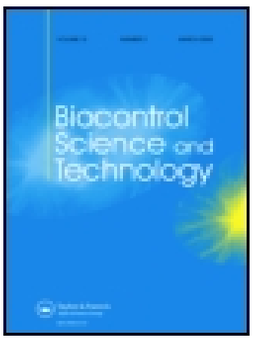

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Ability of *Clonostachys rosea* to Establish and Suppress Sporulation Potential of *Botrytis cinerea* in Deleafed Stems of Hydroponic Greenhouse Tomatoes

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The ability of Clonostachys rosea *to establish and persist in deleafed tomato stems and to suppress sporulation potential of* Botrytis cinerea *was investigated in plots of hydroponic tomatoes in commercial greenhouses. Leaves near lower fruit clusters were removed according to standard practice and deleafed portions of the stems were treated with* C. rosea*, iprodione or water. Inoculum of* B. cinerea *was from natural infections. Stem lesions were not produced by the pathogen during the trials. Development of* C. rosea *and* B. cinerea *in stems was estimated indirectly by quantifying sporulation on excised stem tissues that were incubated on an agar medium containing paraquat. Incidence and area of sporulation of* C. rosea *on tissue pieces were high (76–99%) and moderately high (33–79%), respectively, when stems were treated with the agent at 0, 6, 24 or 48 h after deleafing and sampled 11 to 75 days later. In various instances, the agent also sporulated on tissues from water controls and iprodione treatments, apparently after interplot transmission. In most instances, incidence and area of sporulation of* B. cinerea *on tissue pieces were high (83–100%) and moderate to high (35–76%), respectively, in the water controls, but moderate (31–44%) and moderate to low (5–34%), respectively, for stems treated with* C. rosea *at 0 to 48 h after deleafing and sampled after 11–75 days. Without exception,* C. rosea *suppressed* B. cinerea *as or more effectively than iprodione. Correlations between inoculum density of* C. rosea *(0–10^6 conidia mL^{-1}) and sporulation potential of* B. cinerea *in deleafed stems were strongly negative in each of three tests (*$r = -0.95$ *to* -0.99*). Conidial suspensions and a talc formulation of* C. rosea *were of similar effectiveness against* B. cinerea*. We conclude that* C. rosea *persisted and suppressed sporulation potential of* B. cinerea *in deleafed tomato stems for at least 11 weeks after application.*

Keywords: *biological control,* Botrytis cinerea, Clonostachys rosea, *greenhouse tomatoes, hydroponic crop, deleafing wounds*

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INTRODUCTION

Botrytis cinerea Pers.:Fr. is a destructive pathogen of stems, leaves, flowers and fruits of greenhouse-grown tomatoes (*Lycopersicon esculentum* Mill.) in Canada and elsewhere (Howard *et al*., 1994; Albajes *et al*., 1999). In south-western Ontario, a major production area of hydroponic tomatoes, the principal concern is stem lesions produced by the pathogen. Stem lesions usually originate when *B. cinerea* infects wounds made on stems or petioles during the routine cultural practice of leaf and lateral shoot removal (Verhoeff, 1967; Jarvis, 1992; Peng *et al*., 1996). Lesions that enlarge sufficiently to girdle the stem kill all distal portions of the shoots and thus destroy all fruit and yield potential of affected plants. In Ontario, stem lesions continually threaten productivity of hydroponic tomatoes and often reduce yields despite control measures.

The chief measures employed in the province to control stem lesions are humidity control, removal of crop residues, and fungicide treatments (Howard *et al*., 1994). Heating and ventilating of greenhouses to reduce relative humidity in crop canopies and deter disease progress is difficult under some ambient weather conditions, and cost prohibitive when energy prices are high. Fungicides applied inundatively as sprays, or as pastes to stems after lesions have been excised, continue to be important for control, but a general goal in crop protection in Ontario is to reduce fungicide inputs. Use of microbial agents is an alternative of major potential that has yet to be implemented against *B. cinerea* in commercial greenhouse crops in Canada.

Among several microbes reported to be effective against *B. cinerea* in tomato stems are isolates of *Trichoderma harzianum*, *Cladosporium cladosporioides*, *Aureobasidium pullulans*, and *Clonostachys rosea* (Eden *et al*., 1996; O'Neill *et al*., 1996; Dik & Elad, 1999; Dik *et al*., 1999; Elad, 2000; Liu *et al*., 2001). *Clonostachys rosea* (Link:Fr.) Schroers, Samuels, Siefert, and W. Gams, formerly classified as *Gliocladium roseum* Bainier (Schroers *et al*., 1999), is a fungal agent under development for controlling *B. cinerea* in a range of greenhouse and outdoor crops including tomato (Sutton *et al*., 1997). A non-pathogenic endophyte, *C. rosea* suppresses development and sporulation potential of *B. cinerea* in plants through nutrient competition, hyperparasitism, competitive colonization of senescing and dead tissues, and other control modes (Sutton *et al*., 1997; Yu & Sutton, 1997a; Köhl & Fokkema, 1998; Morandi *et al*., 2000). Previous investigations on control of *B. cinerea* by *C. rosea* in tomato stems were conducted on a small-scale using pot-grown host plants artificially inoculated with the pathogen in a research greenhouse (G. Peng & J.C. Sutton, 1996, unpublished observations; Sutton *et al*., 1997) or stem segments (Dik *et al*., 1999). Studies are lacking on establishment and persistence of the agent in tomato stems and its effectiveness against the pathogen under epidemiological conditions in hydroponic tomato crops.

In the present investigations, the effectiveness of *C. rosea* was compared with that of iprodione, a standard fungicide, for protecting deleafed stems of a commercial crop of greenhouse tomatoes against natural infections of *B. cinerea*. To provide information of practical importance, independent variables included time of *C. rosea* treatment after deleafing, inoculum density of the agent, and agent formulation. Effects of treatments on establishment and persistence of *C. rosea* and *B. cinerea* in host tissues were examined over several weeks during each of several periods of an 11-month crop production season.

MATERIALS AND METHODS

Tomato Plots

Plots were established in commercial crops of 'Rhapsody' tomato grown hydroponically at AMCO Farms Inc., Leamington, Ontario, Canada. The crops were initiated on 24 December 1999 as transplants grown in rockwool blocks (10.0 cm × 10 cm × 6.5 cm). The plants were positioned on rockwool slabs (90 cm × 15 cm × 8 cm), each enclosed in plastic film, and arranged in paired rows on plastic horticultural sheeting on soil of the greenhouse floor.

Rows were 80 cm apart within pairs and 120 cm apart between pairs. Plants within rows were spaced with stems 40–45 cm apart. Plots each comprised nine plants in 4 m of single row, and were spaced 9 m apart within rows. Only one row within each pair of rows was used for experiments. The crops were continuously drip-irrigated with a standard nutrient solution for tomato at pH 5.3–5.5 and EC 2.5–2.8 mS (Huang & Tu, 2001). For the standard practice of deleafing, the lower most six or seven leaves in a 60–70 cm-long zone of the stem bearing lower fruit clusters were periodically cut off with a sharp knife that was surface-disinfested in 2% (v/v) sodium hypochlorite (5.25% Javex) before use in each plot. Petiole stubs about 4–7 mm long remained on the stems after leaf removal.

Inoculum and Inoculations

Plants were inoculated artificially with *C. rosea* and naturally with *B. cinerea*. Inoculum of the pathogen was entirely from natural infections in the greenhouse. Old portions of tomato stems positioned horizontally above the rockwool, and leaf fragments from deleafing practices, were among the inoculum sources throughout the studies.

Inoculum of *C. rosea* isolate PG-A-Fr-88–710 (Peng & Sutton, 1991) was produced on wheat grain in 1-l Mason jars (Home Hardware Stores Ltd, St Jacobs, Ontario, Canada). Grain (200–300 g) in each jar was steamed for 3 h and autoclaved twice, with a 1-day interval, at 121°C and 103 kPa for 20 min. After cooling, grain in each jar was inoculated with 10 mL of *C. rosea* inoculum (10^7 conidia mL^{-1}) from cultures on potato dextrose agar (PDA), and incubated at 20–30°C for 30 days. Jars were shaken every 2–3 days to avoid matting of grain by mycelium, and caps were loosened for 3 s at 4 and 8 days to allow air exchange. At 12 days, when the grain was covered by mycelium, metal lid inserts of the jars were replaced with sterilized photocopy paper to allow cultures to dry slowly and promote spore production. Conidial suspensions were prepared at experimental sites in the commercial greenhouses immediately before use as inoculum. Suspensions were prepared by shaking 30 g of the heavily colonized grain in 1 L of sterile distilled water plus surfactant (0.5 mL Triton X-100 L^{-1} of water) for 15 min, and filtering the suspension through three layers of cheesecloth. This procedure resulted in inoculum densities between 5×10^6 and 1×10^7 conidia mL^{-1} according to conidial counts using a haemacytometer. Germination incidence of conidia after 16 h on PDA at 20–30°C consistently exceeded 96%. Conidial suspensions were applied to deleafed portions of tomato stems, or to leaves, with an air-pressurized backpack sprayer equipped with a single nozzle on a hand-operated lance, or with a 200-mL capacity air-pressurized hand sprayer.

Fungicide

Iprodione (Rovral 50 WP; Rhône-Poulenc Agro BV., 1 g of product L^{-1} water) was used in experiments as a standard treatment presently recommended for controlling *B. cinerea* in greenhouse tomatoes in Ontario. Iprodione was applied with a backpack sprayer of the same type used for *C. rosea*.

***C. rosea* and *B. cinerea* in Host Tissues**

Endophytic establishment and growth of *C. rosea* and *B. cinerea* were estimated indirectly by quantifying potential of the fungi to sporulate on stem and leaf tissues, an established method (e.g. Peng & Sutton 1991, Morandi *et al*., 2000). Tissue pieces, each 25–30 mm long and 4–6 mm deep and bearing a deleafing wound and xylem tissue, were excised from stems. Disks (10-mm diameter) were cut from leaves. Knives and cork borers used for cutting were surface disinfested in sodium hypochlorite and washed in sterile water before each excision. For estimation of sporulation potential, tissues were disinfested in 70% ethanol for 10 s and in 2.0% sodium hypochlorite for 10 s, rinsed three times in sterile distilled water and transferred to paraquat-chloramphenicol agar (PCA) medium (0.1 mL paraquat, 200 mg chloramphenicol, and 12 g agar L^{-1} water) (Peng & Sutton, 1991). The method for surface disinfestation was found in earlier studies to be highly effective against conidia and hyphae

of *B. cinerea* and *C. rosea* on tomato stems and various other plant tissues (e.g. Peng *et al*., 1996; Morandi *et al*., 2000). Sporulation of *C. rosea* and of *B. cinerea* were estimated after the tissues were incubated at 21–22ºC for 14 days. Sporulation of each fungus was assessed on a dissecting microscope as incidence and percent area of tissue pieces with conidiophores. Per cent area was estimated using an equi-incremental scale of 0 to 10 (0, 1–10%, 11–20%...91–100%). Midpoint values of increments were used for data analysis and presentation.

Timing of Stem Wound Treatments

Effects of application time of *C. rosea* to stems after leaves were removed on endophytic establishment and growth of the agent in the wounded stem tissues were examined in two experiments. *C. rosea* treatments were initiated on 4 May and 8 June 2000 in the first experiment and on 7 August and 16 September 2000 in the second. Each time treatments were applied, plants and support strings were lowered so that previously deleafed stem portions were positioned more or less horizontally above the rockwool slabs, and those portions still bearing leaves were oriented almost vertically. The lowermost six or seven leaves on each stem were subsequently cut off, always at 10:30–11:00 h EST. Inoculum of *C. rosea* was applied to deleafed stem portions with the backpack sprayer at 0, 6, 24 and 48 h after deleafing. Iprodione, and water of controls, were applied at 6 h after deleafing. Stem portions of first and second treatment periods were separated by 60–70 cm of deleafed stem that was not treated.

In the first experiment, stem pieces bearing deleafing wounds were excised for estimation of *C. rosea* and *B. cinerea* at 24, 48 and 75 days after treatment initiation on 4 May, and at 13 and 40 days after treatments were initiated on 8 June. A total of 20 1-cm diameter disks were cut from five leaves in the mid canopy region of each treatment replicate at 48 and 75 days after treatments of 4 May (equivalent to 13 and 40 days after treatments of 8 June). In the second experiment, stem pieces were sampled at 25 and 13 days after treatments of 7 August and 16 September, respectively. The stem pieces and leaf disks were surface disinfested, incubated on PCA, and assessed for sporulation of *C. rosea* and *B. cinerea*. In the first experiment, sporulation incidence of *B. cinerea* on deleafing wounds was assessed on 8 August. All wounds in stem portions deleafed on 4 May and 8 June were examined with the aid of a hand lens.

Inoculum Density of *C. rosea*

Inoculum density of *C. rosea* was investigated in relation to ability of the agent to suppress *B. cinerea* in stem tissues with deleafing wounds. Immediately before inoculation, plants were lowered on support strings and lower leaves were cut off as described above. Inoculum densities of 0, 10^3, 10^4, 10^5 and 10^6 conidia of *C. rosea* mL^{-1} were applied to wounded portions of stems 6 h after deleafing by means of the 200-mL capacity hand sprayer on 16 September 2000. A total of 10 pieces of stem tissue from sites with deleafing wounds on two plants of each treatment replicate was excised on 24 September, incubated on PCA, and assessed for sporulation of *C. rosea* and *B. cinerea* as described before. In a repetition of the experiment, plants were inoculated on 29 September and sampled on 13 and 25 October.

Effectiveness of Formulations of *C. rosea*

Inocula of *C. rosea* formulated as a powder (10^4 conidia g^{-1}) using pure talc (baby powder, Johnson and Johnson Inc., Guelph, Ontario, Canada) and prepared as a suspension in water plus surfactant (10^4 conidia mL^{-1}) were compared for effectiveness against *B. cinerea* in deleafed stems. The powder formulation and talc only were applied to deleafing wounds with a 2.5 cm wide paintbrush, and the water suspension and water only were applied to deleafed portions of stems with the hand sprayer. Treatments were applied 6 h after deleafing on 29 September 2000 in two repetitions of the experiment in different ranges of the greenhouse complex. On 13 and 25 October 2000, a tissue piece from each of five wound

sites on each of four plants in each of four replicate plots/treatment in each experimental repetition was excised and placed on PCA medium. Sporulation of *C. rosea* and *B. cinerea* were estimated after 14 days.

Experimental Design and Data Analysis

Each experiment was conducted with a randomized complete block design with four replicate plots per treatment. Statistical computations were performed using Statistical Analysis Systems software (release 6.12, SAS Institute Inc., Cary, NC, USA). Observations on incidence of sporulation of *C. rosea* and of *B. cinerea* on tissue pieces incubated on PCA medium, and on estimated per cent tissue area with sporulation of these fungi, were examined by analysis of variance (ANOVA). Treatment means were compared by the protected least significant difference (LSD) test (Snedecor & Cochran, 1989). Correlation analysis was used to examine relationships between area of sporulation of *C. rosea* and of *B. cinerea* on tissues treated with various inoculum densities of the agent.

RESULTS

Estimation of Treatment Effects

Lesions did not develop around deleafing wounds on treated portions of stems in water controls or in the various treatments during the periods of experiments. Treatment effects are expressed entirely in terms of estimated sporulation potential of *C. rosea* and *B. cinerea* on tissues incubated on PCA. Stem lesions with dense sporulation of *B. cinerea* were frequent throughout the experiments on old portions of deleafed stems positioned horizontally above the rockwool in each greenhouse range used.

Timing of Stem Wound Treatments

Sporulation incidence of *C. rosea* was high (76–99%) on tissue pieces from wound sites on stems that were treated with the agent at 0 to 48 h after deleafing and sampled at times ranging up to 75 and 25 days after treatments were initiated in experiments 1 and 2, respectively (Table 1). Timing of *C. rosea* treatments did not significantly affect sporulation incidence of the agent except for a reduced incidence observed for a 24-h treatment applied on 5 May and sampled on 24 May.

C. rosea also sporulated on numerous tissue pieces from deleafed stems of water controls and of iprodione treatments (Table 1). Mean sporulation incidence in water controls in most

TABLE 1. Effects of treating deleafed tomato stems in a commercial greenhouse with water or iprodione at 6 h after deleafing, or with *C. rosea* at 0 to 48 h after deleafing, on sporulation incidence of the agent in tissue pieces from stem wound sites that were plated on paraquat-chloramphenicol agar medium on various dates after deleafing in 2000

		Incidence of sporulation					
				C. rosea			
Date of deleafing	Date of tissue plating	Water 6 h	Iprodione 6 h	0 h	6 h	24 h	48 h
4 May	24 May	4c[a]	0c	98a	86a	63b	80a
	21 June	32b	71a	91a	98a	78a	93a
	18 July	37b	60ab	96a	96a	98a	84a
8 June	21 June	40b	54b	96a	97a	97a	99a
	18 July	77a	77a	88a	90a	88a	88a
21 August	1 September	47b	56b	98a	92a	98a	94a
16 September	29 September	0c	37b	86a	78a	84a	76a

[a]Treatment means in a row followed by the same letter are not significantly different (protected LSD, $P > 0.05$).

instances, was significantly lower (less than half) than that of *C. rosea* treatments, except for tissues treated on 8 June and sampled on 18 July. Mean sporulation incidence in the iprodione treatments was significantly lower than in *C. rosea* treatments on only four of seven sampling dates. Sporulation incidence of *C. rosea* in stem tissues of water controls was extremely low or zero only on the first and final sampling dates (24 May and 29 September, respectively).

Sporulation incidence of *B. cinerea* was high (60–100%) on plated stem pieces of water controls except on samples of 24 May when no sporulation was observed. Sporulation of the pathogen in most instances began on surfaces of deleafing wounds present on the tissue pieces and subsequently expanded onto surfaces of adjacent tissues that were not wounded. Mean sporulation incidence of *B. cinerea* on tissue pieces from stems that were deleafed/sampled on 4 May/21 June, 4 May/18 July, 8 June/21 June, 8 June/18 July, 7 August/1 September and 16 September/29 September were 85, 100, 60, 93, 95 and 83%, respectively. Treatment of deleafed stems with *C. rosea* significantly reduced sporulation incidence of *B. cinerea* compared to water controls in almost all instances. For the respective dates of deleafing and sampling, *C. rosea* suppressed incidence of the pathogen (mean values for all post-deleafing treatment times) by 54, 66, 33 (not significant), 52, 67 and 60%, respectively, and iprodione did so by 44, 39, 33 (not significant), 7 (not significant), 41 and 40%. Time of application of *C. rosea* up to 48 h after deleafing did not significantly influence effectiveness of the agent in suppressing sporulation incidence of the pathogen.

Estimated per cent area of excised stem pieces with sporulation of *C. rosea* was low or zero for water controls, moderate to high for *C. rosea* treatments, and moderate to low for iprodione treatments (Figures 1 and 2). Time of application of *C. rosea* after deleafing did not significantly affect area of sporulation of the agent, except for tissue pieces excised from stems inoculated with the agent on 4 May or 8 June and sampled on 21 June. Sporulation area of *C. rosea* on stem pieces from iprodione treatments was significantly lower than on pieces from *C. rosea* treatments except for stems treated on 4 May and sampled on 21 June or 18 July, and those treated on 8 June and sampled on 21 June. Sporulation area of *C. rosea* on stem pieces of water controls was significantly lower than in *C. rosea* treatments except for pieces excised on 18 July.

Estimated per cent area of tissue pieces with sporulation of *B. cinerea* at the various sampling times was moderate to high (mean values 35–76%) in water controls, but was significantly and often markedly lower on stem pieces from *C. rosea* treatments and, with one exception, iprodione treatments (Figures 1 and 2). *C. rosea* suppressed the pathogen more effectively than did iprodione in sixteen of twenty four instances, and as effectively in all other instances. Timing of *C. rosea* applications during 0 to 48 h after deleafing significantly influenced effectiveness of the agent against *B. cinerea* only in tissues treated after deleafing on 4 May and sampled on 8 June.

Sporulation incidence of *B. cinerea* on deleafing wounds, estimated in the greenhouse on 8 August, was significantly lower (protected LSD, $P \leqslant 0.05$) in stem portions treated with *C. rosea* or iprodione than in the water controls. For stem portions deleafed on 4 May, the pathogen sporulated on a mean of 20% of deleafing wounds in the controls, on 2–8% of wounds treated with *C. rosea* and on 9% in the iprodione treatment. Respective values for stems deleafed on 8 June were 21, 4–9 and 7%. Time of application of *C. rosea* up to 48 h after deleafing did not significantly affect sporulation incidence of *B. cinerea* (protected LSD, $P \leqslant 0.05$). Effects of *C. rosea* and iprodione treatments did not differ significantly for either deleafing date.

Sporulation was observed of *B. cinerea* but not of *C. rosea* on disks of mid-canopy leaves sampled on 21 June and 18 July from stems treated with the agent or iprodione, and from those of water controls. Sporulation incidence of *B. cinerea* on disks ranged from 13–28% for samples of 21 June, and 30–57% for those of 18 July, but did not differ significantly among controls and treatments for either date (protected LSD, $P \leqslant 0.05$). However, area of the mid-canopy leaf disks with sporulation of the pathogen was significantly lower in plants

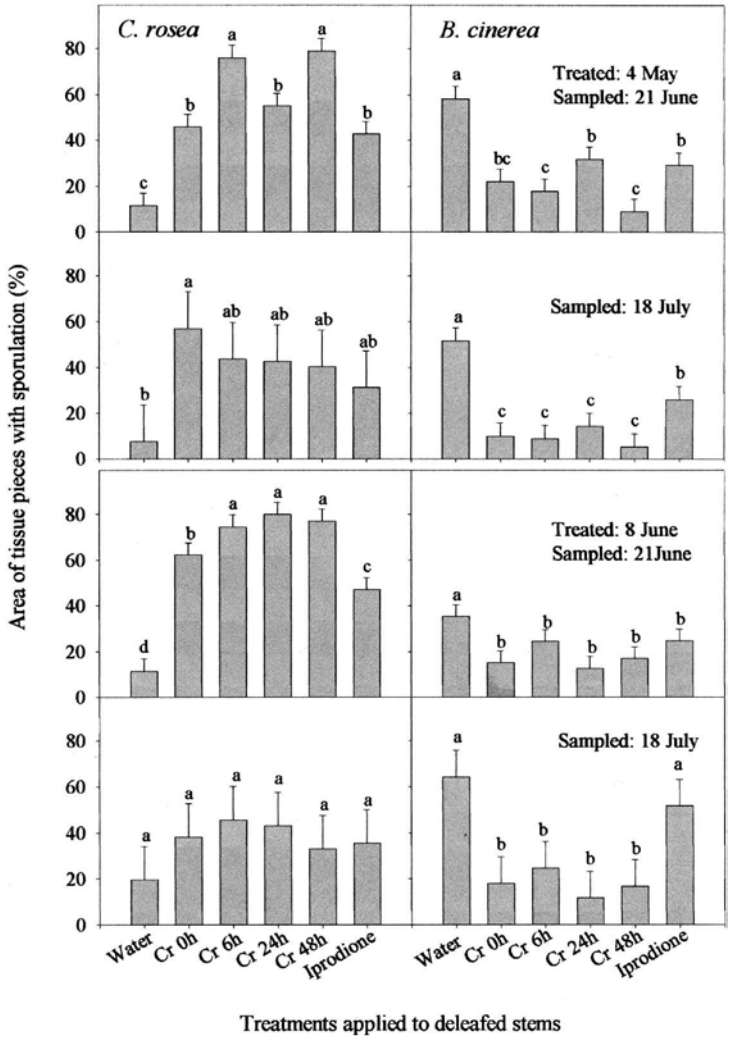

FIGURE 1. Effects of *C. rosea* applied to tomato stems at 0, 6, 24 and 48 h after deleafing, compared to those of water or iprodione each applied 6 h after deleafing, on estimated area of sporulation of the agent and of *B. cinerea* on tissue pieces excised from sites of deleafing wounds at various times after treatment, and incubated on paraquat–chloramphenicol agar medium. Treatments were applied on 4 May and 8 June 2000 in plots in a commercial crop of greenhouse tomatoes. Data bars are mean values each with a standard error (SE) bar. Treatment means for *C. rosea* or *B. cinerea* of given treatment and sampling times, assigned the same letter, are not significantly different (protected LSD, $P \leqslant 0.05$).

treated with *C. rosea* at various times after deleafing or with iprodione than in those of the water controls on both 21 June (means of 4–6% compared to 14%) and 18 July (3–6% compared to 18%) (protected LSD, $P \leqslant 0.05$). Effects of *C. rosea* and iprodione treatments did not differ significantly.

Inoculum Density of *C. rosea*

When inoculum density of *C. rosea* applied to deleafed stems was progressively increased, sporulation incidence of the agent in tissue pieces from sites of deleafing wounds increased,

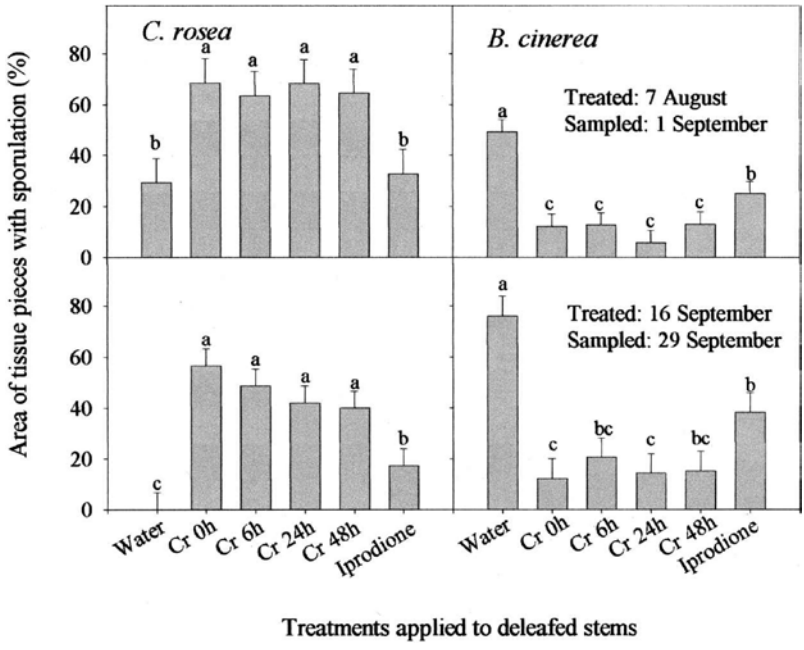

FIGURE 2. Effects of *C. rosea* applied to tomato stems at 0, 6, 24 and 48 h after deleafing, compared to those of water or iprodione each applied 6 h after deleafing, on estimated area of sporulation of the agent and of *B. cinerea* on tissue pieces excised from sites of deleafing wounds at various times after treatment, and incubated on paraquat-chloramphenicol agar medium. Treatments were applied on 7 August and 16 September 2000 in plots in a commercial crop of greenhouse tomatoes. Data bars are mean values each with a SE bar. Treatment means for *C. rosea* or *B. cinerea* of given treatment and sampling times, assigned the same letter, are not significantly different (protected LSD, $P \leqslant 0.05$).

but that of *B. cinerea* decreased. Sporulation incidence of *C. rosea* on stem tissues from noninoculated controls was invariably low (3–7%), while that of *B. cinerea* was high (97–100%). When *C. rosea* was applied in logarithmic increments from 10^3 to 10^6 conidia mL^{-1}, mean sporulation incidence of the agent progressively increased from 53–94% for tissues treated on 16 September and sampled on 29 September. Respective values for tissues treated on 29 September were 45–88% when sampled on 13 October and 34–88% when sampled on 25 October. Mean sporulation incidence of *B. cinerea* progressively decreased on the respective dates from 74–41%, 85–35% and 86–42%.

Progressive increase in inoculum density of *C. rosea* also increased the area of tissue pieces on which the agent sporulated, and decreased the area with sporulation of *B. cinerea* (Figure 3). In control plants that were not artificially inoculated, *C. rosea* sporulated on $\leqslant$ 3%, and *B. cinerea* on 60–89%, of tissue piece surfaces. Application of *C. rosea* inoculum in logarithmic increments from 10^3–10^6 conidia mL^{-1} progressively increased the area of sporulation of the agent on the tissues from 19–26% to 60–66% in the various tests. Correlation analysis indicated that there were strong inverse relationships between the observations for *C. rosea* and those for *B. cinerea* ($r = -0.99$, -0.95 and -0.99 in the three tests). The highest inoculum density of *C. rosea* (10^6 conidia mL^{-1}) suppressed sporulation of *B. cinerea* by 82, 85 and 69% in the successive tests.

Effectiveness of C. rosea Formulations

In the first repetition of the experiment, *C. rosea* sporulated on means of 2 and 5% of tissue pieces sampled on 13 October from stems treated 2 weeks earlier with water or talc only,

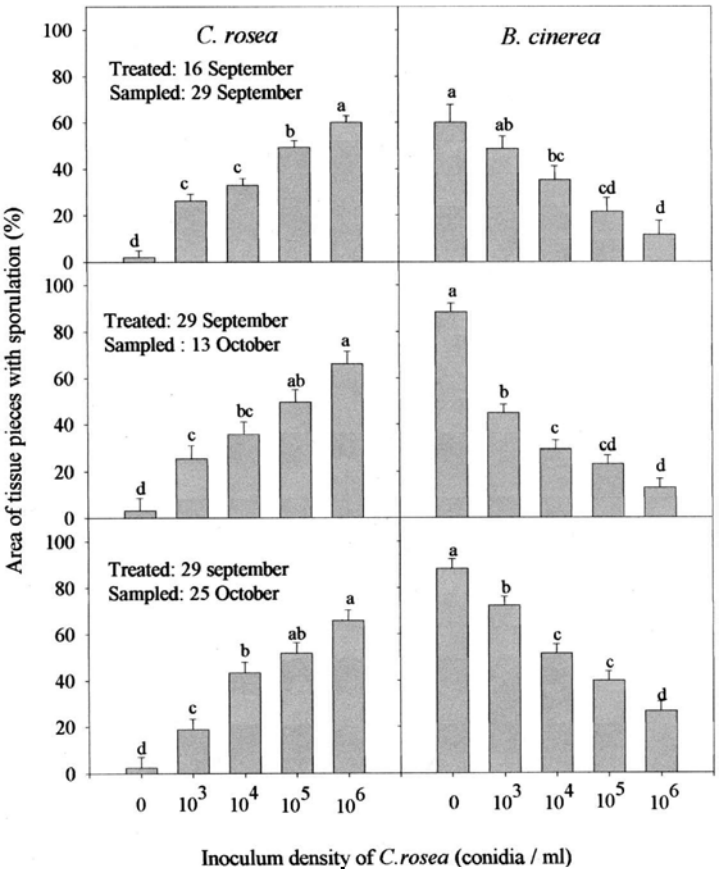

FIGURE 3. Relationships of inoculum density of *C. rosea* applied to deleafed tomato stems to estimated area of sporulation of the agent and of *B. cinerea* on tissue pieces excised from sites of deleafing wounds at various times after treatment and incubated on paraquat-chloramphenicol agar medium. Treatments were applied on 16 and 29 September in plots in a commercial crop of greenhouse tomatoes. Data are mean values each with a SE bar. Treatment means for *C. rosea* or *B. cinerea* of a given treatment and sampling times, assigned the same letter, are not significantly different (protected LSD, $P \leqslant 0.05$).

but on all tissue pieces from stems treated with *C. rosea*, whether as the conidial suspension or talc preparation. Mean values for area of tissue pieces with sporulation of the agent were 7 and 10% for the water and talc controls, respectively, and 80 and 72% for *C. rosea* applied in water or with talc, respectively. In all instances, effects of water and talc did not differ significantly, and the same was found for water plus *C. rosea* and talc plus *C. rosea*, but values obtained with the *C. rosea* treatments were significantly higher than in the controls (all $P \leqslant 0.001$, protected LSD).

In the same experiment, *B. cinerea* sporulated on 91 and 79% of tissue pieces from water and talc controls, and on 23and 27% from stems treated with the conidial suspension or talc preparation of *C. rosea*. Respective values for area of tissue pieces with sporulation of the pathogen were 65, 56, 8 and 12%. Statistical significance for differences in effects of controls and treatments were the same for *B. cinerea* as found for *C. rosea*. Values obtained for *C. rosea* treatments were significantly lower than in the controls.

Observations for stem pieces excised on 25 October (nearly 4 weeks after treatment) in

the first repetition were similar to those obtained on 13 October. Also, observations in the second experimental repetition were similar to those of the first except that incidence and area of sporulation of *B. cinerea* were 12–18% lower.

DISCUSSION

The observations of sporulation by *B. cinerea* on stem tissues that were excised and incubated on the PCA medium, and also on deleafing wounds of plants in the greenhouse plots, indicated that the pathogen frequently infected (i.e. established as a parasite in) stem tissues adjacent to deleafing wounds. These findings agree with earlier reports that deleafing wounds are principal sites of infection by the pathogen in tomato stems (Wilson, 1963; Jarvis, 1992; O'Neill *et al*., 1996; Peng *et al*., 1996). The failure of *B. cinerea* to produce lesions on the stems during the experiments probably was related to the prevalent physiological status of the stem tissues. *B. cinerea* usually remains quiescent in green tissues of host plants infected from conidia, but becomes aggressive, such that lesions may form, when the tissues senesce or are stressed by environmental factors (Jarvis, 1992; Elad & Evensen, 1995; Sutton *et al*., 1997; Shafia *et al*., 2001). Thus, in tomato stems, *B. cinerea* probably remains quiescent, so does not produce lesions, until a particular stage of senescence or stress response is reached. The quiescent state of *B. cinerea* in tomato stems can be long, such as 10–12 weeks (Wilson, 1963; Jarvis, 1992). In the present study, the stem tissues used for treatments possibly did not senesce sufficiently to trigger the aggressive state of *B. cinerea* and allow lesions to form during the periods of the experiments. Development of lesions on old portions of stems positioned horizontally near the rockwool probably reflected aggressive development of the pathogen in response to senescence or stress of the tissues induced by low intensity light or other environmental factors (Shafia *et al*., 2001). Whether or not inoculum density of *B. cinerea* from natural sources in the greenhouse affected ability of the pathogen to become aggressive and produce lesions is unclear. Because stem lesions did not form, assessment of the development and interactions of the pathogen and *C. rosea* were based entirely on estimates of sporulation potential of the fungi on excised tissues killed on the PCA medium.

C. rosea established endophytically and persisted for at least several weeks in stems of commercially-grown hydroponic tomatoes that were treated with the agent soon after deleafing. From observations of sporulation of *C. rosea* on surface-disinfested tissues from sites of deleafing wounds treated with $5 \times 10^6 - 1 \times 10^7$ conidia mL^{-1}, incidence of the agent in wounded tissues was invariably high, usually exceeding 78% and often in the range 91–100%. High incidence was observed whether stems were inoculated at 0 to 48 h after deleafing, and whether stem tissues were assayed at 11–75 days after treatment. Area of stem pieces with sporulation of *C. rosea* was also moderate to high for all times of treatment and sampling. By inference from sporulation data, the agent heavily colonized zones of wounded stems about as large, or possibly larger, than the 25–30-mm long tissue pieces used for sporulation assays. The observations of sporulation on tissues from stems that were inoculated with *C. rosea* require interpretation from the perspective that the agent also sporulated on some tissues that were not inoculated.

The findings that *C. rosea* in some instances sporulated on stem pieces of the water controls and iprodione treatments pointed to probable interplot transmission of the agent. Incidence and area of sporulation in the control and iprodione treatment of various experiments were usually low or zero in May, late September and October, but moderate or high during June to early September. Interplot interference through conidial dispersal during inoculations is considered unlikely in view of substantial spacing of plots, localized and directed application of inoculum, and the wide variation in incidence of *C. rosea* in noninoculated stems after various times of treatment from May–October. Post-inoculation dispersal of the agent from inoculated plots to plants in the water controls and iprodione treatments, and potential secondary dispersals, were possibly effected by water splash, worker activities, and insect vectors. The sticky nature of *C. rosea* conidia (Schroers *et al*.,

1999) would be expected to favour these means of dispersal as opposed to dispersal on air currents. However, because water sprays were not used in the crops and water droplets rarely fell from the greenhouse roof or upper canopy, splash dispersal was probably of little or no importance. Greenhouse workers generally did not handle previously-deleafed portions of stems, so seem unlikely to have been a major factor in transmission of *C. rosea* among plants and plots. Some white flies (*Trialeurodes vaporariorum* Westwood), a few fungus gnats (family Sciaridae), and numerous fruit (or vinegar) flies (family Drosophilidae) were noticed in plot areas of the greenhouse. Among these, fruit flies are considered a possible vector based on feeding habits, and preliminary observations of transmission of *C. rosea* on pot-grown tomatoes (M. Ravensdale & J. C. Sutton, unpublished observations, 2001). The fruit flies were especially numerous in association with overripe fruits during June to early September when incidence of *C. rosea* in noninoculated plants was relatively high. While interplot transmission by one or more means probably accounted for *C. rosea* in plants of the controls and iprodione treatments, transmission from possible background sources of the fungus in the greenhouse cannot be ruled out. Whatever the means, a strong potential for transmission among plants, perhaps in combination with spore production on senescing or dead plant tissues, could contribute to sustained effectiveness of *C. rosea* as a control agent in greenhouse tomatoes.

C. rosea usually suppressed *B. cinerea* in tomato stem tissues under the microclimatic and crop conditions of the experiments, which, based on infection incidence in deleafed stems of control plants, were often highly favorable for the pathogen. Thus, in the experiments to investigate timing of stem treatments after deleafing, sporulation incidence of *B. cinerea* was generally in the range 83–100% in excised tissues of controls, but 52–67% lower in *C. rosea* treated plants. Similarly, area of tissue samples with sporulation of *B. cinerea* was 45–90% lower in plants treated with *C. rosea* (Figures 1 and 2). The chief exception was for stems treated on 8 June and sampled on 21 June, in which pathogen incidence and sporulation area were only moderate (60 and 35%, respectively), and *C. rosea* significantly reduced the area but not the incidence of sporulation of the pathogen. Findings that *C. rosea* markedly suppressed *B. cinerea* in tissues sampled on 18 July, about 11 weeks after a single application of the agent on 4 May, underscored the persistent effectiveness of the agent against the pathogen in tomato stems. Long-term effectiveness of *C. rosea* against *B. cinerea* was reported also in other crops (Sutton & Peng, 1993; Zhang *et al*., 1996; Sutton *et al*., 1997; Yu & Sutton, 1999). Without exception, in the present research, *C. rosea* suppressed *B. cinerea* as or more effectively than did iprodione. As *C. rosea* sporulated on stem pieces of iprodione-treated and control plants, actual effectiveness of the agent probably was greater, and that of iprodione less, than indicated by the data. Reduced sensitivity to iprodione of the *B. cinerea* populations in the test greenhouses was possible, however alternation of iprodione with fungicides of different modes of action had been practiced for several years to reduce this risk (Staub, 1991), and reduced control by iprodione had not been observed in the crops. Although crop conditions did not favor production of lesions by *B. cinerea* on portions of stems used for experiments, at least within time frames of the studies, the marked suppression of pathogen sporulation by *C. rosea* underscored the biocontrol potential of the agent against the pathogen in commercial tomato crops. By reducing inoculum production and tissue colonization by *B. cinerea*, *C. rosea* can be expected to suppress epidemics of disease associated with the pathogen on the leaves, flowers, and fruits as well as stems.

The strong negative correlations between inoculum density of *C. rosea* and sporulation potential of *B. cinerea* in deleafed stems are similar to those reported for the agent in various tissues of other crops (Sutton *et al*., 1997). Increased inoculum density of *C. rosea* possibly favoured establishment and colonization of the agent in deleafed stem tissues in competition with the pathogen. Numerous reports are consistent with the view that substrate competition is a principal mode of suppression of *B. cinerea* by *C. rosea* in various hosts (Sutton *et al*., 1997; Yu & Sutton, 1997a). Inoculum densities of $> 10^7$ conidia mL^{-1} would possibly have

further suppressed *B. cinerea*, but this was not the case in some other hosts (Sutton *et al*., 1997). Densities lower than 10^6 conidia mL^{-1}, such as those used in the comparison of 10^4 conidia mL^{-1} water and 10^4 conidia g^{-1} talc, also strongly suppressed the pathogen in some instances.

A range of other microbes, including filamentous fungi, yeasts, and bacteria, were reported to suppress *B. cinerea* in wounded tomato stems, chiefly in tests that did not closely simulate epidemiologic conditions of commercial greenhouse tomatoes (Eden *et al*., 1996; O'Neill *et al*., 1996; Dik & Elad, 1999; Dik *et al*., 1999). The tests utilized stem segments in the laboratory, or whole plants grown in pots or small hydroponic systems in research greenhouses. Plants or stem-segments were usually inoculated once with *B. cinerea* as opposed to being exposed to a series of natural dispersals as occur in commercial crops. Further, experiments were usually terminated too soon (8–15 days after treatment) to allow long term effectiveness of agents to be explored. However, one investigation, which involved weekly and biweekly applications of microbes, was assessed up to 75 days (Dik & Elad, 1999). Relatively effective microbes included *Cladosporium cladosporioides*, *Trichoderma harzianum* and *Aureobasidium pullulans*. Dik *et al*. (1999) reported that *G. roseum* (= *C. rosea*) was highly effective against *B. cinerea* in tomato stem segments.

Several of the present observations have practical implications for implementing *C. rosea* against *B. cinerea* in commercial hydroponic tomatoes. It is stressed that these implications are based on observations in symptomless tissues that were transferred to PCA medium, and that long-term studies are needed on effects of *C. rosea* or *B. cinerea* under conditions that favor development of stem lesions. As the agent was effective when applied to stems up to 48 h after leaf removal, deleafed stems can be treated on the same day, or on the subsequent two days without concern that possible wound healing, early attack by the pathogen, or other factors might jeopardize treatment effectiveness. The long-term endophytic survival and control effectiveness of *C. rosea* suggested that one application to any given portion of deleafed stem should provide adequate, and possible season-long, protection of treated tissues against *B. cinerea*. From available data, *C. rosea* should be applied to new stem wounds each time leaves are removed. It remains to be clarified whether *C. rosea* can colonize tomatoes sufficiently, or induce sufficient systemic acquired resistance (SAR), to reduce the need to treat distal portions of stems when they are deleafed. However, the reduced area of sporulation by *B. cinerea* compared to water controls combined with lack of sporulation of *C. rosea* on leaf disks taken from mid-canopy leaves pointed to possible SAR in response to stem treatments with the agent, as was reported for *T. harzianum* (De Meyer *et al*., 1998). Such suppression of sporulation potential of *B. cinerea* in the leaves can be expected to reduce inoculum production after the leaves die, and thus rates of disease progress in the crop. Effectiveness of *C. rosea* applied with talc as well as in water pointed to a potential for use of powder formulations on tomato stems, similar to those investigated for treating flowers by means of bee vectors (Yu & Sutton, 1997b). Collectively, the data indicate that *C. rosea* has major potential as an agent against *B. cinerea* in stems of commercial greenhouse tomatoes, at least when used in combination with standard sanitation and ventilation measures. Strategic use of *C. rosea* to minimize the need for heating and ventilating to control *B. cinerea* in an era of rapidly rising energy costs is an enticing possibility in Canadian greenhouses.

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