COMPARISON OF THE BEHAVIOUR OF A TRANSFORMED HYGROMYCIN RESISTANT STRAIN OF *TRICHODERMA ATROVIRIDE* (M1057-HYG^R) WITH THE WILD-TYPE STRAIN (M1057)

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ABSTRACT

The biocontrol isolate *Trichoderma atroviride* M1057 and a transformed hygromycin resistant biotype (M1057-hyg^R) were compared using biological control, rhizosphere competence and antibiosis studies to determine whether the transformed biotype performed in a similar manner to the wild-type strain. In an onion growth chamber trial using soil naturally infested with the onion white rot pathogen, *Sclerotium cepivorum*, there was no significant difference ($P>0.05$) in the level of disease control given by the two *T. atroviride* strains. Similarly, populations of *T. atroviride* M1057 and M1057-hyg^R were equivalent ($P>0.05$) in the rhizosphere of onion seedlings. There was no significant difference ($P>0.05$) between the mycelial growth rates of *S. cepivorum* when grown on agar amended with culture filtrate of *T. atroviride* M1057 and M1057-hyg^R. Thus, *T. atroviride* M1057-hyg^R has similar biological attributes to the wild-type isolate and can be used in future field studies looking at the population ecology of the biological control agent.

Keywords: *Trichoderma atroviride*, transformant, biological control, ecology.

INTRODUCTION

A number of species within the genus *Trichoderma* are well known for their biological control capabilities against a wide range of commercially important plant pathogens (Whipps 1997; Harman & Bjorkman 1998; Whipps 2001; Whipps & Lumsden 2001). In particular, isolates of the species *T. atroviride* P. Karsten have proved effective in controlling the onion white rot pathogen *Sclerotium cepivorum* Berk. (Kay & Stewart 1994; McLean & Stewart 2000). Although numerous potential biological control agents have been reported in the literature over the past 50 years, few have been commercialised because of inconsistency in their ability to reduce plant disease. More recent research has focused on understanding the factors that influence establishment, proliferation and mode(s) of action of such biological control species. This could ultimately lead to better formulations and application strategies, which will enhance the efficiency and reliability of biological control agents. These studies have been hampered in relation to *Trichoderma* species by the inability to distinguish between individual isolates using morphological and cultural characters. Furthermore, *Trichoderma* species appear to be especially abundant in New Zealand soils, thus adding to the problem of monitoring specific biological control agents in the field.

The introduction of molecular based technologies has made it possible to distinguish between *Trichoderma* species and isolates within each species. The ecology of individual isolates can be monitored in the soil environment using two different methods. The identification of an isolate specific segment of DNA sequence allows the researcher to design a molecular probe, which targets the specific region. A second method involves transforming the isolate of interest with a marker gene. The second approach was used to produce a genetic variant of the biological control isolate *T. atroviride* M1057 expressing the hygromycin resistance gene (Bowen et al. 1996).

This paper reports the results of a comparative study examining the behaviour of *T. atroviride* M1057 and M1057-hyg^R. Three biological assays (biological control, rhizosphere competence and antibiosis) were conducted to determine whether the transformed isolate had retained key biological characters exhibited by the wild-type strain. The availability of a marked strain will facilitate soil-based detailed ecological studies aimed at understanding biological control interactions *in situ*.

MATERIALS AND METHODS

Origin of isolates

Trichoderma atroviride M1057 was recovered from a kiwifruit orchard in the Bay of Plenty, NZ in 1989. This isolate was effective as a biological control agent against a number of soil-borne pathogens including *Sclerotium* species (Cutler & Hill 1994; Dodd-Wilson 1996). The hygromycin resistant variant of *T. atroviride* M1057-hyg^R was produced by inserting the *Escherichia coli* hygromycin resistance gene “*hph*” using a pAN7-1 plasmid with a pUC19 vector backbone (Bowen et al. 1996).

Preparation of inoculum

Both isolates were prepared as a solid substrate for the biological control and rhizosphere competence studies. Spore suspensions were prepared from a 10 day old M1057-hyg^R culture grown on potato dextrose agar (PDA) amended with hygromycin (80 µg/ml) and M1057 culture grown on PDA at 20°C in a 12:12 h light:dark incubator. Five millilitres of 0.01% Tween 80 was added to each plate and the culture rubbed with a sterile glass rod to suspend the spores. Each suspension was filtered through lens tissue three times to remove any hyphal fragments and adjusted to 1×10^6 spores/ml. Thirty-five grams of sand (<800 µm) and 35 g of ground wheat bran (<800 µm) were placed together into a conical flask with 17.5 ml of sterile distilled water. The flasks were autoclaved for 30 min, at 121°C and 15 psi on 2 successive days and cooled before 17.5 ml of spore suspension was added. Two flasks per treatment were prepared. Sterile distilled water replaced the spore suspension for the control treatment. The flasks were incubated for 6 days at 25°C in the dark before their immediate application to the soil.

Biological control assay

Sclerotia of *Sclerotium cepivorum* SC3 (isolated from garlic grown in Pukekohe, New Zealand in 1996) were produced on wheat grains (Alexander & Stewart 1994). Sclerotia were harvested using progressive wet sieving through 850 µm and 500 µm sieves (Kay & Stewart 1994). Only sclerotia retained on the 500 µm sieve were used in the trials and the sclerotia were air dried for 24 h before use.

Fifteen 2.5 litre plastic pots with saucers were filled with 2.5 kg of Wakanui silt loam soil infested with *S. cepivorum* sclerotia (1 sclerotium/g soil). There were three treatments, isolate M1057, isolate M1057-hyg^R and a non-*Trichoderma* control. Each treatment had five replicate pots. For each pot in the *Trichoderma* treatments, 30 g of *Trichoderma* infested solid substrate was mixed into the top 5 cm of soil.

Eight captan-coated onion seeds (0.8 g a.i./100 g seed) were sown in each pot at a depth of 1 cm. Seeded pots were placed in a growth chamber at 18°C with 12:12 h light:dark incubator using a randomised block design, and watered when necessary. Emergence was recorded 3 weeks after planting and then the number of diseased seedlings was recorded every 3 weeks.

Rhizosphere competence assay

Nine 1.5 litre plastic pots with saucers were filled with Wakanui silt loam soil and

inoculated solid substrate as described for the biological control assay. Six captan-coated onion seeds (0.8 g a.i./100 g seed) were planted in each pot as above and seeded pots were incubated in a growth chamber at 18°C with 12:12 h light:dark, and watered as required. Rhizosphere competence was assessed at 6, 12 and 18 weeks by removing the six seedlings from each of three pots for each treatment. The roots were severed from the above ground plant parts at the stem base using a scalpel and the roots from each pot were combined and placed in 9 ml of 0.01% water agar (WA) contained in a glass bottle (90 ml in a glass bottle for the 16 week assessment). A 10-fold dilution series was performed (Whipps et al. 1989). Aliquots (0.25 ml) of the M1057 and control treatment dilutions were pipetted onto PDA amended with Triton X (1 drop/litre) and aliquots from the *T. atroviride* M1057-hyg^R treatment were spread on Triton X PDA amended with hygromycin (80 µg/ml). Colony forming units (cfu) were counted after 10 days incubation at 20°C in the dark.

Antibiosis assay

An amended agar assay was used to detect the production of antibiotics by the *T. atroviride* isolates. Conical flasks (250 ml) containing 100 ml of molasses yeast extract broth (10 g molasses, 1.6 g yeast extract and 990 ml distilled water) were autoclaved for 30 min at 15 psi on 2 successive days. Once the broth had cooled, each of two flasks were inoculated with two 5 mm plugs of M1057-hyg^R or M1057 taken from the actively growing edge of a 5 day old colony grown on PDA amended with hygromycin (80 µg/ml) (M1057-hyg^R) or PDA (M1057). Control flasks were inoculated with two sterile agar plugs. The inoculated flasks were incubated at room temperature (18-20°C) in the dark, on a rotary shaker set at 50 rpm (Abd-El-Moity & Shatla 1981).

After 14 days, the two flasks for each treatment were combined. The liquid medium was separated from the mycelium by suction filtration. The resultant culture filtrate was centrifuged at 500 rpm for 10 min. Aliquots (10 ml) of the centrifuged filtrate were filter sterilised with a 0.2 µm Millipore filter, directly into cooled PDA (<55°C) at a concentration of 10% (v:v) (Abd-El-Moity & Shatla 1981; Kay 1991). The amended agar was poured into each of five agar plates, and upon solidification, the plates were centrally inoculated with a 5 mm disk taken from the actively growing edge of a 5 day old *S. cepivorum* culture grown on PDA. Sterile agar plugs acted as the control treatment. The plates were sealed with polythene wrap, inverted and incubated in the dark at 20°C. The radius (mm) of each *S. cepivorum* colony was measured in four directions along pre-drawn lines, each day for 7 days or until the colony reached the plate edge.

Statistical analysis

For the biological control and the rhizosphere competence trials, results were analysed using a one-way ANOVA at each assessment time with disease as the factor for the biological control trial and concentration as the factor for the rhizosphere competence trial. For the antibiosis assay, a one-way ANOVA was used for the growth of *S. cepivorum* on day three (time taken for *S. cepivorum* to reach the plate edge in the control).

RESULTS

Biological control assay

There was no significant difference ($P>0.05$) in the number of emerged seedlings between treatments (Table 1). The progression of onion white rot disease development was similar for both *T. atroviride* strains and there was no significant difference ($P>0.05$) in the number of diseased seedlings between treatments for the duration of the trial (Table 1).

Rhizosphere competence assay

Trichoderma atroviride M1057 was recovered from the rhizosphere of onion plants at 1.3×10^5 , 3.4×10^3 and 2.7×10^4 cfu/g soil at 6, 12 and 18 weeks after planting, respectively. *Trichoderma atroviride* M1057-hyg^R was recovered at 6.5×10^4 , 3.4×10^4 and 4.8×10^4 cfu/g soil for the same time periods. There was no significant difference ($P>0.05$) in the number of colonies recovered between the *T. atroviride* treatments or between the assessment intervals for each treatment.

TABLE 1: Percentage seedling emergence (%) and onion white rot disease after treatment with *Trichoderma atroviride* M1057 and *Trichoderma atroviride* M1057-hyg^R in a soil pot assay.

Treatment	Emergence	Percentage onion white rot disease (weeks after planting)		
		6 weeks	9 weeks	12 weeks
Control	85.0	11.8	38.2	52.9
M1057	70.0	3.6	7.1	35.7
M1057-hyg ^R	72.5	3.4	6.9	13.9
P-value	0.447	0.449	0.063	0.073

Antibiosis assay

The mean radial growth of *S. cepivorum* was 24.3 mm after 3 days growth on unamended PDA. On PDA amended with *T. atroviride* M1057 and *T. atroviride* M1057-hyg^R, *S. cepivorum* colony growth was 20.7 and 22.2 mm, respectively. There was no significant difference ($P > 0.05$) in mycelial growth between the three treatments.

DISCUSSION

The results of the biological control, rhizosphere competence and antibiosis studies conducted in the present study showed no difference in the behaviour of the transformed *T. atroviride* strain compared with the wild-type strain. These results are in accordance with reports in the literature where the behaviour of transformed and wild-type isolates has been compared. Bowen et al. (1996) reported the same level of *Sclerotium rolfsii* Saccardo disease control in lentil with the *T. atroviride* M1057-hyg^R and wild-type strains. In addition, Green & Jensen (1995) reported equivalent *Trichoderma* rhizosphere colonisation of cucumber roots with a GUS transformed *T. harzianum* and its wild-type strain.

The results of the present trial indicate that the transformed *T. atroviride* strain can be used in soil based ecological studies. Conclusive identification of *T. atroviride* M1057-hyg^R can be made on hygromycin amended agar. Such ecological studies will enable a better understanding of biocontrol interactions and population dynamics to be gained.

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