RHIZOCTONIA DISEASE ON POTATOES:

THE EFFECT OF ANASTOMOSIS GROUPS, FUNGICIDES AND ZINC ON DISEASE.

Cathryn Ann Todd

Thesis submitted for the degree of Doctor of Philosophy,

The University of Adelaide

September 2009

Faculty of Sciences,

School of Agriculture, Food and Wine

Table of contents

List of Figures

List of Tables

Abstract

Rhizoctonia solani causes disease on potato crops world-wide. Previous research showed that various anastomosis groups (AGs) caused disease on potato plants in two regions that supply the fresh washed industry in South Australia. However, the AGs of this pathogen and their impact on other potato growing regions of Australia, particularly those that supply the processing industry, was unknown. *R. solani* was isolated from commercial crops and volunteer potato plants in south-eastern South Australia, Kangaroo Island and Tasmania between 2005 and 2007. Both microscopy and AG-specific polymerase chain reaction assays were used to assign the resulting *R. solani* isolates to AGs.

The main AGs found in potato fields, in all three Australian regions, were AG 2-1, AG 2-2 and AG 3. Isolates from all three AGs were associated with disease on root, stolon, stem and tuber tissues of potato plants. Isolates representative of the main AGs found in Australian fields were used in further experiments.

The pathogenicity of selected isolates to potato was investigated. Disease severity was assessed on mature plants in experiments conducted in a shade-house and on potato sprouts grown in a controlled environment. AG 3 caused more sclerotia on tubers than did the other AGs but all three AGs were associated with stem, stolon and root necrosis. Variation in the severity of symptoms was observed among isolates from the same group. For example, inoculation of potato plants with one AG 2-1 isolate resulted in deep necrotic lesions on stems, however, inoculation with two other isolates from the same group produced no or minor lesions on stems.

Current control of Rhizoctonia disease relies on fungicides applied to seed tubers and/or soil at planting. With the aim of optimising fungicide use, the sensitivity of selected Australian isolates to one new and six commercially available fungicides was tested *in vitro* and *in vivo*. At the highest concentration tested most fungicides inhibited mycelial growth of the majority of isolates by over 80%. However, some isolates exhibited fungicide insensitivity. For example, two AG 2-1 isolates were insensitive to an iprodione fungicide *in vitro*. The control of disease symptoms on potato plants by fungicides also differed. The azoxystrobin fungicide significantly reduced stem necrosis caused by AG 2-1 and tuber sclerotia caused by AG 3 *in planta*, however, the other fungicides tested were less effective.

These findings indicate that fungicide application may be tailored to manage specific AGs and disease symptoms.

In other crops, such as medic and wheat, disease severity caused by *R. solani* is increased in micronutrient-deficient conditions. The influence of micronutrients, specifically zinc, on the susceptibility of potato plants to Rhizoctonia disease was investigated. This was explored in a shade-house and a glass-house experiment by growing potato plants in soil in which zinc had been incorporated at four different rates. In both a preliminary shade-house and subsequent glass-house experiment, the concentration of zinc in the stem tissue increased with increasing soil concentration of zinc. Assessment of disease severity was not possible in the preliminary experiment due to early senescence of plants. During a secondary experiment the increased tissue concentrations of zinc were associated with decreased stem necrosis of AG 2-1-inoculated plants after 30 days, however, this was not reflected in AG 3-inoculated plants. At harvest, disease severity could not be linked with zinc concentration due to variation in tissue content of other nutrients.

Effective management of Rhizoctonia disease is essential to produce optimum yield in terms of both quantity and quality of potato tubers. Advances in AG-specific detection of inoculum, prior to planting, provide the opportunity to refine management practices for Rhizoctonia disease, based on observations that AGs may respond differently to some management strategies, such as chemical treatments. The association of AG with Rhizoctonia disease symptoms will allow further investigation of environmental factors that influence symptom severity, including the influence of micronutrients on tolerance of potato plants to Rhizoctonia disease.

Declaration

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

_____ _____/_____/_____

Cathryn Ann Todd

Acknowledgements

I would like to extend my thanks to the many people who provided advice, assistance and support to me during the course of this project.

My supervisors Eileen Scott, Kathy Ophel-Keller and Trevor Wicks, who committed their time and effort whenever needed, thank you for your guidance and patience.

Past and present members of the Scott lab, SARDI Horticulture Pathology lab and Diagnostic Services.

In particular my thanks to Rob Harding, for answering questions on anything plant and field-based, and Herdina for assistance with and providing molecular tools.

Initially the Processing Potato Research and Development (PPR&D) program, now the Australian Potato Research Program (APRP), for funding to support this project and the opportunity to collaborate with the scientists involved in the research program.

Further support was provided by the Potato Industry Advisory Committee, Adelaide University and Alison Lees' lab at the Scottish Crop Research Institute (SCRI), which allowed me to travel to Scotland and experience a different laboratory environment. Thanks to Bob Peake in his assistance with application for funding.

I appreciate the many people who contributed isolates (listed in Table 2.1) which were used in this project and hope you can make use of some I collected.

Kathy Haskard, Dani Shteinberg and Chris Dyson for assistance with statistical analyses.

I would like to thank my friends, family and Trav, who has suffered and celebrated with me,…I hope I've made you all proud.

Abbreviations

AG	anastomosis group
ITS	internal transcribed spacer
IGS	intergenic spacer
SB	sodium borate
TAE	Tris acetate EDTA
PCR	polymerase chain reaction
nt	nucleotide
PDA	potato dextrose agar
WA	water agar
ppm	parts per million
cv.	Cultivar
a.i.	active ingredient

1 Review of Literature

1.1 Introduction

Australian agricultural domestic-use and export crops, including cereals, vegetables and fruits, were valued at $20,088 million in 2005-2006. In this demanding industry, farmers need to manage pathogens and diseases that may affect the quality and yield of their products. *Rhizoctonia* is an agriculturally important fungal genus, which contains diverse species that are found worldwide. These species are associated with disease in a wide range of cultivated crops, such as the world's four main food crops; rice, wheat, potato and maize.

In Australia, potato (*Solanum tuberosum*) is the largest vegetable crop; in 2005-2006 the crop was valued at $465 million. The main Australian potato production regions are South Australia, Tasmania and Victoria (as shown in Figure 1.1).

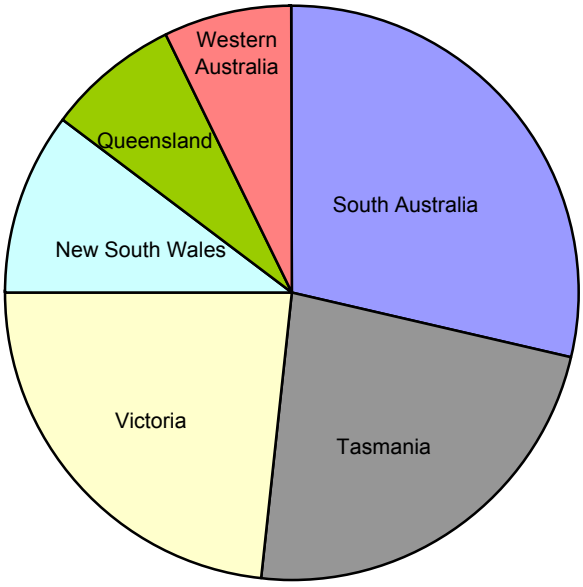


Figure 1.1 Australian potato production by state 2005-2006 (A.B.S. 2005-06)

Of the total potato production, 60% is used for the processing industry and the rest is used in the fresh washed market. In South Australia the fresh washed market is supplied by growers in the Murray Mallee and Riverland regions. The processing industry can be broken into two categories: crisping and fries. The crisping industry is supplied by the Adelaide Hills and Plains potato growing regions. Potatoes for French fries are supplied by the growing regions in the South East of South Australia, near Penola, and this component of the industry supplies 70% of the processing potatoes. Quality is an important issue for potato growers as the fresh washed market grades tubers based on appearance and the processing industry has standards for tuber size and shape.

This project is part of a collaboration called the Australian Potato Research Program (APRP), previously the Processing Potato Research and Development (PPR&D) Program, funded by the Australian government, through Horticulture Australia Limited, and the potato industry. One of the aims of this project is to reduce the impact of disease on the yield and quality of potatoes available for the processing industry. Several diseases reduce tuber yield and quality, they include: powdery scab, common scab and Rhizoctonia disease, all of which are addressed in the current APRP program. Rhizoctonia disease, commonly recognised as black scurf, is one of the most prevalent diseases of potato crops in South Australia (Dillard *et al.* 1993). Therefore black scurf and other disease symptoms caused by *Rhizoctonia* on potato are the focus of the proposed research.

Rhizoctonia disease of potato plants is caused by the fungus *Rhizoctonia solani* Kuhn (teleomorph *Thanatephorus cucumeris* (A.B. Frank) Donk). The most easily observable symptom is commonly called black scurf, where sclerotia form on tubers. This symptom resembles soil that will not wash off and affects the aesthetic value of the tuber for the fresh washed market. *R. solani* also causes sunken brown necrotic lesions on stems, stolons and roots that may contribute to delayed emergence and lower yields. Tubers produced by plants with necrotic lesions on the stolon may also be malformed and the disrupted flow of excess photosynthate results in excessess of large and small tubers (Stevenson *et al.* 2001). Therefore, *R. solani* infection affects both yield and quality of the tuber for the processing industry. As with most fungal diseases, symptoms produced by *R. solani* vary in the different environmental conditions used for potato growing. However, the variation in Rhizoctonia disease symptoms is made more complex by the range of *R. solani* sub-specific groups, which are pathogenic to potatoes. These sub-specific groups have been classified with numerous methods, the most common of which is anastomosis.

The review that follows examines literature on the pathogen *R. solani* and its interactions with potato. It explores the classification systems of the genus, the epidemiology and inoculum potential to provide an understanding of the population structure of *Rhizoctonia*. In particular, this review focuses on pathogenic variability in *R. solani* and mechanisms of disease control.

1.2 Classification of *Rhizoctonia* and sub-specific groups

1.2.1 Morphological classification

Candolle (reviewed by Menzies 1970) first described the taxon *Rhizoctonia* in 1815, however, the definition allowed the inclusion of many unrelated species. The species most studied is *Rhizoctonia solani* Kuhn, for which Parmeter and Whitney (1970) proposed a definition. To clarify the genus *Rhizoctonia*, Ogoshi (reviewed by Sneh *et al.* 1991) elevated components of this description of *R. solani* to genus level in 1975, these include:

- Branching near the distal septum of cells in young vegetative hyphae;
- Constriction of hyphae and formation of septa a short distance from the point of origin of hyphal branches;
- The presence of dolipore septa; and
- Absence of clamp connections, conidia, rhizomorphs and sclerotia differentiated into rind and medulla.

With this revised genus concept, *Rhizoctonia* species were classified into basidiomycete groups based on teleomorphic states. Although there is no single distinguishing cultural characteristic for the separation of *R. solani* from *R. zeae*, *R. oryzae*, *R. repens* and binucleate *Rhizoctonia* species (with *Ceratobasidium* teleomorphs), separation is possible on the basis of the mycelial colour, the number of nuclei in young vegetative hyphae and the morphological characteristics of the teleomorphs (Sneh *et al.* 1991).

1.2.2 *Rhizoctonia solani*

Originally, most agriculturally important isolates of *Rhizoctonia* were classified as *R. solani* (teleomorph: *Thanatephorus cucumeris*) as described by Kuhn (reviewed by Menzies 1970) in 1858. There have been numerous methods proposed for sub-specific classification for *R. solani*. DNA sequence analysis has revealed some support for the anastomosis group (AG) classification of *R. solani* (Gonzalez *et al.* 2001; Kuninaga *et al.* 1997), yet this method still needs development and clarification, for reasons discussed below, before it can be used to identify isolates pathogenic to specific crops.

1.2.3 *Anastomosis groups (AGs)*

R. solani is most commonly subdivided into anastomosis groups on the basis of the frequency of hyphal fusion. When two different, but related, isolates are cultured together the vegetative hyphae tend to fuse (Carling *et al.* 1988). Molecular investigation of the internal transcribed spacer (ITS) sequences and the 28S large subunit (LSU) regions of nuclear-encoded ribosomal DNA shows support for these groupings of *Rhizoctonia* (Gonzalez *et al.* 2001).

Fourteen AGs have been proposed in the literature to date (Table 1.1) and it has been suggested that AGs may be individual biological species (Vilgalys and Cubeta 1994). However, isolates that are part of the bridging group (AG BI) contradict this notion, as they show fusion with two or more other AGs. AG 8 and AG 2 isolates have also been found to fuse with one another, although infrequently (Sneh *et al.* 1996).

Specific AGs preferentially reside on particular host species (Keijer *et al.* 1997) (Table 1.1). AGs also have differing sensitivites to fungicides, the most commonly used Rhizoctonia disease management tools in Australian crops. However, the consistencies among isolates from an AG have allowed the development and application of targeted treatments and disease management strategies for many crops (Balali 1996; Campion *et al.* 2003; Olaya *et al.* 1994).

Table 1.1. Classification of *R. solani* AGs (modified from Sneh *et al.* 1991)

NOTE:
This table is included on page 5 of the print copy of the thesis held in the University of Adelaide Library.

1.2.4 Further physiological sub-groupings

Other methods of sub-grouping *R. solani* have been attempted, including those based on thiamine requirement, host specificity, serological and immunological properties or molecular characteristics. One approach, called intraspecific groups (ISG), attempts to classify *R. solani* into logical groups. ISGs have been described based on host specificity (Ogoshi 1987), by DNA sequence homology (Kuninaga and Yokosawa 1984) and by nutritional requirements (Carling and Kuninaga 1990). Isoenzyme analysis has been used (Liu *et al.* 1990; Liu and Sinclair 1992) but a large number of isolates were required to validate these results. Overall, the ISGs are difficult to use as a classification system because of the variation in anastomosis behaviour, morphology and pathogenicity of isolates within groups (Ogoshi 1987).

In another form of classification, pectic enzymes expressed by *Rhizoctonia* isolates, have been used to determine zymogram groups (ZGs) (Cruickshank and Wade 1980). Correlations have been found between ZGs and AGs. The ZG classification system supports AGs (MacNish *et al.* 1994) and recent studies have revealed that ZGs (Table 1.1) recognise subdivisions of AG 8 (MacNish and O'Brien 2005) and AG 3 (Balali *et al.* 2007) not distinguishable by anastomosis. However, this classification is based on an inducible enzyme system and it is possible that a bias toward the production of some enzymes exists. To overcome any bias standard techniques are used but interpretation by the operator is still required (Cruickshank and Wade 1980). Therefore, further refinement of classification systems and population mapping is still necessary to allow the relationship of sub-groups with pathogenicity to be determined.

1.2.5 Molecular methods of identification and classification

The lack of a well-defined taxonomic classification system may be one reason that pathogenicity of *R. solani* isolates has not yet been definitively established. Rhizoctonia disease is a continual problem, although the AG, ZG and ISG groupings have allowed the development of some targeted disease management strategies. Molecular techniques have been employed to identify the presence of specific AGs in soils (Lees *et al.* 2002; Ophel-Keller *et al.* 2006), which may assist in the understanding of population structures and allow further development of disease management strategies. Molecular techniques based on unique protein expression include the enzyme-linked immunosorbent assay (ELISA) and isozyme analysis. Techniques based on nucleic acids include identification of extrachromosomal RNA,

restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR). These techniques and their applications in Rhizoctonia disease are discussed below.

1.2.5.1 Enzyme linked immuno sorbent assay (ELISA)

ELISA methods have been developed with the intention of producing a fast bioassay for detecting inoculum in naturally infested soils. Antibodies were developed specifically to recognise AG 4 isolates (Thornton *et al.* 1999), however, the technique is reliant on the unique expression of external epitopes and some cross reactivity to other AGs is possible. A double baiting step allowing biological amplification of viable fungi gave an estimation of viable inoculum only (Thornton *et al.* 1999). Therefore, this method may be useful in determining viable soil inoculum prior to planting.

1.2.5.2 Extrachromosomal ribonucleic acid (RNA)

Extrachromosomal double-stranded RNA (dsRNA) has been found in numerous natural populations of *R. solani*, in a range of sizes and a range of AGs. The presence of this dsRNA has also been associated with a condition characterised by hypovirulence, termed Rhizoctonia decline (Castanho *et al.* 1978). The genetic relationship of dsRNA and AG has been studied in an attempt to classify the pathogenicity of *R. solani*. Although Jian *et al.* (1997) confirmed that some virulent strains contained dsRNA, correlation of specific dsRNA with hypovirulence in AG 3 has also been found (Liu *et al.* 2003). Since initial observations of dsRNA in *R. solani*, transmission of dsRNA elements has been observed between somatically compatible (Jian *et al.* 1997; Lakshman *et al.* 1998; Tavantzis *et al.* 2002) and incompatible (Charlton and Cubeta 2007) isolates. Although transfer of hypovirulence (Charlton and Cubeta 2007) is possible, the stability of the resulting isolate is questionable. The current view is that dsRNA found in natural populations has, as yet, an unknown correlation with virulence (Bharathan *et al.* 2005) and requires more research before dsRNA can be considered as an option in the biological control of *R. solani*.

1.2.5.3 Restriction fragment length polymorphism (RFLP)

In RFLP analysis, DNA is digested with specific restriction enzymes and separated into fragments of various sizes by electrophoresis. The resulting banding pattern may be observed when DNA is stained with ethidium bromide. When total genomic DNA is used so many fragments of different sizes are produced that only a smear can be distinguished after electrophoresis. Within this smear the detection of discrete sequences is possible with the use of small DNA fragments as a probe (Jabaji-Hare *et al.* 1990).

RFLP techniques have been used in investigations of the genetic variation between and within *R. solani* AGs (Balali *et al.* 1996; Ceresini *et al.* 2002; Gronberg *et al.* 2003; Weerasena *et al.* 2004). This has led to the ability to discriminate almost all AGs, and some of their sub-groups, by RFLP fingerprint (Guillemaut *et al.* 2003). RFLP techniques have often been combined with PCR techniques, such as when sequences were amplified prior to restriction digestion (PCR-RFLP). RFLP also allowed identification of regions in the genome useful in the design of AG-specific DNA probes (Balali *et al.* 1996; Gronberg *et al.* 2003) and PCR primers (Ceresini *et al.* 2002; Weerasena *et al.* 2004).

1.2.5.4 Polymerase chain reaction (PCR)

PCR methods have been used to amplify non-specific target sequences with universal (Lubeck and Poulsen 2001) or random (Duncan *et al.* 1993) primers to obtain a fingerprint of isolates, similar to that obtained with PCR-RFLP. In an attempt to provide growers with a fast method of inoculum identification, PCR methods have been developed to identify specific AGs via unique DNA sequences (Bounou *et al.* 1999; Boysen *et al.* 1996; Leclerc-Potvin *et al.* 1999; Lees *et al.* 2002; Ophel-Keller *et al.* 2006). Although some AG-specific PCR primers have been produced (Lees *et al.* 2002; Ophel-Keller *et al.* 2006) not all AGs can be specifically identified using PCR techniques. PCR and other methods discussed in this section are useful in determining the presence of inoculum, however, this result is only of value in disease management once a link has been established between an AG (or unique genetic locus) and the disease potential of those isolate(s).

1.3 *Rhizoctonia* disease of potato

1.3.1 The potato crop cycle

The life cycle of potato plants is common to all cultivars (Beukema and van der Zaag 1990) although specific timing of dormancy, emergence and harvest is dependent on a range of factors, including cultivar and environmental conditions. Tubers go through a stage of dormancy where sprouting is inhibited after harvest. Most commercial cultivars of potato are not grown from true seed, but are vegetatively propagated from tubers. Until recently, most commercial growers have used seed pieces, cut from mature tubers, for propagation of potato plants that sprout from the eyes (Figure 1.2). However, cut tubers require healing time to reduce water loss and susceptibility to disease and decay. This healing is a natural wound response that includes the formation of new cork cambium and phellogen layers (Priestley and Woffenden 1923). Healing time is shorter when cut pieces are stored in high temperatures, high relative humidity and sufficient oxygen, yet, temperatures above 15°C and humidity above 95% also favour microbial growth (Beukema and van der Zaag 1990). The difficulties of propagation from cut seed pieces have led to the development of whole seed-tubers or mini-tubers for propagation of potato crops.

Farmers plant mini-tubers or seed pieces at the end of dormancy, which may be broken by chemical methods, to avoid decay in the soil. Emergence of shoots occurs around 10 days after planting and subsequent growth of foliage, called haulm, coincides with growth of roots and stolons. Tuber growth starts 2-4 weeks after emergence when assimilation products from photosynthesis, in above ground parts of the plant, are transferred, via the phloem, to and stored in the underground tubers. Tubers develop at the end of stolons as a proliferation of storage tissue (refer to Figure 1.2). At tuber initiation the skin (periderm) develops. Periderm is 5-15 cell layers thick in a mature tuber and is composed of phellem and phellogen. Phellogen is a cork cambium that develops from the cell layer directly below the epidermis. In immature tubers the cork cambium is still active, cells are thin-walled and can be easily removed during harvest. At maturity the cambium activity stops and skin becomes stronger, after a period called skin “setting”. Due to the potential for damage to immature tubers, harvest occurs in two stages, around 6 months after planting. First the haulm is removed or killed by pulling, cutting, burning or chemical destruction to allow setting of the tuber skin. Tubers are harvested 10 to 20 days after the haulm has been removed, when the skin of the

mature tuber is almost impenetrable to gasses and liquids, and is a good barrier against micro-organisms (Burton 1966).

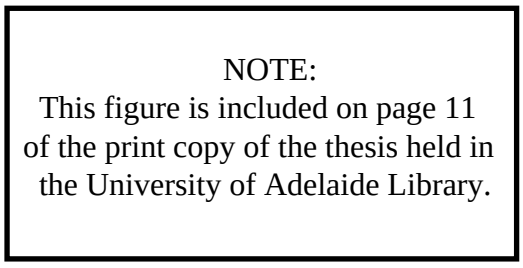
A wide variety of potato cultivars are available with differing resistance to disease and with properties that make them desirable for specific markets (NIAB 2004; Williams 2002; Williams and Dahlenburg 1994). Potato cultivars propagated for the fresh washed market range from the white, smooth-skinned Coliban, through the red-skinned Pontiac to the yellow-fleshed Desiree. The processing industry is more interested in tubers of consistent size and shape, which has led to dominance of specific cultivars. Crisping potatoes, grown in the Adelaide Hills, are commonly cultivar Atlantic whereas potatoes grown in the south east of South Australia, for frying, are often cultivar Russet Burbank (Williams and Dahlenburg 1994).

The majority of South Australian areas are planted in October-December and harvested in February-May, with the warm, dry December-February summer generally unfavourable for disease. Some early planting is done in July-October for harvest in November-February and warm, inland areas can be planted in January-March then harvested May-September (Dillard *et al.* 1993).

1.3.2 Rhizoctonia disease symptoms

R. solani disease on potato tubers was first reproducibly produced, and Koch's postulates were fulfilled, under laboratory conditions in 1861 (reviewed by Sneh *et al.* 1996). Since then, environmental conditions and the susceptibility of the potato cultivar combined with the range of pathogenic AGs have been recognised to cause a variety of Rhizoctonia disease symptoms and severities (Simons and Gilligan 1997; Yanar *et al.* 2005). Above-ground symptoms of Rhizoctonia disease include chlorosis and rolling of leaves. Necrosis on sprouts may cause delayed emergence, or failed emergence if the sprout is girdled. Aerial tubers may form in leaf axils of the stem if necrosis on stems inhibits transfer of assimilation products to underground storage tissues (Beukema and van der Zaag 1990). Sclerotia (black scurf) on tubers are the most obvious symptom of Rhizoctonia disease and, although the fungus generally does not penetrate or damage tubers, these tubers may also be misshapen (Weinhold *et al.* 1982). Necrosis of stems and stolons disrupts the transfer of photosynthate, resulting increased proportions of small and large tubers (Frank and Leach 1980). All of these symptoms lead to yield loss and affect the quality of potatoes for supply of the processing industry.

Figure 1.2 The potato plant (reproduced from Stevenson *et al.* 2001). Note: Old "seed" piece indicated for vegetative propagation of potato plants, the eye from which shoots sprout and tubers developing at the end of stolons



1.4 Pathogenicity of *Rhizoctonia solani*

1.4.1 Inoculum

Rhizoctonia survives saprophytically in soils as either vegetative mycelium or dormant sclerotia (Papavizas 1970). In some crops basidiospores, the perfect stage of *R. solani,* often develops on the lower portions of potato stems near the soil surface (Hill and Anderson 1989; Talbot 1970). Although they are not as hardy as sclerotia, and they may initiate infection of leaf tissue (Sherwood 1970) and act as a source of inoculum. Sclerotia survive in soil, especially in dry conditions, for long periods of time (Sherwood 1970). The fungus grows as a saprophyte when nutrients, such plant debris, are available. There is some evidence that *R. solani* is attracted to host species by exudates produced by the plant (Flentje *et al.* 1963) and that this soil-borne inoculum can cause disease upon contact with a susceptible host. Gilligan and Bailey (1997) investigated the impact of factors in the pathozone on the probability of infection of radish plants by *R. solani*. They determined that host susceptibility dictates the variability in infection when the inoculum source is close but that properties of the inoculum, such as the ability to germinate and grow, dictated the variability in probability of infection when inoculum was more distant. These findings are supported by more recent work of Wilson *et al.* (2008), who observed that inoculum of *R. solani* more damage to 7 day old potato shoots when placed 30 cm than when placed 90 cm away from the plant. Inoculation reduced the yield of tubers of marketable size, regardless of the distance from the plant, highlighting the importance of soil-borne *R. solani* as an inoculum source.

To control soil-borne inoculum fumigation with metham sodium has been investigated, however, this practice showed no decrease in disease at harvest (Wicks *et al.* 1996), indicating that seed-borne inocula was also important in disease. To reduce the inoculum potential from this source the purchase of certified seed (pers. com. R. Harding 2006) and fungicide treatments (Dillard *et al.* 1993) have become general practice in potato crops. With the range of inoculum sources (seed and soil-borne) and host species (Table 1.1), integrated disease management strategies are necessary to control disease in Australian potato crops.

1.4.2 Pathogenic variability of R. solani *AGs*

R. solani isolates appear to have preferred hosts, in artificial inoculation experiments, which is related to their AG (Keijer *et al.* 1997). However, variation in host specificity of

isolates from the same group has allowed further development of sub-groupings within AGs. For example, within AG 3 there is a potato infecting type (PT) and a tobacco infecting type (TB) (Ceresini *et al.* 2007; Kuninaga *et al.* 2000). Isolates from these sub-groups have differences in isozyme and fatty acid composition which are consistent with host specificities (Ceresini *et al.* 2002). This has complicated the determination of pathogenicity of AGs.
Determining the pathogenicity of all *R. solani* groups could be a large undertaking and it would be more realistic to identify the pathogenic AGs on a single crop species or in a region, such as to the potato growing regions of Australia. The study of a specific crop species and region will produce results directly relevant to that industry and may allow development of targeted management strategies.

1.4.3 AGs pathogenic to potato

A range of AGs have been found in fields used for potato cultivation around the world. The groups that have been described as causing disease in potato are summarised below:

1.4.3.1 AG 2-1

AG 2-1 has been found in potato fields in Alaska (Carling *et al.* 1986), France (Campion *et al.* 2003), Turkey (Yanar *et al.* 2005), Great Britain (Woodhall *et al.* 2007) and Finland (Lehtonen *et al.* 2008a). In these studies this group produced some sclerotia on tubers and other skin alterations on susceptible cultivars in the field and in green-house experiments. AG 2 is commonly found on, and is pathogenic to, wheat crops in Australia (MacNish *et al.* 1994; Neate and Warcup 1985), and has been found as mycelium on potato roots in South Australia (Balali 1996) although the pathogenicity of these isolates to potato was not established. In a later study this group was collected from stems of potato plants and sclerotia on tubers from Victoria and in glass-house trials caused both stem necrosis and sclerotia on tubers (de Boer *et al.* 2001).

1.4.3.2 AG 2-2

AG 2-2 isolates were associated with necrosis of stems and sclerotia on tubers from potato crops in Victoria, Australia, however, the pathogenicity of this group was not

investigated (de Boer *et al.* 2001). In the same study, this group was not isolated from brassica or clover crops used in rotation with potatoes.

1.4.3.3 AG 3

AG 3 is the major cause of black scurf worldwide (Bains and Bisht 1995; Balali *et al.* 1995; Bandy *et al.* 1988; Campion *et al.* 2003; Carling *et al.* 1986; de Boer *et al.* 2001; Lehtonen *et al.* 2008a; Virgen-Calleros *et al.* 2000; Woodhall *et al.* 2007). In these studies, AG 3 was the most common group isolated from tuber-borne sclerotia. Isolates of AG 3 generally caused more severe black scurf than isolates of other AGs in inoculation studies. This group is also commonly associated with necrosis of stems, stolons and roots.

1.4.3.4 AG 4

AG 4 has been found in Australian potato fields, on plants and in soils. In pathogenicity tests isolates of this group caused sprout and root burning on potatoes (Balali *et al.* 1995). AG 4 was found in all Canadian regions that produce potatoes and appears to be less pathogenic than AG 3 (Bains and Bisht 1995). AG 4 was also found in Mexico, where it comprised 26.5% of isolates associated with Rhizoctonia disease symptoms but only during the flowering stage of the potato plant (Virgen-Calleros *et al.* 2000).

1.4.3.5 AG 5

In Maine, USA, AG 5 was widespread in soil but infrequently found on the stem, stolon and roots of potato plants and not on the tuber (Bandy *et al.* 1988). In Canada, AG 5 isolates were not restricted to any particular region (Bains and Bisht 1995) but in France AG 5 was found in geographically distinct locations (Campion *et al.* 2003). Considering these results, Campion *et al.* (2003) suggested that survival of AG 5 on stored tubers after harvest is poor, indicating that some studies may underestimate the significance of AG 5. In greenhouse pathogenicity tests of AG 5, lesions were produced on potato stems (Bandy *et al.* 1984; Lehtonen *et al.* 2008a; Woodhall *et al.* 2007). AG 5 isolates have also caused black scurf, although, with less severe symptoms than AG 3 (Bains and Bisht 1995; Balali *et al.* 1995).

1.4.3.6 AG 8

AG 8 has been found in an Australian potato field soil (Balali *et al.* 1995) and symptoms of cankers on potato stems, stolons, roots and decreased numbers of feeder roots were reproduced in glass-house experiments but not sclerotia on tubers. AG 8 caused stolon tip burning (Balali *et al.* 1995) that may affect emergence (Hide and Firmager 1990). Similarly, potato plants inoculated with AG 8 showed delayed emergence and more root and stolon necrosis than uninoculated plants in field trials performed in Great Britain (Woodhall *et al.* 2007). In that experiment AG 8-inoculated plants also produced fewer tubers. AG 8 is a major pathogen of cereal crops in Australia (MacNish and Neate 1996). Therefore the significance of AG 8 in potato crops in Australia but not other countries, may be due to the use of cereal-potato crop rotations in the former.

1.4.3.7 AG 9

AG 9 has been found in Alaskan (Carling *et al.* 1986) and Turkish (Yanar *et al.* 2005) potato fields. It causes slight to moderate tuber damage on susceptible cultivars in the field and in greenhouse experiments. There is no literature reporting AG 9 in Australian potato crops, although in greenhouse tests in Alberta this group shows pathogenicity to wheat, canola and barley crops that are also commonly grown in Australia (Yang *et al.* 1996).

1.4.4 Use of AG-specific PCRs to detect soil inoculum

The range of *R. solani* AGs recovered from fields used to grow potatoes has been investigated for the fresh washed (Balali *et al.* 1995; de Boer *et al.* 2001) but not the processing industry in Australia. The lack of knowledge may have been related to the constraints of determining *R. solani* groups by anastomosis reaction. Hence, recently developed molecular techniques (Ophel-Keller *et al.* 2006) will allow rapid screening of large numbers of isolates, compared with the standard hyphal fusion techniques. Primers specific to AG 2-1, AG 2-2, AG 3 and AG 8 are available and primers for AG 4 and AG 5 are under development by SARDI as their component of the APRP (pers. com. K. Ophel-Keller 2005). These have been developed as TaqMan® MGB^{TM} (Applied Biosystems, Melbourne, Australia) tests for quantification of *R. solani* DNA in soils after initial difficulties with amplification of DNA extracted directly from soil were overcome (Ophel-Keller *et al.* 1999). Herdina *et al.* (2004) also showed that this type of PCR analysis did not, in most situations,

amplify non-viable fungal remnants which would result in an over-estimate of potentially infective biomass. The implementation of these quantitative PCR tests will lead to an understanding of the relative importance of different AGs in disease of potatoes that supply the processing potato industry.

1.4.5 Limitations of and variation within AGs

Variation within AGs was discussed in section 1.2.4 with respect to pectic enzyme expression and, as expected (see section 1.2.4), genetic variation has also been observed within AGs (Carling *et al.* 2002b; Ceresini *et al.* 2007; Godoy-Lutz *et al.* 2008; Jabaji-Hare *et al.* 1990; Kuninaga *et al.* 1997; Kuninaga *et al.* 2000; Salazar *et al.* 1999; Schneider *et al.* 1997). The mechanisms of evolution and maintenance of genetic variation in *R. solani* are unknown although one possible mechanism is heterokaryosis. Heterokaryotic cells contain two dissimilar nuclei and may occur as part of the sexual life cycle or by vegetative fusion. It has been demonstrated that field isolates of *R. solani* are in a heterokaryotic state (Vilgalys and Cubeta 1994) for AG 1 (Parmeter *et al.* 1969a), AG 2 (Stretton and Flentje 1972) and AG 4 (Anderson *et al.* 1972).

In most fungi, a single gene (H factor) determines mating type, which prevents vegetative heterokaryosis between two dissimilar isolates (Leslie and Zeller 1996). This type of incompatibility limits vegetative formation of a heterokaryotic individual but does not rule out sexual conjugation, which is controlled by complex, polygenic factors (Adams 1988). Anderson *et al.* (1972) suggested that in *R. solani,* genetic exchange might be allowed during the somatic cell fusion of anastomosis. In support of this hypothesis, McCabe *et al.* (1999) found that a fungal colony might be a genetic mosaic as the number of nuclei differs per hyphal branch. Although genetic exchange is difficult to assess, Julian *et al.* (1999) used molecular markers to observe genetic exchange in homokaryotic single spore isolates (SSIs), supporting the concept of genetic exchange. It appears that vegetative heterokaryosis, sexual reproduction and somatic recombination may facilitate evolution and the maintenance of genetic variation in *R. solani*. Results of further research into genetic variation may have application in understanding the structure of pathogenic populations of *Rhizoctonia*, however, genetic loci associated with pathogenicity traits have not been identified. Until markers specific to pathogenicity traits are identified, the evolutionary relationship of isolates from the same AG may be useful in assessing pathogenicity.

The internal transcribed spacers (ITS) regions of the genome are multi-copy and have been widely used in the evolutionary studies of *R. solani* mentioned above. The AG-specific primer pairs discussed in 1.4.4 were developed to amplify the ITS region (Ophel-Keller *et al.* 2006; Ophel-Keller *et al.* 2008). Variation within this region, among isolates from the same group, has most recently been investigated in attempts to further sub-group isolates based on pathogenicity. For example, ITS sequence variation was used to distinguished isolates pathogenic to tulips (AG 2-t) from non-pathogenic AG 2 isolates by Salazar *et al.* (1999) and Schneider *et al.* (1997). However, the latter study found the difference too small to use in routine tests. Carling *et al.* (2002b) observed genetic differences between AG 2 isolates which were linked to the severity of damage observed on radish, cauliflower, romaine lettuce and sugar beet plants. However, only one molecular marker was developed for all AG 2-1 isolates and the variation in pathogenicity between isolates from this group was not further investigated.

Genetic variation in another ribosomal DNA region, the intergenic spacer 1 (IGS1), was observed in AG 2-1 isolates from Great Britain and this variation was reflected in the different symptoms caused on potato plants cv. Desiree (Woodhall *et al.* 2007). However, it is not known if genetic variation exists in Australian AG 2-1 isolates, nor if variation is observed in disease symptoms caused by isolates from this same group, on potato cultivars that are grown for supply of the processing industry. Although sequence comparison is not high through-put, further investigation of the variation in these regions may allow for development of primers or probes, which are indicative of pathogenicity and complementary to the current AG-specific tests available for soil samples.

1.5 Disease management

In the past, soil-borne inoculum was believed to be the most important factor in Rhizoctonia disease of potatoes due to the ability of sclerotia to survive for long periods of time (Sherwood 1970). Methods to disrupt mycelial growth and reduce survival have been developed, including mechanical and biological techniques. However, tuber-borne inoculum is also important in Rhizoctonia disease (Wicks *et al.* 1996). Fungicide application to both seed-tubers and soil is currently the most effective (Hide and Cayley 1982) and widely used (Dillard *et al.* 1993) method to control black scurf and other Rhizoctonia disease symptoms.

Resistance of potato crops to disease has long been of interest, and breeding programs have been attempted and genetic modification of crop plants suggested (Bains *et al.* 2002; Huaman *et al.* 2000; Leach and Webb 1993). Tolerance allows growth of the host to continue in the presence of a pathogen and although potato cultivars, with a range of susceptibility to diseases have been developed, none of the cultivars grown for the processing industry has resistance or significant tolerance to Rhizoctonia disease (Yanar *et al.* 2005). Therefore, disease management strategies, such as fungicide treatment, have remained necessary for successful management of soil and seed-borne inoculum in potato crops. However, minimising Rhizoctonia disease by increasing the natural tolerance of plants can also be achieved by providing optimal nutrition (reviewed by Datnoff *et al.* 2007). In the following sections these aspects of inoculum source, methods of control and plant tolerance are further discussed.

1.5.1 Tuber-borne inoculum and fungicides to control R. solani

Chemical methods to control the tuber-borne inoculum of *Rhizoctonia* have been extensively investigated in Australia (Wicks *et al.* 1995; 1996). Dipping tubers in formaldehyde killed most sclerotia but is unlikely to be used in the industry as formaldehyde is dangerous to handle and it is difficult and expensive to dispose of the large volumes required (Wicks *et al.* 1995). Therefore, fungicides are commonly used to control seed-borne inoculum of potatoes. The fungicides currently available are generally effective at controlling Rhizoctonia disease unless high inoculum loads are present (Brewer and Larkin 2005; Hide and Read 1991; Tsror and Peretz-Alon 2005). The relative effectiveness of old, currently available, and new chemistries of fungicides at controlling symptoms in Australia has not been investigated. Research is needed to find the most effective fungicide, with the lowest application rate, to control disease.

As previously discussed (section 1.4.3), more than one AG is associated with Rhizoctonia disease. These AGs have varying sensitivity to fungicides (reviewed in this section) therefore the identification of the group(s) causing disease in any particular field is crucial to fungicide selection. Once the range of AGs pathogenic to Australian potato processing crops is determined, the relative effectiveness of new and commonly used fungicides, such as those discussed below, in controlling the growth and disease severity caused by the different groups could be evaluated.

1.5.1.1 Tolclofos-methyl

Tolclofos-methyl (Rizolex®, Sumitomo) is a form of aromatic hydrocarbon, with a phosphorothionate moiety (Orth *et al.* 1992). Mutational studies have indicated that the target site has a role in osmo-regulation, as strains resistant to this fungicide show osmotic sensitivity (Ochiai *et al.* 2001).

Tolclofos-methyl controls *Rhizoctonia* on almost all crop species in a variety of environments. Wicks *et al.* (1995) showed application of this fungicide to tubers reduced the viability of tuber-borne sclerotia during *in vitro* experiments and reduced incidence and severity in combination with fumigation of soil. Although the quality of tubers was increased with tolclofos-methyl application there was no effect on yield (Wicks *et al.* 1996). These results confirmed previous work that showed no significant difference in yield or size grades, only a decrease in stem canker in some years (Hide and Read 1991) with application of this fungicide to tubers. Tolclofos-methyl is commonly used by South Australian potato growers as a seed treatment specifically to control *R. solani* (Dillard *et al.* 1993) and should be compared with the new fungicides.

1.5.1.2 Iprodione

Iprodione (Rovral®, Bayer) is a dicarboximide fungicide, but its primary target site has not yet been established. Mutational studies have produced species resistant to this fungicide, although the mechanisms are not well understood (Ma and Michailides 2004). Fungi cross-resistant to iprodione and other dicarboximide fungicides have been found in mutational studies, indicating that the target site of iprodione, similar to other dicarboximide fungicides, has a role in osmo-regulation (Ramesh *et al.* 2001).

Iprodione inhibited *Rhizoctonia* species isolated from table beets (Olaya *et al.* 1994), canola and rapeseed *in vitro* (Kataria *et al.* 1991a; Kataria *et al.* 1991b). *R. solani* isolates from potatoes tested *in vitro* were extremely sensitive to Rovral, except two AG 2-1 isolates that showed variable responses (Campion *et al.* 2003). There was no difference in tuber yield of potatoes treated with iprodione, with or without soil fumigation, although tuber quality was increased with fumigation and fungicide treatment (Wicks *et al.* 1996). Tuber size grade was not affected by iprodione treatment, although a decrease in stem canker was evident in some years (Hide and Read 1991). As the above studies indicate contrasting results between *in vivo*

disease control and *in vitro* growth inhibition experiments further investigation is needed into the efficacy of this fungicide, which is commonly used in Australia to control black scurf.

1.5.1.3 Pencycuron

Pencycuron (Monceren®, Bayer) is a non-systemic phenylurea fungicide and was developed specifically for the control of sheath blight of rice and black scurf of potato. The primary target site of this fungicide remains to be established. Pencycuron inhibits growth of *R. solani* (Kataria and Gisi 1989) and, after diseased tubers are treated, the viability of sclerotia was reduced during *in vitro* experiments (Wicks *et al.* 1995). Variation in sensitivity to this fungicide has been found in different AGs during both *in vitro* and *in vivo* experiments (Campion *et al.* 2003; Kataria *et al.* 1991a; Kataria *et al.* 1991b; Olaya *et al.* 1994). This fungicide is registered for use in Australia to control black scurf on potato crops by application to seed.

1.5.1.4 Flutolanil

Flutolanil (Moncut®, Gowan) is a benzanilide fungicide, which is highly specific for basidiomycetes. This fungicide affects the mitochondrial respiratory Complex II, also called the succinate:quinone reductase (SQR) or the succinate dehydrogenase (SDH) complex. This complex is a functional part of the Krebs cycle and respiratory chain (Ito *et al.* 2004). Isolates of AG 3, AG 5 and AG 2-1 from French potato fields were highly sensitive to Flutolanil during *in vitro* tests (Campion *et al.* 2003). This fungicide is registered for use in Australia to control black scurf on potato crops by seed or soil application.

1.5.1.5 Fludioxonil

Fludioxonil (Maxim®, Syngenta) is a phenylpyrrole fungicide that inhibits glucose metabolism. The target site is a transmembrane sugar carrier associated with glucose metabolism (Knight *et al.* 1997). Resistance to fludioxonil is associated with mutation of osmotic-sensitive (Os-1) alleles and the amino acid repeats of Os1p (Ochiai *et al.* 2001). Growth of AG 4, AG 5 and AG 2-2 isolates from table beets was inhibited during *in vitro* trials with fludioxonil (Olaya *et al.* 1994). In Australia it is used as a seed treatment to control black scurf but the relative activity of fludioxonil has not been compared with other fungicides on potatoes.

1.5.1.6 Azoxystrobin

Azoxystrobin (Amistar®, Syngenta) belongs to a relatively new class of broad-spectrum fungicides called Quinone outside (Qo) inhibitors, which prevent energy production in fungal cells (reviewed by Gullino *et al.* 2000). This fungicide inhibits mitochondrial respiration by binding to a quinone site of cytochrome b, part of the cytochrome bc_1 complex on the inner mitochondrial membrane of eukaryotes (Stammler and Klappach 2005). This complex is critical in electron transport in cells and blockage prevents electron transfer and ATP production. However, there is an alternative fungal respiration pathway that must also be inhibited during *in vitro* experimentation (Mizutani *et al.* 1995), simply achieved by adding salicylhydroxamic acid (SHAM) to the culture medium. Resistance to this class of fungicide has been found and can, in most cases, be attributed to a single nucleotide mutation, G143A, in the cytochrome b gene (Gisi *et al.* 2002). This fungicide decreased Rhizoctonia disease, caused by AG 1, in rice fields (Slaton *et al.* 2003). In Australia, azoxystrobin has been recommended for use on potato seed tubers and was recently registered as an in-furrow treatment to control Rhizoctonia disease. However, the degree of Rhizoctonia disease control has not been determined in comparison with currently used fungicides.

1.5.2 Cultural methods of minimising soil-borne inoculum of R. solani

Fungicides alone may be effective in disease management where inoculum is moderate to low but not where there is high inoculum potential (Brewer and Larkin 2005; Hide and Read 1991; Tsror and Peretz-Alon 2005). The soil environment and inoculum potential can influence the growth and pathogenicity of *R. solani* isolates (Harikrishnan and Yang 2004). Wet conditions and low temperatures after planting favour germination of sclerotia, therefore, crops grown through the dry South Australian summer (December to February) are less likely to develop Rhizoctonia disease than crops grown in cold, wet seasons. Rhizoctonia disease severity also varies with soil type and Hill and Anderson (1989) found that sandy, irrigated soils produce the most severe infections of potatoes.

Mechanical methods of controlling disease include cultivation of soil to a depth of 100 mm in an attempt to disturb colonies of *R. solani* AG 8 before sowing cereal crops (MacNish and Neate 1996). However, erosion of sandy soils has led to a decrease in this practice and increased incidence of Rhizoctonia disease (Hill and Anderson 1989). In potato crops a

mechanical method that decreases tuber sclerotia by disrupting fungal colonies in soil is green crop harvesting (Mulder *et al.* 1992). This method involves the separation and removal of haulm and roots from tubers before the period of tuber skin healing in soil, this limits the presence of exudates from decaying tissues which attract *R. solani*, and has been applied and adapted by some farmers.

Crop rotation is widely used to deprive the pathogen of a suitable host. Proliferation of soil-borne inoculum may be reduced along with disease severity in a subsequent crop. However, crop rotation for this purpose may not be useful in the control of AG 8 or AG 2 in Australia as both have wide host ranges (see Table 1.1). Crop rotation can provide additional biological protection, as discussed below.

Kirkegaard *et al.* (1994) investigated the inhibition of *Rhizoctonia* species that damage cereal crops in crop rotations involving *Brassica* species and canola. Products released from the breakdown of brassicas include isothiocyanates (ITC) that are biocidal to fungi, including *Rhizoctonia* (Sarwar *et al.* 1998). Methods used in these studies have been adapted to investigate potato disease, including *Rhizoctonia* (de Boer *et al.* 2001; Wicks *et al.* 1997). Results from *in vitro* experiments show that brassica residues can inhibit the growth of AG 3 and AG 8 isolates, however, their effectiveness varies with brassica species (Wicks *et al.* 1997), tissue type and herbicide application (de Boer *et al.* 2001). Meal produced from Indian mustard reduced viability of sclerotia in the soil by 60% (Wicks *et al.* 1997) but the emergence of plants grown in soil amended with meal in field trials was also inhibited (de Boer *et al.* 2001; Wicks *et al.* 1997). Black scurf on tubers was reduced by 12% in a glasshouse experiment, however, in field trials the production of sclerotia on tubers was not inhibited (de Boer *et al.* 2001; Wicks *et al.* 1997).

1.5.3 Biological control of Rhizoctonia *disease with micro-organisms*

The control of disease, regardless of inoculum source, by biological methods has been attempted since fumigation of soil indicated that both soil and tuber-borne inocula were important in disease (Wicks *et al.* 1996). Biological control of *R. solani* has been attempted using antagonistic organisms that compete for nutrients, space and hosts favoured by *Rhizoctonia* species.

The effect of antagonistic bacteria on *Rhizoctonia* species has been investigated with the aid of the enzyme laccase. The production of laccase potentially has a role in maintaining

homeostasis within fungi and its excretion during *in vitro* experiments has been used as an indicator of stress. For example, when *R. solani* is cultured with different strains of *Pseudomonas fluorescens* the former produces differing quantities of laccase, related to the latter's ability as an growth antagonist (Crowe and Olsson 2001). Some preliminary work has been done, using this method, to determine if biological controls are compatible with chemical controls in potato crops. For example, *Verticillium biguttatum* Gams was tolerant of pencycuron and flutolanil, and when applied in combination with these fungicides showed an additive effect in control of black scurf (van den Boogert and Luttikholt 2004). Isolates of this fungus collected from South Australian fields reduced the incidence and severity of sclerotia on daughter tubers when applied as a spray of resuspended spores to seed-tubers prior to planting in field experiments, with or without pencycuron seed treatment (Wicks *et al.* 1997).

Trichoderma species have also been investigated as biological control agents for Rhizoctonia disease control of potatoes. When this species was used as soil treatment with flutolanil-treated seed early stem necrosis was inhibited and proportions of marketable-sized tubers increased, however, yield losses were not reduced (Wilson *et al.* 2008a; Wilson *et al.* 2008b).

Arbuscular mycorrhizal (AM) fungi have also been investigated as biological control agents of *R. solani* on potato plants. Colonisation of potato plants by *R. solani* and AM fungi was not found to be competitive, however, during dual inoculation plant roots accumulated anti-microbial phytoalexins, indicating that tolerance could be stimulated by AM fungi (Yao *et al.* 2003). Similar findings of tolerance of bean seedling to infection by *R. solani* have been observed during co-inoculation studies with non-pathogenic binucleate *Rhizoctonia* (BNR) species (Xue *et al.* 1998). BNR also provide protection to radish and cotton from infection by *R. solani* (Sneh and Ichielevich-Auster 1989) and signal molecules from BNR that trigger host defence mechanisms in potatoes have been studied (Wolski *et al.* 2006).

In some soils when the environment favours disease, suppression of disease in cereals crops actually occurs (Wiseman *et al.* 1996). This suppressive characteristic was transferred to autoclaved soil, indicating a biological component (Wiseman *et al.* 1996). After further studies in wheat a combination of micro-organisms was shown to reduce disease without altering the concentration of AG 8 DNA in soil (Barnett *et al.* 2006). However, to date no biological products have been developed or are recommended for use by potato growers supplying the processing industry in Australia.

1.5.4 Tolerance of potato plants to Rhizoctonia disease

Although tolerance to *R. solani* can be induced, as discussed in the above section, no individual gene has been found which is associated with true resistance. Most plants selected in breeding programs have an increased tolerance to *R. solani*, which is ineffective under high disease pressure (Scholten *et al.* 2001).

Genetic modification of hosts is one possible method of achieving disease resistance. Therefore, non-native genes have been transformed into plants. For example, the maize *b-32* gene, which encodes a ribosome inhibiting protein (RIP), was transformed into tobacco plants by infection with *Agrobacterium tumefaciens* which harboured a construct contained the gene (Maddaloni *et al.* 1997). This RIP inhibits translation by rendering ribosomes unable to bind elongation factors necessary for this function. Plants processing this gene, and expressing the RIP, resulted in increased tolerance to infection by *R. solani* AG 4. In a separate study, tobacco plants were transformed with a barley chitinase (CHI) gene to degrade the chitin component of the *R. solani* cell wall (O'Brien *et al.* 2001). This study resulted in transgenic tobacco resistant to AG 4 but not to AG 8. Recently, transgenic potatoes were produced which contained a modified demaseptin B1 gene, the protein product of which inhibited mycelial growth of *R. solani* and a number of other pathogens during *in vitro* experiments (Osusky *et al.* 2005). The original gene was isolated from the skin of arboreal frogs (*Phyllomedusa sauvagii*) and produces a broad-spectrum anti-microbial peptide, which is relatively non-toxic to eukaryotic cells at low concentrations. The transgenic potato showed less infection than non-transgenic plants when challenged with *R. solani* in a leaf assay (Osusky *et al.* 2005). However, further experimentation is necessary to determine if Rhizoctonia disease symptoms such as stem necrosis and black scurf are reduced in transgenic plants, when compared to normal plants.

To aid in identifying genes involved in the induction of tolerance to *R. solani,* the differential expression of genes can be investigated with microarray analysis. In two experiments using this method potato plants were used as the host (Lehtonen *et al.* 2008b; Rivard and Jabaji-Hare 2006) and, although a multitude of genes involved in the infection and defence processes were identified (Lehtonen *et al.* 2008b), this technique has not yet produced results, such as individual genes, that would constitute useful genetic material for future breeding programs or modification.

The variability of Rhizoctonia disease from one year to the next means that most farmers in South Australia apply disease control strategies when symptoms appear (Dillard *et*

al. 1993). The most popular disease control strategies may therefore be short-term strategies that control an immediate disease problem, rather than long-term management of field inoculum levels with crop rotation or expensive transgenic crops.

Fertilisers are applied throughout the growing season as agricultural crops, such as potatoes, do not readily mobilise and transfer nutrients from old to new leaves and to tubers (Baghour *et al.* 2002). Plant nutrition has long been established as an important factor in obtaining maximum yields and is also recognised to play a role in tolerance of disease. The essential nutrients for plant growth can be grouped into macronutrients and micronutrients, which are further discussed in the following sections, with emphasis on their possible role in disease development.

1.5.5 Macronutrients

Macronutrients, such as nitrogen, potassium, phosphorus, magnesium, calcium and sulphur, can be applied in large doses and continue to have an effect on plant growth past the plant's "sufficient range". Although deficiency in these nutrients can increase disease severity in many plants (reviewed by Datnoff *et al.* 2007) fertilisation with macronutrients is commonly used to increase crop yields (Graham 1983). Tests to measure the nutrient level of soils and foliar tissue are also commonly used in determining fertilisation requirements for potato crops in Australia (Crump 2006). Applications surplus to the levels necessary for yield increase may result in loss of tuber quality. For example, excess nitrogen promotes excess foliar growth which delays tuber maturity. Therefore, fertiliser application rates and procedures have been optimised to avoid surplus. However, application of one nutrient may not increase growth, yield or disease tolerance if another nutrient is lacking and Australian soils are weathered and often deficient in essential nutrients (Sivasithamparam 1993).

1.5.6 Micronutrients

Small amounts of micronutrients, such as aluminium, boron, chlorine, copper, iron, manganese, molybdenum, nickel and zinc are necessary for optimal plant growth and doses past the sufficient range may be toxic. Micronutrient deficiencies, particularly zinc deficiencies, have been observed in Rhizoctonia disease-affected crops of medic (Streeter *et al.* 2001b), cowpea (Kalim *et al.* 2003) and cereals (Thongbai *et al.* 1993a) in Australia. The addition of micronutrients to wheat, barley and rice showed decreased disease in some cases,

possibly by aiding the plant's natural defence system (Lambert and Manzer 1991; Soltani *et al.* 2002). Micronutrients, discussed below, are often ingredients in fungicides and fertilisers used to promote optimal crop growth, but little is known about the effect of deficiency of these micronutrients on the severity of Rhizoctonia disease.

1.5.6.1 Zinc

Soils in the south east of South Australia, used to cultivate cereal (Thongbai *et al.* 1993a) and medic crops (Streeter *et al.* 2001b), are naturally deficient in zinc. However, nearby potato growing regions have not been studied in relation to zinc deficiency and Rhizoctonia disease severity. Zinc deficiency in *Medicago truncatula* resulted in more severe Rhizoctonia root disease symptoms than on plants with added zinc (Streeter *et al.* 2001b). Application of zinc was not toxic to *R. solani* present in the soil, however, the improved zinc status of the plant increased tolerance to *R. solani,* even though the resulting increase in root length and diameter allowed for more infection points. The interaction of zinc and *R. solani* was also observed to have an effect on phosphorus, manganese and magnesium uptake and transport.

1.5.6.2 Others

Manganese deficiency is common in sandy, calcareous and limed soils (Burton 1966) but potato plants are also very sensitive to excess manganese, which can cause stunting of the plant as the terminal bud dies. Some Australian soils are deficient in manganese, including areas in the south east of South Australia where potatoes are grown (pers. com. P. Frost 2005). The severity of Rhizoctonia disease on manganese-deficient potato plants has not been investigated in Australian conditions. However, application of manganese to deficient cowpea plants decreased Rhizoctonia disease (Kalim *et al.* 2003).

Australian processing potato growing regions, such as the lower south east of South Australia, have soils low in copper (pers. com. P. Frost 2005). Potato crops grown on Prince Edward Island, in Canada, in acidic, organic soils have responded to copper applications (Sanderson and Gupta 1990; Sharma 1990). Although, the ability of application copper to reduce the severity of Rhizoctonia disease has not been investigated for potato crops in

Australian conditions, high levels of copper in USA canola crops were associated with increased disease due to *R. solani* AG 2-1 and AG 4 (Verma 1996).

Boron provided better control of Rhizoctonia disease than other micronutrients tested in cowpea crops and pot trials (Kataria and Sunder 1988). However, boron concentrations in soil in potato fields have only recently been investigated, with respect to disease expression (pers. com. C. Russell 2005). Recent amendment experiments suggest that boron application results in increased common scab (pers. com. T. Wiechel), however the effect of boron application on Rhizoctonia disease has not been investigated.

Very low concentrations of molybdenum and nickel are necessary for plant growth and there are no reports of deficient potato crops. Likewise, iron deficiency is unlikely unless potatoes are grown in highly calcareous soils (Stevenson *et al.* 2001). Possibly because of the widespread use of muriate or potash (KCl) in potato fields, no response to applications of chlorine has been observed in crops (Stevenson *et al.* 2001). Therefore, from a research perspective the interactions of molybdenum, iron and chlorine with pathogens may be of interest; however, the outcome would have limited impact on growing practices as deficiencies of these nutrients are rarely observed in potato crops.

1.6 Summary

Rhizoctonia disease is detrimental to the quality of tubers produced for the processing potato industry. This disease is caused by a range of AGs of *R. solani* with differing pathogenicity, yet the range of AGs associated with Australian processing potato fields has not been investigated. The disease in these fields could be more effectively managed, such as with selected fungicide application, if the pathogenic groups were identified. Molecular techniques could provide an effective means to assign isolates from Australian potato growing fields to an AG. Pathogenicity tests could then be conducted to determine the severity of symptoms caused by that range of AGs.

The most commonly used disease management technique is the application of fungicides to seed tubers. Numerous fungicides are available and recommended for control of black scurf, and new classes of fungicide are being identified yet their activity against *Rhizoctonia* has not been quantified. Australian potato farmers would benefit from an evaluation of the effectiveness of the new and commonly used fungicides. Their relative ability to control disease caused by the range of pathogenic AGs found in Australian potato

crops should be investigated; such information may allow for optimised application rates, selection of fungicides (if resistance is identified) and more effective disease management practices.

Breeding programs have not produced *Rhizoctonia* resistant or tolerant potato cultivars. An important factor in tolerance of disease is optimal crop growth and nutrient status. Micronutrient deficiencies appear to influence the tolerance of plants to disease but their effects have not been investigated in Rhizoctonia disease-affected potato crops. Micronutrients may affect the range of symptom severity, which could result in the use of micronutrients in disease control.

1.7 Aims of the research

1) Determine, using molecular and classical techniques, the range of *R. solani* AGs associated with Australian potato fields that supply the processing industry.
2) Establish the pathogenicity to potatoes of representatives from the various AGs recovered from Australian potato fields.
3) Evaluate the sensitivity of a range of AGs and isolates to selected fungicides.
4) Investigate the effect of micronutrients on the incidence and severity of Rhizoctonia disease of potato.

2 General Methods

2.1 Plant tissue sampling

Organs of potato plants, including stems (green and white), stolons, roots and tubers, were sampled separately as shown in Figure 2.1. Stems and stolons were rinsed with reverse osmosis (RO) water then 2 cm lengths were excised from an area comprising necrotic and the adjacent healthy tissue. Roots were cut into 5 cm lengths and at least one growing tip and one section close to the mother tuber were taken from each plant. Attempts were made to include sections of necrotic tissue from root samples; however, in many cases only healthy tissue was available. Samples from tubers included sclerotia, which were excised without removing the tuber epidermis, and mycelium, observed by microscopy and removed with sterile forceps from the grooves and crevices on the surface of malformed tubers. Some tubers were surface sterilised in a 1 % sodium hypochlorite solution for 5 min then rinsed in sterile RO water three times before sclerotia were removed. Three samples from each organ, from each plant, were placed on a modified Ko and Hora (1971) medium (see Appendix 10.1) in 90 mm Petri dishes. These were incubated in the dark at 25°C and after 48 hrs were observed under a dissecting microscope to identify *Rhizoctonia*-like colonies. Selected colonies were purified by transferring single hyphal tips onto quarter strength potato dextrose agar (PDA, Difco, USA).

2.2 Maintenance and storage of isolate collections

Cultures used as tester isolates were obtained from collaborators in other states of Australia (see Table 2.1 and Figure 2.2). The AG for the tester isolates had been determined by other researchers, independent of the AG-specific primer sets used in this project. All isolates were purified by transferring hyphal tips to PDA and their AG was confirmed with molecular methods as described in the following sections. To confirm the reliability of the AG-specific primer sets, selected isolates were also assigned to AG on the basis of cultural techniques also described in the following sections. All isolates were grown on quarter or full strength PDA for use in the short-term. For medium-term storage two methods were utilised. Firstly, 5 x 5 mm plugs from isolates actively growing on PDA were placed in 2 ml of sterile RO water and stored at 4°C. Secondly, colonised plugs of PDA described above were inserted into a sterilised mixture of UC (University of California) potting mix (Baker 1957) and millet seed (10 % v:v) and stored at 25°C; this was agitated every 2 days for a fortnight to distribute

hyphae evenly through the mix (Sneh *et al.* 1996). Long-term storage of PDA plugs, bearing sclerotia, was in 1.5 ml of 10 % glycerol solution in 2 ml eppendorf tubes which, after incubation overnight at -20°C, were stored at -80°C (Sneh *et al.* 1996).

Figure 2.1. The potato plant showing organs and tissue sections[1] from which *Rhizoctonia* isolates were collected and disease severity scores were taken (reproduced from Stevenson *et al.* 2001)

[1] Circled: Brown = Green (above ground) stem; Pink = White (below ground) stem; Red = Stolon; Blue = Tuber; Yellow = Roots.

Table 2.1. The host plant and region of origin of isolates used[1] in this study (continued on the following pages)

Isolate classification			Origin		
Code	**AG**	**Tester**	**Source**	**Region**	**Host and tissue**
R394	2-1	Yes	Dr. Tonya Wiechel (DPI[2], Knoxfield)	Ballarat, Vic[3]	Potato plant debris
R14	2-1	Yes	Dr. Tonya Wiechel (DPI, Knoxfield)	Colac, Vic	Potato, tuber sclerotia
L51	2-1	Yes	Dr. Herdina (SARDI[4])	Virginia, SA[5]	Potato, tuber sclerotia
L57	2-1	Yes	Dr. Herdina (SARDI)	Virginia, SA	Potato, tuber sclerotia
R107	2-1	No	Dr. James Woodhall (X46) (via SCRI[6])	Cheshire, UK[7]	Potato, stolon
R103	2-1	No	Dr. James Woodhall (Y3) (via SCRI)	Lincolnshire, UK	Potato, stem
R118	2-1	No	Dr. James Woodhall (2023) (via SCRI)	UK	Cauliflower
R72	2-1 IV	No	Dr. Shigeo Naito, Hokkaido National Agricultural Experiment Station (via SCRI)	Japan	Soil
R114	2-1	No	Dr. James Woodhall (1956) (via SCRI)	UK	Cauliflower
R100	2-1	No	Dr. James Woodhall (Y2) (via SCRI)	Shropshire, UK	Potato, tuber sclerotia
R42	2-1	No	Plant Research International (via SCRI)	The Netherlands	Cauliflower
R106	2-1	No	Dr. James Woodhall (X52) (via SCRI)	Scotland	Potato, stem
R117	2-1	No	Dr. James Woodhall (1996) (via SCRI)	Unknown	Cauliflower
SE50	2-1	No	n/a	South East of SA	Potato, root
SE45	2-1	No	n/a	South East of SA	Potato, root
KI65	2-1	No	n/a	Kangaroo Island, SA	Potato, malformed tuber
KI4	2-1	No	n/a	Kangaroo Island, SA	Potato, white stem
Tas6	2-1	No	n/a	Tasmania	Potato, stolon
SE42	2-1	No	n/a	South East of SA	Potato, white stem

[1] Although 329 isolates were assigned an AG during this study the isolates shown in this table were used in further experiments and as tester isolates (previously classified to AG by other researchers) in the preliminary experiment to confirm specificity of primers to target AG (as indicated in the table above)

[2] DPI = the Department of Primary Industries, Victoria

[3] Vic = Victoria, Australia

[4] SARDI = the South Australian Research and Development Institute

[5] SA = South Australia

[6] SCRI = The Scottish Crop Research Institute

[7] UK = United Kingdom

n/a indicates the isolate was collected during the course of this project

T30.1	2-1	No	n/a	Tasmania	Potato, stem
KI61	2-1	No	n/a	Kangaroo Island,SA	Potato, tuber rosetting
730	2-1	Yes	Catherine Hitch (SARDI)	South Australia	Cauliflower, high stem
1030	2-1	Yes	Catherine Hitch (SARDI)	South Australia	Cauliflower
SAR11.18	2-1	No	Dr. Houng Pung (Peracto Pty, Ltd.)	Tasmania	Green bean, hypocotyl
Tas3	2-1	No	n/a	Tasmania	Potato, root
KI43	2-1	No	n/a	Kangaroo Island, SA	Potato, black scurf
SE51	2-1	No	n/a	Kangaroo Island, SA	Potato, white stem
KI41	2-1	No	n/a	Kangaroo Island, SA	Potato, black scurf
WAC-9806	2-1, ZG6	Yes	Dr. Herdina (SARDI)	Unknown	Lupin
WAC-9937	2-2, ZG4	Yes	Dr. Herdina (SARDI)	Doodlakine, WA[8]	Lupin
WAC-9765	2-2, ZG5	Yes	Dr. Herdina (SARDI)	Albany, WA	Lupin
894	2-2	Yes	Catherine Hitch (SARDI)	Bairnsdale, Vic	Cauliflower, stem
R72	2-2	No	Dr. Shigeo Naito, Hokkaido National Agricultural Experiment Station (via SCRI)	Japan	Soil
KI3	2-2	No	n/a	Kangaroo Island, SA	Potato, tuber sclerotia
KI24	3	No	n/a	Kangaroo Island, SA	Potato, stolon
R421	3	Yes	Dr. Tonya Wiechel (DPI, Knoxfield)	Ballarat, Vic	Potato, tuber sclerotia
R44	3	Yes	Dr. Tonya Wiechel (DPI, Knoxfield)	Colac, Vic	Potato, tuber sclerotia
R101	3	Yes	Dr. Tonya Wiechel (DPI, Knoxfield)	Colac, Vic	Potato, tuber sclerotia
R153	3	Yes	Dr. Tonya Wiechel (DPI, Knoxfield)	Ballarat, Vic	Potato, tuber sclerotia
R2	3	Yes	Dr. Tonya Wiechel (DPI, Knoxfield)	Colac, Vic	Potato, tuber sclerotia
R26	3	Yes	Dr. Tonya Wiechel (DPI, Knoxfield)	Colac, Vic	Potato, tuber sclerotia
R7	3	Yes	Dr. Tonya Wiechel (DPI, Knoxfield)	Colac, Vic	Potato, tuber sclerotia
R227	3	Yes	Dr. Tonya Wiechel (DPI, Knoxfield)	Ballarat, Vic	Potato, tuber sclerotia
R242	3	Yes	Dr. Tonya Wiechel (DPI, Knoxfield)	Ballarat, Vic	Potato, tuber sclerotia
R217	3	Yes	Dr. Tonya Wiechel (DPI, Knoxfield)	Ballarat, Vic	Potato, tuber sclerotia
R229	3	Yes	Dr. Tonya Wiechel	Ballarat, Vic	Potato, tuber sclerotia

[8] WA = Western Australia

			(DPI, Knoxfield)		
R214	3	Yes	Dr. Tonya Wiechel (DPI, Knoxfield)	Ballarat, Vic	Potato, tuber sclerotia
R213	3	Yes	Dr. Tonya Wiechel (DPI, Knoxfield)	Ballarat, Vic	Potato, tuber sclerotia
R57	3	Yes	Dr. Tonya Wiechel (DPI, Knoxfield)	Colac, Vic	Potato, tuber sclerotia
R128	3	Yes	Dr. Tonya Wiechel (DPI, Knoxfield)	Colac, Vic	Potato, tuber sclerotia
R404	3	Yes	Dr. Tonya Wiechel (DPI, Knoxfield)	Ballarat, Vic	Potato, stem hyphae
L18	3	Yes	Dr. Herdina (SARDI)	Walkers Flat, SA	Potato, tuber sclerotia
L30	3	Yes	Dr. Herdina (SARDI)	South East of SA	Potato, tuber sclerotia
L41	3	Yes	Dr. Herdina (SARDI)	Walkers Flat, SA	Potato, tuber mycelium
L55	3	Yes	Dr. Herdina (SARDI)	Virginia, SA	Potato, tuber sclerotia
L56	3	Yes	Dr. Herdina (SARDI)	Angle Vale, SA	Potato, tuber sclerotia
L62	3	Yes	Dr. Herdina (SARDI)	Western Australia	Potato, stem lesion
L306	3	Yes	Dr. Herdina (SARDI)	Unknown	Unknown
Tas1	3	No	n/a	Sassafras, Tas[9]	Potato, root
T22	3	No	n/a	Tasmania	Potato, tuber sclerotia
R20	3	No	Unknown (via SCRI)	Lincoln, New Zealand	Potato
R37	3	No	Scottish Agricultural College (via SCRI)	Aberdeen, Scotland	Potato
734	4	Yes	Catherine Hitch (SARDI)	South Australia	Cauliflower, root
R112	4 HG-II	No	Dr. James Woodhall (2037) (via SCRI)	Unknown	Cauliflower
R110	5	No	Dr. James Woodhall (Y55) (via SCRI)	Norfolk, UK	Potato, Sclerotia
WAC-9923	8	Yes	Dr. Herdina (SARDI)	Esperance, WA	Wheat root
RS21	8	Yes	Dr. Herdina (SARDI)	Colac, Vic	Potato, tuber sclerotia
R56	8	Yes	Plant Research International (via SCRI)	The Netherlands	Unknown
L60	AG K	Yes	Dr. Herdina (SARDI)	Virginia, SA	Potato, stem lesion
KI19	AG A	No	n/a	Kangaroo Island, SA	Potato, root

[9] Tas = Tasmania, Australia

Figure 2.2. Australian potato growing regions from which *Rhizoctonia*-affected plants were collected (arrows) and from which isolates were supplied by other researchers

NOTE:
This table is included on page 34 of the print copy of the thesis held in the University of Adelaide Library.

2.3 Molecular methods

2.3.1 DNA extraction

DNA was extracted from mycelia of *Rhizoctonia* isolates produced after 4 to 14 days growth on quarter strength PDA in the dark, at 25ºC. Extractions were performed using either the DNeasy® plant mini kit (Qiagen, Melbourne, Australia) or a quick extraction method modified from Hamelin (2000) (see sections below). DNA was stored at -20°C and used as template in PCR as described in the following section. Concentration of DNA in the template volume varied with extraction.

2.3.1.1 DNeasy® plant mini kit (Qiagen, Melbourne, Australia) extraction

Mycelium was scraped from the surface of the agar medium using a sterile scalpel blade, placed in a sterile mortar and submerged in liquid nitrogen. The frozen mycelium was macerated with a pestle and transferred to a 1.5 ml eppendorf tube in preparation for use in the DNeasy® plant mini kit (Qiagen, Melbourne, Australia) procedure. DNA was extracted using the manufacturer's protocol and eluted in 100 µl.

2.3.1.2 Quick extraction method modified from Hamelin (2000)

Using a sterile, disposable micropipette tip, mycelium, about 1 mm^3, was scraped from the agar surface into a 1.5 ml eppendorf tube containing 100 µl of extraction buffer (0.5M Tris-HCl, pH = 9, 1% Triton X-100). The eppendorf lid was pierced with a needle to allow gas to escape during a 5 min incubation in a heating block at 100°C. The tube was then transferred to ice for a further 5 min before the supernatant was used as template in PCR.

2.3.2 PCR procedure

PCR was used to amplify DNA for multiple purposes. Isolates were assigned to anastomosis groups by use of specific primers designed for AG 2-1, AG 2-2, AG 3, AG 4, AG 5 and AG 8 (Ophel-Keller *et al.* 2006). If isolates could not be classified by AG-specific reactions, PCR with the universal fungal primers ITS1 and ITS4 (White *et al.* 1990) enabled sequence analysis based on genetic variation of the Internal Transcribed Spacer (ITS) region. A second region, the Intergenic Spacer 1 region (IGS1), was also amplified to allow sequence analysis and evaluation of genetic variability between isolates. Each amplification reaction comprised the components shown in Table 2.2 contained in a 0.5 ml tube.

For the AG-specific reactions, the presence of a PCR product indicated that the isolate belonged to the AG corresponding to the primer set used. If a template was not amplified in any AG-specific PCR, the template DNA was checked by PCR with primers ITS1 and ITS4. A positive result in this reaction indicated that PCR inhibitors were not present and that extracted DNA was at sufficient concentration and quality for amplification. Master mixes of the components in Table 2.2 were prepared for use in multiple reactions. Master mixes were tested by the inclusion of one negative control reaction, containing 1 µl of water replacing template DNA, and one positive control reaction, containing template DNA of a known isolates that would produce an amplicon.

Table 2.2. Components and volumes of reagents used in PCR to amplify DNA of *Rhizoctonia* isolates

Component	AG-specific reaction	ITS region	IGS 1 region
MQ water	17.375 µl	17.375 µl	17.375 µl
dNTPs (10 mM) Promega, Sydney, Australia	100 µM	100 µM	100 µM
Forward Primer Geneworks, Adelaide, Australia	180 µM[1]	100 µM[2]	100 µM[3]
Reverse Primer Geneworks, Adelaide, Australia	180 µM[1]	100 µM[2]	100 µM[3]
10 x Buffer Qiagen, Melbourne, Australia	2.5 µl	2.5 µl	2.5 µl
20% PVP	2.5 µl (2%)	2.5 µl (2%)	2.5 µl (2%)
HotStart Taq Qiagen, Melbourne, Australia	0.125 µl	0.125 µl	0.125 µl
Template DNA	1 µl	1 µl	1 µl
Total volume	25 µl	25 µl	25 µl

[1] Final concentrations of primers developed by SARDI
[2] Universal fungal primers: ITS1 and ITS4 (White *et al.* 1990) final concnetrations
[3] Fungal primers for the intergenic spacer 1 (IGS1) region: Seq5S and LR12R (Woodhall *et al.* 2007) final concnetrations

Amplification was performed using a PTC-100^{TM} programmable thermal controller (MJ Research, Inc. Massachusetts, USA).

Conditions for AG-specific and ITS region PCR were as follows. An initial denaturation and enzyme activation at 95°C for 5 min was followed by 30 cycles of denaturation at 94ºC for 1 min, annealing at 60ºC for 1.5 min and extension at 72ºC for 2 min followed by a final extension at 72ºC for 5 min. Conditions for the 35 cycles of IGS1 PCR were at the same temperatures, however, the incubation times were 15 sec for denaturation, 15 sec for annealing and 45 sec for extension, with no final extension step.

PCR products (5 µl) were separated on either a 2% agarose gel with 1 x Tris-acetate-EDTA (TAE) buffer for 60 min at 80 volts or a 2% agarose gel with 1 x sodium borate buffer (Brody and Kern 2004) for 20 min at 200 volts. Gels were subsequently submerged in a 0.4 µg/ml ethidium bromide solution for 20 to 30 min and observed under UV light.

2.3.3 Sequencing

DNA sequences of selected isolates were determined using PCR products following amplification with ITS1 and ITS4 primers. A consensus sequence of each product was obtained by the use of the same forward and reverse primers in separate sequencing reactions. Excess nucleotides and primers were removed from 5 µl of PCR product by incubation at 37 ºC for 15 min with ExoSAP-IT® (GE Healthcare, Buckinghamshire, UK) (1 µl), then incubation at 80ºC for 15 min to inactivate the enzyme. The mixture was centrifuged and the product transferred to a 1.5 ml tube containing 6.4 pmol of primer and 1.35 µl of sterile MQ water. PCR products were submitted for sequencing to the Australian Genome Research Facility (Adelaide, South Australia). Results were viewed with Sequence Scanner software (v1.0 Applied Biosystems) and a text file used for comparison with other sequences using NCBI nucleotide BLAST and ClustalW via the ExPSAy Proteomics server (http://ca.expasy.org/).

2.4 Pathogenicity experiments

To assess the pathogenicity of *R. solani* isolates to potato plants, both long-term shade-house experiments and short-term controlled environment experiments were carried out. Inoculum production and disease assessment were based on the methods of Balali *et al.* (1995; 1996).

2.4.1 Inoculum production

Flasks were filled with millet seed (mixed cultivars), supplied by Seed Services South Australia; the amount of seed per flask was dependent on the number of pots to be inoculated. MQ water was added to each flask and the seed was autoclaved at 121ºC for 20 min on two consecutive days. The autoclaved millet seed was inoculated with five plugs from the margin of a selected *R. solani* culture, actively growing on PDA, and incubated in the dark for 14 days at 25ºC. To ensure seed was evenly colonised, flasks were agitated for about 10 sec every second day. A control was included that comprised *R solani*-colonised seed, subsequently autoclaved. After 12 days of incubation the viability of inoculum was tested by placing five seeds on PDA and incubating at 25ºC. DNA was subsequently extracted from mycelium and PCR performed with the AG-specific primer set as described in section 2.3.2.

2.4.2 Soil preparation

Steam pasteurised (60ºC, 2 hrs) Mt Compass sand (see Table 2.3) was used as the potting mix for eight replicate pots per treatment. This sand was used because of its low nutrient status, with the expectation of using substrate of similar consistency in later zinc experiments (described in chapter 7). The zinc experiments required washing of Mt Compass sand as described by Genc *et al.* (2006) and produced for an intial experiment in this project, however, during for the second zinc experiment an alternative sand was sourced from Sloans (Dry Creek, South Australia). Nutrient status of both of these substrates is also presented in Table 2.3 along with UC mix used in storage of isolates (see section 2.2).

Depending on the experiment, the inoculum was either added directly to the sand of individual pots prior to planting or was mixed into 48 kg of soil using a cement mixer. The weight of inoculum added to each pot varied with the experiment. After inoculation, pots were placed in four rows, with positions randomised in a one-way design with no blocking, along the central length of a shade-house at the Lenswood Research Centre in the Adelaide

Hills, SA. Inoculum was allowed to establish in the pots for a minimum of 4 days prior to planting.

2.4.3 Planting and watering regime

Tubers of Russet Burbank, a cultivar widely used in the processing industry in Australia (section 1.3.1), was used when available to propagate potato plants. If Russet Burbank tuber were unavailable, the cultivar Shepody was used. To propagate potato plants either cut tubers or mini-tubers were used. Both tubers and mini-tubers were surface sterilised with 2% (v/v) formaldehyde for 5 min and rinsed twice in RO water. Mini-tubers were produced from tissue cultured plants, supplied by Department of Primary Industries, Victoria. If mini-tubers were not available, cut tuber (or seed) pieces were produced by removing the "eye" from tubers with a melon ball scoop. Seed pieces were sprouted and the cut surface allowed to suberise under dark, humid conditions in a glasshouse maintained at approximately 25ºC at the South Australian Research and Development Institute for 2 weeks.

Fungicides were applied to seed or soil just prior to planting, at rates and using methods recommended by the supplier, unless otherwise specified. During planting soil adhering to the trowel, between each inoculum treatment, was removed and the trowel was rinsed in 95% ethanol and air dried. In an attempt to replicate furrow applications, a trowel was used to mounded the soil to one side of the pot. This allowed the spray to be targeted to both the soil around the planting site and the soil used to cover the seed. Where furrow sprays were not applied, a trowel was used to displace the soil in the centre of each pot and allow a single seed tuber to be planted at a depth of 5 cm before it was covered with soil. Pots were maintained for 3 months in natural light in the shade-house described above. Pots were watered twice weekly when top-soil was dry to touch (otherwise rain-watered) and fertilised with Thrive® all purpose plant food (Yates) monthly at the recommended rate of 8 g in 4.5 L water.

Table 2.3. Nutrient analysis[1] of Mt Compass sand, before and after washing, and Sloans NT40 sands and UC mix

	Nitrate Nitrogen (mg/kg)	Ammonium Nitrogen (mg/kg)	Cowell Phosphorus (mg/kg)	Cowell Potassium (mg/kg)	KCl 40 Sulfur (mg/kg)	Organic Carbon (%)	Iron (mg/kg)	Electrical Conductivity (dS/m)	pH $CaCl_2$	pH H_2O	DTPA[2] Cu (mg/kg)	DTPA Zn (mg/kg)	DTPA Mn (mg/kg)	DTPA Fe (mg/kg)	Boron HOT (mg/kg)	Chloride (mg/kg)
Mt Compass sand (unwashed)	14	1	5	26	2.7	0.5	194	0.043	5	5.8	0.21	0.44	1.4	35	0.2	6
Mt Compass sand (washed 2008)	3	1	5	45	1	0.26	67	0.23	5.4	6.2	0.28	0.3	0.9	11.4	0.2	n/a
Sloans NT40	27	10	27	109	40	0.05	n/a	0.243	7.6	7.5	0.92	1.92	1.09	6.53	n/a	n/a
UC mix[3]	69	71	31	141	82.9	1.43	79	0.321	4.7	5.3	0.06	0.25	1.49	9.78	0.4	47

[1] performed by Cumming Smith British Petroleum (CSBP) Soil and Plant Laborotory, WA for all 4 substrates
[2] DTPA = diethylenetriaminepentaacetic acid
[3] UC = University of California potting mixture (Baker 1957), high in organic matter, used in storage of isolates.

2.4.4 Disease severity assessment

At harvest plants were carefully removed from their pots and washed with running RO water to remove adhering sand. In shade-house trials yield was measured by tuber number and total weight per plant. Disease symptoms assessed included stem and stolon cankers (necrosis), necrosis of root and stolon tips and sclerotia on tubers. Four classes of infection on stems and stolons were assessed, according to Balali *et al.* (1995) as follows:

1. No disease – no canker present
2. Slightly diseased – superficial canker
3. Moderately diseased – deep canker
4. Severely diseased – sprout or stolon girdled or killed

Four classes of tuber infection were assessed, according to Balali *et al.* (1995) as follows:

1. No disease – no sclerotia present on any tubers
2. Slightly diseased – 1-25 sclerotia on a tuber
3. Moderately diseased – 25-50 sclerotia on a tuber
4. Severely diseased – more than 50 sclerotia on a tuber

Root damage was estimated as a percentage of the plant's total root tissue. To facilitate the association of a group with symptom(s), re-isolation of the pathogen from diseased potato tissues was attempted (see section 2.1) and AG determined (refer to section 2.3). Statistical analysis was performed on results, entered as data sets, using the 10th edition of the program Genstat. Kruskal-Wallis tests (Siegel 1956) were performed on disease severity scores and one-way ANOVA on yield data and percentage of roots damage. Medians and means produced from these analyses, respectively, were compared by least significant differences at the 5 % level (l.s.d 5 %) following significant F values ($P < 0.05$). One-way ANOVA was also performed on disease severity scores, however, based on statistical advice (pers. com. C. Dyson 2008) that this analysis may overestimate differences between treatments, due to the non-linear disease severity scale, these analyses are only presented in appendix 10.2.

2.5 Fungicide experiments

Six commercially available fungicides from different chemical groups, that were registered for use on potato plants to control Rhizoctonia in Australia, were used during *in vitro* and *in planta* experiments (Table 2.4). One experimental fungicide was used during one *in vitro* experiment but, as this fungicide was not to be developed for commercial use in Australia it was not used during *in planta* experiments.

Table 2.4. Fungicides used during *in vitro* and *in planta* experiments

Fungicide	Supplier	Active ingredient (a.i.)	Group [1]	a.i. concentration
Monceren	Bayer	Pencycuron	20	125 g/kg
Moncut	Gowan	Flutolanil	7	400 ml/L
Amistar	Syngenta	Azoxystrobin	11	500 g/kg
Experimental	Bayer	Unknown	?	50 g/kg
Rovral	Bayer	Iprodione	2	500 g/kg
Rizolex	Sumitomo	Tolclofos-methyl	14	100 g/kg
Maxim	Syngenta	Fludioxonil	12	100 ml/L

[1] Australian fungicide groups as at October 2008, CropLife (croplifeaustralia.org.au)

3 Field Sampling, Anastomosis Group Determination and Phylogenetic Comparison

3.1 Introduction

R. solani is subdivided into anastomosis groups on the basis of the frequency of hyphal fusion, as described in section 1.2.3. When two different, but related isolates are cultured together the vegetative hyphae undergo an incompatibility reaction and can be classified based on the tendency of their vegetative hyphae to fuse (or anastomose) with that of other isolates (Carling *et al.* 1988). Although this method is accepted as the standard classification method for *R. solani* isolates, it is time consuming and interpretation of observations can be difficult.

Because the incompatibility reaction minimises genetic recombination between AGs, genetic divergence has occurred between groups. Therefore the AGs are distinct in terms of ribosomal gene sequences (Gonzalez *et al.* 2001; Kuninaga *et al.* 1997). This genetic divergence in the internal transcribed spacer (ITS) region has been used to develop species-specific tests for *R. solani* AGs (Lees *et al.* 2002). As part of the Australian Potato Research Program (APRP) (Ophel-Keller *et al.* 2006), primers specific for AG 2-1, AG 2-2, AG 3, AG 4 and AG 8 have been developed. These tests were designed as TaqMan® MGB^{TM} (Applied Biosystems, Melbourne, Australia) tests for quantification of *R. solani* DNA in soils and are described in section 1.4.4. However, in this project, the AG-specific primer pairs were used in standard PCR with DNA extracted from purified *R. solani* isolates, such that the amplification product has the potential to indicate the AG of the isolate. This method reduces the time required to assign large numbers of isolates to AGs.

R. solani AG 3 is most frequently associated with the development of sclerotia on tubers, a symptom which is called black scurf (see section 1.3.2). However, other AGs (at low frequencies) cause disease on potato plants (see section 1.4.3). A previous study of two sites in South Australia, both of which supply the fresh washed industry (Balali *et al.* 1995), supported findings of international studies (see section 1.4.3). However, investigations in other Australian potato growing regions, particularly those that supply the processing potato industry, are needed to identify which AGs are present and are associated with particular symptoms of Rhizoctonia disease.

AG 2-1 has been associated with Rhizoctonia disease symptoms on potato plants at a low frequency world-wide (refer to section 1.4.3.1). Variation in pathogenicity has been observed among isolates of *R. solani* from potato, particularly those of AG 2-1 (Woodhall *et al.* 2007). The pathogenic variation and origins of these isolates have been linked to genetic groupings determined by amplicon size after PCR of the IGS1 region by Woodhall *et al.* (2007). Isolates originating from necrotic regions of stem and stolon produced a band of 550 bp following PCR of the IGS1 region; whereas those collected from tuber sclerotia produced a band of either 510 bp or 570 bp. Isolates with the smaller amplicon also produce narrower lesions when compared to symptoms caused by isolates with other IGS1 type (Woodhall *et al.* 2008).

Both ITS and IGS1 regions are considered to contain "housekeeping genes" with low genetic variation and intergenic regions with high genetic variation (White *et al.* 1990). Neither of these are directly associated with pathogenicity or fungicide resistance. However, single genes that determine these phenotypic traits have not yet been identified. The IGS and ITS regions are useful for studies of evolution hence were used here to determine if these traits may be linked to a historical evolutionary divergence that can be observed through sequence divergence. In phylogenetic analysis the common genotype should result in clustering of similar phenotypes. Clustering of phenotypes, specifically IGS1 region type, was observed when phylogenetic analysis was performed by Woodhall *et al.* (2007) on the ITS region of the nine British isolates previously mentioned. The ITS region has more commonly been used to determine the relatedness of *Rhizoctonia* isolates (see section 1.4.5) and has been used in development of AG-specific primers (Lees *et al.* 2002).

However, variation in pathogenicity within AG 2-1 cannot be differentiated with the currently available molecular tools for quantifying the DNA of AG 2-1 from field soil samples. It is not known if further distinction of sub-groups, based on the variation among isolates from AG 2-1, will be beneficial for either the researcher or grower. Therefore, in this project the variation in ITS sequence was also investigated and compared with phenotypic information such as IGS1 region type and pathogenicity of AG 2-1 isolates. This information may contribute to the future development of molecular tools used in measuring Rhizoctonia disease potential of field soils.

The aims of the experiments presented in this chapter were to:

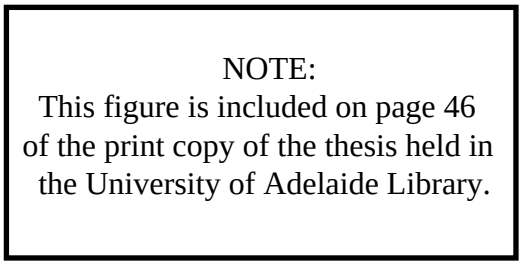
1. Confirm the effectiveness and specificity of the primer sets;
2. Assign isolates to an AG by molecular and, or cultural methods;
3. Determine the *R. solani* anastomosis groups present in Australian potato growing regions that supply the processing industry;
4. Investigate the genetic variation among AG 2-1 isolates from Australia and Great Britain;
5. Determine if IGS1 PCR product size varies in Australian isolates; and
6. If size groupings were observed, determine if they were associated with country, host and tissue of origin or ITS sequence similarity.

3.2 Methods

3.2.1 Field and plant tissue sampling

Potato plants were collected as volunteers from fields previously cropped to potatoes and from commercial crops. Samples were collected from multiple sites in south-eastern South Australia (SE of SA), Kangaroo Island (KI) and Tasmania (Tas) (Figure 3.1) between 2005 and 2007. The South East and Kangaroo Island are both regions of the state of South Australia that supply the processing industry. The South East includes Western Flat, Glenroy, Mt Benson, Lucendale, Naracoorte, Kalangadoo, Mingbool and Penola. Pinaroo and Lameroo may also supply the processing industry, however, also supply the fresh washed industry (summarised by PIRSA 2007). Kangaroo Island provides seed tubers for both the fresh washed and processing industries in Australia. Potato production is a recent industry in this area and fields have rarely been used for growth of potato before (pers. com. R. Harding 2006). Tasmanian samples were supplied by other researchers in the APRP and originated from Sisters Creek, Elliott, Sassafras and Gawler in the North West between Devonport and Sisters Beach Park. This is the region where potatoes are most commonly grown in Tasmania. Due to difficulties in transport from Tasmania, the majority of samples were tubers. Plant tissues were sampled as described in section 2.1. Purification, maintenance and storage of all isolates was as described in sections 2.1 and 2.2.

Figure 3.1. Australian potato growing regions[1] from which plants were collected for isolation of *Rhizoctonia*



3.2.2 Cultural anastomosis grouping by microscopy

Isolates were classified using frequency of hyphal fusion reactions (Parmeter *et al.* 1969b) with tester isolates. To allow mycelial fusion, two isolates were grown, one at each end of a PDA-coated slide (Sanders *et al.* 1977). The slide was incubated in a 90 mm Petri dish, sealed with Gladwrap®, at 25ºC in the dark for a minimum of 48 hrs, until advancing mycelia met. The agar was then allowed to dry in a laminar flow cabinet for 2 or more hrs and mycelia stained with lactophenol trypan blue (see Appendices 10.1). Hyphal fusion was assessed according to the categories described by Carling (1988) as follows:

C0: No interaction between hyphae observed. Isolates not related, different AG.

C1: Contact between hyphae, fusion of walls but not membranes, occasionally cells die. Distantly related isolates, same or different AG.

C2: Obvious wall fusion, uncertain membrane fusion, diameter of anastomosis point less than that of hyphal diameter, anastomosing and adjacent cells always die. Isolates from the same AG.

C3: Cell wall and membrane fuse, diameter of anastomosis point equal to hyphal diameter, adjacent cells generally not killed. Closely related isolates that may be the same isolate, are the same AG.

AG 2-1 and AG 2-2 isolates were differentiated as described by Carling (1988), where unknown isolates were paired independently with tester isolates for both AG 2 sub-groups. When the C2 reaction was produced in more than 50% of interactions with one sub-group (and C1 or C0 reactions with the other sub-group), the unknown isolate was assigned to that same AG. If C2 fusion occurred with tester isolates of both sub-groups the isolate remained unclassified.

3.2.3 AG-specific molecular methods

DNA was extracted from mycelia of each isolate using the DNeasy® plant mini kit (Qiagen, Melbourne, Australia) or the quick extraction method modified from Hamelin (2000) as described in section 2.3.1. PCR assays were performed on template DNA as described in section 2.3.2 using primers specific for AG 2-1, AG 2-2, AG 3, AG 4 and AG 8. Isolates that were not classified using these primer sets were sequenced following amplification with the ITS primer set as described in section 2.3.3. Quantitative PCR assays were performed at SARDI with the TaqMan assay described in section 1.4.4 after DNA extraction by the Root Disease Testing Service (RDTS).

3.2.4 IGS1 region PCR product size from R. solani *isolates*

3.2.4.1 Preliminary experiment in Australia

A preliminary experiment to examine the size of IGS1 amplicons from AG 2-1 isolates was conducted in Adelaide, Australia, using protocols of Woodhall *et al.* (2007). DNA was extracted from 20 isolates of AG 2-1, collected from the South East of South Australia, Tasmania and Kangaroo Island using methods described in section 2.3.1. PCR of the IGS1 region was performed as described in section 2.3.2 and PCR products were separated on a 2% agarose gel run for 11 hrs at 40 volts in TAE buffer and observed as described in section 2.3.2.

3.2.4.2 SCRI, Dundee, Scotland

The IGS1 region of a total of 32 isolates from Australia or supplied in the UK, was examined at SCRI, Dundee, using the protocols of Woodhall *et al.* (2007). DNA was extracted from isolates with a quick extraction method as follows. A 90 mm Petri dish containing potato dextrose broth was inoculated with two 5 mm^2 plugs of agar colonised with the isolate of interest and incubated for 7 days in the dark at 25°C. The mycelial mat was flipped over and the agar plugs removed with sterile tweezers. The excess potato dextrose broth was discarded and the mycelial mat rinsed in the Petri dish with sterile distilled water. The mycelial mat was transferred to a piece of sterile tissue paper and the excess water blotted off before transfer of mycelium into a 1.5 ml tube. The lid remained open but the opening was covered with Parafilm and stored at -4°C for 2 hrs before freeze drying overnight. In a clean 1.5 ml tube, a 1 mm^3 piece of freeze dried tissue was macerated using a Treff (Degersheim, Switzerland) homogeniser with an equivalent amount of sterile sand grains in 23 µl of NaOH. The solution was centrifuged for 5 min at 13,000 rpm then 10 µl removed and mixed with 90 µl of Tris (100 mM, pH = 8) in a clean 0.5 ml tube. The concentration of nucleic acid in this solution was tested using a nanodrop spectrophotometer (Thermo scientific, Delaware, USA) and then a small amount transferred to a sterile 0.5 ml tube and adjusted to 5 µg/µl for use as template in PCR.

The PCR procedure with primer set LR12R and Seq5S is described in section 2.3.2. The PCR products (8 µl) were separated by size on a 2% TAE agarose gel for 90 min then removed from the buffer and an image taken. The gel was then replaced in the buffer and current applied for another 60 min before final visualisation. The method of allowing visualisation of PCR products differed slightly to the method used in Australia (see section 2.3.2) in that SYBRGreen (1%) was added to the gel prior to pouring, to allow visualisation under UV light.

3.2.5 Sequence alignments and phylogenetic comparison of R. solani *AG 2-1 isolates based on IGS1 region*

PCR products of reactions described in section 3.2.4, were sequenced in both directions with primers LR12R and Seq5S by the analytical lab at the Scottish Crop Research Institute. Results were viewed with Sequence Scanner software (v1.0 Applied Biosystems, Victoria, Australia). This allowed consensus sequences to be established from the forward and reverse reactions. Multiple sequence alignments were performed using ClustalW and a neighbour-joining tree was constructed from the results using Kimura Two-Parameter model in MEGA (v4) (Tamura *et al.* 2007). Bootstap values were determined from 1000 replicates of data.

3.2.6 Phylogentic comparison of R. solani *AG 2-1 isolates based on ITS region*

DNA extraction and PCR were performed as described in section 1.1 with primer set ITS 1 and ITS 4. Consensus sequences were determined from both forward and reverse reactions and aligned using MEGA 4.0 as described in section 3.2.5. Sequences for the 15 isolates not available in culture were obtained from the NCBI database, including the AG 1-1 isolate used as an out-group. A neighbour-joining tree was constructed as described in 3.2.5 using the Kimura two-parameter model.

3.3 Results

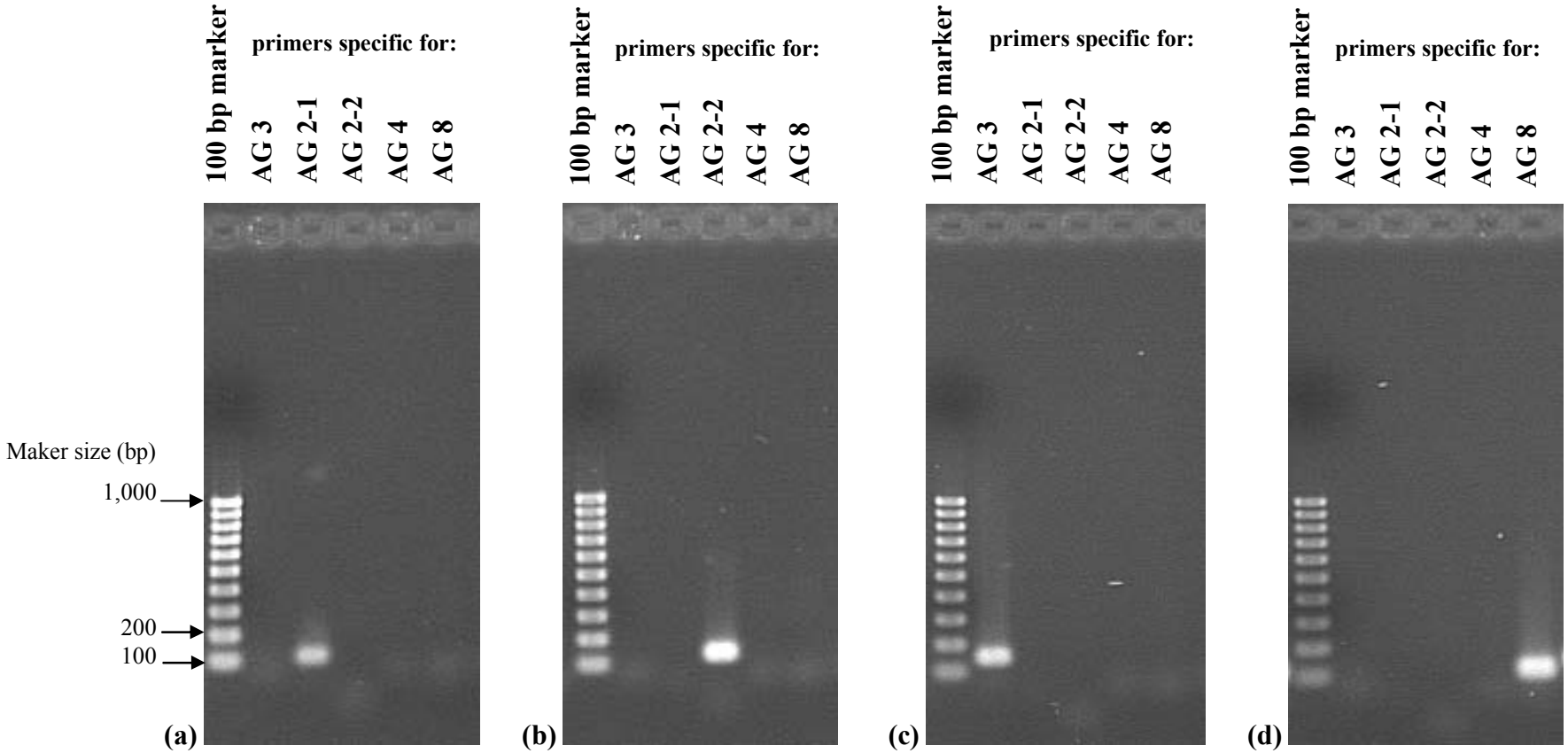
3.3.1 AG-specific molecular classification of tester isolates

Twelve tester isolates were used in a preliminary experiment to confirm the specificity of primers to their target AGs. PCR was performed on DNA extracted from each of these isolates with the five AG-specific primer sets, each in individual reactions. One example of the results for each AG is shown in Figure 3.2. The amplification products, for each primer set, were approximately 150 bp in size, as expected (pers. com. Herdina 2005). An amplification product was obtained for each isolate, with only one of the AG-specific primer sets. Subsequently, the other 26 isolates were amplified but with only three of the five AG-specific primer sets (AG 2-1, AG 2-2 and AG 3).

The AG of 33 of the 38 tester isolates, originating from Australia and previously classified by other researchers, was then confirmed using AG-specific primer sets in PCR. These comprised seven AG 2-1, five AG 2-2, nineteen AG 3 and two AG 8 isolates. The five templates that did not produce an amplicon in any AG-specific PCR reaction included one AG 2-1, three AG 3 and one AG 4 isolate.

To ensure that the boiling DNA extraction method had not influenced the ability of the primer sets to amplify in PCR, for those isolates that were not amplified, DNA was also extracted with the DNeasy® plant mini kit (Qiagen, Melbourne, Australia), however, no amplification was produced with AG-specific primers. Template DNA from these isolates was used in an PCR using primers specific to the ITS region to check that amplification was possible. In ITS PCRs amplicons were produced when either DNA extraction method was used. The PCR product from ITS PCR was then sequenced. This sequence was then compared with other *Rhizoctonia* sequences available in the NCBI database, and the sequences with over 98 % identity confirmed the AG to which the isolates had previously been classified. Isolates were subjected to DNA extraction and QPCR by staff at SARDI and the results confirmed the AG to which the isolates had previously been classified by other researchers, in all cases.

Figure 3.2. Examples of products amplifed from tester isolates[1] by PCR[2] with AG-specific primer sets: (a) Isolate R394, AG 2-1; (b) Isolate WAC-9765, AG 2-2; (c) Isolate L62, AG 3; (d) Isolate WAC-9923, AG 8. Products compared in size with 100 bp ladder as size marker (500 ng).



[1] Isolates which were previously assigned to AG based on methods independent of AG-specific primer sets
[2] Conditions for PCR and subsequent separation of products, on a 2 % agarose gel at 80 V for 1 hr, are described in section 2.3.2

3.3.2 Preliminary experiment: Cultural classification of tester isolates

To confirm the integrity of the assignment to AG by PCR, 11 tester isolates were also assigned to AGs on the basis of cultural methods (Table 3.1). Anastomosis assessments obtained as a result of pairing isolates in culture are shown in Table 3.1. When isolates from different AGs were opposed, C0 reactions were found (e.g. WAC-9937 with RS21). When two isolates from AG 8 were paired a C2 reaction was observed and, similarly, when two AG 3 isolates (e.g. L306 and R229) were paired a C2 reaction was also observed. In most cases when an AG 2-1 isolate was paired with an AG 2-2 isolate a C0 reaction was produced (e.g. WAC-9806 and WAC-9937) and when those isolates were paired with another isolate of the same sub-group a C2 reaction was observed (e.g. WAC-9765 and WAC-9937). However, the pairing of AG 2 isolates from the same sub-group (e.g. L57 and WAC-9806) and different sub-groups (e.g. R394 and WAC 9765) occasionally produced a C1 reaction. In these situations the fusion reaction was repeated, with the same or different isolate, and results were consistent with expectations, of C2 and C0 respectivley.

3.3.3 AG-specific molecular classification of field isolates

The AG-specific primer sets were used to assign isolates collected from the field to an AG. As well as the tester isolates (described in section 3.3.1) a further 307 isolates were assigned to AG 2-1, AG 2-2 and AG 3 based on the amplicons produced in AG-specific PCRs (Table 3.2). An additional 22 isolates did not produce amplicons in the AG-specific PCRs and were grouped based on ITS sequence similarity. Thirteen isolates were classified as binucleate *Rhizoctonia*, with high similarity to AG A and AG K in a search of available sequences. The remaining nine isolates were assigned to AG 2-1 (1), AG 2-2 (2) and AG 3 (6) (Table 3.2).

There was a concern that potentially up to 6% of AG 3 isolates could be misidentified and this led to testing of cultures by SARDI using QPCR. All isolates that did not produce amplicons in standard PCR were successfully amplified by the QPCR for AG 3. These results (data not shown) assigned each isolate to an AG that corresponded to the group determined by ITS sequence.

Table 3.1 Anastomosis categories based on microscopic observations of hyphal fusion[1] frequency of tester isolates in a preliminary experiment

QPCR AG		8	8	2-2	2-2	2-1	2-1	2-1	3	3	3	3
	Isolates	RS21	WAC-9923	WAC-9937	WAC-9765	WAC-9806	L57	R394	L306	L62	R229	R242
8	RS21											
8	WAC-9923	C2										
2-2	WAC-9937	C0						C0				
2-2	WAC-9765			C2								
2-1	WAC-9806	C0		C0	C0		C2	C2				
2-1	L57			C0		C1		C2				
2-1	R394	C0		C0	C1	C1	C1					
3	L306	C0		C0		C0		C0		C1		
3	L62								C2		C1	
3	R229								C2	C1		
3	R242	C0							C2			

C0 showing C0 reaction
C1 showing C1 reaction
C2 showing C2 reaction
C3 showing C3 reaction
self, not paired

Table 3.2 Number of isolates from each region classified by PCR to AGs. Numbers in parenthesis indicate that AG-specific PCR classification failed for that number of isolates and AG was then assigned by ITS sequence similarity in NCBI

	Number of isolates in each AG							
Region	**2-1**	**2-2**	**3**	**4**	**5**	**8**	**Other**	**Total**
KI	44 (1)	44 (2)	27	0	0	0	12	127
Tas	3	2	28 (5)	0	0	0	0	33
SE of SA	51	46	70 (1)	0	0	0	1	168
Lenswood	1	0	0	0	0	0	0	1
Total no.	99	92	125	0	0	0	13	329
% of total	30	28	38	0	0	0	4	100

[1] Isolates were paired on slides coated with PDA and assessed as described in section 3.2.2

3.3.4 Cultural classification of field and tester isolates

Isolates obtained during the course of this project were occasionally opposed with tester isolates and/or each other. Of the total 146 pairings scored, 119 (81%) produced scores consistent with PCR designations of AG; representative results are shown in Table 3.3. Unexpected fusion scores were produced in the other 27 pairings. These unexpected results were resolved for isolates involved in 14 of the 27 pairings. This was done by opposing each isolate with other isolates from AGs that were either the same (11 cases) or different (3 cases). All results from secondary pairings were consistent with PCR designations of AG. However, one isolate (R2) produced multiple (8) unexpected results. The ITS region of R2 was sequenced and the isolate was classed as AG 3. From the 27 pairings that produced unexpected fusion scores one isolate involved in each of five pairings was not paired with any other isolate, hence the AG designation assigned by PCR was not confirmed with cultural techniques.

3.3.5 The relationship among R. solani AG, growing region and plant tissue

Isolates subsequently classified as AG 2-1, AG 2-2 and AG 3 of *R. solani* were recovered from potato plants from all three growing regions sampled. Tubers were the main potato tissue type available for isolation as they were obtained from other researchers after plant tops had been desiccated. Hence, more isolates were obtained from tubers than from any other potato plant tissue. However, during the course of this project *R. solani* was also isolated from other plant tissues. For example, each tissue was sampled from 20 mature plants collected whole from the three South Australian regions sampled. The number of isolates from each AG, plant tissue section and growing region is represented in Figure 3.3.

Seventy nine percent of isolates from Tasmania were classified as AG 3. The majority of these isolates were obtained from tuber samples (Figure 3.3) but one AG 3 isolate was associated with root tissue. Isolates subsequently classified as AG 2-1 were associated with root and stolon tissues from this same region. The one AG 2-2 isolate collected from Tasmanian plants was associated with white (or below-ground) stem tissue (Figure 3.3) but *R. solani* was not isolated from the green stem of plants from Tasmania.

Table 3.3 Anastomosis scores based on microscopic observations of hyphal fusion frequency[1] during interaction of field and tester isolates. Highlighted[2] cells indicate fusion categories that were inconsistent with PCR-based AG designations (continued next page)

AG		8	2-2	2-2	2-2	2-1	2-1	2-1	3	3	3	3	3	3	3	3	3	3	3	3	2-1	3	3	3	3	3	3	3	3	3	3	3	3	3
	Isolates	RS21	R421	WAC-9765	WAC-9937	WAC-9806	L57	R394	R213	R128	R101	R44	L306	L55	L41	L62	R2	R242	R229	R214	R14	R26	R217	SE-1	T1	T5	T7	T9	T10.1	T18	T20	T22	T24.1
8	RS21																																
8	WAC-9923	C2																															
2-2	R421	C0			C0	C0																											
2-2	WAC-9765				C2		C0																										
2-2	WAC-9937	C0	C0					C0							C0																		
2-1	WAC-9806	C0	C0	C0	C0		C2	C2																									
2-1	L57		C0		C0	C1		C2							C0						C2										C0	
2-1	R394	C0		C1	C0	C1	C1		C0												C2											
2-1	R422						C0	C0																									
3	R213	C0						C0							C2		C0																
3	R128	C0	C0		C0			C0	C2																								
3	R101							C0		C1																							
3	R44	C0			C0	C0					C2																						
3	L306	C0			C0	C0		C0			C1	C2				C2																C0	
3	L41				C0		C0	C0	C2	C2			C0	C1			C1																
3	L62												C1		C1				C1														
3	R2	C0	C0		C0				C2	C0			C2		C0						C0	C2	C0										
3	R242	C0											C2																				
3	R229												C1		C2	C1																	
3	R214														C2	C2		C2															
K	L60	C0		C0				C0					C0																				
2-1	R14				C0	C0	C0	C2	C0				C0		C0		C2																
3	R26						C0						C1				C0		C2														
3	R217						C0	C0									C0																

[1] As described in section 3.2.2

[2] Blue - both isolates were opposed with other isolates of the same or different AG and produced expected results. Red – one isolate was not opposed with any other isolate. Grey – isolate R2 as one of pairing.

Table 3.3 cont. Anastomosis scores based on microscopic observations of hyphal fusion frequency[1] during interaction of field and tester isolates. Highlighted cells[2] indicate fusion categories that were inconsistent with PCR-based AG designations

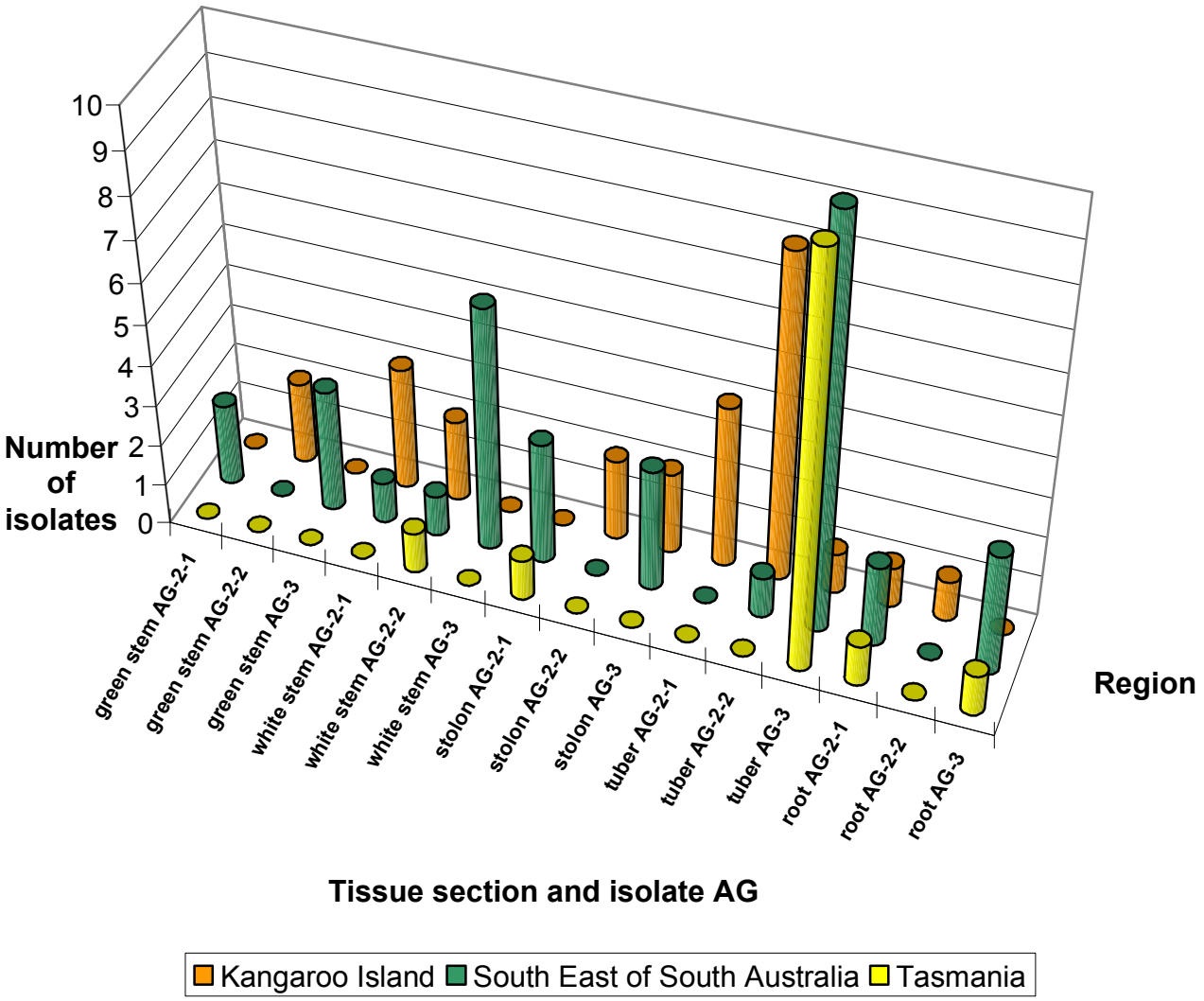
AG		8	2-2	2-2	2-2	2-1	2-1	2-1	3	3	3	3	3	3	3	3	3	3	3	3	3	2-1	3	3	3	3	3	3	3	3	3	3	3
	Isolates	RS21	R421	WAC-9765	WAC-9937	WAC-9806	L57	R394	R213	R128	R101	R44	L306	L55	L41	L62	R2	R242	R229	R214	R14	R26	R217	SE-1	T1	T5	T7	T9	T10.1	T18	T20	T22	T24.1
3	R153												C0				C0																
3	SE-1							C0					C2												C0	C1							
3	KI-1							C0					C1											C0									
2-2	KI-3	C0			C1		C0																										
3	T1							C0																									
3	T3							C0									C2																
3	T6																									C0							
3	T7																										C0						
3	T9	C0																										C0					
3	T10			C0																													
3	T10.1																				C0												
3	T10.2												C1																				
3	T16			C0															C0														
3	T17												C1																				
3	T18																																
3	T19																												C2				
3	T20												C1																				
3	T21																													C3			
3	T22	C0					C1	C0					C1						C2	C2													
3	T23																														C1	C1	
3	T24.1					C1																											
3	T24.2							C0																									C1
3	T25.1												C0																				
3	T25.2																																
3	T26							C0																									

The majority of isolates from tubers from the South East of South Australia were AG 3 (90%) but one AG 2-2 isolate was also collected (Figure 3.3). One AG 2-2 isolate was also associated with the white (below-ground) stem tissue from the same region. The majority of isolates from this tissue type were classified as AG 3, although one AG 2-1 isolate was collected. Green (above-ground) stem sections from plants from the South East of South Australia yielded AG 3 and AG 2-1 isolates at similar frequencies. Similar results were observed for isolates from stolon and root tissues at this site. *R. solani* was isolated from all plant tissues sampled from the South East of South Australia.

R. solani was isolated from all plant tissues from Kangaroo Island. The percentage of isolates classified as AG 3 was lower from plants from Kangaroo Island (12%) than Tasmania (79%) and the South East of South Australia (71%) (Figure 3.3). The majority (92%) of isolates from tubers from Kangaroo Island were classified as AG 2, sub-group AG 2-2. Only one AG 3 isolate and four AG 2-1 isolates were collected. The only other plant tissue from Kangaroo Island from which *R. solani* AG 3 was isolated was the stolon. Also associated with this tissue were AG 2-2 isolates which were collected at frequencies equal to AG 3 isolates. Isolates from both AG 2-1 and AG 2-2 were collected from white stem and root tissues from plants from Kangaroo Island. Only AG 2-2 isolates were found on green stems.

In some cases, two different AGs were isolated from the same plant. For example, this occurred on three plants from Kangaroo Island. Isolates collected from the tuber, stolon and stem (above and below ground) of one of these plants were classified as AG 2-2 whereas one AG 3 isolate was collected from the stolon and a binucleate *Rhizoctonia* from the roots. From one single tuber collected from a crop on Kangaroo Island both AG 2-1 and AG 2-2 isolates were obtained from sclerotia. It is possible that one AG formed the sclerotia and the other group produced only mycelial growth over this structure. This possibility was examined using 10 tubers from two farms. Sclerotia from black scurf were sampled prior to surface sterilising and then from the same tubers after surface sterilising (as described in section 2.1). In this experiment all sclerotia from a tuber yielded a single AG, so no conclusion was drawn regarding the origins of multiple AGs from one tuber.

Figure 3.3. Number of isolates from each AG[1] of *R. solani*, associated with a range of plant tissues[2] and regions that supply the Australian processing potato industry.



[1] Identification of isolates to AG was based on results of PCR with AG-specific primers as described in section 2.2.3

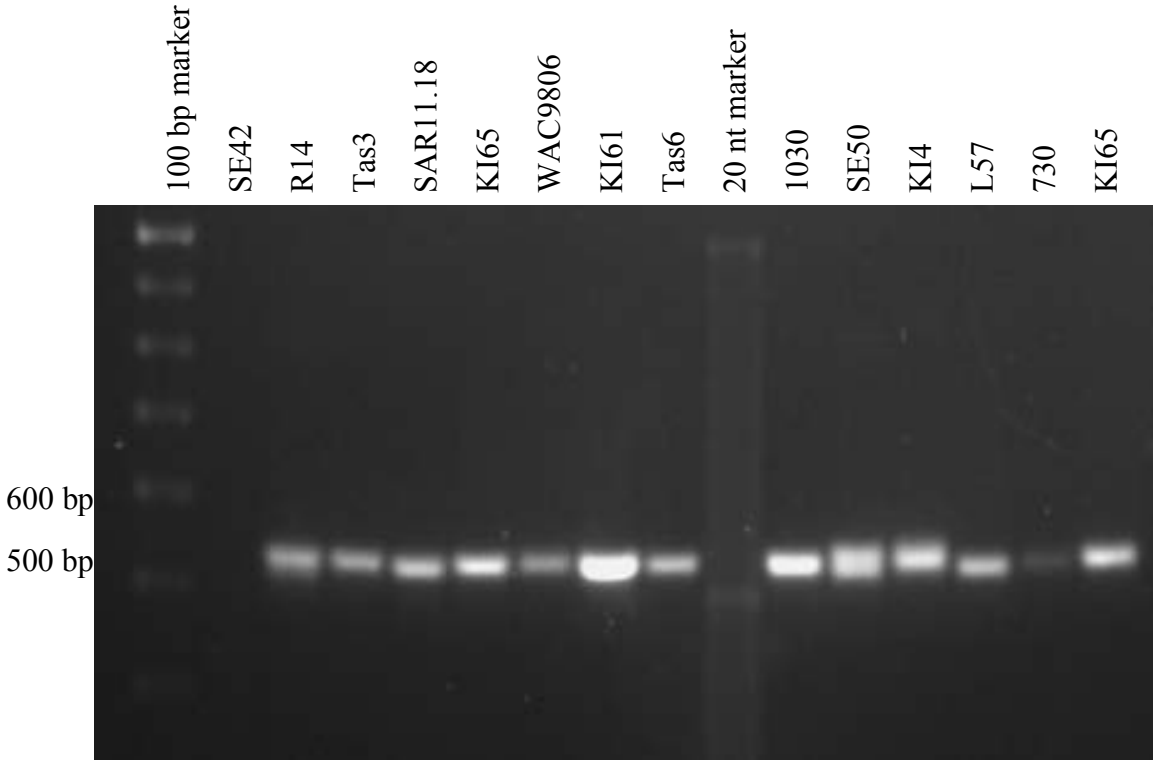
[2] *R. solani* was isolated from the tissues shown as described in section 2.1

3.3.6 IGS1 PCR product size variation

3.3.6.1 Australian AG 2-1 isolates

To investigate the variation in IGS1 sequence, DNA extracted from Australian AG 2-1 isolates collected from various host plant species and sites across Australia was amplified by PCR. Comparison of amplicons, some of which are shown in Figure 3.4, revealed variation in size, however, without positive controls for the size groupings published by Woodhall *et al.* (2007), it was difficult to group Australian AG 2-1 isolates based on visual observation due to small size differences. This experiment was repeated at the Scottish Crop Research Institute in Dundee and the results are presented in the following section.

Figure 3.4. PCR products resulting from amplification of the IGS1 region of Australian *R. solani* AG 2-1 isolates, separated by size in a 2 % agarose gel at 200 V for 20 min and compared to a 100 bp marker. A 20 nt marker was included, however quality of band resolution is not good enough to differentiate sizes



3.3.6.2 Australian and British AG 2-1 isolates

Isolates previously classified as short, intermediate and long IGS1 types by Woodhall *et al.* (2007) were available for use during experiments performed at SCRI in Dundee, Scotland. Representative isolates (five) were used as positive controls for the size categories previously observed in British AG 2-1 isolates and compared with ten Australian and three unclassified British AG 2-1 isolates. Amplicons from isolates previously classified as short (R100), intermediate (R103, R106 and R107) and long (R42) are shown in Figure 3.5. The amplicon for the one isolate classified as short (R100) appears noticeably smaller (estimated 510 bp) than other amplicons (estimated 560 bp) after 90 min of electrophoresis (Figure 3.5), however, no difference was observed between isolates previously classified as intermediate and long. As the PCR products migrated further, after 150 min of electrophoresis (not shown), the florescence of the DNA was decreased, preventing observation of size variation between amplicons. This was in contrast to results published by Woodhall *et al.* (2007) where amplicon sizes of 510, 550 and 570 nt were observed with the aid of a gel documentation system.

Comparison of amplicons by visual observation revealed that the unclassified British and Australian isolates tested (Figure 3.5) were all of similar sizes (between 500 and 600 bp) when separated over 90 min (Figure 3.5) and 150 min (not shown). These amplicons migrated a distance similar to the PCR products from the isolates previously classified as long and intermediate IGS1 type. The amount of PCR product varied in each reaction, although the initial template was standardised to 5 ng of DNA for each PCR. Due to the difficulties in interpreting size differences by visual observation, selected PCR products were directly sequenced and consensus sequences aligned.

3.3.7 Sequence alignments and phylogeny based on IGS1 region

Difficulties in the visual observation of size differences of amplicons from the IGS1 region of *R. solani* AG 2-1 isolates instigated the use alternative methods of investigating the variation in this region. Therefore, the nucleotide length was determined from isolates representative of AG 2-1, including nine originating from Australia and eight from Great Britain, by sequencing PCR products. A representative section is shown in Figure 3.6. Sequences of available isolates that had previously been classified as belonging to the size groups described by Woodhall *et al.* (2007) were included as controls. Size groupings based

on nucleotide sequence were determined by consensus deletions and insertions of two or more nucleotides, ignoring single site deletions and insertions in only one isolate. One consensus sequence was produced for the only available isolates previously classified as "short" (510 nt) and "long" (570 nt) IGS1 type. The nucleotide length of the IGS1 region amplified from the "short" isolate (R100) was similar to the expected length, at 512 nt. However, the length of the "long" isolate (R42) was only 545 nt, shorter than expected. Isolates previously classified by Woodhall *et al.* (2007) as "intermediate" IGS1 type (550 nt) did not produce sequences of consistent length; one such isolate produced a total length of 542 nt (R103) while two others yielded consensus sequences of 545 nt (R107 and R106).

To further support the sub-groupings of AG 2-1 isolates based on sequence length, specific sites of deletion were identified within the IGS1 region sequenced. The one "short" isolate (R100) had three deletion sites (shown in green in Figure 3.6), all 3 nucleotides or more in length, only one of which was unique to this isolate. One of the deletion sites was the same in nucleotide length and position as that found in the "long" isolate (R42 shown in blue in Figure 3.6). Of the nine Australian isolates for which the IGS1 region was sequenced, five (1030, Tas6, KI61, R14 and Tas3) displayed this deletion site but not the other deletions. All three of the unknown British isolates sequenced (R117, R118 and R114) also showed this "long" deletion pattern. The two British isolates (R107 and R106) previously classified by Woodhall *et al.* (2007) as "intermediate" IGS1 type displayed by observation of gel, showed a deletion pattern similar to the "long" isolate. These two isolates lacked the deletion site (shaded red in Figure 3.6) the other "intermediate" isolate (R103) showed (red in Figure 3.6). Four of the nine Australian isolates (SE50, L57, SE45 and SAR11.18) displayed the "intermediate" deletion pattern.

Isolates SE 50 and SE 45 collected from potato plants in the same field in the South East of South Australia displayed the same deletion pattern, as did two isolates collected from potato plants in Tasmania (Tas3 and Tas6). Isolate SAR11.18, also from Tasmania but obtained from a bean plant, had a deletion pattern different from that of Tas3 and Tas6.

To investigate the relationship among AG 2-1 isolates, collected from different locations and of various IGS1 types, a neighbour-joining tree was constructed using IGS1 sequences (Figure 3.7). The majority of "long" IGS1 type isolates clustered together, including isolates from Britain, Australia, from potato and non-potato hosts. Two "long" isolates (R14 and Tas3) did not fall into this cluster. Both of these isolates originated from Australian potato plants, one (Tas3) from the same field as another isolate that fell into the

above-mentioned cluster (Tas6). One isolate of "intermediate" IGS1 type (R107) clustered with the "long" IGS1 isolates, whereas all other intermediate isolates were significantly different from the above-mentioned cluster, as indicated by boot strap values. The one "short" IGS1 type isolate (R100) fell between two "intermediate" isolates (R106 and R103), all of which originated from Great Britain. However, the sequence of this "short" isolate was significantly different from both of the "intermediate" isolates, as indicated by bootstrap values.

Sequence variation was observed between "intermediate" isolates collected from the same region, specifically SE50 and SE45 (Figure 3.7), which were collected from potato plants from the same farm in Australia.

Figure 3.5. IGS1 PCR products of Australian and British AG 2-1 isolates of *R. solani* compared with 100 bp marker by separation on a 2 % agarose gel at 120 V for 90 min

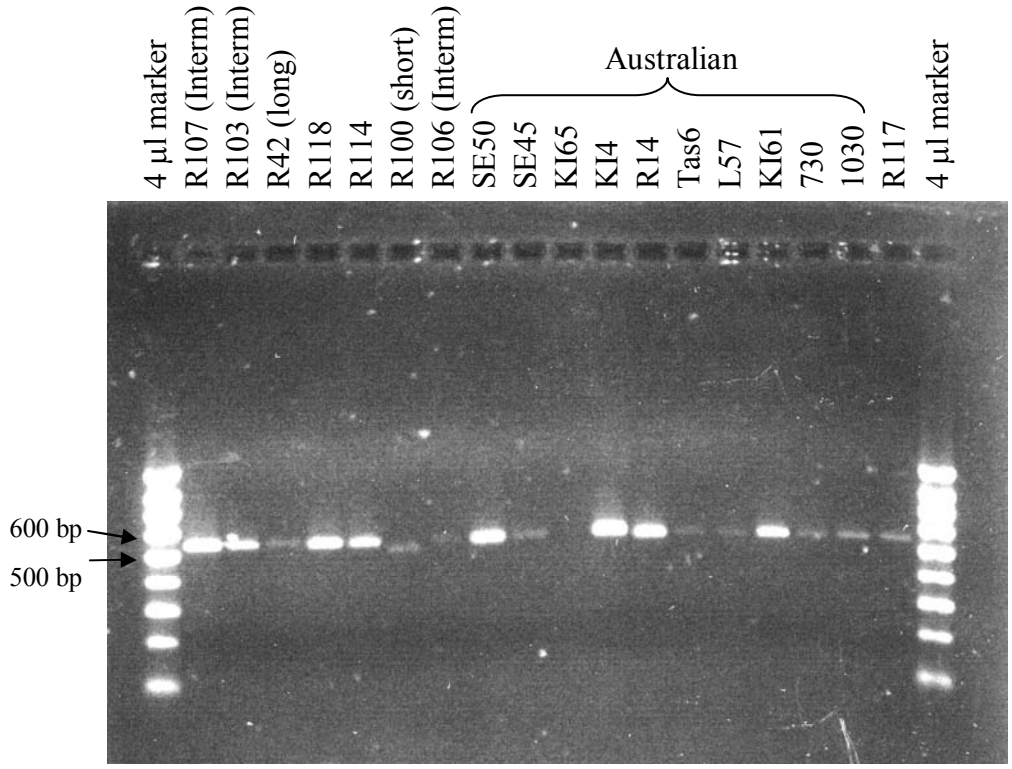


Figure 3.6. Alignment of IGS1 region sequence from Australian and British AG 2-1 isolates of *R. solani*. The size of each amplicon is determined by deletion sites of three or more consecutive nucleotides when compared to consensus sequences of other isolates. Deletion sites outlined with green indicate "short", red indicate "intermediate" and blue indicate "long" classification of isolates. Isolates with deletion patterns inconsistent with classification previously determined by Woodhall *et al*. (2007) are indicated by an opaque red box. (continued next page)

```
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   335        345        355        365        375        385
R42 LONG[1]     TTTCTCTCTT ATTTCTTTTT -TTTATAATT CTTTTTATTT TTTTGCATGG ATGTTGTTCA
R100 SHORT      TTTCTCTCTT ATTATTTTTA -TTTTTA--- TTTTTTATTT TTTTGCATGG ATGTTGTTCA
R103 INTER      TTTCTCTCTT TTTATTTTAT -[---------] -TTTTTATTT TTTTGCATGG ATGTTGTTTA
R107 INTER      TTTCTCTCTT ATTTCTTTTT -[TTTATAATT C]TTTTTATTT TTTTGCATGG ATGTTGTTCA
R106 INTER      TTTCTCTCTT ATTTTATTTT A[TTTTTTATT T]TTTTATTTT TTTTGCATGG ATGTTGTTCA
R117            TTTCTCTCTT ATTTCTTTTT -TTTATAATT CTTTTTATTT TTTTGCATGG ATGTTGTTCA
R118            TTTCTCTCTT ATTTCTTTTT -TTTATAATT CTTTTTATTT TTTTGCATGG ATGTTGTTCA
1030[2]         TTTCTCTCTT ATTTCTTTTT -TTTATAATT CTTTTTATTT TTTTGCATGG ATGTTGTTCA
R114            TTTCTCTCTT ATTTCTTTTT -TTTATAATT CTTTTTATTT TTTTGCATGG ATGTTGTTCA
TAS6[2]         TTTCTCTCTT ATTTCTTTTT -TTTATAATT CTTTTTATTT TTTTGCATGG ATGTTGTTCA
KI61[2]         TTTCTCTCTT ATTTCTTTTT -TTTATAATT CTTTTTATTT TTTTGCATGG ATGTTGTTCA
R14[2]          TTTCTCTCTT ATTTCTTTTT -TATATAATT CTTTTTATTT TTGCGCGGGG ATGTTGCCCG
SE50[2]         TTTCTCTCTC TTT-TTTTAT -[---------] -TTTTTATTT TTTTGCATGG ATGTTGTTTA
L57[2]          TTTCTCTCTC TTTATT-TAT -[---------] -TTTTTATTT TTTTGGAAGG ATTGTTTTGG
TAS3[2]         TTTCTCTCTT ATTTCTTTTT -TATATAATT CTTTTTTTTT TTGCGCGGGG ATGTTGCTCG
SE45[2]         TTTCCCTCTT TTTATTATAT -[---------] -TTTTTATTT TTTAGCATGG AGGATGGATG
SAR11.18[2]     TTTCTCTCTC TTTTTTTCTT -[---------] --TTTTTTTT TTTTGCAAGG ATGTTGTTTA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   395        405        415        425        435        445
R42 LONG        GGGCAGGTTA AATTCCATGA CCTTGGGCAC TCTGTACAAA GAGAGAAGAA [-------]GGT
R100 SHORT      GGGCAGGTCA AATTCCATGA CCTTGAGCAC TCTGTACAAA GAGAGAAAAT [-------]AAT
R103 INTER      GGGCAGGTTA CATTCCATGA CCTTGAGCAC TCTGTACAAA GAGAGAGAAA ATACAATGAG
R107 INTER      GGGCAGGTTA AATTCCATGA CCTTGGGCAC TCTGTACAAA GAGAGAAGAA [-------]GGT
R106 INTER      GGGCAGGTTA AATTCCATGA CCTTGGGCAC TCTGTACAAA GAGAGAAGAA [-------]GAT
R117            GGGCAGGTTA AACTCCATGA CCTTGGGCAC TCTGTACAAA GAGAGAAGAA [-------]GGT
R118            GGGCAGGTTA AATTCCATGA CCTTGGGCAC TCTGTACAAA GAGAGAAGAA [-------]GGT
1030            GGGCAGGTTA AATTCCATGA CCTTGGGCAC TCTGTACAAA GAGAGAAGAA [-------]GGT
R114            GGGCAGGTTA AATTCCATGA CCTTGGGCAC TCTGTACAAA GAGAGAAGAA [-------]GGT
TAS6            GGGCAGGTTA AATTCCATGA CCTTGGGCAC TCTGTACAAA GAGAGAAGAA [-------]GGT
KI61            GGGCAGGTTA AATTCCATGA CCTTGGGCAC TCTGTACAAA GAGAGAAGAA [-------]GGT
R14             GGGCATGATA TTTTCCATGA CCGGGCACTC TCTGTACAAA GAGAGAAGAA [-------]GGT
SE50            GGGCAGGTTA CATTCCATGA CCTTGAGCAC TCTGTACAAA GAGAGAGAAA ATACAATGAG
L57             GGGGAAGTTT CTTTCCATGA CCTTTAACCC TTCGGACAAA GAAAAAAAAA ATCCCATGAA
TAS3            GGGCGTGTTA TTTTCCGTGA CCGGGCCCTC TCTGCACAAA GAGAAAAGAG [-------]GGG
SE45            GGGCAGAAGA CATTCGACGT CCTAGAACCT TCTGCACTCT GAGAAAAAAA ATACAATGAA
SAR11.18        GGGGAGGTTA CATTCCCCGA CCTTGAGCCC TCTCCCCAAA GAGAGAAAAA ATACATTGAG
```

[1] Long (570nt), Short (510 nt) and Intermediate (550 nt) indicate the classification system used by Woodhall *et al*. (2007) based on PCR product size in agarose gel, visualised with GelDoc system.
[2] Australian isolates

```
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   455        465        475        485        495        505
R42 LONG        GGGAGAAATA AATAATCTAT GTGGGAAACC CCCCTATGAA TG-TATGTAT CACCTTAAAC
R100 SHORT      TGTGGAAAAA AAAAAAAAA- ---------- ---------- --+------- CACCTTGAAC
R103 INTER      AAAAGAAATA AGTAGGTGGT GTGAGACAA- CCCATATGAC TG-GACACAT CACCTTGAAC
R107 INTER      GGGAGAAATA AATAAGCTAT GTGGGAAAGC CCCCTATGAA TG-TATGTAT CACCTTAAAC
R106 INTER      GAGA-AAATA AATAAGATAT GTGAGAAAAT CCCCTATGAC TGGCATACAT GAGCTTGAAC
R117            GGGAGAAATA AATAAGCTAT GTGGGAAAAC CCCCTATGAA TG-TATGTAT CACCTTAAAC
R118            GGGAGAAATA AATAAGCTAT GTGGGAAAGC CCCCTATGAA TG-TATGTAT CACCTTAAAC
1030            GGGAGAAATA AATAAGCTAT GTGGGAAAGC CCCCTATGAA TG-TATGTAT CACCTTAAAC
R114            GGGAGAAATA AATAAGCTAT GTGGGAAAGC CCCCTATGAA TG-TATGTAT CACCTTAAAC
TAS6            GGGAGAAATA AATAAGCTAT GTGGGAAAGC CCCCTATGAA TG-TATGTAT CTCCTTAAAC
KI61            GGGAGAAATA AATAAGCTAT GTGGGAAAGC CCCCTATGAA TG-TATGTAT CACCTTAAAC
R14             GGGAGAAATA AATAAGCTAT GTGGGAAAGC CCCCTATGAA TG-TATGTAT CACC-TAAAC
SE50            AGAAGAAATA AGTGGGTGGT GTGAGACAA- CCCATATGAC TG-GATACAT CACCT-GAAC
L57             AAAAAAAATT AGTAGGGGGG GGGAAACCA- CCCATTTGGG TG-GATCCCC C-CCCTAAAC
TAS3            GAGAAAAATA AATATGTTAT GGGAAACCCC CCCCTATGAG TG-TATGTAC CTCCATAAAC
SE45            AAAAGAAATA AGTAGTTGGT GGGAGACGA- CCCACATGAC TG-AACACTG CACCTTGCAC
SAR11.18        AAAAAAAATA AGTGGGGGGG GGGAGACA-C CCCATATGGG GG--GAGACC CCCCCCCAAC
```

3.3.8 Phylogeny based on ITS region

To investigate if sequence variation in the IGS1 region of AG 2-1 isolates was reflected in ITS region, a neighbour-joining tree was constructed for sequences from the latter. Sequence analysis of the ITS region in the British and Australian AG 2-1 isolates tested showed clustering of the "short" IGS type isolates, significantly different from isolates of "intermediate" and "long" types (Figure 3.8). This result was expected, as a similar observation was made by Woodhall *et al.* (2007) when some of the same isolates were used in phylogenetic analysis with AG 2-1 ITS sequences available on the NCBI database.

Isolates belonging to the "long" IGS1 type clustered together and as indicated by a bootstrap value of 93, were significantly different from isolates of both "short" and "intermediate" type.

Isolates belonging to the "intermediate" IGS1 type are significantly different from both the "short" and "long" IGS1 type isolates. However, this group of isolates showed significant differences between isolates belonging to the "intermediate" IGS1 type. One example of this is isolates SE50 and SE45, which were isolated from potato from the same field in Australia, but are separated by a bootstrap value of 88 (Figure 3.8).

Figure 3.7. Neighbour-joining tree constructed from IGS1 sequences from Australian and British *R. solani* AG 2-1 isolates. IGS1 type determined from the length of sequenced amplicons is indicated in brackets following the isolate code and bootstrap values are indicated on branches. Australian isolates are denoted by bold labels and the scale bar indicates 2 base changes per 100 nucleotide positions

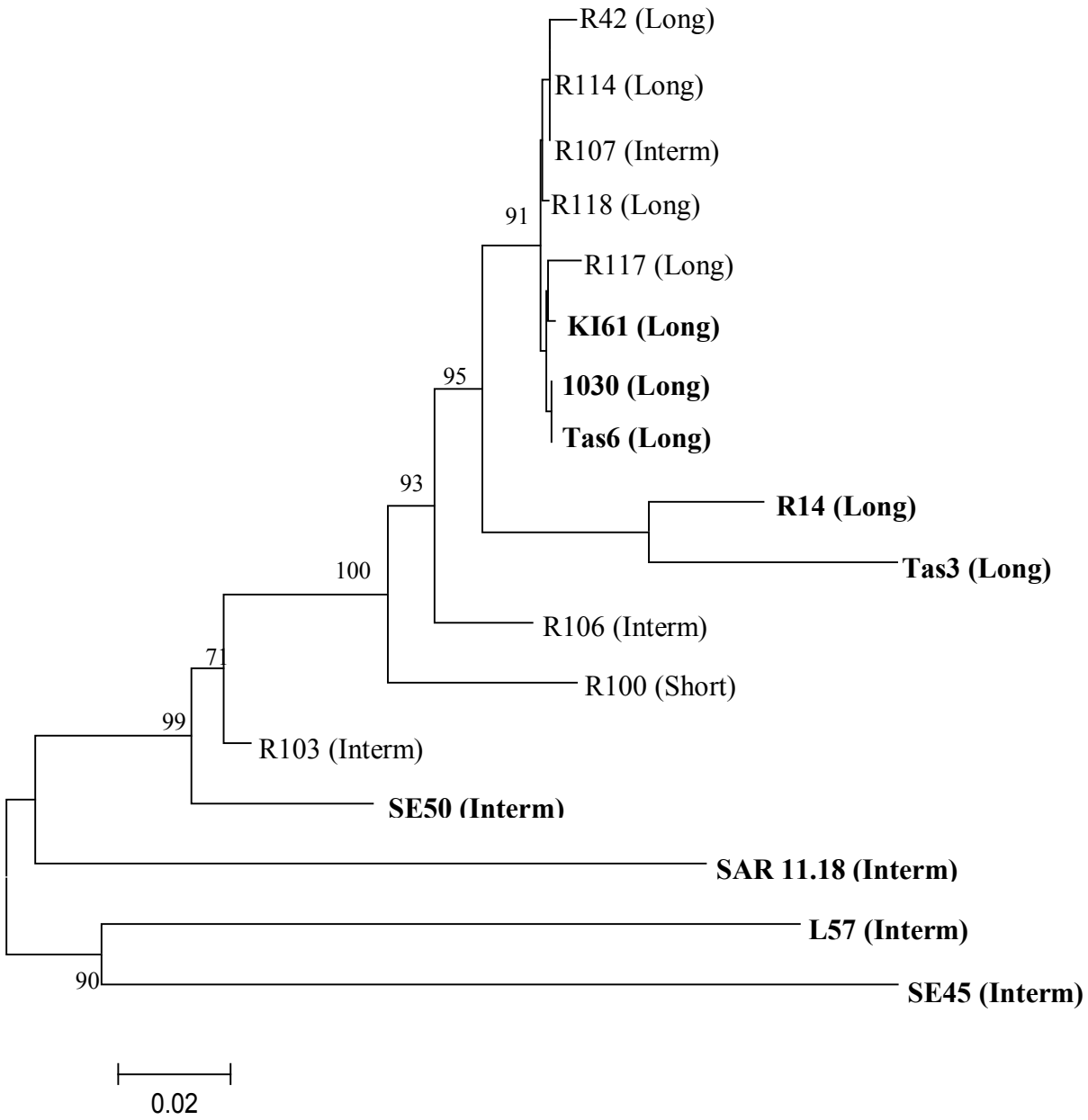
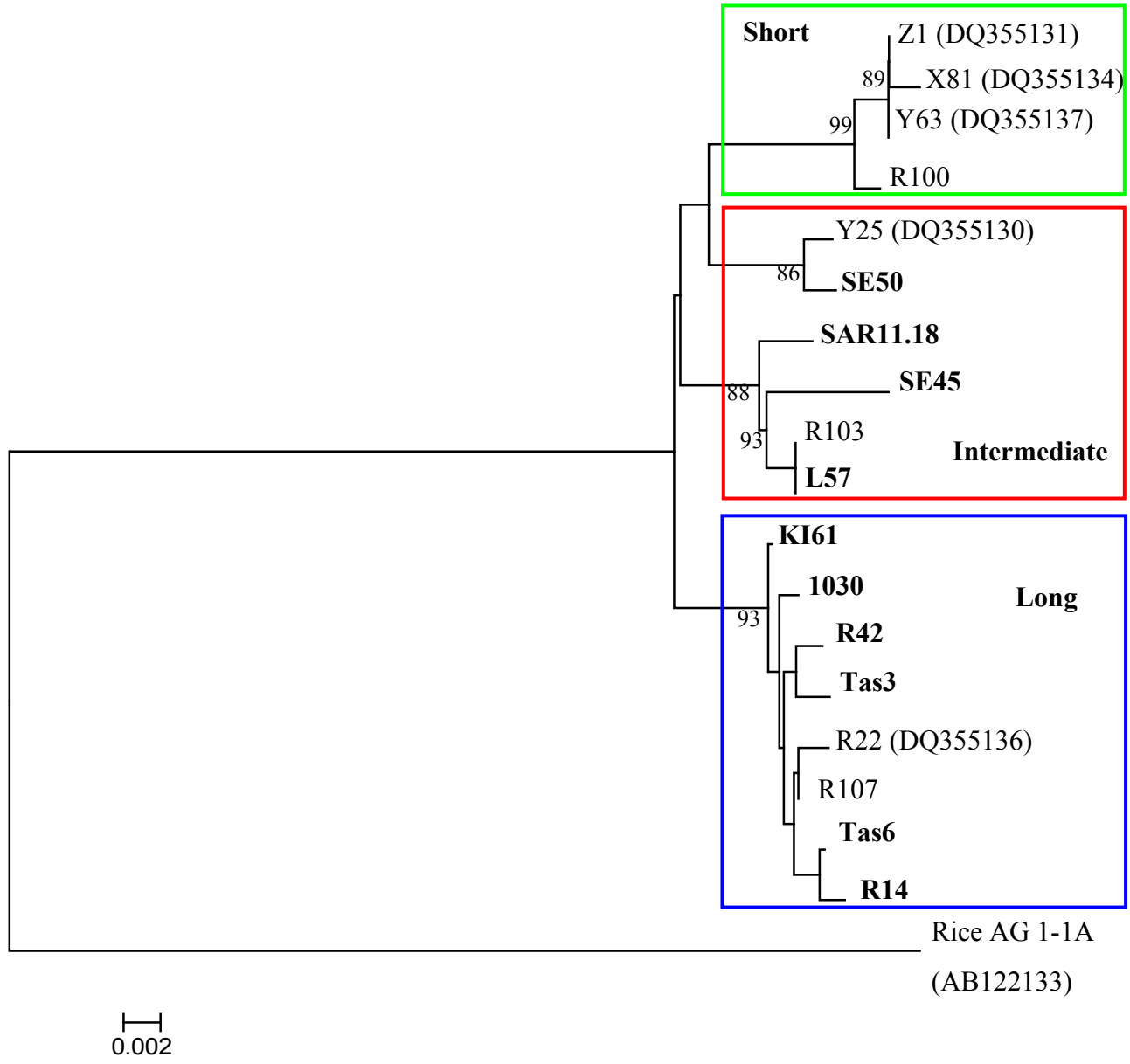


Figure 3.8. **Neighbour-joining tree based on ITS sequence of Australian and British *R. solani* AG 2-1 isolates for comparison to IGS1 tree. Boxes indicate isolates belonging to IGS1 types "short" (green), "intermediate" (red) and "long" (blue) as determined by amplicon sequence for available isolates and by (Woodhall *et al.* 2007) for ITS sequences obtained from NCBI database. The latter have their accession number in parenthesis after isolate code. Australian isolates are indicated by bold labels, scale bar indicates 2 base changes every 1,000 base positions and bootstrap values indicated on branches**



3.4 Discussion

The first aim of the experiments presented in this chapter was to confirm the effectiveness and specificity of the available primer sets, to determine if they would be useful in assigning isolates from Australian potato growing regions to an AG. A preliminary experiment with 12 tester isolates revealed no cross-reactivity when DNA of *R. solani* was amplified by PCR using primer sets specific for AG 2-1, AG 2-2, AG 3, AG 4 or AG 8. The primers, therefore, were considered to be specific to the AG for which they were designed (Ophel-Keller *et al.* 2006).

Further confirmation of specificity of the primers was obtained via the AG designation of an additional 16 isolates, previously classified by other researchers independent of these AG-specific primer sets. However, five isolates could not be classified due to lack of amplification products from DNA, extracted by two different methods (see section 2.3.1), using standard PCR. The classification of these isolates was achieved following the extraction of DNA and qPCR by the RDTS. Furthermore, of the 316 *R. solani* isolates collected from potato fields during this project, 94 % were assigned to AG but 6 % were not identified with standard PCR. These results indicated that although the AG-specific primer sets used in a standard PCR can increase the efficiency of AG assignment for research purposes, the standard PCR procedure is less sensitive than the TaqMan qPCR assay used at the RDTS. This supports results of Lees *et al.* (2002), which also indicated that qPCR was more sensitive than conventional PCR in detecting AG 3 isolates of *R. solani*.

Cultural classification confirmed both the AG previously assigned to isolates by other researchers and those assigned to isolates obtained during this project by AG-specific PCR. Some difficulties were experienced with the consistency of observations via cultural classification, as other researchers have observed (reviewed by Carling 1996). Therefore, the AG-specific PCR method was used routinely for classifying field isolates.

During this project AG 2-1, AG 2-2 and AG 3 were isolated from a range of diseased tissues of potato plants, including tubers, necrotic stem, stolon and roots. These findings support other work in which AG 3 was the major group recovered from sclerotia on infected tubers (Balali *et al.* 1995; Campion *et al.* 2003; Carling and Leiner 1986; de Boer *et al.* 2001; Lehtonen *et al.* 2008a; Virgen-Calleros *et al.* 2000; Woodhall *et al.* 2007) and from stems, stolons and roots of potato plants (Bains and Bisht 1995; Balali *et al.* 1995; Bandy *et al.* 1988; Carling and Leiner 1986; de Boer *et al.* 2001; Lehtonen *et al.* 2008a; Woodhall *et al.* 2007). Likewise, *R. solani* AG 2-1 has been isolated from necrotic sections of stems (de Boer *et al.*

2001; Woodhall *et al.* 2007; Yanar *et al.* 2005), stolons (de Boer *et al.* 2001; Woodhall *et al.* 2007) and sclerotia from tubers (Campion *et al.* 2003; de Boer *et al.* 2001; Woodhall *et al.* 2007). AG 2-2 has previously been isolated from necrotic stems and stolons of potato plants from Victoria, Australia (de Boer *et al.* 2001) and Turkey (Yanar *et al.* 2005).

Binucleate *Rhizoctonia* species were also collected from potato plants and assigned to AG A and AG K by ITS sequence similarity. The 13 binucleate isolates collected were not associated with a particular region or tissue. These species are considered only mildly pathogenic to potato plants (Carling *et al.* 1986; Lehtonen *et al.* 2008a) and may in fact, play a role in disease control (Wolski *et al.* 2004). These aspects of the binucleate *Rhizoctonia* species from potato fields in Australia require further investigation. If they are able to reduce disease caused by *R. solani*, identifying natural populations in field soils may need to be taken into account in the development of Rhizoctonia disease prediction models. Also, such isolates may have potential as biological control agents applied prior to planting potato crops, as reported for *Trichoderma harzianum* by Wilson *et al.* (2008a). They found that *T. harzianum* reduced the severity of potato stem necrosis caused by *R. solani* and the number of tuber-borne sclerotia.

The associations of *R. solani* AGs with specific tissues sampled appeared to be influenced by growing region. For example, isolates found on green stems from potatoes grown on Kangaroo Island were assigned to AG 2-2; however, those from the South East of South Australia were classified as AG 2-1 and AG 3, while no isolates were collected from green stems from Tasmania. The latter result may reflect the small number of samples. This suggests that no single AG is uniquely associated with one region or disease symptom in Australian potato fields that supply the processing industry. These results support evidence from previous studies in which the prevalence of AGs varied between sites within a country or region (Balali *et al.* 1995; Yanar *et al.* 2005). However, site did not influence the association of some combinations of AG with tissues. For Kangaroo Island, the South East of South Australia and Tasmania, AG 2-1 isolates were found on roots, AG 2-2 on the white (below ground) stem and AG 3 on tubers.

Previous studies of Rhizoctonia disease of potatoes have shown that *R. solani* AG 3 is the group most frequently isolated from tubers world-wide (Bains and Bisht 1995; Bandy *et al.* 1988; Campion *et al.* 2003; Carling and Leiner 1986; Virgen-Calleros *et al.* 2000; Woodhall *et al.* 2007; Yanar *et al.* 2005) and in South Australian and Victorian regions that supply the fresh washed industry in Australia (Balali *et al.* 1995; de Boer *et al.* 2001). During

this project AG 3 was most frequently isolated from plants from the South East of South Australia and Tasmania, particularly from tuber sclerotia, however, not from plants from Kangaroo Island. The low frequency of AG 3 at Kangaroo Island sites may be a feature of this growing region, which supplies the seed industry (pers. com. R. Harding 2006). Crops are often grown at sites that have never been used for potato cultivation and the seed used can originate from tissue culture plants, which are unlikely to introduce seed-borne inoculum.

Other researchers (Balali *et al.* 1995; Campion *et al.* 2003; de Boer *et al.* 2001; Lehtonen *et al*. 2008a; Virgen-Calleros *et al.* 2000; Woodhall *et al.* 2007) have reported isolation of AG 2-1 from potato plants, but at frequencies lower than those observed for the Kangaroo Island (35 %) and South East (30 %) sites of South Australia sampled during this project. This high frequency of AG 2-1 in Australia may be a concern, as variation has been observed among AG 2-1 isolates in both pathogenicity (Carling and Leiner 1986; Woodhall *et al.* 2007) and fungicide sensitivity (Campion *et al.* 2003). Therefore, information about the quantity of AG 2-1 inoculum in soils may be insufficient for prediction of disease.

Genetic markers to identify variation in pathogenicity and fungicide (such as iprodione) insensitivity in AG 2-1 would be a useful addition to current field soil tests; however, no single genes have yet been associated with these phenotypes. Therefore, the genetic variation among AG 2-1 isolates collected during this project was further investigated. Initially, this was done to determine if genetic variation among isolates might be linked to origin in terms of: country, region or symptom type on potato plants. Woodhall *et al.* (2007) previously found that nine British AG 2-1 isolates varied in size of PCR product from the IGS1 region, therefore, this genomic region was utilized during investigations of Australian isolates. In preliminary experiments conducted in Australia, genetic variation was observed among AG 2-1 isolates but allocation of isolates to distinct groups, as done by Woodhall *et al.* (2007) was not possible.

Investigation of genetic variation was continued in Scotland, where reference isolates previously classified into size groupings described as “short”, “intermediate” and “long” by Woodhall *et al.* (2007) were available. Results based on amplicon size in an agarose gel showed only one (British) isolate produced a band of “small” size and the “intermediate” and “large” IGS1 types could not be differentiated. These PCR products were sequenced to determine their exact lengths and if loci might be useful in further analysis. Based on sequence variation, three groups were designated: “short”, “intermediate” and “long”. These were based on common sites where three or more nucleotides were deleted. In most cases,

groupings based on sequence analysis were consistent with classifications of the same name by Woodhall *et al*. (2007), although the actual fragment sizes were different. Although, Woodhall *et al.* (2007) originally estimated these fragment sizes by separation of PCR products in an agarose gel, the size differences would be more accurately determined using polyacrylamide gel electrophoresis.

Australian and British isolates fell into both "intermediate" and "long" groups, indicating that the deletion patterns were not indicative of country of origin. Isolates collected from both necrotic tissue and sclerotia on potato plants were classified as either "intermediate" or "long" types, as were isolates collected from other host plants. This suggests that the sequence variation in the IGS1 region was not indicative of host or tissue specificity of the AG 2-1 isolates tested. These results were contrary to the findings of Woodhall *et al.* (2007), where AG 2-1 isolates of "short" IGS1 type were associated with tuber sclerotia and those of "intermediate" type with stem necrosis, and suggest that this molecular test would not be a useful addition to the AG-specific tests currently available to aid decisions on whether or not to plant a potato crop. However, further investigation is required to determine if this molecular test is indicative of the severity of disease likely to be caused by *R. solani* AG 2-1 isolates.

The ITS region, rather than the IGS1 region, has been used in developing *R. solani* AG-specific tools for soil detection (Lees *et al.* 2002; Ophel-Keller *et al.* 2006; Ophel-Keller *et al.* 2008), therefore, phylogenetic comparisons were performed to determine if sequence variation within the IGS1 region reflects that within the ITS region. Groups based on IGS1 region did not cluster in a phylogenetic comparison, indicating that sequence variation in this region was more extensive than the deletion sites on which the groups were based. When sequences from the ITS region were compared clusters of isolates formed, each of which comprised isolates of only one IGS1 group. This indicated that the IGS1 deletion patterns reflected the variation within ITS sequence; therefore, either region may be used to investigate the variation among these AG 2-1 isolates in future experiments. However, during this project the IGS1 region was used because of the genetic markers (deletion sites) identified. The similarity of the ITS and IGS1 regions may also be useful in future evolutionary studies of *R. solani* AG 2-1, as multiple gene regions can be more effective than single sites (Kroon *et al.* 2004).

In conclusion, results from these experiments indicated that the isolates from the various AGs and sub-groups collected from Australian potato fields were associated with the

majority of different symptoms of Rhizoctonia disease on potato plants. However, pathogenicity tests of isolates, representative of those groups found, were still required to investigate the symptoms caused by each group on potato plants.

4 Pathogenicity

4.1 Introduction

A number of anastomosis groups (AGs) of *Rhizoctonia solani* have been found on potato world wide (refer to section 1.4.3) and in fields that supply the fresh washed industry of Australia (Balali *et al.* 1995; de Boer *et al.* 2001). In the previous chapter it was shown that AG 2-1, AG 2-2 and AG 3 were associated with diseased tissues of potato plants in the three regions of Australia (Kangaroo Island, the South East of South Australia and Tasmania) from which potato plants were collected. AG 3 is recognised as the major cause of the black scurf on tubers world-wide (reviewed in section 1.4.3.3), however, the specific association of AGs with stem, stolon and root necrosis, all of which affect yield quantity and quality, is uncertain. Experiments were carried out in pots in a shade-house and in containers in a controlled environment to investigate the association of AGs with disease symptoms on potato plants.

4.2 Methods

4.2.1 Shade-house experiment

To investigate the pathogenicity of isolates representing AG 2-1, AG 2-2, AG 3 and AG 8 of *R. solani* to potato plants, an experiment was carried out in a shade-house in Adelaide, beginning in January 2006. The following isolates were used as inoculum: L57, R394, WAC-9806 (AG 2-1); WAC-9937 (AG 2-2); L62, L306, T22 (AG 3) and RS21 (AG 8). These isolates were representative of the AGs found in Australian potato fields (see Chapter 3). Flasks (250 ml) were filled with 80 g of millet seed and inoculum produced as described in section 2.4.1. Colonised millet seed (10 g) was mixed into each pot containing approximately 6 kg of Mt Compass sand (Table 2.3). Four days after inoculation mini-tubers of cv. Russet Burbank (provided by the Department of Primary Industries, Victoria) were planted as described in section 2.4.3. The experiment was a one-way design with no blocking, with eight replicate plants per inoculum treatment. Plants were grown in the conditions described in section 2.4.3 for 3 months. After harvest, the severity of disease on stems, stolons, roots and tubers was scored as described in section 2.4.4. The size and weight of tubers per plant were also recorded. Data analysis was performed as described in section 2.4.4. *Rhizoctonia* was isolated from roots, necrostic tissue on stems and stolons and sclerotia from tubers and roots as described in section 2.1. To confirm the AG of isolates, DNA was extracted and PCR

preformed with AG-specific primers as described in section 2.3. Tissue samples were also sent to SARDI RDTS for DNA extraction and QPCR.

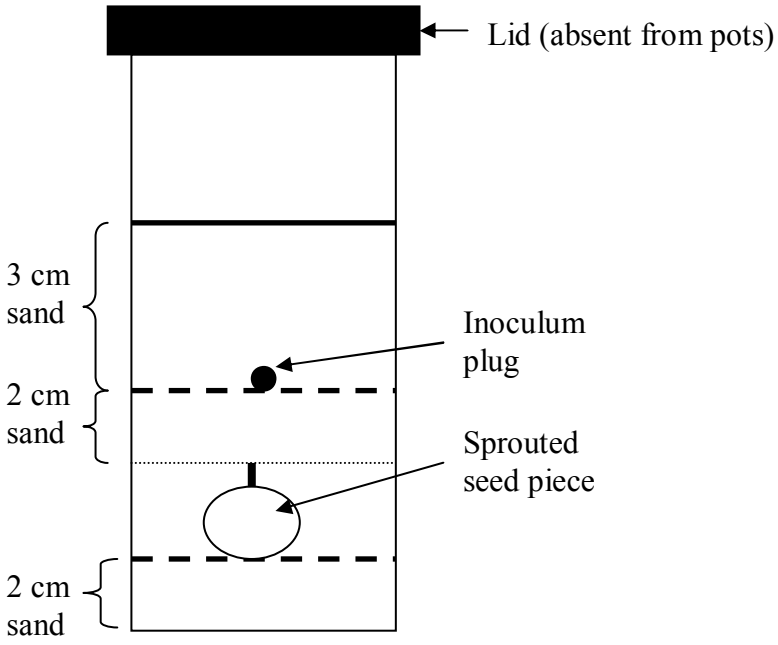
4.2.2 Controlled environment trials

Two experiments were performed to assess the potential of selected isolates to cause necrosis on potato stems. Different substrates, potato seed and growth conditions were used in the two experiments, however, placement of tuber and inoculum were similar to that shown in Figure 4.1. A 2 cm deep layer of steam sterilised sand or autoclaved compost was placed on the bottom of the clean pot or tub. A seed piece, with the sprout facing upwards, was placed in the centre of each container. Sufficient sand or compost was then added to ensure that the sprouts were covered with an additional 2 cm layer. A plug of PDA, taken from the margin of a 7 day old culture of *R. solani* on PDA was placed on this layer of sand or compost, directly above the sprout. Uninoculated controls consisted of uncolonised plugs of PDA in place of inoculum. The inoculum was then covered with a 3 cm deep-layer of sand or compost. After incubation as described in the following sections, plants were washed and disease severity on stems and roots was assessed as described in section 2.4.4.

4.2.2.1 Australian isolates

To enable pathogenicity testing in Adelaide in August, 2007, when the environment in the Adelaide Hills shade-house was not conducive to growth potato plants were grown in a controlled environment in tissue culture tubs, based on the techniques described by Lehtonen *et al*. (2008a). Potato tubers (cv. Russet Burbank) that had been stored at 4ºC since harvest in March of that year were surface sterilised, "eyes" removed with a melon ball scoop and sprouted as described in section 2.4.4. Mt Compass sand, sprouted seed piece and a 5 mm plug of inoculum were layered in polycarbonate tubs (height 15 cm, diameter 6.5 cm) as described in section 4.2.2 and illustrated in Figure 4.1. The pathogenicity of 13 isolates of *R. solani* representing five AGs was tested; Tas6, SAR11.18, KI65, SE50 (AG 2-1), 894, SE42, Tas30.1 (AG 2-2), Tas1, Tas3, KI24, R229 (AG 3), 734 (AG 4) and RS21 (AG 8). The pathogenicity of one binucleate *Rhizoctonia* isolate, with ITS sequence similarity to AG K, was also tested.

Figure 4.1. Assessment of disease severity on potato stems caused by *R. solani* in tissue culture tubs (modified from Lehtonen *et al.* 2008a). Pasturised Mt Compass sand and a 5 mm diameter inoculum plug on PDA was used in tissue culture tubs but a method modified for pots is described in the following section



To maintain humidity during incubation, sterile RO water (10 ml) was pipetted onto the sand in each tub. Tubs were then closed with a polypropylene lid with one 3 mm diameter hole, filled with cotton wool, for gas and moisture exchange. A 5 cm space was left between the top of the sand and the lid. Tubs were placed in trays on three shelves in an incubator (Environ Air, manufactured by SRJ cabinet sales, New South Wales, Australia) in a completely randomised design with eight replicates of each inoculum treatment. Tubs were then incubated in darkness at 25°C for 21 days. After harvest, disease severity on stems was scored as described in section 2.4.4. The severity of root damage was scored as the percentage of the total number of roots with necrosis and/or sclerotia present, these categories comprised:

1 – No damage;

2 – 1 to 25 % of root tissue covered by any combination of damage including necrosis, sheath stripping or sclerotia;

3 – 25 to 50 % coverage as above;

4 – > 50% coverage.

Data analysis was performed as described in section 2.4.4. *R. solani* was isolated from sclerotia from roots, necrotic sections of roots and stems, as described in section 2.1. To confirm the AG of isolates, DNA was extracted and PCR performed with AG-specific primers as described in section 2.3. Stem tissue samples were also sent to SARDI RDTS for DNA extraction and QPCR.

4.2.2.2 British and Australian isolates

To compare the pathogenicity of British and Australian AG 2-1 isolates with different IGS1 types, an experiment based on studies by Woodhall *et al.* (2007) was carried out at the SCRI in July 2008. Potato tubers (cv. Russet Burbank) were surface sterilised in a 1 % bleach solution for 5 min then rinsed three times with tap water. Sprouts were removed and tubers cut with a flame-sterilised kitchen knife on a chopping board rinsed with 100 % ethanol. Seed pieces containing at least one "eye", and with no visible blemishes were cut into cubes of approximately 1 cm. These were placed on the first layer of autoclaved John Innes No. 2, a loam compost, in the base of square pots (10 cm^2 x 20 cm deep) as described in section 4.2.2. The seed piece tissue was allowed to suberise for 24 hrs before covering with 2 cm of loam and an 8 mm diameter inoculum plug (as a 5 mm cork borer was not available) was applied as described in section 4.2.2 and illustrated in Figure 4.1. Pots containing 32 isolates, representing six AGs, were incubated at 25ºC in a growth room. At the same time an experiment using six of the 20 AG 2-1 isolates was prepared with identical conditions except that the pots were incubated in a separate chamber at 16ºC. Three of these six isolates originated from Great Britain and had previously been classified as being of "short" (R100), "intermediate" (R107) and "long" (R42) IGS1 types. The other three isolates originated from a range of host plants and regions of Australia. All eight replicates of each inoculum treatment were placed in one tray to minimise cross-contamination during watering. Trays were arranged in a randomised design in growth chambers under a regime of 16 hrs of light and 8 hrs of dark. Pots were watered with tap water every 2 days until flow-through was observed. Plants, harvested after 21 days, were washed and disease severity was scored on stems as described by Balali *et al.* (1995) (see section 2.4.4). The scoring method described by Carling and Leiner (1990) was also used, where:

0 – No damage or lesions

1 – Minor damage, one to several lesions < 5 mm in size

2 – Moderate damage, lesions > 5 mm in size and some girdling

3 – Major damage, large lesions and girdling or death present on most stems

4 – All stems killed

The size of necrotic lesions on stems was measured with digital callipers. Necrotic lesions, without a clear edge, or stems which displayed multiple small lesions were noted as having a "striped" appearance as described by Woodhall *et al*. (2007). Normal necrotic lesions were regular oblong in shape with a discrete edge.

4.3 Results

4.3.1 Shade-house experiment

The results of disease severity assessment of stems, stolons, roots and tubers are shown in Table 4.1. Uninoculated plants were healthy, showed no necrosis on stems, stolons or roots and no sclerotia developed on roots or tubers. The mean total weight of tubers per plant was 12.7 g; although not the heaviest this result was not significantly different from than that of plants grown in the presence of any inoculum.

Sclerotia were produced on daughter tubers by all of the AG 3 (L62, L306 and T22) isolates tested. Median scores of the severity of sclerotia indicated only slight disease, with the majority of tubers having fewer than 25 sclerotia. The one AG 2-2 isolate (WAC-9937) tested produced sclerotia on remnants of the mother tuber (data not shown), but not on daughter tubers. None of the three AG 2-1 (L57, R394 and WAC-9806) isolates tested produced sclerotia on tubers, however, plants grown in the presence of one isolate (R394) from this group had sclerotia on their roots.

The severity of disease on stems of plants which were inoculated with isolates L57, WAC-9806 (AG 2-1); WAC-9937 (AG 2-2) and all three AG 3 isolates tested was significantly ($P < 0.05$) greater than the uninoculated control plants (Table 4.1). The most severe disease was caused by WAC-9806 (AG 2-1), with a median score of 3, indicating deep necrotic lesions. However, the ranking of this treatment was not significantly ($P < 0.05$) different from that of plants inoculated with isolates WAC-9937, L62 and L306.

Stolon necrosis was significantly ($P < 0.05$) more severe on plants inoculated with the isolates WAC-9937 (AG 2-2), L62 and L306 (AG 3) than the uninoculated control plants. On these plants the median severity (2) indicated only slight, superficial necrosis of stolons.

Assessment of root necrosis was confounded by the development of sclerotia on roots. Hence, root damage was assessed as a percentage of total root area. The isolates that caused significant ($P < 0.05$) root damage were those that produced sclerotia on roots (Table 4.1). These included all three AG 3 isolates, one AG 2-1 isolate (R394) and the AG 2-2 (WAC-9937) isolate.

The severity of disease symptoms varied among isolates from the same AG. Variation was observed in stem necrosis caused by AG 2-1 isolates, where isolate WAC-9806 caused a significantly ($P < 0.05$) higher median score than the other two isolates tested. AG 2-1 isolate R394 damaged roots by producing sclerotia, however, this symptom was not observed on plants inoculated with the two other AG 2-1 isolates tested. Variation in the severity of stolon necrosis caused by AG 3 isolates was also observed where inoculation of plants with isolate L306 caused significantly ($P < 0.05$) higher severity than isolate T22.

Yield, measured as total tuber weight per plant, for three inoculation treatments L57 (AG 2-1), L62 (AG 3) and RS21 (AG 8) was similar to uninoculated control plants. Likewise, plants inoculated with AG 3 isolate L306, which developed significantly ($P < 0.05$) worse necrosis of stem, stolon and sclerotia on tubers and roots, yielded as well as the uninoculated plants. Total tuber weight per plant was significantly ($P < 0.05$) decreased for plants inoculated with isolates R394, WAC-9806 (AG 2-1), WAC-9937 (AG 2-2) and T22 (AG 3) when compared with the uninoculated control.

Isolation from diseased tissue, followed by AG-specific PCR showed that sclerotia on roots and tubers were associated with AG 3 and AG 2-2 isolates (Table 4.2). However, reisolation of *Rhizoctonia* from most sections of necrotic tissue was not achieved. These tissues were stored at 4 ºC, post-harvest, prior to isolation which may have affected the viability of the mycelium.

Table 4.1 Disease severity scores from potato plants grown for 3 months in Mt Compass sand inoculated with different AGs of *R. solani*

Inoculum		Disease severity score ranks[1] and medians[2]				Root sclerotia	Mean[3] total tuber weight (g)/plant l.s.d (5%) = 4.3
AG	Isolate code	Stem necrosis Chi^2 prob. <0.001	Stolon necrosis Chi^2 prob. = 0.002	Root necrosis Chi^2 prob. = 0.019	Tuber sclerotia Chi^2 prob. <0.001		
N/A	control	(15.06) a 1	(17) a 1	(18) a 1	(23) a 1	no	12.69 de
2-1	L57	(31.86) bc 2	(24.57) abc 1	(26.86) ab 1	(23) a 1	no	13.95 e
2-1	R394	(22.19) ab 1	(20.79) ab 1	(33.50) abc 1.5	(23) a 1	yes	7.76 ab
2-1	WAC-9806	(51) d 3	(30.30) abcd 1	(18) a 1	(23) a 1	no	3.48 a
2-2	WAC-9937	(40) cd 2	(38.20) cde 2	(42.80) c 2	(23) a 1	yes	4.57 ab
3	L62	(40) cd 2	(41) de 2	(35.71) bc 2	(53) b 2	yes	8.41 bcd
3	L306	(47.12) cd 2	(48.10) e 2	(41.25) bc 2	(54) b 2.5	yes	12.74 e
3	T22	(35.25) bc 2	(30.25) abcd 1.5	(38.67) bc 2	(44.43) b 2	yes	8.32 bc
8	RS21	(15.06) a 1	(23.62) abc 1	(29.62) abc 1	(23) a 1	no	9.73 cde

[1] Rank as determined by Kruskal-Wallis analysis is indicated in parenthesis and was used to determine significant differences between treatments, with eight replicates (degrees of freedom = 8), which are indicated by a chi squared probability (chi^2 prob.) < 0.05, l.s.d. 5 % = 15.51 and different letters within a column.
[2] Median severity scores are unbracketed values and disease severity was assessed as described in section 2.4.4
[3] Mean values were determined from eight replicates per treatment and ANOVA was used to determine significant differences between them, indicated by the l.s.d. 5 % and different letters in a column

Table 4.2 AG determination of *R. solani* associated with symptoms on potato plants, grown in Mt Compass sand in a shade-house experiment for 3 months, following reisolation and AG-specific PCR or DNA extraction from tissue sections and qPCR

Inoculum AG	Inoculum isolate	Reisolation AG-specific PCR result	Tissue	DNA quantity (fg/PCR) estimated by qPCR				
				AG 2-1	AG 2-2	AG 3	AG 4	AG 8
AG 2-1	R394	AG 2-1	Root sclerotia	n/p	n/p	n/p	n/p	n/p
AG 2-1	WAC-9806	–	root	14244	0	16	0	0
AG 2-1	WAC-9806	–	stem	1544	8	202	0	0
AG 2-1	L57	–	stem	0	0	5	0	0
AG 2-1	L57	–	stem	4	6	5	0	0
AG 2-2	WAC-9923	AG 2-2	Root sclerotia	n/p	n/p	n/p	n/p	n/p
AG 3	L62	AG 3	Tuber sclerotia	n/p	n/p	n/p	n/p	n/p
AG 3	L306	AG 3	Tuber sclerotia	n/p	n/p	n/p	n/p	n/p
AG 3	L306	–	stem	0	0	112231	0	3
AG 3	L306	–	stem	4	6	3794966	0	0
AG 8	RS21	–	stem	21	6	0	0	3
AG 8	RS21	–	root	77	0	0	0	10
AG 8	RS21	–	root	4	0	0	0	0
nil	control	–	stem & root	30	0	7	0	0

– indicates *R. solani* was not reisolated from the tissue section specified.
n/p indicates quantitative PCR was not performed on the specified tissue section

Results for AG-specific, qPCR, conducted by RDTS at SARDI, are shown in Table 4.2. Results of qPCR from stems and roots of uninoculated control plants, showing no disease symptoms, indicated the presence of small amounts of AG 2-1 (30 fg/PCR) and AG 3 (7 fg/PCR) DNA (Table 4.2). When compared with the uninoculated plants, higher values (> 112,000 fg/PCR) of AG 3 DNA were found in the two stem samples from plants inoculated with the AG 3 isolate L306. The two tissue samples from plants inoculated with AG 2-1 isolate WAC-9806 had highest DNA concentrations for AG 2-1 (> 1,500 fg/PCR) although, unexpectedly, a small amount of AG 3 DNA (≤ 202 fg/PCR) was also detected (Table 4.2). In qPCR results no AG 4 DNA was detected in any sample.

Not all qPCR results from plant tissues were consistent with the AG of isolates used as inoculum. Little AG 2-1 DNA (≤ 4 fg/PCR) was detected in samples of necrotic stem tissue from plants inoculated with the AG 2-1 isolate L57. Non-symptomatic stem and root tissue from plants inoculated with the AG 8 isolate RS21 showed low levels of AG 8 DNA (≤ 10 fg/PCR) and AG 2-1 DNA of up to 77 fg/PCR.

4.3.2 Controlled environment experiments

4.3.2.1 Australian isolates

Disease severity on stems and roots of plants inoculated in Australia with Australian *Rhizoctonia* isolates is shown in Table 4.3. The uninoculated control plants showed no necrosis on stems or roots.

Eleven isolate treatments resulted in stem necrosis that was significantly ($P < 0.05$) more severe than that observed on uninoculated control plants. These isolates comprised all four AG 2-1, all three AG 2-2, three of the four AG 3 and the one AG 4 isolate tested. The AG 8 and AG K isolates did not produce stem necrosis, nor did AG 3 isolate Tas3. The most severe stem necrosis, i.e. girdling of sprouts, resulted from isolates SE50, SAR11.18 (AG 2-1) and R229 (AG 3). Median stem necrosis scores, indicating deep necrosis, resulted from inoculation with isolates Tas6 (AG 2-1), 894 and SE42 (AG 2-2), KI24 (AG 3) and 734 (AG 4). However, the severity of stem necrosis caused by isolates SE42 and 734 was not significantly ($P < 0.05$) less than that caused by SAR11.18. When compared with uninoculated controls, plants inoculated with isolates KI65 (AG 2-1), Tas30.1 (AG 2-2) and Tas1 (AG 3) developed moderate root disease (Table 4.3). The isolates of AG 8, AG K and

one AG 3 isolate (Tas3) did not result in stem damage that was significantly ($P < 0.05$) worse than uninoculated plants.

Plants inoculated with three of the four AG 2-1, all three of the AG 2-2, three of the four AG 3 developed significantly ($P < 0.05$) more severe root necrosis than the uninoculated controls. Isolate R229 (AG 3) caused the most severe damage, with more than 50 % of all roots damaged. This result was not significantly different from that found with isolate SE50 (AG 2-1). Inoculation with KI65 (AG 2-1) and KI19 (AG K) resulted in a slight increase in the median severity of root symptom severity, however, this was not significantly ($P < 0.05$) different from uninoculated plants.

Variation in disease severity was observed between isolates of the same AG. For example, one of the AG 3 isolates tested (Tas3) failed to produce necrosis of stems and roots significantly ($P < 0.05$) different from the uninoculated controls (Table 4.3). Also the severity of disease on both stems and roots differed significantly ($P < 0.05$) among the four AG 2-1 isolates tested, with SE50 producing the most severe symptoms.

Isolation from necrotic stem tissue, and subsequent DNA extraction and AG-specific PCR, confirmed the presence of *R. solani* from the same AG as the inoculum to which plants were exposed for isolates SE50 (AG 2-1), SE40 (AG 2-2) and Tas1 (AG 3) (Table 4.4). As reisolation of *Rhizoctonia* was not achieved from all treatments, tissue samples from stems were sent to SARDI for DNA extraction and AG-specific, quantitative PCR. Results for stem tissue of uninoculated control plants, showing no disease symptoms, indicated low levels ($\leq$ 3 fg/PCR) of AG 2-2 and AG 3 DNA. Stems from three inoculated treatments that showed no necrosis (AG K isolate KI19, AG 3 isolate Tas 3 and AG 8 isolate RS21) yielded low DNA concentrations ($\leq$ 21 pg/PCR) for all AGs tested by qPCR.

Of the 14 inoculum treatments, eight produced high DNA concentrations (between 47 and 1,007,386 fg/PCR), consistent with the AG of isolates applied, and small amounts of DNA ($\leq$ 10 fg/PCR) of other AGs (Table 4.4). These treatments were KI65, Tas6, SE50 (AG 2-1), T30.1, SE42 (AG 2-2) Tas1, R229 (AG 3) and 734 (AG 4).

Table 4.3 Disease severity on potato plant stems and roots grown in sand inoculated with different AGs of Australian *Rhizoctonia* isolates. The method used was described in Figure 4.1

AG	Isolate code	Disease severity scores ranks[1] and medians[2]	
		Stem Chi^2 prob. < 0.001	Root Chi^2 prob. < 0.001
2-1	Tas6	(69.81) cde 3	(51.43) bc 1
2-1	SAR11.18	(92.5) fg 3.5	(64.21) c 2
2-1	KI65	(46.5) bc 2	(45.00) abc 1.5
2-1	SE50	(103.38) g 4	(91.43) de 3
2-2	894	(77.69) de 3	(67.88) cd 2
2-2	SE42	(77.38) def 3	(67.75) cd 2.5
2-2	Tas30.1	(46.56) bc 2	(55.31) bc 2
3	Tas1	(58.06) bcd 2	(67.88) cd 2
3	Tas3	(34.69) ab 1	(23.50) a 1
3	KI24	(70.12) cde 3	(66.50) c 2
3	R229	(107.00) g 4	(106.00) e 4
4	734	(73.44) def 3	(45.29) abc 1
8	RS21	(15.50) a 1	(34.25) ab 1
K	KI19	(19.38) a 1	(45.00) abc 1.5
nil	Control	(15.50) a 1	(23.50) a 1

[1] Rank as determined by analysis Kruskal-Wallis analysis is indicated in parenthesis and was used to determine significant differences between treatments. Differences between treatments, with eight replicates (degrees of freedom = 14), are indicated by chi squared probability (chi^2 prob.) < 0.05, an l.s.d 5% = 23.68 and different letters within a column.

[2] Median severity scores are unbracketed values and disease severity was assessed as described in section 2.4.4

Table 4.4 AG associated with necrosis on stems of potato plants in a controlled environment experiment as determined by reisolation and AG-specific PCR or DNA extraction and quantitative PCR directly from tissue

Inoculum AG	Inoculum Isolate	Result of Reisolation & AG-specific PCR	DNA quantity (fg/PCR) estimated by qPCR					
			AG 2-1	AG 2-2	AG 3	AG 4	AG 5	AG 8
nil	**Control**	–	0	1	3	0	0	0
AG 2-1	**KI65**	–	207	0	0	0	0	0
AG 2-1	**Tas6**	AG 2-1	831	3	6	0	0	0
AG 2-1	**SAR11.18**	–	1095606	4	589	0	0	0
AG 2-1	**SE50**	AG 2-1	153727	8	2	0	0	0
AG 2-2	**T30.1**	–	0	47	1	0	0	0
AG 2-2	**SE42**	AG 2-2	3	57470	5	0	0	0
AG 2-2	**894**	–	2	6	9	0	0	0
AG 3	**KI24**	–	2	6291	0	0	0	0
AG 3	**Tas3**	–	0	0	0	0	0	0
AG 3	**Tas1**	AG 3	1	10	3572	0	0	0
AG 3	**R229**	–	5	2	1007386	0	0	0
AG 4	**734**	–	1	0	2	169	0	0
AG 8	**RS21**	–	2	21	17	0	0	1
AG A	**KI19**	–	2	15	2	0	0	0

– indicates that *R. solani* was not reisolated from stem tissue

In three cases, the AG indicated from qPCR results was not consistent with that of the inoculum. Stems inoculated with AG 2-1 isolate SAR11.18 resulted in very high AG 2-1 (1,095,606 fg/PCR) but also moderate amounts of AG 3 DNA (589 fg/PCR). Necrotic tissue from stems of plants inoculated with the AG 2-2 isolate 894 resulted in low DNA concentrations (≤ 9 fg/PCR) for all AGs. Necrotic stem tissue from plants inoculated with the AG 3 isolate KI24 yielded high levels of AG 2-2 DNA (6291 fg/PCR).

4.3.2.2 British and Australian isolates

Of the six isolates incubated at 16ºC in pots in a growth chamber, three isolates caused significantly ($P < 0.05$) more severe stem necrosis than the uninoculated controls (Table 4.5). These three isolates, two of which originated from Great Britain (R100, R42) and the other from Australia (Tas6), produced stems lesions with a striped appearance. The other three isolates did not produce any necrosis. Of the four isolates of long IGS1 type, two (R42 and Tas6) produced significantly ($P < 0.05$) more severe stem necrosis than the uninoculated controls, however the other two isolates (R107 and 1030) tested did not. The pathogenicity of one isolate from each of the short (R100) and intermediate (L57) IGS1 types was tested.

As the summer potato growing environment in Australia can be warmer than that found in the UK, and to be consistent with other experiments, the pathogenicity of more isolates was tested at 25ºC. The severity of stem disease produced by 32 isolates originating from Australia and Great Britain is shown in Table 4.1. Results obtained using the disease severity assessment methods of Balali *et al.* (1995) and Carling and Leiner (1990) were generally consistent. Results from both methods of assessment indicated that stem necrosis was significantly ($P < 0.05$) worse on plants inoculated with five AG 2-1 treatments than uninoculated control plants at 25ºC (Table 4.6.a). These treatments were one isolate originating from Great Britain (R106) and four from Australia (KI4, 730, SAR11.18 and WAC-9806).

Australian AG 2-1 isolates KI4, SAR11.18 produced the most severe symptoms, i.e. girdling of stems, as did isolate WAC-9806, for which statistical tests showed a similarity to isolate 730. The median severity scores of plants inoculated with isolate 730 indicated that deep necrosis was most frequently observed, not girdling of stems. Isolate R106 caused mainly small, superficial, necrotic stem lesions. At 25ºC, stem lesions with a striped appearance were observed on plants inoculated with 13 AG 2-1 isolates, however, isolate R106 caused both lesions of striped appearance and with discrete edges on different plants.

Due to the availability of space the pathogenicity of isolates from groups other than AG 2-1 was also tested at 25ºC (Figure 4.6.b). Of the 12 isolates tested, seven resulted in significantly ($P < 0.05$) more stem necrosis than uninoculated control plants. These comprised all three of the AG 2-2 isolates tested, two of the three AG 3 and one of the two AG 4 isolates tested. As isolates from AG 5 were not available in Australia one isolate from Great Britain was tested and, in this experiment, it caused significantly ($P < 0.05$) more stem necrosis than the uninoculated controls. Variation in stem disease severity was observed among isolates belonging to the same AG. For example, of the three AG 2-2 isolates tested, two caused girdling of sprouts and the other caused only superficial necrosis. Similarly, two of the AG 3 isolates tested caused pruning of stems but the third did not cause any necrosis.

Table 4.5 Assessment of necrosis, using two methods, caused by Australian and British *R. solani* AG 2-1 isolates on stems of potato plants grown in a controlled environment chamber at 16ºC at SCRI.

Isolate			Stem severity rank[1] and median[2]		Striped lesions
Origin	IGS1 type	Code	(Balali *et al.* 1995)	(Carling and Leiner 1990)	
Control	nil	Control	(22) a 1	(22) a 0	n/p
UK	Long	R107	(22) a 1	(22) a 0	n/p
UK	Short	R100	(43.31) b 2	(44.25) b 2	yes
UK	Long	R42	(28.38) ab 1	(28.12) ab 0	yes
Australia	Long	Tas6	(35.56) b 1.5	(34.88) b 0.5	yes
Australia	Inter.	L57	(22) a 1	(22) a 0	n/p
Australia	Long	1030	(22) a 1	(22) a 0	n/p

[1] Rank as determined by analysis Kruskal-Wallis analysis is indicated in parenthesis and was used to determine significant differences between treatments, with eight replicates (degrees of freedom = 32). Differences are indicated by an l.s.d 5 % = 46.17 and different letters within a column.

[2] The median severity score for each treatment is the unbracketed value and disease assessment scales of Balali et al. (1995) and Carling and Leiner (1990) are described in sections 2.4.4 and 4.3.2.2 respectivley. Note that statistical groups were the same for both disease severity assessments

Table 4.6 Assessment of the disease, using two methods, caused by Australian and British *R. solani* isolates on stems of potato plants grown in controlled environment chamber at 25ºC for 21 days

a. AG 2-1 isolates

Isolate				Stem severity rank[1] and median[2]		Striped lesions
Origin	IGS1 type	Code	AG	(Balali *et al.* 1995)	(Carling and Leiner 1990)	
Control	nil	Control	nil	(64.5) a 1	(64) a 0	n/p
UK	Long	R107	2-1	(89.5) ab 1	(89) ab 0	yes
UK	Short	R100	2-1	(102.0) abc 1	(101.5) abc 0	yes
UK	Long	R42	2-1	(75.44) ab 1	(74.94) a 0	yes
Australia	Long	Tas6	2-1	(84.75) ab 1	(78.58) a 0	no
Australia	Inter.	L57	2-1	(97.31) abc 1	(96.81) abc 0	yes
Australia	Long	1030	2-1	(64.5) a 1	(64) a 0	n/p
UK	Long	R103	2-1	(77) ab 1	(76.5) a 0	yes
UK	Long	R118	2-1	(108.25) abc 1.5	(107.75) abc 0.5	yes
UK	Long	R114	2-1	(89.5) ab 1	(89) ab 0	yes
UK	Long	R106	2-1	(114.5) bcd 2	(131.36) bcd 1	yes & no
Australia	Inter.	SE50	2-1	(64.5) a 1	(64) a 0	n/p
Australia	Inter.	SE45	2-1	(102) abc 1	(101.5) abc 0	yes
Australia	n/a	KI65	2-1	(77) ab 1	(76.5) a 0	no
Australia	n/a	KI4	2-1	(208.14) g 4	(215.93) g 4	no
Australia	Long	R14	2-1	(86.38) ab 1	(85.88) ab 0	no
Australia	Long	KI61	2-1	(97.31) abc 1	(96.81) abc 0	yes
Australia	n/a	730	2-1	(155.25) def 3	(156.25) def 2	yes
Australia	Inter.	SAR11.18	2-1	(207.71) g 4	(208.07) g 3	yes
Australia	Long	Tas3	2-1	(86.38) ab 1	(85.88) ab 0	yes
Australia	n/a	WAC-9806	2-1	(171.12) efg 3.5	(179.12) efg 3	no

[1] Rank as determined by analysis Kruskal-Wallis analysis is indicated in parenthesis and was used to determine significant differences between treatments, with eight replicates (degrees of freedom = 32) Differences are indicated by an l.s.d 5 % = 46.17 and different letters within a column

[2] The median severity score for each treatment is the unbracketed value and disease assessment scales of Balali et al. (1995) and Carling and Leiner (1990) are described in sections 2.4.4 and 4.3.2.2 respectivley.

b. Isolates from AGs other than AG 2-1, compared statistically to results from inoculation with AG 2-1 isolates shown in the previous table.

Isolate		Stem severity rank[1] and median[2] (Balali *et al.* 1995)	Stem severity rank[1] and median[2] (Carling and Leiner 1990)
Code	AG		
Control	nil	(64.5) a 1	(64) a 0
R72	2-2	(198.86) fg 4	(195.86) fg 2
SE42	2-2	(190.17) fg 3.5	(201.17) fg 3
T30.1	2-2	(141.3) cde 2	(140.8) cde 1
R20	3	(195.21) fg 4	(183.43) efg 3
R37	3	(89.5) ab 1	(89) ab 0
R229	3	(190.17) fg 1	(181.33) efg 2
R112	4	(97.31) abc 1	(96.81) abc 0
734	4	(217) g 4	(217.5) g 3.5
R110	5	(182.44) efg 3	(181) efg 2
R56	8	(94.36) ab 1	(93.86) ab 0
WAC-9923	8	(94.88) ab 1	(90.12) ab 0
RS21	8	(65.5) a 1	(64) a 0

Root damage was observed only on plants inoculation with isolate WAC-9923 (AG 8) (data not shown). Small necrotic lesions (< 5 mm long) were observed along the length of up to 50% of roots. No sclerotia were observed on roots of plants inoculated with any isolate, from any AG.

Reisolation of *Rhizoctonia* was not possible due to time constraints. Although AG-specific PCR tests were not available at the time, tissue sections from each inoculation treatment were freeze-dried and stored at -20°C for future DNA extraction and PCR analysis.

4.4 Discussion

A series of experiments was undertaken to compare the pathogenicity of isolates of *R. solani* that represent the AGs found in fields that supply the processing potato industry in Australia (refer to Chapter 3) and AGs that have been found on potato plants world-wide (refer to Section 1.4.3). These experiments confirmed other studies that showed that multiple AGs can cause disease on potato plants (Balali *et al.* 1995; Campion *et al.* 2003; Carling and Leiner 1986; Woodhall *et al.* 2008; Yanar *et al.* 2005b).

Experiments conducted in a shade-house in Adelaide showed that of the eight isolates tested, representing four AGs, only AG 3 isolates were associated with the development of sclerotia on tubers. This supports findings world-wide that AG 3 is the major cause of the black scurf symptom (Bains and Bisht 1995; Balali *et al.* 1995; Bandy *et al.* 1988; Campion *et al.* 2003; Carling *et al.* 1986; Virgen-Calleros *et al.* 2000; Woodhall 2004). However, both AG 2-1 and AG 2-2 were isolated from sclerotia on tubers from the field, indicating that these AGs also have the potential to cause black scurf. For AG 2-1 isolates this result is consistent with published findings (Campion *et al.* 2003; Carling and Leiner 1986; Woodhall *et al.* 2007; Yanar *et al.* 2005). AG 2-2 has also been isolated from sclerotia from progeny tubers from field sites in Victoria, Australia at a frequency of 2.4 % from 208 samples collected, however, the pathogenicity of these isolates was not tested (de Boer *et al.* 2001).

In these experiments four AGs were associated with root damage, which included formation of sclerotia and necrotic lesions. AG 2-1, AG 2-2 and AG 3 were isolated from roots collected from the field although symptoms were infrequently observed. However, in the experimental conditions used during this project, necrosis (associated with AG 8 isolates) and sclerotia (of AG 2-2 and AG 3) were frequently observed on root tissues. Necrotic lesions on potato roots have previously been attributed to AG 8 (Balali *et al.* 1995; Woodhall *et al.* 2007; 2008) and AG 3 (Balali *et al.* 1995; Lehtonen *et al.* 2008a; Woodhall *et al.* 2008) but no association has been reported for AG 2 with potato roots. These manifestations of disease may be associated with root exudates from the plant (Dijst 1990) but this suggestion requires further investigation to assess the effect of AG 2 on yields of potatoes.

Isolates representing AG 2-1, AG 2-2 and AG 3 all cause stem and stolon necrosis, supporting the results obtained from field sampling that demonstrated an association between these AGs and tissues infected (see Chapter 3). These findings support results from previous studies in which AG 2-1 isolates were found at low frequencies in potato fields and to cause

stem and stolon necrosis in Britain (Woodhall *et al.* 2007), France (Campion *et al.* 2003), Turkey (Yanar *et al.* 2005), Alaska (Carling *et al.* 1986) and Australia (de Boer *et al.* 2001). In a previous study of necrosis on potato stems and stolons, one AG 2-2 isolate was identified among the 46 *R. solani* isolates collected from Victoria, Australia (de Boer *et al.* 2001). Isolates belonging to AG 3 were the most often isolated from fields that supply the Australian fresh washed industry, and cause stem and stolon necrosis in Australia (Balali *et al.* 1995; de Boer *et al.* 2001) and in Alaska (Carling and Leiner 1986), Canada (Bains and Bisht 1995) Great Britain (Woodhall *et al.* 2007), Finland (Lehtonen *et al.* 2008a) and Maine, USA (Bandy *et al.* 1988).

A limited number of isolates belonging to AGs that were not found on potato plants from the field sites sampled during this project were also assessed for pathogenicity to potato plants. These included isolates belonging to AG 4, AG 5 and AG 8. Three AG 8 isolates were tested, as this group has previously been associated with potato plants in other regions of Australia and shown to cause disease (Balali *et al.* 1995). One AG 8 isolate (WAC-9923), originally from wheat, caused root necrosis on potato plants grown in a controlled environment. *R. solani* AG 8 is associated with bare patch in cereals in Australia (MacNish *et al.* 1994) and preliminary evidence suggests that it may contribute to a new disease of onions ("Mallee Onion Stunt") described in South Australia (Pederick *et al.* 2007). In both crop types, AG 8 damages the roots of the plants, reducing productivity and ultimately yield. Hence, if potatoes are grown in rotation with cereals or onions in these regions the populations of AG 8 may also result in root damage of the potato plants. However, as limited numbers of AG 8 isolates were tested, further investigation is required to determine if damage caused by *R. solani* AG 8 affects yield of potatoes. Also, as this experiment involved only the cultivar Russet Burbank, other cultivars available in the potato industry, need to be tested to determine their susceptibility to root damage caused by AG 8.

Two AG 4 isolates originally from cauliflower were tested for pathogenicity to potatoes in short-term controlled environment experiments. In these experiments, the isolates caused stem necrosis, which is consistent with other reports in which AG 4 isolates were associated with potato disease (Bains and Bisht 1995; Balali *et al.* 1995; Virgen-Calleros *et al.* 2000). In Australia AG 4 has recently been associated with disease in cauliflower (Hitch *et al.* 2007) and green bean (Hoong *et al.* 2007), crops that are grown in rotation with potatoes in the Adelaide Hills and Tasmania, respectively. This group of *R. solani* may, therefore, become an emerging problem in potato crops grown in these regions.

One AG 5 isolate from Great Britain that was pathogenic to potato (cv. Désirée) (Woodhall *et al*. 2007) was tested for pathogenicity to the cultivar Russet Burbank in Scotland. This isolate was associated with stem necrosis (Figure 4.1.b). These results support previous research in which AG 5 was found at low frequencies on potato plants (Bains and Bisht 1995; Balali *et al.* 1995; Bandy *et al.* 1988; Campion *et al.* 2003; de Boer *et al.* 2001; Woodhall *et al.* 2007) and to cause necrosis on potato stems (Bains and Bisht 1995; Balali *et al.* 1995; de Boer *et al.* 2001; Woodhall *et al.* 2007).

Binucleate *Rhizoctonia* species were also isolated from field sites during this project and one isolate was, therefore, tested for pathogenicity. This isolate did not cause necrosis on stems or roots under controlled environment conditions. However, the association of binucleate *Rhizoctonia* species with field plants is of interest as co-inoculation studies using binucleate *Rhizoctonia* species with pathogenic *R. solani* resulted in reduced disease severity on bean (Jabaji-Hare *et al.* 1999) and cotton (Jabaji-Hare and Neate 2005). However, applying microorganisms, such as fungi, to soil as biological control agents is often ineffective because they fail to compete with indigenous microorganisms; this includes binucleate *Rhizoctonia* species (Barnett *et al.* 2006). Instead of applying biological control microorganisms in an innundative manner as biological control agents, their natural populations in soils may be monitored and used as an indicator of disease potential (reviewed by Weller *et al.* 2002). The isolates collected from the field in this study could be investigated for potential in biological control of Rhizoctonia disease as organisms introduced to the Australian soil environment.

The yield of tubers in the only experiment in which plants were grown to maturity was too small to reflect a field situation, however, yields were consistent within the experiment and, therefore, can be associated with disease severity. Plants grown in the presence of isolates WAC-9937 (AG 2-2), L62 and T22 (AG 3) had decreased yields when compared with uninoculated plants. This was expected, as these plants also had increased severity of stem, stolon and root necrosis. This yield reduction was consistent with findings from a study in Great Britain in which infection by AG 3 was associated with decreased yield (Woodhall *et al.* 2008), but inconsistent with results from a study in Australia in which no yield reduction was observed when plants were inoculated with any of the four AG 3 isolates tested (de Boer *et al.* 2001). In this latter study, the pathogenicity of AG 2-2 was not tested and the influence of this group on yield not established. As few isolates from each group were tested during the course of this project and conflicting results have been published in the past, further testing is

required, with more isolates representing each AG, to assess the influence of Rhizoctonia disease on tuber yield.

Stem necrosis without any other disease symptom was associated with a decrease in yield, as shown for plants inoculated with isolate WAC-9806 (AG 2-1). However, yields were reduced only when stem necrosis was severe, for example when stems were girdled. Plants inoculated with isolate L57 also had more severe stem necrosis than uninoculated plants, but without girdling yields did not differ. Woodhall *et al.* (2008) found decreased tuber yields during experiments conducted in Great Britain, where plant stems and stolons were girdled and roots damaged when plants were inoculated with AG 3. In the same experiment, AG 5-inoculated plants with stolon girdling and only 90 % of stem area covered with necrosis (without girdling) also yielded less (Woodhall *et al.* 2008). De Boer *et al.* (2001) found that plants inoculated with AG 2-1 with severe necrosis on stolons and stems may produce lower yields than uninoculated plants, but this was not always the case. Although results from the experiment performed during this project suggested severe stem necrosis alone may decrease yield, the studies mentioned above suggest that damage of stolons and roots may also contribute to yield losses. In the development of disease risk thresholds, it will be important to incorporate the influence of each symptom to predict the impact of infection by *R. solani* on tuber yields. This may require the development of models or bioassays to assess the impact of the severity of each symptom on yield.

Necrosis was not the only symptom to be related to reduced yield, as shown by plants inoculated with isolate R394 which yielded lower tuber weights and an increase in only root sclerotia compared with the uninoculated control. This result suggested that this facet of the disease can reduce yield. The presence of sclerotia on potato roots has been documented (Balali *et al.* 1995; de Boer *et al.* 2001), however, only root necrosis of plants inoculated with *R. solani* AG 3 or AG 8 have previously been associated with lower tuber yield (Woodhall *et al.* 2008). As well as investigating the impact of necrosis on yield it would also be useful to investigate the effect of formation of sclerotia on roots early in the growth of potato plants.

Results from QPCR tests of plant tissues were generally consistent with the AG of the inoculum applied and reisolated from the tissue, suggesting that this technology is a useful research tool to complement isolation of *R. solani* from tissues. However, if the amount of DNA were to be associated with type and severity of symptoms, thresholds values, below which symptoms do not occur, first need to be established. In some cases, values from inoculated, necrotic stem tissue were lower than those from uninoculated control plants

without necrosis (for example see AG 2-2 isolate 894 in Table 4.4), suggesting that a reliable tissue sampling procedure is first needed in the development of a threshold. Some unexpected results were observed following QPCR, for example, a stem infected with AG 2-1 isolate WAC-9806 showed 202 fg DNA/PCR of AG 3 DNA and, although the amount of AG 2-1 DNA was far greater, this result suggested contamination. Reisolation of *R. solani* was not achieved, so contamination cannot be confirmed. In some cases where no disease was observed, QPCR results indicated that no DNA was present, suggesting that inoculation was not successful. Therefore, QPCR could be a useful tool in monitoring colonisation of stems during experiments, such as testing of treatments applied to prevent infection and in breeding programs to develop potato cultivars tolerant of *R. solani*.

Although these experiments have shown that all AGs can cause a range of Rhizoctonia disease symptoms, variation was also observed in severity between isolates from the same AG. Genetic variation among AG 2-1 isolates (see chapter 3) has already been examined in that Woodhall *et al.* (2007) noted a difference in length of the IGS1 region to be associated with isolate origin and pathogenicity in this group in Great Britain. Consistent with results from pathogenicity tests of AG 2-1 isolates from Australia (Balali *et al.* 1995; de Boer *et al.* 2001) and overseas (Campion *et al.* 2003; Carling *et al.* 2002b), variation was observed, particularly in the severity of stem necrosis in short-term controlled environment experiments. However, no link between pathogenicity and region or host of origin was observed, as the five (of 13) AG 2-1 isolates that produced significantly ($P < 0.05$) more severe disease than the controls were from Britain and the rest were from Australia. Disease severity, likewise, was not linked to any one of the IGS1 types as, although one isolate (SAR11.18) of intermediate IGS1 type produced the most severe stem necrosis, five others belonging to the same group did not. Intermediate disease severity was produced by only two of the 11 long IGS1 type isolates tested and the only one isolate of short IGS1 type available (R100) produced no symptoms at 25°C, therefore, it was not possible to attempt to associate this IGS1 type with a pathotype. These results suggested that IGS1 type is not an indicator of the potential to cause severe stem necrosis at 25 °C and would not be an informative tool to complement the AG-specific tests already available for field soil analysis.

The three fastest growing isolates (SE42, R72 both AG 2-2 and 734, AG 4), tested for pathogenicity at SCRI, caused severe disease and stem pruning. However, growth rate was not the only indicator of pathogenicity, as KI4 and WAC-9806 (AG 2-1) had significantly lower growth rates and still pruned stems while R14, KI61, R114 and L57 with

significantly higher growth rates caused no necrosis. This is consistent with findings from Lehtonen *et al.* (2008a), who reported that pathogenicity of *R. solani* AG 3 isolates was not linked to their growth rate.

In conclusion, the results from these experiments showed that several AGs can cause a range of disease symptoms on potato, from necrosis of stems, root and stolons to sclerotia on roots and tubers. AG 3 isolates were associated with the most abundant sclerotia, however, all AGs tested caused necrosis on at least one of the following tissues: stems, stolons or roots. Variation was observed in the severity of symptoms caused by isolates from the same group, however, this could not be linked to a genetic marker in the IGS1 region. These findings have implications for the potato industry as they show that control strategies for Rhizoctonia disease must encompass the multiple plant tissues that can be damaged. Management techniques must also address: the range of pathogenic AGs already present in the regions that supply the processing industry, those that are not, but have the potential to cause disease, and the variation within these AGs. In particular the ability of fungicides, the most common Rhizoctonia disease management strategy used, to control the growth of and disease caused by potato pathogenic AGs will be addressed in the following chapters.

5 Inhibition of Mycelial Growth by Fungicides *In Vitro*

5.1 Introduction

Fungicides are commonly applied to soil and tubers to control Rhizoctonia disease in Australian processing potato crops; some of these are described in section 1.5.1. Application to the seed piece prior to planting has been widely used to control seed-borne inoculum in Australia (Dillard *et al.* 1993). Soil-borne inoculm contributing to Rhizoctonia disease (Carling and Leiner 1986; Carling and Leiner 1990) may be controlled by application of fungicide to the soil at planting (Brewer and Larkin 2005; Hide and Read 1991; Tsror and Peretz-Alon 2005). Both of these inoculum sources can contribute to Rhizoctonia disease on potatoes and their relative importance was discussed in section 1.5. Although the impact of sclerotia as seed inoculum was recognised and minimised (Hide and Cayley 1982; Wicks *et al.* 1995) until the recent development of AG-specific DNA tests growers had no method to measure inoculum levels in field soils. Due to this difficulty in identifying the disease potential of a field, growers who supply the processing potato industry often use fungicides in a preventative manner (Dillard *et al.* 1993). These regular applications may result in costly over-use of fungicides, for example when inoculum of one AG is less sensitive to the fungicide applied than another. However, when necrosis or black scurf symptoms appear, fungicide applications may be too late to minimise yield loss as no systemic fungicides are currently registered in Australia.

A range of *R. solani* AGs has been found in Australian potato fields (refer to section 1.4.3). These groups have been linked to different disease symptoms and severity (Balali *et al.* 1995; Campion *et al.* 2003; Woodhall *et al.* 2008). AG-specific soil DNA assays for detection of *R. solani* allow the possibility to indicate disease potential of field soils. However, once "disease potential" of a field has been determined growers still require an effective disease management options.

Previous research has indicated that isolates belonging to the different AGs differ in fungicide sensitivity (Campion *et al.* 2003; Carling *et al.* 1990; Kataria *et al.* 1991b; Olaya *et al.* 1994). Therefore AG-targeted fungicide application strategies, dictated by the results of soil tests, may allow more effective disease management. However, the sensitivity of the different groups of *R. solani* from Australian processing potato fields to the fungicides used in Australia (refer to section 1.5.1) is unknown.

One method useful in assessing the inhibition of mycelial growth of *R. solani* by chemical fungicides is *in vitro* inhibition assays (Georgopoulos 1982). These methods are useful in monitoring populations and identifying isolates resistant or insensitive to a fungicide. For example, *in vitro* methods were used to detect azoxystrobin-resistant *Alternaria* species from pistachio fields that had no history of application of this fungicide in California (Ma *et al.* 2003).

However, *in vitro* assessment can be problematic as some fungi have alternative pathways for metabolising fungicides. For example, many plant pathogenic fungi can metabolise azoxystrobin (the active ingredient of Amistar) under *in vitro* culture conditions (reviewed by Joseph-Horne and Hollomon 2000). This alternative pathway can be inhibited by incorporating salicylhydroxamic acid (SHAM) (Ziogas *et al.* 1997), however, the effectiveness of the additive on various AGs of *R. solani* is not known.

In vitro assays to assess field soils prior to planting are time consuming and labour intensive. A faster method that could be used to test field soils for the presence of fungicide insensitive isolates might be to use molecular tools specifically designed for these isolates. Molecular technology with the capability of high through-put screening of field soils (previously discussed in section 1.4.4) may potentially be adapted to identify fungicide resistant *R. solani* populations. If a fungicide resistant or insensitive *R. solani* population is recognised in a field prior to planting, a grower could modify their management program by using a fungicide containing a different active ingredient.

Molecular tests require the identification of specific gene regions that distinguish isolates insensitive to particular fungicides. For some fungicides, single sites involved in resistance have been identified (Gisi *et al.* 2002; Knight *et al.* 1997; Ochiai *et al.* 2001; Sierotzki *et al.* 2000), allowing for the development of molecular tools. However, for some commonly used fungicides, such as those containing iprodione, no single gene regions or mutations have been identified that differentiate the variation in sensitivity of *R. solani* isolates (Ma and Michailides 2004). Variation among isolates from AG 2-1 in terms of sensitivity to iprodione fungicides, such as Rovral (Campion *et al.* 2003), has been observed. AG 2-1 has also been associated with Rhizoctonia disease symptoms on potato plants at a low frequency world-wide (Campion *et al.* 2003; Carling and Leiner 1986; Woodhall 2004; Yanar *et al.* 2005). In Australia this group has been isolated from diseased tissue sections more frequently (see section 3.3.3) than in other countries. This variation in iprodione sensitivity within the group cannot be differentiated with the current AG-specific molecular tools for

quantifying AG 2-1 from field soil samples.

Genetic variation has been observed within "housekeeping gene" regions of *R. solani* AG 2-1 isolates (see section 1.4.5). With an IGS1 PCR primer set Woodhall *et al.* (2007) observed variation in amplicon size when British AG 2-1 isolates were used as templates. In the nine British isolates tested, this size variation was associated with position in an ITS phylogeny and pathogenicity on potato plants. However, the association of IGS1 type and fungicide insensitivity was not investigated.

The aims of the experiments reported in this chapter were to determine:

1. The sensitivity of AGs found in Australian potato fields to currently used and experimental fungicides;
2. The effect of SHAM concentration on the inhibitory properties of Amistar *in vitro*;
3. The association of variation in the IGS1 gene region and fungicide sensitivity of AG 2-1 isolates.

5.2 Materials and methods

5.2.2 Effect of fungicide on mycelial growth rate of isolates from various AGs

The following fungicides were used in this experiment: Amistar, Maxim, Monceren, Moncut, Rizolex, Rovral and one experimental fungicide (see Table 2.4). Stock solutions of 10,000 ppm were prepared from commercial formulations to give final concentrations of 0.001, 0.01, 0.1, 1, 5 and 10 ppm a.i. when incorporated into PDA. These concentrations were selected based on those used in previous studies of *R. solani* overseas (Campion *et al.* 2003). Control plates were amended with 180 µl of water but no fungicide. All plates with Amistar treatments also had SHAM (Sigma-Aldrich Pty Ltd, Sydney, Australia) incorporated to a final concentration of 1 ppm a.i.

Details of the eleven isolates used in this experiment, L57, WAC-9806, R394 (AG 2-1); KI3, WAC-9765, WAC-9937 (AG 2-2); L306, L62, R229 (AG 3); WAC-9923, RS21 (AG 8), are given in Table 2.1.

Isolates were grown on PDA for 3 days prior to inoculation of plates. A plug of agar 5 mm diameter was taken from the colony margin and placed, hyphae side down, in the centre of each amended and non-amended plate.

The experimental design was a split-split-plot with randomised blocks. The five

replicates over time represented the blocks. A tray, which contained one isolate, represented a whole plot and a stack, which was designated one concentration, represented a subplot. Agar plates of the seven different fungicides (Table 2.4), plus a no-fungicide control, represented the subsubplots. This extra replication of control plates allowed six observations per isolate to minimise any variation within a stack.

Plates were incubated at 25°C and the colony diameter, subtracting the 5 mm plug, of hyphal growth was measured 24, 48 and 72 hrs after inoculation. The analysis of results was performed in four stages, by Kathy Haskard from BiometricsSA:

Stage 1: Estimate growth rate (mm/hr) for each plate.

Stage 2: ANOVA on growth rates to determine effects of isolate, fungicide, concentration and interactions.

Stage 3: Estimate an EC_{50} for each isolate and fungicide combination.

Stage 4: ANOVA on EC_{50} to determine effects of isolate, fungicide, concentration and interactions.

5.2.3 Effectiveness of Amistar in vitro

PDA plates were prepared as described in the section above, with Amisar incorporated to a final concentration of 1 ppm a.i. and SHAM concentrations varied from 0.5 to 2 ppm a.i. Two isolates of *R. solani* (WAC-9806, AG 2-1 and L306, AG 3) were used as inoculum, as described in the section above. Plates were arranged in a completely randomised design, with five replicates per treatment. All plates were incubated at 25°C for 72 hrs and colony diameter was measured every 12 hrs as described in the section above. The growth rate (mm/hr) was determined for each plate as follows:

$$\text{Growth Rate} = \frac{(\text{diameter at time 2} - \text{diameter at time 1}) - \text{plug size}}{12 \text{ hrs}}$$

Analysis of variance (ANOVA) was performed using Genstat version 7 to produce and compare mean growth rates for each treatment.

5.2.4 Comparison of IGS1 length and Rovral sensitivity of Australian and British isolates

PDA plates were prepared as described in section 5.2.2 and amended with Rovral to

provide final concentrations of 0, 5 and 10 ppm a.i. An 8 mm diameter plug of mycelium was taken from the edge of actively growing cultures of *R. solani* on PDA and used as inoculum as described in section 5.2.2. Three replicates were prepared for each isolate for the two fungicide concentrations and the control. The plates were incubated at 25°C in darkness in a completely randomised design. Colony diameter was measured every 12 hrs. Growth rate was determined for each plate as described in section 5.2.3 and ANOVA was performed with Genstat version 10 to produce mean values for each isolate grown at each concentration.

5.3 Results

5.3.2 Mycelial growth rate of isolates representing various AGs

5.3.2.1 Effect of fungicide concentration on mycelial growth

The percent of growth inhibition by all fungicides tested is shown for AG 2-1 isolates in Figure 5.1, for AG 2-2 isolates in Figure 5.2, for AG 3 isolates in Figure 5.3 and for AG 8 isolates in Figure 5.4. Percent inhibition, for each fungicide concentration, was determined by comparison with the mean of the nil fungicide controls. The least significant difference at 5% (l.s.d. 5%) for each isolate was an approximation, as it was based on the mean growth rate of the nil fungicide controls. This mean value was calculated from six replicate plates per tray, each in a different vertical stack. The purpose of replicated control plates was to confirm that there were no significant differences in the growth of isolates in a vertical stack of plates (data not shown). Although the difference in a stack was not significant (data not shown) it should be noted that the variation in the value for the mean growth rate of the nil fungicide control was not incorporated into the determination of the l.s.d. 5%.

To visualise growth inhibition over a range of concentrations and EC_{50} values on a single graph, values of percent of growth inhibition were converted to probit. An example of this is shown in appendix 10.4, however, many values could not be converted as they were below 0 %, the limit for probit transformation. Values below zero occur when an isolate grows faster in the presence of a fungicide than without. This occured in two situations, when the mean values were not significantly different from the nil-fungicide treatment and when isolates were insensitive to a fungicide and growth was enhanced.

AG 2-1 isolates

Based on variable responses at the fungicide concentrations used, four main trends were observed in the inhibition of mycelial growth of AG 2-1 isolates. The first trend was observed when all three isolates were cultured in the presence of either Monceren or the experimental fungicide (Figure 5.1 a-c). This trend was manifested as increasing inhibition of mycelial growth, to over 60 % at concentrations of 0.1 ppm a.i. At concentrations of 1 ppm a.i. and higher inhibition then increased to over 80 %. A similar pattern was observed when isolates WAC-9806 and R394 were grown in the presence of Maxim (Figure 5.1b and c) and Rizolex inhibited isolate WAC-9806 (Figure 5.2b).

In contrast, the second trend observed was characterised by inhibition of mycelial growth to a lesser extent ($P < 0.05$) than that described for trend one, generally less than 40 %, at 0.1 ppm a.i. At concentrations of 1 ppm a.i. and greater, inhibition was then above 80 %. This trend was observed when one isolate (L57) was grown in the presence of Maxim (Figure 5.1a), all three isolates were cultured in the presence of Moncut (Figure 5.1a-c) and R394 was inhibited by Rizolex and Rovral (Figure 5.1c).

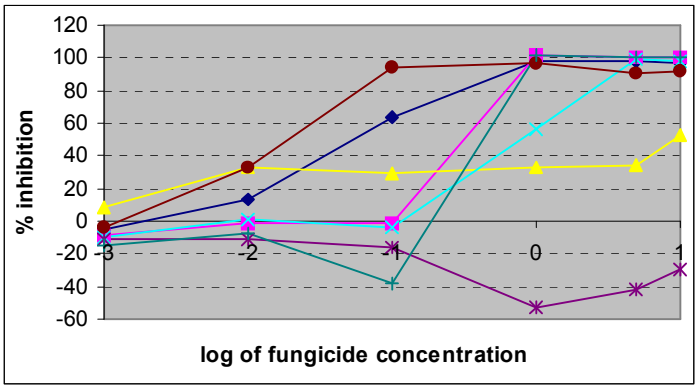
Isolate L57 was inhibited by Rizolex in a manner represented by a third trend. In this trend, inhibition at 0.1 ppm a.i. was significantly ($P < 0.05$) less than that produced by fungicides described in the first trend (below 40 %). At 1 ppm a.i., growth inhibition had increased to close to 60 % but was significantly ($P < 0.05$) less than observed for the first and second trends. At 10 ppm a.i., inhibition was above 80 % (Figure 5.1a). This third trend of inhibition was also observed when isolate R394 was grown in the presence of Rovral (Figure 5.1c), however, inhibition of isolate WAC-9806 grown in the presence of the same fungicide was consistent with the second trend (Figure 5.1b). Isolate L57 was not inhibited by Rovral, rather growth was enhanced (Figure 5.1a).

Only Amistar significantly ($P < 0.05$) inhibited mycelial growth of the three AG 2-1 isolates at 0.001 ppm a.i. (Figure 5.1a-c). This fungicide also produced a pattern of inhibition different from any trend previously described. This fourth trend was described by inhibition of mycelial growth by less than 40 % at 0.1 ppm a.i, as for trends two and three, however, inhibition by this fungicide remained below 50 % at 5 ppm a.i. and below 70 % at 10 ppm a.i., which was significantly ($P < 0.05$) lower than the mycelial inhibition found when AG 2-1 isolates were grown in the presence of any of the fungicides except for L57 and Rovral.

Figure 5.1 The effect of fungicide and concentration[1] on inhibition[2] of mycelial growth of *R. solani* AG 2-1 isolates *in vitro*

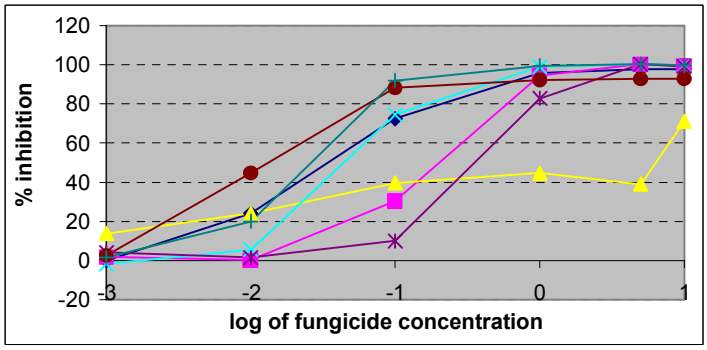
a. Isolate L57

Approximate l.s.d. 5% = 18.3 %



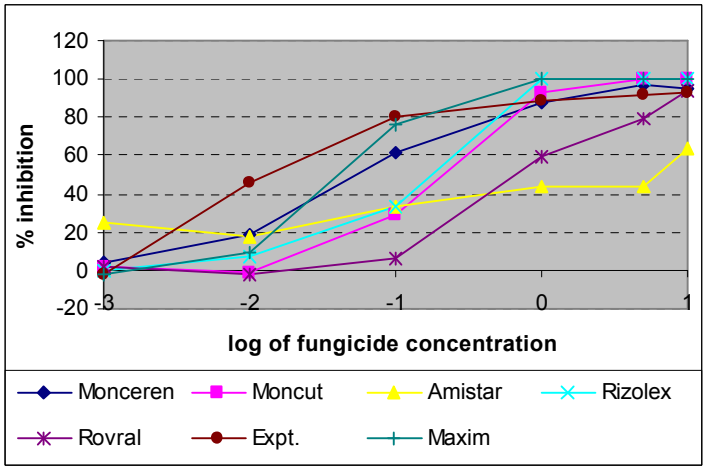
b. Isolate WAC-9806

Approximate l.s.d. 5% = 10.1 %



c. Isolate R394

Approximate l.s.d. 5% = 7.3 %



[1] Log of fungicide concentration values -3, -2, -1, 0, 0.7 and 1 are equivalent to concentrations of 0.001, 0.01, 0.1, 1, 5 and 10 ppm a.i. final concentrations of PDA supplemented with fungicides

[2] Percentage inhibition of isolates, grown on PDA supplemented with fungicides at 25°C in the dark over 72 hrs, was relative to mycelial growth of nil-fungicide controls of each isolate

AG 2-2 isolates

All three AG 2-2 isolates responded in a similar manner to each of the fungicides tested. Based on variation in mycelial growth inhibition at the fungicide concentrations tested, the observations for each fungicide can be described by one of four trends of mycelial growth inhibition. The first trend indicates the fungicides most effective at inhibiting mycelial growth both at the lowest concentration and to the highest percentage at the highest concentration. These fungicides were Monceren, Maxim and Rizolex, which inhibited growth by over 60 % at 0.1 ppm a.i. (Figure 5.2) and from 1 to 10 ppm a.i. growth inhibition was not significantly ($P < 0.05$) different from 100 %.

The second trend was observed in the inhibition of mycelial growth by Moncut and Rovral. At 0.1 ppm a.i., growth of all three AG 2-2 isolates was inhibited by less than 10 % but at 1 ppm, inhibition of the three isolates tested was over 75 %. This latter value was significantly ($P < 0.05$) lower than that produced by Monceren, Rizolex and Maxim at the same concentration. However, at 5 and 10 ppm a.i., inhibition by Moncut and Rovral was not significantly ($P < 0.05$) different from 100%.

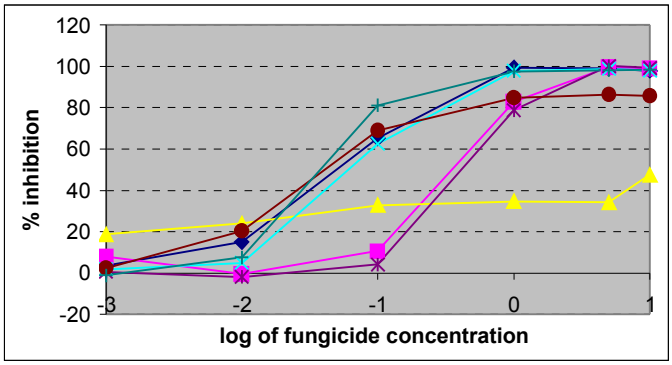
Only the experimental fungicide produced growth inhibition that can be described by a third trend. In the presence of this fungicide, the growth of all three isolates was inhibited by over 60 % at 0.1 ppm a.i. (Figure 5.2) and from 1 to 10 ppm a.i. inhibition was significantly less than 100 %, although above 80 %.

Amistar treatment resulted in a fourth trend. At 0.001 ppm a.i., growth was significantly ($P < 0.05$) inhibited, almost 20 %, whereas no other fungicide produced inhibition greater than the nil-fungicide control at this concentration (Figure 5.2a-c). The inhibition of mycelial growth in the presence of Amistar was significantly ($P < 0.05$) less than for all other fungicides at 0.1 ppm a.i. and remained below 60 % over all concentrations tested.

Figure 5.2 The effect of fungicide and concentration[1] on inhibition[2] of mycelial growth of *R. solani* AG 2-2 isolates *in vitro*

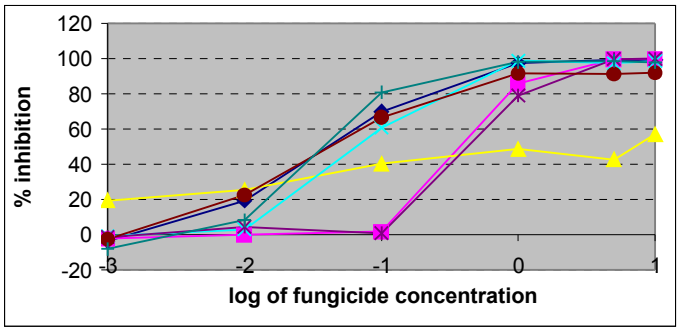
a. Isolate KI-3

Approximate l.s.d. 5% = 6.6 %



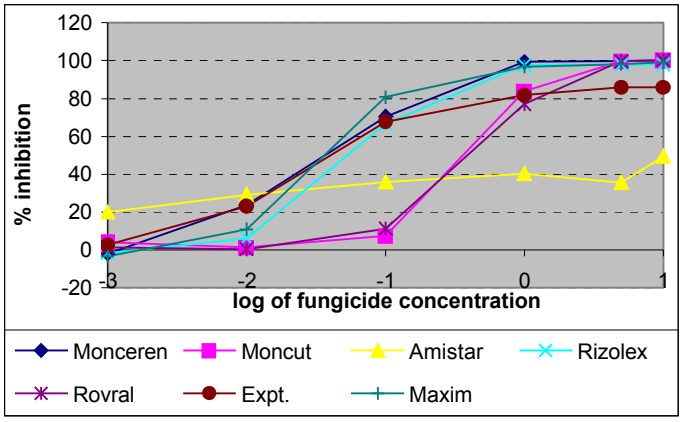
b. Isolate WAC-9765

Approximate l.s.d. 5% = 6.8 %



c. Isolate WAC-9937

Approximate l.s.d. 5% = 7.0 %



[1] Log of fungicide concentration values -3, -2, -1, 0, 0.7 and 1 are equivalent to concentrations of 0.001, 0.01, 0.1, 1, 5 and 10 ppm a.i. final concentrations of PDA supplemented with fungicides

[2] Percentage inhibition of isolates, grown on PDA supplemented with fungicides at 25°C in the dark over 72 hrs, was relative to mycelial growth of nil-fungicide controls of each isolate

AG 3 isolates

Based on the variation in mycelial growth at the different fungicide concentrations, three trends were observed in the response of AG 3 isolates to the fungicides tested (Figure 5.3). The first trend occurred for all three isolates when grown in the presence of Maxim and Rizolex and was characterised by inhibition of mycelial growth by over 60 % at 0.1 ppm a.i. At this concentration, Maxim was significantly ($P < 0.05$) better at inhibiting growth of all three isolates than Rizolex. This trend also consisted of growth inhibition that was not significantly ($P < 0.05$) different from 100 % at fungicide concentrations of 1 to 10 ppm a.i. Monceren also inhibited two isolates (L62; Figure 5.3b and R229; Figure 5.3c) in a manner described by the first trend, with no significant ($P < 0.05$) difference in inhibition from Maxim at 0.1 ppm a.i.

Inhibition of the other isolate (L306; Figure 5.3a) in the presence of Maxim was described by a second trend, as was inhibition of each of the three isolates grown in the presence of the experimental fungicide. This trend showed growth inhibition by over 55 % at 0.1 ppm a.i. When fungicide concentration was increased to 1 ppm a.i. and above, inhibition was greater than 75 % but significantly ($P < 0.05$) less than 100 %.

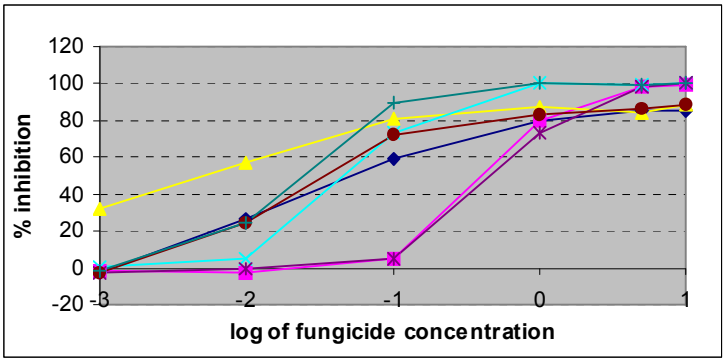
The third trend was observed in the response of mycelial growth of all three isolates in the presence of Moncut and Rovral. At 0.1 ppm a.i., the inhibition of growth was below 20 %. Yet when the fungicide concentration was increased to 1 ppm a.i., inhibition increased to greater than 70 % and at 5 and 10 ppm a.i. the inhibition was not significantly ($P < 0.05$) different from 100 %.

Amistar was the only fungicide to inhibit growth by over 20 % for each of the three isolates, at the lowest concentration tested (0.001 ppm a.i., $P < 0.05$). However, the influence of this fungicide on mycelial growth at higher concentrations was not consistent for all three isolates. At 0.1 to 10 ppm a.i., the growth of isolate L306 (Figure 5.3) was inhibited by over 80 %. At 0.1 ppm a.i., Amistar inhibited the mycelial growth of isolate L62 by 62 % (Figure 5.3b) and R229 by 48 % (Figure 5.3c). At higher concentrations, inhibition of both isolates remained significantly ($P < 0.05$) less than 100 %; inhibition of the latter isolate was significantly ($P < 0.05$) less than than that due to any other fungicide tested.

Figure 5.3 The effect of fungicide and concentration[1] on inhibition[2] of mycelial growth of *R. solani* AG 3 isolates *in vitro*

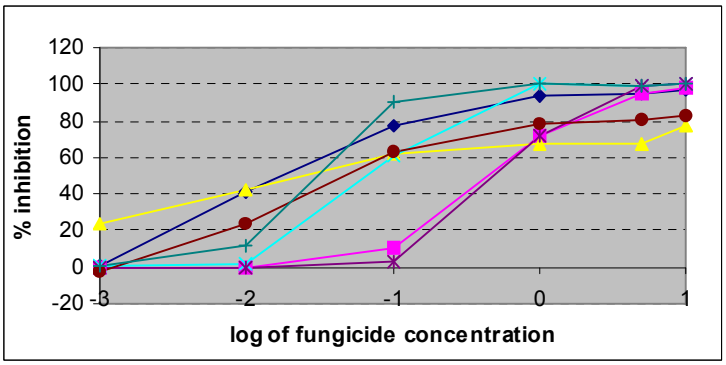
a. Isolate L306

Approximate l.s.d. 5% = 8.1 %



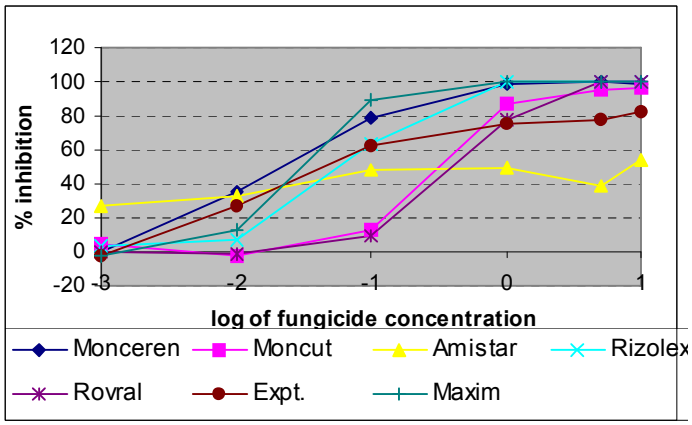
b. Isolate L62

Approximate l.s.d. 5% = 8.0 %



c. Isolate R229

Approximate l.s.d. 5% = 7.7 %



[1] Log of fungicide concentration values -3, -2, -1, 0, 0.7 and 1 are equivalent to concentrations of 0.001, 0.01, 0.1, 1, 5 and 10 ppm a.i. final concentrations of PDA supplemented with fungicides

[2] Percentage inhibition of isolates, grown on PDA supplemented with fungicides at 25°C in the dark over 72 hrs, was relative to mycelial growth of nil-fungicide controls of each isolate

AG 8 isolates

Four trends of growth inhibition were observed, based on variation in response to different fungicide concentrations when the two AG 8 isolates were tested (Figure 5.4).

The first trend was characterised by inhibition of mycelial growth that was significantly ($P < 0.05$) greater than the nil-fungicide control at 0.1 ppm a.i. This occurred when either isolate was grown in the presence of Rizolex, Maxim and the experimental fungicide. In the presence of these same fungicides, at 1 to 10 ppm a.i., mycelial growth of both isolates was above 75 %. The response of the two isolates to Rizolex and the experimental fungicide varied slightly as inhibition of isolate WAC-9923 (Figure 5.4b) was significantly ($P < 0.05$) less than 100 %, whereas that of RS21 (Figure 5.4a) was not.

A second trend was characterised by inhibition of mycelial growth that was not significantly ($P < 0.05$) different from the nil-fungicide control at 0.1 ppm a.i.. At 5 and 10 ppm a.i., the inhibition of mycelial growth produced in the presence of these fungicides was not significantly ($P < 0.05$) different from 100 %. This trend was observed when both isolates were grown in the presence of Rovral and Moncut.

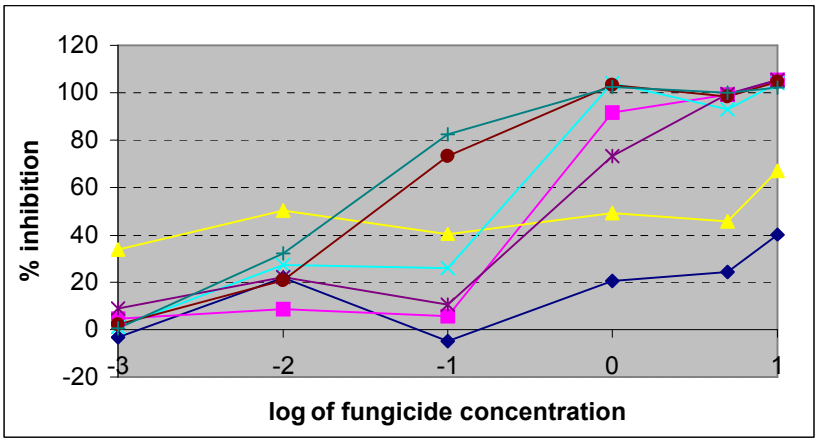
The third trend described mycelial growth inhibition of both isolates in the presence of Amistar. This was the only fungicide 0.001 ppm to inhibit growth by over 25 %. With this fungicide, inhibition of growth remained constant from 0.01 to 5 ppm a.i. for both isolates. Although inhibition reached over 50 % at 10 ppm a.i. for, this was significantly ($P < 0.05$) less than the inhibition produced by the five other fungicides previously mentioned.

The fourth trend described the mycelial growth inhibition of both isolates in the presence of Monceren. At 0.1 ppm a.i., neither isolate was inhibited ($P < 0.05$). At 1 ppm a.i., mycelia were inhibited by less than 28 % significantly ($P < 0.05$) less than that for all other fungicides (excluding Amistar). Growth inhibition of both isolates with Monceren at of 10 ppm a.i. remained below 41 %.

Figure 5.4 The effect of fungicide and concentration[1] on inhibition[2] of mycelial growth of *R. solani* AG 8 isolates *in vitro*

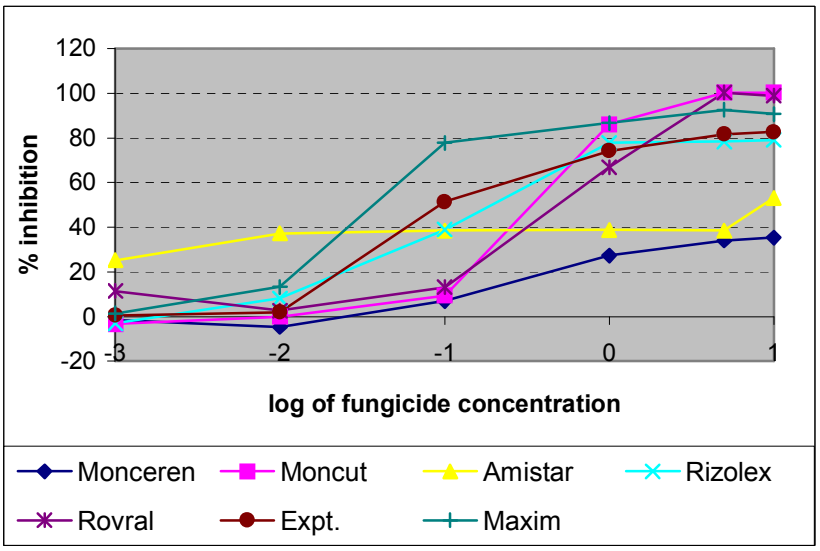
a. Isolate RS21

Approximate l.s.d. 5% = 11.6 %



b. Isolate WAC-9923

Approximate l.s.d. 5% = 14.4 %



[1] Log of fungicide concentration values -3, -2, -1, 0, 0.7 and 1 are equivalent to concentrations of 0.001, 0.01, 0.1, 1, 5 and 10 ppm a.i. final concentrations of PDA supplemented with fungicides

[2] Percentage inhibition of isolates, grown on PDA supplemented with fungicides at 25°C in the dark over 72 hrs, was relative to mycelial growth of nil-fungicide controls of each isolate

5.3.2.2 Determination of 50 % effective concentration (EC50)

At 10 ppm a.i., most fungicides inhibited growth by over 80 %, however, the lowest concentration at which growth was inhibited varied with isolate and fungicide. To compare fungicide activity, the EC_{50} was determined for each isolate x fungicide combination (Figure 5.5), except for Amistar treatments as 50% inhibition was often not reached and data could not be accurately extrapolated. The same situation occurred with AG 8 isolates and Monceren treatments, hence, these results were excluded from Figure 5.5d.

AG 2-1 isolates

EC_{50} values indicated that the experimental fungicide was most effective at inhibiting growth of the three AG 2-1 isolates tested (Figure 5.5a). Maxim was as effective at inhibiting the growth of WAC-9806, however significantly ($P < 0.05$) less effective for the other two AG 2-1 isolates tested. Monceren consistently inhibited the three isolates tested and was the second most effective fungicide, with significantly better inhibition than most isolate x treatment combinations. Moncut also inhibited the three isolates tested, although significantly ($P < 0.05$) less so than Monceren. Rizolex inhibited isolate WAC-9806 to a degree not significantly less than Monceren but inhibition of other isolates was variable.

Rovral inhibited the two of the AG 2-1 isolates tested (R394 and WAC-9806), although, the inhibition was significantly ($P < 0.05$) less than that achieved with other fungicide tested against these isolates. EC_{50} values are missing for the AG 2-1 isolate L57, with Rovral treatment, as 50 % inhibition was not achieved.

AG 2-2 isolates

The EC_{50} values in Figure 5.5b indicated that the three AG 2-2 isolates tested responded in a similar manner to the fungicides tested. The fungicides most effective at inhibiting the three isolates tested were Monceren, Rizolex, Maxim and the experimental fungicide. Moncut and Rovral inhibited the growth of all three AG 2-2 isolates to a lesser extent ($P < 0.05$) than the above-mentioned fungicides.

AG 3 isolates

As indicated by EC_{50} values in Figure 5.5c, Maxim was most effective at inhibiting the

growth of the three AG 3 isolates tested. However, Monceren was as effective at inhibiting the growth of two isolates (R229 and L62) but inhibition of L306 was significantly ($P < 0.05$) less. Inhibition of growth of all isolates by Rizolex and the experimental fungicide was significantly ($P < 0.05$) less than for Maxim. Rovral and Moncut were the least effective at inhibiting growth of the three AG 3 isolates tested ($P < 0.05$).

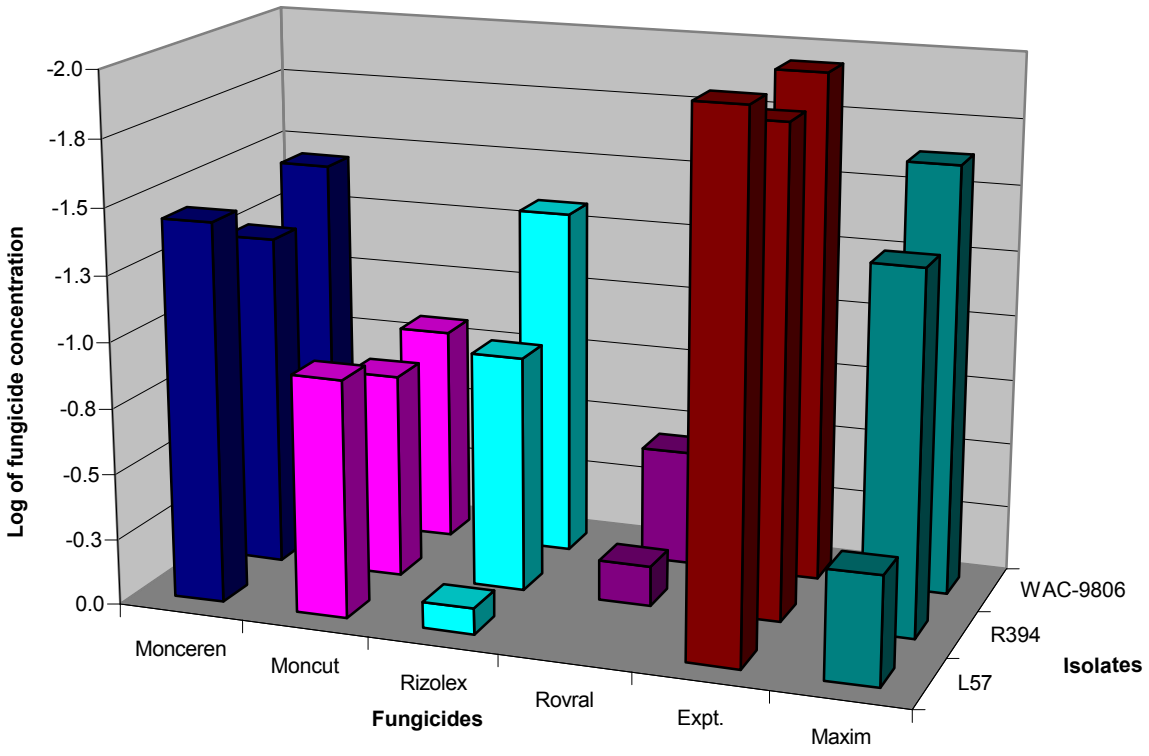
AG 8 isolates

EC_{50} values are not presented in Figure 5.5d for Monceren treatments as 50% inhibition was not reached and data could not be extrapolated with any confidence.

EC_{50} results indicated that Maxim was the most effective at inhibiting both the isolates tested (Figure 5.5d). Inhibition of growth of RS21 by the experimental fungicide was not significantly less than that found with Maxim, whereas isolate WAC-9923 was inhibited more by the experimental fungicide. Rizolex, Moncut and Rovral were significantly ($P < 0.05$) less effective at inhibiting growth of both isolates than the other fungicides presented in Figure 5.5d.

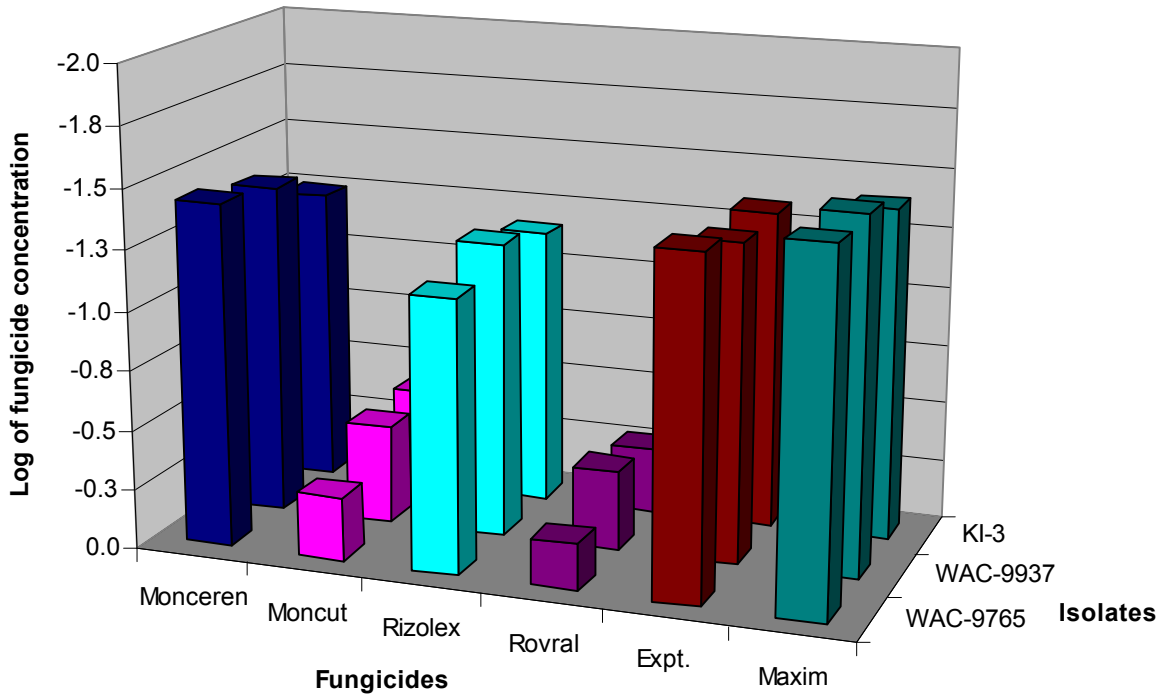
Figure 5.5 Fungicide EC_{50} values[1] for *R. solani* isolates representing potato pathogenic AGs (approximate l.s.d. 5% = 0.26)

a. AG 2-1 isolates

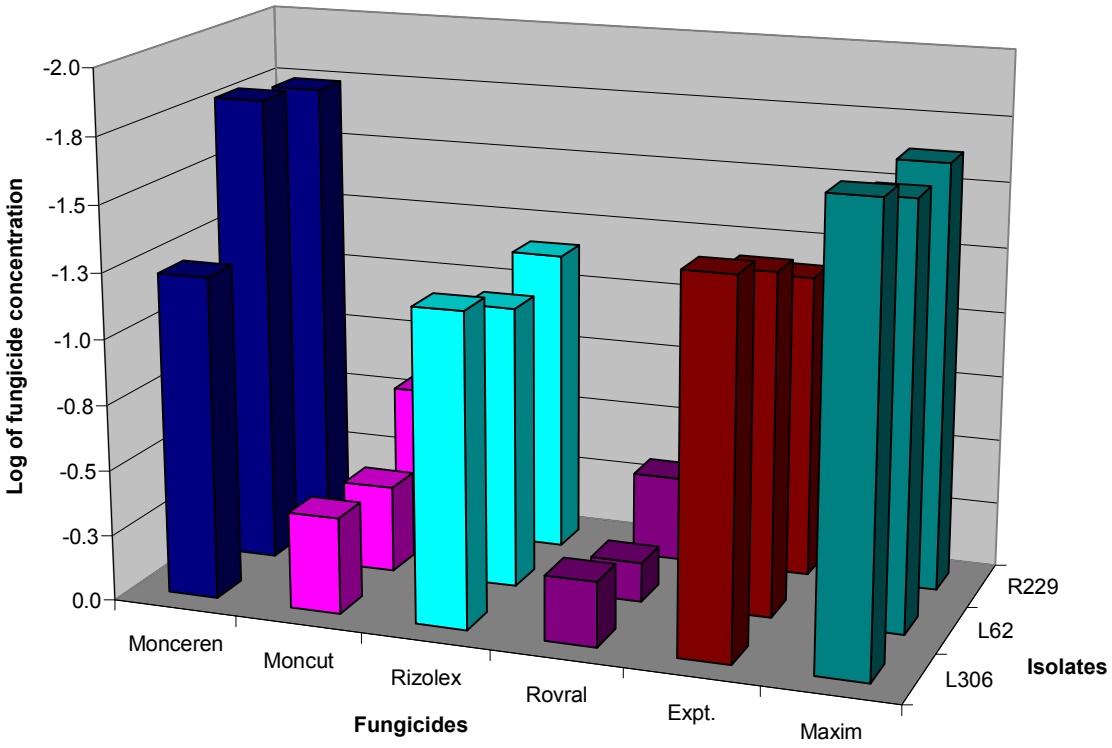


[1] Isolates were grown on PDA supplemented with fungicides at concentrations of 0.001 to 10 ppm a.i. at 25ºC, in the dark, for 72 hrs. The percentage of inhibition, from which EC50 was determined, was relative to mycelial growth of nil-fungicide controls of each isolate

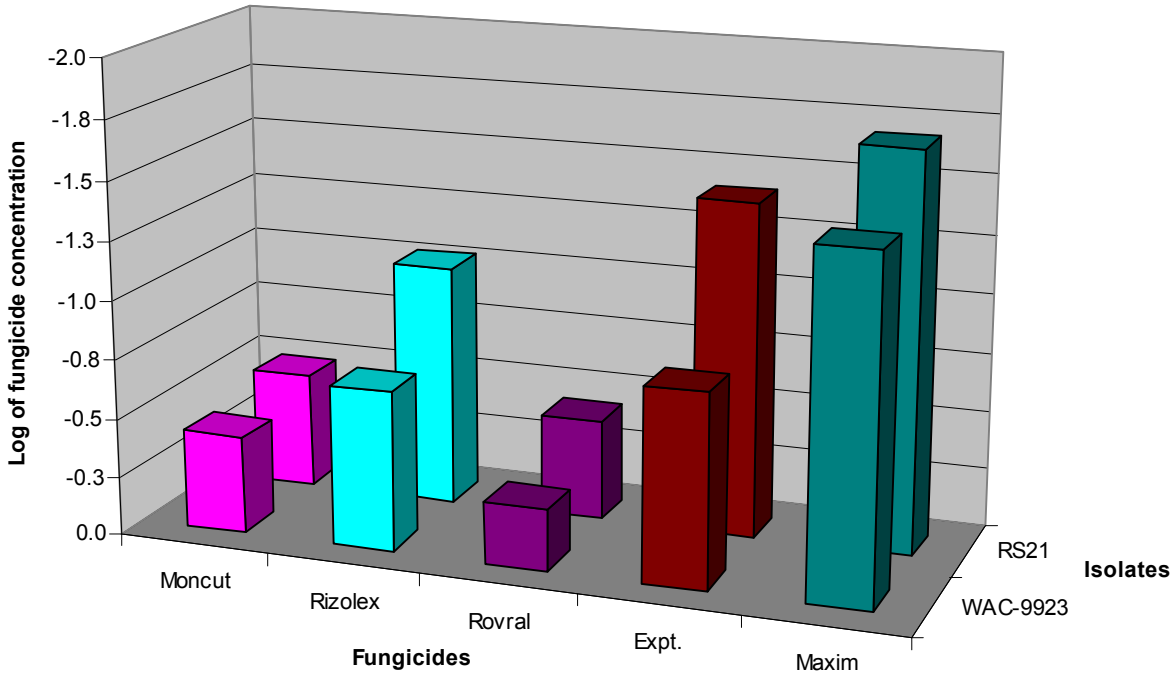
b. AG 2-2 isolates



c. AG 3 isolates



d. AG 8 isolates



5.3.3 *Effectivness of Amistar* in vitro

SHAM is added to media during *in vitro* experiments to inhibit the alternative pathway fungi use to metabolise azoxystrobin (the active ingredient of Amistar) (see section 5.1). To test the influence of SHAM concentration on the effectivness of Amistar, mycelial growth of AG 2-1 and AG 3 isolates was measured *in vitro* and data are presented in Table 5.1. When control plates with no fungicide or no SHAM amendment were compared with plates amended with only Amistar (nil SHAM) growth of both the AG 3 and the AG 2-1 isolates were significantly ($P < 0.05$) inhibited (Table 5.1). The growth of both isolates was inhibited ($P < 0.05$) when 0.5 ml/L SHAM was added to the Amistar-amended plates. Increasing the concentration of SHAM up to 2 ml/L did not affect the inhibition of isolate L306 (Table 5.1). However, as SHAM concentration increased, the mycelial growth rate of isolate WAC-9806 (AG 2-1) decreased. Colonies grown in the presence of Amistar with 2 ml/L of SHAM grew slower ($P < 0.05$) than those with Amistar and 1 ml/L of SHAM. Mycelial growth rate of cultures in the presence of 2 ml/L SHAM only (nil Amistar) was slower ($P < 0.05$) than that of cultures grown without fungicide or SHAM (nil fungicide, nil SHAM).

Table 5.1. Effect of SHAM concentration on inhibition[1] of *R. solani* isolates by Amistar with l.s.d 5 % = 0.1 mm/hr

Plate preparation		Mean mycelial growth rate (mm/hr)									
Treatment	SHAM (ppm a.i.)	Nil	Nil	2	0.5	0.75	1	1.25	1.5	1.75	2
	Amistar (ppm a.i.)	Nil	1	Nil	1	1	1	1	1	1	1
Isolate	L306 (AG 3)	0.61 b	0.37 c	0.26 d	0.17 a	0.19 a	0.14 a	0.15 a	0.15 a	0.14 a	0.13 a
	WAC-9806 (AG 2-1)	0.80 j	0.62 i	0.41 g	0.50 h	0.48 h	0.24 f	0.36 g	0.37 g	0.25 f	0.04 e

[1] Mean values were determined from five replicate plates, of PDA amended with Amistar and/or SHAM, grown at 25ºC, in the dark for 72 hrs, and compared by ANOVA to determine significant differences between treatments. These are indicated by the least significant difference 5 % (l.s.d 5 %) < 0.05 and are represented by different letters within a row

5.3.4 Sensitivity of Australian and British R. solani *AG 2-1 isolates to Rovral*

Mean growth rates for each isolate grown in the presence of 0, 5 and 10 ppm a.i. of Rovral are shown in Table 5.2. The growth rates of 13 isolates of the 27 isolates tested were inhibited by 14 % to 75 % and growth of SE45 was stimulated by 60 % when in the presence of 5 ppm a.i. of Rovral. Five of these 13 isolates originated from Great Britain and eight from Australia. Isolates from each of the IGS1 types were represented in these 13 isolates; this included the only one short, three of the intermediate (all Australian), six of the long (three Australian and three British) and three of unknown (two Australian and one British) length. These 13 isolates included five for which growth was not completely inhibited at 10 ppm a.i. of Rovral.

The growth of 78 % of the 27 isolates tested was completely inhibited by 10 ppm a.i. of Rovral. The exceptions to this were isolates SE50, L57, R14, Tas3 and R117, for which growth rates under Rovral treatment were only inhibited to between 11 % and 67 %, and SE45 for which growth was stimulated by 40 %, when compared to untreated isolates. The growth of isolate L57 was inhibited by 66 % at 10 ppm a.i. of Rovral. This growth rate was significantly ($P < 0.05$) faster than other isolates of the same (SE50, R117) and faster (R14) growth rates on medium without Rovral. Only one of these six isolates was from Great Britain and it was of long IGS1 type. Of the other five Australian isolates; three were of intermediate IGS1 type, one was of long IGS1 type and one of unknown IGS1 length.

Table 5.2. Effect of Rovral on mean growth rate of Australian and British *R. solani* AG 2-1 isolates

Isolate information			Mean growth rate[1] (mm/hr)		
			Rovral concentration (ppm a.i.)		
Isolate	**IGS1 type**	**Country of origin**	**0**	**5**	**10**
R100	short (prev. s, 510 bp)[2]	Great Britain	0.7	0.1	0.0
R103	Intermediate (prev. n, 550 bp)	Great Britain	0.6	0.0	0.0
SE50	Intermediate	Australia	0.6	0.3	0.2
SE45	Intermediate	Australia	0.5	0.8	0.7
L57	Intermediate	Australia	0.6	0.4	0.4
SAR11.18	Intermediate	Australia	0.6	0.0	0.0
R107	Long (prev. n, 550 bp)	Great Britain	0.4	0.3	0.0
R118	Long	Great Britain	0.7	0.2	0.0
R117	Long	Great Britain	0.6	0.2	0.1
R114	Long	Great Britain	0.8	0.0	0.0
R42	long (prev. l, 570 bp)	Great Britain	0.4	0.0	0.0
R106	Long (prev. n, 550 bp)	Great Britain	0.7	0.0	0.0
R14	Long	Australia	0.9	0.2	0.1
TAS6	Long	Australia	0.9	0.1	0.0
KI61	Long	Australia	0.8	0.2	0.0
1030	Long	Australia	0.6	0.0	0.0
R72	Unknown	Great Britain	1.2	0.3	0.0
KI65	Unknown	Australia	0.6	0.0	0.0
KI4	Unknown	Australia	0.7	0.0	0.0
SE42	Unknown	Australia	1.1	0.0	0.0
T30.1	Unknown	Australia	0.7	0.1	0.0
730	Unknown	Australia	0.7	0.0	0.0
WAC9806	Unknown	Australia	0.4	0.0	0.0
TAS3	Unknown	Australia	0.5	0.1	0.1
KI43	Unknown	Australia	1.0	0.0	0.0
SE51	Unknown	Australia	0.5	0.0	0.0
KI41	Unknown	Australia	0.9	0.0	0.0

[1] Mean values were determined from three replicate plates, of PDA amended with Rovral, grown at 25ºC, in the dark for 72 hrs. These were compared by ANOVA to determine significant differences between treatments indicated by the least significant difference 5 % (l.s.d 5 %) =0.1 mm/hr

Yellow highlighted values indicate where isolate grew in the presence of Rovral but was significantly ($P < 0.05$) less than the corresponding control

[2]Notes in parenthesis indicate previous IGS1 classification of isolates by Woodhall *et al.* (2007), based on size of PCR product observed on an agarose gel, s = short, n = intermediate and l = long

5.4 Discussion

The aim of these experiments was to determine the sensitivity of *R. solani* to fungicides commonly used in the Australian potato industry. The *in vitro* experiments initially focused on the *R. solani* isolates representative of the AGs found in Australian potato fields. The fungicides tested included six registered products with recommended applications methods to control either seed-tuber inoculum (Maxim, Monceren, Moncut, Rizolex and Rovral) or soil inoculum (Amistar, Rovral). However, the concentrations tested *in vitro* (0.001 to 10 ppm a.i.) were considerably lower than those recommended for use in the field (25 ppm a.i. of Maxim to 250 ppm a.i. of Monceren for seed treatments). The higher rates are likely to compensate for sorption and degradation of fungicides which occurs in the soil environment. For example, azoxystrobin is reduced in compost amended soils and anaerobic soils or after exposure to ultra-violet light (Ghosh and Singh 2009).

In the first experiment, the fungicides tested generally inhibited the growth of the isolates tested by over 80 % at the highest concentration (10 ppm) tested (section 5.3.2). However, some exceptions were observed with respect to AGs and individual isolates. For ease of viewing, the conversion of data from percentage inhibition to probit was considered, however, probit values resulted in the omission of data points of great interest as certain fungicides are less effective at controlling mycelial growth of the isolate tested. Hence for each isolate, an EC_{50} was determined and used to compare the effectiveness of each fungicide treatment. These results showed that no one fungicide was the most effective at inhibiting growth of representatives of all AGs tested.

Maxim and the experimental fungicide were consistently most effective at inhibiting the mycelial growth of isolates from AG 2-1, AG 2-2, AG 3 and AG 8 at the lowest concentration tested. The latter was the most effective at inhibiting growth of all isolates from AG 2-1, AG 2-2 and one isolate from both AG 3 and AG 8. However, this experimental fungicide is not currently being considered for registration for use on potato in Australia so it was not used in subsequent experiments.

Maxim reduced ($P < 0.05$) the growth of one AG 3 isolate (L306) when compared to other fungicides. Inhibition of the other two AG 3 isolates by Maxim was equivalent to that of Monceren treatment, also significantly ($P < 0.05$) better than other fungicides. Likewise, other *in vitro* studies have shown that isolates belonging to AG 3 are sensitive to fludionxonil (the a.i. in Maxim) (Olaya *et al.* 1994) and pencycuron (the a.i. in Monceren) (Campion *et al.*

2003; Kataria *et al.* 1991b). In another study, two isolates originally from potato, one from each of AG 2 and AG 3, were shown to be sensitive to Pencycuron *in vitro* (Virgen-Calleros *et al.* 2000). Similarly, Maxim and Monceren (as well as the experimental fungicide) inhibited the three AG 2-2 and two AG 2-1 isolates (WAC-9806 and R394) to a greater extent ($P < 0.05$) than the other three fungicides tested. These results for the AG 2-2 isolates were consistent with other *in vitro* studies during which isolates from table beets were shown to be sensitive to fludioxonil (Olaya *et al.* 1994) and isolates from rape seed and canola sensitive to pencycuron (Kataria *et al.* 1991b). The results for AG 2-1 isolates were consistent with other studies that showed isolates from this group collected from potatoes in France were sensitive to Pencycuron (Campion *et al.* 2003). Maxim significantly ($P < 0.05$) reduced the growth of both AG 8 isolates when compared with other fungicides. Sensitivity to this fungicide has not previously been reported for AG 8 isolates from potato.

Although Monceren inhibited AG 2-1, AG 2-2 and AG 3 isolates in the first experiment presented in this chapter, it was significantly ($P < 0.05$) less effective at inhibiting growth of both AG 8 isolates. AG 8 was not isolated from diseased potato plants from any region of Australia during the course of this project (Section 3.3.3). Hence it is unlikely that Monceren-insensitive, AG 8 isolates pathogenic to potato are established in the Australian potato field sampled. However, the Australian wheat belt is severely affected by pathogenic AG 8 isolates (MacNish and Neate 1996), and this group has been associated with disease on onion crops in South Australia (Pederick *et al.* 2007). Therefore, if potato crops are grown in either of these regions, the use of Monceren may need to be reconsidered.

Rizolex was as effective as Maxim, Monceren and the experimental fungicide against two of the AG 2-2 isolates (KI3 and WAC-9937) tested. Although it was less inhibitory ($P < 0.05$) than Maxim for all AG 3, AG 8 isolates and one isolate of AG 2-2 (WAC-9765) and AG 2-1 (WAC-9806), it was more effective ($P < 0.05$) than Moncut and Rovral for these isolates. These results are consistent with other *in vitro* studies in which *R. solani* isolates were shown to be sensitive to tolcofos-methyl (the a.i. in Rizolex). These isolates include one AG 3 isolate (Kataria *et al.* 1991b), AG 2-2 isolates collected from table beets (Olaya *et al.* 1994) and one each of AG 3 and AG 2 from potato (Virgen-Calleros *et al.* 2000).

Moncut inhibited the growth of all isolates of AG 2-1, AG 2-2, AG 3 and AG 8 by over 80 % at the highest concentration tested. This result is consistent with findings of Campion *et al.* (2003) that AG 3 and AG 2-1 isolates were sensitive to flutolanil. However, according to the EC_{50} values presented here, it was one of the three fungicides least effective at inhibiting

isolates from all AGs. Sensitivity to this fungicide has not previously been reported in AG 2-2 and AG 8 isolates from potato. However, yields of barley (Cotterill 1993) and wheat (Cotterill 1991) were increased after flutolanil application reduced root rot caused by AG 8.

Inhibition by Rovral was similar to that found for Moncut in the experiments presented here, supporting results for AG 2-2 from table beets (Olaya *et al.* 1994) and some AG 2-1 isolates from potato (Campion *et al.* 2003) elswhere. However, in the experiment presented in this chapter, one AG 2-1 isolate (L57) was insensitive to this fungicide. Likewise, Campion *et al.* (2003) found that AG 2-1 isolates from potato in France varied in sensitivity to iprodione. As AG 2-1 isolates were commonly associated with diseased potato plants in Australian potato crops in the present study (section 3.3.3), growers may need to limit the use of iprodione fungicides to reduce the possibility of resistant AG 2-1 isolates becoming established in fields. Keeping in mind that this fungicide may also applied to potato crops to control target spot, also known as early blight, caused by *Alternaria solani,* and Sclerotinia rot caused by *Sclerotinia sclerotiorum* (Bayer Crop Bayer *et al.* 2008) there may be considerable selection for resistance.

AG 2-1 isolates were also found to be pathogenic to potato plants in Great Britain and to vary in pathogenicity and IGS1 sequence length (Woodhall *et al.* 2007). The relationship between sensitivity to Rovral and IGS1 length for the 11 British isolates tested was not studied by Woodhall *et al.* (2007), hence, an experiment was performed during this project to investigate this relationship for both British and Australian isolates. However, results from the third experiment presented in this chapter indicated that no single IGS1 sequence type was indicative of iprodione sensitivity, resistance or moderate insensitivity of the isolates tested. Due to time constraints, IGS1 sequence data were not acquired for all isolates tested for Rovral sensitivity.

Fourteen isolates which grew in the presence of 5 ppm a.i. of Rovral from these short, long and intermediate IGS1 sequence type were represented, as were isolates from Britain and Australia. These results suggested that Rovral insensitivity was not related to IGS1 sequence type or country of origin. Hence, the IGS1 sequence was not considered to be a useful addition to the AG-specific molecular tests for field soil samples. Therefore, in Australian potato fields where the presence of *R. solani* AG 2-1 has been confirmed by use of pre-planting soil tests, it may be recommended that growers consider the use of Rhizoctonia disease control measures other than Rovral because of the possibility that the inoculum may be insensitive to this fungicide.

In the first experiment presented in this chapter, Amistar was significantly less effective than most other fungicides tested at inhibiting all but one isolate at the highest two concentrations tested. This was in contrast to results of Virgen-Calleros *et al.* (2000) who showed that azoxystrobin (the a.i. in Amistar) inhibited the growth one isolate of AG 2 and one of AG 3 from potato *in vitro*. This fungicide is commonly used in the field in Australia to control Rhizoctonia disease of potato (Hall *et al.* 2000). Azoxystrobin can be metabolised by many fungal species during *in vitro* growth (reviewed by Joseph-Horne and Hollomon 2000), however, the metabolism pathway can be inhibited with SHAM (Ziogas *et al.* 1997). In the Amistar treatments presented here, SHAM was included at a rate of 1 ppm as recommended for testing *Gaeumannomyces graminis* var. *tritici* (Joseph-Horne et al. 1998). However, the effectiveness of this rate of SHAM at inhibiting metabolism of Amistar by AGs of *R. solani* was unknown. Therefore, different rates of SHAM with a constant concentration of Amistar were tested on one AG 2-1 isolate in a subsequent experiment. Results indicated that SHAM had potentially confounded results of the Amistar treatments in the first experiment presented in this chapter (section 5.3), as this product alone inhibited mycelial growth and also because a higher concentration of SHAM produced greater inhibition.

Inhibition of mycelial growth is important in reducing inoculum density, however, results obtained *in vitro* may not reflect the situation in the field. For example, Amistar was not tested *in vitro* effectively. It is, therefore, important to test the ability of fungicides to inhibit disease caused by the different AGs in a soil environment, for example using plants in pots or in the field.

In summary, the fungicides currently available for control of Rhizoctonia disease in Australian were generally effective at controlling mycelial growth of *R. solani* AG 2-1, AG 2-2, AG 3 and AG 8. However, there was variation in the inhibition of growth of both AGs and individual isolates and complications with the *in vitro* method for Amistar. Therefore, testing these fungicides in soil environments for their ability to control disease was required.

6 *In Vivo* Fungicide Experiments

6.1 Introduction

Multiple *R. solani* AGs have been associated with potato crops world-wide (summarised in section 1.4.3) including those in Australia (Balali *et al.* 1995; de Boer *et al.* 2001 and Chapter 3). These AGs produced a range of symptoms and varying severities (Chapter 3) that affect potato yields. A number of fungicides, with different active ingredients, are registered for use on potato to control Rhizoctonia disease. Variation in sensitivity to fungicides has been observed *in vitro* using isolates representative of the AGs found in potato fields world-wide (Campion *et al.* 2003; Kataria *et al.* 1991b; Olaya *et al.* 1994; Virgen-Calleros *et al.* 2000) and using isolates representative of AGs found in Australia (Chapter 5).

The effectiveness of some fungicides at controlling disease symptoms caused by AG 3 has been investigated under field conditions (Hide and Read 1991; Platt *et al.* 1993; Wicks *et al.* 1995; 1996). However, in Australia new fungicides have been registered and methods of application have been modified, to address the influence of both seed and soil inoculum on disease. The fungicides currently available have not been tested with isolates representative of the AGs found in Australian processing potato fields. The *in vivo* experiments presented in this chapter investigate the effectiveness of commercially available products at controlling the severity of disease symptoms caused by isolates representative of AGs found in Australian fields. The influence of application method, to seed or soil, on disease symptom severity was also assessed.

6.2 Methods

Inoculum was prepared as described in section 2.4.1 and was applied to Mt Compass sand as explained in section 2.4.2. Methods for planting potato seed and watering were as described in section 2.4.3. Information about the active ingredients of fungicides used is presented in Table 2.4 and concentrations applied in the following section. After harvest, scoring and subsequent data analysis of the severity of sclerotia on tubers, necrosis on stems, stolons plus the yield of tubers and percentage of root damage were performed as described in section 2.4.4.

6.2.1 *Effectiveness of commercial fungicides at controlling disease caused by various AGs*

Isolates used as inoculum were WAC-9806 (AG 2-1) and L306 (AG 3). Inoculated millet seed (2.5 g/pot) was mixed into the top 10 cm of soil with a trowel. The effectiveness of Amistar, Maxim and Rizolex *in vivo* was compared. Fungicides were applied as recommended by the supplier; Amistar to soil, Maxim to seed and Rizolex to seed (see Table 6.1 for rates). Seed tubers of the cultivar Shepody were planted in February of 2007, as Russet Burbank was not available. Eight replicate pots were planted and data used in statistical analysis as described in section 2.4.4. However, analysis of disease severity scores was performed with six replicates only. Two replicates per treatment were removed prior to analysis by the Kruskal-Wallis test due to the low rate of emergence, which hindered the comparison of treatments (as the chi^2 approximation did not hold) when all eight replicates were included. The replicates removed from analysis were selected based on lack of emergence and their influence on the median disease severity scores. If two plants did not emerge from a treatment these were both removed from analysis. If only one plant did not emerge this plus a replicate for which disease severity scores fell central in determination of median were removed from analysis. If all plants emerged, replicates for which disease severity scores fell on either side of the median were removed prior to analysis. Maxim treatments were removed from analysis of AG 3-inoculated treatments as low rates of emergence also limited the analysis of other treatments (as the chi squared approximation did not hold).

Table 6.1 Application rates of fungicides used to treat seed or soil for experiments to assess the control of Rhizoctonia disease symptoms

Fungicide	Applied to	Concentration of a.i.	Amount of product	Volume of water
Amistar	soil	250 g/L	10 ml/100 m row[1]	1 L/100 m row
Maxim	seed	100 ml/L	250 ml/tonne of seed	1 L/tonne of seed
Rizolex	seed	500 g/L	0.4 L/tonne of seed	5 L/tonne of seed
Rovral	soil	500g/L	800 ml/hectare	160L/tonne of seed
Rovral	seed	500g/L	400 ml/tonne of seed	80 L/tonne of seed
Amistar	seed	250 g/L	12 ml/tonne of seed	18 L/tonne of seed

[1] Assumes 144 rows of 100 m long in a hectare, requiring 2 tonnes of seed at planting.

6.2.2 Influence of seed and soil application on disease control

6.2.2.1 2007 shade-house experiment

In January of 2007 fungicide efficacy on the control of disease symptoms caused by five different AGs using isolates WAC-9806 (AG 2-1), KI3 (AG 2-2), L306 (AG 3), 734 (AG 4) and RS21 (AG 8) was evaluated. Inoculated millet seed, prepared as decribed in section 2.4.1, was combined with Mt Compass sand in a cement mixer to give 4 g inoculum/pot. The fungicides tested were Amistar as furrow treatment and Rovral as both seed and furrow (see Table 6.1 for rates). Seed of cv. Rusett Burbank were grown in conditions described in section 2.4.3 and, after harvest, disease severity was scored as described in section 2.4.4. Emergence rates in the eight replicates were compared via simple linear regression. Yield, percent of root disease and disease severity were recorded as described in section 2.4.4 and analysed as described in section 6.2.1.

6.2.2.2 2009 shade-house experiment

Due to low rates of plant emergence and resulting difficulties with analysis of disease severity scores in 2008, the experiment was repeated from November 2008 to February 2009 with ten replicates per treatment. Millet seed (200 g) was placed in a 500 ml flask, moistened with 10 ml of water and inoculated with isolates Tas6 (AG 2-1) or R229 (AG 3) as described in section 2.4.1. The control treatment was colonised millet seed subsequently autoclaved. Inoculum was applied by mixing 60 kg of Mt Compass sand in a cement mixer with 40 g of the appropriate millet seed. The fungicides tested were Amistar and Rovral, both applied to either seed or soil at rates shown in Table 6.1. Cut pieces from tubers of cv. Russet Burbank were prepared and planted as described in section 2.4.3. Emergence rates in the ten replicates were compared using with simple linear regression. Yield, percent root disease and disease severity scores were recorded and analysed as described in section 2.4.4.

6.3 Results

6.3.1 Effectiveness of commercial fungicides at controlling disease caused by various AGs

Mean values of tuber yield, number of stems pruned, percentage of roots damaged per plant and number of plants emerged per treatment are presented in Table 6.2. Uninoculated plants produced the greatest tuber yield, had no stems pruned and the least root damage. These mean values were not significantly ($P < 0.05$) different from those observed for uninoculated plants treated with any of the three fungicides tested, although the fungicide treatments in the absence of *R. solani* inoculum did reduce yield slightly.

In the absence of fungicide, the yield of tubers from plants inoculated with the AG 2-1 isolate (WAC-9806) was reduced ($P < 0.05$) when compared to uninoculated plants. Treatment of AG 2-1-inoculated plants with Maxim and Amistar increased yield per plant ($P < 0.05$) when compared with that from similarly inoculated plants grown without fungicide treatment. Both of these fungicides resulted in mean yield values similar to the respective uninoculated, fungicide-treated control, however, only Amistar produced a mean yield value per plant similar to the uninoculated, untreated control.

Plants inoculated with AG 2-1 and not treated with fungicide, had more ($P < 0.05$) pruned stems and a higher percentage of damaged roots than the uninoculated, untreated controls. Treatment with Amistar and Maxim reduced ($P < 0.05$) the number of stems pruned and the percentage of root damage of AG 2-1-inoculated plants to levels found in uninoculated control plants. Rizolex reduced the percentage of root damage, but at 33% this was significantly ($P < 0.05$) higher than that found in the uninoculated control plants.

Tuber yields from AG 3-inoculated, untreated plants were not reduced when compared with the uninoculated control plants ($P < 0.05$). No fungicide treatment altered yields from that of the AG 3-inoculated or fungicide-treated control plants. However, mean yields of AG 3-inoculated, Rizolex and Amistar-treated plants were less ($P < 0.05$) than uninoculated, untreated control plants. Mean values of root damage and stem pruning for plants inoculated with AG 3 were similar to those for uninoculated plants, treated or not with fungicides.

The number of inoculated plants with isolate L306 (AG 3) that emerged was reduced to four following treatment with Maxim, the lowest number with any treatment (Table 6.2). Although this reduction was not significant ($P < 0.05$) when compared to uninoculated controls this result reduced the sample size and affected the validity of the chi squared test.

Therefore, analysis of disease severity scores was performed for each AG individually.

Uninoculated plants had no sclerotia on tubers or necrosis of stolons, however, some superficial necrosis was observed on stems and root damage on the Amistar control.

Table 6.2 Effect of fungicide application on yield, stem pruning, root disease and emergence of plants uninoculated and inoculated with *R. solani*

Treatment	Application	AG	Mean[1] values			
			Yield (g/plant) l.s.d. 5 % = 8.67	Number of stems pruned l.s.d. 5 % = 2.3	Root damage (%) l.s.d. 5 % = 20	Number of plants emerged
Untreated control	**nil**	**nil**	29.20 c	0.0 a	0 a	8
Rizolex control	**seed**	**nil**	21.54 bc	0.0 a	0 a	6
Maxim control	**seed**	**nil**	21.43 bc	0.0 a	0 a	6
Amistar control	**soil**	**nil**	22.20 bc	0.0 a	2 a	6
Untreated control	**nil**	**AG 2-1**	0.89 a	8.8 c	60 c	5
Rizolex	**seed**	**AG 2-1**	8.90 a	2.7 b	33 b	6
Maxim	**seed**	**AG 2-1**	18.65 b	3.2 b	3 a	6
Amistar	**soil**	**AG 2-1**	21.73 bc	0.3 a	5 a	7
Untreated control	**nil**	**AG 3**	24.02 bc	0.0 a	1 a	7
Rizolex	**seed**	**AG 3**	20.02 b	0.3 a	3 a	8
Maxim	**seed**	**AG 3**	21.42 bc	0.0 a	0 a	4
Amistar	**soil**	**AG 3**	19.45 b	0.0 a	0 a	6

[1] Disease was assessed as described in section 6.2.1 and mean values were determined from eight replicates per treatment and ANOVA was used to determine significant differences between means, indicated by the l.s.d. 5 % and different letters in a column.

6.3.1.1 AG 3-inoculated plants

Disease severity scores for plants inoculated with AG 3 isolate L306 are shown in Table 6.3.a. No sclerotia were observed on uninoculated plants, whether or not they had been treated with fungicide. The severity of sclerotia on daughter tubers was significantly ($P < 0.05$) greater on AG 3-inoculated plants than uninoculated plants. Plants treated with Amistar showed no significant difference in severity when compared to the uninoculated control plants, indicating that this treatment reduced sclerotium formation on tubers caused by AG 3. Although treatment of inoculated plants with either Rizolex or Maxim reduced the median score for sclerotia on tubers from 3 (in inoculated controls) to 2, this difference was not significant.

There were no differences in severity of stem necrosis between inoculated or uninoculated controls although the chi squared probability (= 0.029) indicated significance observed. All medians, including those for plants treated with fungicides, indicated minor stem lesions.

Inoculation of plants with AG 3 isolate L306 resulted in significantly ($P < 0.05$) more stolon necrosis than that observed on uninoculated plants (Table 6.3.a). Treatment with Amistar reduced stolon necrosis from a severity of 2 to none, however this was not significantly ($P < 0.05$) different from either inoculated or uninoculated controls. Treatment with Maxim also prevented stolon necrosis; however, there were too few replicates in this treatment to support a statistically significant difference. Treatment with Rizolex had no effect on stolon necrosis.

6.3.1.2 AG 2-1-inoculated plants

Stem necrosis of AG 2-1-inoculated plants treated with Amistar was less ($P < 0.05$) than the inoculated control treatment Table 6.3.b. Stem necrosis on AG 2-1-inoculated plants treated with Rizolex and Maxim was similar to the inoculated untreated control, plants treated with either fungicide still showed pruning of stems.

No necrosis of stolons was observed on uninoculated plants, however, the severity of stolon necrosis could not be analysed with the Kruskal-Wallis method as the sample size was too small for the chi^2 approximation to be valid. However, treatment of AG 2-1-inoculated plants with Amistar reduced the median severity score from 4, indicating that at least one stem was pruned, to 1.5, indicating mild to no symptoms.

Sclerotia were not observed on tubers obtained from uninoculated plants or on those inoculated with the AG 2-1 isolate.

Table 6.3 Effect of fungicide application on severity of disease symptoms[1] on plants of cv. Russet Burbank grown in a shade-house experiment for three months

3.a. Disease symptoms caused by *R. solani* AG 3 isolate L306

Treatment	Application	Disease severity (ranking[2]) and median scores from 1-4		
		Tuber sclerotia chi[2] Pr. < 0.001	stem necrosis chi[2] Pr. = 0.029	stolon necrosis chi[2] Pr. < 0.001
Uninoculated control	Nil	(11.00) a 1	(14.83) a 2	(13.75) a 1
Uninoculated, Rizolex control	Seed	(11.00) a 1	(17.50) a 2	(11.00) a 1
Uninoculated, Amistar control	Soil	(11.00) a 1	(14.83) a 2	(11.00) a 1
Uninoculated, Maxim control[3]	Seed	1	2	1
Inoculated control	Nil	(30.25) b 3	(17.50) a 2	(27.50) bc 2
Inoculated, Rizolex	Seed	(28.75) b 2	(25.75) a 2.5	(27.08) bc 2
Inoculated, Amistar	Soil	(14.83) a 1	(17.50) a 2	(16.50) ab 1
Inoculated, Maxim[3]	Seed	2	2	1

[1] Disease symptoms were assessed as described in section 2.4.4

[2] Rank as determined by Kruskal-Wallis analysis is indicated in parenthesis and was used to determine significant differences between treatments. Differences between treatments, with eight replicates (degrees of freedom = 5), are indicated by an l.s.d 5 % = 11.07 and different letters within a column.

[3] Data not included in analysis due to small number of plants that emerged.

3.b. Disease symptoms caused by *R. solani* AG 2-1 isolate WAC 9806

Treatment	Application	Disease severity (ranking[1]) and median scores from 1-4		
		Tuber sclerotia[2]	Stem necrosis chi[2] Pr. = 0.029	Stolon necrosis[2]
Uninoculated control	Nil	1	(14.08) a 2	1
Uninoculated, Rizolex	Seed	1	(16.50) a 2	1
Uninoculated, Maxim	Seed	1	(14.08) a 2	1
Uninoculated, Amistar	Soil	1	(14.08) a 2	1
Inoculated control	Nil	1	(38.50) b 4	4
Inoculated, Rizolex	Seed	1	(37.08) b 4	4
Inoculated, Maxim	Seed	1	(34.83) b 4	4
Inoculated, Amistar	Soil	1	(20.17) a 2	1.5

[1] Rank as determined by Kruskal-Wallis analysis is indicated in parenthesis and was used to determine significant differences between treatments. Differences between treatments, with eight replicates (degrees of freedom = 7), are indicated by an l.s.d 5 % = 14.07 and different letters within a column.

[2] Sample size was not large enough for the chi squared approximation to be valid.

6.3.2 Influence of seed and soil application on disease control

In the previous experiment, fungicides were applied as recommended by suppliers, hence, Amistar was applied to soil and Maxim and Rizolex to seed. Possible differences due to application method were assessed, with regard to influence on the severity of Rhizoctonia disease symptoms, and the results from two experiments are shown in the following sections.

6.3.2.1 2007 shade-house experiment

In this experiment, Rovral was used to examine the relative effectiveness of seed and soil applications at controlling disease symptoms produced by isolates representing five AGs. Results are presented in Table 6.4 and 6.5. The number of plants that emerged from potting mix is presented in Table 6.4. Linear regression analysis indicated that there were no significant differences between treatments in terms of the number of plants that emerged, even though, in seven of the inoculum x treatment combinations half (or fewer) of the plants emerged. Poor emergence occurred in the untreated, controls, inoculated with AG 2-1, AG 2-2 and AG 4 and also for plants treated as with Rovral uninoculated or inoculated with AG 3, AG 4 and AG 8. Although this result suggests that Rovral reduced emergence, no statistical comparisons were possible as analysis of the severity scores of stem and stolon necrosis was invalid using Kruskal-Wallis one way analysis of variance, as the sample size of these treatments was too small for the chi^2 approximation to hold. The situation was the same for data comprising the number of plants that produced tubers.

R. solani isolates that were collected from necrotic lower stems tissue of two uninoculated plants were classified as AG 2-1 (data not shown). However, classification of isolates collected from stems, root sclerotia and sclerotia from the seed piece confirmed the AG 2-2 inoculum isolate. The AG was not determined for isolates collected from from plants inoculated with AG 3, AG 8 or AG 4; for the latter a primer set specific was not available at the time.

In view of the small numbers of plants that produced tubers, statistical analysis of severity scores for tuber sclerotia was deemed inappropriate (pers. com. C. Dyson 2007). Sclerotia were produced on daughter tubers of untreated plants inoculated with AG 2-2, AG 3 and AG 4 isolates, as indicated by median scores of 1.5, 3.5 and 2, respectively, in Table 6.4. All three fungicide treatments inhibited sclerotium formation on AG 4-inoculated plants and Rovral applied on seed and Amistar to furrow did likewise for AG 2-2-inoculated plants.

None of the fungicides tested eliminated sclerotia caused by AG 3. The AG 2-1 and AG 8 isolates tested did not produce sclerotia on tubers, although data for the untreated, AG 2-1-inoculated control were not obtained.

Mean values of yield and percentage of root damage per plant were compared between treatments using ANOVA and the results are presented in Table 6.5. Total tuber weight per plant was reduced ($P < 0.05$) in plants inoculated with the AG 4 isolate compared to uninoculated control. The application of any of the three fungicides resulted in total tuber weight per plant similar to that of the uninoculated control plants. The mean weight of tubers per plant was similar for uninoculated control plants and those inoculated with the AG 2-2 isolate KI3. Application of Rovral to furrow significantly ($P < 0.05$) increased the yield of AG 2-2-inoculated plants, however, neither value was different from that of uninoculated controls.

The number of tubers per plant (Table 6.5) indicated that only four treatments altered the numbers of tubers when compared to uninoculated control plants. Plants inoculated with AG 3 isolate L306 when the seed was treated with Rovral produced significantly ($P < 0.05$) more tubers than plants similarly inoculated but without fungicide treatment. More tubers were also produced by AG 3-inoculated, Rovral seed-treated plants than uninoculated plants grown with the same, or without any fungicide treatment. Plants inoculated with the AG 4 isolate 734 produced significantly ($P < 0.05$) more tubers than uninoculated plants when grown without fungicide or in the presence of Rovral applied to seed. However, AG 4-inoculated plants produced fewer ($P < 0.05$) tubers when treated with either Rovral or Amistar in-furrow than similarly inoculated plants without fungicide. Plants inoculated with the AG 8 isolate RS21 produced more ($P < 0.05$) tubers when treated with Amistar in-furrow than grown without fungicide treatment. AG 8-inoculated plants with Rovral seed treatment produced fewer tubers than uninoculated plants with the same fungicide treatment.

Root damage was greater ($P < 0.05$) on plants inoculated with AG 2-1 and AG 2-2 isolates than uninoculated controls (Table 6.5). All three fungicide treatments reduced the percentage of roots damaged by AG 2-2 to a level not different from the uninoculated control having the same fungicide treatment. A similar observation was made for both furrow treatments of AG 2-1-inoculated plants. However, root damage of AG 2-1-inoculated plants whose seed was treated with Rovral was significantly ($P < 0.05$) worse than uninoculated control plants, but still lower than AG 2-1-inoculated plants grown without fungicides.

Table 6.4 Effect of method of fungicide application on emergence, disease severity scores[1] and tuber production of uninoculated plants of potato cv Russet Burbank and those inoculated with selected AGs of *R. solani*

Treatment		Number of plants[2] plants[2] emerged	Median[2] severity scores (1-4)			Number of plants[2] that produced tubers
Inoculum AG	Fungicide		stem	stolon	Tuber sclerotia	
Nil	Nil	5	2	1	1	5
Nil	Rovral furrow	8	2	2	1	8
Nil	Rovral seed	4	1.5	1.5	1	4
Nil	Amistar furrow	6	2	2	1	5
AG 2-1	Nil	1	4	*	*	0
AG 2-1	Rovral furrow	6	3	2	1	5
AG 2-1	Rovral seed	5	4	3.5	1	3
AG 2-1	Amistar furrow	8	2	4	1	8
AG 2-2	Nil	2	2	1	1.5	2
AG 2-2	Rovral furrow	6	2	4	2	6
AG 2-2	Rovral seed	6	2	2	1	6
AG 2-2	Amistar furrow	6	2	2	1	6
AG 3	Nil	5	2	2	3.5	4
AG 3	Rovral furrow	6	2	2	3	6
AG 3	Rovral seed	3	2	2.5	4	3
AG 3	Amistar furrow	6	3	3	2	6
AG 4	Nil	4	4	2	2	3
AG 4	Rovral furrow	7	4	3	1	6
AG 4	Rovral seed	1	2	2	1	1
AG 4	Amistar furrow	7	3	2	1	7
AG 8	Nil	6	2	2.5	1	6
AG 8	Rovral furrow	6	2	2	1	6
AG 8	Rovral seed	2	1.5	3	1	2
AG 8	Amistar furrow	6	2	2	1	6

[1] Disease severity was assessed on a scale of 1-4 as described in section 2.4.4 from plants grown in Mt Compass sand, for 3 months, in a shade-house at Lenswood Research Centre

[2] From a total of eight replicate plants per treatment.

* indicates that no scores were collected from this tissue, exposed to this treatment.

Table 6.5 Effect of method of fungicide application on mean[1] values of tuber production and root damage[2] on uninoculated plants of potato cv Russet Burbank and those inoculated with various AGs of *R. solani*

Treatment		Mean[1] values		
AG	Fungicide	Total tuber weight (g) per plant l.s.d 5 % = 7.513	Number of tubers per plant l.s.d 5 % = 1.26	Root damage (%) l.s.d. 5 % = 16.1
nil	nil	24.26 bc	2.00 abc	0 a
nil	Rovral furrow	28.56 bc	2.38 abcd	6.2 abc
nil	Rovral seed	26.30 bc	1.75 b	3.8 ab
nil	Amistar furrow	26.15 bc	3.00 bcde	0 a
AG 2-1	nil	25.17 bc	2.60 abcd	49.3 d
AG 2-1	Rovral furrow	23.98 bc	2.20 abcd	17.5 bc
AG 2-1	Rovral seed	23.57 bc	2.67 abcd	21 c
AG 2-1	Amistar furrow	27.59 bc	2.25 abcd	13.7 abc
AG 2-2	nil	21.26 b	3.00 bcde	77.5 e
AG 2-2	Rovral furrow	29.27 c	2.00 abc	1.7 ab
AG 2-2	Rovral seed	21.27 b	2.50 abcd	16.7 bc
AG 2-2	Amistar furrow	25.40 bc	3.17 cde	3.3 ab
AG 3	nil	27.87 bc	2.00 abc	9 abc
AG 3	Rovral furrow	23.53 bc	2.67 abcd	9.2 abc
AG 3	Rovral seed	28.20 bc	3.33 de	6.7 abc
AG 3	Amistar furrow	27.40 bc	2.67 abcd	6.7 abc
AG 4	nil	12.12 a	4.00 ef	0 a
AG 4	Rovral furrow	26.20 bc	2.17 abcd	10 abc
AG 4	Rovral seed	23.88 bc	4.95 f	0.2 a
AG 4	Amistar furrow	22.74 bc	2.71 abcd	5.7 ab
AG 8	nil	28.02 bc	1.83 ab	1.7 ab
AG 8	Rovral furrow	25.48 bc	1.83 ab	5.8 abc
AG 8	Rovral seed	26.14 bc	1.50 a	7.5 abc
AG 8	Amistar furrow	29.60 c	3.17 cde	10 abc

[11] Mean values were determined from eight replicates per treatment and ANOVA was used to determine significant differences between them, indicated by the l.s.d. 5% and different letters in a column.

[2] Root damage was assessed as described in section 2.4.4 from plants grown in Mt Compass sand, for 3 months, in a shade-house at Lenswood Research Centre

6.3.2.2 2008-09 shade-house experiment

Because of difficulties in assessing disease severity in the experiment presented in section 6.2.2.1 another trial was performed, with more replicates per treatment, to assess the influence of fungicide application method. The results of plant growth parameters are presented in Table 6.6 and disease severity in Table 6.7.

The mean total weight of tubers per uninoculated plant was significantly ($P < 0.05$) greater when seed was treated with either Amistar or Rovral or soil was treated with Rovral, than that from plants grown without fungicides (Table 6.6). The total weight of tubers from AG 2-1 or AG 3-inoculated plants did not differ from that of the uninoculated plants. However, AG 3-inoculated plants with Amistar seed treatment produced significantly ($P < 0.05$) lower weights of tubers per plant than uninoculated plants grown with the same fungicide treatment. When AG 2-1-inoculated plants were compared with uninoculated plants, treated with the same fungicide, only furrow treatment with Amistar maintained mean tuber weights per plant.

The mean number of tubers per uninoculated plant was significantly ($P < 0.05$) higher with any fungicide treatment than without (Table 6.6). This value was also higher in plants inoculated with the AG 2-1 isolate Tas6 than when plants were uninoculated. When compared to AG 2-1-inoculated plants grown without fungicides plants grown in the same inoculum with furrow treatment of Amistar had fewer tubers, whereas those with Rovral seed treatment had more tubers. AG 3-inoculated plants produced significantly ($P < 0.05$) more tubers than uninoculated plants, without fungicide treatment. Seed treatment with Rovral was the only fungicide that altered the mean number of tubers when plants were inoculated with AG 3 isolate R229, increasing the value significantly ($P < 0.05$). All AG 3-inoculated plants produced significantly ($P < 0.05$) fewer tubers per plant that uninoculated plants treated with the same fungicide.

The total mean number of stems produced by uninoculated plant did not vary with fungicide treatments (Table 6.6). However, untreated AG 2-1-inoculated plants produced more ($P < 0.05$) stems than untreated uninoculated plants. Plants grown with AG 2-1 inoculum produced fewer stems with fungicide treatments than those grown without fungicides, with values similar those from uninoculated plants. AG 3-inoculated plants without fungicide treatment produced numbers of stems similar to uninoculated plants. However, significantly ($P < 0.05$) more stems were produced on plants inoculated with AG 3

isolate R229 and also had seed treated with either Amistar or Rovral than for other treatments.

None of the stems of uninoculated plants were pruned (Table 6.6). Inoculation of plants with AG 3 isolate R229 and AG 2-1 isolate Tas6 did not increase the number of stems pruned when compared to uninoculated plants. Although mean values for every treatment were below 1, a significant increase in the number of stems pruned, when compared to uninoculated plants, was observed in AG 3-inoculated plants for which the seed had been treated with Amistar or Rovral.

Table 6.6 Effect of method of fungicide application on mean[1] values of growth parameters of uninoculated plants[2] of potato cv Russet Burbank and those inoculated with *R. solani*

Inoculum	Fungicide	Mean[1] values of plant growth parameters			
		Total weight (g) of tubers per plant (l.s.d. 5 % = 5.5)	Number of tubers per plant (l.s.d 5 % = 0.51)	Total number of stems (l.s.d. 5 % = 0.73)	Number of stems pruned (l.s.d. 5 % = 0.51)
Nil	Nil	22.7 abc	1.4 ab	1.4 a	0.0 a
Nil	Amistar seed	33.4 f	2.9 g	1.7 ab	0.0 a
Nil	Amistar furrow	23.2 abc	2.1 cde	1.8 abc	0.0 a
Nil	Rovral seed	29.9 ef	4.1 h	1.6 ab	0.0 a
Nil	Rovral furrow	29.3 def	2.3 def	1.3 a	0.0 a
AG 2-1	Nil	26.0 bcde	2.1 cde	2.2 b	0.5 abc
AG 2-1	Amistar seed	27.8 cde	1.8 bcd	1.5 ab	0.1 a
AG 2-1	Amistar furrow	18.3 a	1.1 a	1.6 ab	0.0 a
AG 2-1	Rovral seed	24.3 bcd	2.8 fg	2.0 ab	0.2 ab
AG 2-1	Rovral furrow	20.9 ab	1.6 abc	1.4 a	0.1 a
AG 3	Nil	22.4 abc	1.8 bc	1.6 ab	0.4 abc
AG 3	Amistar seed	24.2 bcd	2.0 cde	2.5 c	0.6 bc
AG 3	Amistar furrow	20.8 ab	1.4 ab	1.3 a	0.2 ab
AG 3	Rovral seed	30.2 ef	2.4 efg	2.2 b	0.5 abc
AG 3	Rovral furrow	24.3 bcd	1.3 ab	2.0 ab	0.8 c

[1] Mean values were determined from ten replicates per treatment and ANOVA was used to determine significant differences between them, indicated by the l.s.d. 5 % and different letters in a column.
[2] Plants were grown in Mt Compass sand, for 3 months, in a shade-house at Lenswood Research Centre

No sclerotia were observed on daughter tubers of uninoculated plants when grown with or without fungicides (Table 6.7). Inoculation of plants with AG 2-1 isolate Tas6 did not increase the severity of tuber sclerotia when grown without fungicides. However, AG 2-1-inoculated plants for which seed was treated with Rovral had significantly more sclerotia on tubers than uninoculated plants. AG 3-inoculated plants grown without fungicides had the most sclerotia on tubers, significantly ($P < 0.05$) more than uninoculated plants. The only fungicide treatment that reduced sclerotia on AG 3-inoculated plants was Amistar applied to the furrow, which reduced the severity to a level similar to that observed on uninoculated plants.

Although superficial necrosis was observed on some stems of uninoculated plants, the median was 0 for plants grown with or without fungicides (Table 6.7). Similar observations were made for plants inoculated with AG 2-1 isolate Tas6, however, plants inoculated with AG 3 isolate R229 and not treated with fungicides had significantly ($P < 0.05$) more severe stem necrosis than uninoculated plants. The only fungicide treatment to significantly ($P < 0.05$) reduce the severity of stem necrosis of AG 3-inoculated plants was Amistar to furrow, which reduced the necrosis to a level similar to that observed on uninoculated plants.

Median values of stolon necrosis were 0 for uninoculated plants, grown with or without fungicides (Table 6.7). Plants inoculated with AG 2-1-isolate Tas6 or AG 3 isolate R229 and grown without fungicides had a stolon necrosis value not significantly ($P < 0.05$) different from uninoculated plants. AG 2-1-inoculated plants grown with Rovral furrow treatment showed significantly ($P < 0.05$) more severe stolon necrosis than either AG 2-1-inoculated or uninoculated plants grown with any fungicide, however, this value was not different from that observed on inoculated or uninoculated plants grown without fungicides. The only treatment to reduce stolon necrosis of AG 3-inoculated plants was Amistar to furrow, this value was not significantly ($P < 0.05$) different from that observed on uninoculated plants grown with the same fungicide.

No root damage nor sclerotia were observed on uninoculated or AG 2-1-inoculated plants grown with or without fungicides. Sclerotia were observed on the roots of all AG 3-inoculated plants and root damage was significantly ($P < 0.05$) worse than that of uninoculated plants, for plants with and without fungicide treatments. Plants inoculated with AG 3 and treated with Amistar in-furrow produced the most severe root necrosis.

Table 6.7 Effect of fungicide application method on disease severity of uninoculated potato plants[1] cv Russet Burbank and those grown in *R. solani* inoculated soil

Fungicide	Inoculum	Rank[2] and median disease severity scores (1-4)			Mean[3] root damage (%) (l.s.d. 5 % = 2.0)	Root sclerotia
		Tuber sclerotia	White stem necrosis	Stolon necrosis		
Nil	**Nil**	(47) a 0	(64.67) a 0	(59.83) ab 0	0 a	N
Amistar seed	**Nil**	(47) a 0	(63.2) a 0	(50.5) a 0	0 a	N
Amistar furrow	**Nil**	(47) a 0	(62) a 0	(50.5) a 0	0 a	N
Rovral seed	**Nil**	(47) a 0	(50) a 0	(57.05) a 0	0 a	N
Rovral furrow	**Nil**	(47) a 0	(56) a 0	(50.5) a 0	0 a	N
Nil	**AG 2-1**	(58.6) ab 0	(69.45) a 0	(65.06) ab 0	0 a	N
Amistar seed	**AG 2-1**	(52.8) ab 0	(56) a 0	(57.05) a 0	0 a	N
Amistar furrow	**AG 2-1**	(47) a 0	(56) a 0	(50.5) a 0	0 a	N
Rovral seed	**AG 2-1**	(70.8) b 0	(68.78) a 0	(57.78) a 0	0 a	N
Rovral furrow	**AG 2-1**	(52.2) ab 0	(58.45) a 0	(83.25) b 0.5	0 a	N
Nil	**AG 3**	(121.3) c 4	(101.12) cd 1	(81.93) b 0	4.4 b	Y
Amistar seed	**AG 3**	(123.5) c 4	(99.75) c 2.5	(121.25) c 1	3.1 b	Y
Amistar furrow	**AG 3**	(67.8) ab 0	(71.65) ab 0	(50.5) a 0	7.0 c	Y
Rovral seed	**AG 3**	(123.5) c 4	(94.8) bc 1	(125.75) c 1.5	4.3 b	Y
Rovral furrow	**AG 3**	(123.5) c 4	(123.61) d 4	(118.61) c 1	3.3 b	Y

[1] Plants were grown in Mt Compass sand, for 3 months, in a shade-house at Lenswood Research Centre

[2] Rank as determined by Kruskal-Wallis analysis is indicated in parenthesis and was used to determine significant differences between treatments, with eight replicates (degrees of freedom = 14). Differences are indicated by an l.s.d. 5 % = 23.68 and different letters within a column.

[3] Mean values were determined from ten replicates per treatment and ANOVA was used to determine significant differences between them, indicated by the l.s.d. 5 % and different letters in a column.

Isolation of *R. solani* was not acchieved from these stems and tissue samples were sent to SARDI for DNA extraction and AG-specific quantitative PCR. Results from three different plants revealed the absence of AG 2-1 and AG 3 DNA from uninoculated controls and the presence of high levels of AG 2-1 and AG 3 DNA on the appropriate plants (Table 6.8).

Table 6.8 Amount of *R. solani* DNA detected from stems of uninoculated potato plants cv Russet Burbank or those inoculated with *R. solani* and grown without fungicide treatment

Inoculum	DNA quantity (pg/PCR) from quantitative PCR	
	AG 2-1	AG 3
nil	0	0
nil	0	0
nil	0	0
AG 3	0	119,807
AG 3	0	120,365
AG 3	0	2,804,969
AG 2-1	1,702	0
AG 2-1	42,530	0
AG 2-1	6,989	0

6.4 Discussion

The experiments presented in this chapter were undertaken to evaluate the efficacy of fungicides for the control of disease of potato caused by *R. solani*. Amistar but not Maxim and Rovral applied at rates recommend by the supplier reduced the severity of tuber sclerotia caused by AG 3. The results for Amistar were consistent with reports of furrow applications reduced black scurf (Hall *et al.* 2000). Maxim and Rovral are registered for use in Australia to control black scurf, however, Rizolex applied to seed-tubers did not significantly reduce black scurf and too few plants emerged from the Maxim treatments to permit analysis. However, Rizolex and Maxim reduced the severity of black scurf in previous research (Hall *et al.* 2000; Wicks *et al.* 1996), suggesting that further investigation, with better replication of treatments, is required to assess the effectiveness of these two fungicides at reducing formation of sclerotia on tubers.

Results from the first experiment presented in this chapter showed that inoculation of plants with AG 3 isolate L306 did not increase the severity of stem necrosis, which was inconsistent with results presented in chapter 4 and other studies (Bains and Bisht 1995; Balali *et al.* 1995; Bandy *et al.* 1988; Carling and Leiner 1986; de Boer *et al.* 2001; Lehtonen *et al.* 2008a; Woodhall *et al.* 2007). Therefore, further investigation of the influence of these fungicides on necrosis symptoms caused by AG 3 is required.

Plants inoculated with AG 2-1 isolate WAC-9806 had significantly more severe stem necrosis and root damage than uninoculated plants, which was consistent with results reported in chapter 4. Amistar treatment in-furrow was the only treatment in this experiment to reduce stem necrosis. However, fewer stems were pruned on plants treated with fungicides than on control plants. Unexpectedly, root damage was decreased with application of all of the three fungicides tested, but higher yields resulted only from Maxim and Amistar treatments; the latter was particularly effective. The application of Maxim to seed-tubers increased tuber yields as a result of a reduction in root damage, as was reported in chapter 4, emphasising the importance of healthy roots for maximum yield. The application of fungicides to seed tubers would not be expected to protect the potato root system and it is possible that leaching of the fungicide through the soil profile may have produced this result. Rizolex reduced root damage only half as well as Maxim and this may be explained by the variable rates of leaching, based on solubility in water (1.1 and 1.8 ppm respectively (Tomlin 1997)) and persistence in soil.

Amistar was also the only fungicide applied to soil, as a furrow spray in the first

experiment, whereas the other two fungicides were applied to seed pieces. Further experiments were undertaken to investigate if the method of application influences the control of disease symptoms caused by soil-borne inoculum. The first such experiment was performed over the summer of 2007 and showed low emergence rates in the Rovral seed treatment and, more importantly, in the inoculated controls. As it was not possible to confirm that any damage was caused by *R. solani*, statistical analysis of control of these disease symptoms by the fungicide treatments was invalid. Therefore, a second experiment was performed over the summer of 2008/09, with fewer AGs and more replicates of each treatment. However, it is important to note that this was the first test of the pathogenicity of AG 2-2 and AG 4 isolates on plants grown to maturity and both were associated with the formation of sclerotia on tubers, as was AG 2-1. This supports reports that AG 2-1 (Carling and Leiner 1986; de Boer *et al.* 2001; Woodhall *et al.* 2007), AG 2-2 (de Boer *et al.* 2001) and AG 4 (Balali *et al.* 1995; de Boer *et al.* 2001) have been isolated from tuber-borne sclerotia. All of these three AGs produced fewer (median) sclerotia on tubers than did AG 3 isolates. Although the values of yield and root damage for each treatment could be compared statistically, it is necessary to recognise that these values are ultimately determined by the number of plants that emerged and produced roots and tubers.

In the experiment conducted in the summer of 2008/09, the fungicide treatments altered the mean total weight and numbers of tubers per plant in uninoculated control and AG 2-1-inoculated plants, although median values for these yield values indicated that these plants had no stem or stolon necrosis. Rovral applied to seed was the only treatment to increase the weight of tubers per plant inoculated with AG 3 compared with similarly inoculated, fungicide-free controls, however, neither value differed from that of uninoculated plants. Also, disease symptoms and the number of stems per plant were unchanged. Likewise, although reports of previous field trials (Hide and Cayley 1982) in which Rovral was applied to seed, showed no increase in yield, Hide and Cayley (1982) did find decreases in the number of tubers with sclerotia and severity of stem necrosis.

The AG 3 isolate R229 again produced sclerotia on progeny tubers and the only fungicide treatment to reduce this symptom was Amistar, applied to furrow. This is consistent with results of Hall *et al.* (2000), where in furrow applications at rates of over 56 g a.i./ha in shadehouse trials and 112 g a.i./ha in field experiments reduced the incidence and severity of black scurf. Because Amistar did not control black scurf when applied to seed, these results suggest that application method can affect control of black scurf. However, neither Rovral

treatment reduced the severity of black scurf, suggesting that chemistry of the fungicide may also influence control of sclerotium formation.

Increased severity of stem necrosis was observed on AG 3-inoculated plants compared with uninoculated plants and, again, furrow treatment with Amistar decreased this symptom. This is consistent with findings from field trials reported by Hall *et al.* (2000). However, neither seed treatment reduced stem necrosis, suggesting that furrow treatments are more effective at reducing this symptom. In comparison stems of plants grown in furrows treated with Rovral, displayed more necrosis, which was inconsistent with findings by Hall *et al.* (2000), where stem necrosis did not differ significantly from the untreated controls. Hide and Cayley (1982) observed reduced stem necrosis in plants treated with Rovral when the inoculum source was either soil or seed-borne, however, when the inoculum was seed-borne only stolon necrosis was reduced by application of Rovral. In the experiment presented in this chapter the severity of stolon necrosis of AG 3-inoculated plants also reflected results of stem necrosis, again suggesting that chemistry of the fungicide, more than application method, influenced Rhizoctonia disease control.

Root damage of AG 3-inoculated plants mainly consisted of sclerotium formation on roots. Amistar in furrow was the only treatment that altered this symptom, exacerbating disease. That Amistar did not reduce root damage of AG 3-inoculated plants was contradictory to results from the first experiment, in which roots of AG 2-1-inoculated potato plants appeared to be protected by the fungicides. In all treatments, root damage was minimal (below 7 %) and neither AG 2-1 or AG 3 inoculation produce lower yields than uninoculated control plants. Hide and Firmager (1990) showed root necrosis caused by AG 8 can reduce height and tuber production by potato plants, however, root necrosis does not appear to have been assessed in previous research on the efficacy of treatments. Of more concern may be that sclerotia on roots left in soil after harvest could provide an inoculum source for subsequent crops as has been found for sclerotia on tubers (Tsror and Peretz-Alon 2005; Wicks *et al.* 1996).

The results presented in this chapter suggest that some fungicides can reduce the severity of some symptoms caused by *R. solani* on potato plants. However, many factors affect disease control, including the AG of inoculum, and method of fungicide application. Therefore, alternatives to fungicide applications, to increase the tolerance of the plant to a wide range of *R. solani* AGs, are being investigated. Such alternatives include the effect of plant nutrition status on the severity of disease.

7 Influence of Micronutrients on Rhizoctonia Disease

7.1 Introduction

Numerous methods of controlling Rhizoctonia disease have been investigated (reviewed in section 1.5) since *R. solani* was recognised as a pathogen of potato plants (reviewed by Menzies 1970). Breeding programs have attempted to increase the tolerance of potato cultivars to *R. solani* (Bains *et al.* 2002; Leach and Webb 1993; Yanar *et al.* 2005) but this has not been successful (Leach and Webb 1993; NIAB 2004). A number of fungicides are currently registered in Australia, to control soil and seed-borne inoculum of *R. solani* and limit Rhizoctonia disease of potato crops (reviewed in section 1.5.1). However, where inoculum levels are high, yield losses can still occur (Brewer and Larkin 2005; Scholten *et al.* 2001; Tsror and Peretz-Alon 2005).

Adequate nutrition of crop plants has long been recognised to improve yield and to increase tolerance to diseases (reviewed by Datnoff *et al.* 2007). Macronutrients, such as nitrogen, potassium and phosphorus, are well monitored in Australian potato crops by plant tissue and soil analysis (Williams *et al.* 1993). However, the soils used for growth of potato crops in Australia are commonly deficient in micronutrients, including zinc, manganese, copper and boron (Donald and Prescott 1975; Halloway *et al.* 2008). In some cases, damage due to Rhizoctonia disease can be limited by supply of nutrients. This has been documented for a range of nutrients in different crops, such as zinc in medic (Streeter *et al.* 2001a) and cereals (Thongbai *et al.* 1993a; b), manganese in medic (Kalim *et al.* 2003) and silicon in rice (Datnoff *et al.* 1992; Hooda and Srivastava 1996; Rodrigues *et al.* 2003) and creeping bent grass (Uriarte *et al.* 2004). The role of zinc is discussed below.

7.1.1 Zinc

Zinc is required for the maintenance of the enzyme, superoxide dismutase, that is involved in reducing the levels of superoxide radicals in plants (Cakmak and Marschner 1988b). In zinc-deficient plants the activity of this enzyme is reduced and the radicals increased. These radicals contribute to the peroxidation of lipids in membranes, leading to reduced membrane integrity and increased permeability (Cakmak and Marschner 1988a; Cakmak and Marschner 1988b; Welch and Norvell 1993). Zinc is also involved in protein synthesis, which is also inhibited in deficient plants, resulting in accumulation of free amino

acids and amides. In zinc-deficient plants these may be exuded from the leaky membranes (Cakmak and Marschner 1988a) and accelerate infection by *R. solani*, as this fungus is attracted by root exudates (Flentje *et al.* 1963).

A relationship between zinc nutrition and the severity of Rhizoctonia disease was observed in Australian wheat by Thongbai *et al.* (1993b) and medic by Streeter *et al.* (2001a). Siddiqui *et al.* (2002) reported similar observations in experiments performed on tomato plants in Pakistan. A survey of field soils in South Australia and Victoria intended for use in growing potato crops in 2005, showed a range of zinc levels such that 17 out of 27 sites had below the recommended rate of 3 ppm of zinc (Nigel Crump (2007), unpublished data). The critical value of zinc for optimal growth of potato plants is 20 ppm in a petiole of a plant at the flowering and early tuber bulking stage (Maier and Shepard 1998). However, the relationship of the zinc status of potato plants and severity of Rhizoctonia disease has not been studied.

In the research described in this chapter potato plants were grown in sand containing different zinc concentrations to produce plants with tissue concentrations of zinc spanning the deficiency and sufficiency ranges. A subset of these soils was also inoculated with *R. solani* to allow investigation of the severity of necrosis and sclerotia symptoms formed on plants of different zinc status.

7.2 Methods

7.2.1 First zinc experiment - "closed-pot" (2008)

This preliminary experiment was conducted from February to March 2008 to investigate the effect of amendment of soil with zinc on the severity of Rhizoctonia disease of potatoes. Methods were based on those used to investigate the zinc efficency of bread wheat by Genc *et al.* (2006). Mt Compass sand was washed to minimise the organic matter content (see Table 2.3). A cement mixer was half-filled with Mt Compass sand and RO water added until full, and the drum was rotated for about 2 min, until the sand at the bottom was well mixed with water. The drum was stopped and water and organic matter were slowly tipped off, leaving the heavier sand at the bottom. This washing process was repeated five to six times until the water was almost clear. The final rinse was performed with MQ water. The washed sand was spread on a bench in a glasshouse at the University Adelaide to air dry before use.

Pots of 20 cm diameter were lined with polyethylene bags to avoid leaching of nutrients and each filled with 6 kg of sand. Calcium carbonate powder (0.5 %) was added to the sand to increase the pH to 8 and reduce zinc availability to the plant (Silber *et al.* 2004). Basal nutrient solutions (volumes shown in Table 7.1) were mixed into the sand in each pot, as was the zinc sulfate solution to give final concentrations of 0, 0.1, 1 or 3 ppm of zinc in the solution applied. The sand was left to dry, by standing in pots in natural light in the Lenswood shade-house for one week, then inoculum was added. Inoculum consisted of the AG 3 *R. solani* isolate R229, in the form of infested millet seed (Section 2.4.1), and uninoculated controls comprised infested millet seed subsequently autoclaved; each was added to the appropriate pots at a rate of 4 g of seed / 6 kg soil. To distribute the inoculum and nutrients evenly through the sand the plastic bags were closed, removed from the pots, and inverted five times then replaced into the pot to create the "closed-pot" system. Inoculum was allowed to establish in the soil for 4 days before the potato seed was planted.

Cut seed pieces, of cultivar Russet Burbank, were prepared and planted as described in section 2.4.3. Pots were maintained in natural light at the Lenswood shade-house; placement in four rows along the central length of the shade-house was randomised using GenStat. Each treatment was replicated eight times to account for variation between plants. Moisture content was maintained by watering with MQ water to 12 % w/v, which was measured with a hand-held theta probe and theta probe (TP) reader (Measurement Engineering Australia, Magill, SA) on Monday, Wednesday and Friday of each week over 2 months. Extra amendments of basal nutrient solution 1 (at half the concentration shown in Table 7.1) were applied monthly as a drench and topped up to 12% w/v by standard watering practice.

Table 7.1 Basal nutrient solutions added as equivalent per kg of dry sand

component	NH_4NO_3	KH_2PO_4	K_2SO_4	$MgSO_4.7H_2O$	$MnSO_4.7H_2O$	$CuSO_4.5H_2O$	H_3BO_3	$CoSO_4.7H_2O$	$FeSO_4.7H_2O$	$H_2Mo.H_2O$	$NiSO_4.6H_2O$
mg/kg soil	350	90	120	90	3	5	0.1	1	1.4	0.005	0.15
solution	1	2			3					4	
volume ml/kg soil	25	25			10					10	

After harvest, plants were washed with MQ water and disease was assessed as described in section 2.4.4. Two stems from uninoculated plants were excised at soil level, leaves removed and the stems were submitted to Waite Analytical Services (Adelaide, Australia) for drying and nutrient content analysis. Attempts were made to reisolate *R. solani* from stems, roots and sclerotia as described in section 2.1. The AG of these cultures was established using the AG-specific PCR methods described in section 2.3.

7.2.2 Second zinc experiment – "contained flow-through" (2008-09)

The main experiment to investigate the relationship of the zinc status of potato plants with Rhizoctonia disease severity was conducted from October 2008 to January 2009. Methods were as for the preliminary experiment described in section 7.2.1 with the following changes. A fine-grade sand (NT40) purchased from Sloans, Adelaide, Australia (see Table 2.3) was used. Inoculum comprised AG 2-1 isolate Tas6 and AG 3 isolate R229, as well as autoclaved millet seed for uninoculated controls. Inoculum was added to each pot at a rate of 5 g of seed / 6 kg soil. After nutrient amendment and inoculation, the sand was mixed then transferred to clean pots that had drainage holes lined with paper towel to reduce loss of sand. Pots were placed in individual plastic plates to create a contained flow-through system when watered. After planting potato seed, pots were maintained in natural light in the Lenswood glasshouse, their placement on four benches along the north side of the glasshouse was randomised using GenStat. Changes to assessment of disease and nutrient content are documented below.

The severity of necrosis on the above-ground portion of stems was assessed 30 days after planting. At the same time, the number of stems per plant and number of plants per treatment were recorded and height of plants estimated as; below 10 cm, 15 to 25 cm, or 30 cm and over. The percentage of the stem circumference with necrosis was also estimated as follows:

1 – no necrosis

2 – less than 10 %, all necrosis below the first leaf

3 – 50 % coverage, may extend past the first leaf

4 – greater than 50 %, extending past the first leaf

At 30 days after planting the fifth leaf from the growing tip was excised from three control plants grown in each of the four zinc concentrations and submitted to Waite

Analytical Services (Adelaide, Australia) for nutrient analysis. Beginning after these leaves were removed, all pots were amended monthly with solution 3 and fortnightly with solutions 1 and 2, all at 1/3 the basal concentrations (see Table 7.1). Solutions were applied as described in section 7.2.1.

Three months after planting plants were harvested, washed with MQ water and disease was assessed as described in section 2.4.4. Tubers were removed and stems were cut at soil level to separate whole shoots from below ground stems, roots and stolons. Fresh weights were recorded of whole shoots, below ground stems, roots and stolons and each tuber, as was the width of each tuber at its widest point. Isolation of *R. solani* was attempted (as described in section 2.1) from necrotic tissue on stems and sclerotia on roots and tubers from three plants from each treatment (inoculum x zinc concentration).

Tubers, roots and whole shoots were placed separately in yellow paper envelopes (Spicers, Officeworks) and dried. Yellow envelopes were used to avoid contamination with the trace amounts of zinc present in white envelopes (pers. com. L. Palmer 2008). The dry weight of roots, whole shoots and tubers from each plant was recorded before these tissues were ground. Roots and whole shoots were dried for one week at 40°C and then ground for 30 sec using an A10 IKA® mill (IKA, Petaling Jaya, Malaysia). Tubers were dried for an additional week at 60°C and initially ground in a Toolrite mill (Toolrite, Wisconsin, USA) before using the A10 IKA® mill for 30 sec. Four samples of whole shoots, roots and tubers from uninoculated plants grown in each of the zinc concentrations were then submitted to Waite Analytical Services (Adelaide, Australia) for nutrient content analysis.

7.3 Results

7.3.1 First zinc experiment - "closed-pot" (2008)

Plants were harvested after only 2 months due to early senescence caused by hot weather (15 days with maximum over 35ºC) in March 2008.

7.3.1.1 Disease symptom severity and plant growth parameters

Because of the damage to plant tops, including moisture loss and sun-browned stems, the severity of stem necrosis could not be assessed accurately. DNA of *R. solani* AG 2-1, AG 2-2 and AG 3 was not detected in soil samples taken from control pots prior to planting and analysed by QPCR (Table 7.2). Root tissue had begun to deteriorate, confounding measurement of root weights. No tubers were produced; hence the influence of treatments on the severity of sclerotia on tubers could not be evaluated.

Table 7.2 Amount of *R. solani* DNA in qPCR after DNA was extracted by SARDI from samples of inoculated and uninoculated sand, prior to planting potato seed in the preliminary zinc experiment

Inoculum	DNA of *R. solani* (pg/g soil)		
	AG 2-1	AG 2-2	AG 3
control	0	0	0
AG 3	0	0	293

7.3.1.2 Analysis of zinc content of stem tissue

Results of nutrient analysis of stem tissue sections, with leaves removed, from two uninoculated plants are presented in Table 7.3. The concentration of zinc differed significantly in plants grown in the various zinc treatments. The mean stem tissue concentration of plants grown in sand amended with 3 ppm zinc (470 ppm) was significantly ($P < 0.05$) greater than those grown in sand unamended (20 ppm), amended with 0.1 ppm (17 ppm) and amended with 1 ppm (49 ppm).

7.3.1.3 Analysis of stem tissue for the content of nutrients other than zinc

Iron content varied significantly ($P < 0.05$) in the stems of the plants grown in sand amended with different concentrations of zinc (Table 7.3b). Plants grown in sand amended with 1 ppm zinc had significantly ($P < 0.05$) higher mean stem tissue iron concentration than plants grown in either unamended sand or sand amendment with 3 ppm of zinc.

None of the other elements tested differed significantly ($P < 0.05$) among the plants grown in different zinc concentrations. The mean values determined from all plants tested (grand mean) are shown in Table 7.3b and compared with the "sufficient" range of elements for potato plant growth. Elements that were low compared with the normal range recommended by Maier and Shepard (1998) included: boron (B), cobalt (Co), iron (Fe), manganese (Mn) and potassium (P). Elements that were high compared with the normal range recommended by Maier and Shepard (1998) included: calcium (Ca), potassium (K), magnesium (Mg) and sulfur (S).

Recommended ranges of aluminium, copper and sodium were not available for whole shoots of potato plants at the vegetative stage of growth. Nitrogen content was not assessed as the method of sample preparation did not allow accurate determination. Attempts were made to analyse titanium, chromium, cadmium, lead, arsenic and selenium content of the stem tissue, however, the concentrations of these elements were below the limit of the detection methods used.

Table 7.3 Mean nutrient content of stems collected from uninoculated plants cv Rusett Burbank grown for 2 months in sand amended with different concentrations of zinc in the first experiment. Two stems were analysed per zinc treatment.

a. Zinc and iron content where differences between values in a column were determined by the least significant difference at 5 % (l.s.d. 5 %) from ANOVA, as indicated by different letter.

Zinc soil addition	Nutrient concentrations (mg/kg)	
	Zn (l.s.d. 5 % = 139.4)	Fe (l.s.d. 5 % = 35.19)
0 ppm	20 a	75.2 a
0.1 ppm	17 a	90.6 ab
1 ppm	49 a	120.1 b
3 ppm	470 c	60.3 a

b. Content of nutrients other than zinc and iron where the grand mean[1] is compared with recommendations for potato whole shoots at the vegetative growth stage (of the cv. Russet Burbank if available) published in the "Plant analysis and interpretation manual for potato" (Maier and Shepard 1998).

[1] The grand mean was determined from all stems (8) analysed, with a mean dry weight of 0.27 g.
[2] The F probability (F Pr.) above 0.05 indicates no significant difference between plants grown in different concentrations of zinc hence the grand mean is used.

7.3.2 Second zinc experiment - "contained flow-through" (2008-09)

Because disease symptoms and accurate plant tissue weights were not adequately assessed in the preliminary experiment, a second experiment was carried out using the same rates of zinc as soil amendments.

7.3.2.1 Emergence of plants grown in different zinc concentrations and *R. solani* inocula

All uninoculated and AG 2-1-inoculated plants had emerged 10 days after planting (Table 7.4). Plants grown in the presence of AG 3 (R229) inoculum and zinc concentrations of 0.1 ppm or higher emerged significantly ($P < 0.05$) later. Mean dates of emergence in AG 3-inoculated treatments did not appear to be related to soil zinc concentration. The mean date of emergence was increased by 1.5 days in the presence of 0.1 ppm zinc, 4.4 days in the presence of 1 ppm zinc and 2.5 days in the presence of 3 ppm zinc. Thirty days after planting, all eight replicate plants had emerged from all treatments except those growing in AG 3 inoculum and 3 ppm zinc (Table 7.4). In this treatment two plants did not emerge, although one did emerge prior to harvest.

The number of stems that emerged from the control plants did not differ among zinc concentrations (Table 7.4). At any given zinc concentration no differences were observed in mean number of stems per plant between inoculated and uninoculated treatments (all treatment means > 1 stem). However, plants inoculated with the AG 2-1 isolate (Tas6) produced significantly ($P < 0.05$) fewer stems in 0 and 1 ppm than in 0.1 ppm zinc, and fewer in 1 ppm than in 3 ppm zinc. Conversely, plants inoculated with the AG 3 isolate (R229) had significantly ($P < 0.05$) fewer stems when grown in any zinc-amended sand than sand without zinc amendment.

7.3.2.2 Mean nutrient content of the fifth leaf from the growing tip at 30 days after planting potatoes in uninoculated sand containing different zinc concentrations

The mean nutrient content of the fifth leaf excised from three uninoculated plants, after 30 days of growth, is shown in Table 7.5. The concentration of zinc varied significantly ($P < 0.05$) among the plants grown in sand amended with different zinc concentrations (Table

7.5a). The mean zinc concentration of plants grown without zinc amendment (15 ppm) was less than the critical value for potato plant growth (20 ppm) (Maier and Shepard 1998). The seed piece (mean weight = 0.3 g) used to propagate these plants also had a zinc content (13 ppm) lower than this critical value (Table 7.5b). The mean concentration of zinc in the fifth leaf increased in proportion to the amount of zinc with which the soil was amended (Table 7.5a). The mean concentration of zinc in this tissue from plants grown in sand amended with 1 ppm zinc (37 ppm) was significantly ($P < 0.05$) greater than that of plants grown in sand with 0.1 ppm (15 ppm), and that of plants grown in 3 ppm (68 ppm) zinc was significantly ($P < 0.05$) greater than both of the lower concentrations.

Table 7.4 Emergence data for potato plants cv Russet Burbank grown for 3 months in different concentrations of zinc and either uninoculated or inoculated with *R. solani*

Inoculum	Zinc soil addition	Mean[1] values		
		Number of days before emergence (l.s.d. 5 % = 1.24 days)	Number of plants emerged at 30 days after planting (F Pr. = 0.69)	Mean no. of stems (l.s.d 5 % = 0.69)
control	0 ppm	10 a	8	1.6 abc
	0.1 ppm	10 a	8	2 bcd
	1 ppm	10 a	8	1.9 abcd
	3 ppm	10 a	8	2 bcd
AG 2-1	0 ppm	10 a	8	1.4 ab
	0.1 ppm	10 a	8	2.1 cd
	1 ppm	10 a	8	1.3 a
	3 ppm	10 a	8	2 bcd
AG 3	0 ppm	11.1 ab	8	2.5 d
	0.1 ppm	11.5 bc	8	1.5 abc
	1 ppm	14.4 d	8	1.6 abc
	3 ppm	12.5 c	6	1.8 abc

[1] Means of eight replicates were compared by ANOVA in which an F probability (F Pr.) < 0.05 indicates differences between treatments. These are indicated by the least significant difference at 5 % (l.s.d 5 %) and different letters between values in a column.

For nutrients other than zinc, the mean nutrient values, from the fifth leaf after 30 days of growth, from all 11 plants (grand mean) are shown in Table 7.5c. These are compared with the sufficient range of elements for potato plant growth as reported by Maier and Shepard (1998). Nutrients that were low when compared with these normal ranges included: boron (B), calcium, (Ca), cobalt (Co), iron (Fe) and magnesium (Mg). Nutrients that were high for the normal range included: potassium (K), phosphorus (P) and sulfur (S). Manganese (Mn) was within the normal range for optimal growth of potato plants during the vegetative stage. Based on these results of petiole analysis, drenches of the basal amendment solutions (excluding zinc) were applied (refer to section 7.2.2).

Recommended ranges of aluminium, copper and sodium were not available and nitrogen, titanium, chromium, cadmium, lead, arsenic and selenium content was not assessed, for reasons noted in section 7.3.1.3.

Table 7.5 Mean nutrient content of the fifth leaf from the growing tip of uninoculated potato plants after 30 days of growth and seed pieces used to propagate potato plants cv Russet Burbank grown in sand amended with various zinc concentrations

a. Mean content[1] of zinc of the fifth leaf from uninoculated plants after 30 days growth.

Sample type	Zinc soil addition	Mean tissue concentrations (mg/kg)
		Zn (l.s.d. 5 % = 5.77)
5th leaf	0 ppm	15 a
	0.1 ppm	15 a
	1 ppm	37 b
	3 ppm	68 c

[1] Three replicates were used to find the means of zinc treatments 0, 0.1 and 1 ppm while two were used to determine the mean for 3 ppm zinc.

b. Nutrient content seed piece of potato cv Russet Burbank for which four replicates were used to find mean values

Sample type	Mean weight	Mean tissue concentrations (mg/kg) of elements												
		Zn	Al	B	Ca	Co	Cu	Fe	K	Mg	Mn	Na	P	S
seed piece	0.3 g	13	3	5	373	< 0.9	1	22	16500	933	8	1397	3100	1590

c. Content of nutrients other than zinc from fith leaf of uninoculated plants after 30 days of growth for which grand means[1] were determined and compared with recommendations for whole shoots of potato at the vegetative growth stage (of the cv. Russet Burbank if available) published in the "Plant analysis and interpretation manual for potato" (Maier and Shepard 1998)

NOTE:
This table is included on page 151
of the print copy of the thesis held in
the University of Adelaide Library.

[1] Grand means were determined from all 11 plants (with a mean leaf dry weight of 0.22 g) as F probibility (F Pr) values > 0.05 indicated no significant differences between zinc treatments for these nutrients.

7.3.2.3 Severity of necrosis on above-ground stems at 30 days after planting in soils with various zinc concentrations and inoculation

The percentage of girdling by necrosis of the above-ground stem 30 days after planting is presented in Table 7.6. Some stem damage was observed on uninoculated plants. This was unexpected as DNA of *R. solani* AG 2-1, AG 2-2 or AG 3 was not detected in tests performed on the sand prior to planting (Table 7.7). However, isolation of *R. solani* from these lesions was not attempted until after plants were harvested.

AG 2-1-inoculated plants grown in sand with 0 ppm zinc had more necrotic area on the above-ground stem than similarly inoculated plants grown in 1 or 3 ppm zinc. All plants inoculated with the AG 3 isolate (R229) had necrosis circling the stem. All necrosis at this stage of growth was superficial; no deep lesions were observed and most plants produced healthy stem tissue in the following weeks.

Table 7.6 The percentage of above-ground stem area with necrosis on potato plants cv Russet Burbank uninoculated and inoculated with *R. solani* at 30 days after planting in sand ammended with zinc

Inoculum	Zinc soil addition	Mean[1] % of necrosis coverage of stem area[2] (l.s.d 5 % = 18.15)
control	0 ppm	16 a
	0.1 ppm	43 c
	1 ppm	3 a
	3 ppm	17 a
AG 2-1	0 ppm	36 bc
	0.1 ppm	20 ab
	1 ppm	14 a
	3 ppm	16 a
AG 3	0 ppm	96 d
	0.1 ppm	100 d
	1 ppm	94 d
	3 ppm	100 d

[1] Means were determined from eight replicates and variation between treatments, established from the least significant difference 5 % (l.s.d. 5 %), is indicated by different letters in the columns.
[2] Disease was assessed as described in section 7.2.2

Table 7.7 Amount of *R. solani* DNA present in inoculated and uninioculated sand used in the contained flow-through zinc experiment, assessed prior to planting potato seed

Inoculum	DNA of *R. solani* (pg/g soil)		
	AG 2-1	AG 2-2	AG 3
control	0	0	0
AG 3	4	0	3,374
AG 2-1	746	0	0

7.3.2.4 Growth parameters at harvest of plants grown in soils with various zinc concentrations and inocula

Plants in this experiment grew to maturity and were harvested 90 days after planting when two plants had begun to senesce, making it necessary to score stem disease before the stems browned. Uninoculated plants had significantly ($P < 0.05$) fewer tubers per plant when grown in sand amended with 3 ppm of zinc than those grown in lower zinc concentrations (Table 7.8). For uninoculated plants grown in 1 and 3 ppm of zinc, the mean weight of tubers per plant was significantly ($P < 0.05$) less than that of plants grown in 0 and 0.1 ppm zinc. Likewise, mean fresh weights of whole shoots were significantly less for plants grown in 1 and 3 ppm zinc than 0 and 0.1 ppm zinc, however, no differences were observed in mean fresh root weight of uninoculated plants.

Plants inoculated with the AG 2-1 isolate (Tas6) produced fewer tubers when grown in sand amended with 3 ppm, and the mean weight of tubers per plant was less ($P < 0.05$) when plants were grown in 1 and 3 ppm of zinc, than those grown in lower zinc concentrations. Mean fresh weights of whole shoots of AG 2-1-inoculated plants were lower ($P < 0.05$) when grown in 1 and 3 ppm than in 0.1 ppm and those grown in 0 ppm were the highest. However, mean fresh root weights were heavier ($P < 0.05$) in AG 2-1-inoculated than uninoculated plants grown in 0 ppm zinc. For AG 2-1-inoculated plants mean fresh root weight in 0 ppm zinc was larger ($P < 0.05$) than that observed in plants grown in 1 and 3 ppm zinc. Plants grown in 0.1 ppm also had larger mean root weight values than 3 ppm.

Plants inoculated with the AG 3 isolate (R229) and grown in 3 ppm zinc produced significantly ($P < 0.05$) fewer tubers than plants grown in 1 ppm zinc. However, the mean

weight of tubers per plant was significantly ($P < 0.05$) lower in 1 and 3 ppm than in 0 and 0.1 ppm. Mean fresh weights of whole shoots and roots were significantly ($P < 0.05$) heavier in plants grown without zinc than with all levels of zinc amendment. At some zinc concentrations, values of these growth parameters were lower than those observed in uninoculated plants, for example all parameters at 0.1 ppm zinc. However, in other concentrations there were no differences between values from uninoculated and AG 3-inoculated plants, for example all parameters at 1 ppm zinc.

Table 7.8 Mean values[1] at harvest of tissue weights and tuber numbers of plants of potato cv Russet Burbank grown for 3 months in the presence of different zinc concentrations and *R. solani* inoculum

Treatment			Mean weight (g)		
Inoculum	Zinc soil addition	Mean number of tubers per plant l.s.d 5 % = 1.429	Tubers per plant l.s.d. 5 % = 17.43	Fresh whole shoots l.s.d. 5 % = 11.97	Fresh root l.s.d. 5 % = 5.1
control	0 ppm	6.4 e	112 e	62.4 bc	16.2 cd
control	0.1 ppm	6 de	102.6 de	56 b	17.5 d
control	1 ppm	5.9 de	69.1 ab	38.1 a	15.1 bcd
control	3 ppm	5.5 bc	78.5 abc	34.5 a	15.9 bcd
AG 2-1	0 ppm	6.1 e	117.2 e	71.2 c	24.9 e
AG 2-1	0.1 ppm	6.4 e	106.2 de	62.5 b	19.9 de
AG 2-1	1 ppm	6 de	81.6 bc	37.1 a	19.3 d
AG 2-1	3 ppm	5.3 bc	72.9 ab	41.1 a	10.3 ab
AG 3	0 ppm	4.1 ab	93.8 cd	68 c	19.5 d
AG 3	0.1 ppm	4.4 abc	91.9 cd	37.7 a	11.9 abc
AG 3	1 ppm	5.6 cd	73 ab	35.4 a	10.8 ab
AG 3	3 ppm	3.6 a	61.3 a	37.5 a	8.8 a

[1] Means values were determined from eight replicate plants and compared by ANOVA to determine significant differences between treatments. These are indicated by the least significant differences (l.s.d) 5 % < 0.05 and are represented by different letters within a column.

7.3.2.5 Severity of Rhizoctonia disease at harvest of plants grown in soils with various zinc concentrations and inocula

The severity of Rhizoctonia disease symptoms observed on potato plants grown in the different zinc concentrations is presented in Table 7.9. No sclerotia were observed on tubers or roots from uninoculated plants grown in any zinc concentration. However, some necrosis was observed on stems and stolons, but there was no trend in response to concentration of zinc. Necrosis was less severe at 1 ppm zinc than 0 or 0.1, but was not significantly ($P > 0.05$) different from 3 ppm. *R. solani* was not isolated from these tissues and results from quantitative PCR indicate no AG 2-1 or AG 3 DNA was present.

Plants inoculated with the AG 2-1 isolate (Tas6) had significantly ($P < 0.05$) more tuber sclerotia in zinc concentrations of 1 and 3 ppm than 0 and 0.1 ppm. The median severity of stem necrosis indicated superficial to deep necrosis, however the differences were not significant between zinc or inoculum treatments. The least stolon disease observed on AG 2-1-inoculated plants was on those grown in 1 ppm zinc. The median value of this treatment indicated superficial necrosis, however, in other zinc concentrations deep necrosis was observed. At a zinc concentration of 3 ppm the severity of stolon necrosis of AG 2-1-inoculated plants was significantly ($P < 0.05$) greater than that observed on the equivalent uninoculated plants. Sclerotia were observed on the roots of all inoculated plants making it difficult to score symptoms of necrosis accurately.

Sclerotia were observed on tubers of all AG 3-inoculated plants. The median severity of AG 3-inoculated plants was more than 50 sclerotia on a tuber, significantly ($P < 0.05$) more than observed on any AG 2-1-inoculated or uninoculated plants. The median values of stem necrosis of AG 3-inoculated plants indicate superficial lesions, however, those observed on stolons were deep and caused pruning off. Stolon necrosis was most severe in AG 3-inoculated plants grown in 0.1 ppm zinc but this was not significantly ($P < 0.05$) different to those grown in 3 ppm zinc. Stolon necrosis was significantly ($P < 0.05$) more severe on AG 3-inoculated plants, at equivalent zinc concentrations, than observed for uninoculated plants grown in zinc concentrations of 0, 1 and 3 ppm and AG 2-1 inoculated plants grown in 0.1 and 1 ppm zinc.

Comparison of necrosis on stems resulted in a chi^2 probability of 0.066, indicating a lack of significant difference, although median values varied from 1.5 to 3 (data not shown). *R. solani* was isolated from the stem, stolon and root of AG 2-1 inoculated and AG 3-

inoculated but not uninoculated plants.

Table 7.9 Rhizoctonia disease[1] symptoms on plants of potato cv Russet Burbank at harvest after growth in the presence of different zinc concentrations and *R. solani* inoculum

Treatment		Sclerotia severity		Necrosis severity rank[2] and median[3]	
inoculum	Zinc soil addition	Tubers rank[2] and median[3]	Roots Y/N[4]	Stem chi[2] Pr. = 0.066	Stolon
control	**0 ppm**	(25.5) a 1	n	2	(41.9) bc 3
control	**0.1 ppm**	(25.5) a 1	n	2	(62.4) cde 3
control	**1 ppm**	(25.5) a 1	n	1.5	(19.6) a 1
control	**3 ppm**	(25.5) a 1	n	2	(27.3) ab 2
AG 2-1	**0 ppm**	(25.5) a 1	y	3	(52.7) cd 3
AG 2-1	**0.1 ppm**	(29.56) a 1	y	2	(53.6) cd 3
AG 2-1	**1 ppm**	(58) bc 2	y	2	(19.5) a 2
AG 2-1	**3 ppm**	(49.9) b 2	y	2	(57.6) cd 3
AG 3	**0 ppm**	(79.25) d 4	y	2	(48.8) cd 3
AG 3	**0.1 ppm**	(79.25) d 4	y	2	(78.2) e 4
AG 3	**1 ppm**	(81.12) d 4	y	2	(53.3) cd 3
AG 3	**3 ppm**	(74.79) cd 4	y	2	(63) de 3

[1] Disease was assessed as described in section 7.2.2
[2] Rank is the value in parenthesis, determined from Kruskal-Wallis analysis of eight replicate plants (8 degrees of freedom), for which the least significant differences (l.s.d) 5 % = 19.68 is indicated by different letters between values in a column.
[3] Median values were determined from eight replicate plants
[4] Y indicates the presence of sclerotia on roots, n indicates the absence of sclerotia on roots

7.3.2.6 Nutrients in whole shoots, roots and tubers after harvest from uninoculated plants grown in various zinc concentrations

The mean nutrient content from four uninoculated plants per zinc treatment is shown in Table 7.10. Two trends were observed in the change of concentration of nutrients in potato plant tissues, which were either directly or indirectly proportional to soil concentration of zinc.

Concentrations of nutrients in whole shoots are shown in Table 7.10a. The change in concentration of zinc (Zn), potassium (K) and manganese (Mn) was generally directly proportional to soil concentration of zinc. For example, plants grown in 3 ppm Zn had the highest concentrations of Zn in whole shoots. The change in concentration of aluminium (Al), calcium (Ca), magnesium (Mg) and titanium (Ti) was, in general, inversely proportional to soil concentration of Zn. For example, plants grown in 3 ppm Zn had the lowest concentrations of Ca in whole shoots.

The same two trends were also observed in the concentrations of nutrients in potato roots, which are shown in Table 7.10b. The concentration of Zn in soil was directly proportional to the root concentration of Zn, with plants grown in 3 ppm Zn having the highest root concentrations of Zn. Concentrations of sodium (Na) and sulfur (S) were generally, inversely proportional to soil concentrations of Zn, with plants grown in sand amended with 3 ppm Zn having the lowest concentrations of S in roots. The concentration of Al and Ti in roots of potato plants did not appear to be proportional to Zn levels in soils, as plants grown in sand amended with 1 ppm Zn had the lowest root concentrations of Al and Ti.

Concentrations of nutrients in potato tubers are shown in Table 7.1a. This tissue showed variation of most nutrients in relation to soil concentration of Zn. Nutrients that tended to increase along with the soil concentration of Zn were boron (B), copper (Cu), K, Mg, Mn, phosphorus (P), S and Zn. Concentrations of cobalt (Co) in tubers were inversely proportional to soil concentration of Zn as the plants grown in 3 ppm Zn had significantly ($P < 0.05$) less Co than plants grown in unamended soil. The concentration of Ca in tubers did not appear to be related to soil concentrations of Zn as plants grown in 1 ppm Zn had the highest concentration of Ca in tubers.

The concentrations of some nutrients did not vary when plants were grown in sand amended with different concentrations of Zn; these are shown in Table 7.11. Grand means of these nutrients, determined from plants grown in all Zn concentrations, were compared to the

normal range for potato tissues at maturity or harvest as recommended by Maier and Shepard (1998). Sulfur (0.62 % dry weight, equivalent to 6150 mg/kg of 0.3 g sample) was within the range reported for normal plant growth (0.34 to 0.8 % dry weight). In whole shoots B (12 mg/kg) was low compared to concentrations in Russet Burbank at maturity, which has been reported as 27 – 45 mg/kg (Maier and Shepard 1998). Iron (Fe) in tubers (19 mg/kg) was less than the values of 30 and 78 mg/kg reported for plants of the same cultivar grown in glasshouse conditions by Maier and Shepard (1998), although in other cultivars concentrations as low as 15 have been reported (Maier and Shepard 1998). The highest value reported for normal plant growth of P was 0.45 % of dry weight (Maier and Shepard 1998), therefore at 0.48 % (equivalent to 4875 mg/kg of 0.3 g sample) in whole shoots after harvest these plants had excess.

Although an attempt was made to measure tissue concentrations of other elements, results could not be determined for Mo in whole shoots and tubers, Ni, Ti and Cr in tubers and Cd, Pb and Se in all tissues as their concentrations were below accurate detection thresholds.

Table 7.10 Concentrations of elements in whole shoots, roots and tuber tissues from uninoculated, mature potato plants cv Russet Burbank grown in the presence of different zinc concentrations. Means were determined by ANOVA from four replicate plants from each zinc concentration and least significant differences (l.s.d.) 5 % are indicated by different letters in a column

a. Whole shoots

Sample type	Zinc soil addition	Mean tissue concentrations (mg/kg)						
		Zn l.s.d. 5 % = 38.3	Al l.s.d. 5 % = 10.7	Ca l.s.d. 5 % = 4326	K l.s.d. 5 % = 5196	Mg l.s.d. 5 % = 1176	Mn l.s.d. 5 % = 217	Ti l.s.d. 5 % = 0.304
Whole shoots	0 ppm	12.8 a	30.2 bc	30750 b	44250 a	8150 b	572 a	0.744 ab
	0.1 ppm	13.9 a	39.1 c	30750 b	45500 a	7875 b	612 a	0.922 b
	1 ppm	48 a	16.3 a	22800 a	55250 b	5575 a	940 b	0.347 a
	3 ppm	112.4 b	20.2 ab	20975 a	55000 b	5475 a	1360 c	0.455 a

b. Roots

Sample type	Zinc soil addition	Mean tissue concentrations (mg/kg)				
		Zn l.s.d. 5 % = 53.8	Al l.s.d. 5 % = 24.0	Na l.s.d. 5 % = 1431	S l.s.d. 5 % = 5534	Ti l.s.d. 5 % = 0.465
Roots	0 ppm	9.1 a	190.1 ab	4650 b	12575 b	4.214 b
	0.1 ppm	7.7 a	212.7 b	3325 ab	9775 ab	4.835 c
	1 ppm	46 a	174.4 a	2438 a	5325 a	3.507 a
	3 ppm	114.2 b	209.6 b	2698 a	4350 a	4.498 bc

c. Tubers

Sample type	Zinc soil addition	Mean tissue concentrations (mg/kg)									
		Zn l.s.d. 5 % = 5.2	B l.s.d. 5 % = 0.66	Ca l.s.d. 5 % = 184	Co l.s.d. 5 % = 0.359	Cu l.s.d. 5 % = 2.57	K l.s.d. 5 % = 2005	Mg l.s.d. 5 % = 117	Mn l.s.d. 5 % = 29.4	P l.s.d. 5 % = 490	S l.s.d. 5 % = 178
Tubers	0 ppm	3.42 a	2.08 a	540 a	1.991 b	6.20 a	18525 a	1050 a	52.7 a	3050 a	1590 a
	0.1 ppm	5.05 a	1.96 a	495 a	1.864 b	5.44 a	18875 a	1120 a	54.4 a	3450 a	1888 b
	1 ppm	23.35 b	3.05 b	842 b	1.224 a	11.37 b	21250 b	1450 b	63.9 a	4925 b	2650 c
	3 ppm	25.05 b	3.34 b	612 a	1.119 a	11.68 b	21375 b	1350 b	102.2 b	5075 b	2600 c

Table 7.11 Concentrations of elements from the tissue sections (mg/kg) tested from plants of potato cv Russet Burbank grown in the contained flow-through experiment. The grand mean was determined from all 16 plants grown in sand containing different concentrations of zinc as the F probability 5% (F Pr. > 0.05) indicated the values from each treatment were not significantly different from each other.

Tissue	Values	Elements												
		Al	B	Ca	Co	Cu	Fe	K	Mg	Mn	Na	Ni	P	S
Whole shoots	F Pr.		0.11		0.05	0.12	0.55				0.532	0.606	0.556	0.062
	Grand Mean		11.92		6.74	27.95	514				609	3.37	4875	6150
Roots	F Pr.		0.68	0.22	0.17	0.25	0.89	0.87	0.67	0.76		0.81	0.064	
	Grand Mean		6.7	27812	23.2	58.7	4212	16075	2851	957		38.6	1584	
Tubers	F Pr.	0.19					0.39				0.126			
	Grand Mean	9.6					18.95				22.2			

7.4 Discussion

The effect of zinc concentration on the severity of Rhizoctonia disease of potatoes was investigated.

7.4.1 First zinc experiment: "Closed-pot" (2007)

In a preliminary "closed-pot" experiment, zinc concentration in stems varied in proportion to the increase of soil amendment with zinc. The zinc content of tissues ranged from deficiency to sufficiency as intended, however, difficulties in assessing disease symptoms led to a subsequent "contained flow-through" experiment.

7.4.2 Second zinc experiment: "contained flow-through" (2008-09)

In this second experiment, delayed emergence was observed in plants grown in higher zinc concentrations when inoculated with *R. solani* AG 3. Previous research (Baker 1970; Errampalli *et al.* 2006; Wilson *et al.* 2008a) has shown that if necrosis girdles sprouts prior to emergence, secondary sprouts may subsequently emerge. Although this was a possible explanation for the delayed emergence in this experiment, examination after harvest showed that no girdling had developed on below-ground stems.

The emergence of uninoculated and AG 2-1-inoculated plants did not differ with soil concentration of zinc. Similarly, Langille and Batteese (1974) did not observe deficiency symptoms or reduced plant height or dry weights of plants grown in a solution culture system with the lowest zinc concentration applied (0 ppm).

7.4.2.1 At 30 days after planting

The nutrient content of whole shoots, roots and tubers was assessed after 30 days of growth to determine fertilisation regimes for the following months. Results indicated that zinc concentration varied in all tissues in proportion with the zinc concentration of soil, and ranged from deficiency to sufficiency as reported by Maier and Shepard (1998). At the same time, necrosis on above-ground stems of AG 2-1-inoculated plants, grown in sand amended with 1 and 3 ppm zinc was less severe than equivalent plants in sand without zinc, suggesting that amendment of soil with zinc may inhibit the development of early necrosis. However, this result was not reflected in AG 3-inoculated plants, where over 90% of the stem was covered

in superficial necrosis irrespective of zinc concentration. The above-ground stems of control plants also showed some necrosis, up to 43 % coverage, however, no *R. solani* was subsequently isolated from these plants after harvest.

Nutrient analysis of plants after 30 days of growth indicated that boron, calcium and magnesium were low, yet phosphorus and potassium were high when compared with the ranges recommended by Maier and Shepard (1998). The possible influence and interaction of these nutrients on Rhizoctonia disease is discussed in the following sections and their variation from that required for healthy potato plant growth may have influenced the disease observed on AG 3-inoculated potato plants after 30 days of growth. This explains the lack of variation between symptoms of inoculated plants grown in different zinc concentrations. To determine if zinc concentration affects emergence or early development of necrosis, repetition of this experiment would be required with management of other nutrients.

7.4.2.2 After harvest

After potato plants were harvested, nutrient analysis of whole shoots, roots and tubers showed that levels of zinc in plant tissue varied with soil concentration of zinc, covering the deficiency and sufficiency ranges documented by Maier and Shepard (1998). AG 2-1-inoculated plants grown in the higher zinc concentrations (1 and 3 ppm) had more sclerotia on tubers than those grown in the lower zinc concentrations (0 and 0.1 ppm), suggesting that higher rates of zinc may stimulate formation of sclerotia on tubers. This is consistent with *in vitro* experiments of Dijst (1988) where cultures maintained on nutrient rich media (malt peptone agar) produced more sclerotia than those on nutrient deficent media (water agar). However, this was not reflected in results for AG 3-inoculated plants as large numbers of sclerotia formed on tubers irrespective of zinc concentration.

No relationship was observed between severity of necrosis on stems and stolons after harvest and soil concentration of zinc. This differs from results from field studies of cereals (Thongbai *et al.* 1993a) and medic (Streeter *et al.* 2001a) where Rhizoctonia root disease was most severe in plants with low tissue concentrations of zinc. Further investigations are required to determine if zinc concentration affects the severity of necrosis on potato plants as some damage was observed on both stems and stolons of uninoculated plants, although *R. solani* was not isolated from these plants.

Growth parameters, such as whole shoot and root fresh and dry weights, of potato

plants were also assessed after harvest, to test the assumption that increased soil concentrations of zinc would improve growth and yield in both uninoculated and inoculated plants. In this experiment, uninoculated plants grown in unamended sand produced most tubers by number and weight and also the highest shoot fresh weight. This result differed from Puzina (2004) which found that application of a zinc sulphate solution resulted in increased number and weight of progeny tubers and the formation of tubers grown in low zinc soils by potato cv Skoroplodnyi. Further investigations of the nutrient status of plant tissues in this experiment indicated that several nutrients varied in either direct or indirect proportion with soil concentration of zinc, discussed in the following sections. Some of these nutrients are reported to affect both the growth of potato plants and disease severity. For example, both calcium and magnesium concentrations were highest in plants growing in the lower zinc concentrations and their role in disease tolerance (described below) suggests they may have confounded the severity of disease in this experiment.

The variation in concentrations of nutrients in potato tissues at harvest may be associated with the differences in tissue zinc concentration observed as early as 30 days after planting. For example, high soil concentations of zinc can decrease boron uptake of tomato plants and the associated symptoms of boron toxicity (Gunes *et al*. 1999). The soil environment in this experiment may also have influenced uptake of nutrients as a negative correlation has been observed between the concentration of zinc and cation exchange capacity of soils (Ownley *et al.* 2003). Therefore, in future experiments to assess the severity of stem necrosis steps hould be taken to minimise the impact of other nutrients, for example, by making experiments short-term. However, influence on fertilise regimes must also be investigated in plants grown to maturity, to assess the impact on tuber yield and impact on fertiliser usage.

7.4.2.3 Influence of nutrients on disease

Previous research (reviewed by Datnoff *et al.* 2007) has shown that boron, calcium, magnesium, phosphorus and potassium all influence plant growth and disease. Kataria and Sunder (1985) increased boron application to cowpea and Kataria and Grover (1987) did likewise to mungbean and both treatments reduced Rhizoctonia disease. These authors suggested that boron contributes to a physical barrier to pathogen penetration, based on previous findings of Lewis (1980) who showed that boron is involved in lignin biosynthesis.

Calcium is abundant in healthy cell walls and strawberry plants deficient in this nutrient show collapsed cell wall structure (Chiu and Bould 1976). Therefore, in calcium-deficient conditions the leakiness of membranes may increase, attracting pathogens such as *R. solani,* and resulting in more disease, as was observed for peanuts by Hallock and Garren (1986). High levels of potassium may also have contributed to reduced calcium uptake, as found in corn seedlings by Claassen and Wilcox (1974). Kruistufek *et al.* (2000) showed that calcium accumulates to higher levels in tuber periderm of potato cultivars more susceptible to common scab (caused by *Streptomyces scabies*) than tolerant cultivars, but this has not been studied for black scurf of potato caused by *R. solani*.

Magnesium is not associated with a physical barrier to penetration by *R. solani* but is associated with rapid growth of plant cells, and 90 % of the magnesium in plant tissues is associated with ribosomes (Huber 1981). High levels of magnesium have been associated with tolerance of cotton, bean and soybean to Rhizoctonia disease, but contributed to Rhizoctonia disease of peanut (reviewed by Datnoff *et al.* 2007).

Potassium has been shown to have variable effects on potato canker caused by *R. solani* (reviewed by Datnoff *et al.* 2007), however, it is unknown what influence excess potassium, as found in this experiment, will have on disease severity.

Pathogenicity can be influenced by fungal growth rate, therefore, to verify that addition of nutrients to soil altered the plant's tolerance of disease and not simply the growth of the pathogen, a soil baiting experiment was attempted (data not shown). This experiment consisted of sand prepared with nutrient solutions as described in 7.2.2. A single point of inoculation was achieved with three AG 3-colonised millet seeds and mycelium advancing from that focus was to be detected with sterilised toothpicks, placed at 1 to 2 cm intervals, as described by (Siwek 1997). These toothpicks were removed daily and replaced with new ones, however, over the 10 days of this experiment no mycelial growth of *R. solani* from the toothpicks plated on Ko & Hora media was observed. It is possible that the sand was too dry to allow colonisation by *R. solani*, as toothpick baiting techniques are commonly used to isolate *R. solani* from field soils (pers. comm. S. Pederick 2008). During *in vitro* experiments, Jacobs *et al.* (2002) found that although an *R. solani* AG 4 isolate was able to solubilise zinc phosphate no difference in growth or final biomass (dry weight) was observed in cultures grown with or without this phosphate. However, zinc fertilisers are normally inorganic salts such as zinc sulphate (Havlin *et al.* 1999) and, if plant tolerance to *R. solani* in response to zinc application was to be confirmed in future studies, any effects of this treatment on fungal

growth rate would need to be accounted for.

7.4.2.4 Conclusion

Sand culture procedures used for cereal experiments by Genc *et al.* (2006) were adapted to grow potatoes. Tissues, such as whole shoots, petioles, roots and tubers from the plants grown in these experiments contained different concentrations of zinc, based on the amount of zinc applied to the sand prior to planting. In these experiments, the range of tissue concentrations spanned deficiency and sufficiency ranges previously documented from potato plants grown in a solution culture system (Langille and Batteese 1974). However, interpretation of the effect of zinc concentration in potato tissue on disease severity was confounded by the variation of other nutrients. Therefore, further studies are required before any conclusion can be drawn on the effect of soil concentration of zinc on Rhizoctonia disease of potato.

8 General discussion

Three anastomosis groups of *Rhizoctonia* (AG 2-1, AG 2-2 and AG 3) were recovered and identified from potato plants from fields sampled in South Australia and Tasmania. Classification using AG-specific primer sets in PCR was consistent with classification via classical techniques. Isolates of AG 2-1, AG 2-2 and AG 3 all caused stem and stolon necrosis in pathogenicity experiments, reflecting the tissues from which these groups were recovered. AG 3 formed the most sclerotia on tubers. Although AG 4, AG 5 and AG 8 were not recovered from these field samples, AG 4 and AG 5 caused stem necrosis and AG 8 root necrosis on inoculated plants. Both AG 2-1 and AG 3-inoculated plants produced lower yields than uninoculated plants; however, plants exposed to the different inocula displayed different combinations of necrosis and sclerotium development that contributed to these reduced yields. These results suggest that no one AG or symptom is linked to reductions in tuber yield and quality. Variation was also observed in the severity of symptoms caused by isolates from the same AG. Links between pathogenic and genetic variation previously identified among AG 2-1 isolates (Woodhall *et al.* 2007) were explored and significant sequence variation within this group was observed for the IGS1 and ITS regions of DNA. Sequence variation was generally consistent between the two DNA regions, but this variation was not linked to pathogenicity.

Early detection of soil-borne pathogens, prior to planting, is a necessary component of assessing the risk of crop loss, in terms of yield quality or quantity, as this is when disease management strategies are instigated. Species-specific molecular tests have been developed to facilitate the detection and quantification of soil-borne pathogens of potato (Lees *et al.* 2005) and are available as research tools, however, they have not been adapted for commercial delivery to industry. In contrast, the Australian cereal industry has had access to DNA-based testing services since 1997, to assess the risk of soil pathogens prior to planting (Ophel-Keller *et al.* 2008).

An ability to predict the disease risk of fields used for potato production based on inoculum levels would be useful, as there is currently no simple approach to do this. Ophel-Keller *et al.* (2008) specified the requirements necessary to deliver this technology to cereal growers and, of this list of requirements, the following three have been fulfilled (i) systems to track samples, manage data and generate reports; (ii) sampling strategies that result in a soil sample representing the pathogen level in a field; and (iii) a DNA extraction system capable

of processing relatively large samples. The present project has contributed to fulfilling further requirements for achieving a soil diagnostic test for potato growers (Ophel-Keller *et al.* 2008) through the validation of AG-specific DNA tests for the *R. solani* groups pathogenic to potato. Although tests specific to *R. solani* AGs are available, two requirements have yet to be addressed before soil diagnostic tests can be developed. The quantitative AG-specific assays need to be sensitive enough to detect the AGs of *R. solani* at levels below the economic threshold. Also, an understanding is needed of the relationship between the amount of each *R. solani* AG and the risk of disease (Ophel-Keller *et al.* 2008). These requirements are more difficult to address for the potato industry than the cereal industry, because of the multiple pathogenic AGs of *R. solani* and inoculum sources (soil and seed) that must be taken into account for potato plants, as discussed below.

One of the barriers to using a soil test in the assessment of the risk of Rhizoctonia disease in potato crops is the current inability to evaluate the contribution of seed-borne inoculum to disease. Wicks *et al.* (1995) showed that sclerotia on seed-tubers in Australia, generally caused by AG 3 (de Boer *et al.* 2001), contributed to Rhizoctonia disease. This project supported the findings of Campion *et al.* (2003) and de Boer *et al.* (2001) that AG 2-1 isolates were also present on tubers and seed-tubers, respectively, and that this group could contribute to disease symptoms on progeny tubers. Results such as these endorse the current seed certification standards in Australia, which are designed to ensure that seed tubers are free from contamination with visible sclerotia (Holland and Spencer 2007).

The contribution of *R. solani* mycelium associated with tubers, but not visible with the naked eye, is not considered in current seed certification standards. Superficial mycelium is common on tubers and was observed on the surface of tubers collected from South Australian fields during this study. However, isolation of the fungus was not achieved and consequently AG was not determined (data not shown). This supports results of Hide (1981), who microscopically observed hyphae adhering to 99 % of seed tubers collected in England and Wales, even when sclerotia were sparse. In an experiment conducted in 1965, researchers investigated Rhizoctonia disease and yield of potato plants grown from sclerotia-free seed-tubers treated with an organo-mercury fungicide to eliminate mycelia, compared with similarly treated tubers inoculated with a mycelial suspension before planting (Hide *et al.* 1973). Hide *et al.* (1973) found that plants originating from the seed tubers with mycelial inoculum produced more progeny tubers with sclerotia than did plants originating from seed

tubers without mycelia, showing that this inoculum source can contribute to Rhizoctonia disease. The research described above implies that the current standards should be revised. For this reason, detection and quantification of seed tuber-borne inoculum of *R. solani* will be a necessary component of the development of pre-plant tests, to complement soil detection, for potato crops. As *R. solani* generally does not damage the tuber surface (Weinhold *et al.* 1982), it is possible to detect and quantify inoculum on peel using PCR techniques similar to those used to quantify DNA in soil (pers. com. K. Ophel-Keller 2009). However, further experiments would be required to link the amount of pathogen DNA in peel to disease severity and economic loss in different soils and for various potato cultivars. To make this link, experiments should be conducted with varying amounts of mycelium and sclerotia on seed tubers, a sub-sample of which could be tested using quantitative PCR then planted in sterilised soil and grown to allow subsequent assessment of disease symptoms on plant tissues and progeny tubers.

Even if the above methodology for detection of seed and soil-borne inoculum of *R. solani* allowed estimation of disease potential, it is necessary to understand the relationship between amount of inoculum, risk of disease and the subsequent threshold at which the impact on yield becomes a significant economic cost, as discussed for seed above. However, multiple AGs are involved in Rhizoctonia disease of potato plants (as discussed in chapter 4) and, hence, these need to be considered when developing disease risk thresholds. The contribution of inoculum of individual AGs to disease severity and, ultimately, yield needs to be investigated further. The influence of various AGs and inoculum sources could be assessed individually in experiments conducted in a shade-house or controlled environment conditions or could be monitored in the field, the latter incorporating data on environmental factors that influence the expression of disease (discussed later).

One cultural practice that can affect Rhizoctonia disease of potato, by influencing the amount of soil-borne inoculum, is crop rotation. For example, brassica crops can reduce Rhizoctonia disease of potatoes, a response attributed to the production of isothiocyanates during decomposition of brassica tissue (de Boer *et al.* 2001; Kirkegaard *et al.* 1994; Larkin and Griffin 2007). However, the impact of brassicas on quantity of inoculum is yet to be investigated. Similarly, the impact of rotational crops that are also alternative hosts of potato-pathogenic AGs (Hitch *et al.* 2007; Hoong *et al.* 2007; Pederick *et al.* 2007) has not been investigated. This suggests that future field experiments could involve long-term experiments

to investigate crop rotation practices involving potato, to identify those which minimise soil-borne inoculum of all relevant AGs.

Fungicide application to soil and tubers is commonly used to reduce inoculum of *R. solani* and associated disease symptoms. Most of the fungicides registered in Australia to control Rhizoctonia disease of potato crops have been developed for application to seed tubers. However, limitations in the control of soil-borne inoculum have led to the development of targeted in-furrow applications (Taylor 1993). In this project, fungicides were tested for their ability to inhibit mycelial growth of *R. solani* AGs and, for most isolates tested, all fungicides reduced growth *in vitro* by more than 80 %. However, there were a few exceptions, including reduced sensitivity of AG 8 isolates to Monceren (a.i. pencycuron) and of AG 2-1 isolates to Rovral (a.i. iprodione).

In shade-house experiments, stem necrosis caused by AG 2-1 and tuber sclerotia caused by AG 3 were most effectively controlled by Amistar applied in-furrow. Initially Amistar was the only fungicide applied in-furrow while other fungicides were applied to seed tubers. However, further experiments showed that disease control achieved by the same fungicide can be influenced by application method (ie. when both Rovral and Amistar were applied to seed and in-furrow). A similar response was reported for Rhizoctonia disease of barley when control using furrow application of fungicides was influenced by cultivation method; fenbuconazole exacerbated disease in cultivated plots but controlled it in direct drilled sites (Cotterill 1993). Further experiments are required to evaluate the most cost-effective combinations of seed and furrow applications that reduce *Rhizoctonia*-associated yield loss of potato, while minimising fungicide usage. The application of fungicides to both furrow and seed has long been used to control Rhizoctonia disease of cotton (Bell and Owen 1963; Bird *et al.* 1957). However, the cost of these practices has prompted research to reduce the use of fungicides. For example, Hancock *et al.* (2004) reduced fungicide use by 50 % while maintaining equivalent disease control when fungicide sprays were pulsed to surround and cover individual cotton seed, rather than employing a continuous spray method. This method of application may be adapted to suit the machinery used for planting potato crops.

It has become apparent in the course of this project (see chapter 5) and in research elsewhere (Campion *et al.* 2003) that some *R. solani* isolates and AGs are less sensitive to some fungicides than to others. Monitoring of inoculum levels of fungicide-insensitive isolates may be as useful as detecting AGs in helping growers to selecting chemicals for

disease management. In this study, variation was observed in fungicide sensitivity of AG 2-1 isolates but this was not related to genetic variation in the IGS1 region. This suggests that IGS variation is not a useful predictor of sensitivity to iprodione fungicide. This region was investigated as the mechanism that confers resistance to iprodione has not yet been well described, and it was hoped that IGS sequence information may be diagnostic of fungicide-insensitive isolates. However, other fungicides, such as Amistar, a Qo inhibitor for which mutations that confer resistance have been described, may be more amenable to the development of molecular tools for monitoring resistance, as was described by Zhang *et al.* (2009) for *Penicillium digitatum*, which causes postharvest decay of citrus. Resistance to Qo inhibitors is conferred by a single site mutation in the cytochrome *b* gene (see section 1.5.1.6), however has not yet been reported in *R. solani*. As such there is currently little incentive to develop molecular tools for detecting Amistar resistant isolates.

Another strategy for disease management is to increase the tolerance of plants to infection, which means that the plant can maintain growth and yield in spite of being infected. Increased tolerance to Rhizoctonia disease in cereal (Thongbai *et al.* 1993b) and medic (Streeter *et al.* 2001a) crops has been associated with optimal nutrition of the plants. This reduces the attraction to and penetration of hosts by *R. solani*, by limiting the membrane leakage that occurs in micronutrient-deficient plants (Cakmak and Marschner 1988a; Cakmak and Marschner 1988b; Welch and Norvell 1993). The feasibility of increasing the tolerance of potato plants to Rhizoctonia disease by manipulating zinc nutrition was investigated. Although zinc application reduced the mild stem necrosis caused by AG 2-1 at 30 days after planting, it had no effect on the more severe stem necrosis caused by AG 3, the levels of sclerotia developing on tubers or yield at maturity. These results are in contrast to those studies of cereal and medic mentioned above but do support the findings of Cooke (2000) that zinc content, as applied to soil or initially in the seed, had little effect on the tolerance of wheat to infection by *Rhizoctonia*.

In the same experiments, Cooke (2000) showed that zinc-deficient wheat plants had decreased root length, although not dry matter content, compared with controls, suggesting that growth of fibrous roots was impaired. *R. solani* can also damage roots of potato and reduce yield (chapter 4) and, although visual assessments of roots were conducted, the focus was on damage caused by necrosis and the presence of sclerotia, while root length and architecture were not addressed. Root architecture is important in the uptake of nutrients by

plants; for example, Silberbush and Barber (1983) found phosphorus uptake of soybean plants was increased more by a larger root surface area than a number of other factors, including root length and the initial concentration of the nutrient in soil solution. Root architecture is also a factor that contributes to the uptake of manganese in wheat (Sadana *et al.* 2002) and, more specifically, fine roots have been implicated in uptake of zinc in wheat (Dong *et al.* 1995). Current methods for assessment of root damage are time consuming and, to allow for more detailed investigation of root architecture in future experiments, high resolution scanners and computer software may be useful. For example, the commercially available program, WinRHIZO, has been used to link the loss of wheat roots, due to damage caused by *Pythium* species, with stunting of the plants (Higginbotham *et al.* 2004). Using this technology, studies of potato plant root morphology may provide an indicator of tuber yield losses. Subsequent experiments might then allow scientists to infer the impact of environmental factors, including nutrient deficiencies, on yield in short-term experiments. Such short-term experiments would avoid complications resulting from growing plants to maturity, such as the nutrient interactions associated with altered manganese uptake during zinc amendment experiments in this project (see chapter 7).

The success of any crop management strategy at limiting disease is a complex process, influenced by the environment, plant vigour and pathogen population, as previously discussed. A key factor influencing the severity of Rhizoctonia disease is the virulence of the pathogen. Pathogenic variation has been observed not only among AGs but also among isolates of the same AG. As described in section 1.2.3, the AG classification system is not based on pathogenicity determinants. No single gene has been associated with pathogenicity of *R. solani*, in fact, multiple proteins have been implicated in the infection process (Govozdeva *et al.* 2006). In contrast, an assay has been developed which identifies a virulence determinant in inoculum of *Streptomyces* species. This assay detects *nec1* (Cullen and Lees 2007), a gene closely linked to those responsible for biosynthesis of the phytotoxin thaxtomin, which confers pathogenicity to potato and causes the symptoms of common scab (Healy *et al.* 2000). It is possible that molecular tests for the detection of *R. solani* could be refined to produce more accurate disease risk thresholds if genes directly associated with pathogenicity were targeted. Therefore research is underway to identify candidate pathogenicity-related genes. One research group has used microarray technology to evaluate pathogenicity genes involved during various stages of the infection of potato sprouts by *R. solani* (Rivard and Jabaji-Hare 2006). The genes and gene- products identified during this research, such

as the fungal genes encoding β-glucosidase and volvatoxin (Jabaji 2008), the latter a pore-forming toxin that may insert into a membrane (Lin *et al.* 2004), could be considered as targets for future diagnostic tests and disease control strategies.

Genes involved in defence of the potato plant are being studied (Jabaji 2008) and these findings could contribute to breeding programs also. Natural resistance, where *R. solani* fails to establish an infection, has not yet been identified in potato breeding programs (Leach and Webb 1993). However, the gene, *snakin-1*, has been identified as a component of the potato plant's limited natural defence system against microbial infection (Segura *et al.* 1999). The low molecular-weight peptide encoded by the *snakin-1* gene was found in tubers, stems, axillary and young floral buds but did not respond to biotic or abiotic stimuli (Segura *et al.* 1999). Constitutive up-regulation of the expression of this gene produced plants that were less susceptible to stem damage than normal plants (Almasia *et al.* 2008). These genetically modified potato plants are not being used in commercial breeding programs due to adverse attitudes of consumers and restrictive legislation of many countries, including Australia. However, these problems could be circumvented by targeting genes in traditional breeding programs. One way of altering gene expression is by using mutagenesis, for example, the wheat cultivar Scarlet-Rz1 has resistance to *R. solani* AG 8 induced by ethyl methane sulphonate (EMS) mutagenesis (Okubara *et al.* 2009). The inheritance of resistance over four subsequent generations appears to be as a single co-dominant gene (Okubara *et al.* 2009) and is the first *Rhizoctonia*-resistance available to wheat breeding programs. Therefore, although little natural resistance has been identified in potato plants the possibility exists to create *Rhizoctonia*-resistant breeding material using mutagenesis technologies. However, if this technique is used in future potato plant breeding programs it will be necessary to consider the multiple AGs that are present and have the potential to cause disease both in Australia and world-wide.

In conclusion, the research presented here has expanded knowledge of the association of *R. solani* AGs with potato crops in Australia and the disease symptoms they can cause. Newly developed diagnostic tests were successfully used to identify the AGs of isolates from Australian sites that supply the processing potato industry. Fungicides were shown to vary in effectiveness at controlling both mycelial growth and disease caused by the various AGs, and micronutrient supply was explored as a means for non-chemical control of Rhizoctonia disease in potato crops.

9 References

Adams GC (1988) *Thanatephorus cucumeris* (*Rhizoctonia solani*), A species complex of wide host range. In 'Advances in Plant Pathology'. (Eds DS Ingram, PH Williams) pp. 535-550. (Academic Press Limited).

Almasia NI, Bazzini AA, Hopp HE, Vazquez-Rovere C (2008) Overexpression of *snakin-1* gene enhances resistance to *Rhizoctonia solani* and *Erwinia carotovora* in transgenic potato plants. *Molecular Plant Pathology* **9**, 329-338.

Anderson NA, Stretton HM, Groth JV, Flentje NT (1972) Genetics of Heterokaryosis in *Thanatephorus cucumeris*. *Phytopathology* **62**, 1057-1065.

Australian, Bureau, of, Statistics, (A.B.S.) (2005-06) http://www.abs.gov.au/AUSSTATS/abs@.nsf/DetailsPage/7112.02005-06?OpenDocument. (Agricultural Commodities, Australia (7121.0)).

Baghour M, Moreno DA, Villora G, Lopez-Cantarero I, Hernandez J, Castilla N, Romero L (2002) Root-zone temperature influences the distribution of Cu and Zn in potato-plant organs. *Journal of Agricultural and Food Chemistry* **50**, 140-146.

Bains PS, Bennypaul HS, Lynch DR, Kawchuk LM, Schaupmeyer CA (2002) Rhizoctonia disease of potatoes (*Rhizoctonia solani*): Fungicidal efficacy and cultivar susceptibility. *American Journal of PotatoResearch* **79**, 99-106.

Bains PS, Bisht VS (1995) Anastomosis group identity and virulence of *Rhizoctonia solani* isolates collected from potato plants in Alberta, Canada. *Plant Disease* **79**, 241-242.

Baker KF (1957) The U.C. System for producing healthy container-grown plants. (University of California Press, Berkeley).

Baker KF (1970) Types of Rhizoctonia diseases and their occurrence. In '*Rhizoctonia solani*: Biology and pathology'. (Ed. JR Parmeter) pp. 125-148. (University of California Press: Berkeley).

Balali GR (1996) Genetic variation of Rhizoctonia solani AG-3 in South Australia. PhD thesis, University of Adelaide, Australia.

Balali GR, Neate S, Kasalkheh AM, Stodart BJ, Melanson DL, Scott ES (2007) Intraspecific variation of *Rhizoctonia solani* AG 3 isolates recovered from potato fields in Central Iran and South Australia. *Mycopathologia* **163**, 105-115.

Balali GR, Neate SM, Scott ES, Whisson DL, Wicks TJ (1995) Anastomosis group and pathogenicity of isolates of *Rhizoctonia solani* from potato crops in South Australia. *Plant Pathology* **44**, 1050-1057.

Balali GR, Whisson DL, Scott ES, Neate SM (1996) DNA fingerprinting probe specific to isolates of *Rhizoctonia solani* AG-3. *Mycological Research* **100**, 467-470.

Bandy BP, Leach SS, Tavantzis SM (1988) Anastomosis group 3 is the major cause of Rhizoctonia disease of potato in Maine. *Plant Disease* **72**, 596-598.

Bandy BP, Zanzinger DH, Tavantzis SM (1984) Isolation of anastomosis group 5 of *Rhizoctonia solani* from potato field soils in Maine. *Phytopathology* **74**, 1220-1224.

Barnett SJ, Roget DK, Ryder MH (2006) Suppression of *Rhizoctonia solani* AG-8 induced disease on wheat by the interaction between *Pantoea*, *Exiguobacterium*, and Microbacteria. *Australian Journal of Soil Research* **44**, 331-342.

Bell DK, Owen JH (1963) Effect of soil temperature and fungicide placement on cotton seedling damping-off caused by *Rhizoctonia solani*. *Plant Disease Reporter* **47**, 1016-1021.

Beukema HP, van der Zaag DE (1990) 'Introduction to potato production.' (Centre for Agricultural Publishing and Documentation (Pubdoc): The Netherlands).

Bharathan N, Saso H, Gudipati L, Bharathan S, Whited K, Anthony K (2005) Double-stranded RNA: distribution and analysis among isolates of *Rhizoctonia solani* AG-2 to -13. *Plant Pathology* **54**, 196-203.

Bird LS, Ranney CD, Watkins GM (1957) Evaluation of fungicides mixed with the covering-soil at planting as a control measure for the cotton seedling-disease complex. *Plant Disease Reporter* **41**, 165-173.

Bounou S, Jabaji-Hare SH, Hogue R, Charest PM (1999) Polymerase chain reaction-based assay for specific detection of *Rhizoctonia solani* AG-3 isolates. *Mycological Research* **103**, 1-8.

Boysen M, Borja M, Moral C, Salazar O, Rubio V (1996) Identification at strain level of *Rhizoctonia solani* AG4 isolates by direct sequence of asymmetric PCR products of the ITS regions. *Current Genetics* **29**, 174-181.

Brewer MT, Larkin RP (2005) Efficacy of several potential biocontrol organisms against *Rhizoctonia solani* on potato. *Crop Protection* **24**, 939-950.

Brody JR, Kern SE (2004) Sodium boric acid: a Tris-free, cooler conductive medium for DNA electrophoresis. *BioTechniques* **36**, 214-216.

Burton WG (1966) 'The Potato.' (Longman Scientific & Technical: England).

Cakmak I, Marschner H (1988a) Enhanced superoxide radical production in roots of zinc-deficient plants. *Journal of Experimental Botany* **39**, 1449-1460.

Cakmak I, Marschner H (1988b) Zinc-dependent changes in ESR signals, NAPDH oxidase and plasma membrane permeability in cotton roots. *Physiologia Plantarum* **73**, 182-186.

Campion C, Chatot C, Perraton B, Andrivon D (2003) Anastomosis groups, pathogenicity and sensitivity to fungicides of *Rhizoctonia solani* isolates collected on potato crops in France. *European Journal of Plant Pathology* **109**, 983-992.

Carling DE (1996) Grouping in *Rhizoctonia solani* by hyphal anastomosis reaction. In *'Rhizoctonia* species: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control'. (Eds B Sneh, S Jabaji-Hare, S Neate, G Dijst) pp. 37-47. (Kluwer Academic Publishers: the Netherlands).

Carling DE, Baird RE, Gitaitis RD, Brainard KA, Kuninaga S (2002a) Characterisation of AG-13, a newly reported anastomosis group of *Rhizoctonia solani*. *Disease Control and Pest Management* **92**, 893-899.

Carling DE, Helm DJ, Leiner RH (1990) *In vitro* sensitivity of *Rhizoctonia solani* and other multinucleate and binucleate *Rhizoctonia* to selected fungicides. *Plant Disease* **74,** 860-863.

Carling DE, Kuninaga S (1990) DNA base sequence homology in *Rhizoctonia solani* Kuhn: inter- and intragroup relatedness of anastomosis group-9. *Phytopathology* **80**, 1362-1364.

Carling DE, Kuninaga S, Brainard KA (2002b) Hyphal anastomosis reactions, rDNA-internal transcribed spacer sequences, and virulence levels among subsets of *Rhizoctonia solani* ansatomosis group-2 (AG-2) and AG-BI. *Phytopathology* **92**, 43-50.

Carling DE, Kuninaga S, Leiner RH (1988) Relatedness within and among intra-specific groups of *Rhizoctonia solani* - a comparison of grouping by anastomosis and by DNA hybridisation. *Phytoparasitica* **16**, 209-210.

Carling DE, Leiner RH (1986) Isolation and characterization of *Rhizoctonia solani* and binucleate *R. solani*-like fungi from aerial stems and subterranean organs of potato plants. *Phytopathology* **76**, 725-729.

Carling DE, Leiner RH (1990) Virulence of isolates of *Rhizoctonia solani* AG-3 collected from potato plant organs and soil. *Plant Disease* **74**, 901-903.

Carling DE, Leiner RH, Kebler KM (1986) Characterization of *Rhizoctonia solani* and binuleate rhizoctonia-like fungi collected from Alaskan soils with varied crop histories. *Canadian Journal of Plant Pathology* **8**, 305-310.

Carling DE, Leiner RH, Kebler KM (1987) Characterization of a new anastomosis group (AG-9) of *Rhizoctonia solani*. *Phytopathology* **77**, 1609-1612.

Carling DE, Pope EJ, Brainard KA, Carter DA (1999) Characterisation of mycorrhizal isolates of *Rhizoctonia solani* from an orchid, including AG-12, a new anastomosis group. *Ecology and Population Biology* **89**, 942-946.

Carling DE, Rothrock CS, MacNish GC, Sweetingham MW, Brainard KA, Winters SW (1994) Characterisation of anastomosis group 11 (AG-11) of *Rhizoctonia solani*. *Phytopathology* **84**, 1387-1393.

Castanho B, Butler EE, Shepard RJ (1978) The association of double-stranded RNA with Rhizoctonia decline. *Phytopathology* **68**, 1515-1519.

Ceresini PC, Shew HD, James TY, Vilgalys RJ, Cubeta MA (2007) Phylogeography of the Solanaceae-infecting Basidiomycota fungus *Rhizoctonia solani* AG 3 based on sequence analysis of two nuclear DNA loci. *BMC Evolutionary Biology* **7**, 163-184.

Ceresini PC, Shew HD, Vilgalys RJ, Cubeta MA (2002) Genetic diversity of *Rhizoctonia solani* AG-3 from potato and tobacco in North Carolina. *Mycologia* **94**, 437-449.

Charlton ND, Cubeta MA (2007) Transmission of the M2 double-stranded RNA in *Rhizoctonia solani* anastomosis group 3 (AG-3). *Mycologia* **99**, 859-867.

Chiu TF, Bould C (1976) Effects of calcium and pottassium on ^{45}C mobility, growth and nutritional disorders of strawberry plants (*Fragaria* spp.). *Journal of Horticultural Science* **51**, 525-531.

Claassen ME, Wilcox GE (1974) Comparative reduction of calcium and magnesium composition of corn tissue by NH_4-N and K fertilization. *Agronomy Journal* **66**, 5221-5522.

Cooke JA (2000) Nutritional requirements of wheat in relation to tolerance to *Rhizoctonia solani* Kuhn. PhD thesis, University of Adelaide.

Cotterill PJ (1991) Evaluation of in-furrow fungicide treatment to control rhizoctonia root rot of wheat. *Crop Protection* **10**, 473-478.

Cotterill PJ (1993) Fungicide treatment, applied in furrow to undisturbed or cultivated soils, for control of rhizoctoni root rot of barley. *Crop Protection* **12**, 273-278.

Crowe JD, Olsson S (2001) Induction of laccase activity in *Rhizoctonia solani* by antagonistic *Pseudomonas fluorescens* strains and a range of chemical treatments. *Applied and Environmental Microbiology* **67**, 2088-2094.

Cruickshank RH, Wade GC (1980) Detection of pectic enzymes in pectin-acrylamide gels. *Analytical Biochemistry* **107**, 177-181.

Crump N (2006) Managing Advanced Soil Health (MASH) research program. (Department of Primary Industries: Victoria, Australia).

Cullen DW, Lees AK (2007) Detection of the *nec1* virulence gene and its correlation with pathogenicity in *Streptomyces* species on potato tubers and in soil using conventional and real-time PCR. *Journal of Applied Microbiology* **102**, 1082-1096.

Datnoff LE, Elmer WH, Huber DM (2007) 'Mineral nutrition and plant disease.' (The American Phytopathological Society: Minnesota).

Datnoff LE, Snyder GH, Deren CW (1992) Influence of silicon fertiliser grades on blast and brown spot development and on rice yields. *Plant Disease* **76**, 1011-1013.

de Boer R, Petkowski J, Wicks TJ, Harding R, Watson A (2001) Influence of rotation and biofumigation on soil-borne diseases on potatoes. Horticulture Australia Final Report PT96032, Department of Primary Industries, Victoria.

Dijst G (1988) Formation of sclerotia by *Rhizoctonia solani* on artificial media and tubers. *Netherlands Journal of Plant Pathology* **94**, 233-242.

Dijst G (1990) Effect of volatile and unstable exudates from underground potato plant parts on sclerotium formation by *Rhizoctonia solani* AG 3 before and after haulm destruction. *Netherlands Journal of Plant Pathology* **96**, 155-170.

Dillard HR, Wicks TJ, Philp B (1993) A grower survey of diseases, invertebrate pests, and pesticide use on potatoes grown in South Australia. *Australian Journal of Experimental Agriculture* **33**, 653-661.

Donald CM, Prescott JA (1975) Trace elements in Australian crop and pasture production. In 'Trace Elements in Soil-Plant-Animal Systems'. (Eds DJD Nicholoas, AR Egan) pp. 7-38. (Academic Press, Inc.: New York).

Dong B, Rengel Z, Graham RD (1995) Root morphology of wheat genotypes differing in zinc efficiency. *Journal of Plant Nutrition* **18**, 2761-2773.

Duncan S, Barton JE, O'Brien PA (1993) Analysis of variation in isolates of *Rhizoctonia solani* by random amplified polymorphic DNA assay. *Mycological Research* **97**, 1075-1082.

Errampalli D, Peters RD, MacIsaac K, Darrach D, Boswall P (2006) Effect of a combination of chlorine dioxide and thiophanate-methyl pre-planting seed tuber treatment on the control of black scurf of potatoes. *Crop Protection* **25**, 1231-1237.

Flentje NT, Dodman RL, Kerr A (1963) The mechanism of host penetration by *Thanatephorus cucumeris*. *Australian Journal of Biological Science* **16**, 784-799.

Frank JA, Leach SS (1980) Comparison of tuber borne and soil borne inoculum in the rhizoctonia disease of potato. *Phytopathology* **70**, 51-53.

Genc Y, McDonald GM, Graham RD (2006) Contribution of different mechanisms to zinc efficiency in bread wheat during early vegetative stage. *Plant and Soil* **281**, 353-367.

Georgopoulos SG (1982) Detection and measurment of fungicide resistance. In 'Fungicide resistance in crop protection'. (Eds J Dekker, SG Georgopoulos) pp. 24-31. (PUDOC: Wageningen, The Netherlands).

Ghosh RK, Singh N (2009) Effect of organic matter on sorption and degradation of azoxystrobin in soil. *Journal of Agricultural and Food Chemistry* **57**, 632-636.

Gisi U, Sierotzki H, Cook A, McCaffery A (2002) Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. *Pest Management Science* **58**, 859-867.

Godoy-Lutz G, Kuninaga S, Steadman JR, Powers K (2008) Phylogenetic analysis of *Rhizoctoni solani* subgroups associated with web blight symptoms on common bean based on ITS-5.8S rDNA. *Journal of General Plant Pathology* **74**, 32-40.

Gonzalez D, Carling DE, Kuninaga S, Vilgalys RJ, Cubeta MA (2001) Ribosomal DNA systematics of *Ceratobasidium* and *Thanatephorus* with *Rhizoctonia* anamorphs. *Mycologia* **93**, 1138-1150.

Govozdeva EL, Volotskaya AV, Sof'in AV, Kudryavtseva NN, Revina TA, Valueva TA (2006) Interaction of proteinases secreted by the fungal plant pathogen *Rhizoctonia solani* with natural proteinase inhibitors produced by plants. *Applied Biochemistry and Microbiology* **42**, 502-507.

Graham RD (1983) Effects of nutrient stress on susceptibility of plants to disease with particular reference to the trace elements. *Advances in Botanical Research* **10**, 221-276.

Gronberg H, Paulin L, Sen R (2003) ITS probe development for specific detection of *Rhizoctonia* spp. and *Suillus bovinus* based on Southern blot and liquid hybridisation-fragment length polymorphism. *Mycological Research* **107**, 428-438.

Guillemaut C, Edel-Hermann V, Camporota P, Alabouvette C, Richard-Molard M, Steinberg C (2003) Typing of anastomosis groups of *Rhizoctonia solani* by restriction analysis of ribosomal DNA. *Canadian Journal of Microbiology* **49**, 556-568.

Gullino ML, Leroux P, Smith CM (2000) Uses and challenges of novel compounds for plant disease control. *Crop Protection* **19**, 1-11.

Gunes A, Alpaslan M, Cikili Y, Ozcan H (1999) Effect of zinc on the alleviation of boron toxicity in tomato. *Journal of Plant Nutrition* **22**, 1061-1068.

Hall B, Davies K, Wicks TJ (2000) Horticulture Australia Ltd. Report: PT98036. Biological and chemical control of *Rhizoctonia,* South Australaian Research and Development Institute, Adelaide, Australia.

Hallock DL, Garren KH (1986) Pod breakdown, yield and grade of Virginia type peanuts as affected by Ca, Mg and K sulfates. *Agronomy Journal* **60**, 253-257.

Halloway RE, Graham RD, Stacey SP (2008) Micronutrient defeciencies in Australian field crops. In 'Micronutrient Deficiencies in Global Crop Production'. (Ed. BJ Alloway) p. 63. (Springer, New York, London).

Hamelin RC, Bourassa M, Rail J, Dusabenyagasani M, Jacobi V, Laflamme G (2000) PCR detection of *Gremmeniella abietina*, the causal agent of scleroderris canker of pine. *Mycological Research* **104**, 527-532.

Hancock JH, Wilkerson JB, Moody FH, Newman MA (2004) Seed-specific placement of in-furrow fungicides for control of seedling disease in cotton. *Crop Protection* **23**, 789-794.

Harikrishnan R, Yang XB (2004) Recovery of anastomosis groups of *Rhizoctonia solani* from different latitudinal positions and influence of temperatures on their growth and survival. *Plant Disease* **88**, 817-823.

Havlin JL, Beaton JD, Tisdale SL, Nelson WL (1999) 'Soil Fertility and Fertilisers: An introduction to Nutrient Management.' (Prentice Hall, Upper Saddle River, New Jersey).

Healy FG, Wach M, Krasnoff SB, Gibson DM, Loria R (2000) The *txtAB* genes of the plant pathogen *Streptomyces acidiscabies* encode a peptide synthetase required for phytotoxin thaxtomin A production and pathogenicity. *Molecular Microbiology* **38**, 794-804.

Herdina, Neate SM, Jabaji-Hare S, Ophel-Keller K (2004) Persistance of DNA of *Gaeumannomyces graminis* var. *tritici* in soil as measured by a DNA-based assay. *FEMS Microbiology Ecology* **47**, 143-152.

Hide GA (1981) Fungus diseases on potato seed tubers planted in England and Wales, 1963-76. *Annals of Applied Biology* **98**, 377-393.

Hide GA, Cayley GR (1982) Chemical techniques for control of stem canker and black scurf (*Rhizoctonia solani*) disease of potatoes. *Annals of Applied Biology* **100**, 105-116.

Hide GA, Firmager JP (1990) Effects of an isolate of *Rhizoctonia solani* Kuhn AG8 from diseased barley on the growth and infection of potatoes (*Solanum tuberosum* L.). *Potato Research* **33**, 229-234.

Hide GA, Hirst JM, Stedman OJ (1973) Effects of black scurf (*Rhizoctonia solani*) on potatoes. *Annals of Applied Biology* **74**, 139-148.

Hide GA, Read PJ (1991) Effects of rotation length, fungicide treatment of seed tubers and nematicide on diseases and the quality of potato tubers. *Annals of Applied Biology* **119**, 77-87.

Higginbotham RW, Paulitz T, Campbell KG, Kidwell K (2004) Evaluation of adapted wheat cultivars for tolerance to Pythium root rot. *Plant Disease* **88**, 1027-1032.

Hill CB, Anderson NA (1989) An evaluation of potato disease caused by isolates of *Rhizoctonia solani* AG-3. *American Potato Journal* **66**, 709-720.

Hitch CJ, Hall BH, Wicks TJ (2007) Brassica stem cancer - The role of *Rhizoctonia*. In 'Proceedings of the 16th Australasian Plant Pathology Society Conference' p. 34. (Australasian Plant Pathology Society: Adelaide).

Holland M, Spencer D (2007) Western Australian certified seed potato production rules: Incorporating the National standard for the certification of seed potatoes. (Ed. Western Australian seed advisory committee). (*AGWEST* Plant Laboratories, Western Australia).

Homma Y, Yamashita Y, Ishii M (1983) A new anastomosis group (AG 7) of *Rhizoctonia solani* Kuhn from Japanese radish fields. *Annals of the Phytopathological Society of Japan* **49**, 184-190.

Hooda KS, Srivastava MP (1996) Role of silicon in the management of rice blast. *Indian Phytopathology* **49**, 26-31.

Hoong P, Cross S, Ophel-Keller K, McKay A (2007) Industry report HVG05090 R#1. *Rhizoctonia solani* in cropping soils and vegetable crops p. 13. (Peracto Pty. Ltd, Tasmania, Australia).

Huaman Z, Hoekstra R, Bamberg JB (2000) The inter-genebank potato database and the dimensions of available wild potato germplasm. *American Journal of Potato Research* **77**, 353-362.

Huber DM (1981) The role of nutrients and chemicals. In 'Biology and control of take-all'. (Eds MJC Asher, PJ Shipton). (Academic Press: London).

Ito Y, Muraguchi H, Seshime Y, Oita S, Yanagi SO (2004) Flutolanil and carboxin resistance in *Coprinus cinereus* conferred by a mutation in the cytochrome b_{560} subunit of succinate dehydrogenase complex (complex II). *Molecular Genetics and Genomics* **272**, 328-335.

Jabaji-Hare S, Chamberland H, Charest PM (1999) Cell wall alterations in hypocotyls of bean seedlings protected from Rhizoctonia stem canker by a binucleate *Rhizoctonia* isolate. *Mycological Research* **103**, 1035-1043.

Jabaji-Hare S, Meller Y, Gill S, Charest PM (1990) Investigation of genetic relatedness among anastomosis groups of *Rhizoctonia solani* using cloned DNA probes. *Canadian Journal of Plant Pathology* **12**, 395-404.

Jabaji-Hare S, Neate S (2005) Nonpathogenic binucleate *Rhizoctonia* spp. and benzothiadiazole protect cotton seedlings against Rhizoctonia damping-off and Alternaria leaf spot in cotton. *Phytopathology* **95**, 1030-1036.

Jabaji S (2008) Microarray analysis of differentially expressed genes of *Rhizoctonia solani* AG 3 during the pathogenic interaction with potato. In '4th International Symposium on Rhizoctonia'. Berlin, Germany p. 11.

Jacobs H, Boswell GP, Harper FA, Ritz K, Davidson FA, Gadd GM (2002) Solubilization of metal phosphates by *Rhizoctonia solani*. *Mycological Research* **106**, 1468-1479.

Jian J, Lakshman DK, Tavantzis SM (1997) Association of distinct double-stranded RNAs with enhanced or diminished virulence in *Rhizoctonia solani* infecting potato. *Molecular Plant-Microbe Interactions* **10**, 1002-1009.

Joseph-Horne T, Hollomon DW (2000) Functional diversity within the mitochondrial electron transport chain of plant pathogenic fungi. *Pest Management Science* **56**, 24-30.

Joseph-Horne T, Wood PM, Wood CK, Moore AL, Headrick J, Hollomon D (1998) Characterisation of a split respiratory pathway in the wheat "Take-all" fungus, *Gaeumannomyces graminis* var. *tritici*. *The Journal of Biological Chemistry* **273**, 11127-11133.

Julian MC, Acero J, Salazar O, Keijer J, Rubio V (1999) Mating type-correlated molecular markers and demonstration of heterokaryosis in the phytopathogenic fungus *Thanatephorus cucumeris (Rhizoctonia solani)* AG 1-IC by AFLP DNA fingerprinting analysis. *Journal of*

Biotechnology **67**, 49-56.

Kalim S, Luthra YP, Gandhi SK (2003) Cowpea root rot severity and metabolic changes in relation to manganese application. *Journal of Phytopathology* **151**, 92-97.

Kataria HR, Gisi U (1989) Recovery from soil and sensitivity to fungicides of *Rhizoctonia cerealis* and *R. solani*. *Mycological Research* **92**, 458-462.

Kataria HR, Grover RK (1987) Influence of soil factors, fertilisers and manures on pathogenicity of *Rhizoctonia solani* on *Vigna* species. *Plant and Soil* **103**, 57-66.

Kataria HR, Hugelshofer U, Gisi U (1991a) Sensitivity of *Rhizoctonia* species to different fungicides. *Plant Pathology* **40**, 203-211.

Kataria HR, Sunder S (1985) Effect of micronutrients on the efficacy of fungicides against *Rhizoctonia solani* on cowpea seedlings. *Pesticide Science* **16**, 453-456.

Kataria HR, Sunder S (1988) A comparison of in vitro and in vivo effects of clay minerals, humic acid and micronutrients on the activity of fungicides against *Rhizoctonia solani*. *Plant and Soil* **111**, 95-104.

Kataria HR, Verma PR, Gisi U (1991b) Variability in the sensitivity of *Rhizoctonia solani* anastomosis groups to fungicides. *Journal of Phytopathology* **133**, 121-133.

Keijer J, Korsman GM, Dullemans AM, Houterman PM, De Bree J, Van Silfhout CH (1997) In vitro analysis of host plant specificity in *Rhizoctonia solani*. *Plant Pathology* **46**, 659-669.

Kirkegaard JA, Gardner PA, Angus JF, Koetz E (1994) Effect of *Brassica* break crops on the growth and yield of wheat. *Australian Journal of Agricultural Research* **45**, 529-545.

Knight SC, Anthony VM, Brady AM, Greenland AJ, Heany SP, Murray DC, Powell KA, Schulz MA, Spinks CA, Worthington PA, Youle D (1997) Rationale and perspectives on the development of fungicides. *Annual Review of Phytopathology* **35**, 349-372.

Ko W, Hora FK (1971) A selective medium for the quantitative determination of *Rhizoctonia solani* in soil. *Phytopathology* **61**, 707-710.

Kroon LPNM, Bakker FT, van den Bosch GBM, Bonants PJM, Flier WG (2004) Phylogenetic analysis of *Phytophthora* species based on mitochondrial and nuclear DNA sequences. *Fungal Genetics and Biology* **41**, 766-782.

Kruistufek V, Divis J, Dostalkova I, Kalcik J (2000) Accumulation of mineral elements in tuber periderm of potato cultivars differing in susceptibility to common scab. *Potato Research* **43**, 107-114.

Kuninaga S, Carling DE, Takeuchi T, Yokosawa R (2000) Comparison of rDNA-ITS sequences between potato and tobacco strains in *Rhizoctonia solani* AG 3. *Journal of General Plant Pathology* **66**, 2-11.

Kuninaga S, Natsuaki T, Takeuchi T, Yokosawa R (1997) Sequence variation of the rDNA ITS regions within and between anastomosis groups in *Rhizoctonia solani*. *Current Genetics* **32**, 237-243.

Kuninaga S, Yokosawa R (1984) DNA base sequence homology in *Rhizoctonia solani* Kuhn IV. Genetic relatedness within AG 4. *Annals of the Phytopathological society of Japan* **50**, 322-330.

Kuninaga S, Yokosawa R, Ogoshi A (1978) Anastomosis grouping of *Rhizoctonia solani* Kuhn isolated from non-cultivated soils. *Annals of the Phytopathological Society of Japan* **44**, 591-598.

Lakshman DK, Jian J, Tavantzis SM (1998) A double-stranded RNA element from a hypovirulent strain of *Rhizoctonia solani* occurs in DNA form and is genetically related to the pentafunctional AROM protein of the shikimate pathway. *Proceedings of the National Academy of Science* **95**, 6425-6429.

Lambert DH, Manzer FE (1991) Relationship of calcium to potato scab. *Phytopathology* **81**, 632-636.

Langille AR, Batteese RI (1974) Influence of zinc concentration in nutrient solution on growth and elemental content of the "Katahdin" potato plant. *American Potato Journal* **51**, 345-354.

Larkin RP, Griffin TS (2007) Control of soilborne potato diseases using *Brassica* green manures. *Crop Protection* **26**, 1067-1077.

Leach SS, Webb RE (1993) Evaluation of potato cultivars, clones and true seed populations for resistance to *Rhizoctonia solani*. *American Potato Journal* **70**, 317-328.

Leclerc-Potvin C, Balmas V, Charest PM, Jabaji-Hare S (1999) Development of reliable molecular markers to detect non-pathogenic binucleate *Rhizoctonia* isolates (AG-G) using PCR. *Mycological Research* **103**, 1165-1172.

Lees AK, Cullen DW, Sullivan L, Nicolson MJ (2002) Development of conventional and quantitative real-time PCR assays for the detection and identification of *Rhizoctonia solani* AG-3 in potato and soil. *Plant Pathology* **51**, 293-302.

Lees AK, Wale SJ, van de Graaf P, Brierley JL (2005) The use of molecular diagnostics to investigate the epidemiology of potato diseases. *Australasian Plant Pathology* **34**, 449-455.

Lehtonen ML, Ahvenniemi P, Wilson PS, German-Kinnari M, Valkonen JPT (2008a) Biological diversity of *Rhizoctonia solani* (AG-3) in a northern potato-cultivation environment in Finland. *Plant Pathology* **57**, 141-151.

Lehtonen ML, Somervuo P, Valkonen JPT (2008b) Infection with *Rhizoctonia solani* induces defence genes and systemic resistance in potato sprouts grown without light. *Phytopathology* **98**, 1190-1198.

Leslie JF, Zeller KA (1996) Heterokaryon incompatibility in fungi - more than just another way to die. *Journal of Genetics* **75**, 415-424.

Lewis DH (1980) Boron, lignification and the origin of vascular plants - a unified hypothesis. *New Phytopathologist* **84**, 209-229.

Lin S-C, Lo Y-C, Lin J-Y, Liaw Y-C (2004) Crystal structures and electron micrographs of fungal volvatoxin A2. *Journal of Molecular Biology* **343**, 477-491.

Liu C, Lakshman DK, Tavantzis SM (2003) Quinic acid influences hypovirulence and expression of a hypovirulence-associated double-stranded RNA in *Rhizoctonia solani*. *Current Genetics* **43**, 103-111.

Liu Z, Nickrent DL, Sinclair JB (1990) Genetic relationships among isolates of *Rhizoctonia solani* anastomosis group-2 based on isozyme analysis. *Canadian Journal of Plant Pathology* **12**, 376-382.

Liu ZL, Sinclair JB (1992) Genetic diversity of *Rhizoctonia solani* anastomosis group 2. *Phytopathology* **82**, 778-787.

Lubeck M, Poulsen H (2001) UP-PCR cross blot hybridisation as a tool for identification of anastomosis groups in the *Rhizoctonia solani* complex. *FEMS Microbiology Letters* **201**, 83-89.

Ma Z, Felts D, Michailides TJ (2003) Resistance to azoxystrobin in *Alternaria* isolates from pistachio in California. *Pesticide Biochemistry and Physiology* **77**, 66-74.

Ma Z, Michailides TJ (2004) Characterization of iprodione-resistant *Alternaria* isolates from pistachio in California. *Pesticide Biochemistry and Physiology* **80**, 75-84.

MacNish GC, Carling DE, Sweetingham MW, Brainard KA (1994) Anastomosis group (AG) affinity of pectic isozyme (zymogram) groups (ZG) of *Rhizoctonia solani* from the Western Australian cereal-belt. *Mycological Research* **98**, 1369-1375.

MacNish GC, Carling DE, Sweetingham MW, Ogoshi A, Brainard KA (1995) Characterisation of anastomosis group-10 (AG-10) of *Rhizoctonia solani*. *Australasian Plant Pathology* **24**, 252-260.

MacNish GC, Neate SM (1996) Rhizoctonia bare patch of cereals. *Plant Disease* **80**, 965-971.

MacNish GC, O'Brien PA (2005) RAPD-PCR used to confirm that four pectic isozyme (zymogram) groups within the Australian *Rhizoctonia solani* AG 8 population are true intraspecific groups. *Australasian Plant Pathology* **34**, 245-250.

Maddaloni M, Forlani F, Balmas V, Donini G, Stasse L, Corazza L, Motto M (1997) Tolerance to the fungal pathogen *Rhizoctonia solani* AG4 of transgenic tobacco expressing the maize ribosome-inactivating protein b-32. *Transgenic Research* **6**, 393-402.

Maier NA, Shepard KL (1998) 'Plant analysis and interpretation manual for potato.' (South Australian Research and Development Institute: Adelaide).

McCabe PM, Gallagher MP, Deacon JW (1999) Microscopic observation of perfect hyphal fusion in *Rhizoctonia solani*. *Mycological Research* **103**, 487-490.

Menzies JD (1970) Introduction: The first century of Rhizoctonia solani. In *'Rhizoctonia solani*, Biology and Pathology'. (Ed. J Parmeter, J.R.). (University of California Press: Berkeley).

Mizutani A, Yukiota H, Tamura H, Miki N, Masuko M, Takeda R (1995) Respiratory characteristics in *Pyricularia oryzae* exposed to a novel alkoxyiminoacetamide fungicide. *Phytopathology* **85**, 306-311.

Mulder A, Turkensteen LJ, Bouluman A (1992) Perspectives of green-crop-harvesting to control soil-borne and storage diseases of seed potatoes. *Netherlands Journal of Plant Pathology* **98**, 103-114.

Neate SM, Warcup JH (1985) Anastomosis grouping of some isolates of *Thanatephorus cucumeris* from agricultural soils in South Australia. *Transactions of the British Mycological Society* **85**, 615-620.

NIAB (2004) 'Pocket guide to varieties of potatoes.' (Cambridge Marketing Limited: Cambridge, UK).

O'Brien PA, MacNish GC, Milton NMK (2001) Transgenic tobacco plants show different resistance to *Rhizoctonia solani* AG-4 and AG-8. *Australasian Plant Pathology* **30**, 221-225.

Ochiai N, Fujimura M, Motoyama T, Ichiishi A, Usami R, Horikoshi K, Yamaguchi I (2001) Characterisation of mutations in the two-component histidine kinase gene that confer fludioxonil resistance and osmotic sensitivity in the *os-1* mutants of *Neurospora crassa*. *Pest Management Science* **57**, 437-442.

Ogoshi A (1975) Grouping of *Rhizoctonia solani* Kuhn and their perfect stages. *Review of Plant Protection Research Japan* **8**, 98-103.

Ogoshi A (1987) Ecology and pathogenicity of anastomosis and intraspecific groups of *Rhizoctonia solani* Kuhn. *Annual Review of Phytopathology* **25**, 125-143.

Okubara PA, Steber CM, DeMacon VL, Walter NL, Paulitz T, Kidwell K (2009) Scarlet-Rz1, an EMS-generated hexaploid wheat with tolerance to the soilborne necrotrophic pathogens *Rhizoctonia solani* AG-8 and *R. oryzae*. *Theoretical Applied Genetics* **119**, 293-303.

Olaya G, Abawi GS, Barnard J (1994) Response of *Rhizoctonia solani* and binucleate *Rhizoctonia* to five fungicides and control of pocket rot of table beets with foliar sprays. *Plant Disease* **78**, 1033-1037.

Ophel-Keller K, Herdina, Hartley D, Wiechel T, Harding R, Crump N, Bulman SR, Marshall J, Hall B, Wicks T, McKay A (2006) DNA monitoring tools for soilborne diseases of potato. 'Proceedings of the 4th Australasian Soilborne Diseases Symposium'. p 66. Queenstown, New Zealand. (Eds RE Falloon, MG Cromey, A Stewart, EE Jones).

Ophel-Keller K, McKay A, Driver F, Curran J (1999) The cereal root disease testing service. 'Proceedings of the 1st Australasian Soilborne Disease Symposium' p. 63 (Brisbane, Australia) (Ed Magarey RC).

Ophel-Keller K, McKay A, Hartley D, Herdina, Curran J (2008) Development of a routine DNA-based testing service for soilborne diseases in Australia. *Australasian Plant Pathology* **37**, 243-253.

Orth AB, Sfarra A, Pell EJ, Tien M (1992) An investigation into the role of lipid peroxidation in the mode of action of aromatic hydrocarbon and dicarboximide fungicides. *Pesticide Biochemistry and Physiology* **44**, 91-100.

Osusky M, Osuska L, Kay W, Misra S (2005) Genetic modification of potato against microbial diseases: in vitro and in planta activity of a dermaseptin B1 derivative, MsrA2. *Theoretical Applied Genetics* **111**, 711-722.

Ownley BH, Duffy BK, Weller DM (2003) Identification and manipulation of soil properties to improve the biological control performance of phenazine-producing *Pseudomonas fluorescens*. *Applied and Environmental Microbiology* **69**, 3333-3343.

Papavizas GC (1970) Colonization and growth of *Rhizoctonia solani* in soil. In 'Rhizoctonia solani, Biology and Pathology'. (Ed. Parmeter J). (University of California Press: Berkeley).

Parmeter J, J.R., Sherwood RT, Platt DW (1969) Anastomosis grouping among isolates of Thanatephorus cucumeris. *Phytopathology* **59**, 1270-1278.

Parmeter Jr JR, Whitney HS (1970) Taxonomy and nomenclature of the imperfect state. In '*Rhizoctonia solani*, Biology and Pathology'. (Ed. J Parmeter, J.R.). (University of California Press: Berkeley).

Pederick SJ, Wicks TJ, Walker GE, Hall BH, Walter A (2007) Studies on the cause of Mallee onion stunt in South Australia. 'Proceedings of the 16th Australasian Plant Pathology Society Conference' p. 59. (Australasian Plant Pathology Society: Adelaide).

PIRSA (2007) South Australian industry profile. (Government of South Australia, Primary Industries and Resources of South Australia).

Platt HW, Canale F, Gimenez G (1993) Effects of tuber-borne inoculum of *Rhizoctonia solani* and fungicidal seed potato treatment on plant growth and Rhizoctonia disease in Canada and Uruguay. *American Potato Journal* **70**, 553-558.

Priestley JH, Woffenden LM (1923) The healing of wounds in potato tubers and their propagation by cut seed sets. *Annals of Applied Biology* **10**, 96-115.

Puzina TI (2004) Effect of Zinc sulfate and Boric acid on the hormonal status of potato plants in relation to tuberization. *Russian Journal of Plant Physiology* **51**, 234-240.

Ramesh MA, Laidlaw RD, Durrenberger F, Orth AB, Kronstad JW (2001) The cAMP signal transduction pathway mediates resistance to dicarboximide and aromatic hydrocarbon fungicides in *Ustilago maydis*. *Fungal Genomics and Biology* **32**, 183-193.

Rivard D, Jabaji-Hare S (2006) Microarray analysis of differentially expressed genes of Rhizoctonia solani strain AG 3 during the pathogenic interaction with potato. *Canadian Journal of Plant Pathology* **28**, 364-364.

Rodrigues FA, Vale FXR, Korndorfer GH, Prabhu AS, Datnoff LE, Oliveira AMA, Zambolim L (2003) Influence of silicon on sheath blight of rice in Brazil. *Crop Protection* **22**, 23-29.

Sadana US, Lata K, Claassen N (2002) Manganese efficiency of wheat cultivars as related to root growth and internal manganese requirement. *Journal of Plant Nutrition* **25**, 2677-2688.

Salazar O, Schneider JHM, Julian MC, Keijer J, Rubio V (1999) Phylogenetic subgrouping of *Rhizoctonia solani* AG 2 isolates based on ribosomal ITS sequences. *Mycologia* **91**, 459-467.

Sanders PL, Burpee L, Cole HJ (1977) Preliminary studies on binucleate turfgrass pathogens that resemble *Rhizoctonia solani*. *Phytopathology* **68**, 145-148.

Sanderson JB, Gupta UC (1990) Copper and zinc nutrition of Russet Burbank potatoes grown on Prince Edward Island. *Canadian Journal of Plant Science* **70**, 357-362.

Sarwar M, Kirkegaard JA, Wong PTW, Desmarchelier JM (1998) Biofumigation potential of brassicas III. In vitro toxicity of isothiocyanates to soil-borne fungal pathogens. *Plant and Soil* **201**, 103-112.

Schneider JHM, Salazar O, Rubio V, Keijer J (1997) Identification of *Rhizoctonia solani* associated with field-grown tulips using ITS rDNA polymorphism and pectic zymograms. *European Journal of Plant Pathology* **103**, 607-622.

Scholten OE, Panella LW, De Bock TSM, Lange W (2001) A greenhouse test for screening sugar beet (*Beta vulgaris*) for resistance to *Rhizoctonia solani*. *European Journal of Plant Pathology* **107**, 161-166.

Segura A, Moreno M, Madueno F, Molina A, Garcia-Olmedo F (1999) Snakin-1, a peptide from potato that is active against plant pathogens. *Molecular Plant-Microbe Interactions* **12**, 16-23.

Sharma UC (1990) relative efficacy of methods of zinc, manganese, iron and copper application to potato (*Solanum tuberosum*). *Indian Journal of Agricultural Sciences* **60**, 806-809.

Sherwood RT (1970) Physiology of Rhizoctonia solani. In 'Rhizoctonia solani, Biology and

Pathology'. (Ed. J Parmeter, J.R.). (University of California Press: Berkeley).

Siddiqui IA, Shaukat SS, Hamid M (2002) Role of zinc in rhizobacteria-mediated suppression of root-infecting fungi and root-knot nematode. *Phytopathology* **150**, 569-575.

Siegel S (1956) 'Nonparametric statistics for the Behavioral Sciences.' (McGraw-Hill: New York).

Sierotzki H, Wullschleger J, Gisi U (2000) Point mutation in cytochrome *b* gene conferring resistance to strobulurin fungicides in *Erysiphe graminis* f. sp. *trici* fireld isolates. *Pesticide Biochemistry and Physiology* **68**, 107-112.

Silber A, Yones LB, Dori I (2004) Rhizosphere pH as a result of nitrogen levels and NH_4/NO_3 ratio and its effect on zinc availibility and on growth of rice flower (*Ozothamnus diosmifolius*). *Plant and Soil* **262**, 205-213.

Silberbush M, Barber SA (1983) Sensitivity of stimulated phosphorus uptake to parameters used by a mechanistic-mathematical model. *Plant and Soil* **74**, 93-100.

Simons SA, Gilligan CA (1997) Relationships between stem canker, stolon canker, black scurf (*Rhizoctonia solani*) and yield of potato (*Solanum tuberosum*) under different agronomic conditions. *Plant Pathology* **46**, 651-658.

Sivasithamparam K (1993) Ecology of root infecting pathogenic fungi in mediterranean environments. *Advances in Plant Pathology* **10**, 245-280.

Siwek K (1997) Mechanisms of biological control of the damping-off fungus, *Pythium ultimum*, by binucleate Rhizoctonia. PhD thesis, University of Adelaide.

Slaton NA, Cartwright RD, Meng J, Gbur Jr. EE, Norman RJ (2003) Sheath blight severity and rice yield as affected by nitrogen fertilizer rate, application method, and fungicide. *Agronomy Journal* **95**, 1489-1496.

Sneh B, Burpee L, Ogoshi A (1991) 'Identification of *Rhizoctonia* Species.' (The American Phytopathological Society: Minnesota).

Sneh B, Ichielevich-Auster M (1989) Mechanism of seedling protection induced by a hypovirulent isolate of *Rhizoctonia solani*. *Canadian Journal of Botany* **67**, 2135-2141.

Sneh B, Jabaji-Hare S, Neate S, Dijst G (1996) '*Rhizoctonia* Species: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control.' (Kluwer Academic Publishers: Dordrecht, Boston).

Soltani N, Conn KL, Abbasi PA, Lazarovits G (2002) Reduction of potato scab and verticillium wilt with ammonium lignosulfonate soil amendment in four Ontario potato fields. *Canadian Journal of Plant Pathology* **24**, 332-339.

Stammler G, Klappach K (2005) Development, monitoring and management of QoI-resistance in plant pathogenic fungi. 'Proceedings of the 15th biennial Austrilasian Plant

Pathology Society Conference', p216.

Stevenson WR, Loria R, Franc GD, Weingartner DP (2001) 'Compendium of Potato Diseases.' (The American Phytopathological Society: Minnesota).

Streeter TC, Rengel Z, Neate SM, Graham RD (2001a) Zinc fertilisation increases tolerance to *Rhizoctonia solani* (AG 8) in *Medicago truncatula*. *Plant and Soil* **228**, 233-242.

Streeter TC, Rengel Z, Neate SM, Graham RD (2001b) Zinc fertilisation increases tolerance to *Rhizoctonia solani* (AG 8) in *Megicago truncatula*. *Plant and Soil* **228**, 233-242.

Stretton HM, Flentje NT (1972) Inter-isolate heterokaryosis in *Thanatephorus cucumeris*. *Australian Journal of Biological Science* **25**, 293-303.

Talbot PHB (1970) Taxonomy and nomenclature of the perfect state. In '*Rhizoctonia solani*, Biology and Pathology'. (Ed. Parmeter J). (University of California Press: Berkeley).

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. In 'Molecular Biology and Evolution ' pp. 1596-1599. (http://www.kumarlab.net/publications accessed 2007).

Tavantzis SM, Lakshman DK, Liu C (2002) Double-stranded RNA elements modulating virulence in *Rhizoctonia solani*. In 'dsRNA genetic elements: Concepts & applications in agriculture, forestry and medicine'. (Ed. SM Tavantzis) pp. 191-207. (CRC press, Florida, USA).

Taylor PA (1993) New and future approaches for control of soilborne plant pathogens with chemicals. 'Proceedings of the International Congress of Plant Pathology' p. S1.3 Montreal, Canada).

Thongbai P, Hannam RJ, Graham RD, Webb RE (1993a) Interaction between zinc nutritional status of cereals and *Rhizoctonia* root rot severity. I. Field observations. *Plant and Soil* **153**, 207-214.

Thongbai P, Hannam RJ, Graham RD, Webb RE (1993b) Interaction between zinc nutritional status of cereals and *Rhizoctonia* root rot severity. II. Effect of Zn on disease severity of wheat under controlled conditions. *Plant and Soil* **153**, 215-222.

Thornton CR, O'Neill TM, Hilton G, Gilligan CA (1999) Detection and recovery of *Rhizoctonia solani* in naturally infested glasshouse soils using a combined baiting, double monoclonal antibody ELISA. *Plant Pathology* **48**, 627-634.

Tomlin CDS (1997) 'The Pesticide Manual.' (British Crop Protection Council, UK).

Tsror L, Peretz-Alon I (2005) The influence of the inoculum source of *Rhizoctonia solani* on development of black scurf on potato. *Journal of Phytopathology* **153**, 240-244.

Uriarte RF, Shew HD, Bowman DC (2004) Effect of soluble silica on brown patch and dollar

spot of creeping bentgrass. *Journal of Plant Nutrition* **27**, 325-339.

van den Boogert PHJF, Luttikholt AJG (2004) Compatible biological and chemical control systems for *Rhizoctonia solani* in potato. *European Journal of Plant Pathology* **110**, 111-118.

Verma PR (1996) Biology and control of *Rhizoctonia solani* on rapeseed: A review. *Phytoprotection* **77**, 99-111.

Vilgalys R, Cubeta MA (1994) Molecular systematics and population biology of *Rhizoctonia*. *Annual Review of Phytopathology* **32**, 135-155.

Virgen-Calleros G, Olalde-Portugal V, Carling DE (2000) Anastomosis groups of *Rhizoctonia solani* on potato in central Mexico and potential for biological and chemical control. *Journal of Potato Research* **77**, 219-224.

Weerasena OVDSJ, Chandrasekharan NV, Wijesundera RLC, Karunanayake EH (2004a) development of a DNA probe and a PCR based diagnostic assay for *Rhizoctonia solani* using a repetitive DNA sequence cloned from a Sri Lankan isolate. *Mycological Research* **108**, 649-653.

Weinhold AR, Bowman T, Hall DH (1982) Rhizoctonia disease of potato: effect on yield and control by seed tuber treatment. *Plant Disease* **66**, 815-818.

Welch RM, Norvell WA (1993) Growth and nutrient uptake by barley (*Hordeum vulgare* L. cv Herta): Studies using an N-(2-Hydroxyethyl)ethylenedinitrilotriacetic acid-buffered nutrient solution technique II. Role of zinc in the uptake and root leakage of mineral nutrients. *Plant Physiology* **101**, 627-631.

Weller DM, Raaijmakers JM, McSpadden Gardner BB, Thomashow LS (2002) Microbial populations responsible for specific soil suppressiveness to plant pathogens. *Annual Review of Phytopathology* **40**, 309-348.

White TJ, Bruns T, Lee S, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In 'PCR protocols: A guide to methods and applications'. (Eds MA Innis, DH Gelfand, JJ Sninsky, TJ White) pp. 315-322. (Academic Press: New York, USA).

Wicks TJ, McMahon R, Morgan B, Hall B (1997) HRDC report: SARDIPT315. Control of *Rhizoctonia* on fresh market potatoes. South Australian Research and Development Institute, Adelaide, Australia.

Wicks TJ, Morgan B, Hall B (1995) Chemical and biological control of *Rhizoctonia solani* on potato seed tubers. *Australian Journal of Experimental Agriculture* **35**, 661-664.

Wicks TJ, Morgan B, Hall B (1996) Influence of soil fumigation and seed tuber treatment on the control of *Rhizoctonia solani* on potatoes. *Australian Journal of Experimental Agriculture* **36**, 1339-1345.

Williams CMJ (2002) Eyes on potatoes. In 'Australian potato industry council newsletter' p.

18. AusVeg, Victoria, Australia.

Williams CMJ, Dahlenburg AP (1994) Potato quality for fresh and processing markets in Australia. *Food Australia* **46**, 34-36.

Williams CMJ, Maier NA, Dahlenburg AP (1993) Improved strategies of nutrient management to obtain the competitive edge using calibrated soil and sap quick tests. In 'National Potato Research Workshop'. Ulverstone, Tasmania pp. 131-136.

Wilson PS, Ahvenniemi PM, Lehtonen ML, Kukkonen M, Rita H, Valkonen JPT (2008a) Biological control and chemical control and their combined use to control different stages of the Rhizoctonia disease complex on potato through the growing season. *Annals of Applied Biology* **153**, 307-320.

Wilson PS, Ketola EO, Ahvenniemi P, Lehtonen ML, Valkonen JPT (2008b) Dynamics of soilborne *Rhizoctonia solani* in the presence of *Trichoderma harzianum*: effects on stem canker, black scurf and progeny tubers of potato. *Plant Pathology* **57**, 152-161.

Wiseman BM, Neate SM, Ophel-Keller K, Smith SE (1996) Suppression of *Rhizoctonia solani* anastomosis group 8 in Australia and its biological nature. *Soil Biology & Biochemistry* **28**, 727-732.

Wolski EA, Daleo GR, Andreu AB (2004) Induction of pathogenisis-related proteins on potato sprouts by an alpha-(1,3)-glucan from cell wall of binucleate non-pathogenic *Rhizoctonia* sp. isolate. *Phytopathology* **94**, S111.

Wolski EA, Maldonado S, Daleo GR, Andreu AB (2006) A novel a-1, 3-glucan elicits plant defence responses in potato and induces protection against *Rhizoctonia solani* AG 3 and *Fusarium solani* f. sp. *eumartii*. *Physiological and Molecular Plant Pathology* **69**, 93-103.

Woodhall JW (2004) Characterisation of *Rhizoctonia solani* anastomosis groups and their pathogenicty to potato. PhD thesis, Harper Adams University, UK.

Woodhall JW, Lees AK, Edwards SG, Jenkinson P (2007) Characterisation of *Rhizoctonia solani* from potato in Great Britain. *Plant Pathology* **56**, 286-295.

Woodhall JW, Lees AK, Edwards SG, Jenkinson P (2008) Infection of potato by *Rhizoctonia solani*: effect of anastomosis group. *Plant Pathology* **57**, 897-905.

Xue L, Charest PM, Jabaji-Hare S (1998) Systemic induction of peroxidases, 1,3-beta-glucanases, chitinases, and resistance in bean plants by binucleate *Rhizoctonia* species. *Phytopathology* **88**, 359-365.

Yanar Y, Yilmaz G, Cesmeli I, Coskum S (2005) Characterization of *Rhizoctonia solani* isolates from potatoes in Turkey and screening potato cultivars for resistance to AG-3 isolates. *Phytoparasitica* **33**, 370-376.

Yang J, Kharbanda PD, Wang H, McAndrew DW (1996) Characterisation, virulence, and genetic variation of *Rhizoctonia solani* AG-9 in Alberta. *Plant Disease* **80**, 513-518.

Yao MK, Desilets H, Charles MT, Boulanger R, Tweddell RJ (2003) Effect of mycorrhization on the accumulation of rishitin and solavetivone in potato plantlets challenged with *Rhizoctonia solani*. *Mycorrhiza* **13**, 333-336.

Zhang Z, Zhu Z, Ma Z, Li H (2009) A molecular mechanism of azoxystrobin resistance in *Penicillium digitatum* UV mutants and a PCR-based assay for detection of azoxystrobin-resistant strains in packing- or store-house isolates. *International Journal of Food Microbiology* **131**, 157-161.

Ziogas BN, Baldwin BC, Young JE (1997) Alternative respiration: a biochemical mechanism of resistance to azoxystrobin (ICIA5504) in S*eptoria tritici*. *Pesticide Science* **50**, 28-34.

10 Appendices

10.1 Media List

Ko & Hora - Modified from (Ko and Hora 1971)

NOTE:
This list is included on page 192
of the print copy of the thesis held in
the University of Adelaide Library.

10.2 Tables of ANOVA using disease severity scores

Table 10.1. Disease severity scores from potato plants grown in sand inoculated with different AGs of *R. solani* compared to analysis presented in Table 4.1.

Inoculum		Disease severity score (means) and ranges (1-4)			
AG	Isolate code	Stem necrosis l.s.d. 5 % = 0.51	Stolon necrosis l.s.d. 5 % = 0.58	Root necrosis l.s.d. 5 % = 0.45	Tuber sclerotia l.s.d. 5 % = 0.37
N/A	control	(1.1) a 1-2	(1.0) a 1	(1.0) a 1	(1.0) a 1
2-1	L57	(1.7) bc 1-2	(1.3) ab 1-2	(1.3) ab 1-2	(1.0) a 1-2
2-1	R394	(1.4) ab 1-2	(1.1) a 1-2	(1.5) bc 1-2	(1.0) a 1
2-1	WAC-9806	(3.0) e 2-4	(1.8) bc 1-4	(1.0) a 1-2	(1.0) a 1
2-2	WAC-9937	(2.0) cd 2	(1.8) bc 1-2	(1.8) c 1-2	(1.0) a 1
3	L62	(2.0) cd 2	(2.0) cd 1-3	(1.6) bc 1-2	(2.0) b 2
3	L306	(2.4) d 2-3	(2.4) d 2-3	(1.8) c 1-2	(2.5) c 1-4
3	T22	(1.8) bc 1-2	(1.5) abc 1-2	(1.7) bc 1-2	(1.7) b 1-2
8	RS21	(1.1) a 1-2	(1.3) ab 1-2	(1.4) abc 1-2	(1.0) a 1

Differences in disease severity implied by ANOVA (Table 10.1) that were not observed in results from Kruskal-Wallis analysis presented in Table 4.1 include:

- Stem necrosis caused by AG 3 isolate L306 was worse ($P < 0.05$) than that caused by isolates T22 (AG 3) and L57 (AG 2-1), but not ($P < 0.05$) WAC-9806 (AG 2-1), which produced more severe ($P < 0.05$) stem necrosis than any other inoculum treatment.
- Stolon necrosis caused by AG 2-1 isolate WAC-9806 was worse ($P < 0.05$) than the uninoculated control. AG 3 isolate L306 produced the most severe ($P < 0.05$) stem necrosis, but which was not different ($P > 0.05$) to that caused by L62 (AG 3) and WAC-9806 (AG 2-1).
- Root necrosis caused by AG 2-1 isolate R394 was worse ($P < 0.05$) than

uninoculated controls and AG 3 isolate L306 was worse ($P < 0.05$) than that caused by AG 2-1 isolate L57.

- More ($P < 0.05$) sclerotia formed on tubers of plants inoculated with AG 3 isolate L306 than any other treatment.

Differences in disease severity implied by ANOVA (Table 10.2) that were not observed in results from Kruskal-Wallis analysis presented in Table 4.3 include:

- Stem necrosis caused by AG 3 isolate Tas3 was worse ($P < 0.05$) than uninoculated controls.
- The most severe ($P < 0.05$) root disease was caused by AG 3 isolate R229 and that caused by AG 4 isolate 734 was worse ($P < 0.05$) than the uninoculated control.

Differences in disease severity implied by ANOVA (Table 10.3) that were not observed in results from Kruskal-Wallis analysis presented in Table 4.5 include:

- Stem necrosis caused by AG 2-1 isolate of short IGS1 type R100 caused more severe necrosis ($P < 0.05$) than any other treatment with the Caling and Leiner (1990) scoring method and to all but isolate Tas6 with the scoring method of Balali *et al.* (1995).

Differences in disease severity implied by ANOVA (Table 10.4.a) that were not observed in results from Kruskal-Wallis analysis presented in Table 4.6.a include:

- The severity scoring method of Balali et al. (1995) suggested that stem necrosis caused by isolate R106 was worse ($P < 0.05$) than uninoculated controls and that caused by WAC-9806 was not as severe ($P < 0.05$) as that caused by SAR11.18 and KI4.
- The severity scoring method of Caling and Leiner (1990) suggested that stem necrosis caused by isolate KI4 was more severe ($P < 0.05$) than that caused by WAC-9806.

Table 10.2. Disease severity on potato plant stems and roots grown in sand inoculated with different AGs of Australian *Rhizoctonia* isolates compared to analysis presented in Table 4.3.

AG	Isolate code	Disease severity scores means and ranges (1-4)	
		Stem l.s.d. 5 % = 0.58	Root l.s.d. 5 % = 0.70
2-1	**Tas6**	(2.8) ef 2-4	(1.9) bcd 1-4
2-1	**SAR11.18**	(3.5) gh 3-4	(2.0) cd 1-3
2-1	**KI65**	(2.0) cd 2	(1.5) abc 1-2
2-1	**SE50**	(3.9) h 3-4	(3.0) e 2-4
2-2	**894**	(3.0) fg 2-4	(2.1) cd 1-3
2-2	**SE42**	(3.0) fg 2-4	(2.3) de 1-4
2-2	**Tas30.1**	(2.0) cd 1-3	(1.9) bc 1-4
3	**Tas 1**	(2.4) de 1-4	(2.1) cd 1-4
3	**Tas 3**	(1.6) bc 1-4	(1.0) a 1
3	**KI24**	(2.8) ef 2-3	(2.0) cd 2
3	**R229**	(4.0) h 4	(4.0) f 4
4	**734**	(2.9) ef 2-4	(1.7) bcd 1-4
8	**RS21**	(1.0) a 1	(1.3) ab 1-2
K	**KI19**	(1.1) ab 1-2	(1.5) abc 1-2
nil	**Control**	(1.0) a 1	(1.0) a 1

Table 10.3. Assessment of necrosis, using two methods, caused by Australian and British *R. solani* AG 2-1 isolates on stems of potato plants grown in controlled environment conditions at 16ºC compared to analysis shown in Table 4.5.

Isolate				Stem severity scores means and ranges	
Origin	IGS1 type	Code	AG	Scores from 1 to 4 (Balali *et al.* 1995) l.s.d. 5 % = 0.63	Scores from 0 to 4 (Carling and Leiner 1990) l.s.d. 5 % = 0.57
Control	nil	Control	nil	(1.0) a 1	(0.0) a 0
UK	Long	R107	2-1	(1.0) a 1	(0.0) a 0
UK	Short	R100	2-1	(2.4) c 1-4	(1.6) c 0-3
UK	Long	R42	2-1	(1.3) ab 1-2	(0.3) ab 0-1
Australia	Long	Tas6	2-1	(1.8) bc 1-4	(0.6) b 0-2
Australia	Inter.	L57	2-1	(1.0) a 1	(0.0) a 0
Australia	Long	1030	2-1	(1.0) a 1	(0.0) a 0

Differences in disease severity implied by ANOVA (Table 10.4.b) that were not observed in results from Kruskal-Wallis analysis presented in Table 4.6.b include:

- The severity scoring method of Balali et al. (1995) suggested that stem necrosis caused by R37 was less ($P < 0.05$) than those inoculated with T30.1.
- The severity scoring method of Caling and Leiner (1990) suggested that stem necrosis caused by isolate T30.1 was less severe ($P < 0.05$) than that caused by R20, R229 and R110.

Table 10.4. Assessment of the disease, using two methods, caused by Australian and British *R. solani* isolates on stems of potato plants grown under controlled environment conditions at 25ºC for 2 ½ weeks compared to analysis presented in Table 4.6

a. AG 2-1 isolates compared with analysis presented in Table 4.6.a

Isolate				Stem severity scores means and ranges	
Origin	IGS1 type	Code	AG	Scores from 1 to 4 (Balali *et al.* 1995) l.s.d. 5 % = 0.66	Scores from 0 to 4 (Carling and Leiner 1990) l.s.d. 5 % = 0.74
Control	nil	Control	nil	(1.0) a 1	(0.0) a 0
UK	Long	R107	2-1	(1.3) a 1-2	(0.3) abc 0-1
UK	Short	R100	2-1	(1.4) ab 1-2	(0.4) abc 0-1
UK	Long	R42	2-1	(1.1) a 1-2	(0.1) ab 0-1
Australia	Long	Tas6	2-1	(1.3) a 1-3	(0.2) ab 0-1
Australia	Inter.	L57	2-1	(1.4) ab 1-2	(0.4) abc 0-1
Australia	Long	1030	2-1	(1.1) ab 1	(0.0) a 0
UK	Long	R103	2-1	(1.1) ab 1-2	(0.1) ab 0-1
UK	Long	R118	2-1	(1.5) ab 1-2	(0.5) abc 0-1
UK	Long	R114	2-1	(1.3) a 1-2	(0.3) abc 0-1
UK	Long	R106	2-1	(1.6) ab 1-2	(0.9) bc 0-1
Australia	Inter.	SE50	2-1	(1.0) a 1	(0.0) a 0
Australia	Inter.	SE45	2-1	(1.4) ab 1-2	(0.4) abc 0-1
Australia	n/a	KI65	2-1	(1.1) a 1-2	(0.1) ab 0-1
Australia	n/a	KI4	2-1	(3.7) fg 3-4	(3.4) i 2-4
Australia	Long	R14	2-1	(1.3) a 1-2	(0.3) ab 0-1
Australia	Long	KI61	2-1	(1.4) ab 1-2	(0.4) abc 0-1
Australia	n/a	730	2-1	(2.5) cd 1-4	(1.6) de 0-3
Australia	Inter.	SAR11.18	2-1	(3.7) fg 2-4	(3.1) hi 1-4
Australia	Long	Tas3	2-1	(1.3) a 1-2	(0.3) ab 0-1
Australia	n/a	WAC-9806	2-1	(3.0) de 1-4	(2.6) fgh 0-4

b. Isolates from AGs other than AG 2-1, compared statistically to results from inoculation with AG 2-1 isolates shown in the previous table and analysis presented in Table 4.6.b

Isolate		Stem severity scores mean and range (see Table 10.4.a. for l.s.d 5 %)	
Code	AG	Scores from 1 to 4 (Balali *et al.* 1995)	Scores from 0 to 4 (Carling and Leiner 1990)
Control	nil	(1.0) a 1	(0.0) a 0
R72	2-2	(3.4) efg 2-4	(2.6) fgh 1-4
SE42	2-2	(3.2) ef 2-4	(2.8) ghi 1-4
T30.1	2-2	(2.0) bc 1-3	(1.0) cd 0-2
R20	3	(3.6) efg 1-4	(2.4) fgh 0-4
R37	3	(1.3) a 1-2	(0.3) abc 0-1
R229	3	(3.2) def 2-4	(2.0) ef 1-3
R112	4	(1.4) ab 1-2	(0.4) abc 0-1
734	4	(4.0) g 4	(3.5) i 3-4
R110	5	(3.1) def 1-4	(2.3) efg 0-4
R56	8	(1.4) ab 1-3	(0.4) abc 0-2
WAC-9923	8	(1.5) ab 1-3	(0.4) abc 0-2
RS21	8	(1.0) a 1	(0.0) a 0

Differences in disease severity implied by ANOVA (Table 10.5) that were not observed in results from Kruskal-Wallis analysis presented in Table 6.3 include:

- For AG 3 inoculated plants the numbers of sclerotia on tubers were fewer ($P < 0.05$) when seed was treated with Rizolex and Maxim than inoculated controls. Amistar application to furrow and Maxim application to seed reduced ($P < 0.05$) the severity of stolon necrosis when compared to inoculated controls.
- For AG 2-1 inoculated plants more severe ($P < 0.05$) stolon necrosis was observed on plants that has seed treatment with Rizolex or Maxim than inoculated controls.

Results of disease severity implied by ANOVA are not presented for comparison to those results presented in Table 6.4 or Table 6.6.

Table 10.5. Effect of fungicide application on severity of disease symptoms in shade-house experiments compared to analysis presented in Table 6.3.a & 6.3.b.

Inoculum AG	Treatment Fungicide	Disease severity (mean) and range scores (1-4)		
		Tuber sclerotia l.s.d. 5 % = 0.3	stem necrosis l.s.d. 5 % = 0.7	stolon necrosis l.s.d. 5 % = 0.5
nil	**nil control**	(1.0) a	(1.0) a	(1.0) a
AG 2-1	**Nil control**	(1.0) a	(4.0) c	(2.0) b
AG 2-1	**Rizolex**	(1.0) a	(3.7) c	(3.8) c
AG 2-1	**Maxim**	(1.0) a	(3.8) c	(3.8) c
AG 2-1	**Amistar**	(1.0) a	(1.7) ab	(1.9) b
AG 3	**nil control**	(2.5) c	(2.0) b	(2.0) b
AG 3	**Rizolex**	(2.1) b	(2.3) b	(1.9) b
AG 3	**Amistar**	(1.0) a	(1.5) a	(1.3) a
AG 3	**Maxim**	(2.0) b	(1.5) a	(1.3) a

Differences in disease severity implied by ANOVA (Table 10.6) that were not observed in results from Kruskal-Wallis analysis presented in Table 7.9 include:

- Stem necrosis was worse ($P < 0.05$) on AG 3-inoculate plants grown in 3 ppm zinc than uninoculated plants grown in all concentration of zinc except 0.1 ppm.

Table 10.6. Rhizoctonia disease symptoms on plants at harvest after growth in the presence of different zinc concentrations and *R. solani* inoculum compared to anaylsis presented inTable 7.9.

Treatment		Disease severity scores mean and range		
Inoculum	**Zinc soil addition**	**Tuber Sclerotia l.s.d 5 % = 0.4**	**Stem necrosis l.s.d 5 % = 0.7**	**Stolon necrosis l.s.d 5 % = 0.6**
control	**0 ppm**	(1.0) a 1	(2.0) abc 1-3	(2.5) bc 1-3
control	**0.1 ppm**	(1.0) a 1	(2.3) abcd 1-3	(3.1) de 3-4
control	**1 ppm**	(1.0) a 1	(1.6) a 1-3	(1.6) a 1-3
control	**3 ppm**	(1.0) a 1	(1.9) ab 1-3	(2.1) ab 1-3
AG 2-1	**0 ppm**	(1.0) a 1	(2.6) cd 2-3	(2.9) cd 2-4
AG 2-1	**0.1 ppm**	(1.1) a 1-2	(2.1) abcd 1-3	(2.9) cd 2-3
AG 2-1	**1 ppm**	(2.0) b 1-2	(2.0) abc 2	(2.0) abc 2
AG 2-1	**3 ppm**	(1.8) b 1-2	(2.0) abc 2	(3.0) cd 2-4
AG 3	**0 ppm**	(3.8) c 3-4	(2.4) bcd 2-3	(2.8) cd 2-3
AG 3	**0.1 ppm**	(3.8) c 3-4	(2.8) d 2-4	(3.6) e 3-4
AG 3	**1 ppm**	(3.9) c 3-4	(2.1) abcd 2-3	(3.0) cd 2-4
AG 3	**3 ppm**	(3.6) c 4	(2.7) d 2-4	(3.1) de 3-4

10.3 Inhibition of mycelial growth of *R. solani* isolates by fungicides *in vitro*

Table 10.7. EC_{50} values of each fungicide for each isolate with l.s.d. 5% = 0.26

Isolate		EC_{50} values represented as log of fungicide concentration (mg a.i./L)					
AG	Name	Monceren	Moncut	Rizolex	Rovral	Expt.	Maxim
2-1	L57	-1.442 efgh	-0.903 mnop	-0.099 w	*	-2.052 a	-0.409 stuv
	WAC-9806	-1.448 efgh	-0.825 nopq	-1.335 ghijk	-0.442 rstu	-1.940 ab	-1.633 cdef
	R394	-1.267 ghijkl	-0.781 opq	-0.900 mnop	-0.152 vw	-1.850 abc	-1.370 fghij
2-2	KI-3	-1.253 ghijkl	-0.416 rstu	-1.166 ijkl	-0.273 tuvw	-1.339 ghijk	-1.394 fghij
	WAC-9765	-1.436 efgh	-0.264 tuvw	-1.138 jklm	-0.194 uvw	-1.413 fghi	-1.484 defg
	WAC-9937	-1.392 fghij	-0.416 rstu	-1.239 ghijkl	-0.333 stuvw	-1.329 ghijk	-1.487 defg
3	L306	-1.228 ghijkl	-0.367 stuv	-1.192 hijkl	-0.248 tuvw	-1.410 fghi	-1.715 bcd
	L62	-1.787 bc	-0.331 stuvw	-1.077 klmn	-0.151 vw	-1.301 ghijk	-1.608 cdef
	R229	-1.742 bcd	-0.568 qrs	-1.161 ijklm	-0.324 stuvw	-1.161 ijklm	-1.633 cdef
8	WAC-9923	*[1]	-0.413 stuv	-0.678 pqr	-0.256 tuvw	-0.813 nopq	-1.439 efgh
	RS21	*	-0.492 rst	-1.026 lmno	-0.425 rstu	-1.413 fghi	-1.684 bcde

Values from Table 10.7 were converted from log of fungicide concentration to fungicide concentration as shown in Table 10.8. This showed the volume of active ingredient in the presence of which mycelial growth was inhibited. However, this also resulted in an l.s.d 5% that is no longer reflected the significant differences between treatments.

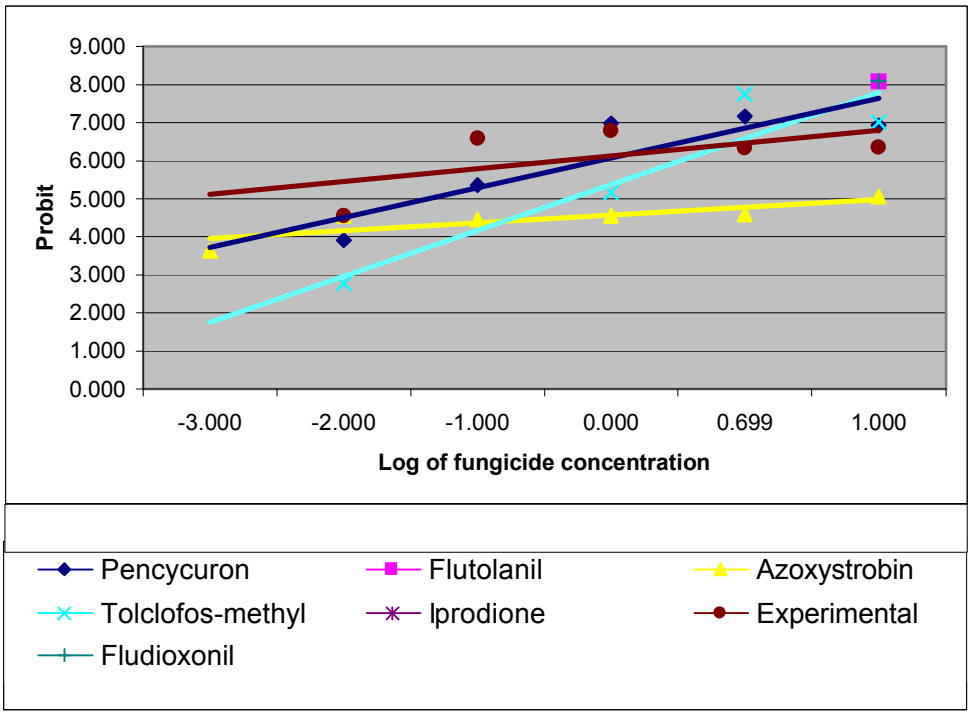
Table 10.8. EC_{50} values of each fungicide for each isolate

Isolate		EC50 values represented as fungicide concentration (mg a.i./L)					
AG	Name	Monceren	Moncut	Rizolex	Rovral	Expt.	Maxim
2-1	L57	0.036	0.128	0.796	*	0.008	0.390
	WAC-9806	0.053	0.166	0.126	0.704	0.014	0.043
	R394	0.036	0.151	0.044	0.362	0.011	0.023
2-2	KI-3	0.054	0.383	0.068	0.516	0.046	0.040
	WAC-9765	0.036	0.384	0.056	0.464	0.047	0.033
	WAC-9937	0.036	0.506	0.072	0.603	0.038	0.033
3	L306	0.059	0.430	0.064	0.565	0.040	0.019
	L62	0.016	0.466	0.083	0.696	0.050	0.025
	R229	0.018	0.271	0.065	0.474	0.069	0.023
8	WAC-9923	*	0.322	0.094	0.335	0.039	0.021
	RS21	*	0.386	0.210	0.554	0.154	0.036

[1] * indicates no EC_{50} value as the inhibition did not reach 50% as shown in graphs

10.4 Conversion of percentage of inhibition to probit values

Figures 10.1 **Probit transformation of values of percent of inhibition of AG 2-1 isolate L57 by fungicides as compared to Figure a .**



Log of fungicide concentration values below 1 and above 99 simply indicate that the mean growth was less or more than the control, although in most cases not significantly different, they could not be converted to probit values. For any given fungicide if less than three data points were available from the concentrations tested a line of best fit and EC50s could not be determined. For fungicides that could be plotted the loss of data resulted in a lower confidence in EC50 values obtained from these graphs. Although graphing of probit values allows easy visualisation of inhibition over the range of concentrations by observation of slope of the line of best fit log of concentration better represents more values required for determining EC50 values for each fungicide.

Table 10.9. Conversion of data from log of concentration values to probit[78].

		% of mycelial growth inhibition					
	logConc	**-3**	**-2**	**-1**	**0**	**0.7**	**1**
	Probit	**-3.000**	**-2.000**	**-1.000**	**0.000**	**0.699**	**1.000**
	AG 2-1 (L57)						
Fungicide	Pencycuron		3.911	5.361	6.977	7.170	6.927
	Flutolanil						8.090
	Azoxysrobin	3.634	4.552	4.455	4.549	4.585	5.053
	Tolclofos-methyl		2.774		5.164	7.748	7.014
	Iprodione						
	Experimental		4.557	6.598	6.787	6.341	6.360
	Fludioxonil						8.090
	AG 2-1 (R394)						
Fungicide	Pencycuron	3.213	4.100	5.295	6.146	6.866	6.665
	Flutolanil	2.925		4.447	6.491		
	Azoxysrobin	4.326	4.069	4.557	4.847	4.852	5.345
	Tolclofos-methyl		3.502	4.574			
	Iprodione	2.946		3.420	5.248	5.810	6.589
	Experimental		4.907	5.863	6.237	6.372	6.476
	Fludioxonil		3.701	5.710			
	AG 2-1 (WAC 9806)						
Fungicide	Pencycuron		4.297	5.595	6.717	6.977	6.995
	Flutolanil	2.946	2.252	4.490	6.607		7.652
	Azoxysrobin	3.911	4.294	4.736	4.869	4.721	5.556
	Tolclofos-methyl		3.411	5.656	7.326		7.652
	Iprodione	3.272	2.830	3.724	5.946		7.366
	Experimental	3.040	4.864	6.180	6.426	6.454	6.468
	Fludioxonil	2.856	4.162	6.398	7.576		7.652
	AG 2-2 (KI-3)						
Fungicide	Pencycuron	3.213	3.964	5.391	7.512	7.290	7.014
	Flutolanil	3.595		3.757	5.950	7.878	7.366
	Azoxysrobin	4.115	4.294	4.552	4.604	4.593	4.937
	Tolclofos-methyl	2.925	3.345	5.319	7.014	7.226	7.034
	Iprodione	2.543		3.272	5.800		7.457
	Experimental	3.023	4.176	5.499	6.028	6.099	6.071
	Fludioxonil		3.568	5.882	6.960	7.097	7.170
	AG 2-2 (WAC 9937)						
Fungicide	Pencycuron		4.281	5.536	7.457	7.878	
	Flutolanil	3.249	2.743	3.546	5.982	7.878	
	Azoxysrobin	4.158	4.452	4.639	4.757	4.631	4.985
	Tolclofos-methyl		3.454	5.429	6.977	7.014	7.054
	Iprodione	2.743	2.252	3.779	5.742	7.878	
	Experimental	3.023	4.261	5.459	5.904	6.071	6.076
	Fludioxonil		3.763	5.871	6.852	7.075	7.366
	AG 2-2 (WAC 9765)						
Fungicide	Pencycuron		4.140	5.524	6.960	7.457	7.409
	Flutolanil			2.925	6.067	8.090	8.090
	Azoxysrobin	4.140	4.341	4.757	4.965	4.819	5.176
	Tolclofos-methyl		3.134	5.277	7.290	6.943	7.226
	Iprodione		3.305	2.634	5.813	7.878	8.090
	Experimental		4.248	5.459	6.385	6.360	6.405
	Fludioxonil		3.615	5.871	7.060	7.226	7.034

[78] Grey shading indicates log of concentration values below 1 (light) or above 99 (darker) that could not be converted to probit.

Table 10.1 Continued

		% of mycelial growth inhibition					
	logConc	**-3**	**-2**	**-1**	**0**	**0.7**	**1**
	Probit	**-3.000**	**-2.000**	**-1.000**	**0.000**	**0.699**	**1.000**
	AG 3 (L306)						
Fungicide	**Pencycuron**		4.366	5.228	5.849	6.028	6.058
	Flutolanil			3.305	5.838	7.226	7.878
	Azoxysrobin	4.532	5.184	5.882	6.165	6.015	6.221
	Tolclofos-methyl	2.591	3.365	5.607		7.652	
	Iprodione			3.325	5.625	7.226	
	Experimental		4.316	5.598	5.958	6.103	6.211
	Fludioxonil		4.322	6.276		7.652	
	AG 3 (L62)						
Fungicide	**Pencycuron**	2.543	4.793	5.762	6.572	6.655	6.927
	Flutolanil			3.757	5.568	6.635	7.034
	Azoxysrobin	4.284	4.808	5.311	5.459	5.448	5.762
	Tolclofos-methyl	2.424	2.880	5.274		7.576	
	Iprodione			3.040	5.568	7.457	
	Experimental		4.297	5.324	5.779	5.871	5.966
	Fludioxonil	2.710	3.820	6.305		7.576	
	AG 3 (R229)						
Fungicide	**Pencycuron**		4.617	5.789	7.257	7.652	7.409
	Flutolanil	3.374		3.850	6.146	6.685	6.866
	Azoxysrobin	4.384	4.571	4.945	4.993	4.721	5.108
	Tolclofos-methyl	3.261	3.532	5.335			7.576
	Iprodione			3.689	5.769	7.652	7.576
	Experimental		4.375	5.324	5.694	5.759	5.946
	Fludioxonil		3.892	6.221			7.576
	AG 8 (RS21)						
Fungicide	**Pencycuron**		4.224		4.176	4.303	4.754
	Flutolanil	3.315	3.641	3.420	6.385	7.409	
	Azoxysrobin	4.577	5.010	4.757	4.983	4.895	5.443
	Tolclofos-methyl	2.674	4.396	4.351		6.483	
	Iprodione	3.653	4.231	3.741	5.619	7.652	
	Experimental	2.946	4.183	5.619		7.144	
	Fludioxonil	2.424	4.538	5.935			
	AG 8 (WAC 9923)						
Fungicide	**Pencycuron**			3.524	4.396	4.590	4.626
	Flutolanil			3.678	6.089		
	Azoxysrobin	4.332	4.673	4.708	4.716	4.708	5.080
	Tolclofos-methyl		3.602	4.718	5.769	5.786	5.810
	Iprodione	3.795	3.057	3.878	5.437		7.326
	Experimental	2.424	2.925	5.128	5.650	5.904	5.942
	Fludioxonil	2.803	3.878	5.766	6.117	6.447	6.323