Biological Control of Blossom Blight of Alfalfa Caused by *Botrytis cinerea* Under Environmentally Controlled and Field Conditions

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ABSTRACT

Li, G. Q., Huang, H. C., Acharya, S. N., and Erickson, R. S. 2004. Biological control of blossom blight of alfalfa caused by *Botrytis cinerea* under environmentally controlled and field conditions. Plant Dis. 88:1246-1251.

Fungal and bacterial antagonists were tested for their inhibition of sporulation of *Botrytis cinerea* on detached alfalfa florets. *Clonostachys rosea*, *Gliocladium catenulatum*, and *Trichoderma atroviride* were evaluated for protecting young blossoms and pods of alfalfa from infection by *B. cinerea* in vitro. *C. rosea* was further tested to control pod rot and seed rot caused by *B. cinerea* under field conditions. The results showed that four of the tested antagonists, *C. rosea*, *G. catenulatum*, *T. atroviride*, and *Trichothecium roseum*, could inhibit sporulation by *B. cinerea* on detached alfalfa florets. Both *C. rosea* and *G. catenulatum* were effective in suppression of infection of alfalfa pods by *B. cinerea* when inoculated on fresh petals of alfalfa at the anthesis stage, and their efficacy was greater than that of *Trichoderma atroviride*. A significant suppression of *B. cinerea* by *C. rosea* and *G. catenulatum* on pods and seed of alfalfa was observed when they were inoculated on senescent petals at the pod-development stage. Results of a field trial indicated that *C. rosea* applied to upper parts of alfalfa plants significantly suppressed pod rot and seed rot caused by *B. cinerea*, and significantly increased seed production of alfalfa in each of 3 years. These studies show that *C. rosea* has potential as a biocontrol agent for control of alfalfa blossom blight caused by *B. cinerea*.

Botrytis cinerea Pers.:Fr. is a ubiquitous phytopathogenic fungus that infects a wide range of plants, including many economically important crops (16). On alfalfa, it can cause shoot and stem blight (33) and blossom blight (14). This pathogen, together with *Sclerotinia sclerotiorum* (Lib.) de Bary, has become a severe problem for alfalfa seed production, especially in irrigated regions in western Canada (9–11).

Senescent petals of alfalfa usually remain attached to pods during pod development. This botanical characteristic predisposes pods and seed of alfalfa to infection by *B. cinerea* as well as by *S. sclerotiorum* (14) under certain conditions, because the

two pathogens require exogenous nutrients for spore germination, growth of germ tubes, formation of appressoria, and infection of healthy plant tissues. Huang et al. (14) and Li et al. (23) showed that pollen grains of alfalfa, which can be found on petals in large quantities, could stimulate conidial germination and growth of germ tubes of pathogens such as *B. cinerea* and antagonists such as *Coniothyrium minitans* (23) and *Clonostachys rosea* (24). We hypothesized that use of biocontrol agents (BCAs) to compete with *B. cinerea* for the nutrients on petals may be effective in suppression of alfalfa blossom blight caused by this pathogen.

Several species of BCAs have been tested for control of *B. cinerea* in previous studies. They include *Trichoderma harzianum* (5), *Clonostachys rosea* (= *Gliocladium roseum* Bainier; 2,29,31,34,35), *Ulocladium atrum* (7,21,22,30), *Candida* spp. (1,8), *Rhodosporidium toruloides* (4), and *Paenibacillus polymyxa* (12). Commercial products based on *T. harzianum*, such as Trichodex (T₃₉), and on *Candida oleophila*, such as Aspire (I-182), now are available for protection of greenhouse vegetables and post-harvest fruits, respectively, from infection by *B. cinerea* (5). However, there is no report on biocontrol of alfalfa blossom blight caused by *B. cinerea* in the literature.

A few fungal and bacterial antagonists of *B. cinerea*, which had been reported in

other studies, were isolated in our previous research (15). However, their potential as antagonists or BCAs for suppression of alfalfa blossom blight caused by *B. cinerea* remained unknown. Therefore, the objectives of this study were to (i) compare the effect of the fungal and bacterial antagonists of our collections on sporulation of *B. cinerea* on detached alfalfa florets, (ii) compare the efficacy of selected antagonists for suppression of alfalfa pod rot or seed rot caused by *B. cinerea* under environmentally controlled conditions, and (iii) determine the efficacy of *Clonostachys rosea* to control alfalfa blossom blight under field conditions.

MATERIALS AND METHODS

Fungal and bacterial strains. Twelve strains, comprising nine fungi and three bacteria, of candidate antagonists and a strain of *B. cinerea* were used in this study (Table 1). Each of the strains was stored in 10% glycerol solution at –20°C. Working cultures of each strain were established by transferring a stock agar plug onto a potato dextrose agar (PDA) plate (9 cm in diameter) and incubating at 20°C for 1 to 4 weeks.

Inoculum preparation. Mycelial agar plugs of each strain from 3-day-old colony margins were placed onto petri dishes (9 cm in diameter), each containing approximately 20 ml of PDA, one plug per dish. The dishes were incubated at 20°C under fluorescent light (15.2 µE s⁻¹ m⁻²) for 20 days. Spores were washed off by flooding the colony in each dish with 10 ml of sterile distilled water and scraping the colony surface using a glass spatula. The spore suspensions of each strain were pooled from dishes and filtered through four layers of cheesecloth. Spore concentrations were determined using a hemacytometer and a compound microscope. Similar procedures were used to obtain bacterial suspensions. Bacterial concentration of each strain was determined by plating serially diluted bacterial suspensions onto PDA and counting the number of colonies on each dish after incubation for 4 days at room temperature (20 ± 2°C). The fungal and bacterial suspensions were stored for a short period of time at 4°C until used.

Spore powder inoculum of *C. rosea* GR-8 was prepared for the field trials. Agar plugs (5 mm in diameter) containing conidia and mycelia of this fungus were

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This research was funded by the Alberta Agriculture Research Institute, Farming for the Future, Project No. 19990070.

Clonostachys rosea strain GR-8 and *Trichoderma atroviride* strain Tw-1 were obtained from China with the Import Permit No. P-2000-01296 issued by the Canadian Food Inspection Agency.

This manuscript is Lethbridge Research Centre contribution number 38703077.

Accepted for publication 25 June 2004.

Publication no. D-2004-0901-01R
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inoculated on autoclaved wheat bran-cornmeal mixture (wheat bran:cornmeal: water = 150:150:300, wt/wt/vol) in aluminium trays (30 by 25 by 8 cm, length by width by height), six plugs per tray. The trays were covered with 2-mm meshed screen (Thomas Scientific, Philadelphia). Spore concentration in the powder was determined by dilution plating on PDA. Non-inoculated wheat bran-cornmeal mixture was dried and ground to powder as a control.

Experiment 1: suppression of *B. cinerea* sporulation on detached alfalfa florets by antagonists. Seed of alfalfa (*Medicago sativa* L. cv. Barrier) were sown in Cornell Peat-Lite mixes (3) in plastic pots (15 cm in diameter) in a greenhouse and seedlings were watered as required. Six months later, these plants began to flower. They were trimmed once a month. Racemes that formed on 20-day-old shoots of alfalfa (after trimming) were used for all of the environmentally controlled experiments of this study. For this experiment, newly opened florets were removed from racemes of alfalfa and the pedicel of each floret was inserted in moist autoclaved vermiculite in plastic petri dishes (9 cm in diameter), 12 florets per dish. Aliquots of 20 μ l of aqueous fungal spore or bacterial cell suspension of each tested antagonist (2.2 to 7.4×10^6 spores or CFU/ml) were individually inoculated on florets in each dish. Two control treatments, the *B. cinerea* alone and the water alone, were included in this experiment. Florets in these control treatments were treated with sterile distilled water, also at 20 μ l per floret. Two hours later, the florets for the treatment of antagonists plus *B. cinerea* or *B. cinerea* alone were inoculated with the conidial suspension of *B. cinerea* (5×10^5 conidia/ml) at 20 μ l per floret. The florets for the treatment of the water alone were treated with sterile distilled water. There were three dishes for each treatment. The dishes for all treatments were individually sealed with parafilm and incubated in a growth chamber at 20°C under continuous fluorescent light (15.2 μ E s⁻¹ m⁻²) in a growth chamber. Sporulation of *B. cinerea* on each floret of alfalfa was determined after incubation at 20°C for 10 days. The percentage of alfalfa florets with *B. cinerea* sporulation was calculated based on the number of florets with sporulation of *B. cinerea* and the total number ($n = 12$) of alfalfa florets in each dish. The experiment was repeated once.

Experiment 2: colonization of young petals by selected antagonists and development of Botrytis pod rot. An experiment was conducted to show the efficacy

of *C. rosea* strains GR-8 and LRC 2090, *G. catenulatum* LRC 2087, and *T. atroviride* Tw-1 for suppression of infection of attached alfalfa pods or seed by *B. cinerea*. *Trichothecium roseum* LRC 2424 was not used in this experiment, because it produced fewer spores that were not enough for the test.

At the anthesis stage, 10 shoots of greenhouse-grown alfalfa plants with newly opened florets in a pot (20 days old after trimming) were selected randomly and 15 florets in a raceme on each shoot were individually pollinated by tripping the keel petal with a sterilized wooden toothpick using the method described by Huang et al. (14). The tripped florets in each pot were used for the following treatments: (i) water alone, (ii) *B. cinerea* alone, (iii) antagonist alone (GR-8, LRC 2090, LRC 2087, or Tw-1), or (iv) antagonist (GR-8, LRC 2090, LRC 2087, or Tw-1) + *B. cinerea*. For the treatment of antagonist + *B. cinerea*, aliquots of 15 μ l of aqueous spore suspension of each antagonist (5.0×10^6 conidia/ml) were individually inoculated onto the keel petal of each tripped floret of alfalfa with a pipette and, 2 h later, spore suspension of *B. cinerea* (4×10^5 conidia/ml) was challenge-inoculated at 15 μ l per floret. In the other treatments, the tripped florets on each raceme were inoculated with water alone; *B. cinerea* alone (4×10^5 conidia/ml), or each antagonist alone (5.0×10^6 conidia/ml) at 15 μ l per floret, and the challenge inoculation was water alone instead of the spore suspension of *B. cinerea*. The treated racemes were individually covered with clear plastic bags. The alfalfa plants were moved to and maintained in a growth chamber (20°C) under fluorescent light for 12 h per day. Pod formation and infection of pods by *B. cinerea* on each inoculated raceme were determined at 7 and 30 days after inoculation, respectively. The experiment was repeated once with three replicates for each treatment.

Experiment 3: colonization of senescent petals by selected antagonists and development of Botrytis pod and seed rot. To study the efficacy of selected antagonists *C. rosea* GR-8 and *G. catenulatum* LRC 2087 for suppression of *B. cinerea* at the pod-development stage, alfalfa florets were tripped as described previously. *Trichoderma atroviride* Tw-1 was not tested, because it was not as effective as GR-8 or LRC 2087 in suppression of *B. cinerea* when applied on petals of alfalfa at the anthesis stage. Eight racemes on eight alfalfa shoots (20 days old after trimming) in plants of each pot were tripped and the pollinated alfalfa florets were used for the inoculation test. The plants were maintained in a growth chamber (20°C) for 14 days to allow the formation and the development of alfalfa pods. During this period, petals on the tripped florets became senescent but remained attached to the pods.

The senescent petals in each raceme were individually treated with (i) water alone, (ii) *B. cinerea* alone, (iii) antagonist (GR-8 or LRC 2087) alone, or (iv) antagonist (GR-8 or LRC 2087) + *B. cinerea*. In the treatment of antagonist + *B. cinerea*, aliquots of 15 μ l of spore suspension of each antagonist (5.0×10^6 conidia/ml) were individually inoculated onto a senescent petal attached to an alfalfa pod in a raceme using a pipette and, 2 h later, spore suspension of *B. cinerea* (4×10^5 conidia/ml) was challenge inoculated on the same petals, also at 15 μ l per petal. In the other treatments, the senescent petals attached to alfalfa pods in a raceme were inoculated with water alone, *B. cinerea* alone (4×10^5 conidia/ml), or the conidial suspension of GR-8 or LRC 2087 (5.0×10^6 conidia/ml) at 15 μ l per petal; the challenge inoculation was water alone instead of the spore suspension of *B. cinerea*. The treated racemes were individually covered with clear plastic bags and the plants were maintained in the same growth chamber as previously described. The number of infected pods, number of seed formed per pod, and the number of infected seed in each treated raceme were determined 30 days after inoculation. The experiment was repeated once with three replicates for each treatment.

Experiment 4: efficacy of *C. rosea* for suppression of Botrytis blossom blight of alfalfa under field conditions. A field trial was conducted in each of 3 years (2000 to 2002) in different parts of an alfalfa field to test the efficacy of *C. rosea* GR-8 in the suppression of pod rot and seed rot of alfalfa caused by *B. cinerea*. The experiment was independently carried out each year in nylon mesh tents (2 by 1.5 by 1.5 m, length by width by height), which were set up in an alfalfa field when about 20% of alfalfa plants were flowering. Scarified seed of alfalfa (cv. AC Blue J) were broadcast to the experiment field at a rate of 500 kg/ha. The seed were harrowed into soil using a harrow packer in June 1999. Therefore, alfalfa plants established in that field were not arranged in rows. The plot of alfalfa plants inside each tent served as a replicate for each treatment. Three treatments each with three replicates were included: (i) water alone, (ii) *B. cinerea* alone, and (iii) *C. rosea* GR-8 + *B. cinerea*. The treatments in the trial of each year were arranged in a randomized complete block design.

In the treatment of *C. rosea* GR-8 + *B. cinerea*, conidia of GR-8 were prepared on PDA as described previously. Spore suspensions (6.7×10^6 conidia/ml) of GR-8 were amended with Pelgel (0.5%, wt/vol; LiphaTech, Milwaukee, WI) and Tween 20 (0.03%, vol/vol) and sprayed onto the blossoms of alfalfa in the tents to the point of runoff (approximately 1,000 ml per tent). The spray was applied three times each year (29 June, 7 July, and 13 July 2000; 26 July, 31 July, and 8 August 2001;

and 3 July, 17 July, and 1 August 2002). Conidial suspensions of *B. cinerea* (5×10^5 conidia/ml) amended with Pelgel and Tween 20 were sprayed 1 day after each application of *C. rosea* (approximately 1,000 ml/tent). In the treatment of *B. cinerea*, tap water plus Pelgel (0.05%, wt/vol) and Tween 20 (0.03%, wt/vol) was sprayed first and the conidial suspensions of *B. cinerea* (5×10^5 conidia/ml) were sprayed 1 day later. In the treatment of water, the two sprays were tap water plus Pelgel (0.05%, wt/vol) and Tween 20 (0.03%, wt/vol) instead of spore suspensions of GR-8 or *B. cinerea*.

In order to pollinate the alfalfa florets in the tents, leafcutter bees (*Megachile rotundata*) were released once a year into each tent. The bees were provided by MR Pollination Services of Coaldale, Alberta, Canada. In a cold room (10°C), bees were sorted in petri dishes (9 cm in diameter) into males and females, with 23 female and 12 male bees per plot in 2000, and 40 female and 20 male bees per plot in 2001 and 2002. Before releasing, the bees were coated with ground powder of wheat bran–cornmeal mixture (control powder) for the treatments of water and *B. cinerea* alone or the spore powder of GR-8 (6.1×10^8 conidia/g) for the treatment of *C. rosea* GR-8 + *B. cinerea*. The coatings were done by adding 5 g of the control powder or the spore powder of *C. rosea* to each petri dish and gently shaking for about 5 min. Bees were released to corresponding field plots immediately after coating, 2 days after the first application of *B. cinerea*. The tents were closed with zippers after the release of leaf-cutter bees. The natural rain precipitations were 29.8, 6.4, and 51.2 mm/month during the experiment period for 2000, 2001, and 2002, respectively. Alfalfa plants in the tents were sprinkle irrigated 14, 10, and 2 times with

an average interval of 5 days for the trial of 2000, 2001, and 2002, respectively. The water precipitation of each irrigation was 17.2, 20.3, and 63.5 mm, respectively, for the trials of these years.

When most of the alfalfa pods were mature, tents were removed and racemes of alfalfa pods were randomly sampled on 26 September 2000, 10 October 2001, and 18 October 2002 from the middle part of each plot (five points, one on each of the four sides and one in the middle of each plot) with a minimum of 100 alfalfa racemes/plot. The number of healthy and diseased pods and the number of healthy and diseased seed were determined from the sampled racemes. Alfalfa seed were harvested approximately 1 week after sampling using a Wintersteiger small plot combine (Ried im Innkreis, Austria), and seed were dried at room temperature ($20 \pm 2^\circ\text{C}$), cleaned, and weighed.

Data analyses. Analysis of variance (ANOVA) was performed to determine the efficacy of *C. rosea* as well as other antagonists in the suppression of *B. cinerea* under different conditions. Data for the same treatment in different trials were pooled for each treatment in the experiments on in vitro (experiment 1) and in vivo (experiment 2) florets of alfalfa, because they were not significantly different in the analyses ($P > 0.05$). Data on incidence of diseased pods, incidence of diseased seed, and number of seed per pod collected in the two runs of experiment 3 were analyzed separately because they differed greatly between these two runs. Data on incidence of diseased pods, incidence of diseased seed, number of seed per pod, and seed yield of alfalfa collected in different years of experiment 4 also were analyzed independently. Percentage data of all the experiments were arcsine transformed prior to analysis. Means were

separated using Duncan's multiple range tests at $P = 0.05$.

RESULTS

Experiment 1: suppression of *B. cinerea* sporulation on alfalfa florets. Water-soaked lesions appeared on detached alfalfa florets after incubation for 3 days in the treatment of *B. cinerea* as well as all the treatments of antagonist + *B. cinerea*. Sporulation of *B. cinerea* occurred after incubation for 10 days in the treatment of *B. cinerea* and some of the treatments of antagonist + *B. cinerea*. No conidiophore production by *B. cinerea* was observed in the treatments of *G. catenulatum* LRC 2087 + *B. cinerea*, *C. rosea* LRC 2090 + *B. cinerea*, *C. rosea* GR-8 + *B. cinerea*, *T. atroviride* Tw-1 + *B. cinerea*, and *Trichothecium roseum* LRC 2424 + *B. cinerea*. The efficacy of BCAs LRC 2087, LRC 2090, Gr-8, Tw-1, and LRC 2424 for suppression of sporulation of *B. cinerea* was 100% (Table 1). The efficacy of *Talaromyces flavus*, *Trichoderma harzianum*, *T. virens*, *T. viride*, *Pseudomonas putida*, *P. fluorescens*, and *Bacillus subtilis* ranged from 11.1 to 44.4%. *Coniothyrium minitans* did not show any inhibition of *B. cinerea* sporulation (Table 1).

Experiment 2: colonization of young petals by selected antagonists and development of Botrytis pod rot. Young pods of alfalfa were visible 7 days after tripping in all of the treatments except for that with *Botrytis cinerea*. The frequency of pod formation for the treatment of water was 70.3% (Table 2). This value was not significantly different ($P > 0.05$) from the treatments of *Clonostachys rosea* LRC 2090 alone, *C. rosea* GR-8 alone, *G. catenulatum* LRC 2087 alone, *T. atroviride* Tw-1 alone, and LRC 2090 + *B. cinerea*. For the treatments of Tw-1 + *B. cinerea* and LRC 2087 + *B. cinerea*, the frequency

Table 1. Origin of the fungal and bacterial antagonists and *Botrytis cinerea* used in this study and in vitro suppression of *B. cinerea* sporulation on alfalfa florets by the fungal and bacterial antagonists (experiment 1)

Strain ^x	Species	Origin	Suppression of sporulation (%) ^y
LRC 2087	<i>Gliocladium catenulatum</i>	A sclerotium of <i>Sclerotinia sclerotiorum</i> , Manitoba, Canada, DAOM 149586	100.0 a
LRC 2090	<i>Clonostachys rosea</i> ^z	Dr. J. Bissett (ECORC-AAFC, Canada) DAOM 174998	100.0 a
GR-8	<i>C. rosea</i>	A sclerotium of <i>S. sclerotiorum</i> , Wuhan, China, 1993	100.0 a
Tw-1	<i>Trichoderma atroviride</i>	Canola rhizosphere, Wuhan, China, 1998	100.0 a
LRC 2424	<i>Trichothecium roseum</i>	A sclerotium of <i>S. sclerotiorum</i> , Alberta, Canada, 1987	100.0 a
LRC 2152	<i>Talaromyces flavus</i>	Sunflower rhizosphere, Manitoba, Canada, 1976, DAOM 172557	44.4 b
LRC 2428	<i>Trichoderma harzianum</i>	Dr. B. J. R. Alexander (University of Auckland, New Zealand), NZ 4208	36.1 b
LRC 2425	<i>T. virens</i>	A sclerotium of <i>B. cinerea</i> on sunflower, Manitoba, Canada, 1978	22.2 c
LRC 941	<i>Pseudomonas fluorescens</i>	Alfalfa rhizosphere, Alberta, Canada, 1993	22.2 c
LRC 944	<i>Bacillus subtilis</i>	Sunflower rhizosphere, Alberta, Canada, 1993	19.4 c
LRC 2429	<i>T. viride</i>	A sclerotium of <i>S. sclerotiorum</i> , Manitoba, Canada, 1975	16.7 d
LRC 956	<i>P. putida</i>	Safflower rhizosphere, Alberta, Canada, 1993	11.1 d
LRC 2137	<i>Coniothyrium minitans</i>	A sclerotium of <i>S. sclerotiorum</i> in Manitoba, Canada, 1977	0.0 e
LRC 2421	<i>Botrytis cinerea</i>	Diseased lentil plant, Alberta, Canada	0.0 e

^x LRC stands for Lethbridge Research Centre, GR stands for *Gliocladium roseum*, and Tw stands for *Trichoderma* isolated from Wuhan. Strains LRC 941, LRC 944, and LRC 956 are bacteria, whereas other strains are fungi.

^y Suppression (S) of *B. cinerea* sporulation (BCS) on florets was calculated as: $S = (\text{percentage of florets with BCS in the } B. \text{ cinerea treatment} - \text{percentage of alfalfa florets with BCS in the treatment of antagonist plus } B. \text{ cinerea}) / \text{percentage of alfalfa florets with BCS in the } B. \text{ cinerea treatment} \times 100\%$. These data are an average of the two runs of the experiment for each antagonist. Means followed by the same letter in column are not significantly different at $P = 0.05$ according to Duncan's multiple range test.

^z *Clonostachys rosea* = *Gliocladium roseum* Bainer.

of pod formation was reduced compared with the water-control treatment. Although petals of alfalfa treated with water or each of the four biocontrol agents alone remained healthy, water-soaked lesions appeared after incubation for 3 days on petals treated with *B. cinerea* alone. After 30 days, all of the senescent petals in the treatment of *B. cinerea* alone were colonized by *B. cinerea* with the formation of profuse conidiophores and conidia. In the treatments of LRC 2087 + *B. cinerea*, LRC 2090 + *B. cinerea*, and GR-8 + *B. cinerea*, most of the senescent petals were colonized by the treated antagonists. Small lesions on pods were observed in these treatments and the frequency of infection of pods by *B. cinerea* was 10.4, 12.2, and

12.6%, respectively (Table 2). In the treatment of Tw-1 + *B. cinerea*, colonization of senescent petals by this fungus was rarely observed and the pod infection was 55.0% (Table 2).

Experiment 3: colonization of senescent petals by selected antagonists and development of Botrytis rod rot. Application of *C. rosea* GR-8 and *G. catenulatum* LRC 2087 to senescent petals resulted in a significant reduction of pod rot of alfalfa caused by *B. cinerea* and a significant increase in alfalfa seed per pod compared with the treatment of *B. cinerea* alone (Table 3). After incubation for 14 days, water-soaked lesions developed on the pods of alfalfa treated with *B. cinerea* alone, whereas the pods treated with water,

GR-8 alone, LRC 2087 alone, GR-8 + *B. cinerea*, or LRC 2087 + *B. cinerea* remained healthy. After incubation for 30 days, most of the pods treated with LRC 2087 + *B. cinerea* or GR-8 + *B. cinerea* and all of the pods treated with water, LRC 2087 alone, or GR-8 alone were still healthy and brown or black in color. In contrast, the pods treated with *B. cinerea* alone became white with the formation of profuse conidiophores of *B. cinerea* on the surface of the pods. In experiment 3, frequency of the diseased alfalfa pods was low in the treatments of water (untreated control), LRC 2087 alone, GR-8 alone, LRC 2087 + *B. cinerea*, and GR-8 + *B. cinerea*. However, frequency of diseased pods was high in the inoculated control treatment of *B. cinerea* alone. Seed per pod in the water treatment, LRC 2087 alone, GR-8 alone, LRC 2087 + *B. cinerea*, and GR-8 + *B. cinerea* was significantly ($P < 0.05$) higher than the control treatment of *B. cinerea* alone in both runs of the experiment (Table 3).

Experiment 4: efficacy of *C. rosea* against alfalfa blossom blight under field conditions. A field experiment conducted during 2000 to 2002 showed that the biocontrol agent *C. rosea* GR-8 was effective in suppressing pod and seed rots of alfalfa caused by *B. cinerea* (Table 4), thereby increasing seed production (Table 5). The frequency of diseased alfalfa pods was significantly lower ($P < 0.05$) in the *C. rosea* GR-8 + *B. cinerea* treatment than that for the treatment of *B. cinerea* alone, and it was significantly higher ($P < 0.05$) than that for the treatment of water alone (Table 4). A similar trend was observed with respect to the frequency of diseased seed of alfalfa (Table 4). Alfalfa seed yield was significantly increased ($P < 0.05$) in the treatment of GR-8 + *B. cinerea* by 168% in 2000, 230% in 2001, and 181% in 2002, compared with the treatment of *B. cinerea* alone. This reflected an increase in number of pods per raceme for the trials of 2000 and 2002, and in number of seed per pod in the trials over 3 years. Seed yield for the treatment of GR-8 + *B. cinerea* were similar to that in the uninoculated treatment of water alone (Table 5).

DISCUSSION

Suppression of fungal sporulation has been proven as a valid strategy for control

Table 2. Effect of the selected antagonists applied to young blossoms on pod formation and development of pod rot caused by *Botrytis cinerea* (experiment 2)^a

Treatment	Pod formation (%) ^y	Infected pods (%)
<i>B. cinerea</i> alone	0.0 e	... ^z
Water control	70.3 ab	0.0 c
<i>Clonostachys rosea</i> LRC 2090 + <i>B. cinerea</i>	55.1 bcd	12.2 b
<i>C. rosea</i> LRC 2090	79.3 a	0.0 c
<i>C. rosea</i> GR-8 + <i>B. cinerea</i>	61.1 bcd	12.6 b
<i>C. rosea</i> GR-8	64.6 bc	0.0 c
<i>Gliocladium catenulatum</i> LRC 2087 + <i>B. cinerea</i>	50.0 cd	10.4 b
<i>G. catenulatum</i> LRC 2087	69.7 ab	0.0 c
<i>Trichoderma atroviride</i> Tw-1 + <i>B. cinerea</i>	44.4 d	55.0 a
<i>T. atroviride</i> Tw-1	67.3 ab	0.0 c

^a These data are an average of two runs of the experiment for each treatment. Means followed by the same letter in each column are not significantly different at $P = 0.05$ according to Duncan's multiple range test.

^y Pod formation were determined 7 days after pollination, whereas pod infection by *B. cinerea* was determined 30 days after pollination.

^z No pod was formed, all florets were colonized by *B. cinerea* and conidiophores were produced.

Table 3. Effect of selected antagonists applied to senescent petals of alfalfa on infection of pods and seed by *Botrytis cinerea* and on alfalfa seed per pod (experiment 3)^y

Treatment ^z	First run		Second run	
	Diseased pods (%)	Seed per pod	Diseased pods (%)	Seed per pod
Water alone	6.3 c	2.5 ab	0.0 b	2.4 a
<i>B. cinerea</i> alone	100.0 a	0.7 c	96.7 a	0.0 c
<i>G. catenulatum</i> LRC 2087 + <i>B. cinerea</i>	0.0 c	2.8 ab	8.3 b	2.2 ab
<i>G. catenulatum</i> LRC 2087	0.0 c	2.1 b	0.0 b	2.1 ab
<i>C. rosea</i> GR-8 + <i>B. cinerea</i>	16.5 b	2.4 ab	8.3 b	1.9 b
<i>C. rosea</i> GR-8	0.0 c	3.0 a	0.0 b	2.2 ab

^y Means followed by the same letter in each column are not significantly different at $P = 0.05$ according to Duncan's multiple range test.

^z Alfalfa florets were pollinated and maintained in a growth chamber (20°C) for 14 days for the development of pods and then water, *B. cinerea* alone, *Gliocladium catenulatum* alone, *G. catenulatum* + *B. cinerea*, *Clonostachys rosea* alone, or *C. rosea* + *B. cinerea* was inoculated on the senescent petals and evaluated after 30 days.

Table 4. Efficacy of *Clonostachys rosea* in control of pod rot and seed rot of alfalfa caused by *Botrytis cinerea* under field conditions (experiment 4)^y

Treatment ^z	Diseased pods (%)			Diseased seed (%)		
	2000	2001	2002	2000	2001	2002
Water alone	22.8 c	11.9 c	24.1 c	4.6 b	8.2 b	7.3 b
<i>B. cinerea</i> alone	67.4 a	51.4 a	69.8 a	16.0 a	35.3 a	29.2 a
<i>C. rosea</i> GR-8 + <i>B. cinerea</i>	43.1 b	33.6 b	34.5 b	7.2 b	10.4 b	8.8 b

^y Data on diseased pods and diseased seeds of alfalfa were collected at the maturity stage in each year. Means followed by the same letter in each column were not significantly different at $P = 0.05$ according to Duncan's multiple range test.

^z Water alone, or aqueous spore suspension of *C. rosea* (6.7×10^6 conidia/ml) or *B. cinerea* (5.0×10^5 conidia/ml) was sprayed at approximately 1,000 ml/plot (3 m²) three times each year.

Table 5. Production of pods and seed of alfalfa treated with water, *Botrytis cinerea* alone, or *Clonostachys rosea* plus *Botrytis cinerea* under field conditions^y

Treatment ^z	No. of pods/raceme			No. of seed/pod			Yield (kg/ha)		
	2000	2001	2002	2000	2001	2002	2000	2001	2002
Water alone	11.0 a	8.4 a	9.9 a	3.5 a	2.4 ab	3.6 a	190 ab	131 a	184 a
<i>B. cinerea</i> alone	8.1 b	6.1 b	7.4 b	1.6 b	1.7 b	1.5 b	88 b	40 b	63 b
<i>C. rosea</i> GR-8 + <i>B. cinerea</i>	11.5 a	6.9 b	9.3 a	3.3 a	3.0 a	3.4 a	236 a	132 a	177 a

^y Data on number of pods per raceme, number of seed per pod, and seed yield of alfalfa of each treatment were collected at the maturity stage each year. Means followed by the same letter in each column were not significantly different at $P = 0.05$ according to Duncan's multiple range test.

^z Water alone, or aqueous spore suspension of *C. rosea* (6.7×10^6 conidia/ml) or *B. cinerea* (5.0×10^5 conidia/ml) was sprayed at approximately 1,000 ml/plot (3 m²) three times each year.

of polycyclic pathogens such as *Botrytis* spp. (6,19). This study demonstrates that *C. rosea*, *G. catenulatum*, *Trichothecium roseum*, and *Trichoderma atroviride* can effectively inhibit sporulation of *B. cinerea* on detached alfalfa florets. The result confirms previous studies that screened for antagonists to suppress *B. cinerea* on other host plants (21,28,29,34,37). *C. rosea* and *G. catenulatum* were effective both on young petals of alfalfa at the anthesis stage to improve pod formation and protect young pods from infection by *B. cinerea*, and on senescent petals of alfalfa at the pod-development stage to protect pods and seed of alfalfa from infection by this pathogen. On the other hand, *T. atroviride* was as effective as *C. rosea* and *G. catenulatum* on detached alfalfa florets, but was less effective than these two antagonists when inoculated on alfalfa florets in vivo at the anthesis stage. Profuse colonization by *C. rosea*, *G. catenulatum*, and *T. atroviride* occurred on detached alfalfa florets, but only *C. rosea* and *G. catenulatum* could colonize senescent petals of alfalfa in vivo, as evidenced by production of conidiophores and conidia of the antagonists. Most species of *Trichoderma* have been reported as secondary, but not primary, colonizers of plants (26), whereas *C. rosea* has been shown to colonize senescent tissues of raspberry (38). Therefore, *C. rosea* and *G. catenulatum* may have higher saprophytic colonization ability on senescent petals of alfalfa than *T. atroviride*. The difference in this study between the in vivo test at the anthesis stage and the in vitro test using fresh florets of alfalfa was related to the quantity of water available for the fungi, with more water in vitro than in vivo. This implies that *C. rosea* and *G. catenulatum* may be relatively less sensitive to a lack of available water than *T. atroviride* during colonization of alfalfa petals.

The effectiveness of *C. rosea* for suppression of pod and seed rots of alfalfa and for increasing alfalfa seed production under field conditions was demonstrated in the field trial of each year in the present study. Previous reports showed that *C. rosea* was an effective biocontrol agent for *B. cinerea* on other plants, including cyclamen (21), strawberry (29,34), and raspberry (37); for *Fusarium culmorum* on cereal crops (17); for *Bipolaris so-*

rokiniana on barley (18); for *Phomopsis sclerotoides* on cucumber (25); and for multiple root pathogens on peas (36). Therefore, *C. rosea* has great potential for use as a biocontrol agent for control of certain plant diseases.

Leafcutter bees are natural pollinators of alfalfa and they are used in commercial production of alfalfa seed. The results of this study indicate that bees coated with conidia of *C. rosea* still can pollinate alfalfa florets in normal fashion. Therefore, leafcutter bees may be used as a carrier like honey bees (29,37) to deliver *C. rosea* to alfalfa flowers. However, the leafcutter bee is solitary in behavior, and may not deliver fungal spores repeatedly as do honey bees (29,37). Additional studies to evaluate the efficiency of leafcutter bees in delivering *C. rosea* to alfalfa blossoms are warranted.

G. catenulatum has been reported as a mycoparasite of *Fusarium* spp. and *Sclerotinia sclerotiorum* (13), as well as *Botrytis cinerea* (32). It also was used as a biocontrol agent against soilborne pathogens such as *Rhizoctonia solani* (27). The current study shows that *G. catenulatum* can significantly suppress sporulation of *Botrytis cinerea*, and the efficacy of this antagonist in both in vitro and in vivo tests was similar to *C. rosea*. This is similar to the findings of Köhl et al. (20) that *G. catenulatum* can suppress *B. allii* and *B. cinerea* on onion. Further studies to evaluate the efficacy of *G. catenulatum* for suppression of *B. cinerea* in commercial alfalfa seed fields are needed.

ACKNOWLEDGMENTS

We thank J. Bissett (Eastern Cereal and Oilseeds Research Center, AAFC) for identification of *Trichoderma atroviride* Tw-1, and D. Friebe for technical assistance.

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