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Bacillus amyloliquefaciens PKM16 acts as an antagonist of white mold and an inducer of defense enzymes in tomato plants

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ABSTRACT. This study aimed to investigate the potential of rhizobacteria isolated from tomato plants to control *Sclerotinia sclerotiorum* and induce the activity of pathogenesis-related enzymes in Micro-Tom tomato plants. Three rhizobacterial isolates were evaluated to determine the most efficient antagonist agent, which was later identified by gene sequencing as *Bacillus amyloliquefaciens* PKM16. The antagonistic effects of *B. amyloliquefaciens* against *S. sclerotiorum* were assessed *in vivo* and *in vitro* using live and autoclaved cultures at concentrations of 0% (control), 20%, 30%, and 40% (v/v). The residual effects of four treatments (20% live culture, 20% autoclaved culture, a *Bacillus subtilis*-based commercial product, and autoclaved distilled water) on tomato plants inoculated with *S. sclerotiorum* were determined. The same treatments were also used to assess the myceliogenic germination of sclerotia and induction of plant defense enzymes (peroxidase, catalase, polyphenol oxidase, phenylalanine ammonia-lyase, and β-1,3-glucanase) in tomato plants. The live culture had a residual effect for 4 days and inhibited sclerotial germination by approximately 30%. Furthermore, live and autoclaved bacterial growth cultures stimulated enzyme activity. Therefore, *B. amyloliquefaciens* PKM16 was antagonistic to *S. sclerotiorum*, effectively inhibiting mycelial growth and activating defense mechanisms in Micro-Tom tomato plants.

Keywords: biological control; defense mechanisms; micro-tom; pathogenesis-related proteins; rhizobacteria; *Sclerotinia sclerotiorum.*

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Introduction

Tomato plants (*Solanum lycopersicum*) are the second most cultivated Solanaceae plants in Brazil with an estimated production of 3,956,687 t per year on 55,034 ha, mainly in the southeastern region (IBGE, 2021). Tomato production has great social and economic importance in the country as it generates jobs, income, and food and helps strengthen the family agriculture sector (Carvalho, Ponciano, Souza, Souza, & Sousa, 2014). However, tomato plants are susceptible to diseases and pests, which drastically reduce crop quality and yield. The fungus *Sclerotinia sclerotiorum* (Lib.) de Bary, the causal agent of white mold disease, is one of the main pathogens of tomato plants. This pathogen is difficult to control in infected fields, particularly when predisposing factors are present, such as high crop density, long periods of rainfall, high air humidity, and mild temperatures (15-21°C) (Reis, Costa, & Lopes, 2007).

The control of white mold is largely based on preventive measures to avoid field contamination. Crop rotation has limited efficacy as there are no resistant varieties or hybrids, and the pathogen is polyphagous (*S. sclerotiorum* can affect 75 plant families and more than 400 species) (Jaccoud Filho, Henneberg, & Grabicoski, 2017). Chemical control is not always efficient due to the difficulty in treating the soil, where the fungus forms survival structures known as sclerotia (Reis et al., 2007). Integrated management strategies that combine chemical, biological, and cultural control are the most commonly used approaches (Jaccoud Filho et al., 2017).

Plant growth-promoting rhizobacteria have been studied for their ability to produce phytohormones and antimicrobial compounds and activate latent plant defense mechanisms involving pathogenesis-related proteins (Li et al., 2015; Singh, Yadav, Chaudhary, Rana, & Sharma, 2016; Pascholati & Dalio, 2018). Motivated by the increasing demand for control agents that can be applied to agroecological farming systems,

several studies have been conducted in recent years on the use of rhizobacteria as biological control agents and elicitors of plant resistance (Toledo, Costa, Bacci, Fernandes, & Souza, 2011). This study aimed to investigate the potential of rhizobacterial isolates to control *S. sclerotiorum* and activate resistance mechanisms in tomato plants.

Material and methods

Experiments were carried out in a greenhouse from March 2018 to December 2019. Rhizobacteria were isolated from the rhizosphere of tomato plants by serial dilution and plating on nutrient agar. Three selected isolates (RB1, RB2, and RB3) stood out among others that produced large inhibition zones against microorganisms, such as fungi and bacteria. Colonies were streaked on nutrient agar until pure cultures were obtained. Isolates were maintained in nutrient broth and nutrient agar in a biochemical oxygen demand (BOD) chamber at 28 ± 2°C in the dark.

S. sclerotiorum was obtained from the Phytopathology Laboratory of the State University of Ponta Grossa (Ponta Grossa, Paraná State, Brazil), identified by the code CMM-2969 in the Culture Collection of Phytopathogenic Fungi Maria Menezes. The fungus was reactivated in potato dextrose agar (PDA) in a BOD chamber at 24 ± 2°C with a 12h photoperiod. The control treatment consisted of only phytopathogens on one side of the plate.

The antagonistic effects of rhizobacterial isolates against *S. sclerotiorum* were assessed using the direct confrontation method (Dennis & Webster, 1971). A disk of 8 mm in diameter was cut from the fungal culture and placed approximately 1 cm from the margin of a PDA plate, and isolates were streaked close to the opposite margin. The plate was incubated in a BOD chamber at 24 ± 2°C with a 12h photoperiod.

A similar test was performed to assess the inhibitory effects of the volatile compounds produced by the isolates; however, the PDA plate was subdivided into sections to avoid direct contact between microorganisms. The plate was incubated in a BOD chamber at 24 ± 2°C with a 12h photoperiod. In both tests, mycelial growth was measured daily at two diametrically opposite points after 24h of incubation. These measurements were used to calculate the area under the mycelial growth curve (AUMGC) (Shaner & Finney, 1977) and the percentage of mycelial growth inhibition.

The experiments were repeated and conducted in a completely randomized design with seven replicates. Each plate was considered an experimental unit. Data were subjected to analysis of variance, and means were compared using the Scott-Knott test at $p < 0.05$ with R software (R Core Team, 2020).

Bacterial isolate identification

The isolate with the highest inhibitory activity against *S. sclerotiorum* in the direct confrontation and volatile compound assays was sent to the Laboratory of Microbial Physiology and Genetics of the Federal University of Lavras (Minas Gerais State, Brazil) for identification by gene sequencing. The isolate was identified as *Bacillus amyloliquefaciens* PKM16 (98% similarity, GenBank accession number KF732989.1) and deposited in the Culture Collection of Agricultural Microbiology (code CCMA-2020). The isolate was maintained in nutrient broth and nutrient agar in a BOD chamber at 28 ± 2°C in the dark.

In vitro mycelial inhibition of *S. sclerotiorum* and reduction of white mold severity in tomato plants with different live and autoclaved culture concentrations

Nutrient broth was inoculated with the rhizobacterial strain and incubated at 28 ± 2°C in the dark for 48h. Then, 1 mL of rhizobacterial culture was adjusted to an optical density (OD_{600}) of 0.6 (1×10^8 ufc), mixed with 210 mL of nutrient broth, and incubated at 28 ± 2°C and 180 rpm for 48h. *In vitro* and *in vivo* assays were performed to assess the antagonistic action of the live culture (produced metabolites + viable bacterial cells) and autoclaved culture (produced metabolites + non-viable bacterial cells) of *B. amyloliquefaciens* PKM16 at different concentrations (0, 20, 30, 40, and 50%) against *S. sclerotiorum*.

For the *in vitro* assay, the live culture was added and homogenized with autoclaved PDA before solidification. The autoclaved culture was obtained by mixing active cultures with PDA and autoclaving at 121°C (1 atm) for 25 min. The media were poured into Petri dishes, and the plates were allowed to solidify. An 8 mm mycelial disc of *S. sclerotiorum* (previously grown in PDA for 7 days) was placed at the center of each plate, and the plates were incubated at 20 ± 2°C. Mycelial growth was analyzed every 24h by measuring the distance between diametrically opposite points. Measurements were conducted until the pathogen reached the edges of the control plate. The results are presented as the AUMGC and the percentage of mycelial growth inhibition.

In vivo experiments were conducted using Micro-Tom tomato plants, which are considered an excellent model for the study of biological processes. Micro-Tom tomato plants are a miniature tomato cultivar that produces viable fruits and seeds in 70-90 days when grown in 50–150 mL pots (Meissner et al., 1997). Seeds from the State University of Western Paraná were sown in 128-cell polystyrene trays containing a commercial substrate (MecPlant®; Telêmaco Borba, Paraná State, Brazil). At 22 days after sowing, seedlings were transplanted to 500 mL plastic pots containing a 1:1:2 (v/v/v) mixture of soil, sand, and potting substrate fertilized with 0.5 g of 10-10-10 NPK fertilizer. Live and autoclaved cultures were diluted to the appropriate concentrations with autoclaved distilled water containing 0.01% Tween 20. Treatments were applied when the plants had developed five fully developed true leaves (29 days after sowing). The entire plant was sprayed to the point of runoff (200 mL).

Fungal cultures grown for 7 days on PDA were used as inocula. Inoculation was performed at 72h after treatment (HAT) with bacterial cultures. The third true leaf was cut 2 cm from the main stem, and a micropipette tip containing the mycelial disc was inserted into the cut stem. After inoculation, the plants were kept in a humidity chamber at 18 ± 2°C to stimulate disease development (Barros, Fonseca, Balbi-Peña, Pascholati, & Peitl, 2015).

Disease progression was analyzed after 2 days of inoculation (when the disease had reached the main stem). The lesion length was measured with a caliper every 24h for 5 days. Results are expressed as the area under the disease progress curve (AUDPC) and the percentage of lesion inhibition.

The experiments were repeated and conducted in a completely randomized design with six replicates. Each plate was considered an experimental unit, and each plant was considered an experimental unit. Data were subjected to analysis of variance, and means were compared using the Scott-Knott test at $p < 0.05$ with R software (R Core Team, 2020).

Residual effects of live and autoclaved culture treatments

Treatments were applied at 29 days after sowing (when the tomato plants had five true leaves). The following four treatments were used: autoclaved distilled water (control), 20% live culture, 20% autoclaved culture, and a biological commercial product containing *Bacillus subtilis* (minimum of 1×10^9 CFU g^{-1} asset), indicated for *S. sclerotiorum* control. The commercial product was applied at the manufacturer's recommended dose (2–4 L ha^{-1}; spray volume of 500–1,000 L ha^{-1}). Plants were inoculated with *S. sclerotiorum* at 0, 24, 48, 72, 96, 122, and 144 HAT using the micropipette tip method and placed in a humidity chamber at 18 ± 2°C. Disease progression was analyzed after 2 days of inoculation (when the disease had reached the main stem). The lesion length was measured with a caliper every 24h for 5 days, (a total of 11 assessments per plant). Results are expressed as the AUDPC.

The experiments were repeated and conducted in a completely randomized design with six replicates. Each plant was considered an experimental unit. Data were subjected to analysis of variance, and means were compared using the Scott-Knott test at $p < 0.05$ with R software (R Core Team, 2020).

Myceliogenic germination of sclerotia

S. sclerotiorum was grown on PDA for 7 days at 20°C with a 12h photoperiod. Then, the fungus was transferred to new PDA plates and kept under the same conditions for 20 days to induce the production of sclerotia. Sclerotia were collected, disinfected with 70% alcohol for 60 s followed by 2% (v/v) sodium hypochlorite for another 60 s, and rinsed with autoclaved distilled water (Marcuzzo & Schuller, 2014). Disinfected sclerotia were immersed for 10 min in one of the following treatments: 20% live culture, 20% autoclaved culture, commercial product, and autoclaved distilled water. After treatment, sclerotia were placed in Petri dishes containing Neon medium (1 L PDA + 50 mg bromophenol blue) and incubated at 20 ± 2°C with a 12h photoperiod to stimulate germination (adapted from Napoleão, Nasser, Lopes, & Cafe Filho, 2006). The number of sclerotia that germinated and produced hyphae was recorded daily.

The experiment was repeated and carried out in a completely randomized design with six replicates. Each plate containing 10 sclerotia was used as the experimental unit. Data were subjected to Kaplan-Meier (1958) survival analysis (Dudley, Wickham, & Coombs, 2016) using the "survfit" function of the "survival" package in R software. Survival curves were compared using the G-rho test (log-rank test) (Ihaka & Gentleman, 1996).

Activity of plant defense enzymes

Treatments (20% live culture, 20% autoclaved culture, commercial product, and autoclaved distilled water) were applied when plants had five true leaves (29 days after sowing), and fungal inoculation was performed at 48 HAT using the micropipette tip method. For the analysis of enzyme activity, one leaf from each plant was collected before treatment application and at 24, 48, 72, 96, 120, and 144 HAT (always at the same time in the morning). During sampling, the collected leaves were placed between two sheets of aluminum foil and stored on ice. In the laboratory, the samples were weighed and stored at −80°C until enzyme extraction.

For enzyme extraction, 100 mg of leaves were ground in liquid nitrogen with a mortar and pestle and homogenized with 4 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 1% (w/w) polyvinylpyrrolidone. The macerate was transferred to microtubes and centrifuged at 14,500 rpm for 30 min. at 4°C. The supernatant was transferred to new microtubes and stored at −80°C (Lusso & Pascholati, 1999).

Total protein was quantified using the Bradford (1976) method. Guaiacol peroxidase (POD), catalase (CAT), polyphenol oxidase (PPO), β-1,3-glucanase (GLU), and phenylalanine ammonia-lyase (PAL) activities were determined as described by Lusso and Pascholati (1999), Góth (1991) with modification by Tománková, Luhová, Petrivalský, Peè, and Lebeda (2006), Duangmal and Apenten (1999), Vogelsang and Braz (1993), and Umesha (2006), respectively.

The experiment was carried out in a completely randomized design using a [(4 × 6) + 1] factorial arrangement, consisting of water (control), live culture, autoclaved culture, and a commercial product at different times (24, 48, 72, 96, 120, and 144 HAT) with an additional condition designed to assess the effect of collection time on non-treated plants. Seven replications were performed, and each plant was considered an experimental unit. Enzyme assays were performed in duplicate. Data were subjected to analysis of variance. Differences ($p < 0.05$) between treatments were assessed using the Scott-Knott test, and differences ($p < 0.05$) between treated and non-treated plants were analyzed using Dunnett's test. All statistical analyses were performed with R software (R Core Team 2020).

Results

In vitro mycelial inhibition of *S. sclerotiorum*

Direct confrontation between rhizobacteria and *S. sclerotiorum* revealed that two isolates (RB1 and RB2) were able to inhibit mycelial growth. Their AUMGC values (RB1 = 9.50, RB2 = 12.21) differed from each other and from the value of the control (15.23). RB3 had an AUMGC value of 14.04, which was not significantly different from the value of the control. The inhibition zone of RB3 was much smaller than that of other isolates, equivalent to a mycelial growth inhibition of less than 10%. RB1 showed the largest inhibition zone and inhibition percentage (40.27%) (Table 1). This isolate was also the most effective in reducing the final mycelial growth (Figure 1a).

Under the tested conditions, rhizobacterial isolates did not produce volatile compounds capable of inhibiting *S. sclerotiorum* mycelial growth, as demonstrated by the lack of difference in AUMGC values between the isolates and the control (Table 1).

Table 1. Area under the mycelial growth curve (AUMG) of *Sclerotinia sclerotiorum* grown in potato dextrose agar in direct confrontation with rhizobacterial isolates or under exposure to rhizobacterial volatile compounds.

Treatments	Volatile compounds	Direct confrontation	
	AUMGC	AUMGC	IMG*
RB1	4.55 ^{ns}	9.5 ^{a}	40.27
RB2	5.95 ^{ns}	12.21 ^{b}	25.97
RB3	5.50 ^{ns}	14.04 ^{c}	9.38
Control	5.20 ^{ns}	15.23 ^{c}	-
CV %	18.56	11.38	

Means followed by the same letter in the column did not differ by the Scott-Knott test ($p < 0.05$). *Inhibition of mycelial growth.

Based on the analysis of growth characteristics, cell morphology, colony morphology, and gram-stained cells, RB1 and RB2 belonged to the genus *Bacillus*. Cells were rod-shaped, elongated, and gram positive, forming white, mucoid, and rough colonies with irregular edges. The cells multiplied rapidly in a liquid medium (Rabinovitch & Oliveira, 2015). As RB1 showed the best results against *S. sclerotiorum*, it was analyzed by gene sequencing, which confirmed its identity as *Bacillus amyloliquefaciens*. The isolate was then

assessed in further experiments. RB3 was characterized by coccus-shaped cells, non-mucoid white colonies, and slow growth, differing greatly from other isolates. The results indicated that it likely belonged to another genus of soil-dwelling bacteria. However, RB2 and RB3 were not identified in the present study.

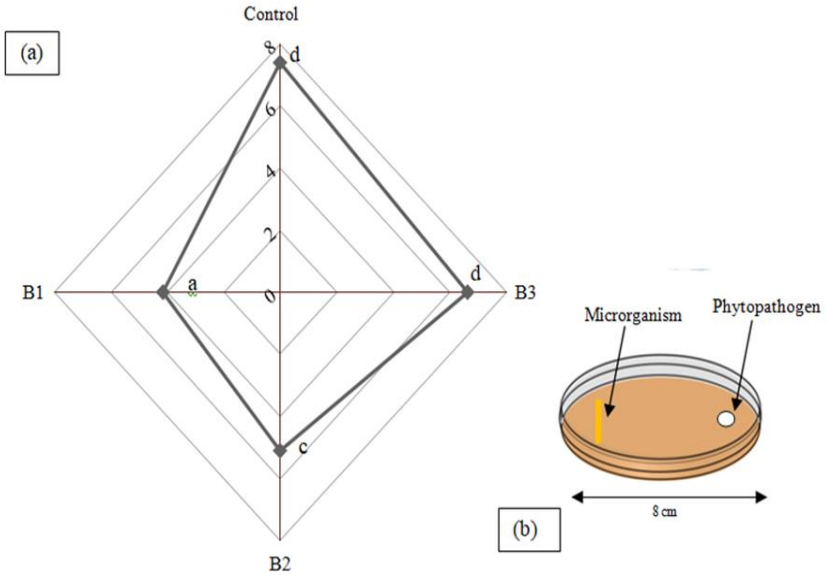

Figure 1. (a) Growth of the phytopathogen *S. sclerotiorum* in the presence of rhizobacterial isolates. Means followed by the same letter did not differ by the Scott-Knott test ($p < 0.05$). (b) Direct confrontation test method.

In vitro mycelial inhibition of *S. sclerotiorum* and reduction of white mold severity in tomato plants with different live and autoclaved culture concentrations

In the *in vitro* assay, the live culture completely inhibited fungal development at all concentrations (data not shown). The autoclaved culture inhibited fungal development only at concentrations above 30%; at 50%, the treatment resulted in the lowest AUMGC value (7.50) and the highest growth inhibition (32%) (Figure 2).

In the *in vivo* assay, the control had an AUDPC value of 16. The AUDPC was significantly different between the live culture and the control but not between different live culture concentrations. The mean AUDPC value and growth inhibition rate were 11 and 32%, respectively. The autoclaved culture decreased the AUDPC to 12 at 20% and to 9 at 30%, resulting in a growth inhibition of 33 and 25%, respectively. At these concentrations, the effect of the autoclaved culture was similar to that of the live culture. However, the autoclaved culture at 40 and 50% allowed disease development, which was not significantly different from the effect of the control (Figure 3).

The live culture of *B. amyloliquefaciens* PKM16 did not completely inhibit disease progression in tomato plants. The dose-dependent response of *S. sclerotiorum* to the autoclaved culture observed in the *in vitro* assay was not observed in the *in vivo* assay. The results showed that 20% was the optimal concentration for live and autoclaved cultures.

Residual effects of live and autoclaved culture treatments

The AUDPC was low for plants inoculated with the pathogen at 48 HAT. The live culture and the commercial product showed the best results (inhibition of 35%); they did not differ from each other but differed from the control (autoclaved distilled water). The autoclaved culture also reduced the AUDPC in plants inoculated with the pathogen at 48 HAT. For plants inoculated at 24 HAT, the commercial product showed the best results. Treatment with the live culture resulted in the greatest reduction in disease development in plants inoculated at 72 HAT. Disease progression was slow in plants inoculated at 96 HAT; however, only the live culture and the commercial product differed from the control. In plants inoculated before treatment and at 120 HAT, no differences were observed between treatments (Table 2).

The results showed that the residual effect of the live culture persisted for 48 to 96h. Therefore, the live culture should be reapplied every 2 to 4 days for white mold management in Micro-Tom tomato plants. After 120h, the live culture did not affect white mold severity. The residual effect of the autoclaved culture lasted for 48h; thus, it should be applied every 2 days.

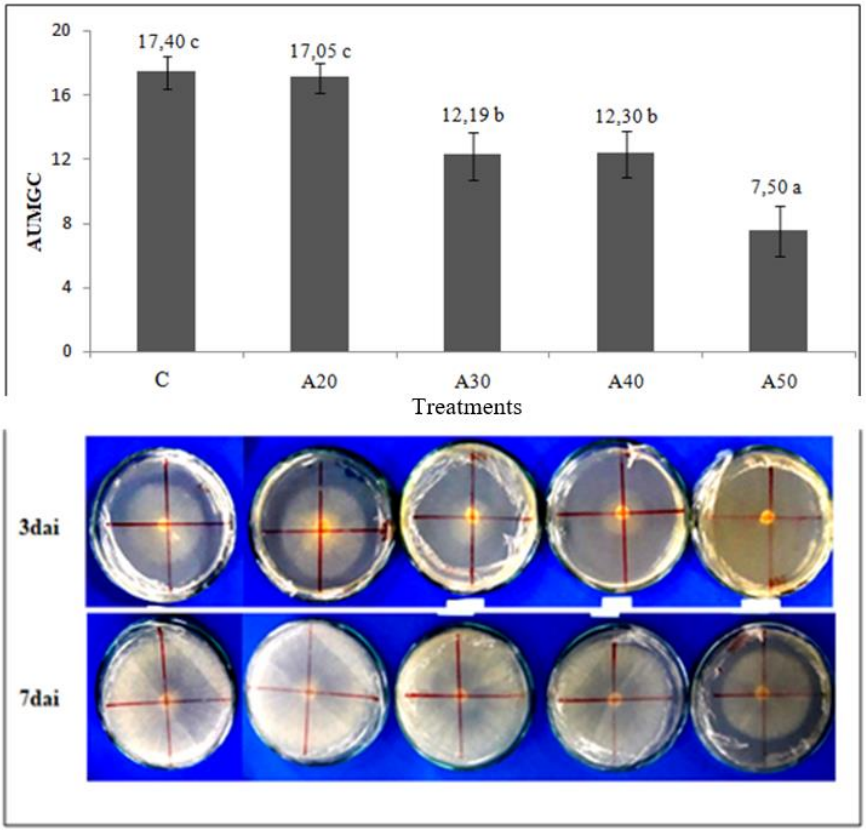

Figure 2. Area under the mycelial growth curve (AUMGC) (7 days after incubation) of *S. sclerotiorum* inoculated in potato dextrose agar in the presence of the autoclaved culture of *B. amyloliquefaciens* PKM16. Means followed by the same letter did not differ by the Scott-Knott test ($p < 0.05$). CV(%) = 19.73.

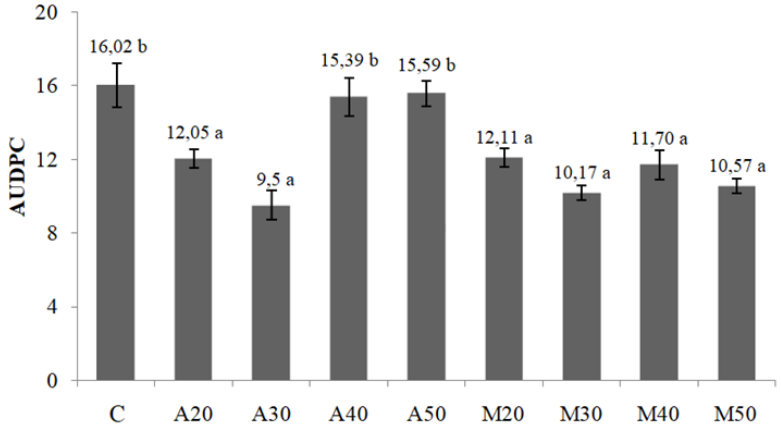

Figure 3. Area under the disease progress curve (AUDPC) of white mold (*S. sclerotiorum*) in tomato plants treated with live (M) or autoclaved (A) cultures of *B. amyloliquefaciens* PKM16. Means followed by the same letter did not differ by the Scott-Knott test ($p < 0.05$). CV(%) = 22.51.

Table 2. Area under the disease progress curve (AUDPC) of white mold in tomato plants treated with live and autoclaved cultures of *B. amyloliquefaciens* PKM16, a *B. subtilis*-based commercial product, or autoclaved distilled water and inoculated with *S. sclerotiorum* at different times after treatment.

Inoculation time (HAT)	Autoclaved culture	Live culture	Commercial product	Control (water)
0h	19.14 ^{Ba}	18.80 ^{Ca}	17.42 ^{Ca}	19.50 ^{Ba}
24h	15.67 ^{Ab}	14.98 ^{Bb}	12.93 ^{Aa}	16.88 ^{Ab}
48h	13.88 ^{Ab}	11.19 ^{Aa}	11.36 ^{Aa}	17.52 ^{Ac}
72h	16.31 ^{Ab}	13.71 ^{Ba}	15.96 ^{Bb}	17.79 ^{Ab}
96h	16.31 ^{Ab}	13.04 ^{Ba}	14.56 ^{Ba}	16.32 ^{Ab}
120h	18.65 ^{Ba}	19.99 ^{Ca}	20.77 ^{Da}	20.82 ^{Ba}
144h	17.20 ^{Ba}	20.72 ^{Cb}	20.77 ^{Db}	18.75 ^{Ba}
C.V. (%)		12.66%		

Means followed by the same uppercase letter in a column and lowercase letters in a row did not differ by the Scott-Knott test ($p < 0.05$).

Myceliogenic germination of sclerotia

Sclerotia treated with the control (autoclaved distilled water) began to germinate on day 3 after inoculation and achieved almost complete germination by day 5 (Figure 4a). Sclerotia treated with the autoclaved culture began to germinate later than the control but was completely germinated by day 7 (Figure 4b), which was statistically significant. Sclerotia treated with the commercial product based on *B. subtilis* showed a curve similar to that of sclerotia treated with the autoclaved culture despite a lower germination rate (Figure 4c); both of them differed from the control. Treatment with the live culture showed the best results; at 7 days after inoculation, the germination rate was approximately 30% compared with the complete sclerotial germination of the other treatment groups (Figure 4d).

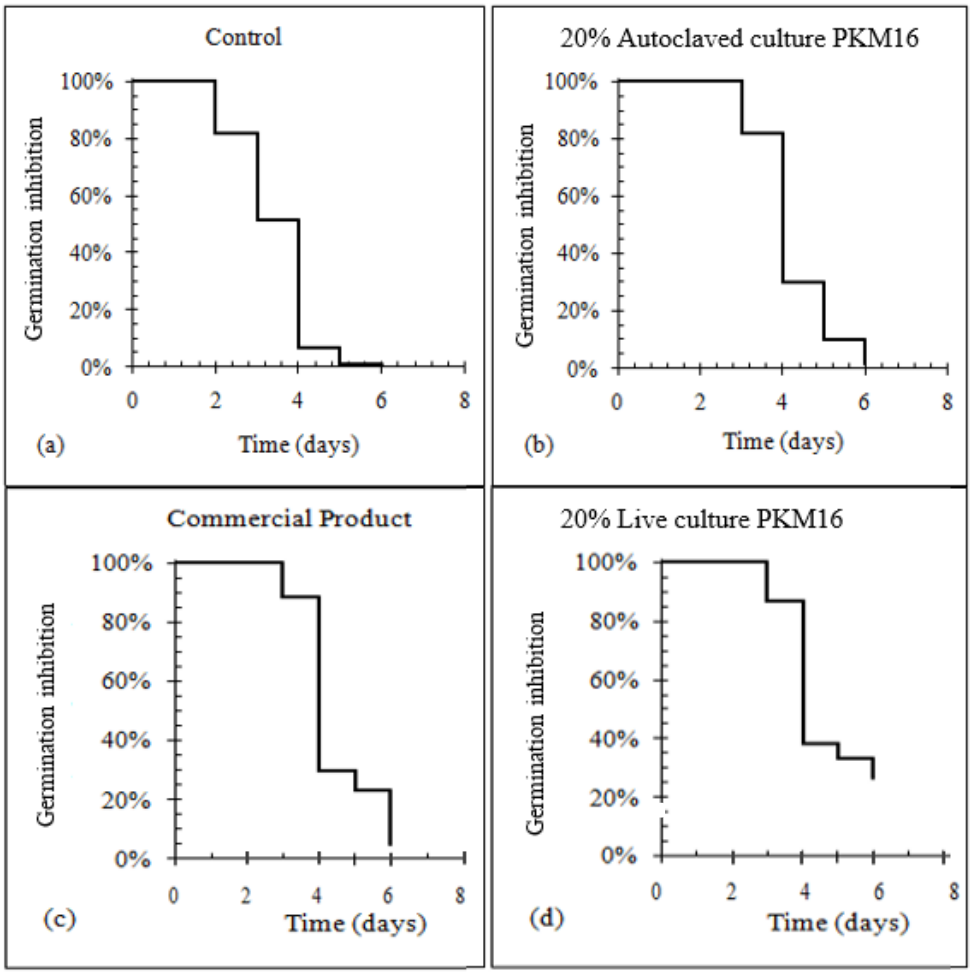

Figure 4. Kaplan-Meier curves of the inhibition of the myceliogenic germination of the sclerotia of *S. sclerotiorum* by treatment with (a) autoclaved distilled water, (b) 20% autoclaved culture of *B. amyloliquefaciens* PKM16, (c) a *B. subtilis*-based commercial product, or (d) 20% live culture of *B. amyloliquefaciens* PKM16.

Activity of plant defense enzymes

POD activity was considerably higher at 48h after pathogen inoculation (96 HAT) when fungal colonization began. The highest activity was observed in plants treated with the autoclaved culture, followed by water, the commercial product, and the live culture. Enzyme activity was markedly increased at 96h after pathogen inoculation (144 HAT) and was the highest in plants treated with the commercial product, followed by the autoclaved culture, water, and the live culture (Figure 5a). As the POD activity pattern of plants treated with water was similar to that of plants treated with biological agents, POD activity may be associated with pathogen colonization (Amorim & Pascholati, 2018).

CAT activity was also increased significantly after pathogen inoculation. At 24h after inoculation (72 HAT), higher CAT activity was observed in plants treated with the commercial product, live culture, and autoclaved culture. However, the highest CAT activity was detected at 96h after inoculation (144 HAT) in plants treated with the commercial product, followed by plants treated with the autoclaved culture (Figure 5b).

PAL activity was the highest in plants treated with water and autoclaved culture at 96h after inoculation (144 HAT). Although this peak may be attributed to pathogen infection, PAL activity was also significantly induced on day 3 after treatment with *B. amyloliquefaciens* (Figure 5c). Peak PPO activity was observed at 24h after inoculation (72 HAT) in plants treated with the live culture. Live culture treatment also resulted in high enzyme activity at 48h after inoculation (96 HAT); however, the effects of other treatments were not significantly different (Figure 5d).

In addition to POD, CAT, PPO, and PAL, GLU may play an important role in disease resistance signaling. GLU activity was increased from 48 HAT. At 24h after inoculation, plants treated with the commercial product showed the highest GLU activity. Peak GLU activity was observed at 96h after inoculation (144 HAT) in plants treated with the live culture (Figure 5e).

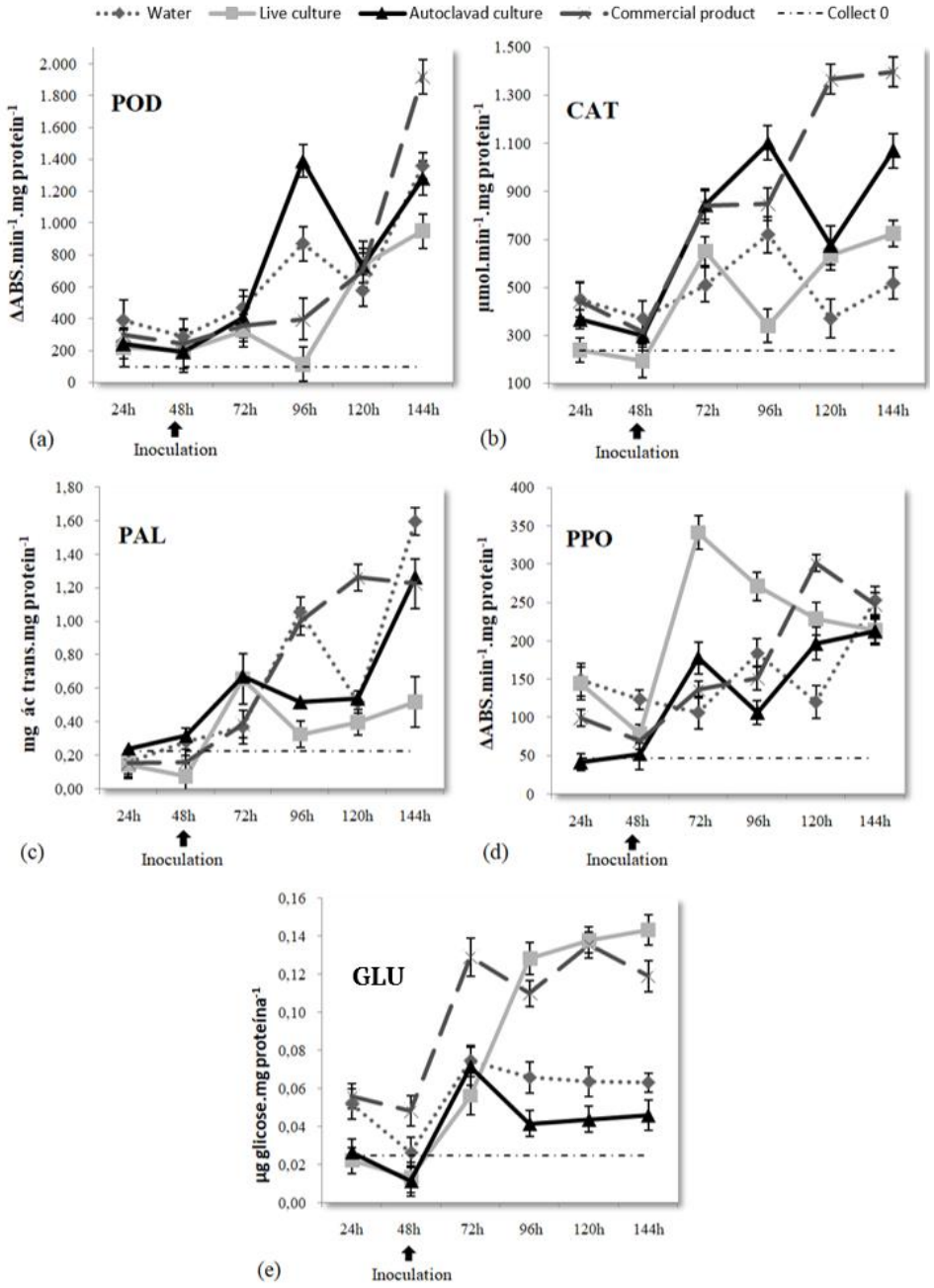

Figure 5. (a) Guaiacol peroxidase (POD) (ΔABS.min^{-1}.mg protein^{-1}), (b) catalase (CAT) (µmol.min^{-1}.mg protein^{-1}), (c) phenylalanine ammonia-lyase (PAL) (mg transcinnamic acid mg protein^{-1}), (d) polyphenol oxidase (PPO) (ΔABS.min^{-1}.mg protein^{-1}), and (e) β-1,3-glucanase (GLU) (µg glucose.mg protein^{-1}) activities in Micro-Tom tomato plants at 24, 48, 72, 96, 120, and 144h after treatment (HAT) with 20% live culture of *B. amyloliquefaciens* PKM16, 20% autoclaved culture of *B. amyloliquefaciens* PKM16, a *B. subtilis*-based commercial product, or autoclaved distilled water (control). Tomato plants were inoculated with *S. sclerotiorum* at 48 HAT. Error bars represent the standard deviation. The comparison of each treatment group at a specific time point and each time point in the same treatment group was performed using the Scott-Knott test ($p < 0.05$).

Discussion

Direct confrontation and volatile compounds

Rhizobacteria can inhibit the development and spread of pathogens by competing for resources and producing soluble or volatile antimicrobial compounds (Vinodkumar, Nakkeeran, Renukadevi, & Malathi, 2017; Guevara-Avendaño et al., 2018). Several studies have reported the emission of volatile antimicrobial compounds by rhizobacteria (Méndez-Bravo et al., 2018, Guevara-Avendaño et al., 2019), including against *S. sclerotiorum* (Giorgio, Angelo, Pietro, & Nicola, 2015). Nevertheless, as highlighted by Mariano (1993), test sensitivities may differ according to the type of analysis conducted and the variables analyzed, and control efficiency may vary with the phytopathogen and antagonistic isolates. Braga Junior et al. (2017) observed that the *in vitro* effects of *B. subtilis* on *Fusarium subglutinans*, *Curvularia lunata*, and *Bipolaris* sp. differed according to the test performed. The authors performed four assays and found that in some tests, bacteria were able to inhibit mycelial growth by producing volatile and/or thermostable metabolites; however, in other tests, these effects were not observed.

Vieira, Vieira, Sousa, and Mendonça (2016) investigated the action of *B. subtilis* against *Fusarium solani* f. sp. *phaseoli*, *Fusarium oxysporum* f. sp. *phaseoli*, *Macrophomina phaseolina*, and *Rhizoctonia solani* and found that the bacterial isolate BSV-05 did not produce volatile metabolites. Michereff, Silveira, and Mariano (1994) evaluated the activity of *B. subtilis* (BSV-05) isolates against *Colletotrichum graminicola* and observed that thermostable, soluble, and non-volatile bacterial metabolites were antagonistic to phytopathogens. These studies demonstrated that *Bacillus* isolates could produce metabolites with different properties and modes of action.

Soluble antibiotics, such as lipopeptides, are produced at different concentrations by a variety of rhizobacteria. These compounds may vary in their pathogen control efficiency (Yarzabal & Chica, 2019). Li et al. (2015) showed that different strains of *B. amyloliquefaciens* produced different types of lipopeptides in the presence of different pathogens. An *in vitro* study (Chowdhury, Hartmann, Gao, & Borriss, 2015) found that the antifungal activity of some *Bacillus* species, including *B. amyloliquefaciens*, may be attributed to the non-ribosomal synthesis of the lipopeptides bacillomycin D, fengycin, and surfactin. Bacillomycin D was observed to have high *in vitro* activity against *Fusarium* pathogens (Li et al., 2015). Li et al. (2014) showed that the activity of *B. amyloliquefaciens* SQR9 against *S. sclerotiorum*, *R. solani*, and *F. solani* may be mediated by surfactin, whereas the activity of the strain against *Verticillium dahliae*, *F. oxysporum*, *F. solani*, and *Phytophthora parasitica* may be mainly mediated by fengycin.

The action of rhizobacteria against phytopathogens may also be attributed to the activity of hydrolytic enzymes on the microbial cell wall. Rocha and Moura (2013) observed that the rhizobacteria of the genus *Pseudomonas*, *Bacillus*, and *Streptomyces* produced antimicrobial enzymes that reduced the *in vitro* mycelial growth of *R. solanacearum* and *F. oxysporum* f. sp. *lycopersici*. Some *Bacillus* isolates produced chitinase and lipase and were able to solubilize calcium phosphate.

The preliminary *in vitro* assay revealed that RB1, identified as *B. amyloliquefaciens* PKM16, had significant fungitoxic activity. Biocontrol agents may not only affect phytopathogens but may also induce disease resistance in plants (Huang et al., 2016; Singh et al., 2016).

In vitro mycelial inhibition of *S. sclerotiorum* and reduction of white mold severity in tomato plants with different live and autoclaved culture concentrations

Around 8.5% of the genome of *B. amyloliquefaciens* is involved in the synthesis of secondary metabolites, such as lipopeptides (surfactin, iturin, bacillomycin D, and fengycin), polyketides (macrolactin and bacillaene), volatile compounds (acetoin), and hydrolytic enzymes (cellulase, amylase, protease, and chitinase). These compounds may be directly associated with the pathogen-inhibiting and resistance-inducing effects of *B. amyloliquefaciens* in plants (Chen et al., 2009).

Live and autoclaved cultures had different effects on *S. sclerotiorum* mycelial growth. It is possible that some of the antimicrobial compounds produced by *B. amyloliquefaciens* PKM16 were thermolabile, i.e., they may have degraded during autoclaving (121°C, 1 atm, 25 min.). As a result, the autoclaved culture likely contained lower concentrations of these compounds. The live culture may also have performed better than the autoclaved culture because live bacteria can compete with fungi for nutrients and space. Furlani, Camargo, Panizzi, and Pereira (2007) showed that autoclaved microbial cultures had no *in vitro* effects on *Colletotrichum acutatum*. On the other hand, a filtered culture solution was highly effective in inhibiting

mycelial growth. In this case, as the solution was filtered, antagonistic effects may most likely be attributed to the action of thermolabile metabolites instead of microbial competition for resources.

Gomes, Grigoletti Junior, and Auer (2001) investigated the *in vitro* effects of autoclaved and non-autoclaved *B. subtilis* broth cultures at concentrations of 10, 50, and 100% on the germination of *Cylindrocladium spathulatum* conidia; inhibition was greater at culture concentrations of 50% and 100%. In contrast, in the present study, a bacterial culture concentration of 20% was sufficient to completely inhibit *S. sclerotiorum*. However, Gomes et al. (2001) found that the non-autoclaved culture had a higher inhibitory activity, as observed in the current study. Li et al. (2015) quantified *F. oxysporum*, *Botrytis cinerea*, and *Alternaria* spp. in the cucumber rhizosphere treated with 1 and 10% *Bacillus* and obtained better results with 10% *Bacillus*.

In vitro results showed that *B. amyloliquefaciens* PKM16 was effective in inhibiting the growth of *S. sclerotiorum*. Although *in vitro* assays are valuable preliminary analyses, *in vivo* assays are essential for choosing the best microbial agent concentration as pathogen-host interactions and uncontrollable variables, such as temperature and humidity, are taken into account (Amorim, Rezende, & Bergamin Filho, 2018). Maciel, Walker, Muniz, and Araujo (2014) investigated the antagonistic action of *B. subtilis* against *Fusarium sambucinum* in *Pinus elliottii*. *In vitro* and *in vivo* assays confirmed its antagonistic effects; however, the antifungal activity of *B. subtilis* was lower in the *in vivo* assay. Rocha and Moura (2013) performed *in vivo* assays in two different seasons and found that a *Bacillus* isolate was effective only under mild climatic conditions (less favorable to the pathogen). This result demonstrated the influence of climate and plant factors on the action of biocontrol agents (Amorim et al., 2018). In the present study, *in vitro* and *in vivo* assays showed that 20% was the optimal concentration for live and autoclaved cultures.

Residual effects and myceliogenic germination of sclerotia

The study was conducted under temperature and humidity conditions that were favorable for pathogen growth. Therefore, it is likely that the residual effect would have been greater under less favorable conditions. In comparison with the autoclaved culture, the live culture of *B. amyloliquefaciens* PKM16 had a higher residual effect. As discussed above, this may likely be attributed to the thermosensitivity of metabolites and competition with live microorganisms for resources.

The live culture was also highly effective in inhibiting the myceliogenic germination of sclerotia. Giorgio et al. (2015) found that *Pseudomonas* and *Bacillus* isolates produced volatile compounds with the potential to inhibit *S. sclerotiorum* sclerotial germination. Overall, the *B. subtilis*-based commercial product and *B. amyloliquefaciens* PKM16 culture showed high inhibition efficiencies, demonstrating the antimicrobial potential of *Bacillus*.

Sclerotia are highly resistant to adverse environmental factors and may survive in the soil for several years even in the absence of a host (Lane, Denton-Giles, Derbyshire, & Kamphuis, 2019), which make it difficult for white mold management in the field. Therefore, the inhibitory effects of *B. amyloliquefaciens* PKM16 on sclerotial germination suggest that this strain has great potential to control the fungus under field conditions.

Plant defense enzymes

As a plant defense mechanism, pathogenesis-related proteins are activated by biotic or abiotic inducers. Phytopathogens can also trigger defense responses when they infect plants (Pascholati & Dalio, 2018). Previous studies have demonstrated the efficiency of different strains of *B. amyloliquefaciens* in inducing the expression of genes related to plant protection in the absence or presence of pathogens. PR-1 genes were highly expressed in cucumber leaves on days 2 and 3 after treatment with *B. amyloliquefaciens* FJ02 (Li et al., 2015). Micro-Tom tomato plants treated with *B. amyloliquefaciens* WF02 highly expressed pathogenesis-related genes 1 day after inoculation with *R. solanacearum* (Huang et al., 2016).

In the present study, both CAT and POD showed peak activity at 96h after pathogen inoculation (144 HAT). Peak enzyme activity indicated that the fungus was growing rapidly. Treatments induced CAT and POD activities; however, their effects were masked by the influence of *S. sclerotiorum*, which is extremely aggressive towards Micro-Tom tomato plants. The fungus secretes cell wall-degrading enzymes, leading to severe stress conditions. As a result, the plants synthesize reactive oxygen species (ROS), such as H_2O_2, which cause oxidative damage to proteins, lipids, and nucleic acids (Pascholati & Dalio, 2018).

Plants use enzymatic and non-enzymatic antioxidant systems to combat oxidative stress caused by ROS (Reczek & Chandel, 2015). According to Sharma, Jha, Dubey, and Pessarakli (2012), the oxidative stress

response is the initial plant defense response to phytopathogens. The synthesis of antioxidant enzymes, such as CAT and POD, increases under these conditions.

POD oxidizes organic material by eliminating H_2O_2 in the cytosol and chloroplasts using phenolic compounds as electron donors (Locato, Pinto, Paradiso, & Gara, 2010). An increase in POD activity from day 3 after treatment was observed by Li et al. (2015). The authors reported peak POD activity on day 7, probably due to excess H_2O_2 production. Similarly, CAT reduces excess ROS by converting H_2O_2 to oxygen and water. CAT activity is crucial under severe stress conditions.

S. sclerotiorum secretes dicarboxylic acid oxalate to facilitate colonization. This compound induces host cell death and regulates ROS generation. At the beginning of infection, oxalate maintains stable levels of ROS so that plants do not recognize the fungus, leading to successful colonization. In the later stages of infection, oxalate induces ROS generation and host cell apoptosis (Kabbage, Yarden, & Dickman, 2015). ROS have distinct biological functions depending on their concentrations in the host. For instance, when present at high concentrations, they cause severe damage to cellular components; however, at low concentrations, they act as signaling molecules in defense pathways (Sharma et al., 2012).

PAL activity was the highest at 72 HAT. In the study by Li et al. (2015), PAL activity was also significantly higher in cucumber plants treated with *B. amyloliquefaciens* LJ02 than in the control on day 3. Similar to the activity of other enzymes, PAL activity in plants can be affected by pathogen infection. Huang et al. (2016) treated tomato plants with *B. amyloliquefaciens* and exposed the plants to *Ralstonia solanacearum*; PAL activity was considerably higher on day 3 after pathogen inoculation in control plants.

PAL contributes to plant defense by stimulating the synthesis of phenolic compounds (Oliveira**,** Varanda, & Félix, 2016). Phenolic compounds exert vital antioxidant activity and may serve as substrates for lignin synthesis (Gerasimova et al., 2005).

PPO is strongly associated with PAL as it oxidizes phenolic compounds, favoring the formation of H_2O_2 and contributing to lignin biosynthesis. For example, the oxidation of phenol chlorogenic acid hinders pathogen penetration in plants due to the polymerization of phenolic barriers in the cell wall and the generation of an unfavorable environment for pathogen development (Yuan, Li, Hu, & Wu, 2002). Li et al. (2015) found that PPO activity was increased rapidly on day 3 after treatment with 1% *B. amyloliquefaciens* LJ02. An increase in PPO activity indicates cell damage and pathogen penetration.

GLU is associated with the hydrolysis of the fungal cell wall. Pathogen infection stimulates GLU synthesis in plants. The enzyme acts on the cell middle lamella, degrading fungal hyphae. These hydrolyzed structures induce higher concentrations of GLU. The greater the extent of colonization, the greater the synthesis of GLU in plants as a means to contain the infection (Stangarlin et al., 2011).

In this study, enzyme activity may have been induced by the bacterium and its metabolites, which may be related to plant defense and biotic stress. Micro-Tom tomato plants have a short life cycle (Meissner et al., 1997) and a potentially rapid metabolism, and *S. sclerotiorum* is aggressive (Jaccoud Filho et al., 2017) and can completely destroy the plants in less than a week, which may explain the effect of pathogen infection on enzyme activity after inoculation. This effect might have masked the action of *Bacillus amyloliquefaciens* PKM16. Plants treated with the control and autoclaved culture, which was not as effective in controlling white mold as the live culture, had the highest enzyme activity on day 6. Therefore, it can be concluded that because the control and autoclaved culture were not able to control the fungus, the pathogen was able to develop and damage the plants, causing an increase in enzyme activity.

Conclusion

The results showed that *B. amyloliquefaciens* PKM16 was antagonistic to *S. sclerotiorum*. The strain effectively inhibited *in vitro* mycelial growth, *in vivo* disease severity, and *in vitro* sclerotial germination. The residual effect of the live culture (which contains live bacterial cells and metabolites) lasted up to 4 days. In addition to its effect on the phytopathogen, *B. amyloliquefaciens* PKM16, as well as its metabolites, induced POD, CAT, PAL, POX, and GLU activities.

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