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Sawai Boukaew, Poonsuk Prasertsan, Claire Troulet, Marc Bardin. Biological control of tomato gray mold caused by *Botrytis cinerea* by using *Streptomyces* spp.. *BioControl*, 2017, 62, pp.793-803. 10.1007/s10526-017-9825-9 . hal-01564548

**HAL Id: hal-01564548**

**<https://hal.science/hal-01564548>**

Submitted on 24 Feb 2022

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# Biological control of tomato gray mold caused by *Botrytis cinerea* by using *Streptomyces* spp.

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## Abstract

*Streptomyces* is a genus known for its ability to protect plants against many pathogens and various strains of this bacteria have been used as biological control agents. In this study, the efficacy of *Streptomyces philanthi* RM-1-138, *S. philanthi* RL-1-178, and *S. mycarofaciens* SS-2-243 to control various strains of *Botrytis cinerea* was evaluated both *in vitro* and *in vivo*. *In vitro* studies using confrontation tests on PDA plates indicated that the three strains of *Streptomyces* spp. inhibited the growth of 41 strains of *B. cinerea*. Volatile compounds produced by *Streptomyces* spp. had an influence on the growth of 10 strains of *B. cinerea* while its culture filtrate at low concentration (diluted at  $10^{-3}$ ) showed a complete inhibition (100%) of spore germination of *B. cinerea* strain BC1. A significant protection efficacy of tomato against *B. cinerea* was observed on both whole-plant test (57.4%) and detached leaf test (60.1%) with *S. philanti* RM-1-138. Moreover, this antagonistic strain had a preventive and a curative effect.

These results indicated that *S. philanthi* RM-1-138 may have the potential to control gray mold caused by *B. cinerea* on tomato but further work is required to enhance its efficacy and its survival *in planta*.

**Keywords:** *Botrytis cinerea* \* Biological control \* Durability \* Sensitivity \* *Streptomyces* spp.

## Introduction

Gray mold disease caused by the fungus *Botrytis cinerea* Pers.: (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel) is an economically important disease on numerous vegetable including tomato (Jarvis 1980; Helbig 2002; Elad et al. 2015). *Botrytis cinerea* can infect the plants either by direct penetration or through wounds caused by cultivation practices. Infection is promoted by high humidity, free moisture on the plant surface, and low temperatures (Lee et al. 2006). In general, disease management strategy largely relies on the use of chemical fungicides that are generally considered harmful to the environment and can result in fungicide resistance as previously reported (Locke and Fletcher 1988; Yourman and Jeffers 1999; Chung et al. 2006). The development of efficient antifungal microbial agents could be an alternative method to control the disease.

Species of *Streptomyces* are potential biocontrol agents since they are ubiquitous in the environment and many of them produce secondary metabolites such as enzyme inhibitors and antibiotics with diverse biological activities, including the ability to inhibit plant pathogenic fungi (Omura 1992; Lange and Sanchez 1996; Vaz Jauri et al. 2016). Several species of *Streptomyces* have been isolated and used to control plant pathogens on various crops, such as *Pyricularia oryzae* and *Rhizoctonia solani* on rice (Prabavathy et al. 2006), *R. solani* and *Sclerotium rolfsii* on sugar beet (Sadeghi et al. 2006; Errakhi et al. 2007) and *Sclerotinia*

*sclerotiorum* on sunflower (Baniasadi et al. 2009). In particular, strain RM-1-138 and RL-1-178 of *S. philanthi* and strain SS-2-243 of *S. mycarofaciens*, isolated from the rhizosphere of chili pepper in southern Thailand (Boukaew et al. 2011), have shown a good efficacy to control *S. rolfsii*, *Ralstonia solanacearum* on chili (Boukaew et al. 2011), and *R. solani* on rice (Boukaew et al. 2013, 2014a; 2014b). However, few studies explored the effect of *Streptomyces* sp. on *B. cinerea*. For instance, the strain A01 of *Streptomyces lydicus* produces natamycin and chitinase that have the potential to control gray mold caused by *B. cinerea* (Wu et al. 2013).

In all the studies cited above, the effect of the biocontrol agent *Streptomyces* spp. was tested against a single strain of the plant pathogen considered. However, some studies highlight differences in the sensitivity of various isolates of plant pathogens to biocontrol agents, with the existence of less sensitive isolates in natural populations of plant pathogens and even the capacity of plant pathogens to adapt to biocontrol agents (Bardin et al. 2015). Diversity of sensitivity of plant pathogens to biocontrol agents may result in a reduction of efficacy of the biocontrol agent in the field and if selection pressure would increase, *i.e.* intensive use of biocontrol agents by growers, we can expect a selection of the less sensitive isolates and consequently a reduced efficacy of biocontrol agents against plant pathogens in the field.

The first objective of the present study was to evaluate the ability of three strains of *Streptomyces* spp. to inhibit the growth of *B. cinerea in vitro* and on tomato plants. The three tested strains, RM-1-138 and RL-1-178 of *Streptomyces philanthi* and SS-2-243 of *S. mycarofaciens*, are known for their antifungal effect on various plant pathogens (Boukaew et al. 2011, 2013, 2014a; 2014b), but they have never been tested against *B. cinerea*. The second objective of the study was to assess the possible variability in susceptibility of various strains of *B. cinerea* to these antagonistic strains. To this end, 41 strains of *B. cinerea* differing in their date of isolation, geographic and host or substrate of origin were used.

76

77 **Materials and Methods**

## 78 Microorganisms and inoculum preparation

79 Strains RM-1-138 and RL-1-178 of *S. philanthi* and strain SS-2-243 of *S. mycarofaciens*  
80 were isolated in a previous study from the rhizosphere of chili pepper in southern Thailand  
81 (Boukaew et al. 2011). Spores of *Streptomyces* were collected in 10 ml water from 10-day-old  
82 culture on glucose yeast-malt agar at 30°C and counted using a hemacytometer. Inoculum  
83 was prepared by dilution in distilled sterilized water to achieve the required concentration.

84 Forty-one strains of *Botrytis cinerea* differing in their date of isolation, geographic and  
85 plant hosts or substrate of origin were used in this study. All isolates were single-spored and  
86 conserved at -20°C before use. In all tests, the *B. cinerea* strain BC1, known to be very  
87 aggressive on tomato (Decognet et al. 2009), was used. An inoculum of *B. cinerea* was prepared  
88 by cultivating the strain on potato dextrose agar (PDA) plates under cool white fluorescent light  
89 (14-h photoperiod at 65  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) in a growth chamber at 21°C. Spores were collected in 5  
90 ml water from 14-day-old culture and counted using a hemacytometer. Inoculum was  
91 prepared by dilution in distilled sterilized water to achieve the required concentration.

92

## 93 Dual culture

94 The three strains of *Streptomyces* spp. were evaluated for their antagonistic properties  
95 against forty-one strains of *B. cinerea* using a dual culture technique (Islam et al. 2009). For  
96 each strain of *Streptomyces* spp., a streak of spore suspension at  $10^7$  spores.ml<sup>-1</sup> was deposited  
97 on one side of a PDA medium in Petri dishes. In previous study, Boukaew et al. (2011) have  
98 shown that the antagonistic activity of the 3 strains of *Streptomyces* by dual culture on agar was  
99 observed after 7 days of incubation at 28°C. For RM-1-138 the optimal activity of antifungal  
100 metabolites was observed for culture filtrate obtained after 9 to 11 days of incubation at 28°C

(Boukaew and Prasertsan 2014b). Plates were then incubated in a growth chamber for 10 days at 21°C in the dark.

A 5-mm-diameter mycelial plug, excised from a 3-day-old *B. cinerea* colony, was transferred to the center of each plate. As a control, a mycelial plug of *B. cinerea* was placed on a PDA plate without any *Streptomyces* strain. The dual culture plates were further incubated in a growth chamber (21°C, dark) for 2 days, after which the radial mycelial growth of *B. cinerea* was measured and compared to that of the control. Three replicates were conducted for each *Streptomyces*–*B. cinerea* strain combination. The colony size in each treatment was recorded and the percentage inhibition of hyphal growth was calculated.

#### Volatile effect

*Streptomyces philanthi* RM-1-138 and RL-1-178, and *S. mycarofaciens* SS-2-243 were evaluated for the effect of their volatile substances on the mycelial development of ten strains of *B. cinerea*. Strains of *B. cinerea* tested were selected based on their susceptibility to *Streptomyces* spp. in the dual culture assay: the 5 most sensitive strains and the 5 less sensitive strains to *Streptomyces* spp. were tested. For each strain of *Streptomyces* spp., a streak of a spore suspension at  $10^7$  spores.ml<sup>-1</sup> was deposited on PDA medium in a Petri dish. After a ten days incubation in a growth chamber (21°C, dark) a Petri dish containing *Streptomyces* spp. was covered with another Petri dish containing PDA medium inoculated with a 5 mm-diameter mycelial plug of *B. cinerea*. The two plates were then sealed using double layers of Parafilm to obtain a double-plate chamber (Raza et al. 2015). Double plates were incubated in a growth chamber at 21°C (14 h day and 10 h night). After 2 days of incubation, the diameters of the colonies of *B. cinerea* were recorded, and the percentage inhibition of hyphal growth was calculated. For each treatment, three replicates were realized.

## 126 Culture filtrate effect

127 *Streptomyces philanthi* RM-1-138 and RL-1-178, and *S. mycarofaciens* SS-2-243 were  
 128 each grown at 30°C in a 250 ml flask containing 100 ml liquid GYM medium (glucose yeast-  
 129 malt extract broth, pH adjusted at 7.0 before autoclaving) on a rotary shaker at 150 rpm. After  
 130 3 days of incubation, 5 ml aliquots of this culture was transferred into 100 ml fresh GYM  
 131 medium, and incubated for 10 days under the same conditions. The culture broth was  
 132 centrifuged (8880 g for 20 min), then filtered through a 0.45 mm Millipore membrane to recover  
 133 the culture filtrate.

134 Serial dilutions of the culture filtrate (1/10, 1/100 and 1/1000) were prepared in distilled  
 135 sterile water. Ten µl of the different dilutions were then mixed with 2 µl of a spore suspension  
 136 of the strain BC1 of *B. cinerea* dosed at  $10^5$  spores.ml<sup>-1</sup> and dropped on a glass-slide. Sterile  
 137 Potato Dextrose Broth (PDB) was used instead of the culture filtrate as a positive control. The  
 138 slides were further placed in Petri dishes with humid absorbent paper to maintain high relative  
 139 humidity and incubated under cool white fluorescent light (14-h photoperiod-65 µmol m<sup>-2</sup> s<sup>-1</sup>)  
 140 in a growth chamber at 21°C for 24 h. Spores that germinate were counted under the microscope  
 141 and the inhibition of spore germination (in percentage) was calculated.

142

## 143 Efficacy of *Streptomyces* spp. against *B. cinerea* on tomato

144 Seed of tomato cv. Monalbo (INRA Plant Pathology, Avignon, France) were sown in  
 145 compost and transplanted after 1 week in an individual pot containing a horticulture compost  
 146 mix (De Baat, Coevorden, The Netherlands). Plants were grown in a glasshouse for 7 to 8 weeks  
 147 where they received a standard commercial nutrient solution once or twice a day, depending on  
 148 needs. They had at least 8 fully expended leaves when used.

149 Two types of test were performed to evaluate the efficacy of *Streptomyces* spp. to  
 150 control *B. cinerea* on tomato plants: a detached-leaf assay and a whole-plant assay. Tests were

first carried out with the three strains of *Streptomyces* spp. (RM-1-138, RL-1-178 and SS-2-243) against the strain BC1 of *B. cinerea*. Additional tests with RM-1-138 were realized with 3 strains of *B. cinerea* (BC1, C10 and CEN12-13) having different level of sensitivity to RM-1-138 in dual culture tests.

For the detached-leaf assay, leaves were sprayed with a spore suspension of *Streptomyces* spp. at the concentration of  $10^6$  or  $10^7$  spores.ml<sup>-1</sup>. When the leaves were dried (between 30 and 60 minutes after treatment), they were removed, and two leaflets were placed in a transparent plastic box with humid absorbent paper to maintain high relative humidity (close to 100%). Mycelial plugs (5-mm in diameter) of *B. cinerea* excised from the growing margin of a 3-day-old PDA cultures were deposited onto the leaflets of tomato. For each treatment, 6 leaflets of tomato (3 plastic boxes) were inoculated. Following inoculation, the detached-leaves were incubated in a growth chamber in conditions conducive to disease development (21°C, 14 h-photoperiod). Leaves removed from non-treated plants were inoculated as a control. The lesion area was determined with ImageJ software 2 days after inoculation. Three independent repetitions of the test were realized.

For the whole-plant test, three leaves per plant were removed leaving 5-10 mm petiole stubs on the stems and each pruning wound was inoculated with 10 µl of a spore suspension of *B. cinerea* dosed at  $10^6$  spores.ml<sup>-1</sup>, in order to place *Botrytis* in the most favorable conditions for infestation of the stem. *Streptomyces* spp. was then applied to pruning wounds with 10 µl of a spore suspension dosed at  $10^6$  or  $10^7$  spores.ml<sup>-1</sup>, 5 min after the inoculation of *B. cinerea*, the time that the drop was absorbed by the petiole. The pathogen was inoculated alone as a control. All plants were incubated in a growth chamber with a photoperiod of 14 h and maintained at 21°C with a relative humidity above 80% to favor disease development. The experiment was repeated three times independently, each with three replicates per treatment.



Lesion expansion on tomato stem was recorded daily for 7 days after inoculation (DAI). To take into account the kinetics of disease development, we computed the area under the disease progress curves (AUDPC) as described by Decognet et al. (2009), as:

$$AUDPC = \left[ \frac{Y_1}{2} + \sum_{j=2}^{n-1} Y_j + \frac{Y_n}{2} \right] \cdot [I]$$

where  $Y_j$  was the observed lesion length (in mm) at the  $j^{\text{th}}$  observation time,  $n$  was the total number of observations, and  $I$  the interval between each observation (in days). These values were computed for individual pruning lesions for  $n=5$  observations at daily intervals during the period from the 3<sup>rd</sup> to the 7<sup>th</sup> day after inoculation. For both tests, a percentage of protection of tomato generated by the bacteria, compared to the control, was computed.

#### Preventive and curative effect of *Streptomyces* against *B. cinerea* on tomato plants

To test a potential preventive effect of the 3 strains of *Streptomyces* against *B. cinerea* on tomato plants, three leaves per plant were removed, leaving 5-10 mm petiole stubs on the stems. Each pruning wound was treated with 10  $\mu$ l of a spore suspension at  $10^7$  spores.ml<sup>-1</sup> of a strain of *Streptomyces* sp. and then inoculated with 10  $\mu$ l of a spore suspension at  $10^6$  spores.ml<sup>-1</sup> of the strain BC1 of *B. cinerea*. Inoculation of BC1 was realized 0, 2, 4 or 6 h after treatment with the selected strain of *Streptomyces*.

To test a potential curative effect of the 3 strains of *Streptomyces* against *B. cinerea*, the same method was used except that inoculation of *B. cinerea* at  $10^6$  spores.ml<sup>-1</sup> was realized before the treatment with a spore suspension at  $10^7$  spores.ml<sup>-1</sup> of a strain of *Streptomyces* sp. In this case, treatment with *Streptomyces* was realized 0, 2, 4 or 6 h after the inoculation with the strains BC1 of *B. cinerea*.

All plants were incubated in a growth chamber with a photoperiod of 14 h and maintained at 21°C with a relative humidity above 80% to favor disease development. The

experiment was repeated three times independently, each with three replicates (3 plants each with 3 pruning wounds) per treatment. Lesion expansion on tomato stem was recorded from the 3<sup>rd</sup> to the 7<sup>th</sup> day after inoculation (DAI) and AUDPC and protection index were computed as described above.

## Statistical analysis

The data were subjected to one- or two-factor analysis of variance (ANOVA) using Statistical Package for the Social Sciences (SPSS) version 15 for Windows. When appropriate, the means were compared with the Duncan's Multiple Range Test (DMRT) and a  $P < 0.05$  was considered as being significantly different.

## Results

### *In vitro* antagonism of *Streptomyces* spp. against different strains of *B. cinerea*

The *in vitro* dual culture assay shows that the three strains of *Streptomyces* spp. inhibited the mycelial growth of the forty-one strains of *B. cinerea* in the range of 73% to 100% inhibition on PDA medium after two days of incubation at 21°C, compared with the control treatment without *Streptomyces* (Fig. 1). Significant differences in sensitivity to the 3 strains of *Streptomyces* sp. were observed between strains of *B. cinerea* (*S. philanthi* RM-1-138;  $F = 4.36$ ,  $df = 40, 82$ ,  $P < 0.0001$ ; *S. philanthi* RL-1-178;  $F = 3.45$ ,  $df = 40, 82$ ,  $P < 0.0001$ ; *S. mycarofaciens* SS-2-243;  $F = 3.03$ ,  $df = 40, 82$ ,  $P < 0.0001$ ). The strains RM-1-138 and RL-1-178 of *S. philanthi* completely inhibit the mycelial growth of 31 out of the 41 strains of *B. cinerea* tested and the strain SS-2-243 of *S. mycarofaciens* completely inhibited the mycelial growth of 25 strains of *B. cinerea*. Culture filtrates of each strain of *Streptomyces* have a strong inhibitory effect on the spore germination of the strains BC1 of *B. cinerea* (Fig. S1;

Supplementary data). This inhibitory effect is very effective (100% inhibition of spore germination) even when the culture filtrate is diluted at  $10^{-3}$ , suggesting a strong antifungal effect of the 3 strains of *Streptomyces* spp. tested.

To test the effect of volatile compounds produced by *Streptomyces* spp. on the mycelial growth of *B. cinerea*, the *in vitro* inhibitory action of volatiles was tested on ten isolates of *B. cinerea* having various level of sensitivity to the bacteria based on dual culture assay (Fig. 2). Significant differences between strains of *Streptomyces* spp. was observed (*S. philanthi* RM-1-138;  $F = 63.64$ ,  $df = 9, 20$ ,  $P < 0.0001$ ; *S. philanthi* RL-1-178;  $F = 92.53$ ,  $df = 9, 20$ ,  $P < 0.0001$ ; *S. mycarofaciens* SS-2-243;  $F = 51.28$ ,  $df = 9, 20$ ,  $P < 0.0001$ ) with an inhibition ranging from 6 to 30% depending both on the strain of *B. cinerea* and on the strain of *Streptomyces* spp. Some strains of *B. cinerea* are significantly less sensitive to *Streptomyces* than others. The strain E258 of *B. cinerea* is for instance among the least sensitive strain against the three strains of *Streptomyces*.

Efficacy of *Streptomyces* spp. against *B. cinerea* on tomato

The protective efficacy of the three strains of *Streptomyces* spp. against the strain BC1 of *B. cinerea* was evaluated on tomato plants, both on a detached-leaf test and on a whole-plant test. Two spore concentrations of *Streptomyces* spp. ( $10^6$  or  $10^7$  spores.ml<sup>-1</sup>) were evaluated. Globally, the highest is the spore concentration of *Streptomyces* spp. the most effective is the control of *B. cinerea* on plant organs (Fig. 3). A 2-way analysis of variance (strain of *Streptomyces* spp. x dose of bacteria done on the protection of the biological control agent) was realized independently on detached-leaf and on whole-plant bioassays. It reveals a *Streptomyces* strain effect ( $F = 15.62$ ,  $df = 2$ ,  $P < 0.0001$ ) and a dose effect ( $F = 49.04$ ,  $df = 1$ ,  $P < 0.0001$ ) on detached-leaf bioassay and a *Streptomyces* strain effect ( $F = 9.47$ ,  $df = 2$ ,  $P = 0.003$ ) and a dose effect ( $F = 7.95$ ,  $df = 1$ ,  $P = 0.015$ ) on whole-plant bioassay, with no interaction effect ( $F$

= 0.82,  $df = 2$ ,  $P = 0.464$  and  $F = 1.58$ ,  $df = 2$ ,  $P = 0.246$  for detached-leaf and whole plant bioassays, respectively). The strain RM-1-138 of *S. philanthi* at  $10^7$  spores.ml<sup>-1</sup> exhibits the strongest protection effect against *B. cinerea* on tomato plants with 57% protection on whole plants and 60% protection on detached leaves. The two other strains of *Streptomyces* sp. provide a lower protective effect against *B. cinerea* on tomato.

Efficacy of the strain RM-1-138 of *S. philanthi* against 3 different strains of *B. cinerea* on tomato

Three isolates of *B. cinerea* having various levels of *in vitro* sensitivity to the strain RM-1-138 were tested on tomato plants to estimate the efficacy of protection generated by this strain (Fig. 4). On both bioassays (whole plant test and detached leaf test) and whatever the dose of RM-1-138 applied ( $10^6$  or  $10^7$  spores.ml<sup>-1</sup>), lesions caused by *B. cinerea* were significantly reduced compared to the control without the biocontrol agent. A 2-way analysis of variance (strain of *B. cinerea* X dose of RM-1-138 on the protection of the biological control agent) was realized for each bioassay. On detached-leaf bioassay, analysis reveals a *B. cinerea* strain effect ( $F = 5.08$ ,  $df = 1$ ,  $P = 0.025$ ) and a dose effect ( $F = 17.02$ ,  $df = 2$ ,  $P = 0.001$ ) with no interaction ( $F = 0.36$ ,  $df = 2$ ,  $P = 0.703$ ). On whole-plant bioassay, an absence of *B. cinerea* strain effect ( $F = 1.40$ ,  $df = 2$ ,  $P = 0.284$ ), a dose effect ( $F = 4.22$ ,  $df = 2$ ,  $P = 0.05$ ) and no interaction effect ( $F = 0.28$ ,  $df = 2$ ,  $P = 0.764$ ) were observed. *S. philanthi* RM-1-138 at  $10^7$  spores.ml<sup>-1</sup> displays a protection of tomato plants comprised between 45% and 52% on whole plant tests and between 44% and 60% on detached-leaf tests, depending on the strain of *B. cinerea* tested.

Preventive and curative effect of *S. philanthi* RM-1-138 against *B. cinerea* on tomato plant

Treatment of pruning wounds of tomato with a spore suspension of *S. philanthi* RM-1-138 concomitantly with an inoculation of a spore suspension of *B. cinerea* gives a protection index of approximately 50% (Fig. 5). When the plants are treated with *S. philanthi* few hours before the inoculation with *B. cinerea* the protective effect decreases significantly (ANOVA on the protection index;  $F = 342.06$ ,  $df = 3, 8$ ,  $P < 0.0001$ ). A treatment realized on pruning wounds with *S. philanthi*, 6 hours before the inoculation with *B. cinerea* tends to favor the development of *B. cinerea* (protection index = -23%). When the plants are treated with *S. philanthi* few hours after the inoculation with *B. cinerea*, the protective effect also decreases significantly (ANOVA on the protection index;  $F = 172.14$ ,  $df = 3, 8$ ,  $P < 0.0001$ ). The protection index attained less than 5% when *S. philanthi* was inoculated 6 hours after the fungal pathogen *B. cinerea*.

## Discussion

The three strains of *Streptomyces* spp. tested in this study had a significant effect on the mycelial growth of all strains of *B. cinerea* tested on PDA agar plates, suggesting a strong direct antifungal effect. Inhibition of germination of *B. cinerea* conidia is also achieved by the culture filtrate of the three strains of *Streptomyces* spp. tested. This result was also observed for other strains of *Streptomyces* such as the culture filtrate of *S. globisporus* JK-1 that inhibit the spore germination of *Magnaporthe oryzae* and reduced its appressorial formation on rice leaves (Li et al. 2011). Moreover, volatile compounds produced by the strains *Streptomyces* spp. have also an inhibitory effect against *B. cinerea* mycelial growth. The genus *Streptomyces* is known to produce volatile antifungal compounds (Vaz Jauri et al. 2016). Volatile compounds produced by *S. globisporus* JK-1 was for instance able to control *Penicillium italicum* on *Citrus microcarpa* and *B. cinerea* on tomato fruit (Li et al. 2010; Li et al. 2012). *Streptomyces albulus* NJZJSA2 also produced volatile compounds effective against *Fusarium oxysporum* and *Sclerotinia sclerotiorum* (Wu et al. 2015).

Testing 41 strains of *B. cinerea* for their degree of sensitivity to the three strains of *Streptomyces* spp. revealed that some strains of *B. cinerea* are less sensitive to the inhibitory effect of antimicrobial compounds produced by these bacteria. Studies highlighting the diversity in sensitivity of plant pathogens to antimicrobial compounds produced by biocontrol agents have been listed by Bardin et al. (2015). However to our knowledge, this study is the first one revealing the diversity of sensitivity of a plant pathogen to volatile compounds produced by a biocontrol agent. Detection of less sensitive isolates to these compounds may indicate a risk of developing a resistance in case of increased selection pressure exerted by the given biocontrol agents. Even if a limited diversity in the sensitivity of the different strains of *B. cinerea* was observed, further studies are needed to evaluate the capacity of *B. cinerea* to become resistant to these compounds. Indeed, previous studies have demonstrated that *B. cinerea* can evolve and become resistant to antibiotic produced by biocontrol agents (Ajouz et al. 2010; Fillinger et al. 2012).

In this study, we demonstrated that spore suspension of the three isolates of *S. philanthi* and *S. mycarofaciens* tested in this study can significantly control *B. cinerea* on leaves and stem of tomato plants. Among the three strains tested, *S. philanthi* RM-1-138 exhibit the strongest protective efficacy against *B. cinerea* with 57% protection on whole-plant and 60% protection on the detached leaf, suggesting that this strain may have the best potential to be used for protection of tomato grown in greenhouses against gray mold. These results confirm the potential of strains belonging to the genus *Streptomyces* to control *B. cinerea* (Ge et al. 2015; Jiang et al. 2016). The best protection of tomato against *B. cinerea* was observed when spore suspension of *S. philanthi* were inoculated concomitantly with spores of *B. cinerea*, given a protection index superior to 50% both on leaves and on the stem. However, when the plants were treated with *S. philanthi* before or after the inoculation of *B. cinerea*, the protective effect decreased dramatically. The absence of a preventive effect by *S. philanthi* suggested that this

bacteria is not able to induce a strong systemic resistance in tomato plants and also that the strain may not be able to survive in the plant. This low capacity to survive within the plant could be due to its sensitivity to molecules produced by the plant. It could also be due to the temperature of the tests (21°C) which is not favorable to the growth of *S. philanthi* (Boukaew and Prasertsan 2014b). Experiments will be done to test these hypothesis and to measure the ability of this strain to survive in tomato tissues. The absence of curative effect suggests that the bacteria is not able to produce enough amount of antifungal compounds to limit the development of *B. cinerea* on the plant. It reinforces the hypothesis that this bacteria is not able to survive and to develop on the plant. More work is therefore needed to develop a formulation to consider using this strain preventively or curatively.

Our results demonstrated that the 3 strains of *Streptomyces* spp. produce very effective anti-*Botrytis* substances. The antagonistic mechanism of *Streptomyces* spp. is therefore probably related to the production of inhibitory antifungal volatile and non-volatile compounds. These results confirm that species of *Streptomyces* spp. can produce a wide spectrum of antifungal compounds such as antibiotics and volatile compounds (Vaz Jauri et al. 2016). The isolation and the characterization of these substances could lead to the development of effective and stable molecule-based product. Other modes of action could be tested, and a variety of fungal cell wall-degrading enzymes associated to biocontrol activity, such as chitinases and  $\beta$ -1,3-glucanase are known to be synthesized by *Streptomyces* (de Boer et al. 1998; Mahadevan and Crawford 1997; Mukherjee and Sen 2006; Vaz Jauri et al. 2016).

In conclusion, the strains of *Streptomyces* tested in this study may have the potential to control gray mold caused by *B. cinerea* on tomato but further work is definitely required to enhance its efficacy *in planta*. For instance improvement in the production process and development of a suitable formulation of the bacteria may result in a more effective product.

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## Figure Captions

**Fig. 1** Mycelial growth inhibition of the forty-one strains of *Botrytis cinerea* caused by the strains RM-1-138 (a) and RL-1-178 (b) of *S. philanthi* and the strain SS-2-243 (c) of *S. mycarofaciens* revealed by dual culture technique on PDA agar plates and incubated at 21°C for 2 days. Values are mean of three replications ( $\pm$  SE). Values with the same letter are not significantly different (ANOVA,  $P < 0.05$ ; Duncan multiple range test).

**Fig. 2** Mycelial growth inhibition of ten strains of *B. cinerea* caused by volatile substances produced by each of the strains RM-1-138 (a) and RL-1-178 (b) of *S. philanthi* and the strain SS-2-243 (c) of *S. mycarofaciens* on PDA agar plates and incubated at 21°C for 2 days. Values are mean of three replications ( $\pm$  SE). Values with the same letter are not significantly different (ANOVA,  $P < 0.05$ ; Duncan multiple range test).

**Fig. 3** Protection of tomato plants (in percentage) on whole-plant assay (a) and detached-leaf assay (b) against the strain BC1 of *B. cinerea* with the strains RM-1-138 and RL-1-178 of *S. philanthi* and the strain SS-2-243 of *S. mycarofaciens* at two spore concentrations ( $10^6$  and  $10^7$  spores.ml<sup>-1</sup>). Values are mean of three repetitions ( $\pm$  SE). To test the hypothesis that the efficacy of a biocontrol agent was not dependent on the strain of *Streptomyces* spp. and on the dose of bacteria used, a 2-way analysis of variance (strain of *Streptomyces* spp. X dose of bacteria) was realized for each bioassay and the Duncan's Multiple Range Test was used to compare the percentage of protection of tomato against *B. cinerea*. Values with the same letter are not significantly different for each bioassay independently (2-way ANOVA done on protection,  $P < 0.05$ ; Duncan multiple range test).

**Fig. 4** Protection of tomato plants (in percentage) on whole-plant assay (a) and detached-leaf assay (b) against 3 different strains of *B. cinerea* with the strain RM-1-138 of *S. philanthi* at two spore concentrations ( $10^6$  and  $10^7$  spores.ml<sup>-1</sup>). Values are mean of three repetitions ( $\pm$  SE). To test the hypothesis that the efficacy of a biocontrol agent was not dependent on the strain of *B. cinerea* and on the dose of the strain of RM-1-138 of *S. philanthi*, a 2-way analysis of variance was realized and the Duncan's Multiple Range Test was used to compare the means of the percentage of protection of tomato against *B. cinerea*. Values with the same letter are not significantly different at  $P < 0.05$ .

**Fig. 5** Timing of preventive (a) or curative (b) measures by the strain RM-1-138 of *S. philanthi* on the protection of tomato plants inoculated with *B. cinerea*. Values are mean of three replications ( $\pm$  SE). Values with the same letter are not significantly different (ANOVA,  $P < 0.05$ ; Duncan multiple range test).

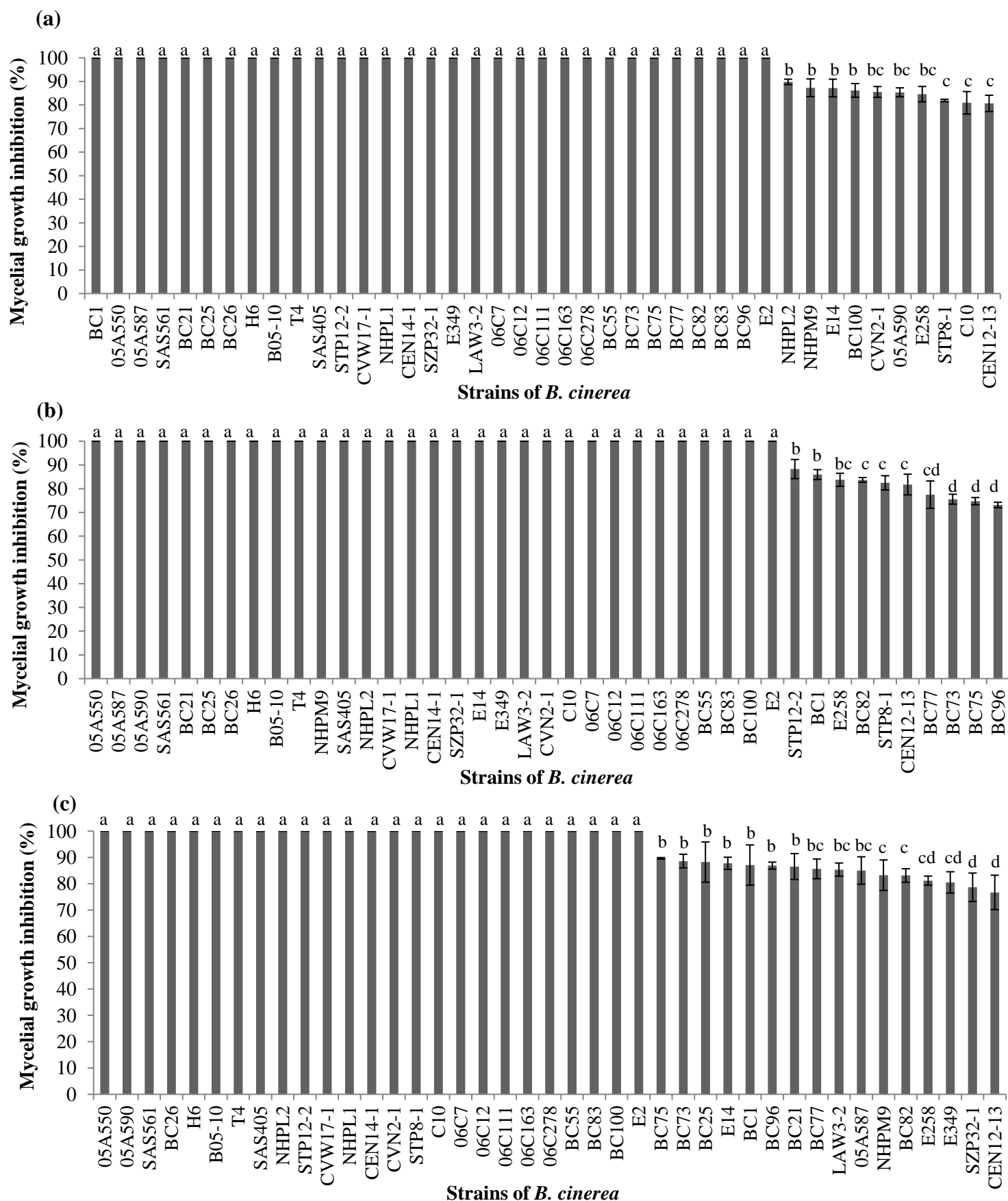
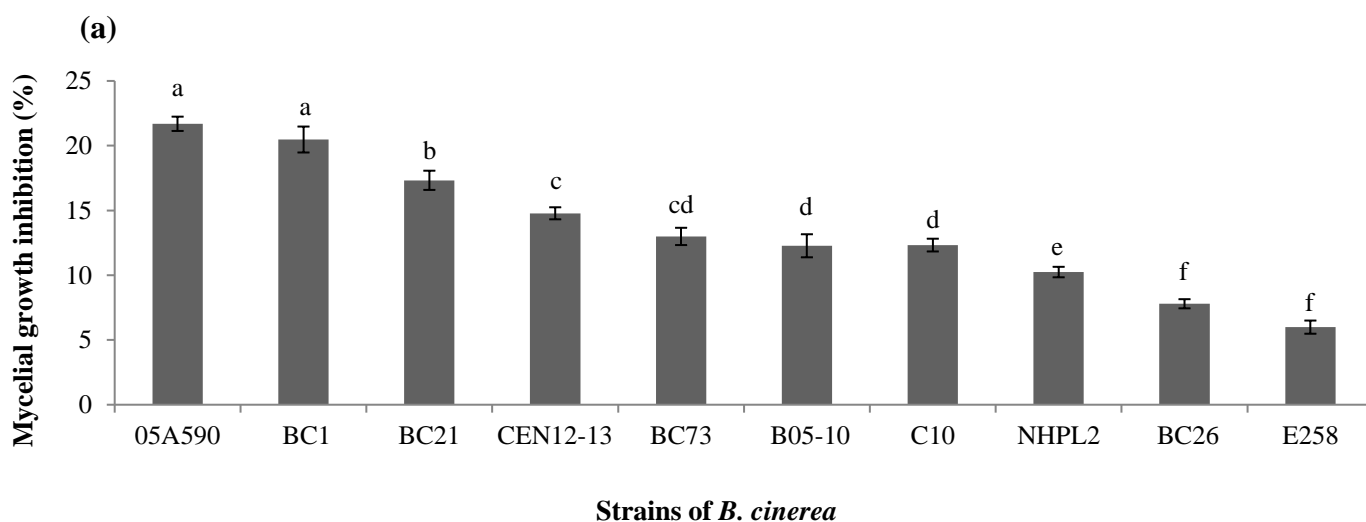
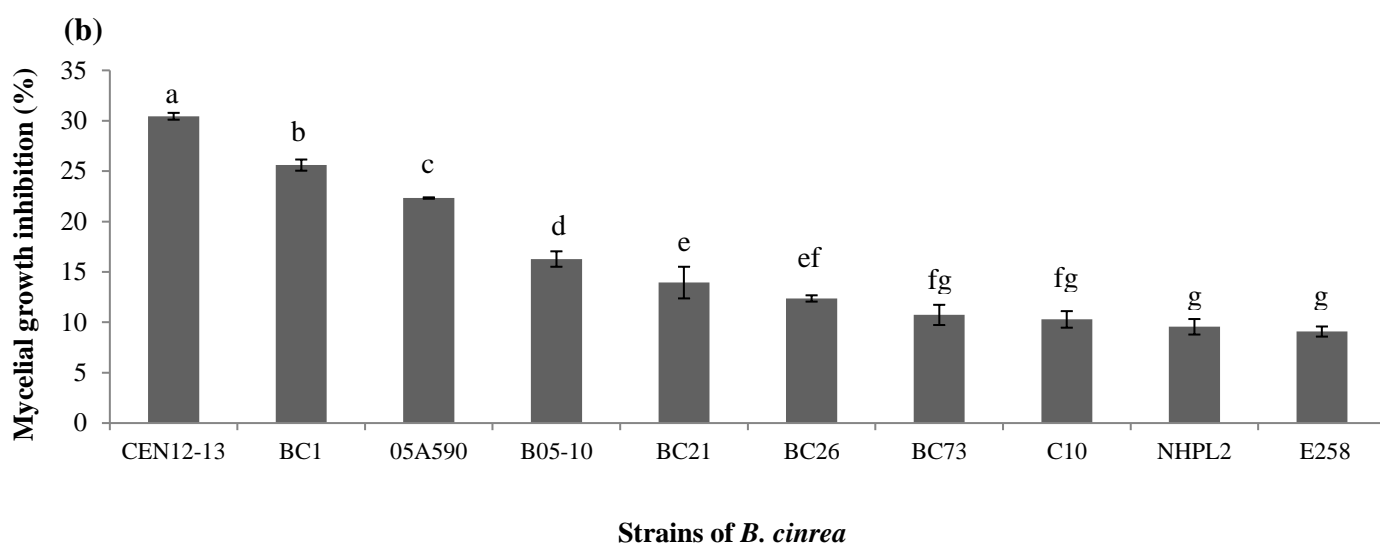


Fig. 1

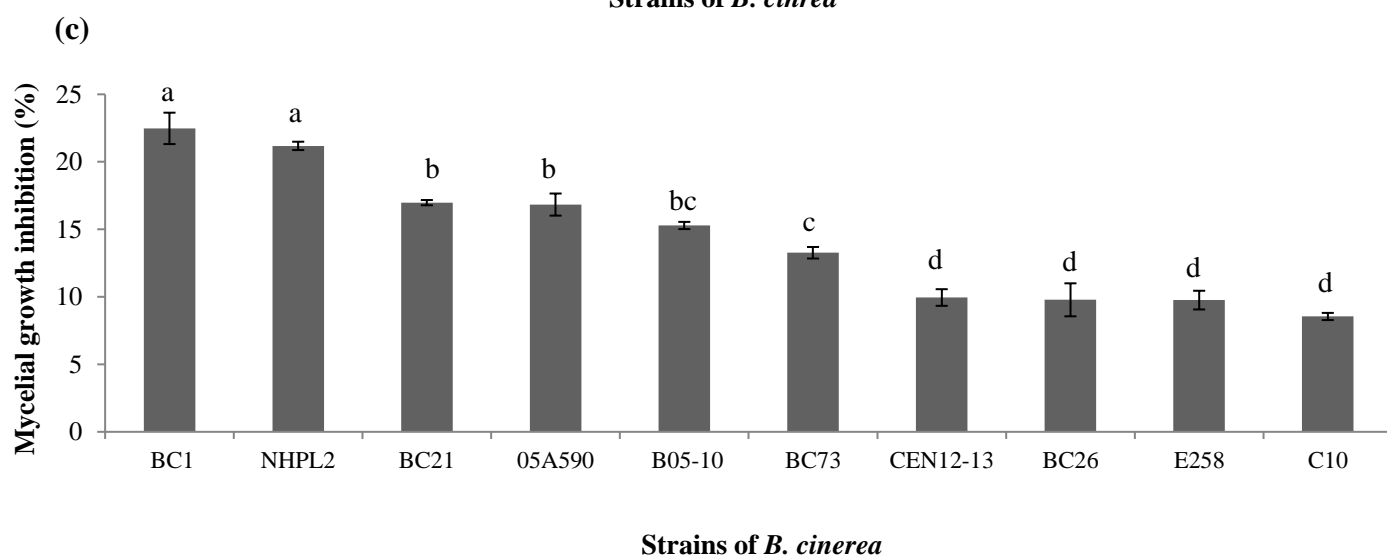
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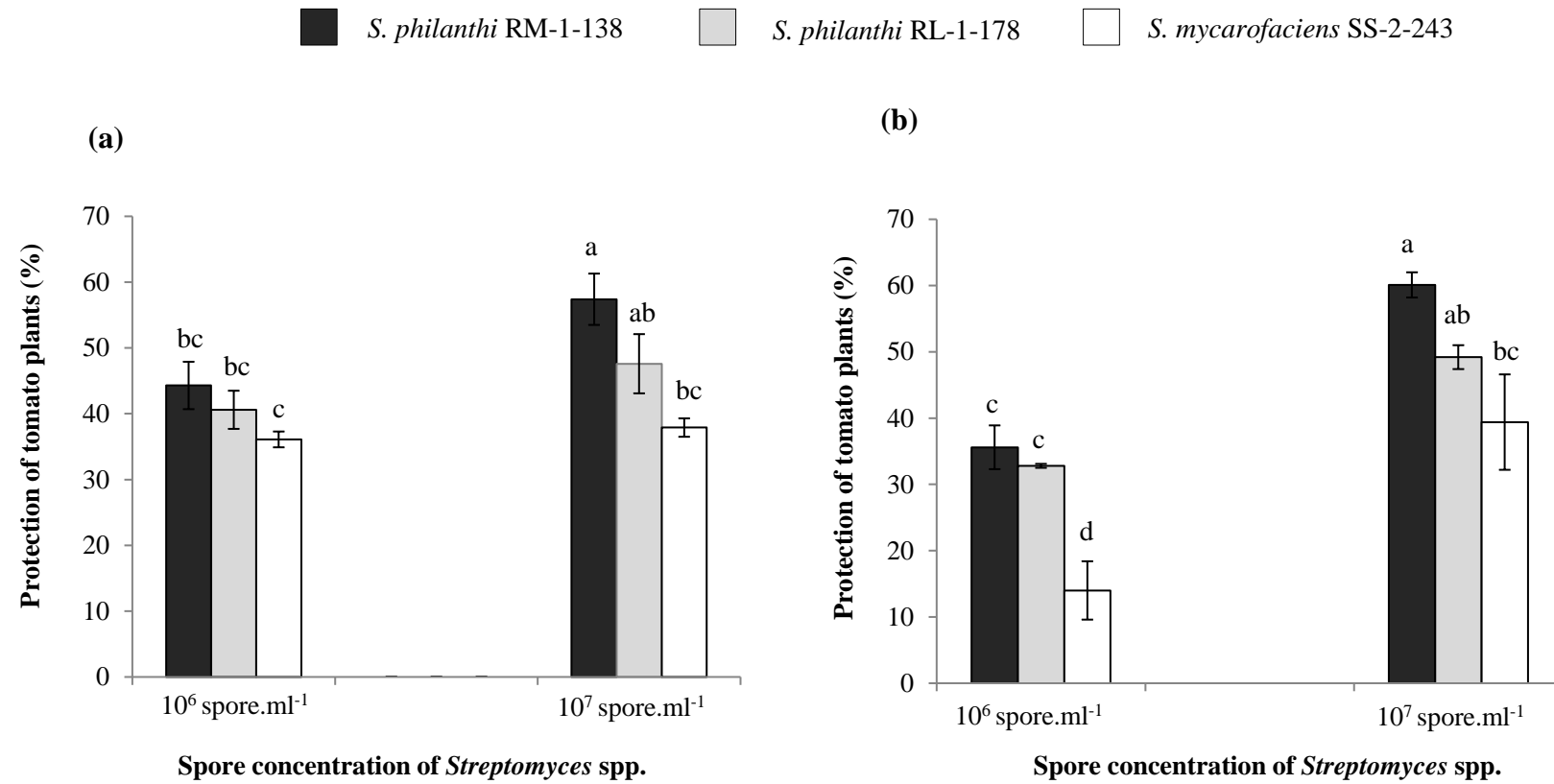


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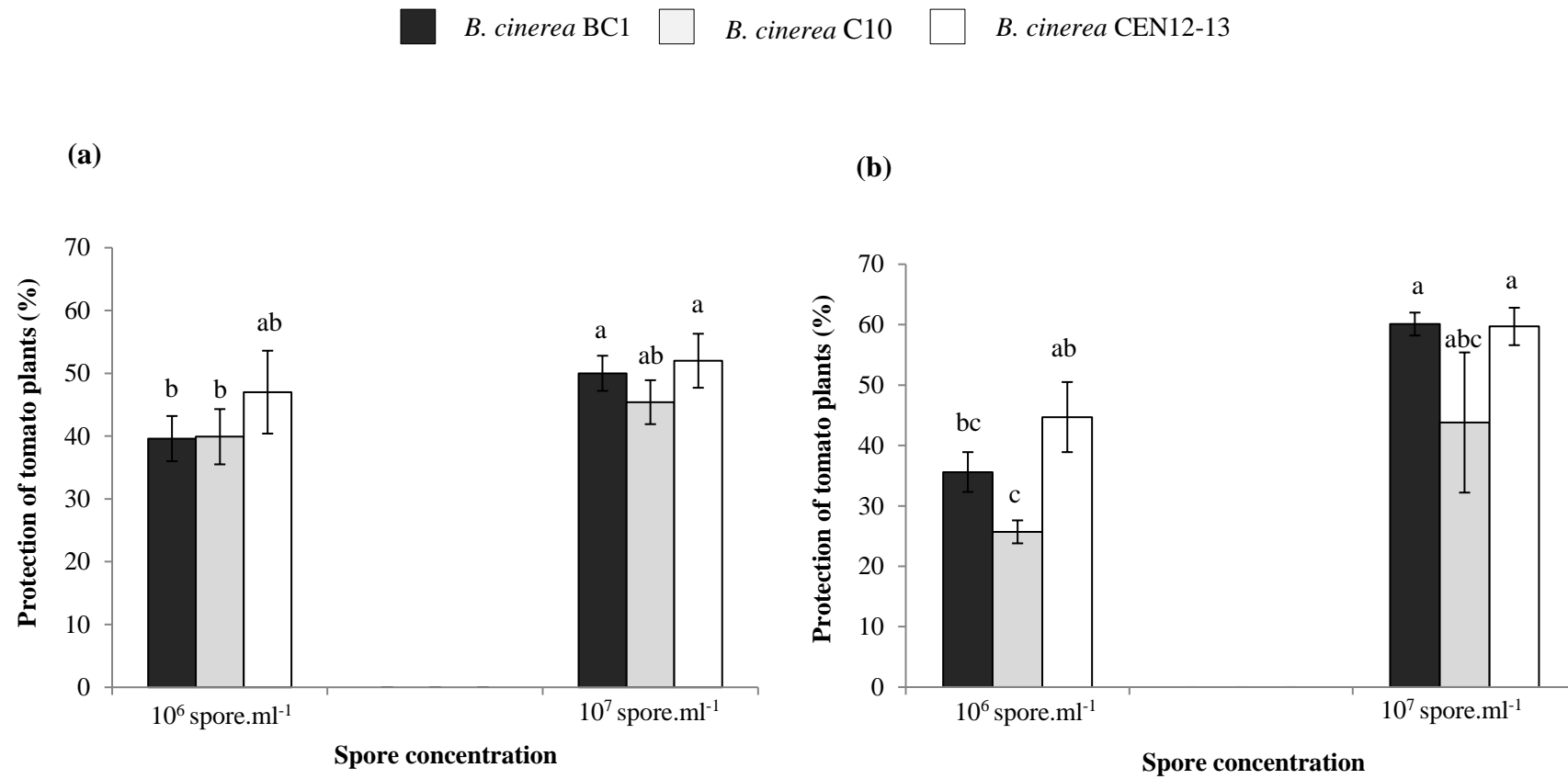
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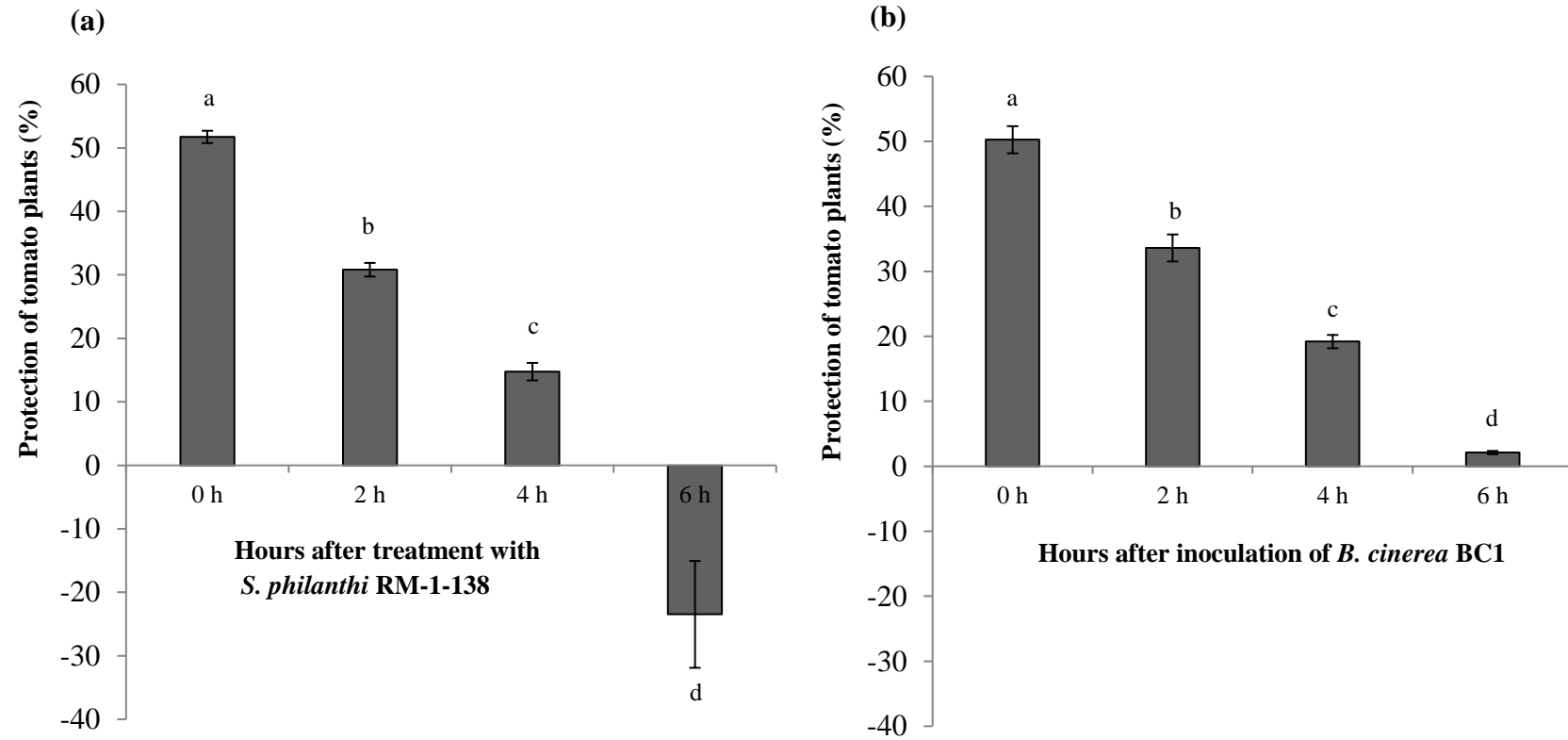
514 **Fig. 2**



**Fig. 3**



**Fig. 4**

**Fig. 5**