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Biological control of strawberry gray mold by *Clonostachys rosea* under field conditions

Luciano V. Cota, Luiz A. Maffia *, Eduardo S.G. Mizubuti, Paulo E.F. Macedo, Ricardo F. Antunes

Departamento de Fitopatologia, Universidade Federal de Viçosa, 36570-000 Viçosa, MG, Brazil

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ABSTRACT

Gray mold, caused by *Botrytis cinerea*, is an important strawberry disease in Brazil. As a component of a disease management program, we have been evaluating pathogen biological control with *Clonostachys rosea*, and selected four isolates as potential antagonists to *B. cinerea*. In 2006 and 2007, under field conditions, we compared the efficiency of the four *C. rosea* isolates (applied once or twice a week) with a weekly spray of procymidone alternated with captan in controlling gray mold. Following the applications and up to harvest, we evaluated weekly: leaf area colonization by *C. rosea* (LAC), average number of *B. cinerea* conidiophores on leaves (ANC), incidence of gray mold on both flowers (Iflower) and fruits (Ifruit), incidence of latent infections on fruits (Ilat), and yield. The applications of *C. rosea* twice a week provided higher LAC (16.97%), smaller ANC (10.28; 78.22 in the check treatment, sprayed with water), smaller IFlower (10.02%; 50.55% in the check treatment), and smaller IFruit (5.95%; 25.10% in the check treatment). Yield ranged between 3490 and 3750 g plot^{-1} with applications of *C. rosea* twice a week and between 1740 and 1910 g plot^{-1} in the check treatment. Ilat was 20% in the check treatment and less than 10% in the other treatments. Based on this 2-year study, at least two weekly applications of *C. rosea* are required for a successful gray mold management program.

1. Introduction

Gray mold, caused by *Botrytis cinerea* Pers.: Fr. (perfect stage = *Botryotinia fuckeliana* (de Bary) Whetzel) is an important strawberry disease that causes losses before or after harvest wherever strawberry (*Fragaria x ananassa* Duch.) is grown (Boff et al., 2001; Elad et al., 2004; Valdebenito-Sanhuenza et al., 1997; Williamson et al., 2007; Xiao et al., 2001; Zhang et al., 2007).

Botrytis cinerea has a wide host range and is a necrotrophic fungus that sporulates on dead tissue or on the surface of crop debris (Jarvis, 1992; Williamson et al., 2007). Optimum temperature for fungal sporulation on strawberry leaf debris is 17–18 °C (Sosa-Alvarez et al., 1995). Gray mold epidemics usually start with conidia either produced on infested crop debris or wind dispersed from other producing areas (Boff et al., 2001; Braun and Sutton, 1988; Sutton, 1990). Although *B. cinerea* can infect almost all aerial plant parts, the most damaging infections occur at flowering (Mertely et al., 2002; Powelson, 1960). Infection starts on petals, stamens, and pistils, and the fungus can colonize the fruits. Fruit infection occurs in the field, but disease symptoms will be seen at ripening when the fungus sporulates (Bristow et al., 1986). Leaves can also be infected by the pathogen that stays in a quiescent stage until the leaves senesce and die (Braun and Sutton, 1988). Leaf infection is important in yielding inoculum for secondary disease cycles, as the fungus profusely sporulates on dead leaves (Braun and Sutton, 1988; Sosa-Alvarez et al., 1995; Sutton, 1990). Disease intensity in post-harvest is highly correlated with duration of both relative humidity (RH) above 80% and temperature between 15 and 25 °C at flowering (Wilcox and Seem, 1994).

Gray mold management is based principally on chemical control, but fungicide application may cause problems such as toxic residues on the fruits and selection of resistant isolates of the pathogen (Dianez et al., 2002; LaMondia and Douglas, 1997; Myresiotis et al., 2007; Rabolle et al., 2006; Yourman and Jeffers, 1999). Additionally, fungicide application at flowering may reduce pollen viability and consequently hinder fruit formation (Kovach et al., 2000). Disease may be controlled through cultural practices, such as increasing planting spacing to promote conditions less conducive to gray mold, removal of crop debris to reduce initial inoculum, or planting less susceptible cultivars (Legard et al., 2000, 2001; Xiao et al., 2001). However these measures have limited effects on disease control.

Biological control is being studied as an alternative for strawberry gray mold management. Although biological control may be used in post-harvest, when environmental conditions could be more controlled or modified to favor biological control agents, these agents are most efficient against infections that are established during the post-harvest stage (Janisiewicz and Korsten, 2002). To reduce losses to disease in post-harvest, control

* Corresponding author. Fax: +55 31 3899 2240.
E-mail address: lamaffia@ufv.br (L.A. Maffia).

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measures must be applied under field conditions (Ippolito and Nigro, 2000; Sutton et al., 1997). Biological control under field conditions may be used with antagonists that reduce pathogen sporulation on crop debris and consequently flower and fruit infection. The fungus *Clonostachys rosea* (Link: Fr.) Schroers, Samuels, Siefert & Gams (formerly *Gliocladium roseum* Bainier) is an antagonist that controls *B. cinerea* efficiently. *C. rosea* isolate Pg 88–710 suppressed *B. cinerea* sporulation on strawberry leaves, petals, and stamens as efficiently as captan, a standard fungicide for gray mold control (Peng and Sutton, 1991; Sutton et al., 1997; Sutton and Peng, 1993). The isolate Pg 88–710 was efficient in suppressing pathogen sporulation on rose and strawberry plants in experiments conducted in Brazil (Morandi et al., 2003, 2000; Valdebenito-Sanhuenza et al., 1997). Under greenhouse conditions, *C. rosea* efficiently suppressed *B. cinerea* sporulation on rose crop debris, even under conditions favorable to the pathogen (Morandi et al., 2003, 2006).

A program aiming to find *C. rosea* isolates adapted to Brazilian ecosystems was established some years ago, and four isolates were selected (Nobre et al., 2005). These isolates were as efficient as the Pg 88–710 in colonizing leaf tissues under short or long periods of leaf wetness, optimal temperatures for the pathogen, and in suppressing *B. cinerea* sporulation even when applied 14 days before or after pathogen inoculation (Cota et al., 2007, 2008). Although intensively studied under controlled conditions, these isolates have not been evaluated in the field. Evaluations under field conditions must be conducted, as there are reports of biological control agents being efficient under controlled conditions but ineffective under commercial crop conditions (Elad and Stewart, 2004; Elmer and Reglinski, 2006; Fravel, 2005). Therefore, the objective of this study was to evaluate the efficiency of the four *C. rosea* isolates in the biological control of strawberry gray mold under field conditions.

2. Materials and methods

2.1. Isolates and inoculum production

We used the four Brazilian isolates of *C. rosea*, selected based on the capacity of establishing and suppressing *B. cinerea* sporulation on leaves of strawberry, tomato, rose, and eucalyptus (Nobre et al., 2005). The isolates were grown in potato dextrose agar medium (PDA) at 25 °C, 12 h photoperiod (15 μmol cm^{-2} s^{-1}). Spore concentration was adjusted to 1×10^6 conidia mL^{-1} with the aid of a hemacytometer. At the moment of application, the surfactant Tween 80 (0.05% v/v) was added to the conidial suspension.

2.2. Experimental plots

The experiment was conducted in 2006 and repeated in 2007 at the experimental area of the Departamento de Fitopatologia, Universidade Federal de Viçosa (20°44′47″ S and 42°50′55″ W). Each plot was a bed with four 3-m long rows and 'Camarosa' plants were spaced 0.3 m within a row. Plots were spaced 2 m apart from each other. The two external rows and the last plant of each row were considered as borders. Plantings were on April 13, 2006 and April 18, 2007. At 20 days after planting (dap), each plant received 15 g of NPK (4-14-8). After 40 dap, the beds were covered with black plastic film (25-μm thick). Plants were sprinkler irrigated for 90 min at every 2 days. To control leaf spots, the fungicide tebuconazole (Folicur 200 EC; 0.375 mL i.a. L^{-1}) was sprayed twice in 2006 (at 30 and 45 dap) and once in 2007 (at 40 dap). Weeds were controlled in the areas around the plots with glyphosate (Glifos; 4.8 mL i.a. L^{-1}). Temperature, leaf wetness, and RH were registered in a data logger (CR10X, Campbell Scientific, Inc.). Application of treatments to the plots started as soon as the beds were plastic covered (at 43 and 45 dap, in 2006 and 2007, respectively). Both 2006 and 2007 experiments included ten treatments: each of the four *C. rosea* isolate applied either once or twice a week; a fungicide treatment consisting of weekly applications of procymidone (Sialex 500; 0.5 g i.a. L^{-1}) alternated with captan (Orthocide 500; 1.2g i.a. L^{-1}), and a check [application of water + Tween 80 (0.05% v/v)]. The treatments were applied with a backpack sprayer. Each experiment was in a randomized complete block design with four repetitions.

2.3. Evaluations

We evaluated colonization of strawberry leaf tissues by *C. rosea* isolates and their efficiency in suppressing *B. cinerea* sporulation, as well as flower colonization by the pathogen, gray mold incidence on fruits, and fruit production.

To evaluate colonization of *C. rosea* and suppression of *B. cinerea* on leaves, five leaflets were weekly sampled in each plot. Colonization of both pathogen and antagonist was indirectly evaluated by assessing intensity of sporulation on leaf tissues. We cut 15 leaf disks (1-cm diameter) and plated them in paraquat–chloramphenicol–agar medium (PCA) (Peng and Sutton, 1991) in Petri dishes. After 10–12 days incubation at 20 °C, percentage of disk area with *C. rosea* sporulation and the number of *B. cinerea* conidiophores were assessed under a stereoscope with diagrammatic scales. For *C. rosea*, we used the grading scale: 0 = 0; 1 = >0–3; 2 = >3–6; 3 = >6–12; 4 = >12–25; 5 = >25–37; 6 = >37–50; and 7 = >50% of leaf area disk colonized (Nobre et al., 2005). For *B. cinerea*, we used the grading scale: 0 = 0; 1 = 1–12; 2 = 13–24; 3 = 25–48; 4 = 49–100; 5 = 101–200; 6 = 201–300; and 7 = 301–400 conidiophores/leaf disk (Peng and Sutton, 1991). For statistical analysis, values of each class were transformed to the average value of the corresponding percentage of colonized area or conidiophores range. For instance, if a disk was rated as 3 (for *C. rosea*), its corresponding average value was 9.0%. Data for each experimental unit was the average of the 15 disks.

To evaluate flower colonization by the pathogen, ten flowers were weekly taken from each plot and plated in PCA, incubating at 20 °C, 12 h photoperiod. After 5 days, the flowers were observed under the stereoscope and checked for pathogen sporulation.

Ripe fruits were harvested weekly. Following harvest, fruits were weighted and gray mold incidence on fruits (number of diseased/total number of fruits) was evaluated. To account for latent infections, 10 fruits were sampled from each plot, set inside a plastic box (11 cm width × 11 cm length × 3.5 cm depth), incubating at 20 °C and observed daily for *B. cinerea* sporulation (during 10 days).

2.4. Data analysis

Six variables were gathered: disk area colonized by *C. rosea* (LAC), average number of *B. cinerea* conidiophores/disk (ANC), incidence of diseased flowers (Iflower), incidence of diseased fruits (Ifruit), incidence of latent infections (Ilat), and yield. Theses variables were analyzed considering the model of randomized blocks with repeated measures, using the Proc Mixed (Littell et al., 2006). Three models of covariance structures were considered: compound symmetry, unstructured, and autoregressive 1. The smallest Akaike information criterion (AIC) was used to select the model that better fitted the model covariance structure among evaluations. As most comparisons among isolates generated not statistical differences, we pooled the data across all isolates. Therefore, the effect of treatments was assessed with the following contrasts comparisons: application of *C. rosea* once a week (CR1) versus application of *C. rosea* twice a week (CR2); CR1 versus fungicide sprays; CR1 versus check; CR2 versus fungicide sprays; and

CR2 versus check. When the interaction term was significant, contrasts were applied to each evaluation date. To facilitate interpretation, only the contrast with the highest *P*-value across all evaluation dates assessed is reported.

Standardized areas under the curves of LAC (AUCLAC), ANC (AUCANC), Iflower (AUCIflower), of Ifruit (AUCIfruit), and yield (AUCYield) were estimated. The use of standardized areas was required to allow comparisons among curves of different durations (Fry, 1977). With the area values estimated in 2006 and 2007, homogeneity of error variances was analyzed through Levene's test. When there was homogeneity, data of both years were pooled and subjected to the analysis of variance. Treatment means were compared through the least square significant difference (protected LSD, = 0.05). All statistical analyses were conducted using SAS v. 9.1.

3. Results

3.1. Leaf colonization by Clonostachys rosea

In 2006, average LAC values were 13.03% and 17.5% with CR1 and CR2, respectively. LAC values tended to decrease with CR1 and to increase with CR2 (Fig. 1A). The interaction between treatment and evaluation interval was significant ($P = 0.0118$). By applying the contrast procedure, at most evaluations LAC was higher with CR2 than with CR1 ($P < 0.0335$). At 55 dap, both treatments did not differ.

In 2007, average LAC values were 11.54% and 16.39% with CR1 and CR2, respectively. LAC tended to remain steady with CR1 and to increase with CR2 (Fig. 1B). The interaction between treatment and evaluation interval was not significant ($P = 0.6000$), whereas evaluation interval and treatment were significant ($P < 0.0001$ and $P = 0.0009$, respectively). LAC was greater with CR2 than with CR1 ($P < 0.0001$).

Error variance for AUCLAC was homogenous between experiments. AUCLAC values were higher for all isolates applied twice a week and did not differ among isolates in each application time (Table 1).

3.2. Average number of Botrytis cinerea conidiophores

In 2006, average reductions of ANC were 86.67%, 67.68%, and 32.67%, with CR2, CR1, and fungicide, respectively (Fig. 2A). ANC values tended to decrease in check, increase with fungicide and remain steady with both CR1 and CR2 (Fig. 2A). The interaction between treatment and evaluation interval was significant ($P = 0.0001$). At all evaluations, ANC was smaller with CR2 than with CR1 ($P < 0.0007$), fungicide ($P < 0.0001$), and in the check ($P < 0.0001$); smaller with CR1 than with fungicide ($P < 0.0001$) and in the check ($P < 0.0001$). At most evaluations, ANC was smaller with fungicide than in the check ($P < 0.0029$); at 84 dap, both treatments did not differ.

In 2007, average reductions of ANC were 84.54%, 67.34%, and 47.56%, with CR2, CR1, and fungicide, respectively (Fig. 2B). ANC values tended to increase in the check and remain steady with fungicide, CR1, and CR2 (Fig. 2B). The interaction between treatment and evaluation interval was significant ($P < 0.0001$). At all evaluations, ANC was smaller with CR2 than with CR1 ($P < 0.0001$), fungicide ($P < 0.0001$), and in the check ($P < 0.0001$); smaller with CR1 than fungicide ($P < 0.0001$) and in the check ($P < 0.0001$); and smaller with fungicide than in the check ($P < 0.0001$).

Error variance for AUCANC was homogenous between experiments. AUCANC values were higher for all isolates applied twice a week, followed by the values when the isolates were applied once a week. No difference regarding AUCANC was found among isolates in each application time (Table 1).

3.3. Incidence of diseased flowers

In 2006, average reductions of Iflower were 80.33%, 47.52%, and 34.81%, with CR2, CR1, and fungicide, respectively (Fig. 3A). Iflower values tended to remain steady with fungicide and to decrease with CR1, CR2, and in the check (Fig. 3A). The interaction between treatment and evaluation interval was not significant ($P = 0.9886$), whereas evaluation interval and treatment were significant ($P = 0.0005$ and $P < 0.0001$, respectively). Iflower was smaller with CR2 than with CR1 ($P < 0.0002$), fungicide ($P < 0.0001$), and in the check ($P < 0.0001$); with CR1 than with fungicide ($P < 0.0001$), and in the check ($P < 0.0001$); and with fungicide than in the check ($P < 0.0001$).

In 2007, average reductions of Iflower were 79.70%, 43.33%, and 37.79% with CR2, fungicide, and CR1, respectively (Fig. 3B). Iflower values tended to decrease in all treatments (Fig. 3B). The interaction between treatment and evaluation interval was significant ($P < 0.0001$). At most evaluations, IFlower was smaller with CR2 than with CR1 ($P < 0.0479$), and did not differ at 137 and 145 dap. IFlower was smaller with CR2 than with fungicide at 55, 69, 77, 84, 92, 107, 127, and 137 dap ($P < 0.0476$); and in the check at most evaluations ($P < 0.0068$) except on 137 and 145 dap. Iflower with CR1 and fungicide did not differ at most evaluations; at 62 and 69 dap it was smaller with fungicide ($P < 0.0371$). Iflower was smaller in CR1 than in the check ($P < 0.0125$) except on 100, 122, 129, 137, and 145 dap; and with fungicide than in the check ($P < 0.0211$), except on 92, 100, 107, 129, 137, and 145 dap.

Error variance for AUCIflower was not homogenous between experiments ($P = 0.0025$). In 2006, AUCIflower values were smaller for all isolates applied twice a week, followed by the values when

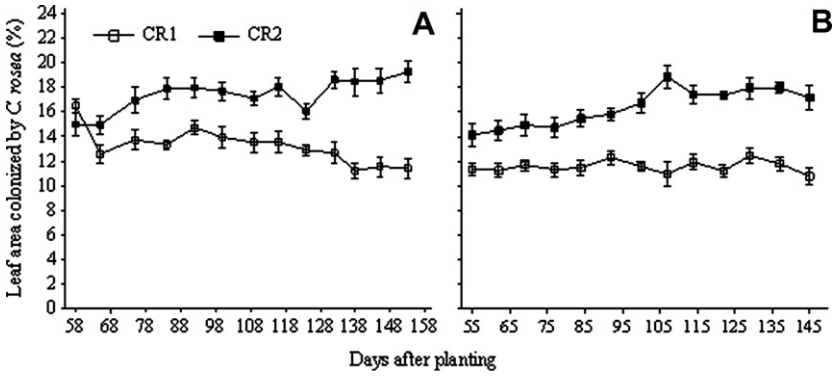

Fig. 1. *Clonostachys rosea* colonization of strawberry leaves that were sampled weekly in experiments conducted in 2006 (A) and 2007 (B). Planting was on 13 April and 18 April in 2006 and 2007, respectively. Average of four *C. rosea* isolates applied once (CR1) or twice a week (CR2). Vertical bars are mean standard errors.

Table 1
Standardized areas under the curves of: leaf area colonized by *Clonostachys rosea* (AUCLAC), average number of *Botrytis cinerea* conidiophores (AUCANC), incidence of diseased flowers (AUCIflower), incidence of diseased fruits (AUCIfruit), and Yield (AUCYield)

Treatment	AUCLAC	AUCANC	AUCIflower		AUCIfruit		AUCYield
			2006	2007	2006	2007	
NCR61/F[2]	17.21A[*]	11.20D	11.86D	7.94 D	6.24D	5.19CD	326.35A
NCR62/F[2]	17.03A	10.33D	13.20D	7.78 D	6.85D	4.98CD	322.40A
NCR60/F[2]	16.81A	11.15D	12.18D	7.19 D	7.29D	3.23D	330.35A
NCR19/F[2]	16.70A	10.21D	14.58D	7.88 D	10.37C	6.04C	306.43B
NCR19/F[1]	12.67B	22.60C	35.29C	22.26BC	22.36B	11.99B	211.40F
NCR60/F[1]	12.48B	24.74C	37.47C	26.31B	22.80B	12.97B	228.17D
NCR61/F[1]	12.22B	23.46C	35.25C	22.86BC	22.44B	12.91B	225.98DE
NCR62/F[1]	12.15B	22.64C	34.43C	23.26BC	22.82B	11.16B	214.16FE
Fungicide	[**]	46.42B	46.45B	19.96C	20.12B	7.28C	248.57C
Check		78.49A	66.96A	37.39A	30.70A	20.36A	157.95G

The areas were estimated for ten treatments: applications of four isolates of *C. rosea*, fungicides (procymidone weekly alternated with captan), and check (water sprays) in experiments conducted in 2006 and 2007. As there was homogeneity of variances for data of AUCLAC, AUCANC, and AUCYield, data of both years were pooled.
[*] In each column, means followed by the same letter do not differ (protected LSD, $\alpha = 0.05$).
[**] *C. rosea* was not detected.
[1] Once a week.
[2] Twice a week.

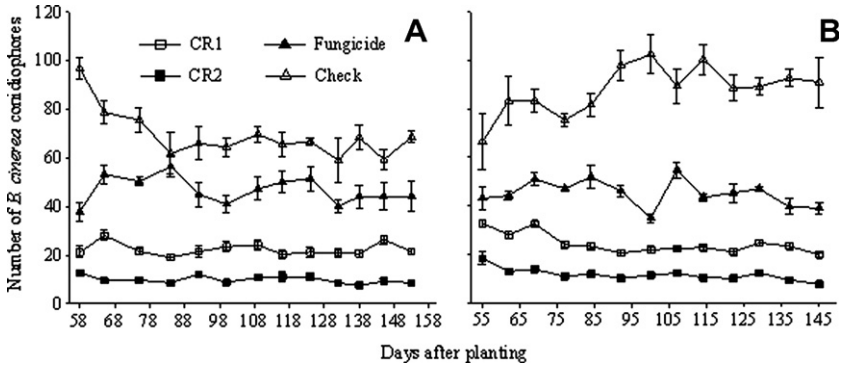

Fig. 2. Number of *Botrytis cinerea* conidiophores on strawberry leaves that were sampled weekly in experiments conducted in 2006 (A) and 2007 (B). Planting was on 13 April and 18 April in 2006 and 2007, respectively. Average of four *C. rosea* isolates applied once (CR1) or twice a week (CR2), fungicide (procymidone weekly alternated with captan), or check (water sprays). Vertical bars are mean standard errors.

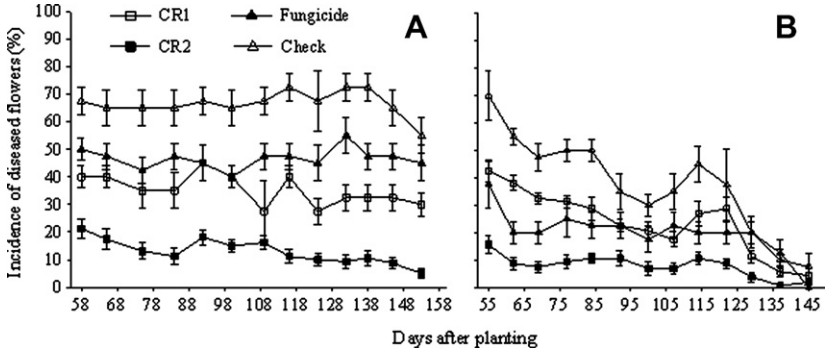

Fig. 3. Gray mold incidence on strawberry flowers that were sampled weekly in experiments conducted in 2006 (A) and 2007 (B). Planting was on 13 April and 18 April in 2006 and 2007, respectively. Average of four *C. rosea* isolates applied once (CR1) or twice a week (CR2), fungicide (procymidone weekly alternated with captan), or check (water sprays). Vertical bars are mean standard errors.

the isolates were applied once a week. In 2007, AUCIflower values were smaller for all isolates applied twice a week, followed by the values with fungicide and when the isolates were applied once a week. In both years, no difference regarding AUCIflower was found among isolates in each application time (Table 1).

3.4. Incidence of diseased fruits

In 2006, average reductions of Ifruit were 77.25%, 34.86%, and 27.23% with CR2, fungicide, and CR1, respectively (Fig. 4A). Ifruit values tended to decrease in all treatments (Fig. 4A). The interaction between treatment and evaluation interval was not significant ($P = 0.3447$), whereas evaluation interval and treatment were significant ($P = 0.0005$ and $P < 0.0001$, respectively). Ifruit was smaller with CR2 than CR1 ($P < 0.0001$), fungicide ($P < 0.0058$) and in the check ($P < 0.0001$); with CR1 than fungicide ($P < 0.0001$), and in the check ($P < 0.0001$); and with fungicide than in the check ($P < 0.0001$). Values of Ilat were 2.34%, 4.37%, 7.29%, and 18.54% with CR2, CR1, fungicide, and in the check, respectively.

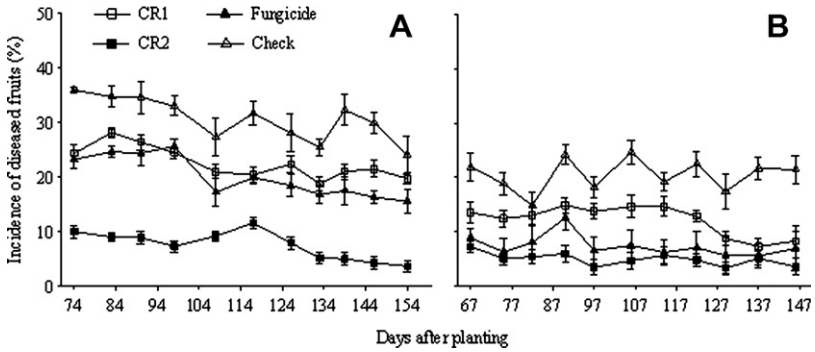

Fig. 4. Gray mold incidence on strawberry fruits that were sampled weekly in experiments conducted in 2006 (A) and 2007 (B). Planting was on 13 April and 18 April in 2006 and 2007, respectively. Average of four *C. rosea* isolates applied once (CR1) or twice a week (CR2), fungicide (procymidone weekly alternated with captan), or check (water sprays). Vertical bars are mean standard errors.

In 2007, average reductions of Ifruit were 75.62%, 63.69%, and 39.29%, with CR2, fungicide and CR1, respectively (Fig. 4B). Ifruit values tended to decrease with CR2 and CR1 and to remain steady in the check and with fungicide (Fig. 4B). The interaction between treatment and evaluation interval was not significant ($P = 0.8174$), whereas evaluation interval and treatment were significant ($P = 0.001$ and $P < 0.0001$, respectively). Ifruit was smaller with CR2 than CR1 ($P < 0.0001$), fungicide ($P < 0.0196$), and in the check ($P < 0.0001$); with fungicide than CR1 ($P < 0.0001$) and in the check ($P < 0.0001$); and with CR1 than in the check ($P < 0.0001$). Ilat values were 0.68%, 1.42%, 2.78%, and 15.45% with fungicide, CR2, CR1, and in the check.

Error variance for AUCIfruit was not homogenous between experiments ($P = 0.0003$). In both years, AUCIfruit values were smaller for all isolates applied twice a week, followed by the fungicide treatment and when the isolates were applied once a week. In both years, in general no difference regarding AUCIfruit was found among isolates in each application time (Table 1).

3.5. Yield

In 2006, the average increase in yield when compared to the untreated check were 98.01%, 58.83%, and 30.00%, with CR2, fungicide, and CR1 treatments, respectively (Fig. 5A). Yield values tended to decrease with CR2 and CR1 and to remain steady with fungicide and in the check (Fig. 5A). The interaction between treatment and evaluation interval was significant ($P < 0.0001$). Yield was higher with CR2 than with CR1 ($P < 0.0001$) at all evaluation times, fungicide ($P < 0.0117$) except at 126 and 154 dap, and in the check ($P < 0.0001$) except at 161 dap. Yield was higher with fungicide than with CR1 ($P < 0.0365$) except at 83, 108, 117, and 133 dap; and than in the check at all evaluations ($P < 0.0036$). Yield with CR1 was higher than in the check ($P < 0.0317$) except at 146 and 154 dap.

In 2007, yield increases were 102.01%, 53.25%, and 45.19% with CR2, fungicide, and CR1, respectively (Fig. 5B). Yield values tended to decrease with CR2, increase with CR1 and fungicide, and to remain steady in the check (Fig. 5B). The interaction between treatment and evaluation interval was significant ($P < 0.0001$). In all evaluation times, yield was higher with CR2 than with CR1 ($P < 0.0001$); fungicide ($P < 0.0143$) except at 146 dap; and in the check ($P < 0.0001$). CR1 was as efficient as fungicide in all evaluations ($P > 0.2880$), except on 82 dap ($P = 0.0181$), and provided higher yield than check ($P < 0.0019$). Fungicide application increased yield in all evaluations ($P < 0.0156$).

Error variance for AUCYield was homogenous between experiments. AUCYield values were higher for all isolates applied twice a week, followed by the values with fungicide. In general, no difference regarding AUCYield was found among isolates in each application time (Table 1).

3.6. Meteorological data

During the experiments conducted in 2006 and 2007, average air temperature was 16.29 and 18.52 °C, RH was 83.61% and 79.29%, average maximum temperature was 23.89 and 24.61 °C, average minimum temperature was 10.98 and 13.72 °C, and average daily leaf wetness duration period was 12.07 h and 11.68 h, respectively. In 2006, after 110 dap temperature tended to increase and RH to decrease (Fig. 6).

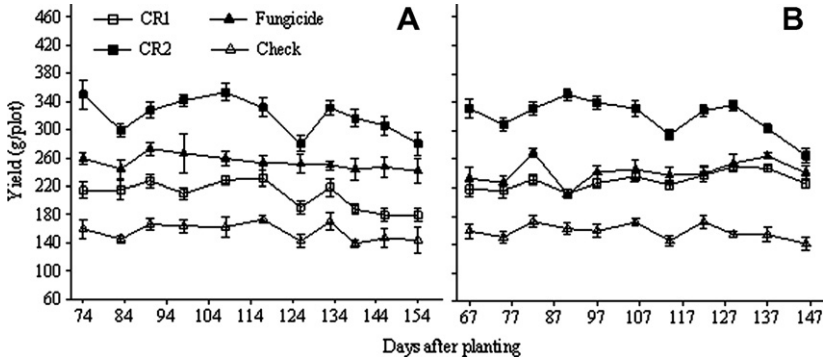

Fig. 5. Yield of strawberry fruits harvested weekly in experiments conducted in 2006 (A) and 2007 (B). Planting was on 13 April and 18 April in 2006 and 2007, respectively. Average of four *C. rosea* isolates applied once (CR1) or twice a week (CR2), fungicide (procymidone weekly alternated with captan), or check (water sprays). Vertical bars are mean standard errors.

4. Discussion

One of the most challenging stages of biological control of plant diseases is the field test. Biological control of *B. cinerea* has been thoroughly investigated (Elad and Stewart, 2004). However, according to these authors, most studies were conducted *in vitro* or under controlled conditions. At our laboratory, biological control studies were initiated in the 90s (Tatagiba et al., 1998), and we got promising results on the control of rose gray mold under greenhouse conditions with isolate Pg 88–710 (Morandi et al., 2003). Despite the efficiency of isolate Pg 88–710, we searched for *C. rosea* from Brazilian conditions and found four isolates efficient in colonizing leaves of rose, eucalyptus, and tomato (Nobre et al., 2005), as well as suppressing *B. cinerea* on these hosts. These isolates were also efficient to suppress *B. cinerea* on strawberries under controlled conditions (Cota et al., 2007, 2008). Here we report on the efficiency of biological control of gray mold on strawberry under field conditions.

In this 2-year experiment, the isolates of *C. rosea* were efficient in colonizing strawberry leaves and suppressing *B. cinerea* sporulation, results similar to those we got under greenhouse conditions, where leaf colonization and suppression of pathogen sporulation occurred at temperatures between 15 to 30 °C, with the optimum at 25 °C, with or without long periods of leaf wetness (Cota et al., 2007, 2008). Under field conditions, mostly in 2006, average temperature was 16.3 °C, but both colonization and suppression were high. However, average maximum temperature at day time was about 24 °C. Thus, *C. rosea* growth at day time, when temperatures were optimal, could have compensated unfavorable temperatures for colonization at night time when temperature was low (11 °C), as observed in experiments with rose plants (Morandi et al., 2003, 2006). Colonization of both flowers and fruits by *C. rosea* was also observed and gray mold incidence on both organs was low (data not quantified).

Antagonists that compete with saprophytic growth of *Botrytis* spp. may reduce pathogen growth and/or sporulation in crop debris (Köhl et al., 1995; Morandi et al., 2003), resulting in the reduction of disease progress rate. Using these antagonists is advantageous because of the continuity of the interaction between pathogen and antagonist in the crop debris (Fokkema, 1993). Suppressing either colonization or sporulation of *B. cinerea* is a valid strategy to biologically control the pathogen in strawberry and other hosts (Köhl and Fokkema, 1994, 1998; Köhl et al., 1995; Morandi et al., 2003; Sutton and Peng, 1993). All *C. rosea* isolates competed efficiently with *B. cinerea* in colonizing strawberry leaf tissues. Under crop conditions, leaves are infected at the young stage by *B. cinerea* that stays in a quiescent stage until the leaves senesce and die; then the pathogen resumes the growth and sporulates abundantly (Braun and Sutton, 1988; Sosa-Alvarez et al., 1995; Sutton, 1990). Therefore, the high levels of suppression of pathogen sporulation in leaves will effectively reduce inoculum produced in crop debris and consequently contribute to reduced disease incidence on both flowers and fruits. Strawberry flowers must be protected to avoid fruit infection and to successfully manage gray mold (Legard et al., 2005; Mertely et al., 2002; Powelson, 1960). Flower protection demands a large number of chemical

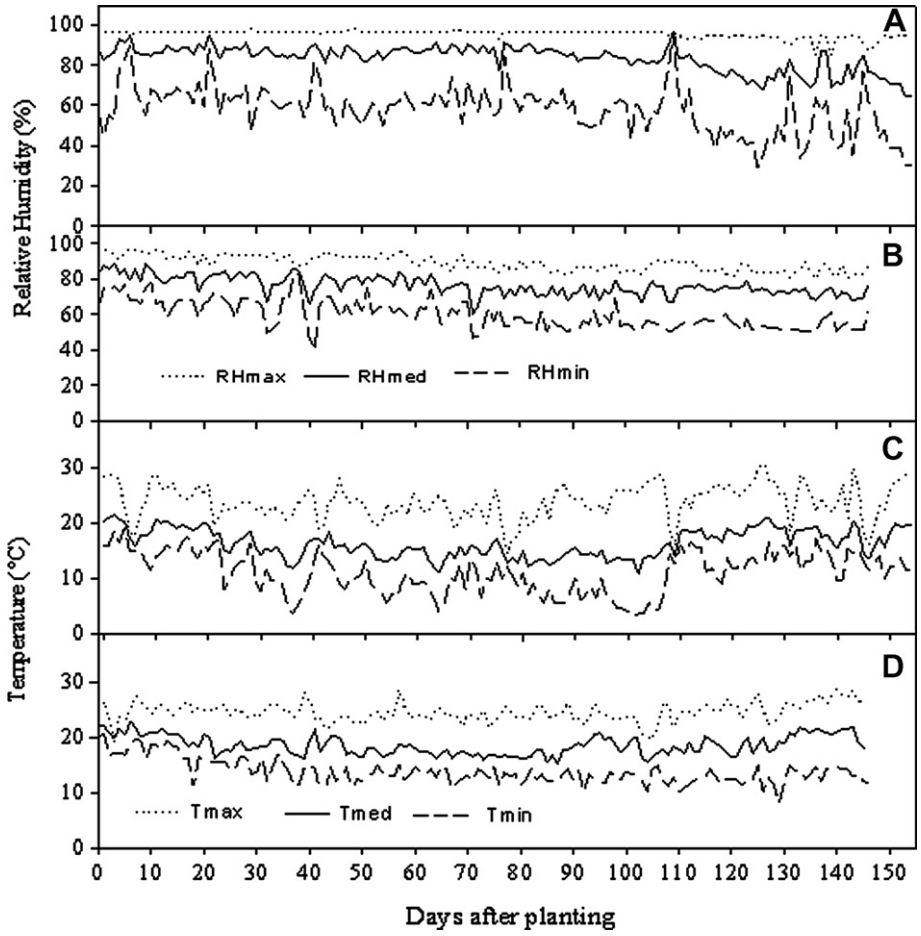

Fig. 6. Climatic variables registered during the experiments conducted in 2006 (A and C) and 2007 (B and D). A and B: maximum, mean, and minimum daily relative humidity (RHmax, RHmed, and RHmin, respectively); (C and D) maximum, mean, and minimum daily temperature (Tmax, Tmed, and Tmin, respectively).

sprays as the extended flowering period. Also flowering and fruit setting are not synchronized. It is also possible to have plants with green fruits and fruits ready to be harvested. Thus, in addition to being costly, fungicide applications can result in problems with chemical residues in fruits. Application of a biological control agent would be an attractive solution to this problem and an alternative to keep flowers and fruits constantly protected is using pollinator insects that would carry antagonists to the flowers. The use of bees as dispersing agents of *C. rosea* or *Trichoderma harzianum* Rifai controlled gray mold as efficiently as fungicide or as antagonist sprays on strawberry and other hosts (Kovach et al., 2000; Peng et al., 1992; Shafir et al., 2006; Sutton et al., 1997).

In general in our experiments, applying *C. rosea* was as efficient as or more efficient than fungicide in reducing leaf colonization by *B. cinerea*, as well as of gray mold incidence in both flowers and fruits. The effect was most striking when the isolates were applied twice a week, probably because the antagonist reached a larger amount of flowers at several developmental stages. The application of *C. rosea* isolates twice a week was more efficient in controlling gray mold than weekly application of fungicide. The fungicides used in this experiment had limited (procymidone) or no (captan) systemicity. Protection of plant tissues must be accomplished with frequent sprays. On the other hand, there is evidence that *C. rosea* can establish endophytically in plant tissues (Morandi et al., 2001; Sutton et al., 1997), and this could allow growth to newly formed plant tissues and removal by water would be less critical compared to the fungicides. Control efficiency achieved with our isolates was similar or greater than that reported in other studies with different *C. rosea* isolates in Canada and Brazil (Peng and Sutton, 1991; Peng et al., 1992; Sutton et al., 1997; Valdebenito-Sanhuenza et al., 1997) and with biological control agents such as *Ulocladium atrum* Preuss, *Bacillus licheniformis* Weigmann, and *T. harzianum* (Boff et al., 2002; Kim et al., 2007; Kovach et al., 2000; Shafir et al., 2006). Thus, these four isolates deserve further studies under field conditions at other production areas. In gray mold management programs that include *C. rosea*, it is expected that biological control efficiency would improve by increasing the number of sprays, considering the better results we got by applying the antagonist twice a week.

It is expected that applying *C. rosea* as soon as the crop is set in the field may contribute to increase control efficiency. In experiments with roses in greenhouses, *C. rosea* reduced pathogen sporulation on crop debris, but disease incidence in rose buds was not reduced, probably because the sprays started when disease incidence was high (Morandi et al., 2003). In experiments under field conditions in Holland, the efficiency of *U. atrum* in the biological control of strawberry gray mold was increased when applications of the antagonist started at transplanting time (Boff et al., 2002). Although not quantified, we observed *B. cinerea* sporulation on dead parts of transplants, which most likely were inoculum sources of the pathogen to other plant parts. Early application of the antagonist is expected to reduce the efficiency of this inoculum source.

There is potential to develop a commercial product based on *C. rosea*. The efficiency of control we got, under environmental conditions favorable to gray mold, and the consistency of the results in both years, are indicators that there is a high chance of success of biological control in commercial crops. Additionally, although drip irrigation is the most commonly used system in strawberry fields, the plants in our experiments were sprinkler irrigated, and infection by B. *cinerea* was favored. Thus one may expect that under commercial conditions control efficiency can be increased, because environmental conditions are less favorable to infection by *B. cinerea* without affecting colonization by *C. rosea* as it can establish in the absence of leaf wetness (Cota et al., 2007, 2008; Morandi et al., 2001). For most variables analyzed and in both years the experiment was conducted, the four isolates did not differ among themselves in biological control efficiency. These isolates were compared regarding temperature and moisture requirements in colonizing strawberry leaves and did not differ (Cota et al., 2008). Other environmental variables not evaluated may have had additional effects on them and need to be assessed. Under controlled conditions, these four isolates share ecological requirements (Cota et al., 2008) and do not differ regarding antagonism to *B. cinerea* (Cota et al., 2007). Thus, a single isolate or a mixture of the four can be used in a commercial formulation, although the use of mixture should be preferred. A formulation based on the four isolates will likely be effective in controlling gray mold on other plant species besides strawberry, because of the capacity of the four isolates in colonizing leaves of rose, eucalyptus, and tomato, as well as suppressing *B. cinerea* on these hosts (Nobre et al., 2005).

Yield increased more by applying *C. rosea* twice a week than with weekly fungicide application. This strengthens the viability of adopting gray mold biological control with *C. rosea* under field conditions. Before using the biological control under crop conditions, compatibility of *C. rosea* with other control measures needs to be assessed, as well as the integration of control measures. In our laboratory, we are testing the compatibility of the four isolates with pesticides registered for strawberry crops in Brazil. According to preliminary results, they are compatible with herbicides and most insecticides used in strawberry (Macedo et al., 2007), but are sensitive to fungicides (Macedo et al., 2006). Considering the use in other production systems (Shtienberg and Elad, 1997), application of the biological control agents can be rotated with fungicides under conditions of higher disease pressure. Defining these conditions is the next step to be pursued.

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