Biocontrol of *Botrytis cinerea* in Strawberry Leaves

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ABSTRACT

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In biocontrol tests, strawberry leaves were inoculated with *Botrytis cinerea*, were treated 2–5 wk later with antagonistic fungi or with chlorothalonil, and were kept on paraquat-chloramphenicol agar (PCA) or were sprayed with paraquat to kill the tissues and allow the pathogen to sporulate. *Gliocladium roseum*, a *Penicillium* sp., and *Trichoderma viride* suppressed the number of conidiophores of *B. cinerea* by 97–100% in attached leaves in the greenhouse. The antagonists also suppressed sporulation incidence of the pathogen by 58, 64, and 48%, respectively, in semisenescent overwintered leaves in field plots and by 81–100, 59–100, and 53–87%, respectively, in green leaves in field plots. *G. roseum* suppressed the pathogen as effectively as did chlorothalonil in all tests, and *Penicillium* sp. and *T. viride* were as effective in the greenhouse and in three of six field tests. Biosuppression generally increased as temperature increased from 10 to 25 C, but only *G. roseum* was highly suppressive at 10 and 15 C. Germination rate of conidia and growth of germ tubes of each antagonist on the leaves increased with temperature. When applied to infected green leaves and incubated on PCA, the antagonists suppressed hyphal growth of *B. cinerea* in leaf tissues. Hyperparasitism was not observed in leaves or in a separation-membrane assay. The antagonists markedly suppressed the number of conidiophores of *B. cinerea* when applied after the leaves had been incubated on PCA for 0 or 24 h but only slightly or not at all in leaves incubated on PCA for 48 or 72 h. *G. roseum* also was nonsuppressive in leaves that had died naturally. The antagonists suppressed *B. cinerea* primarily when applied to living green leaves.

Additional keywords: *Fragaria* × *ananassa*, gray mold, *Myrothecium verrucaria*, *Rhodotorula glutinis*, substrate competition.

Key strategies for managing gray mold fruit rot of strawberry (*Fragaria* × *ananassa* Duchesne), caused by *Botrytis cinerea* Pers.:Fr., include suppression of conidial production by the pathogen in inoculum sources and protection of flowers against infection by conidia of the fungus (13,26,29,30). In Ontario, Canada, strawberry fields, the principal source of *B. cinerea* is mycelium in dead strawberry leaves (6), but in other areas, mummified strawberry fruits, straw mulch, and weeds also may be important (13,14, 29). Conidia dispersed from inoculum sources can infect flowers, from which the pathogen is able to invade contiguous fruits (8, 14,27). Although fungicides are widely used to protect flowers and fruits, fungicide resistance in the *B. cinerea* population is widespread, and fungicide use is opposed by the public, in part because of residues in strawberries and the environment (29). However, chlorothalonil applied to the foliage before flowering suppressed inoculum production of *B. cinerea* in dead leaves and controlled fruit rot without leaving residues in the fruits (4,5,29). Recently, biological methods have been developed as alternatives to fungicides for fruit rot control (22,24). Although flower protection was the primary strategy explored, several fungal antagonists also suppressed *B. cinerea* in leaves used in screening tests. The potential for biocontrol of the pathogen in leaves, however, has not been explored.

To examine and understand biocontrol of *B. cinerea* in strawberry leaves, it is important to consider known relationships between the pathogen and the leaf tissues. Strawberry leaves are highly susceptible to infection by *B. cinerea* at the bud stage and when expanding, but susceptibility declines in fully expanded and senescent leaves (7,21). Although the pathogen infects green leaves, symptoms normally are not produced. The fungus remains quiescent in the epidermal cells until the leaves senesce, after which it may progressively colonize the leaves and later sporulate (7). The pathogen can survive and sporulate in dead strawberry leaves for several months in the field (7).

In the present study, selected antagonists were evaluated for effectiveness in suppressing growth and sporulation of *B. cinerea* in strawberry leaves in the greenhouse and in the field. Relationships of temperature and leaf death to efficacy of biocontrol, and interactions between antagonists and the pathogen were investigated under controlled conditions.

MATERIALS AND METHODS

Inoculum production. Isolate PG-A-Fr-87-001 of *B. cinerea* (24) was used in all studies. Conidia of the pathogen were produced on a strawberry agar medium (7) and recovered by aspiration into sterile distilled water plus surfactant (50 μl of Triton XR/100 ml of water). Conidial suspensions were diluted to 10^6 spores/ml of water plus surfactant and used immediately for inoculating strawberry leaves in the laboratory or greenhouse and were diluted to 10^5 spores/ml of water plus surfactant and used within 90 min for inoculations in the field.

To produce inoculum of the antagonists, the mycelial fungi and the yeast *Rhodotorula glutinis* (Fresen.) F.C. Harrison were grown on potato-dextrose agar (PDA) for 10–15 and 3–5 days, respectively, at 20–23 C. For application in the field, the yeast was grown in a liquid medium prepared with 10 g of proteose peptone and 1 g of yeast extract in 1 L of water. Liquid cultures were shaken continuously at 150 rpm for 3–4 days at 20–23 C. Conidia and yeast cells were recovered in sterile distilled water, centrifuged at 2,520 *g* for 5 min, and resuspended in sterile distilled water plus surfactant. Propagule concentrations were adjusted to 10^7 spores or cells/ml of water for use in biocontrol studies.

Plant materials. For greenhouse and laboratory studies, commercial transplants of strawberry cv. Redcoat were stored at −1 C and grown as needed in 12-cm pots containing artificial soil (Promix, Plant Products Co. Ltd., Brampton, ON, Canada), in a greenhouse at 20–30 C without supplemental lighting. Soluble 20-20-20 N-P-K fertilizer was applied (5 g/L of water) at 2-wk intervals.

Field studies were conducted in matted-row plantings of straw-

berry cv. Redcoat at the Arkell Research Station, located near Guelph, ON, Canada. Rows were 0.5 m wide and spaced 1 m apart. Each plot consisted of a single row 4-m long. Plots were spaced 1 m apart within the row. Wheat straw was placed over the rows during late November as winter protection and was moved to between the rows during early April. Plots were trickle-irrigated during dry weather. Fluazifop-butyl (Fusilade 250 EC, Chipman Inc., Stoney Creek, ON, Canada) was applied (3 L/ha) during early May to control grasses; no other chemical pesticides were used.

Inoculations. Leaves for greenhouse and laboratory studies were tagged at the bud stage and inoculated 10 days later, when they were green and fully expanded. Inocula of *B. cinerea* and of the antagonists were applied to attached leaves or to 1-cm-diameter leaf disks of strawberry with a small, aerosol sprayer (Spra-Tool, Crown Industrial Products Co., Hebron, IL).

In field studies, the various inocula were applied to individual leaves with the aerosol sprayer and to entire plots with a compressed-air sprayer, 7.6-L capacity and equipped with a single nozzle. Inoculum was applied to form a thin film on the adaxial surface of leaves. Plants in the field were inoculated 1–2 h before dusk.

Estimation of gray mold. Infection and colonization of leaves by *B. cinerea* were quantified indirectly by estimating the incidence or number of conidiophores of the pathogen on the tissues. Treated leaf disks and disks cut from treated leaves were incubated on paraquat-chloramphenicol agar (PCA) at 20–23 C for 7 days to kill the tissues and favor sporulation (24). Conidiophores of *B. cinerea* on the disks were counted, or their incidence was estimated by examinination with a dissecting microscope.

In field studies in 1990, leaflets of treated leaves were cut lengthwise and crosswise into quarters, were placed on galvanized wire mesh approximately 1 cm above wet paper towels in translucent-plastic boxes (30 × 20 × 12 cm deep), and were sprayed with paraquat (20 mg of a.i./L of water). Boxes were sealed with lids and kept in the laboratory at 20–23 C without supplemental light for 15 days, after which the incidence of quarter leaflets with conidiophores of *B. cinerea* was estimated.

Biocontrol tests. Six fungal antagonists (with isolate numbers) were used in biocontrol studies of *B. cinerea* in the greenhouse and in the field: a *Fusarium* sp. (701), *Gliocladium roseum* Bainier (710), *Myrothecium verrucaria* (Albertini & Schwein) Ditmar:Fr. (104), a *Penicillium* sp. (705), *R. glutinis* (428), and *Trichoderma viride* Pers.:Fr. (004). The origin of each antagonist was reported previously (24). The effectiveness of the antagonists in suppressing *B. cinerea* in strawberry leaves was compared with that of chlorothalonil 50 F (1 ml/100 ml of water).

In the greenhouse study, 10-day-old leaves (one attached leaf per plant) were inoculated with *B. cinerea*, and the plants were kept in a humidity chamber for 24 h at 20–21 C. Relative humidity (RH) >95% was maintained in the chamber by means of an ultrasonic humidifier that operated in response to a control signal from a data logger (model 21X, Campbell Scientific Inc., Logan, UT) connected in a switch relay to a humidity sensor (model 207, Campbell Scientific Inc.) positioned among the leaves in the chamber. After the humid period, plants were kept in a greenhouse at 20–30 C for 2 wk. Leaves previously inoculated with *B. cinerea* were sprayed with inocula of the antagonists, chlorothalonil, or water plus surfactant. Ten leaves were randomly assigned to each of three replicates of each treatment, which were arranged in a completely randomized design. The treated plants were kept in the humidity chamber for 24 h and in the greenhouse for 2 days. Forty disks were cut from leaves from each replicate of each treatment (for a total of 120 disks per treatment) and were incubated on PCA, and the number of conidiophores per disk was estimated. The study was repeated once.

Treatments in the field were arranged in a completely randomized block design with four replicate plots per treatment. In a study in 1989, the six antagonists, chlorothalonil, or water plus surfactant was applied to 10 overwintered leaves in each replicate plot on 26 May and to the same leaves on 3 June. The leaves were not artificially inoculated with *B. cinerea*. In a second study in 1989, 10 green and expanded leaves of the early-spring flush (7) in each replicate plot were inoculated with *B. cinerea* on 13 May and were treated with the antagonists, chlorothalonil, or water plus surfactant on 13 and 20 June. All treated leaves in the two studies were harvested on 10 and 27 June, respectively. Approximately 150 disks were cut from leaves from each plot and were placed on PCA to estimate sporulation incidence of *B. cinerea* in the disks.

In 1990, isolates of *G. roseum*, *T. viride*, and *Penicillium* sp. and chlorothalonil were evaluated for gray mold suppression by treating 10 attached and fully expanded green leaves in each replicate plot and by inundatively treating entire plots. In a first experimental repetition of each method, *B. cinerea* was applied on 2 August, and the antagonists, fungicide, or water plus surfactant was applied on 2 and 9 September. In a second repetition, the pathogen was applied on 4 September, and the treatment was applied on 1 and 6 October. All leaflets of individually treated leaves and approximately 100 randomly sampled leaflets of a similar shade of green in inundatively treated plots were collected on 16 September (first repetition) and 13 October (second repetition). Collected leaflets were quartered and sporulation incidence of *B. cinerea* in the quarter leaflets was estimated.

Microclimate. Weather variables were monitored in the strawberry plantings at the time of each field study and were recorded with a data logger (model 21X, Campbell Scientific Inc.). Air temperature and RH were measured with shielded thermistors and RH probes, respectively (models 107 and 207, Campbell Scientific Inc.), positioned 30 cm above the soil. Rain was measured with a tipping bucket gauge (Weathertronics Inc., Sacramento, CA).

Effects of temperature. A leaf disk assay (24) was used to examine temperature in relation to suppression of gray mold by isolates of *G. roseum*, *T. viride*, and *Penicillium* sp. and by chlorothalonil. Leaf disks were washed twice in sterile distilled water, inoculated with *B. cinerea*, and incubated at 20–23 C for 48 h on a fiberglass screen (1-mm mesh) that overlaid three layers of moist paper towel in petri dishes in the laboratory. The antagonists, chlorothalonil, or water plus surfactant was applied to the disks, which were transferred immediately to PCA in petri dishes. The dishes were sealed with Parafilm and placed randomly in dark incubators maintained at 10, 15, 20, or 25 C. Conidiophores of *B. cinerea* on the disks were estimated after 7 days. The experimental design was a split plot, with temperature as the main plot factor and the antagonists and chlorothalonil as subplot factors. The treatments were replicated (blocked) six times over time with one petri dish containing 10 disks for each replicate of each treatment. In each repetition, temperatures were assigned randomly to each incubator. The air temperature of the incubators was adjusted 2 days before each test and was measured at 0800, 1600, and 2300 each day with calibrated mercury-in-glass thermometers. Relative suppression (RS) of pathogen sporulation in the treatments was calculated prior to statistical computation, from $RS = (C - c) / C$, in which C and c were the estimated mean number of conidiophores in the check and the treatments, respectively.

Temperature also was studied in relation to conidial germination and germ-tube elongation of the three antagonistic fungi on leaves. Leaf disks sprayed with conidial suspensions of the fungi were placed on PCA in petri dishes. Three replicate dishes of each fungus were incubated at 10, 15, 20, and 25 C. After 24, 48, 72, 96, and 120 h of incubation, one disk from each dish was placed on a microscope slide. Drops of fixative (3:97 glacial acetic acid and 95% ethanol, v/v), fungal stain (0.01 g of cotton blue and 0.01 g of aniline blue in 100 ml of lactophenol), and mountant (lactophenol) were placed successively on the disks, which were examined microscopically. Percent germination and length of germ tubes were estimated based on observations of 100–150 spores and 20 spores, respectively, from each replicate. A spore was considered germinated when the length of the germ tube equaled or exceeded the diameter of the spore. Germ tubes up to 250 μm in length were measured with the aid of an eyepiece reticule. To facilitate statistical computations, germ-tube measure-

ments were transformed to a 0–11 equi-spaced scale (18) in which 0 and 11 represented germ-tube lengths of 0 and ≥250 μm, respectively. Only nontransformed data are reported in the results. The experiment was arranged as a 4 × 5 × 3 split-split plot with three replicated disks per treatment. Temperature, incubation time, and fungi were the main plot, subplot, and sub-subplot factors, respectively.

Effects of leaf senescence and death. Leaf disks were inoculated with *B. cinerea*, incubated in high humidity in petri dishes, as described above, for 24, 48, 72, or 96 h, and kept on PCA for 72, 48, 24, or 0 h, respectively. Ninety-six hours after inoculation, disks were treated with the antagonists, fungicide, or water plus surfactant and transferred to fresh PCA; the number of conidiophores of the pathogen was estimated after 7 days at 20–23 C. The experiment was a 4 × 5 factorial in a completely randomized arrangement, with three replicate dishes, each with 10 disks for a total of 30 disks in each treatment.

In a related study, disks from green, senescent, and dead leaves of plants in the greenhouse (repetition 1) or field (repetition 2) were inoculated with *B. cinerea*, incubated in petri dishes for 48 h, treated with *G. roseum*, chlorothalonil, or water plus surfactant, and incubated on PCA. The number of conidiophores was estimated as before. The study was a 3 × 3 factorial in a completely randomized design, with three replicate dishes, each with five disks for a total of 15 disks per treatment.

Hyphal interactions. Possible interactions between *B. cinerea* and *G. roseum*, between *B. cinerea* and *Penicillium* sp. and between *B. cinerea* and *T. viride* were examined by an assay adapted from earlier studies (3,10,11). Plugs, 7-mm in diameter, from near the advancing edge of the pathogen and antagonist colonies on PDA were positioned 3 cm apart on separation membrane (MW [molecular weight] cut-off = 10 kDa, Fisher Scientific Inc., Pittsburgh, PA) that overlaid a simple agar medium (1 g of sucrose, 0.1 g of aspartic acid, 0.1 g of glutamic acid, and 20 g of agar per 1 L of distilled water) in petri dishes. Paired plugs of *B. cinerea* were used as checks. The paired cultures were kept at 20–23 C and were examined microscopically at intervals during 5 days. To prepare specimens for light microscopy, pieces of membrane were cut, placed on glass slides, fixed in acetic acid plus ethanol, and stained in 2% aniline blue in lactic acid. Nonstained hyphae were examined by phase-contrast microscopy.

TABLE 1. Effects of antagonistic fungi and of chlorothalonil on the estimated density of conidiophores of *Botrytis cinerea* in leaves of strawberry in the greenhouse and on the incidence of conidiophores of the pathogen in overwintered leaves and leaves of the spring flush in a strawberry field plot in 1989[x]

Treatments	No. of conidiophores/ leaf disk[y]	Incidence of conidiophores in leaf disks from field plots (%)	
		Overwintered leaves	Spring leaves
Water + surfactant	120 a[z]	25.7 a	18.7 a
Rhodotorula glutinis	112 a	27.0 a	16.4 a
Fusarium sp.	28 b	23.0 a	23.6 a
Myrothecium verrucaria	2 c	22.0 a	14.0 a
Trichoderma viride	4 c	13.3 b	2.8 b
Penicillium sp.	3 c	9.3 b	0.0 b
Gliocladium roseum	0 c	10.7 b	0.0 b
Chlorothalonil	3 c	9.0 b	3.5 b

[x]The leaves in the greenhouse and those of the spring flush were artificially inoculated with *B. cinerea* approximately 2 and 4 wk, respectively, before the treatments were applied. Overwintered leaves were treated with antagonists on 26 May and 3 June and were harvested on 10 June. Leaves of the spring flush were treated on 13 and 20 June and were harvested on 27 June. Disks of leaves harvested in the greenhouse or in the field were placed on paraquat-chloramphenicol agar and were examined for conidiophores of *B. cinerea*.

[y]From plants treated in the greenhouse; observations of two repetitions of this study were pooled.

[z]Means in a column followed by the same letter are not significantly different ($P \leq 0.05$, protected LSD).

Hyphal interactions also were examined in leaf disks inoculated with *B. cinerea*, incubated on a fiberglass screen in moist petri dishes at 20–23 C for 3 days, and sprayed with the antagonists or water plus surfactant. Treated disks were immediately transferred to PCA, incubated at 20–23 C for 3 days, and cleared by immersion in 95% ethanol at 60 C for 2 h. Cleared disks were dried on filter paper for 10 min and immersed in stain (0.01 g of cotton blue, 0.01 g of aniline blue in 100 ml of lactophenol) under vacuum for 12–24 h. Stained disks were mounted in lactophenol on microscope slides. Pieces of disks, each approximately 4 × 4 mm, were rapidly frozen to −20 C in Cryoform (International Equipment Co., Needham Heights, MA) and sectioned with a freezing microtome. Sections 14-μm thick were examined with a light microscope for growth and distribution of hyphae of *B. cinerea* and the antagonists and for evidence of hyperparasitism.

Data analyses. All experiments were repeated at least once. Statistical computations were performed using the Statistical Analysis System (SAS Institute Inc., Cary, NC). Observations of repeated experiments were subjected to analyses of homogeneity and were pooled accordingly. Observations of pathogen sporulation in biocontrol studies were subjected to analysis of variance (ANOVA). Observations on sporulation incidence of *B. cinerea* were transformed by the arcsine function; however, nontransformed values are reported. Treatment means were separated by protected LSD. In experiments of factorial design, the general linear model (GLM) procedure was used to identify the significance of interactions and main effects of the factors, and orthogonal contrast was used to compare effects of different levels of a factor (unless otherwise specified in the results).

RESULTS

Biocontrol tests. *M. verrucaria*, *T. viride*, *Penicillium* sp., and *G. roseum* suppressed the density of conidiophores of *B. cinerea* by 97–100% in attached strawberry leaves in the greenhouse and were as effective as chlorothalonil (Table 1). The *Fusarium* sp. was only moderately suppressive, and *R. glutinis* did not significantly suppress the pathogen.

In the field in 1989, *T. viride*, *Penicillium* sp., *G. roseum*, and chlorothalonil suppressed sporulation incidence of *B. cinerea* by 48–64% in overwintered leaves and by 85–100% in leaves produced in spring (Table 1). However, *R. glutinis*, *M. verrucaria*, and the *Fusarium* sp. did not suppress sporulation incidence of the pathogen in either test. At the times of treatment application, overwintered leaves were semisenescent and appeared pale green to yellowish or reddish-brown; leaves from the spring flush were green and appeared vigorous.

TABLE 2. Effects of antagonistic fungi and of chlorothalonil on estimated incidence of conidiophores of *Botrytis cinerea* in strawberry leaves in 1990[x]

Treatment	Incidence of quarter leaflets with conidiophores of *B. cinerea* (%)[y]			
	Leaves treated individually		Leaves treated inundatively	
	2 and 9 September	1 and 6 October	2 and 9 September	1 and 6 October
Water + surfactant	49 a[z]	38 a	64 a	31 a
Trichoderma viride	20 b	5 b	30 b	7 b
Penicillium sp.	20 b	6 b	22 bc	7 b
Gliocladium roseum	0 c	1 c	7 d	6 b
Chlorothalonil	5 c	0 c	11 cd	1 b

[x]Leaves in strawberry field plots were treated individually or inundatively approximately 1 mo after inoculation with *B. cinerea*.

[y]Leaves treated on 2 and 9 September were sampled on 16 September; those treated on 1 and 6 October were sampled on 13 October. Quarter leaflets of sampled leaves were kept at high humidity and were examined for conidiophores of *B. cinerea*.

[z]Means in a column followed by the same letter are not significantly different ($P \leq 0.05$, protected LSD).

In field studies in 1990, *T. viride*, *Penicillium* sp., *G. roseum*, and chlorothalonil each suppressed sporulation incidence of *B. cinerea* in strawberry leaves when applied to individual leaves or when inundatively applied to entire plots during early September or early October (Table 2). *G. roseum* suppressed *B. cinerea* as effectively as did chlorothalonil in all tests, and, in comparison to the water-plus-surfactant check, *G. roseum* reduced sporulation incidence of the pathogen by 97–100% after application to individual leaves and by 81–89% when inundatively applied. *G. roseum* was more effective than *T. viride* and *Penicillium* sp., except in plots inundatively treated during October. *T. viride* and *Penicillium* sp. were each proportionately more suppressive to *B. cinerea* after treatments applied during October (77–88% and 77–84%, respectively) than after applications during September (57–59% and 59–66%, respectively). Leaves in all treatments emerged primarily during August and September and were midgreen. Old leaves had been removed by mowing during crop renovation during late July.

In the field studies, humid periods (RH $\geq$ 90%) and mean hourly temperatures were obtained daily for 7 days after each inoculation with *B. cinerea* and after each treatment was applied. During the 7-day periods after the pathogen was inoculated on 13 May 1989 and on 2 August and 4 September 1990, humid periods (with means) ranged from 9 to 18 (14.5), 8 to 16 (13.1), and 10 to 16 h (13.9), respectively, and temperatures ranged from 8 to 14 (11.4), 16 to 23 (21.3), and 10 to 14 C (12.6), respectively. In the 7-day periods after the treatments on 26 May and 3 June and 10 and 27 June 1989 and on 2 and 9 September and 1 and 6 October 1990, mean humid periods were 10.8 and 14.8, 15.1 and 13.3, 14.0 and 14.3, and 8.6 and 17.1 h, respectively, and mean temperatures were 16.1 and 21.0, 17.2 and 24.1, 12.2 and 13.3, and 14.0 and 9.2 C, respectively. Rain ($\geq$5 mm) did not fall within 3 days of treatment, except on 8 October 1990.

Temperature effects. The three antagonists generally suppressed *B. cinerea* in leaf disks with increasing effectiveness as temperatures increased in the tested range of 10–25 C ($P < 0.001$, orthogonal contrast) (Fig. 1). The pattern of increase in suppressing conidiophore production of the pathogen was quadratic ($P < 0.002$, quadratic contrast). At 10 and 15 C, only *G. roseum* markedly suppressed the pathogen; *G. roseum* was significantly more effective at these temperatures than was *Penicillium* sp. or *T. viride* ($P < 0.01$, one-sided, Student's *t* test). *Penicillium* sp. and *T. viride* were ineffective at 10 C and were moderately effective at 15 C. At 20 and 25 C, however, each of the antagonists markedly suppressed conidiophore production of *B. cinerea*, from 92 to 100%. The antagonists suppressed the number of conidiophores as effectively, or almost as effectively, as did chlorothalonil at 20 and 25 C. *G. roseum* was also as effective at 15 C. Suppression of conidiophore production of *B. cinerea* by chlorothalonil was high and was not influenced significantly by temperature ($P > 0.1$, protected LSD).

Temperature and time of incubation interactively affected percent germination of conidia of *G. roseum*, *T. viride*, and *Penicillium* sp. on leaf disks incubated on PCA ($P < 0.001$, GLM). The rate of germination of each fungus generally increased with temperature in the range tested and was most rapid at 25 C ($P < 0.001$, orthogonal contrast) (Fig. 2). *G. roseum* germinated faster than did *T. viride* and *Penicillium* sp. ($P < 0.001$, orthogonal contrast), especially at 10–20 C.

Temperature and time of incubation also interactively affected germ-tube growth of the antagonists on leaf disks ($P < 0.001$, GLM). Rate of growth of germ tubes of each fungus generally increased with temperature ($P \leq 0.01$, orthogonal contrast). After various incubation times at various test temperatures, germ tubes were significantly and often markedly longer in *G. roseum* than in *T. viride* and *Penicillium* sp. (Table 3). Differences in germ-tube length among the antagonists were greatest at 10 C.

Effects of leaf senescence and death. Incubation of leaf disks inoculated with *B. cinerea* on PCA for 0–72 h immediately before application of the control agents progressively reduced pathogen suppression by the antagonists ($P < 0.001$, GLM) but did not affect suppression by the fungicide (Fig. 3). The ability of the

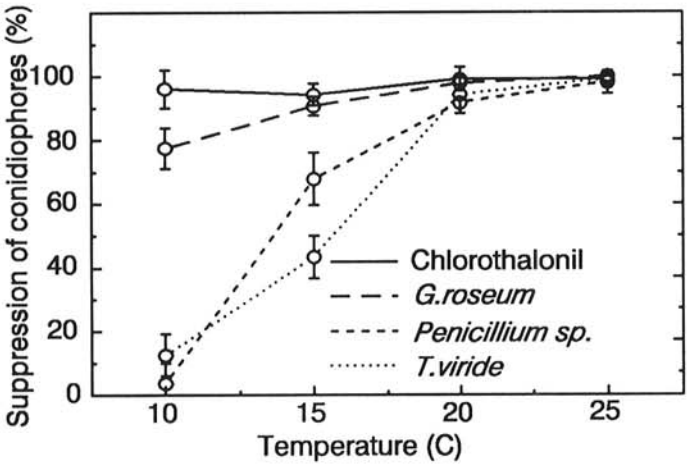

Fig. 1. Suppression of conidiophore production of Botrytis cinerea in strawberry leaf disks (1-cm diameter) by chlorothalonil, *Gliocladium roseum*, *Penicillium* sp., and *Trichoderma viride* at various temperatures. Values, with standard error bars, are relative to those obtained in water-plus-surfactant checks.

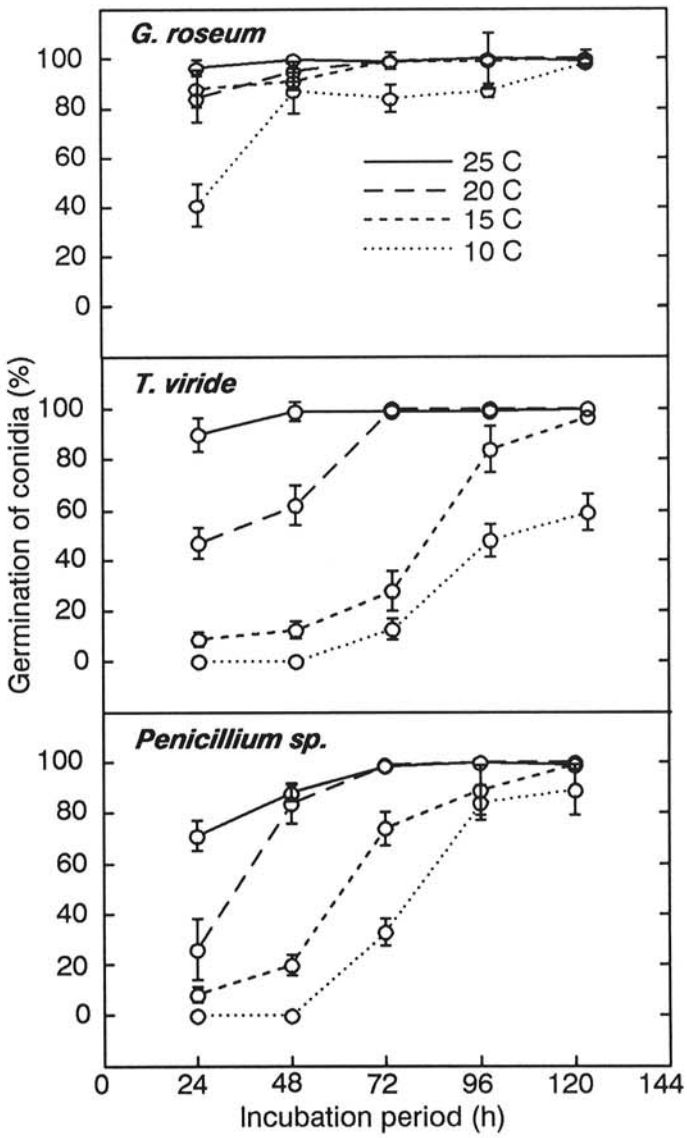

Fig. 2. Percent germination of conidia of *Gliocladium roseum*, *Trichoderma viride*, and *Penicillium* sp. on strawberry leaf disks incubated on paraquat-chloramphenicol agar as a function of time and temperature. Standard errors are shown.

antagonists to suppress conidiophore production of *B. cinerea* was only slightly affected when the disks were incubated on PCA for 24 h ($P > 0.28$, orthogonal contrast) but was markedly reduced or eliminated in disks incubated for 48 or 72 h ($P \leq 0.05$, orthogonal contrast). The PCA treatments did not significantly affect conidiophore production in the water-plus-surfactant and chlorothalonil treatments ($P > 0.05$, PLSD). Chlorothalonil suppressed conidiophores almost completely in all PCA treatments. Leaf disks incubated on PCA for 0 or 24 h appeared midgreen, and those incubated for 48 and 72 h were pale green and pale green to brown, respectively.

G. roseum suppressed conidiophore production of *B. cinerea* by 88–100% and was as effective as chlorothalonil when green or senescent leaves were inoculated with the pathogen, treated 48 h later with the antagonist or the fungicide, and placed on PCA. The antagonist failed to suppress conidiophore production, but chlorothalonil suppressed the pathogen by >98% in similarly treated dead leaves.

Hyphal interactions. In the separation-membrane assay, *G. roseum* and *Penicillium* sp. inhibited growth of *B. cinerea* compared to that of the check, and their hyphae did not contact those of the pathogen during 5 days of observations. No morphological changes were observed in hyphae of the pathogen in the presence of *G. roseum* and *Penicillium* sp. *T. viride* did not suppress growth of *B. cinerea*, and paired colonies of the two fungi grew together. Hyphae of *T. viride* occasionally coiled around those of *B. cinerea* but did not form penetration structures or penetrate hyphae of the pathogen.

Hyphae of *G. roseum*, *T. viride*, and *Penicillium* sp. grew profusely on surfaces of inoculated leaf disks incubated on PCA and often penetrated the epidermis and colonized the mesophyll. Colonies of *G. roseum* were frequent in epidermal cells. Hyphae of the antagonists did not coil around or penetrate those of *B. cinerea*, neither on the leaf surface nor within the leaf tissues. Growth of *B. cinerea* was sparse in epidermal cells that also were invaded by antagonists but often were abundant in similar, noninvaded cells. Relatively long hyphae (>100 μm) of *B. cinerea* were infrequent in leaf disks treated with the antagonists but were frequent in water-plus-surfactant checks. After 3 days on PCA, *B. cinerea* sometimes began to sporulate in the checks but not in disks treated with the antagonists.

DISCUSSION

The isolates of *G. roseum*, *T. viride*, and *Penicillium* sp. markedly suppressed sporulation of *B. cinerea* in strawberry leaves under a wide range of conditions. The antagonists suppressed the pathogen almost completely in the greenhouse, confirming earlier observations (22,24). When applied in field tests to green leaves inoculated 27–38 days earlier with *B. cinerea*, the antagonists suppressed the pathogen by 57–100%. *T. viride* and *Penicillium* sp. were highly effective when evaluated during the spring and early fall and were moderately effective during late summer. *G. roseum*, however, effectively suppressed *B. cinerea* in all field studies and was consistently as effective as chlorothalonil, which in earlier studies, suppressed the pathogen in strawberry leaves better than other commercially available fungicides (4,5,29). The antagonists generally performed well against *B. cinerea* when applied to infected leaves that were green but performed only moderately well in leaves that had overwintered and were semisenescent. The greater suppression of *B. cinerea* in individually treated leaves compared to leaves of inundatively treated plots may indicate that the inundative treatments did not provide adequate coverage of all leaves.

A notable feature of the biocontrol tests was the application of antagonists 2–5 wk after the leaves were inoculated with the pathogen or after the leaves were exposed to natural inoculum of *B. cinerea* during several months. From the postinoculation conditions and high incidences of *B. cinerea* in leaves of water checks, the pathogen probably infected the leaves shortly after inoculation in the field as well as under controlled conditions. The postinoculation humid periods and temperatures observed in each field test frequently were conducive to infection (7; J. C. Sutton, *unpublished data*). Based on previous observations (7), the pathogen probably was quiescent in the epidermal cells when treatments were applied to green leaves but progressively colonized the tissues of the semisenescent overwintered leaves treated in 1989. The procedures used in the biocontrol tests contrasted those of earlier studies in which healthy leaves were treated with antagonists and were later inoculated with *B. cinerea* (22,24). However, the isolates of *G. roseum*, *T. viride*, and *Penicillium* sp. suppressed the pathogen when either procedure was used.

The *G. roseum* isolate was effective against *B. cinerea* in a wider temperature range than were the isolates of *Penicillium* sp. and *T. viride*. In the controlled studies, *G. roseum* effectively suppressed sporulation of *B. cinerea* in leaf disks in the temperature range tested (10–25 C), but *Penicillium* sp. and *T. viride* were effective only at 15–25 and 20–25 C, respectively. In the field, each of the fungi effectively suppressed *B. cinerea* during late May and June, when the weather was warm. However, under cooler conditions during September and October, *G. roseum* generally was more effective than *Penicillium* sp. and *T. viride*. Because of faster rates of germination and of germ-tube elongation

TABLE 3. Effects of temperature and incubation time on growth of germ tubes of *Gliocladium roseum*, *Trichoderma viride*, and *Penicillium* sp. on strawberry leaf disks incubated on paraquat-chloramphenicol agar

Temp. (C)	Incubation time (h)	Mean length of germ tubes (μm)		
		G. roseum	*T. viride*	*Penicillium* sp.
10	24	13	0	0
	48	38	0	0
	72	113	14	36
	96	238	23	72
	120	>250	35	66
15	24	68	13	14
	48	226	58	71
	72	>250	>250	114
	96	...[z]	...	>250
20	24	94	74	83
	48	>250	>250	189
	72	...	...	>250
25	24	>250	117	136
	48	...	>250	228
	72	...	...	>250

[z] Lengths >250 μm were not recorded.

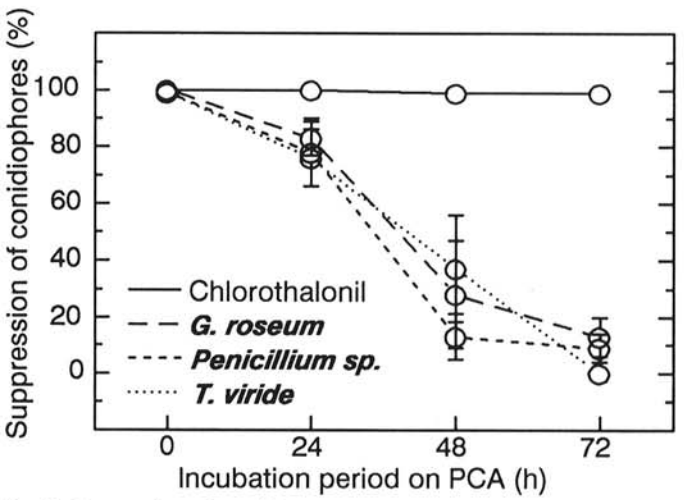

Fig. 3. Suppression of conidiophore production of *Botrytis cinerea* in infected strawberry leaf disks (1-cm diameter) incubated for varying periods on paraquat-chloramphenicol agar (PCA) and immediately treated with water, chlorothalonil, *Trichoderma viride*, *Penicillium* sp., or *Gliocladium roseum*. The fungicide and biocontrol agents were applied 96 h after the disks were inoculated with *B. cinerea*. Standard errors are shown.

on leaves at 10–25 C, *G. roseum* may penetrate and invade the leaf tissues more rapidly and suppress *B. cinerea* more effectively than can *T. viride* or *Penicillium* sp., especially under cool conditions. The increased rate of germination and growth of *T. viride* and *Penicillium* sp. as temperatures increased may have contributed to the more effective biocontrol of *B. cinerea* by these fungi at higher temperatures.

The antagonists suppressed *B. cinerea* in infected leaves primarily when applied while the leaves were green or senescent. All three antagonists were ineffective against *B. cinerea* when applied to leaves killed by incubation on PCA for ≥48 h, and *G. roseum* failed to suppress the pathogen when tested on leaves that had died naturally. Incubation of infected leaves on PCA for only 24 h prior to treatment with the antagonists, however, only slightly reduced biosuppression. Effectiveness of biocontrol declined sharply, apparently a few hours before or after the leaves died. A similar decline may explain the lower effectiveness of the antagonists in suppressing *B. cinerea* in semisenescent overwintered leaves compared to green leaves in the field studies.

Collectively, the observations support the concept that the antagonists suppressed *B. cinerea* when the pathogen invaded senescent or dead leaf tissues. Previous reports indicated that colonization is initiated from sites of quiescent infection in the epidermis (7), and the pathogen normally is able to colonize and potentially sporulate in the leaves 3–5 days after the leaves die or are killed on PCA (22–24). In the present study, *G. roseum*, *Penicillium* sp., and *T. viride* applied to infected leaves subsequently killed on PCA grew profusely on the leaf surface, invaded the tissues, reduced incidence and length of *B. cinerea* hyphae within the tissues, and suppressed sporulation by the pathogen. The antagonists also suppressed sporulation when applied after the infected tissues were incubated on PCA for 24 h but were only marginally suppressive when applied after 48 h of incubation. In addition, *G. roseum* suppressed sporulation potential when applied to infected leaves that were green or senescent but not after the leaves had died naturally. From these observations, the antagonists may suppress *B. cinerea* only while the pathogen is colonizing the tissues and probably are ineffective in tissues already colonized or possessed (9) by the pathogen. The faster germination and growth of the antagonists and generally increased suppression of *B. cinerea* at higher temperatures studied indicate that effective biocontrol probably depends on the rate as well as the timeliness of tissue invasion by the antagonists.

Competition between the antagonists and *B. cinerea* for leaf substrate was probably a key mechanism of the observed biocontrol. Based on the frequent occurrence of *G. roseum*, *T. viride*, and *Penicillium* sp. on strawberry leaves in the field (20; J. C. Sutton, *unpublished data*) and on the rapid growth of these fungi observed in dead leaves in the present study, the antagonists may be ecologically well adapted and have high exploitive potential in strawberry leaves. Observations in the separation-membrane assay indicated the isolates of *G. roseum* and *Penicillium* sp. can suppress growth of *B. cinerea* through antibiosis, a possibility supported in an earlier study in which an antifungal metabolite from cultures of *G. roseum* inhibited growth of *B. cinerea* (21). However, a mutant of G. roseum with enhanced production of the metabolite and a second mutant that was completely deficient for the metabolite both suppressed *B. cinerea* in strawberry leaves as effectively as did the wild type, indicating that antibiosis may not be important for biocontrol by *G. roseum*, at least in leaves already infected by *B. cinerea*. Similar observations were made in biocontrol of other pathogens (15,17). Mycoparasitism of fungi has been reported for species of *Gliocladium* and *Trichoderma*, including *G. roseum* and *T. viride* (1,2,10,12,16,28,31,32), but no evidence was found in the present study for extracellular or intracellular hyperparasitism of *B. cinerea*. The observations of suppressed hyphal development and sporulation of *B. cinerea* in leaves treated with *G. roseum*, *T. viride*, and *Penicillium* sp. were compatible with concepts of exploitive competition, in which the biocontrol organism depletes the resources of the leaf, and interfering competition, in which the control organism inhibits access to the resources (19,33).

Applications of *G. roseum*, *T. viride*, and *Penicillium* sp. to strawberry leaves may be useful for managing gray mold fruit rot in the field. These fungi suppressed sporulation by *B. cinerea* as effectively, or almost as effectively, as did chlorothalonil in field studies. Although the effect of foliar applications of the antagonists on fruit rot was not measured, in earlier studies, chlorothalonil applied during the fall and early spring suppressed fruit rot as effectively as conventional captan sprays applied when the strawberries were flowering and fruiting (4,5,29). From the effectiveness of the antagonists when applied to green or senescent as opposed to dead leaves, applications timed to precede the death of leaves of successive flushes (7) may be prudent. Subsequent sporulation by the antagonists in the dead leaves may help to perpetuate biocontrol in subsequent flushes of leaves or on flowers and fruits. Inundative applications to foliage could be integrated with delivery of inoculum to the flowers by bees (25). In Ontario, application of *G. roseum*, *T. viride*, and *Penicillium* sp. to strawberry crops would generally augment indigenous populations of these fungi (20,21).

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