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Biological Control 26 (2003) 311–317

Biological Control

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Suppression of *Botrytis cinerea* sporulation by *Clonostachys rosea* on rose debris: a valuable component in Botrytis blight management in commercial greenhouses

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Received 6 May 2002; accepted 25 September 2002

Abstract

Botrytis blight, caused by *Botrytis cinerea* (Bc), is an important disease on roses grown in plastic greenhouses in Brazil. Biocontrol with *Clonostachys rosea* (Cr) applied to leaves and crop debris to reduce pathogen sporulation can complement other control measures for disease management. Two experiments, each with a rose cultivar, were conducted in a plastic greenhouse. For 'Red Success,' four treatments were compared: (1) control; (2) fortnightly sprays of Cr; (3) weekly sprays of mancozeb; and (4) weekly sprays of either Cr or mancozeb to the lower third of the plants and the debris. For 'Sonia,' treatment 4 was not included. Samples were taken from debris (leaves and petals) at ten 15-day intervals and plated on PCA medium. Sporulation of fungi and incidence of Botrytis blight on buds were assessed. For both cultivars, C treatments significantly ($P = 0.05$) reduced Bc sporulation. However, disease incidence was not consistently reduced, probably because the applications of *C. rosea* started when Botrytis blight epidemic was advanced and no sanitation practices were performed on nontreated plots. From the present and previous studies, continuous application of Cr on debris, associated with sanitation practices, has the potential to reduce Bc sporulation and disease incidence in the buds.

Keywords: Biological control; *Botrytis cinerea*; *Clonostachys rosea*; Crop residues; Necrotrophic pathogens; *Rosa hybrida*

1. Introduction

Rose (*Rosa hybrida* L.) is one of the most appreciated flowers around the world. In Brazil, the crop is grown as a long-term perennial in ground beds in polyethylene-covered greenhouses and the annual production is estimated at 88 million cuttings. Under such conditions, Botrytis blight, caused by *Botrytis cinerea* Pers.:Fr., is responsible for severe pre- and post-harvest losses. The pathogen produces spreading necrotic lesions on leaves, shoots, and renewal canes as well as small lesions and blighting of petals of the cut flowers during storage and transit (Elad, 1988; Horst, 1983; Tatagiba et al., 1998). Economic losses are associated chiefly with the development of the disease on the petals.

The abundant *B. cinerea* sporulation on senescent and dead plant tissues contributes to the development and the maintenance of an epidemic within a crop (Hausbeck and Moorman, 1996; Monteiro et al., 1996; Sosa-Alvarez et al., 1995). The suppression of pathogen sporulation on crop debris was proposed as a potential strategy of biological control (Köhl and Fokkema, 1998; Köhl et al., 1995; Sutton and Peng, 1993). The non-pathogenic, saprophytic fungus *Clonostachys rosea* (Link: Fr.) Schroers, Samuels, Siefert, and W. Gams [*Gliocladium roseum* Bainier, teleomorph, *Bionectria ochroleuca* (Schw.) Schroers and Samuels] (Schroers

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1049-9644/02/$ - see front matter © 2002 Elsevier Science (USA). All rights reserved.
doi:10.1016/S1049-9644(02)00134-2

et al., 1999) was selected as an efficient antagonist against *B. cinerea*. It suppresses development of the pathogen in plant tissues as well as on the leaf and flower surface of rose and other hosts through competitive colonization of senescing and dead tissues, nutrient competition, and hyperparasitism (Morandi et al., 2001; Sutton et al., 1997; Yu and Sutton, 1997).

In recent studies under controlled conditions, *C. rosea* markedly suppressed sporulation of *B. cinerea* in rose leaves and petals regardless of developmental stage of the tissues, minor wounds, natural densities of microflora, and aphid and mite infestations (Morandi et al., 2000a,b). *C. rosea* can easily colonize rose leaves under a broad range of microclimatic conditions. Further, it can control sporulation of *B. cinerea* regardless of the time of its arrival in relation to the pathogen (Morandi et al., 2001).

Most studies reporting high efficacy of *C. rosea* against *B. cinerea* on roses were conducted under controlled environments. Thus, information on the effectiveness of its use under commercial greenhouse conditions is lacking. Occasionally, introduction of antagonists that have been highly effective in controlled environments to the phyllosphere of commercially grown plants is only moderately effective and sometimes totally ineffective (Guetsky et al., 2001). Under commercial conditions, the phylloplane is subjected to fluctuating temperatures, relative humidity, surface wetness, gases, and air movement. These conditions may adversely affect the phyllosphere microflora, including the biocontrol agents, or may have an indirect influence by modifying the characteristics of the host tissues, such as the metabolic state and surface chemistry of the leaves (Burrage, 1971; Hannush and Boland, 1996). The objective of this study was to investigate the capacity of *C. rosea* to establish on rose debris and control *B. cinerea* sporulation under a commercial greenhouse environment. This information will contribute to a better understanding of the antagonist's population dynamics under uncontrolled environments and may lead to a reliable and feasible biocontrol strategy for *B. cinerea* on roses.

2. Materials and methods

2.1. Rose plants and cultural procedures

From January to May 2000 two experiments, each one with a rose cultivar, were conducted in a commercial, plastic-covered greenhouse without climatic control. Three-year-old plants of cultivars 'Sonia' and 'Red Success' were grown in ground beds (30×1.2 m). There were five beds for 'Sonia' and seven beds for 'Red Success.' The distance between plants on each bed was 0.25×0.50 m.

Fertilization and commercial pesticide were applied according to standard practice of growers in southeast Brazil. Each plant was fertilized with 200 g N:P:K (4:14:8) in December 1999 and then at two-month intervals. Mite and aphids were controlled by periodic application of malathion (Malatol 500 CE) and acephate (Orthene 750 BR). Periodic sprays of sulfur (Thiovit BR), folpet (Folpet 500PM), and metalaxyl + chorothalonil (Folio) controlled powdery and downy mildews. The plants were base-irrigated one to three times a week. Buds for marketing were harvested weekly, and the damaged buds and open flowers were cut and left on the ground, as practiced by the growers.

2.2. Inocula and inoculations

Botrytis cinerea inoculum existed naturally in the greenhouse at levels sufficient to cause epidemics and was used for quantification of pathogen colonization and sporulation on rose debris. The biological control agent *C. rosea* (PG-88-710 isolate, Peng and Sutton, 1991) was applied as a spore and mycelium suspension. Mass production of *C. rosea* conidia was obtained on wheat grains, according to a protocol developed by M.A.B. Morandi and L.A. Maffia (unpublished). Wheat grains were steam cooked (1 h) and autoclaved (121 °C, 30 min). After cooling down, 10 ml *C. rosea* suspension (10^7 conidia/ml) was applied to 300 g wheat grains and incubated at 22 °C. After being colonized by the fungus, the grains were slowly air dried for 15 days at 22–25 °C, ground, and sieved on a 1 mm screen. The final powder averaged 10^9 conidia/g. For spray applications, 10 g of the powder was suspended on 1 liter of water plus surfactant (Tween 80, 0.5 ml/liter water), achieving a final suspension of 10^7 conidia/ml. The suspension of *C. rosea* was applied at the rate of 1-liter suspension/10 m^2 by a hand spray directed toward the rose debris on the bed surface.

2.3. Treatments and samples collection

The experiments were conducted in a complete randomized design with three replications. Experimental plots were 3×1.2 m wide (24 plants/plot) and were 1.5 m apart. One bed on each side of the greenhouse was kept as border.

For 'Red Success' there were four treatments: (i) application of *C. rosea* (Cr) biweekly; (ii) application of mancozeb weekly at a ratio of 200 g c.f. per 100 liter of water (MC); (iii) application of *C. rosea* and mancozeb (CrM) in alternate weeks; and (iv) water control (CK). For 'Sonia,' the same treatments were applied, except CrM.

For estimation of *B. cinerea* and *C. rosea* colonization and sporulation rate on rose debris and the suppression of *B. cinerea* sporulation by *C. rosea*, six

sub-samples of 20–30 fallen yellowish leaves and/or recently fallen petals were taken from the surface of each plot at 13–15 day intervals. Twelve disks of 1-cm diameter were cut from each sub-sample and placed on paraquat–chloramphenicol–agar medium (PCA) in petri dishes (Peng and Sutton, 1991). The percentage of disks with *B. cinerea* and/or *C. rosea* sporulation was estimated 7–10 days after incubation at 22 ± 2 °C and 12-h photoperiod. A sample collection was performed prior to treatment applications to quantify *B. cinerea* inoculum and to check for the presence of any native strain of *C. rosea* in the experimental area.

A sample of 20 rose buds at harvest stage was taken from each plot at 13–15 day intervals to evaluate Botrytis blight incidence. A bud was considered diseased if there was at least one fleck spot on each external petal. In the same manner as used for sporulation evaluation, a sample was collected prior to treatment applications.

2.4. Weather data

During the period of the experiment, air temperature and relative humidity were monitored with a hygrothermograph. It was kept in a white, wooden shelter placed inside the rose canopy at a height of about 1.5 m. Rainfall was measured with a rain gauge placed outside the greenhouse.

The hourly values of air temperature and relative humidity were used to calculate the daily mean temperature, the mean relative humidity, and the daily maximum and minimum temperatures and relative humidity. The vapor pressure deficit (VPD) was calculated for each

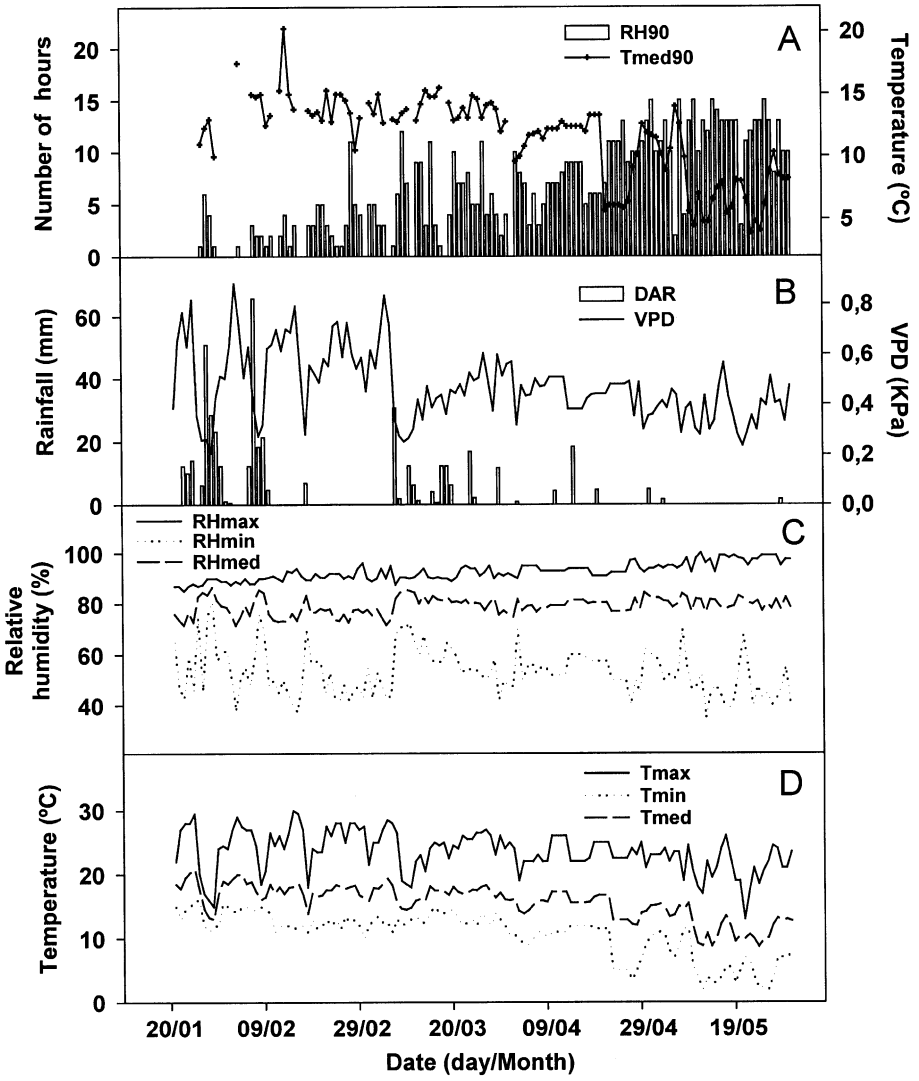

Fig. 1. Climatic variables registered in the greenhouse during the period of the experiment. (A) Daily number of hours with RH >90% (RH90) and the average temperature during RH90 ($T_{med}90$); (B) daily accumulated rainfall outside the greenhouse (DAR) and daily vapor pressure deficit average (VPD); (C) maximum, medium, and minimum daily relative humidity (RH_{max}, RH_{med}, and RH_{min}, respectively); (D) maximum, medium, and minimum daily temperature (T_{max}, T_{med}, and T_{min}, respectively).

hour and a daily average obtained by the following equation: $VPD = 0.6108e^{17.269T/(T+237.3)}(1 - RH/100)$; where, T is the temperature (°C) and RH is the relative humidity (%) (Snyder and Shaw, 1984).

2.5. Data analysis

Data for fungal sporulation and Botrytis blight incidence on rose buds were examined by analysis of variance (ANOVA) (GLM Procedures, SAS Institute, Cary, NC). Since there was no significant effect of subsampling in each plot, the average value of each replication was used for subsequent analysis. The area under the fungal sporulation curve (AUFSC) was calculated for both *C. rosea* and *B. cinerea* on 'Sonia' and 'Red Success.' The treatment means were compared by the protected least significant difference test (PLSD; Snedecor and Cochran, 1989).

3. Results

3.1. Weather

From January to May 2000, most days had weather conditions favorable to Botrytis blight development inside the greenhouse (Fig. 1). Daily average temperature varied from 15 to 20 °C. Maximum temperature ranged from 23 to 29 °C and minimum temperature from 10 to 13 °C. Relative humidity remained above 85% for at least 10 h on 70% of days, and rainfall was recorded on 28% of the days. The average daily VPD ranged from 0.2 to 1.0 kPa and was favorable to *B. cinerea* development on most of the days during the experiments.

3.2. Sporulation of Botrytis cinerea and Clonostachys rosea on rose debris

Sporulation intensity and AUFSC of *B. cinerea* on rose debris of 'Sonia' and 'Red Success' were consistently reduced by 30–50% on plots treated with *C. rosea* or *C. rosea* plus mancozeb (Figs. 2A, B and 3A, B). Compared to the control, weekly applications of mancozeb only moderately affected pathogen sporulation on 'Red Success' (Figs. 2A and 3A) and did not affect sporulation on 'Sonia' (Figs. 2B and 3B).

Sporulation intensity and AUFSC of *C. rosea* increased steadily on the plots treated with *C. rosea* (Cr) or in combination with mancozeb (CrM). In the CrM treatment, a slight reduction (10–15%) on *C. rosea* sporulation was observed (Figs. 2C and 3C), but the capacity of the antagonist to reduce *B. cinerea* sporulation was not affected (Figs. 2A and 3A). *C. rosea* was

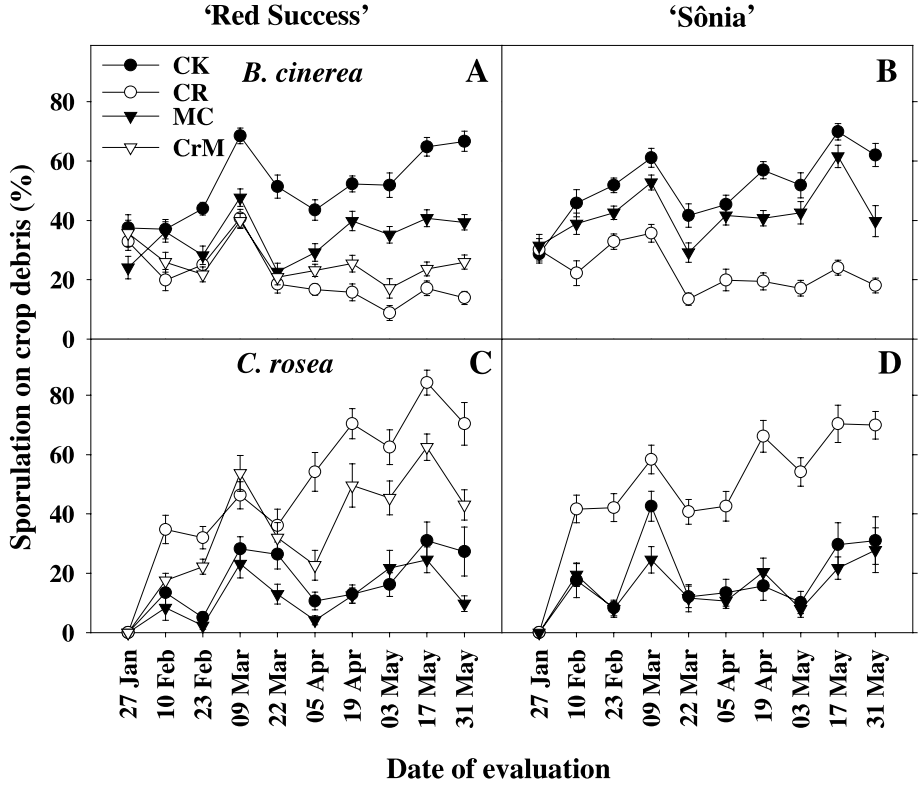

Fig. 2. Intensity of *B. cinerea* and *C. rosea* sporulation on petal and leaf disks from rose debris treated with *C. rosea* applied biweekly (Cr), mancozeb applied weekly (MC), *C. rosea* and mancozeb applied in alternate weeks (CrM), and a water control (CK). The samples were taken at 13–15 day intervals. (A and B) *B. cinerea* on cv. 'Red Success' and 'Sonia,' respectively; (C and D) *C. rosea* on cv. 'Red Success' and 'Sonia,' respectively. The treatment CrM was not applied on 'Sonia.' Curves show mean values with standard error bars.

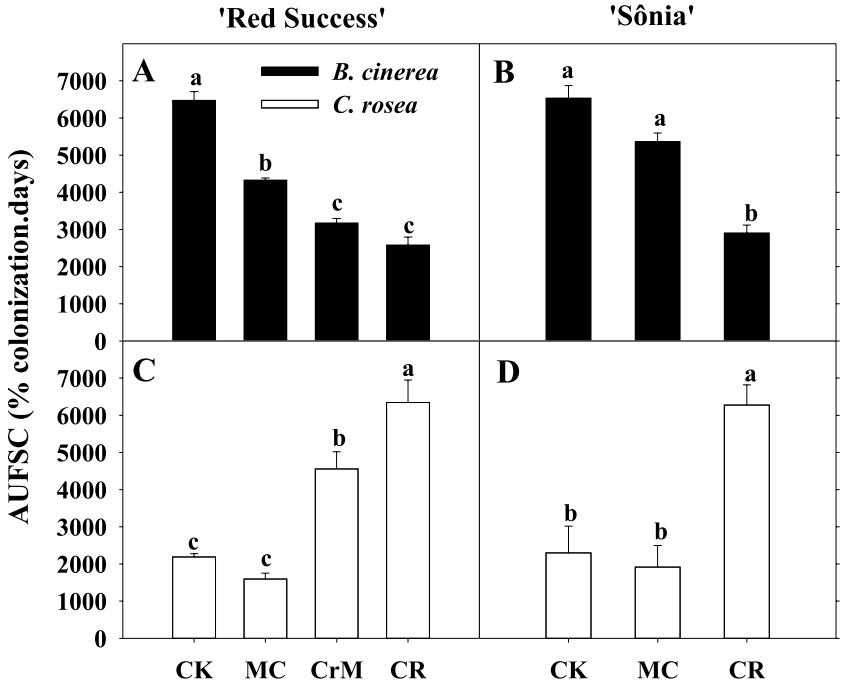

Fig. 3. Area under fungus sporulation curve (AUFSC) of *B. cinerea* and *C. rosea* on rose debris treated with *C. rosea* applied biweekly (Cr), mancozeb applied weekly (MC), *C. rosea* and mancozeb applied in alternate weeks (CrM), and a water control (CK). The samples were taken at 13–15 day intervals. (A and B) AUFSC of *B. cinerea* on cv. 'Red Success' and 'Sonia,' respectively; (C and D) AUFSC of *C. rosea* on cv. 'Red Success' and 'Sonia,' respectively. The treatment CrM was not applied for 'Sonia.' Data bars are mean values each with a standard error bar. Treatment means followed by the same letter are not significantly different (Protected LSD, $P \leqslant 0.05$).

recovered sporadically and in minor amounts on the control plots (CK) and on plots treated with mancozeb (MC) (Figs. 2C and D). As *C. rosea* was not detected in the greenhouse before the experiment was initiated, contamination from the treated plots possibly occurred.

3.3. Botrytis blight incidence on rose buds

The incidence of Botrytis blight on rose buds of both cultivars in the experimental plots before the treatment applications ranged from 62 to 100%. The Cr and CrM treatments significantly reduced (PLSD, $P \leqslant 0.05$) disease incidence on five of eight sample dates for 'Red Success' (Table 1). The MC treatment was efficient only on three sample dates. For 'Sonia,' the Cr treatment reduced the disease significantly only on two sampling dates and the MC on none (Table 1). However, in all cases the reduction of Botrytis blight incidence was not superior to 25% in relation to the control plots.

Table 1
Botrytis blight incidence (%) on rose buds of cv. 'Red Success' and 'Sonia' on eight sample dates from January 25th to May 10th of 2000

Sample date	'Red Success'				'Sonia'		
	CK	Cr	MC	CrM	CK	Cr	MC
1[a]	72.0	73.1	80.9	81.9	81.8	79.7	81.5
2	86.7	75.0[b]	83.3	70.0[b]	70.0	76.7	71.7
3	96.7	81.7[b]	83.3[b]	85.0[b]	98.3	91.7	88.3
4	91.1	82.0	88.9	85.2	93.3	81.7	83.3
5	93.3	75.0[b]	86.7[b]	81.7[b]	91.7	78.3	81.7
6	93.3	81.7[b]	91.7	80.0[b]	93.3	76.7[b]	86.7
7	91.7	80.0[b]	81.7[b]	78.3[b]	95.0	68.3[b]	85.0
8	93.3	81.7	91.7	85.0	96.7	81.7	95.0

Cr, application of *C. rosea* biweekly; MC, application of mancozeb weekly at the ratio of 200 g c.f. per 100 liter of water; CrM, application of *C. rosea* and mancozeb in alternate weeks; and CK, water control.

[a] Prior to treatment applications.

[b] Significantly different values in relation to the control on the same sample date (PLSD, $P \leqslant 0.05$).

4. Discussion

Necrotrophic, nonspecialized pathogens such as *B. cinerea* infect healthy plant tissues but the mycelial growth and sporulation occur only after the senescence of the tissues (Köhl et al., 1995). Thus, rose debris inside the greenhouse is the main inoculum source of the pathogen (Araújo et al., 1995). Because the progress of Botrytis blight epidemics on rose production systems depends chiefly on the inoculum produced inside the greenhouse (Monteiro et al., 1996), the use of an antagonist that competes efficiently with the saprophytic growth and sporulation of the pathogen is a valid control strategy (Köhl and Fokkema, 1998). However the effectiveness of *C. rosea* against *B. cinerea* is directly related to the capacity of the antagonist to colonize host senescent tissues faster than the pathogen (Morandi et al., 2001; Morandi et al., 2000a). Thus, the developmental stage of the host tissue, the microbial community associated with the debris, and the microclimatic conditions during the colonization events are factors that determine the success of *C. rosea* and other antagonists as biological control agents of *B. cinerea* (Guetsky et al., 2001; Morandi et al., 2001; Sutton et al., 1997; Yu and Sutton, 1997).

In our study, *C. rosea* established efficiently on rose debris as verified by the increment of its sporulation at each sample date, the high values of AUFSC, and the reduction of *B. cinerea* sporulation on the plots treated with the biocontrol agent. Other evidence of the successful establishment of *C. rosea* on rose debris was the recovery of the fungus from the nontreated plots. Before application in the greenhouse, *C. rosea* was not detected in soil and debris samples. It is reasonable to suppose that the isolation of *C. rosea* on the CK and MC plots resulted from contamination that originated in plots treated with the antagonist. The occurrence of drift during *C. rosea* application could also explain the contamination of CK and MC plots. However, this is not likely the main reason for the contamination, as the fungal suspension was sprayed with a low-pressure hand sprayer directed toward the surface of the rose beds and with a relative low volume of suspension per area (0.1 liter/m^2). Most likely, the contamination was caused by conidia of *C. rosea* produced within the treated plots and carried to the other plots by the irrigation water. This hypothesis is corroborated by the fact that the contamination occurred initially and predominantly on the plots located adjacent to and downward from those treated with the antagonist.

The successful establishment of *C. rosea*, notwithstanding conditions favorable to Botrytis blight development inside the greenhouse, reinforces prior reports that environmental requirements for *C. rosea* and *B. cinerea* are similar (Morandi et al., 2001). This finding can be of paramount importance when establishing a biocontrol strategy against the pathogen, as *C. rosea* could be effectively applied to reduce *B. cinerea* sporulation not only in marginal situations but also under conditions favorable to the pathogen. As already indicated, our data stress the importance of understanding the ecological attributes of the biocontrol agent to prevent its failure (Guetsky et al., 2001; Köhl and Fokkema, 1994; Köhl et al., 1999; Shtienberg and Elad, 1997; Sutton et al., 1997).

Compared to the control, weekly applications of mancozeb moderately affected pathogen sporulation on 'Red Success' and did not affect sporulation on 'Sonia' plants. Under high inoculum pressure, the efficiency of fungicides to control *B. cinerea* is reduced (Gullino, 1992; Monteiro et al., 1996; Hausbeck and Moorman, 1996). In our study, the use of alternate applications of mancozeb with *C. rosea* did not increase the efficiency of the antagonist against *B. cinerea*. This was probably due to the noticeable reduction of *C. rosea* growth on these plots. In a previous study, Morandi et al. (1996) reported up to 40% reduction of mycelial growth and sporulation of *C. rosea* on agar medium containing mancozeb.

Despite the reduction of *B. cinerea* sporulation on the treated plots, Botrytis blight incidence on rose buds was not consistently reduced, probably due to three main reasons: (i) the applications of *C. rosea* were begun by the end of January when Botrytis blight epidemic was advanced; (ii) no sanitation practices were performed during the experiment; and (iii) the production of *B. cinerea* inoculum was found on the nontreated border plants.

Most studies that have indicated high efficacy of biocontrol agents were conducted under controlled conditions. It has been suggested that environmental conditions that are not fully controlled in commercial productions systems might influence the survival, establishment, and activity of the biocontrol agents (Burrage, 1971; Hannush and Boland, 1996) and consequently reduce the efficacy of biological control. However, according to our results, *C. rosea* was able to establish on rose tissues and reduce *B. cinerea* sporulation in a commercial greenhouse without climatic control. Accordingly, the present and previous studies indicate that continuous applications of *C. rosea*, associated with sanitation practices, can be expected to markedly reduce inoculum production by *B. cinerea* and consequently Botrytis blight incidence on rose buds.

Acknowledgments

This article was part of a Ph.D. dissertation of the first author. He was supported by a scholarship provided by the Brazilian Coordination of Training of Higher Education Graduate (CAPES). The research was

partially founded by CNPq ('Conselho Nacional de Desenvolvimento Científico e Tecnológico') and FAPEMIG ('Fundação de Apoio à Pesquisa do Estado de Minas Gerais'). This paper was partially founded by FAPESP ('Fundação de Amparo à Pesquisa do Estado de São Paulo').

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