ASPECTS OF THE BIOLOGY, EPIDEMIOLOGY AND CONTROL OF *RHIZOCTONIA SOLANI* (KÜHN) ON POTATO

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ABSTRACT

Aspects of the biology, epidemiology and control of *Rhizoctonia solani* from potato were investigated using a range of laboratory and field-based experiments. *In vitro* experiments revealed nutritional factors including a range of carbon sources, and inorganic and organic nitrogen did not affect significantly mycelial growth or sclerotial germination. Carbon and nitrogen sources including cellobiose, glucose, glycerol and potassium nitrate significantly increased sclerotial biomass production *in vitro*. Mycelial growth, sclerotial production and germination occurred over a temperature range of 10 – 30°C, with an optimum of 25°C for both AG 2-1 and AG 3 isolates. Mycelial growth and sclerotial germination occurred at pH 4 – 9 with an optimum of pH 5.6, whereas sclerotial production occurred between pH 4 – 6 for AG 2-1 isolates and pH 4 – 8 for AG 3 isolates. Mycelial growth, sclerotial biomass production and germination declined with decreasing osmotic, matric and soil water potential, with mycelial growth prevented between –3.5 MPa and –4.0MPa on osmotically adjusted media, at –2.0 MPa on matrically adjusted media and –6.3 MPa in soil. Sclerotial production ceased prior to the limits for mycelial growth and germination for all isolates, between –1.5 MPa and –3.5 MPa on osmotically adjusted media and –1.5 MPa on matrically adjusted media. AG 3 isolates produced significantly more well-formed sclerotia during all *in vitro* experiments compared to the loosely constructed sclerotia produced by AG 2-1 isolates.

A pathogenicity bioassay, coupled with staining and microscopic examination of stem tissues, showed all AGs formed infection cushions as a prerequisite to infection, with clear differences in the extent of infection cushion formation and subsequent stem lesion severity. AG 2-1 produced small, infrequent infection cushions, causing stem lesions only 1 – 2 mm in length which did not increase in size or severity after initial formation. AG 3 was significantly more pathogenic than AG 2-1 and AG 5 isolates, however, both AG 3 and AG 5 isolates both produced extensive infection cushions,

which coincided with extensive stem lesion formation, resulting in sunken brown lesions and ultimately stem pruning and death of the developing plant. *In vitro* enzyme assays revealed that isolates of all AGs produced a range of cell-wall degrading enzymes, including cutinase, endo-1,4-β-glucanase and polygalacturonase.

Sclerotial viability declined under field conditions over 18 months regardless of the method of sclerotial production, soil type or burial depth. Burial depth (5, 10 and 20 cm) had no significant effect, whereas sclerotial viability was up to four times lower in a sand (Dreghorn series) compared to a loam-sand (Bargour series) soil. Sclerotial infection by potential antagonistic fungi was not a contributory factor in the decline in sclerotial viability in this study. Increased soil-borne inoculum density was found to increase disease incidence and severity on stems, stolons and progeny tubers whether incorporated into soil as mycelial or sclerotial inoculum. A combination of planting population and soil-borne inoculum density significantly affected black scurf incidence and severity, however, there were no clear patterns of disease development on progeny tubers.

Potential antagonistic fungi isolated from sclerotia harvested during sclerotial viability studies and known antagonists grown in dual culture with *R. solani in vitro*, including *Verticillium biguttatum* and a *Gliocladium* sp. both reduced radial mycelial growth rates and sclerotial biomass production of *R. solani* AG 3. Similarly, antifungal volatiles produced by the same isolates prevented sclerotial biomass production. Sclerotial germination was reduced by antifungal volatiles produced by *Gliocladium* sp., *Penicillium* sp., *Mauginiella* sp. and *Truncatella angustata*. Spore suspensions of all the above isolates applied to sclerotia infested soil did not protect against stem canker and black scurf formation in a glasshouse trial. Pot trials to test novel control treatments revealed neem cake, and commerical products containing garlic extract, *Trichoderma* sp. and *Gliocladium catenulatum* reduced black scurf formation on progeny tubers, but not as effectively as the currently available fungicide azoxystrobin.

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PUBLICATIONS

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CONTENTS

LIST OF ABBREVIATIONS

ADAS	Agricultural and Development Advisory Service
AG	anastomosis group
ANOVA	analysis of variance
C:N ratio	carbon to nitrogen ratio
cv	cultivar
d	day(s)
diam	diameter
DF	degrees of freedom
h	hour(s)
HRI	Horticulture Research Institute
LSD	Least significant difference
MYA	Malt yeast extract agar
MPa	megapascals
PDA	potato dextrose agar
SEA	soil extract agar
SAC	Scottish Agricultural College
spp.	species
WA	water agar

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CHAPTER 1

THE BIOLOGY, EPIDEMIOLOGY AND CONTROL OF *RHIZOCTONIA SOLANI* ON POTATO

1.1 The potato crop in the UK

The arrival of the potato from South America to mainland Europe and the UK is estimated to have occurred in the late 16th Century (Salaman, 1989). The exact date is unclear, with the first record of potatoes being sold in Spain dating back to 1570, and the first record of potatoes in the UK from 1596 (Salaman, 1989). In Scotland, potato production started to increase dramatically between 1795 and 1840 in response to growing industrialisation and urbanisation, with greater demands for food supplies (Salaman, 1989). Potato production accounted for over 3 % of the total land in the UK used for growing crops in 2003 (Anon, 2004a). In 2003, 124,800 hectares of potatoes were planted in the UK, down from 138,700 hectares in 2002. Over a fifth of the total area for planting potatoes in the UK is in Scotland (Nix, 2003). From the total land planted with potatoes in 2002, 61.9 % was planted with main crop varieties, 34.0 % with second early varieties and the remaining 4.1 % first early varieties. For England and Wales, the percentage of land planted with the same 3 categories of potatoes was 65.0 %, 29.8 % and 5.2 % (Nix, 2003). An average of 44.0 tonnes of potatoes ha^{-1} were produced in 2002 and 40.7 tonnes in 2003, with prices for early and maincrop potatoes averaging £132.50 and £99.10 per tonne in 2003, respectively (Anon, 2004a).

In 2003, it was reported that between 70 and 85 % of seed tubers were infected with *R. solani* and was estimated the cost to growers in losses was between £50 and £75 million a year (Anon, 2003). The control of *R. solani* is also important to the processing and fresh markets, with the UK now consuming these products in almost equal proportions. The ability for growers to control *R. solani* is vital to meet the quality specifications required for both markets, as sclerotia infested and misshapen tubers can reduce the value of a crop to as little as £10 to £20 per tonne (Anon, 2003).

1.2 The pathogen - *Rhizoctonia solani*

Rhizoctonia solani Kühn [teleomorph *Thanatephorus cucumeris* (Frank) Donk] was initially observed on diseased potato tubers almost 150 years ago by Julius Kühn in 1858 and was subsequently found to be a destructive and widespread fungal pathogen of many plant species (Menzies, 1970). The fungus targets the underground stems and stolons of the developing plant, causing brown sunken lesions known as cankers (Plates 1.1a and 1.1b). This can cause tuber development to be disrupted, with misshapen tubers a common occurrence in *R. solani* infected crops (Hide *et al.*, 1973; Anderson, 1982; Jeger *et al.*, 1996). The presence of survival structures called sclerotia (black scurf) as well as russetting (elephant hide) on the tuber skin are also indicative of *R. solani* infection (Plates 1.1c and 1.1d) (Secor & Gudmestad, 1999; Carling & Leiner, 1986). Sources of *R. solani* infection include seed tubers covered in sclerotia and mycelium as well as the soil itself, which can contain different types and densities of inoculum (Jager *et al.*, 1991).

1.2.1 Inoculum sources

The importance of seed-borne inoculum in causing disease has been demonstrated in many studies. The use of sclerotia-infested seed has been demonstrated to reduce plant emergence by almost a quarter compared to using seed visibly free of *R. solani* (Carling *et al.*, 1989). Stem canker severity was also greater when seed tubers infested with sclerotia were planted (Carling *et al.*, 1989). Different densities of inoculum on the tuber surface have also been demonstrated to affect stem canker development, with low inoculum densities resulting in negligible levels of stem infection and high inoculum levels resulting in severe stem infection (Simons & Gilligan, 1997a). Similar experiments comparing the use of sclerotia-free seed with seed with 20 % sclerotial coverage showed that stem canker was more severe in the latter

a

b

c

d

Plate 1.1. Disease symptoms caused by *R. solani* on potato. a) stem canker; b) stolon canker; c) black scurf (sclerotia) and d) russetting (elephant hide).

(Gudmestad *et al.*, 1979). Stolon numbers can be reduced by high inoculum densities of *R. solani*, resulting in an increased frquency of oversized tubers on infected plants (Simons & Gilligan, 1997b). The planting of sclerotia-infested seed pieces can also significantly reduce tuber yield (Banville, 1989; Carling *et al.*, 1989).

Soil-borne *R. solani* is another important inoculum source, and increases in mycelial soil-borne inoculum density cause increases in stem canker incidence and severity (Kyritsis & Wale, 2002a). Severe stem canker infection caused by soil-borne *R. solani* has caused reductions in tuber yields and increased proportions of misshapen tubers. Five year rotations have been shown to reduce the percentage of severely infected plants to negligible levels compared to continuous potato cropping, where increases in the proportions of misshapen and undersized tubers were found (Scholte, 1989). Soil inoculum densities of *R. solani* decline when potatoes are not cultivated, however, the introduction of another crop susceptible to *R. solani*, for example sugar beet, can result in a rapid increase in soil inoculum (Gilligan *et al.*, 1996). Recent research has shown that the severity of stem canker and black scurf is higher when high densities of both seed and soil borne inoculum were present, compared to low levels. This illustrates the additive effect of these sources on disease severity, and highlights the need to control both seed and soil-borne inoculum (Tsror & Peretz-Alon, 2005). Much of the research into *R. solani* has foccussed on the effect of mycelial inoculum in soil or sclerotia on seed tubers, without considering soil-borne sclerotia as a possible inoculum source.

1.2.2 The lifecycle of *R. solani*

The disease cycle of this pathogen consists of two stages; a rarely observed basidiomycetous perfect stage where the teleomorph is known as *Thanatephorus cucumeris* and an imperfect stage known as *Rhizoctonia solani*, where the fungus survives in the soil as a sterile mycelium (Figure 1.1) (Parmeter & Whitney, 1970).

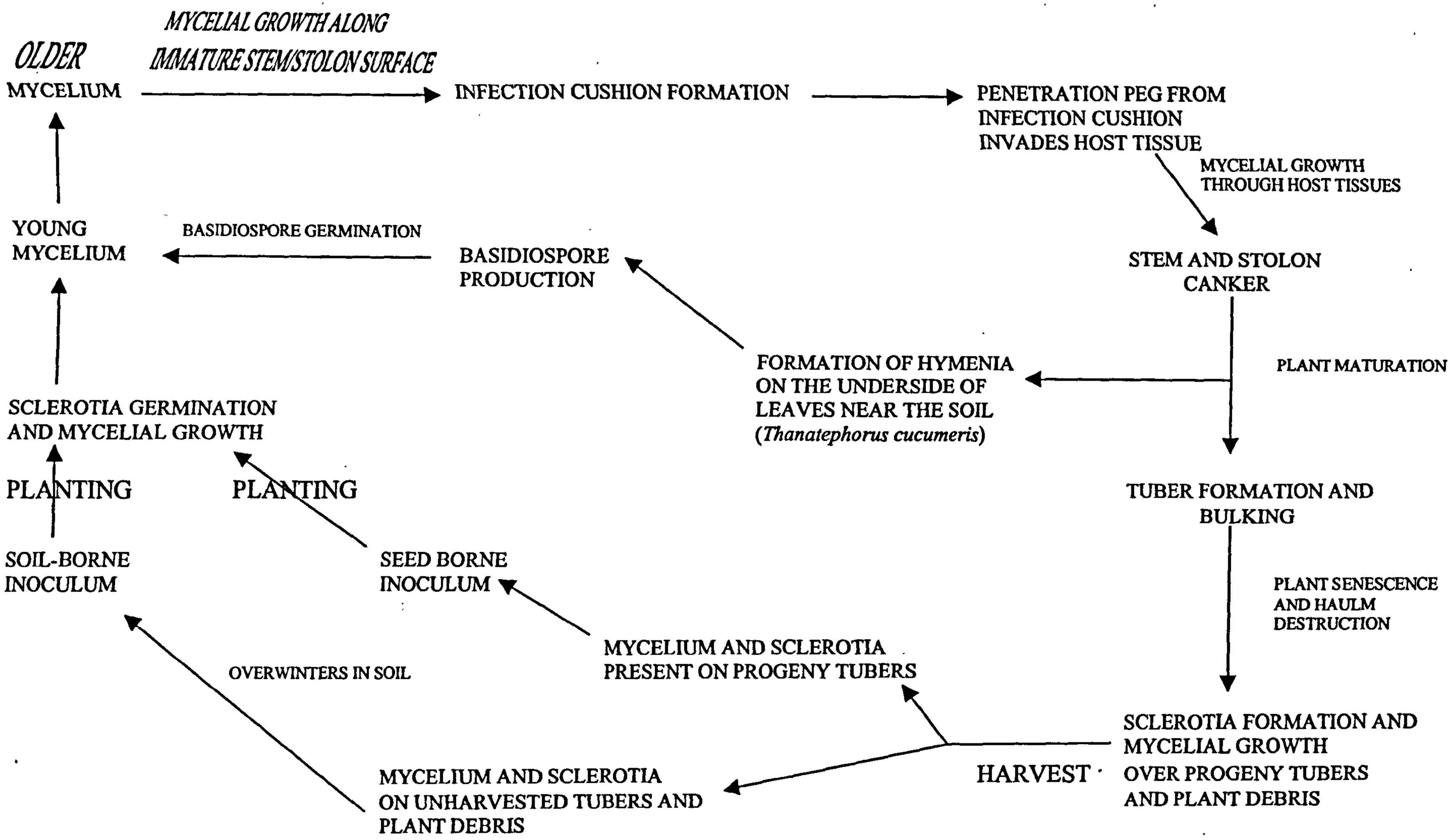


Figure 1.1. Life cycle of *Rhizoctonia solani* Kühn [teleomorph *Thanatephorus cucumeris* Frank (Donk)] on potato. (adapted from Agrios, 1996).

The perfect stage *T. cucumeris* is rarely observed in the laboratory or nature, so the production of basidiospores is not used as a diagnostic feature of *R. solani* (Agrios, 1996). The current species concept of *R. solani* outlines the main characteristics used to distinguish it from other fungi, with the basis for taxonomic classification derived from the morphology of the sterile mycelium in culture as well as hyphal cytomorphology (Sneh *et al.*, 1991; Parmeter & Whitney, 1970).

1.2.3 The perfect state – *Thanatephorus cucumeris*

When exposed to certain environmental conditions, some isolates of *R. solani* will produce the teleomorph, *T. cucumeris*, which is characterised by the production of basidiospores (Talbot, 1970). The hymenium consists of basidia produced on mats of interwoven branched hyphal cells, most often located on the lower aerial parts of the host plant near the soil surface (Sneh *et al.*, 1991). Sterigmata are produced from the basidia, with numbers ranging from 1 to 7 observed, and basidiospores produced at the tips of the sterigmata (Sneh *et al.*, 1991). A combination of these characters, as well as a more detailed description of the hymenia, including the size and shape of basidia, sterigmata and basidiospores are required to distinguish *T. cucumeris* from morphologically similar basidiospore-producing fungi (Talbot, 1970; Sneh *et al.*, 1991). Hymenial derived isolates of *T. cucumeris* removed from potato stems have been found to cause disease, but the predominant form causing disease symptoms on potato is the imperfect state, *R. solani* (Carling & Leiner, 1990b).

1.2.4 The imperfect state – *R. solani*

In general, *R. solani* hyphae can be identified by pale/dark brown hyphal pigmentation, branching near the distal septum of young hyphal cells and the constriction

of branch hyphae at the origin, where the branch attaches to the main hyphae (Plate 1.2). Septum formation occurs near the origin of hyphal branches, with the presence of dolipore septa and multinucleate cells in young, actively growing hyphae characteristic of *R. solani*. Branch hyphae form at 45° and 90° angles to the main hyphae (Parmeter & Whitney, 1970; Sneh *et al.*, 1991; Butler & Bracker, 1970). Other features often present in isolates of *R. solani*, but not universal, include a rapid growth rate, the presence of monolloid cells and sclerotia, with many strains exhibiting varying levels of host specificity and pathogenicity (Sneh *et al.*, 1991). Monolloid cells, also called barrel-shaped cells, are formed from buds or at the ends of pre-existing cells, and can form into infection cushions and sclerotia. Characteristics never present in a fungus belonging to the genus *Rhizoctonia* include clamp connections, conidia, rhizomorphs and sclerotia that are differentiated into a rind and medulla (Sneh *et al.*, 1991).

1.3 Stem infection and sclerotia production

1.3.1 Stem infection

Rhizoctonia solani infects developing sprouts and stolons on potato plants, causing brown lesions (stem and stolon canker). Damage to potato plants by *R. solani* occurs primarily beneath the soil surface, where young subterranean and meristematic tissues are particularly susceptible to attack (Banville *et al.*, 1996). Prior to infection, *R. solani* makes contact with the plant and runner hyphae grow longiditudinally along the sprout, following the junctions between the epidermal cell walls (Hofman & Jongebloed, 1988). After this initial contact, the original hyphae produce primary mycelial branches consisting of swollen cells shorter than those observed in the original hyphae. Secondary branches develop from these primary branches, which themselves branch again several times to form infection cushions (Chand *et al.*, 1985; Hofman & Jongebloed, 1988).

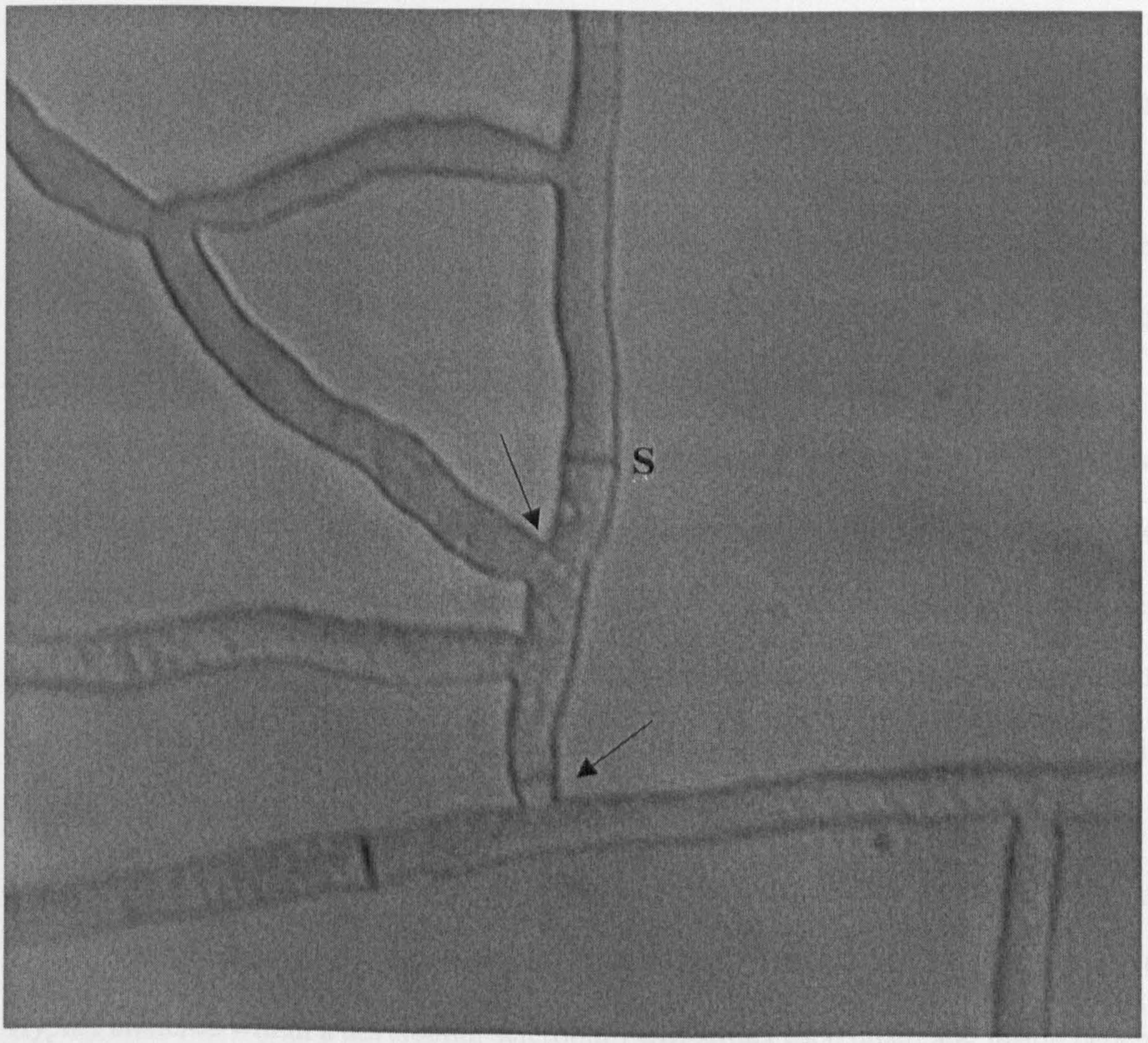


Plate 1.2. Hyphae of *R. solani* showing the characteristic constriction at the ends of hyphal branches (➔) and clearly visible septa (**S**) separating the hyphal cells. (x 132 magnification).

1.3.2 Sclerotia formation

Sclerotia are asexual, vegetative structures that are produced by fungi to allow survival during conditions unsuitable for mycelial growth and conserve mycelial nutrients until conditions are more suitable (Deacon, 1984; Townsend & Willets, 1954; Chet & Henis, 1975). Sclerotia are generally divided into two types, those that produce a rind and those that do not (Coley-Smith & Cooke, 1971). *R. solani* produces the latter, a loosely developed sclerotium consisting of knots of melanised hyphae with no thickening of hyphal walls at the periphery to form a rind or any cellular differentiation at all within the sclerotium (Webster, 1980; Townsend & Willets, 1954; Deacon, 1984).

Sclerotial initials can form from older elongate hyphal cells as well as newly grown cells (Sanford, 1956). These cells are shorter as well as broader (20-22 μm) compared to ordinary vegetative hyphal cells (7-8 μm). Extensive septation of these cells results in short barrel-shaped cells (30-35 μm long) containing vacuoles and dense cellular contents (Townsend & Willets, 1954). Most sclerotial initials will form along vegetative hyphae, and increased and localised irregular branching of ordinary mycelium results in numerous septa. This provides the basis for the formation of the undifferentiated and loosely constructed sclerotia characteristic of *R. solani* (Hudson, 1986; Sherwood, 1970). Hyphal branches within a developing sclerotium will attract each other, and fusion between these branches increases the mechanical strength of the tissues and assists nutrient flow throughout the entire sclerotium (Hudson, 1986). The continued branching and dividing of sclerotial initials results in the aggregation of hyphal cells, and the immature pale sclerotium begins to darken and become red-brown in colour (Townsend & Willets, 1954).

Mature sclerotia are composed primarily of loosely arranged chains of monilliod cells that contain no vacuoles, and this arrangement results in the production of a soft, homogeneous, loosely constructed sclerotium (Hudson, 1986; Townsend & Willets, 1954;

Butler & Bracker, 1970; Webster, 1980). The central section of the sclerotium is a pseudo-parenchymous mass of hyphae, however, closer to the outer edge of the structure, the hyphae have a loose cellular arrangement (Townsend & Willets, 1954). Immature sclerotia are initially white, turning to brown as they mature, with the centre of sclerotia often darker than the surface (Butler & Bracker, 1970).

Sclerotial initials formed *in vitro* tend to form around the site where the agar plate was inoculated, but mature sclerotia have been found to develop at random over the plate surface (Sanford, 1956). When found on potato tubers, sclerotia tend not to penetrate more than 5-6 cell layers into the periderm, which can be between 8-14 cell layers thick (Chand & Logan, 1984). The size of individual sclerotia varies as differences existing between natural and laboratory conditions affect formation, with a range of sizes observed from < 1 mm to several millimetres in diam (Banville *et al.*, 1996). Developing sclerotial crusts *in vitro* have been found to exude large brown droplets of liquid (Dijst, 1988).

The exact triggers for sclerotial formation are unknown, but it has been observed that after haulm destruction or when the mother plant begins to senesce, the infestation of progeny tubers by sclerotia is initiated (Banville *et al.*, 1996; Dijst *et al.*, 1986). Sclerotia can also form on the root systems of potato plants (Tsror & Peretz-Alon, 2005). The *in vitro* production of sclerotia on agar media was found not to be initiated until the mycelium had reached the edge of the plate, and it was therefore thought that the termination of linear hyphal growth was a trigger in the formation of sclerotia (Allington, 1936). Subsequent research revealed that this is not a universal feature of all *R. solani* isolates, and that sclerotia development will occur prior to this (Dijst, 1988). Chemical substances such as adenosine 3', 5'-cyclic monophosphate (cyclic AMP) have been implicated in controlling the regulatory events that induce sclerotia development in *R. solani* isolates pathogenic to

rice (Hashiba & Ishikawa, 1978), but it is not known whether this is a universal feature of *R. solani* isolates.

1.4 Anastomosis groups and pathogenicity

Rhizoctonia solani consists of many different sub-groups of closely related fungal isolates, and early attempts to place isolates into intra-specific groups were based on variations in pathogenicity, cultural appearance and morphology, physiology and ecology (Chand & Logan, 1983; Neate & Warcup, 1985; Ogoshi, 1987). Hyphal anastomosis has been used to determine intra-specific groups within *R. solani* known as anastomosis groups (AGs). Genetically similar isolates will recognise and fuse with other isolates, whereas genetically unrelated isolates will not (Ogoshi, 1987; Parmeter & Whitney, 1970). The grouping of the different strains of *R. solani* is necessary as sub-groups can vary in host specificity and the severity of infection they cause, and these groups are indistinguishable using colony morphology alone (Sherwood, 1969).

1.4.1 AG determination

AGs have been determined traditionally by placing an isolate of a known AG of *R. solani* opposite an unknown isolate on agar media, and observing the presence/absence of hyphal anastomosis observed at the interaction zone of the colonies (Parmeter *et al.*, 1969). This conventional method for AG determination is labour-intensive and time-consuming, especially as there are currently 13 different AGs of *R. solani* (Carling *et al.*, 2002; Thornton *et al.*, 1993). Grouping of *R. solani* isolates into AGs is based on the hyphal anastomosis reaction between an isolate from a known AG and an unknown one, and determines whether or not the isolates are closely related genetically.

1.4.1.1 Determination of AGs using hyphal anastomosis

Hyphal fusion is divided into four categories: perfect (C3), imperfect (C2), contact (C1) and no interaction (C0) (Sneh *et al.*, 1991; Cubeta & Vilgalys, 1997). Genetically similar isolates of the same AG will undergo perfect fusion (C3), with complete fusion of the cytoplasm and cell walls occurring between opposing isolates. Imperfect fusion or the "killing" reaction (C2) is observed when the hyphal walls fuse, with plasmolysis of anastomosing cells and surrounding cells also evident. This indicates that the isolates belong to the same AG, but are genetically distinct within that group. When hyphal contact is observed between the fungal walls, and there is no penetration of the walls or membrane-membrane contact (C1), this indicates that the genetic relationship of the two isolates is distinct and the isolates probably belong to a different AG. No hyphal contact (C0) shows that the two isolates are genetically distinct and belong to different AGs (Anderson, 1982; Ogoshi, 1987; Sneh *et al.*, 1991; Cubeta & Vilgalys, 1997).

1.4.1.2 Biochemical and molecular methods to determine AG

Many different biochemical and molecular techniques have been used to demonstrate the genetic differences that exist within *R. solani* (Balali *et al.*, 1996). Research investigating random fragment length polymorphisms (RFLPs) within *R. solani* demonstrated that significant variation exists in ribosomal DNA of isolates both between and within AG (Vilgalys & Gonzalez, 1990; Balali *et al.*, 1996; Balali *et al.*, 1996). Similar genetic differences have been demonstrated by examining pectic zymograms of *R. solani* isolates taken from various agricultural regions of South Australia (Neate *et al.*, 1988). Much of this research has focussed on the development of immunological, DNA fingerprinting, DNA probes and PCR based techniques for the identification of different AGs of *R. solani*.

Several studies have been undertaken to determine whether individual AGs can be determined serologically. A monoclonal antibody-based immunological assay was developed to detect the presence of live *R. solani* propagules in the soil, but the antibodies were not AG specific (Thornton *et al.*, 1993). Monoclonal antibodies have been developed to distinguish AG 8 isolates from other AGs but were also found to weakly hybridise to isolates representing AG2-1, AG 3 and AG 4. Although the antibodies were cross-reactive within *R. solani*, the proteins detected by the antibodies in AG2-1, AG 3 and AG 4 isolates all had molecular weights different to those representing AG 8, which could be used to identify intraspecific groups (Matthew & Brooker, 1991).

Analysis of ribosomal DNA restriction fragment length polymorphisms between different AGs of *R. solani* revealed that AG 3, AG 4, AG 7, AG 8 and binucleate isolates could be characterised by unique restriction fragment patterns (Vilgalys & Gonzalez, 1990). Other DNA hybridisation techniques have revealed differences within AGs that could not have been distinguished via conventional hyphal anastomosis techniques (Carling & Kuninaga, 1990). The discovery of these genetic differences have lead to research into the development of DNA probes to specifically identify individual AGs of *R. solani*, which would contribute to epidemiological and control strategies (Matthew *et al.*, 1995). A DNA fingerprinting probe prepared from an isolate representing AG 3 was found to hybridise strongly to Southern blot digests containing the DNA of other AG 3 isolates from potato fields in Australia and Japan, with no distinct hybridisation patterns observed when other AGs were tested using the same procedure (Balali *et al.*, 1996). Another probe developed for the detection of AG 8 isolates was shown to hybridise strongly to isolates representing AG 8 and react minimally with isolates from other AGs (Matthew *et al.*, 1995). Other research has focussed on the use of PCR-based methods to identify AGs.

A polymerase chain reaction (PCR) restriction mapping method has been developed for the identification of AG 3 isolates (Bounou *et al.*, 1999). Southern blot analysis revealed a sequence found in the AG 3 genome which was almost unique (Bounou *et al.*, 1999). Other PCR assays have been developed specifically to identify and quantify levels of AG 3 in potato and in soil (Lees *et al.*, 2002). The primers that were developed were tested against total DNA extracted from other potato pathogens, other AGs of *R. solani* and on isolates of AG 3 from around the world. It was found that the amplification of the particular sequence was only observed in AG 3 isolates (Lees *et al.*, 2002).

It is well documented that variations in nutritional requirements and pathogenicity exist within and between different AGs of *R. solani* (Ogoshi, 1987; Carling & Leiner, 1990b; Bains & Bisht, 1995; Balali *et al.*, 1995; Bounou *et al.*, 1999). The ability to rapidly distinguish between anastmosis groups and quantify soil inoculum levels would be useful in disease control, as the presence, predominance and pathogenicity of particular AGs is often determined by geographical location and environmental conditions.

1.4.2 Global distribution and frequency of AGs

Field isolates have been collected from all over the world and associated with various stages of disease caused by *R. solani* on potato plants (Table 1.1). Numerous studies have revealed significant differences in the virulence of isolates both between and within AGs (Balali *et al.*, 1995; Carling *et al.*, 1986; Carling & Kuninaga, 1990).

Table 1.1. AGs of *Rhizoctonia solani* found to be pathogenic to potato and the part of the potato plant they damage.

Anastomosis group	Infection caused by *R. solani* on potato	Geographical Origin	Reference
1	Root lesions	USA	Bandy *et al.*, 1988
2-1	Stem canker, sclerotia on tubers	N. Ireland	Chand & Logan, 1983
2-1	Stolon and root lesions	Alaska	Carling & Leiner, 1986
2-1	Stem canker, tuber sclerotia, stolons	Australia	Petkowski & de Boer, 2001
3	Stem canker, tuber sclerotia	N. Ireland	Chand & Logan, 1983
3	Stolon, stem and root lesions	Alaska	Carling & Leiner, 1986
3	Sclerotia on tubers, stem/ root canker	USA	Bandy *et al.*, 1988
3	Tuber sclerotia	Peru	Anguiz & Martin, 1989
3	Subterranean and aerial stem lesions	USA	Carling *et al.*, 1989
3	Stem lesions, tuber sclerotia	Alaska	Carling & Leiner, 1990a
3	Root and sprout lesions	Alaska	Carling & Leiner, 1990b
3	Isolated from a potato plant	Canada	Bains & Bisht, 1995
3	Tuber/root sclerotia, stem/root canker	S. Australia	Balali *et al.*, 1995
3	Stem and stolons, tuber sclerotia	Australia	Petkowski & de Boer, 2001
4	Stem canker	Peru	Anguiz & Martin, 1989
4	Isolated from a potato plant	Alaska	Bains & Bisht, 1995
4	Tuber and root sclerotia, root canker	S. Australia	Balali *et al.*, 1995
5	Isolated from soil	USA	Bandy *et al.*, 1985
5	Stem/stolon canker	USA	Bandy *et al.*, 1988
5	Sprout lesions	Alaska	Carling & Leiner, 1990b
5	Isolated from a potato plant	Canada	Bains &Bisht, 1995
5	Tuber sclerotia, stem canker	S. Australia	Balali *et al.*, 1995
5	Stem and root lesions	UK	Woodhall & Lees, 2004
7	Tuber sclerotia, root/shoot lesions	Mexico	Carling *et al.*, 1998
8	Root and sprout lesions	Alaska	Carling & Leiner, 1990b
8	Root canker	UK	Hide & Firmager, 1990
8	Isolated from soil	S. Australia	Balali *et al.*, 1995
9	Stem lesions	Alaska	Carling *et al.*, 1987
11	Sprout lesions	W. Australia	Carling *et al.*, 1994

R. solani of different AGs have been found to vary in the disease symptoms they cause, with variations in the plant part targeted and the stage at which disease occurs during plant development (Carling *et al.*, 1986; Carling *et al.*, 1987; Dijst *et al.*, 1986). Soil samples from potato growing areas and infected individual potato plants from around the world have shown that the most abundant AG present is AG 3.

Sampling of potato fields in Alaska revealed that nearly three-quarters of *R. solani* isolated were AG 3 and a fifth were AG 2-1. These were recovered from stem lesions, sclerotia and hymenia, and the overwhelming majority of sclerotial isolates obtained belonged to AG 3 (Carling & Leiner, 1986). A later study showed nearly all the isolates retrieved from potato plants were AG 3, however, just over half of the isolates retrieved from the soil were AG 3 (Carling & Leiner, 1990b). Isolates of *R. solani* obtained from stems, stolons and roots in Maine were nearly 80 % from AG 3, 13% AG 5 and a very small percentage were AG 1, with all samples isolated from sclerotia on tubers found to be AG 3 (Bandy & Leach, 1988). Isolates of *R. solani* taken from potato plants in Peru were predominately AG 3 and AG 4 (Anguiz & Martin, 1989).

Ninety percent of the isolates collected from the soil, stems, roots and tuber-borne sclerotia in South Austraila were AG 3, with the remaining representing AG 4, AG 5 and binucleate isolates (Balali *et al.*, 1995). In Alberta, Canada, isolates collected from potato plants were three-quarters AG 3, with the remaining isolates AG 4 and AG 5 (Bains & Bisht, 1995). The predominance of particular AGs is significant as variations in pathogenicity between and within AGs of *R. solani* have been demonstrated in numerous *in vitro* and field trials.

1.4.3 Variations in pathogenicity of different AGs

Pathogenicity tests on *R. solani* isolates derived from tuber-borne sclerotia taken from seed potatoes in a laboratory-based study demonstrated that nearly 60 % of sclerotial isolates were pathogenic, and responsible for damage ranging from superficial stem lesions to girdling and killing of potato sprouts (Bolkan & Wenham, 1973). Chand and Logan (1983) used laboratory and field tests to show that AG 3 isolates from tuber-borne sclerotia could differ in virulence from one another as well from other AGs. Out of 5 groups of AG 3 isolates tested, one group caused significantly higher levels of stem canker. Inter AG differences in virulence were illustrated by AG 2-1 isolates grown from tuber-borne sclerotia that caused significantly less stem canker than the AG 3 isolates examined (Chand & Logan, 1983). Further laboratory research revealed all AG 3 isolates recovered from potato plants in Alaskan fields were capable of damaging potato sprouts, although isolates of sclerotial origin were found to be less aggressive than lesion or mycelial derived isolates. In contrast, AG 2-1 isolates were associated with minimal damage to stems, with 38 % of the AG 2-1 isolates tested producing no symptoms of *R. solani* infection (Carling & Leiner, 1986).

Pot experiments to test the virulence of AG 3 isolates from different cropping systems revealed significant differences in pathogenicity within this AG (Carling & Leiner, 1990b). AG 3 isolates from potato fields with a history of continuous vegetable and potato cropping were significantly more virulent than those taken from fields where potato had been grown in rotation with grain and forage grasses. Hymenial isolates were also found to be more virulent than isolates taken from lesions on potato plants (Carling & Leiner, 1990b). Pathogenicity tests in glasshouse trials comparing the damage caused by AG 3, AG 4, AG 5 and AG 8 isolates all taken from infected potato plants found there were significant differences between the four AGs tested. The development of tuber-borne sclerotia was

moderate to severe when plants were inoculated with AG 3 but only moderate to slight when inoculated with AG 5. All AG 3 and AG 4 isolates caused moderate stem canker with AG 5 causing slight to moderate stem canker, and AG 8 causing slight stem canker. AG 4 and AG 8 were responsible for severe root cankers, with severely pruned roots also observed on potato plants inoculated with AG 8 mycelium (Balali *et al.*, 1995). An earlier pot experiment using a different isolate of AG 8 as inoculum revealed a 70 % reduction in yield in tubers compared to uninfected controls, with this isolate predominantly responsible for infecting and pruning the root system (Hide & Firmager, 1990).

An investigation into potato-derived sclerotial AG 3 and AG 2-1 isolates and an AG 2-1 isolate from a potato stem lesion in glasshouse trials showed all isolates were pathogenic to potato, producing symptoms of stem canker on underground stems, stolons and tuber-borne sclerotia (Petkowski & de Boer, 2001). Inoculation with the AG 2-1 isolate taken from a sclerotium caused a low incidence and severity of stem canker on potato plants, whereas a higher incidence and severity of stem canker was observed on plants inoculated with the sclerotial AG 3 and stem lesion AG 2-1 isolates (Petkowski & de Boer, 2001). Other AGs have been identified as pathogenic to potato, but are less commonly reported. Isolates of AG 7 from around the world have been found to cause different symptoms with some causing superficial discolouration to shoots, and others causing 1 mm diam lesions (Carling & Brainard, 1998). When the virulence of AG 7 isolates was compared to the virulence of AG 3 isolates, the latter caused major damage to roots and shoots, whereas disease caused by AG 7 was much less severe (Carling & Brainard, 1998). AG 9 has been isolated from lesions on potato stems and was found not damage root systems (Carling *et al.*, 1987). AG 11 was found to produce very small stem lesions on the developing sprouts of potato plants and no damage to the roots, with AG 4 isolates typically more virulent (Carling *et al.*, 1994).

1.4.4 Cross-species infection by *R. solani* isolates pathogenic to potato

Research has also focussed on the ability of *R. solani* isolates taken from potato plants to infect other crops, as crop rotation is often used to prevent the accumulation of inoculum in soil and avoid severe outbreaks of the disease (Anderson, 1982). Greenhouse pathogenicity tests showed that AG 5 isolates from potato stem lesions produced sunken brown stem lesions on both potato and tomato plant stems (Bandy *et al.*, 1984). An AG 8 isolate from barley was found to reduce the fresh weight of potato plants by nearly three - quarters compared to controls by pruning the roots and reducing the number of progeny tubers (Hide & Firmager, 1990). Isolates representing AG 3 and AG 2-1 from tuber-borne sclerotia and potato stem canker lesions were found to damage hypocotyls, cause crown rot, wire stem symptoms, damping off and produce sclerotia on fodder rape, Indian mustard and red clover plants (Petkowski & de Boer, 2001). An isolate of AG 5 from couch grass originating from a potato field in the UK was found to be pathogenic to potato, with moderate stem infection and root infection observed on inoculated potato plants (Woodhall & Lees, 2004).

Although different AGs of *R. solani* have been found to infect multiple crop species, the damage is not always as severe as was observed on the original host plant. Isolates of AG 11 from fields in Western Australia and the US were found to cause severe lesions on wheat, rice and soybean coleoptiles, but caused only minor damage to developing sprouts of potato plants and no damage to any other part of the plant (Carling *et al.*, 1994). There is currently evidence to suggest that particular AGs of *R. solani* are more host specific than those outlined previously. *R. solani* AG 3 isolates from diseased potatoes were found to induce stem and root disease on other potato plants, but not on tobacco plants (Stevens Johnk *et al.*, 1993). Similarly, *R. solani* AG 3 isolates taken from target spot

lesions on tobacco caused disease on other tobacco plants, but induced little or no damage to potato sprouts and did not damage root systems (Stevens Johnk et al., 1993).

The observations described previously show that AG 2-1, AG 3, AG 4, AG 5 and AG 8 are responsible for much of the damage observed on potato plants in the field globally, although the extent of the disease observed on host plants is significantly different. (Bandy & Leach, 1988; Carling & Leiner, 1990a; Hide & Firmager, 1990; Carling & Leiner, 1990b; Chand & Logan, 1983). Differences in the predominance of anastmosis groups were also apparent depending on the geographical region surveyed, and research into the virulence of *R. solani* has revealed that environmental conditions can have a significant effect on the incidence and severity of *R. solani* disease caused by different AGs.

1.4.5 Effect of environmental factors on *R. solani* biology and pathogenicity

Studies on the *in vitro* mycelial growth of AG 3 and AG 2-1 isolates *in vitro* found the optimum temperature for growth of all these isolates was between 22 and 25°C (Chand & Logan, 1983). The optimum temperature for *in vitro* mycelial growth of different AG 3 isolates taken from different agroecological zones of Peru was 20 to 25°C, whereas for AG 4 it was 25 to 28 °C (Anguiz & Martin, 1989). Large numbers of sclerotia were produced *in vitro* by AG 3 isolates at 20 to 25°C but very few formed at 28°C. AG 4 isolates produced only very few sclerotia at 25°C. The virulence of these isolates in glasshouse trials showed that AG 3 and AG 4 isolates reduced seedling survival to between 22 – 45 % at 9 to 18°C, with seedling survival much higher, between 57 – 65 %, at 18 to 24°C, (Anguiz & Martin, 1989). Balali *et al.* (1995) conducted a similar experiment to compare the mycelial growth rates of AG 3, AG 4 and AG 5 at 15°C and 25°C, and found that AG 3 grew faster at 15°C

than 25°C. Both AG 4 and AG 5 isolates grew faster at 25°C than 15°C with only AG 3 isolates producing sclerotia after 5 d incubation (Balali *et al.*, 1995).

Growth chamber studies at 10, 15 and 21°C showed that at 21 °C, AG 3 and AG 5 caused significantly more damage to sprouts than AG 4 or AG 8 (Carling & Leiner, 1990a). At both 10 and 21°C, AG 3 isolates from the US and Mexico were shown to cause significant damage to roots and shoots of potato plants, and AG 7 isolates from the US caused discolouration of shoots at both temperatures. An AG 7 isolate from Mexico was more aggressive at lower temperautre, producing small root and shoot lesions at 10°C but not at 21°C (Carling & Brainard, 1998).

Water potential is one of many environmental factors shown to have a significant influence on disease caused by *R. solani* on various hosts (Blair, 1943; Doornik, 1980; Dorrance *et al.*, 2003; Grosch & Kofoet, 2003; Harikrishnan & Yang, 2004; Chang *et al.*, 2004). Total water potential is the sum of four main components: matric, osmotic, gravitational and pressure potential (Cook & Duniway, 1980). In soil systems, osmotic and matric potential contribute the most to water potential, with matric potential being the major component and determining the availability of soil water to organisms (Papendick & Campbell, 1980). Fungi have to maintain an internal water potential lower than that of the total water potential of the environment to maintain turgidity of cells and allow continuation of mycelial growth (Cook, 1973; Eamus & Jennings, 1986). The internal water potential of the fungus is, therefore, determined by the surrounding environment, with exposure to changes in the availability of water within the solid matrix itself (matric potential) and the presence of dissolved substances (osmotic potential) (Carlile *et al.*, 2001; Cook, 1973). The influence of osmotic potential on mycelial growth of *R. solani in vitro* has been investigated previously for isolates infecting cereals (Dubé *et al.*, 1971) and cotton

(Sterne & McCarver, 1978). The effect of osmotic and matric potential on mycelial growth, sclerotia production and germination of *R. solani* from potato are unknown.

1.4.6 Nutritional factors affecting mycelial growth and sclerotia production by *R. solani*

Sclerotia produced by *R. solani* isolates studied previously appear small and infrequent when grown on a nutritionally poor media, and much larger and more frequent when grown on nutrient-rich media (Sherwood, 1970; Dijst, 1988). Several *in vitro* and growth chamber studies have revealed that the potato tuber and other nutritional factors can influence sclerotia formation (Allington, 1936; Dijst, 1988; Dijst, 1990). An early *in vitro* study showed that potato tubers placed on agar media have a temporary inhibitory effect on mycelial growth, with hyphal colonization and sclerotial formation occurring after a delay (Allington, 1936). Isolates of *R. solani* also differ in their requirement for thiamine and many AG 3 isolates taken from potato have been shown to be prototrophic for thiamine (Stevens Johnk *et al.*, 1993). AG 9 isolates have also been found to vary in their requirement for thiamine for mycelial growth, as have AG 2, AG 4 and AG 1 (Sherwood, 1969).

In vitro studies on the production of *R. solani* sclerotia on different carbon sources showed that the fungus can utilize glucose, sucrose and potato starch for the formation of sclerotia (Allington, 1936). Similar results were observed in later experiments, and it was concluded that the greatest number of sclerotia developed on a media that also favoured mycelial growth (Townsend, 1957). It was found that a decrease in carbohydrate in an agar medium stimulates sclerotial development, whereas a reduction in nitrogen inhibits the same process (Townsend, 1957; Allington, 1936). High concentrations of carbohydrate and nitrogen tended to favour the initiation of sclerotia, but these higher levels inhibited their maturation (Townsend, 1957). The carbon : nitrogen ratio of subterranean potato plant parts

in growth chamber studies were found to increase during plant maturation and following haulm destruction (Dijst, 1990).

1.5 Control of disease caused by *R. solani*.

Due to the wide range of AGs pathogenic to potato, the development of control strategies to curb crop infection by all these groups would be desirable. Many different control methods have been evaluated and are currently in use, or are being developed in an attempt to control *R. solani* disease on potato.

1.5.1 Cultivar resistance

The prevention or control of *R. solani* infection using resistant cultivars of potatoes offers an economical and efficient method of growing crops as well as reducing the currently required need for fungicidal disease control (Chand & Logan, 1982). Field studies carried out on sandy soils revealed that two cultivars of potato, Element and Mirka, varied in their susceptibility to *R. solani* infection (Scholte, 1989). On average, the incidence of damage to plants derived from cv. Mirka was over twice as high as the damage observed on cv. Element. Subsequent experiments revealed that early maturing cultivars Eersteling and Ostara were less severely attacked than the late maturing cultivars Multa and Alpha (Scholte, 1989).

Continuous cultivation of potato resulted in the percentage of severely infected plants grown from clean seed tubers of two different cultivars being 40 % for cv. Element and 78 % for cv. Mirka compared to 10 % infection on cv. Element. The rate of severely infected plants was reduced to 27 % on cv. Mirka when potato cultivation was interrupted with a rotational crop of maize (Scholte, 1989). Earlier trials conducted under field conditions with ten cultivars of potato found no significant differences between the

cultivars with respect to their susceptibility to stem canker (Chand & Logan, 1982). Black scurf development significantly varied between cultivars during a field trial investigating the effect of different harvesting methods on the incidence of black scurf on tubers. It was found that there was a significant difference in black scurf development between cultivars, with tubers of cv. Astarte having significantly less sclerotial coverage than cvs Prominent and Bintje (Dijst *et al.*, 1986).

A pot-based investigation into the susceptibility of seven commercially grown potato cultivars to *R. solani* infection revealed significant differences in the percentage area of stems damaged by lesions and with the development of black scurf on seed tubers (Kyritsis & Wale, 2002a). Stem canker severity on the least susceptible cultivars, Sante and Maris Piper, was nearly 40 %, whereas the more susceptible cultivars, Estima, Nadine and Osprey, had values nearer 50 %. The incidence of black scurf was also less on the cultivars with lower stem canker at around 40 %, and the more susceptible cultivars were found to have almost 60 % of seed tubers infected with black scurf. Although there were differences in susceptibility to infection, none of the cultivars tested showed adequate resistance to *R. solani* infection (Kyritsis & Wale, 2002a). With no known cultivars of potato resistant to *R. solani* infection, the use of resistant cultivars as part of a disease management strategy is not currently a viable alternative to the chemical control methods presently used to control *R. solani* on seed potatoes and in soil (Secor & Gudmestad, 1999).

1.5.2 Chemical control

The main sources of *R. solani* inoculum are derived from existing populations of the fungus present in soil and from sclerotia and/or mycelium present on seed tubers (Keijer, 1996). It has been observed that plants derived from severely infested seed are slower to emerge than chemically treated seed or seed without visible *R. solani* infection

(Hide *et al.*, 1973). The most widely used mechanism for the control of *R. solani* by farmers worldwide is the application of synthetic fungicides as seed and soil treatments (Kataria & Gisi, 1996).

1.5.2.1 Chemical seed treatment

Fungicidal seed treatments will protect from damping-off and seedling root rot for between 2 - 3 weeks, whereas fungicidal soil applications as a dust, drench or granules, will protect from soil-borne inoculum throughout the duration of the growing season (Kataria & Gisi, 1996). Any fungicides used must be compatible with others applied to the same area to control other pests and pathogens thus the selection, timing of application and the precise targeting of fungicides to control *R. solani* is important (Jeger *et al.*, 1996). Soil type and other factors such as the agro-ecological region, can also affect the success of preventative chemical treatments (Jager *et al.*, 1991; Kataria & Gisi, 1996).

The first study to document successful control of *R. solani* on potatoes used steam-formalin treatment to control the development of sclerotia on progeny tubers (Winston, 1913). Since then, a wide variety of chemicals has been evaluated and is currently in use to control stem, stolon and tuber infection from seed-borne and soil borne inoculum. Early chemical treatments included organic mercury, however, the phasing out of mercury-containing compounds from agricultural applications lead to new research into alternatives to control the disease (Wilson, 1974).

In vitro studies on harvested seed potato tubers have shown that the concentrations of sodium hypochlorite and formaldehyde as well as the dipping time of the tubers in these chemicals affected the viability of sclerotia present on the potato surface (Wicks *et al.*, 1995). Eighty percent of the sclerotia removed from harvested tubers treated with water and subsequently grown on water agar were viable, whereas most sclerotia were killed when

tubers were submerged in 2 % formaldehyde for 20 min or 4 % formaldehyde for 10 min. Treatment with 2 % sodium hypochlorite for 20 min destroyed just over half of sclerotia present on the seed tuber surface (Wicks *et al.*, 1995). The effectiveness of sodium hypochlorite as a pre-planting seed tuber treatment for the management of black scurf was demonstrated both *in vitro* and in field studies (Errampalli & Johnston, 2001). Laboratory studies revealed that only 2 % of sclerotia removed from seed tubers dipped in 500 ppm sodium hypochlorite for 8 min or 1000 ppm sodium hypochlorite for 2 min germinated after 48 h on potato dextrose agar (PDA). Field trials where clean, moderate and severely diseased seed were treated showed that dipping tubers in 500 ppm sodium hypochlorite for 8 min or thiophanate-methyl at 0.005kg kg^{-1} individually was ineffective. The use of these two treatments in combination on seed potatoes gave significant reductions on black scurf in consecutive years (Errampalli & Johnston, 2001).

Investigations into the effect of seed tuber fungicidal treatment and short potato growing rotations revealed that pre-planting seed tuber treatments with iprodione or tolclofos-methyl reduced the severity of stem canker (Hide & Read, 1991). The decline in stem canker severity was not uniform over a 7 year period of field trials and this was attributed to levels of soil-borne inoculum that were not controlled by any fungicide treatment (Hide & Read, 1991). Seed tuber treatments with 2 and 6 % thiabendazole and iprodione suspensions were all found to reduce the incidence of stem and stolon canker, with the latter concentration being the most effective (Hide & Cayley, 1982). Current chemical seed treatments available commericially include RhiNo (flutolanil), Amistar (azoxystrobin) and Monceren (pencycuron).

1.5.2.2 Chemical soil treatment

Differences in the efficacy of chemical fungicides to control black scurf have been found depending on the soil type they were applied to prior to planting (Jager *et al.*, 1991). Application of thiabendazole, iprodione and benodanil as soil treatments have been found to be effective in field trials to control stem canker, stolon pruning and black scurf when applied at rate of 12 kg a.i/ha^{-1} (Hide & Cayley, 1982). When applied to an acidic soil, thiabendazole, which is easily protonated and strongly adsorbed onto acidic soils, was more effective at reducing disease levels than in neutral soils (Hide & Cayley, 1982).

Chemical control in field trials with pencycuron and tolclofos-methyl at fractions of the recommended application rates was found to differ significantly depending on the soil type the potatoes were grown in. These two fungicides are currently the most popular to control *Rhizoctonia* diseases, with pencycuron specifically developed for the control of black scurf and tolclofos-methyl controlling almost every type of disease on numerous crops and under many environmental conditions (Kataria & Gisi, 1996). It was found that sclerotia numbers on tubers and the losses due to grading were lower with the recommended dose (20 kg ha^{-1}) of pencycuron. Application of pencycuron to neutral loam soils at rates of $^{1}/_{8}$ and $^{1}/_{4}$ of the recommended amount gave similar results to the control achieved with the recommended application rate. Similar levels of pencycuron applied to slightly acid sand and reclaimed peat soils were not as effective in controlling black scurf development (Jager *et al.*, 1991). Field trials over 2 years where soils were treated with pencycuron prior to planting consistently resulted in a lower incidence of progeny tubers with black scurf compared to untreated controls (Lootsma & Scholte, 1996). Application of azoxystrobin as a pre-planting soil treatment and in-furrow treatment was successful in controlling disease caused by *R. solani* on potato, but at high inoculum pressure, the effectiveness of disease control declined (Wale *et al.*, 2004). It was suggested that this

chemical would be more effective in controlling disease where the inoculum densities of *R. solani* were lower (Hilton *et al.*, 2004). In furrow treatment with flutolanil at planting has been shown to be effective in reducing black scurf observed on progeny tubers, however, the planting of heavily infested seed in severely infected soil reduced the efficacy of this type of treatment (Tsror & Peretz-Alon, 2005).

There is also the possibility of fungicides being selective regarding the AGs they target, as studies have found that the genetic diversity within AG 3 is more homogenous than AG 2-1 (Woodhall & Jenkinson, 2002). Pencycuron was found to be effective against AG 3 and AG 2-1 at very low concentrations during *in vitro* agar tests, whereas the incorporation of 250 ppm pencycuron had little effect on the growth of isolates representing AG 5 and AG 8 (Woodhall & Jenkinson, 2002). The presence of genetically distinct isolates within AGs as well as resistant strains of *R. solani* could potentially make developing future chemical treatments more problematic. The development of resistance of *R. solani* to current chemical treatments, as well as a growth in organic farming practices, has led to research into new, more environmentally sound methods of disease control. Farming under organic management requires strict regulations to be followed and most synthetic chemicals available to control *R. solani* cannot be used. It would, therefore, be desirable to develop products to control fungal pathogens as alternatives to fungicides (Tsror *et al.*, 2001).

1.5.3 Biological control

Sclerotia produced by *R. solani* on potato tubers are known to be colonised and destroyed by mycoparasitic fungi (Jager & Velvis, 1983a). The use of these fungi and other mycoparasitic organisms as biological control agents could reduce the dependence of commercial farming on fungicides by reducing application rates or replacing them

altogether (Herr, 1995). To be a success commercially, a biocontrol agent should be effective over a range of environmental conditions and applied at rates that would be affordable to the farming industry (Escande & Echandi, 1991a). At present, there are many biological control systems under investigation utilising mycoparasitic fungi, mycophagous soil organisms and hypovirulent strains of *R. solani* that could be beneficial to the potato growing industry (Jeger *et al.*, 1996).

1.5.3.1 Mycoparasitic fungi

Mycoparasitic (antagonistic) fungi can be divided into two groups: (1) biotrophic fungi, where nutrients are obtained from living host cells; (2) necrotrophic fungi, where nutrients are obtained from host cells that have been killed (van den Boogert, 1996). Many recent studies have focused on *Verticillium biguttatum* (W. Gams), an ecologically obligate mycoparasite (EOM) that can colonise living mycelium of *R. solani* (Morris *et al.*, 1995a). EOMs are non-competitive and their ability to be biocontrol agents is dependent on obtaining all the necessary nutrients from their living fungal host (van den Boogert, 1996). Ecologically facultative (saprophytic) mycoparasites (EFMs), such as *Gliocladium* spp. and *Trichoderma* spp., can utilise nutrients released by the host plant and compete with *R. solani*, which may lead to subsequent exclusion of this pathogen (van den Boogert, 1996).

Small plot studies and field trials in the Netherlands on different soil types revealed that slightly acid sandy soils suppressed the onset of *R. solani* disease. Many of the sclerotia sampled from tubers grown in these soils were infected with mycoparasitic fungi including *Gliocladium* spp. and *Verticillium* spp. Seed potatoes planted in the same soils produced plants that suffered from less *R. solani* infection (Jager & Velvis, 1983b). These variations were attributed to differences in the species of antagonists present on the

surface of seed potatoes and sclerotia, as well as differences in the proportion of the strains located on sclerotia attached to seed potatoes (Jager & Velvis, 1983b).

Van den Boogert and Jager (1984) investigated the effects of *V. biguttatum* M73 and other hyperparasites, *Clonostachys rosea* (Link: Fr) [*Gliocladium roseum* (Bainer)] M36, *Trichoderma hamatum* (Bon.) Bainer M37 and *Hormiactis fimicola* M58 that were known to colonise *R. solani* on potato *in vitro*, under green house conditions and in the field. It was found that application of *V. biguttatum* alone and a mixture of all 4 of these hyperparasites in field trials significantly reduced sclerotial development on seed and ware potatoes, with tuber yield and plant growth unaffected (van den Boogert & Jager, 1984). *V. biguttatum* in particular has been the subject of many studies to determine its effect on *R. solani* populations in both growth chamber and *in vitro* studies as well as experimental field trials in different soil types.

1.5.3.2 V. biguttatum and control of R. solani

The presence of *Verticillium* sp. was first reported in the Netherlands by Jager *et al.*, (1979), however, the faster growing *C. rosea* over grew the specimen and it was identified as the latter species (Jager & Velvis, 1985). *V. biguttatum* has also been isolated from field soil in the UK (Morris & Coley-Smith, 1992). Subsequent research has revealed *V. biguttatum* to be an important antagonist of *R. solani* and it has been studied extensively. Inoculation of sclerotia covered seed with *V. biguttatum* isolate M73 grown in acidic soil was found to produce emerging sprouts with a longer average length than those grown from non-inoculated seed tubers during growth chamber trials (Jager & Velvis, 1984). *V. biguttatum* colonised non-inoculated tubers as it was naturally present in the soil, however, there was little evidence to suggest that this very low level of naturally available soil-borne inoculum suppressed the growth of *R. solani* (Jager & Velvis, 1984). Sprouted seed tubers

inoculated with *V. biguttatum* had, on average, significantly reduced sclerotia infection severity at harvest. Reductions in sclerotia infection observed on potato plants grown in Holocene marine soils and slightly acid sandy soils were 50 % and 35 % respectively, compared to untreated tubers (Jager & Velvis, 1986).

Studies where *V. biguttatum* was used to inoculate different plant species revealed this mycoparasite had a strong preference for potato, and was found to almost completely colonise the stolons of potato plants grown from seed inoculated with *V. biguttatum* (van den Boogert, 1989). Field studies showed that by applying a suspension of *V. biguttatum* spores onto both acid and neutral loam soils led to lower grading losses and sclerotium indices on progeny tubers (Jager et al., 1991). Field studies comparing plots with high and low incidences of black scurf revealed there were no antagonistic mycoparasitic factors present that could be responsible for the variations observed in the occurrence of *R. solani* infection (Jager & Velvis, 1995). It was found that *R. solani* AG 3, a major cause of black scurf, had been replaced by AG 5, a weak competitor of AG 3 in regions where black scurf levels were low, however the reasons for this is unknown. Levels of V. biguttatum determined from the same areas were regarded too low to cause *R. solani* disappearance (Jager & Velvis, 1995).

Research has found that poor numbers of *V. biguttatum* spores germinated in the absence of a host, but the presence of *R. solani* increased germination to 100 % (van den Boogert & Deacon, 1994). Microscopy has revealed the ability of the mycoparasitic hypha to penetrate the host cell walls and grow along the inside the host mycelium. Infection structures were observed on some side branches of *V. biguttatum*, with the appressoria located at the hyphal tips (van den Boogert & Deacon, 1994). Investigations into the nutritional requirements of *V. biguttatum* revealed that the fungus was unable to utilise nitrates as a nitrogen source or degrade cellulose (van den Boogert & Deacon, 1994). In the

presence of the same substances plus *R. solani* AG 3 or AG 4, *V. biguttatum* growth occurred, demonstrating that it has the ability to obtain the required nutrients for growth from *R. solani*. Growing *V. biguttatum* and *R. solani* in agar culture together also resulted in reduced sclerotia production compared with *R. solani* isolates grown alone (van den Boogert & Deacon, 1994).

Between 50 and 90 % of *R. solani* sclerotia treated with conidial suspensions of three isolates of *V. biguttatum* were found not to germinate (Velvis & Jager, 1983) .Further investigation showed that sclerotia treatment with *V. biguttatum* not to be as effective at 10°C with 86 % of sclerotia germinating compared only 5 % at both 15 and 20°C (Velvis & Jager, 1983). Sclerotial survival in different soil types *in vitro* showed that at 20°C, germinating sclerotia were greatly reduced in most soils where a high density of *V. biguttatum* was also observed. When sclerotia survival was tested under field conditions, no hyperparasites were found to accumulate on the sclerotia, however, the viability of sclerotia recovered decreased over a two-year period (Velvis *et al.*, 1989). Laboratory-based experiments in Australia showed that *V. biguttatum* reduced sclerotia viability to levels which were 40 % lower than the controls, and killed significantly more sclerotia than *G. roseum*, *Trichoderma* sp. and *Bacillus* sp. based treatments. After 35 d incubation, the treatment of *R. solani* sclerotia with *V. biguttatum* spores did not differ significantly from treatment with the fungicide pencycuron (Wicks *et al.*, 1995).

1.5.3.3 Mycophagous nematodes

Other research into the control of *R. solani* diseases on potato has focused on the effects that mycophagous nematode and springtail populations have in preventing stem canker. Combined application of the nematode *Aphelenchus avenae* (Bastian) and the springtail *Folsomia fimetaria* L. to the soil was found to be particularly effective at

reducing stem canker in growth chamber studies where the soil population of *R. solani* was high (Lootsma & Scholte, 1997b). Glasshouse bioassays showed that when soil moisture content (7 %) and *R. solani* soil population were low, *A. avenae* was more effective at preventing stem infection and *F. fimetaria* reduced stem canker severity at higher *R. solani* soil populations and soil moisture levels (14 %) (Lootsma & Scholte, 1997a).

Different organic soil amendments and the soil pH have been shown to affect populations of mycophagous soil organism populations and their ability to reduce the occurrence of potato stem canker. Growth chamber trials found *A. avenae* to reduce stem infection significantly when oil-seed rape material consisting of the leaves and stems from seven month-old plants cut into pieces <5 cm was incorporated into the soil (Lootsma & Scholte, 1998). *F. fimetaria* alone was found to suppress the incidence of *R. solani* disease better at a low pH (4.8) without any organic amendments having been applied to the soil (Lootsma & Scholte, 1998). Field trials examining the effects of mycophagous soil organisms on the incidence stem canker on potato showed that treatment of plots with products containing aldicarb significantly reduced the populations of saprophagous and mycophagous nematodes, as well as increasing the severity of *R. solani* stem canker (Scholte & Lootsma, 1998). The addition of oats as a soil amendment significantly reduced disease severity and levels of mycophagous nematodes were the highest after this treatment, particularly when lower levels of *R. solani* inoculum were present in the soil (Scholte & Lootsma, 1998).

The control of *R. solani* in this manner requires an understanding of the complex relationship between the pathogen and antagonists and the various environmental and nutritional factors present in the soil. The soil inoculum density of *R. solani*, the variety of different species and numbers of mycophagous soil fauna, an availability of alternative

food sources for mycophagous organisms and application of organic soil amendments to the soil could all potentially affect the control of *R. solani* disease.

1.5.3.4 Other biological control methods

Other potential agents for biological control are naturally occurring non-pathogenic or hypovirulent strains of *R. solani*. These isolates form no appressoria or other structures to penetrate their host, however, they compete with virulent strains for plant surfaces and nutrients within the rhizosphere (Herr, 1995). Also currently under investigation is whether or not hypovirulence can be conferred onto virulent strains (Jeger *et al.*, 1996). *Rhizoctonia* sp. are known to become infected by double-stranded RNAs (ds-RNAs) which have been associated with reducing or increasing the ability of *R. solani* to cause disease (Lakshman & Tavanzis, 1994; Jian *et al.*; 1997). Hypovirulent ds-RNAs could be transferred to virulent strains via hyphal anastomosis, and may offer a way of controlling the disease in potato.

Glasshouse experiments using binucleate *Rhizoctonia* isolates showed they could protect from stem canker at temperatures ranging from 11 – 23 °C (Escande & Echandi, 1991b). Disease severity on plants inoculated with these isolates was more than halved compared to the untreated controls. Field trials using binucleate isolates to protect against *R. solani* AG 3 showed that a combination of three binucleate isolates reduced the severity of stolon canker to around one quarter of that observed on the controls. This reduction in disease using the binucleate isolates was similar to the reduction in disease caused by chemical fungicidal treatment pentachloronitrobenzene (PCNB) (Escande & Echandi, 1991b). Tsror *et al.* (2001) found that in two consecutive years of field trials, two binucleate non-pathogenic isolates of *Rhizoctonia* significantly reduced black scurf severity, with various isolates reducing disease incidence by between 60 – 80 % in the first

year of cropping and 45 – 55 % in the subsequent year. Alternative methods of biological control using the coelomycete *Microsphaeropsis* sp. strain P130A have been tested to evaluate the effect of this anti-microbial antagonist on the viability and production of tuber-borne sclerotia. The germination of sclerotia produced *in vitro* and progeny tubers were both significantly reduced from an average of 82 % following 1 day of *Microsphaeropsis* sp. treatment, down to an average of 5.8 % after 35 d (Carisse *et al.*, 2001).

1.5.4 Cultural control

To prevent the transmission of viruses via flying aphids, the haulm of the potato is destroyed usually during the second half of tuber development, however, it has been observed that haulm destruction stimulates sclerotia production on tubers (Dijst *et al.*, 1986). Immature crop harvesting, where the haulm pulling and tubers collection is done by hand, has been shown to be an effective way to control black scurf giving results similar or lower than soil treatment with pencycuron before seed planting (Lootsma & Scholte, 1996). This method was not as effective if the seed tubers used were severely infested with black scurf as black scurf begins to form on progeny tubers before they are harvested (Lootsma & Scholte, 1996).

Crop rotation has also been found to influence the incidence and severity of stem canker caused by soil-borne *R. solani* present in soil (Gilligan *et al.*, 1996). Field trials revealed that stem canker was more prevalent when potato was grown more frequently (2 year rotation) compared to a longer period (6 year rotation). During intercrop periods, the lowest levels of stem canker on bait plants were observed in the 4 to 6 year rotations. It was also found that growing a susceptible crop allowed rapid replenishment of soil inoculum with over 70% of plants infected after seed was grown after a single crop was grown in a 6 year rotation (Gilligan *et al.*, 1996).

Soil-borne inoculum is considered difficult to eliminate from fields, but 3 to 4 year rotation may help prevent or lessen the severity of infection (Secor & Gudmestad, 1999). The best rotational crops to grow between potato crops have been found to be barley and oats, with sugar beet and dry beans to be avoided. The use of clean seed is recommended to prevent further disease, as planting of seed with 5% of the tuber surface covered in sclerotia has been shown to reduce emergence (Secor & Gudmestad, 1999).

1.5.5 Intergrated management strategies

The use of integrated management strategies to control *R. solani* is often advantageous as isolated procedures to control this disease may provide varying levels of control depending on soil type or environmental conditions as discussed previously. When combining chemical and biological control methods, it is necessary to determine whether the biological control agents are compatible with any chemicals which will also be applied (Jager *et al.*, 1991). Integrated approaches to *R. solani* disease control could employ combinations of chemical, biological and cultural control measures to suppress the pathogen (Sweetingham, 1996).

1.5.5.1 Harvesting and cultural strategies

The use of crop rotation has been shown to be effective in reducing the levels of soil borne *R. solani* in the soil, however, shorter rotation periods were found to coincide with a reduction in the efficacy of chemical seed tuber treatment with tolclofos-methyl. An increase of *R. solani* inoculum densities in the soil as a result of the shorter rotations was thought to be the main cause, as seed-derived disease was being eradicated chemically, and the soil therefore became the predominant source of inoculum (Hide & Read, 1991). Combining chemical haulm destruction with root severing was found to slightly reduce the

incidence of black scurf on progeny tubers compared to haulm pulling alone. Black scurf was also found to develop more slowly after haulm destruction if the root had been severed, which would allow a longer period in which to harvest before black scurf infestation becomes significant (Dijst *et al.*, 1986). Despite the range of work carried out on many control methods, it is likely that a combination of the control measures described previously would offer a solution to manage *R. solani* disease on potato.

1.5.5.2 Integrated control and V. biguttatum

Integrated control of *R. solani* using a combination of pencycuron at less than the recommended rate and *V. biguttatum* caused significant reductions in sclerotial development on progeny tubers and lower grading losses, with the level of control similar to those when using pencycuron at the recommended application rate (Jager *et al.*, 1991). Research into commonly used fungicides used to control many potato diseases showed that selected chemicals could improve the efficacy of *R. solani* control when combined with *V. biguttatum* (van den Boogert & Luttikholt, 2004). Broad-spectrum fungicides azoxystrobin and thiabendazole had a detrimental effect on the control of black scurf formation by *V. biguttatum* in a mini tuber bioassay, but co-application of the *R. solani* specific fungicides pencycuron and flutalonil with *V. biguttatum* showed additive effects for black scurf control (van den Boogert & Luttikholt, 2004).

1.6 Objective and aims

The overall objective of this study was to investigate the survival, formation and germination of *R. solani* sclerotia, as well as the pathogenicity and control of *R. solani* on potato. Although there is a large body of research on *R. solani* in general, many questions still remain unanswered regarding this plant pathogen, particularly with regards to isolates pathogenic to potato. The main aims were to investigate the:

1. environmental and nutritional factors affecting mycelial growth, sclerotia production and sclerotia germination *in vitro*;
2. infection process of *R. solani* by different AGs on potato stems;
3. survival of *R. solani* sclerotia in soil and the presence of mycoparasitic infection;
4. effect of soil-borne sclerotial inoculum levels on disease incidence and severity;
6. potential for control of soil-borne *R. solani* inoculum using commerically available biological control products, novel soil treatments and potential and known antagonistic fungi.

CHAPTER 2

ORIGIN AND MAINTENANCE OF FUNGAL CULTURES

2.1 *R. solani* and *V. biguttatum* isolate collection and storage

Details of the *R. solani* and *V. biguttatum* isolates used throughout this study and their origin are described in Table 2.1. All fungal isolates were stored on potato dextrose agar (PDA) (Sigma) slopes covered in paraffin oil (BDH laboratories) in the dark at room temperature until required. Both *R. solani* and *V. biguttatum* isolates were maintained on PDA (approximately 20 ml) dispensed into 9 cm Petri dishes prior to use in experiments. All media were autoclaved at 121°C and 103.4 kPa for 15 min unless otherwise stated. Isolates obtained from harvested sclerotia and evaluated as potential antagonists of *R. solani* are described in Table 7.2.

Table 2.1. Origin of fungal isolates used throughout this study.

Isolate code	Species	Geographical origin	Plant part isolated from	Provider
x46	*R. solani* AG 2-1	NW England	Stolon	HAUC
x81	*R. solani* AG 2-1	Scotland	Tuber	HAUC
PK	*R. solani* AG 3	Aberdeen	Tuber	SAC Aberdeen
x72	*R. solani* AG 3	Scotland	Tuber	HAUC
UN	*R. solani* AG 3	Shropshire	Tuber	HAUC
T1	*R. solani* AG 5	Shropshire	*Agropyron repens*[a]	HAUC
M73	*V. biguttatum*	The Netherlands	N/A	Warwick HRI, Wellesbourne
M92	*V. biguttatum*	The Netherlands	N/A	Warwick HRI, Wellesbourne

[a]isolated from couch grass found alongside a potato plant.

CHAPTER 3

FACTORS AFFECTING THE MYCELIAL GROWTH AND SCLEROTIAL PRODUCTION OF *R. SOLANI*

3.1 Introduction

The importance of *R. solani* as a destructive and widespread seed and soil-borne plant pathogen of potato is well established (Ogoshi, 1987; Tsror & Peretz-Alon, 2005). Despite this, there have been very few studies investigating the effect of environmental and nutritional factors on mycelial growth and sclerotial formation by *R. solani* isolates pathogenic to potato. Many studies on *R. solani* pathogenic to other plant species have focussed on the effect of environmental factors such as pH and osmotic potential on mycelial growth with no reference to sclerotial production (Sherwood, 1970; Dubé *et al.*, 1971; Sterne & McCarver, 1978). The effect of nutritional factors on mycelial growth and sclerotial production *in vitro* have been investigated for various isolates of *R. solani* (Allington, 1936; Townsend, 1957; Sanford, 1956). Again, these studies were completed using either isolates of *R. solani* from potato where the AG was unspecified, or the original host plant from which the isolate was taken was not reported.

There are currently limited data available regarding the effect of environmental and nutritional factors on *R. solani* pathogenic to potato, particularly regarding sclerotium yield. Previous methods employed when investigating the effect of different carbon and nitrogen sources on sclerotial production were predominantly qualitative, with sclerotial production recorded as one of several categories; poor, moderate or high (Allington, 1936; Townsend, 1957). The lack of a quantitative measurement of sclerotial production, such as sclerotium yield, in these previous studies makes it difficult to compare the effects of the different treatments. Further investigation into the effect of environmental and nutritional factors on *R. solani* isolates from different AGs on mycelial growth and sclerotium yield would therefore contribute to the existing knowledge of this plant pathogen on potato.

3.2 Materials and methods

Five isolates of *R. solani*, three AG 3 (UN, PK and x72) and two AG 2-1 (x46 and x81) were used in all mycelial growth and sclerotium yield experiments (section 2.1).

3.2.1 *In vitro* production of sclerotia on different media

To investigate the effect of nutrient availability on sclerotium yield and establish a suitable medium on which to mass produce sclerotia for future field and pot trials, all isolates were grown on four media. The effect of nutrient rich media was tested on potato dextrose agar (PDA) (Sigma) and malt yeast extract agar (MYA), containing (l^{-1} distilled water) 15 g malt extract (Sigma), 12 g Technical no. 3 agar (Oxoid), 5 g yeast extract (Oxoid). The effect of nutrient poor media was tested on soil extract agar (SEA) containing (l^{-1} distilled water) 250 ml soil extract, 12 g Technical agar no. 3 and distilled water agar (WA) containing (l^{-1} distilled water) 12 g Technical no. 3 agar. Soil extract was prepared by taking 100 g of a loam-sand soil (pH 6.0: Diamond Field, SAC Auchincruive) and adding it to 1-l of distilled water. This was left at room temperature for 3 d and the suspension filtered through Whatman no. 1 filter paper to remove soil and other debris. Finally, 250 ml of the filtered extract was made up to 1^{-1} with distilled water and the appropriate amount of agar added prior to autoclaving. All media were autoclaved at 121°C for 15 min and then poured into 9 cm diam Petri dishes when it had cooled to 50°C.

To inoculate Petri dishes, mycelial plugs with a diam of 3 mm were removed with a cork borer from the periphery of 3-d old colonies of *R. solani* grown on PDA at 25°C. These were placed centrally in Petri dishes. These were then sealed with Parafilm and arranged in a randomised block design within an incubator (25°C). There were four replicates per treatment.

To determine the radial mycelial growth rate, two lines were drawn across the base of the Petri dishes crossing at the central inoculation site of the plate at right angles. Measurements were taken along the four axes 2 to 6-d after initial inoculation at 24 h intervals. These were used to calculate the average radial mycelial growth rate in mm d^{-1}.

Sclerotium yield (total dry weight biomass (mg) per dish) was determined after 21 d by removing the mature brown sclerotial mats from the surface of each individual agar plate using a sharp scalpel. The mats were placed on a sieve (mesh size 250 μm), and thoroughly washed under running water to remove the agar. Sclerotia were subsequently filtered through pre-weighed filter papers (Whatman no. 1) which had been oven-dried at 70°C for 48 h. Filter papers plus sclerotia were then placed in the same oven (70°C) and the mass of sclerotia produced was determined after 48 h.

3.2.2 Effect of nutrient availability on sclerotium yield

The effect of nutrient availability on sclerotium yield was investigated using a modification of an established method previously used by Dijst (1988). Petri dishes containing PDA and WA were covered in autoclaved (121°C and 103.4 KPa for 15 min) 90 mm diam cellophane discs (PT 600; British Cellophane Co.). Petri dishes were inoculated as described previously (section 3.2.1), and incubated at 25°C for 4 d, with four replicates for each treatment. After 4 d, the Petri dishes were removed from incubation, and six treatments applied (Table 3.1). Cellophane discs and mycelial mats were aseptically removed and transferred to fresh media using forceps. Petri dishes were incubated at 25°C and sclerotium yield determined after 21 d as described previously (section 3.2.1).

Table 3.1. Treatments applied to investigate the effect of nutrient availability on sclerotium yield by *R. solani*.

Initial growth media	Fresh media
PDA	PDA[a]
PDA	WA
PDA	-[b]
WA	WA
WA	PDA
WA	-

[a]isolate transferred to new growth media after 4 d.
[b]no transfer to fresh media was made.

3.2.3 Effect of carbon source on sclerotium yield

A basal growth medium containing (l^{-1} distilled water) 1.75 g KH_2PO_4 (Sigma), 0.75 g, $MgSO_4 \cdot 7H_2O$ (Sigma) and 15 g Technical no. 3 agar (Oxoid) was used routinely (Townsend, 1957). It was prepared by mixing appropriate amounts of individual chemical solutions that had been autoclaved (15 min at 121 °C and 103.4 KPa) separately. The ability of *R. solani* to utilise several carbon sources (Table 3.2) for mycelial growth and sclerotial production was tested on the basal medium with KNO_3 (3.5 g l^{-1}, 0.48 g N l^{-1}) as the N source. All carbon sources were autoclaved separately and added to the batches of sterile medium, except for disacchaarides which were added to the medium after filter sterilisation. The total amount of carbon added in each case was 16 g l^{-1}. Allowance for the carbon and nitrogen content of agar was not made. Media were adjusted to pH 5.6 using 1 M NaOH and 20 ml of each was dispensed into four replicate 9 cm diam Petri dishes. Petri dishes were inoculated and incubated at 25°C, with sclerotium yield determined as described previously (section 3.2.1).

3.2.4 Effect of nitrogen source on sclerotium yield

The effect of different nitrogen sources (Table 3.3) was tested in the basal medium with D-glucose (40 g l^{-1}, 16 g C l^{-1}) as the carbon source. All inorganic and organic sources were autoclaved separately and added to batches of sterile medium to give a nitrogen level of 0.48 g l^{-1}. The pH of all media was adjusted to pH 5.6. There were four replicates per treatment. Petri dishes were inoculated, incubated at 25°C, and sclerotium yield determined as described previously (section 3.2.1).

Table 3.2. Carbon sources

Type of sugar	Carbon source
Pentose	D - Xylose
Hexose	D - Glucose
Disaccharides	Maltose
	Sucrose
	Cellobiose
Sugar alcohols	D - Mannitol
	Glycerol

Table 3.3. Nitrogen sources

Type of nitrogen	Nitrogen source
Inorganic	Ammonium chloride
	Potassium nitrate
Organic	L - Alanine
	L - Arginine
	L - Asparagine
	L - Glycine
	L - Proline

3.2.5 Effect of carbon to nitrogen (C:N) ratio on mycelial growth and sclerotium yield

To study the effect of C:N ratio, the basal medium was supplemented with different amounts of D-glucose and KNO_3. The C:N ratio of the medium was adjusted between 4 : 1 and 128 : 1 either by varying the concentration of D-glucose with KNO_3 fixed at 3.5 g l^{-1} (0.48 g nitrogen l^{-1}), or by adjusting the concentration of KNO_3 with D-glucose fixed at 40 g l^{-1} (16 g carbon l^{-1}). In calculating the C:N ratios, allowance for the carbon and nitrogen contents of the agar was not made. The pH of all media was adjusted to pH 5.6 using 1 M NaOH. Petri dishes were inoculated, incubated at 25°C and growth rates and sclerotium yields determined as described previously (section 3.2.1).

3.2.6 Effect of pH on mycelial growth and sclerotium yield

To examine the effects of medium pH, batches of sterile double-strength PDA were adjusted with 1 M NaOH or HCl to the required pH and an equal volume of buffer was added to give the correct concentration of medium. The pH was maintained over the ranges of 5 – 7 and 8 – 9 with citrate phosphate (0.05 M citric acid, 0.1 M $Na_2HPO_4 \cdot 7H_2O$) and Tris (hydroymethyl) aminoethane (0.1 M Tris, 0.1 M HCl) buffers, respectively (Gomori, 1955). Petri dishes were inoculated, incubated at 25°C and growth rates and sclerotium yield determined as described previously (section 3.2.1).

3.2.7 Effect of temperature on mycelial growth and sclerotium yield

3.2.7.1 Mycelial growth and sclerotium yield on agar

The effect of temperature and growth medium on radial mycelial growth and sclerotium yield by *R. solani* was determined at 5, 10, 15, 20, 25 and 30°C on PDA, MYA and WA. Media were prepared as described previously (section 3.2.1) and approximately 20 ml was poured into individual 9 cm diam Petri dishes. Inoculation of Petri dishes, radial

mycelial growth rate and sclerotium yield were all carried out as described previously (section 3.2.1). The Petri dishes were arranged in each incubator in a randomized block design with four replicates for each temperature.

3.2.7.2 Mycelial growth in soil

A soil sandwich technique modified from Grose *et al.*, (1984) was used to investigate the effect of temperature on mycelial growth. A brown earth soil (Dunnington Heath series) was passed through a 3 mm sieve and air-dried for 7 d. The soil was adjusted to – 0.5 MPa (0.070 g of water per g^{-1} of soil) in accordance with a soil moisture retention curve (w/w). Water was added as a fine spray and mixed thoroughly through the soil. Soil at each matric potential was then added to four replicate 9 cm diam. Petri dishes and a cellulose nitrate filter (85 mm diam, pore size 0.45 μm; Sartorius, Germany) was placed over the surface to act as a platform for mycelial growth (Grose *et al.*, 1984). Two black lines were drawn across the filter at right angles through the centre using a marker pen. The centre of each filter was inoculated with a 3 mm diam mycelial disc of PDA and covered with a 9 x 9 cm square piece of nylon netting. Further soil was added to cover the nylon netting and dishes were sealed with parafilm, weighed and incubated at 5, 10, 15, 20, 25 and 30 °C. Dishes were re-weighed every 3 d to maintain the desired matric potential. After 10 d, the filters were retrieved and the radial mycelial growth across the surface of the filter determined along the four axes marked on the filter.

3.2.8 Effect of water potential on mycelial growth and sclerotium yield

3.2.8.1 Mycelial growth and sclerotium yield on agar

PDA was adjusted osmotically over the range of – 0.8 MPa to – 5.0 MPa with sodium chloride (NaCl, Sigma) (Lang, 1967), potassium chloride (KCl, Sigma) (Campbell & Gardener, 1971) and glycerol (Sigma) (Dallyn & Fox, 1980). The total water potential was the sum of the water potential of the PDA (– 0.4 MPa) and the osmotic potential of the added osmotia. Sterile medium (20 ml) adjusted with NaCl, KCl or glycerol was dispensed into 9 cm diam Petri dishes. Four replicate dishes were used for each treatment. All Petri dishes were incubated at 25°C and the inoculation method, mycelial growth rate and sclerotium yield were determined as described previously (section. 3.2.1).

The matric potential of PDA was adjusted using polyethylene glycol (PEG 6000) (Steuter *et al.*, 1981) PEG 6000 (g kg^{-1} liquid) of different concentrations (equivalent to – 0.4, – 1.1, – 1.6 & – 2.1 MPa) for incubation temperatures of 25°C were determined using a formula derived by (Michel & Kaufmann, 1973). The total water potential was the sum of the water potential of PDA and the matric potential of the added PEG 6000. Concentrations of PEG 6000 were autoclaved separately, cooled to 50°C and added to the PDA to give the required matric potential. Sterile medium (20 ml) of PDA/PEG 6000 at each matric potential was dispensed into four replicate 9 cm diam Petri dishes. Autoclaved 9 cm diam cellophane discs (PT 600; British Cellophane Co.) were placed on the matrically adjusted media to provide a suitable platform for growth and sclerotial production, as solutions of PDA/PEG 6000 do not solidify completely below –1.5 MPa. Petri dishes were sealed in plastic bags over the course of the experiment to reduce water loss. Inoculation, mycelial growth rate and sclerotium yield were determined as described previously (section 3.2.1).

3.2.8.2 Total soil water potential and mycelial growth

Appropriate amounts of soil were mixed with sterile distilled water (0.070, 0.063, 0.057, 0.050, 0.046, 0.044 g of water per g^{-1} of soil) to obtain six matric potentials (– 0.5, – 1.0, – 2.0, – 4.1, – 6.3 and – 7.8 MPa) in accordance with a soil moisture retention curve (w/w). Petri dishes were then prepared and inoculated as described previously (section 3.9.2.2). There were four replicates for each treatment and Petri dishes were incubated at 20°C for 10 d. Filters were retrieved and the mycelial growth rate across the filter surface determined as described previously (section 3.2.7.2).

3.2.9 Statistical analysis

Histograms of the residuals and plots of the residuals versus fitted values were determined to check homogeneity of variance and the normality of the distribution of the data. Data found in violation of the normality assumption were log transformed prior to an analysis of variance (ANOVA), where treatment means were compared using the least significant difference (LSD) at a probability of 5 % ($P = 0.05$). The effect of temperature on mycelial growth and sclerotium yield on agar was further analysed using multiple linear regression. A quadratic model term of the form log (mycelial growth rate or sclerotium yield) = $\alpha + \beta t + \chi t^2$ was fitted where t = temperature to determine whether there were significant differences in the effect of temperature between different isolates and, if applicable, media. Parameter estimates were calculated using isolate PK and medium MYA as the base line reference level and pairwise t probabilities were calculated. These were used to establish significance levels of difference between the effects of the different isolates and media on mycelial growth and sclerotium yield. The effect of temperature on mycelial growth in soil was analysed in the same way, using PK as the baseline reference level. Data were analysed using Genstat® for windows, 7th Edition.

3.3 Results

3.3.1 Mycelial growth rate and i*n vitro* production of sclerotia on different media

Mycelial growth occurred on all media tested, with significantly slower mycelial growth on SEA for several isolates (Table 3.4). There were significant differences (P = 0.05) between sclerotium yield on nutrient poor and nutrient rich media (Table 3.5). Sclerotial production was minimal on WA and SEA, with significantly more sclerotia produced on PDA and MYA by most isolates, particularly those from AG 3. MYA was the better medium for production of sclerotia by AG 3 isolates, whereas AG 2-1 isolates produced more sclerotia on PDA.

3.3.2 Effect of nutrient availability on sclerotium yield

There were significant differences in sclerotium yield with regard to nutritent availability (Table 3.6). Both AG 2-1 and AG 3 isolates produced fewer sclerotia when grown on nutrient poor WA, and there were no further increases in sclerotium yield when growing mycelium was transferred to fresh WA or left on WA for 21 d. When growing mycelia of AG 3 isolates were transferred to PDA from WA, sclerotium yields increased significantly. This effect, however, was not observed for AG 2-1 isolates. Isolates grown on PDA initially and transferred to nutrient poor WA produced higher sclerotium yields than those observed when *R. solani* was grown on WA alone. All *R. solani* isolates produced significantly (P = 0.05) more sclerotia when mycelium was initially grown on or transferred to a nutrient rich media, compared to mycelium that was grown on nutrient poor media alone.

Table 3.4. Radial mycelial growth rate (mm d^{-1}) of five isolates of *R. solani* on different types of media at 25°C.

	Media			
Isolate (AG)	WA	SEA	MYA	PDA
x81 (2-1)	10.2[a]	7.4	7.3	8.3
x46 (2-1)	7.8	5.1	6.9	8.4
UN (3)	10.4	7.7	9.5	9.7
PK (3)	10.7	6.4	10.0	10.2
x72 (3)	8.1	6.0	7.3	8.3
P value		0.002		
LSD ($P = 0.05$), DF = 57[b]		2.03		

[a]Values are mean mycelial growth rates (mm d^{-1}) of four replicates measured between 2 and 6 d.
[b]LSD is the least significant difference at 5 % ($P = 0.05$), DF = degrees of freedom in ANOVA.

Table 3.5. Sclerotium yield [total dry weight biomass (mg) per dish] by five isolates of *R. solani* after 21 d growth on different media at 25°C.

	Media			
Isolate (AG)	WA	SEA	MYA	PDA
x81 (2-1)	1.0[a]	0.8	2.03	13.5
x46 (2-1)	0.0	0.9	1.7	14.7
UN (3)	0.0	0.8	46.9	46.4
PK (3)	0.0	0.5	47.2	35.8
x72 (3)	0.0	0.4	70.7	53.8
P value		<0.001		
LSD ($P = 0.05$), DF = 57[b]		7.99		

[a]Values are the total dry weight biomass of sclerotia from four replicates after drying for 48 h at 70°C.
[b]LSD is the least significant difference at 5 % ($P = 0.05$), DF = degrees of freedom in ANOVA.

Table 3.6. Effect of transferring mycelial mats of five isolates of *R. solani* to high and low nutrient media on sclerotium yield [total dry weight biomass (mg) per plate].

	Transfers					
	WA			PDA		
Isolate (AG)	No transfer	WA	PDA	No transfer	PDA	WA
x81 (2-1)	3.0[a]	2.0	5.2	16.0	10.4	10.7
x46 (2-1)	0.7	0.4	9.2	14.6	7.0	7.1
UN (3)	4.4	4.6	55.6	36.4	48.4	28.0
PK (3)	8.2	9.8	32.6	38.6	56.0	29.9
x72 (3)	3.0	2.1	36.4	67.9	36.7	48.2
P value	<0.001					
LSD ($P = 0.05$), DF = 116[b]	18.41					

[a]Values are the total dry weight biomass of sclerotia from four replicates after drying for 48 h at 70°C.
[b]LSD is the least significant difference at 5 % ($P = 0.05$), DF = degrees of freedom in ANOVA.

3.3.3 Effect of carbon source on sclerotium yield

Sclerotium yields of AG 2-1 isolate x46 were minimal on all carbon sources tested and none were produced on xylose and the carbon-free control, whereas AG 2-1 isolate x81 produced sclerotia only on the medium containing glycerol (Table 3.7). All AG 3 isolates produced sclerotia on every carbon source tested, with sclerotium yields significantly greater ($P = 0.05$) than the carbon-free control on cellobiose, glucose and glycerol. Glycerol was the only carbon source on which all AG 2-1 and AG 3 isolates of *R. solani* produced sclerotia.

3.3.4 Effect of nitrogen source on sclerotium yield

No sclerotia were produced on the control medium by any AG 2-1 or AG 3 isolate, when nitrogen was absent (Table 3.8). Isolate x81 (AG 2-1) produced no sclerotia on any of the nitrogen sources tested, whereas x46 (AG 2-1) produced small sclerotium yields on potassium nitrate and L-arginine only. All AG 3 isolates produced the greatest sclerotium yields when potassium nitrate was the nitrogen source, with x72 the only AG 3 isolate producing significantly greater ($P = 0.05$) sclerotium yields than the control when L-proline, L-alanine, L-arginine and L-glycine were the nitrogen sources.

Table 3.7. Effect of carbon source on sclerotium yield [total dry weight biomass (mg) per dish] by five isolates of *R. solani* at 25°C.

Isolate	Carbon source							
(AG)	Control	D - Glucose	Maltose	D - Xylose	Sucrose	Cellobiose	D - Mannitol	Glycerol
x81 (2-1)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	9.4
x46 (2-1)	0.0	4.1	4.0	0.0	1.6	4.2	3.1	15.4
x72 (3)	3.1	23.8	10.9	6.1	14.2	21.1	9.5	28.8
UN (3)	2.3	18.0	7.1	12.4	6.7	16.1	6.7	14.8
PK (3)	3.6	17.9	11.0	9.6	5.2	21.6	9.8	17.4
P value				<0.001				
LSD ($P = 0.05$), DF = 117[b]				8.07				

[a]Values are the mean total dry weight biomass of sclerotia from four replicate dishes (mg per dish).
[b]LSD is the least significant difference at 5 % ($P = 0.05$), DF = degrees of freedom in ANOVA.

Table 3.8. Effect of nitrogen source on sclerotium yield [total dry weight biomass (mg) per dish] by five isolates of *R. solani* at 25°C.

Isolate (AG)	Nitrogen source							
	Control	L-Proline	L-Alanine	L-Asparagine	L-arginine	Ammonium chloride	Potassium nitrate	L-Glycine
x81 (2-1)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
x46 (2-1)	0.0	0.0	0.0	0.0	1.9	8.1	7.1	0.0
x72 (3)	0.0	32.6	28.4	12.5	17.5	8.8	50.3	28.0
UN (3)	0.0	8.4	5.5	10.0	7.8	16.0	19.7	0.0
PK (3)	0.0	0.0	0.0	10.5	0.0	15.5	27.0	6.0
P value				<0.001				
LSD ($P = 0.05$), DF = 117[b]				9.92				

[a]Values are the mean total dry weight biomass of sclerotia from four replicate dishes (mg per dish).
[b]LSD is the least significant difference at 5 % ($P = 0.05$), DF = degrees of freedom in ANOVA.

3.3.5 Effect of carbon to nitrogen (C:N) ratio on mycelial growth and sclerotium yield

The effect of C:N ratio on mycelial growth of *R. solani* depended on whether the carbon or nitrogen concentration was altered. When the carbon concentration was kept constant and the nitrogen concentration altered, mycelial growth rate increased for all isolates from C:N ratios of 4:1 to 16:1, with no further increases in mycelial growth beyond this (Table 3.9). Isolate x81 (AG 2-1) produced very few sclerotia at 64:1, and no sclerotia were produced by AG 2-1 isolate x46 (Table 3.9). Sclerotial production occurred from 4:1 to 64:1 for all AG 3 isolates with no sclerotia formed at 128:1 (Table 3.10).

When the nitrogen concentration was kept constant and the carbon concentration altered, increasing C:N ratios resulted in reductions in mycelial growth rate from ratios of 16:1 to 128:1 (Table 3.11). No sclerotia were produced by the AG 2-1 isolate x81, with x46 producing few sclerotia at C:N ratios of 4:1 and 8:1 (Table 3.12). All AG 3 isolates produced the greatest sclerotium yields on media with a C:N ratio between 4:1 and 16:1, with sclerotia production declining significantly ($P = 0.05$) from 16:1 to 128:1.

3.3.6 Effect of pH on mycelial growth and sclerotium yield

All isolates of *R. solani* were able to grow over a pH range of 4 to 9, with the optimum for mycelial growth at pH 5.6 (Figure 3.1). Mycelial growth was significantly restricted ($P = 0.05$) at pH 4 and 9 for all isolates compared to the optimum pH 5.6. Sclerotial production was observed between pH 4 and 8 for AG 3 isolates, with sclerotial production by AG 2-1 isolates observed between pH 5 and 6 (Table 3.13).

Table 3.9. Effect of C:N ratio on the radial mycelial growth rate (mm d^{-1}) of five isolates of *R. solani* at 25°C [Carbon source constant (40 g glucose l^{-1} = 16 g C l^{-1})].

	C:N ratio [amount of N (g l^{-1})]					
	4:1	8:1	16:1	32:1	64:1	128:1
Isolate (AG)	[1.92]	[0.96]	[0.48]	[0.24]	[0.12]	[0.06]
x81 (2-1)	5.6[a]	8.1	10.0	10.4	11.3	10.4
x46 (2-1)	3.7	7.5	8.9	10.1	10.2	9.9
x72 (3)	1.6	4.7	5.9	5.8	5.7	6.2
UN (3)	3.3	6.8	8.4	9.2	9.9	10.2
PK (3)	2.8	6.3	7.5	6.8	7.5	7.5
P value			<0.001			
LSD (*P* = 0.05),			0.82			
DF = 87[b]						

[a]Values are mean mycelial growth rates (mm d^{-1}) of four replicates measured between 2 and 6 d.
[b]LSD is the least significant difference at 5 % (*P* = 0.05), DF = degrees of freedom in ANOVA.

Table 3.10. Effect of C:N ratio on sclerotium yield [total dry weight biomass (mg) per dish] of five isolates of *R. solani* at 25°C [Carbon source constant (40 g glucose l^{-1} = 16 g C l^{-1})].

	C:N ratio [amount of N (g l^{-1})]					
	4:1	8:1	16:1	32:1	64:1	128:1
Isolate (AG)	[1.92]	[0.96]	[0.48]	[0.24]	[0.12]	[0.06]
x81 (2-1)	0.0[a]	0.0	0.0	0.0	4.1	0.0
x46 (2-1)	0.0	0.0	0.0	0.0	0.0	0.0
x72 (3)	16.8	14.2	21.2	21.5	8.2	0.0
UN (3)	16.0	9.5	13.4	12.8	13.4	0.0
PK (3)	4.5	3.8	16.7	10.9	4.4	0.0
P value			0.003			
LSD (*P* = 0.05), DF =			10.89			
87[b]						

[a]Values are the mean total dry weight biomass of sclerotia from four replicate dishes (mg per dish).
[b]LSD is the least significant difference at 5 % (*P* = 0.05), DF = degrees of freedom in ANOVA.

Table 3.11. Effect carbon to nitrogen ratio on radial mycelial growth rate of five isolates of *R. solani* at 25°C [Nitrogen source constant (3.5 g KNO_3 l^{-1}= 0.48 g N l^{-1})].

	Carbon to nitrogen ratio [amount of C (g l^{-1})]					
	4:1	8:1	16:1	32:1	64:1	128:1
Isolate (AG)	[2.0]	[4.0]	[8.0]	[16.0]	[32.0]	[64.0]
x81 (2-1)	12.2[a]	12.0	12.1	10.1	8.7	3.4
x46 (2-1)	12.2	11.5	11.9	9.5	8.2	2.5
x72 (3)	8.8	8.1	9.1	7.8	5.0	1.9
UN (3)	12.8	12.0	11.2	9.1	9.2	2.4
PK (3)	8.4	8.9	9.1	8.3	6.4	2.5
P value			<0.001			
LSD (*P* = 0.05), DF = 87[b]			1.16			

[a]Values are mean mycelial growth rates (mm d^{-1}) of four replicates measured between 2 and 6 d.
[b]LSD is the least significant difference at 5 % (*P* = 0.05), DF = degrees of freedom in ANOVA.

Table 3.12. Effect of C:N ratio on sclerotium yield [total dry weight biomass (mg) per dish] of five isolates of *R. solani* at 25°C [Nitrogen source constant (3.5 g KNO_3 l^{-1}= 0.48 g N l^{-1})].

	Carbon to nitrogen ratio [amount of C (g l^{-1})]					
	4:1	8:1	16:1	32:1	64:1	128:1
Isolate (AG)	[2.0]	[4.0]	[8.0]	[16.0]	[32.0]	[64.0]
x81 (2-1)	0.0[a]	0.0	0.0	0.0	0.0	0.0
x46 (2-1)	10.4	9.4	0.0	0.0	0.0	0.0
x72 (3)	30.5	23.3	36.5	16.7	7.2	0.0
UN (3)	33.4	26.2	33.0	9.8	5.1	0.0
PK (3)	27.8	26.3	10.0	10.6	6.4	4.7
P value			<0.001			
LSD (*P* = 0.05), DF = 87[b]			15.20			

[a]Values are the mean total dry weight biomass of sclerotia from four replicate dishes (mg per dish).
[b]LSD is the least significant difference at 5 % (*P* = 0.05), DF = degrees of freedom in ANOVA.

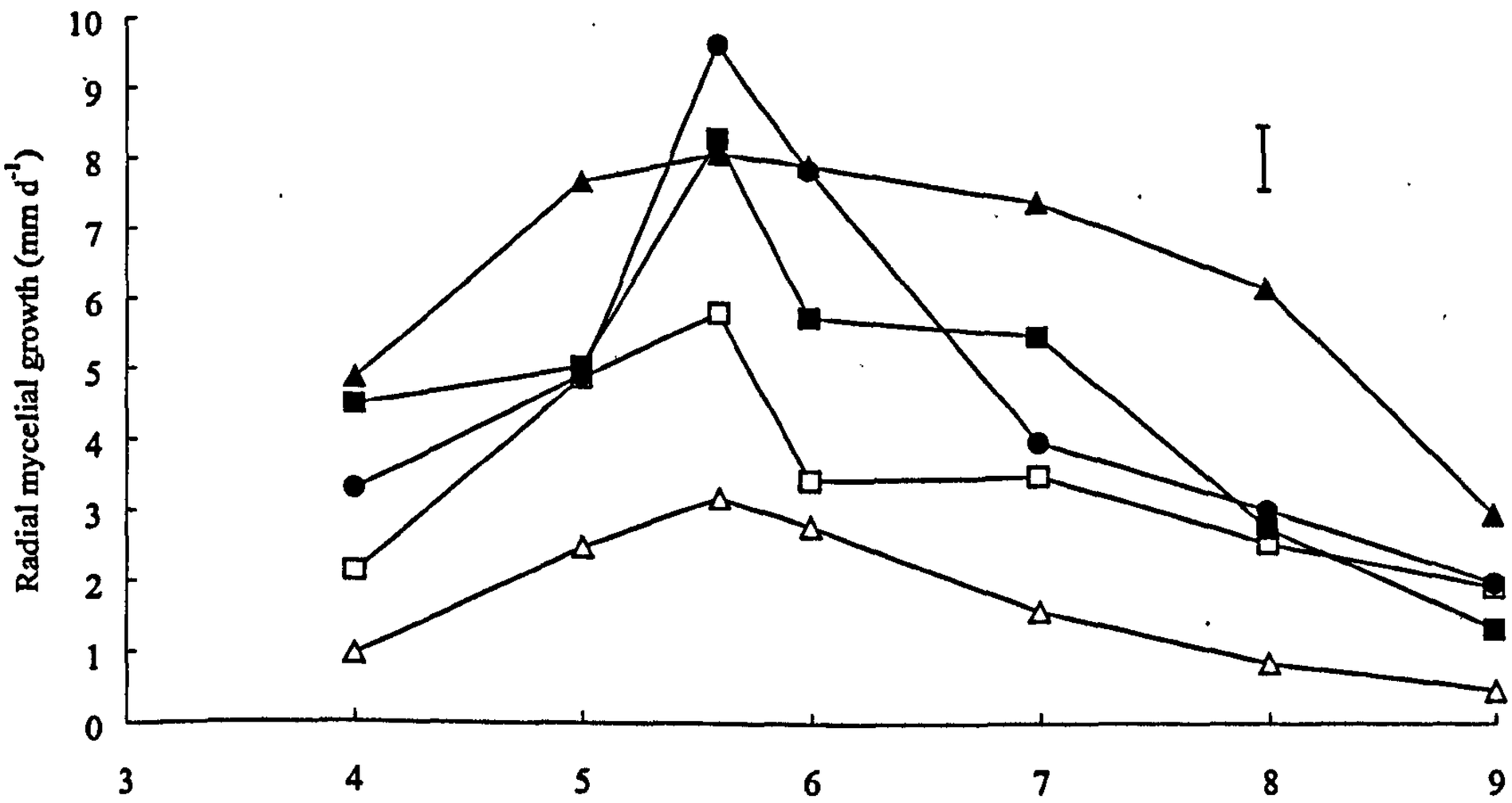


Figure 3.1. Effect of pH on radial mycelial growth rate of five isolates [x46 □, x81 Δ, x72 ■, UN ▲ and PK ●] of *R. solani* on buffered PDA at 25°C. Values are the mean radial mycelial growth rates (mm d^{-1}) of four replicates between 2 and 6 d. Bar = LSD at $P = 0.05$ (DF =102).

Table 3.13. Effect of pH on sclerotial yield [total dry weight biomass (mg) per dish] by five isolates of *R. solani* after 21 d growth at 25°C on PDA.

	pH						
Isolate (AG)	4.0	5.0	5.6	6.0	7.0	8.0	9.0
x81 (2-1)	0.0[a]	0.8	6.3	1.1	0.0	0.0	0.0
x46 (2-1)	0.0	1.2	3.7	3.4	0.0	0.0	0.0
UN (3)	5.9	8.3	27.2	18.2	12.2	0.0	0.0
PK (3)	0.0	15.1	22.0	15.0	6.5	0.0	0.0
x72 (3)	8.5	9.6	28.9	11.2	0.0	10.5	0.0
P value	<0.001						
LSD (*P* = 0.05),	7.51						
DF = 102[b]							

[a]Values are mean total dry weight biomass (mg) from four replicate dishes after drying for 48 h at 70°C.
[b]LSD is the least significant difference at 5 % (*P* =0.05), DF = degrees of freedom in ANOVA.

3.3.7 Effect of temperature on mycelial growth and sclerotium yield

3.3.7.1 Mycelial growth and sclerotial production on agar

Temperature had a significant effect on mycelial growth rate (P = <0.001), with the optimum observed for all isolates between 20 – 25°C on all media tested (Figure 3.2). Multiple linear regression analysis showed there were no significant differences between the growth rates of any AG 3 isolates at each temperature on all media tested. Further analysis revealed that the mycelial growth of isolate x81 (AG 2-1) on WA (P = 0.030) and isolate x46 (AG 2-1) on PDA (P = 0.002) was significantly different to the isolates on all other media tested.

There was a marked effect of temperature on sclerotial production with the optimum between 20 – 25°C for all isolates (Figure 3.3). Sclerotium yield on WA was almost zero for all isolates at all temperatures and the data were excluded from further analysis. Multiple linear regression analysis of isolate growth on PDA and MYA revealed there was a significant effect of temperature (P = <0.001) on sclerotium yield. Sclerotia were not produced by any isolate on any media at 5°C, and there were no significant differences between AG 3 isolates with regards to sclerotium yield. The AG 2-1 isolates x81 and x46 produced significantly less sclerotia (P = 0.007 and 0.002, respectively) than AG 3 isolates.

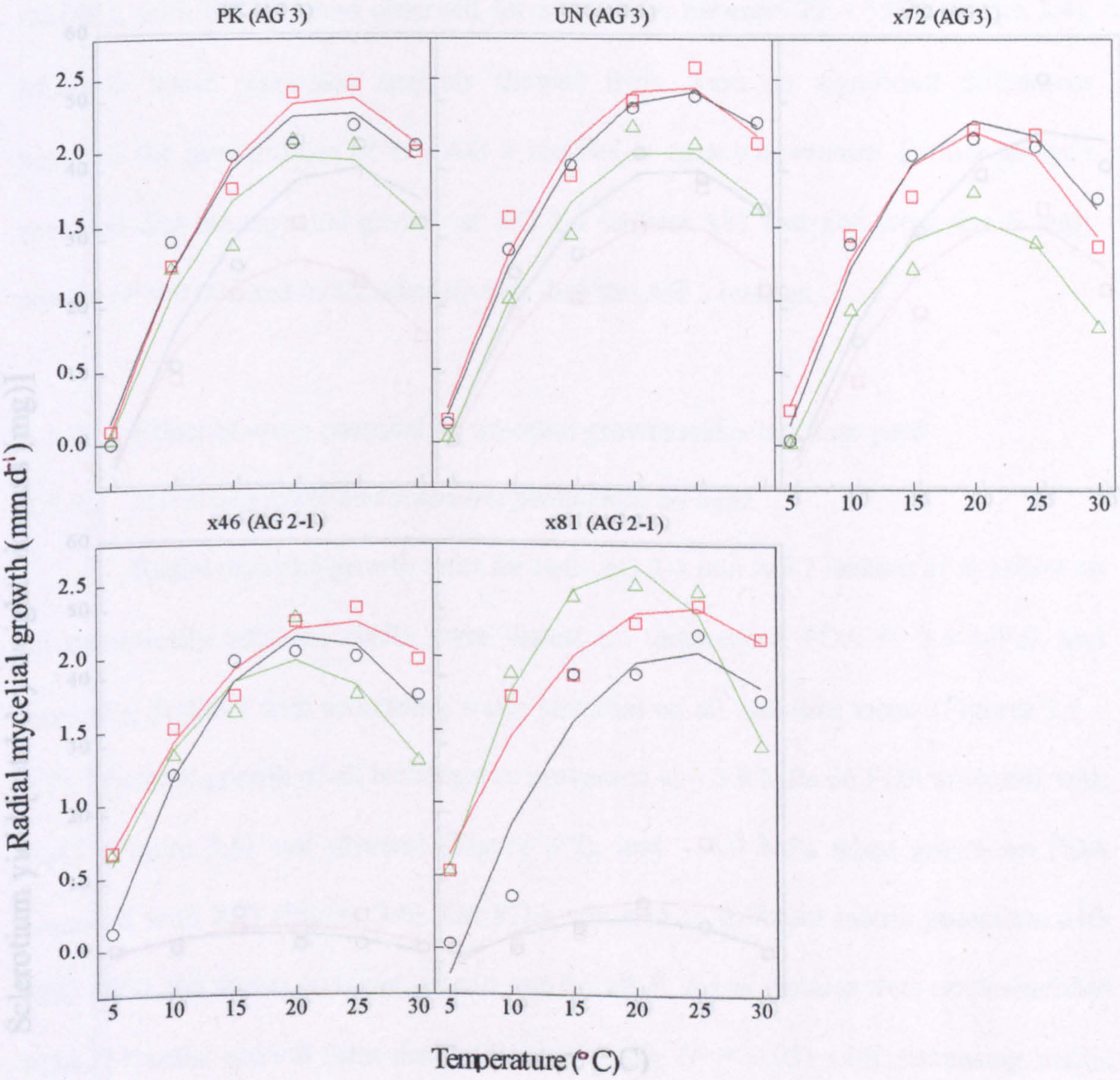


Figure 3.2. Multiple linear regression analysis on the effect of temperature and media on mycelial growth rate by five isolates of *R. solani* at 25 °C. Points represent the log transformed means of the original data values and lines show the fitted model for each medium tested (green = WA, red = PDA, black = MYA). $R^2 = 93.2$, $P = <0.001$ (DF = 44).

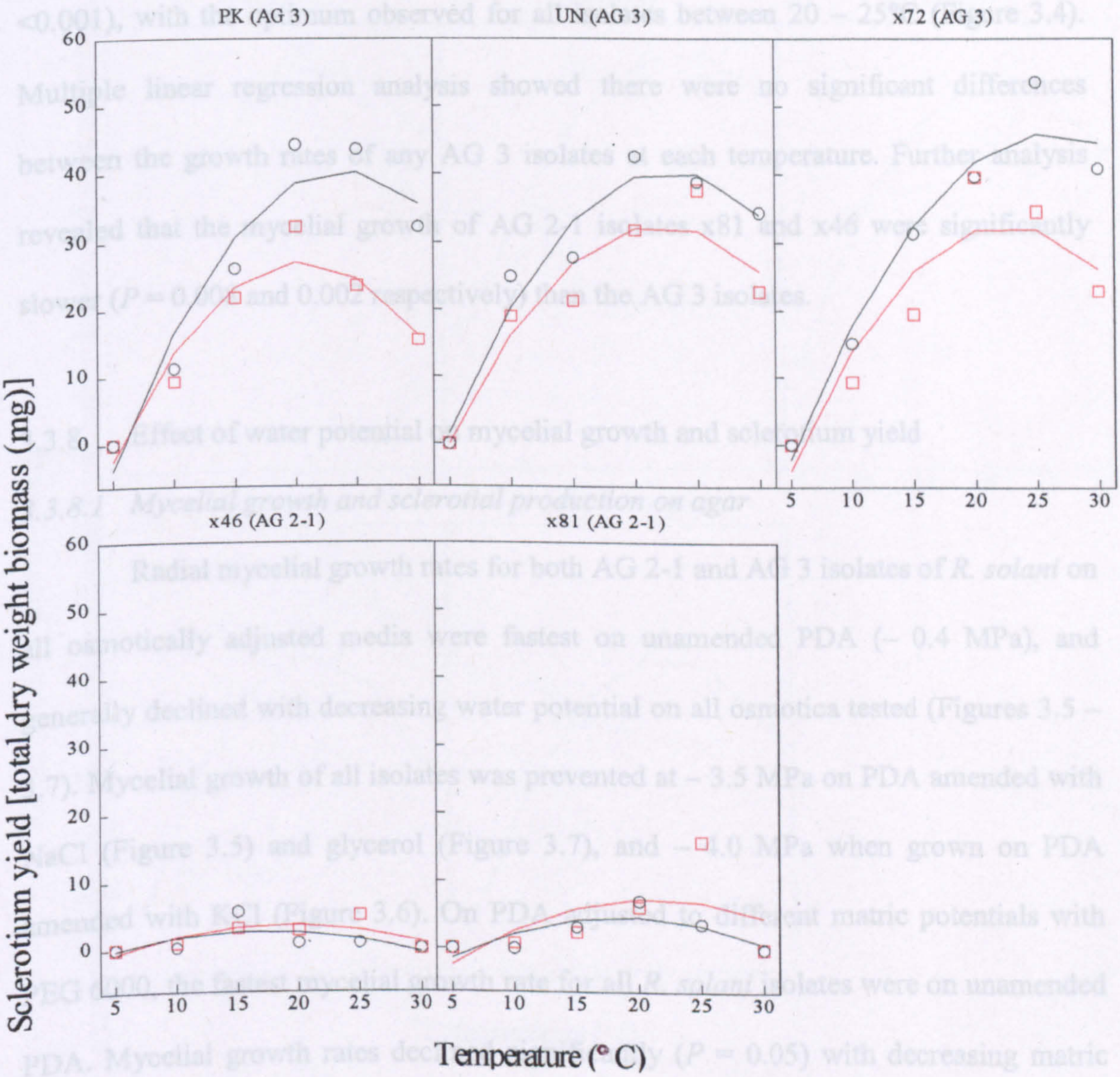


Figure 3.3. Multiple linear regression analysis on the effect of temperature and media on sclerotium yield [total dry weight biomass (mg) per plate] by five isolates of *R. solani* at 25°C. Points represent the log transformed means of the original data values and lines show the fitted model for each media tested (red = PDA, black = MYA). $R^2 = 80.9$, $P = <0.001$ (DF = 29).

3.3.7.2 Temperature and mycelial growth in soil

Temperature had a significant effect on mycelial growth rate through soil (P = <0.001), with the optimum observed for all isolates between 20 – 25°C (Figure 3.4). Multiple linear regression analysis showed there were no significant differences between the growth rates of any AG 3 isolates at each temperature. Further analysis revealed that the mycelial growth of AG 2-1 isolates x81 and x46 were significantly slower (P = 0.008 and 0.002 respectively) than the AG 3 isolates.

3.3.8 Effect of water potential on mycelial growth and sclerotium yield

3.3.8.1 Mycelial growth and sclerotial production on agar

Radial mycelial growth rates for both AG 2-1 and AG 3 isolates of *R. solani* on all osmotically adjusted media were fastest on unamended PDA (– 0.4 MPa), and generally declined with decreasing water potential on all osmotica tested (Figures 3.5 – 3.7). Mycelial growth of all isolates was prevented at – 3.5 MPa on PDA amended with NaCl (Figure 3.5) and glycerol (Figure 3.7), and – 4.0 MPa when grown on PDA amended with KCl (Figure 3.6). On PDA adjusted to different matric potentials with PEG 6000, the fastest mycelial growth rate for all *R. solani* isolates were on unamended PDA. Mycelial growth rates declined significantly (P = 0.05) with decreasing matric potential over the range of – 0.4 to – 0.8 MPa. Between – 0.8 to – 1.5 MPa, growth of all isolates decreased slightly, and complete inhibition was observed at – 2.0 MPa for all isolates (Figure 3.8).

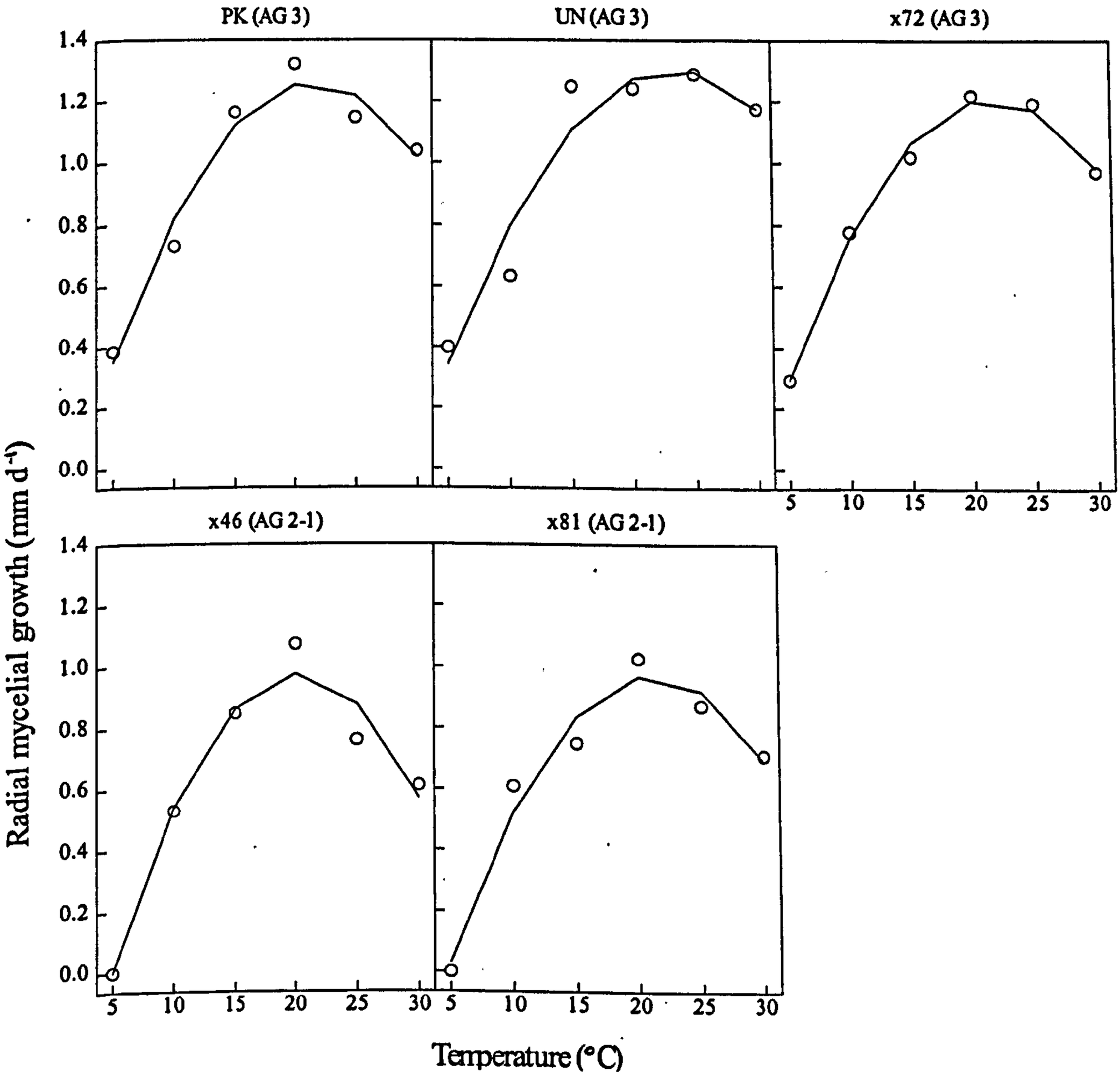


Figure 3.4. Multiple linear regression analysis on the effect of temperature on mycelial growth rate through unsterile soil by five isolates of *R. solani* at 20°C. Points represent the log transformed means of the original data values and lines show the fitted model for each isolate. $R^2 = 85.8$, $P = <0.001$ (DF = 119).

Figure 3.5. (NaCl)

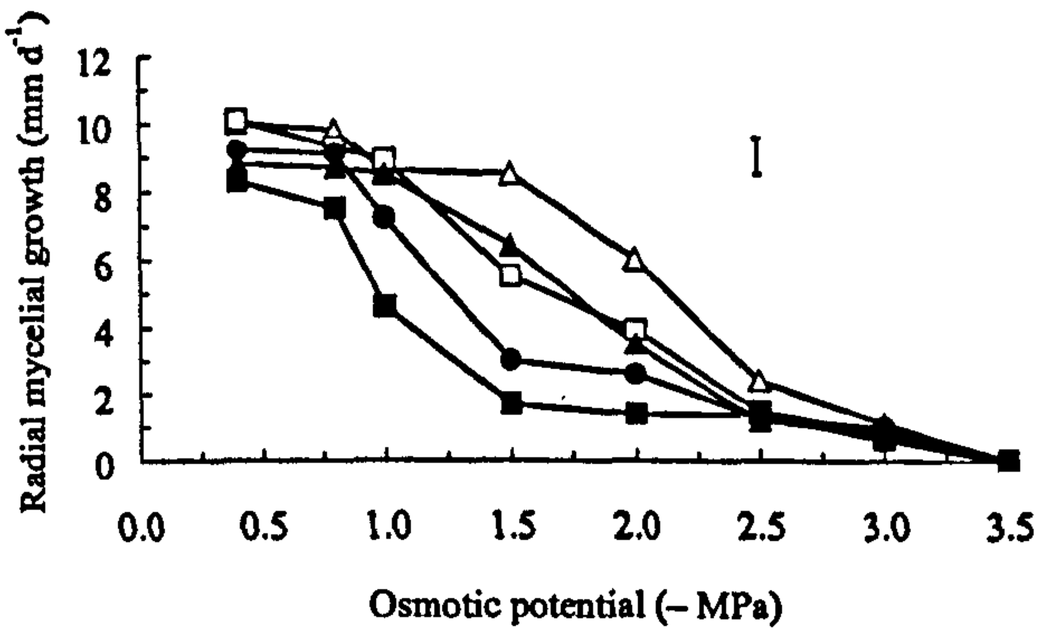


Figure 3.6. (KCl)

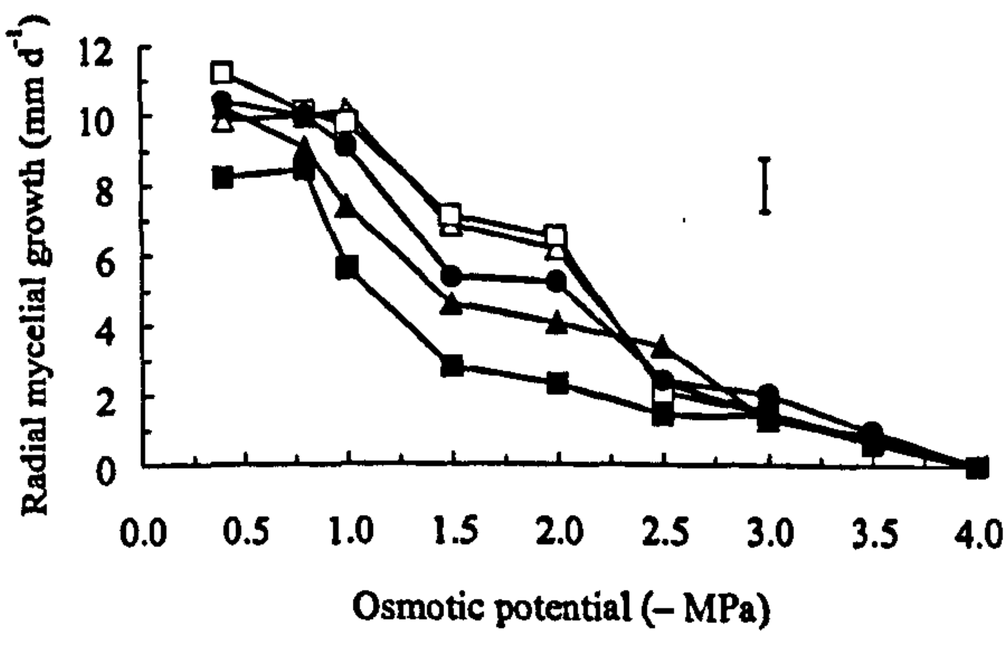
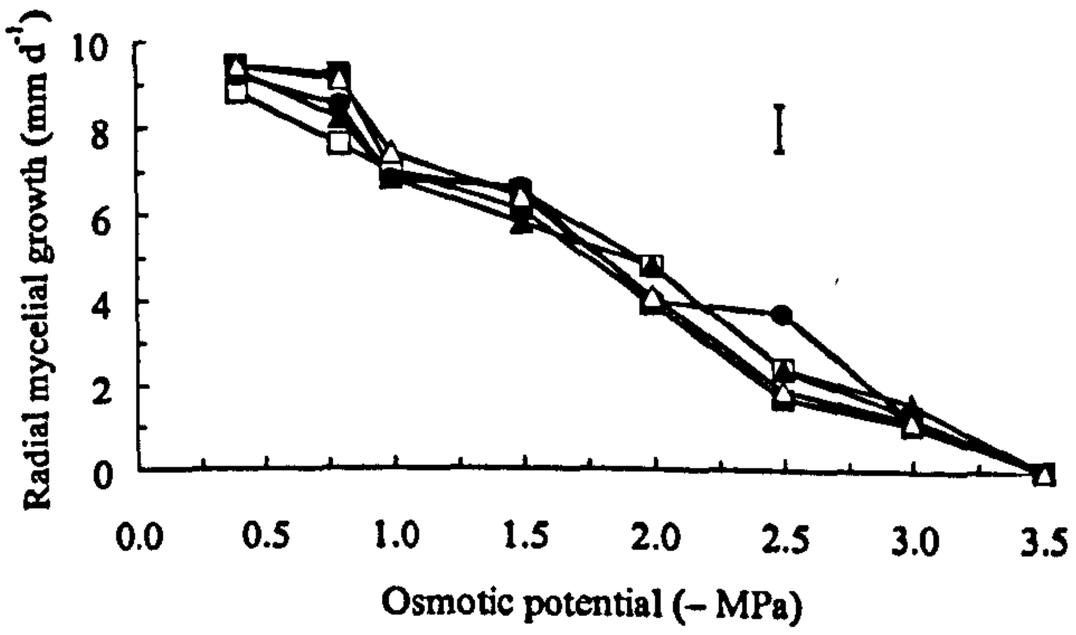


Figure 3.7. (glycerol)



Figures 3.5 – 3.7. Effect of osmotic potential of PDA, adjusted with different osmotica, on radial mycelial growth rates of five isolates [x46 □, x81 Δ, x72 ■, UN ▲ and PK ●] of *R. solani*. Figure 3.5, NaCl; Figure 3.6, KCl; Figure 3.7; glycerol. Values are the mean radial mycelial growth rates (mm d^{-1}) of four replicates between 2 and 6 d at 25°C. Bar = LSD at $P = 0.05$ (DF = 28, 32 and 28 respectively).

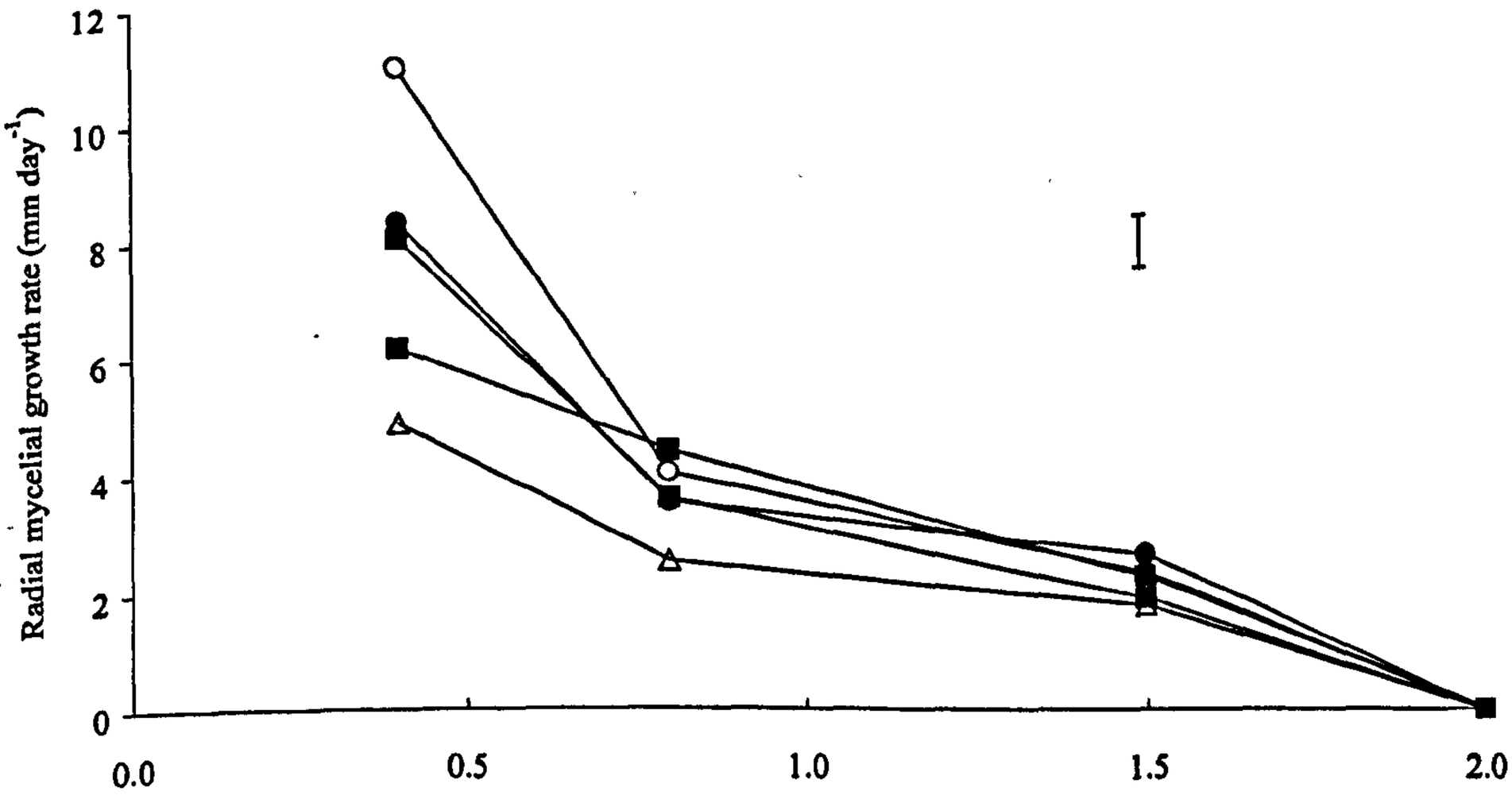


Figure 3.8. Effect of matric potential of PDA, adjusted with PEG 6000 on radial mycelial growth rate of five isolates [x46 □, x81 Δ, x72 ■, UN ▲ and PK ●] of *R. solani*. Values are the mean radial mycelial growth rates (mm d^{-1}) of four replicates between 2 and 6 d at 25°C. Bar = LSD at $P = 0.05$ (DF = 57).

In osmotic potential studies, sclerotium yield by both *R. solani* AG 2-1 and AG 3 isolates was generally the greatest on unamended PDA (– 0.4 MPa). Sclerotium yield on either NaCl, KCl or glycerol-amended PDA declined with decreasing osmotic potential over the range – 0.4 to – 3.5 MPa (Tables 3.14 – 3.16). Sclerotial formation by AG 2-1 isolates (x81 and x46) was prevented over the range of – 1.5 to – 2.5 MPa, whereas formation by AG 3 isolates (UN, PK and x72) was prevented over the range of – 2.5 to – 3.5 MPa. In most cases, sclerotium yield by AG 3 isolates was greater than AG 2-1 isolates at all osmotic potentials tested.

In matric potential studies, sclerotium yield by both AG 2-1 isolates was greatest on unamended PDA (– 0.4 MPa), and completely inhibited at – 0.8 MPa on PEG 6000-amended PDA (Table 3.17). Sclerotium yield by all three AG 3 isolates was also greatest on unamended PDA (– 0.4 MPa). However, with further decreases in matric potential, sclerotium yield of the AG 3 isolates declined significantly ($P = 0.05$) and formation was completely inhibited at – 1.5 MPa (Table 3.17).

3.3.8.2 Total soil water potential and mycelial growth

In soil, the fastest mycelial growth rates for all *R. solani* isolates occurred at – 0.5 MPa (Figure 3.9). With further decreases in soil water potential, mycelial growth rates declined slightly until growth of all isolates was inhibited at – 7.5 MPa. At any of the water potentials tested, growth rates of AG 3 isolates were generally faster than those of AG 2-1 isolates (Figure 3.9).

Table 3.14. Sclerotium yield [total dry weight biomass (mg) per dish] by five isolates of *R. solani* at 25°C on PDA adjusted to various osmotic potentials with NaCl.

	Osmotic potential (– MPa)					
Isolate (AG)	0.4	0.8	1.5	2.0	2.5	3.0
x81 (2-1)	16.4[a]	1.12	2.8	1.4	0.0	0.0
x46 (2-1)	18.0	14.6	0.0	0.0	0.0	0.0
x72 (3)	41.0	25.9	7.9	2.1	2.4	0.0
UN (3)	35.3	7.6	8.7	5.2	2.1	0.0
PK (3)	38.4	7.9	2.1	2.6	0.0	0.0
P value			0.012			
LSD ($P = 0.05$), DF = 147[b]			13.99			

[a]Values are the mean total dry weight biomass of sclerotia from four replicate dishes (mg per dish).
[b]LSD is the least significant difference at 5 % ($P = 0.05$), DF = degrees of freedom in ANOVA.

Table 3.15. Sclerotium yield [total dry weight biomass (mg) per dish] by five isolates of *R. solani* at 25°C on PDA adjusted to various osmotic potentials with KCl.

	Osmotic potential (– MPa)					
Isolate (AG)	0.4	0.8	1.5	2.0	2.5	3.0
x81 (2-1)	16.4[a]	1.12	2.8	1.4	0.0	0.0
x46 (2-1)	0.0	25.7	6.2	4.1	0.0	0.0
x72 (3)	55.6	28.0	15.8	1.7	5.4	0.0
UN (3)	49.8	42.9	33.2	32.7	24.2	0.0
PK (3)	31.5	32.8	34.1	23.1	21.6	0.0
P value			<0.001			
LSD ($P = 0.05$), DF = 147[b]			14.94			

[a]Values are the mean total dry weight biomass of sclerotia from four replicate dishes (mg per dish).
[b]LSD is the least significant difference at 5 % ($P = 0.05$), DF = degrees of freedom in ANOVA

Table 3.16. Sclerotium yield [total dry weight biomass (mg) per dish] by five isolates of *R. solani* at 25°C on PDA adjusted to various osmotic potentials with glycerol.

	Osmotic potential (– MPa)						
Isolate (AG)	0.4	0.8	1.5	2.0	2.5	3.0	3.5
x81 (2-1)	4.2[a]	12.1	0.0	0.0	0.0	0.0	0.0
x46 (2-1)	5.5	18.0	4.5	1.5	0.0	0.0	0.0
x72 (3)	74.0	52.7	54.6	46.3	50.6	11.7	0.0
UN (3)	57.1	19.0	21.6	4.4	12.0	0.0	0.0
PK (3)	40.2	31.6	18.4	21.2	16.5	16.5	0.0
P value				<0.001			
LSD ($P = 0.05$),				19.36			
DF = 147[b]							

[a]Values are the mean total dry weight biomass of sclerotia from four replicate dishes (mg per dish).
[b]LSD is the least significant difference at 5 % ($P = 0.05$), DF = degrees of freedom in ANOVA.

Table 3.17. Sclerotium yield [total dry weight biomass (mg) per dish] by five isolates of *R. solani* at 25°C on PDA adjusted to various matric potentials with PEG 6000.

	Matric Potential (– MPa)		
Isolate (AG)	0.4	0.8	1.5
x81 (2-1)	6.4[a]	0.0	0.00
x46 (2-1)	3.7	0.0	0.00
x72 (3)	28.2	9.2	0.00
UN (3)	27.8	19.2	0.00
PK (3)	24.7	13.7	0.00
P value		<0.001	
LSD ($P = 0.05$),		6.65	
DF = 117[b]			

[a] Values are the mean total dry weight biomass of sclerotia from four replicate dishes (mg per dish).
[b]LSD is the least significant difference at 5 % ($P = 0.05$), DF = degrees of freedom in ANOVA.

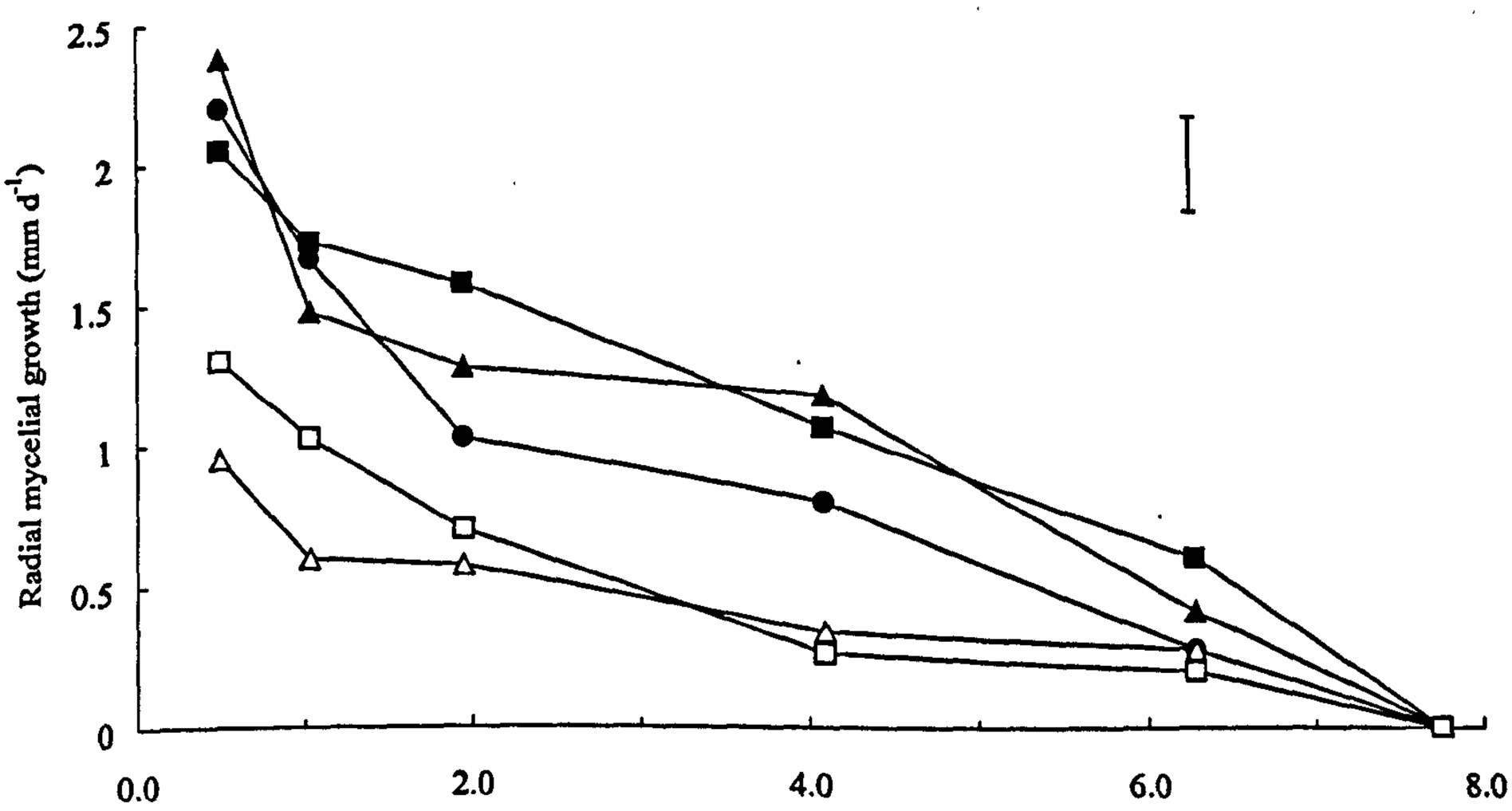


Figure 3.9. Effect of soil water potential on radial mycelial growth rates of five isolates [x46 □, x81 Δ, x72 ■, UN ▲ and PK ●] of *R. solani*. Values are the mean radial mycelial growth rates (mm d^{-1}) of four replicates after 10 d at 20°C. Bar = LSD at P = 0.05 (DF = 87).

3.4 Discussion

This study has identified differences in the effect of environmental and nutritional factors on mycelial growth and sclerotium yields of *R. solani* isolates both between and within different AGs pathogenic to potato. It was demonstrated that nutrients are essential for sclerotial production, as very few or no sclerotia formed on nutrient poor WA compared to greater sclerotium yields on PDA and MYA. Differences in sclerotium yields were also apparent between AGs, with AG 3 isolates producing significantly greater sclerotium yields than AG 2-1 isolates. The initial growth or subsequent transfer of cultures to nutrient rich PDA resulted in greater sclerotium yield for AG 3 isolates and AG 2-1 isolates, although sclerotium yields were far greater for AG 3 isolates. A similar study using MYA and WA as the test media, and an *R. solani* AG 3 isolate from potato, showed similar results, with sclerotia production greatest when the isolates had been grown on or transferred from a high nutrient medium (Dijst, 1988).

Many of the respiratory substrates in soil utilised by fungi are derived from the cell walls of plants and are primarily carbohydrates consisting of sugar containing molecules or sugar complexes (Griffin, 1972; Deacon, 1984). For external carbohydrates to act as substrates for respiration, the passage of the carbohydrate or subsequently produced breakdown products through the cell wall and membrane is essential (Griffin, 1972). Most fungi growing in culture are able to utilise hexoses and disaccharides, but this ability becomes progressively reduced as the carbon sources become more complex (Griffin, 1972). The utilisation of different carbon sources by *R. solani* has been shown to vary significantly in this study, both between and within AGs.

Vegetative mycelial growth is known to precede sclerotial initiation and it is during this growth phase that any available nutrients in the substrate are absorbed by the fungus (Willets & Bullock, 1992). All *R. solani* AG 3 isolates grew well on D-glucose

(monosaccharide) and cellobiose (disaccharide), with both carbohydrates supporting increased sclerotia production compared to the other carbon sources tested. Cellobiose was utilised more readily for mycelial growth and subsequent sclerotial production than the other disaccharides tested (sucrose and maltose). Glucose provides the basis for cellulose, starch and other carbohydrates, including cellobiose (Griffin, 1980; Deacon, 1984). Both glucose and cellobiose were good carbon sources for mycelial growth and sclerotial production. It is unknown whether *R. solani* can utilise cellobiose directly or has to produce an enzyme to convert it into glucose first. *R. solani* isolates from rice were able to grow on a wide variety of carbon sources with maltose, glucose, fructose and galactose supporting mycelial growth and extensive sclerotial production (Madhusudan *et al.*, 1977).

As it is rare for the external environment to contain the appropriate nutritional balance for fungal growth, carbon compounds are often taken up and converted into other compounds, before storage as carbohydrate reserves. These include the polyhydric alcohols (polyols) which include glycerol and mannitol, which are also accumulated in fungal tissues in response to increases in osmotic stress (Carlile *et al.*, 2001). When glycerol was the sole carbon source, sclerotium yield was significantly greater than the control for all AG 2-1 and AG 3 isolates. Small yields of sclerotia were produced on mannitol by all isolates, apart from isolate x81. These results, however, were not significantly different from the sclerotium yields produced on the control medium containing no carbon source. Mannitol was also found to support poor sclerotial production of *R. solani* isolates from rice (Madhusudan *et al.*, 1977). Another study found that *R. solani* was unable to produce sclerotia on a medium containing exogenous mannitol (Townsend, 1957).

Potassium nitrate supported abundant sclerotial production by all isolates except AG 2-1 isolate x81, which produced no sclerotia on any media tested. Asparagine was

the only organic nitrogen source which supported sclerotial production by all AG 3 isolates, whereas arginine supported sclerotial production by all isolates except x81. Asparagine and potassium nitrate also supported sclerotial production by *R. solani* isolates from rice (Madhusudan *et al.*, 1977). Only one AG 3 isolate (x72) produced sclerotia on all nitrogen sources tested. Moromizato *et al.* (1980) found that growing AG 1 isolates from rice, sugar cane and *Cyperus rotundus* on media containing asparagine, alanine, arginine and proline all resulted in the production of well formed sclerotia. In this study, the presence of these amino acids was found to support sclerotia production for two or more of the isolates tested, in particular those from AG 3. Other amino acids, such as glycine, resulted in the production of sclerotia by only one AG 3 isolate (PK). Glycine was shown previously to be a poor nitrogen source for sclerotia production in *R. solani* pathogenic to rice (Moromizato *et al.*, 1980).

In the laboratory, fungi are often grown under conditions where nutrients are in excess, and although a wide range of nutrient rich habitats are available in nature, fungi often face the problem of starvation (Cooke & Whipps, 1993). Most fungi can assimilate inorganic nitrogen in the form of ammonia or nitrate, as well as utilising a wide range of organic compounds (Carlile *et al.*, 2001). Amino acids can also serve as carbon sources as well as nitrogen sources for many fungi, and many *R. solani* isolates were able to utilise nitrate, which is present in many agricultural soils via the application of artificial fertilisers (Carlile *et al.*, 2001). All the amino acids tested here have been found in the root exudates of various plant species (Dakora & Phillips, 2002). Consequently, it is possible that both AG 2-1 and AG 3 could utilise these as substrates for mycelial growth and subsequent sclerotia production under natural conditions. Amino acids, such as cysteine, methionine, histidine and leucine, have also been found to negatively affect sclerotial differentiation on an AG 1 isolate from sugar cane

(Moromizato & Matsuyama, 1980). Similar effects of these amino acids on sclerotia differentiation in *R. solani* isolates from potato are currently unknown.

The C:N ratio of the glucose-potassium nitrate medium had a significant effect on the mycelial growth of all *R. solani* isolates tested. There was a direct relationship between mycelial growth and increasing carbon and nitrogen content until maximum mycelial growth rates were reached. The C:N ratio of the growth medium was also found to affect sclerotium yields, particularly for AG 3 isolates. Although the C:N ratios were identical, sclerotium yields differed depending on whether the carbon or nitrogen source was kept constant. This suggests that it is the concentration of carbon or nitrogen on the media that may have a greater influence on subsequent sclerotium yield rather than the actual C:N ratio. In general, lower C:N ratios (4:1 – 32:1) were more favourable for sclerotial biomass production, whereas high C:N ratios (64:1 – 128:1) supported the production of few or no sclerotia.

Many fungi are able to grow over a wide pH range, with an optimum between pH 5.5 to 8 (Deacon, 1984). It has been demonstrated previously that if *R. solani* is able to initiate growth on moderately acid or alkaline media, it will usually modify the pH to a range more favourable for itself in order to grow successfully (Sherwood, 1970). In this study, *R. solani* AG 2-1 and AG 3 isolates from potato grew over a wide pH range, from pH 4 - 8 on buffered media. This corresponds well with the optimum pH range for mycelial growth of other *R. solani* isolates. For example, a similar pH range (pH 3.5 – 7.5) was most favourable for mycelial growth of an isolate of *R. solani* pathogenic to poinsettia (Bateman, 1962). A more recent study on the effect of pH on mycelial growth of *R. solani* isolates from AG 1-1B pathogenic to lettuce showed that the optimum pH was between pH 5 – 6 depending on the isolate tested (Grosch & Kofoet, 2003). In the current study, mycelial growth rates of *R. solani* isolates belonging to both AG 2-1 and

AG 3 was optimal at pH 5.6. A pH of 5.8 was optimal for mycelial growth of a poinsettia isolate of *R. solani* (Bateman, 1962).

It was apparent that AG 2-1 isolates produced sclerotia over a narrower range (pH 5 – 6), compared with the AG 3 isolates (pH 4 – 8). Differences in sclerotial production in response to pH have been demonstrated previously, with AG 11 isolates from lupin producing sclerotia between pH 4 – 9, and AG 8 producing no sclerotia at any pH (Kumar *et al.*, 1999). The reasons for the effect of pH on sclerotia production by AG 2-1 and AG 3 is unclear and merits further investigation. Membrane permeability is affected by pH and, therefore, the ability of the fungus to take up nutrients required for mycelial growth and sclerotial production may have been affected at different media pH (Deacon, 1984). It is also possible that the buffers may have been toxic at the concentration used for efficient buffering, and may have had an effect on both mycelial growth and sclerotium yield (Carlile *et al.*, 2001).

The optimum temperature for mycelial growth of both AG 2-1 and AG 3 isolates of *R. solani* was between 20 and 25°C on all media tested. Previous studies on *in vitro* mycelial growth of AG 3 and AG 2-1 isolates found the optimum temperature for growth of *R. solani* from potato to be similar, and between 22 and 25°C (Chand & Logan, 1983). The optimum temperature for *in vitro* mycelial growth of different AG 3 isolates from potato in Peru was between 20 to 25°C, whereas for AG 4 it was 25 to 28°C (Anguiz & Martin, 1989). Other researchers have found that potato isolates of AG 3 grew more rapidly at 15 °C than 25°C (Balali *et al.*, 1995). Isolates of *R. solani* AG 8 and AG 11 pathogenic to lupin were also found to grow optimally at 25°C (Kumar *et al.*, 1999). *R. solani* AG 1-1B from lettuce was found to grow optimally between 20 and 30°C (Grosch & Kofoet, 2003).

Sclerotium yield was optimal between 20 and 25°C for both AG 3 and 2-1 isolates in this study, with AG 3 producing significantly more sclerotia than AG 2-1.

Differences in sclerotial production between AGs has been observed previously, with AG 3 isolates producing numerous sclerotia at 20 to 25°C and AG 4 isolates producing very few at 25 °C *in vitro* (Anguiz & Martin, 1989). Another study on the comparison of sclerotial production by AG 3, AG 4 and AG 5 isolates from potato found that only AG 3 had produced any sclerotia at 15 and 25°C after 5 d (Balali *et al.*, 1995).

Temperature had a significant effect on mycelial growth rate of all isolates of *R. solani* through soil. The growth curves produced were similar to those observed when isolates were grown on agar, however, mycelial growth rates were much slower in soil. It was shown that AG 2-1 isolates grew significantly slower than the AG 3 isolates tested, and the optimum temperature for mycelial growth ranged between 20 – 25°C for all isolates.

The mycelial growth rates of both AG 2-1 and AG 3 isolates to different osmotic potentials in this study were similar to those previously observed for other isolates of the pathogen. A wide range of water potentials allowed mycelial growth of *R. solani*, with optimum growth observed at – 0.4 MPa on agar. There was a marked decline in the mycelial growth rate of *R. solani* in response to decreasing water potentials *in vitro* which were similar to those observed previously for *R. solani* strains belonging to other AGs. For example, the mycelial growth of isolates belonging to AG-8 and AG-11 and pathogenic to lupin occurred at osmotic potentials ranging from – 0.4 MPa to – 3.1 MPa (Kumar *et al.*, 1999). Similarly, Dubé *et al.* (1971) demonstrated that an isolate belonging to an unknown AG, causing bare patch on cereals, was capable of *in vitro* growth from – 0.7 MPa to – 4.5 MPa.

Formation of sclerotia by isolates of *R. solani* belonging to both AGs occurred on PDA only at osmotic and matric potentials higher than the minimum required for mycelial growth, with formation reduced more in response to a decline in matric compared to osmotic potential. In previous studies, decreasing the osmotic potential

caused similar declines in sclerotial production in other fungi, with sclerotia production ceasing before the limits for mycelial growth in *Sclerotinia minor* (Imolehin *et al.*, 1980) and *Macrophomina phaseolina* (Shokes *et al.*, 1977). Decreasing the substratum osmotic and matric potential *in vitro* reduced and eventually inhibited both mycelial growth and sclerotium yield in this study.

Mycelial growth of both AG 2-1 and AG 3 isolates in soil declined with decreasing total water potential, with a minimum potential permitting both growth and germination of – 6.3 MPa. Fungal mycelial growth on matrically adjusted media *in vitro* normally occurs over a wider range than fungal mycelial growth in soil adjusted to different water potentials (Cooke & Whipps, 1993). However, in this study, isolates of *R. solani* belonging to AG 3 grew over a wider range of soil water potentials (– 0.4 to – 6.3 MPa) than matric (– 0.4 to – 2.0 MPa) and osmotic potentials (– 0.4 to – 4.0 MPa) on PDA. This observation contrasts with previous studies on many soil-borne fungi (Adebayo & Harris, 1971; Cook & Duniway, 1980; Brownell & Schneider, 1985; Cooke & Whipps, 1993). These results indicate that a wide range of nutritional and environmental factors have an effect on the mycelial growth rate and sclerotium yield of *R. solani* from potato.

CHAPTER 4

NUTRITIONAL AND ENVIRONMENTAL FACTORS AFFECTING SCLEROTIAL GERMINATION BY *R. SOLANI*

4.1 Introduction

It was demonstrated in the previous chapter that mycelial growth and sclerotia production of *R. solani* were affected by various environmental and nutritional factors *in vitro*. Sclerotia produced by *R. solani* are soft, and are constructed of loosely arranged chains of monilliod cells containing no vacuoles (Hudson, 1986; Townsend & Willets, 1954; Butler & Bracker, 1970; Webster, 1980). The hyphae have a loose cellular arrangement at the outer edge of the sclerotium, with a pseudo-parenchymous mass of hyphae in the centre (Townsend & Willets, 1954). The production of sclerotia enables the fungus to survive adverse environmental conditions and *R. solani* is the only fungus known to produce loosely interwoven sclerotia that have no cellular differentiation into a rind and medulla (Townsend & Willets, 1954; Willets & Bullock, 1992).

Sclerotia of *R. solani* are known to be an important source of inoculum for infection of potato when present on seed tubers (Tsror & Peretz-Alon, 2005). *R. solani* sclerotia undergo direct myceliogenic germination, whereby vegetative hyphae capable of infecting the host grow directly out of the sclerotium (Coley-Smith & Cooke, 1971). There are currently no known studies on any aspect of the germination of sclerotia produced by *R. solani* from potato. The contribution of sclerotia present on seed tubers to disease development on subsequent plants is well established, therefore it would be desirable to ascertain the extent to which various environmental and nutritional factors may influence sclerotial germination.

4.2 Materials and methods

4.2.1 Production and aseptic harvest of *R. solani* sclerotia i*n vitro*

Sclerotia produced by three AG 3 isolates of *R. solani*, UN, x72 & PK (Section 2.1) were used in all germination experiments. Sclerotia were harvested aseptically by scraping them from the surface of 21-d-old *R. solani* cultures grown on MYA at 25°C (Section 3.2.1). The sclerotia were washed five times in sterile distilled water to remove excess agar. Sclerotia were then washed through a 1000 μm sieve and collected on a 425 μm sieve. Individual sclerotia were washed out of the lower sieve and transferred to a container filled with 200 ml distilled water. The water plus sclerotia were then poured through Whatman no.1 filter papers, transferred to sterile plastic Petri dishes and left to dry in a laminar flow cabinet overnight. To obtain a specific size range, sclerotia were dry sieved so that all were in the size range between 425 and 1000 μm. Sclerotia were checked using a stereo binocular microscope (Gallenkamp) at x45 magnification to ensure there were no outgrowing hyphae before use in experiments. Sclerotia were stored at 5°C after harvesting for a maximum of 7 d prior to use in experiments.

For all experiments, ten sclerotia were placed aseptically, using forceps, at least 1.5 cm apart on 9 cm diam Petri dishes containing approximately 20 ml of the appropriate media. Sclerotial germination was assessed as the percentage of sclerotia germinated per plate. A sclerotium was considered to have germinated when any outgrowing hyphae were equal to or greater than the diam of the sclerotium. Germination was determined by assessing individual sclerotia for outgrowing hyphae under a stereo binocular microscope at x 45 magnification.

4.2.2 Effect of temperature and nutrient availability on sclerotial germination

4.2.2.1 Temperature and sclerotial germination on agar

To determine the effect of temperature and nutrient availabiltiy on sclerotial germination, Petri dishes containing PDA, MYA and WA were prepared as described previously (section 3.2.3). Ten sclerotia were placed onto each plate and incubated at 5, 10, 15, 20, 25 and 30°C (section 4.2.1). The Petri dishes were arranged in a randomized block design with four replicates for each treatment at each temperature. Germination at 24 and 72 h was determined as described previously (section 4.2.1).

4.2.2.2 Temperature and sclerotial germination in soil

A soil sandwich technique modified from Grose *et al.* (1984) was used to investigate the effect of temperature on sclerotial germination in soil. A brown earth soil (Dunnington Heath series) was passed through a 3 mm sieve, air-dried for 7 d and adjusted to – 0.5 MPa as described previously (section 3.2.7.2). Petri dishes (9 cm diam) were half-filled with soil, covered with a 5 x 5 cm piece of nylon netting approximately 1 cm apart. A larger (8 x 8 cm) square piece of netting was placed over the top of the sclerotia, further soil was added to cover the netting. All dishes were sealed with Parafilm and incubated at 20°C. Dishes were re-weighed and sterile distilled water was added as required every 3 d to maintain the constant matric potential. Sclerotial germination was assessed after 3 and 10 d as described previously (section 4.2.1).

4.2.3 Effect of carbon source on sclerotial germination

To determine the effect of carbon source on germination, a basal growth medium was prepared (section 3.2.3) with potassium nitrate added as a nitrogen source at a concentration of 3.5 g l^{-1}. Individual components of the media were autoclaved separately. The different carbon sources were added so that each medium had a final concentration of 16 g carbon l^{-1} (section 3.2.3). The final pH was adjusted to 5.6 using 1 M NaOH and approximately 20 ml media poured into 9 cm diam Petri dishes. Ten sclerotia were placed on each plate and there were four replicate Petri dishes for each treatment. Petri dishes were incubated at 25°C and germination determined after 24 and 72 h as described previously (section 4.2.1).

4.2.4 Effect of nitrogen source on sclerotial germination

To establish the effect of nitrogen source on germination, a basal growth medium containing 10 g l^{-1} D-glucose as the carbon source was prepared (section 3.2.4). Different inorganic and organic nitrogen sources were added individually to this medium so each contained a final concentration of 0.48 g N l^{-1} (section 3.2.4). All solutions were autoclaved separately and combined when cooled to approximately 50°C. All media were adjusted to pH 5.6 with NaOH. Ten sclerotia were placed on each plate, with four replicates per treatment. Petri dishes were incubated at 25°C and sclerotial germination determined after 24 and 72 h as described previously (section 4.2.1).

4.2.5 Effect of C:N ratio on sclerotial germination

The effect of C:N ratio on germination was determined on a basal medium (section 3.2.3) containing different concentrations of D-glucose and KNO_3 in two parallel experiments as described previously (section 3.2.5). The D-glucose

concentration was maintained at 40g l^{-1} (16 g carbon l^{-1}) and the KNO_3 concentration adjusted to give the following C:N ratios; 4:1, 8:1, 16:1, 32:1, 64:1, 128:1. In a parallel experiment, the KNO_3 concentration was maintained at 3.5 g l^{-1} (0.48 g nitrogen l^{-1}) and the D-glucose concentration amended to produce the same C:N ratios outlined previously (section 3.2.5). There were four replicates for each treatment and Petri dishes were incubated at 25°C. Sclerotial germination was determined after 24 and 72 h as described previously (section 4.2.1).

4.2.6 Effect of pH on sclerotial germination

The effect of pH on sclerotial germination was investigated using buffered PDA adjusted over the range pH 4 - 9. Batches of PDA were prepared and buffered using citrate-phosphate and tris-hydroxymethyl-aminoethane buffers as described previously (section 3.2.6). Approximately 20 ml of buffered media was dispensed into 9 cm diam Petri dishes with four replicates for each treatment. Ten sclerotia were placed onto each plate and these were incubated at 25°C. Sclerotial germination was determined as described previously after 24 and 72 h (section 4.2.1).

4.2.7 Effect of water potential on sclerotial germination

4.2.7.1 Sclerotial germination on agar

The effects of osmotic and matric potential on sclerotial germination were determined on PDA using NaCl, KCl and glycerol as described in section 3.2.8.1. PDA was adjusted osmotically over the range of – 0.8 MPa to – 5.0 MPa with sodium chloride (NaCl, Sigma) (Lang, 1967), potassium chloride (KCl, Sigma) (Campbell & Gardner, 1971) and glycerol (Sigma) (Dallyn & Fox, 1980). Total water potential was calculated as the water potential of the PDA (– 0.4 MPa) plus the osmotic potential of the added osmotica as described previously (section 3.2.8.1).

The effect of matric potential was determined using a formula as described previously (Michel & Kaufmann, 1973) (section 3.2.8.1). The media were poured into 9 cm Petri dishes, allowed to solidify overnight and the Petri dishes overlain with a 90 mm cellophane disc (PT 600; British Cellphane Co). There were four replicates for each treatment with Petri dishes kept in sealed plastic bags in the incubator to reduce water loss. Ten sclerotia were placed on each plate and all treatments were incubated at 25 °C, with germination determined after 24 and 72 h as described previously (section 4.2.1).

4.2.7.2 Sclerotial germination in soil

The effect of soil water potential was determined using the same method outlined in section 3.2.8.2. Appropriate amounts of soil were mixed with sterile distilled water to obtain six matric potentials (0.5, 1.0, 2.0, 4.1, 6.3 and 7.8 – MPa) in accordance with a soil moisture retention curve (w/w). Petri dishes were prepared as described previously (section 3.2.8.2). Sclerotial germination was assessed after 3 and 10 d as described previously (section 4.2.1).

4.2.8 Statistical analysis

Histograms of the residual and plots of the residuals versus fitted values were produced as described previously, with all treatment means compared using analysis of variance (ANOVA) using the least significant difference (LSD) at a probability of 5 % ($P = 0.05$) (section 3.2.9). The effects of temperature and media, if applicable, on sclerotial germination were further analysed using multiple linear regression as described previously (section 3.2.9).

4.3 Results

4.3.1 Effect of temperature and nutrient availability on sclerotial germination

4.3.1.1 Temperature and sclerotial germination on agar

There was little or no germination when sclerotia were incubated at 5°C after both 24 and 72 h. Sclerotial germination was not significantly affected by temperature or media for all isolates tested after 24 h (Figure A1 in Appendix A). After 72 h, however, temperature was found to significantly affect germination ($P = <0.001$), with the optimum temperature for germination of all isolates between 20 – 30°C on all media tested (Figure 4.1).

4.2.1.2 Temperature and sclerotial germination in soil

No sclerotial germination was observed after 3 d at 5°C, and there was no significant effect of incubation temperature on germination in soil of all isolates (Figure A2 in Appendix A). Incubation temperature had a significant effect on sclerotial germination in soil ($P = 0.003$) after 10 d, with the optimum between 15 – 25°C for all isolates. There were no significant differences observed in sclerotial germination in response to temperature between the different isolates tested (Figure 4.2).

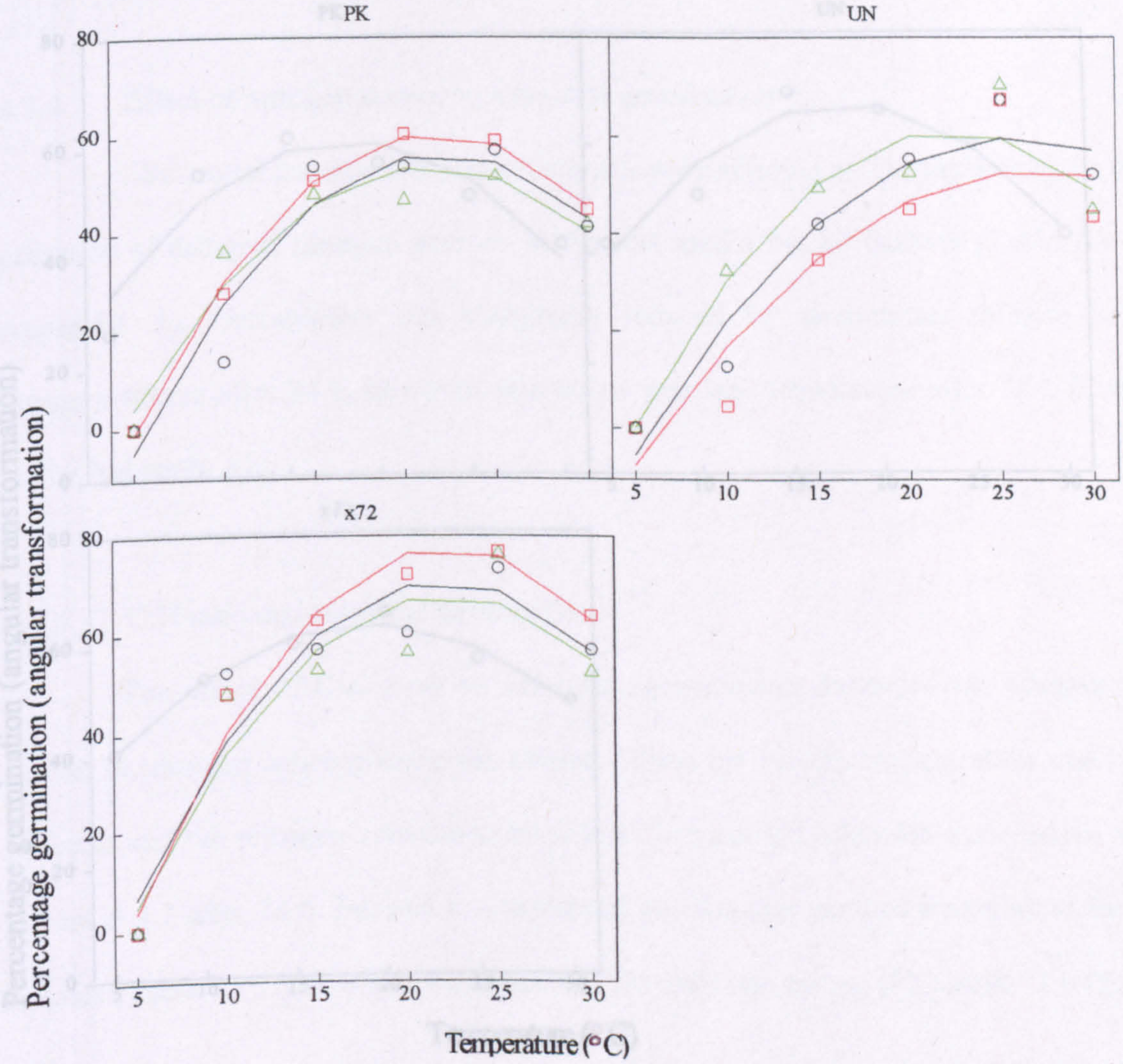


Figure 4.1. Multiple linear regression analysis of the effect of temperature and media on germination (%) of *R. solani* at 25°C after 72 h. Points represent angularly transformed mean data values and lines show the fitted model for each media tested (green = WA, red = PDA, black = MYA). $R^2 = 80.0$, $P = 0.022$ (DF = 215).

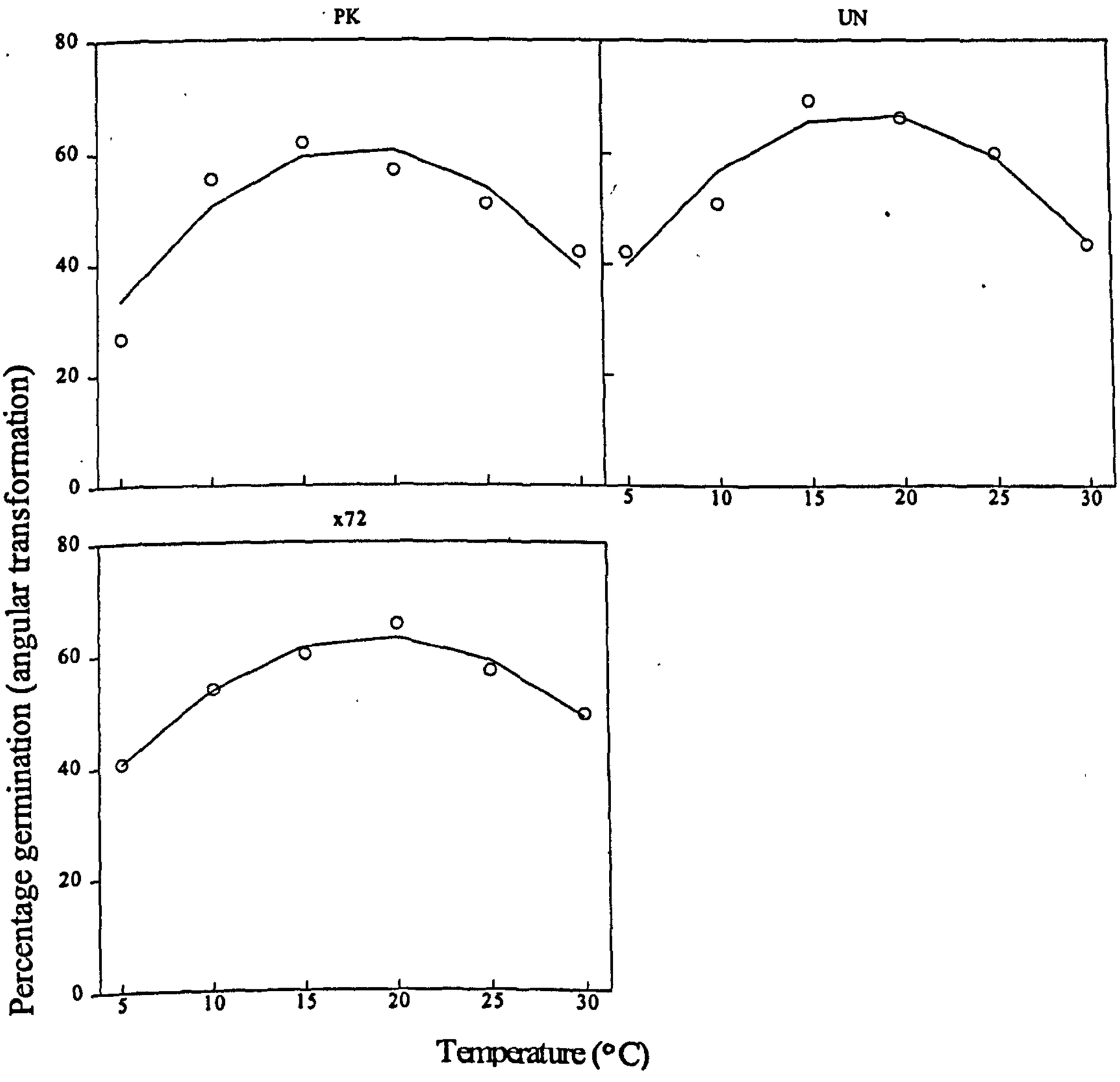


Figure 4.2. Multiple linear regression analysis of the effect of temperature on germination (%) in soil of *R. solani* at 20°C after 10 d. Points represent angularly transformed mean data values and lines show the fitted model for each isolate tested. $R^2 = 70.3$, $P = 0.003$ (DF = 69).

4.3.3 Effect of carbon source on sclerotial germination

All carbon sources and the carbon free control supported sclerotial germination, and there were no significant differences between the treatments after 24 and 72 h (Tables A1 and A2 in Appendix A).

4.3.4 Effect of nitrogen source on sclerotial germination

Sclerotial germination was not significantly affected by the absence of, or the presence of different nitrogen sources in the test media for all isolates (Table A3 in Appendix A). Germination was marginally reduced by ammonium chloride as a nitrogen source after 24 h, however, this effect was less pronounced after 72 h (Table A4 in Appendix A).

4.3.5 C:N ratio and sclerotial germination

The effect of C:N ratio on sclerotial germination depended on whether the carbon or nitrogen concentration was altered. When the carbon concentration was kept constant and the nitrogen concentration altered, percentage sclerotial germination was lowest at 4:1 after 24 h. Beyond 4:1, sclerotial germination reached a maximum for all isolates (Table 4.1). This effect was observed for only one isolate (PK) after 72 h (Table 4.2).

When the nitrogen concentration was kept constant and the carbon concentration altered, a C:N ratio of 128:1 prevented sclerotial germination of one isolate (x72), whilst restricting the germination of the other isolates (Table 4.3). When germination was assessed after 72 h, germination was 100 % for isolates x72 and UN, with isolate PK germinating significantly less than the other isolates, particularly at 128:1 (Table 4.4).

Table 4.1. Effect of C:N ratio on sclerotial germination by three isolates of *R. solani* AG 3 at 25°C after 24 h [Carbon source kept constant (40 g glucose l^{-1} = 16 g C l^{-1})].

	C:N ratio [amount of N (g l^{-1})]					
	4:1	8:1	16:1	32:1	64:1	128:1
Isolate	[1.92]	[0.96]	[0.48]	[0.24]	[0.12]	[0,06]
x72	25.0	90.0	97.5	97.5	97.5	97.5
	(22.5)[a]	(77.1)	(85.4)	(85.4)	(85.4)	(85.4)
UN	77.5	97.5	100.0	100.0	100.0	100.0
	(62.7)	(85.4)	(90.0)	(90.0)	(90.0)	(90.0)
PK	20.0	87.5	90.0	92.5	95.0	92.5
	(29.9)	(69.5)	(76.2)	(76.6)	(80.8)	(76.2)
P value			(0.013)			
LSD (P = 0.05), DF = 51[b]			(12.99)			

[a]Values are the mean percentage of sclerotia germinated (angularly transformed data) of four replicates. Analysis was carried out on the transformed data.
[b] LSD is the least significant difference at 5 % (*P* = 0.05), DF = degrees of freedom in ANOVA.

Table 4.2. Effect of C:N ratio on sclerotial germination by three isolates of *R. solani* AG 3 at 25°C after 72 h [Carbon source kept constant (40 g glucose l^{-1} = 16 g C l^{-1})].

	C:N ratio [amount of N (g l^{-1})]					
	4:1	8:1	16:1	32:1	64:1	128:1
Isolate	[1.92]	[0.96]	[0.48]	[0.24]	[0.12]	[0.06]
x72	85.0	97.5	100.0	100.0	100.0	100.0
	(70.5)[a]	(85.4)	(90.0)	(90.0)	(90.0)	(90.0)
UN	97.5	100.0	100.0	100.0	100.0	100.0
	(85.4)	(90.0)	(90.0)	(90.0)	(90.0)	(90.0)
PK	70.0	90.0	97.5	92.5	95.0	92.5
	(57.0)	(71.6)	(85.4)	(76.2)	(80.8)	(76.2)
P value			(0.024)			
LSD (P = 0.05), DF = 51[b]			(9.10)			

[a]Values are the mean percentage of sclerotia germinated (angularly transformed data) of four replicates. Analysis was carried out on the transformed data.
[b] LSD is the least significant difference at 5 % (*P* = 0.05), DF = degrees of freedom in ANOVA.

Table 4.3. Effect of C:N ratio on sclerotial germination by three isolates of *R. solani* AG 3 at 25°C after 24 h [Nitrogen source kept constant (3.5 g KNO_3 l^{-1}= 0.48 g N l^{-1})].

	C:N ratio [amount of C (g l^{-1})]					
	4:1	8:1	16:1	32:1	64:1	128:1
Isolate	[2.0]	[4.0]	[8.0]	[16.0]	[32.0]	[64.0]
x72	97.5	97.5	85.0	82.5	80.0	0.0
	(85.4)[a]	(85.3)	(67.9)	(68.7)	(63.8)	(0.0)
UN	100.0	100.0	100.0	100.0	90.0	67.5
	(90.0)	(90.0)	(90.0)	(90.0)	(74.1)	(56.1)
PK	92.5	85.0	75.0	67.5	52.5	10.0
	(76.2)	(67.5)	(67.9)	(68.8)	(63.8)	(13.28)
P value			(<0.001)			
LSD (P = 0.05), DF = 51[b]			(12.86)			

[a]Values are the mean percentage of sclerotia germinated (angularly transformed data) of four replicates. Analysis was carried out on the transformed data.
[b] LSD is the least significant difference at 5 % (*P* = 0.05), DF = degrees of freedom in ANOVA.

Table 4.4. Effect of C:N ratio sclerotial germination by three isolates of *R. solani* AG 3 at 25°C after 72 h [Nitrogen source kept constant (3.5 g KNO_3 l^{-1}= 0.48 g N l^{-1})].

	C:N ratio [amount of C (g l^{-1})]					
	4:1	8:1	16:1	32:1	64:1	128:1
Isolate	[2.0]	[4.0]	[8.0]	[16.0]	[32.0]	[64.0]
x72	100.0	100.0	100.0	100.0	100.0	100.0
	(90.0)	(90.0)	(90.0)	(90.0)	(90.0)	(90.0)
UN	100.0	100.0	100.0	100.0	100.0	100.0
	(90.0)	(90.0)	(90.0)	(90.0)	(90.0)	(90.0)
PK	100.0	87.5	82.5	80.0	82.5	45.0
	(90.0)	(72.1)	(68.4)	(67.3)	(52.7)	(42.1)
P value			(<0.001)			
LSD (*P* = 0.05), DF = 51[b]			(9.28)			

[a]Values are the mean percentage of sclerotia germinated (angularly transformed data) of four replicates. Analysis was carried out on the transformed data.
[b] LSD is the least significant difference at 5 % (*P* = 0.05), DF = degrees of freedom in ANOVA.

4.3.4. Effect of pH on sclerotial germination

The optimal pH for germination was found to occur between pH 5 and 6 for all isolates after 24 and 72 h (Figure 4.3 and 4.4). Sclerotial germination was severely restricted ($P = 0.05$) at pH 4 after 24 h, with only 20 – 50 % of sclerotia germinating, compared to over 80 % at the optimum (pH 5.6) (Figure 4.3). Less than 20 % of sclerotia for all isolates germinated at pH 7, with no germination recorded at pH 9 for any isolate (Figure 4.3). The effect of pH on sclerotial germination became much less pronounced after sclerotia had been on the amended media for 72 h (Figure 4.4). Sclerotial germination was still lower at pH 4 (between 45 – 60 %), and between pH 8 and 9 (45 – 65 %) than the optimum (65 – 100 %) for all isolates.

4.3.5 Effect of water potential on sclerotial germination

The effect of osmotic potential on germination of *R. solani* sclerotia depended on the isolate, the osmoticum used and the time allowed for germination (Figures 4.5 – 4.6). When NaCl was used as the osmoticum, sclerotial germination was maximum on – 0.4 MPa (unamended PDA) for all isolates after 24 h (Figure 4.5). There was a significant decline in sclerotial germination for all isolates between – 0.8 to – 4.0 MPa, with UN and x72 prevented from germinating at – 3.5 MPa and PK at – 4.0 MPa. After 72 h, germination was at a maximum for all isolates between – 0.4 MPa (unamended PDA) and – 0.8 MPa (Figure 4.6). There was still a significant decline ($P = 0.05$) in sclerotial germination between – 0.8 to – 4.0 MPa after 72 h , with UN and PK prevented from germinating at – 4.0 MPa, and x72 ceasing at – 3.5 MPa.

Figure 4.3. (24 h)

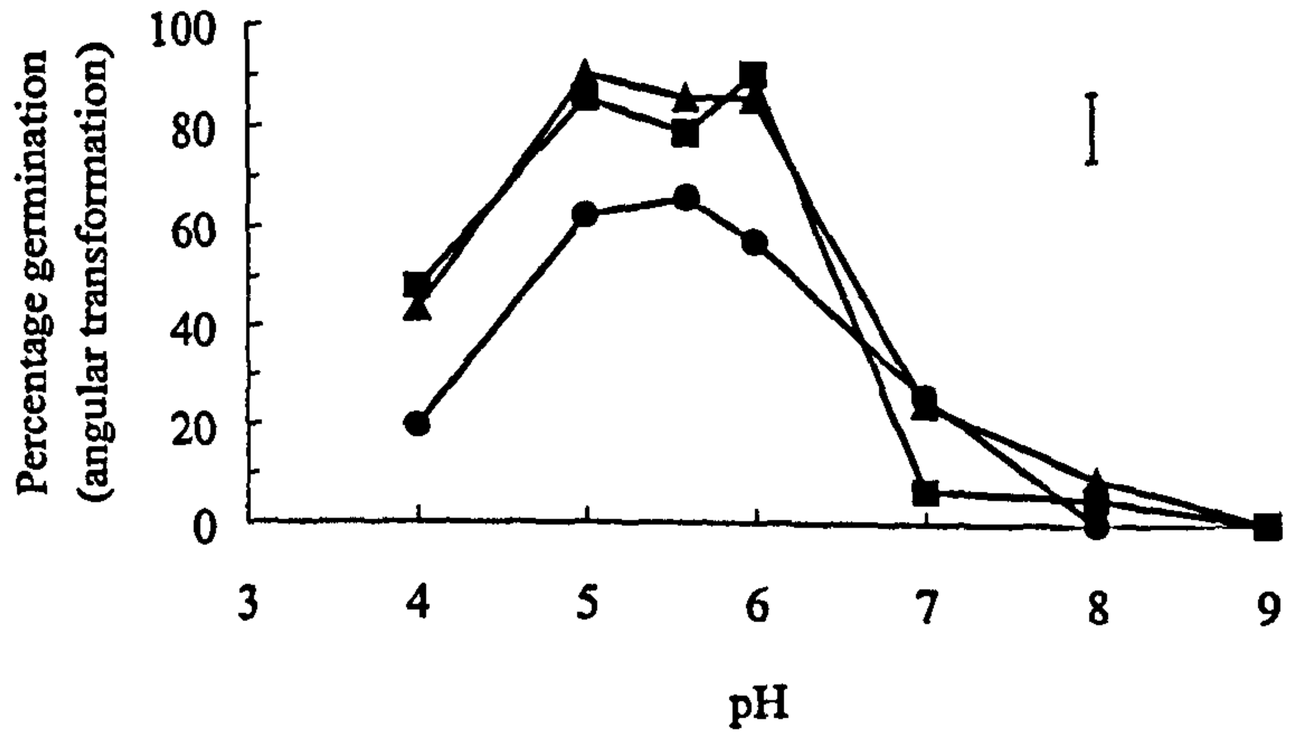


Figure 4.4. (72 h)

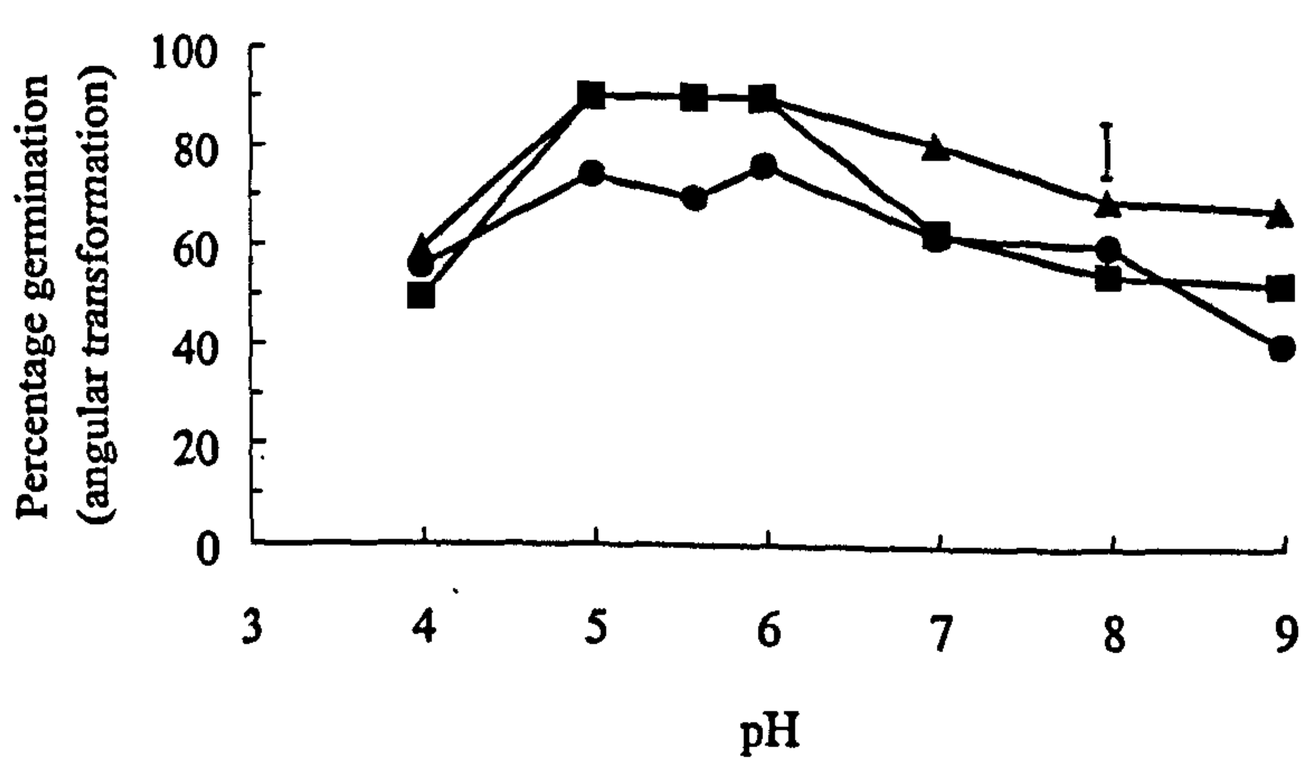


Figure 4.3 – 4.4. Effect of pH on sclerotial germination by 3 isolates (x72 ■, UN ▲ and PK ●) of *R. solani* on buffered PDA at 25°C after 24 and 72 h. Values are the mean percentage of sclerotia germinated per treatment. Bar = LSD at $P = 0.05$ (DF = 60).

Figure 4.5. (24 h)

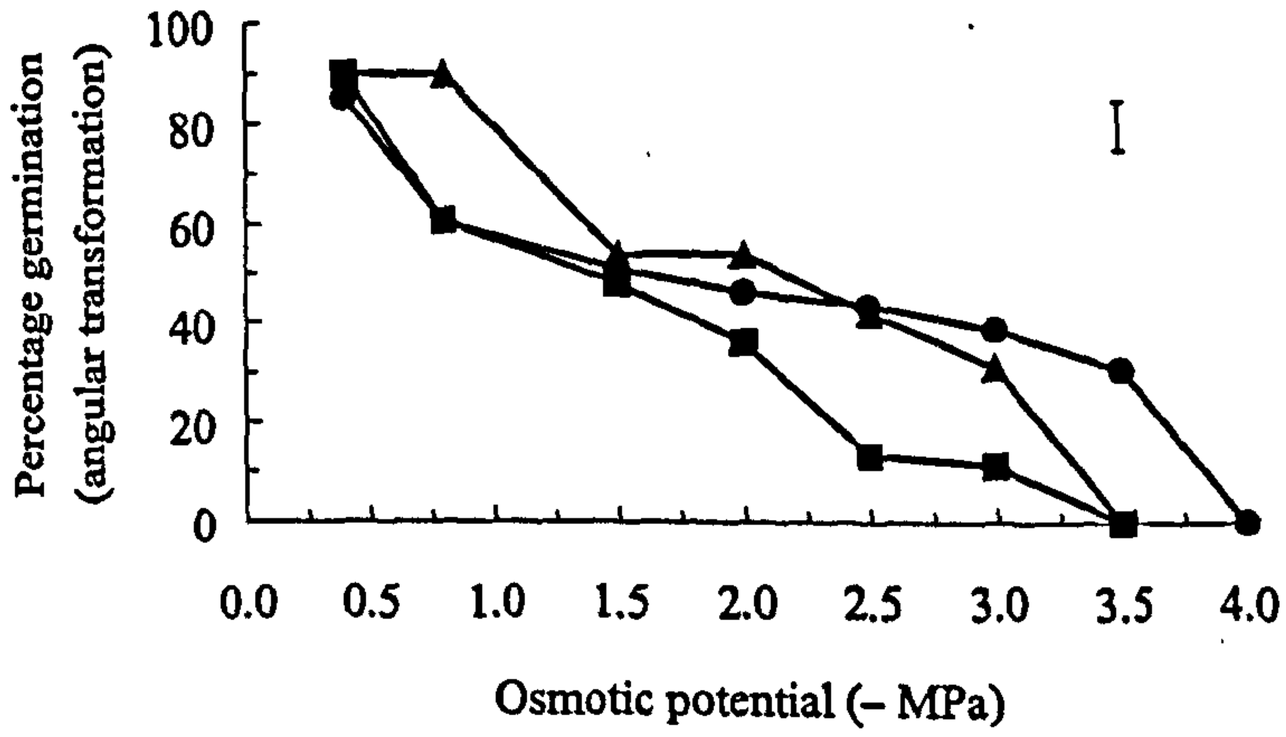


Figure 4.6. (72 h)

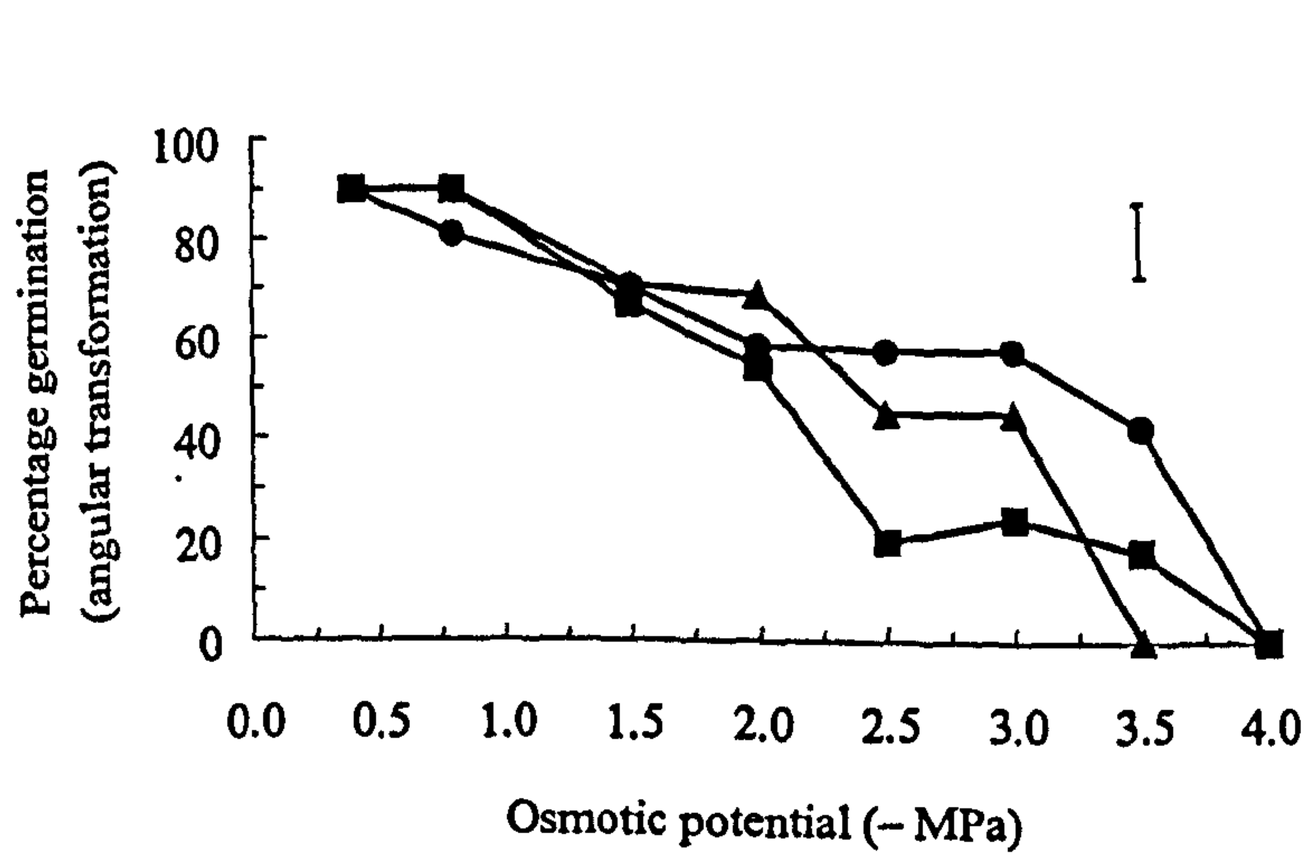


Figure 4.5 – 4.6. Effect of osmotic potential of PDA, adjusted with NaCl, on sclerotial germination of three isolates (x72 ■, UN ▲ and PK ●) of *R. solani* on buffered PDA at 25°C after 24 and 72 h. Values are the mean percentage of sclerotia germinated per treatment. Bar = LSD at $P = 0.05$ (DF = 69).

Sclerotial germination on PDA osmotically adjusted with KCl again showed a significant decline in response to decreasing osmotic potential, with maximum germination on – 0.4 MPa (unamended PDA) after 24 h (Figure 4.7). Germination was prevented at – 2.5 MPa for x72, and – 3.0 MPa for PK and UN. When germination of the same sclerotia was determined after 72 h, there was no significant difference between germination on – 0.4 MPa (unamended PDA) and PDA osmotically adjusted to – 0.8 MPa for all isolates (Figure 4.8). Declining osmotic potential beyond – 0.8 MPa significantly reduced sclerotial germination , until germination was prevented at – 3.0 MPa for both PK and x72, and – 3.5 MPa for UN.

Using glycerol as the osmoticum had a significant effect on sclerotial germination, especially for isolate x72, with germination of this isolate significantly slower ($P = 0.05$) after 24 h. Germination was prevented between – 2.5 and – 3.5 MPa depending on the isolate (Figure 4.9). After 72 h, over 50 % of sclerotia had germinated at – 3.0 MPa for isolates UN and PK, with less than 10 % of sclerotia of isolate x72 germinated. No sclerotial germination was observed for any isolate at – 3.5 MPa (Figure 4.10).

Figure 4.7. (24 h)

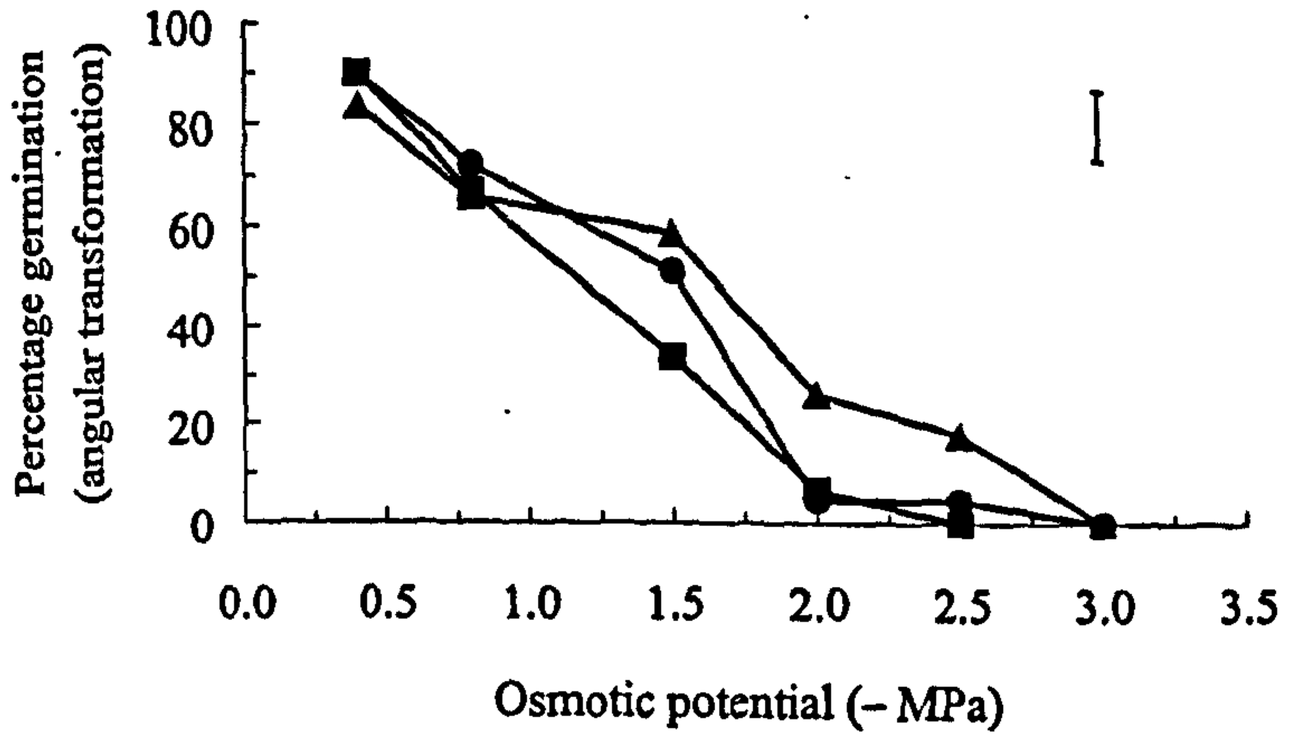


Figure 4.8. (72 h)

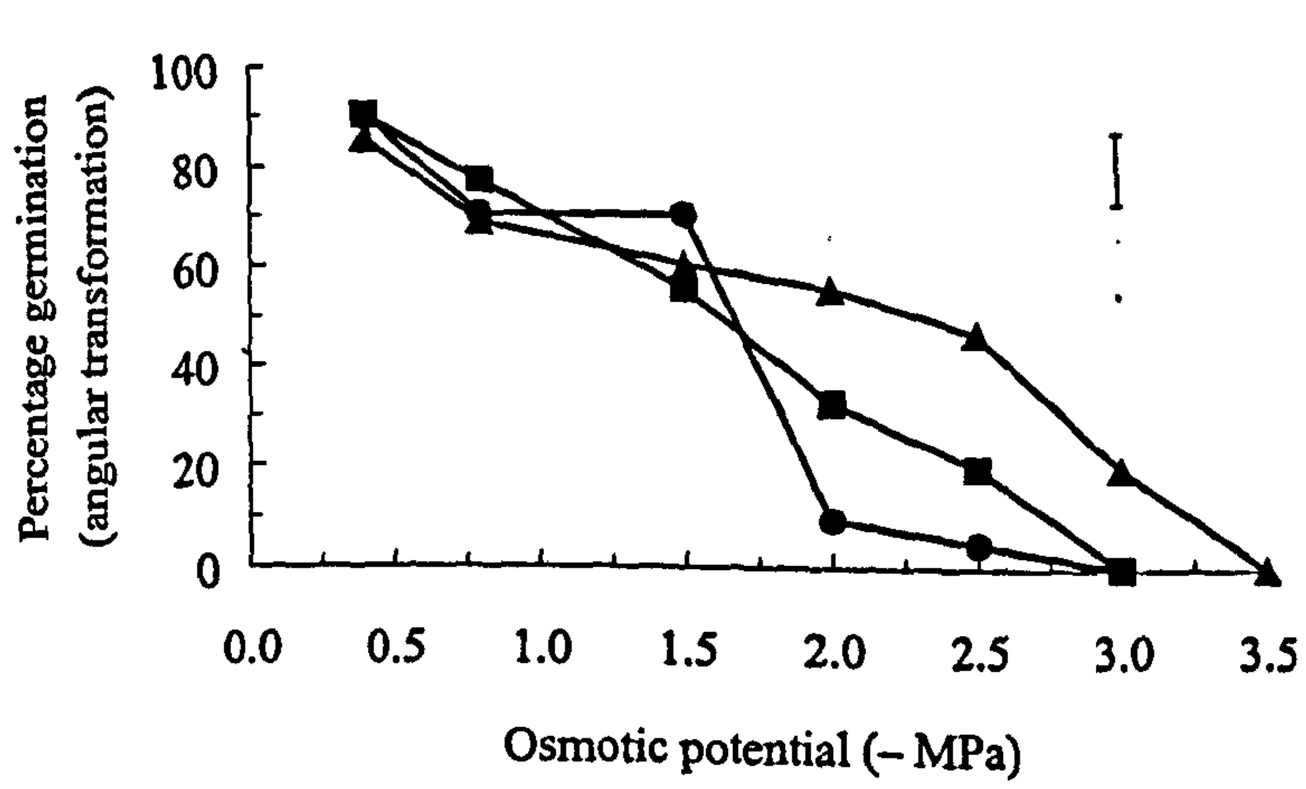


Figure 4.7 – 4.8. Effect of osmotic potential of PDA, adjusted with KCl, on sclerotial germination of three isolates (x72 ■, UN ▲ and PK ●) of *R. solani* on buffered PDA at 25°C after 24 and 72 h. Values are the mean percentage of sclerotia germinated per treatment. Bar = LSD at $P = 0.05$ (DF = 60).

Figure 4.9. (24 h)

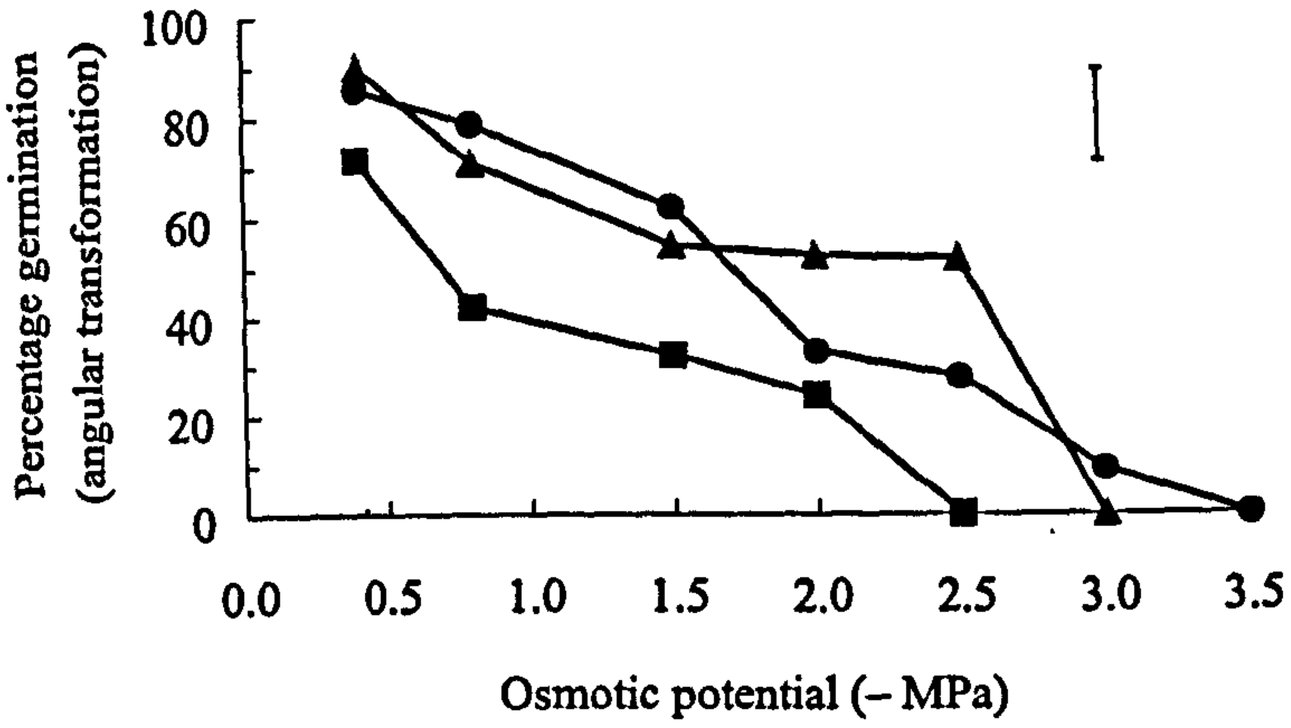


Figure 4.10 (72 h)

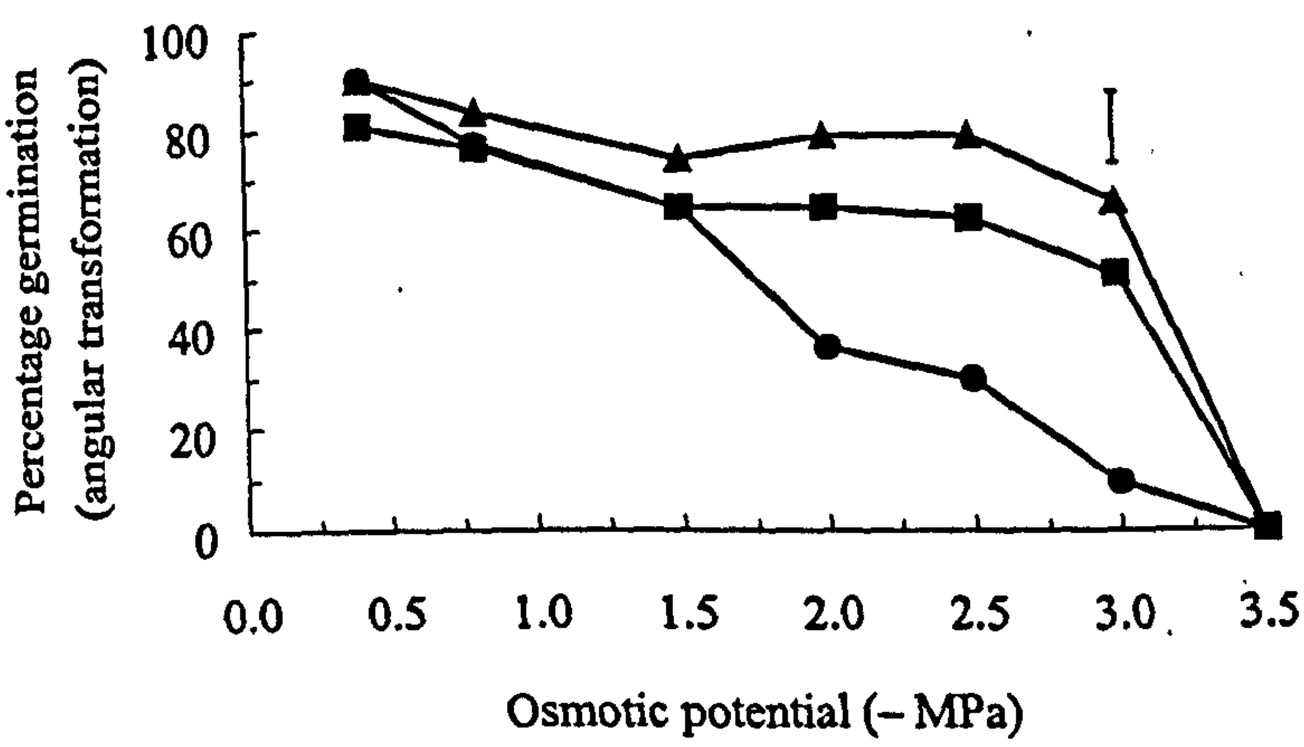


Figure 4.9 – 4.10. Effect of osmotic potential of PDA, adjusted with glycerol, on sclerotial germination of three isolates (x72 ■, UN ▲ and PK ●) of *R. solani* on buffered PDA at 25°C after 24 and 72 h. Values are the mean percentage of sclerotia germinated per treatment. Bar = LSD at $P = 0.05$ (DF = 60).

4.3.6 Effect of matric potential on sclerotial germination

When sclerotia of the three isolates were tested for the effect of matric potential on germination *in vitro*, it was found that were were no significant differences between isolates after 24 h (Figure 4.11). Germination occurred only on unamended PDA (– 0.4 MPa) and PDA adjusted to – 1.0 MPa with PEG 6000. After 72 h, there were still no significant differences in the effect of matric potential between the isolates (Figure 4.12). The range of matric potentials allowing sclerotial germination had increased after 72 h, with between 20 and 30 % of sclerotia germinating at – 1.5 MPa. No germination was observed at – 2.0 MPa or below.

4.3.7 Effect of total soil water potential on sclerotial germination

Germination of sclerotia after 3 d in soil was maximum between – 0.5 to – 2.0 MPa for all isolates, with germination was less than 10 % at – 6.1 MPa. No germination was observed at – 7.8 MPa (Figure 4.13). After 10 d, there was still little difference in the effect of soil water potential on sclerotial germination between the three isolates (Figure 4.14). Sclerotial germination was still maximum between 0.5 and – 2.0 MPa with no germination at – 7.8 MPa.

Figure 4.11. (24 h)

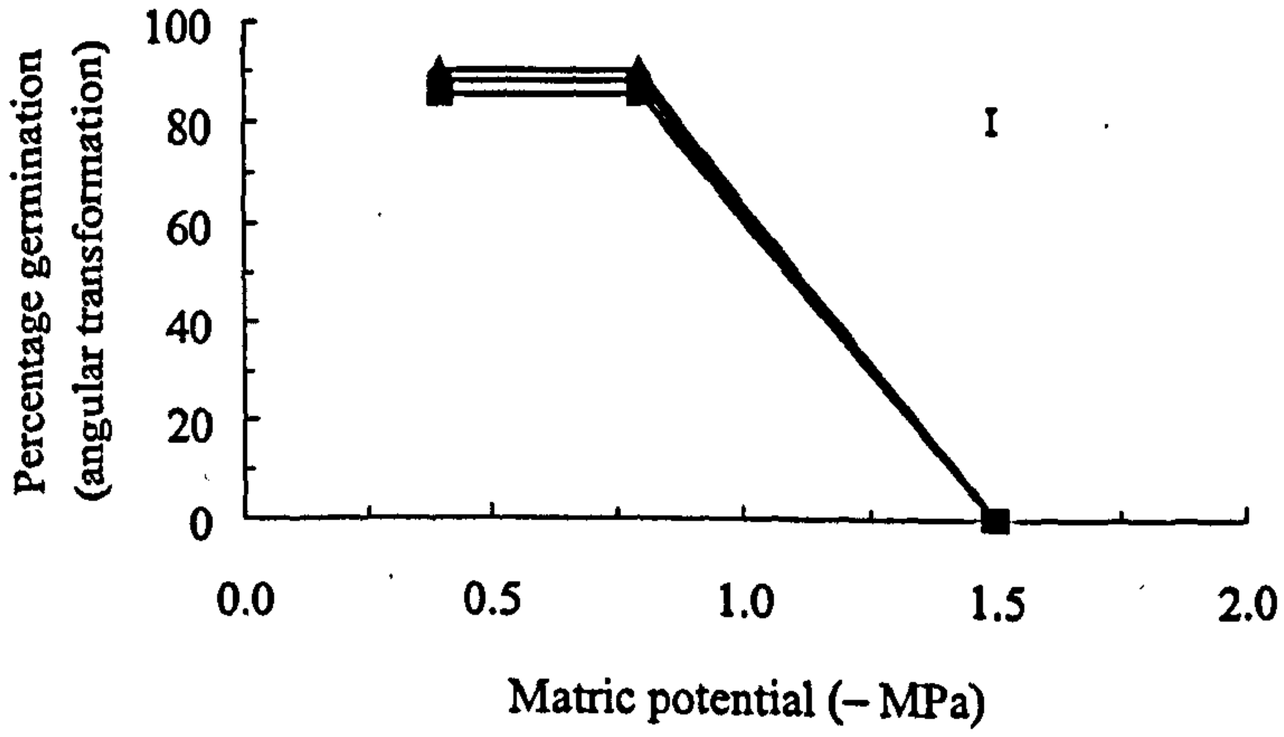


Figure 4.12. (72 h)

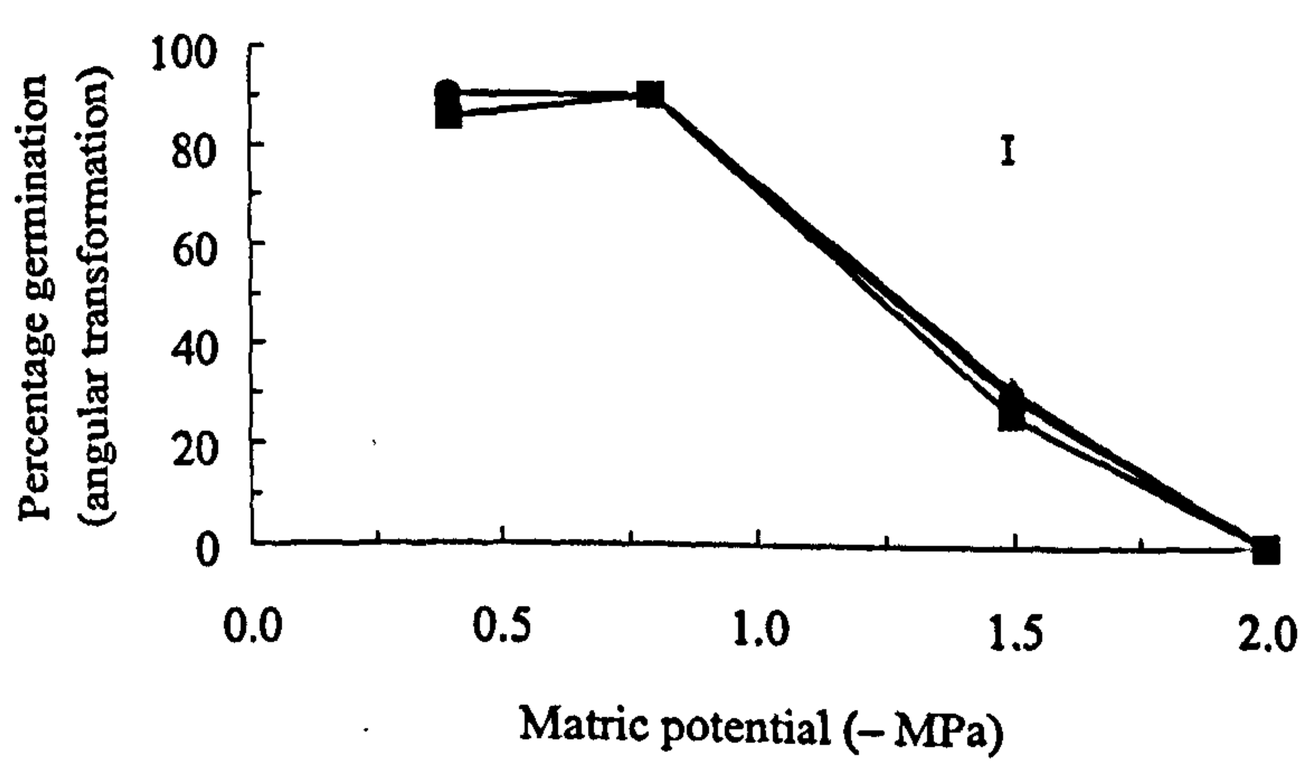


Figure 4.11– 4.12. Effect of matric potential of PDA, adjusted with PEG 6000, on sclerotial germination of three isolates (x72 ■, UN ▲ and PK ●) of *R. solani* on buffered PDA at 25°C after 24 and 72 h. Values are the mean percentage of sclerotia germinated per treatment. Bar = LSD at $P = 0.05$ (DF = 33).

Figure 4.13. (24 h)

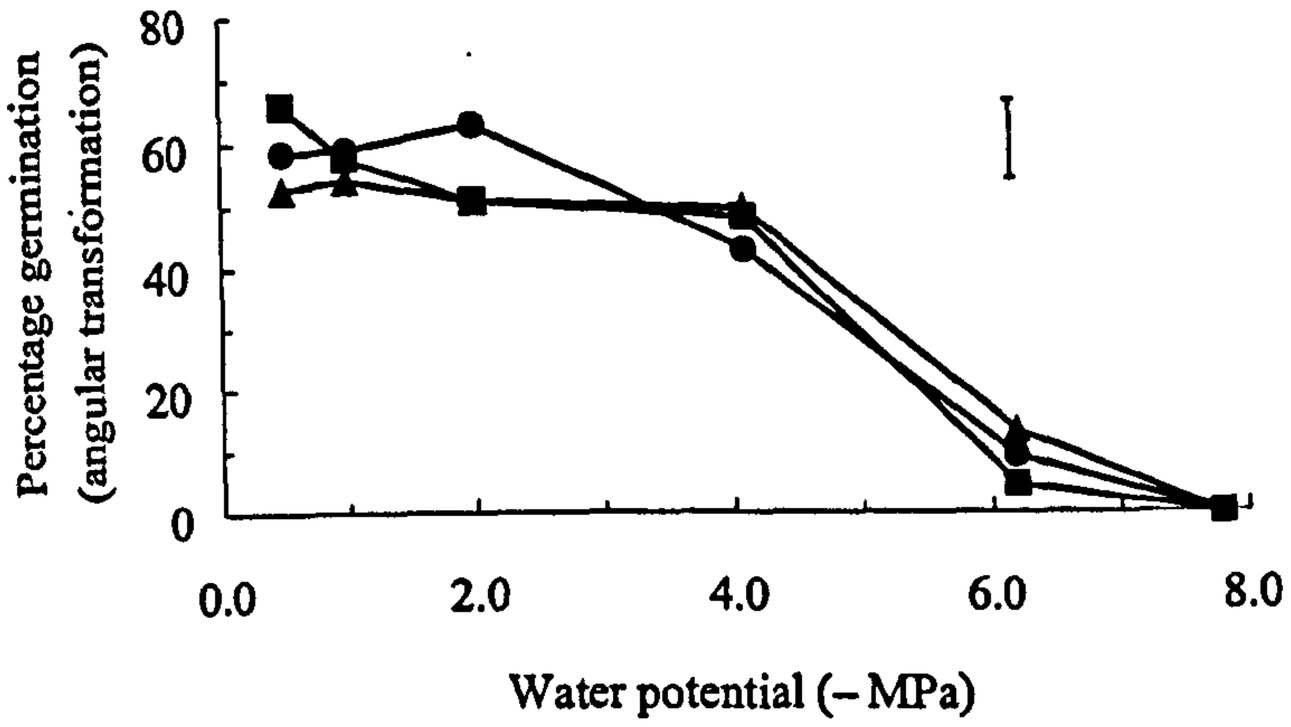


Figure 4.14. (72 h)

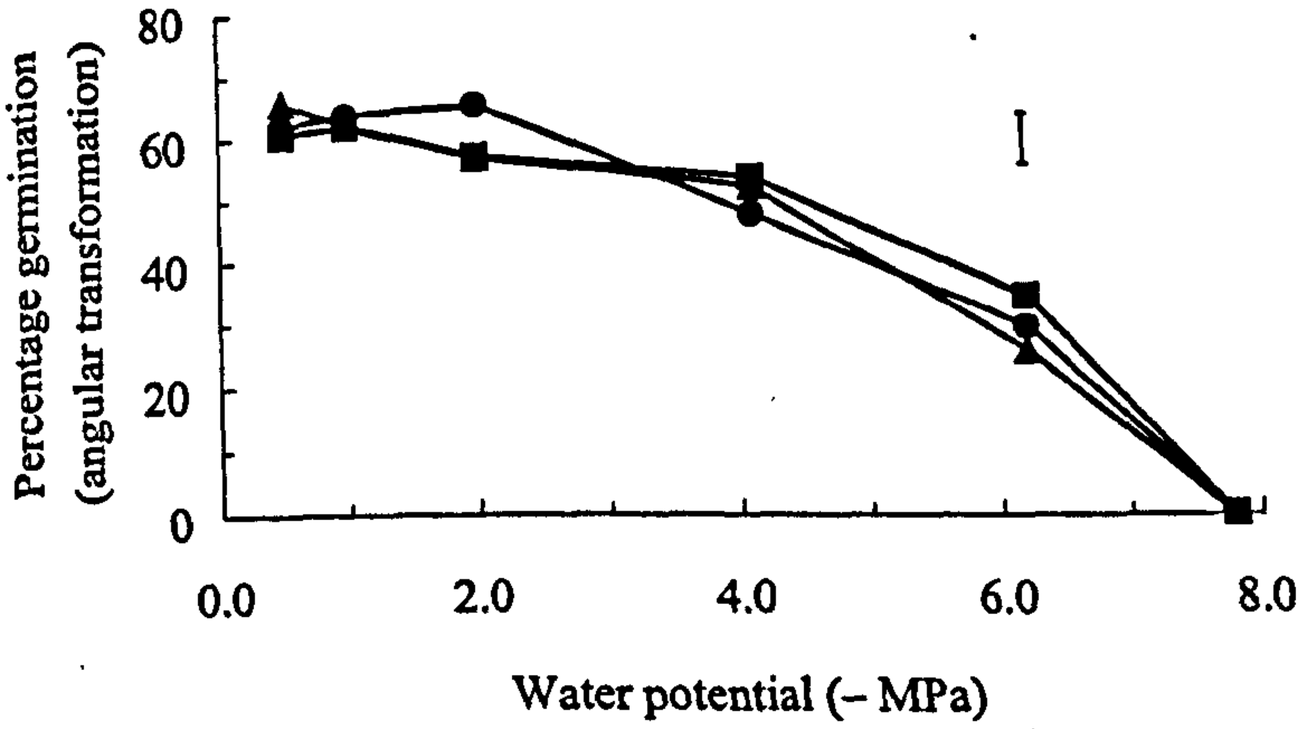


Figure 4.13 – 4.14. Effect of soil water potential on sclerotial germination of three isolates (x72 ■, UN ▲ and PK ●) of *R. solani* at 20°C after 24 and 72 h. Values are the mean percentage of sclerotia germinated per treatment. Bar = LSD at $P = 0.05$ (DF = 48).

4.4 Discussion

There appears to be no previously published studies concerning the effects of nutritional and environmental factors on the germination of *R. solani* sclerotia pathogenic to potato. This study has identified, for the first time, numerous factors that have an effect on germination of *R. solani* AG 3 isolates *in vitro*. Although the *in vitro* studies reported here do not directly simulate the conditions of the natural environment, the results provide an insight to the likely behaviour and growth of the pathogen in soil.

Sclerotial germination occurred over a broad temperature range (10 – 30°C). The isolates were typical mesophiles with optimum sclerotial germination between 20 – 30°C for all isolates. The results suggest that *R. solani* isolates from potato are well suited as pathogens of potato grown in temperate conditions, such as Europe, and disease is likely to be inhibitied at high and low temperature extremes. There was also no significant effect of the type of test media on germination. A similar non-specific response was observed using sclerotia from wheat and potato derived isolates of *R. solani* on a range of solid media, including water and malt extract agar, with germination almost 100 % on all media tested after several days (Pitt, 1964).

Sclerotial germination occurred on all carbon and nitrogen sources tested, with no differences in germination observed with regards to the carbon or nitrogen sources present in the media. The carbon : nitrogen (C:N) ratio of the glucose – potassium nitrate medium had a slight effect on the germination of sclerotia of *R. solani*. When the carbon source was kept constant, sclerotia were up to four times slower to germinate when placed on media with a C:N ratio of 4:1 compared to the higher C:N ratio treatments after 24 h. This effect was much less pronouced after 72 h. When the nitrogen source was kept constant, a C:N ratio of 128:1 resulted in reduced sclerotial germination after 24 h compared to other C:N ratios for all isolates. Germination was 100 % for two isolates (x72 and UN) after 72 h on all C:N ratios, but one isolate (PK)

showed significantly less germination. This phenomenon may be ascribed to constituents of the medium, especially nitrogen, becoming limiting at high carbon concentrations and/or to the development of unfavourable osmotic potential of the culture medium for this isolate. Germination of sclerotia was also found to occur over a broad pH range, from pH 4 – 9, with an optimum between pH 5 – 6, suggesting that the pathogen is likely to proliferate in most agricultural soils. Further experiments are required to investigate the effect of soil pH on the germination of *R. solani* sclerotia.

The water potential of the environment is recognised as an important factor in the ecology of plant pathogenic fungi (Cook & Duniway, 1980). In soil, pathogens are exposed mainly to matric forces, but as they infect plants they become dependent on the water relations of the surrounding plant tissues. This study has revealed for the first time the effects of both osmotic and matric potentials on the sclerotial germination of *R. solani*. Sclerotial germination of all isolates declined with decreasing osmotic potential, with the minimum for germination between – 3.0 and – 4.0 MPa, depending on the isolate and osmoticum used. These germination responses to osmotic potential are similar to those previously observed for other sclerotium-forming fungi, including plant pathogens such as *Sclerotinia minor* (Hao *et al.*, 2003). The isolates in this study were also more tolerant of osmotic than matric potential *in* vitro, with no germination occurring at – 2.0 MPa on media adjusted with PEG 6000. A wider range of osmotic (– 0.4 to – 4.0 MPa) than matric potentials (– 0.4 to – 2.0 MPa) supported the germination of *R. solani* on agar. Sclerotial germination in soil also declined with decreasing soil water potential, with no sclerotial germination occurring below – 6.3 MPa.

It has been demonstrated that a range of environmental and nutritional factors can affect mycelial growth, sclerotial production and germination of these AG 3 isolates. Another important aspect in disease development by *R. solani* is also the pathogenicity of different anastomosis groups of *R. solani* on potato.

CHAPTER 5

THE PATHOGENICITY OF AND THE PRODUCTION OF CELL-WALL DEGRADING ENZYMES BY DIFFERENT ANASTOMOSIS GROUPS

5.1 Introduction

R. solani disease of potato occurs primarily beneath the soil surface, with young stems and stolons particularly susceptible to attack (Banville *et al.*, 1996). Developing sprouts can be attacked and killed by the fungus prior to emergence and this often causes the plant to make several attempts to emerge (Banville *et al.*, 1996). Penetration of the host tissue by *R. solani* involves contact with the host plant which results in the formation of infection cushions on the surface of the stem or stolon (Weinhold & Sinclair, 1996). (Figure 5.1).

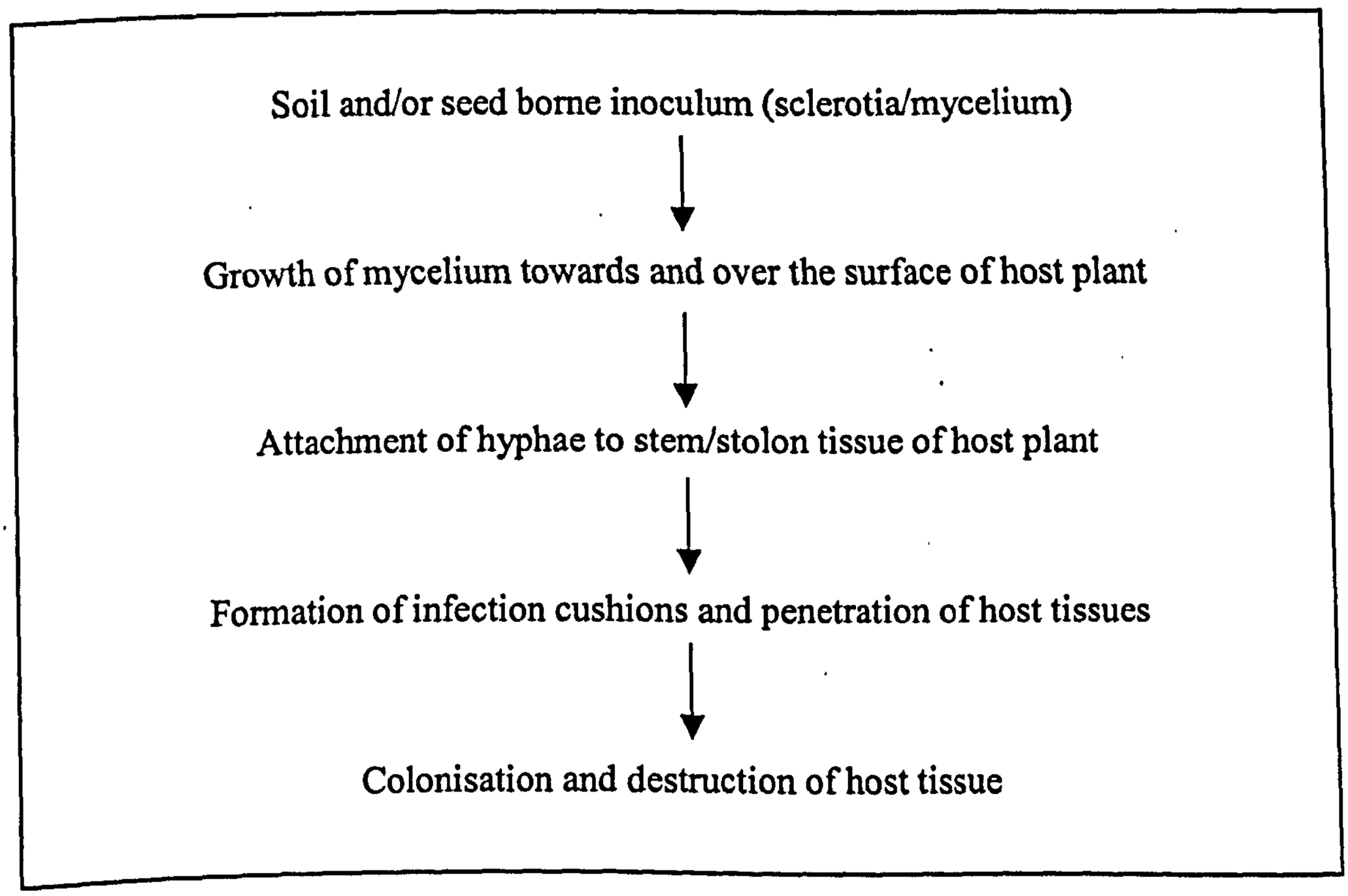


Figure 5.1. The infection process of *R. solani* on potato stems (adapted from Keijer, 1996).

Infection cushions are tightly packed hyphal aggregates, and their diameter and height can vary. At the base of the infection cushion, the fungal cells lie perpendicular to the stem surface and force their way between epidermal cell walls by forming penetration pegs (Chand *et al.*, 1985). After penetration of the host cells in this manner, hyphae penetrate the tissue directly via the resulting pore. Once the fungus is within the

cortex tissue of the host, it can penetrate intercellularly without the formation of further infection pegs (Chand *et al.*, 1985). Stem lesions have been found to develop underneath infection cushions and penetrate up to twelve cell layers deep into susceptible tissue. These lesions can reach the vascular bundle and cause the death of young sprouts, also known as stem pruning (Hofman & Jongebloed, 1988).

Investigation of *R. solani* stem infection by scanning electron microscopy revealed regions of light coloured tissue encircling penetration sites. It has been suggested that the penetration of potato stems by *R. solani* may be partly enzymatic, but the range of cell wall degrading enzymes produced by *R. solani* isolates pathogenic to potato is currently unknown (Hofman & Jongebloed, 1988).

The pathogenicity of different AGs of *R. solani* with regards to stem infection. was investigated, along with the timing of onset of stem canker symptoms and infection cushion formation from soil-borne inoculum. The production of cell-wall degrading enzymes by the different *R. solani* isolates *in vitro* was also determined.

5.2 Materials and methods

Four isolates of *R. solani* representing three different AGs [x72 (AG 3), UN (AG 3), x81 (AG 2-1) and T1 (AG 5)] were used in a pathogenicity bioassay and all enzyme assays (Table 2.1).

5.2.1 Pathogenicity of different AGs of *R. solani* from potato

Mycelial inoculum was prepared by centrally inoculating MYA Petri dishes with 3 mm diam plugs of mycelium from actively growing 3-d-old *R. solani* cultures (section 3.2.1). Inoculum was grown for 3 d, macerated in 500 ml of distilled water and added to John Innes no. 3 at a rate of $^1/_2$ Petri dish of mycelium kg d.w.$^{-1}$ growth medium (w/w) and mixed thouroughly.

Infested compost was transferred into 1-l pots and all pots were individually weighed. One mini tuber (15–20 mm cv. Kondor), which had been pre-sprouted for 7 weeks in the dark at 15°C, was planted 8 cm down in each pot using a dibber. Stem lengths were measured (mm) prior to planting. The experiment was arranged in a split-plot design comprising of four replicates, with five sampling dates. The experiment was split between two growth cabinets (Conviron) with two replicates per cabinet. Pots were incubated at 20°C, re-weighed every 2 d with additional distilled water added to keep the soil moisture content constant.

The pots were sampled over a 10 d period at 1, 2, 3, 6, and 10 d intervals. Mini tubers were removed from the pot by tipping the contents into a tray and carefully retrieving the tuber with the stem attached. The length of the stem was measured (mm) and removed carefully from the potato at the stem base. The incidence of infected stems per pot (%) was determined, and severity of stem canker lesions present was recorded using a categorical scale (Table 5.1).

Table 5.1. Four point scale (0–3) for categorising infection by *R. solani* on potato stems.

Severity category	Disease symptoms present
0	No visible stem lesions
1	1 – 33 % stem covered in lesions
2	34 – 66 % of stem covered in lesions
3	67 – 100 % of stem covered in lesions or stem destroyed

These categories were then converted into a disease severity index using the following formula:

$$\frac{[(1 \times \text{no. stems in category 1}) + (2 \times \text{no. stems in category 2}) + (3 \times \text{no. stems in category 3})]}{\text{total no. of stems}}$$

To investigate infection cushion formation microscopically, harvested individual stems were rinsed in sterile distilled water and cut in half lengthways using a

scalpel blade. Tissue from inside the stem was scraped from the epidermis, and the resulting strips of tissue placed in a container and submerged in 10 % potassium hydroxide (w/v). The strips were autoclaved for 15 min at 121°C, rinsed gently in sterile distilled water and placed into 0.03% chlorazol black E solution [1:1:1 (v/v) chlorazol black E, glycerol, lactic acid]. These were autoclaved for a further 15 min, after which the resulting stained sections were removed and placed in a 50 % (v/v) glycerol solution to de-stain for 2 d. The strips were then placed on microscope slides, covered in several drops of lactoglycerol [1:1 (v/v) lactic acid, glycerol] and overlain with a coverslip. The epidermal strips were viewed using an Olympus Vanox microscope (x33, 66 and 132 magnification) connected via a camera (JVC TK-S350) to Optimas 3.01 image analysis software. They were examined for the presence of infection cushions and whether the presence of microscopic infection cushions coincided with the onset of stem lesions.

5.2.2 The production of extracellular cell-wall degrading enzymes by different AGs of *Rhizoctonia solani*

For all enzyme assays, isolates were grown in a liquid basal medium as according to Townsend (1957) [3.5g KNO_3, 1.75g KH_2PO_4, 0.75g $MgSO_4.7H_2O$] adjusted to pH 5.5 with NaOH, and supplemented with a carbon source or cell walls as appropriate.

Cell walls were isolated using a modified method originally described by Hu and Brown (1994). Potato plants were grown in 3-l pots containing John Innes no. 3 compost for 15 d in a glasshouse at 15°C and the stems harvested. The lower portions of stems, which had been under the soil, were excised and 20 g of this tissue was cut into smaller 5 mm pieces. The tissue was rapidly frozen in liquid nitrogen and ground in a pestle and mortar. The tissue was resuspended in 30 ml HEPES buffer (pH 7.0) containing 2 mM $K_2O_5S_2$ and ultrasonicated in a Biosonic III sonifier for 6 min at 50 %

full power. The solution was kept below 10°C by immersing the vessel containing the cell wall suspension in ice. Sonication was repeated five times until microscopic examination revealed no cytoplasmic residue attached to the cell wall fragments. Cell wall fragments were centrifuged at 1000 g for 10 min and the precipitate resuspended, washed with cold water and centrifuged again. The resulting precipitate was washed three times with 80 % (v/v) ethanol and once with a 1:1 methanol:chloroform (v/v) solution. This was followed by washing in acetone. After several washes in sterile distilled water, cell walls were immediately added to the basal medium.

The liquid medium was decanted into 9 cm diam Petri dishes (~20 ml) and inoculated by adding a 3 mm diam mycelial plug taken from the edge of a 3-d-old colony of *R. solani* grown on MYA. All Petri dishes were incubated at 25°C and the production of cell-wall degrading enzymes determined after 1, 2, 3, 6, and 10 d. Prior to all enzyme assays, the mycelial mats were removed from the cultures, washed and placed onto Whatman no. 1 filter papers, which had been oven-dried at 70°C and pre-weighed. The mycelium plus filter paper was then placed in the same oven at 70°C for a further 48 h to determine the dry weight of the mycelial biomass. The remaining culture solutions were filtered through sterile Whatman no. 1 filter papers to remove any remaining cell wall/substrate material prior to use in assays.

5.2.2.1 In vitro *production of extracellular cutinase*

The cutinase [cutin hydrolase, EC 3.1.1.74] assay measured the release of 4-nitrophenol from 4-nitrophenol esters of short chain fatty acids spectrophotometrically at 405 nm from the model substrate. All four isolates of *R. solani* were grown in the basal medium alone, basal medium plus (g l^{-1}) cutin powder (10 g), and basal medium plus cell walls (10 g), and prepared for assaying as described previously (section 5.2.2).

Cutin powder was prepared from apple peels, cv. Golden Delicious (Bostock *et al.*, 1999). The apples were peeled and the skins autoclaved (121°C, 103.4 kPa) in distilled water for 15 min. After cooling, any excess pulp was removed from the peels by scraping with a scalpel. The peels were placed in a beaker containing a 2:1 (v/v) chloroform:methanol solution for 24 h, removed and placed in a laminar flow cabinet until dry. Residual pectin and cellulose were removed from the peels by incubation overnight at 30°C in a solution containing cellulase (Sigma: 44 units ml^{-1}) and pectinase (Sigma: 0.05 mg protein ml^{-1}) in 0.05 M sodium acetate buffer (pH 4). The peels were washed in several rinses of deionized water followed by further washing in a solution of 95 % (v/v) ethanol. Immersion in the chloroform:methanol solution through to the ethanol rinse was repeated, and the peels were dried as before. Once dried thoroughly, peels were ground into a powder using a Wiley mill equipped with a 60 mesh filter.

To prepare the substrate solution, 21 mg of 4-nitrophenol butyrate and 160 mg of Triton X-100 were transferred to a 100 ml beaker with ethyl ether as a solvent (Kolattukudy *et al.*, 1981). The solvent was evaporated off with a stream of N_2 and 50 ml of distilled water added. The beaker was heated on a steam bath until a liquid layer was formed by the substrate and the mixture subjected to ultrasonic treatment with the large probe of Biosonic III for 1 min at 40 % full power. Once cooled, a clear solution was obtained and used in the assay (Kolattukudy *et al.*, 1981).

For the assay, 1.6 ml of 0.1 M sodium phosphate buffer (pH 8.0), 0.2 ml of Triton X-100 solution, 1.0 ml of substrate solution and 0.2 ml of enzyme solution were pipetted into a cuvette. The contents were mixed by inverting the cuvette several times and the absorbance change measured at 405 nm from zero to 5 min. A standard curve was produced by plotting absorbance at 405 nm against µmoles of 4-nitrophenol present (Appendix B). One unit of activity (U) was defined as the amount of enzyme required to produce 1 µmole of 4-nitrophenol min^{-1}. The assay was carried out at 18°C,

pH 8.0 with 4-nitrophenol butyrate as the substrate. Enzyme activity per Petri dish was subsequently converted into the activity per mg (dry weight) of mycelium (U mg^{-1} d.w. mycelium).

5.2.2.2 In vitro *production of extracellular pectin lyase*

The presence of pectin lyase [(1→4)-6-*O*-methyl-α-D-galacturonan lyase, EC 4.2.2.10] in culture filtrates was determined by measuring the reaction between the unsaturated end product of pectin degradation and thiobarbituric acid (Pitt, 1988). All *R. solani* isolates were grown on the basal medium alone, basal medium containing 1 % (w/v) citrus pectin (Sigma) and basal medium plus 10 g l^{-1} cell walls, and prepared for assaying as described previously (section 5.2.2).

The reaction mixture contained 5.0 ml of 1 % (w/v) pectin solution in 0.05 M Tris-HCl buffer (pH 8.5), 1.0 ml of 0.01 M $CaCl_2$ solution and 1.0 ml of enzyme solution with 3 ml of distilled water added to make a final reaction volume of 10 ml. The mixture was incubated for 2 h at 30°C in a water bath, after which 0.6 ml of 9 % $ZnSO_4$ solution, followed by 0.6 ml of 0.5 M NaOH were added. The precipitated protein and unused substrate were removed by centrifugation (Centaur) at 3000 g for 10 min, and 5 ml of the supernatant added to a mixture of 3.0 ml of 0.04 M thiobarbituric acid, 1.5 ml 0.1 M HCl and 0.5 ml distilled water. The mixture was heated in a boiling water bath for 30 min, and the absorbance read at 550 nm against a reference cuvette once the solution had cooled to room temperature (18–21°C). One unit of activity (U) was defined as the amount of enzyme causing a change in absorbance of 0.01 under the conditions of the assay. Enzyme activity per Petri dish was converted into activity per mg (dry weight) of mycelium (U mg^{-1} d.w. mycelium) at 18°C, pH8.5 with citrus pectin as the substrate.

5.2.2.3 *In vitro* production of extracellular polygalacturonase

Polygalacturonase activity was determined using a method which measured the increase in reducing groups resulting from the release of oligogalacturonates from polygalacturonate (Collmer *et al.*, 1988). All *R. solani* isolates were grown on the basal medium alone, basal medium containing 1 % (w/v) citrus pectin (Sigma) and basal medium plus 10 g l^{-1} of cell walls, and prepared for assaying as described previously (section 5.2.2). Three stock solutions were prepared for this assay: a substrate solution, copper reagent and arsenomolybdate reagent. To prepare the substrate solution, 20 ml of 0.6 M NaCl was added to 80 ml of a solution containing 75 mM sodium acetate buffer (pH 5.3), 7.5 mM of EDTA and 0.3 % (w/v) of polygalacturonic acid. The final pH was adjusted to 5.3 with 1 M NaOH, 0.02 % (w/v) sodium azide added and the solution stored at 4°C until required.

The copper reagent was prepared by dissolving 12 g of sodium potassium tartrate and 24 g anhydrous Na_2CO_3 in 250 ml water. During stirring and in the following sequence, a solution of 4 g cupric sulphate pentahydrate in 100 ml water, 16 g of sodium hydrogen carbonate and a solution of 180 g of anhydrous sodium sulphate in 500 ml boiled water were added. The final solution was made up to 1-l with the addition of 150 ml of distilled water. Any precipitate that formed was removed by filtration through Whatman no. 1 filter papers, and the resulting clear blue filtrate retained.

The arsenomolybdate reagent was prepared by dissolving 25 g of ammonium molybdate in 450 ml of water together with 21 ml of 96 % (v/v) sulphuric acid. A solution of 3.0 g disodium hydrogen arsenate heptahydrate in 25 ml water was also added, and the final solution stored in a brown bottle. The solution was incubated at 37°C for 24 h prior to use.

The assay was initiated by mixing 2.5 ml of substrate solution and 0.5 ml of a four-fold diluted enzyme sample at 30°C. After 10 min, 0.5 ml samples were removed

from the reaction mixture and immediately mixed with 0.5 ml of copper reagent to stop the reaction. The sample tubes were each covered with a marble and placed in a boiling water bath for 10 min. Once the tubes had cooled to room temperature, 1.0 ml of arsenomolybdate reagent was added. After 40 min at room temperature (18-21°C), the assay mixture was centrifuged (3000g; Centaur) to remove any precipitated residual substrate and absorbance of the supernatant was read at 500 nm.

The increase in absorbance over time in relation to a substrate blank was then calculated and these values were used in reference to a D-galacturonic acid standard curve to determine the rate of oligogalacturonic acid formation (Appendix B). One unit of enzyme (U) formed 1 µmol of oligogalacturonate in 1 min under the assay conditions described. Enzyme activity per plate was converted into the activity per mg (dry weight) of mycelium (U mg^{-1} d.w. mycelium). The assay was carried out at 30°C, pH 5.3 on citrus pectin as the substrate.

5.2.2.4 In vitro *production of extracellular endo-β-1,4-glucanase*

The production of cellulase [1,4-(1,3;1,4)-b-D-glucan-4-glucanohydrolase, EC 3.4.2.1] was measured by estimating the amount of reducing sugar liberated from the substrate (Wood & Bhat, 1988). All *R. solani* isolates were grown on the basal medium alone, basal medium containing 1 % (w/v) carboxymethylcellulose (CMC; low viscosity, Sigma), and basal medium containing 10 g l^{-1} of cell walls and were prepared for assaying as described previously (section 5.2.2). To prepare the dinitrosalicyclic acid (DNS) reagent, 8 g of dinitrosalicyclic acid, 1 g of phenol, 0.25 g of Na_2SO_3 and 100 g of Rochelle salt were dissolved in 250 ml of 2% (w/v) NaOH solution and diluted to 500 ml with 250 ml distilled water. For the assay, the enzyme solution was heated in a water bath for 5 min at 37°C and thereafter 0.25 ml of 2 % (w/v) of CMC in 0.05 M sodium citrate buffer (pH 4.8) was added. The solution was mixed well on a vortex

mixer, and placed in an incubator for 30 min at 37°C. DNS reagent (1.5 ml) was added and the solution mixed by inverting the tubes completely so that the solution separated from the bottom of the tube each time.

The absorbance was measured at 540 nm and translated into µg glucose produced using a standard curve of glucose (µg) against absorbance (Appendix B). The units of enzyme activity (U) were calculated as 1µmol of hydrolysis product released per min. Enzyme activity per plate was converted into the activity per mg (dry weight) of mycelium (U mg^{-1} d.w. mycelium) when the assay was carried out at 37°C, pH 4.8 with CMC as the substrate.

5.2.2.5 In vitro *production of extracellular β-glucosidase*

This assay measured the production of β-glucosidase [β-D-glucoside glucohydrolase, EC 3.2.1.21] by determining the liberation of nitrophenol from the 4-nitrophenyl-β-D-glucoside substrate colorimetrically (Wood & Bhat, 1988). All *R. solani* isolates were grown on the basal medium alone, basal medium containing (g l^{-1}) 10 g of cellobiose (Sigma), and basal medium containing 10 g of cell walls, and prepared for assaying as described previously (section 5.2.2).

To begin the assay, 1 ml of 5 mM 4-nitrophenyl-β-D-glucoside in 0.1 M sodium acetate buffer (pH 4.8) was pipetted into a test tube together with 1.8 ml of 0.1 M acetate buffer (pH 4.8), and equilibrated to 37°C in a water bath. The enzyme solution was diluted five-fold and 200 µl of this solution was added to the reaction mixture. The tube contents were thoroughly mixed using a vortex mixer and incubated at 37°C for 30 min. Immediately after removal from the incubator, 4 ml of 0.4 M glycine buffer (pH 10.8), was added to stop the reaction, and any liberated 4-nitrophenol was measured at 430 nm once the tubes had cooled to room temperature (18–21°C). A standard curve was produced using 4-nitrophenol as the standard, and the absorbance

values obtained were translated into μmoles of nitrophenol (Appendix B). Enzyme activity (U) was calculated as the amount of enzyme required to release 1 μmol of 4-nitrophenol per min under the conditions of the assay. Enzyme activity per plate was converted into the activity per mg (dry weight) of mycelium (U mg^{-1} d.w. mycelium) where assay conditions were 37°C, pH 10.8 and 4-nitrophenyl-β-D-glucoside as the substrate.

5.2.3 Statistical analysis

All data were analysed using analysis of variance (ANOVA), and treatment means were compared using the least significant difference (LSD) at a probability of 5 % ($P = 0.05$) as described previously (section 3.2.9). The standard errors of the treatment means were also calculated for all enzyme assays. Data were analysed using Genstat® for Windows, 7th Edition.

5.3 Results

5.3.1 Pathogenicity of different anastomosis groups of *R. solani* from potato

No infection cushions were detected microscopically, and no visible stem lesions formed on any stems grown in uninfested growth medium (Plate 5.1). There were significant differences in the disease symptoms caused by different AGs of *R. solani*, and these are summarised in Table 5.2. Isolate x81 (AG 2-1) formed small, isolated infection cushions between 1–2 d after planting, producing tiny lesions no more than 3–5 mm in length (Plate 5.1). These lesions were yellow-brown in colour, unlike the deep dark brown lesions found on stem tissue infected by AG 3 and 5 isolates. After 10 d, the stem lesions had not increased in size, and only small infection cushions were detectable microscopically (Plate 5.2).

The onset of infection cushion formation also occurred between 1–2 d for isolate T1 (AG 5). In three of the four replicates sampled after 2 d, hyphal branching and infection cushions were apparent on the surface of the stem microscopically, but no visible lesions were present on the stems. After 3 d, the infection cushions had become more extensive (Plate 5.3) and stem infection was clearly visible on the stem, primarily as superficial brown surface lesions. In several instances, lesions girdled the stem and penetrated the vascular bundle, causing stem death (Plate 5.1). The short molloloid cells typical of infection cushions formed by *R. solani* AG 3 were clearly visible (Plate 5.4) and hyphal cells were shorter and wider compared to the long and narrow cells observed during mycelial growth observed *in vitro* (Plate 5.5).

Both AG 3 isolates (x72 and UN) caused no stem infection until between 2–3 d, when extensive infection cushion formation and stem canker lesions were observed (Plate 5.1). Although the initiation of infection was more than 1 d later than the other AGs, extensive infection cushions and deep sunken lesions developed rapidly on the stems. Dark brown lesions covered the entire stem, with the majority of the tissue

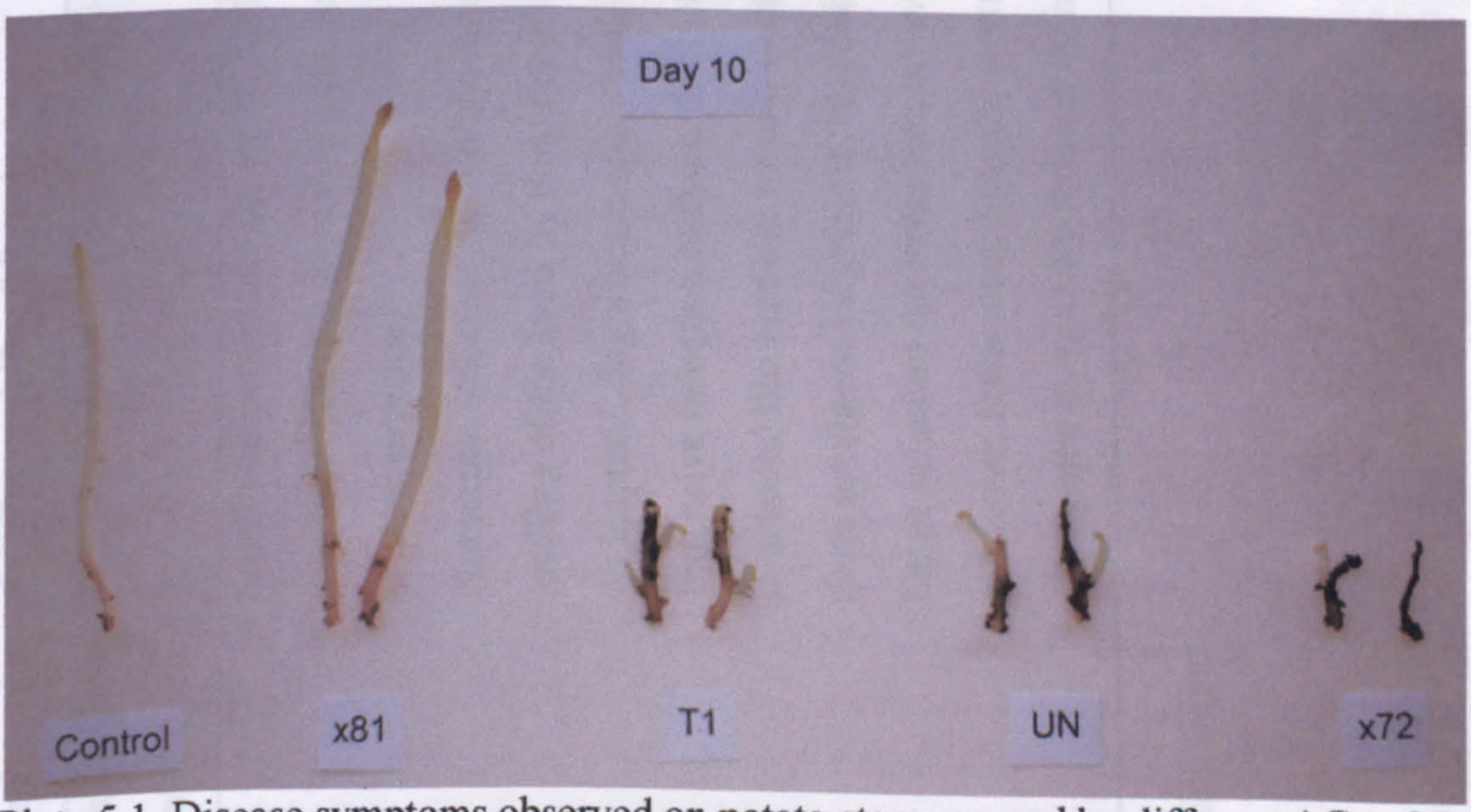


Plate 5.1. Disease symptoms observed on potato stems caused by different AGs of *R. solani* pathogenic to potato after 10 d [Control (uninfected), x81 (AG 2-1), T1 (AG 5), x72 and UN (both AG 3)].

Table 5.2. Summary of the stem symptoms caused by soil-borne inoculum of different AGs of *R. solani*.

Isolate (AG)	Infection cushion formation (d after inoculation)	Onset of visible stem lesions (d after inoculation)	Typical disease symptoms after 10 d
x81 (2-1)[a]	2–3[b]	2–3[b]	Small compact infection cushions formed, stem lesions no more than 3–4 mm in length. No stem death as a result of infection and no reduction in stem emergence.
x72 (3)	3–4	3–4	Numerous infection cushions over stem surface; stem lesions extensive, girdling of the stem by deep necrotic lesions common; stem death widespread. Significant reduction in stem emergence.
UN (3)	3–4	3–4	Extensive infection cushion formation over stem surface; necrotic lesions penetrated the vascular bundle causing tissue shrinkage and stem death. Stem emergence significantly reduced by infection.
T1 (5)	2–3	3–4	Large infection cushions formed over stem surface; surface lesions extensive; fewer deep necrotic stem lesions causing stem death. Stem emergence marginally reduced by infection.

[a]anastomosis group of isolate.
[b]onset of disease symptoms occurred between these two sampling times.

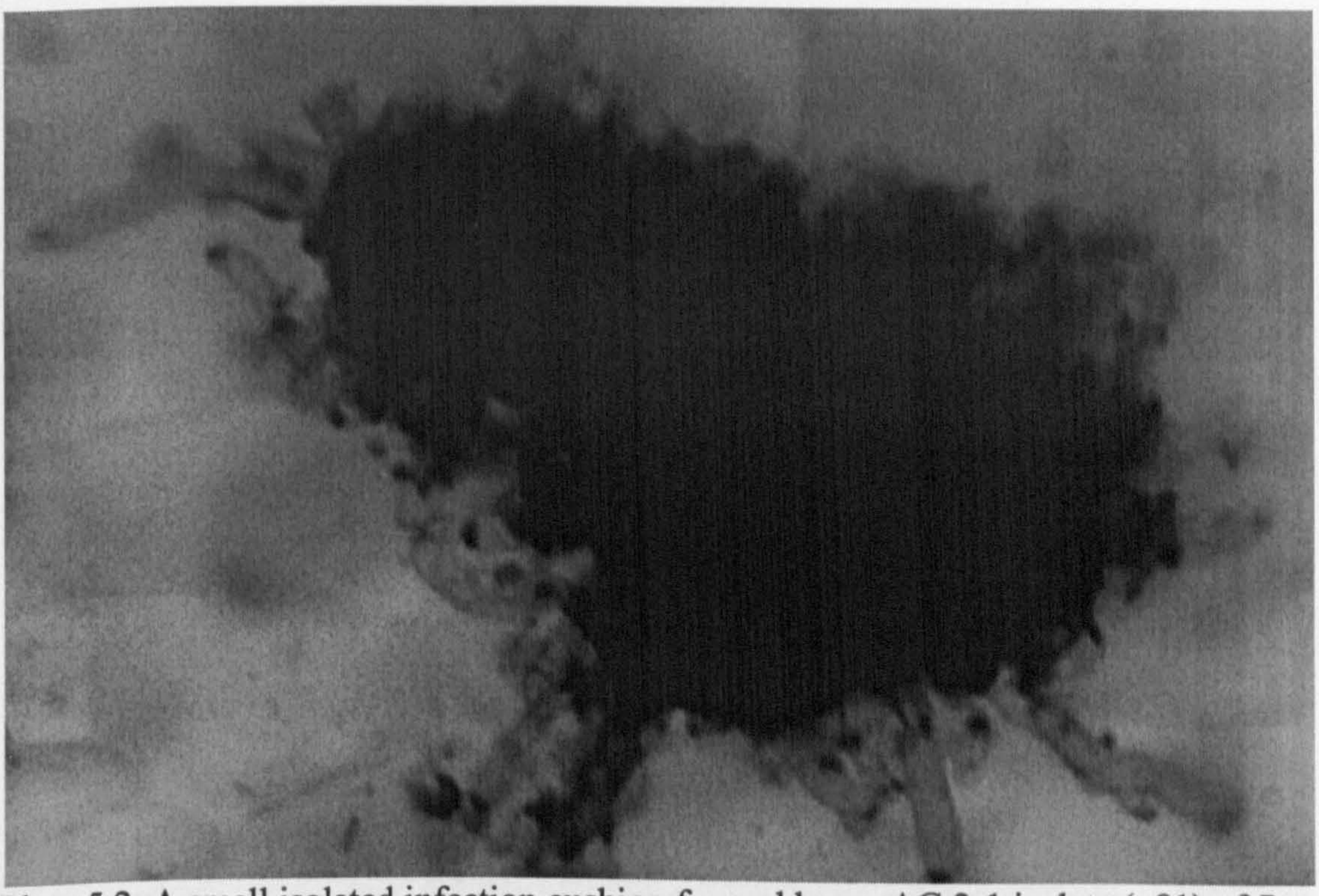


Plate 5.2. A small isolated infection cushion formed by an AG 2-1 isolate (x81) of *R. solani* after 10 d. (x 132 magnification)

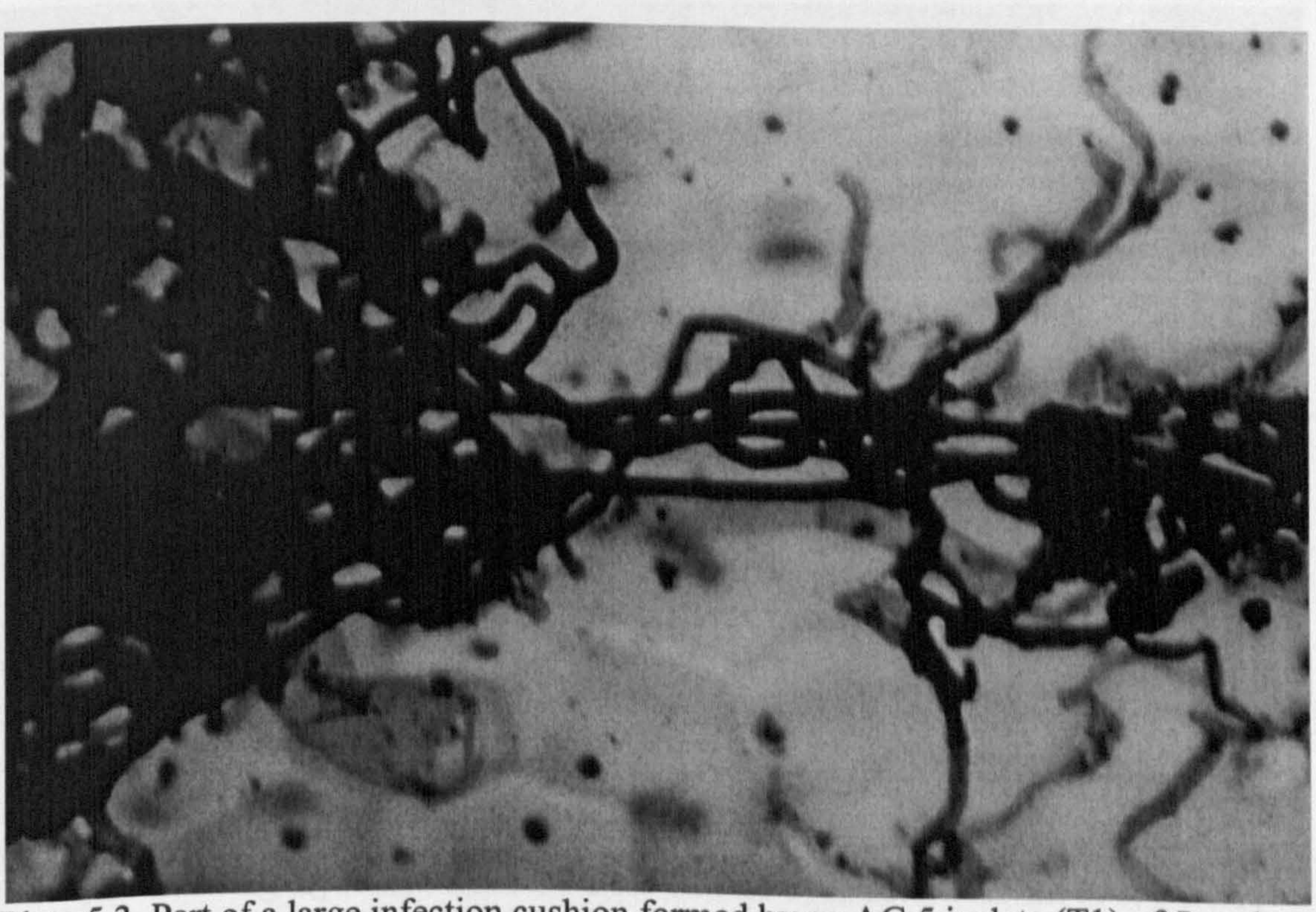


Plate 5.3. Part of a large infection cushion formed by an AG 5 isolate (T1) of *R. solani* after 10 d (x 33 magnification).

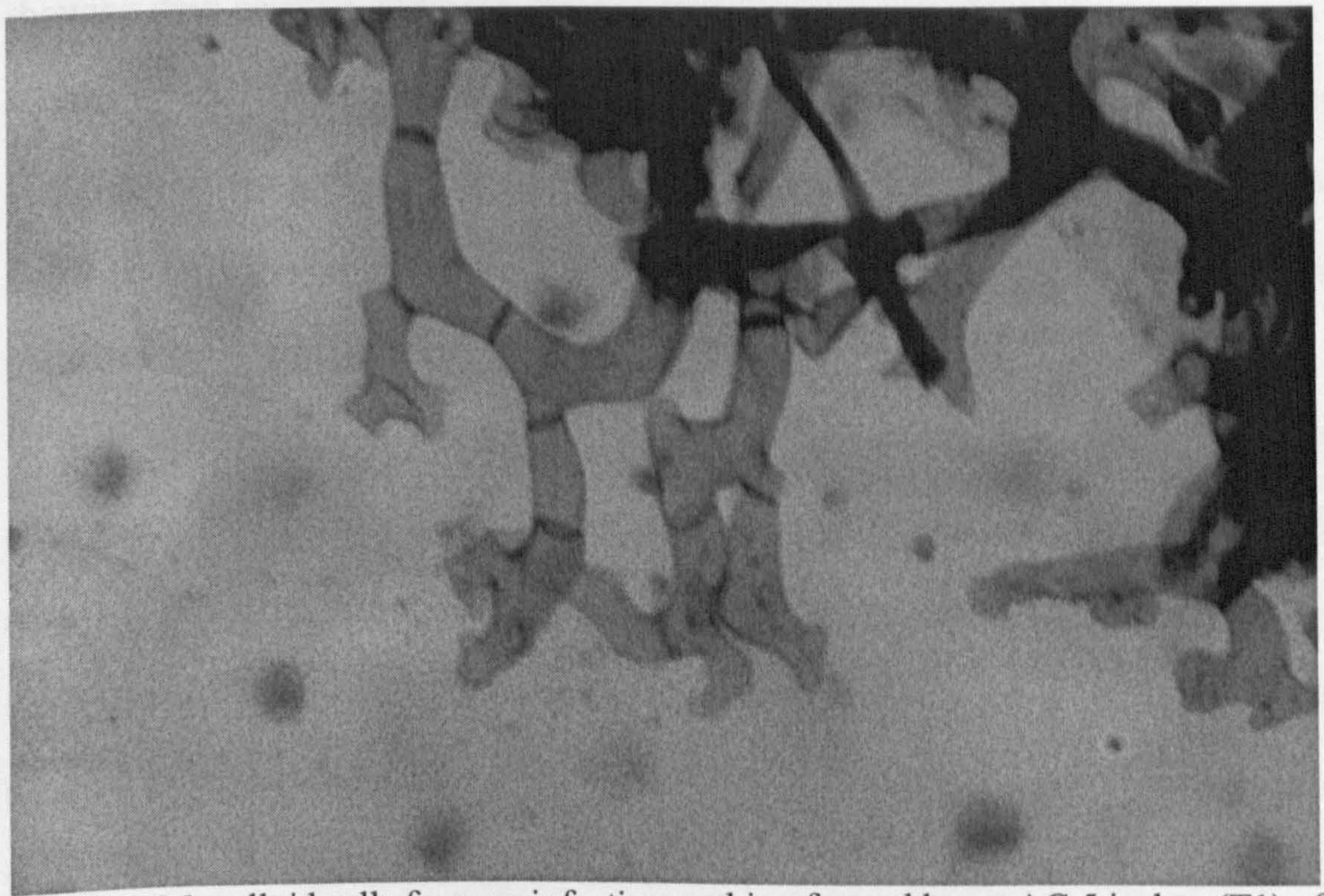


Plate 5.4. Monolloid cells from an infection cushion formed by an AG 5 isolate (T1) of *R. solani* on an immature potato stem after 10 d (x 132 magnification).

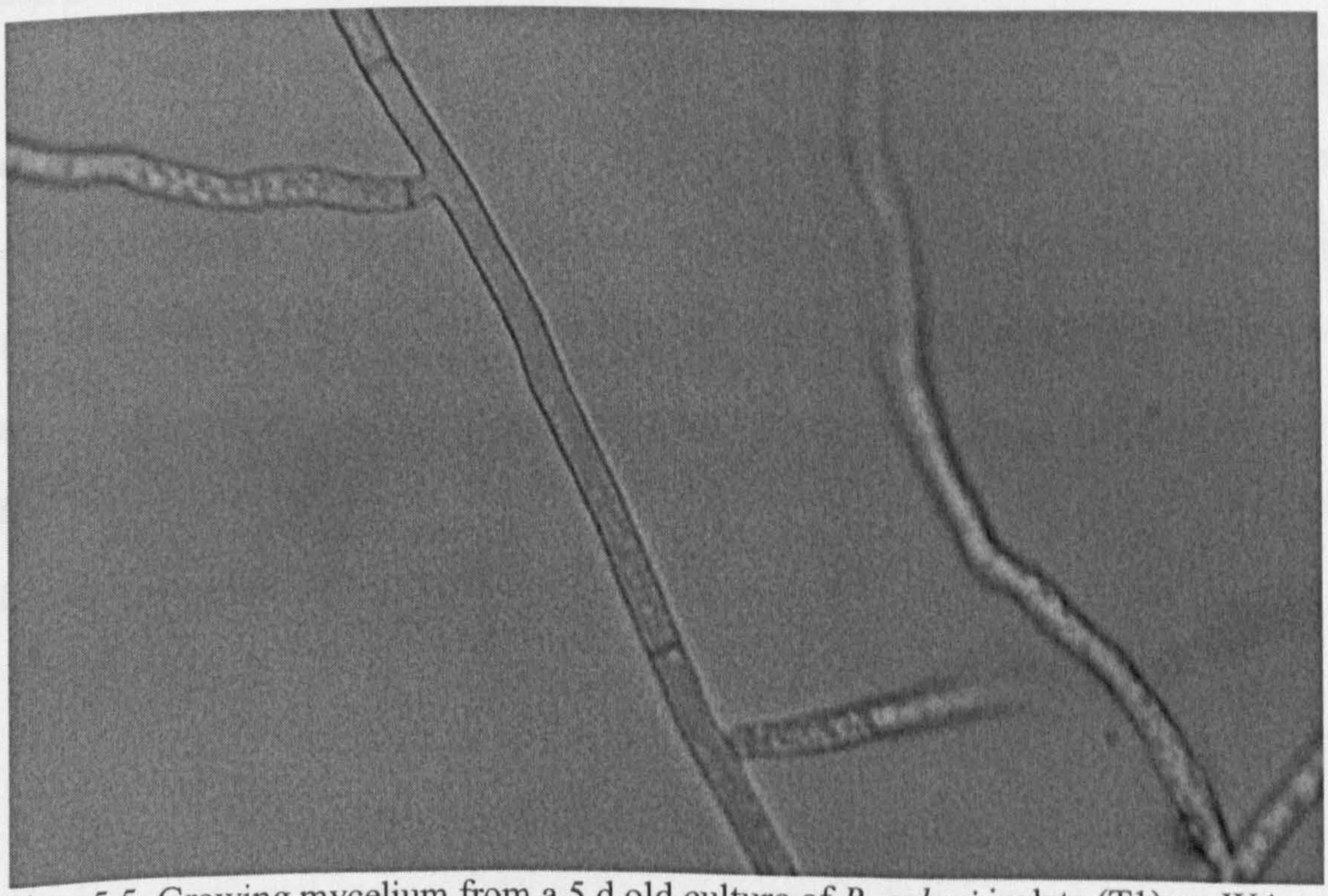


Plate 5.5. Growing mycelium from a 5 d old culture of *R. solani* isolate (T1) on WA (x 66 magnification).

showing deep necrosis. Extensive tissue destruction was caused by both AG 3 isolates, and the lesions routinely penetrated the vascular bundle, causing stem death. After 6 and 10 d, new sprouts were observed growing out from uninfected healthy tissue on infected stems (Plate 5.1). This occurred on the healthy tissue beneath severe lesions that had destroyed the original growing tip.

Stem emergence was affected significantly ($P = 0.05$) by the AG of *R. solani* present as soil-borne inoculum (Figure 5.2). Both the uninfested control and isolate x81 (AG 2-1) had 100 % emergence 6 d after planting. Less than half of the stems planted in soil infested with T1 (AG 5) had emerged after 6 d, and no emergence was observed for plants growing on soil infected with isolates UN and x72 (AG 3). After 10 d, emergence was > 60 % for plants growing in soil infected with T1. Stem emergence in AG 3 infested soil was poor after 10 d, with just under 20 % emergence for plants growing in soil inoculated with UN, and no emergence for plants grown in soil infested with x72.

The AGs of *R. solani* used as soil-borne inoculum also had a significant effect on stem canker severity (Figure 5.3). No stem canker was observed in the uninfested control at any time throughout the duration of the experiment. Stem lesions were visible for all isolates between 2–3 d after planting. Once stem canker was visible, most isolates showed an increase in stem canker severity over 10 d, except AG 2-1 isolate x81. Moderate disease was observed on stems planted in soil infested with isolate T1 (AG 5), however, the most severe stem canker was caused by AG 3 isolates UN and x72.

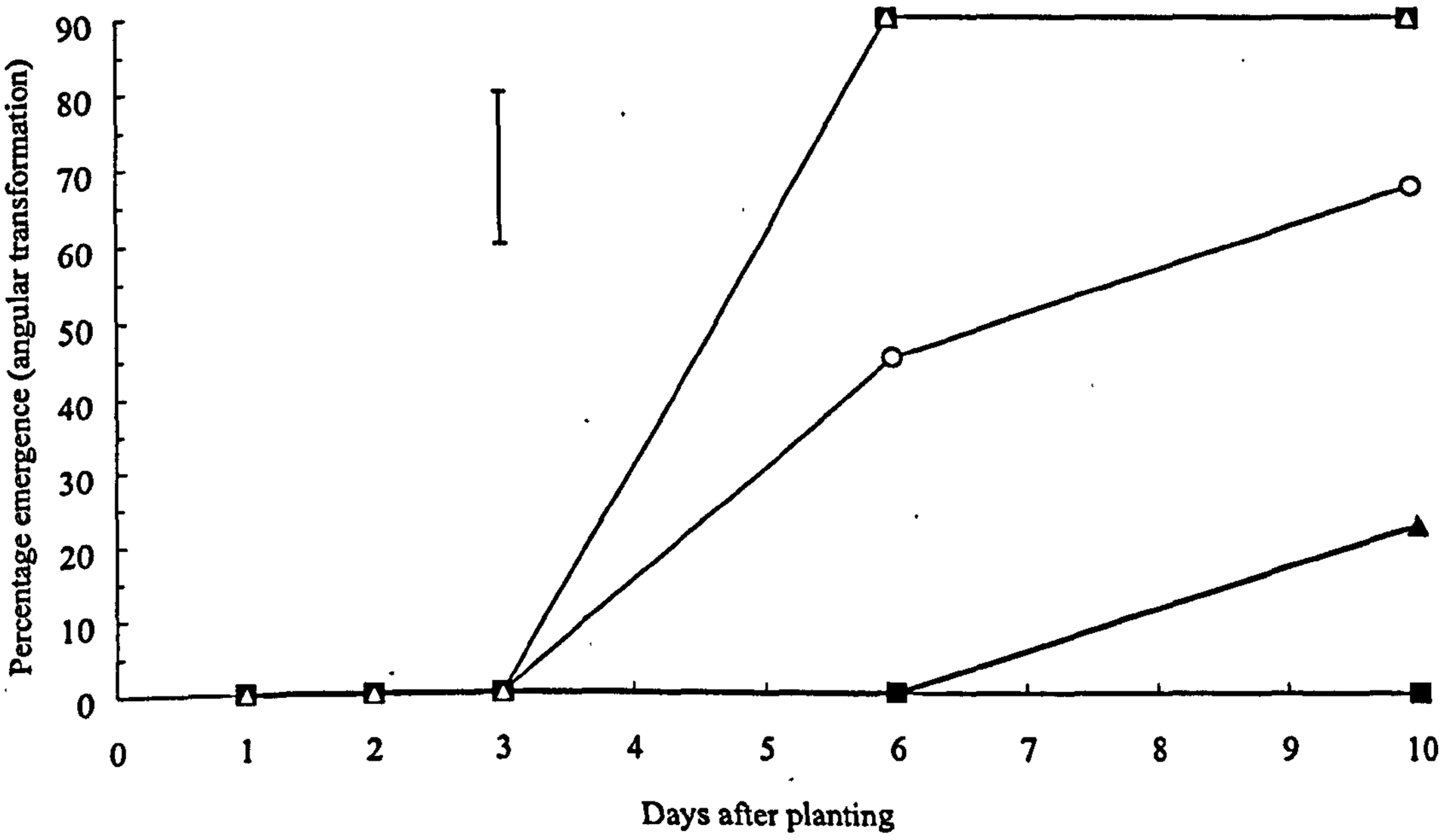


Figure 5.2. Effect of soil-borne inoculum from different AGs (uninfested control □, x81 [AG 2-1] Δ, x72 [AG 3]■, UN [AG 3]▲ and T1 [AG 5] o) of *R. solani* on plant emergence. Values are the angularly transformed means of the percentage emergence of four replicates. Bar = LSD at $P = 0.05$ (DF = 59).

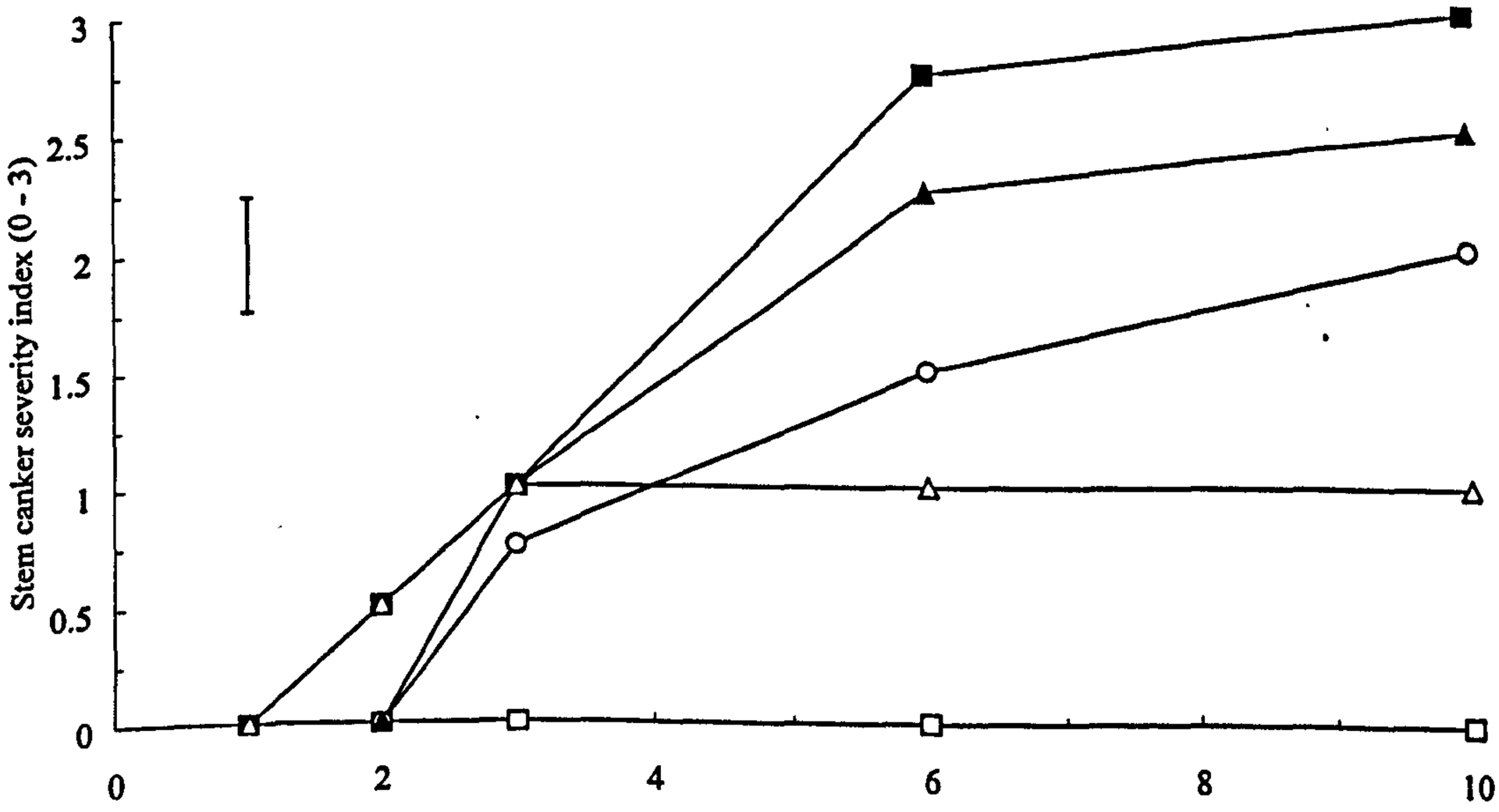


Figure 5.3. Effect of soil-borne inoculum from different AGs (uninfested control □, x81 [AG 2-1] Δ, x72 [AG 3]■, UN [AG 3]▲ and T1 [AG 5] o) of *R. solani* on stem canker severity. Values are the mean stem canker severity indices observed of four replicates. Bar = LSD at $P = 0.05$ (DF = 99).

Stem length was significantly affected by the AGs present as soil-borne inoculum (Figure 5.4). Uninfected control plants increased > 400 % in length after 10 d, with stems grown in soil infected with isolates T1 (AG 5) and x81 (AG 2-1) increasing in length > 300 %. Stems grown in soil inoculated with AG 3 isolates (x72 and UN) showed an average increase in stem length of just over 50 % after 10 d (Figure 5.4). This reduction was caused by the AG 3 isolates attacking the growing stems, the resulting lesions destroying the tissue, killing the sprout and preventing further growth.

5.2.3 The production of extracellular cell-wall degrading enzymes by different AGs of *Rhizoctonia solani*.

5.2.3.1 In vitro *production of extracellular cutinase.*

All isolates of *R. solani* produced cutinase on all culture media tested (Table 5.3). Between 6 to 10 d, cutinase production was significantly greater ($P = 0.05$) when isolates T1 (AG 5) and UN (AG 3) were grown on cutin-containing media compared to the basal media alone, or media containing cell walls (Table 5.3). The AG 2-1 isolate produced significantly less ($P = 0.05$) cutinase than all other isolates after 10 d on cutin-containing media.

5.2.3.2 In vitro production of extracellular pectin lyase

Pectin lyase activity was not detected in any culture media until cultures had been incubated for 3 d. (Table 5.4). Slightly more pectin lyase activity was detected in cultures containing citrus pectin as a substrate after 10 d growth for most isolates. There were no significant differences between the isolates on the different media tested with regards to the production of pectin lyase.

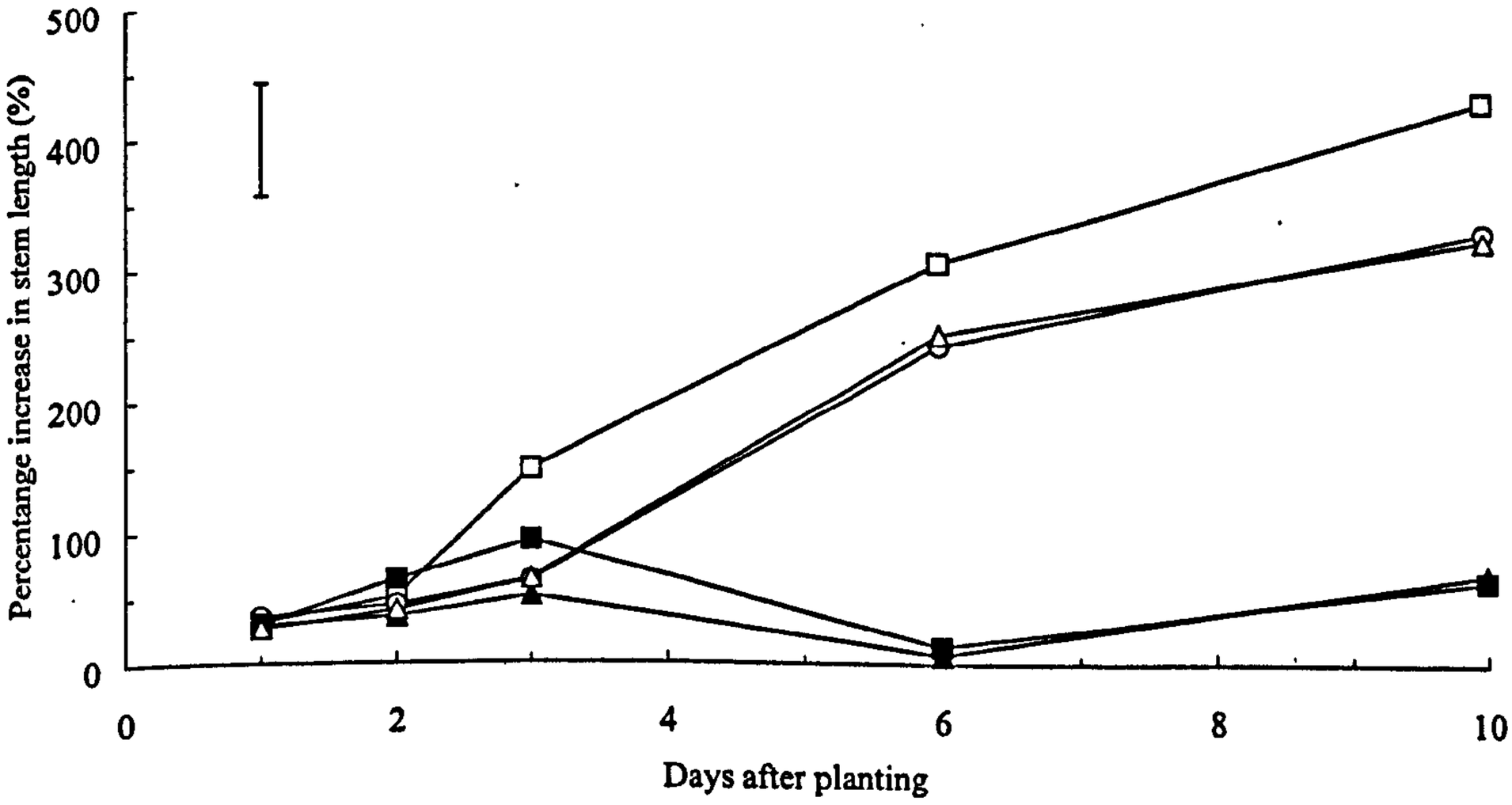


Figure 5.4. Effect of different AGs (uninfested control □, x81 [AG 2-1] Δ, x72 [AG 3]■, UN [AG 3]▲ and T1 [AG 5] o) of *R. solani*. Values are the mean increases in stem length of four replicates. Bar = LSD at $P = 0.05$ (DF = 99).

Table 5.3. The production of extracellular cutinase by five isolates of *R. solani* representing three AGs pathogenic to potato.

	Incubation time (d)											
	1			3			6			10		
Isolate (AG)	basal	cell walls	cutin	basal	cell walls	cutin	basal	cell walls	cutin	basal	cell walls	cutin
T1 (5)	8.6 ± 2.6[a]	3.7 ± 6.3	11.3 ± 2.8	7.5 ± 2.0	5.3 ± 1.2	11.1 ± 2.2	6.2 ± 4.8	9.7 ± 3.0	37.7 ± 6.6	12.2 ± 5.3	13.4 ± 3.1	37.2 ± 9.3
UN (3)	8.7 ± 2.8	2.6 ± 11.1	7.4 ± 0.9	11.0 ± 4.8	15.1 ± 5.0	14.7 ± 4.6	9.8 ± 1.6	11.1 ± 3.8	36.3 ± 7.6	8.9 ± 7.1	12.6 ± 3.7	33.6 ± 12.5
x72 (3)	5.9 ± 1.4	11:9 ± 4.3	5.9 ± 0.7	7.9 ± 1.4	8.9 ± 5.8	17.7 ± 6.4	6.1 ± 1.6	20.0 ± 6.6	26.3 ± 6.3	14.7 ± 3.4	17.0 ± 1.8	23.4 ± 4.3
x81 (2-1)	2.7 ± 1.8	3.0 ± 0.7	14.2 ± 7.5	3.2 ± 1.3	5.4 ± 3.9	14.9 ± 6.3	7.5 ± 2.8	13.3 ± 5.8	16.5 ± 5.7	10.5 ± 4.2	9.6 ± 2.8	9.7 ± 1.4
P value	0.033			0.001			0.039			0.049		
LSD	5.80			11.74			14.06			14.63		
($P = 0.05$), DF = 33[b]												

[a]Values are the mean enzyme activities (U mg^{-1} d.w. mycelium) of four replicates ± standard error (SE).
[b]LSD is the least significant difference at 5 % ($P = 0.05$), DF = degrees of freedom in ANOVA.

Table 5.4. The production of extracellular pectin lyase by five isolates of *R. solani* representing three AGs pathogenic to potato.

	Incubation time (d)											
	1			3			6			10		
Isolate (AG)	basal	cell walls	citrus pectin	basal	cell walls	citrus pectin	basal	cell walls	citrus pectin	basal	cell walls	citrus pectin
T1 (5)	0.0[a]	0.0	0.0	2.0 ± 0.7	1.4 ± 1.1	5.3 ± 1.0	3.7 ± 0.7	3.6 ± 0.7	8.5 ± 2.2	13.5 ± 2.9	6.4 ± 1.2	13.6 ± 3.9
UN (3)	0.0	0.0	0.0	3.0 ± 0.7	2.6 ± 0.2	8.0 ± 0.9	6.3 ± 1.2	10.3 ± 1.1	4.2 ± 1.8	7.9 ± 1.9	7.1 ± 1.1	10.9 ± 3.6
x72 (3)	0.0	0.0	0.0	4.0 ± 1.3	1.14 ± 0.3	3.2 ± 0.8	9.0 ± 1.6	5.9 ± 3.1	5.7 ± 1.8	8.5 ± 1.8	7.2 ± 1.5	13.7 ± 3.0
x81 (2-1)	0.0	0.0	0.0	2.2 ± 0.4	2.4 ± 0.5	6.2 ± 0.1	7.3 ± 3.3	3.4 ± 0.4	6.5 ± 0.6	5.8 ± 0.6	5.8 ± 0.8	10.2 ± 2.0
P value	*			0.202			0.128			0.641		
LSD ($P = 0.05$), DF = 33[b]	*			10.23			5.25			6.05		

[a]Values are the mean enzyme activities (U mg^{-1} d.w. mycelium) of four replicates ± standard error (SE).
[b]LSD is the least significant difference at 5 % ($P = 0.05$), DF = degrees of freedom in ANOVA.
*not calculated.

5.2.2.3 In vitro *production of extracellular polygalacturonase*

No polygalacturonase activity was detected after 1 d growth on culture media containing cell walls for all isolates (Table 5.5). There were no significant differences in the production of polygalacturonase on the basal cell wall-containing medium for any isolate on any assay date. Polygalacturonase production by T1 (AG 5), UN and x72 (both AG 3) was significantly greater ($P = 0.05$) than that detected on the basal medium alone after 10 d.

5.2.2.4 In vitro production of extracellular endo-1,4-β-glucanase

No endo-1,4-β-glucanase activity was detected on the basal medium at any time during the 10 d incubation period (Table 5.6). Enzyme activity on the medium containing cell walls was not detected until after 6 d incubation. Endo-1,4-glucanase activity was detected after 3 d incubation on a medium containing CMC. After 10 d, significantly greater ($P = 0.05$) enzyme activity was found in the medium containing CMC compared to cell walls, except for isolate x81 (AG 2-1).

5.2.2.5 In vitro production of extracellularβ-glucosidase

Activity of β-glucosidase on basal media was negligible for all isolates, with slightly greater activity found on the media containing cell walls. Extracellular β-glucosidase was detected after 1 d incubation on media containing cellobiose, but not until 3 d after on basal and cell wall containing media (Table 5.11). Significantly greater ($P = 0.05$) β-glucosidase production was observed when isolates were grown on cellobiose compared to the other treatments after 10 d, except isolate x81 (AG 2-1).

Table 5.5. The production of extracellular polygalacturonase by five isolates of *R. solani* representing three AGs pathogenic to potato.

	Incubation time (d)											
	1			3			6			10		
Isolate (AG)	basal	cell walls	citrus pectin	basal	cell walls	citrus pectin	basal	cell walls	citrus pectin	basal	cell walls	citrus pectin
T1 (5)	0.04 ± 0.03	0.00	0.35 ± 0.06	0.03 ± 0.01	0.17 ± 0.14	1.07 ± 0.22	0.07 ± 0.04	0.18 ± 0.09	1.70 ± 0.06	0.22 ± 0.04	0.91 ± 0.14	2.26 ± 0.38
UN (3)	0.06 ± 0.06	0.00	0.56 ± 0.14	0.00	0.23 ± 0.11	1.24 ± 0.24	0.13 ± 0.07	0.31 ± 0.11	1.15 ± 0.09	0.32 ± 0.02	0.70 ± 0.21	1.95 ± 0.42
x72 (3)	0.16 ± 0.07	0.00	0.32 ± 0.09	0.03 ± 0.02	0.37 ± 0.08	0.85 ± 0.12	0.13 ± 0.02	0.18 ± 0.08	0.63 ± 0.13	0.27 ± 0.08	0.87 ± 0.24	2.94 ± 1.13
x81 (2-1)	0.22 ± 0.04	0.00	1.09 ± 0.41	0.04 ± 0.04	0.44 ± 0.03	1.15 ± 0.21	0.21 ± 0.06	0.36 ± 0.07	1.10 ± 0.08	0.09 ± 0.04	0.47 ± 0.04	0.96 ± 0.23
P value		0.072			0.442			0.049			0.026	
LSD		0.378			0.367			0.973			1.38	
(*P* = 0.05), DF = 33[b]												

[a]Values are the mean enzyme activities (U mg^{-1} d.w. mycelium) of four replicates ± standard error (SE).
[b]LSD is the least significant difference at 5 % (*P* = 0.05), DF = degrees of freedom in ANOVA.

Table 5.6. The production of extracellular endo-1,4-β-glucanase by five isolates of *R. solani* representing three AGs pathogenic to potato.

	Incubation time (d)											
	1			3			6			10		
Isolate (AG)	basal	cell walls	CMC	basal	cell walls	CMC	basal	cell walls	CMC	basal	cell walls	CMC
T1 (5)	0.0[a]	0.0	0.0	0.0	0.0	4.4 ± 3.4	0.0	5.6 ± 2.5	16.8 ± 5.8	0.0	23.1 ± 9.4	79.6 ± 4.8
UN (3)	0.0	0.0	0.0	0.0	0.0	14.5 ± 3.7	0.0	16.3 ± 3.2	21.6 ± 4.4	0.0	31.4 ± 4.2	57.9 ± 3.1
x72 (3)	0.0	0.0	0.0	0.0	0.0	3.8 ± 1.4	0.0	3.1 ± 1.3	24.3 ± 3.5	0.0	12.1 ± 5.7	44.7 ± 5.9
x81 (2-1)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	12.6 ± 7.7	5.8 ± 1.4	0.0	8.9 ± 2.3	18.8 ± 2.4
P value	*		<0.001			0.018			<0.001			
LSD ($P = 0.05$), DF = 33[b]	*		4.08			10.44			12.68			

[a]Values are the mean enzyme activities (U mg^{-1} d.w. mycelium) of four replicates ± standard error (SE).
[b]LSD is the least significant difference at 5 % ($P = 0.05$), DF = degrees of freedom in ANOVA.
*not calculated.

Table 5.7. The production of extracellular β-glucosidase by five isolates of *R. solani* representing three AGs pathogenic to potato.

	Incubation time (d)											
	1			3			6			10		
Isolate (AG)	basal	cell walls	cellobiose	basal	cell walls	cellobiose	basal	cell walls	cellobiose	basal	cell walls	cellobiose
T1 (5)	0.00[a]	0.00	0.94 ± 0.10	0.01 ± 0.10	0.11 ± 0.10	0.35 ± 0.03	0.00	0.05 ± 0.01	2.02 ± 0.12	0.41 ± 0.09	0.14 ± 0.08	1.24 ± 0.08
UN (3)	0.00	0.00	0.77 ± 0.16	0.00	0.54 ± 0.13	0.94 ± 0.04	0.01 ± 0.01	0.96 ± 0.34	1.34 ± 1.19	0.02 ± 0.01	0.89 ± 0.02	2.47 ± 1.24
x72 (3)	0.00	0.00	0.85 ± 0.25	0.08 ± 0.02	0.14 ± 0.02	0.57 ± 0.02	0.00	0.13 ± 0.02	1.69 ± 0.22	0.21 ± 0.06	0.60 ± 0.12	1.33 ± 0.15
x81 (2-1)	0.00	0.00	0.68 ± 0.34	0.00	0.62 ± 0.32	0.31 ± 0.14	0.06 ± 0.01	0.31 ± 0.11	0.73 ± 0.18	0.06 ± 0.06	0.29 ± 0.06	0.45 ± 0.05
P value		0.964			0.003			<0.001			0.181	
LSD (*P* = 0.05), DF = 33[b]		0.40			0.34			0.44			1.09	

[a]Values are the mean enzyme activities (U mg^{-1} d.w. mycelium) of four replicates ± standard error (SE).
[b]LSD is the least significant difference at 5 % (*P* = 0.05), DF = degrees of freedom in ANOVA.

5.3 Discussion

The formation of infection cushions via the branching and aggregation of fungal hyphae occurred in all AGs and was similar to that of other isolates of *R. solani* pathogenic to bean, cotton, tomato, radish, crucifers, rice and potato (Christou, 1962; Flentje, 1957; Khadga *et al.*, 1963; Dodman *et al.*, 1968; Marshall & Rush, 1980a; Armentout & Downer, 1987; Weinhold & Motta, 1973). No tissue discolouration and necrosis was observed unless infection cushions were present on the stems in all AGs. There were clear differences between AGs with regard to the extent of infection cushion formation on the stem surface, and the severity of stem lesions that subsequently developed. The AG 2-1 isolate (x81) rapidly formed (1–2 d after planting) small infrequent infection cushions consisting of tightly coiled hyphae over the stem surface. Only minor superficial damage was visible, with small areas of light brown discolouration no more than 1–2 mm across forming over the stem surface. These lesions did not increase in size or severity over 10 d, and the lesions did not penetrate the vascular tissues and cause stem death. AG 3 and AG 5 isolates, on the other hand, initially formed much more extensive infection cushions, with loosely connected hyphae forming the infection cushions. Stem canker severity then increased along with further infection cushion formation over 10 d until the tissue was completely destroyed. It was found in a previous study that there is a relationship between the frequency infection cushion formation and lesion severity in rice, with the severity of lesions increasing as the number of infection cushions on the stem surface increased (Marshall & Rush, 1980b).

A delay between infection cushion formation and lesion development was observed with the AG 5 isolate. Infection cushions formed 1–2 d after planting, whereas visible stem lesions were only apparent 2–3 d after planting. The time interval between infection cushion formation and stem lesion development was, therefore, less than 24 h, and *R. solani* isolates from other hosts have shown a rapid onset of lesion formation

following infection cushion initiation and development (Weinhold & Motta, 1973). AG 5 produced large, extensive infection cushions similar to those observed with AG 3 isolates, in contrast to the small, isolated and tightly-coiled cushions produced by AG 2-1. In this study, AG 3 isolates were significantly more virulent than both the AG 5 and AG 2-1 isolates tested. There were differences in virulence of different AGs of *R. solani* on potato stems, however, the reasons for this are unclear. Other studies have found that AG 2-1 isolates from potato caused significantly less stem canker than AG 3 isolates, and that AG 2-1 isolates can also vary within this AG with respect to virulence on potato (Chand & Logan, 1983; Petkowski & de Boer, 2001). A previous study with *R. solani* isolates originally from cotton found that, prior to lesion formation and penetration of the host via infection cushions, hyphae were tightly appressed to the cuticle prior with no evidence of penetration (Weinhold & Motta, 1973). In a more recent study with AG 4 isolates pathogenic to cotton, a mucilage material was observed on the hyphal surface and a role in hyphal adhesion to the stem prior to infection cushion formation was suggested (Armentout & Downer, 1987). The production of this mucilage by *R. solani* from potato, and any role it may have in hyphal adhesion or virulence of the different anastmosis groups, is unknown and merits further investigation.

The small lesions produced by the AG 2-1 isolates in this study may be attributed to a hypersensitive response, which was previously observed when a strain of *R. solani* pathogenic to crucifers was used to infect lettuce (Flentje, 1957). Infection cushion formation progressed as normal, and localised small yellow-brown lesions developed on the stems. Further examination revealed that the infection peg had penetrated the cell wall, however, the invading hyphae were dead and failed to grow when dissected out of the lesions (Flentje, 1957). Cross-species infection by *R. solani* from potato has been reported previously with AG 11 isolates severely virulent to wheat and rice producing minor lesions on developing potato stems (Carling *et al.*, 1984).

Whether the AG 2-1 isolate used in this study is pathogenic to other crops, or the symptoms observed were due to such a hypersensitive response is unknown.

This study has revealed, for the first time, that cutinase, pectin lyase, polygalacturonase, endo-1,4-β-glucanase and β-glucosidase are all produced by different AGs of *R. solani* pathogenic to potato. The insoluble polymers of hydroxy and hydroxyepoxy fatty acids of the cuticle (cutin) embedded in a complex of non-polar lipids (waxes) have to be breached prior to infection (Kolattukudy, 1985). Cutinase was produced extracellularly by all isolates when grown in liquid culture on the basal medium, cell walls and cutin powder, suggesting cutinase production is constitutive. There was little difference in cutinase production whether isolates were grown on basal medium alone or cell walls. An *R. solani* isolate of unknown host and AG was previously found to produce cutinase when grown in liquid culture with cutin powder as a carbon source (Baker & Bateman, 1978). Cutinase production differed between the isolates when grown on a liquid medium containing cutin as a carbon source. The AG 5 isolate (T1) and both AG 3 isolates (x72 and UN) excreted more cutinase than the AG 2-1 isolate (x81). Cutinase has been implicated in the pathogenicity of *Fusarium oxysporum* f. sp *pisi* on pea (*Pisum savitum*), having been detected in germinating spores during host tissue penetration and demonstrated immunologically to be present in the infection peg (Shaykh, *et al.*, 1977; Köller, *et al.*, 1982).

Beneath the cuticle is the cell-wall, consisting of a range of pectic polysaccharides, hemicelluloses and celluloses (Brett & Waldron, 1996). Many enzymes involved in the attack of higher plants by micro-organisms degrade pectin (Brett & Waldron, 1996). All AGs of *R. solani* tested in this study produced the pectin degrading enzymes polygalacturonase and pectin lyase on the basal medium and when cell walls and citrus pectin were available as a carbon source. After 10 d, there were differences in the production of polygalacturonase between isolates, with the AG 2-1

(x81) isolate excreting less polygalacturonase than the other isolates tested, particularly when citrus pectin was the carbon source.

A previous study on *R. solani* infection of cotton seedlings found that pectic substances were removed from the cell walls during infection cushion formation and was followed several hours later by cell wall degradation (Weinhold & Motta, 1973). In another study, endopolygalacturonase (endoPG) was produced in culture by *R. solani* from cotton and an endoPG similar in catalytic properties and molecular weight, but with different ionic properties, was extracted from infected tissues (Brookhauser *et al.*, 1980). More recently, both highly (HV) and weakly virulent (WV) isolates of *R. solani* AG 8 synthesised multiple forms of pectin-degrading enzymes when grown on a citrus pectin medium as a carbon source, with no differences in the pectic enzyme profiles produced by HV and WV strains (O'Brien & Zamani, 2003).

All *R. solani* isolates excreted endo-1,4-β-glucanase in a liquid medium containing CMC and cell walls as carbon sources, but no endo-1,4-β-glucanase activity was detected when isolates were grown on the basal medium alone. The absence of endo-1,4-β-glucanase activity in the basal medium suggests that production of this enzyme occurs is induced in response to a substrate in the cells walls and CMC. β-glucosidase activity was barely detectable on the basal medium, with significantly higher levels produced on a cellobiose medium. In a recent study, β-glucosidase was found to be produced by the *R. solani* teleomorph *Thanatephorus cucumeris* pathogenic to rubber trees (*Hevea brasiliensis*) in liquid media (Jayasinghe *et al.*, 2004).

R. solani isolates from potato have been shown to produce a range of cell-wall degrading enzymes *in vitro*, but their precise role, if any, in pathogenesis is currently unknown. Many other factors have the potential to contribute to the pathogenicity of *R. solani* on potato. SDS-PAGE analysis has revealed differences in the range of proteins produced by both HV and WV AG 8 isolates of *R. solani* (O'Brien & Zamani, 2003). A host specific toxin partially purified from *R. solani* pathogenic to rice produced disease

symptoms indistinguishable from those arising from natural infection with the fungus (Vidhyasekaran *et al.*, 1997). Host resistance to *R. solani* infection has also been attributed to the presence of abundant wax deposits on the cuticles of rice stems, with the removal of these deposits rendering previously resistant cultivars unable to withstand *R. solani* infection (Marshall & Rush, 1980a).

This *in vitro* study has demonstrated differences in the pathogenicity of and cell-wall degrading enzyme production of different AGs of *R. solani*. AG 3 and 5 isolates initiated a rapid and massive invasion of fungal tissues, whereas the AG 2-1 isolate was less pathogenic. Samples taken from potato plants from around the world have shown that the most abundant AG isolated was AG 3, which may be due, in part, to the greater survival potential or aggressiveness of this AG compared to others (Carling & Leiner, 1986; Bandy & Leach, 1988; Bains & Bisht, 1995; Balali *et al.*, 1995; Woodhall, 2004). It would be useful to purify and characterise the range of enzymes produced by different AGs of *R. solani* from potato and determine the role of cell-wall degrading enzymes in the infection process. Further studies using stains to monitor changes in the cell wall tissue, alongside immunocytochemical techniques, would determine the precise nature and location of cell-wall enzyme production during *R. solani* infection on potato.

CHAPTER 6

SCLEROTIAL SURVIVAL AND THE EFFECT OF INOCULUM DENSITY AND HAULM DESTRUCTION ON DISEASE INCIDENCE AND SEVERITY

6.1 Introduction

Rhizoctonia solani forms sclerotia in soil, on potato tubers and plant residues, enabling the fungus to survive conditions which would be unsuitable for mycelial growth (Anderson, 1982; Jeger *et al.*, 1996). Sclerotia germinate when conditions improve, with the resulting hyphae causing stem/stolon canker and forming new sclerotia (black scurf) on progeny tubers (Menzies, 1970). Misshapen tubers are a common feature of crops infected with *R. solani*, as the stolons are targeted and destroyed by the fungus (Hide *et al.*, 1973; Banville, 1989; Carling *et al.*, 1989).

R. solani infection can be caused by both soil- and seed-borne inoculum (Jeger *et al.*, 1996). Seed visibly free from sclerotia was found to produce progeny tubers covered in less black scurf than those grown from sclerotia-covered seed (Small, 1943). High sclerotium densities of *R. solani* on tubers were found to delay plant emergence and increase the incidence of black scurf on progeny tubers (Carling *et al.*, 1989; Simons & Gilligan, 1997a,b). Significant reductions in total and marketable yield, and increases in tuber numbers infested with black scurf have also been attributed to the planting of infested seed tuber pieces compared to *R. solani*-free pieces (Banville, 1989; Carling *et al.*, 1989). Sclerotia formation on progeny tubers has been found to be less severe on potatoes harvested soon after haulm destruction compared to those harvested several weeks later (Small, 1943, 1945; Chand & Logan, 1986). Black scurf on potato tubers destined for both the seed and pre-pack ware markets is undesirable, as it reduces the quality of seed potatoes and the overall value of the crop (Jager *et al.*, 1991).

The soil itself is known to be a potential source of *R. solani* inoculum. Sclerotial viability during burial in field soil has been found to decline over 2 years, however, many harvested sclerotia still underwent germination (Velvis *et al.*, 1989). Plants grown from disinfected seed have exhibited reduced yields and tuber quality in response to severe attacks of soil-borne inoculum (Scholte, 1989). High levels of soil-borne mycelial inoculum have reduced emergence, and caused severe stem canker and

black scurf development on plants grown from disease-free micro-propagated mini tubers (Kyritsis & Wale, 2002a, b). A combination of soil- and seed-borne inoculum has been found to cause a greater incidence of black scurf compared with either inoculum source separately (Tsror & Peretz-Alon, 2005).

Previous work has concentrated on the effect of soil-borne mycelial inoculum and sclerotial seed-borne inoculum on disease incidence and severity, with no studies investigating the effect of soil-borne sclerotial inoculum on disease development. The objectives of the work described in this chapter were to determine whether sclerotia produced on potato tubers and *in vitro* have similar survival characteristics in the field and the effect of soil type and depth on sclerotial survival. The effect of sclerotial soil-borne inoculum density and planting density on disease development was also determined, as was the effect of haulm destruction.

6.2 Materials and Methods

6.2.1 Effect of burial in soil on sclerotial viability over 18 months

6.2.1.1 Effect of method of sclerotial production on sclerotial germination

An AG 3 isolate of *R. solani* (x72) (section 2.3.2) was used to produce sclerotia both *in vitro* and on potato tubers. Sclerotia were produced *in vitro* as described previously (section 4.2.1). To produce sclerotia on potato tubers, sclerotial inoculum was used to infest John Innes No. 3 compost (Clydeside Trading Society Ltd, Strathclyde) at a density of 100 mg kg^{-1} d.w. growing medium. The contaminated growing medium was used to fill 3-l pots and a mini tuber (cv Hermes, 15-20 mm diam) (Agrico Ltd UK, Angus, UK) was planted 8 cm deep using a dibber. Forty potato plants were grown in a glasshouse at 15°C for 90 d, and the haulms were removed approximately 5 cm above soil level by cutting stems using secateurs. Tubers were harvested 4 weeks later, and washed thoroughly with sterile distilled water to remove any adhering growing medium. Sclerotia were scraped from the surface of the tubers

with a sharp scalpel into a 425 µm sieve, washed again and dried overnight in a laminar flow cabinet. Sclerotia were dry sieved to give a size range 425 – 1000 µm and checked at x 45 magnification using a stereo binocular microscope (Gallenkamp) to ensure there were no outgrowing hyphae before burial. Harvested sclerotia were stored at 5°C for a maximum of 7 d prior to use in experiments.

Small bags were made from a fine nylon mesh fabric (mesh size 200 µm). Two 5 x 5 cm squares of fabric were sealed together 0.5 cm from the edges along three sides using a heated bag sealer. Twenty sclerotia were placed in each bag using forceps and the remaining side heat-sealed as described previously. Each individual bag was enclosed in a larger wide meshed nylon net bag for easy retrieval when sampling. An 8 x 10 m plot located at Diamond Field, SAC, Auchincruive was rotovated twice and 1-l pots dug into the ground so the upper edge of the pot was level with the soil surface. A gap of 100 cm was left between replicates and 30 cm between the pots within replicates. The experiment was set up in Oct 2003 and laid out in a split-plot design with five replicates per treatment. Pots were filled with sieved (<5 mm) loam-sand soil (Bargour series) from Diamond Field, SAC, Auchincruive and an individual bag was buried in each pot at a standard depth of 5 cm beneath the soil surface. Sclerotia were recovered after 1, 3, 6, 9, 12, 15 and 18 months.

Following recovery, sclerotia were thoroughly washed in sterile distilled water and dried overnight in a laminar flow cabinet. Germination of retrieved sclerotia was tested by plating individual sclerotia onto 8 mm diam discs cut from Petri dishes containing approximately 20 ml of basal medium (section 3.2.3) plus (l^{-1}): 10 g D-glucose; 0.1 g, chloramphenicol (Sigma); and 0.1 g, streptomycin sulphate (Sigma). Ten agar discs of D-glucose medium with sclerotia were placed into sterile 9 cm Petri dishes. Petri dishes were sealed with Parafilm and incubated at 25°C for 72 h. Sclerotial germination was assessed by viewing individual sclerotia for outgrowing hyphae under a stereo binocular microscope at x 45 magnification. A sclerotium was considered to

have germinated when any outgrowing hyphae were equal to or greater than the diam of the sclerotium. The percentage of sclerotia germinated per bag sampled was determined for each treatment.

The presence of potential antagonists was investigated by rinsing all recovered sclerotia following assessment for germination in sterile distilled water. They were dried on sterile filter papers in a laminar flow cabinet and placed onto overgrown 7-d-old cultures of *R. solani* (isolate x72) grown on PDA. The sclerotia were transferred from these cultures to PDA after 72 h and the percentage of sclerotia exhibiting outgrowth of potential fungal antagonists following incubation at 25°C for 72 h was recorded. Mycelia from these potential antagonistic colonies were transferred onto PDA containing (l^{-1}): 0.1 g, chloramphenicol (Sigma); 0.1 g, streptomycin sulphate (Sigma). The most commonly isolated potential antagonists were sub-cultured until pure cultures were obtained and identified tentatively. Confirmation of identification was made by experts at CABI Bioscience (Egham, Surrey, UK).

6.2.1.2 Effect of soil type on sclerotial germination

An experiment to investigate the effect of soil type on sclerotial survival was set up at Diamond Field, SAC, Auchincruive in Oct 2003 in a split-plot design with five replicates per treatment. Sclerotia were produced *in vitro* (section 4.2.1) and placed in bags as described previously (section 6.2.1.1). The plots were prepared by burying 1-l pots in a rotovated plot as described previously (section 6.2.1.1). The pots were filled with either a loam-sand soil [Bargour series (pH 6.0): Diamond Field, SAC Auchincruive] or a sand soil [Dreghorn series (pH 5.7) GI unit, SAC, Auchincruive]. Sclerotial germination and the presence of potential antagonists were assessed over 18 months as described previously (section 6.2.1.1).

6.2.1.3 Effect of burial depth on sclerotial germination

An experiment to investigate the effect of burial depth on sclerotial survival was set up at Diamond Field, SAC, Auchincruive in Oct 2003 and laid out in a split-plot design with five replicates per treatment. Sclerotia were produced *in vitro* (section 4.2.1) and placed in bags as described previously (section 6.2.1.1). A separate 10 x 8 m plot was rotovated twice and the bags buried at three different depths: 5, 10 and 20 cm. A 1 m gap was left between replicates with 30 cm between bags within replicates. Sclerotial germination and the presence of potential antagonists were assessed over 18 months as described previously (section 6.2.1.1).

6.2.2 Effect of inoculum type and density on disease incidence and severity

6.2.2.1 Different densities of mycelial and sclerotial inoculum

To investigate the effect of different soil-borne inoculum types and densities on disease incidence and severity, a pot experiment was set up outside at Mansion Field, SAC, Auchincruive on Monday 19 May 2003. The growing medium used was John Innes no. 3 compost, and potatoes were grown in 3-l pots. The growing medium was tested for *R. solani* using an established baiting method (Appendix C), with none detected in any of ten replicates. Each pot contained one unsprouted micro-propagated seed tuber (cv. Hermes, 15 – 20 mm, Agrico UK Ltd, Angus) planted 8 cm deep using a dibber. The trial consisted of eight treatments with twelve replicates per treatment for each of five harvest dates.

To prepare the mycelial inoculum, actively growing 3-d-old cultures of *R. solani* isolate x72 (AG 3) were sub-cultured onto fresh PDA in 9 cm diam Petri dishes and the new cultures incubated for 7 d at 25°C. Both mycelia and agar were macerated in a blender and the growing medium infested with four different mycelial inoculum densities (0, $^1/_8$, $^1/_4$ and $^1/_2$ plate mycelium per kg^{-1} d.w. growing medium). Sclerotial inoculum of isolate x72 was prepared as described previously (section 4.2.1) and used

to inoculate the growing medium with four sclerotial inoculum densities (0, 25, 50 and 100 mg sclerotia kg^{-1} d.w growing medium). All inocula were mixed through the growing medium thoroughly prior to filling the 3-l pots. A sample of unplanted minitubers was tested to confirm that they were free of *R. solani*. Twenty seed tubers were cut in half and placed cut side down onto modified Ko and Hora selective medium (see Appendix C). No *R. solani* mycelium grew from the plated tubers confirming there was no seed-borne inoculum. Pots were spaced 10 cm apart and were watered overhead using an impulse sprinkler twice a week for one hour, with each pot receiving approximately 500 ml.

Twelve plants per treatment were harvested and assessed on each of five sampling dates. The trial was sampled 4 (19 Jun), 6 (3 Jul) and 12 weeks (14 Aug) after planting. Haulm destruction was carried out by cutting the stems approximately 5 cm above soil level with secateurs. This followed the third harvest on 14 Aug. Two further harvests were taken, 1 (21 Aug) and 3 (15 Sep) weeks following haulm destruction. Plant emergence was assessed weekly from the date the first plants emerged. Stem canker incidence and severity were assessed 4, 6, and 12 weeks after planting. The incidence of stem canker was assessed per plant as the percentage of infected stems. Stem canker severity per plant was determined by placing each stem into one of four categories depending on the surface area of the stem covered by lesions. The categorical data were converted into a severity index as described previously (section 5.2.1).

For each plant, the total tuber yield, black scurf and elephant hide incidence and severity were determined 12 weeks after planting, and 1 and 3 weeks following haulm destruction. The incidences of black scurf and elephant hide were recorded separately as the percentage of tubers showing disease symptoms. The severities of black scurf and elephant hide on progeny tubers was determined using the ADAS disease assessment key 2.4.1 (Table 6.1). The number of tubers per plant in each of

these categories was then converted into a disease severity index for elephant hide and black scurf using the following formula:

$$\frac{\left[\left(0.55 \times \text{no. of tubers in category 2}\right)+\left(3.5 \times \text{no. tubers in category 3}\right)+\left(8 \times \text{no. tubers in category 4}\right)+\left(18 \times \text{no. tubers in category 5}\right)+\left(38 \times \text{no. tubers in category 6}\right)+\left(63 \times \text{no. tubers in category 7}\right)+\left(88 \times \text{no. tubers in category 8}\right)\right]}{\text{total number of tubers on the plant}}$$

Table 6.1. Eight point scale for categorising black scurf/elephant hide symptoms.

Severity category	Disease symptoms present (% surface area of tuber covered)
1	0
2	0.1 – 1
3	2 – 5
4	6 – 10
5	11 – 25
6	26 – 50
7	51 – 75
8	76 - 100

6.2.2.2 Different densities of sclerotial inoculum in soil

The trial was conducted at Mansion Field, SAC, Auchincruive and was planted on 24 May 2004. A loam-sand soil (Bargour series) was used and the experiment conducted in 3-l pots outdoors. Fertiliser (16:16:16 N:P:K) was added at a rate of 1250 kg ha^{-1}. Micropropagated seed tubers (cv. Maris Piper, 4–8 g, Gentech Propagation Ltd, Dundee) and the soil were initially tested for the presence of *R. solani* using the previous testing method on seed (section 6.2.2.1) and an established baiting method for the soil (Appendix C). No *R. solani* was detected on seed tubers, and ten replicates of the baiting method revealed 3 % of beet seeds were colonised per 75 g soil. Sclerotial inoculum was prepared as described previously (section 4.2.1) and used to inoculate the soil at six inoculum densities (0, 6, 12, 25, 50 and 100 mg sclerotia kg^{-1} d.w soil). All

treatments were mixed through the soil thoroughly and the soil placed in the 3-l pots. A single unsprouted micropropagated tuber was planted 8 cm deep in each pot using a dibber.

The experiment was set up in a split-plot design with eight replicates for each of the six treatments. Eight plants were sampled on each of four sampling dates for each treatment. The trial was sampled 6 (28 Jun) and 12 (16 Aug) weeks after planting. Haulm destruction was carried out by cutting the stems as described previously (section 6.2.1.1), followed by the second harvest on 16 Aug. Two further harvests were taken, 2 (30 Aug) and 4 (13 Sep) weeks following haulm destruction. Plant emergence was assessed weekly as before from the date the first plants emerged. Stem canker incidence and severity were assessed 6 and 12 weeks after planting as described previously (section 6.2.2.1). Black scurf and elephant hide incidence and severity were determined immediately prior to haulm destruction (12 weeks after planting), and also 2 and 4 weeks after haulm destruction as described previously (6.2.2.1). Total tuber yield, black scurf and elephant hide symptoms were also assessed as described previously in section 6.2.2.1.

6.2.2.3 The effect of soil-borne inoculum density and plant population density

The field trial was planted at Diamond Field, SAC, Auchincruive, on 19 Jul 2004. Fertiliser (16:16:16 N:P:K) was applied at a rate of 1250 kg ha^{-1}. Seed tubers with no visible black scurf (cv. Maris Peer, 35–55 mm) were surface sterilised in 0.1% (v/v) sodium hypochlorite (15–20 % available chlorine) for 10 min and subsequently thoroughly rinsed with tap water. Twenty-five skin samples (2 x 2 cm) from surface sterilised tubers and 25 from unsterlised tubers were plated onto Ko and Hora selective medium and viewed microscopically at x 40 magnification (Gallenkamp) to determine the presence of *R. solani* mycelium after 48 h as described previously (section 6.2.2.1). Out of 50 tuber skin segments tested for the presence of *R. solani*, 8% of samples from

the surface sterilised tubers showed evidence of outgrowing hyphae, whereas 54 % of pieces from unsterilised tubers showed evidence of *R. solani* infection. Background soil-borne *R. solani* was assessed as described previously and was found to contain 7 % of beet seeds colonised per 75 g soil (section 6.2.2.2).

To simulate soil-borne inoculum, individual plots were infested using pieces of seed (cv. Maris Peer, 35–55 mm) with a severity of black scurf between 11 and 25 %. The eyes of the infested tubers were removed and tubers cut into six pieces. One piece from each blind tuber was placed into a tray for each replicate to ensure the inoculum was distributed as evenly as possible between the treatments. Pieces from blind surface sterilised tubers were used as a control. The seed pieces were applied evenly to the individual plots by hand at different inoculum densities (Table 6.2). The pieces were added to the plots 14 d prior to planting and rotovated into the soil prior to the formation of the drills.

Table 6.2. Treatments to investigate the effect of plant population and soil inoculum density.

Treatment	Inoculum level (tuber pieces/plot)	Plant density (number of potatoes planted/plot)
1	775 (surface sterilised)	52 (41,000 ha^{-1})
2	775 (surface sterilised)	104 (80,000 ha^{-1})
3	194	52 (41,000 ha^{-1})
4	194	104 (80,000 ha^{-1})
5	775	52 (41,000 ha^{-1})
6	775	104 (80,000 ha^{-1})

Treatment plots (19.38m^2) consisted of four drills, with a 1 m length-wise gap between sub-plots and a 6 m length-wise gap between different treatments. Each treatment plot had four sub-plots, one for each harvesting date. Unplanted paths, 3.4 m wide, separated each group of four drills. Each drill was 85 cm wide with 18 or 37 cm spacing between the mid-point of each tuber (104 and 52 plants per plot respectively). All tubers were planted by hand 16 cm deep using a dibber. The experiment was set up in a split plot design of six treatments with six replicate plots. The plants in the outer drills acted as guards and 24 plants per treatment (4 per replicate) were harvested from the central two drills on each harvesting date.

Soil was tested for soil-borne *R. solani* immediately prior to planting, 1 week following planting, then at two weekly intervals until the end of the trial. Soil samples (50 g) were taken from between two plants on each drill for each treatment and samples from each treatment bulked together and assessed using the bait seed method described previously (Appendix C). Stem canker incidence and severity were assessed 4 weeks (16 Aug) after planting. The plants in this trial began to senesce after 65 days, so the second sampling date was made 9 weeks (27 Sep) after planting. Haulm destruction was carried out following the second harvest as described previously (section 6.2.1.1). Black scurf and elephant hide incidence and severity were determined 8 weeks after

planting, and 2 (11 Oct) and 4 (25 Oct) weeks after haulm destruction. Plant emergence, stem canker incidence and severity, total tuber yield, black scurf and elephant hide symptoms were assessed as described previously (section 6.2.2.1).

6.2.3 Effect of haulm destruction on black scurf formation on progeny tubers

To investigate the effect of haulm destruction on black scurf formation on progeny tubers, two field trials were conducted in consecutive years (2004: Main Holm; 2005: Mid Field, both SAC Auchincruive). In both trials, fertiliser was applied (16:16:16 NPK) at a rate of 1250 kg ha^{-1}. In 2004, treatment plots were infested with seed tuber pieces (cv. Maris Peer, 35 – 55 mm) with a severity of black scurf between 6 – 10 %. In 2005, treatment plots were infested with seed tuber pieces (cv. Pentland Dell, 25 – 35 mm) with a severity of black scurf between 11 – 25 %. Seed pieces were prepared and applied to plots 2 weeks prior to planting as described previously (section 6.2.2.3) at a rate of 10 seed pieces m^{-2}. The entire trial area was rotovated to incorporate the seed pieces into the soil. Treatment plots in both years consisted of 5 drills, with a 1 m length wise gap between plots. Each treatment plot was divided into four sub-plots, one for each sampling date. Unplanted paths 3.4 m wide separated each group of five drills and each drill was 85 cm wide. There were six replicates per treatments in 2004, and eight replicates in 2005.

Seed tubers with no visible black scurf were surface sterilised and skin samples tested for the presence of *R. solani* infection as described previously (section 6.2.2.3). Out of 50 tuber skin segments tested in 2004, 10 % of samples from surface sterilised tubers tested positive for *R. solani*, compared to 28 % from unsterilised tubers. The same tests in 2005 showed 12 % of surface sterilised tubers tested positive for *R. solani* compared to 30 % of unsterilised tubers. Seed tubers were planted on 8 Jun in 2004 (cv. Maris Peer, 35 – 55 mm) and 26 May in 2005 (cv. Pentland Dell, 35 – 55 mm, with 30 cm between the mid-point of each tuber. One hundred and eighty tubers were planted in each treatment plot.

The trial consisted of two treatments, one where the haulms of neighbouring (N) plants were cut immediately following harvest as described previously (6.2.1.1) and the haulms of central (C) plants were left attached, and the other where the haulms of all plants were left attached (Figure 6.1). The first harvest occurred 12 weeks following planting, 18 Aug in 2004 and 31 Aug in 2005, with further harvests 2, 4.5 and 7 weeks following haulm destruction.

N	N	N	N	N	N	N	N	N
N	C	N	C	N	C	N	C	N
N	N	N	N	N	N	N	N	N
N	C	N	C	N	C	N	C	N
N	N	N	N	N	N	N	N	N

Figure 6.1. Layout of sub-plots for each harvest date. Highlighted letters indicate which plants were harvested.

Stem and stolon canker incidence and severity were assessed on the first harvest only, with tuber yield, black scurf and elephant hide incidence and severity assessed as described previously (section 6.2.2.1) on all harvest dates.

6.2.4 Statistical Analysis

Histograms of the residuals and plots of the residuals versus fitted values were examined to check homogeneity of variance and the normality of the distribution of the data. Percentages were routinely angularly transformed prior to an analysis of variance (ANOVA). Treatment means were compared using the least significant difference at a probability of 5 % ($P = 0.05$). For sclerotial germination, the interactions between the fixed factors (e.g. soil type and sampling day) were analysed using the residual maximum likelihood (REML) method. The significance of any interactions was

obtained from the Wald statistics (chi-squared distribution), and pair-wise comparisons of the data made using the least significant difference (LSD) at a probability of 5 % (P = 0.05). A linear regression was carried out on the combined data from all sclerotial burial trials to evaluate the overall effect of burial time on sclerotial germination after harvest. Data were analysed using Genstat for Windows, 7th Edition.

6.3 Results

6.3.1. Effect of burial in soil on sclerotial germination over 18 months

6.3.1.1 Effect of method of sclerotial production on sclerotial germination

REML analysis revealed significant differences ($P = 0.008$) in germination between sclerotia produced on potato tubers and sclerotia produced *in vitro*, and the dates on which the sclerotia were recovered (Figure 6.2). Sclerotia harvested after 18 months were 60 % less likely to undergo germination after harvest compared to those tested prior to burial (Figure 6.2). These differences were particularly apparent 3 – 6 months following burial, when sclerotial germination by laboratory-produced sclerotia was significantly less than those from potato tubers. Laboratory and potato produced sclerotia recovered earlier than 3 months or later than 6 months showed no significant differences in germination. Infection of sclerotia with potential mycoparasites was low, with no more than 4 % of sclerotia showing infection on any harvest date. The potential antagonists obtained from harvested sclerotia included *Penicillium* sp. and *Clonostachys rosea*.

6.3.1.2 Effect of soil type on sclerotial germination

Soil type and sampling date both had a significant effect ($P = 0.022$) on sclerotial germination (Figure 6.3). Sclerotial germination was between 48 – 87 % less after 18 months than sclerotial germination recorded prior to burial. After 6 months burial (Apr 2005) sclerotial viability had only reduced by approximately 20 %. There was little difference in survival between the two soil types until the final harvest at 18 months, when approximately 6 % of sclerotia harvested from the sand soil germinated, compared to over 30 % of those harvested from the loam-sand soil. Sclerotia harbouring potential antagonistic fungi were few, with no more than 3 % of sclerotia showing infection at any single harvest. The predominant antagonists isolated were *Penicillium* sp. (sand soil) and *C. rosea* (loam-sand soil).

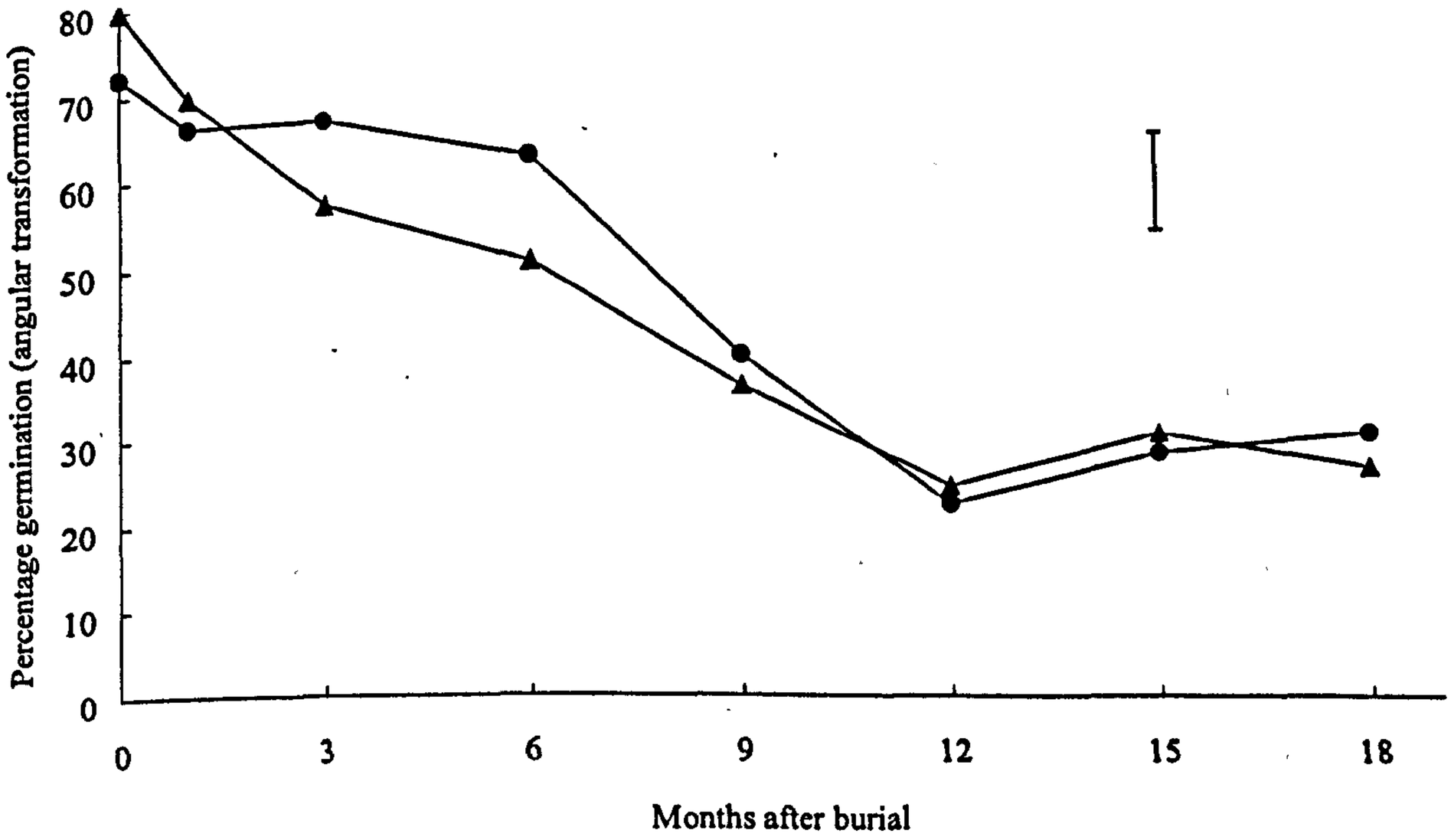


Figure 6.2. Effect of the method of production of sclerotia on sclerotial germination of an AG 3 isolate (x72) of *R. solani* over an 18 month period (potato-produced sclerotia ●, *in vitro*-produced sclerotia ▲). Values are the angularly transformed means of the percentage of germinated sclerotia after 72 h on PDA at 25 °C. Bar = LSD at $P = 0.05$ (DF = 7).

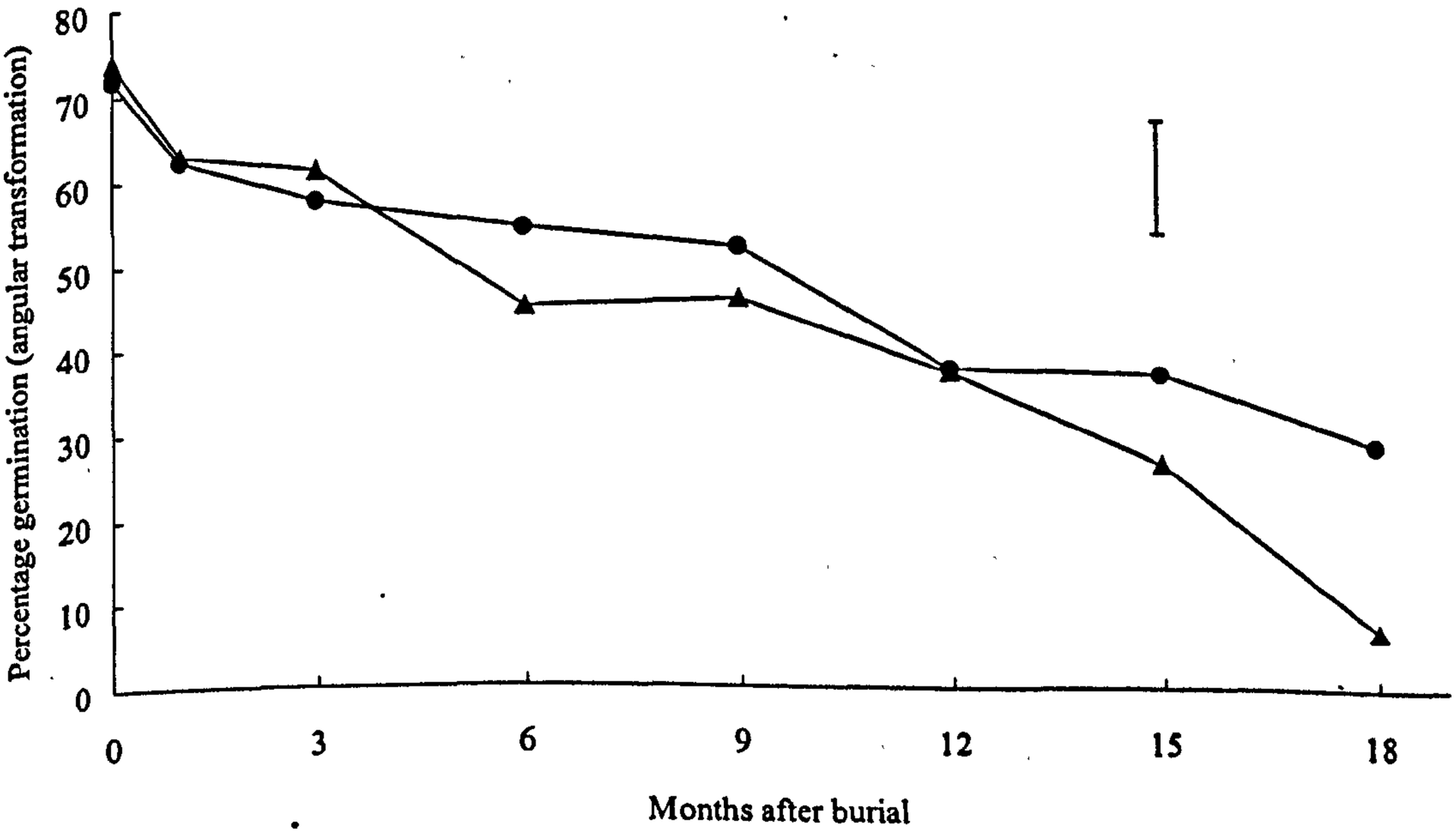


Figure 6.3. Effect of soil type on sclerotial germination of an AG 3 isolate (x72) of *R. solani* over an 18 month period (loam-sand soil ●, sand soil ▲). Values are the angularly transformed means of the percentage of germinated sclerotia after 72 h on PDA at 25 °C. Bar = LSD at $P = 0.05$ (DF = 7).

6.3.1.3 Effect of burial depth on sclerotial germination

There was no statistically significant effect of burial depth on germination (P = 0.388). There was, however, a significant reduction in sclerotial germination between 1 and 18 months of around 50 % compared to germination prior to recovery (Figure 6.4). There was little decline in sclerotial viability recorded 6 months after burial (Apr 2005), with reductions in sclerotial germination of approximately 10 %. The greatest decline in sclerotial germination occurred between 6 and 15 months burial in soil, with little change between 15 and 18 months. Antagonistic infection was minimal, with a maximum of 10 % of sclerotia showing infection at any one harvest. Potential mycoparasites isolated from all depths included *Mauginella* sp. and *Truncatella angustata* (Pers:) S.J. Hughes.

By combining all the data from the previous three experiments, a sclerotial viabiltiy decline curve was plotted, showing the general decline in sclerotial viability over 18 months regardless of the treatment applied (Figure 6.5).

6.3.2. Effect of inoculum type and density on disease incidence and severity

6.3.2.1 Different densities of mycelial and sclerotial inoculum in compost

Plant emergence was first observed 5 weeks after planting on the 18 Jun (Figure 6.6). Inoculum density had a significant effect (P = 0.05) on initial emergence, with nearly 20 % of uninfested control plants emerged after 5 weeks compared to the 0 – 10 % of plants that had emerged in infested treatments. By 25 Jun, emergence of the uninfested control and plants treated with the lowest soil-borne inoculum densities was double that observed for plants exposed to the highest inoculum densities. By 2 Jul, plant emergence was almost maximum for all treatments, and there were no significant differences between them.

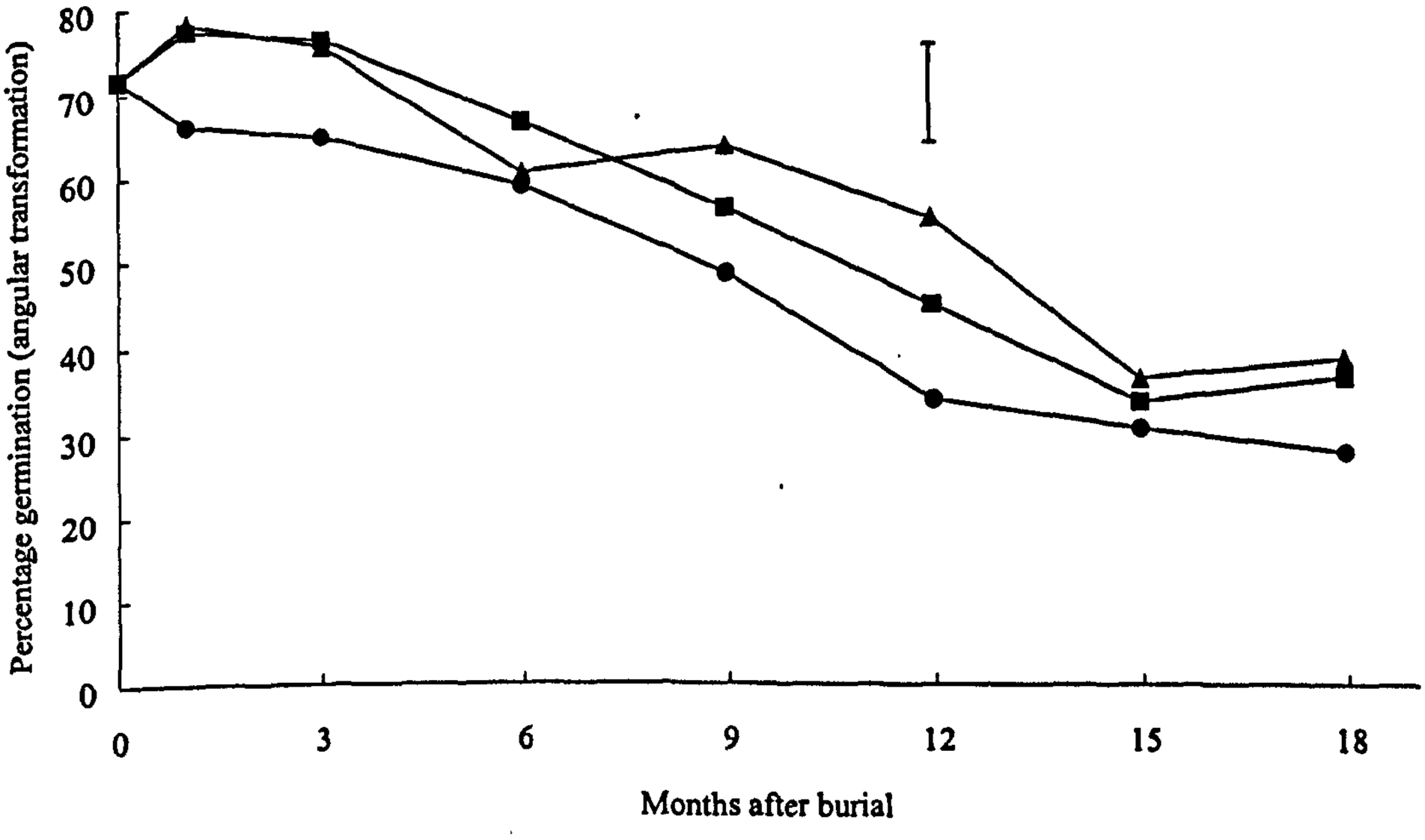


Figure 6.4. Effect of burial depth on sclerotial germination of an AG 3 isolate (x72) of *R. solani* over an 18 month period (5 cm ▲, 10 cm ● and 20 cm ■). Values are the angularly transformed means of the percentage of germinated sclerotia after 72 h on PDA at 25 °C. Bar = LSD at $P = 0.05$ (DF = 14).

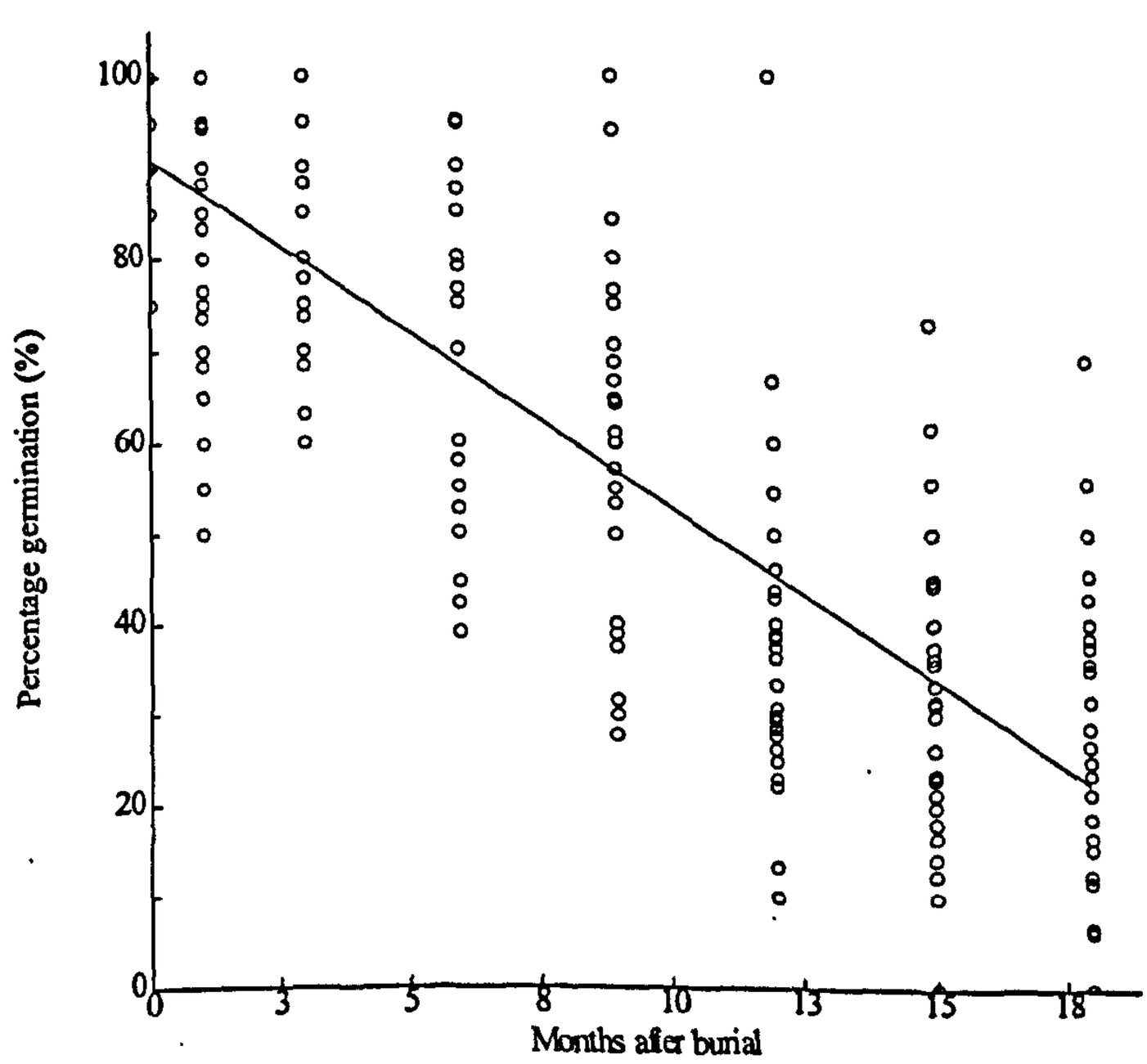


Figure 6.5. Sclerotial viability decline curve showing the effect of prolonged burial on sclerotial germination *in vitro* after harvest over 18 months. Open circles represent the individual values at each time point. The line represents the model best fitted to the data, $P = <0.001$, $R^2 = 72.1$.

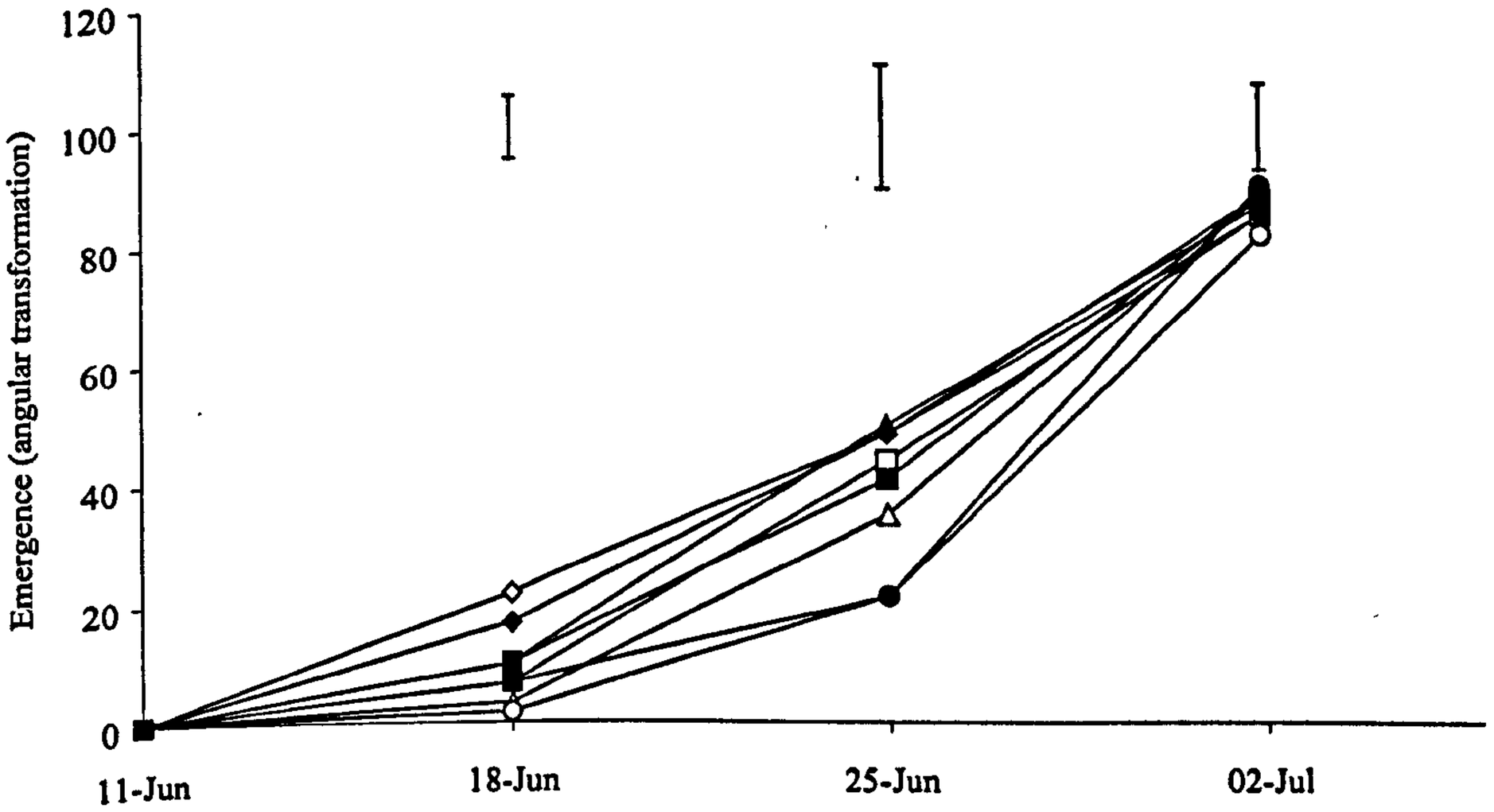


Figure 6.6. Effect of inoculum density on stem emergence [mycelial inoculum: control ◇, 1/8 Δ, 1/4 □ and 1/2 o plate mycelium; sclerotial inoculum: control ♦, 25 mg ▲, 50 mg ■ and 100 mg ● kg^{-1} d.w. growing medium]. Values are the angular transformations of the mean percentage of plants emerged of twelve replicates. Bars = LSD at $P = 0.05$ (DF = 77).

There were no significant effects between mycelial and sclerotial inoculum densities at different inoculum levels throughout the experiment. Low ($^1/_8$ plate; 25 mg sclerotia), medium ($^1/_4$ plate; 50 mg sclerotia) and high ($^1/_2$ plate; 100 mg sclerotia) inoculum densities resulted in similar incidences and severity indices for stem and stolon canker, and black scurf. Reductions in tuber number and yield were observed as inoculum density increased. The percentage of pruned stems observed in each treatment was generally greater at the first harvest (19 Jun) compared to the final harvest (14 Aug) and consistently increased with inoculum density regardless of inoculum type (Table 6.3). No stem canker was observed in the uninfested control (Figure 6.7). In general, the effect of inoculum density on stem canker incidence was most pronounced at the beginning of the growing season (19 Jun), with the lowest mycelial and sclerotial inoculum densities causing significantly less stem canker than the highest inoculum densities. Six weeks after planting (3 Jul), between 32 and 68 % of stems showed stem canker symptoms, with a reduction in the differences between the infested treatments. By the 12 week harvest (14 Aug), between 27 and 43 % of stems were infected regardless of treatment, and any differences between treatments were no longer statistically significant (Figure 6.7).

Plants harvested from growing medium infested with the lowest density of mycelial inoculum had significantly less ($P = 0.05$) stem canker than any of the other infested treatments at the first (19 Jun) and second (3 Jul) harvests (Figure 6.8). Stem canker severity generally declined over the growing season, with the severity of disease reduced 26 and 44 % between 3 Jul and 14 Aug, depending on the treatment.

Table 6.3. Effect of mycelial and sclerotial inoculum density on the percentage of pruned stems 4 (19 Jun), 6 (3 Jul) and 12 (14 Aug) weeks after planting.

Inoculum type	Inoculum level (kg^{-1} d.w growing medium)	Date of harvest		
		19 Jun	3 Jul	14 Aug
Mycelial	$^1/_2$ agar (control)	0.0 (0.0)[a]	0.0 (0.0)	0.0 (0.0)
	$^1/_8$ plate mycelium	0.0 (0.0)	4.2 (4.9)	0.0 (0.0)
	$^1/_4$ plate mycelium	30.1 (27.7)	12.5 (13.8)	20.0 (20.9)
	$^1/_2$ plate mycelium	38.4 (36.7)	23.6 (22.3)	29.7 (28.5)
Sclerotial	0 (control)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	25 mg	21.2 (18.7)	8.1 (7.2)	2.2 (2.5)
	50 mg	25.3 (25.4)	7.0 (6.7)	10.0 (9.6)
	100 mg	34.0 (33.0)	21.1 (22.7)	16.6 (18.4)
P value		(<0.001)	(<0.001)	(<0.001)
LSD (P = 0.05), DF = 77[b]		(15.67)	(11.75)	(10.69)

[a]Values in parenthesis are the angular transformations of the percentage means of pruned stems of twelve replicates.
[b]Pairs of means that differ by more than the LSD are significantly different (P = 0.05).

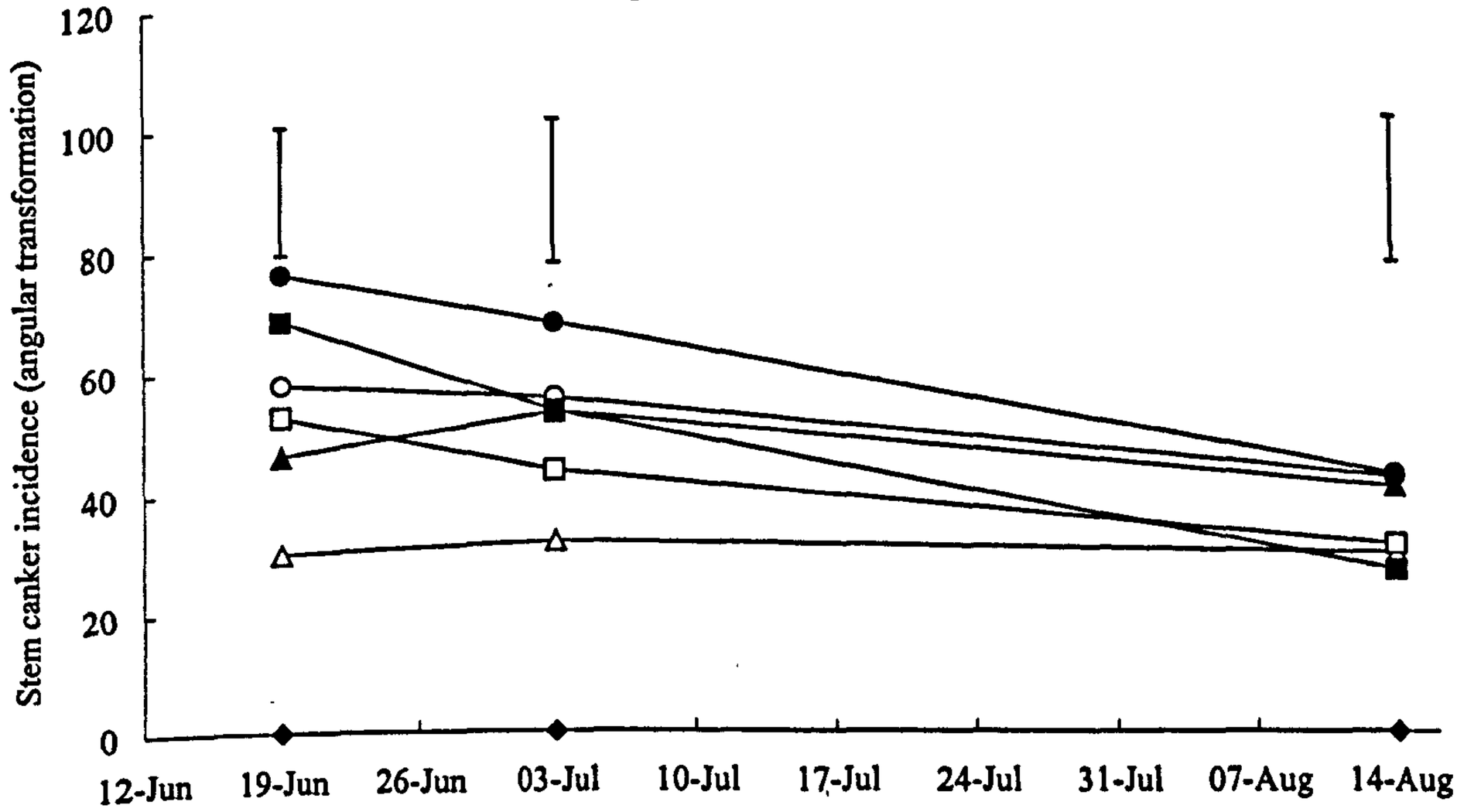


Figure 6.7. Effect of inoculum density on the incidence (%) of stem canker [mycelial inoculum: control ◇, 1/8 Δ, 1/4 □ and 1/2 o plate mycelium; sclerotial inoculum: control ♦, 25 mg ▲, 50 mg ■ and 100 mg ● kg^{-1} d.w. growing medium]. Values are the angular transformations of the percentage means of twelve replicates. Bars = LSD at $P = 0.05$ (DF = 77).

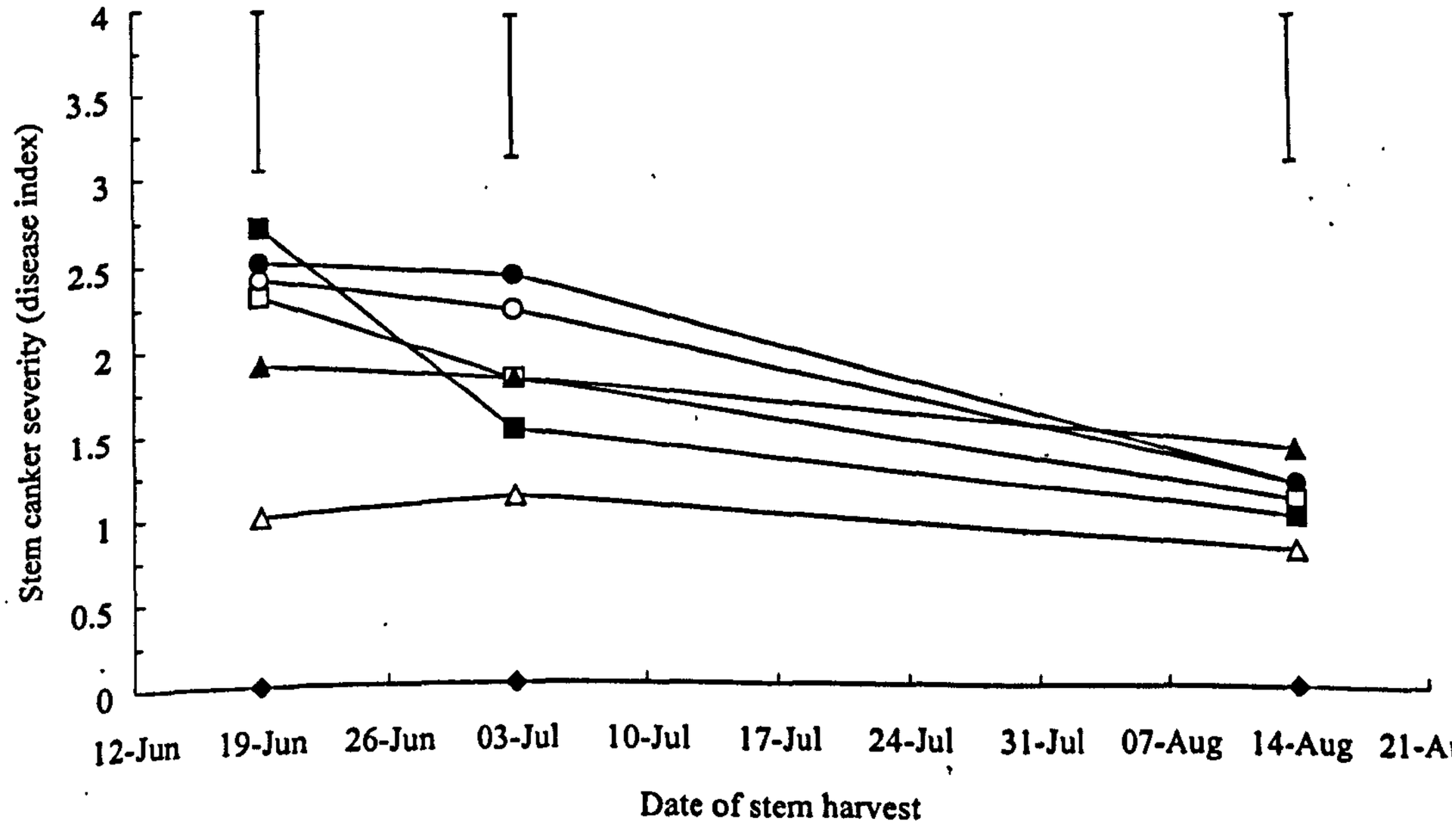


Figure 6.8. Effect of inoculum density on the severity of stem canker [mycelial inoculum: control ◇, 1/8 Δ, 1/4 □ and 1/2 o plate mycelium; sclerotial inoculum: control ♦, 25 mg ▲, 50 mg ■ and 100 mg ● kg^{-1} d.w. growing medium]. Values are the mean stem canker severity indices of twelve replicates. Bars = LSD at $P = 0.05$ (DF = 77).

The effect of disease inoculum density on the stolons (Table 6.4) was similar to that observed on stems. There were no significant differences between treatments with regards to the number of stolons observed on the potato plants (Table 6.4). Stolon length was significantly reduced ($P = 0.05$) particularly at the highest mycelial and sclerotial inoculum densities tested. Stolons from the uninfested control pots were nearly 70 % longer than stolons from plants in growing medium containing the highest inoculum densities (Table 6.4).

No stolon disease was observed on the control plants, and there was a clear increase in the incidence of stolon infection as the inoculum density increased (Table 6.4). Around 70 % of the stolons on plants grown in growing medium at the highest mycelial and sclerotial inoculum densities were infected, compared to between 32 and 54 % on plants from growing medium containing the lowest inoculum densities (Table 6.4). Stolon canker severity observed on plants grown at the lowest mycelial inoculum density was significantly lower ($P = 0.05$) than all other infested treatments (Table 6.4). The percentage of pruned stolons also increased significantly ($P = 0.05$) as both mycelial and sclerotial inoculum density in the soil increased (Table 6.4).

Table 6.4. Effect of mycelial and sclerotial inoculum density on the percentage of infected stolons 12 weeks after planting (14 Aug).

Inoculum type	Inoculum level (kg^{-1} d.w growing medium)	Number of stolons	Average stolon length (mm)	Incidence (%) of stolon infection	Stolon canker severity	Incidence (%) of pruned stolons
Mycelial	$^1/_2$ agar (control)	7.3 (2.7)[a]	73.2	0.0 (0.0)[b]	0.0[c]	0.0 (0.0)[d]
	$^1/_8$ plate mycelium	5.6 (2.3)	64.1	32 (31.3)	0.5	12 (11.9)
	$^1/_4$ plate mycelium	6.8 (2.6)	35.2	56 (51.2)	1.7	43 (39.3)
	$^1/_2$ plate mycelium	6.4 (2.5)	22.7	68 (58.5)	2.1	45 (49.1)
Sclerotial	0 (control)	7.0 (2.6)	79.4	0 (0.0)	0.0	0 (0.0)
	25 mg	5.4 (2.3)	42.8	54 (47.3)	1.7	40 (37.4)
	50 mg	6.3 (2.5)	25.5	62 (53.5)	1.8	46 (41.0)
	100 mg	6.5 (2.5)	24.0	74 (61.9)	2.2	57 (50.6)
P value		(0.346)	<0.001	(<0.001)	<0.001	(<0.001)
LSD ($P = 0.05$), DF = 77[e]		(0.366)	15.11	(15.07)	0.70	(15.56)

[a]Values in parentheses are the square roots of number of stolons from twelve replicates.
[b]Values in parentheses are the angular transformations of the percentage means of infected stolons from twelve replicates.
[c]Values are the mean stolon canker severity indices from twelve replicates.
[d]Values in parentheses are the angular transformations of the percentage means of pruned stolons from twelve replicates.
[e]Pairs of means that differ by more than the LSD are significantly different ($P = 0.05$).

The presence of sclerotial soil-borne *R. solani* resulted in significantly reduced ($P = 0.05$) yields of progeny tubers at all inoculum densities, with the lowest sclerotial inoculum density resulting in a 25 % reduction in yields compared to plants grown in non-infested growing medium (Table 6.5). Only the highest mycelial inoculum density tested consistently reduced tubers yields in this trial. Tuber yields were most severely affected at the highest sclerotial and mycelial inoculum densities tested, with approximately a 35 % reduction in tuber yields compared to uninfested controls.

The relationship between inoculum density and elephant hide incidence on progeny tubers was not straight forward (Table 6.6). There were significant differences ($P = 0.05$) in the incidence of elephant hide between the control and several treatments at the two earlier harvests, however, incidences were not clearly linked to inoculum density. Elephant hide severity generally increased with inoculum density, however, differences were only occasionally significantly different (Table 6.7). Severity of elephant hide was greatest 12 weeks after planting (14 Aug) and generally declined thereafter.

The incidence and severity of black scurf in the control treatments was negligible (Figure 6.9). Black scurf incidence was significantly lower on all harvest dates ($P = 0.05$) only when plants had been grown at the lowest mycelial inoculum density. There was little difference in the incidence of black scurf between the other inoculum densities, apart from the plants grown in growing medium containing the lowest sclerotial inoculum density, where black scurf incidence was significantly lower ($P = 0.05$) 1 week following haulm destruction (21 Aug). Both black scurf severity and incidence increased between haulm destruction (14 Aug) and 1 week following haulm destruction (21 Aug), with no further increases in disease severity observed between 1 (21 Aug) and 3 (11 Sep) weeks following haulm destruction.

Table 6.5. Effect of mycelial and sclerotial inoculum density on total tuber yield (g) immediately prior to haulm destruction (14 Aug), 1 (21 Aug) and 3 (11 Sep) weeks following haulm destruction.

Inoculum type	Inoculum level (kg^{-1} d.w. growing medium)	Date of harvest		
		14 Aug	21 Aug	11 Sep
Mycelial	$^1/_2$ agar (control)	158.7[a]	164.2	165.7
	$^1/_8$ plate	144.1	145.1	142.4
	$^1/_4$ plate	129.8	134.0	136.5
	$^1/_2$ plate	117.2	122.3	128.9
Sclerotial	0 (control)	161.4	183.6	182.7
	25 mg	121.5	134.8	135.2
	50 mg	125.9	136.9	116.1
	100 mg	102.6	116.0	103.9
P value		<0.001	<0.001	<0.001
LSD ($P = 0.05$), DF = 77[b]		22.79	26.87	29.28

[a]Values are the total weight of tubers produced from twelve replicates.
[b]Pairs of means that differ by more than the LSD are significantly different ($P = 0.05$).

Table 6.6. Effect of mycelial and sclerotial inoculum density.on elephant hide incidence immediately prior to haulm destruction (14 Aug), 1 (21 Aug) and 3 (11 Sep) weeks following haulm destruction.

Inoculum type	Inoculum level (kg^{-1} d.w. growing medium)	Date of harvest		
		14 Aug	21 Aug	11 Sep
Mycelial	$^1/_2$ agar (control)	1.0 (1.6)[a]	1.5 (1.9)	0.0 (0.0)
	$^1/_8$ plate mycelium	13.5 (16.0)	12.4 (15.6)	5.9 (6.3)
	$^1/_4$ plate mycelium	18.4 (22.3)	13.8 (16.3)	8.4 (11.7)
	$^1/_2$ plate mycelium	8.3 (10.5)	24.5 (25.3)	9.7 (12.4)
Sclerotial	0 (control)	0.0 (0.0)	4.1 (4.5)	10.0 (9.5)
	25 mg	18.9 (20.6)	10.6 (12.8)	13.3 (14.8)
	50 mg	16.6 (20.9)	19.4 (21.2)	19.8 (18.6)
	100 mg	11.3 (15.0)	26.9 (27.7)	8.1 (9.1)
P value		(<0.001)	(<0.001)	(0.413)
LSD (P = 0.05), DF = 77[b]		(10.06)	(12.05)	(14.35)

[a]Values in parentheses are the angular transformations of the percentage means from twelve replicate pots.
[b]Pairs of means that differ by more than the LSD are significantly different (P = 0.05).

Table 6.7. Effect of mycelial and sclerotial inoculum density on elephant hide severity immediately prior to haulm destruction (14 Aug), 1 (21 Aug) and 3 (11 Sep) weeks following haulm destruction.

Inoculum type	Inoculum level (kg^{-1} d.w. growing medium)	Date of harvest		
		14 Aug	21 Aug	11 Sep
Mycelial	$^1/_2$ agar (control)	0.01[a]	0.01	0.00
	$^1/_8$ plate	0.51	0.79	0.21
	$^1/_4$ plate	0.74	0.96	0.23
	$^1/_2$ plate	0.57	4.93	0.72
Sclerotial	0 (control)	0.00	0.18	0.11
	25 mg	0.56	0.10	0.10
	50 mg	1.27	2.63	1.11
	100 mg	0.78	2.32	1.81
P value		0.300	<0.001	0.093
LSD ($P = 0.05$), DF = 77[b]		1.115	2.200	1.279

[a]Values are the mean elephant hide severity indices of twelve replicates.
[b]Pairs of means that differ by more than the LSD are significantly different ($P = 0.05$).

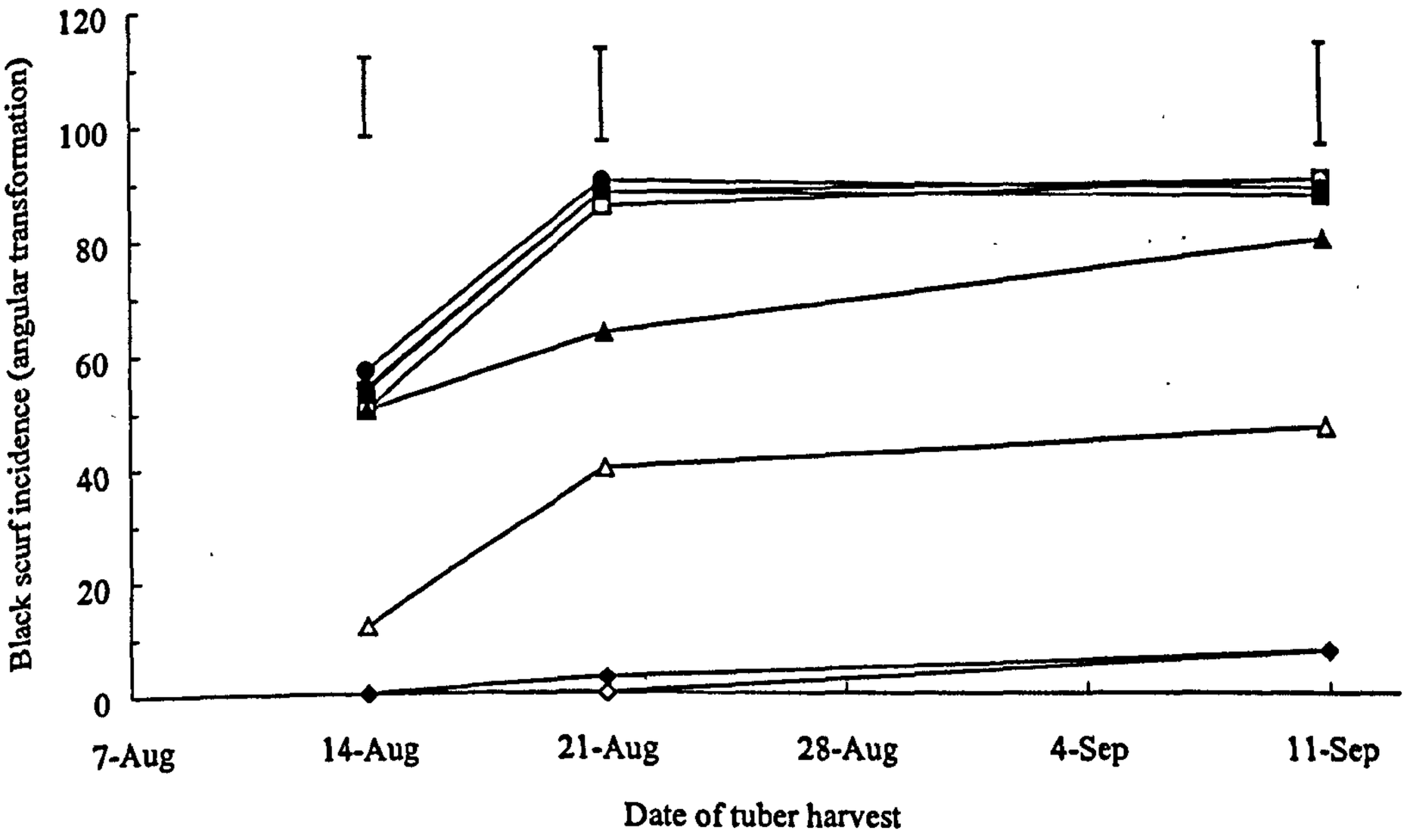


Figure 6.9. Effect of mycelial and sclerotial inoculum density on the incidence of black scurf [mycelial inoculum: control ◇, 1/8 Δ, 1/4 □ and 1/2 o, plate mycelium; sclerotial inoculum: control ♦, 25 mg ▲, 50 mg ■ and 100 mg ● kg^{-1} d.w. growing medium]. Values are the angularly transformed percentages of tubers showing black scurf symptoms of twelve replicates. Bars = LSDs. at $P = 0.05$ (DF = 77).

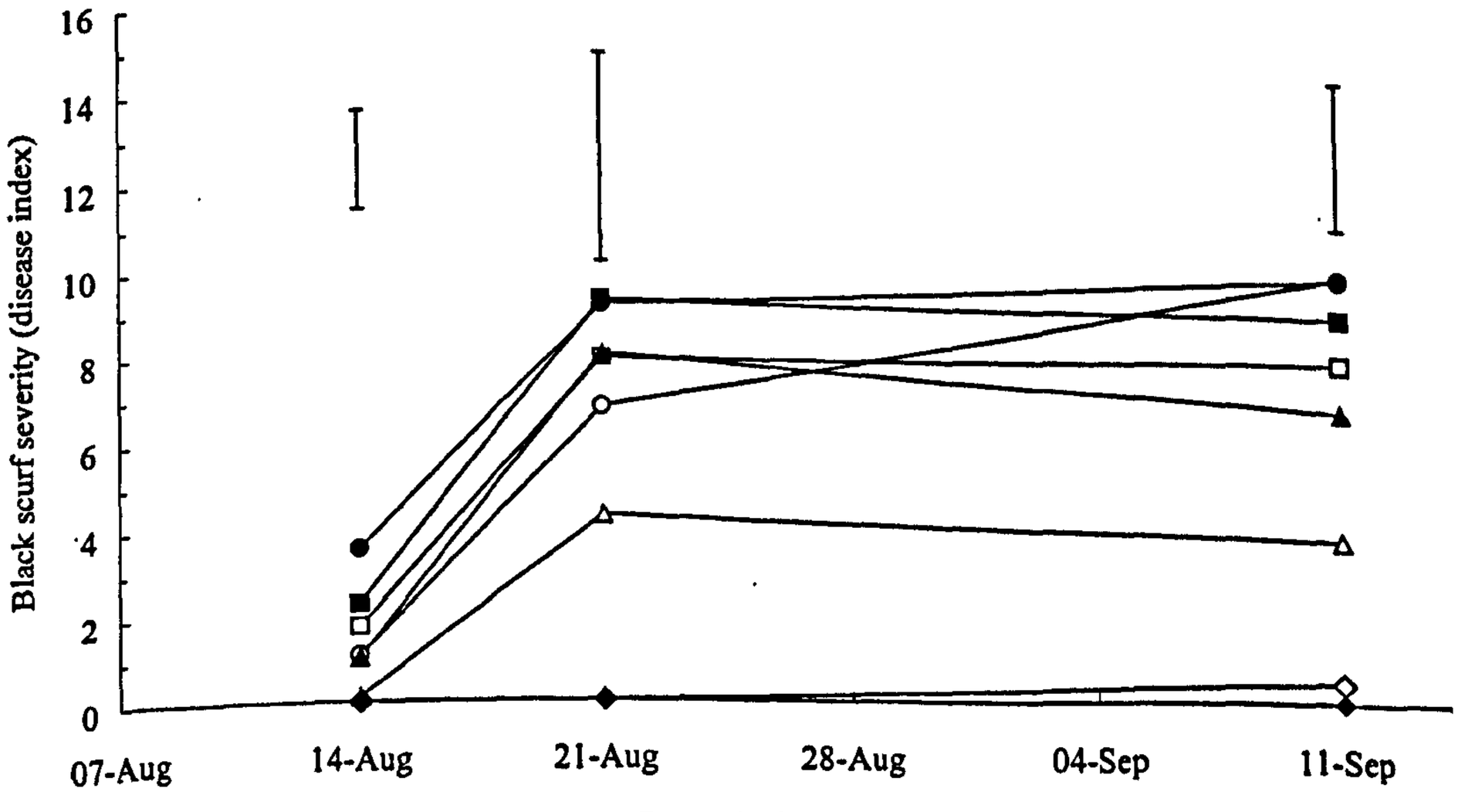


Figure 6.10. Effect of mycelial and sclerotial inoculum density on the severity of black scurf [mycelial inoculum: control ◇, 1/8 Δ, 1/4 □ and 1/2 o, plate mycelium; sclerotial inoculum: control ♦, 25 mg ▲, 50 mg ■ and 100 mg ● kg^{-1} d.w. growing medium]. Values are the mean black scurf severity indices observed of twelve replicates. Bars = LSDs. at $P = 0.05$ (DF = 77).

6.3.2.2 Different densities of sclerotial inoculum in soil

Plant emergence occurred between 3–4 weeks following planting (Figure 6.11). Both medium and low inoculum densities (12 and 25 mg kg^{-1} d.w. soil) had a negligible effect on emergence 3 weeks following planting (10 Jun). High inoculum densities (50–100 mg kg^{-1} d.w. soil) significantly reduced ($P = 0.05$) emergence 4 and 5 weeks after planting (17 and 24 Jun), with plant emergence almost 20 % lower than that observed in the infested control.

Stem canker incidence observed on plants exposed to the lowest sclerotial inoculum densities tested (6 and 12 mg kg^{-1} d.w. soil) was between 42 and 57 % lower than those grown in soil infested with the highest sclerotial inoculum densities (Table 6.8). Twelve weeks after planting (16 Aug), there was little difference in stem canker incidence at the three highest inoculum densities tested (25, 50 and 100 mg kg^{-1} d.w. soil). No pruned stems were observed on any harvested plants and no stem canker was found in the uninfested control. At the earliest assessment (28 Jun), the incidence and severity of stem canker increased as inoculum density increased over the range tested (Tables 6.8 and 6.9). At the later assessment (16 Aug), there were no significant differences instem canker incidence and severity between the highest inoculum densities tested (25, 50 and 100 mg kg^{-1} d.w. soil). Stem canker severity was over 30 % lower on plants grown in soil containing the lowest sclerotial inoculum density (6 mg kg^{-1} d.w. soil), compared to those grown in soil containing the highest sclerotial inoculum density (100 mg kg^{-1} d.w. soil).

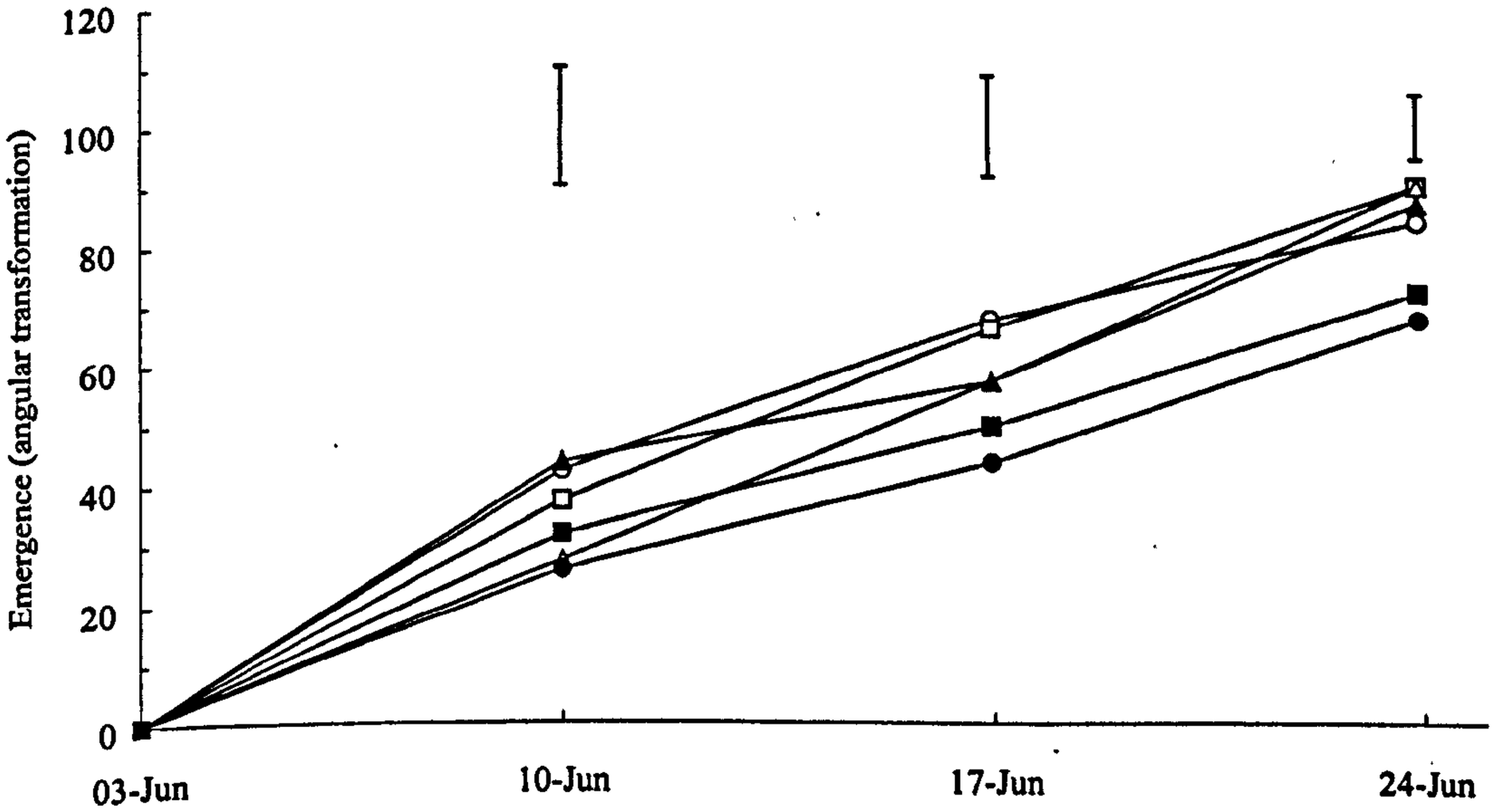


Figure 6.11. Effect of sclerotial inoculum density on stem emergence [control ○, 6 mg □, 12 mg Δ, 25 mg ▲, 50 mg ■ and 100 mg ● kg^{-1} d.w. soil]. Values are the angular transformations of the mean percentage of plants emerged of eight replicates. Bars = LSD at $P = 0.05$ (DF = 77).

Table 6.8. Effect of sclerotial inoculum density on the incidence of stem canker 6 (28 Jun) and 12 (16 Aug) weeks after planting.

Inoculum density (mg kg^{-1} d.w. soil)	Date of harvest	
	28 Jun	16 Aug
0	0 (0.0)[a]	0 (0.0)
6	40.0 (36.0)	42.5 (37.5)
12	55.0 (49.5)	65.0 (58.5)
25	75.0 (67.5)	95.0 (85.5)
50	94.0 (84.9)	90.0 (81.0)
100	96.7 (86.5)	83.3 (73.9)
P value	(< 0.001)	(< 0.001)
LSD ($P = 0.05$), DF = 45[c]	(26.07)	(28.86)

[a]Values in parentheses are the angular transformations of the percentage means of eight replicates.
[b]Pairs of means that differ by more than the LSD are significantly different ($P = 0.05$).

Table 6.9. Effect of sclerotial inoculum density on the severity of stem canker 6 (28 Jun) and 12 (16 Aug) weeks after planting.

Inoculum density (mg kg^{-1} d.w. soil)	Date of harvest	
	28 Jun	16 Aug
0	0.00[a]	0.00
6	0.65	0.43
12	0.60	1.00
25	0.90	1.35
50	1.65	2.05
100	1.89	1.57
P value	< 0.001	< 0.001
LSD ($P = 0.05$), DF = 45[b]	0.562	0.725

[a]Values are the mean stem canker severity indices of eight replicates.
[b]Pairs of means that differ by more than the LSD are significantly different ($P = 0.05$).

In general, stolon infection followed a similar pattern to that observed with stem canker, with disease incidence and severity increasing as sclerotial inoculum density increased. Four weeks after planting (28 Jun), there were no significant differences between the stolon length of control plants and those exposed to low and medium inoculum densities between 6 and 25 mg kg^{-1} d.w. soil (Table 6.10). By the second harvest (16 Aug), stolons from plants grown at the lowest inoculum density (6 mg kg^{-1} d.w. soil) were 34 % shorter than those on plants grown in uninfested soil. Plants grown at the highest inoculum density (100 mg kg^{-1} d.w. soil) had stolons that were up to 70 % shorter than plants from the uninfested control.

The incidence of pruned stolons was greatest at inoculum densities between 25 and 100 mg kg^{-1} d.w. soil on both harvest dates (Table 6.11). Four weeks after planting (28 Jun), stolon canker incidence at the lowest inoculum density was one quarter that observed at the highest inoculum density. Eight weeks after planting (16 Aug), this difference had reduced to just under 50 % (Table 6.12). Stolon canker severity increased with increasing sclerotial inoculum density (Table 6.13). Disease severity was generally significantly less at ($P = 0.05$) on plants grown in soil containing the lower sclerotial inoculum densities (6, 12 and 25 mg kg^{-1} d.w. soil) compared to those grown in soil containing the higher inoculum densities (50 and 100 mg kg^{-1} d.w. soil) (Table 6.13).

Tuber number significantly declined as sclerotial inoculum density in soil increased (Table 6.14). Plants grown in soil contaminated with the highest inoculum density produced 43 – 68 % fewer tubers than the control. Lower sclerotial inoculum densities (6 and 12 mg kg^{-1} d.w. soil) had no significant effect on tuber yields at the first harvest prior to haulm destruction, but further harvests revealed even the lowest inoculum density tested caused significant reductions in tuber yield compared to the control (Table 6.15).

Table 6.10. Effect of sclerotial inoculum density on stolon length 6 (28 Jun) and 12 (16 Aug) weeks after planting.

Inoculum density (mg kg^{-1} d.w. soil)	Date of harvest	
	28 Jun	16 Aug
0	56.1[a]	64.7
6	53.9	42.1
12	53.1	34.1
25	41.5	25.1
50	29.2	21.6
100	17.2	18.8
P value	< 0.001	< 0.001
LSD ($P = 0.05$), DF = 45[b]	17.52	13.86

[a]Values are the average stolon lengths (mm) of eight replicates.
[b]Pairs of means that differ by more than the LSD are significantly different ($P = 0.05$).

Table 6.11. Effect of sclerotial inoculum density of the incidence (%) of pruned stolons 6 (28 Jun) and 12 (16 Aug) weeks after planting.

Inoculum density (mg kg^{-1} d.w. soil)	Date of harvest	
	28 Jun	16 Aug
0	0.0 (0.0)[a]	0.0 (0.0)
6	6.4 (6.7)	20.2 (21.0)
12	7.3 (8.6)	26.0 (22.9)
25	33.7 (32.1)	49.5 (44.8)
50	63.8 (57.9)	57.8 (51.2)
100	55.3 (45.7)	63.3 (56.0)
P value	(<0.001)	(<0.001)
LSD ($P = 0.05$), DF = 45[b]	(21.44)	(19.24)

[a]Values in parentheses are the angular transformations of the percentage means from eight replicates.
[b]Pairs of means that differ by more than the LSD are significantly different ($P = 0.05$).

Table 6.12. Effect of sclerotial inoculum density of the incidence (%) of stolon canker 6 (28 Jun) and 12 (16 Aug) weeks after planting.

Inoculum density (mg kg^{-1} d.w. soil)	Date of harvest	
	28 Jun	16 Aug
0	0.0 (0.0)[b]	0.0 (0.0)
6	15.7 (16.7)	31.3 (29.4)
12	38.5 (36.4)	50.8 (45.4)
25	49.8 (46.5)	71.1 (60.9)
50	78.7 (70.0)	73.7 (63.9)
100	80.0 (67.9)	69.3 (59.6)
P value	(< 0.001)	(< 0.001)
LSD ($P = 0.05$), DF = 45[b]	(23.24)	(18.79)

[a]Values in parentheses are the angular transformations of the percentage means of eight replicates.
[b]Pairs of means that differ by more than the LSD are significantly different ($P = 0.05$).

Table 6.13. Effect of sclerotial inoculum density on disease severity observed on stolons 6 (28 Jun) and 12 (16 Aug) weeks after planting.

Inoculum density (mg kg^{-1} d.w. soil)	Date of harvest	
	28 Jun	16 Aug
0	0.000[b]	0.000
6	0.174	0.387
12	0.572	0.640
25	0.688	1.339
50	1.024	1.953
100	0.771	1.980
P value	< 0.001	< 0.001
LSD ($P = 0.05$), DF = 45[b]	0.4449	0.5166

[a] Values are the mean stolon canker severity indices of eight replicates.
[b]Pairs of means that differ by more than the LSD are significantly different ($P = 0.05$).

Table 6.14. Effect of sclerotial inoculum density on tuber number 6 (28 Jun) weeks after planting, immediately prior to haulm destruction (16 Aug), 2 (30 Aug) and 4 (13 Sep) weeks after haulm destruction.

Inoculum density	Date of harvest			
(mg kg^{-1} d.w. soil)	28 Jun	16 Aug	30 Aug	13 Sep
0	9.6[a]	8.7	9.4	10.9
6	8.2	7.2	7.6	8.1
12	7.0	5.7	8.1	7.7
25	5.7	5.8	7.00	7.9
50	4.3	4.7	7.2	5.7
100	3.1	4.4	4.6	6.2
P value	< 0.001	0.002	0.004	0.001
LSD ($P = 0.05$), DF = 45[b]	3.02	2.127	2.217	2.391

[a] Values shown are the mean tuber numbers per plant of ten replicates.
[b] Pairs of means that differ by more than the LSD are significantly different ($P = 0.05$).

Table 6.15. Effect of sclerotial inoculum density on tuber yield 6 (28 Jun) weeks after planting, immediately prior to haulm destruction (16 Aug), 2 (30 Aug) and 4 (13 Sep) weeks after haulm destruction.

Inoculum density	Date of harvest			
(mg kg^{-1} d.w. soil)	28 Jun	16 Aug	30 Aug	13 Sep
0	36.1[a]	115.3	105.2	112.8
6	28.9	83.6	82.2	89.9
12	24.8	73.6	87.2	87.9
25	18.3	69.3	90.4	72.3
50	12.8	58.8	73.0	70.0
100	2.6	61.4	64.3	70.2
P value	< 0.001	< 0.001	< 0.001	< 0.001
LSD ($P = 0.05$), DF = 45[b]	9.18	20.65	18.98	18.40

[a] Values are mean tuber yields per plant of ten replicates.
[b] Pairs of means that differ by more than the LSD are significantly different ($P = 0.05$).

There was no clear pattern with regards to sclerotial inoculum density in soil and the incidence of elephant hide on progeny tubers, although the greatest incidence of elephant hide was observed at the first harvest and subsequently declined for most treatments (Table 6.16). Elephant hide severity was generally less severe 4 weeks following haulm destruction (13 Sep), however, there were no significant differences between treatments (Table 6.17).

No black scurf was observed on progeny tubers grown in the control treatment containing uninfested soil (Figure 6.12). At haulm destruction (16 Aug), lower soil-borne sclerotial inoculum density (6 and 12 mg kg^{-1} d.w. soil) resulted in significantly lower ($P = 0.05$) black scurf incidence on progeny tubers compared to tubers grown from plants grown in high inoculum densities (25 – 100 mg kg^{-1} d.w. soil). Two weeks following haulm destruction (30 Aug), only plants grown in soil containing an inoculum density of 6 mg kg^{-1} d.w. soil had significantly less black scurf ($P = 0.05$) than the other inoculum densities (Figure 6.12). Four weeks following haulm destruction (13 Sep), there were no longer any significant differences between treatments with regards to black scurf incidence on progeny tubers.

Black scurf severity increased on progeny tubers following haulm destruction (Figure 6.13). Two weeks following haulm destruction (30 Aug), the lower the soil-borne sclerotial inoculum density, the lower the severity of black scurf observed on progeny tubers. Four weeks following haulm destruction (13 Sep), black scurf severity was greatest at an inoculum density of 100 mg kg^{-1} d.w. soil and significantly higher ($P = 0.05$) than the disease severity observed on tubers from plants grown at 6 and 12 mg kg^{-1} d.w. soil.

Table 6.16. Effect of sclerotial inoculum density on incidence of elephant hide immediately prior to haulm destruction (16 Aug), 2 (30 Aug) and 4 (13 Sep) weeks following haulm destruction.

Inoculum density (mg kg^{-1} d.w. soil)	Date of harvest		
	16 Aug	30 Aug	13 Sep
0	0.0 (0.0)[a]	0.0 (0.00)	0.0 (0.0)
6	29.0 (30.7)	2.0 (2.7)	3.7 (5.1)
12	34.2 (32.7)	6.7 (7.1)	0.0 (0.0)
25	1.4 (2.2)	5.6 (6.3)	9.2 (12.6)
50	9.2 (8.5)	4.5 (5.6)	8.3 (9.5)
100	18.4 (18.6)	0.0 (0.0)	2.5 (3.0)
P value	(<0.001)	(0.476)	(0.059)
LSD ($P = 0.05$), DF = 45[b]	(13.55)	(9.42)	(9.64)

[a]Values in parentheses are the angularly transformed percentages of tubers showing elephant hide symptoms of eight replicates.
[b]Pairs of means that differ by more than the LSD are significantly different ($P = 0.05$).

Table 6.17. Effect of sclerotial inoculum density on elephant hide severity immediately prior to haulm destruction (16 Aug), 2 (30 Aug) and 4 (13 Sep) weeks following haulm destruction.

Inoculum density (mg kg^{-1} d.w. soil)	Date of harvest		
	16 Aug	30 Aug	13 Sep
0	0.00[a]	0.00	0.00
6	1.12	0.07	0.13
12	0.79	0.95	0.00
25	0.01	0.15	0.38
50	1.48	1.31	0.23
100	0.28	0.00	0.27
P value	0.115	0.460	0.497
LSD ($P = 0.05$), DF = 45[b]	1.277	1.673	0.458

[a]Values are the mean elephant hide severity indices of eight replicates.
[b]Pairs of means that differ by more than the LSD are significantly different ($P = 0.05$).

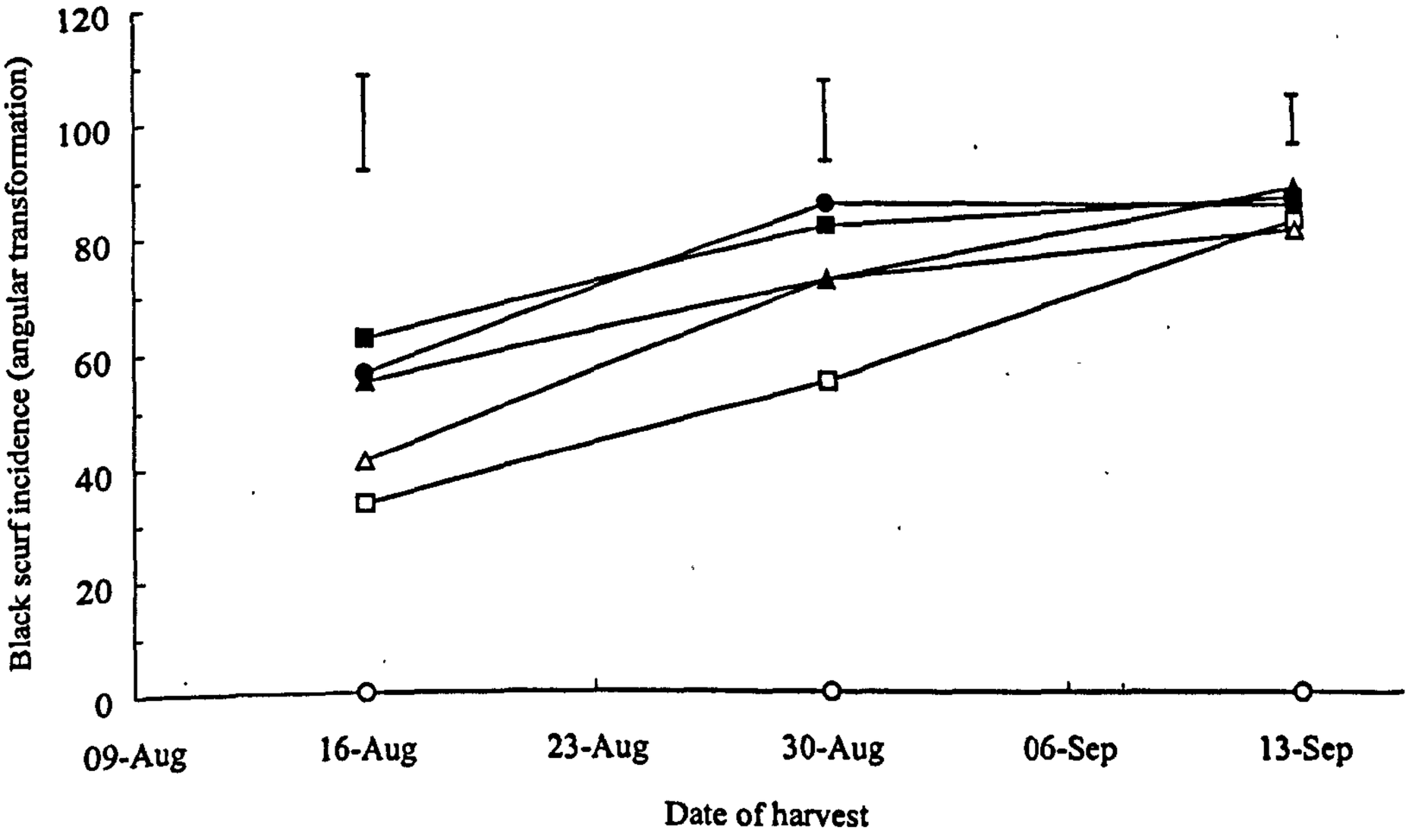


Figure 6.12. Effect of sclerotial inoculum density on black scurf incidence [control ○, 6 mg □, 12 mg Δ, 25 mg ▲, 50 mg ■ and 100 mg ● kg^{-1} d.w. soil]. Values are the angularly transformed percentages of tubers showing black scurf symptoms of eight replicates. Bars = LSD at $P = 0.05$ (DF = 77).

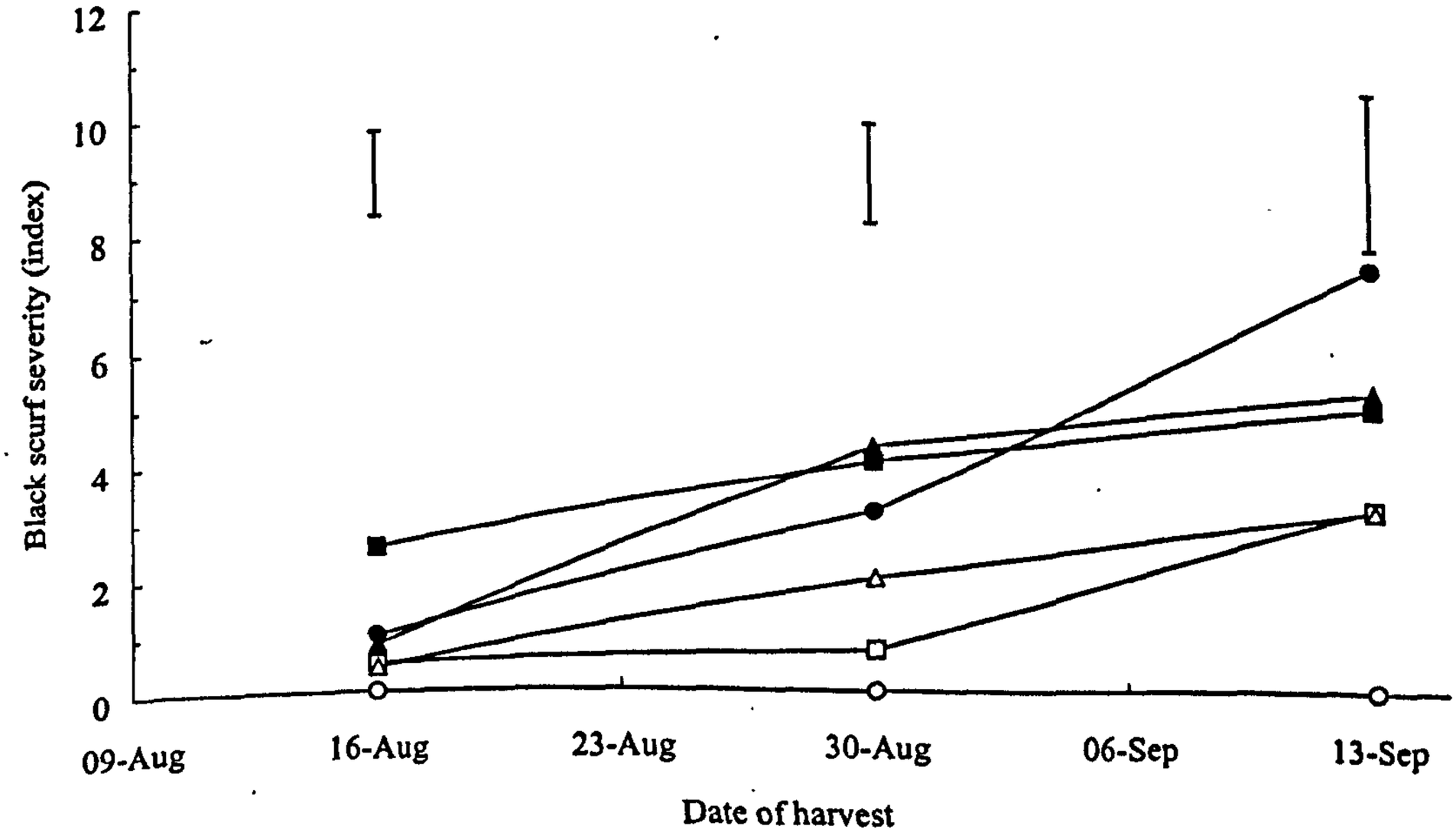


Figure 6.13. Effect of sclerotial inoculum density on black scurf severity [control ○, 6 mg □, 12 mg Δ, 25 mg ▲, 50 mg ■ and 100 mg ● kg^{-1} d.w. soil]. Values are the mean black scurf severity indices of eight replicates. Bars = LSD at $P = 0.05$ (DF = 77).

6.3.2.3 The effect of soil-borne inoculum density and plant population density

Soil-borne inoculum density and plant density had no significant effect on plant emergence, with almost all plants having emerged by 12 Jul regardless of the treatment applied (Figure 6.14). From planting until 15 Aug (5 weeks after planting), there were no significant differences in the concentrations of *R. solani* in soil (Figure 6.15). By 29 Aug, beet seed colonisation was significantly greater for plots infested with 775 seed pieces compared with the control, and this trend generally continued for the duration of the experiment. There were no significant differences in the incidence of bait seed colonisation between soils from control plots (surface sterilised tuber pieces) and those contaminated with 194 seed pieces.

Stem canker was present in all treatments, including control plots infested with surface sterilised seed tuber pieces (Table 6.18). There were no significant interactions between inoculum density and plant population on stem canker incidence or severity. The two factors individually did have significant effects on disease levels observed on stems. Soil-borne inoculum density had a significant effect ($P = 0.05$) on stem canker incidence 4 and 12 weeks after planting (16 Aug), with more stem canker observed in plots infested at the higher inoculum density than the control or the lower inoculum density. There was significantly greater stem canker incidence ($P = 0.05$) in plots containing 52 compared to 104 plants on 16 Aug, but this was no longer significant 8 weeks later (27 Sep). Stem canker severity was significantly greater ($P = 0.05$) on plants harvested from plots containing 775 contaminated seed pieces compared to 194 pieces both 4 and 12 weeks following planting (27 Sep). Stem canker severity was over 3 times greater at the higher compared to the lower inoculum density 4 weeks after planting (16 Aug). Planting population did not significantly affect stem canker severity (Table 6.18).

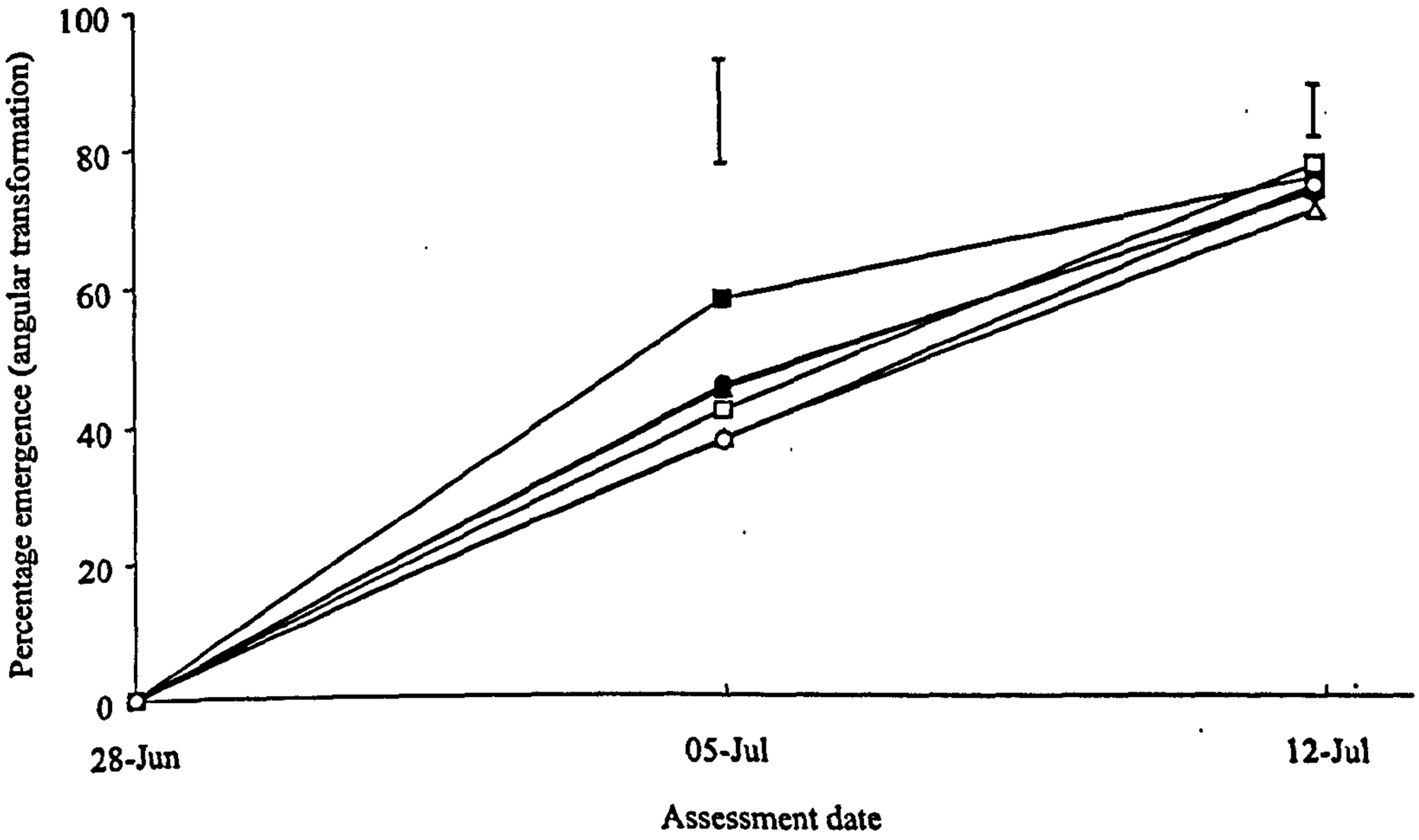


Figure 6.14. Effect of sclerotial inoculum density and plant population density on stem emergence [104 plants per plot, inoculum density (pieces per plot): 775 (control) □, 194 Δ and 775 o; 52 plants per plot, inoculum density (pieces per plot): 775 (control) ■, 194 ▲ and 775 ●]. Values are the angular transformations of the mean percentage of plants emerged of six replicates. Bars = LSD at $P = 0.05$ (DF = 35).

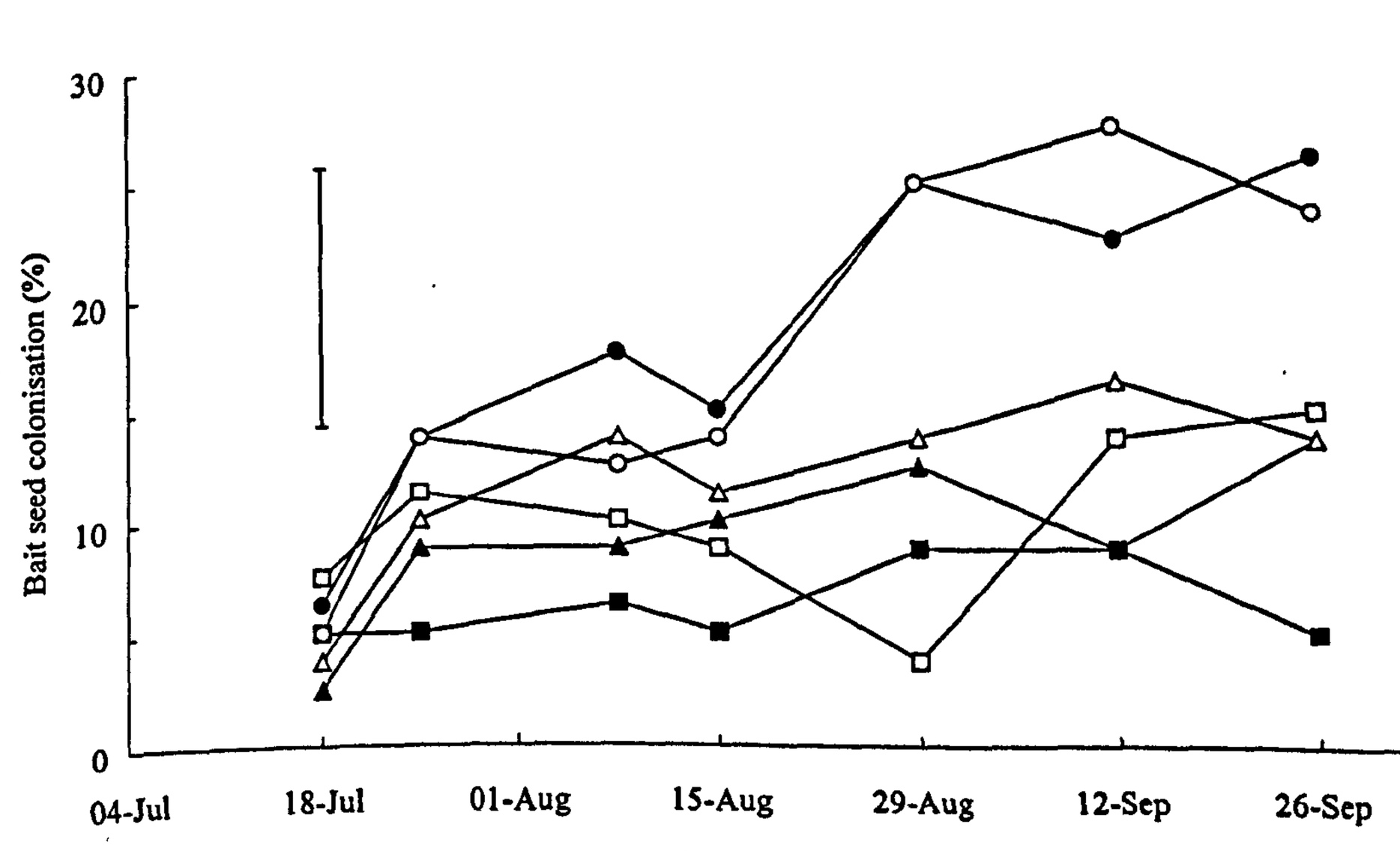


Figure 6.15. Changes in *R. solani* in soil throughout the growing season [104 plants per plot, inoculum density (pieces per plot): 775 (control) □, 194 Δ and 775 o; 52 plants per plot, inoculum density (pieces per plot): 775 (control) ■, 194 ▲ and 775 ●]. Values are the angular transformations of the mean percentage of bait seeds colonised from six replicates. Bars = LSD at $P = 0.05$ (DF = 329).

Table 6.18. Effect of inoculum density and planting population on stem canker incidence and severity 4 (16 Aug) and 9 weeks (27 Sep) after planting.

	Stem canker incidence		Stem canker severity	
Plant population (plants per plot)	16 Aug	27 Sep	16 Aug	27 Sep
52	43.5 (39.9)[a]	56.9 (50.4)	0.642[b]	0.897
104	23.1 (22.3)	47.0 (42.5)	0.368	0.648
P value	(0.020)	(0.292)	0.063	0.199
LSD ($P = 0.05$), DF = 12[c]	(14.39)	(18.07)	0.3660	0.4006
Inoculum density (pieces per plot)				
control	25.5 (25.1)	34.2 (31.0)	0.298	0.409
194	17.7 (18.1)	51.5 (46.1)	0.295	0.734
775	56.8 (50.1)	70.1 (62.2)	0.921	1.175
P value	(0.017)	(0.004)	0.011	0.004
LSD ($P = 0.05$), DF = 9[d]	(20.98)	(14.12)	0.4391	0.3525

[a]Values in parentheses are the angular transformations of the mean incidence (%) of stem canker observed in six replicates.
[b]Values are the mean stem canker indices of six replicates.
[c]LSD is the least significant difference at 5 % ($P = 0.05$) between planting population means, DF = degrees of freedom in ANOVA.
[d]LSD is the least significant difference at 5 % ($P = 0.05$) between inoculum density means, DF = degrees of freedom in ANOVA.

There was no significant interaction between soil-borne inoculum density and plant population on stolon canker incidence and severity. Both factors individually had an effect on disease development on stolons. Four weeks after planting, stolon canker incidence was significantly lower ($P = 0.05$) where the plant population was 104 plants per plot compared with 52, but this was not apparent 12 weeks after planting (Table 6.19). On 16 Aug, stolon canker severity was significantly higher at the greater inoculum density, almost double that observed on plants grown in plots infested with 194 seed pieces and control plots.

Planting density had a significant effect ($P = 0.05$) on stolon canker severity, with plants grown in plots containing 775 seed pieces having disease indices almost twice those observed on plants from plots infested with 194 seed pieces. By 27 Sep, there was no longer a significant effect of planting density on disease severity. Inoculum density had a significant ($P = 0.05$) effect on disease severity on 16 Aug, with severity indices almost 70 % greater on plants grown at the greater compared to the lower inoculum density. By 27 Sep, inoculum density still had a significant effect on disease severity, however, the difference between the severity indices of the two inoculum densities was reduced to 37 %.

Table 6.19. Effect of inoculum density and planting population on stolon canker incidence and severity 4 weeks (16 Aug) and 9 weeks (27 Sep) after planting.

	Stolon canker incidence		Stolon canker severity	
Plant population (plants per plot)	16 Aug	27 Sep	16 Aug	27 Sep
52	48.8 (43.3)[a]	66.1 (55.8)	0.642[b]	0.897
104	32.7 (30.1)	59.0 (50.8)	0.368	0.648
P value	(0.014)	(0.476)	0.005	0.372
LSD ($P = 0.05$), DF = 12[c]	(9.98)	(15.09)	0.1250	0.4006
Inoculum density (pieces per plot)				
control	25.5 (24.4)	44.4 (38.7)	0.298	0.409
194	17.7 (32.8)	60.6 (50.8)	0.295	0.734
775	56.8 (52.8)	82.6 (70.4)	0.921	1.175
P value	(0.004)	(<0.001)	<0.001	<0.001
LSD ($P = 0.05$), DF = 9[d]	(14.08)	(8.04)	0.4391	0.3525

[a]Values in parentheses are the angular transformations of the mean incidence (%) of stolon canker observed in six replicates.
[b]Values are the mean stolon canker indices of six replicates.
[c]LSD is the least significant difference at 5 % ($P = 0.05$) between planting population means, DF = degrees of freedom in ANOVA.
[d]LSD is the least significant difference at 5 % ($P = 0.05$) between inoculum density means. DF = degrees of freedom in ANOVA.

Tuber yield did not differ significantly between treatments on either of the harvest dates (Table D1 in Appendix D). The incidence of elephant hide was low and symptoms were not severe. Although the incidence of elephant hide was consistently greater at all four sampling times for tubers from plots to which 775 seed pieces had been added compared with 194, the differences were not significant (Table 6.20). The severity of elephant hide was generally greater at the higher inoculum density, and at the two later harvests the difference was statistically significant.

There was a significant interaction ($P = 0.05$) between plant population and soil-borne inoculum density on black scurf incidence and severity (Figure 6.16). On 16 Aug, black scurf incidence was significantly greater ($P = 0.05$) on potatoes harvested from plots infested with 775 seed pieces than those with 194 pieces or control plots. By 27 Sep (12 weeks after planting), there was significantly less black scurf on tubers harvested from plots infested with 194 pieces containing 104 plants compared to all other infested plots. Two weeks following haulm destruction (11 Oct), black scurf incidence had fallen in plots containing 52 plants, and increased in plots containing 104, however, there were no significant differences two weeks later (25 Oct).

Black scurf severity increased only slightly in infested treatments during plant growing and prior to haulm destruction (16 Aug and 27 Sep) (Figure 6.17). Two weeks following haulm destruction (11 Oct), the severity of black scurf on tubers harvested from plots containing 104 plants was significantly greater than that on tubers from plots containing 52 plants. Four weeks following haulm destruction (25 Oct), there were no longer any significant differences between the treatments.

Table 6.20. Effect of inoculum density and planting population on elephant hide incidence and severity 4 (16 Aug) and 9 weeks (27 Sep) after planting, and 2 (11 Oct) and 4 (25 October) weeks following haulm destruction.

Inoculum density	Elephant hide incidence				Elephant hide severity			
(pieces per plot)	16 Aug	27 Sep	11 Oct	25 Oct	16 Aug	27 Sep	11 Oct	25 Oct
control	3.84 (2.64)[a]	2.02 (2.64)	4.16(6.63)	1.05 (2.09)	0.065	0.161	0.072	0.043
194	5.32 (6.94)	5.08 (6.94)	2.67 (3.61)	2.92 (4.39)	0.167	0.340	0.060	0.133
775	8.23 (11.01)	8.62 (11.01)	4.10 (6.07)	7.16 (9.90)	0.092	0.430	0.215	0.428
P value	(0.554)	(0.131)	(0.678)	(0.049)	0.447	0.133	0.022	0.002
LSD ($P = 0.05$)	(9.001)	(8.442)	(8.084)	(6.201)	0.1644	9.2672	0.1214	0.220
DF = 127[c]								

[a] Values in parentheses are the angularly transformed percentages of tubers showing elephant hide symptoms of six replicates.
[b] Values are the mean elephant hide indices of six replicates.
[c] Pairs of means that differ by more than the LSD are significantly different ($P = 0.05$).

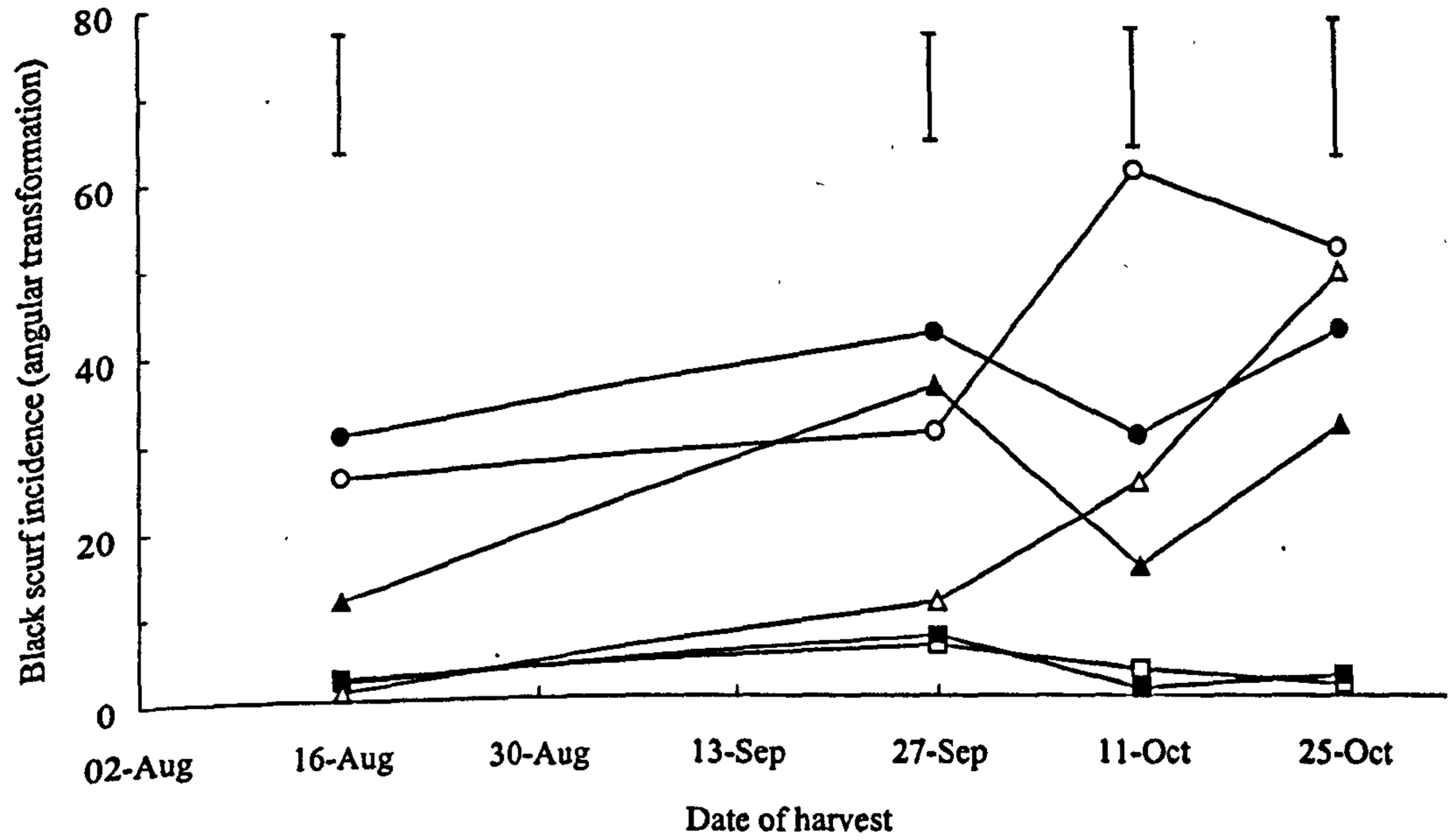


Figure 6.16. Effect of sclerotial inoculum density and plant population density on black scurf incidence [104 plants per plot, inoculum density (pieces per plot): 775 (control) □, 194 Δ and 775 o; 52 plants per plot, inoculum density (pieces per plot): 775 (control) ■, 194 ▲ and 775 ●]. Values are the angularly transformed percentages of tubers showing black scurf symptoms of six replicates. Bars = LSD at $P = 0.05$ (DF = 127).

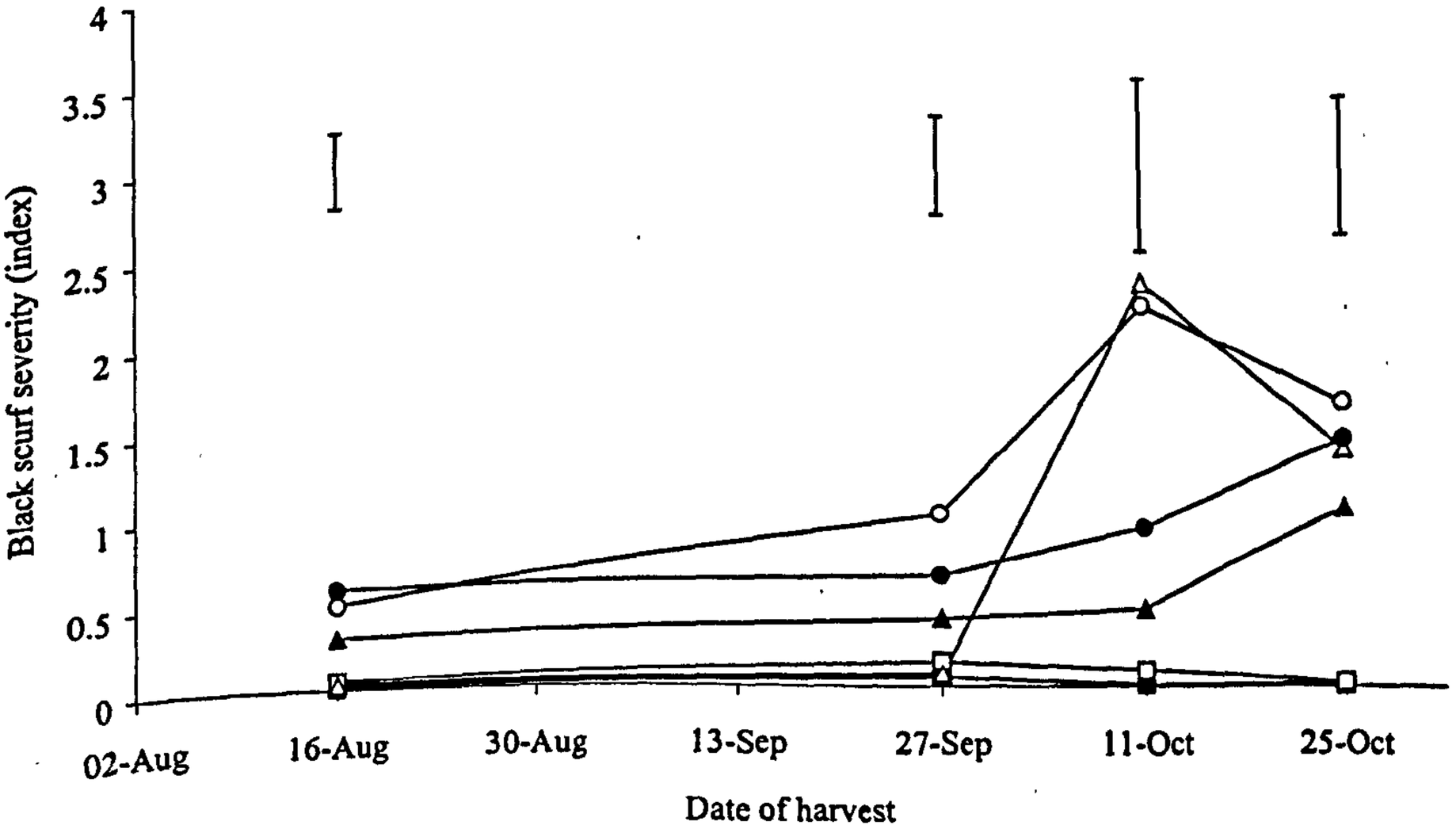


Figure 6.17. Effect of sclerotial inoculum density and plant population density on black scurf severity [104 plants per plot, inoculum density (pieces per plot): 775 (control) □, 194 Δ and 775 o; 52 plants per plot, inoculum density (pieces per plot): 775 (control) ■, 194 ▲ and 775 ●]. Values are black scurf disease indices of six replicates. Bars = LSD at $P = 0.05$ (DF = 127).

6.3.3 Effect of haulm destruction on black scurf formation on progeny tubers

In 2004 and 2005, there were no significant differences between treatments, or C or N plants, with regards to stem and stolon canker incidence and severity, as well as tuber yield and elephant hide incidence and severity (data not shown). The only significant results were on black scurf development in 2004 (Tables 6.21 and 6.22). Prior to haulm destruction, there were no significant treatment differences. Two weeks later (1 Sep), black scurf incidence was 15 % greater on tubers harvested from plants where the haulms had been cut and removed compared to those with uncut haulms in the same plots and the control (Table 6.21). Seven weeks after haulm destruction (6 Oct), tubers from plants with cut haulms had a significantly greater incidence (P = 0.05), up to 50 %, of black scurf compared to the control. Plants with haulms still attached and growing adjacent to plants with cut haulms also had a significantly greater incidence ($P = 0.05$) of black scurf (35 %) on progeny tubers.

Two weeks after haulm destruction, black scurf severity was significantly greater (P = 0.05) on plants with haulms still attached and growing adjacent to those with the haulms cut (Table 6.22). Seven weeks following haulm destruction, there was a significant difference ($P = 0.05$) in black scurf severity between control plants and those with haulms removed. Two to three times more black scurf was observed on progeny tubers from plants where the haulm had been cut compared to uncut controls. Comparing the data from 2004 and 2005, it is clear that black scurf incidence and severity was, on average, almost double in 2005 compared to 2004 (Tables 6.21 – 6.24). Black scurf incidence and severity in 2005 2 weeks after haulm destruction were similar to those found 7 weeks after haulm destruction in 2004. No further disease development occurred between 4.5 and 8 weeks after haulm destruction in any treatment in 2005, whereas incidence of black scurf increased by 37 – 45% during the same interval on progeny tubers from plots with cut haulms in 2004.

Table 6.21. The effect of haulm destruction on black scurf incidence prior to haulm destruction (18 Aug), 2 (1 Sep), 4.5 (23 Sep) and 7 (6 Oct) weeks following haulm destruction in 2004.

Treatment	N or C plants	18 Aug	1 Sep	23 Sep	6 Oct
N haulm uncut (control)	N[a]	9.6 (9.8)[b]	34.9 (33.1)	35.7 (34.7)	28.7 (28.5)
	C	9.0 (9.7)	27.3 (27.8)	28.1 (30.3)	34.1 (32.1)
N haulm cut	N	12.2 (12.9)	41.3 (38.6)	28.9 (29.2)	60.4 (53.8)
	C	7.4 (7.1)	27.8 (28.3)	28.5 (28.1)	49.9 (44.9)
P value		(0.294)	(0.035)	(0.601)	(0.049)
LSD ($P = 0.05$), DF = 72[c]		(10.67)	(13.11)	(10.09)	(9.10)

[a]N and C plants were harvested as outlined in Figure 6.1. The haulms of the N plants were left uncut in the control treatment to determine the effect of haulm cutting on black scurf development on the tuber.

[b]Values in parentheses are the angular transformations of the percentage means tubers showing black scurf symptoms of six replicates.

[c]Pairs of means that differ by more than the LSD are significantly different ($P = 0.05$).

Table 6.22. The effect of haulm destruction on black scurf severity prior to haulm destruction (18 Aug), 2 (1 Sep), 4.5 (23 Sep) and 7 (6 Oct) weeks following haulm destruction in 2004.

Treatment	N or C plants	18 Aug	1 Sep	23 Sep	6 Oct
N haulm uncut (control)	N[a]	0.226[b]	0.245	0.634	0.328
	C	0.226	0.226	0.478	0.468
N haulm cut	N	0.324	0.186	0.726	1.223
	C	0.337	0.599	0.687	0.832
P value		0.967	0.015	0.722	0.033
LSD ($P = 0.05$), DF = 72[c]		0.5429	0.3431	19.43	0.6466

[a]N and C plants were harvested as outlined in Figure 6.1. The haulms of the N plants were left uncut in the control treatment to determine the effect of haulm cutting on black scurf development on the tuber.
[b]Values in parentheses are the mean black scurf indices of six replicates.
[c]Pairs of means that differ by more than the LSD are significantly different ($P = 0.05$).

Table 6.23. The effect of haulm destruction on black scurf incidence prior to haulm destruction (31 Aug), 2 (14 Sep), 4.5 (7 Oct) and 7 (2 Nov) weeks following haulm destruction in 2005.

Treatment	N or C plants	31 Aug	14 Sep	7 Oct	2 Nov
N haulm uncut (control)	N[a]	15.6 (15.7)[b]	26.3 (25.5)	48.6 (41.7)	60.3 (51.5)
	C	16.7 (16.9)	29.0 (26.4)	44.3 (39.2)	56.8 (49.6)
N haulm cut	N	21.2 (22.1)	43.7 (37.6)	46.5 (40.5)	58.4 (50.6)
	C	24.8 (23.5)	37.9 (33.4)	54.0 (46.2)	55.1 (47.9)
P value		(0.975)	(0.513)	(0.348)	(0.934)
LSD ($P = 0.05$), DF = 72[c]		(16.54)	(21.96)	(14.80)	(26.07)

[a]N and C plants were harvested as outlined in Figure 6.1. The haulms of the N plants were left uncut in the control treatment to determine the effect of haulm cutting on black scurf development on the tuber.
[b]Values in parentheses are the angular transformations of the percentage means tubers showing black scurf symptoms of eight replicates.
[c]Pairs of means that differ by more than the LSD are significantly different ($P = 0.05$).

Table 6.24. The effect of haulm destruction on black scurf severity prior to haulm destruction (31 Aug), 2 (14 Sep), 4.5 (7 Oct) and 7 (2 Nov) weeks following haulm destruction in 2005.

Treatment	N or C plants	31 Aug	14 Sep	7 Oct	2 Nov
N haulm uncut (control)	N[a]	0.362[b]	0.521	1.912	1.52
	C	0.464	0.646	1.924	1.67
N haulm cut	N	0.488	1.463	1.645	1.96
	C	0.609	0.863	1.633	1.57
P value		0.945	0.030	0.986	0.291
LSD ($P = 0.05$), DF = 72[c]		0.6760	0.6333	0.8901	9.83

[a]N and C plants were harvested as outlined in Figure 6.1. The haulms of the N plants were left uncut in the control treatment to determine the effect of haulm cutting on black scurf development on the tuber.
[b]Values in parentheses are the mean black scurf indices of eight replicates.
[c]Pairs of means that differ by more than the LSD are significantly different ($P = 0.05$).

6.4 Discussion

It has been demonstrated in this study that after burial in field soil, sclerotial germination of *R. solani in vitro* after recovery declined over 18 months. This decline in sclerotial germination was not complete, with between 10 and 35 % of sclerotia remaining viable after 18 months burial. A previous study demonstrated sclerotial germination to reduce to between 25 and 50 % after 24 months burial in field soil (Velvis *et al.*, 1989). A common feature of all sclerotial burial trials in this study was the reduction in germination of *R. solani* over the 18 month period. *R. solani* sclerotia germinated with 72 h of harvest and did not appear to require a dormancy period. Other sclerotial-forming pathogens, such as *Sclerotium cepivorum*, are known to require a period of constitutive dormancy prior to germination and be stimulated into germination by the presence of extracts and exudates obtained from the host plant (Coley-Smith *et al.*, 1968; McLean *et al.*, 2005).

It was assumed that any recovered sclerotia not undergoing germination had not survived the burial period. An investigation into the germination of *R. solani* sclerotia in soil following prolonged burial, as well as the effect of potato plant extracts on sclerotial germination, would provide further information of the survival and germination of this plant pathogen in its natural environment. A comparison of laboratory produced and naturally produced sclerotia revealed slight differences in germination of the two sclerotial types. These were primarily in the early months following burial, and there was little difference in sclerotial germination thereafter. Sclerotia can be produced more rapidly and in greater numbers *in vitro* than on potato tubers. As only minor differences were observed between the germination of the two sclerotial types, it could be concluded that sclerotia produced *in vitro* would be a suitable alternative to using sclerotia from potato tubers for futher studies on sclerotia.

The soil type that sclerotia were buried in also affected germination of sclerotia after recovery, with the percentage germination of sclerotia buried in sand soil almost four times lower than those from loam-sand soil. Previous studies on the prolonged burial of sclerotia of *R. solani* in different soils showed sclerotial viability to decline more rapidly in sand-based soils as opposed to clay soils (Velvis *et al.*, 1989). Whether the markedly greater decline in sclerotial viability in sand soil is a direct result of soil structure is unknown and warrants further investigation. Previous studies demonstrated that 80% of sclerotia remained viable after 6 months when stored in field soil at 15–18°C with regular watering (Pitt, 1964). Further research into the effect of soil moisture and temperature would determine what factors are important for sclerotial survival in soil. The effect of three burial depths (5, 10 and 20 cm) was investigated in the present study as sclerotial formation occurs primarily on progeny tubers, which form at depths similar to these. Once formed, sclerotia may remain at these depths for prolonged periods, and soil disturbance during harvest and cultivation would also affect the position of sclerotia in the soil. The present study demonstrated there was no effect of burial depth on sclerotial germination following recovery. As a parallel study revealed that soil type could significantly affect sclerotial survival, the effect of burial depth in different soil types should be also be investigated.

Potential antagonistic infection of sclerotia did not exceed 8 % on any sampling date in any of the experiments. It is clear that the reduction in sclerotial viability was not linked to the presence of antagonists, as the percentage of infected sclerotia at each harvest remained relatively stable throughout the duration of all experiments. Both *Penicillium* sp. and *Clonostachys rosea* were isolated from retrieved sclerotia in this study, and sclerotia samples taken from Dutch potato fields have also been found to harbour these potential antagonists (Jager & Velvis, 1983). Relatively little is known about *C. rosea* as an antagonist, although previous studies have shown it

did not reduce sclerotial production *in vitro* when grown in dual culture with *R. solani* (van den Boogert & Deacon, 1994). A recent study demonstrated that several isolates of *Penicillium* sp. were effective in reducing the incidence and severity of black scurf in pot trials, as individual treatments and in combination with other biocontrol organisms including *Bacillus subtilis* and *Trichoderma* (*Gliocladium*) *virens* (Brewer & Larkin, 2005). The most well-known antagonist of *R. solani*, *Verticillium biguttatum*, was not isolated from any sclerotia in this study. Other fungi isolated from sclerotia included *Mauginiella* sp. and *T. angustata*, which have not been isolated from *R. solani* sclerotia previously. It is unknown whether these fungi are potential antagonists of *R. solani* sclerotia, and this warrants further investigation.

Many studies have highlighted the importance of soil-borne inoculum, which cannot be easily eradicated once it is established (Frank & Leach, 1980; Tsror & Peretz-Alon, 2005). The type of soil-borne inoculum did not appear to affect disease caused by *R. solani*, with mycelial and sclerotial sources causing similar disease symptoms. In the present study, regardless of inoculum type, the greater the inoculum density in the soil, the greater the disease incidence and severity observed on stems, stolons and tubers, and this corroborates findings from previous studies. For example, the relationship between mycelial soil-borne inoculum density and disease development was demonstrated previously, with low densities resulting in limited disease and higher inoculum densities causing severe stem canker and greater black scurf formation (Kyritsis & Wale, 2002a). The present study is the first to show the significance of sclerotial soil-borne inoculum density on disease development, with the highest inoculum densities reducing tuber yields, as well as increasing the incidence and severity of stem and stolon canker, and black scurf and elephant hide formation on progeny tubers.

Both inoculum types were applied to the soil 2 weeks prior to planting, however, soil-borne inoculum would, most probably, have been in the soil for a much

longer period of time prior to planting than this. Under field conditions, there is likely to be a combination of the two types of inoculum present. Previous laboratory studies have demonstrated that the addition of mycelial inoculum of *R. solani* to soil results in an initial increase in inoculum density in the absence of a host, followed by a reduction and stabilisation of inoculum density after 2 months, which remained stable for the 8 month duration of the experiment (Velvis *et al.*, 1989). This suggests that mycelial inoculum may have the potential for long-term survival between potato crops, however, the reduction in viability of mycelial inoculum in the field in the absence of a host is unknown.

The current study has shown sclerotial viability to decline with extended burial in soil. It is possible that the soil-borne sclerotial inoculum densities investigated in this study may not be as virulent six months after they were established in the field. For example, the reduction in sclerotial germination between initial burial in Oct 2003 and the following May (2004), when planting of new potato crops would occur, were between 10 and 30 % whereas the difference in sclerotial germination in May 2005 compared to germination prior to burial was over 50 %. Some growers will plant a second potato crop shortly after the first is harvested, whereas others, such as seed producers, have rotations of seven years between potato crops to prevent the build up of soil-borne pests and diseases. Some studies have demonstrated rapid declines in soil-borne inoculum with short rotations (one year), of up to 50 % (Hide & Read, 1991). Others have found soil-borne inoculum levels to reduce more rapidly during a 6 year than a 4 year rotation (Gilligan *et al.*, 1996). They also found the planting of a potato crop resulted in the detection of relatively high densities of soil-borne inoculum, even after a 6 year rotation (Gilligan *et al.*, 1996).

Both planting population and sclerotial inoculum density were investigated to determine their effect on disease development, however, the latter was the main factor

responsible for differences in disease incidence and severity on stems and progeny tubers in both pot and field trials. Sclerotial inoculum density in pot trials had a different effect on plant emergence depending on the growing medium. When grown in pots using John Innes No. 3 as the growing medium, emergence was slower, and the highest inoculum densities tested only had a significant effect early in the growing season, with no effect 7 weeks after planting. When a similar experiment was done using field soil as the growing medium, emergence occurred 1 week following planting and the highest inoculum densities consistently reduced emergence over the following 3 weeks. No effect of inoculum density or plant population was found on plant emergence in the field trial. There was a clear decline in stem canker incidence and severity over the growing season when plants were grown in John Innes no. 3, whereas there was no definite upwards or downwards trend when plants were grown in pots in field soil. In the field, stem canker incidence and severity clearly increased with increasing inoculum density over the entire growing season, in contrast to both pot experiments.

In the field trial, plant population only had a limited effect on stem canker incidence and severity. Tuber-borne disease symptoms varied in response to inoculum density and growing media. Elephant hide symptoms on progeny tubers in trials done in John Innes no. 3 and soil in pots and in the field showed no definite trends in response to differences in inoculum density or dates following haulm destruction regardless of the growing medium. This is in contrast to previous work which demonstrated elephant hide to be most severe prior to haulm destruction, with reductions in incidence of up to 50 % and a 6-fold reduction in disease severity 4 weeks after haulm destruction (R. Bain, personal communication).

One week following haulm destruction, black scurf incidence on progeny tubers from both pot trials at the highest inoculum densities reached a maximum, with no further development in incidence and severity of symptoms thereafter. Black scurf

incidence at the lowest inoculum densities was more gradual, with a steady increase in the incidence of symptoms, reaching similar levels to those at the highest inoculum densities 4 weeks after haulm destruction. In John Innes no. 3, black scurf severity indices reached a maximum 1 week following haulm destruction, whereas those in soil were much less severe and showed a more gradual increase in disease severity over the 4 weeks following haulm destruction. The results from the trial using field soil are more likely to be representative of what would occur under naturally, as *R. solani* would face competition from other soil-borne fungi and attack from mycoparasitic fungi which would not be present in composted growing media.

An established baiting method was used during the trial investigating soil-borne inoculum density and plant population, where a percentage colonisation of beet seeds <10 % suggests low risk, 10 to 20 % intermediate risk and >20 % high risk from soil-borne *R. solani* (A. Hilton, personal communication). Surveys of commercial fields have shown 70 % to be in the low risk category, with 20 % and 10 % on intermediate and high risk categories respectively (A. Hilton, personal communication). In this trial, the maximum percentage colonisation of beet seed over the course of the entire trial was between 2 to 25 %, which suggests the inoculum densities obtained in this trial were representative of the range routinely found on commercial farms.

Both inoculum density and plant population had a significant effect on black scurf formation in the field trial, however, the results were inconsistent. Following haulm destruction, black scurf incidence increased in plots containing 104 plants (80,000 potatoes ha^{-1}), whereas those containing 52 plants per plot (41,000 potatoes ha^{-1}) decreased regardless of inoculum density. Black scurf severity in plots containing 104 plants reached a peak 2 weeks following haulm destruction with a decline thereafter, whereas black scurf severity in plots containing 52 plants was steadily increasing. These results made it difficult to draw any sufficient conclusions as to the true effects of these

factors on black scurf formation from this study. Field trials from 2002 and 2003 using cv. Maris Peer found higher planting population densities resulted in increased black scurf development on progeny tubers. Black scurf severity in 2003 was almost double on potatoes harvested from plots grown at higher (73,500 potatoes ha^{-1}) compared to lower (49,000 potatoes ha^{-1}) planting populations in 2003 (R. Bain, personal communication). Another study using cv. Marfona, also in 2003, found no difference in black scurf severity between high (48,000 potatoes ha^{-1}) and low (33,000 potatoes ha^{-1}) planting populations (R. Bain, personal communication).

Planting of seed tubers covered in black scurf is known to cause greater levels of disease on the resulting potato crops (Hide *et al.*, 1973; Gudmestad *et al.*, 1979; Carling *et al.*, 1989). This study has revealed that inoculum from seed tuber pieces can contribute to soil-borne inoculum, and affect the incidence and severity of stem canker and black scurf development. It was found previously that planting seed tubers with 5–15 % of their surface area covered in black scurf would result in minor stem canker development on the crop (James & McKenzie, 1972). In contrast, the incorporation of potato pieces from tubers with between 11–25 % black scurf coverage into field soil was enough to contribute significantly to soil-borne inoculum, resulting in increased incidence and severity of stem canker and black scurf compared to untreated plots. This highlights the potential for unharvested tubers to act as a source of soil-borne inoculum for future crops.

It has been widely reported that black scurf formation on progeny tubers accelerates following haulm destruction, with maximum sclerotial development occurring 3 – 4 weeks later (Chand & Logan, 1984; Chand & Logan, 1986; Gudmestad *et al.*, 1979; Dijst *et al.*, 1986). The results on the effect of haulm destruction in this study were inconsistent, with a significant effect observed in 2004 but not in 2005. The 2004 trial demonstrated haulm removal to increase sclerotial formation on progeny

tubers in contrast to 2005, where there were no differences between treatments. Harvesting seasons in 2004 and 2005 were very different, with average rainfall after haulm destruction (Aug – Oct) 200 to 250 mm and 75 to 150 mm respectively (Anon, 2004b). Average monthly temperatures during the same period also varied in range, at 8 to 14°C in 2004 and 10 to 12°C in 2005 (Anon, 2005b). In the current study, black scurf development on progeny tubers was much greater in 2005 than 2004. Black scurf incidence and severity at 2 weeks following haulm destruction in 2005 were similar to that found 7 weeks following haulm destruction in 2004. Seven weeks following haulm destruction in the drier season of 2005, black scurf development was almost double that found in 2004, which had more rainfall over the same period. Previous work found that significantly more black scurf was present on progeny tubers with a mean soil temperature around harvesting of 17.3°C with 193 mm rainfall compared to 20.9°C with 48 mm rainfall (Lootsma & Scholte, 1996). In contrast, soil moisture levels of 60 % WHC have been found to cause significant reductions in the formation of black scurf on seed tubers (Kyritsis & Wale, 2002b). Whether the differences between the two trials in this study are due to environmental factors warrants further investigation.

Results from this study indicate that sclerotia of *R. solani* can remain viable after prolonged burial of 18 months in field soil irrespective of burial depth or presence of potential antagonists on sclerotia. Increased incidence and severity of stem canker was shown to be a direct result of increased soil-borne sclerotial inoculum densities. Although it has been demonstrated that sclerotia of *R. solani* can germinate *in vitro* after burial in soil, it remains unknown if prolonged burial in field soil in the absence of the host would affect the pathogenicity of *R. solani* sclerotia. An investigation into sclerotial burial duration and the pathogenicity of sclerotia after prolonged burial on potato crops would contribute to a greater understanding of the effect of sclerotial survival in soil and the consequences of this on soil-borne inoculum densities.

CHAPTER 7

CONTROL OF SOIL-BORNE *R. SOLANI* ON POTATO

7.1 Introduction

Concern over the effect of agricultural pesticides on the environment, as well as greater restrictions placed on currently available fungicides has increased the need for alternative methods of controlling plant pathogens (Whipps & Lumsden, 2001). There are also strict regulations governing the use of chemicals in organic farming and, therefore, alternative control methods are required (Brown & Haward, 2001). The use of organic soil amendments has been shown to have a positive effect on crop health and productivity in many crops (Bailey & Lazarovits, 2003). Farmyard and green manures have been investigated as a means of controlling *R. solani* disease incidence and severity on potato with varying degrees of success (Lootsma & Scholte, 1998; Scholte & Lootsma, 1998; Tsror *et al.*, 2001). The viability of other sclerotial-forming plant pathogens, such as *Sclerotium cepivorum*, have been found to decrease in response to incorporation of composted wastes into soil (Coventry *et al.*, 2002).

The use of biological control methods, whereby organisms are used to control or eliminate plant pathogens, is another means of control without fungicides (Whipps & Davies, 2000). Fungal antagonists have been found to colonise *R. solani* sclerotia present on progeny tubers and buried in soil (Jager *et al.*, 1979; van den Boogert & Jager, 1983). The most extensively studied fungus with potential for the biological control of *R. solani* on potato is *Verticillium biguttatum*, which has been evaluated as a seed tuber treatment as part of an integrated disease management approach to *R. solani* (Jager & Velvis, 1984; Jager & Velvis, 1986; van den Boogert & Luttikholt, 2004). Commercially available formulations of biological control organisms, including *Gliocladium* sp., *Trichoderma* sp. and *Bacillus subtilis*, have been evaluated and shown to vary widely in their ability to control *R. solani* on potato and other hosts (Lewis & Lumsden, 2001; Brewer & Larkin, 2005).

This chapter investigates the effect of soil-amendments and commercially available products on stem and tuber disease caused by sclerotial soil-borne inoculum of *R. solani*. A range of potential antagonists isolated from the sclerotia survival trial (Chapter 6) were evaluated *in vitro*. A glasshouse bioassay compared the efficacy of these potential antagonists to known antagonistic fungi and current chemical control methods to determine their potential to protect against disease caused by soil-borne *R. solani* on potato.

7.2 Materials and Methods

7.2.1 Effect of soil amendments on disease caused by sclerotial soil-borne *R. solani*

7.2.1.1 Effect of timing of soil amendment application on disease

One isolate (x72) of *R. solani* AG 3 (section 2.1) was used to produce sclerotial inoculum as described previously (section 4.2.1). The experiment was conducted at Mansion Field, SAC, Auchincruive, and the growth medium was a loam-sand soil (Bargour Series). NPK (16:16:16) fertiliser was added to the soil at a rate of 1250 kg ha^{-1} and the soil infested with 50 mg sclerotia kg^{-1} d.w. soil 4 weeks prior to planting on 13 May 2004. The soil was used to fill 3-l pots (Plantpak) and onion waste compost (Emma Coventry, Warwick Horticulture Research International, Wellesbourne), green waste compost (Fife Council, Dunfermline) or neem cake (Kalpesh Shah, Knightel Ltd, London) added to the soil 2 weeks prior to planting (27 May 2004) or on the day of planting at a rate of 10 % w/w d.w. compost (10 Jun 2004). These dates were chosen to simulate adding the soil amendment whilst the field was being prepared for planting, or directly at planting. A single mini-tuber (cv. Kondor, 15 – 20 mm, Agrico Ltd, Angus) was planted 8 cm deep in each pot using a dibber. The trial was laid out in a split plot design consisting of seven treatments, with eight replicate pots per treatment.

Plant emergence was assessed weekly from the date the first plants emerged. Stem canker incidence and severity were assessed 4 and 12 weeks after planting as described previously (section 6.2.2). The total tuber yield per plant, black scurf and elephant hide incidence and severity were all determined 12 weeks after planting, and 2 and 4 weeks following haulm destruction as described previously (section 6.2.2).

7.2.1.2 Efficacy of commercially available products against soil-borne R. solani

This experiment was conducted to compare the efficacy of commercial products in controlling disease caused by *R. solani* AG 3 (isolate x72) on potato. The trial was set up in a glasshouse at Diamond Field, SAC, Auchincruive. The growth medium was John Innes no. 3 (Clydeside Trading Society Ltd, Strathclyde) and pots were infested with 50 mg sclerotia kg^{-1} d.w. growth medium and prepared as described previously (section 6.2.2.1). Compost was infested with sclerotia on 17 Jan 2005, with the application of seed and soil treatments 2 weeks later (31st Jan) at planting, according to the manufacturers' recommendations (Table 7.1). A single tuber (cv. Maris Piper, 4 to 8 g, Gentech Propagation Ltd, Dundee) was planted 8 cm down in each 3-l pot using a dibber. The trial was set up in a split-plot design with seven replicates for each of the 10 treatments. The glasshouse temperature was 15 ± 5 °C for the duration of the experiment.

Table 7.1. Seed and soil treatments evaluated in the glasshouse trial.

Product Name (formulation)	Supplier	Main constituent	Recommended application rate	Application method
Control	-	uninfested	-	-
Control	-	Infested (no treatment)	-	
RhiNo (liquid)	Certis, UK	460g l^{-1} flutolanil	200 ml $tonne^{-1}$ potatoes	seed
Biofungus Instant $^{(R)}$ (granules)		*Trichoderma* sp.	200 g l^{-1} soil	soil
Microgran (granules)	*P.P.* Products, Norwich	Calcium cyanamide (40 %)	400 g ha^{-1}	soil
Cultimide (liquid)	*P.P.* Products, Norwich	Calcium cyanamide (24 – 26 %)	225 l ha^{-1} in 600 l water; 100 ml diluted product per pot	soil
Garlic (granules)	Garlic Farms UK Ltd, Surrey	Garlic	100 g per 100 m^2	soil
Neem cake (granules)	Knightel Ltd, London	Azadirachtin (2500 ppm)	10 % w/w	soil
Prestop WP (granules)	Verdera Oy, Finland	*Gliocladium catenulatum*	1 % w/v solution	seed
Bactolife (granules)	Biotechnica, Reading	*B.subtilis*, *Trichoderma* sp.	24 g in 10 l water; 300 ml per pot	soil

Plant emergence was assessed weekly from the date the first plants emerged. Stem canker incidence and severity were assessed 4 and 12 weeks after planting as described previously (section 6.2.2). The total tuber yield per plant, black scurf and elephant hide incidence and severity were all determined 12 weeks after planting, and 2 and 4 weeks following haulm destruction as described previously (section 6.2.2).

7.2.2. Investigation into the biological control potential of fungi isolated from buried sclerotia

Potential antagonists (Table 7.2) were isolated from sclerotia retrieved at various intervals over 18 months in previous sclerotial survival studies (Chapter 6). In addition, two isolates of *V. biguttatum* were included, one from potato (M73) and another from tulip (M92) (Table 2.1), as this fungus is a known antagonist of *R. solani* on potato. *R. solani* AG 3 isolate x72 was used in all experiments (section 2.1). All fungal isolates were grown on PDA from 3 mm plugs taken from the edge of actively growing colonies and sub-cultured centrally onto fresh PDA as described previously (section 3.2.1). All cultures were incubated at 25°C prior to use in experiments and for the duration of all experiments.

Table 7.2. Potential and known antagonists tested *in vitro*.

Potential antagonists	Known antagonists
Gliocladium sp. IMI 393299	*Verticillium biguttatum* M73
Clonostachys rosea Link:Fr (*Gliocladium roseum* Bainer) IMI 393300	*Verticillium biguttatum* M92
Truncatella angustata (Pers.) S. Hughes IMI 393301	
Mauginiella sp. IMI 393302	
Penicillium sp.	

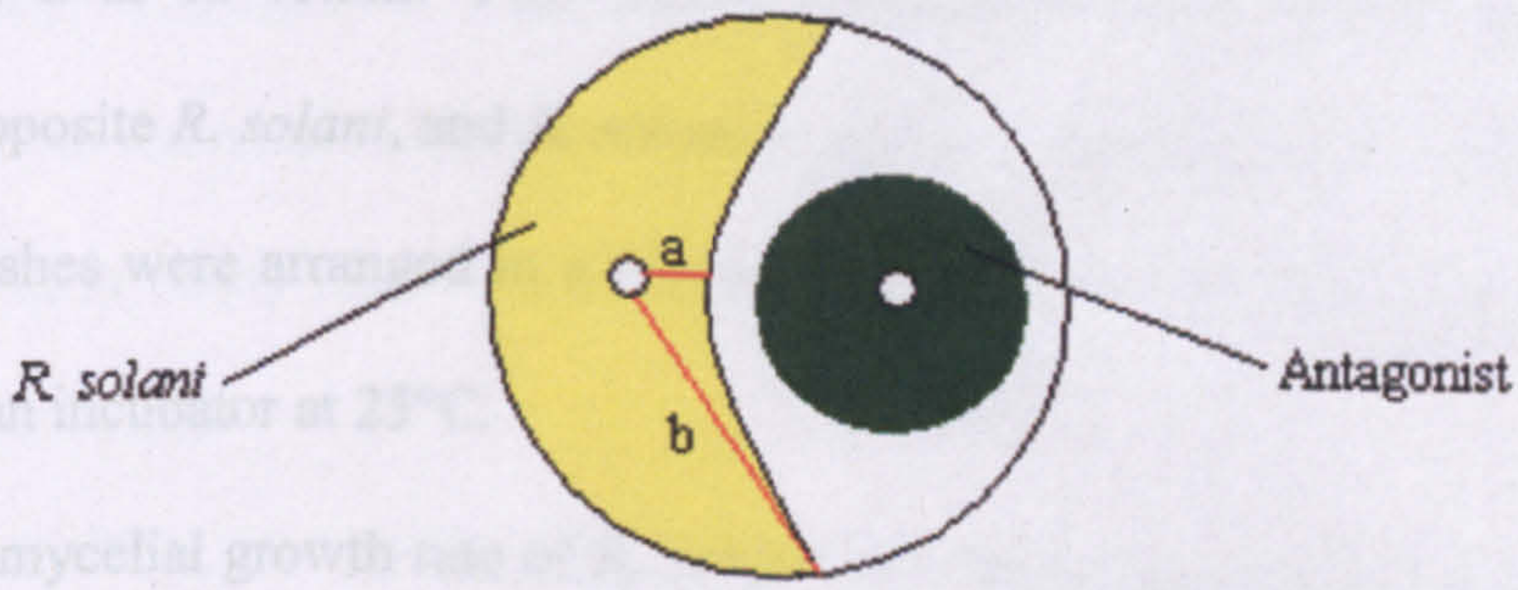


Figure 7.1. Diagram representing the inoculation of Petri dishes with *R. solani* and the antagonist. The shortest line between *R. solani* and the antagonist. (a) represents the radial mycelial growth of *R. solani* directly opposite the antagonist and (b) the furthest radial mycelial growth.

7.2.2.1 Growth of potential antagonists in dual culture with R. solani and the effects on mycelial growth and sclerotial biomass production

The interaction of potential antagonists and *R. solani* was studied using a technique based on Royse & Ries (1978) and Whipps (1987). Petri dishes containing PDA or WA were prepared as described previously (section 3.2.1). A 3 mm mycelial disc of each of the potential and known antagonistic fungi was placed 4 cm across from a 3 mm disc of *R. solani* mycelium, with all pairings carried out on both WA and PDA. Due to differences in the mycelial growth rates of the potential and known antagonists, *Gliocladium* sp., *C. rosea*, *Penicillium* sp. and both *V. biguttatum* isolates M73 and M92 were inoculated 3 d prior to *R. solani*. *Mauginiella* sp. and *T. angustata* were inoculated on the same d as *R. solani*. Two control treatments were set up, with *R. solani* inoculated opposite *R. solani*, and *R. solani* inoculated opposite a 3 mm disc of PDA or WA. Petri dishes were arranged in a randomised block design with five replicates per treatment in an incubator at 25°C.

The mycelial growth rate of *R. solani* was determined along two axes between 2 and 5 d following inoculation, during which time all colonies had undergone contact with each other (Figure 7.1). The percentage inhibition (%) of mycelial growth was calculated using these values for radial mycelial growth in the following formula: [(b – a)/b] x 100 (Whipps, 1987). Sclerotial biomass was determined by harvesting sclerotia after 21 d as described previously (section 3.2.1).

7.2.2.2 Production of antifungal volatiles by potential antagonists and effects on mycelial growth, sclerotial production and germination of R. solani

The effect of antifungal volatiles produced by potential antagonists on mycelial growth, sclerotia formation and germination was investigated using methods adapted from Dennis & Webster (1971), in which Petri dishes of the potential antagonists were sealed to Petri dishes containing actively growing cultures of *R. solani*. Petri dishes of PDA or WA were prepared as described previously (section 3.2.1). Mycelial plugs (3 mm diam) of *Gliocladium* sp., *C. rosea*, *Penicillium* sp. and both *V. biguttatum* M73 and M92 were used to inoculate Petri dishes of PDA and WA centrally. All of these potential and known antagonistic fungal colonies were grown at 25°C for 3 d. Petri dishes of *Mauginiella* sp. and *T. angustata* were prepared on the same day as *R. solani* Petri dishes were inoculated. Control Petri dishes of *R. solani* opposite *R. solani* and *R. solani* opposite an empty plate were also prepared, and Petri dishes sealed together with Parafilm (Figure 7.2).

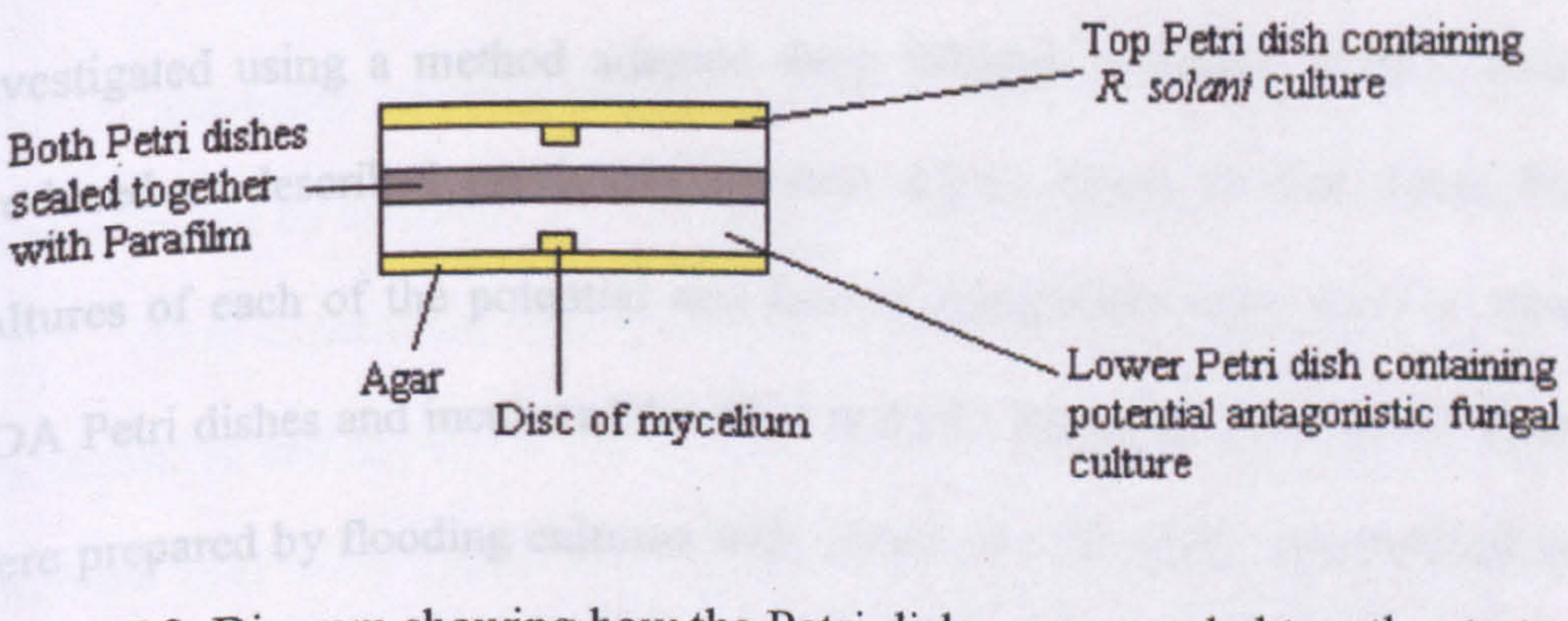


Figure 7.2. Diagram showing how the Petri dishes were sealed together to test the effect of fungal volatiles on the mycelial growth and sclerotium yield of *R. solani*.

Petri dishes were incubated at 25°C with the plate containing the antagonist below the plate of *R. solani*. Percentage inhibition (%) of mycelial growth was calculated by comparing the mycelial growth of *R. solani* in the presence of a test isolate with the mycelial growth of *R. solani* in the controls between 2 and 5 d following inoculation of Petri dishes with *R. solani*. Sclerotial production was determined after 21 d as described previously (section 3.2.1).

To determine the effect of antifungal volatiles produced by potential antagonists on sclerotial germination, PDA and WA cultures of the antagonists were prepared as described earlier in this section. Sclerotia of *R. solani* were produced as described previously (section 4.2.1). Ten individual sclerotia were placed on PDA and WA Petri dishes and sealed to the antagonist Petri dishes with Parafilm. Petri dishes were incubated at 25°C, with the plate containing the antagonist at the base. Percentage sclerotial germination (%) per treatment was determined after 72 h.

7.2.2.3 Colonisation of sclerotia by potential antagonists of R. solani

The ability of potential antagonists to colonise *R. solani* sclerotia was investigated using a method adapted from Whipps & Budge (1990). Sclerotia were produced as described previously (section 4.2.1). Discs (3 mm diam) from 3-d-old cultures of each of the potential and known antagonists were used to inoculate fresh PDA Petri dishes and incubated for 10 d at 25°C. Spore suspensions of each antagonist were prepared by flooding cultures with 10 ml of 1 % sterile mycological peptone (1% w/v (Sigma) in distilled water) and rubbing the culture surface with a glass spreader. Spore suspensions were filtered through muslin to remove most of the mycelium. Spore concentrations were determined using a haemocytometer and dilutions prepared to give suspensions containing approximately 1.5×10^6 spores ml^{-1}.

Fifty sclerotia of *R. solani* were placed in 20 ml of spore suspension of each antagonist in universal bottles and gently shaken for 10 min. Bottles were left to stand

for 10 min and the sclerotia removed using sterile forceps and touching them against the side of the bottle to remove excess liquid. Sclerotia were transferred to 9 cm diam Petri dishes containing 40 g autoclaved horticultural grade sand (Silvaperl silver sand, lime-free washed silica sand particle size >1mm, William Sinclair Horticulture Ltd, Lincoln, UK) sieved to <425 μm. Ten sclerotia were pressed into the surface of the sand, which had been moistened to 75% water holding capacity (WHC). Petri dishes were sealed with Parafilm and incubated for 2 weeks at 20°C. Petri dishes were re-weighed every week to monitor and maintain original water content. Petri dishes were arranged in a randomised block design with five replicates per treatment.

Following 2 weeks incubation, sclerotia were examined at x40 magnification using a binocular dissecting microscope for evidence of sporulation of antagonists. Once this was assessed, sclerotia were removed from the sand by dry sieving, washed three times in sterile distilled water and re-plated (10 sclerotia per plate) onto D-glucose medium (section 3.2.4). Sclerotial germination (%) per treatment was assessed after 72 h.

7.2.3 Pot-based bioassay to determine the effect of potential antagonists in soil on disease caused by sclerotial soil-borne inoculum of *R. solani*

This experiment was located in a glasshouse at Diamond Field Unit, SAC, Auchincruive and set up on Jul 13 2005. Sclerotial inoculum was prepared *in vitro* as described previously (section 4.2.1). Sclerotia were used to infest John Innes no. 3 growth medium (Clydeside Trading Society Ltd, Strathclyde) at a rate of 50 mg sclerotia kg^{-1} d.w. compost) and used to fill 3-l pots. Individual spore suspensions of *Gliocladium* sp., *C. rosea*, *T. angustata* and *Mauginiella* sp. containing 4 x 10^6 spores ml^{-1} were prepared as described earlier in this section. A spore suspension of *V. biguttatum* M73 containing 4 x 10^6 conidia ml^{-1} was also prepared, with uninfested and infested growth medium treated with distilled water included as control treatments. Two

fungicides were also included for comparison. A soil treatment containing azoxystrobin (as Amistar; 25 % a.i. SC; Syngenta; 250g a.i. l^{-1}) was applied at a rate of 6-l ha^{-1} using dibber to make a hole 8 cm deep in the soil and the fungicide sprayed directly into it prior to planting of the seed tuber. A seed treatment containing flutolanil (as RhiNo, 46% a.i. FS; Certis; 460 g a.i. l^{-1}) was applied as a seed tuber treatment prior to planting as described previously (Table 7.2). The trial was set up in a split-plot design with eight replicates per treatment.

Seed tuber pieces (cv. Pentland Dell, 35 - 55 mm) were prepared by halving tubers transversely using a knife surface sterilised with industrial methylated spirit (IMS). Tuber halves were placed into trays and left uncovered for 1 week at room temperature to allow the cut ends to heal. Pots (3-l) were filled with growing medium and a hole 8 cm deep made in each pot using a dibber. Four hundred ml of spore suspension was sprayed into the hole immediately prior to planting seed tuber pieces 8 cm deep in each pot. Pots were watered weekly with approximately 300 ml water until 8 weeks after planting, when watering increased to twice weekly. Pots were supplemented weekly with 100 ml of Miracle Gro® (NPK 24:8:16 – 15ml product diluted in 4.5 l before application).

Plant emergence was assessed weekly from the date the first plant emerged. Stem canker incidence and severity were assessed immediately prior to haulm destruction (21 Sep) as described previously (section 6.2.2.1). Eight plants per treatment were destructively harvested and assessed on each of four sampling dates. The trial was sampled immediately prior to haulm destruction (21 Sep), 1 (28 Sep), 3 (12 Oct) and 6 (2 Nov) weeks following haulm destruction. Glasshouse temperature averaged 19 ± 6°C for the duration of the experiment. Total tuber yield, black scurf and elephant hide incidence and severity were also assessed as described previously (section 6.2.2.1).

7.3 Results

7.3.1 Effect of soil amendments on disease caused by sclerotial soil-borne *R. solani*

7.3.1.1 Effect of timing of soil amendment application on disease

Plant emergence was initially observed approximately 4 weeks after planting (14 Jul). Emergence was slightly higher, although not significantly in the infested control compared to the uninfested control. The addition to the soil of neem cake or green waste compost 2 weeks before planting, or onion compost at either time, resulted in significantly lower ($P = 0.05$) plant emergence on 14 Jul compared with the infested control. Five weeks after planting (21 Jul), there was over 35 % greater plant emergence where green waste compost had been added at planting compared to the infested control (Table 7.3). By 28 Jul, any differences in plant emergence between treatments were not statistically significant.

The addition of neem cake to soil had a significant effect ($P = 0.05$) on stem height, with the heights recorded being approximately double those observed in the infested control on both harvest dates (8 Jul and 2 Sep) (Table 7.4). Stem canker severity on plants grown in soil where green waste compost was applied 2 weeks prior to planting was significantly higher than the infested control on 8 Jul. No significant reduction in disease was observed in any organic soil amendment treatment and only azoxystrobin reduced stem canker incidence and severity significantly on all but one harvest date (2 Sep).

Neem cake had a significant effect on stolon length, with stolons between 2 and 4 times longer than those grown in infested soil alone, depending on the harvest date (Table 7.5). The effect of onion compost was inconsistent, with stolon length only significantly greater 12 weeks after planting (2 Sep). Only azoxystrobin soil treatment resulted in significant reductions in stolon canker incidence and severity, with no disease symptoms recorded occurred 12 weeks after planting (Table 7.5).

Table 7.3. Effect of timing of soil amendment application on plant emergence.

Soil Amendment	Timing of application	Assessment date 14 Jul	21 Jul	28 Jul
Uninfested soil (control)	-	40.6 (36.6)[a]	56.2 (50.6)	100.0 (90.0)
Infested soil (control)	-	50.0 (45.0)	53.1 (47.8)	93.8 (84.4)
Azoxystrobin	At planting	40.6 (36.6)	78.1 (70.3)	96.9 (87.2)
Onion compost	2 weeks before planting	18.8 (16.9)	37.5 (33.8)	93.8 (84.4)
Onion compost	At planting	15.6 (14.1)	43.8 (39.4)	81.2 (73.1)
Neem cake	2 weeks before planting	6.3 (5.6)	35.5 (33.8)	87.5 (78.8)
Neem cake	At planting	34.4 (30.9)	40.6 (36.6)	96.9 (87.2)
Green waste compost	2 weeks before planting	12.5 (11.2)	31.2 (28.1)	90.6 (81.6)
Green waste compost	At planting	68.8 (61.9)	84.4 (75.9)	100.0 (90.0)
P value		(<0.001)	(0.003)	(0.143)
LSD ($P = 0.05$), DF= 56[b]		(19.20)	(25.59)	(12.35)

[a]Values in parentheses are the angular transformations of the percentage emergence of eight replicates.
[b]LSD is the least significant difference at 5 % ($P = 0.05$), DF = degrees of freedom in ANOVA.

Table 7.4. Effect of timing of soil amendment application on stem height, and stem canker incidence and severity 4 (8 Jul) and 12 (2 Sep) weeks after planting.

		Stem height (mm)		Stem canker incidence		Stem canker severity	
Soil Amendment	Timing of application	8 Jul	2 Sep	8 Jul	2 Sep	8 Jul	2 Sep
Uninfested soil (control)	-	124.5[a]	134.8	0.0 (0.0)[b]	0.0 (0.0)	0.000[c]	0.000
Infested soil (control)	-	127.1	140.1	75.0 (67.5)[b]	41.5 (40.0)	0.906	1.025
Azoxystrobin	At planting	119.2	137.4	6.3 (5.6)	6.3 (5.6)	0.063	0.063
Onion compost	2 weeks before planting	141.3	139.6	41.7 (38.2)	57.5 (51.3)	1.031	0.946
Onion compost	At planting	132.8	134.9	61.6 (55.7)	43.8 (39.4)	1.536	1.083
Neem cake	2 weeks before planting	228.5	287.1	66.7 (60.7)	43.8 (39.4)	0.958	0.750
Neem cake	At planting	242.8	290.4	62.5 (56.2)	70.8 (63.1)	0.688	1.417
Green waste compost	2 weeks before planting	120.1	151.8	62.5 (58.2)	50.0 (45.0)	1.667	1.052
Green waste compost	At planting	117.3	151.9	49.6 (44.9)	47.1 (41.3)	1.275	1.988
P value		< 0.001	< 0.001	(< 0.001)	(< 0.001)	< 0.001	0.045
LSD ($P = 0.05$), DF= 56[d]		30.97	11.96	(30.86)	(29.33)	0.6760	1.193

[a]Values are the mean stem heights (mm) of eight replicates.
[b]Values in parentheses are the angular transformations of the percentage means of eight replicates.
[c]Values are the mean stem canker severity indices of eight replicates.
[d]LSD is the least significant difference at 5 % ($P = 0.05$), DF = degrees of freedom in ANOVA.

Table 7.5. Effect of soil amendment application on stolon length, and stolon canker incidence and severity 4 (8 Jul) and 12 (2 Sep) weeks after planting.

Soil Amendment	Timing of application	Stolon length		Stolon canker incidence		Stolon canker severity	
		8 Jul	2 Sep	8 Jul	2 Sep	8 Jul	2 Sep
Uninfested soil	-	20.49[a]	18.52	0.0 (0.0)[b]	0.0 (0.0)	0.000[c]	0.000
Infested soil	-	16.70	7.61	28.1 (24.4)	36.9 (35.6)	0.802	0.565
Azoxystrobin	At planting	12.21	15.33	0.0 (0.0)	0.0 (0.0)	0.000	0.000
Onion compost	2 weeks before planting	12.56	14.23	29.2 (26.9)	32.3 (28.7)	0.802	0.802
Onion compost	At planting	11.19	17.30	18.8 (16.9)	48.1 (45.8)	0.312	0.838
Neem cake	2 weeks before planting	37.88	40.28	26.7 (28.9)	37.3 (35.6)	0.414	0.808
Neem cake	At planting	57.44	33.65	28.0 (27.8)	39.7 (36.8)	0.525	0.781
Green waste compost	2 weeks before planting	11.54	11.61	21.7 (20.6)	34.4 (33.8)	0.594	0.427
Green waste compost	At planting	10.87	9.79	50.0 (45.0)	25.0 (22.5)	1.219	0.500
P value		< 0.001	< 0.001	(0.062)	(< 0.001)	0.109	0.002
LSD ($P = 0.05$), DF= 56[d]		11.771	8.532	(28.62)	(21.75)	0.8486	0.5001

[a]Values are the mean stolon lengths (mm) of eight replicates.
[b]Values in parentheses are the angular transformations of the percentage means of eight replicates.
[c]Values are the mean stolon canker severity indices of eight replicates.
[d]LSD is the least significant difference at 5 % ($P = 0.05$), DF = degrees of freedom in ANOVA.

The addition of soil amendments had a significant effect ($P = 0.05$) on tuber yield on 8 Jul, with the addition of green waste compost 2 weeks before planting resulting in reduced yields of tubers compared to the infested control (Table 7.6). At the three later harvests, plants grown in soil containing neem cake produced significantly higher yields, on average nearly three times greater than those observed in the infested control.

Prior to haulm destruction (2 Sep), black scurf incidence in all onion compost and neem cake treatments were significantly lower ($P = 0.05$) than the infested control (Table 7.7). Two and 4 weeks later (16 and 30 Sep), black scurf incidence was significantly lower ($P = 0.05$) where the soil had been treated with azoxystrobin or neem cake added at planting. A similar but non-significant effect was observed for black scurf severity with these treatments. Black scurf severity was generally greatest where the soil had been amended with onion or green waste compost (Table 7.7).

7.3.1.2 Efficacy of commercially available and novel products against soil-borne sclerotia of R. solani

Plant emergence was first recorded just over 2 weeks following planting (17 Feb). On all assessment dates, there was a significant reduction in the percentage of plants emerged when Microgran or neem cake were incorporated into the soil, compared to the infested control (Table 7.8).

Table 7.6. Effect of timing of soil amendment application on tuber yield 4 (8 Jul) and 12 (2 Sep) weeks after planting, and 2 (16 Sep) and 4 (30 Sep) weeks after haulm destruction.

Soil Amendment	Timing of application	Date of Harvest			
		8 Jul	2 Sep	16 Sep	30 Sep
Uninfested soil	-	45.2[a]	65.4	77.8	73.2
Infested soil	-	41.0	72.3	89.5	74.5
Azoxystrobin	At planting	28.5	59.8	77.1	76.3
Onion compost	2 weeks before planting	41.3	91.1	90.8	83.6
Onion compost	At planting	22.0	66.6	83.7	63.6
Neem cake	2 weeks before planting	33.9	190.7	185.1	197.0
Neem cake	At planting	55.3	174.7	197.4	179.9
Green waste compost	2 weeks before planting	20.4	62.8	54.9	36.6
Green waste compost	At planting	24.1	48.6	62.1	61.5
P value		0.014	< 0.001	< 0.001	< 0.001
LSD ($P = 0.05$), DF= 56[b]		20.54	33.55	43.31	36.95

[a]Values are the mean total tuber yields (g) of eight replicates.
[b]LSD is the least significant difference at 5 % ($P = 0.05$), DF = degrees of freedom in ANOVA.

Table 7.7. Effect of timing of soil amendment application on black scurf incidence and severity immediately prior to haulm destruction (2 Sep), and 2 (16 Sep) and 4 (30 Sep) weeks after haulm destruction.

		Black scurf incidence			Black scurf severity		
Soil Amendment	Timing of application	2 Sep	16 Sep	30 Sep	2 Sep	16 Sep	30 Sep
Uninfested soil	-	0.0 (0.0)[a]	0.0 (0.0)	9.4 (9.4)	0.00[b]	0.00	0.03
Infested soil	-	83.3 (74.3)	60.4 (53.2)	93.8 (84.4)	1.62	2.33	1.56
Azoxystrobin	At planting	0.0 (0.0)	6.2 (5.6)	12.5 (11.3)	0.00	0.03	0.07
Onion compost	2 weeks before planting	37.5 (33.8)	75.2 (67.6)	85.0 (75.4)	0.67	2.24	3.29
Onion compost	At planting	18.7 (16.9)	49.3 (44.6)	89.6 (80.0)	0.29	2.64	2.84
Neem cake	2 weeks before planting	37.9 (34.0)	57.4 (67.6)	76.8 (65.1)	0.75	0.68	1.56
Neem cake	At planting	8.7 (8.9)	14.1 (13.8)	44.8 (40.0)	0.23	0.12	0.29
Green waste compost	2 weeks before planting	65.4 (59.9)	75.0 (59.9)	84.4 (75.0)	2.57	3.66	1.66
Green waste compost	At planting	64.6 (57.5)	71.9 (63.8)	83.3 (74.3)	2.23	1.73	2.16
P value		(< 0.001)	(< 0.001)	(< 0.001)	0.016	0.250	0.002
LSD (*P* = 0.05), DF= 56[c]		(28.74)	(29.81)	(27.84)	1.699	3.281	1.751

[a]Values in parentheses are the angularly transformed percentage mean incidence of tubers with black scurf symptoms of eight replicates.
[b]Values are the mean black scurf severity indices observed of eight replicates.
[c] LSD is the least significant difference at 5 % (*P* = 0.05), DF = degrees of freedom in ANOVA.

Table 7.8. Effect of seed or soil treatment with commercially available or novel products on plant emergence.

Product	Assessment date		
	17 Feb	24 Feb	3 Mar
Uninfested control	46.9 (41.2)[a]	87.5 (76.9)	100.0 (90.0)
Infested control	34.4 (33.8)	53.1 (48.8)	93.8 (82.5)
Flutolanil	28.1 (31.9)	75.0 (65.6)	100.0 (90.0)
Azoxystrobin	37.5 (35.6)	68.8 (61.6)	100.0 (90.0)
Cultimide	21.9 (24.4)	37.5 (35.6)	78.1 (69.4)
Microgran	0.0 (0.0)	3.1 (3.7)	9.4 (11.3)
Biofungus instant	40.6 (37.5)	71.9 (61.9)	93.8 (82.5)
Bactolife	28.1 (28.1)	53.1 (46.9)	93.8 (82.5)
Neem Cake	12.5 (13.1)	25.0 (24.4)	40.6 (37.5)
Prestop	34.4 (33.8)	71.9 (61.9)	96.9 (86.2)
Garlic Barrier AG	37.5 (35.6)	68.8 (60.0)	100.0 (90.0)
P value	(<0.001)	(<0.001)	(<0.001)
LSD ($P = 0.05$), DF = 87[b]	(16.53)	(18.29)	(13.23)

[a]Values in parentheses are the angular transformations of the percentage emergence of seven replicates.
[b]LSD is the least significant difference at 5 % ($P = 0.05$), DF = degrees of freedom in ANOVA.

At the first harvest (28 Feb), stem height was significantly lower for plants grown in soil treated with Microgran whereas those grown in soil amended with neem cake were almost twice the height of the controls (Table 7.9). By 25 Apr, plants grown in soil treated with Cultimide and Microgran were significantly taller than the controls. Stem canker incidence was only affected by treatment on 28 Feb, where it was significantly lower on plants grown in soil treated with Microgran or the fungicides flutolanil and azoxystrobin. Stem canker severity was significantly reduced by both fungicides and Garlic Barrier AG on 28 Feb, but by 25 Apr only azoxystrobin still reduced stem canker severity significantly.

Stolon length was significantly greater on 28 Feb when plants had been grown in soil amended with neem cake, however, by 25 Apr there were no significant differences between treatments (Table 7.10). Microgran and Prestop significantly reduced stolon length, but only at the earliest assessment (28 Feb). On 28 Feb, stolon canker was significantly greater than the infested control where the soil had been amended with Biofungus instant, neem cake and Garlic Barrier AG. By 25 Apr, any differences between treatments were no longer statistically significant.

Table 7.9. Effect of seed or soil treatment with commercially available or novel products on stem height, and stem canker incidence and severity 4 (28 Feb) and 12 (25 Apr) weeks after planting.

	Stem height		Stem canker incidence		Stem canker severity	
Product	28 Feb	25 Apr	28 Feb	25 Apr	28 Feb	25 Apr
Uninfested control	123.8[a]	278.2	0.0 (0.0)[b]	0.0 (0.0)	0.000[c]	0.000
Infested control	112.5	293.6	48.1 (50.4)	45.8 (42.6)	1.050	0.938
Flutolanil	134.2	306.4	29.1 (13.5)	44.6 (41.8)	0.135	0.683
Azoxystrobin	136.7	267.4	16.3 (13.8)	18.8 (16.9)	0.163	0.250
Cultimide	139.2	366.6	53.1 (37.5)	68.8 (61.9)	0.656	0.875
Microgran	19.0	421.6	15.6 (0.0)	31.3 (28.1)	0.564	0.438
Biofungus instant	104.4	278.6	49.5 (54.2)	44.8 (40.0)	0.667	0.625
Bactolife	125.6	266.7	36.7 (47.3)	26.0 (25.0)	1.106	0.302
Neem Cake	221.3	279.8	55.2 (43.8)	66.7 (25.0)	0.542	1.33
Prestop	107.2	280.8	44.3 (34.4)	54.2 (49.4)	0.490	1.000
Garlic Barrier AG	139.9	315.8	58.3 (37.5)	79.2 (71.9)	0.375	1.5
P value	<0.001	<0.001	(0.008)	(<0.001)	0.002	<0.001
LSD ($P = 0.05$), DF = 87[d]	29.68	55.52	(34.34)	(31.31)	0.5971	0.6500

[a]Values are the mean stem heights (mm) of seven replicates.
[b]Values in parentheses are the angular transformations of the percentage means of seven replicates.
[c]Values are the mean stem canker indices of seven replicates.
[d]LSD is the least significant difference at 5 % ($P = 0.05$), DF = degrees of freedom in ANOVA.

Table 7.10. Effect of seed or soil treatment with commercially available or novel products on stolon length, and stolon canker incidence and severity 4 (28 Feb) and 12 (25 Apr) weeks after planting.

	Stolon length		Stolon canker incidence		Stolon canker severity	
Product	28 Feb	25 Apr	28 Feb	25 Apr	28 Feb	25 Apr
Uninfested control	18.5[a]	29.6	0.0 (0.0)[b]	0.0 (0.0)	0.000[c]	0.000
Infested control	23.8	21.9	1.2 (1.8)	17.9 (18.4)	0.144	0.398
Flutolanil	23.2	24.4	8.8 (10.7)	8.3 (8.8)	0.263	0.125
Azoxystrobinr	19.0	21.8	4.2 (4.4)	10.4 (11.9)	0.042	0.219
Cultimide	17.5	19.3	10.4 (10.0)	29.2 (26.9)	0.104	0.354
Microgran	0.0	20.7	0.0 (0.0)	0.0 (0.0)	0.000	0.000
Biofungus instant	15.9	18.0	21.6 (21.9)	22.9 (23.1)	0.287	0.463
Bactolife	26.6	28.6	19.2 (19.0)	16.2 (13.6)	0.503	0.425
Neem Cake	53.2	33.3	29.3 (32.3)	36.0 (34.8)	0.483	0.835
Prestop	12.9	26.6	6.3 (7.2)	25.4 (22.6)	0.144	0.481
Garlic Barrier AG	16.9	14.5	23.9 (23.4)	22.9 (21.3)	0.653	0.604
P value	<0.001	0.147	(0.004)	(0.010)	0.049	0.055
LSD ($P = 0.05$), DF = 87[d]	10.05	12.75	(17.91)	(20.86)	0.4739	0.5593

[a]Values are the mean stolon lengths (mm) of seven replicates.
[b]Values in parentheses are the angular transformations of the percentage means of seven replicates.
[c]Values are the mean stolon canker indices of seven replicates.
[d]LSD is the least significant difference at 5 % ($P = 0.05$), DF = degrees of freedom in ANOVA.

Tuber yields were significantly affected by several products tested (Table 7.11). Microgran and Cultimide consistently and significantly reduced tuber yield, with neem cake significantly increasing yield only on 9 May. The effect of Prestop on yield was inconsistent, where it reduced tuber yield on 9 May. No black scurf was observed at the first harvest on 28 Feb, and there were no significant differences between treatments until after haulm destruction on 25 Apr (Table 7.11). Two weeks following haulm destruction (9 May), all treatments except flutolanil, Biofungus instant and Bactolife gave significantly reduced incidences of black scurf than the infested control. Four weeks after haulm destruction (23 May), significantly better control was still obtained using azoxystrobin, Microgran, Prestop and Garlic Barrier AG.

There were no significant differences between treatments with regard to black scurf severity prior to haulm destruction (25 Apr) (Table 7.11). The azoxystrobin, Prestop, neem cake and Microgran treatments consistently and significantly reduced black scurf severity at the two later assessments (2 and 4 weeks after haulm destruction). Although Microgran appeared to reduce black scurf severity, it had a negative effect on tuber development, with significantly lower tuber yields than the infested control.

Table 7.11. Effect of seed or soil treatment with commercially available and novel products on tuber yield, and black scurf incidence and severity immediately prior to haulm destruction (25 Apr), 2 (9 May) and 4 (23 May) weeks after haulm destruction.

	Tuber yield			Black scurf incidence			Black scurf severity		
Product	25 Apr	9 May	23 May	25 Apr	9 May	23 May	25 Apr	9 May	23 May
Uninfested control	177.6[a]	201.7	182.3	0.0 (0.0)[b]	0.0 (0.0)	0.0 (0.0)	0.000[c]	0.000	0.000
Infested control	159.7	172.8	173.9	0.0 (0.0)	71.9 (63.8)	68.3 (61.6)	0.000	1.860	2.212
Flutolanil	155.7	174.1	177.6	0.0 (0.0)	50.6 (43.8)	33.3 (31.3)	0.000	1.465	0.580
Azoxystrobin	167.3	173.4	183.4	0.0 (0.0)	3.1 (3.8)	17.9 (16.4)	0.000	0.155	0.477
Cultimide	120.8	132.4	132.1	0.0 (0.0)	13.5 (11.9)	51.1 (45.4)	0.000	0.751	0.920
Microgran	34.4	53.7	71.1	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.000	0.000	0.000
Biofungus instant	160.6	166.7	168.6	6.2 (5.6)	65.0 (56.0)	58.8 (54.2)	0.034	0.630	1.000
Bactolife	132.3	165.2	158.7	9.8 (9.7)	58.3 (50.0)	81.4 (71.4)	0.238	1.451	1.921
Neem Cake	173.8	197.9	180.2	4.3 (6.1)	8.9 (8.9)	42.6 (40.5)	0.097	0.150	0.681
Prestop	161.7	151.1	177.7	0.0 (0.0)	14.6 (12.5)	33.6 (31.1)	0.000	0.300	0.520
Garlic Barrier AG	132.7	180.1	176.4	12.5 (11.2)	37.5 (33.8)	37.5 (32.0)	0.069	1.188	0.672
P value	<0.001	<0.001	<0.001	(0.460)	(<0.001)	(<0.001)	0.458	0.021	0.002
LSD ($P = 0.05$), DF = 87[d]	31.06	15.66	30.41	(12.42)	(26.35)	(23.89)	0.2094	1.2411	1.0851

[a]Values are the mean total tuber yields (g) of seven replicates.
[b]Values in parentheses are the angularly transformed percentage means of tubers with black scurf symptoms of seven replicates.
[c]Values are the mean black scurf severity indices observed of seven replicates.
[d]LSD is the least significant difference at 5 % ($P = 0.05$), DF = degrees of freedom in ANOVA.

7.3.2 Investigation into the biological control potential of fungi isolated from buried sclerotia

7.3.2.1 Growth of potential antagonists in dual culture with R. solani and the effects on mycelial growth and sclerotial biomass production

There were no differences between the two control treatments, therefore the percentage inhibition of mycelial growth was calculated using the control where *R. solani* was placed opposite a 3 mm plug of PDA or WA alone. The percentage inhibition of mycelial growth *in vitro* was greatest (between 32 – 34 %) when *R. solani* was grown opposite the known antagonist *V. biguttatum* on PDA (Table 7.12). All other fungi tested on PDA caused reductions in mycelial growth of between 8 – 26 %, depending on the isolate. There were no significant differences between the fungi with regards to percentage inhibition of mycelial growth when fungi were grown on WA.

Sclerotia were not produced when cultures were grown on WA, however, sclerotial biomass was significantly affected ($P = 0.05$) by growth in dual culture on PDA (Table 7.12). No sclerotia were observed when *R. solani* was grown in dual culture with *V. biguttatum* or *Mauginiella* sp., and reductions in sclerotial biomass of up to 99 % occurred when grown in dual culture with *Gliocladium* sp. or *C. rosea*. *Penicillium* sp. and *T. angustata* did not appear to have any influence on sclerotial biomass.

7.3.2.2 Production of antifungal volatiles by potential antagonists and effects on mycelial growth, sclerotial production and germination of R. solani

There were no significant differences between the two controls where exposure to antifungal volatiles was evaluated, therefore, the control where *R. solani* was grown opposite an empty plate was used to calculate the percentage inhibition of mycelial growth. Exposure to antifungal volatiles produced by *Gliocladium* sp., *C. rosea*

Table 7.12. Percentage inhibition of radial mycelial growth and sclerotium yield [total dry weight biomass (mg) per dish)] by *R. solani* grown in dual culture, and the response to antifungal volatiles produced by known and potential antagonists on PDA and WA at 25°C.

Known/potential antagonists	Dual culture				Antifungal volatiles			
	Percentage inhibition[a]		Sclerotial biomass[b]		Percentage inhibition[a]		Sclerotial biomass[b]	
	PDA	WA	PDA	WA	PDA	WA	PDA	WA
R. solani alone	-	-	40.0	-[c]	-	-	75.0	-[c]
V. biguttatum M73	28.8 (32.0)	17.7 (24.6)	0.0	-	7.7 (7.2)	10.8 (18.8)	0.0	-
V. biguttatum M92	33.2 (34.3)	17.0 (23.9)	0.0	-	0.0 (0.0)	12.2 (20.4)	0.0	-
Gliocladium sp.	12.3 (20.2)	19.6 (22.1)	0.4	-	37.5 (37.6))	42.2 (40.5)	0.0	-
C. rosea	4.8 (8.7)	8.3 (14.4)	2.9	-	23.5 (25.5)	45.7 (42.5)	63.6	-
Mauginiella sp.	13.0 (26.4)	6.9 (21.9)	0.0	-	0.6 (2.3)	24.6 (29.7)	27.5	-
Penicillium sp.	13.0 (20.3)	6.9 (13.2)	40.6	-	14.1 (21.0)	49.0 (44.4)	42.1	-
T. angustata	16.9 (20.9)	10.7 (16.4)	41.8	-	5.9 (11.9)	24.8 (29.9)	38.5	-
P value	(0.035)	(0.146)	<0.001	-	(<0.001)	(<0.001)	0.018	-
LSD ($P = 0.05$), DF = 45; 24[d]	(14.23)	(15.62)	15.71	-	(11.18)	(3.832)	54.1	-

[a]Values in parentheses are the angular transformations of percentage inhibition of mycelial growth calculated as described by Whipps (1987).
[b]Values are the mean dry weight biomass (mg per dish) of sclerotia of four replicate dishes.
[c]No sclerotial formation was observed.
[d]LSD is the least significant difference at 5 % ($P = 0.05$), DF = degrees of freedom in ANOVA – dual culture; antifungal volatiles.

and *Penicillium* sp. resulted in significantly greater ($P = 0.05$) percentage inhibition of mycelial growth of between 21 – 37 % compared to other fungi tested. *T. angustata*, *V. biguttatum* and *Mauginiella* sp. caused significantly lower ($P = 0.05$) percentage inhibition of mycelial growth, with reductions between 0 – 12 % than other fungi tested. On PDA, sclerotial biomass production was only significantly less ($P = 0.05$) than the control when *R. solani* was exposed to antifungal volatiles from *V. biguttatum*. When grown on WA, *Gliocladium* sp. *Penicillium* sp. and *C. rosea* all reduced mycelial growth (40 – 44 %) significantly more ($P = 0.05$) than *V. biguttatum*, *Mauginiella* sp. and *T. angustata* (18 – 30 %) when grown on WA (Table 7.12). No sclerotial production occurred on WA

Sclerotial germination was significantly affected by antifungal volatiles produced by several of the potential antagonists tested, with similar results observed on both PDA and WA (Table 7.13). Exposure of sclerotia to known and potential antagonists for 24 h resulted in significant reductions in sclerotial germination compared to the control, except for *V. biguttatum* isolate M92. On both PDA and WA, sclerotia germination was reduced by the presence of the other antagonists by between 22 – 44 %. After 72 h, antifungal volatiles produced by *Gliocladium* sp., *C. rosea*, *Penicillium* sp. and *T. angustata* all significantly reduced sclerotial germination compared to the control on PDA and WA. The greatest reduction in germination was observed when sclerotia were exposed to *Gliocladium* sp., with germination reduced by 58 % compared to the control on PDA.

7.3.2.3 Colonisation of sclerotia by potential antagonists of R. solani

In vitro colonisation of *R. solani* sclerotia by known and potential antagonists revealed that exposure to all test fungi, except *Penicillium* sp. and *T. angustata* resulted in a significant reduction ($P = 0.05$) in sclerotial germination compared to the uninoculated controls (Table 7.14). Exposure to the spores of both *V. biguttatum*

Table 7.13. Effect of antifungal volatiles produced by known and potential antagonists on sclerotial germination of *R. solani* at 25°C.

Known/potential antagonists	Time after exposure to potential antagonists			
	24 h		72 h	
	PDA	WA	PDA	WA
No antagonist	91.2 (65.5)[a]	87.5 (87.5)	100.0 (90.0)	87.5 (69.5)
V. biguttatum M73	73.8 (50.8)	70.0 (60.0)	87.5 (75.1)	80.0 (64.3)
V. biguttatum M92	90.0 (72.5)	83.8 (87.5)	92.5 (76.2)	80.0 (67.3)
Gliocladium sp.	38.7 (36.9)	42.5 (37.5)	40.0 (38.1)	47.5 (43.6)
C. rosea	53.8 (40.4)	43.8 (40.0)	65.0 (57.7)	47.5 (43.6)
Mauginiella sp.	61.2 (47.9)	63.8 (55.0)	67.5 (59.2)	72.5 (59.2)
Penicillium sp.	70.0 (48.1)	56.3 (52.5)	85.0 (67.5)	60.0 (50.8)
T. angustata	48.8 (38.4)	38.8 (25.0)	57.5 (49.8)	52.5 (46.4)
P value	(<0.001)	(<0.001)	(0.001)	(<0.001)
LSD ($P = 0.05$), DF = 24[b]	(13.57)	(15.73)	(21.37)	(12.62)

[a]Values in parentheses are the angular transformations of the mean percentage of sclerotial germination of five replicates

[b]LSD is the least significant difference at 5 % ($P = 0.05$), DF = degrees of freedom in ANOVA.

Table 7.14. Effect of inoculation of *R. solani* sclerotia with potential antagonists on sclerotial germination and colonisation of sclerotia by known and potential antagonists.

Inoculum	Percentage germination of sclerotia	Percentage colonisation of all sclerotia
No antagonist	85.0 (67.9)[a]	0.0 (0.0)[b]
V. biguttatum M73	22.5 (27.9)	52.5 (46.4)
V. biguttatum M92	27.5 (31.0)	52.5 (46.5)
Gliocladium sp.	55.0 (51.2)	15.0 (19.6)
C. rosea	57.5 (49.6)	17.5 (24.2)
Mauginiella sp.	30.0 (33.1)	40.0 (38.9)
Penicillium sp.	67.5 (59.5)	10.0 (15.9)
T. angustata	67.5 (59.4)	15.0 (19.0)
P value	(<0.001)	(<0.001)
LSD ($P = 0.05$), DF = 21[c]	(14.21)	(18.57)

[a]Values in parentheses are the angular transformations of the mean percentage of sclerotia germinated of five replicates.
[b]Values in parentheses are the angular transformations of the mean percentage of sclerotia colonised of five replicates.
[c]LSD is the least significant difference at 5 % ($P = 0.05$), DF = degrees of freedom in ANOVA.

isolates and *Mauginiella* sp. and caused the greatest reductions in sclerotial germination of between 42 – 49 %. Colonisation of sclerotia was significant for all antagonistic fungi, except *Penicillium* sp.. Both *V. biguttatum* isolates and *Mauginiella* sp. were also responsible for the greatest percentage colonisation of *R. solani* sclerotia, with between 40 – 50 % of sclerotia colonised by these fungi.

7.3.3 Pot-based bioassay to determine the effect of potential antagonists in soil on disease caused by sclerotial soil-borne inoculum of *R. solani*

Plant emergence was initially observed on 22 Jul (2 weeks following planting), and soil application with known and potential antagonistic fungi was not found to have statistically significant effects on the emergence of plants on this, or any of the subsequent emergence assessments (Table 7.15). There were no significant differences between treatments with regards to stem and stolon canker incidence and severity (Table 7.16). Tuber yield was not significantly affected by the application of fungal spores to the soil (Table 7.17). None of the fungi tested, including *V. biguttatum*, was found to significantly reduce black scurf incidence and severity on progeny tubers (Table 7.18). Six weeks following haulm destruction, Azoxystrobin was the only soil treatment that had significantly reduced black scurf incidence compared to infested control (Table 7.18).

Table 7.15. Effect of application of potential antagonists to soil on plant emergence.

Known/potential antagonists	Date of assessment		
	22 Jul	29 Jul	5 Aug
Uninfested growth medium	14.3 (12.9)[a]	78.6 (70.7)	82.1 (73.9)
Infested growth medium	10.7 (9.6)	78.6 (70.7)	85.7 (77.1)
Azoxystrobin	3.6 (3.2)	75.0 (67.5)	71.4 (64.3)
V. biguttatum M73	10.7 (9.6)	85.7 (77.1)	92.9 (83.6)
Gliocladium sp.	17.9 (16.1)	57.1 (51.4)	60.7 (54.6)
C. rosea	21.4 (19.3)	89.3 (80.4)	92.9 (83.6)
Mauginiella sp.	25.0 (22.5)	75.0 (67.5)	78.6 (70.7)
T. angustata	14.3 (12.9)	64.3 (57.9)	78.6 (70.7)
P value	(0.453)	(0.316)	(0.237)
LSD ($P = 0.05$), DF = 21[b]	(17.39)	(24.54)	(23.57)

[a]Values in parentheses are the angular transformations of the percentage emergence of eight replicates.
[b]LSD is the least significant difference at 5 % ($P = 0.05$), DF = degrees of freedom in ANOVA.

Table 7.16. Effect of application of potential antagonists to soil on stem and stolon canker incidence and severity 12 weeks after planting (21 Sep).

	Stem disease		Stolon disease	
Known/potential antagonists	Stem canker incidence	Stem canker severity	Stolon canker incidence	Stolon canker severity
Uninfested growth medium	19.0 (17.9)[a]	0.262[b]	18.9 (21.9)[a]	0.195[c]
Infested growth medium	56.0 (48.5)	0.810	35.7 (30.0)	0.990
Azoxystrobin	5.7 (5.6)	0.086	19.6 (22.4)	0.292
V. biguttatum M73	17.1 (16.7)	0.336	38.1 (33.5)	0.774
Gliocladium sp.	17.9 (17.1)	0.357	25.2 (23.7)	0.415
C. rosea	4.8 (5.0)	0.048	33.8 (23.7))	0.755
Mauginiella sp.	35.7 (32.1)	0.571	26.1 (33.2)	0.567
T. angustata	17.9 (17.1)	0.036	20.2 (28.1)	0.392
P value	(0.122)	0.077	(0.895)	0.128
LSD ($P = 0.05$), DF = 42[d]	(30.72)	0.5478	(22.04)	0.5925

[a]Values in parentheses are the angular transformations of the percentage means of eight replicates.
[b]Values are the mean stem canker indices of eight replicates.
[c]Values are the mean stolon canker indices of eight replicates.
[d]LSD is the least significant difference at 5 % ($P = 0.05$), DF = degrees of freedom in ANOVA.

Table 7.17. Effect of application of potential antagonists to soil on tuber yield (g per pot) immediately prior to haulm destruction (21 Sep) and 1 (28 Sep), 3 (12 Oct) and 6 (2 Nov) weeks after haulm destruction.

Known/potential antagonists	Date of harvest			
	21 Sept	28 Sept	12 Oct	2 Nov
Uninfested growth medium	135.6[a]	140.3	148.8	145.0
Infested growth medium	136.7	142.4	159.3	149.3
Azoxystrobin	136.5	147.5	140.1	146.8
V. biguttatum M73	139.3	147.1	146.3	139.7
Gliocladium sp.	145.5	139.5	134.0	152.7
C. rosea	147.7	153.3	151.8	145.0
Mauginella sp.	142.1	140.9	160.5	139.8
T. angustata	147.6	148.7	149.5	142.5
P value	0.846	0.883	0.360	0.938
LSD ($P = 0.05$), DF = 42[b]	20.93	21.59	23.94	22.55

[a]Values are the mean tuber yields (g) of seven replicates.
[b]LSD is the least significant difference at 5 % ($P = 0.05$), DF = degrees of freedom in ANOVA.

Table 7.18. Effect of application of potential antagonists to soil on black scurf incidence and severity immediately prior to haulm destruction (21 Sep) and 1 (28 Sep), 3 (12 (Oct) and 6 (2 Nov) weeks after haulm destruction.

	Black scurf incidence				Black scurf severity			
Known/potential antagonists	21 Sept	28 Sept	12 Oct	2 Nov	21 Sept	28 Sept	12 Oct	2 Nov
Uninfested growth medium	0.0 (0.0)[a]	7.1 (6.4)	0.0 (0.0)	0.0 (0.0)	0.000[b]	0.040	0.000	0.000
Infested growth medium	36.1 (33.0)	66.7 (59.2)	50.0 (45.0)	45.7 (42.4)	1.240	3.091	1.822	1.688
Azoxystrobin	9.5 (10.1)	10.2 (8.2)	23.2 (20.3)	13.6 (12.4)	0.291	0.399	0.177	0.330
V. biguttatum M73	47.6 (41.4))	26.2 (24.3)	18.0 (17.3)	58.3 (52.2)	1.252	0.822	0.371	2.308
Gliocladium sp.	28.6 (25.7)	46.4 (42.9)	45.7 (40.3)	56.1 (48.6)	0.698	2.700	3.754	1.384
C. rosea	2.9 (3.8)	59.9 (53.3)	48.8 (46.9)	60.7 (55.7)	0.101	2.231	1.483	2.311
Mauginiella sp.	23.8 (20.7)	40.8 (35.4)	31.9 (29.9)	35.0 (33.8)	1.150	2.133	2.066	0.762
T. angustata	25.0 (21.4)	28.6 (25.7)	36.7 (32.9)	42.3 (38.0)	1.103	1.343	1.148	1.458
P value	(0.312)	(0.068)	(0.122)	(0.002)	0.567	0.309	0.545	0.208
LSD ($P = 0.05$), DF = 42[c]	(36.22)	(38.53)	(34.04)	(28.31)	1.6672	2.8692	3.8091	2.019

[a]Values in parentheses are the angularly transformed percentage means of tubers with black scurf symptoms of eight replicates.
[b]Values are the mean black scurf severity indices of eight replicates.
[c]LSD is the least significant difference at 5 % ($P = 0.05$), DF = degrees of freedom in ANOVA.

7.4 Discussion

Organic amendments have been used for centuries to alter soil ecology, and their incorporation is known to have a positive impact on crop productivity and health (Bailey & Lazarovits, 2003). In this study, it was clear that soil amendments can have a range of effects on plant development and disease suppression. Rapid plant emergence was observed when plants were grown in green waste compost compared to onion compost and neem cake. Although rapid emergence is generally accepted as a factor in reducing the presence of *R. solani* stem lesions, there was no reduction in stem canker incidence and severity with the addition of green waste compost. Composted onion waste has been evaluated recently and found to reduce the viability of sclerotia produced by *S. cepivorum*, responsible for causing *Allium* white rot (Coventry *et al.*, 2002). However, no suppressive effect was found on soil-borne sclerotia of *R. solani* in this study. Other research on the suppression of *R. solani* disease has demonstrated that mycophagous soil organism populations were increased through soil amendment incorporation and were responsible for reductions in stem canker severity caused by *R. solani* on potato (Scholte & Lootsma, 1998; Lootsma & Scholte, 1998).

It is apparent from the results in this study that neem cake application had a significant effect on plant development and black scurf control when it was applied at planting compared with application 2 weeks prior to planting. Neem cake had a significant effect on the overall development of the plant in one of the two trials, with increased stem heights and higher tuber yields than control plants grown in uninfested growth medium. The addition of neem cake to growth media was previously found to increase the biomass of shoot and roots in mung bean (*Vigna radiata*), as well increasing the number of pods produced per plant (Tiyagi & Alam, 1995). Although neem cake was not as effective at controlling *R. solani* disease as the chemical treatment, it had a suppressive effect on black scurf development in this study, with

significant reductions observed when it was applied to the soil at planting. An earlier study into the effect of neem cake on the incidence of black scurf on potato tubers showed neem cake reduced the incidence of diseased tubers, as well as disease severity (Singh, 1968). Treatment of soil with neem cake prior to growing a mung bean crop was found to reduce the occurrence of *R. solani* in the rhizosphere (Tiyagi & Alam, 1995). Although none of the composted wastes in this study had a significant effect on disease control, previous work on other plant species susceptible to *R. solani* has shown that other composted wastes can reduce the incidence and severity of *R. solani* infection. For example, the use of composted hardwood bark in container media was found to reduce the incidence of *R. solani* damping-off in celosia (*Celosia argenta*) and radish (*Raphanus savitus*) (Nelson & Hoitink, 1982).

None of the commercially available or novel treatments tested in this study were as effective at controlling stem canker or black scurf formation as the currently available fungicide products (azoxystrobin and flutolanil). Commercial products containing *G. catenulatum* (Prestop) and *Trichoderma* sp. (Biofungus Instant), as well as Garlic Barrier AG were the most effective products tested at reducing black scurf severity on progeny tubers. Both *Gliocladium* sp. and *Trichoderma* sp. are known antagonists of *R. solani* on potato (Jager *et al.*, 1979; Velvis & Jager, 1983; van den Boogert & Jager, 1984) and have been shown previously to produce a range of cell-wall degrading enzymes and antibiotics *in vitro* (Worasatit *et al.*, 1994; Howell & Stipanovic, 1995). *G. catenlulatum* has been demonstrated previously to sporulate and form appressorium-like structures on *R. solani* AG 4 hyphae *in vitro* (McQuilken *et al.*, 2001). Garlic Barrier AG did not reduce stem canker incidence and severity, although black scurf severity was reduced. Previous ultrastructural studies on the effect of garlic extract during mycelial growth of *R. solani in vitro* have shown that the mycelium appear to collapse inward and there are increases in the thickening of fungal cell walls

(Bianchi *et al.*, 1997). Whether the reduction in black scurf severity was a direct result of a fungicidal effect of the garlic extract on *R. solani* in this study is currently unknown and deserves further study.

In this study, *V. biguttatum* reduced mycelial growth rates and prevented sclerotial production both in dual culture and when *R. solani* was exposed to antifungal volatiles. *V. biguttatum* has been extensively studied, and is known to be a biotrophic antagonist of *R. solani* on potato (van den Boogert & Deacon, 1994). *V. biguttatum* has been found previously to excrete chitinase, β-1,3-glucanase and protease *in vitro*, which may have a role in the cell wall dissolution and penetration of *R. solani* (McQuilken & Gemmell, 2004). Growth of *R. solani* in dual culture with *V. biguttatum* in the present study resulted in reduced mycelial growth and sclerotial formation by *R. solani*. Sclerotial production was inhibited over the entire plate (9 cm diam) of *R. solani* and not just along the line of contact between the two colonies. The inhibition of sclerotial production by *V. biguttatum* grown in dual culture with *R. solani* has been demonstrated previously, with suppression shown to be mediated via the hyphae of *R. solani* (van den Boogert & Deacon, 1994).

Sclerotia production by *R. solani* is known to occur in response to depletion of nutrients and sclerotial development requires the remobilisation of nutrients in the mycelium (Christias & Lockwood, 1973). It has been suggested that *V. biguttatum* may act as a nutrient sink via contact with *R. solani* mycelium, which is sufficient to divert nutrients away from sclerotia production, and not via the production of a suppresive factor that is distributed throughout the mycelium (van den Boogert & Deacon, 1994). In the current study, sclerotial production was also inhibited in response to antifungal volatiles, where no colony interaction occurred. The inhibition of mycelial growth of *R. solani* in response to hydroxymethyl-phenols produced by *V. biguttatum* has been demonstrated previously (Morris *et al.*, 1995). However, it is unknown whether *V.*

biguttatum produces a specific volatile toxic metabolite responsible for inhibiting mycelial growth and/or sclerotial production.

Tests on antagonists directly isolated from buried sclerotia of *R. solani* (Chapter 6), revealed *Gliocladium* sp. reduced sclerotial production almost completely, both in dual culture and in response to antifungal volatiles, whereas *C. rosea* was only effective when grown in dual culture. In contrast, a previous *in vitro* study found *C. rosea* only affected sclerotial production along the line of contact between the antagonist and *R. solani*, and did not significantly reduce sclerotial production (van den Boogert & Deacon, 1994). Previous work has demonstrated that *C. rosea* can cause the collapse of fungal cells and produce water-diffusible inhibitors of mycelial growth (Deacon & Berry, 1992). A recent study has shown *C. rosea* to grow around the hyphae of *R. solani*, but not penetrate and grow intracellularly (Xue, 2003). *C. rosea* has been shown to infect conidia through direct penetration of hyphal tips when grown in culture with *Botrytis cinerea*, with evidence of cytoplasmic degradation and presence of *C. rosea* within the hyphal cells (Li *et al.*, 2002). Whether any of these factors is responsible for the inhibition of sclerotial production of *R. solani in vitro* remains unknown and warrants further investigation.

This is the first report of *T. angustata* and a *Mauginiella* sp. as potential antagonists of *R. solani* from potato. *Mauginiella* spp. reduced sclerotial formation when *R. solani* was exposed to antifungal volatiles *in vitro*, and prevented sclerotial formation *in vitro* when grown in dual culture with *R. solani*. Exposure to antifungal volatiles of *T. angustata* resulted in reductions in sclerotial production *in vitro*. *T. angustata* has previously been isolated from non-symptomatic grape berries and grape vines (Dugan *et al.*, 2002; Sergeeva *et al.*, 2005), whereas *Mauginiella* spp. have been studied primarily for the production of laccase (Palonen *et al.*, 2003). Neither of these fungi has been evaluated as a potential antagonist previously and, consequently,

interactions with the mycelium of *R. solani* and the production of any mycotoxins or other metabolites are currently unknown.

Significant reductions in sclerotial germination were found after exposure to antifungal volatiles produced by *Gliocladium* sp., *C. rosea*, *Mauginiella* sp. and *T. angustata*. Sclerotial germination was not affected by antifungal volatiles produced by *V. biguttatum*, however, inoculation of sclerotia with *V. biguttatum* spores and *Mauginiella* sp.conidia resulted in significant colonisation of sclerotia and reductions in sclerotial germination. Reductions in the viability of *R. solani* sclerotia in response to inoculation of sclerotia with conidia of *V. biguttatum* has been demonstrated previously, where inoculated sclerotia were six times less likely to undergo germination than non-inoculated sclerotia (Jager & Velvis, 1988).

The mechanism by which *V. biguttatum* inhibits sclerotial germination is unclear. *V. biguttatum* hyphae have been found previously to only penetrate *R. solani* sclerotia intercellularly (Jager & Velvis, 1988). Therefore it was concluded that sclerotial inactivation was a result of antifungals produced by *V. biguttatum*, and not via direct penetration of the monoloid cells of the sclerotium (Jager & Velvis, 1988). Whether antifungal hydroxymethyl-phenols (Morris *et al.*, 1995) have any role in sclerotial germination by *V. biguttatum* is unknown and warrants futher investigation. Previous studies on the sclerotia of *R. solani* have found two antibiotics produced by *Trichoderma* (*Gliocladium*) *virens*, gliotoxin and viridin, to prevent sclerotial germination (Aluko & Hering, 1970). *Microsphaeropsis* sp. spores used to inoculate a culture of *R. solani* were found to germinate, penetrate *R. solani* hyphae and produce fungitoxic metabolites, which reduced sclerotial viability significantly *in vitro* (Carisse *et al.*, 2001).

Although all known and potential antagonists tested had a detrimental effect on one growth parameter, or a combination, including mycelial growth, sclerotial

production and germination *in vitro*, none exhibited successful control of *R. solani* disease on stems, stolons and tubers in a pot bioassay when applied as a soil treatment at planting. Previous work on *V. biguttatum* found it to be successful in reducing the incidence and severity of stem canker and sclerotia formation on progeny tubers when applied as a seed treatment, which was later attributed, in part, to its transport through the soil on growing stolons, stems and roots (Jager & Velvis, 1984; Jager & Velvis, 1986; van den Boogert, 1989). Sandy soils have been shown to reduce the effectiveness of *V. biguttatum* in controlling *R. solani* disease, which was attributed to higher inoculum densities of the pathogen and the presence of wild *V. biguttatum* strains (Jager & Velvis, 1985). It is possible that the growth medium used in the bioassay (John Innes No. 3; 17 % sand) may have had an influence on the efficacy of *V. biguttatum*, and that of the other antagonists tested in this study. Futher research into the nutrient requirements of these fungi would determine their specific requirements for growth in soil, and any effect these requirements may have on their ability as fungal antagonists.

Although stem canker and black scurf formation were not effectively controlled by any of the soil amendments or fungal antagonists in this study, several demonstrated the potential for control of *R. solani in vitro*. As very little is known about several of the potential antagonistic fungi investigated in this study (e.g. *Mauginiella* sp. and *T. angustata*) further investigation into the precise suppressive mechanisms of these fungi would be required to evaluate any benefits they may offer as part of a disease management strategy. It has long been established that antagonistic fungi can be less effective when compared with existing chemical control measures, and present difficulties with regards to production, storage and application methods (Whipps, 1997). Recent studies on the efficacy of potential biocontrol organisms for the control of *R. solani* on potato have demonstrated that combinations of antagonists may provide improved disease control as opposed to the application of individual microorganisms

(Brewer & Larkin, 2005). Further research will be required to determine whether the soil amendments and potential antagonistic fungi investigated in this study could have a future as part of an integrated management strategy for the control of *R. solani* on potato.

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

8.1 General discussion and conclusions

In the UK in 2004, over 5.5 million tonnes of potatoes were produced with a market value of almost £482 million (Anon, 2005b). *R. solani* has long been established as the casual agent of stem and stolon canker, and black scurf on potato tubers (Banville *et al.*, 1996). Previously in 2003, 70 and 85 % of seed tubers in the UK were infected with *R. solani* and the resultant reduction in seed quality was estimated to have cost growers between £50 and £75 million (Anon, 2003). The prevention and control of *R. solani* on potato therefore has serious financial implications for the potato industry. With no potato cultivars resistant to *R. solani* infection, current control measures primarily involve the use of long rotations, treatment of seed prior to/ at planting with fungicides, coupled with the use of *R. solani*-free seed (Banville *et al.*, 1996; Kataria & Gisi, 1996). Fungicidal treatment of seed cannot prevent *R. solani* infection completely, as inoculum is also present in the soil (Tsror & Peretz-Alon, 2005). Comparisons of disease symptoms caused by both seed and soil-borne inoculum have demonstrated that both sources can contribute significantly to disease development on potato plants and progeny tubers (Tsror & Peretz-Alon, 2005). Although *R. solani* on potato has been investigated since its discovery, many aspects of this plant pathogen remain unknown.

The significance of sclerotia on progeny tubers (black scurf) in reducing tuber quality and as a source of inoculum when infected seed potatoes were planted has long been established (Gudmestad *et al.*, 1979; Hide *et al.*, 1985), however, relatively little was known about nutritional and environmental factors affecting sclerotial production. This study was the first to investigate the effect of environmental and nutritional factors on mycelial growth and sclerotial biomass production of different AGs from potato *in vitro*. Although these experiments did not simulate the environment in which they would occur, they provide an insight into factors which may affect the pathogen under field conditions. Previous *in vitro* studies on sclerotial production by *R. solani* from

potato used arbitrary categories (poor, moderate and high) to define sclerotial production (Allington, 1936; Sanford, 1956; Townsend, 1957) whereas, in this study, sclerotial biomass was used here as a quantitative indicator of sclerotial production.

This study demonstrated differences in sclerotial production both within and between AGs 2-1 and 3, suggesting that within *R. solani* there are many ecotypes with variability in their response to environmental and nutritional factors. Previous global studies on the population distribution of the different AGs of *R. solani* have shown that most isolates from potato belong to AG 3 (Carling & Leiner, 1986; Bandy & Leach, 1988; Bains & Bisht, 1995). Similarly, a recent investigation into the population structure of *R. solani* infecting potato plants in the UK revealed that AG 3 accounted for 92% of isolates retrieved from sampled plants, with 7 and <1% belonging to AG 2 and AG 5, respectively (Woodhall, 2004). In this study, isolates belonging to AG 3 produced significantly greater sclerotial biomass in all experiments investigating environmental and nutritional factors, than isolates belonging to AG 2-1. This ability of isolates belonging to AG 3 to produce greater sclerotial biomass in general, and over a wider range of nutritional and environmental conditions may be a contributing factor in the dominance of this AG over others in causing disease on potato. This merits further investigation.

The range of temperatures (10 to 30°C), pH (4 – 8) and osmotic (–0.4 to –4.0 MPa) and matric potentials (–0.4 to –2.0 MPa) *in vitro* and soil water potential (–0.5 to –6.3 MPa) allowing mycelial growth (Chapter 3) were similar to those permitting sclerotial germination (Chapter 4). This suggests that sclerotial germination will only occur when conditions are suitable for mycelial growth, however, the preseice mechanism by which this occurs is unknown and warrants further investigation. Also, the range of water potentials allowing the mycelial growth and germination of *R. solani* has important practical implications as the majority of agricultural soils are maintained

naturally or artificially by irrigation at water potentials higher than the permanent wilting point of mesophytic higher plants, approximately – 1.5 MPa (Slayter, 1967). It is clear that mycelial growth and sclerotial germination of *R. solani* can occur at water potentials in soil far lower than –1.5 MPa. Therefore this is probably one of many factors involved in its success as a plant pathogen. Further work is required to establish the effect of environmental and nutritional factors on sclerotial production on progeny tubers and sclerotial germination in field-based studies.

Some fungicides available to control *R. solani*, such as those containing pencycuron, have been specifically developed to target AG 3 and have proven effective against other AGs, including AG 2-1, but not AG 5 (Kataria & Gisi, 1996; Woodhall & Jenkinson, 2002). The use of AG specific fungicides may to reduce populations of AG 3, but may indirectly increase populations of other AGs with the potential to be as pathogenic and damaging to potato crops. Therefore, research into the pathogenicity of other AGs is necessary to determine their potential for causing disease on potato. A virulence bioassay using isolates of AG 2-1, 3 and 5 (Chapter 5) showed that AG 2-1 caused very few lesions on potato stems, with AG 5 reducing emergence and causing stem canker symptoms similar in severity to those caused by AG 3. In this study, the virulence of only one isolate of AG 2-1 and one of AG 5 were tested. It is probable that a similar investigation into other isolates from these AGs would vary in virulence. For example, a previous study showed that two AG 2-1 isolates from potato differed in virulence, with one causing extensive stem lesions comparable to those caused by AG 3, and the other resulting in limited disease symptoms (Petkowski & de Boer, 2001). This study has demonstrated the potential for AG 5 isolates to reduce emergence and result in severe stem canker comparable to that caused by AG 3. The bioassay was, however, on a relatively small scale and, therefore, further field trials would be useful to evaluate the full extent to which AG 5 infects developing plants.

All AG 2-1, 3 and 5 isolates tested in this study produced a range of cell wall degrading enzymes including cutinase, pectin lyase, polygalacturonase, endo-1,4-β-glucanase and β-glucosidase (Chapter 5). The results suggest that these enzymes may play a previously unforseen role during the infection process of potato by *R. solani* in dissolving and penetrating the sell walls of potato. However, further experimental work using immunocytochemistry would confirm their location and role, if any, in the infection of potato.

A significant percentage of sclerotia were found to germinate after 18 months burial in field soil, regardless of soil type or burial depth (Chapter 6). The contribution of soil-borne sclerotia in causing disease was previously unknown and this study has shown, in pots trials, that sclerotial soil-borne inoculum can contribute significantly to disease development on plants and progeny tubers (Chapter 6). High inoculum densities of soil-borne sclerotia were responsible for a higher incidence and severity of stem and stolon canker, as well as black scurf incidence and severity compared to low inoculum densities. Further investigation inoculating field soil with high and low numbers of black scurf infected seed pieces as soil-borne inoculum (Chapter 6) showed increased black scurf incidence and severity on progeny tubers when the number of infected seed pieces was higher.

A previous study on the presence of volunteer potatoes in UK crops found 66 % of fields surveyed contained groundkeepers where were potatoes had been cropped previously (Davies *et al.*, 1999). The presence of seed tuber pieces infected with *R. solani* sclerotia was sufficient to increase the levels of *R. solani* detectable in the soil and cause a reduction in the quality of progeny tubers, increasing the potential for *R. solani* inoculum to remain in the soil for prolonged periods. The survival duration of *R. solani* sclerotia, coupled with the effect of sclerotia-covered seed pieces on soil-borne inoculum, may lead to unharvested sclerotia-covered tubers having the potential to

contribute to the soil-borne inoculum and infect future crops. As no single cultural or chemical treatment can eradicate groundkeepers, optimising the pick-up of potatoes during harvest, combined with sufficient rotations and herbicide treatment as part of an integrated control programme would reduce their numbers (Davies *et al.*, 1999).

The increase in black scurf formation on progeny tubers following haulm destruction is well documented (Gudmestad *et al.*, 1979; Chand & Logan, 1984; Chand & Logan, 1986; Dijst *et al.*, 1986). Previous work had suggested that sclerotial formation on progeny tubers is accelerated in response to changes in the exudation of volatile exudates from tubers and underground potato plant parts after haulm destruction (Dijst, 1990). This study investigated the effect of cutting the haulm on plants grown alongside those with the haulm remaining intact. Increased black scurf incidence and severity on progeny tubers occurred in plots containing plants with their haulms removed compared to control plots, but this effect was only observed in 2004, and not when the trial was repeated in 2005. The warmer, wetter environmental conditions in 2004 may have hindered black scurf development, making the effects of haulm destruction more apparent, as opposed to the more favourable cooler, drier conditions in 2005. Whether volatile tuber exudates are responsible for sclerotial development in the field following haulm destruction remains unknown, but whatever their role, these trials suggest environmental conditions may also affect black scurf formation following haulm destruction and this deserves further investigation.

This study has demonstrated, for the first time, the significance of soil-borne sclerotia of *R. solani* in causing and contributing to disease development on potato. As soil-borne sclerotia have been shown to contribute significantly to disease development, reducing sclerotia formation on progeny tubers and in soil, as well as controlling inoculum already in the soil is necessary. Current methods to control seed and soil-borne *R. solani* include fungicide application, the use of black scurf-free seed and long

rotations between crops. However, with the incidence of high levels of toxic chemicals in plants and the environment, the development of resistance in pathogen populations to fungicides and changes in legislation resulting in the withdrawal of chemicals, alternative means of control are required (Spadaro & Gullino, 2005). In this study, commercial products containing *G. catenulatum* (Prestop) and *Trichoderma* sp. (Biofungus Instant), and Garlic Barrier AG (garlic extract), as well as neem cake, were the most effective at reducing black scurf incidence and severity on progeny tubers, but were not as effective as the commercially available fungicide azoxystrobin. These novel products could possibly be integrated with existing crop protection practices or used in low fungicide input systems to control the development of *R. solani* on progeny tubers. Further trials are recommended to investigate such an approach.

Potential and known fungal antagonists isolated from recovered sclerotia (Chapters 6 and 7), including *Gliocladium* spp. and *Gliocladium roseum*, caused significant reductions in mycelial growth, sclerotial production and germination *in vitro*, but did not control soil-borne sclerotia in glasshouse trials when applied as individual spore suspensions. This is commonly found with potential biological control agents, as they often perform successfully in laboratory studies, with variable success in the field (Clarkson & Whipps, 2002). Little is known about the environmental and nutritional factors affecting the mycelial growth, sporulation and survival of the fungi with biological control potential isolated in this study. It is possible that application of a spore suspension containing the correct nutrients for sporulation and mycelial growth would have increased efficacy of these potential biological control fungi, therefore, further research into the precise environmental and nutritional requirements for each species would be useful. Successful control of *R. solani* using biological control organisms has been demonstrated recently using a combination of *Bacillus subtilis* and *Trichoderma* (*Gliocladium*) *virens* (Brewer & Larkin, 2005). Previous studies on the

use of biological control agents on potato found that reducing the recommended fungicide dosage (pencycuron) and applying a spore suspension of *V. biguttatum* could reduce black scurf formation on progeny tubers (Jager & Velvis, 1986; Jager *et al.*, 1991). Application of biological control agents in conjunction with reduced fungicide application rates offers a more environmentally sound approach to disease control. However, many other factors have to be considered, including the effect of fungicides on the antagonist. For example, *R. solani* specific fungicides (pencycuron and flutolanil) were found not to affect the mycelial growth of *V. biguttatum in vitro*, whereas broad-spectrum fungicides (azoxystrobin) were fungitoxic to the antagonist (van den Boogert & Luttikholt, 2004).

This study has highlighted the significance of many factors in the development of *R. solani in vitro* and under field conditions which were unknown previously. Continued research into this pathogen is necessary as the presence of stem and stolon canker, and sclerotia on progeny tubers can severely affect plant development, with reductions in tuber quality caused by misshapen and sclerotia-covered tubers affecting the price farmers can obtain for their crop. Also, recent research has suggested *R. solani* infected potato plants are more likely to become infected with the potato cyst nematode *Globodera rostochiensis* (Back *et al.*, 2006), suggesting that *R. solani* may indirectly increase crop susceptibility to other diseases. Continued research into aspects of the biology, epidemiology and control of *R. solani* will increase the knowledge of this soil-borne plant pathogen, and contribute to the development of new approaches to control this plant pathogen in soil.

APPENDICES

APPENDIX A

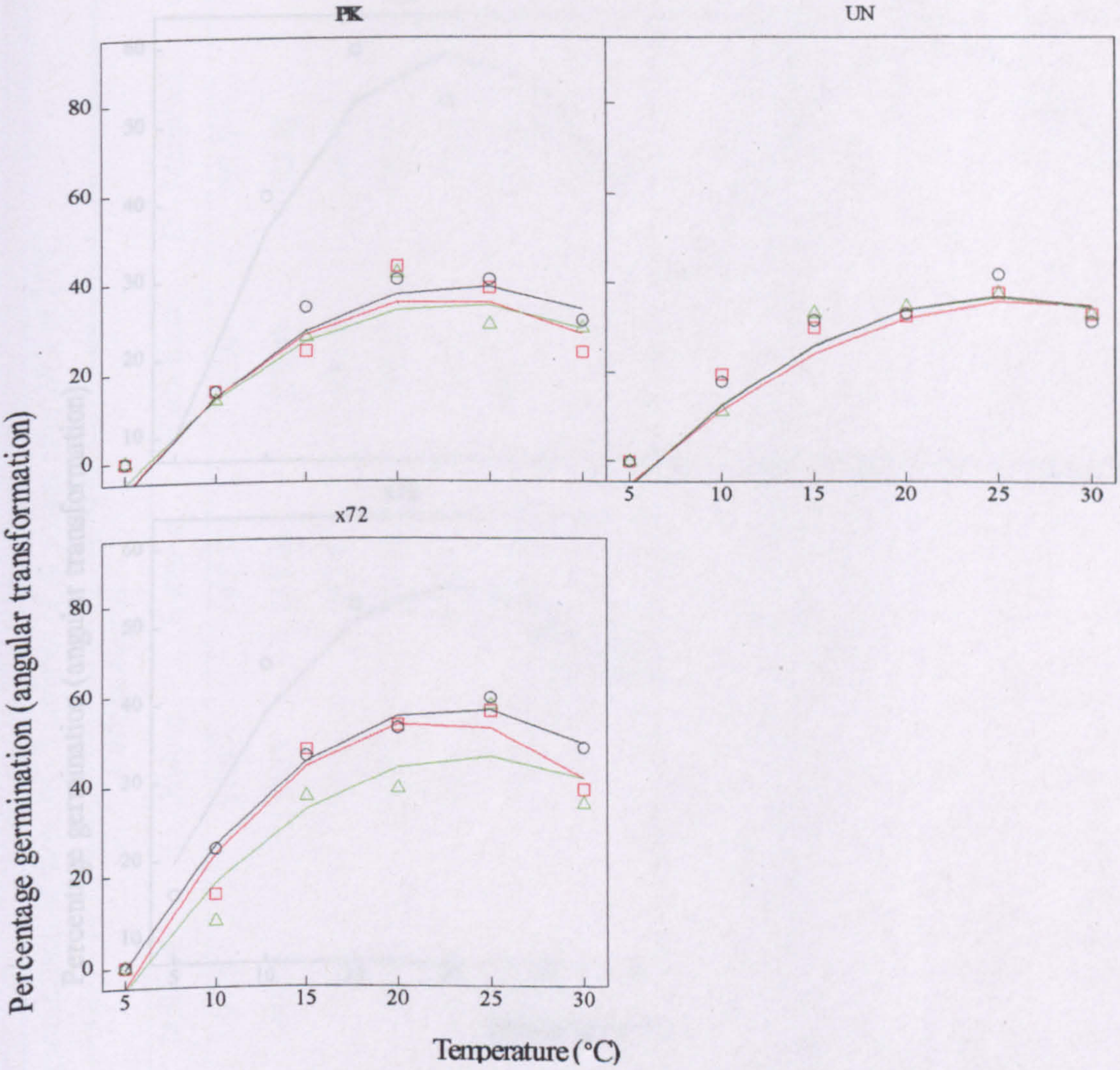


Figure A1. Multiple linear regression analysis of the effect of temperature & media on germination (%) of *R. solani* at 25°C after 24 h. Points represent angularly transformed mean data values & lines show the fitted model for each media tested (green = WA, red = PDA, black = MYA). $R^2 = 71.4$, $P = 0.884$ (DF = 215).

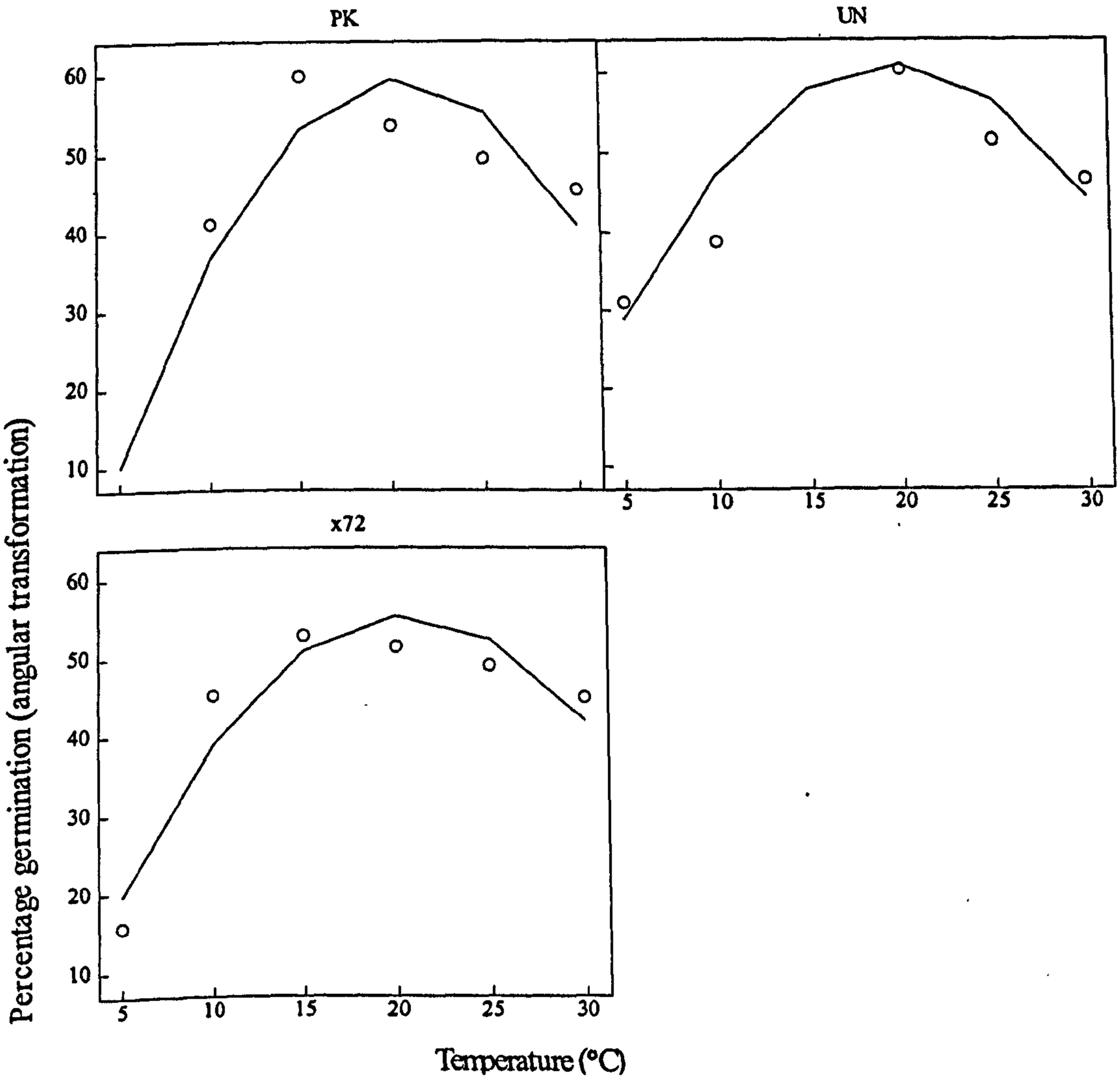


Figure A2. Multiple linear regression analysis of the effect of temperature on germination (%) in soil of *R. solani* at 20°C after 3 d. Points represent angularly transformed mean data values & lines show the fitted model for each isolate tested. $R^2 = 54.9$, $P = 0.156$ (DF = 215).

Table A1. Effect of carbon source on sclerotial germination of three AG 3 isolates of *R. solani* after 24 h at 25°C.

Isolate (AG)	Carbon source							
	Control	D - Glucose	Maltose	D - Xylose	Sucrose	Cellobiose	D - Mannitol	Glycerol
x72 (3)	85.0[a]	100.0	97.5	87.5	97.5	85.0	97.5	90.0
	(70.4)	(90.0)	(85.4)	(75.1)	(85.4)	(70.4)	(83.4)	(80.2)
UN (3)	100.0	100.0	100.0	100.0	97.5	100.0	100.0	97.5
	(90.0)	(90.0)	(90.0)	(90.0)	(90.0)	(90.0)	(90.0)	(85.4)
PK (3)	95.0	87.5	90.0	92.5	92.5	85.0	97.5	92.5
	(80.8)	(75.1)	(76.7)	(78.8)	(78.8)	(67.5)	(85.4)	(76.2)
P value	(0.585)							
LSD	(14.87)							
(*P* = 0.05),								
DF = 117[b]								

[a]Values are the mean percentage of sclerotia germinated (angularly transformed data) of four replicates. Analysis was carried out on the transformed data.
[b]LSD is the least significant difference at 5 % (*P* = 0.05), DF = degrees of freedom in ANOVA.

Table A2. Effect of carbon source on sclerotial germination of three AG 3 isolates of *R. solani* after 72 h at 25°C.

Isolate (AG)	Carbon source							
	Control	D - Glucose	Maltose	D - Xylose	Sucrose	Cellobiose	D - Mannitol	Glycerol
x72 (3)	95.0[a]	100.0	97.5	92.5	97.5	92.5	97.5	95.0
	(83.4)	(90.0)	(85.4)	(78.8)	(85.4)	(76.2)	(85.4)	(83.4)
UN (3)	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	(90.0)	(90.0)	(90.0)	(90.0)	(90.0)	(90.0)	(90.0)	(90.0)
PK (3)	95.0	97.5	95.0	95.0	92.5	92.5	100.0	95.0
	(80.8)	(85.4)	(80.8)	(80.8)	(78.8)	(76.2)	(90.0)	(80.8)
P value				(0.941)				
LSD ($P = 0.05$), DF = 117[b]				(12.09)				

[a]Values are the mean percentage of sclerotia germinated (angularly transformed data) of four replicates. Analysis was carried out on the transformed data.
[b]LSD is the least significant difference at 5 % ($P = 0.05$), DF = degrees of freedom in ANOVA.

Table A3. Effect of nitrogen source on sclerotial germination of three AG 3 isolates of *R. solani* after 24 h at 25°C.

Isolate (AG)	Nitrogen source							
	Control	L-Proline	L-Alanine	L-Asparagine	L-arginine	Ammonium chloride	Potassium nitrate	L-Glycine
x72 (3)	92.5[a]	87.5	95.0	97.5	100.0	87.5	95.0	92.5
	(81.7)	(72.1)	(83.4)	(85.4)	(90.0)	(69.5)	(80.8)	(78.8)
UN (3)	100.0	100.0	95.0	100.0	100.0	67.5	100.0	97.5
	(90.0)	(90.0)	(83.4)	(90.0)	(90.0)	(59.2)	(90.0)	(85.4)
PK (3)	90.0	92.5	90.0	80.0	95.0	77.5	90.0	92.5
	(73.7)	(78.8)	(74.1)	(63.8)	(80.8)	(62.3)	(76.7)	(76.2)
P value	(0.305)							
LSD ($P = 0.05$), DF = 117[b]	15.03							

[a]Values are the mean percentage of sclerotia germinated (angularly transformed data) of four replicates. Analysis was carried out on the transformed data.
[b]LSD is the least significant difference at 5 % ($P = 0.05$), DF = degrees of freedom in ANOVA.

Table A4. Effect of nitrogen source on sclerotial germination of three AG 3 isolates of *R. solani* after 72 h at 25°C.

Isolate (AG)	Nitrogen source							
	Control	L-Proline	L-Alanine	L-Asparagine	L-arginine	Ammonium chloride	Potassium nitrate	L-Glycine
x72 (3)	95.0[a]	90.0	100.0	100.0	100.0	90.0	97.5	92.5
	(83.4)	(74.1)	(90.0)	(90.0)	(90.0)	(74.1)	(85.4)	(78.8)
UN (3)	100.0	100.0	100.0	100.0	100.0	92.5	100.0	100.0
	(90.0)	(90.0)	(90.0)	(90.0)	(90.0)	(81.7)	(90.0)	(90.0)
PK (3)	95.0	92.5	100.0	97.5	97.5	82.5	97.5	95.0
	(80.8)	(81.7)	(90.0)	(85.4)	(85.4)	(65.5)	(85.4)	(80.8)
P value	(0.719)							
LSD ($P = 0.05$), DF = 117[b]	(12.02)							

[a]Values are the mean percentage of sclerotia germinated (angularly transformed data) of four replicates. Analysis was carried out on the transformed data.
[b]LSD is the least significant difference at 5 % ($P = 0.05$), DF = degrees of freedom in ANOVA.

APPENDIX B

Standard curves were prepared on the day of the assay, one for each medium being tested. They were produced using solutions of each medium that had been incubated paralell to the relevant experiment, sampled and stored in the same manner as the solutions being assayed (section 5.2.2).

1. Cutinase

The standard curve was produced by taking solutions of each medium and measuring the absorbance of 0, 5, 10 and 20 µmol of 4-nitrophenol in relation to a substrate blank. The resulting data was entered into an excel spreadsheet and absorbance plotted against mg of 4-nitrophenol present in the solution. The equations of the lines obtained, including the R-square (R^2) value of the line obtained are shown in table A1. The absorbance (nm) of the samples was used in the relevant standard curve equation to determine the µmol of 4-nitrophenol present in the assay samples. This value was then used to calculate cutinase activity as described previously (section 5.2.2.1).

Table B1. Standard curves and R^2 values for the cutinase assay.

Media	Incubation time (d)			
	1	2	6	10
Cutin	y = 13.56x R^2 = 0.980	y = 12.962x R^2 = 0.984	y = 13.238x R^2 = 0.988	y = 13.853x R^2 = 0.987
Basal	y = 19.654x R^2 = 0.977	y = 10.921x R^2 = 0.9921	y = 20.915x R^2 = 0.9993	y = 21.157x R^2 = 0.9936
Cell walls	y = 19.183x R^2 = 0.9931	y = 18.421x R^2 = 0.9936	y = 21.209x R^2 = 0.9875	y = 19.904x R^2 = 0.9667

2. Polygalacturonase

The standard curve was produced by taking solutions of each medium and measuring the absorbance of 0, 5, 10 and 20 μmol solutions of D-galacturonic acid 1 in relation to a substrate blank. The resulting data was entered into an excel spreadsheet and absorbance plotted against mg of D-galacturonic acid present in the solution. The equations of the lines obtained, including the R^2 value of the line obtained are shown in table A2. The absorbance (nm) of the samples was used in the relevant standard curve equation to determine the mg of D-galacturonic acid present in the assay samples. This value was then used to calculate polygalacturonase activity as described previously (section 5.2.2.3).

Table B2. Standard curves and R^2 values for the polygalacturonase assay.

	Incubation time (d)			
Media	1	2	6	10
Citrus pectin	$y = 14.899x$ $R^2 = 0.9924$	$y = 15.077x$ $R^2 = 0.9909$	$y = 15.642x$ $R^2 = 0.9915$	$y = 15.502x$ $R^2 = 0.9925$
Basal	$y = 16.883x$ $R^2 = 0.9725$	$y = 16.506x$ $R^2 = 0.9701$	$y = 16.588x$ $R^2 = 0.9747$	$y = 15.714x$ $R^2 = 0.9857$
Cell walls	$y = 15.714x$ $R^2 = 0.9857$	$y = 15.437x$ $R^2 = 0.9799$	$y = 15.419x$ $R^2 = 0.9772$	$y = 16.680x$ $R^2 = 0.9969$

3. Endo-1,4-β-glucanase

The standard curve was produced by taking solutions of each medium and measuring the absorbance of 0, 10, 20, 40 and 80 μmol solutions of glucose in relation to a substrate blank. The resulting data was entered into an excel spreadsheet and absorbance plotted against μmol of glucose present in the solution. The equations of the lines obtained, including the R^2 value of the line obtained are shown in table A3. The absorbance (nm) of the samples was used in the relevant standard curve equation to determine the μmol of glucose present in the assay samples. This value was then used to calculate endo-1,4-β-glucanase activity as described previously (section 5.2.2.4).

Table B3. Standard curves and R^2 values for the endo-1,4-β-glucanase assay.

	Incubation time (d)			
Media	1	2	6	10
CMC	$y = 137.43x$	$y = 141.56x$	$y = 142.89x$	$y = 146.85x$
	$R^2 = 0.9846$	$R^2 = 0.9891$	$R^2 = 0.9891$	$R^2 = 0.9787$
Basal	$y = 136.62x$	$y = 127.70x$	$y = 121.99x$	$y = 120.97x$
	$R^2 = 0.9824$	$R^2 = 0.9823$	$R^2 = 0.9831$	$R^2 = 0.9866$
Cell walls	$y = 156.47x$	$y = 158.88x$	$y = 153.62x$	$y = 158.66x$
	$R^2 = 0.9760$	$R^2 = 0.9880$	$R^2 = 0.9894$	$R^2 = 0.9954$

4. β-glucosidase

The standard curve was produced by taking solutions of each medium and measuring the absorbance of 0, 10, 20, 40 and 80 μmol solutions of 4-nitrophenol in relation to a substrate blank. The resulting data was entered into an excel spreadsheet and absorbance plotted against μmol of 4-nitrophenol present in the solution. The equations of the lines obtained, including the R^2 value of the line obtained are shown in table A4. The absorbance (nm) of the samples was used in the relevant standard curve equation to determine the μmol of 4-nitrophenol present in the assay samples. This value was then used to calculate β-glucosidase activity as described previously (section 5.2.2.5).

Table B4. Standard curves and R^2 values for the β-glucosidase assay.

	Incubation time (d)			
Media	1	2	6	10
Cellobiose	$y = 135.54x$	$y = 128.78x$	$y = 114.99x$	$y = 112.97x$
	$R^2 = 0.9884$	$R^2 = 0.9924$	$R^2 = 0.9935$	$R^2 = 0.9867$
Basal	$y = 110.71x$	$y = 111.76x$	$y = 115.18x$	$y = 110.77x$
	$R^2 = 0.9921$	$R^2 = 0.593$	$R^2 = 0.9897$	$R^2 = 0.9985$
Cell walls	$y = 148.76x$	$y = 149.88x$	$y = 150.75x$	$y = 154.07x$
	$R^2 = 0.9874$	$R^2 = 0.9930$	$R^2 = 0.9820$	$R^2 = 0.9880$

APPENDIX C

Baiting method for isolating *R. solani* from soil – from Kyritsis (2003)

Preparation of modified Ko and Hora medium

Modified Ko and Hora medium was prepared containing (l^{-1} distilled water) 1 g di-potassium phosphate (K_2HPO_4), 0.5 g magnesium sulphate heptahydtrate ($MgSO_4 \cdot 7H_2O$), 0.5 g potassium chloride (KCl), 0.01 g ferrous sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$), 0.2 g sodium nitrite ($NaNO_2$), 0.05g chloramphenicol and 20 g Agar technical no. 3. The medium was autoclaved and cooled to 50°C. Each of the following were mixed in 10 ml sterle distilled water; 0.05 g streptomycin, 0.4 g gallic acid, 0.0633 g metalaxyl (as Ridomil, 25%, WP = 0.2532 g) and 0.005 ml prochloraz (as Poraz, 45%, EC = 0.0111 ml), filter sterilised and added to the sterile medium. Gallic acid was dissolved by heating on a hot plate stirrer for 2 - 5 min. Media was poured into 9 cm Petri dishes under sterile conditions and stored at 5°C until required.

Baiting method from soil

Soil samples were air-dried and sieved through a 2 mm sieve. Beetroot seeds (*Beta vulgaris* var. *crassa*) were autoclaved three times at 24 h intervals. The soil moisture content of the smples were adjusted to 40 % WHC by adding appropriate amounts of sterile distilled water. Ten beetroot seeds were added, using forceps, to approximately 75 g of soil in a 9 cm diam Petri dish, and all seeds covered with soil. The Petri dish was sealed with Parafilm and all dishes placed in a plastic bag to retain moisture. All dishes were incubated in the dark at 25°C for 48 h. Seeds were recovered by sieving, washed for 10 – 15 s and blotted dry on sterile filter paper. They were placed on the selective medium and pushed

approximately 3 –4 mm into the agar. Dishes were sealed with Parafilm and incubated at 25°C. After 48 h, outgrowing hyphae were viewed at x40 magnification to detect *R. solani* hyphae. The percentage of beetroot seeds colonised by *R. solani* was determined per plate.

APPENDIX D

Table D1. Effect of inoculum density on tuber yield.

Inoculum density (potato pieces m^{-2})	Tuber yield	
	16 August	27 September
0	145.8[a]	203.2
194	129.9	254.6
775	145.6	229.5
P value	0.524	0.122
LSD ($P = 0.05$)	31.79	49.22
DF = 127[b]		

[a] Values are mean tuber yields per plant of ten replicates.
[b] Pairs of means that differ by more than the LSD are significantly different ($P = 0.05$), DF = degrees of freedom in ANOVA.

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