MOLECULAR CHARACTERISATION OF *RHIZOCTONIA SOLANI* ANASTOMOSIS GROUP 2

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Abstract

The soil-borne fungal pathogen *Rhizoctonia solani* has a broad host range, causing disease on agriculturally important crops in many regions of the world. This species complex has been classified into 12 genetically distinct groups, termed anastomosis groups (AG), based on the frequency of hyphal fusion. Further subdivision within groups has been achieved using zymogram grouping (ZG). *R. solani* AG 2 was originally subdivided into two subgroups, AG 2-1 and AG 2-2, by frequency of anastomosis. However, more recently it has been recognised that AG 2 consists of AG 2-1, AG 2-2 and AG 2-3 based on anastomosis frequency, thiamine requirement and pathogenicity. Zymogram analysis has been used to subdivide AG 2-1 into ZG 5 and ZG 6, while AG 2-2 was divided into ZG 4 and ZG 10. In addition, subgroups of AG 2 have been distinguished on the basis of ribosomal ITS sequences (Salazar *et al.*, 1999). The discovery of subgroups within the AG has led to the recognition that AG 2 is more complex and variable than originally thought. Clarification of the AG 2 complex has been attempted using methods such as PCR and isozyme analyses, however, little research has been conducted on Australian isolates to determine the extent of genetic variability.

A collection of isolates of *R. solani* AG 2 was established from various agricultural regions in Australia, along with representative isolates from Japan and the Netherlands. These isolates were classified as either AG 2-1 or AG 2-2 by anastomosis reaction, with four isolates being classed as "AG 2 undefined", based on an inconclusive reaction. In addition, isolates were examined for morphological differences. In order to clairfy further the grouping of AG 2 isolates and to begin to examine genetic variation, zymogram analysis using vertical poly-acrylamide gel electrophoresis was conducted. The technique identified 25 patterns which were used to group isolates of both AG 2-1 and 2-2 into ZG. Following UPGMA analysis, isolates of AG 2-1 could be separated into ZG 5 and ZG 6. However, isolates belonging to AG 2-2, ZG 4 or ZG 10 could not be separated. Instead, two groups, termed AG

2-2 group I and group II, were identified. Both AG 2-1 and AG 2-2 were highly variable and the possibility of the existence of further subdivisions (the s is OK here, don't know why you deleted it?) is discussed.

Random genomic cloning was used in an attempt to identify specific molecular markers for AG 2-1 and AG 2-2. From a total of 1587 clones, 368 were selected for further examination as markers for AG 2-1 and AG 2-2. Two of these clones, pRAG21-413 and pRAG22-39, were used to assess genetic variation and were shown to be informative RFLP markers for the isolates of AG 2-1 and AG 2-2, respectively. Furthemore, these two clones were used as putative markers for their respective subgroups. While both clones hybridised to DNA from other AG, neither showed hybridisation to DNA from the other AG 2 subgroup. It is suggested that these two clones be examined further to determine if they may provide a basis for molecular markers specific to AG 2-1 and AG 2-2.

Genetic varation was examined further using microsatellite-PCR analysis. Two microsatellite primers and the intron-splice junction primer revealed polymorphisms in reproducible banding patterns. High levels of genetic diversity were observed among isolates of AG 2. While no subgroup-specific amplification patterns were found, UPGMA analysis was able to separate isolates of AG 2-1 into the subgroups of ZG 5 and ZG 6, while AG 2-2 isolates were grouped more closely in geographic regions, without the recognition of ZG or group I and group II.

The genetic data were combined in an attempt to clarify the relationships between isolates of *R. solani* AG 2. Zymogram, RFLP and PCR data were analysed using both the heirarchical UPGMA cluster analysis and the non-heriarchical approach of principal coordinates analysis. Again, isolates of AG 2-1 could be separated into ZG 5 and ZG 6 when either analysis was used. In contrast, isolates of AG 2-2 could not be separated into the accepted ZG 4 or ZG 10. Neither could the isolates be placed into group I or group II as observed for vertical gel zymogram analysis. The isolates of AG 2-2 appeared to separate more into geographic groups when the RFLP and PCR data were incorporated with the zymogram data.

Correlations of the distance matrices produced by the different analysis methods by the Pearson product moment coefficient, resulted in a strong correlation score.

The relevance of the AG 2 subgroupings, the use of molecular methods to exmine genetic diversity and the proposal for extensiveve population analysis are discussed.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is given. I give consent to this thesis being made available for photocopying and loan from the University library.

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Abbreviations

AG -	anastomosis group
bp -	base pairs
EDTA -	ethylenediaminetetra acetic acid
h -	hour(s)
IPTG -	isopropyl β-D-thiogalactopyranoside
ITS -	internal transcribed spacer
kb -	kilobase pairs
min -	minute(s)
mJ -	millijoules
PCR -	polymerase chain reaction
sec -	second(s)
SDS -	sodium dodecyl sulphate
Tris -	tris (hydroxymethyl) amino methane
TE -	tris-EDTA
v -	volts
XGal -	5-bromo-4-chloro-3-indolyly- β -D-galactopyranoside
ZG -	zymogram group

Chapter 1

Review of Literature

1.1. Introduction

Primary production remains as one of Australia's largest export earners. The estimated gross value of the crops produced by Australian growers for 1999-2000 was A$ 16.6 billion (Australian Bureau of Statistics, 2001). Primary producers need to be aware of the many plant pathogens, including viruses, bacteria, nematodes and fungi, which may affect both the quality and yield of their crops. Definitive identification of these pathogens and an increased understanding of their biology will aid in the development of more efficient and effective control practices, the adoption of which may reduce the risk of yield loss for the producer.

Rhizoctonia is an agriculturally important fungal genus that occurs worldwide. Some members of this genus are the asexual stages (anamorphs) of *Thanatephorus, Waitea* and *Ceratobasidium*, in the division Basidiomycota. These can exist as both soil saprophytes and plant parasites, and are responsible for some of the most devastating diseases in crops. Sheath blight of rice, caused by *R. solani* for example, has been estimated to cause annual yield losses of US $15 million in South India and US $72 million in South and South East Asia (Sriram *et al.*, 1997).

The species *R. solani* consists of a large number of functionally heterogeneous, morphologically similar fungi, with a near unlimited host range (Anderson, 1982). Isolates of *R. solani* are currently grouped into anastomosis groups (AG), based on the frequency with which vegetative hyphae interact. Members of different AG either do not interact, or do so with very low frequency. Within Australia, one of the most important fungal diseases is bare patch of cereals, caused by *R. solani* anastomosis group 8 (AG 8). Reports suggest that 30% of a cereal crop can be affected, which may result in a yield loss of up to 25% (Yang *et al.*, 1994; MacNish and Neate, 1996). Although AG 8 has been shown to be the causal agent, AG

2 and AG 4 have also been isolated from patches. While only limited research has been conducted on these two groups within Australia, the data suggest that AG 2 may be an important pathogen to a number of field crops, including canola, tulips and sugar beet. The following review will concentrate on *R. solani* and the relevance of the pathogen to crop production in Australia. It will also cover methods used for identification of the fungus and the techniques used in investigating its biology and genetics.

This literature review was compiled largely during 1997. The references used throughout this review were current at the time of writing. More recent references relating to specific research and methods are discussed in each of the relevant chapters.

1.2. General concepts and characteristics of *Rhizoctonia* spp.

The anamorphic genus *Rhizoctonia* has been characterised and placed in a number of different genera based on classification of the teleomorph. The current, accepted taxonomic model for the genus is that proposed by Ogoshi (1987). The following characteristics are used to define the genus *Rhizoctonia*: 1) Branching near the distal septum of young, vegetative cells; 2) formation of a septum in the branch near the point of origin; 3) constriction of the branch; 4) dolipore septum; 5) no clamp connection; 6) no conidium, except monilioid cells; 7) sclerotium, termed pseudo-sclerotium, not differentiated into rind and medulla; and 8) no rhizomorph.

The simplest method of characterisation has been by cellular nuclear number, established by staining, which is used to separate the genus into binucleate and multinucleate species (Sneh *et al.*, 1991). In addition, the characteristics of teleomorphs (sexual states) have enabled three major groups to be identified: 1) multinucleate species, teleomorph in the genus *Thanatephorus* (Frank) Donk; 2) multinucleate species, teleomorph in the genus *Waitea* Warcup and Talbot; and 3) binucleate species, teleomorph in the genus *Ceratobasidium* Rogers (Ogoshi, 1987).

Classification of *Rhizoctonia* spp., based on morphological characteristics, has led to the view that the species consist of anamorphs representing a diverse collection of teleomorphs, which belong to different families, orders and classes (reviewed in Ogoshi, 1987). Classification of isolates using the structure of the septal pore has provided a means to define *Rhizoctonia* species further. Moore (1987) placed isolates referred to as *Rhizoctonia* into the genera *Thanatephorus, Ceratobasidium, Waitea,* as outlined above, *Tulasnella* (binucleate, anamorph in the *R. repens* group) *Helicobasidium* (multinucleate, anamorph *R. crocorum*), and *Sebacina* (multinucleate and binucleate, anamorph type species *R. globularis*) using the characteristics of both the teleomorph and the septal pore structure.

1.2.1. Morphology of *R. solani*

Separation of *R. solani* from other *Rhizoctonia* spp., such as *R. zeae*, *R. oryzae* and *R. repens*, is based on the following morphological characteristics: 1) hyphae, septa and sclerotia are typical of *Rhizoctonia,* as outlined above; 2) teleomorph is *Thanatephorus cucumeris*; 3) multinucleate hyphae; and 4) shade of pigmentation in culture (Parmeter and Whitney, 1970; Ogoshi, 1987). However, individual isolates of *R. solani* exhibit no distinctive morphological characters on which they can be distinguished (Sneh *et al*., 1991). Various other methods have been used to classify *R. solani* isolates. These have included host specificity (Watanake and Matsuda, 1966), serological and immunological properties (Adams and Butler, 1979; Matthew and Brooker, 1991; Thornton *et al*., 1993; Thornton, 1996) and a number of molecular techniques (see section 1.5). The most powerful and commonly used approach is based on hyphal fusion (Ogoshi, 1975; Vilgalys and Cubeta, 1994), which resulted in the subdivision of *R. solani* into AG.

1.2.2. Anastomosis groups

Two different isolates of *R. solani*, co-cultured on agar, undergo an incompatibility reaction, based on the tendency of vegetative hyphae to fuse with one another. This is termed hyphal

anastomosis and has been used to place isolates into AG (Carling *et al.*, 1987; Sneh *et al.*, 1991). Several systems have been proposed to classify the reaction types observed (reviewed in Carling, 1996). The most commonly used and accepted system is that proposed by Carling *et al.* (1988), as follows:

C3 ~ closely related isolates, same AG, often the same isolate; walls and membrane fuse; diameter of anastomosis point is equal to hyphal diameter; generally adjacent cells are not killed.

C2 ~ related isolates of the same AG; wall fusion obvious, membrane fusion uncertain; diameter of anastomosis point less than that of hyphae; anastomosing cells and those adjacent always die.

C1 ~ distantly related isolates, can be from either different or the same AG; contact between hyphae; fusion of walls, but not of membranes; occasionally cells die.

C0 ~ isolates not related, from different AG; no interaction between hyphae observed.

It has been suggested that anastomosis, or somatic cell fusion, allows gene exchange in *R. solani* (Anderson, 1982). However, this is difficult to assess. In a recent study, McCabe *et al.* (1999) used light microscopy to examine the anastomosis reaction. Their results suggest that, in general, anastomosis was limited to hyphal tips and that major runner hyphae were not involved, only the tips of side branches. Further observations suggested that while perfect fusion allowed cytoplasmic streaming to occur, movement of nuclei or mitochondria could not be detected with this method. The authors suggest the use of organelle labelling systems to assess the movement of nuclei and mitochondria during anastomosis (McCabe *et al.*, 1999).

To date 12 AGs have been proposed. AG 1 to AG 4 were described by Parmeter *et al.* in 1969; AG 2 was separated into AG 2-1 and AG 2-2, and AG 5 was described by Ogoshi in 1975; AG 6 and the bridging isolates (AG BI) by Kuninaga *et al.* in 1978; AG 7 by Homma *et al.* in 1983; AG 8 by Neate and Warcup in 1985; AG 9 by Carling *et al.* in 1987; AG 10 by MacNish *et al.* in 1995; and AG 11 by Carling *et al.* in 1994.

Generally, anastomosis occurs between isolates of the same AG (Anderson, 1982). However, a bridging isolate may be seen to anastomose with isolates from other AGs. For isolates to be classed in AG BI, anastomosis must occur with at least two other AGs. In addition, some AGs, such as AG 2 and AG 8, are able to undergo infrequent anastomosis reactions with isolates from a different AG. This illustrates that AG separations are not always definitive (Carling, 1996), which may lead to some difficulty in assigning isolates to a single group. Furthermore, the genetic state of the isolates, either homokaryotic or heterokaryotic, may interfere with the anastomosis reaction, thereby complicating the interpretation (Cubeta and Vilgalys, 1997). There is no doubt that grouping isolates based on anastomosis is useful, but interpretation is sometimes difficult and the procedure is time consuming. The ability of a few anastomosis groups to cross-react with isolates from other AGs, adds to the difficulty of the procedure. For example, Adams (1988) argues for the placement of AG 2-1, AG 2-2 and AG BI into the one group, AG 2. Consequently, much time and effort has gone into finding other criteria to classify *R. solani* into separate groups, which may complement the AG approach.

1.2.3. AG and intra-specific groups

Intra-specific grouping (ISG) within AGs was first proposed by Ogoshi (1987). He was able to divide AG 1 and AG 2 into subgroups based on differences in host specificity. Since then, researchers have examined various other techniques to divide AGs into ISGs. For example, Kuninaga and Yokosawa (1984 a&b) divided AG 4 and AG 6 into two ISGs based on DNA sequence homology, these being AG 4 HG-1 and HG-II, and AG 6 HG-I and HG-II. AG 9 was divided into two subgroups based on nutritional requirements (Carling and Kuninaga, 1990). Isozyme analysis of metabolic enzymes has been used by Liu *et al.* (1990) and Liu and Sinclair (1992) to subdivide AG 2 into five ISGs, termed 2A-2E. Isolates from 2A and 2E correlated with those previously identified as AG 2-1, isolates from 2B correlated with AG 2-2IIIB, group 2C isolates corresponded with AG 2-2IV and 2D isolates correlated with an

AG 2-2 unspecialised group. However, one of the isolates placed into an AG 2 subgroup was later found to belong to AG 3, indicating that a large number of isolates needs to be examined to validate the use of the isozyme technique.

The general consensus of researchers now is that the term ISG has become too broad and confusing. This has led many to believe the term to be redundant (Carling, 1996). Other approaches, based on the use of a single method, such as zymogram and isozyme analysis, have been proposed for use in future classification systems, and may prove to be less confusing and more informative.

1.2.4. AG and zymogram groups

Zymogram grouping (ZG) is based on the detection of pectic enzymes expressed by isolates, using polyacrylamide gel electrophoresis (Cruickshank and Wade, 1980). This technique has been used to place isolates of some AGs into subgroups, based on the resultant banding pattern seen on a gel.

Cruickshank (1983) was able to distinguish between species of *Sclerotinia* by examining pectic enzymes. Since then, virulent strains of *R. solani* have been distinguished from hypovirulent strains, based on the activities of pectin esterase and polygalacturonase (Marcus *et al.*, 1986). Using similar techniques, both binucleate and multinucleate species of *Rhizoctonia* have been grouped into several ZG each. For example, AG 8 has been divided into five ZGs, ZG 1-1 to ZG 1-5, based on the different pectic enzyme fingerprints observed among isolates (Neate and Cruickshank, 1988; MacNish *et al.*, 1993). In addition, a study conducted on AG 2 isolates in the Netherlands suggested that AG 2-1 isolates could display five distinct zymogram patterns (Schneider *et al.,* 1997). The authors proposed the placement of Netherlands AG 2-1 isolates into ZG 5-1 to ZG 5-5 based on pectic enzyme analysis and host specificity.

While there can be no doubt that pectic zymograms provide a useful and acceptable system with which to subdivide AGs, problems do occur. The technique is based on an

inducible enzyme system; thus, it could be expected that some bias towards particular enzymes may exist. This is overcome, to some extent, by the induction and staining of individual enzymes using standard experimental techniques. In addition, the interpretation of results can differ depending upon the experience of the researchers. The observed patterns are not always clear and are, therefore, open to differing interpretations. There is not sufficient information available on the genetics of this fungus to determine what a zymogram group defines. Furthermore, the anastomosis reactions observed further complicate the analysis of genetic data generated by zymogram analysis. This suggests the need for more definitive approaches for taxonomic purposes.

1.3. Genetic variation: evolution and maintenance

Evidence from anastomosis grouping and DNA similarity studies suggests that different AG are, in fact, individual biological species (Adams, 1988; Vigalys, 1988). If this is taken into account, along with the classification of teleomorphs into different genera, this expands the concept of *Rhizoctonia* to one that comprises genetically diverse groups existing at several levels of organisation (Vilgalys and Cubeta, 1994). The mechanisms that enable the evolution and maintenance of genetic variation within the genus *Rhizoctonia* are unknown. A number of processes have been proposed which may contribute to the genetic structure of this diverse fungal genus.

1.3.1. Heterokaryosis

Heterokaryotic cells contain dissimilar nuclei and may form vegetatively through anastomosis and mutation, or as a part of the sexual reproduction cycle. A difference at a single gene that determines mating type, simultaneously precludes formation of a vegetative heterokaryon between two dissimilar strains, yet is essential for formation of a sexual heterokaryon between the same two strains (Leslie and Zeller, 1996). Within *R. solani* AG 1 and AG 4,

heterokaryon formation is controlled by two closely linked loci (H factors), found in genes that act like the incompatibility loci of other basidiomycetes (Julian *et al.,* 1996). Incompatibility defines heterokaryotic individuals but does not rule out sexual conjugation of isolates. This is governed by more complex polygenic and heterogenic somatic recognition systems (Adams, 1988).

The genetic state of most field isolates of *R. solani* is generally accepted as being heterokaryotic (Vilgalys and Cubeta, 1994). However, this has been proved for AG 1 (Parmeter *et al.*, 1969), AG 2 (Stretton and Flentje, 1972) and AG 4 (Anderson *et al.,* 1972) only, and proposed for AG 8 (Yang *et al.*, 1994). Furthermore, the role of the homokaryon, individuals with similar nuclei in each cell, is not well understood and requires more research (Adams, 1996). The isolation of uninucleate, single-spore isolates (SSI) from field isolates for the production of homokaryons, has provided a useful research tool with which to study the formation of heterokaryons. Stretton and Flentje (1972 a&b) performed several studies using SSI from AG 2 field isolates. Their observation of differences in morphology and growth of the progeny compared to the parental isolates suggested that the parental isolates were heterokaryotic. When 20 single-spore derived progeny from four parental isolates from the same host were paired, 75% of the resultant heterokaryotes were restored to the wild-type growth and were able to sporulate, and remained stable in culture (Stretton and Flentje, 1972 a). This is in contrast to SSI derived from parental isolates originating from different hosts, where the resulting heterokaryons were unable to sporulate and were not stable in culture (Stretton and Flentje, 1972 b). It was suggested that the genetic differences, between isolates from different hosts were too great to enable stable heterokaryotisation to occur. In more recent studies, Julian *et al.* (1997) noticed that the 45 SSI obtained from one AG 1-1C field isolate all differed in their cultural morphology when compared to the original parent, suggesting the heterokaryotic nature of the isolate. While 40 of the 45 SSI were able to produce fruiting bodies that contained spores, only three of these were able to expel the basidiospores successfully. However, this occurred at a rate 500 of the original parent (Julian

et al., 1997). When the homokaryotic SSI were paired, the reconstituted heterokaryons were found to be able to sporulate at rates similar to that of the original parent isolate.

Some of the characteristics used to determine heterokaryosis do not always concur with genetic studies, which has led to some confusion. Cubeta *et al*. (1993) observed that hyphal tufts, often associated with heterokaryon formation via anastomosis of two homokaryons, did not always result in the production of a heterokaryon. Thus, the validity of some heterokaryons formed in the laboratory is in doubt. In addition, when tuft formation was not observed, heterokaryotic isolates could still be obtained (Cubeta *et al*., 1993). Julian *et al*. (1999) used tuft formation to place AG 1-1C SSI into two "mating" types. When paired, those SSI that produced tufts were placed into different groups and those that did not were placed into the same group. By using nine different primer combinations in amplified fragment length polymorphism analysis (AFLP), 91 markers could be identified which segregated distinctively with the SSI. In addition, four markers were found which correlated with the defined "mating" types, two for each type. When the parental isolates and reconstituted heterokaryons were examined, the four "mating" type markers were present in each isolate (Julian *et al*., 1999), indicating that genetic exchange had occurred between two homokaryons.

1.3.2. The sexual species

Depending on the view taken as to the reproductive system present in *R. solani* (sexual or asexual), the role of the heterokaryon may change. In an asexual species, the role of the heterokaryon would allow for genetic recombination via fusion of dissimilar nuclei within the cell, prior to mitosis. In a sexual species, outbreeding between compatible isolates needs to be considered. Here, heterokaryon formation may follow fusion of dissimilar nuclei within the fruiting body, prior to meiosis, to form a recombinant spore. Thus, the constraints of incompatibility need to be considered (reviewed in Adams, 1988). In a study conducted by Julian *et al*. (1997), homokaryotic individuals of AG 1-1C were produced following isolation

of single basidiospores. These were then used to track the progress of nuclei during induced sporulation, and heterokaryon production. The results suggested that within the pro-basidium, two haploid nuclei fuse to form one diploid nucleus, which undergoes meiosis to give rise to four haploid nuclei (Julian *et al.,* 1997). If this is then considered to be the case for heterokaryotic individuals, then fusion must occur between two genetically different nuclei, in order to give rise to recombinant basidiospores.

The sexual state of *R. solani* has been observed for all AGs under laboratory conditions (Carling *et al*., 1994). However, sporulation is not readily observed in nature and, therefore, the role of sexual recombination in the generation and maintenance of genetic variation is the subject of considerable debate. Molecular markers have been developed which allow the assessment of segregation in fungal species. The use of such markers in examining the sexual state of *Rhizoctonia* is yet to be fully assessed. The extent to which sexual reproduction occurs in natural populations can now be measured, although some caution is required, as the theories put forward thus far have been based on much studied and characterised model fungal systems, with which many fungal species do not comply.

1.3.3. Somatic incompatibility

Incompatibility reactions have been observed in culture for isolates of *R. solani*. Cubeta *et al.* (1993) observed two somatic incompatibility reaction types when heterokaryotic progeny were opposed to their homokaryotic parents, in culture. One reaction was seen as a cleared zone of inhibition, similar to those seen in antagonistic responses, while the other appeared as a white line along the confrontation zone.

It has been proposed that the killing reaction, seen in C2 anastomosis reactions, is also an incompatibility reaction (Carling, 1996; Leslie and Zeller, 1996). This may delimit AG and their ISG into vegetative compatibility groups (VCG), which is, perhaps, a more appropriate term to replace ISG (Carling, 1996). Strains of one VCG are genetically isolated from strains of any another VCG during asexual reproduction. In addition, it is thought that

VCG arise through genetic recombination (Leslie and Zeller, 1996). However, there remains some debate as to whether or not somatic recombination occurs in *R. solani*. An interesting observation was made by McCabe *et al.* (1999) in regard to somatic incompatibility. Seventy-eight single hyphal tip cultures were isolated from an individual AG 4 parent. When the cultures were paired with the parent, six were found to somatically incompatible, as determined by the presence of the killing reaction. When these six were paired with one another, the killing reaction placed them into two incompatibility groups (McCabe *et al.*, 1999). The authors noted that the numbers of nuclei in the cells of the parent culture differed between the branches. They suggest that a colony may consist of a genetic mosaic, where the numbers and type of nuclei differ between branches.

In summary, it appears that the processes of vegetative heterokaryosis, sexual reproduction and perhaps somatic recombination, could provide the means for both the evolution and maintenance of genetic variation in *R. solani*. The suggestion that sporulation is reduced in homokaryotic isolates of AG 1-1C may have dramatic implications for sexually reproducing strains of *R. solani*. If this were to occur in the field, then homokaryotic individuals would need to find compatible "mates" with which to interact and produce heterokaryons, prior to successful sporulation. This may not be difficult in an agricultural field where the host is readily available, nor if, as observed by Julian *et al.* (1997), fusion of dissimilar nuclei can occur in the basidiospore. However, in cases where sexual reproduction is limited, somatic incompatibility may constrain genetic variation. This may be where the transfer of genetic material during anastomosis of compatible strains plays a significant role. If, as was observed by McCabe *et al.* (1999), individual colonies exist as a genetic mosaic, then separate branches may interact with other local colonies in differing manners. For example, while one branch may anastomose with a nearby branch of a separate but compatible colony, a second branch may be unable to find a compatible match. Thus, genetic transfer may occur for one part of the colony, resulting in expansion and diversity, at the same time as incompatibility limits another part of the colony. It may be that the heterokaryotic

nature of *R. solani* field isolates enables somatic incompatibility to be overcome to some degree, ensuring the viability and diversity of the fungus. It is here that much more work is required in order to understand fully the processes by which heterokaryons are formed. This will greatly advance the knowledge of the genetics and evolution *R. solani*.

1.4. Pathogenicity of *R. solani*

R. solani inhabits the soil of many parts of the world, and causes both pre- and post-emergence damping off of seedlings, root, crown and stem rots and foliar blights (Adams, 1988). Research to date has shown that, generally, each AG has a defined host range (Watanake and Matsuda, 1966; Anderson, 1982; Sneh *et al*., 1991).

The population level of *R. solani* in soil is believed to influence both disease incidence and severity. This is dependent on the AG, the number of viable propagules in the soil, as well as their size and distribution, the host, and the environmental conditions at the time of infection (Henis, 1979). Three major components which give rise to infection efficiency were identified by Gilligan and Bailey (1997): i) germinability of inoculum; ii) growth towards the host and; iii) the net effect of host susceptibility and mycelium infectivity upon host contact. Inoculum may occur as basidiospores, which are responsible for a wide range of leaf diseases, but are fragile and not suited to long term survival (Baker, 1970), and sclerotia which are much more hardy and have been reported to survive in soils for several years (Sherwood, 1970). As sclerotia are the primary survival structure, germination would occur in the soil and on plant debris. In both cases, this is dependent on temperature and moisture (Sherwood, 1970), and the resulting mycelial growth has been suggested to be stimulated by plant exudates (Kerr and Flentje, 1957). The fungus then proceeds to grow saprophytically from the source of inoculum towards a susceptible host (Bailey *et al.,* 2000). While germination of inoculum is not affected by the distance from the host, the further the expanding colony must grow to contact the host, the fewer hyphae reach there. Thus, as distance from inoculum to

host increases, the overall infectivity of the fungus decreases (Gilligan and Bailey, 1997). As the fungal colony expands through the soil medium, a number of limiting factors determine the size and rate of expansion. For saprophytic growth to proceed uncolonized sites need to be available to provide adequate nutrition for expanding and actively growing hyphae (Bailey *et al.*, 2000). In addition, the soil itself limits the growth. The effect of air-filled soil pore volume on colony expansion of *R. solani* AG 4, was examined by Otten *et al.* (1999). As the pore volume decreased and pores became less connected, the fungal colonies were small and dense. As the pore volume increased and pores began to connect, a sharp increase in colony spread was observed, resulting in large, less dense colonies (Otten *et al.*, 1999).

The ability of *R. solani* to survive in the soil for extended periods and saprophytic growth are important factors in the infectivity of the fungus. Once germination of inoculum occurs, the survival of the colony relies upon its ability to reach a susceptible host. In areas where hosts are separated by some distance, the ability of the fungus to reach nutrient pockets and survive saprophytically would be an additional benefit for the expansion of the colony.

1.4.1. *R. solani* in Australian agriculture

Australian soils tend to develop from nutrient poor parent rock and are heavily weathered, leached and reduced in nutrients (Sivasithamparam, 1993). The southern regions of Australia are the major cereal growing areas and are in a Mediterranean climate (MacNish and Neate, 1996). As crop production and other primary industries still provide for a large part of Australia's economy, it is important that maximum yields and cost efficiency are maintained. One way to achieve this is to obtain information on the types of pathogens likely to interfere with crop production, and how effective control of the diseases they cause can be achieved.

The following AGs are known to inhabit the soils of Australia, and are currently thought to pose the most threat to agricultural production:

AG 2: This AG is divided into AG 2-1 and AG 2-2 based on the frequency of hyphal fusion and thiamine requirement. AG 2-2 has been further subdivided into AG 2-2-IIIB and

AG 2-2-IV based on host specificity and the ability to grow at 35°C (Ogoshi, 1987; Sneh *et al.*, 1991). AG 2-1 has been isolated from onion seedlings in South Australian nurseries (Masuhara *et al.*, 1994) and causes pre-emergence damping off in *Brassica* spp., including canola (*B. napus*), in Canada with some species being more susceptible than others (Kataria *et al.*, 1993). In a study conducted in Western Australia, Khangura *et al.* (1999) reported that, of 112 *Rhizoctonia* isolates recovered from diseased canola plants, 62% belonged to AG 2-1. Schneider *et al.* (1997) identified a new cultural type of AG 2-1, which is pathogenic to tulip in the Netherlands. This subgrouping, termed AG 2t, is supported by both zymogram and PCR analysis. AG 2-2 has caused pre- and post-emergence damping off, and root and stem rot in soybean (Liu and Sinclair, 1991) and some isolates were varied in virulence (Muyolo *et al.*, 1993). Although isolates of AG 2, in general, are not known to be serious cereal pathogens, both AG 2-1 and AG 2-2 have been isolated from bare patches in wheat crops (Roberts and Sivasithamparm, 1986; Brisbane *et al.*, 1995). In addition, both AG 2-1 and AG 2-2 have been isolated from turf grass species affected by brown patch, and both cause severe pre- and post-emergence damping off of subterranean clover. However, isolates of AG 2-2 were found to differ in virulence on both these hosts (Wong *et al.*, 1984; Burpee and Martin, 1992). Based on cultural morphology, growth rate, pathogenicity to turf grass and restriction fragment length polymorphism (RFLP) analysis of ribosomal DNA, Hyakumachi *et al.* (1998) identified a new cultural type of AG 2-2, termed AG 2-2LP.

AG 3: Isolates of this AG are known as the "potato type" and cause black scurf on potato, a serious disease in many regions of the world. They are also pathogenic to tobacco and tomato plants. AG 3 isolates are auxotrophic for thiamine and at first appeared to be a genetically uniform group (Anderson, 1982; reviewed in Sneh *et al.*, 1991). However, recent research indicates that considerable heterogeneity exists among isolates from South Australian potato crops (Balali *et al.*, 1996).

AG 4: Little research has been performed on this AG in Australia. It has been isolated from wheat bare patches, but is not considered highly virulent to cereal crops

(Roberts and Sivasithamparam, 1986; Brisbane *et al.*, 1995). However, AG 4 has a broad host range and is highly virulent on many crops, worldwide. AG 4 has been isolated from both cauliflower and cucumber seedlings in South Australian nurseries (Masuhara *et al.*, 1994), and from field-grown potato (Balali *et al.*, 1995). Keinath (1995) examined the incidence of wirestem disease on cabbage caused by AG 4 and reported that severity of disease decreased as the inoculum density (sclerotia/kg soil) increased. In addition, disease incidence increased when temperatures were in the range of 18-21°C but decreased at higher temperatures. More recently, AG 4 has been isolated from rootlings of grapevine, along with AG 2-1 and the nematode *Meloidogyne incognita* (Walker, 1997). Data from pot-based experiments showed that both AG were able to infect the roots of grapevine, with AG 4 being more virulent. In addition, it was found that infection by AG 4 was increased when *M. incognita* was also present (Walker, 1997).

AG 8: This is an important cereal pathogen and is associated with bare patch of cereals in Australia and other regions of the world (reviewed in MacNish and Neate, 1996). AG 8 is often isolated with other AGs, as well as other fungal species; however, results suggest that AG 8 is the causal agent of bare patch. Yang *et al.* (1994) collected isolates from both the centre and perimeter of bare patches, and examined the level of virulence of individual isolates on seedling roots, *in vitro*. Their results suggest that highly virulent isolates are located at the centre of the bare patch, and that these are generally *R. solani* AG 8, while less virulent AG, such as AG 2 and AG 4, are present at the patch perimeter.

Of the AGs outlined above, most research in Australia has concentrated on AG 8. However, the presence of AG 2, AG 3 and AG 4, and their association with disease in crops throughout the world, suggests that these, too, may be important pathogens in Australian agriculture. Further research is needed to establish the importance of AG 2, AG 3 and AG 4 in Australian agriculture systems.

1.4.2. Disease control

Several approaches have been examined for the control of disease caused by *R. solani*, primarily AG 8, in the field. One major method is the utilisation of appropriate cultivation techniques. It has been suggested that deep ripping to a depth of 10 cm. to disturb the soil and fungal colonies, reduces the effects of *Rhizoctonia* root rot. Nutritional supplements for crops, such as nitrogen and zinc, also reduce damage, and rotation of pulses, medics and some lupins with cereals appears to help reduce damage caused by *R. solani* on cereals (reviewed in; MacNish and Neate, 1996).

Applications of fungicides and nitrogen, and timing of watering have also reduced disease damage. Perennial ryegrass turf, used on many golf courses, was shown to be more susceptible to brown patch caused by *R. solani* when high levels of nitrogen (240 kg/ha/year), in the form of 50% isobutylidene urea (31N - 0P - 0K) or 50 % urea (46N - 0P - 0K), were applied along with the dispersal of grass clippings after mowing (Dunn *et al.*, 1996). In regions with no grass clippings, brown patch was less severe but still evident. Plots with lower nitrogen levels (120 kg/ha/year) were less susceptible to brown patch, even when grass clippings remained. In a second study, the application at low levels of sulphur-coated urea as a nitrogen source, in conjunction with early watering (prior to sunrise), reduced the severity of brown patch on the same grass type (Fidanza and Dernoeden, 1996). In a further trial, sodium nitrate was used as the nitrogen source, resulting in severe blighting of the turf which was independent of the level at which it was applied (Fidanza and Dernoeden, 1996). The conclusions were that the source of nitrogen is an important component of turf management, as is the removal of grass clippings, which may act as a source of inoculum of *R. solani*. Fungicides have given almost total control (75-100%) of damping off of canola caused by AG 2. The level of control achieved can vary according to the type of fungicide used and the crop and cultivar to which it is applied (Kataria *et al.*, 1993; Kataria and Verma, 1993).

The development of plant hosts with resistance to disease caused by *Rhizoctonia* has been attempted in various regions of the world. Some success has been achieved with sugar

beet in the United States of America, where resistant cultivars have been available since 1968 (Gaskill, 1968; Scholten *et al.*, 2001). Some difficulties are still encountered, as the resistant sugar beet cultivars are not genetically homogeneous. Moderate resistance has been identified in tall fescue accessions (Green *et al.*, 1999) and tulip cultivars (Schneider *et al.*, 1999), however, under high disease pressure unacceptable disease levels occur. Using 122 cultivars of *Brassica* spp., Yang and Verma (1992) were able to show significant differences between and within species, but failed to identify immunity to *R. solani* in trials conducted in growth chambers and field plots. Breeding for resistance in crops such as sugar beet, has provided some control of disease caused by *R. solani.* In most cases, however, the hosts display tolerance, which enables growth in the presence of the pathogen rather than a true resistance (Panella and Ruppel, 1996). This, used in conjunction with fungicide application and other control techniques, generally provides suitable disease control.

One novel approach that appears promising for the control of *Rhizoctonia* diseases is biofumigation. *Brassica* species have been reported to increase wheat yields by up to 15% when used as a break crop (Kirkegaard *et al.*, 1994). This effect has been attributed to better root growth, increased nitrogen and water availability, and the presence of isothiocyanates (ITCs), a product from brassica debris decomposing in the soil. Such residues have an inhibitory effect on the growth, not only of *R. solani*, but also of species of *Fusarium*, *Bipolaris* and *Pythium.* These compounds may have important properties which can be exploited in the control of fungal pathogens. Sarwar *et al.* (1998) showed that the suppression of some fungi by propenyl-ITC and 2-phenylethyl-ITC, products of glucosinolate hydrolysis in brassica, was superior to that of the synthetically produced methyl-ITC. The authors suggested that the selection of *Brassica* species that produce large quantities of the glucosinolate precursors to the more effective ITCs, may provide the most benefit for the biofumigation processes. However, AG 2-1 is known to cause disease on canola and has been found to be capable of the breakdown of phytoalexins produced by cruciferous crops (Pedras

and Khan, 2000; Pedras and Okanga, 1999). Research into how the different ITCs affect each AG is required in order to determine their effectiveness.

1.5. Molecular techniques in taxonomy

Classic taxonomic techniques have generally failed to identify species diversity within *Rhizoctonia* (Vilgalys and Cubeta, 1994). The advent of anastomosis grouping of both *R. solani* and binucleate *Rhizoctonia*, in conjunction with zymogram grouping, has proven to be successful, but is time consuming and, at times, inconclusive. Recently, techniques of molecular biology have been applied to fungal systems, in order to clarify some of the problems encountered with classic taxonomic techniques. In all instances, information about the biology of the pathogen, its host specificity and pathogenicity is essential in forming classification systems for taxonomic purposes. However, the naturally occurring variation in the pathogen population must also be considered, and cannot always be determined using classical taxonomy. Molecular biology provides the tools required to elucidate genetic variation, both within and among populations. The techniques may help to establish definitive markers for classification purposes and may provide valuable information for use in control strategies.

1.5.1. Nucleic acid techniques in fungal systems

Molecular biological techniques have been applied to a diverse range of fungal species. The genetic diversity of *Fusarium* species has been studied extensively using RFLP analysis. RFLP analysis in conjunction with species-specific DNA probes, allowed two species, *F. culmorum* and *F. graminearum,* to be distinguished from one another. Although not all isolates tested could be placed into species, the probes were of most use when applied to distinguish *Fusarium* species from other organisms (Koopmann *et al.*, 1996). Further studies on *Fusarium* examined random amplified polymorphic DNA polymerase chain reaction

(RAPD-PCR). Schilling *et al.* (1996) used RAPD analysis to differentiate three species, *F. culmoram*, *F. graminearum* and *F. avenaceum.* By isolating unique PCR fragments, the authors were able to produce PCR primers capable of distinguishing the three species. A combination of PCR and RFLP analysis was used by Woo *et al.* (1996) to examine diversity within *F. oxysporum* f.sp. *phaseoli.* Results suggested that the genetic fingerprints generated by both PCR and RFLP analysis correlated well with the groupings determined by pathogenicity and vegetative incompatibility.

Like *R. solani*, some *Phytophthora* species lack distinguishing morphological features capable of distinguishing *P. parasitica* and *P. citrophthora* from other *Phytophthora* species. PCR has been used to amplify DNA sequences specific to *Phytophthora* spp., and these sequences were then used to develop species-specific DNA probes (Goodwin *et al.*, 1989; Goodwin *et al.*, 1990 a&b). Furthermore, Ersek *et al.* (1994) synthesised PCR primers derived from a *Phytophthora* species-specific plasmid clone, and used the primers to identify *P. parasitica* and *P. citrophthora* in diseased plant tissue. More recently, Goodwin *et al.* (1999) used the combined data generated from RFLP fingerprint analysis, isozyme analysis, RAPD-PCR and a DNA probe specific to ribosomal DNA (rDNA), to distinguish *P. mirabilis* from the closely related *P. infestans*. In more recent studies targeting rDNA, Fouly and Wilkinson (2000) amplified the mid-region of 18s rDNA of *Gaeumannomyces graminis* varieties, and cloned and sequenced the resulting PCR fragments. By designing primers specific to *G. graminis* varieties, the authors were able to identify and separate *G. graminis* var. *tritici*, *G. graminis* var. *avenae* and *G. graminis* var. *graminis* using both pure culture and field material (Fouly and Wilkinson, 2000).

Although there are many more nucleic acid based techniques than have been discussed above, it is clear that such methods can be used in taxonomic studies to identify isolates to the species level, particularly when definitive morphological characters are lacking. However, not all techniques are equally useful and, as is often the case, new techniques are continually being devised and applied to fungal genetics with varying degrees of success. The following

section deals with the more commonly used techniques and reviews their application to *R. solani*.

1.5.2. Plasmids in *R. solani*

Both double-stranded RNA (dsRNA) and linear, extrachromosomal DNA fragments are common in field isolates of *R. solani* (Bharathan and Tavantzis, 1990). The isolation of dsRNA from *R. solani* isolates affected by degenerative disease led researchers to believe that the presence of these elements resulted in the isolates being hypovirulent. However, no correlation between the presence of these plasmids and low virulence of isolates could be found (Hashiba, 1987). Miyasaka *et al.* (1990) were able to isolate plasmid-like DNA from field isolates of AG 1-1B, AG 2-2, AG 3, AG 4, AG 5 and AG 6. Their results suggest that these elements are homogeneous within each AG and that their presence may correlate with host specificity. More recently, a study conducted with AG 3 isolates identified five dsRNA elements, L1 (25Kb), L2 (23Kb), M1 (6.4Kb), M2 (3.6Kb) and S1 (1.2Kb), in three cultures sectored from a virulent parental field isolate (Jian *et al.*, 1997). The parental isolate, Rhs 1AP, carried two dsRNAs, L2 and M1. Of the sector-derived isolates, Rhs 1A1 carried L1, L2, M1, M2 and S1, Rhs 1A2 carried M2 and S1, while Rhs 1A3 carried L1 and S1. During anastomosis, the dsRNA elements were transmitted between isolates and after several cycles of hyphal tip isolation, elements could be lost. This allowed the effects of the five dsRNAs to be examined individually. The results suggested that, for these AG 3 isolates, dsRNA M1 is essential for virulence to potato shoots while M2 counteracts M1 (Jian *et al.*, 1997). While the results are promising and informative, more research is required to determine the role of extra-chromosomal elements within each AG of *R. solani*.

1.5.3. G/C content and DNA complementarity

Classification based on guanine and cytosine (G/C) content of DNA has been carried out on fungal systems, but provides little useful taxonomic information. In addition, contradictory

results have been obtained (reviewed in Klich and Mullaney, 1992). G/C content is, however, useful for demonstrating differences between isolates. When the G/C content of two isolates differs, they are not clonally related, but when the G/C content is similar, two isolates may or may not be closely related. Although this technique generally examines the entire genome of a fungus, it relies on the determination of a small difference in the base content between isolates. In addition, differences can not be located to specific areas of the genome.

An early molecular technique to be applied to a number of systems, including plants and fungi, was DNA complementarity. While this is also based on DNA base composition, it provides a more accurate measure of genetic relatedness than does G/C content. The technique is based on the ability of two genomes to reassociate by complementation (reviewed in Klich and Mullaney, 1992). Both the rate and extent of reassociation provide an indication of genetic relatedness. DNA reassociation is generally measured by use of radioisotopes or spectrophotometrically. Results for *R. solani* suggest that reassociation within AGs is high, while there is less reassociation between AGs. Some AGs, such as AG 1 and AG 2, display considerable heterogeneity among isolates within the AG, and a correlation was seen for the subgroups previously identified by various ISG techniques (Vilgalys, 1987). Using DNA complementarity, Carling and Kuninaga (1990) were able to identify subgroups of AG 9 previously separated by nutritional requirements, while other AGs examined, such as AG 3, AG 4, AG 7 and AG BI, displayed no variation. As with many methods, some results from different species appear to be contradictory; this could be due to the different procedures used by research groups. In addition, the locations from where the isolates were obtained and host specificity may have a causal effect upon genetic diversity (Klich and Mullaney, 1992).

Although DNA complementarity provides useful information on the relatedness of *R. solani* isolates, the information provided is still limited. No indication as to where in the genome these differences occur, or indeed, as to why they occur, can be obtained. This leads to the requirement for techniques that are able to provide information on discrete regions of the fungal genome.

1.5.4. Restriction fragment length polymorphism analysis and specific probes

RFLP analysis is relatively simple to apply in taxonomic studies. In most applications, DNA is digested with restriction enzymes in order to obtain discrete fragments of DNA, which are then separated by electrophoresis, and the resultant banding pattern observed. Heterogeneous DNA, such as chromosomal DNA, is so large and diverse that it gives rise to so many fragments that a continuous smear is formed. In this case, a small DNA fragment or probe is required to detect sequences within the smear. Discrete bands or polymorphisms from less heterogeneous DNA, such as ribosomal or mitochondrial DNA, can also be detected with sequence-specific probes, allowing non-homologous sequences of similar size to be distinguished (Klich and Mullaney, 1992).

Identification of AG-specific probes for *R. solani* has greatly enhanced the power of RFLP analysis as a taxonomic tool. Vilgalys and Gonzalez (1990 a&b) were able to clone an entire rDNA repeat from an AG 4 isolate. Using a heterologous 18S-RNA fungal-specific probe to screen an AG 4 genomic library, a fragment was identified and cloned for use in Southern hybridisation analysis. Analysis of genomic DNA, digested with the restriction endonucleases *Bam*HI and *Eco*RI, revealed 55 unique restriction patterns from 87 isolates of various AG. In addition, the 87 isolates could be placed into nine AGs based on the patterns obtained. Isolates belonging to AG 3, 4, 7, 8 and BI possessed a single RFLP pattern, whereas isolates belonging to AG 1, 2, 5 and 6 possessed multiple patterns, suggesting the presence of genetic variability at this region within these four AG (Vilgalys and Gonzalez, 1990b).

More recently, a probe specific to AG 8 has been developed. Genomic DNA from an AG 8 isolate, 1512, was digested with *Bam*HI and cloned into the plasmid vector, pUC19. A multi-copy clone, pRAG12, was selected and used to screen isolates of *R. solani* AG 2-1, AG 3, AG 4 and AG 8 for DNA polymorphisms (Matthew *et al.*, 1995). The clone showed strong homology to all AG 8 isolates tested, and little or no homology to isolates from the other AGs. The fragment is highly repeated within the genome of AG 8 and can be used to detect

AG 8 DNA directly from soil samples (Whisson *et al.*, 1995). Similarly, an AG 3 specific multi-locus probe, pR3781, was isolated (Balali *et al.,* 1996). Screening of genomic DNA from isolates of AG 1-1A to AG 9 and AG BI with this probe, revealed little or no homology to any of the AGs other than AG 3. Furthermore, all AG 3 field isolates collected were confirmed as true to type with the probe.

RFLP analysis can provide detailed information for the determination of genetic relatedness both within and between the AGs of *R. solani*. Discrete genomic regions, such as the 18S region of the rDNA repeat, may be examined for genetic variability. However, RFLP analysis is hindered by time constraints and is technically demanding. Diagnostic laboratories are expected to provide quality analysis with high output and, therefore, require procedures which are rapid and reliable. It may be feasible that a PCR-based procedure could be developed as a diagnostic test, using the sequence data of an RFLP-derived specific probe.

1.5.5. Polymerase chain reaction

PCR technology has become a powerful approach in molecular biology and genetic engineering. It is based upon the amplification of a specific DNA segment, flanked by a pair of synthesised primers of known sequence (Sano *et al.*, 1992). This procedure provides large amounts of specific DNA fragments for analysis.

Modification of the PCR process has allowed specific applications and almost complete automation. RAPD-PCR has been one of the most popular techniques in use. This technique generally utilises a single 10 – 20 nucleotide primer of arbitrary sequence, to randomly amplify sequences within the genome (Williams *et al.*, 1990; Caetano-Anolles, 1996). Duncan *et al.* (1993) examined 30 isolates of *R. solani* with seven primers and found that at least four of these primers were able to identify isolates belonging to different AGs. Other RAPD primers used by Brisbane *et al.* (1995) were used to differentiate isolates of AG 4 and AG 8. In this case, the primers generated fragments that were specific to each of the two AGs. These fragments were excised from electrophoretic gels, sequenced and used to

design specific PCR primers for identification of AG 4 and AG 8 field isolates. More recently, Yang *et al.* (1996) used RAPD primers to demonstrate genetic diversity among isolates of AG 9.

While RAPD-PCR is a useful technique, there are a number of problems that arise with its use. Firstly, RAPD-PCR identifies only extremely small regions of the genome, thus the full extent of genetic variation is not revealed. Secondly, the random nature of the primers used in this process means that sequences from non-related organisms can be amplified. This is an important point when developing diagnostic tests for a soil-borne organism, such as *R. solani*. The ideal PCR procedure would need to amplify a sequence specific to *R. solani*, without prior isolation of the fungus from the soil.

Recent approaches have concentrated on rRNA coding sequences. Nuclear small sub-unit DNA evolves slowly and is useful for examining distantly related organisms. Mitochondrial rDNA evolves more rapidly and has been successfully used in taxonomy at the family level. Finally, the internal transcribed spacer (ITS) and intergenic spacer (IGS) regions of nuclear rDNA repeats evolve most rapidly and vary among species within a genus (reviewed in White *et al.*, 1990). Boysen *et al.* (1996) used ITS sequence primers to amplify DNA directly from isolates of AG 4. Specific fragments could be identified with different combinations of the primers, suggesting that these sequences may be unique to AG 4 isolates. Further investigation may reveal sequences unique to other AGs.

In a study of the rDNA ITS region of *R. solani*, Kuninaga *et al.* (1997) observed that the 5.8S rDNA gene sequence was conserved across all AGs tested. However, the ITS regions displayed considerable variation. Using this variation, Kuninaga *et al.* (1997) were able to distinguish between the subgroups of AG 1 and AG 4 and, by the use of phylogenetic analysis, found genetic evidence for the proposed genetic isolation of AGs.

Different molecular techniques provide different levels of information, which can be correlated and used to extend the value of individual techniques. For example, AG 3 to AG 10 was distinguished by PCR amplification of the ITS-5.8S domain, followed by RFLP

analysis of amplified products (Liu *et al.,* 1993). Using a similar approach, binucleate AG of *Rhizoctonia* was also characterised (Cubeta *et al*., 1991). Liu *et al.* (1995) amplified the 18S rDNA region of DNA prepared from *R. solani* isolates of 10 AGs. Following digestion of the products with combinations of 11 different restriction enzymes, all 10 AGs could be identified using the resultant genetic fingerprints.

PCR has proved to be a valuable technique in taxonomy. The relative ease of application and sensitivity of PCR have allowed researchers to gain insights into the diversity of a number of fungal systems. Results from studies on *R. solani* suggest that this species is genetically diverse and complex. In diagnostic applications, PCR may be used to distinguish the different AGs, based on the observed genetic variation between groups. However, as with the other techniques, some difficulties may arise when interpreting results. Faint products may or may not be interpreted as a specific fragment. These could be the result of a number of problems, including carry-over DNA contamination, non-specific priming or an imbalance in the reaction conditions. Stringent controls are required to ensure that results reflect the true nature of the genome. In addition, questions about the reproducibility and reliability of the PCR procedure have been raised.

As with RFLP analysis, PCR only provides information on the composition of discrete regions of the fungal genome. For more complete genetic information, other techniques must be used in conjunction with the PCR procedure. Current approaches in both taxonomic and diagnostic studies are tending to use two or more techniques, such as RFLP and isozyme analysis. The results from both techniques are then correlated, and theories discussed as to the meaning of differences between AGs and their subgroups. For *Rhizoctonia* and other soil-borne organisms, the ideal PCR and RFLP procedure needs to detect DNA extracted from soils. Separation and amplification of DNA from soils is hampered by soil impurities, the loss of DNA by degradation and adsorption, and contamination by other organisms (Steffan *et al*., 1988; Smalla *et al*., 1993; Volossiouke *et al*., 1995). PCR and some restriction enzymes are inhibited by humic acids in soil, thus it is essential to remove these from any DNA

preparation. Attempts to separate fungal material by centrifugation are inappropriate, as the fungi pellet out with a large proportion of the bio-mass (Holben *et al.*, 1988). However, the use of extraction procedures incorporating products such as polyvinyl-pyrollidone (PVP), skim milk and chaotropic agents to remove DNA from humic acids, and protect against degradation, has improved the quality of DNA extracts and the efficiency of PCR and RFLP probes in detection of bacterial and fungal species in soil (Volossiouke *et al.*, 1995; Whisson *et al.*, 1995).

1.5.6. Immunological techniques

The production of monoclonal and polyclonal antibodies has provided researchers with techniques such as Western analysis and enzyme-linked immunosorbent assay (ELISA), for the examination of the properties and roles of enzymes in a diverse range of organisms.

Adams and Butler (1979) purified antiserum from rabbits inoculated with mycelial extracts of *R. solani* and used this to identify isolates of AG 1 to AG 4 on gel diffusion plates. However, AG 2-1 and AG 2-2 could not be separated, and it was difficult to distinguish AG 1 from AG 4. More recently, polyclonal and monoclonal antibodies raised against secreted protein extracts from mycelia of *R. solani* AG 8, were used to characterise this AG (Matthew and Brooker, 1991). The results suggested that polyclonal antibodies could be used to distinguish an isolate of unknown AG from one of a known AG. Monoclonal antibodies raised to protein extracts of AG 8 were able to react not only with AG 8, but also with isolates from AG 2-1, AG 3 and AG 4, although the intensity of the reaction differed for each AG.

Other data suggest that immunological techniques will be of use when identifying fungal isolates to the species level only. Thornton *et al.* (1993) successfully purified monoclonal antibodies for use in detecting *R. solani* in soils. Further purification of the antibody by magnetic beads allowed detection of AG 1, AG 2-1, AG 3, AG 4 and AG 7 (Thornton, 1996). The fact that considerable cross-reactivity exists amongst AG, particularly

with monoclonal antibodies, casts some doubt on the usefulness of immunological techniques for distinguishing between AGs of *R. solani*.

1.5.7. Isozyme analysis

Apart from the use of pectic enzymes to classify *R. solani* isolates into ZG (discussed in section 2.4), other enzymes have been examined for use in fungal taxonomy (Goodwin *et al.*, 1993). Electrophoretic separation of enzymes exploits polymorphisms of detectable isozyme forms (Damaj *et al.*, 1993). Each isozyme can be considered as a direct expression of genotype, therefore, the observed patterns can be used as an indicator of genetic relatedness (Liu and Sinclair, 1992).

Liu *et al.* (1990) examined isolates of *R. solani* for the expression of 29 metabolic enzymes. Eleven of these enzymes were found to be active and provided useful polymorphisms for 10 AG. Further research using the same approach revealed 58 electrophoretic phenotypes with 11 of the enzymes. Although some bands were common, each AG could be distinguished by the presence of specific bands (Liu and Sinclair, 1992).

Among binucleate *Rhizoctonia,* nine out of 23 metabolic enzymes examined gave useful banding patterns. Damaj *et al.* (1993) found that each of the nine enzymes gave different phenotypes for isolates belonging to different binucleate AG. Analysis of phenotypic similarity revealed the presence of four genetically distinct groups. Isolates of the same AG were clustered at higher similarity levels than those from different groups.

The use of isozymes has provided valuable information on the genetic relatedness of field isolates of *R. solani*. Isozymes examined for many fungal systems have tended to be those that are constitutively expressed, such as metabolic enzymes, rather than the induced enzyme system used for zymogram grouping (refer to section 2.4). This may help rule out any bias towards one isozyme. Analysis of metabolic enzymes to determine genetic variation between isolates of *R. solani* is becoming more commonly used, partly because the technique is cheap and relatively simple to perform. However, the technique needs to be refined in

order to achieve consistency and to provide a standard protocol, such as that used for zymogram grouping. While both of these enzyme-based techniques provide valuable information as to genetic variation, they are time consuming and require a large number of samples to be examined in order to obtain statistically reliable results. In addition, isozyme markers reveal only low levels of polymorphisms in pathogenic fungi, and are difficult to analyse in multinucleate organisms (May, 1992).

1.6. Summary

Isolates of *Rhizoctonia solani* belonging to AG 2-1, AG 2-2, AG 3, AG 4 and AG 8 have been identified as important plant pathogens in Australian agriculture and horticulture. AG 3 and AG 8 cause disease in potato and cereal crops, respectively, particularly in South Australia and Western Australia (see section 4.1). Research has identified molecular markers specific for both AG 3 and AG 8 (Matthew *et al.*, 1995; Whisson *et al.*, 1995; Balali *et al.*, 1996), with the AG 8 probe now being used commercially to detect and quantify AG 8 DNA in soil samples. However, little attention has been paid to AG 2 and it's subgroups in Australia.

AG 2 subgroups have been reported as being pathogenic to soybean, turf grass, tulip, tobacco and canola in different regions of the world. In South Australia and Western Australia, AG 2-1 and AG 2-2 are associated with wheat roots and medics, and are pathogenic to turf grass (see section 4.1). Agricultural practices are tending towards low impact strategies to reduce environmental degradation. Crop rotations are changing to involve more suitable and economically viable crops. However, some of these practices, such as low impact tillage, may in fact promote *Rhizoctonia* disease (Neate, 1984). The inclusion of canola in rotation strategies may allow for the promotion of AG 2-1 inoculum in soils and the use of pasture cover crops in vegetable growing regions may have a similar effect.

The identification of molecular markers specific to AG 2-1 and AG 2-2, along with a determination of host range, will increase the knowledge of the biology and pathogenicity of

this group in Australian agriculture. This is an important step towards control of disease caused by *R. solani* AG 2. Molecular markers will allow rapid and definitive identification of field isolates, and assist in the provision of appropriate control measures. The genetic information will provide a valuable insight into the interactions between AG 2 and the other AGs known to occur in Australian soils.

1.7. Aims of research

The main aim of this project was to characterise a collection of AG 2 isolates in order to identify and isolate molecular markers specific to *R. solani* AG 2-1 and AG 2-2. Such markers will enable the rapid identification of isolates belonging to each AG, and provide putative tools to develop a diagnostic test for these groups in soil. In addition, DNA markers may provide information on the extent of genetic variation both within and between populations of AG 2-1 and AG 2-2 and provide an insight into the means by which the fungus maintains this variation. Second, the host range of Australian AG 2 isolates was examined to determine any relationship between genetics and host preference. The information on the host range of AG 2 isolates needs to be determined to allow appropriate rotation strategies to be developed.

Chapter 2

Morphological Characterisation of AG 2 Isolates and Pectic Zymography Using Vertical Gel Electrophoresis

2.1. Introduction

The morphology of the genus *Rhizoctonia* has been well documented and its separation into species based on morphological characters achieved (Sneh *et al.,* 1991). The most studied species, *R. solani,* is multinucleate with variable cultural morphology and an extensive host range. This has made necessary the use of more definitive techniques to determine isolate grouping within the species. The anastomosis technique, described by Carling *et al.* (1988), is the most widely accepted method by which isolates of *R. solani* are placed into groups. However, the anastomosis groups themselves can consist of genotypically diverse individuals and evidence from DNA sequences and isozyme analysis has, in many cases, provided the additional information required to group isolates.

Electrophoretic separation of naturally occurring polymorphisms of detectable enzymes, called isozymes, has been used widely to characterise strains of various fungi (Micales *et al.*, 1986; Mills *et al.*, 1991). Each isozyme can be considered as a direct expression of genotype; therefore, the patterns observed can be used as an indicator of genetic variability (Liu and Sinclair, 1992). The separation of pectic enzymes by polyacrylamide gel electrophoresis (Cruickshank and Wade, 1980) has been used to group isolates of *R. solani* within an AG into zymogram groups (ZG). While some AGs have been shown to contain a number of zymogram groups, the same ZG has not been found in different AG. For example, Neate *et al.* (1988) found that isolates which were classified as *R. solani* AG 8, could be subgrouped into ZG 1-1 to 1-4, with the subsequent addition of ZG 1-5 (Sweetingham *et al.*, 1986). Initially, four ZG were proposed for AG 2; ZG 5 and 6 fall within AG 2-1, and ZG 4

and 10 belong to AG 2-2IIIB and AG 2-2IV (MacNish *et al.,* 1993). The improved resolution of pectic isozymes obtained with vertical gel electrophoresis, as opposed to the traditional horizontal method, has suggested the existence of additional groups. Schneider *et al.* (1997) proposed that ZG 5 isolates be subgrouped following a structure similar to that used for AG 8, in which AG 2-1 isolates are subdivided into ZG 5-1 to 5-5. They assigned isolates able to infect both crucifers and tulips in the Netherlands to ZG 5-1 based on both the observed host preference and ZG patterns, and to distinguish these from AG 2-1 isolates not pathogenic to tulip (Schneider *et al.* 1997). Further use of the vertical zymogram technique by Nicoletti *et al.* (1999) has suggested that *R. solani* isolates from tobacco show considerable homogeneity. The patterns observed show similarity to both AG 2-1 and AG BI, and suggested that these isolates may be related to both of these AG. Consequently, Nicoletti *et al.* (1999) have proposed that tobacco isolates may form a new zymogram subgroup within AG 2-1.

The following experiments were conducted to document the morphological characters of the *R. solani* isolates used in this study and to verify their placement into AG and ZG. In addition, experiments were conducted to examine the extent of genetic variability among AG 2 isolates from Australia, Japan and the Netherlands, using vertical gel electrophoretic separation of pectic isozymes.

2.2. Materials and methods

2.2.1. Culture of fungal isolates

Isolates of *R. solani* AG 2 and representative isolates of various other AG were obtained from research groups in Australia, the Netherlands and Japan (Table 2.1). Isolates were given a code to denote AG (first two digits or letters); R to denote *Rhizoctonia*; a code to denote country of origin (Au, J, Nz or N; Australia, Japan, New Zealand or the Netherlands, respectively); a code to denote host from which the isolate was obtained (B, Br, C, Cf, L, M, P, Pe, Pr, O or S; barley, *Brassica*, canola, cauliflower, lupin, medic, potato, pea, pine, oat

and soil, respectively); and an identifying digit. For example, isolate 21RAuP08 is an AG 2-1 *Rhizoctonia* from Australia, originally isolated from potato. All isolates were cultured on Difco Potato Dextrose Agar (PDA; see appendix A) in 9 cm Petri dishes, at room temperature (approximately 23°C) in darkness. For long-term storage, isolates were maintained on PDA slopes covered with 5 ml sterile paraffin oil and these long-term isolates were sub-cultured annually to check viability.

2.2.2. Anastomosis reaction and determination of nuclear number

The method of Carling *et al.* (1988) was used to assign *R. solani* isolates to an anastomosis group, by pairing them with 'tester isolates' and determining the frequency of hyphal fusion. Isolates were assigned to the following categories:

C0 Not related and belong to different AG

C1 Distantly related and belong to different AG

C2 Related and belong to the same AG

C3 Closely related belonging to the same AG

Two AG 2-1 and two AG 2-2 isolates, plus an AG 3, AG 4, AG 8, a binucleate and a bridging isolate were used as testers for the anastomosis procedure. These isolates were well characterised by the research groups from which they were obtained. The "PDA coated slide" method of Sanders *et al.* (1978)was used to oppose isolates and to determine the nuclear numbers of AG 2 isolates (Yang *et al.*, 1991). For each isolate examined, two replicate slides were used. A 5 mm^2 plug of PDA was taken from the growth front of 3-day-old cultures of two different isolates and placed at opposite ends of PDA-coated microscope slides. The slides were placed in 9 cm Petri dishes containing sterile, moist filter paper and incubated at 23°C in darkness. Once hyphae reached confrontation (24–72 h), the agar was dried on the slides in a laminar flow cabinet and the transfer plug removed.

Table 2.1: The collection of *R. solani* isolates used in this study. Isolates were obtained from various research groups and given an isolate code for the purposes of this research. Missing information is represented by a question mark (?).

Isolate code	AG[1]	ZG[1]	Original Designation	Origin[2]	Host
BIR01	BI	?	?	Ogoshi / Japan	?
BNR03	BN	?	1396	?	?
03RAuP01	3	7	R 37	Balali / SA	Potato
04RAuP01	4	?	R.49	Balali / SA	Potato
05RJSb01	5	?	GM-10	Ogoshi / Japan	Soybean
06RJS01	6	?	OHT-1-1	Ogoshi / Japan	Soil
08RAuB08	8	1	1512	Neate / SA	Barley
09R01	9	?	AG 9	O'Brien / ?	?
02RAu01	2	?	1341	?	?
02RAuCf02	2	?	C5959	Sweetingham / WA	Cauliflower
02RAuCf03	2	?	C6596	Sweetingham / WA	Cauliflower
02RAuL04	2	?	R456	Sweetingham / WA	Lupin
21RJ01	2-1	5	Ps-4	Ogoshi / Japan	Pea
21RAuC02	2-1	5	200-C	Dennis / SA	Canola
21RAuM03	2-1	5	201-M	Dennis / SA	Medic
21RAuM04	2-1	5	202-M	Dennis / SA	Medic
21RN06	2-1	5	2-1R31	Schneider / Netherlands	Crucifer
21RN07	2-1	5	2-1R81	Schneider / Netherlands	Crucifer
21RAuP08	2-1	6	R 20	Balali / SA	Potato
21RAuW09	2-1	5	1344	Warcup / SA	Wheat
21RAuW10	2-1	5	189	Kelly / SA	Wheat
21RAuB11	2-1	6	376	Kelly / SA	Barley
21RAuB12	2-1	6	377	Kelly / SA	Barley
21RAuB13	2-1	6	378	Kelly / SA	Barley
21RAuB14	2-1	5	434	Kelly / SA	Barley
21RAuC15	2-1	5	WAC 9283	Sweetingham / WA	Canola
21RAuPe16	2-1	5	R1670	Sweetingham / WA	Pea
21RAuL17	2-1	5	R2224	Sweetingham / WA	Lupin
21RAu18	2-1	5	SCR113	O'Brien / SA	?
21RAu19	2-1	5	SCR114	O'Brien / SA	?
21RAuPr20	2-1	6	Tas D	O'Brien / Tas	Pine
21RNzBr21	2-1	6	NZT 7	O'Brien / NZ	Brassica
22RJ01	2-2 IV	4	1627	Ogoshi / Japan	?
22RJ02	2-2 IIIB	10	C-96	Ogoshi / Japan	Mat rush
22RN03	2-2 IIIB	10	2-2R52	Schneider / Netherlands	Sugar beet
22RN04	2-2 IIIB	10	2-2R54	Schneider / Netherlands	Sugar beet
22RN06	2-2 IIIB	10	2-2R80	Schneider / Netherlands	Sugar beet
22RAuO08	2-2	10	319	Kelly / SA	Oats
22RAuL09	2-2 IV	4	R614	Sweetingham / WA	Lupin
22RAuS10	2-2 IV	10	R939	Sweetingham / WA	Soil
22RAuL11	2-2 IIIB	4	R56	O'Brien / WA	Lupin
22RAuL12	2-2 IIIB	4	R57	O'Brien / WA	Lupin
2tRN01	2t	5-1	2tR144	Schneider / Netherlands	Tulip

1 AG and ZG as determined by research group which provided the isolate; BI = bridging isolate; BN = binucleate isolate

2 SA = South Australia; WA = Western Australia; Tas = Tasmania, Australia; NZ = New Zealand.

The hyphae, embedded in the dried agar, were stained with lactophenol-trypan blue to assess anastomosis by light microscopy, or Hoechst 33258 dye (4.8 µg/ml, pH 7.8) to determine the number of nuclei per hyphal cell by fluorescence, using a Zeiss Standard 16 Lab microscope with excitation filter BP 450-490 and barrier filter LP 520. Anastomosis reactions were determined by calculating the frequency of each reaction type observed in five fields of view and the isolate classified by the most frequent reaction observed. The nuclear number was recorded as the average number of nuclei counted in 10 random cells, of varying age.

2.2.3. Morphology of AG 2 isolates

A 5 mm^2 plug of AG 2 inoculum was transferred to PDA and incubated at room temperature, in darkness. The morphological characteristics of each isolate were recorded after 4 weeks of incubation. The colour of the upper and lower surface of each colony was determined by reference to a mycological colour chart (Rayner, 1970). The colony diameter of each isolate, excluding the colony transfer plug, was recorded after 72 h incubation at 23°C. In addition, growth at 35°C was determined as another indicator for separating AG 2-2IIIB and AG 2-2IV isolates (Sneh *et al.*, 1991). A 5 mm^2 plug of AG 2-2 inoculum was transferred to the centre of PDA plates and incubated at 35°C for 72 h, in darkness. Emergence of hyphae from the transfer plug was used to indicate the ability of isolates to grow at 35°C.

2.2.4. Induction of pectic isozymes

Pectic isozymes were induced in culture using a modification of the method of Sweetingham *et al.* (1986). A 5 mm^2 plug from the colony margin of a 3-day-old PDA culture was transferred to 2 ml of induction medium (appendix A), held in 24-well cell culture plates (Flow Laboratories Inc., Virginia, USA) and incubated at 23°C in darkness for 9 days. Each isolate was prepared in duplicate. The culture fluids were removed and duplicates pooled, before being centrifuged at 5,000 *g* for 5 min to remove hyphal debris. Approximately 3.5 ml

of the supernatant was added to 1 ml of pectin PAGE sample buffer (appendix A) and stored at -20°C prior to determining total protein content and subsequent electrophoresis.

2.2.5. Protein assay

The total protein content in each sample was determined using the Biuret procedure (Alexander and Griffiths, 1993). Briefly, 1.5 ml Biuret reagent (12 mM $CuSO_4.5H_20$ and 42 mM NaK tartrate: 1.5 M NaOH, mixed 1:1) and 1.5 ml diluted culture sample, described above, were mixed by vortexing and incubated at 37°C for 15 min. Absorbance at 540 nm was recorded using a Beckman DU-68 spectrophotometer with the Protein Assay Soft-Pac Module (Beckman Instruments, California, USA). Total protein was calculated using a standard curve obtained with 0 to 1.2 mg/ml of bovine serum albumin (BSA) (Sigma).

2.2.6. Vertical gel electrophoresis

A modified version of the vertical gel technique of Schneider *et al.* (1997) was used for zymogram analysis of AG 2 isolates. Pectic enzymes, produced by each isolate, were separated in non-denaturing, pectin polyacrylamide gels using a vertical gel system (Hoeffer Scientific, Inc.). The resolving gels contained 0.375 M Tris–HCl (pH 8.8), 12% (w/v) acrylamide:bisacrylamide (36.5:1; Bio-Rad), 0.2% (w/v) citrus pectin (Sigma, lot 27F-0030) 0.05% (v/v) ammonium persulfate and 0.1% (v/v) TEMED (N,N,N',N'–tetramethylethylenediamine). Stacking gels contained 0.125 M Tris–HCl (pH 6.8), 4% acrylamide:bisacrylamide (36.5:1), 0.2% citrus pectin, 0.05% ammonium persulfate and 0.2% TEMED. Gels were cast between 16 cm x 18 cm glass plates, separated by 0.75 mm plastic spacers. The stacking gel was cast to 2 cm below the sample well depth. Aliquots containing 15 μg total protein were removed from sample preparations and bromophenol blue was added to a final concentration of 0.0125% (v:v), before being loaded into wells. Each gel contained the samples to be tested, along with protein preparations of isolates for which the ZG had been determined previously. Electrophoresis was conducted in running buffer containing 250

mM Tris-HCl and 1.92 M glycine (pH 8.3), for 4 h at 250 V. The electrophoresis system was cooled to 5°C using a refrigerated circulating water cooler (Thermoline Scientific Equipment, Australia). Following electrophoresis, gels were incubated in 0.1 M DL–malic acid (Ajax Chemicals, Australia) for 90 min with gentle agitation at room temperature. Gels were rinsed twice in ddH_2O and stained with 0.02% (w/v) ruthenium red (ICN) for 4 h with gentle agitation at room temperature. After rinsing the gels twice with ddH_2O, they were destained in 3 mM Na_2CO_3 overnight with gentle agitation at room temperature. Gels were photographed over a light box containing fluorescent lights to produce both positive and negative prints (Polaroid 665 film).

2.2.7. Data analysis

The electrophoretic patterns were scored as alleles present at the loci coding for polygalacturonase (dark bands) and pectin esterase (light bands) according to Cruickshank and Wade (1980). The R_f values were calculated for consistently present bands (alleles) to enable an accurate comparison of patterns across isolates and gels. Bands were scored as present (1) or absent (0), to form a binomial representation of the banding pattern. Genetic similarity was calculated using the similarity index of Nei and Li (1979), and pairwise comparisons were made between all isolates to generate a similarity matrix. Cluster analysis was performed on percent similarity using the group average analysis method (UPGMA, Sneath and Sokal, 1973), using the GENSTAT 5, release 4.1, statistical package (Rothamsted Experimental Station, 1998).

2.3. Results

2.3.1. Anastomosis grouping

Anastomosis reactions, as described by Carling *et al.* (1988), were recorded for all AG 2 isolates to confirm their placement in AG 2-1 or AG 2-2 (Table 2.2A and B). Examples of the anastomosis reaction categories observed are presented in Figure 2.1. The majority of the isolates in the collection were classified as either AG 2-1 or AG 2-2 based on the frequency of the C2 reaction. Isolates 02RAu01, 02RAuCf02, 02RAuCf03 and 02RAuL04, could not be classified by anastomosis, as the C2 reaction occurred frequently when these isolates were opposed to both AG 2-1 and AG 2-2 tester isolates. Therefore, these isolates were termed "AG 2 undefined" for the purposes of this thesis. None of the AG 2 isolates gave a C2 reaction with the AG 3, 4, or 8 tester isolates. However, both the C1 and C2 reaction occurred between the Australian AG 2-1 isolate, 21RAuC02, and the bridging isolate, BIR01. No interaction (C0) between hyphae of the binucleate tester BNR01 and the AG 2 isolates was observed.

2.3.2. Morphology of AG 2 isolates

The common features associated with *R. solani*, such as hyphae with regular septa and multinucleate cells, were present among all the AG 2 isolates examined. No morphological characters could be used to distinguish the AG 2 subgroups. The morphological characters of the Australian isolates were similar to the isolates from other countries (Table 2.3A and B). Aerial hyphae occurred for a number of isolates and ranged from low to high density. The pigmentation of cultures varied from buff, as seen for isolate 21RAuB11, to dark brick described for isolates such as 22RJ02 (Figure 2.2). Pseudo-sclerotia, termed sclerotia for this thesis, were observed for the majority of isolates examined and, where present, were either light or dark brown and ranged in size from less than 1mm to approximately 5mm in diameter (Table 2.3 A&B). The positioning of sclerotia varied amongst the isolates. For instance,

ordered positioning seen when sclerotia occurred at the centre and outer edges of the colony, such as for isolate 21RAuP08, or when sclerotia occurred in concentric rings following the pattern of aerial hyphae, such as for isolate 22RN03. For isolates such as 02RAuCf02, the sclerotia were scattered amongst the colony. The nuclear number for all isolates varied between 4 to 16 nuclei per cell, with the AG 2-1 isolates averaging 8.6 (± 0.59) nuclei per cell and AG 2-2 isolates averaging 6.7 (± 0.54) nuclei per cell. The hyphal width for all the isolates ranged between 7 to 12 μm, which fell within the expected range for isolates of *R. solani*.

Considerable variation in colony diameter among the isolates was observed. The majority of the isolates grew at a rate above 0.40mm/h (Table 2.3A & B). The Japanese isolate21RJ01 had the slowest growth (0.14mm/h) of all isolates examined, while for the Netherlands AG 2-2 isolates, 22RN03, 22RN04 and 22RN06, the rate of growth was approximately 1.3 to three times faster than the majority of AG 2-1 and AG 2-2 isolates, at approximately 0.90 – 0.95 mm/h (Table 2.3B). The Australian isolates varied in growth rate with the slowest rate being 0.21 mm/h for the "AG 2 undefined" isolates 02RAuCf03 and 02RAuL04, while the faster growing isolates had a rate of above 0.45 mm/h. The growth of isolates at 35°C was used to place the AG 2-2 isolates 22RJ02, 22RN03, 22RN04, 22RN06, 22RAuL11 and 22RAuL12 into AG 2-2IIIB.

2.3.3. Zymogram analysis

Evidence to support the classification of AG 2 isolates into subgroups and to assess genetic variation was obtained from pectic zymogram analysis. Vertical gel electrophoresis of pectic isozymes from 31 *R. solani* isolates belonging to AG 2 revealed the presence of 27 distinct electrophoretic patterns. The banding pattern obtained for the isolates was affected by the amount of total protein loaded into the wells of the gel. Flaring and smearing of the bands on the vertical acrylamide gel was limited by optimising the loading of total protein to 15 μg per

Table 2.2A: Anastomosis reactions observed for AG 2-1 isolates when opposed to tester isolates from various AG. The frequency of anastomosis reactions in five random fields of view was used to determine the grouping of the isolates. NT = not tested

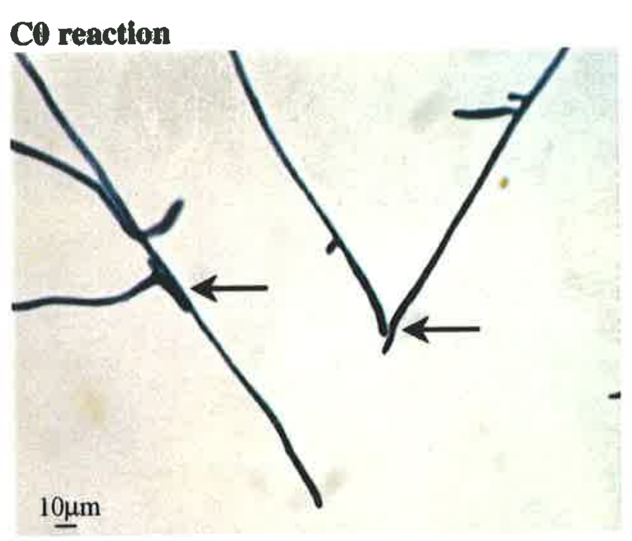
	Test isolate								
Isolate code	21RJ01	21RAuC02	22RJ02	22RAuL11	03RAuP01	04RAu01	08RAuB08	BNR01	BIR01
21RJ01	C3	C2	C1	C1	C1	C0	C0	C0	C0
21RAuC02	C2	C3	C0	C1	C0	C0	C1	C0	C1/C2
21RAuM03	C2	C2	C1	C0	C0	C0	C1	C0	C1
21RAuM04	C2	C1	C1	C1	C0	C0	C0	C0	C0
21RN06	C2	C2	C0	C0	C0	C0	C0	C0	C0
21RN07	C2	C2	C1	C0	C1	C0	C0	C0	C1
21RAuP08	C1	C2	C1	C0	C0	C0	C0	C0	C0
21RAuW09	C2	C2	C0	C1	C0	C1	C1	C0	NT
21RAuW10	C2	C2	C0	C1	C0	C0	C0	C0	NT
21RAuB11	C2	C1	C0	C0	C1	C0	C1	C0	NT
21RAuB12	C1	C2	C1	C1	C0	C0	C1	C0	NT
21RAuB14	C2	C2	C1	C0	C0	C0	C0	C0	NT
21RAuC15	C2	C1	C0	C1	C0	C0	C1	C0	C1
21RAuPe16	C1	C2	C1	C0	C0	C0	C0	C0	NT
21RAuL17	C1	C2	C0	C0	C0	C0	C1	C0	NT
21RAu18	C2	C1	C0	C0	C0	C0	C1	C0	NT
21RAu19	C2	C2	C1	C1	C0	C0	C0	C0	NT
21RAuPr20	C2	C2	C1	C0	C0	C0	C0	C0	NT
21RNzBr21	C2	C2	C0	C0	C0	C0	C0	C0	NT
2tRN01	C1	C2	C0	C0	C0	C0	C0	C0	C0

Table 2.2B: Anastomosis reactions observed for AG 2-2 isolates and isolates termed "AG 2 undefined", when opposed to tester isolates from various AG. The frequency of anastomosis reactions in five random fields of view was used to determine the grouping of the isolates. NT = not tested

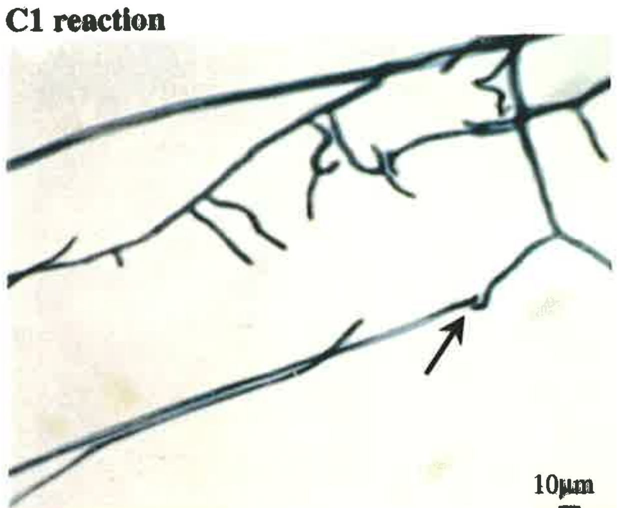
	Test isolate								
Isolate code	**21RJ01**	**21RAuC02**	**22RJ02**	**22RAuL11**	**03RAuP01**	**04RAu01**	**08RAuB08**	**BNR01**	**BIR01**
22RJ01	C1	C0	C2	C2	C0	C0	C0	C0	C0
22RJ02	C1	C1	C3	C2	C0	C0	C0	C0	C1
22RN03	C1	C0	C2	C1	C0	C1	C0	C0	C1
22RN04	C0	C0	C1	C2	C0	C0	C0	C0	C0
22RN06	C0	C0	C2	C1	C0	C0	C1	C0	NT
22RAuL09	C1	C0	C2	C2	C0	C0	C0	C0	NT
22RAuS10	C0	C1	C1	C2	C0	C0	C1	C0	NT
22RAuL11	C0	C1	C2	C3	C0	C0	C1	C0	C0
22RAuL12	C0	C1	C1	C2	C0	C0	C0	C0	C0
02RAu01	C1	C2	C2	C1	C0	C0	C0	C0	NT
02RAuCf02	C2	C1	C1	C2	C0	C1	C1	C0	NT
02RAuCf03	C1	C2	C1	C2	C0	C0	C0	C0	NT
02RAuLO4	C2	C1	C1	C2	C0	C0	C0	C0	NT

Figure 2.1: Examples of the anastomosis reactions used to group AG 2 isolates. Hyphae along the confrontation zone on agar-coated slides were stained with trypan blue and covered with a glass cover slip. The interaction between hyphae of two opposing isolates was observed using a light microscope at x200, x400 and x1000 magnification. The number and types of reactions in five random fields of view were observed and the frequency of anastomosis determined. Arrows indicate the reaction site for each of the classifications.

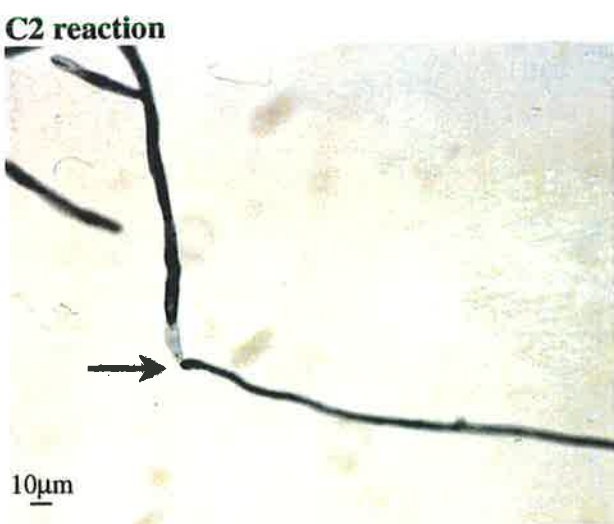
C0 reaction



C1 reaction



C2 reaction



C3 reaction

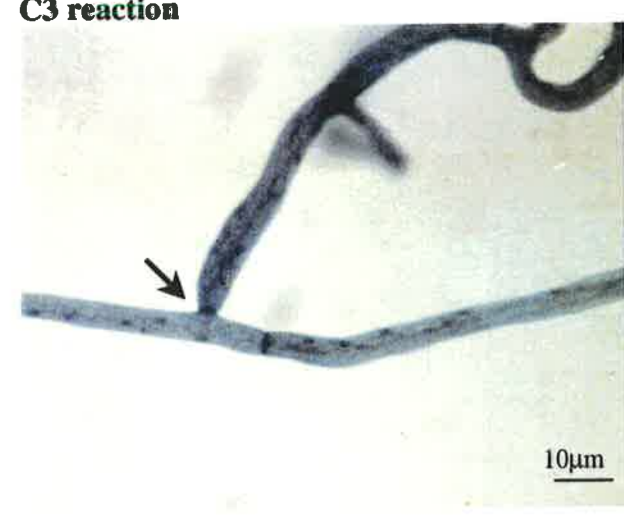


Table 2.3A: Morphological characters of *R. solani* AG 2-1 isolates used in this study. Cultures were grown at 23°C in darkness, for 4 weeks, unless otherwise stated. The growth rate is presented as the mean of three replicates, with the standard error in parenthesis.

Isolate code	Macroscopic features: Aerial hyphae		Cultural appearance on surface			Sclerotia			Growth rate (mm/hour)	Growth at 35°C
	Presence	Density	Upper[a]	Lower[a]	Zonation	Colour	Size	Position		
21RJ01	no	np	buff	dark buff	concentric	dark brown	5mm	concentric	0.14 (0.01)	no
21RAuC02	yes	medium	buff	cinnamon	concentric	dark brown	< 1mm	concentric	0.38 (0.05)	no
21RAuM03	yes	low	umber	dark umber	concentric	light brown	< 1mm	scattered	0.69 (0.02)	no
21RAuM04	yes	high	buff	cinnamon	radial	dark brown	3mm	scattered	0.56 (0.01)	no
21RN06	yes	medium	dark brick	sepia	concentric	light brown	< 1mm	concentric	0.63 (0.03)	no
21RN07	no	np	dark brick	sepia	radial	light brown	< 1mm	scattered	0.73 (0.02)	no
21RAuP08	yes	medium	buff	honey	radial	dark brown	5mm	c/e	0.42 (0.04)	no
21RAuW09	no	np	honey	cinnamon	radial	np	np	np	0.35 (0.02)	no
21RAuW10	yes	Low	honey	cinnamon	radial	np	np	np	0.42 (0.01)	no
21RAuB11	no	np	buff	buff	radial	dark brown	5mm	central	0.53 (0.02)	no
21RAuB12	no	np	buff	buff	radial	np	np	np	0.48 (0.01)	no
21RAuB14	yes	Low	buff	cinnamon	concentric	light brown	< 1mm	concentric	0.53 (0.02)	no
21RAuC15	no	np	buff	cinnamon	concentric	dark brown	< 1mm	c/e	0.52 (0.02)	no
21RAuPe16	yes	low	umber	umber	concentric	dark brown	5mm	c/e	0.63 (0.02)	no
21RAuL17	yes	medium	buff	umber	concentric	dark brown	< 1mm	scattered	0.31 (0.02)	no
21AuR18	yes	medium	dark buff	umber	concentric	dark brown	< 1mm	c/e	0.42 (0.01)	no
21AuR19	yes	low	buff	umber	concentric	dark brown	< 1mm	c/e	0.31 (0.01)	no
21RAuPr20	yes	low	buff	cinnamon	concentric	dark brown	< 1mm	c/e	0.48 (0.01)	no
21RNzBr21	no	np	buff	cinnamon	concentric	np	np	np	0.45 (0.01)	no
2tRN01	yes	medium	umber	sepia	concentric	light brown	3mm	scattered	0.33 (0.02)	no

[a] Colour based on Rayner's Mycological Colour Chart (1970)
np = not present; c/e = centre and edges

Table 2.3B: Morphological characters of *R. solani* AG 2-2 and "AG 2 undefined"* isolates used in this study. Cultures were grown at 23°C in darkness, for 4 weeks, unless otherwise stated. The growth rate is presented as the mean of three replicates, with the standard error in parenthesis.

Isolate code	Macroscopic features: Aerial hyphae		Cultural appearance on surface			Sclerotia			Growth rate (mm/hour)	Growth at 35°C
	Presence	Density	Upper[a]	Lower[a]	Zonation	Colour	Size	Position		
22RJ01	yes	low	buff	honey	concentric	light brown	< 1mm	scattered	0.42 (0.02)	no
22RJ02	yes	low	dark brick	sepia	concentric	dark brown	< 1mm	concentric	0.45 (0.03)	yes
22RN03	yes	medium	honey	cinnamon	concentric	dark brown	5 mm	concentric	0.94 (0.02)	yes
22RN04	yes	medium	dark brick	sepia	concentric	dark brown	< 1mm	concentric	0.92 (0.03)	yes
22RN06	yes	low	buff	sepia	concentric	dark brown	< 1mm	concentric	0.94 (0.02)	yes
22RAuL09	yes	high	white	vinaceous buff	radial	np	np	np	0.63 (0.03)	no
22RAuS10	no	np	white	vinaceous grey	radial	np	np	np	0.31 (0.02)	no
22RAuL11	no	np	umber	dark sepia	radial	np	np	np	0.72 (0.01)	yes
22RAuL12	yes	medium	buff	buff	radial	dark brown	5 mm	central	0.64 (0.02)	yes
02RAu01*	no	np	buff	buff	radial	np	np	np	0.21 (0.01)	no
02RAuCf02*	yes	high	buff	sepia	np	dark brown	< 1mm	scattered	0.64 (0.01)	no
02RAuCf03*	yes	low	sepia	sepia	np	dark brown	5 mm	scattered	0.21 (0.02)	no
02RAuL04*	no	np	buff	cinnamon	np	np	np	np	0.42 (0.01)	no

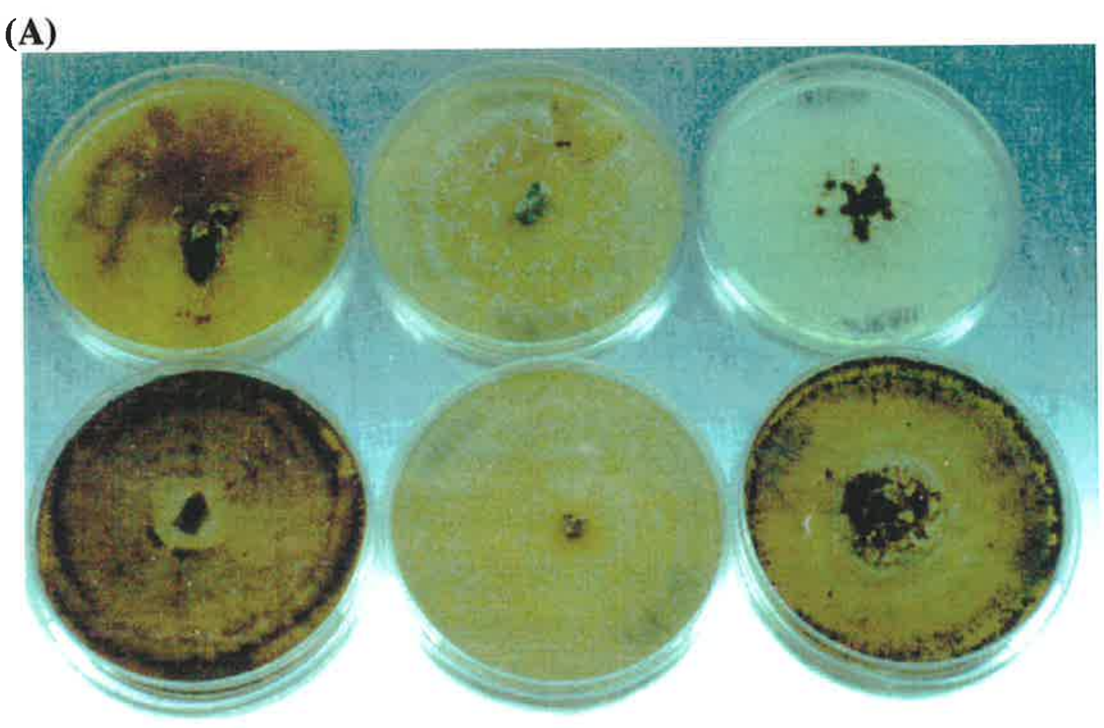
[a] Colour based on Rayner's Mycological Colour Chart (1970)
np = not present; c/e = centre and edges

Figure 2.2: Examples of colony morphology for isolates of *R. solani* AG 2-1, AG 2-2 and "AG 2 undefined". Isolates were grown on Difco PDA at 23°C for 4 weeks in darkness in a controlled environment cabinet and were assessed for culture colour, hyphal type and the presence and placement of sclerotia.

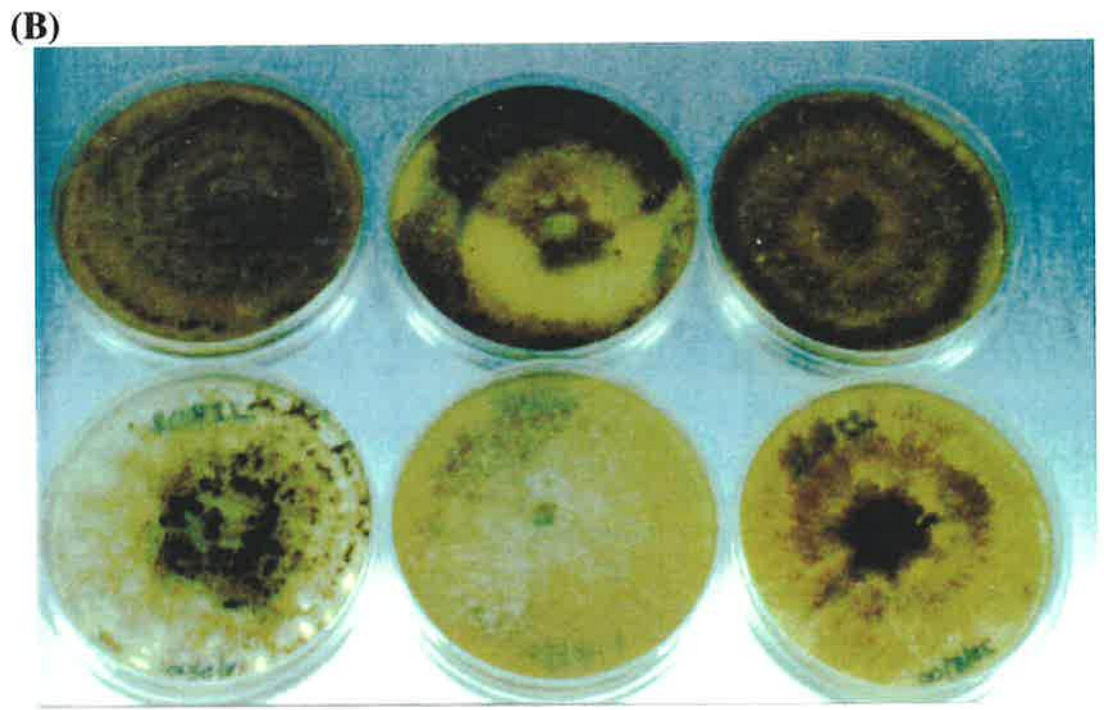
A) From top left – 21RAuM03, 21RAuW10, 21RAuB11, 21RN06, 21RNzBr21, 21RAu19.

B) From top left – 22RN04, 22RN06, 22RJ02, 22RAuL12, 02RAu01, 22RJ01.

(A)



(B)



well (Figure 2.3). The banding profiles of the pectic isozymes produced by the isolates of AG 2 used in this study are presented in Figure 2.4. The analysis separated the AG 2-1 isolates from Australia, the Netherlands and Japan into the previously identified zymogram groups of ZG 5 and ZG 6. Genetic similarity among the AG 2-1 isolates ranged from 10% to 100% (appendix B), with the two major groups, AG 2-1, ZG 5 and AG 2-1, ZG 6, displaying approximately 10% similarity (Figure 2.5). The AG 2-1, ZG 5 isolates appeared to consist of seven different electrophoretic patterns (Figure 2.4, top gel). Two subgroups were apparent and were distinguished by the presence of a single band, '*K*'. The larger group consisted of eight isolates from various locations and hosts, all of which had the common band, '*J*', and the lower band, '*K*'. Isolate 21RJ01, from Japan, also displayed the bands '*J*' and '*K*' (Figure 2.3, Lane 1). The smaller group of three isolates consisted of two Australian isolates, 21RAu18 and 21RAu19, and the AG 2t isolate, 2tRN01, from the Netherlands. The two Australian isolates displayed the band '*J*', in common with the other ZG 5 isolates, along with the band '*I*', which was also present in the AG 2t isolate and isolate 21RN06, originally from crucifer in the Netherlands (Figure 2.4, top gel). The AG 2t isolate appeared to have a unique banding pattern when compared to the other isolates.

Similarly, for AG 2-1 isolates belonging to ZG 6, six electrophoretic patterns were observed amongst the six isolates examined. All isolates grouped as ZG 6 shared the common bands '*A*' and '*B*' (Figure 2.4, middle gel). In addition, ZG 6 isolates could be further sub-divided into two groups with approximately 30% similarity, based on the presence of two additional bands, '*F*' and '*H*'. One group consisted of three Australian isolates from barley, 21RAuB11, 21RAuB12 and 21RAuB13, and an "AG 2 undefined" isolate 02RAu01, while the second group contained two Australian isolates, 21RAuP08 and 21RAuPr20, and the New Zealand isolate 21RNzBr21, all from various hosts (Figure 2.5).

Figure 2.3: Pectin PAGE of isolates belonging to AG 2-1. Lanes 1 - 5 were loaded with 15 μg of total protein, while lanes 6 - 10 were loaded with 30 μg total protein. A polyacrylamide gel (12%) was used to separate pectic isozymes at 250 v, using Tris/glycine running buffer.

15 μg loads - Lane 1, 21RJ01; lane 2, 21RAu19; lane 3, 21RAuC02; lane 4, blank; lane 5, 21RAuPr20.

30 μg loads - Lane 6, 21RJ01; lane 7, 21RAu19; lane 8, 21RAuC02; lane 9, blank; lane 10, 21RAuPr20.

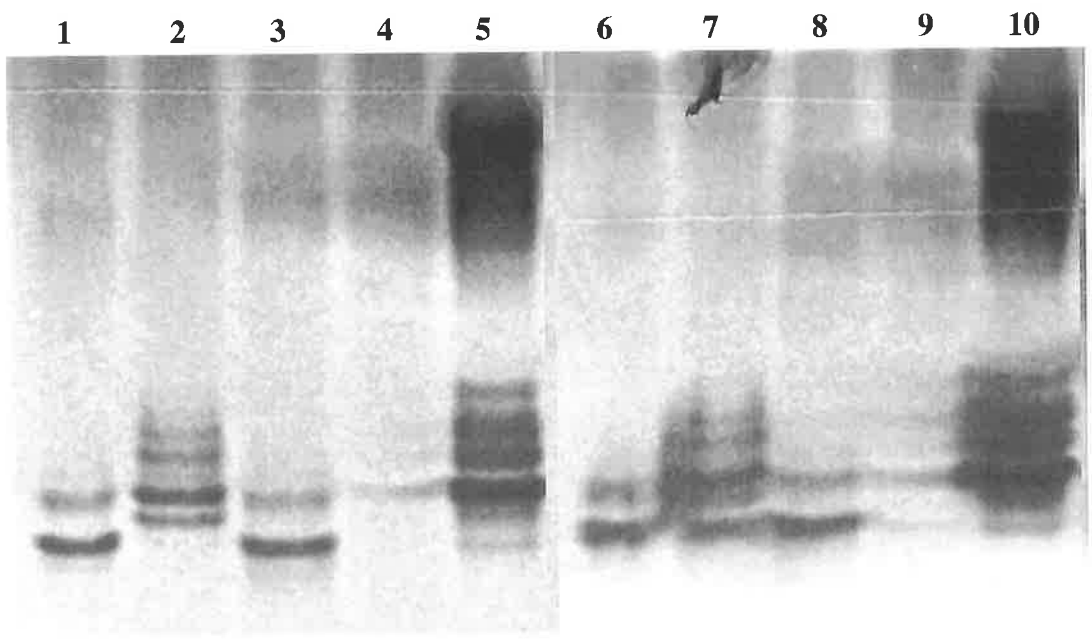
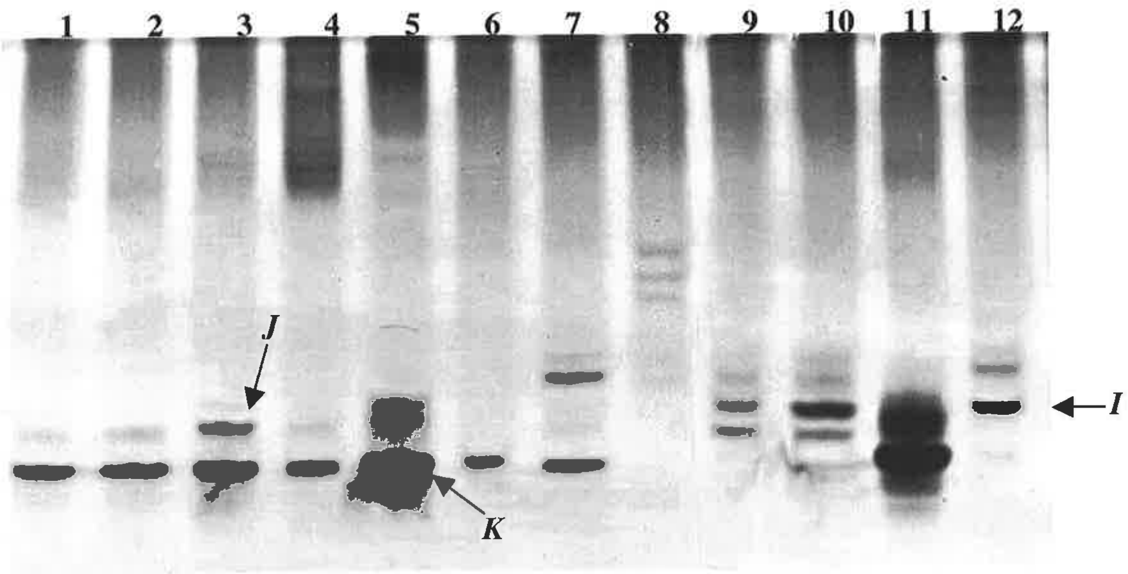

1
2
3
4
5
6
7
8
9
10

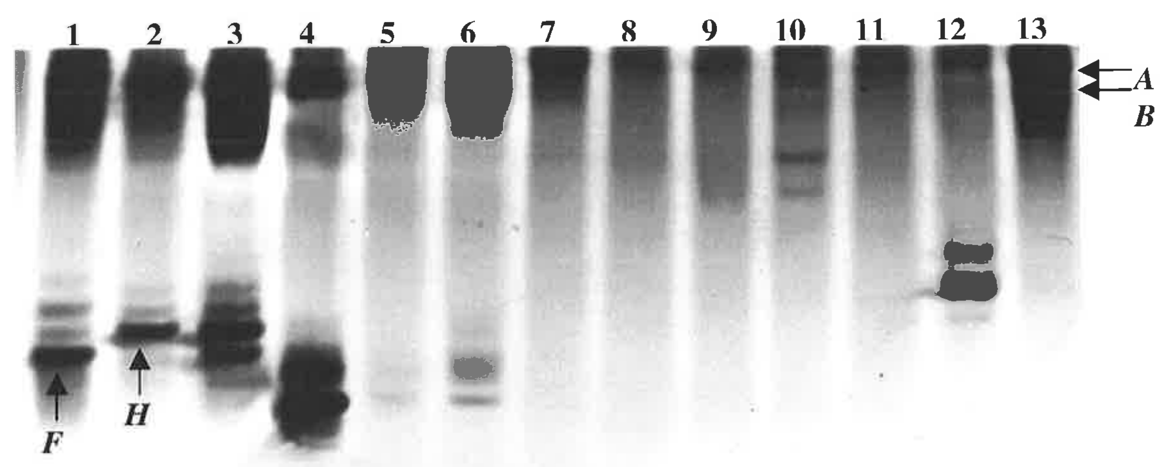
Figure 2.4: Pectin PAGE of isolates belonging to AG 2-1, AG 2-2, AG 2t and "AG 2 undefined". Polyacrylamide gels (12%) were used to separate pectic isozymes at 250 v, using Tris/glycine running buffer. Isolates, which had been previously grouped into ZG by horizontal gel analysis (test isolates), were used as controls to assign a ZG to each of the AG 2 isolates.

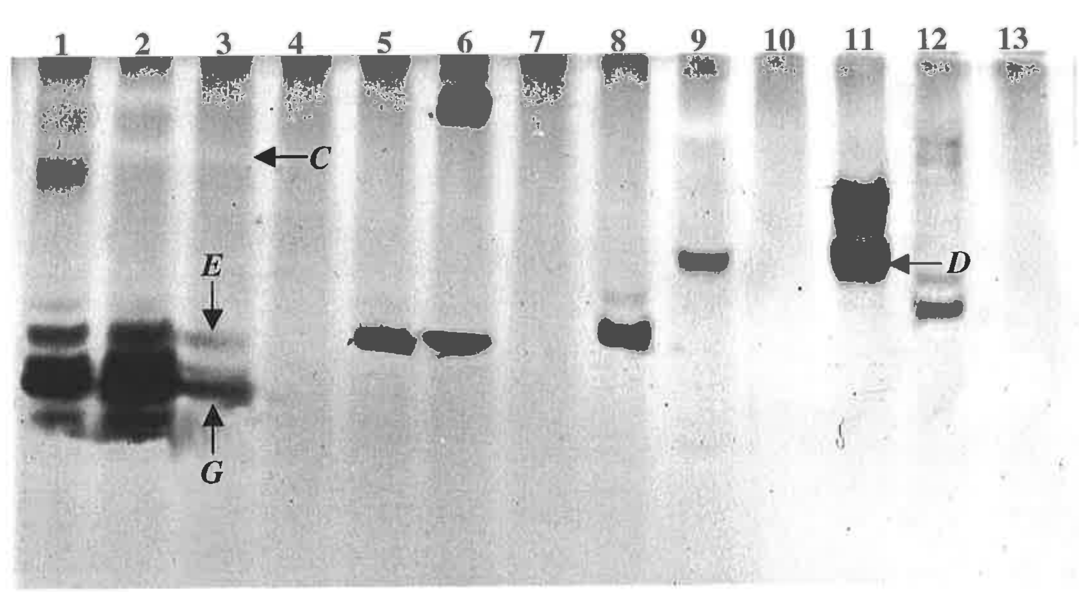
Top: AG 2-1 isolates belonging to ZG 5. Lane 1, 21RAuC02 (ZG 5 test isolate); lane 2, 21RAuC15; lane 3, 21RAuPe16; lane 4, 21RAuM03; lane 5, 21RAuM04; lane 6, 21RAuL17; lane 7, 21RN06 (ZG 5 test isolate); lane 8, 21RN07; lane 9, 21RAu18 (ZG 5 test isolate); lane 10, 21RAu19 (ZG 5 test isolate); lane 11, 21RAuW10 (ZG 5 test isolate); lane 12, 2tRN01 (AG 2t test isolate).

Centre: AG 2-1 isolates belonging to ZG 6 and "AG 2 undefined". Lane 1, 21RAuP08 (ZG 6 test isolate); lane 2, 21RNzBr21; lane 3, 21RAuPr20; lane 4, 21RAuW10 (ZG 5 test isolate); lane 5, 21RAuB11; lane 6, 21RAuB12 (ZG 6 test isolate); lane 7, 21RAuB13 (ZG 6 test isolate); lane 8, blank; lane 9, 02RAu01; lane 10,02RAuCf02 ; lane 11, blank; lane 12, 02RAuCf03; lane 13, 02RAuL04.

Bottom: AG 2-2 isolates belonging to ZG 4 and 10. Lane 1, 22RAuL09 (ZG 4 test isolate); lane 2, 22RAuL11; lane 3, 22RAuL12; lane 4, blank; lane 5, 22RJ02 (ZG 10 test isolate); lane 6, 22RAuS10; lane 7, blank; lane 8, 22RJ01 (ZG 10 test isolate); lane 9, 22RN03; lane 10, blank; lane 11, 22RN06 (ZG 4 test isolate); lane 12, 22RAuO08; lane 13, blank.


1 2 3 4 5 6 7 8 9 10 11 12
J
K
I


1 2 3 4 5 6 7 8 9 10 11 12 13
A
B
F
H


1 2 3 4 5 6 7 8 9 10 11 12 13
C
E
G
D

Nine electrophoretic patterns were observed for the nine AG 2-2 isolates examined (Figure 2.4, bottom gel), with a number of different patterns for both the ZG 4 and ZG 10 isolates. A comparison of banding patterns revealed the existence of two genetically isolated groups (Figure 2.5). AG 2-2 Group I isolates comprised four Australian isolates, 22RAuL09, 22RAuS10, 22RAuL11 and 22RAuL12, and one Japanese isolate (22RJ02), all which shared a common band, '*E*'. This group could be further sub-divided into both ZG 4 and ZG 10, with the ZG 4 isolates having two bands, '*C*' and '*G*', in common between isolates 22RAuL09, 22RAuL11 and 22RAuL12. The second group consisted of four isolates, one from Japan (22RJ01), two from the Netherlands (22RN03 and 22RN06) and one from Australia (22RAuO08). Again, the two subdivisions of ZG 4 and 10 were evident in this group, with the three ZG 10 isolates, 22RN03, 22RN06 and 22RAuO08, sharing band '*D*' (Figure 2.4, bottom gel; Figure 2.5).

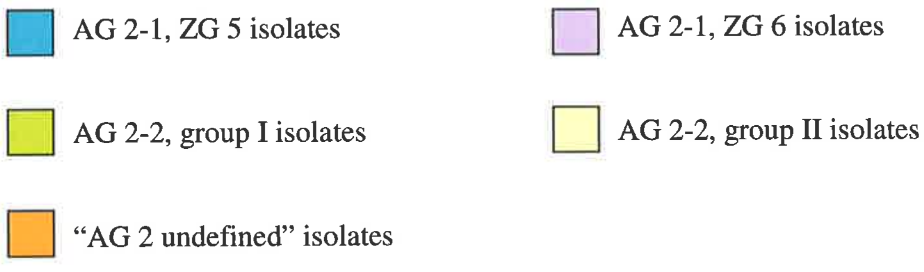
Analysis of similarity coefficients for the banding patterns produced by the 31 AG 2 isolates tested revealed the presence of five groups with an average similarity of approximately 10% (Figure 2.5). The genetic similarity across all isolates ranged from 0% to 100% (appendix B). The results suggested that three of the isolates classed as "AG 2 undefined", 02RauCf02, 02RauCf03 and 02RAuL04, are more similar to AG 2-2 group I isolates than to AG 2-1 or AG 2-2 group II isolates. However, since the similarity between these 'undefined' isolates, as well as between the AG 2-2 group 1 isolates was only 5 - 10%, it was not possible to group the isolates. One 'undefined' isolate, 02RAuCf02, appeared to be genetically related to isolate 21RN07, identified as an AG 2-1 by anastomosis, with 50%similarity. The remaining 'undefined' isolate, 02RAu01, appeared to be genetically related to AG 2-1 isolates belonging to ZG 6, as it had a genetic similarity greater than 50%.

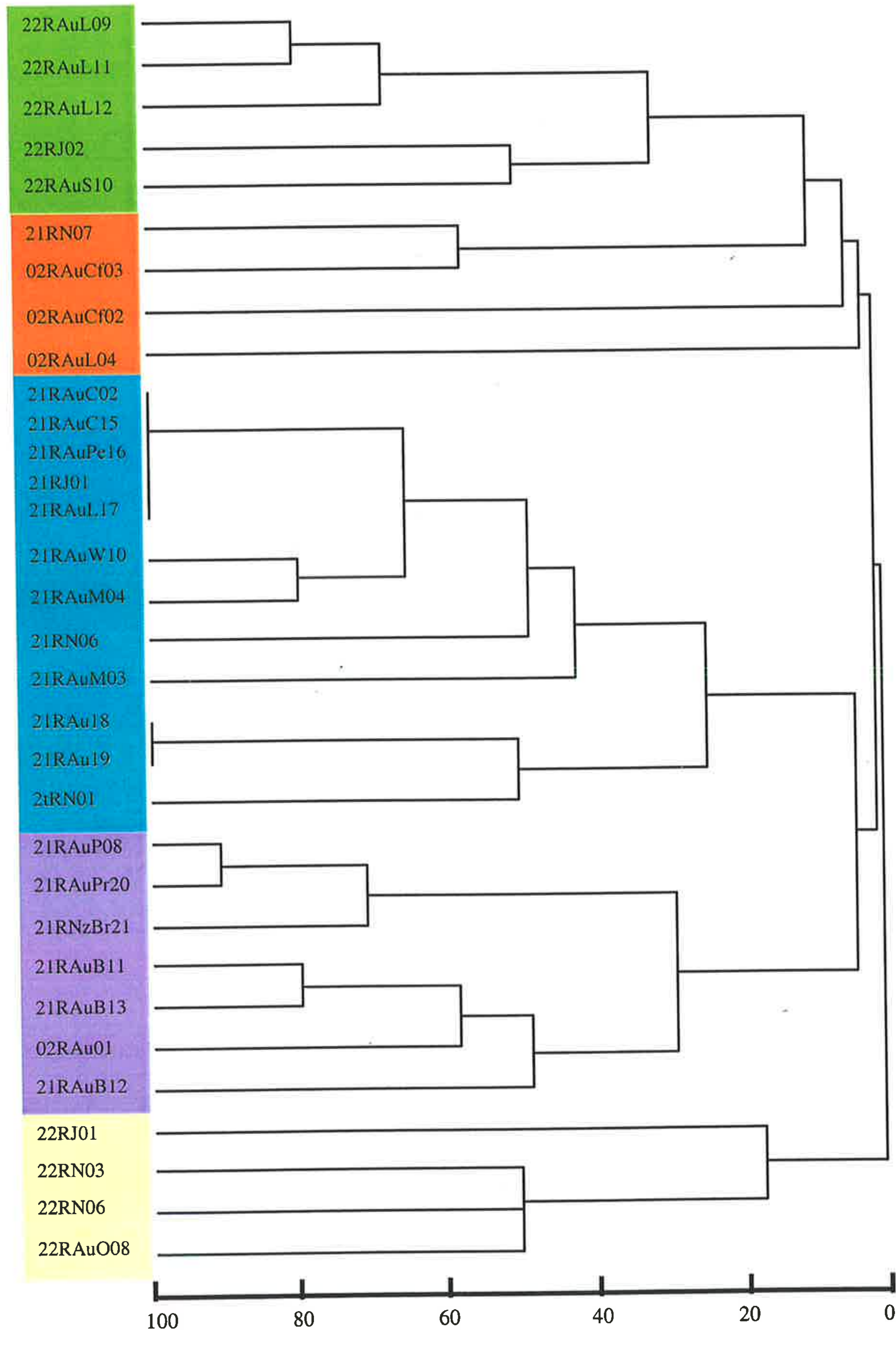
2.3. Discussion

Analysis of the morphological characters of 33 *R. solani* AG 2 isolates confirmed the general features associated with the species, however, no consistent characters could be identified for the AG 2 group as a whole, or for the established subgroups. Anastomosis reactions between the isolate collection and the tester isolates of various AG confirmed the placement of the majority of the isolates obtained from other researchers into either AG 2-1 or AG 2-2. However, four isolates from Australia remained unclassified by anastomosis, with the C2 reaction being observed frequently with both AG 2-1 and AG 2-2 testers. In addition, there was a mixed C1/C2 reaction between the AG 2-1 isolate, 21RAuC02, and the AG BI isolate, BIR01. This indicates a limitation in using anastomosis as the definitive means for classifying isolates into subgroups. Thus, to assign isolates into subgroups with confidence it is necessary to include other techniques such as zymogram analysis. An advantage of zymography over anastomosis is that an insight into the genetic relatedness of isolates may also be determined.

Vertical gel electrophoresis of pectic enzymes produced by AG 2 isolates from different locations within Australia and overseas revealed a low to high level of similarity. Based on UPGMA clustering analysis of zymogram patterns, AG 2 isolates could be placed into five groups. Two groups corresponded to ZG 5 and 6 for AG 2-1 isolates, respectively, while AG 2-2 isolates comprised two genetically distinct groups, with both ZG 4 and 10 being represented in each. The fifth group consisted of the three undefined AG 2 isolates from Australia and one isolate, 21RN07 from the Netherlands, which was previously allocated to AG 2-1 by anastomosis. Isolates within this group shared little similarity with any of the remaining isolates. The final undefined AG 2 isolate, 02RAu01, fell within the AG 2-1, ZG 6 cluster. There was no correlation between zymogram pattern and the location from which the isolates originated. However, the zymogram analysis of the AG 2-1 group was conducted on

Figure 2.5: Dendrogram showing genetic similarity of AG 2 isolates based on zymogram analysis. Similarities were calculated using the UPGMA method, following comparison of banding patterns, by Nei and Li's genetic index (1979).




22RAuL09
22RAuL11
22RAuL12
22RJ02
22RAuS10
21RN07
02RAuCf03
02RAuCf02
02RAuL04
21RAuC02
21RAuC15
21RAuPe16
21RJ01
21RAuL17
21RAuW10
21RAuM04
21RN06
21RAuM03
21RAu18
21RAu19
2tRN01
21RAuP08
21RAuPr20
21RNzBr21
21RAuB11
21RAuB13
02RAu01
21RAuB12
22RJ01
22RN03
22RN06
22RAuO08
100
80
60
40
20
0
Nei and Li's genetic similarity (%)

14 isolates from Australia, two from the Netherlands and one from both New Zealand and Japan whereas, of the AG 2-2 isolates, five were from Australia and two each from Japan and the Netherlands. Therefore, insufficient isolates were tested to draw conclusions from the use of zymograms to distinguish isolates from different locations. In addition, the use of more isolates from within Australia may provide more information on the existence of further zymogram subgroups, as was observed for the Netherlands isolates tested by Schneider *et al.* (1997).

The results obtained using a vertical gel system were comparable to published results from the traditional horizontal slab gel approach, suggesting that the isolates examined and the test isolates were true to type. For instance, in this study the three AG 2-1 isolates from barley displayed a ZG 6 pattern. This was previously reported by MacNish *et al.* (1993) using the horizontal gel system and their results suggested that AG 2-1 isolates from legumes belong to ZG 6. However, the results presented here suggested that a lupin isolate, 21RAuL17, and a pea isolate, 21RAuPe16, fell within ZG 5. It was evident that more isolates need to be tested before a true correlation between ZG and host can be determined. It also indicated that the zymogram groups of AG 2 were highly variable and that isolates within a ZG had the ability to infect a range of host types.

The vertical gel technique has been used previously to examine the relationship between AG 2 isolates from tulip and other plant hosts (Schneider *et al.*, 1997). Schneider *et al.* (1997) suggested that AG 2 isolates from tulip, designated AG 2t, can be grouped separately from other AG 2-1 isolates. They also suggested that AG 2-1 isolates pathogenic to both crucifer and tulip be classified as one group, proposing ZG 5-1 be allocated to describe the isolates. In the present study, the Netherlands AG 2t isolate, 2tRN01, displayed a unique zymogram pattern, with approximately 50% similarity to other AG 2-1, ZG 5 isolates. If this criterion for subgrouping is applied, then the remaining ZG 5 isolates could also be placed into subgroups, according to the data generated here. For example, isolates

21RAuC02, 21RAuC15, 21RAuPe16, 21RAuL17, 21RAuW10 and 21RAuM04 formed a large subgroup while isolates 21RN06 and 21RAuM03 formed subgroups as individuals. As only a limited number of ZG 5 isolates have been examined in this study, it was not possible to assign isolates to true subgroups, particularly when a single isolate was seen to constitute a subgroup. However, based on zymogram analysis, AG 2-2 isolates appeared to fall into two distinct groups, which have been referred to as groups I and II. What is evident from the vertical gel analysis of pectic isozymes is that AG 2 isolates display a high degree of genetic variability. It may be that different geographic regions will have a differing subset of ZG patterns within each population. Thus, the subgrouping of isolates based on zymogram analysis needs to be conducted with a large number of isolates from one region.

While vertical gel electrophoresis of pectic enzymes has given an insight into the variability of AG 2 isolates, molecular techniques may provide a more accurate measure of diversity. This is because zymogram analysis targets a limited number of genes within the fungal genome. In addition, there is some conjecture as to the role that pectinases play in the pathogenicity of *R. solani*. If a high degree of variability is obtained in zymogram analysis, as was observed here, then it is necessary to examine other regions of the genome to assist in the determination of AG 2 subgroups. The presence of a group of 'undefined' AG 2 isolates suggested that zymogram analysis may not give a full description of the genetic diversity present within this complex group, as the coding and expression of pectinases is probably only a part of the genetic information that defines an AG 2 isolate.

Chapter 3

Pathogenicity of AG 2 Isolates

3.1. Introduction

The broad host range of *R. solani* often makes it difficult to assign individual AG to a particular host or to define pathotypes within an AG. Disease symptoms can include root and hypocotyl rots, pre- and post-emergence damping off of seedlings, aerial blights, foliar lesions and cankering of underground tubers. *R. solani* isolates belonging to AG 2 also have a broad host range, infecting plants such as canola and rapeseed (Kataria and Verma, 1992), soybean (Liu and Sinclair, 1991; Naito and Kanematsu, 1994) and turf grass species (Burpee and Martin, 1992). Recent reports have identified subgroups of AG 2 isolates that appear to have more defined host preferences. For example, AG 2t isolates from the Netherlands are able to infect tulip bulbs and roots, whereas other AG 2-1 isolates do not. The AG 2t isolates, while grouped with AG 2-1, ZG 5, have a unique zymogram pattern and differ in ITS-RFLP phenotype when compared to other AG 2-1 isolates (Schneider *et al.*, 1997). Isolates specific to turf grass disease have been identified within AG 2-2. In addition to being relatively host specific, these groups of isolates, termed AG 2-2 LP (large patch), also appear to be genetically different to other AG 2-2, as determined by RFLP analysis (Hyakumachi *et al.*, 1998).

The aim of the experiments reported in this chapter was to determine if AG 2 isolates, which differed in zymogram patterns, also had different or unique host preferences. Three plant hosts were chosen, canola and medic as crops used in rotation with cereals in Australia, and turf grass. None of the imported isolates were examined for pathogenicity due to quarantine restrictions.

3.2. Materials and methods

3.2.1. Preparation of isolates

The *R. solani* AG 2 isolates 21RAuC02, 21RAuM03, 21RAuM04, 21RAuP08, 22RJ01, 22RAuL09, 22RAuS10, 22RAuL11, the AG 3 isolate 03RP03 and the AG 8 isolate 08RAuB08, were grown on sterile millet seed for use as inoculum in pathogenicity trials (McDonald and Rovira, 1985). Millet seed (French White) was soaked in ddH_2O overnight at room temperature. The seed (approximately 500 for each isolate) was then drained on paper towel, placed in 50 ml conical flasks and 5 ml of ddH_2O was added to each flask. The flasks were then autoclaved at 121°C for 15 min. The autoclaving procedure was repeated two times over consecutive days. The sterilised seed was then air-dried by placing open flasks within a laminar flow cabinet overnight. Seed was inoculated with a 5 mm^2 plug of an isolate taken from the edge of a colony on PDA and incubated at 23°C in darkness for 3 weeks. Following the incubation period, a sample of five infested seed was removed and placed on PDA to check the viability of the pathogen and to confirm the absence of contaminants. The control inoculum for each trial was prepared from 21RAuC02-infested millet seed. The infested seed was soaked in 5 ml ddH_2O and was autoclaved, at 121° for 20 min, a total of three times on consecutive days. After the autoclaving procedure, the seed was air-dried in open conical flasks, within a laminar flow cabinet. A sample of five seed was placed on PDA to confirm that the *R. solani* isolate was dead and no contamination was present.

3.2.2. Canola trial

Three canola cultivars, Monty, Oscar and Rainbow, were used for the following experiments. Canola seed was surface sterilised using a 0.1% sodium hypochlorite solution (1.0% available chlorine) for 1 min and rinsed four times with ddH_2O. The seed was then placed in a beaker

containing 500 ml ddH_2O and non-viable seed, those that rose to the surface, was removed. The remaining seed was drained and air-dried in a laminar flow on sterile paper towel.

Preliminary experiments were carried out to determine a suitable inoculum rate. Black plastic pots (10 cm diameter) were filled to a depth of 5 cm with steam-pasteurised UC (University of California) potting mix (Baker, 1957) and the surface was lightly packed. Millet seed infested with 21RAuC02, 08RAuB08, and control millet were used at 5, 10 or 20 infested millet seed per 100 ml UC mix. The inoculum was mixed with 100 ml UC potting mix, then added to the pots and the surface was lightly packed. Ten surface sterilised canola seed were placed onto the surface and overlaid with UC mix to a depth of 1.5 cm. For each of the canola cultivars, the inoculum rates were replicated three times. Pots were maintained in the glasshouse under natural light, with a day temperature of 22°C ± 3°C and a night temperature of 15°C ± 3°C. Pots were watered to saturation as needed. Seedlings were assessed at 7 and 14 days after sowing for emergence and the presence of disease symptoms on the hypocotyl. After 21 days, seedling survival was assessed and the seedlings were removed from the pots and the roots washed free of potting mix. Disease severity was scored using the index of Sweetingham *et al.* (1994). Hypocotyl rot was rated on a 0 to 3 scale, where:

0 = no lesions.

1 = necrosis affecting less than 25% of the cross-sectional area.

2 = necrosis affecting 25 - 75% of the cross-sectional area.

3 = necrosis of more than 75% of the cross-sectional area.

Percent disease index was calculated as: [Σ(number of plants in disease category) x (numerical value of the disease category) x 100]/[(number of plants in all categories) x (maximum value on the rating scale)] (Khangura *et al.*, 1999).

Once a suitable inoculum rate was established, millet seeds infested with the isolates 21RAuCO2, 21RAuMO3, 21RAuMO4, 21RAuP08, 22RAuL09, 03RAuP01, 08RAuB08 and control millet were used for the pathogenicity trial. A total of 10 infested millet seed per pot was required for visible disease expression, and the trial was conducted and assessed as outlined above. Pots were placed in a randomised complete block design, with three replicates per isolate/cultivar combination. The experiment was repeated once. Since disease caused by AG 2 isolates on canola occurs on the roots and low on the hypocotyl, cross-contamination of isolates between pots was considered highly unlikely.

3.2.3. Medic trial

The medic cultivars, *Medicago truncatula* cv. Caliph and *M. littoralis* cv. Herald, were used in the pathogenicity trials. As for the canola trial, an initial experiment was performed to determine a suitable inoculum level. Both the soil and medic seed were prepared as described for canola in section 3.2.2. The same inoculum rates of 5, 10 or 20 infested millet seed per 100 ml UC potting mix were used and the pots were prepared in a similar manner, using 10 surface sterilised medic seed per pot. Pots were maintained in the glasshouse under natural light, with a day temperature of 17°C ± 3°C and a night temperature of 12°C ± 3°C and watered to saturation as necessary. Plants were assessed for emergence after 7 and 14 days, while seedling survival and disease severity was determined 28 days after sowing.

Based on the results of the preliminary trial, the pathogenicity test was conducted with 10 infested millet seed to achieve suitable disease expression. Isolates 21RAuC02, 21RAuP08 21RAuM03, 21RAuM04, 22RAuL09, 08RAuB08, and control millet were used as

inoculum (see section 3.2.1). Three replicate pots per isolate/cultivar combination were used in a randomised complete block design. The experiment was repeated once. Seedling emergence, seedling survival and disease severity were assessed as above. The 1 to 4 scale of Barbetti and MacNish (1984) was used to assess rot on the tap root, where:

1 = healthy roots.

2 = rot of less than 10%.

3 = 10 – 70% root tissue affected by rot.

4 = 70 – 100% root tissue affected.

3.2.4. Turf grass

The cultivar Saharan couch (*Cynodon dactylon*) and the tall fescue (*Festuca arundinacea*) cv. Marathon were used in each of three experiments to determine pathogenicity of *R. solani* isolates. Pot experiments were established following the methods described by Burpee and Martin (1992). In two experiments, 10 cm black plastic pots were filled with UC potting mix to 1.5 cm below the pot lip. In the first experiment, ten millet seed infested with the isolates 21RAuC02, 21RAuM03, 21RAuP08, 22RAuL09, 22RAuS10, 22RAuL11, 08RAuB08, and the control inoculum were prepared as for the canola and medic trials (see Section 3.2.1). The inoculum was mixed with 100 ml of UC and added to the pots. The surface was gently packed and the turf seed was sown evenly across the surface at a rate of 40 g/m^2. In the second of these experiments, UC mix was added to bring the surface to 0.5 cm below the pot lip. The inoculum was then placed randomly on the soil surface and covered to a depth of 0.5 cm with UC mix, before turf seed was sown on top. Pots were watered to saturation and then were watered twice daily for the first 15 days to allow turf establishment, then once every

second day for the remainder of the trial. All pots were maintained under natural light in the glasshouse, with day/night temperatures of 25°C ± 3°C and 18°C ± 3°C, respectively. A relative humidity of 80% was maintained using a humidity chamber placed within the glasshouse.

In the third experiment, the turf grass cultivars were inoculated after thatch establishment. Black 10 cm plastic pots were filled with UC mix and the surface gently packed. Turf seed was sown as previously described and maintained as above, for 21 days. The inoculum, ten seed per pot, was then placed at the centre of the established turf thatch, and the pots were maintained for a further 2 weeks to allow disease development.

After 5 weeks, each of the three experiments was assessed for disease expression within the thatch. After this initial assessment, pots were removed from the humidity chamber and left in dry conditions for 48 h to induce water stress. In all experiments, the turf grass was re-assessed for thatch disease. In addition, the turf from the first two experiments was removed from the pots, the roots washed free from potting mix and assessed for root disease.

3.2.5. Data analysis

All data were entered as data sets into the Statistix for Windows program. Data were subjected to analysis of variance (one-way and two-way ANOVA) without transformation. Means were compared by least significant differences (Fisher's protected LSD), following significant F values. Data for repeated experiments were combined as there were no significant differences between repetitions.

3.3. Results

3.3.1. Canola trials

The experimental layout of the canola trial is shown in Figure 3.1A. Disease symptoms were assessed 21 days after sowing, but were observed on the seedlings after 10 days. The occurrence of brown lesions on the hypocotyl and the stunting of seedlings were evident in those pots inoculated with viable *R. solani* (Figure 3.1 B and C). Seven days after sowing, the canola plants were assessed for seedling emergence, with a total count taken for each pot and recorded as a percentage of seed emerged in the control pots (Table 3.1). Two-way ANOVA revealed significant differences between isolates ($F_{6, 42} = 10.44$, $p<0.001$), with no significant difference between cultivars or in the interaction between isolate and cultivar. The emergence of seedlings in the controls was adjusted to 100% for all three cultivars. Inoculation of canola cvs. with the AG 2-1 isolate 21RAuC02 from canola, significantly reduced the emergence of seedlings of all three cvs. to approximately 17 - 58%, compared to the controls ($p = 0.05$). For the cultivar Oscar, inoculation with isolate 21RAuC02 resulted in the significant reduction of seedling emergence compared to all other treatments ($p = 0.05$). A second AG 2-1 isolate, 21RAuM03 originally from medic, resulted in emergence of between 73% and 89% across all cultivars, and caused a significant decrease in emergence of Rainbow when compared to all other treatments, except isolate 21RAuC02. The remaining isolates did not significantly reduce the emergence of any cultivar (Table 3.1). The data were combined for the three canola cvs. to show the effects of inoculation with *R. solani* on seedling emergence (Table 3.1). One-way ANOVA revealed a significant difference between the effects of isolates on the emergence of canola cultivars ($F_{6, 56} = 11.3$, $p<0.001$),

Figure 3.1: Pathogenicity trials conducted on the *B. napus* cvs. Monty, Oscar and Rainbow.

A) Pots (10 cm diameter) were placed in a randomised complete block design in a glasshouse.

B) Cv. Monty 21 days after sowing in potting mix inoculated with isolate 21RAuC02 (lower three pots) and control (upper three pots).

(A)

(B)

Figure 3.1 cont.

C) Hypocotyl rot and stunting of cv. Oscar. The top plant is from a control, lower two plants are from pots inoculated with isolate 21RAuC02. Insert shows a magnified view of lesions and girdling of the hypocotyl (arrows).

(C)

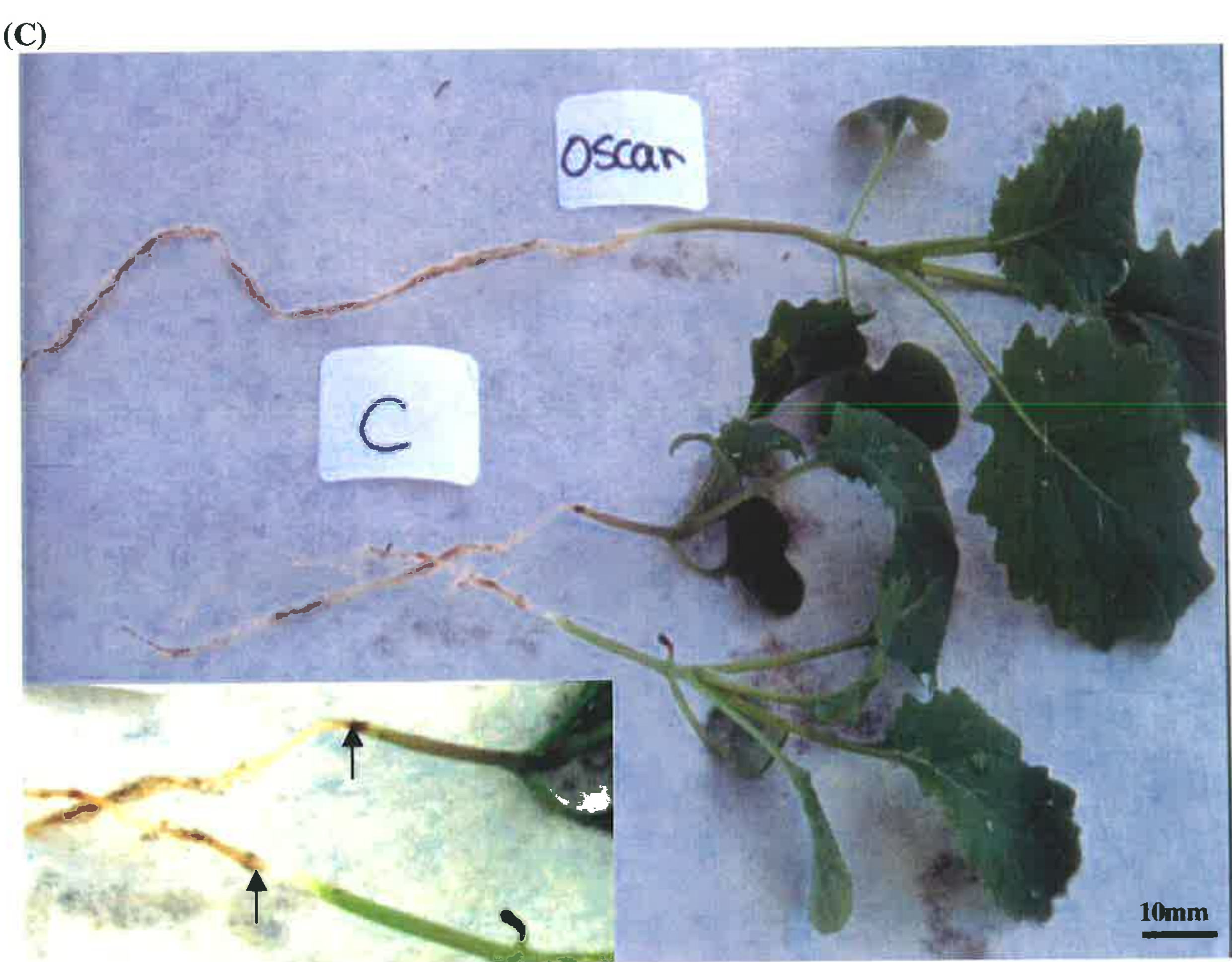


Table 3.1: Percentage of canola seed emerged after 7 days in controls was adjusted to 100%. The emergence of seed in potting mix infested with isolates of *R. solani* was recorded as a percentage of the controls. Means of six replicates are shown with standard errors in parenthesis.

	% Canola seedling emergence			
Treatment	**Monty**	**Oscar**	**Rainbow**	**Mean of cultivars**
Control	100.00 (0)	100.00 (0)	100.00 (0)	100.00 (0)
21RAuP08	116.66 (16.66)	101.66 (13.01)	90.00 (5.77)	102.78 (7.41)
21RAuC02	41.66 (30.04)	57.68 (12.31)	16.67 (8.82)	38.67 (11.40)
21RAuM03	80.56 (10.02)	88.88 (11.11)	73.33 (3.33)	80.93 (4.96)
22RAuL09	97.22 (12.11)	108.33 (8.33)	100.00 (0)	101.85 (4.55)
03RAuP01	94.44 (24.22)	104.63 (10.67)	96.67 (3.33)	98.58 (7.85)
08RAuB08	102.78 (23.73)	112.04 (7.23)	96.67 (3.33)	103.83 (7.56)
$LSD_{0.05}$	**58.20**	**29.90**	**13.78**	**20.01**

with isolate 21RAuC02 causing a significant decrease in emergence (39%) when compared to the controls and other isolates, while isolate 21RAuM03 caused a significant decrease in emergence compared to isolates 21RAuP08, 22RAuL09 and 08RAuB08 ($LSD_{0.05} = 20.01$, $p = 0.05$).

Assessments of percentage seedling survival and disease severity, on remaining seedlings, conducted 21 days after sowing are shown in Tables 3.2 and 3.3, respectively. Two-way ANOVA showed there was no significant difference between the cultivars and no significant interaction was found between isolate and cultivar. However, there was a significant difference between isolates ($F_{6, 42} = 12.1$, $p<0.001$). Seedling survival in the controls was approximately 93 - 97%, while inoculation with isolates of *R. solani* resulted in seedling survival of approximately 45 - 100%. For the cvs. Monty and Rainbow, the AG 2-1 isolate, 21RAuC02, caused a significant decrease in the survival of emerged seedlings compared to all other isolates ($p = 0.05$). The results for each cv. were combined to produce a mean for cvs. and the data presented in table 3.2. One-way ANOVA for the combined data revealed that isolate 21RAuC02 caused a significant decrease in the survival of canola seedlings, when compared to the controls and the other isolates ($p = 0.05$; Table 3.2).

The *R. solani* isolates caused disease on the hypocotyls of the canola cultivars, but not on the roots (Figure 3.1C). Therefore, disease severity was measured as the amount of hypocotyl rot observed, using the scale described in section 4.2.1 (Table 3.3). . In addition, percent disease index was calculated (Figure 3.2). Two-way ANOVA revealed a significant difference between cultivars ($F_{2, 42} = 3.4$, $p = 0.04$), a highly significant difference between isolates ($F_{6, 42} = 73.6$, $p<0.001$), with no significant interaction of isolate and cultivar. A comparison of scores for plants inoculated with isolates 21RAuC02, 21RAuM03 and

Table 3.2: Percentage of emerged canola seed surviving after 21 days growth in potting mix infested with isolates of *R. solani*. Means of six replicates are presented for each cultivar and a combined mean for all three cultivars. Figures in parenthesis represent the standard error of the mean.

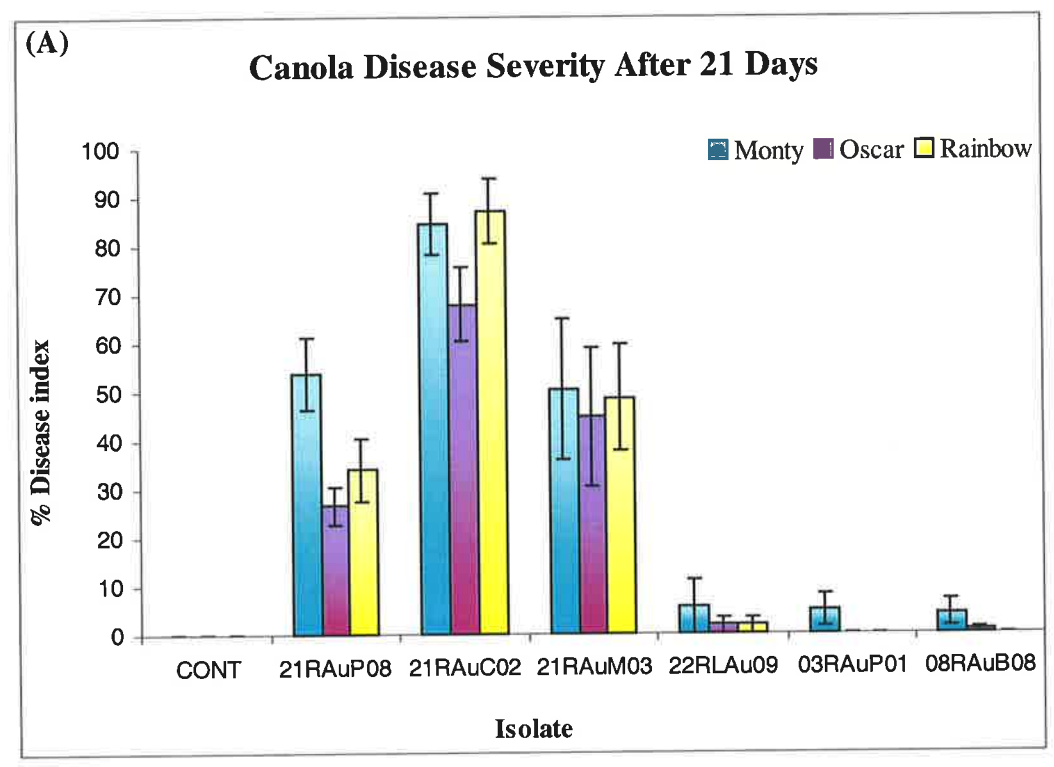
	% Canola seedlings surviving			
Treatment	**Monty**	**Oscar**	**Rainbow**	**Mean of cultivars**
Control	95.23 (4.76)	93.33 (3.33)	96.67 (3.33)	95.08 (0.97)
21RAuP08	93.33 (6.67)	92.97 (6.49)	87.70 (7.22)	88.00 (2.99)
21RAuC02	45.00 (22.91)	71.62 (7.14)	50.00 (9.62)	55.54 (8.17)
21RAuM03	88.89 (11.11)	82.37 (12.96)	80.00 (10.00)	83.75 (2.66)
22RAuL09	100.00 (0)	96.67 (3.33)	100.00 (0)	98.89 (1.11)
03RAuP01	100.00 (0)	100.00 (0)	100.00 (0)	100.00 (0)
08RAuB08	91.60 (4.29)	100.00 (0)	100.00 (0)	97.2 (2.80)
$LSD_{0.05}$	**43.6**	**NS**	**26.1**	**12.18**

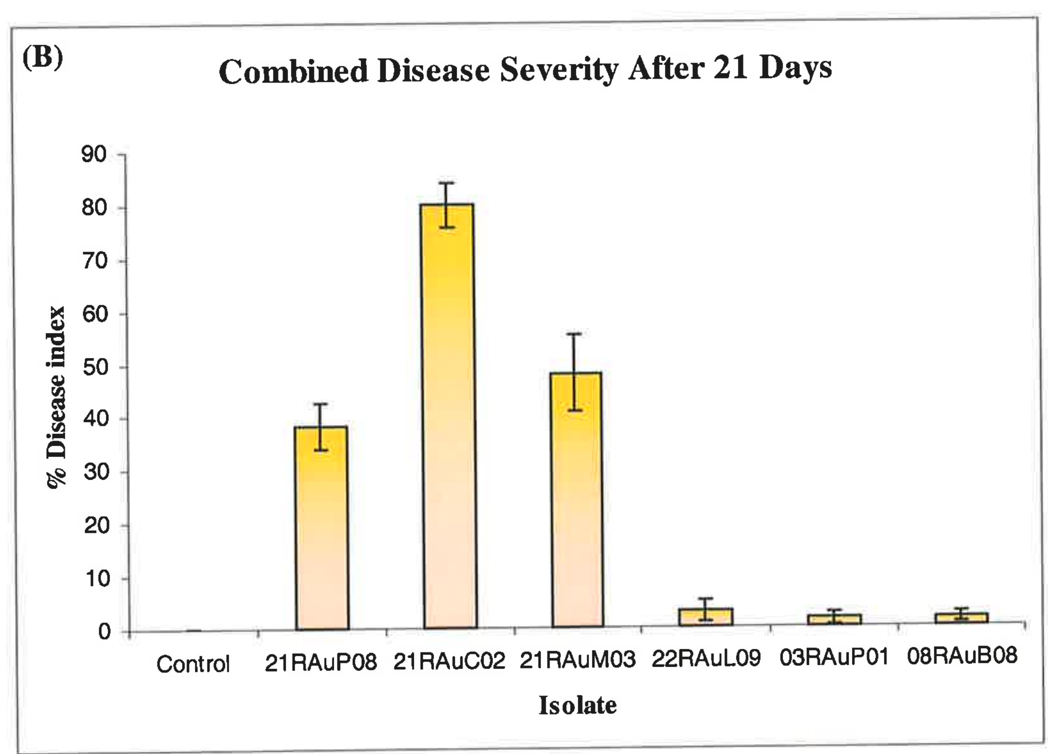
NS = not significantly different

Table 3.3: Disease severity was measured as the degree of hypocotyl rot for the surviving seedlings of canola cultivars Monty, Oscar and Rainbow, where 0 = no disease and 3 = severe disease (see Section 3.2.2). Assessment was made 21 days after sowing in infested potting mix. The means of six replicates and a combined mean for the two cultivars are shown, with standard errors in parenthesis.

	Canola disease severity			
Treatment	**Monty**	**Oscar**	**Rainbow**	**Mean of Cultivars**
Control	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)
21RAuP08	1.82 (0.41)	1.41 (0.20)	0.69 (0.11)	1.30 (0.21)
21RAuC02	2.60 (0.40)	2.72 (0.15)	2.30 (0.37)	2.54 (0.17)
21RAuM03	0.80 (0.61)	2.23 (0.25)	1.05 (0.40)	1.36 (0.31)
22RAuL09	0.33 (0.12)	0.33 (0.33)	0.1 (0.1)	0.25 (0.11)
03RAuP01	0.30 (0.12)	0.00 (0)	0.00 (0)	0.1 (0.07)
08RAuB08	0.25 (0.13)	0.28 (0.17)	0.00 (0)	0.18 (0.06)
$LSD_{0.05}$	**1.48**	**0.88**	**0.96**	**0.71**

Figure 3.2: Disease severity on canola cultivars as measured by percent disease index. Three canola cultivars exposed to *R. solani* isolates for 21 days were assessed for hypocotyl rot (A). Disease scores for each cultivar were combined to compare isolate effects (B). Scores are presented as means of three replicates (A) and nine replicates (B), with bars representing standard error of means.


(A)
Canola Disease Severity After 21 Days
Monty
Oscar
Rainbow
% Disease index
100
90
80
70
60
50
40
30
20
10
0
CONT
21RAuP08
21RAuC02
21RAuM03
22RLAu09
03RAuP01
08RAuB08
Isolate


(B)
Combined Disease Severity After 21 Days
% Disease index
90
80
70
60
50
40
30
20
10
0
Control
21RAuP08
21RAuC02
21RAuM03
22RAuL09
03RAuP01
08RAuB08
Isolate

21RAuP08, from canola, medic and potato, respectively, showed that these isolates caused moderate to severe disease in all three cultivars examined, while the other isolates caused little or no disease (Figure 3.2 A and B). Isolate 21RAuC02 caused significantly more disease on cultivars Monty and Rainbow, than did all other isolates, while for the cultivar Oscar, only isolates 21RAuC02 and 21RAuM03 caused significantly higher disease than the other isolates (Table 3.3). When the data for the three cultivars were combined, one-way ANOVA showed a significant difference between the levels of disease caused by the isolates examined ($F_{6, 56} = 34.00$, $p<0.001$). The AG 2-1 isolate, 21RAuC02 caused significantly more disease than all other isolates, while isolates 21RAuM03 and 21RAuP08 caused more disease than isolates of the other AG examined ($p = 0.05$; Table 3.3).

3.3.2. Medic trials

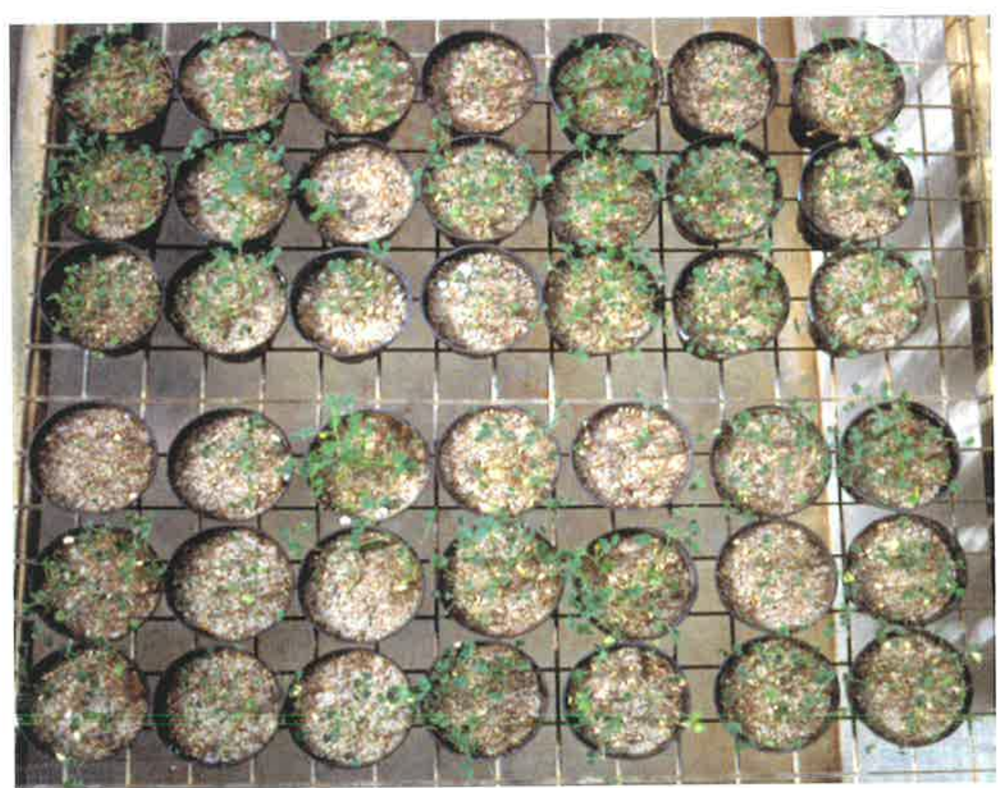
The experimental layout for the inoculation of medic cultivars is shown in Figure 3.3A. Disease was assessed 28 days after sowing, however, disease was evident after 14 days on seedlings grown in potting mix inoculated with isolate of *R. solani*. Seedlings were stunted compared to the control plants and often did not survive to 28 days (Figure 3.3B). After 7 days, seedling emergence was recorded in all pots. The seedling emergence in the controls was adjusted to 100% and the treatment pots were recorded as a percentage of the controls. Two-way ANOVA showed no difference between cultivars in terms of emergence 7 days after sowing into infested potting mix, and no significant interaction between cultivar and isolate. However, there was a significant difference observed between isolates ($F_{6, 28} = 10.44$, $p<0.001$). The AG 2-1 isolate 21RAuM04, originating from medic, reduced emergence for Caliph and Herald seed to approximately 60% and 38%, respectively (Table 3.4). Data were combined to produce a mean for cultivars. One-way ANOVA revealed a statistically

Figure 3.3: Pathogenicity trials conducted on *Medicago* cvs. Caliph (*M. truncatula*) and Herald (*M. littoralis*).

A) Pots (10 cm diameter) were placed in a randomised complete block design, in a glasshouse.

B) Cultivar Caliph 28 days after sowing in potting mix inoculated with isolate 21RAuM04 (upper three pots) and controls (lower pots).

(A)



(B)

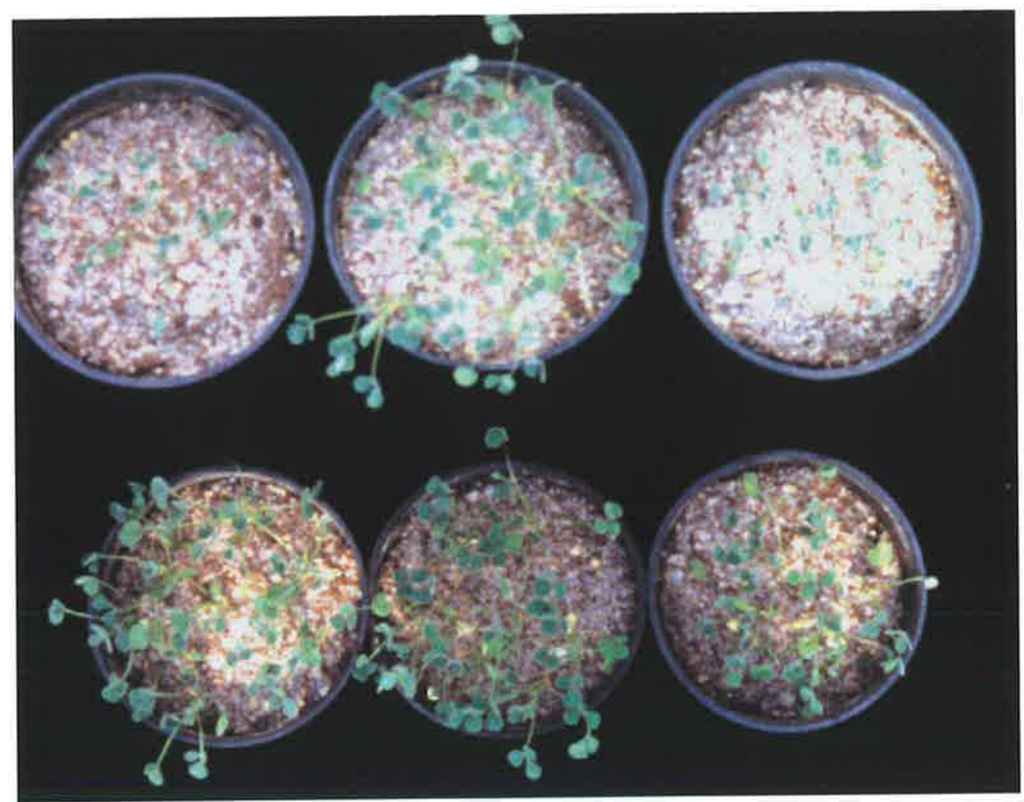


Figure 3.3 cont

C) Complete plants of cultivar Herald. To the left is a control plant rated 1 on the disease scale. Disease ratings of 2, 3 and 4 progress to the right. Arrows indicate lesions and root rotting.

(C)

1
2
3
4

significant difference between the effects of isolates on seedling emergence ($F_{6,\ 35}$ = 7.57, p<0.001). Isolate 21RAuM04 from medic resulted in the reduction of seedemergence (49%) when compared to the controls and the other isolates ($LSD_{0.05}$ = 21.2, p = 0.05). The other three AG 2-1 isolates, including another from medic, 21RAuM03, had little effect on emergence.

Statistical analysis of survival of the seedlings 28 days after sowing, showed no significant difference in the reaction of cultivars to the treatments, nor any significant interaction between isolates and cultivars. There was a slight difference was seen in the effect of isolates ($F_{6,\ 28}$ = 2.84, p = 0.03). In the control treatments, Caliph had a survival rate of 100%, while approximately 97% of emerged seed of cv. Herald survived. For cv. Caliph, isolate 21RAuM04, from medic, reduced seedling survival compared to all other isolates, while isolates 21RAuP08 (86%) and 08RAuB08 (84%) reduced survival of seedlings when compared to the control (p = 0.05; Table 3.5). None of the isolates reduced seedling survival in Herald. Again, the data for cultivars were combined to show the trend of isolate treatments (Table 3.5). One-way ANOVA revealed a significant difference in the effect of isolates ($F_{6,\ 35}$ = 3.2, p = 0.01), with isolate 21RAuM04 causing a reduction in the survival of medic seedlings compared to the control (p = 0.05; Table 3.5).

Disease severity was assessed on surviving seedlings as root damage after 28 days. In addition, percent disease severity was calculated as for canola. Dark brown lesions observed on taproots were assessed using the scale given in section 4.2.3 (Figure 3.4C). All *R. solani* AG 2-1, 2-2 and 8 isolates tested caused significant root disease on both medic cultivars when compared with the controls, which remained disease-free (Table 3.6; Figure 3.4A). Two-way ANOVA showed a significant difference between the severities of disease observed on each cultivar ($F_{1,\ 28}$ = 10.46, p = 0.003). This was due to the difference between disease severity caused by isolates 21RAuC02 and 21RAuM03 on Caliph and Herald. There was a significant difference in disease severity caused by all isolates when compared to the control on both

Table 3.4: Percentage of medic seed emerged after 7 days in control pots was adjusted to 100%. For seed sown in infested potting mix, the emergence was recorded as a percentage of the controls. The means for six replicates are shown and a combined mean for both cultivars, with standard errors in parenthesis.

	% Medic seedling emergence		
Treatment	**Caliph**	**Herald**	**Mean of cultivars**
Control	100.00 (0)	100.00 (0)	100.00 (0)
21RAuC02	126.32 (9.19)	92.96 (3.53)	109.64 (8.66)
21RAuM03	105.69 (14.29)	85.56 (3.90)	95.62 (8.01)
21RAuM04	60.05 (12.93)	38.51 (11.21)	49.29 (9.04)
21RAuP08	118.46 (15.87)	96.66 (7.22)	107.79 (9.25)
22RAuL09	100.46 (6.81)	92.59 (7.22)	96.53 (3.89)
08RAuB08	107.27 (11.50)	82.22 (3.39)	94.75 (7.76)
$LSD_{0.05}$	**34.12**	**17.42**	**21.20**

Table 3.5: Percent of emerged medic seed surviving after 28 days growth in potting mix infested with isolates of *R. solani*. The means of six replicates are presented, along with a combined mean for the two cultivars. Standard errors are in parenthesis.

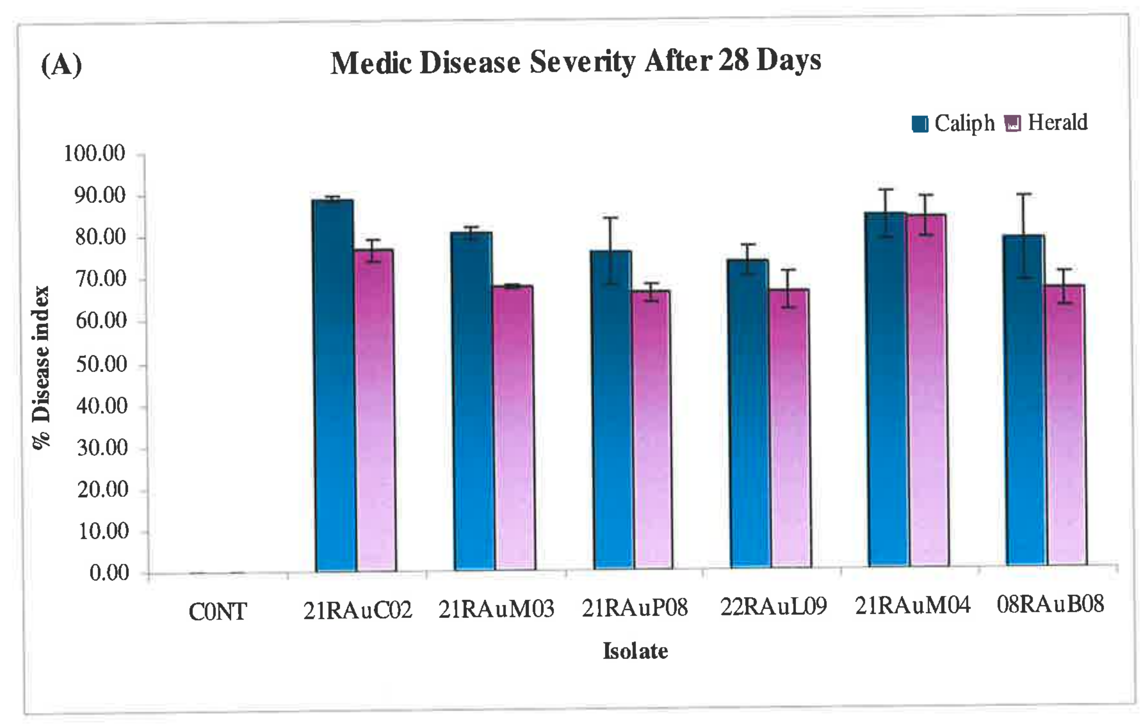
	% Medic seedlings surviving		
Treatment	**Caliph**	**Herald**	**Mean of cultivars**
Control	100.00 (0)	96.67 (3.33)	98.33 (1.67)
21RAuC02	96.67 (3.33)	89.33 (5.81)	93.00 (3.41
21RAuM03	92.00 (4.16)	88.67 (11.33)	90.33 (5.45
21RAuM04	69.33 (9.94)	77.67 (14.68)	73.50 (8.14)
21RAuP08	86.00 (3.06)	91.67 (8.33)	88.83 (4.17)
22RAuL09	100.00 (0)	90.00 (10.0)	95.00 (5.00)
08RAuB08	84.33 (2.96)	78.67 (3.67)	81.50 (2.46)
$LSD_{0.05}$	**16.3**	**NS**	**13.68**

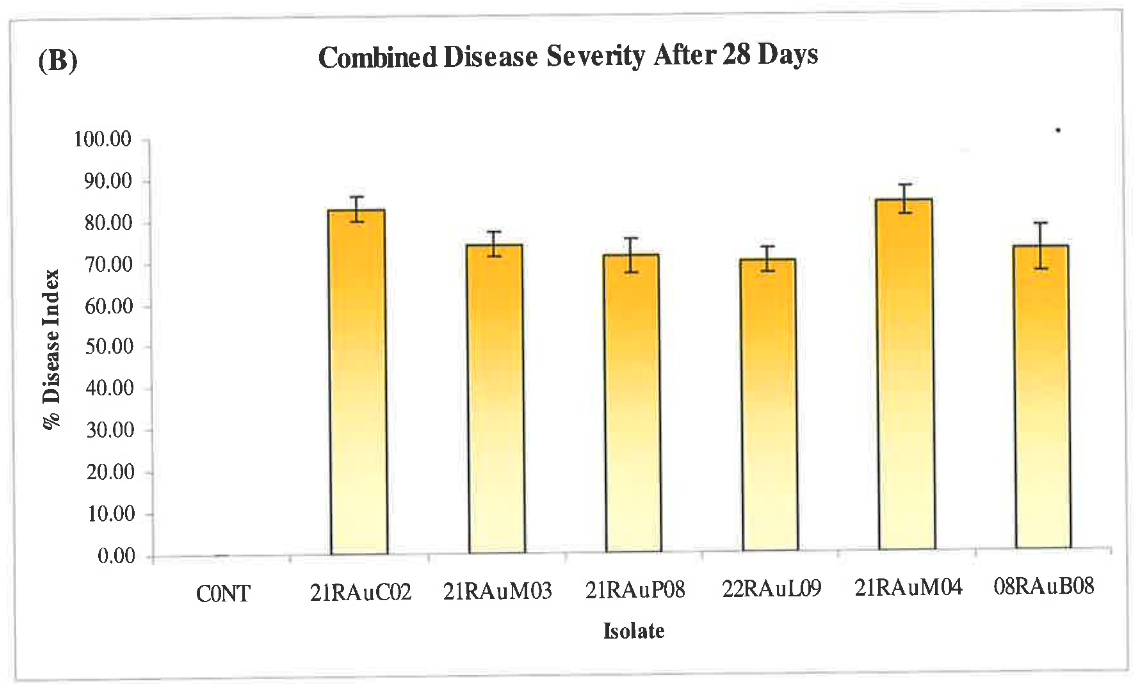
NS = not significantly different

Table 3.6: Disease severity was measured as the degree of root rot for the surviving seedlings of medic cultivars Caliph and Herald, where 1 = no disease and 4 = severe disease. Assessment was made 28 days after sowing in infested potting mix. The means of six replicates and a combined mean for the two cultivars are shown, with standard errors in parenthesis.

	Medic disease severity		
Treatment	**Caliph**	**Herald**	**Mean of cultivars**
Control	1.00 (0)	1.00 (0)	1.00 (0)
21RAuC02	3.55 (0.03)	3.06 (0.10)	3.31 (0.12)
21RAuM03	3.21 (0.06)	2.71 (0.02)	2.96 (0.12)
21RAuM04	3.37 (0.23)	3.35 (0.19)	3.37 (0.14)
21RAuP08	3.04 (0.31	2.65 (0.08)	2.85 (0.18)
22RAuL09	2.94 (0.14)	2.66 (0.17)	2.80 (0.12)
08RAuB08	3.14 (0.40)	2.67 (0.17)	2.91 (0.22)
$LSD_{0.05}$	**0.73**	**0.43**	**0.40**

Figure 3.4: Percent disease severity index on two cultivars of medic grown in soil infested with *R. solani* isolates for 28 days (A). Scores for each cultivar were combined to show the general effects of isolates (B). Scores are presented as means of six replicates, with bars representing standard errors.


(A)
Medic Disease Severity After 28 Days
Caliph
Herald
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
% Disease index
CONT
21RAuC02
21RAuM03
21RAuP08
22RAuL09
21RAuM04
08RAuB08
Isolate


(B)
Combined Disease Severity After 28 Days
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
% Disease Index
CONT
21RAuC02
21RAuM03
21RAuP08
22RAuL09
21RAuM04
08RAuB08
Isolate

cultivars ($F_{6, 28}$ = 39.64, p<0.001). Comparison of means showed no significant differences between isolates for disease severity on the cultivar Caliph. However,for the cultivar Herald, isolate 21RAuM04 caused significantly more severe disease than all other isolates, except 21RAuC02 and 21RAuM03 (Table 3.6). The data for each cultivar were combined to create a mean for cultivars (Table 3.6 and Figure 3.4). The AG 2-1 isolates 21RAuC02 from canola, 21RAuM03 and 21RAuM04, both from medic, all caused more disease than the remaining AG 2-1 isolate, 21RAuP08 from potato and the AG 2-2 isolate, 22RAuL09 from lupin. A significant difference was seen with the isolates 21RAuC02 and 21RAuM04 when compared to isolate 22RAuL09 (p = 0.005; Table 3.6). In addition, the two isolates from medic, 21RAuM03 and 21RAuM04, were significantly different from one another in their effects, suggesting that even though these two isolates originated from the same host type their genetic differences may result in differing pathogenicity and virulence. The AG 8 isolate, 08RAuB08 originating from barley, caused significantly less disease than isolate 21RAuM04. These results suggest that AG 2-1 isolates are more virulent to the medic cultivars tested in these experiments than the other isolates examined. In addition, isolate 21RAuM04, an AG 2-1 from medic was more pathogenic than the other AG 2-1 isolates and may have some advantage on this particular host.

3.3.3. Turf grass trials

The Saharan couch and Marathon turf grass cultivars were used in a final experiment to examine pathogenicity (Figure 3.5A). Both were grown in humid conditions and inoculated with *R. solani* isolates, using three methods. In each trial, no disease as a result of inoculation with *R. solani* isolates could be identified in the thatch. During the trial, plants developed a white hyphal growth in the thatch, which occurred in both controls, treated with autoclaved, colonised millet seed, and *R. solani*-inoculated treatments and, ultimately, caused the

Figure 3.5: Turf grass cvs. Saharan couch and Marathon inoculated with various isolates of *R. solani.*

A) Established turf thatch in a randomised block design, in a glasshouse.

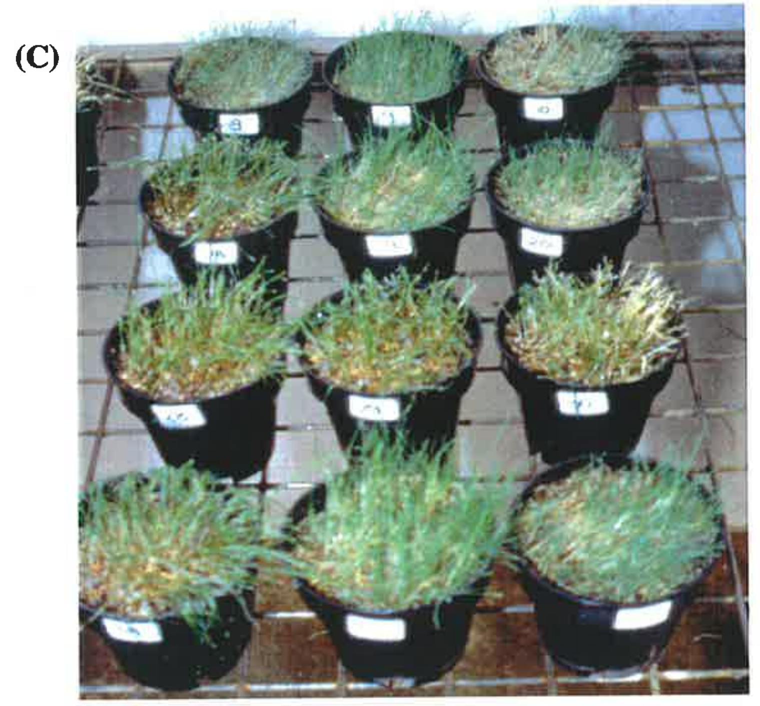
B) Blackening of the thatch of the couch variety, caused by an unidentified fungus. This was seen in inoculated pots and un-inoculated controls. *R. solani* could not be isolated from blackened leaves nor roots.

C) Pots containing fescue variety after 48 h of water stress. Although browning of the thatch was evident, *R. solani* could not be isolated from any part of the plants. Roots remained healthy.

(A)

(B)

(C)



blackening of an area of thatch (Figure 3.5B). Samples of the thatch affected by the white hyphae and of unaffected thatch were taken and placed on both water agar and PDA. A fungus with cultural morphology similar to *Penicillium* was isolated, but *R. solani* could not be detected. No root disease was detected. Water-stressed plants (Figure 3.5°C) did not develop disease and, while browning and wilting occurred, this did not appear to be associated with infection by *R. solani*.

3.4. Discussion

Differences in pathogenicity of *R. solani* AG 2 isolates were examined in a series of glasshouse trials, using canola, medic and turf grass cultivars as hosts. In addition, isolates from AG 3 and AG 8 were included in some trials to compare the incidence and severity of disease. The canola cultivars Monty, Oscar and Rainbow and the medic cultivars Caliph and Herald were susceptible to disease caused by the AG 2 isolates tested. In addition, the medic cultivars were susceptible to the AG 8 isolate, 08RAuB08.

Three canola cultivars were used to examine the host response to different AG 2 isolates. Isolates 21RAuC02 and 21RAuM03 belong to ZG 5 and were originally isolated from canola and medic, respectively. Isolate 21RAuP08 belongs to ZG 6 and was isolated from potato. These isolates were the only three of the seven examined to cause significant disease on all three canola cultivars. AG 2-1 and AG 4 have been reported as the causal agents of both pre- and post-emergence damping off of canola seedlings in Canada (Kaminski and Verma, 1985; Yitbarek *et al.*, 1987). Within Western Australia, Khangura *et al.* (1999) recently isolated mainly AG 2-1, ZG 5 from canola fields and found both AG 2-1, ZG 5 and AG 8, ZG 1-1 to be highly pathogenic to canola in glasshouse trials, causing both hypocotyl rot and root rot. Our results with 21RAuP08 indicate that an isolate of AG 2-1, ZG 6, also, can cause moderate to severe disease on canola seedlings, in glasshouse conditions.

Of interest in the experiments reported here was the lack of root disease observed on canola following inoculation with any *R. solani* isolate. The canola cultivars developed only hypocotyl rot when inoculated with the AG 2-1 isolates, and not root rot, as was reported previously (Khangura *et al.*, 1999). This difference may be due to conditions in the potting mix not being conducive to root disease. A possible change to the method would be the infestation of potting mix for a period prior to sowing seed. This would allow colonisation of organic matter by the fungus and an expanded region of hyphal growth for the roots to pass through. In addition, it may be possible to assess the ability of the isolates to infect canola root tissue and, subsequently, to examine any host tissue/isolate interactions that may occur. Another approach would be to establish small plots in the field to assess disease development under natural conditions to determine if root rot and hypocotyl rot can occur simultaneously in the field.

The medic cultivars used in these trials were susceptible to all *R. solani* isolates tested. Rotting of the taproot on each medic cultivar was evident as a result of inoculation with *R. solani*, with the AG 2-1 isolates 21RAuM04 and 21RAuC02 causing the most severe disease on roots. Previous studies have examined the effect of *R. solani* and other fungal isolates on medic cultivars (Pankhurst *et al.*, 1998). While it was determined that *R. solani* isolates caused severe disease on medic cultivars, the anastomosis grouping of the isolates was not determined by the authors.

The pathogenicity trials conducted on medic showed a lack of correlation between AG, ZG, host and location from which the isolates originated, and disease severity. Unlike the canola trial, in which only AG 2-1 isolates caused significant disease, all of the isolates examined caused significant root rot of medic cultivars compared to the control. In addition, it was interesting to observe that the AG 2-1 isolates that caused significant hypocotyl rot, but not root rot, of canola, caused root rot of medic. This does not explain why root disease was not observed for canola, but suggests that these isolates are virulent and have the ability to

infect different host tissue. In addition, it demonstrated that while the AG 2-1 group has been described as a '*Crucifer* type', isolates are able to extend beyond the bounds of plant species.

The lack of disease in the turf grass species examined was unexpected, although in preliminary experiments, *R. solani* could not be isolated from diseased turfgrass samples collected in South Australia (data not shown). Many reports from other countries have suggested that isolates of AG 2, particularly AG 2-2, are of concern to the turf industry. There are a number of possible explanations for the failure of the *R. solani* isolates to cause disease on the turf grass. The most obvious would be that the isolates tested were simply non-pathogenic to this host. However, the tall fescue cultivar used was originally derived from rye grass, so that the AG 8 isolate from cereal would be expected to have caused at least some root disease. It is possible that the conditions under which the trial was conducted were not conducive to infection by *R. solani*, but allowed infection by the *Penicillium*-like fungus. This may have resulted in competition and/or an induced resistance type of response, resulting in the prevention of infection by *R. solani*. Finally, the turf grasses used may be resistant to infection by *R. solani*. If further testing established this to be the case, the resistance mechanism that prevented infection by *R. solani* isolates might be examined to see if any benefit could be gained toward incorporating resistance into other crops.

The mechanism by which a host range is defined for a particular AG has not been investigated in detail. It is generally accepted that AG 2 has a diverse host range, with the AG 2-1 subgroup being linked mainly with disease of *Brassica* species. The emergence of what appear to be host-related subgroups of AG 2 suggests that the pathogen is able to adapt to different hosts. This, in turn, provides the pathogen with an opportunity to gain a survival advantage. The breeding of *R. solani*-resistant crops has been successful in some crops, such as sugar beet (Scholten *et al.*, 2001). However, there are still a large number of agriculturally important crops that are susceptible to disease caused by *R. solani* and a range of other fungi. The identification of more tolerant breeding lines for susceptible crops will enhance the

ability to minimise yield and quality loss under high disease pressure. In addition, much more work needs to be done to determine the extent of development of new pathotypes of AG 2 isolates with the ability to infect different hosts. This is an area of research where information on the development of new pathotypes with extended host range or enhanced virulence, would directly benefit the agricultural and horticultural industries. A better understanding of the genetic relationships between *R. solani* and hosts may lead to strategies for more successful breeding of crops, promoting a more strategic approach to disease management.

Chapter 4

Development of RFLP Markers for Anastomosis Group 2

4.1. Introduction

Since the identification of highly polymorphic loci in humans by Jeffreys *et al.* in 1985, dispersed repetitive DNA sequences have been found in a range of organisms, including fungi. RFLP analysis also termed DNA fingerprinting, in fungi has advanced the classification of taxonomic relationships and allowed genetic studies of species belonging to *Phytophthora* (Goodwin *et al.*, 1993), *Fusarium* (Koopmann *et al.*, 1996; Woo *et al.*, 1996), *Gaeumannomyces* (O'Dell *et al.*, 1992) and *Pythium* (Harvey *et al.*, 2001), as well as species belonging to *Rhizoctonia*. For example, Rosewich *et al.* (1999) used seven single-copy nuclear RFLP markers to examine isolates of *R. solani* AG 1-1A from rice. Thirty-six multi-locus genotypes were identified amongst 182 isolates collected from six separate field populations throughout Texas.

The isolation of sequence-specific probes has further enhanced the usefulness of the RFLP technique in separating closely related fungal species. Many of the sequences used have been from rDNA genes. For example, Jabaji-Hare *et al.* (1990) used a cloned rDNA sequence from *Armillaria ostoyae* as a probe in Southern hybridisations to restriction enzyme digested DNA of isolates belonging to *R. solani*. AGs 1, 2, 3 and 6 yielded one or more RFLP patterns that were not found in other AG, whilst AGs 4, 5, 7, 8, 9 and BI showed a number of RFLP patterns, some of which were shared among the groups. In further studies, conducted in Australia, O'Brien (1994) examined Southern blots of restriction enzyme digested *R. solani* DNA using a cloned 18S rRNA gene and randomly cloned fragments of *R. solani* DNA as probes. The ribosomal sequence showed that some AG, such as AG 2-1, had more genetic variation within the group than others, but group-specific RFLP patterns were

not detected. In contrast, the randomly cloned probes revealed less variation, but AG specific patterns were distinguished.

Evidence for the existence of AG-specific sequences comes from studies conducted on AG 8 and AG 3 isolates. A multi-copy DNA clone was selected from a random genomic library constructed from an AG 8 isolate, and used to screen isolates of *R. solani* AG 2-1, AG 3, AG 4 and AG 8 for DNA polymorphisms (Matthew *et al*., 1995). The clone showed strong homology to all AG 8 isolates tested, and little or no homology to isolates from the other AGs. Similarly, an AG 3-specific multi-locus probe was isolated by Balali *et al.* (1996). Screening genomic DNA from isolates of AG 1-1A to AG 9 and AG BI with this probe, revealed little or no homology to groups other than AG 3.

RFLP analysis can provide detailed information for the determination of genetic relatedness both within and between the AGs of *R. solani*. Given the existing evidence for genetic variation within AG 2, RFLP analysis of this complex group may provide further insight into the genetic relationships that exist between members of the AG 2 subgroups. The aim of the following experiments was to isolate random DNA fragments from AG 2 isolates for use in the analysis of genetic variation of this group and to obtain DNA sequences specific to the major subgroups, AG 2-1 and AG 2-2.

4.2. Materials and methods

4.2.1. General extraction of DNA from isolates of *R. solani*

Isolates of *R. solani* AG 2 were grown on PDA for 4 days at 23°C in darkness. A 5 mm^2 piece of inoculum was taken from the colony edge, transferred to each 9 cm Petri dish containing 15 ml of potato dextrose broth (PDB, Difco, see appendix A) and incubated for 48–72 h. The inoculum plug was excised and mycelial mats were harvested by filtration. The mats were patted dry with filter paper, weighed into 0.1 g amounts and used fresh or stored at

-80°C. Each sample of hyphae was ground to a fine powder with a mortar and pestle in liquid nitrogen. Hyphal powder was brushed into 1 ml of DNA extraction buffer (Appendix A) in a 2 ml Eppendorf tube, and mixed gently by inversion to form a slurry. An equal volume of phenol/chloroform (1:1) was added, mixed gently to form an emulsion and centrifuged at 15,800 *g* for 15 min. The aqueous phase was removed and re-extracted with an equal volume of chloroform/isoamyl alcohol (24:1) and centrifuged as before. The aqueous phase was recovered, 50 μl RNase A (0.25 mg/ml stock solution) was added and the mixture incubated at 37°C for 30 min. The solution was re-extracted with an equal volume of chloroform/isoamyl alcohol (24:1) and centrifuged as before. The aqueous phase was recovered and the DNA was precipitated at –20°C for 2 h with the addition of 0.4 volumes of 1 M ammonium acetate (final concentration) and 0.6 volumes of cold isopropanol. DNA was recovered by centrifugation at 15,800g for 20 min and the pellet was then washed with 70% ethanol for 10 min at room temperature. Following centrifugation at 15 800 *g* for 10 min, the DNA pellet was air-dried for 20 min, suspended in 20-50 μl TE and stored at –20°C.

4.2.2. Cesium chloride gradient purification of *R. solani* DNA used for cloning

Cesium chloride (CsCl)-purified DNA from isolates 21RJ01, 21RAuC02, 21RAuM04, 22RJ02, 22RAuL09 and 22RAuS10 was prepared for cloning into plasmid vectors as follows. Approximately 0.1 g of mycelium was ground to a fine powder in liquid nitrogen, and suspended in 1 ml CsCl-DNA extraction buffer (2x SSC; 20 mM EDTA; 3% sarkosyl). Pre-digested pronase (100 μg/ml; Sambrook *et al.*, 1989) was added, and the mixture was incubated for 20 min at 37°C prior to centrifugation at 15,800 *g* for 5 min. The supernatant was removed to a clean tube and the pellet resuspended in CsCl-DNA extraction buffer, mixed and centrifuged as before. The supernatant was combined with that from the first centrifugation. An equal amount (w/v) of CsCl was added and the tubes warmed gently to

dissolve the salt. The mixture was transferred to 3.5 ml heat-sealable ultracentrifuge tubes (Beckman) and 80 μl ethidium bromide (10 mg/ml) was added to each and mixed. The tubes were filled with a 1:1 (w/v) solution of CsCl, balanced by weight, heat-sealed and centrifuged at 100,000 rpm for 24 h using a TL-100 ultracentrifuge with a TLA-100.3 fixed angle rotor (Beckman Instruments). The position of the DNA band within the gradient was marked under UV light (312nm). A hole was pierced at the top of the tube and an 18-gauge needle, attached to a syringe, was inserted below the marked level of the band. The DNA was drawn from the tubes and transferred to a clean 2 ml centrifuge tube. The DNA samples were extracted twice with water-saturated butanol to remove the ethidium bromide. The lower aqueous phase was removed and 2 volumes of TE, pH 8 (Appendix A) followed by 2 volumes of cold absolute ethanol were added. The solution was mixed gently and left at -20°C overnight to precipitate the DNA. The samples were centrifuged at 8,000 *g* for 20 min, and the DNA pellet was washed in 70% ethanol, 10 mM magnesium acetate, air-dried, resuspended in 50 μl TE and stored at -20°C.

4.2.3. Preparation of *R. solani* insert DNA

Approximately 1 μg of CsCl-purified total DNA was digested with 20 units of the restriction enzyme, *Hin*dIII, overnight at 37°C in a volume of 50 μl, according to the manufacturer's directions (Boehringer Mannheim). The volume was increased to 200 μl with sterile ddH_2O and the DNA was extracted with an equal volume of phenol/chloroform (1:1). Following centrifugation at 12,000 *g* for 2 min, the upper aqueous phase was re-extracted with an equal volume of chloroform/isoamyl alcohol (24:1), and centrifuged as before. The aqueous phase was removed and the digested DNA was precipitated at -20°C overnight, by the addition of 0.5 volumes of 7.5 M ammonium acetate and 2 volumes of cold absolute ethanol. The DNA was collected by centrifugation at 15,800 *g* for 10 min, washed in 70% ethanol, 10 mM

magnesium acetate, re-centrifuged and air-dried. The final DNA pellet was dissolved in 20 μl ddH_2O and stored at -20°C.

4.2.4. Preparation of pUC19 vector DNA

A 10 ml culture of *Escherichia coli* JM101 containing the plasmid vector, pUC19, was prepared in Luria Bertani (LB, see Appendix A) broth containing 50 mg/ml ampicillin, by shaking at 37°C, overnight. Aliquots (2 ml) of the culture were centrifuged sequentially at 15,800 *g* for 30 sec to collect the cells in one tube. The pellet was drained thoroughly over paper towels and the cells were resuspended in 1 ml Tris wash (10 mM Tris, pH 8.0) by vortexing at low speed for 10 sec. The cells were collected by centrifugation as before, the supernatant poured off and the wash repeated. The final cell pellet was drained thoroughly over a paper towel and resuspended in 100 μl buffer, containing 50 mM glucose, 25 mM Tris-HCl, pH 8.0 and 10 mM EDTA. A 200 μl volume of cell lysing/DNA denaturing solution (0.2 M NaOH; 1% SDS, prepared fresh) was added and the suspension mixed by inversion before being placed on ice. The DNA was renatured by adding 150 μl of 3 M potassium acetate (pH 5.4). The lysate was mixed by inversion and cell debris and chromosomal DNA were removed by centrifugation at 12,000 *g* for 5 min. The supernatant was transferred to a clean tube and protein was removed by phenol/chloroform extraction. After vortexing briefly, the phases were separated by centrifugation at 12,000 *g* for 2 min. The upper aqueous phase was removed to a clean tube and the DNA re-extracted with an equal volume of chloroform/isoamyl alcohol (24:1), and centrifuged as before. The upper aqueous phase was transferred to a clean tube and the DNA was precipitated by the addition of 2 volumes of cold absolute ethanol. After mixing and standing the suspension for 2 min at room temperature, the DNA was collected by centrifugation at 12,000 *g* for 5 min, washed twice with 70%

ethanol and centrifuged as before. The pellet was air-dried, suspended in 50 µl RTE (2 mg RNase A in 10 ml sterile TE), and stored at -20°C.

Ten µg of pUC19 was digested with 20 units of *Hin*dIII (Boehringer Mannheim) in a final volume of 20 µl for 4h at 37°C, according to the manufacturer's directions. A 2 µl aliquot was removed and analysed on a 0.8% agarose gel to verify that the plasmid had been linearised. A 10 µl volume of 10X Sure Cutter Buffer H (Boehringer Mannheim, Appendix A) was added to the remaining 18 µl of digest and the linearised plasmid was dephosphorylated with 0.2 units of calf intestinal phosphatase (CIP, Boehringer Mannheim) in a final volume of 100 µl, at 37°C for 60 min. The reaction was stopped by the addition of 0.5 M EDTA (pH 8) to a final concentration of 5 mM and incubating at 65°C for 10 min.

The dephosphorylated plasmid was cooled to room temperature, the volume adjusted to 200 µl with sterile ddH_2O, and extracted with an equal volume of phenol/chloroform. Following centrifugation at 12,000 *g* for 2 min, the aqueous phase was removed, re-extracted with an equal volume of chloroform/isoamyl alcohol and centrifuged as before. The upper aqueous phase was transferred to a clean tube and the DNA precipitated overnight with 0.5 volumes of 7.5 M ammonium acetate and two volumes of cold absolute ethanol at -20°C. The DNA was collected by centrifugation at 15,800 *g* for 10 min, and the pellet was washed with 70% ethanol, 10 mM magnesium acetate, re-centrifuged and air-dried. The final DNA pellet was resuspended in 20 µl sterile ddH_2O.

4.2.5. Ligation of *R. solani* DNA into the pUC19 vector

Aliquots containing 25 ng and 75 ng of insert and vector DNA, respectively, were used in the ligation reaction. The DNA was mixed with 2 µl of 10x ligase buffer (Boehringer Mannheim), 1 mole ATP, 0.1 unit of T4 DNA ligase (Boehringer Mannheim), adjusted to a final volume of 20 µl with ddH_2O and incubated at 4°C overnight, according to the

manufacturer's directions. The DNA ligations were diluted to 1 ng DNA/µl in TE and stored at -20°C.

4.2.6. Preparation and transformation of competent cells of *Escherichia coli*

A 1 ml aliquot of an overnight culture of *E. coli* JM101 cells was inoculated into 50 ml of LB broth. The flask was shaken vigorously at 37°C until the OD_{650} reached 0.6 units. A 20 ml aliquot of the cell suspension was centrifuged at 5,000 rpm for 5 min at 4°C. The supernatant was poured off and the bacterial cell pellet was suspended in 10 ml ice-cold 0.1 M magnesium chloride. The cells were pelleted by centrifugation as before, resuspended in 1.5 ml of ice-cold 0.1 M calcium chloride, and placed on ice for 30 min. The cells were pelleted as before, resuspended in 5 ml 0.1 M calcium chloride plus 5 ml of 30% glycerol and stored in 1.3 ml aliquots at –80°C.

A 50 µl volume of the diluted ligation reaction mixture containing 50 ng of DNA was mixed with 200 µl of competent cells in an Eppendorf tube by gentle pipetting. The mixture was incubated on ice for 30 min, heat shocked at 37°C for 2 min, and cooled on ice for 1 min before adding 1.5 ml of LB broth and incubating at 37°C for 1 h to allow cell recovery. Cells were collected by centrifugation at 5,000 *g* for 5 min, and the pellet was resuspended in 100 µl LB broth.

Luria Bertani agar (LBA, Appendix A) plates containing 50 mg/ml ampicillin were overlaid with 3 ml 'top agar' containing 80 ng/µl IPTG, 0.013 % X-GAL (see Appendix A) and 50 mg/ml ampicillin. A 50 µl aliquot of transformed cells was spread on each plate and incubated at 37°C overnight. White colonies, which are predicted to contain cells with recombinant plasmids, were transferred, in a 46-square grid pattern, to fresh LBA plates containing 50 mg/ml ampicillin and incubated overnight at 37°C. A second transfer was made to fresh LBA/ampicillin plates and to nitrocellulose membranes overlaid on

LBA/ampicillin plates, using the grid numbers as a reference (Sambrook *et al.*, 1989) and incubated at 37°C overnight.

4.2.7. Screening and selection of recombinant cells

Colonies that had formed on the nitrocellulose membranes were prepared for hybridisation using the Grunstein method (Sambrook *et al.*, 1989). Filters were washed with 10% SDS for 5 min to lyse the bacterial cells and the DNA was denatured with two changes of 0.5 M NaOH, 0.5 M NaCl, each for 5 min. The DNA was then renatured with three changes of 1.5 M NaCl, 0.5 M Tris (pH 7.5), for 5 min each, and the filters were washed with 2 x SSC (Appendix A) for 5 min. The membranes were allowed to dry briefly on paper towel and the cell debris was cleaned from the filters in 2 x SSC using non-absorbent cotton wool, before being air-dried. The DNA was fixed to filters by UV cross-linking as recommended by the manufacturer (Bio Rad GS Gene Linker).

Colonies containing cloned *R. solani* DNA inserts were identified by hybridisation with ^{32}P-labelled total *R. solani* DNA, extracted from the original isolates from which the fragments were derived, as follows. The filters were incubated in 20 ml of prehybridisation buffer (4 x SSC, 5 x Denhardt's solution, 100 μg/ml sonicated salmon sperm DNA (SSS) and 0.1% SDS, with an additional 2 mg of SSS that had been denatured at 95°C for 5 min), in bottles using a Hybaid rotating bottle oven for 4 h at 65°C. Total genomic DNA from the *R. solani* isolates selected for cloning was radiolabelled with [α-32P]-dCTP using the Amersham Megaprime labelling kit. Approximately 25 ng of genomic DNA and 75 ng random hexamer primers were combined with ddH_2O in a final volume of 20 μl and denatured at 95°C for 5 min. The DNA/primer mixture was cooled on ice, centrifuged briefly, and 5 μl of 10 x labelling buffer was added (Appendix A). Following the addition of 20 μl ddH_2O, a 3 μl volume of [α-^{32}P]-dCTP was added and the reaction started with 2 units of exonuclease-free

Klenow fragment (Pharmacia Biotech, USA). The tubes were incubated at 37°C for 30 min and the reaction stopped by the addition of 2 μl of 0.5 M EDTA (pH 8) and 48 μl of 1 x TEN buffer (Appendix A). Unincorporated nucleotides were removed by applying the reaction mixture to 1 ml Bio-Spin Chromatography columns (Bio-Rad). Columns had been prepared previously by adding Bio-Gel P60 (50-100 mesh, 90-180 μm bead size range, Bio-Rad) hydrated in 1 x TEN buffer to the column, and centrifuging for 3 min at 1,000 rpm. The column was packed until the mesh volume reached 1 ml, before being equilibrated with 100 μl of 1 x TEN buffer and centrifuged as before. The labelling reaction mixture (100 μl) was applied to the packed column and centrifuged at 1,000 rpm for 3 min to collect the labelled DNA in a clean 1 ml tube.

The prehybridisation fluid was poured from the bottles and 10 ml hybridisation solution containing 4 x SSC, 2 x Denhardt's solution, 0.1% SDS and 100 μg/ml SSS in sterile ddH_2O, plus a further 2 mg denatured SSS, was added. The labelled DNA (100 μl) was denatured at 95°C for 5 min, and added to the solution. Membranes were incubated at 65°C overnight in the rotating bottle oven. The hybridisation solution was poured off and membranes were washed with 2 x SSC, 0.1% SDS at 65°C for 30 min, in the oven. A second wash was performed using 1 x SSC, 0.1% SDS at 65°C in a shaking water bath for 30 min. The third and final wash was carried out with 0.5 x SSC, 0.1% SDS using the same conditions for the second wash. The membranes were allowed to air-dry before being placed in a plastic sleeve and exposed to x-ray film (Kodak Diagnostic Film, X-Omat KX-1) at -80°C for 72 – 120 h. Colonies were assessed as having high, medium or low hybridisation intensity and selected from the replica grid plates for further screening.

4.2.8. Assessment of selected clones as RFLP markers

Plasmids containing inserts of *R. solani* DNA were isolated from selected colonies following the alkaline lysis method outlined in section 4.2.4.

Genomic DNA was extracted from isolates of *R. solani* using the procedure outlined in section 4.2.1. Approximately 300 ng of total DNA from a range of isolates was digested to completion with 20 units of the restriction enzyme *Hin*dIII at 37°C overnight. The DNA fragments were size-fractionated on a 1% agarose gel in TAE buffer (Appendix A) at 60 V and visualised under UV light (312nm) using ethidium bromide staining. Gels were then incubated at room temperature in denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 30 min with gentle agitation. Following a brief rinse in water, the gels were neutralised in 1 M Tris-HCl, pH 7.0; 1.5 M NaCl for 30 min at room temperature with gentle agitation. After a brief rinse in water, the nucleic acids were transferred to positively charged nylon membranes (Boehringer Mannheim) by the method of Southern (1975).

Selected plasmid clones were radiolabelled with [α-32P]-dCTP as described previously (section 4.2.7) and used as probes to examine DNA polymorphisms within and between AG 2 subgroups.

4.2.9. Slot blot analysis

The slot blot procedure was used to examine the specificity of the probes derived from *R. solani* AG 2, pRAG21-413 and pRAG22-39, towards DNA from various AG. DNA was extracted from representative isolates of *R. solani* AGs 1, 2-1, 2-2, 3, 4, 5, 6, 7, 8, 9, BI and "AG 2 undefined" following the procedure in section 4.2.1. Aliquots containing approximately 100 and 200 ng of DNA extracted from isolates of AGs 1, 3, 4, 5, 6, 7, 8, 9 and BI were adjusted to 200 μl with sterile ddH_2O. For the AG 2 isolates, aliquots containing 100 ng of DNA were adjusted to 200 μl with sterile ddH_2O. An additional 100 ng of DNA from

isolates 21RAuC02 and 22RAuL11 was diluted to form two sets of standards containing 50, 25, 12, 6, 3 and 1 ng of DNA, in 200 μl. An equal volume of 0.8 M NaOH, 20 mM EDTA was added to each sample before denaturing at 100°C for 10 min. The samples were allowed to cool before being loaded into the individual wells of the assembled slot blot apparatus and applied to the positively charged nylon membrane, by vacuum as outlined by the manufacture (BioRad). Slot blot wells were rinsed once with 0.4 M NaOH under vacuum, before removing the membrane and rinsing in 2 x SSC. The membrane was then UV-cross-linked at 30 mJ before being used in hybridisation procedures, as described in section 4.2.7.

4.2.10. Data analysis

AG 2-1 and AG 2-2 isolates used in Southern blot analysis with the DNA probes, pRAG21-413 and pRAG22-39, respectively, were scored for the presence and absence of bands, with 1 representing the presence of a band and 0 representing its absence. Genetic similarities were calculated between all pairs of isolates using the formula of Nei and Li (1979) to form a similarity matrix (appendix D), and dendrograms showing genetic similarities among isolates were constructed using the unweighted pair group method using arithmetic averages (UPGMA) (Sneath and Sokal, 1973). Calculations were performed using the statistical package Genstat version 5 release 4.1 (Lawes Agricultural Trust, Rothamsted Experimental Station).

4.3. Results

A total of 1587 white colonies containing putative DNA fragments from six Australian and Japanese isolates of AG 2 were selected for colony blot analysis. Following hybridisation of the membranes with total genomic DNA from either the AG 2-1 isolates, 21RJ01, 21RAuC02 and 21RAuM04, or the AG 2-2 isolates, 22RJ02, 22RAuL09 and 22RAuS10, a total of 368

recombinants were identified as carrying AG 2 DNA inserts from the six isolates. On the resulting autoradiograph, colonies displayed either high, medium or low signal strengths and 40 of each type were randomly chosen for further assessment. Plasmids were isolated from the colonies as outlined in section 4.2.4 and the inserts liberated by digestion with *Hin*dIII. The fragments were separated by electrophoresis (Figures 4.1A and 4.2A), and their Southern blots were hybridised with total genomic DNA from either 21RAuC02 or 22RAuL09 (Figures 4.1B and 4.2B, respectively). One hundred fragments that showed hybridisation to the respective DNA were selected for RFLP analysis. These fragments represented DNA from each of the six isolates used in the cloning methods.

The selected clones were hybridised to Southern blots of *Hin*dIII-digested genomic DNA. Only a few clones hybridised to DNA from both isolates of AG 2-1 and AG 2-2. One clone, termed pRAG21-413 (Figure 4.1A and B, lane 6), carrying a 2.2 kb DNA insert from isolate 21RAuC02, hybridised to a repeated DNA sequence in the AG 2-1 isolates, but not to DNA from isolates of AG 2-2 (Figure 4.3). However, the clone hybridised strongly to a single 9.5 kb fragment of *Hin*dIII-digested AG 4 DNA and 2.5 kb fragment from digested AG 8 DNA, respectively (Figure 4.4, lanes 10 and 11). When hybridised to DNA from AG 2-1 isolates originating from Australia, the Netherlands, Japan and New Zealand (Figure 4.4), pRAG21-413 identified 42 polymorphic fragments and 15 RFLP patterns amongst the 15 isolates examined. The sequence of the clone appeared to be highly repetitive for the majority of the AG 2-1 isolates. However, only one or two bands were evident for the Netherlands isolates, 21RN06 and 21RN07 and the Australian isolate 21RAu19 (Figure 4.4, lanes 7, 9, and 8). The AG 2-1, ZG 6 isolates (Figure 4.4, lanes 12 - 15) appeared more variable in the RFLP patterns produced.

A dendrogram was produced by UPGMA cluster analysis using the 42 polymorphic fragments identified by pRAG21-413 amongst the AG 2-1 isolates (Figure 4.5). Analysis of the similarity coefficients (appendix D) indicated that the AG 2-1 isolates ranged from 3.0 –

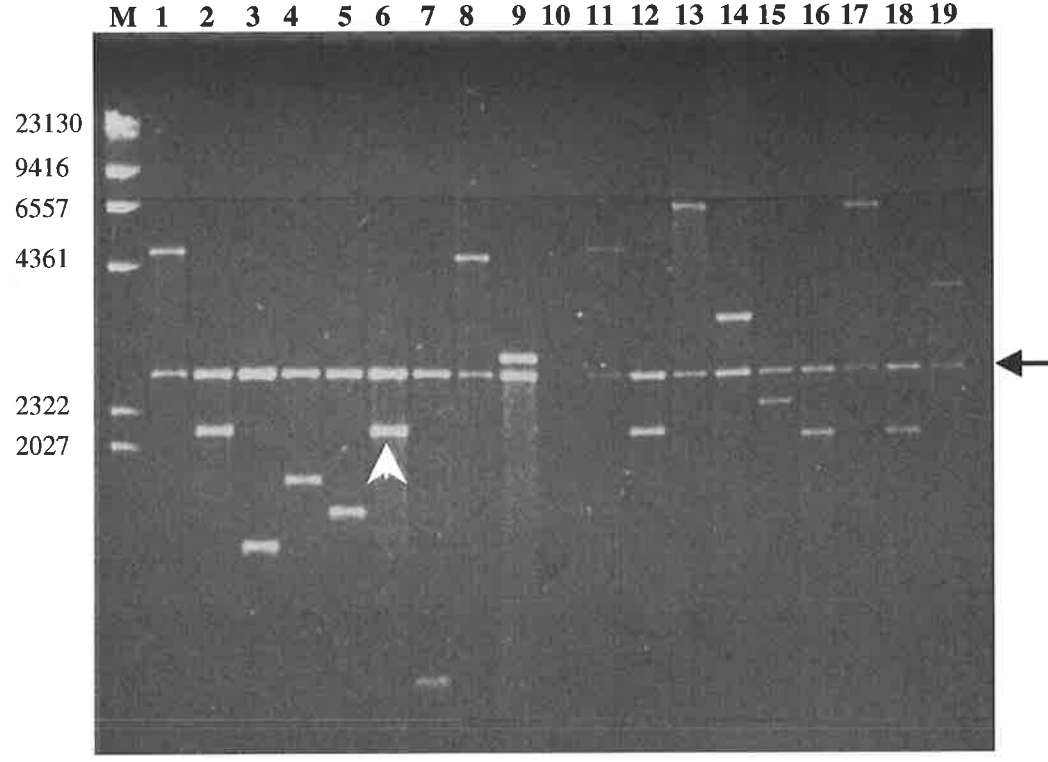
90.5% similarity. The Netherlands isolate, 21RN07, was distantly related to the other AG 2-1 isolates, with only 3% genetic similarity, whereas isolates 21RAuPe16 and 21RAuC15 were 90% similar to one another (Figure 4.5). The RFLP marker pRAG21-413 was unable to distinguish the isolates classified as AG 2-1, ZG 5 from those of AG 2-1, ZG 6. In addition, isolates from various locations and hosts were not clearly differentiated.

As for the AG 2-1 clones, the majority of AG 2-2 recombinant plasmids selected from 22RAuL09, 22RAuS10 or 22RJ02 DNA, did not hybridise to all the AG 2-2 isolates tested. One construct, carrying a 3 kb fragment from isolate 22RJ02 (Figure 4.2A and B, lane 6), termed pRAG22-39, hybridised to a repeated sequence in AG 2-2 isolates, but not to DNA from the AG 2-1 isolates (Figures 4.6 and 4.7). However, this AG 2-2 fragment did hybridise to a single, but different, DNA fragment of digested DNA prepared from an AG 8 and AG 4 isolate (Figure 4.6, lanes 15 and 16). When pRAG22-39 was hybridised to AG 2-2 DNA, four RFLP patterns were identified amongst the seven isolates tested (Figure 4.7). The Japanese isolate, 22RJ02 (Figure 4.7, lane 3), shows a pattern distinct from the other AG 2-2 isolates examined, while the second Japanese isolate, 22RJ01 (Figure 4.7, lane 4) is near identical to the two Netherlands isolates, 22RN06 and 22RN04 (Figure 4.7, lanes 5 and 6). The final Netherlands isolate, 22RN03, shares an identical banding pattern with the two Australian isolates, 22RAuL11 and 22RAuL12 (Figure 4.7, lanes 7, 8, and 9), as well as with the Australian isolate 22RAuL09, seen in lane 14 of Figure 4.6. Unlike the AG 2-1 clone, pRAG22-39 hybridised strongly to the *Hin*dIII digests of all the AG 2-2 isolates examined and, despite the differences in host and geographic region, the patterns of hybridisation were quite similar.

Figure 4.1: Example of an agarose gel with a sample of *Hin*dIII digested pUC19 constructs containing AG 2-1 DNA fragments. The molecular weight size marker, *Hin*dIII-digested lambda (λ) DNA, is shown to the left (bp).

A) DNA fragments cloned from AG 2-1 isolates 21RAuC02 (lanes 1-9) and 21RJ01 (lanes 11-19). The arrow indicates the linear pUC19 plasmid (2686 bp). The pRAG21-413 clone from 21RAuC02 is present in lane 6 (arrow).

B) Southern hybridisation of the above agarose gel with ^{32}P-labelled genomic DNA from isolate 21RAuC02, showing the level of hybridisation of each clone.


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
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9416
6557
4361
2322
2027

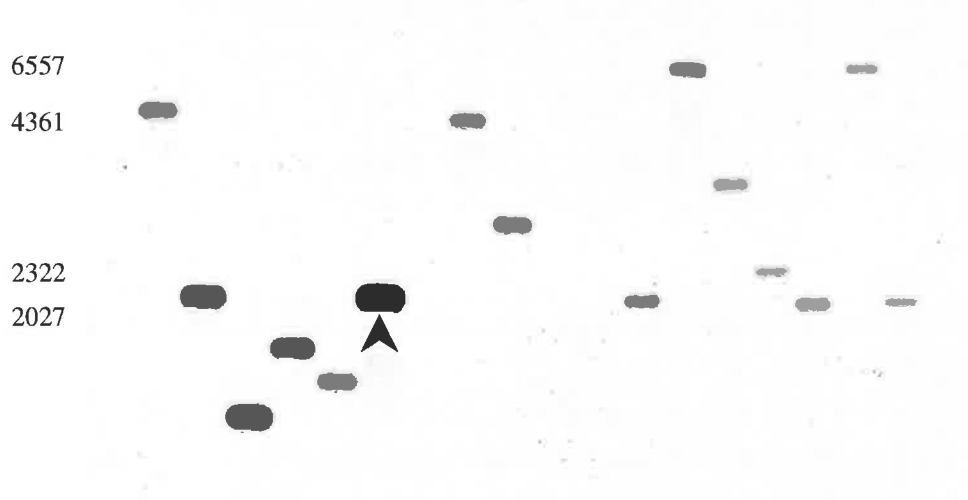
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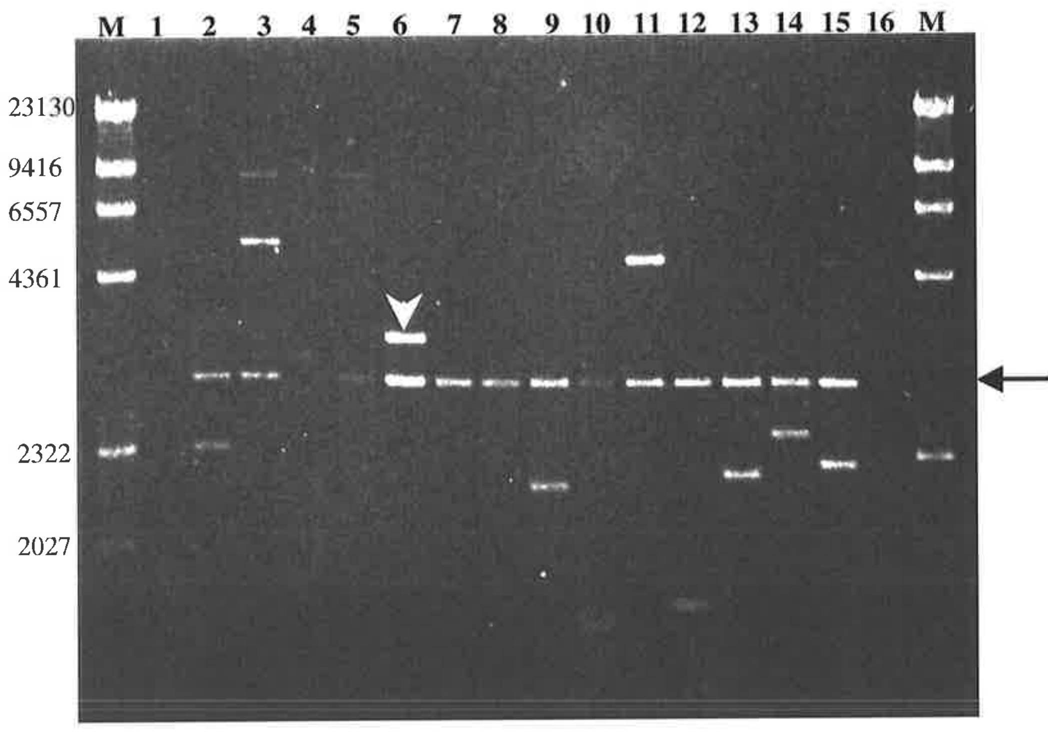
Figure 4.2: Example of an agarose gel with a sample of *Hin*dIII digested pUC19 constructs containing AG 2-2 DNA fragments. The molecular weight size marker, *Hin*dIII-digested λ DNA, is shown to the left (bp).

A) DNA fragments cloned from AG 2-2 isolates 22RAuL09 (lanes 2-8) and 22RJ02 (lanes 9-15). The arrow indicates the linear pUC19 plasmid (2686 bp). The pRAG22-39 clone from 22RJ02 is present in lane 6 (arrow)

B) Southern hybridisation of the above gel with ^{32}P-labelled genomic DNA from isolate 22RAuL09 showing the level of hybridisation of each clone.


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 M
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M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

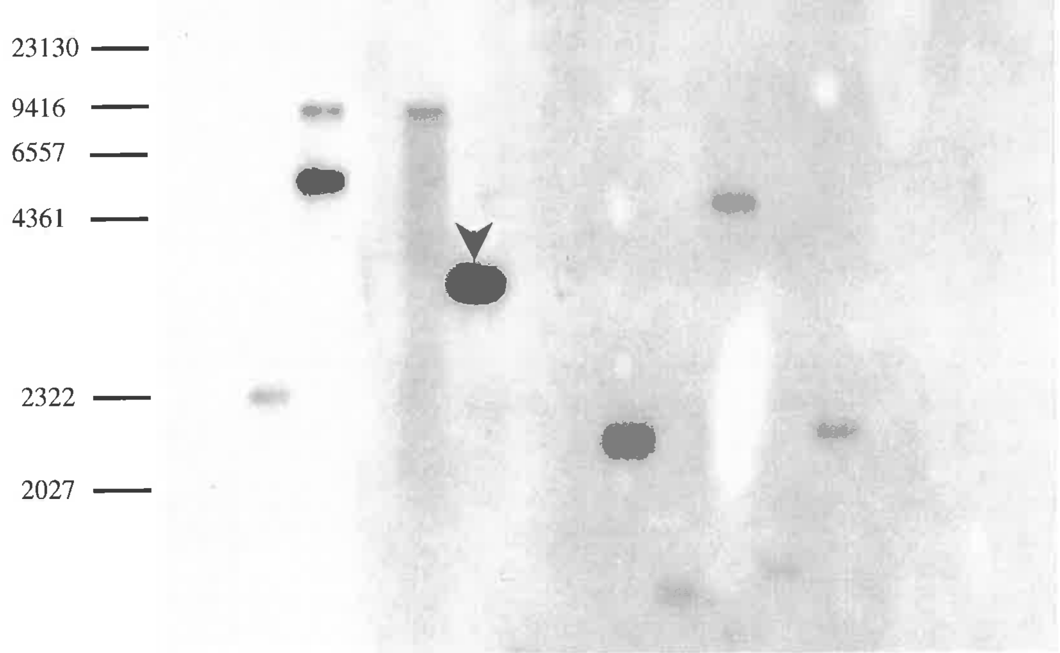
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Figure 4.3: Southern blot of *Hin*dIII digested genomic DNA from isolates of AG 2-1, 2-2, 4 and 8, hybridised with the ^{32}P-labelled pRAG21-413 clone. The sizes in base pairs for the marker, *Hin*dIII- digested λ DNA, are shown to the left.

Lane 1, 21RAuC02; Lane 2, 21RAuM04; Lane 3, empty; Lane 4, 22RJ02; Lane 5, 22RJ01; Lane 6, 22RN06; Lane 7, 22RN04; Lane 8, 22RN03; Lane 9, 22RAuL11; Lane 10, 22RAuL12; Lane 11, 22RAuL09; Lane 12, 04RAuP01; Lane 13, 08RAuB08.

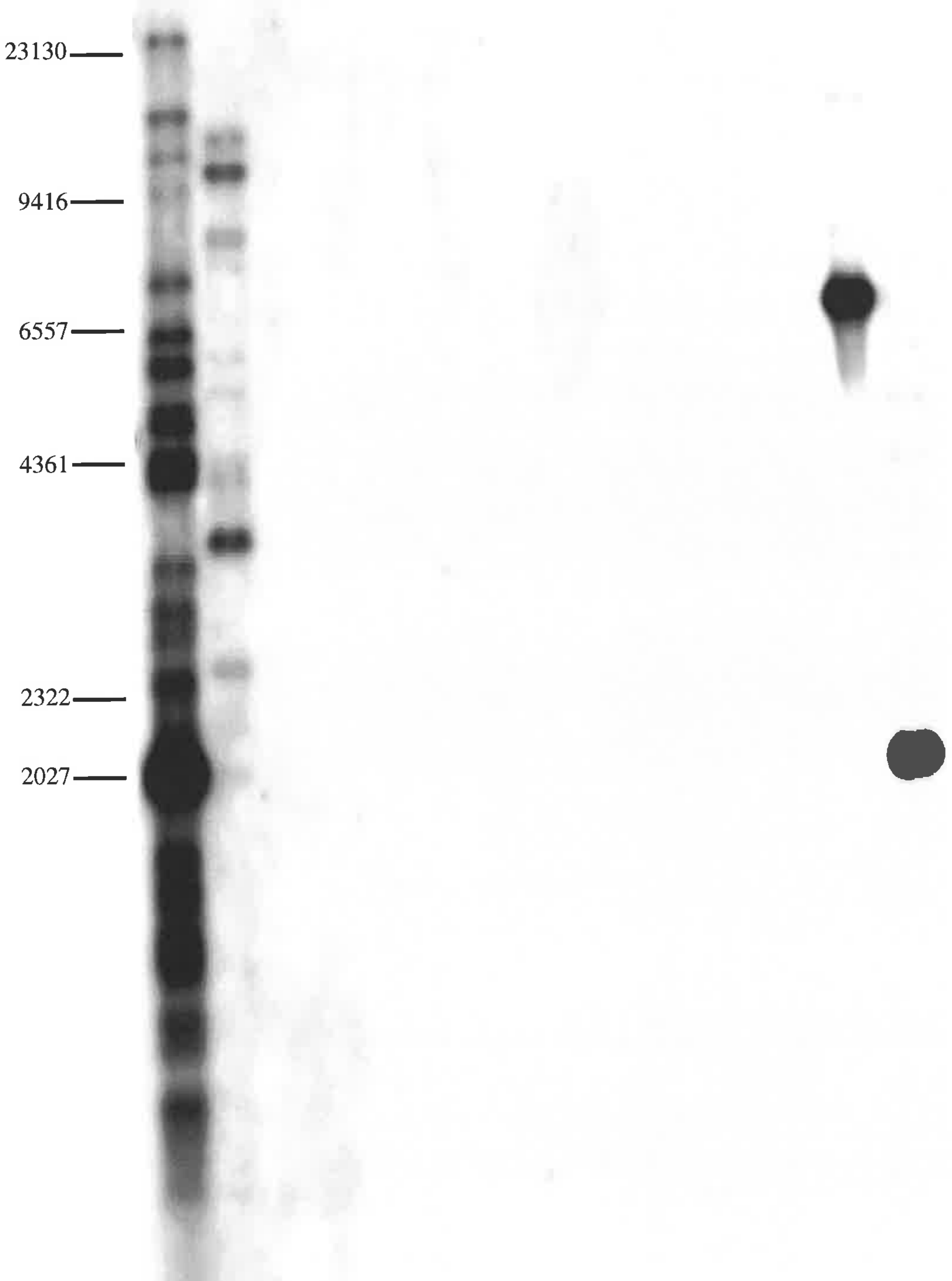

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Figure 4.4: Southern blot of *Hind*III-digested genomic DNA from AG 2-1 isolates, hybridised with the ^{32}P-labelled pRAG21-413 clone. The sizes in base pairs for the marker, *Hind*III-digested λ DNA, are shown to the left.

Lane 1, 21RAuC02; Lane 2, 21RAuC15; Lane 3, 21RAuPe16; Lane 4, 21RAuL17; Lane 5, 21RAuW09; Lane 6, 21RAuM04; Lane 7, 21RN06; Lane 8, 21RAu19; Lane 9, 21RN07; Lane 10, 21RJ01; Lane 11, 21RAuW10; Lane 12, 21RAuB11; Lane 13, 21RAuB12; Lane 14, 21RAuP08; Lane 15, 21RNzPr20.

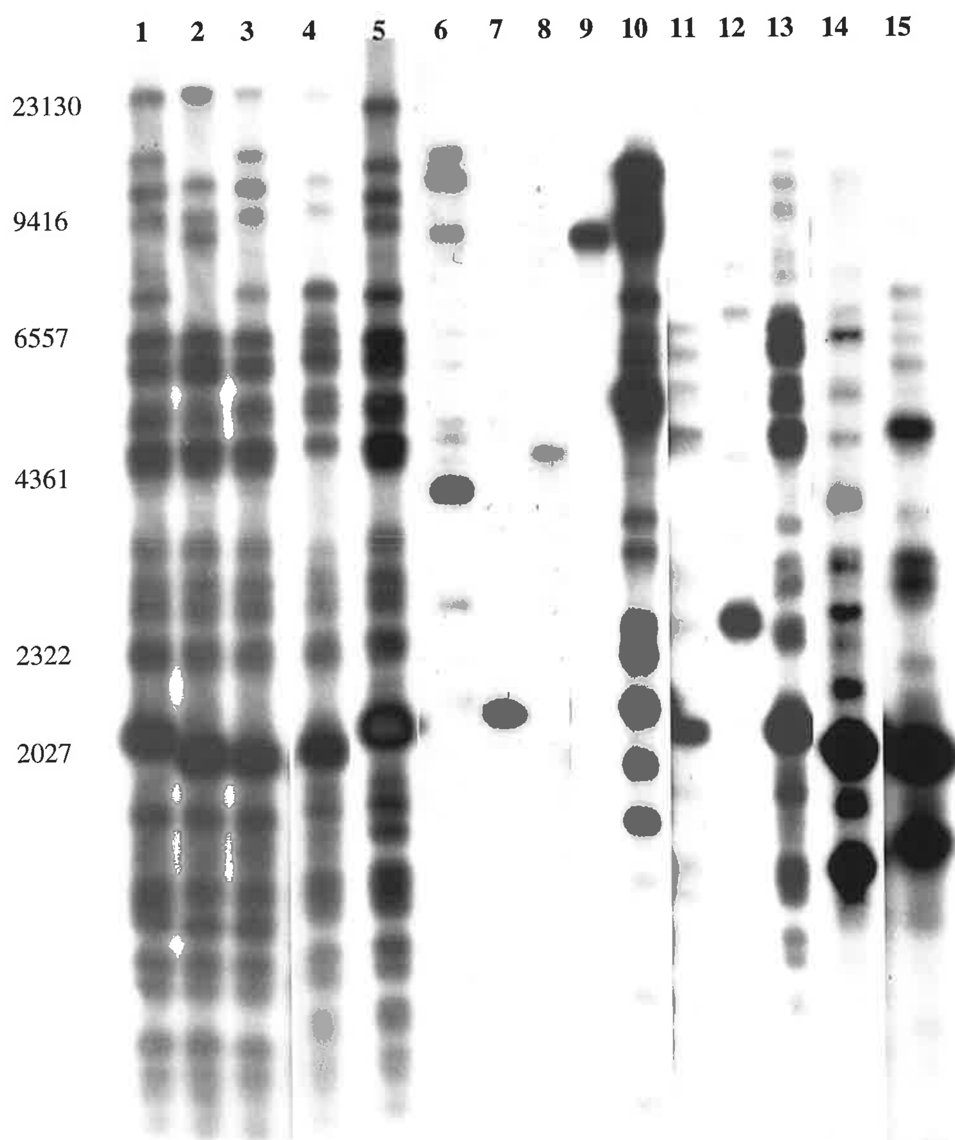

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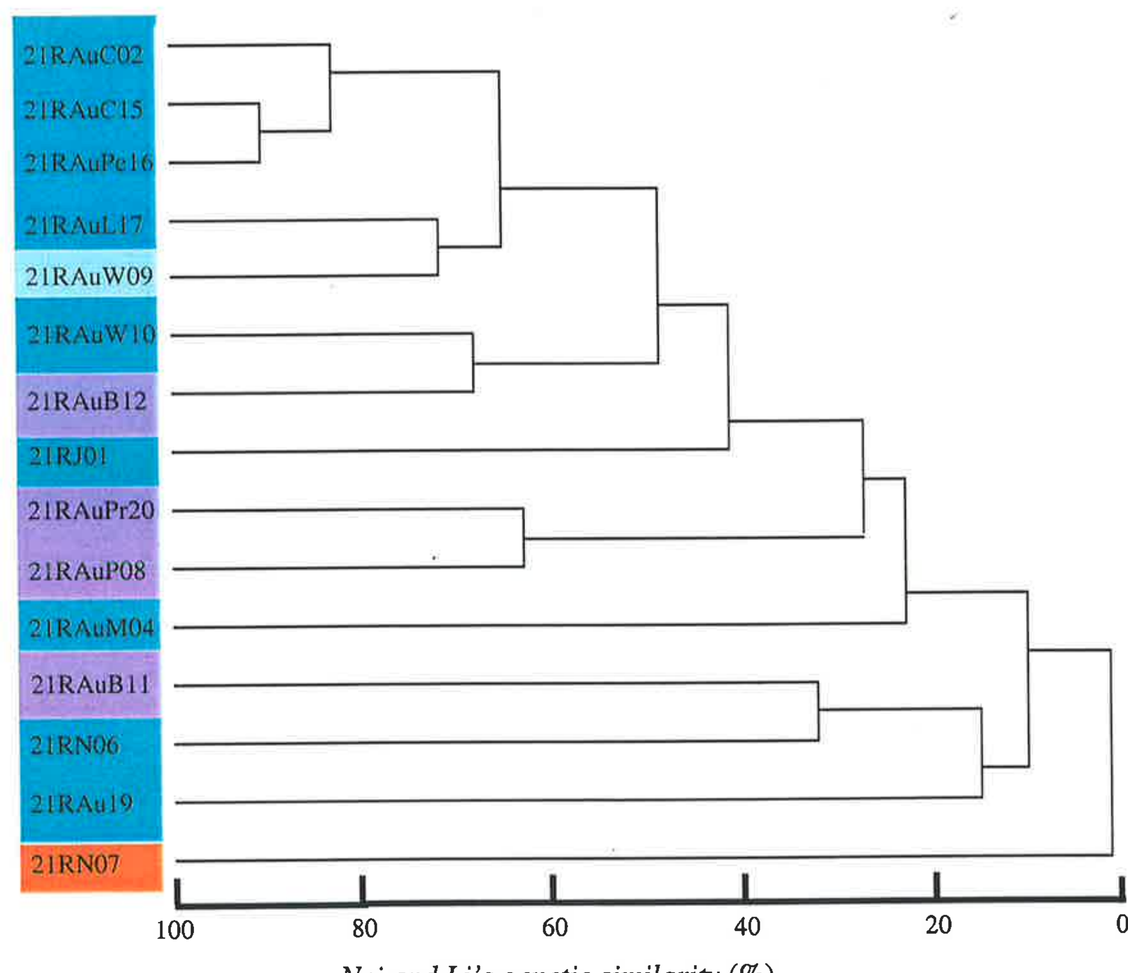
Figure 4.5: Dendrogram representing genetic similarity of AG 2-1 isolates based on hybridisation with the pRAG21-413 clone to *Hin*dIII digested genomic DNA. Similarities were calculated by the UPGMA method after the comparison of banding patterns by Nei and Li's genetic index (1979).

AG 2-1, ZG 5 isolates

AG 2-1, ZG 6 isolates

"AG 2 undefined" isolates


21RAuC02
21RAuC15
21RAuPe16
21RAuL17
21RAuW09
21RAuW10
21RAuB12
21RJ01
21RAuPr20
21RAuP08
21RAuM04
21RAuB11
21RN06
21RAu19
21RN07
100
80
60
40
20
0
Nei and Li's genetic similarity (%)

A second dendrogram was produced using UPGMA cluster analysis to examine the similarities among AG 2-2 isolates using RFLPs identified by pRAG22-39 (Figure 4.8). Analysis of the similarity coefficients (appendix D) indicated that the eight AG 2-2 isolates examined showed between 36 - 100% similarity. The isolates clustered into three groups, with the Netherlands isolate 22RN03 and the three Australian isolates, 22RAuL09, 22RAuL11 and 22RAuL12, being identical (Figure 4.8). Similarly, the Netherlands isolates 22RN04 and 22RN06 were identical and formed a second group (61% similarity) with the Japanese isolate, 22RJ01. The Netherlands and Japanese isolates showed 40% similarity with the group of four isolates mentioned previously. The final AG 2-2 isolate, 22RJ02 from Japan, shared 36% similarity with the other AG 2-2 isolates examined. The RFLP marker, pRAG22-39, was unable to distinguish between AG 2-2, ZG 4 or ZG 10 isolates and could not discriminate between isolates identified as AG 2-2 group I or group II by zymogram analysis (reported in chapter 2).

The specificity of the clones, pRAG21-413 and pRAG22-39, was examined by slot blot analysis as described in section 4.2.9 and the resulting autoradiograms are presented (Figures 4.9A and B). Both membranes contained 0.5 to 200 ng of AG 2 DNA and larger amounts (200 and 400 ng) of DNA from the other AG. The AG 2-1 clone, pRAG21-413, hybridised weakly to DNA from AGs 1, 4 and 7, moderately to DNA from AGs 3, 5 and 6, and strongly to DNA from the AG 2-1 isolates as well as AGs 8, 9 and BI (Figure 4.9A). The strong level of hybridisation observed for 400 ng of DNA from the AG 8, 9 and BI isolates, was approximately equal to that for 100 ng of AG 2-1 DNA. DNA from isolates of AG 2t, AG 2-2 or "AG 2 undefined" did not hybridise with the pRAG21-413 clone (Figure 4.9A). The AG 2-2 pRAG22-39 clone hybridised weakly to DNA from AGs 1, 4, and 7, moderately to DNA from AGs 3, 5, 6, 8, 9, BI and "AG 2 undefined" and strongly to DNA from the AG

Figure 4.6: Southern blot of *Hin*dIII digested genomic DNA from isolates of AG 2-1, 2-2, 4 and 8, hybridised with ^{32}P-labelled pRAG22-39 clone. The sizes (bp) for the marker, *Hin*dIII-digested λ DNA, are shown to the left.

Lane 1, 21RAuC02; Lane 2, 21RAuC15; Lane 3, 21RAuPe16; Lane 4, 21RAuM03; Lane 5, 21RAuM04; Lane 6, 21RN06; Lane 7, 21RAu19; Lane 8, 21RAuP08; Lane 9, 21RAuBr21; Lane 10, 21RNzPr21; Lane 11, 21RAuB11; Lane 12, 21RAuB12; Lane 13, 21RAuB13; Lane 14, 22RAuL09; Lane 15, 08RB08; Lane 16, 04R01.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

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Figure 4.7: Southern blot of *Hin*dIII digested genomic DNA from AG 2-2 isolates and hybridised with ^{32}P-labelled pRAG22-39 clone. The sizes (bp) for the marker, *Hin*dIII-digested λ DNA, are shown to the left.

Lane 1, 21RAuC02; Lane 2, 21RAuM04; Lane 3, 22RJ02; Lane 4, 22RJ01; Lane 5, 22RN06; Lane 6, 22RN04; Lane 7, 22RN03; Lane 8, 22RAuL11; Lane 9, 22RAuL12.

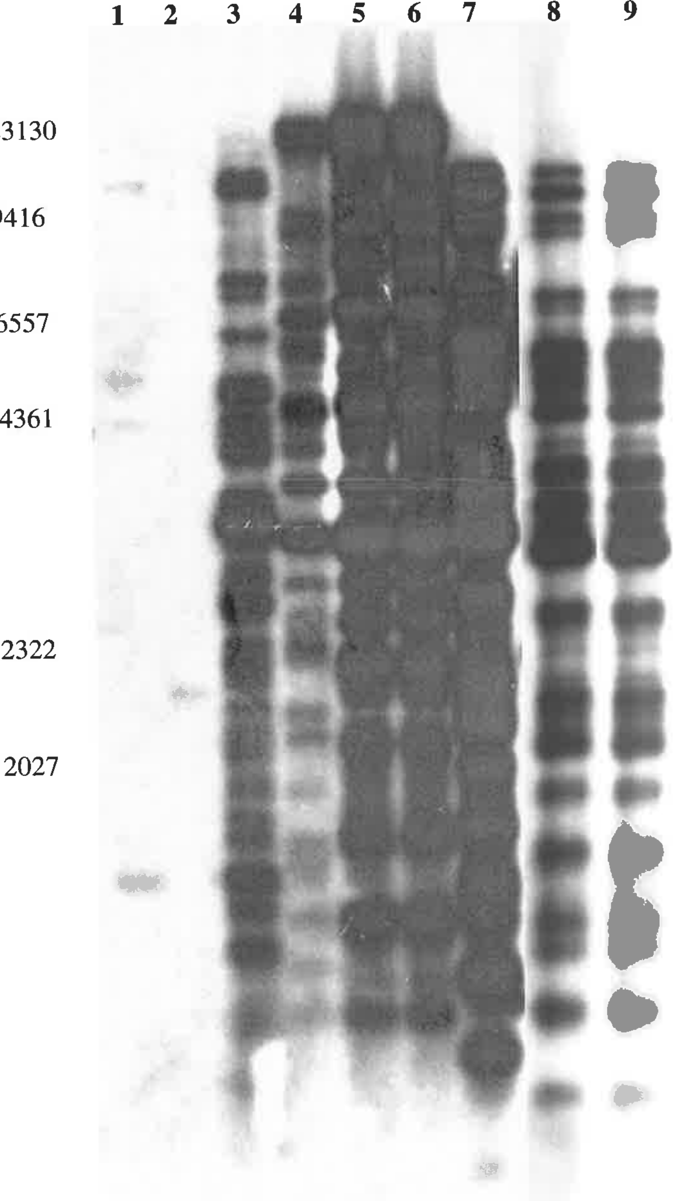

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Figure 4.8: Dendrogram representing genetic similarity of AG 2-2 isolates based on hybridisation with the pRAG22-39 clone to *Hin*dIII digested genomic DNA. Similarities were calculated using the UPGMA method after comparison of the banding patterns by Nei and Li's genetic similarity (1979).

AG 2-2, group I isolates

AG 2-2, group II isolates

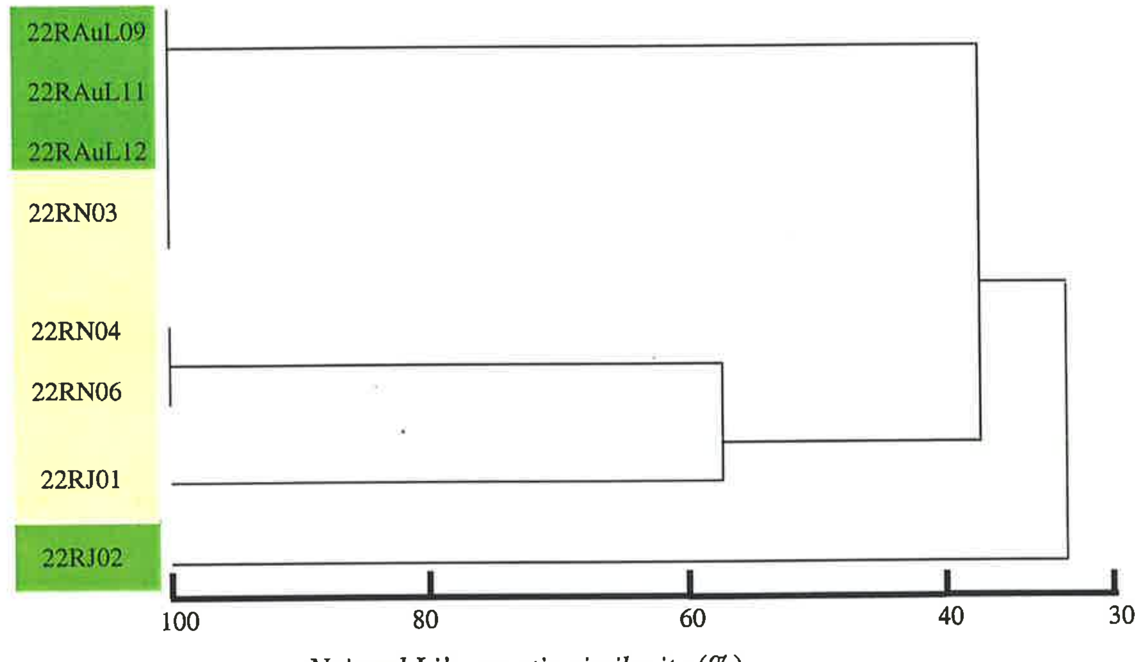

22RAuL09
22RAuL11
22RAuL12
22RN03
22RN04
22RN06
22RJ01
22RJ02
100
80
60
40
30
Nei and Li's genetic similarity (%)

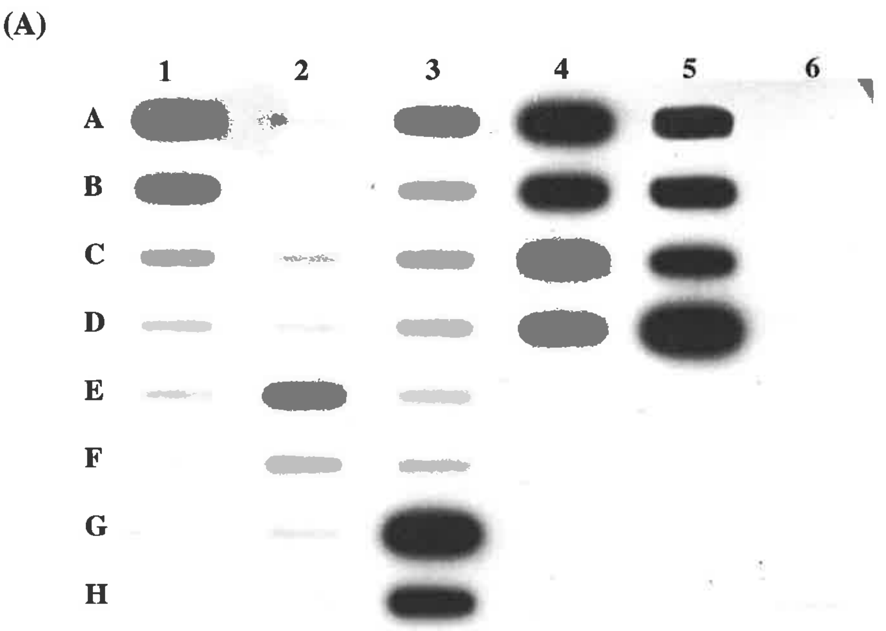
Figure 4.9: Slot blot of DNA from various *R. solani* AGs probed with the two putative AG 2-specific probes.

A) Slot blot membrane hybridised with pRAG21-413

	1	2	3	4	5	6
A	AG 2-1 50ng	AG 1-1A 400ng	AG 5 400ng	AG 9 400ng	21RAuC02 100ng	-
B	AG 2-1 25ng	AG 1-1A 200ng	AG 5 200ng	AG 9 200ng	21RN07 100ng	AG 2-2 0.5ng
C	AG 2-1 12ng	AG 1-1B 400ng	AG 6 400ng	AG BI 400ng	21RAuP08 100ng	AG 2-2 1ng
D	AG 2-1 6ng	AG 1-1B 200ng	AG 6 200ng	AG BI 200ng	21RAuM04 100ng	AG 2-2 3ng
E	AG 2-1 3ng	AG 3 400ng	AG 7 400ng	AG 2t 200ng	-	AG 2-2 6ng
F	AG 2-1 1ng	AG 3 200ng	AG 7 200ng	AG 2t 100ng	02RAuCf03 100ng	AG 2-2 12ng
G	AG 2-1 0.5ng	AG 4 400ng	AG 8 400ng	AG 2-2 200ng	02RAuL04 100ng	AG 2-2 25ng
H	-	AG 4 200ng	AG 8 200ng	AG 2-2 100ng	-	AG 2-2 50ng

B) Slot blot membrane hybridised with pRAG22-39

	1	2	3	4	5	6
A	AG 2-1 50ng	AG 1-1A 400ng	AG 5 400ng	AG 9 400ng	22RAuL09 100ng	-
B	AG 2-1 25ng	AG 1-1A 200ng	AG 5 200ng	AG 9 200ng	22RN03 100ng	AG 2-2 0.5ng
C	AG 2-1 12ng	AG 1-1B 400ng	AG 6 400ng	AG BI 400ng	22RJ01 100ng	AG 2-2 1ng
D	AG 2-1 6ng	AG 1-1B 200ng	AG 6 200ng	AG BI 200ng	22RN06 100ng	AG 2-2 3ng
E	AG 2-1 3ng	AG 3 400ng	AG 7 400ng	AG 2t 200ng	-	AG 2-2 6ng
F	AG 2-1 1ng	AG 3 200ng	AG 7 200ng	AG 2t 100ng	02RAuCf03 100ng	AG 2-2 12ng
G	AG 2-1 0.5ng	AG 4 400ng	AG 8 400ng	AG 2-1 200ng	02RAuL04 100ng	AG 2-2 25ng
H	-	AG 4 200ng	AG 8 200ng	AG 2-1 100ng	-	AG 2-2 50ng


(A)
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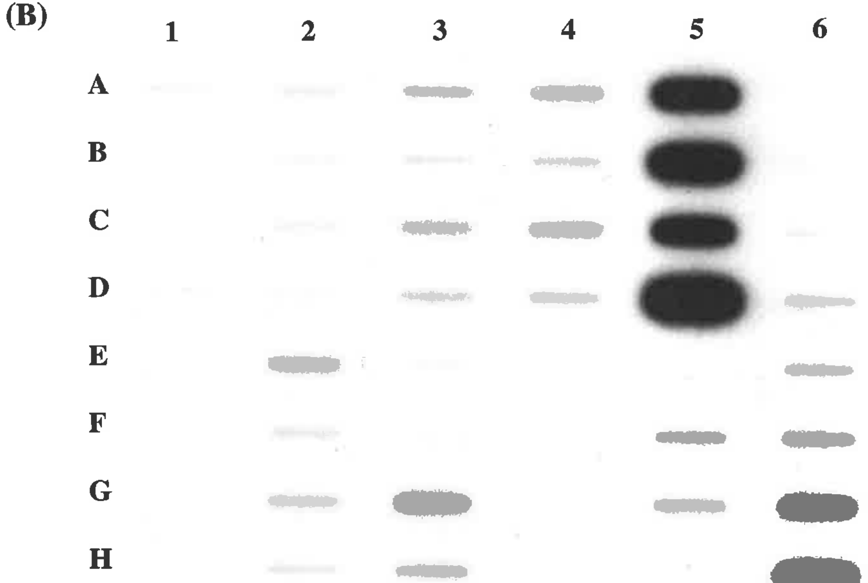
(B)
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2-2 isolates (Figure 4.9B). The hybridisation signal obtained for 400 ng of AG 3, 5, 6, 8, 9 or BI DNA was similar to the hybridisation strength observed with 12 ng of AG 2-2 DNA. For the two "AG 2 undefined" isolates, 02AuCf03 and 02AuL04, the 100 ng loading was equivalent to approximately 12 ng of AG 2-2 DNA. The pRAG22-39 clone did not hybridise to DNA from isolates of AG 2-1 (Figure 4.9B), nor to DNA from AG 2t (data not shown).

No clones specific to either AG 2-1 or AG 2-2 were identified. More importantly for genetic analysis, no clones which recognised all isolates of either subgroup were obtained in these experiments and no clones that recognised DNA from all AG 2-1 and AG 2-2 isolates were found. Therefore, it was not possible to examine the relationship between AG 2-1 and AG 2-2 with RFLP analysis and it was difficult to resolve the relationship of all isolates within each respective subgroup using the two probes.

4.4. Discussion

Random cloning resulted in the identification of two putative DNA markers for AG 2-1 and AG 2-2 isolates of *R. solani*. Clone pRAG21-413 contained a 2.2 kb fragment of AG 2-1 DNA from the Australian isolate 21RAuC02, while pRAG22-39 carried a 3 kb fragment of AG 2-2 DNA from the Japanese isolate 22RJ02. Neither fragment hybridised to DNA of the other AG 2 subgroup, however, both fragments hybridised to DNA from other *R. solani* AGs in both slot blots and Southern blots.

The clone pRAG21-413 was used in RFLP analyses of 15 AG 2-1 isolates from various hosts and locations. For the majority of isolates examined, the clone identified a number of bands, indicating that the sequence occurs in multiple copies in the genomes of these isolates. However, no distinct patterns were obtained which could separate AG 2-1, ZG 5 isolates from AG 2-1, ZG 6 isolates. UPGMA cluster analysis of the data failed to distinguish the two AG 2-1 zymogram groups and there was no distinction between isolates

from different locations or hosts. However, with further work it may be possible to identify DNA sequences that are able to differentiate between the groups. Due to the time constraints it was not possible to screen all clones originally identified. The analysis did reveal that the Netherlands isolate, 21RN07, classified as "AG 2 undefined" by the zymogram procedure in chapter 2, is only distantly related to other AG 2-1 isolates, sharing approximately 3% similarity. In addition, isolates such as 21RAuC02, 21RAuC15 and 21RAuPe16, which shared 100% similarity by zymogram analysis, also shared high similarity using RFLP analysis with pRAG21-413 as the probe. Furthermore, differences in the repetitiveness and extent of similarity between AG 2-1 isolates were observed, using the pRAG21-413 clone. There are two possible explanations for this broad range of genetic similarity. The first is that the collection of AG 2-1 isolates examined was from geographically diverse regions and from various hosts, therefore the genetic diversity may be attributed to differences in evolution. While isolates such as 21RAuC02 and 21RAuPe16 shared high similarity (approximately 84%) but came from geographically distant locations (South Australia and Western Australia, respectively), it is possible that this is a result of recent introductions, either naturally or via agricultural practices. The second explanation is that the pRAG21-413 sequence represents a region of the DNA that is not highly conserved. This is supported to some degree by the fact that isolates from different countries are quite distinct from one another whereas, in general, isolates from the same country are more closely related.

The AG 2-2 clone, pRAG22-39, revealed four distinct banding patterns amongst the seven AG 2-2 isolates examined which shared an average similarity of 36%. The clone was not able to distinguish between AG 2-2, ZG 4 and ZG 10 isolates, nor could isolates be placed into AG 2-2 groups I and II identified in chapter 2 of this thesis. The three lupin isolates from Australia shared 100% similarity with one of the Netherlands isolates from sugar beet. It does not seem likely that the regions of the genome identified by pRAG22-39 are conserved amongst these AG 2-2 isolates, as the Japanese isolate 22RJ02 shares only 36% similarity

with the other AG 2-2 isolates examined, including a second Japanese isolate of AG 2-2. It is possible that the region represented by pRAG22-39 is undergoing slow evolutionary change.

Both the pRAG21-413 and pRAG22-39 clones need to be sequenced and genetic data bases searched to identify the sequences represented by the clones. Genomic mapping would identify the region of the genome from which the clones are derived. Both processes would enable further assessment of their use as markers for isolates of AG 2-1 and AG 2-2.

Only the AG 2-2 clone, pRAG22-39, hybridised to the "AG 2 undefined" isolates, 02RAuCf03 or 02RAuL04 in the slot blot procedure. In addition, pRAG21-413 did not hybridise to the Netherlands AG 2t isolate, 2tRN01. This suggests that both the "AG 2 undefined" isolates from Australia and the tulip isolate, 2tRN01, from the Netherlands represent genetically distinct identities. In the case of 2tRN01, the results presented here support the proposal by Schneider *et al.*, (1997) that AG 2t represents a genetically distinct subgroup within AG 2-1, ZG 5. For the "AG 2 undefined" isolates, this may suggest that they are distantly related to AG 2-2 isolates. Further study is required to determine the identity of the "AG 2 undefined" isolates identified in this research and to establish their role in the evolution of the AG 2 complex.

It is noteworthy that the cloning experiment did not identify DNA fragments that hybridised to all AG 2 isolates examined. Even more unexpected was the similarity of the AG 2-1 and AG 2-2 derived sequences to DNA from isolates of other AGs. It has been assumed from previous studies, particularly anastomosis, that AG 2-1 and AG 2-2 are more closely related to one another than they are to the other groups. In studies conducted with randomly generated genomic DNA clones, O'Brien (1994) found that of six fragments originating from an AG 11, ZG 3 isolate, four were able to hybridise to DNA from other AG and ZG. It may be that the AG 11 group has recently evolved and still has close genetic relationships to other AG. In addition, O'Brien (1994) reported that the banding pattern obtained for the original AG 11, ZG 3 isolate was more complex than the patterns of isolates

from other AG and ZG. This was also observed in the results presented in this chapter, where *Hin*dIII digested DNA from isolates of AG 4 and AG 8 gave only one or two bands when probed with either pRAG21-413 or pRAG22-39. In addition, the hybridisation to DNA from other AG in the slot blot procedures suggest that there exists a relationship between the groups, some of which may be more closely related to one another than others. Whether this is due to the occurrence of infrequent anastomosis between groups or some other phenomenon, needs to be determined to produce a more complete picture of the genetic relationships that exist between the different AG. The identification of DNA probes that hybridise to repeated sequences in all AG would be a useful genetic tool, when combined with other molecular techniques, for such studies.

As mentioned previously, the sequencing of the putative markers would assist in their development as markers for AG 2-1 and AG 2-2. By identifying discrete sequences and restriction sites, it may be possible not only to reduce the size of the fragments, but also to identify sequences which are specific to AG 2-1 or AG 2-2, similar to those identified for AG 3 (Balali *et al*., 1996) and AG 8 (Matthew *et al*., 1995). This would also facilitate identification of primer sites for use in PCR. The repetitive nature of the probes is a feature that needs to be retained, as this would allow both identification to AG level and the assessment of genetic diversity within the AG to be conducted in the one process. In addition, it would facilitate analysis of the relationships that exist between AG 2-1 and AG 2-2. Whether these two subgroups are closely related is questionable according to the results presented in this chapter. The fact that the two groups displayed distinct zymogram patterns also suggests that the genetic relationship between the two is not close. Research with other molecular markers may help to clarify the classification of the AG 2 subgroups.

Chapter 5

Amplification of DNA Polymorphisms in *R. solani* AG 2

5.1. Introduction

The sensitivity and specificity of the polymerase chain reaction (PCR), and its use in combination with sequencing techniques, have facilitated rapid analysis of the genomes of many organisms. Primer sequences now available enable the comparison of organisms for genetic relatedness and recent advances have resulted in the development of species-specific primer sets for identification to, at or below the species level.

PCR has been used to study the genetic diversity of *R. solani*, both within and between the various AG, and to examine the relationships of the AGs to one another. The two most common techniques used in these studies have been 1) RAPD-PCR and 2) amplification of the internal ITS regions of rDNA repeats. RAPD analysis has been used to generate primer sets capable of distinguishing AG 4 and 8 (Brisbane *et al.*, 1995), and to examine the diversity amongst isolates from AG 8, 2-1, 2-2, 3 and 4 (Duncan *et al.*, 1993). Recently, MacNish (1999) reported that RAPD-PCR can be used to identify particular genotypes belonging to each ZG, within AG 8. A large amount of research has also been directed at the identification of group-specific primers generated from the ITS regions. ITS-PCR has been used to identify AG 2 subgroups for a number of years. Liu and Sinclair (1992) demonstrated that sequence variability in ITS regions could be used to distinguish five intra-specific groups within AG 2, and these correlated closely with the established ZG. More recently, Salazar *et al.* (1999) examined the ITS sequence of 31 AG 2 isolates, mainly from Japan and the Netherlands. They reported that the entire ITS region among AG 2 isolates share 78–100% sequence similarity. However, ITS-1 varied between 18 and 100%, while the ITS-2 region was 39–

100% similar. This high level of genetic variation in the ITS region of AG 2 DNA is an indication of the variable nature of the group. Following on from these studies, Salazar *et al.* (2000) reported the development of primer sets specific for AG 2 DNA. One set, AG2sp/ITS 4B was shown to identify a 680 bp fragment in DNA extracted from pure cultures of isolates of AG 2-1, 2-2, 2-3 and 2t from Japan, Alaska and the Netherlands. However, when the primer set was used to identify DNA extracted from radish infected with a range of AG 2 isolates, only AG 2-1 and 2t could be amplified (Salazar *et al.*, 2000). In addition, the authors noted that to achieve amplification, 10 µg of total DNA (i.e. plant and fungal DNA combined) was required for a 50 µl reaction volume.

Microsatellite-PCR has also been used to detect polymorphisms in fungi. Microsatellites, also termed simple sequence repeats, are abundant and uniformly distributed throughout the genome of many organisms . In addition, the repeats are highly polymorphic and co-dominant, making them ideal for PCR analysis (Jarne and Lagoda, 1996). Primers have been designed to amplify the microsatellite regions present in chromosomal DNA of fungi (Bridge and Arora, 1998). In most instances, these primers have provided a level of phylogenetic information similar to that obtained using the RAPD technique, allowing the grouping of fungi at infraspecific levels (Bridge *et al.*, 1997). In addition, it may be possible to identify species-specific patterns or group-specific fragments for use in diagnostics. Microsatellite primers can also be used to provide insights into the genetic relatedness of isolates within a species. For example, Anderson *et al.* (1998) used microsatellite-PCR to amplify polymorphisms of the basidiomycete, *Pisolithus tinctorius*, in order to examine colony size and distribution of isolates within a field. A large number of genetically distinct *P. tinctorius* isolates were identified from the single field population, indicating that a high level of genetic diversity exists within this species in a small geographic area.

In the research reported here, experiments were designed to 1) examine the genetic diversity of a collection of AG 2 isolates using microsatellite-PCR, and 2) examine the ability

of published ITS primers, specific to AG 2 isolates from Japan and the Netherlands, to identify Australian AG 2 isolates.

5.2. Materials and methods

5.2.1 Extraction of DNA

Total DNA was extracted from isolates of *R. solani* using the method described in section 4.2.1. In some instances, DNA was purified further using the GENECLEAN® SPIN kit (Bio 101, Inc., California).

5.2.2. Amplification with the intron-exon splice junction (ISJ) primer, R1

The R1 primer, specific to ISJ regions of plant DNA (Weining and Langridge, 1991) was used to detect genetic variation between AG 2 isolates. Reactions were performed using a MJ Research PTC-100 thermal controller (MJ Research, Inc.). Each reaction consisted of 1 x amplification buffer (Qiagen), 0.2 mM each dNTP, 75 pmole of primer, approximately 60 ng of template DNA and 0.75 units of *Taq* DNA polymerase (Qiagen) in a reaction volume of 25 μl. The cycling conditions consisted of an initial denaturation step of 94°C for 5 min followed by 10 cycles at 94°C for 1 min, 40°C for 1 min, and 72°C for 2 min. This was followed by 28 cycles at 94°C for 1 min, 57°C for 1 min and 72°C for 2 min. The procedure was completed with a 5 min final extension at 72°C. Amplification products were separated by electrophoresis at 30 V overnight in 0.5 x TBE buffer (Appendix A), using 2% agarose gels. Gels were stained with ethidium bromide and amplification products visualised under UV.

5.2.3. Microsatellite-primed PCR

Microsatellite primers $(GACA)_4$, $(GATA)_4$, $(TCT)_5$, $(CAC)_5$, $(GGAT)_4$, $(GAA)_6$ and the minisatellite primer M13 (5'-GAGGGTGGCGGTTCT-3') were used in PCR to assess their ability to reveal DNA polymorphisms among the AG 2 isolates. In preliminary experiments, the $(GACA)_4$, and $(CAC)_5$ primers provided reproducible amplification products and were used in subsequent experiments. The reaction and thermocycling conditions were similar to those described in Section 5.2.2, except that the primer concentration was 25 pmole per reaction and 1 ng of template DNA was used. Amplification products were separated by electrophoresis at 30 V overnight using 2% agarose gels in 0.5 x TBE buffer, stained with ethidium bromide and viewed under UV.

5.2.4. Amplification using ITS primers

PCR and Southern analysis

The ability of the AG 2-specific primer developed by Salazar *et al.* (2000) to identify Australian AG 2 isolates was examined by comparing the amplification of DNA from several Australian AG 2-1 and AG 2-2 isolates, along with that of representative isolates from the Netherlands. In addition, AG 2 isolates from New Zealand and Japan, as well as a bridging isolate and an AG 3, 4 and 8 isolate, were included in the analysis to confirm specificity. The AG2-specific primer, AG2sp (5'-ATTATTGAATTTAAACAAAG-3') was used in combination with the ITS4B primer (5'-CAGGACACTTTGTACACGGTCCAC-3') in amplification reactions. Reactions were carried out in 25 μl using an MJ Research PTC-100 thermal cycler. Each reaction consisted of 1 x Qiagen reaction buffer, 2 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 μM of each primer, 0.75 units of *Taq* polymerase and approximately 10 ng of template DNA. Cycling conditions consisted of an initial denaturation at 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 50°C for 45 sec and an extension step at 72°C for 1 min. The reaction was completed with a final extension step at 72°C for 5 min.

Amplification products were separated on 1% agarose gels at 60 V, using 0.5 x TBE running buffer. Gels were stained with ethidium bromide and products visualised under UV. Amplification products were then transferred to a positively charged nylon membrane, following the method of Southern (1975; see Section 4.2.7). An AG 2-specific probe was prepared by amplifying DNA from the Netherlands isolate 22RN06 by PCR amplification with the AG2sp/ITS4B primer set. The 860 bp fragment was purified using the GENECLEAN® SPIN Kit (Bio-101, Inc.) and 30 ng was labelled with α^{32}P-dCTP and used to probe the membranes in a hybridisation reaction (see Section 4.2.7). Both pre-hybridisation and hybridisation were carried out at 65°C in a rotating bottle oven (Hybaid, Inc.. The membranes were washed at 65°C with a total of four consecutive 30 min washes, increasing in stringency, as follows: 2 x SSC / 0.1% SDS, 1 x SSC / 0.1% SDS, 0.5 x SSC / 0.1% SDS, 0.2 x SSC / 0.1% SDS. Membranes were air-dried and exposed to x-ray film at -80°C for 12 h.

5.2.5. Data analysis

Isolates analysed using microsatellite- and R1-primed PCR, were scored for the presence and absence of amplified fragments, with 1 representing the presence of a band and 0 representing its absence. All amplifications were performed at least twice and a combined binary matrix was constructed for all three primers. Across the range of isolates tested, the frequency with which each band occurred was calculated as the sum of the number of times the band was present / the total number of isolates. Any bands occurring with a frequency of less than 0.1 were excluded from the analysis. Genetic similarities were calculated between all pairs of isolates using the formula of Nei and Li (1979) to form a similarity matrix (appendix C), and a dendrogram was constructed using UPGMA (Sneath and Sokal, 1973) to show clustering. Calculations were performed using the statistical package Genstat version 5 release 4.1

(Lawes Agricultural Trust, Rothamsted Experimental Station). Only reproducible bands were assessed.

For each primer and the combined data set, isolates were placed into phenotypic groups based on the banding patterns. Diversity among isolates was examined using Shannon's diversity index, H and normalised index, H', which were calculated as follows;

$$H = -\Sigma\, Pi.(\ln Pi)$$

$$H' = H/\ln.n$$

where Pi is the frequency of the ith phenotype at the jth locus and n is the number of isolates (Peet, 1974). H' was used to describe diversity on a 0 to 1 scale, where 0 = no diversity and 1 = maximum diversity.

In addition, isolates were divided into three groups based on their original host (crucifer, legume and miscellaneous), and another three groups based on geographic region (South Australia, Western Australia and Japan / the Netherlands / New Zealand). Nei's genetic differentiation (G_{st}) was determined from Shannon's diversity index for each primer (locus) examined, to determine the distribution of genetic similarity between isolates from different hosts or locations using the following formula;

$$G_{st} = (Hc - Hg)/Hc$$

where Hc is the diversity of the collection and Hg is the diversity of the particular group. G_{st} ranges between 1 and 0, where 0 = no differentiation and 1 = maximum differentiation (Brown, 1996; Goodwin *et al.,* 1993).

5.3. Results

5.3.1. Detection of DNA polymorphisms using PCR

The ISJ primer, R1, amplified between five and 12 DNA fragments, which ranged in size from 400 to 2,700 bp for the AG 2 isolates examined (Figure 5.1). While R1-primed PCR

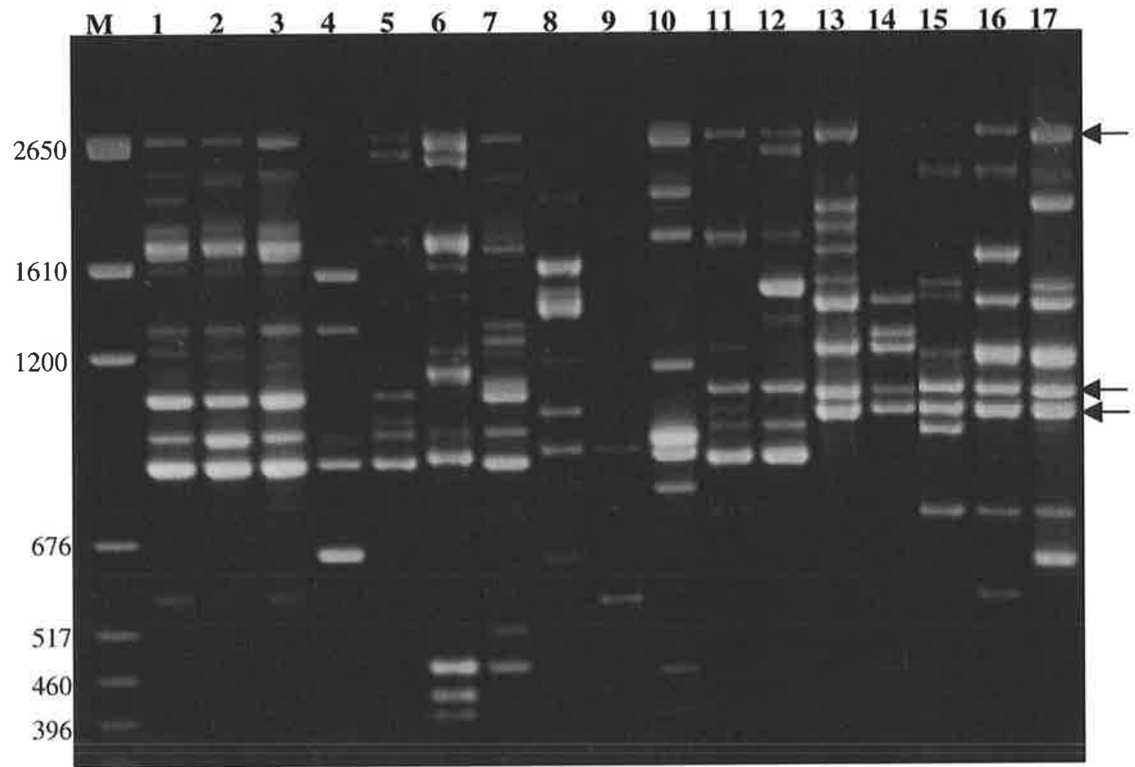
revealed considerable diversity, similar banding patterns were obtained for different isolates. For example, Australian isolates 21RAuC02, 21RAuC15 and 21RAuPe16 (Figure 5.1, gel A, lanes 1, 2 and 3 respectively) had near-identical banding patterns, but originated from different regions and two different crops. The R1 primer amplified a common 2,650 bp fragment (Figure 51A, top arrow) in the majority of the AG 2-1 isolates. In addition, the PCR using this primer was able to distinguish the AG 2-1, ZG 5 isolates (Figure 5.1A, lanes 1 – 12) from the AG 2-1, ZG 6 isolates (Figure 5.1A, lanes 13 - 17). While no characteristic bands were present for the ZG 5 isolates examined, the ZG 6 isolates showed a common pair of bands at approximately 1,000 and 1,100 bp (Figure 5.1A, middle and lowest arrow) that did not occur in the ZG 5 isolates. Similarly, the AG 2-2 isolates (Figure 5.1B, lanes 1 - 9) appeared genetically diverse, and the Australian isolates examined (lanes 6 - 9) displayed banding patterns distinct from those of Japan and the Netherlands (lanes 1 –5). In addition, the AG 2-2 isolates did not display the common band at 2,650 bp seen amongst the majority of AG 2-1 isolates. The two Japanese isolates (Figure 5.1B, lanes 1 and 2), one from mat rush and the other from sugar beet, shared some bands, but were genetically different to one another, whereas the three Netherlands isolates (Figure 5.1B, lanes 3 – 5) from sugar beet showed a greater degree of similarity. The Netherlands AG 2 isolate from tulip, 2tRN01 (Figure 5.1B, lane 13), displayed a unique banding pattern. Isolates 22RAuL09 and 22RAuS10 (Figure 5.1B, lanes 6 and 7) collected from lupin and soil, respectively, appeared identical to one another, as did two other lupin isolates, 22RAuL11 and 22RAuL12 (Figure 5.1B, lanes 8 and 9), collected from Western Australia. The isolates classified as "AG 2 undefined" (Figure 5.1B, lanes 10 – 12) by zymogram analysis had few bands in common with the AG 2-1 isolates tested, however, a number of bands were shared with the AG 2-2 isolates.

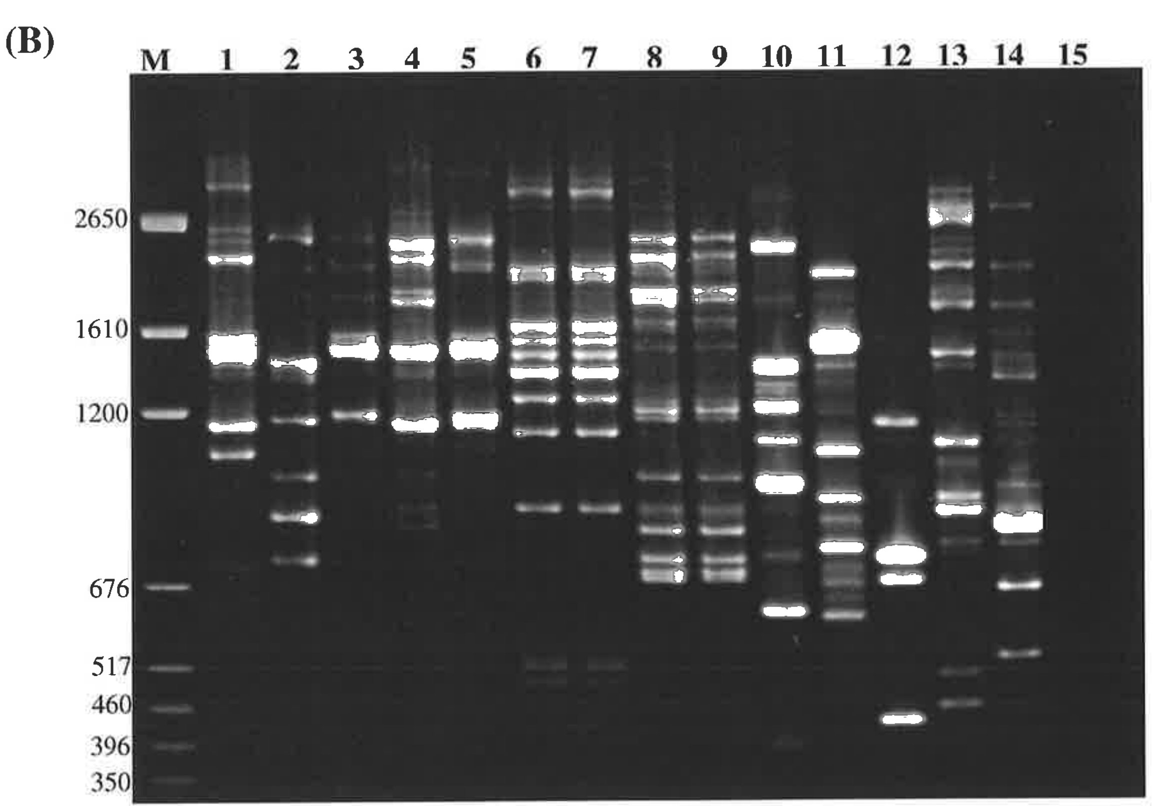
Figure 5.1: PCR amplification of AG 2 DNA using the ISJ primer, R1. Fragments were separated on 2% agarose gels in 0.5 x TBE buffer. The molecular weight marker, pGEM, is shown to the left (bp). The upper arrow indicates a band common to the majority of AG 2-1 isolates, the two lower arrows indicate the ZG 6-related bands.

A) AG 2-1 isolates.
Lane 1, 21RAuC02; lane 2, 21RAuC15; lane 3, 21RAuPe16; lane 4, 21RAuM03; lane 5, 21RAuL17; lane 6, 21RAuM04; lane 7, 21RN06; lane 8, 21RN07; lane 9, 21RAu18; lane 10, 21RAu19; lane 11, 21RAuW09; lane 12, 21RAuW10; lane 13, 21RAuB11; lane 14, 21RAuB12; lane 15, 21RAuP08; lane 16, 21RNzBr21; lane 17, 21RAuPr20.

B) AG 2-2 isolates, 'undefined AG 2', tulip isolate and bridging isolate.
Lane 1, 22RJ02; lane 2, 22RJ01; lane 3, 22RN06; lane 4, 22RN03; lane 5, 22RN04 ; lane 6, 22RAuL09; lane 7, 22RAuS10; lane 8, 22RAuL11; lane 9, 22RAuL12; lane 10, 02RAu01; lane 11, 02RAuCf03; lane 12, 02RAuL04; lane 13, 2tRN01; lane 14, BIR01; lane 15, negative control.

(A)





The two microsatellite primers also revealed a high level of genetic diversity amongst the AG 2 isolates tested (Figures 5.2 and 5.3). Within AG 2-1, a number of different banding patterns were obtained using the primer $(GACA)_4$, but no bands could be used to distinguish the group as a whole (Figure 5.2), or to separate the ZG 5 (Figure 5.2A, lanes 1- 7 and 9 –10) from the ZG 6 isolates (Figure 5.2A, lanes 13 - 16).

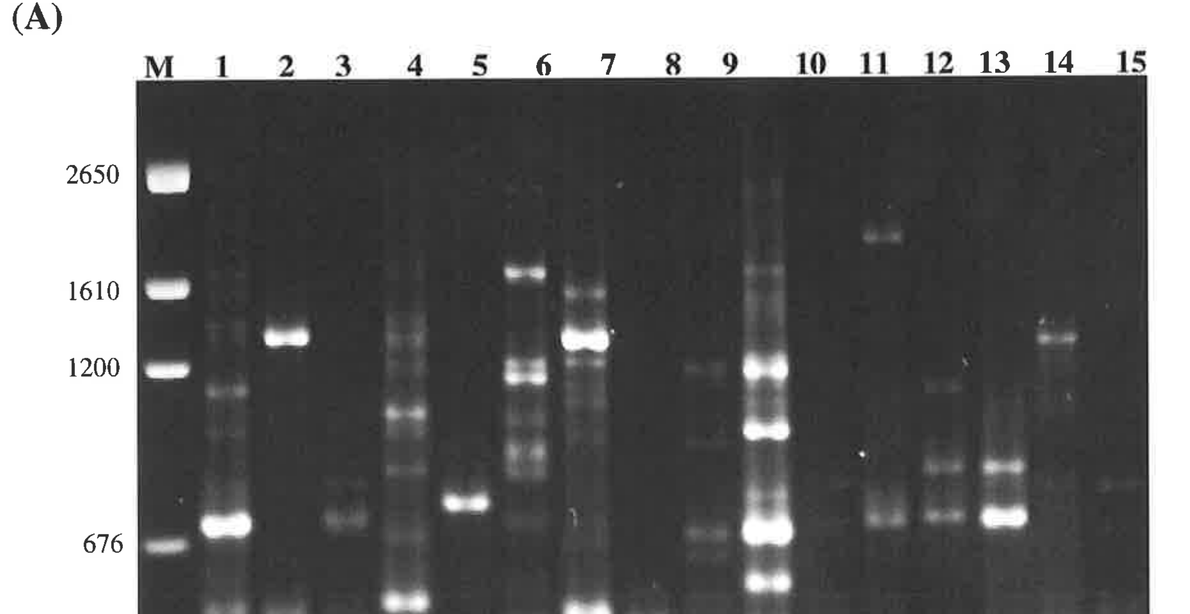
Microsatellite analysis of the AG 2-2 isolates (Figure 5.2B, lanes 1 – 9), revealed a situation similar to that observed for the R1 primer. The Japanese isolates (Figure 5.2B, lanes 1 and 2) shared some similarities but were genetically distinct from one another, while the Netherlands isolates (Figure 5.2B, lanes 3 – 5) again had a high level of similarity to one another. The Australian isolates 22RAuL09, 22RAuS10, 22RAuL11 and 22RAuL12 (Figure 5.2B, lanes 6 – 9) shared two patterns amongst the four isolates. As for the R1 primer, isolates termed "AG 2 undefined" shared more bands in common with the AG 2-2 than with the AG 2-1 isolates. Amplification of AG 2 DNA with $(GACA)_4$, produced a double band at approximately 460 and 500 bp (Figure 5.2A and B, arrows) for the majority of the isolates examined. Although these bands also occurred in the bridging isolate (Figure 5.2B, lane 14), further analysis was carried out to determine if this doublet could be used to distinguish AG 2 from AG 3, AG 4 and AG 8. However, these fragments also appeared for these AG (Figure 5.2, gel C).

The $(CAC)_5$-primed PCR also revealed differences among AG 2-1 isolates (Figure 5.3A), but the ZG 5 and ZG 6 isolates could not be distinguished. The banding patterns for isolates 21RAuC02, 21RAuC15 and 21RAuPe16 were nearly identical (Figure 5.3A, lanes 1-3), as was found for the R1 and $(GACA)_4$ primers. The two wheat isolates, 21RAuW09 and 21RAuW10 (Figure 5.3A, lanes 11 and 12), showed more similarity when amplified with $(CAC)_5$ than with the other primers. Within AG 2-2, the Japanese isolates were similar to one another, as were the Netherlands isolates (Figure 5.3B, lanes 2 and 3, and 4 – 6, respectively). The Australian isolates, 22RAuL09 and 22RAuS10, from lupins and soil in Western

Figure 5.2: PCR amplification of AG 2 DNA using the microsatellite primer $(GACA)_4$. Products were separated on 2% agarose gels at 30 V. The arrows in parts (A), (B) and (C) indicate the bands present in the majority of the isolates examined. The molecular weight marker, pGEM, is shown to the left (bp).

A) AG 2-1 isolates.
Lane 1, 21RAuC02; lane 2, 21RAuC15; lane 3, 21RAuPe16; lane 4, 21RAuM03; lane 5, 21RAuL17; lane 6, 21RAuM04; lane 7, 21RN06; lane 8, 21RN07; lane 9, 21RAu18; lane 10, 21RAu19; lane 11, 21RAuW09; lane 12, 21RAuW10; lane 13, 21RAuB11; lane 14, 21RAuB12; lane 15, 21RAuP08; lane 16, 21RNzBr21.

B) AG 2-2 isolates, "AG 2 undefined" isolates, tulip isolate and bridging isolate.
Lane 1, 22RJ02; lane 2, 22RJ01; lane 3, 22RN06; lane 4, 22RN03; lane 5, 22RN04 ; lane 6, 22RAuL09; lane 7, 22RAuS10; lane 8, 22RAuL11; lane 9, 22RAuL12; lane 10, 02RAu01; lane 11, 02RAuCf03; lane 12, 02RAuL04; lane 13, 2tRN01; lane 14, BIR01; lane 15, negative control.


(A)
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
2650
1610
1200
676
517
460
396
350

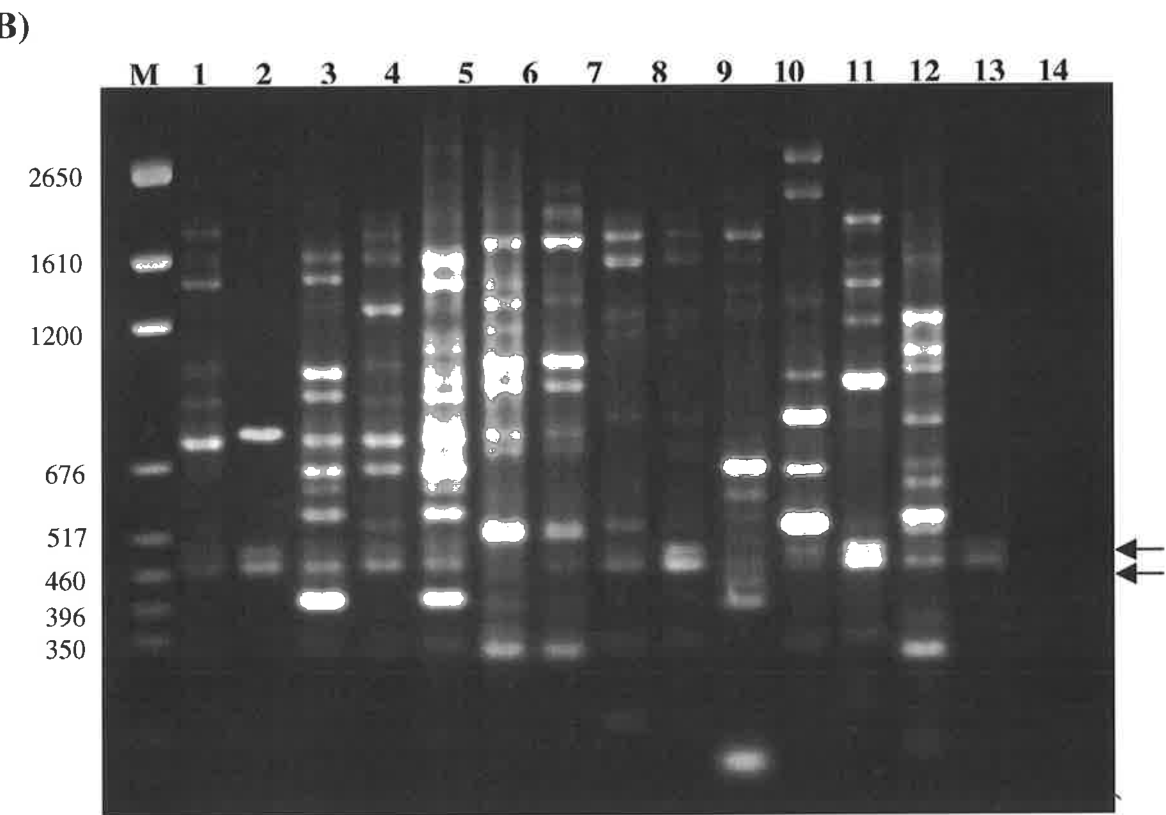

(B)
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14
2650
1610
1200
676
517
460
396
350

Figure 5.2 continued

C) Various AG.

Lane 1, 21RAuL17; lane 2, 21RAuW09; lane 3, 21RAuPr20; lane 4, 2tRN01; lane 5, AG 8 isolate 08RAuB08; lane 6, AG 4 isolate 04RAuP01; lane 7, AG 3 isolate 03RAuP01; lane 8, negative control.

(C)

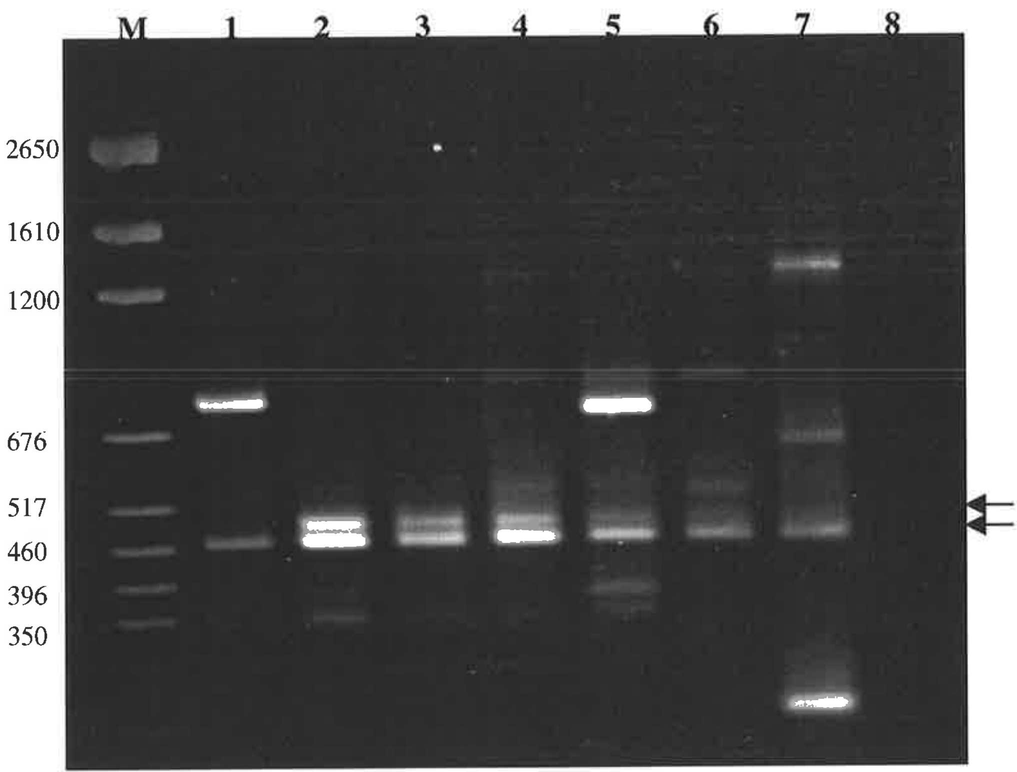


Figure 5.3: PCR amplification of AG 2 DNA using the microsatellite primer $(CAC)_5$. Products were separated on 2% agarose gels at 30 V using 0.5 x TBE running buffer. The molecular weight marker, pGEM, is shown to the left (bp).

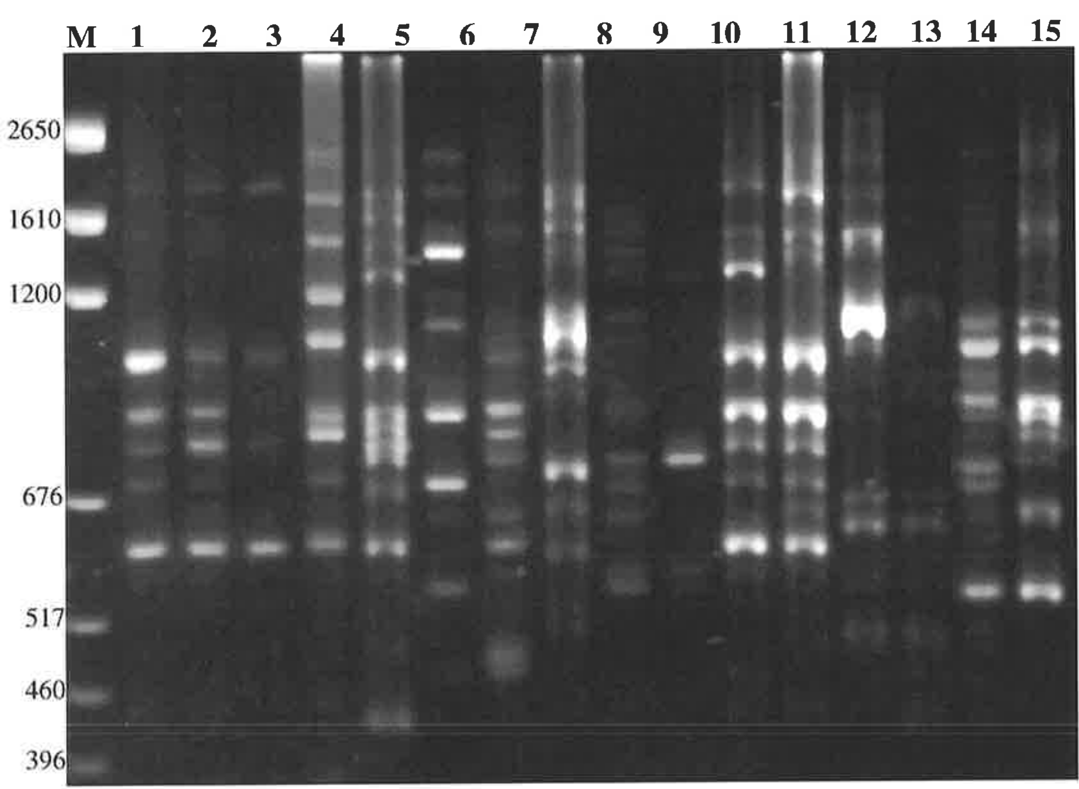
A) AG 2-1 isolates.

Lane 1, 21RAuC02; lane 2, 21RAuC15; lane 3, 21RAuPe16; lane 4, 21RAuM03; lane 5, 21RAuL17; lane 6, 21RAuM04; lane 7, 21RN06; lane 8, 21RN07; lane 9, 21RAu18; lane 10, 21RAu19; lane 11, 21RAuW09; lane 12, 21RAuW10; lane 13, 21RAuB11; lane 14, 21RAuB12; lane 15, 21RAuP08; lane 16, 21RNzBr21.

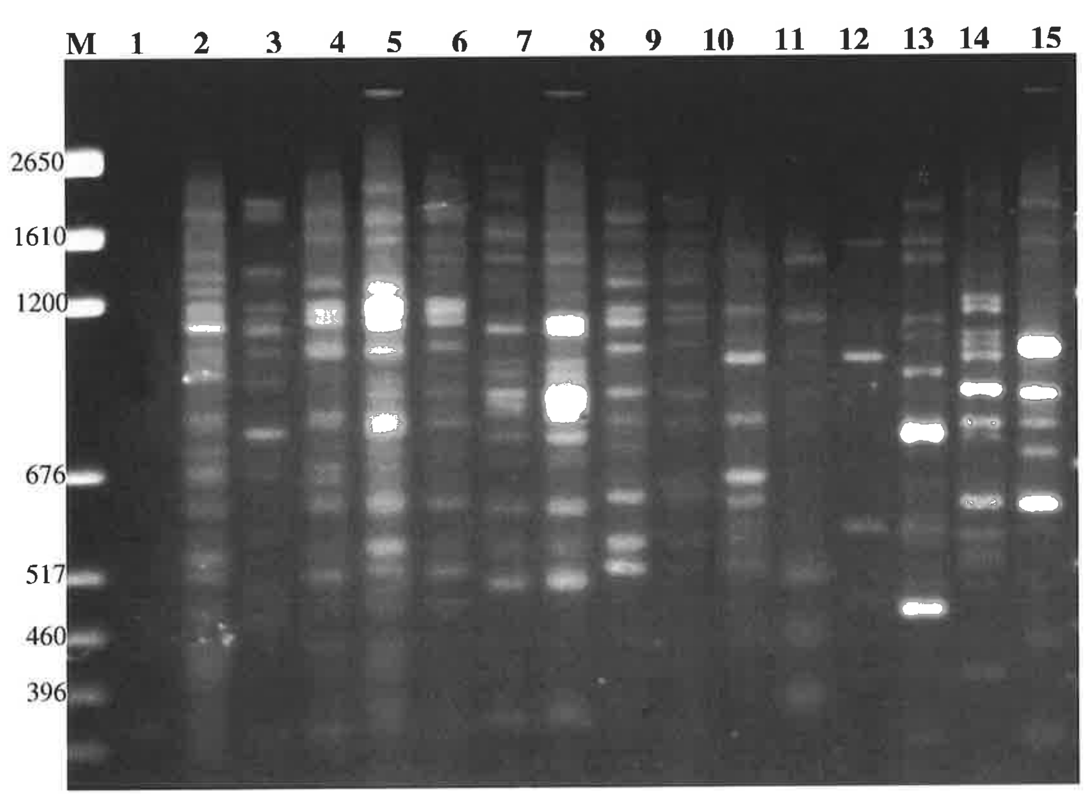
B) AG 2-2 isolates, "undefined AG 2", tulip isolate and bridging isolate.

Lane 1, negative control; lane 2, 22RJ02; lane 3, 22RJ01; lane 4, 22RN06; lane 5, 22RN03; lane 6, 22RN04; lane 7, 22RAuL09; lane 8, 22RAuS10; lane 9, 22RAuL11; lane 10, 22RAuL12; lane 11, 02RAu01; lane 12, 02RAuCf03; lane 13, 02RAuL04; lane 14, 2tRN01; lane 15, BIR01; lane 16, 21RAuC02.

(A)



(B)



Australia, respectively, were similar to each other (Figure 5.3B, lanes 7 and 8), but two other lupin isolates from Western Australia, 22RAuL11 and 22RAuL12 (Figure 5.3B, lanes 9 and 10), displayed banding patterns different from one another, in contrast to banding patterns obtained with both the R1 and $(GACA)_4$ primers. As for the other primers, amplification with the $(CAC)_5$ primer could not distinguish group-specific patterns among the AG 2 isolates.

5.3.1.1. Genetic similarity analysis

Analysis of the similarity coefficients from the combined PCR data revealed two major groups that could be distinguished with an average similarity of 20% (Figure 5.4, Appendix B). One group consisted of AG 2-1, ZG 5 isolates, the tulip isolate 2tRN01 (originally described as 2tR144 and designated ZG 5-1 by Schneider *et al.* (1997)), the bridging isolate, BIR01, and isolate 21RN07, classed as AG 2-1 by anastomosis but as "AG2 undefined" using the vertical zymogram technique. The second group contained the AG 2-1, ZG 6 isolates, the AG 2-2 isolates and the remaining "AG 2 undefined" isolates. The AG 2-1, ZG 5 cluster divided at approximately 30% to form two subgroups containing isolates from various regions and crops.

At approximately 25% relatedness, the second major group divided into two subgroups, one of which split further, separating the AG 2-1, ZG 6 isolates from the two Japanese AG 2-2 isolates and an undefined lupin isolate. Within the AG 2-2 group, it was evident that the isolates from Japan, 22RJ01 and 22RJ02, and the Australian undefined isolate, 02RAuL04, are distantly related to the other AG 2-2 isolates and appeared to cluster with the AG 2-1, ZG 6 isolates. The Netherlands isolates 22RN03, 22RN04 and 22RN06, appeared to be genetically similar (65 - 80%) to one another but dismilar to other AG 2-2 isolates. Similarly, the Australian isolates 22RAuL11 and 22RAuL12 were closely related (approximately 70%), while isolates 22RAuL09 and 22RAuS10 shared approximately 95%

similarity. However, these isolates did not share close similarity to any of the other AG 2-2 isolates examined.

Table 5.1: Genetic diversity of AG 2 isolates as determined by PCR amplification. Genetic diversity was calculated using Shannon's diversity index (H') from phenotypic frequencies at each locus. Isolates were separated into groups based on host (crucifer, legume and miscellaneous) and location (South Australia, Western Australia and "overseas") to examine diversity within the categories. Each locus was assessed for its ability to distinguish between groups using the genetic differentiation statistic (G_{st}).

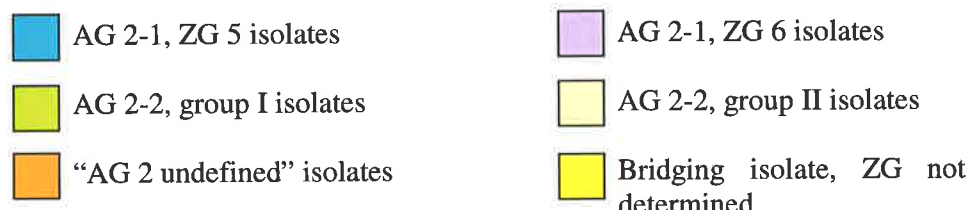
	Collection			Host		Location	
Locus	**Isolates** [a]	**Phenotypes** [b]	**H'**	**H'**	**G_{st}**	**H'**	**G_{st}**
$(CAC)_5$	31	28	0.93	0.98	0.34	0.96	0.30
R1	31	26	0.96	0.93	0.35	0.93	0.35
$(GACA)_4$	31	26	0.93	0.98	0.31	1.0	0.34
Combined	31	31	0.94	0.96	0.33	0.96	0.33

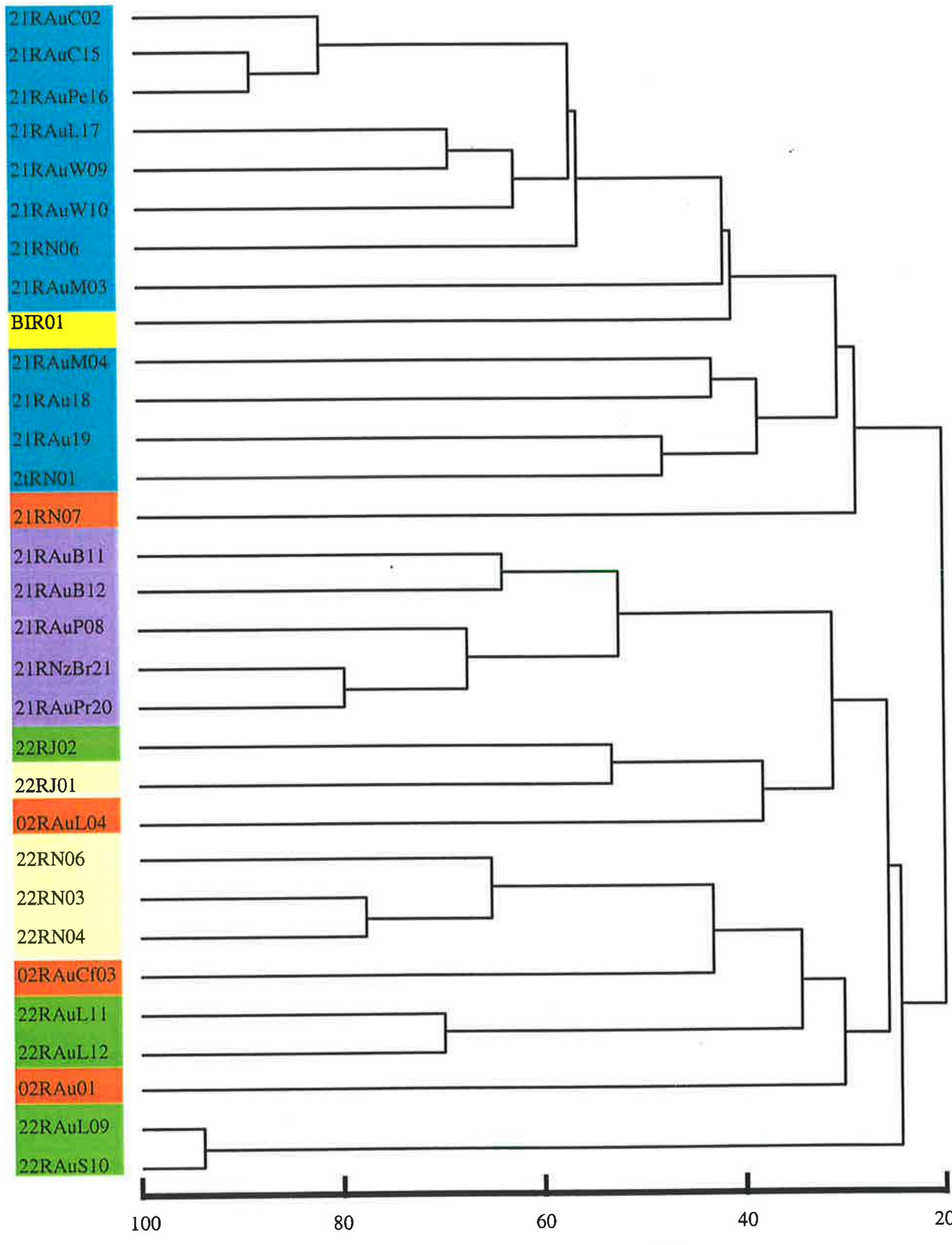
[a] number of isolates amplified by each primer.
[b] number of unique banding patterns distinguished by each primer.

5.3.1.2. Genetic diversity analysis

The 31 isolates examined were placed into phenotypic groups based on the banding patterns produced by the three PCR primers. A total of 28 phenotypic groups were identified for $(CAC)_5$, and 26 for both R1 and $(GACA)_4$ (Table 5.1). When data for the three primers were combined, each isolate belonged to a unique phenotypic group, as represented in the dendrogram. Shannon's diversity index (H') was used to quantify the diversity present within the isolate collection as a whole, and in groups of isolates from different hosts and locations. In addition, genetic differentiation (G_{st}) was calculated for each primer, to examine how useful each was in differentiating between isolates from different hosts or locations (Table 5.1). The PCR primers enabled detection of high levels of diversity within the collection as a whole (H' = 0.94). When isolates were divided into groups based on location

Figure 5.4: Dendrogram showing genetic similarity of AG 2 isolates based on PCR analysis using three microsatellite and the R1 primers. Similarities were calculated using the UPGMA method after comparison of banding patterns by Nei and Li's genetic similarity (1979).





21RAuC02
21RAuC15
21RAuPe16
21RAuL17
21RAuW09
21RAuW10
21RN06
21RAuM03
BIR01
21RAuM04
21RAu18
21RAu19
21RN01
21RN07
21RAuB11
21RAuB12
21RAuP08
21RNzBr21
21RAuPr20
22RJ02
22RJ01
02RAuL04
22RN06
22RN03
22RN04
02RAuCf03
22RAuL11
22RAuL12
02RAu01
22RAuL09
22RAuS10
100
80
60
40
20
Nei and Li's genetic similarity (%)

and host, a similar level of diversity was detected, with H'= 0.94 for location and H' = 0.97 for host. However, for each locus the genetic differentiation (G_{st}) was low (G_{st}host = 0.33; G_{st}location= 0.33), indicating that these loci could not be used to group the isolates into "host types" nor to identify isolates from the different locations. This was evident when the AG 2-1 isolates from canola, 21RC02 from South Australia, and 21RAuC15 from Western Australia, were compared. These isolates had an average similarity of approximately 85%, indicating that there is a high level of similarity between two geographically distant isolates. This similarity may be related to host type. However, the trend seen across the collection suggests that isolates from different hosts and geographically distant locations share high similarity. For example, 21RNzBr21 from brassica and 21RAuPr20 from pine showed 85% similarity. This was quite surprising given that the isolates are from very different geographical regions and from different hosts.

5.3.2. AG 2-specific ITS-PCR

Amplification of the ITS regions of 11 AG 2 isolates and the bridging isolate, BIR01, was performed using the primer set, AG2sp/ITS4B, published by Salazar *et al.* (2000). An amplified fragment of approximately 860 bp was detected for all AG 2 isolates tested, and in the bridging isolate (Figure 5.5A). This contrasts with the 680 bp fragment reported by Salazar *et al.* (2000). Since the primers also amplified a number of extra bands, Southern hybridisation of the PCR gel was conducted using the AG2sp/ITS 4B-primed PCR amplification product from the Netherlands isolate 22RN06, as the labelled probe, to test for similarity (see section 5.2.4). Figure 5.5B shows that the PCR product hybridised to the 860 bp DNA fragment in all isolates. There was also strong hybridisation to a 1,500 bp fragment in isolate 21RAuB11 (Figure 5.5B, lane 5), and moderate hybridisation to bands produced for other isolates. However, some bands that were amplified did not hybridise with the probe. For example, a 700 bp amplification product that was observed for the bridging isolate,

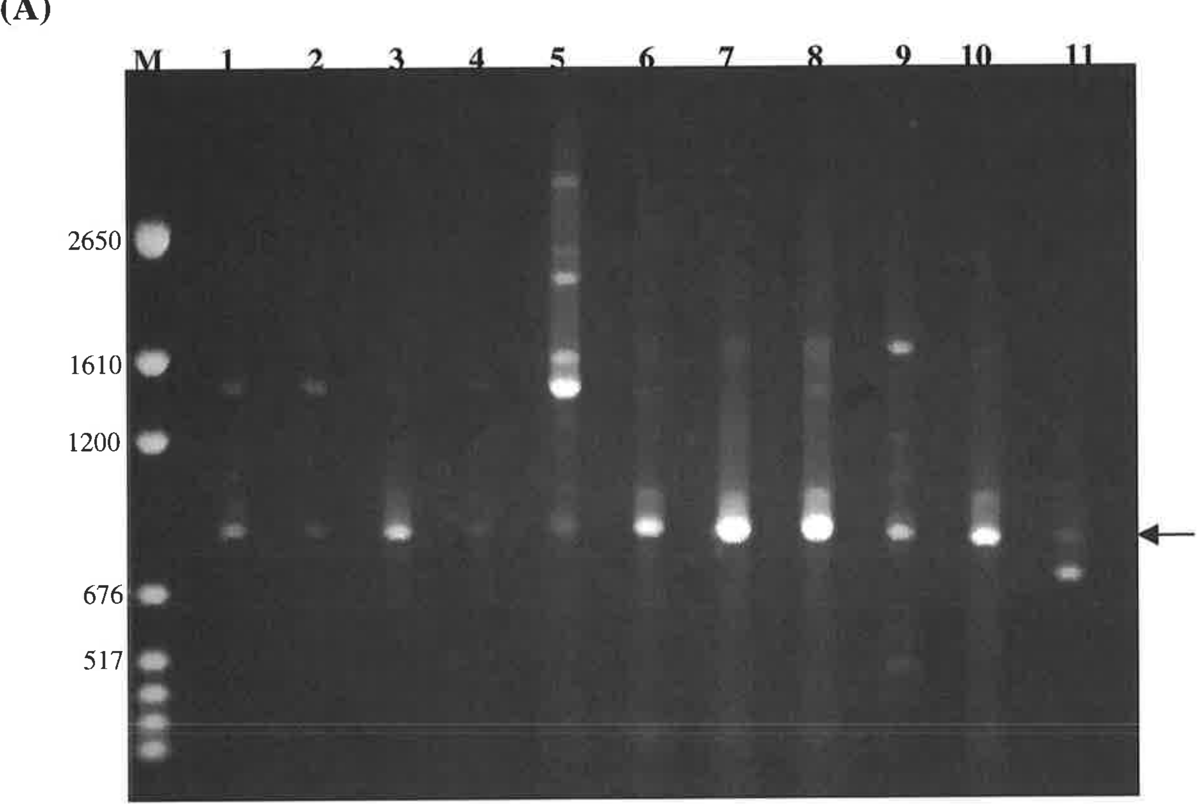
Figure 5.5: PCR amplification using the primer set AG2sp/ITS 4B (Salazar *et al.*, 2000). Amplification products were separated on 1% agarose gels in 0.5 x TBE running buffer (A). Fragments were then transferred to a positively charged nylon membrane by the method of Southern (1975) and hybridised with the ^{32}P – labelled fragment amplified from isolate 22RN06 (B). Arrows indicate the common 860 bp fragment in each isolate. The size marker, pGEM, is shown to the left (bp).

A) Electrophoretic gel of amplified DNA.

Lane 1, 21RC02; lane 2, 21RN07; lane 3, 21RPr20; lane 4, 21RBr21; lane 5, 21RB11; lane 6, 22R02; lane 7, 22RN06; lane 8, 22RN03; lane 9, 22RS10; lane 10, 22RL11; lane 11, BIR01.

B) Autoradiograph of PCR gel. Lanes correspond to those marked in Figure 5.5A.

(A)



(B)

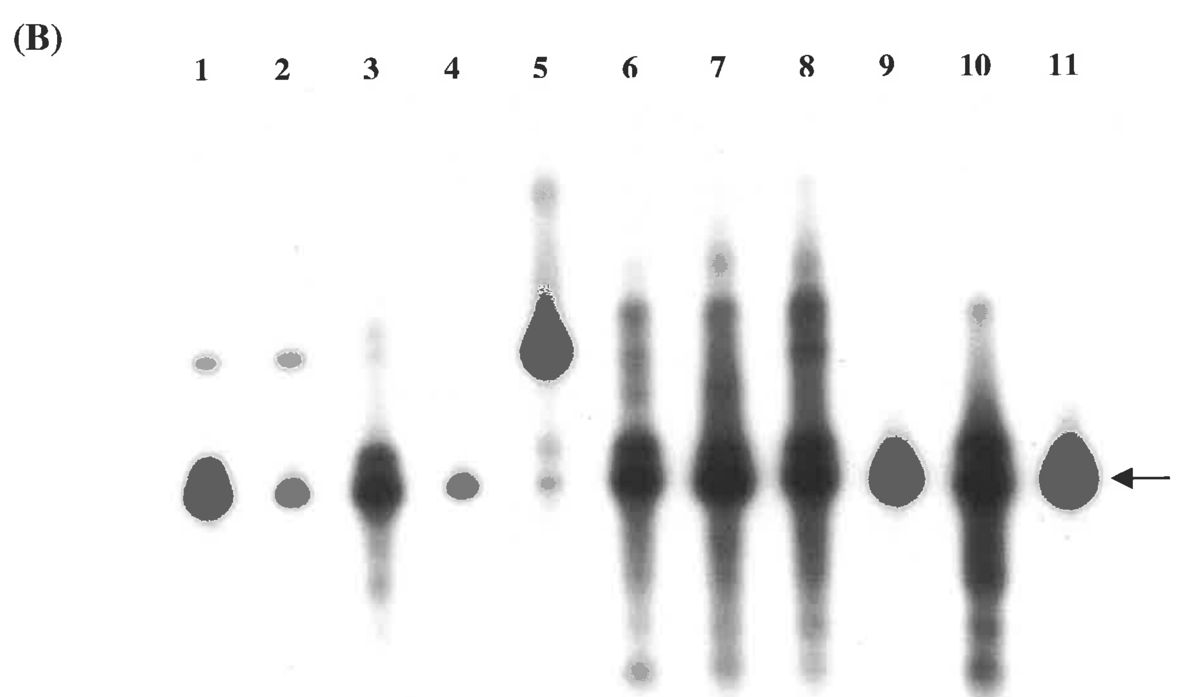
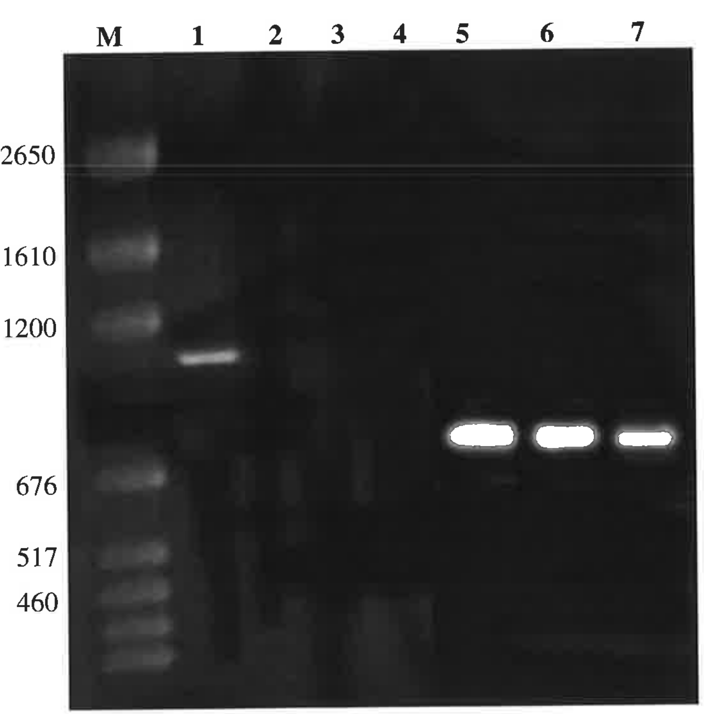


Figure 5.6: PCR amplification of DNA extracted from isolates of *R. solani* representing AG 2, 3, 4 and 8, using the primer set AG2sp/ITS 4B (Salazar *et al*., 2000). The DNA size marker, pGEM™, is shown to the left (bp).

Lane 1, 03RAuP01; lane 2, 04RAuP01; lane 3, 08RAuB08; lane 4, empty: lane 5, 22RJ02; lane 6, 22RN06; lane 7, 22RN03.


M
1
2
3
4
5
6
7
2650
1610
1200
676
517
460

BIR01 (Figure 5.5A, lane 11), did not hybridise to the probe. This was also the case for the 1,600 bp amplification fragment observed for isolate 22RAuS10 (lane 9). This may indicate diversity within the amplified region or that the primer set amplified sequences which were not related to the ITS region. Experiments were carried out to determine whether the amplification conditions may have been responsible for this anomaly. Annealing temperatures and times were adjusted to between 52 and 56°C for 30 – 45 sec, which resulted in the amplification of multiple fragments. Both increasing and decreasing the $MgCl_2$ concentration had no effect on amplification, whereas an increase in template DNA to 15 ng resulted in multiple bands. In addition to the anomalies produced for the AG 2 isolates, the primer set also amplified a 1200 bp fragment in an AG 3 isolate (Figure 5.6, lane 1). No amplification of DNA from isolates of AG 4 or 8 was observed. It appeared that the AG2sp/ITS4B primer sequences were not specific enough to allow AG 2 DNA to be distinguished from that of other AGs. It will be necessary to examine a large range of AG 2 isolates from various locations as well as isolates from other AG to determine if these primers are suitable for distinguishing this complex group. Due to the anomalies produced in PCRs using the AG2sp/ITS 4B primer pair, analysis of AG 2 DNA using this approach was not continued.

5.4. Discussion

Amplification of DNA of *R.* solani AG 2 using the ISJ primer, R1, and the microsatellite primers $(GACA)_4$ and $(CAC)_5$, produced multiple bands for all isolates tested. No single primer was able to distinguish between AG 2 isolates belonging to the different ZG, however, analysis by UPGMA of the combined data from all three primers allowed for the separation of the isolates into subgroups. Within AG 2-1, the combined data could be used to distinguish clearly between ZG 5 and ZG 6 isolates. The ZG 5 isolates clustered as one large diverse

group, including the bridging isolate and the tulip isolate 2tRN01, and were separated from the other ZG. Similarly, the AG 2-1 ZG 6 isolates formed another cluster, which appeared to be more closely related to AG 2-2 than AG 2-1, at these loci.

The grouping of the bridging isolate with the AG 2-1, ZG 5 isolates is an interesting feature. A recent report by Nicoletti *et al.* (1999) suggested that isolates of *R. solani* from tobacco are capable of high frequency anastomosis with AG BI but low frequency with AG 2 isolates. However, zymogram analysis placed the tobacco isolates in a unique group, while morphology, growth requirements and RFLP analysis of rDNA-ITS regions showed they were more similar to AG 2-1 than to AG BI (Nicoletti *et al.*, 1999). The PCR data generated in the present study also indicated that the bridging isolate was related to AG 2-1 isolates, however, only one bridging isolate was tested. While additional bridging isolates would need to be tested using PCR techniques, other methods, such as isozyme and morphological studies, would help to clarify identification. Not only do the results indicate the difficulty associated with using single methods to resolve the complexity of the AG 2 group, they also highlight the apparent genetic relationship that exists between AG 2 and AG BI. The fact that both bridging isolates and AG 2 isolates can anastomose at a low frequency with other AG, adds a level of uncertainty when placing isolates into anastomosis groups. This is where further research using methods such as PCR and RFLP analysis, in combination with the established anastomosis and zymogram techniques, will be useful in defining groups at the genetic level.

The primers used in these experiments could not be used to separate the AG 2-2 isolates examined into ZG 4 or ZG 10. This may indicate that the zymogram distinction is more ambiguous for this group than for AG 2-1, and that the generic primers used are unable clearly to differentiate ZGs. Furthermore, the isolates designated as AG 2-2 group I and II by zymogram analysis in Chapter 2, were not distinguished by PCR analysis, indicating again that the loci amplified by these primers were not linked to zymogram grouping in AG 2-2.

Although genetic diversity was observed, there appeared to be a clustering of isolates from similar locations, which did not occur for the AG 2-1 isolates. However, it is necessary to examine more isolates with these and other primers, such as RAPD-primers, before any correlation between genetic relatedness, and host or geographic region, can be determined.

Analysis of DNA polymorphisms amplified by both the R1 and microsatellite primers, revealed considerable variation within the collection of isolates of AG 2 (H'= 0.94), possibly due to a combination of differences in host and location. It might be assumed that the origin of the isolates contributes more than the host towards the observed diversity. The collection of individual isolates from many varied locations may outweigh any common host effects, thus obscuring any relationship. However, because only a limited number of isolates from each location were examined in this study, it is not possible to determine population structure, the origin of diversity, nor the mechanisms by which diversity is maintained. In addition, as microsatellites appear to be randomly distributed within the genome of many organisms (Jarne and Lagada, 1996), and detect high levels of heterogeneity (Davies *et al.*, 1999), it is possible that the loci examined do not reflect differences in either host or location, but are a measure of genetic hypervariability. Therefore, they may provide efficient markers with which to assess genetic diversity within single field populations, and to assess differences between field populations in a small geographic region. A recent report on diversity of AG 1-1A field isolates revealed that isolates from a single field showed common zymogram and RFLP patterns, yet when the M13 and $(TCT)_5$ simple sequence repeat primers were used, a high degree of variation was observed (Banniza and Rutherford, 2001). Markers such as these, which are randomly distributed throughout the genome, would be useful in assessing the overall population structure of isolates of *R. solani* from different AG. This area has received little attention. It has been suggested that microsatellite loci are under selective pressure (Jarne and Lagoda, 1996). Therefore, these markers could be used to monitor changes in populations over time, the effect of different crops on diversity and the rate of gene

flow in and between populations. An examination of microsatellite loci needs to be carried out on single populations in order to determine their true value as markers for population analysis in *R. solani.* However, the research conducted here and elsewhere suggests they have considerable potential.

Examination of the rDNA-ITS region of the AG 2 isolates used in this study, with primers published by Salazar *et al.* (2000), provided interesting results. Since the AG2sp primer was designed from sequence alignments of ITS regions from Japanese and Netherlands isolates, three Netherlands isolates were used as “positive controls” for the PCR. Although a 680 bp product was expected using the AG 2sp/ITS4B primer set (Salazar *et al.*, 2000), a band of approximately 860 bp was amplified in all isolates tested, including the bridging isolate. In addition, other bands were amplified in several isolates of AG 2. Furthermore, a 1200 bp fragment was amplified when DNA from an AG 3 isolate was used in the reaction. Several changes to the amplification reaction were made in an attempt to eliminate the multiple bands and to achieve the fragment of the expected size. However, increasing the annealing temperature and time, changing the concentrations of primer and $MgCl_2$ and altering the amount of template DNA resulted in either failure of amplification or increased the number of bands. The use of the amplification product from the Netherlands isolate 22RN06, as a labelled probe, resulted in strong hybridisation to the common 860bp fragment as well as hybridisation to various other fragments. In recent communications, Dr. Hans Schneider suggested that the AG2sp/ITS 4B primer set was not as specific as had been reported. The AG 2-2 specific primer sets described in the paper by Salazar *et al.* (2000) only amplified between 75-80% of the DNA prepared from a collection of AG 2-2 isolates taken from sugar beet in the Netherlands (J.H.M. Schneider, *pers. comm.* 2001). In addition, Dr Schneider's group also found anomalies in the size of fragments amplified by one of the AG 2-2 primer sets.

While a prominent 860 bp fragment was produced with the AG2sp/ITS 4B primer set, the amplification of a number of other bands indicated that either the AG 2 ITS regions vary considerably or, the primers target other regions of the genome. When producing species-specific PCR primers from variable regions of DNA, it is important to include isolates from a range of geographic areas, along with the DNA from other AGs, to ensure that appropriate sequences are used. It also illustrates the difficulty faced by researchers when designing and producing reliable markers for the identification of genetically diverse species such as *R. solani*.

PCR analysis revealed informative DNA polymorphisms for all of the AG 2 isolates examined in this study. Examination of the combined data from three primers determined that a high degree of genetic variation existed in the collection, with each isolate displaying a unique multilocus phenotype. While the individual primers could not be used to identify group-specific patterns for the known AG 2 subgroups, hierarchical clustering analysis of the combined data showed that PCR with these primers could distinguish between AG 2-1, ZG 5, and AG 2-1, ZG 6, while isolates from AG 2-2 were more difficult to distinguish. In addition, the combined data placed the isolates classed as “AG 2 undefined” into clusters containing AG 2-1 and AG 2-2 isolates. However, in order to clarify the relationship between AG 2 isolates and other AG, additional microsatellite primers need to be examined as potential markers. In addition, it would be necessary to combine data from other forms of genetic analysis, such as that generated from RFLP, AFLP and RAPD analysis, to understand fully how the subgroups within the AG 2 complex relate to one another.

Chapter 6

Analysis of Combined Molecular Data

6.1. Introduction

Hierarchical cluster analysis of the molecular data generated for the isolates of *R. solani* used in this study may not be appropriate for a number of reasons. Firstly, the isolates examined were obtained from different regions of the world and from geographically distinct locations within Australia. In addition, isolates such as those from Japan, were collected many years ago and retrieved from collections held in Australia. Furthermore, long-term storage and periodic transfers of isolates on artificial media may induce genetic changes that do not reflect the natural state of the isolates. Finally, data on the sampling strategies used to collect the isolates, was not available. Therefore, there was little or no basis for the assumption that a hierarchy of relatedness existed in the collection. It is possible that the concept of hierarchy does not exist for the AGs of *R. solani*, as genotypes from specific groups within a single species, or from closely related species, may arise as a result of genetic processes such as recombination and segregation, co-evolution and specific host adaptation, which occur concurrently and independently (Sneath and Sokal, 1973).

Cluster analysis is a hierarchical technique which will always link isolates, regardless of similarity or distance and, when used to examine between-group relationships, may provide inaccurate representations, as these are likely to be non-hierarchical (Bridge, 1998). Given the many differences in the isolates of AG 2 investigated here, along with the absence of a clear indication of hierarchy, it may be more appropriate to analyse the molecular data reported in chapters 2, 4 and 5, using a non-hierarchical multivariate procedure such as principal coordinate (PCO) analysis. PCO analysis was first shown to be suitable for binary data by Gower (1966) and thus, it can used to interpret data comprising DNA fingerprints.

The method places isolates as individuals based on the overall variation within the data, providing a more accurate representation of between-group relationships (Anderson, 1985). In the case of a large number of markers (variables), PCO analysis reduces the data so that the two or three principal sources of variation are shown and a reasonable proportion of the overall variability present among the isolates. Put more simply, cluster analysis groups the most similar isolates and then re-examines the remaining isolates as to their extent of similarity to this group, not to the original isolates, thus each isolate is linked to all other isolates. PCO analysis places each individual isolate as a coordinate of the principal axes, thus isolates with similar coordinates are more likely to be closely related to one another than isolates with different coordinates, but no one isolate is linked to any other.

This chapter describes the application of both cluster and PCO analysis to a diverse range of isolates of *R. solani* AG 2, using the banding data generated from zymogram, RFLP and PCR analyses.

6.2. Methods

The data generated from zymogram and PCR analyses were combined to form a single binary matrix to facilitate the comparison of all AG 2 isolates. The data from the zymogram, RFLP and PCR analyses were combined to form a single binary matrix to allow the comparison of AG 2-1 isolates and AG 2-2 isolates, separately. Only isolates for which a complete data set was available were used. As no RFLP marker was found that hybridised to both AG 2-1 and AG 2-2 isolates, it was not possible to combine the data from the three methods to analyse the group as a whole. The data set was entered into the Multi Variate Statistical Package version 3.1 (MVSP, Kovach Computing Systems, Wales, UK). Cluster analysis was performed using Nei and Li's (1979) genetic index, while PCO analysis was performed using Gower's general

similarity index (Gower, 1966), which compares two cases (isolates) i and j at the kth variable (marker), and is defined as follows:

$$GGSC_{ij} = \Sigma_k (W_{ijk} S_{ijk}) / \Sigma_k W_{ijk}$$

where: $S_{ijk} = 0$ for all mismatches and 1 for matches of binary data.

$W_{ijk} = 0$ for negative matches in binary data and 1 in all other situations.

In order to compare the different statistical procedures, the similarity matrices generated were correlated using the Pearson product moment correlation coefficient (Walpole and Myers, 1978).

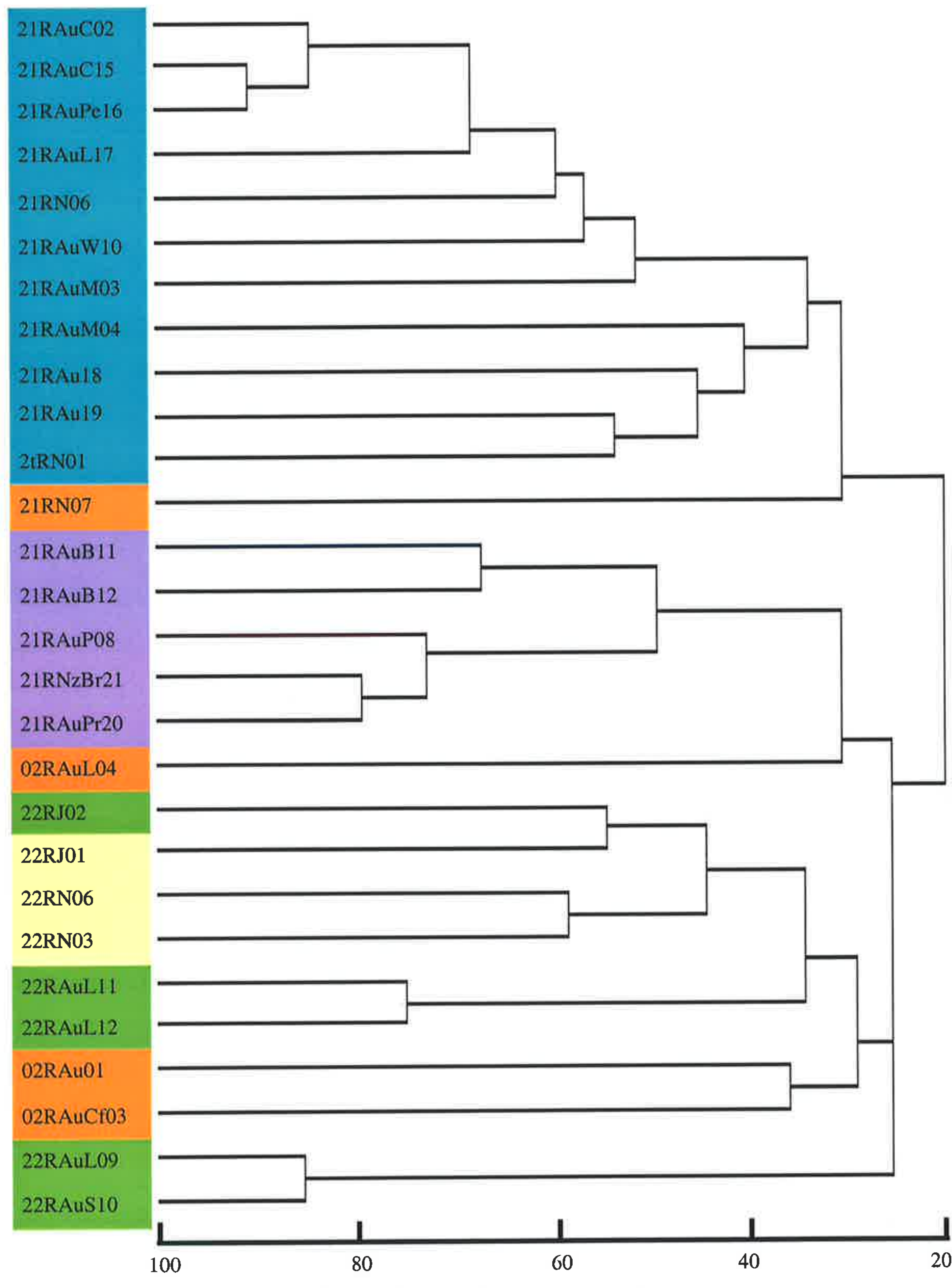
6.3. Results

6.3.1. Analysis of AG 2 isolates using combined zymogram and PCR data

The data from zymogram analysis and microsatellite-primed PCR of DNA from 28 isolates of AG 2 consisted of 81 assessable markers, and was used to generate a binary matrix. UPGMA cluster analysis of the matrix using Nei and Li's genetic similarity coefficient (Nei and Li, 1979) produced a dendrogram which showed a clear separation of AG 2-1, ZG 5 isolates and the "AG 2 undefined" isolate 21RN07, from all other AG 2 isolates, at approximately 20% similarity (Figure 6.1). Within this AG 2-1, ZG 5 group, isolate 21RN07 had an average similarity to the remaining isolates of approximately 30%. Within the former group, a division occurred at 33%, and placed the isolates 21RAuM04, 21RAu18, 21RAu19 and 2tRN01 in a distinct, but diverse subgroup. The remaining seven AG 2-1, ZG 5 isolates formed a group in which similarity ranged from 51 to 90% (Figure 6.1; Appendix E).

The AG 2-1, ZG 6 isolates formed a separate group of five isolates, which divide into two groups at approximately 49% similarity. One of these groups consisted of two barley isolates, 21RAuB11 and 21RAuB12, from Australia, and the second group contained three isolates which originated from different hosts, two from Australia, 21RAuP08 and

Figure 6.1: Dendrogram produced from UPGMA cluster analysis representing similarities among isolates of *R. solani* AG 2. Nei and Li's (1979) genetic similarity coefficient was used to construct the similarity matrix from 81 markers representing zymogram and PCR data.



21RAuC02
21RAuC15
21RAuPe16
21RAuL17
21RN06
21RAuW10
21RAuM03
21RAuM04
21RAu18
21RAu19
2tRN01
21RN07
21RAuB11
21RAuB12
21RAuP08
21RNzBr21
21RAuPr20
02RAuL04
22RJ02
22RJ01
22RN06
22RN03
22RAuL11
22RAuL12
02RAu01
02RAuCf03
22RAuL09
22RAuS10
100
80
60
40
20
Nei and Li's genetic similarity (%)

21RAuPr20, and one from New Zealand, 21RNzBr21. The "AG 2 undefined" isolate 02RAuL04 was related to the ZG 6 isolates at approximately 30% similarity, and these ZG 6 isolates, in turn, were related to the AG 2-2 and remaining undefined isolates, at a level of 25% similarity.

The AG 2-2 isolates, including the "AG 2 undefined" isolates 02RAu01 and 02RAuCf03, formed a diverse group (Figure 6.1). It was not possible to distinguish the AG 2-2, ZG 4 isolates (22RJ01, 22RAuL11, 22RAuL12 and 22RAuL09) from the AG 2-2, ZG 10 isolates (22RN03, 22RN06, and 22RAuL10). In addition, the isolates characterised as AG 2-2 group I and AG 2-2 group II by vertical gel zymogram analysis (Chapter 2, page 50) did not form distinct groups. The Australian isolates, 22RAuL09 and 22RAuL10, were easily discernable from the remaining AG 2-2 isolates, sharing approximately 85% similarity with one another and only 25% similarity with the other AG 2-2 isolates. The remaining two Australian isolates, 22RAuL11 and 22RAuL12, were also closely related to one another, sharing 75% similarity, but were only 34% similar to the AG 2-2 isolates from Japan and the Netherlands. The two Japanese isolates, 22RJ01 and 22RJ02, shared 54% similarity to one another, while the two isolates from the Netherlands, 22RN03 and 22RN06, shared 58% similarity (Figure 6.1). In general, AG 2-2 isolates from the same geographic region were more closely related to one another than to AG 2-2 isolates from geographically distinct regions.

PCO analysis of the 81 zymogram and PCR markers for the 28 isolates of AG 2 generated 28 axes (Appendix E) and resulted in distinct groupings when plotted on a two-dimensional scatter plot (Figure 6.2). The axes represent approximately 30% of the total variation detected by the markers, with axis 1 and axis 2 showing approximately 18% and 12% of the total variability represented by the data, respectively (Appendix E). The AG 2-1, ZG 5 isolates formed a diverse group (Figure 6.2, right of centre line), which contained the

Figure 6.2: Scatter plot derived from principal coordinates analysis of 81 markers obtained from zymogram and PCR analysis of 28 isolates of *R. solani* AG 2. Gower's general similarity coefficient (Gower, 1966) was used to generate the similarity matrices to determine genetic relationships and the two most variable axes are plotted.

AG 2-1, ZG 5 isolates

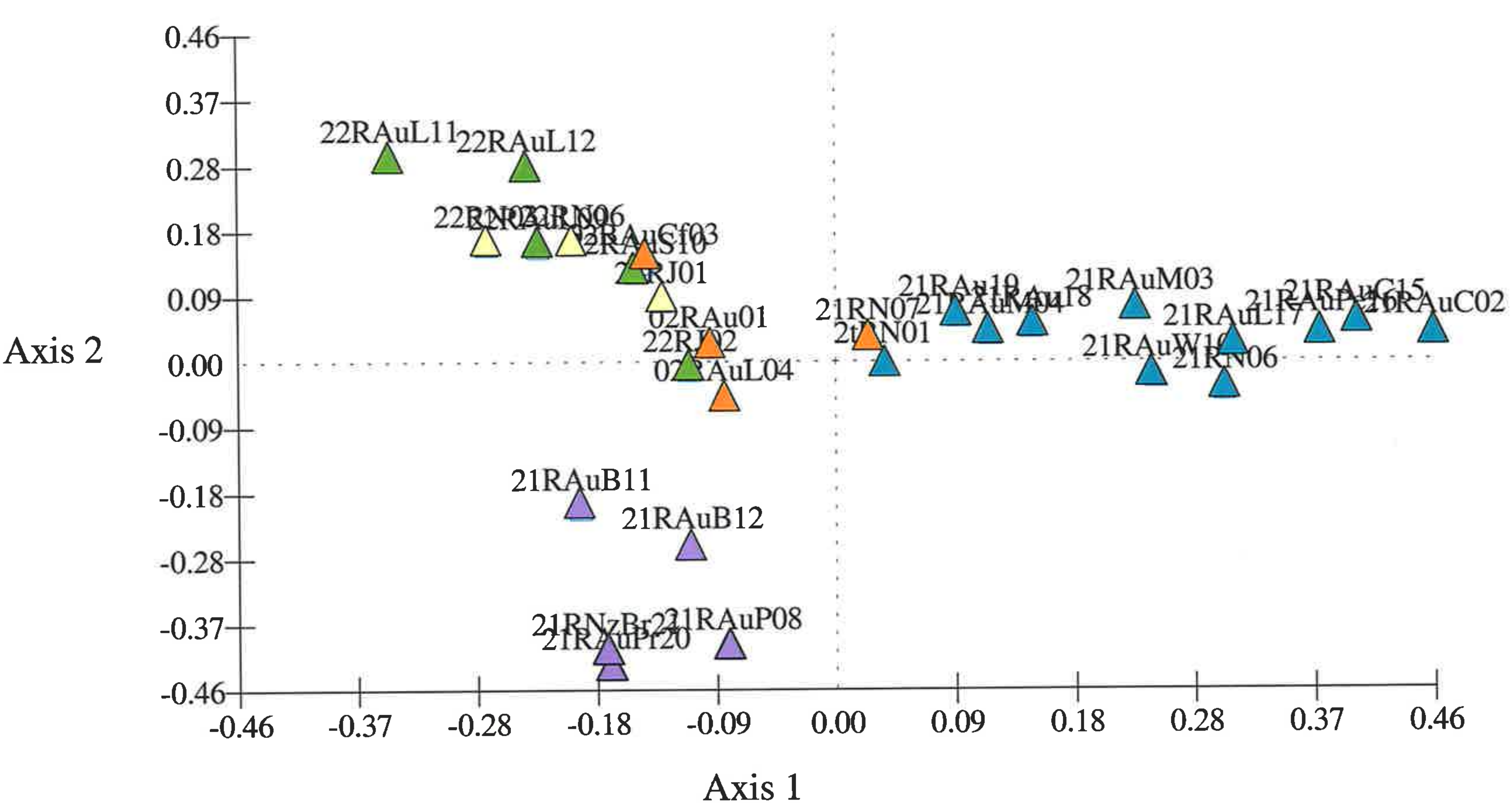
AG 2-1, ZG 6 isolates

"AG 2 undefined" isolates

AG 2-2, group I isolates

AG 2-2, group II isolates

PCO case scores (Gower General Similarity Coefficient)



"AG 2 undefined" isolate 21RN07. Three of the AG 2-1, ZG 6 isolates, 21RNzBr21, 21RAuP08 and 21RAuPr20, formed a tight group (Figure 6.1, lower left quadrant), distinct from all other isolates, whereas the two barley isolates, 21RAuB11 and 21RAuB12, were separated from this group, as was shown by UPGMA analysis. The AG 2-2 isolates and the remaining undefined isolates formed a large, diverse group (Figure 6.2). As was observed with cluster analysis, AG 2-2 isolates from similar geographic regions were more closely related to one another than they were to those isolates from different regions (Appendix E). For example, the Australian isolates 22RAuL09 and 22RAuL10 shared approximately 93% similarity to one another, while isolate 22RAuL09 was approximately 67% similar to the two Japanese isolates 22RJ01 and 22RJ02.

The Pearson product moment correlation coefficient was applied to correlate the results obtained by the two statistical methods (Walpole and Myers, 1978). The correlation between the two similarity matrices was determined to be 0.886, which indicated the existence of a good correlation between the genetic relationships represented by the two statistical methods.

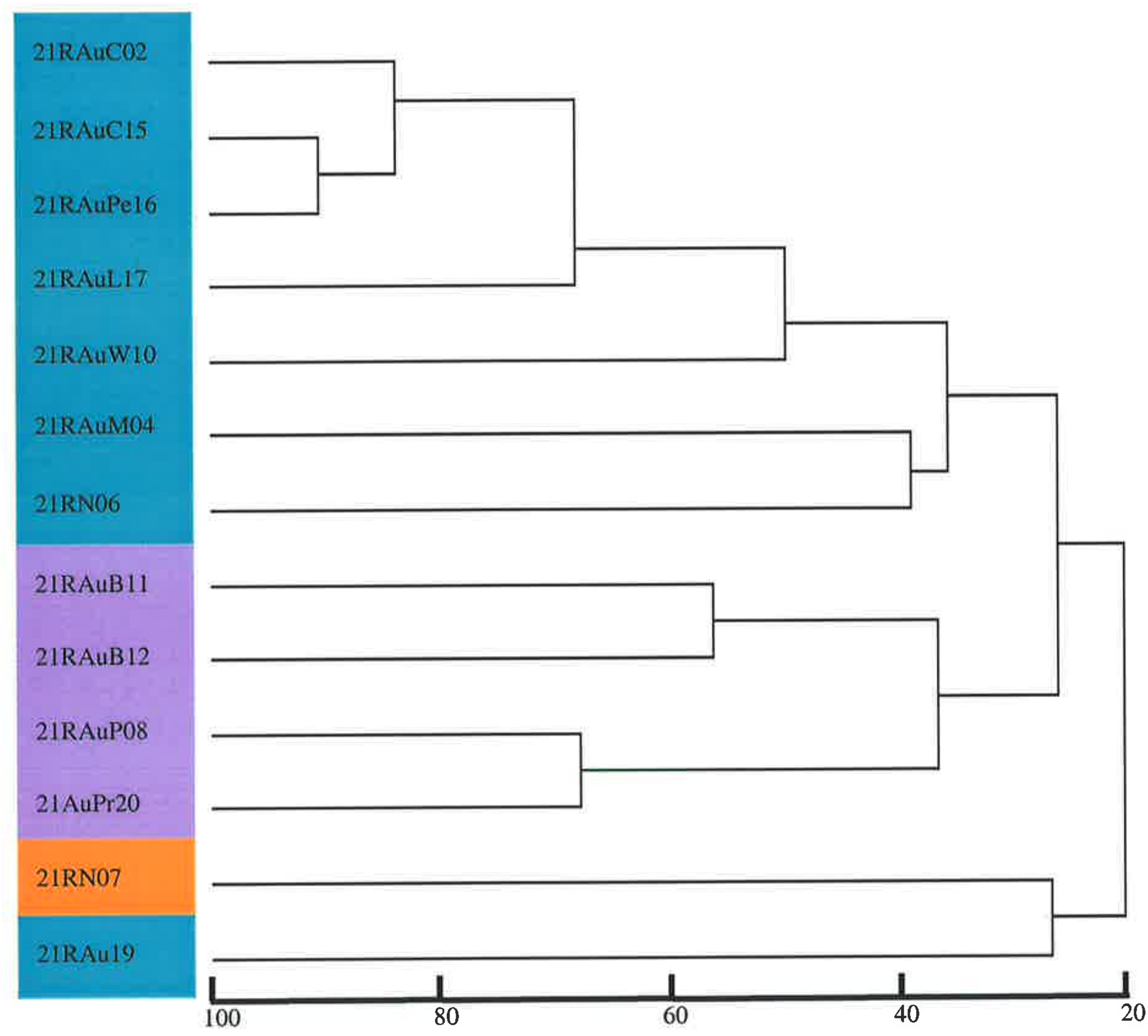
6.3.2. Analysis of AG 2-1 isolates using combined zymogram, PCR and RFLP data

The data from zymogram, PCR and RFLP analyses of 13 isolates of AG 2-1 were combined to produce a binary matrix consisting of 122 assessable markers. UPGMA cluster analysis using Nei and Li's (1979) genetic similarity coefficient resulted in the clear separation of AG 2-1, ZG 5 from ZG 6 isolates (Figure 6.3). However, one ZG 5 isolate, 21RAu19, and the "AG 2 undefined" isolate 21RN07 were not closely related to the other isolates, with which they shared an average similarity of approximately 20%. The remaining AG 2-1, ZG 5 isolates shared approximately 26% similarity with the AG 2-1, ZG 6 isolates, and divided into two subgroups at 36% similarity. The larger group consisted of five Australian isolates from

Figure 6.3: Dendrogram representing the genetic similarities of 13 isolates of *R. solani* AG 2-1. Nei and Li's (1979) genetic similarity coefficient was used to form a similarity matrix from 122 markers generated by zymogram, PCR and RFLP analyses.

AG 2-1, ZG 5 isolates

AG 2-1, ZG 6 isolates


21RAuC02
21RAuC15
21RAuPe16
21RAuL17
21RAuW10
21RAuM04
21RN06
21RAuB11
21RAuB12
21RAuP08
21AuPr20
21RN07
21RAu19
100
80
60
40
20
Nei and Li's genetic similarity (%)

various hosts, while the second group comprised the Australian isolate 21RAuM04 and isolate 21RN06 from the Netherlands. These two isolates were approximately 39% similar to one another (Figure 6.3; Appendix E). Within the ZG 6 group, the two Australian barley isolates, 21RAuB11 and 21RAuB12, shared higher similarity (56%) to one another than to the other two ZG 6 isolates from potato and pine (21RAuP08 and 21RAuPr20). The genetic relationships of the AG 2-1 isolates determined by combining data from zymogram, PCR and RFLP analyses was similar to that produced using only two methods, zymogram and PCR.

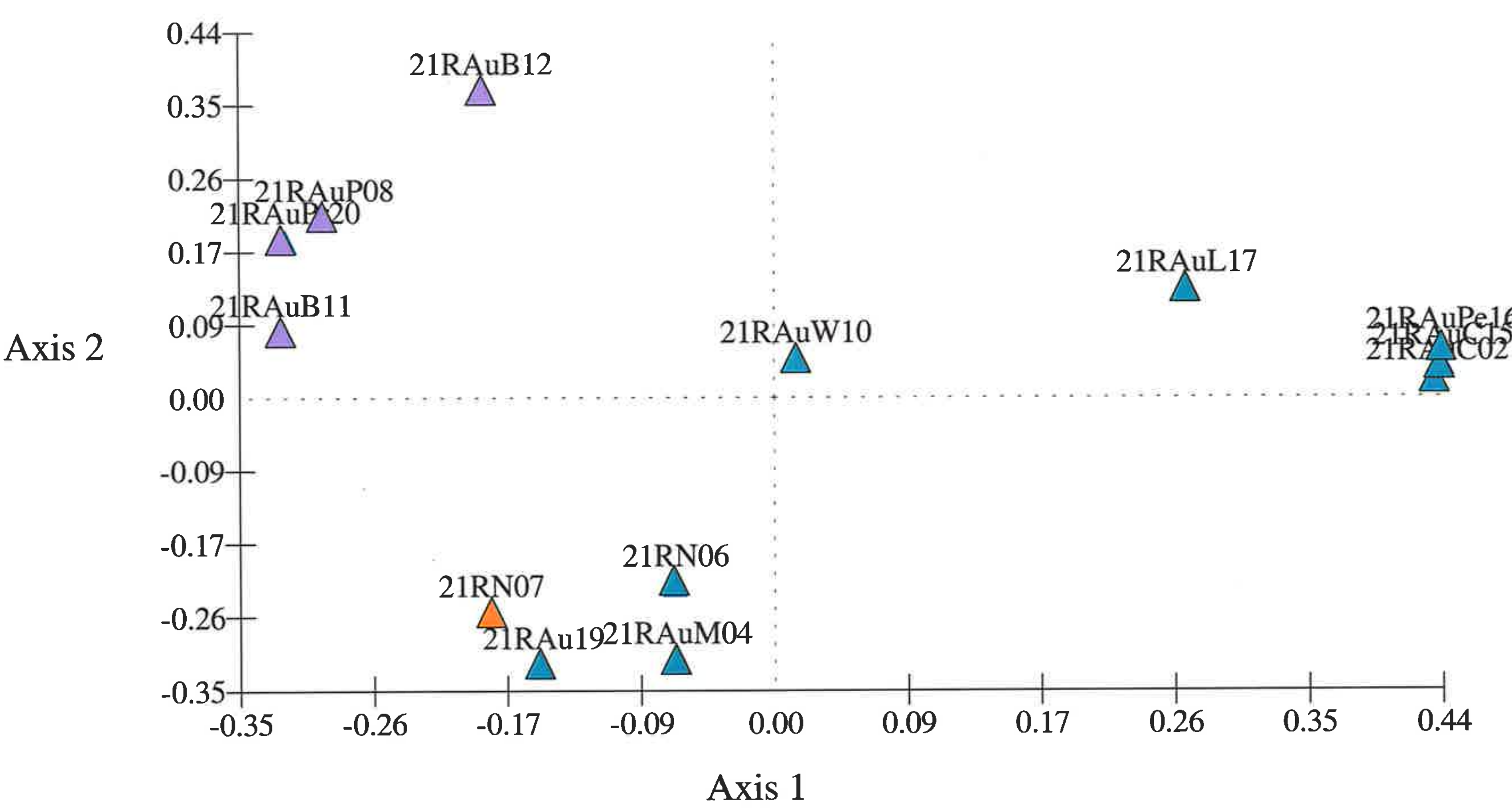
PCO analysis of the 122 markers by Gower's general similarity coefficient (Gower, 1966), produced 13 axes for the 13 isolates examined (Appendix E) which resulted in the distinct grouping of isolates when plotted on a two-dimensional scatter plot (Figure 6.4). The two axes plotted represent approximately 45% of the variation detected by the markers, with axis 1 showing approximately 29% and axis 2 approximately 16% of that variation (Appendix E). While the isolates tended to have similarities of above 50% (Appendix E), the plot indicates that the isolates separated into three groups (Figure 6.4), which do not correspond to the dendrogram constructed by cluster analysis (Figure 6.3). The ZG 5 isolates, 21RAuC02, 21RAuC15 and 21RAuPe16, formed a tight group (Figure 6.4, far right) with isolates 21RAuL17 and 21RAuW10, while belonging to the group, more distantly related. The remaining ZG 5 isolates (21RAuM04, 21RAu19 and 21RN06) formed a distinct group (lower left quadrant) along with the "AG 2 undefined" isolate, 21RN07. The ZG 6 isolates, 21RAuP08 and 21RAuPr20, appear to be closely related and shared approximately 72% similarity with 21RAuB11. The final ZG 6 isolate, 21RAuB12, shared approximately 80% similarity to 21RAuB11, but shared only 67% similarity with the other two isolates, making the ZG 6 isolates a diverse group.

A comparison of the two similarity matrices using the Pearson product moment coefficient resulted in a strong correlation score of 0.860, suggesting that the genetic relationships determined by the two statistical methods were in agreement.

Figure 6.4: Scatter plot derived from principal coordinates analysis of 122 markers obtained from zymogram, PCR and RFLP analyses of 13 isolates of *R. solani* AG 2-1. Gower's general similarity coefficient (Gower, 1966), was used to generate a similarity matrix to determine the genetic relationships between isolates. The two most variable axes are plotted.

AG 2-1, ZG 5 isolates

AG 2-1, ZG 6 isolates



PCO case scores (Gower General Similarity Coefficient)
Axis 2
Axis 1
0.44
0.35
0.26
0.17
0.09
0.00
-0.09
-0.17
-0.26
-0.35
-0.35
-0.26
-0.17
-0.09
0.00
0.09
0.17
0.26
0.35
0.44
21RAuB12
21RAuP08
21RAuB11
21RAuW10
21RAuL17
21RAuPe16
21RN06
21RN07
21RAu19
21RAuM04

6.3.3. Analysis of AG 2-2 isolates using combined zymogram, PCR and RFLP data

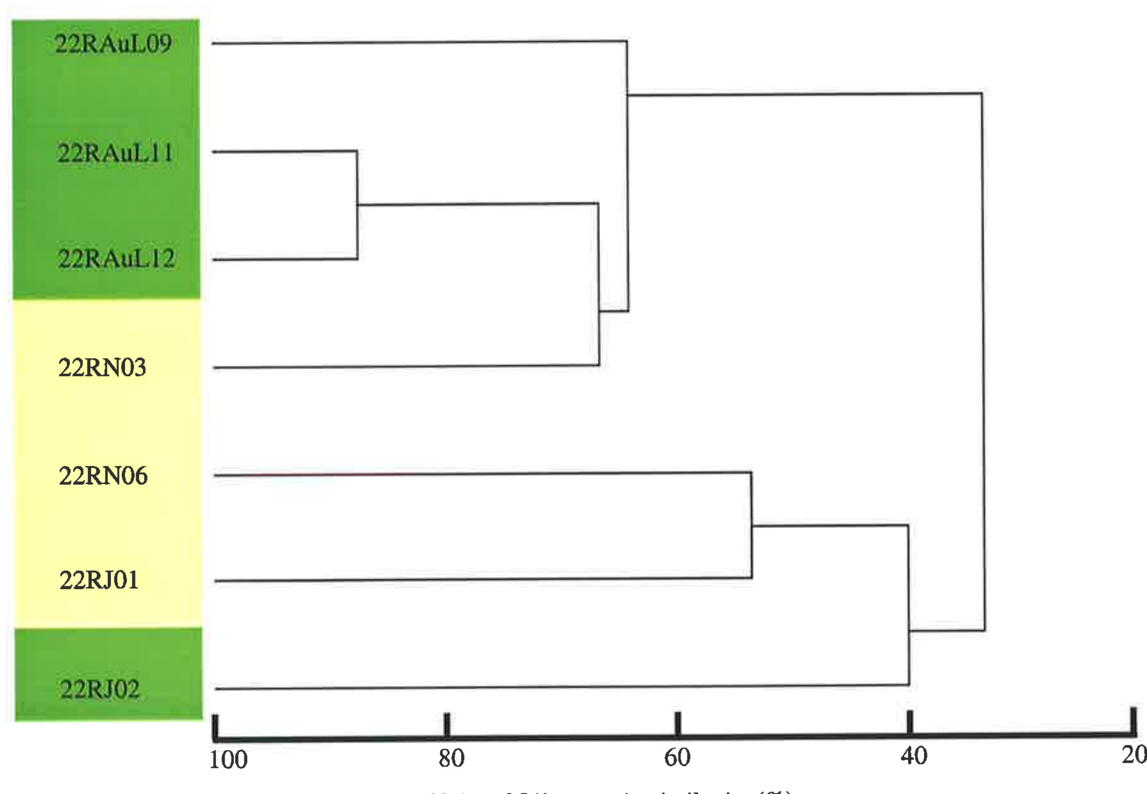
Data generated from zymogram, PCR and RFLP analyses of seven AG 2-2 isolates was combined to form a binary matrix consisting of 132 assessable markers. UPGMA cluster analysis of the markers was performed using Nei and Li's (1979) genetic similarity coefficient, which generated a dendrogram that separated the isolates into two distinct groups sharing 36% similarity. One group (Figure 6.5, upper section) consisted of three Australian isolates, 22RAuL09 (ZG 4), 22RAuL11 (ZG 10) and 22RAuL12 (ZG 4) from lupin, along with the Netherlands isolate 22RN03 (ZG 10), originally isolated from sugar beet. Isolates 22RAuL11 and 22RAuL12 shared approximately 88% similarity with one another, while 22RAuL09 and 22RN03 were related to these two isolates with 65% and 68% similarity, respectively. In the second group (Figure 6.5, lower section), the Netherlands isolate 22RN06 shared approximately 55% similarity with Japanese isolate 22RJ01 while the second Japanese isolate, 22RJ02, was approximately 42% similar to these isolates. In contrast to the results for the AG 2-1 isolates, different similarity relationships were observed using data from all three methods than when only data from zymogram and PCR analyses were combined. With the addition of the RFLP markers, the AG 2-2 isolates from Japan and the Netherlands were less similar to one another, whereas the Australian AG 2-2 isolates were more closely related to each other than to either the Netherlands or Japanese isolates.

PCO analysis of the 132 markers by Gower's general similarity coefficient (Gower, 1966) produced seven axes for the seven isolates examined (Appendix E), and revealed distinct groups of isolates when plotted on a two-dimensional scatter plot (Figure 6.6). The two axes represent approximately 59% of the variation associated with the 132 markers, with 40% and 19% of that variation shown by axis 1 and axis 2, respectively. All isolates shared greater than or equal to 54% similarity when analysed with this coefficient (Appendix E). The results suggested that two groups exist, and these were quite distinct. One group

Figure 6.5: Dendrogram representing the genetic similarities of seven isolates of *R. solani* AG 2-2. Nei and Li's (1979) genetic similarity coefficient was used to form a similarity matrix from 132 markers generated from zymogram, PCR and RFLP analyses.

AG 2-2, group I isolates

AG 2-2, group II isolates

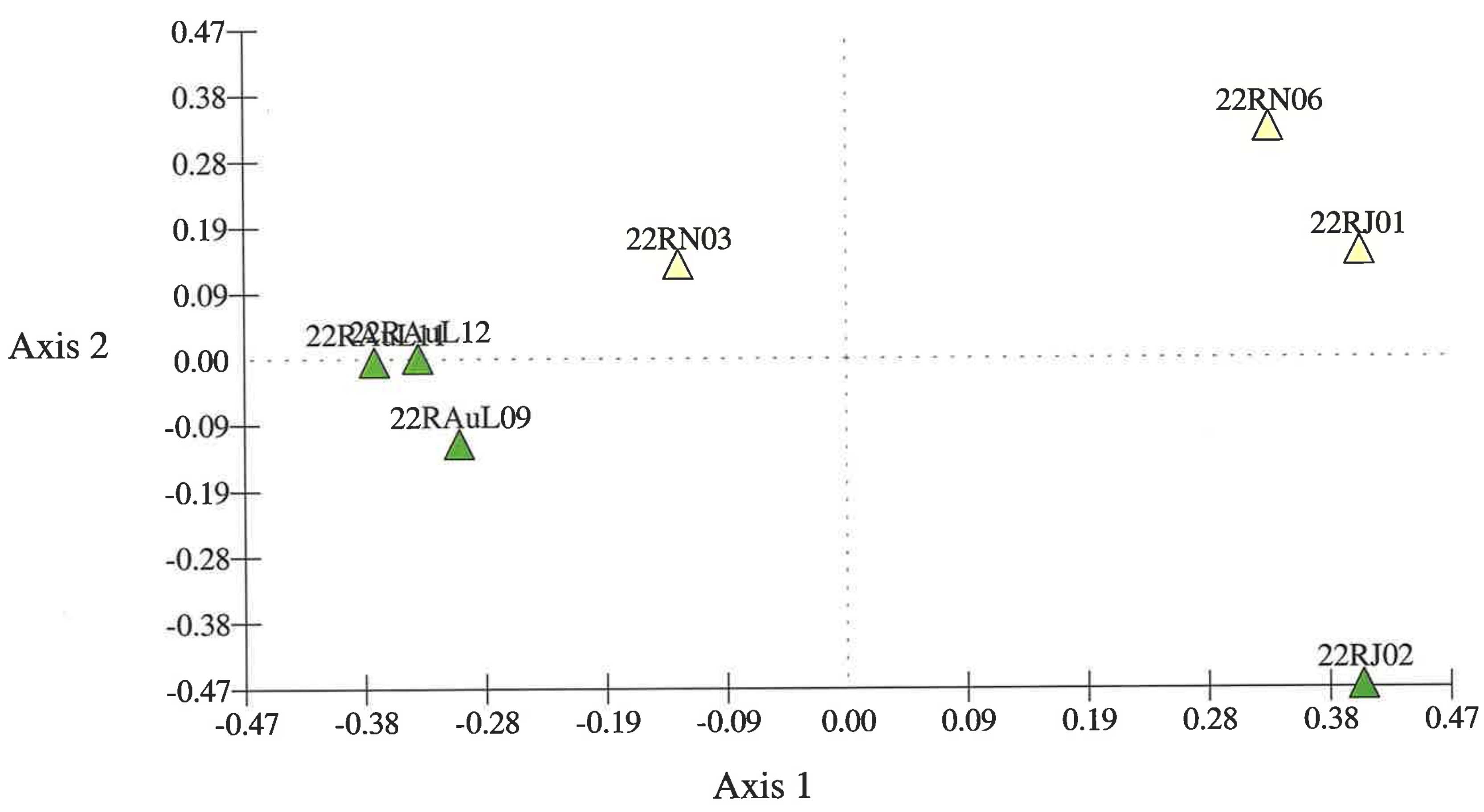


22RAuL09
22RAuL11
22RAuL12
22RN03
22RN06
22RJ01
22RJ02
100
80
60
40
20
Nei and Li's genetic similarity (%)

Figure 6.6: Scatter plot derived from principal coordinates analysis of 132 markers obtained from zymogram, PCR and RFLP analyses for seven isolates of *R. solani* AG 2-2. Gower's general similarity coefficient (Gower, 1966) was used to generate a similarity matrix for the determination of the genetic relationships between isolates and the two most variable axes were plotted.

AG 2-2, group I isolates

AG 2-2, group II isolates


PCO case scores (Gower General Similarity Coefficient)
Axis 2
Axis 1
0.47
0.38
0.28
0.19
0.09
0.00
-0.09
-0.19
-0.28
-0.38
-0.47
-0.47
-0.38
-0.28
-0.19
-0.09
0.00
0.09
0.19
0.28
0.38
0.47
22RN06
22RJ01
22RN03
22RAuL12
22RAuL09
22RJ02

contained the isolates 22RAuL09, 22RAuL11, 22RAuL12 and the more distantly related 22RN03 (left of centre), while the second, more diverse group consisted of 22RJ01, 22RJ02 and 22RN06 (right of centre). This corresponded directly with the dendrogram produced by cluster analysis (Figure 6.5). Isolates 22RJ01, 22RJ02 and 22RN06 formed a diverse group, sharing an average similarity of greater than 64% with one another (Appendix E). The remaining isolates comprised the second group, and shared an average similarity of greater than 75%.

Correlation of the two distance matrices by the Pearson product moment coefficient resulted in a strong correlation score of 0.994, suggesting that a high level of agreement exists between the genetic relationships determined by the two statistical methods.

6.4. Discussion

Two statistical methods were used to examine the genetic relationships among isolates of *R. solani* AG 2. UPGMA cluster analysis, using Nei and Li's (1979) genetic similarity coefficient is classed as a hierarchical, multivariate method, which will link all isolates based on similarities, and assumes that a hierarchy exists among the isolates. PCO analysis is classed as a non-hierarchical, multivariate method, which, while still linking isolates based on similarity, does not assume a hierarchical structure. The results obtained using the Pearson product moment coefficient showed that there was a good agreement between the two statistical methods, for each of the combined data sets examined, despite the fact that the methods use different similarity coefficients, giving an increased confidence in the relationships determined by these methods.

A clear separation of the anastomosis groups was observed when the AG 2-1 and AG 2-2 isolates were examined on the basis of the combination of zymogram and PCR data. In addition, the separation of AG 2-1, ZG 6 from AG 2-1, ZG 5 and AG 2-2 isolates on the basis

of the combined markers, was evident. When the RFLP data were included in the analysis of the AG 2-1 isolates, the ZG 5 and ZG 6 isolates remained distinct. This suggested that the ZG subgroups that exist within AG 2-1 are genetically distinct from one another, despite the ability of isolates from the two ZG to undergo anastomosis. Both cluster analysis and PCO analysis of the data sets confirmed this observation.

In contrast, the AG 2-2 isolates were not separated into ZG 4 and ZG 10 when any of the data sets were analysed. Furthermore, isolates identified as belonging to AG 2-2 group I and group II were not separated by the combination of zymogram and PCR markers, with or without the inclusion of the RFLP data. When only the zymogram and PCR data were combined, did the AG 2-2 isolates separate into geographic groups, with the Australian isolates being separated from isolates obtained from Japan and the Netherlands. This geographic grouping was also observed for two "AG 2 undefined" isolates from Australia, which clustered with AG 2-2. However, with the addition of the RFLP data, the isolates resolved into different groups, with one Netherlands isolate (ZG 10) more closely related to the Australian isolates (ZG 4), and a Japanese isolate (ZG 4) being more closely related to a second Netherlands isolate (ZG 10). Both the hierarchical and non-hierarchical statistical methods supported the shift to the new groups. This suggested that, within this collection of AG 2-2 isolates, the markers examined do not recognise the accepted AG 2-2 zymograms groups, ZG 4 and ZG 10. In addition, while the combination of zymogram and PCR data showed two Australian isolates, 22RAuL09 and 22RAuS10, to be closely related, a more in-depth analysis with a third set of markers, such as those provided by RFLP analysis, may reveal far less similarity between the isolates.

Analysis of combinations of data obtained by several procedures has been used previously to separate closely related species. For example, *Phytophthora mirabilis* has been named *P. infestans* var. *mirabilis*, *P. infestans* forma specialis *mirabilis* and *P. mirabilis*. Goodwin *et al.* (1999) combined data generated from isozyme loci of glucose-6-phosphate

isomerase, RAPD-PCR using 15 primers, RFLP fingerprinting of genomic DNA and rDNA-RFLP to determine the relationship of *P. mirabilis* to *P. infestans*. The combination of isozyme data, RAPD and genomic RFLP fingerprinting separated *P. mirabilis* from *P. infestans*, whereas the rDNA-RFLP analysis was unable to distinguish the two (Goodwin *et al*., 1999). The authors suggested that the combination of dominant (RAPD) and co-dominant (isozymes and genomic RFLP) data provided a more accurate determination of species due to the sampling of a larger portion of the genome, when compared to phylogenies derived from single gene sequences, such as those derived from rDNA. This may add some credence to the suggestion by Schneider *et al.* (1997) that AG 2-1 consists of more zymogram groups than the accepted ZG 5 and ZG 6. Their results indicated that at least five groups were evident within AG 2-1, ZG 5, alone. The results obtained for this thesis also indicated that subdivisions may exist in AG 2-1, ZG 5 and perhaps among the AG 2-1, ZG 6 isolates. In addition, the results presented here suggest the AG 2-2 isolates also consist of more than two subgroups. The identification of subgroups such as AG 2-2LP (Hyakumachi *et al*., 1998) supports this suggestion.

The combination of data generated by three different approaches has enabled the determination of genetic relationships amongst the AG 2-1 and AG 2-2 isolates investigated here. In addition, the statistical methods applied to determine the genetic relationships that exist among the isolates examined, showed a high level of agreement. This is important when considering the manner in which to group isolates, and for determining at what level of relatedness a group is formed. A dendrogram produced from hierarchical cluster analysis shows all sources of variation between isolates, and may only show two isolates as being similar due to the fact that they are dissimilar to all other isolates. In addition, the average similarity obtained for isolates is usually representative only if isolates are closely related, whereas the average values obtained from distantly related isolates might not be truly representative (Abbott *et* al., 1985), and may result in the clustering of unrelated isolates.

While PCO analysis plots only the two or three major sources of variation that occur, the isolates are placed within the plot on the basis of the overall variation in the data, which provides an accurate representation of inter-group relationships (Alderson, 1985). The close correspondence of the two methods presented here suggests that the both methods are suitable for analysis of the types of data generated here, and that the genetic relationships presented are reliable and robust.

Chapter 7

General Discussion

A collection of isolates of *R. solani* anastomosis group 2 (AG 2) from four countries was examined using anastomosis grouping, colony morphology and pathogenicity. A modified pectic zymogram technique and PCR and RFLP analyses of the DNA were used to investigate genetic diversity. In addition, molecular markers that were able to distinguish the AG 2-1 isolates from the AG 2-2 isolates were identified. Very little is known about the highly variable AG 2 group in Australia, in particular in regard to the genetic diversity present within natural populations, the potential host range and the evolution of the group. By identifying suitable genetic markers it would be possible to further examine the group in Australia and other regions of the world where disease caused by AG 2 isolates is common.

The colony morphology of the collection of isolates was variable, with no morphological features which distinguish AG 2-1 and AG 2-2. The isolates were classified as either AG 2-1 or AG 2-2 based on hyphal fusion (Carling *et al.*, 1988), although four of the 33 isolates examined produced both C1 and C2 reactions with opposing isolates from AG 2-1 and AG 2-2. These isolates were classified as "AG 2 undefined". In addition, when a bridging isolate was opposed to one of the AG 2-1 isolates, a mixed C1 / C2 reaction was observed. Thus, while the majority of the isolates could be placed into AG, hyphal fusion was not sufficient to classify all isolates into established groups. It is suggested that an additional level of complexity may be involved and the inconsistent and ambiguous reactions observed here agree with the results of McCabe *et al.* (1999). If, as these authors suggested, a mosaic of genetically different hyphae occur in a single colony, then anastomosis reactions may differ when genetically differing hyphae from the one colony are opposed to those of a second colony. It may be possible not only to classify an isolate incorrectly as a particular

AG, but also to produce isolates genetically different from the parental isolate, following hyphal tip subculture. Furthermore, this does indicate the need for additional techniques to be used when classifying isolates of *R. solani* into groups. Anastomosis is the main technique by which isolates of *R. solani* are classified. However, it is imperative to note that the classification into AG needs to be undertaken immediately after an isolate has been established in pure culture. It is possible that, over time, genetic changes may occur which affect the ability of isolates to anastomose. This in turn raises the question of using "tester isolates" collected many years ago in determining AG. It may be beneficial for research groups periodically to update their collections to contain recently isolated standards for use in anastomosis classification. An understanding of the molecular basis and biochemical mechanisms governing anastomosis (vegetative compatibility), would also be beneficial as it would allow isolation of genes responsible for regulating the process, resulting in a better understanding of the role anastomosis plays in the evolution of *Rhizoctonia* and similar fungi.

The modification of the horizontal zymogram gel electrophoresis technique to utilise commercially available vertical gel electrophoresis equipment facilitated the separation and detection of pectic isozymes from 31 isolates of AG 2. The data obtained were used to define the isolates into AG 2-1, ZG 5, AG 2-1, ZG 6 and AG 2-2, with the collection sharing an average genetic similarity of less than 1%. This high diversity and low level of similarity between isolates in their pectic enzyme profile was probably due in part to the differing geographic locations from which the isolates were obtained, the different times at which they were isolated and the host types from which the isolates originated from. Both AG 2-1, ZG 5 and AG 2-1, ZG 6 appeared to consist of subgroups, supporting the proposal of Schneider *et al.* (1997), that AG 2-1, ZG 5 isolates from the Netherlands constitute five ZG 5 subgroups.

In this study the established zymogram groups of AG 2-2, ZG 4 and ZG 10, could not be identified. Again, as for anastomosis, it is possible that genetic changes within the isolates occurred over time, which may have resulted in changes to the production of pectic isozymes.

Furthermore, the fact that isolates in culture are not exposed to a host may affect the production of pectic isozymes, which are secreted during the early stages of the infection process. While "tester isolates" from each ZG were used as markers to determine ZG, these may not necessarily reflect all the variation that is present within each of the AG 2-2 subgroups. Every attempt was made to maintain similar culturing conditions to those previously reported, but it is possible that the conditions had some effect on the expression of isozymes. However, two distinct groups were observed for AG 2-2 isolates, which were genetically diverse. In general the low genetic similarity between some isolates made it difficult to distinguish true groupings of AG 2 isolates. Vertical gel electrophoresis for pectic enzymes should be carried out on more isolates of AG 2 and other AG as it may provide informative markers for the determination of genetic relationships both within and between the AG of *R. solani* and other *Rhizoctonia* species.

The pectic enzymes produced by *Rhizoctonia* species may be associated with pathogenicity. Consequently, eight AG 2 isolates that showed variation in their zymogram patterns, along with an AG 3 and AG 8 isolate, were selected for pathogenicity trials on three hosts; canola, medic and turf grass. The three AG 2-1 isolates examined on canola, which were originally isolates from canola, medic and potato, caused much higher levels of disease than the other AG, suggesting that *R. solani* AG 2-1 may be a potential threat to canola production in Australia. This is of concern in areas where *Rhizoctonia* disease is prevalent and wheat and canola are grown in rotation. Both AG 2 and AG 8 isolates have been associated with bare patches in wheat. It is possible that rotations of crops that are susceptible to the different AG of *R. solani* will result in the persistence and build up of pathogenic types, resulting in high disease pressures during the growing season. For example, while AG 8 was not seen to be pathogenic to canola, it is likely that the fungus can survive in the soil during the canola growing season. When wheat is then planted, the level of inoculum may increase during the growing season, which, with conducive environmental conditions, would place the

crop under high disease pressure. While canola exhibited a much higher level of susceptibility to AG 2-1 isolates than to isolates from the other AGs, this was not the case for medic cultivars. All isolates examined caused significant disease on the medic cultivars examined, which suggests that the use of medic in a wheat rotation system in some regions of Australia would result in poor medic establishment and may also act to promote the increase in inoculum levels of pathogenic AG, such as AG 2 and AG 8.

No disease associated with *R. solani* was observed in the pathogenicity trials carried out on turf grass cultivars. Since AG 2-2 is known to be pathogenic to turf grass in the USA and Japan (Burpee and Martin, 1992; Hyakumachi *et al.,* 1998), the results suggest that the isolates used were not pathogenic to this host species, the conditions used were not suitable for disease expression or that the cultivars used were resistant to *R. solani.* Further examination of the host/pathogen relationship between AG 2-2 and turf grass is needed to determine which groups cause damage to grasses commonly used in gardens and recreational and ornamental facilities, in Australia.

Isolates of AG 2-1, ZG 5 and ZG 6 were able to cause disease on two of the hosts, indicating that ZG plays a limited role in defining host range within AG 2-1. Some isolates were more pathogenic than others, indicating variation in virulence and possibly suggesting that pathotypes exist within ZG. However, only a small number of isolates were examined and the pathogenicity of additional isolates from both ZG is required in order to draw further conclusions. Studies in which pathogenic and non-pathogenic isolates of *R. solani* have been used to examine host infection processes have suggested that non-pathogenic isolates, while still attracted to the host, do not adhere to the host or proceed in infection (Keijer 1997). Thus, it is possible that the production of pectic enzymes, which are exuded after the initial penetration event, are not associated with host specificity, the process must be controlled by other means. The identification of the biochemical mechanisms and DNA sequences

associated with host recognition would help us to understand the process and more accurately identify pathotypes within *R. solani* and other agriculturally important fungi.

Research carried out on fungal pathogens, such as *Magnaporthe grisea* (Hamer *et al.*, 1989), *Phytophthora sojae* (Ryley *et al.*, 1998) and *Pythium irregulare* (Harvey *et al.*, 2001), has suggested that selective pressure may be exerted on pathogens due to the interaction between the host and the pathogen. This host-mediated selection may result in the evolution of pathotypes adapted to the different hosts to which they have been exposed. Indeed, the existence of host-specific subgroups such as AG 2-2LP and AG 2t in *Rhizoctonia* suggests that an event such as host-adaptation, may be occurring in AG 2. However, in the results presented in this research, there appeared to be only limited host adaptation. The isolates of AG 2-1 were the only isolates which proved pathogenic to canola, with an AG 2-1 originating from a canola host displaying an increased disease severity compared to the other isolates. Furthermore, all of the *R. solani* isolates examined were pathogenic to two medic cultivars, suggesting that with the isolates examined, there is no clear definition of host-specificity. The examination of more AG 2 isolates on hosts such as canola, tobacco and tulip, would help to determine the prevalence of host specific pathotypes similar to that of AG 2t. In addition, the examination over time of a large number of isolates from field plots with different crop rotations should be carried out to determine if the genetic and pathogenic structure of the populations shifts in response to crop type. For a direct comparison, plots within the same fields in which continuous monoculture occurs would need to be included to see if a population shift occurs due to other factors. The study would provide much needed information on the evolution of *R. solani* in agro-ecosystems.

Although *R. solani* AG 2-1 appears to be a more economically important pathogen than AG 2-2 in Australian agriculture, the potential exists in the turf grass industry for AG 2-2 to become a problem. The role of AG 2-2 in Australian agriculture is yet to be defined. AG 2-2 has been isolated from fields in which lupins and wheat have been grown, but their

pathogenicity on the respective crops has not yet been studied. Since research conducted elsewhere has shown that the AG 2-2 subgroups are severe pathogens of turf grass in other regions of the world, it is important to determine whether the turf grass industry in Australia is at risk from this pathogen and the extent to which damage occurs.

The research described above identified considerable genetic and pathogenic variability within the collection of AG 2 isolates, especially given that only a relatively small number of genetic loci were examined. In addition, the results at times were contradictory to those previously published. One of the major aims of the study reported here was to identify molecular markers specific to AG 2-1 and AG 2-2. Random cloning resulted in the identification of two clones, pRAG21-413, which showed homology to isolates of AG 2-1 but not AG 2-2, and pRAG22-39, which identified isolates of AG 2-2 but not AG 2-1. However, both these DNA clones were not specific to their respective AG as homology to DNA from isolates of other AG was observed. The reason for this homology is not known, but may be due to phenomena such as infrequent anastomosis, which may result in the loss of nuclei or the combining of genetically different nuclei within the hyphae, although the transfer of nuclei has not been observed. In addition, specific chromosomal deletions in isolates of AG 2-1 and AG 2-2 and the existence of extra chromosomal elements, such as plasmids (Miyasaka *et al.*, 1990) and dsRNA (Jian *et al.*, 1997) may also be responsible. The observed hybridisation to AG 4 and AG 8 was to a single band when DNA from these AG was digested with *Hin*dIII. This suggests that the regions represented by the clones are present as a single copy in the genome of these AG. Furthermore, hybridisation to other AG in the slot blot procedure was observed, but the signal strength obtained from high levels of DNA from these AG was equivalent to low levels of either AG 2-1 or AG 2-2 DNA. In-depth analysis of the sequences of both pRAG21-413 and pRAG22-39 is required to investigate the genomic origin of the two clones and to attempt to develop specific sequences for AG 2-1 and AG 2-2 DNA. In addition, the use of techniques such as protoplasting and single spore isolations would be

beneficial in determining the extent of variability contained within a single colony. An important point to note was the inability to isolate a DNA clone that was able to identify isolates of AG 2 in general. This raises the question as to how closely related AG 2-1 and AG 2-2 actually are. Both zymogram and PCR analyses suggested that the two groups were genetically distinct, in that they shared low genetic similarities to one another, using those two markers. The fact that pRAG21-413 and pRAG22-39 did not hybridise to either AG 2-2 or AG 2-1, respectively, but had minimal hybridisation to other AG, indicates that perhaps AG 2-1 and AG 2-2 are more distantly related than expected. It has been shown previously that AG 2-1 and AG 2-2 undergo low frequency anastomosis with one another and with other isolates (Carling *et al.,* 1988). However, for isolates to be classified as AG BI, by definition, they must undergo low frequency anastomosis with at least two other AG. The evidence suggests that AG 2-1 and AG 2-2 are possibly two distinct groups, with the possibility that either one or both are acting as a bridging type. Nicoletti *et al.* (1999) suggested that isolates of AG BI originating from tobacco were genetically related to isolates of AG 2-1. This needs to be examined further with a broad range of isolates from both groups, as well as with isolates from AG 2-2, to determine what relationship, if any, exists between these groups.

Both the DNA clones (see chapter 4) identified repetitive sequences in the DNA of their respective groups and were used to examine further the genetic relationships that existed between isolates within each of the respective AG. Similar to the pectic zymogram data, RFLP analysis of the AG 2-1 isolates revealed considerable genetic variation within the group, and the DNA hybridisation results indicated that both ZG 5 and ZG 6 consist of additional subgroups. However, this was not the case for the AG 2-2 isolates. While zymogram analysis showed that considerable variation existed amongst the isolates, the data from RFLP analysis with pRAG22-39 indicated that the isolates shared high genetic similarity. This suggests that pRAG22-39 contains a DNA fragment from a conserved region

of the AG 2-2 genome, especially since the AG 2-2 isolates were from diverse host and geographic origins.

Intron splice junction and microsatellite-primed PCR was also investigated as a means of identifying AG-specific markers and to determine the extent of genetic variation within the collection. Isolates of AG 2-1 could be placed into ZG 5 and ZG 6 based on the combined banding patterns obtained with the three primers used. Again, AG 2-2 isolates could not be placed into ZG 4 or ZG 10, as determined by other researches (MacNish *et al.*, 1993), nor could they be placed into AG 2-2 group I or group II, as was the case for vertical gel zymogram analysis. The primers used for genetic similarity analyses were not able to group isolates from different geographic regions, or identify isolates originating from different host species. It may be necessary to use more primers of similar type to achieve this and to examine greater numbers of isolates from individual regions before a relationship between genetics and origin can be achieved. The PCR approach provided a rapid means of determining genetic diversity among isolates of *R. solani*, compared to the cloning and isolation of DNA probes. While the PCR and RFLP data for the isolates of AG 2-1 examined corresponded reasonably well with one another, the RFLP probe, pRAG22-39, revealed less genetic variation within the isolates of AG 2-2 than did the PCR primers. This suggests that, when choosing techniques to determine genetic relationships among isolates, it is necessary to use a range of different markers to derive a more accurate picture of the inter-relationships. Analysis of more isolates of both AG 2-1 and AG 2-2 from individual field populations would help to clarify the usefulness of microsatellite primers in discerning genetic relationships of these groups. Microsatellite-primed PCR has been beneficial in determining population diversity of various fungi, and in the separation of closely related species. For example, Fisher *et al.* (2000) found that microsatellite loci could be used to resolve the population structure of *Coccidiodes immitits* in various regions of the USA. Czembor and Arseniuk (1999) found microsatellite-primed PCR to be the more sensitive technique for detecting

DNA polymorphisms in *Stagonospora* spp. and *Septoria tritici,* when compared with RAPD-PCR and the amplification of dispersed repetitive elements (rep-PCR). Likewise, the evidence presented here for *R. solani* AG 2 suggests that microsatellite-primed PCR is a technique that would help to resolve the extent of genetic variability that exists within the AG 2 subgroups and for the study of genetic variability within all of the current AG.

It was interesting to note that for the three approaches used here, zymogram, RFLP and PCR analysis, a number of the relationships among the isolates of AG 2 remained constant. For instance, the average genetic similarity of three Australian isolates, 21RAuC02, 21RAuC15 and 21RAuPe16, was greater than 80% regardless of the approach used. While these isolates are genetically similar, they originated from two geographic regions that are separated by thousands of kilometres. Isolate 21RAuC02 originated from the mid north of South Australia, and both isolates 21RAuC15 and 21RAuPe16 from the south west of Western Australia. In addition, 21RAuPe16 was isolated from pea whereas the two other isolates were from canola. This not only suggests that migration has occurred, but that it may play an important role in population structure and diversity. Migration may have occurred via "man made" mechanisms, such as the transfer of infected seed or infested soil, or through natural mechanisms such as wind dispersal of infected organic material. The latter would infer that migration is a continual process and maybe of more significance. It would be interesting to apply the techniques of microsatellite PCR and RFLP analysis to examine the distribution of genotypes within and between fields in Australia over time. This would provide information on the genotypes that occur in each region and how rapidly they may migrate.

What appears to be lacking in research on *Rhizoctonia* is the study of population genetics. The aims of the research reported here were to identify techniques and develop tools for diagnosing a particular AG. However, diagnosis of a group is only one step towards the goal of devising suitable disease control strategies. Without in-depth knowledge of the

biology of the pathogen, it is difficult to predict the likely emergence of new pathotypes. The identification of the two RFLP markers described here may be of assistance. With refinement and further research to eliminate the cross-hybridisation to other AG, it may be possible to use the markers to detect isolates of AG 2-1 or AG 2-2 in soil and plant matter, and in turn the RFLP markers may be applied to more detailed analysis of the genetic variability of populations, as would microsatellite-primed PCR. In Australia, it is also necessary to determine the presence or absence of the sexual state of *R. solani*, *Thanatephorus cucumeris*, and its contribution to maintaining and dispersing the observed genetic diversity in the field. The isolation of co-dominant markers is crucial to the study of *R. solani* populations. Recent studies have indicated that sexual recombination and migration play a significant role in generating genetic diversity in *R. solani*. Rosewich *et al.* (1999) identified seven single copy probes, which were used to separate 182 isolates of AG 1-1A from six field populations in Texas, into 36 multilocus genotypes. While the multilocus genotypes were mainly location specific, several were present in distantly separate populations. Statistical analysis of the results indicated a high degree of gene flow between the six populations. In addition, a high number of the genotypes at all the loci examined were heterozygotes, suggesting that *R. solani* AG 1-1A is an actively outbreeding group in Texan rice-fields (Rosewich *et al.*, 1999).

The further refinement of the two genomic clones identified in this study is required in order to examine their potential as markers for AG 2 isolates. Determination of the sequences and sub-cloning to generate smaller probes may help to increase specificity to AG 2. This in turn may assist in the identification of sequences that hybridise to distantly related AG, and lead to the development of probes spcific to *R. solani*, in general. These sequence data could be used to develop PCR primers, enabling the development of a PCR-based assays. Again, strict testing procedures would be necessary to ensure that the primers are capable of discriminating between DNA from other AG, other fungal species and plant DNA. The clones may be of use in studies on the biology of AG 2, and the identification of co-dominant

multi-copy and single copy DNA markers, are crucial for determing the population diversity and genetic structure of AG 2 in Australia. RFLP analysis and microsatellite-primed PCR would enable the examination of isolates both within and between field populations, as well as the genetic relationships between anastomosis groups. The determination of genetic diversity and an expanded knowledge of the rate at which populations change, the levels of gene flow among locations and host-adaptation may assist the development of disease control strategies and the forecasting of disease outbreaks. I believe the push towards the development of markers purely for diagnostic purposes needs to be revisited. For example, markers developed from the ITS regions of *R. solani* rDNA are inconclusive due to the existence of high sequence variation within the region. Goodwin *et al.* (1999) suggested that the combination of dominant and co-dominant data can provide a more accurate determination of species by sampling a large portion of the genome, when compared to phylogenies derived from single gene sequences, such as those derived from rDNA. Furthernore, the use of functional genes in taxonomic studies is becoming more common. De Jong *et al.* (2001) used DNA sequencing of the ITS nuclear rDNA, mitochonrial rDNA and the β-tubulin gene to examine the phylogeny of five *Neofabraea* species. Their results using 56 isolates indicated that mitochondrial rDNA was not informative with no variation present among the species. The ITS rDNA was able to separate four of the five species examined, while the β-tubulin gene was the most infomative marker, being able to separate the five species from one another. While the ITS region has provided informative data in regard to the separation of fungal genera and species, its lack of functionality limits the data that can be obtained from its analysis. Markers that will assist in the assessment of the population structure need to be developed in conjunction with diagnostic markers. Without knowledge on rates of genetic change in a population, it is possible that diagnostic markers developed to target discrete regions of the genome, such as the ITS regions of rDNA will miss "newly" evolved genotypes and pathotypes in a population. For this reason, I believe that the use of a

combination of co-dominant multilocus markers along with the examination of functional genes, will be of benefit to biologists studying soil-borne pathogens such as *R. solani*.

Chapter 8

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Appendices

Appendix A

Media, Buffers and Reagents

Culture Media

Potato Dextrose Agar (PDA):

Difco Bacto potato dextrose agar	39g
Nanopure water	1lt
Autoclave at 121°C for 15min	

Potato Dextrose Broth (PDB):

Difco Bacto potato dextrose broth	24g
Nanopure water	1lt
Autoclave at 121°C for 15min	

Pectinase Induction Medium:

$(NH_4)_2SO_4$	2.64g
K_2HPO_4	0.34g
$MgSO_4 . 7H_2O$	0.14g
Citrus pectin (Sigma, P-9135)	10.0g
Nanopure water	1lt
Adjust to pH 5.5 with NaOH	
Autoclave at 121°C for 12min	

Luria Bertani (LB) Broth and Agar:

Difco Bacto tryptone	10g
Difco Bacto yeast extract	5g
NaCl	10g
Difco agar (technical #1)	15g (for LB agar)
Nanopure water	1lt
pH to 7.5 with NaOH	
Autoclave at 121°C for 15min.	

Top Agar:

Difco Bacto tryptone	10g
Difco Bacto yeast extract	5g
NaCl	10g
Oxoid Agar Technical #1	7.5g
Nanopure water	1lt
pH 7.5 with NaOH	
Autoclave at 121°C for 15min.	

Buffers and Reagents

Pectin PAGE Sample/Loading Buffer:

Tris	20mM
Sucrose	40%
2-B-mercaptoethanol (BDH)	10mM
Adjust pH to 6.8 with NaOH	
0.5% Bromophenol blue (prior to use)	2.5%

General DNA Extraction Buffer:

NaCl	1.5M
NaOAc	0.15M
Sarkosyl	2.5%
$EDTANa_2$	0.05M
Nanopure water	100ml
pH 5.7	

RNase A:

10mg/ml solution prepared in:

Tris-HCl, pH7.5	10mM
NaCl	15mM
Heat at 100°C for 15min.	
Cool and store at -20°C	

Tris/EDTA Buffer (TE):

Tris	10mM
$EDTANa_2$	1mM
Adjust pH to 7.5 with HCl	

Cesium Chloride Extraction Buffer:

SSC	2 x
EDTA	20mM
Sarkosyl	3% (w:v)

Boehringer Mannheim 10 x De-phosphorylation Buffer:

$ZnCl_2$	10mM
$MgCl_2$	10mM
Tris-HCl	100mM
pH 8.3	

Boehringer Mannheim 10 x Ligation Buffer:

Tris-HCl	100mM
Dithiothreitol	50mM
BSA	500μg/ml
pH 7.6	

20 X Saline-Sodium Citrate (SSC):

NaCl	175.3g
Sodium citrate	88.2g
Nanopure water	1Lt
Adjust pH to 7.0 with NaOH	

Amersham 10 x Labelling Buffer:

Tris-HCl, pH 7.5	500mM
$MgCl_2$	100mM
Dithiothreitol	10mM
Acetylated BSA	0.5mg/ml

100 x Denhardts Reagent:

BSA	2g
Ficoll	2g
Polyvinyl-pyrrolidone-360	2g
Nanopure water	100ml
Store at -20°C	

1 x TEN Buffer:

Tris-HCl, pH 8	10mM
EDTA	1mM
NaCl	100mM
Nanopure water	150ml
Autoclave at 121°C for 15min.	

TAE Electrophoresis Buffer (50X):

Tris base	2M
Na acetate	1M
$EDTANa_2$	0.05M
pH to 7.8 with acetic acid	
Autoclave at 121°C for 12min. Store at 4°C	

Agarose Gel Loading Buffer (10X):

Urea	4M
Sucrose	40% (w:v)
EDTA, pH7.0	50mM
Bromophenol blue	0.1% (w:v)
Xylene cyanol	0.1% (w:v)

Appendix B

Similarity matrix for AG 2 isolates classified by zymogram analysis. Pair wise comparisons between isolates were calculated by Nei and Li's genetic similarity index (1979) using the Genstat 5 version 4.1 statistical package (Lawes Institute, Rothamsted Experimental Station)

	22RAuL09	22RAuL11	22RAuL12	22RJ02	22RAuS10	22RJ01	22RN03	22RN06	22RAuO08	21RAuC02
22RAuL09	1.0000									
22RAuL11	0.8000	1.0000								
22RAuL12	0.6000	0.7500	1.0000							
22RJ02	0.2857	0.4000	0.4000	1.0000						
22RAuS10	0.2222	0.2857	0.2857	0.5000	1.0000					
22RJ01	0.0000	0.0000	0.0000	0.0000	0.0000	1.0000				
22RN03	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.0000			
22RN06	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.5000	1.0000		
22RAuO08	0.0000	0.0000	0.0000	0.0000	0.0000	0.5000	0.5000	0.5000	1.0000	
21RAuC02	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.0000
21RAUC15	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.0000
21RAuPe16	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.0000
21RAuM03	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.4444
21RAuM04	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.6667
21RAuL17	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.6667
21RN06	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.5000
21RN07	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
21RAu18	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.5000
21RAu19	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.5000
21RAuW10	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.8000
2tRN01	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
21RAuP08	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
21RNzBr21	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
21RAuPr20	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
21RAuB11	0.0000	0.0000	0.0000	0.0000	0.3333	0.0000	0.0000	0.0000	0.0000	0.0000
21RAuB12	0.1667	0.0000	0.0000	0.0000	0.2222	0.0000	0.0000	0.0000	0.0000	0.2500
21RAuB13	0.0000	0.0000	0.0000	0.0000	0.4000	0.0000	0.0000	0.0000	0.0000	0.0000
02RAu01	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
02RAuCf02	0.0000	0.0000	0.3333	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
02RAuCf03	0.2000	0.2500	0.2500	0.0000	0.2857	0.0000	0.0000	0.0000	0.0000	0.0000
02RAuL04	0.2222	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000

	21RAuC15	21RAuPe16	21RAuM03	21RAuM04	21RAuL17	21RN06	21RN07	21RAu18	21RAu19	21RAuW10
21RAuC15	1.0000									
21RAuPe16	1.0000	1.0000								
21RAuM03	0.4444	0.4444	1.0000							
21RAuM04	0.6667	0.6667	0.2500	1.0000						
21RAuL17	0.6667	0.6667	0.2500	1.0000	1.0000					
21RN06	0.5000	0.5000	0.2222	0.6667	0.6667	1.0000				
21RN07	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.0000			
21RAu18	0.5000	0.5000	0.2222	0.0000	0.0000	0.0000	0.0000	1.0000		
21RAu19	0.5000	0.5000	0.2222	0.0000	0.0000	0.0000	0.0000	1.0000	1.0000	
21RAuW10	0.8000	0.8000	0.6000	0.5000	0.5000	0.4000	0.0000	0.4000	0.4000	1.0000
2tRN01	0.0000	0.0000	0.0000	0.0000	0.0000	0.5000	0.0000	0.5000	0.5000	0.0000
21RAuP08	0.0000	0.0000	0.0000	0.0000	0.0000	0.5000	0.0000	0.5000	0.5000	0.0000
21RNzBr21	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
21RAuPr20	0.0000	0.0000	0.0000	0.0000	0.0000	0.2500	0.2222	0.0000	0.0000	0.0000
21RAuB11	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
21RAuB12	0.2500	0.2500	0.3077	0.0000	0.0000	0.0000	0.0000	0.2500	0.2500	0.2222
21RAuB13	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
02RAu01	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
02RAuCf02	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
02RAuCf03	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.5714	0.0000	0.0000	0.0000
02RAuL04	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000

	2tRN01	21RAuP08	21RNzBr21	21RAuPr20	21RAuB11	21RAuB12	21RAuB13	02RAu01	02RAuCf02	02RAuCf03	02RAuL04
2tRN01	1.0000										
21RAuP08	0.2857	1.0000									
21RNzBr21	0.0000	0.7500	1.0000								
21RAuPr20	0.2500	0.9091	0.6667	1.0000							
21RAuB11	0.0000	0.2500	0.3333	0.2222	1.0000						
21RAuB12	0.0000	0.1818	0.2222	0.1667	0.6667	1.0000					
21RAuB13	0.0000	0.2857	0.4000	0.2500	0.8000	0.5000	1.0000				
02RAu01	0.0000	0.3333	0.5000	0.2857	0.5000	0.2857	0.6667	1.0000			
02RAuCf02	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.0000		
02RAuCf03	0.0000	0.0000	0.0000	0.2000	0.0000	0.0000	0.0000	0.0000	0.0000	1.0000	
02RAuL04	0.0000	0.0000	0.0000	0.0000	0.0000	0.2222	0.0000	0.0000	0.0000	0.0000	1.0000

Appendix C

Similarity matrix for AG 2 isolates classified by PCR analysis. Pair wise comparisons between isolates were calculated by Nei and Li's genetic similarity index (1979) using the Genstat 5 version 4.1 statistical package (Lawes Institute, Rothamsted Experimental Station)

	21RAuC02	21RAuC15	21RAuPe16	21RAuM03	21RAuL17	21RAuM04	21RN06	21RN07	21RAu18	21RAu19
21RAuC02	1.0000									
21RAuC15	0.8387	1.0000								
21RAuPe16	0.8000	0.8889	1.0000							
21RAuM03	0.5000	0.4800	0.4167	1.0000						
21RAuL17	0.5517	0.6923	0.6400	0.3478	1.0000					
21RAuM04	0.3226	0.3571	0.3704	0.2400	0.3846	1.0000				
21RN06	0.6471	0.5161	0.5333	0.4286	0.5517	0.4516	1.0000			
21RN07	0.3571	0.3200	0.3333	0.3636	0.2609	0.3200	0.2857	1.0000		
21RAu18	0.3846	0.4348	0.4545	0.3000	0.2857	0.4348	0.3846	0.2000	1.0000	
21RAu19	0.2941	0.2581	0.2667	0.1429	0.2069	0.3871	0.2941	0.2857	0.4615	1.0000
21RAuW09	0.5806	0.6429	0.6667	0.4000	0.6923	0.4286	0.6452	0.2400	0.4348	0.2581
21RAuW10	0.5161	0.4286	0.4444	0.4000	0.6154	0.3571	0.5161	0.3200	0.2609	0.2581
21RAuB11	0.1250	0.1379	0.2143	0.0769	0.2222	0.1379	0.1875	0.3846	0.1667	0.1250
21RAuB12	0.2667	0.2222	0.2308	0.0833	0.2400	0.2222	0.3333	0.2500	0.1818	0.1333
21RAuP08	0.2667	0.2963	0.3077	0.1667	0.3200	0.2222	0.3333	0.1667	0.1818	0.2000
21RNzBr21	0.1875	0.2069	0.2143	0.1538	0.2222	0.3448	0.2500	0.2308	0.1667	0.2500
21RAuPr20	0.1875	0.2069	0.2143	0.2308	0.2222	0.2759	0.2500	0.3077	0.2500	0.2500
22RJ02	0.1935	0.2143	0.2222	0.0800	0.3077	0.5000	0.2581	0.3200	0.1739	0.2581
22RJ01	0.1379	0.1538	0.2400	0.0870	0.1667	0.3077	0.1379	0.1739	0.1905	0.1379
22RN06	0.1667	0.2424	0.1250	0.2000	0.1290	0.2424	0.1111	0.2667	0.1429	0.1667
22RN03	0.1081	0.1765	0.1818	0.1290	0.1875	0.2353	0.1081	0.2581	0.1379	0.2162
22RN04	0.1579	0.2286	0.1765	0.1875	0.1212	0.2286	0.1053	0.2500	0.1333	0.1579
22RAuL09	0.1176	0.1290	0.2000	0.2143	0.1379	0.1935	0.1765	0.2857	0.2308	0.2353
22RAuS10	0.1765	0.1935	0.2667	0.2857	0.2069	0.1935	0.2353	0.2857	0.2308	0.2353
22RAuL11	0.0556	0.0606	0.0625	0.0667	0.1290	0.1818	0.1111	0.1333	0.0714	0.1667
22RAuL12	0.1714	0.1875	0.1935	0.1379	0.1333	0.3125	0.2286	0.2069	0.2222	0.2286
02RAu01	0.1714	0.2500	0.1935	0.2069	0.2667	0.1250	0.2857	0.2069	0.0741	0.1143
02RAuCf03	0.1250	0.2069	0.1429	0.2308	0.2963	0.0690	0.1250	0.2308	0.1667	0.2500
02RAuL04	0.1481	0.1667	0.1739	0.0952	0.0909	0.3333	0.1481	0.0952	0.2105	0.2222
2tRN01	0.3030	0.2667	0.2759	0.1481	0.2143	0.4000	0.3636	0.3704	0.3200	0.4848
BIR01	0.3704	0.4167	0.4348	0.3810	0.3636	0.4167	0.4444	0.1905	0.3158	0.2222

	21RAuW09	21RAuW10	21RAuB11	21RAuB12	21RAuP08	21RNzBr21	21RAuPr20	22RJ02	22RJ01	22RN06
21RAuW09	1.0000									
21RAuW10	0.6429	1.0000								
21RAuB11	0.2069	0.2759	1.0000							
21RAuB12	0.2963	0.2963	0.6429	1.0000						
21RAuP08	0.4444	0.3704	0.4286	0.5385	1.0000					
21RNzBr21	0.3448	0.2759	0.4667	0.6429	0.7143	1.0000				
21RAuPr20	0.3448	0.3448	0.5333	0.5714	0.6429	0.8000	1.0000			
22RJ02	0.2857	0.3571	0.3448	0.3704	0.3704	0.4138	0.4138	1.0000		
22RJ01	0.1538	0.2308	0.2963	0.3200	0.2400	0.2222	0.2222	0.5385	1.0000	
22RN06	0.1818	0.1818	0.2941	0.3125	0.1875	0.2353	0.2353	0.4848	0.4516	1.0000
22RN03	0.2353	0.1765	0.2857	0.2424	0.2424	0.3429	0.3429	0.4118	0.3750	0.5641
22RN04	0.1714	0.1714	0.3333	0.2353	0.1176	0.2222	0.2222	0.4571	0.3636	0.7500
22RAuL09	0.1290	0.1935	0.4375	0.2667	0.2000	0.2500	0.3125	0.1935	0.2069	0.2222
22RAuS10	0.1935	0.2581	0.4375	0.2667	0.1333	0.2500	0.3125	0.1935	0.2069	0.2222
22RAuL11	0.1818	0.1212	0.2353	0.2500	0.2500	0.2941	0.1765	0.1818	0.3871	0.3158
22RAuL12	0.2500	0.1875	0.1818	0.3226	0.1935	0.3030	0.2424	0.2500	0.3333	0.3784
02RAu01	0.2500	0.3125	0.2424	0.3226	0.2581	0.1818	0.2424	0.1875	0.2667	0.3243
02RAuCf03	0.2069	0.2759	0.3333	0.2143	0.1429	0.1333	0.3333	0.2759	0.1481	0.4706
02RAuL04	0.1667	0.0833	0.1600	0.2609	0.3478	0.4000	0.4000	0.4167	0.3636	0.2069
2tRN01	0.2667	0.2667	0.1935	0.2069	0.2759	0.2581	0.3226	0.2667	0.1429	0.2857
BIR01	0.5000	0.4167	0.1600	0.2609	0.2609	0.2400	0.2400	0.2500	0.1818	0.2069

	22RN03	22RN04	22RAuL09	22RAuS10	22RAuL11	22RAuL12	02RAu01	02RAuCf03	02RAuL04	2tRN01	BIR01
22RN03	1.0000										
22RN04	0.7805	1.0000									
22RAuL09	0.3243	0.3158	1.0000								
22RAuS10	0.2703	0.3158	0.9412	1.0000							
22RAuL11	0.4615	0.3500	0.2778	0.2778	1.0000						
22RAuL12	0.4737	0.4103	0.1143	0.1143	0.7027	1.0000					
02RAu01	0.2632	0.2564	0.1714	0.2286	0.3243	0.3333	1.0000				
02RAuCf03	0.4000	0.4444	0.3750	0.3750	0.1765	0.2424	0.3636	1.0000			
02RAuL04	0.3333	0.3226	0.1481	0.1481	0.2069	0.1429	0.2143	0.1600	1.0000		
2tRN01	0.2222	0.2162	0.2424	0.2424	0.1714	0.2941	0.1176	0.1935	0.3077	1.0000	
BIR01	0.2000	0.1935	0.2222	0.2963	0.2069	0.2857	0.2857	0.1600	0.3000	0.3846	1.0000

Appendix D

Similarity matrix for AG 2 isolates classified by RFLP analysis. Pair wise comparisons between isolates were calculated by Nei and Li's genetic similarity index (1979) using the Genstat 5 version 4.1 statistical package (Lawes Institute, Rothamsted Experimental Station)

A) AG 2-1 isolates hybridised with the plasmid construct pRAG21-413.

	21RAuC02	21RAuC15	21RAuPe16	21RAuL17	21RAuM04	21RN06	21RN07	21RAu19	21RAuW10
21RAuC02	1.000								
21RAuC15	0.829	1.000							
21RAuPe16	0.837	0.905	1.000						
21RAuL17	0.600	0.718	0.732	1.000					
21RAuM04	0.267	0.345	0.323	0.214	1.000				
21RN06	0.091	0.000	0.000	0.000	0.200	1.000			
21RN07	0.000	0.095	0.000	0.000	0.200	0.000	1.000		
21RAu19	0.091	0.095	0.087	0.100	0.000	0.000	0.000	1.000	
21RAuW10	0.412	0.364	0.343	0.625	0.182	0.143	0.000	0.143	1.000
21RAuB11	0.231	0.160	0.148	0.167	0.143	0.333	0.000	0.333	0.444
21RAuB12	0.450	0.410	0.390	0.632	0.214	0.100	0.000	0.100	0.688
21RAuP08	0.250	0.323	0.303	0.333	0.200	0.000	0.000	0.000	0.333
21RAuPr20	0.375	0.387	0.424	0.333	0.200	0.000	0.000	0.000	0.167
21RJ01	0.486	0.444	0.421	0.343	0.320	0.118	0.118	0.000	0.345
21RAuW09	0.700	0.564	0.585	0.737	0.143	0.100	0.000	0.100	0.625

	21RAuB11	21RAuB12	21RAuP08	21RAuPr20	21RJ01	21RAuW09
21RAuB11	1.000					
21RAuB12	0.417	1.000				
21RAuP08	0.125	0.333	1.000			
21RAuPr20	0.000	0.267	0.636	1.000		
21RJ01	0.286	0.514	0.148	0.222	1.000	
21RAuW09	0.250	0.632	0.200	0.133	0.457	1.000

B) AG 2-2 isolates hybridised with the plasmid construct pRAG22-39

	22RJ02	22RJ01	22RN04	22RN06	22RN03	22RAuL11	22RAuL09	22RAuL12
22RJ02	1.000							
22RJ01	0.367	1.000						
22RN04	0.367	0.609	1.000					
22RN06	0.367	0.609	1.000	1.000				
22RN03	0.360	0.340	0.468	0.468	1.000			
22RAuL11	0.360	0.340	0.468	0.468	1.000	1.000		
22RAuL09	0.360	0.340	0.468	0.468	1.000	1.000	1.000	
22RAuL12	0.360	0.340	0.468	0.468	1.000	1.000	1.000	1.000

Appendix E

Similarity matrix for AG 2 isolates classified by combinations of zymogram analysis, PCR analysis and RFLP analysis. Pair wise comparisons between isolates were calculated by Nei and Li's similarity index (UPGMA cluster analysis) and Gower's similarity index (principal coordinates analysis), using the Multi Variate Statistical Package (MVSP, Kovach Computing Systems,).

CLUSTER ANALYSIS-AG 2 PCR+ZG
Similarity matrix

	21RAuC02	21RAuC15	21RAuPe16	21RAuM03	21RAuL17	21RAuM04	21RN06	21RN07	21RAu18	21RAu19
21RAuC02	1.000									
21RAuC15	0.857	1.000								
21RAuPe16	0.824	0.903	1.000							
21RAuM03	0.581	0.571	0.519	1.000						
21RAuL17	0.606	0.733	0.690	0.462	1.000					
21RAuM04	0.364	0.400	0.414	0.308	0.429	1.000				
21RN06	0.667	0.545	0.563	0.414	0.581	0.452	1.000			
21RN07	0.333	0.296	0.308	0.348	0.240	0.320	0.286	1.000		
21RAu18	0.414	0.462	0.480	0.364	0.333	0.417	0.370	0.190	1.000	
21RAu19	0.333	0.303	0.313	0.207	0.258	0.387	0.294	0.286	0.519	1.000
21RAuW10	0.571	0.500	0.516	0.500	0.667	0.400	0.545	0.296	0.308	0.303
21RAuB11	0.114	0.125	0.194	0.071	0.200	0.133	0.182	0.296	0.154	0.121
21RAuB12	0.278	0.242	0.250	0.138	0.258	0.194	0.294	0.214	0.222	0.176
21RAuP08	0.222	0.242	0.250	0.138	0.258	0.194	0.353	0.143	0.148	0.176
21RNzBr21	0.162	0.176	0.182	0.133	0.188	0.313	0.229	0.207	0.143	0.229
21RAuPr20	0.154	0.167	0.171	0.188	0.176	0.235	0.270	0.323	0.200	0.216
22RJ02	0.182	0.200	0.207	0.077	0.286	0.500	0.258	0.320	0.167	0.258
22RJ01	0.129	0.143	0.222	0.083	0.154	0.308	0.138	0.174	0.182	0.138
22RN06	0.162	0.235	0.121	0.200	0.125	0.250	0.114	0.276	0.143	0.171
22RN03	0.103	0.167	0.171	0.125	0.176	0.235	0.108	0.258	0.133	0.216
22RAuL09	0.100	0.108	0.167	0.182	0.114	0.171	0.158	0.250	0.194	0.211
22RAuS10	0.158	0.171	0.235	0.258	0.182	0.182	0.222	0.267	0.207	0.222
22RAuL11	0.049	0.053	0.054	0.059	0.111	0.167	0.103	0.121	0.063	0.154
22RAuL12	0.150	0.162	0.167	0.121	0.114	0.286	0.211	0.188	0.194	0.211
02RAu01	0.167	0.242	0.188	0.207	0.258	0.129	0.294	0.214	0.074	0.059
02RAuCf03	0.111	0.182	0.125	0.207	0.258	0.065	0.118	0.286	0.148	0.235
02RAuL04	0.143	0.160	0.167	0.095	0.087	0.348	0.154	0.100	0.211	0.231
2tRN01	0.278	0.242	0.250	0.138	0.194	0.387	0.412	0.357	0.370	0.529

	21RAuW10	21RAuB11	21RAuB12	21RAuP08	21RNzBr21	21RAuPr20	22RJ02	22RJ01	22RN06
21RAuW10	1.000								
21RAuB1	0.250	1.000							
21RAuB12	0.303	0.667	1.000						
21RAuP08	0.303	0.424	0.471	1.000					
21RNzBr21	0.235	0.471	0.571	0.743	1.000				
21RAuPr20	0.278	0.500	0.486	0.703	0.789	1.000			
22RJ02	0.333	0.333	0.323	0.323	0.375	0.353	1.000		
22RJ01	0.214	0.286	0.276	0.207	0.200	0.188	0.538	1.000	
22RN06	0.176	0.294	0.286	0.171	0.222	0.211	0.500	0.467	1.000
22RN03	0.167	0.278	0.216	0.216	0.316	0.300	0.412	0.375	0.579
22RAuL09	0.162	0.378	0.263	0.158	0.205	0.244	0.229	0.182	0.205
22RAuS10	0.229	0.457	0.278	0.111	0.216	0.256	0.242	0.194	0.216
22RAuL11	0.105	0.211	0.205	0.205	0.250	0.143	0.222	0.353	0.300
22RAuL12	0.162	0.162	0.263	0.158	0.256	0.195	0.286	0.303	0.359
02RAu01	0.303	0.303	0.353	0.294	0.229	0.270	0.194	0.276	0.343
02RAuCf03	0.242	0.303	0.176	0.118	0.114	0.324	0.258	0.138	0.457
02RAuL04	0.080	0.160	0.308	0.308	0.370	0.345	0.348	0.381	0.222
2tRN01	0.242	0.182	0.176	0.294	0.229	0.324	0.258	0.138	0.229

	22RN03	22RAuL09	22RAuS10	22RAuL11	22RAuL12	02RAu01	02RAuCf03	02RAuL04	2tRN01
22RN03	1.000								
22RAuL09	0.293	1.000							
22RAuS10	0.256	0.850	1.000						
22RAuL11	0.429	0.372	0.293	1.000					
22RAuL12	0.439	0.238	0.150	0.744	1.000				
02RAu01	0.270	0.158	0.222	0.308	0.316	1.000			
02RAuCf03	0.378	0.368	0.333	0.205	0.263	0.353	1.000		
02RAuL04	0.345	0.200	0.143	0.194	0.133	0.154	0.154	1.000	
2tRN01	0.216	0.211	0.222	0.154	0.263	0.118	0.176	0.308	1.000

PRINCIPAL COORDINATES ANALYSIS- AG2 PCR+ZG
Similarity matrix

	21RAuC02	21RAuC15	21RAuPe16	21RAuM03	21RAuL17	21RAuM04	21RN06	21RN07	21RAu18
21RAuC02	1.000								
21RAuC15	0.938	1.000							
21RAuPe16	0.926	0.963	1.000						
21RAuM03	0.840	0.852	0.840	1.000					
21RAuL17	0.840	0.901	0.889	0.827	1.000				
21RAuM04	0.741	0.778	0.790	0.778	0.802	1.000			
21RN06	0.852	0.815	0.827	0.790	0.840	0.790	1.000		
21RN07	0.753	0.765	0.778	0.815	0.765	0.790	0.753	1.000	
21RAu18	0.790	0.827	0.840	0.827	0.802	0.827	0.790	0.790	1.000
21RAu19	0.704	0.716	0.728	0.716	0.716	0.765	0.704	0.753	0.840
21RAuW10	0.815	0.802	0.815	0.827	0.877	0.778	0.815	0.765	0.778
21RAuB11	0.617	0.654	0.691	0.679	0.704	0.679	0.667	0.765	0.728
21RAuB12	0.679	0.691	0.704	0.691	0.716	0.691	0.704	0.728	0.741
21RAuP08	0.654	0.691	0.704	0.691	0.716	0.691	0.728	0.704	0.716
21RNzBr21	0.617	0.654	0.667	0.679	0.679	0.728	0.667	0.716	0.704
21RAuPr20	0.593	0.630	0.642	0.679	0.654	0.679	0.667	0.741	0.704
22RJ02	0.667	0.704	0.716	0.704	0.753	0.827	0.716	0.790	0.753
22RJ01	0.667	0.704	0.741	0.728	0.728	0.778	0.691	0.765	0.778
22RN06	0.617	0.679	0.642	0.704	0.654	0.704	0.617	0.741	0.704
22RN03	0.568	0.630	0.642	0.654	0.654	0.679	0.593	0.716	0.679
22RAuL09	0.556	0.593	0.630	0.667	0.617	0.642	0.605	0.704	0.691
22RAuS10	0.605	0.642	0.679	0.716	0.667	0.667	0.654	0.728	0.716
22RAuL11	0.519	0.556	0.568	0.605	0.605	0.630	0.568	0.642	0.630
22RAuL12	0.580	0.617	0.630	0.642	0.617	0.691	0.630	0.679	0.691
02RAu01	0.630	0.691	0.679	0.716	0.716	0.667	0.704	0.728	0.691
02RAuCf03	0.605	0.667	0.654	0.716	0.716	0.642	0.630	0.753	0.716
02RAuL04	0.704	0.741	0.753	0.765	0.741	0.815	0.728	0.778	0.815
2tRN01	0.679	0.691	0.704	0.691	0.691	0.765	0.753	0.778	0.790

	21RAu19	21RAuW10	21RAuB11	21RAuB12	21RAuP08	21RNzBr21	21RAuPr20	22RJ02	22RJ01	22RN06
21RAu19	1.000									
21RAuW10	0.716	1.000								
21RAuB11	0.642	0.704	1.000							
21RAuB12	0.654	0.716	0.864	1.000						
21RAuP08	0.654	0.716	0.765	0.778	1.000					
21RNzBr21	0.667	0.679	0.778	0.815	0.889	1.000				
21RAuPr20	0.642	0.679	0.778	0.765	0.864	0.901	1.000			
22RJ02	0.716	0.753	0.753	0.741	0.741	0.753	0.728	1.000		
22RJ01	0.691	0.728	0.753	0.741	0.716	0.704	0.679	0.852	1.000	
22RN06	0.642	0.654	0.704	0.691	0.642	0.654	0.630	0.802	0.802	1.000
22RN03	0.642	0.630	0.679	0.642	0.642	0.679	0.654	0.753	0.753	0.802
22RAuL09	0.630	0.617	0.716	0.654	0.605	0.617	0.617	0.667	0.667	0.617
22RAuS10	0.654	0.667	0.765	0.679	0.605	0.642	0.642	0.691	0.691	0.642
22RAuL11	0.593	0.580	0.630	0.617	0.617	0.630	0.556	0.654	0.728	0.654
22RAuL12	0.630	0.617	0.617	0.654	0.605	0.642	0.593	0.691	0.716	0.691
02RAu01	0.605	0.716	0.716	0.728	0.704	0.667	0.667	0.691	0.741	0.716
02RAuCf03	0.679	0.691	0.716	0.654	0.630	0.617	0.691	0.716	0.691	0.765
02RAuL04	0.753	0.716	0.741	0.778	0.778	0.790	0.765	0.815	0.840	0.741
2tRN01	0.802	0.691	0.667	0.654	0.704	0.667	0.691	0.716	0.691	0.667

	22RN03	22RAuL09	22RAuS10	22RAuL11	22RAuL12	02RAu01	02RAuCf03	02RAuL04	2tRN01
22RN03	1.000								
22RAuL09	0.642	1.000							
22RAuS10	0.642	0.926	1.000						
22RAuL11	0.704	0.667	0.642	1.000					
22RAuL12	0.716	0.605	0.580	0.864	1.000				
02RAu01	0.667	0.605	0.654	0.667	0.679	1.000			
02RAuCf03	0.716	0.704	0.704	0.617	0.654	0.728	1.000		
02RAuL04	0.765	0.704	0.704	0.691	0.679	0.728	0.728	1.000	
2tRN01	0.642	0.630	0.654	0.593	0.654	0.630	0.654	0.778	1.000

Eigenvalues

	Axis 1	Axis 2	Axis 3	Axis 4	Axis 5	Axis 6	Axis 7	Axis 8	Axis 9	Axis 10	Axis 11	Axis 12	Axis 13	Axis 14	Axis 15	Axis 16	Axis 17	Axis 18
Eigenvalues	1.418	0.935	0.753	0.609	0.553	0.418	0.353	0.321	0.277	0.265	0.245	0.207	0.198	0.197	0.178	0.158	0.150	0.131
Percentage	17.838	11.763	9.474	7.669	6.963	5.257	4.444	4.041	3.485	3.328	3.084	2.605	2.489	2.473	2.234	1.988	1.892	1.653
Cum. Percentage	17.838	29.601	39.075	46.744	53.707	58.964	63.408	67.449	70.934	74.263	77.347	79.952	82.441	84.914	87.148	89.137	91.029	92.682

	Axis 19	Axis 20	Axis 21	Axis 22	Axis 23	Axis 24	Axis 25	Axis 26	Axis 27
Eigenvalues	0.107	0.093	0.092	0.083	0.060	0.054	0.044	0.032	0.018
Percentage	1.348	1.175	1.152	1.043	0.749	0.675	0.556	0.397	0.225
Cum. Percentage	94.030	95.205	96.356	97.399	98.148	98.823	99.378	99.775	100.000

CLUSTER ANALYSIS- AG2-1 combined data
Similarity matrix

	21RAuC02	21RAuC15	21RAuPe16	21RAuL17	21RAuM04	21RN06	21RN07	21RAu19	21RAuW10	21RAuB11
21RAuC02	1.000									
21RAuC15	0.842	1.000								
21RAuPe16	0.831	0.904	1.000							
21RAuL17	0.603	0.725	0.714	1.000						
21RAuM04	0.317	0.373	0.367	0.321	1.000					
21RN06	0.448	0.333	0.327	0.353	0.390	1.000				
21RN07	0.192	0.208	0.163	0.133	0.286	0.267	1.000			
21RAu19	0.241	0.222	0.218	0.196	0.293	0.278	0.267	1.000		
21RAuW10	0.493	0.431	0.424	0.645	0.308	0.426	0.195	0.255	1.000	
21RAuB11	0.164	0.140	0.172	0.185	0.136	0.205	0.242	0.154	0.320	1.000
21RAuB12	0.368	0.333	0.329	0.464	0.203	0.222	0.125	0.148	0.492	0.561
21RAuP08	0.235	0.281	0.277	0.295	0.196	0.261	0.100	0.130	0.316	0.327
21RAuPr20	0.254	0.269	0.294	0.250	0.222	0.204	0.233	0.163	0.233	0.346

	21RAuB12	21RAuP08	21RAuPr20
21RAuB12	1.000		
21RAuP08	0.406	1.000	
21RAuPr20	0.388	0.678	1.000

PRINCIPAL COORDINATES ANALYSIS- AG2-1 combined data
Similarity matrix

	21RAuC02	21RAuC15	21RAuPe16	21RAuL17	21RAuM04	21RN06	21RN07	21RAu19	21RAuW10
21RAuC02	1.000								
21RAuC15	0.902	1.000							
21RAuPe16	0.893	0.943	1.000						
21RAuL17	0.762	0.844	0.836	1.000					
21RAuM04	0.648	0.697	0.689	0.689	1.000				
21RN06	0.738	0.705	0.697	0.730	0.795	1.000			
21RN07	0.656	0.689	0.664	0.680	0.795	0.820	1.000		
21RAu19	0.639	0.656	0.648	0.664	0.762	0.787	0.820	1.000	
21RAuW10	0.713	0.697	0.689	0.820	0.705	0.779	0.730	0.713	1.000
21RAuB11	0.582	0.598	0.607	0.639	0.689	0.746	0.795	0.730	0.721
21RAuB12	0.607	0.607	0.598	0.697	0.615	0.656	0.656	0.623	0.730
21RAuP08	0.574	0.623	0.615	0.648	0.664	0.721	0.705	0.672	0.680
21RAuPr20	0.566	0.598	0.607	0.607	0.656	0.680	0.730	0.664	0.623

	21RAuB11	21RAuB12	21RAuP08	21RAuPr20
21RAuB11	1.000			
21RAuB12	0.795	1.000		
21RAuP08	0.730	0.689	1.000	
21RAuPr20	0.721	0.664	0.844	1.000

Eigenvalues

	Axis 1	Axis 2	Axis 3	Axis 4	Axis 5	Axis 6	Axis 7	Axis 8	Axis 9	Axis 10	Axis 11	Axis 12	Axis 13
Eigenvalues	1.033	0.562	0.482	0.310	0.247	0.224	0.191	0.161	0.144	0.127	0.059	0.051	0.000
Percentage	28.776	15.648	13.417	8.639	6.875	6.247	5.332	4.485	4.011	3.526	1.633	1.412	0.000
Cum. Percentage	28.776	44.424	57.841	66.480	73.354	79.601	84.933	89.418	93.429	96.955	98.588	100.000	100.000

CLUSTER ANALYSIS- AG 2-2 combined data
Similarity matrix

	22RJ02	22RJ01	22RN06	22RN03	22RAuL11	22RAuL09	22RAuL12
22RJ02	1.000						
22RJ01	0.427	1.000					
22RN06	0.420	0.553	1.000				
22RN03	0.350	0.373	0.543	1.000			
22RAuL11	0.302	0.346	0.391	0.674	1.000		
22RAuL09	0.306	0.275	0.349	0.612	0.703	1.000	
22RAuL12	0.329	0.325	0.419	0.682	0.879	0.644	1.000

PRINCIPAL COORDINATES ANALYSIS- AG 2-2 combined data
Similarity matrix

	22RJ02	22RJ01	22RN06	22RN03	22RAuL11	22RAuL09	22RAuL12
22RJ02	1.000						
22RJ01	0.674	1.000					
22RN06	0.644	0.742	1.000				
22RN03	0.606	0.644	0.720	1.000			
22RAuL11	0.545	0.598	0.598	0.788	1.000		
22RAuL09	0.553	0.561	0.576	0.750	0.795	1.000	
22RAuL12	0.568	0.591	0.621	0.795	0.917	0.758	1.000

Eigenvalues

	Axis 1	Axis 2	Axis 3	Axis 4	Axis 5	Axis 6	Axis 7
Eigenvalues	0.790	0.383	0.279	0.267	0.190	0.078	0.000
Percentage	39.746	19.292	14.047	13.455	9.541	3.919	0.000
Cum. Percentage	39.746	59.038	73.085	86.540	96.081	100.000	100.000