European Journal of Plant Pathology **109:** 489–502, 2003.

Quantitative aspects of infection of *Sclerotinia sclerotiorum* sclerotia by *Coniothyrium minitans* – timing of application, concentration and quality of conidial suspension of the mycoparasite

M. Gerlagh, H.M. Goossen-van de Geijn, A.E. Hoogland and P.F.G. Vereijken
Plant Research International, P.O. Box 16, NL-6700 AA Wageningen, The Netherlands
(Phone: +31 317 476084; Fax: +31 317 423110; E-mail: M.Gerlagh@plant.wag-ur.nl

Accepted 29 January 2003

Key words: white mould, epidemiology, biological control

Abstract

White mould disease leads to production of sclerotia, which subsequently survive in soil and may be responsible for future epidemics. The effect of the mycoparasite *Coniothyrium minitans* in decreasing survival of sclerotia of *Sclerotinia sclerotiorum* was studied. Infection of sclerotia of *S. sclerotiorum* by *C. minitans* can be achieved by a single conidium. Under optimal conditions, 2 conidia per sclerotium produced 63% of the maximum infection (ca. 90%) of sclerotia produced by up to 1000 conidia. Similar results were observed on the infection of stem pieces infected by *S. sclerotiorum*. In field trials, application of conidial suspensions of *C. minitans* to a bean crop soon after white mould outbreak led to a higher percentage of sclerotial infection than later applications. Ninety per cent infection of sclerotia was obtained within 3 weeks of application by *C. minitans* suspensions in the range of 5×10^5 and 5×10^6 conidia ml^{-1} at 1000 l ha^{-1}. The concentration of the conidial suspensions and the isolate used were of less importance. The result was marginally affected by the germinability of the conidia (75% against 61% infected sclerotia at 91% and 16% viability of isolate IVT1, respectively). Less apothecia of *S. sclerotiorum* developed in soil samples collected after 2 months from plots sprayed immediately after disease outbreak than from those treated 11–18 days later. It is concluded that a suspension of 10^6 conidia ml^{-1} in 1000 l ha^{-1} ($= 10^{12}$ conidia ha^{-1}) sprayed immediately after the first symptoms of disease are observed, results in >90% infection of sclerotia of *S. sclerotiorum.* The infection of sclerotia, which prevents their carry-over, occurs within a broad range of inoculum quality.

Introduction

White mould, caused by the fungus *Sclerotinia sclerotiorum*, is one of the most damaging plant diseases of the temperate zone. Due to the pathogen's wide host range and its survival as sclerotia, the control of white mould largely relies on the use of fungicides. Public pressure for less dependence on chemical crop protection has stimulated research on biological control. Two specific fungal antagonists of *S. sclerotiorum*, *Sporidesmium sclerotivorum* and *Coniothyrium minitans* have drawn special attention in this respect (Adams and Ayers, 1981; Whipps and Gerlagh, 1992). *Coniothyrium minitans* was registered as 'Contans' for white mould control in lettuce in Germany in 1997 and as 'Koni' in Hungary in 1998 (Whipps and Lumsden, 2001). Recently, Contans has been registered in many other countries and for various crops (www.Prophyta.de). *Coniothyrium minitans* is highly specific to *Sclerotinia* spp. and *Sclerotium cepivorum*. Specificity has the advantage of excluding undesirable side-effects, but limits the market size, making the product less attractive to producers and more expensive to customers. Consequently, the user of such an expensive product aims at a good effect, while using the minimal quantity, although the realistic dosage of such a biocontrol agent is also a function of the price of chemical alternatives.

There are two main approaches in the application of *C. minitans*: (1) to the soil to kill sclerotia, the source of apothecia (the fruiting bodies), and (2) to crop debris to infect mycelium and sclerotia of *S. sclerotiorum,* which consequently prevents contamination of the soil with viable sclerotia (Gerlagh et al., 1999).

The mycoparasite lacks active growth through the soil which limits the effect of application of *C. minitans* conidia to soil (Adams, 1990). To compensate, large quantities of inoculum and thorough mixing may enhance contact between sclerotia and the mycoparasite. However, Williams et al. (1998) have shown that the soil mesofauna can transport conidia of *C. minitans* in the soil. This could result in a substantial reduction in numbers of sclerotia, even with lower densities of inoculum. When a diseased crop or crop debris is treated with a suspension of *C. minitans* conidia, the conidia have direct access to the survival structures of *S. sclerotiorum*. The question arises whether this would allow the use of much lower dosages of the biological control agent (BCA) than with soil application, without jeopardising its effect.

The present study addresses two aspects of this problem. Firstly, elucidation of the minimum number of conidia of the mycoparasite necessary to cause substantial infection and subsequent death of sclerotia of *S. sclerotiorum* in *in vitro* experiments. This study was extended to include plant tissue pervaded by *S. sclerotiorum*. Secondly, the effect of concentration, spore batch (quality and isolate) and application time on sclerotial infection and apothecial production was studied. Field plots were sprayed with realistic concentrations of conidial suspensions after the appearance of first disease symptoms with the aim to induce a high degree of infection of sclerotia in order to guarantee a reduced soil contamination and therefore control of future white mould epidemics.

Materials and methods

Spore suspensions of C. minitans

Coniothyrium minitans was grown in spawn bags on oats for 3–4 weeks at 20 °C (Gerlagh et al., 1999). After incubation, the culture was used immediately or stored at 4 °C after drying for 24 h at room temperature in a sterile airstream. Spore suspensions were prepared by macerating the oat grain inoculum in a household blender. About 100 g (dry weight) of the inoculum was added to 1 litre tap water, macerated for 10 s, stored for 30 min at 4 °C, and then macerated for 2 min at full speed. The thick suspension was passed through a sieve to eliminate chaff and subsequently through double cheesecloth. The concentration of the remaining suspension was counted with a haemacytometer and the suspension diluted according to the needs. The concentration commonly used for application as a BCA was 5×10^6 conidia ml^{-1}.

Depending on the experiment, three isolates of *C. minitans*, IVT1, C15 (Gerlagh et al., 1996) and a German isolate received from Dr. P. Lüth (Prophyta), were used.

Infection of sclerotia by C. minitans

Laboratory experiments

Production of sclerotia and plant tissue colonised by S. sclerotiorum. A culture of *S. sclerotiorum* was obtained from infected carrot. Sclerotia were produced on potato dextrose agar (PDA, Oxoid, Basingstroke, UK) in 9 cm diameter Petri dishes incubated in darkness at 20 °C. After 1 week the first ripe (black) sclerotia of >2 mm diameter were collected and stored at 4 °C in a Petri dish sealed with parafilm. Every second day new sclerotia were collected from the same dishes until production had stopped.

Snapbean, *Phaseolus vulgaris*, cv. Groffy, was sown in 14 cm pots filled with potting soil and grown in the greenhouse at 20 °C under natural light conditions. Stems of 6-week-old plants were cut in 1.2 cm segments, surface sterilised in 1% sodium hypochlorite for 30 s, and rinsed three times in sterilised tap water. The segments were arranged in a concentric circle in Petri dishes containing PDA and inoculated with *S. sclerotiorum* 24 h earlier. After 3 days of incubation at 16 °C the segments had been colonised by the pathogen. They were collected and put individually in a sterile 24-well cell culture plate (COSTAR, Cambridge, MA, USA). They were further incubated for 3 days at 20 °C in the dark, with a moist filter paper in the cover of the plate to maintain humid conditions.

Inoculation with C. minitans *and assessment of infection.* Sclerotia were individually applied in a sterile 24-well cell culture plate. A 25 µl spore suspension of *C. minitans* was applied on top of each sclerotium. Spore suspensions were diluted to yield 1, 5, 10, 50, 100, 500 and 1000 conidia per 25 µl droplet. Real numbers per droplet were checked on a microscope slide, 6–8 drops per concentration. Cell

culture plates were incubated at 20 °C for 2 weeks with moist filter paper in the cover to create optimal conditions for infection. Subsequently, sclerotia were screened for infection by *C. minitans*. They were surface sterilised, bisected and plated on PDA plus tetracycline (Whipps and Budge, 1990) and the percentage of infected sclerotia was scored after 2 weeks of incubation in the dark at 20 °C. Other plates were incubated for 3 weeks and screened by visual observation at 50× under the dissecting microscope, which was repeated after one more week of incubation. The presence of *C. minitans* was evident as black pycnidia with conidia oozing out in black, shiny slime.

Sclerotinia sclerotiorum-infected stem segments were inoculated with *C. minitans* in the same way as sclerotia. Care was taken not to inoculate sclerotia whenever they were formed on the tissue. Infection of *S. sclerotiorum* on the stem pieces by *C. minitans* was assessed visually after 3 weeks of incubation.

The experiments were set up as a randomised block (block = 24-well plate) design. In each 24-well plate 10 sclerotia were inoculated with a specific spore concentration of isolate IVT1, and 10 with the same concentration of isolate C15. Four control sclerotia in each plate remained uninoculated. The sclerotial randomised block design had three plates (replicates) per conidial concentration. This experiment was repeated twice, with the 5 conidia per 25 µl droplet treatment missing in the first repetition. The stem segment assay consisted of five plates (replicates) per conidial concentration, each containing per plate ten stem segments per isolate, and again four uninoculated control segments.

Additional experiments were performed with *S. sclerotiorum*-infected stem segments at incubation temperatures of 15 and 10 °C .

Field trials

Field trials were performed in 1995 and 1996 to compare the effect of timing of *C. minitans* application, of different conidial concentrations of suspensions, and different qualities (isolates and batches) on sclerotial infection by the mycoparasite.

Lay-out trial one, 1995. A split-plot design field trial was laid out on May 22, 1995 with 156 subplots of $5 \times 5\,m^2$ with snapbean, cv. Groffy, separated from each other by a 3 m wide maize buffer to prevent reciprocal contamination with *C. minitans* between plots with different treatments. Rows of bean were 50 cm apart; 15 untreated bean seeds m^{-1}. Crop management was limited to hand weeding, and fertilising according to normal agricultural practice. No crop protectants were applied. The field contained the following treatments in four replicate blocks.

The three main plots per block represented soil inoculation with laboratory-grown sclerotia of *S. sclerotiorum* at three levels, 0, 1 and 5 g sclerotia m^{-2}, randomised within the blocks. The sclerotia were grown on beans, washed free from bean residues, and incubated for at least 6 weeks at 4 °C to stimulate germination with apothecia. They were spread on the field immediately before sowing the bean crop and subsequently raked in to obtain a distribution within the superficial layers of the soil in order to assure production of apothecia after canopy closure of the bean crop.

Within each plot, 13 treatments were randomly allocated to subplots. After the appearance of symptoms of white mould, subplots were sprayed with a suspension of *C. minitans* conidia in water at a rate equivalent of 1000 l ha^{-1} (2.5 l per subplot). The application time of *C. minitans* (early, August 8 (immediately after the first symptoms of white mould had been found); moderately late, August 16; or late, August 24), the isolate (IVT1, C15 or German isolate) used and the concentration (5×10^5, 10^6 or 5×10^6 conidia ml^{-1}) varied:

1. Early, IVT1, 5×10^5;
2. Early, IVT1, 10^6;
3. Early, IVT1, 5×10^6;
4. Early + moderately late, IVT1, 5×10^5;
5. Early + moderately late, IVT1, 10^6;
6. Early + moderately late, IVT1, 5×10^6;
7. Moderately late, IVT1, 5×10^5;
8. Moderately late, IVT1, 10^6;
9. Moderately late, IVT1, 5×10^6;
10. Late, IVT1, 10^6;
11. Late, C15, 10^6;
12. Late, German isolate, 10^6;
13. Control (untreated)

To follow the infection of sclerotia by *C. minitans,* a collection of 5 sclerotia of *S. sclerotiorum* per subplot took place weekly, beginning 8 days after the spray application. Sampling within the subplots was random from different plants, and continued until the crop was overripe and drying. The number of sclerotia sampled was reduced when too few (< 5) bean plants with white mould occurred in the plot. Infection of sclerotia by *C. minitans* was assessed by plating surface sterilised sclerotia onto PDA as described above (Laboratory experiments).

Lay-out trial two, 1996. In 1996, a comparable trial was laid out, with minor modifications. Plot size was $5 \times 4.5\,m^2$, and sowing date was May 30. The trial set-up was the same as in 1995 with four replicate blocks, each consisting of three main plots with inoculation of 0, 1 and 5 g sclerotia m^{-2}.

Subplots were sprayed with a suspension in water of *C. minitans*, 1000 l ha^{-1} (2.25 l per subplot). There were 12 different treatments (subplots), differing in time of application of *C. minitans* (early, August 9; moderately late, August 20, or late, August 27), in isolate of *C. minitans* (IVT1 or C15) used, in the quality of the conidia (% viability), and in concentration of the spore suspension (10^6 conidia ml^{-1} for treatments 1–3 and 6–11 and 5×10^5 and 5×10^6 conidia ml^{-1} for treatments 4 and 5, respectively). The quality of conidia was influenced by the duration of incubation of the culture of *C. minitans*. Three weeks of incubation led to production of conidia with 91% and 83% germination on PDA within 48 h for IVT1 and C15, respectively. Six weeks of incubation of IVT1 yielded conidia with 16% germination. The treatments were:

1. Early, IVT1, 91% viability;
2. Early, IVT1, 16% viability;
3. Early, C15, 83% viability;
4. Early and moderately late, IVT1, 91% viability, 5×10^5 conidia ml^{-1};
5. Early and moderately late, IVT1, 91% viability, 5×10^6 conidia ml^{-1};
6. Moderately late, IVT1, 91% viability;
7. Moderately late, IVT1, 16% viability;
8. Moderately late, C15, 83% viability;
9. Late, IVT1, 91% viability;
10. Late, IVT1, 16% viability;
11. Late, C15, 83% viability;
12. Control (untreated).

To stimulate germination of *S. sclerotiorum* sclerotia and production of apothecia on the soil, irrigation was applied two times per week, beginning by the middle of July. Irrigation was discontinued by the middle of August when natural rainfall became sufficient (at least two rainy periods per week). First apothecia were observed on July 29. Weekly collection of sclerotia from the subplots was as in 1995.

Survival of sclerotia; soil samples

Based on experience (Gerlagh et al., 1999) sclerotia infected by *C. minitans* were believed not to survive in the soil and produce apothecia in the next season. To assess the effect of the *C. minitans* treatments on the numbers of sclerotia surviving in the soil after the 1996 bean crop, composite soil samples were collected from all subplots by taking five superficial (10 cm deep) soil samples of 1–1.5 kg (dry soil) per subplot at the end of the 1996 growing season. Each composite sample was stored over winter in a cold warehouse in a plastic tray $30 \times 40 \times 8\,cm^3$. The soil was kept moist by regular watering. With the rise of temperature in spring, apothecia developed on the soil. Numbers of apothecia were counted weekly until no new apothecia appeared in June. The number of apothecia per tray was considered proportional to the number of surviving sclerotia in the soil samples.

Statistics

Laboratory experiments

The relationship between percentage of infected sclerotia and the density of *C. minitans* (numbers per droplet) was described by the exponential model

$$y = A[1 - \exp(-cz)]$$

where y is the percentage of the pathogen population infected, z is the density of *C. minitans*, c is a constant that governs the efficiency of the biological control agent and A represents the maximum percentage that can be infected by *C. minitans* (Johnson, 1994; formula 4). This model allows less than 100% infection of sclerotia with high inoculum doses. In separate analyses, both target density values and realised densities were used in fitting the exponential model to observed percentages of infection of sclerotia and of stem pieces by *C. minitans* as scored in the laboratory experiments. Under the exponential model the response is 63%, 86% and 91% of the maximum value at densities of $z = 1/c, 2/c$ and $3/c$, respectively. An approximate 95% confidence interval for $1/c$ was computed as $1/c \pm 2 \times \mathrm{SE}(1/c)$, where SE denotes the standard error. The exponential model was fitted to the data of each substrate $*$ isolate $*$ screening method combination, using the non-linear procedure in GENSTAT (Genstat 5 Committee, 1993; 1997).

Field trials; infection of sclerotia

For a visual assessment of differences in mean percentages of infected sclerotia with time between the spray treatments and the control treatment, means were

plotted against the number of weeks after spraying. This was done separately for the set of early spray treatments and the control, the set of moderately late spray treatments and the control and for the set of late spray treatments and the control. The treatments which were sprayed twice, early and moderately late, were analysed as early sprays.

For the 1996 experiment, differences between treatments in percentage of infected sclerotia at 4 weeks after spraying were investigated by fitting logistic regression models for over-dispersed proportions (McCullagh and Nelder, 1989) to the observed counts of infected sclerotia. For comparisons with the control treatment a logistic model with replicate and treatment as explanatory variables was fitted to the observed counts of infected sclerotia of each set separately. Effects of different levels of time of application and of 'type of isolate' (IVT1 with 91% viability, IVT1 with 16% viability and C15) were investigated by fitting a logistic model with replicate, time of application, isolate and the interaction between time of application and isolate to the observed counts of infected sclerotia of the set of 9 factorial treatment combinations of time of application (early, middle late and late) and isolate.

In the model, the relationship between the probability P, $0 < P < 1$, that a random sclerotium will be infected and the explanatory variables was described by the logit-link function, i.e.

$$\text{Logit}(P) = \ln\left(\frac{P}{1-P}\right) = \text{constant} + \text{replicate} + \text{effects explanatory variables}$$

In the models the variance of the observed counts (Y) of infected sclerotia was assumed to be proportional to binomial variance, i.e.

$$\text{Var}(Y) = \varphi n P(1-P),$$

where n is the number of sclerotia examined and φ denotes the dispersion parameter, with $\varphi > 1$ in case of over-dispersion.

Apothecial counts

Differences in total counts of observed apothecia were examined using a generalised linear model for gamma distributed counts (McCullagh and Nelder, 1989). In the model, the relationship between mean counts and the explanatory variables was described by the log-link function. The log-link function reads

$$\ln(\mu) = \text{constant} + \text{replicate} + \text{effects explanatory variables}$$

The log-link function was used, assuming proportional treatment effects are more appropriate than additive effects. The model assumed that the coefficient of variation in the observed counts is approximately constant. The validity of this assumption was checked by investigating the relationship between sample variance and sample mean on log–log scale. Two models were used: in model 1 replicate, time of application and treatments within time of application were taken as explanatory variables. In model 2 replicate, time of application, isolate (IVT1 with 91% viability, IVT1 with 16% viability and C15) and their interaction were taken as explanatory variables. Model 1 was fitted to the observed counts of all treatments. Model 2 was fitted to the data of the 9 factorial treatment combinations of time of application and isolate. Effects due to differences between different levels of artificial contamination of the plots were ignored, because they did not show any effects.

For generalised linear models an analysis of deviance is used as a generalisation of the analysis of variance for data with Normal errors. In an analysis of deviance table each deviance represents the variation accounted for by its corresponding term having eliminated the effects of terms above it, but ignoring any effects of those terms below it. The criterion for inclusion of a term in the model is an F-test for the ratio of the mean deviance for the particular term to the mean deviance of the rest. The mean deviance of the rest is an estimate for the over-dispersion of the observed proportions or an estimate of the square of CV for the observed counts of apothecia. Pairwise comparisons between treatment means on the logit or log-scale were tested using t-tests. All statistical analyses were performed with GENSTAT 5 (Genstat 5 Committee, 1993; 1997).

Results

Laboratory experiments

Infection of sclerotia and stem segments gave virtually the same results. Experiments with stem segments incubated at 15 and 10 °C also yielded the same levels of infection by *C. minitans* (data not shown). The data were analysed both for the targeted numbers of conidia per droplet (0, 1, 5, 10, 50, etc. conidia per droplet) and the numbers realised (as counted in the drops sampled on the microscope slides), but this led to essentially the same results. Data from two experiments with sclerotia and one with infected stem pieces

Table 1. Parameter estimates related to percentage infection by *C. minitans* of both sclerotia of *Sclerotinia sclerotiorum* and bean stem pieces infected by *S. sclerotiorum.* The infection was caused by a variable number of conidia (range 1–1000), isolates IVT1 and C15, and assessed by plating on agar and by visual inspection

Substrate	Isolate	Screening method	Trial number	Analysis according to realised number of conidia per droplet		% accounted for*	Estimate of droplet concentration for 63% of maximum response (# per 25 µl suspension)		
				A	c		$1/c$	Confidence interval $1/c$ (95%)	
								Lower limit	Upper limit
Sclerotia	IVT1	Plating	1	78.0 (5.67)	0.50 (0.244)	75	2.0 (0.96)	0.1	3.9
Sclerotia	IVT1	Plating	2	81.3 (3.58)	0.62 (0.188)	68	1.6 (0.49)	0.6	2.6
Sclerotia	C15	Plating	1	95.3 (2.49)	0.50 (0.082)	86	2.0 (0.33)	1.3	2.7
Sclerotia	C15	Plating	2	95.7 (4.62)	0.59 (0.124)	86	1.7 (0.36)	1.0	2.4
Sclerotia	IVT1	Visual	1	79.4 (5.46)	0.13 (0.048)	87	7.8 (2.92)	2.0	13.7
Sclerotia	IVT1	Visual	2	68.7 (1.90)	0.43 (0.081)	91	2.3 (0.44)	1.4	3.2
Sclerotia	C15	Visual	1	58.6 (10.68)	0.03 (0.018)	4	34.9 (22.47)	0	79.8
Sclerotia	C15	Visual	2	74.6 (2.05)	0.45 (0.051)	96	2.2 (0.25)	1.7	2.7
Stem pieces	IVT1	Visual	1	87.5 (5.18)	0.42 (0.154)	61	2.4 (0.88)	0.6	4.2
Stem pieces	C15	Visual	1	90.6 (2.66)	0.77 (0.158)	85	1.3 (0.26)	0.8	1.8

*By exponential model. The parameter estimates are based on the equation $y = A[1 - \exp(-cz)]$, in which: y = percentage infection; A = the asymptote value; maximum % infection at high inoculum dose; c = efficiency parameter; z = number of conidia (per 25 µl droplet inoculum). The SEs are indicated between brackets. At $z = 1/c$, $2/c$ and $3/c$ the % infection is 63%, 86% and 91%, respectively of A.

(analysed according to realised number of conidia) are summarised in Table 1 and Figure 1.

The asymptote value A, the maximum percentage infection by *C. minitans* obtained with high numbers of conidia per droplet, ranged from 58.6% to 95.7%. The lowest values were generally obtained with visual examination of treated sclerotia. The percentage accounted for by the model varied between 61% and 96%, with the exception of visually assessed sclerotia of C15 in the first experiment (Figure 1d). In the latter case only 4% of the variance was explained, and no fitted line was drawn in the graph. Even at 2 conidia per droplet, 63% (column $1/c$) of the asymptote infection percentage was already reached. Only in the first experiment, at this low number of applied conidia, visual inspection of infection of sclerotia led to aberrant figures for both IVT1 and C15 compared to diagnostic plating of the sclerotia. Visual inspection of stem pieces gave similar results as the plating of sclerotia. Four or six conidia per droplet led to 86% and 91% of the maximum infection.

Field trials

The number of apothecia in the field were often low ($<1\ m^{-2}$) in 1995. No relation was found between the appearance of apothecia and the artificial contamination of the field, indicating a poor germination of the applied sclerotia of *S. sclerotiorum*, which nevertheless could be seen on the soil. In most subplots, the disease incidence (number of bean plants infected) was low in 1995 in contrast to infection in 1996.

The percentage of sclerotium infection by *C. minitans* increased with time after application. One week after spraying it was still low in 1995 and only 70% infection was reached 4 weeks after the early spray treatment with the highest concentration (Figure 2). Plots which received early plus moderately late sprays had similar infections to those which only received early sprays. They were therefore presented in the same graph as the early spray. Except for the late spraying and the moderately late spraying with lowest concentration (5×10^5), the infection percentage finally reached a level of 50–80%. Infection of sclerotia from control plots remained low.

In 1996, 30–60% of the sclerotia were infected with *C. minitans* at 1 week after spraying. One or two weeks later the infection had reached about 80% for all early spraying treatments, and the overall infection finally reached 90%. The effects of application time was highly significant (Table 2). Based on the calculated P-values of the t-tests for pairwise differences early application resulted in a significantly higher infection

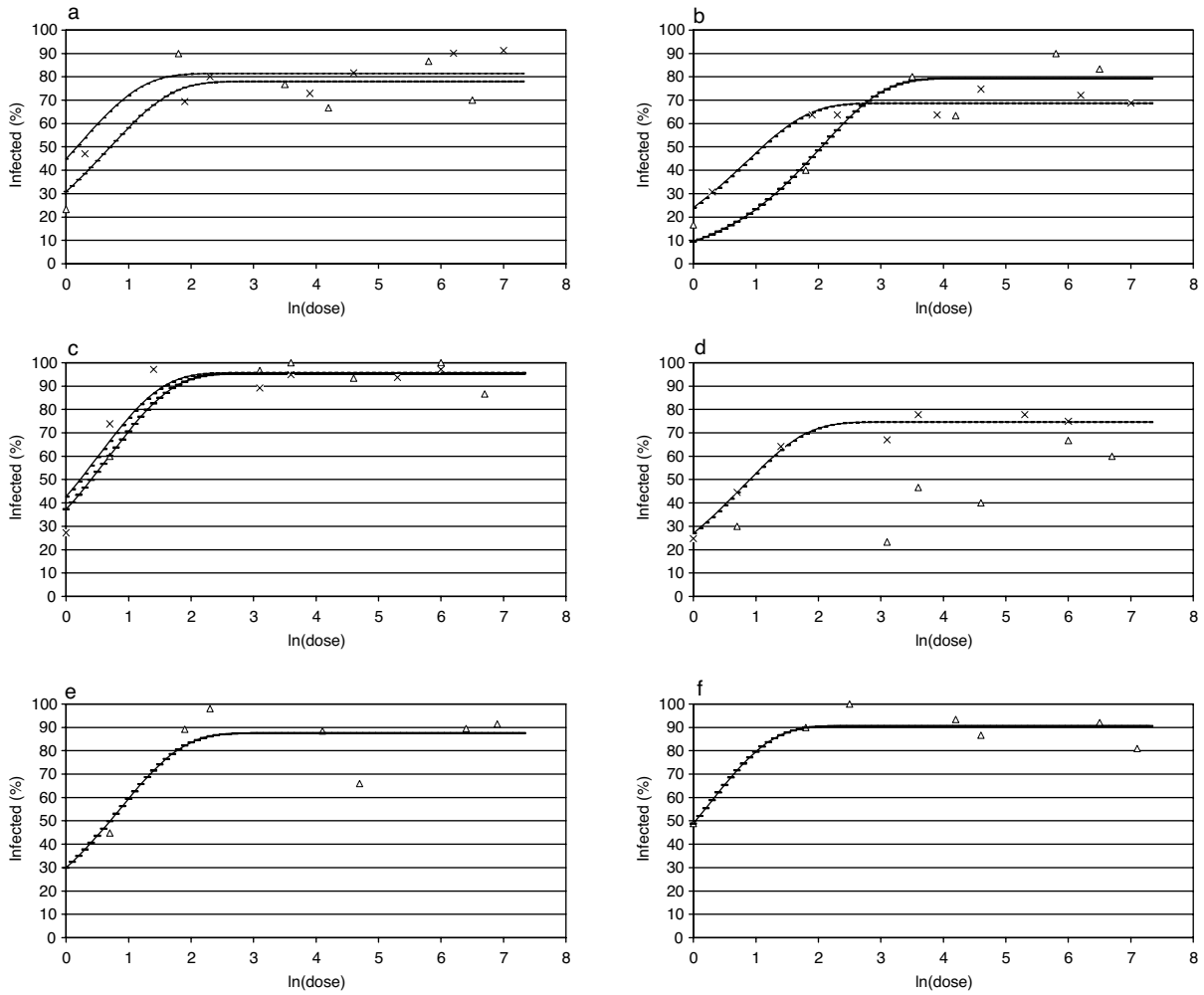

Figure 1. Effect of increasing numbers of *C. minitans* conidia on the percentage infection of sclerotia and *S. sclerotiorum*-colonised stem segments realised in laboratory trials. Fitted lines are based on the exponential model $y{=}A[1-\exp(-cz)]$, and realised conidial numbers. Graphs a–d contain data from two trials (trial 1Δ; trial 2×). The fitted line for d, trial 1, was unacceptable as only 4% of the variation was accounted for. a = *C. minitans* isolate IVT1; infection of sclerotia scored by diagnostic plating; b = *C. minitans* isolate IVT1; infection of sclerotia scored by visual inspection; c = *C. minitans* isolate C15; infection of sclerotia scored by diagnostic plating; d = *C. minitans* isolate C15; infection of sclerotia scored by visual inspection; e = *C. minitans* isolate IVT1; infection of stem pieces scored by visual inspection; f = *C. minitans* isolate C15; infection of stem pieces scored by visual inspection.

rate (84%) than moderately late (64%) and late (53%), which did not differ significantly. Late treatment did not differ from the control (50% infection).

There was slight evidence for isolate (IVT1, 91% viability as opposed to IVT1, 16% and C15, 83%) effects ($0.05 < P < 0.10$). IVT1, 91% viability resulted in significantly higher infection rate (75%) than IVT1, 16% (61% infected sclerotia; $P = 0.02$), while the remaining pairwise differences were not significant (66% infected sclerotia for C15). The effects of isolate and viability and their interaction with time of application were extremely limited. Lower concentrations of spray suspension (5×10^5 *versus* 1×10^6 or 5×10^6 conidia ml^{-1}) resulted in a slight delay of infection, but after 3 weeks the same level was attained for all concentrations (Figure 3A). Later spraying delayed high infection percentages more than lower number or quality of conidia. Even by the end of the experiment moderately late and late spraying did not result in the level of infection realised with earlier spraying. The

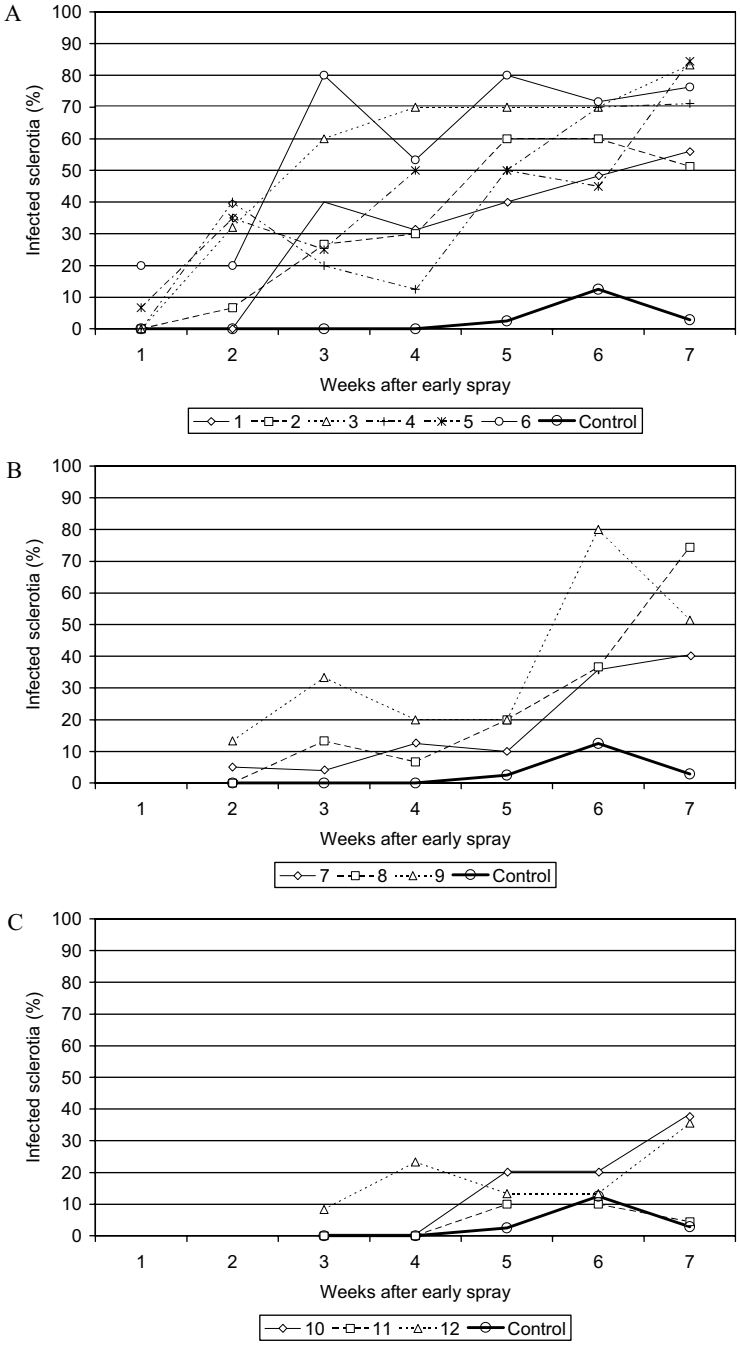

Figure 2. Effect of various treatments (2.51 conidial suspension of *C. minitans* per plot) of a *S. sclerotiorum*-infected bean crop on the percentage of sclerotia infected by *C. minitans* compared to the control. (Trial 1995) Treatments were performed immediately after appearance of the first symptoms (A, early spray, in week 0, isolate IVT1, at concentrations of 5×10^5, 10^6 or 5×10^6 conidia ml^{-1}, treatments 1–3, respectively), 8 days later (B, moderately late, in week 1, isolate IVT1, at concentrations of 5×10^5, 10^6 or 5×10^6 conidia ml^{-1}, treatments 7–9, respectively), or 16 days later (C, late, in week 2, isolates IVT1, C15 and a German isolate at 10^6 conidia ml^{-1}, treatments 10–12, respectively). Three treatments received both an early as well as a moderately late spray (A, treatments 4–6) at concentrations mentioned above. In all treatments weekly sampling of sclerotia started 1 week after spraying and continued until the end of the experiment.

Table 2. Deviance table for counts of infected sclerotia at 4 weeks after spraying. The effect of time of application (early, moderately late and late) and isolate of *C. minitans* (IVT1 with 91% viability, IVT1 with 16% viability, and C15 with 83% viability) in the nine factorial combinations on infection of sclerotia of *S. sclerotiorum* was analysed

Source	Degrees of freedom	Deviance	Mean deviance	Deviance ratio	F probability*
Replicate	3	0.53	0.18	0.1	0.949
Time of application (T)	2	43.17	21.59	14.6	<0.001
Isolate (I)	2	8.47	4.23	2.9	0.062
Interaction $T*$I	4	2.87	0.72	0.5	0.746
Residual	96	141.60	1.48		
Total	107	196.63	1.84		

*According to F-distribution, ignoring effect in the following rows.

control plots also gradually developed quite high levels of infection by *C. minitans*, stabilising at about 40%, and not statistically different from the infection of late sprayed plots (Table 3; Figure 3).

Apothecia

The validity of the model assumption of approximately constant CV was investigated by linear regression of ln(sample variance) on ln(sample mean) of the treatments. The variance accounted for by the fitted line was 94% and the slope of the fitted line was 1.78 (SE = 0.13). Since for data with a constant CV the relationship between ln(variance) and ln(mean) is linear with a slope of 2 the validity of the assumption was not doubted. Some treatments showed rather low mean counts, suggesting a low incidence of apothecia. At low incidence, zero counts for individual plots are quite probable. Since apothecia were found for all treatments we considered zero counts to be due to sampling and not to be structural. Since the generalised linear model for gamma distributed data cannot cope with zero counts we added a small positive value to all counts prior to analysis. The robustness of this procedure was investigated by adding 0.02, 0.1 and 0.5 to the observed counts. In all cases conclusions on treatment effects were the same.

The effect of explanatory variables on the number of apothecia was analysed according to models 1 and 2 (Deviance Tables 4 and 5, respectively). Both analyses led to the same conclusions.

The number of apothecia scored on the trays with soil samples from the 1996 trial field was only influenced by the time of spraying. Control trays produced 28.1 apothecia on average, late sprays 20.3, moderately late sprays 13.0, early sprays 2.4, and early plus moderately late sprays 0.9. The latter figure was significantly different from all others. Early sprays resulted in significantly lower numbers of apothecia than moderately late or late sprays and the control. The control did not differ from moderately late or late sprays. The effect of concentration of the spray suspension or spore batch (viability) was insignificant (Figure 4 and Tables 3–5).

Discussion

Trials with BCAs to control plant pathogens generally use initially high BCA concentrations in order to prove the potential benefit. Hundreds of kilograms of inoculum per hectare as a soil application of *C. minitans* (Whipps and Gerlagh, 1992) is common. McQuilken et al. (1997) film-coated *S. sclerotiorum*-infected seeds of sunflower and sclerotia with 5×10^6 and $7–14 \times 10^6$ conidia of *C. minitans* per gram respectively. These numbers were still insufficient to improve seedling survival, but effectively prevented development of apothecia from the sclerotia. With inoculum dosages in the order of 500–3000 kg ha^{-1} (depending on the substrate), McLaren et al. (1996) succeeded in strongly reducing the development of apothecia in soil. At such concentrations, a quantitative dose effect was established with dose variations 1 : 3. Tu (1999) also reported a much better suppression of apothecia with 10^6 than with 10^4 conidia of *C. minitans* per gram of experimental soil. Budge et al. (1995), as well as McQuilken and Whipps (1995), found high percentages (80–100%) of infected sclerotia in plots treated with high doses of *C. minitans* (2300 kg ha^{-1}). But even in control plots, some of the sclerotia were infected by *C. minitans*. McQuilken et al. (1995) showed a strong (>90%)

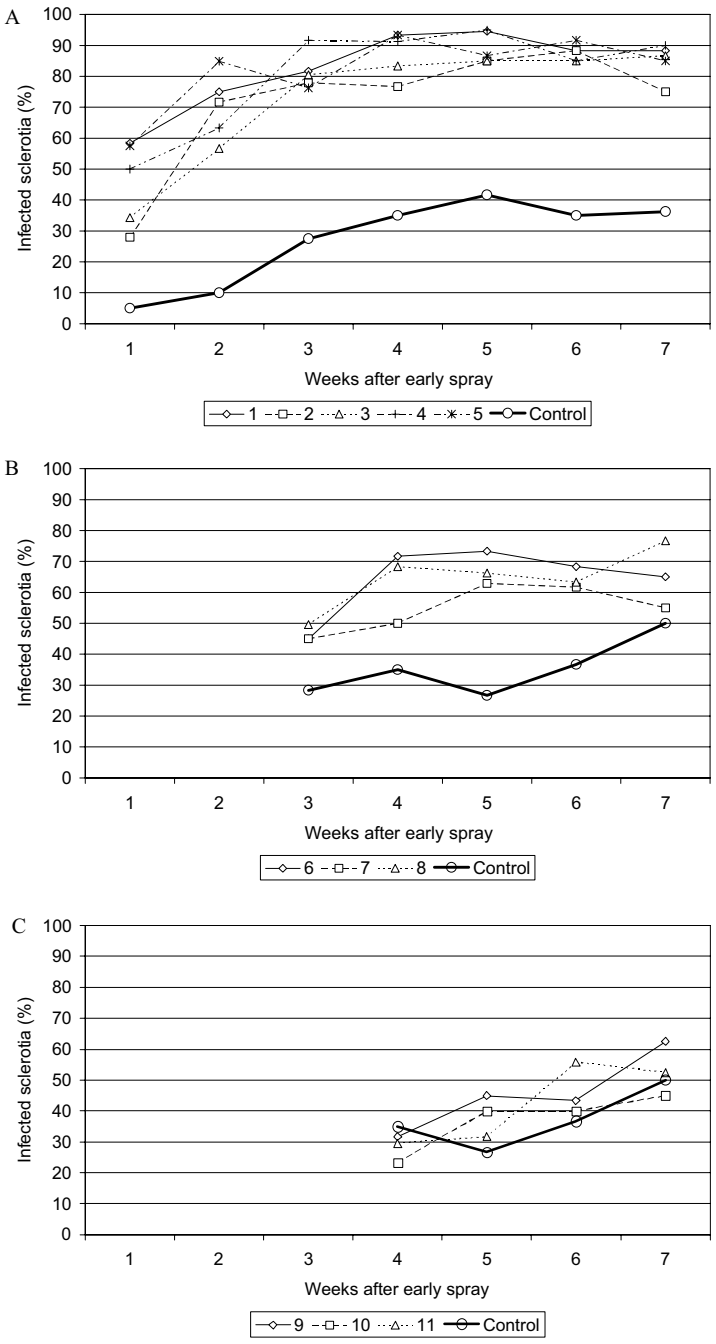

Figure 3. Effect of various treatments (2.25 l conidial suspension of *C. minitans* per plot) of a *S. sclerotiorum*-infected bean crop on the percentage of sclerotia infected by *C. minitans* compared to the control. (Trial 1996). Treatments were performed immediately after appearance of the first symptoms (A, early spray, in week 0, isolate IVT1, 91% viability; IVT1, 16% viability; C15, 83% viability; treatments 1–3, respectively), 11 days later (B, moderately late, in week 1, batches of isolates as above, treatments 6–8, respectively) or 18 days later (C, late, in week 2, batches of isolates as above, treatments 9–11, respectively). Early, moderately late and late sprays were at a concentration of 10^6 conidia ml^{-1}. Two treatments received both an early as well as a moderately late spray with IVT1, 91% viability at concentrations of 5×10^5, and 5×10^6 conidia ml^{-1}, respectively (A, treatments 4 and 5). In all treatments weekly sampling of sclerotia started 1 week after spraying and continued until the end of the experiment.

Table 3. Comparison of percentage infection of sclerotia at 4 weeks after spraying in 1996 in relation to apothecia development in the following year. Treatments consisted of single sprays of a *S. sclerotiorum*-infected crop with conidial suspensions of *C. minitans* varying in isolate, quality and concentration and applied at three periods after the start of the epidemic. Only treatments 4 and 5 received two sprays. The effect of treatments on percentage sclerotial infection was grouped per spraying time to account for the increase of infection of the control 4 weeks after gradually later spraying

Treatment number	Description				% infected sclerotia		Numbers of apothecia	
	Application time	Isolate	Viability (%)	Concentration	Real value	Logit	Real value	In scale
12	Early	−(Control)			35.0	−0.62 a*	28.1	3.34 e
1		IVT1	91	10^6	95.3	3.26 c	1.5	0.41 b
2		IVT1	16	10^6	76.7	1.81 b	3.3	1.21 bc
3		C15	83	10^6	83.3	2.23 bc	2.5	0.92 b
4	(+moderately late)	IVT1	91	5×10^5	91.2	2.96 bc	1.6	0.47 b
5	(+moderately late)	IVT1	91	5×10^6	93.3	3.26 c	0.3	−1.32 a
12	Moderately late	− (Control)			36.7	−0.55 a	28.1	3.34 e
6		IVT1	91	10^6	68.3	1.32 b	21.7	3.08 de
7		IVT1	16	10^6	61.7	1.02 b	11.5	2.44 cde
8		C15	83	10^6	63.3	1.09 b	5.7	1.74 bcd
12	Late	−(Control)			50.0	0.00 a	28.1	3.34 e
9		IVT1	91	10^6	62.5	0.51 a	20.6	3.03 de
10		IVT1	16	10^6	45.0	−0.20 a	19.3	2.96 de
11		C15	83	10^6	52.5	0.10 a	20.9	3.04 de

*Figures within the same column, and with respect to the % infected sclerotia within the same spraying time-group, not followed by the same letter are significantly different ($P < 0.05$).

Table 4. Deviance table of the analysis of effects of replicate, time of application and treatments within time of application on apothecial counts. Time of application consisted of four treatments and control; *C. minitans* was applied to sclerotia of *S. sclerotiorum* on a bean crop early, moderately late and late as a single application, and early plus moderately late. Treatments within time of application consisted of IVT1 with 91% viability, IVT1 with 16% viability, or C15 with 83% viability applied as a single spray at 10^6 conidia ml^{-1} and IVT1, 91% viability applied at 5×10^5 or 5×10^6 conidia ml^{-1} twice (early and moderately late)

Source	Degrees of freedom	Deviance	Mean deviance	Deviance ratio	F probability*
Replicate	3	33.64	11.21	3.53	0.017
Time of application (T)	4	159.96	39.99	12.58	<0.001
Treatment within T	7	26.25	3.75	1.18	0.319
Residual	129	410.16	3.18		
Total	143	630.01	4.41		

*According to F-distribution, ignoring effect in the following rows.

Table 5. Deviance table of the analysis of effects of replicate, time of application, isolate treatments and their interaction on apothecial counts. The analysis was restricted to the factorial combinations of time of application (early, moderately late and late) of *C. minitans*, isolates IVT1 with 91% viability, IVT1 with 16% viability, or C15 with 83% viability to sclerotia of *S. sclerotiorum* on a bean crop

Source	Degrees of freedom	Deviance	Mean deviance	Deviance ratio	F probability*
Replicate	3	21.70	7.23	2.32	0.081
Time of application (T)	2	71.81	35.91	11.50	<0.001
Isolate (I)	2	1.90	0.95	0.30	0.738
Interaction $T*$I	4	12.36	3.09	0.99	0.417
Residual	96	299.77	3.12		
Total	107	407.55	3.81		

*According to F-distribution, ignoring effect in the following rows.

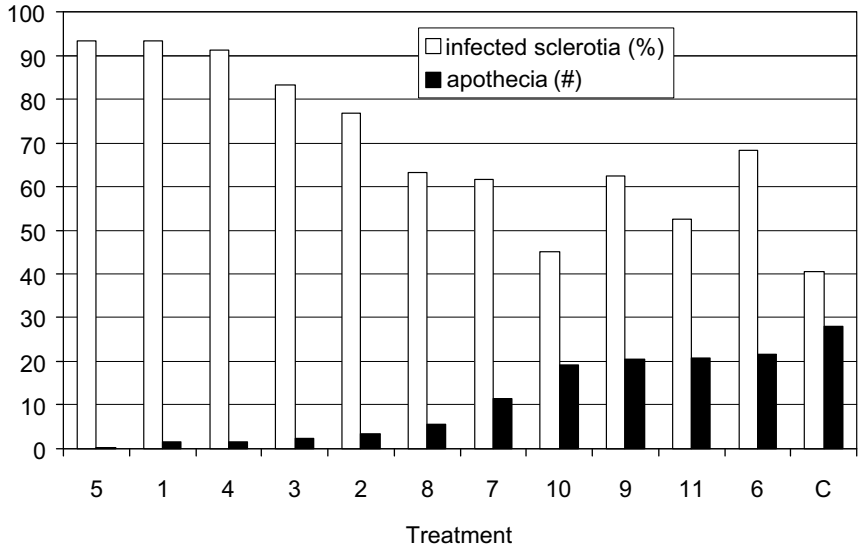

Figure 4. Effect of various treatments (described in Figure 3) of a *S. sclerotiorum*-infected bean crop on the percentage of sclerotia infected by *C. minitans* at 4 weeks after the spray application and on the number of apothecia developing on soil samples collected from differently treated subplots. Soil samples were collected in autumn following the experiment, stored during the winter, after which the apothecia were counted in spring.

suppression of apothecial germination in spring after application of 0.8 and 1.6 l m^{-2} *C. minitans* inoculum to soil in the preceding autumn. Spring application, however, was less effective; a significant suppression of apothecia started only at >2 months after application of *C. minitans*. Hedke and Tiedemann (1999), working with 'Contans' also confirmed that soil application of the BCA in autumn was effective. The concentration was low (only 1 or 2 kg ha^{-1}) in their trials and the difference in quantity was less relevant.

It is obvious that the environment (disregarding nutrients) in the soil is much more stable and generally favourable for the activity of micro-organisms than above ground and may possibly lead to more efficient exploitation of the applied antagonist in soil. However, it is much easier to reach sclerotia on above ground crop parts than in the soil. This would limit the need for a large quantity of inoculum in above ground application compared to soil incorporation. In order to better understand the weight of each of these factors, we first examined the minimal number of conidia required for infection under near optimal conditions in the laboratory. The application of *C. minitans* to the above ground crop was then performed in field trials with inoculum doses much lower than normally used for soil application. The scores reflected not only the effect of the amount of *C. minitans* applied, but also its reproduction within the sclerotia for new infections. The results obtained in laboratory tests show the remarkable effectiveness of just 2 conidia, suggesting that the application of very low amounts of *C. minitans* on a diseased crop can effectively infect the sclerotia, and thus control the pathogen under otherwise optimal conditions. The field trials largely confirm this idea.

The field experiments showed sclerotial infections by *C. minitans* in the order of 90% 4 weeks after spraying when performed immediately after the first observation of disease symptoms. When sprayed later, the infection rate was slower, perhaps due in part to the lower temperatures later in the season. The importance of the natural population build-up of *C. minitans* after introduction to the crop can be clearly seen from the similarity in sclerotial infection following treatments differing in isolate, concentration, or germinability of conidia. Even accidental infection of sclerotia by *C. minitans* in the control reached the same level of infection as the late application of poor quality conidia (treatment 10, Figure 3). The effect of late spraying with high quality inoculum was not even statistically different from control (Table 3). Huang et al. (2000) found a similar recovery of *C. minitans* in all treatments (control included) in a biocontrol trial with several potential antagonists to *S. sclerotiorum*, underlining its unique capacity to be dispersed in a diseased crop.

All data indicate that an early application of the antagonist, as soon as the first disease symptoms appear, is the best way to reduce survival of newly formed sclerotia. When the crop persists in the field for at least 3 weeks after application, the inoculum quality and dosage are less decisive, at least under the conditions of these trials.

The final percentages of sclerotial infection scored during these trials considerably underestimate the effect of *C. minitans*. As the sampling period progressed, it became more difficult to collect sclerotia in the majority of the treatments, due to the fact, that sclerotia had completely decomposed and disappeared after infection by *C. minitans*. The rare sclerotia which could be collected may have escaped early infection, or were produced by recent progress of *S. sclerotiorum* on the bean crop. A 90% disappearance of sclerotia after early treatment with *C. minitans* is certainly not exaggerated, given the difficulty in collecting sclerotia in such plots. If 90% infection occurred in the remaining 10%, this represents 99% suppression. When sprayed later however, part of the sclerotia will have dropped on the soil, and might escape infection. This would result in higher percentages survival than expected on the basis of estimated percentages infection of sclerotial samples. A difference of a factor 100 was observed for apothecia recovered from control plots and from plots sprayed early and moderately late with 5×10^6 conidia ml^{-1} (Table 3). This supports the order of magnitude mentioned for suppression of sclerotial survival. In these experiments the reduction of sclerotia due to diverse other biological mechanisms is taken for granted. Bourdôt et al. (2000) calculated a half-life time of about 1 year for sclerotia of *S. sclerotiorum* in soil. Clearly this general decline is many orders of magnitude less than the effect of *C. minitans*.

With a concentration of 10^6 conidia ml^{-1}, applied at a rate of 1000 l ha^{-1} (10^{12} conidia ha^{-1}) as soon as the first symptoms appear, satisfactory results can be obtained within broad ranges of inoculum quality. This result might have been expected, since 2 conidia were sufficient to infect the majority of sclerotia (under optimal conditions). Since sclerotia are generally larger than 1 mm (diameter), 10^{12} conidia ha^{-1} (100 conidia mm^{-2}) provide sufficient potential overdose to account for less optimal conditions, low germinability of conidia and/or a leaf area index of the crop of >1. This makes biocontrol of *S. sclerotiorum* by *C. minitans* a reliable, robust practice.

Acknowledgements

Part of the work was supported by funds of the Dutch Technology Foundation (Project WBI44.3318) and the Commission of the European Communities, Biotechnology Programme (CT95-0250). We are grateful to Dr. J. Köhl for critically commenting earlier drafts of the manuscript, and to Dr. T.A. Dueck for improving the English text.

References

Adams PB (1990) The potential of mycoparasites for biological control of plant diseases. Annual Review of Phytopathology 28: 59–72

Adams PB and Ayers WA (1981) *Sporidesmium sclerotivorum*: Distribution and function in natural biological control of sclerotial fungi. Phytopathology 71: 90–93

Bourdôt GW, Saville DJ, Hurrell GA, Harvey IC and de Jong MD (2000) Risk analysis of *Sclerotinia sclerotiorum* for biological control of *Cirsium arvense* in pasture: Sclerotium survival. Biocontrol Science and Technology 10: 411–425

Budge SP, McQuilken MP, Fenlon JS and Whipps JM (1995) Use of *Coniothyrium minitans* and *Gliocladium virens* for biological control of *Sclerotinia sclerotiorum* in glasshouse lettuce. Biological Control 5: 513–522

Gerlagh M, Whipps JM, Budge SP and Goossen-van de Geijn HM (1996) Efficiency of isolates of *Coniothyrium minitans* as mycoparasites of *Sclerotinia sclerotiorum, Sclerotium cepivorum* and *Botrytis cinerea* on tomato stem pieces. European Journal of Plant Pathology 102: 787–793

Gerlagh M, Goossen-van de Geijn HM, Fokkema NJ and Vereijken PFG (1999) Long-term biosanitation by application of *Coniothyrium minitans* on *Sclerotinia sclerotiorum*-infected crops. Phytopathology 89: 141–147

GENSTAT 5 Committee (1993) GENSTAT 5 Reference Manual, R.W. Payne (Chairman) and P.W. Lane (Secretary), Oxford: Clarendon Press

GENSTAT 5 Committee (1997) GENSTAT 5, Release 4.1. Reference Manual Supplement, R.W. Payne (Chairman) and P.W.Lane (Secretary), Oxford, Numerical Algorithms Group

Hedke K and von Tiedemann A (1999) Erste Ergebnisse des grossflächigen Einsatzes von CONTANS® (*Coniothyrium minitans*) zur biologischen *Sclerotinia*-Bekämpfung im Raps unter Praxisbedingungen. Phytomedizin 29(4): 24–25

Huang HC, Bremer E, Hynes RK and Erickson RS (2000) Foliar application of fungal biocontrol agents for the control of white mold of dry bean caused by *Sclerotinia sclerotiorum*. Biological Control 18: 270–276

Johnson KB (1994) Dose–response relationships and inundative biological control. Phytopathology 84: 780–784

McCullagh P and Nelder JA (1989) Generalized Linear Models, 2nd edn, Chapman and Hall, London

McLaren DL, Huang HC and Rimmer SR (1996) Control of apothecial production of *Sclerotinia sclerotiorum* by *Coniothyrium minitans* and *Talaromyces flavus*. Plant Disease 80: 1373–1378

McQuilken MP and Whipps JM (1995) Production, survival and evaluation of solid-substrate inocula of *Coniothyrium minitans* against *Sclerotinia sclerotiorum*. European Journal of Plant Pathology 101: 101–110

McQuilken MP, Budge SP and Whipps JM (1997) Biological control of *Sclerotinia sclerotiorum* by film-coating *Coniothyrium minitans* on to sunflower seed and sclerotia. Plant Pathology 46: 919–929

McQuilken MP, Mitchell SJ, Budge SP, Whipps JM, Fenlon JS and Archer SA (1995) Effect of *Coniothyrium minitans* on sclerotial survival and apothecial production of *Sclerotinia sclerotiorum* in field-grown oilseed rape. Plant Pathology 44: 883–896

Tu JC (1999) Effect of temperature and moisture on the destruction of sclerotia of *Sclerotinia sclerotiorum* by *Coniothyrium minitans*. Mededelingen Faculteit Landbouwwetenschappen Universiteit Gent 64/3b: 411–417

Whipps JM and Budge SP (1990) Screening for sclerotial mycoparasites of *Sclerotinia sclerotiorum*. Mycological Research 94: 607–612

Whipps JM and Gerlagh M (1992) Biology of *Coniothyrium minitans* and its potential for use in disease biocontrol. Mycological Research 96: 897–907

Whipps JM and Lumsden RD (2001) Commercial use of fungi as plant disease biocontrol agents: Status and prospects. In: Butt T, Jackson C and Magan N (eds), Fungal Biocontrol Agents – Progress, Problems and Potential (pp 9–22), CAB International, Wallingford

Williams RH, Whipps JM and Cooke RC (1998) Role of soil mesofauna in dispersal of *Coniothyrium minitans*: Transmission to sclerotia of *Sclerotinia sclerotiorum*. Soil Biology and Biochemistry 30: 1929–1935

www.Prophyta.de. Contans®, das neue Fungizid gegen die Weissstängeligkeit