European Journal of Plant Pathology (2005) 113: 197–209
DOI 10.1007/s10658-005-2331-4

Effect of combined treatment of pasteurisation and *Coniothyrium minitans* on sclerotia of *Sclerotinia sclerotiorum* in soil

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Accepted 16 August 2005

Key words: fungal ecology, integrated biocontrol, methyl bromide, pasteurization, steam sterilisation

Abstract

Integrated control of soil-borne plant pathogens such as *Sclerotinia sclerotiorum* is becoming more important as the soil fumigant methyl bromide is being phased out of use. Two alternative methods of control that have been found to reduce viability of sclerotia are steam sterilisation (pasteurisation) of soil or the application of the mycoparasite *Coniothyrium minitans*. This work investigated the possibility of integrating these two control measures. Soil was pasteurised in an autoclave, using a temperature of 80 °C for 3 min to simulate the possible temperatures reached by soil steaming machines for field use. *Coniothyrium minitans* was subsequently applied to the pasteurised soil to assess the effects of the combination of control measures in reducing sclerotial viability of *S. sclerotiorum*. Similar results were found in two soil types. Either method used individually was effective in decreasing the number of viable sclerotia, but no further reduction in sclerotial viability was seen when the two methods were combined. *Coniothyrium minitans* was found to colonise pasteurised sclerotia significantly quicker than untreated sclerotia, and it was seen that there was an increase in number of *C. minitans* in pasteurised soil in the presence of sclerotia. Experiments were also conducted to investigate the effect of application timing of the biocontrol agent to soil following pasteurisation, in relation to sclerotial infection. Here, two different isolates of *S. sclerotiorum* were used, with similar results. Application of *C. minitans* to soil immediately following pasteurisation resulted in sclerotial infection by the mycoparasite, but application 7 days or more after soil pasteurisation resulted in low recovery of the biocontrol agent from sclerotia, possibly due to the mycoparasite being masked by the presence of other fungi which colonised the sclerotia first.

Introduction

Methyl bromide has been relied upon for many years as a broad-spectrum soil fumigant to control soil-borne pathogens. However, this chemical has been recognised as a contributory factor to the depletion of the ozone layer and is scheduled to be phased out of use in industrialised countries by 2005, and in developing countries by 2015 (Duniway, 2002). The use and availability of other chemical pesticides is also being reduced, and as such, alternative control measures for soil-borne pathogens need to be developed. Interest in integrated control measures is increasing, and combinations of different methods could allow reduced or sublethal rates to be used. Examples of these include combinations of disinfested or heat-treated soil, chemicals applied at reduced rates, and soil amendments with organic residues or biological control agents (Marois and Locke, 1985; Sivan and Chet, 1993; Fravel, 1996; Stapleton and Duncan, 1998). Combinations of different control

measures could provide better control than either method used independently. For example, combining the use of a heat treatment and the application of a biological control agent (*Talaromyces flavus*) increased the mortality of microsclerotia of the soil-borne pathogen *Verticillium dahliae*, compared to the heat treatment alone (Tjamos and Fravel, 1995).

This approach could be used for the integrated control of *Sclerotinia sclerotiorum*. *Sclerotinia sclerotiorum* is a fungal pathogen of worldwide importance, causing significant losses in agriculture and horticulture and affecting over 400 plant species including lettuce, sunflower, beans and oilseed rape (Boland and Hall, 1994). Sclerotia of this pathogen can remain viable in the soil for several years, before germinating either myceliogenically, or carpogenically to produce apothecia and airborne ascospores. Breaking the life-cycle of the pathogen by attacking the sclerotial stage is a good method of control, and steam sterilisation (pasteurisation) of soil has been found to reduce the viability of buried sclerotia of *S. sclerotiorum* (Pinel et al., 2000; van Loenen et al., 2003). The term 'steam sterilisation' is often used to describe treating soil with steam to kill plant pathogens. However, true sterilisation of the soil is not possible under field conditions, and thus the term 'pasteurisation' is used throughout this paper, referring to the situation where some but not all microorganisms are killed by the heat treatment. Pasteurisation usually refers to temperatures of 50–60 °C being applied, although here a temperature of 80 °C was used because this study was part of a larger project developing a novel steam machine for field use, capable of attaining 80 °C. Previous work on steam machines for field use has shown that soil temperatures up to 100 °C can be reached (Pinel et al., 2000).

However, not all sclerotia may be affected by the heat or steam treatment, and could be problematic as the soil is cultivated and they are brought to the surface. An alternative strategy is the use of a biocontrol agent to attack sclerotia buried in the soil. *Coniothyrium minitans* is a mycoparasite of *S. sclerotiorum* and the addition of *C. minitans* to soil reduces sclerotial viability of the pathogen and can reduce disease incidence over time (Budge and Whipps, 1991; Gerlagh et al., 1999; Jones and Whipps, 2002). However, although sclerotial numbers may be reduced in soil following the addition of *C. minitans*, the level of disease may not always be reduced significantly, particularly if the pathogen inoculum is high (Budge et al., 1995).

The combined use of soil pasteurisation and the application of the biological control agent *C. minitans* may be a strategy that is applicable for the control of Sclerotinia disease aimed at destruction of sclerotia. Thus, *C. minitans* added to pasteurised soil has the potential to target any sclerotia that are unaffected by the heat treatment. Also, the biocontrol agent can survive and remain viable in the soil to parasitise any new sclerotia that fall onto the soil from plants infected by airborne ascospores, and thereby prevent a build-up of pathogen inoculum for future crops.

Another potential benefit of the addition of a beneficial microorganism to pasteurised soil is that it may prevent or delay the reinvasion of the soil by other potentially deleterious microorganisms. Importantly, as freshly steamed soil can quickly be recolonised by a range of microorganisms (Rowe et al., 1977; Marois and Locke, 1985), it may be necessary to apply beneficial microorganisms directly to the soil immediately after the steam treatment to allow them to colonise the soil first, before other microorganisms reinvade and become established. Thus, application timing is an important consideration.

The aim of the work presented here was to investigate the integration of soil pasteurisation with the application of *C. minitans* to reduce the viability of sclerotia of *S. sclerotiorum*. Laboratory experiments were conducted in two soil types to assess the effects of pasteurisation on sclerotia buried in soil, combined with the use of *C. minitans* as a soil amendment. Experiments were also conducted to assess whether the application timing of the biocontrol agent following soil pasteurisation influenced the success of sclerotial infection by *C. minitans*.

Materials and methods

Fungal culture and maintenance

Coniothyrium minitans IMI 134523 (Conio) was originally isolated from a sclerotium of *S. sclerotiorum* (Turner and Tribe, 1976). Cultures were obtained from an isolate stored in liquid nitrogen at Warwick HRI, Wellesbourne, and grown on

potato dextrose agar (PDA) (Oxoid). Plates were inoculated centrally with an agar plug taken from the growing margin of a colony and the fungus was grown at 20 °C in the dark until the colony nearly covered the plate and pycnidia had formed. Subcultures of this isolate were then grown in the same way and maintained on PDA at 20 °C.

Sclerotia were produced following the methods of Budge and Whipps (2001). In the experiments investigating the effect of pasteurisation on sclerotial infection, a single sclerotium of a standard glasshouse isolate of *S. sclerotiorum* (isolate SB) was taken from stocks at Warwick HRI, Wellesbourne, UK, and surface-sterilised using a mix of ethanol and 15% sodium hypochlorite solution (50:50 v:v), followed by three rinses in sterile distilled water (SDW). The sclerotium was bisected and the two halves plated onto PDA and incubated at 20 °C. After 1 week, agar plugs were taken from the edge of the growing colony and inoculated onto sterile wheat grain in flasks (25 g wheat grain, 50 ml water, autoclaved at 121 °C for 15 min). The inoculated flasks were incubated at 18 °C for four weeks and shaken periodically to disperse the mycelium. Subsequently, the flasks were transferred to 4 °C for a further four weeks to condition the sclerotia. Finally sclerotia were harvested from the flasks by wet-sieving the contents, and sclerotia of size 2.0–5.6 mm were retained for use. Sclerotia were air-dried overnight in a laminar flow cabinet and either used immediately, or stored at room temperature until required (no longer than two weeks).

In the experiments investigating the application timing of *C. minitans* to soil, the standard *S. sclerotiorum* isolate SB was used in Experiment 1, and *S. sclerotiorum* isolate 13 (from field lettuce; provided by Dr John Clarkson, Warwick HRI, UK) was used in Experiment 2. The sclerotia for Experiment 2 were prepared in the same way as described above, except they were conditioned and stored at 4 °C for 20 months.

Inoculum preparation

For inoculum production of *C. minitans*, SDW (10 ml) was added to cover a 3–6 week-old PDA culture, and the conidia scraped into suspension using a sterile glass spreader. An aliquot (0.1 ml) of this suspension was then spread onto a fresh PDA plate, and incubated at 20 °C. This ensured that the colonies that resulted were of the same age, and the pycnidia would therefore also be of an even age. This second spread plate was used to prepare the inoculum for the experiment. SDW (10 ml) was added to the spread plates once colonies had matured (usually 2–4 weeks old), and the conidia were scraped into suspension. A dilution series of the resulting suspension was carried out to facilitate haemacytometer counts and the number of conidia ml^{-1} of original suspension was calculated. This suspension was used as the inoculum source for adding *C. minitans* to soil samples.

Pasteurisation of soil and sclerotia

Dalcross (very fine sandy loam) and Wellesbourne (sandy clay loam) soils were used in these experiments. Samples of field soil were left to stand at room temperature for 2–3 days before being sieved to pass through a 2 mm diam mesh to obtain an even particle size. Sclerotia to be pasteurised were placed in gauze bags, made by heat sealing nylon gauze (150 μm aperture) into squares, containing 20 sclerotia per bag, and buried in 200 g soil in wide-necked jars during the pasteurisation process. Jars containing soil and sclerotia were autoclaved at 80 °C for 3 min to simulate soil pasteurisation that may be achieved by a steam machine for field use. The thermal lock on the autoclave was manually overridden after this time and the jars of soil and sclerotia were removed. Immediately following the pasteurisation process, the gauze bags containing sclerotia were removed from the soil, and the sclerotia were transferred to sterile Petri dishes under aseptic conditions. The sclerotia remained in the Petri dishes in a laminar flow cabinet until the experiment was set up the following day. Similarly, the pasteurised soil was left in the jars (with the lids replaced) and kept in the laminar flow cabinet overnight. Non-sterile soil and sclerotia to be used in non-sterile soil were left untreated at room temperature until required. Soil moisture contents were determined to allow for the adjustment of moisture to 25% field capacity for the Wellesbourne soil, and 40% field capacity for the Dalcross soil. The water potentials at these moisture contents were found to be similar (> -0.1 MPa).

Effect of pasteurisation on sclerotial infection by C. minitans

To assess the effect of pasteurisation on sclerotial infection two experiments were conducted, one in Dalcross soil and one in Wellesbourne soil. Each experiment consisted of four treatments: untreated sclerotia in non-sterile soil; untreated sclerotia in non-sterile soil amended with *C. minitans*; pasteurised sclerotia in pasteurised soil; and pasteurised sclerotia in pasteurised soil amended with *C. minitans*. For *C. minitans* inoculated samples, a suspension of conidia was incorporated into the previously prepared soil in the jars using a sterile spatula, before being transferred to Petri dishes. An inoculum rate of 1×10^6 cfu g^{-1} soil was routinely used. Soil (between 40 and 50 g) was placed in a Petri dish and tamped down before 20 sclerotia were lightly pressed into the soil surface in each dish. Each treatment had four replicate dishes for each time interval, and enough dishes were set up initially to allow for destructive harvesting after 3, 7, 14, 30, 60, 90 and 120 days for Dalcross soil, and 3, 7, 14, 30 and 60 days for Wellesbourne soil. Results from the experiment with Dalcross soil showed that by 90 days a large number of infected sclerotia had disintegrated, which is why the Wellesbourne soil experiment was stopped earlier.

The dishes were individually weighed and the soil moisture content was maintained throughout the experiment by topping up the dishes with SDW (pipetted onto the soil surface) every week as needed. The dishes were grouped according to treatment in plastic bags and the tops loosely sealed with masking tape. Within each of the groupings the dishes were arranged randomly in terms of harvesting intervals, and a four-by-four random block design was set up for the 16 groupings (4 replicates by 4 treatments). Dishes were incubated in the dark at 18 °C.

Effect of C. minitans *application timing on sclerotial infection*

Two experiments were set up in Dalcross soil to investigate the effect of application timing of *C. minitans* on sclerotial infection following soil pasteurisation, using two different isolates of *S. sclerotiorum* – isolate SB and isolate 13 from the Warwick HRI collection. For each experiment there were four treatments as described above, with 40 g of soil tamped down in each Petri dish. All samples were set up initially without the addition of *C. minitans* inoculum. An aliquot (1 ml) of conidial suspension was subsequently evenly pipetted onto the soil surface of four replicate dishes in the inoculated samples at each time interval (on setting up the experiment, and after 3, 7, 14 and 30 days) to give an inoculum rate of 1×10^6 cfu g^{-1} soil. Non-inoculated samples had SDW pipetted onto the soil surface at the application times. The moisture content in the dishes was maintained throughout the experiment with the addition of SDW as needed. The dishes were grouped according to treatment in plastic bags as before, with the *C. minitans*-amended dishes being transferred to a separate bag following each application time. As before, within each of the groupings the dishes were arranged randomly in terms of harvesting intervals, and a four-by-four random block design was set up for the 16 groupings (4 replicates by 4 treatments). Dishes were incubated in the dark at 18 °C, and destructive harvesting took place 30 days following application of *C. minitans* or the SDW control.

Determination of sclerotial infection

The sampling procedure was the same for all experiments. Sclerotia were assessed for viability and infection by other microorganisms using the technique of Budge and Whipps (2001). Sclerotia were surface-sterilised for 3 min in a mixture of 100% ethanol and 15% sodium hypochlorite (50:50 v:v) in a beaker, with intermittent agitation to ensure total coverage by the sterilant. Following surface-sterilisation, the sclerotia were individually transferred to a separate well of a multi-well plate, and rinsed twice in SDW (2 ml) for 1 min each. The sclerotia were transferred to fresh SDW for the second rinse using flamed forceps to prevent cross-contamination. Following the second rinse the sclerotia were transferred to empty Petri dishes to be bisected before the halves were individually plated onto PDA plugs amended with chlortetracycline (25 μg ml^{-1}). The plugs were prepared in advance by cutting 12 mm disks out of thickly-set PDA plates amended with chlortetracycline, using a sterilised core-borer. The plugs were transferred to a fresh Petri dish and ten plugs were placed in each dish, allowing for five separate sclerotia to be plated per dish.

Sclerotia were sampled immediately after pasteurisation at 80 °C to check their viability following the heating process. As a control, untreated (non-sterile) sclerotia were also plated in the same way. Subsequently, at each time interval throughout the experiment, four replicates of each treatment were destructively harvested and sampled. A total of 80 sclerotia were plated at each time interval for each of the four treatments and incubated at 20 °C for 7 days before assessment. Assessments were as follows: Sclero = *S. sclerotiorum* growth, indicating viability of sclerotia; Conio = *C. minitans* growth, indicating infection by the biocontrol agent; Other = Other fungal or bacterial growth; Conio + Other = A mixed infection of *C. minitans* and another microorganism; Disintegrated = Sclerotia did not withstand the surface-sterilisation procedure; None = No growth of any microorganism from the sclerotia.

Soil sampling

The soil remaining in the dish, once the sclerotia had been removed, was mixed with a sterile spatula, and a soil sample (1 g) was taken. The soil sample was subjected to a dilution series (in SDW) before appropriate dilutions were plated onto PDA amended with chlortetracycline (25 μg ml^{-1}) and Triton X-100 (2 ml l^{-1}) (Budge and Whipps, 2001). Plates were incubated at 20 °C for 7 days before assessment, where *C. minitans* counts were recorded for the inoculated samples and other fungi were recorded for the non-inoculated (control) samples. At the final sampling time (120 days in Dalcross soil and 60 days in Wellesbourne soil) the infected sclerotia in soil amended with *C. minitans* were incorporated into the soil using a sterile spatula before the soil sampling took place to allow an estimate of the total number of *C. minitans* cfu present. Infected sclerotia were relatively soft by this stage, and could be easily broken up for distribution through the soil before sampling took place. Non-inoculated control treatments were not sampled at this time.

Statistical analyses

For each treatment at each sampling time, the number of sclerotia with microbial growth was determined according to the categories stated above (Sclero; Conio; Other; Conio + Other; Disintegrated; None). The counts were converted to percentage data, which were subsequently arcsine-transformed and subjected to analysis of variance in GenStat for Windows. In the analyses, sclerotia with a mixed infection of *C. minitans* and other fungi ('Conio + Other') were also included with the category 'Conio' to give the total number of sclerotia where *C. minitans* infection had occurred, and this is presented as 'Total Conio' in the tables in the results section. Similarly, the category 'Conio + Other' was also included with the category 'Other' to give the total number of sclerotia where secondary invaders were established, and this is presented as 'Total Other' in the tables. As such, the percentage data in the tables do not sum to 100%. Count data for numbers of *C. minitans* in amended soil, and other fungi in unamended soil, were $\log_{10}$ transformed following the addition of a constant of 0.375 to cope with zero counts. Data were then subjected to an analysis of variance in GenStat for Windows. Significant differences between treatment means at the 5% significance level were assessed against the appropriate least significant difference (LSD), where LSD = $t_v \times$ SED. SED is the standard error of the difference between two means derived from the residual mean square obtained from each analysis, and t_v is the critical value ($P = 0.05$) of the Student's t distribution on v degrees of freedom.

Results

Effect of pasteurisation on sclerotial infection by C. minitans

Experiment 1 – Dalcross soil

No growth was recorded from the sclerotia plated immediately after the pasteurisation process, whereas all the untreated sclerotia produced mycelium and secondary sclerotia. All the untreated sclerotia in non-sterile soil (non-inoculated control) remained viable for the duration of the experiment and produced *S. sclerotiorum* growth on the agar plugs (Table 1). Untreated sclerotia in non-sterile soil where *C. minitans* was added (inoculated control) became infected with the mycoparasite over time (Table 1), and the application of *C. minitans* caused a significant reduction in sclerotial viability ($P < 0.001$). The first sign of sclerotial infection was seen at 7 days when

Table 1. Growth of microorganisms from *Sclerotinia sclerotiorum* isolate SB sclerotia incubated in non-sterile or pasteurised Dalcross soil, with or without the addition of *Coniothyrium minitans*. Percentage data is back-transformed and values in parentheses are means after arcsine transformation of data. Analyses were carried out on arcsine-transformed data

Soil state	*C. minitans* application	Assessment (days)	Sclero[a]	Total Conio[b]	Total other[b]	Conio + other	Disintegrated	None[c]
Non-sterile	No	3	100.0 (90.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Non-sterile	No	7	100.0 (90.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Non-sterile	No	14	100.0 (90.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Non-sterile	No	30	100.0 (90.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Non-sterile*	No	60	99.7 (86.8)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Non-sterile	No	90	100.0 (90.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Non-sterile*	Yes	3	99.7 (86.8)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Non-sterile	Yes	7	64.2 (53.2)	35.9 (36.8)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Non-sterile	Yes	14	17.7 (24.9)	82.3 (65.1)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Non-sterile	Yes	30	7.6 (16.0)	91.4 (72.9)	1.0 (5.7)	0.7 (4.6)	0.0 (0.0)	0.0 (0.0)
Non-sterile	Yes	60	7.5 (15.9)	45.0 (42.1)	70.3 (57.0)	22.33 (28.2)	1.9 (7.8)	0.0 (0.0)
Non-sterile	Yes	90	9.3 (17.7)	0.0 (0.0)	14.6 (22.5)	0.0 (0.0)	75.1 (60.1)	0.0 (0.0)
Pasteurised	No	3	0.0 (0.0)	0.0 (0.0)	43.5 (41.3)	0.0 (0.0)	0.0 (0.0)	56.5 (48.8)
Pasteurised	No	7	0.0 (0.0)	0.0 (0.0)	100.0 (90.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Pasteurised	No	14	0.0 (0.0)	0.0 (0.0)	100.0 (90.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Pasteurised	No	30	0.0 (0.0)	0.0 (0.0)	100.0 (90.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Pasteurised	No	60	0.0 (0.0)	0.0 (0.0)	99.7 (86.8)	0.0 (0.0)	0.3 (3.2)	0.0 (0.0)
Pasteurised	No	90	0.0 (0.0)	0.0 (0.0)	84.1 (66.5)	0.0 (0.0)	12.4 (20.6)	1.9 (7.8)
Pasteurised	Yes	3	0.0 (0.0)	0.0 (0.0)	76.1 (60.8)	0.0 (0.0)	0.0 (0.0)	23.9 (29.3)
Pasteurised	Yes	7	0.0 (0.0)	98.7 (83.4)	16.8 (24.2)	13.1 (21.2)	0.0 (0.0)	0.0 (0.0)
Pasteurised	Yes	14	0.0 (0.0)	97.4 (80.8)	29.3 (32.8)	23.2 (28.8)	0.0 (0.0)	0.0 (0.0)
Pasteurised	Yes	30	0.0 (0.0)	90.3 (71.9)	25.9 (30.6)	15.2 (22.9)	0.0 (0.0)	0.0 (0.0)
Pasteurised	Yes	60	0.0 (0.0)	69.1 (56.3)	20.3 (26.8)	9.3 (17.7)	20.6 (27.0)	0.0 (0.0)
Pasteurised	Yes	90	0.0 (0.0)	9.4 (17.9)	31.2 (33.9)	2.8 (9.7)	62.5 (52.3)	0.0 (0.0)
LSD_1 (5%)			(6.76)	(8.54)	(21.12)	(11.33)	(4.08)	(13.14)
LSD_2 (5%)			(6.44)	(8.74)	(20.64)	(10.77)	(4.15)	(13.75)

Significant differences between any treatment mean are calculated from the least significant difference (LSD), where LSD = $t_v \times$ SED, and SED = Standard error of the difference between the means derived from the residual mean square from the analysis of variance, and t = critical value ($P = 0.05$) of Student's t distribution for v degrees of freedom (d.f.). LSD_1 is used for comparing pairs of means with the same combination of soil state and *C. minitans* application. LSD_2 is used for comparing pairs of means at the same assessment time.

[a]Indicates viable sclerotia.

[b]Sum across categories at each assessment time for each treatment may total >100% as Total Conio and Total Other both include the category Conio + Other, which is therefore counted twice.

[c]No growth of any microorganism.

*Missing sclerotium.

36% of sclerotia were infected by *C. minitans*, and the number of sclerotia infected by *C. minitans* increased significantly up to 30 days (Table 1). By 30 days mixed growth was also found where *C. minitans* and other fungi were isolated from the same sclerotium. Less than 10% of the sclerotia remained viable by this stage, although this viable proportion did persist to 90 days. By 60 days the number of sclerotia where *C. minitans* alone was recovered had decreased, although the number of sclerotia producing mixed fungal growth increased by this stage, as did those where other fungi were isolated alone. At this stage, some sclerotia were also found to disintegrate, and by 90 days the majority of sclerotia had disintegrated, although other fungi were still recovered from some sclerotia.

In pasteurised soil, nearly half of the sclerotia (44%) showed bacterial growth on the surface by 3 days, with the remainder showing no recovery of microorganisms at all (Table 1). Sclerotia were completely colonised by other microorganisms (fungi) by 7 days, and other fungi were consistently isolated from sclerotia until 60 days. By 90 days the number of disintegrated sclerotia increased significantly, although the majority were still intact and colonised by other fungi at this time

(Table 1). In pasteurised soil with the *C. minitans* amendment, a high number of sclerotia had bacterial growth at 3 days (76%), or had no growth at all (Table 1). By 7 days, *C. minitans* had colonised most of the sclerotia, and significantly more so than the equivalent treatment in non-sterile soil (Table 1). Mixed infections of *C. minitans* and other fungi were also found in the pasteurised treatment at 7 days, and a few sclerotia were colonised by other fungi only. This pattern remained until 60 days when a proportion of the sclerotia had disintegrated. By 90 days the number of sclerotia where *C. minitans* was isolated had dropped significantly, and other fungi were recovered from the remainder of the intact sclerotia. However, the majority of sclerotia had disintegrated by this time (Table 1).

Colony counts from soil samples showed *C. minitans* remained in high numbers in the soil following application, with numbers significantly higher in pasteurised than non-sterile soil at all sampling times ($P < 0.05$; Figure 1). At 120 days (when the infected sclerotia were incorporated into the soil before sampling), numbers of *C. minitans* in pasteurised soil were significantly higher than at other sampling times. A similar pattern was seen in non-sterile soil. Soil sampling results also showed that the numbers of other (indigenous) fungi remained at a constant level of about 5 $\log_{10}$ cfu g^{-1} soil in non-sterile soil (without *C. minitans* amendment), but were significantly lower in pasteurised soil (without *C. minitans* amendment) at all sampling times ($P < 0.001$). (Data not shown).

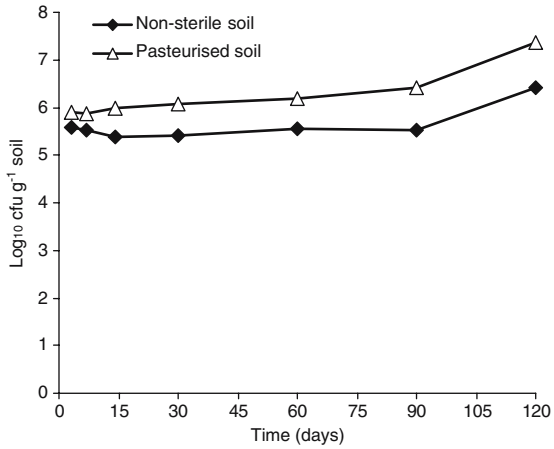

Figure 1. Numbers of *Coniothyrium minitans* in non-sterile and pasteurised Dalcross soil, where sclerotia of *Sclerotinia sclerotiorum* isolate SB have been incubated. Sclerotia were removed from the soil before sampling at all times except the final one (120 days), where they were incorporated into the soil before sampling occurred. LSD (5%) = 0.294.

Experiment 2 – Wellesbourne soil

Results from the experiment in Wellesbourne soil were similar to those from the Dalcross soil and infection of sclerotia by *C. minitans* and other fungi followed the same pattern (data not shown).

Effect of C. minitans *application timing on sclerotial infection*

Experiment 1 – Sclerotinia sclerotiorum *isolate SB*

No growth was recorded from sclerotia plated immediately after the pasteurisation process, whereas all the untreated sclerotia produced mycelium and secondary sclerotia. In this experiment, untreated sclerotia in non-sterile soil (non-inoculated control) became infected with other microorganisms, irrespective of when the SDW control was added to the dishes (Table 2). In general, the number of viable sclerotia ranged between 71 and 86%, with the remainder of the sclerotia at each sampling time being infected by other fungi or disintegrating during the sampling procedure.

In non-sterile soil amended with *C. minitans* (inoculated control) a trend was noted that as the application time of *C. minitans* became later, fewer sclerotia became infected with the biocontrol agent (Table 2). When *C. minitans* was added on setting up the experiment (0 days application), the majority of sclerotia at the sampling time (30 days) were found to have been infected by *C. minitans* and other fungi. A few sclerotia had disintegrated by the time the sampling was carried out. With the later application times of *C. minitans*, the number of viable sclerotia was high compared to earlier application times, and infection by *C. minitans* and other fungi decreased significantly (Table 2). The majority of pasteurised sclerotia in pasteurised soil became colonised by other fungi at all application times of the SDW control (Table 2). A proportion of sclerotia were found to disintegrate at each application time, with the exception of 3 days, when no sclerotia disintegrated (Table 2). Similarly, at each application time a proportion of sclerotia showed no growth of any microorganism when plated out (None).

In pasteurised soil where *C. minitans* had been added, sclerotia in the first application times (0 and 3 days) became infected by the mycoparasite (Table 2). However, application after 3 days did not result in *C. minitans* infection of sclerotia. Remaining sclerotia were colonised by varying proportions of other fungi, or disintegrated, or no growth was recovered at all.

Results from the soil sampling showed that numbers of *C. minitans* were approximately 6 $\log_{10}$ cfu g^{-1} soil in pasteurised soil throughout the experiment. In contrast, in non-sterile soil the recovered numbers of *C. minitans* fluctuated, and were consistently lower than those seen in pasteurised soil (data not shown). Although small but significant changes in other fungi were found to occur in non-sterile and pasteurised soil without *C. minitans* amendment, the numbers remained relatively constant at approximately 5 $\log_{10}$ cfu g^{-1} soil at all assessment times.

Experiment 2 – Sclerotinia sclerotiorum *isolate 13*
In this experiment, the pasteurisation process caused a significant reduction in sclerotial viability ($P < 0.001$), as only a single sclerotium yielded *S. sclerotiorum* growth when plated out (Table 3). All 20 untreated sclerotia produced *S. sclerotiorum* growth. Untreated sclerotia in non-sterile soil (non-inoculated control) remained largely uninfected in this experiment, with a few exceptions of

Table 2. Growth of microorganisms from *Sclerotinia sclerotiorum* isolate SB sclerotia incubated in non-sterile or pasteurised Dalcross soil, where *Coniothyrium minitans* was added at different application times. Controls had SDW added. Percentage data is back-transformed and values in parentheses are means after arcsine transformation of data. Analyses were carried out on arcsine-transformed data (Experiment 1)

Soil state	*C. minitans* application	Application time (days)	Assessment time (days)	Sclero[a]	Total Conio[b]	Total other[b]	Conio + Other	Disintegrated	None[c]
Non-sterile	No	0	30	71.5 (57.8)	0.0 (0.0)	25.8 (30.5)	0.0 (0.0)	0.3 (3.2)	0.3 (3.2)
Non-sterile	No	3	33	76.4 (60.9)	0.0 (0.0)	17.3 (24.6)	0.0 (0.0)	6.1 (14.3)	0.0 (0.0)
Non-sterile*	No	7	37	86.0 (68.0)	0.0 (0.0)	7.0 (15.3)	0.0 (0.0)	1.0 (5.7)	0.0 (0.0)
Non-sterile	No	14	44	71.7 (57.8)	0.0 (0.0)	5.5 (13.5)	0.0 (0.0)	19.9 (26.5)	0.0 (0.0)
Non-sterile	No	30	60	85.8 (67.9)	0.0 (0.0)	4.4 (12.2)	0.0 (0.0)	8.6 (17.1)	0.0 (0.0)
Non-sterile	Yes	0	30	7.3 (15.7)	48.7 (44.3)	40.9 (39.8)	11.6 (19.9)	14.8 (22.6)	0.0 (0.0)
Non-sterile	Yes	3	33	42.2 (40.5)	37.1 (37.5)	20.8 (27.1)	2.8 (9.7)	1.3 (6.5)	0.0 (0.0)
Non-sterile	Yes	7	37	57.5 (49.3)	18.4 (25.4)	24.9 (30.0)	5.5 (13.5)	4.7 (12.5)	0.0 (0.0)
Non-sterile	Yes	14	44	68.0 (55.5)	7.2 (15.6)	14.5 (22.4)	2.8 (9.7)	10.9 (19.2)	0.0 (0.0)
Non-sterile*	Yes	30	60	76.5 (61.0)	6.1 (14.3)	8.3 (16.8)	0.7 (4.6)	7.5 (15.9)	0.0 (0.0)
Pasteurised	No	0	30	0.0 (0.0)	0.0 (0.0)	94.5 (76.5)	0.0 (0.0)	0.7 (4.6)	3.7 (11.1)
Pasteurised	No	3	33	0.0 (0.0)	0.0 (0.0)	82.2 (65.1)	0.0 (0.0)	0.0 (0.0)	17.8 (24.9)
Pasteurised	No	7	37	0.0 (0.0)	0.0 (0.0)	84.3 (66.6)	0.0 (0.0)	3.7 (11.1)	8.7 (17.2)
Pasteurised	No	14	44	0.0 (0.0)	0.0 (0.0)	68.4 (55.8)	0.0 (0.0)	16.6 (24.0)	8.0 (16.5)
Pasteurised	No	30	60	0.0 (0.0)	0.0 (0.0)	72.8 (58.6)	0.0 (0.0)	18.6 (25.6)	5.1 (13.1)
Pasteurised	Yes	0	30	0.0 (0.0)	35.8 (36.8)	85.7 (67.8)	28.3 (32.2)	1.0 (5.7)	1.9 (7.8)
Pasteurised	Yes	3	33	0.0 (0.0)	1.9 (7.8)	72.8 (58.6)	1.9 (7.8)	2.9 (9.9)	21.2 (27.4)
Pasteurised	Yes	7	37	0.0 (0.0)	0.0 (0.0)	73.5 (59.0)	0.0 (0.0)	6.4 (14.6)	17.7 (24.9)
Pasteurised	Yes	14	44	0.0 (0.0)	0.0 (0.0)	85.8 (67.9)	0.0 (0.0)	4.6 (12.3)	7.2 (15.6)
Pasteurised	Yes	30	60	0.0 (0.0)	0.0 (0.0)	70.3 (57.0)	0.0 (0.0)	28.3 (32.2)	0.3 (3.2)
LSD_1 (5%)				(6.05)	(6.65)	(13.29)	(7.14)	(13.39)	(10.65)
LSD_2 (5%)				(5.96)	(6.75)	(13.65)	(6.70)	(12.45)	(12.35)

Significant differences between any treatment mean are calculated from the least significant difference (LSD), where LSD = $t_v \times$ SED, and SED = Standard error of the difference between the means derived from the residual mean square from the analysis of variance, and t = critical value (P = 0.05) of Student's t distribution for v degrees of freedom (d.f.). LSD_1 is used for comparing pairs of means with the same combination of soil state and *C. minitans* application. LSD_2 is used for comparing pairs of means at the same assessment time.

[a]Indicates viable sclerotia.

[b]Sum across categories at each assessment time for each treatment may total > 100% as Total Conio and Total Other both include the category Conio + Other, which is therefore counted twice.

[c]No growth of any microorganism.

*Missing sclerotium.

infection by naturally occurring *C. minitans* or other fungi (Table 3). Where *C. minitans* was added to non-sterile soil (inoculated control), a trend was found that later application times of the biocontrol agent resulted in low infection of the sclerotia. Recovery of *C. minitans* was highest at application time 0 days, and was significantly less at 3 days and subsequent application times (Table 3). *Sclerotinia sclerotiorum* growth was found from a high proportion of the sclerotia (>91%) at all application times after 3 days.

In pasteurised soil without *C. minitans* amendment it was found that some sclerotia survived the pasteurisation process and produced *S. sclerotiorum* growth at 0 and 30 days application time, although this was only significant at 30 days application time (Table 3). Generally the sclerotia were largely colonised by other fungi at all application times, with no significant difference found with application time. A significant number of sclerotia had disintegrated by 30 days application time ($P < 0.05$; Table 3). Pasteurised sclerotia in pasteurised soil where *C. minitans* had been added were largely colonised by other fungi at all application times (Table 3). *Coniothyrium minitans* was isolated from sclerotia at 0, 3, 7 and 14 days application. At 30 days application a significant number of sclerotia had disintegrated ($P < 0.05$; Table 3).

Results from the soil sampling showed that although there were small and sometimes significant differences in numbers of *C. minitans*

Table 3. Growth of microorganisms from *Sclerotinia sclerotiorum* isolate 13 sclerotia incubated in non-sterile or pasteurised Dalcross soil, where *Coniothyrium minitans* was added at different application times. Controls had SDW added. Percentage data is back-transformed and values in parentheses are means after arcsine transformation of data. Analyses were carried out on arcsine-transformed data (Experiment 2)

Soil state	*C. minitans* application	Application time (days)	Assessment time (days)	Sclero[a]	Total Conio[b]	Total other[b]	Conio + Other	Disintegrated	None[c]
Non-sterile	No	0	30	98.7 (83.5)	1.3 (6.5)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Non-sterile	No	3	33	99.7 (86.8)	0.0 (0.0)	0.3 (3.2)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Non-sterile	No	7	37	98.7 (83.5)	0.3 (3.2)	0.3 (3.2)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Non-sterile	No	14	44	100.0 (90.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Non-sterile	No	30	60	99.7 (86.8)	0.3 (3.2)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Non-sterile	Yes	0	30	37.3 (37.6)	60.2 (50.9)	2.8 (9.7)	0.3 (3.2)	0.0 (0.0)	0.0 (0.0)
Non-sterile	Yes	3	33	93.8 (75.5)	6.3 (14.5)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Non-sterile	Yes	7	37	91.4 (72.9)	7.3 (15.7)	0.3 (3.2)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Non-sterile	Yes	14	44	97.2 (80.3)	2.8 (9.7)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Non-sterile	Yes	30	60	97.6 (81.1)	2.4 (8.9)	0.3 (3.2)	0.3 (3.2)	0.0 (0.0)	0.0 (0.0)
Pasteurised	No	0	30	2.5 (9.1)	0.0 (0.0)	97.5 (80.9)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Pasteurised	No	3	33	0.0 (0.0)	0.0 (0.0)	98.7 (83.5)	0.0 (0.0)	0.0 (0.0)	1.3 (6.5)
Pasteurised	No	7	37	0.0 (0.0)	0.0 (0.0)	99.7 (86.8)	0.0 (0.0)	0.0 (0.0)	0.3 (3.2)
Pasteurised*	No	14	44	0.0 (0.0)	0.0 (0.0)	99.7 (86.8)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Pasteurised	No	30	60	4.3 (12.0)	0.0 (0.0)	86.1 (68.1)	0.0 (0.0)	4.6 (12.5)	0.0 (0.0)
Pasteurised	Yes	0	30	0.0 (0.0)	27.1 (31.4)	91.1 (72.6)	15.5 (23.2)	0.0 (0.0)	0.0 (0.0)
Pasteurised*	Yes	3	33	0.0 (0.0)	1.0 (5.7)	98.7 (83.5)	1.0 (5.7)	0.0 (0.0)	0.3 (3.2)
Pasteurised	Yes	7	37	0.0 (0.0)	5.4 (13.4)	96.2 (78.8)	1.0 (5.7)	0.0 (0.0)	0.0 (0.0)
Pasteurised	Yes	14	44	0.0 (0.0)	0.7 (4.6)	97.2 (80.3)	0.7 (4.6)	0.3 (3.2)	1.3 (6.5)
Pasteurised	Yes	30	60	0.3 (3.2)	0.0 (0.0)	93.8 (75.5)	0.0 (0.0)	5.1 (13.1)	0.0 (0.0)
LSD_1 (5%)				(11.29)	(13.37)	(14.90)	(7.35)	(5.16)	(4.44)
LSD_2 (5%)				(12.41)	(12.79)	(14.26)	(6.88)	(4.87)	(4.46)

Significant differences between any treatment mean are calculated from the least significant difference (LSD), where LSD = $t_v \times$ SED, and SED = Standard error of the difference between the means derived from the residual mean square from the analysis of variance, and t = critical value ($P = 0.05$) of Student's t distribution for v degrees of freedom (d.f.). LSD_1 is used for comparing pairs of means with the same combination of soil state and *C. minitans* application. LSD_2 is used for comparing pairs of means at the same assessment time.

[a]Indicates viable sclerotia.

[b]Sum across categories at each assessment time for each treatment may total > 100% as Total Conio and Total Other both include the category Conio + Other, which is therefore counted twice.

[c]No growth of any microorganism.

*Missing sclerotium.

throughout the experiment in both non-sterile and pasteurised soil, overall the numbers remained at approximately 6 $\log_{10}$ cfu g^{-1} soil. Other fungi were found consistently at approximately 5 $\log_{10}$ cfu g^{-1} soil in non-sterile and pasteurised soil (data not shown).

Discussion

As the soil fumigant methyl bromide is phased out of use, alternative measures to control soil-borne pathogens are becoming increasingly sought after. It is important to investigate measures to eradicate *S. sclerotiorum* from soil, as sclerotia can survive and remain viable for considerable periods, despite losses through natural degradation. Alexander and Stewart (1994) found that numbers of *S. sclerotiorum* sclerotia buried in field soil dropped significantly over 6 months, but even after 11 months viable sclerotia were found amongst those remaining. An example of one potential integrated control strategy for this pathogen in the glasshouse is the application of *C. minitans* to soil, combined with a single spray application of the fungicide iprodione (Budge and Whipps, 2001). This method was found to give equivalent disease control to fortnightly sprays of iprodione alone.

The possibility of combining soil pasteurisation and the application of the mycoparasite *C. minitans* to control sclerotia of *S. sclerotiorum* was investigated in this work. Although pasteurisation (steam sterilisation) of soil is common in glasshouses, the use of steam machines to pasteurise field soil is also a possibility, and several different field steaming machines are available (Quarles, 1997). Pinel et al., (2000) reported the use of a self-propelled steam machine for use on raised beds, reaching temperatures of up to 100 °C in the top 10 cm of the soil and killing a variety of pathogen propagules. However, sclerotia of *S. sclerotiorum* buried at depths of 15 cm or more remained viable following steaming. It is these sclerotia, and those in marginal areas not affected by the heat treatment, that may be problematic for future crops. The addition of a biocontrol agent may allow further eradication of soil-borne sclerotia unaffected by the heat treatment. *Coniothyrium minitans* is effective in reducing viability of *S. sclerotiorum* sclerotia in soil and can therefore reduce disease incidence, although not if disease pressure is high (Budge et al., 1995). Thus, combining these two measures may be an effective way of controlling *S. sclerotiorum* disease in the field.

In the first experiments investigating the integrated use of soil pasteurisation and the application of *C. minitans*, the two potential control measures separately gave similar results in the reduction of sclerotial viability. In this laboratory-based study, a temperature of 80 °C was used for pasteurisation as this was within the range of target temperatures of a novel steam machine under development for field use. It was found by van Loenen et al. (2003) that lower temperatures of 50–60 °C reduced viability of moist sclerotia in a laboratory test, although higher temperatures of 80 °C were necessary to significantly affect dry sclerotia of *S. sclerotiorum*. In the current study it was also found that dry sclerotia were killed at a temperature of 80 °C, and were subsequently colonised by other fungi in the soil and ultimately disintegrated. Soil samples showed that the numbers of other soil fungi decreased significantly with the pasteurisation process, but some survived the heat treatment and were able to utilise the pasteurised sclerotia as a food base, increasing in number again.

The application of *C. minitans* to untreated sclerotia in non-sterile soil resulted in the majority of sclerotia being infected by the mycoparasite over time, followed by colonisation by secondary invaders and sclerotial disintegration. Jones et al. (2003) found a similar pattern of sclerotial infection in four different non-sterile soil types, where sclerotial viability decreased over an 8-week period in soils amended with *C. minitans*. Similar to this study, recovery of a wild-type isolate of *C. minitans* (A69) was found to decrease over time and other fungi were increasingly isolated from the sclerotia. However, using a hygromycin B resistant isolate (*C. minitans* T3) it was shown that *C. minitans* was still recoverable from the sclerotia when agar amended with hygromycin B was used as the isolation medium. This illustrated that secondary fungal invaders do not necessarily displace *C. minitans* in sclerotia, but rather mask the recovery of the mycoparasite (Jones et al., 2003). In the current study, a small percentage of untreated sclerotia in non-sterile soil amended with *C. minitans* survived and remained viable until the final sampling time. As *C. minitans* has not been found to exhibit mycelial growth through

non-sterile soil (Williams, 1996), direct contact between the conidia and sclerotia may be necessary for infection to occur.

Combining the use of pasteurisation at 80 °C followed by soil amendment with *C. minitans* did not have any further effect on reducing sclerotial viability compared to the separate treatments. However, it was found that *C. minitans* colonised pasteurised sclerotia significantly faster than untreated (non-sterile) sclerotia, suggesting that live sclerotia do exhibit some resistance to *C. minitans*. Also, the addition of *C. minitans* to pasteurised soil meant that the mycoparasite was the primary invader of the pasteurised sclerotia as opposed to other fungi in pasteurised soil without the *C. minitans* amendment.

Soil samples showed the numbers of the introduced *C. minitans* remained high, particularly in pasteurised soil where an increase in numbers was seen over time. It has previously been found that *C. minitans* does not increase in numbers in pasteurised soil (Bennett et al., 2003), although this study was done in the absence of sclerotia. In the current study, the presence of sclerotia may have allowed an increase in numbers of *C. minitans* as the sclerotia became infected by the mycoparasite and conidia were subsequently exuded from pycnidia, providing a further inoculum source of *C. minitans*. More recent work has shown that infected sclerotia can be reservoirs for the survival of *C. minitans* in soil (Bennett et al., 2005), and in the current study it was also seen that when infected sclerotia were incorporated into the soil before the final sampling time, numbers of *C. minitans* were significantly higher than when sclerotia were not incorporated.

In the first set of experiments, the application of *C. minitans* to the soil immediately following pasteurisation allowed the biocontrol agent to colonise sclerotia and increase the inoculum potential of the mycoparasite above that which was applied to the soil initially. In order to further assess the importance of an immediate application of the biocontrol agent to soil following pasteurisation, the application timing experiments were conducted. The first of these, using isolate SB of *S. sclerotiorum* resulted in unexpectedly high losses of sclerotia due to infection by indigenous soil fungi and disintegration in non-sterile soil. Consequently, the experiment was repeated with another isolate 13. This apparent weak batch of isolate SB sclerotia may reflect the situation for the field or glasshouse, where sclerotia are not produced under aseptic conditions and may naturally be weaker or susceptible to infection. Trutmann et al. (1980) found field sclerotia decayed more rapidly than culture-produced sclerotia when exposed to *C. minitans*, due to infection of field sclerotia by numerous other microorganisms. Nevertheless, despite the different batches of sclerotia used in this work, the overall results were similar in both experiments.

As before, the temperature of 80 °C used to pasteurise samples typically killed most of the sclerotia, although there were a few survivors in Experiment 2 (isolate 13), possibly reflecting inherent differences in the sclerotial isolates. The fact that a few sclerotia survived the 80 °C treatment also illustrates the possibility of sclerotia surviving a heat treatment in field or glasshouse soil, particularly if they are in marginal areas and less affected by the heat. As before, sclerotia that were killed by the pasteurisation process were subsequently colonised by other fungi and ultimately started to disintegrate.

The two application timing experiments both illustrated that later application timings of *C. minitans* to sclerotia in non-sterile soil resulted in fewer sclerotia being infected by the mycoparasite over time. It has been shown that drying and wetting of sclerotia causes a release of exudates that promotes attack by microorganisms (Smith, 1972). However, in this laboratory-based system there was no drying and wetting of sclerotia, which would occur naturally in field or glasshouse situations. Consequently, it may be that the sclerotia in the earlier application times were more susceptible to attack by microorganisms (including *C. minitans*) as they became remoistened on addition to the soil. At later application times, the sclerotia had already been *in situ* in the soil (maintained at a constant moisture content) for up to 30 days before *C. minitans* was added. As such, they were not subjected to the drying and wetting and associated nutrient loss resulting in the decreased sclerotial infection. It has been shown that *C. minitans* requires an external nutrient source for conidia to germinate. Li et al. (2003) showed that conidia of *C. minitans* placed in water did not germinate, whereas those placed in water

amended with pollen as a nutrient source did. It may be that a similar situation occurred here with insufficient nutrients being available at later application times for *C. minitans* to germinate and infect the sclerotia. Similarly, in pasteurised samples amended with *C. minitans* the highest recovery of the biocontrol agent from sclerotia was found with earlier application times, followed by recovery in low numbers or no recovery at all at later application times. Unless *C. minitans* was applied promptly after pasteurisation, other indigenous fungi in the soil appeared to colonise the pasteurised sclerotia before *C. minitans* could infect. However, it may be that the presence of other fungi masked the true level of infection by *C. minitans* (Jones et al., 2003).

Integrated control for Sclerotinia disease using pasteurisation of soil and the application of *C. minitans* may only be realistically viewed as a long-term strategy as the cost of both repeatedly pasteurising soil and applying a biocontrol agent may be prohibitive. Soil pasteurisation on its own has the added advantage that other soil-borne pathogens can also be killed (Bollen, 1969; White et al., 2000), whereas *C. minitans* is specific to a limited number of sclerotial-forming pathogens (Punithalingam, 1982) and may not reduce disease if it does not come into direct contact with sclerotia for infection to occur. However, soil could be initially pasteurised and *C. minitans* applied not to eradicate sclerotia that are already present, but to prevent future problems developing from sclerotia that subsequently land on the soil. The life cycle of *S. sclerotiorum* means that even if no sclerotia are present in the soil where a crop is planted, airborne ascospores may be blown in from further afield and cause disease. Sclerotia formed on plants infected in this way will eventually return to the soil.

Applying *C. minitans* to glasshouse soil contaminated with sclerotia, Jones et al. (2004) found that an 8 week period was required before a lettuce crop was planted to ensure the biocontrol agent had sufficient time to destroy the sclerotial inoculum. If soil was initially pasteurised to reduce the sclerotial numbers, *C. minitans* could be applied immediately afterwards to build up an antagonistic population in the soil to protect future crops and there need be no delay in planting. The same could be true for use in field soil. If *C. minitans* is already present, this may reduce the impact of new sclerotia introduced into the soil, and reduce disease in the long-term, particularly in view of the considerable survival potential of *C. minitans* in soil.

A different approach may be to target soil-borne sclerotia with steam pasteurisation, and reduce future infections of crops by applying *C. minitans* as a conidial spray to foliar plant parts. Gerlagh et al. (2003) found that early applications of *C. minitans* conidial suspensions to a bean crop infected with *S. sclerotiorum* resulted in significantly higher sclerotial infection than later application of the biocontrol agent. Ultimately, applying *C. minitans* to a crop early would mean that fewer sclerotia are returned to the soil following a disease outbreak. Similarly, Li et al. (2003) suggested that applying *C. minitans* as a conidial suspension to alfalfa before *S. sclerotiorum* disease occurred may allow the biocontrol agent to be the primary coloniser of pollen grains. Both *S. sclerotiorum* and *C. minitans* can use the nutrients in pollen grains to germinate, and if a population of *C. minitans* is established first, this may reduce the incidence of disease experienced subsequently by *S. sclerotiorum* (Li et al., 2003).

In conclusion, although *C. minitans* application to pasteurised soil did not further reduce the viability of the sclerotia present, the combination of soil pasterurisation and *C. minitans* amendment may have a two-fold effect in the field: the biocontrol agent could immediately target any sclerotia that survived the heat treatment, and a population of the biocontrol agent could be established in the soil to infect any sclerotia that subsequently land on the soil surface, thus providing a long-term strategy for the control of Sclerotinia disease.

Acknowledgements

We thank Andrew Mead for help with the statistical analyses. This work was funded by DEFRA under the UK Horticulture LINK programme. Consortium members including funders are: Bioquell Food Ltd., Berry World Ltd, East Malling Trust, Greenvale AP, Horticultural Development Council (Project CP6), Langmead Farms Ltd, The MicroBio Group Ltd, Regero SA, Tesco Stores Ltd, TIO Ltd.

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