Coniothyrium minitans survival in soil and ability to infect sclerotia of *Sclerotinia sclerotiorum*

E.E. Jones¹ and A. Stewart²

¹Faculty of Agriculture and Life Sciences, P.O. Box 84, Lincoln University, New Zealand

²Bio-Protection Research Centre, P.O. Box 84, Lincoln University, New Zealand

Corresponding author: Eirian.Jones@lincoln.ac.nz

Abstract Survival of the sclerotial parasite *Coniothyrium minitans* in soil, when applied as spore suspension or colonised solid substrate (maizemeal-perlite) inocula, and ability to infect *Sclerotinia sclerotiorum* sclerotia incorporated into the soil after different times was assessed over 6 months. Unambiguous detection of the *C. minitans* isolate from the indigenous *C. minitans* soil population was achieved using a hygromycin B resistant transformant (T3), which was similar in behaviour to the wild type LU112. *Coniothyrium minitans* was recovered from soil by dilution plating at all assessment times, with higher recovery from spore suspension compared with maizemeal-perlite amended soil. *Coniothyrium minitans* was able to infect and reduce viability of sclerotia incorporated into the amended soil over the 6 month experiment, with spore suspension significantly increasing infection compared with maizemeal-perlite inoculum. Hygromycin B amendment of the agar significantly increased *C. minitans* recovery from sclerotia, especially when the population of secondary fungal colonisers was high.

Keywords *Coniothyrium minitans*, *Sclerotinia sclerotiorum*, sclerotial parasites, biological control.

INTRODUCTION

The sclerotial parasite *Coniothyrium minitans* has been shown to control *Sclerotinia* spp. both in glasshouse and field crops (Jones et al. 2004; Li et al. 2006; Rabeendran et al. 2006). Control is often achieved by applying *C. minitans* inocula to soil to target the sclerotia, with two main inoculum types used – colonised solid substrate and spore suspension inocula (Jones et al. 2004; Rabeendran et al. 2006). When applied as a solid substrate inoculum (maizemeal-perlite), *C. minitans* was shown to survive in soil for at least 2 years when assessed by dilution plating onto agar (McQuilken et al. 1995). However, care must be taken when extrapolating from

germination and growth on nutrient-rich agar to activity in soil, especially for stressed spores. It is unclear whether *C. minitans* inoculum is able to not only survive in soil but also infect new sclerotia incorporated into soil from subsequently diseased crops.

To enable unambiguous detection of the applied *C. minitans*, a hygromycin-resistant transformant, T3, which has been verified to have similar activity to the wild type isolate LU112, was used (Jones et al. 1999, 2003b). The use of the hygromycin phosphotransferase gene allows the selective isolation of the transformant from soil on medium containing hygromycin B,

allowing survival and sclerotial colonisation to be monitored. It has also been shown to increase the sensitivity of detection as *C. minitans* recovery from sclerotia can be masked by other contaminating fungi (Jones et al. 2003b).

The aim of this study was to assess the ability of *C. minitans* LU112 and transformant T3, applied as either solid substrate inoculum or spore suspension, to survive in soil over a 6 month period and subsequently infect sclerotia.

MATERIALS AND METHODS

Source and maintenance of fungi

Coniothyrium minitans isolate LU112 (A69) was isolated from a parasitized sclerotium of *Sclerotinia sclerotiorum*, New Zealand (Jones & Stewart 2000) with transformant T3 being a *uidA* and *hph* transformant of LU112 (Jones et al. 1999). *Sclerotinia sclerotiorum* isolate G18 was isolated from diseased carrot, Christchurch, New Zealand (Jones & Stewart 2000). The fungi were stored in 20% glycerol at -80°C and routinely cultured at 18–20°C on potato dextrose agar (PDA; Oxoid, UK).

Sclerotial production

Sclerotia of *S. sclerotiorum* isolate G18 were produced on sterilised wheat grain (Jones et al. 2003a). Wheat grains (25 g; Australian Standard White, Champion Flourmills, Christchurch, New Zealand) and 60 ml distilled water were autoclaved in 250 ml flasks and once cooled inoculated with three discs (10 mm diameter) taken from the colony margin of 2–3 day old PDA cultures of *S. sclerotiorum* isolate G18. Flasks were incubated at 20°C for 3 weeks in darkness. Each flask was shaken after 1 week to facilitate colonisation. The sclerotia were then washed, air-dried overnight in a laminar flow hood and those of 2–4 mm diameter selected, stored at 4°C and used within 4 weeks.

Coniothyrium minitans maize meal-perlite inoculum

Coniothyrium minitans LU112 and transformant T3 inoculum were grown in 250 ml flasks containing 100 ml of autoclaved maize meal-

perlite (MP; 15%:75% v/v) moistened with 20% distilled water (Jones et al. 2003b). Each flask was inoculated with 5 ml of 1×10^6 spores/ml produced from 21-day-old PDA cultures of either *C. minitans* LU112 or T3 and incubated at 20°C in the dark for 4 weeks. To facilitate colonisation the flasks were shaken once a week. The *C. minitans* inoculum level of the MP was determined using a standard dilution plating technique (Jones et al. 2003b). Ten ml of the colonised MP was ground using a mortar and pestle, shaken in 90 ml of 0.01% water agar for 10 min using a wrist action shaker, and then allowed to stand for 20 min. A dilution series was made and 0.2 ml of each dilution spread on three plates of both Czapek Dox agar (CDA; Oxoid) supplemented with chlortetracycline hydrochloride (200 µg/litre; Sigma Chemicals, Poole) and Triton X-100 (2 ml/litre) (CAT) and CDA supplemented with chlortetracycline hydrochloride and hygromycin B (80 µg/ml; Boehringer Mannheim Biochemicals, Mannheim) (CAH). The number of colonies was counted after incubation at 20°C in the dark for 14 days and used to calculate colony forming units (CFU) per ml of MP inoculum. Inoculum concentration for *C. minitans* A69 MP inoculum was $7.0 \log_{10}$ CFU/ml MP and T3 was $7.1 \log_{10}$ CFU/ml MP on both CAT and CAH.

Coniothyrium minitans soil bioassay

A modification of the method of Jones et al. (2003b) was used to assess the survival of *C. minitans* inoculum in soil and ability to parasitise sclerotia after different periods. *Coniothyrium minitans* was applied to soil as either spore suspension (10^6 spores/g soil) or colonised MP (1%) and after different incubation times sclerotia were added, incubated for a further 2 weeks before sclerotial viability and *C. minitans* infection was assessed.

For spore suspension-inoculated soil, 9 ml of diluted spore suspension (5.6×10^6 spores/ml) prepared from 21-day-old PDA cultures of either *C. minitans* LU112 or T3, was applied to each of 48 Petri dishes containing 50 g of air-dried soil (Wakanui silt loam, sieved 4 mm) and mixed

thoroughly, to give a final concentration of 1×10^6 spores/g soil and a soil water potential of -0.1 MPa. Control plates were inoculated with 9 ml SDW.

For MP-inoculated soil, for each isolate, 25 ml of the colonised MP was ground using a mortar and pestle and mixed thoroughly into 2475 ml of air-dried soil. Fifty grams of this mixture was placed in each of 48 Petri dishes and moistened to -0.1 MPa (9.0 ml/50 g soil) with SDW.

The plates were incubated for 24 weeks with the plates watered to their original content using SDW each week. After 0, 1, 2, 4, 8, 12, 16 and 24 weeks, six plates per treatment were randomly selected, three for CFU determination and three for assessing sclerotial parasitism.

Coniothyrium minitans survival in soil was determined using the soil dilution technique as described. A 10 g soil sample taken from each of three Petri dishes per treatment per assessment time was diluted with 90 ml sterile 0.01% agar, and treated as described previously. The plates were incubated at 20°C in the dark for 14 days, after which the number of colonies was counted and used to calculate CFU/g soil.

For the sclerotial parasitism assay, 60 sclerotia (2–4 mm diameter) were pressed gently into the surface of the soil, 20 sclerotia in each dish. The Petri dishes were then reincubated at 20°C in the dark, with the plates being watered to their original content using SDW after 1 week. After 2 weeks, all sclerotia were harvested and assessed for viability and infection by *C. minitans* as described by Jones et al. (2003b). The sclerotia were surface sterilised by agitation in 20 ml of a 50:50 solution of sodium hypochlorite (NaOCl, 13–15%):ethanol for 3 min and then rinsed in 10 ml of SDW for 3 min. This rinsing was repeated a further two times. The sclerotia were then bisected, with one half being placed on a PDA plus chlortetracycline hydrochloride (200 µg/litre) disc and one on a PDA plus hygromycin B (80 µg/ml) disc. These were then incubated at 20°C for 10 days, after which the number of sclerotia showing mycelial growth (viable) and/or infection by *C. minitans* were recorded.

Statistical analysis

Colony forming unit data were $\log_{10}$ transformed prior to analysis. The data were subject to Analysis of Variance using GenStat 8.2 with the treatment means compared using the LSD test at $P=0.05$. Pair-wise comparisons were made between *C. minitans* recovery from T3-amended soil on unamended and hygromycin B-amended agar using the Chi-square analysis.

RESULTS

Coniothyrium minitans survival in soil

No *C. minitans* was recovered from the untreated control soil at any of the assessment times (data not shown). Similarly, no *C. minitans* colonies were recovered on hygromycin B-amended agar from soil amended with LU112 as either MP or spore suspension inocula (data not shown). In all cases there was a significant decrease in *C. minitans* recovery from soil between the levels immediately after application (0 weeks) and subsequent assessments (1–24 weeks) (Figure 1). Although for all treatments some fluctuation in *C. minitans* recovery level was seen between assessment times, in general over the whole experimental period recovery was relatively constant, with no significant difference in *C. minitans* recovery between 1 and 24 weeks. At most assessment times, *C. minitans* recovery on CAT or CAH from soil amended with T3 spore suspension was significantly higher compared with recovery on the corresponding agar from soil amended with T3 colonised MP. A similar trend was seen for LU112 although in most cases this was not significant. Overall, agar supplemented with hygromycin B increased the recovery of *C. minitans* from soil amended with both T3 spore suspension and MP.

Sclerotial parasitism

There was no significant difference in viability of sclerotia incubated in the untreated control at the different assessment times, with viability being high (85–95%) throughout (Table 1). Across all *C. minitans* treatments and assessment times, incubation in *C. minitans*-amended soil significantly decreased sclerotia viability

Figure 1 *Coniothyrium minitans* recovery ($\log_{10}$ CFU/g soil) from soil on unamended or hygromycin-amended (hyg) agar when incorporated as maize meal-perlite (MP) solid substrate or spore suspension (Spore) inocula over time. Values are the mean of three replicates per treatment and the bar represents LSD ($P=0.05$). * No count due to contamination of plates with other fungi.

compared with the untreated control. For both LU112 and T3, sclerotial viability at the later assessment times (16 and 24 weeks) was significantly lower in soil amended with spore suspension compared with MP inocula.

Coniothyrium minitans infection of sclerotia incubated in the untreated control soil was low (0–10%) throughout, with none recovered on hygromycin B-amended agar. Similarly, no *C. minitans* was recovered on hygromycin B-amended agar from sclerotia incubated in LU112-amended soil. Across all treatments apart from T3 spore suspension at 16 weeks, *C. minitans* infection of sclerotia assessed on unamended agar was significantly lower at the later assessment times (16 and 24 weeks) compared with that observed initially (0 and 1 weeks). In general, *C. minitans* infection of sclerotia incubated in soil amended with spore suspension was higher compared to those incubated in MP amended soil. For T3-amended soils across both incubation time and inoculum type, there was a significant difference ($P<0.001$) in the detection of *C. minitans* from sclerotia on hygromycin

B-amended agar compared with unamended agar. Overall, addition of hygromycin B to the agar increased detection of *C. minitans* from sclerotia compared with unamended agar, with *C. minitans* infection of sclerotia, as assessed on hygromycin B-amended agar, being over 70% for all assessment times.

Other fungi, such as *Trichoderma* spp., were recovered from the sclerotia, with significantly greater numbers isolated from sclerotia on unamended agar at the later assessment time (24 weeks) compared with initially (0–4 weeks). None were isolated on hygromycin B-amended agar.

DISCUSSION

Coniothyrium minitans was seen to survive in soil, and infect and reduce viability of *S. sclerotiorum* sclerotia over the 6-month experimental period, with the behaviour of T3 and LU112 being similar. For both inoculum types there was an initial reduction in *C. minitans* recovery from soil onto dilution plates. This is in contrast to other reports where *C. minitans* level (10^6 CFU/ml soil) remained similar to that applied (McQuilken et

Table 1 Percentage of sclerotia viable (V) and percentage infection of *S. sclerotiorum* sclerotia by *C. minitans* (Cm) or other fungi (other) incubated in soil amended with maize meal-perlite (MP) or spore suspension (Spore) inocula of *C. minitans* isolate LU112 (wild type) or transformant T3 over time. Values are the mean of three replicate plates of 20 sclerotia per assessment time per treatment.

Treatment		0 wk		1 wks		2 wks		4 wks		8 wks		12 wks		16 wks		24 wks	
		-hyg ¹	+hyg ²	-hyg	+hyg	-hyg	+hyg	-hyg	+hyg	-hyg	+hyg	-hyg	+hyg	-hyg	+hyg	-hyg	+hyg
Untreated Control	V	95.0	NA ³	85.0	NA	93.3	NA	86.7	NA	91.7	NA	90.0	NA	95.0	NA	88.3	NA
	Cm ⁴	0	0	6.7	0	3.3	0	10.0	0	3.3	0	5.0	0	1.7	0	6.7	0
	Other	6.7	0	8.3	0	5.0	0 ^c	23.3	0	20.0	0	16.7	0	5.0	0	31.7	0
LU112 MP	V	15.0	NA	15.0	NA	20.0	NA	15.0	NA	20.0	NA	31.7	NA	40.0	NA	35.0	NA
	Cm ⁴	81.7	0	86.7	0	68.3	0	91.7	0	86.7	0	76.7	0	66.7	0	56.7	0
	Other	5.0	0	3.3	0	5.0	0	1.7	0	8.3	0	3.3	0	3.3	0	43.3	0
LU112 Spore	V	5.0	NA	3.3	NA	6.7	NA	20.0	NA	26.7	NA	20.0	NA	15.0	NA	16.7	NA
	Cm ⁴	100	0	98.3	0	88.3	0	95.0	0	86.7	0	93.3	0	76.7	0	56.7	0
	Other	0	0	1.7	0	8.3	0	0	0	5.0	0	0	0	20.0	0	45.0	0
T3 MP	V	6.7	NA	6.7	NA	15.0	NA	30.0	NA	21.7	NA	15.0	NA	30.0	NA	23.3	NA
	Cm ⁴	95.0	95.0	93.3	88.3	86.7	96.7	76.7	96.7	71.7	93.3	91.7	78.3	70.0	78.3	63.3	90.4
	Other	0	0	0	0	0	0	1.7	0	16.7	0	0	0	5.0	0	25.0	0
T3 Spore	V	6.7	NA	0	NA	6.7	NA	1.7	NA	10.0	NA	8.3	NA	8.3	NA	8.3	NA
	Cm ⁴	98.3	100	100	100	88.3	100	75.0	91.7	93.3	95.0	95.0	100	86.7	81.7	35.0	95.8
	Other	0	0	0	0	8.3	0	25.0	0	3.3	0	0	0	10	0	56.7	0
LSD	V	12.87 ⁵															
	Cm	13.02 ⁶															
	Cm + hyg	10.87 ⁷															
	Other	16.20 ⁸															

¹PDA with chlortetracycline.

²PDA with hygromycin B and chlortetracycline.

³NA not applicable.

⁴Cm data for the –hyg plates can include any *C. minitans* strain, but for +hyg plates only T3 can be detected.

⁵LSD for comparison of *S. sclerotiorum* recovery across weeks or treatments on unamended agar.

⁶LSD for comparison of *C. minitans* recovery on unamended agar across weeks or treatments.

⁷LSD for comparison of *C. minitans* on hygromycin amended agar across weeks or treatments; control, LU112 MP, LU112 Spore data omitted from Cm + hyg analysis due to zero counts.

⁸LSD for comparison of other fungi recovery across weeks or treatments for unamended agar; none recovered on hygromycin B amended agar.

al. 1995; Jones et al. 2004). However, unlike the current study, a natural sclerotial population was present in the experimental plots in both these studies and this may have affected both *C. minitans* population levels and survival. Bennett et al. (2006) reported that *C. minitans* spores survived in soil for up to 10 months within pycnidia that had formed within parasitised sclerotia. Additionally, whereas the current study was conducted in the absence of plants, in both of these studies the experimental plots contained plants and again this may have affected *C. minitans* survival. After the initial decline, recovery level thereafter remained relatively constant, being around $4.0 \log_{10}$ CFU/g soil. This inoculum level was reported by Jones et al. (2003a) to be the minimum *C. minitans* inoculum concentration required to give any reduction in sclerotial viability and carpogenic germination. Although *C. minitans* infection was seen throughout the experiment, the *C. minitans* inoculum level in soil was close to the threshold for biocontrol activity and may therefore partly explain the variability in reduction of sclerotial viability observed at the different assessment times.

In general, although *C. minitans* infection of sclerotia when assessed on hygromycin B-amended agar was similar for the two inoculum types, spore incorporation resulted in a higher loss of sclerotial viability compared with MP amendment. While this may partly be explained by the higher *C. minitans* inoculum level seen with the spore- compared with MP-amended soil, at the later assessment times where the difference in sclerotial viability was clearly seen, there was no significant difference in the *C. minitans* levels in the two inoculum treatments. Since *C. minitans* is not reported to grow myceliogenically through soil, infection relies on direct contact between *C. minitans* spores and the sclerotia (Adams 1990; Williams & Whipps 1995). Spore suspension inocula may therefore allow more even distribution of the inoculum in soil compared with MP amendment, thereby increasing the chance of contact between *C. minitans* inoculum and sclerotia. Additionally, more even distribution of the inoculum would potentially result in an individual sclerotium being in contact

with multiple spores and may result in multiple infections compared with a single infection site for MP inoculum. This may have resulted in a more rapid reduction in sclerotial viability in spore-amended soil, although with increased incubation time a comparable decrease in sclerotial viability would be seen with the MP inoculum. In contrast to the current study, Jones et al. (2003a) reported that applied at a range of inoculum concentrations (10^7 - 10^3 CFU/ml) MP inocula was more effective than spore suspension inocula at reducing sclerotial germination in a glasshouse box bioassay. In these experiments, both the *C. minitans* inocula and sclerotia were exposed to fluctuating soil moisture and temperatures whereas in the present experiment a constant incubation temperature (20°C) and soil moisture level (-0.1 MPa) was used. This may indicate better survival of *C. minitans* as solid substrate inocula under fluctuating environmental conditions with *C. minitans* spores being protected both within pycnidia and the solid substrate inoculum itself. The effect of fluctuating environmental conditions, as would be the case in soil in field and glasshouse situations, on survival and subsequent sclerotial parasitism by *C. minitans* when applied as different inocula warrants further study.

Hygromycin B amendment of the agar increased the recovery of *C. minitans* isolate T3 from infected sclerotia, especially at the last assessment when the levels of other contaminating fungi were high on unamended agar. These contaminating fungi are often secondary colonisers of sclerotia weakened by *C. minitans* and have been shown to mask the detection of *C. minitans* from sclerotia (Jones et al. 2003b).

Hygromycin B sensitive *C. minitans* isolates were isolated from sclerotia incubated in the unamended control soil, indicating the presence of an indigenous hygromycin sensitive population in this soil. However, no corresponding decrease in sclerotial viability was observed which may indicate that the indigenous *C. minitans* isolates may be weak parasites, only colonising the outer surface of sclerotia but not being able to kill them. Using the same soil, Jones et al. (2003b) showed that the indigenous *C. minitans* isolates were unable to reduce sclerotial viability even

after 8 weeks incubation. *Coniothyrium minitans* isolates recovered from New Zealand soil have been shown to vary in their ability to infect and reduce sclerotial viability (Jones & Stewart 2000). Further, *C. minitans* was not recovered on the soil dilution plates, indicating the population is below the detection level (10^2 - 10^3 CFU/g soil) for this method and therefore contributing to the low level of sclerotial parasitism seen. In addition, the use of a hygromycin B resistant isolate allowed the applied isolate (T3) to be distinguished from the indigenous hygromycin sensitive *C. minitans* in soil.

The study has shown that *C. minitans* inoculum as either colonised solid substrate (MP) or spore suspension amendments, remains viable in soil for at least 6 months and is able to infect and reduce the viability of sclerotia that are subsequently introduced. Soil incorporation of inoculum therefore has potential to provide long term control of *S. sclerotiorum* especially in crops such as lettuce with a relatively short cropping cycle between planting and harvest (2-3 months). Not only would *C. minitans* inoculum application infect and reduce the viability of sclerotial inoculum already present in the soil but also any sclerotia subsequently incorporated into the soil from diseased plant debris reducing any carry over of inoculum to subsequent crops.

ACKNOWLEDGEMENTS

The work was supported by a grant from the New Zealand Foundation for Research, Science and Technology (contract no. LIN 503).

REFERENCES

- Adams PB 1990. The potential of mycoparasites for biological control of plant diseases. *Annual Review of Phytopathology* 28: 59-72.
- Bennet AJ, Leifert C, Whipps JM 2006. Survival of *Coniothyrium minitans* associated with sclerotia of *Sclerotinia sclerotiorum* in soil. *Soil Biology and Biochemistry* 38: 164-172.
- Jones EE, Stewart A 2000. Selection of mycoparasites of sclerotia of *Sclerotinia sclerotiorum* isolated from New Zealand soils. *New Zealand Journal of Crop and Horticultural Science* 28: 105-114.
- Jones EE, Carpenter M, Fong D, Goldstein A, Thrush A, Crowhurst R, Stewart A 1999. Co-transformation of the sclerotial mycoparasite *Coniothyrium minitans* with hygromycin B resistance and β -glucuronidase markers. *Mycological Research* 103: 929-937.
- Jones EE, Mead A, Whipps JM 2003a. Evaluation of different *Coniothyrium minitans* inoculum sources and application rates on apothecial production and infection of *Sclerotinia sclerotiorum* sclerotia. *Soil Biology and Biochemistry* 35: 409-419.
- Jones EE, Stewart A, Whipps JM 2003b. Use of *Coniothyrium minitans* transformed with the hygromycin B resistance gene to study survival and infection of *Sclerotinia sclerotiorum* sclerotia in soil. *Mycological Research* 107: 267-276.
- Jones EE, Mead A, Whipps JM 2004. Effect of quality and timing of application of *Coniothyrium minitans* on *Sclerotinia sclerotiorum*: control of *Sclerotinia* in glasshouse lettuce. *Plant Pathology* 53: 611-620.
- Li GQ, Huang HC, Acharya SN, Erickson RS 2006. Effectiveness of *Coniothyrium minitans* and *Trichoderma atroviride* in suppression of sclerotinia blossom blight of alfalfa. *European Journal of Plant Pathology* 114: 345-355.
- McQuilken MP, Mitchell SJ, Budge SP, Whipps JM, Fenlon JS, 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.
- Rabeendran N, Jones EE, Moot D, Stewart A 2006. Biocontrol of *Sclerotinia* lettuce drop by *Coniothyrium minitans* and *Trichoderma hamatum*. *Biological Control* 39: 352-362.
- Williams RG, Whipps JM 1995. Growth and transmission of *Coniothyrium minitans* in soil. *Bulletin International Organisation for Biological control* 18 (3): 39-43.