

**Genetic Variation of *Rhizoctonia solani* AG-3 in
South Australia**

by

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To my wife and my dear sons Arsalan and Ardalan

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Abstract

Rhizoctonia solani is a complex species comprising morphologically similar basidiomycetous imperfect fungi. Its broad host range and the lack of diagnostic characters create difficulties in the taxonomy and in the study of population genetics of this fungus. The aim of this study was to determine the level of genetic diversity within *R. solani*^{AG-3} causing rhizoctonia disease of potato in the major potato growing areas of South Australia. For this purpose, pectic zymogram, PCR, DNA fingerprinting and RFLP techniques were used in conjunction with traditional plant pathology procedures.

Isolates of *Rhizoctonia* collected from the stems, roots, tuber-borne sclerotia and soil of potato crops in Virginia and Lenswood, South Australia, were identified on the basis of the number of nuclei per cell and the anastomosis reaction. AG-3 was most commonly associated with rhizoctonia disease, with AG2-1, AG-4 and AG-5 also present. In glasshouse tests, both AG-3 and AG-5 isolates caused black scurf and stem cankers, although symptoms of black scurf were more severe with AG-3. AG-4 isolates produced severe stem and stolon cankers. This is the first report of AG-4 and AG-5 isolates causing disease in potato crops in South Australia. AG-8 isolates recovered from diseased barley and wheat also caused disease on potato plants. Rhizoctonia disease in potato crops in South Australia appears to be caused by a combination of anastomosis groups.

Intraspecific variation among *R. solani* AG-3 isolates was examined by pectic zymogram analyses. Three proposed new zymogram sub-groups, ZG7-1, ZG7-2 and ZG7-3, were identified within AG-3. Two loci, one coding for pectin esterase (monomorphic) and the other coding for polygalacturonase (polymorphic) were observed for all isolates. Isolates originating from morphologically different

sclerotia were assigned to different zymogram sub-groups. The majority of the isolates were ZG7-1 and it was the most common ZG in the areas sampled. ZG7-1 appeared to be a canker pathotype, whereas ZG7-2 and ZG7-3 were identified as black scurf pathotypes. RAPD-PCR fingerprint analysis provided additional evidence for sub-group identification. In a comparison of PCR profiles, polymorphisms were detected between and within the zymogram sub-groups, and some bands were group-specific.

Variation in *R. solani* AG-3 was studied using RFLP analysis. A highly repeated AG-3 specific DNA fingerprinting probe, pR3718, originating from isolate R37 (AG-3) was obtained. This probe showed little or no homology with DNA of isolates from other anastomosis groups. Another probe, pR4918, originating from the AG-4 isolate R49, showed homology to DNA from AG1-1A, AG1-1B, AG2-1, AG2-2, AG-3, AG-4, AG-5, AG-6, AG-7, AG-8, AG-9 and AG-BI isolates and detected polymorphisms within and between anastomosis groups.

Genetic relationships of AG-3 isolates, based on RFLP, PCR and pectic zymogram data, were determined by constructing dendrograms using the unweighted pair-group method with arithmetic average (UPGMA). There was good agreement, with respect to relationships between zymogram sub-groups, between these three approaches. The results also showed that AG-3 is not a homogenous taxon and that the proportion of genetic variation between the AG-3 zymogram sub-groups is higher than within the sub-groups.

The AG-3 specific multi-locus probe, pR3718, showed that the *R. solani* AG-3 isolates were highly variable and genetic diversity, either within or between zymogram sub-groups, was more precisely resolved with this probe. This AG-3 specific probe has the potential to be used as a diagnostic tool for disease and as a sensitive means of studying ecology, epidemiology and genetic diversity of the plant pathogen.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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.....DATE: 15/1/96.....

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Chapter 1

Introduction and Review of Literature

1.1. INTRODUCTION

The potato is Australia's largest horticulture crop in terms of value and volume of production. Potato growing is concentrated in the high rainfall areas and in some irrigated farms along the Murray River system (Federson, 1973). In South Australia it is the most economically important vegetable crop (Lomman, 1988; Dillard *et al.*, 1993). Most of the potato production in this State is in three main regions: the Adelaide Plains, the Adelaide Hills and the South East (Lomman, 1988). The Adelaide Hills and the South East are the main regions producing processing potatoes and potatoes from other areas are for fresh-washed markets (Lomman, 1988). Due to the suitable climatic conditions, potatoes can be grown almost all year round in South Australia, and two crops per year are generally grown. The main crop is planted in the Adelaide Hills and the South East from October to December, for harvest from February to May. In addition, early crops are planted in the Adelaide plains (metropolitan areas, Virginia, Angle Vale, Gawler River and Salisbury) from June until September, for harvest about December until February (Federson, 1973; Lomman, 1988).

In 1992 and 1993, of the total of 10,635 hectares under cultivated vegetables in South Australia, 2,971 hectares and 3,305 hectares were devoted to early and main potato crops, respectively (Gardner, 1995). Most of the varieties grown in Australia are of American origin, with European potato cultivars less common (Federson, 1971).

- Black scurf or rhizoctonia disease, caused by *Rhizoctonia solani* Kühn, is the most important fungal disease in potato crops worldwide. *R. solani* was first observed on potato tubers by Kühn in 1858. Black to brown sclerotia on the surface of tubers and sunken necrotic lesions on roots, stems and underground parts of potato plants, are the most common symptoms of the disease (Carling & Leiner, 1986; Frank, 1981). This disease causes a reduction in tuber size and yield (Carling & Leiner, 1986; Dillard *et al.*, 1993; Leach & Webb, 1993). Isolates of *R. solani* are generally assigned to anastomosis groups based on hyphal fusion *in vitro*. Historically, isolates of *R. solani* associated with black scurf disease have been characterised as anastomosis group 3 (AG-3) (Anderson, 1982; Carling & Leiner, 1986). Other anastomosis groups have also been isolated from potato, but largely due to difficulties in identifying anastomosis groups, relatively little is known about pathogenicity, epidemiology and ecology of *R. solani* on this host in Australia. In the remainder of this chapter, the literature on *R. solani* is reviewed, with emphasis on pathology and taxonomy.

1. 2. BIOLOGY OF RHIZOCTONIA DISEASE ON POTATO

The rhizoctonia disease of potatoes occurs world-wide wherever potatoes are grown. *R. solani* overwinters as sclerotia on potato tubers and in soil or as mycelium on plant debris. The perfect stage of the pathogen has been observed on the stems near the soil surface without causing disease (Frank, 1981; Hill & Anderson, 1989). High moisture levels in soils and low temperature (about 18°C) are favourable environmental conditions for disease development (Frank, 1981; Banville, 1989). Potato plants in the fields become infected by either tuber-borne sclerotia or soil-borne inoculum. Sclerotia germinate in favourable conditions and invade potato stems or sprouts (Frank, 1981). Penetration of plants may occur either through the intact epidermal tissues, natural openings or through wounds (Dodman & Flentje, 1970).

1. 3. ECOLOGY AND HOST RANGE

Ecologically, *R. solani* is diverse; it may be pathogenic on many hosts, saprophytic in soil, or form mycorrhizal associations with orchidaceous plants (Ogoshi, 1987). *R. solani* consists of a wide range of pathogenic strains, some of which are specific to only one host species while others attack a wide range of hosts. Some of the anastomosis groups, such as AG-1, AG-2, AG-3 and AG-4, have been reported in all areas of the world, while other anastomosis groups are less prevalent (Ogoshi, 1987) and their distribution has not been thoroughly investigated.

Ogoshi & Ui (1983) concluded that cultivated crops have a strong influence on the prevalence of a given anastomosis group in certain areas. Isolates of AG-1 have been recovered from species of the *Leguminosae* and *Graminae*, AG2-1 from members of the *Cruciferae*, whereas AG2-2 isolates have been isolated from the *Chenopodiaceae*, mainly sugar beet. Most isolates of AG-3 have been recovered from the *Solanaceae*, while AG-4 isolates are associated with the *Chenopodiaceae*, *Leguminosae* and *Solanaceae*, and those of AG-5 with the *Leguminosae* and from soils (Ogoshi, 1987). AG-6 and AG-7 are considered as non-pathogenic and are usually isolated from soils (Sneh *et al.*, 1991). AG-8 isolates are pathogenic on cereals (Neate & Warcup, 1985) and lupins (Sweetingham, 1990). AG-9 isolates are weak pathogens on the *Cruciferae* and *Solanaceae*, including potato (Carling *et al.*, 1987), and non-pathogenic AG-10 has been isolated from barley (Ogoshi *et al.*, 1990). See Addendum 1

1. 4 THE ROLE OF INOCULUM IN DISEASE CAUSED BY *R. SOLANI*

Disease incidence and disease severity are directly related to inoculum potential (Baker & Martinson, 1970). The population of *R. solani* in soil in many areas is higher than that of many other soil pathogens (Takahashi & Kawase, 1964).

Inoculum of *R. solani* consists of hyphae, sclerotia, and also basidiospores in some instances.

Hyphae of *R. solani* have been observed on the surface of, and within, plant debris from soil, providing a significant source of natural inoculum (Boosalis & Scharen, 1959; Neate, 1987). The fungus can grow rapidly in suitable conditions; Blair (1943) estimated growth rate to be more than 1 cm per day in soil. He also found that *R. solani* in natural soil grows more horizontally than vertically thus colonisation is greater in a lateral direction.

Sclerotia are frequently found on the surface of potato tubers and plant debris (Allison, 1951). It has been thought that inoculum of *R. solani* originated from the soil and infected the underground parts of plants, but inoculum may also be carried on the seed coat, the endosperm or the embryo of weed plants and some crops (Sanford, 1952; Baker & Martinson, 1970). Some infected weeds and rotation crops may act as sources of inoculum (Boosalis & Scharen, 1960). Basidiospores have been observed on a wide range of hosts such as wheat, potato, tomato, parsnip, cotton, bean and flax. Therefore basidiospores might be important inoculum in the field (Baker & Martinson, 1970).

Pitt (1964a) showed evidence that units of inoculum of *R. solani* store significant energy. Sclerotia have high energy resources and it has been observed that one sclerotium produced 80 germ tubes (Boosalis & Scharen, 1959) and that some sclerotia survived even after 25 successive germinations (Shurtleff, 1953).

The propagules of *R. solani* can survive in the soil, and maintain inoculum potential, in the absence of a specific host (Baker & Martinson, 1970). Pitt (1964b) reported that dry sclerotia can survive in an inactive state for more than 2 years in pure culture. It has been reported that stock cultures in dry soil have remained viable when stored at room temperature for 6 years (Baker & Martinson, 1970).

Newton (1931) mentioned that longevity of sclerotia is decreased by high temperature or moist conditions during storage.

The incidence of rhizoctonia disease in some crops, following a susceptible crop in the rotation, could probably be related to the presence of inoculum remaining in the soil from the previous crop. Sclerotia originating from potato tubers and stolons have been observed to produce sharp-eyespot disease on wheat (Pitt, 1964b). Therefore, active and inactive mechanisms are both involved in survival of *R. solani*.

1.5. GENERAL CONCEPTS OF THE GENUS *RHIZOCTONIA*

The genus *Rhizoctonia* was described by de Candolle in 1815 as a non-sporulating root pathogen (Parmeter, 1970). *Rhizoctonia* contains heterogenous species comprising morphologically similar basidiomycetous imperfect fungi. On the basis of sexual reproduction, the majority of *Rhizoctonia* isolates found in agricultural soils can be divided into one of three Basidiomycete groups: one is the multinucleate *Rhizoctonia*, containing three or more nuclei per cell, with hyphae 6-10 μm in diameter, and a teleomorph in the genus *Thanatephorus* Donk. The second is the binucleate *Rhizoctonia*, hyphae 4-7 μm in diameter, containing two nuclei per cell (rarely one or three), and a teleomorph, where known, in the genus *Ceratobasidium* Rogers. The third includes *R. oryzae* and *R. zaeae*, multinucleate isolates with rosy or brownish cylindrical sclerotia with a teleomorph in the genus *Waitea* Warcup & Talbot (Ogoshi, 1987). Distinguishing between species of *Rhizoctonia* is difficult because of the lack of reliable morphological characters. Unfortunately, the teleomorph, which is the best distinguishing characteristic for identifying *Rhizoctonia* taxa, is difficult to induce (Mordue *et al.*, 1989).

Thanatephorus cucumeris (Frank) Donk is the teleomorph of *Rhizoctonia solani* Kühn. The teleomorph was first described by Frank in 1883, as *Hypochnus*

cucumeris Frank (Talbot, 1970). The species belongs to the family *Ceratobasidiaceae*, order *Tulasnellales*, class *Holobasidiomycetes* and sub-division Basidiomycotina (Talbot, 1970).

1. 6. CHARACTERISTICS OF *R. SOLANI*

Isolates of *R. solani* are widespread throughout the world, with considerable variation in morphological, biochemical, cultural and pathogenic characteristics. Isolates of *R. solani* are similar with respect to hyphal morphology (Flentje *et al.*, 1970). Mycelia of *R. solani* are aerial on the surface of culture media, such as potato dextrose agar (PDA), and possess thick walled multinucleate cells. Branching of hyphae occurs at right angles near the distal septum of the cells. The hyphae are also typified by constriction of the branch base, formation of a septum in the branch near the point of origin of that branch and a prominent septal pore apparatus (Butler & Bracker, 1970). Clamp connections, conidia and rhizomorphs have never been observed in *R. solani* (Parmeter & Whitney, 1970). The diameter of vegetative hyphae ranges from approximately 3 to 17 μm , with most between 8 and 12 μm (Parmeter, 1965). Many isolates which were shown to be different in cultural and pathogenic characters, had a common perfect state (*T. cucumeris* (Frank) Donk) (Flentje, 1956; Whitney & Parmeter, 1963; Papavizas, 1965). These isolates of *R. solani* were, therefore, considered to comprise a collective species. Single basidiospore-derived isolates of *T. cucumeris* showed variation in cultural morphology, anastomosis and other characteristics (Parmeter, 1970). The vegetative cells of *R. solani* usually contain 6-18 haploid nuclei (Flentje *et al.*, 1963). These nuclei divide simultaneously and segregate evenly with half of the daughter nuclei moving into the new cell. The nuclei in the vegetative cells pair and are separated by septa. The binucleate cells form basidia wherein the nuclei fuse to become diploid and then undergo meiosis to form four haploid nuclei. Each of the haploid nuclei forms a basidiospore, or two nuclei may move into one spore. In

addition to recombination, irregularities in the division and segregation of daughter nuclei may contribute to variation (Flentje *et al.*, 1970). Mechanisms of variation may also be governed by the factors discussed in the following sections of this review.

1. 6. 1. Heterokaryosis

The study of variability of single basidiospore-derived progeny has proved that field isolates of certain anastomosis groups are heterokaryotic (Whitney & Parmeter, 1963; Garza-Chapa & Anderson, 1966; Anderson *et al.*, 1972; Yang *et al.*, 1992). Heterokaryosis may occur through binucleate spores, mutation or by anastomosis (Flentje *et al.*, 1970). It has been shown that a percentage of basidiospores in some isolates of *R. solani* are binucleate (Flentje & Stretton, 1964) and there is evidence that two genetically different nuclei may move into one basidiospore (McKenzie, 1966). Flentje *et al.* (1967) showed that mutation occurred in homokaryotic single basidiospore-derived cultures of *R. solani* and that the resulting mutants were heterokaryotic. Heterokaryon formation by anastomosing single basidiospore-derived cultures was demonstrated by Garza-Chapa & Anderson (1966).

Heterokaryon formation in AG-1 and AG-4 isolates is controlled by a nuclear gene locus, the H-factor, with multiple alleles (Anderson *et al.*, 1972); two homokaryons containing different H factors can produce heterokaryons. Puhalla & Carter (1976) concluded that H-factors in AG-4 isolates are not involved in hyphal fusion but they do control sexual compatibility, nuclear pairing and the stability of the heterokaryon. Heterokaryons were obtained when single basidiospore-derived, homokaryotic cultures of isolates with different H-factors were paired on culture media. The resultant heterokaryon contained both H-factors and was morphologically different from the paired homokaryons (Whitney & Parmeter,

1963; Vest & Anderson, 1968; Anderson, 1982). Although natural isolates have not been identified to be homokaryotic (Anderson, 1982; Adams, 1988), heterokaryosis in AG-3 has not been confirmed and information on AG-5, AG-6, AG-7, AG-8, AG-9 AG-10 and AG-BI is not available (Yang, 1993).

1. 6. 2. Pathogenicity and heterokaryosis

The pathogenicity of *R. solani* has been investigated using synthesised heterokaryons and single basidiospore progenies. Vest & Anderson (1968) found that synthesised heterokaryons of AG1-1 derived from pairing of high and low virulence homokaryons were as virulent as the single basidiospore-derived parent of highest virulence on flax. Also, heterokaryons from low virulence homokaryons were much more virulent than either parent. They concluded that the virulence controlling factor seems to be dominant and a number of genes are involved, with dominance or epistatic effects. It has been reported that heterokaryons of AG1-1 resulting from two non-pathogenic homokaryons or from one virulent and one avirulent homokaryon were all highly pathogenic on flax hypocotyls (Anderson & Stretton, 1978).

Synthesised heterokaryons of AG-4 were obtained through hyphal anastomosis by pairing homokaryons (Phillips, 1990). In most of the combinations, synthesised heterokaryons were much more virulent on the host than were the least virulent homokaryons from which they were synthesised.

1. 6. 3. The sexual stage in *R. solani*

R. solani has been reported to be homothallic, based on observation of self-fertile single basidiospore-derived progenies, however, it has been suggested that some isolates tend to be heterothallic (Flentje & Stretton, 1964). In heterokaryotic isolates, unlike nuclei may fuse and form diploid recombinant progenies (Flentje *et*

al., 1970). Whitney & Parmeter (1963) demonstrated that some homothallic isolates were able to form heterokaryons when fused with other homokaryons. In some heterokaryotic isolates, unlike or like nuclei may migrate into one spore. Consequently, the basidiospores forming a heterokaryon will contain identical or recombinant nuclei and a spore may contain two unlike nuclei (McKenzie, 1966).

It has been suggested that sexual recombination may play a role in producing variation in *R. solani in vitro* (Flentje *et al.*, 1970). Papavizas & Ayers (1965) suggested that single basidiospore-derived progeny can be different from parental isolates in virulence. There is no evidence of parasexuality in *R. solani* (Flentje *et al.*, 1970).

1.7. TAXONOMY OF *R. SOLANI*

Lack of diagnostic characters creates difficulties in the taxonomy and genetic studies of populations of *R. solani*. The identification of *R. solani* has been attempted by several researchers using different techniques. Early research workers recognised intra-specific groups within *R. solani* based on culture characters, pathogenic behaviour and ecological criteria (Vilgalys & Gonzales, 1990). Isolates of *R. solani* are divided into eighteen groups and sub-groups based on anastomosis reaction (Parmeter *et al.*, 1969; Anderson, 1982; Ogoshi, 1987; Ogoshi *et al.*, 1990). Biochemical techniques, such as antigen analysis (Adams & Butler, 1979), soluble protein electrophoresis and isoenzyme analysis (Reynolds *et al.*, 1983; Mordue *et al.*, 1989), monoclonal and polyclonal antibodies (Matthew & Brooker, 1991) and pectic zymograms (Sweetingham *et al.*, 1986; Neate *et al.*, 1988) have been used to classify and study genetic diversity of the fungus. More recently, molecular techniques such as DNA-DNA hybridisation (Kuninaga & Yokosawa, 1984a & 1984b; Vilgalys, 1988; Carling & Kuninaga, 1990), restriction fragment length polymorphisms (Vilgalys & Gonzales, 1990; Jabaji-Hare *et al.*, 1990) and random

amplified polymorphic DNA (Duncan *et al.*, 1993) have been used to study genetic variation and population genetics of *R. solani*.

1. 7. 1. Anastomosis groups and sub-groups of *R. solani*

Schultz (1937) first divided *R. solani* into groups according to hyphal anastomosis (i.e. physiological behaviour). Anastomosis is determined by opposing isolates on a slide coated with 2% water agar or on cellophane overlaid on 2% water agar in Petri dishes (Parmeter *et al.*, 1969). Two discs containing mycelia of the isolates to be paired are cut from the margins of young PDA cultures and placed 2 cm apart on the agar-coated slide or the Petri dishes. Mycelia grow and overlap and then the intersects of advanced mycelia can be checked for fusion. If hyphal fusion occurs between two isolates they belong to the same anastomosis group (AG), otherwise they are in different anastomosis groups. Usually, when fusion occurs a few cells in opposing isolates show a killing reaction, as described by Flentje *et al.* (1967). Anastomosis group determination is time consuming (Neate *et al.*, 1988) and the use of morphological characters, and anastomosis grouping are of limited value in studying genetic variability of *R. solani*.

Carling *et al.* (1988) have illustrated and interpreted four categories of hyphal fusion. In "category 0" hyphal strands from different anastomosis groups make contact with one another but there is no reaction. In "Category 1", hyphal contact occurs, hyphal attachment is apparent, and no membrane fusion occurs. Some isolates from the same or different anastomosis groups react toward one another in this way, indicating a distant relationship. In "category 2", wall fusion is obvious, membrane fusion is probable and anastomosing cells frequently die. Different clones within same anastomosis groups interact in this way. In "category 3" wall and membrane of anastomosing cells fuse, and anastomosing cells

frequently remain alive. This reaction occurs between isolates belonging to the same anastomosis groups, the same clones and between isolates paired with self.

It is believed that the anastomosis reaction is governed by a vegetative incompatibility system within an anastomosis group (Anderson, 1982). Vegetative incompatibility in fungi involves one of two recognition systems. In a heterogenic system, fusion of hyphae occurs when alleles are identical at governing loci, whereas in a homogenic system, isolates from the same species or subspecies have different alleles at governing loci (Adams, 1988). Different anastomosis groups do not interact sexually and usually anastomosis does not occur between groups as stated above. AG-BI, however, is able to anastomose frequently with AG-2-1, AG-2-2, AG-3, AG-4 and AG-8 (Adams, 1988). It has been suggested that secretion of one or more attracting substances is involved in fusion (Ogoshi, 1987).

The ability to anastomose is considered to be the most useful grouping method for *R. solani*. However, the behaviour of the fungus cannot be understood only in terms of anastomosis groups. Therefore, studies on vitamin requirements, serological relationships, isoenzyme electrophoresis, DNA base composition (guanine plus cytosine content), DNA base and sequence homology have been undertaken in attempts to elucidate the biological basis of anastomosis and pathogenicity of *R. solani* (Ogoshi, 1987). On the basis of these criteria, further sub-groups have been identified among isolates of the same anastomosis group.

In addition, sub-groups are identified according to host specificity. AG-1 was divided into three subgroups; AG-1-1A (rice-infecting isolates), AG-1-1B (attacking sugar beet) and AG-1-1C (causing disease of buckwheat) (Ogoshi, 1987). AG-2 was classified into AG-2-1 and AG-2-2 based on frequency of hyphal anastomosis, and AG-2-2 was further divided into AG-2-2IIIB and AG-2-2IV based on host specificity (Ogoshi, 1987). Intra-groups have been recognised in AG-4 (AG-4 GH-1, AG-4 GH-II) and AG-6 (AG-6 HG-1, AG-6 GV) on the basis of DNA

homology (Sneh *et al.*, 1991). AG-9TP and AG-9TX were derived from AG-9 based on thiamine requirement (Carling & Kuninaga, 1990).

Isolates of AG-1 (Carling & Leiner, 1986), AG-2-1 and AG-2-2 (Chand & Logan, 1983), AG-3 (Carling & Leiner, 1986), AG-4 (Burpee & Goulty, 1984), AG-5 (Abe & Tsuboki, 1978; Weinhold, 1977) and AG-9 (Carling *et al.*, 1987) have been recovered from potato plants. Isolates of AG-3 (Carling & Leiner, 1986), AG-4 (Burpee & Goulty, 1984) and AG-5 (Abe & Tsuboki, 1978) have been more frequently recovered from potato crops affected by black scurf or black canker, with AG-3 being the most common (Gudmestad *et al.*, 1989). These groups are discussed in more detailed in the following sections.

1. 7. 1. 1. Anastomosis group 3 (AG-3)

AG-3 isolates cause 'black scurf' disease of potatoes (Carling & Leiner, 1986; Bandy *et al.*, 1984; Anguiz & Martin, 1989) and can also cause tomato leaf blight (Date *et al.*, 1984), tobacco leaf spot (Meyer *et al.*, 1990; Stevens Johnk *et al.*, 1993) and egg plant brown spot (Kodama *et al.*, 1982). In addition, the fungus has been recovered from diseased sugar beet seedlings (Sneh *et al.*, 1991). This group is defined as the "potato type" and consists of slow growing isolates (Sneh *et al.*, 1991). ^{Watanabe & Matsuda, 1966, cited in} AG-3 isolates are thiamine autotrophic with high DNA base sequence homology among isolates and are able to anastomose with members of AG-3, AG-8 and AG-BI (Sneh *et al.*, 1991).

AG-3 has been known as a genetically homogenous group (Anderson, 1982; Vilgalys & Gonzales, 1990), but Jabaji-Hare *et al.* (1990) indicated that intra-group variation occurs within AG-3 isolates originating from Canada, USA, Britain and Japan. Heterogeneity has also been demonstrated in AG-3 isolates from South Australia and Tasmania, using random amplified polymorphic DNA (Duncan *et al.*, 1993). However, the cause of this heterogeneity has not been determined.

1. 7. 1. 2. Anastomosis group 4 (AG-4)

AG-4 isolates are commonly recovered from soil or diseased plants, and cause tomato fruit rot (Lewis & Papavizas, 1985), foot rot of spinach, stem rot of pea, stem canker of potato (Sneh *et al.*, 1991) and damping-off on a wide range of vegetable crops (Anderson, 1982). AG-4 consists of thiamine autotrophic isolates and is subdivided into groups according to sclerotial form and DNA base sequence homology. AG-4 HG-I is recognised by producing dark brown sclerotia on PDA and having 89 - 93% DNA base sequence homology, whereas AG-4 HG-II is typified by whitish-brown sclerotia and high DNA base sequence homology (93-100%) among isolates, and low homology (31-42%) with members of AG-4 HG-I (Kuninaga & Yokosawa, 1984a).

1. 7. 1. 3. Anastomosis group 5 (AG-5)

AG-5 isolates have been reported to cause potato 'black scurf' and it has been found that 3% of the isolates of *R. solani* recovered in Japan from potato plants and sclerotia were members of AG-5 (Abe & Tsuboki, 1978). Also, 18% of the isolates of *R. solani* collected from potato fields in Maine, USA, were recognised as AG-5 (Bandy *et al.*, 1984). However, AG-5 isolates have been recovered from turf grass (Martin & Lucas, 1984), bean and soybean also, and some isolates form mycorrhizal associations with orchids (Sneh *et al.*, 1991). In general, this group is reported to be weakly pathogenic. AG-5 isolates are thiamine auxotrophic with a high DNA homology of 94 - 98% within the group (Kuninaga & Yokosawa, 1983).

1. 8. PECTIC ZYMOGRAMS IN FUNGAL CLASSIFICATION

A polyacrylamide gel electrophoretic method applicable to study of pectic enzymes was developed by Cruickshank & Wade (1980) for species of *Monilinia*,

Botrytis, *Penicillium*, *Aspergillus* and *Sclerotinia* (Cruickshank, 1983a). Cruickshank & Wade (1980) used polyacrylamide gel containing pectin as the substrate and subsequent staining with ruthenium red to detect activity of isozymes of polygalacturonase, pectin esterase and pectin lyase, simultaneously. This was possible due to the differing staining reactions and independent action of the enzymes on the same substrate. This technique has been recommended for following enzyme production in culture, determining thermal tolerance of pectin enzymes and for studying fungal enzymes in relation to fungal taxonomy (Cruickshank & Wade, 1980).

Pectic zymograms have been used for identifying species of fungi. Cruickshank (1983a) showed that extracellular pectic isozymes provide a simple, fast and reliable method for identifying *Sclerotinia* species. Pectic zymograms have also been used successfully for identifying species of *Botrytis* (Cruickshank, 1983b) and *Penicillium* (Cruickshank & Pitt, 1987).

Sweetingham *et al.* (1986) described 11 distinct zymogram groups in the *Ceratobasidiaceae*, designated ZG for multinucleate isolates of *Thanatephorus*, CZG for binucleate isolates of *Ceratobasidium* and WZG for multinucleate isolates of *Waitea*. They also found that isolates within a zymogram group had a similar cultural morphology and pathogenicity. The technique was used by other workers (Neate & Cruickshank, 1988; Neate *et al.*, 1988; Cruickshank, 1990; MacNish & Sweetingham, 1993) to identify species and pathogenic strains of *Rhizoctonia*.

Neate *et al.* (1988) produced zymogram groups for isolates of *R. solani* from different hosts and localities in South Australia. They described new zymogram groups of ZG6 (AG-2-1), ZG7 (AG-3) and ZG8 (AG-4) in addition to the zymogram groups reported by Sweetingham *et al.* (1986). They also sub-divided the ZG1 (AG-8) (Sweetingham *et al.*, 1986) into three subgroups; ZG1-1, ZG1-2 and ZG1-3 based on zymogram patterns. These sub-groups were not distinguishable by anastomosis reaction. These findings were confirmed by

MacNish & Sweetingham (1993), furthermore, they introduced more subgroups for AG-8. They have identified five zymogram groups, ZG1-1 to ZG1-5, within *R. solani* AG-8, the cause of rhizoctonia bare patch disease of cereals, legumes and pasture and they provided evidence that these five zymogram groups are very stable. Neate & Cruickshank (1988) have also investigated isolates of binucleate *Rhizoctonia* spp. in South Australia. They recovered five zymogram groups, CZG-1 to CZG-5, previously described in Western Australia (Sweetingham *et al.*, 1986).

Yang (1993) attempted to characterise field isolates of *R. solani* AG-8 obtained from three bare patches and to investigate the genetic background of the pectic enzymes by comparing the asexual homokaryon and the sexual progenies derived from field isolates. He found that all the field isolates tested produced the same zymogram pattern. Asexual sibling homokaryons and single basidiospore-derived progenies of a field isolate segregated widely for pectic phenotypes. Yang (1993) concluded that expression of pectic enzymes involved multiple genetic factors. However, it is not known whether or not field isolates of other anastomosis groups, including AG-3, contain genetically isolated zymogram groups.

1. 9. MOLECULAR TAXONOMY OF PLANT PATHOGENIC FUNGI

In spite of their importance, little is known about evolutionary relationships within fungi. Simple morphology, lack of fossil records and diversity have been major obstacles in fungal taxonomy (Bruns *et al.*, 1991). Common techniques for identifying taxa are based on morphology and sexual reproduction. Such methods are not applicable to fungi which are closely related and to fungi that do not reproduce sexually (Klich & Mullaney, 1987). Therefore, data at the molecular level could be valuable to discriminate between morphologically similar fungi and to assess taxonomic relationships. Recently, there has been progress in the

molecular tools available to address biological questions. The elucidation of evolutionary relationships among the major groups of fungi and delineation of species concepts are the most widespread applications of molecular techniques in classification of fungi (Klich & Mullaney, 1987).

In the next section the literature on the application of molecular biology to characterise plant pathogenic fungi is reviewed, with emphasis on *R. solani*.

1.9.1. DNA-DNA Hybridisation

Hybridisation of denatured DNA from one isolate to denatured DNA from another provides a measure of the relatedness of the two genomes. DNA nucleotide sequence homology, DNA complementarity and DNA reassociation are related to this technique (Klich & Mullaney, 1987), which was one of the first molecular approaches in classification of organisms (Hoyer *et al.*, 1964). Kurtzman (1985) first used DNA-DNA hybridisation to study relationships in fungi.

Different methods have been used to assess DNA relatedness, using radioisotopes and measuring the level of radioactivity in the reassociated DNA, and spectrometrically measuring the extent of renaturation between two DNAs as the temperature was decreased, as indicators of the level of complementarity (Dutta & Ojha, 1972; Ojha *et al.*, 1975; Dutta, 1976; Ellis, 1985; Vilgalys, 1988).

The complementarity between *Rhizopus arrhizus* and *Rhizopus oryzae*, which are difficult to distinguish morphologically, was examined by Ellis (1985). He found that *R. oryzae* X *R. arrhizus* reassociations were all more than 95% and concluded that the two were conspecific. Also, reassociation experiments on *Fusarium* spp. confirmed that the presence or absence of chlamydospores was a reliable character for distinguishing among species (Ellis, 1988).

DNA-DNA reassociation has been studied in the following anastomosis groups of *R. solani*; AG-1, AG-2, AG-3, AG-4, AG-5, AG-6, AG-7, AG-BI (Kuninaga & Yokosawa, 1980; 1982a; 1982b; 1983; 1984a; 1984b; 1985a; 1985b), and AG-9 (Carling & Kuninaga, 1990). A high degree of reassociation was observed for isolates within an anastomosis group, whereas low intergroup reassociation values were detected. These studies confirmed that there are close relationships among isolates within most anastomosis groups and more distant relationships among isolates of different anastomosis groups. Also, genetic relationships among isolates of anastomosis groups 1, 2, 3, 4 and 5 of *R. solani* were confirmed by Vilgalys (1988) using DNA-DNA hybridisation techniques. Carling & Kuninaga (1990) found that intra-specific groups of AG-9, determined on the basis of thiamine requirement, can be distinguished from one another by DNA hybridisation techniques but not by hyphal anastomosis. However, variation within AG-3, AG-4, AG-7 and AG-BI was not detected using this technique. Also, the requirement for large quantities of DNA for precise hybridisation studies can be a major disadvantage of using this technique.

1. 9. 2. Restriction fragment length polymorphism (RFLP) analysis

Restriction fragment length polymorphisms (RFLP) are powerful molecular markers that can be used to characterise the genotypes of an organism. They can be generated by the loss or gain of a restriction endonuclease recognition site at points of mutation, or by rearrangement of the DNA sequence by deletions or insertions of one or more restriction sites (Anderson *et al.*, 1987). Unlike morphological characteristics and isozymes, RFLPs exist in unlimited number and they tend to be variable in most crosses (Michelmore & Hulbert, 1987).

Polymorphisms in the total DNA restriction fragment lengths (Manicom & Baayen, 1993; Levesque *et al.*, 1993; Kohn *et al.*, 1988; Anderson *et al.*, 1987;

Bates & Buck, 1993; Moller *et al.*, 1993; McDonald & Martinez, 1990) and polymorphisms in mitochondrial DNA restriction fragment lengths (Sock *et al.*, 1994; Kawasaki *et al.*, 1990; Kozlowski *et al.*, 1982; Foster & Coffey, 1992; Foster & Coffey, 1993; Varga *et al.*, 1993; Jeng *et al.*, 1991; Ko *et al.*, 1993) have been used in systematics of various fungi.

RFLP analysis of isolates of *R. solani*, representing 10 anastomosis groups from diverse geographic origins, was reported by Jabaji-Hare *et al.* (1990). They used a ribosomal DNA (rDNA) probe originating from *Armillaria ostoyae* in Southern blot hybridisation and found one or more unique patterns for anastomosis groups 1, 2, 3, 6, and subgroups AG-1-1 and AG-2-1. Intra-group RFLPs were observed in all anastomosis groups, especially among isolates of AG-2 and its subgroups AG-2-1 and AG-2-2. On the basis of highly polymorphic patterns in *R. solani* rDNA, detected with *EcoRI*, Jabaji-Hare *et al.* (1990) concluded that *R. solani* isolates appear to fall into groups, some of which are similar to anastomosis groups. Their results did not provide clear evidence to support division of AG-2 isolates into AG-2-1 and AG-2-2, nor to segregate AG-2-2 into culture types AG-2-2 IIIB and AG-2-2 IVB. These authors observed that intra-group variation occurs within AG-3 isolates and there is a low level of intra-group variation in AG-7 and AG-BI isolates. Also, they studied the RFLPs in nuclear DNA of isolates of different anastomosis groups using five recombinant clones generated from the nuclear DNA of an isolate of AG-3. These probes hybridised specifically with AG-3 isolates. However, the potential of these probes has not been evaluated for studying genetic population or intra-group variations.

RFLPs in the nuclear encoded rDNA repeat of *R. solani* were also investigated by Vilgalys & Gonzales (1990). They found a considerable variation within and among intra-specific groups, using an rDNA probe originating from AG-4. Based on analysed data, AG-3, AG-4, AG-7, AG-8 and AG-BI all revealed a single, invariant RFLP unique to each intra-specific group. Low level variation was

detected within AG1-1B, AG1-1C, AG2-2 and AG-9, whereas a high level of genetic diversity was observed among isolates of AG1-1A, AG2-1, AG-5 and AG-6.

1. 9. 3. DNA fingerprinting

Ecological and evolutionary studies employing DNA-based techniques have been limited due to the lack of suitable probes (Heath *et al.*, 1993). Recently, the discovery of hypervariable VNTR (Variable Number Tandem Repeat) loci has given much hope that DNA fingerprinting techniques may become a powerful tool to estimate relationships among members of groups (Lynch, 1988) and to determine levels of genetic variation in populations (Lynch, 1990; Parkin & Wetton, 1991). Jeffreys *et al.* (1985) were the first to use the term "DNA fingerprinting" for using probes capable of hybridising to multiple loci.

DNA fingerprinting is a useful tool to assess the genetic variation in natural and domesticated populations. It relies on the existence of families of minisatellites dispersed throughout the genome in a large number of hypervariable loci (Lynch, 1988). Fingerprint loci may share a core sequence, in which case multiple RFLPs can be visualised simultaneously on the same gel. These loci tend to exhibit high allelic diversity, therefore members of an outbred population rarely have the same fingerprint patterns. This suggests that similarity in DNA-fingerprinting may provide a sensitive marker of relative levels of population homozygosity (Lynch, 1990).

DNA fingerprinting based on highly variable RFLP patterns can be generated either by using several different single or low copy DNA probes to make multiple locus haplotypes, in which alleles are present at each RFLP locus, or by using a DNA probe that hybridises simultaneously with variable RFLP loci

dispersed throughout the genome to provide unique patterns of restriction fragments for every clone in the population (McDonald & Martinez, 1991).

Many probes have been developed to generate DNA fingerprints in fungi (Goodwin *et al.*, 1990; Levy *et al.*, 1991; McDonald & Martinez, 1991; Meyer *et al.*, 1990; Jeng *et al.*, 1991; Whisson *et al.*, 1992). These probes have been employed to study genetic variation in the fungal populations (McDonald & Martinez, 1991; Karlsson, 1994; DeScenzo & Harrington, 1994) and pathotype diversity (Levy *et al.*, 1991). Also this technique has been recommended for use in studies of fungal ecology, population biology, taxonomy (DeScenzo & Harrington, 1994), gametic disequilibrium and variation in natural populations (McDonald & Martinez, 1991).

A DNA fingerprinting probe, (CAT)5, was used to detect variation in higher fungi by DeScenzo & Harrington (1994). The probe hybridised with VNTR loci of *Heterobasidium annosum* and *Ophiostoma piliferum* and was useful in delineating genotypes, identifying intra-specific variants and quantifying genetic variation. Also, genetic variation in *H. annosum* has been detected using M13 fingerprinting and rDNA probes, and intersterility groups of this fungus were differentiated based on M13 minisatellite bands (Karlsson, 1994). A dispersed repeated DNA sequence, called MGR, was employed to indicate the pathotypes of isolates of the rice blast fungus, *Magnaporthe grisea*, by Levy *et al.* (1991). They showed that the MGR-DNA fingerprinting probe could be used to determine the current geographic organisation of pathotypic diversity, to monitor its change over time and to study the dispersal ranges of specific genotypes. Also, genomic DNA fingerprint markers have been used to indicate genetic diversity among isolates of *Colletotricum orbiculare* (Correll *et al.*, 1993).

The DNA fingerprinting technique has been little used to study the levels of variation in *R. solani*. There is only one report of a dispersed repeated DNA sequence in *R. solani* AG-8 isolates. The probe showed a high degree of

polymorphism between AG-8 isolates and has considerable potential for field detection of AG-8 (Matthew, 1992). Such a probe could be useful to quantify the levels of soil-borne inoculum of rhizoctonia disease of potato.

1.9.4. RAPD-PCR

Random amplified polymorphic DNA based on the polymerase chain reaction (RAPD-PCR) is a useful molecular approach in many diverse fields, including clinical diagnosis, forensic analysis, population genetics (Tsai & Olson, 1992), development of genetic maps, targeting genetic markers and phylogeny (Grajal-Martin *et al.*, 1993).

In the RAPD method, a single arbitrarily chosen oligonucleotide sequence is used to prime DNA synthesis at low stringency from pairs of sites to which an oligonucleotide is matched (Wang *et al.*, 1993). In this reaction, a single primer binds to the genomic DNA at two different sites on opposite strands of the DNA template. Then the primed sites will be amplified and produce a discrete DNA through thermocyclic amplification (Tingey & Tufo, 1993). Different primers generate different patterns of RAPD markers, detecting polymorphic loci in segregated progenies (Doudrick *et al.*, 1993). There are several applications of the RAPD assay to detect DNA base-sequence polymorphisms. RAPD polymorphisms are the result of either a nucleotide base change, or an insertion or deletion within the amplified region (Williams *et al.*, 1990). Therefore, polymorphisms are usually denoted by the presence or absence of an amplification product from a single locus.

Use of the RAPD technique for identification, characterisation and genetic analysis of fungi has recently increased (Doudrick *et al.*, 1993; Grajal-Martin, *et al.*, 1993; Duncan *et al.*, 1993; Van Der Vlugt-Bergmans *et al.*, 1993). Pathogenic and nonpathogenic isolates of *Fusarium oxysporum* f. sp. *dianthi* were distinguished using RAPD patterns (Manulis *et al.*, 1994).

RAPD-PCR has also been applied to isolates of some anastomosis groups of *R. solani* to detect genetic variation (Duncan *et al.*, 1993; Yang, 1993; Liu & Sinclair, 1992). Yang (1993) evaluated the RAPD-PCR technique for detecting genetic variation among field isolates from the five pectic zymogram groups (ZG) within AG-8, and also to investigate the possibility of molecular variation among asexual homokaryons derived from protoplast-generated cultures. Based on RAPD profiles, he found that each ZG has a specific amplified DNA pattern and that homokaryon types derived from the field isolates differed from each other. Genetic variation in isolates of *R. solani* from Australia, representing AG-2-1, AG-2-2, AG-3, AG-4 and AG-8, was analysed by RAPD assay (Duncan *et al.*, 1993). Variation between isolates, especially between isolates from different geographic locations and also between anastomosis groups and pectic zymogram groups tested, was distinguishable by the RAPD markers. The authors also found that isolates from different zymogram groups and isolates belonging to the same zymogram groups can be distinguished by RAPD markers. In spite of the variation between the isolates tested, they observed a high degree of similarity between the isolates of ZG7 (AG-3) pathogenic on members of the *Solanaceae*. AG-3 isolates from the same geographical regions were reported to be more closely related to each other than to the isolates from other regions, however, there were few AG-3 isolates, and only one from South Australia. Therefore, variation among South Australian isolates of AG-3 has not been evaluated precisely. Also, Duncan *et al.* (1993) did not state the host species of their AG-3 isolates, therefore, the variation detected between the isolates cannot be interpreted on the basis of host specificity.

The literature was reviewed prior to June 1995.

1. 10. OBJECTIVES

The objectives of this study are:

- 1) to characterise the range of the *Rhizoctonia* spp. recovered from potato crops from different locations in South Australia.
- 2) to determine the pathogenicity of different anastomosis groups of *R. solani* recovered from diseased potato crops.
- 3) to evaluate, using pectic zymogram analysis, the variation within *R. solani* AG-3 which is the major cause of rhizoctonia disease of potato.
- 4) to evaluate genetic variation within *R. solani* AG-3 recovered from diseased potato crops, using molecular markers.

Chapter 2

Anastomosis group and pathogenicity of isolates of *Rhizoctonia solani* from potato crops in South Australia

2. 1. INTRODUCTION

Rhizoctonia disease, caused by *R. solani*, is one of the most important fungal diseases in potato fields in South Australia (Dillard *et al.*, 1993). *R. solani* is found in the soil and on tubers wherever potatoes are grown and the disease has been considered important in certified potato seed production (De Boer & Wicks, 1994).

The typical sunken necrotic lesions on roots, stems and underground parts lead to reduction in size of progeny tubers as well as reduction in yield (Carling & Leiner, 1986; Dillard *et al.*, 1993; Leach & Webb, 1993). In addition, the presence of black sclerotia on the surface of tubers lowers the quality of the crop and results in economic loss to the growers. Crop rotation has been only moderately successful as a control measure (Emmond & Ledinghan, 1972; Frank & Murphy, 1977), and a 3-year rotation using small grains and planting tubers surface-sterilised with formaldehyde have been recommended to reduce the population of *R. solani* in soil and hence disease incidence (Anderson, 1982, 1993).

Sclerotia on the surface of tubers are the most important source of inoculum for the next season, especially if potatoes are grown continuously (Anderson, 1993). Several workers concluded that tuber-borne sclerotia play a major role in the development of rhizoctonia disease and a large percentage of tuber-borne sclerotia are pathogenic on potatoes (Frank & Leach, 1980).

- A range of anastomosis groups of *R. solani* has been isolated from potato plants (see Section 1. 7. 1) and AG-3 is by far the most common (e.g. Carling & Leiner, 1986; Gudmestad *et al.*, 1989). ^{Carling & Leiner, 1990a} Isolation of binucleate *Rhizoctonia* spp. from potato crops has not been reported to date.

The experiments described in this chapter were conducted to determine the anastomosis groups of *R. solani* associated with potato plants at two sites in South Australia and the pathogenicity of these isolates.

2. 2. MATERIALS AND METHODS

2. 2. 1. Sampling

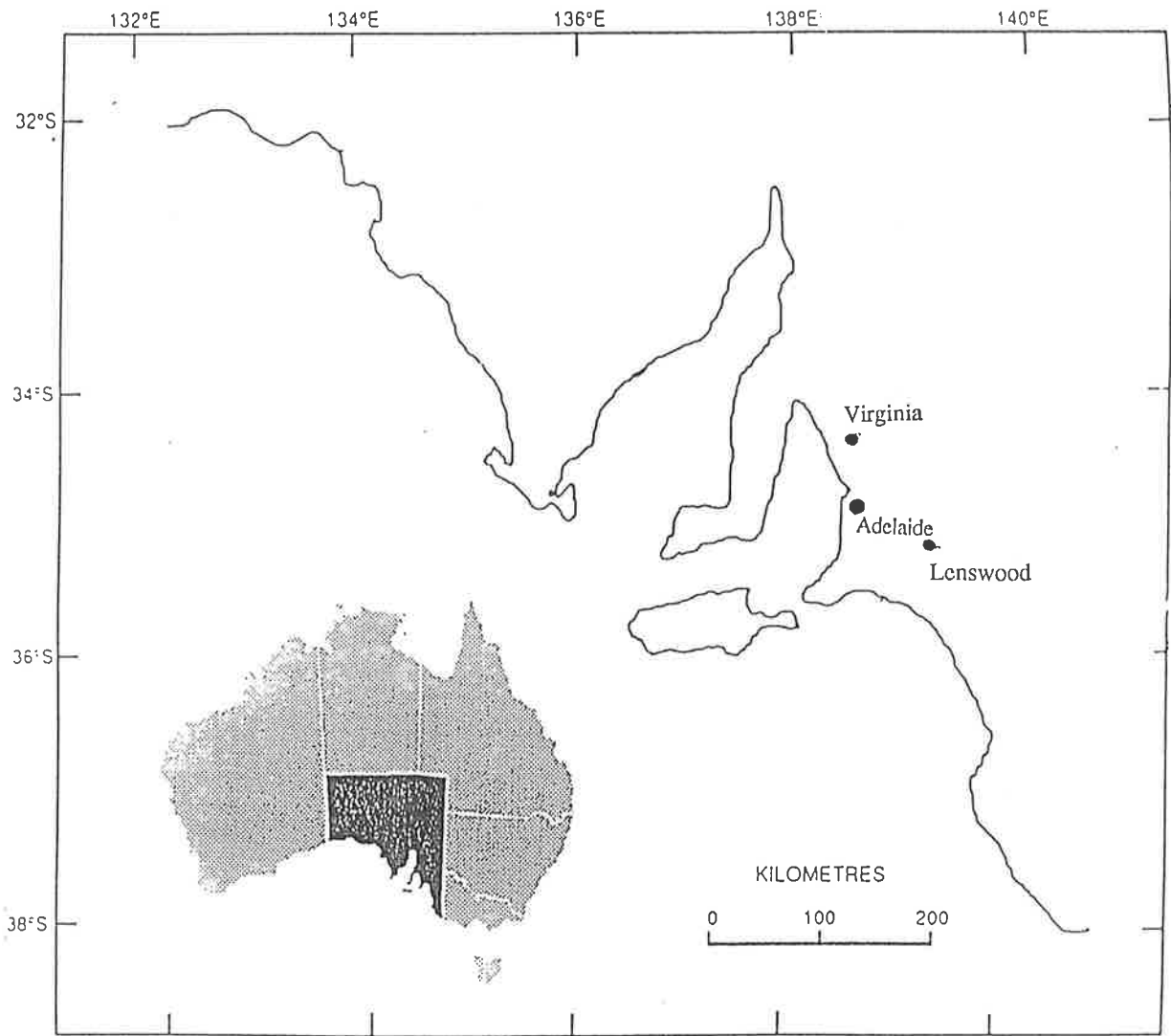
Samples were obtained from Lenswood and Virginia which represent potato growing areas located at high elevation (450 m) and low elevation (15 m), respectively (Fig 2. 1). *Rhizoctonia* was isolated at different times, mostly at flowering stage, from 10 field sites. Lenswood, with mean annual rainfall of 1070 mm and 18 °C mean annual maximum temperature, has a cooler climate than Virginia with mean annual rainfall of 453 mm and a 22.5 °C mean annual maximum temperature. Potato plants with root and stem cankers, tubers with sclerotia and soil from around infected roots, all randomly selected from material collected, were used for isolation. Material was stored at 4°C and sampled within 24 h of collection.

2. 2. 2. Isolation of *Rhizoctonia* from potato plants

Stems and roots with cankers were cut into 3-5 mm pieces, washed in tap water for 20 min, surface-sterilised in 5% sodium hypochlorite for 5 min and rinsed twice in sterilised double distilled water. The disinfected pieces were placed on slightly acidified tap water agar (AWA; 20 g agar (Difco) plus 1 ml l⁻¹lactic acid)

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Figure 2. 1. Map showing location of Virginia and Lenswood, South Australia, the two major sampling areas.



and incubated at 25°C in the dark. A total of 205 tubers with sclerotia were collected from the potato fields, washed in running tap water for 30 min and surface-sterilised with 70% ethanol for 30 s, then rinsed in sterilised double distilled water. A minimum of four sclerotia was then removed from each of the tubers, placed on AWA, and incubated at 25°C in the dark.

2. 2. 3. Isolation of *Rhizoctonia* from soil

Soil samples (1 kg each) collected from potato fields were mixed thoroughly, and two sub-samples (100 g each) were used for fungal isolation by the modified sieve screening method of Weinhold (1977). Each sub-sample was washed through a 411 µm mesh sieve (Endecotts (filters) Ltd, England). The material which passed through the sieve was collected in a beaker and then passed through a 211 µm mesh sieve. The collected slurry was then passed through a 105 µm mesh sieve and the materials retained on the sieve were washed into a beaker with sterilised distilled water. The soil particles were evenly distributed onto Whatman No. 1 filter paper discs (3 cm diameter) by filtering through a Millipore filter apparatus, then the particles trapped on six to eight Whatman filter paper discs were distributed over 20 ml AWA in 9-cm diameter Petri dish. Sclerotia were recovered from the soil samples as described by Ui *et al.* (1976).

2. 2. 4. Purifying and storage the cultures

Fungal colonies were observed under a dissecting microscope every 24 h and hyphal tips of *Rhizoctonia*-like fungi were transferred to potato dextrose agar (PDA, Difco) containing 1 ml l⁻¹ lactic acid and incubated as described above. Pure cultures of the isolates were kept on PDA at 10 °C as stock cultures. For long term

preservation, *R. solani* isolates were grown in Bijoux bottles containing sterile, dry, sandy loam soil plus 4% (w/w) wheat bran (Butler, 1980).

2. 2. 5. Isolate identification

The number of nuclei per cell was determined using a modified fluorescence technique (Yang *et al.*, 1991). A PDA plug of 2-day-old hyphal tips was placed directly on a microscope slide and squashed in a drop of Hoechst Dye 33258 (Sigma), 4.8 ng ml⁻¹ (pH 7.8), and observed immediately at 1000X on a Zeiss Standard Lab 16 microscope equipped for fluorescence microscopy, with excitation filter BP 450-490 and barrier filter LP 520.

Anastomosis groups were determined using the method of Parmeter *et al.* (1969). Each isolate was paired with tester strains of AG-1 1A, AG-1 1B, AG-1 1C, AG-2-1, AG-2-2, AG-3, AG-4, AG-5, AG-6, AG-7, AG-8 and AG-9 on sterilised Cellophane overlaying tap water agar (WA) in 3.5 cm diameter Petri dishes and incubated at 25°C for 24-48 h. Hyphal intersects of the advancing mycelia were observed microscopically and four or five anastomosis reactions considered for each sample, as described by Carling *et al.* (1988).

2. 2. 6. Growth rate

Six representative isolates each of AG-3, AG-4 and AG-5 were grown on PDA in Petri dishes (9 cm diameter), at 15°C or 25°C in the dark. The radial growth was measured at 12 h intervals, using the method of Bandy *et al.* (1984). There were three replicates per isolate and the Petri dishes were arranged in a randomised block design. Growth rate of isolates of AG-2-1 was not measured in this experiment.

2. 2: 7. Pathogenicity test

Representative, randomly selected isolates of AG-3, AG-4 and AG-5 of *R. solani* from potato crops and of AG-8 from diseased wheat and barley plants were used in pathogenicity tests on potatoes grown in the glasshouse (Bandy *et al.*, 1984; Bolkan & Wenham, 1973). Inoculum was grown in 250 ml flasks containing 100 g wheat grain plus 120 ml double distilled water which was autoclaved for 1 h at 121°C on three consecutive days. Each flask was inoculated with five plugs (5mm diameter) taken from the margin of a 1 week-old culture grown on PDA. Flasks were then incubated at 25°C in the dark for 10 days.

Plastic pots (20 cm diameter) were filled with pasteurised UC potting mix (Baker, 1957). Each pot was inoculated with 10 g of wheat inoculum of either AG-3, AG-4, AG-5 or AG-8 which was mixed with UC potting mix to a depth of 5 cm 4 days prior to tubers being planted. For controls, tubers were planted in uninoculated potting mix. There were eight replicate pots per treatment, including controls, and pots were arranged in a randomised block design.

Tubers of Coliban, a common cultivar grown in South Australia, were surface-sterilised in 2% (v/v) formaldehyde for 5 min and then treated with 2 mg l⁻¹ gibberellic acid (GA₃) to break dormancy (Bryan *et al.*, 1981). The tubers were placed in the dark at 25°C until sprouted then exposed to light for 2 weeks. In each pot, the soil was prised back with a trowel which had been surface-disinfected with 95% ethanol, one sprouted tuber was placed in the hole, and the soil allowed to fall back over the tuber, then pots were watered weekly as required. The potatoes were kept in a glasshouse at 25°C in natural light and harvested after 3 months. Disease symptoms were scored using the modified method of Bolkan & Wenham (1973). The disease symptoms were stem and root cankers (sunken necrosis), stolon and root tip burning (necrosis of the tips of stems, roots and stolon) and black scurf (black sclerotia). Four classes of pathogenicity were distinguished on roots and stems: non-diseased (no canker present), slightly diseased (superficial canker

present), moderately diseased (deep canker present) and severely pathogenic (sprouts and stolon girdled or killed). Also, four classes of black scurf disease were distinguished for harvested potato tubers: non-diseased (no sclerotia present), slightly diseased (1-25 sclerotia present on each tuber), moderately diseased (25-50 sclerotia present on each tuber) and severely diseased (more than 50 sclerotia present on each tuber). All tubers, regardless of size, were assessed.

Potato tubers with 25 or more sclerotia on each tuber and which were harvested during the first pathogenicity test, were used in a subsequent test to determine the role of sclerotia as tuber-borne inoculum. Tubers infested with AG-3 and AG-5 isolates were planted under the conditions described above and after 3 months plants were harvested and symptoms assessed. There were three representative isolates and eight replicates per isolate for each anastomosis group.

2. 3. RESULTS

2. 3. 1. Isolate identification

From the samples collected from potato fields in Virginia and Lenswood, 316 isolates showing characteristics typical of *Rhizoctonia* spp. (Table 2. 1) were transferred to PDA. Isolates were grouped into those possessing binucleate (Fig. 2. 2) or multinucleate cells (Fig. 2. 3); 12 were binucleate and the remainder were multinucleate. Based on anastomosis reaction (Figs 2. 4 & 2. 5), four anastomosis groups, AG2-1, AG-3, AG-4 and AG-5, were identified among the multinucleate isolates collected from potatoes and soil, with one isolate recovered from a soil sample identified as AG-8.

Isolates of AG2-1 were recovered from root mycelia only from Virginia (three isolates). In both Virginia and Lenswood, AG-3 isolates were predominant, comprising 272 of the 304 isolates of *R. solani* recovered from potato plants and

Table 2. 1. Anastomosis groups of *Rhizoctonia solani* isolates from potato fields in Virginia and Lenswood in South Australia

Location	Anastomosis group	Origin and number of isolates						total
		tuber sclerotium	root sclerotium	stem canker	root canker	root mycelium	soil	
Virginia	2-1	0	0	0	0	3	0	3
	3	117	5	12	13	0	11	158
	4	1	4	0	2	8	2	17
	5	4	0	3	0	0	0	7
	8	0	0	0	0	0	1	1
	binucleate	0	0	0	0	1	7	8
Lenswood	3	94	0	9	6	4	1	114
	4	0	2	0	0	0	2	4
	5	0	0	0	0	0	0	0
	binucleate	0	0	0	0	0	4	4

facing page

Figure 2. 2. Binucleate *Rhizoctonia* (*Ceratobasidium* sp.) recovered from potato crops, stained with Hoechst Dye 33258 at pH 7.8. Stained nuclei (N) and septa (S) are apparent.

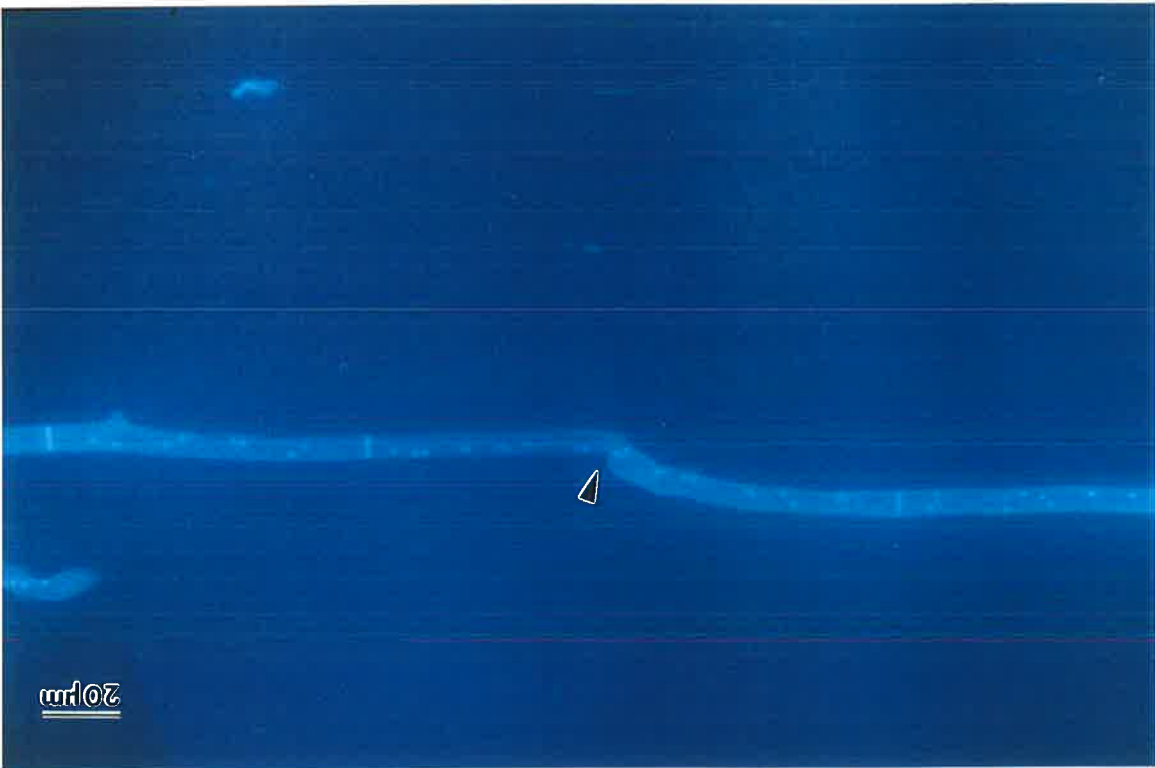
Figure 2. 3. Multinucleate isolate of *Rhizoctonia solani* (*Thanatephorus cucumeris*) AG-3 recovered from diseased potato, stained with Hoechst Dye 33258 at pH 7.8. Stained nuclei (N) and septa (S) are apparent.



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Figure 2. 4. Tip by tip anastomosis reaction between the tester strain of AG-3 (ST-11-10) and one multinucleate *Rhizoctonia solani* isolate recovered from a potato plant, shown by fluorescence microscopy, using Hoechst Dye 33258.

Figure 2. 5. Tip by side anastomosis reaction between two AG-3 isolates of *Rhizoctonia solani* recovered from potato plants shown by fluorescence microscopy, using Hoechst Dye 33258.



soil (Table 2. 1). AG-3 isolates were recovered from stem cankers, tuber sclerotia, mycelium on tubers and roots and also from soil samples from fields with a long history of potato planting. PDA cultures of the AG-3 isolates, after 2 weeks, were brown to dark brown with many sclerotia which were concentrated at the centre or the edge of Petri dishes. Aerial mycelia were common and zonation was observed in some cultures (Fig. 2. 6).

Isolates of AG-4 recovered from potato plants and soil from Virginia (17 isolates) and Lenswood (4 isolates) were associated mostly with roots and underground stems of the potato plants, with the main runner hyphae visible to the naked eye. Some irregular and large sclerotia, recovered from roots and lower parts of stems of infected plants, were AG-4, whereas only one AG-4 isolate was obtained from a tuber-borne sclerotium. The AG-4 isolates on PDA appeared white to tan-coloured with a few embedded small sclerotia and aerial mycelia (Fig. 2. 6).

AG-5 isolates were recovered from stem cankers and tuber sclerotia, only at Virginia, and comprised seven (2%) of the total isolates. Isolates of AG-5 were cream to light brown on PDA and produced brown to dark brown sclerotia which were irregularly distributed from the centre to the edge of Petri dishes (Fig. 2. 6).

2. 3. 2. Growth rate

The growth rates of the AG-3, AG-4 and AG-5 isolates of *R. solani* at 15°C and 25°C, measured at 12 h intervals over 60 h, showed significant differences (Table 2. 2). AG-3 isolates grew faster at 15°C than at 25°C. In contrast, the AG-4 and AG-5 isolates grew faster at 25°C than at 15°C. Sclerotia in AG-3 cultures were observed at day 5 at both 15°C and 25°C. Neither AG-4 nor AG-5 isolates produced sclerotia at this time. When the same isolates were incubated at 10°C, only AG-3 isolates grew initially, although the growth rate was much slower than at either 15°C or 25°C and sclerotia developed at day 12. Growth of AG-4 and AG-5

facing page

Figure 2. 6. Two-week old PDA cultures of different anastomosis groups of *Rhizoctonia solani* isolates recovered from diseased potato plants. Morphological differences are apparent among anastomosis groups. AG-3 (a), AG2-1 (b), AG-4 (c) and AG-5 (d).

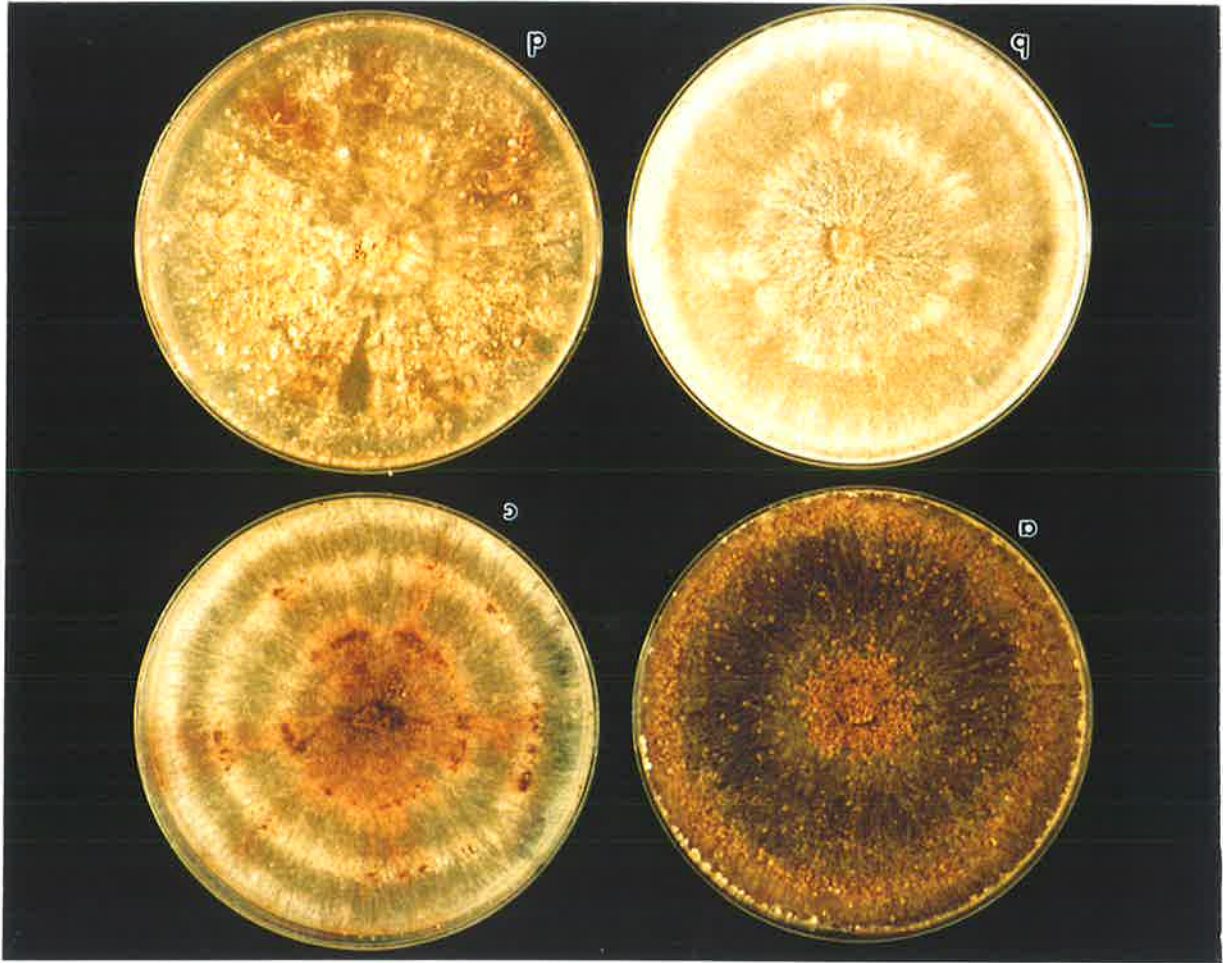


Table 2. 2. Mean of growth rate (mm h^{-1}) of representative isolates of AG-3, AG-4 and AG-5 of *Rhizoctonia solani* at 15°C and 25°C measured at 12 h intervals

Anastomosis group	Temperature	Growth rate (mm h^{-1})				
		12 h	24 h	36 h	48 h	60 h
3	15	3.6	7.1	11.8	15.2	21.7
3	25	1.3	3.7	9.1	12.8	17.6
4	15	1.2	3.8	7.4	10.0	14.0
4	25	3.7	8.4	21.7	28.9	45.0
5	15	2.3	6.8	10.1	12.8	17.3
5	25	3.8	7.0	17.3	23.0	34.6
LSD^a		0.3	0.5	1.3	1.2	1.3
LSD^b		0.4	0.7	1.6	1.5	1.6

-LSD^a ($p < 0.05$) value for comparison between temperature within each anastomosis group

-LSD^b ($p < 0.05$) value for comparison between anastomosis groups at the same temperature

-In this experiment six representative isolates each of AG-3, AG-4 and AG-5 were used. There were three replicates of each isolate.

isolates commenced at day 4 and subsequent growth was much slower than that of AG-3 isolates (data not shown).

2. 3. 3. Pathogenicity

Glasshouse trials, conducted in 1992 and 1993, indicated that representative isolates of AG-3, AG-4, AG-5 and AG-8 recovered from different sources were pathogenic on potato plants (Table 2. 3). Uninoculated control plants remained healthy throughout the experiment. Isolates of AG-3 and AG-5 caused stem and root cankers and black scurf. Isolates of AG-4 and AG-8 caused cankers on the stem, stolon and root, a decrease in number of feeder roots, but no black scurf. Black scurf was generally moderate to severe on plants inoculated with AG-3 (Fig. 2. 7) and slight to moderate on those inoculated with AG-5 (Fig. 2. 8). All isolates of AG-3 caused moderate stem canker on at least one plant and some caused slight or moderate root canker. AG-4 was capable of causing severe stolon canker (Fig. 2. 9) and root canker (Fig 2. 10), whereas isolates of AG-5 caused only moderate root canker.

Two of the three AG-8 isolates tested for pathogenicity produced some severe root cankers (Table 2. 3, Figs 2. 11 & 2. 12) and all caused very weak stem cankers on at least one plant. All plants inoculated with AG-8 exhibited severely pruned roots and stolon tip burning was observed on some. Visible root symptoms on potato plants inoculated with AG-8 were very similar to those produced by AG-4 isolates. Only AG-4 and AG-8 caused severe root canker.

Burning of feeder root tips, sprouts and stolons was observed on most plants inoculated with isolates of AG-3, AG-4, AG-5 and AG-8. Isolates of AG-4 caused more severe stolon cankers and stolon tip burning than did isolates of the other anastomosis groups. Stunting and leaf chlorosis were observed in some potato plants inoculated with isolates of AG-4 and AG-8.

Table 2. 3. Pathogenicity on potato of representative isolates of *Rhizoctonia solani* AG-3, AG-4 and AG-5 recovered from potato fields and AG-8 isolated from cereal crops in South Australia

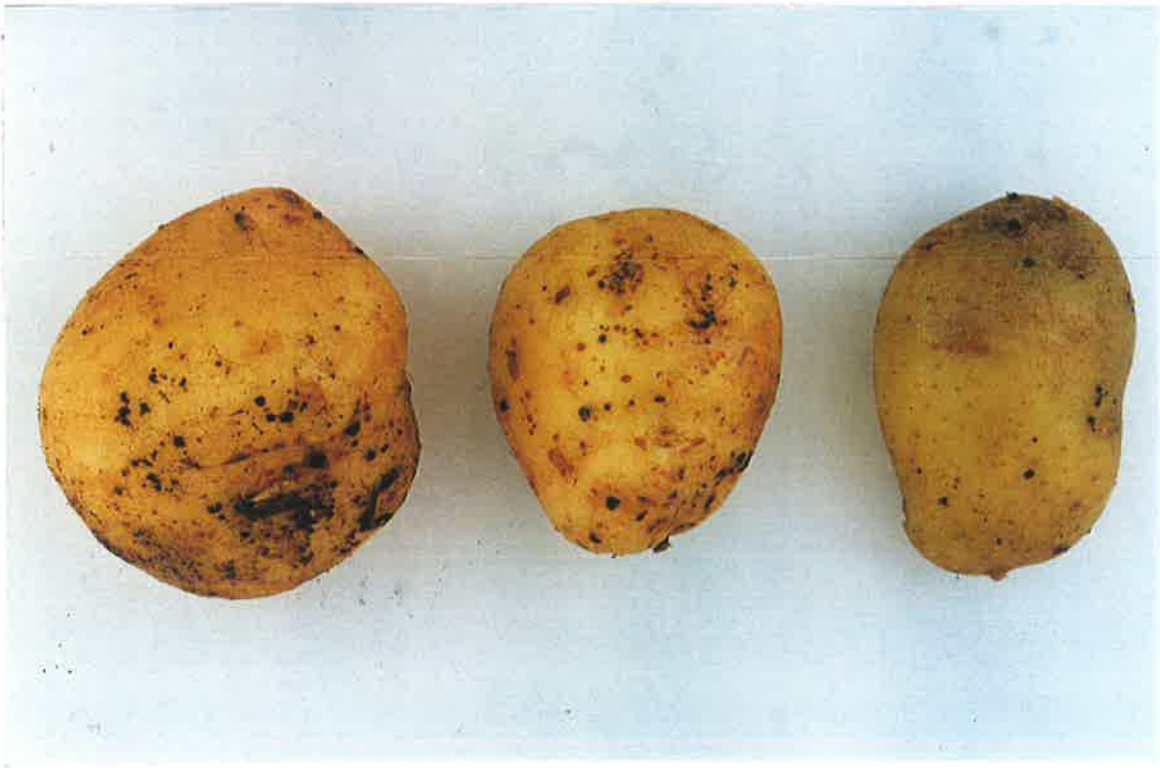
Anastomosis group	Isolate code	Origin	Pathogenicity ^a											
			Black scurf				Stem canker				Root canker			
			N	Sl	M	S	N	Sl	M	S	N	Sl	M	S
3	R21	Stem canker	0	2	4	2	0	3	5	0	4	4	0	0
3	R29	Root canker	0	5	2	1	1	6	1	0	2	5	1	0
3	R32	Root canker	0	3	5	0	2	4	2	0	8	0	0	0
3	R37	Stem canker	0	1	5	1	1	5	1	0	7	0	0	0
3	R46	Stem canker	2	0	4	2	0	6	2	0	6	2	0	0
3	R47	Stem canker	0	0	0	8	0	4	4	0	8	0	0	0
3	R48	Tuber sclerotium	0	2	3	3	0	5	3	0	2	4	2	0
3	R55	Tuber sclerotium	0	1	6	1	4	3	1	0	8	0	0	0
3	R65	Soil	4	0	2	0	0	4	2	0	6	0	0	0
4	R3	Root canker	6	0	0	0	0	0	2	4	1	5	0	0
4	R49	Stem canker	8	0	0	0	0	0	5	3	5	3	0	0
4	R67	Soil	6	0	0	0	0	1	5	0	2	4	0	0
4	R671	Soil	7	0	0	0	0	1	3	3	2	4	1	0
4	R672	Stem canker	7	0	0	0	1	0	6	0	1	4	1	1
4	R673	Stem canker	8	0	0	0	0	0	6	2	0	4	3	1
4	R674	Root canker	7	0	0	0	0	1	6	0	3	3	1	0
5	R51	Tuber sclerotium	0	4	4	0	0	6	2	0	0	5	3	0
5	R515	Stem canker	1	1	6	0	0	3	5	0	0	6	2	0
5	R516	Stem canker	0	5	3	0	0	4	4	0	2	3	3	0
5	R517	Tuber sclerotium	2	0	6	0	1	2	4	1	3	2	3	0
8	R1438	Barley	8	0	0	0	7	1	0	0	0	2	4	2
8	R1477	Barley	8	0	0	0	5	3	0	0	1	1	4	2
8	R1491	Wheat	8	0	0	0	5	3	0	0	1	2	5	0
Control	-	-	8	0	0	0	8	0	0	0	8	0	0	0

a: Pathogenicity calculated as the number of plants tested which fell into the four severity categories. Non-pathogenic (N), Slight (Sl), Moderate (M), Severe (S) for the three disease symptoms, black scurf, stem cankers or root cankers. There were eight replicate plants per treatment, but in some cases one or two tubers did not emerge.

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Figure 2. 7 (top). Potato infected by an AG-3 isolate of *Rhizoctonia solani* (left), black sclerotia are visible on the tuber (a) and stem (b) but no stem or root cankers are apparent. The potato on the right is an uninoculated control (c).

Figure 2. 8 (bottom). Black scurf on three potato tubers caused by an AG-5 isolate of *Rhizoctonia solani*.



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Figure 2. 9 (top). Potato stolon tip burning (a) and cankers (b), caused by an AG-4 isolate of recovered from diseased potato plants. Uninoculated control (c, group of three).

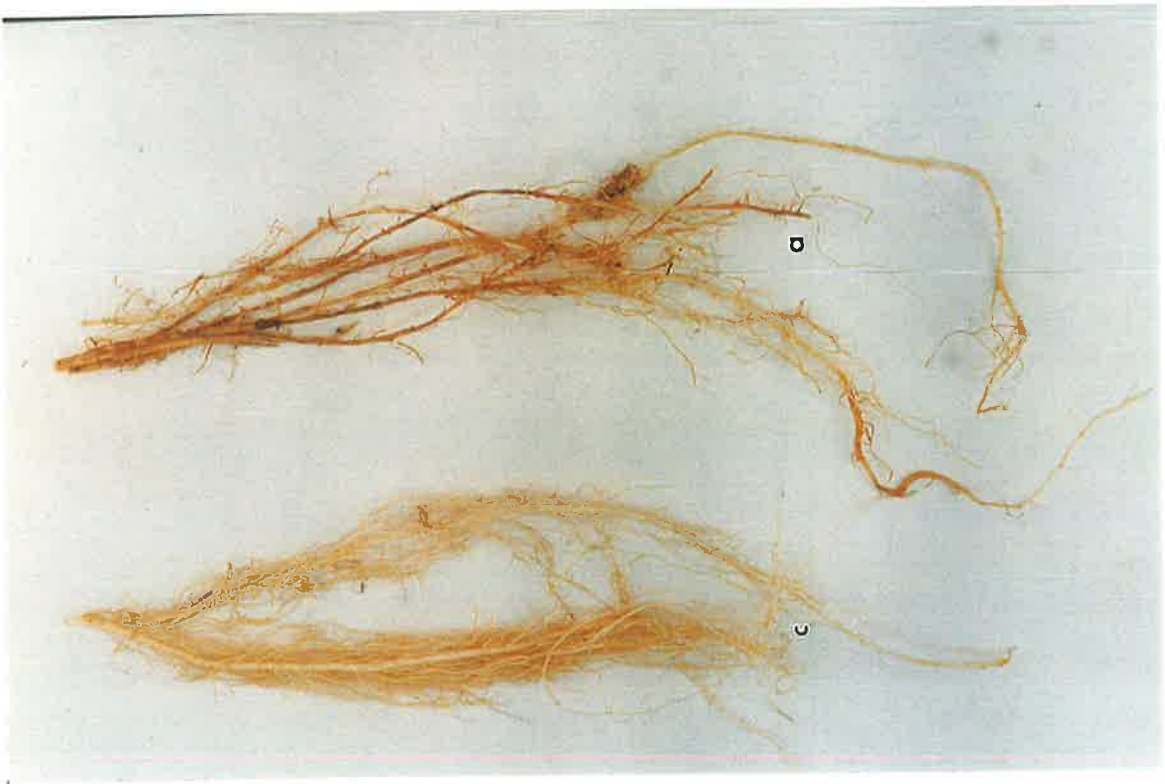
Figure 2. 10 (bottom). Cankers and pruned roots caused by an AG-4 isolate of *Rhizoctonia solani* recovered from diseased potato plant (a). Uninoculated control (c).



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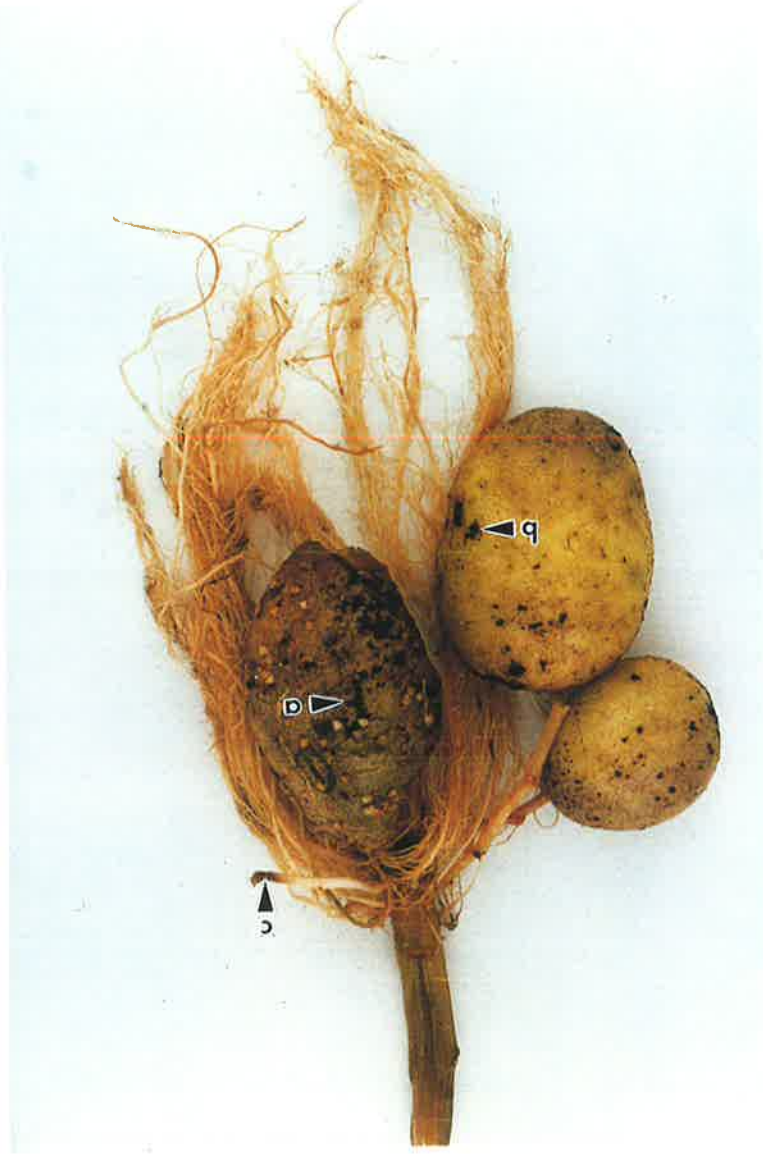
Figure 2. 11 (top). Potato plant infected by an AG-8 isolate of *Rhizoctonia solani* recovered from barley, showing stolon tip burning (a), and root cankers (b). Uninoculated control (c).

Figure 2. 12 (bottom). Potato with pruned root symptoms and cankers, caused by an AG-8 isolate of *Rhizoctonia solani* recovered from barley (a). Uninoculated control (c).



facing page

Figure 2. 13. Disease symptoms on progeny tubers arising from a mother seed tuber infected with *Rhizoctonia solani* AG-3. Note; sclerotia on mother seed (a) and progeny tubers (b), and some stolon tip burning (c) are visible.



All potato plants grown from infested tubers obtained from this pathogenicity test showed moderate to severe black scurf on progeny tubers and weak to moderate stem and stolon cankers (Fig. 2. 13). The *R. solani* isolates recovered from diseased tubers were the same as those on the mother tubers which had been used to inoculate the mother tubers.

2. 4. DISCUSSION

AG-3 isolates of *R. solani* were predominant in potato crops in Lenswood and Virginia, South Australia, however, AG-4 and AG-5 were also recovered. This is the first report of the isolation of AG-4 and AG-5 from potato plants in South Australia. Although AG-5 comprised only seven of the 186 isolates of *R. solani* from five fields sampled at Virginia, it is interesting that it was not found in the other major potato growing area, at Lenswood. Isolates of AG-3, AG-4 and AG-5 collected from potatoes and isolates of AG-8 from cereal crops were pathogenic on potato plants. Thus it is clear that several anastomosis groups are associated with rhizoctonia disease on potatoes in South Australia.

Isolates of *R. solani* AG-3 have also been obtained from potato crops in Victoria, the major potato growing state in Australia (R. DeBoer, 1993, personal communication), and Western Australia (M. Sweetingham, 1994, personal communication); however, there is no other information about the range of anastomosis groups associated with potatoes in these states. Bandy *et al.* (1984) found that 18% of the isolates of *R. solani* collected from potato fields in Maine, USA, were AG-5 and the majority were identified as other anastomosis groups, predominantly AG-3. Abe & Tsuboki (1978) reported that 96% and 3% of isolates of *R. solani* recovered from potato plants and tuber-borne sclerotia in Japan were AG-3 and AG-5, respectively. In Northern Ireland, rhizoctonia disease is caused by a combination of AG-1, AG-2, AG-3 and AG-4 (Chand & Logan, 1983). The

results presented here support previous observations that rhizoctonia disease in potato crops can be caused by several anastomosis groups of *R. solani*. Isolates of *R. solani* AG-3 have been reported to cause black scurf (Frank, 1981). However, AG-5 isolates are reported to be weak pathogens (Section 1. 7. 1. 3), but observation of plant material from field collections and pathogenicity experiments in this study showed that isolates of AG-5 can also produce these symptoms; thus AG-5, as well as AG-3, is implicated in causing black scurf in South Australia. Furthermore, the isolates of AG-5 isolates tested had a greater ability to produce stem and root cankers than did the AG-3 isolates. However, they are probably less important, as they comprised only 2% of the isolates obtained. Similarly, Abe & Tsuboki (1978) reported that isolates of *R. solani* AG-3 and AG-5 can be associated with tuber sclerotia formation. Gudmestad *et al.* (1989) also found these to be the predominant groups recovered from infected potato plants but did not discuss the role of isolates of AG-5 in black scurf formation.

It is possible that potato plants are infected by tuber-borne inoculum of isolates such as AG-3 and AG-5 more rapidly than by soil-borne inoculum, due to the close proximity of the inoculum to the host. Banville (1989) showed that seed pieces infested with sclerotia of AG-3 produced smaller total yield and smaller progeny tubers, and were significantly more heavily infested with sclerotia than were progeny tubers derived from uninfested seed pieces. Other anastomosis groups such as AG-4, which are usually soil-borne, may be less able to compete with tuber-borne inoculum. The pathogenicity of tuber-borne inoculum (Frank & Leach, 1980; Bolkan & Wenham, 1973; Platt *et al.*, 1993) and its importance in aetiology (Emmond & Ledingham, 1972^{); Carling *et al.*, 1989}) of rhizoctonia disease have been confirmed. It seems that the presence of tuber-borne inoculum may be the most important factor in pathogenicity, epidemiology and population dynamics of AG-3 isolates. James & McKenzie (1972) concluded that black scurf on seed tubers did not result in seed decay or stem cankers and did not affect tuber yield in the subsequent crop whereas Frank & Leach (1980) found that both tuber-borne and

soil-borne inoculum of *R. solani* were important in the development of rhizoctonia disease. Davis & Groskopp (1979) showed that rhizoctonia disease reduced potato production. In the experiments reported here, the AG-3 isolates varied in pathogenicity and in the disease symptoms produced. Hence, the different conclusions reached by previous workers in respect to pathogenicity of AG-3 isolates, especially tuber-borne inoculum, could be related to genotype variation in the isolates tested.

AG-4 isolates did not cause black scurf (Table 2. 3) and were not isolated from tuber-borne sclerotia; however, a few sclerotia recovered from underground parts of stem, and mycelia associated with the same parts and with tubers, were identified as AG-4. AG-4 was dominant over AG-3 isolates originating from mycelia isolated from the stem and root cankers. These findings, along with observations of pathogenicity, suggest that AG-4 isolates may be important in producing stem and root cankers, whereas AG-3 isolates cause both stem and stolon cankers and black scurf. AG-4 isolates are probably distributed as soil-borne inoculum or resting mycelia, especially on the surface of tubers. Therefore, sclerotia-free tubers should be used by potato growers in order to minimise inoculum potential and disease incidence.

Climate in South Australia could be an important factor in determining the geographical distribution of anastomosis groups. In this state, two potato crops may be grown each year. The main crop is planted in late spring to early summer (October to December) and harvested in late summer to early autumn (February to May) whereas, in some areas, an early crop may be planted during winter to early spring (June to September) (Lomman, 1988). AG-5 was found only in the warmer Virginia area. The climate in Virginia allows the cultivation of early and main potato crops on different parts of a farm whereas, in Lenswood, usually there is only a main crop. Using infested early crop potato tubers as "seed" for main cultivation or using main crop potato tubers as "seed" for early crops may expose potato plants to

a range of different anastomosis groups originating in different environmental conditions. As reported here, both isolates of AG-3 and AG-5 cause black scurf and their sclerotia can act as tuber-borne inoculum to infect the potato plants and progeny tubers. It is possible that mixed inoculum of AG-3 and AG-5 may be present as sclerotia but that AG-5 will grow only under warmer conditions. It has been shown previously that cool temperatures favour AG-3 (Anguiz & Martin, 1989; Carling & Leiner, 1990^a) and in this study it was observed that in warmer conditions *in vitro* the growth rate of isolates of AG-4 and AG-5 is significantly faster than AG-3. In such conditions in the field, isolates of AG-4 and AG-5 may develop faster and be able to compete with the isolates of AG-3. Therefore, the amount of AG-3 inoculum, especially tuber-borne inoculum, could play an important role in causing primary infection in potato plants. Isolates of AG-3 may be predominant at cooler temperatures, whereas in warmer areas, or later in the growing season, other anastomosis groups such as AG-4 and AG-5 are able to grow along with AG-3.

Some isolates of *R. solani* AG-8 recovered from diseased wheat and barley caused severe cankers and severe pruning of feeder roots on potato. In South Australia, "bare patch" caused by AG-8 is a serious disease of cereals (Neate & Warcup, 1985) and in some areas cereals are commonly grown in rotation with potatoes because it is believed that potatoes have fewer diseases in common with cereals than other crops (Anderson, 1993). Hide & Firmager (1990) showed that an isolate of AG-8, recovered from stunted barley, caused severe stunting of Desiree potatoes grown in pots and that most main and lateral roots were pruned. ^{See} *Addendum 2*

Furthermore, isolates of AG-3 have been recovered from wheat and barley fields (Ogoshi *et al.*, 1990). On the basis of the results presented here, using this type of rotation in potato fields should be reconsidered, as it is possible that isolates of AG-8 may attack potato roots in the field, especially in the year following a cereal crop. In addition, cereals grown in rotation with potatoes do not totally eliminate isolates of AG-3, the major pathogen of potatoes.

- The results presented here suggest that *R. solani* AG-3, AG-4, AG-5 and AG-8 may attack potato crops in different seasons and locations in South Australia. However, a more comprehensive survey, and studies of the epidemiology, quantification and population diversity are necessary to elucidate the interaction among these anastomosis groups.

Chapter 3

Pectic zymogram analyses of field isolates of *Rhizoctonia solani* AG-3

3. 1. INTRODUCTION

Isozyme electrophoresis has been used to characterise strains within natural populations of various fungi (Mills *et al.*, 1991; Oudemans & Coffey, 1991; Bonde *et al.*, 1991). In particular, it has been valuable for identification of microorganisms in epidemiological, ecological and systematic studies (Caugant *et al.*, 1981).

Pectic isozymes, or zymograms, have been used to distinguish species within fungal genera (Cruickshank, 1983a, 1983b; Cruickshank, 1990; Lecours *et al.*, 1994). Sweetingham *et al.* (1986) used pectic zymograms to assign field isolates of *R. solani*, recovered from wheat and lupin in Western Australia, to one of two zymogram groups, ZG1 or ZG2. They also demonstrated that the isolates within a zymogram group had a similar cultural and morphological appearance. Neate *et al.* (1988) studied pectic enzymes produced by members of *R. solani* recovered from many hosts and localities in South Australia and showed that zymogram groups are related to the established anastomosis groups. Thus AG-8 isolates belonged to ZG1-1, ZG1-2, ZG1-3 and ZG1-4 and, subsequently, a fifth ZG for AG-8 was designated ZG1-5 (Sweetingham, 1990). Neate *et al.* (1988) also established a correlation between ZG7 and AG-3 isolates recovered from potato plants and that ZG6 and ZG8 belonged to the AG-2-1 and AG-4 isolates, respectively. MacNish *et al.* (1994) showed that ZG5 and ZG6 isolates both belonged to AG-2-1 but differed in host range and pathogenicity.

Although, to date, all isolates in a zymogram group belong to a single anastomosis group, there is more than one zymogram group for each anastomosis group

(MacNish & Sweetingham, 1993). The pectic zymogram technique provides a quick and reliable tool for determining the anastomosis group of *R. solani* strains (Sweetingham *et al.*, 1986; Neate *et al.*, 1988; Cruickshank, 1990; MacNish & Sweetingham, 1993).

The following experiment was conducted to determine the genetic heterogeneity of AG-3 isolates of *R. solani* by comparing isolates originating from different locations in South Australia.

3. 2. MATERIALS AND METHODS

3. 2. 1. Isolates of *R. solani* and morphology of sclerotia

Isolates of *R. solani* AG-3, obtained from stem cankers, root cankers and tuber-borne sclerotia (see Chapter 2) were used in this experiment. They were examined for the presence of tuber-borne sclerotia. These sclerotia on potato tubers collected from the field sites were classified into three morphological groups, domed, flat or intermediate shaped. Pure cultures of tuber-borne sclerotia from each group were established on PDA as described previously (Section 2. 2. 4) and used for pectic enzyme analysis.

3. 2. 2. Pectic enzyme preparation

One 5 mm diameter plug was taken from each colony margin and transferred to 2 ml of sterile liquid medium in a 24-well Nunc disposable multidish (Nunc, Denmark). The medium contained 1% citrus pectin (P-9135, Sigma) as the sole carbon source (Sweetingham *et al.*, 1986). Cultures were grown at 25°C in the dark without shaking for 10-12 days. Mycelium was then removed by filtration using sterile 0.45 µm syringe filters and 100 µl of each culture filtrate was mixed with 0.01 g Sephadex G-150

Superfine to make a slurry which was kept at room temperature for up to 30 min before electrophoresis.

3. 2. 3. Electrophoresis

The method of Cruickshank & Wade (1980) as modified by Cruickshank (1983a) was used. Horizontal pectin-acrylamide gels were prepared as recommended by Sweetingham *et al.* (1986), whereas the gel moulds were adapted from those described by Cruickshank & Wade (1980), and were made of glass and Perspex plates (8x16 cm) separated by glass strips 2 mm thick by 3 mm wide. The culture filtrate/Sephadex slurries were loaded into the wells (10 μ l each) and 3 μ l of 0.05% bromophenol blue were applied to each of the first and last wells as tracking dye. Wettex cloth (Bunzel) was used as a wick at the cathodic and anodic ends of the gel. Power was supplied using a constant 16 mA per gel and electrophoresis was stopped when the tracking dye had migrated 5 cm toward the anodic end (1.5 h). An aqueous solution of boric acid (7.2 g l⁻¹) and sodium tetraborate dehydrate (15.75 g l⁻¹) was used as electrode tank buffer and ice water was circulated underneath the gel supporter plate during electrophoresis. An isolate belonging to ZG7 (Neate *et al.*, 1988) was used as control.

3. 2. 4. Gel developing, staining and printing

The buffer front region (0.5 cm) of each gel was removed and the remaining gel was rinsed briefly with distilled water. The gels were incubated for 1 h in 0.1 M malic acid at 25°C (Cruickshank & Wade, 1980) before staining overnight at 5°C in 0.02% ruthenium red to visualise the bands. Stained gels were washed with three changes of distilled water and incubated in 0.05% ammonium persulphate for 20 min at 25°C to

increase the contrast for photography. The gels were rinsed briefly with distilled water and contact printed on high contrast photographic paper (Ilfospeed RC 1S5.M).

3. 2. 5. Data analysis

The electrophoretic types were interpreted in terms of loci coding for polygalacturonase (PG, black zones in the print) and pectin esterase (PE, white zones in the print) (Cruickshank & Wade, 1980). Presence or absence of a band (allele) was determined by calculating the R_f value, the ratio of the distance travelled by an enzyme to that travelled by the bromophenol blue. The phenotype frequencies, based on the two loci, were calculated.

Zymogram patterns were transferred into a binary character matrix; 1 for presence, 0 for absence of a band with a particular R_f value. Based on reproducible zymogram banding patterns, test isolates were assigned to zymogram groups. Each zymogram group was considered as a sub-group of either Virginia or Lenswood populations.

Genetic similarity among the electrophoretic phenotypes was calculated using the similarity statistic (Lynch, 1990). Pairwise comparisons were made between all isolates and a similarity matrix was generated. A cluster analysis was performed based on similarity values using the group average analysis (UPGMA) method (Sneath & Sokal, 1973) and the GENSTAT 5 statistical program, release 1.3 (Rothamsted Experimental Station, 1989) was used for data analyses.

3. 3. RESULTS

A total of 101 isolates of *R. solani* AG-3 (Table 3. 1), ^{including the nine isolates used in pathogenicity tests, listed in Table 2.} originating from root cankers, stem cankers and tuber-borne sclerotia, was examined by pectic zymogram

Table 3. 1. Zymogram patterns (ZP) and zymogram groups (ZG) of isolates of *Rhizoctonia solani* recovered from diseased potato plants

Location	Source ^a	Number of isolates	ZP	ZG
Lenswood	stem canker	3	1	7-1
Lenswood	stem canker	2	7	7-1
Lenswood	domed sclerotium	9	1	7-1
Lenswood	domed sclerotium	2	7	7-1
Lenswood	intermediate sclerotium ^b	7	1	7-1
Lenswood	domed sclerotium	3	2	7-2
Lenswood	domed sclerotium	2	4	7-2
Lenswood	intermediate sclerotium	2	2	7-2
Lenswood	flat sclerotium	5	3	7-3
Lenswood	flat sclerotium	1	5	7-3
Lenswood	intermediate sclerotium	2	3	7-3
Lenswood	intermediate sclerotium	2	5	7-3
Virginia	stem canker	10	1	7-1
Virginia	root canker	5	1	7-1
Virginia	root canker	1	7	7-1
Virginia	intermediate sclerotium	10	1	7-1
Virginia	domed sclerotium	18	2	7-2
Virginia	domed sclerotium	1	4	7-2
Virginia	intermediate sclerotium	6	2	7-2
Virginia	flat sclerotium	7	3	7-3
Virginia	intermediate sclerotium	1	3	7-3
Virginia	domed sclerotium	1	6	U
South East ^c	intermediate sclerotium	1	1	7-1

U= not determined

^a *Rhizoctonia solani* isolated from stem canker, root canker or tuber borne sclerotia

^b sclerotia intermediate between domed and flat

^c South East South Australia

electrophoresis. Several zymogram phenotypes were produced (Fig. 3. 1). PG and PE isoenzymes were observed in all AG-3 isolates tested. Ten bands were identified and designated 'a' to 'j' (Figs 3. 1 & 3. 2). Only one band, 'c', corresponded to the PE isoenzyme. It had the same R_f value in all isolates. The other nine bands corresponded to the PG isoenzyme. Six new zymogram patterns (ZP) were identified, ZP1 to ZP6 (Fig. 3. 2), for the isolates tested, with the remaining pattern identified as zymogram group 7 (ZG7), previously reported by Neate *et al.* (1988).

The total number of bands in each ZP varied from three to six. Three characteristic bands; 'c', 'd', and 'f' were common to all AG-3 isolates tested except the ZP6 isolate, which lacked 'd'. ZP6 occurred in only one isolate, so was omitted from the genetic similarity analyses.

For the purposes of this thesis, certain bands were termed 'inconsistent bands'. These inconsistent bands appeared in some gels but not in others prepared from a single isolate, whereas they were always absent from gels of other isolates.

ZP1 was identified by the common PG bands, 'd' and 'f', ZP2 had bands 'd', 'f', and 'g', while ZP3 contained four PG bands, 'd', 'e', 'f' and 'g' (Fig. 3. 2). The pattern of ZP4 had bands 'd', 'f', 'g' and 'h' and ZP5 'd', 'e', 'f', 'g' and 'h'. While 'h' is a distinguishing character between ZP3 and ZP5 and between ZP2 and ZP4, this band was not consistently present. The presence of the inconsistent band, 'a', close to the cathode in ZG-7, was the only distinguishable character between this pattern and ZP1. The unique bands 'b', 'i' and 'j' were found only in ZP6.

The inconsistent, backward migrating enzyme 'a' ZG7 also occurred in some old cultures of ZP1 isolates and some concentrated samples, whereas the band did not appear in cultures younger than 10 days or in diluted samples (data not shown). In addition to the ten bands routinely detected in AG-3 isolates, there were some weakly stained and inconsistent bands which were not considered in the zymogram pattern analyses.

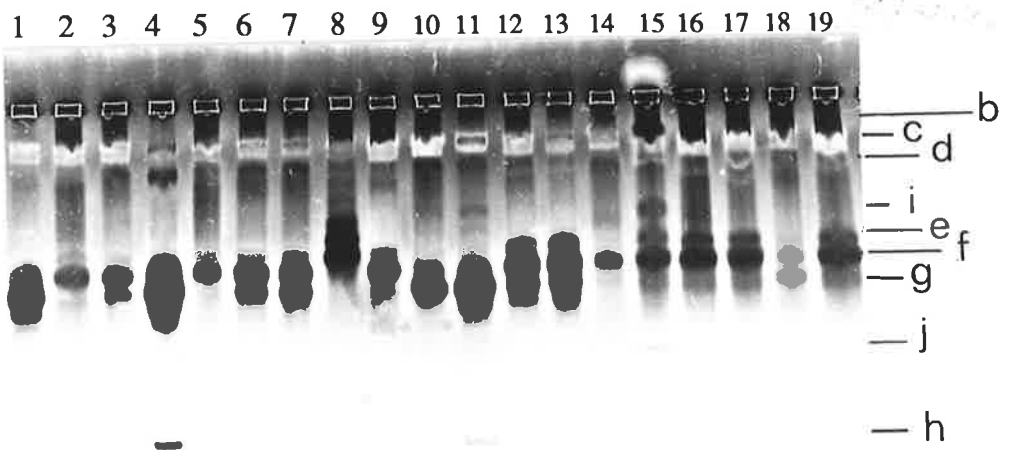
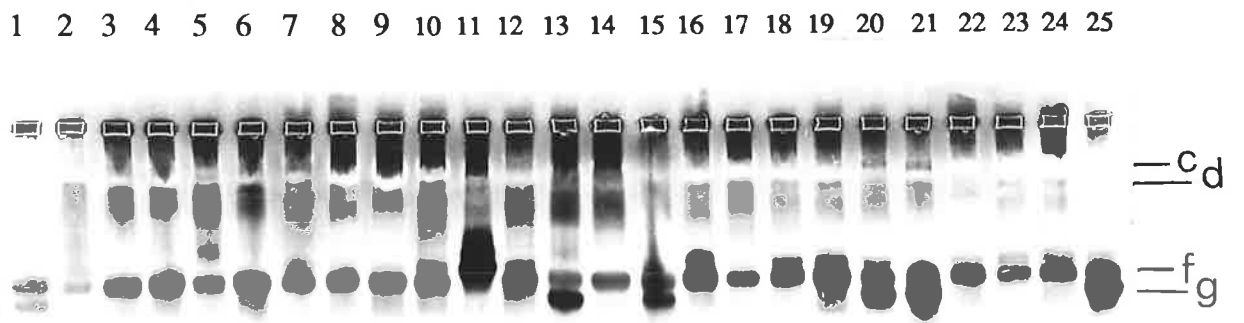
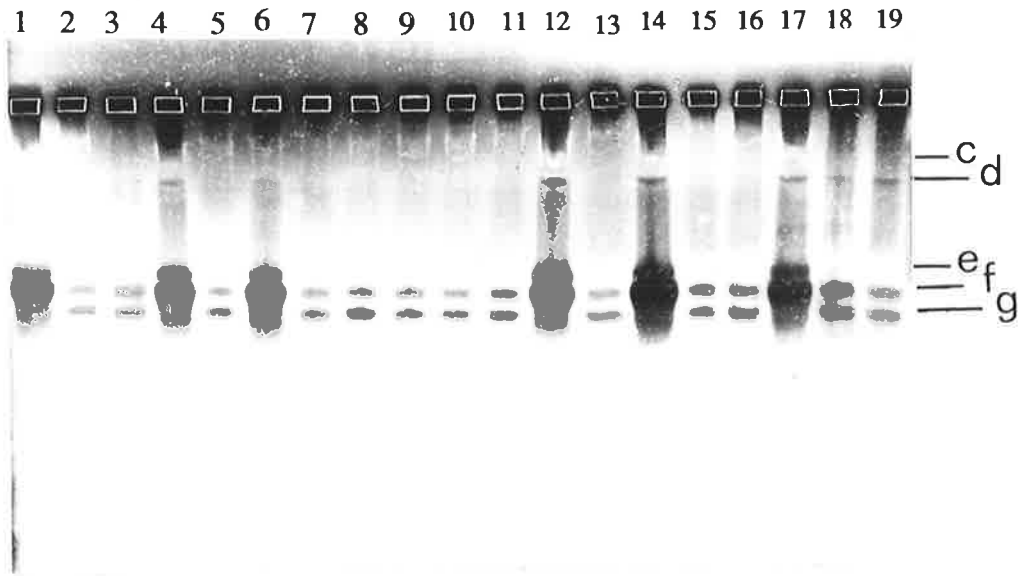
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Figure 3. 1 Pectic zymogram of the field isolates of *R. solani* AG-3 recovered from potato crops in South Australia. In these gels there is no sample with band 'a'.

Top. Lane 1, R883; lane 2, R900; lane 3, R901; lane 4, R902; lane 5, R903; lane 6, R904; lane 7, R905; lane 8, R906; lane 9, R907; lane 10, R908; lane 11, R909; lane 12, R910; lane 13, R911; lane 14, R912; lane 15, R913; lane 16, R914; lane 17, R915; lane 18, R916; lane 19, R917.

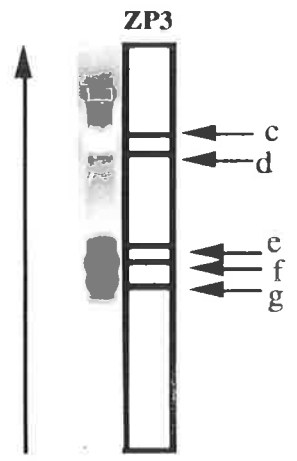
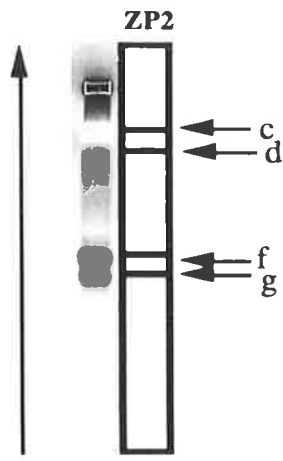
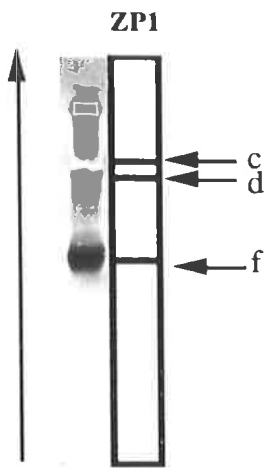
Middle. Lane 1, R802; lane 2, R814; lane 3, R820; lane 4, R827; lane 5, R840; lane 6, R841; lane 7, R851; lane 8, R852; lane 9, R853; lane 10, R856; lane 11, R862; lane 12, R863; lane 13, R864; lane 14, R866; lane 15, R871; lane 16, R872; lane 17, R873; lane 18, R874; lane 19, R875; lane 20, R876; lane 21, R877; lane 22, R878; lane 23, R879; lane 24, R880; lane 25, R882.

Bottom. Lane 1, R802; lane 2, R804; lane 3, R807; lane 4, R9813; lane 5, R830; lane 6, R844; lane 7, R847; lane 8, R850; lane 9, R857; lane 10, R858; lane 11, R859; lane 12, R862; lane 13, R863; lane 14, R870; lane 15, R935; lane 16, R932; lane 17, R938; lane 18, R939; lane 19, R936.

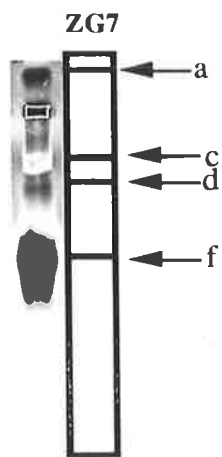


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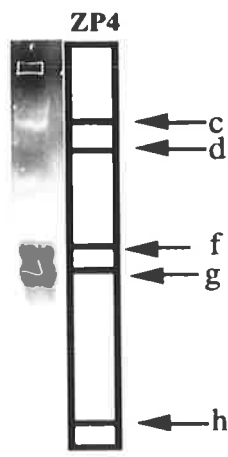
Figure 3. 2. Pectic zymogram patterns (ZP) and pectic zymogram groups (ZG) of AG-3 isolates of *Rhizoctonia solani*. ZP1 to ZP6 were determined based on total bands. ZG7-1, ZG7-2 and ZG7-3 were assigned according to the following bands: pectic esterase, 'c' and polygalacturonase, 'a', 'b', 'd', 'e', 'f', 'g', 'h', 'i' and 'j'.



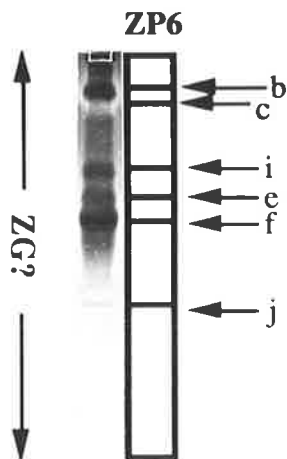
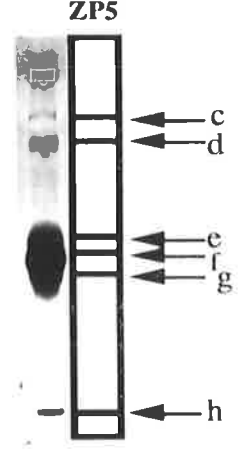
ZG7-1



ZG7-2



ZG7-3



ZG?

- Dome shaped sclerotia were more common than flat sclerotia in both Virginia and Lenswood, but 38% of sclerotia were intermediate in shape. Isolates from domed and flat sclerotia gave different zymogram patterns. The isolates from flat sclerotia were all ZP3 whereas the isolates with domed sclerotia were either ZP1 or ZP2. Isolates from both stem and root cankers gave a single zymogram pattern, ZP1. All AG-3 isolates used in pathogenicity tests described previously (Chapter 2, Balali *et al.*, 1995a), produced ZP1.

Zymogram profiles occurred at different frequencies throughout the sampled areas. ZP1 occurred in 25 of 60 isolations from Virginia and 19 of 40 isolations from Lenswood, whereas ZP2 appeared in 24 of 60 isolates from Virginia and 5 of 40 isolations from Lenswood (Table 3. 1). ZP3 was recovered with approximately the same frequency, 8 of 60 isolations and 7 of 40 isolations in both Virginia and Lenswood, respectively. Other ZPs were found only sporadically.

The similarities among the electrophoretic phenotypes were calculated and pairwise comparisons were made between all isolates to generate a similarity matrix. Intergroup relationships were deduced from the similarity matrices. The genetic similarity, based on band frequencies, ranged from 50% to 87.5% (Table 3. 2).

The data were subjected to UPGMA and expressed in a dendrogram to show the genetic similarity between isolates tested (Fig. 3. 3). The dendrogram displayed two main branches. In one branch, ZP1 and ZG7 were clustered together with 85.7% similarity. The other branch was split into two sub-branches, one containing ZP2 and ZP4 and the other ZP3 and ZP5.

The zymogram patterns were grouped according to consistent diagnostic PG bands and new zymogram sub-groups were created. ZP1 isolates, which consisted of canker pathotypes (Chapter 2, Balali *et al.*, 1995a), can be grouped with ZG7 as they displayed the same banding pattern (Neate *et al.*, 1988). This new sub-group was designated ZG7-1. The presence of two PG diagnostic bands 'f' and 'g' in conjunction

Table 3. 2. Genetic similarity (%) revealed for zymogram patterns (ZP) based on the total bands

	ZP1	ZP2	ZP3	ZP4	ZP5	ZG7
ZP1	100					
ZP2	85.7	100				
ZP3	71.4	85.7	100			
ZP4	71.4	85.7	71.4	100		
ZP5	57.1	71.4	85.7	85.7	100	
ZG7	85.7	71.4	57.1	57.1	42.9	100

facing page

Figure 3. 3. Dendrogram based on pectic zymogram pattern (ZP) analysis showing genetic diversity among AG-3 isolates of *Rhizoctonia solani* recovered from potato crops in Virginia and Lenswood, South Australia. The dendrogram was constructed using the method of group average analysis (UPGMA).

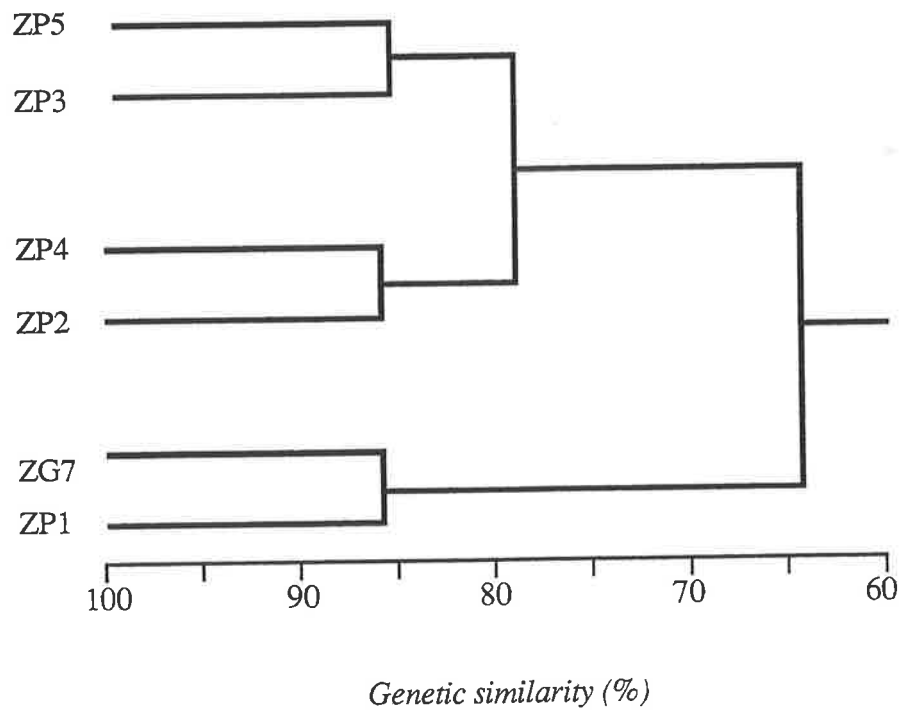


Table 3. 3. Genetic similarity (%) revealed for zymogram groups (ZG) based on consistent reproducible bands

	ZG7-1	ZG7-2	ZG7-3
ZG7-1	100		
ZG7-2	57.1	100	
ZG7-3	42.9	85.7	100

facing page

Figure 3. 4. Dendrogram based on pectic zymogram groups (ZG) analysis showing inter-group genetic diversity among AG-3 isolates of *Rhizoctonia solani* recovered from potato crops in Virginia and Lenswood, South Australia. The dendrogram was constructed based on diagnostic bands using the method of group average analysis (UPGMA).

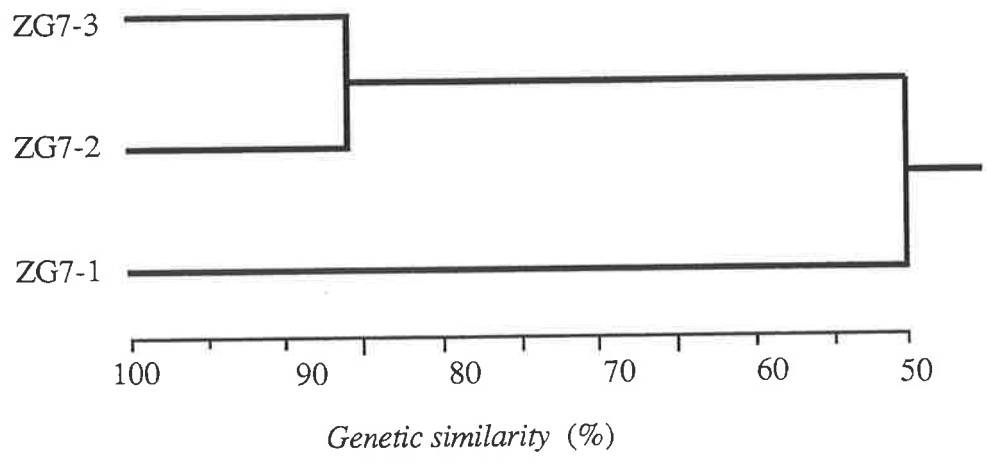


Table 3. 4. Allele frequency in zymogram sub-populations of AG-3 *Rhizoctonia solani* at two electrophoretic loci in geographically different locations

Locus	Allele	Viginia	Lenswood
PE	c	1.00	100
PG (ZG7-1)	d, f	0.575	0.440
PG (ZG7-2)	d, e, f	0.175	0.424
PG (ZG7-3)	d, e, f, g	0.250	0.136

with the inconsistent band 'h' was used as the criterion to group ZP2 and ZP4 isolates together as ZG7-2. Similarly, the presence of three PG bands 'e', 'f' and 'g' and the inconsistent 'h' band, was used to group ZP3 and ZP5 isolates as ZG7-3. As ZG7-2 and ZG7-3 were isolated from tuber-borne sclerotia, these patterns were considered to be black scurf pathotypes (Balali *et al.*, 1995a). Similarly, the ZP6 produced by a single isolate can tentatively be re-designated ZG7-4.

The three zymogram groups; ZG7-1, ZG7-2 and ZG7-3 were considered as sub-groups and their genetic similarities (Table 3. 3) were re-expressed in a second dendrogram (Fig. 3. 4). Zymogram sub-groups ZG7-2 and ZG7-3, designating the black scurf pathotype, were clustered together whereas ZG7-1, associated with stem and root canker, was on a separate branch. Phenotype frequencies based on PE and PG loci for zymogram sub-groups are given in Table 3. 4. All three zymogram groups were identified in both Virginia and Lenswood. The frequency of ZG7-1 was almost the same as ZG7-2 in Lenswood, the two comprising approximately 90% of the AG-3 population examined. In Virginia, however, the frequency of ZG7-1 was 58% of the population, with ZG7-2 and ZG7-3 comprising 18% and 25%, respectively.

All nine AG-3 isolates used in pathogenicity tests (Chapter 2) were found to belong to ZG7-

3. 4. DISCUSSION

Based on electrophoresis of pectic enzymes, isolates of *R. solani* AG-3 obtained from potato plants in South Australia were initially grouped into seven zymogram patterns. One of these zymogram patterns corresponded to the previously described ZG7 (Neate *et al.*, 1988). With the exception of ZP6, which was found only once in the isolates tested, the zymogram patterns were reduced to three zymogram sub-groups; ZG7-1, ZG7-2, ZG7-3 on the basis of consistent bands. Also, ZP6 was tentatively allocated to ZG7-4. Zymogram analyses demonstrated that variation was considerable among the 101 AG-3 isolates tested. Pectic enzyme electrophoresis can, therefore, be used to determine genetic variation within *R. solani* AG-3. Variation in pectic enzymes between isolates of AG-3 reported here agrees with the report of heterogeneity in this anastomosis group based on RAPD-PCR studies by Duncan *et al.* (1993).

Yang *et al.* (1994) suggested that expression of the pectic enzymes in field isolates of *R. solani* is governed by multiple gene loci, and that there was possible linkage among these loci in ZG1-1 of AG-8. The presence of multiple polygalacturonase bands, observed in this study, suggests that expression of alleles is governed at different loci. Also, the presence of the common bands 'c', 'd' and 'f' may indicate that some of these loci are conserved in *R. solani* AG-3. Multiple gene loci could generate several zymogram patterns among the isolates of one anastomosis group. Therefore, a general pattern in the constant bands should be considered as the diagnostic zymogram pattern for closely related isolates, and minor differences in R_f values and weak bands should be ignored. Because pectic enzyme production is induced in zymogram techniques, it is possible that it may be influenced by cultural or nutritional conditions or the physiological state of the fungus. Unequal amounts of pectic enzymes in samples loaded may affect the resolution, R_f values and reproducibility of the bands produced. Genotypes of *R. solani* isolates may change due to heterokaryon formation, anastomosis and mutation and these could affect the loci coding for pectic enzymes and generate new zymogram patterns. Therefore, if the zymogram technique is used to identify *R. solani* strains, the relationship between genetic variability and zymogram group stability should be established.

All isolates from stem and root cankers and some isolates from tuber-borne sclerotia produced ZG7-1, whereas ZG7-2 and ZG7-3-type isolates were recovered only from tuber-borne sclerotia. The canker pathotype, ZG7-1, was more commonly recovered from diseased potato plants than were the ZG7-2 and ZG7-3 were typically black scurf pathotypes. There is evidence that pectic enzyme patterns in isolates of AG-8 can be associated with differences in virulence (Sweetingham *et al.*, 1986) and the role of pectic enzymes in the pathogenicity of *R. solani* isolates has been discussed (Barker & Walker, 1962). The variability in pathogenicity reported here suggests that there is a relationship between pectic enzyme activities of, and disease symptoms caused by, different AG-3 isolates.

See Addendum 3.

- Contradictory data have been presented on the pathogenicity of tuber-borne inoculum of AG-3. James & McKenzie (1972) found that tuber-borne inoculum did not result in seed decay or stem cankers, and Gudmestad *et al.* (1979) concluded that tuber-borne sclerotia did not significantly increase disease severity. In other studies, tuber-borne inoculum has been shown to be the major cause of both cankers and black scurf (Weinhold *et al.*, 1982; Hill & Anderson, 1989; Balali *et al.*, 1995a). It is possible that these observations could be related to variation in pectic enzymes produced by the isolates causing disease.

Sclerotia collected from field-grown tubers varied in shape from domed to flat. Isolates from dome shaped tuber-borne sclerotia were placed into ZG7-1 and ZG7-2, whereas isolates with flat tuber-borne sclerotia were placed into ZG7-3. The latter was less common than ZG7-1 and ZG7-2 and was not recovered from either stem or root cankers.

Host specificity for each anastomosis group could be explained by divergence of each anastomosis group from a common gene pool. Therefore, genetic differentiation may occur gradually in each anastomosis group and different zymogram sub-groups result. Hierarchical clustering of the zymogram sub-groups according to their similarities implied that ZG7-2 and ZG7-3 populations showed a closer relationship to each other than they did to ZG7-1 (Fig 3. 4). Based on isolation frequency it appears that ZG7-1 is a common sub-population for AG-3, and ZG7-2 and ZG7-3 have diverged from this population. The frequency of ZG7-2 in Virginia was considerably higher than in Lenswood, perhaps indicating that ZG7-2 is better adapted to the warmer conditions and or soil type of Virginia or it may be the result of recent introduction on infected seed tubers (Section 2. 4). Conversely, ZG7-1 is more frequent in Lenswood, and may be better suited to cooler conditions.

Pectic zymogram variability may provide a useful tool for analysing pathogen populations and the taxonomic composition of *R. solani* AG-3 populations. This

relationship could be interpreted more precisely using molecular markers in addition to pectic zymograms.

Chapter 4

PCR analysis of variation in pectic zymogram subgroups of *Rhizoctonia solani* AG-3

4. 1. INTRODUCTION

The genetic population structure of *R. solani* is difficult to study because of the lack of suitable techniques. However, a number of molecular markers has recently been used to determine genetic population structure of fungi, including *R. solani*. Isozyme electrophoresis, polymerase chain reaction (PCR) and restriction fragment length polymorphisms (RFLPs) are three methods which have potential to provide markers (Meijer *et al.*, 1994). Pectic enzyme electrophoresis revealed considerable variation within AG-3 isolates of *R. solani* recovered from diseased potato plants (Chapter 3), indicating the usefulness of this method for identification of intra-specific groups and for population genetic study of this pathogen.

RAPD-PCR random markers offer a useful approach for genetic studies, especially for organisms with limited morphological characters (Williams *et al.*, 1990). This technique has proved to be suitable for providing easily obtainable dominant molecular markers for detecting genetic variation. RAPD-PCR has also been used to track plant resistance genes (Paran *et al.*, 1991), for systematics of plant pathogens (Assigbetse *et al.*, 1994; Huang *et al.*, 1995) and for determining evolutionary relationships between plant pathogens (Chen *et al.*, 1993; Doudrick *et al.*, 1993) and also to establish the relative degree of similarity between individuals, populations and species (Guirao *et al.*, 1995).

RAPD markers are polymorphic DNA sequences generated using random oligonucleotide primers and separated by gel electrophoresis. Each primer gives a

different pattern of RAPD markers, and has the potential to detect one or more polymorphic loci in segregating progeny (Doudrick *et al.*, 1993).

Some researchers have demonstrated good agreement between clustering obtained from RAPD-PCR data and that deduced from isozyme data in black *Aspergillus* spp. (Megnegneau *et al.*, 1993). Meijer *et al.* (1994) showed that RAPD-PCR markers provided a classification which was highly correlated with isozyme data and ribosomal DNA RFLPs obtained from *Phomopsis subordinaria*.

Genetic variation in Australian isolates of *R. solani* was analysed by PCR using several primers, including the R1 specific to the intron-exon splice junction region of the consensus sequence for cereals (Weining & Langridge, 1991), and RAPD-PCR by Duncan *et al.* (1993). They found that different anastomosis groups and zymogram groups could be distinguished based on fingerprint patterns generated by RAPD markers. They also reported that fingerprint patterns for the ZG7 (AG-3) isolates differed depending on geographical origins.

The work reported in this chapter aimed to evaluate the ability of the PCR technique to identify zymogram sub-groups, ZG7-1, ZG7-2 and ZG7-3, of *R. solani* AG-3 recovered from potato crops in South Australia.

4. 2. MATERIALS AND METHODS

4. 2. 1. Fungal culture and preparation of mycelia

Isolates of *R. solani* (see Chapter 2) were grown on PDA for 3 days at 25°C in the dark. One disc (5 mm diameter) from the colony margin of each isolate was transferred to 15 ml of potato dextrose broth (PDB, Difco) in Petri dishes (9 cm diameter) and incubated at 25°C in the dark without shaking. After 2-4 days, the mycelia were harvested by filtration through a Millipore vacuum apparatus, washed with sterile distilled water, blotted dry, frozen in liquid nitrogen and stored at -80°C.

4. 2. 2. DNA preparation

Total genomic DNA from each isolate was extracted using a method modified from Raeder & Broda (1985). Frozen mycelia (2g) of each isolate were ground to a fine powder in liquid nitrogen. The ground mycelium was resuspended in five volumes (10 ml) of extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) and mixed gently. The slurry was dispensed (700 μ l) into 1.5 ml Eppendorf tubes and extracted with an equal volume of phenol/chloroform and the phases separated by centrifugation at 15,800 g (r_{av} 7.2 cm) for 1 h. The upper phase was transferred immediately to a new Eppendorf tube and treated with 100 μ g ml⁻¹ RNAase at 37°C for 30 min, then extracted with an equal volume of chloroform/isoamylalcohol (24:1) as described previously. The upper phase was transferred to a new Eppendorf tube and DNA was precipitated with either a 0.54 volume of cold absolute isopropanol or two volumes of cold absolute ethanol. The DNA precipitate was hooked out of solution and transferred to a new tube, washed with cold 70% ethanol containing 10 mM magnesium acetate, vacuum dried and resuspended in TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8). The DNA concentration was estimated by running an aliquot on a 1% agarose gel and comparing the intensity of the band with a Lambda DNA standard after ethidium bromide staining. The DNA was stored at -20°C.

4. 2. 3. PCR primers and conditions

Twenty oligonucleotide primers, OPD-1 to OPD-20, from an Operon 10-mer kit (Operon Technologist, Inc) containing 10-base oligonucleotide primers, and the R1 primer (5'-GTCCATTCAGTCGGTGCT-3') specific to the intron-exon splice junction region of the consensus sequence for cereals (Weining & Langridge, 1991), were used for DNA amplification. The PCR reaction was carried out in 25 μ l vol

containing 67 mM Tris HCl pH 8.8, 16.6 mM (NH₄)SO₄, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 40 ng of genomic DNA, 4 mM MgCl₂, 60 ng of R1 primer or 50pg of oligonucleotide primer and 1U of *Taq* polymerase (Promega). Each reaction was covered with 25µl of light mineral oil (M-3516, Sigma). Amplifications were performed in a FTS-1C thermocycler (Corbett Research). The temperature profile for amplification, using R1 primer, was one cycle at 94°C for 1 min; 6 cycles of 94°C for 1 min, 40°C for 1 min, and 72°C for 2 min; 26 cycles of 94°C for 1 min, 58°C for 1 min, and 72 °C for 10 min (Duncan *et al.*, 1993), followed by one cycle of 25°C for 5 min, and the temperature profile for the Operon 10-mer primers was one cycle at 94°C for 1 min; 40 cycles of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min; one cycle of 72°C for 5 min and one cycle of 25°C for 5 min. Each PCR product was mixed with 5 µl of loading buffer (see appendix) and the products were separated in a 3% agarose gel in TAE buffer (see appendix). Electrophoresis was carried out at 30 V overnight and the gel was stained with ethidium bromide and photographed under UV light. Reproducibility of the bands was tested with two or three replicates for each isolate. The DNA template was omitted from the control reaction to confirm that amplified bands were not primer artefacts.

4. 2. 4. Data analysis

Polymorphisms among isolates within zymogram sub-groups, generated by the R1 primer, were assessed by comparing the PCR profiles of their amplification products. The band sizes were used to estimate genetic similarities between zymogram sub-groups using a similarity matrix. A dendrogram was then constructed based on this matrix to illustrate the relationships among zymogram sub-groups. The methods used are as described in Section 3. 2. 6.

4. 3. RESULTS

DNA from isolates of *R. solani* AG-3 belonging to zymogram sub-groups ZG7-1, ZG7-2 and ZG7-3 was used in PCR reactions (Table 4. 1). The Operon primers, OPD-10, OPD-11 and OPD-12, gave one or two bands in some isolates but DNA amplification was not reproducible for the isolates of AG-3 tested. The other 17 Operon primers did not amplify the DNA from the isolates of AG-3 (data not shown). The R1 primer produced fingerprint patterns in each of the isolates examined and the size of amplified DNA sequences varied from 600 bp to 2500 bp. The weak bands were ignored and 17 bands were scored for R1. The pattern of bands obtained with the R1 primer was consistent for all DNA concentrations, ranging from 10 ng to 60 ng, and was independent of DNA extraction. The fingerprint profiles of R1-amplified DNA of 14 isolates, representing the three different zymogram sub-groups, are shown in Fig. 4. 1.

The 14 AG-3 isolates could be classified into three groups according to the fingerprint patterns obtained with the R1 primer. However, some bands were common among isolates belonging to the different zymogram sub-groups. The isolates corresponding to ZG7-1 were characterised by the presence of a ^{[See Addendum 4}

Although no particular band was characteristic of ZG7-3 isolates, the banding pattern was unique, with a different combination of bands to that displayed by the ZG7-1 and ZG7-2 isolates.

Polymorphisms were detected not only between zymogram sub-groups but also within each sub-group. Some bands were observed in some isolates of a group which were not present in the other isolates of the same group. For example, the fingerprint pattern obtained with R1 for isolate R812 (Fig 4. 1, lane 3), belonging to ZG7-1, was characterised by a sharp band 1080 bp in size. Similarly, isolates R802 and R803 (Fig 4. 1, lanes 6 & 7) were distinguished from the other members of ZG7-

Table 4. 1. Isolates of *R. solani* AG-3 used in the PCR analysis

Isolate code	Zymogram sub-group	Host symptoms	Isolate origin	Location
R37	ZG7-1	stem canker	canker	Virginia
R810	ZG7-1	black scurf	d-sclerotium ^a	Lenswood
R812	ZG7-1	stem canker	canker	Lenswood
R866	ZG7-1	black scurf	d-sclerotium	Lenswood
R873	ZG7-1	black scurf	black scurf	Virginia
R802	ZG7-2	black scurf	i-sclerotium ^b	Lenswood
R803	ZG7-2	black scurf	i-sclerotium	Lenswood
R903	ZG7-2	black scurf	d-sclerotium	Virginia
R911	ZG7-2	black scurf	d-sclerotium	Virginia
R916	ZG7-2	black scurf	d-sclerotium	Virginia
R902	ZG7-3	black scurf	f-sclerotium ^c	Virginia
R904	ZG7-3	black scurf	f-sclerotium	Virginia
R912	ZG7-3	black scurf	f-sclerotium	Virginia
R915	ZG7-3	black scurf	f-sclerotium	Virginia

a= dome-shaped sclerotium

b= sclerotium intermediate between dome shaped and flat

c= flat sclerotium

facing page

Figure 4. 1. PCR fingerprint patterns generated with primer R1 for isolates of *Rhizoctonia solani* AG-3 belonging to three different zymogram sub-groups; ZG7-1, ZG7-2 and ZG7-3, originating from potato crops in South Australia. Lane 1, R37; lane 2, R810; lane 3, R812; lane 4, R866; lane 5, R873; lane 6, R802, lane 7, R803; lane 8, R903; lane 9, R911; lane 10, R916; lane 11, R902; lane 12, R904; lane 13, R912; lane 14, R915. Arrows show: ^{characteristic of} bands ^{and ZG7-2} ZG7-1 isolates.

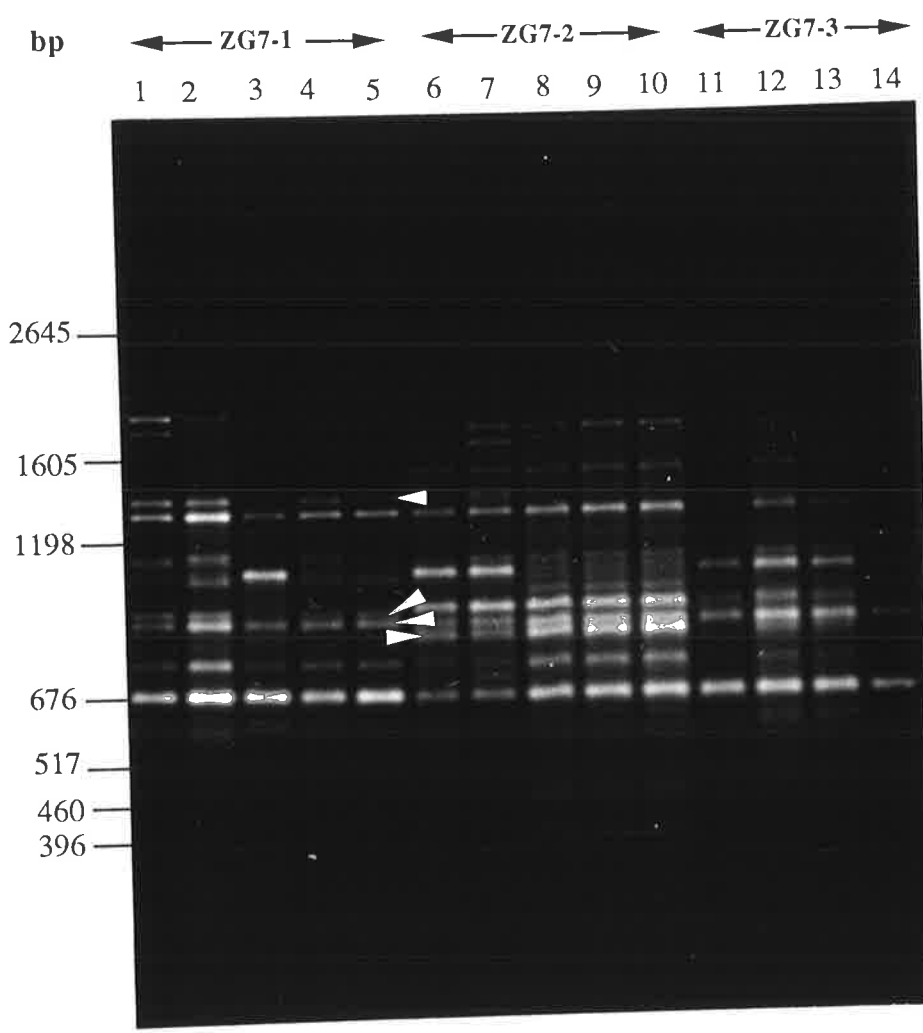
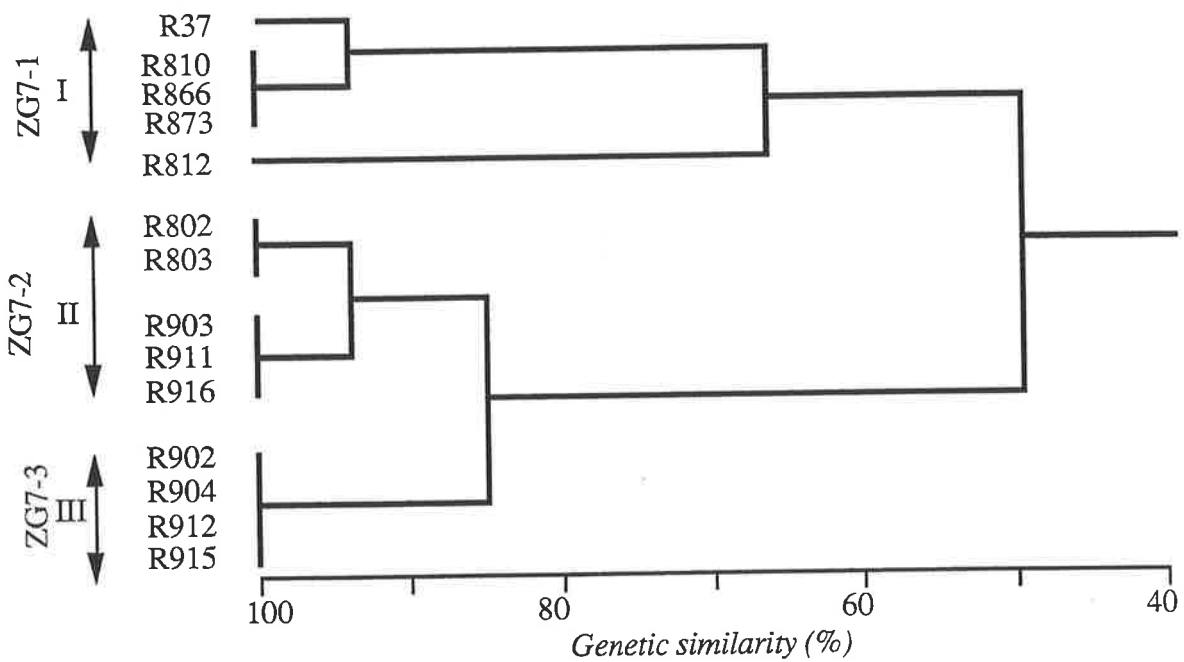


Table 4. 2. Genetic similarity (%) between amplified DNA fragments of isolates of *Rhizoctonia solani* AG-3 revealed by the R1 primer

	R37	R810	R812	R866	R873	R802	R803	R903	R911	R916	R902	R904	R912	R915
R37	100													
R810	94.1	100												
R812	70.6	64.7	100											
R866	94.1	100	64.7	100										
R873	94.1	100	64.7	100	100									
R802	47.1	41.2	41.2	41.2	41.2	100								
R803	47.1	41.2	41.2	41.2	41.2	100	100							
R903	52.9	47.1	47.1	47.1	47.1	94.1	94.1	100						
R911	52.9	47.1	47.1	47.1	47.1	94.1	94.1	100.	100					
R916	52.9	47.1	47.1	47.1	47.1	94.1	94.1	100	100	100				
R902	58.8	52.9	52.9	52.9	52.9	88.2	88.2	82.4	82.4	82.4	100			
R904	58.8	52.9	52.9	52.9	52.9	88.2	88.2	82.4	82.4	82.4	100	100		
R912	58.8	52.9	52.9	52.9	52.9	88.2	88.2	82.4	82.4	82.4	100	100	100	
R915	58.8	52.9	52.9	52.9	52.9	88.2	88.2	82.4	82.4	82.4	100	100	100	100

facing page

Figure 4. 2. Dendrogram of genetic similarities between isolates of zymogram subgroups of *Rhizoctonia solani* AG-3 derived from the amplified PCR products of the R1 primer.



2 by the same band. This band was also present in the members of ZG7-3. One sharp band 676 bp in size was highly conserved in all of the AG-3 isolates tested (Fig 4. 1).

Genetic similarity between representative isolates of zymogram sub-groups obtained with the R1 primer was calculated (Table 4. 2). A dendrogram was constructed based on band similarity (Fig. 4. 2). The dendrogram showed two primary clusters, containing three different groups of isolates. One primary cluster, representing group I isolates, contained only ZG7-1 isolates. The other cluster was sub-divided into groups II and III, containing isolates of ZG7-2 and ZG7-3 respectively (Fig 4. 2). The average similarity between ZG7-2 and ZG7-3 zymogram sub-groups was approximately 85%. However, the similarity value between these two sub-groups and ZG7-1 was about 50%.

Based on the total DNA fingerprint pattern, the R1 primer differentiated three fingerprint types for ZG7-1, two for ZG7-2 and one for ZG7-3 among the isolates of AG-3 tested. Differences in fingerprint profiles between the genotypes of zymogram sub-groups suggested that these sub-groups are genetically distinguishable. There was no overlapping fingerprint pattern between zymogram sub-groups. However, some bands were common to all three sub-groups.

4. 4. DISCUSSION

Field isolates of *R. solani* AG-3 belonging to different pectic zymogram sub-groups were analysed by PCR using the R1 18-base primer and 12 oligonucleotide (10-base primers). Isolates from different pectic zymogram sub-groups were differentiated based on patterns of amplified DNA polymorphisms generated by the R1 primer, whereas the oligonucleotide primers did not produce definite patterns. The results indicate that pectic zymogram sub-groups, ZG7-1, ZG7-2 and ZG7-3 of

R. solani AG-3 may be distinguished by PCR and further support the hypothesis that AG-3 is a heterogenous group.

The pattern of isozyme electrophoresis variation among *R. solani* AG-3 isolates using seven enzymes, α -esterase, diaphorase, fumarase, hexokinase, leucine aminopeptidase, malate dehydrogenase and mannose-6-phosphate isomerase, provided evidence for three sub-groups within AG-3 (Laroche *et al.*, 1992). However, there is no information as to whether these sub-groups are related to the sub-groups reported here. Also, different intra-specific groups within *R. solani* AG-8 have been recognised on the basis of pectic zymogram patterns corresponding with grouping based on RAPD-PCR markers (Duncan *et al.*, 1993). RAPD analysis has been known to be useful in studying intra- and inter-specific variation in black *Aspergillus* spp. (Megnegneau *et al.*, 1993), and is also applicable for isolate typing in *Fusarium solani* f. sp. *cucurbitae* (Crowhurst *et al.*, 1991). Genetic differences among inter-specific groups have been demonstrated in fungi, using RAPD-PCR (Welsh & McClelland, 1990; Williams *et al.*, 1990), including *R. solani* (Duncan *et al.*, 1993). Therefore, it appears that the PCR technique could be useful for studying intra-specific groups of *R. solani* and it provides a rapid means to discriminate isolates of different zymogram sub-groups.

The PCR data obtained in these experiments confirmed that heterogeneity exists between and within zymogram sub-groups of AG-3. Heterogeneity within zymogram group 7 (here renamed ZG7-1) has previously been observed between isolates originating from the same area (Duncan *et al.*, 1993) and the results presented here agreed with these findings, however, these workers examined only 5 isolates of AG-3 and, therefore, were not able to identify sub-groups. The heterogeneity within AG-3 isolates may be related to heterokaryon formation, anastomosis reaction or sexual reproduction of the fungus. During this study basidiospore formation was not observed although basidia and basidiospores have

been reported to be common on diseased potato stems at the end of the growing season (Frank, 1981). The sexual stage has not been reported on potato in Australia.

The results show that it is possible to use PCR technology to identify the major pectic zymogram groups of isolates of *R. solani* AG-3. It seems that in addition to its taxonomic application, PCR may be useful for the detection of genetic variability between and within groups. Cenis (1993) concluded that DNA polymorphisms revealed by RAPD markers were somewhat similar to these shown by RFLPs, consisting of insertions or deletions between priming sites and single base changes that cause mismatches in priming sites. There was no banding profile specific to zymogram sub-groups, but some bands were group-specific. Additional primers and a larger number of isolates could give more accurate identification of AG-3 zymogram sub-groups.

Therefore, the PCR work would need to be repeated with more isolates representing all three zymogram sub-groups, and fingerprinting patterns compared, to ascertain the extent of variation within the zymogram sub-groups described.

Chapter 5

DNA Fingerprinting and RFLP Markers of

Rhizoctonia solani

5. 1. INTRODUCTION

Restriction fragment length polymorphism (RFLP) analysis is a molecular approach which has been used to determine variation at the DNA level in many organisms, including fungi (Anderson *et al.*, 1987; Michelmore & Hulbert, 1987). RFLPs have also been used successfully as genetic markers to determine taxonomic relationships between closely related groups of phytopathogenic fungi (Michelmore & Hulbert, 1987; Braithwaite *et al.*, 1990). These polymorphisms are the result of DNA sequence variations which are detected as a change in the size of fragments obtained after digestion of genomic DNA with a restriction endonuclease enzyme. The RFLP technique has provided a large number of genetic markers in fungi (Anderson *et al.*, 1987; Kohn *et al.*, 1988; Nicholson *et al.*, 1993; McDonald & Martinez, 1990), but little work has been done on *R. solani* (Jabaji-Hare *et al.*, 1990; Vilgalys & Gonzales, 1990; Matthew *et al.*, 1995; O' Brien, 1994).

Various molecular markers, such as ribosomal DNA (rDNA) and random DNA sequences, have been used as probes to study genetic divergence among *R. solani* isolates. Vilgalys & Gonzales (1990) detected considerable molecular variation in the nuclear DNA among and within intra-specific groups of *R. solani* using a rDNA probe. RFLPs in nuclear DNA of different isolates of anastomosis groups also were observed by Jabaji-Hare *et al.* (1990) using a rDNA probe from *Armillaria ostoyae* and a nuclear DNA probe originating from an AG-3 isolate. They observed intra-group RFLP variation in all anastomosis groups using the

rDNA probe, but probes originating from the AG-3 isolate, with one or a few copies per genome, were group specific.

Polymorphisms at the DNA level have been used recently in the analysis of genetic identity, variability and relatedness in fungi (Morjane *et al.*, 1994). In this regard, DNA fingerprinting with multi-locus probes that detect numerous restriction fragment length polymorphisms simultaneously on the same gel (Lynch, 1988; 1990) and also RAPD-PCR (Welsh & McClelland, 1990; Duncan *et al.*, 1993) have received most attention.

Repeated sequences have been used to study clonal variation in fungi such as *Erysiphe graminis* (O'Dell *et al.*, 1989) *Phytophthora* spp. (Panabieres *et al.*, 1989), *Fusarium oxysporum* (Manicom *et al.*, 1987) and *Sclerotinia sclerotiorum* (Kohli *et al.*, 1992). However, little is known about the amount of genetic diversity present in populations of *R. solani*. The first report of the cloning of a dispersed repeated sequence in *R. solani* was for AG-8 isolates (Matthew *et al.*, 1995). This sequence revealed a high degree of polymorphism between AG-8 isolates from South Australia. In addition, Duncan *et al.* (1993) reported genetic diversity for a range of *R. solani* isolates, including AG-3, using RAPD-PCR. Because repeated sequences occur in many fungi, these loci could be useful markers in ecological and evolutionary studies and in the determination of the relationships in a wide range of strains or species.

In this portion of the study the objectives were to develop fingerprinting and RFLP markers which could be used to differentiate polymorphisms among isolates of *R. solani* AG-3, and to use these markers to study population genetics and epidemiology of this group. An AG-3-specific multicopy DNA probe, originating from an AG-3 isolate, which recognised a repeated DNA sequence, and a general low copy DNA probe, originating from an AG-4 isolate, were employed to detect RFLPs between and within anastomosis groups. The term genotype is used, in preference to phenotype, to describe individual banding patterns.

5. 2. MATERIALS AND METHODS

5. 2. 1. Fungal isolates

Isolates of *R. solani* AG-3 recovered from stem and root cankers, tuber-borne sclerotia and soil from potato fields in South Australia, Western Australia and N.S.W (see Table 5. 1), and DNA from the tester strains; isolates R1506 (AG-8) and R84 (binucleate *Rhizoctonia* sp.) from South Australia, S-21 (AG-9) from Alaska, U.S.A. (source, D. Carling) and isolates CS-Ka (AG-1 1A), B-19 (AG-1 1B), PS-4 (AG-2-1), C-96 (AG-2-2), ST-11-6 (AG-3), RH-165 (AG-4), GH-10 (AG-5), OHT-1-1 (AG-6), HO-1556 (AG-7), TS-2-4 (AG-BI) from Japan (source, P. O'Brien), were used in these experiments.

5. 2. 2. Fungal culture and DNA preparation

Isolates of *R. solani* and binucleate *Rhizoctonia* sp. (Chapter 2) were grown in liquid culture and DNA extracted from the mycelia as described in Section 4. 2. 2.

5. 2. 3. Construction of DNA library

5. 2. 3. 1. Insert DNA preparation

The DNA (0.5 µg) from isolates R37 and R49 was digested with the restriction enzyme *Sac*I as recommended by the manufacturer (Boehringer-Mannheim). Digested DNA was extracted with an equal volume of phenol/chloroform as described in Section 4. 2. 2, then ethanol precipitated and the DNA was resuspended in 20 µl TE buffer and stored at -20°C.

5. 2. 3. 2. Vector preparation

Approximately 20 µg of the plasmid vector, pUC19, was digested with *Sac*I, extracted with an equal volume of phenol/chloroform and centrifuged at 15,800 g for 5 min in an Eppendorf tube. The supernatant was transferred to a new tube and extracted with an equal volume of chloroform/isoamylalcohol (24:1) for 2 min, ethanol precipitated, washed as described in Section 4. 2. 2, resuspended in 20 µl of TE buffer and stored at -20°C.

Linearised vector DNA was dephosphorylated with CIP (Calf Intestinal Alkaline Phosphatase) as described by the manufacturer (Boehringer-Mannheim). Approximately 6 µg (9 µl) of the vector DNA was mixed with 5 µl of 10X CIP buffer, 0.5 µl (3 U) CIP and 36.5 µl sterilised distilled water (SDW) to make a total volume of 50 µl and incubated at 37°C for 30 min. The reaction was stopped by adding 2 µl of 0.5 M EDTA, and the mixture was adjusted to 100 µl with SDW and incubated at 65°C for 10 min. The mixture was centrifuged briefly, extracted with phenol/chloroform and chloroform/isoamylalcohol and precipitated overnight with two volumes of cold absolute ethanol at -20°C. The plasmid DNA was recovered by centrifugation at 15,800 g for 20 min. The pellet was washed with cold 70% ethanol, 10 mM magnesium acetate, then resuspended in 10 µl of TE buffer and stored at -20°C.

5. 2. 3. 3. Ligation

A 2 µl volume (0.3 µg) of pUC19 vector DNA and 1 µl (0.2 µg) of insert DNA were combined to make a final concentration of 10 ng DNA ml⁻¹. The final volume of the reaction was adjusted to 50 µl with 5 µl of 10 mM ATP, 1 µl T4 DNA ligase (5 U), 5 µl of 10X ligase buffer (0.5 M Tris, 100 mM MgCl₂, 100 mM dithiothreitol and 500 µg ml⁻¹ bovine serum albumin) and 37.5 µl SDW and incubated at 4°C overnight.

5. 2. 4. Transformation

5. 2. 4. 1. Preparation of competent cells

Escherichia coli strain *JM 101* was grown overnight on Luria Bertani agar (LBA, see appendix) with ampicillin ($50 \mu\text{g ml}^{-1}$) at 37°C . A single colony was removed with a sterile toothpick and suspended into 10 ml of Luria Bertani (LB) broth with ampicillin ($50 \mu\text{g ml}^{-1}$) and incubated overnight on a 37°C shaker incubator. A volume of 500 μl of this culture was added to 100 ml of LB containing ampicillin ($50 \mu\text{g ml}^{-1}$) and placed in a 37°C shaker incubator for approximately 3 h until the $\text{OD}_{600\text{nm}}$ was between 0.2 and 0.3. The cells were pelleted by centrifugation at 3000 g for 5 min at 4°C , the supernatant poured off and the cells resuspended in 40 ml of cold, sterile 0.1 M MgCl_2 . The cells were centrifuged as described above and the pellet resuspended in 20 ml of cold, sterile 0.05 M CaCl_2 and placed on ice. After 30 min, the cells were centrifuged as above and the pellet resuspended in 8 ml 0.05 M CaCl_2 . The cells were used immediately or mixed with sterile glycerol (150 μl glycerol, 850 μl cell suspension), divided into 500 μl aliquots, frozen in liquid nitrogen and stored at -80°C .

5. 2. 4. 2. Transformation of *E. coli*

Ligated DNA (4 μl = 40 ng) was added to 200 μl of fresh competent cells. The mixture was incubated on ice for 30 min and then heat-shocked in a 37°C water bath for 2 min. A 5 ml volume of LB was added and the cell suspension was incubated at 37°C for 1 h. The cells were centrifuged at 2719 g to obtain a pellet which was then resuspended in 400 μl of LB.

Each LBA plate containing ampicillin ($50 \mu\text{g ml}^{-1}$) was overlaid with 3 ml of top agar (see appendix) containing $50 \mu\text{g ml}^{-1}$ ampicillin, 2% X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside) and 24 mg ml^{-1} IPTG (isopropylthio- β -D-

galactoside). A 100 μ l volume of the cell suspension was spread on each plate and the cultures were incubated overnight at 37°C. White colonies containing recombinant DNA were selected and grown on LBA containing 50 μ g ml⁻¹ ampicillin at 37°C.

5. 2. 5. Screening bacterial colonies for the presence of cloned fragments

A slightly modified Grunstein and Hogness (1975) method, described in Sambrook *et al.* (1989), was used to screen bacterial colonies for the presence of cloned fragments. The white recombinant colonies were replica-plated to a nitrocellulose filter (0.45 μ m, BA 85 Schleicher & Schuell membrane filter in a grid pattern) which had been overlaid on LBA plates containing ampicillin (50 μ g ml⁻¹). The plates were incubated overnight at 37°C. The filters, with colonies, were removed and placed in Petri dishes containing 10% SDS for 5 min to lyse the cells. The filters were denatured in two changes of 0.5 M NaOH, 1.5 M NaCl, neutralised in three changes of 1 M Tris-HCl, 1.5 M NaCl, transferred to 2X SSC (see appendix) and dead bacterial cells wiped off gently with non-absorbent cotton wool. The filters were left to air dry at room temperature for 30 min and the liberated single stranded DNA was fixed to the filter using a Gene-Linker UV chamber (Bio-Rad) set at 30 mJoules.

Membranes were pre-hybridised for at least 2 h at 65°C in 20 ml of pre-hybridisation buffer (4X SSC, 5X Denhardt's solution, 0.1% SDS and 100 μ g ml⁻¹ sonicated, denatured herring sperm DNA) in a Hybaid rolling bottle in a Hybaid oven. Hybridisation was performed in 10 ml hybridisation buffer (4X SSC, 2X Denhardt's solution, 0.1% SDS and 100 μ g ml⁻¹ sonicated, denatured herring sperm DNA) containing 100 μ l of freshly denatured ³²P labelled DNA probe at 65°C in the

hybridisation oven overnight. At the end of hybridisation, the filters were washed in 2X SSC, 0.1% SDS at 65°C (3 X 30 min) and then left on paper towels to air dry.

The filters were exposed to X-ray film (Fuji) for 1 to several days at -80°C using intensifying screens. The film was developed in Ilford (Penisol) X-ray developer for 3 min and fixed in Ilford rapid fixer.

5. 2. 6 Storage of DNA library

Bacteria containing DNA inserts were grown overnight at 37°C on LBA containing ampicillin (50 µg ml⁻¹). Each colony was removed with a sterilised toothpick, mixed with 750 µl LB and 250 µl sterile glycerol in 1.5 ml Eppendorf tubes. The tubes were vortexed, frozen in liquid nitrogen and stored at -80°C.

5. 2. 7. Plasmid DNA mini-preparation

Transformed bacteria containing putative low to high copy clones were grown overnight on LBA containing ampicillin (50 µg ml⁻¹) at 37°C. Cells were removed from single colonies with the blunt end of a sterile toothpick and suspended in 300 µl sucrose buffer (15% sucrose, 50 mM Tris-HCl, 50 mM EDTA) in a sterile Eppendorf tube. 30 µl of 10 mg ml⁻¹ lysosyme solution (freshly made in sucrose buffer) was added to a final concentration of 1 mg ml⁻¹ and the suspension incubated on ice for 10 min. A volume of 40 µl of 5% Triton buffer was added to a final concentration of 0.5% and mixed very gently. The mixture was boiled for 40 s and placed immediately on ice for 3 to 5 min, then centrifuged at 15,800 g for 20 min. The supernatant was treated with RNaseA (10 mg ml⁻¹) at room temperature for 20 min and extracted with an equal volume of phenol/chloroform followed by chloroform/isoamylalcohol as described in Section 4. 2. 2. Finally, DNA was precipitated with two volumes of cold absolute ethanol, incubated at -20°C for 2 h,

centrifuged at 15,800 g for 5 min and the DNA pellet was resuspended in 20 μ l of TE buffer. The sizes of the cloned fragments were estimated by digestion with *Sac*I and separation of the vector and insert fragments by electrophoresis on 1% agarose gels.

5. 2. 8. Preparation of labelled DNA probes

Plasmid clones were labelled with [α -³²P]dCTP by the random primer method, using a Megaprime labelling kit (Amersham). In a 1.5 ml Eppendorf tube, 3 μ l (25 ng) of template DNA was mixed with 5 μ l of primer solution containing random 9-mer primers and 15 μ l SDW. The mixture was denatured on a hot plate at 100°C for 5 min, placed on ice and centrifuged a few seconds. Then, 10 μ l of labelling buffer (Amersham) containing dATP, dGTP and dTTP in Tris-HCl, pH 7.5, MgCl₂ and 2-mercaptoethanol, 10 μ l SDW, 5 μ l [α -³²P]dCTP (50 microcuries) and 2 μ l (2 U) Klenow enzyme were added, and the mixture incubated in a 37°C water bath for 25 min. The reaction was stopped by the addition of 2 μ l of 0.5 M EDTA and the volume of the reaction mixture was made to 100 μ l with equilibration buffer containing 2% SDS, 10 mM Tris-HCl and 1 mM EDTA.

Unincorporated nucleotides were separated from the labelled DNA on a Bio-Gel (Bio-Rad) column, made in a 400 μ l Eppendorf tube. A hole was made in the bottom of the tube using a syringe (27G x 1/2'), then the tube was filled with 100 μ l of 50-100 mesh Bio-Gel P-60 then overlaid with 400 μ l of 100-200 mesh Bio-Gel P-60 (Bio-Rad) which had been equilibrated with 2% SDS, 10 mM Tris-HCl and 1 mM EDTA. The tube was placed in a 1.5 ml Eppendorf tube and centrifuged for 2 min at low speed and the column was washed with 100 μ l of equilibration buffer. The column was placed in a new 1.5 ml Eppendorf tube. The labelling reaction mixture was added to the top of the column and the tube was centrifuged at low

speed (1000 r.p.m.) for 2 min. Approximately 100 µl eluate was collected and used in the hybridisation reaction.

5. 2. 9. DNA electrophoresis

Agarose gel electrophoresis was carried out using the method of Sambrook *et al.* (1989). The *Sac*I restricted DNA was size fractionated in a 1% agarose gel in TAE buffer. The DNA was mixed with loading buffer (see appendix) and loaded into the wells. *Hind* III digested Lambda fragments were used as size markers. Electrophoresis was carried out at 30 V overnight, after which the gels were stained with ethidium bromide (Sambrook *et al.*, 1989) to visualise DNA bands using a UV transilluminator.

5. 2. 10. Southern blotting

Southern blotting was carried out according to Southern (1975, cited in Sambrook *et al.*, 1989) with minor modification, as described below, to immobilise single stranded DNA fragments to a nitrocellulose membrane.

Gels containing DNA fragments were denatured in two volumes of denaturing buffer (0.5 M NaOH, 1.5 M NaCl) on a rocking platform for 30 min. The denaturing buffer was poured off and the gel was gently shaken in two volumes of neutralising buffer (1 M Tris, 1.5 M NaCl) at room temperature for 30 min. The denatured DNA was transferred to nitrocellulose membranes (Schleicher & Schuell) overnight using the capillary transfer method (Sambrook *et al.*, 1989). The gel was removed from the neutralisation solution and inverted on the support so that it was centred on wet Whatman 3 MM filter papers. A pre-wetted nitrocellulose filter, cut to fit the gel, was placed on the top of the gel and three pieces of 3 MM filter papers were pre-wetted in 2X SSC and placed on top of the wet nitrocellulose filter. A

stack of paper towels was placed on the 3 MM filter papers. A glass plate and a 400g weight were placed on the top of the paper towels. Transfer of DNA was allowed to proceed for 12-16 h. The gel was turned over and the position of the gel slots marked on the filter with a soft graphite pencil. The membrane was peeled off, rinsed briefly with 2X SSC and air-dried on tissue paper for 30 min at room temperature. The DNA was fixed into the filter using a Gene-Linker UV Chamber (Bio-Rad) set at 150 mJoules (for wet membranes).

5. 2. 11. Slot blot analysis

Soil samples were collected, to a depth of 20 cm, from infested potato fields in Virginia and Lenswood. Four soil samples (1 kg each) from the same field were mixed and one sub-sample (100 g) was taken. Each sub-sample was washed through three sieves, 411, 211 and 105 μm mesh size and organic matter of particle size 105-211 μm was collected as described in Section 2. 2. 3, air dried at room temperature and stored at -80°C . Total DNA was extracted and purified from the soil organic matter as described by Whisson *et al.* (1995). A Bio-dot slot format micro-filtration unit with a Zeta-probe nylon membrane was assembled according to the manufacturer's instructions (Bio-Rad) and DNA samples were loaded into the unit.

5. 2. 12. Data analysis

Similarities among the RFLP genotypes were calculated as described in Section 3. 2. 5. The cluster analysis was performed based on these similarity values using the group average method (UPGMA, Sneath & Sokal, 1973). The similarity between two genotypes is the average similarity of all points of unit involving a

member of each group (Harvey, 1993). The GENSTAT 5 statistical program, release 1.3 (Rothamsted Experimental Station, 1989) was used for data analysis.

5. 3. RESULTS

5. 3. 1 DNA fingerprinting and RFLP markers originating from AG-3 isolate R37

From a total of 400 recombinant clones, 35 were screened for insert *SacI* fragments by gel electrophoresis. Insert sizes ranged from 0.5 to 5.5 kbp. A number of clones was selected for hybridisation to Southern blots of *SacI* digested total DNA of the source isolate R37. Only one clone, pR3718, which contained an insert of 3 kbp in size, recognised a repeated DNA sequence in the R37 isolate, while the other clones recognised low copy DNA sequences.

The pR3718 clone was hybridised to Southern blots of *SacI* digested total DNA from the tester strains listed in Section 5. 2. 1 as well as several South Australian AG-3 isolates, including R37. Clone pR3718 hybridised strongly to DNA of AG-3 isolates only, whereas no or only weak reactions were detected for DNA of isolates of other anastomosis groups and for *Rhizoctonia* sp. (Fig. 5. 1).

5. 3. 2. Fingerprint genotypes identified with pR3718 and *SacI*

Fingerprint analysis was carried out on *R. solani* AG-3 isolates which had been recovered from potato growing areas in South Australia. DNA isolated from 152 AG-3 isolates of *R. solani* (Table 5. 1), was cut with *SacI* and hybridised with the dispersed, repetitive DNA probe pR3718. The majority of the isolates (137) tested was recovered from Virginia and Lenswood.

The probe hybridised strongly to total DNA of all 152 AG-3 isolates, regardless of origin, and revealed a total of 48 different fingerprint patterns, designated 'S1' to 'S48' (Figs 5. 2a, 5. 2b, 5. 2c & 5. 2d). In all isolates tested, 12-19 *SacI* fragments,

ranging size from 0.1 to 23 kbp, hybridised with the pR3718 insert, indicating the presence of a highly repeated and dispersed DNA sequence. Of the 48 different genotypes, 14 were identified from Virginia and 23 from Lenswood. In addition, more than 50% (28/48) of the fingerprint patterns were unique. One fingerprint genotype, 'S1' was common to both Virginia and Lenswood and another fingerprint genotype, 'S9' was common to Lenswood and Walkers Flat (Table 5. 1). 'S18' was found only in Virginia and at a very high frequency. The remaining genotypes originated from locations other than South Australia. From 107 characteristic bands, one band (3 kbp) was common to 95% of the isolates, while 22 bands were unique to individuals and the remainder were shared among different fingerprint genotypes (Table 5. 1). Of the 22 unique bands, 15 belonged to the unique fingerprint genotypes. The large number of distinctive restriction fragment patterns generated by the probe, pR3718, indicated that a high degree of variation, with many different genotypes, exists for AG-3 isolates of *R. solani* present in the potato growing areas of South Australia. The autoradiograms showed that the R37 DNA fragment contained in the pR3718 clone hybridised to itself (3 kbp band) and was also present in the majority of the AG-3 isolates tested. However, as the probe hybridised to many other fragments, part or all of this sequence is found elsewhere in the genome, where it lacks or has additional *SacI* restriction sites. The fingerprint analysis also showed that five *SacI* fragments, with sizes of 0.8, 1.6, 3, 3.4 and 4.3 kbp, were common to all the AG-3 isolates tested while the standard AG-3 isolate from Japan (ST-11-6) exhibited only three common bands (1.6, 3 and 3.4 kbp). Most polymorphisms were observed among fragments more than 5 kbp in size.

Cluster analysis of the similarity values (Table 5. 2) assigned the 48 genotypes into four primary groups, each of which was divided into a number of sub-clusters (Fig. 5. 3). Group 1 was divided into two sub-clusters containing isolates of the same geographical origin (Lenswood) except for genotype 'S4' originating from Virginia. Group 2 consisted mainly of isolates originating from Virginia, except for genotype 'S21' from Western Australia and genotype 'S11' from Lenswood. Groups 3 and 4 were more sub-divided, containing isolates from diverse geographical regions. However, in

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Figure 5. 1. Southern hybridisation analysis of total DNA from different anastomosis groups of *Rhizoctonia solani*, generated by the clone pR3718. The clone pR3718 was used to probe *Sac*I digests of 5 µg of DNA from representatives of anastomosis groups. Lane 1, AG-1 1A; lane 2, AG-1 1B; lane 3, AG-2-1; lane 4, AG-2-2; lane 5, AG-4; lane 6, AG-3 (Japan); lane 7, AG-5; lane 8, AG-3 (R47); lane 9, AG-6; lane 10, AG-7; lane 11, AG-8; lane 12, AG-9; lane 13, AG-BI; lane 14, AG-3 (R37, origin of probe); lane 15, AG-4 (R49); lane 16, AG-3 (R55); lane 17, binucleate *Rhizoctonia* sp. (R84). Molecular size markers in kilobase pairs (kbp) are shown on the left.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

23.1 —

9.4 —

6.5 —

4.3 —

2.3 —

2.0 —

0.5 —

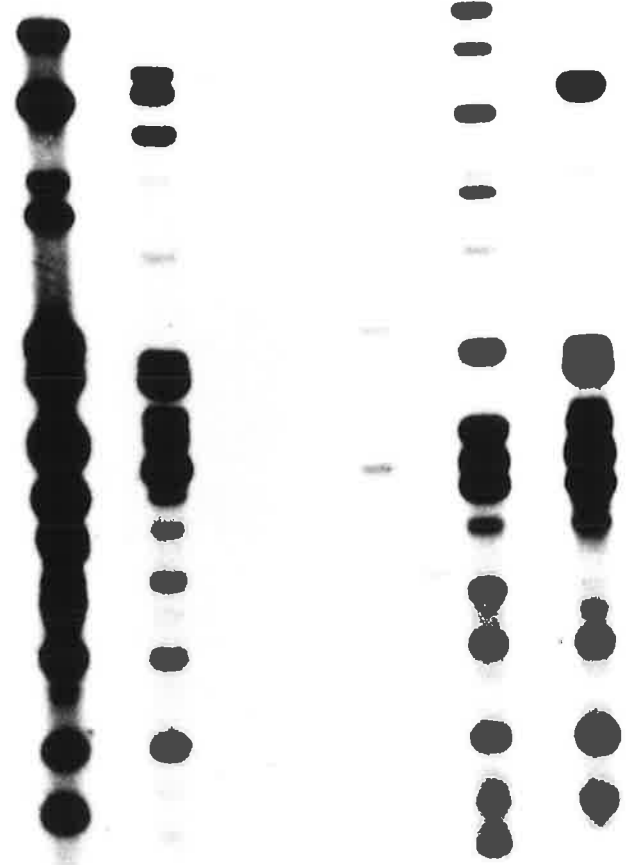


Table 5. 1. Number and frequency of *SacI* fingerprint genotypes, total of *SacI* fragments and unique fragments detected among AG-3 isolates of *Rhizoctonia solani* by the probe pR3718

<i>SacI</i> fingerprint pattern	No. of isolates	Total <i>SacI</i> fragments	Location	Unique <i>SacI</i> fragment (kbp)	Frequency
S1	15	19	Lenswood	19.6	0.099
S1	23	19	Virginia	19.6	0.152
S2	3	13	Lenswood	-	0.019
S3	3	16	Lenswood	6.7 & 3.9	0.019
S4	1	10	Lenswood	-	0.006
S5	1	12	Virginia	-	0.006
S6	1	16	Lenswood	4.5	0.006
S7	2	16	Lenswood	-	0.013
S8	2	15	Lenswood	-	0.013
S9	4	14	Lenswood	-	0.026
S9	1	14	Walkers Flat	-	0.006
S10	8	11	Lenswood	1.9	0.052
S11	2	13	Lenswood	-	0.013
S12	1	13	Lenswood	-	0.006
S13	1	13	Lenswood	4.7	0.006
S14	1	17	Lenswood	23.0 & 17.0	0.006
S15	1	12	Virginia	15.4	0.006
S16	7	17	Virginia	13.8	0.046
S17	1	13	Virginia	-	0.006
S18	22	13	Virginia	-	0.145
S19	9	14	Virginia	5.2	0.059
S20	1	11	Lenswood	-	0.006
S21	1	17	Western Australia	-	0.006
S22	4	11	Virginia	-	0.026
S23	4	14	Lenswood	-	0.026
S24	1	13	Lenswood	-	0.006
S25	1	14	Virginia	12.8	0.006
S26	2	17	Lenswood	-	0.013
S27	1	13	Lenswood	17.6	0.006
S28	1	15	Lenswood	-	0.006
S29	1	13	Lenswood	9.9 & 9.2	0.006
S30	1	19	South East	-	0.006
S31	1	19	South East	6.5	0.006
S32	1	18	Virginia	-	0.006
S33	1	14	WalkersFlat	-	0.006
S34	4	19	Virginia	-	0.026
S35	1	17	South East	7.8	0.006
S36	2	12	Virginia	-	0.013
S37	1	18	Riverland	-	0.006
S38	1	16	Currency Creek	8.0	0.006
S39	1	14	Virginia	-	0.006
S40	2	17	Waite Campus	-	0.013
S41	1	18	Lenswood	-	0.006
S42	1	15	N.S.W. ^a	-	0.006
S43	1	11	N.S.W.	5.1	0.006
S44	2	18	Lenswood	20.8 & 8.3	0.013
S45	1	14	Virginia	13.4	0.006
S46	1	17	Lenswood	-	0.006
S47	1	11	South Australia	-	0.006
S48	2	16	Japan	-	0.013

Lenswood, Virginia, Walkers Flat, South East, River Land, Currency Creek and Waite Campus are all locations in South Australia

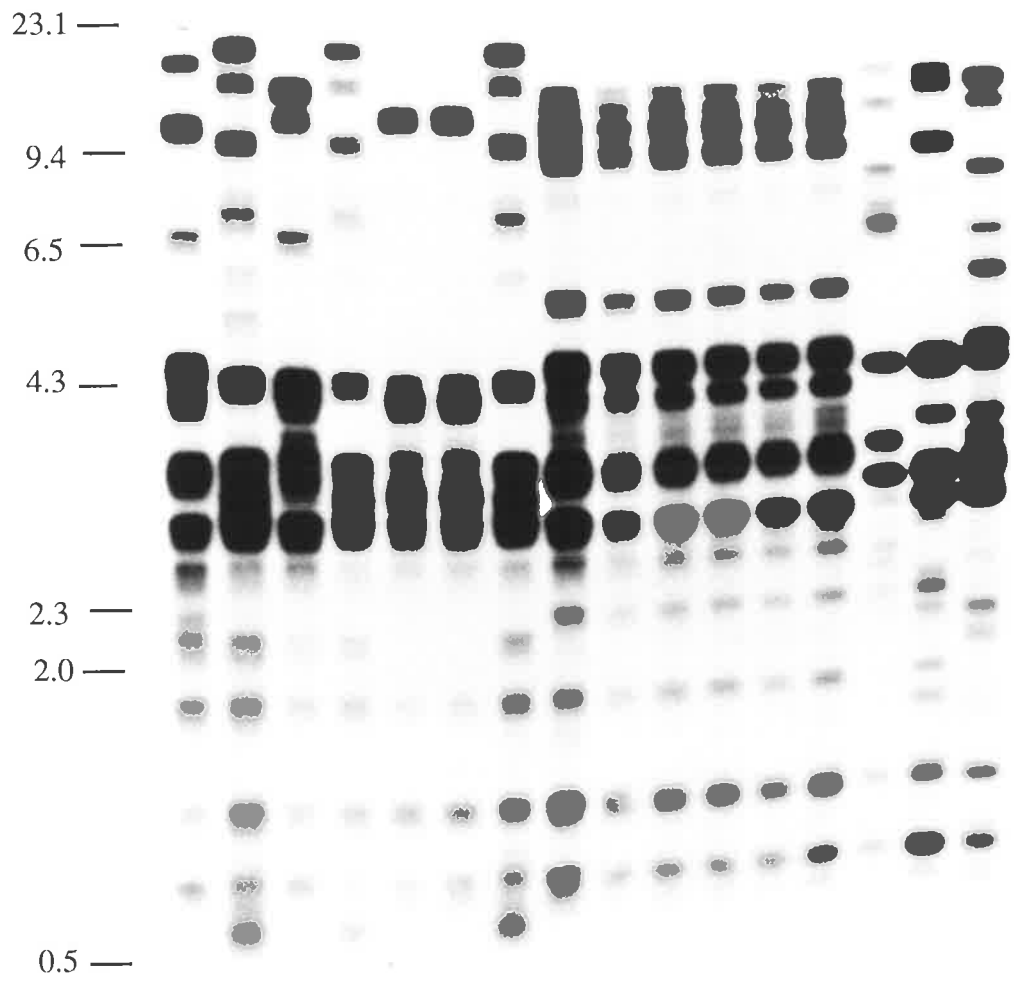
^a= New South Wales, Australia

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Figure 5. 2. Examples of Southern blot hybridisation analysis of total DNA from different isolates of *R. solani* AG-3 recovered from potato crops in South Australia, generated by pR3718. The clone pR3718 was used to probe *SacI* digests of 5 µg of DNA from isolates of AG-3.

Figure 5. 2a. Lane 1, ST-11-6 (AG-3 standard, genotype 'S48'); lane 2, R37 (AG-3, origin of probe, genotype 'S1'); lane 3, R870 (genotype 'S17'); lane 4, R873 (genotype 'S1'); lanes; 5, R874 and 6, R875 (genotype 'S22'); lane 7, R877 (genotype 'S1'); lanes 8, R879; 9, 880; 10, R881; 11, R882; 12, R883 and 13, R884 (all genotype 'S16'); lane 14, R885 (genotype 'S15'); lane 15, R51 (genotype 'S11') and lane 16, L62 (genotype 'S21'). Lambda size markers (kbp) are shown on the left.

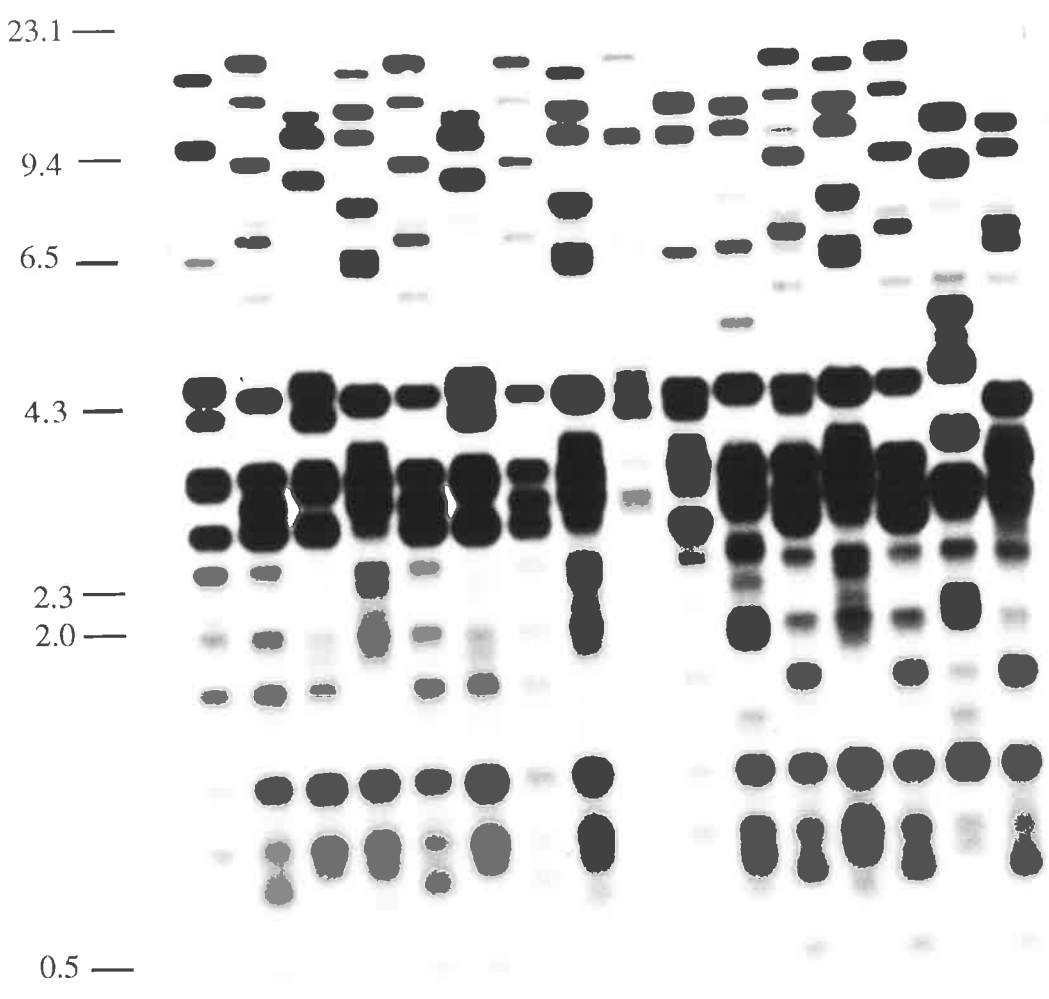
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16



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Figure 5. 2b. Lane 1, ST-11-6 (AG-3 standard, genotype 'S48'); lane 2, R37 (AG-3, origin of probe, genotype 'S1'); lane 3, R823 (genotype 'S2'); lane 4, R824 (genotype 'S3'); lane 5, R825 (genotype 'S1'); lane 6, R826 (genotype 'S2'); lane 7, R827 (genotype 'S1'); lane 8, R828 (genotype 'S3'); lane 9, R829 (genotype 'S4'); lane 10, R830 (genotype 'S5'); lane 11, R840 (genotype 'S6'); lane 12, R842 (genotype 'S1'); lane 13, R843 (genotype 'S3'); lane 14, R844 (genotype 'S1'); lane 15, R845 (genotype 'S8') and lane 16, R846 (genotype 'S9'). Lambda size markers (kbp) are shown on the left.

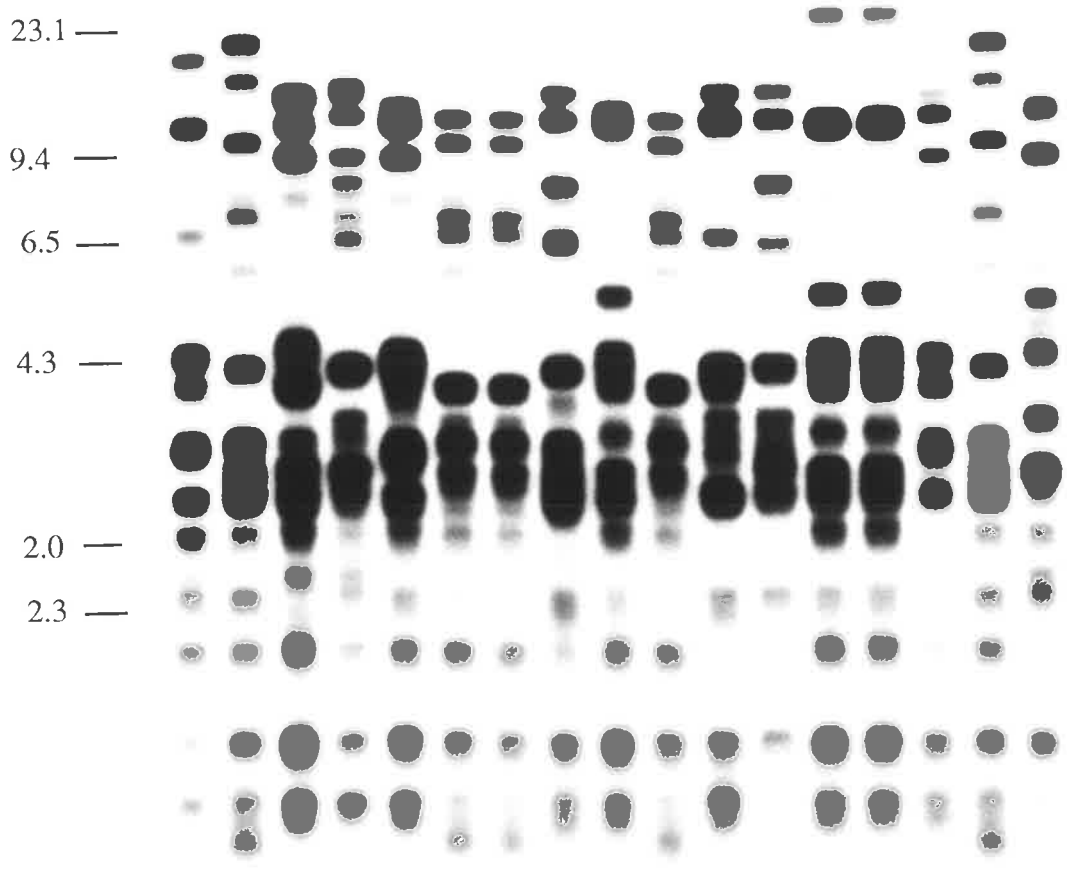
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16



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Figure 5. 2c. Lane 1, ST-11-6 (AG-3 standard, genotype 'S48'); lane 2, R37 (AG-3, origin of probe, genotype 'S1'); lane 3, R816 (genotype 'S26'); lane 4, R850 (genotype 'S29'); lane 5, R851 (genotype 'S26'); lane 6, R852 (genotype 'S9'); lane 7, R853 (genotype 'S9'); lane 8, R854 (genotype 'S30'); lane 9, R856 (genotype 'S28'); lane 10, R857 (genotype 'S9'); lane 11, R858 (genotype 'S27'); lane 12, R861 (genotype 'S30'); lane 13, R862 (genotype 'S23'); lane 14, R863 (genotype 'S23'); lane 15, R865 (genotype 'S2') and lane 16, R866 (genotype 'S1') and lane 17, L1 (genotype 'S8'). Lambda size markers (kbp) are shown on the left.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17



0.5 —

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Figure 5. 2d. Lane 1, ST-11-6 (AG-3 standard, genotype 'S48'); lane 2, R37 (AG-3 origion of probe, genotype 'S1'); lane 3, R801 (genotype 'S12'); lanes 4, R802 and 5, R803 (genotype 'S11'); lane 6, R804 (genotype 'S8'); lane 7, R805 (genotype 'S10'); lanes 8, R806; 9, R807; 10, R808; 11, R809 and 12, R810 (all genotype 'S1") lanes 13, R812; 14, R813 and 15, R814 (genotype 'S10'); lane 16, R817 (genotype 'S13'); lanes 17, R820 and 18, R822 (genotype 'S1'). Lambda size markers (kbp) are shown on the left.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

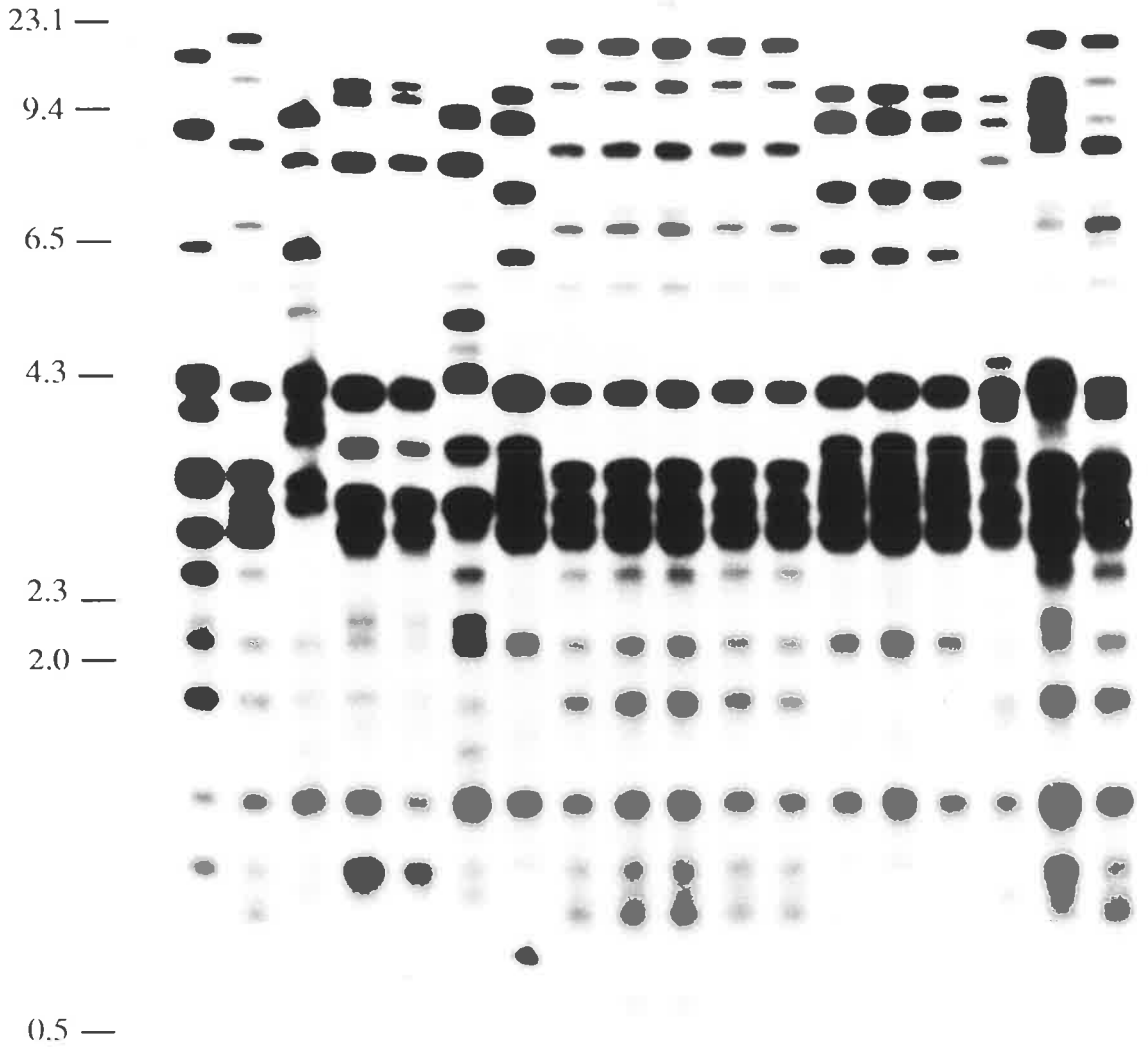


Table 5. 2. Genetic similarity (%) between fingerprint genotypes of *Rhizoctonia solani* AG-3 isolates revealed by the probe pR3718 and *SacI*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
S1	100																								
S2	75.7	100																							
S3	72.9	87.9	100																						
S4	76.6	89.7	83.2	100																					
S5	76.6	89.7	86.9	86.9	100																				
S6	74.8	86.0	86.9	81.3	83.2	100																			
S7	76.6	78.5	73.8	81.3	79.4	77.6	100																		
S8	75.7	86.9	82.2	86.0	86.0	86.0	80.4	100																	
S9	76.6	84.1	83.2	86.9	85.0	83.2	86.9	82.2	100																
S10	77.6	79.4	78.5	80.4	84.1	78.5	78.5	77.6	82.2	100															
S11	77.6	75.7	74.8	78.5	78.5	76.6	80.4	79.4	78.5	81.3	100														
S12	77.6	79.4	78.5	82.2	84.1	78.5	78.5	79.4	78.5	83.2	79.4	100													
S13	77.6	85.0	80.4	82.2	82.2	80.4	80.4	75.7	80.4	86.9	75.7	86.9	100												
S14	75.4	79.4	78.5	78.5	78.5	80.4	71.0	77.6	74.8	81.3	77.6	79.4	85.0	100											
S15	76.6	78.5	79.4	81.3	81.3	81.3	83.2	86.0	79.4	80.4	86.0	78.5	76.6	78.5	100										
S16	77.6	77.6	74.8	76.6	80.4	78.5	74.8	79.4	76.6	79.4	85.0	77.6	73.8	77.6	80.4	100									
S17	79.4	81.3	78.5	82.2	82.2	80.4	80.4	81.3	80.4	81.3	85.0	81.3	81.3	81.3	84.1	88.8	100								
S18	81.3	77.6	74.8	80.4	82.2	76.6	82.2	77.6	82.2	85.0	81.3	77.6	79.4	77.6	84.1	79.4	83.2	100							
S19	78.5	84.1	79.4	81.3	83.2	81.3	79.4	78.5	79.4	82.2	78.5	78.5	80.4	78.5	83.2	80.4	82.2	87.9	100						
S20	83.2	81.3	78.5	84.1	86.0	80.4	82.2	81.3	86.0	85.0	85.0	83.2	81.3	77.6	84.1	79.4	81.3	88.8	82.2	100					
S21	79.4	75.7	71.0	78.5	74.8	71.0	78.5	75.7	74.8	77.6	85.0	77.6	75.7	75.7	84.1	81.3	83.2	81.3	78.5	79.4	100				
S22	80.4	84.1	81.3	85.0	85.0	83.2	79.4	82.2	79.4	82.2	84.1	82.2	82.2	82.2	85.0	86.0	93.5	82.2	85.0	82.2	80.4	100			
S23	82.2	80.4	75.7	83.2	85.0	79.4	81.3	82.2	83.2	82.2	78.5	80.4	80.4	74.8	79.4	76.6	80.4	82.2	77.6	89.7	74.8	83.2	100		
S24	87.9	78.5	77.6	81.3	79.4	81.3	79.4	78.5	79.4	82.2	80.4	82.2	82.2	80.4	81.3	78.5	84.1	80.4	79.4	86.0	80.4	86.9	88.8	100	

Table 5. 2. continued 1

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
S25	86.9	77.6	74.8	78.5	78.5	80.4	74.8	81.3	76.6	79.4	79.4	79.4	77.6	79.4	80.4	81.3	81.3	83.2	76.6	85.0	81.3	82.2	82.2	87.9
S26	85.0	81.3	80.4	84.1	84.1	82.2	84.1	85.0	86.0	81.3	83.2	83.2	81.3	83.2	86.0	81.3	85.0	85.0	82.2	88.8	81.3	84.1	87.9	89.7
S27	78.5	82.2	79.4	81.3	81.3	81.3	75.7	82.2	79.4	80.4	78.5	78.5	80.4	78.5	77.6	76.6	82.2	80.4	77.6	80.4	72.9	83.2	83.2	81.3
S28	81.3	77.6	76.6	80.4	82.2	78.5	78.5	79.4	82.2	81.3	79.4	81.3	81.3	79.4	80.4	79.4	81.3	81.3	80.4	83.2	73.8	82.2	86.0	87.9
S29	80.4	80.4	77.6	83.2	81.3	77.6	75.7	78.5	81.3	82.2	80.4	84.1	84.1	78.5	79.4	74.8	80.4	78.5	77.6	86.0	76.6	81.3	83.2	81.3
S30	79.4	75.7	74.8	78.5	80.4	78.5	78.5	79.4	80.4	81.3	77.6	79.4	79.4	73.8	80.4	73.8	75.7	81.3	76.6	92.5	72.0	76.6	89.7	84.1
S31	79.4	77.6	76.6	80.4	84.1	78.5	80.4	79.4	84.1	85.0	83.2	81.3	83.2	79.4	82.2	79.4	81.3	83.2	80.4	88.8	75.7	82.2	87.9	86.0
S32	81.3	79.4	74.8	82.2	84.1	78.5	80.4	81.3	82.2	83.2	77.6	77.6	81.3	75.7	78.5	75.7	79.4	85.0	78.5	88.8	73.8	82.2	95.3	87.9
S33	79.4	79.4	76.6	82.2	84.1	78.5	78.5	79.4	82.2	81.3	77.6	81.3	81.3	77.6	78.5	75.7	79.4	81.3	80.4	85.0	73.8	82.2	86.0	84.1
S34	84.1	78.5	75.7	81.3	83.2	77.6	81.3	80.4	85.0	84.1	80.4	78.5	82.2	78.5	79.4	76.6	80.4	84.1	79.4	87.9	76.6	81.3	90.7	88.8
S35	81.3	75.7	72.9	76.6	76.6	74.8	80.4	75.7	82.2	75.7	79.4	75.7	75.7	72.0	80.4	77.6	79.4	79.4	76.6	81.3	79.4	78.5	80.4	78.5
S36	77.6	80.4	77.6	77.6	77.6	79.4	81.3	78.5	83.2	77.6	73.8	72.9	76.6	72.9	77.6	75.7	76.6	80.4	79.4	81.3	79.4	75.7	76.6	76.6
S37	80.4	78.5	75.7	77.6	75.7	83.2	79.4	78.5	79.4	78.5	82.2	80.4	82.2	78.5	79.4	80.4	84.1	80.4	81.3	78.5	80.4	83.2	77.6	81.3
S38	86.9	75.7	72.9	78.5	78.5	76.6	76.6	75.7	76.6	77.6	81.3	81.3	77.6	77.6	76.6	81.3	83.2	81.3	80.4	83.2	83.2	86.0	82.2	87.9
S39	81.3	77.6	74.8	80.4	78.5	76.6	84.1	77.6	84.1	77.6	83.2	77.6	79.4	73.8	84.1	79.4	86.9	81.3	80.4	85.0	81.3	84.1	80.4	82.2
S40	76.6	78.5	79.4	83.2	79.4	85.0	79.4	84.1	85.0	80.4	86.0	80.4	78.5	78.5	85.0	78.5	84.1	82.2	79.4	86.0	76.6	83.2	81.3	81.3
S41	80.4	71.0	70.1	73.8	75.7	75.7	77.6	74.8	77.6	82.2	80.4	78.5	76.6	76.6	81.3	76.6	78.5	82.2	73.8	82.2	78.5	79.4	83.2	85.0
S42	74.8	76.6	75.7	81.3	77.6	79.4	79.4	78.5	83.2	76.6	80.4	84.1	78.5	74.8	77.6	76.7	82.2	78.5	75.7	82.2	78.5	81.3	77.6	81.3
S43	76.6	80.4	77.6	81.3	77.6	81.3	79.4	78.5	79.4	80.4	78.5	84.1	82.2	76.6	77.6	74.8	80.4	80.4	79.4	82.2	82.2	81.3	77.6	83.2
S44	83.2	81.3	80.4	78.5	84.1	76.6	76.6	79.4	80.4	79.4	79.4	81.3	79.4	81.3	78.5	79.4	83.2	79.4	78.5	81.3	79.4	82.2	78.5	82.2
S45	82.2	78.5	73.8	77.6	81.3	75.7	77.6	82.2	79.4	78.5	80.4	76.6	76.6	76.6	79.4	76.6	78.5	80.4	73.8	86.0	80.4	77.6	85.0	85.0
S46	77.6	79.4	76.6	80.4	82.2	80.4	86.0	83.2	86.6	81.3	79.4	77.6	81.3	77.6	82.2	75.7	77.6	86.9	78.5	86.9	79.4	76.6	84.1	80.4
S47	81.3	85.0	80.4	86.0	82.2	84.1	86.0	85.0	87.9	81.3	79.4	81.3	83.2	79.4	84.1	77.6	81.3	83.2	82.2	86.9	83.2	80.4	84.1	86.0
S48	86.0	76.6	73.8	77.6	79.4	73.8	75.7	78.5	77.6	78.5	78.5	78.5	74.8	76.6	79.4	76.6	78.5	80.4	75.7	84.1	78.5	83.2	86.9	88.8

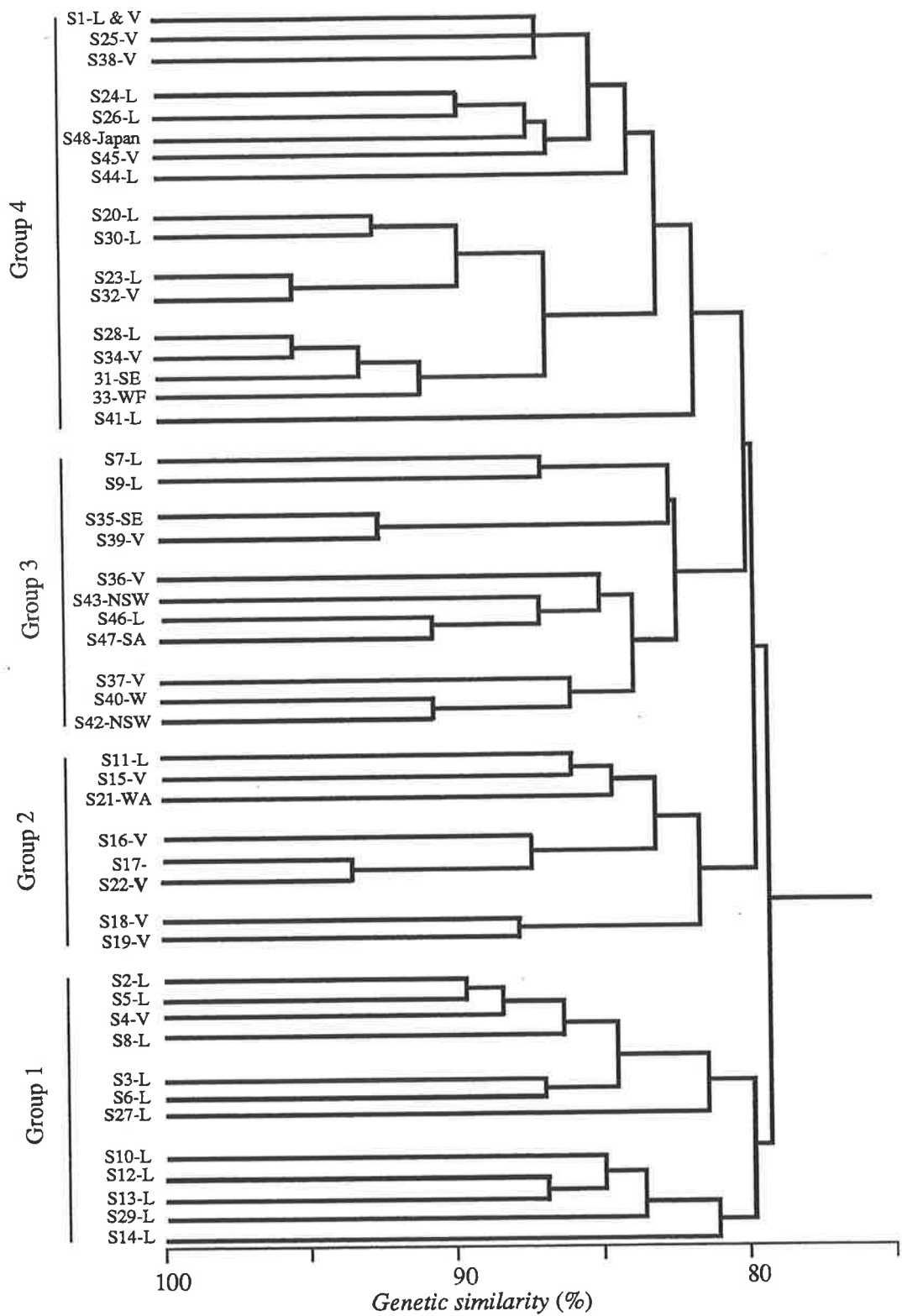
Table 5. 2. continued 2

	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	
S25	100																								
S26	86.9	100																							
S27	80.4	82.2	100																						
S28	79.4	86.9	82.2	100																					
S29	80.4	84.1	79.4	80.4	100																				
S30	81.3	85.0	76.6	83.2	82.2	100																			
S31	79.4	86.9	82.2	92.5	82.2	85.0	100																		
S32	83.2	86.9	82.2	86.9	80.4	90.7	88.8	100																	
S33	79.4	85.0	80.4	92.5	82.2	83.2	88.8	86.9	100																
S34	82.2	87.9	81.3	95.3	81.3	86.0	93.5	91.6	91.6	100															
S35	77.6	83.2	72.9	77.6	82.2	77.6	81.3	79.4	75.7	80.4	100														
S36	77.6	80.4	73.8	76.6	75.7	77.6	77.6	77.6	74.8	80.4	85.0	100													
S37	80.4	84.1	79.4	80.4	81.3	74.8	80.4	76.6	78.5	79.4	82.2	79.4	100												
S38	86.9	85.0	78.5	81.3	78.5	77.6	83.2	81.3	81.3	82.2	79.4	75.7	80.4	100											
S39	77.6	85.0	74.8	79.4	82.2	79.4	83.2	79.4	77.6	82.2	92.5	81.3	82.2	81.3	100										
S40	80.4	86.0	83.2	84.1	85.0	84.1	86.0	82.2	82.2	83.2	82.2	81.3	86.9	78.5	84.1	100									
S41	84.1	84.1	81.3	78.5	73.8	82.2	80.4	82.2	76.6	81.3	78.5	76.6	77.6	80.4	78.5	79.4	100								
S42	78.5	84.1	79.4	82.2	79.4	78.5	82.2	78.5	80.4	79.4	82.2	81.3	85.0	80.4	84.1	90.7	79.4	100							
S43	80.4	82.2	79.4	78.5	79.4	76.6	78.5	78.5	76.6	77.6	76.6	81.3	86.9	80.4	78.5	83.2	75.7	86.9	100						
S44	85.0	86.9	80.4	79.4	80.4	77.6	79.4	77.6	79.4	80.4	79.4	78.5	78.5	83.2	79.4	78.5	80.4	76.6	76.6	100					
S45	84.1	87.9	75.7	80.4	77.6	84.1	82.2	84.1	84.1	85.0	78.5	76.6	77.6	82.2	78.5	79.4	83.2	77.6	73.8	84.1	100				
S46	81.3	86.9	78.5	85.0	80.4	83.2	86.9	86.9	83.2	87.9	83.2	86.0	80.4	77.6	83.2	84.1	80.4	84.1	84.1	79.4	82.2	100			
S47	85.0	90.7	80.4	83.2	84.1	83.2	83.2	85.0	81.3	84.1	85.0	87.9	86.0	81.3	85.0	86.0	78.5	86.0	89.7	83.2	82.2	90.7	100		
S48	84.1	86.0	77.6	80.4	79.4	82.2	80.4	84.1	82.2	83.2	78.5	72.9	75.7	84.1	78.5	75.7	83.2	73.8	73.8	82.2	86.9	78.5	80.4	100	

facing page

Figure 5. 3. Dendrogram showing overall relationships between the 48 fingerprint genotypes detected in AG-3 populations of *R. solani* using the probe pR3718 and *SacI* restriction enzyme.

L= Lenswood; N.S.W.=New South Wales; SA= South Australia; SE=South East of South Australia; V= Virginia ; WA=Western Australia; WF=Walkers Flat, South Australia



these groups each sub-cluster consisted mostly of isolates from the same geographical region. Genotype 'S48', which corresponded to the standard AG-3 isolate from Japan, was related to the isolates from Lenswood, with a similarity of 88.8%. Fingerprint genotypes 'S23' and 'S28' from Lenswood showed the highest similarity (95.3%) with fingerprint genotypes 'S32' and 'S34' from Virginia, respectively. In each sub-cluster the range of similarity values varied. In group 1 the isolates showed an average similarity of 84.1%, whereas the average similarity in groups 2, 3 and 4 was 89.2%, 87.3% and 88.7%, respectively. Of the two representative isolates from N.S.W., one genotype, 'S42', was grouped with the isolates from Waite Campus (fingerprint genotype 'S40') and showed a high similarity of 90.7%, despite their having been isolated thousands of kilometres apart. The other isolate from N. S. W., fingerprint genotype 'S43', was more similar to the isolates recovered from Lenswood (fingerprint genotype '46').

Cluster analyses of the similarity values were also performed separately for the isolates recovered from Virginia (Table 5. 3) and Lenswood (Table 5. 4). The isolates of Virginia grouped in one primary cluster, which was divided into different sub-clusters (Fig. 5. 4) whereas, based on genetic similarity, isolates recovered from Lenswood were grouped in a more complicated dendrogram (Fig. 5. 5). The dendrogram showed two primary clusters, but one was divided into several sub-clusters. Of 78 isolates tested from Virginia, 7 of 14 genotypes were identified as unique, whereas, for 58 isolates tested from Lenswood, 12 of 23 genotypes were unique (Table 5. 1).

5. 3. 3. Low copy RFLP markers originating from AG-3 isolate R37

Two randomly selected low copy clones, pR3733 and pR3725, containing 3.6 and 4.3 kbp *SacI* inserts, respectively, were hybridised with DNA of isolates from different anastomosis groups. pR3733 hybridised strongly with DNA from

Table 5. 3. Genetic similarities (%) between 14 *SacI* fingerprint genotypes detected in the AG-3 population in Virginia using the probe pR3718

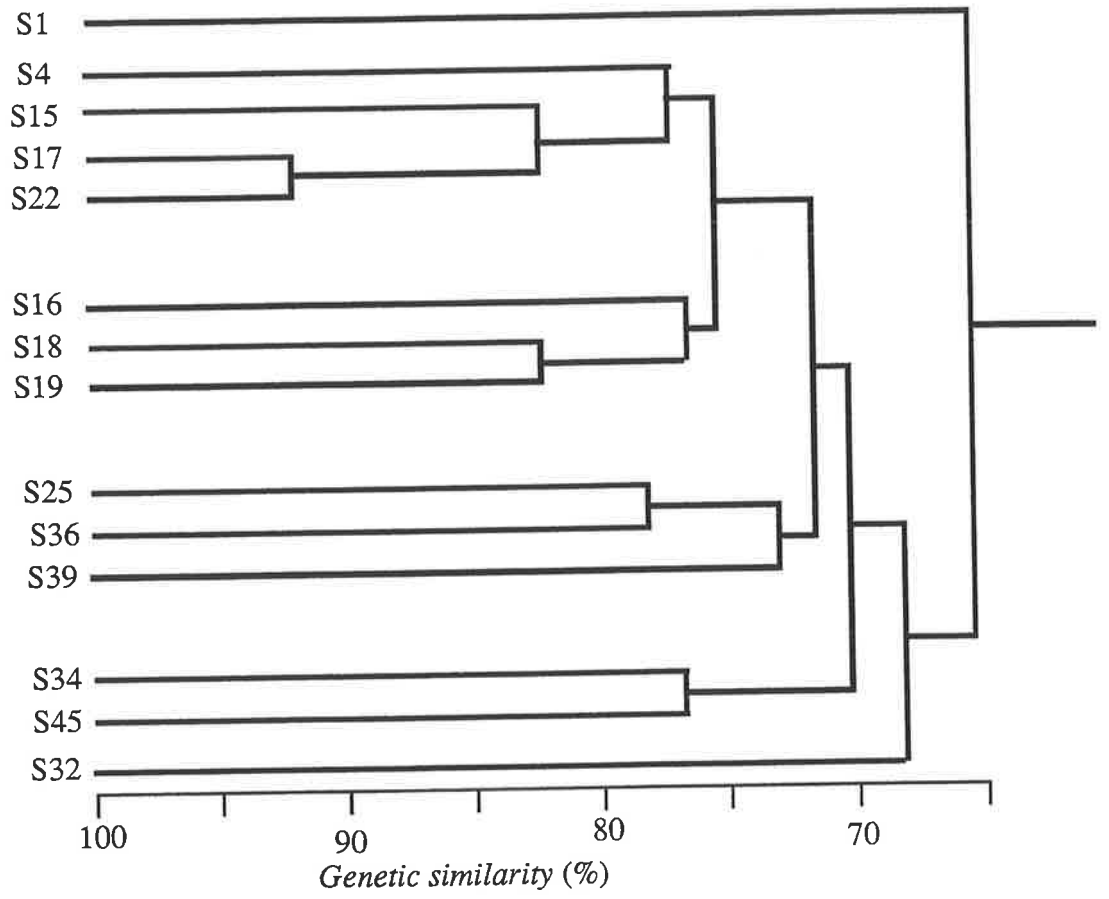
	S1	S4	S15	S16	S17	S18	S19	S22	S25	S32	S34	S36	S39	S45
S1	100													
S4	66.2	100												
S15	71.6	75.7	100											
S16	62.2	74.3	71.6	100										
S17	70.3	77.0	77.0	83.8	100									
S18	64.9	77.0	77.0	70.3	75.7	100								
S19	66.2	75.7	75.7	71.6	74.3	82.4	100							
S22	73.0	79.7	79.7	81.1	91.9	75.7	79.7	100						
S25	55.4	73.0	70.3	63.5	71.6	68.9	67.6	74.3	100					
S32	66.2	70.3	67.6	66.2	66.2	71.6	70.3	66.2	64.9	100				
S34	62.2	74.3	66.2	73.0	75.7	73.0	71.6	78.4	68.9	66.2	100			
S36	66.2	78.4	78.4	68.9	77.0	74.3	70.3	77.0	78.4	73.0	68.9	100		
S39	66.2	75.7	67.6	63.5	71.6	71.6	70.3	74.3	70.3	73.0	71.6	75.7	100	
S45	60.8	70.3	70.3	66.2	68.9	71.6	64.9	74.3	70.3	62.2	77.0	64.9	62.2	100

Table 5. 4. Genetic similarity (%) between 23 *SacI* fingerprint genotypes detected in the Lenswood AG-3 population using the probe pR3718

	S1	S2	S3	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S20	S23	S24	S26	S27	S28	S29	S30	S41	S44	
S1	100																							
S2	84.5	100																						
S3	84.5	83.3	100																					
S5	82.1	85.7	81.0	100																				
S6	72.6	66.7	76.2	71.4	100																			
S7	83.3	77.4	79.8	82.1	75.0	100																		
S8	79.8	78.6	81.0	78.6	83.3	77.4	100																	
S9	73.8	72.6	79.8	72.6	72.6	71.4	77.4	100																
S10	69.0	67.9	72.6	70.2	75.0	73.8	72.6	76.2	100															
S11	73.8	72.6	82.1	75.0	72.6	73.8	72.6	78.6	73.8	100														
S12	81.0	75.0	79.8	75.0	75.0	69.0	75.0	83.3	69.0	83.3	100													
S13	73.8	72.6	72.6	72.6	63.1	71.4	67.9	76.2	71.4	73.8	81.0	100												
S14	76.2	72.6	82.1	75.0	77.4	76.2	82.1	81.0	81.0	78.6	76.2	71.4	100											
S20	75.0	69.0	78.6	73.8	76.2	77.4	78.6	77.4	72.6	75.0	75.0	67.9	86.9	100										
S23	71.4	70.2	77.4	72.6	72.6	73.8	77.4	76.2	73.8	76.2	76.2	73.8	78.6	82.1	100									
S24	69.0	67.9	75.0	72.6	75.0	73.8	77.4	76.2	71.4	73.8	73.8	66.7	88.1	86.9	78.6	100								
S26	71.4	70.2	79.8	72.6	75.0	73.8	79.8	81.0	78.6	76.2	78.6	73.8	85.7	84.5	90.5	81.0	100							
S27	73.8	67.9	77.4	72.6	75.0	76.2	77.4	78.6	71.4	71.4	76.2	69.0	85.7	94.0	83.3	88.1	85.7	100						
S28	73.8	70.2	79.8	75.0	75.0	73.8	79.8	76.2	71.4	76.2	76.2	71.4	83.3	82.1	90.5	76.2	88.1	83.3	100					
S29	72.6	69.0	78.6	71.4	76.2	75.0	81.0	79.8	75.0	72.6	77.4	72.6	84.5	88.1	94.0	82.1	91.7	89.3	89.3	100				
S30	72.6	66.7	76.2	69.0	71.4	77.4	73.8	72.6	75.0	70.2	70.2	70.2	82.1	81.0	75.0	77.4	77.4	79.8	77.4	81.0	100			
S41	70.2	66.7	73.8	66.7	69.0	72.6	71.4	72.6	72.6	72.6	67.9	70.2	79.8	83.3	75.0	77.4	75.0	79.8	75.0	78.6	83.3	100		
S44	71.4	67.9	72.6	75.0	67.9	76.2	70.2	73.8	73.8	73.8	71.4	73.8	81.0	77.4	73.8	76.2	73.8	78.6	73.8	77.4	79.8	79.8	100	

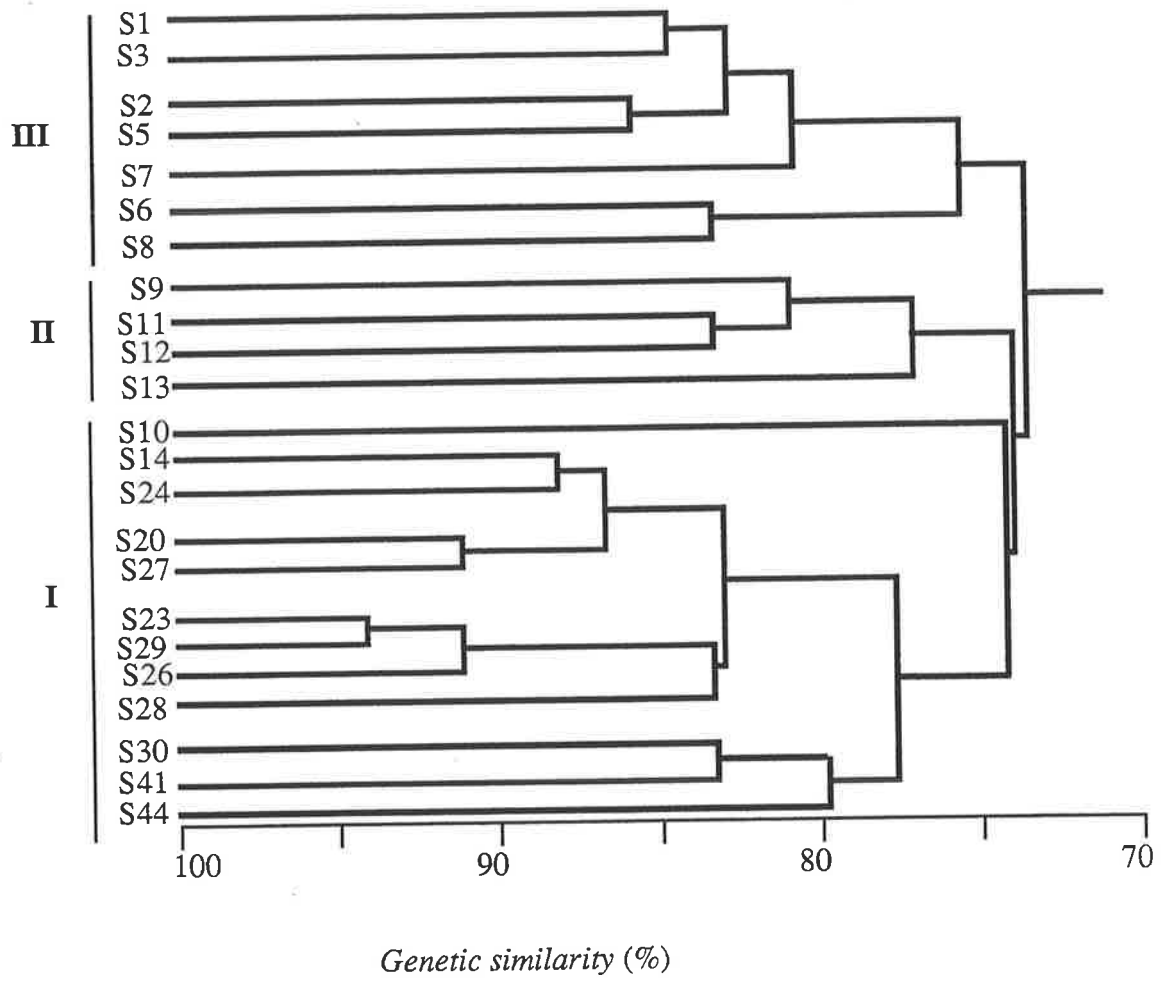
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Figure 5. 4. Dendrogram showing relationships between 14 fingerprint genotypes detected in the *R. solani* AG-3 population in Virginia, generated by group average analysis of the similarity values obtained using the probe pR3718 and *SacI* restriction enzyme.



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Figure 5. 5. Dendrogram showing relationships between 23 fingerprint genotypes detected in the *R. solani* AG-3 population in Lenswood, generated by group average analysis of the similarity values obtained using the probe pR3718 and *SacI* restriction enzyme.



isolates of different anastomosis groups. Polymorphisms were detected within AG-4 and AG-5 isolates from different locations, whereas isolates of AG-3 were identical. In addition, polymorphisms were observed between AG-3, AG-4, AG-5, AG-8 and AG-9 and the binucleate isolate of *Rhizoctonia* sp. tested (Fig. 5. 6a). Polymorphisms between anastomosis groups were also detected with the single copy clone, pR3725. This clone showed polymorphisms within AG-4 and AG-5 isolates but polymorphisms were not observed between AG-3 isolates from Virginia and the AG-3 isolate from Japan (Fig. 5. 6b).

5. 3. 4. Fingerprint genotypes identified with pR3718 and *Hind* III

DNA from 97 isolates, representing 23 *Sac*I fingerprint genotypes (Table 5. 5), was cut with *Hind* III restriction enzyme, blotted and hybridised with the pR3718 probe. The *Hind* III enzyme revealed a greater number of high molecular weight homologous fragments than did *Sac*I. The number of total bands detected in each isolate after Southern blot hybridisation ranged from 9 to 17, and 23 *Hind* III fingerprint genotypes were identified which corresponded to the data obtained for *Sac*I. The average of the total discernable bands was slightly less than with *Sac*I digestion, but a *Hind* III digest probed with pR3718 also revealed a high degree of RFLPs between the isolates tested (Figs 5. 7a & 5. 7b). The number of discernable fingerprint genotypes obtained using pR3718/*Hind* III combination was the same as that obtained with the pR3718/*Sac*I combination.

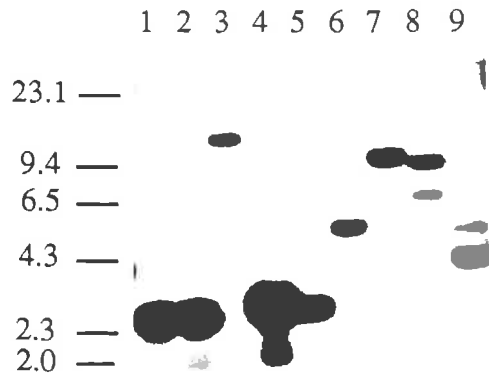
Group average cluster analysis based on similarity values (Table 5. 6) assigned the *Hind* III digested isolates into three groups (Fig. 5. 8). Group 1 mainly included isolates from Lenswood, with only one genotype, 'H4', from Virginia. Group 2 was the most diverse group and was divided into four sub-clusters; 'a', 'b', 'c' and 'd'. Sub-groups 'a', 'b' and 'c' consisted mainly of isolates from Virginia, whereas sub-group 'd', contained mainly isolates from Lenswood. Group 3 contained isolates recovered from

facing page

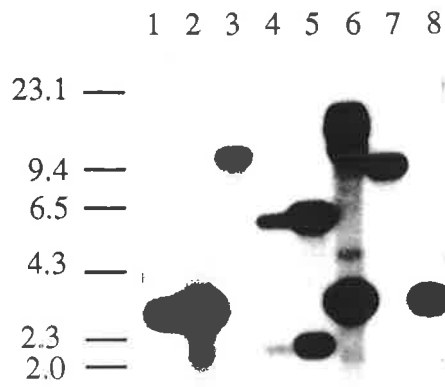
Figure 5. 6. Southern hybridisation analysis of total DNA from different anastomosis groups of *Rhizoctonia solani*, generated by the low copy RFLP markers pR3733 and pR3725.

Figure 5. 6a. Probe pR3733. This clone was used to probe *SacI* digests of 2 µg of DNA of isolates from different anastomosis groups and different locations. Lane 1, R37 (AG-3 from Virginia); lane 2, St-11-6 (AG-3 from Japan); lane 3, R67 (AG-4 from Virginia); lane 4, RH-165 (AG-4 from Japan); lane 5, R51 (AG-5 from Virginia); lane 6, GH-10 (AG-5 from Japan); lane 7, 1506 (AG-8 from unknown location in South Australia); lane 8, S-21 (AG-9 from Alaska, USA) and lane 9, R84 (binucleate *Rhizoctonia* sp. from Virginia).

Fig 5. 6b. Probe pR3725. This clone was used to probe *SacI* digests of 2 µg of DNA of isolates from different anastomosis groups and different locations. Lane 1, R37 (AG-3 from Virginia), lane 2, St-11-6 (AG-3 from Japan); lane 3, RH-165 (AG-4 from Japan); lane 4, R67 (AG-4 from Virginia); lane 5, R671 (AG-4 from Virginia); lane 6, GH-10 (AG-5 from Japan); lane 7, R51 (AG-5 from Virginia); lane 8, R515 (AG-5 from Virginia).



a



b

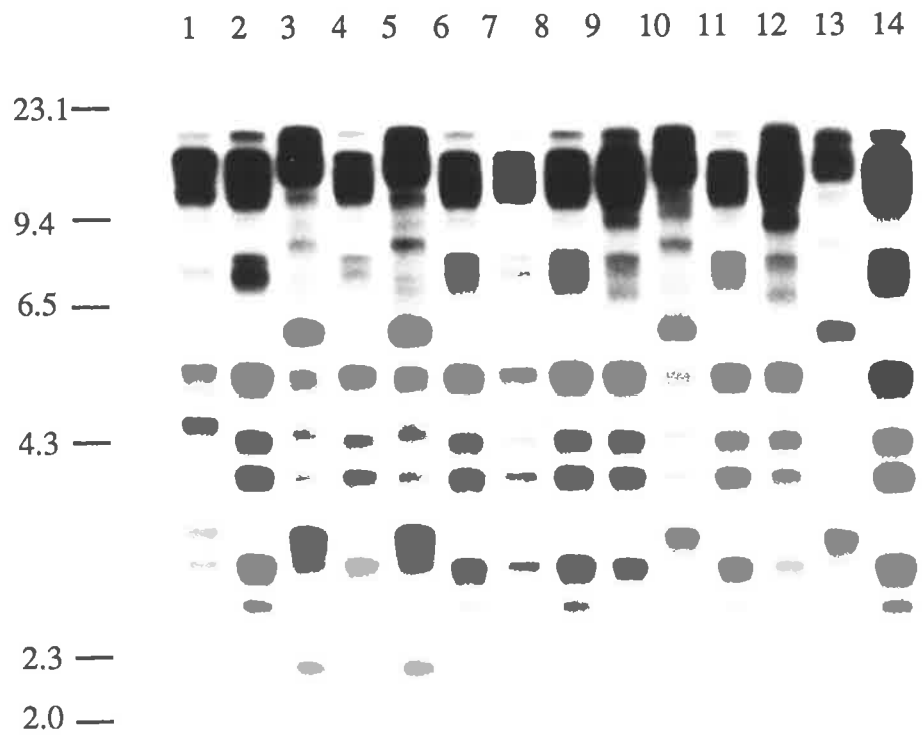
Table 5. 5. Number and frequency of *Hind*III fingerprint genotypes detected among AG-3 isolates of *R. solani* revealed using the probe pR3718.

<i>Hind</i> III genotype	<i>Hind</i> III fragments	<i>Sac</i> I genotype	No. of isolates	Location
H1	17	S1	17	Virginia
H1	17	S1	15	Lenswood
H2	13	S2	2	Lenswood
H3	14	S3	3	Lenswood
H4	13	S4	1	Virginia
H5	12	S5	1	Lenswood
H7	17	S7	2	Lenswood
H8	11	S8	3	Lenswood
H9	14	S9	1	Lenswood
H10	12	S10	4	Lenswood
H11	13	S11	3	Lenswood
H13	10	S13	1	Lenswood
H14	15	S14	1	Lenswood
H15	12	S15	1	Virginia
H16	14	S16	6	Virginia
H18	12	S18	21	Virginia
H19	13	S19	6	Virginia
H23	11	S23	1	Virginia
H24	11	S24	2	Virginia
H27	11	S27	1	Lenswood
H42	10	S42	1	Virginia
H44	9	S44	2	Lenswood
H45	11	S45	1	Virginia
H46	11	S46	1	Virginia

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Figure 5. 7. Examples of Southern blot hybridisation analysis of total DNA from different isolates of *R. solani* AG-3 recovered from potato crops in South Australia, generated by pR3718. The clone pR3718 was used to probe *Hind*III digests of 5 µg of DNA from isolates of AG-3.

Figure 5. 7a. Lane 1, ST-11-6 (AG-3 standard, genotype 'H48'); lane 2, R900 (genotype, 'H18'); lane 3, R902 (genotype 'H19'); lanes 4, R903 (genotype 'H18'); lane 5, R904 (genotype 'H19'); lane 6, R905 (genotype 'H18'); lane 7, R906 (genotype 'H18'); lane 8, R907 (genotype 'H18'); lane 9, R909 (genotype 'H18'); lane 10, R910 (genotype 'H19'); lane 11, R911 (genotype 'H18'); lane 12, R908 (genotype 'H18'); lane 13, R912 (genotype 'H19') and lane 14, R913 (genotype 'H18'). Lambda size markers (kbp) are shown on the left.



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Figure 5. 7b. Lane 1, ST-11-6 (AG-3 standard, genotype 'R48'); lane 2, R823 (genotype 'H2'); lane 3, R824 (genotype 'H3'); lanes 4, R825 (genotype 'H1'); lane 5, R826 (genotype 'H2'); lane 6, R827 (genotype 'H1'); lane 7, R828 (genotype 'H3'); lane 8, R829 (genotype 'H4'); lane 9, R830 (genotype 'H5'); lane 10, R840 (genotype 'H5'); lane 11, R842 (genotype 'H7'); lane 12, R843 (genotype 'H3'); lane 13, R844 (genotype 'H1'); lane 14, R845 (genotype 'H8') and lane 15, R846 (genotype 'H9'). Lambda size markers (kbp) are shown on the left.

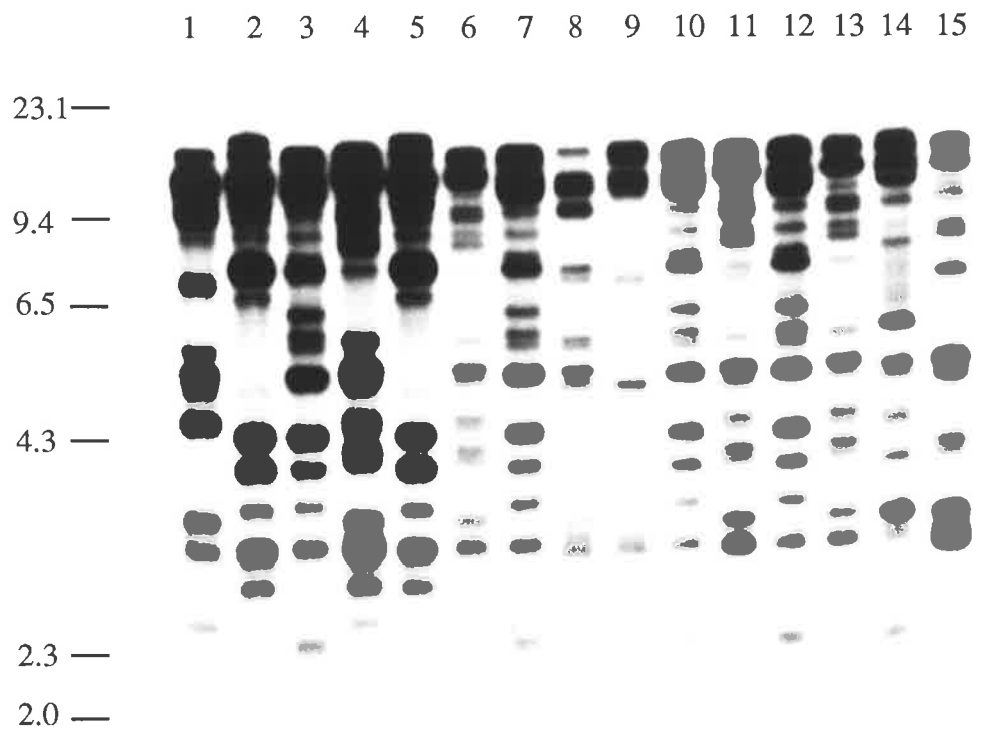


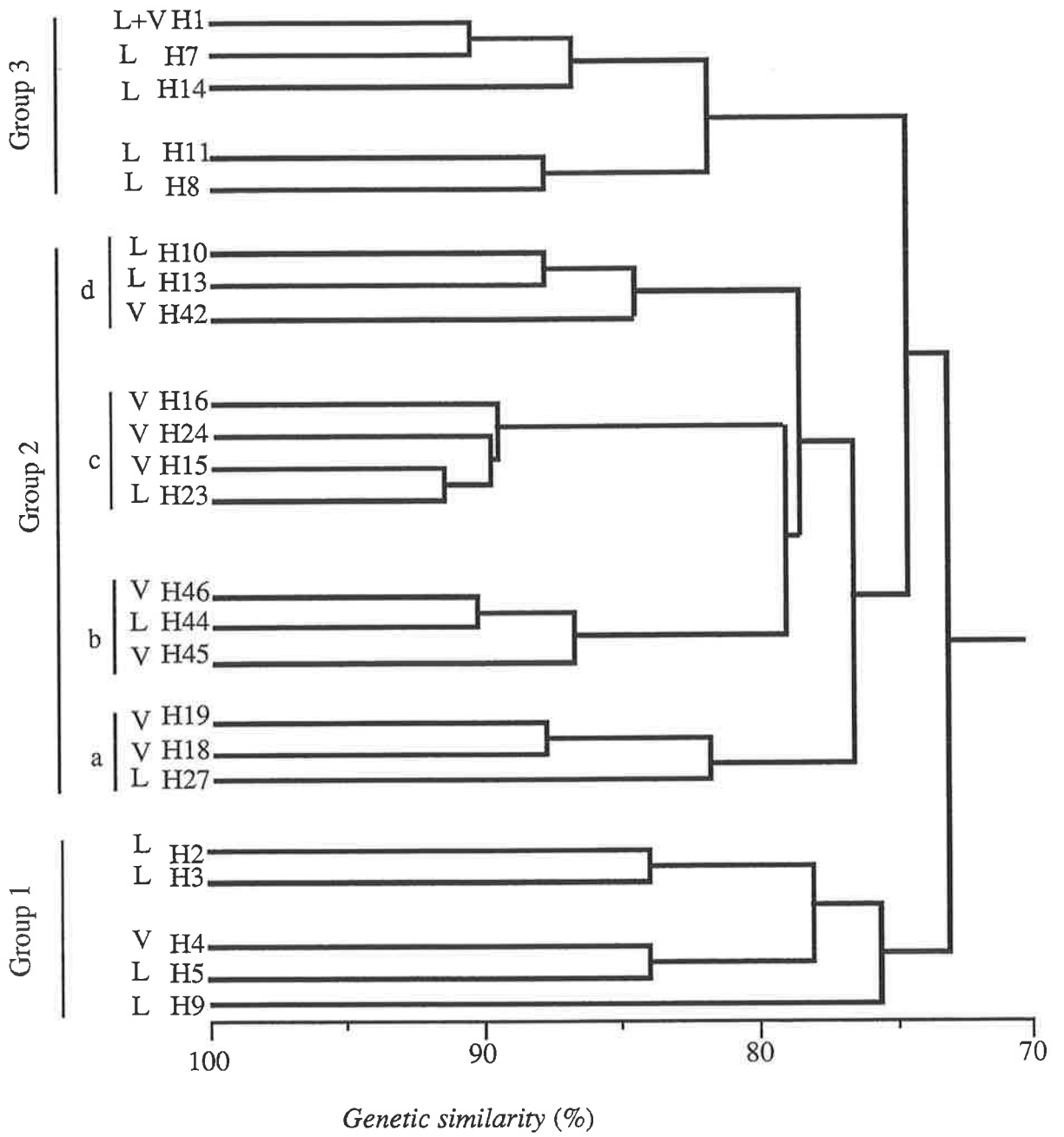
Table 5. 6. Genetic similarity (%) between fingerprint genotypes detected using the probe pR3718 and *Hind*III restriction enzyme digest of isolates from Virginia and Lenswood.

	H1	H10	H11	H13	H14	H7	H8	H2	H3	H4	H5	H9	H16	H24	H15	H23	H18	H19	H27	H42	H46	H44	H45	
H1	100																							
H10	76.8	100																						
H11	80.5	79.3	100																					
H13	74.4	87.8	79.3	100																				
H14	85.4	72.0	78.0	76.8	100																			
H7	90.2	76.8	78.0	76.8	87.8	100																		
H8	82.9	81.7	87.8	81.7	85.4	85.4	100																	
H2	70.7	72.0	75.6	76.8	70.7	68.3	78.0	100																
H3	72.0	73.2	72.0	70.7	69.5	69.5	74.4	84.1	100															
H4	70.7	76.8	75.6	76.8	75.6	70.7	75.6	78.0	81.7	100														
H5	64.6	75.6	74.4	75.6	67.1	67.1	76.8	76.8	75.6	84.1	100													
H9	69.5	70.7	74.4	73.2	72.0	72.0	74.4	76.8	73.2	76.8	75.6	100												
H16	72.0	80.5	76.8	73.2	64.6	67.1	74.4	69.5	70.7	72.0	73.2	68.3	100											
H24	73.2	79.3	80.5	76.8	68.3	70.7	78.0	75.6	74.4	73.2	79.3	72.0	89.0	100										
H15	74.4	80.5	74.4	78.0	67.1	72.0	74.4	74.4	75.6	76.8	78.0	73.2	90.2	89.0	100									
H23	75.6	81.7	78.0	79.3	68.3	73.2	78.0	73.2	74.4	73.2	76.8	72.0	89.0	90.2	91.5	100								
H18	72.0	75.6	76.8	75.6	69.5	72.0	74.4	69.5	73.2	74.4	75.6	73.2	85.4	86.6	87.8	86.6	100							
H19	73.2	76.8	75.6	79.3	68.3	70.7	75.6	70.7	67.1	73.2	69.5	69.5	79.3	73.2	76.8	75.6	72.0	100						
H27	70.7	72.0	80.5	76.8	70.7	70.7	80.5	78.0	72.0	73.2	79.3	74.4	79.3	78.0	81.7	80.5	74.4	82.9	100					
H42	74.4	82.9	76.8	85.4	72.0	76.8	76.8	72.0	73.2	74.4	73.2	75.6	75.6	79.3	80.5	79.3	80.5	79.3	74.4	100				
H46	73.2	74.4	75.6	76.8	75.6	70.7	78.0	70.7	72.0	70.7	76.8	74.4	76.8	80.5	79.3	80.5	79.3	70.7	75.6	79.3	100			
H44	73.2	76.8	75.6	79.3	78.0	70.7	78.0	73.2	72.0	73.2	76.8	74.4	79.3	82.9	79.3	80.5	79.3	73.2	75.6	81.7	90.2	100		
H45	78.0	81.7	73.2	79.3	75.6	75.6	82.9	73.2	72.0	73.2	76.8	72.0	74.4	80.5	79.3	78.0	74.4	75.6	73.2	84.1	87.8	85.4	100	

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Figure 5. 8. Dendrogram showing overall relationships between fingerprint genotypes detected in *R. solani* AG-3 recovered from Virginia and Lenswood, generated by group average cluster analysis of the similarity values obtained using probe pR3718 and *Hind*III restriction enzyme.

V= Virginia; L=Lenswood; H= *Hind*III



Lenswood, with an average similarity of 87.1%. However, the majority of the isolates in each sub-cluster originated from either Virginia or Lenswood. The cluster analysis showed that genotypes originating from the same location clustered together (Fig. 5. 8).

5. 3. 5. DNA slot blot analysis

A total of 1 and 2 µg DNA was extracted from 0.1 g dried soil organic matter from Virginia and Lenswood, respectively. The ability of the AG-3 specific multi-copy probe, pR3718, to quantify *R. solani* AG-3 in potato fields was examined using the slot blot technique. Approximately 0.5 to 500 ng of organic matter DNA from potato field soil samples was applied to a nitrocellulose membrane and compared to a dilution series of purified AG-3 DNA from isolate R37, using radiolabelled pR3718 as the probe. AG-3 DNA was detected in all of the soil samples. The probe showed strong homology to DNA extracted from soil organic matter, at all concentrations, whereas there was no homology with the DNA of AG-4 isolate R67 (Fig. 5. 9). Approximately 0.5 ng DNA extracted from soil organic matter was detected by the probe pR3718.

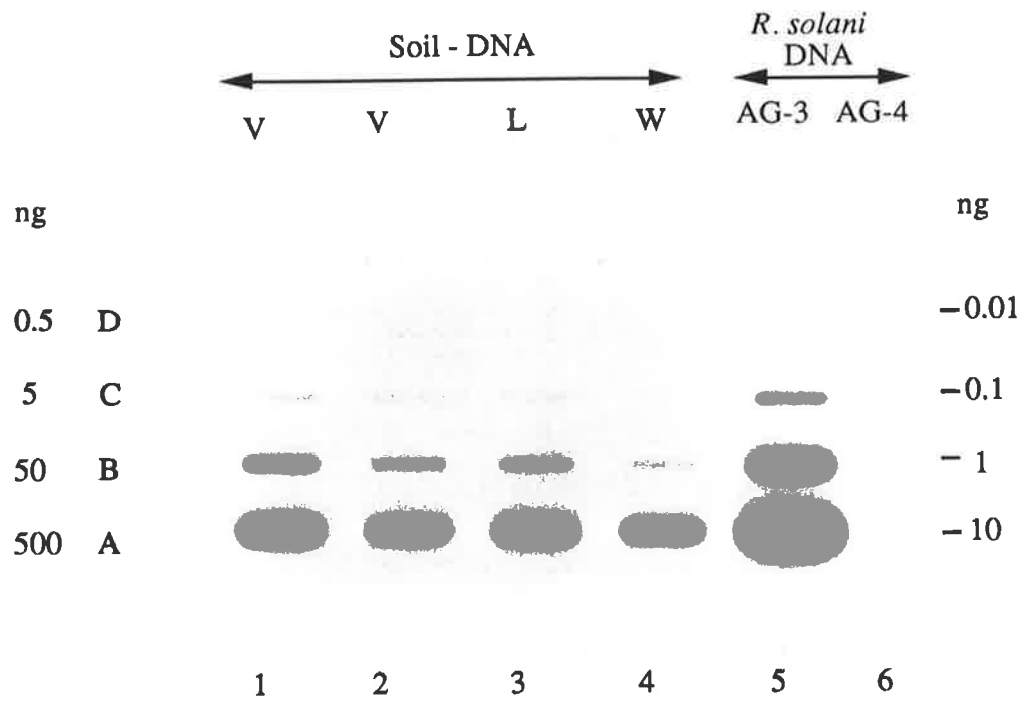
5. 3. 6. RFLP markers originating from AG-4 isolate R49

A total of 700 putative recombinant colonies was screened for *R. solani* DNA inserts as described in Section 5. 2. 5. The clones, which hybridised strongly with total DNA from the AG-4 isolate R49, were selected and the plasmid prepared as described in Section 5. 2. 7. DNA insert sizes ranged from 0.5 to 7 kbp. Restriction enzymes *EcoRI*, *Hind III* and *SacI* were used in the RFLP analyses and most polymorphisms were generated by *Hind III* digestion.

Twenty five clones were tested in Southern blot hybridisations of *Hind III* digested DNA from R49 (AG-4) and representative isolates of AG-3, AG-5 and AG-9.

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Figure 5. 9. Detection of *R. solani* AG-3 DNA in potato field soil organic matter by the slot blot technique using the pR3718 probe. Lanes 1 to 4 show the dilution series of total DNA, 500ng (A1-A4), 50ng (B1-B4), 5ng (C1-C4) and 0.5ng (D1-D4) isolated from soil organic matter collected from potato fields in Virginia (V), Lenswood (L) and Waite Campus (W). Lanes 5 and 6 show a dilution series of DNA (0.01 to 10 ng, shown on right) from *R. solani* AG-3 isolate R37 and AG-4 isolate R49, respectively.



Some clones revealed multiple bands and while others gave low or single copy patterns.

One clone, pR4918, containing a 7 kbp *SacI* insert, which showed homology to DNA from AG-3, AG-4, AG-5 and AG-9, was selected for further analyses. The clone was used to probe Southern blots of *Hind III* digested DNA from representative isolates of different anastomosis groups of *R. solani*. The probe was homologous to DNA from all tester strains of *R. solani*, representing different anastomosis groups, and to all AG-3 isolates recovered from potato crops in South Australia. pR4918 revealed 10 bands when hybridised to its source, isolate R49 (AG-4), whereas it revealed 1 to 4 bands for *Hind III* digested DNA from isolates belonging to other anastomosis groups. Southern blot hybridisation of DNA from representative isolates of anastomosis groups: AG-1 1A; AG-1 1B; AG-2-1; AG-2-2; AG-3; AG-4; AG-5; AG-6; AG-7; AG-8; AG-9; AG-BI and binucleate *Rhizoctonia* sp. revealed different banding patterns (Fig. 5. 10). Also, the sub-groups, AG-1 1A and AG-1-1B, were distinguished by RFLPs. Similarly, the AG-2-1 and AG-2-2 isolates displayed unique patterns. Although no common bands were observed between all anastomosis groups, some anastomosis groups shared one or two bands. A common band was shared by AG-5 and AG-1 1B isolates and, similarly, a band was shared between AG-7 and both AG-2-1 and AG-2-2 isolates. The AG-9 standard isolate and the R84 binucleate isolate also showed a common band. A common 4.0 kbp band was observed for AG-3 isolates R37, R48 and R55, recovered from diseased potato plants, and the AG-3 tester strain (ST-11-6).

The pR4918 clone was evaluated as an RFLP marker for AG-3 isolates by Southern hybridisation using *Hind III* digested DNA from different isolates of AG-3 originating from potato fields in South Australia. The *Hind III* digests of DNA from 101 AG-3 isolates probed with pR4918 revealed ten distinct RFLP genotypes designated '1' to '10' (Figs 5. 11a, 5. 11b & 5. 11c). A total of 16 RFLP loci (bands), ranging from 1.4 to 11 kbp, were considered in RFLP analyses. Two to five *Hind III*

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Figure 5. 10. Southern hybridisation analysis of total DNA from different anastomosis groups of *Rhizoctonia solani* generated by the probe pR4918. pR4918 was used to probe *Hind*III digest of 5 µg of DNA from representatives of anastomosis groups. Lanes ; 1, AG1-1A; 2, AG1-1B, 3; AG2-1, 4; AG2-2; 5, AG-4, 6; AG-3; 7, AG-5; 8, AG-6, 9; AG-7, 10; AG-8; 11, AG-9; 12, AG-BI; 13, R37 (AG-3); 14, R48 (AG-3); 15, R49 (AG-4, origin of probe); 16, R55 (AG-3) and 17, R84 (binucleate *Rhizoctonia* sp.).

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

23.1-

9.4-

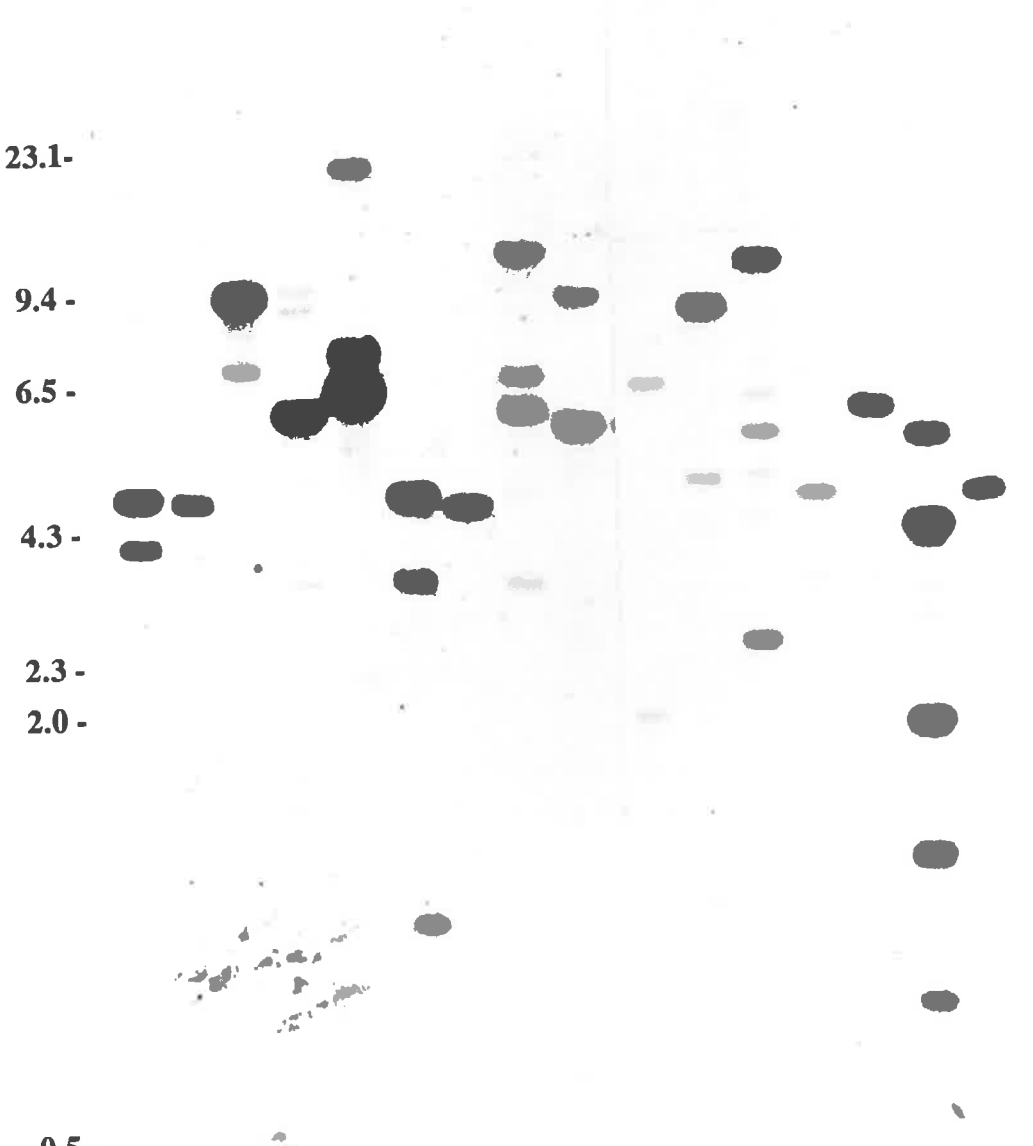
6.5-

4.3-

2.3-

2.0-

0.5-



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Figure 5. 11 Southern hybridisation analysis of total DNA from isolates of *Rhizoctonia solani* AG-3, generated by the probe pR4918. The clone was used to probe *Hind*III digests of 5 µg of DNA of AG-3 isolates recovered from potato crops in different locations in South Australia.

Figure 5. 11a. Lanes 1, R49 (AG-4, origin of probe); lane 2, ST-11-6 (AG-3, standard, genotype '1'); lane 3, RC5 (genotype '2'); lanes 4, R18 and 5, R21 (both genotype 3); 6, R29 (genotype '6'); lanes 7, R31; 8, R321; 9, R33 and 10, R37 (all genotype '1"); lanes 11, R44; 12, R462; 13, R47 and 14, R48 (all genotype '4'); lane 15, R55 (genotype '7'); lanes 16, R65 and 17, R73 (both genotype '1') and 18, R1325 (genotype '6'). Lambda size markers in kbp are shown on the left.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

23.1—

9.4 —

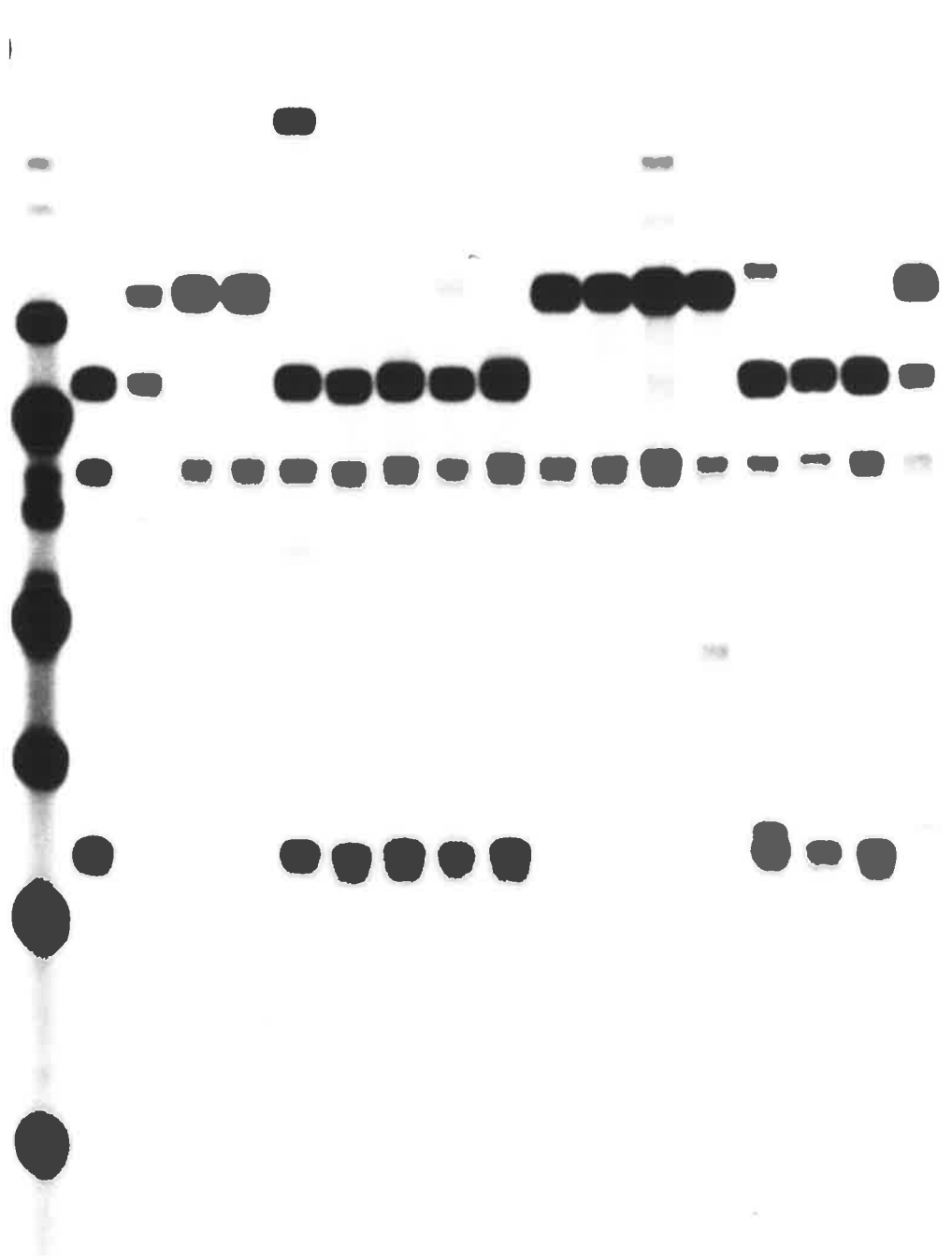
6.5 —

4.3 —

2.3 —

2.0 —

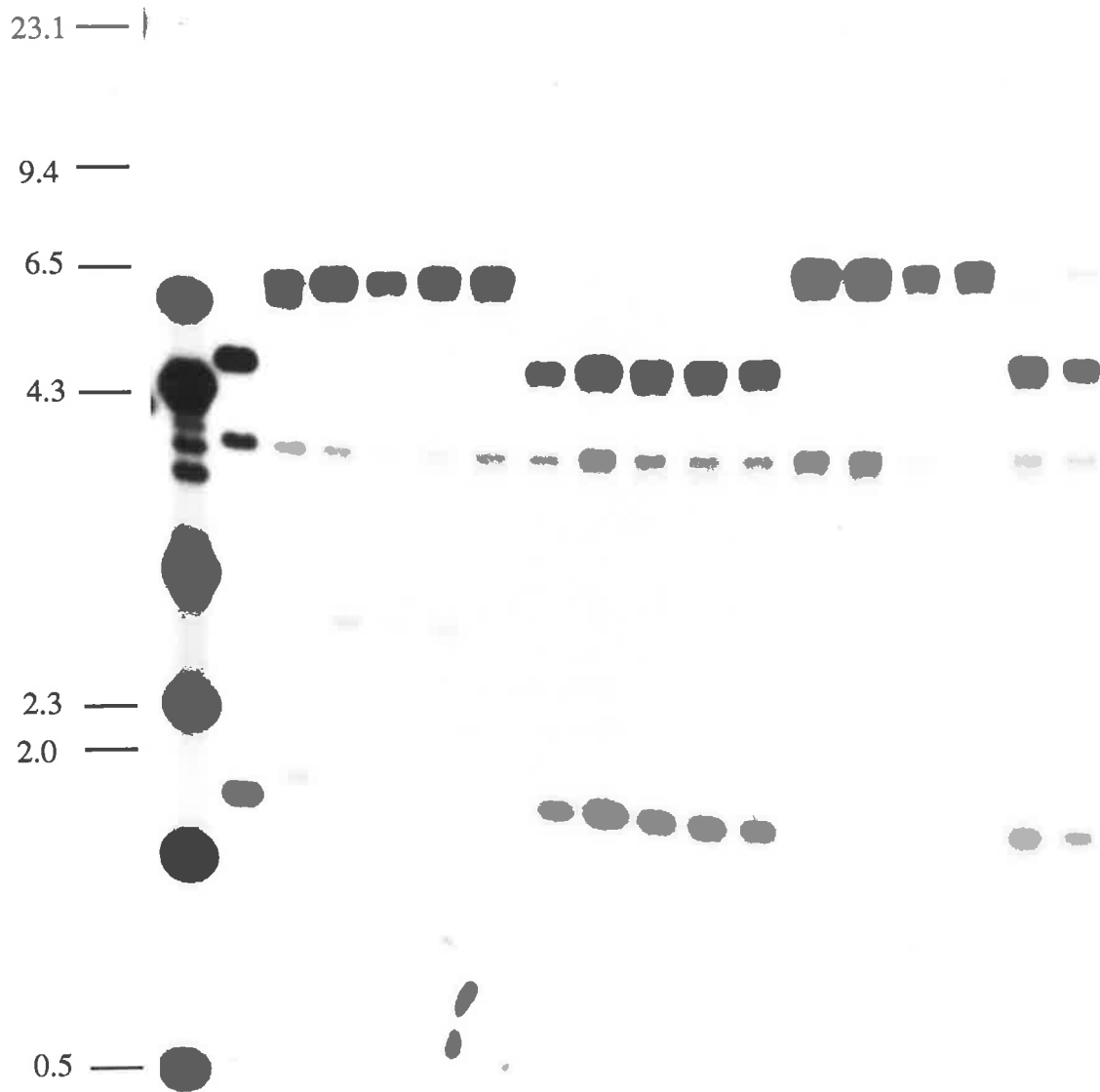
0.5 —



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Figure 5. 11b. Lane1, R49 (AG-4, origin of probe); Lane 2, ST-11-6 (AG-3 standard, genotype '1'); lane 3, R801 (genotype '3'); lanes 4, R802; 5, R803; 6, R804 and 7, 805 (all genotype '4'); lanes 8, R806; 9, R807; 10, R808; 11, R809 and 12, R810 (all genotype '1'); lanes 13, R812; 14, R813; 15, R814 and 16, R817 (all genotype '4'); lane 17, R820 and 18, R822 (both genotype '1'). Lambda size markers in kbp are shown on the left.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18



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Figure 5. 11c. Lane1, R49 (AG-4, origin of probe); Lane 2, ST-11-6 (AG-3 standard, genotype '1'); lane 3, R870 (genotype '2'); lanes 4, R873 (genotype '1'); lane 5, R874 (genotype '10'); lane 6, R875 (genotype '10') lane 7, R877 (genotype '1'); lanes 8, R879; 9, R880; 10, R881; 11, R882; 12, R883; 13, R884, and 14, R885 (genotype '5'); lane 15, R51 (genotype '2') and lane 16, L62 (genotype '5'). Lambda size markers in kbp are shown on the left.

fragments, which ranged in size from 1.5 to 13.2 kbp, were detected among the AG-3 isolates. One band of 4.0 kbp was common to all RFLP genotypes except genotypes '2', '5' and '7'. Fragments of 1.5, 4.8, 5.4, 6.5 and 7.1 kbp were shared by some genotypes, whereas approximately 56% of the fragments were unique to individual groups.

Of the isolates from Virginia, 66 of 101 were assigned to RFLP genotypes '1', '2', '4', '5', '6', '7', '8', '9' and '10', whereas 35 of 101 isolates from Lenswood belonged to RFLP genotypes '1', '2', '3', '5' and '10' (Table 5. 7). In both areas, RFLP genotype '5' was the most common genotype. Two representative isolates of AG-3, one from New South Wales and the other from the South East of South Australia, were also assigned to genotype '5' (data not shown). RFLP genotype '1', which was characterised by three fragments, was observed at both Virginia and Lenswood. RFLP genotype '2' was also recovered from both areas, but its frequency in Virginia was higher than in Lenswood. Isolates of this genotype originated from stem cankers, root cankers and tuber-borne sclerotia. Also, two isolates from Lenswood and nine isolates from Virginia, characterised by five fragments, were determined as RFLP genotype '10'. In general, three RFLP genotypes, '1', '2' and '5', were found in about 80% of the isolates tested. RFLP genotypes '3' from Lenswood and '6', '7', '8' and '9' from Virginia were unique.

Cluster analysis of genetic similarity values (Table 5. 8) showed two primary groups for RFLP genotypes (Fig. 5. 12). One primary group (upper branch of dendrogram), containing 94 isolates, was represented by six RFLP genotypes; genotype '3' occurred only at Lenswood, '6' only at Virginia, the other four genotypes were commonly recovered from both areas. The other primary group (lower branch of dendrogram) containing 7 isolates, was represented by four RFLP genotypes which were recovered only from Virginia.

Other DNA clones also showed potential for use in RFLP analyses of *R. solani* isolates. The clone pR4914, presumed to be low copy and containing a 7.2

Table 5. 7. RFLP genotypes, obtained with the pR4918 probe, represented by isolates of *Rhizoctonia solani* AG-3 recovered from potato fields in Virginia and Lenswood

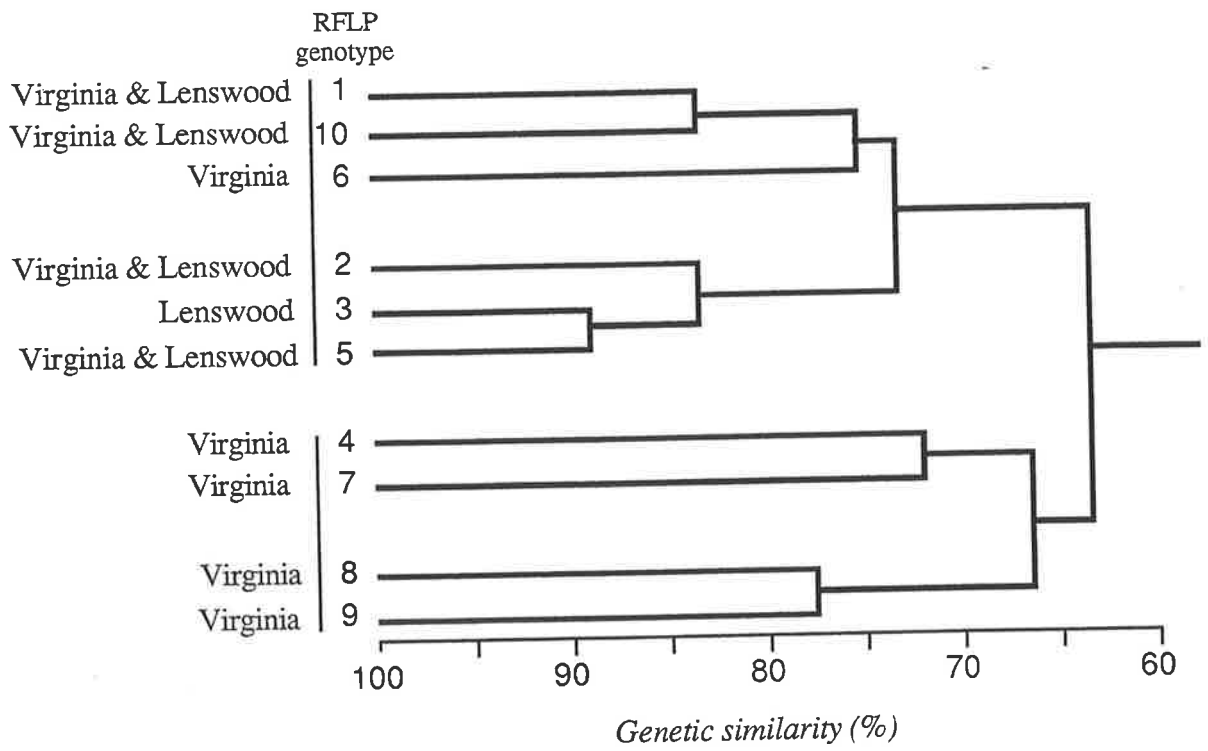
<i>Hind</i> III RFLP genotype	<i>Hind</i> III fragment sizes (kbp)					Location	Number of isolates
	1	2	3	4	5		
1	4.8	4.0	1.5	-	-	Virginia	11
						Lenswood	10
2	7.1	4.3	2.8	-	-	Virginia	14
						Lenswood	8
3	7.1	5.8	4.0	1.5	-	Virginia	0
						Lenswood	1
4	7.0	4.0	-	-	-	Virginia	4
						Lenswood	0
5	7.1	5.4	1.5	-	-	Virginia	24
						Lenswood	14
6	6.5	5.4	4.0	2.7	1.5	Virginia	1
						Lenswood	0
7	7.4	2.5	-	-	-	Virginia	1
						Lenswood	0
8	6.5	4.8	4.0	2.4	1.4	Virginia	1
						Lenswood	0
9	11.0	6.5	4.0	-	-	Virginia	1
						Lenswood	0
10	6.5	4.8	4.0	1.6	1.5	Virginia	9
						Lenswood	2

Table 5. 8. Genetic similarity (%) between RFLP genotypes of *R. solani* AG-3 isolates revealed by the low copy probe pR4918 and *Hind*III restriction enzyme

	1	2	3	4	5	6	7	8	9	10
1	100									
2	77.8	100								
3	83.3	83.3	100							
4	66.7	66.7	61.1	100						
5	83.3	83.3	88.9	61.1	100					
6	77.8	66.7	72.2	55.6	83.3	100				
7	72.2	72.2	66.7	72.2	66.7	61.1	100			
8	66.7	55.6	50.0	55.6	50.0	55.6	61.1	100		
9	66.7	66.7	61.1	77.8	61.1	66.7	72.2	77.8	100	
10	83.3	61.7	66.7	61.1	66.7	72.2	66.7	72.2	72.2	100

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Figure 5. 12. Dendrogram showing relationships between ten RFLP genotypes, revealed by pR4918, among isolates of *R. solani* AG-3 recovered from Virginia and Lenswood.



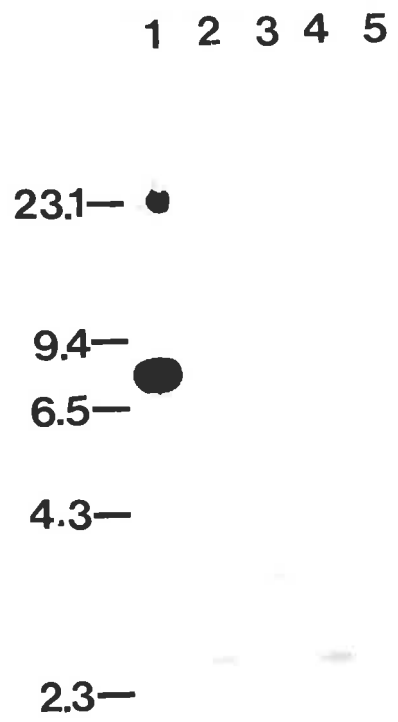
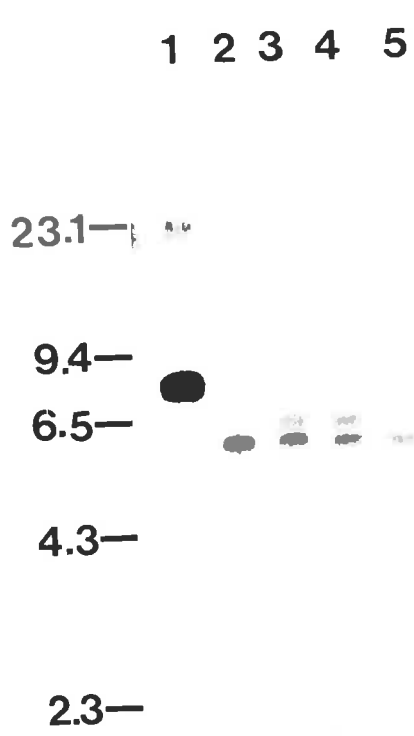
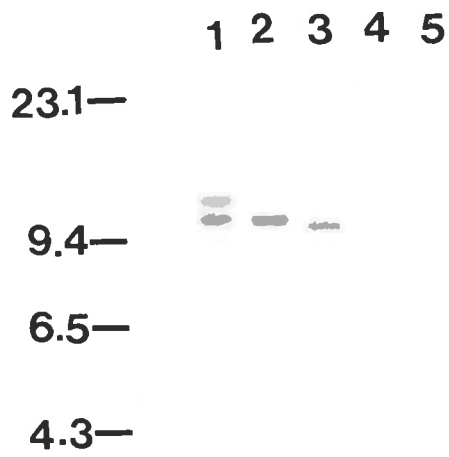
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Figure 5. 13. Southern hybridisation analysis of total DNA from isolates of four anastomosis groups of *R. solani* generated by the probe pR4914 using different restriction enzymes. Lambda size markers are shown on the left.

Figure 5. 13a. The clone was used to probe *Hind*III digests of 5 µg of DNA from different isolates. Lane 1, R49 (AG-4); lane 2, R67 (AG-4); lane 3, ST-11-6 (AG-3), lane 4, GH-10 (AG-5) and lane 5, S-21 (AG-9).

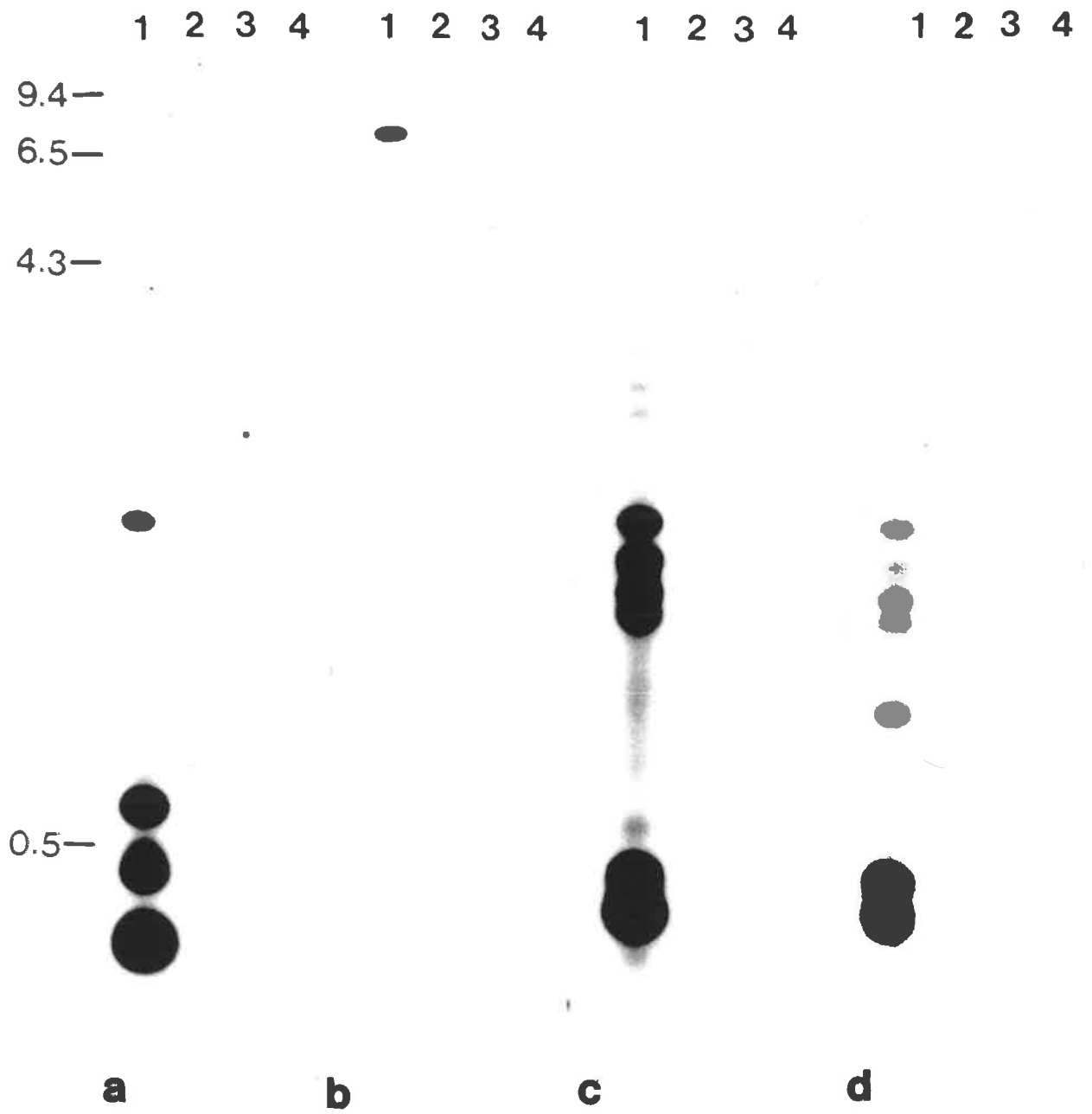
Figure 5. 13b. The clone was used to probe *Eco*R1 digests of 5 µg of DNA from different isolates. Lane 1, R49 (AG-4, origin of probe); lane 2, R37 (AG-3); lane 3, R46 (AG-3); lane 4, R48 (AG-3) and lane 5, R55 (AG-3).

Figure 5. 13c. The clone was used to probe *Sac*I digests of 5 µg of DNA of AG-3 isolates recovered from diseased potato crops in South Australia. Lane 1, R49 (AG-4, origin of probe); lane 2, R37 (AG-3); lane 3, R46 (AG-3); lane 4, R48 (AG-3) and lane 5, R55 (AG-3).



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Figure 5. 14. Southern blot hybridisation of *Hind*III digested DNA samples of isolates from four anastomosis groups. Lane 1, R49 (AG-4, origin of probe); lane 2, R37 (AG-3); lane 3, R51 (AG-5) and lane 4, S-21 (AG-9). a, probe pR497; b, pR4919; c, probe pR4920 and d, probe pR491. Lambda size markers in kbp are shown on the left.



kbp *SacI* fragment, showed a limited level of polymorphism for digested DNA from R49 (AG-4), R67 (AG-4) and ST-11-6 (AG-3), GH-10 (AG-5) and S-21 (AG-9). The clone pR4914 hybridised to two *SacI* DNA fragments of the isolates R49 and S-21 but gave a single band for the isolates ST-11-6 (AG-3), R67 (AG-4) and GH-10 (AG-5) (Fig. 5. 13a). Southern blots of *EcoRI* digested DNA from one AG-4 (R49) and four AG-3 (R37, R48, R55 and R462) isolates also gave single or two homologous fragments (Fig. 5. 13b). RFLPs were also observed for *SacI* digests of DNA from AG-3 isolates using the pR4918 probe (Fig. 5. 13c). Two of the isolates, R37 and R48, generated a single band, while R46 and R55 gave two bands each. The clones pR4919, pR4920, pR491 and pR497, containing 1.4, 0.8, 1.1 and 1.8 kbp *SacI* inserts, respectively, hybridised only with DNA from AG-4 isolate R49. pR491, pR497 and pR4920 revealed four to six bands, whereas pR4919 gave a single band (Fig. 5. 14). These clones exhibited a high degree of specificity to AG-4 isolates.

5. 4. DISCUSSION

5. 4. 1. AG-3 specific DNA fingerprinting probe

Southern blot hybridisation of genomic DNA from AG-3 isolates of *R. solani* using the multi-copy clone pR3718 revealed different DNA fingerprint patterns among isolates. Furthermore, results presented indicate that the clone is specific to AG-3 isolates and that there is a high degree of genotype variation among the AG-3 isolates collected from South Australia. The probe gave highly variable banding patterns that were reproducible and easy to score. On average, the multi-copy clone hybridised to 16 bands of which five *SacI* fragments were common to all the isolates tested, except the Japanese standard AG-3 isolate. The banding patterns did not change during repeated subculturing of isolates (data not shown). This suggests that the probe detects a stable sequence in the genome of AG-3 isolates and that this

repetitive sequence is widely distributed throughout the genome. Therefore, it may be possible to estimate genotype diversity and genetic similarity of AG-3 isolates using the molecular marker pR3718. The relatively large number of loci detected by the probe may be adequate to differentiate among clones within and between AG-3 populations. AG-3 had been known previously as a genetically homogenous group, based on RFLP analysis using a marker in the nuclear encoded-rDNA originating from an AG-4 isolate (Vilgalys & Gonzales, 1990), but research conducted by Jabaji-Hare *et al.* (1990) showed that intra-group variation occurred within AG-3 isolates of *R. solani* from Canada, USA, Britain and Japan, and Duncan *et al.* (1993) demonstrated heterogeneity in zymogram group 7 (AG-3) isolates from South Australia and Tasmania, using random amplified polymorphic DNA. These data support the latter findings.

Genotype variation in the isolates was detected by hybridisation of pR3718 to *SacI* digests. Polymorphisms between isolates of AG-3 were more evident with the probe pR3718 than with pR4918. The isolates which showed identical patterns with pR4918 were found to belong to different genotypes when hybridised with pR3718. The high level of genetic variation detected by the pR3718 probe is in agreement with the results of Jabaji-Hare *et al.* (1990) and Duncan *et al.* (1993) and indicates that there is considerable polymorphism within AG-3 isolates in South Australia. This variation may be due to outbreeding or heterokaryosis. Outbreeding in isolates of AG-3 (Anderson & Butler, 1982) and heterokaryosis in some anastomosis groups of *R. solani* (Anderson, 1982; Dodman, 1972) have been reported previously.

No overlap of banding patterns was observed between the isolates recovered from different locations and several genetically distinct isolates which were recovered from the same area. In Virginia, one fingerprint pattern was dominant, but a more comprehensive sampling is required to investigate the distribution of different genotypes. This genetic diversity may be related to the introduction of

different sources of contaminated seed. Potato tubers obtained from different locations may carry different genotypes of AG-3 isolates and black scurf disease may develop in one field from a combination of genetically different isolates. Consequently, one could expect to find identical genotypes across different potato growing areas, but the local populations may be dominated by one or two genotypes. It is possible, therefore, that ecological conditions or seed sources may contribute to the genetic diversity detected among AG-3 isolates in potato fields in South Australia. The presence of several polymorphic and common bands in multi-locus RFLPs in each isolate tested suggests that natural selection or gene flow may be operating within the population. Therefore, the ability to detect, reproducibly, a large number of variable loci in AG-3 isolates makes the probe potentially very useful in the study of ecology, population dynamics, epidemiology and taxonomy of AG-3 isolates of *R. solani*.

Detection of *R. solani* AG-3 using the slot blot technique takes advantage of the repeated sequence of the *R. solani* AG-3 specific pR3718 clone. The sensitivity needed for a quantitative diagnostic test using such a probe has been discussed by Whisson *et al.* (1995). In a DNA-based diagnostic test, levels of *R. solani* AG-3 in soil can be determined by measuring the amount of AG-3 DNA, using the pR3718 specific probe. The assay could be valuable in detection of AG-3 levels in natural soil in potato fields and also from host tissues.

5.4.2. RFLP markers originating from AG-4 isolate R49

The low copy DNA probe, pR4918, originating from AG-4 isolate R49 hybridised to total DNA of representative isolates of 12 anastomosis groups of *R. solani*. DNA homology exists between sequences of different anastomosis groups and polymorphisms also occurred within the AG-3 isolates. The results showed that

the pR4918 clone is a general probe, able to hybridise to DNA of isolates from different anastomosis groups.

Polymorphisms between anastomosis groups were distinguished by Vilgalys & Gonzales (1990) using an rDNA probe originating from an AG-4 isolate. They found considerable molecular variation between and within anastomosis groups. However, they observed that AG-3, AG-4, AG-7 and AG-BI isolates were invariant groups and that each group produced a single diagnostic RFLP. In contrast, Vilgalys & Gonzales (1990) showed that each of the established anastomosis and intra-specific groups possessed a distinct rDNA RFLP pattern. They also observed that restriction analysis of ribosomal RNA genes revealed little variation between different anastomosis groups of *R. solani*. O'Brien (1994) showed that random cloned fragments of *R. solani* DNA can be used to identify different anastomosis groups. However, these analyses failed to distinguish the pectic zymogram groups of AG-8. RFLP results obtained in the current study, for 12 representative isolates of different anastomosis groups and different isolates of *R. solani* AG-3 using the probe pR4918, confirmed that polymorphisms exist among isolates belonging to the same anastomosis groups.

The AG-3 isolates originating from tuber-borne sclerotia displayed more unique RFLP patterns, whereas isolates originating from cankers had fewer RFLP patterns. It could be that the tuber-borne sclerotia isolates are more genetically diverse than canker pathotype isolates. Alternatively, this may reflect the sampling pattern, in that more isolates from tuber-borne sclerotia than from cankers, were examined. The higher degree of variation in sclerotial isolates could be related to the ecologically different sources of isolates which are associated with infested tubers. However, RFLP data from both Virginia and Lenswood were not sufficient to show a correlation in diversity between genotypes and geographical locations or disease symptoms. The field isolates of canker and black scurf pathotypes seem to be genetically different. Some genotypes seem to be involved mostly in producing

cankers on potato plants, but some other genotypes could be black scurf pathotypes. However, a larger number of isolates need to be examined to establish any correlation.

AG-1 isolates have been divided into subgroups based on sclerotial form, cultural characteristics and DNA base sequence homology (Sneh *et al.*, 1991). Based on the results presented here, sub-groups AG-1 1A and AG-1 1B were characterised by two variant RFLPs using the pR4918 probe. Similarly, AG-2, subdivided according to frequency of anastomosis and cultural characteristics (Ogoshi, 1975), also gave two different banding patterns. Therefore, results presented here using the probe pR4918 support the sub-division of these groups. The probe pR4918 could be a useful means for detecting genetic variation between and within anastomosis groups and their sub-groups.

Genetic variation between the different pathotypes may indicate that each of the putative pathotypes causing stem canker and root canker or black scurf is derived from a polyphyletic origin selected from a common ancestor. These genetic divergences may be investigated more precisely using repetitive dispersed sequences as DNA probes. Such highly variable probes are very useful for analysing relationships between groups of organisms and levels of variation within these groups (O'Brien, 1994). Probes of this kind, capable of detecting and identifying isolates of *R. solani* AG-3, could be useful tools for determining the correlation between isolates and disease symptoms in potato crops. Also, a combination of RFLP markers and pectic zymograms, which are direct expressions of genotypes and found to be related to disease symptoms caused by *R. solani* (Chapter 3), could be another approach to addressing the relationships between isolates in terms of damage to the crops. Although, the fingerprinting technique visualises several RFLPs on the same gel simultaneously, a larger number of probes and enzyme digestions, either low or high copy, should be used to obtain more precise data for fingerprinting and

constructing dendrograms for more detailed analysis of genetic variation within AG-3.

In summary, a specific DNA probe recognising polymorphisms among AG-3 isolates was developed. Another probe originating from an AG-4 isolate was hybridised with DNA from isolates of various anastomosis groups of *R. solani* and showed polymorphisms within and among anastomosis groups. In addition, some single and low copy probes were found to be specific to AG-4 isolates.

Chapter 6

Analysis of variability using combined pectic zymogram and RFLP data

6. 1. INTRODUCTION

RFLP analyses have been reported to detect greater diversity than isoenzymes in a number of fungi (O'Dell *et al.*, 1989; Harvey, 1993). In contrast to isoenzyme analysis, the number of RFLP markers is unlimited because any piece of DNA can be used as a probe and several restriction enzymes can be assayed to identify RFLPs with each probe (Michelmore & Hulbert, 1987).

A considerable level of genetic variation was detected, using pectic enzyme analyses, among isolates of *R. solani* AG-3 recovered from potato crops (Chapter 3). This approach was found to be useful for identifying sub-groups within AG-3 and provided markers for use in the study of genetic variation in this fungus. Likewise, RFLP analysis using the low copy probe pR4918 showed that polymorphisms exist within AG-3. Fingerprinting analysis using the repeated sequence probe pR3718 (Chapter 5, Balali *et al.*, 1995b) showed a high degree of genotype variation among the AG-3 isolates and genetic variation was resolved more precisely by using pR3718 than by pR4918 (Chapter 5). These markers could be useful for studying genetic diversity within and among zymogram sub-groups.

The objective of this section of the research was to evaluate the use of the pectic isoenzyme assay in combination with RFLP analysis using the low copy probe pR4918 and the repeated-sequence pR3718, to determine the genetic variability of *R. solani* AG-3 associated with potato crops in South Australia.

6. 2. MATERIALS AND METHODS

6. 2. 1. Experimental data

Data on zymogram groupings for the 100 of the isolates, reported in Chapter 3, were used. The single isolate tentatively assigned to ZG7-4 was not used in this analysis. An additional 27 isolates were subjected to zymogram analysis in an attempt to increase the number of ZG7-2 and ZG7-3 in the study. Therefore, RFLP data for zymogram sub-groupings of the 127 isolates obtained using pR4918/*Hind*III, pR3718/*Sac*I and pR3718/*Hind*III combinations described in Chapter 5 were used in data analysis.

6. 2. 2. Data analysis

Hybridisation patterns of isolates belonging to the zymogram sub-groups, generated by the probe/restriction enzyme combinations: pR4918/*Hind* III; pR3718/*Sac*I and pR3718/*Hind* III, were used to provide estimates of genetic similarities between isolates as described in Section 3. 2. 5.

The frequencies of the electrophoretic genotypes of zymogram sub-groups were calculated and the genetic diversity for each genotype was estimated with the Shannon information statistic (Bowman *et al.*, 1971), using $M = -\sum gi \ln gi$, where gi is the frequency of a particular genotype. To infer genetic similarities between isolates, cluster analyses were performed on similarity values using the group average method (Sneath & Sokal, 1973). The results were used to generate dendrograms and data were analysed using GENSTAT 5, release 1. 3 (Rothamsted Experiment Station, 1989).

6. 3. RESULTS

6. 3. 1. Variation within zymogram sub-groups of AG-3 based on the low copy RFLP marker, pR4918.

Zymogram sub-groups of the 101 isolates of *R. solani* AG-3 used in the RFLP analysis described in Table 5. 7 (Chapter 5) were determined; of these 58 were ZG7-1, 31 were ZG7-2 and 12 were ZG7-3 (Table 6. 1). When *Hind* III digests were probed with pR4918, the three pectic zymogram sub-groups were found to be genetically diverse. Isolates ascribed to ZG7-1 showed very diverse patterns and consisted of nine RFLP genotypes, '1', '2', '4', '5', '6', '7', '8', '9' and '10'. ZG7-2 isolates displayed RFLP genotypes '1', '2', '5' and '10', whereas ZG7-3 isolates contained genotypes '2', '3', '5' and '10'. RFLP genotypes '2', '5' and '10' were, therefore, common to all three zymogram sub-groups.

Cluster analysis of genetic similarity values showed two primary groups of related zymogram phenotypes (Fig. 6. 1). The group of six RFLP genotypes; '1', '2', '3', '5', '6' and '10', with an average similarity of 81.1%, contained representatives of all three zymogram sub-groups. The other group consisted of four RFLP genotypes; '4', '7', '8' and '9', that were strictly related to ZG7-1. The average similarity was 72.2% and all were recovered from Virginia. The highest similarity of 88.9% was observed for RFLP genotypes '3' and '5'.

The dendrogram (Fig. 6.1) showed that most genotypes representing one zymogram sub-group clustered together. The RFLP data obtained by using pR4918 to probe *Hind*III digests of *R. solani* DNA indicated that 60% of the genotypes were either ZG7-1 or ZG7-3 and 40% were determined to be a mixed population containing ZG7-1, ZG7-2 and ZG7-3. Genotypes containing more than one zymogram sub-group were recovered from both Virginia and Lenswood. The frequency of ZG7-1 in both Virginia and Lenswood was considerably higher than that of other zymogram sub-groups (Table

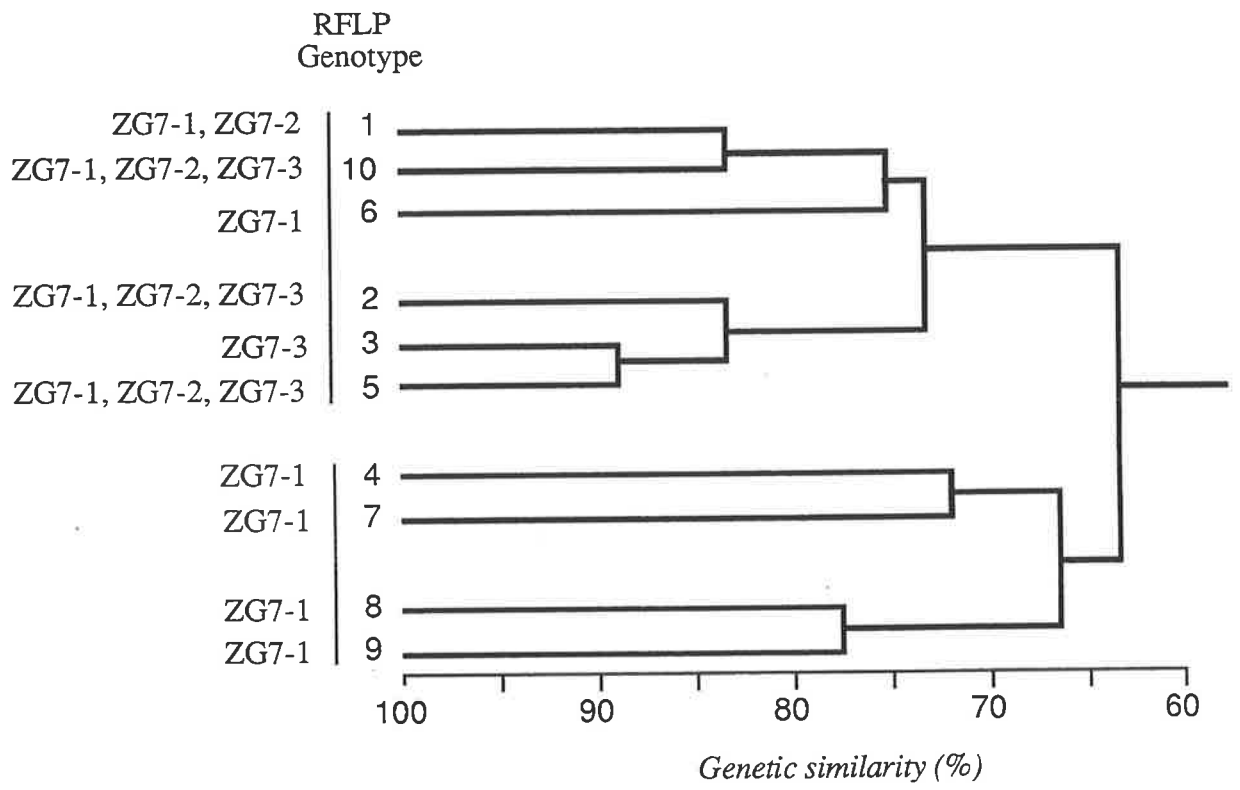
Table 6. 1. RFLP genotypes obtained with pR4918 for three zymogram sub-groups of isolates of *Rhizoctonia solani* AG-3 recovered from potato fields in Virginia and Lenswood

<i>Hind</i> III RFLP genotype	Location	No. of isolates			Frequency of			Overall frequency
		ZG7-1	ZG7-2	ZG7-3	ZG7-1	ZG7-2	ZG7-3	
1	Virginia	10	1	0	0.099	0.009	0.000	0.108
	Lenswood	9	1	0	0.085	0.009	0.000	0.094
2	Virginia	7	1	6	0.069	0.009	0.059	0.137
	Lenswood	5	3	0	0.049	0.029	0.000	0.078
3	Virginia	0	0	0	-	-	-	-
	Lenswood	0	0	1	0.000	0.000	0.009	0.009
4	Virginia	4	0	0	0.039	0.000	0.000	0.039
	Lenswood	0	0	0	-	-	-	-
5	Virginia	5	17	2	0.049	0.168	0.019	0.236
	Lenswood	11	2	1	0.108	0.019	0.009	0.136
6	Virginia	1	0	0	0.009	0.000	0.000	0.009
	Lenswood	0	0	0	-	-	-	-
7	Virginia	1	0	0	0.009	0.000	0.000	0.009
	Lenswood	0	0	0	-	-	-	-
8	Virginia	1	0	0	0.009	0.000	0.000	0.009
	Lenswood	0	0	0	-	-	-	-
9	Virginia	1	0	0	0.009	0.000	0.000	0.009
	Lenswood	0	0	0	-	-	-	-
10	Virginia	3	6	0	0.029	0.059	0.000	0.088
	Lenswood	0	0	2	0.000	0.000	0.019	0.019

- Frequency = 0.000

facing page

Figure 6. 1. Dendrogram showing relationships between RFLP genotypes, revealed by pR4918, and zymogram sub-groups ZG7-1, ZG7-2 and ZG7-3 assigned to *R. solani* AG-3 isolates collected from Virginia and Lenswood, South Australia. (This dendrogram is the same as Fig. 5. 12, with zymogram sub-groups superimposed).



6. 1). pR4918 did not detect any characteristic band specific to any of the zymogram sub-groups, but RFLPs were detected between isolates tested.

6. 3. 2. Variation within zymogram sub-groups of AG-3 based on the highly repeated probe pR3718

Genetic variation within zymogram sub-groups was determined for the 127 isolates tested in the fingerprint analysis using a combination of pR3718/*SacI* and pR3718/*HindIII* (Section 5. 3. 2). Although ZG7-1, ZG7-2 and ZG7-3 were found in both Virginia and Lenswood, the ZG7-1 sub-group was found most frequently. However, ZG7-2 was found to be more common in Virginia than in Lenswood. Two isolates, one from New South Wales and the other from Western Australia were found to belong to ZG7-1.

6. 3. 2. 1. Variation within the ZG7-1 sub-group

The greatest genetic diversity was observed within the ZG7-1 sub-group when the DNA was cut with either *SacI* or *HindIII* restriction enzyme and probed with pR3718. Of the 127 isolates of AG-3 tested by zymogram analysis, 80 belonged to ZG7-1 and 22 distinct *SacI* fingerprint genotypes were identified (Table 6. 2). Southern blots of DNA from 37 isolates from Virginia revealed 8 fingerprint genotypes while 12 genotypes were identified for 40 isolates from Lenswood (Table 6. 2). Of the Virginia and Lenswood isolates examined, 60% (77) belonged to ZG7-1 and 49% (38) of these belonged to fingerprint genotype 'S1'. Also, the representative isolates from Western Australia (L62), N.S.W. (41443) and the South East of South Australia (L36) were identified as ZG7-1. Fingerprint genotype 'S10' was found to be next most common in the ZG7-1 sub-group and was recovered only from Lenswood. Other genotypes were unique or represented by two to five isolates.

Table 6. 2. Frequency of *SacI* fingerprint genotypes, revealed by the probe pR3718, for three zymogram sub-groups of *R. solani* AG-3

Zymogram sub-groups	<i>SacI</i> fingerprint genotype	No. of isolates	Source*	Location	Frequency
ZG7-1	S1	15	SC+RC+TS	Lenswood	0.118
ZG7-1	S1	23	SC+RC+TS	Virginia	0.181
ZG7-1	S5	1	TS	Lenswood	0.007
ZG7-1	S6	1	TS	Lenswood	0.007
ZG7-1	S7	1	TS	Lenswood	0.007
ZG7-1	S8	2	TS	Lenswood	0.015
ZG7-1	S9	5	TS	Lenswood	0.039
ZG7-1	S10	8	TS	Lenswood	0.062
ZG7-1	S14	1	TS	Lenswood	0.007
ZG7-1	S15	1	TS	Virginia	0.007
ZG7-1	S17	1	TS	Virginia	0.007
ZG7-1	S21	1	SC	Western Australia	0.007
ZG7-1	S22	4	TS	Virginia	0.031
ZG7-1	S26	2	TS	Lenswood	0.015
ZG7-1	S27	1	TM	Lenswood	0.007
ZG7-1	S29	1	TS	Lenswood	0.007
ZG7-1	S31	1	TM	South East	0.007
ZG7-1	S34	4	SC+TS	Virginia	0.031
ZG7-1	S40	1	TS	Virginia	0.007
ZG7-1	S41	2	SC	Lenswood	0.015
ZG7-1	S42	1	unknown	New South Wales	0.007
ZG7-1	S44	2	TS	Virginia	0.015
ZG7-1	S45	1	TS	Virginia	0.007
ZG7-2	S1	3	TS	Virginia	0.023
ZG7-2	S1	2	TS	Lenswood	0.015
ZG7-2	S11	2	TS	Lenswood	0.015
ZG7-2	S16	7	TS	Virginia	0.055
ZG7-2	S18	22	TS	Virginia	0.173
ZG7-3	S12	1	TS	Lenswood	0.007
ZG7-3	S19	9	TS	Virginia	0.070
ZG7-3	S28	1	TS	Lenswood	0.007

*SC= stem canker, RC= root canker, TS= tuber sclerotia, TM= tuber mycelium

Genetic variation was also determined for 52 representative isolates of ZG7-1 belonging to 16 *SacI* fingerprint genotypes, using a combination of *HindIII*/pR3718. The same number of genotypes was identified and no overlapping was observed between *SacI* and *Hind III* fingerprint genotypes of ZG7-1 (Table 6. 3).

6. 3. 2. 2. Variation within the ZG7-2 sub-group

Of the 127 isolates of *R. solani* AG-3 examined, 28% (36) belonged to ZG7-2 and four *SacI* fingerprint genotypes were identified within this sub-group (Table 6. 2). Fingerprint genotype 'S1', identified for ZG7-1, was also observed in ZG7-2 and was found in both Virginia and Lenswood. Fingerprint genotypes 'S16' and 'S18' were found in Virginia and 'S11' was associated with Lenswood. Genotype 'S18' was observed more commonly (22 of 36) than other genotypes (Table 6. 2) and was found only in Virginia. Similar results were also obtained for 33 isolates of the ZG7-2 sub-group belonging to four *SacI* fingerprint genotypes obtained using a combination of the probe pR3718 and *HindIII* (Table 6. 3).

6. 3. 2. 3. Variation within the ZG7-3 sub-group

Of the 127 isolates tested, 8% (11) were assigned to ZG7-3 (Table 6. 2). The combination of *SacI* and probe pR3718 detected three fingerprint genotypes, 'S12', 'S19' and 'S28', within the ZG7-3 sub-population. These fingerprint genotypes were not detected in the ZG7-1 or ZG7-2 sub-groups. Fingerprint genotype 'S19' was found most commonly and was detected only in Virginia. Genetic variation was also determined for six representative isolates of ZG7-3 belonging to fingerprint genotype 'S19' using a combination of probe pR3718 and *HindIII*. The six isolates tested were identified as fingerprint genotype 'H19' (Table 6. 3).

Table 6. 3. Frequency of *Hind*III fingerprint genotypes, revealed by the probe pR3718, detected for three zymogram sub-groups of *R. solani* AG-3

Zymogram sub-group	<i>Hind</i> III fingerprint genotype	No. of isolates	Source*	Location	Frequency
ZG7-1	H1	15	SC+RC+TS	Virginia	0.164
ZG7-1	H1	14	SC+RC+TS	Lenswood	0.153
ZG7-1	H2	2	TS	Lenswood	0.021
ZG7-1	H4	1	SC	Virginia	0.010
ZG7-1	H5	1	TS	Lenswood	0.010
ZG7-1	H7	2	TS	Lenswood	0.021
ZG7-1	H8	2	TS	Lenswood	0.021
ZG7-1	H9	1	TS	Lenswood	0.010
ZG7-1	H10	4	TS	Lenswood	0.043
ZG7-1	H11	2	TS	Virginia	0.021
ZG7-1	H13	1	TS	Lenswood	0.010
ZG7-1	H14	1	TS	Lenswood	0.010
ZG7-1	H21	1	SC	Western Australia	0.010
ZG7-1	H27	1	TM	Lenswood	0.010
ZG7-1	H40	1	TS	Virginia	0.010
ZG7-1	H44	2	TS+Soil	Lenswood	0.021
ZG7-1	H45	1	TS	Virginia	0.010
ZG7-2	H1	2	TS	Virginia	0.020
ZG7-2	H1	1	TS	Lenswood	0.010
ZG7-2	H11	3	TM	Lenswood	0.032
ZG7-2	H16	6	TS	Virginia	0.065
ZG7-2	H18	21	TS	Virginia	0.229
ZG7-3	H19	6	TS	Virginia	0.065

*SC= stem canker, RC= root canker, TS= tuber sclerotia, TM= tuber mycelium

6. 3. 2. 4. Variation between zymogram sub-groups

Of 28 *SacI* fingerprint genotypes detected for all the zymogram sub-groups, 15 were unique. Fingerprint genotype 'S1' was found within two sub-groups (ZG7-1 and ZG7-2), and other genotypes were sub-group specific.

In general, cluster analyses using the molecular data showed that genotypes represented by ZG7-1 usually clustered together and genotypes corresponding to ZG7-2 and ZG7-3 were more related to each other than to ZG7-1 and clustered together or branched as close neighbours (Fig. 6. 2).

A similarity matrix was generated by pairwise comparison of restriction profiles and zymogram sub-groups (Table 6. 4) and a dendrogram was constructed (Fig. 6. 2). The dendrogram showed that the overall relationships between genotypes corresponded to zymogram sub-groups. In the dendrogram, genotypes related to ZG7-2 and ZG7-3 were generally clustered in the same branch as very close neighbours. However, genotypes 'S12' and 'S28', representing unique genotypes for ZG7-3, did not cluster with genotype 'S19'. Genotype 'S19', which was the most common genotype, corresponded to ZG7-3 and clustered with 'S18' which is the most common genotype for ZG7-2 (Fig. 6. 2).

6. 3. 3. Genetic diversity within and between zymogram sub-groups

Genetic diversity, calculated with the Shannon information statistic, within and between zymogram sub-groups was estimated based on different combinations of probe and restriction enzyme, pR4918/*HindIII*, pR3718/*SacI*, pR3718/*HindIII* and pectic zymograms. The level of genetic diversity within ZG7-1 in Virginia (0.362) and Lenswood (0.341) was higher than for the other zymogram sub-groups (Table 6. 5). The different combinations of probe and restriction enzyme showed different levels of variation within the populations in both these areas. The mean of genetic diversity within the sub-groups in Virginia was slightly higher than that for Lenswood. In contrast, the

Table 6. 4. Genetic similarity matrix (%) between fingerprint genotypes detected with repeated sequence pR3718 and *SacI* restriction enzyme and zymogram sub-groups of *R. solani* AG-3 isolated from Virginia and Lenswood

	1	5	6	7	8	9	10	11	12	14	15	16	17	18	19	22	26	27	28	29	31	34	40	41	44	45	
S1	100																										
S5	74.2	100																									
S6	72.2	81.4	100																								
S7	74.2	77.3	75.3	100																							
S8	73.2	84.5	84.5	78.4	100																						
S9	74.2	83.5	81.4	85.6	80.4	100																					
S10	75.3	82.5	76.3	76.3	75.3	80.4	100																				
S11	74.2	75.3	73.2	77.3	76.3	75.3	78.4	100																			
S12	73.2	80.4	74.2	74.2	75.3	74.2	79.4	76.3	100																		
S14	73.2	76.3	78.4	68.0	75.3	72.2	79.4	74.2	75.3	100																	
S15	74.2	79.4	79.4	81.4	84.5	77.3	78.4	83.5	74.2	76.3	100																
S16	75.3	78.4	76.3	72.2	77.3	74.2	77.3	84.5	75.3	75.3	78.4	100															
S17	77.3	80.4	78.4	78.4	79.4	78.4	79.4	82.5	77.3	79.4	82.5	87.6	100														
S18	79.4	80.4	74.2	80.4	75.3	80.4	83.5	80.4	75.3	75.3	82.5	79.8	81.4	100													
S19	75.3	80.4	78.4	76.3	75.3	76.3	79.4	76.3	77.3	75.3	80.4	79.4	79.4	85.6	100												
S22	78.4	83.5	81.4	77.3	80.4	77.3	80.4	81.4	78.4	80.4	83.5	84.5	92.8	80.4	82.5	100											
S26	84.5	83.5	81.4	83.5	84.5	85.6	80.4	81.4	80.4	82.5	85.6	78.4	84.5	84.5	80.4	83.5	100										
S27	79.4	82.5	82.5	76.3	83.5	80.4	81.4	78.4	77.3	79.4	78.4	77.3	83.5	81.4	77.3	84.5	84.5	100									
S28	77.3	78.4	74.2	74.2	75.3	78.4	77.3	76.3	79.4	75.3	76.3	77.3	77.3	79.4	79.4	78.4	84.5	81.4	100								
S29	80.4	81.4	77.3	75.3	78.4	81.4	82.5	79.4	82.5	78.4	79.4	74.2	80.4	78.4	76.3	81.4	85.6	82.5	78.4	100							
S31	77.3	82.5	76.3	78.4	77.3	82.5	83.5	80.4	77.3	77.3	80.4	77.3	79.4	81.4	77.3	80.4	86.6	83.5	89.7	82.5	100						
S34	82.5	81.4	75.3	79.4	78.4	83.5	82.5	77.3	74.2	76.3	77.3	74.2	78.4	82.5	76.3	79.4	87.6	82.5	92.8	81.4	92.8	100					
S40	74.2	77.3	83.5	77.3	82.5	83.5	78.4	83.5	76.3	76.3	83.5	76.3	82.5	80.4	76.3	81.4	85.6	84.5	80.4	85.6	84.5	81.4	100				
S41	79.4	74.2	74.2	76.3	73.2	76.3	81.4	78.4	75.3	75.3	80.4	75.3	77.3	81.4	71.1	78.4	84.5	81.4	75.3	74.2	79.4	80.4	78.4	100			
S44	81.4	82.5	74.2	74.2	77.3	78.4	77.3	76.3	77.3	79.4	76.3	77.3	81.4	77.3	75.3	80.4	86.6	81.4	75.3	80.4	77.3	78.4	76.3	79.4	100		
S45	80.4	79.4	73.2	75.3	80.4	77.3	76.3	77.3	72.2	74.2	77.3	74.2	76.3	78.4	70.1	75.3	87.6	76.3	76.3	77.3	80.4	83.5	77.3	82.5	82.5	100	

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Figure 6. 2. Dendrogram generated by group average analysis of the similarity values showing relationships between 26 fingerprint genotypes detected with probe pR3718 and *SacI* restriction enzyme in zymogram sub-groups of *R. solani* AG-3 in Virginia and Lenswood.

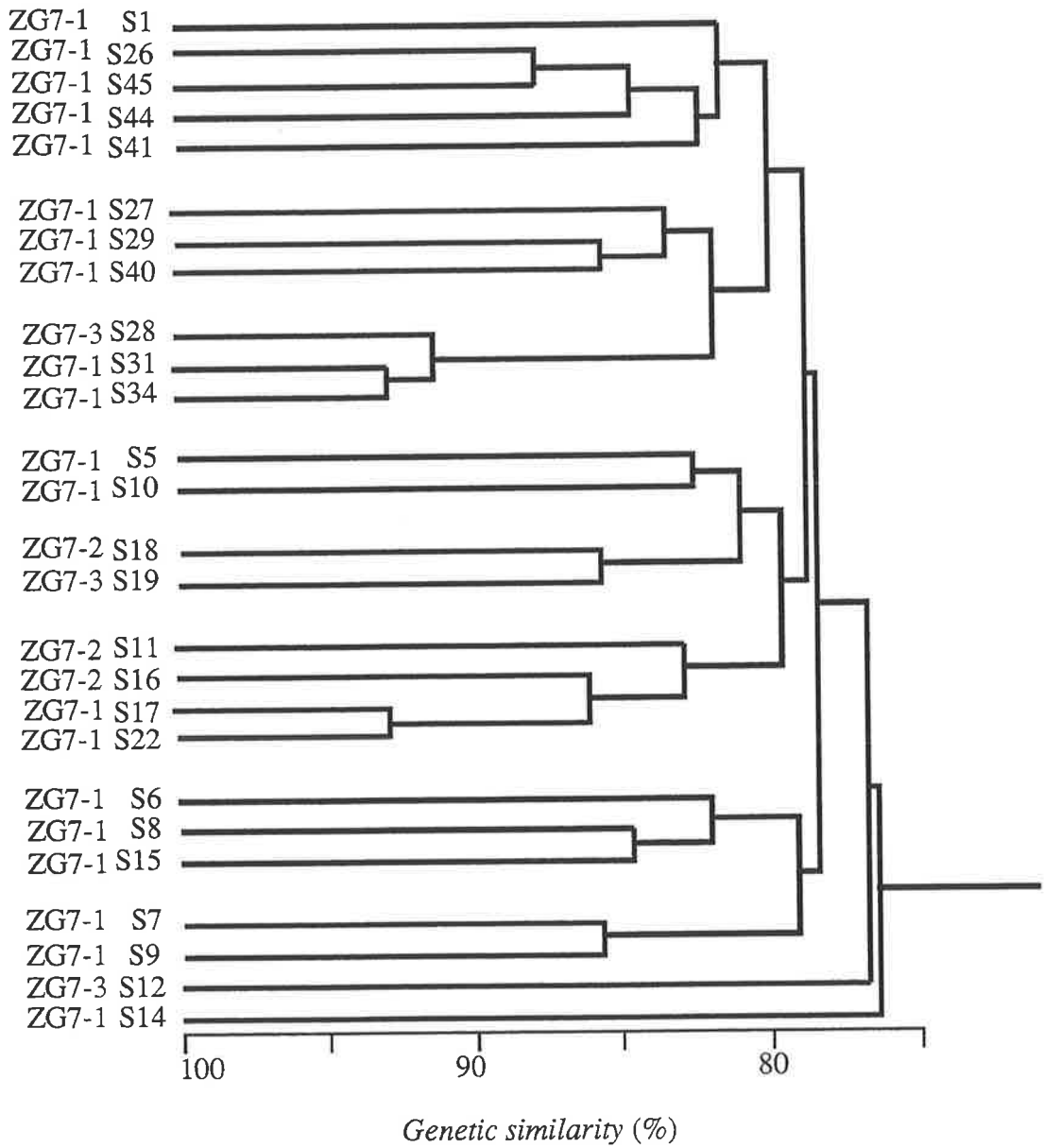


Table 6.5. Estimates of genetic diversity^a within and between zymogram sub-groups of *R. solani* AG-3 calculated from RFLP (determined with probe pR4918) and DNA fingerprint (determined with pR3718) frequencies

Population	Locus	Within sub-groups			Average within sub-groups	Total within sub-groups	Proportion within sub-groups	Proportion between sub-groups
		ZG7-1	ZG7-2	ZG7-3				
Virginia	pR4918 /HindIII	0.365	0.345	0.200	0.303	0.952	0.318	0.681
	pR3718 /HindIII	0.367	0.321	0.202	0.296	0.945	0.313	0.689
	pR3718 /SacI	0.355	0.159	0.159	0.224	0.983	0.227	0.772
	Mean	0.362	0.275	0.187	0.274	0.960	0.286	0.714
Lenswood	pR4918 /HindIII	0.345	0.166	0.102	0.204	0.987	0.200	0.793
	pR3718 /HindIII	0.340	0.202	0.202	0.248	0.948	0.261	0.738
	pR3718 /SacI	0.360	0.159	0.206	0.241	0.962	0.250	0.749
	Mean	0.341	0.175	0.170	0.231	0.965	0.237	0.760
	Mean in both areas	0.351	0.225	0.178	0.252	0.962	0.261	0.737

^a= determined using the Shanon information statistic

level of genetic diversity between the sub-groups in Lenswood was higher than for Virginia. The combination of pR3718/*SacI* detected a greater level of diversity between sub-groups than the other combinations in Virginia (0.772). In addition, the highest level of genetic diversity within sub-groups was found for Virginia isolates.

Genotypes detected by the pR3718 probe clearly showed that the three zymogram sub-groups are genetically distinguishable. Both the 'S1' (*SacI*) and 'H1' (*HindIII*) fingerprint genotypes revealed by pR3718 correlated with ZG7-1 and ZG7-2 in Virginia and Lenswood (Tables 6. 2 and 6. 3).

Although several genotypes were identified for each zymogram sub-group, it appeared that each sub-group had a predominant genotype. Fingerprint genotypes 'S1' corresponded to 'H1', 'S18' to 'H18', and 'S19' to 'H19', therefore, these genotypes could be considered as predominant representative fingerprint genotypes for ZG7-1, ZG7-2 and ZG7-3, respectively. Therefore, a dendrogram was constructed based on similarity values between predominant genotypes (Table 6. 6). The dendrogram showed that ZG7-2 and ZG7-3 were more related to each other than to ZG7-1 (Fig. 6. 3). This dendrogram was in agreement with that dendrogram generated for zymogram sub-groups in Sections 3. 3 and 4. 3.

Dome shaped and flat shaped sclerotia (described in Chapter 3), exhibited by ZG7-2 and ZG7-3 respectively, were also found to be genetically distinguishable from each other using the pR3718 probe in combination with either *SacI* or *HindIII*. Dome shaped sclerotia belonged to fingerprint genotype 'S18' or 'H18' and flat shaped to "S19" or 'H19' (data not shown).

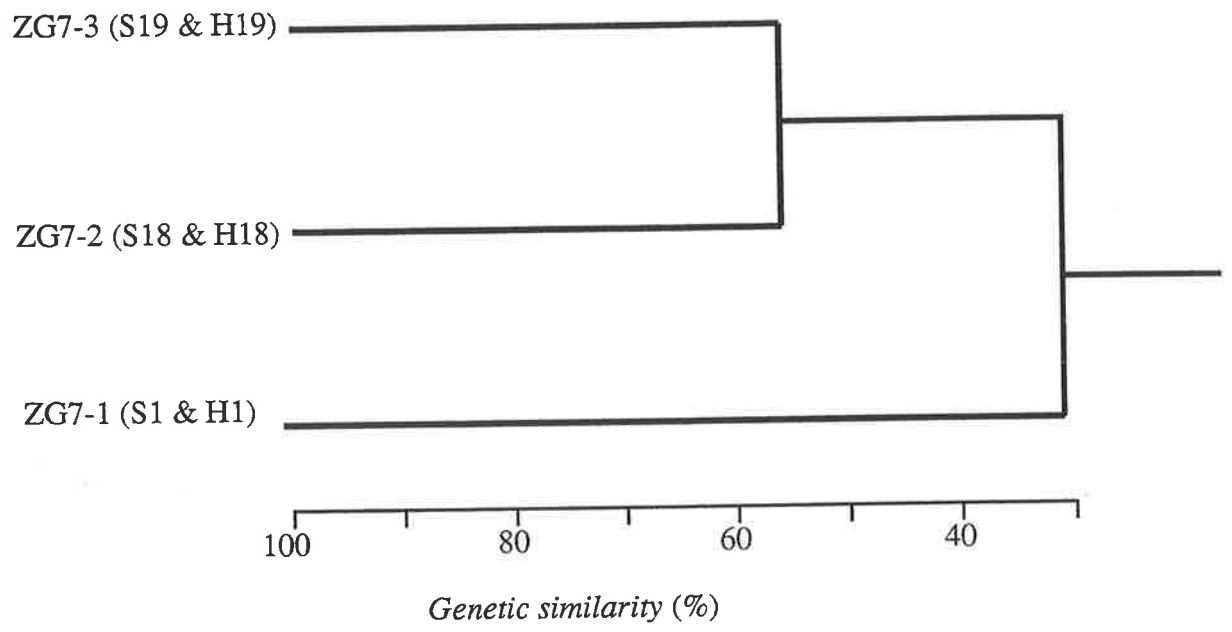
In general, there was agreement between fingerprint genotypes and the classification of isolates belonging to ZG7-1, ZG7-2 and ZG7-3. Most of the genetic diversity occurred between zymogram sub-groups rather than within sub-groups. The proportion of genetic diversity partitioned within and between zymogram groups varied

Table 6. 6. Genetic similarities (%) between three predominant *SacI* and *HindIII* genotypes revealed by pR3718 related to zymogram sub-groups of *R. solani* AG-3

	ZG7-1 (S1 & H1)	ZG7-2 (S18 & H18)	ZG7-3 (S19 & H19)
ZG7-1 (S1 & H1)	100		
ZG7-2 (S18 & H18)	37.5	100	
ZG7-3 (S19 & H19)	25.0	56.2	100

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Figure 6. 3. Dendrogram showing relationships between representative predominant genotypes (S1, 18, 19 and H1, 18, 19) of three different zymogram subgroups of *R. solani* AG-3 in Virginia and Lenswood. Data were generated by group average analysis of the similarity values obtained with probe pR3718 in combination with *Sac*I or *Hind*III restriction enzyme.



depending on geographical location. This value was higher within isolates recovered from Lenswood than from Virginia (Table 6. 5).

6. 4. DISCUSSION

6. 4. 1. RFLP marker pR4918

Isolates of *R. solani* AG-3 displayed more variation at the DNA level, as detected by the RFLP marker pR4918, than at pectic enzyme loci. Genetic similarities between different zymogram sub-groups of *R. solani* AG-3 based on the hybridisation of this marker to *Hind*III digests did not reveal any correlation between the geographical location and population specificity of genotypes. As shown in Chapter 5, pR4918 could be used as a means to analyse genetic variation within AG-3 and, perhaps, within or between other anastomosis groups. pR4918 showed that several genotypes of *R. solani* AG-3 are commonly distributed in Virginia and Lenswood, however, this probe did not discriminate different zymogram sub-groups.

Cluster analysis of genetic similarities among genotypes obtained with pR4918 indicated that RFLP genotypes '1', '2', '5' and '10', represented by more than one zymogram sub-group, grouped together on a monophyletic branch and the remainder of the genotypes, with only one zymogram sub-group, were clustered as a separate group (Fig. 6. 1). It is possible that genotypes related to different zymogram sub-groups are more genetically diverse and clustered apart from genotypes related to only one zymogram group. Isolates identified as ZG7-2 or ZG7-3 showed a closer genetic similarity to each other than to those in ZG7-1. Higher frequency of ZG7-1 isolates in the two locations suggests that this group is the dominant zymogram population of AG-3. Consequently, the higher level of genotype diversity within this group may imply it is more subjected to gene flow than are the other two zymogram sub-groups. Thus AG-3 isolates in South Australia may be composed of a series of populations in which variation is generated by

either mutation or genomic rearrangement followed by selection of particular pathotypes suited to environmental conditions.

Based on the frequency of genotypes revealed by pR4918, RFLP genotypes '1' and '5' were found to be more common in the ZG7-1 sub-group. Similarly, RFLP genotypes '5' and '2' were most frequent for ZG7-2 and ZG7-3 isolates, respectively (Table 6. 1). This suggests that each zymogram sub-group may have a common genotype resulting from selection operating on several distinct ancestral genotypes. It is also possible that the common genotypes of each sub-group may result from common environmental selection or host-mediation.

The 4.0 kbp fragment resolved by pR4918/*Hind*III was detected in 7 out of 10 RFLP genotypes and the 7.1 kbp fragment was observed in 3 out of 4 genotypes represented by ZG7-3. Some other bands were found to be group specific. The pR4918/*Hind*III combination did not resolve any genotypically characteristic bands in the zymogram sub-groups. The variation between different zymogram sub-groups implies that the low-copy RFLP marker, pR4918, shows homology with different types of genomic sequences and detects the genotypic frequencies either between or within the zymogram sub-groups. Therefore, analysis of the AG-3 isolates by using group specific DNA probes could be useful for distinguishing zymogram sub-groups. It has been shown that group-specific DNA probes can distinguish polymorphisms existing between intra-specific groups of anastomosis groups of *R. solani* (Vilgalys & Cubeta, 1994).

6. 4. 2. Fingerprinting marker pR3718

A comparison of the genetic similarities detected in the zymogram sub-groups with the repeated sequence probe pR3718 showed different relationships to those observed with the low-copy clone, pR4918. Also, genetic diversity either within or between sub-groups was more precisely resolved with pR3718. Some isolates, identified by use of pR4918 to be the same RFLP genotype, were different when tested with pR3718. ZG7-

1, ZG7-2 and ZG7-3 were different when tested with pR3718, but each zymogram sub-group was dominated by one fingerprint pattern. For instance, ZG7-1, ZG7-2 and ZG7-3 were dominated by fingerprint genotypes 'S1', 'S18' and 'S19' respectively.

The probability that any two isolates will have identical DNA fingerprints is low and isolates with the same DNA fingerprint are most likely to be clonally related (McDonald & Martinez, 1991). The results obtained for isolates of *R. solani* AG-3 using the pR3718 probe support these conclusions. Results presented here suggest that genetic variation between closely related organisms could be revealed with the repeat-sequence probe pR3718 whereas this was not the case for pR4918. However, pR3718 with either *SacI* or *HindIII* revealed that high levels of genetic diversity occurred within and between zymogram sub-groups of *R. solani* AG-3. This may indicate that isolates with identical fingerprint genotypes could be clonally related.

pR3718 revealed 36 *SacI* fingerprint genotypes in Virginia and Lenswood. Only fingerprint genotype 'S1', which occurred at the highest frequency, was found to be common in both areas and it could be that this fingerprint genotype is less influenced by the host cultivar and environmental conditions in South Australia than are other genotypes. Although it is possible that isolates with fingerprint genotype 'S1' are well adapted to South Australia environmental conditions, the distribution of this genotype at both locations sampled may also indicate that the source of initial inoculum has been the same. No data are presented to support this hypothesis, but it is possible that there is an influence of host genotype on the population diversity of different fingerprint genotypes. Yearly sampling was performed twice, from different potato cultivars in both areas, but more sampling over a number of years would provide more precise information on the regional distribution of the various fingerprint genotypes. In addition, there were some other *SacI* fingerprint genotypes; 'S11' and 'S23' in Lenswood and 'S19', 'S34' and 'S36' in Virginia, which occurred frequently and these *SacI* fingerprint genotypes could be different populations. Cluster analysis of genetic similarities revealed by the pR3718 probe indicated that zymogram sub-groups were grouped geographically.

Approximately 90% of the isolates belonging to fingerprint genotype 'S1' were represented by the ZG7-1 sub-group and the remainder belonged to ZG7-2. This may suggest that fingerprint genotype 'S1' is a collective genotype containing isolates relating to different zymogram sub-groups from the same gene pool. The amount of similarity in repeat sequences, detected between isolates of ZG7-2 and ZG7-3 sub-groups, was higher than for ZG7-1. Again, this, implies that ZG7-2 and ZG7-3 are more related to each other than either is to ZG7-1. According to the different analyses conducted here, ZG7-2 and ZG7-3 sub-groups showed a high level of similarity and were clustered together or branched as very close neighbours in cluster analysis. Existence of the dominant fingerprint genotype 'S1' in both areas sampled and the presence of common genotypes between ZG7-1 and ZG7-2 sub-groups may suggest that fingerprint genotype 'S1', mostly represented by ZG7-1 isolates, could be the ancestor from which ZG7-2 and ZG7-3 have diverged.

Harvey (1993) concluded that the ability of repeat sequences to differentiate between taxa of *Gaeumannomyces graminis* appeared to be population-dependent and that they could not be applied as taxonomic markers within species. He also provided an assessment of the taxonomic composition of pathogen populations using low-copy RFLP markers. Other workers have reported that repetitive DNA sequences, which have been used to fingerprint plant pathogens, have been found to be taxon-specific and could, therefore, be used in assessing the taxonomy of pathogens (O'Dell *et al.*, 1989; Levy *et al.*, 1991). Therefore, the detection of several fingerprint genotypes within one zymogram sub-group and the lack of genotypic overlapping between isolates belonging to different sub-groups suggests that the repeat-sequence pR3718 may be a suitable marker for use in combination with zymogram analysis. Genetic diversity in repeat-sequence DNA could be caused by either transposition, gene conversion or unequal crossing over, and these may result in differentiation between populations (Dover, 1982). Also, variation in disease symptoms, which could be relevant to gene expression, may reflect genetic diversity within *R. solani* AG-3.

The data obtained from pR3718 indicated that the similarities between zymogram sub-groups are less than those within the sub-groups. It appears that each zymogram sub-group has a predominant fingerprint genotype in each sub-population. The group average cluster analysis was performed for the predominant fingerprint genotypes to determine their relationships to the zymogram sub-groups. The genetic similarity of the representative fingerprint types obtained following digestion with *SacI* and *HindIII* was the same as the relationships exhibited by pectic zymograms. This again suggests that the probe pR3718 could be a useful marker to differentiate zymogram sub-groups and also to determine genetic variability between and within the AG-3 zymogram sub-groups.

Chapter 7

General Discussion

At the commencement of this study, in spite of the world-wide importance of *R. solani* AG-3 little was known about the genetic diversity of this pathogen. In this project, a series of experiments was conducted to provide information on identification, pathology and genetic diversity of *R. solani* causing rhizoctonia disease of potato.

The majority of the isolates of *R. solani* collected from diseased potato plants and soil from potato crops in South Australia were identified as AG-3. Although *R. solani* AG-3 is known as the "potato type" and is reported to exist wherever potatoes are grown (Laroche *et al.*, 1992), other anastomosis groups are associated with rhizoctonia disease of potatoes. The results presented here showed that rhizoctonia disease of potato in South Australia involves a combination of anastomosis groups (mainly AG-3, AG-4 and AG-5), similar to those reported in Alberta, Canada (Bains & Bisht, 1995) and elsewhere (Chand & Logan, 1983). This is the first report of AG-4 and AG-5 in potato fields in South Australia. Isolates of AG-4 grew faster than AG-3 and AG-5, but AG-3 was isolated most frequently. This finding supports the hypothesis that, in this pathogen, host specificity is more important than growth rate of an anastomosis group or environmental conditions in determining the ability of an isolate of AG-3 to infect the host. Disease development may also be influenced by other factors such as source of initial inoculum. AG-3 is probably the only group that is commonly distributed as tuber-borne inoculum, because it forms black sclerotia on potato tubers. Potato growers usually obtain seed tubers from up to three sources: replanting of harvested tubers, obtaining them from other local growers or purchasing certified seed tubers. Seed tubers from the first two sources

are usually contaminated with *R. solani*. Although freedom from *Rhizoctonia* damage is one of the requirements for first grade unwashed potato seed tubers (Johansen, 1992), certified seeds are often infested with mycelia or sclerotia (Tsrer *et al.*, 1993). Therefore certified seed or fresh potatoes could be the most important factor in the spread of AG-3. There are contradictory reports in the literature about the pathogenicity of tuber-borne inoculum, and this study showed that the presence of tuber-borne inoculum could be an important factor in disease incidence, epidemiology, pathogenicity and population dynamics of AG-3 isolates.

Sub-groups, comprising isolates that anastomose more frequently with one another, and that differ from other isolates in host range specificity, nutrient utilisation and molecular markers (Vilgalys & Cubeta, 1994), have not been reported for AG-3 isolates of *R. solani*, in contrast with other anastomosis groups. Zymogram groups are related to the established anastomosis groups (Neate *et al.*, 1988) and zymogram analysis has been used in the identification of intra-specific groups of *R. solani* AG-8 (Sweetingham *et al.*, 1986; Neate *et al.*, 1988). Variation among isolates of *R. solani* AG-3 allowed them to be assigned to one of three zymogram sub-groups, ZG7-1, ZG7-2 and ZG7-3. Based on pectic zymogram data, AG-3 has previously been described as a distinct and homogenous anastomosis group (Cruickshank, 1990) and, although Duncan *et al.* (1994) reported variation within AG-3, this is the first report of distinct sub-groups within AG-3 based on zymogram electrophoresis. The variation in pectic enzyme activities of *R. solani* AG-3 isolates reported here may be related to different alleles or genetic loci, the products of which possess different electrophoretic mobilities. Based on the considerable variation detected by zymogram analysis, it is likely that at least three sub-groups occur within AG-3. Indeed, the single isolate, tentatively designated ZG7-4, provides preliminary evidence for the existence of additional sub-groups. A comprehensive survey, including material from other major potato growing areas in South Australia, such as the Riverland and the South East, and also from other states, would provide more information about variation within AG-3. In addition, such a

study may provide sufficient information to test the hypothesis, proposed here, that ZG7-1 is a canker pathotype and ZG7-2 and ZG7-3 are black scurf pathotypes, and also may be helpful for studying the possible correlation between the genotype of an isolate and the morphology of the tuber-borne sclerotia from which it originated.

There is considerable interest in the role of pectolytic enzymes in pathogenicity. The role of pectic enzymes in fungal infection of plants has been studied in *Aspergillus nidulans* (Dean & Timberlake, 1989). In preliminary studies conducted by O'Brien *et al.* (1995) the role of pectic enzymes as virulence factors in AG-8 isolates has been investigated. These workers observed that two isozymes of polygalacturonase were prominently represented in extracts from highly virulent strains of AG-8. The apparent differences in pathogenicity of different zymogram sub-groups of AG-3, especially with respect to ability to produce cankers, may be related to differences in expression of pectolytic enzymes. Therefore, cloning of the genes coding for pectolytic enzymes, and further studies on activity of the isozymes, could provide valuable information on the aggressiveness of isolates of *R. solani* AG-3.

The genetic diversity of *R. solani* AG-3 zymogram sub-groups was studied using the highly repeated fingerprinting (pR3718) and low copy RFLP (pR4918) markers. [The development of such probes makes possible a comprehensive study of genetic variation within AG-3 and of the similarities between AG-3 and the other anastomosis groups. Probing DNA from AG-3 isolates with pR3718 revealed a high level of polymorphisms. Repeated sequences have been shown to be suitable for generating polymorphisms in fungi and such polymorphisms are thought to be a consequence of gene conversion or recombination (Scherer & Stevens, 1988), differences in the internal structure of repeat sequences and heterokaryosis (Matthew, 1992) or the presence of nuclear transposons (Cameron *et al.*, 1979). Matthew (1992) showed that restriction sites present in the repeat of one isolate may not be present in any repeat sequences of another isolate of *R. solani* AG-8. Similar

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results were obtained for AG-3 isolates in this study, and this may suggest that a high level of polymorphism occurs for restriction sites in repeated sequences detected with pR3718. *See Addendum 6*

The high level of genetic variation observed within AG-3 may be attributable, at least in part, to the occurrence of sexual reproduction, which can allow the transposition of a sequence to a new location in the genome and cause polymorphisms. The sexual stage of the fungus was not observed in the areas sampled in this study. Any future studies should include a comprehensive survey for the existence of the sexual stage, however, at present it appears that asexual mechanisms may be more important in the areas sampled. The local movement of clones from one sub-population to another, for example, can also affect the genetic structure of a fungal population. Also, long distance dispersal could cause genetic change in populations by introducing new alleles into geographically isolated fungal populations (McDonald & Martinez, 1991).

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It has been concluded that genome rearrangements evolve more rapidly in repetitive sequences than in single or low copy sequences and this repeat-sequence variation may spread through partially isolated breeding groups (Dover, 1982; Harvey, 1993). Therefore, groups of individuals belonging to separate reproductive or geographical populations accumulate different variants of repeat sequences (Harvey, 1993). Matthew (1992) concluded that, in AG-8, such genetic processing plays a role in the variation observed between isolates, based on repeat sequences. Therefore, the presence of different repeat sequence genotypes within and between zymogram sub-groups of AG-3 may be due to the existence of gene flow within and between these sub-groups. Knowledge of the amount of gene flow is limited because of the lack of genetic markers suitable for differentiating between, and tracking, populations which are morphologically identical. It appears that the highly repetitive marker pR3718 has the potential to distinguish between isolates belonging

to different populations, and, therefore, may be particularly valuable in studies of gene flow.

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Based on the high level of genetic diversity observed, it can be assumed that *R. solani* AG-3 has the potential to adapt to different environmental conditions, cultivars and soil types, and this may be reflected in its world-wide distribution. Correlation between genetic diversity and geographical distribution has been demonstrated previously in some plant pathogenic fungi (Goodwin & Allard, 1992). There was some evidence that certain isolates of AG-3 were associated with particular geographic areas, and further studies of genotypes identified using pR3718 and other molecular markers, such as RAPDs or microsatellites, and their geographic location are necessary to establish whether such a correlation exists for this fungus.

RFLP analysis of AG-3 isolates, using the low copy clone pR4918, gave multiple banding patterns and polymorphisms were detected within AG-3. Previously, random DNA probes corresponding to low copy sequences have been used successfully to detect polymorphisms within AG-3 (Jabaji-Hare *et al.*, 1990). Although pR4918 detected polymorphisms between and within the anastomosis groups (Chapter 5), it was less discriminative for identification of zymogram sub-groups, and there were genotypes common to different zymogram sub-groups. The lack of RFLP markers for zymogram sub-groups suggests that sequences detected by pR4918 are not linked to the genes for pectic enzyme activities. However, it is suggested that pR4918 is a suitable marker for studying genetic relationships within and between anastomosis groups.

The cluster analyses based on pectic zymogram (Chapter 3), PCR (Chapter 4) and fingerprint data (Chapter 6) were in good agreement and showed that ZG7-2 and ZG7-3 clustered together. This suggests that these two sub-groups are genetically more related to each other than to ZG7-1. Isoenzymes and RFLPs have been also used by the other workers to assign taxonomic groupings or infer genetic relatedness among geographically diverse collections of isolates of fungal populations (O'Dell *et*

al., 1989). MacDonald & Martinez (1991) showed that single locus and multi-locus probes revealed the same results, in terms of fingerprinting patterns of individual isolates in a *Mycosphaella graminicola* population. They observed a good agreement between multilocus haplotypes and DNA fingerprints for identification of clones. Genetic similarities measured between and within zymogram sub-groups of AG-3 using pR3718 and pR4918 were not comparable. More genetic variation was resolved by pR3718. However, more probe-enzyme combinations need to be used for fingerprint analyses to obtain a more accurate estimation of genetic similarity. DNA fingerprinting for AG-3 isolates could be also accomplished using several single locus probes, such as pR3725 and pR3733 which were developed in this study.

The nucleotide sequence of pR3718 has not yet been determined, and the function of this repeated sequence in the genome is unknown. Sequence information would allow comparison to functional genes described previously in other organisms. Characterisation of the sequence could be combined with karyotype analysis of *R. solani* AG-3 in order to provide information on the distribution of the sequence pR3718 in the genome. Studies of this nature have allowed the genes for cellulase and xylanase in *Trichoderma reesei* to be mapped (Carter *et al.*, 1992). Karyotype analysis would also provide valuable information on the number of chromosomes present in the fungus and any detectable variation in the number and size of chromosomes between strains. Knowledge of the nucleotide sequence of pR3718 would allow the design of primers suitable for further PCR-based studies. Preliminary PCR analysis of representative isolates of ZG7-1, ZG7-2 and ZG7-3 (Chapter 4) showed each zymogram sub-group to be genetically diverse, and that some bands were sub-group specific and one band was highly conserved in all isolates tested. Although this suggests that PCR-based techniques could be a suitable and quick means of detecting genetic variation between and within zymogram sub-groups, further studies using a larger number of isolates and more primers are required to provide a more accurate estimation of genetic variability

between and within zymogram sub-groups. However, PCR analyses, using two oligonucleotide primers, have shown that isolates from the same pectic zymogram group of AG-8 had the same pattern of DNA amplification and that one pattern was unique to one zymogram group (Yang, 1993). Irrespective of the use of PCR for assessing genetic variation, PCR with primers complementary to the nucleotide sequence flanking the pR3718 region would be valuable for the specific detection of AG-3 using non-radioactive methods.

Although experiments using isolates from a range of anastomosis groups of *R. solani* indicated that the probe pR3718 was specific to AG-3, in future studies the specificity of this probe, with respect to other pathogenic and non-pathogenic soil-borne fungi, should be examined.

The results obtained by slot blot hybridisation showed that pR3718 can be used in detection and identification of DNA from AG-3 isolates in soil samples. Therefore, pR3718 may provide a means for estimating amounts of hyphae of AG-3 in soil, and may give information for use in developing control measures, including crop rotation. The application of such probes in detection and estimation of AG-8 in agricultural soil has been described previously (Matthew *et al.*, 1995; Whisson *et al.*, 1995).

Rotation is known to be the most effective method to control potato rhizoctonia disease and reduce soil inoculum, and should be used in combination with sclerotia-free seed tubers, surface sterilisation of tubers, avoiding deep ploughing prior to planting, avoiding irrigation until emergence and avoiding planting in cool wet soils (Anderson, 1982, 1993). South Australian farmers often rotate wheat, barley and legumes with potato crops. AG-4 and AG-5 isolates, which are associated with legume plants (Sneh *et al.*, 1991), and AG-8 isolates which are cereal pathogens with a wide host range, were all found to be pathogenic on potato plants. Therefore, rotations with plant species which are the primary hosts of these anastomosis groups should be reconsidered by potato growers. The specific DNA

probe pR3718, which recognises AG-3 isolates causing potato black scurf disease, and pRAG12, which recognises AG-8 isolates that cause cereal bare patch disease (Matthew *et al.*, 1995), provide the tools to study population dynamics and interactions between AG-3 and AG-8 isolates relating to rhizoctonia disease incidence, especially in cereal-potato rotations, and such information could be useful in developing crop rotations to minimise losses due to the *R. solani*.

Thus studies on the identification, genetic structure, ecology and epidemiology of AG-3 populations in potato fields in South Australia and elsewhere are now possible using the probe pR3718 and other markers, including pectic zymogram sub-grouping, developed in this study. Such studies could provide basic information of the biology of *R. solani* AG-3 and its interaction with other anastomosis groups and, ultimately, lead to the development of improved disease management practices:

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Appendix

1. Culture media and reagents

Double distilled water was used in the preparation of all culture media and reagents.

All media were autoclaved at 121°C for 20 min and approximately 20 ml poured into each 9 cm diameter Petri dish.

1. 1. Acidified potato dextrose agar (APDA)

Potato dextrose agar (Difco): 39 g per L double distilled water supplemented with lactic acid (0.1% final concentration) after autoclaving.

1. 3. Water agar (WA)

Water agar: 20 g agar (Difco) per L double distilled water.

1. 4. Acidified water agar (AWA)

Acidified water agar: 20 g agar per L double distilled water supplemented with 0.1% lactic acid after autoclaving.

1. 5. Potato dextrose yeast broth (PDY)

Potato dextrose broth: 24 g potato dextrose broth (Difco) and 0.5 g yeast extract (Oxoid) per L double distilled water.

1. 6. Luria-Bertani broth (LB, Sambrook *et al.*, 1989).

Tryptone (Oxoid) 10 g

Yeast extract (Oxoid)	5 g
NaCl	10 g
Double distilled water	up to 1 L

pH adjusted to 7.0 with 5 M NaOH

1. 7. Luria-Bertani agar (LBA)

For Luria Bertani Agar, LB was solidified with 15 g agar (Difco) per L.

1. 8. Pectic enzyme inducing medium

$(\text{NH}_4)_2\text{SO}_4$	2.64 g
K_2HPO_4	0.34 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.14 g
Citrus pectin (Sigma, P-9135)	10.0 g
Double distilled H_2O	up to 1L

pH adjusted to 5.5 with 1N NaOH

2. Phenol/Chloroform

500 g phenol (BDH) was melted at 68°C using a water bath. 8-hydroxyquinoline was added to a final concentration of 0.1% and equilibrated with an equal volume of 1 M Tris-HCl (pH 8) overnight. This procedure was repeated until the pH of the aqueous phase was approximately 7.5. The phenol was then equilibrated with TE buffer for two changes and then an equal volume of chloroform was added. Fresh TE buffer was added and the phenol/chloroform was stored at 4°C in a brown glass bottle.

3. Salmon sperm DNA

100 mg of salmon sperm DNA was dissolved in 10 ml of distilled water, sonicated for 3 min at setting 5, boiled and stored at -20°C.

4. 50X TAE buffer

Tris-HCl	2 M
Na acetate	1 M
EDTA	0.05 M

pH adjusted to 7.8 with glacial acetic acid

5. 5% Triton buffer

Triton X-100	5%
Tris-HCl pH 8.5	50 mM
EDTA	50 mM

6. 10X CIP buffer

ZnCl ₂	10 mM
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MgCl₂ 10 mM

Tris-HCl pH, 8.3 100mM

7. 20X SSC

NaCl 3 M

Na citrate 0.3 M

8. 100X Denhardt's solution

Bovine Serum Albumin (Sigma) 2 g

Ficol 2 g

Polyvinyl-polypyrrolidone (360, Sigma) 2 g

9. 1X Gel buffer for pectic zymogram electrophoresis

Tris 4.60 g

Citric acid monohydrate 0.53 g

Double distilled H₂O up to 1 L

pH adjusted to 8.7 with 1N NaOH

10. 1X Pectic gel incubation buffer

DL-Malic acid 6.71 g

Double distilled water up to 500 ml

11. 1X Tank buffer for zymogram electrophoresis

HBO₃ 7.22 g

Borax (sodium tetraborate decahydrate) 15.75 g

Double distilled water up to 1 L

12. 1X Pectin-acrylamide gel staining solution

Ruthenium red (Sigma) 0.04 g

Distilled water 500 ml

Stir without heating 20 min

13. Pectin-acrylamide gel mixture for two gels

Gel buffer (section 2. 8) 60 ml

Citrus pectin (Sigma, P-9135) 0.1 g

Acrylamide 6.0 g

N, N¹Methylene bis acrylamide 0.15 g

N, N, N¹, N¹, Tetra-methylethylenediamine (TEMED) 60 μ l

Ammonium persulphate 0.06 g

Citrus pectin was added gradually, over 30 min, to the gel buffer while stirring, then the other reagents were added in the above order. After adding ammonium persulphate the mixture was immediately poured into the gel moulds, 2 mm thick, 8 cm wide and 16 cm long.

14. Stock preparation for top agar

Ampicillin 25 mg ml⁻¹ in SDW (stored at -20°C)

X-gal 2% in DMF (Dimethylformamide) (stored at -20°C)

IPTG 24 mg ml⁻¹ in SDW (stored at -20°C)

Agar 7 g L⁻¹ in SDW (stored in 4°C)

14. 1. Top agar for each plate

Melted agar	3 ml
Ampicillin	50 μ l
X-gal	40 μ l
IPTG	20 μ l

15. 10X loading buffer

Urea	4 M
Sucrose	50%
EDTA pH, 7.0	50 mM
Bromophenol blue	0.1% (w/v)

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Addenda to be inserted as follows:

1. Page 3, 1.3, last line. "Isolates of AG-11 have been recovered from Western Australia and Arkansas, USA and were found to be pathogenic on lupin, soybean and other field crops and to cause disease of cotton, radish and wheat in the glasshouse (Carling *et al.*, 1994). AG-BI, which is termed the "bridging isolate" and was found in Japan, is a soil-borne thiamine auxotrophic group (Kuninaga *et al.*, 1979)."
2. Page 46, paragraph 2, line 8. "In the present study only one AG-8 isolate was obtained from soil samples. The scarcity of AG-8 isolates is probably due to the fact that isolation was predominantly from plant organs in potato fields in rotations not involving wheat, the main host for AG-8. Also, the environmental conditions in sampled areas were unlikely to be conducive for the growth of AG-8. Therefore, more soil samples from potato fields involving wheat in the rotation could provide more information about the epidemiology of AG-8 in potato crops."
3. Page 63, below last line. "The pathogenicity experiments were conducted prior to zymogram analyses and the isolates used as inoculum were selected as representative of the anastomosis groups. Although the AG-3 isolates used in these experiments were obtained from different sources, all were subsequently found to belong to ZG7-1. Therefore, it would be informative to conduct pathogenicity tests using a larger number of isolates of AG-3 representing ZG7-1, ZG7-2 and ZG7-3. Such an experiment would provide more information regarding relationships among different ZGs and the symptoms they produce on inoculated potato plants."
4. Page 70, paragraph 2, line 4. "sharp but weak band at 1400 bp (Fig. 4. 1, shown by arrow) with the possible exception of isolate R812 (lane 3). The presence of two bands, at 920 and 980 bp (arrowed), together with the 1400 bp band appears to be typical of ZG7-1 isolates. A band at 800 bp was common to all isolates of ZG7-2 tested (Fig. 4. 1, shown by arrow)."
5. Page 154, paragraph 3, line 3. "The use of a multicopy probe, such as pR3718, has the advantage that many loci are visible on the same gel. Because of the large number of bands to be compared and the number of isolates involved, it was decided to express genetic relatedness in terms of percent similarity, in dendrogram form. Therefore, an estimate of relatedness was calculated from the proportion of shared bands; it was not the intention to determine genetic distance. Christiansen & Giese (1990) stated that multicopy clones in

general recognise sequences spread throughout the genome and that no close linkage was detected between any of these loci. Therefore, calculations were done based on the assumption that marked fingerprint loci were unlinked, and these loci were free to undergo independent assortment.”

6. Page 155, line 3. “In addition, correlation between banding patterns observed following digestion with *Hind*III and *Sac*I suggests that some of the polymorphisms are due to insertion and/or deletion events rather than to changes in restriction enzyme sites. Confidence in the analysis of the RFLP data would be increased by using more probes and probe-enzyme combinations.”

7. Page 155, paragraph 2, below last line. “Polymorphisms in *R. solani* could be a consequence of heterokaryosis. It has been shown that field isolates of certain anastomosis groups of *R. solani* are heterokaryotic, with multinucleate cells. In a heterokaryon, nuclei of different genotypes may co-exist in the same mycelia and in the same cell of a hypha. In a heterokaryotic individual, each nucleus is independent of the other nuclei, but the individual could be affected by the interaction of all nuclear types, which appears to be controlled by the genes and their relative frequencies. Therefore, genetic analysis in heterokaryotic individuals is more complicated than in homokaryotic individuals. Consequently, the multinucleate condition along with heterokaryosis may result in a lower estimate of genetic similarity than the true value.”

8. Page 156, line 2. “Fingerprinting probes, in general, provide high resolution of genetic data for natural populations. Such probes were found to be very useful in detecting frequent polymorphisms (Christiansen & Giese, 1990). This is due to the fact that, in cases when the variation is sufficient, the probability that two randomly selected individuals would have the same fingerprint would be quite low. An important result obtained in fingerprint analyses, using pR3718, was that the dominant fingerprint patterns in both sampled areas and also the major fingerprint patterns within each ZG were recognised. These are depicted clearly in graph form.”

9. Page 159, after paragraph 2. “In general, surveys of genetic variation have been conducted using a limited number of isolates collected from a large geographical area. The parameters concerned in such surveys provide data about the range of variability in large geographical areas, but the samples may have been collected from a large number of discrete populations, therefore, they are probably inadequate to provide accurate results on genetic



populations of plant pathogen (McDonald & Martinez, 1990). An alternative strategy is to take a larger number of samples from individual populations over a small area. In this case, sampling and sample size are adequate to quantify genetic variation within pathogen populations from a single location (McDonald & Martinez, 1990). In the present study, samples were taken from five properties in Virginia and five in Lenswood. Except for one property, belonging to the Lenswood Experimental Station, the other nine were growers' properties. To obtain more information about genetic variation within AG-3, these existing isolates could be subjected to analysis with more probe/enzyme combinations. Also, isolates could be obtained from more potato growing areas such as the Riverland, Walkers Flat and the south east of South Australia as well as from other states, in particular Victoria and Tasmania."

10. Page 170. Carling, D.E. & Leiner, R.H. (1990b). Virulence of isolates of *Rhizoctonia solani* AG-3 collected from potato plant organs and soil. *Plant Disease* **74**, 901-903.

11. Page 170. Carling, D.E., Leiner, R.H. & Westphale, P.C. (1989). Symptoms, signs and yield reduction associated with rhizoctonia disease of potato induced by tuber-borne inoculum of *Rhizoctonia solani* AG-3. *American Potato Journal* **66**, 693-702.

12. Page 170. Carling, D.E., Rothrock, C.S., MacNish, G.C., Sweetingham, M.W., Brainard, K.A. & Winters, S.W. (1994). Characterisation of anastomosis group 11 (AG-11) of *Rhizoctonia solani*. *Phytopathology* **84**, 1387-1393.

13. Page 170. Christiansen, S.K. & Giese, H. (1990). Genetic analysis of the obligate parasitic barley powdery mildew fungus based on RFLP and virulence loci. *Theoretical and Applied Genetics* **79**, 705-712.

14. Page 178. Kuninaga, S., Yokosawa, R. & Ogoshi, A. (1979). Some properties of anastomosis groups 6 and BI in *Rhizoctonia solani* Kuhn. *Annals of the Phytopathological Society of Japan* **45**, 207-217.