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Biological and integrated control of plant pathogens
IOBC-WPRS Bulletin Vol. 115, 2016
pp. 89-94

Efficacy of *Streptomyces* spp. strains against different strains of *Botrytis cinerea*

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Abstract: Strains RM-1-138 and RL-1-178 of *Streptomyces philanthi* and SS-2-243 of *S. mycarofaciens*, isolated from the rhizosphere of chili peppers grown in southern Thailand, have shown a good efficacy to control *Sclerotium rolfsii*, *Ralstonia solanacearum* and *Rhizoctonia solani* in previous studies but their effect against *Botrytis cinerea* is not known. In this study we evaluated the efficacy of the three strains of *Streptomyces* spp. against *B. cinerea in vitro* and on tomato plants. Results indicate that the three strains inhibit the growth of *B. cinerea* in Petri plates and have a significant protective efficacy, although variable between strains of *Streptomyces* spp., against *B. cinerea* on tomato plant. To assess the possible variability in susceptibility to these antagonistic strains in populations of *B. cinerea*, the effect of these bacteria were evaluated against 41 strains differing in their geographic origin, host of isolation and level of aggressiveness. Results based on confrontation tests in Petri plates suggest a limited diversity in the sensitivity of the different strains of *B. cinerea* to these biological control agents.

Key words: *Botrytis cinerea*, biological control, durability, sensitivity, *Streptomyces* spp.

Introduction

Grey mould caused by the fungus *Botrytis cinerea* is an economically important disease on numerous crops plants. In general, the disease management strategy for plants largely relies on the use of chemical fungicides. Biocontrol is a promising method to control the disease. Species of *Streptomyces* are potential biocontrol agents since they are ubiquitous in the environment and many of them produce various secondary metabolites with diverse biological activities including the ability to inhibit plant pathogenic fungi. Several species of *Streptomyces* have been isolated and used to control plant diseases such as sunflower head and stem rot caused by *Sclerotinia sclerotiorun* (Baniasadi *et al*., 2009), sugar beet damping-off caused by *Sclerotium rolfsii* (Errakhi *et al*., 2007), rice blast and sheath blight caused by *Pyricularia oryzae* and *Rhizoctonia solani*, respectively (Prabavathy *et al*., 2006). In a previous study, the strains RM-1-138 and RL-1-178 of *Streptomyces philanthi* and SS-2-243 of *S. mycarofaciens* were shown to produce both volatile and non-volatile antifungal metabolites that inhibited a range of plant pathogens (Boukaew *et al*., 2011; 2013; 2014).

The objectives of the present study were (i) to evaluate the efficacy of these three strains of *Streptomyces* spp. against *B. cinerea in vitro* and on tomato plants and (ii) to assess the possible variability in susceptibility to these antagonistic strains in populations of *B. cinerea*.

Material and methods

Microorganisms and inoculum production

Streptomyces spp. strains used in this study were isolated from the rhizosphere of chilli pepper in southern Thailand (Boukaew *et al*., 2011). The strains were maintained in 20% glycerol at -20 °C as stock culture. Inoculum was produced on glucose yeast-malt agar (GYM) medium at 21 °C. After 10 days of incubation, spores were collected by washing the culture in sterile distilled water. The spore concentrations of *Streptomyces* spp. was determined with a haemacytometer and adjusted to 10^6 and 10^7 spores/ml.

Forty-one strains of *B. cinerea* differing in their geographic origin and host of isolation were used in this study. All isolates were single-spored and conserved at -20 °C before use. Inoculum was produced on potato dextrose agar (PDA) medium in a growth chamber at 21 °C. After 14 days of incubation, conidia were collected by washing the culture in sterile distilled water. The cell suspension was filtered through a sterile 30 µm mesh filter to remove mycelial fragments. The concentration of the spore suspensions was determined with a haemacytometer and adjusted to 10^6 conidia/ml.

In vitro *experiment*

The three strains of *Streptomyces* spp. were evaluated for their antagonism against forty-one strains of *B. cinerea* using a dual culture technique (Islam *et al*., 2009). For each strain of *Streptomyces* spp., a spore suspension was one-line streaked on one side of a PDA plate and incubated in a growth chamber (21 °C, dark) for 10 days. After 10 days of incubation of the Streptomycete cultures, 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 was placed on a PDA plate not inoculated with *Streptomyces* spp. 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 realized for each *Streptomyces – B. cinerea* strain combination. The colony size in each treatment was recorded and the percentage of inhibition of mycelial growth was calculated as follows:

$$\text{Inhibition (\%)} = [\{(\text{Control - treatment})/\text{Control}\} \times 100].$$

Evaluation of the efficacy of S. philanthi RM-1-138 against B. cinerea on tomato

Plants of tomato cv. ‘Monalbo’ were grown in a glasshouse for 7 to 8 weeks where they received a standard commercial nutrient solution once or twice a day, depending on needs. They had at least 8 fully expanded leaves when used. Two types of test were realized to evaluate the efficacy of *Streptomyces* spp. to control *B. cinerea* on tomato: a detached-leaf assay and a whole-plant test.

Tomato leaves were sprayed with a spore suspension of *Streptomyces* spp. at the concentration 10^6 or 10^7 spores7ml, and then placed in clear plastic boxes with humid absorbent paper to maintain high relative humidity. Mycelial plugs (5-mm in diameter) of strains of *B. cinerea* excised from the growing margin of three-day old PDA cultures were inoculated onto the leaves. Following inoculation, the detached-leaves were incubated in a growth chamber in conditions conducive to disease development (21 °C, 14 h-photoperiod). Leaves not treated with *Streptomyces* spp. were also inoculated as a control. Three replicates were realized for each treatment and two independent repetitions of the test were done. The lesion area was determined with ImageJ at 2, 3 and 4 days after inoculation.

For the whole-plant test, three leaves were removed from each of 5 plants, leaving 5-10 mm petiole stubs on the stems. Plants were concomitantly inoculated with a spore suspension dosed at 10^6 conidia7ml of *B. cinerea* and a 10-µl spore suspension of *Streptomyces* spp. at the concentration of 10^6 or 10^7 spores/ml. As a control, each pruning wound was inoculated with 10 µl of a spore suspension of *B. cinerea* alone. Plants were incubated in a growth chamber in conditions conducive to disease development. Two repetitions of the tests were realised. Lesion expansion on tomato stem was recorded from the 3rd to the 7th day after inoculation (DAI) and the areas under the disease progress curves (AUDPC) were calculated.

For both tests a protection index was computed as follows:

$$\text{Protection (\%)} = [\{(\text{Control - treatment})/\text{Control}\} \times 100].$$

The data were subjected to analyses of variance (ANOVA) using Statistica software. Statistical significance was evaluated using Newman Keuls test and a $P < 0.05$ was considered as being significantly different.

Results and discussion

In vitro *antagonism of* Streptomyces *spp. against forty-one strains of* B. cinerea

The three strains of *Streptomyces* spp. inhibited the mycelial growth of all strains of *B. cinerea* in the range of 73% to 100%, suggesting all three produced an anti-*Botrytis* substance, probably secreted into the medium. Among the 41 strains of *B. cinerea* tested, 31, 31 and 25 were completely inhibited by strains RM-1-138, RL-1-178 and SS-2-243, respectively, revealing that some strains of *B. cinerea* were less sensitive to *Streptomyces* spp. As an example, the inhibition of *B. cinerea* mycelial growth by strain RM-1-138 of *S. philanthi* is presented in Figure 1.

Evaluation of the efficacy of three strains of* Streptomyces spp. *against* Botrytis cinerea *on tomato plant

The efficacy of three strains of *Streptomyces* spp. (RM-1-138, RL-1-178 and SS-2-243) against strain BC1 of *B. cinerea* was evaluated both on a detached leaf test and on a whole-plant test (Table 1). Two spore concentrations of *Streptomyces* spp. were tested, 10^6 or 10^7 spores/ml. All strains of *Streptomyces* spp. had a significant protective efficacy, although it varied between strains and dose of *Streptomyces* spp., against *B. cinerea* on tomato plant. At 10^6 spores/ml, there are no statistical differences between strains of *Streptomyces* spp. (ANOVA, $p = 0.063$ for detached-leaves test and $p = 0.54$ for whole-plant test). Further repetitions are needed to precisely evaluate a possible antagonist strain effect.

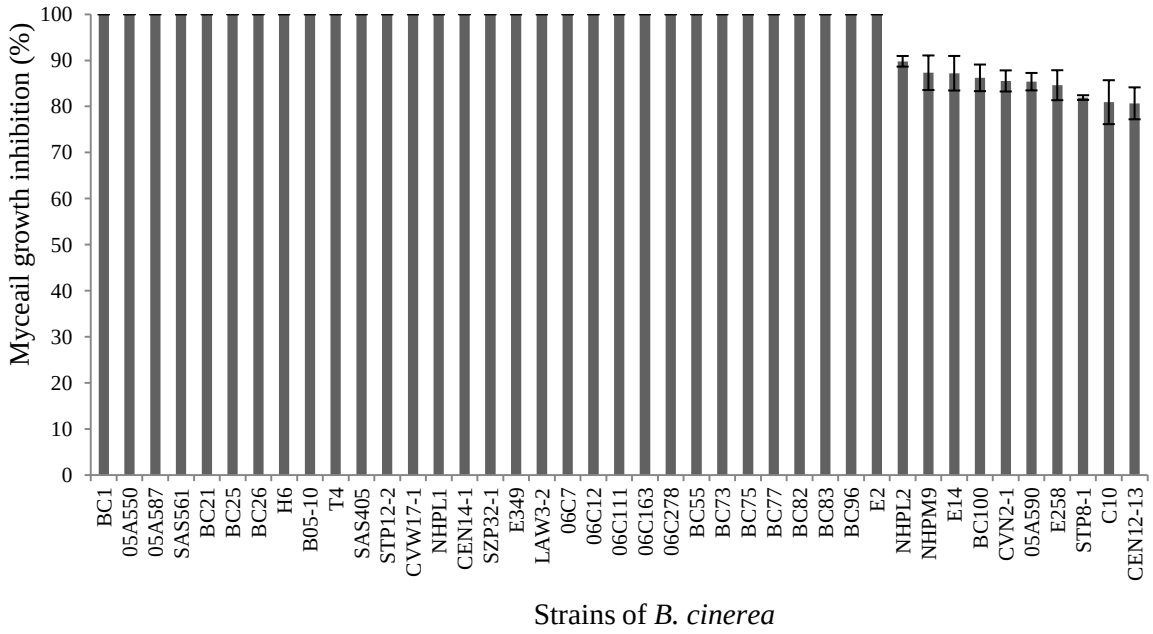

Figure 1. Mycelial growth inhibition of the forty-one strains of *B. cinerea* caused by *S. philanthi* strain RM-1-138, revealed by dual culture technique on PDA agar plates incubated at 21 ºC for 2 days. Data are the mean of three replicates ± SE.

Table 1. Protection of tomato (in percentage) against *Botrytis cinerea* BC1 with the biocontrol agents RM-1-138, RL-1-178 and SS-2-243. At 10^6 spores/ml, data are the mean of two replicates ± SE. At 10^7 spores/ml, only one replicate of the experiment was done.

	Dose (sp/ml)	**Whole-plant test**	**Detached-leaves test**
RM-1-138	10^6	42.3 ± 5.2	38.1 ± 3.8
	10^7	50	62.3
RL-1-178	10^6	39.0 ± 4.1	32.7 ± 0.5
	10^7	40	52
SS-2-243	10^6	35.4 ± 1.6	17.0 ± 5.6
	10^7	39	51

Evaluation of the efficacy of* Streptomyces philanthi *RM-1-138 against selected strains of* Botrytis cinerea *on tomato plant

The effect of *S. philanthi* RM-1-138 to control *B. cinerea* was evaluated on a detached leaf test and on whole-plant test against three strains of *B. cinerea* (Table 2). Strains BC1, C10 and CEN12-13 were chosen as they present diverse levels of sensitivity to *S. philanthi* RM-1-138 (Figure 1). On both bioassays and at a spore concentration of 10^6 or 10^7 spores/ml of *S. philanthi* RM-1-138, lesions caused by *B. cinerea* were reduced compared to the control without the biocontrol agent. A 2 way-analysis of variance (dose x strains) did not reveal any significant differences for both bioassays. Further repetitions are needed to precisely evaluate a possible dose and strain effect.

Table 2. Protection of tomato (in percentage) against 3 strains of *Botrytis cinerea* with the biocontrol agent RM-1-138 of *S. philanthi*. Data are the mean of two replicates ± SE.

	Whole-plant test		**Detached-leaves test**	
	10^6 sp/ml	10^7 sp/ml	10^6 sp/ml	10^7 sp/ml
BC1	25.5 ± 9.3	38.0 ± 2.3	38.1 ± 3.8	62.3*
C10	38.2 ± 7.0	44.3 ± 5.7	28.8 ± 0.1	57.6 ± 9.7
CEN12-13	44.7 ± 12.5	50.8 ± 8.2	45.1 ± 2.6	58.2 ± 6.2

* only one replicate of the experiment was done.

Bacteria of the species *Streptomyces* ssp. are known to produce several antimicrobials and have been frequently identified as effective biocontrol agents against various plant pathogens including *B. cinerea* (Li *et al*., 2012; Wan *et al*., 2008). This study reveals that these strains of *Streptomyces* spp. are potential biocontrol agents against *B. cinerea* on tomato, probably acting through antibiosis. Even if a limited diversity in the sensitivity of the different strains of *B. cinerea* was observed, further studies are now needed to evaluate the capacity of *B. cinerea* to become resistant to these antibiotic-producing biocontrol bacteria and thus jeopardise their durability. Indeed, previous studies have demonstrated that *B. cinerea* is able to evolve and become resistant to antibiotic-producing biocontrol agents (Ajouz *et al*., 2010; Fillinger *et al*., 2012).

Acknowledgements

This work was financially supported by a scholarship from the Office of the Higher Education Commission to Mr. Sawai Boukaew under the CHE-PhD Scholarship Program and Graduate School of Prince of Songkla University.

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