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Diversity in the susceptibility of *Botrytis cinerea* strains to the biological control agent *Pseudomonas helmanticensis*

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Abstract: The strain *Pseudomonas helmanticensis* CT22 isolated from tomato stem in a tomato greenhouse in Bejaia (Algeria) has shown a good protective efficacy against *Botrytis cinerea* in controlled conditions but its effect against various strains of *B. cinerea* was not assessed. In this study, we evaluated the diversity in the sensitivity of sixty two strains of *B. cinerea* strains to *P. helmanticensis* CT22 both *in vitro* and on tomato. *In vitro* effect of the biocontrol agent is variable according to the strain of *B. cinerea*, both in dual culture assay or in volatile assay. Due to the very high level of efficacy against all tested strains of *B. cinerea* on detached tomato stem when *P. helmanticensis* CT22 was applied at 10^7 CFU/ml, the dose of application was reduced 10-fold at 10^6 CFU/ml. In this condition, the protective efficacy of the biocontrol agent is significantly influenced by *B. cinerea* strains with protection levels ranging from 24% to 100%. These results suggest a diversity in the sensibility of the different strains of *B. cinerea* to the biological control agent and emphasize the importance to consider several strains of a given plant pathogen species when screening a biocontrol agent.

Key words: *Botrytis cinerea*, *Pseudomonas helmanticensis*, efficacy, diversity, durability

Introduction

Gray mold caused by *Botrytis cinerea* is one of the most damaging diseases in greenhouse tomato production throughout the word including Algeria (Aissat *et al.*, 2008). This fungus infects aerial parts of tomato, and the infection of stems can kill the entire plant and cause substantial yield losses. Biocontrol agents constitute an alternative to fungicide use for the protection of crops against this plant pathogenic fungus.

Biological control is generally considered as inconsistent in field conditions, especially because of climatic fluctuation and lack of ecological competence of the antagonistic microorganisms (Nicot *et al.*, 2016). The variation in sensitivity of fungal pathogen populations to biological products is another factor that may explain the inconstancy of the efficacy of biocontrol agents (Bardin *et al.*, 2015).

This study aimed to evaluate the diversity in the susceptibility of various strains of *B. cinerea* to the biocontrol agent *Pseudomonas helmanticensis* CT22, recently isolated in Algeria (Bouaoud *et al.*, 2017). To this end, sixty two strains of *B. cinerea* sampled from various fields in France and Algeria and having different level of aggressiveness on tomato were tested for their susceptibility on tomato plants and in *vitro* on Petri plates against strain CT22 of *P. helmanticensis*.

Material and methods

Fungal strains

Sixty two strains of *B. cinerea* collected in Algeria and France from different plant hosts (tomato, grapevine, strawberry, poinsettia, gerbera) or from the environment (water, rainfall, snow) were used throughout this study. These strains were maintained as spore suspensions in glycerol phosphate buffer at -20 °C. To prepare inoculum, strains were grown in Petri plates on potato dextrose agar medium (PDA, Difco Laboratory Detroit, 39 g/l) and incubated in a growth chamber (21 °C, photoperiod of 14 hours) for 14 days. Conidia produced on PDA medium were then collected in sterile distilled water and filtered through a 30 µm sterile filter to remove mycelial fragments. Conidia concentration was determined with a hemocytometer and adjusted to 10^6spores/ml for further use.

Biocontrol agent

Pseudomonas helmanticensis CT22 used in this study was isolated from tomato stem in tomato greenhouse situated in Bejaia region, Algeria (Bouaoud *et al.*, 2017). The bacterial strain was grown on Tryptic Soy Agar medium (TSA; Trypton Caseine Soja, 30 g/l and Agar, 15 g/l, Fisher Scientific) at 25 °C for 48 hours. For experimental purposes, cell suspension was realized by adding bacterial cells to distilled sterile water. The final concentration achieved for each bacterial suspension was assessed by dilution plating on TSA medium.

Dual culture assay

A 5-mm mycelial plug collected from the peripheral region of a 3-days old colony of *B. cinerea* was placed in the center of a Petri plate (PDA medium) and a 5-mm plug of the bacteria *P. helmanticensis* CT22 collected from TSA medium was placed in the same Petri plate at 2.5 cm apart. Plates were incubated in a growth chamber during 3 to 4 days depending on *B. cinerea* strain (21 °C, 14 h photoperiod). The experiment was conducted with three replicates. The percentage of mycelial growth inhibition of *B. cinerea* (PI) was then calculated for each of the 62 strains tested according to the following formula:

$$PI\ (\%) = 100 \times (R_{control} - R_{treatment}) / R_{control,}$$

with $R_{control}$, the mycelial radial growth of a given strain of *B. cinerea* in the control plate without bacteria and $R_{treatment}$, the mycelial radial growth of a given strain of *B. cinerea* in the presence of the bacteria CT22.

Volatile effect assay

To evaluate the effect of volatiles produced by CT22 on the mycelial development of the 62 strains of *B. cinerea*, two Petri dish plates sealed with parafilm, each containing a microorganism (*B. cinerea* on one plate and *P. helmanticensis* CT22 on the other) were prepared according to the protocol described by Jamalizadeh *et al.* (2008). Sealed plates were incubated in controlled conditions (21 °C, 14h photoperiod) during 3 to 4 days. Evaluation of the volatile effect was determined by measuring the diameter of fungal colony (mm). The experiment was conducted with three replicates. The percentage of mycelial growth inhibition (PIv) was then calculated as following:

$$PIv\ (\%) = 100 \times (D_{control} - D_{treatment}) / D_{control},$$

with $D_{control}$, the diameter of the mycelial growth of *B. cinerea* in the control plate without bacteria and $D_{treatment}$, the diameter of the mycelial growth of *B. cinerea* in the presence of the bacteria CT22.

Tomato plant bioassay

To evaluate the effect of CT22 on the 62 strains of *B. cinerea*, a bioassay was realized on detached stem of 8-10 weeks old tomato plants cultivar Monalbo, according to the protocol described by Bouaoud *et al.* (2017). Detached stems were incubated in a growth chamber (21 °C, 14-h photoperiod; 114 µmol/$m^{2/}$s). The length of lesion (in mm) was measured between the 3rd and the 7th day after inoculation and the area under the disease progress curve (AUDPC) was computed as described by Decognet *et al.* (2009). For each strain of *B. cinerea* tested, six replicates were done and a control without CT22 was assessed. A percentage of protection of tomato generated by the bacteria was computed for each isolate as followed:

$$PP\ (\%) = 100 \times (AUDPC_{control} - AUDPC_{treatment}) / AUDPC_{control,}$$

with $AUDPC_{control}$ the disease severity due to *B. cinerea* measured on the stem without the bacteria CT22 and $AUDPC_{treatment}$ the disease severity due to *B. cinerea* measured on the stem in the presence of CT22.

Results and discussion

In vitro effect of* P. helmanticensis *on the development of* B. cinerea *strains

In vitro assays reveal that the strain CT22 of *P. helmanticensis* inhibits the mycelial growth of the 62 strains of *B. cinerea* on PDA medium in the range of 33-66% in dual culture assay (PI) and in the range of 40-89% in volatile assay (PIv), compared with the control treatment without CT22. Analysis of variance performed respectively on PI and PIv revealed a significant *B. cinerea* strains effect ($P < 0.0001$), suggesting a diversity of sensitivity of *B. cinerea* strains to *P. helmanticensis* CT22.

Diversity in the susceptibility of* B. cinerea *strains to* P helmanticensis *on tomato

At the highest dose of bacteria (10^7 CFU/ml), protection of tomato against *B. cinerea* is high: the protection index for the 62 strains of *B. cinerea* tested is comprised between 75 and 100%. At this concentration, the protection is thus weakly influenced by the strain of *B. cinerea* tested. At 10^6 CFU/ml, the protection is more variable with a protection comprised between 24 and 100% with a significant strain effect (ANOVA, $P < 0.0001$). A significant correlation was established between the level of protection conferred by *P. helmanticensis* at the concentration of 10^7 CFU/ml and the level of protection at 10^6 CFU/ml (Spearman test, $R = 0.46$, $P < 0.05$), suggesting the existence of strains of *B. cinerea* less sensitive to CT22. These results show that the protective efficacy of this biocontrol agent can vary depending on the strain of *B. cinerea*.

Conclusions and perspectives

In this study, the diversity in the sensitivity of *B. cinerea* strains to the biocontrol agent *P. helmanticensis* CT22 was evaluated through *in vitro* (dual culture and volatiles) and *in planta* assays. Significant variability in sensitivity of *B. cinerea* strains to CT22 was detected revealing the existence of *B. cinerea* strains less sensitive to the biocontrol agent. Other studies have also revealed a wide range of sensitivity of plant pathogens to various biocontrol agents (Bardin *et al.*, 2015). It emphasizes the importance of considering several strains of a given plant pathogen species when screening a biocontrol agent.

The establishment of a baseline sensitivity would be helpful in future studies to monitor possible shifts in the sensitivity to this biocontrol agent in *B. cinerea* populations and thus verify that the presence of less sensitive strains does not affect its efficacy in the field. Moreover, it will be necessary to ensure that the use of this biocontrol agent in commercial greenhouses will not lead to the selection of even less sensitive strains. The possible decrease in sensitivity of *B. cinerea* strains to *P. helmanticensis* CT22 will be tested in future studies by producing successive generations of *B. cinerea* in the presence of this biocontrol agent.

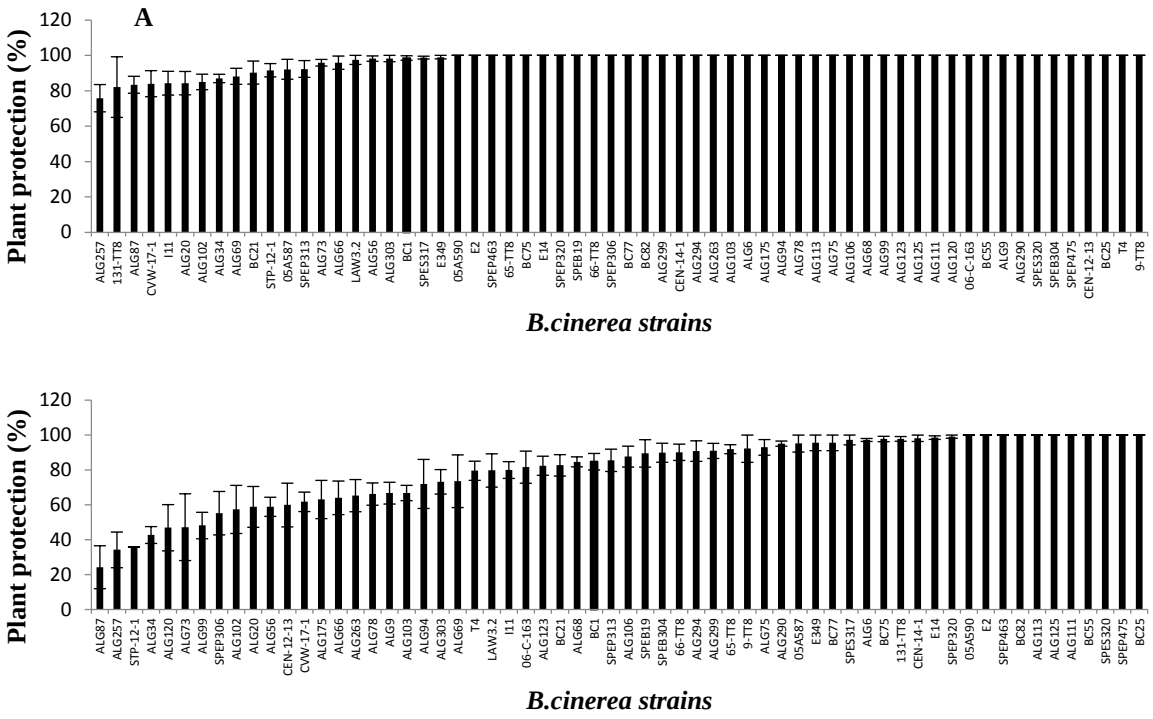

Figure 1. Protection of detached tomato stem conferred by *P. helmanticensis* CT22 at two concentrations (A) 10^7 CFU/ml and (B) 10^6 CFU/ml against 62 strains of *B. cinerea*. Each value corresponds to the mean of 6 replicates ± standard error.

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