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OF

MASTER OF AGRICULTURAL SCIENCE

Investigation of *Fusarium oxysporum* f. sp. *cepae* and *Phoma terrestris* on onion (*Allium cepa)* in the Mid Murray region of South Australia

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Abstract

Two fungi that cause disease on onion in the Mid Murray region of South Australia were investigated. These were *Fusarium oxysporum* f. sp. *cepae,* the cause of damping off of seedlings, wilting in the field, and basal rot in storage, and *Phoma terrestris,* which causes pink root. The aim of the study was to investigate resistant or tolerant cultivars and crop rotations as possible management strategies for these diseases.

Several *Fusarium* spp. were isolated from onion roots and bulbs in the field, onion bulbs in storage, soil, and onion seed. All isolates were tested for pathogenicity by various means, however, none caused disease and therefore could not be identified conclusively as *F. oxysporum* f. sp. *cepae*. As a result research on *Fusarium* was discontinued.

Pink discolouration was detected on onion roots in commercial crops at Bowhill, South Australia, and isolation of *P. terrestris* from roots was attempted. Several published and novel methods were employed with variable success. Only one of the published methods yielded *P. terrestris,* but results were inconsistent. Two methods developed in this study permitted isolation of *P. terrestris.* In both cases, infested plant material was incubated on wheat straw agar (WSA), which turns pink in the presence of *P. terrestris*. One method involved soaking surface sterilised pink-pigmented wheat straw in solution, at approximately 24ºC for 2 days, and then spreading 1 ml of the resulting suspension on acidified potato dextrose agar (APDA) and incubating for a further 7 to 10 days. The second method involved dispensing warm sterile water into a plate of pink-pigmented WSA, agitating the plate, and spreading 1 ml aliquots onto APDA, and incubating for 7 to 10 days. One isolate of *P. terrestris*, shown to be pathogenic to onion was used in subsequent experiments.

Several brown onion cultivars commonly grown in the Mid Murray region of South Australia, or marketed as tolerant to pink root, were examined for susceptibility to infection by *P. terrestris*. Seven cultivars were tested *in vitro,* and seedlings of eight cultivars were grown in a greenhouse for 4 months in soil naturally infested with *P. terrestris*. Selected cultivars were also evaluated in the field. Of the cultivars tested, none was significantly less susceptible to infection by *P. terrestris* than any other as indicated by pink pigment on WSA.

Cereals such as wheat, oat and barley, as well as lucerne and canola, are grown in rotation with onion to provide ground cover, to minimise soil erosion and reduce inoculum of pathogens between onion crops. As the susceptibility of these rotation crops to *P. terrestris* was not known, several were tested in the field and in the greenhouse for their ability to act as hosts for *P. terrestris*. Likewise, the potential of weeds commonly found in onion fields to act as hosts of *P. terrestris* was investigated. Less *P. terrestris* was detected in the roots of plants of oat cultivars Marloo and Swan, as well as barley cultivars Galleon and Schooner than of most other crops tested and, thus, these cvs were considered less likely to act as hosts of *P. terrestris*. Weeds such as *Brassica tournefortii, Portulaca oleracea* and *Oxalis pes-caprae* collected from the field were infected by *P. terrestris*, whereas *P. terrestris* was not detected in the roots of *Chenopodium album.*

In summary, new methods for isolation of *P. terrestris* were identified and may, with further development, aid in research on pink root. The onion cultivars tested in this study did not differ in susceptibility to pink root, but more cultivars should be tested. *P. terrestris* infected a range of other crops and weeds, and it is recommended that further work is conducted in this area to facilitate cultural management of onion crops to minimise build up of inoculum in the soil.

Declaration

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

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Yvonne J. Smith

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1 Introduction and literature review

1.1 Introduction

Onion (*Allium cepa*) is an important horticultural crop in Australia. The crop, approximately 200,000 tonnes per year, grown on about 4,000 hectares, is worth around $80 million per annum to the Australian economy. Indirect revenue from seed, fertiliser and chemical companies, together with wholesale and retail markets, increases this figure significantly.

Onions are grown in every state of Australia. The industry in South Australia has grown rapidly in the last 20 years, to account for almost 40% of the total production in Australia (Australian Onion Industry Association 2002). The major onion growing regions in South Australia include the Mid Murray region and the South East of the state.

In Tasmania, the onion industry produces 30% to 40% of the Australian total. Major diseases of onion in Tasmania include white rot (*Sclerotium cepivorum*) and Botrytis neck/bulb rot (*Botrytis allii*). Both of these diseases have caused significant losses of yield of onion in conditions that favour disease development (Dennis 1995). Little research has been conducted on onion in South Australia, although this state is the largest producer of onion in the country. This is almost entirely due to the lack of research funds. As the climate in South Australia is markedly different from the other onion growing regions in Australia, research conducted in other states is often of little relevance to the disease problems in South Australia.

Major soil borne fungal pathogens of onion world-wide include *Pythium* spp., *Rhizoctonia solani, Phoma terrestris* (syn. *Pyrenochaeta terrestris*), *Sclerotium cepivorum* and *Fusarium* spp. (Gabor 1997); (Lomman 1991). Of these pathogens, *S. cepivorum* mainly occurs in the lower South East of South Australia (Lomman 1991),

where the colder and wetter climatic conditions can be suitable for white rot. *R. solani* is believed to be the causal organism of damping-off in many onion crops in the Mid Murray region (Trevor Twigden, personal communication, October 2002, (Lomman 1991). Onions with damping-off symptoms were observed on a farm in the Mid Murray region of South Australia in 1994 and 1995, and *Pythium* and *Fusarium* were found to be the causal organisms (Barbara Hall, personal communication, 1995).

In 2004 many onion crops in the Mid Murray region were devastated by a condition of unknown aetiology, which caused widespread stunting in young crops, resulting in small bulbs at maturity. This problem is currently the focus of a research project at the South Australian Research and Development Institute (SARDI).

Previous scientific research conducted in the Mid Murray region indicated that *F. oxysporum* f. sp. *cepae* was present in areas of damping-off of onion (Smith 1999) and Fusarium basal rot of onion can be present in fields and in storage (Lomman 1991). *Fusarium* spp., however, are not listed as pathogens of onion in the Host-Pathogen Index of Plant Diseases in South Australia (Cook and Dubé 1989).

Control of Fusarium basal rot of onion involves the practice of long rotations with non-hosts of the pathogen. However, it is unclear which rotation crops are non-hosts of *Fusarium*, particularly as many species of *Fusarium* exist. *F. oxysporum* f. sp. *cepae* causes Fusarium basal rot of onion (Lorbeer and Stone 1965), although a number of *Fusarium* spp. may be responsible for damping-off symptoms (Sumner 2008a). The sowing of tolerant or resistant cultivars may be beneficial (Gabor 1997) however, few cultivars of onion are marketed in South Australia as having tolerance or resistance to *Fusarium.* Furthermore, waterlogging of soil must be avoided and damage to bulbs during harvest minimised (Lomman 1991).

Pink root is a major soil borne disease of onion in South Australia (Lomman 1991) and is caused by the fungus *Phoma terrestris* (syn. *Pyrenochaeta terrestris*). Growers are familiar with the disease and find symptoms in onion crops in most seasons, in many different cultivars (Kevin Smith, personal communication, 2000). Control measures for this pathogen involve long rotations with non-hosts of the pathogen between onion crops, early sowing to avoid heat during seedling establishment and hygiene to avoid spread of the propagules (Lomman 1991). However, economic considerations make long rotations unlikely, many cultivars cannot be sown early or they will not develop properly, and it is not clear which rotation crops are non-hosts of the pink root pathogen.

Very little is known of the incidence and effects of the above-mentioned soil borne pathogens on onion in South Australia. This study, initiated in 2000, was designed to gain an understanding of *F. oxysporum* f. sp. *cepae* and *P. terrestris*, and the diseases they cause on onion in South Australia to enable appropriate management practices to be implemented, and yield and profitability to be improved.

1.2 Growing onions in Australia

In the Mid Murray region of South Australia onions are grown from seed that is directly sown into fine seedbeds. In fields where soil erosion and sand-blasting may occur, cover or “nurse crops”, typically cereals, are sown prior to seeding onions, and the cover crop is then killed with herbicide when onion seedlings are sufficiently well established (Lomman 1991). In some areas of the world, onion sets are planted to produce a commercial or garden crop. Onion sets are produced by sowing onion seeds densely to produce small bulbs less than 2.5 mm in diameter. Planting sets reduces the length of the growing period or produces very large onion bulbs when planted.

In the Mid Murray region, onions are sown from May to September, depending on the cultivar, for harvest in December to April (Kevin Smith, personal communication, 2002). Cultivars sown include both hybrid and open pollinated seed of white, brown and red Spanish onion, and mild/sweet onion cultivars are becoming more common.

The onion plant grows slowly, taking over 6 months from sowing to maturity, and competes poorly with weeds. Seedlings grow through a series of leaf stages prior to "bulbing", the process of stem expansion that results in the onion bulb, and then the bulb continues to grow until leaf regression and maturity (fig 1.1). When the onion plant matures, the bulb is firm, the green leaf wilts, dries out and turns a brown colour, and the onion is ready to harvest (Lovatt *et al.* 1997). Generally, onions of the same cultivar in a field mature at the same time. There are times, however, when a small patch of onions within the field remains erect for much longer than the rest of the crop. These onions typically have experienced disrupted growth and are less mature than the rest of the field. Disruption to the growth could be attributed to disease, soil type or aspect (Kevin Smith, personal communication, 2000).

Onions seed crops are grown from selected bulbs planted into the soil (Brewster 1994). Bulbs are selected on traits such as desirable size, firm skin, consistent colour, etc, in order to reproduce these traits in the new seed stock. The bulb will break dormancy and produce an inflorescence stalk or a "scape", about 1-2 m long, and a flower head at the apex of the stalk, which is termed the "umbel", that produces seed at maturity. However, an onion crop may flower and produce inflorescence stalks during growth from seed (bolting), particularly if unseasonably cold weather occurs during growth, or cultivars are sown at inappropriate times of the year. Bulbs that produce umbels in this way are unmarketable (Lovatt *et al.* 1997).

In the Mid Murray region of South Australia, most onions produced are 'storage onions'. This means that the bulbs can be stored for many months, compared with 'fresh onions' that need to be sold within weeks of harvest. The storage onions are placed in wooden 1-tonne or 0.5-tonne bins, and placed under cover at ambient temperature. Cultivars vary in the length of time for which they can be stored, but 6 months is common for many brown cultivars. Red and white cultivars cannot be stored for quite so long. Placing bins of onions in cold storage rooms set at 0– 1ºC extends storage life, and some cultivars of onion are sold following cold storage for 8-10 months after harvest (Kevin Smith, personal communication, 2000).

Onions are graded according to size, shape, colour, lack of blemishes and skin retention. Onions are usually packed into 10 or 20-kg net bags, or into 10 or 15-kg cartons for sale. Most onions from South Australia are sold domestically in the eastern states of Australia, but there is also an export market to Europe, South East Asia and Japan (Lomman 1991).

Crops commonly grown in rotation with onion in South Australia include *Triticum aestivum* (wheat), *Hordeum vulgare* (barley), *Avena* spp. (oat), *Triticum x secale* (triticale), *Medicago sativa* (lucerne), *Solanum tuberosum* (potato), *Cucurbita* spp. (pumpkin), *Zea mays* (sweetcorn) and *Vicia sativa* (vetch). Weeds commonly found in onion fields in South Australia include *Heliotropium europaeum* (heliotrope), *Portulaca oleracea* (purslane), *Arctotheca calendula* (capeweed), *Polygonum aviculare* (wireweed), *Brassica tournefortii* (turnip), *Chenopodium album* (fathen), *Tribulus terrestris* (caltrop), *Chondrilla juncea* (skeleton weed) and many grasses (Kevin Smith, personal communication, 2002).

NOTE:
This figure is included on page 6
of the print copy of the thesis held in
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Fig 1.1 Growth stages of an onion, from seed germination to maturity. The process takes approximately 6 months. Drawings from http:www.ics-agri.com/onion-cycle.htm

Various soil-borne pathogens can infect onion, as discussed above (section 1.1). Two of these pathogens were the focus of this project and will be discussed in depth.

1.3 Pathogens of onion: *Fusarium*

1.3.1 Taxonomy

The genus *Fusarium* Link. is of the form-class Hyphomycetes in the group Mitosporic Fungi (formerly Deuteromycotina). Most *Fusarium* spp. produce macroconidia, microconidia and chlamydospores. The chlamydospore is the resting structure of the fungus (Booth 1971). The shape of the septate macroconidium, with a foot-shaped basal cell is the main criterion used for taxonomic purposes. Some species, have a teleomorph stage in the Hypocreales (Burgess *et al.* 1994; Windels 1992, Burgess, 1994 #101).

The taxonomy of the genus *Fusarium* has been a topic of research and debate since the discovery of the genus by Link in 1809. The first real attempt to develop a key for the species was in 1913 by Wollenweber (1913). This research led to the publication of a taxonomic reference for *Fusarium* in 1935 (Wollenweber and Reinking 1935). However, the system devised by Wollenweber, with 65 species, was regarded as too complex for practical purposes, due to the reliance on spore measurements and septation to differentiate species. There is large variation in these characteristics within a single species.

An endeavour was made to simplify the taxonomic system for *Fusarium.* Snyder and Hansen (1940) used the section *Elegans,* developed by Wollenweber, as an example of the changes they proposed. The three original subsections of *Elegans* were placed in the one subsection, *oxysporum*, to account for the variation that exists within a species in regard to cultural appearance, morphology and physiology. The subsection, in this

system, would contain various biological forms, where parasitic fungi would be named based on their pathogenicity to specific hosts. Snyder and Toussoun (1965) applied this system to other sections of *Fusarium,* to reduce the number of species in the genus to nine.

Booth (1971) proposed a scheme with 44 species of *Fusarium,* whereas Nelson *et al.* (1983) developed another taxonomic system with 30 species of *Fusarium,* regarded as a compromise between the complex and the simple. Burgess (1994) favours the system developed by Nelson *et al.* (1983). However, the reduction of the section *Elegans* to a single species, *F. oxysporum,* by Snyder and Hansen (1940) is accepted widely by plant pathologists.

Parasitic strains of *F. oxysporum,* according to the system of Snyder and Hansen (1940), are differentiated by their relative pathogenicity to host plants under the concept of *forma speciales*. This allows differentiation between strains that are morphologically similar. However, not all *forma speciales* are host specific; some have a broad host range, which complicates the system (Windels 1991). New approaches involving aspects of physiology, genetics and molecular biology now provide useful identification tools for differentiation among species of *Fusarium*.

Efforts have been made to classify strains of pathogenic *F. oxysporum* into groups according to their vegetative compatibility (VCG), or the ability to form heterokaryons (Puhalla 1985). This method of classification correlates well with *forma speciales* and, if proved accurate, would provide a laboratory testing procedure for classification that may be more manageable than pathogenicity testing, which has limitations, particularly due to inconsistency in technique. However, it has been reported that the relationship between VCGs and *forma speciales* can be complex in some cases (Correll 1991). To date, no races have been characterized based on vegetative compatibility (Havey 2008).

A review of the literature on Fusarium basal rot of onion shows that taxonomy is confusing. Early reports refer to *F. vasinfectum* var. *zonatum* (Kreutzer 1941) as causing basal rot of onion. Another species, *F. zonatum forma* 1, is mentioned by Davis and Henderson (1937) as being present in the roots and the bulb of onion, and to cause bulb rot. Subsequently, *F. vasinfectum* var. *zonatum* was described as a synonym for *F. oxysporum* f. sp. *cepae* (Lorbeer and Stone 1965). It has now been established that all the *Fusarium* taxa mentioned above are synonyms of *F. oxysporum* f. sp. *cepae* (Entwhistle 1990).

Difficulties with classification and subsequent identification of *Fusarium* spp. promote caution that reference to a particular species of *Fusarium* in early publications must not be assumed correct. It is likely that degenerated cultural variants were named as new species (Windels 1992). In this study, the causal organism of Fusarium basal rot of onion will be referred to as *F. oxysporum* f. sp. *cepae*.

1.3.2 *Fusarium* disease on onion – infection and symptoms

F. oxysporum f. sp. *cepae* can infect onion at all stages of the plant's growth. It causes damping-off of seedlings, root rot in older plants that may result in the death of the plant, and basal rot of bulbs, which can cause breakdown of the bulb in the field or in storage (Abawi and Lorbeer 1972). The fungus can penetrate either as a primary or secondary invader (Abawi and Lorbeer 1971a).

Once *F. oxysporum* f. sp. *cepae* has penetrated a root, growth is mainly confined to the intercellular spaces of the cortex. The hyphae will then branch and invade adjacent cells. The pathogen continues into the vascular system of the plant near the root tip. The fungus will progress to the stem plate area and, if conditions are favourable, will colonise the bulb tissue (Abawi and Lorbeer 1971a). If disease develops in the bulb, rotting starts at the stem plate, and spreads upwards in the scale. Infected brown bulbs

appear reddish-brown. The bulb tissue will be watery in appearance initially and, later, desiccate completely and shrink. There is no evidence that infection will spread from infected to healthy bulbs in the field or in storage (Tahvonen 1981).

F. oxysporum f. sp. *cepae* releases pectic enzymes that break down the pectin in the onion cell wall (Cramer 2000). The bulb scales and the leaf sheaths of the onion have high levels of total sugars; the sugar content has been shown to be a factor in the repression of synthesis of the pectic enzymes and may retard growth of the fungus (Holz and Knox-Davies 1985). The stem plate and the apoplast have less sugar and this may explain why the pathogen appears in these areas of the plant first. Therefore, it would seem that parts of the onion plant vary in susceptibility to *F. oxysporum* f. sp. *cepae*.

There is a significant increase in Fusarium basal rot of onions where mechanical wounding has occurred (Everts *et al.* 1985) and this has led some researchers to believe that *F. oxysporum* f. sp. *cepae* is a secondary pathogen of onion. Mechanical wounding can occur readily in the field through activities such as hand weeding, particularly when roots of plants are intertwined with weeds. Nevertheless, Lorbeer (1965) found that *F. oxysporum* f. sp. *cepae* is a primary invader of onion and that wounding or damage is not necessary for infection to occur. *F. oxysporum* f. sp. *cepae,* therefore, is considered to be a primary pathogen of onion.

Whilst a correlation exists between amount of inoculum of *F. oxysporum* f. sp. *cepae* and disease development in artificial conditions (Abawi and Lorbeer 1972), the same result has not been demonstrated in field trials (Abawi and Lorbeer 1971b). It appears that factors other than inoculum density are involved, whether biotic or abiotic, that influence the amount of disease that will occur. For example, studies have shown that irrigation of the onion crop after leaf fall aided the entry of *F. solani* and resulted in an

increase in storage rots of bulbs (Singh *et al.* 1992). Whilst this study named *F. solani* as the pathogen, it is possible that, due to taxonomic confusion, the pathogen under investigation was *F. oxysporum* f. sp. *cepae.*

There are some reports that infection of onion by *F. oxysporum* f. sp. *cepae* will give rise to pink roots (Dennis *et al.* 1997). Davis and Henderson (1937) reported that all roots infected with *F. oxysporum* f. sp. c*epae* eventually changed from white to a dark red colour. However, other authors report that pinking of roots never occurs on onion when *F. oxysporum* f. sp. c*epae* is the sole pathogen, and that this symptom only occurs in association with the pink root disease caused by *Phoma terrestris* (Abawi and Lorbeer 1971a; Awuah and Lorbeer 1989).

1.3.3 Characteristics of *F. oxysporum* f. sp. *cepae*

The pathogen *F. oxysporum* f. sp. *cepa,* is one of over a hundred formae speciales of *F. oxysporum* (Burgess *et al.* 1994), and produces chlamydospores, macroconidia, and, less often, microconidia (Havey 2008). Chlamydospores are the primary source of inoculum in the field (Burgess *et al.* 1994). Macroconidia are curved and have three to four septa (Havey 2008).

Fusarium species are difficult to preserve for any length of time. Isolates generally mutate quickly in nutrient-rich environments, and these mutants are usually less pathogenic than the original culture (Burgess *et al.* 1994).

There are several methods of maintenance, one of which involves the use of silica gel (Windels *et al.* 1993). Isolates are grown on potato dextrose agar (PDA) or carnation leaf agar (CLA) until actively sporulating, then spores are washed into a milk solution and pipetted onto silica gel for long-term storage. This method has been useful for maintaining isolates for up to 10 years without detectable mutation.

Methods such as that described by Windels *et al.* (1993) are now common practice in maintaining *Fusarium* isolates. However, in the past storage methods may have been detrimental to the maintenance of the isolate, resulting in mutation and loss of virulence, which may have affected experiments conducted to evaluate pathogenicity of isolates on host crops. This possibility must be considered when reviewing previous research.

1.3.4 Distribution

F. oxysporum f. sp. *cepae* has been reported to cause damage to onion in many geographical locations of the world. For example, *F. oxysporum* f. sp. *cepae* was reported to cause basal rot of onion in storage in Arizona (Marlatt 1958). The pathogen was also identified in Japan (Takakuwa *et al.* 1977) and in Israel (Joffe *et al.* 1972), where several isolates of *Fusarium* were shown to be pathogenic on onion. *F. oxysporum* f. sp. *cepae* has contributed to poor stand establishment in Zambia (Naik and Burden 1981) and is the main bulb rot pathogen of onions both in the field and in storage in Italy (Fantino and Schiavi 1987). There are also reports of *F. oxysporum* f. sp. *cepae* causing storage rots in Turkey (Koycu and Özer 1997), Finland (Tahvonen 1981) and Nigeria (Wilcox and Balogh 1998). Brayford (1996), in the IMI description of pathogenic fungi and bacteria, also included Egypt, Ethiopia, South Africa, India, the Philippines, Greece, Hungary, USA and Brazil. Tasmania is the only state of Australia that is included in the IMI index as a location for *F. oxysporum* f. sp. *cepae*. The Host-Pathogen Index of Plant Diseases in South Australia (Cook and Dubé 1989) makes no mention of any *Fusarium* species on onion. However, *F. oxysporum* f. sp. *cepae* has been isolated from onion fields in South Australia (Smith 1999), and reports of wilt in the field and bulb rots in storage provide anecdotal evidence that it has been present for many years (Kevin Smith, personal communication, 2002). Smith (1999) suggested that

further research be undertaken to improve our understanding of the pathogen and the disease.

1.3.5 Alternative hosts

F. oxysporum f. sp. *cepae* is not specific to *Allium cepa*. Other *Allium* species listed as hosts of *F. oxysporum* f. sp. *cepae* are *A. oschaninii* (shallots), *A. sativum* (garlic), *A fistulosum* (Japanese bunching onion) and *A. chinense* (rakkyo). *Asparagus officinalis* (asparagus) is listed as a non-*Allium* host of the pathogen (Brayford 1996). Other crops that may be infected by *F. oxysporum* f. sp. *cepae* include *Zea mays* (corn), *Triticum aestivum* (wheat), *Oryza sativa* (rice), *Glycine max* (soybean), *Cucumis sativus* (cucumber), *Pisum sativum* (pea) and *Cucurbita maxima* (squash) (Cramer 2000).

Armstong and Armstrong (1948) suggested the possibility of symptomless hosts after experiments with several Fusarium wilt pathogens. Following inoculation of *Ipomea batatas* (sweet potato) with the cotton wilt pathogen, *F. oxysporum* f. sp. *vasinfectum. F. oxysporum* f. sp. *vasinfectum* was consistently isolated from sweet potato, which exhibited no external symptoms of disease. Further experiments with this strain of *Fusarium* indicated that it could also be isolated from wilting plants of *Medicago sativa* (lucerne), *Glycine max* (soybean) and *Nicotiana tabacum* (tobacco). Likewise, Tomato-wilt Fusaria were reisolated from inoculated sweet potato and *Albizia julibrissin* (mimosa), although the plants showed no wilt symptoms (Armstrong and Armstrong 1948). Extensive research to investigate the host specificity of other *F. oxysporum* strains showed that many strains also had a broad host range or were a primary pathogen of one crop and a secondary pathogen of others (Armstrong and Armstrong 1975).

Oxalis spp. are weeds common to agricultural areas worldwide and are symptomless hosts of *F. oxysporum* f. sp. *cepae* in New York (Abawi and Lorbeer 1972). Isolates of

F. oxysporum obtained from *Oxalis* spp. were highly pathogenic when applied to onion slices and to onion seedlings in soil. As a result, these isolates were classified as *F. oxysporum* f. sp. *cepae*. This report is supported by work conducted in South Africa (Holz and Knox-Davies 1976) where it is believed that *Oxalis* spp. play an important role in maintaining high inoculum levels of *F. oxysporum* f. sp. *cepae* between onion crops.

All weeds in an onion crop pose a threat to yield, as onions are poor competitors for water and nutrients. *Oxalis pes-caprae* (soursob) occurs in onion fields in Australia. Dense populations are controlled by either spraying with a herbicide such as glyphosate before the onions emerge, or by handweeding during the growth of the crop. However, a farmer may not be so diligent in controlling moderate populations of soursob as they pose few problems at harvest, by which time the soursob will have senesced. In instances such as this, a seemingly unthreatening weed may be allowed to proliferate, particularly in rotation crops between onion crops. If *O. pes-caprae* is an alternative host in Australia, it may be an important factor in maintaining soil populations of *F. oxysporum* f. sp. *cepae* in some farms.

There is little published research on the host range of *F. oxysporum* f. sp. *cepae* in Australia, and common break crops such as cereals and lucerne may be important carriers of disease. This is a key area for investigation as farmers may inadvertently maintain, or even increase, the populations of *F. oxysporum* f. sp. *cepae* in the soil between onion crops.

1.3.6 Source of inoculum

Seed may be an important source of inoculum of *Fusarium* (Katan *et al.* 1980). *Fusarium* spp. are often detected in onion seed that is screened for *Botrytis allii* in Tasmania (Dean Metcalf, personal communication, 2001) However, it may be difficult

to detect *F. oxysporum* f. sp. *cepae* in seed when *Aspergillus niger* is present, as the latter organism can either inhibit or disguise the presence of the *Fusarium* spp. (Koycu and Özer 1997). It has not been determined if the existence of a *Fusarium* sp. in the seed will progress to infection of the seedling or mature plant, and whether the species commonly found is *F. oxysporum* f. sp. *cepae.*

Soil is a major source of inoculum of *F. oxysporum* f. sp. *cepae.* However, many *Fusarium* species exist in the soil, so difficulties exist in specifically isolating *F. oxysporum* f. sp. *cepae,* as current selective media such as Peptone PCNB Agar (PPA) (Nelson *et al.* 1983) do not allow differentiation between species and races. *Fusarium* spp. isolated on PDA need to be subcultured onto other media for identification (Windels 1992). Komada (1975) established a medium that is useful for isolating *F. oxysporum* from soil and plant tissue.

1.3.7 Control of *F. oxysporum* f. sp. *cepae*

1.3.7.1 Chemical control

Fungicidal treatments of seed may be effective in reducing the incidence of Fusarium basal rot of onion. A mixture of benomyl (15%) and mancozeb (60%) or benomyl dust alone on sets, improved stand establishment and reduced incidence of basal rot in harvested bulbs and in storage in Zambia (Naik and Burden 1981). Dipping of sets in benomyl (100 µg/ml) for 15 minutes reduced rots in stored onion bulbs and increased yields by over 50% compared to untreated sets (Komada 1975). Similar results have been obtained in Finland, resulting in good protection of sets, and the crop, from infection by *F. oxysporum* f. sp. *cepae* (Tahvonen 1981).

In Australia, sets are not used as crop is grown from seed. Where soil was inoculated with *F. oxysporum* f. sp. *cepae* and seed treated with benomyl (1g a.i./kg seed), an improvement in onion seedling stands was observed (Smith, 1999). Many growers

planted onion seed pre-coated with Benlate® but this is no longer possible as this product has recently been removed from the market.

The widespread use of fungicides may not be the answer to control of *F. oxysporum* f. sp. *cepae*, as fungicides may have negative effects on mycorrhizal fungi, and the symbiotic relationship with the onion root may be impaired (Sukarno *et al.* 1993), resulting in reduced nutrient transfer between the symbionts and possibly less yield.

The concept of dipping the harvested onion bulbs in fungicides rather dipping than the propagating material is considered ineffective, particularly due to the high humidity in storage that would result from the dipping procedure (Grinstein *et al.* 1992). A Reduced Volume Application (RVA) of fungicides allows deposition of droplets on the surface of the onion; a fine mist spray, at such reduced volumes that the bulbs dry almost immediately, is applied instead of total submersion. Carbendazim + diethofencarb and iprodione were the most effective chemicals of those tested in reducing the incidence of storage rots (Grinstein *et al.* 1992). The use of chemicals on harvested products, however, is a contentious issue, due to consumer preferences for chemical free produce, and application on a commercial basis would need further investigation.

Currently, little is done in the industry to attempt to reduce losses due to *F. oxysporum* f. sp. *cepae.* Few options are available, and research is needed to provide the industry with a viable chemical control to reduce the loss of yield caused by infection by *F. oxysporum* f. sp. *cepae.*

1.3.7.2 Biological control

Biological control is a term used to describe the "reduction of inoculum or disease-producing activity of a pathogen accomplished by or through one or more organisms other than man" (Baker and Cook 1974). Market demands are forcing growers to

reduce the use of pesticides and yet improve the quality of produce. Alternative means of pest and disease control need to be investigated.

In vitro testing showed that the bacterium *Pseudomonas cepacia,* which causes "sour skin" on onion bulbs, protected onion seedlings from damage by *F. oxysporum* f. sp. *cepae* (Kawamoto and Lorbeer 1976). Also, studies in Iran (Tehrani and Ramezani 2003) showed that species of *Bacillus* were antagonistic to *Fusarium* on onion, and that *B. subtilis* was the most antagonistic of all tested (Tehrani and Riseh 2004). Antagonism of *F. oxysporum* f. sp. *cepae* by *Trichoderma* spp. *in vitro* has been investigated. *T. virens* DAR 74290 was more effective in suppressing growth of *F. oxysporum* f. sp. *cepae* on PDA by antibiosis than were wild type isolates of *Trichoderma* obtained from fields in the Mid Murray district of South Australia (Smith 1999). *T. virens* isolate DAR 74290has been effective in controlling *Phytophthora erythroseptica*, the causal agent of pink rot on potato, both *in vitro* and in the glasshouse (Etebarian *et al.* 2000). Further investigations in this area could be beneficial.

F. oxysporum f. sp. *cepae* does not compete well with microbiota found naturally in the soil (Abawi and Lorbeer 1972). Therefore, it should be possible to find an effective biological agent that can be used to control *F. oxysporum* f. sp. *cepae.* However, production of an economical and practical commercial product would require considerable research.

1.3.7.3 Soil solarisation

Solar heating of soil or soil solarisation has been tested for the control of many soil borne pathogens, including nematodes (Katan *et al.* 1980). The process involves covering moist soil with polyethylene sheets for several weeks during the hottest time of the year. Soil temperatures can be as high as 50 - 60ºC (Hartz *et al.* 1989; Porter and

Merriman 1983; Porter *et al.* 1989), which will either kill the pathogen or alter the biological balance in the soil. Populations of *Fusarium* spp. in onion fields were greatly reduced by the technique in Israel (Katan *et al.* 1980). Trials in Victoria, Australia, however, indicated that *F. oxysporum* in soil could withstand temperatures of 50ºC for 6 hours (Porter and Merriman 1983).

The effects of solarisation may last for more than one season, and subsequent crops will benefit from the reduced pathogen population (Katan *et al.* 1980). Natural reinfestation by *Fusarium* spp. was slower in solarised soil than in soils treated with other methods, such as fumigation (Katan *et al.* 1980). This is thought to be due to the alteration in the biological balance of the soil and an increase in microbes antagonistic to the pathogen. However, the cost and feasibility of laying, removing and disposing of large amounts of plastic would limit the use of this method for the control of any pest or disease.

1.3.7.4 Resistance breeding

Breeding for resistance to, or tolerance of, *F. oxysporum* f. sp. *cepae* offers another means to minimise yield loss due to this pathogen or other *Fusarium* spp. Unfortunately, only one cultivar, Macarena (Henderson Seed Group Pty Ltd, Bulleen, Victoria, Australia) is marketed as having tolerance to *Fusarium* in South Australia. This cultivar has a tendency to bolt (produce inflorescence stalks) readily, and for this reason is considered unsuitable for cultivation in the Mid Murray area (Kevin Smith, personal communication, 2001).

Methods for screening potentially resistant or tolerant cultivars involve field trials or greenhouse trials. Researchers in the USA (Guiterrez and Cramer 2005; Guiterrez *et al.* 2006) tested over 20 different local cultivars for resistance to *F. oxysporum* f. sp. *cepae* in field trials over 2 years. Results from field tests have proved to be consistently reliable, but such tests are very slow when compared with the methods that have been

developed for screening in the greenhouse (Retig *et al.* 1970). The ability to infest soil or seedlings with *F. oxysporum* f. sp. *cepae* artificially in the greenhouse, and then control the environment, may allow faster and more reliable comparison of resistance than would sowing of seed in naturally infested soil and relying on disease development in an uncontrolled environment. Retig *et al.* (1970) found that results for screening of onion for resistance to *F. oxysporum* f. sp. *cepae* in the greenhouse correlated well with results from field trials. Greenhouse screening also proved useful when only limited amounts of seed were available, as there was more likelihood that each seedling would be infected by *F. oxysporum* f. sp. *cepae* (Fantino and Schiavi 1987). However, other studies have indicated that whilst an association exists between field and greenhouse results, the correlation is low (Holz and Knox-Davies 1974).

Krueger *et al.* (1989) used a greenhouse screening method, modified from that of Retig *et al.* (1970), to evaluate inbred onion cultivars for resistance to *F. oxysporum.* An interaction between nuclear genes and cytoplasm appeared to be involved in the genetic control of *Fusarium* resistance in onions (Krueger *et al.* 1989). Cramer (2000) reported, in a review of resistance to *F. oxysporum* f. sp. *cepae* in onion, that two partially dominant genes control resistance. Özer *et al.* (2003) found that the degree of infection of onion by *F. oxysporum* f. sp. *cepae* was dependent on the production of different amounts, and distinctive patterns of, pectolytic enzymes by the pathogen, as well as the expression of distinct antifungal compounds. Further research is required into the mechanism and inheritance of genes for resistance to *Fusarium* in onion cultivars and to develop genotypes that are resistant to *F. oxysporum* f. sp. *cepae* and suitable for growth in differing climatic and agronomic conditions.

1.3.7.5 Other management practices

The addition of certain crop residues to soil can improve the yield of onion (Latham and Watson 1966). Residues of oat, alfalfa and wheat incorporated into sterilised soil in pots increased the growth of onion compared with unamended soil. Onions grown in soil amended with alfalfa residues developed less bulb rot (caused by *F. oxysporum* f. sp. *cepae*) in storage than onion grown in soils amended with wheat or oat, or unamended soils. Increase in the microbial population in the soil may be responsible for the reduction in storage rots, due to the poor competitive ability of *F. oxysporum* f. sp. *cepae* in soil, or fungi toxic by-products from residue breakdown may have reduced the *Fusarium* population (Latham and Watson 1967).

The use of cruciferous plant residues that release toxic isothiocyanates (ITCs) into the soil that suppress the activity of many pathogens has been termed biofumigation (Matthiessen *et al.* 2000). Glucosinolates (GSLs) that exist in many brassica plants will produce ITCs when the plant is disrupted, particularly when plant residues are incorporated into soil. The degradation of GSLs to ITCs is believed to be partly responsible for reduced pathogen activity. The increase in populations of other soil microbes also plays a role in controlling plant pathogens, possible due to increased competition (Smolinska 2000). *F. graminearum* was sensitive to some ITCs that were released from Brassica crops *in vitro,* and reduced growth in agar was observed (Sarwar *et al.* 1998). The incorporation of air-dried and crushed plants of *Brassica juncea* into soil reduced the chlamydospore population of *F. oxysporum* f. sp. *lycopersici* in the soil, and subsequently reduced disease symptoms in tomato (Smolinska 2000). Whilst research will continue in the area of biofumigation (Matthiessen *et al.* 2000), no work has been done to determine which *Brassica* spp., if any, will produce the toxic compounds necessary to suppress growth of *F. oxysporum* f. sp. *cepae*, and what other

plant species may induce benefits such as pathogen suppression when incorporated into the soil.

1.4 Pathogens of onion: *Phoma*

1.4.1 Taxonomy

The genus *Phoma* is of the form-class Coelomycetes in the Mitosporic Fungi (Deuteromycotina) (Leath 1992). The distinguishing characteristic of *Phoma* spp. is the smooth, dark pycnidia produced on diseased tissue (Barnett and Hunter 1998; Gorenz *et al.* 1948), although these structures may be difficult to generate in culture (Kulik and Tims 1960a). For most species of *Phoma,* pycnidia and conidia are produced on potato dextrose agar (PDA) at 20-22ºC and often in darkness (Leath 1992). Macroscopic features in culture often include dark red/purple, velvety, small colonies, which grow very slowly (Hansen 1929). Microsclerotia are also produced by some *Phoma* spp. and may be a major factor in the overwintering of the species (Biles *et al.* 1992). Condia range in size from 4 – 7 x 1.5 – 2.5 µm, are oblong to ellipsoid, aseptate and hyaline, although appear whitish in mass exudation (Kinsey 2002).

Originally, the causal organism of pink root disease of onion was thought to be a species of *Fusarium,* referred to as *F. mali* (Taubenhaus and Mally 1921). Subsequent studies by Hansen (1929) showed that the organism responsible for causing pink root was not a *Fusarium* but rather a fungus he referred to as *Phoma terrestris.* The earlier confusion could easily have stemmed from the abundance of *Fusarium* spp. found in association with pink pigmented, necrotic root systems. Isolations from such roots often yield *Fusarium* spp. and, due to its slow growth in comparison, *P. terrestris* is difficult to isolate (Awuah and Lorbeer 1989).

Hansen did not describe the pycnidia produced by *P. terrestris* in detail and, when the causal organism of pink root was investigated subsequently (Gorenz *et al.* 1948), it was

found that the fungus had been incorrectly classified. Due to the setose nature of the pycnidia when mature, the pathogen was reclassified as *Pyrenochaeta terrestris* and described by Punithalingam and Holliday (1973) as the causal organism of pink root of onion.

More recent literature refers to *Phoma terrestris* as a synonym for *Pyrenochaeta terrestris* (McGrath and Campbell 1983; Sumner *et al.* 1997; Thornton and Mohan 1996). Also, more recently, Sumner (2008b) stated *Phoma terrestris* as the causal organism of pink root of onion, but noted *Pyrenochaeta terrestris* is a synonym. Both *Phoma* and *Pyrenochaeta* are still referred to in the literature as the causal organism of pink root, however, Shishkoff (1992) clearly stated that *Phoma terrestris* is now excluded from the genus *Pyrenochaeta.* Likewise, Kinsey (2002) described the setae on the pycnidia and classified the fungus as *Phoma terrestris*. In this thesis the pink root pathogen will be referred to as *Phoma terrestris.*

1.4.2 Disease on onion

Pink root of onion is a disease caused by *P. terrestris* that results in pink discoloration of roots, which eventually die. This symptom is generally diagnostic of the disease. The pink root fungus infects the onion roots early in the growing season, and is present in the roots before any pigmentation is evident (Coleman *et al.* 1997). Therefore, if the pink pigment is absent from the roots of diseased onion plants, *P. terrestris* should not necessarily be disregarded as the pathogen (Kreutzer 1941). Generally, root discolouration is most obvious at a later stage of crop development, when roots are already severely affected. Therefore, alternative means of disease detection are required rather than simply screening for pink roots (Rabinowitch *et al.* 1981).

P. terrestris initially colonises the surface of the root. Upon entering the root, the hyphae grow inter and intracellulary through the cortex. Pink pigmentation is generally

confined to the hyphae, although some diffusion into invaded cells will occur. Roots will eventually shrivel and die, denying the bulb access to water and nutrients. *P. terrestris* does not invade the living tissues of onion bulbs (Kreutzer 1941), however, can cause reddening of dry outer scales in white onions (Shock *et al.* 2000; Sumner 2008b). Reddening of the scales is common in white onions in South Australia (Kevin Smith, personal communication, 2001) and this pigmentation is a problem particularly with onions grown for export, where "snow-white" onions are required. In addition, it has been observed that areas of reddening on the outer scale may progress to rotting of adjacent bulb tissue over time. However, this rotting may be due to a *Fusarium* sp., which has been found to cause pink discolouration of white onion bulbs (Trevor Wicks, personal communication, 2002).

The optimum temperature for infection by *P. terrestris* on onion is between 24 and 28ºC (Gabor 1997; Sumner 2008b). There are reports that commercial cultivars tolerant to pink root will be more susceptible to infection by *P. terrestris* at temperatures of 28ºC or above (Sumner 2008b).

It is believed that onion plants subject to certain stresses, particularly moisture deficiency, are most susceptible to pink root (Kevin Smith, personal communication, 2001). If this is the case, then severe damage to the bulb could be avoided by supplying onion plants with more than the usual amount of water to compensate for the poor root system. Coleman *et al.* (1997) found, however, that whilst all onions benefit from extra water, diseased onions do not reach their full potential.

The ability of isolates of *P. terrestris* to produce pycnidia is variable (Kulik and Tims 1960a). The virulence of *P. terrestris* isolates is also variable (Gorenz *et al.* 1949). Virulence, according to Gorenz *et al.* (1949) was measured by the amount of pink pigmentation and pink root symptoms, where each set of onion roots was classified

according to a scale (free of pink root, 0; slightly affected, 25; moderately affected, 50; severely affected, 75; dead, 100). A correlation has been found between the production of pycnidial structures *in vitro* and the ability of that isolate to produce pink root symptoms on onion seedlings (Kulik and Tims 1960a). Therefore, the presence of pycnidia may be a useful determinant of pathogenicity.

1.4.3 Isolation of *P. terrestris*

Watson (1961) developed a method for rapid identification of the pink root fungus. The procedure involves incorporation of sterile wheat straw into a low nutrient agar. When *P. terrestris* is grown on this medium, the wheat straw turns pink after several days. Another technique for identification of *P. terrestris* is the use of sterile cheesecloth in a mineral medium supplemented with chloramphenicol. The growth of the fungus results in a pink pigment on the cheesecloth (Sumner *et al.* 1997). Both of these techniques indicate the presence of *P. terrestris*.

Sneh *et al.* (1974) developed a method for the isolation and identification of the pink root fungus from soil using soil dilution plates. This method enabled quantification of the inoculum in the soil. The medium, Watson's medium (Watson 1961) without the wheat straw, initially permits unrestricted and unpigmented growth of *P. terrestris* on the agar plate. After 4 days, 2-(4-thiazolyl) benzamidazole (TBZ) is added to the agar plate, and after a further 48 hours of incubation, the presence of restricted, pink colonies of *P. terrestris* is visible on the plate.

Another technique, developed by Awuah and Lorbeer (1989), involves isolating *P. terrestris* from onion roots, by plating surface sterilised stelar root tissue on water agar. This procedure was developed to prevent *P. terrestris* being overrun on agar plates by faster growing fungi such as *Fusarium* spp.

Ferriera (1990) describes a procedure that was developed to isolate *P. terrestris* from onion roots. Pink pigmented roots are placed on moist filter paper, incubated and pycnidia develop on the paper, from which the fungus can be isolated.

The techniques described here are quite varied and do not seem to be extensively used. There has been limited research conducted in this area and Sumner (2008b) does not recommend a standard method.

1.4.4 Distribution

Lindsey and Corgan (1976) found the pink root disease of onions to be prevalent in New Mexico. In their survey, it was found in 90% of onion fields, affecting 40% of plants. In Georgia, Sumner (1997) found the pathogen to be present in 74% of the fields assessed, correlating significantly (f = 0.05) with loss of plant stand. In the well-known onion growing region of Treasure Valley in Eastern Oregon and South West Idaho, USA, pink root is a serious disease affecting onion size and yield (Vaughan *et al.* 1971). The first report of pink root in Sudan was in the late 1970s (Yassin *et al.* 1982) after an outbreak that was distributed widely and uniformly, devastating many crops. *P. terrestris* has also been reported in Argentina, Australia, Brazil, Canada, Egypt, Germany, Hong Kong, Mauritius, New Zealand, Pakistan, Sierra Leone, South Africa, Uganda, UK and Venezuela (Punithalingam and Holliday 1973).

Pink root has been recorded on onion in South Australia (Cook and Dubé 1989; Lomman 1991). Growers are very familiar with the symptoms and effects of pink root in the Mid Murray region of South Australia (Kevin Smith, personal communication, 2001).

1.4.5 Alternative hosts

Ground never sown to onion is not necessarily free of *P. terrestris.* It appears that the fungus survives on a wide variety of plants (Coleman *et al.* 1992). *P. terrestris* affects some varieties of *Sorghum* spp. (millet), *Cucumis sativus* (cucumber), *Daucus carota* subsp. *sativus* (carrot) and *Spinacia oleracea* (spinach), severely or moderately (Kreutzer 1941). Furthermore, *P. terrestris* has been observed to damage roots of some varieties of *Pisum sativa* (pea), *Avena sativa* (oat), *Hordeum vulgare* (barley), *Triticum* spp. (wheat), *Zea mays* (corn), *Cucurbita* spp. (squash), *Cucumis melo* (cantaloupe/muskmelon), *Solanum lycopersicum* (tomato), *Solanum melongena* (eggplant) and *Brassica oleracea* (cauliflower) (Kreutzer 1941). There is a report of poor crops of onion when planted immediately after sweet corn, where it was believed that the population of *P. terrestris* grew in the presence of the sweet corn, and increased the incidence of pink root on the subsequent onion crop (Latham and Watson 1967).

Other species of *Allium,* such as *A. sativum* (garlic) and *A. oschaninii* (shallot), are reported as hosts of *P. terrestris*. The fungus had no effect on, but has been isolated from, varieties of *Piper nigrum* (pepper) and *Glycine max* (soybean). Whilst no symptoms of pink root existed on the roots of the above plants when grown in *P. terrestris*-infested fields, the *P. terrestris* isolates obtained from the roots were found to be pathogenic on onion (Tims 1955).

P. terrestris has been isolated from over 50 Gramineous hosts, including *Triticum* spp. (wheat), *Hordeum vulgare* (barley) and *Avena sativa* (oats), but shown to cause little damage to any of these crops. It is a weak parasite on grass species in conditions that favour its development. *P. terrestris* is commonly found in roots of wheat in dry sandy soil in mid-late season on the northern plains of the USA (Sprague 1944). Grasses may

be an important alternative host of *P. terrestris*, and an important factor in maintaining populations between onion crops.

Siemer and Vaughan (1971) believed there was a need to determine if there are crops that will increase or decrease the inoculum of *P. terrestris* in the soil. Many crops in other countries are carriers of *P. terrestris,* as mentioned above. An investigation of potential alternative hosts of *P. terrestris* in South Australian conditions is required. This would allow modification of crop rotations, by planting of non-hosts, which could minimise inoculum in the soil and, perhaps, provide a useful tool in an integrated pest management strategy.

1.4.6 Inoculum source

P. terrestris can survive for many years in the soil, on alternative hosts or on crop debris (Gabor 1997). Therefore, soil is considered a very important source of inoculum of *P. terrestris*. The pathogen is not known to be seed-borne (Kinsey 2002; Sumner *et al.* 1997).

Onion bulbs are reported to carry *P. terrestris* and reinfest soil when used as sets (Katan *et al.* 1980). However, as noted above, *P. terrestris* will not invade the living tissue of the onion bulb, only the dead outer scale (Kreutzer 1941), and bulbs are not recorded in the literature to be an important source of inoculum of *P. terrestris* (Kinsey 2002).

1.4.7 Control of *P. terrestris*

1.4.7.1 Chemical control

Laboratory screening has indicated that some chemicals are inhibitory to *P. terrestris* and have the potential to reduce effects of *P. terrestris* on onion (Kulik and Tims 1960b). However, there is no evidence to demonstrate that chemicals other than fumigants control pink root in the field (Miller 1987).

Fumigation of soil with metham sodium (356 kg a.i./ha) effectively controlled pink root disease of onion in Texas, USA (Hartz *et al.* 1989). Similarly, Sumner (1997) found fumigation with metham sodium a useful alternative to fumigation with methyl bromide, and effective in reducing populations of the pink root pathogen in onion fields in Georgia, USA. Fumigation of soil with chloropicrin (330 kg/ha) for cultivation of onion for seed production has maintained yields in soil infested with *P. terrestris* in California, USA (Ahmed and Harrington 1974). Furthermore, in Senegal, dazomet (40 g/m^2) injected into the soil increased production of onion by as much as 90% (Pages and Notteghem 1996). Dazomet decomposes quickly in the soil to methyl isothiocyanate, which is toxic to most microorganisms. The use of dazomet, or similar chemicals, in an integrated pest management regime with other management practices may improve yield and bulb size in onion.

Fumigation reduces the population density of *P. terrestris* in the soil but does not eradicate the pathogen. However, fumigation provides the grower with the ability to grow onions in a previously infested field, and to be confident that *P. terrestris* will not devastate the crop. *P. terrestris* will reinfest a field sown to onion before the end of the growing season, therefore, onion cultivars susceptible to pink root may be affected late in the growing season (Vaughan *et al.* 1971).

Fumigation is a costly method of control, and the potential benefits need to be considered in terms of the cost of the possible loss of yield.

1.4.7.2 Biological control

A biological control product exists that vendors claim has “virtually eliminated the incidence of pink root disease” in trial areas. The product, Nutri-life 4/20 (Lawrie and Co., Wingfield, South Australia) contains four fungal species, including *Trichoderma* spp., together with 20 bacterial species which, in “optimum” conditions, is claimed to

reduce the effectiveness of the pathogens present in the soil (Lawrie 2001). The product has been trialled in onion crops in the Mid Murray region, and there are claims from the supplier that improved yields have been observed, along with reduction in disease caused by pathogens such as *P. terrestris*, *Pythium, Rhizoctonia, Aspergillus* and nematodes. However, no data were presented in the paper to support this claim (Lawrie 2001). Other researchers that have conducted studies in this area., found that the measurement of disease severity was lower where selected *Trichoderma* spp. were added to soil naturally infested with *P. terrestris*, but there was no yield benefit recorded for the onion crop (Donayre *et al.* 2004).

The use of certain crop residues as a soil amendment has been shown to be a possible tool in the control of pink root of onion. However, wheat and oat residues, if not properly decomposed, can restrict the nitrogen available to the onion crop, whereas lucerne residue caused less interference (Latham and Watson 1966). The growth of the pathogen was either inhibited by antibiosis by microbiota in the soil, or by competition for nutrients (Latham and Watson 1967). Further studies of biological control agents and their mode of action are required. There is a need to develop commercially viable biological control products for use on a broad scale.

1.4.7.3 Soil solarisation

Solarisation has proved to be effective in reducing the population of *P. terrestris* in soil (Hartz *et al.* 1989; Porter *et al.* 1989). However, as mentioned in section 1.3.7.3, the practicalities of handling large amounts of plastic limit the use of this method.

1.4.7.4 Resistance breeding

As mentioned, the visual rating of pink pigmentation of the roots is an important technique for screening for the presence of *P. terrestris.* This method is often used in screening varieties for resistance to *P. terrestris.* Whilst this method has limitations,

timing of the inspection can be important. Coleman *et al.* (1997) found that by inspecting onion roots for pink pigment just prior to maturity, before leaf fall, when the root system is normal and functioning well, enables differentiation between onion lines in their ability to resist infection by *P. terrestris* (Coleman *et al.* 1997). A rating scale of 1-10 (1 = 2.5% pink roots; 2 = 10%; 3 = 21%; 4 = 35%; 5 = 50%; 6 = 65%; 7 = 79%; 8 = 90%; 9 = 97.5%; 10 = 100%) was used to determine the severity of pink root in onion.

P. terrestris causes problems in onion seed production as well as in bulb production. The use of resistant cultivars (instead of susceptible cultivars) as mother bulbs increased yield of onion seed when sown in fields in California naturally infested with *P. terrestris* (Ahmed and Harrington 1974).

Thornton (1996) suspected that extensive root replacement is a main characteristic of onion cultivars that are pink root resistant. Some cultivars have been observed to grow well compared to other cultivars in the Mid Murray area, despite disease pressure due to *P. terrestris* (Kevin Smith, personal communication, March 2002), and anecdotal evidence suggests that these cultivars replace damaged roots very quickly.

One factor that may prevent infection of onion by *P. terrestris* is early maturation or early harvest to avoid weather favourable to the pathogen. Late-maturing cultivars, which grow through the summer, are more exposed to the activity of the pathogen, which is favoured in the hotter months of summer and, thus, disease is likely to occur more readily (Levy and Gornik 1981).

The existence of a single recessive gene controlling pink root resistance has been established, although another gene may have a role (Nicols *et al.* 1965). *Allium fistulom* is genetically resistant to pink root disease, and attempts have been made to cross this

species with *A. cepa*, to produce a pink root resistant onion line, however, the hybrids are usually sterile (Brewster 1994).

Various studies have been conducted to investigate the role a range of chemicals in the onion bulb that may be indicators of susceptibility to infection. Secalonic acid A has been isolated from liquid cultures of *P. terrestris* and found to accumulate in infected onion tissue (Steffens and Robeson 1987). Hess and Weber (1988) attempted to isolate the specific toxins responsible for disease caused by *P. terrestris* in order to evaluate onion seedlings for resistance *in vitro* and, whilst they did not identify specific toxins, found that a significant variation in toxic compounds could be obtained from different isolates. Gourd *et al.* (1988) used tissue cultures to examine the role of various toxins from filtrates of *P. terrestris* and effects on different cultivars of onion and found that damage observed in the callus tissue was probably due to the unidentified toxin in the filtrate, and would be a useful screening method for susceptibility of onion lines to infection from *P. terrestris*. Zappacosta *et al.* (2003) further developed the methods of Gourd *et al*. (1988) and examined the role of peroxidases, chitinases and glucanases in resistance to infection by *P. terrestris*; found that a high activity of glucanases and possibly chitanses may be involved in resistance to *P. terrestris* in *Allium* species. Extension of this work may allow development of *in vitro* screening methods for resistance to *P. terrestris.*

Currently, very few onion cultivars are marketed as either tolerant of, or resistant to, *P. terrestris* and some of these cultivars have undesirable qualities, such as a tendency to bolt. Further research is required to produce resistant or tolerant cultivars with desirable agronomic qualities.

1.5 Interaction of *F. oxysporum* f. sp. *cepae* and *P. terrestris*

Resistance to either *F. oxysporum* f. sp. *cepae* or *P. terrestris* provides no protection from the other and, in combination, both pathogens can cause more injury than either organism alone. Kehr *et al.* (1962) believed there was a need to determine the prevalence of *P. terrestris* and *F. oxysporum* f. sp. *cepae* individually and together in major onion growing districts in order for effective management practices to be implemented.

Lacy and Roberts (1982) found that *Fusarium* appeared to be the more important pathogen in fields infested with both *Fusarium* spp. and *P. terrestris.* For the 32 onion cultivars tested, yield was most significantly reduced in soil either naturally or artificially infested with *F. oxysporum* f. sp. *cepae*, whereas *P. terrestris* reduced yield to a lesser extent. When both pathogens were present in the same soil, there was no significant difference in yield of onion compared with those grown in soil with F. *oxysporum* f. sp. *cepae* alone (Lacy and Roberts 1982). Likewise, Kruetzer (1941), who conducted pot trials, found that invasion of the root by *P. terrestris* did not enhance entry of *F. oxysporum* f. sp. *cepae* into the onion bulb. However, Hartz *et al.* (1989) considered that infection by *P. terrestris* may facilitate the entry of *Fusarium* spp., resulting in bulb rots. This view is supported by earlier work of Davis and Henderson (1937), who found that when both pathogens were present in the soil, *P. terrestris* was the primary invader, followed by *Fusarium* spp.

F. oxysporum f. sp. *cepae* and *P. terrestris* are two of the most important pathogens on onion in the Idaho-Oregon region of northwest USA. Thornton and Mohan (1996) found that the incidence of basal rot in this region was not closely correlated with pink root severity. Some cultivars tested had very low incidence of basal rot, good yield, but severe pink root.

It appears that whilst the incidence of disease caused by *F. oxysporum* f. sp. *cepae* and *P. terrestris* may not be correlated, the two pathogens are often found together. Evidence in the literature indicates the possibility of interaction between *F. oxysporum* f. sp. *cepae* and *P. terrestris* on onion, but further research is required in this area, particularly in Australian conditions.

1.5.1 Management

In light sandy soils, like those in the Mid Murray region of South Australia, methods have been adopted to prevent wind erosion in the last two decades. For example, planting a cereal crop prior to sowing onions, to reduce soil erosion during the seedling development phase, is common practice. Studies have been conducted in order to determine the optimum height of barley plants to provide maximum yield of onion, before the barley is sprayed with a selective herbicide (Greenland 2000). Yield of onions can be affected by the use of barley as a "nurse crop" because of competition for nutrients if the barley is allowed to grow for too long. However, an examination of the potential of barley to increase inoculum of pathogens such as *F. oxysporum* f. sp. *cepae* and *P. terrestris* has not been undertaken.

1.6 Summary

Rot and death of onion seedlings in the field, and rots of bulbs in storage, occur in most seasons in South Australia. *F. oxysporum* f. sp. *cepae* is a possible cause of these diseases, and research is required to understand and develop strategies to control this pathogen in Australian conditions.

Very little is known of the epidemiology of *P. terrestris* in Australia. However, the pathogen has the potential to damage onion crops, particularly in the hot regions of the Mid Murray of South Australia, where the disease is common.

Aspects such as cultivar resistance and host specificity of *F. oxysporum* f. sp. *cepae* and *P. terrestris* require further research. Information on the biology of, and damage caused by, *F. oxysporum* f. sp. *cepae* and *P. terrestris*, will provide a basis for a sound integrated pest management system, and help sustain the onion industry in Australia.

1.7 Aims of the research

This study was undertaken to

- Isolate and identify the pathogens *F. oxysporum* f. sp. *cepae* and *P. terrestris* and the diseases caused on onion in South Australia
- Determine the susceptibility of local commercial onion cultivars to *P. terrestris*
- Evaluate the role of management of "nurse crops" in maintaining inoculum of the pathogens in the soil
- Identify crops and weeds that might act as alternative hosts of *F. oxysporum* f. sp. *cepae* and *P. terrestris* in South Australia.

2 General Materials and Methods

2.1 Trial site

A property at Bowhill (S 34º 53', E 139º 38', WGS 84) in the Mid Murray region of South Australia was used as a trial site for this study (see fig 2.1).

NOTE:
This figure is included on page 35 of the print copy of the thesis held in the University of Adelaide Library.

Figure 2.1 Map of South Australia showing location of trial site. The blue line is the River Murray and the Bowhill area is circled.

(http://www. southaustralia.co.nz/SiteResources/Data/Templates/satc_normal.asp?docid=275).

Onions have been grown on the property for over 20 years, under centre pivot irrigation (fig 2.2) with water from the River Murray. Soil on the property is generally sandy

loam to a depth of 20-40 cm, over sandy clay loam, often to a depth of 80 mm or more, with a pH between 7.5 and 8.5. Average annual rainfall is approximately 350 mm, most falling between April and October. Average maximum temperatures in the major onion-growing season, between August and February, range from 17ºC to 30ºC, with extended periods of days with maximum over 40ºC common between November and February.

Figure 2.2 A centre pivot in a field on the property at Bowhill in the Mid Murray region of South Australia.

2.2 Media for culturing

Media commonly used throughout this project are described below.

2.2.1 Acidified Potato Dextrose Agar (APDA)

Potato Dextrose Agar (Difco®), 39 g, was added to 1 L of reverse osmosis (RO) water, boiled until dissolved, poured into Schott® bottles and autoclaved at 121ºC for 15

minutes. The medium was cooled to approximately 50ºC then 1 ml/L of lactic acid was added and the solution mixed by inverting the bottle. The medium was poured immediately into 90 mm Petri dishes, approximately 20 ml per plate.

2.2.2 Wheat Straw Agar (WSA)

WSA (Watson 1961) was prepared by gathering wheat straw from fields of wheat stubble at the trial site (section 2.1). The straw was cut into 5 mm-long segments, placed in polycarbonate tubs (65 mm diameter x 80 mm high), closed and autoclaved for 20 minutes at 121ºC. The medium was prepared by dissolving 1 g $MgSO_4$, 3 g $NaNO_3$ and 20 g agar (Difco®) in 1 L RO water and poured into Schott® bottles. The mixture was then autoclaved for 20 mins at 121ºC. The medium was cooled slightly and poured into 90 mm plates as above. Segments of autoclaved wheat straw, approximately 15-20 pieces per plate, were sprinkled on the medium before setting.

2.2.3 Water Agar (WA)

To 1 L RO water, 20 g of agar (Difco®) was added, dissolved, autoclaved and dispensed into Petri dishes as described above.

2.2.4 Carnation Leaf Agar (CLA)

Fresh carnation leaves were sourced from a local florist (CJ's Flower Basket, Murray Bridge, South Australia). Leaves were cut into approximately 5 mm-long segments and dried in an fan-forced oven at approximately 70ºC for 3 hours. The carnation leaf segments were wrapped in aluminium foil and autoclaved twice at 121ºC for 20 minutes. The carnation leaf pieces were scattered in Petri dishes, approximately 20 pieces per plate, and 20 ml autoclaved, cooled WA (section 2.2.3) was added to each dish (Fisher *et al.* 1982).

2.3 Isolation of fungi from onion roots

Onion roots collected from the field were washed in tap water to remove soil, and soaked in surface sterilising solution (250 ml 5% sodium hypochlorite, 9 ml ethanol and sterile RO water to 1 L) for 3 minutes (Sukarno *et al.* 1993). The roots were removed and rinsed three times in sterile RO water, and then dried between sterile paper towels. Roots were cut with a sterilised scalpel into approximately 5 mm lengths, before placing on APDA or WSA. Plates were incubated at room temperature (approx. 24ºC) in natural daylight for 7 to 10 days.

2.4 Preparation of onion seedlings *in vitro*

Onion seed for experiments was sourced from commercial stocks from the owner of the trial site (Kevin Smith, Bowhill Produce Pty Ltd) or directly from seed companies, in trial size packs (500-1000g).

Onion seed was surface sterilised for 3 minutes in surface sterilising solution (section 2.3), rinsed three times in sterile RO water and dried between sterile paper towels. Seeds were then placed aseptically on sterile moist filter paper in 90 mm Petri plates, 25 seeds per plate, then incubated at room temperature (approx. 24ºC) in natural light for 7 to 10 days.

2.5 Cultures of *Phoma terrestris*

Cultures of *P. terrestris* were maintained on APDA and stored at 4ºC until use.

When fresh isolates were required for experiments, 5 x 5 mm discs, from the edge of *P. terrestris* isolates stored on APDA, were placed in the centre of fresh plates of WA and incubated in natural light at room temperature (approx. 24ºC) for 7 to 10 days.

2.6 Collection of soil

When required for experiments, soil was collected from several random locations within onion fields at the trial site (section 2.1) up to a depth up to 15 cm, and mixed to provide a representative sample of the soil in the field.

2.7 Sterilisation of soil

Soon after the project began, the trial site was proclaimed a quarantine area for the control of *Orabanche ramosa* (branched broomrape) (Kerin 2001). Subsequently, it was not possible to move soil from the trial site to facilities at Waite Campus to be steam-sterilised for use in experiments. Therefore, alternative methods of soil sterilisation were attempted on site.

2.7.1 Microwave

Soil was spread in plastic trays to a depth of 30 mm, then microwaved in a 700 w oven on high power for 150 seconds per 1 kg (Ferriss 1984). Microwaved soil was placed in 12 plastic pots, 0.55 L (100 x 85 x 95 mm deep) each and another 12 pots were filled with untreated soil for comparison. Onion seed, cv. Patrick, was surface sterilised (section 2.4) and approximately 15 – 20 seeds were sown at a depth of approximately 15 mm in each pot. The seed was surface sterilised to avoid introducing seed-borne micro-organisms or pathogens. Pots were placed in a greenhouse in natural daylight in August 2002 at approximately 26ºC and watered daily. After 8 weeks the seedlings were removed, and the roots rinsed, surface sterilised and placed on APDA (section 2.3).

Micro-organisms that grew from the seedlings in the microwave-treated soil, on APDA, were similar to those that grew on APDA from the seedlings grown in the untreated

soil. It was concluded that the microwave treatment had been ineffective in reducing the microbial population and that this method was not used further.

2.7.2 Autoclave

Soil was autoclaved (Bioclean, Falcon SA-250A) at the trial site. Soil (250g) was placed into a double layer of autoclave bags and autoclaved twice, on consecutive days, at 121ºC for 20 minutes at 1.2kg/cm^2. Soil was kept in the sealed bags at ambient temperature until use. Autoclaved soil was used in certain experiments as a control.

2.8 Statistical analysis

Data were analysed using analysis of variance (ANOVA) utilising the GENSTAT® (6th edition, Lowes Agricultural Trust, Rothamsted, UK) statistical package unless otherwise stated. Means were separated by the least significant difference test (LSD) $P < 0.05$ (Zar 1999).

3 *Fusarium*

3.1 Introduction

Fusarium belongs to a large and complex taxonomic group of fungi and many species are soil borne pathogens (section 1.3.1). Several species may infect onion as secondary pathogens after wounding or damage (Burgess *et al.* 1994). However, *Fusarium oxysporum* f. sp. *cepae* is a primary pathogen of onion that causes damping off and rots in the field, and rot of bulbs in storage (fig 3.1) (Snyder and Hansen 1940).

Figure 3.1 Basal rot caused by *Fusarium oxysporum* f. sp. c*epae* (arrows) on brown onion from a field in the Mid Murray region of South Australia.

Very little is known about the incidence and effects of *F. oxysporum* f. sp. *cepae* on onion in South Australia (see section 1.3.4) but evidence suggests that the pathogen has been present in onion fields in the Mid Murray region for many years (Kevin Smith, personal communication, 2000), and an understanding of the pathogen and the disease is required.

Stunted onions collected from the Mid Murray region had been taken to the South Australian Research and Development Institute (SARDI), Adelaide, in 1995 and *Fusarium* was diagnosed as the probable causal organism (Barbara Hall, personal communication, 1995). *F. oxysporum* f. sp. *cepae* was later isolated from onion fields in South Australia and established as a primary pathogen of onion (Smith 1999). This study in 1999 was the only documented report of *Fusarium* as a pathogen of onion in South Australia prior to this project.

As noted in section 1.3.6, *F. oxysporum* f. sp. *cepae* can be transmitted in soil, seed and planting material (Brayford 1996; Windels 1992) but can be difficult to isolate. The lack of a selective medium for *F. oxysporum* f. sp. *cepae* is an impediment to research, and reliable protocols are required to isolate the pathogen. Methods to isolate and identify the *Fusarium* species pathogenic to onion are necessary before the importance of the pathogens and disease can be determined and, if warranted, control methods developed.

The aim of the experiments reported here was to isolate *F. oxysporum* f. sp. *cepae* from soil, seed and plant material, and confirm the fungus as a primary pathogen of onion.

3.2 Isolation of *Fusarium* species

3.2.1 Materials and Methods

3.2.1.1 Isolation from onion roots and bulbs in the field

A crop of onion, cv. Patrick, near maturity at the trial site in the Mid Murray region (section 2.1) was selected in March 2001, and small patches within the field were identified where the plants were immature, possibly due to infection by *Fusarium* spp. (Smith 1999). Five immature plants were collected from each of four areas within the

crop, ensuring that each plant was removed intact. All samples were stored in clean paper bags at 4ºC until used.

Roots were prepared as described in section 2.3 and placed on APDA in 90 mm Petri dishes. Sections of the bulb from the stem plate of the onion were surface sterilised in a similar manner to the roots. Stem plates of the bulb were then sliced into quarters and placed on 90 mm plates of APDA. The plates were incubated at room temperature (approx. 24ºC) in natural light for 4 days. White, fluffy mycelium was subcultured onto ¼ strength APDA to obtain pure cultures for identification.

3.2.1.2 Isolation from bulbs in storage

Onion bulbs cv. Patrick that had been in storage at ambient temperature in wooden crates for 10 weeks were inspected for basal rot (fig 3.1). Ten bulbs with basal rot and 10 bulbs with no symptoms of basal rot were taken from the crates.

The basal plate of each onion was cut from the bulb and surface sterilised as described in section 2.3. Each basal plate was cut into four sections and each section placed on APDA in 90 mm Petri plates and incubated at room temperature (approx. 24ºC) in natural light for 4 days.

3.2.1.3 Isolation from soil by baiting

A sample of approximately 1000 seeds of onion cv. Patrick was collected from commercial stocks supplied by the owner of the trial site (section 2.1). The seed was surface sterilised as above (section 2.4) for 5 minutes, dried in sterile paper towels and then stored in an autoclaved McCartney bottle.

A range of sites were identified for collection of soil (section 2.6) as described below. All sites had been sown to onion at the time of collection of soil, and represented a range of rotation programs as described below:

A: Sown to onion cv. Early creamgold (Yates, Smithfield, NSW); previously sown to onion cv. Patrick 6 years earlier, and various cereal crops grown in rotation since that time. This treatment was designated ECG6.

B: Sown to onion cv. Patrick (Magnus Kahl Seeds, Lancefield, Vic); sown to onion cv. Early creamgold in the previous year. Soil had been treated with metham sodium between onion crops at 400 L/ha. This site was designated PF.

C: Sown to onion cv. Patrick (Magnus Kahl Seeds, Lancefield, Vic); site treated as B, but with no application of metham sodium between onion crops. Designated PNF.

D: Sown to onion cv. Sandridge (Magnus Kahl Seeds, Lancefield, Vic); onions not planted on this site previously. Designated SNG.

Soil was sampled to a depth of approximately 15 cm, and stored in plastic bags at 4ºC for 1 day before use. The soil samples were kept separate and placed in 0.55 L (100 x 85 x 95 mm deep) pots. There were three blocks of 12 pots for each soil sample. The surface sterilised onion seed (cv. Patrick) was sown into the soil at a depth of 10 to 15 mm, 10 seeds per pot, and pots placed in a greenhouse (29/10/2001). As a control treatment, a mixed sample of soil from the four sites above was autoclaved for 20 minutes at 121ºC (1.2 kg/cm^3), placed in 12 pots as described above (section 2.6.2) and sown with onion seeds cv. Patrick. The temperature in the greenhouse was maintained between approximately 27 and 35ºC.

Pots were watered regularly and fertilised weekly with All Purpose Water Soluble Plant Food (Miracle-Gro® Plant Food, N:P:K 15:13:12, Mitre 10, Murray Bridge, South Australia) at the recommended rate.

After 6 weeks, 10 seedlings per soil type were collected from the pots. The seedlings were washed under slow running water to remove excess soil from the roots. The roots were removed from the seedling and surface sterilised as described in section 2.3. Roots were then cut into 5 mm sections and plated on APDA, one whole root per plate. Plates were incubated at room temperature (approx. 24ºC) in natural light.

Plates were inspected 7 days later and colonies with white, fluffy mycelia were selected for subculturing on ¼ strength APDA and on carnation leaf agar (CLA) (Windels 1992).

3.2.1.4 Isolation from roots collected from the field

From the field sites described in section 3.2.1.3, 10 onion plants were collected at three different stages of the crop's growth. The growth stages were; stage 1 – 2nd true leaf, stage 2 – 4th true leaf, stage 3 – bulb formation. Roots were prepared as described in section 2.3 and plated on APDA, one root system per plate. Plates were incubated at room temperature (approx. 24ºC) in natural light.

Plates were inspected 7 days later and fluffy, white colonies were selected for subculturing on ¼ strength APDA and on CLA.

3.2.1.5 Isolation from soil

Soil sampled to a depth of 10 cm (section 2.6) was collected from the four sites described above (section 3.2.1.3). Overhead irrigation, of 10 mm, had been applied to the area the previous evening. The four samples were mixed together in a bucket, placed in a plastic bag and stored in a refrigerator at 4ºC until used.

Soil samples were added to 1/10 strength WA (section 2.2.3) at a dilution of 1 g/100 ml, and shaken to suspend the soil particles evenly through the agar. Aliquots of the suspension, 1 ml, were spread on APDA. Twenty plates were prepared. The plates were

incubated at room temperature (approx. 24ºC) in natural light, and white, fluffy fungal colonies were selected for subculturing as above.

3.2.1.6 Isolation from seed

Bowhill

To determine if *Fusarium* is seed borne, seed cv. Patrick from commercial stocks was surface sterilised as described in section 2.4 and plated on to agar medium.

Seeds were placed on 10 plates of APDA, 25 seeds per plate, and incubated at room temperature (approx. 24°C) in natural light for 4 days. Plates were inspected for colonies of white, fluffy mycelium.

Tasmania

Twenty six cultures of *Fusarium* spp. isolated from seed whilst screening onion seed for infection by *Botrytis allii* were provided by Dr Dean Metcalf (Department of Primary Industries, Tasmania). In September 2001 the cultures were subcultured onto ¼ strength PDA (Difco®) and WA (Difco®) for maintenance, incubated at room temperature (approx. 24ºC) and checked regularly for contamination. Seven subcultures of the original 26 cultures supplied were infested with mites and discarded. The remaining 19 isolates were examined macroscopically for features of *Fusarium,* and microscopically for the distinctive foot-shaped basal cells of macroconidia. Isolates without macroconidia were not considered to be *Fusarium* spp. and were discarded.

3.2.1.7 Isolate maintenance

Pure cultures of *Fusarium* spp. were maintained on silica gel using the following method, modified from Windels *et al.* (1993). Sterile RO water 1-3 ml at room temperature was placed on each culture of *Fusarium*, the plate shaken, and the resulting suspension siphoned off with a syringe into a sterile McCartney bottle. Autoclaved

skim milk (Pura, 1.5% fat), 2 ml, was added to each McCartney bottle and the contents mixed with a vortex mixer. Kimble tubes (100 mm) were half filled with silica gel crystals and autoclaved. Once cooled, tubes were placed on ice. Kimble tubes were removed from the ice, 0.3 ml of the milk suspension added, placed on the vortex mixer, and placed on ice again. Several crystals from each tube were immediately placed on PDA, two plates per tube, sealed with Parafilm™ and incubated at room temperature (approx. 24ºC) in natural light, to check for viability. The remainder were stored at 4ºC until used.

3.2.1.8 Identification

Fusarium isolates collected from soil, seed, roots and bulbs were subcultured onto plates of CLA (section 2.2.4) and incubated for 7 days in natural light at room temperature (approx. 24ºC) to induce sporulation for identification purposes.

3.2.2 Results

3.2.2.1 Isolation from onion roots and bulbs in the field

Pure cultures could not be obtained from the roots and bulbs collected in the field in March 2001. The plates were contaminated with many different fungal species, including *Penicillum*, *Mucor* and *Trichoderma* that overgrew any *Fusarium* spp. that may have been present.

3.2.2.2 Isolation from bulbs in storage

There were up to five fungal colonies on each plate that were white and fluffy like *Fusarium* spp. The fungal colonies on each plate were similar in appearance whether obtained from a symptomless bulb, or a bulb with basal rot. Subculturing for *Fusarium* spp. was not successful in most cases due to contamination on the plates. However, one colony from a bulb with basal rot was successfully subcultured to produce a pure culture. The isolate was designated B1 and confirmed as *Fusarium* due to the abundant

macroconidia with hooked apical and barely notched basal cells, and abundant reniform microconidia.

3.2.2.3 Isolation from soil by baiting

Between 60 and 90% of the seedlings grown in soil from each of the four field sites produced colonies of *Fusarium* when the roots were plated on APDA. *Fusarium* was not isolated from seedling roots grown in the control soil (table 3.1).

Seedlings grown in the soil from the site designated PNF produced most colonies of *Fusarium* spp. on APDA. The two field sites designated ECG6 and PF produced *Fusarium* colonies on APDA from 80% of the seedlings, whereas only 60% of the seedlings grown in soil from the field site labelled SNG produced colonies of *Fusarium* on APDA. Subculture from the *Fusarium* colonies yielded only three pure cultures for further study, designated SNG, ECG6, and PF.

Table 3.1 The percentage of seedlings that yielded colonies of *Fusarium* on APDA after growth for 6 weeks in pots of soil, and the number of pure cultures subsequently obtained. There were 10 replicate seedlings per soil type.

[A]Codes for soil samples are explained in section 3.2.1.3, and briefly below.

Soil history [A]	Number of seedlings with *Fusarium* (%)	Pure cultures
ECG6 cv. Early creamgold, 6 years since previous onion crop	80	1
PF cv. Patrick, soil fumigated	80	1
PNF cv. Patrick, soil not fumigated	90	0
SNG cv. Sandridge, soil never sown to onion previously	60	1
Control	0	0

3.2.2.4 Isolation from roots in the field

Fusarium spp. were isolated from root systems of onion plants collected at three growth stages from four field sites (table 3.2).

Between 40 and 80% of the roots of seedlings obtained from the site designated ECG6 produced *Fusarium* colonies on APDA. Between 30 and 90 % of the seedlings obtained from the site designated PNF produced *Fusarium* colonies on APDA, and in only 10 – 50% of cases from the site designated PF. Between 0 and 80% of the seedlings obtained from the site designated SNG produced *Fusarium* colonies on APDA.

There was no consistent change in the percentage of seedlings that produced *Fusarium* on APDA as the onion plant reached each growth stage. Only for plants grown in the site designated SNG did the incidence of *F. oxysporum* f. sp. *cepae* increase with age.

There were generally too many fungal contaminants to obtain pure cultures, but from the site designated ECG6, three pure isolates were obtained. These isolates were designated E381, E373 and E323.

Table 3.2 The percentage of onion seedlings that yielded *Fusarium* following culture of root systems obtained from different growth stages at four different sites at Bowhill, SA. There were 10 replicate plants per growth stage per soil type.

[A] Code for soil samples are explained in section 3.2.1.3, and briefly below.

Soil history [A]	Percentage of sampled root systems that yielded *Fusarium* spp.		
	2nd true leaf	4th true leaf	bulbing
ECG6 cv. Early creamgold, 6 years since previous onion crop	**50**	40	80
PF cv. Patrick, soil fumigated	50	10	30
PNF cv. Patrick, soil not fumigated	30	90	**80**
SNG cv. Sandridge, soil never sown to onion previously	0	30	80

3.2.2.5 Isolation from soil

Of the 20 agar plates inoculated with soil, eight yielded colonies typical of *Fusarium* spp. However, only one pure culture was obtained by subculturing on APDA due to fungal contamination. The culture was designated isolate S1.

3.2.2.6 Isolation from seed

Bowhill

Colonies typical of *Fusarium* were not obtained from surface sterilised seeds cultured on APDA. Instead a diverse range of fast growing fungal colonies was present. *Fusarium* spp. were not isolated from seed by this method.

Tasmania

Macroscopic examination of the fungi on PDA suggested that seven isolates were not *Fusarium* spp. so these were discarded. The 12 remaining isolates were confirmed as *Fusarium* spp. following microscopic examination.

All 12 produced abundant macro and microconidia, but were difficult to distinguish from each other on the basis of microscopic features. Based on macroscopic morphological features, as shown in table 3.3, the isolates were placed into three distinct groups.

One representative isolate from each of the groups was used for further pathogenicity testing; group 1 - DM2, group 2 - DMG, and group 3 – DMO.

Table 3.3 Macroscopic features of colonies of *Fusarium* isolated from seed on APDA.

	Macroscopic features upper side	Macroscopic features underside
Group 1	white fluffy	cream/orange
Group 2	dark pink, not white fluffy	pink
Group 3	white fluffy	pale purple

3.2.2.7 Summary of isolates

Details of the *Fusarium* cultures isolated during the experiments described in section 3.2, with regard to macroscopic and morphological features, are summarised in table 3.4. The pathogenicity of these cultures to onions was assessed in subsequent experiments.

Table 3.4 The source and macroscopic features of the 11 isolates of *Fusarium* spp. collected in this study and used in pathogenicity tests.

Isolate	Source and section	Macroscopic features upper side	Macroscopic features underside
B1	Bulb, 3.2.2.2	white, fluffy	purple/pink
SNG	Soil, 3.2.2.3	white, fluffy	pale orange
ECG6	Soil, 3.2.2.3	white, slightly fluffy	cream/pale orange
PF	Soil, 3.2.2.3	white fluffy	cream/pale orange
E381	Roots, 3.2.2.4	white, slightly fluffy	cream/orange, dark centre
E373	Roots, 3.2.2.4	white, fluffy	cream/light pink
E323	Roots, 3.2.2.4	white, not fluffy	cream/orange
S1	Soil, 3.2.2.5	very white, fluffy	pale pink/orange
DM2	Seed, 3.2.2.6	white fluffy	cream/orange
DMG	Seed, 3.2.2.6	dark pink, not fluffy	pink
DMO	Seed, 3.2.2.6	white fluffy	pale purple

3.2.2.8 Isolate maintenance

Each of the *Fusarium* isolates (table 3.4) was stored on silica gel as described in section 3.2.1.7. The inoculated silica gel, placed on APDA before storage, from each isolate, produced colonies with morphological features consistent with those recorded for each isolate.

3.2.2.9 Identification

All of the *Fusarium* isolates (table 3.4) were grown on CLA as described in section 2.2.4. None of the isolates sporulated on CLA, and no distinguishing features were detected by microscopic examination. Identification to species based on morphology was not possible.

3.2.3 Discussion

The focus of this study was to recover isolates of *F. oxysporum* f. sp. *cepae* from the trial area. An isolate identified as *F. oxysporum* f. sp. *cepae* found in the same area in a study by Smith (1999), was no longer available for comparison with the fresh isolates obtained in this study.

In the present study, the most effective method for isolating *Fusarium* was baiting soil with onion seedlings, either in pots or in the field. The initial isolations from roots and bulbs were not successful but subsequent isolation from roots yielded *Fusarium* spp. on ¼ strength APDA.

Isolating directly from bulbs did not yield pure cultures. Whilst *Fusarium* was isolated from most bulbs sampled, contamination by a range of faster growing fungi made it impossible to obtain a pure isolate. This also occurred with the direct soil isolation where *Fusarium* was growing on the medium, but far slower than other fungi, and it proved impossible to obtain a pure culture.

Fusarium was not detected in the seed. The method employed yielded a range of fungi, but it was not possible to detect *Fusarium* at all, as fast growing colonies, including *Mucor* and *Penicillium,* dominated growth on the plates. The *Fusarium* cultures were obtained with a method used by Dr. Dean Metcalf (Department of Primary Industries 2002) that involves placing surface sterilised seed on ½ strength APDA to screen onion

seed for *Botrytis allii*. This may be a useful procedure for isolation of *Fusarium* from seed, and should be investigated for its suitability for this task.

The baiting of *Fusarium* from soil using onion seedlings was the most effective method for obtaining pure cultures. The fungal colonies that grew from surface sterilised roots on APDA were assumed to be confined to the range that could infect the onion roots, which probably contributed to minimising contamination by other microorganisms.

Isolation from seedlings of onions that were sown in soil from the four field sites (section 3.2.1.3.) yielded colonies of *Fusarium* spp., with results similar for those grown in pots and those collected from the field. Both trials showed that the soil from the site sown to onion cv. Sandridge with no onions ever sown previously (SNG) resulted in the lowest yield of *Fusarium* on APDA. Both experiments showed that onion crops sown in consecutive seasons, with no fumigation between crops, frequently yielded *Fusarium* spp. on APDA.

Prior fumigation of the soil did not show any benefit compared with the other non fumigated soil samples at the seedling stage (table 3.2) in lowering incidence of *Fusarium*, but as the plant grew, the field data indicated a lower incidence of *Fusarium* in the roots of plants from the fumigated area (PF) compared with that found in the roots of plants from the other areas. The soil that was sown to onion cv. Early creamgold and not planted to onion for 6 years prior (ECG6), yielded a frequency of *Fusarium* in the roots comparable with other soils in both the pot trial (table 3.1) and in plants collected from the field (table 3.2). In contrast, Gabor (1997) considered that a rotation between onion crops of 4 years or more with non-host crops may reduce the incidence of *Fusarium* in the soil. As this site had never been planted to onion before, it is possible that other crops had acted as hosts for the *Fusarium* spp. and maintained the

inoculum levels in the soil, that *F. oxysporum* f. sp. *cepae* had grown saprophytically in the soil, or that the soil had been infested with wind blown inoculum.

The use of CLA as a means of inducing the spores required for identification of *Fusarium* species is well documented (Burgess *et al.* 1989; Burgess *et al.* 1994; Burgess *et al.* 1991; Fisher *et al.* 1982). However, the CLA was often overgrown with Zygomycete fungi, and sporulation of *Fusarium* did not occur on the substrate, despite many repetitions, so identification by this method was not possible.

The two main sources of *Fusarium* inoculum are diseased host tissue and infested soil (Windels 1992). In this study *Fusarium* was isolated from both sources. The microscopic features of the isolates were similar, with all producing abundant macroconidia. The macroscopic characteristics, such as colour and fluffiness, of the isolates allowed differentiation between them. Eleven distinctly different isolates were grouped according to their macroscopic features.

3.3 Pathogenicity tests for identification of *Fusarium oxysporum* f. sp. *cepae*

3.3.1 Introduction

Fusarium species are commonly found in, but difficult to obtain as a pure culture from, necrotic plant tissue, debris and soil. As noted above, the pathogenic species of *Fusarium* are slow growing and easily overrun by saprophytic fungi (Windels 1992).

Many *Fusarium* spp. are secondary pathogens that infect plants through wounds. *F. oxysporum* f. sp. *cepae* is a primary pathogen of onion which does not require a wound to enter the plant (Snyder and Hansen 1940) (see section 1.3.2.). Attempts were made to infect onion seedlings with the *Fusarium* isolates described in table 3.4, without wounding, thus enabling the primary pathogens to be distinguished from those likely to

be secondary pathogens. Numerous methods were evaluated to assess the pathogenicity of the *Fusarium* isolates to onion.

3.3.2 Materials and methods

3.3.2.1 Pathogenicity testing

1. *Fusarium*-impregnated filter paper

Eleven isolates of *Fusarium* (table 3.4) were subcultured in duplicate onto APDA and grown at room temperature (approx. 24ºC) in natural light for 7 days. Sterile RO water, 20 ml, was added to each culture and the plates were agitated by hand briefly. Filter paper (Whatmans® 42, 90 mm) was autoclaved (20 mins at 121ºC) and one disc placed in each plate, soaked for 2 hours, then transferred to a sterile 90 mm Petri dish. Seedlings (cv. Patrick), grown from surface sterilised seed on sterile moist filter paper for 10 days (section 2.4), were placed on the filter paper, five per plate. The control comprised sterilised filter paper soaked in sterile RO water. The plates were incubated for 7 days at room temperature (approx. 24ºC) in natural light. After incubation the roots of the seedlings were examined for lesions, discolouration or any other damage (modified from (Mesterházy 1978).

2. *Fusarium* mycelia placed directly on seedlings

Seedlings (cv. Patrick), grown from surface sterilised seed on sterile moist filter paper for 10 days (section 2.4), were placed on autoclaved, moist filter paper (Whatmans® 42, 90 mm), three seedlings per plate, in a method modified from Robertson (1968). A 5 mm diameter disc, taken from the margin of each culture of *Fusarium* (table 3.4) grown on APDA for 7 days at room temperature (approx. 24ºC) in natural light, was placed on the root of each seedling. There were four replicate Petri dishes for each of the 11 isolates. Controls were sham-inoculated with 5 mm discs of APDA. Plates were

incubated at room temperature (approx. 24ºC) for 7 days in natural light as above. The seedlings were inspected after this period for necrosis or other damage.

3. Inoculation of roots on bulbs collected from the field

Near mature onion bulbs (cv. Patrick) were dug up and collected, root and bulb intact, from the trial site at Bowhill (section 2.1). Whilst leaving the plant intact, the root and stem plate were placed in 1% NaOCl surface sterilising solution for 1 minute (section 2.3), then rinsed three times with sterile water. A mycelial suspension of each *Fusarium* isolate (table 3.4) was prepared by homogenising cultures grown for 7 days on PDA as above, in a food processor (Goldair® FP570) with 100 ml of sterile water per plate. The food processor was sterilised with 70% ethanol between isolates. Each suspension was placed in a 2 L plastic square container previously sterilised with 70% ethanol, and three intact onion plants with surface sterilised roots and stems (prepared above) were placed in each container, with the roots and stem plate immersed in the mycelial suspension. The control comprised a Petri dish of PDA mixed with 100 ml of water. The onions were left in the suspension for 48 hours. The onions were then removed from the container, taking care to avoid cross contamination. The suspension was discarded, the container thoroughly cleaned and sterilised with 70% ethanol. Enough sterile water was placed in the container to cover the roots and stem plate of the onions, before the onions were replaced for 2 days. Roots were then removed from the bulb, placed in 1% NaOCl surface sterilising solution for 3 minutes, rinsed three times with sterile RO water and then cut into 5 mm sections and plated on APDA (section 2.3). Plates were incubated at room temperature (approx. 24ºC) for 7 days in natural light. Plates were inspected for the presence of *Fusarium,* and the morphological features of any isolate compared with the inoculum.

4. Onion scale assay

The onion scale assay technique of Earnshaw *et al.* (2000) was used as follows. Dry, outer scales of onions were removed. Segments of onion flesh (50 x 50 mm) were cut from the side of mature, stored onion bulbs cv. Patrick, and placed in surface sterilising solution for 1 minute (section 2.3). Each piece was then rinsed three times in sterile water. Each segment was placed, concave side up, in a sterile, 90 mm Petri dish lined with autoclaved moist filter paper (Whatmans® 42, 90 mm). A 5 mm diameter disc from the margin of each actively growing isolate of *Fusarium* (table 3.4) on APDA was placed mycelial side down on the centre of the bulb section. Controls were prepared using a 5 mm disc of APDA placed on the onion section. There were three replicates segments per isolate. The diameter of necrosis was measured using Vernier callipers, after 7 days of incubation at room temperature (approx. 24ºC) in natural light.

5. Direct inoculation of bulbs

A 5 mm diameter disc taken from the margin of each 7-day old culture of *Fusarium* grown on APDA was placed on whole, mature onion bulbs (cv. Patrick), by peeling back the outer dry scales from the side of the bulb to expose flesh and replacing the dry scale after inoculation, to hold the plug in place. Five onions were inoculated with each *Fusarium* isolate (table 3.4). The inoculation point was marked on the outer scale with permanent marker and the bulb placed in a sterile plastic bag and incubated at room temperature (approx. 24ºC) in natural light for 7 days. A 5 mm plug of APDA was used to sham-inoculate the controls. After incubation the tissue around the inoculation point was assessed for necrosis.

6. Inoculation of seedlings in pots

The method used was similar to that developed by Oxspring (2004) to inoculate leeks with *F. avenaceum* and *F. oxysporum*. Onion seed (cv. Patrick) were sown in Nu Erth® general purpose potting mix (Nu-Erth Horticultural Supplies, South Australia) in pots, and grown in the greenhouse for 4 weeks in September 2002. Fresh pots, 5 cm diameter x 15 cm deep, were filled with Nu Erth® general purpose potting mix to 4 cm below the rim. Cultures of *Fusarium* (table 3.4) grown on APDA for 7 days as above were cut radially into five sections. Each section was then chopped and placed into one of five pots per treatment. Immediately following, the 4-week-old onion seedlings were carefully removed from the potting mix, placed in surface sterilising solution (section 2.3) for 3 minutes, rinsed three times in sterile water, then dried between sterile sheets of paper towel. The seedlings were planted into the infested pots, one plant per pot, with the roots placed in the soil that received the inoculum, and the pot then filled to the rim with fresh potting mix as above. Controls were prepared using a plate of APDA as described above. All pots were placed randomly in free draining trays in the greenhouse to avoid cross contamination. Temperatures fluctuated diurnally between 20ºC and 30ºC, and the pots were carefully watered to avoid splash every second day for 10 weeks (fig 3.2). Plant height was recorded at 8 and 10 weeks to determine the effects of the different treatments on growth of the plants. Roots were inspected and necrotic tissue surface sterilised and plated on APDA. Plates were incubated and inspected as described in section 3.3.2.1.1.

Figure 3.2 Pots with onion seedlings grown in soil inoculated with eleven isolates of *Fusarium.*

7. Seed germinated on *Fusarium* cultures

The following method was modified from Sirois (1998). Subcultures of each isolate of *Fusarium* (table 3.4) were grown on APDA for 7 days at room temperature (approx. 24ºC) in natural light. Approximately 250 onion seeds, cv. Patrick, were surface sterilised (section 2.3) and five seeds then placed on each 7-day old *Fusarium* culture, with four replicate plates per isolate. A control was prepared with surface sterilised seeds placed on fresh APDA. Plates were incubated at room temperature (approx. 24ºC) in natural light for 7 to 10 days, and germination and necrosis of seedlings and plant growth was recorded. Necrotic tissue was surface sterilised and plated on APDA to isolate *Fusarium*.

3.3.3 Results

1. *Fusarium*-impregnated filter paper

Control seedlings appeared healthy, as did those on filter paper exposed to *Fusarium* cultures. Although there was more mycelial growth on some seedlings than on others, lesions or necrosis did not develop on the seedlings inoculated with *Fusarium* or the control. Seedling growth in all treatments was similar.

2. *Fusarium* mycelia placed directly on seedlings

Growth of the inoculated seedlings was similar to that of the control. No necrosis developed on the seedlings. Small amounts of mycelial growth were visible on the roots of some of the plants inoculated with the DM2 isolate, but growth of the seedlings was not affected.

3. Inoculation of roots on bulbs collected from the field

Onion roots previously soaked in the mycelial suspension did not develop lesions and *Fusarium* was not isolated from surface sterilised roots.

4. Onion scale assay

The controls did not develop necrosis on the onion scale. In addition, none of the inoculated segments of onion scale developed necrosis and no mycelial growth was evident on the onion tissue.

5. Direct inoculation of bulbs

None of the onion bulbs developed necrosis following inoculation with the isolates of *Fusarium,* nor did the control. There was no mycelial growth around the inoculum plug or into the flesh of the onion for any of the treatments.

6. Inoculation of seedlings in pots

The mean plant height for onions from each treatment after 10 weeks of growth is shown in fig 3.3. The mean plant heights for eight of the 11 treatments were significantly (P > 0.05) greater than the mean height of the plants in the control. The mean heights of onions from the remaining three treatments were not significantly different from the control.

No necrosis was found on any of the plant roots, so no tissue was plated on APDA.

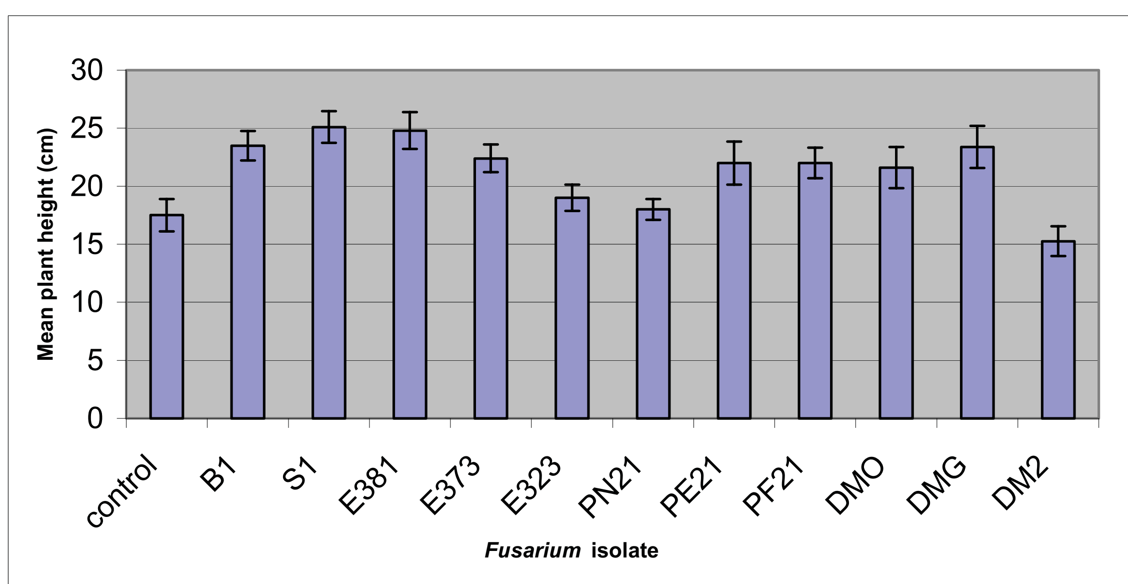

Figure 3.3 Mean plant height of onion seedlings inoculated with *Fusarium* isolates (table 3.4) grown in pots in the greenhouse for 10 weeks. Bars indicate standard error.

7. Seed germinated on *Fusarium* cultures

Ninety-five percent of seeds germinated in the control, as did 90 percent or more of the seeds in most of the plates inoculated with *Fusarium* (table 3.5). Only 85 percent of seeds placed on cultures of *Fusarium* isolate DM2 and 75 percent of seeds placed on *Fusarium* isolate E323 germinated.

Necrosis was found in only 10% of seedlings grown on agar inoculated with isolates B1, E381, DMO and DMG. Seedlings grown on all other isolates did not yield necrotic

tissue. Necrotic tissue that was observed was placed on APDA, but no colonies of *Fusarium* grew on the medium after 7 days incubation.

Table 3.5 Seedlings grown from surface sterilised seed on *Fusarium* cultures (table 3.4). Percentage germination of onion seeds following exposure to isolates of *Fusarium* and the percentage of seedlings with signs of necrosis after 10 days incubation. There were 4 replicate plates per *Fusarium* isolate per treatment.

Isolate Code	Germination %	% Necrosis
Control	95	0
B1	90	10
S1	90	0
E381	95	10
E373	95	0
E323	75	0
PN21	90	0
PE21	90	0
PF21	95	0
DMO	90	10
DMG	90	10
DM2	85	0

3.3.4 Discussion

As no disease symptoms developed in any plant in the seven methods used to test pathogenicity of the *Fusarium* isolates, none of the *Fusarium* isolates tested could be considered a primary pathogen of onion.

The use of *Fusarium* - impregnated filter paper (method 1) required a sporulating culture to provide a spore suspension with which to inoculate the filter paper. The cultures were not checked microscopically for the presence of conidia, however, cultures of the *Fusarium* isolates previously prepared were known to produce conidia within the same period. It was expected that conidia would have been adsorbed onto the filter paper with the water. It is possible therefore, that the culture either did not produce conidia, or the conidia were not adsorbed onto the filter paper. Either reason could explain why there was no incidence of disease for this experiment.

Placing mycelia of *Fusarium* directly on seedlings (method 2) was modified from a method used by Robertson (1968) for the determination of pathogenicity of *Phytophthora* spp. on tomato. Whilst it was considered that this method might be appropriate for testing pathogenicity of *Fusarium* on onion, there were no positive results. One of the isolates, DM2, produced mycelium on a seedling, but did not affect growth of the seedling. This method did not provide any evidence to suggest that any of the isolates of *Fusarium* was a primary pathogen of onion.

The third method, inoculation of bulbs collected from the field, was developed to expose an intact, actively growing plant to a large amount of *Fusarium* inoculum. That this method did not result in disease suggests that none of the isolates was a primary pathogen. There was no evidence of infection, nor was any *Fusarium* sp. isolated from the roots. As there was no wound to allow entry, secondary pathogens are unlikely to have infected onion in this experiment.

The onion scale assay, method 4, was published by Earnshaw (2000) to evaluate the ability of *Sclerotium cepivorum* to produce white rot on onion. Although *Fusarium* f. sp. *cepae* can directly penetrate the bulb (Havey 2008), there was no evidence that any of the isolates tested was capable of infecting the tissue of mature bulbs directly. As

this experiment exposed the flesh of the bulb, it was expected that even a secondary pathogen would cause necrosis to develop. This experiment should be repeated with more replicates to rule out any of the isolates used as pathogenic on onion.

Direct inoculation of bulbs (method 5) was designed to determine a primary pathogen, as the bulb flesh was not damaged. Either a primary pathogen was not among the isolates tested, or *Fusarium* could not infect bulbs directly as noted for method 4 above.

Inoculation of seedlings in pots, method 6, was modified from Oxspring's (2004) methods for assessing the pathogenicity of *Fusarium* to leek. There was no indication from plant height data that onion seedlings were negatively affected by inoculation with any of the *Fusarium* isolates. Although this experiment did not result in disease or stunting of onion, it may be useful to repeat the trial in future studies with more replicates and other isolates. As this method was very useful in determining pathogenicity of *Fusarium* spp. on leeks, it is reasonable to consider that the method should be successful in determining a *Fusarium* pathogen of onion.

Finally, germinating onion seed on *Fusarium* cultures (method 7) was modified from the work by Sirois (1998), where inoculated seeds were transferred to a soil medium for further growth. In this study the seedlings were left to grow on the *Fusarium* culture *in vitro*. Sirois (1998) detected *Fusarium* in the seedlings as they grew, however, could not be certain this originated from inoculation or if the fungus existed in the soil before seeding. In this study there were no visible signs of infection by *Fusarium* on any of the seedlings and no attempts were made to isolate the fungus from the seedlings.

The methods described here were either modified from published methods or developed to minimise any damage to plants and to detect a primary pathogen, however, none of the methods was successful. Previously, Smith (1999) inoculated onion seedlings with a

suspension prepared by macerating 7-day old cultures of *F. oxysporum* f. sp. *cepae* on PDA in RO water. Eight ml of this inoculum was mixed in 500 g of soil, and seeds of onion cv. Patrick sown at a depth of 15 mm. *Fusarium* was reisolated from over 75% of all the onion seedlings treated, and, whilst compared to the controls this result was significant, there was no significant difference between the isolates in ability to infect onion and the pathogenicity of the isolates could not be differentiated. As the inoculum load in the experiments may have been very high (Smith 1999), it was recommended that the quantity of inoculum to use in future experiments would need further investigation to ensure reliable results. As it was considered that the method published by Oxspring *et al.* (2004) and tested in this project was similar and more recently successful, the experiments by Smith (1999) were not repeated here. However, the method of Smith (1999) should be evaluated in any future studies.

Several inferences are possible from this study. There is evidence for *Fusarium* as a cause of disease in onions in fields in the Mid Murray region (Smith 1999) and onions with symptoms of *Fusarium,* such as damping off, wilt and bulb rot, have been found regularly in onion crops (Kevin Smith, personal communication, 2001). In this study, whilst pure cultures were difficult to obtain, *Fusarium* spp. was readily isolated from onions. The methods used in this study may not have been adequate to identify the primary pathogen, *F. oxysporum* f. sp. *cepae,* from the range of isolates tested. Further experiments are necessary to determine if any of the isolates is *F. oxysporum* f. sp. *cepae.* However, it is possible that none of the isolates was *F. oxysporum* f. sp. *cepae*. If this is the case, the primary pathogen may be difficult to isolate by these methods, or the population in the soil may be small compared with other *Fusarium* spp.

The aim of this study was to isolate and identify *Fusarium* cultures pathogenic to onion in order to determine if alternative hosts exist or examine possible control measures.

However, due to the lack of a confirmed primary pathogen, the decision was made to focus on pink root disease of onion.

4 *Phoma terrestris*

4.1 Introduction

Phoma terrestris is a common pathogen of onion in the Mid Murray area of South Australia, causing pink root disease. The pathogen can infest most plants in a field of onion when the plants are under stress (section 1.4.2) (Sumner 2008b).The effect on the crop yield is likely to depend on the severity and the timing of infection. Infection by *P. terrestris* can severely inhibit the growth, and reduce the subsequent size of the bulb, particularly if infection occurs early in the crop's growth (Sumner 2008b). The size of the mature onion bulb is a critical factor in the marketable value of the crop.

Despite the importance of this disease, to date little research has been conducted on the impact of *P. terrestris* on onion in Australia. One of the factors contributing to this may be the fact that *P. terrestris* grows very slowly in culture and therefore is difficult to isolate and identify, as more vigorous fungi easily overgrow it. It is more practical to try detect the presence of *P. terrestris* using wheat straw agar (Watson 1961) (see section 1.4.3) where the fungus produces a distinctive pink pigment (fig 4.1).

NOTE:
This figure is included on page 69
of the print copy of the thesis held in
the University of Adelaide Library.

Figure 4.1 Wheat straw agar (Watson 1961). The plate on the right was inoculated with *Phoma terrestris,* resulting in a pink pigment on the wheat straw. The plate on the left is a control where *P. terrestris* was not present and the wheat straw has not changed colour.

A simple and reliable method for isolation of *P. terrestris* from onion roots or soil is required, in order to examine the pathogen further. Studies to determine the host range of the fungus and to study other aspects of epidemiology, such as susceptibility of onion cultivars, and suitable methods of control, require pure cultures of the pathogen. Research on these factors may improve management of the onion crop.

4.2 Materials and Methods

4.2.1 Isolation of *Phoma terrestris*

Several techniques were utilised in an attempt to isolate *P. terrestris* from onion roots and soil:

1. Isolation from pink roots

The following method was modified from Engelhard (1978) and reported by Dhingra and Sinclair (1994) for the isolation of *Ascochyta chrysanthemi* from infected chrysanthemum flowers and stems. Pink roots (cv Sandridge, MKS), were collected from approximately 100 onion plants from a field at the trial site (section 2.1) near crop

maturation in February 2002. The roots were washed in tap water to remove excess soil and dried with paper towels. Roots were placed between sheets of sterilised paper towel and kept for several weeks at room temperature (approx. 24ºC). Ten individual, dried roots were chosen at random and rehydrated in sterile RO water in a Petri dish for 30 mins. Petri dishes were placed on a compound microscope (40x) to observe extrusion of conidia from pycnidia. Aliquots of the resulting suspension, 1 ml, were spread over six plates of APDA per root sample. Plates were incubated at room temperature (approx. 24ºC) for 2 weeks. Mycelia from colonies with macroscopic features as described in section 1.4.1 were subcultured onto APDA (section 2.2.1) to establish pure cultures of *P. terrestris*. Once established, subcultures of the colonies were transferred to WSA to confirm the identity of the isolate as *P. terrestris.* These isolates were examined microscopically to assess distinguishing features, such as pycnidia.

2. Isolation from stelar sections of onion roots

The method developed by Awuah and Lorbeer (1989) was used to minimise the contamination of plates by *Fusarium* spp. and other fungi when attempting to isolate *P. terrestris* from roots. The root systems from 20 onion plants, cv. Patrick, with pink pigmentation were collected from a field within the trial site (section 2.1) in February 2002 and washed in tap water to remove soil. Stelar sections were prepared by removing the cortex of one representative onion root per system using a dissecting needle and a scalpel. The stelar material was cut into 5 mm lengths and surface sterilised by placing in 0.1% sodium hypochlorite solution for 10 seconds (section 2.3). The sections were then rinsed three times in sterile RO water and dried between sterilised paper towels. The sections of each root were placed together in 90 mm plates of water agar (WA). There were 20 plates in total, one for each plant sampled. The

plates were sealed with Parafilm™ and incubated at room temperature (approx. 24ºC) in natural light and examined after 5 days. Plates were discarded if contaminated with *Fusarium* or other fungi. Plates that were free of contamination were inspected for fungal growth similar to that of *P. terrestris* and 5 x 5 mm sections of such colonies were subcultured onto cornmeal agar (Sigma®) supplemented with chloramphenicol (Sigma®) (500 ppm) (CCMA) for further examination.

3. Pycnidium development on moist filter paper

Pink discoloured roots from 30 onion plants, cv. Patrick, were collected from the trial site (section 2.1), then surface sterilised (section 2.3) and placed on autoclaved filter paper moistened with sterile RO water in Petri dishes (Ferreira 1990). There were 30 plates with one root, cut into sections, per plate. The plates were incubated at room temperature (approx. 24ºC) in the dark for 14 days to allow pycnidia to develop on the root segments or the filter paper. Pycnidia that developed were to be subcultured to APDA to establish a pure culture.

4. Isolation from soil

The method described by Sneh *et al.* (1974) for isolation of *P. terrestris* was tested. A base medium consisting of 3 g $NaNO_3$, 1 g $MgSO_4$, 20 g water agar (Difco®) in 1 litre RO water was prepared and autoclaved, cooled and approximately 20 ml poured into each 90 mm Petri dish. There were 20 dishes prepared in all. Ten soil samples collected from fields within the trial site (section 2.6), to a depth of 15 cm and known to be infested with *P. terrestris,* were mixed and diluted 1:100 in 0.1% sterile, cooled WA (section 2.2.3). Aliquots, 1 ml, of this suspension were spread across the base medium. The plates were incubated at room temperature (approx. 24ºC) for 4 days. Thiabendazole (2-(4-thiazolyl) benzamidazole, (TBZ), Syngenta Australia, MacQuarie Park, New South Wales) (20% a.i. in 7% hypophosphorous acid) was diluted in sterile

distilled water (TBZ final concentration 1μg/ml) and aliquots, 0.3 ml, were spread evenly over the surface of the base medium. Plates were incubated for a further 2 days. The purpose of the TBZ was to restrict the growth of all fungal colonies to allow pink colonies of *P. terrestris* to be identified and counted.

5. Isolation from pink-pigmented wheat straw

Onion root segments with pink discolouration were prepared as described in section 2.3 and plated on WSA. Plates were sealed with Parafilm™ and incubated at room temperature (approx. 24°C) in the dark for 3 weeks. Pink discolouration was observed on wheat straw fragments. Pieces of the pink-pigmented wheat straw were used in the experiments described below.

5(a). Pink-pigmented wheat straw was taken from plates of WSA and directly placed in the centre of plates of APDA. Plates were incubated for 7 days at room temperature (approx. 24°C).

5(b). Numerous pieces of pink wheat straw were removed from 10 plates of pink-pigmented WSA, surface sterilised (section 2.3) and dried between sterilised paper towels for 3 weeks. Another sample of similar size was collected in the same way 3 weeks later from the same plates of pink-pigmented wheat straw, surface sterilised, and briefly dried between paper towels to remove excess moisture. The latter constituted a "fresh" sample. Of the dried and the fresh sample, half of the pieces were homogenised separately with sterilised water in a food processor (Goldair® FP570) that had been rinsed with 70% ethanol and with sterilised water. As a result there were four treatments plus a control; *viz.* wheat straw pieces

- fresh and intact;
- fresh and homogenised;
- dried and intact;

- dried and homogenised;
- control – not exposed to *P. terrestris* but surface sterilised and dried between paper towels.

All samples were placed separately in 500 ml Schott® Bottles and filled to 250 ml with sterile RO water, shaken for a minute and incubated in the dark at room temperature (approx. 24ºC). After 1, 2 and 5 days incubation, the bottles were shaken, and 1 ml aliquots of suspension were spread on 10 plates each of WSA and APDA. The WSA plates were inspected to confirm the presence of viable *P. terrestris* in suspension, and *P. terrestris* colonies that developed on APDA were subcultured to PDA to obtain a pure culture.

5(c). Sterile water at approximately 30ºC was poured into WSA plates with pink pigmentation. Each plate was agitated for 20 seconds then incubated at approximately 30ºC for 2 hours. A syringe was used to take 1 ml aliquots, which were spread on APDA (for isolation) and WSA (for identification). There were six plates of each medium and three attempts made. The plates were incubated at room temperature (approx. 24ºC) in the dark for 7-10 days.

4.2.2 Pathogenicity tests

Onion seedlings were prepared as in section 2.4. Seedlings were placed on 7-day old cultures of *P. terrestris* on WA (section 2.5) that had been grown from the isolates obtained on APDA (section 4.2.1). Five seedlings were placed on each culture plate and there were ten plates per isolate. A control plate was prepared comprising seedlings placed on sterile WA. The plates were incubated for a further 7 days at approximately 24ºC in the dark.

After incubation the roots of the seedlings were inspected for signs of pink root.

4.3 Results

4.3.1 Isolation of *Phoma terrestris*

1. Isolation from pink roots

The first attempt to isolate *P. terrestris* from pink onion roots by this method was successful. Microscopic examination showed that conidia were discharged from pycnidia on one of the 10 onion roots. Neither the pycnidia nor the conidia were measured for comparison with published information. Conidial suspension from the one root was spread on APDA and produced several colonies of *P. terrestris*. The colonies were approx. 2 cm in diameter, with a fluffy, greyish upper surface, and a dark red colour underneath. These colonies were subcultured onto APDA and four isolates were obtained from the one root. This method was repeated several times with fresh root samples, but no pycnidia were observed under the microscope, and further isolates were not recovered.

2. Isolation from stelar sections of onion roots

Contamination by *Fusarium* and other fungal colonies developed on all plates inoculated with stelar root sections and no colonies of *P. terrestris* could be identified. The method was repeated once, but with the same results.

3. Pycnidium development on moist filter paper

The method of Ferreira (1990) did not yield any pycnidia on the filter paper although the onion roots produced a pink pigment on the paper, indicating the presence of *P. terrestris*. Consequently, no isolates of *P. terrestris* were obtained by this method. This method was repeated several times, but with the same results.

4. Isolation from soil

The method of Sneh *et al* (1974) was unsuccessful for isolation of *P. terrestris* from soil. No pink colonies developed on any of the plates, and no isolates were obtained from soil. This experiment was repeated, but with the same results.

5. Culture of isolates from pink-pigmented wheat straw

5(a). All APDA plates inoculated with pink-pigmented wheat straw were over-grown with fungal contaminants. No isolates of *P. terrestris* could be obtained by this method.

5(b). Pink discolouration of WSA indicated that viable *P. terrestris* had been transferred from the original pink-pigmented WSA to suspension with some of the treatments (table 4.1). *P. terrestris* was not detected in the control treatment as indicated by the lack of pink pigment on WSA plates. There was no indication that viable *P. terrestris* was present in suspension prepared from fresh and intact wheat straw after 1 day incubation. All of the ten plates inoculated indicated the presence of viable *P. terrestris* when the fresh, intact wheat straw had been incubated in water for 2 days, whereas after 5 days incubation only 20% of the plates indicated that viable *P. terrestris* had been present in suspension. In the fresh and homogenised treatment, the number of plates of WSA with pink pigment (indicating the presence of viable *P. terrestris*) was similar after incubation in water for 1 and 2 days, but there was no indication of the presence of *P. terrestris* in suspension after 5 days incubation in the water.

In the treatment where the wheat straw was dried and intact then incubated in water, *P. terrestris* was not detected in the suspension after any incubation period. Where the wheat straw was dried and then homogenised viable *P. terrestris* was detected in suspension after incubation for 1 and 2 days, but was not detected in the suspension obtained after incubation for 5 days.

Many fungi grew on the APDA plates. Only *P. terrestris* colonies that grew on APDA plates inoculated with suspension from pink-pigmented wheat straw that was used fresh and intact and incubated in water for 2 days were sufficiently free of contaminants to yield any isolates after subculturing. Five pure cultures were collected in this way.

Table 4.1 The number of plates of WSA, from 10 replicates, which had pink pigment indicating the presence of *P. terrestris*, after 1, 2 and 5 days of incubation of pink-pigmented wheat straw in sterile RO water. * indicates the only plates to yield pure isolates.

Treatment	1 day	2 day	5 day
None	0	10*	2
Fresh and homogenised	7	8	0
Dried and intact	0	0	0
Dried and homogenised	2	1	0

5(c). At the first attempt, pink discolouration did not develop on WSA, and only fluffy, white fungal growth covered the plates of APDA. In the second attempt, four of six plates of WSA developed pink pigmentation, indicating the presence of viable *P. terrestris*. Fungal growth on APDA that was considered *P. terrestris* was subcultured to obtain three isolates. This method was repeated once more and three more isolates obtained.

4.3.1.1 Isolates obtained

The four isolates obtained by method 1, five isolates obtained by method 5(b) and six isolates obtained by method 5(c) were subcultured on WSA plates to confirm identification as *P. terrestris.* All were confirmed as *P. terrestris* due to the pink pigmentation on the wheat straw (Watson 1961).

The isolates obtained by using methods 5(b) and 5(c) above were collected late in the project. Isolates obtained using method 1 were collected early in the project and were the only isolates available for use in pathogenicity testing.

4.3.2 Pathogenicity tests

All isolates used in the pathogenicity tests were obtained from the one onion root. The isolates were coded according to the plate from which they were retrieved; A_1, A_2, B and C.

None of the seedlings on the control plate had pink pigmentation and all were healthy. Of the four isolates tested for pathogenicity, three (A_1, B and C) produced pink roots on the onion seedlings (fig 4.2). Isolate A_1 produced pink roots on 50% of the seedlings, isolate B on 20%, and isolate C resulted in pink discolouration of all of the onion seedlings (Table 4.2).

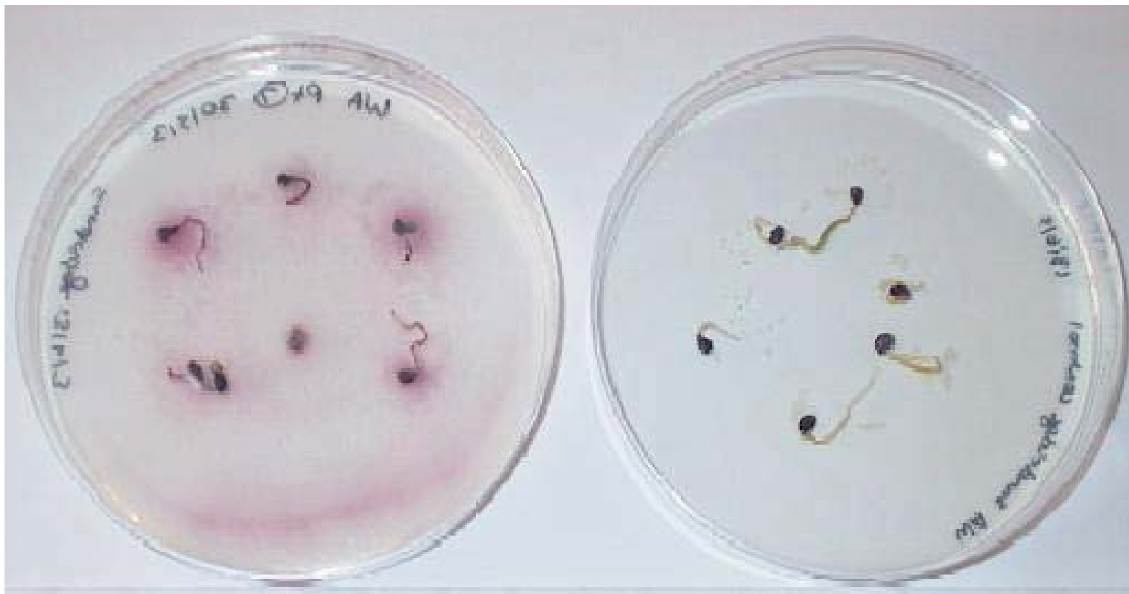

Figure 4.2 Seedlings of onion cv. Sandridge incubated for 7 days at approx. 24°C in the dark on a culture of *P. terrestris*, isolate C, on water agar (left) and on water agar alone (control, right). Pink pigment indicates that seedlings had been infected by *P. terrestris*.

Table 4.2 Number of onion seedlings, as a percentage, which developed pink discolouration when inoculated with one of four isolates of *P. terrestris in vitro.* There were 50 seedlings per treatment.

Isolate	Pink root
A_1	50%
A_2	0%
B	20%
C	100%

4.4 Discussion

Few of the isolation methods yielded cultures of *P. terrestris*. Methods involving isolation of the pathogen from pink-pigmented wheat straw (5(b) and 5(c)) gave the most reliable results.

Method 1, involving isolation from onion roots, yielded four pure cultures of *P. terrestris* from one root of ten used. This method was repeated several times, but no more isolates could be obtained. Possible improvements to this method may include a shorter or longer period of drying of the roots prior to rehydration, determination of the optimum temperature for release of conidia from pycnidia on the roots, or different culture media that encourage growth of *P. terrestris*. However, due to time constraints and the evaluation of other methods, no further modifications were made to method 1 in this project.

Isolation of *P. terrestris* from stelar sections (Awuah and Lorbeer 1989) was not successful, as all attempts to isolate *P. terrestris* resulted in plates overgrown with fungal contaminants. Most contaminants were *Fusarium* sp. although they were not formally identified. It is possible that the population of *Fusarium* in the soil at the trial

sites was larger than that in the soils used by Awuah and Lorbeer (1989), which may explain why the method was not successful in this study. The method was very time consuming and was only attempted twice.

The method described by Ferreira (1990) (method 3) also failed to yield isolates of *P. terrestris*, despite many attempts. *P. terrestris* was detected in the soil and root segments as indicated by pink pigment produced by duplicate samples on moist filter paper. No pycnidia developed however. Previous studies (Coleman *et al.* 1997); (Kulik and Tims 1960a) showed that it is rare for *P. terrestris* to produce pycnidia *in vitro.*

Method 4, as described by Sneh *et al.* (1974) was not dependent on the formation of pycnidia, only on the existence of mycelial fragments of *P. terrestris* in the soil for colonies to be established. However, this method was also unsuccessful, as it did not detect *P. terrestris* in naturally infested soils and no pink-pigmented colonies were formed. Sneh *et al.* (1974) isolated and identified *P. terrestris* from artificially inoculated soils. Porter *et al.* (1989) attempted to use the method of Sneh *et al.* (1974) in their study of control of pink root but with inconsistent results. They reported that in some of the sample soils where *P. terrestris* was present at densities causing serious damage to onion crops, the population was still often too small (< 1000 propagules/g) to recover the fungus and the plates were overgrown by other fungal species, such as *Fusarium* and *Penicillium.* The amount of inoculum present in soils in this trial was sufficient to cause significant damage to onion roots in the crop. It is possible, as in the studies by Porter *et al.* (1989), that the density of *P. terrestris* present in the soil in this study was too low (< 1000 propagules/g) to be detected or recovered by the method of Sneh *et al.* (1974). If the number of colony forming units of *P. terrestris* in the soil is too small to be determined by the method of Sneh *et al.* (1974), yet large enough to

cause serious damage to an onion crop, there could be limitations to the usefulness of the method.

Each of the published methods used in this study were unsuccessful in consistently isolating *P. terrestris*. The last methods (5a-c), involving isolation from pink-pigmented wheat straw, were developed as part of this project, towards a more consistent method for isolation of *P. terrestris*. Placing pink wheat straw directly onto APDA yielded too many fungal contaminants to allow the identification of *P. terrestris* colonies. Methods 5(b) and 5(c) were developed in order to dilute the fungi sufficiently to form separate colonies and allow *P. terrestris* to be identified.

Method 5(b) relied upon the release of conidia or mycelial fragments from the wheat straw in suspension and dilution rates suitable to minimise overgrowth of fungal contaminants. The most successful treatment was to transfer the wheat straw directly into water after surface sterilisation, and incubate at room temperature for 2 days before plating the resulting suspension on agar. It appeared that incubation for 1 day was not long enough to allow release of *P. terrestris* propagules into suspension and that the *P. terrestris* propagules were not viable after 5 days. On APDA plates on which had been placed wheat straw that was fresh and intact, few fungal contaminants grew after any incubation period, compared with the other treatments. Homogenising the fresh wheat straw pieces yielded a high percentage of WSA plates with *P. terrestris* colonies after 1 and 2 days of incubation but, due to fungal contamination, the pink-pigmented wheat straw used fresh and intact yielded the only pure cultures. Drying the wheat straw, either intact or homogenised, was of no benefit, perhaps because propagules of *P. terrestris* were damaged or destroyed by this process.

Method 5(c), in which water was added directly to the WSA plates, also gave promising results. In the successful attempts, there was little contamination by other

fungi, allowing the establishment of pure cultures of *P. terrestris*, and further experimentation may improve the success rate. The temperature could be varied to determine the optimum for release of propagules and the least contamination. The incubation period may have been too short, in view of the results of 5(b) that 2 days of incubation at room temperature yielded more colonies than did incubation for 1 or 5 days.

Unfortunately, the last two methods were developed late in the project and the resulting isolates could not be used in subsequent experiments.

The results of the pathogenicity tests were unexpected. All four isolates were recovered from the same onion root, yet they differed in the ability to cause pink pigmentation of onion seedlings. Previous studies have shown wide variability in isolates obtained from the same field, in terms of their ability to infect onion roots (Gorenz *et al.* 1949); (Kulik and Tims 1960a). It is possible that different strains of *P. terrestris* had infected the same onion root, but confirmation would require further investigation, using more isolates and a range of techniques including molecular biology. Further work of this nature was outside the scope of this project.

Kulik and Tims (1960a) based their assessment of pathogenic variation on a collection of 91 isolates from the same field, although they do not describe in detail the method used for isolation. Ferreira *et al.* (1991), who isolated *P. terrestris* by the use of moist filter paper (Ferreira 1990), reported genetic variation within *P. terrestris* and noted that it was unusually large. In both studies variability was assessed in terms of symptoms visible on onion roots and cultural characteristics on beanpod agar and PDA.

Improved methods for isolation of *P. terrestris* are required and further trials using several of the methods developed in this study would be useful. Numerous isolates are required to investigate variability in *P. terrestris*.

4.5 Additional Information

In this project, the aims of the experiments in relation to *P. terrestris* were to:

- Isolate and identify the pathogen causing pink root in the field in the Mid Murray region of South Australia
- Determine the susceptibility of locally grown commercial onion cultivars to *P. terrestris*.
- Evaluate the role of "nurse crops" in maintaining inoculum in the soil
- Identify crops and weeds that may act as alternative hosts of *P. terrestris* in fields in South Australia.

Historically, infection of onion by *P. terrestris* has been confused with that by *Fusarium* spp. For example, in early studies, it was thought that *Fusarium* spp. caused pink root disease (Sideris 1924; Sideris 1929; Taubenhaus and Mally 1921), as *Fusarium* was regularly isolated from pink discoloured onion roots. Hansen (1929) however, extensively tested over 15 different isolates of *Fusarium* obtained from pink pigmented onion roots, as well as cultures of *Fusarium* believed to cause pink root disease (Sideris 1924), and none of the isolates produced a pink pigment on onion roots. Further inoculation studies by Hansen (1929) with species of *Phoma* and *Fusarium*, yielded pink roots only in those plants inoculated with *Phoma* spp. Later studies showed that although both *Phoma* and *Fusarium* spp. could be isolated from pink pigmented roots, only *P. terrestris* induced pink root discolouration in onion roots when inoculated with these fungi (Abawi and Lorbeer 1971a; Awuah and Lorbeer 1989; Davis and Henderson 1937; Marlatt 1958; Sumner *et al.* 1997).

The first step in this project was to confirm, by isolation and identification, that *P. terrestris* existed in the soil in the Mid Murray region of South Australia. However, isolation of *P. terrestris* proved difficult in this project (section 4.3); several published methods were used, but without consistent success. Other researchers, likewise, noted that *P. terrestris* is difficult to isolate and is easily overgrown by other, faster growing fungi (Awuah and Lorbeer 1989; Blok and Bollen 1995). Nevertheless, isolations early in this project (see section 4.3.1) allowed verification of the presence of *P. terrestris* in fields in the Mid Murray region. Verification was achieved by colony morphology and the use of Watson's (1961) rapid identification procedure.

Colonies of *P. terrestris* are distinctive. As described in section 4.3.1, colonies found in the study had a fluffy, greyish, upper surface, and a dark red colour underneath on PDA. Figure 4.3, published recently by Luong *et al.* (2008), illustrates a typical culture of *P. terrestris* on PDA and is similar to cultures obtained in this study. The isolates found in the present study also matched the morphological descriptions of *P. terrestris* in culture by Gorenz *et al.* (1948), Hansen (1929), Kinsey (2002) and Sprague (1944), providing some confidence that the isolates were *P. terrestris*.

NOTE:
This figure is included on page 83 of the print copy of the thesis held in the University of Adelaide Library.

Figure 4.3 Colonies of *P. terrestris* grown on PDA. Upper surface (right), lower surface (left) (Luong *et al.* 2008)

Watson's (1961) technique to identify *P. terrestris* is well recognized by researchers. The technique has been used to identify isolates of *P. terrestris* as the pathogen associated with pink pigmented onion roots sampled from the field (Biles *et al.* 1992; Newby *et al.* 1997; Yassin *et al.* 1982), or to determine incidence of *P. terrestris* in the field (Coleman *et al.* 1997; Esfahani and Pour 2008; Hartz *et al.* 1989). Carroll (2003), when discussing *P. terrestris* causing red root rot of corn, stated that production of pink pigment on WSA (Watson 1961) is a positive test for identification of *P. terrestris*. Furthermore, Sumner (2008b), in the most recent edition of the Compendium of Onion and Garlic Diseases and Pests, states that a characteristic pink pigment is produced when the fungus is grown on wheat straw or cheese cloth (see section 4.1, Fig 4.1).

When Watson's (1961) technique was developed, *P. terrestris* was the only known fungus to produce pink pigment on WSA. Porter *et al.* (1989) reported that species of *Penicillium* and *Fusarium* colour the agar, but that the diffuse, red, almost brown colour was easily distinguishable from the intense, pink pigment produced by *P. terrestris*. They also stated that pink discolouration of WSA was a more reliable method to indicate the presence of *P. terrestris* than the formation of fruiting bodies. Watson's technique was adopted widely in this study as other methods proved unsuitable, as explained below.

The identification of *P. terrestris* via inspection of fruiting bodies was not possible in this project. Although the intention was to use pycnidia for identification of *P. terrestris*, pycnidia were not observed *in vitro* despite using a range of techniques (Biles *et al.* 1992; Ferreira 1990; Hansen 1929; Sneh *et al.* 1974). Gorenz *et al.* (1948), Kulik and Tims (1960a), Porter *et al.* (1989) and Biles *et al.* (1992) reported that many isolates of *P. terrestris* either did not form pycnidia or did so rarely in culture. However, Kulik and Tims (1960a) found that isolates which formed pycnidia were

more virulent. Blok and Bollen (1995) also reported that *P. terrestris* rarely sporulated in culture and identified the pathogen by colony morphology and by Watson's (1961) method.

A common method of diagnosing pink root in the field or in the greenhouse is to determine the presence of *P. terrestris* by visual assessment of the pink pigment in the roots of the onions (Alberto *et al.* 2002; Coleman *et al.* 1992; Kulik and Tims 1960a; Lacy and Roberts 1982; Levy and Gornik 1981; Pages and Notteghem 1996; Rabinowitch *et al.* 1981; Sumner *et al.* 1997; Thornton and Mohan 1996; Vaughan *et al.* 1971). *P. terrestris* causes the pink pigmentation of onion roots, however, this method of assessment can underestimate the level of infection as *P. terrestris* can be isolated from non-pigmented roots (Kreutzer 1939; Siemer and Vaughan 1971). In the current project, *P. terrestris* could be detected in roots early in the growth of an onion crop, based on pigmentation of WSA and before pink discoloured roots developed. It was believed that the assessment of the presence of *P. terrestris* in this project would be conducted most accurately and quickly using Watson's (1961) method.

To confirm the findings reported in this thesis, 10 isolates of *P. terrestris* on WA and 20 pigmented WSA plates that had been refrigerated at 3ºC for 5 years were re-examined in June 2009 for the presence of pycnidia. Only one pycnidium could be found on pink pigmented wheat straw on WSA. The structure was approximately 180 µm in diameter (figure 4.4), conforming to the published description of pycnidia of *P. terrestris* (Kinsey 2002; Punithalingam and Holliday 1973), although no setae were observed. In addition, approximately 100 pink pigmented roots, collected from the field in 2006, that had been dried between sterile paper towels and stored at ambient temperature, were rehydrated in sterilised RO water for several hours, then infected root segments were placed on slides, gently macerated, and inspected using a

compound microscope. Although mature pycnidia were not observed, bodies similar to those described as pycnidial primordia (Kreutzer 1941; Struckmeyer *et al.* 1962), pycnidium-like structures (Kulik and Tims 1960a), microsclerotia (Biles *et al.* 1992), or resting bodies (Hansen 1929; Sneh *et al.* 1974) were commonly found. These structures were found among the cortical cells of pink pigmented onion roots (figures 4.5 and 4.6). The bodies varied in size from 20-30 µm wide to 50-80 µm long, and appeared as a mass of dark, thick-walled cells. These observations support the assumption that the isolates described in the thesis are *P. terrestris*.

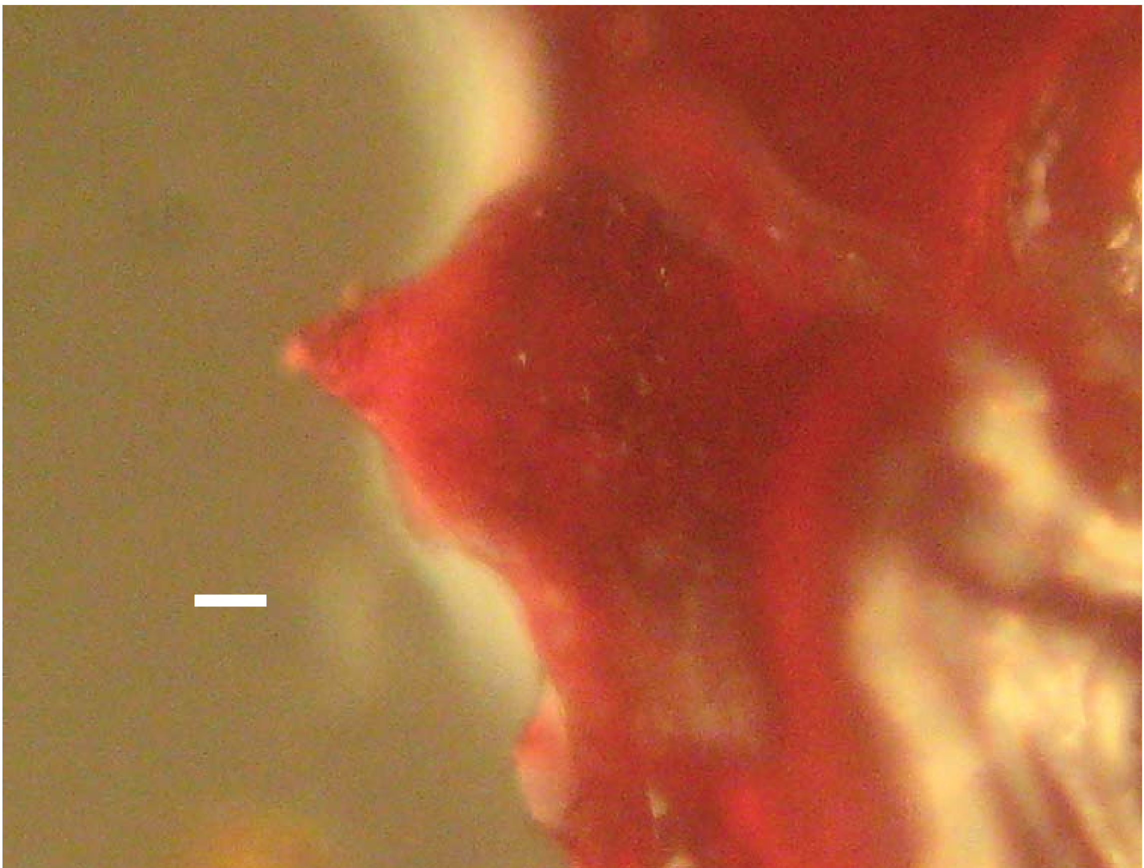

Figure 4. 4 Pycnidium found on wheat straw. Bar approx. 30 µm

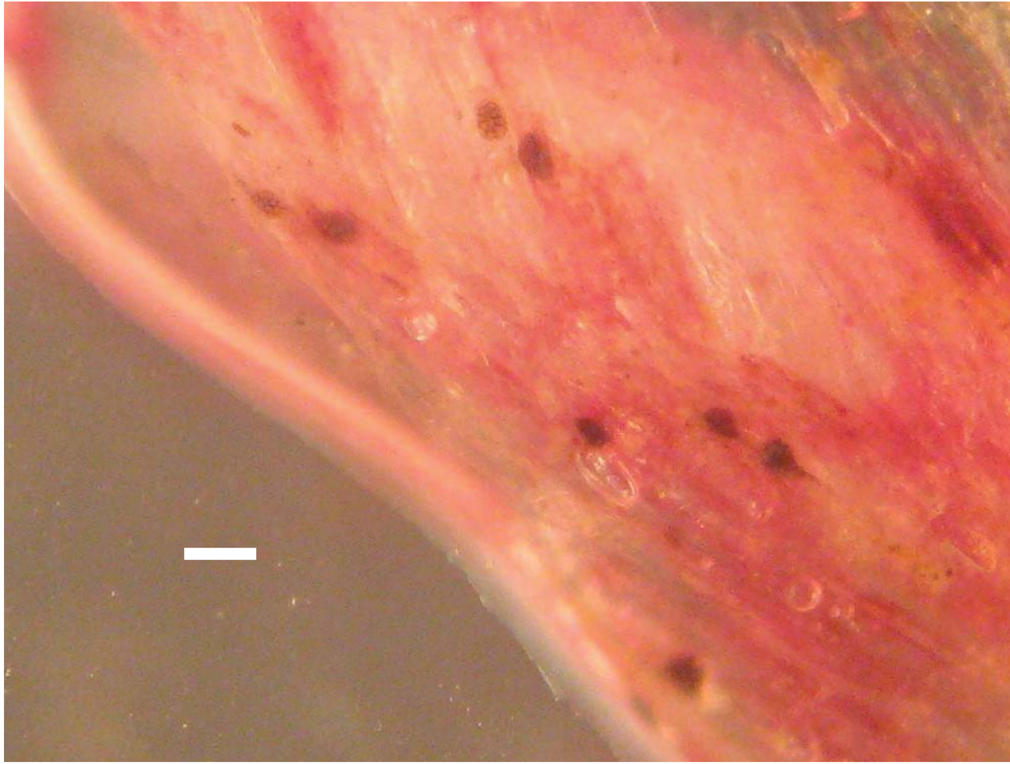

Figure 4.5 Putative pycnidial primordia found among cortical cells of pink pigmented onion root. Bar approx 100 µm

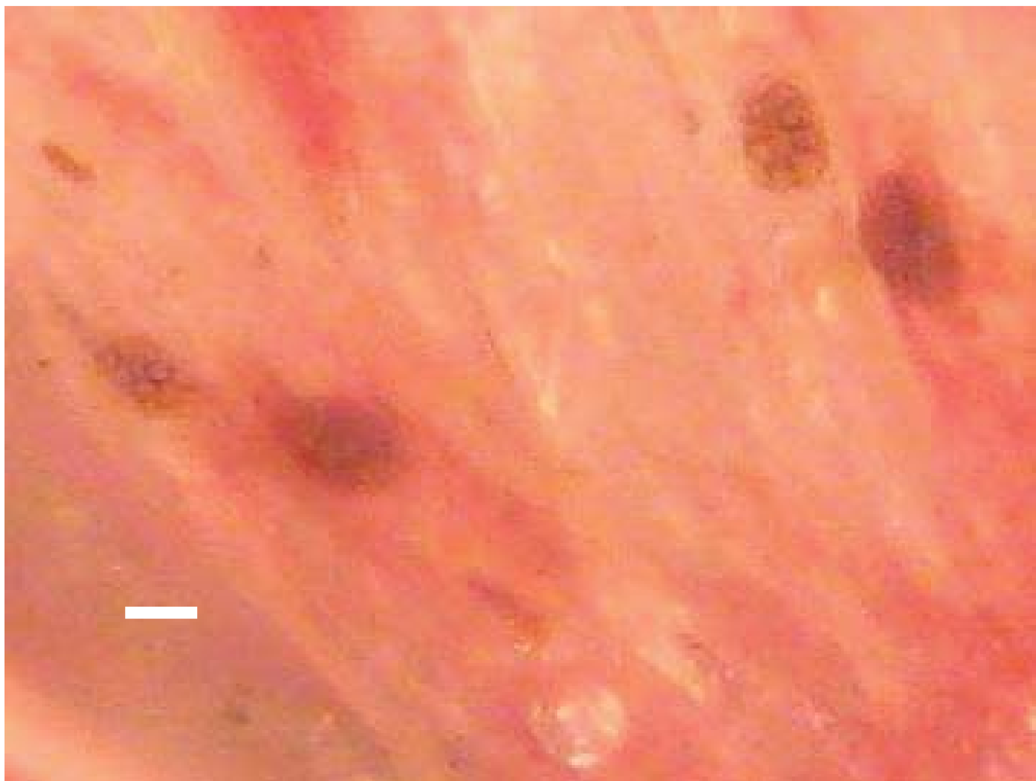

Figure 4.6 Putative pycnidial primordia found among cortical cells of pink pigmented onion root. Bar approx 30 µm

During this project, in order to progress with the aims, it was decided that:

- the most reliable and efficient method of detection of the presence of *P. terrestris* was to use Watson's (1961) method rather than attempt examination of fruiting bodies that may or may not be produced in culture;
- a new method should be developed to isolate pure cultures of *P. terrestris* as the WSA method only identifies or detects the presence of the pathogen. There was some success with developing a new procedure in this project, as described in section 4.3.1, method 5(b) and 5(c).

5 Screening onion cultivars for susceptibility to infection by *Phoma terrestris*

5.1 Introduction

Farmers may grow several different onion cultivars in one season, for reasons including different coloured cultivars (i.e. brown, white and red), harvest timing and suitability for the area, in consideration of local climatic and soil conditions (Kevin Smith, personal communication, March 2001). Onion cultivars grown in the Mid Murray area include Patrick, Sandridge (Magnus Kahl Seeds, Lancefield, Vic), Early creamgold (Terranova Seeds, Smithfield, NSW), Brownkeep (Seminis Vegetable Seeds NZ, Fairfield, Vic), Colleen and Rave (Henderson Seeds, Bulleen, Vic) (Kevin Smith, personal communication, March 2001). New cultivars are continually being developed and some cultivars are promoted by seed companies as tolerant of pink root disease. Brown cultivars that are promoted as tolerant of pink root include Wilkar, Lawan, Coppertone (Jarit Australia, Gosford, NSW), Macarena (Henderson Seeds, Bulleen, Vic), Perez (Seminis Vegetable Seeds NZ, Fairfield, Vic), Patagonia F1 (Bejo, Cranbourne, Vic), Southern Gold (Syngenta Seeds, Dandenong, Vic), Taurus and Valiant (Terranova Seeds, Smithfield, NSW).

There have been few studies to determine the susceptibility of onion cultivars to *P. terrestris*. Coleman *et al.* (1992) tested a range of cultivars suitable for the State of New York in the USA, including some cultivars promoted as pink root resistant, and other cultivars with no information about response to pink root. Coleman *et al.* (1997) subsequently reported lower incidence of pink root in the roots of mature plants of cultivars promoted as tolerant of the disease than in those not tolerant of pink root. Thornton and Mohan (1996) tested yellow sweet onions suitable for growth in the Idaho region in the USA and found that hybrid lines had the least incidence of *P.*

terrestris, primarily due to extensive replacement of damaged roots rather than resistance to infection.

True resistance of a plant to a disease occurs when the host and the pathogen are essentially incompatible and infection cannot occur (Agrios 1997). Resistance can be confused with disease escape, where the host may either have inherited traits that aid in avoiding infection, or tolerance (Agrios 1997). In tolerant cultivars, infection will occur but there is little effect on yield or quality (Kirk *et al.* 2001).

This study was conducted to examine the resistance or tolerance of onion cultivars commonly grown in the Mid Murray area to *P. terrestris,* by assessing the susceptibility to infection, to aid in the selection of cultivars to minimise the impact of pink root disease.

5.2 Materials and Methods

5.2.1 Cultivars of onions tested

Brown cultivars were selected for the screening trials, as they are the predominant type grown in the Mid Murray area. The following cultivars were selected from different seed companies:

Colleen (Henderson Seeds, Bulleen, Vic); new cultivar being grown in the area;

Coppertone (Jarit Australia, Gosford, NSW); marketed as suitable for growth in the area and pink root tolerant;

Lawan (Jarit Australia, Gosford, NSW); marketed as suitable for growth in the area and pink root tolerant;

Patrick (Magnus Kahl Seeds, Lancefield, Vic); major cultivar grown in the area for many years;

Rave (Henderson Seeds, Bulleen, Vic); new cultivar being marketed as suitable for growth in the area;

Sandridge (Magnus Kahl Seeds, Lancefield, Vic); major cultivar grown in the area for many years;

Wilkar (Jarit Australia, Gosford, NSW); new cultivar, marketed as pink root tolerant.

Seed was sourced from commercial stocks or from the seed companies indicated above, in trial size packs (500 – 1000 g).

5.2.2 *In vitro* screening of onion cultivars for susceptibility to infection by *Phoma terrestris*

The seven cultivars described above were tested *in vitro* for susceptibility to infection by *P. terrestris.*

Cultures of *P. terrestris,* isolate C (section 4.4.2), on PDA were prepared as described in section 2.5. and used at 10 days old.

Surface sterilised seed, approximately 100, of each onion cultivar (prepared as in section 2.4), were placed on sterilised moist filter paper (Whatman® No 42, Ashless) in 90 mm Petri dishes in order to prepare seedlings for trials. The plates were incubated in natural light and the filter paper was kept moist with sterile water for 10 days. After incubation, healthy seedlings were identified, removed aseptically and five seedlings were placed on each culture of *P. terrestris*. There were 10 plates for each onion cultivar. Three plates of PDA with no *P. terrestris* were prepared for each onion cultivar, with five seedlings per plate, as a control. All plates were incubated in the dark, at room temperature (approx. 24ºC) for 7 days. Each seedling was examined individually for presence of pink pigmentation, and those with pinking of the roots were considered infected with *P. terrestris*.

5.2.3 Greenhouse screening of onion cultivars

The susceptibility of the seven cultivars to pink root was tested by growing seedlings in soil naturally infested with *P. terrestris* in pots in the greenhouse.

Soil naturally infested with *P. terrestris* was collected from the trial site (section 2.6) and placed in sterile 0.55 L pots, 100 x 85 x 95 mm deep). Seeds of each of the onion cultivars listed in section 5.2.1 were surface sterilised as in section 2.4.

Twelve pots were prepared for each onion cultivar and 15 seeds were planted per pot, to a depth of 15 mm. The plants were regularly watered and fertilised (Miracle-Gro® Plant Food, Mitre 10, Murray Bridge) during the 4 month growth period. The pots were kept in a 4 x 7 x 2.5 m polytunnel greenhouse (Fenlow Tunnels, Mount Barker, South Australia) with a green mesh cover to provide shade and reduce heat when in full sun (fig 5.1).

Figure 5.1 Polytunnel greenhouse in which pot trials were conducted to evaluate cultivar susceptibility and possible alternative hosts of *P. terrestris*.

The pots were randomly placed in the greenhouse in groups of 12 (fig 5.2). Controls were prepared using soil sterilised as described in section 2.7.2.

Figure 5.2 Six week old onion seedlings growing in soil naturally infested with *P. terrestris* in the greenhouse.

Ten sample plants were collected of each onion cultivar from different pots, at intervals beginning at 6 weeks after sowing, with minimum disturbance of the remaining plants. The height of each plant was measured, then plants were washed in running water to remove soil and surface sterilised as described in section 2.3. Roots were inspected for pink pigment. The root segments of each of the ten seedlings per cultivar were placed on WSA, one plate for each plant, and incubated at room temperature (approx. 24ºC) in the dark for 14 days. After incubation, the WSA plates were inspected for pink discolouration that indicated the presence of *P. terrestris*, and the number of plates per cultivar that contained pink pigment was recorded. A further three sets of samples were collected at 8, 10 and 12 weeks of growth, and the plants processed as above.

5.2.4 Greenhouse screening of onion cultivars using metham sodium to vary the population of *P. terrestris* in naturally infested soil

The greenhouse trial was conducted again, with the addition of a range of soil treatments using the soil fumigant metham sodium to vary the population of *P. terrestris* in the naturally infested soil.

Screening of onion cultivars described in section 5.2.1. was conducted, with the addition of another brown onion cultivar, cv. Belvedere (Magnus Kahl Seeds, Lancefield, Vic). This cultivar was included as seed had become available since the previous trial. This cultivar had not been grown extensively in the Mid Murray region, but was marketed as suitable for the area. Naturally infested soil was fumigated and mixed with untreated soil to examine if reducing the inoculum level in the soil could reduce the incidence of infection by *P. terrestris*.

Soil naturally infested with the pink root pathogen was collected from the trial site (section 2.6). Two clean trays, 1 x 1 m and 20 cm deep, were loosely filled with the soil and moistened with tap water until damp throughout. The fumigant, Metham® (metham sodium a.i. 423g/L, Nufarm, Laverton North, Victoria), was applied at the recommended rate of 450 L/Ha, by adding 90 ml of fumigant to 4.5 L water, and spraying both trays evenly with the mixture, and the soil covered with a tarpaulin for 3 weeks before use. Fumigated soil and non-fumigated soil were mixed in a clean bucket as follows and placed in pots:

1. Soil not fumigated (NF)
2. 25% fumigated soil, 75% non-fumigated soil (F1:NF3)
3. 50% fumigated soil, 50% non-fumigated soil (F1:NF1)
4. 75% fumigated soil, 25% non-fumigated soil (F3:NF1)

5. Fumigated soil (F)

There were 24 pots for each soil treatment, and three of each were sown with each of the eight cultivars. The onions were grown in the greenhouse (fig 5.1) for 8 weeks. After that time three plants were collected per three replicate pots, to give a sample of nine plants per cultivar per treatment, treated as described in section 2.4 and plated on WSA, one plant per plate (section 2.2.2). The plates were incubated for 10 days and inspected for pink pigmentation to indicate the presence of *P. terrestris*. Presence of *P. terrestris* was assessed as for the previous greenhouse trial (section 5.2.3).

5.2.5 Screening of onion cultivars in the field

This experiment was undertaken to compare the susceptibility to *P. terrestris* of Colleen (C), Rave (R) and Patrick (P) (section 5.2) in the field.

The three cultivars were sown in a field trial within a commercial crop. Onion beds were approximately 1 m wide, with three triple rows of plants per bed. Each cultivar occupied a full bed and cultivars were sown in adjacent beds in the order C, R, P, C, R, P. Ten intact plants were collected at random within the same 20 m length of the beds every 2 weeks from October 2003 to April 2004. Each fresh, whole onion plant was weighed, and the roots were checked for symptoms of pink root. The whole root system was assessed for pink discolouration, and where this was observed the plant was considered to be infected with *P. terrestris*. The roots were then prepared as in section 2.3, with each of the ten root systems per cultivar plated onto individual plates of WSA (Watson 1961). The plates were assessed for pink discolouration as per the greenhouse trials. As the root systems were larger later in the trial, only a third of each root system was plated. Air temperature was monitored over the period and the average temperature per week recorded.

5.3 Results

5.3.1 *In vitro* screening of onion cultivars for susceptibility to infection by *Phoma terrestris*

No pink discolouration developed on the seedlings of any cultivar placed on the control plates of PDA. Discoloured roots developed on 48% of more of the onion cultivars tested. The exception was the cv. Sandridge in which less than 20% of plants were discoloured by *P. terrestris*, significantly ($P < 0.05$) less than all other cultivars in the trial (fig 5.2). All other cultivars had similar, and not significantly different, incidence of pink pigment in roots when incubated on PDA cultures of the pathogen. No obvious difference was detected in the growth of the roots in terms of their length, whether or not symptoms of pink root were present.

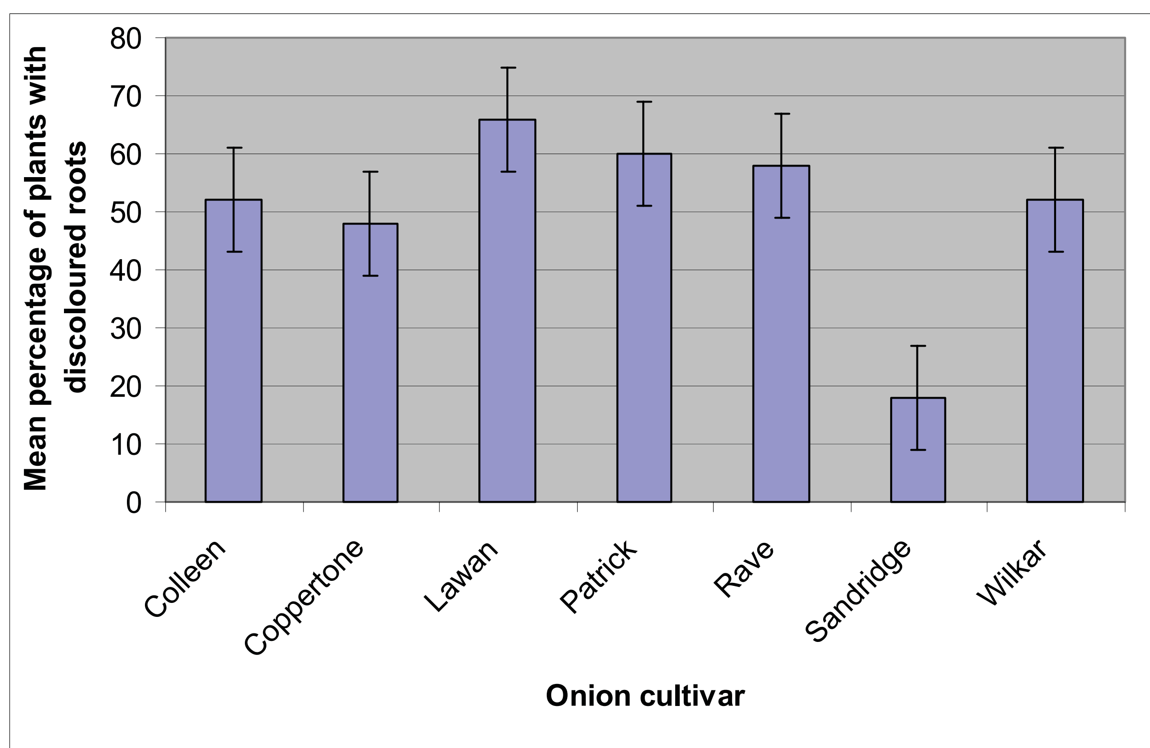

Figure 5.3 Mean incidence of discoloured roots shown as a percentage of total number of roots on different onion cultivars incubated on cultures of *P. terrestris* on PDA for 7 days at 24°C (Bars = standard error of the mean of 10 replicate plates containing five seedlings each).

5.3.2 Greenhouse screening of onion cultivars

The growth of the plants in pots appeared consistent across all cultivars at all sampling times. No pink pigment was observed on any root systems of plants at any sampling date. The incidence of pink pigment on WSA did not consistently increase or decrease over time and no cultivar had significantly lower incidence of pink pigmentation on WSA than any other over time (fig 5.4). At the initial sampling, between 50 and 100% (mean values) of root systems from all cultivars exhibited pink pigmentation on WSA. Cv. Patrick exhibited significantly ($P < 0.05$) less pink pigment on WSA than did cvs Colleen, Coppertone and Rave. At the second and third sampling dates, 80-100% of root systems exhibited pink pigmentation on WSA and there was no significant difference between any of the cultivars. At the final sampling, cv. Sandridge exhibited significantly ($P < 0.05$) less pink pigment on WSA than cvs Colleen, Coppertone and Wilkar.

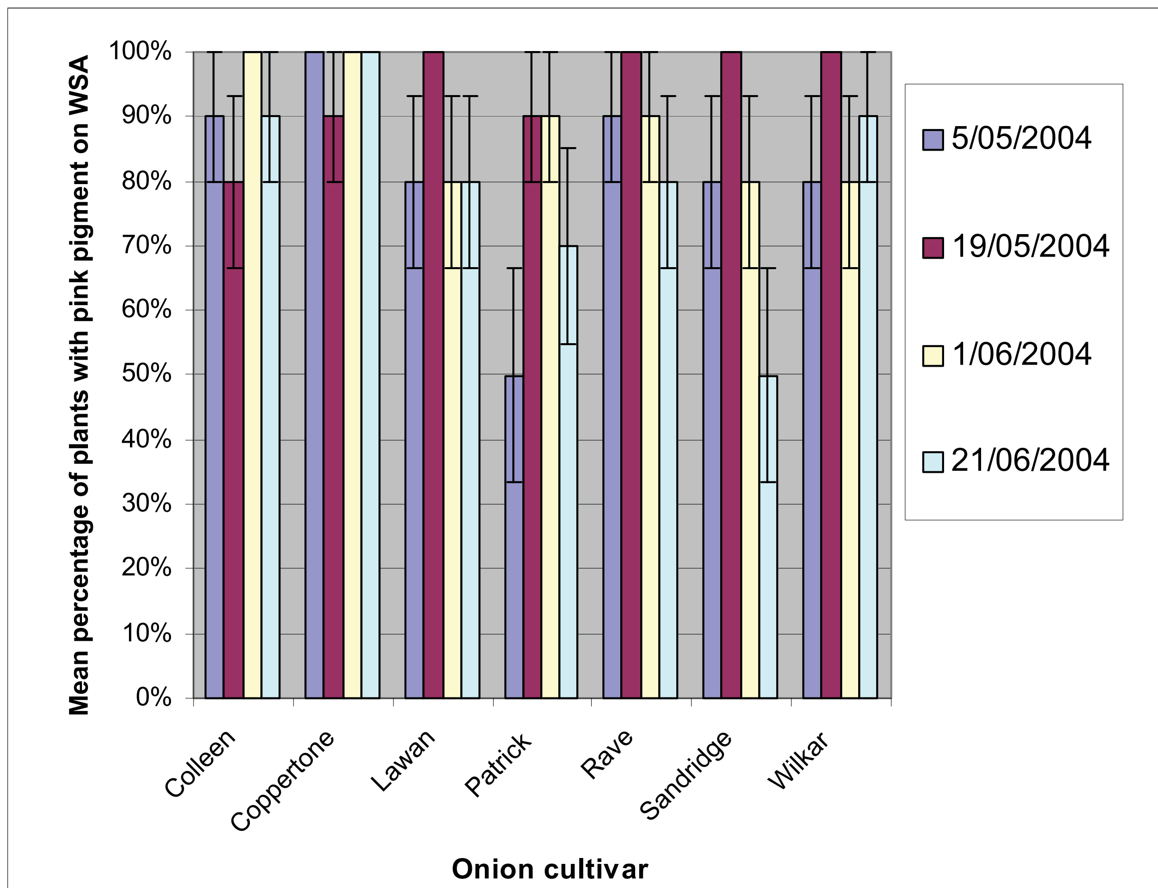

Figure 5.4 Mean percentage of onion plants with pink pigment on WSA for each cultivar grown in naturally infested soil in pots in the greenhouse trial at four sampling times. Bars = standard error (SE) of 10 replicates per cultivar.

For each cultivar, the data from all sampling periods (fig 5.4) were combined and are displayed in figure 5.5. There was no significant difference in incidence of pink pigment on WSA between the cultivars.

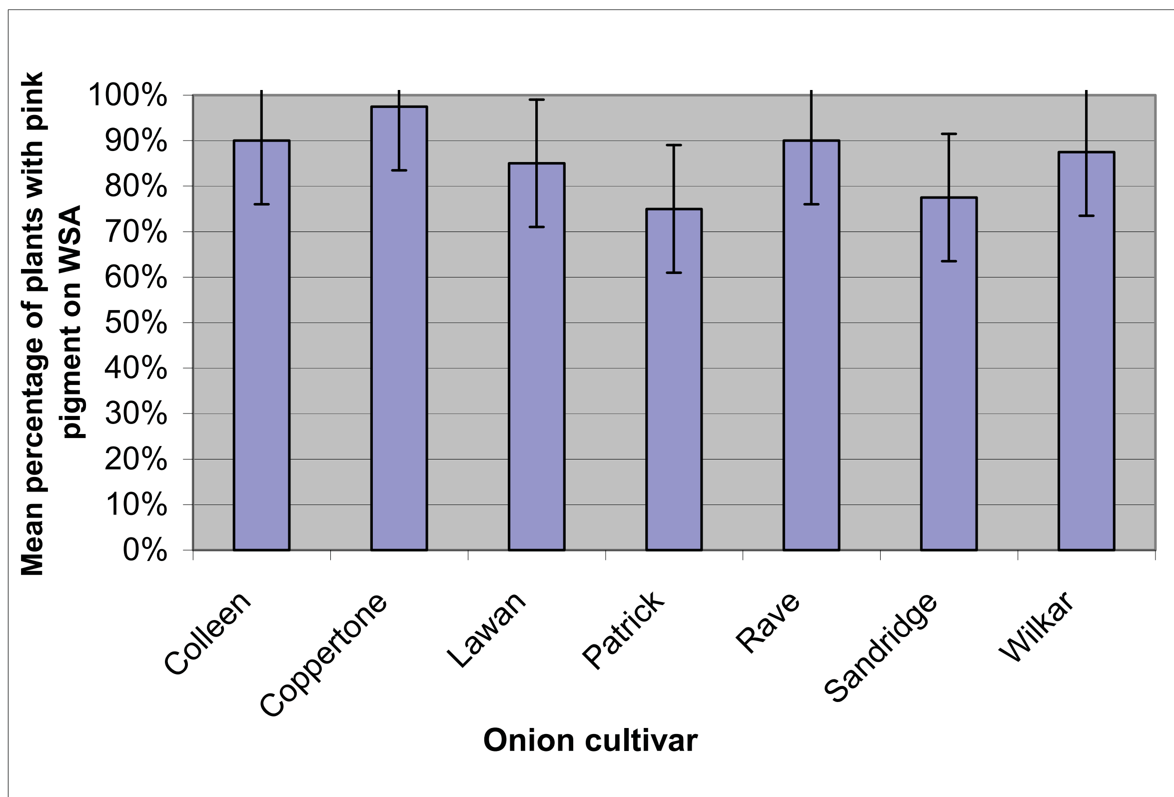

Figure 5.5 Mean percentage of onion plants with pink pigment on WSA for each cultivar grown in naturally infested soil in pots in the greenhouse trial. Data for samples on dates shown in fig 5.4 were combined. Bars = SE ($P < 0.05$) of 40 replicates per cultivar.

Results for mean plant height for the last sampling date (21/06/2004) are shown in figure 5.6. Plants of cv. Patrick were the tallest and significantly ($P < 0.05$) taller than those of Wilkar.

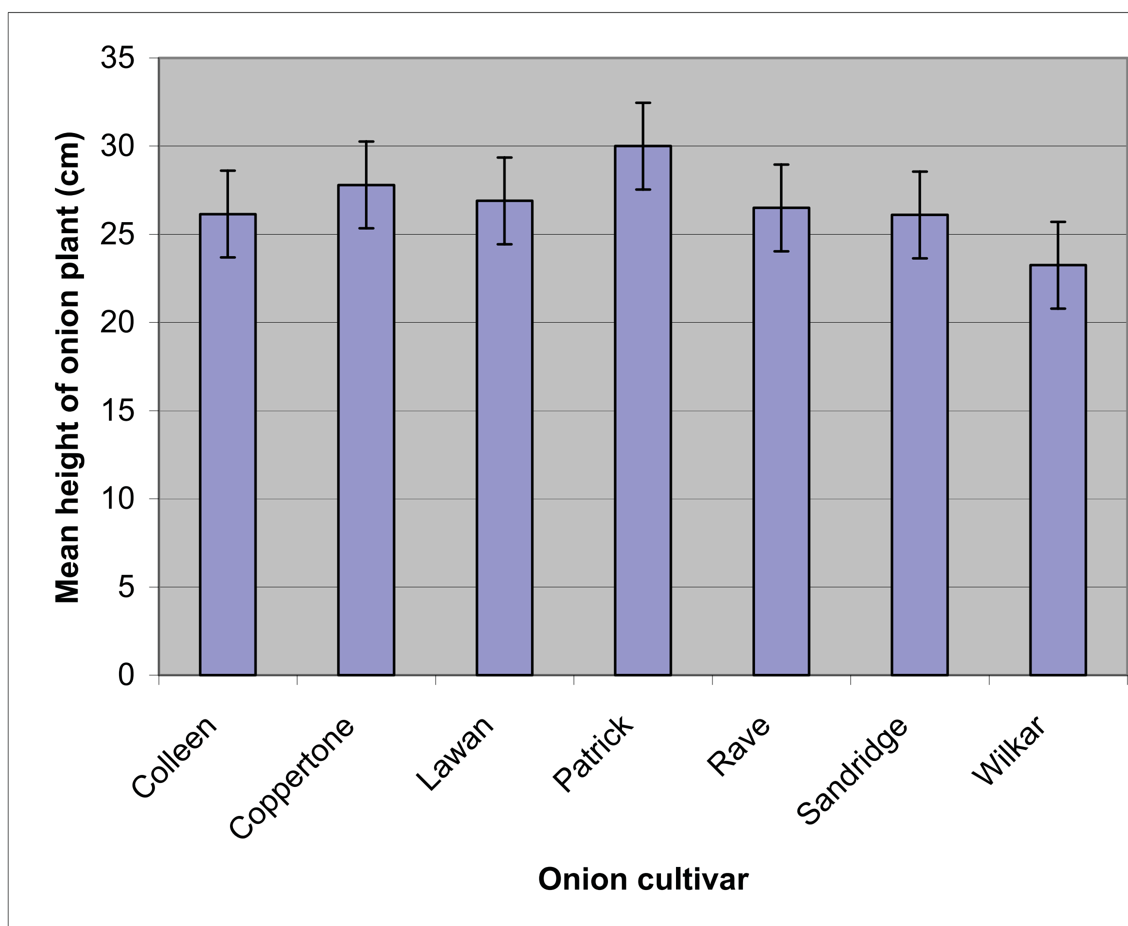

Figure 5.6 Average height (cm) of plants of seven onion cultivars grown in pots for 4 months in soil naturally infested with *P. terrestris* in the greenhouse. Data are means of 10 replicate plants sampled after 12 weeks of growth. Bars =LSD (P < 0.05).

5.3.3 Greenhouse screening of onion cultivars using metham sodium to vary the population of *P. terrestris* in naturally infested soil

Figure 5.7 shows the proportion of plants that displayed pink roots on WSA after growth for 8 weeks in the various combinations of fumigated and non-fumigated soil. Roots of the cultivar Lawan showed significantly (P < 0.05) more discolouration than did roots of cv. Rave in fumigated soil (F), whereas none of the other cultivars exhibited pink discolouration following growth in the fumigated soil.

For the plants grown in non-fumigated soil (NF), pink pigmentation of roots on WSA was significantly (P < 0.05) more common on individuals of cvs Lawan and Rave than on all other cvs tested. Pink pigmentation of roots on WSA was significantly (P < 0.05)

less frequent on cv. Wilkar than all other cvs except Patrick and Belvedere. For plants grown in non-fumigated soil, the incidence of pink pigmentation on roots of cvs Patrick and Belvedere on WSA was similar to that for Coppertone, but was significantly less ($P < 0.05$) than for Colleen, Sandridge, Lawan and Rave.

There was no pattern of reduction in occurrence of pink discolouration on WSA as the ratio of fumigated to non-fumigated soil increased (F1:NF3, F1:NF1, F3:NF1). In some instances, more pink discolouration was observed on the roots of plants grown in the various combinations of fumigated and non-fumigated soils (F:NF) than in soil that had not been fumigated.

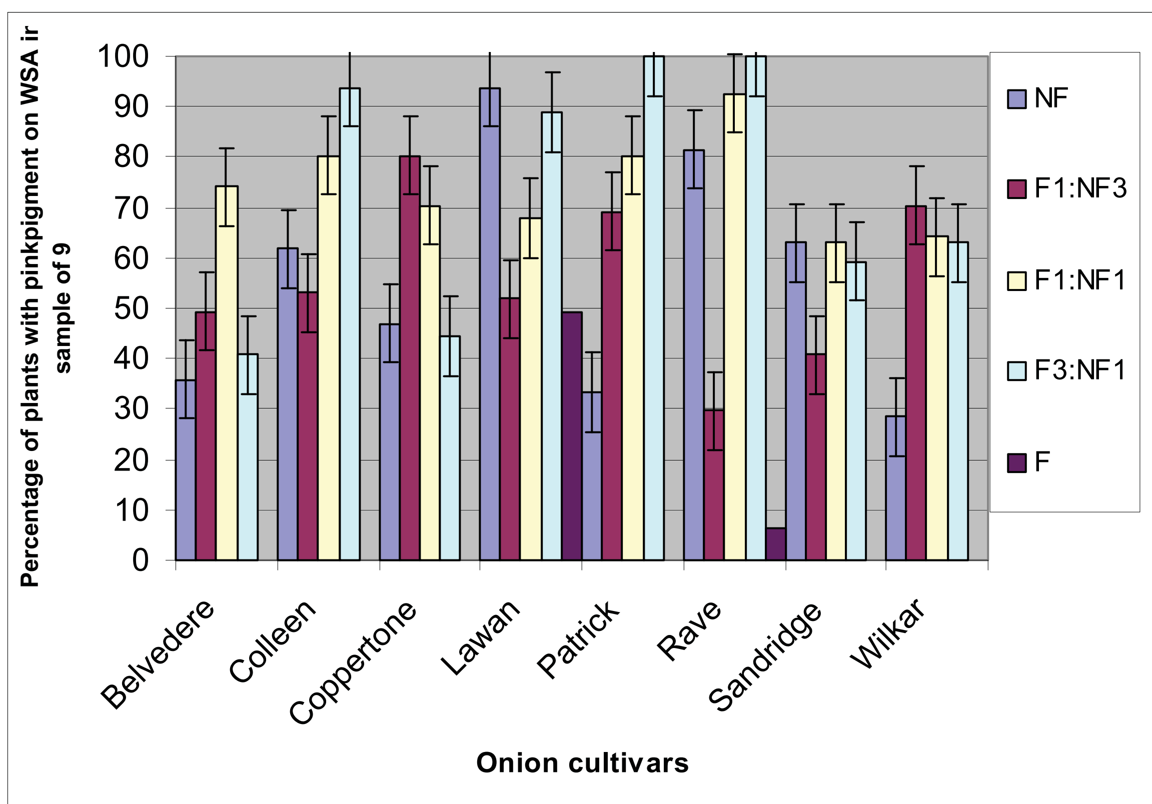

Figure 5.7 The percentage of onion plants of the eight brown cultivars grown in fumigated or non-fumigated soil with evidence of pink pigment after incubating roots for 7-10 days on WSA, after 8 weeks growth in the greenhouse. NF = soil not fumigated, F1:NF3 = 25% fumigated soil, 75% non-fumigated soil, F1:NF1 = 50% fumigated soil, 50% non-fumigated soil, F3:NF1 = 75% fumigated soil, 25% non-fumigated soil, F = fumigated soil. Bars =LSD ($P < 0.05$).

5.3.4 Screening of onion cultivars in the field

All three cultivars developed pink roots in samples collected from 19/11/2003 onwards (fig 5.8). The incidence (% of plants) with pink roots, of the three cultivars over time, with the average weekly temperature over the sampling period, shows that peaks in the percentage of discoloured roots appeared to follow periods of average weekly temperatures above 28ºC. No data for rainfall or irrigation were collected for this period. There was no obvious trend in the incidence of pink root recorded over time, and no differences were observed between cultivars in the amount of pink pigment on the roots systems.

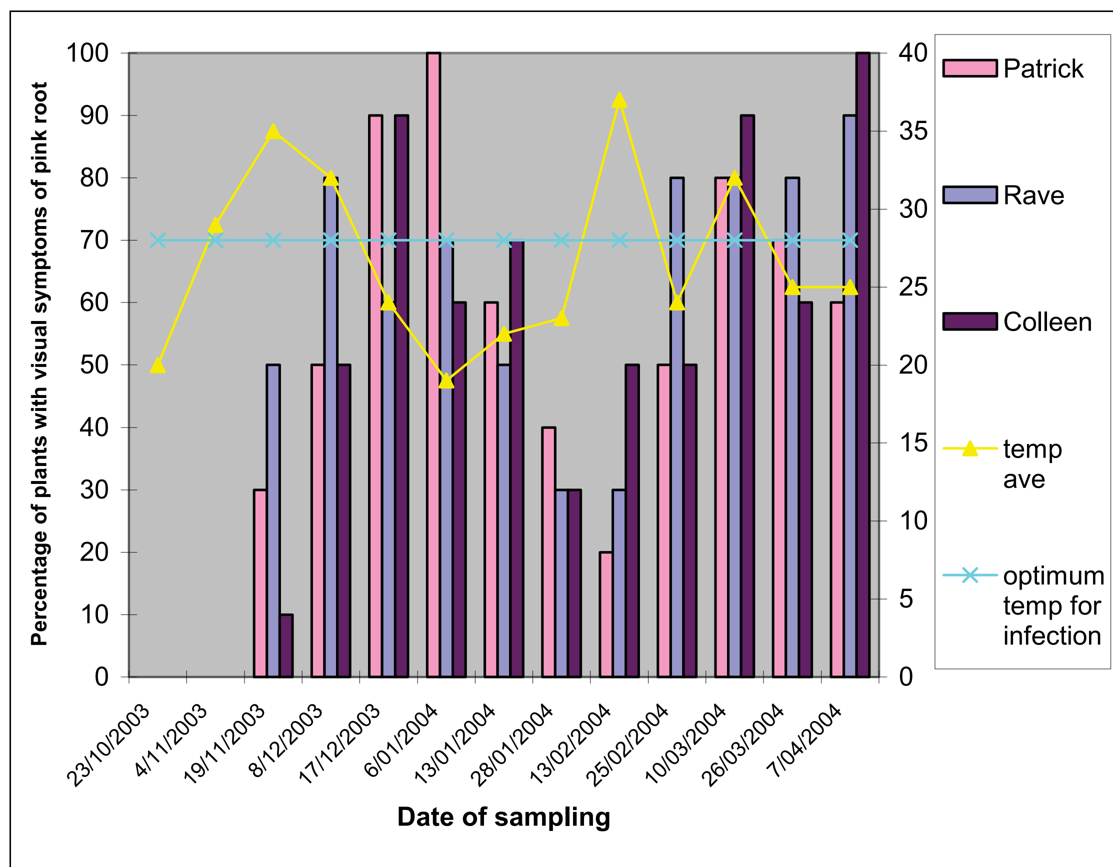

Figure 5.8 Percentage of plants of three onion cultivars with visual symptoms of pink pigment on roots collected from the field over time and the average weekly temperatures recorded for the sampling period. The optimum temperature for infection by *P. terrestris* (Sumner 2008b) is shown for comparison.

Figure 5.9 shows the percentage of plants at each sampling period that had *P. terrestris* in the roots as indicated by pink pigment on WSA. The fungus was detected in the roots

of most of the plants sampled over the trial period. The data indicate that *P. terrestris* was present in the roots of plants even before the temperature increases occurred.

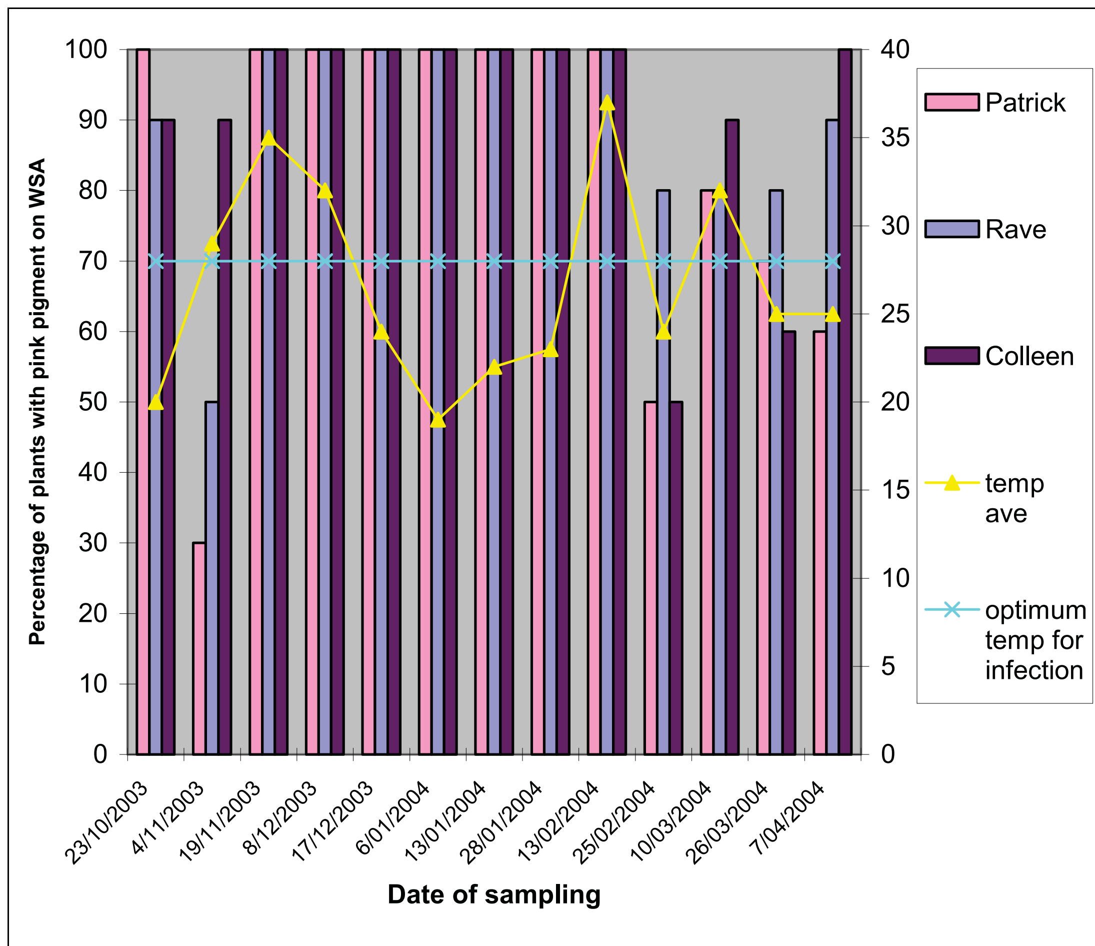

Figure 5.9 Percentage of plants of the three onion cultivars with pink pigment on WSA collected from the field over time and the average weekly temperatures recorded for the sampling period. The optimum temperature for infection by *P. terrestris* (Sumner 2008b) is shown for comparison.

The mean values for pink root on freshly collected plants and pink pigmentation on WSA for all times of sampling (data combined) for the three cultivars are shown in figure 5.10. There was no significant difference between cultivars in terms of pink discolouration of roots. Likewise, there was no significant difference in pink pigmentation on WSA.

Overall, there was a significantly (P < 0.05) higher incidence of *P. terrestris* in the roots of plants of all cultivars than could be determined by visual observation of pink discolouration of fresh roots.

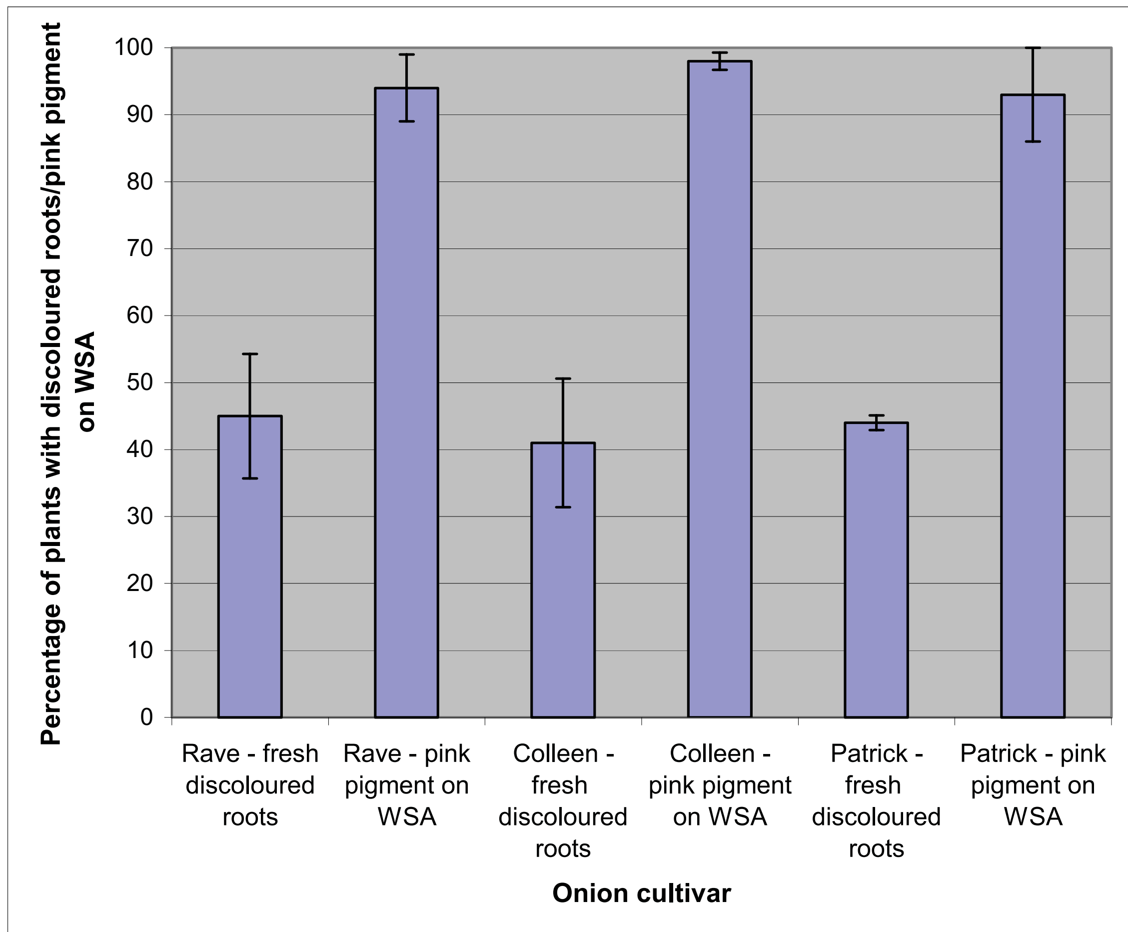

Figure 5.10 Comparison of percentage of plants with pink pigmentation of roots immediately after removal of soil at collection and percentage of roots of plants producing pink pigment on WSA from three cultivars grown for 7 months in the field. Collections were every 2 weeks throughout the growing period and data for all sampling times were combined. Bars = LSD (P < 0.05).

No significant difference was found in the fresh weight of the ten whole plants collected at each sampling period among the three onion cultivars assessed (data not presented).

5.4 Discussion

The aim of these trials was to determine if onion cultivars suitable for the Mid Murray region of South Australia are resistant to or tolerant of the pink root pathogen.

In the *in vitro* trial where seedlings were grown on PDA in the presence of *P. terrestris* the control plates did not turn pink. The cv. Sandridge was less susceptible to infection by *P. terrestris* than the other six cvs tested. However, in the first greenhouse screening of the same seven onion cultivars, the results were not as clear. Only for plants assessed 12 weeks after sowing, was the amount of pink root evident on WSA from the roots of onion cv. Sandridge significantly ($P < 0.05$) less than any other cultivar (namely cv. Coppertone). At the first sampling date, 6 weeks after sowing, roots of cv. Patrick had significantly ($P < 0.05$) less pink pigment on WSA than did cv. Coppertone. Likewise, when data for the four sampling dates were combined, roots of cvs Patrick and Sandridge tended to produce least pink pigment on WSA, although this was significantly ($P < 0.05$) less only than cv. Coppertone. Seedlings of cv. Patrick were slightly taller, on average, than all the other cultivars tested, but significantly ($P < 0.05$) taller than only cv. Wilkar. Therefore, no cultivar tested in these two experiments was consistently less susceptible to infection by *P. terrestris* than any other.

Results for the second greenhouse trial indicated that in non-fumigated soil, and hence a high inoculum level of *P. terrestris*, onion cv. Wilkar had significantly ($P < 0.05$) less pink pigment on WSA than most other onion cultivars tested. However, cvs Belvedere and Patrick, which are not marketed as pink root tolerant as is cv. Wilkar, were not significantly different from cv. Wilkar in terms of pink pigment on WSA. Cv. Lawan, which is marketed as pink root tolerant, was more susceptible to infection by *P. terrestris* than the other cultivars tested except Rave in non-fumigated soil. Likewise, of the eight cultivars grown in fumigated soil, only Lawan and Rave appeared to be

infected by *P. terrestris* based on pink pigmentation on WSA, but at a reduced rate compared with non-fumigated soil. The pink pigment on cvs Lawan and Rave is unlikely to be a physiological response of the two cultivars, as pink pigment was not evident on the roots of seedlings on the PDA control plates. Fumigation appeared to be very effective in preventing infection by *P. terrestris* in the remaining cultivars. The results suggest that although pink root inoculum was likely to have been reduced in fumigated soil, it was not eradicated completely. Likewise, Hartz *et al.* (1989) found that fumigation of soil with metham sodium improved stand establishment of onion and reduced the incidence of pink root disease, such that less than 5% of plants from the fumigated treatment showed pink discolouration of roots, but it did not eradicate *P. terrestris* from the soil.

It was expected that the incidence of *P. terrestris* in the root systems of all the onion cultivars would decrease as the proportion of fumigated soil increased. However, this was not the case, and there was no obvious relationship between inoculum of *P. terrestris,* as inferred from proportion of fumigated soil, and the incidence of pink pigmentation on WSA except where 100% fumigated soil was used. The incidence of pink pigment on WSA in many cases was less in non-fumigated soil than in soil diluted with fumigated soil, indicating the possibility that fumigation may reduce the population of organisms antagonistic to *P. terrestris*, thus allowing an increase in the number of plants infected. The results indicate that the pathogen may have the ability to multiply and spread quickly within soil, particularly in the presence of a host such as onion, or that only a small amount of inoculum is required to stimulate pink pigmentation in roots. Overall, these findings suggest that the metham sodium should be applied at least at 450 L/Ha, a recommended label rate for fumigation, to prevent or reduce pink root, as lower rates may be ineffective.

From the field trials (fig 5.8), it appeared that the pink discolouration of roots was more evident after an average weekly temperature above the optimum temperature for infection of around 28°C (Sumner 2008b). Results from previous studies (Kehr *et al.* 1962) indicated that temperatures of 28ºC and above reduced resistance of most onion lines tested to infection by *P. terrestris,* and also that pink pigment on onion roots did not occur unless *P. terrestris* had been present in the soil. The reduced number of roots with visual symptoms of pink pigment in cooler weather could indicate that the previously pink pigmented roots had senesced between sampling dates, as pink pigmented roots become brittle and die (Coleman *et al.* 1997). As *P. terrestris* was present in the roots (as indicated by pink pigment on WSA) throughout the trial, even when pink pigmented roots were not present early in the season, or uncommon (fig 5.9), it could be postulated that the warmer weather triggered the expression of the disease in the roots of onion plants, and resulted in the pink pigment. The observations from the field supported those from both greenhouse trials, and from the *in vitro* screening trial, that the incidence in pink pigment on roots of cvs Rave, Colleen and Patrick was similar.

As noted in section 1.4.2, visual symptoms of pink root have been used as a key indicator of the presence of the pathogen (Alberto *et al.* 2002). Data collected from the field and presented in figure 5.10 indicated that appearance of pink roots immediately after collection from soil may underestimate infection by *P. terrestris*, as roots not pink at collection resulted in pink pigmentation on WSA in many cases. Likewise, Coleman *et al.* (1997) found *P. terrestris* to be commonly present in the roots of onion plants as indicated by pink pigment on WSA, even when no pink pigment was present on the roots, and Kruetzer (1939) reported that lack of pink pigment in the roots did not mean that *P. terrestris* was not present. Likewise, Siemer and Vaughan (1971) found that

when using bioassays to determine amount of infection by *P. terrestris* in onion seedlings, visual symptoms gave a conservative measure of actual infection. Therefore, inspecting for pink pigment on roots at the time of collection from soil as an indication of the presence of *P. terrestris* is unreliable.

There was no consistent evidence across the experiments conducted in this study that any cultivar was less susceptible to *P. terrestris*. Although the cultivars Patrick and Sandridge tended to be less infected, or taller, than the other cvs tested, this was generally not statistically significant. The cultivars Coppertone, Lawan and Wilkar are marketed as tolerant of pink root, however, there was no evidence in the current study to support that claim. That cv. Lawan was found to be infected following growth in fumigated soil, indicates that, even in the presence of small populations of *P. terrestris*, the cultivar is susceptible to infection. Experiments conducted to support the claim of tolerance to pink root may have been conducted in different climatic regions, or with different isolates of *P. terrestris* but, based on this study, no particular cultivar of those tested could be recommended for growth in the Mid Murray area of South Australia. However, further trials to compare infection and yield would need to be done on an extensive scale, and with a wider range of cultivars and isolates, before any recommendations could be made for the industry as a whole.

6 Susceptibility of alternative hosts to *Phoma terrestris*

6.1 Introduction

Onions are generally not sown in the same field in consecutive seasons, as it is necessary to minimise the accumulation of inoculum of pathogens that infect onions by rotating crops over a period of about three to four years. *Phoma terrestris* has been found to have a wide host range (Sprague 1944; Tims 1955). Therefore, it is necessary to ensure that the crops grown in rotation with onion do not increase inoculum levels of *P. terrestris* and that pink root disease does not increase in severity as a result. At the trial site (section 2.1), crops commonly grown in rotation with onion include cereals such as barley, triticale, oats and wheat, and also cultivars of canola, vetch and lucerne.

Previous studies have investigated possible alternative hosts of *P. terrestris* that are used in rotation with onions. Kruetzer (1941) investigated a range of crops (see section 1.4.5) and found that oats, barley and wheat are susceptible to infection by *P. terrestris*. Latham and Watson (1967) used various crop residues as soil amendments, including alfalfa (lucerne), barley, oat and wheat, and determined that *P. terrestris* was regularly isolated from amended soil, but at a lesser frequency than in unamended, control soil infested with *P. terrestris*.

Another factor in the management of onion and rotation crops that might affect soil borne inoculum in the Mid Murray region is the need to plant "nurse crops" (Lomman 1991). The light, sandy soils of the Mid Murray region are very susceptible to wind erosion. To minimise potential damage and reduce the need for irrigation to stabilise soil after sowing, the onion seed is sown into an established cereal "nurse crop", which commonly consists of cultivars of barley, triticale, oats or wheat. The cereal nurse crop

may be sown up to 8 weeks prior to sowing onions, be seeded at a heavy rate and will establish quickly with only minimal irrigation before it is sufficiently developed to be safe from wind damage. If well established before the emergence of the onion seedlings, the nurse crop can be killed with a broad-spectrum herbicide, such as glyphosate, and the onions will grow through the cereal residue and be sufficiently established to avoid damage by wind before the cereal residue is completely degraded. If the nurse crop is slow to become established, the crop is sprayed with a grass-selective herbicide after the onion seedlings have emerged, so that there is enough crop matter to protect the onion seedlings from wind damage.

It is not known if these “nurse crop” management practices have any impact on the amount of inoculum of pathogens or pests in the soil. An investigation of the effect of “nurse crops” on the build up of inoculum of *P. terrestris* in soil was conducted over an onion-growing season, and the two management practices outlined above were examined to determine if there was any difference in the number of onion roots with pink pigment (to indicate the presence of *P. terrestris*).

Weeds may also contribute to changes in populations of soil-borne pathogens. As only a small number of plant species are generally studied for susceptibility to a pathogen, it is possible that the host range in nature is much larger than is currently known (Agrios 1997). Although *P. terrestris* is known to have a wide host range, few weed species have been investigated as hosts of the pathogen. The pink root fungus has been isolated from pig weed (*Amaranthus retroflexus*), crab grass (*Digitaria sanguinalis*), crow foot grass (*Eleusine indica*) and jungle rice (*Echinochloa colona*) in Louisiana, USA (Tims 1955).

Areas to be planted to onions are often left fallow between onion crops. The plant species grown will depend to some extent on the populations that existed before, but

will comprise primarily weed species. The role of weeds as hosts of *P. terrestris* in Australia is not known and this aspect needs to be examined as weeds may provide a potential means of increasing the inoculum population in fields between onion crops.

The aim of the experiments reported in this chapter was to determine if there are plants other than onions that are likely to be significant hosts of *P. terrestris*. This investigation included crops grown in rotation with onion, nurse crops, and weed species. This knowledge will aid in management of onion crops to minimise populations of *P. terrestris* in the soil.

6.2 Materials and Methods

6.2.1 Preliminary screening of species other than onion, grown in the field, as alternative hosts of *P. terrestris*

6.2.1.1 Rotation crops – lucerne and oats

A crop of lucerne mixed with oats was sown in April 2003 at the trial site (section 2.1) immediately after the harvest of an onion crop (cv. White Spanish, Southland, Mypolonga, South Australia). The landowner, to prevent soil erosion and to establish a long-term stand of lucerne, sowed the mixed crop. The prior onion crop had been sampled for presence of *P. terrestris* and the pathogen appeared to be abundant (section 6.2.1.2). The field was sown on 20/4/2003 in two halves, each of approximately 11 hectares. One half was sown with lucerne cv. Hunterfield (Daish Irrigation and Fodder, Murray Bridge, South Australia) mixed with oat cv. Swan (Hamilton Ackland, Farmer, Bowhill, South Australia). The other half of the field was sown with lucerne cv. Hallmark (Keith Seeds, Keith, South Australia) and oat cv. Marloo (Hamilton Ackland). The lucerne cultivars were sown at a rate of 12 kg seed/hectare and the oat cultivars at 75 kg/hectare. Mono ammonium phosphate fertiliser (HiFert, Murray

Bridge, South Australia) was applied to the field at 50 kg/ha before sowing, and 5 mm of water applied twice, a day after sowing the crop and again a week later, via centre pivot irrigation for crop establishment.

Ten plants of each species were collected every 4 weeks from a central area of the field from August 2003 to February 2004, when oats had senesced, and the period of sampling reflected the normal season for onion growing. Areas sampled were adjacent to each other to minimise any differences in soil type. Temperature was recorded daily using a maximum/minimum thermometer at a regular monitoring point at the residence near the trial site. Over the trial period the average daily maximum per week was calculated and compared with the optimum temperature for pink root, *viz*. 28ºC (Sumner 2008b). Roots were removed, washed in tap water to remove excess soil and then the roots were placed in surface sterilising solution (section 2.3) for 3 minutes and rinsed three times in sterilised RO water. Each root was then cut into 5 mm segments and all segments from each root sample were placed on a plate of WSA. The plates were incubated in the dark at room temperature (approx. 24ºC) for up to 4 weeks and the number of plates with pink pigment recorded. The number of plates with pink pigment was analysed and the results converted to a percentage to indicate the number of plants with pink pigment overall.

6.2.1.2 Effect of nurse crop management on pink root of onion

Two fields were selected on the trial site at Bowhill (section 2.1), both with established onion crops. Both crops had been sown into an unknown cultivar of oats. The onion crop cv. Sandridge (MKS, Lancefield, Victoria) was sown on 20/8/2002 (field 1) into an oat crop that had been had sown on 25/7/2002. The cv. White Spanish (Southland, Murray Bridge, South Australia) was sown on 29/8/2002 (field 2) into an oat crop that had been sown on 1/8/2002. Drought conditions in 2002 combined with persistent

strong wind in the onion-sowing season contributed to the likelihood of soil erosion and crop damage. As a result, two management practices for removal of the nurse crop were implemented. The farm manager applied glyphosate (Round-up Power Max®, a.i. 540 g/L, 1 L/Ha, Landmark, Murray Bridge, South Australia) to kill off the oat nurse crop in most of the field (field 1: 30/08/2002, field 2: 9/9/2002) but exposed areas on tops of hills were not sprayed at that time, to extend the protection of the onion seedlings. These hills were sprayed 6 weeks later with the grass-selective herbicide fluazifop-p (Fusilade®, a.i. 212 g/L, 750 ml/Ha, Crop Care, Landmark, Murray Bridge, South Australia).

A plot of approx. 30 cm long by 1 m across an onion bed, for each of the above management practices, was marked out with bamboo stakes. The height of every onion plant in a 5 cm by 1 m section of each plot was measured at the beginning of the trial (field 1: 30/10/2002, field 2: 6/11/2002) and, in the same section, again 4 weeks later (field 1: 29/11/2002, field 2: 5/12/2002). Samples were collected from field 1 at monthly intervals from October 2002 until January 2003, and from field 2 at monthly intervals from November 2002 and January 2003. Each sample involved removal of 10 onion plants, with minimal disturbance to the remaining plants, from each plot in both the glyphosate and fluazifop-p treated areas. Symptoms of pink roots were recorded and all roots were prepared as described in section 2.3 and plated on WSA then incubated in the dark for 4 weeks at room temperature (approx. 24ºC). WSA plates were inspected for pink pigmentation to indicate the presence of *P. terrestris* in the roots. Data were collected and analysed as described in section 6.2.1.1.

6.2.1.3 Weed species

Samples of weeds were collected from onion fields to determine if *P. terrestris* could infect or was associated with roots of these plants.

Weeds growing in beds of onions (cv. Patrick, MKS, Lancefield, Victoria) were collected in November 2001. The species were typical of the populations normally found in onion crops in the Mid Murray region. The onions were at approximately the four-leaf stage of growth and the weeds collected were near maturity. Species collected were *Malva parviflora* (marshmallow), *Polygonum aviculare* (wireweed), *Heliotropium europaeum* (common heliotrope), *Oxalis pes-caprae* (soursob), *Chenopodium album* (fat hen), *Portulaca oleracea* (purslane) and *Brassica tournefortii* (turnip weed). Ten plants of each species were collected randomly from within a 100 m x 100 m area of the onion field. Roots were inspected for lesions and pink colour, then portions of the root system were washed and surface sterilised for 3 minutes in surface sterilising solution (section 2.3). Roots were cut into 5 mm segments, placed on WSA and incubated in the dark for 14 days at room temperature (approx. 24 ºC). After incubation the plates were inspected for the presence of pink pigment on the wheat straw (Watson 1961).

6.2.2 Screening of potential hosts of *P. terrestris in vitro* and in the greenhouse

A range of crops was screened for susceptibility to infection by *P. terrestris*. Crop species selected were those commonly grown in the area and used for break crops between onion crops. All seed was sourced from a neighbouring cereal farmer, Mr. Hamilton Ackland, and had not been treated with fungicides or any other seed treatments. The cultivars were: vetch cv. Blanchfleur, lucerne cv. Hunterfield, mustard cv. Nemcon, canola cv. 501TT, canola cv. Karoo, triticale cv. Tahara, oat cv. Marloo, oat cv. Swan, wheat cv. H45, wheat cv. Yinti, barley cv. Galleon, and barley cv. Schooner.

Approximately 1000 seeds were surface sterilised, dried as for onion seed (section 2.4) and stored in airtight sterile containers until used. Seeds were stored for no longer than one month.

6.2.2.1 In vitro

Cultures of *P. terrestris,* isolate C (section 4.4.2), were grown on PDA in the dark at room temperature (approx. 24ºC) for 10 days.

Surface sterilised seed of each cultivar was placed on sterilised moist filter paper (Whatman® No 42, Ashless) in 90 mm Petri dishes. There were approximately 100 seeds per cultivar. Onions (cv. Patrick) were also prepared as a positive control. The filter paper was kept moist with sterile water for the incubation period of 10 days in natural light at room temperature (approx. 24ºC). After 10 days healthy seedlings, between 2 and 4 cm long, were removed aseptically and five intact seedlings were placed evenly around the perimeter of each plate of *P. terrestris*. Ten plates were prepared for each cultivar including cv. Patrick. Three plates of PDA with no *P. terrestris* were prepared with five seedlings of each cultivar as negative controls. All plates were incubated in the dark at room temperature (approx. 24ºC) for 7 days.

The roots of all seedlings were inspected for pink discolouration. All seedlings were removed from the culture plates, surface sterilised (see section 2.3) and placed on plates of WSA. There were five seedlings per plate, and 10 plates for each cultivar and three plates per control. All plates were incubated in the dark at room temperature (approx. 24ºC) for 14 days, and then inspected for discolouration of the wheat straw and the percentage of plants with pink pigmented roots determined.

6.2.2.2 Greenhouse trials

Soil naturally infested with *P. terrestris* was collected from the trial site (see section 2.6), mixed well and placed in 0.55 L pots (100 x 85 x 95 mm deep). The soil was sandy loam, and collected from a field where onions had been grown in the previous season and harvested in February 2004, and pink pigment on the roots of onion plants had been evident near maturity. Seeds of the various cultivars were retrieved from storage (see section 6.2.2) except for the vetch, of which there were insufficient seeds. Onion was included as a positive control.

Nine pots were prepared for each cultivar and 20 seeds were planted per pot at a depth of approximately 15-20 mm. The plants were regularly watered and fertilised (Miracle-Gro® Plant Food, Mitre 10, Murray Bridge, South Australia) during the 4-month trial period.

A plant was taken from each pot fortnightly with minimum disturbance of the remaining plants, which gave a total of nine plants per cultivar at each sampling time, beginning at 6 weeks after sowing, and repeated four times (18/8/2004, 6/9/2004, 22/9/2004 and 19/10/2004). Each plant was washed in running water to remove soil and surface sterilised as described in section 2.3. When the plants were small the whole root system was cut into approximately 5 mm lengths, and the entire system placed on WSA. As the plants became larger during the trial only a random sample of each root system was placed on the WSA, again in 5 mm segments. After incubation the WSA plates were inspected for signs of pink discolouration, indicative of the presence of *P. terrestris*. Data were collected and analysed as in section 6.2.1.1.

6.3 Results

6.3.1 Preliminary screening of species other than onion as alternative hosts of *P. terrestris*

6.3.1.1 Rotation crops – lucerne and oats

The number of plates of WSA with pink pigment from the 10 plants sampled as a percentage for each plant species is shown in figure 6.1. Initially the amount of pink pigmentation was slight for the plant species and cultivars sampled. Over time, however, the pink pigment detected on WSA increased for all cultivars of oats and lucerne, and followed a trend similar to the average daily maximum temperature for each week. In November 2003 the temperature for the week of the sampling period exceeded the optimum of 28ºC for infection (Sumner 2008b) and the number of roots detected as having *P. terrestris* peaked in all species at this time. By the next sampling date the average maximum had decreased below the optimum temperature for infection by *P. terrestris*, and the number of plants with pink pigmentation on WSA also declined. At the final sample date the pigmentation increased again, and the average daily maximum for the week again exceeded the optimum temperature for infection by *P. terrestris*. There was no obvious difference among the plant species and the cultivars tested in the amount of pink pigmentation on WSA from roots over time.

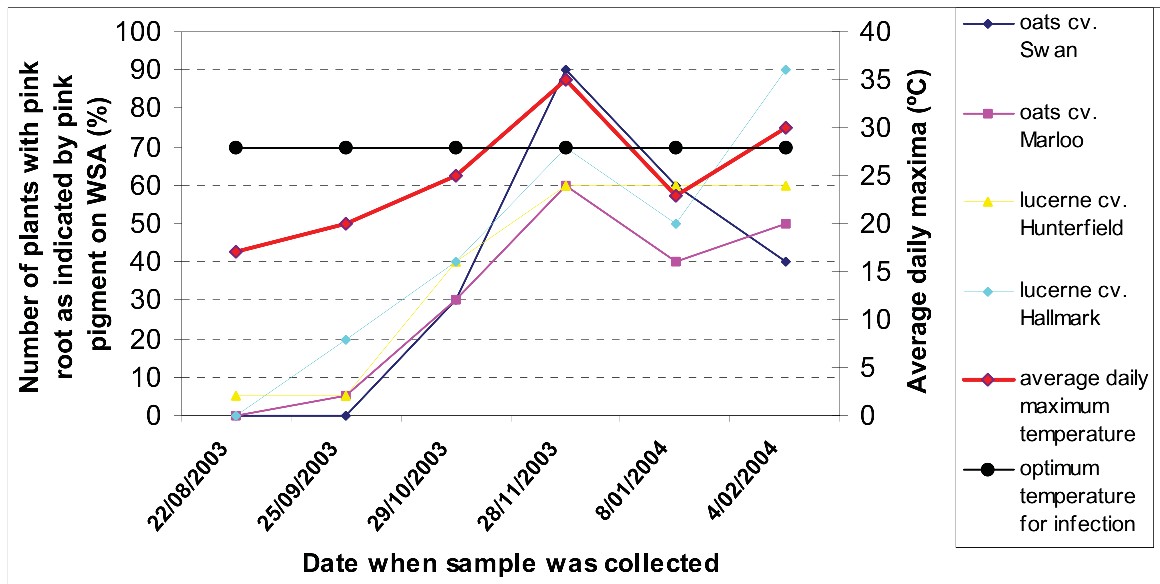

Figure 6.1 Number of lucerne and oat plants (%) with pink root as indicated by pink pigment on plates on WSA from the roots over time. The average daily maximum temperature for the week in which the samples were collected during the trial period is shown in red and the optimum temperature for infection by *P. terrestris* in black.

The data for each sampling time and cultivar were pooled and subjected to analysis of variance to examine differences between cultivars and between plant species (fig 6.2). There was no significant difference in the number of plants with pink root, as indicated by pink pigment on WSA, between cultivars and between plant species.

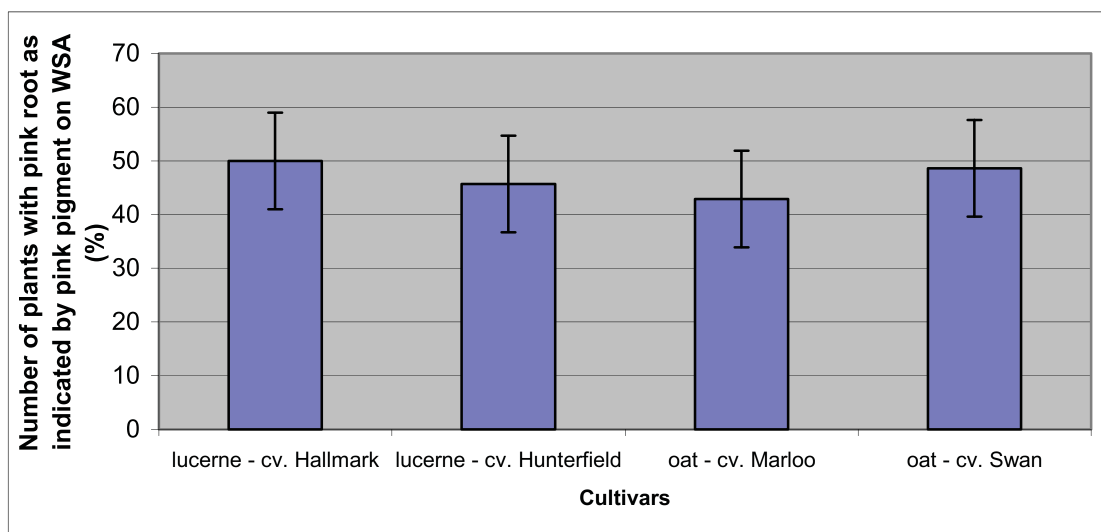

Figure 6.2 Number of plants with pink root as indicated by pink pigment on WSA from root samples of two cultivars each of oats and lucerne collected from Bowhill from 22/8/2003 to 4/2/2004. Data collected over time are pooled. Data are means of pink pigment on WSA from samples of ten plants per sample time. Bars = standard error.

Data from plants collected on 28/11/2003, when the average weekly temperature was 34ºC, were independently subjected to analysis of variance to determine if there was a difference in the number of plants infected at this time. There was more slightly pink pigmentation on WSA plates on which roots of oat cv. Swan were cultured than on plates with cv. Marloo (fig 6.3) although not statistically significant, and the lucerne cvs were similar.

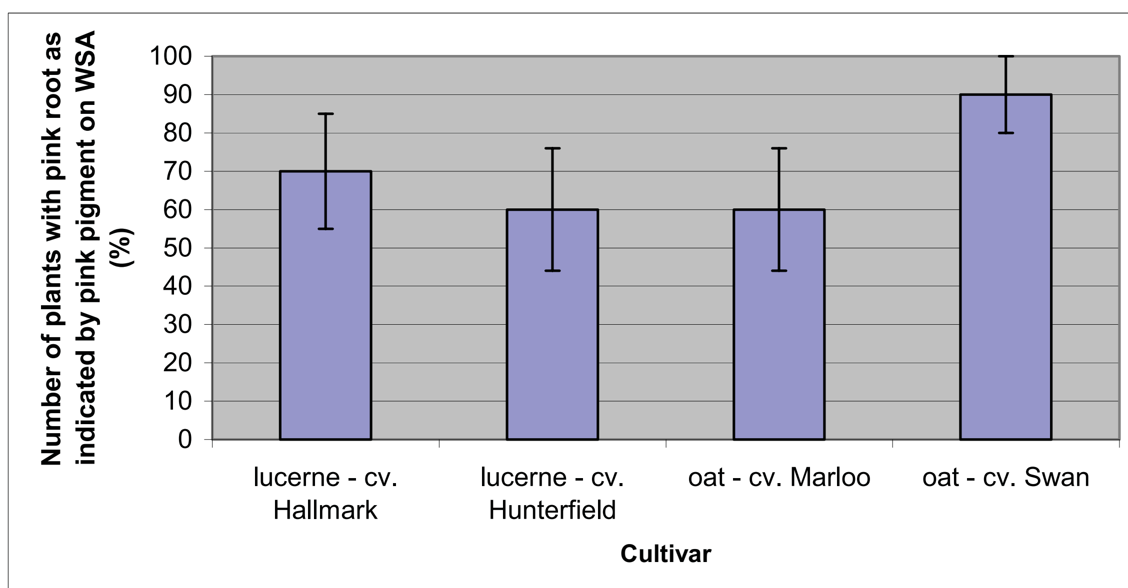

Figure 6.3 Number of plants (%) with pink root as indicated by pink pigment on WSA from root samples of two cultivars each of oats and lucerne at one sample date, 28/11/2006, when the average daily maximum for the week was 35ºC. Bars = standard error.

6.3.1.2 Effect of nurse crop management on pink root of onion

Of the onions collected from both trial sites pink pigment was only found on the roots at the final sampling date respectively. For both cvs Sandridge and White Spanish, 40% of the plants sampled from the fluazifop-p treated site had pink pigment on up to 30% of each root system, whilst 80% of the plants sampled from the crop treated with glyphosate had pink pigment on up to 30% of each root system.

In the trial where cv. Sandridge onions were established with an oat crop, the onions sampled from the fluazifop-p treated site showed a steady increase in pink pigment on WSA over the trial period (fig 6.4). The onions sampled from the area that was treated with glyphosate tended to have more roots that produced pink pigment on WSA than plants sampled from the fluazifop-p site. However, there was no significant difference in the number of plants that produced pink pigment on WSA following culture of onions collected from areas treated on 30/8/2002 with glyphosate or 11/10/2002 with fluazifop-p at each sampling time.

The plant height data (fig 6.5) indicated that the Sandridge onion plants tended to grow more vigorously in the area where the oat crop was treated with glyphosate on 30/8/2002 than in the area treated with fluazifop-p on 11/10/2002. However, the difference was not significant ($P > 0.05$) between the two treated areas at each of the sampling times.

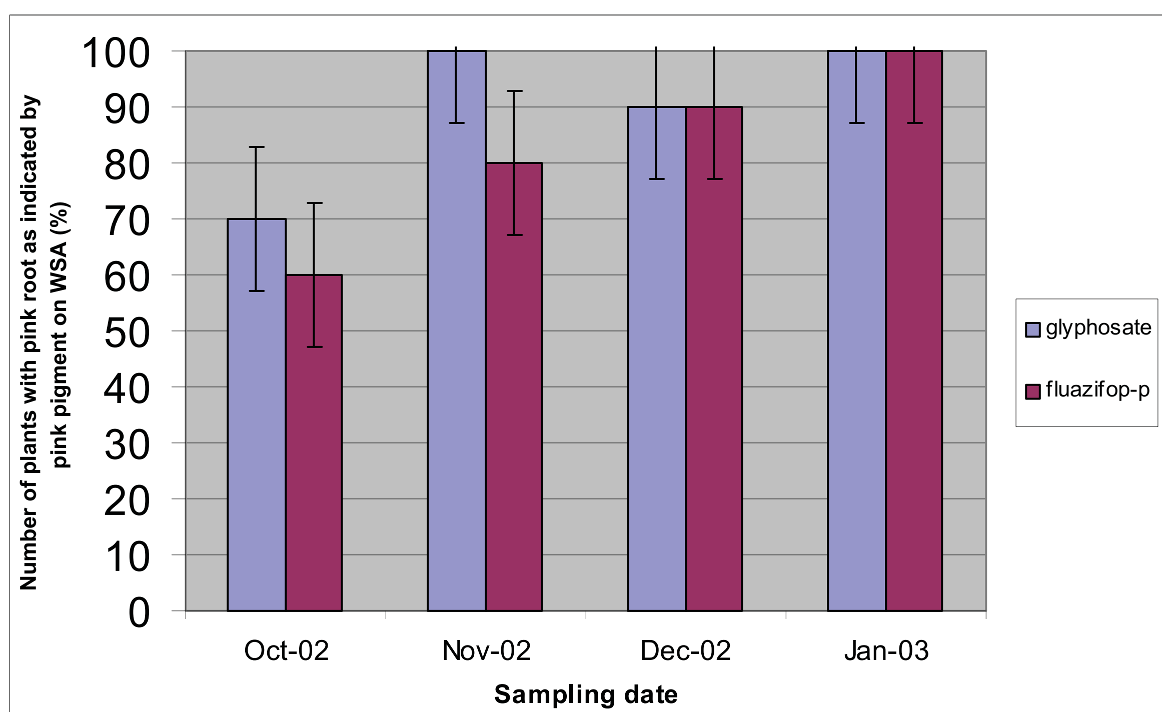

Figure 6.4 Number of plants with pink root as indicated by pink pigment on WSA (%) developed from plants of cv. Sandridge as a percentage of 10 plants sampled every month for 4 months in nurse crops treated with either glyphosate (applied within 10 days after sowing onions) or fluazifop-p (applied approx. 6 weeks after sowing onion). Bars = standard error.

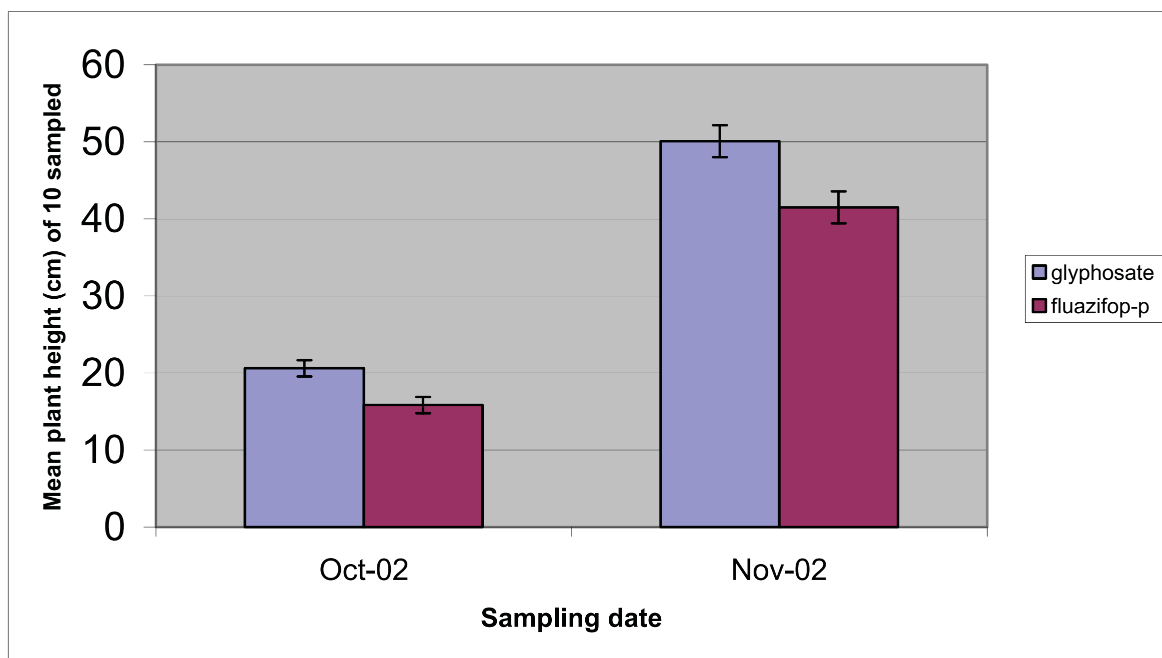

Figure 6.5 Mean height of 10 plants of onion cv. Sandridge sampled from the field treated with glyphosate (applied within 10 days after sowing onion) and fluazifop-p (applied approx. 6 weeks after sowing onion). Bars = standard error.

In the crop of onion cv. White Spanish, pink pigment was found on WSA only for onion roots collected in November and January and there was no evidence of *P. terrestris* in samples collected in December (fig 6.6). The number of plants collected in January that produced pink pigment on WSA after incubation was significantly greater ($P < 0.05$) than that observed in November. In January the number of plants that produced pink pigment on WSA, was similar for onions collected from the area where the nurse crop had been treated with glyphosate on 9/9/2002 and from the area treated with fluazifop-p on 15/10/2002 ($P < 0.05$).

The plant height data for onion cv. White Spanish (fig 6.7) indicated that there was no difference in terms of plant vigour between the onions sampled from areas treated with glyphosate or fluazifop-p at either sampling time.

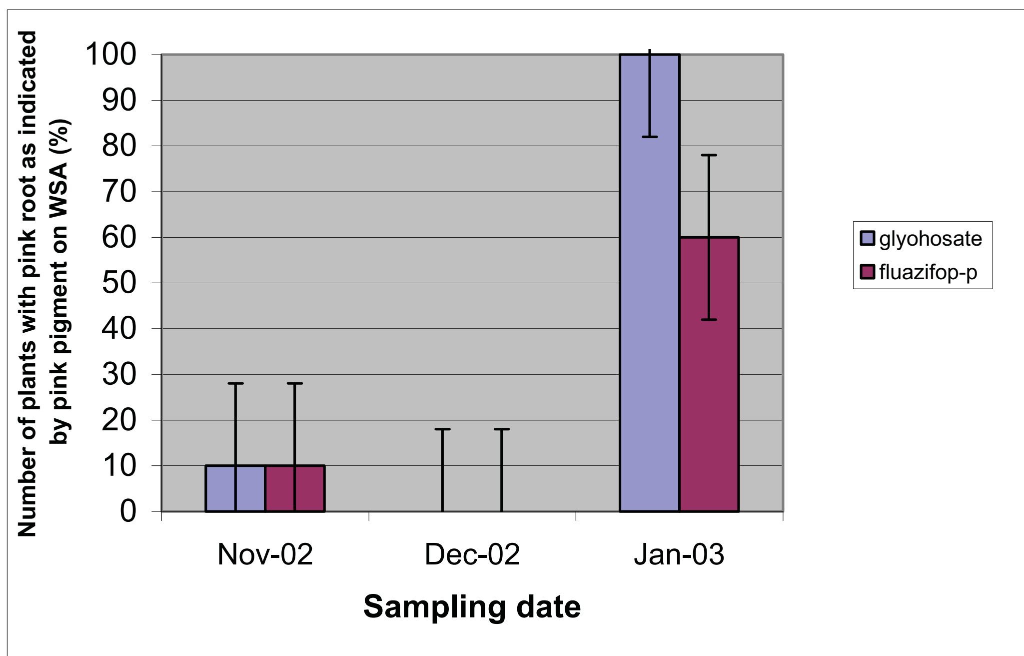

Figure 6.6 Number of plants with pink root as indicated by pink pigment on WSA (%) developed from plants of cv. White Spanish as a percentage of 10 plants sampled every month for 3 months in nurse crops treated with either glyphosate (applied within 10 days after sowing onion) or fluazifop-p (applied approx. 6 weeks after sowing onion). Bars = standard error.

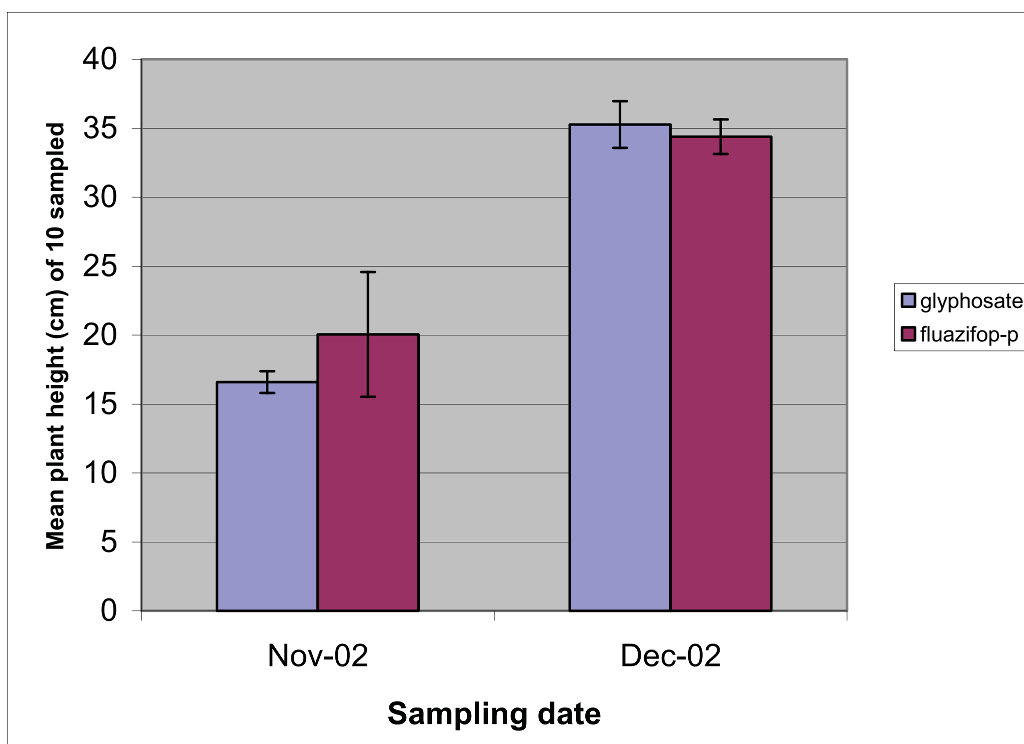

Figure 6.7 Mean height of 10 plants of onion cv. White Spanish sampled from the field treated with glyphosate (applied within 10 days after sowing onion) and fluazifop-p (applied approx. 6 weeks after sowing onion). Bars = standard error.

6.3.1.3 Weed species

Pink discolouration of roots was not visible in any of the samples at the time of collection from the field. Faint pinking of wheat straw was found on the plates of WSA from the roots of some individuals of most species (table 6.1).

Over half of the root samples of the following species resulted in pink pigment on WSA; *Polygonum aviculare, Oxalis pes-caprae, Portulaca oleracea* and *Brassica tournefortii.* Less than half the root samples from *Malva parviflora* and *Heliotropium europaeum* resulted in pink pigment on WSA. The roots of *Chenopodium album* did not produce any pink pigment on WSA indicating that *P. terrestris* was not present.

Table 6.1 The number of plants for which pink pigment was detected on WSA from a sample of roots of weed species collected from an onion field (cv. Patrick) at the trial site at Bowhill. 10 plants per species were collected in November 2001.

Species	Number, from 10 plant samples, which produced pink pigment on WSA
Brassica tournefortii	8
Portulaca oleracea	8
Oxalis pes-caprae	7
Polygonum aviculare	6
Malva parviflora	4
Heliotropium europaeum	2
Chenopodium album	0

6.3.2 Screening of potential hosts of *P. terrestris in vitro* and in the greenhouse

6.3.2.1 In vitro

Pink discolouration of seedlings was not found on any of the negative control plates (PDA). Only 20% of the seedlings of onion cv. Patrick (positive control) gave rise to pink pigment when incubated on the cultures of *P. terrestris* on PDA, which was significantly ($P < 0.05$) fewer than mustard cv. Nemcon, lucerne cv. Hunterfield, canola cvs 501TT and Karoo, and wheat cv. H45 (fig 6.8). Seedlings of canola cv. Karoo on PDA had significantly ($P < 0.05$) more pink discolouration on PDA than onion cv. Patrick, oat cvs Marloo and Swan, and barley cv. Schooner.

None of the negative control seedlings on WSA resulted in pink pigment. For the onion positive control, 71% of the seedlings yielded pink pigment on WSA (fig 6.8). Only the oat cvs Marloo and Swan had a pink pigment score on WSA that was significantly ($P < 0.05$) less than the onion control. Pigmentation on WSA from canola cvs Karoo and 501TT was similar. Oat cvs Marloo and Swan produced significantly ($P < 0.05$) less pink pigment on WSA than lucerne cv. Hunterfield, canola cv. Karoo, and wheat cv. H45. The two oat cultivars were similar in terms of pink pigment production on WSA. Likewise, barley cvs Galleon and Schooner were similar in terms of pink pigment score on WSA, however seedlings of wheat cv. Yinti, although not statistically significant, yielded slightly less pink pigment on WSA than did wheat cv. H45.

Generally, there was no significant difference ($P > 0.05$) between the number of seedlings with pink roots on PDA for each cultivar and the number of root samples that produced pink pigment on WSA. Only mustard cv. Nemcon had significantly ($P < 0.05$) more pink discolouration of roots on PDA than on WSA, and onion cv. Patrick had less pink discolouration of roots on PDA than produced on WSA.

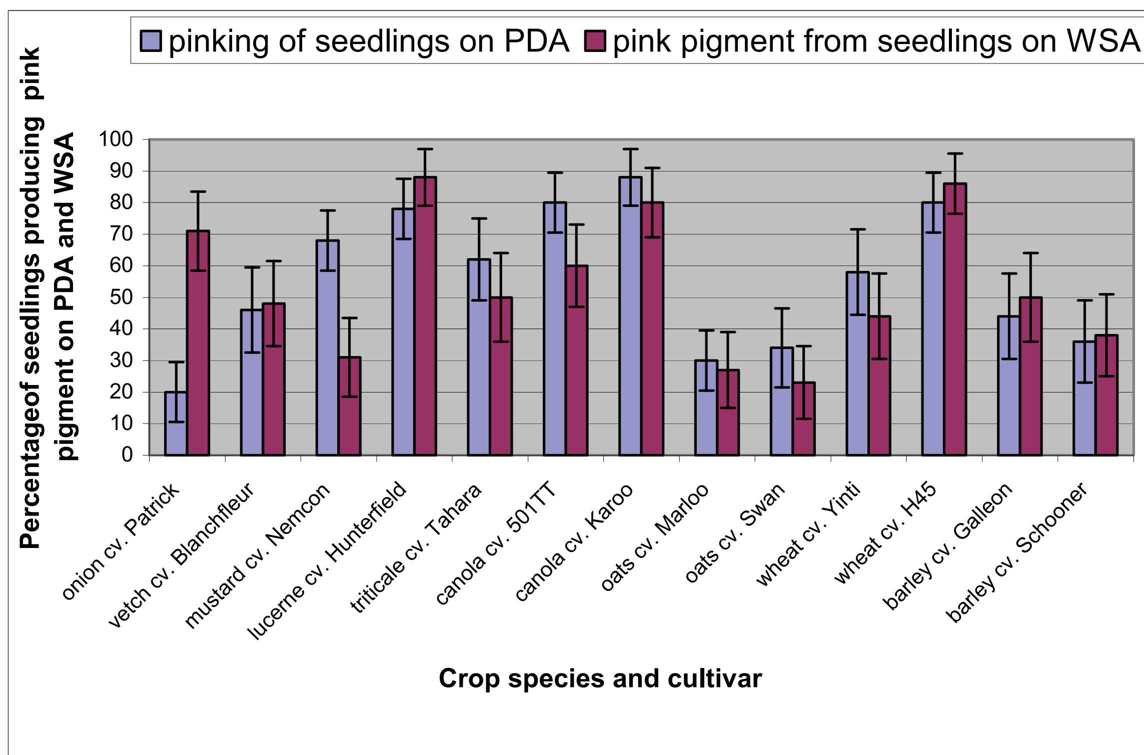

Figure 6.8 The percentage of seedlings of various crop species and cultivars that produced pink pigment on PDA colonised by *P. terrestris* and subsequently cultured on WSA. There were 50 seedlings per species or cultivar and onion, cv. Patrick, was included as a positive control. Bars = standard error.

6.3.2.2 Greenhouse trials

The results of the greenhouse trial to test the susceptibility of various plant species and cultivars to infection by *P. terrestris* are shown in figure 6.9. Only seedlings of wheat cv. H45 produced any pink pigment on WSA at the first sampling time (4/8/2004). The onion plants had significantly ($P < 0.05$) more pink pigment on WSA for three of the five sampling periods than the crop species or cultivars tested, except for mustard cv. Nemcon and canola cv. Karoo, which had a pink pigment score on WSA not significantly different from onion at the sampling time of 22/9/2004. Most other crops species and cultivars had a variable degree of pink discolouration on WSA and there was no consistent trend over time. Neither barley cultivar exhibited any pink pigment on WSA over the whole period.

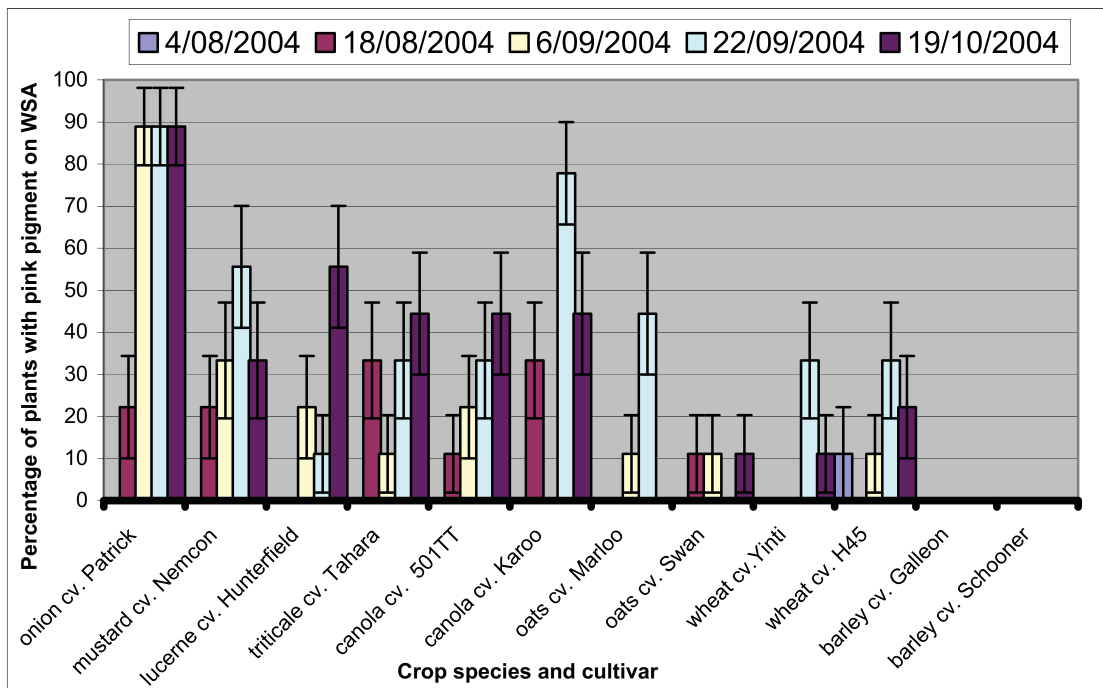

Figure 6.9 The incidence of pink pigmentation on WSA from the roots of different crop species and cultivars grown in the greenhouse in soil naturally infested with *P. terrestris*. Data are means of nine replicates. Bars = standard error.

Data for the sequential sampling times were combined and the mean percent of root systems producing pink pigment on WSA for the five sampling times for each species and cultivars tested is shown in fig 6.10. As noted above, barley cvs Galleon and Schooner did not produce any pink pigment on WSA. All other crop species and cultivars produced pink pigment on WSA, with no significant differences ($P > 0.05$).

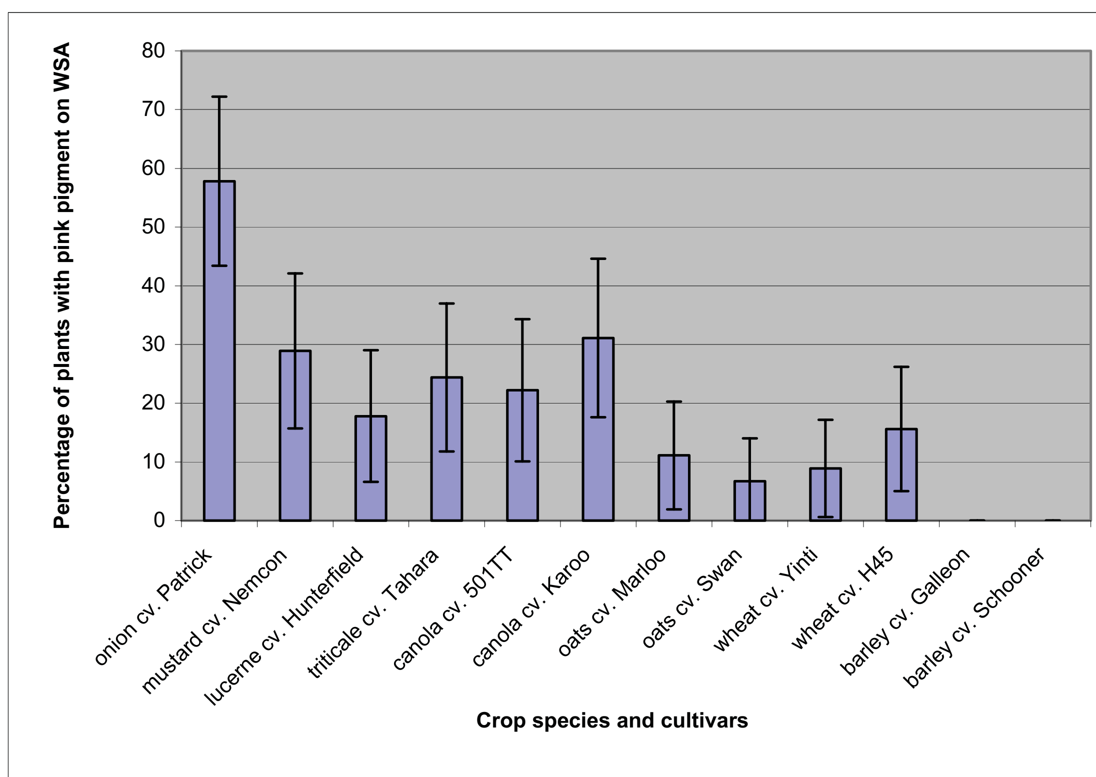

Figure 6.10 The percentage of seedlings sampled of various crop species and cultivars that produced pink pigment on WSA after growing in soil naturally infested with *P. terrestris* in a greenhouse. Data for the five sampling periods (4/8/2004 to 19/10/2004) were combined. Data are means of 45 replicates. Bars = standard error.

6.4 Discussion

Detection of pink pigment on WSA indicated the presence of *P. terrestris* in the roots of all cultivars of lucerne and oats collected from the field site. In this trial the incidence of *P. terrestris* in the roots of the lucerne and oat cultivars examined, as indicated by the pink pigment on WSA, increased as the average daily maximum temperature for the week of sampling increased over time. No cultivar had significantly more pigmented plants than any other overall, however, there were some differences at certain sampling times. The samples examined in November 2003 were collected during a week when the average daily maximum was almost 35ºC and the number of plants that produced pink pigment on WSA increased in all cultivars. Therefore, it is possible that activity of *P. terrestris* increased as the optimum temperature threshold for disease development of 28ºC (Sumner 2008b) was exceeded at certain times in that

period. There was a decrease in the number of plants yielding pink pigment on WSA in January when the temperature dropped from that recorded at the sampling date in November, indicating that the roots infected with *P. terrestris* in November may have become brittle and detached (Coleman *et al.* 1997). The observation that more plants of the oat cv. Swan yielded pink pigment on WSA than those of oat cv. Marloo and lucerne cv. Hunterfield, although not significant, indicated that some of the crops used in rotation with onion may be more susceptible to infection by *P. terrestris* than others. While this suggestion requires further investigation, such crop species and cultivars could contribute to increase in inoculum in the soil between onion crops.

The lucerne and oat cultivars examined in this study were only a sample of the types of crops grown in rotation with onion, so it would be useful to extend this investigation to include other crops used in the Mid Murray region and other areas. A range of cereal crops, including cultivars of wheat, barley and triticale, and other crops, including cultivars of mustard, vetch and canola, are commonly rotated with onion. As it was not possible to examine a wider range of crop species and cultivars in rotation with onion in the field, *in vitro* and greenhouse experiments were conducted to examine additional potential alternative hosts of *P. terrestris*. These experiments will be discussed later.

Results obtained in the study of the management of nurse crop as a factor in increasing inoculum of *P. terrestris* in the soil were inconclusive. The experiment was designed to determine if the different nurse crop management practices often used in the field, could affect inoculum of *P. terrestris* in the soil and the number of plants with pink root. The incidence of *P. terrestris* in the roots of onion cv. Sandridge in the oat nurse crop treated with fluazifop-p did not differ from that detected in onion roots from the area treated with glyphosate at any sampling time. The same result occurred in the onion cv. White Spanish. In comparing the height of the two cultivars tested, seedlings

of cv. Sandridge treated with glyphosate were not significantly ($P > 0.05$) taller than those treated later with fluazifop-p, likewise, those of cv. White Spanish did not differ in height from either treatment site. As the nurse crop remained alive and hence able to host pathogens for longer when sprayed with fluazifop-p than with glyphosate, it was expected that if there were to be any difference in the amount of *P. terrestris* inoculum in the soil, that onions from the areas treated with fluazifop-p would result in more pink pigment on WSA than those from the glyphosate-treated areas. The data did not support this hypothesis.

This experiment provided little evidence to indicate that the type and timing of the herbicide to remove the nurse crop are factors in the amount of inoculum of *P. terrestris* in the soil and, hence, the degree of pink root in the onion crop. This may be because the time between the applications of the two herbicide treatments to the nurse crop was not long enough to detect any difference, particularly as growth of the nurse crop is primarily in the cold winter months when activity of *P. terrestris* is expected to be low (Davis and Henderson 1937). Therefore, it is unlikely that the different approaches in management of the nurse crop would affect the amount of *P. terrestris* in the soil. In this trial the nurse crop of oats was an unknown cultivar, but it is possible that different cereals or cultivars utilised as nurse crops may increase or decrease the amount of inoculum of *P. terrestris* in the soil and, hence, potentially cause more or less damage to the onion crop. Further work is necessary to determine which of the plant species suitable for use as nurse crops may be infected by *P. terrestris* and thus may contribute to the production of inoculum in the soil. Factors that need to be considered in planning future investigations in this area include determining if herbicide treatment, different species or cultivars as nurse crops, or interactions

between onion cultivars and the nurse crop, may affect amount of inoculum in the soil. Such work was outside of the scope of this project.

Many of the weed species commonly found in onion fields were shown to be likely to act as hosts of the pink root pathogen. *P. terrestris* was detected using Watson's method (1961) in all but one of the weed species sampled. This trial represented a preliminary investigation of weed species that might be hosts for *P. terrestris,* and would need to be repeated to determine with confidence which species are most likely to act as hosts of *P. terrestris*. Further work, including an investigation of a wider range of weed species and sampling at various times of the year from this property and others in the Bowhill area, as well as other regions, may determine which weed species are susceptible to infection by *P. terrestris,* and if they might contribute to inoculum in the soil and subsequent infection of onion roots.

The *in vitro* trial was established to allow preliminary screening before undertaking further greenhouse trials. This trial enabled the use of a large number of replicates to reduce variation within crop species and cultivars. In addition, infection of susceptible crop species or cultivars arose from direct contact with *P. terrestris* on PDA. The results of the *in vitro* trial indicated that some crop species and cultivars were more susceptible to infection by *P. terrestris* than others. For example, wheat cv. H45 produced more pink pigment on WSA than did oat cv. Swan in the *in vitro* trial. However, this result was not supported by the results of the greenhouse trial, indicating further research is necessary to provide conclusive results.

Overall the results from the *in vitro* trial indicated that all crop species and cultivars screened could act as hosts of *P. terrestris*, and all should be tested in further greenhouse trials. Also it was noted that for each crop or cultivar, the number of seedlings with pink pigmentation observed on roots incubated on cultures of *P.*

terrestris on PDA, in most cases, was not significantly different from the number of roots that yielded pink pigment on WSA. Onion cv. Patrick was an exception, in that fewer root systems produced pink pigment on PDA than on WSA, an observation that is similar to that discussed in section 5.4. The result for mustard cv. Nemcon, however, may mean that the pink discolouration of the roots on PDA was not due to the presence of *P. terrestris* in the roots of plants, as indicated by the smaller number of root systems that produced pink pigment on WSA than on PDA, again supporting the hypothesis that visual pinking on the roots of plants is not a reliable indication of the presence of *P. terrestris* as discussed in section 5.4.

The results from both the *in vitro* and the greenhouse trials indicated variation between crop species and cultivars in ability to act as hosts of *P. terrestris* as indicated by pink pigment on WSA. In both trials the oat and the barley cultivars appeared to be less susceptible to infection by *P. terrestris* than all the other crop types, whereas both of the canola cultivars and the lucerne were more susceptible.

Kreutzer (1941) screened a range of crops in a series of pot trials to identify alternative hosts of *P. terrestris*. Seeds were planted into soil naturally infested with the pink root pathogen, and it was found that oats, wheat and barley seedlings became infected and *P. terrestris* could be isolated from the roots. Isolation was achieved by incubation of roots on nutrient agar. There was no indication, from Kreutzer's work, of any differences in amount of infection in oats, wheat and barley, or which cultivars were used. Kreutzer (1941) also reported that alfalfa (lucerne) was not infected by *P. terrestris,* whereas in the current study, lucerne was found to be susceptible to infection by *P. terrestris*.

Latham and Watson (1967) amended soil by incorporating ground plants, including oats, barley, wheat and alfalfa (lucerne), into soil naturally infested with the pink root

pathogen (10% dry weight of soil). They found that the incidence of *P. terrestris* in soil amended with barley, wheat and alfalfa residues, in terms of the symptoms of pink root on the roots of onion grown in the amended soils, was significantly higher than that for oat residues. In comparison, the current greenhouse study showed that both oat and barley cultivars were generally less infected by *P. terrestris* than were wheat and lucerne cultivars. However, the methodology for detection of *P. terrestris* was different in the two studies, so a direct comparison is inappropriate.

The studies of Kruetzer (1941) and Latham and Watson (1967) produced results slightly different from the current study in terms of determining the crop species that were susceptible to *P. terrestris*. However, the present study raised the possibility that cultivars within a crop species may vary in susceptibility to infection by *P. terrestris* and this factor should be considered in the cultural management of pink root disease. Further investigation of the range of cultivars within crop species grown in rotation with onion is necessary to determine which crop species and cultivars are the least susceptible to infection by *P. terrestris* and, hence, would be suitable for use in areas infested with this pathogen.

This study supported previous reports that *P. terrestris* has a large host range (Punithalingam and Holliday 1973); (Sumner 2008b). Examination of lucerne and oat in the field, the nurse crop of oat used in onion, and of weeds growing within an onion field in the Mid Murray region, suggested that various plant species and cultivars may act as hosts for *P. terrestris.* The preliminary screening conducted in this study should be repeated, with more replicates to improve reliability. Whilst further investigation is necessary, oat and barley cultivars may be suitable as break crops between onion crops. The use of these crops in rotation with onion may help to reduce the incidence and severity of pink root.

7 General Discussion

The primary findings achieved in relation to the aims of this project were: 1. Confirmation that *P. terrestris* infects onion in the Mid Murray region of South Australia; 2. Development of potential new methods for isolation of *P. terrestris* from onion roots; 3. Demonstrating that cultivars marketed as tolerant of pink root disease were not significantly less susceptible to infection by *P. terrestris* than cultivars currently being grown in the region; 4 (a). Crop species varied in ability to act as hosts of *P. terrestris*, 4 (b). Many weeds common to the Mid Murray region were infected by *P. terrestris*; and 5. Pink pigmentation on roots was not a reliable indicator of the presence of *P. terrestris.*

Farmers in the area have known that pink root disease of onion was present in the Mid Murray region for years (Kevin Smith, personal communication, 2003). However, no scientific research had been reported to confirm the disease and identify the causal organism. Therefore, there has been little information available on which to base strategies for management of the disease. The isolation, identification and confirmation of *P. terrestris* as the causal organism of the disease in the region provided the basis for further work to be conducted in this project towards understanding and managing the disease.

None of the published methods for isolation of *P. terrestris* tested (Awuah and Lorbeer 1989; Ferreira 1990; Sneh *et al.* 1974) was consistently successful in this project. Netzer *et al*. (1985) utilised the method of Sneh *et al*. (1974) to isolate *P. terrestris* from soil, but with modifications, as they found that thiabendazole (TBZ) was not necessary to produce pink colonies. Porter *et al.* (1989), likewise, used the method of Sneh *et al*. (1974), but were not consistently successful in isolating *P. terrestris* from soil. Porter *et al.* (1989) found that when there were fewer than 1000 propagules of the

pathogen per g of soil, isolation was not possible, and also many plates were overgrown by other fungal species, such as *Fusarium* spp. and *Penicillum* spp. Due to the difficulty of isolating the pathogen in this study, the pathogenicity testing conducted was limited to the one isolate collected early in the project, and the remainder of the project focused on the presence of *P. terrestris* as indicated on WSA. However, the new methods for isolation of *P. terrestris* that were developed during the course of this study may, perhaps with further development, facilitate further research to examine the presence, diversity and virulence of *P. terrestris* in different regions of Australia. The information gathered would provide an insight into a poorly understood disease in Australia.

Kulik and Tims (1960a) showed that *P. terrestris* isolates collected from the same area or field can be diverse in many features, including virulence and production of pycnidia. Likewise, Ferriera (1991) examined the variability of *P. terrestris* isolates from South Africa and the United States of America, in terms of isozyme polymorphism, cultural characteristics and virulence on onion and found that there was extensive variation in the pigment of the culture and the ability to form pycnidia in culture. Similarly, Biles *et al.* (1992) identified high variability in a selection of *P. terrestris* isolates from Texas and New Mexico in terms of variation in microsclerotium production *in vitro.* The ability to collect large numbers of isolates from soil would allow testing, preferably involving molecular techniques, for such diversity within an area or field. This information would enhance an assessment of the variation that exists among isolates, in terms of pathogenicity on onion, from the Mid Murray region of South Australia. Also, further studies could be conducted to investigate susceptibility of various onion cultivars or alternative hosts, by exposing the plants to the most pathogenic strains present. Furthermore an examination of eradication strategies could

be undertaken, as isolates may vary in their reaction to fumigants or other methods of control.

In research prior to the current project, in which naturally infested soil from onion fields was used to evaluate cultivar susceptibility or examine control measures for *P. terrestris* (Hartz *et al.* 1989; Coleman *et al.* 1997; Sumner *et al.* 1997; Alberto *et al.* 2002), the inoculum in the soil was not quantified. The method developed by Sneh *et al*. (1974) as described in section 1.4.3 and 4.2.1 does not appear to have been adopted widely and there is evidently a need to develop and standardise a method for quantification of *P. terrestris* inoculum in the soil. If the quantity of inoculum in the soil were known, results would be more widely applicable and a comparison between studies would be possible. Porter (1989) had some success quantifying *P. terrestris* in the soil with the method of Sneh *et al*. (1974) when the number of propagules per gram of soil was over 1000, and further examination and modification of these techniques may help develop a standard method to assay *P. terrestris* in the soil. The information that could be generated with reliable methods for quantification of *P. terrestris* in the soil may facilitate examination of field management practices, selection of cultivars and other crop species for resistance or tolerance or test efficacy of potential control measures.

A standard, quantitative method to produce inoculum of *P. terrestris* could not be found in the literature, suggesting a need for a method to be developed. Although various techniques have been used to produce inoculum for experiments (Kulik and Tims 1960a; Kehr *et al.* 1962; Netzer *et al.* 1985; Mao *et al.* 1998), no standard method has been adopted. In the current project, a method published by Lacy and Roberts (1982), involving the colonisation of wheat seed with the pathogen, was utilised without success, as the growth of the onion appeared to be improved by the presence of the

wheat seed in soil rather than decreased due to the presence of the pathogen (data not presented). Evaluation of a range of published methods should be conducted to develop a method to produce inoculum based on known and quantifiable propagules such as conidia. However, the inability to produce pycnidia of *P. terrestris in vitro* experienced in this study would make this process difficult.

Symptoms of pink root have been prevalent on onion in the Mid Murray region for many years (Kevin Smith, personal communication, March 2003) and one approach that has been implemented in the past to minimise disease, is to sow cultivars that are marketed as tolerant of pink root in fields where pink root is likely to be a problem. However, the results of this study, although limited to the few cultivars tested, showed that the cultivars marketed as tolerant of *P. terrestris* were just as susceptible to infection as other cultivars commonly grown in the area. Kehr *et al*. (1962) found that screening cultivars for resistance to infection by *P. terrestris* was more effective in differentiating resistant and susceptible responses at temperatures closer to 20ºC than at 28ºC. However, if lower temperatures were necessary for the expression of resistance, this would not necessarily translate to resistance in the field in South Australia, where temperatures routinely exceed 28ºC during the crop growth. It is possible that pink root tolerant onion cultivars developed for early to mid winter sowing escape infection by having most of their growth in the relatively cooler, late winter - early spring months. However, screening of spring-sown cultivars, that have a long period of growth in the hot summer months, would need to be conducted at warmer temperatures to ensure that expression of resistance could be detected, and that resistance or tolerance of infection by *P. terrestris* would occur in the field.

Onion cultivars, globally, are bred for specific climatic and soil conditions and research is relevant mainly for local cultivars (S. Nicola, personal communication, 2007). For

example, Alberto *et al.* (2002) and Coleman *et al.* (1997) conducted experiments to screen local cultivars of onion in Nueva Ecija (The Philippines) and New York (USA) respectively, for susceptibility to local isolates of *P. terrestris*. Likewise, at the 5th International Society for Horticultural Science Symposium on Edible Alliaceae held at Dronten, the Netherlands (2007), many presentations concerning onion involved local cultivars suitable for the area in which the research was conducted (Nicola 2007; Sorenson 2007; Seetohul 2007). The cultivars of onions and other crops used in this study were either currently used in the Mid Murray area of South Australia, or selected to be suitable for the area. The results of this study, therefore, are relevant to the Mid Murray area, and further screening is required to identify local cultivars suitable for other onion growing areas where pink root is a problem. The screening process would involve collecting local isolates of *P. terrestris*, inoculating soil and using pot trials with a range of local onion cultivars to assess susceptibility. Standardising inoculum procedures and refining isolation techniques would assist in such experiments.

Another management strategy to minimise pink root disease is to use long rotations between onion crops, which is a standard practice employed to prevent or reduce pests and diseases in a range of crops (Agrios 1997). However, as *P. terrestris* is known to have a wide range of hosts (Sumner 2008b), it is likely that long rotations would have little effect in reducing inoculum in the soil between onion crops, particularly in soils heavily infested with *P. terrestris*. The results from this study provide preliminary information that many of the crops commonly grown in rotation with onion in the Mid Murray region act as hosts of *P. terrestris*, but to varying degrees. Future research must include investigation of the susceptibility to *P. terrestris* of a wide range of crop types and cultivars, particularly using standard methods for inoculation and quantification, as discussed earlier.

Similarly, the preliminary results from this study indicate that various weeds have the ability to act as hosts of *P. terrestris.* However, as *P. terrestris* was not detected in all of the weed species sampled, it appears that weeds differ in susceptibility to infection. Extensive sampling and screening of a wide range of weed species for susceptibility to infection by *P. terrestris,* using a larger number of replicates than utilised in this project, is required in order to make recommendations for commercial use.

Fumigation with metham sodium reduced the incidence of pink root in onion plants in this study. Whilst eradication of the pathogen from the soil was not achieved, infection was reduced when soil was treated with the recommended rate of metham sodium. However, metham sodium is not widely used in commercial agricultural practice, particularly in large, broad acre plantings, as fumigants are dangerous, difficult to apply, and not always commercially viable unless used in a high value crop where high disease pressure is expected. There is a need to investigate alternative treatments that can be used to control pink root in commercial onion cropping situations.

The solarisation of soil to reduce the inoculum of *P. terrestris* has been tested in conjunction with the use of a fumigant on onions (Hartz *et al.* 1989; Porter *et al.* 1989). Hartz *et al*. (1989) found solarisation of soil to be as effective as fumigation in controlling pathogens but only when the season was warm enough to generate temperatures of >60ºC in the top 2cm of soil for more than 120 cumulative hours, compared to a cooler season where the maximum temperature reached in the top 2cm was 59ºC but prolonged exposure was only >40ºC. Likewise, Porter *et al*. (1989) found that the average maximum air temperatures in southern Victoria, Australia, in the summer that the trial was conducted, were 4ºC cooler than the long term average of approximately 27ºC and were a factor in the lack of effectiveness of the treatment in eradicating *P. terrestris* without the use of fumigation as well. However, Porter *et al.*

(1989) did not consider the combined treatment of solarization and fumigation to be economically viable in commercial onion crops for control of *P. terrestris* populations unless other pests and diseases were to be targeted as well. Previously, Katan *et al.* (1980) found that in Israel solarisation alone was effective, and that reinfestation of soil by pathogens was delayed after treatment due to the shift in the biological balance in favour of antagonists of the pathogen. The effectiveness of solarisation of soil to prevent disease is therefore likely to depend on the climatic conditions of the region, and this method is only suitable for use in consistently warm onion growing regions. Also, the economic benefit of solarization would need to be considered in conjunction with the potential value of the crop, the predicted risk of disease and the practicalities related to the use and disposal of large quantities of plastic.

The use of biofumigants, where incorporation of some *Brassica* crops into the soil as green manure has the potential to release toxic isothiocyanates, may suppress certain pest and diseases in the soil (Kirkegaard and Sarwar 1998). Some tests have been conducted in onion fields at the trial site in Bowhill, where *Brassica* crops have been used as green manure, and although there was anecdotal evidence to suggest an improvement in the stand of onions that followed such treatment (Kevin Smith, personal communication, March 2003), the effects of the treatment were not evaluated scientifically. Biofumigation is likely to be a viable and practical treatment (Matthiessen *et al.* 2000) and, if proved to be effective against pink root and other diseases and pests in onion crops, would be a management tool that would be utilised by growers. However, in this study, canola cvs 501TT and Karoo, mustard cv. Nemcon and the weed *B. tournefortii* were found to act as hosts of *P. terrestris,* and Kruetzer (1941) found that *P. terrestris* invaded the roots of *B. oleracea*. Therefore, screening of

Brassica cvs specifically for susceptibility to infection by *P. terrestris* with potential for use as a biofumigant crop is recommended.

Visual observation of pink pigment on roots of plants to indicate presence of *P. terrestris* was unreliable. In this study, *P. terrestris* was often present in onion roots even when no pink pigment was observed on fresh roots. Likewise, other researchers have reported that observation of pink pigment on onion roots often underestimates the amount of infection (Kreutzer 1939; Siemer and Vaughan 1971; Coleman *et al.* 1997; Rabinowitch *et al.* 1981; Biles *et al.* 1992; Alberto *et al.* 2002), whereas others believe that the observation of pink pigment on roots provides a good indication of the presence of *P. terrestris* in the roots of plants (Hartz *et al.* 1989; Porter *et al.* 1989). However, Hartz *et al*. (1989) evaluated pink root close to maturity of the onion plants, and Porter *et al*. (1989) found that plants examined less than 6 weeks after sowing often had no visible signs of pinking on the roots even when *P. terrestris* was present, but after this stage of growth the correlation between pink pigment observed on roots and the presence of *P. terrestris* in those roots was good. In this project it was observed that pink roots were abundant on senescing roots of mature bulbs (data not presented), but during active growth of the plants *P. terrestris* was frequently detected in roots using WSA where no pink pigment on fresh roots had been observed, whereas pink pigmented onion roots always gave rise to pink pigment on WSA.

As the presence of *P. terrestris* in roots is not reliably determined by visual observation of roots, there is a need to develop new methods to assay roots for infection by *P. terrestris*. Likewise, the ability to assay soil for the presence of *P. terrestris* would aid in field selection and help reduce the impact of the disease. DNA-based testing methods are available for some pests and pathogens of cereal crops through the Root Disease Testing Service offered by The South Australian Research and Development Institute,

and the possibility of extending the service to Australia's horticultural industries has been discussed (Ophel-Keller *et al.* 2008). The Australian Potato industry has shown an interest in the technology, but a major barrier to the commercial development of such tests in horticulture is the relatively small size of the Australian market (Ophel-Keller *et al.* 2008). However, there is potential to increase the size of the market if tests could be developed for pathogens that are common in several horticultural crops, such as those already developed for *Meloidogyne* spp.(Ophel-Keller *et al.* 2008). Development of a targeted DNA assay might provide a reliable means of detecting and quantifying *P. terrestris* in roots and soil.

It was intended to examine the relationship and potential effect on onion of the presence of both *P. terrestris* and *F. oxysporum* f. sp. *cepae*. Kehr *et al*. (1962) found that yield loss resulting from infection by both pathogens was greater than that caused by either pathogen alone. Lacy and Roberts (1982) found that *F. oxysporum* f. sp. *cepae* appeared to have more negative effect on yields than *P. terrestris*, but that view was not supported by Porter *et al*. (1989). *F. oxysporum* f. sp. *cepae* has been found in the Mid Murray area of South Australia (Smith 1999), and the potential interaction and effect on yield of onion should be investigated. However, a pre-requisite for further research is the development of suitable methods for isolating, identifying and quantifying *F. oxysporum* f. sp. *cepae*.

Pink root disease of onion has become a major problem in mainland Australia (Tony Gurcuillo, Peter Ivankovich, Kevin Smith, personal communication, February 2008). Results from this study indicate that there is still much to be learned before management recommendations can be made. The development of new techniques to isolate *P. terrestris*, presented here, will facilitate further research to develop better disease management practices for the onion industry in Australia.

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