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Antifungal effect of the entomopathogenic fungi *Beauveria bassiana* on the phytopathogenic fungi *Botrytis cinerea*

Antifungalni učinak entomopatogene gljive *Beauveria bassiana* na fitopatogenu gljivu *Botrytis cinerea*

Katarina MARTINKO (✉), Anton MIHOVILOVIĆ

University of Zagreb Faculty of Agriculture, Division of Phytomedicine, Department of Plant Pathology, Svetošimunska 25, 10000 Zagreb, Croatia

✉ Corresponding author: kmartinko@agr.hr

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ABSTRACT

The use of antagonistic microorganisms in the suppression of phytopathogenic fungi represents the ultimate approach to biological control, and because of their significant beneficial effects, antagonistic microorganisms are increasingly being researched. The necrotrophic fungus *Botrytis cinerea* known as the causative agent of gray mold, leads to significant global yield and post-harvest storage losses. It is a polyphagous of high risk for fungicide resistance development, which is why the use of antagonistic microorganisms a promising biocontrol strategy. Among the antagonistic microorganisms, the entomopathogenic fungus *Beauveria bassiana* is considered to have antagonistic properties in controlling phytopathogenic fungi due to the wide range of secondary metabolites. In order to maximize the antagonistic fungi effectiveness in field conditions, special attention was paid to researching the antagonistic mechanisms under optimal conditions for their development. The aim of this study is to test the antifungal effect of *B. bassiana* on pathogen *B. cinerea* by dual cultures method and test the production of volatile metabolites on a medium with two different pH values (pH = 5.6 and 7.2). The results of the dual culture method and testing production of volatile metabolites confirmed that the antagonistic effect of *B. bassiana* is significantly higher on a neutral pH medium, where the inhibition of the *B. cinerea* was 36%, i.e. 38% which was achieved by different antagonistic mechanisms. Microscopic analysis confirmed inhibition mechanisms (mycoparasitism and antibiosis), more significant on a neutral pH medium by *B. bassiana*, and *B. cinerea* microstructures deformations. *B. bassiana* isolate has a significant antagonistic potential in suppressing *B. cinerea*, which is the starting point for further research *in vivo*.

Keywords: antagonism, antibiosis, *Beauveria bassiana*, *Botrytis cinerea*, mycoparasitism, inhibition

SAŽETAK

Primjena antagonističkih mikroorganizama u supresiji fitopatogenih gljiva predstavlja suvremeni pristup biološke borbe, a zbog značajnih beneficijskih učinaka, antagonistički mikroorganizmi se sve više istražuju. Nekrotrofna gljiva *Botrytis cinerea* poznata kao uzročnik sive plijesni, dovodi do značajnih globalnih gubitaka prinosa u polju i skladištu nakon berbe zbog čega se primjena antagonističkih mikroorganizama smatra obećavajućom strategijom biološkog suzbijanja. Među antagonističkim mikroorganizmima spominje se entomopatogena gljiva *Beauveria bassiana* koja posjeduje i antagonistička svojstva u suzbijanju fitopatogenih gljiva zbog širokog spektra sekundarnih metabolita koje proizvodi. Radi što veće učinkovitosti antagonističkih gljiva u poljskim uvjetima, u novijim laboratorijskim istraživanjima posebna pažnja se pridaje istraživanju antagonističkih mehanizama u optimalnim uvjetima za njihov razvoj. Cilj istraživanja je testirati antifugalni učinak *B. bassiana* na patogena *B. cinerea* metodom dvojnih kultura i testiranjem produkcije volatila na standardnoj PDA podlozi s dvije različite pH vrijednosti (pH = 5,6 i 7,2). Rezultati testova dvojnih kultura i testiranja produkcije volatila su potvrdili da je antagonistički učinak *B. bassiana* značajno viši na hranjivom mediju neutralne pH vrijednosti gdje je inhibicija patogena *B. cinerea* iznosila 36 % tj. 38 % što je postignuto različitim mehanizmima borbe. Mikroskopskom analizom potvrđeni su mehanizmi antagonističke borbe (mikoparazitizam i antibioza), značajan na mediju neutralne pH vrijednosti te deformacije na mikrostrukturama *B. cinerea*. Izolat *B. bassiana* ima značajan antagonistički potencijal u suzbijanju uzročnika sive plijesni što je polazišna točka za daljnja istraživanja u uvjetima *in vivo*.

Ključne riječi: antagonizam, antibioza, *Beauveria bassiana*, *Botryts cinerea*, mikoparazitizam, inhibicija

INTRODUCTION

Plant production is hindered by several obstacles in controlling plant pathogens, and the demand for an additional 70% of food to sustain the world's rapidly rising population, as well as climate change adaptations (FAO, 2009), contributes to the increasing pressure in the field of phytomedicine. These issues require more effective use of natural resources, with minimum use of agrochemicals. Phytopathogenic fungi have adapted to the frequent fungicide application and developed resistance. The bacteria resistance development to antibiotics is a major problem in human medicine and veterinary medicine, just as the phytopathogenic fungi resistance development to fungicides in phytomedicine (Ivić and Cvjetković, 2017). It is necessary to continue the research line of microbial antagonists as potential biocontrol agents for suppressing fungal diseases because regulations of existing fungicides become more striking (Menzler-Hokkanen, 2006). Necrotrophic fungus *Botrytis cinerea* Pers. It's known to cause grey mould that infects a large number of economically important agricultural and horticultural plants. This pathogen leads to significant global production yield and post-harvest storage losses (Williamson et al., 2007; Haidar et al., 2016). Despite the availability of various fungicides for suppression of *B. cinerea*, a long-term application before and/or after harvest is not considered sustainable for possible harmful effects on consumers' health (Komárek et al., 2010; Haidar et al., 2016) and the emergence of resistant pathogens (Hahn, 2014; Haidar et al., 2016). Due to its great genetic variability, short life cycle and reproduction, *B. cinerea* is considered a high-risk pathogen for fungicide resistance development (Leroux et al., 2010; Haidar et al., 2016). Therefore, alternative methods (such as the use of antagonistic microorganisms) to *B. cinerea* fungicide control are considered a promising strategy (Haidar et al., 2016). The most famous representative species from the genus *Beauveria* is *Beauveria bassiana* (Bal.-Criv.) Vuill., which has been used for many years as a biological insecticide worldwide. It is a polyphagous fungus that is naturally found in the soil and has a proven ability to endophytically colonize plants, which is why the fungus is present in different plant species (Wagner and Lewis, 2000; Vidal and Jaber, 2015; Rondot and Reineke, 2018). Besides its ability to regulate the populations of harmful insects, this fungus can also be a phytopathogen antagonist, plant endophyte and plant growth promoter (Vega et al. 2009; Jaber and Ownley, 2018; Barra-Bucarei et al., 2019).

In the global market, there are biological control preparations based on entomopathogenic fungi. In 2007, a total of 171 products were registered, and 40% of preparations were based on *Beauveria* species, of which 34% were based on *B. bassiana* (Faria and Wraight 2007; Kovač, 2021). In the meantime, some preparations have been removed from the global market, and with the arrival of new ones, a total of 59 preparations based on *B. bassiana* (Mascarin and Jaronski, 2016) were recorded in 2016. In numerous countries, including Croatia (FIS, 2023), such products are not yet available, and due to limitations, high production costs and insufficiently regulated regulations, development, registration and implementation of these products is very difficult (Kovač, 2021). Such availability of *Beauveria* species preparations in the world market refers to the use of these preparations as insecticides, while the use of *B. bassiana* as an antagonist is poorly investigated. Interestingly, *B. bassiana* produces enzymes that are recently studied because of their fungicidal, fungistatic and bactericidal properties (Wang et al., 2012), and it was found that certain *B. bassiana* isolates can significantly inhibit the growth and development of *B. cinerea* (Barra-Bucarei et al., 2019). The effectiveness of biological control using antagonistic fungi has mainly been evaluated under *in vitro* conditions, and, interestingly, most fungi do not show significant control results in *in vivo* conditions. For this reason, special attention is paid to the research of fungi antagonistic mechanisms under conditions in which these mechanisms are optimal (Haidar et al., 2016).

Therefore, the aim of this study is to test an antifungal effect of entomopathogenic isolate *B. bassiana* on phytopathogen *B. cinerea* on a nutrient medium with moderately acidic and neutral pH; and microscope analysis of the antagonistic effect on *B. cinerea* microstructure.

MATERIALS AND METHODS

Pathogen isolation and cultivation

The antagonistic fungi *B. bassiana*, isolated from a dead adult Colorado potato beetle, and the pathogenic fungus *B. cinerea*, isolated from strawberry fruit with gray mold symptoms, were used for the experiment.

Both isolates were molecularly identified by a conventional PCR and sequenced to the species level at Macrogen Europe (Amsterdam, the Netherlands). Isolate cultures grown on PDA (Liofilchem, Italy) and incubated in a climate chamber, at 22 °C, in the dark are kept in the collection of the Department of Plant Pathology at the University of Zagreb, Faculty of Agriculture.

Preparation of nutrient media

The *B. bassiana* antagonistic potential against *B. cinerea* was tested on a nutrient medium with two different pH values. Based on a preliminary experiment and literature review of optimal conditions for test fungi growth and development, the experiments were carried out on a standard Potato dextrose agar (PDA, Liofilchem, Italy) with pH values of 5.6 and 7.2.

For this purpose, the following was prepared: i) a standard PDA medium according to the manufacturer's instructions with a pH value of 5.6 +/- 0.2 and ii) a standard PDA medium in which a certain volume of 10% aqueous potassium hydroxide (KOH) solution was applied in accordance to the volume of the nutrient medium in order to achieve a pH value of 7.2. The pH value of previously prepared and sterilized PDA medium was checked using a pH meter (Voltcraft PH – 100 ATC, Slovakia) in a sterile chamber.

Testing the antifungal potential of Beauveria bassiana isolate

In order to investigate the antagonistic potential of the species *B. bassiana* against the pathogen *B. cinerea*, a total of four experiments were conducted under laboratory conditions.

To test antagonism *in vitro*, micellar discs of antagonist *B. bassiana* and pathogen *B. cinerea* (Ø 5 mm) were cut from the edge of growing fungal colonies and placed on previously poured and cooled PDA medium in sterile Petri dishes (Ø 9 cm). Inoculated Petri dishes containing fungi were incubated in a climate chamber for 4 days, in the dark, at 22 °C.

All experiments were set up in two variants and five repetitions individually on PDA with pH values of 5.6 and 7.2.

Dual culture method

The dual culture method was performed according to the modified method of Yun et al. (2017).

Micellar discs of antagonistic and pathogen fungi (Ø 5 mm) were used, which were cut with a circular cutter from the edge of growing 5-day-old colonies. The micellar disk of *B. bassiana* was placed 2 cm from the edge of the Petri dish (Ø 9 cm) on the bottom of the previously poured PDA medium. The micellar disk of pathogen *B. cinerea* was placed at an equal distance on the opposite side from the antagonist and incubated at 22 °C for 5 days, in the dark. Control Petri dishes contained only a disc of pathogen *B. cinerea*.

Testing the production of volatile metabolites

Testing the production of volatile metabolites of the antagonist *B. bassiana* against pathogen *B. cinerea* was carried out according to the method of Dennis and Webster (1971b).

The micellar disk of antagonistic *B. bassiana* was placed in the centre of the cover of the test Petri dish containing the poured PDA medium, while the micellar disk of pathogen *B. cinerea* was placed in the centre of the bottom of the same Petri dish. In the control Petri dish, a PDA disc was placed instead of the micellar disc of antagonist *B. bassiana*. The Petri dishes were double-wrapped with parafilm tape and placed for incubation in a climate chamber for 5 days, at 22 °C, in the dark.

Microscopic analysis

The microscope observation of the effect of antagonist *B. bassiana* on the pathogen *B. cinerea* was carried out according to the modified method of Al-Shibli et al. (2019) using a light microscope (BH2, Olympus, Japan) and a stereomicroscope (SZX7, Olympus, Japan).

To quantify the antagonistic effect on the pathogen's microstructures, microscopic preparations stained with lactophenol blue solution, contained: i) pathogen hyphae from the zone of inhibition, ii) pathogen hyphae exposed to volatiles, iii) pathogen hyphae from the control variant. The antagonistic effect was quantified according to observed pathogen structural changes.

Micellar growth area measurement

After 5 days, the results of all experiments were read. Photographs of test and control Petri dishes were processed using the computer program ImageJ (Schneider et al., 2012) according to the modified method of Martinko et al. (2022). Mean values (cm^2) of the micellar growth area of *B. cinerea* were obtained, from which the inhibition index (%) was calculated and the antagonistic effect of the antagonist *B. bassiana* on the pathogen *B. cinerea* was quantified.

Statistical analysis

The results of testing the antagonistic potential of *B. bassiana* are presented as mean values and standard deviations. The difference between the mean values of the test and control groups was determined using the Student t-test in the statistical program IBM SPSS Statistics for Windows Version 21.0 (IBM Corp. Armonk, NY, USA) and was considered statistically significant at $P < 0.05$.

RESULTS

Results of the dual culture method

The micellar growth of the pathogen *B. cinerea* on the PDA medium with a pH value of 5.6 was inhibited by 4%, while on the PDA medium with a pH value of 7.2, the growth of the fungus was reduced by 36% compared to the growth of the pathogen in the control (Table 1, Figure 1).

Table 1. Antifungal effect of *Beauveria bassiana* on the *Botrytis cinerea* growth using the dual culture method on PDA medium with pH 5.6 and 7.2 after 5 days

Dual culture method				
	ph 5,6		ph 7,2	
	Control	*Test*	Control	Test
	B. cinerea	*B. bassiana* + *B. cinerea*	*B. cinerea*	*B. bassiana* + *B. cinerea*
x̄ (%) ± SD	99 ± 0,4	94,6 ± 1^{ns}	99,2 ± 0,4	63,3 ± 2,9*
I (%)	4,4		35,9	

Data are presented as mean (x̄) growth values expressed as a percentage and standard deviation (SD).
* - significant difference in the mean growth values of *B. cinerea* compared to the control according to the t-test ($P < 0.05$).
^{ns} - no significant difference between mean growth values of *B. cinerea* compared to control by t-test ($P < 0.05$).

The mean values of the *B. cinerea* growth were not significantly reduced in the variant with a medium pH of 5.6, while the mean values of the growth were significantly reduced in the variant with a medium pH of 7.2 compared to the mean values of the control group (t-test, $P < 0.05$).

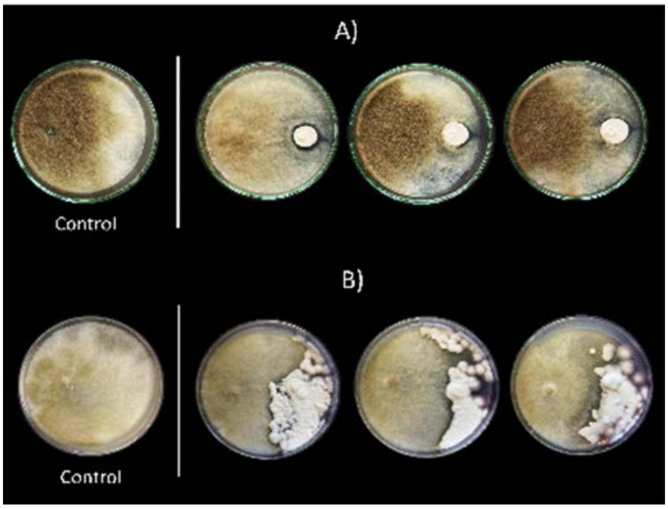

Figure 1. Antifungal effect of *Beauveria bassiana* on the pathogen *Botrytis cinerea* compared to the control after 5 days, dual culture method: A) pH 5.6; B) pH 7.2

Results of volatile production testing

The growth of *B. cinerea* mycelium on the PDA medium with a pH value of 5.6 was inhibited by 13%, while on the PDA medium with a pH value of 7.2, the growth of the fungus was reduced by 38% compared to the pathogen growth in the control (Table 2, Figure 2).

Table 2. Antifungal effect of *Beauveria bassiana* on the *Botrytis cinerea* growth using the testing a production of volatile metabolites method on PDA media with pH 5.6 and 7.2 after 5 days

Testing the production of volatile metabolites				
	ph 5,6		ph 7,2	
	Control	Test	Control	Test
	B. cinerea	*B. bassiana* + *B. cinerea*	*B. cinerea*	*B. bassiana* + *B. cinerea*
x̄ (%) ± SD	99,1 ± 0,5	86,6 ± 3,2*	99,5 ± 0,4	62 ± 4,4*
I (%)	12,5		37,5	

Data are presented as mean (x̄) growth values expressed as a percentage and standard deviation (SD).
* - significant difference in the mean growth values of *B. cinerea* compared to the control according to the t-test ($P < 0.05$).
ns - no significant difference between mean growth values of *B. cinerea* compared to control by t-test ($P < 0.05$).

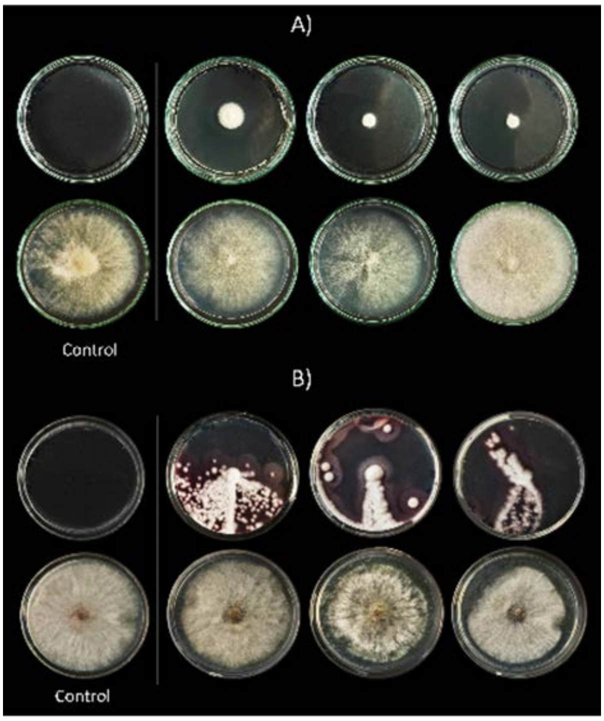

Figure 2. Antifungal effect of *Beauveria bassiana* on the pathogen *Botrytis cinerea* compared to the control after 5 days, testing production of volatile metabolites: A) pH 5.6; B) pH 7.2

The mean values of the pathogen growth area were significantly reduced in the variant with pH values of PDA medium 5.6 and 7.2 compared to the mean values of the control group according to the t-test ($P < 0.05$).

Results of microscope analysis

Microscopic analysis showed that the antagonist *B. bassiana* caused a significant change in *B. cinerea* hyphal morphology and spore production after 5 days compared to the control variant due to direct mycelium contact and during volatile fumigation on parts of the inhibitory zones (Figure 3).

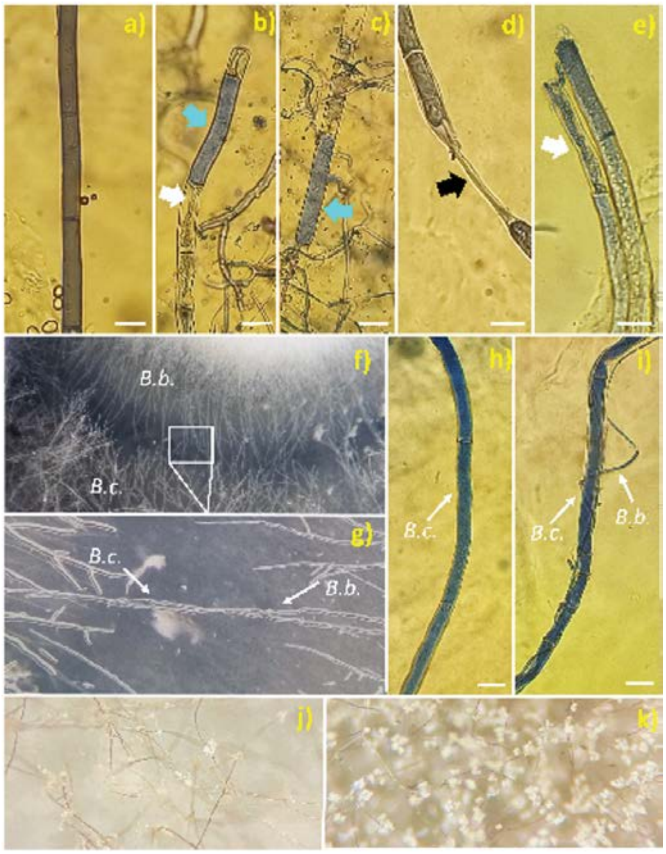

Figure 3. Antagonistic effect of *Beauveria bassiana (B.b.)* on hyphae and sporulation of the pathogen *Botrytis cinerea (B.c.)* after 5 days: a) control; (b-e) hyphal deformations – blue arrow; intense blue coloration with lactophenol in individual hyphal fragments; white arrow - leakage and vacuolization of hyphal content; black arrow – hyphal constriction; (f-i) hyphae from the inhibition zone; (f-g) *B.b.* hyphae wrapping around the *B.c.* hyphae; (h) *B.b.* hyphae wrapping around the B.c. hyphae; (g) *B.b.* hyphae wrapping around the *B.c.* hyphae; h) control; i) parasitized hyphae; j) reduction of sporulation by fumigation, k) production of spores of the pathogen *B.c.* on the control. Scale bar 10 µm.

Pathogen microstructural changes were recorded in all variants regardless of the pH value of the nutrient medium, but the changes are significant in the variants with a nutrient medium with a neutral pH value. Pathogen hyphae from the area of the inhibition zone showed deformations, collection, vacuolization and leakage of contents from individual hyphal fragments and necrosis. Penetration of lactophenol blue (with which hyphae are stained during microscopy) is more intense in certain fragments of hyphae than in others, which indicates the permeability of certain parts, which makes the hyphae appear empty. Using a stereomicroscope, reduced sporulation of the pathogen *B. cinerea* was observed due to the *B. bassiana* antibiosis compared to the control.

DISCUSSION

The results of dual culture and volatile production tests show that the antagonistic effect of *B. bassiana* isolates after 5 days is significantly higher on the neutral pH medium (pH = 7.2) where the inhibition of the pathogen *B. cinerea* was 36% ie 38%. On a medium with a moderately acidic pH (pH = 5.6), the pathogen inhibition was achieved about 8 times (4 %) in dual cultures and 3 times lower (13 %) in testing production of volatile metabolites compared to the inhibition achieved on a medium with neutral pH values. The antagonism of *B. bassiana* isolates against the pathogen *B. cinerea* is also confirmed by the results of microscopic analysis, which prove visible antagonistic mechanisms such as mycoparasitism (twisting of antagonist hyphae around pathogen hyphae) and antibiosis (suppression of sporulation and appearance of red pigment in the medium). The above-mentioned mechanisms are clearly represented, weakly or not at all, depending on the applied method and the pH value of the nutrient medium, which is confirmed by various studies.

The results of Yun et al. (2017) confirm the significant *B. bassiana* antifungal activity in inhibition of the pathogen *B. cinerea* using a dual culture method on a standard PDA medium (pH = 5.6) on which *B. bassiana* isolate with less micellar growth developed a significant inhibition zone which limited the growth of the pathogenic fungus. The authors report the *B. bassiana* antibiosis as the reason for the significant inhibition of *B. cinerea*. In order to evaluate the antifungal effect, in the study of Barra-Bucarei et al. (2019) and Tomilova et al. (2020), a dual culture method was carried out in such a way that the atnagonist *B. bassiana* was inoculated two days before the inoculation of the pathogen *B. cinerea*, which led to significant inhibition of the pathogenic species by antibiosis, as evidenced by the inhibition zone that prevented direct contact of both mycelia. By modifying the method, the authors of the paper gave a time advantage to *B. bassiana* in order to produce secondary metabolites and release them into the medium before the development of the pathogen *B. cinerea*. Interestingly, the authors describe *B. bassiana* as a weak colonizer and competitor in the soil microbiota. They also state that the *B. bassiana* is a weak mycoparasite, but a strong antibiotic factor due to the wide range of secondary metabolites it produces.

In our study, it was observed that it is not necessary to inoculate antagonists and pathogens at different periods but to adjust the pH value of the nutrient medium. That the *B. bassiana* isolate produced and released enzymes into the nutrient medium is proven by the appearance of a red pigment in both methods after 5 days on a neutral pH medium, which was not the case with the acidic medium. According to De Hoog (1972) and Strasser et al. (2000), the appearance of a red pigment confirms the presence of a significant enzyme oosporein produced by *B. bassiana* as a result of an antagonistic defence mechanism. The above is confirmed by many studies that bring results about the importance of the pH medium value in the case of entomopathogenic fungi (Wang and Feng, 2014; Zhu et al., 2016) and their enzyme production (St Leger et al., 1997; Luo et al., 2014; Zhu et al., 2016). Although a wide pH range of media has been reported for the species *B. bassiana* (Galani 1988; Shimazu and Sato 1996) it has been proven that this fungus requires an appropriate pH in order to function properly and be able to parasitize insects, but also phytopathogenic fungi (Prusky and Yacoby, 2003; Parine et al., 2010; Sahab, 2012). That the antagonism of *B. bassiana* has a greater potential on a neutral nutrient medium is confirmed by study of Zhu et al. (2016) whose results prove the importance of a neutral medium (pH = 7) for the proper functioning and production of *B. bassiana* enzymes. Also, research by Zibaea et al. (2011) confirms that the optimal pH medium value for the highest enzymatic *B. bassiana* activity is 7.2, which is attributed to the pH of the insect cuticle on which it parasitizes (St. Leger et al., 1998). For the species *B. bassiana*, according to Padmavathi et al. (2003), a slightly acidic medium (such as the standard PDA medium) leads to a negative effect on the initial growth of some *B. bassiana* isolates which is slowed down, although after 10 days the fungus produces significant biomass. A review of the literature revealed contradictions when it comes to *B. bassiana* antagonism. For example, Lee et al. (1999) and Shternshis et al. (2014) observed the ability of *B. bassiana* to mycoparasitic fungi, while Veselý and Koubová (1994) showed that species of the genus *Beauveria* do not show any antagonistic activity against pathogen *Rizoctonia solani*.

The research of Deb and Dutta (2021) brings the results of testing different isolates of *B. bassiana* for inhibition of the causative agent of seedling decay, i.e. species of the pseudofungal genus *Pythium*, where pathogen inhibition of 62-83% was recorded, depending on *B. bassiana* isolate. It is significant that testing using the dual culture method was performed on the standard PDA medium (pH 5.6±0.2) used in our research as well. Deb and Dutta (2021) recorded mycoparasitism by *B. bassiana* through microscopic analysis and a significant antibiotic effect attributed to the action of secondary metabolites of the species. Since it is a pathogenic pseudofungus, they assume that cellulase enzyme production was key in suppressing the target pathogen. The same results were confirmed by Vesely and Koubova (1994), who proved the ability of *B. bassiana* mycoparasitism in combination with antibiosis. It is also obvious according to the results of our research that the degree of antagonism of *B. bassiana* differs depending on the isolate. On the other hand, the pathogen *B. cinerea* has been found to modulate its environmental pH by producing and releasing acid or ammonia compounds, on which the virulence of the pathogen depends (Rascle et al., 2018). Also, it was confirmed that the pathogen *B. cinerea* produces oxalic acid on a neutral medium, which modifies the pH of the substrate. Considering the B. cinere defence mechanisms and toxin arsenal, the antagonism of different *B. bassiana* isolates are variable, often combined with mechanisms (Ownley et al., 2008; Vega et al., 2009) and still insufficiently investigated.

CONCLUSION

The tested autochthonous *B. bassiana* isolate can act antifungally on the phytopathogenic fungus *B. cinerea* under *in vitro* conditions. A significant antifungal effect was observed using the method of dual cultures (36%) and volatile metabolites production test (36%) on a medium with neutral pH (pH = 7.2) values. On medium with a moderately acidic pH (pH = 5.6), the antifungal effect of the fungus *B. bassiana* was not significant in the dual culture method (4%), while pathogen inhibition by testing the production of volatiles showed significant results (13%). The antifungal activity of the *B. bassiana* isolate causes significant changes in the microstructures of the phytopathogenic fungi *B. cinerea* in conditions of neutral pH value (deformation of hyphae and reduced sporulation), while the indicated changes are insignificant in the moderately acidic pH medium. Isolate *B. bassiana* has the potential to reduce the development of the causative agent of grey mould *in vitro*, but additional research is needed to optimize the methods and conditions for the production of enzymes of this insufficiently researched antagonist.

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