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Abstract: The development of BCAs represents an attractive alternative to fungicides for the protection of crops against plant pathogens but the durability of this method has not been studied in details. The objective of the present work was to estimate the risk of loss of biocontrol efficacy towards *Botrytis cinerea*, by evaluating the sensibility of various isolates of the pathogen to the biocontrol agent *Microdochium dimerum*. The protective efficacy of *M. dimerum* was evaluated on tomato plants against 41 strains of *B. cinerea* differing in their geographic origin and host of isolation. To this end, whole tomato pruning wounds and detached stem sections were concomitantly inoculated with *B. cinerea* and with *M. dimerum*. Lesion expansion was recorded daily from the 3rd to the 7th day after inoculation. Due to the very high level of efficacy against all tested strains of *B. cinerea* when *M. dimerum* was used at the recommended dose, it was necessary to reduce the dose of application 10-fold to assess the diversity of sensitivity of *B. cinerea* to this BCA. In these conditions, a wide range of sensitivities were observed among strains of the pathogen tested with protection levels ranging from 0 to 100% (mean = 53 ± 4%; median = 49%). A correlation was observed between the level of aggressiveness of a strain to tomato and its sensitivity to the biocontrol agent (assessed by the protection level). It reveals the importance of considering several strains of the pathogen when screening for biocontrol agents, to obtain a good representation of the pathogen population and thus take into account the potential durability of biocontrol.

Key words: *Botrytis cinerea*, biological control, durability, sensitivity, *Microdochium dimerum*

Introduction

The development of biocontrol agents (BCAs) represents an attractive alternative to fungicides for the protection of crops against plant pathogens. Various microbial agents have shown a significant efficacy in different plant species against various plant pathogens and particularly against *Botrytis cinerea* (Nicot *et al.*, 2011). Although biological control against plant pathogens has been widely studied in the past years leading to the marketing of several BCAs, little is known on the durability of efficacy of this control method. Even though knowledge on the baseline sensitivity of plant pathogens to BCAs appears to be necessary to determine the risk of possible adaptation of pathogen populations in response to selection pressure exerted by the BCAs in the field. To our knowledge, the diversity of the efficacy of biocontrol agents against plant pathogens was mainly studied for BCAs that produce metabolites with a direct effect on patnogens (Ajouz *et al.*, 2011; Buck & Jeffers, 2004; Mazzola *et al.*, 1995; Schouten *et al.*, 2004). Not much is known about BCAs which do not produce toxins or antagonistic compounds.

The purpose of the present study was (i) to estimate the diversity in susceptibility of *B. cinerea* to the BCA *Microdochium dimerum* in order to detect any differences in sensitivity between isolates that might lead to development of resistance and (ii) to evaluate the

correlation between the level of aggressiveness of strains of *B. cinerea* and the efficacy of the BCA. No evidence of a direct effect of *M. dimerum* on *B. cinerea* has been made so far. The mode of action of this BCA is probably related to indirect effects such as nutrient competition, interference with the pathogenic mechanisms of *B. cinerea* or induction of plant defense.

Material and methods

Collection of fungal isolates and inoculum production

Forty-one strains of *B. cinerea* differing in their geographic origin and host of isolation were tested. All isolates were single-spored and conserved at -20°C before use. Inoculum was produced in Petri plates containing Potato Dextrose Agar medium and incubated in a growth chamber (21°C, 14 hours light). After 14 days of incubation, conidia were collected by washing the culture in sterile distilled water. The cell suspension was filtered through a 30µm mesh sterile filter to remove mycelium fragments. The conidial concentrations was determined with a haemacytometer and adjusted to 10^6 conidia/ml. Inoculum of *M. dimerum* was produced on LPGA agar medium (7g/l yeast extract, 7g/l biogelytone, 7g/l glucose and 15g/l agar) in Petri dishes. After 14 days of incubation, conidia were collected in sterile water and spore suspension was adjusted to 10^6 or 10^7 conidia/ml with a haemacytometer.

Plant production and treatments

Seeds of tomato ('Monalbo') were sown in compost and transplanted after 1 week in individual pots. Plants 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.

Aggressiveness of* B. cinerea *on tomato plants and efficacy of protection

Two types of bioassays were developed on tomato to test for the aggressiveness of *B. cinerea* strains and the efficacy of *M. dimerum* against *B. cinerea* strains.

Detached stem bioassay: sections of tomato stems (40mm in length) were removed from tomato plants and placed in clear polystyrene boxes with humid absorbent paper to maintain high relative humidity. On each stem section, a 5-10mm petiole stub was left in order to inoculate spore suspension of the microorganisms. A set of stem sections was inoculated with *B. cinerea* at 10^6 conidia/ml in order to estimate the aggressiveness of the different strains of *B. cinerea*. Another set of stem sections was concomitantly inoculated with *B. cinerea* and *M. dimerum* at the desired dose (10^6 conidia/ml or 10^7 conidia/ml) to evaluate the efficacy of protection. We used three replicate polystyrene boxes, each containing 2 stem sections per treatment for each strain. Following inoculation, the clear polystyrene boxes were incubated in a growth chamber in conditions conducive to disease development (21°C, 14h-photoperiod, 114µmol/ s*m^2). Two to three independent repetitions of the test were done for each strain.

Whole plant bioassay: A set of plants was used as untreated control in order to estimate the aggressiveness of *B. cinerea* strains. For a given strain, three leaves were removed from each of the 3 plants tested, leaving 5-10mm petiole stubs on the stems. Each pruning wound was inoculated with 10 µl of the spore suspension of *B. cinerea*. Another set of plants was concomitantly inoculated with the spore suspension of *B. cinerea* and a spore suspension of *M. dimerum* at the desired dose (10^6 conidia/ml or 10^7 conidia/ml). Plants were incubated in a growth chamber in conditions conducive to disease development (20°C, 16h-photoperiod, 162µmol/s*m^2, relative humidity > 80%). Two to three independent repetitions of the test were done for each strain.

Disease assessment and data analysis

Lesion expansion on tomato stem was recorded daily from the 3rd to the 7th day after inoculation (DAI). The areas under the disease progress curves (AUDPC) were used to compare the different treatments and strains of *B. cinerea*.

To compare the aggressiveness of *B. cinerea* strains, an aggressive index, relative to reference strain BC1, was computed as $100 \times (AUDPC_{BC}/AUDPC_{BC1})$, where $AUDPC_{BC}$ was the average AUDPC for a given strain and $AUDPC_{BC1}$ is the average AUDPC for the reference strain BC1. To compare the efficacy of biocontrol against the different strains of *B. cinerea*, a protection index was computed as $100 \times (AUDPC_{BC} - AUDPC_{Md})/AUDPC_{BC}$, where $AUDPC_{BC}$ was the average AUDPC for a given strain and $AUDPC_{Md}$ was the average AUDPC for the same strain in presence of the BCA *M. dimerum*. Analyses of variance were used to test for an effect of strain on treatment efficacy. When appropriate, the means were compared with the test of Newman and Keuls.

Results and discussion

Aggressiveness of* Botrytis cinerea *strains

All *B. cinerea* strains were able to infect tomato petiole stubs and thereafter to generate symptoms on the stem of the plant. The aggressive index for the 41 strains ranged from 1 to 100% on potted tomato plants (Fig. 1). Results obtained on stem sections were similar, and a significant correlation was obtained between the two types of test (Spearman rank test $R = 0.92$, $P < 0.001$). Three strains of *B. cinerea* generated small lesions with each of the two types of tests, suggesting that they are hypo-aggressive on tomato stems. For both types of test, significant differences were observed among strains (ANOVA, $P < 0.001$).

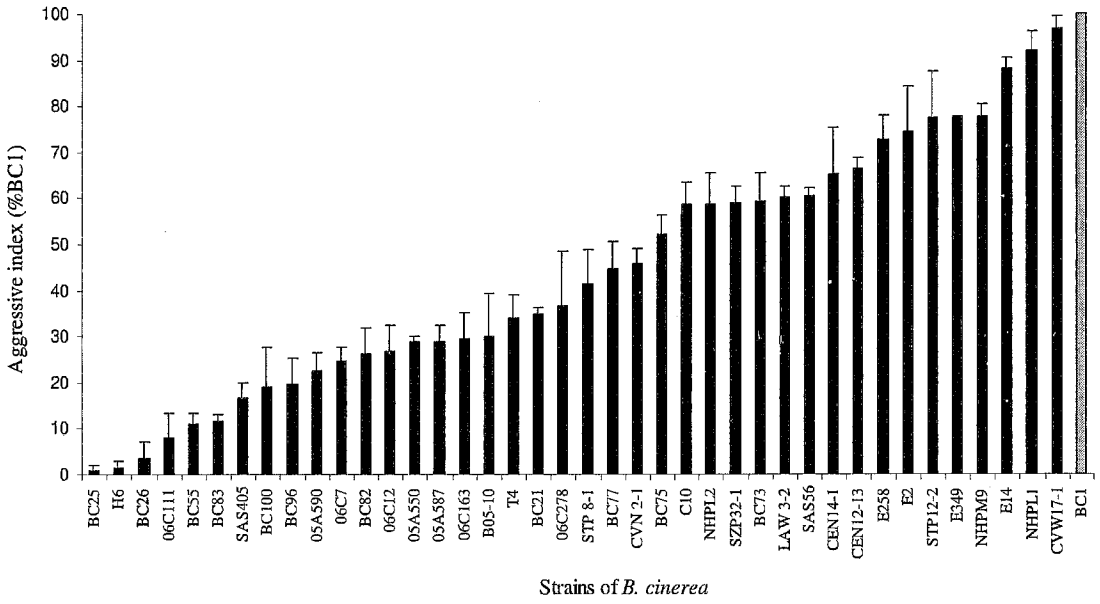

Figure 1. Aggressiveness of the 41 strains of *B. cinerea* estimated on whole tomato plants, relative to reference strain BC1.

Effect of strain of* B. cinerea *on the efficacy of biocontrol

Efficacy of *M. dimerum* was tested against 41 strains of *B. cinerea*. For the normal application dose of *M. dimerum* (10^7 conidia/ml) the protection provided by the BCA was high. The average protection index for the 41 strains of *B. cinerea* tested was 88 ± 2% and the median was 91%. Under normal conditions of use of the BCA, the protection is thus weakly influenced by the strain of *B. cinerea* tested.

For the 10-fold reduced dose of application, the average protection index on potted plants was still substantial (average of 53 ± 4% and median of 49% for the whole plant bioassay), but diversity was revealed among the 41 strains of *B. cinerea*., In this case, the protection was significantly influenced by the strain of *B. cinerea* (ANOVA, $P < 0.001$) and the level of protection ranged from 0 to 100% (Fig. 2). On tomato stem sections, the results were equivalent and a significant correlation was obtained between the two types of bioassay for the protection index at the 10-fold reduced dose of application of *M. dimerum* (Spearman rank test R = 0.42, $P = 0.007$).

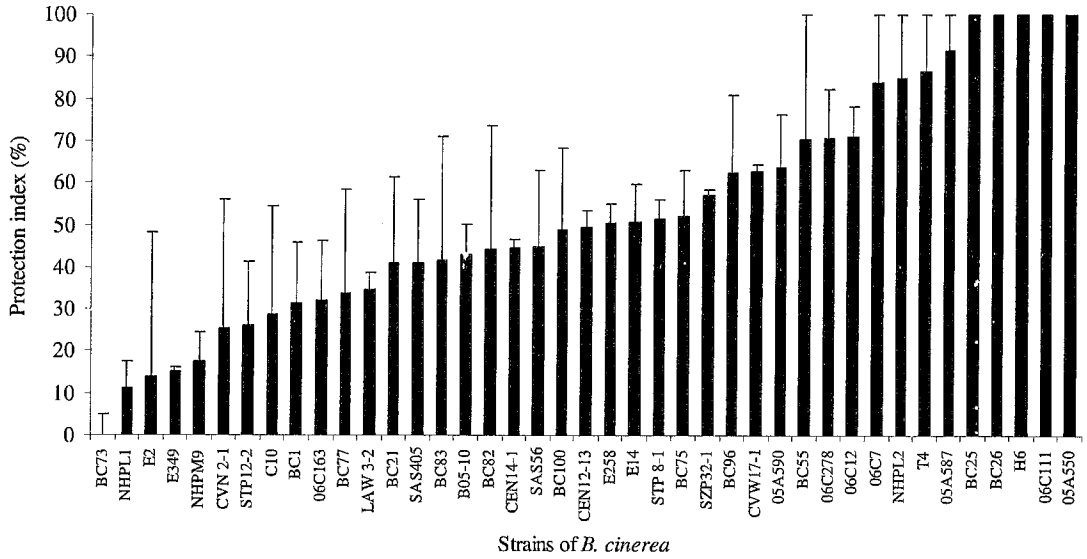

Figure 2. Distribution of the 41 strains of *Botrytis cinerea* according to the level of protection of whole tomato stem with a 10-fold reduced application dose of the biocontrol agent *Microdochium dimerum*.

Correlation between aggressiveness of* B. cinerea *and efficacy of biocontrol

The level of protection conferred by *M. dimerum* was significantly correlated with the level of aggressiveness of the 41 strains of *B. cinerea* both for the whole-plant bioassay (Spearman rank test, R = -0.58, $P < 0.0001$) and for the stem-section bioassay (Spearman rank test, R = -0.68, $P < 0.0001$) (Fig. 3).

These results clearly show that the protective efficacy of a BCA, in certain conditions, can vary depending on the strain of *B. cinerea*. It reveals the importance of considering several strains when screening for BCAs to obtain a good representation of the pathogen population and thus increase the durability of the biological control.

One may now wonder whether the use of this BCA on commercial fields or greenhouses could lead to the selection of less sensitive strains which may eventually jeopardise the

efficacy of this control method as already observed for antibiotic-producing BCAs (Ajouz *et al.*, 2009; Li & Leifert, 1994; Mazzola *et al.*, 1995). The probable complex mode of action of *M. dimerum* and its high level of protection provided at the recommended dose may reduce the risk of resistance development of *B. cinerea* strains. Anyway, the establishment of the baseline sensitivity would be helpful in future studies aiming at monitoring possible shifts in the sensitivity to this BCA in *B. cinerea* populations.

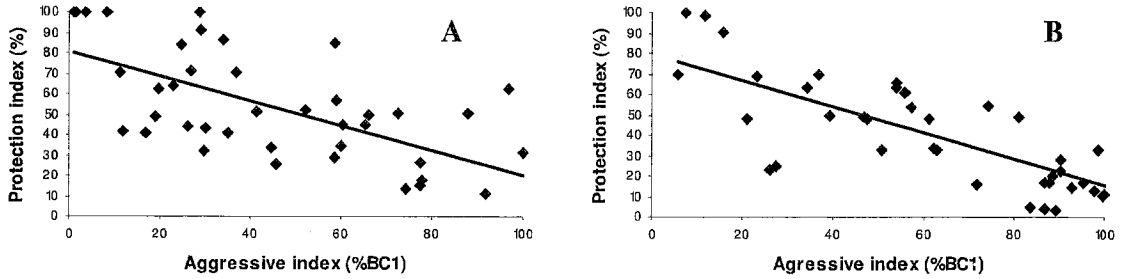

Figure 3. Correlation between the aggressiveness of the 41 strains of *Botrytis cinerea* estimated by the aggressive index (%BC1) and the protection by the 10-fold reduced application dose of the BCA *Microdochium dimerum* with the whole plant bioassay (A) and with the stem section bioassay (B).

Acknowledgements

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