GENETIC COMPARISON OF *SCLEROTINIA SCLEROTIORUM* ISOLATES FROM NEW ZEALAND AND USA

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ABSTRACT

The use of the fungus, *Sclerotinia sclerotiorum*, as a biological control agent for weeds in New Zealand pastures may be dependent upon specific strain identification to distinguish applied from background strains of the pathogen. Using RAPD and rDNA analysis, four New Zealand strains and four derivatives of a USA isolate of *S. sclerotiorum* were compared with single isolates of *S. minor* and *S. trifoliorum*. Few differences were detected between any strains of *S. sclerotiorum*, although a single band out of 94 scored on RAPDs, consistently divided the isolates into USA and New Zealand strains. Isolates of *S. minor* and *S. trifoliorum* were clearly separate from *S. sclerotiorum* using RAPDs, but no differences were detected in rDNA.

Keywords: *Sclerotinia sclerotiorum*, mycoherbicides, RAPD analysis, ribosomal DNA, molecular comparison

INTRODUCTION

Plant pathogenic fungi can cause problems in agriculture, due to crop losses occurring through infections. However, many weed species are also attacked by such fungi, and this has led to the investigation of some species as biological control agents. One such fungus, *Sclerotinia sclerotiorum* (Lib.) de Bary (Discomycetes: Sclerotiniaceae), has been shown to be pathogenic to pasture weeds such as Californian thistle (*Cirsium arvense*), giant buttercup (*Ranunculus acris*), ragwort (*Senecio jacobaea*), Scotch thistle (*Cirsium vulgare*), winged thistle (*Carduus tenuiflorus*) and nodding thistle (*Carduus nutans*) (Waipara *et al.* 1993). Unfortunately *S. sclerotiorum* has a broad host range and of the 101 host species listed in Pennycook (1989), only 14 can be classed as weeds. This wide host range would preclude use as a mycoherbicide if displayed by all strains, but individual strains do vary in their ability to infect different hosts. Several isolates are under investigation in New Zealand for pasture weed control (Hurrell and Bourdôt 1993; Harvey 1996).

Development of formulations based on selected isolates may well require their rapid and unambiguous identification. The separation of applied isolates from background inoculum and infections and specific strain identification would be a valuable tool in risk assessment. Species separation within the genus *Sclerotinia* is presently based mainly on morphological differences in the sclerotia and ascospores (Kohn 1979). There is no physiological specialisation found in this species (Mordue and Holliday 1976) but strains can differ in their reaction to various hosts (Harvey 1996) and one strain can be highly virulent to some hosts but mildly so against others (Waipara *et al.* 1993). No reliable method of separating strains on host reaction has been developed. Molecular techniques which focus directly on genetic information may provide more discrimination. Techniques which discriminate at different taxon levels have been developed. A technique which has proved useful at the species and subspecies level with other fungal genera is random amplified polymorphic DNA (RAPD) analysis (eg. Zimand *et al.* 1994), whereas examination of regions of ribosomal DNA has proved useful at strain, species or higher levels (Curran *et al.* 1994).

This study investigated genetic differences between isolates of three species of *Sclerotinia*.

MATERIALS AND METHOD

Fungal isolates and methods of culture

Fungal isolates (Table 1) were maintained on malt extract agar (MEA: 25g malt extract, 20g agar per litre) at 20°C. Isolates S1 through S4 were from Montana, USA. S2 and S3 were auxotrophic mutants derived from the S1 wild-type (Miller *et al.* 1992), while S4 was a sclerotium-less mutant from the same wild type (Miller *et al.* 1989). For the DNA extraction, a 1 cm square of actively growing mycelium on agar was cut into approximately 100 pieces and placed in 100 ml of potato dextrose broth (PDB: 27g PDB, 1% yeast extract per litre). The flasks were placed on a shaker in a 20°C room for 3 days. Approximately 10 ml of mycelium was then subcultured into 40 ml of PDB and left shaking at 20°C overnight, to ensure actively growing mycelium for the DNA isolation.

TABLE 1: *Sclerotinia* isolates used in the study.

Genus and species	Isolate	Source	Host
<i>Sclerotinia sclerotiorum</i>	S1	USA	<i>Centurea maculosa</i> (spotted knapweed) (Wild type)
	S2	USA	Arginine deficient mutant of S1
	S3	USA	Leucine deficient mutant of S1
	S4	USA	Sclerotium-less mutant of S1
	S6	NZ	<i>Actinidia deliciosa</i> (kiwifruit)
	S9W1	NZ	<i>Cirsium arvense</i> (California thistle)
	S15	NZ	<i>Cucurbita maxima</i> (Squash)
	S31	NZ	<i>C. arvense</i>
<i>S. minor</i>	SM	NZ	<i>Carduus nutans</i> (nodding thistle)
<i>S. trifoliorum</i>	S20	NZ	<i>Medicago sativa</i> (lucerne)
<i>Metarhizium anisopliae</i>	F80	NZ	Soil isolation

Isolation of DNA from fungal mycelium

DNA was isolated using a micropreparation variation on the cellulase assisted lysis method of Pfeifer and Khachatourians (1993). DNA was isolated from 500 ml of liquid culture and eventually resuspended in 100 ml of TE (10mM Tris-HCl, pH 8.0, 1mM EDTA) and stored at 4°C. DNA was isolated on three separate occasions from actively growing mycelia. The concentration of DNA used ranged from 2-20 mg/ml, and was estimated using a TKO 100 Dedicated Mini Fluorometer (Hoefer Scientific). The concentration of the DNA used in the PCR reactions was determined empirically and ranged from a 1 to 10 x dilution of the initial isolation.

RAPD analysis

Amplifications were performed in 25 ml containing 100 mM dNTP, 0.1 mM primer (Operon Technologies Ltd, Table 2), 0.55 units of *Taq* DNA polymerase (Boehringer Mannheim) in the reaction buffer (containing 0.038 mM Mg) supplied. Each tube was overlaid with a drop of mineral oil. PCR was executed in a Perkin Elmer thermal cycler using 45 cycles of one minute at 94°C, one minute at 36°C, and two minutes at 72°C. A previously amplified DNA control, *Metarhizium anisopliae* isolate F80 (van der Wielen and Glare unpublished data), and a negative (water) control were included in every experiment. The variables of the PCR reaction were optimised at the start of experimentation.

RAPD products were resolved on 1.4% agarose in 0.5 x TBE electrophoresis buffer. (TBE: 50 mM Tris, 50 mM Boric acid and 1 mM EDTA). A standard (100 bp DNA Ladder, Gibco, BRL) was included on each gel. Banding patterns from three separate isolations were compared and only bands which occurred repeatedly were included in the assessment. A binomial comparison using Genstat (Genstat 1987) was then made between all bands. Segregation analysis was performed with three statistics; analysis of shared fragments (Gower 1985), Jacquard similarity (Gower 1985), Nei-Lei genetic distance (Nei and Lei 1979).

TABLE 2: Random primers (Operon Technologies) and specific primers for rDNA (Curran *et al.* 1993) used in the analysis.

Primers used	Sequence 5' to 3'
OPF-06	GGGAATTCGG
OPF-07	CCGATATCCC
OPF-08	GGGATATCGG
OPF-09	CCAAGCTTCC
OPF-10	GGAAGCTTGG
OPF-11	TTGGTACCCC
OPF-12	ACGGTACCAG
OPF-13	GGCTGCAGAA
OPF-14	TGCTGCAGGT
OPF-15	CCAGTACTCC
OPF-16	GGAGTACTGG
OPF-17	AACCCGGGAA
TW81	GTTTCCGTAGGTGAACCTGC
AB28	ATATGCTTAAGTTCAGCGGGT

Analysis of rDNA

Using the DNA extracted by the above method, the region of the ribosomal repeat from the 3' end of 16S rDNA across internal transcribed spacer 1 (ITS 1), the 5.8S rDNA, and ITS 2, to the 5' end of the 28S rDNA was amplified using specific primers (Table 2). The PCR reactions were performed in 50 µl reaction volumes containing 4 µl of extracted DNA, 2.5 µl of 2 mM dNTPs, 5 µl of reaction buffer, 1.25 units of *Taq* polymerase and 0.6 µl of each 0.027 mM primer. PCR was executed in a Perkin Elmer thermal cycler at 95°C for 5 min. and then 30 cycles of 94°C for 1 min., 52°C for 1 min. and 72°C for 2 min. (Curran *et al.* 1994). Amplified rDNA was digested with 9 restriction endonuclease enzymes (*Mse*I, *Alu*I, *Hae*III, *Cla*I, *Eco*RI, *Sau*3a, *Hpa*II, *Rsa*I and *Taq*I) using standard methods (Sambrook *et al.* 1989) and visualised on a 2% agarose gel.

RESULTS

Morphological comparisons of *Sclerotinia* spp.

On MEA, *S. minor* developed many small sclerotia laterally on hyphae throughout the colony. *S. trifoliorum* and *S. sclerotiorum* isolates develop fewer and larger sclerotia arranged radially at the growing margins. *S. trifoliorum* was slower growing than *S. sclerotiorum* and produced pigmented hyphae near the agar underside. *S. trifoliorum* and *S. sclerotiorum* can only be positively differentiated through differences in the location and the size of the ascospores produced on the apothecia grown from the sclerotia. The isolates of *S. sclerotiorum* were not distinguishable on morphological criteria, with the exception of S4, which is a sclerotium-less mutant. All other *S. sclerotiorum* isolates used in this project produced similar sized sclerotia produced randomly over the agar surface.

RAPD analysis

The ten primers used produced 149 scorable bands on the agarose gels after the first DNA isolation, 94 of these were confirmed when the amplifications were repeated with the second and third DNA isolations. Banding patterns were very similar for all the *S. sclerotiorum* isolates. Primer OPF-08 produced a band of approximately 2000 bp in the New Zealand isolates which was not present in the USA isolates (Fig. 1). Although this was only a single band, it resulted in the isolates of *S. sclerotiorum* dividing into two groups in subsequent analysis (Fig. 2). Dendrograms produced when analysed using 3 statistical methods (Shared fragments, Nei and Lei, and Jacquard Similarity) confirmed similarity between the *S. sclerotiorum* isolates, while clearly distinguishing *S. minor* and *S. trifoliorum* from *S. sclerotiorum* (Fig. 2). *M. anisopliae*, used as an outgroup for comparison, had few similarities to *Sclerotinia* spp.

FIGURE 1: Schematic representation of an electrophoresis gel of the PCR products produced using primer OPF-08 (hatched bands indicate the distinguishing band between USA and New Zealand *S. sclerotiorum* isolates).

FIGURE 2: Dendrogram showing the relationship based on RAPD analysis between species and strains (produced using Genstat analysis, Nei and Lei statistic. Results of 94 scorable bands using 10 random primers).

Amplification of rDNA and analysis by restriction enzymes

The ribosomal DNA sequence containing the highly conserved 5.8s region and two more rapidly evolving ITS regions were investigated indirectly through restriction digests. The nine restriction enzymes used found no differences in the ITS and 5.8s region of the DNA. Restriction enzymes used account for about 13% of the whole region that was investigated, as determined by comparison with the published sequence of this region for *S. sclerotiorum* (Morales *et al.* 1993).

DISCUSSION

RAPD analysis of 10 isolates of *Sclerotinia* spp. confirmed the species distinctions determined on morphological criteria. In addition, analysis showed that the isolates of *S. minor* and *S. trifoliorum* were more closely related to each other than to the *S. sclerotiorum* isolates. The eight *S. sclerotiorum* isolates were placed into two groups based on the presence or absence of a single band of 94 scored, corresponding to USA and New Zealand groups.

Given the geographic and pathotypic differences, this homogeneity within *S. sclerotiorum* is surprising. Similarities between the USA isolates was not surprising, as the four isolates were derived from the same wild-type. The New Zealand material, however, originally came from 3 hosts, from both the North and the South Islands, and could be expected to show more variation. Similar studies on insect pathogenic fungi have found large variation using RAPDs within geographic regions (eg. Fegan *et al.* 1993).

Ribosomal DNA is well suited for use in phylogenetic studies, as the coding regions (ie. 5.8s) change very slowly and are useful for comparison of relatively distant organisms, whereas the internal transcribed spacers (ITS1 and ITS2) evolve more rapidly. Therefore, the coding and ITS regions may be useful for different levels of discrimination. Comparison of the published sequence of the ITS regions of *S. sclerotiorum* (Morales *et al.* 1993) and *M. anisopliae* (Curran *et al.* 1994) showed a 96% similarity in the 5.8s region and approximately 23% in the ITS regions, demonstrating the conserved nature of the 5.8s region and the more rapidly evolving ITS regions. Using restriction digestions, about 13% of the total region was indirectly investigated in the present study. No differences were found between any of the isolates or species.

As no distinct pattern differentiated between isolates, it is difficult to develop specific identification necessary for tracking of released strains of the pathogen, as required for mycoherbicide experiments. Even the difference shown in Fig. 1 only distinguished between USA and New Zealand strains. This study has indicated that it will be difficult to produce isolate-specific probes for *S. sclerotiorum*. It may be possible to develop specific probes using genes encoding functions responsible for the host specific exhibited by the isolates; however, this will require further research.

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