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Competence of *Coniothyrium minitans* in Preventing Infection of Bean Leaves by *Sclerotinia sclerotiorum*

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ABSTRACT

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The effectiveness of applying pycnidiospores of *Coniothyrium minitans* to bean flowers to protect bean leaves from infection by ascospores of *Sclerotinia sclerotiorum* was studied using a detached leaf assay. *C. minitans* applied to bean flowers at 8, 80 or 800 spores mm^{-2} at 24 or 48 h prior to that of ascospores of *S. sclerotiorum* reduced lesion development on bean leaves caused by the pathogen. When applied at the same time or 24 h after *S. sclerotiorum*, the efficacy of *C. minitans* to suppress lesion development by *S. sclerotiorum* was reduced, especially at low application rates. *C. minitans* at 800 spores mm^{-2} applied to bean flowers suppressed lesion development by *S. sclerotiorum* at 15, 20 or 25℃. *C. minitans* applied to the surface of bean leaves also reduced the diameter of *S. sclerotiorum* lesions formed when flowers inoculated with ascospores of *S. sclerotiorum* were placed on the leaf surface, though less effectively than when *C. minitans* was applied directly to the bean flower. Water-soluble extracts of bean leaves or flowers stimulated spore germination and hyphal growth of *C. minitans*. Thus, colonization of senescent tissues such as bean flowers by *C. minitans* is an effective mechanism for suppression of white mold of bean caused by *S. sclerotiorum*.

Key words : White mold of bean, biological control, *Sclerotinia sclerotiorum*, ascospores, *Coniothyrium minitans*, pycnidiospores, detached leaf assay

INTRODUCTION

Ascospores of *Sclerotinia sclerotiorum* (Lib.) de Bary are the primary source of inoculum for white mold of bean (*Phaseolus vulgaris* L.) [1]. Infection of healthy plant tissues by ascospores occurs in the presence of exogenous nutrients available from petals [23, 24], anthers [16] and pollen [9, 17, 27]. Preventing colonization of senescent flowers by ascospores of *S. sclerotiorum* is the key to the successful control of the disease in bean and other crops. Hunter et al. [20] found that application of chemical fungicide to bean flowers effectively reduced incidence of white mold of bean. Naturally occurring microorganisms can also prevent colonization of flowers by *S. sclerotiorum* [5, 18, 22].

Coniothyrium minitans Campbell [7] is a mycoparasite capable of attacking hyphae [15] and sclerotia [14] of *S. sclerotiorum*. It is able to survive and spread in the plant canopy [3, 8, 28], and has been evaluated as a potential foliar-applied biocontrol agent for white mold of bean [8, 28]. In a three-year field study in southern Alberta, comparing five fungal agents, *C. minitans* was the most effective agent in the suppression of white mold in bean [12], but its efficacy of white mold control in bean was often inconsistent. Our objective was to evaluate the effectiveness of *C. minitans* in protecting healthy bean leaf tissues from infection by ascospores of *S. sclerotiorum* using a detached leaf assay.

MATERIALS AND METHODS

Preparation of spore suspensions

Two isolates of *C. minitans*, LRS 2137 and Cm#2, were used in this study. LRS 2137 is a wild type isolated from an infected sclerotium of *S. sclerotiorum* collected in a sunflower field in Manitoba [11]. Cm#2 is a single-spore isolate selected from strain LRS 2137 for its stability and sporulation

characteristics. To produce pycnidiospores from each isolate, agar plugs containing mycelia were inoculated on potato dextrose agar (PDA) in Petri dishes, 1 plug/dish. After incubation for 3 weeks at 20°C under continuous fluorescent light, pycnidiospores were harvested by flooding the colony with sterile water and gently rubbing the colony surface to dislodge spores. Concentrations of the pycnidiospore suspension were determined using a hemacytometer (Hausser Scientific Company, Horshan, PA, 19044-1286, USA).

Spores of *S. sclerotiorum* were obtained from isolate sun-87, a single ascospore isolate collected from a diseased head of sunflower in Alberta [19]. To produce ascospores, agar plugs containing mycelial mats were removed from 5-day-old PDA cultures and inoculated on PDA in Petri dishes, 1 agar plug/dish. The dishes were sealed with parafilm and kept in the dark at 10°C for 8 to 10 weeks. Sclerotia produced in each dish were removed, transferred to Petri dishes containing moist sterile vermiculite and incubated in a growth cabinet at 20°C under continuous fluorescent light for 2 to 3 weeks. Mature apothecia produced on the sclerotia were collected and used to make ascospore suspensions for the experiments immediately or they were placed on filter paper in a desiccant and stored at -20°C for future use [21].

Timing of flower inoculation

This experiment evaluated the effect of timing *C. minitans* application to flowers of bean, cultivar NW 63, on control of *S. sclerotiorum*. Young bean flowers were removed from plants, cultivar NW63, grown in the greenhouse in pots containing Cornell mix [6]. The flowers were placed on moistened filter paper in Petri dishes and used immediately or stored at 4°C and used within 3 days. Suspensions of pycnidiospores of *C. minitans* isolate LRS 2137 were prepared from PDA cultures by the method described above and sterile water was added to adjust the concentrations to 10^6, 10^7 and 10^8 spores ml^{-1}. Spore suspensions, including a water control, were atomized onto whole flowers for 5 minutes at 0.5 liter per minute (LPM) in a 4-L chamber using a nebulizer (Aero Mist Neb Set, VitalAire, Edmonton, Alberta). This procedure was conducted 48 h before, 24 h before, just before or 24 h after inoculation of the bean flowers with ascospores of *S. sclerotiorum*. Flowers were inoculated with *S. sclerotiorum* by atomizing a suspension containing 10^6 ascospores ml^{-1} for 10 minutes at 0.5 LPM in a 15-L chamber. In each run with *C. minitans* or *S. sclerotiorum*, the density and viability of applied spores was determined by placing an uncovered Petri dish containing PDA among the inoculated flowers and directly examining the plate under 400x magnification immediately after atomization for spore density and again after 30 h for number of viable spores. During the period between inoculation of *C. minitans* and *S. sclerotiorum*, flowers were placed in a plastic bag with wet paper towels and incubated in a growth cabinet at 20°C with a 16 h light/8 h dark cycle. Once the flower had received its full treatment, it was placed onto a fully-expanded trifoliate bean leaf collected from greenhouse-grown plants. To maintain high humidity, the leaves were placed on wet paper towels in trays with the petiole of each leaf covered with wet paper towel. The surface of the leaves was misted with water prior to placement of 1 or 2 flowers on each leaf. Trays containing 4 to 6 leaves were sealed within clear plastic bags and placed in a growth cabinet at 20°C with a 16 h light/8 h dark cycle. Lesions that developed on leaves were measured after 7 days. Each treatment was replicated eight times in a randomized complete block design. The experiment was repeated twice and results were combined after testing for homogeneity of variances.

Leaf inoculation

This experiment determined if *C. minitans* could suppress infection by *S. sclerotiorum* when applied to the surface of the bean leaf instead of the flower. The same procedures were followed as described previously, except that pycnidiospores of *C. minitans*, isolate LRS 2137, were atomized directly onto bean leaves just before or 48 h before placing *S. sclerotiorum*- treated flowers onto the leaves. Each treatment was replicated eight times in a randomized complete block design.

Temperature

This experiment determined the effect of temperature on the effectiveness of *C. minitans* to suppress infection by *S. sclerotiorum*. The same methods were used as described previously, except that bean flowers were inoculated with *C. minitans* isolate LRS 2137 at 24 h before inoculation with *S. sclerotiorum* and flowers and leaves were incubated in controlled temperature chambers in the dark at 15, 20, 25 and 30°C for 6 days. Each treatment was replicated eight times in a randomized complete block design.

Germination of pycnidiospores

This experiment determined if soluble nutrients from bean flowers or leaves could stimulate the germination of pycnidiospores of *C. minitans*. Bean flowers or leaves were obtained from greenhouse-grown plants. One gram of plant material was ground with 10 ml of water in a glass grinder. The sample was immediately passed through a Whatman #1 filter paper to remove large particles and then passed through an 8-μm filter. The extracts or water were applied (200 μL) to membrane filters (Millipore 0.4 μm HTTP, 5 cm diameter) placed in small Petri dishes. A pycnidiospore suspension containing 10^5 spores ml^{-1} was prepared using *C. minitans* isolate Cm#2. The suspension was atomized onto the membranes by the same method described previously for application of pycnidiospores to bean flowers. The

membranes were incubated under humid conditions at 20°C for 24 h. The samples were then rapidly dried on the laboratory bench. After staining with 0.01% acid fuchsin in 85% lactic acid, pycnidiospores on the membranes were examined for germination under a compound microscope.

RESULTS

Based on spore counts, 800 to 1000 spores mm^{-2} were applied when a pycnidiospore suspension of *C. minitans* containing 10^8 spores ml^{-1} was atomized for 5 min in a 4 L chamber. Preliminary experiments demonstrated that spore concentration was proportional to density of applied spores. For *S. sclerotiorum*, 8 to 16 ascospores mm^{-2} were applied when a suspension containing 10^6 ascospores ml^{-1} was atomized for 10 minutes in a 15 L chamber. Thus, the spore ratio of *C. minitans* to *S. sclerotiorum* applied to the bean tissues ranged from about 0.75 at the low concentration of 10^6 pycnidiospores ml^{-1} to 75 at the high concentration of 10^8 pycnidiospores ml^{-1}. All the spore suspensions of *C. minitans* and *S. sclerotiorum* used in this study had a spore germination rate higher than 85%.

The effectiveness of applying *C. minitans* to bean flowers in protecting bean leaves from *S. sclerotiorum* infection was affected by the time between application of *C. minitans* and *S. sclerotiorum* (Fig. 1). Application of *C. minitans* 48 h before *S. sclerotiorum* reduced lesion diameters in detached bean leaves by an average of 79% at all application rates of *C. minitans*, (significant at $P<0.05$). When *C. minitans* was applied 24 h before *S. sclerotiorum*, all three application rates reduced lesion diameter, but effectiveness increased with increasing application rate (Fig. 1). When *C. minitans* was applied just before or 24 h after *S. sclerotiorum*, application of *C. minitans* at 8 pycnidiospores mm^{-2} did not reduce lesion diameter while application of *C. minitans* at 80 pycnidiospores mm-2 was only effective when applied just before *S. sclerotiorum*. Although application of *C. minitans* at 800 pycnidiospores mm^{-2} significantly reduced lesion diameter when applied just before or 24 h after *S. sclerotiorum*, the reduction in lesion diameter was less than when pycnidiospores were applied 24 or 48 h prior to *S. sclerotiorum*. New pycnidia of *C. minitans* were often observed on flowers after 7 days when treated with *C. minitans* at 800 pycnidiospores mm^{-2}.

Application of *C. minitans* to the surface of detached bean leaves also reduced the diameter of *S. sclerotiorum* lesions, but less effectively than when applied directly to bean flowers (Figs. 1 and 2). Lesions developed in all replicates of this experiment (data not presented). The high application rate of 800 pycnidiospores mm^{-2} of *C. minitans* was the most effective, reducing lesion diameter by an average of 42%. The timing of *C. minitans* application to bean leaves did not affect the efficacy of *C. minitans* (Fig. 2).

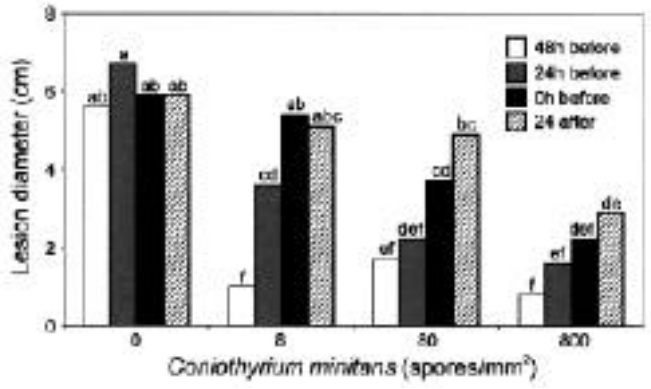

Fig. 1. Effect of applying *C. minitans* to bean flowers on the development of lesions of *S. sclerotiorum* in detached bean leaves. Pycnidiospores of *C. minitans* were atomized onto bean flowers 48 h before, 24 h before, just before (0 h), or 24 h after inoculation of the bean flowers with *S. sclerotiorum*. Flowers and leaves were incubated in a moist chamber for 7 days after application of *S. sclerotiorum*. Values labeled with a common letter are not significantly ($P>0.05$) different from each other based on the Least Significant Difference test.

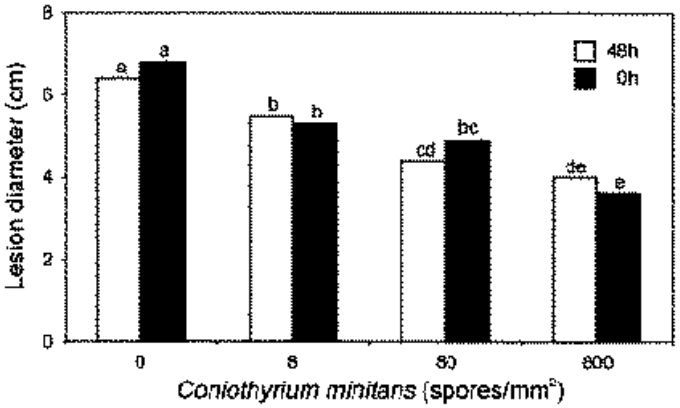

Fig. 2. Effect of applying *C. minitans* to the surface of detached bean leaves on the development of lesions of *S. sclerotiorum*. Pycnidiospores of *C. minitans* were atomized onto the surface of detached bean leaves 48 h before or just before placement of *S. sclerotiorum*- treated flowers on the leaves. Flowers and leaves were incubated in a moist chamber for 7 days after application of *S. sclerotiorum*. Values labeled with a common letter are not significantly ($P>0.05$) different from each other based on the Least Significant Difference test.

The effectiveness of *C. minitans* in preventing infection of bean leaves by *S. sclerotiorum* was affected by temperature. At 15 and 20°C, all three application rates of *C. minitans* significantly ($P < 0.05$) reduced the diameter of *S. sclerotiorum* lesions, but only the high rate of 800 pycnidiospores mm^{-2} significantly reduced lesion diameter at 25°C (Fig. 3). At 30°C, no leaf lesions developed in any of the treatments. For the treatment of *S. sclerotiorum* alone

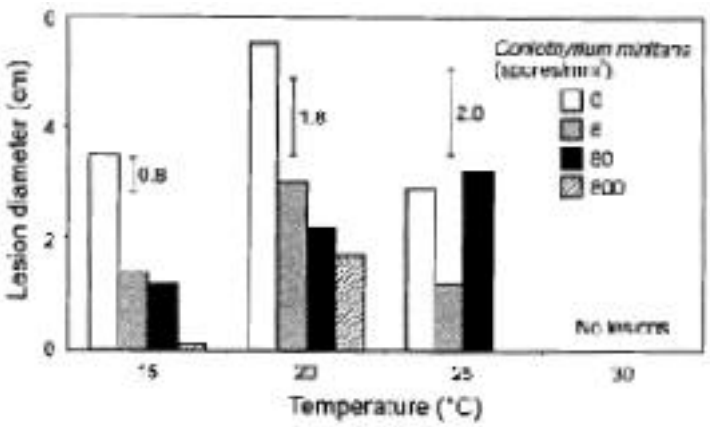

Fig. 3. Effect of temperature on the efficacy of *C. minitans* to suppress lesion development of *S. sclerotiorum* on detached bean leaves. Pycnidiospores of *C. minitans* were atomized onto bean flowers 24 h before inoculation of the bean flowers with *S. sclerotiorum*. Flowers and leaves were incubated in a moist chamber for 6 days after application of *S. sclerotiorum*. Error bars are the Least Significant Differences (P=0.05) between application rates of *C. minitans* at each temperature.

(controls), leaf lesions developed at 20℃ were larger than those developed at 15 or 25℃.

Water extracts from bean flowers or leaves stimulated germination of pycnidiospores of *C. minitans* (Table 1). Whereas only 0.3% of pycnidiospores germinated in the water control, 91% of pycnidiospores germinated in flower or leaf extracts. Moreover, water-soluble extracts of bean flowers or leaves also stimulated the growth of germ tubes of germinated pycnidiospores (Table 1). The average length of the germ tube after 24 h was 137 μm for spores placed on membrane filters treated with flower extract and 59 μm for spores placed on membrane filters treated with bean leaf extract.

DISCUSSION

Application of pycnidiospores of *C. minitans* to bean flowers effectively reduced infection and lesion development on bean leaves by ascospores of *S. sclerotiorum* in this study. The effectiveness depended on inoculum density, timing of application and temperature.

Table 1. Effect of water extracts of bean flowers or leaves on germination of *C. minitans* pycnidiospores

Substrate	Germination[1] (%)	Germ tube length (μm)
Water	0.3±0.4	NA
Bean flower extract [2]	91.3±0.4	137±25
Bean leaf extract [2]	91.3±p 1.8	59±2

1. *C. minitans* pycnidiospores were atomized onto membranes moistened with substrate. Germination and germ tube length were observed after 30 h. Values are means± standard deviations.
2. One gram of fresh plant material was mixed with 10 ml of de-ionized water, ground in a tissue grinder, and filtered through Whatman No. 1 and 8-μm filters.

An approximate spore ratio of 75:1 of *C. minitans* to *S. sclerotiorum* provided a consistent reduction in lesion development by *S. sclerotiorum* when *C. minitans* was applied to non- sterilized bean flowers (Figs. 1&3). In comparison, Boland and Hunter [(5)] found that co- inoculation of autoclaved bean flowers with spores of *Alternaria alternata* (Fr.) Keissler or *Cladosporium cladosporioides* (Fr.) De Vries and *S. sclerotium* at a spore ratio of 1:1 significantly reduced the number of lesions formed on bean leaves compared to the number that occurred with *S. sclerotium* alone. Inglis and Boland [(22)] obtained almost complete inhibition of lesion development with 9 out of 10 fungal strains when applied to sterilized bean flowers as a spore mixture with a ratio of 6:1 between *S. sclerotiorum* and the test fungus. The effectiveness of fungal agents to suppress lesion development at a lower ratio in these studies than in our study may be due to the use of sterilized bean flowers, different fungal agents or differences in the absolute amount of spores applied.

The effective temperature range for suppression of *S. sclerotiorum* by *C. minitans* appears to coincide with the temperature range for the growth of these two organisms. McQuilken et al. [(25)] showed that the temperature range for spore germination and mycelial growth of *C. minitans* was 10-25℃, with an optimum of 20℃, similar to the temperature range for suppression of lesion development of *S. sclerotiorum* by *C. minitans* observed in the present study. The present study also confirms earlier reports that infection of bean plants by ascospores of *S. sclerotiorum* fails to occur at 30℃ [(1, 4)].

The suppressive effect of *C. minitans* on infection and lesion development by *S. sclerotiorum* can be attributed to competition for exogenous nutrients and parasitism or other antagonistic activities by *C. minitans*. The availability of exogenous nutrients is essential for infection of bean plants by ascospores of *S. sclerotiorum* [(1, 26)] and was reduced by pre-emptive colonization by *C. minitans*. Efficacy of *C. minitans* improved when applied 24 or 48 h before *S. sclerotiorum* than when applied at the same time or 24 h after *S. sclerotiorum* (Fig. 1). Availability of exogenous nutrients from bean flowers supported the formation of pycnidia of *C. minitans*. Water-soluble extracts from bean flowers also stimulated germination and growth of *C. minitans* (Table 1). *C. minitans* may also suppress lesion development by attacking hyphae of *S. sclerotiorum*, as observed in pure culture [(13, 15)] and on plant tissue [(28)]. The efficacy of the high application rate of *C.*

minitans to reduce lesion size when applied 24 h after *S. sclerotiorum* (Fig. 1) or to the surface of the leaf (Fig. 2) may be due to parasitic activities of *C. minitans*; other studies with non-parasitic biocontrol agents have observed much poorer control when the biocontrol agent was applied 24 h after *S. sclerotiorum* [22, 29]. However, Huang [10] reported that *C. minitans* was ineffective in preventing secondary spread of Sclerotinia wilt of sunflower (*Helianthus annuus* L.) due to rapid mycelial growth of *S. sclerotiorum* in host tissues.

Previous reports indicate that control of white mold in the field by application of *C. minitans* at rates of 10^{11} to 2 x 10^{12} spores ha^{-1} was less effective than application of the systemic fungicide, benomyl [3, 12, 28]. This reflects the difference in mode of action of a biocontrol agent and a chemical fungicide. While a chemical fungicide can be active against the target pathogen immediately after application, biocontrol agents require a certain period of time under suitable environmental conditions for the agent to become established and prevent colonization by the pathogen [2]. Although *C. minitans* successfully colonized bean flowers and suppressed leaf lesion development by *S. sclerotiorum* in the present study, improved control under field conditions may require higher dosages or further development of an isolate and formulation that allows *C. minitans* to persist and colonize flowers under field conditions.

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摘　要

Bremer, E. [1], 黃鴻章[2,4], Selinger, L.J.[2], Davies, J.S.[3] 2000. *Coniothyrium minitans* 抑制菌核病菌危害菜豆葉片的效果. 植病會刊 9:69-74. ([1.] Present address: Symbio Ag Consulting, 1703-18 Street S., Lethbridge, Alberta, Canada T1K 2B5; [2.] 加拿大農業及食品部, Lethbridge Research Centre, PO Box 3000, Lethbridge, Alberta, Canada T1J 4B1; [3.] Apotex Fermentation Inc., 40 Scurfield Boulevard, Winnipeg, Manitoba, Canada R3Y 1G4; [4.] 聯絡作者：電子郵件 huangh@em.agr.ca; 傳真 (403) 382-3156)

本研究旨在探討應用離葉測定法來撿驗拮抗真菌 *Coniothyrium minitans* 柄孢子 (pycnidospores) 對控制菌核病菌 (*Sclerotinia sclerotiorum*) 子囊孢子 (ascospores) 危害菜豆葉片的效果。將每平方毫米 (mm^{-2}) 含有 8, 80, 或 800 個 *C. minitans* 柄孢子的懸浮液接種於菜豆花瓣上，經 24 或 48 小時之後，再接種菌核病菌之子囊孢子懸浮液。經這種處理的花瓣放在菜豆之切離葉片上時，有顯著抑制菌核病菌的病斑在葉片擴展的效果。但如果將菌核病菌之子囊孢子先接種於花瓣上，經 24 小時之後再接種拮抗微真菌之柄孢子，或者將兩種孢子同時接種於花瓣上，則 *C. minitans* 抑制葉片病斑擴展的效果就顯著降低。這種防治效果減低的現象尤以低濃度的 *C. minitans* 柄孢子之處理時為最。經 *C. minitans* 高濃度 (每平方毫米 800 個孢子) 之處理的花瓣，於 15, 20, 及 25℃ 的環境下，均有顯著抑制葉片病斑擴展的效果；但是溫度升高到 30℃ 時，因已接近臨界高溫而沒有病斑形成。將 *C. minitans* 柄孢子直接噴布於菜豆葉片上，也有降低菌核病菌囊孢子入侵的作用，但效果遠不如將柄孢子先接種於花器再放置葉片上之處理。進一步實驗證實葉片或花器的水浸出液有刺激 *C. minitans* 柄孢子發芽及菌絲生長的功效。本研究證實用花器之類凋謝組織可以增進 *C. minitans* 防治菌核病菌子囊孢子危害菜豆的效果。

關鍵詞：菜豆菌核病、生物防治、*Sclerotinia sclerotiorum*、子囊孢子、*Coniothyrium minitans*、柄孢子、離葉檢定法