Agar and of *P. syringae* pv. *syringae* and *X. arboricola* pv. *corylina* for medium King B. It was determined that the antibacterial activity of Biosept Active and BioZell biopreparations and *H. perforatum* extract against *Agrobacterium tumefaciens* (strain C58), *Pseudomonas syringae* pv. *syringae* (strain 760) and *Xanthomonas arboricola* pv. *corylina* (strain RIPF-x13) were dependent on the strain of pathogen as well as the growth medium used. According to the research results obtained, the Biosept Active preparation and *H. perforatum* extract demonstrated high bacteriostatic activity against three bacterial strains grown on the Nutrient Agar medium.

Key words: anti-microbial activity, biotechnical preparation, plant extract, *Hypericum perforatum*, pathogenic bacteria

INTRODUCTION

Integrated plant protection (IPP) is a means of plant protection against pathogens, which involves the application of all methods of plant protection available, in particular non-chemical methods, in a manner that minimizes the risk to human and animal health as well as to the environment.

Since January 1, 2014, the Republic of Poland and other European Union Member States have been obliged to apply the system of integrated protection in their plant production according to the Regulation (EC) No 1107/2009 of the European Commission and of the Council of October 21, 2009 concerning the placing of plant protection products on the market and repealing the Council Regulations 79/117/EEC and 91/414/EEC (Dz.U. No 309, of 24 November 2009, pp. 1–50) – Art. 55 and Regulation (EC) No, 2009/128/EC of 21 October 2009 establishing a framework for Community action to achieve a sustainable use of pesticides (Dz.U. No 309, of 24 November 2009, pp. 71–86) Art. 14 and Annex III.

✉ elzbieta_paduch_cichal@sggw.pl

General principles of integrated plant protection include the eradication of harmful organisms using primarily biological, physical and agrotechnical methods, whereas the use of chemical plant protection should be limited to a strict minimum, in particular by reducing the fungicide dose and limiting the number of operations performed. When selecting the plant protection products, it is necessary to select the fungicides causing the least side effects on the human and animal health as well as the environment and apply the products in a manner that minimizes the risk of resistance in pathogens. The general principles of integrated plant protection allow, on one hand, to reduce the use of chemical plant protection products to a strict minimum and, on the other, to reduce the pressure on the natural environment and protect the biodiversity of the agricultural environment [Jemiołkowska et al. 2017].

Due to the need to protect the natural environment as well as growing demand for preparations of biological origin, the search for new and safe plant protection products have become necessary as well as popular [Panasiewicz et al. 2007]. At the end of the 20th century, an intensive research has been initiated into producing pesticides containing a biological active substance [Carvalho 2006]. The biopreparations being currently in use contain active substances belonging to three groups, i.e. microorganisms, biochemicals and semiochemicals. The American Environmental Protection Agency identifies a fourth group of these products, i.e. transgenes [Chandler et al. 2011]. Jamiołkowska and Hetman [2016] have used the expression of biotechnical preparations to describe products of antibacterial and antifungal activities. These preparations are produced based on, among others, extracts from grapefruit, chitosane and garlic. The natural plant protection products can be applied in a conventional, integrated and ecological growing system. The role of biopesticides is greater in organic farming [Piekutowska 2017].

The aim of this study was to evaluate the antimicrobial activity of selected biotechnical preparations and *Hypericum perforatum* extract against the following bacteria: *Agrobacterium tumefaciens* – bacterial crown gall, *Pseudomonas syringae* pv. *syringae* – bacterial canker or blast of stone and pome fruits and *Xanthomonas arboricola* pv. *corylina* – bacterial blight of hazelnut.

MATERIALS AND METHODS

In the research, three strains of bacteria were used: *Agrobacterium tumefaciens* (strain C58), *Pseudomonas syringae* pv. *syringae* (strain 760) and *Xanthomonas arboricola* pv. *corylina* (strain RIPF-x13). They were obtained from the Collection of the Bacteriological Laboratory of the Research Institute of Horticulture in Skierniewice. Three bacteria were seeded on three media: Nutrient Agar (NA Difco), Pseudomonas Agar F (Merck) – analogue of King B and 523 [Kado 1979].

The agar plate method was used to determine the antimicrobial activity of the following preparations: Biosept Active (Cintamani Poland Majewscy i Koć Sp.J.) – based on a grapefruit extract, BioZell (www.biozell-2000b.com) – based on a thyme oil, Hypericum perforatum extract – obtained from the Bacteriological Laboratory of the Research Institute of Horticulture in Skierniewice, a streptomycin solution (200 ppm) and fungicide Champion 50WP (active ingredient – 50% copper hydroxide).

Three sterile Petri dishes (Ø 10 cm) were filled with one of the media mentioned above. Each dish was spread with a suspension of the bacteria tested (100 µl of 24-hour-old cultures of concentration 107 CFU/ml). A cork borer was used to cut four wells (Ø 7 mm) in the medium. Each two wells were filled with 200 µl of the preparation (Biosept Active, BioZell) or the *Hypericum perforatum* extract. The remaining two wells were filled with streptomycin or fungicide Champion 50WP. The plates were incubated at room temperature for 24 h. The diameter of the inhibition zones was measured (in millimeters). The measurements were performed for three strains of bacteria (*A. tumefaciens*, *P. syringae* pv. *syringae* and *X. arboricola* pv. *corylina*), three media and two biotechnical preparations (Biosept Active, BioZell), streptomycin and fungicide Champion 50WP in three repetitions for each combination.

Statistical analysis was performed by means of one-way analysis for variance (ANOVA) for three media, on which the inhibition of bacterial growth was dependent on the bacterium strain and the preparation/extract used as well as for three bacterial strains, the growth of which was inhibited depending on the medium and the preparation/extract used.

The average growth inhibition rates were divided into homogeneous group using Tukey test for the interaction of preparation/extract × bacterial strain and medium × preparation/extract at the significance level of p = 0.05. The calculations were performed using Statistica 13.1 software.

RESULTS

In this study, no significant statistical differences were observed in the influence of streptomycin and Champion 50WP on the growth inhibition of the three bacteria strains for medium 523 and Nutrient Agar (Tabs. 1a, c).

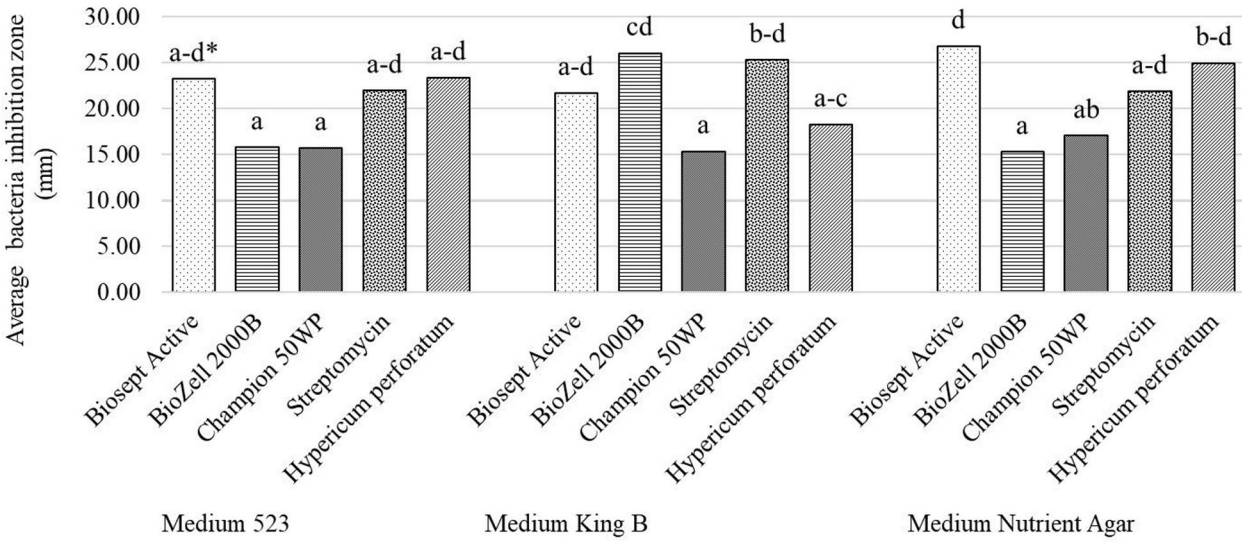
For medium King B, no statistically significant differences were noticed in the effect of streptomycin and Champion 50WP on *P. syringae* pv. *syringae* and *X. arboricola* pv. *corylina.* A stronger bacteriostatic effect against *A. tumefaciens* was exhibited by streptomycin (Tab. 1b).

No significant statistical differences were observed in the influence of Biosept Active, BioZell and the *Hypericum perforatum* extract on the growth inhibition of *P. syringae* pv. *syringae* and *X. arboricola* pv. *corylina* bacteria strains growing on medium 523. Biosept and the *Hypericum perforatum* extract demonstrated stronger antibacterial effect against *A. tumefaciens,* than BioZell (Tab. 1a).

The strongest bacteriostatic effect against *A. tumefaciens* growing on King B was exhibited by BioZell and Biosept Active, while the effect demonstrated by the *Hypericum perforatum* extract was weaker. No significant statistical differences were observed in the influence of the biopreparations and *Hypericum perforatum* extract on the growth inhibition of *P. syringae* pv. *syringae* and *X. arboricola pv. corylina* (Tab. 1b).

No statistically significant differences were observed in the antimicrobial activity of Biosept Active, BioZell and the *Hypericum perforatum* extract against *X. arboricola pv. corylina* on Nutrient Agar. Biosept Active and the *Hypericum perforatum* extract showed stronger bacteriostatic effect against *A. tumefaciens* than BioZell, while the antibacterial activity against *P. syringae* pv. *syringae* of *Hypericum perforatum* extract was stronger than that of Biozell (Tab. 1c).

According to the analysis of the growth inhibition zones of the *Xanthomonas arboricola* pv. *corylina* strain, all the preparations applied and *Hypericum perforatum* extract exhibited similar effects regardless of the medium used (Fig. 3).



*Values followed by the same letter do not differ significantly on the significance level p = 0.05. Homogeneous groups acc. to Tukey test

Fig. 1. Effect of the medium and the preparations/extract on the growth inhibition zone of *Agrobacterium tumefaciens*

Table 1. Effectiveness of biochemical preparations and the extract from *Hypericum perforatum* against *Agrobacterium tumefaciens*, *Pseudomonas syringae* pv. *syringae*, *Xanthomonas arboricola* pv. *corylina*

a)

Medium 523					
Bacteria	Average bacteria inhibition zone size (mm)				
	Preparation/antibiotic/extract				
	Biosept Active	BioZell 2000B	Champion 50WP	streptomycin	extract of *Hypericum perforatum*
Agrobacterium tumefaciens	23,2c	15,8ab	15,7ab	21,9b–c	23,3c
Pseudomonas syringae pv. *syringae*	16,5a–c	19,1a–c	19,4a–c	20,1a–c	19,3a–c
Xanthomonas arboricola pv. *corylina*	17,7a–c	15,8ab	13,5a	13,7a	19,7a–c

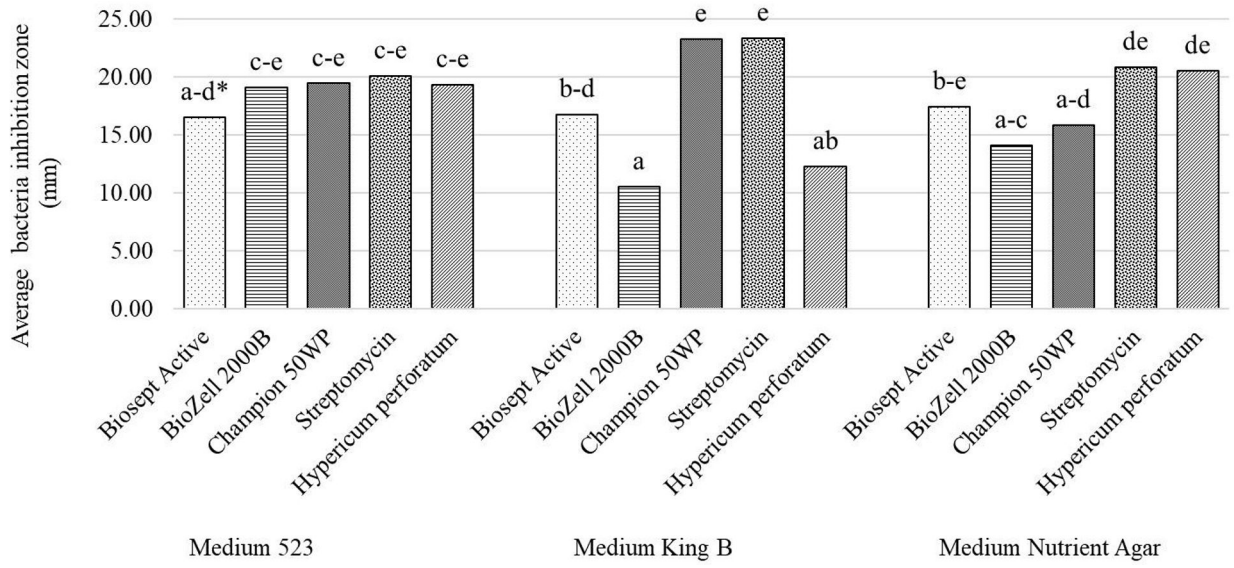
b)

Medium King B					
Bacteria	Average bacteria inhibition zone size (mm)				
	Preparation/antibiotic/extract				
	Biosept Active	BioZell 2000B	Champion 50WP	streptomycin	extract of *Hypericum perforatum*
Agrobacterium tumefaciens	21,7c–f	26f	15,3a–c	25,3ef	18,3b–e
Pseudomonas syringae pv. *syringae*	16,8a–d	10,5a	23,3d–f	23,3d–f	12,3ab
Xanthomonas arboricola pv. *corylina*	16,4a–d	16a–d	11,7ab	17,8a–e	17,9a–e

c)

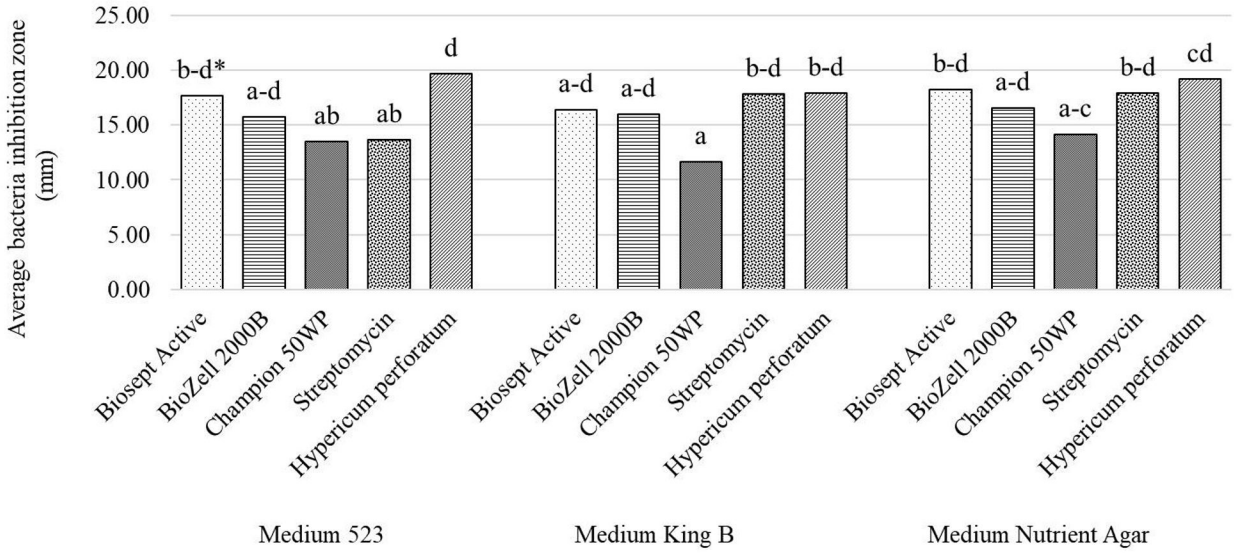
Medium Nutrient Agar					
Bacteria	Average bacteria inhibition zone size (mm)				
	Preparation/antibiotic/extract				
	Biosept Active	BioZell 2000B	Champion 50WP	streptomycin	extract of *Hypericum perforatum*
Agrobacterium tumefaciens	26,8e	15,3a	17a–c	21,8c–e	24,9de
Pseudomonas syringae pv. *syringae*	17,4a–c	14,1a	15,8ab	20,8b–d	20,5b–d
Xanthomonas arboricola pv. *corylina*	18,3a–c	16,6ab	14,2a	17,9a–c	19,2a–c

*Values followed by the same letter in each row do not differ significantly on the significance level of p = 0.05. Homogeneous groups were distinguished using Tukey test for the interaction of bacterium × preparation



*Values followed by the same letter do not differ significantly on the significance level p = 0.05. Homogeneous groups acc. to Tukey test

Fig. 2. Effect of the medium and the preparations/extract on the growth inhibition zone of *Pseudomonas syringae* pv. *syringae*



*Values followed by the same letter do not differ significantly on the significance level p = 0.05. Homogeneous groups acc. to Tukey test

Fig. 3. Effect of the medium and the preparations/extract on the growth inhibition zone of *Xanthomonas arboricola* pv. *corylina*

A stronger antimicrobial activity against *A. tumefaciens* on the King B medium was shown by BioZell and Biosept Active (Fig. 1).

No significant differences in the efficiency of BioZell and the *Hypericum perforatum* extract against *P. syringae* pv. *syringae* were observed (the 523 medium). The strongest activity against *P. syringae* pv. *syringae* was demonstrated by Biosept Active and *Hypericum perforatum* extract (the Nutrient Agar medium) and King B medium (Fig. 2).

DISCUSSION

The fungicide [Champion 50WP] and the antibiotic (streptomycin) applied in this study showed antimicrobial activity against *A. tumefaciens, P. syringae* pv. s*yringae* and *X. arboricola* pv. *corylina.* The results obtained are corroborated by data found in the research literature. Mikiciński et al. [2012] have proven that copper oxychloride inhibited the growth of *Erwinia amylovora*, *X. arboricola* pv. *corylina*, *X. arboricola* pv. *juglandis* and *A. tumefaciens.* The bacteriostatic effect of copper hydroxide and streptomycin against *Ralstonia solanacearum*, *X. campestris* pv. *vesicatoria* has been described by Adaskaveg and Hine [1985], Richie and Dittapongpitch [1991] and Marko and Stall [1983], while their antibacterial effect against *P. syringae* pv. *tomato* has been mentioned by Lee et al. [2012] and Conlin and McCarter [1983]. The chemical plant protection methods against phytobacteria are known to be based on the application of preventive fungicides containing copper hydroxide or copper oxychloride. A disadvantage of these fungicides is their unreliability and risk of phytotoxicity. Moreover, there are bacteria strains resistant to copper preparations. Lee and Cho [1996] have observed five strains of *X. campestris* pv. *vesicatoria* isolated from peppers resistant to copper hydroxide. For plant protection against bacterioses in non-European countries antibiotics, e.g. streptomycin, are used. In Europe, antibiotics are no longer applied in plant protection due to a risk of the spread of antibiotics-resistant bacterial strains pathogenic to humans and animals as a result of increased environmental pressure. Therefore, it is recommended to search for alternative methods of plant protection against bacterioses. Recently, many science centers have undertaken intensive research under *in vitro* conditions into the use of natural products of plant origin having antimicrobial properties (essential oils, products of secondary metabolism of plants) in plant protection against bacterioses. Iscan et al. [2002] have shown that peppermint essential oil exhibited the highest activity against *P. syringae* pv. *phaseolicola*, while squaw mint (*Mentha pulegium)* essential oil effectively inhibited the growth of *Clavibacter michiganensis* subsp. *michiganensi* [Daferera et al. 2003] and *E. amylovora* [Kokoškovă and Pavela 2007]. According to a study by Yanmis et al. [2012], essential oil obtained from *Mentha longifolia* spp. *longifolia* had strong antibacterial properties against *C. michiganensis* subsp. *michiganensis*. The bacteriostatic activity against *P. syringae* pv. *syringae* of essential oils obtained from *Origanum vulgare* has been described by Vasinauskiene et al. [2006] and Kokoskova et al. [2011], while their activity against *Origanum compactum* and *Mellisa officinalis* has been mentioned by Kokoskova et al. [2011]. Paret et al. [2010] have proven the antibacterial activity of essential oils from *Cymbopogon martini*, *C. citratus* and eucalyptus against *Ralstonia solanacearum*. Essential oil from *Thymus vulgaris* has exhibited antimicrobial activity against *A. tumefaciens* [Abo-Elyousr, 2006, El-Zemity et al. 2008], *E. amylovora* and *P. syringae* pv. *syringae* [Kokoskova and Papavela, 2007, Kokoskova et al. 2011] as well as *X. campestris* pv. *campestris*, *X. campestris* pv. *vesicatoria* [Bajpai et al. 2011]. Abo-Elyousr [2006] has stated that the biotechnical preparation BioZell, which contains 70% thyme oil, 20% corn oil and 10% sesame oil, effectively inhibited the growth of *X. campestris* pv. *phaseoli* grown on King B. According to Mikiciński et al. [2012], essential oils from *Salvia fructicosa*, *Syzygium aromaticum* and *Thymus vulgaris* (the BioZell preparation) demonstrated antibacterial activity against *Erwinia amylovora*, *Xanthomonas arboricola* pv. *corylina*, *X. arboricola* pv. *juglandis* and *A. tumefaciens.* BioZell exhibited the highest activity against *A. tumefaciens* and *X. arboricola* pv. *corylina* grown on King B. Similarly, the authors of this study obtained results indicating a high bacteriostatic activity of the BioZell preparation against *A. tumefaciens* and *X. arboricola* pv. *corylina* grown on the King B medium. Another biotechnical preparation used in this study, Biosept Active, contains 33% grapefruit seed and pulp extract. The active compounds present in the grapefruit ex-

tract: 7-geranoxycumarin, benzotonin and triclosan, inhibited the growth of bacteria and fungi [Angloni et al. 1998, Woedlke e al. 1999]. According to Caccioni et al. [1998], the substances dominant in grapefruit: aliphatic aldehydes, nutkakon and monoterpenes, cooperated in inhibiting a specific pathogen. Saniewska [2002] has shown that protective measures can also involve the presence of many biologically active compounds found in pulp and outer skin, i.e. endogenous flavonoids and glycosides as well as terpenes, coumarins and furanocoumarins.

Numerous studies have proven a high efficiency of Biosept Active against plant diseases caused by fungi, e.g. noble rot, alternariosis, powdery mildew and rust in crops of vegetables and ornamental plants [Jamiałkowska and Hetman 2016]. Currently, no studies have been published on the use of the Biosept Active preparation for plant protection against bacterioses. However, there is research upon a high antibacterial activity of the grapefruit seed and pulp extract against bacteria pathogenic to humans. Okungbowa et al. [2011] Han et al. [2015] have shown the high activity of against *Escherichia coli*, *Micrococcus luteus*, *Staphyloccocus aureus*, *Klebsiella aerogenesis* or *Salomonella* sp. *In vitro* tests conducted by Cvetni and Vladimir-Knezevi [2004] have revealed the bacteriostatic activity of a Grapefruit Seed Extract (GSE) preparation (33% grapefruit seed and pulp extract) against Gram-positive bacteria (*Bacillus cereus*, *B. subtilis*, *Sarcina flava*, *S. lutea*, *Staphylococcus aureus*, *S. epidermidis*, *Streptococcus faecalis*, *Listeria monocytogenes*). On the other hand, GSE exhibited no antibacterial activity against Gram-negative bacteria (*Shigella sonnei*, *Yersinia enterocolitica*, *Citrobacter freundi*, *Proteus mirabilis*, *P. vulgaris*, *P. aeruginosa*). The Biosept Active preparation applied in this study demonstrated large potential for antimicrobial activity against Gram-negative phytobacteria: *A. tumefaciens*, *P. syringae* pv. *syringae* and *X. arboricola* pv. *corylina*, in particular on the Nutrient Agar medium.

Hypericum perforatum is a medicinal plant known since antiquity and used widely due to its biological properties [Turek 2005]. Chemical studies of *H. perforatum* have revealed a number of constituents, including hypericins, flavonoids, hyperforin as an antibiotic substance, essential oil, tannins and procyanidins [Medic-Saric et al. 1996, Jurgenliemk and Nahrstedt 2002]. Antimicrobial tests of this plant have shown its high activity. The Perforate St John's-wort has demonstrated a high bacteriostatic activity under *in vitro* conditions against Gram-positive bacteria (*Corynebacterium diphteriae*, *Enterococcus faecalis*, *S. aureus*, *S. epidermidis*) and Gram-negative bacteria (*Enterobacter cloaceae*, *E. coli*, *Klebsiella pneumoniae*, *P. aeruginosa*) [Gibbons 2002, Males et al. 2006, Meral and Karabay, 2012, Feyzioglu et al. 2013, Shalibeik et al. 2016].

Currently, there is no commercially available biotechnical preparation applicable for plant protection against pathogens based on the *H. perforatum* extract. According to a study by Milosevic et al. [2007], the Perforate St John's-wort extract can be used as an effective antibacterial product against Gram-negative phytobacteria *P. phaseolicola*, *P. glycinea*, *E. carotovora* and *A. tumefaciens*. Similar research results presented in this study demonstrated the antimicrobial properties of *H. perforatum* extract against the strains tested, i.e. *A. tumefaciens* (strain C58), *P. syringae* pv. *syringae* (strain 760) and *X. arboricola* pv. *corylina* (strain RIPF-x13). In the majority of cases, its effect was similar to that of Biosept Active and BioZell. Furthermore, in some cases, the Perforate St John's-wort extract showed an activity higher than that of the fungicide applied and similar to that of streptomycin. Meral and Karabay [2012] have mentioned a higher activity of *H. perforatum* extract against *Staphylococcus aureus*, *S. epidermidis*, *Enterobacter cloaceae*, *P. aeruginosa* and *E. faecalis* than the activity of an antibiotic (ampicillin) against these bacteria. Tests performed by Milosevic et al. [2007], in which *P. fluorescens* and *Bacillus mycoides* as well as the antibiotic saincilin, have revealed similar results. In conclusion, it can be argued that the biotechnical preparation BioZell used in this study exhibits bacteriostatic activity against the Gram-negative bacteria tested, which corresponds with the data published by Mikiciński et al. [2012].

The research results presented in this study constitute the first report on the application of the Biosept Active preparation and the Perforate St John's-wort extract in plant protection against bacterial crown gall caused by *A. tumefaciens*, bacterial canker or blast stone and pome fruits caused by *P. syringae* pv.

syringae and bacterial blight of hazelnut caused by *X. arboricola* pv. *corylina*.

The analysis of the research results presented, in particular of the use of grapefruit seed and pulp extract and *H. perforatum* extract, provides arguments in favor of further research, which will enable the future application of the Biosept Active preparation or the Perforate St John's-wort extract in the integrated plant protection system against bacterial crown gall, bacterial canker or blast stone and pome fruits and bacterial blight of hazelnut. Due to the lack of efficient chemical protection and difficulties in obtaining the cultivars resistant to bacteria, currently the only preventative measures of plant protection applied against bacterioses have been phytosanitary procedures in combination with proper agrotechnics. Therefore, all further research on the application in plant protection against bacterioses of alternative plant protection products, i.e. natural products of plant origin having antimicrobial properties, should be considered fully justified and will certainly contribute to widely understood protection of the environment, which comprises the protection of human life and health.

CONCLUSIONS

1. No differences were observed in the effect of streptomycin and Champion 50WP on the growth inhibition of three bacterial strains.

2. It was established that the biotechnical preparations Biosept Active and BioZell as well as the *Hypericum perforatum* extract exhibited varied antimicrobial activity against *Agrobacterium tumefaciens*, *Pseudomonas syringae* pv. *syringae* and *Xanthomonas arboricola* pv. *corylina* depending on the medium, on which they were grown.

3. The BioseptActive preparation and *H. perforatum* extract showed strong antimicrobial activity against the bacteria strain tested grown on the Nutrient Agar medium.

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