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CONTROL OF GRAY MOLD DISEASE OF TOMATO CAUSED BY *Botrytis cinerea* USING BACTERIAL SECONDARY METABOLITES

ARAFAT HASHIM[1], SITI IZERA ISMAIL[1], WAEL ALSULTAN[1], IDRIS ABU SEMAN[2], OSAMAH RASHED[1] and KHAIRULMAZMI AHMAD[1,3*]

[1]Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor Darul-Ehsan, Malaysia
[2]Malaysian Palm Oil Board, 6, Persiaran Institusi, Kawasan Institusi Bangi, 40300 Kajang, Selangor
[3]Institute of Plantation Studies. Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor Darul Ehsan, Malaysia
[*]E-mail: khairulmazmi@upm.edu.my

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ABSTRACT

Gray mold disease is caused by *Botrytis cinerea* and it is the most significant and devastating disease affecting tomato cultivation worldwide. This disease was recently reported infecting tomato fruits in Cameroon Highland, Pahang. Biocontrol agents have been proven to be effective towards many phytopathogens. In this study, 43 bacterial isolates were screened against *B. cinerea in-vitro*. Five isolates with more than 65% inhibition of radial growth (PIRG) were selected for the subsequent experiments. Findings in dual culture assay revealed that BM11 and BC4 were the most effective biocontrol agent, with PIRG of 82.5% and 71.8%, respectively. BM11 and BC4 isolates were identified using the 16S rDNA gene. The identification results found that BM11 and BC4 were *Pseudomonas protegens* and *Brevibacterium casei,* respectively. Ten major bioactive compounds were successfully identified by Gas chromatography-mass spectrometry (GC-MS) from the culture filtrate of both biological control agent (BCA) isolates. These bioactive compounds may responsible for antifungal activity. *In vivo* study revealed that culture filtrate extract manages to suppress disease lesions on the treated tomato fruit from 0.86 -1.03 cM, 30 days after inoculation. Based on overall findings suggested that *P. protegens* and *B. casei* were the promising BCA in controlling gray mold disease of tomato fruit.

Key words: Tomato fruit, gray mold disease, secondary metabolites, GC-MS, biological control

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is known as one of the most substantial food crops in the world including eggplant, chili peppers, and potato. According to the report of the US National Agriculture Statistics Services, the value of the fresh market of tomatoes in 2005 was over US$1.6 billion. Moreover, approximately 160 million tonnes of fresh tomato are produced annually. In comparison, triple times more than potatoes production and six times more than rice are cultivated worldwide (FAO, 2016). The major fungal pathogens on tomatoes either in the field or after harvesting are *Botrytis*, *Geotrichum*, *Phytophthora*, *Penicillium*, and *Fusarium*. Tomato fruit is threatened by one of the most devastating diseases called grey mold disease caused by *Botrytis cinerea*. The disease causes serious yield losses and reduces fruit quality (Sarwar & Frankenberger, 1995). *Botrytis cinerea* infection could take place in the field during the harvesting process or earlier than that, but the adverse impact of the disease commonly happens in the post-harvesting time (Emmert & Handelsman, 1999). The pathogen can attack a wide range of hosts and different parts of the plant like flowers, leaves, fruit, and stems. The economic losses due to the pathogen easily exceed 10$ billion worldwide annually (Weiberg *et al*., 2013). *Botrytis cinerea* has been categorized as the second significant phytopathogen after *Magnaporthe oryzae* the casual pathogen of rice blast disease (Dean *et al*., 2012). It is difficult to control *B. cinerea* in the field as well as in the storage room. This is because it has a broad host

* To whom correspondence should be addressed.

range, various attack modes, and both asexual and sexual stages to survive in favorable or unfavorable conditions (Fillinger & Elad, 2016).

Diverse methods are being used to limit gray mold disease and reduce yield loss. To date, the principal means in controlling grey mold disease in the field by the application of synthetic fungicides. Besides that good agronomic and horticultural practices, physical and biological control methods are also being used. However, the control effects of fungicides are not satisfactory on *B. cinerea* whose genome is plasticity and prone to develop drug resistance genes. More importantly, the overuse fungicides are not safe for human and environment (Droby *et al*., 2009). Therefore, it is very important to deeply understand the biological control system. Biological control is an impressive alternative strategy due to harmless to the environment and effective against many plant diseases. The microbes particularly bacteria species have commonly been known biologically as a rich source of unique and efficient metabolites. Biological control of *Botrytis* infections was reported by many researchers since the mid-1950s. Various bacteria such as *Pseudomonas* (Lavermicocca *et al*., 2000; Kumar *et al*., 2014)*, Bacillus, Erwinia, Serratia, Paenibacillus,* and *Rhizobium* (NikuPaavola *et al*., 1999; Slininger *et al*., 2004) are considered as potential biocontrol agents that can inhibit the growth of gray mold pathogen. The beneficial effects of biological control agent's bacteria have been attributed to the production of metabolites, antifungals, and antibiotics. The objectives of the present study were to isolate and screen biocontrol agents against *B. cinerea* and to evaluate the efficacy of selected culture filtrate extract against *B. cinerea in-vivo*.

MATERIALS AND METHODS

Sample collection and BCA isolation from ripe and diseased tomato

A total of 43 samples of ripe and diseased tomato fruits were collected from four local wet markets in Sri Serdang, Selangor. The fruit was then rinsed under running tap water for 5 min, surface-sterilized by dipping successively into 10% sodium hypochlorite solution for 1 min then rinsed three times for three min with sterilized distilled water to remove the chemicals and allowed to dry on a sterilized filter paper in the sterile chamber.

Then each tomato fruit was cut by sterile scalpel 0.5 cM^2 before transferred onto nutrient agar (NA) medium for culture surface and incubated at 30°C for 2 days in an incubator. Similarly, small pieces (0.5 cM) of tomato cuts were then transferred to nutrient broth (NB) (250 mL) and placed on an orbital shaker (Tech-Lab Scientific) under room temperature for three days. One loop-full of bacterial supernatant was taken and streaked on the NA surface and incubated at 30°C for 2 days. The pure culture of BCA isolates was maintained on nutrient agar (NA) for a subsequent experiment.

In vitro screening of BCA against *Botrytis cinerea*

The pure culture of *B. cinerea* was obtained from the culture collection unit of the Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia. A total of 43 BCA isolates were screened for their antagonistic activity against *B. cinerea in vitro* by the dual culture, culture filtrate based on percentage inhibition of radial growth (PIRG), and spore germination tests.

a) Dual culture assay

A mycelial plug (5 mM in diameter) was taken from a 7-days-old PDA culture of *B. cinerea* was placed in the center of a freshly prepared NA plate. A full loop of BCA from 2-days old NA culture was streaked in two parallel straight lines, (2 cM away from *B. cinerea* plug and 4 cM length for each streaking line). Control petri dishes were maintained by inoculating with pathogen only. All plates were incubated at 25°C for 7 days. The antagonistic activity of the BCA against *B. cinerea* was evaluated 7 days after incubation by measuring the radial growth of *B. cinerea* towards the bacteria (Chaurasia *et al*., 2005). The results were transformed into PIRG about the radial growth of *B. cinerea* in the control plate (R1), using the following formula:

$$\text{PIRG (\%)} = \frac{\text{R1} - \text{R2}}{R1} \times 100$$

Where, R1 and R2 are the radial of *B. cinerea* in control and dual culture plates, respectively

b) Culture filtrate assay

This test detects the production of diffusible compounds or inhibitors produced by BCA in standing liquid culture. The isolates with stronger repressive activity (suppression rates of more than 60%) in dual culture test were inoculated in 250 mL nutrient broth and incubated at 30°C for 2 days in the dark. After incubation, the filtrate was then centrifuged for 5 min at 10,000 rpm, the supernatant was collected. The supernatant was filtered through a 0.25 µM membrane filter in sterile conditions. The filtrate was incorporated into sterilized double strength PDA (50°C) in a ratio of 2:1, approximately 20 mL of the amended agar was poured into each plate and allowed to solidify. A mycelial plug of

B. cinerea was centrally inoculated and each of the plates was incubated for 7 days at 25°C. The control plate was inoculated with pathogen only. The antagonistic activity was expressed as PIRG about the mycelia growth of *B. cinerea* in the control plate (Chaurasia *et al*., 2005).

c) Inhibition of spore germination

A volume of 1 mL of spore suspension 10^7 spores mL^{-1} was prepared and transferred in a series of micro-tubes and a volume of 20 µL of the BCA filtrates was added carefully. The tubes were prepared in triplicates and incubated for 24 hr at room temperature. After incubation, the inhibition of spore germination was observed under a microscope (Olympus) using a Malassez cell. The number of spores germinated and non-germinated were recorded. The percentage of non-germinated spores was calculated according to the formula:

$$\text{Percentage Inhibition in Spore Germination} = \frac{SG - tSG}{SG} \times 100$$

Where, SG- number of spores germinated in the control plate, tSG- total number of spores germinated in treatments plate

Data analysis

Dual culture tests, culture filtrate tests, and spore suppression tests were carried out in a completely randomized design (CRD) with three replications. Statistical analysis software (SAS) version 9.4 was used to achieve one-way Analysis of Variance (ANOVA) to identify the significant differences in the mean of inhibition mycelial growth of fungus in each replicate. The percentage data were transformed into Arcsine values before subjected to ANOVA. Mean comparison was tested using Tukey's Studentised Range HSD test by considering significance for $p \leq 0.05$.

Molecular identification of potential antagonistic bacteria

Four days old of bacteria isolates were subcultured into 20 mL (NB) and incubated for 2 days at 25±°C on the orbital shaker. Each isolate was harvested by taking 1 mL from NB into 1.5 tubes then centrifuged for 1 – 2 min to obtain the pellet. The DNA of the bacteria was extracted using MyTACG Bioscience Extraction Kits. The forward primer 27F 5'-AGAGTTTGATCCTGGTCAG-3' and reverse primer 1492R 5'-GGTTACCTTGTTA CGACTT-3' (Lane, 1991) were used to amplify the 16S rDNA gene of the selected BCA isolates. The PCR reaction was carried out by ready master-mix (MyTaq™ Mix 2x) (http://www.bioline.com/) in total volume 25 µL (12.5 ready master-mix, 2 µL DNA template (20 – 50 mg), 1 µL (10 µM) primers and complete to 25 µL with free nucleate water). PCR program began with an initial denaturation at 95°C, for 5 min followed by 34 cycles of denaturation for 30 sec; annealing at 57°C for 30 sec and extension for 1 min. followed by a final extension at 72°C for 10 min. PCR product was checked using 1% agarose and run electrophoresis at 100 V for 40 min then visualized under ultraviolet (UV) illumination. PCR products were sent for purification and sequencing at the commercial laboratory, First BASE Laboratory Sdn. Bhd. The gene sequences of each isolate were then edited using Bioedit Software and blasted using Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI).

Determination of bioactive compounds of culture filtrate extract

Preparation culture filtrate extract

BM11 and BC4 isolates were cultivated in NB for 2 days at 110 rpm on a rotary shaker under room temperature. The bacteria cells were then removed by centrifugation at 10000 rpm for 10 min. Antibacterial compounds in the supernatant were extracted with an equal volume of organic solvent of ethyl acetate in a separating funnel. The concentrated products were regarded as crude samples and stored at -20°C until further use.

GC-MS analysis of BCA volatile components

Gas chromatography coupled mass spectrometry (GC-MS) was used to identify the volatile compounds by Shimadzu GC-MS-QP2010 plus system as a component of the investigation. The column used was fused Silica capillary column [GC-2010] COLUMN: ZB-5MS 30 M x 0.25mM ID x 0.25µM film thickness. An injection volume of 2µL, 0.5µL from BM11, BC4 respectively were employed (a split ratio of 5.0). The injector temperature was maintained at 250°C, the column oven temperature was programmed at 50°C, the ion-source temperature was 240°C, and the column temperature was kept up at 50°C for 3 min, followed by modified programming at 300°C for 10 min.

Determination of the minimum inhibitory concentration (MIC)

1 mL of culture filtrate containing antimicrobial substances of BM11 and BC4 was placed in a 10 mL test tube then diluted by adding sterile distilled water to the test tubes to obtain four different concentrations 3%, 2%, 1%, 0.5%, and 0% (control) of bacteria filtrates. The filtrate was incorporated into sterilized double strength PDA (50°C) in a ratio

of 2:1, approximately 20 mL of the amended agar was poured into each plate and allowed to solidify. A mycelial plug of *B. cinerea* was centrally inoculated in each plate incubated for 7 days at 25°C. The control plate was inoculated with pathogen only. The test was conducted in three replicates, in a completely randomized design (CRD). Inhibition effect was calculated with the formula as follows:

$$P\ (\%) = \frac{(C - d) - (T - d)}{(C - d)}$$

Where P: inhibitory rate, C: diameter of the colony of control, T: diameter of the colony of treatment, d: diameter of the mycelial plug.

***In vivo* assay on tomato fruits**

40 ripe tomato fruits of the same size, weight, and maturity were used. Tomato fruit was rinsed under running tap water for 5 min, surface-sterilized by dipping successively into 10% sodium hypochlorite solution for 1 min then rinsed three times for three min with sterilized distilled water to remove any chemicals and allowed to dry on a sterilized filter paper in the sterile chamber. Tomato fruits were sprayed carefully with 10 mL of BCA culture extract at a concentration of 1% (based on MIC test) mixed with Tween 80 and left to dry inside the laminar flow. After that, the fruits were artificially injured using a sterile fine needle and put the mycelial plug (5 mM, 5-days-old culture) of *B. cinerea* on the injured skin of the tomato. The tomato fruits were incubated at 17°C for 30 days in the storage room (Chan *et al*., 2007; Zhang *et al*., 2015). Negative controls without inoculation and spray with sterile distilled water only. While positive control was inoculated with pathogen only. The experiment was repeated twice. Lesion area was calculated and analysis of variance (ANOVA) was used to determine the differences between means. Least Significant Difference (LSD) test was used to examine differences between treatment means.

RESULTS AND DISCUSSION

***In vitro* assay**

The dual culture test is used to distinguish isolates with antagonistic potential from a large population. From the 43 isolates tested, 31 isolates recorded PIRG less than 50%, 12 isolates were more than 50%. The minimum inhibition zones against the pathogen of the best five isolates (more than 60%) are shown in Table 1. The best reduction of fungal pathogen growth among BCA isolates was 82.50% and 71.75% by isolates BM11 and BC4, respectively. Further evaluation of these five BCA isolates by culture filtrate and spore germination tests. The data of the culture filtrate test showed that there were significant differences ($p \leq 0.05$) for *B. cinerea* at different BCA isolates tested. For the BC4, BC23, and BM11, the percentage diameters of mycelial growth *B. cinerea* were 47.13%, 43.75%, and 48.38%, respectively (Table 1). BM4 and BM13 were showed weak activity in culture filtrate with the percentages of inhibition growth of 13% and 23.75%, respectively. Based on data in Table 1, it was revealed that all the BCA tested can suppress the spore germination of *Botrytis.* BM11 isolate was the best BCA towards *B. cinerea* (87.22%). However, BC4, BC23, BM4, and BM13 had the least effect with percentages of inhibition ranging from 73 – 77%.

Molecular identification of antagonistic bacteria

PCR amplification of the 16SrDNA gene region yielded a product size of 1500 bp. The results of Blast analysis revealed that BM11 isolate was identified as *Pseudomonas protegens*, while BC4 isolate was identified as *Brevibacterium casei* with similarity index ranged from 99 – 100% compared with references isolates KJ396833.1 and KU550195.1, respectively. The accession number of both isolates obtained from Genbanck were MG190379 and MG190378 for *P. protegens* and *B. casei,* respectively.

Table 1. Potential isolates of antagonistic bacteria tested using dual culture, culture filtrate and spore germination tests against *B. cinerea*

Bacteria isolates	Percentage Inhibition Growth (PIRG) of *Botrytis cinerea*		
	Dual Culture (%)	Culture Filtrate (%)	Spore Germination (%)
BM13	68.25c	23.75c	75.94b
BC4	71.75bc	47.13a	77.22b
BM4	65.00cd	31.00b	73.69bc
B23	65.83d	43.75ab	76.53b
BM11	82.50a	48.38a	87.22a

Determining the chemical constituents by GC-MS

The GC-MS results showed that various bioactive compounds from BCA culture filtrate extract were detected. The GC-MS spectrum range confirmed the presence of 48 and 42 bioactive components derived from *P. protegens* and *B. casei*, respectively. The diverse of the bioactive components are shown by the different retention times and chemical structures. The major five compounds with the highest peak of each bacterium are summarized in Table 2 and 3.

Pseudomonas and *Brevibacterium* are known to have diverse secondary metabolites with varied biological activities; many studies have combined various analytical methods such as GC-MS to support the analysis of chemicals (Slininger *et al*., 2004; Jog *et al*., 2014). five major compounds have been found by *P. protegens* which are pyrrolo[1,2-a]pyrazine-1,4- dione, hexahydro- (32%); (5-methyl-2, 4- imidazolidine dione) (9%); 2,5-Piperazinedione, 3,6-bis(2-methylpropyl)(5.2%); actinomycin C2(4.74%); Pyrrolo[1,2 a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) (3.13%). Likewise, Five major compounds have been found by *B. casei* which are Benzeneacetic acid or Phenylacetic acid (23.11%); pyrrolo[1,2-a]pyrazine-1,4- dione, hexahydro-(10.5%); Thiamin (6.51%); 3-Deoxy-d-mannonic acid (7.74%) and Methyl 2-furoate (3.41%).

Pyrrolo[1,2-a]pyrazine-1,4- dione, hexahydro in the extract *Pseudomonas* and *Brevibacterium* proved the potential of the isolates. The recent results carried out on this compound have anti-oxidant and similar finding was were also reported by (Janisiewicz *et al*., 1997). These outcomes have evidence that pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- was capable to scavenge or reduce the number of free radicals as estimated by using decreasing power test. In brief, an antioxidant is the potential to play substantial roles in the inhibition and remedy of diseases (Nunes *et al*., 2002). Methylhydantoine (5-methyl-2, 4- imidazolidine dione) was identified as antifungal by (Niku Paavola *et al*., 1999; Meziane *et al*., 2006; Li *et al*., 2012). While, 2, 5-Piperazinedione, 3, 6-bis (2-methylpropyl) the compound has been reported to have antimicrobial against fungi (Kumar *et al*., 2014). Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) may be used as the potential antifungal compound. The composite actinomycin C2 produced by *P. casei* has been described to have antioxidant (Arda-Pirincci *et al*., 2011), anti-microbial (Chen *et al*., 2012), and anti-cancerous properties including Gestational trophoblastic neoplasia, Wilms' tumor, Rhabdomyosarcoma, Ewing's sarcoma, Malignant hydatidiform mole. Benzeneacetic acid or Phenylacetic acid, deamination produce of phenylalanine, has been

Table 2. Five of the highest peak components exhibited in the crude of *Pseudomonas protegens* as analyzed using a GC-MS

Compound	Formula	Rt (min.)	Area %	High	WM	Biological activity
Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro	$C_7H_{10}N_2O_2$	12.192	32.47	35.97	154	antioxidant
2,4-Imidazolidinedione, 5-methyl-	$C_4H_6N_2O_2$	8.883	9	6.49	114	antifungal
Actinomycin C2	$C_{63}H_{88}N_{12}O_{16}$	13.2	4.74	4.38	1268	antimicrobial
2,5-Piperazinedione, 3,6-bis(2-methylpropyl)	$C_{12}H_{22}N_2O_2$	14.225	5.2	4.63	226	antimicrobial
Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)	$C_{11}H_{18}N_2O_2$	14.592	0.26	0.32	210	antifungal

Table 3. Five of the highest peak components exhibited in the crude of *Brevibacterium casei* as analyzed using a GC-MS

Compound	Formula	Rt (min.)	Area %	High	WM	Biological activity
Benzeneacetic acid	$C_8H_8O_2$	8.767	23.11	29.61	136	antifungal
Pyrrolo[1,2-a]pyrazine-1, 4-dione, hexahydro-	$C_7H_{10}N_2O_2$	12.192	10.5	15.21	154	antioxidant
3-Deoxy-d-mannonic acid	$C_6H_{12}O_6$	11.033	7.74	4.18	180	antifungal
Methyl 2-furoate	$C_6H_6O_3$	7.483	3.41	1.64	126	antifungal
Thymine	$C_5H_6N_2O_2$	11.208	6.51	2.81	126	antioxidant

known to have a positive impact on the development and growth of maize. The microorganisms and plants which create phenylalanine ammonia-lyase can obtain phenylacetic acid from phenylalanine in nature (Sarwar & Frankenberger, 1995). Benzeneacetic acid was demonstrated to have antifungal effectiveness, against the dry rot, caused by *Gibberella pulicaris*, and to inhibit dry rot disease of injured potato (Sudha & Masilamani, 2012) and it suppressed the development of *Rhizoctonia solani, Saccharomyces cerevisiae Phytophthora capsici,* and *Pythium ultimum* (Lee *et al*., 2006).

MIC determination

Based on the data of the MIC test showed that *Brevibacterium* and *Pseudomonas* suppressed the fungal pathogen growth at different concentrations. At 3% and 2% concentrations, the percentage of PIRG were 96% and 91% for *Brevibacterium* and 95% and 89.25%, for *Pseudomonas,* respectively. The effect of both concentrations was almost similar to mycelial inhibition where concentration at 3% showed high inhibition activity against *B. cinerea* for both BCA isolates. Concentration at 1% showed 87.75% and 84.25% for *Pseudomonas* and *Brevibacterium,* respectively, but 0.5% showed the lowest activity (Table 4).

In vivo* trial of bacterial metabolites against *Botrytis cinerea

The in-vivo assay revealed that *P. protegens* and *B. casei* significantly reduced lesion development compared to a positive control ($p \leq 0.05$). Both BCA agents showed almost similar effect on lesion development. In positive control, lesion formation on tomato fruit at 4 days after treatment (DAT) showed significant different. Disease lesions development during the trial period of 12 DAT to 30 DAT was sharply increased (Figure 1). However, disease lesions formations on treated fruit with *P. protegens* and *B. casei* at 12 DAT were slowly increased. Disease lesion continuously increased slowly at 20 DAT for both bacteria. Finally, at day 30 *P. protegens* showed the greatest disease reduction in lesion area around 81.2% while, *B. casei* showed a reduction of 84.3 % (Figure 2 and Table 5).

However, insufficient information on gray mold disease control approaches is found particularly using bacterial secondary metabolites in the public databases. Today, the majority of growers are more in favor to use synthetic fungicides to control plant diseases but this method causes many setbacks to

Table 4. The minimum inhibitory concentration (MIC) of culture filtrate containing antimicrobial substances of *P. protegens* (BM11) and *B. casei* (BC4)

Concentration (%)	PIRG (%)	
	BC4	BM11
0	0c	0d
0.5	84.25b	72.5c
1	87.75b	84.25ab
2	95a	89.25a
3	96a	91a

Mean value with the same letter are not significantly different using Tukey's Studentised Range HSD test at $p \leq 0.05$.

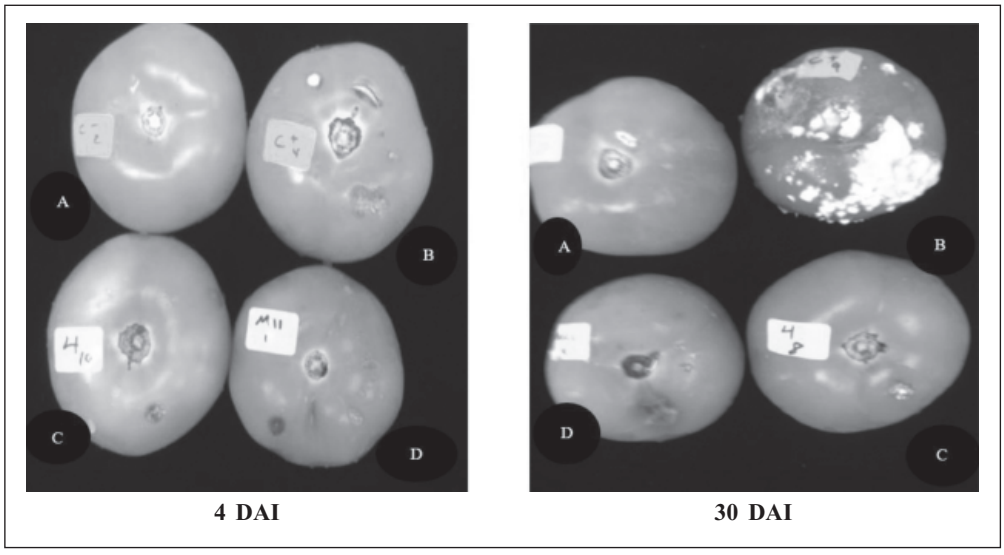

Fig. 1. Comparison of disease severity of gray mold disease on tomato fruit at 4 DAI and 30 DAI after being treated with culture filtrate extract. A) Negative control, B) Positive control, C) Treated with *B. casei* and D) treated with *P. protegens.*

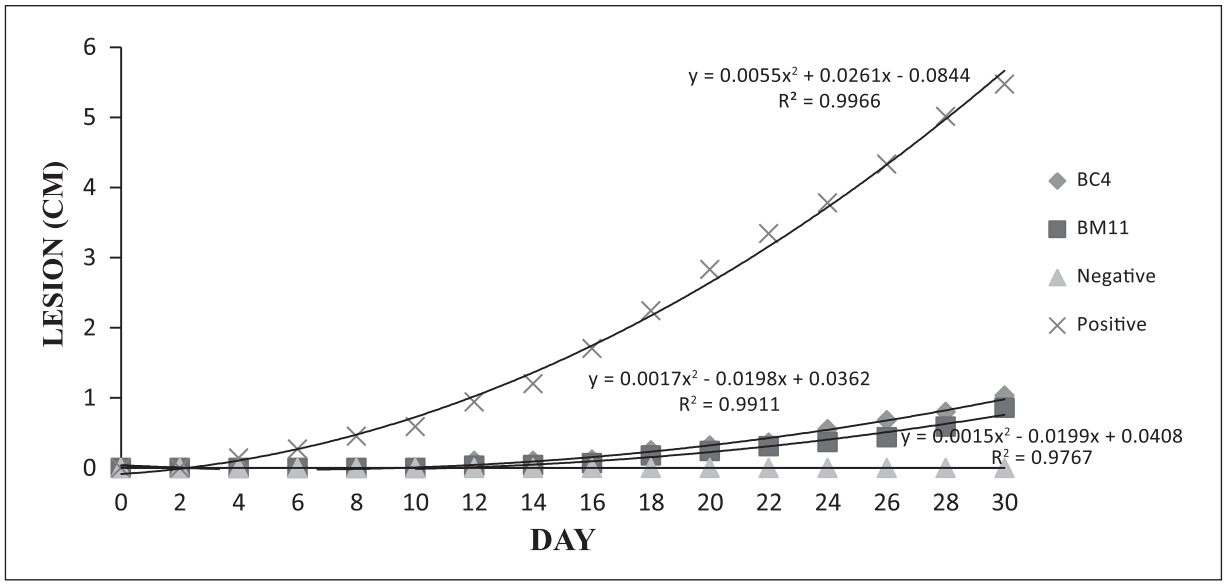

Fig. 2. Effect of culture filtrate extracts on disease lesion diameter caused by *Botrytis cinerea* compared with the positive and negative control.

Table 5. Disease reduction percentage (DR %) of treated tomato with secondary metabolites cured extract of *P. protegens* (BM11) and *B. casei* (BC4)

Days	BC4	BM11
2	0	0
4	100	100
6	100	100
8	100	100
10	100	100
12	96.8	89.4
14	96.7	91.7
16	95.9	92.9
18	92.0	88.8
20	91.5	88.7
22	90.7	89.2
24	90.2	85.4
26	89.8	84.3
28	88.2	84.0
30	84.3	81.2

the consumers, growers, and environment. The establishment of a safe disease control approach particularly using bacterial secondary metabolites is a novel approach. Exploration and identification of potentially active compounds are easier and convenient with the introduction of new technology.

Future investigation will be focusing on establishing new and convenient technology for the application of biological control agents in large-scale product and at research on the control mechanisms of *P. protegens* and *B. casei* against *B. cinerea*. The present study contributes to future investigation programs that target to promote *P. protegens* and *B. casei* as a potential biopesticide for augmentative biocontrol of numerous diseases of crops. Nevertheless, requires a better understanding of the factors that affect the relationship among antagonistic bacteria, soil dwellers, pathogens and plants, are yet to be explored to enhance the understanding of biological control systems as vast applicable biopesticides in the future.

CONCLUSION

In vitro investigation results revealed that the five bacteria isolates were isolated from tomato fruit were potent in inhibition of growth of *Botrytis cinerea* in dual culture, culture filtrate, and spore germination tests. Two isolates (BM11 and BC4) showing the highest antifungal activities against *B. cinerea* were chosen as biological control agents. Based on gene sequencing results BM11 isolate was identified as *P. protegens* and BC4 was *B. casei* with similarity index ranging between 99 – 100% compared with references from GenBank. The findings of this study showed that *P. protegens* and *B. casei* produced strong antifungal activity against *B. cinerea* pathogenic strain. GC-MS analysis showed that *P. protegens* and *B.casei* compound extract contained different bioactive compounds with diverse concentrations. The results of *in vivo* test support the use of *P. protegens* and *B. casei* as a biological control agent against *B. cinerea* in tomato fruit where they reduced the disease lesion area compared to the untreated control.

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