LUCIANO VIANA COTA

Clonostachys rosea NO CONTROLE BIOLÓGICO E MANEJO INTEGRADO DO MOFO CINZENTO DO MORANGUEIRO

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Fitopatologia, para obtenção do título de *Doctor Scientiae*.

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APROVADA: 29 de fevereiro de 2008.

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Prof. Luiz Antonio Maffia
(Orientador)

À minha família

DEDICO

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BIOGRAFIA

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SUMÁRIO

RESUMO

COTA, Luciano Viana, D.Sc., Universidade Federal de Viçosa, fevereiro de 2008. ***Clonostachys rosea* no controle biológico e manejo integrado do mofo cinzento do morangueiro**. Orientador: Luiz Antonio Maffia. Co-Orientadores: Eduardo Seiti Gomide Mizubuti e Fabrício de Ávila Rodrigues.

O mofo cinzento, causado por *Botrytis cinerea* (Bc) é uma das principais doenças do morangueiro. Considerando a dificuldade de controle da doença, avaliou-se o potencial de *Clonostachys rosea* (Cr) no controle biológico e no manejo integrado da doença, em experimentos de campo, em 2006 e 2007. Comparou-se a eficiência de quatro isolados de Cr (com uma ou duas aplicações semanais) à aplicação de fungicidas (procymidone alternado semanalmente com captan). Da aplicação à colheita, avaliaram-se: colonização foliar por Cr (AFC), número médio de conidióforos de Bc em folhas (NMC), incidência do mofo cinzento em flores (IFlor) e em frutos (IFruto), incidência de infecções latentes em frutos (IL) e produção (Prod). A aplicação de Cr duas vezes por semana resultou em maior AFC (16,97%), menor NMC (10,28; 78,22 na testemunha, com aplicação de água), menor IFlor (10,02%; 50,55% na testemunha) e menor Ifruto (5,95; 25,10% na testemunha). A Prod variou entre 3490 e 3750 g/parcela com a aplicação de Cr duas vezes por semana e entre 1740 e 1910 g/parcela na testemunha. A IL foi maior na testemunha (20%) e inferior a 10% nos demais tratamentos. A aplicação de Cr foi mais eficiente que a de fungicidas no controle do mofo cinzento. Em programas de manejo da doença, deve-se priorizar maior número de aplicações de Cr, visto os melhores resultados obtidos com a aplicação do antagonista duas vezes por semana. Estudou-se a integração do controle biológico (CR) por Cr, aplicação de fungicidas (AF) e remoção de restos de cultura (ER) no manejo do mofo cinzento. Em ambos os anos, AFC foi maior nas parcelas sem AF. Maiores reduções do NMC ocorreram com CR (92,01%), CR + ER (94,8%) e CR + ER + AF (96,62%). Maiores reduções na IFlor foram com CR (68,48%), CR + AF (67,82%), CR + ER (77,58%) e CR + ER + AF (86,54). Maiores reduções da IFruto foram com AF + ER (69,20%), CR (65,33%), CR + ER (76,47%) e CR + ER + AF (83,64%). Houve maiores incrementos da Prod com CR (75,15%), CR + AF (78,39%), CR + ER (79,83%) e CR + ER + AF (103,14%). A medida mais eficiente no manejo de Bc foi o controle biológico, cuja associação às outras medidas levou ao aumento da eficiência de controle. Assim, o controle biológico por Cr é estratégia chave no manejo do mofo cinzento do

morangueiro. Estudaram-se, também, os gradientes de dispersão de Cr e Bc, em experimentos independentes. Para Cr, a fonte de inóculo constituía-se de grãos de trigo colonizados e moídos e, para Bc, de frutos e hastes de morangueiros com esporulação. Em cada experimento, depositou-se a fonte no centro de parcelas (10 e 20 m de comprimento para Cr e Bc, respectivamente) e semanalmente até a colheita realizaram-se as amostragens a cada 30 cm de distânca da fonte, nos sentidos predominante e contrário do vento. Para, Cr, quantificou-se a AFC. As distâncias máximas alcançadas por Cr foram 45 e 105 cm, nos sentidos contrário e do vento, respectivamente. Para Bc, avaliaram-se o NMC, IFlor e IFruto. Em todas as avaliações, maiores valores de NMC, IFlor e IFruto ocorreram próximo à fonte. Na última avaliação (104 dias do plantio), detectaram-se folhas, flores e frutos doentes até 975 cm da fonte. Houve tendência de achatamento do gradiente em flores e frutos, mas não em folhas. Não houve efeito pronunciado do vento na dispersão de Bc. Os restos de cultura foram importantes para as epidemias do mofo cinzento do morangueiro e o antagonista Cr teve baixa capacidade de dispersão em cultivos de morangueiro.

ABSTRACT

COTA, Luciano Viana, D.Sc., Universidade Federal de Viçosa, February, 2008. ***Clonostachys rosea* in the biological control and integrated management of strawberry gray mold**. Adviser: Luiz Antonio Maffia. Co-advisers: Eduardo Seiti Gomide Mizubuti and Fabrício de Ávila Rodrigues.

Gray mold, caused by *Botrytis cinerea* (Bc), is an important strawberry disease in Brazil. As a component of a disease management program, we have been evaluating pathogen biocontrol with *Clonostachys rosea* (Cr). In 2006 and 2007, we set field experiments to evaluate the potential of Cr on biological control and on integrated management of gray mold. We compared the efficiency of four Cr isolates (applied once or twice a week) with a weekly spray of procymidone alternated with captan in controlling gray mold. Following the applications and until the harvest, we evaluated weekly: leaf area colonized by Cr (LAC), average number of Bc 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 Cr 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, with applications of Cr twice a week and between 1740 and 1910 g/plot in the check treatment. Ilat was 20% in the check treatment and less than 10% in the other treatments. Biological control by Cr was most efficient than fungicide in control of gray mold and, at least two weekly applications of Cr are required for a successful gray mold management. We studied the integration of biological control (CR) by Cr, removal of crop debris (DE), and fungicide sprays (FS) to manage gray mold. In both years, LAC was higher in the treatments with no FS. Most reductions of ANC were with CR (92.01%), CR + DE (94.80%), and CR + DE + FS (96.62%). Iflower was most reduced with application of CR (68.48%), CR + FS (67.82%), CR + DE (77.58%), and CR + DE + FS (86.54%). IFruit was most reduced with FS + DE (69.20%), CR (65.33%), CR + CD (76.47%) and CR + DE + FS (83.64%). Yield was most increased with CR (75.15%), CR + FS (78.39%), CR + DE (79.83%) and CR + DE + FS (103.14%). The most efficient method to manage Bc was biocontrol, that when associated to the other methods increased efficiency in reducing gray mold intensity. Thus the use of CR is a key strategy to manage gray mold of

strawberry. We also studied dispersal gradients of both Bc and Cr, in independent experiments. Inoculum sources for Cr were wheat grains colonized and ground and for Bc were strawberry fruits and stems with pathogen sporulation. Each source was set in the center of 10 m or 20 m-length plots for Cr and Bc, respectively, and weekly until the harvest we assessed several variables at each 30 cm distance, at both downwind and upwind directions from the sources. For Cr, we quantified LAC, and the maximum distances the antagonist reached were 45 and 105 cm, at upwind and downwind directions, respectively. For Bc, we evaluated ANC, IFlowers and IFruits. At all evaluations, the three variables were higher closer the source. At the last evaluation (104 days after planting), diseased leaves, flowers and fruits were detected up to 975 cm from the source. Gray mold gradients in flowers and fruits flattened, but gradients in leaves did not. Wind direction had no effect on Bc dispersal. Crop debris are important inoculum source for gray mold epidemics. Bc was efficient in dispersing under field conditions whereas Cr was not.

INTRODUÇÃO GERAL

O morangueiro (*Fragaria x ananassa* Duch.), fruteira de clima temperado, passou a ser cultivada extensivamente no Brasil a partir da década de 60, com a introdução de cultivares nacionais produtivos. Geralmente, os plantios são realizados por pequenos agricultores e em áreas inferiores a 5 ha. Os principais estados produtores são Minas Gerais (32,3% da produção nacional), São Paulo (31,4%) e o Rio Grande do Sul (16,5%). A produtividade média brasileira é de aproximadamente 24.269kg/ha, enquanto que a produtividade média dos Estados Unidos (maior produtor mundial, 740 mil toneladas/ano) é de 40.960kg/ha. Entre os fatores que reduzem a produtividade brasileira, destacam-se as doenças (Reichert and Madail, 2003; Santos and Medeiros, 2003). O mofo cinzento, causado por *Botrytis cinerea* Pers.: Fr. [forma perfeita= *Botryotinia fuckeliana* (de Bary) Whetzel] é uma das principais doenças do morangueiro, e causa perdas acentuadas em pré e pós-colheita em diferentes partes do mundo (Boff et al., 2001; Legard et al., 2001).

Botrytis cinerea tem ampla gama de hospedeiros e é um fungo necrotrófico que esporula em tecidos mortos ou restos culturais localizados na superfície do solo (Jarvis, 1992). Assim, as epidemias do mofo cinzento originam-se, na maioria das vezes, dos conídios produzidos nos restos culturais infestados. A contribuição dos conídios produzidos nos restos varia com o sistema de produção. Enquanto no Canadá, em sistema de cultivo perene, a principal fonte de inóculo são os restos (Braun and Sutton, 1988; Sutton, 1990), na Holanda, em sistemas de cultivo anual, os conídios produzidos em outras áreas e dispersos pelo vento são mais importantes para o desenvolvimento de epidemias (Boff et al., 2001). Em condições brasileiras, desconhece-se a contribuição dos restos de cultura como fonte de inóculo. Esta informação é importante para implementar estratégias de manejo da doença. Por exemplo, se os restos forem importantes, a supressão da esporulação do patógeno nesses restos poderá ser uma estratégia viável de controle biológico (Morandi et al., 2003).

O controle biológico de *B. cinerea* já foi avaliado em várias culturas. Um dos principais agentes de biocontrole, cuja eficiência foi comprovada no controle de *B. cinerea*, é o fungo *Clonostachys rosea* (Link: Fr.) Schroers, Samuels, Siefert & Gams (sin. *Gliocladium roseum* Bainier). Diante do sucesso obtido com esse organismo em outros países, selecionaram-se quatro isolados adaptados a ecossistemas brasileiros para uso no biocontrole de *B. cinerea* (Nobre et al., 2005). Esses quatro isolados foram tão

eficientes quanto o isolado Pg 88-710, obtido no Canadá (Peng and Sutton, 1991), em colonizar tecidos foliares, na ausência ou em períodos prolongados de molhamento foliar, em temperaturas ótimas para o patógeno, bem como suprimiram a esporulação de *B. cinerea* em intervalo de aplicação de até 14 dias antes ou após a inoculação do patógeno (Cota, 2004; Cota et al., 2008). É necessário estudar estes isolados no controle do mofo cinzneto em condições de campo, pois há relatos de agentes de biocontrole que foram eficientes em condições controladas, mas ineficientes em condições de cultivo comercial (Fravel, 2005).

O estabelecimento nas plantas tratadas é importante para o sucesso de agentes de biocontrole. A dispersão do antagonista é relevante para que ele chegue a partes não tratadas da planta. Alguns autores utilizaram abelhas como agente de dispersão do antagonista (Sutton et al., 1997), enquanto outros detectaram-no em parcelas não tratadas, o que sugere que o antagonista se dispersa após seu estabelecimento em áreas tratadas (Morandi et al., 2003; Sutton et al., 2002). Desconhece-se, por sua vez, a dinâmica de dispersão do antagonista no campo.

Considerando a necessidade de manejo do mofo cinzento do morangueiro, delineou-se esse trabalho que objetivou avaliar em condições de campo: o controle biológico do mofo cinzento por *C. rosea*; a eficiência de medidas integradas para o manejo do mofo cinzento; a contribuição dos restos de cultura para o desenvolvimento de epidemias do mofo cinzento; e os gradientes de dispersão de *B. cinerea* e *C. rosea.*

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

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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 biocontrol 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 two-year study, at least two weekly applications of *C. rosea* are required for a successful gray mold management program.

Keywords: *Botrytis cinerea; Gliocladium roseum; Clonostachys rosea; Fragaria x ananassa*; biocontrol.

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).

B. cinerea has a wide host range and is a necrotrophic fungus that sporulates on dead tissue or on crop debris surface (Jarvis, 1992; Williamson et al., 2007). Optimum temperature for fungal sporulation on strawberry leaf debris is 17 - 18^{o}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 disease secondary 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 15º and 25^{o}C at flowering (Wilcox and Seem, 1994).

Gray mold management is strongly based on chemical control, but fungicide application may cause problems as toxic residues in 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 fruits 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; Legard et al., 2001; Xiao et al., 2001). However, these measures have limited effects on disease control.

Biological control has been studied as an alternative for strawberry gray mold management. Although biocontrol may be used in post-harvest, when environmental

conditions could be more controlled or modified to favor biocontrol agents, these agents are efficient only against infections that are established during the post-harvest stage. To reduce losses to disease in post-harvest, control measures must be applied under field conditions (Ippolito and Nigro, 2000; Janisiewicz and Korsten, 2002; Sutton et al., 1997). Biocontrol 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; Morandi et al., 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; Morandi et al., 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; Cota et al., 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 biocontrol 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 (15μmol cm-2 s-1). Conidia from 12 to 14-day old colonies were suspended in water, filtered through two layers of cheesecloth. With a haemocytometer, conidia concentration was account and adjusted to $1x10^6$ conidia mL-1. At the moment of application, the surfactant Tween 80 (0.05% v/v) was added to the conidial suspension. The volume applied by plot ranged from 0.2 to 0.4 L.

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 minutes at every two days. To control leaf spots, the fungicide tebuconazole (Folicur 200 EC; 0.375 mL a.i. 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 a.i. 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 (procymidone (Sialex 500; 0.5 g a.i. L-1) weekly alternated with captan (Orthocide 500; 1.2 g a.i. L-1), and a check [application of water + Tween 80 (0.05% v/v)]. The treatments were applied with a backpack sprayer (Jacto® model PJH), nozzle type cone, and pressure of 75 psi.

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 (from fully expanded leaves) 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 discs (1cm-diameter) and planted them in Petri dishes containing paraquat-chloramphenicol-agar medium (PCA) (Peng and Sutton, 1991). After 10-12 days incubation at 20^{o}C, the percentage of disc 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 disc 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 discs.

To evaluate gray mold incidence on flowers, ten flowers (open flower with all petals and sepals) were weekly taken from each plot and plated on PCA, incubating at 20^{o} C, 12 h photoperiod. After 5 days, the flowers were observed under the stereoscope and checked for gray mold symptoms and signals.

Ripe fruits were harvested weekly. Healthy and diseased fruits were always picked separately to avoid contamination of healthy during harvesting. Following harvest, both yield and gray mold incidence (number of diseased/total number of fruits) was evaluated. To account the latent infections, ten symptomless fruits were randomly chosen from each plot, set inside a plastic box (11 cm width x 11 cm length x 3.5 cm depth), incubating at 20^{o}C and observed daily for *B. cinerea* sporulation (during 10 days).

2.4 Data analysis

Each experiment was in a randomized block design with four blocks. Six variables were assessed: disc area colonized by *C. rosea* (LAC), average number of *B. cinerea* conidiophores/disc (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. 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; CR2 versus check; and fungicide sprays versus check. When the interaction term was significant, contrasts were applied for comparisons at 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 (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, α=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 (Figure 1A). The interaction treatment - 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 (Figure 1B). The interaction treatment - 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 largest 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 (Figure 2A). ANC values tended to decrease in check, increase with fungicide until 84 dap and remain steady with both CR1 and CR2 (Figure 2A). The interaction treatment - 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 at the check (P<0.0001); smaller with CR1 than with fungicide (P<0.0001) and at the check (P<0.0001). At most evaluations, ANC was smaller with fungicide than at 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 (Figure 2B). ANC values tended to increase in the check and remain steady with fungicide, CR1, and CR2 (Figure 2B). The interaction treatment - 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 at the check (P<0.0001); smaller with CR1 than fungicide (P<0.0001) and at the check (P<0.0001); and smaller with fungicide than at 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 (Figure 3A). Iflower values tended to remain steady with fungicide and to decrease with CR1, CR2, and at the check (Figure 3A). The interaction treatment - 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<0.0001), fungicide

(P<0.0001), and at the check (P<0.0001); with CR1 than with fungicide (P<0.0001), and at the check (P<0.0001); and with fungicide than at the check (P<0.0001).

In 2007, average reductions of Iflower were 79.7, 43.33, and 37.79% with CR2, fungicide, and CR1, respectively (Figure 3B). Iflower values tended to decrease in all treatments (Figure 3B). The interaction treatment - evaluation interval was significant (P<0.0001). At most evaluations, IFlower was smaller with CR**2** than with CR**1** (P<0.0479), and did not differ at 137 and 145 dap. IFlower was smaller with CR**2** than with fungicide at 55, 69, 77, 84, 92, 107, 127, and 137 dap (P<0.0476); and at 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 at 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 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 (Figure 4A). Ifruit values tended to decrease at all treatments (Figure 4A). The interaction treatment - 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<0.0001) and at the check (P<0.0001); with fungicide than CR1 (P<0.0058), and at the check (P<0.0001); and with fungicide than at the check (P<0.0001). Values of Ilat were, respectively, 2.34, 4.37, 7.29, and 18.54% with CR2, CR1, fungicide, and at the check, respectively.

In 2007, average reductions of Ifruit were 75.62, 63.69, and 39.29%, with CR2, fungicide and CR1, respectively (Figure 4B). Ifruit values tended to decrease with CR2

and CR1 and to remain steady at the check and with fungicide (Figure 4B). The interaction treatment - 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 at the check (P<0.0001); with fungicide than CR1 (P<0.0001) and at the check (P<0.0001); and with CR1 than at the check (P<0.0001). Ilat values were 0.68, 1.42, 2.78, and 15.45% with fungicide, CR2, CR1, and at 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 increments in yield were 98.01, 58.83 and 30%, with CR2, fungicide, and CR1 treatments, respectively (Figure 5A). Yield values tended to decrease with CR2 and CR1 and to remain steady with fungicide and in the check (Figure 5A). The interaction treatment - 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 at 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 check at all evaluations (P<0.0036). Yield with CR1 was higher than 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 (Figure 5B). Yield values tended to decrease with CR2, increase with CR1 and fungicide, and to remain steady in the check (Figure 5B). The interaction treatment - 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 at 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

In 2006 and 2007, average air temperature was 16.3 and 18.5°C, RH was 83.6 and 79.3%, average maximum temperature was 23.9 and 24.6°C, average minimum temperature was 11.0°C and 13.7°C, and average daily hours with leaf wetness were 12.1 h and 11.7 h, respectively. In 2006, after 110 dap temperature tended to increase and RH to decrease (Figure 6).

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, biocontrol studies were initiated in the 90's (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; Cota et al., 2008). Here we report on the efficiency of biocontrol of gray mold on strawberry under field conditions.

In this two-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; Cota et al., 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 temperature was above average, 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; Morandi et al., 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 for biocontrol the pathogen in strawberry and other hosts (Köhl and Fokkema, 1994; Köhl and Fokkema, 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 starts growing 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 imply in reducing inoculum production in crop debris and, consequently, will contribute to reduce 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 sprays as the flowering period lasts long. 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, besides the high cost protecting flowers and fruits, fungicide applications can result in problems with chemical residues on them. Application of a biocontrol 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 antagonists sprays on strawberry and other hosts (Kovach et al., 2000; Peng et al., 1992; Shafir et al., 2006; Sutton et al., 1997).

In general at 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 cells and removal by water would be less critical compared to the fungicides. Control efficiency achieved with our isolates was similar to 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 biocontrol 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 biocontrol 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 biocontrolling of strawberry gray mold was increased when the antagonist was applied since planting time (Boff et al., 2002). Although not quantified, we observed *B. cinerea* sporulation on dead parts of transplants, which most likely provided inoculum 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 biocontrol in commercial crops. Additionally, although drip irrigation is commonly used system in strawberry fields (mainly at south Brazil region), 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; Cota et al., 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 biocontrol 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 biocontrol with *C. rosea* under field conditions. Before using the biocontrol 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 biocontrol agents can be rotated with fungicides under condition of higher disease pressure. Defining these conditions is the next step to be pursued.

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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). The areas were estimated for ten treatments: applications of four isolates of *C. rosea* ([1]once or [2]twice a week), 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.

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

*In each column, means followed by the same letter do not differ (Protected LSD, α=0.05).

** *C. rosea* was not detected.

Figures

Figure 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.

Figure 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.

Figure 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.

Figure 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.

Figure 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.

Figure 6 Climatic variables registered during the experiments conducted in 2006 (A, C) and 2007 (B, 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).

Figure 1

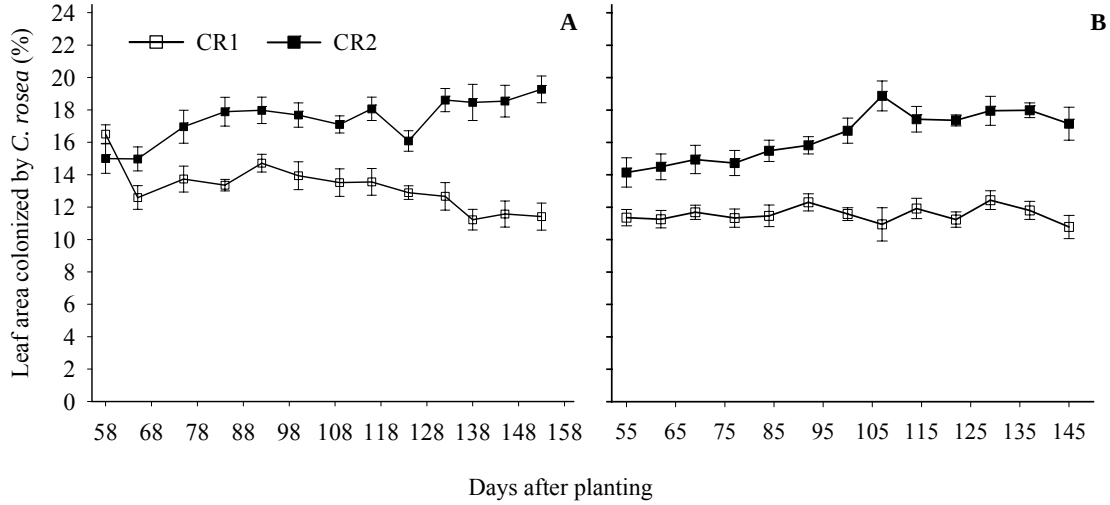

A
B
CR1
CR2
Leaf area colonized by C. rosea (%)
0
2
4
6
8
10
12
14
16
18
20
22
24
58 68 78 88 98 108 118 128 138 148 158
55 65 75 85 95 105 115 125 135 145
Days after planting

Figure 2

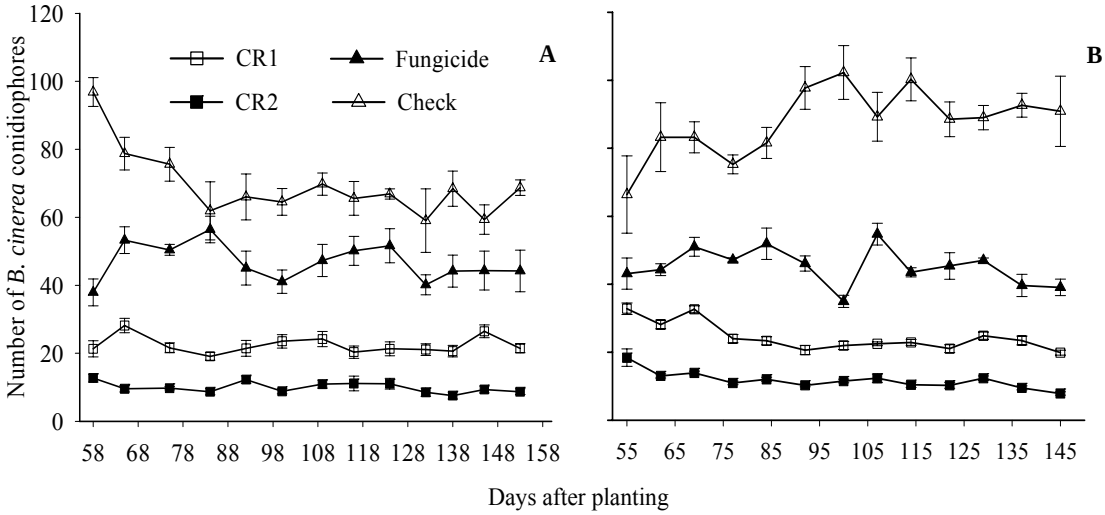

Number of B. cinerea conidiophores
120
100
80
60
40
20
0
CR1
CR2
Fungicide
Check
A
B
58 68 78 88 98 108 118 128 138 148 158
55 65 75 85 95 105 115 125 135 145
Days after planting

Figure 3

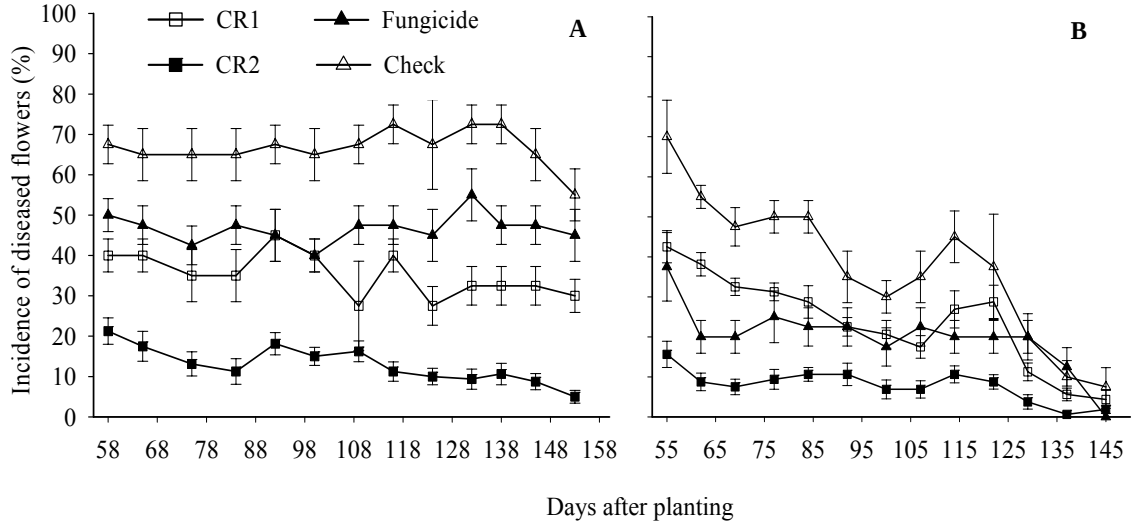

CR1
Fungicide
CR2
Check
A
B
Incidence of diseased flowers (%)
100
90
80
70
60
50
40
30
20
10
0
58 68 78 88 98 108 118 128 138 148 158
55 65 75 85 95 105 115 125 135 145
Days after planting

Figure 4

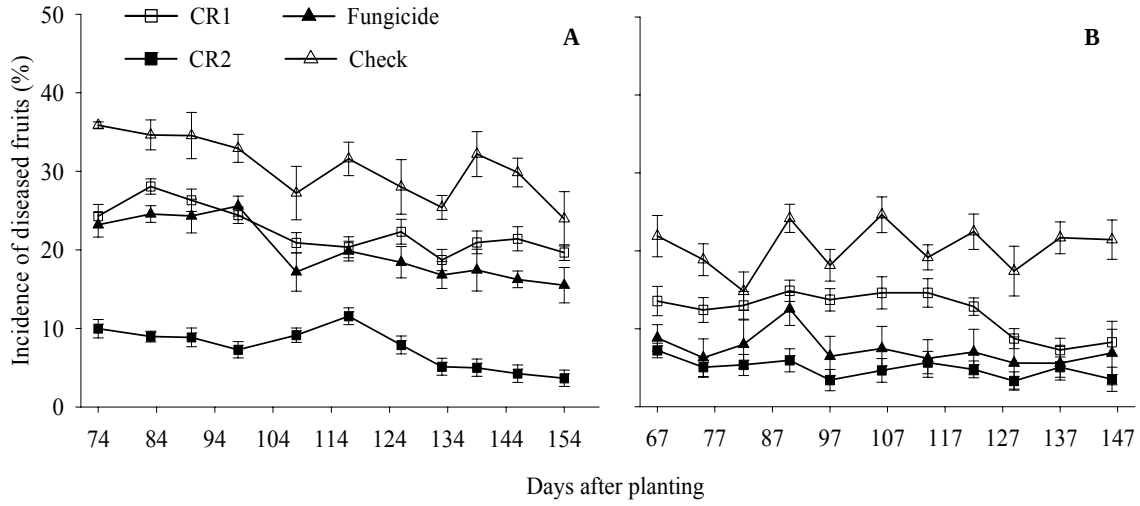

CR1
Fungicide
CR2
Check
A
B
Incidence of diseased fruits (%)
50
40
30
20
10
0
74
84
94
104
114
124
134
144
154
67
77
87
97
107
117
127
137
147
Days after planting

Figure 5

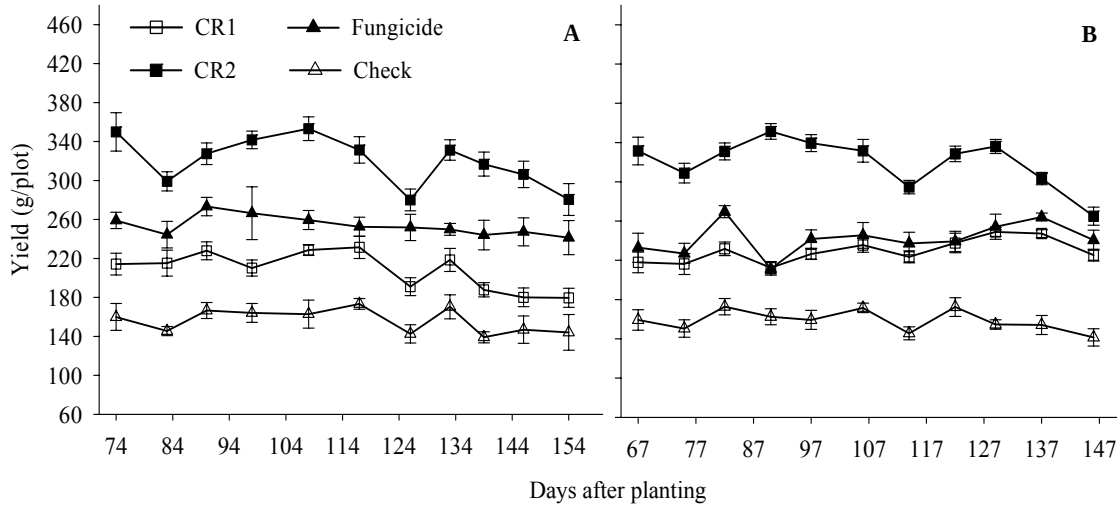
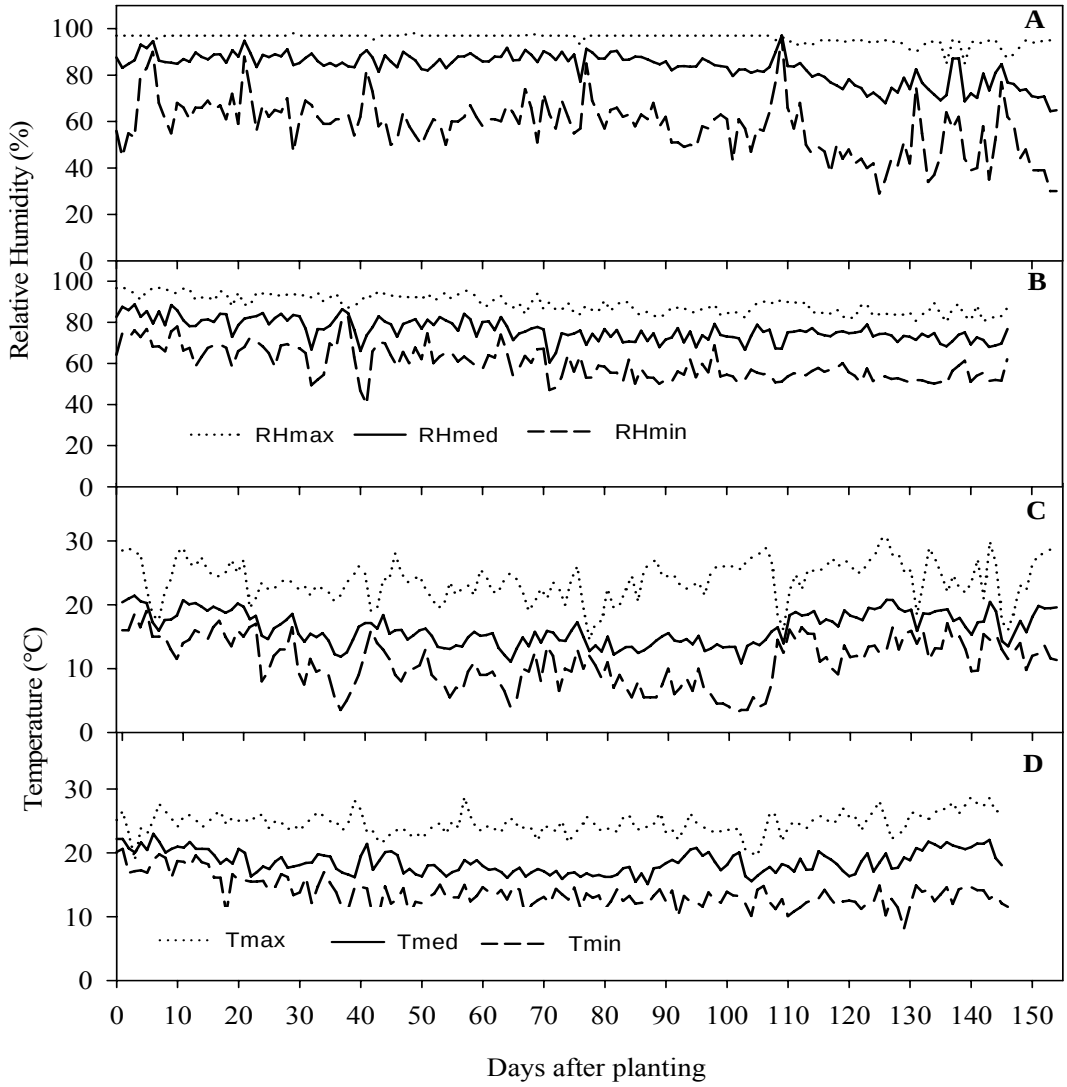

CR1
CR2
Fungicide
Check
A
B
Yield (g/plot)
460
420
380
340
300
260
220
180
140
100
60
74
84
94
104
114
124
134
144
154
67
77
87
97
107
117
127
137
147
Days after planting

Figure 6


A
B
C
D
Relative Humidity (%)
Temperature (°C)
RHmax
RHmed
RHmin
Tmax
Tmed
Tmin
Days after planting

Integrated management of gray mold of strawberry under field conditions

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Abstract

Gray mold, caused by *Botrytis cinerea* (Bc) is an important strawberry disease. As gray mold control is difficult, there is a need to evaluate integrated methods to successfully manage the disease. We studied the efficiency of integrating *Clonostachys rosea* sprays (CR), fungicide sprays (FS), and crop debris removal (DE) to manage gray mold in field experiments conducted in 2006 and 2007. Leaf colonization by *C. rosea* (LAC), average number of Bc conidiophores (ANC), gray mold incidence in both flowers (Iflower) and fruits (Ifruit), and yield were weekly evaluated. In both years, LAC was higher in the treatments with no fungicide. ANC was most reduced in the treatments with CR (92.01% reduction), CR + DE (94.80%), and CR + DE + FS (96.62%). Iflower was most reduced with CR + FS (67.82%), CR (68.48%), CR + DE (77.58%), and CR + DE + FS (86.54%). IFruit was most reduced with CR (65.33%), FS + DE (69.20%), CR + DE (76.47%), and CR + DE + FS (83.64%). Yield was most increased with CR (75.15% increase), CR + FS (78.39%), CR + DE (79.83%) and CR + DE + FS (103.14%). The most efficient method to manage Bc was the biocontrol, which when associated to other methods increased control efficiency. Thus, the use of CR is a key strategy to manage gray mold of strawberry under field conditions.

Keywords: *Botrytis cinerea; Clonostachys rosea; Fragaria x ananassa*; biological control; cultural practices; chemical control.

1 Introduction

Botrytis cinerea Pers.: Fr. [perfect stage = *Botryotinia fuckeliana* (de Bary) Whetzel] is a plant pathogen widely distributed in the world that can infect plant species in more than 200 genera (Jarvis, 1992). In strawberry (*Fragaria x ananassa* Duch.), *B. cinerea* causes losses up to 50% of yield considering the damages that occur both before and after harvest (Blanco et al., 2006).

Control of strawberry gray mold is difficult, because the pathogen has a wide host range, multiple mechanisms of attack, and can infect all plant organs at most development stages (Kars and van Kan, 2004; Sutton, 1990; Williamson et al., 2007). Additionally, the use of resistant plants is difficult because *B. cinerea* has high genetic variability and is a necrotrophic pathogen (Beever and Weeds, 2004; Williamson et al., 2007). Gray mold is mostly controlled with fungicides, but the high number of fungicide sprays required to achieve a satisfactory control may lead to problems with fungicides residues in fruits (Rabolle et al., 2006; Legard et al., 2005; Legard et al., 2001) and selection of fungicide-resistant individuals (Brent and Hollomon, 2007). Resistance to the most used active principles, benzimidazoles and dicarboximides, is widely spread (Dianez et al., 2002; Ghini, 1996; LaMondia and Douglas, 1997; Yourman and Jeffers, 1999). There are also reports of resistance even to newly developed compounds, as anilinopyrimidine, phenylpyrrole, and hydroxyanilide fungicides (Ma and Michailides, 2005; Myresiotis et al., 2007). Reduction on the number of fungicide sprays may be achieved by either concentrating the sprays at the flowering peaks or applying reduced amounts of protectant fungicides throughout crop cycle. However, both strategies are less efficient than the conventional calendar sprays (Legard et al., 2005; Legard et al., 2001; Mertely et al., 2002). Other control methods must be used as alternatives to reduce the high number of fungicide sprays.

Many alternatives have been evaluated, as increasing plant spacing and removal of crop debris (Legard et al., 2001; Mertely et al., 2000; Xiao, 2001). The effect of these cultural practices on disease control is limited, but when combined to other methods, control efficiency can be increased. Crop debris removal reduced gray mold severity, but not incidence on rose buds after harvest (Monteiro, 1996). According the author, when the removal was associated to mancozeb applications, both incidence and severity were reduced. However, the integration of debris removal and fungicide sprays did not improve the efficiency of controlling strawberry gray mold in different conditions

(Mertely et al., 2000). Therefore, as the outcome of using cultural practices is somewhat variable, biological control is being also evaluated.

Biological control is an interesting alternative to manage strawberry gray mold (Boff et al., 2002; Kim et al., 2007; Peng and Sutton, 1991; Sutton et al., 1997; Sutton and Peng, 1993; Valdebenito-Sanhuenza et al., 1997). Many microorganisms have been evaluated as biocontrol agents, as the fungus *Clonostachys rosea* (Link: Fr.) Schroers, Samuels, Siefert & Gams (formerly *Gliocladium roseum* Bainier). *C. rosea* has been efficient in controlling the disease by protecting leaves, flowers, and fruits from *B. cinerea* infection (Peng and Sutton, 1991; Sutton and Peng, 1993) and by suppressing pathogen sporulation on crop debris (Morandi et al., 2003; Nobre et al., 2005; Sutton et al., 1997; Valdebenito-Sanhuenza et al., 1997). The antagonist established and competed with *B. cinerea* in leaves of both strawberry and rose (*Rosa hybrida* L.) under either absence of wetting period or with 48 h of wetness (Cota et al., 2008a; Cota et al., 2007; Morandi et al., 2001). Under field conditions, *C. rosea* was more efficient than fungicides in controlling strawberry gray mold (Cota et al., 2008b).

The integration of methods for strawberry gray mold management has not been evaluated under Brazilian conditions. Through this integration, the efficiency of biological control could be increased and contamination of both environment and fruits with fungicides be reduced (Elad and Stewart, 2004; Fravel, 2005; Shtienberg and Elad, 1997). Under greenhouse conditions, integrating *Trichoderma harzianum* Rifai and fungicides increased the efficiency of both chemical and biological controls (Shtienberg and Elad, 1997). Considering this approach, we evaluated the integration of *C. rosea*, fungicides, and removal of crop debris to manage strawberry gray mold under field conditions.

2 Materials and Methods

2.1 Isolates and inoculum production

We used the four Brazilian *C. rosea* isolates, selected by the ability 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^{o}C, 12 h photoperiod (15μmol cm-2 s-1). Conidia from 12 to 14-day old colonies were suspended in water, filtered through two layers of cheesecloth. With a haemocytometer, conidia concentration was adjusted to 4x10^{6} conidia mL-1. At

the moment of application, the surfactant Tween 80 (0.05% v/v) was added to the conidial suspension. The volume applied by experimental plot ranged from 0.2 to 0.4 L.

2.2 Experimental Plots

The experiments were conducted in 2006 and 2007 at the field plots of the Departamento de Fitopatologia, Universidade Federal de Viçosa (20°44'47'' S and 42°50'55'' W). Plots were spaced 2 m and each had four 3 m-long lines of 'Camarosa' plants spaced 0.3 x 0.3 m. The two external lines and the last plant of each line were considered as borders. Plantings were on 13/04/2006 and 18/04/2007. At 20 days after planting (dap), each transplant was fertilized with 15 g NPK (4-14-8). After 43 dap, the beds were covered with a black plastic film (25 μm-thick). Plants were sprinkler irrigated for 90 minutes at every two days. To control leaf spots, the fungicide tebuconazole (Folicur 200 EC; 0.375 mL a.i. L-1) was spread 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 a.i. L-1). Temperature, leaf wetness, and relative humidity were registered in a data logger (CR10X, Campbell Scientific, Inc.).

Application of treatments started as soon as the beds were plastic covered (at 45 and 47 dap, in 2006 and 2007, respectively). We evaluated the combination of the following treatments: removal of crop debris or not; fungicide sprays or not; and application of *C. rosea* or not. Crop debris (diseased organs and dead parts of plants) were removed daily. The fungicide procymidone (Sialex 500; 0.5 g a.i. L-1) was sprayed weekly alternated with captan (Orthocide 500; 1.2 g a.i L-1). Application of *C. rosea* was based on a mixture of the four isolates of *C. rosea* (each at the concentration $4x10^6$) plus the surfactant Tween 80 (0.05% v/v) and applied once a week. Application of *C. rosea* and fungicides were spaced 3 days apart. The treatments were applied with a backpack sprayer (Jacto® model PJH), nozzle type cone, and pressure of 75 psi.

2.3 Evaluations

We evaluated colonization of strawberry leaf tissues by *C. rosea* isolates and their efficiency in suppressing *B. cinerea* sporulation, incidence of gray mold on both flowers and fruits, and yield.

To evaluate colonization of *C. rosea* and suppression of *B. cinerea* on leaves, five leaflets (from fully expanded leaves) were weekly sampled in each plot.

Colonization of pathogen and antagonist was indirectly evaluated by assessing intensity of sporulation on leaf tissues. Fifteen leaf discs (1 cm diameter) were cut and platted in Preti dishes containing paraquat-chloramphenicol-agar medium (PCA) (Peng and Sutton, 1991). After 10-12 days incubation at 20°C, percentage of disc area with *C. rosea* sporulation and the number of *B. cinerea* conidiophores were assessed under the 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 the disc was rated as 3 for *B. cinerea*, its corresponding average value was 36.5 conidiophores. Data for each replicate were the average of the 15 discs.

To evaluate gray mold incidence on flowers, ten flowers (open flower with all petals and sepals) were weekly sampled from each plot and platted in PCA, incubating at 20° C, 12 h photoperiod. After 5 days, the flowers were observed under the stereoscope and checked for gray mold symptoms and signals.

Ripe fruits were weekly harvested. Healthy and diseased fruits were always picked separately to avoid contamination of healthy during harvesting. Following harvest, both yield and gray mold incidence (number of diseased/total number of fruits) were evaluated. To account for latent infections, from each plot 10 symptomless fruits were randomly chosen, set inside a plastic box (11 cm width x 11 cm length x 3.5 cm depth), incubating at 20°C, and observed for *B. cinerea* sporulation daily, until 10 days.

2.4 Data analysis

Each experiment was in the factorial scheme in the randomized block design with four blocks. Six variables were assessed: disc area colonized by *C. rosea* (LAC), average number of *B. cinerea* conidiophores/disc (ANC), incidence of diseased flowers (Iflower), incidence of diseased fruits (Ifruit), incidence of latent infections (ILat), and yield. Standardized areas under the curves (Fry, 1977) of LAC (AUCLAC), ANC (AUCANC), Iflower (AUCIflower), of Ifruit (AUCIfruit), and yield (AUCYield) were estimated. With the area values estimated in 2006 and 2007, homogeneity of error

variances was analyzed with Levene's Test. As there was homogeneity of error variance between the two-year experiments for all areas-under-curve values ($P>0.21$), the data were pooled and subjected to the analysis of variance. To study interactions effects, the Lsmeans procedure was used to evaluate the effect of each factor level in the presence/absence (Sliced statement) of the other factors. All statistical analyses were conducted using SAS v. 9.1.

To help understand interactions in the factorial experiment, each factor was abbreviated: *C. rosea* application as CR, debris removal as DE, and fungicide sprays as FS. Each level of a factor received a + or – superscript, implying that the factor was either present or not, respectively. For example, $CR^{+}/DE^{-}/FS^{+}$ means that the treatment combination included *C. rosea* application, no debris removal, and fungicide spray.

3 Results

3.1 Leaf colonization by *Clonostachys rosea*

In both years, *C. rosea* was not detected in the untreated plots. LAC was higher in the plots not sprayed with fungicide (Figure 1). Throughout the experiments in 2006 and 2007, average LAC values were, respectively, 12.77 and 11.96 in $CR^{+}/DE^{-}/FS^{-}$; 13.35 and 15.16 in $CR^{+}/DE^{+}/FS^{-}$; 6.21 and 7.98 in $CR^{+}/FS^{+}/DE^{-}$; 5.90 and 7.87 in $CR^{+}/DE^{+}/FS^{+}$. In both years, LAC values tended to remain steady in the plots unsprayed with fungicide. In 2006, LAC values tended to decreased in the plots sprayed. In 2007, this tendency also occurred up to 90 dap (Figure 1).

C. rosea was detected only in the plots it was applied. Thus, all statistical comparisons were in the CR^{+} treatments. AUCLAC values were: 14.25 in DE^{+}/FS^{-}, 14.07 in DE^{-}/FS^{-}, 6.95 in DE^{-}/FS^{+}, and 6.74 in DE^{+}/FS^{+}, always significantly lower in FS^{+} treatments ($P<0.0001$).

3.2 Average number of conidiophores of *Botrytis cinerea*

Overall, in both experiments, the ANC values were smaller in all combinations that included CR^{+} (Figure 2B, D). Without CR, smaller values were for $CR^{-}/DE^{+}/FS^{+}$ combination (Figure 2A, C). Largest average reductions on ANC relative to the control in 2006 and 2007 were, respectively, 92.68 and 91.38% in $CR^{+}/DE^{-}/FS^{-}$; 86.60% and

86.78% in $CR^+/DE^-/FS^+$; 95.48 and 94.12% in $CR^+/DE^+/FS^-$; 97.82 and 95.41% in $CR^+/DE^+/FS^+$; and 87.18 and 86.35% in $CR^-/DE^+/FS^+$.

Largest (94.64) and smallest (3.28) AUCANC values were in the control ($CR^-/DE^-/FS^-$) and in the $CR^+/DE^+/FS^+$ treatment, respectively. The triple interaction CR x DE x FS was significant (F=342.47, P<0.0001). To study this interaction, the effect of each factor level was evaluated in the presence/absence of the other two factors (Table 1). When fungicide was sprayed, AUCANC was significantly reduced in CR^+/DE^-, CR^-/DE^+, CR^-/DE^-, but not in CR^+/DE^+. When debris were removed, AUCANC was significantly reduced in CR^-/FS^-, CR^-/FS^+, and CR^+/FS^+, but not in CR^+/FS^-. When *C. rosea* was applied, AUCANC was significantly reduced in all combinations of FS/DE levels.

3.3 Incidence of gray mold in flowers

Overall, in both experiments, Iflower values were smaller in all combinations that included CR^+ (Figure 3B, D). Without CR, smaller values were for $CR^-/DE^+/FS^+$ combination (Figure 3A, C). Largest average reductions on Iflower relative to the control in 2006 and 2007 were, respectively, 68.34 and 68.62% in $CR^+/DE^-/FS^-$; 70.31 and 65.32 in $CR^+/DE^-/FS^+$; 80.25 and 74.9 in $CR^+/DE^+/FS^-$; and 86.27 and 86.21 in $CR^+/DE^+/FS^+$**;** 53.69 and 50.78 in $CR^-/DE^+/FS^+$.

Largest (37.42) and smallest (4.94) AUCIflower values were in the control ($CR^-/DE^-/FS^-$) and in the $CR^+/DE^+/FS^+$ treatment, respectively. The triple interaction CB x DE x FS was significant (F=15.69, P= 0.0002). When fungicide was sprayed, AUCIflower was significantly reduced in CR^-/DE^-**,** CR^-/DE^+, CR^+/DE^+, but not in CR^+/DE^-. When debris were removed, AUCIflower was significantly reduced in all combinations of CR/FS levels. When *C. rosea* was applied, AUCIflower was significantly reduced in all combinations of FS/DE levels.

3.4 Incidence of gray mold in fruits

Overall, in 2006 experiments, Ifruit was smaller in CR^+/DE^+, DE^+/FS^+, and $CR^+/DE^+/FS^+$ combination (Figure 4A, B). In 2007, Ifruit was smaller in all treatments

that included CR^+ (Figure 4D). Without CR, smaller values were for $CR^-/DE^+/FS^+$ combination (Figure 4C). Largest average reductions on Ifruit relative to the control in 2006 and 2007 were, respectively, 60.58 and 70.07 with $CR^+/DE^-/FS^-$; 70.39 and 48.36 and 71.33 $CR^+/DE^-/FS^+$; 80.69 and 76.00% with $CR^+/DE^+/FS^-$; and 88.48 and 78.80% with $CR^+/DE^+/FS^+$; and 68.00 with $CR^-/DE^+/FS^+$. Average reductions on ILat in 2006 and 2007 were, respectively, 81.04 and 92.45% with $CR^-/DE^-/FS^+$; 76.01 and 81.94% with $CR^-/DE^+/FS$; 91.34 and 98.61% with $CR^+/DE^-/FS^-$; 86.62 and 95.49% with $CR^-/DE^+/FS^+$; 88.82 and 98.61% with $CR^+/DE^-/FS^+$; 80.69 and 95.14% with $CR^+/DE^+/FS^-$; and 86.26 and 91.67% with $CR^+/DE^+/FS^+$.

AUCIfruit values were: 23.87 in $CR^-/DE^-/FS^-$; 14.76 in $CR^-/DE^-/FS^+$; 20.56 in $CR^-/DE^+/FS^-$; 7.31 in $CR^-/DE^+/FS^+$; 8.36 in $CR^+/DE^-/FS^-$; 9.70 in $CR^+/DE^-/FS^+$; 5.53 in $CR^+/DE^+/FS^-$; and 3.86 in $CR^+/DE^+/FS^+$. Significant effects on AUCIfruit were found for the interactions CR x FS ($F=114.06$, $P<0.0001$) and DE x FS ($F= 12.09$, $P=0.0011$), but not for the interactions CR x DE ($F=1.03$, $P=0.3151$) and CR x DE x FS ($F=0.31$, $P=0.5814$). When fungicides were sprayed, AUCIfruit was significantly reduced in CR^- ($F=235.12$, $P<0.0001$), but not in CR^+ ($F=0.05$, $P=0.8189$). When *C. rosea* was applied, AUCIfruit was significantly reduced in FS^- ($F=438.5$, $P<0.0001$) and FS^+ ($F=34.07$, $P<0.0001$). Fungicide sprays resulted in smaller AUCIfruit in DE^- ($F=28.34$, $P<0.0001$) and DE^+ ($F=104.87$, $P<0.0001$). Debris removal resulted in smaller AUCIfruit in FS^- ($F=17.65$, $P=0.0001$) and FS^+ ($F=83.15$, $F<0.0001$).

3.5 Yield

Yield was higher in $CR^-/DE^-/FS^+$, $CR^-/DE^+/FS^+$, and with all combinations that included CR^+ in 2006 (Figure 5A,B) and in $CR^-/DE^-/FS^+$, $CR^-/DE^+/FS^+$, $CR^+/DE^+/FS^-$, and $CR^+/DE^+/FS^+$ in 2007 (Figure 5C, D). Largest average increases in yield relative to the control in 2006 and 2007 were, respectively, 64.96 and 51.86 with $CR^-/DE^-/FS^+$; 72.48 and 77.82 with $CR^+/DE^-/FS^-$; 69.93 and 72.41 with $CR^-/DE^+/FS^+$; 75.16 and 81.62 in $CR^+/DE^-/FS^+$; 75.17 and 84.86 with $CR^+/DE^+/FS^-$; and 101.61 and 104.67 with $CR^+/DE^+/FS^+$.

Largest (282.03) and smallest (142.83) AUCYield values were in the $CR^+/DE^+/FS^+$ treatment and in the control treatment ($CR^-/DE^-/FS^-$), respectively. The triple interaction CB x DE x FS was significant (F=8.11, P= 0.0064). When fungicide was sprayed, AUCYield was significantly increased in CR^-/DE^-, CR^-/DE^+, and CR^+/DE^+, but not in CR^+/DE^-. When debris were removed, AUCYield was significantly increased in CR^-/FS^-, CR^-/FS^+, and CR^+/FS^+, but not in CR^+/FS^-. When *C. rosea* was applied, AUCYield was significantly increased in all combinations of FS/DE levels (Table 1).

3.6 Meteorological data

In 2006 and 2007, average air temperatures were respectively 16.4 and 18.6°C, RH were 83.0 and 76.1%, average maximum temperatures were 24.1 and 24.7°C, average minimum temperatures were 11.1°C and 13.7°C, and average daily hours with leaf wetness was 12.1 h and 11.4 h, respectively. In 2006, after 110 dap temperature tended to increase and RH to decrease (Figure 6).

4 Discussion

Control of strawberry gray mold is difficult because *B. cinerea* can infect several plant organs in almost all phenological stages, causes latent infection, and flower protection requires high number of chemical sprays (Boff et al., 2003; Legard et al., 2005; Legard et al., 2001; Mertely et al., 2002; Sutton, 1998). Furthermore, resistance to commonly applied fungicides is widely disseminated (Barak and Edgington, 1984; Dianez et al., 2002; Ghini, 1996; LaMondia and Douglas, 1997; Myresiotis et al., 2007; Rabolle et al., 2006; Yourman and Jeffers, 1999). Integration of control methods is needed to disease management either to reduce chemical residues or to increase chemicals efficiency. Also, integration can contribute to enhance efficiency of other disease control methods such as cultural and biological. Here, we report on the efficiency of the integration of biological control with *C. rosea* with crop debris removal and chemical control to manage strawberry gray mold under field conditions.

When executed singly, crop debris removal was the least effective treatment in reducing incidence of gray mold in both flowers and fruits and in increasing yield.

Similar results were reported in experiments conducted in The Netherlands for management of gray mold in perennial strawberry crops (Boff et al., 2002). In The United States, removal of crop debris reduced incidence in fruits, but there was no increase in strawberry yield (Mertely et al., 2000). Under Brazilian conditions, removal of crop debris in greenhouse-grown rose crops contributed to reduce severity of gray mold in flowers, but not to reduce disease incidence (Monteiro et al., 1996). In our experiments, debris removal was more effective than fungicides in reducing leaf colonization by *B. cinerea,* however was not efficient in reducing disease in both flowers and fruits. Differences in efficacy associated with plant organ could be due to a differential susceptibility of host tissues to *B. cinerea*. Often *B. cinerea* profusely sporulated on crop debris and on removed fruits. It is expected that spore dispersal that occurred during fruits removal contributed for new infections on leaves and flowers. Only young strawberry leaves are susceptible to *B. cinerea*, whereas flowers can be infected during most of their development stages (Sutton, 1990; Mertely et al., 2002). The association of crop debris removal and chemical control had a synergistic effect that enhanced the effect of both components. Fungicide sprays protected leaves and flowers from infection by airborne spores in suspension during debris removal. Furthermore, debris removal reduced the amount of inoculum in the plots. These results diverge from those obtained in the United States, where disease control was not improved by integration of crop debris removal and chemical control (Mertely et al., 2000). In roses grown in greenhouse, debris removal associated to fungicide sprays increased the efficiency of disease control in flower buds, by reducing 30 and 50% of incidence and severity of gray mold, respectively (Monteiro, 1996). Thus, debris removal must be associated with other control methods to assure a satisfactory disease control.

When executed singly, biological control by *C. rosea* was the most effective method in reducing the production of conidiophores of *B. cinerea* in leaves and disease incidence in flowers and fruits and in increasing yield. Efficiency of disease control with our isolates was higher than with other isolates of *C. rosea* in Canada and in other Brazilian states (Peng and Sutton, 1991; Peng et al., 1992; Sutton et al., 1997; Valdebenito-Sanhuenza et al., 1997) as well as with other biocontrol agents such as *Ulocladium atrum* Preuss, *Bacillus lichenformis* Weigmann and *T. harzianum* (Boff et al., 2002; Kim et al., 2007; Kovach et al., 2000; Shafir et al., 2006). These results were similar to those obtained with biocontrol of gray mold with the four *C. rosea* isolates

applied separately (Cota et al., 2008b). This strengthens the viability of production of a commercial formulation based in a single isolate or a mixture of the four isolates. Biological control with our *C. rosea* isolates was independent of environmental conditions. Commonly, biocontrol agents are effective in controlling the disease only under marginally favorable environmental conditions (Boff et al., 2002; Shtienberg and Elad, 1997). Under low disease pressure (maximum incidence in the control treatment = 12.3%), *U. atrum* reduced the intensity of strawberry gray mold in four of seven field experiments conducted in The Netherlands (Boff et al., 2002). The efficiency of control achieved in our study, in which environmental conditions were conducive to gray mold epidemics, and the consistency of results in the two-year experiments are indicators of the chances of success of biological control under commercial crop conditions in Brazil.

Chemical control when executed singly was efficient in reducing the production of conidiophores of *B. cinerea* in leaves and disease incidence in flowers and fruits and in increasing yield, however less efficiently than biocontrol. However, chemical control hindered the biological control. The negative effect occurred due to the sensitivity of *C. rosea* to the applied fungicides (Macedo et al., 2006). Selection of fungicide-resistant isolates of the antagonist can increase the efficiency of biological control when associated with chemical control (Tarantino et al., 2007). A reduction in the number of fungicide sprays or the increase in the number of *C. rosea* applications should be evaluated, once control levels for antagonist alone was similar to those of the combination of biological control and fungicide. However, when comparing the results of the treatments $CR^-/DE^-/FS^+$ and $CR^+/DE^-/FS^+$, the efficiency of chemical control increased. This was expected because the fungicides are not effective in reduce pathogen sporulation in crop debris (Köhl and Fokkema, 1998; Morandi et al., 2003). Therefore, the application of *C. rosea* reduced the amount of inoculum produced and the sprays of fungicides protected leaves, flowers and fruits against infection by *B. cinerea*.

Using the integrated control approach enhanced the efficacy of the individual methods of disease control. Crop debris removal or application of *C. rosea* increased the efficacy of chemical control. Debris removal as well as biological control by *C. rosea* reduced leaf colonization by *B. cinerea*, which in turn decreased the amount of inoculum for infection of flowers and fruits. This strategy has been effective in reducing pathogen sporulation in crop debris (Köhl et al., 1995; Köhl and Fokkema, 1994; Köhl

and Fokkema, 1998; Morandi et al., 2003; Sutton and Peng, 1993). Therefore, it is likely to be successful if applied by strawberry growers.

The biological control by *C. rosea* is advantageous because it protects leaves and flowers from infection by *B. cinerea* and can reduce pathogen sporulation in crop debris. Consequently, application of *C. rosea* increased the efficiency of crop debris removal by reducing the availability of inoculum produced in an area. Another action to reduce this source of inoculum is the removal of crop debris, but this is a cumbersome and labor-intense practice. In our experiments, application of *C. rosea* was more efficient in reducing pathogen sporulation than removal of crop debris. The application of *C. rosea* does not contribute to dispersal of conidia normally associated with manual elimination of crop debris or during other cultural practices (Hausbeck and Pennypacker, 1991). Therefore the application of the antagonist should be preferred.

Reduced leaf colonization by *B. cinerea*, low gray mold incidence in flowers and fruits and higher increments in yields were recorded with $CR^{+}/DE^{+}/FS^{+}$. When the three methods were applied simultaneously, the negative effect of fungicide on the antagonist was not observed. Probably, removal of crop debris counterbalanced the negative effects of the fungicide on *C. rosea*. In our experiments, fungicide was applied weekly. However, it is possible to reduce the number of fungicide sprays or limit application during flowering peaks (Legard et al., 2005; Legard et al., 2001; Mertely et al., 2002). Integration of control methods was as effective as the application of *C. rosea* twice a week (Cota et al., 2008b). More frequent applications of the biocontrol agent may allow applying fungicide only under environmental conditions highly favorable to gray mold, in a management scheme similar to the adopted for greenhouse crops in Israel (Shtienberg and Elad, 1997). Studies to evaluate this approach are required in Brazilian conditions.

The integration of control methods is potentially valid to successfully manage strawberry gray mold. By integrating control methods, it is expected a reduction on the selection pressure towards fungicide resistance and, consequently, a decrease in the chance of selecting isolates resistant to fungicides. To reduce both environmental contamination and the amount of fungicides residues in fruits, it is needed to evaluate fungicide application intervals longer that the week interval we adopted in this study. Considering the methods we evaluated, biocontrol with *C. rosea* was the most important in defining the success of integrating control methods. Therefore, by increasing the

intervals between fungicide sprays, the conditions of *C. rosea* to be most effective is likely to be increased.

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Table 1 Standardized areas under the curves of: average number of *Botrytis cinerea* conidiophores (AUCANC), incidence of diseased flowers (AUCIflower), and yield (AUCYield); F values from analysis of variance and levels of significance (P). Area values were estimated for the treatment combinations: *C. rosea* (CR) weekly applied (+) or not (-), fungicide (FS) weekly sprayed (+) or not (-), and crop debris (DE) removal (+) or not (-). Fungicide treatments consisted of weekly sprays of procymidone alternated with captan. Data were pooled from experiments conducted in 2006 and 2007. For the three area values, the triple interaction effect was significant (P<0.0064).

Effect	Combination		AUCANC		F (P)	AUCIFlower		F (P)	AUCYield		F (P)
	CR	DE	FS^+	FS^-		FS^+	FS^-		FS^+	FS^-	
	-	-	30.61	94.64	1455.04 (<0.0001)	20.79	37.42	122.67 (<0.0001)	220.23	142.83	268.43 (<0.0001)
Fungicide	-	+	12.56	20.86	24.44 (<0.0001)	17.01	25.37	30.99 (<0.0001)	238.89	163.43	255.13 (<0.0001)
spray	+	-	7.47	12.26	8.16 (0.0063)	11.71	11.58	0.01 (0.9322)	251.52	244.81	2.02 (0.1616)
	+	+	3.28	4.88	0.91 (0.3443)	4.94	8.43	5.42 (0.0241)	282.03	250.35	44.95 (<0.0001)
	CR	FS	DE^+	DE^-		DE^+	DE^-		DE^+	DE^-	
	-	-	20.86	94.64	1931.44 (<0.0001)	25.37	37.42	64.42 (<0.0001)	163.43	142.83	19.02 (<0.0001)
Debris	-	+	12.56	30.61	115.51 (<0.0001)	17.01	20.79	6.34 (0.0151)	238.89	220.23	15.60 (0.0003)
removal	+	-	4.88	7.47	2.37 (0.1298)	8.43	11.58	4.39 (0.0413)	250.35	244.81	1.38 (0.2460)
	+	+	3.28	12.26	28.64 (<0.0001)	4.94	11.71	20.33 (<0.0001)	282.03	251.52	41.70 (<0.0001)
	DE	FS	CR^+	CR^-		CR^+	CR^-		CR^+	CR^-	
	-	-	7.47	94.64	2696.43 (<0.0001)	11.58	37.42	296.01 (<0.0001)	244.81	142.83	465.94 (<0.0001)
C. rosea	-	+	12.26	30.61	119.38 (<0.0001)	11.71	20.79	36.53 (<0.0001)	251.52	220.23	43.86 (<0.0001)
application	+	-	4.88	20.86	90.63 (<0.0001)	8.43	25.37	127.11 (<0.0001)	250.35	163.43	338.52 (<0.0001)
	+	+	3.28	12.56	30.59 (<0.0001)	4.94	17.01	64.56 (<0.0001)	282.03	238.89	465.94 (<0.0001)

Figures

Figure 1 Leaf surface colonized by *Clonostachys rosea* in strawberry leaves sampled weekly in experiments conducted in 2006 (A) and 2007 (B). Plantings were on April 13th and 18th, 2006 and 2007, respectively. Treatments consisted of application of *C. rosea* (CR +), crop debris removal (DE +) or not (DE -); and fungicide sprays, application of captan alternated weekly with procymidone (FS +) or no fungicide sprays (FS -). Vertical bars represent the standard error of the mean.

Figure 2 Average number of conidiophores of *Botrytis cinerea* in strawberry leaves sampled weekly during experiments conducted in 2006 (A, B) and 2007 (C, D). Plantings were on April 13th and 18th, 2006 and 2007, respectively. Treatments consisted of application (CR +) or not of *C. rosea* (CR -), removal (DE +) or no removal (DE -) of crop debris; and fungicide sprays, application of captan alternated weekly with procymidone (FS +) or no fungicide application (FS -). Vertical bars represent the standard error of the mean.

Figure 3 Gray mold incidence on strawberry flowers that were sampled weekly during experiments conducted in 2006 (A, B) and 2007 (C, D). Plantings were on April 13th and 18th, 2006 and 2007, respectively. Treatments consisted of application (CR +) or not of *C. rosea* (CR -), removal (DE +) or no removal (DE -) of crop debris; and fungicide sprays, application of captan alternated weekly with procymidone (FS +) or no fungicide application (FS -). Vertical bars represent the standard error of the mean.

Figure 4 Gray mold incidence on strawberry fruits that were sampled weekly during experiments conducted in 2006 (A, B) and 2007 (C, D). Plantings were on April 13th and 18th, 2006 and 2007, respectively. Treatments consisted of application (CR +) or not of *C. rosea* (CR -), removal (DE +) or no removal (DE -) of crop debris; and fungicide sprays, application of captan alternated weekly with procymidone (FS +) or no fungicide application (FS -). Vertical bars represent the standard error of the mean.

Figure 5 Yield of strawberry fruits harvested weekly during experiments conducted in 2006 (A, B) and 2007 (C, D). Plantings were on April 13th and 18th, 2006 and 2007, respectively. Treatments consisted of application (CR +) or not of *C. rosea* (CR -), removal (DE +) or no removal (DE -) of crop debris; and fungicide sprays, application of captan alternated weekly with procymidone (FS +) or no fungicide application (FS -). Vertical bars represent the standard error of the mean.

Figure 6 Climatic variables registered during the experiments conducted in 2006 (A, C) and 2007 (B, 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).

Figure 1

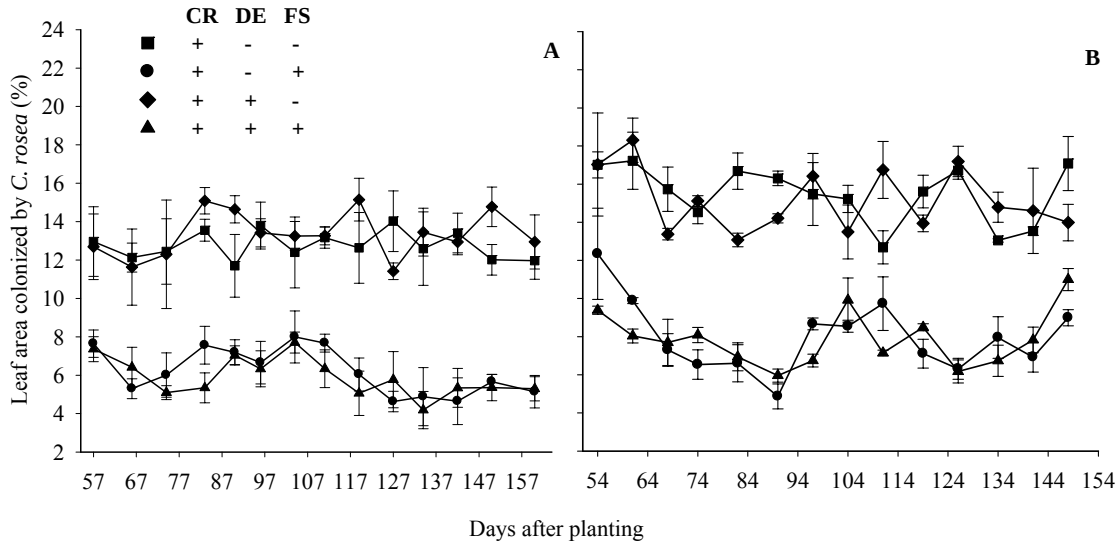

CR DE FS
+ - -
+ - +
+ + -
+ + +
A
B
Leaf area colonized by C. rosea (%)
Days after planting

Figure 2

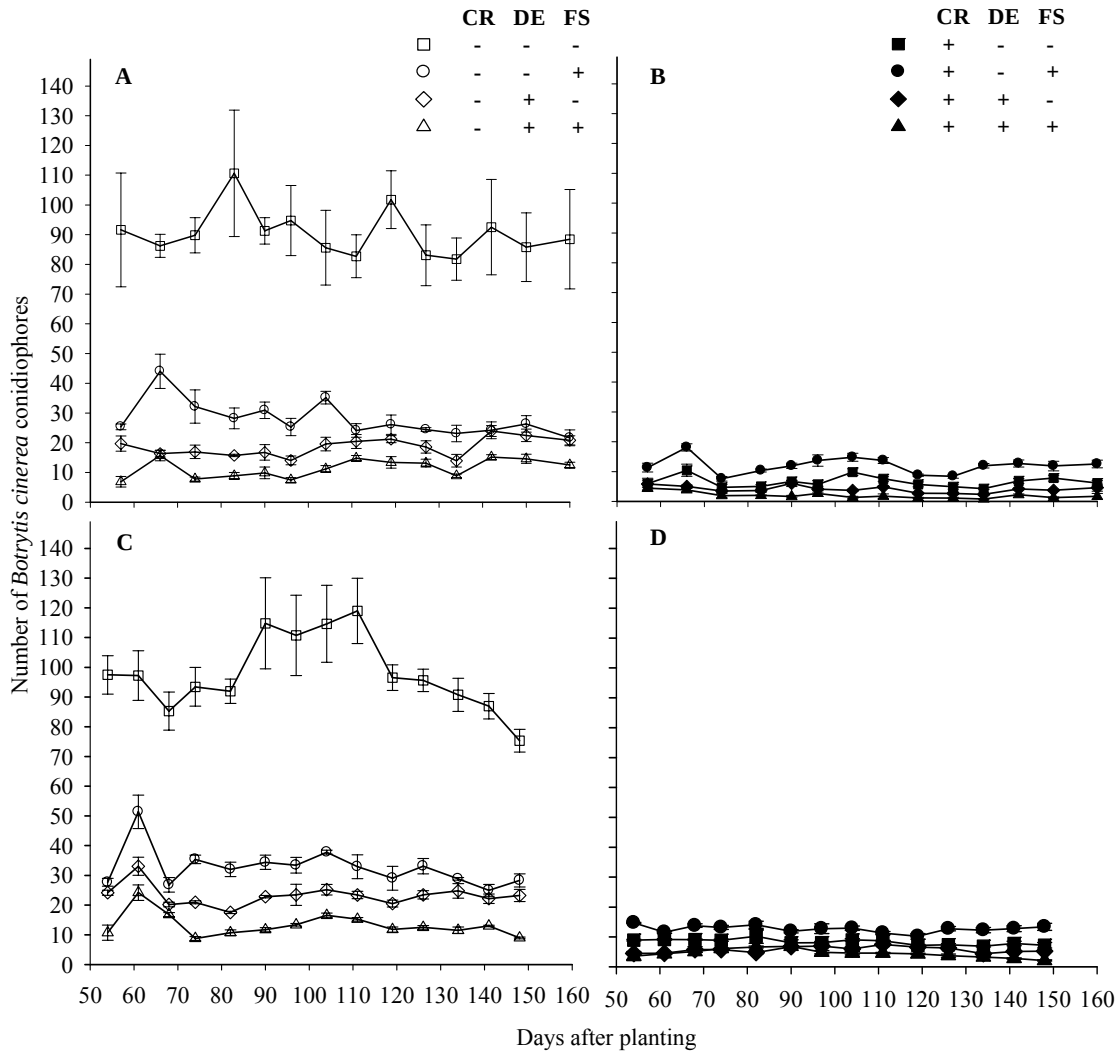

CR DE FS
A
B
C
D
Number of Botrytis cinerea conidiophores
Days after planting

Figure 3

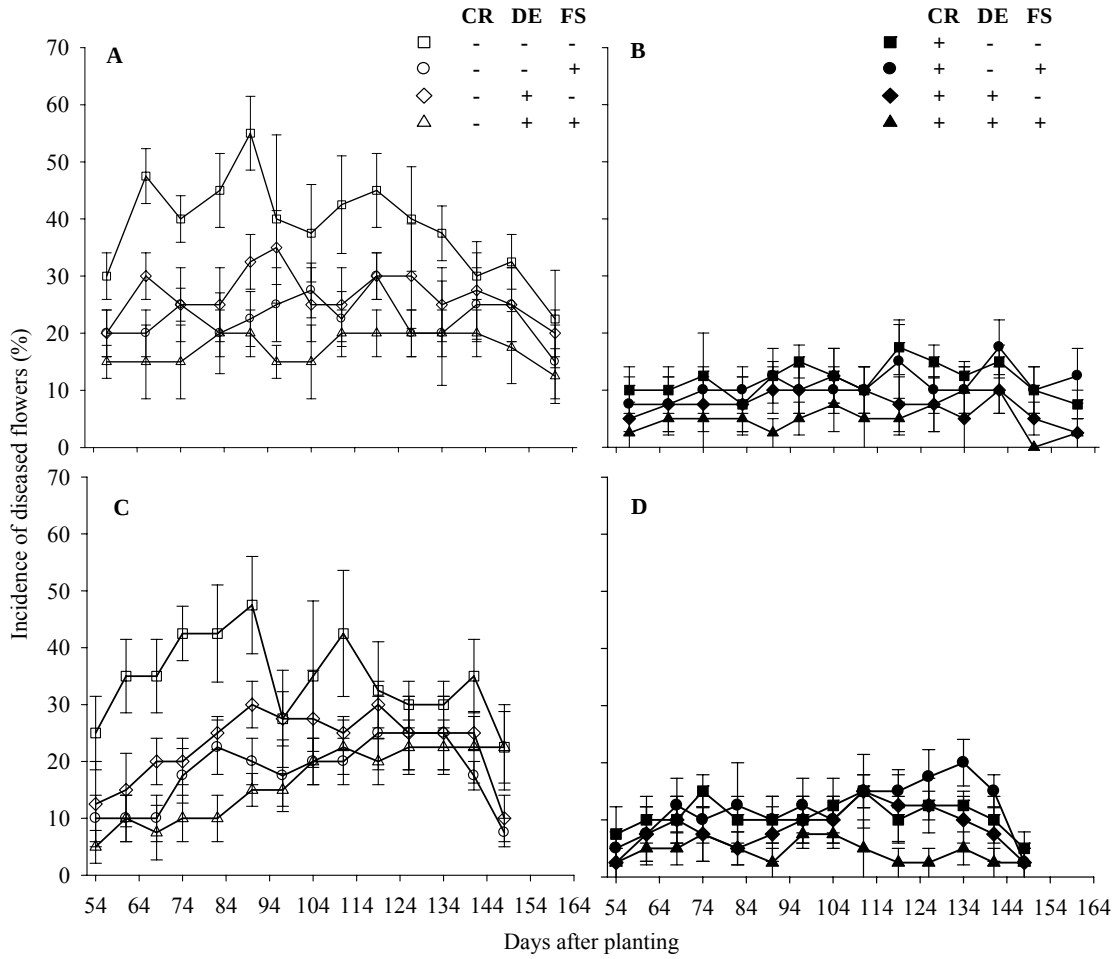

CR DE FS
A
B
C
D
Incidence of diseased flowers (%)
Days after planting
0 10 20 30 40 50 60 70
54 64 74 84 94 104 114 124 134 144 154 164

Figure 4

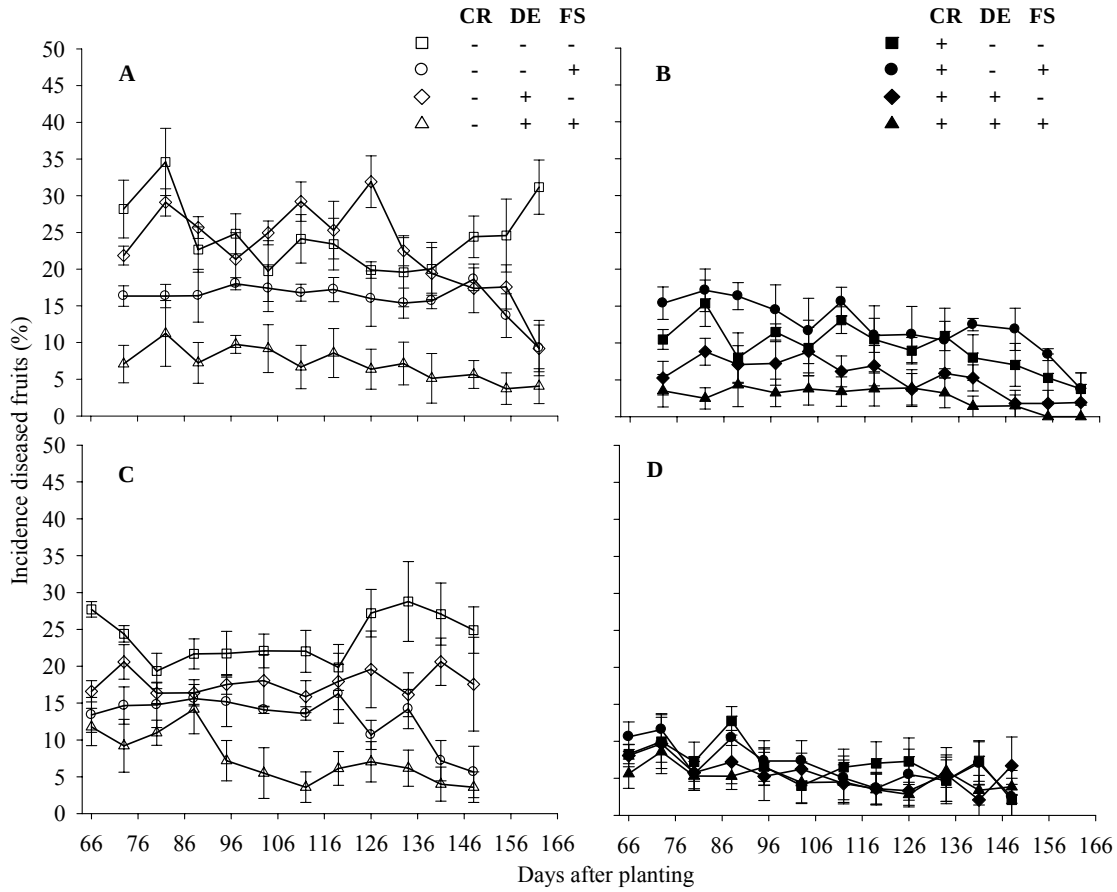

CR DE FS
A
B
C
D
Incidence diseased fruits (%)
Days after planting
0 5 10 15 20 25 30 35 40 45 50
66 76 86 96 106 116 126 136 146 156 166

Figure 5

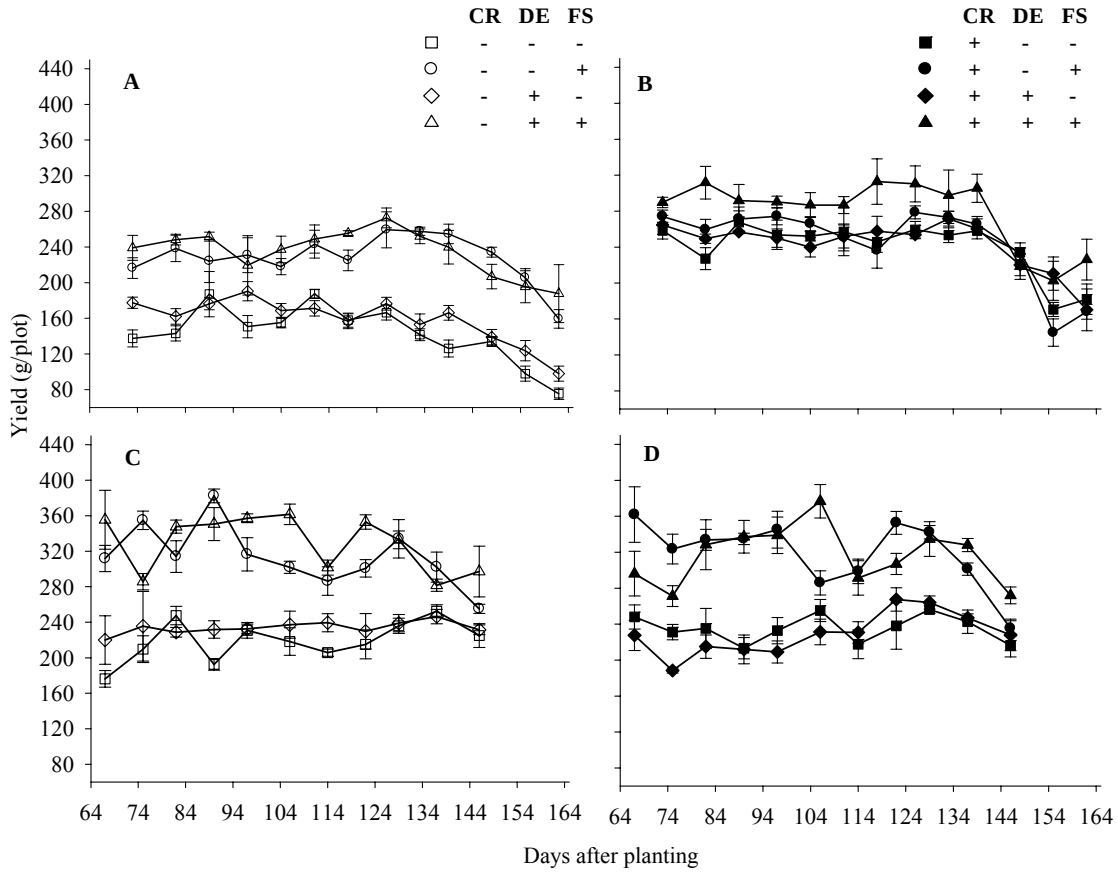
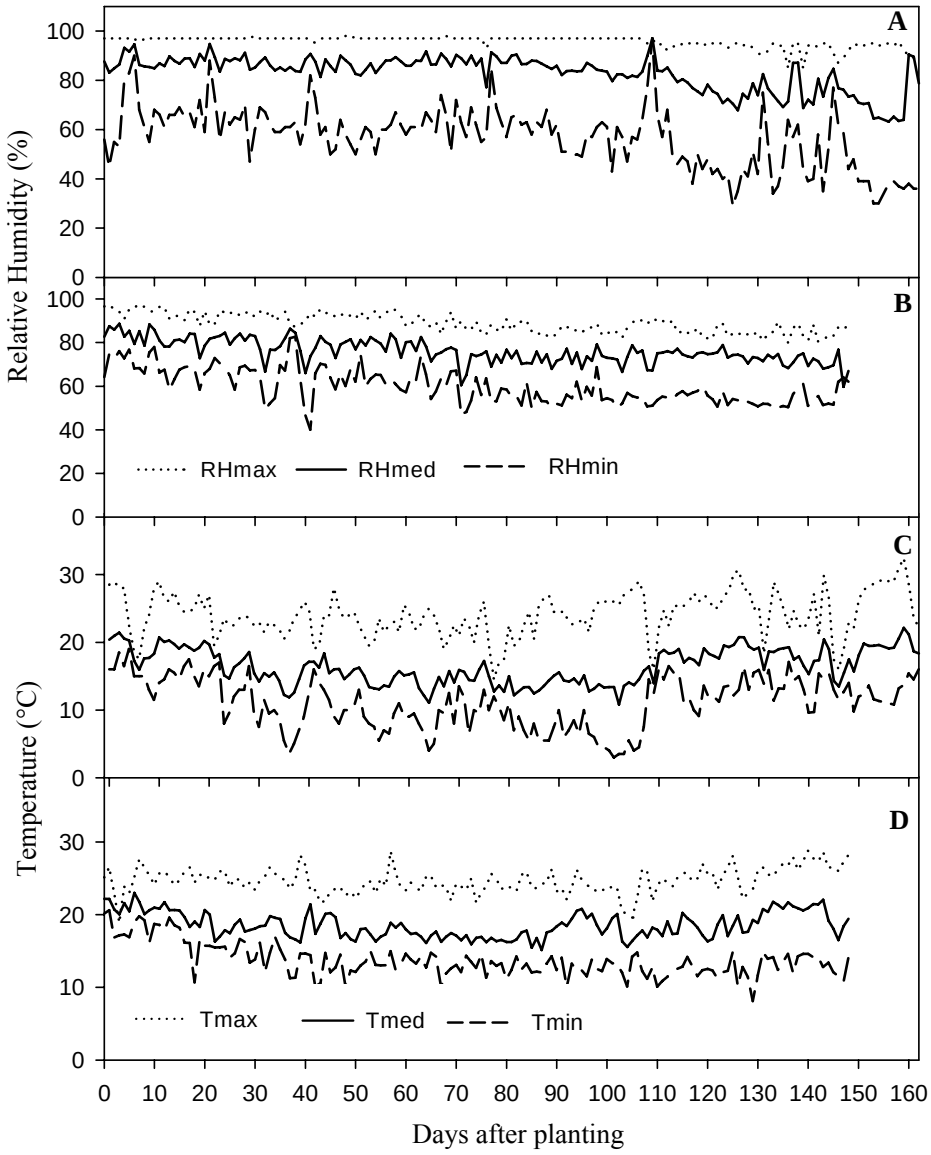


Figure 6


A
B
C
D
Relative Humidity (%)
Temperature (°C)
RHmax
RHmed
RHmin
Tmax
Tmed
Tmin
Days after planting

Dispersal gradients of *Botrytis cinerea* and *Clonostachys rosea* in a strawberry crop

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Abstract

Considering the importance of gray mold (*Botrytis cinerea*) to strawberry, we are evaluating pathogen biocontrol with *Clonostachys rosea* as a component of a disease management program. We studied dispersal gradients of both pathogen and antagonist under field conditions. For *C. rosea*, an inoculum source (wheat grains colonized and ground) was set in the center of 10 m-long experimental plots, and leaf colonization at several distances at both downwind and upwind directions was evaluated weekly. The maximum distances *C. rosea* reached were 45 and 105 cm, at upwind and downwind directions, respectively. For *B. cinerea*, the inoculum source (fruits and stems of strawberry fully colonized and with pathogen sporulation) was set in the center of 20 m-long experimental plots. Weekly, at several distances and at both directions, we evaluated leaf colonization and gray mold incidence in both flowers and fruits. At all evaluations, values of the three variables were higher close to the inoculum source. At the last evaluations (104 days after planting), diseased leaves, flowers and fruits were detected up to 975 cm from the inoculum source. Gray mold gradients in flowers and fruits were flat, but gradients in leaves were steep. Wind direction has no effect on *B. cinerea* dispersal. Crop debris was the main inoculum source for gray mold epidemics. *B. cinerea* was efficiently dispersed in the field, whereas *C. rosea* had low dispersal capacity on strawberry crops. Implications of these findings for disease management are discussed.

Keywords: Gray mold*; Fragaria x ananassa*; *Gliocladium roseum;* Biological control; management.

1 Introduction

Gray mold caused by *Botrytis cinerea* Pers.: Fr., teliomorph *Botryotinia fuckeliana* (de Bary) Whetzel is a disease widely spread throughout the world. The pathogen can infect plant species included in more than 200 genera, in temperate and subtropical regions as in Brazil (Elad et al., 2004; Jarvis, 1992). The disease is one of the most damaging to strawberry crop (Blanco et al., 2006; Mertely et al., 2000; Valdebenito-Sanhuenza et al., 1997; Williamson et al., 2007).

Survival strategies of *B. cinerea* can involve production of sclerotia or saprophytic development in crop debris. In strawberry crop debris in Ontario, Canada, sclerotia were produced in less than 7% of the samples analyzed, whereas sporulation in plant material was detected in more than 99% of the samples (Braun and Sutton, 1987). The authors detected differences in conidia production according to plant organs; sporulation was higher on leaf blade than on petioles. In Spain and Israel, no sclerotia were produced in debris of tomato, cucurbit, bean and cucumber crops grown in greenhouses, and the fungus survived as mycelium in decomposing plant materials (Raposo et al., 2001; Elad et al., 1992). *B. cinerea* sporulates profusely on crop debris: in strawberry, about 10^5 to 10^7 conidia are produced by cm^2 of leaf tissue (Sosa-Alvarez et al., 1995). Although *B. cinerea* can sporulate on crop debris, the effectiveness of this inoculum source to epidemics under field conditions is variable. In a perennial crop system of strawberry, debris is the main inoculum source (Braun and Sutton, 1988; Sutton, 1990), whereas in an annual system conidia formed in other areas and wind dispersed are most important (Boff et al., 2001). In Brazilian conditions, the contribution of strawberry crop debris for epidemics development is unknown. This information is important for control strategies: if debris are important inoculum sources, then their removal or suppression of pathogen sporulation on them could be a viable management practice (Köhl et al., 1995; Morandi et al., 2003). Although debris removal can be efficient, it is a labor intense and costly opperation. Thus, an interesting alternative would be the suppression of pathogen sporulation on the debris. Suppressing pathogen sporulation without fungicide application is highly recommended and can be achieved through biological control.

The fungus *Clonostachys rosea* (Link: Fr.) Schroers, Samuels, Siefert & Gams (sin. *Gliocladium roseum* Bainier) can compete with *B. cinerea* and inhibit its sporulation in different host parts, through hyperparasitism and nutrient competition in

colonizing senescing and dead tissues (Sutton et al., 1997; Yu & Sutton, 1997; Köhl & Fokkema, 1998; Morandi et al. 2001). Biocontrol of *B. cinerea* by *C. rosea* has been successful because of the capacity of both establishment and competition of the antagonist. In rose plants, *C. rosea* survived for one month in crop debris that were kept from 10 to 30^{o}C and was not affected by absence of leaf wetness for up to 48 h after being applied (Morandi et al., 2001). It also established and suppressed pathogen sporulation regardless the stage of tissue development (Morandi et al., 2000). In greenhouse experiments, *C. rosea* suppressed sporulation of *B. cinerea* on crop debris, even under favorable conditions to the pathogen (Morandi et al., 2003; Morandi et al., 2006). *C. rosea* colonized strawberry leaf tissues without leaf wetness or under optimum temperature to the pathogen, and efficiently suppressed *B. cinerea* sporulation when applied up to 14 days before or after pathogen inoculation (Cota et al., 2008a; Cota et al., 2007). Under field experiments to manage strawberry gray mold, *C. rosea* when applied once or twice a week was as efficient as or more efficient than fungicides, respectively (Cota et al., 2008b). *C. rosea* can establish in green leaves, which is an important competitive advantage for the antagonist (Köhl and Fokkema, 1998; Morandi et al., 2001; Sutton et al., 1997; Yu and Sutton, 1997). The antagonist established in tomato stems and suppressed *B. cinerea* sporulation after 11 weeks of application (Sutton *et al.,* 2002).

The dispersal ability of *C. rosea* was still not elucidated. The antagonist was detected in untreated plots, which suggests that it can be dispersed after being established (Morandi et al., 2003; Sutton et al., 2002). It would be interesting to explore this ability, if it exists. However, the dynamics of *C. rosea* dispersal under field conditions is unknown. Also many basic aspects of gray mold epidemics on strawberry in Brazilian conditions are unknown. Therefore, we assessed the contribution of crop debris to the development of gray mold epidemics in strawberry, by evaluating dispersal gradients of *B. cinerea* from these inoculum sources. We also evaluated dispersal gradient of *C. rosea*, to generate knowledge for establishing pathogen biocontrol under field conditions

2 Materials and Methods

Two experiments aimed to study dispersal gradients of either *B. cinerea* or *C. rosea* were conducted in the experimental area of the Departamento de Fitopatologia, Universidade Federal de Viçosa (20°44'47''S and 42°50'55''W). For each fungus three plots were established (Figure 1). At about 40 m south of both experiments, there were two other experiments dealing with management of *B. cinerea*, and strawberry was not grown in the remaining experimental area. In each experiment, transplants of 'Camarosa' were planted spaced 0.3 x 0.3 m on beds on 13 April 2006. After planted and at 2-day-intervals, plants were sprinkler irrigated for 90 min each time. At 15 days after planting (dap), dead parts and senescing leaves of all plants were removed, and each plant was fertilized with 15 g of NPK (4-14-8). Fungicides sprays were at 20 and 34 dap with procymidone (Sialex 500; 0.5 g a.i. L-1) and at 27 and 41 dap with captan (Orthocide 500; 1.2 g a.i. L-1), to preventively control gray mold, and at 30 dap with tebuconazole (Folicur 200 EC; 0.375 mL a.i. L-1), to control leaf spots. At 43 dap, beds were covered with black plastic film (25 μm-thick) as mulching.

2.1 Dispersal gradient of *Botrytis cinerea*

Isolate MoB1 of *B. cinerea*, aggressive to strawberry (Nobre, 2003), was grown in potato dextrose agar medium (PDA), at 20^{o}C, 12 h photoperiod. Conidia from 10 to 12-day old colonies were suspended in water + Tween 20 (0.05% v/v), filtered through two layers of cheesecloth and adjusted to a concentration of $1x10^{5}$ conidia mL^{-1}. The suspension was sprayed on strawberry fruits and stems pieces (5 cm length) set above wet paper towel in plastic boxes (11 cm width x 11 cm length x 3.5 cm depth). The boxes were covered with a lid, incubated at 20^{o} C 12 h photoperiod for 15 days, when pathogen had profusely sporulated. One box (with approximately 20 fruits and 15 stems) was set in the center of each plot, at 54 dap. Inoculum loss was avoided by closing the boxes before the sprinklers were turned on. One day before setting the boxes, one leaflet and one flower/plant/plot were sampled to detect *B. cinerea* infections. This sample scheme continued at week intervals, after setting the inoculum sources until 104 dap.

From each leaflet, five 1-cm diameter disks were cut and plated on containing paraquat-chloramphenicol-agar medium (PCA) in 9-cm Petri dishes (Peng and Sutton, 1991). Each dish was divided into four sectors, and each sector received the five disks

from one plant. The dishes were kept at 20° C, 12 h photoperiod. After 10 to 12 days, intensity of tissue colonization by *B. cinerea* was assessed through the scale: 0=0; 1=1-12; 2=13-24; 3=25-48; 4=49-100; 5=101-200; 6=201-300; 7=301-400 conidiophores/ leaf disc (Peng and Sutton, 1991). For statistical analysis, values of each class were transformed to the average value of the corresponding percentage of conidiophores range. For instance, if the disc was rated as 3, its corresponding average value was 36.5 conidiophores. For each plant, the mean number of conidiophores was the average of the five leaf discs.

To evaluate the gray mold incidence on flowers (open flowers with all petals and sepals), they were plated on PCA. Each Petri dish was divided into four sectors, each sector receiving one flower from one plant. The dishes were kept at 20°C, 12 h photoperiod for 5 days, when incidence of diseased flowers was assessed.

Harvest started at 83 dap and was repeated at 7-days intervals. Each time, incidence of diseased fruits per plant (number of diseased fruits with pathogen sporulation /total number of fruits harvested) was assessed at the moment of harvest.

2.2 Dispersal gradient of *Clonostachys rosea*

The inoculum was constituted by wheat grains completely colonized by and with high sporulation of *C. rosea* (10^8 conidia/g substrate) (Morandi et al., 2003). A plastic box (11 cm width x 11 cm length x 3.5 cm depth) with a mixture of 15 g of inoculum of each of the four isolates obtained by Nobre et al. (2005) was set in the center of each plot at 53 dap. One day before setting the boxes, one leaflet/plant/plot was sampled to detect *C. rosea* colonization. This sample scheme continued at weekly intervals, after setting the inoculum sources, until 89 dap.

To detect *C. rosea*, the same procedures adopted for *B. cinerea* were followed, except that incubation was at 25°C. After 12 days, intensity of tissue colonization by *C. rosea* was assessed through the scale: 0= 0; 1= 0-3; 2= 3-6; 3= 6-12; 4= 12-25; 5= 25-37; 6 = 37-50, and 7 = more than 50% of disc area colonized (Nobre et al., 2005). For statistical analysis, values of each class were transformed to the average value of the corresponding percentage of colonized area. For instance, if a disc was rated as 1, its corresponding average value was 1.5%. Data for each plant corresponded to the average of the disc area colonized by *C. rosea* from the five leaf discs.

2.3 Data analysis

The trends of dispersal at the three plots, either of *B. cinerea* or *C. rosea*, were similar and data of the three were pooled for statistical analysis. The following variables were calculated as proportions relative to the plants closest (15 cm distance) to the respective inoculum source: average number of *B. cinerea* conidiophores (PNMC), diseased flowers (PFD), diseased fruits (PFrD) and leaf area colonized by *C. rosea* (PAFC). For the variable PFD, we calculated the incidence of diseased flowers in sampling units (quadrats 2x2), and the midpoints in distances of these quadrats were used in the analyses. Variables PNMC, PFD, PFrD and PAFC (y) were non-linearly regressed on distance in cm (d) from the source with Proc Nlin of SAS (SAS v. 9.1). The exponential (Kiyosawa and Shiyomi, 1972), in which $y = a*\exp(-b*d)$, and power law (Gregory, 1968), in which $y = a*d^{-b}$, models were fit to the data. In each model, "a" is related to source strength and "b" is the gradient slope. The decision regarding the model that best fitted the data was based on error mean square, significance of parameters, normality in errors distribution and on the pattern of distribution of the residuals (Madden et al., 2007). To compare downwind to upwind gradients at each evaluation in time, the confidence interval for b was calculated through the equation: $IC = (b_d - b_u) \pm ep\ t\ (p/2; n_d + n_u - 4)$, where b_d and b_u= gradient slopes downwind and upwind, respectively; ep= standard error of the difference of the two parameters, t= t statistics; p= probability at 95% confidence; n_d and n_u= degrees of freedom associated to b_d and b_u, respectively. Based on the exponential model, the distance in which PNMC, PFD, PFrD and PAFC were reduced by 50% (D_{50}) was calculated: D_{50}= [(ln(a) + 0.693)/b] (Fitt et al., 1987).

3 Results

The exponential model best fitted the data for the dispersal gradients of both fungi, at both wind directions and at all evaluation times.

3.1 Dispersal gradient of *Botrytis cinerea* in leaves

. Disregarding direction, in the first assessment date, *B. cinerea* was detected only on leaves located next to the inoculum source (Figures 2 and 3). At 68 dap, *B.*

cinerea was detected until 135 and 165 cm upwind and downwind, respectively. At 104 dap, it was detected at 765 and 975 cm from the source upwind and downwind, respectively. Although *B. cinerea* has been detected in almost all distances, at the last two evaluations largest PNMC values were recorded in the plants next to the inoculum source (Figures 2 and 3). D_{50} values were higher in the downwind direction, and increased throughout evaluations (Figures 2 and 3). According to the confidence interval, the b parameter was larger downwind than upwind, in all evaluations, except on 61 on 90 dap. In both directions, the gradients had no trend of flattening (Figures 2 and 3).

3.2 Dispersal gradient of *Botrytis cinerea* in flowers

Upwind, diseased flowers were detected up to 195 cm from inoculum source in the first evaluation and, from the fourth evaluation on diseased flowers were found at all distances from the inoculum source (Figure 4). Incidence of diseased flowers tended to increase from 25.25% (76 dap) to 65.40% in the last evaluation (104 dap). D_{50} increased as evaluations proceeded (Figure 4).

Downwind, diseased flowers were detected up to 135 cm from the inoculum source in the first and second. After 76 dap diseased flowers were detected up to 975 cm. Incidence of diseased flowers tended to increase from 31.86% (at 76 dap) to 63.13% in the last evaluation (104 dap). At both directions, D_{50} values increased throughout the evaluations (Figure 5). The b parameter did not differ between downwind and upwind at all evaluations, except at 76 dap.

3.3 Dispersal gradient of *Botrytis cinerea* in fruits

Upwind, diseased fruits were detected up to 315 cm in the first evaluations and up to 975 cm in the last evaluations (Figure 6). Downwind, diseased fruits were detected up 435 cm at 83 and 90 dap. At the last evaluations diseased fruits were detected up to 975 cm (Figure 6). At both directions, D_{50} values increased throughout the evaluations (Figure 6). The b parameter was larger downwind than upwind in 83 and 90 dap, and did not differ at 97 and 104 dap.

3.4 Dispersal gradient of *Clonostachys rosea*

The prevailing wind direction positively affected leaf colonization by *C. rosea* (Figure 7). Maximum distance from the source in which *C. rosea* was detected was 45 and 105 cm, upwind and downwind, respectively (Figure 7). At all evaluations, D_{50} values were largest at downwind direction, in which D_{50} tended to increase throughout evaluations (Figure 7). Based on the confidence interval of the b parameter, the gradient was flatter in the downwind at all evaluations.

4 Discussion

To proper manage diseases, the inoculum sources of pathogens must be known. No studies were conducted regarding dispersal of *B. cinerea* on strawberry under field conditions in Brazil. *B. cinerea* sporulates profusely on diseased strawberry fruits and stems, where it can survive more than 4 months (unpublished data). Therefore, these plant parts were "potential" inoculum sources. In our gradient study, we found that under field conditions they are "effective" inoculum sources, as gray mold progressed in time and space from them.

Gradients of *B. cinerea* dispersal in flowers and fruits flattened in time, but not in leaves. This is probably because flowers and fruits are more susceptible to *B. cinerea* than leaves that are infected only when young (Braun and Sutton, 1988; Mertely et al., 2002; Sutton, 1990). Flower infections are important for disease to develop in fruits, as *B. cinerea* penetrates petals, stamens and pistil and follows colonizing through the fruits (Bristow et al., 1986; Mertely et al., 2002; Powelson, 1960). Therefore, high disease incidence in flowers results in high incidence in fruits. As fruits were harvested only once a week, a high number of conidia were produced, originating the secondary cycles of the disease. A similar pattern of *B. cinerea* dispersal was found on bean (*Phaseolus vulgaris* L.) plots, and flattening of gradient was observed at harvest time when the amount of inoculum increased (Johnson and Powelson, 1983). In our strawberry experiment, *B.cinerea* dispersed farther from the source (until 720cm) than in the bean experiment (300 cm) (Johnson and Powelson, 1983). Although no quantitative comparison can be made because host plants, inoculum sources and environmental conditions were different between experiments, the point that should be stressed is the dispersal ability of *B. cinerea*.

Wind dispersal of the antagonist, *C. rosea*, was not as efficient as the pathogen, *B. cinerea*. The antagonist was found just 45 cm from the source, even at 5 weeks after the inoculum source was introduced. The low efficiency of the wind in removing conidia from the inoculum source was probably due to the way the source was set over plant bed. The plants were about 30 cm high when the experiment started and the leaves restricted wind movement over conidia. This effect could be minimized by keeping the sources at the same height of leaves and/or using a source with finer granulometry. We used wheat colonized grains that were ground and sieved in 1 mm mesh. Probably with the powder formulation (mixture of talc plus conidia) used for bee dispersal (Peng et al., 1992; Sutton et al., 1997) the conidia would be more easily removed by wind. Additionally, no rainfall occurred throughout the experiment and for sprinkler-irrigation we closed the boxes, to avoid loss of inoculum. The conidia probably would be dispersed farther with the combined action of wind and water drops. Nevertheless, as discussed below, secondary cycles of *C. rosea*, if they have occured, probably lasted longer and were in lower number than of *B. cinerea*.

The low ability of dispersal/multiplication of *C. rosea* in the field must be compensated by the antagonist be successful as biocontrol agent. Therefore, it is expected that more sprays of *C. rosea* propagules would be required for an efficient control of *B. cinerea*. We found that two weekly sprays were more efficient than one to control strawberry gray mold in leaves, flowers and fruits (Cota et al., 2008b). Bees are an alternative way to deliver the antagonist to strawberry flowers. Bees were shown to be efficient dispersal agents of *C. rosea* (Peng et al., 1992; Sutton et al., 1997) and *Trichoderma harzianum* Rifai (Kovach et al., 2000; Shafir et al., 2006). Under field conditions, bees dispersed conidia of *T. harzianum* up to 200 m from the source (Shafir et al., 2006). As paradoxical as it may sound, the pathogen is also an alternative to disperse the biocontrol agents, as observed with yeasts (Cook, 2002a;b). As *B. cinerea* conidia have about 10 x 8.5 µm and *C. rosea* 8.1 x 2.7 µm (Schroers, et al., 1999; Tenberge, 2004) this alternative is feasible and should be considered in gray mold management, by applying *C. rosea* on infested crop debris where *B. cinerea* sporulates profusely. As *C. rosea* is an hyperparasite, conidia might be attached to *B. cinerea* conidia.

When comparing dispersal gradients of *C. rosea* and *B. cinerea*, the pathogen dispersed farther than the antagonist. Conidia of *B. cinerea* are released from conidiophores by a hygroscopic mechanism. When relative humidity decreases and

temperature increases, the conidia are released and thus are more easily transported by wind gusts (Chastagner et al., 1978). The secondary cycles of *B. cinerea*, that probably flattens its dispersal gradient, favors the gradual pathogen dispersal to farther distances than *C. rosea*. As we did not observe *C. rosea* sporulating in strawberry plants in the field, we assumed that no secondary cycles of the antagonist occurred. Probably *C. rosea* established endophytically in strawberry tissues (Morandi et al., 2001; Sutton et al., 1997) and started sporulating only in the senescing/dead tissues. Solar radiation may have restricted the establishment of *C. rosea*, because conidia germination and rose leaf colonization reduced as the time of exposure of leaves to sunshine increased (Morandi et al., 2008). However, the authors found that the ability of suppressing pathogen sporulation was not affected. Thus we expect that even with the levels of leaf area colonized by *C. rosea* we got, the antagonist can compete efficiently with the pathogen.

Fruits and stems debris are important inoculum sources for gray mold epidemics under field conditions. At the first assessment date, *B. cinerea* was detected only in the plants next to the inoculum source and, even at the last evaluations, highest values of numbers of conidiophores on leaves, incidence of both diseased fruits and flowers, were found next to the source. These results are similar to those found with gray mold epidemics in strawberries (Braun and Sutton, 1988; Sutton, 1990) and greenhouse-grown roses (Monteiro, 1996). Therefore, diseased fruits and crop debris must be removed to manage gray mold. The removal applied solely has no significant effect in reducing the disease in fruits (Mertely et al., 2000; Chap. 2), but when associated with sprays of *C. rosea* and/or fungicides disease intensity in flowers and fruits was significantly reduced (Chap. 2). As wind dispersal of *C. rosea* is not efficient and the fungus does not sporulate in green tissues, plant organs, mostly flowers, should be always treated with the antagonist either by sprays or by vectors to assure a continuous protection against *B. cinerea*.

Acknowledgments

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Figures

Figure 1 Schematic layout of the field experimental plots to study dispersal gradients of *Botrytis cinerea* and *Clonostachys rosea* in strawberry plants.

Figure 2 Dispersal gradient of *Botrytis cinerea* in strawberry leaves sampled at the upwind direction. The proportion of the average number of conidiophores was calculated relative to the largest values of average number of conidiophores in plants at 15 cm from the inoculum source. D_{50} = estimated distance in which the proportion of average number of conidiophores was reduced in 50%.

Figure 3 Dispersal gradient of *Botrytis cinerea* in strawberry leaves sampled at the downwind direction. The proportion of the average number of conidiophores was calculated relative to the largest values of average number of conidiophores in plants at 15 cm from the inoculum source. D_{50} = estimated distance in which the proportion of average number of conidiophores was reduced in 50%.

Figure 4 Dispersal gradient of *Botrytis cinerea* in strawberry flowers sampled at the upwind direction. The proportion of diseased flowers was calculated relative to the largest values of diseased flowers in plants at 15 cm from the inoculum source. D_{50} = estimated distance in which the proportion of diseased flowers was reduced in 50%.

Figure 5 Dispersal gradient of *Botrytis cinerea* in strawberry flowers sampled at the downwind direction. The proportion of diseased flowers was calculated relative to the largest values of diseased flowers in plants at 15 cm from the inoculum source. D_{50} = estimated distance in which the proportion of diseased flowers was reduced in 50%.

Figure 6 Dispersal gradient of *Botrytis cinerea* in strawberry fruits sampled at both downwind and upwind directions. The proportion of diseased fruits was calculated relative to the largest values of diseased fruits in plants at 15 cm from the inoculum source. D_{50} = estimated distance in which the proportion of diseased fruits was reduced in 50%.

Figure 7 Dispersal gradient of *Clonostachys rosea* in strawberry leaves at both downwind and upwind directions. The proportion of leaf area colonized was calculated relative to the largest values of leaf area colonized in plants at 15 cm from the inoculum source. D_{50} = estimated distance in which the proportion of leaf area colonized was reduced in 50%.

Figure 1

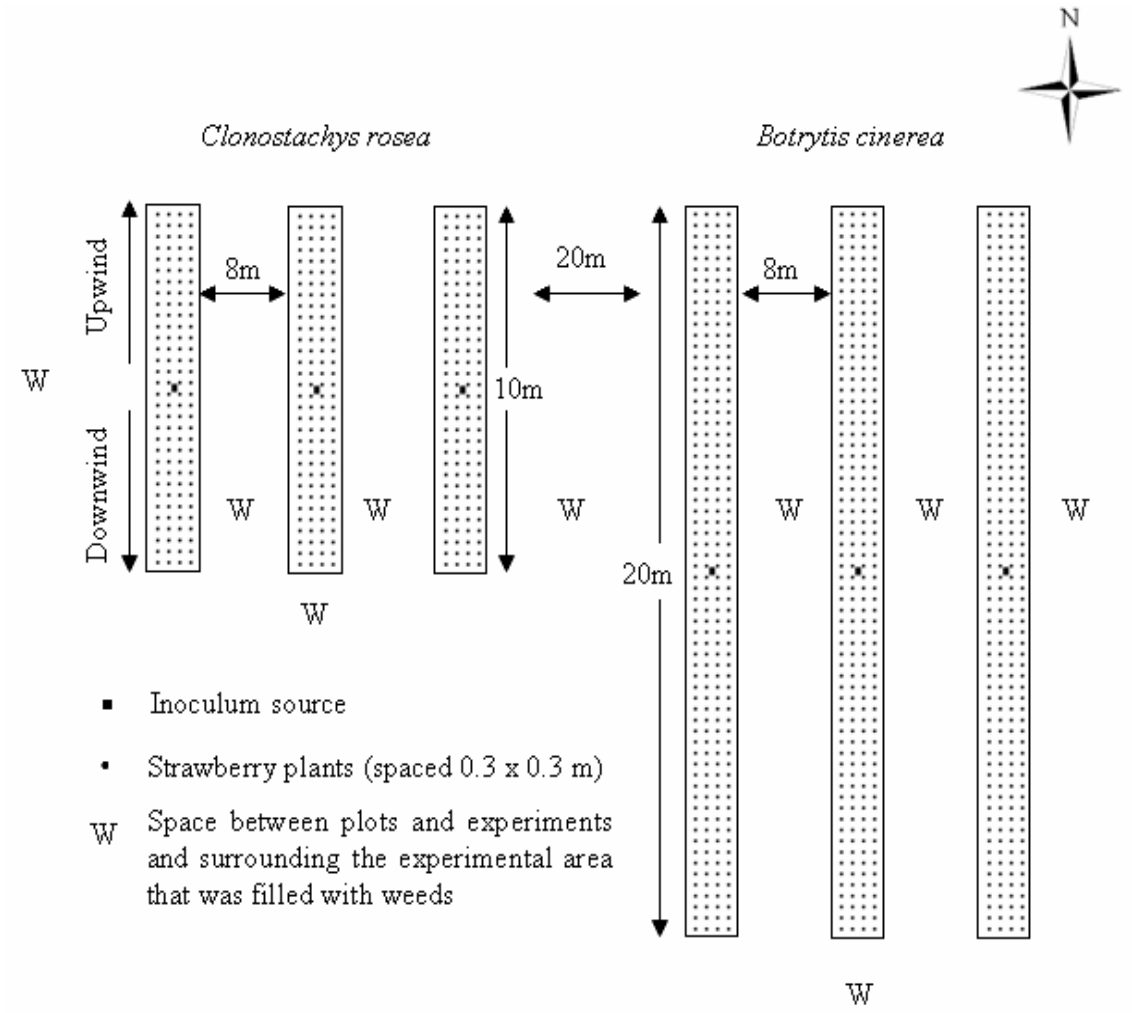

N
Clonostachys rosea
Botrytis cinerea
Upwind
Downwind
8m
20m
10m
W
Inoculum source
Strawberry plants (spaced 0.3 x 0.3 m)
Space between plots and experiments and surrounding the experimental area that was filled with weeds

Figure 2

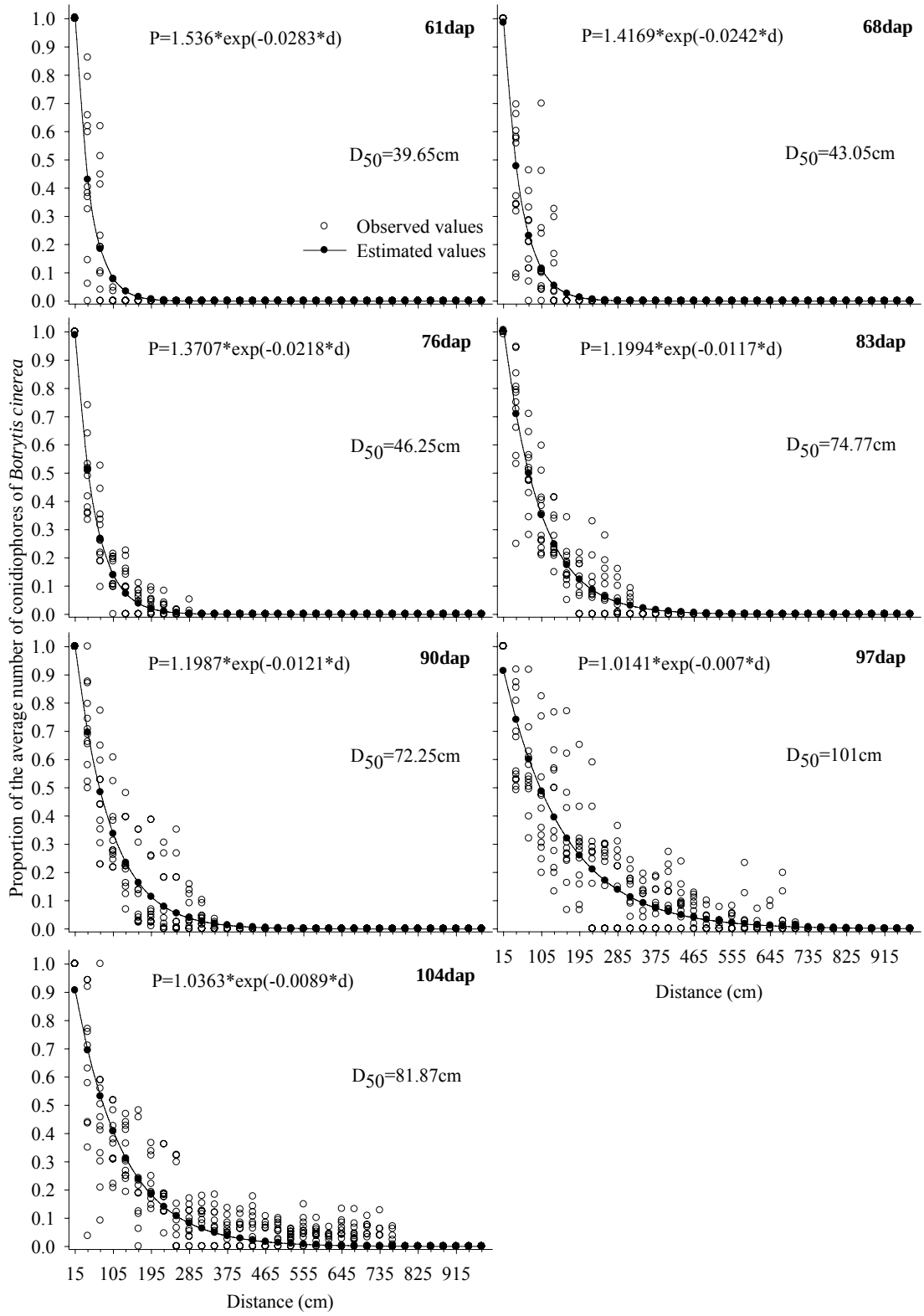

61dap
P=1.536*exp(-0.0283*d)
D50=39.65cm
68dap
P=1.4169*exp(-0.0242*d)
D50=43.05cm
76dap
P=1.3707*exp(-0.0218*d)
D50=46.25cm
83dap
P=1.1994*exp(-0.0117*d)
D50=74.77cm
90dap
P=1.1987*exp(-0.0121*d)
D50=72.25cm
97dap
P=1.0141*exp(-0.007*d)
D50=101cm
104dap
P=1.0363*exp(-0.0089*d)
D50=81.87cm
Observed values
Estimated values
Proportion of the average number of conidiophores of Botrytis cinerea
Distance (cm)
15 105 195 285 375 465 555 645 735 825 915

Figure 3

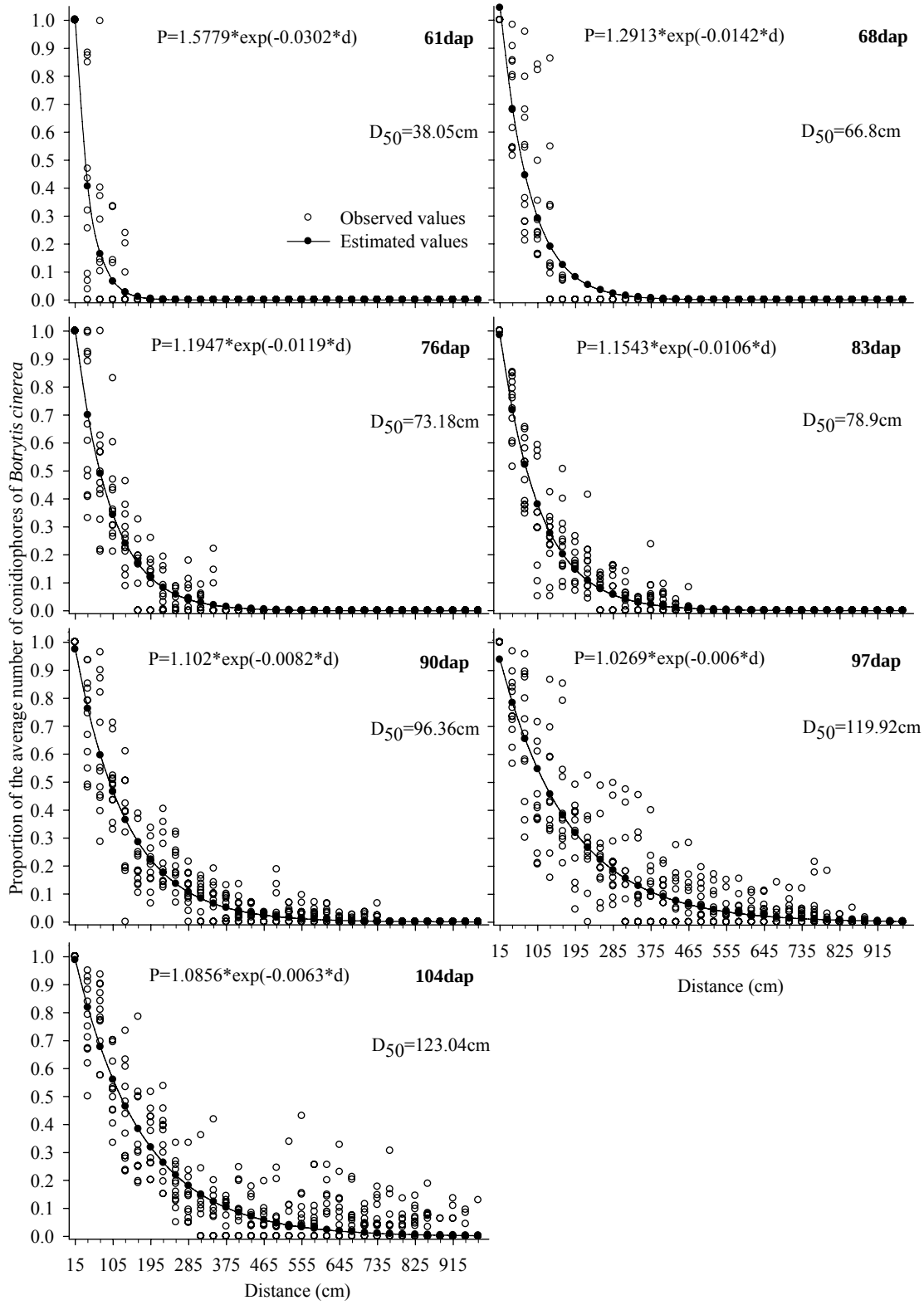

P=1.5779*exp(-0.0302*d)
61dap
D50=38.05cm
P=1.2913*exp(-0.0142*d)
68dap
D50=66.8cm
Observed values
Estimated values
P=1.1947*exp(-0.0119*d)
76dap
D50=73.18cm
P=1.1543*exp(-0.0106*d)
83dap
D50=78.9cm
P=1.102*exp(-0.0082*d)
90dap
D50=96.36cm
P=1.0269*exp(-0.006*d)
97dap
D50=119.92cm
P=1.0856*exp(-0.0063*d)
104dap
D50=123.04cm
Proportion of the average number of conidiophores of Botrytis cinerea
Distance (cm)
15 105 195 285 375 465 555 645 735 825 915

Figure 4

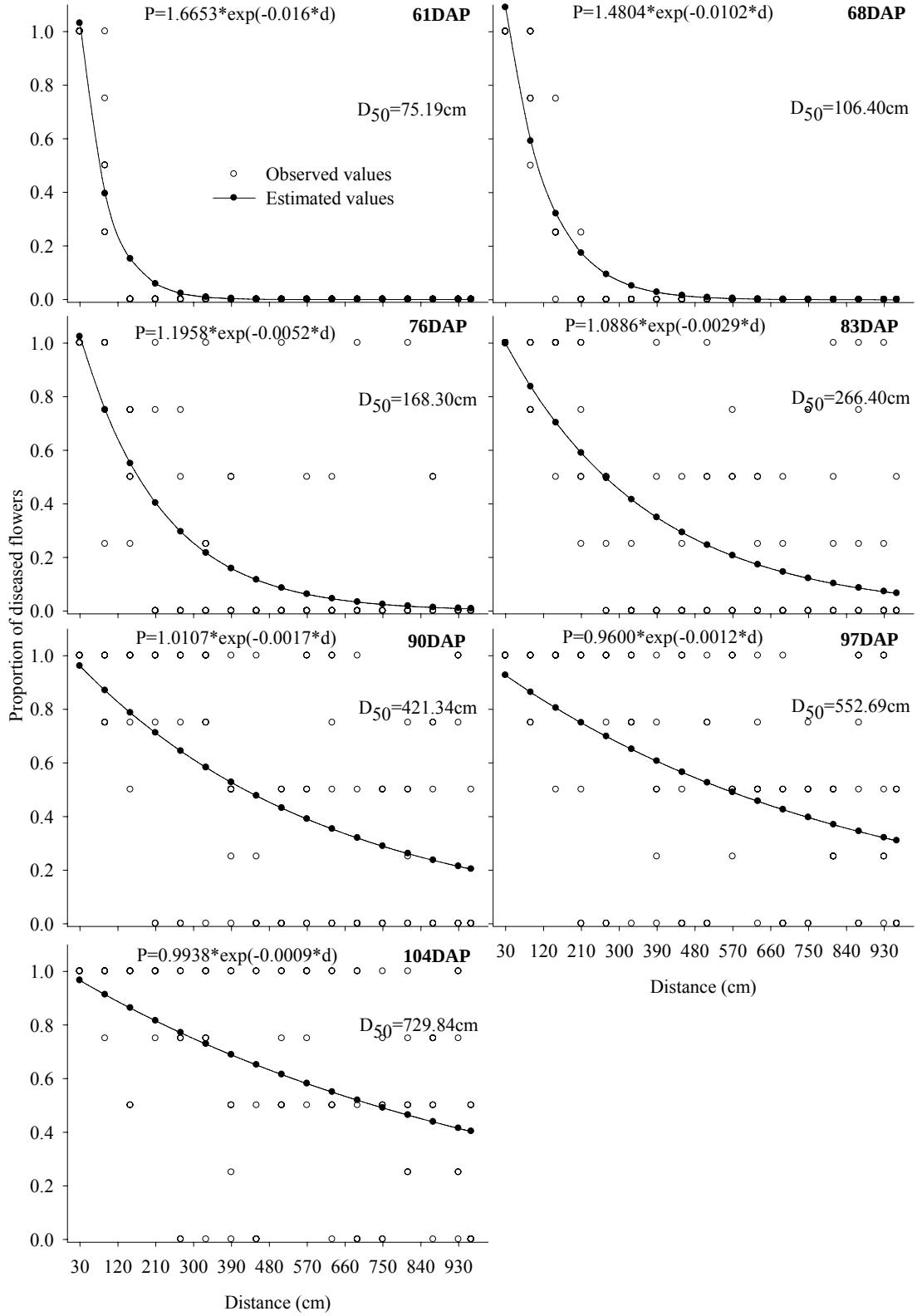

P=1.6653*exp(-0.016*d)
61DAP
D50=75.19cm
Observed values
Estimated values
P=1.4804*exp(-0.0102*d)
68DAP
D50=106.40cm
P=1.1958*exp(-0.0052*d)
76DAP
D50=168.30cm
P=1.0886*exp(-0.0029*d)
83DAP
D50=266.40cm
P=1.0107*exp(-0.0017*d)
90DAP
D50=421.34cm
P=0.9600*exp(-0.0012*d)
97DAP
D50=552.69cm
P=0.9938*exp(-0.0009*d)
104DAP
D50=729.84cm
Proportion of diseased flowers
0.0
0.2
0.4
0.6
0.8
1.0
30 120 210 300 390 480 570 660 750 840 930
Distance (cm)

Figure 5

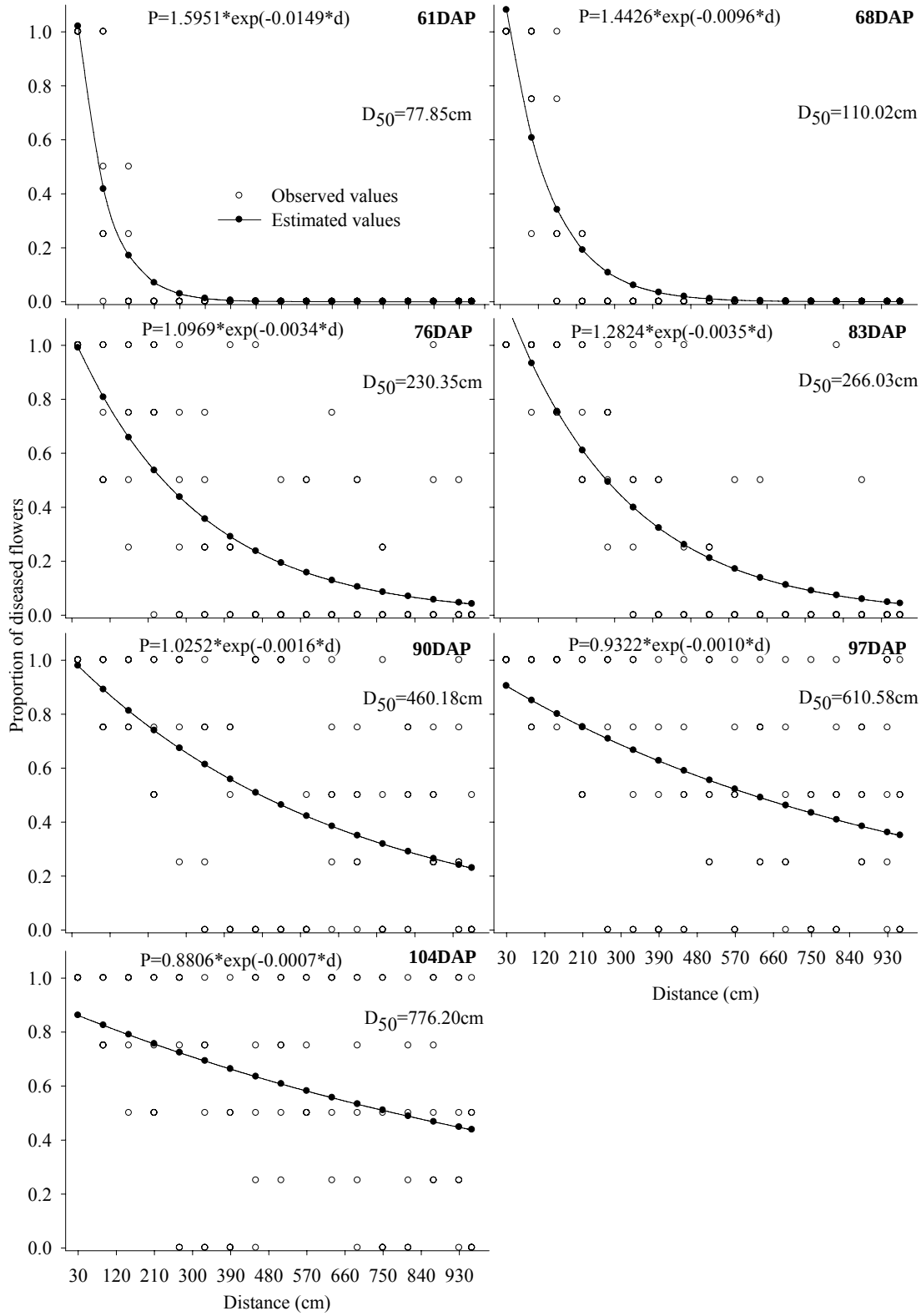

P=1.5951*exp(-0.0149*d)
61DAP
D50=77.85cm
Observed values
Estimated values
P=1.4426*exp(-0.0096*d)
68DAP
D50=110.02cm
P=1.0969*exp(-0.0034*d)
76DAP
D50=230.35cm
P=1.2824*exp(-0.0035*d)
83DAP
D50=266.03cm
P=1.0252*exp(-0.0016*d)
90DAP
D50=460.18cm
P=0.9322*exp(-0.0010*d)
97DAP
D50=610.58cm
P=0.8806*exp(-0.0007*d)
104DAP
D50=776.20cm
Proportion of diseased flowers
0.0
0.2
0.4
0.6
0.8
1.0
30 120 210 300 390 480 570 660 750 840 930
Distance (cm)

Figure 6

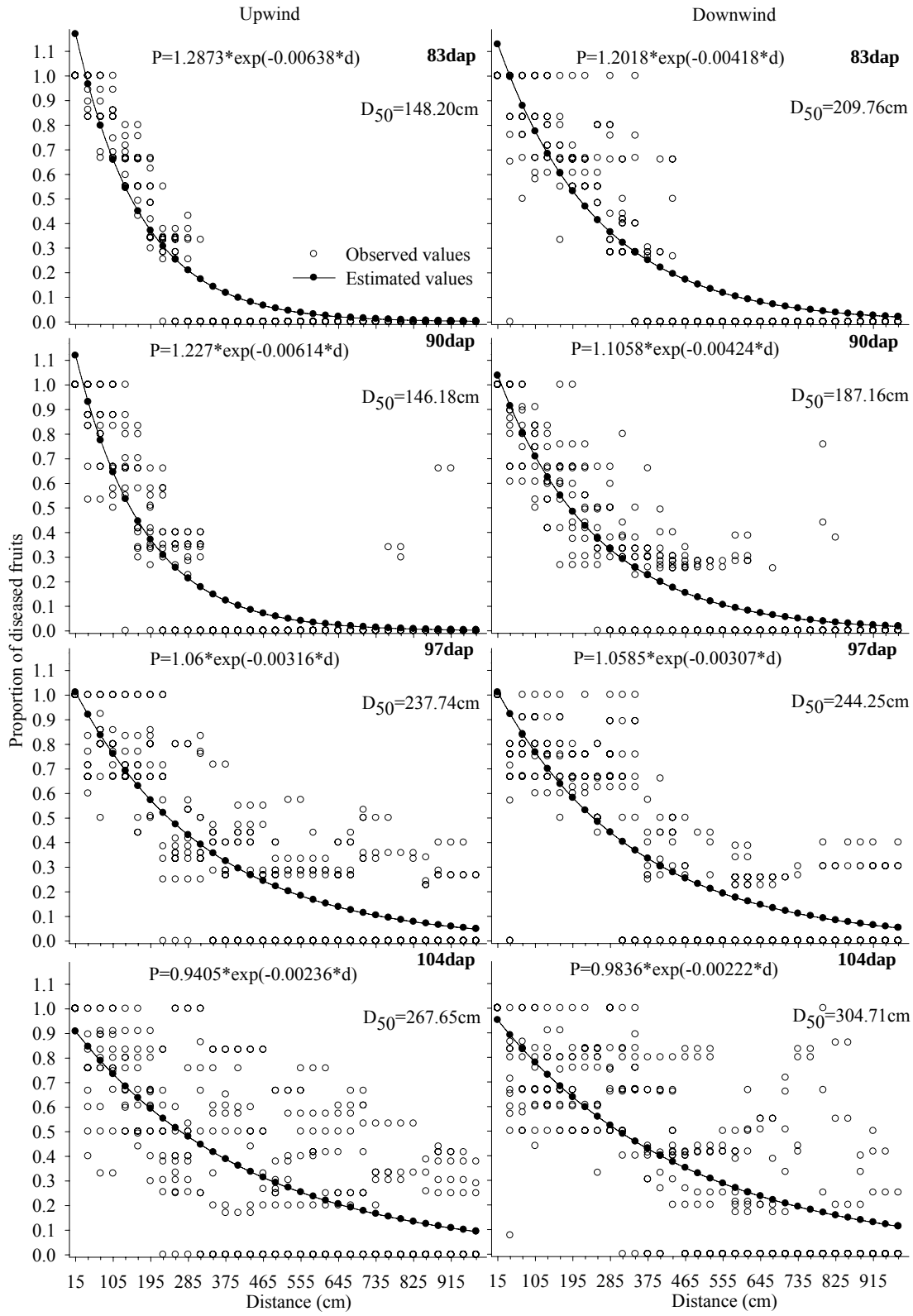
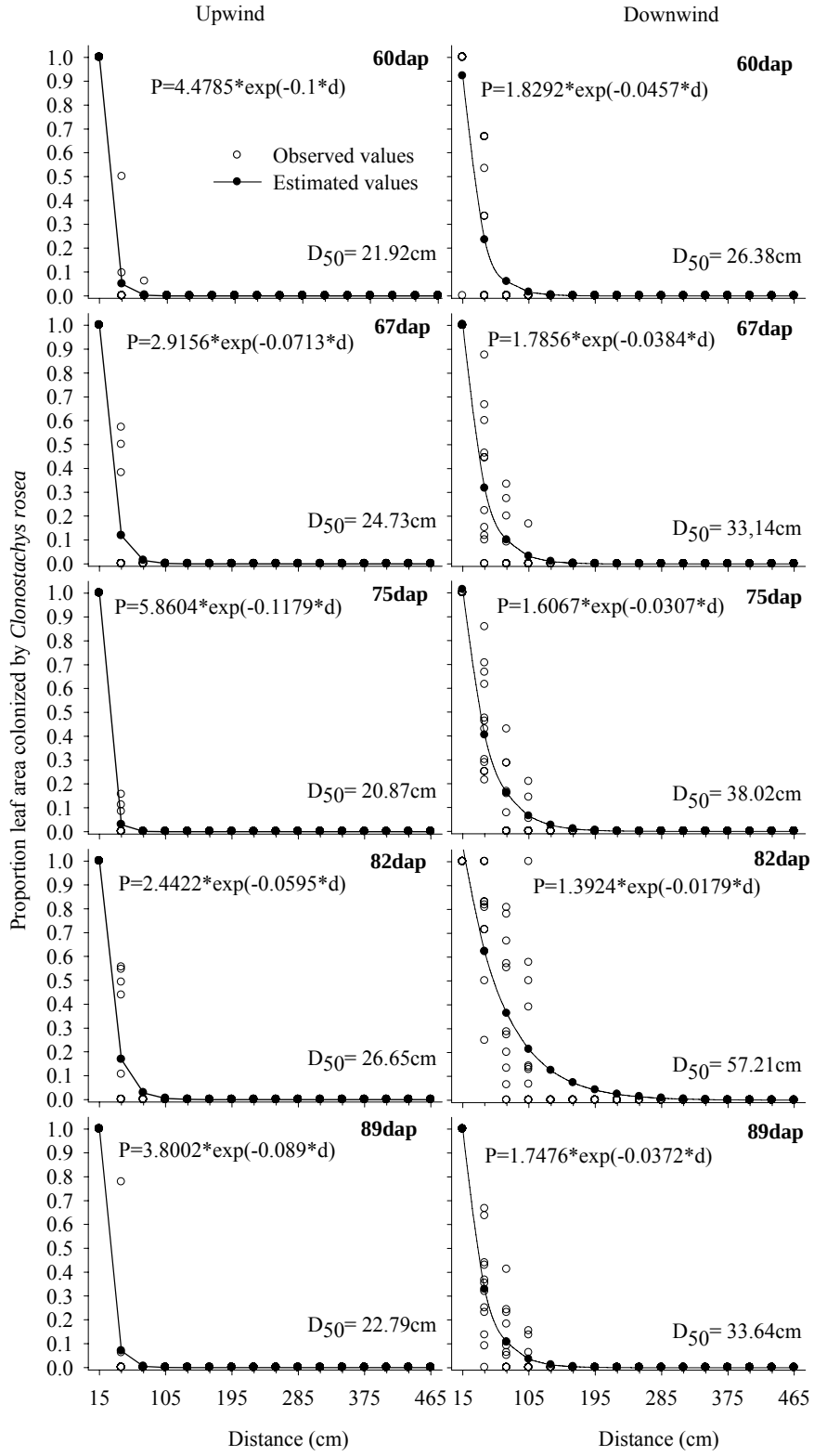

Upwind
Downwind
P=1.2873*exp(-0.00638*d)
83dap
D50=148.20cm
P=1.2018*exp(-0.00418*d)
83dap
D50=209.76cm
Observed values
Estimated values
P=1.227*exp(-0.00614*d)
90dap
D50=146.18cm
P=1.1058*exp(-0.00424*d)
90dap
D50=187.16cm
P=1.06*exp(-0.00316*d)
97dap
D50=237.74cm
P=1.0585*exp(-0.00307*d)
97dap
D50=244.25cm
P=0.9405*exp(-0.00236*d)
104dap
D50=267.65cm
P=0.9836*exp(-0.00222*d)
104dap
D50=304.71cm
Proportion of diseased fruits
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0 1.1
15 105 195 285 375 465 555 645 735 825 915
Distance (cm)

Figure 7


Upwind
Downwind
60dap
P=4.4785*exp(-0.1*d)
Observed values
Estimated values
D50= 21.92cm
60dap
P=1.8292*exp(-0.0457*d)
D50= 26.38cm
67dap
P=2.9156*exp(-0.0713*d)
D50= 24.73cm
67dap
P=1.7856*exp(-0.0384*d)
D50= 33,14cm
75dap
P=5.8604*exp(-0.1179*d)
D50= 20.87cm
75dap
P=1.6067*exp(-0.0307*d)
D50= 38.02cm
82dap
P=2.4422*exp(-0.0595*d)
D50= 26.65cm
82dap
P=1.3924*exp(-0.0179*d)
D50= 57.21cm
89dap
P=3.8002*exp(-0.089*d)
D50= 22.79cm
89dap
P=1.7476*exp(-0.0372*d)
D50= 33.64cm
Proportion leaf area colonized by Clonostachys rosea
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
15 105 195 285 375 465
Distance (cm)
Distance (cm)

CONCLUSÕES GERAIS

Com base nos resultados obtidos, concluiu-se que:

- O controle biológico por *C. rosea* foi eficiente no manejo do mofo cinzento do morangueiro;
- *C. rosea*, em duas aplicações semanais, foi mais eficiente no controle do mofo cinzento que os fungicidas testados;
- Em programas de manejo da doença, deve-se priorizar maior número de aplicações do antagonista, visto os melhores resultados obtidos com sua aplicação duas vezes por semana;
- Medidas integradas de controle foram eficientes no manejo do mofo cinzento;
- A aplicação de *C. rosea* aumentou a eficiência do controle químico e da eliminação de restos culturais;
- *C. rosea* tem baixa capacidade de dispersão em cultivos de morangueiro;
- Fontes de inóculo importantes para *B. cinerea*, são os restos de cultura, de onde o patógeno dispersa-se eficientemente.