VG511
Molecular identification of strains of sclerotinia species

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Know-how for Horticulture™

VG511

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Final Report

Molecular Identification of Strains of *Sclerotinia* spp. VG318 (1993-95) continued as VG511 (1995-97)

1. Introduction

In Australia, Sclerotina head, root and stem rots are caused by one of two fungal species: *Sclerotinia sclerotiorum* (Lib.) de Bary and *S.minor* Jagger. A third species, *S.trifoliorum* Erikss. which attacks clover, is of minor concern. *Sclerotina* represents an economically important group of plant pathogens, causing severe losses to many crop species including: tomato, *Brassica* spp., lettuce and various grain crops including sunflowers and peanut. *Sclerotinia* spp. are amongst of the most intractable groups of plant pathogens to control, as genetic resistance is not available in host crop species and chemical control is extremely expensive and generally ineffective.

Both *S.sclerotiorum* and *S.minor* produce modified vegetative resting structures known as "sclerotia" which comprise of compacted fungal mycelia covered in a hard melanised rind. It is these structures which frequently remain viable in the soil for up to twenty years allowing the disease to be carried over from one year to the next. Consequently, once a field is infested with *Sclerotinia* spp. it is hard to eradicate this pathogen. The sclerotia produced by *S.sclerotiorum* are generally larger that those produced by *S.minor*, however environmental conditions can influence the size of the sclerotia, often making it difficult to tell the species apart.

S.sclerotiorum and *S.minor* are both ascomycete fungi. This means that they are capable of producing ascospores following sexual fertilisation. These spores are produced in cup-like fruiting structures which form on the sclerotia.

The lack of genetic resistance amongst the host species and the ineffectiveness of chemical control of *Sclerotina* spp. has lead to a search for alternative means of control. One such means is being researched at the CRC for Tropical Plant Pathology (CRCTPP). Novel strategies for resistance are being developed using genes encoding antifungal proteins which are normally expressed in the seeds of a range of plants including some Australian native plants (CRCTPP 1997). The genes encoding these

proteins can be manipulated by linking them to specific promoters (gene switches) enabling the proteins to be expressed in tissue encountered by plant pathogens such as *Sclerotina*. Such gene constructs can then be transformed into *Sclerotinia* host species. However, it is imperative when testing such novel forms of resistance that the widest range of genotypes of the pathogen is assessed, otherwise plants considered resistant may fail when released into the field because of some unrecognised genetic variant of the pathogen.

The diversity of plant pathogen populations has traditionally been assessed by studying morphological features of the pathogen in culture and by testing the pathogenicity on a host species. However, since fungi such as *Sclerotina* spp. have very few features which can be assessed in culture and produce a narrow range of rapidly expressed symptoms in pathological tests, it is difficult to find sufficient characters for such analyses. Recent advances in molecular biology allow us to study fungal populations at the DNA level thereby identifying different genotypic groups that may exist. We can do this using molecular markers such as RAPDs (random amplified polymorphic DNA, Williams *et al.* 1990) and RFLPs (restriction fragment length polymorphisms). Both these systems allow us to screen the DNA of the fungal population and look for differences in resultant banding patterns which represent differences in the DNA sequences between individuals in the population. This type of analysis been used to study the populations of a wide range of plant pathogens (Braithwaite *et al.* 1991; Foster *et al.* 1993; Hayden *et al.* 1994)

The molecular analysis can be used to distinguish within and between fungal species. (i.e *S.sclerotiorum* from *S.minor*) and identify different genetic groups within a species. Once identified, representatives from different genetic groups can be used to screen putative resistant plants, ensuring a wide genetic range of the pathogen is being used in any screening tests.

Another important aspect with regard to control of *Sclerotinia* is to identify crop species which are not hosts to *Sclerotinia*. Such species could then be used in crop rotations, thus preventing further build up of *Sclerotinia* inoculum within a field.

Sclerotinia sclerotiorum is a pathogen of a range of both vegetable and grain crops. For this reason financial support was also sought and obtained from the GRDC to include analysis of *Sclerotinia* on grains. The GRDC funding finished in June 1996. Mr Merrick Ekins commenced working on this project during his honours year of study in 1993. He then continued to work on this project for his PhD studies and was awarded a GRDC Junior Research Fellowship for this purpose. The funding of this scholarship finished in June 1997 and since then Mr Ekins has obtained some financial support from a UQ Dept of Botany scholarship with consumables being provided by the Dr Aitken's Department of Botany research account. Mr Ekins is expected to submit his PhD thesis in April 1998. ***Consequently, certain aspects of this research are not yet complete and a supplement to this final report will be forwarded to the HRDC at the completion of Mr Ekins thesis.***

2. Materials and Methods

2.1. Variation between Sclerotinia spp.

2.1.1. Initial Studies

Since 1993 the following isolates of *Sclerotinia* spp. have been collected from various crops including capsicum, celery, lettuce, tomato, sunflower, peanut, canola, soybean and navy bean from different areas of Queensland, NSW and Victoria : 290 *Sclerotinia minor*; 572 *S.sclerotiorum* and 15 putatively *S.trifoliorum*.

For the initial studies a total of 27 *S.sclerotiorum* and 21 *S.minor* isolates collected from various crops in SE Queensland were subjected to RAPD analysis to determine whether this form of genetic analysis would be suitable for distinguishing between the two species and for detecting variation within the species.

RAPDs (Random Amplified Polymorphic DNA) This method, as developed by Williams *et al.* (1990), allows the amplification *in vitro* of small fragments of DNA using the polymerisation chain reaction (PCR). Oligonucleotide primers, generally 10 bases long, recognise corresponding sites on the DNA of the organism being tested and from these points the amplification of DNA is initiated. In the procedure of RAPDs, when two such sites are sufficiently close on the DNA chain (generally less than 2kb), the DNA between these two sites is amplified using the PCR process. This fragment is then amplified exponentially by applying multiple cycles of rapid temperature changes (from 37-95C) allowing denaturation, annealing and amplification of the DNA to proceed. Anything up to 40 different fragments can be amplified using one primer. The benefit of RAPDs compared with some other molecular marker systems is that no prior knowledge is required of the genome of the organism being tested.

The amplified bands are visualised by size fractionating in an agarose gel to which an electric current is applied. The smaller the fragments the further they move through the gel. The gels are then stained with ethidium bromide which binds to the DNA and fluoresces under UV light. Figure 1 shows such a stained agarose gel.

Variations occur between individuals when specific primer sites differ, either by not being present or by the distance between the sites being too large to allow *in vitro* amplification of DNA. This is noted by the absence of a band or the addition of band on the agarose gels. These variations are called **polymorphisms**. The more polymorphisms that occur between any two individuals the more distantly related their DNA and consequently the more distantly related the individuals.

Each isolate was grown in liquid culture, mycelia was removed after 4 days and freeze dried. Using a modification of the method of Yoon *et al.* (1991) the DNA was extracted and subject to RAPD analysis. A total of eight 10-base oligonucleotide primers were used. The primers were supplied by Operon. The PCR products were run on an agarose gel, stained with ethidium bromide and photographed under UV light. For each isolate the presence or absence of amplified DNA bands using all primers was assessed. The data were analysed using the SAS program, which on the basis of dissimilarity clustered the isolates into groups.

2.1.2. Distinguishing Different *Sclerotinia* species

S.sclerotiorum and *S.minor* are widespread in Australia. However, the distribution of *S.trifoliorum* is less understood if indeed it is present at all. *Sclerotinia* sp. has been identified on clover within Australia but it can not be assumed that this is *S.trifoliorum* as *S.sclerotiorum* has also been recorded on clover. It is possible to distinguish the different *Sclerotinia* species using an RFLP probe developed in the lab of Bob Metzenberg in N.America (Free *et al.* 1979).

RFLP (restriction fragment length polymorphism). In this process the DNA of an organism is digested with restriction enzymes. These enzymes recognise a particular sequence of DNA (4-6 bases long) along the chain of the DNA molecule and cut the chain at these points. The DNA is then fractionated up into numerous fragments of varying lengths. When run through an agarose gel there are so many of these fragments of different lengths that it just appears as a smear. However, it is possible to detect particular fragments by using a probe which recognises and binds to certain DNA sequences. By labelling the probe with radioactivity, it is possible to detect where that fragment occurs in a size fractionated gel. Differences in the size of fragment recognised by the probe can occur when the distances between restriction enzyme sites differ. When two individuals are distantly related the fragments recognised by the probe are more likely to differ in length, due to the chances of mutations having occurred between or at the restriction enzyme sites. Whereas closely related individuals would be expected to have more fragment lengths in common. These differences are also called polymorphisms as they refer to differences in the DNA, similar to that which occurs with RAPDs.

The DNA of isolates of known *S.sclerotiorum*, *S.minor* and of 15 isolates of *Sclerotinia* sp. collected from clover will be assessed using the RFLP probe. DNA of the isolates will be extracted as above, digested with restriction enzymes, subject to Southern analysis using the radioactively labelled DNA fragment specific for *Sclerotinia* sp. Consequently, we can determine whether or not *S.trifoliorum* is found in Australia.

Southern analysis is the procedure by which the restriction enzyme digested DNA which has been run on a agarose gel is transferred to a nylon membrane and then radioactively probed. An X-ray photograph shows the area on the membrane where the radioactively labelled DNA probe has detected the complementary DNA sequence.

2.2. *Geographical and Temporal Variation in S.sclerotiorum* populations

In identifying different genotypes for use in resistance breeding programs it is essential to determine how variable *S.sclerotiorum* populations are and if that variation is changing with time. Without taking this into account we may find that putative resistant cultivars fail due to the development in the pathogen population of variants which can overcome the resistance. By increasing our understanding of the pathogen population and by ensuring that any new cultivar shows resistance to as wide a range of genetic variants within the pathogen population, we can increase the chances of obtaining cultivars which possess durable resistance over time and location.

For this purpose, hierarchal samples of *S.sclerotiorum* isolates were collected from: within a plant; within a field and within a locality and over a wide geographic range for the same crops species and over several years.

It was intended that a vegetable crop would be sampled. However, the lack of rain during the 1993/94 and 1994/95 growing seasons prevented widespread development of *S.sclerotiorum,* the consequence being that we were unable to sample to the level required from any vegetable species. The sampling has proceeded from sunflower only. Trends within a sunflower crop are expected to directly relate to trends within any other *Sclerotinia* susceptible crop.

Variations in plant pathogen populations have traditionally been assessed using morphological characters, however there are only a few actual characters that can be analysed such as spore shape, size etc. In some fungal species incompatibility systems exist within the species which has the effect of dividing the population into sub-species groupings. The number and frequency of these sub-species groupings gives us an indication of the level of genetic diversity within the population. More recently the use of molecular techniques have been used to look at the level of genetic variation within and between species.

In the following experiments variation between isolates has been assessed using molecular genetic analysis (RAPDs, RFLP-multicopy probe, RFLP-single copy probes) and /or by mycelial compatibility groupings (MCGs).

RFLP-multicopy probes recognise several restriction enzyme digested fragments of DNA at the same time. It is likely that the DNA being recognised by the probe is from an area of repeated DNA within the genome which has been fractionated into different lengths by the restriction enzyme. The resultant pattern visualised by the X-ray film can give a "fingerprint" for a particular genetic variant of the organism.

RFLP-single copy probes only recognise a single site on the DNA and as a consequence are more likely to be within a transcribed region of the DNA, i.e. a region which actually encodes a gene. Providing that the restriction enzyme digests the DNA outside the probe recognition site, only one fragment (in a haploid organism such as *Sclerotinia*) is apparent. By using several single copy RFLP probes and assessing for polymorphisms using these probes, the level of relatedness between isolates can be established i.e. distantly related individuals would show more polymorphisms between each other than closely related organisms.

MCGs-mycelial compatibility groupings. Within certain fungal species barriers exist which prevent the fungal hyphae of one genetic variant intermingling and possibly fusing with another genetic variant. These systems are often only under the control of one gene and one theory about their existence is that they prevent the spread of mycoviruses within the whole fungal population (Caten 1981). Consequently, such fungal species are effectively divided into sub-species between which there is no genetic exchange. Such a system exists in *Sclerotinia sclerotiorum*. Determining whether two isolates are from the same compatibility group or not, is quite simple. The two isolates are sub-cultured onto opposite sides of a Petri dish containing nutrient media with red food colouring. When the two colonies meet in the middle they can repel each other which results in an accumulation of the red food colouring, visualised as a strong red band, if this is the case they are said to be **incompatible**. If they are **compatible**, the hyphae intermingle with each other and there is no accumulation of the red food colouring at the colony fronts. It is thought that the red food colouring creates a band in the incompatible interaction due to breakage of the hyphal tips where the colonies meet.

For use in RFLPs and RAPDs, DNA has been extracted from freeze dried mycelium using a method derived from Zolan and Pukkila (1986).

2.2.1. Variation within a Location

S.sclerotiorum isolates were collected from two sunflower fields in S.E. Queensland in June 1994. Isolates were collected from a field operated by Pacific Seeds at Gatton and a field operated by Pioneer Seeds at Wyreema near Toowoomba. From each field, sclerotia were randomly collected from ten separate sunflower plants for head rots and ten separate plants for basal stem rots, with three sclerotia being collected from each plant. This gave a total of 120 isolates.

On collecting *S.sclerotiorum*, isolates were axenically cultured, stored in liquid nitrogen and under water.

In order to establish the MCGs, each of the 120 isolates were paired with each other at least twice (a total of >14,460 pairings). Based on the method of Kohn *et al.* (1990), all isolates were paired on modified Patterson's media (MPM) which included six drops of red food colouring (McCormick Foods, Australia Pty. Ltd) per litre of media and 1.5% agar. Each isolate tested was initially grown up on MPM for 7 days before 4mm mycelial plugs were taken from the margin and placed 5cm apart on a 9cm Petri dish of MPM. The plates were assessed after 10 days for the presence or absence of interaction zones (red bands).

RAPD analysis was conducted as described before (section 2.1.1). Eighteen primers were used all which were supplied by Operon. Data were analysed using POPGENE (Francis Yeh, University of Alberta, Canada) and GenALEX (Rod Peakall, ANU, Canberra).

RFLP analysis was conducted using six multicopy probes (of which one was supplied by Linda Kohn, University of Toronto) and 11 single copy probes. For each isolate the DNA was extracted as described before and digested with *Bam*HI and *Hind*III. The digested DNA was run on a 0.8% agarose gel along with a DNA ladder of known fragment lengths. The gel was acid treated to denature the DNA and then Southern blotted onto a nylon membrane after which the membrane was hybridised with the radioactively (P^{32}) labelled probe and placed against an X-ray film for 1-7days. For each isolate the size of each of the hybridising bands was assessed.

For the single copy probes a genomic library of *S.sclerotiorum* was established. Genomic DNA was partially digested with *Sau*3AI and subcloned. The library was screened for inserts which gave single bands when hybridised onto restriction enzyme digested DNA of sample isolates of *S.sclerotiorum*. (see Appendix 1 for details of cloning method).

2.2.2. Temporal variation

By repeating the hierarchal sampling at a specific location over three seasons it was possible to determine if the pathogen population was changing over time. For this purpose it was necessary to choose a crop which would be grown in the same location

in consecutive years and over several localities. The hierarchal sample of *S.sclerotiorum* isolates collected from two sunflower fields in S.E. Queensland in June 1994 (see section 2.2.1) was repeated in June 1995 and again in 1996.

Single and multicopy RFLP probes were prepared and used to assess the sunflower isolates collected over the three year period. A total of 11 single copy probes were and six multicopy probes were used as in section 2.2.1. above.

2.2.3. Variation within Australia

Samples have been collected over a wider geographical area to determine the genetic variation of *S.sclerotiorum* in Australia. Late rains during the late summer of 95/96 promoted development of *Sclerotinia* spp. making possible sampling from sunflower crops from localities throughout eastern Australia during April and May 1996.

One hundred and seventy isolates were hierarchically sampled from Queensland and New South Wales. Areas surveyed include: Gatton, Toowoomba, Oakey, Pittsworth, Allora, Warwick, Goondiwindi, Boggabilla, Yallori, Moore, Croppa Creek, Gurley, Edgeroi, Narrabri, Gunnedah, Tambar Springs, Premer, Spring Ridge, Quirindi, Breeza and Tamworth. Areas further south including Southern NSW and Victoria contained smaller sunflower crops and rare occurrences of *S.sclerotiorum*. These isolates have also been axenically cultured, stored in liquid nitrogen and under water. The mycelium was grown up in liquid culture, extracted and freeze dried. DNA was extracted as before.

These isolates were analysed using the 11 single copy probes and six multicopy probes as in sections 2.2.1. and 2.2.2.

2.2.4. Comparisons between Australian Populations and Overseas Populations

Comparisons have also been made between isolates collected in Australia with those from Canada. For this purpose Merrick Ekins travelled to the University of Toronto in Canada and conducted RFLP multicopy probe analysis on a sample of 120 Australian isolates representing the 20 distinct genotypes he took with him and compared the results with analysis already collated from Canadian isolates.

2.3. Pathogenicity tests

2.3.1. Assess for Host Specificity

Comparisons were made in the pathogenicity of *S.minor* and *S.sclerotiorum* isolates originating from different host species. A total of 18 *S.minor* isolates (five from sunflower, ten from peanut, one from tomato, one from globe artichoke and one from Bathurst burr) and 24 *S.sclerotiorum* isolates (12 from sunflower, two from canola, one from sowthistle, one from cotton, three from peanut, one from cauliflower, one

from lettuce, one from celery and two from tomato) were compared by inoculating sunflower and peanut plants.

2.3.1.1.Sunflower Inoculations

Helianthus annus (sunflower) seedlings (cv. Hysun 33, Pacific Seeds) were grown in a glasshouse at temperatures of 20-35C. Inoculation was conducted at 8 weeks post emergence at the closed flower bud stage.

Each isolate was initially grown on V8 agar at 20C for 7 days, then a 6mm mycelial block was transferred to 20g shelled oats which had previously been autoclaved in 15ml distilled water in a 150ml flask. Each of these cultures were grown for 7 days at 20C and then used as inoculum by placing 2g of colonised oats in the soil of the sunflower stem, without wounding the plant. The plants were transferred to a controlled cabinet set at 15-21C and the resultant lesions were measured daily. Inoculation with sterile oats was used as controls.

2.3.1.2.Peanut Inoculations

Pathogenicity tests on peanut were conducted using detached stems (Brenneman *et al.* 1989). The cultivar Virginia bunch was grown in a glasshouse at 20-35C. At eight weeks after emergence, 14cm stem sections were excised from the plants and all leaves except the primordial leaves were removed. The stems were supported by foam plugs in 14cm test tubes containing 5ml nutrient solution (Dhingra and Sinclair 1985).

Each isolate was initially grown on V8 agar, as above, then subcultured onto another V8 agar plate. After 2 days a 4mm mycelial plug was taken from the margin of the actively growing culture and placed onto the middle of the stem in the axil between a petiole and the stem. No wounding was involved. The stems were transferred to a temperature controlled cabinet set at 15-21C. The resultant lesions were measured daily. Sterile agar was used for control inoculations.

2.3.2. Assess for Differences in Isolate Pathogenicity

The sunflower hybrid Hysun 36 (Pacific Seeds) used is the product of crossing two highly inbred parents. This homogenity was required to prevent any physiological differences amongst the plants which may mask differences between the isolates. The sunflowers were inoculated four weeks after sowing. The 120 isolates of *S.sclerotiorum* collected from the diseased sunflower heads and basal stem rots in two locations Gatton, Queensland and Wyreema, Queensland (see section 2.2.1) were used. The sclerotia were removed from sunflower tissue, immersed in 4% Bleach and 70% ethanol for 2 minutes before being rinsed in sterile distilled water and blotted dry and then plated out onto V8 agar plates and advancing mycelia were subcultured and subsequently hyphal tipped. The isolates were stored under liquid nitrogen as soon as possible to maintain virulence for the virulence testing. The isolates were restored from liquid nitrogen onto V8 plates and freshly subcultured from the growing edge of

the colony 2 days before inoculation. For inoculation 3 mm mycelial plugs were cut from the growing edge of the colony and these were placed against the stem of the sunflower plant equidistant between the internodes, with the mycelium facing the stem. The mycelial plug and adjacent stem were wrapped in parafilm to preserve moisture at the inoculation site. The lesions were then measured daily from 48 hours post inoculation. These inoculation experiments were conducted twice.

2.4. Variation within a Sclerotium

A sclerotium is made up of a mass of dense mycelia surrounded by a hard melanised rind, which allows it to survive dormant over long periods. It is generally assumed that a single sclerotium is derived from one genotype but this has not been tested. In order to verify this, five separate cultures were taken from each of three different sclerotia and their DNA subject to molecular analysis using five multicopy RFLP probes and 11 single copy probes. Comparisons were made with the level of variation obtained in the geographic and temporal studies (see section 2.2.).

2.5. Mating Systems of S.minor and S.sclerotiorum

Fungal Mating Systems In the same way that plant species can have breeding systems that promote either outbreeding or inbreeding, so do fungi. Fungi can be **heterothallic**, where an opposite mating type (which equates roughly to an opposite sex) has to be present in order for sexual reproduction to occur. Whereas fungi which are **homothallic** are self-fertile, i.e. do not require the presence of another mating type. Those which are heterothallic clearly promote outbreeding and such populations are generally more varied as genetic recombination is frequently occurring.

Previous overseas studies have indicated that *S.sclerotiorum* is homothallic, whereas no work has been reported on the genetic mating system for *S.minor*. In understanding the level of variability in a fungal population and determining its propensity to overcome new resistance, it is important to know whether that population is undergoing outbreeding or inbreeding processes as the former will promote better means of overcoming new resistances.

For both species the eight ascospores within a single ascus were isolated and cultured. Each isolate was then paired on nutrient media on a Petri dish with each of the other isolates from the same ascus. After four months these plates were examined for the presence of new ascocarps (sexual fruiting bodies). If ascocarps were present in all combinations, then the fungus would be homothallic (i.e all pairings were sexually fertile). If however, ascocarps only developed in half the combinations then the fungus would be heterothallic (outbreeding).

The ascocarps of *Sclerotinia* species are known as apothecia, for these to develop in culture it is necessary to subject the cultures to specific light, humidity and temperature regimes. Apothecia were raised from *S.sclerotiorum* and *S.minor* using the method outlined by Hawthorne (1973). Sclerotia were air-dried for 2-3 days after four weeks growth on wholemeal agar. The sclerotia were transferred to deep Petri dishes (96mm x 25 mm) with 10 ml of sterile water and incubated for 6-8 weeks in the dark at 15 C for 8 hrs and 10 C for 16 hrs. When stipes were formed the Petri dishes were

transferred to another incubator and the fruit bodies were matured by illumination for 8 hrs/day at 15C -18C for 14-21 days under daylight fluorescent tubes.

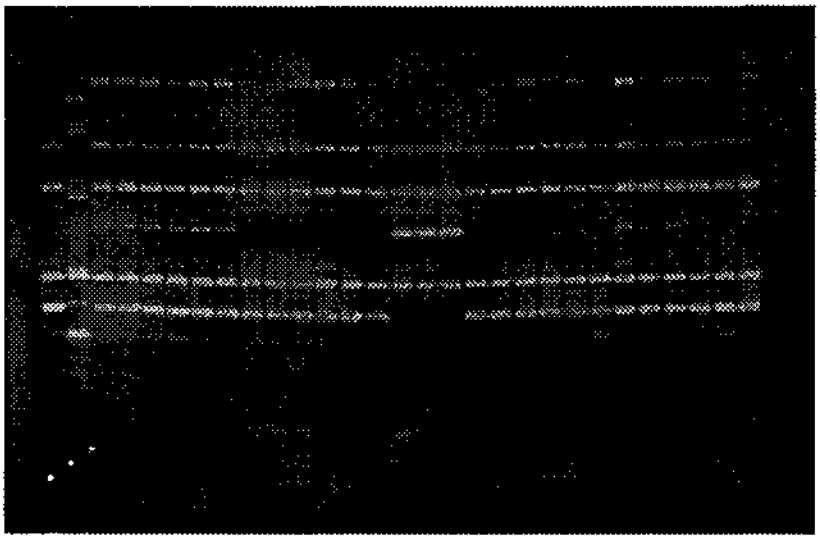
Within each ascus, the four pairs of ascospores (tetrads) were separated by releasing the ascospores by slicing off the tip and stem of the ascus, using a Leica Leitz DMIL micromanipulator (Leica instruments Pty., Ltd. Queensland, Australia). The single spores were grown into individual cultures and the above procedure was repeated until apothecia were again raised.

3. Results

3.1. Variation between Sclerotinia spp.

3.1.1. Initial Studies

Initial genetic analyses using the molecular marker system of RAPDs showed that there was a large variation in amplified bands between the two species *S.sclerotiorum* and *S.minor* indicating a genetic dissimilarity of 90% between the two species. Figure 1 shows a photograph of a RAPD gel showing the different banding pattern between the two species. Analyses within species indicated that variation was greater among the *S.sclerotiorum* isolates (with 5.5% level of dissimilarity) than among the *S.minor* isolates (with 2.5% level of dissimilarity). It was thought that the greater level of variation evident among the *S.sclerotiorum* isolates, compared with the *S.minor* isolates reflected the fact that *S.sclerotiorum* infections arise from both ascospores (sexual spores) and from direct germination of the sclerotia (vegetative resting structure) whereas *S.minor* infections only appear to arise from direct germination of the sclerotia. It would be expected that the continual involvement of the sexual spore stage in the disease cycle would lead to a high level of genetic diversity, which our initial results show.



An RAPD gel showing polymorphisms (differences in banding patterns) between isolates of *Sclerotinia sclerotiorum*.

3.1.2. Distinguishing Different *Sclerotinia* species

Results of the tests on the putative *S.trifoliorum* isolates will be discussed in the supplemental report.

3.2. *Geographical and Temporal Variation in* S.sclerotiorum populations

3.2.1. Variation within a Location

The isolates collected in the hierarchal sample have been tested for genetic similarity using RFLPs, mycelial compatibility groups (MCGs) and RAPDs. From a total of 120 samples collected from the two fields of sunflower at Gatton and Wyreema, a total of 20 distinct genotypes of *S.sclerotiorum* were identified. Isolates collected from the same plant tended to be of the same genotype.

The 120 isolates of *S.sclerotiorum* collected from sunflower were divided into similar genotypic groupings by the three methods used in this study. Figure 2 shows a comparison of groupings of different isolates using the three methods. Each of these methods have their advantages and disadvantages. RFLP analysis using the single multicopy probe divided the isolates into 20 groups. RAPDs using 10 primers divided the isolates into 17 groups. On the basis of 14,460 separate interactions, MCGs divided the 120 isolates into a total of 13 groups. In terms of the information obtained, all give similar results, with both molecular methods screening larger numbers of isolates in a shorter amount of time. However, RFLPs could identify slightly more genotypes than MCGs. Consequently RFLP analysis should be used in preference when assessing overall genetic variation.

All three methods identified some identical genotypes from both head and basal stem rots. This indicated that genotypes were not specialised for forming apothecia and subsequent head rot as opposed to basal stem rot. Several genotypes were also identified from both Gatton and Wyreema, indicating that genotypes are widely dispersed. Some genotypes were also discovered to be more common than others. The genotypes have the potential to disperse over large distances. Dispersal may be by airborne ascospores, or mechanical movement of sclerotia. How far dispersal of genotypes has occurred around Australia is not known.

3.2.2. Temporal variation

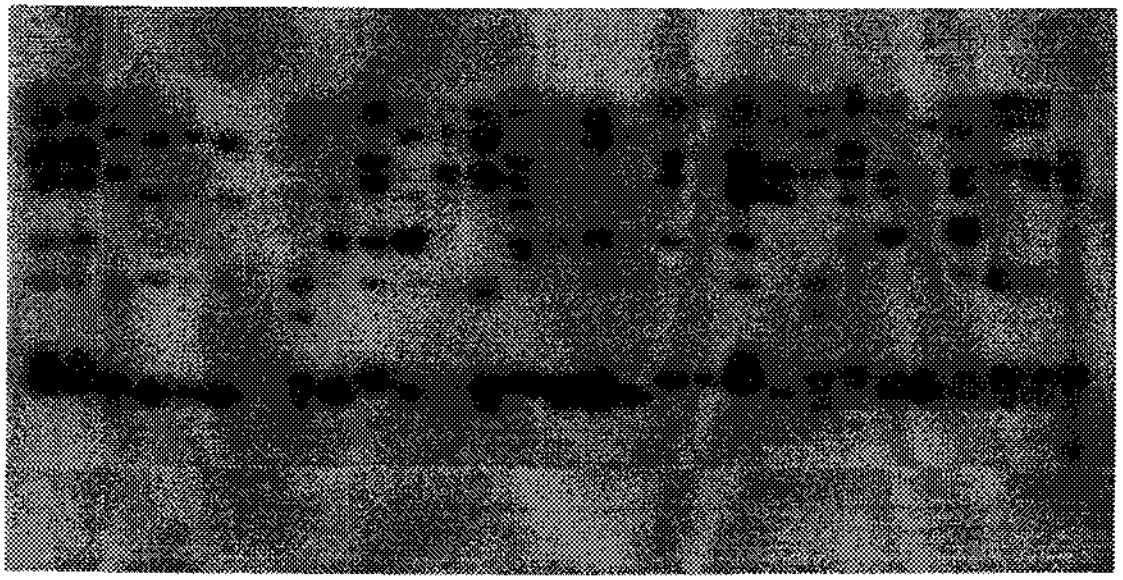
This will be reported in the supplementary report in 1998.

3.2.3. Variation within Australia

The molecular analysis of this collection of isolates will be reported on in the supplemental report in 1998.

3.2.4. Comparisons between Australian Populations and Overseas Populations

Southern blotting of the 120 Australian isolates in Canada revealed 20 different clones. Each clone is regarded as a separate genetically distinct identity or genotype. The 20 Australian clones were compared to 659 clones previously identified from Canadian collections of *Sclerotinia sclerotiorum* isolated from canola and sunflower by Yatika Kohli (eg Kohli *et al.* 1992, Kohli *et al.* 1995). None of the 20 Australian clones were identical to Canadian clones. Most of the Australian clones clustered together eg clones: 2, 3, 4, 5, 6, 7, 8, 11, 13, 14, 20. The remaining clones were scattered throughout the Canadian clones although not closely related to any of them, but not as distantly related as to each other. Most of the clones had a small fragment, known as 12.1 of the 53 *Bam*H1 fragments using this probe. Clone number 17 containing isolates 1333-1, 1333-2 and 1333-3 was the only Australian clone lacking the 12.1 fragment. This 12.1 fragment had only previously been obtained on the West Coast of Canada by Y. Kohli and has not identified in the other provinces of Canada.



An RFLP autoradiograph separating genotypes of *Sclerotinia sclerotiorum* with a multi-copy probe.

In summary, comparisons between these 20 Australian clones and those of 659 clones previously identified in Canada showed that the Australian clones were distinct. The genetically nearest Canadian isolates to the Australian isolates were those collected in western Canada.

3.3. Pathogenicity tests

3.3.1. Assess for Host Specificity

3.3.1.1.Sunflower Inoculations

Symptoms produced by all 42 isolates were identical. Lesions developed along the stem, usually girdling the stem within three days at which time the plants wilted. Some of the isolates did differ significantly in the linear lesion development rate from others, but there was no correlation between either isolate host origin or location origin with regard to rate of lesion extension. This indicates a lack of host specificity amongst the isolates tested.

3.3.1.2.Peanut Inoculations

There were significant differences between some of the isolates with regard to the rate of lesion development on the peanut stems. However, there was no apparent correlation between either location or host origin of the isolates. Thus again indicating a lack of host specificity for the isolates of either *S.sclerotiorum* or *S.minor*.

3.3.2. Assess for differences in isolate pathogenicity

Pathogenicity testing did reveal differences in the aggressiveness of different isolates to infect sunflowers. The significant differences between isolates indicated that the pathogenicity testing method would be good for virulence testing and assessment of lesion length after two days is more reliable than after three days or the linear rate of lesion development. Significant differences found between the isolates could not be directly correlated with other genotypic groupings (ie MCGs, RAPDs and RFLPs). This however does not rule out any correlation between virulence and genotype as this may be indicated with another pathogenicity test such as ascospore inoculation. Pathogenicity testing revealed isolates collected from both head and basal stem rots were equally capable of causing stem infections so no correlation between mode of reproduction and aggressiveness exists.

3.4. Variation within a Sclerotium

Ten sclerotia, were hyphal tipped to produce four isolates per sclerotia, and a total of forty isolates. From each set of four isolates derived from a single sclerotia all four had identical genotypes, ie no variation within a sclerotium. From a total of ten sclerotia and thus ten different plants there were five different genotypes present. Indicating that sclerotia are derived from mycelium resulting from the same single infection unit, whether it be originally from a basal stem rot or an ascospore derived head rot.

3.5. Genetic Systems of S.minor and S.sclerotiorum

Asci were harvested from isolates of *S.sclerotiorum* and *S.minor* were induced to produce apothecia. From the eight excised ascospores from a single ascus , eight cultures resulted which were induced to produce apothecia. The formation of eight sibling apothecial bearing cultures indicates the fungus is self fertile and thus homothallic. This was confirmed for both species. A strictly heterothallic fungus would be unable to produce any sibling apothecia without cross fertilisation from spermatia. Another possibility is that of bipolar heterothallism as illustrated by *S. trifoliorum* in which case four daughter cultures would produce apothecia, whilst the remaining four would not, as half (four) of the ascospores are sterile and the remaining four are self fertile (Uhm and Fuji 1983). Results indicate that *S.sclerotiorum* and *S.minor* are both homothallic. Therefore inbreeding is the normal means of sexual reproduction within these species within Australia.

4. Discussion

4.1. Extension / Adoption by Industry

S.sclerotiorum and *S.minor* are species which are capable of infecting a wide range of vegetable and grain crops. Molecular analysis indicates that these two species are quite distinct, with hybridisation very unlikely between the species. Within these two species the populations are divided into sub-species groupings or clones. Each clonal group is effectively genetically isolated due to mycelial incompatibility which prevents intermingling of mycelial of different isolates and due to the homothallic sexual system which favours inbreeding. Our studies, which were focused mainly on *S.sclerotiorum*, show that each of these clonal groups are widespread geographically and that certain clones seem to predominate. Pathogenicity tests indicated that certain isolates may be more aggressive than others but that no host specificity existed.

What this means to the industry is that *S.sclerotiorum* is a genetically diverse and highly pathogenic fungus with a wide host range. In developing resistant cultivars care should be taken to assess a wide sample of isolates representing the different clonal groups which we have identified in order to secure the durability of resistance.

The clonal groups we have found in Australia are quite distinct from those found in Canada. This emphasises the need for continued care in quarantine to prevent the introduction of any new genetic variants into Australia. The widespread nature of the different genotypes we already have present in Australia does indicate that this pathogen has in the past been easily distributed throughout the cropping areas and any new introduction could also spread as equally easily.

The genetic isolation which occurs within *Sclerotinia* populations due to the vegetative incompatibility systems, may reduce spread among the whole population of genes for: fungicide resistance, tolerance to anti-fungal peptides and those that can overcome host resistance. If mutations giving rise to such genes occur in less aggressive strains then the impact will be lessened especially if biocidal sensitive aggressive strains are

removed from the population. Of course the opposite is true; biocidal tolerant aggressive clones may quickly dominate but hopefully they will have some other loss of fitness.

The lack of host specificity of the different isolates does mean that the only non-host crops should be used in crop rotations.

The verification of the presence or absence of *S.trifoliorum* in Australia and the quarantine implications will be reported on in the supplemental report.

4.2. Directions for Future Research

We have identified several clones of *S.sclerotiorum* in our analyses. All these clones are widespread in the crop growing areas of eastern Australia. Representatives of each of these clones will be used in screening putative resistant cultivars which are products of either conventional breeding programmes or those which are products of genetic manipulation such as the anti-fungal peptide project being conducted at the CRC for Tropical Plant Pathology.

4.3. Financial / Commercial Benefits

The financial and commercial benefits of this project will come when cultivars have been developed with resistance that has a great chance of being durable because it has been tested against the different genotypes identified in this project. The current cost of Sclerotinia rots to the industry is immeasurable, as not only are yields reduced or crops fail as a consequence of severe outbreaks, often alternative less lucrative crops have to be grown when the land is infested with sclerotia of *Sclerotinia* spp. The production of durable resistant cultivars of susceptible species would give the industry the option of continuing to grow host species in infested soil.

5. Appendix I

The production of single copy probes

A partial digestion pilot experiment was carried out by digesting DNA from *S.sclerotiorum* (isolate UQ 2554) at varying enzyme/DNA ratios ranging from 1 unit of *Sau*3AI (New England Biolabs, Genesearch Pty., Ltd, Queensland, Australia) to 1/256 of a unit of *Sau*3A per mg DNA. A ratio of 1/8 of a unit *Sau*3A per mg fungal DNA was selected and the proper digest was performed. The digest consisted of 50 ml of UQ 2573 DNA (at 250 ng/ml), 30 ml 10X buffer, 30 ml 10X BSA, 190 ml of 10 mM TrisCl and 0.195 ml *Sau*3AI, at 37 C for 1 hour and then a 20 min stop reaction at 65 C. The resulting digested DNA was electrophoresed on a 0.3% agarose gel at 25 Volts for 24 hours at 4 C. (see Gel 1 p239 book 4). The bands between 3kb and 0.5 kb were excised from the gel and the DNA was extracted from the agarose using QIAquick Gel Extraction Kit (Qiagen Pty., Ltd. Victoria, Australia).

Cloning of the DNA was carried out using a *Bam* H I pre-digested and CIAP treated Lambda Zap express vector (Stratagene, Integrated Sciences, New South Wales, Australia). Pilot test ligations were carried out varying the ratio of insert/vector from 5:1 to 0.2:1. The DNA was ligated with the following reaction: 1 ml Zap express vector (1 mg/ml), 1 ml DNA (100ng/ml), 0.5 ml 10X ligase buffer, 2 ml H20 and 0.5 ml T4 ligase. All 5 ml of the substrate DNA was subsequently added to MaxPlax lambda packaging extract (Epicentre Technologies, Astral Scientific, New South Wales, Australia), incubated at room temperature for two hours. The packaged lambda vector was then diluted from 10-1 to 10-6. A dilution of 10-1 was found to give a titre of 1.46 x 105 pfu/ml giving a recombination efficiency of 90% to 98%.

The packaged library was mixed with XL1-Blue MRF strain of *E.coli* and incubated for 15 minutes at 37C and then plated onto large NZY plates (15 cm). Some of the plaques were transferred to nylon membranes in an attempt to differentiate plaques containing high and low copy DNA inserts. The large NZY plates (15 cm) containing the plaques were overlaid with nylon membranes (Hybond-N+ Amersham Australia Pty., Ltd, Australia) for 2 minutes. The membranes were denatured in 1.5M NaCl and 0.5M NaOH for 2 minutes, followed by neutralisation in 1.5M NaCl and 0.5 M Tris-HCl (pH 8.0) for five minutes. The membranes were then rinsed for thirty seconds in 0.2 M Tris-HCl (pH 7.5) and 2 X SSC, then blotted and UV crosslinked. The nylon disks were hybridised 25 ng of genomic DNA using the same protocol as for hybridisations carried out in the detection of single copy probes.

The remainder of the plaques not transferred to membranes were used for amplification of the lambda library. The large NZY plates were overlaid with 10 ml of SM buffer and gently rocked overnight, allowing the phage to diffuse out of the agarose. The recovered bacteriophage suspension was centrifuged for 10 minutes at 500 X g with 5% chloroform and the resulting supernatant was stored at -70 C with 7% DMSO (dimethylsulfoxide).

For mass excision of the plasmids, the library was added to XL1-Blue MRF strain of *E.coli* at a ratio of 1:10 lambda phage to bacterial cells, ie 10 ml of 1.46 E105 pfu/ml to 100 ml bacterial cells giving 8 E107 cells. To this 1.48 ml of Exassist helper phage (Stratagene, Integrated Sciences, New South Wales, Australia) was added to give a 1:1 Phage to cell ratio. This was then incubated for 15 minutes at 37C before addition of NZY broth and a three hour incubation at 37C, followed by 20 minutes at 65C. The solution was centrifuged for 10 minutes at 1000 X g and 1 ml of the decanted supernatant was added to 200 ml of XLOLR strain of *E.coli* cells and incubated at 37 C for 15 minutes, to which 40 ml of 5 X NZY was added and incubated at 37 C for 45 minutes and then plated onto LB kanamycin agar plates (50 mg/ml) with IPTG and Xgal. White recombinant colonies were picked off and used for plasmid minipreps. Blue non-recombinant colonies made up only 46% of the total number of colonies. The single colonies were added to 2 ml of LB broth with kanamycin and incubated overnight at 37 C. The pellet was dried and plasmid DNA extraction was carried out by the alkaline lysis method as in Sambrook *et al.* (1989).

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7. Investigators and Acknowledgments

7.1. Investigators

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7.2. Acknowledgments

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HRDC Technical Summary
Molecular Identification of Strains of *Sclerotinia* spp.
VG318 (1993-95) continued as VG511 (1995-97)

A supplemental report will follow the completion of Mr Merrick Ekins PhD thesisin April 1998

Sclerotinia rots are caused by one of two fungal species: *Sclerotinia sclerotiorum* and *S.minor*. *Sclerotinia* spp. are amongst of the most intractable groups of plant pathogens to control, as genetic resistance is not available in host crop species and chemical control is extremely expensive and generally ineffective.

Sclerotinia spp. produce sclerotia which frequently remain viable in the soil for up to 20 years allowing the disease to be carried over from one year to the next. The sclerotia produced by *S.sclerotiorum* are generally larger that those produced by *S.minor*, however environmental conditions can influence the size of the sclerotia sometimes making it difficult to tell the species apart. Both species are ascomycete fungi with the ascocarps forming on the sclerotia.

The lack of genetic resistance amongst the host species and the ineffectiveness of chemical control of *Sclerotina* spp. has lead to a search for alternative means of control. At the CRC for Tropical Plant Pathology novel strategies for resistance are being developed using antifungal proteins. Current work is underway to manipulate these genes, enabling their expression in susceptible host species. However, it is imperative when testing such novel forms of resistance that the widest range of genotypes of the pathogen is assessed, otherwise plants considered resistant may fail when released into the field because of some unrecognised genetic variants of the pathogen.

We have compared isolates of *Sclerotinia* species collected from a range of crops and locations throughout eastern Australia. This research will establish the extent of genetic variation between and within species of *Sclerotinia* and allow correct selection of isolates for pathogenicity testing in the development of *Sclerotinia* resistant plant varieties. Both molecular techniques (RAPDs and RFLPs) and mycelial compatibility grouping (MCGs) have been used in these studies. Once identified representatives from different genetic groups can be used to screen putative resistant plants, ensuring a wide genetic range of the pathogen is being used in any screening tests.

- We have established that *S.sclerotiorum* and *S.minor* are easily distinguished using molecular analysis such as RAPDs, illustrating that they are two quite distinct species. We have also demonstrated that the three different methods we used to analysis genetic diversity (MCGs, RAPDs and RFLPs) were suitable methods to obtain genotypic information about a fungal population.

  S.sclerotiorum has been shown to exist within a single field several distinct genotypes. Although a perfect species, *S.sclerotiorum* is forced to exist as a "clonal" organism because of the mycelial compatibility groups, consequently there is little or no genetic exchange between groups. For this reason the Australian isolates could have been monomorphic having derived from only a single or very few introductions. However, the initial analysis has shown that at least 20 genotypes were present within a field suggesting that either sexual exchange is occurring and/or several introductions of the fungus have occurred in the past.

- We also found that the same genotype can be found at different geographical locations and that is studies so far the Australian population appears to be quite distinct from isolates in a previous study carried out in Canada.

- The research outcomes from this project will feed into other projects where the aim is to utilise resistant germplasm for use in the field. Consequently, the transfer of results to the growers will be indirect via the breeders and other researchers. Such interaction is already in place through the CRCTPP.

HRDC Industry Summary

Molecular Identification of Strains of *Sclerotinia* spp. VG318 (1993-95) continued as VG511 (1995-97)

A supplement to this report will follow at the completion of Mr Merrick Ekins PhD thesis which is expected to be submitted in April 1998

Sclerotinia rots are caused by one of two fungal species: *Sclerotinia sclerotiorum* and *S.minor*. *Sclerotinia* represents an economically important group of plant pathogens, causing severe losses to many crop species including: tomato; capsicum; carrot; celery; lettuce; brassica; bean; pea; soybean; sunflower; peanut and canola. *Sclerotinia* spp. are amongst of the most intractable groups of plant pathogens to control, as genetic resistance is not available in host crop species and chemical control is extremely expensive and generally ineffective.

The lack of genetic resistance amongst the host species and the ineffectiveness of chemical control of *Sclerotina* spp. has lead to a search for alternative means of control. At the CRC for Tropical Plant Pathology novel strategies for resistance are being developed using antifungal proteins which are normally expressed in the seeds of a range of certain plants including Australian natives such as macadamia. Current work is underway to manipulate these genes, enabling their expression in susceptible host species. However, it is imperative when testing such novel forms of resistance that the widest range of genotypes of the pathogen is assessed, otherwise plants considered resistant may fail when released into the field because of some unknown genetic variant of the pathogen.

Molecular techniques have been used to compare isolates of *Sclerotinia* species collected from a range of crops. This research will establish the extent of genetic variation between and within species of *Sclerotinia*. A knowledge of the differences between *Sclerotinia* from different regions and crop species will allow correct selection of isolates for pathogenicity testing in the development of *Sclerotinia* resistant plant varieties.

Results

- We have established that *S.sclerotiorum* and *S.minor* are easily distinguished using molecular analysis, illustrating that they are two quite distinct species.

- *S.sclerotiorum* has been shown to exist within a single field as several distinct genetically isolated genotypes suggesting that either sexual exchange is occurring and/or several introductions of the fungus have occurred in the past.

- We also found that the same genotype can be found at different geographical locations and that is studies so far the Australian population appears to be quite distinct from isolates in a previous study carried out in Canada.

- The research outcomes from this project will feed into other projects where the aim is to utilise resistant germplasm for use in the field. Consequently, the transfer of results to the growers will be indirect via the breeders and other researchers. Such interaction is already in place through the CRCTPP.

Publications and Publication Schedule

Published

1. M. G. Ekins, K. C. Goulter, and E. A. B. Aitken. (1994). Genetic diversity in *Sclerotinia* species. Seventh International Symposium on Molecular Plant-Microbe Interactions. Edinburgh, Scotland.

2. M. G. Ekins, K. C. Goulter, and E. A. B. Aitken. (1994). Genetic diversity of *Sclerotinia* species. Australian Peanut Conference. Gladstone, Queensland.

3. M. G. Ekins, K. C. Goulter, and E. A. B. Aitken. (1994). Genetic diversity in *Sclerotinia* species attacking sunflower. Proceedings 10th Conference Australian Sunflower Association. Gold Coast. Queensland.

4. M. G. Ekins, K. C. Goulter, L. M. Kohn and E. A. B. Aitken. (1995). *Sclerotinia sclerotiorum* populations affecting sunflower fields in Australia. Botany Postgraduate Conference. Brisbane, Queensland.

5. M. G. Ekins, K. C. Goulter, L. M. Kohn and E. A. B. Aitken. (1995). *Sclerotinia sclerotiorum* populations affecting sunflower fields in South east Queensland. 10th Biennial Australasian Plant Pathology Society Conference. Lincoln, New Zealand.

6. M. G. Ekins, K. C. Goulter, L. M. Kohn and E. A. B. Aitken. (1995). Molecular identification of *Sclerotinia* species. Sclerotinia Workshop. 10th Biennial Australasian Plant Pathology Society Conference. Lincoln, New Zealand.

7. M. G. Ekins, E. A. B. Aitken. and K. C. Goulter. (1996). Molecular identification of strains of *Sclerotinia* species. VEGTEC 2000 - National Vegetable and Potato Industry Conference. Brisbane, Queensland. [Poster]

8. M. G. Ekins. (1996). *Sclerotinia sclerotiorum* populations affecting sunflower fields in Australia. Botany Postgraduate Conference. Brisbane, Queensland.

9. M. G. Ekins, K. C. Goulter, E. A. B. Aitken. (1997). *Sclerotinia sclerotiorum* populations affecting sunflower fields in Australia. 11th Biennial Australasian Plant Pathology Society Conference. Perth, Australia.

10. M. G. Ekins. (1997). *Sclerotinia sclerotiorum* populations affecting sunflower fields in Australia. Botany Postgraduate Conference. Brisbane, Queensland.

To be published...

M.G. Ekins PhD thesis: expected mid 1998

At least five papers are expected to be published from this work in international journals, these papers will be on:

1. Head rot of sunflowers caused by *Sclerotinia minor* (draft prepared)

2. Pathogenicity testing of different genotypes of *S.sclerotiorum* (draft prepared)

3. Geographical studies of populations of *S.sclerotiorum* in Australia

4. Temporal studies of populations of *S.sclerotiorum* in Australia

5. Comparative use of RAPD, RFLP and MCGs for assessing populations of *S.sclerotiorum*