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Biological control of sclerotinia diseases of rapeseed by aerial applications of the mycoparasite *Coniothyrium minitans*

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Abstract

Indoor and field experiments were conducted to evaluate the efficacy of applying the mycoparasite *Coniothyrium minitans* to the aerial parts of rapeseed plants at the flowering stage to control sclerotinia diseases caused by *Sclerotinia sclerotiorum*. Under controlled conditions, a petal inoculation technique was used to determine the effect of conidial suspensions of *C. minitans* on suppression of sclerotinia leaf blight. Results showed that *C. minitans* was effective in inhibiting infection initiated by ascospores of *S. sclerotiorum* on flower petals by restricting mycelial growth of the pathogen. Suppression of lesion development was related to the conidial concentration of *C. minitans*, with larger lesions at low concentration (5×10^3conidia ml^{-1}), but smaller lesions at high concentration (5×10^4 conidia ml^{-1} or higher). When *C. minitans*-treated rapeseed leaves were inoculated with mycelia of *S. sclerotiorum*, *C. minitans* failed to prevent infection of leaves, but caused a significant reduction in number of sclerotia produced on the diseased leaves. No significant difference in efficacy was detected between the two isolates of *C. minitans*, LRC 2137 and Chy-1, on the two rapeseed cultivars, Westar (spring type) and Zhongyou 821 (winter type). Results of field trials showed a significant reduction of stem rot of rapeseed in four (1997, 1999, 2003 and 2004) out of five years by aerial application of *C. minitans*, compared with controls. No significant difference in suppressive efficacy was observed between the treatments of *C. minitans* (10^6 conidia ml^{-1}), *C. minitans* (10^6 conidia ml^{-1}) + benomyl (50 μg ml^{-1}) and benomyl (100 μg ml^{-1}) in 2003, and between the treatments of *C. minitans* (10^6 conidia ml^{-1}), *C. minitans* (10^6 conidia ml^{-1}) + vinclozolin (100 μg ml^{-1}) and vinclozolin (500 μg ml^{-1}) in 2004. Sclerotia of *S. sclerotiorum* collected from diseased plants in plots treated with *C. minitans* in 1999, 2000 and 2003, or with *C. minitans* + benomyl in 2003 were infected by *C. minitans* at frequencies ranging from 21.3 to 54.5%. This study concludes that aerial spraying of *C. minitans* is an effective method for controlling sclerotinia diseases of rapeseed.

Introduction

Sclerotinia sclerotiorum is a cosmopolitan pathogen on many economically important crops including rapeseed (*Brassica* spp.) (Boland and Hall, 1994). It occurs in all rapeseed-growing areas in China, especially in the provinces of the Yangtze River region where the temperature and moisture are conducive to outbreaks of this disease (Yang, 1959). The incidence of sclerotinia stem rot in this region was estimated at 10–20% on average and reached up to 80% in some instances (Yang,

1959). Control of sclerotinia diseases of rapeseed in China is mainly by the use of fungicides and the practice of rapeseed-rice rotation. No resistant rapeseed cultivars are available. Increasing evidence of fungicide resistance in populations of *S. sclerotiorum* and concerns about the lack of environmental sustainability of chemical pesticide use have created a need to find other sustainable measures as alternatives to chemical control. Crop rotation alone was ineffective as rapeseed plants can be infected by airborne ascospores of *S. sclerotiorum* from other areas and neighbouring host crops (Yang, 1959).

Rapeseed petals and pollen play an important role in the development of pod rot, leaf blight and stem rot of rapeseed, as *S. sclerotiorum* requires exogenous nutrients to trigger germination of ascospores, and support the development of germ tubes and infection cushions for infection of healthy tissues (Yang, 1959; Jamaux et al., 1995). Thus, prevention of colonization of senescent flower tissues by *S. sclerotiorum* might be a rational strategy for management of this disease on rapeseed. Previous reports indicate that fungi such as *Epicoccum purpurascens* (Zhou and Reeleder, 1989), *Alternaria alternata* (Boland and Hunter, 1988), *Cladosporium cladosporioides* (Boland and Hunter, 1988) and *Ulocladium atrum* Preuss (Li et al., 2003), and bacterial antagonists such as *Bacillus cereus* (Huang et al., 1993) can effectively suppress the colonization of *S. sclerotiorum* on flower petals of plants.

Coniothyrium minitans is a mycoparasite of *S. sclerotiorum* (Campbell, 1947) that is widely distributed in China (Li et al., 1995) and many other countries (Whipps and Gerlagh, 1992). This organism has been developed into commercial products for control of *S. sclerotiorum* in Europe (de Vrije et al., 2001). Traditionally, *C. minitans* is applied to the soil where it can interact with, infect, and destroy sclerotia of *S. sclerotiorum*, resulting in a reduction in apothecial production (McLaren et al., 1996; Huang and Erickson, 2000, 2004). This strategy was successful in suppression of wilt of sunflower (*Helianthus annuus*) due to myceliogenic germination of sclerotia of *S. sclerotiorum* (Huang, 1980), but unsuccessful for controlling sclerotinia stem rot of rapeseed due to ascospore infection by carpogenic germination of sclerotia of *S. sclerotiorum* (McQuilken et al., 1995).

Turner and Tribe (1976) suggested that *C. minitans* might be a saprophyte on aerial parts of plants, and this was confirmed by Whipps et al. (1993) who isolated *C. minitans* from petals collected from rapeseed fields. Trutmann et al. (1982) found that spraying bean (*Phaseolus vulgaris*) plants with *C. minitans* did not effectively reduce the severity of white mould caused by *S. sclerotiorum*, but it significantly reduced the number of sclerotia produced on diseased plants. Sclerotia collected from *C. minitans*-treated plots formed fewer stipes and produced less apothecia than the sclerotia collected from untreated or benomyl-treated plots (Trutmann et al., 1982). Gerlagh et al. (1999) showed that spraying *C. minitans* on the canopies of crops susceptible to *S. sclerotiorum* resulted in a high rate (>90%) of infection of sclerotia by the mycoparasite, and a significant reduction in apothecial production. Bremer et al. (2000) reported that early colonization of bean flower petals by *C. minitans* could effectively reduce petal-mediated infections of bean leaves by ascospores of *S. sclerotiorum* when the petals were placed on bean leaves. Huang et al. (2000) further indicated that application of *C. minitans* on the foliage of bean plants during the early flowering period could reduce the incidence and severity of white mould caused by *S. sclerotiorum* under field conditions.

The objectives of this study were: (1) to determine the suppressive effect of *C. minitans* on colonization/infection of rapeseed petals and leaves by *S. sclerotiorum*; and (2) to determine the effectiveness of controlling sclerotinia stem rot of rapeseed by aerial applications of *C. minitans* in central China.

Materials and methods

Fungal isolates

The biocontrol agents used in this study were *C. minitans* isolates LRC 2137 (syn.Cm54) and Chy-1, and *Trichoderma viride* Pers. isolate Hn-1-1. The *C. minitans* isolate LRC 2137 was obtained from a decayed sclerotium of *S. sclerotiorum* collected from a diseased sunflower plant in Manitoba, Canada (Huang, 1977), and was used for the indoor studies (Bioassays 1–4) only. The *C. minitans* isolate Chy-1 and the *T. viride* isolate Hn-1-1

were both obtained from a soil sample collected in Changyang County of Hubei Province, China (Li et al., 1995). Chy-1was used for comparison of the biocontrol efficacy with LRC 2137 in an indoor bioassay, and it was then used for field trials. Hn-1-1 was used just for the field trial in 1997. The pathogen used for the indoor studies was *S. sclerotiorum* isolate LRC 2148 (syn. sun-87), which was isolated from a sclerotium collected from a diseased sunflower plant in Alberta, Canada (Huang and Kozub, 1989).

Preparation of inoculum

For the production of conidia, *C. minitans* isolates LRC 2137 and Chy-1, and *T. viride* isolate Hn-1-1 were grown on potato dextrose agar (PDA) and incubated at 20 °C in a growth chamber under fluorescent light for 4 weeks. Conidial suspensions of LRC 2137, Chy-1and Hn-1-1 were prepared by adding 10 ml of sterile distilled water (SDW) to each dish of the 4-week-old cultures and rubbing the surface of each colony with a sterile glass rod. The spore suspensions were filtered through four layers of cheesecloth, and the concentrations of the filtered spore suspensions were determined using a haemocytometer. To obtain ascospores of *S. sclerotiorum*, apothecia were produced from sclerotia of LRC 2148 according to the method described by Huang and Kozub (1989), and fresh, mature apothecia were harvested and ground to slurry using sterilized pestle and mortar. SDW was added to the slurry at a volume ratio of 3:2 (apothecial slurry:water), and the mixtures were filtered through double-layered cheesecloth. The concentration of the resulting ascospore suspensions was determined as previously described. The viability of conidia of *C. minitans* and *T. viride*, and ascospores of *S. sclerotiorum* was higher than 95% before use.

Bioassay 1: Colonization of petals by C. minitans *and suppression of* S. sclerotiorum

An indoor experiment was conducted to test the effect of *C. minitans* (LRC 2137) on saprophytic growth of *S. sclerotiorum* on rapeseed petals. Seeds of rapeseed (*Brassica napus* cv. Westar) were sown in Cornell Peat-Lite mix in plastic pots (15 cm diam.) as described in our previous studies (Li et al., 2003, 2005) and kept in a greenhouse (15–20 °C) until flowering. Newly opened flower petals were removed from the plants, washed twice in 30 ml SDW containing 0.01% Tween 20 (Sigma, St Louis, MO, USA) and placed on the surface of moist autoclaved vermiculite in Petri dishes (9 cm diam.), 10 petals per dish. Petals in each dish were inoculated with one of the following treatments: (1) *C. minitans* + *S. sclerotiorum*; (2) *S. sclerotiorum*; (3) *C. minitans*; and (4) water. For the treatment of *C. minitans* + *S. sclerotiorum*, aliquots of 20 μl conidial suspension of *C. minitans* (2.7×10^6 conidia ml^{-1}) were pipetted onto the centre of each petal, and the Petri dish was left open under a laminar flow hood for 2 h to allow evaporation of the excess water on petals. Then, 20 μl ascospore suspension of *S. sclerotiorum* (2.7×10^5 ascospores ml^{-1}) was inoculated onto each petal. For the treatments of *C. minitans*, *S. sclerotiorum* and water, rapeseed petals were treated first with water at 20 μl per petal, and then inoculated with *C. minitans* (2.7×10^6 conidia ml^{-1}), *S. sclerotiorum* (2.7×10^5 ascospores ml^{-1}) or water, at 20 μl per petal. There were three replicates (dishes) for each treatment. Dishes were individually sealed with parafilm and incubated at 20 °C for 4 days in the growth chamber. Saprophytic growth of *S. sclerotiorum* on each petal was determined by the formation of white cottony mycelia (Boland and Hunter, 1988; Li et al., 2005). The experiment was performed twice.

Bioassay 2: Effect of C. minitans *conidial concentration on suppression of* S. sclerotiorum

Three experiments were conducted to determine the effect of conidial concentration of *C. minitans* on suppression of *S. sclerotiorum* using a petal-mediated infection technique (Bremer et al., 2000; Li et al., 2003). *Coniothyrium minitans* isolate LRC 2137 was used in this assay. The first experiment determined the effect of conidial concentration of *C. minitans* applied to rapeseed petals on infection of rapeseed leaves by ascospores of *S. sclerotiorum*. Rapeseed plants, cv. Westar, were grown in the greenhouse. At the flowering stage, racemes in full bloom were selected for the experiment. Unopened florets were removed, and the racemes with open florets were individually tagged and sprayed until run-off with conidial suspensions of *C. minitans* containing 5.3×10^3, 5.3×10^4, 5.3×10^5, 5.3×10^6 or 5.3×10^7 conidia ml^{-1}. Racemes

sprayed with water were used as controls. All of the conidial suspensions and water contained 0.01% (v/v) Tween 20 (Sigma, St Louis, MO, USA) and 0.5% Pelgel (w/v, LiphaTech, Milwaukee, WI, USA). Two days later, the petals were removed from racemes and used as inoculum. For inoculation, rapeseed leaves, cv. Westar, were excised from 30-day-old plants, kept on moist towels in aluminium trays ($30\times25\times8$ cm^3, L×W×H), 4 leaves per tray, and inoculated with *C. minitans*-treated or water-treated petals by placing four petals on each leaf, with 3–4 cm between petals. There were four leaves in each tray and four replicates (trays) for each treatment. Each petal was inoculated with an aliquot of 20 μl of the *S. sclerotiorum* ascospore suspension (2.7×10^5 ascospores ml^{-1}). Trays were covered with clear plastic films and incubated in the growth chamber under fluorescent light. The diameter of the leaf lesion surrounding each petal was measured after incubation at 20 °C for 6 days. The experiment was performed twice.

The second experiment determined the effect of conidial concentration of *C. minitans* applied to rapeseed leaves for control of ascospore infection of *S. sclerotiorum*. Rapeseed plants, cv.Westar, were grown in pots (3 plants per pot) in the greenhouse for 30 days. Leaves in the centre of the plants in each pot were sprayed until run-off with conidial suspensions of *C. minitans* containing 5×10^3, 5×10^4, 5×10^5, 5×10^6, or 5×10^7 conidia ml^{-1}. Leaves sprayed with water were used as controls. All of the conidial suspensions and water contained 0.01% Tween 20 and 0.5% Pelgel. Two hours later, *C. minitans*-treated and water-treated leaves were excised, and placed on wet paper towels in aluminium trays, 4 leaves per tray. Four fresh flower petals collected from 45-day-old rapeseed plants, cv. Westar, were placed on each leaf, with 3–4 cm between petals. Aliquots of 20 μl of the ascospore suspension of *S. sclerotiorum* (2.7×10^5 ascospores ml^{-1}) were inoculated onto the centre of each petal. There were 4 trays (replicates) for each treatment. Trays were covered with clear plastic films and incubated in the growth chamber. The diameter of the leaf lesion surrounding each petal was measured after incubation at 20 °C for 6 days. The experiment was performed twice.

The third experiment determined the effect of conidial concentration of *C. minitans* applied to rapeseed leaves for control of infection by mycelia of *S. sclerotiorum*. Rapeseed leaves were treated with *C. minitans* conidial suspensions containing 0, 5×10^3, 5×10^4,5×10^5, 5×10^6, and 5×10^7 conidia ml^{-1} by the same method described in the second experiment of this bioassay. Two hours later, leaves for each treatment were excised and placed in aluminium trays, 4 leaves per tray, and 4 trays (replicates) per treatment. Agar plugs (6 mm diam.) containing mycelia of *S. sclerotiorum* (LRC2148) were removed from the margin of 3-day-old PDA cultures and inoculated onto the rapeseed leaves in each tray, two plugs per leaf with 4–5 cm between plugs. Trays were covered with clear plastic films and incubated in the growth chamber at 20 °C. Commencing at 48 h after inoculation, the diameter of the leaf lesion surrounding each agar plug was measured daily. Number of sclerotia formed on each leaf was recorded after incubation for 30 days. The experiment was performed twice.

Bioassay 3: Efficacy of two C. minitans *isolates*

Two isolates of *C. minitans*, LRC 2137 and Chy-1, were compared for suppression of *S. sclerotiorum* on leaves of rapeseed, cv. Westar, using the method of petal-mediated infection as described previously. The treatments included: (1) Chy-1 + *S. sclerotiorum* (Ss); (2) LRC 2137 + Ss; and (3) Ss. The conidial concentration of the spore suspension for LRC2137 and Chy-1 was 1.9×10^6 conidia ml^{-1} and each petal was inoculated at 20 μl. The ascospore concentration for *S. sclerotiorum* was still 2.7×10^5 ascospores $ml^{-1}$and each petal was inoculated at 20 μl. There were 4 replicates (trays) for each treatment. The diameter of the leaf lesion surrounding each petal was measured after incubation in the growth chamber at 20 °C for 6 days. The experiment was run twice.

Bioassay 4: Effect of rapeseed cultivars on efficacy of C. minitans

Two cultivars of rapeseed, Westar (north American origin, spring type) and Zhongyou 821 (Chinese origin, winter type), were used in this study. Both cultivars were grown in Cornell Peat-Mix in pots in the greenhouse. At the 3-leaf stage, seedlings of cv. Zhongyou 821 were vernalized at 10 °C for 8 weeks, and then maintained in the

greenhouse (15–20 °C). At the full bloom stage, flowers of the two rapeseed cultivars were sprayed until run-off with conidial suspensions of *C. minitans* (LRC 2137) containing 5×10^5 or 5×10^6 conidia ml^{-1}. Flowers sprayed with water were used as controls. Meanwhile, leaves of Westar or Zhongyou 821 were excised from the centre of 30-day-old plants and placed on wet paper towels in aluminium trays, 4 leaves per tray. The *C. minitans*-treated and water-treated petals of Westar and Zhongyou 821 were placed on leaves of corresponding rapeseed cultivars with 4 petals per leaf. Aliquots of 20 μl of the ascospore suspension of *S. sclerotiorum* (2.7×10^5 ascospores ml^{-1}) were inoculated onto the centre of each petal, and the trays were then covered with plastic films and incubated at 20 °C in the growth chamber. The diameter of the resulting leaf lesion surrounding each petal was measured after incubation for 6 days. There were four replicates (trays) for each treatment and the experiment was run twice.

Study 5: Field trials on control of stem rot of rapeseed by C. minitans

Field trials to determine the efficacy of *C. minitans* for suppression of sclerotinia stem rot of rapeseed by *C. minitans* were conducted for five years (1997, 1999, 2000, 2003 and 2004) in Hubei Province, China. The 1997, 1999 and 2000 trials were conducted near the city of Wuhan using rapeseed cv. Zhongyou 821. At that location, radish (*Raphanus sativus*) was successively planted in this field for more than 10 years before the 1997 trial. The 2003 and 2004 trials were conducted in two different fields, one for each year, in Xiannin County, about 200 km south of Wuhan, using rapeseed cv. Huaza No. 6. In both fields, watermelon (*Citrullus lanatus*) was planted in summer, followed by planting rapeseed in autumn. For the trials of 1997, 1999 and 2000, fields were artificially infested with sclerotia collected from diseased rapeseed plants, by applying them to each plot at a rate of 500 sclerotia per plot, 2 days before rapeseed seedlings were transplanted from nursery to field. For the trials of 2003 and 2004, fields were naturally infested with *S. sclerotiorum*. In each field trial, seeds were sown in a nursery in early October and transplanted to the field in early November before each trial year. The size of each field plot was 2.0×5.0 m (W×L). Rapeseed plants were spaced by $10–15\times25–30$ cm^2, and there were 350–380 plants for each plot. Rapeseed plants were sprinkle-irrigated for three weeks after transplanting, and weeds in plots were removed by hand. The trial of 1997 tested the efficacy of *C. minitans* for control of sclerotinia stem rot of rapeseed. There were three treatments: *C. minitans* isolate Chy-1 (1×10^7 conidia ml^{-1}); *T. viride* (1×10^8 conidia ml^{-1}); and water. The trials of 1999 and 2000 compared rates of application of *C. minitans* on control of the disease. There were four treatments: *C. minitans* Chy-1 at 1×10^5, 1×10^6, and 1×10^7 conidia ml^{-1}, and water. The trials of 2003 and 2004 tested the feasibility of integrated application of *C. minitans* and fungicides for the control of sclerotinia stem rot of rapeseed. There were four treatments in 2003 including *C. minitans* Chy-1 (1×10^6 conidia ml^{-1}) (CM), CM + benomyl (50 μg ml^{-1}) and benomyl (200 μg ml^{-1}), and five treatments in 2004 including CM (1×10^6 conidia ml^{-1}), CM + vinclozolin (100 μg ml^{-1}), vinclozolin (100 μg ml^{-1}), vinclozolin (500 μg ml^{-1}) and water. For the trials of 1997, 1999 and 2000, treatments were applied by spraying the agent or water with a hand sprayer onto aerial parts (mainly flowers) of rapeseed plants three times at weekly intervals, starting at the early flowering stage (20–30% of plants in bloom). For the trials of 2003 and 2004, treatments were applied also twice at weekly intervals, and starting at the early flowering stage. The amount of biocontrol agent or water used for each spray was 3 l per plot. The treatments in each trial were arranged in a randomised complete block design with three replicates for each treatment. At the maturity stage, the disease incidence and severity of all rapeseed plants in each plot were assessed. Disease incidence was defined as the percentage of infected plants. Disease severity of each plant was rated using a value scale of 0 to 4, where 0 = no lesion; 1 = less than 1/3 of branches had lesions, or all branches were healthy but the main stem had lesions of less than 3 cm long; 2 = 1/3 to 2/3 of branches had lesions, or less than 1/3 of branches were infected, but the lesion on the main stem was longer than 3 cm; 3 = 2/3 of branches were diseased, or less than 2/3 of branches were diseased, but the lesion on the main stem was longer than 3 cm; and 4 = more than 2/3 of branches were diseased, or less than 2/3 of branches were

diseased, but the lesion on the main stem was longer than 10 cm. Disease severity (DS) for each plot was calculated by the formula (Fang, 1998):

$$DS = 100 \times (P_0 \times 0 + P_1 \times 1 + P_2 \times 2 + P_3 \times 3 + P_4 \times 4)/4 (P_0 + P_1 + P_2 + P_3 + P_4)$$

where P_0, P_1, P_2, P_3 and P_4 were the number of rapeseed plants corresponding to each disease rating for each plot, and 4 represents the maximum disease rating value. For the trials in 1999, 2000 and 2003, sclerotia formed on diseased plants of each plot were collected at the end of the growing season, surface-sterilized in 70% ethanol for 90 s, rinsed with SDW twice and placed on sterile moistened sand in Petri dishes (15 cm diam.), 50 sclerotia per dish. After incubation at 20 °C for 7 days, the number of sclerotia parasitized by *C. minitans* in each dish was determined under a stereomicroscope by the appearance of characteristic black pycnidia on the surface of the infected sclerotia.

Data analyses

Analysis of variance (ANOVA) (SAS Institute, Cary, NC, USA, version 6.0, 1989) was used to determine the statistical significance of differences among treatments in the indoor and field experiments. Data for the same treatment, but collected from corresponding runs of the same experiment, were pooled when they were not significantly different in the F-test in ANOVA ($P < 0.05$). The percentage data of disease incidence and of sclerotial infection by *C. minitans* were arcsine-transformed prior to analysis, and the means were back transformed to percentage values after analysis. Treatment means in each experiment were separated using Duncan's multiple range test at the $P = 0.05$ level.

Results

Bioassay 1: Colonization of petals by C. minitans *and suppression of* S. sclerotiorum

After incubation at 20 °C for 4 days, no mycelial growth of *S. sclerotiorum* was observed on the petals inoculated with *C. minitans* + *S. sclerotiorum*, *C. minitans* alone or water alone. Petals of these three treatments remained firm and appeared healthy. In contrast, white, cottony mycelial masses of *S. sclerotiorum* appeared on and around each petal as a result of vigorous growth of the pathogen. These petals became soft and eventually disintegrated.

Bioassay 2: Effect of conidial concentrations of C. minitans *on suppression of* S. sclerotiorum

Results of the first experiment showed that treatment of rapeseed petals with *C. minitans* reduced number and size of lesions of *S. sclerotiorum* developed on rapeseed leaves. An average of 85 and 72% of the petals inoculated with ascospores of *S. sclerotiorum* alone and *C. minitans* (5.3×10^3 conidia ml^{-1}) + *S. sclerotiorum*, respectively, developed lesions on rapeseed leaves after incubation for 6 days. The lesion diameter was 2.6 and 1.8 cm, respectively, for these two treatments. There was no significant difference between these two treatments regarding the lesion diameter ($P < 0.05$). For the treatments of *C. minitans* (5.3×10^4 conidia ml^{-1}) + *S. sclerotiorum*, *C. minitans* (5.3×10^5 conidia ml^{-1}) + *S. sclerotiorum*, *C. minitans* (5.3×10^6 conidia ml^{-1}) + *S. sclerotiorum*, and *C. minitans* (5.3×10^7 conidia ml^{-1}) + *S. sclerotiorum*, the percentage of petals caused lesions on leaves was 48, 25, 4, and 0%, respectively. The mean lesion diameter for these treatments was 0.6, 0.3, 0.2 and 0 cm, respectively (Figure 1). These values were significantly lower than those for the treatments of *S. sclerotiorum* alone and *C. minitans* (5.3×10^3 conidia ml^{-1}) + *S. sclerotiorum* ($P < 0.05$). Moreover, the mean lesion diameter (Y) was inversely proportional to the conidial concentration (X) of *C. minitans*, $Y = -0.5029 \log_{10} X + 2.6933$ ($R^2 = 0.8392$, $P < 0.01$).

Results of the second experiment showed that more than 90% of petals inoculated with ascospores of *S. sclerotiorum* developed lesions on rapeseed leaves treated with water (95%) or with low spore concentration of *C. minitans* at 5.0×10^3 conidia ml^{-1}(91%). The mean lesion diameters for these two treatments were 2.9 and 3.3 cm, respectively (Figure 2). In comparison, the percentages of petals resulting in lesions on rapeseed leaves inoculated with *C. minitans* conidia at 5.0×10^4, 5.0×10^5, 5.0×10^6, and 5.0×10^7 conidia ml^{-1} were reduced to 36, 25, 16, and 11%,

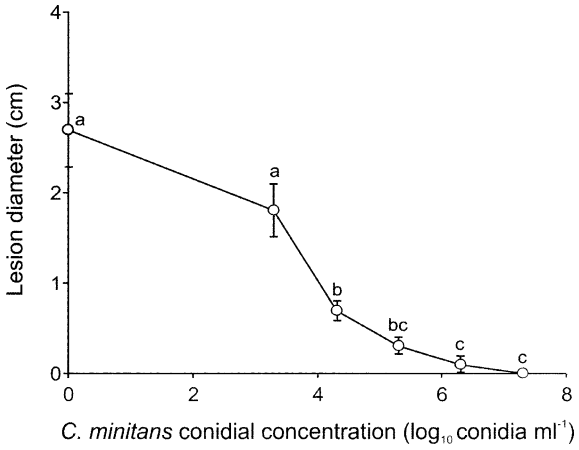

Figure 1. Effect of conidial concentrations of *Coniothyrium minitans* applied to rapeseed flowers on suppression of leaf blight caused by *Sclerotinia sclerotiorum*. The diameter of leaf lesion derived from each petal inoculated with ascospores of *S. sclerotiorum* was measured after incubation for 6 days at 20 °C. Vertical bars represent standard errors of by *Sclerotinia sclerotiorum* (Bioassay 2). Means followed by the same letter are not significantly different ($P \geq 0.05$).

respectively. The mean lesion diameters for these treatments were reduced to 2.5, 1.0, 0.7, and 0.6 cm, respectively (Figure 2), significantly different from those for the treatments of water or *C. minitans* at 5.0×10^3 conidia ml^{-1} ($P<0.05$). Moreover, the mean diameter (Y) was inversely proportional to the applied conidial concentration (X) of *C. minitans*, $Y=-0.5314\ \log_{10}X+3.6267$ ($R^2=0.8358$, $P<0.01$).

Results of the third experiment showed that lesions developed around the point of inoculation with mycelial mats of *S. sclerotiorum* on rapeseed leaves inoculated with water or with any of the tested concentrations of *C. minitans* conidial suspensions after incubation at 20 °C for 24 h. Seven days later, leaves of all treatments became completely rotted. After 30 days, an average of 7 sclerotia of *S. sclerotiorum* was produced on each diseased rapeseed leaf treated with water alone. Sclerotial production (Y) was significantly ($P<0.05$) decreased when *C. minitans* was inoculated (Figure 3). The suppression was increased with the increase in the applied conidial concentration (X) of *C. minitans*, $Y=-1.2543\ \log_{10}X+6.0733$ ($R^2=0.6675$, $P<0.05$).

Bioassay 3: Effect of isolates of C. minitans *on control of* S. sclerotiorum

Treatment of *C. minitans* on rapeseed petals was effective in suppression of lesion development of *S. sclerotiorum* on rapeseed leaves. Rapeseed petals treated with *C. minitans* Chy-1 + *S. sclerotiorum*

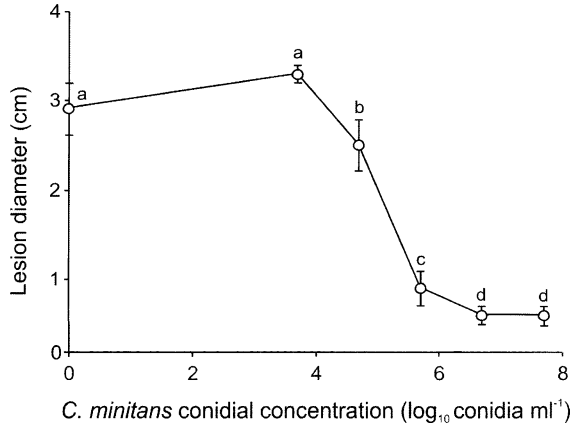

Figure 2. Effect of conidial concentrations of *Coniothyrium minitans* applied to rapeseed leaves on suppression of leaf blight caused by *Sclerotinia sclerotiorum* (Bioassay 2). The diameter of leaf lesion derived from each petal inoculated with ascospores of *S. sclerotiorum* was measured after incubation for 6 days at 20 °C. Vertical bars represent standard errors of means. Means followed by the same letter are not significantly different ($P \geq 0.05$).

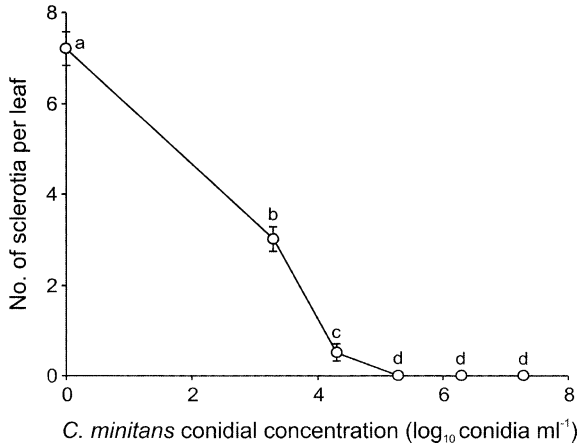

Figure 3. Effect of conidial concentrations of *Coniothyrium minitans* applied to rapeseed leaves on suppression of sclerotial production by *Sclerotinia sclerotiorum* (Bioassay 2). Water or *C. minitans*-treated rapeseed leaves were inoculated with mycelial agar plugs of *S. sclerotiorum* and incubated at 20 °C. The number of sclerotia formed on each rapeseed leaf inoculated with mycelia of *S. sclerotiorum* was recorded after incubation for 30 days. Vertical bars represent standard errors of means. Means followed by the same letter are not significantly different ($P \geq 0.05$).

and *C. minitans* LRC 2137 + *S. sclerotiorum* produced small lesions on rapeseed leaves (0.3 and 0.5 cm on average, respectively), compared with large lesions (4.5 cm on average) for the treatment of *S. sclerotiorum* alone (Figure 4). The difference in size of lesions on rapeseed leaves between the treatments Chy-1 + or LRC 2137 + *S. sclerotiorum* and *S. sclerotiorum* alone was significant ($P < 0.05$). However, there was no significant difference ($P > 0.05$) between the treatments of Chy-1 + *S. sclerotiorum* and LRC 2137 + *S. sclerotiorum* (Figure 4).

Bioassay 4: Effect rapeseed cultivars on suppressive efficacy of C. minitans *to* S. sclerotiorum

Inoculation of *C. minitans* on rapeseed petals was effective in suppression of lesion development of *S. sclerotiorum* on rapeseed leaves of both cultivars. For the two *B. napus* cultivars, Westar and Zhongyou 821, a low percentage (< 20%) of disease lesions developed from flower petals treated with *C. minitans* + *S. sclerotiorum*, compared with high percentages (> 85%) for flower petals treated with *S. sclerotiorum* alone. The mean lesion diameter for the treatment of *C. minitans* was less than 0.6 cm on Westar and 0.7 cm on Zhongyou 821, significantly lower than that for the treatment of

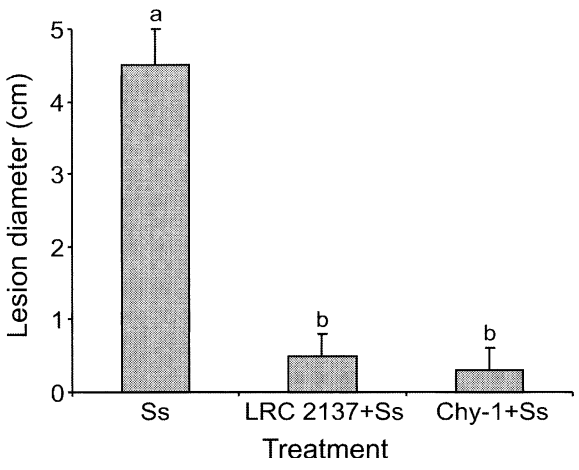

Figure 4. Comparison of efficacy of *Coniothyrium minitans* isolates in suppression of leaf blight of rapeseed caused by *Sclerotinia sclerotiorum* (Bioassay 3). The diameter of leaf lesion derived from each petal inoculated with ascospores of *S. sclerotiorum* was measured after incubation for 6 days at 20 °C. Ss = *Sclerotinia sclerotiorum*. Vertical bars represent standard errors of means. Means followed by the same letter are not significantly different ($P \geq 0.05$).

S. sclerotiorum alone on Westar (2.5 cm) or on Zhongyou 821 (3.8 cm) (Table 1). There was no significant difference in lesion diameter between the treatments of *C. minitans* at 1×10^5 and 1×10^6 conidia ml^{-1} + *S. sclerotiorum* on each tested rapeseed cultivars.

Study 5: Field trials on control of scleroti stem rot by C. minitans

Results of the field trials revealed a significant ($P < 0.05$) suppression of sclerotinia stem rot by aerial application of *C. minitans* in four (1997, 1999, 2003 and 2004) out of five years (Table 2). In the 1997 trial, both *C. minitans* (1×10^7 conidia ml^{-1}) and *T. viride* (1×10^8 conidia ml^{-1}) were effective in reducing the disease severity of rapeseed compared with the control treatment. However, disease incidence and severity were significantly lower ($P < 0.05$) for the treatment of *C. minitans* (1×10^7 conidia ml^{-1}) than those for the treatment of *T. viride* (1×10^8 conidia ml^{-1}) (Table 2). Effective suppression of the disease was achieved by using *C. minitans* conidial suspensions ranging from 1×10^5 to 1×10^7 conidia ml^{-1} in 1999, but no significant differences were observed between treatments of different conidial concentrations (Table 2). Both *C. minitans* alone (1×10^6 conidia ml^{-1}) and *C. minitans* + low dosage of the fungicides benomyl (50 μg ml^{-1}) in 2003 or *C. minitans* + vinclozolin (100 μg ml^{-1}) in 2004 was effective in reduction of disease incidence and severity of rapeseed (Table 2). The efficacy for these treatments was not significantly different ($P > 0.05$) from the recommended application rate of benomyl (200 μg ml^{-1}) in 2003 or vinclozolin (500 μg ml^{-1}) in 2004 treatments (Table 2).

Coniothyrium minitans-infected sclerotia of *S. sclerotiorum* were found inside the diseased rapeseed plants treated with *C. minitans* alone in 1999, 2000 and 2003, or treated with *C. minitans* + benomyl in 2003. The percentages of *C. minitans*-infected sclerotia for the treatments of *C. minitans* alone (1×10^5–1×10^7 conidia ml^{-1}) in 1999 (36.8–40.6%) and in 2003 (46.0%), and for the treatment of *C. minitans* + benomyl in 2003 (45.3%) were significantly higher ($P < 0.05$) than those for the water control (0.0–31.1%). No significant difference in the percentage of *C. minitans*-infected sclerotia was observed between *C. minitans* alone and *C. minitans* + benomyl in 2003 (Table 2).

Table 1. Efficacy of *Coniothyrium minitans* (Cm) for suppression of infection by *Sclerotinia sclerotiorum* (Ss) on leaves of different rapeseed cultivars (Bioassay 4)

Treatment	Lesion diameter (cm)	
	Westar	Zhongyou 821
Ss	2.5 a*	3.8 a*
Cm (1×10^5 conidia ml^{-1}) + Ss	0.6 b	0.7 b
Cm (1×10^6 conidia ml^{-1}) + Ss	0.3 b	0.4 b

*Means followed by the same letters within each column are not significantly different at the $P<0.05$ level of confidence according to Duncan's multiple range test.

Discussion

This study demonstrates that aerial application of *C. minitans* is an effective strategy for biocontrol of sclerotinia leaf blight and stem rot of rapeseed caused by *S. sclerotiorum*. The findings confirm previous reports on biocontrol of white mold of bean (Huang et al., 2000) and blossom blight of alfalfa (Li et al., 2005) by aerial application of *C. minitans*. The indoor experiments also confirm the report of Bremer et al. (2000) that *C. minitans* can directly suppress petal-mediated infection of plant leaves by ascospores of *S. sclerotiorum*. Li et al. (2003) reported that ascospores of *S. sclerotiorum* germinated more quickly than conidia of *C. minitans*, when spores of the two organisms were mixed and incubated on agar media or rapeseed flower petals. However, mycelia of *S. sclerotiorum* in mixed cultures showed signs of cytoplasmic aggregation and cell wall disintegration after incubation for four days even without direct contact between the two organisms. This implies that cell wall degradation enzymes (Giczey et al., 2001) or antifungal substances (McQuilken et al., 2003) secreted by *C. minitans* may be responsible for suppression of saprophytic growth of *S. sclerotiorum* on rapeseed petals observed in this study.

Results of the indoor bioassays showed that among the factors (*C. minitans* conidial concentration, *C. minitans* isolate and rapeseed cultivar)

Table 2. Efficacy of *Coniothyrium minitans* for suppression of sclerotinia stem rot of rapeseed under field conditions (Study 5)

Year	Treatment[a]	% Disease incidence	Disease severity[b]	Selerotia infected by *C. minitans* (%)
1997	*C. minitans* 1×10^7 conidia ml^{-1}	10.2 c[c]	6.5 b[c]	ND
	T. viride 1×10^8 conidia ml^{-1}	29.3 b	23.5 a	ND
	Water	37.3 a	28.9 a	ND
1999	*C. minitans* 1×10^7 conidia ml^{-1}	25.7 b	15.0 b	38.5 a[b]
	C. minitans 1×10^6 conidia ml^{-1}	29.5 b	17.2 b	36.8 a
	C. minitans 1×10^5 conidia ml^{-1}	25.8 b	14.1 b	40.6 a
	Water	40.9 a	26.5 a	11.2 b
2000	*C. minitans* 1×10^7 conidia ml^{-1}	7.0 a	4.4 a	54.5 a
	C. minitans 1×10^6 conidia ml^{-1}	8.6 a	6.1 a	21.3 c
	C. minitans 1×10^5 conidia ml^{-1}	7.4 a	4.7 a	35.5 ab
	Water	6.4 a	4.1 a	31.1 b
2003	*C. minitans* 1×10^6 conidia ml^{-1}	11.7 b	5.8 b	46.0 a
	C. minitans 1×10^6 conidia ml^{-1} + benomyl 50 μg ml^{-1}	11.7 b	5.2 b	45.3 a
	Benomyl 200 μg ml^{-1}	17.7 a	7.8 b	3.1 b
	Water	21.0 a	9.6 a	0.0 b
2004	*C. minitans* 1×10^6 conidia ml^{-1}	10.6 b	3.9 b	ND
	C. minitans 1×10^6 conidia ml^{-1} + vinclozolin 100 μg ml^{-1}	9.5 b	3.2 b	ND
	vinclozolin 100 μg ml^{-1}	19.1 b	5.9 b	ND
	Vinclozolin 500 μg ml^{-1}	27.6 ab	7.8 ab	ND
	Water	39.5 a	12.9 a	ND

ND, not determined.

[a]There were three sprays for treatments in 1997, 1999 and 2000, and two sprays in 2003 and 2004.

[b]The disease severity was on a 0–100 scale. It was calculated on the basis of rating disease symptoms of each plant from 0 (healthy) to 4 (serious stem rot) (see Materials and methods).

[c]Means followed by the same letters within each column for each year are not significantly different at the $P<0.05$ level of confidence according to Duncan's multiple range test.

examined, conidial concentration of *C. minitans* is the most important. This confirms the findings of a previous study by Bremer et al. (2000), and emphasizes the importance of establishing and maintaining *C. minitans* populations on the phyllosphere and anthoplane of rapeseed to a level that is adequate for suppression of sclerotinia diseases. Improvement of spray efficiency by using appropriate equipment and enhancement of conidial adhesion to the surface of rapeseed tissues may increase the population of *C. minitans* on rapeseed plants. Meanwhile, addition of protective agents in formulations of *C. minitans* to improve its tolerance to ultraviolet light irradiation and desiccation may increase the survival of this fungus, and thereby enhance its efficacy.

The results of field trials in this study reveal that *C. minitans* applied to rapeseed plants has the potential to reduce the inoculum potential of *S. sclerotiorum* in the subsequent growing seasons, as numerous sclerotia collected from diseased plants were infected by the mycoparasite and would not likely survive to produce apothecia (Table 2). Previous reports determined that *C. minitans*-infected sclerotia germinated poorly to produce fewer apothecia (Trutmann et al., 1982; Gerlagh et al., 1999; Gerlagh et al., 2003) and infection of sclerotia by *C. minitans* was responsible for reduction of sclerotial population in the soil (Gerlagh et al., 1999) and for inducing soil suppression of sclerotinia wilt of sunflower (Huang and Kozub, 1991). Gerlagh et al. (2003) reported that about 90% of sclerotia of *S. sclerotiorum* became infected when *C. minitans* was sprayed onto the canopy of bean. This percentage is much higher than that achieved in the present study on rapeseed in China, as well as in a previous study by Huang et al. (2000) on bean in Canada. Different environmental conditions and different timing of applications may be the reasons for the different results in various reports. Additional studies are required on factors that influence infection of *S. sclerotiorum* sclerotia by *C. minitans* in the rapeseed production regions in China.

In the 2000 field trial, both the disease incidence and the disease severity were relatively lower than those for other years, even for the control treatment (water). The reason for this result might be the environmental factors (temperature and rainfall precipitation) affecting sclerotial survival, sclerotial germination or development of mycelia of *S. sclerotiorum* on rapeseed plants (Yang, 1959). Previous studies showed that the disease pressure may affect the efficacy of biocontrol agents including *C. minitans* (Trutmann et al., 1982; Huang et al., 2000). This study readily indicated that *C. minitans* could effectively suppress the infection by ascospores of *S. sclerotiorum via* petals, but could not suppress the infection by mycelia of this pathogen. Under the conditions of low disease pressure, *C. minitans* could aggressively colonize lesions caused by *S. sclerotiorum*, resulting in infection of sclerotia of this pathogen. Also in the 2000 trial, an average of 31.1% of sclerotia of *S. sclerotiorum* collected from the water treatment was infected by *C. minitans*. This may be caused by the aerially drifted conidia of *C. minitans* from sprayed neighbouring plots with conidial suspensions of this mycoparasite, by the use of a *C. minitans*-contaminated sprayer to treat the control plots, or by the secondary transmissions of conidia by some insects.

Combination of biocontrol agents with low doses of fungicides is a strategy used to synergistically improve the efficacy of control of plant diseases including those caused by *S. sclerotiorum* (Zhou and Reeleder, 1989; Budge and Whipps, 2001). Although *C. minitans* was sensitive to the fungicides benomyl and vinclozolin (Li et al., 2002), the present field trials showed that the treatments of *C. minitans* plus benomyl (low dosage) in 2003 or *C. minitans* plus vinclozolin (low dosage) in 2004 were as effective as *C. minitans* alone in the suppression of sclerotinia stem rot of rapeseed and in the infection of sclerotia. Therefore, *C. minitans* has the potential to be integrated with fungicides for management of sclerotinia diseases of rapeseed. Further field trials on combined application of *C. minitans* with fungicides on a large scale are warranted.

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