MOLECULAR MARKER ASSISTED DETECTION OF THE MYCOPARASITE *CONIOTHYRIUM MINITANS* A69 IN SOIL

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ABSTRACT

Coniothyrium minitans A69 has been shown to have biological control activity against the plant pathogen *Sclerotinia sclerotiorum* and a PCR based assay has been developed to specifically identify this isolate. The practical application of this PCR assay for detection of *C. minitans* from soil was assessed. Sterile and non-sterile soil was inoculated with spores from *C. minitans* A69 at five different concentrations and DNA recovered using a SDS/Phenol/Chloroform method. A number of factors affected DNA recovery and subsequent PCR, with a maximum sensitivity of down to $1x10^2$ spores/g soil achieved in sterile soil. Detection of *C. minitans* in non-sterile soil was hampered by failure of the fungus to germinate. However, this method has improved throughput and cost effectiveness compared with conventional detection methods involving quantitative colony recovery.
Keywords: *Coniothyrium minitans*, DNA extraction, soil, molecular marker.

INTRODUCTION

The fungal plant pathogen *Sclerotinia sclerotiorum* causes significant production losses of vegetable crops in many parts of the world, including New Zealand (Purdy 1979). This pathogen forms hardy survival structures called sclerotia which are the source of infection for subsequent growing seasons. Traditional control of this pathogen has been with fungicides, however, due to reduced effectiveness and environmental/health impacts, this form of control has become increasingly unpopular. Recent research has been directed at finding alternatives to chemical based control.

The mycoparasite *Coniothyrium minitans* is a filamentous fungus and has potential for use as a biological control agent against the sclerotia of *Sclerotinia sclerotiorum* (Whipps and Gerlagh 1992). Effective biological control activity has been demonstrated to occur in both field and glasshouse trials (Huang 1980; Budge and Whipps 1991). However, efficient use of *C. minitans* as a biological control agent relies on in-depth knowledge of its growth, dispersal and survival in soil ecosystems. Such ecological understanding requires the ability to detect and differentiate a single experimental strain from the numerous micro-organisms that inhabit the soil.

Analysis of the interaction of *C. minitans* with host plants and the environment has been facilitated by the development of a strain specific PCR based molecular marker (Goldstein *et al.* 2000). This molecular marker involves amplification of 1.7, 1.6, 0.5 and 0.35 kb bands from a dispersed repetitive element creating a band profile specific for *C. minitans* A69. Amplification of this band profile has been used in recent experiments to confirm the survival of *C. minitans* at the site of inoculation for over one year (K. Eade, pers. comm.). Traditional quantitative colony recovery, strain purification and subsequent PCR analysis using the molecular marker is laborious and expensive. Ideally, confirmation of the presence of *C. minitans* A69 in field plots would be achieved by PCR of DNA extracted directly from soil. The following investigation describes the recovery of *C. minitans* A69 DNA from soil and subsequent PCR analysis of this DNA.

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METHODS

Preparation of a spore suspension and soil inoculation

A spore suspension was prepared from 21 day old potato dextrose agar (Roche Diagnostics N.Z. Ltd) plates and collected in sterile Universals containing approximately 10 ml water and 0.01% Tween-80 (Sigma, NSW, Australia). Spores were counted using a haemocytometer and diluted to appropriate concentrations. Lismore soil was sterilised by autoclaving (20 min, 121°C, 15 p.s.i.) three times on consecutive days. Sterile and non-sterile soil (30 g) was weighed into 25 mm deep petri dishes and a total volume of 6 ml of liquid added to each. Each 6 ml liquid aliquot contained 3 ml of potato dextrose broth (Roche Diagnostics N.Z. Ltd) and 3 ml of the appropriate spore suspension to give final concentrations of $1x10^7$ to $1x10^2$ spores/g soil. A control was prepared containing 3 ml potato dextrose broth and 3 ml of sterile water. Soil and liquid was mixed thoroughly using a sterile pipette tip and then incubated at 22°C for 2, 5 and 10 days.

DNA extraction

DNA was extracted using a modification of the method of Volossiouk *et al.* (1995). Briefly, 2 g soil was ground to a fine powder in liquid nitrogen and transferred to a pre-cooled tube. Extraction buffer (5 ml) (0.3 % SDS, 0.14 M NaCl, 50 mM NaOAc, pH 5.1) was added and the mixture incubated at 37°C for 20-40 min followed by centrifugation at 1000 g for 5 min. The supernatant was transferred to a clean tube and extracted six times with 2 ml phenol and 2 ml chloroform, followed by a single extraction with 4 ml chloroform. DNA was precipitated by addition of 60% volume of ice-cold isopropanol and the pellet washed with 70% ethanol. The pellet was then air dried and resuspended in 500 ml sterile water. Resuspended DNA was diluted 1:10 immediately prior to amplification with the diagnostic PCR primers.

Polymerase chain reaction

Amplification was performed with the previously described DRE300 ($^{5'}$GGAATTCCTTCTTACCCTTTGCACGATAG$^{3'}$) and DRE400 ($^{5'}$GCTCTAGAGCTTGCGGACGTCCCTTA GG$^{3'}$) primers using a modification of the method of Goldstein *et al.* 2000. These primers amplify 1.7, 1.6, 0.5 and 0.35 kb products from a dispersed repetitive element. Each 25 ml amplification reaction contained 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 100 mM each of dATP, dTTP, dGTP and dCTP, 10 mg of bovine serum albumin (Roche Diagnostics N.Z. Ltd), 1 ml of diluted DNA extract and 0.25 U Taq DNA polymerase (Roche Diagnostics N.Z. Ltd). The reactions were performed in an Eppendorf Mastercycler® Gradient PCR machine and consisted of 35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 3 min. PCR products were separated on 1.0% agarose gels and visualised using ethidium bromide.

RESULTS

DNA was successfully extracted from sterile soil both 2 and 5 days after inoculation. The sensitivity of detection was improved by leaving the soil until 5 days had elapsed. DNA (>50 ng/g soil) was extracted from 2 day old soil which had originally been inoculated with spore concentrations greater than 1×10^4 spores/g soil and from all soil samples, except the control, 5 days after inoculation. White mycelium was visible on the soil from which DNA (>50 ng/g soil) was recovered. Amplification of the *C. mintans* A69 molecular marker was achieved from 2 day old soil when the original inoculation had been at a spore concentration of 1×10^5 or higher and at all concentrations 5 days after inoculation (Fig. 1).

In inoculated non-sterile soil, DNA (>50 ng/g soil) was recovered from all samples at both 2 and 10 days. However, amplification with DRE300 and DRE400 failed to generate the characteristic molecular marker pattern in any soil sample (results not shown). Furthermore, the mycelium visible in the sterile soil treatments was not visible on these samples.

DISCUSSION

Any successful molecular marker for a soil-borne biological control agent must be robust enough to detect the fungus in field samples. This allows the investigation of ecological aspects such as persistence in soil and spread from the site of application. This investigation describes a method for the detection of the biological control agent *C. minitans* A69 in soil. Extraction of *C. minitans* A69 DNA from dormant spores relies on disruption of the spore coat. This is particularly important given the size and robust nature (3.1-7.7 mm x 2.0-5.1 mm) of *C. minitans* spores (Sandys-Winsch *et al.* 1993) which make breaching the spore coat by grinding with a mortar and pestle difficult. However, after germination the spore coat is disrupted by the emergence of the germ tube facilitating entry of the nuclei extraction media. For this reason, a nutrient source was added to soil to encourage *C. minitans* A69 germination. DNA was successfully recovered from germinated spores in sterile soil using the SDS/Phenol/Chloroform method, however germination stage and inoculum concentration had a direct effect on the sensitivity of detection (Fig. 1). Inoculum levels below 1 x 10^5 spores/g required up to 5 days incubation at 22°C for a positive PCR result.

Despite the length of the incubation period this method was much faster and more cost-effective than the traditional method of quantitative colony recovery. Furthermore, this rationale suggests that only those spores capable of germinating will have their structural integrity disrupted and produce a positive PCR result. This is an advantage over many other DNA extraction procedures which fail to discriminate between DNA from viable and non-viable spores.

Amplification of the molecular marker using DNA purified from soil was difficult. The DNA was contaminated with humic acid and metal ions co-extracted during the purification process. The inhibitory effect of these contaminants was overcome by the addition of the macromolecule bovine serum albumin (BSA) to the PCR reaction. BSA is believed to act as a carrier that stabilises the Taq DNA polymerase and promotes the formation of longer amplimers (Volossiouk *et al.* 1995). However, the effects of inhibitors were still apparent in the reduced concentration of the large 1.7 kb product of the marker pattern (Fig. 1). These results suggest that molecular markers which involve the production of large (>1 kb) amplimers are unsuitable for the detection of soil-borne fungi.

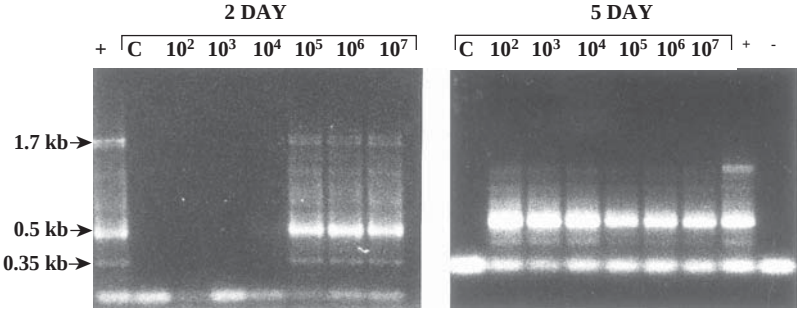

FIGURE 1: Agarose gel of DNA extracted from sterile soil and amplified using the primers DRE300 and DRE400. The sampling day and spore concentration/g soil are designated above the gel, together with the positive (+) and negative (-) controls. Arrows designate the size of the amplimers.

Although DNA was successfully recovered from non-sterile soil, amplification with the DRE primers failed to produce the characteristic *C. minitans* A69 pattern. This suggested that little, if any, *C. minitans* A69 DNA was present. The lack of amplification with the DRE primers even at the highest spore concentration and the absence of visible white mycelium suggest that *C. minitans* A69 failed to germinate.

The large amount of DNA extracted was probably from plant organic matter, bacteria and other fungi present in the soil. Lack of germination may be the result of inhibitory factors secreted by these other soil inhabitants or a poor competitive saprophytic ability. This is in marked contrast to other biological control agents, such as *Trichoderma harzianum,* which have excellent saprophytic ability.

In summary, further work is required to find an additive that stimulates *C. minitans* A69 germination in non-sterile soil, despite competition from other organisms. This may be a particular substrate, sclerotial extract, temperature or specific pH. Alternatively, other means of disrupting the spore coat such as enzymatic degradation may prove successful. This is however, a rapid and cost-effective method for DNA extraction directly from soil samples with subsequent amplification of the molecular marker.

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