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Bacillus subtilis BS-2 AND PEPPERMINT OIL AS BIOCONTROL AGENTS AGAINST *Botrytis cinerea*

Bacillus subtilis BS-2 ORAZ OLEJEK MIĘTOWY JAKO CZYNNIKI BIOKONTROLI WOBEC *Botrytis cinerea*

Abstract: The purpose of this study was to assess the activity of *Bacillus subtilis* BS-2 and peppermint oil against *Botrytis cinerea*. In this study parameters such as the age and the density of the bacterial culture and the incubation temperature were taken into consideration. Furthermore, the cellulolytic activity of the bacterium was determined. The effect of peppermint oil was evaluated at a concentration range of 0.5-4.0 %. The research was conducted with a dual culture plate method. The influence of *B. subtilis* BS-2 and peppermint oil on the growth of *B. cinerea* was evaluated based on the growth rate index. It was noted that the bacterial culture occurred at an initial density of OD_{560} = 1.0, cultivated at 30 °C for 48 hours demonstrated the strongest antagonistic effect (57.07 % inhibition). Furthermore, it was observed that the highest cellulolytic activity occurred on the bacteria incubated for 48 hours at 37 °C. The effect of mint oil, at the lowest concentration of 0.5-1.0 %, was much weaker on bacterial activity (1.1-12.1 % inhibition). The highest concentration (4.0 %) of mint oil caused the maximum inhibition (31.9 %) of the mycelial growth. *B. subtilis* BS-2 may be environmental-friendly alternatives for protecting plants against *B. cinerea*

Keywords: *Bacillus subtilis* BS-2, CMCase activity, peppermint oil, antifungal activity, *Botrytis cinerea*

Introduction

Grey mould, caused by the fungus *B. cinerea*, is one of the most significant agricultural phytopathogens that can infect plants throughout all stages of their growth. For example, in cold and damp weather conditions it can affect strawberry plants, attacking the flowers, leaves, and mature fruit [1, 2]. *B. cinerea* produces large amounts of conidia that are widespread in the air and can be readily transported by wind over long distances. It also produces long-lived structures called sclerotia which are able to survive in the absence of a host plant [3]. For this reason, *B. cinerea* may cause serious pre- and post-harvest diseases and can be one of the most difficult plant diseases to prevent or control [4-6]. Control of grey mould disease is usually carried out by the application of chemical fungicides, which are generally not effective and lead to contamination of the environment, accumulation of toxic residues in fruits, and can cause toxic effects on human health [2, 7, 8]. Several

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studies have shown that the best alternatives with the potential to control *Botrytis cinerea* include filamentous fungi (*Trichoderma* sp., *Gliocladium* sp.), yeast (*Pichia* sp., *Candida* sp.), bacteria (*Pseudomonas* sp., *Bacillus* sp.), and certain essential oils [9-11]. Several mechanisms have been indicated as responsible for their antagonistic activity, including: mycoparasitism [9], secretion of bioactive compounds (trichozianins A1 and B1 from *T. harzianum*, gliotoxin from *G. virens*, pyrolnitrin from *P. cepacia*, and flagelin-like protein from *B. subtilis*) [9, 12], competition for space and nutrients, promotion of plant growth (the growth of plant seedlings, increased the biomass seedlings) [2, 13] and induction of systemic resistance (ISR) [14]. These microorganisms can cause inhibition of mycelium growth, morphological changes of mycelium, weakness or inhibition of spore germination, and inhibition of hyphal elongation [2, 4, 11].

The search for antagonists for plant protection has intensified in recent years. There have been many studies on the effectiveness and potential of various bacteria from the genera *Bacillus* and *Pseudomonas* or essential oils from the *Lamiaceae* family against various fungal pathogens e.g. *Fusarium* sp., *Penicillium* sp., *Rhizoctonia solani*, *Alternaria* sp., *Botrytis cinerea* [15-19]. The *Bacillus* genus, particularly *B. subtilis* and the closely related *B. amyloliquefaciens* species, are being widely researched for their potential to function as biocontrol agents. Their beneficial attributes for plant protection and growth promotion include the synthesis of broad spectrum active metabolites and the ability to adapt in various environmental conditions. In contrast, essential oils contain a range of biologically active components, most of which exhibit different effects on the pathogens e.g. inhibiting the growth of mycelium and spore germination by membrane disruption, inhibiting the action of enzymes and/or causing their complete inactivation. Thus, biological methods of protection against *Botrytis* sp., such as using microorganisms or their metabolites and plant extracts or essential oils, offer an effective alternative or complement to the use of conventional disease control methods [2, 3, 9, 14].

The aim of this study was to assess the suppressing effect of *Bacillus subtilis* BS-2 and peppermint oil (*Mentha piperita* L.) on the mycelium growth of *Botrytis cinerea* isolated from strawberries.

Materials and methods

Preparation of microbial cultures

The *Bacillus subtilis* strain, encoded as BS-2, was cultured in a broth medium on a rotary shaker at 100 rpm at 35 °C for 48 h. It was then inoculated into a flask containing both the nutrient broth and CMC liquid medium containing (g/dm^3) KH_2PO_4 1.0, $MgSO_4 \cdot 7H_2O$ 0.5, NaCl 0.5, $FeSO_4 \cdot 7H_2O$ 0.01, $MnSO_4 \cdot H_2O$ 0.01, NH_4NO_3 0.3, CMC 10.0 [20], with bacterial cultures at a density of OD_{560} = 1.0 ($1.5 \cdot 10^6$ CFU/cm^3) and OD_{560} = 2.0 ($1.8 \cdot 10^8$ CFU/cm^3). Then these cultures were incubated at 30 and 37 °C for different lengths of time in order to attain 6, 24 and 48-hour cultures. After each time period, the cultures were centrifuged at 10000 rpm for 20 min at 4 °C. The cell-free supernatants (CFs) containing the metabolite and the crude enzyme were analyzed in order to detect the degree of antifungal and carboxymethyl cellulase activities. The *B. cinerea* strain isolated from strawberries was cultivated on a PDA medium at 25 °C for 7 days.

Determination of the antagonistic activity of *B. subtilis* BS-2 and peppermint oil against *B. cinerea*

The fungistatic activity of *B. subtilis* and peppermint oil (*Mentha piperita* L.) was determined using the culture-plate method on potato dextrose (PDA) medium. The supernatants and the essential oil were spread thoroughly all over the surface of PDA plates. Fungal mycelial-discs (8.0 mm in diameter) obtained from the growing cultures of test fungal isolate were placed at the centre of the medium inoculated with the supernatants. The control plates contained only *B. cinerea* cultures and aseptic broth medium in place of the supernatants. In order to evaluate the effect of the peppermint oil on the mycelial growth, the following concentrations of EO were used: - 0.5, 1.0, 2.0, 4.0 %. Initially, in order to enhance the essential oil solubility, the oil was dissolved with 0.5 % dimethyl sulfoxide (DMSO). In the control tests, the essential oil was replaced with 0.5 % DMSO solution and inoculated in the same manner as in the tests evaluating the antifungal activity of *B. subtilis* BS-2. All plates were incubated at 25 ± 2 °C for 8 days and the fungal linear growth was measured every 1-2 days until the mycelium of *B. cinerea* in the control plate reached the edge of the plate. The experiments were performed in four replicates, where one repeat was represented by one plate containing the growth medium with one mycelia disc. The influence of metabolites produced by *B. subtilis* BS-2 and essential oil on the growth of this fungus was determined based on the growth rate index (*GRI*) [21].

$$GRI = \frac{A}{D} + \frac{b_1}{d_1} + ... + \frac{b_x}{d_x} \quad (1)$$

where: A - mean value of colony diameter measurements [mm], D - duration of the experiment [days], $b_1...b_x$ - colony diameter increase since last measurement [mm], $d_1...d_x$ - number of days since the last measurement.

The inhibition of fungal growth was evaluated as the percentage reduction of the growth rate index of the treated plate versus the growth rate index of the control plate.

Carboxymethyl cellulase activity assay

The activity of carboxymethylcellulase (CMCase) was determined by the colorimetric method measuring the release of reducing sugars by the 3,5-dinitrosalicylic acid (DNS) [22]. The enzyme assay was conducted by incubating the reaction mixture containing the suitable CFs and 1 % (w/v) final concentration of CMC, prepared in sodium acetate buffer (pH = 5.0). After incubation for 30 min at 50 °C, the DNS reagent was added to the above mixture to stop the reaction. The control for the enzyme assay was run simultaneously. The colour of the reaction mixture was developed in a boiling water bath for 10 minutes. After cooling, the absorbance was measured at 540 nm. Standard curves were prepared for D-glucose. One unit of CMCase activity was determined as the amount of enzyme that catalyzes the release of 1 µmol of glucose per cm^3, per minute under the assay conditions [U/cm^3].

Determination of protein content and specific enzyme activity

The crude protein concentration was determined according to the Lowry method [23]. Bovine serum albumin (BSA) was used as the standard to calculate the specific enzyme activity [U/mg protein]. The hydrolytic ability of the crude enzyme against 0.5 % CMC was determined to evaluate substrate specificity.

Statistical analysis

All experiments were conducted in four replicates. The data are expressed as the mean ±standard deviation. Differences between the treatments and analyzed parameters were performed using two-way analysis of variance with a Tukey's composition test (statistic program MegaStat, Two-factor ANOVA). Significant differences were determined with 99 % confidence intervals ($p \leq 0.01$).

Results and discussion

The studies show that bacterial density, culturing time, and the temperature of cultivation all had a significant impact on the growth of mycelium and the degree of inhibition. The antifungal activity of various supernatants of *B. subtilis* BS-2 on the growth of *B. cinerea* on PDA medium is presented in Table 1 and Figure 1. In these tests, we observed that in the presence of supernatants the mycelium grew slower and was morphologically altered compared to the mycelium in the control tests. Statistical differences were found ($p \leq 0.01$) between the control and other supernatants (Table 1).

Table 1

Effects of *B. subtilis* BS-2 on the growth rate index (*GRI*) of *B. cinerea*

Treatment (*)	**Culturing time [hours]**			**Mean**
	6	**24**	**48**	
Control	77.60±0.26 ^{A}	77.60±0.26 ^{A}	77.60±0.26 ^{A}	77.60 ^{A}
OD 1.0/*T* 30 °C	51.5±1.5 ^{B}	40.5±1.69 ^{C}	33.31±0.82 ^{D}	41.77 ^{C}
OD 2.0/*T* 30 °C	50.6±1.1 ^{B}	40.18±0.16 ^{C}	42.2± 1.7 ^{C}	44.33 ^{B}
OD 1.0/*T* 37 °C	41.2±1.2 ^{C}	48.1±1.4 ^{B}	46.35±0.67 ^{B}	45.22 ^{B}
OD 2.0/*T* 37 °C	40.0±1.9 ^{C}	37.1±2.0 ^{D}	46.7±1.97 ^{B}	41.27 ^{C}
Mean	52.18 ^{A}	48.70 ^{B}	49.23 ^{B}	50.04

Values represent mean±standard devition of four replicates; values with the different capital letters are significant at $p < 0.01$; (*) *OD* - optical density; *T* - temperature

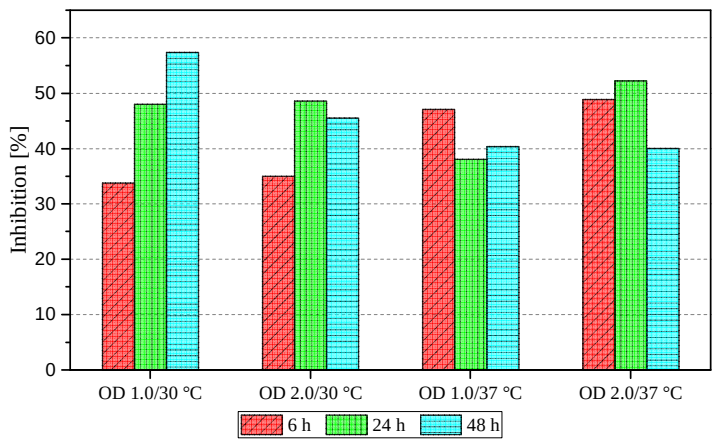

Fig. 1. Effects of *B. subtilis* BS-2 on mycelial growth of *B. cinerea*; *OD* - optical density; 30, 37 °C - incubation temperature; 6 h, 24 h, 48 h - time of the bacterial culture

The strongest antifungal activity in all supernatants against *B. cinerea* was observed in the 48 h culture at initial OD_{560} = 1.0 incubated at 30 °C with a growth rate index (*GRI*) of 33.31. Close results were observed for the antifungal activity of supernatants in the 24-hour and 6-hour cultures at initial OD_{560} = 2.0 incubated at 37 °C, with a *GRI* of 37.15 and 40.02, respectively (Table 1). The inhibitory effect of *B. cinerea* in the presence of the above-mentioned supernatant reached the values 57.07, 52.13 and 48.43 %, respectively (Fig. 1).

It was observed that in order to attain a similar antifungal effect, the higher density of bacterial culture (*OD* = 2.0) incubated at the higher temperature (37 °C) needed a shorter incubation time (6 h, 24 h) than the lower density of bacterial culture (OD_{560} = 1.0) incubated at the lower temperature (30 °C) (Fig. 1). The high antifungal activity of *B. subtilis* BS-2 observed may be due to its ability to produce cellulolytic enzymes, peptides, and another metabolites which can play an important role in limiting the growth of phytopathogenic fungi.

Table 2

Production of CMCase from cell-free culture of *B. subtilis* BS-2

Treatment (*)	**CMCase activity [U/cm^3]**			
	6 hour	**24 hour**	**48 hour**	**Mean**
OD 1.0/ *T* 30 °C	0.020±0.0004	0.022±0.0006	0.023±0.0007	0.021 ^{C}
OD 2.0/ *T* 30 °C	0.019±0.0006	0.021±0.0005	0.021±0.0004	0.020 ^{C}
OD 1.0/ *T* 37 °C	0.023±0.0005	0.030±0.0004	0.035±0.0003	0.029 ^{A}
OD 2.0/ *T* 37 °C	0.021±0.0004	0.023±0.0006	0.029±0.0003	0.024 ^{B}
Mean	0.021 ^{C}	0.024 ^{B}	0.027 ^{A}	

Values represent mean ± standard deviation of four replicates; values with the different capital letters are significant at $p < 0.01$; (*) *OD* - optical density; *T* - temperature

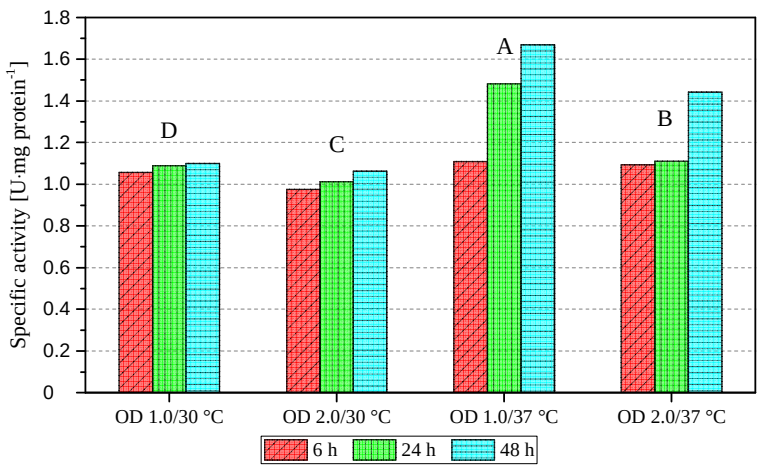

Fig. 2. Specific activity (CMCase) of *B. subtilis* BS-2 grown in CMC medium; Values with the different capital letters are significant at $p < 0.01$; *OD* - optical density; 30 °C, 37 °C - incubation temperature; 6 h, 24 h, 48 h - time of the bacterial culture

In this study, the *Bacillus subtilis* B-2 was able to produce cellulolytic enzymes. The CMCase activity and specific enzyme activity were recorded based on the U/cm^3 and

U/mg protein, respectively (data analysis shown in Table 2, Fig. 2). The temperature had the greatest influence on the CMCase production by the BS-2 strain. The highest values were obtained when the strain was grown at 37 °C. Furthermore, an increase of activity was found in supernatants obtained from the bacterial culture at initial density OD_{560} = 1.0. The level of CMCase activity ranged from 0.023 U/cm^3 (for 6 hour of CFs) to 0.035 U/cm^3 (for 48 hour of CFs) and from 0.83 U/mg protein to 1.31 U/mg protein (specific enzyme activity). Also, with the increase of incubation time of the bacterial culture from 6 o 48 h, there was a slight increase in CMCase activity and the specific activity was recorded (Table 2, Fig. 2).

Among all of the variants the highest level of activity up to 0.035 U/cm^3 (CMCase) and up to 1.31 U/mg protein was obtained for *B. subtilis* BS-2 culture at initial density 1.0 incubated at 37 °C for 48 h. No significant relationship between the antagonistic activity of *B. subtilis* BS-2 strain and the level of CMCase production was observed. The cell-free culture at OD_{560}= 2.0 incubated at 37 °C, which recorded the highest antifungal activity, exhibited the lowest CMCase activity (Table 2, Fig. 2).

Compared to the bacterial activity, the effect of *Mentha piperita* L. on mycelial growth was much weaker. It was observed that with the increase of peppermint oil concentration there was a deceleration of *B. cinerea* mycelial growth (Fig. 3).

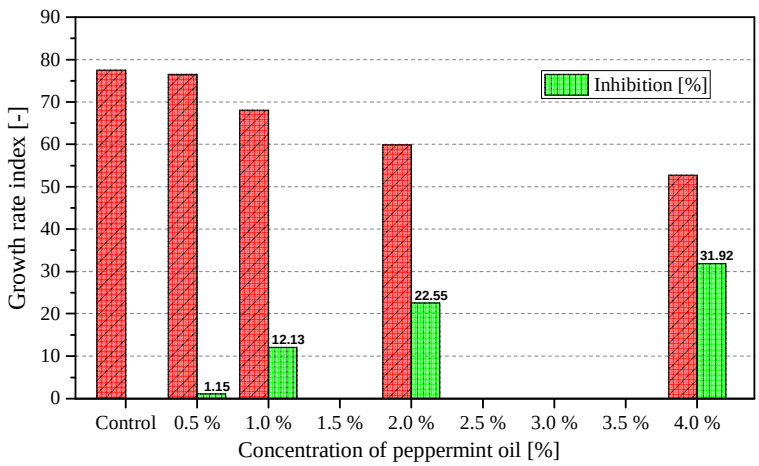

Fig. 3. Mycelial growth and inhibition of *B. cinerea* by peppermint oils at different concentrations; Values with the different capital letters are significant at $p < 0.01$; 0.5, 1.0, 2.0, 4.0 % - concentrations of peppermint oils

The growth rate index of mycelium was between 76.71 (at 0.5 % essential oil) and 52.83 (at 4.0 % essential oil) compared to the control 77.6. The peppermint oil reached maximum efficiency only at the highest concentration (4.0 %), where the mycelial growth of *B. cinerea* was reduced by 31.9 %. The lowest concentration of the peppermint oil (0.5 %) caused the least inhibition of the mycelial growth at only 1.15 % (Fig. 3).

The results obtained from our study are in agreement with the results of other authors who showed in vitro suppression of the growth of the *B. cinerea* in interaction with bacteria of the genus *Bacillus* [12, 24]. *B. subtilis* S1-0210 was found to be effective on the fungus and inhibited the mycelial growth of *B. cinerea* in kiwi fruit at about 66 % and in

strawberries at about 80 % [25]. *B. amyloliquefaciens* IMAUB1034 proved to have even greater inhibitory properties against *Botrytis* sp. (95.3 %) due to the synthesis of surfactin [26]. Shternshis et al. [2] tested strains of *Bacillus* under laboratory conditions and found they suppressed the growth of the phytopathogenic fungus *B. cinerea* in the range of 70 % (for *B. licheniformis*) to 100% (for *B. subtilis*, *B. amyloliquefaciens*). The inhibitory effect depended on the concentration of the tested bacterial suspension and was achieved at the highest concentration (10^6 CFU/ cm^3). Research undertaken by Wang et al. [27] showed that the inhibitory effect of *B. subtilis* EB-28 on mycelial growth of *Botrytis cinerea* reached about 70 %, similar to that observed by Torue et al. [18] as well as in this present study. Such a low mycelial growth could be related to the production of individual lipopeptides in the various bacterial cell growth phases [18]. Numerous scientific studies have reported that some *Bacillus* strains are capable of producing antibiotics with antifungal properties. These include the cyclic lipopeptides such as surfactin, subtilin, fengicyn [18, 28-30], and kanosamine [14, 31]. Furthermore these compounds were found capable of degrading the cell structure and increasing the permeability of the cell fungi. Some studies have shown that fengycins are synthesized by *B. subtilis* GA1 in the late logarithmic or early stationary phase [18], in contrast with surfactins which are produced during exponential growth, and iturins which are produced in the later stationary phase [32]. Recent evidence shows that *Bacillus* strains are capable also of producing volatile compounds with an antifungal nature against the plant pathogen [14, 33, 34]. Chen et al. [4] confirm this in their results, identifying 14 components produced by *B. subtilis* JA (including, among others, 2-ethyl-hexanol, ammonium acetate, tetramethyl-pyrazine). They show that the spore germination and the germ tube elongation of *B. cinerea* were significantly inhibited. Liu et al. [33] reported that most of *B. cinerea* spores exposed to the volatile compounds of the bacteria were cracked and the inclusions were browned. This may have been related to the water solubility of the volatile compounds diffused and dissolved in the culture medium.

Moreover, several *Bacillus* sp. strains are able to produce bioactive mycolytic enzymes, including proteases [34-36], β-1,3-glucanase, cellulose and chitinase [14, 34, 37], that inhibit the growth of phytopathogenic fungi through the lysis of cell walls. Several authors have reported on the effect of different growth parameters (the inoculum concentration, temperature, growth time, pH value) on carboxymethylcellulase activity of *Bacillus* sp. The extremely low level of CMCase activity was detected in the culture supernatant at early logarithmic phase of growth presumably due to the low density of cells and a rapid increase of enzyme level during log phase [38-41]. In this present study the maximum enzyme was found when the cultures were incubated at 37 °C. Similar observations have also been made by other researchers. In this study, the maximum cellulolytic efficiency was obtained at 40 °C by *B. subtilis*, *B. circulans* [40] and by *B. brevis* [42]. These findings are congruous with Dias et al. [43] who observed an increase in carboxymethylcellulase activity of *Bacillus* C1AC55.07 with an increase in temperature.

Several studies on the use of essential oils as fungicides have been published, and have suggested that natural plants could be used instead of synthetic fungicides or as fumigants [16, 44]. Essential oils from plants of the *Lamiaceae* family, or some of their constituents, were found to possess antifungal properties against the frequently tested phytopathogenic fungi [45]. Bouchra et al. [16] reported that essential oil from *M. piperita* was not effective at doses lower than 250 ppm, whereas *M. pulegium* inhibited the mycelial growth of *B. cinerea* by 4.1 % (50 ppm) to 58.5 % (250 ppm). Sesan et al. [17] observed the greatest

inhibition of mycelial growth and the sporulation of *B. cinerea* in the blackcurrant, ranging from 37.5 to 100 % (in vitro) and up to 70 % (in vivo). Among *Mentha* species, *M. pulegium* was shown to be the most potent in comparison to *M. spicata.* This study found that 100 % inhibition of *B. cinerea* growth occurred at concentrations above 350 ppm for *M. spicata* and 100 ppm for *M. pulegium*. Below these concentrations, essential oil efficacy ranged from 19.9 to 93.58 % and amounted to 45.6 %, respectively [46]. Multiple factors play an important role in determining antifungal activity including the origin of the plant and the conditions of its cultivation as well as its composition, and the structure and the amount of essential oil. Many scientists have confirmed that there is a relationship between the chemical structure of the most abundant compounds in the essential oils and antimicrobial activity [16, 45-47]. *Mentha piperita* L. and other *Mentha* species essential oils mainly contain menthol, menthon, carvone, and methyl acetate in different concentrations [16, 48, 49]. Earlier investigations have shown differences in antifungal activities of various mint oils as a result of the different percentages of these components. The inhibitory effect of this oil can be explained by a high content of carvone and menthol, which possess very strong fungistatic and fungicidal activities. On the other hand, a lower efficacy of this essential oil may be due to a high content of methyl acetate which causes a decrease of antifungal properties [44, 50-53]. Other studies suggest that menthol alone was found to be the compound responsible for the antifungal properties of peppermint oil, whereas menthon alone did not show any effect at all on the concentrations [54]. The chemical composition of the oil depends largely on plant cultivation. The content of the individual components may be affected by the origin of the plant, its age, and cultivation conditions, among other factors [50].

Conclusions

Among the biological agents tested, the supernatants obtained from *Bacillus subtilis* BS-2 were found to be more effective to a higher degree in the suppression of mycelial growth than peppermint oil. The mycelial growth was most inhibited by the 48 h bacterial culture at initial OD_{560} = 1.0 incubated at 30 °C (57.07 % maximum inhibition). Furthermore, the highest cellulolytic activity was demonstrated by the bacteria incubated for 48 hours at 37 °C. In contrast, the peppermint oil (*Mentha piperita* L.) had much less of an impact on the linear mycelial growth of *B. cinerea*. Use of the highest dose of oil (4 %) reduced the linear growth of mycelium by 31.9 %. Therefore, this strain of *B. subtilis* BS-2 may be an environmentally friendly agent for plant protection against *Botrytis cinerea.*

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