24/9/96

# CHARACTERISATION OF RHIZOCTONIA BAREPATCH DECLINE

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Thesis submitted for the degree of
**DOCTOR OF PHILOSOPHY**
in the
Faculty of Agricultural and Natural Resource Sciences
The University of Adelaide

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February 1996

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## SUMMARY

Bronwyn Meg Wiseman. 1996. **Characterisation of Rhizoctonia barepatch decline**. PhD thesis, Department of Soil Science, The University of Adelaide, Australia.

Rhizoctonia barepatch is a major root rotting disease of cereals and pastures in southern Australia and the Pacific North-West of the United States. The causal agent of the disease is the fungus *Rhizoctonia solani* Kühn anastomosis group (AG) 8 (teleomorph *Thanatephorus cucumeris* (Frank) Donk). This disease is favoured by reduced tillage and control is currently based on physical disturbance of the soil. The suggested control mechanism is fragmentation of particles of debris and disturbance of the hyphal network which reduces the size and / or pathogenicity of propagules of *R. solani*.

A unique result has been observed at a long term trial at Avon in South Australia where Rhizoctonia barepatch has declined over time in a direct drilled farming system. Disease severity initially rose as expected with the introduction of reduced tillage, but began declining until there were no detectable symptoms eight years later. Initial experiments suggested this suppression was due to biological factors. A second trial at Avon still has significant severity of Rhizoctonia barepatch present. The two trials formed the basis of a comparative study of a "suppressive" (where disease has declined) and a "non-suppressive" (disease present) soil.

This project was initiated with the aim of characterising the disease decline and determining likely mechanisms by which the disease was being controlled.

The first hypothesis tested was whether the suppression observed at Avon was biological, chemical or physical in nature. Methods to demonstrate biological suppression were based on transfer of the suppressive characteristics to another soil, and elimination of suppressive characteristics with biocidal treatments. Suppressive characteristics could be transferred to an autoclaved or steam pasteurised soil by adding 10 % (w/w) of the unsterilised soil. Gamma irradiation or pasteurisation at 60, 70 or 80°C for 30 min eliminated the ability of the soil to suppress disease and also eliminated differences in the soil microflora of suppressive and non-suppressive soil which were observed for untreated and 50°C pasteurised soil. Different responses to pasteurisation at 50°C indicated different microbial populations were present in the two soils. This indicated the suppression was due to both the general soil biota and the action of specific organisms.

Disease severity in suppressive and non-suppressive soil following inoculation with increasing doses of *R. solani* AG-8, or one of a range of other fungal pathogens was used as a measure of the soils' receptivity to these pathogens. Disease caused by high and low doses of *R. solani* AG-8, and those caused by *Gaeumannomyces graminis* var. *tritici* and *Fusarium graminearum*, were suppressed. The presence of chitin-glucan in the pathogens correlated with suppression in pot experiments, supporting the potential role of specific antagonism.

The resident pathogen population in suppressive and non-suppressive soil was not known. Absence of disease does not always imply absence of pathogen so it needed to be determined if the two soils differed in amount of pathogen as well as severity of disease. Total, viable, and pathogenic

*Rhizoctonia* were quantified using a specific DNA probe, fungal isolations, and disease severity bioassays, with a good correlation between the latter two parameters. Further quantification of the populations of *Rhizoctonia* using direct observation of hyphae in soil suggested suppression of both the saprophytic and parasitic phases was occurring. Length of *Rhizoctonia*-like hyphae tended to be greater in non-suppressive compared to suppressive soil both in the presence or absence of living plant roots.

Initial characterisation of the microbial populations in suppressive and non-suppressive soil was done to compare communities and identify organisms that may be involved in this suppression. Microbial activity and biomass were higher in suppressive compared to non-suppressive soil.

This thesis describes the occurrence of natural, biologically based suppression of Rhizoctonia barepatch in a direct drilled system at Avon, South Australia. The suppressive characteristics are transferable, removed by biocidal treatments, and active against increasing doses of *R. solani* AG-8, *G. graminis* var. *tritici* and *F. graminearum*. Disease severity and the viable population of *Rhizoctonia* are reduced in suppressive soil but the causal agent is still present. The microbial populations in suppressive and non-suppressive soil appear to differ both in their functioning and composition. The development of this suppression, through manipulation of the existing soil biota with farming practices, represents a significant breakthrough in the search for control strategies for a major disease such as Rhizoctonia barepatch.

## DECLARATION

*I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university or tertiary institution. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference has been made in the text.*

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

February, 1996 Signed:

Bronwyn Meg Wiseman

# PUBLICATIONS FROM THE THESIS

The following papers have arisen from work done in this thesis.

**Journal Article:**

**Wiseman, B. M., Neate, S. M., Ophel Keller, K., and Smith, S. E.** Suppression of *Rhizoctonia solani* anastomosis group 8 and its biological nature. Soil Biology and Biochemistry. Accepted. (Results of parts of Chapter 3 and 7).

**Conference Articles**

1. **Wiseman, B. M., Neate, S. M., Ophel Keller, K., and Smith, S. E.** 1995. Suppression of *Rhizoctonia solani* anastomosis group 8 and its biological nature. (poster paper) International Symposium on *Rhizoctonia*, Noordwijkerhout, The Netherlands.

2. **Wiseman, B. M., Neate, S. M., Ophel Keller, K., and Smith, S. E.** Biological suppression of Rhizoctonia root rot of wheat. 10 th Biennial Australasian Plant Pathology Society Conference, Lincoln University, New Zealand.

## ACKNOWLEDGMENTS

I would firstly like to thank my supervisors, Dr Stephen Neate, Dr Kathy Ophel Keller and Professor Sally Smith, who in their own particular ways have all been a great source of assistance, knowledge, and support throughout this project.

To the many people I have dealt with throughout the past three years I owe many thanks: Herdina, Sue Wiebkin and Sonya Dyer for their friendship and help with all things molecular, Ipi Sukarno for her memorable friendship and support, Richard Inwood for being there to talk to, Bruce Hawke and Dan Kluepfel for assistance with the FAME experiments, and members of the mycorrhizal lab for the many day to day things. Thanks also go to David Roget for his work leading up to my project and helpful discussion during it. Also, I am grateful to all members of CSIRO Soil Biology, the Department of Soil Science, and others who have helped either with words of advice, techniques, or supplies of seeds, cultures and whatever else was required.

Finally, thanks must go to my parents and family who have continued their tremendous support, and to Sam, for helping to make me realise what really was important.

# Chapter 1: Literature Review

## 1.1 Introduction

Rhizoctonia barepatch is a major root rotting disease of cereals and pastures in southern Australia and the Pacific Northwest of the United States. The causal agent of the disease is the fungus *Rhizoctonia solani* Kühn anastomosis group 8 (teleomorph: *Thanatephorus cucumeris* (Frank) Donk). This disease is favoured by reduced tillage systems and control is currently based on physical disturbance of the soil by cultivation. *R. solani* characteristically persists as thick-walled hyphae and sclerotia in association with particles of plant debris, and hyphae ramifying thoughout the soil. It is thought that cultivation causes fragmentation of particles of debris and disturbance of the hyphal network, which reduces the viability of propagules of *R. solani* and / or its pathogenicity.

A long term trial in South Australia has produced a unique result of declining Rhizoctonia barepatch severity in a direct drilled system. Disease severity initially increased as expected with the introduction of direct drilling but began declining after a peak in disease in the fourth year of the trial. Disease severity continued to decline until there were no detectable symptoms eight years later. Initial experiments (S. Neate, A. Rovira, and M. Ryder, pers. comm.) suggested this suppression of disease was due to biological factor(s).

For the development of soil-borne plant disease, several conditions need to be met. The pathogen must be able to survive in the soil environment in the absence of a plant host and retain its viability. Propagules must then be able

to germinate and grow towards susceptible roots and successfully infect them. If any of these phases are unsuccessful disease severity will be reduced or disease will be absent. Hence, reduced severity of disease can be due to either a lack of propagules of the pathogen or limitations in the infection process. The infection process can be limited due to innate characteristics of the pathogen and / or inhibition by other organisms. To explain the observed decline in Rhizoctonia barepatch, the disease needs to be examined in terms of these phases of disease development. Possible explanations for declined disease severity are:

•absence of propagules of *R. solani* AG-8 due to long-term low survival and viability,

•inability of propagules of *R. solani* AG-8 to infect host plants successfully due to:

a) reduced viability / pathogenicity of propagules,

b) adverse interactions with other soil organisms,

c) change in the hosts' ability to be infected. This can not be an explanation for the disease decline at Avon because new seed was introduced at each sowing.

This project is an investigation of the basis of a decline in Rhizoctonia barepatch severity in a direct drilled system. The options outlined above were used as a basis for further experimental work.

## 1.2 Rhizoctonia barepatch

### 1.2.1 Description

*Rhizoctonia solani* Kühn was first reported as causing barepatch in cereals in South Australia by Samuel in 1928. The disease was later more fully described by Samuel and Garrett (1932). Affected plants are obvious at an early stage of crop growth (often before tillering), appear spindly and stunted and may die at this stage or as the temperature rises at the end of winter. Stunting is thought to be due to rotting of the seminal roots, resulting in the plant being unable to form an adequate secondary root system (Samuel and Garrett, 1932).

The root rotting disease of cereals caused by *R. solani* has been referred to as Rhizoctonia root rot, Rhizoctonia patch or barepatch, purple patch, and barley stunt disorder due to the characteristic symptoms of stunting, formation of patches of severely affected plants, and frequent purpling of leaves and stems (of oats in particular) (Hynes, 1937; Samuel and Garrett, 1932; Hynes, 1933; Murray, 1981).

Smiley and Uddin (1993) conclude that Rhizoctonia barepatch primarily affects crop yields by suppressing tiller development. They also concluded that the overall effect of barepatch on yield depends on the interaction of the disease with "ever changing environmental influences that also affect tillering".

*R. solani* characteristically exists in the soil as thick-walled hyphae and sclerotia in association with particles of plant debris (Boosalis and Scharen,

1959). Investigations of South Australian soils have shown that sclerotia are not important for survival of this fungus (De Beer, 1965; Warcup and Talbot, 1962). In a detailed study of important sources of *R. solani* inoculum in a South Australian cereal growing soil, Neate (1987) concluded that *R. solani* propagules were concentrated in the top 10 cm of soil in particles of plant debris of size range 750 µm - 2 mm.

### 1.2.2 Taxa of *Rhizoctonia* and those found in southern Australian wheat fields

Neate (1985) reported there to be at least four species of *Rhizoctonia* in South Australian wheat fields based on teleomorphs: *T. cucumeris* (anamorph *R. solani*), *Ceratobasidium cornigerum* (Bourd.) Rogers, *Waitea circinata* Warcup and Talbot, and *Iodophanus carneus* (Pers.:Fr) Korf.

As well as classification into teleomorphs, *Rhizoctonia* isolates can be further sub-divided into hyphal anastomosis groups (AG's). Isolates are assigned to anastomosis groups according to their "affinity for hyphal fusion with members of designated anastomosis groups" (Sneh *et al.*, 1991). Anastomosis, or hyphal fusion, is a manifestation of somatic or vegetative incompatibility between isolates (Anderson *et al.*, 1972). Carling *et al.* (1990) described four categories of anastomosis (C0 - C3). The C0 category involves no interaction between opposed hyphae and infers they are from different AG's. The C1 category involves hyphal contact, no wall penetration or membrane-membrane contact, and anastomosing and adjacent cells may die and infers that the isolates are distantly related and may be from different or similar AG's. The C2 category involves wall penetration, with the diameter of the pore at the point of penetration being less than hyphal

diameter, and frequent death of anastomosing and adjacent cells and infers that the isolates are from the same AG. The C3 category involves fusion of walls and membrane, the diameter of the pore at the point of penetration being equal or nearly equal to hyphal diameter. In this case there is generally not death of anastomosing and adjacent cells and this interaction infers that the isolates are from the same AG and are actually the same isolate.

Fourteen AG's have currently been described for multinucleate species of *Rhizoctonia*. *R. solani* isolates fall into one of 12 groups (1 - 11 and BI) and *R. zeae* and *R. oryzae* comprise one group each (WAG-Z and WAG-O respectively) (Sneh *et al.*, 1991; Carling *et al.*, 1994). Binucleate *Rhizoctonia* species are currently assigned to 17 AG's (A - Q) (Sneh *et al.*, 1991 after Ogoshi).

Within *T. cucumeris* four anastomosis groups have been identified in South Australia, AG 2-1, 3, 4 and 8 (Neate, 1985; Neate and Warcup, 1985). Binucleate anastomosis groups reported in southern Australia include: C, D, E and K (Roberts and Sivasithamparam, 1986). Hence, non-specific quantification of multinucleate populations of *R. solani* in South Australian soils may include isolates from 4 AGs. Isolates from AGs 4 and 8 are pathogenic on cereals but isolates from AGs 2-1 and 3 are not (Sneh *et al*, 1991; Carling and Sumner, 1992). To date, only fungi from AG D have been found to be pathogenic on cereals, fungi from AG E are non-pathogenic on cereals, and fungi from AG's C and K are non-pathogenic (Sneh *et al*, 1991; Carling and Sumner, 1992).

Both binucleate and multinucleate *Rhizoctonia* can exist in a barepatch, but the relative amounts at different positions within the patch vary (Roberts and Sivasithamparam, 1986). Within a barepatch, 90 % of the isolates were

multinucleate and the remaining 10 % binucleate. Multinucleate isolates were more predominant in the centre of the patches, with the relative frequency of binucleate isolates increasing towards the edge of the patch.

*Rhizoctonia* isolates can be further sub-divided into zymogram groups (ZG's) based on separation and characterisation of pectic enzymes in pectin-acrylamide gels. The technique, first described by Cruickshank and Wade (1980), has been used in several studies of variation among isolates of *R. solani* (Sweetingham *et al.*, 1986; Neate *et al.*, 1988; MacNish and Sweetingham, 1993b; MacNish *et al.*, 1993). Within *R. solani* AG-8 there are currently four ZG's : 1-1, 1-2, 1-3, 1-4 and 1-5 (previously ZG 2) (Sweetingham *et al.*, 1986; Neate *et al.*, 1988; MacNish and Sweetingham 1993a). Each barepatch is dominated by a single zymogram group of *R. solani* AG-8 (MacNish and Sweetingham, 1993b; MacNish *et al.*, 1993). In South Australia, ZG's 1-1, 1-2, 1-3 and 1-5 have been identified (Neate *et al.*, 1988).

1.2.3 Hosts and Distribution

*R. solani* AG-8 ('root strain') has a wide host range (Table 1.1). All plants growing in a broadacre cropping system could be infected by *R. solani* AG-8, although the possibility that different extents of colonisation may occur needs to be considered.

Rhizoctonia barepatch is found throughout the cereal production zones of southern Australia. Rhizoctonia barepatch was initially thought to be restricted to alkaline mallee soils in South Australia (Samuel and Garrett, 1932), but it is now known to have become established throughout the cereal districts of the state. Rhizoctonia barepatch was observed in Western Australia in 1971 and is now a disease of major importance in the calcareous

mallee soils of that state (MacNish, 1983). Annual production losses due to Rhizoctonia barepatch occur in New South Wales, Victoria, South Australia, and Western Australia, with potential losses in these states of $43m annually (Brennan and Murray, 1988).

Rhizoctonia root rot has also been reported in the United States, England, Scotland, and Iran (Weller *et al.*, 1986; Dillon-Weston and Garrett, 1943; Murray and Nicolson, 1979; Behroozin and Assadi, 1993).

### 1.2.4 Control of Rhizoctonia barepatch

Both the severity of Rhizoctonia barepatch and extent of the patches increase in reduced tillage farming systems compared to when the soil is cultivated (Neate, 1984; MacNish, 1985; Rovira, 1986; Jarvis and Brennan, 1986; Weller *et al.*, 1986; de Boer *et al.*, 1991). Control can therefore be achieved by physical disturbance of the soil. The suggested mechanisms of this control is fragmentation of particles of plant debris and disturbance of the hyphal network, reducing propagule size and / or pathogenicity of *R. solani* (Rovira, 1986). Cultivation prior to seeding each year is necessary (MacNish, 1985). Cultivation each year controlled disease but as soon as this practice ceased there was an immediate increase in disease severity. This supports the conclusion of Rovira (1986) that at least annual physical disturbance is required to control Rhizoctonia barepatch. The transitory effect of cultivation on Rhizoctonia barepatch is similar to the effect on mycorrhizal fungi reported by McGonigle and Miller (1993).

| | common name | scientific name |
|---|---|---|
| cereals | barley | *Hordeum vulgare* L. |
| | cereal rye | *Secale cereale* L. |
| | oat | *Avena sativa* L. |
| | wheat | *Triticum aestivum* L. |
| pasture grasses | Arabian grass | *Schimus barbatus* (L.) Juel. |
| | barley grass | *Hordeum murinum* L. |
| | brome grass | *Bromus* spp. |
| | soft brome | *Bromus mollis* L. |
| | perennial veldt grass | *Ehrharta calycina* Smith |
| | phalaris | *Phalaris tuberosa* L. |
| | ryegrass | *Lolium rigidum* Gaudin. |
| legumes | hare's foot clover | *Trifolium arvense* L. s. lat. |
| | sub-terranean clover | *T. subterraneum* L. |
| | lucerne | *Medicago sativa* L. |
| | lupin | *Lupinus* spp. |
| | medic | *M. rugosa* Desr. |
| | barrel medic | *M. truncuIata* Gaertn. |
| | burr medic | *M. polymorpha* L. |
| | small woolly burr medic | *M. minima* (L.) Bartal. |
| | snail medic | *M. scutellata* (L.) Miller |
| | garden pea | *Pisum sativum* L. |
| other | brussel sprouts | *Brassica oleraceae* L. var. *gemmifera* DC |
| | cabbage | *B. oleraceae* L. var. *capitata* Alef. |
| | capeweed | *Arctotheca calendula* (L.) Levyns |
| | evening primrose | *Oenothera stricta* Ledeb. ex Link |
| | lettuce | *Lactuca sativa* L. |
| | radish | *Raphanus sativus* L. |
| | storksbill | *Erodium* spp. |
| | sugar beet | *Beta vulgaris* L. |
| | tomato | *Lycopsersicon esculentum* Mill. |

From: Ludbrook *et al*., 1953; Kerr, 1955; Flentje and Saksena, 1957; Neate, 1985; Schisler *et al*., 1994.

Table 1.1: Common and scientific names of hosts of *R. solani* AG-8 (root-strain)

Disease severity is also increased by the presence of volunteer pasture growth just prior to sowing, possibly due to a build up of fungal inoculum on the roots of these plants (Roget *et al.*, 1987). Chemical removal of pasture prior to direct-drilling has been shown to reduce disease in South Australia (Roget *et al.*, 1987) but not Western Australia (MacNish and Fang, 1987).

Direct effects of certain herbicides on *R. solani* are unclear. Patch area in a field trial and disease severity under controlled conditions increased following application of the sulfonylurea herbicide, chlorsulfuron. (Rovira and McDonald, 1986; Smiley and Wilkens 1992). In contrast, chlorsulfuron has had no effect on disease severity (Wong *et al.*, 1993). These differences may be explained by variation in pH of the various test soils used and the increase in persistence of chlorsulfuron in soil as pH increases (Smiley and Wilkens, 1992). Hence, chlorsulfuron may remain in some soils for considerably longer than others , leading to greater impact on *R. solani*.

Various fungicides have been tested for control of Rhizoctonia barepatch in field trials, pot bioassays and *in vitro* against *R. solani* AG-8. Results have shown potential for fungicidal control of Rhizoctonia barepatch, but inconsistencies over different soil types and cultivation methods and the monetary costs involved are major limiting factors. The triazole fungicide, flutriafol has been shown to reduce severity of Rhizoctonia barepatch when applied directly to the soil, combined with phosphate fertiliser (double superphosphate), coated onto clay granules, and less effectively, as a seed coating (Cotterill and Ballinger, 1989; Cotterill *et al.*, 1990; Cotterill, 1991; Cotterill 1993a). Flutriafol reduces the saprophytic survival and growth of *Gaeumannomyces graminis* (Sacc.) Arx &Olivier var. *tritici* Walker in soil

but *in vitro* tests indicated it did not have a similar effect on *R. solani* AG-8 (Cotterill, 1993b).

In summary, due to the lack of resistant crop and pasture plants or a viable chemical control method, control of Rhizoctonia barepatch is based on cultivation and removal of volunteer pasture prior to sowing, . However, this is not entirely satisfactory due to this disease being common in areas prone to wind erosion where cultivation is discouraged. As well, cultivation does not always provide control (Cotterill, 1990; Cotterill *et al.*, 1990; Henry and Brown, 1991).

### 1.3 Rhizoctonia barepatch decline under direct drilling

The first positive case of natural decline of Rhizoctonia root rot in the field was reported by Roget (1995). Two key findings of this work were: a) a decline in severity of Rhizoctonia root rot in direct drilled wheat following an initial phase of increasing disease severity; b) a similar trend of disease severity initially increasing, then decreasing, for all four rotations with wheat. (See Section 2.1 for trial details). The trial began in 1978 with a two year rotation of wheat with wheat, peas, medic (*Medicago scutella*) or volunteer grass pasture (PhI). Identical plots were established in 1979 with the rotations one year out of phase (PhII). Two cultivation treatments were used, conventional cultivation (representing common farm practice of the area) and direct drilling which involved no cultivation prior to seeding. From 1979-1986, eight to ten weeks after sowing wheat plants were sampled annually from PhI / PhII and assessed for Rhizoctonia root rot severity. PhI ceased in 1985 so after 1986 wheat samples were taken from PhII every second year. Disease severity in direct drilled wheat peaked in 1982 - 83, declined to negligible levels by 1990 and has remained at that level since

(Fig. 1.1). This decline was unexpected and is not explained by the current theories of the effect of reduced tillage on Rhizoctonia barepatch.

In unpublished experiments Neate, Rovira and Ryder (pers. comm.) cycled suppressive soil through a series of plantings of wheat and then assessed its ability to suppress Rhizoctonia root rot. The cycled soil was compared with a non-suppressive soil sampled at an earlier stage of the trial. Suppressive characteristics were successfully transferred to another soil and there was significantly lower severity of disease in pots containing the cycled soil compared to the non-suppressive soil. The work of Neate *et al.* provided preliminary evidence that the observed decline is due to biological factors.

The treatments applied at the Avon trial site have been similar over the time in which Rhizoctonia barepatch severity has been monitored. Hence, there have been no effects from external alterations of conditions due to management (eg alterations in treatment of stubble, herbicide application). The seed drill was changed in 1987 (Roget, 1995), but this was 4 - 5 years after disease severity began declining and had no effect on this trend. Conditions such as seasonal rainfall and temperature, soil carbon and phosphorus, pH, cation exchange capacity, and available phosphorus are all reported as having no apparent relationship with the development of this decline (Roget, 1995).

Following long-term exposure to a pathogen, plant populations may develop or exhibit resistance mechanisms, resulting in inhibition of the infection process. Two facts disprove this as a possibility in the observed decline: 1) the seed cultivar and source at this trial has varied over time, and 2) the decline has not been observed in an adjacent trial established at the site in 1990 with some treatments similar to PhI / PhII.

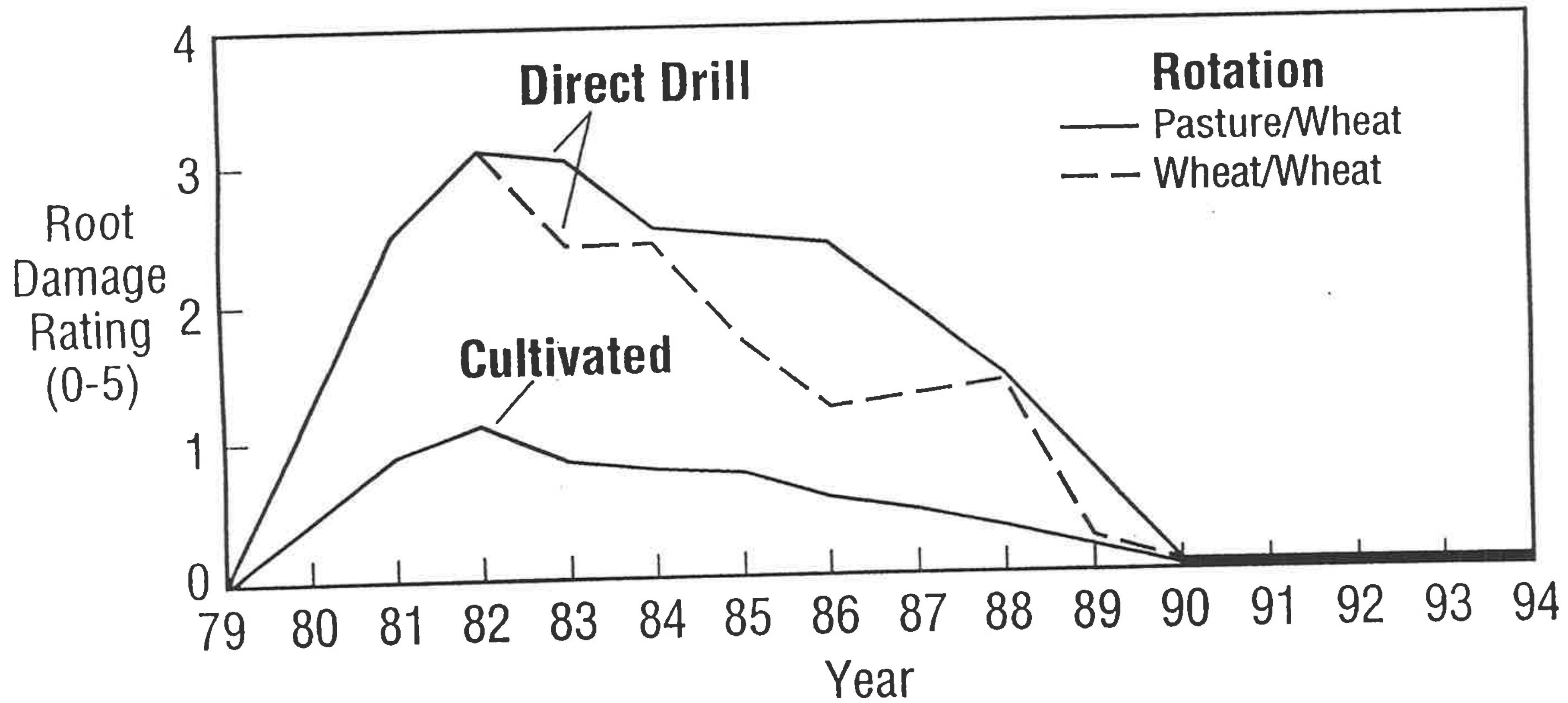




Figure 1.1: Decline in Rhizoctonia root damage in direct drilled wheat at Avon, South Australia (1982 - 1994)
(data and figure of David Roget, CSIRO Division of Soils, Adelaide, South Australia, pers. comm., see Roget, 1995)

Wheat cultivars used at the Avon trial site have been: Condor 1979 - 1985, Spear 1986 - 1993 and Stiletto 1994 (Roget, 1995).

Decline of Rhizoctonia barepatch occurred in all four rotations with wheat used at the Avon site. Significant differences occurred between rotations in most years (prior to 1990) with generally higher disease severity in wheat following grass pasture than following peas, medic or wheat (Roget, 1995). There was no effect of rotation on disease severity in cultivated plots. Lack of significant differences between rotations in cultivated plots may have been due to the low disease severity occurring. Disease severity was negligible for all treatments from 1990 onwards.

## 1.4 Disease suppression

### 1.4.1 Introduction and definitions

In a review on disease suppression, Hornby (1983) highlighted the difficulties in precisely defining this topic and also the variety of terms used by authors to describe particular situations. As an introduction to this thesis, a number of definitions will be given as future reference for later experimental and discussion sections. Extensive work dealing with soils suppressive to the take-all fungus (*G. graminis* var. *tritici*, Ggt) and the associated Take-all Decline (TAD) phenomenon, the *Fusarium* wilt pathogen, *Fusarium oxysporum* Schlect. and the tobacco root rot pathogen, *Thielaviopsis basicola* (Berk. & Br.) Ferr. has been reported and many definitions are based on these works.

A suppressive soil is one in which, despite favourable conditions, the pathogen either does not become established in the soil, establishes but does

not produce disease, or establishes and initially produces disease, which then declines (Baker and Cook, 1974). Alabouvette (1986) defined suppressive soils as those "in which disease severity remains limited in spite of a high pathogen inoculum density". The opposite situation to a suppressive soil is a conducive soil, defined as a soil which "allows severe disease in the presence of a relatively low pathogen inoculum density".

Both pathogen suppressive (PS) and disease suppressive (DS) soils have been reported, with Hornby (1983) suggesting that they may be distinguished by suppression of the saprophytic (PS) and parasitic (DS) phase of the pathogens' life-cycle. Schnieder (1984) distinguished "parasitic competition" from "saprophytic competition" between pathogenic and non-pathogenic *Fusarium* species based on an antagonistic interaction at the root cortex and prior to root colonisation respectively. Depending on the complexity of the mechanisms of suppression in a soil these two phases in disease development may be both affected, thus the term "suppressive soil" will be used in this thesis for the situation in which both, or either, phases are suppressed in some way.

An alternative terminology, described by Alabouvette (1986), is the useful concept of "soil receptivity" which reflects the capacity of a soil to allow pathogen establishment, development, persistence and disease incitement and relates to both the biotic and abiotic characteristics of the soil. A measure of soil receptivity can be used to identify and compare suppressive soils. Pathogen inoculum is added to soil at increasing doses and the disease severity at equivalent doses compared between soils. Disease will be low in soils with low receptivity compared with more receptive soils at the same inoculum dose and can be considered to have a higher level of suppression.

## 1.4.2 Biotic and abiotic mechanisms of suppression

Biotic and abiotic mechanisms of suppression are possible and evidence for combined biotic and abiotic effects resulting in suppression are known. From the three well documented examples of suppressive soils many mechanisms have been shown, but, as described by Alabouvette (1993) there are several common properties demonstrating that "suppressiveness is fundamentally microbial in nature". This may be the situation in these established cases of suppressive soils, but clearly the underlying mechanism(s) for each new case must be determined before such a conclusion can be made.

A biological basis for suppression can be shown by the successful transfer of the suppressive characteristics to another soil (Rovira and Wildermuth, 1981; Rovira, 1982). An alternative method of demonstrating the involvement of a biological factor in a suppressive soil is to apply a biocidal treatment which results in the elimination of suppressive characteristics of the soil (Gerlagh, 1968; Alabouvette, 1986).

The terms general and specific suppression were established from investigations of decline of the take-all disease and relate to the ability of the biologically based suppression to be transferred to another soil, survive biocidal treatments and its likely site of action (Gerlagh, 1968; Cook and Rovira, 1976). General suppression is defined as being not transferable, probably operating in the bulk soil, not eliminated by 70°C moist heat, methyl bromide or chloropicrin, but eliminated by autoclaving. This form of suppression is caused by general antagonism of the pathogen found in all soils and is the effect of the general soil biota on disease severity. Specific suppression is defined as being transferable, operating on or near the roots, and eliminated by 60°C moist heat, methyl bromide or chloropicrin. This

form of suppression is caused by specific antagonism and is the effect of particular groups of the soil biota. These two forms of suppression are not alternatives and both can operate in the same soil, as is thought to be the case for suppression of take-all in the Pacific Northwest of the USA (Cook and Rovira, 1976).

Biotic mechanisms previously reported in suppressive soils are based on competition for nutrients and direct antagonism such as antibiosis and hyperparasitism. A well characterised case of nutrient competition is the immobilisation of iron by siderophore-producing fluorescent *Pseudomonas* spp of bacteria involved in suppression of *Fusarium* (Scher and Baker, 1982). Competition for nutrients, primarily carbon, is also a mechanism in suppression of *Fusarium* wilt (Alabouvette, 1986). An example of direct antagonism occurring in suppressive soils involves members of the genus *Trichoderma* Pers. ex Fr. that can cause both hyphal parasitism and lysis (Chet and Baker, 1980; Lui and Baker, 1980). The basis of general suppression can be high levels of soil microbial biomass and activity. Garrett (1933) found a negative correlation between bacterial numbers and severity of disease due to *Ophiobolus* Sacc.(now *G. graminis* var. *tritici*), and the level of soil suppressiveness to *Fusarium* wilt correlates positively with microbial biomass and respiration rate following glucose addition (Alabouvette, 1986).

Soil suppressiveness has been correlated with abiotic characteristics such as soil pH, texture, moisture and nutrient levels. For example, low incidence of clubroot of brassicas (caused by *Plasmodiophora brassicae* Woronin) is associated with increased calcium or sodium ions and high pH (Fletcher *et al.*, 1982). Suppression of avocado root rot (*Phytophthora cinnamoni* Rands) is eliminated by water logging of the soil, and soil suppressiveness to

*Fusarium* wilt has been correlated with clay addition and increased pH (Baker and Cook, 1974; Höper *et al.*, 1995). Soil type is important for suppression of black root rot of tobacco (*T. basicola*), with only soils derived from moraine and containing vermiculitic clays being suppressive (Stutz *et al.*, 1989).

### 1.4.3 The role of organic matter in disease suppression

Inputs of organic matter potentially affect both pathogenic and beneficial non-pathogenic organisms and it is the relative influence of inputs on these two populations that will determine if disease suppression occurs or not. An effect of addition of organic matter is the potentially increased microbial biomass (Doran, 1980) and its reported correlation with soil suppressiveness (Section 1.4.2).

A great deal of evidence exists for the suppression of *R. solani* following soil amendments with organic matter. Various types of dry and green plant material, when incorporated into potting media induced suppression of damping-off caused by *R. solani* (Davey and Papavizas, 1960; Papavizas and Davey, 1960; Papavizas *et al.*, 1962; Hadar and Mandelbaum, 1992; Voland and Epstein, 1994). In most cases, suppression was active against disease of plants sown directly into the amended soil, but occasionally suppression was not apparent until second plantings were made (Voland and Epstein, 1994).

Reduced disease severity following amendment with organic matter has been reported for a number of other pathogens including *Fusarium oxysporum f. sp. raphani*, *F. moniliforme* Sheldon, and *Verticillium dahliae* Kleb. (Huang *et al.*, 1986; Osunlaja, 1990a ; 1990b; Hawke, 1993).

There is a relationship between suppression and the general microbial activity in the soil following amendment (Davey and Papavizas, 1960). The importance of saprophytic microflora associated with the decomposition of organic matter was highlighted by Papavizas and Davey (1960) who observed increases in soil and rhizosphere fungi, streptomyces, and bacteria in association with suppression of damping-off caused by *R. solani*. *R. solani* is an effective saprophytic coloniser of plant residues (Blair, 1943; Garrett, 1962) which enables the fungus to survive in the absence of living hosts. Amendment with organic matter may therefore affect *R. solani* through increased competitive saprophytism. An example of competitive saprophytism in another pathogen is the competition between pathogenic and non-pathogenic strains of *Fusarium oxysporum*. The ability of non-pathogenic strains of *F. oxysporum* to control pathogenic strains relates to competition for carbon (Couteaudier and Alabouvette, 1990a). The superior competitiveness of the non-pathogenic strains for carbon was assumed to be the result of effectiveness in rapidly immobilising carbon compounds in exudates and effective utilisation of limited carbon supplies.

When considering the overall effect of organic matter on disease severity there are a number of factors that need to be considered. As mentioned above, there are a number of reports of reduced disease severity following the addition of organic amendments, but in many cases the underlying mechanisms are not known. Suppression induced by amendment with organic matter due to increased microbial activity would be classed as a form of general suppression, and by the definition of Gerlagh (1968) would not be transferable. A possible explanation for the variable effects of addition of organic matter is the carbon : nitrogen ratio of the material. Papavizas *et al.* (1962) found oat straw was more effective in reducing saprophytic survival of *R. solani* when its carbon : nitrogen ratio had been modified to 80 : 1 or

30 : 1 compared to 10 : 1. It has also been proposed that suppression is a function of the extent of decomposition of organic matter and microbial species-diversity (Hoitink and Fahy, 1986; Hoitink *et al.*, 1993). Over time, suppression of *Pythium ultimum* Trow. was lost as the organic matter decomposed. This was associated with chemical changes of the organic matter and shifts in the bacterial population away from predominantly pseudomonads in suppressive soils, to *Arthrobacter* Conn & Dimmick and *Micrococcus* Cohn in conducive soils. Although the precise organisms involved in each case of suppression will vary, succession of organisms would always exist. Hence, the organisms and biochemical processes involved in suppression may not be constant, but variable over time.

In a system as complex as the soil it is likely that more than one mechanism would be involved in disease suppression. Competitive colonisation of nutrient-rich substrates by a non-pathogenic fungus can result in the depletion of nitrogen or the supply of other nutrients (Wicklow, 1981). Secondary metabolites can then be produced which restrict invasion of competitors. In addition to affecting competitors during their saprophytic phase these secondary metabolites may also inhibit organisms in their pathogenic phase. If this is the case, both general and specific suppression would be observed.

Clearly, evidence exists for the role of inputs of organic matter in the suppression of plant diseases in general. Whether the effect is due to physical or chemical modification of the soil environment, stimulation of the growth of saprophytic competitors or induction of the production of metabolites inhibitory to the saprophytic and/or pathogenic phase of the life-cycle of the pathogen is not yet known. Potential mechanisms include both general and specific suppression, and in some cases both.

### 1.4.4 Discussion of definitions and their suitability

Definitions used to describe suppression and its possible mechanisms were developed from a few individual examples of suppressive soils, so, while useful for describing those soils and the mechanisms involved, they may be limited for fully describing new cases of suppression. Definitions of specific vs general suppression (Gerlagh, 1968) and parasitic vs saprophytic competition (Schnieder, 1984) are perhaps the most limited, due to the great detail in which they are defined. In contrast, the definition for soil receptivity (Alabouvette, 1986) is more flexible as the overall capacity of the soil to suppress disease is described. Hence, while detailed definitions provide a strong framework on which to base experimental work, new or modified definitions may need to be formulated for each new case of suppression.

While limited, the definitions of Gerlagh provide several parameters by which suppression in different soils can be compared: transferability, reaction to biocidal treatments (moist heat, chemical biocides) and site of action (bulk soil vs on or near roots). In addition to describing suppression in terms of a specific and / or general nature, comparison of soils using these three parameters provides insight into how and where the suppression is taking place.

In addition to definitions relating to suppression, the distinction between suppression and biological control will be briefly mentioned here. The development of suppression in soils involves changing the soil environment to be deleterious towards the pathogen, either by changing the ratio of organism(s) that are antagonistic toward the pathogen and/or by changing the physico-chemical environment. Biological control involves changing the balance of organisms by introducing a foreign, or native, organism into the soil in massive numbers where it controls the pathogen by mechanisms such as hyperparasitism, exclusion of preferred niches, antibiotic production or substrate acquisition.

### 1.4.5 Scope of suppression

As described in Section 1.4.2, numerous examples of naturally occurring and induced suppressive soils are known. In general, these examples report the ability of a suppressive soil to reduce disease caused by a single pathogen. The ability of a suppressive soil to reduce disease caused by a number of pathogens has important implications for determining the possible mechanism(s) of suppression. Two contrasting examples are *Fusarium*- and take-all-suppressive soils. An Australian soil suppressive to take-all also suppressed disease caused by *R. solani*, *P. irregulare*, *Gibberella zeae* (*F. graminearum*) and *F. culmorum* (Wildermuth, 1977). The degree of suppressiveness to these pathogens varied, implying that different mechanisms were involved in suppression of the causal agents, or that the mechanisms were effective to varying degrees for the different pathogens. In contrast, soil suppressive to *Fusarium* was not capable of suppressing other diseases (Alabouvette, 1986). Other suppressive soils (eg other take-all suppressive soils and amended potting mixes suppressive to *Rhizoctonia*) may be capable of suppressing a wider range of pathogens, but such investigations have not been reported.

Broad spectrum mechanisms of suppression (eg exclusion of common infection sites, depletion of substances required for microbial growth, release of widely acting biocidal compounds) allow suppression of a number of pathogens by a single mechanism. In contrast, narrowly acting mechanisms of suppression, such as hyperparasitism, may be very effective against single pathogens but have no effect on other pathogens. Similar severity of disease control may result from one broad spectrum mechanism or a number of narrowly acting mechanisms.

1.4.6 Suppression of *R. solani*

There is only one reported case of the induction of suppression of *R. solani* AG-8 (Lucas *et al.*, 1993). In contrast there has been fairly extensive literature published on the suppression of damping-off and root rots caused by *R. solani* from other AG's. Lucas *et al.* (1993) used field soils for 6 successive plantings of wheat with or without AG-8 inoculum under glasshouse conditions. Five successive plantings of wheat in the presence of *R. solani* AG-8 caused a disease decline. For the decline to occur, both the susceptible host (wheat) and the pathogen had to be present. *R. oryzae* was unable to cause a decline of disease due to *R. solani* AG-8. Micro-organisms were presumed to be involved in this decline but no mechanisms were suggested. A decline of Rhizoctonia root rot severity following an intial period of increase has been found in Western Australia, but this was not attributed to a build up of suppressiveness to *R. solani* (MacNish, 1988).

Inducement of disease decline with successive plantings of host and pathogen also occurs with disease due to *R. solani* AG-2-2 on sugarbeet, *R. solani* AG-2 on cauliflower, and *R. solani* AG-4 on radish and cucumber (Henis *et al.*, 1978b; Liu and Baker, 1980; Davik and Sundheim, 1984; Hyakumachi *et al.*, 1990).

Disease severity in a sugarbeet monoculture was constantly low for the first 3 years of a trial, increased from year 4-6, then declined back to the original severity by year 9 (Hyakumachi *et al.*, 1990). No correlation was found between disease severity and soil physico-chemical characteristics, or qualitative changes in the pathogen. Two important factors were highlighted by these authors: the rapid decline in the inoculum potential of the pathogen

and the role of antagonistic micro-organisms in the decline. The mechanism proposed was that in a monoculture, the population density of the pathogen initially increases as the incidence and severity of the disease increases. However, this increase induces an increase in the population of antagonistic micro-organisms which subsequently reduces the inoculum potential of the pathogen. The time-scale of the initial increase in disease and subsequent decline is very similar to that described in Section 1.3 for Rhizoctonia barepatch decline.

A major difference between induced suppression of disease caused by *R. solani* AG-2-2 and AG-8 (Hyakumachi *et al.*, 1990; Lucas *et al.*, 1993) and Rhizoctonia barepatch decline (Roget, 1995) is the need for monoculture. For both cases of induced suppression continued monoculture was necessary. However, Rhizoctonia barepatch decline was observed in 4 different rotations. Although *R. solani* AG-8 has a wide host range (Section 1.2.3) and all plants in these rotations could be infected, continued plantings of one particular host was not required for disease decline.

Suppression of disease caused by *R. solani* has also been induced in potting mixes containing relatively high amounts of composted hardwood bark (Chet and Baker, 1980; Nelson and Hoitink, 1982; Nelson *et al.*, 1983; Nelson and Hoitink, 1983). This suppression was eliminated by heat (60°C) and gamma irradiation (25 krad) and can therefore be assumed to be of biological origin (Nelson and Hoitink, 1983). Of direct relevance to the suppression of Rhizoctonia barepatch is the fact that the hardwood bark became more suppressive with increasing age / degree of decomposition (Nelson and Hoitink, 1983). This relates to the possible role of plant residues to disease suppression described in Section 1.4.3.

As the development of suppression of Rhizoctonia root rot at Avon in South Australia (Roget, 1995) occurred following the adoption of direct drilling practices, it is relevant to consider briefly the possible effects of tillage on the soil biota and the subsequent effect on disease suppression.

It is well illustrated that reduced tillage practices lead to an increase in populations of bacteria, fungi, actinomycetes, earthworms, VA mycorrhizal fungi and soil fauna (Jasper *et al.*, 1989a; 1989b; 1991; Gupta, 1994; Rovira, 1987). While total populations increase with reduced tillage, effects on specific groups within these populations will vary between soils. Development of suppression will be dependant on the relative effects of tillage on beneficial and deleterious populations. Tillage can affect these populations through physical modification of the soil (eg increased bulk density, pore size distribution, physical damage to the organisms), chemical modification of the soil (eg increased levels organic carbon, levels of extractable cations) and modification of weed populations and plant residue levels.

Trends for effects of tillage on specific diseases are known but conflicting results occur. Inconsistencies in the effects of tillage are presumably due to the complex interactions possible in different environments. Direct drill or reduced tillage are associated with consistent reductions of Rhizoctonia root rot and control of *Heterodera avenae*, but the response of take-all to tillage is inconsistent (Neate, 1994). Mechanisms explaining the consistent reaction of Rhizoctonia root rot and *H. avenae* to tillage are known, but in general the final outcome of the complex interaction between host, pathogen and environment are unknown. Interactions likely to occur between *R. solani* and sections of the soil biota enhanced by reduced tillage are discussed in more detail in Section 1.5.

1.4.7 Biological suppression of *R. solani*

The fungal genera most often described as potential biological control agents of *Rhizoctonia* are *Trichoderma*, *Gliocladium* Corda and *Penicillium* Link ex Fr (Naiki, 1986; Maplestone *et al.*, 1991; Huang and Kuhlman, 1992; Hwang and Chakravarty, 1993). All three genera are described as being involved in the suppression of damping-off induced by the application of composted hardwood bark (Nelson *et al.*, 1983). Direct antagonism of *R. solani* by *Trichoderma harzianum* has been observed (Ridout *et al.*, 1986). The biocontrol fungus initially coils around the hyphae of the pathogen and then degrades its cell-wall (Benhamou and Chet, 1993). The existence of populations of *Trichoderma* in Rhizoctonia barepatch decline soils is not known, but due to their ability to produce cellulases (Mandels, 1975) *Trichoderma* populations may be enhanced by the continued inputs of organic matter into these soils.

Control of disease due to *R. solani* on hosts including creeping bentgrass, snap beans, sugar beet, potato and capsicum has been achieved with addition of binucleate *Rhizoctonia* spp. (Burpee and Goulty, 1984; Cardoso and Echandi, 1987; Herr, 1988; Escande and Echandi, 1991; Harris *et al.*, 1993a; 1993b; 1994). These fungi have not been reported as antagonists of *R. solani* AG-8, but as binucleate fungi are known to exist in southern Australian cereal fields (Neate, 1985; Roberts and Sivasithamparam, 1986; Section 1.2.2) it is possible they may suppress *R. solani* AG-8 in these areas.

Other fungi reported as antagonists of *Rhizoctonia* include: *Laetisaria arvalis* Burdsall, *Arthrobotrys superba* Corda sensu Dreschler, *Verticillium biguttatum* Nees ex Link, and *Stachybotrys elegans* (Pidopl.) W. Gams, comb. nov. (Lewis and Papavizas, 1992; Saxena *et al.*, 1992; Den Braver *et*

*al.*, 1993; Tweddell *et al.*, 1994). Antagonism of *Rhizoctonia* by bacteria such as *Bacillus spp.* Cohn and *Pseudomonas spp.* Migula (now *Burkholderia* spp. ex Burkholder) has also been observed (Schmiedeknecht, 1993; Zaspel and Suss, 1993).

All, or some of these previously reported antagonists of *Rhizoctonia* may be involved in the suppression of Rhizoctonia barepatch. However, their overall impact depends on their indigenous populations in the soils in question, their ability to survive in a relatively harsh environment and their competitive ability or rhizosphere competence. While it is unlikely that a single organism is responsible for such a dramatic decline as observed in the described field soil, this possibility can not be ruled out until the microbial population has been investigated in detail.

**1.5 Soil biotic interactions in reduced tillage systems**

In Sections 1.4.6 and 1.4.7 observed cases of suppression and biological control of *Rhizoctonia* were described. Many organisms are capable of inhibiting *Rhizoctonia* in some way, so in this section discussion focuses on interactions likely to occur in reduced tillage systems. Interactions between *R. solani* and other soil biota can be divided into two groups, direct antagonism (Section 1.4.5) and competition, and indirect effects, such as the physical disruption of the mycelium of *R. solani* AG-8 through movement of meso or macro-fauna through the soil.

Populations of beneficial vesicular arbuscular (VA) mycorrhizal fungi are enhanced by reduced soil disturbance (Jasper *et al.*, 1989a; 1989b; 1991) and have been implicated in disease suppression (Linderman, 1992). Likely mechanisms are improved plant vigour due to increased nutrient uptake,

greater ability of mycorrhizal plants to tolerate environmental stress such as drought (Linderman, 1992) and increased tolerance to root damage (S. Smith, pers. comm.). Prior colonisation of the root by VA fungi appears to be necessary for an effect on disease severity (Stewart and Pfleger, 1977; Graham and Menge, 1982; Caron *et al.*, 1980). Root colonisation by VA fungi is known to decrease when soil phosphorus levels are relatively high. The inhibition of colonisation is actually due to plant phosphorus concentrations rather than P concentrations in soil (Menge *et al.*, 1978; Levy and Krikun, 1980). A role of VA fungi in suppression at Avon is unlikely due to annual application of phosphorus (10 kg P / ha as superphosphate) and a more than adequate soil P content of 65 mg/kg ( Roget, 1995).

The small number of reports on bacterial populations in reduced tillage systems indicate increased numbers compared to cultivated soils (Gupta and Germida, 1988; Hassink *et al.*, 1991). Potential interactions with *R. solani* AG-8 which might contribute to suppression therefore include competition for nutrients due to the increased populations (Section 1.4.2) or direct interactions. Direct interactions are involved in the suppression of damping-off caused by *R. solani* by *Bacillus* and *Pseudomonas* spp. and reduced disease severity caused by *R. solani* on potato by *Bacillus* spp. (Schmiedeknecht, 1993; Zaspel and Suss, 1993).

In addition to other micro-flora, interactions of *R. solani* with soil fauna would also occur. Limited information is available on nematode populations in reduced tillage systems compared to cultivated systems but control of disease caused by *R. solani* with a nematode (*Aphelenchus avenae* Bastian) under controlled conditions has been observed (Barnes *et al.*, 1981). The general response of nematode populations to tillage at Avon was higher populations in direct drilled soil compared to cultivated soil (Yeates and

Bird, 1994). The involvement of a deleterious interaction between *R. solani* AG-8 and nematodes such as *A. avenae* in suppression of Rhizoctonia barepatch is possible, but very large nematode populations would be necessary (ca. 0.25 - 1m per 1.5 kg after Barnes *et al.*, 1981).

The mycophagous nature of protozoa, amoebae in particular, is well documented. Mycophagous amoebae have been shown to attack oospores of *Pythium ultimum*, conidia of *Cochliobolus sativus* (Ito & Kuribayashi) Dreschler ex Dastur, hyphae and microconidia of *Fusarium oxysporum* and spores of *Chalara elegans* (*T. basicola*) (Dreschler, 1936; Old, 1977a; 1977b; 1978). Of particular relevance to the suppression of Rhizoctonia barepatch is the proposed role of mycophagous amoebae in decline of take-all (Chakraborty, 1983a; b). Numbers of mycophagous amoebae were higher in take-all suppressive soil compared to non-suppressive soil, and disease severity in a pot bioassay was reduced by the addition of mycophagous amoebae. Physical damage to hyphae occurred with extensive erosion and discrete perforations of the hyphal walls observed. With the reported interactions between protozoa and fungal pathogens and the ubiquitous presence of protozoa in soil (Cutler, 1919 ; 1920), potential exists for a role of protozoa in suppression of Rhizoctonia barepatch. Evidence for the role of protozoa would be higher populations in suppressive soil compared to non-suppressive soil and physical damage of *R. solani* AG-8 hyphae.

Earthworm numbers have been demonstrated to double within three years of adoption of direct drilling in Australia (Rovira *et al.*, 1987). Similar observations have been made by Marinissen (1992) who observed earthworms in reduced cultivation plots but not in conventionally cultivated plots in a trial in The Netherlands. Evidence for the potential role of earthworms in suppression of Rhizoctonia barepatch is provided by Stephens

*et al.* (1994) who showed reduced disease severity due to *R. solani* AG-8 in the presence of the earthworm *Aporrectodea trapezoides* Dugés under glasshouse conditions. Reduced disease severity has also be reported due to interactions between *R. solani* and two collembolan species, *Proisotoma minuta* Tullberg and *Onychiurus encarpatus* Denis (Curl *et al.*, 1985).The role of earthworms and other meso and macro-fauna in suppression is therefore possible and could be indicated by reduced levels of suppression in situations where this section of the soil biota is unlikely to be present.

## 1.6 *Rhizoctonia* populations : quantity and quality

### 1.6.1 Quantification of *Rhizoctonia* populations in soil

Quantification of *Rhizoctonia* populations in suppressive and non-suppressive soil is vital for understanding the mechanisms of the suppression. Initially, disease severity in the Rhizoctonia barepatch decline soil increased (Roget, 1995; Section 1.3), but whether the subsequent decline was associated with a decline in pathogen population is not known. From the definition of soil receptivity of Alabouvette (1986), disease severity is lower in suppressive soil compared to non-suppressive soil at similar inoculum densities (Section 1.4.1). Alabouvette (1986) also noted for *Fusarium* wilt that "absence of disease could not always be accounted for by absence of the pathogen". Hence, for an understanding of disease suppression it is important to quantify both disease severity and the pathogen population as a correlation between them may not always exist.

Quantification of *Rhizoctonia* in soil is difficult due to a lack of discrete structures. Sclerotia of *R. solani* AG-8 exist but they are not important for the survival of this fungus (Section 1.2.1) so quantification of sclerotia would

not be relevant. Rather than separating the population into distinct morphological types, the *Rhizoctonia* population can be separated into distinct functional groups (Neate and Schneider, in press). Four types are defined : total, viable, active and pathogenic. To quantify these types adequately, different methods are clearly required.

Quantification of the total population requires a method that does not rely on fungal growth; appropriate methodology may be nucleic acid or serological detection. Viable *Rhizoctonia* are the part of the total population that are culturable. Active *Rhizoctonia* are the part of the total population that are growing in the soil (ie metabolically active). Pathogenic *Rhizoctonia* are the part of the total population that will incite disease. The choice of method is dependent on the population type in question but the use of different methods will elucidate the functional capacity of a particular soil *Rhizoctonia* population as well as quantifying single components of the population. The advantage of using different methods has been discussed, with Davey and Papavizas (1962) concluding that use of the debris particle method in conjunction with the buckwheat colonisation method gave the most complete information as they reflected the status of the quiescent and active *Rhizoctonia* in soil respectively.

Recent developments in molecular-based detection procedures have resulted in the development of a DNA probe specific to *R. solani* AG-8 (Matthew *et al.*, 1995) and a procedure for extraction of DNA from soil organic matter (Whisson *et al.*, 1996 after Raeder and Broder, 1985). The quantity of DNA of *R. solani* AG-8 present in soil organic matter can be determined by comparison of the signal (radio-active or chemi-luminescent) from total DNA extracted from organic matter with diluted DNA extracted from pure culture

of *R. solani* AG-8 following slot-blot hybridisation with the AG-8 specific probe.

Estimation of viable *Rhizoctonia* populations in soil can be done using small clumps of soil (Ko and Hora, 1971), soil formed into uniform pellets (Henis *et al.*, 1978b; van Bruggen and Arneson, 1986) and organic matter sieved from soil (Weinhold, 1977; Neate, 1987), followed by incubation on selective or semi-selective agar media. Quantification of viable *Rhizoctonia* was expressed as the number of *Rhizoctonia* colonies formed per unit weight of soil, or number of debris particles or soil pellets from which *Rhizoctonia* grew.

Saprophytic colonisation of plant material or other substrate as a measure of the active *Rhizoctonia* population has been determined using plant segments, seeds, and filter paper immersed in nutrient solution and antibiotics (Blair, 1945; Papavizas and Davey, 1959; Sneh *et al.*, 1966, Papavizas *et al.*, 1975; Huang and Kuhlman, 1989). The *Rhizoctonia* population was then expressed as the number of pieces of plant material or filter paper colonised by *Rhizoctonia*.

Growing susceptible plants in soil and assessing disease severity as a measure of the pathogenic *Rhizoctonia* population is a well established method (Davey and Papavizas, 1962; Sneh *et al.*, 1966; MacNish, 1984). A measure of the pathogenic *Rhizoctonia* population is therefore expressed in terms of disease severity or incidence rather than amount of fungus in the soil.

### 1.6.2 Qualification of *Rhizoctonia* populations in soil

Quantification of *Rhizoctonia* populations will determine how much pathogen is present as well as the amount of disease it can cause. However, when quantifying the population it is assumed that the pathogenicity of propagules is similar to that in non-suppressive soil. For example, a suppressive soil may have reduced disease severity but similar numbers of active propagules of pathogen as a non-suppressive soil. This could imply antagonism by other organisms and / or, it could imply reduced pathogenicity of the isolates. Hence, following quantitative isolation of *Rhizoctonia* from soil, pathogenicity tests can provide additional information on the mechanisms of suppression.

Pathogenicity tests are based on inoculation of sterile or partially sterile soil with *Rhizoctonia*, sowing of susceptible host plants, and assessment of disease severity following growth under controlled conditions (McDonald and Rovira, 1985; Carling and Sumner, 1992; Sweetingham and MacNish, 1994). Fungal inoculum can be mycelial mats or infested substrates such as cereal grain or millet seed. Standardisation of these tests is important to eliminate variability caused by differences in incubation conditions, test plants and assessment method, and also because the nutrition of the fungus has been reported to influence virulence of *Rhizoctonia* (Weinhold *et al*., 1969; Khotin and Polyakova, 1992).

Qualitative assessment such as the pathogenicity test (McDonald and Rovira, 1985; Carling and Sumner, 1992; Sweetingham and MacNish, 1994), or assessment of growth *in vitro* or in soil (Neate and Benger, 1995), are suitable methods for study of *Rhizoctonia* isolates following isolation from

soil. To fully explain Rhizoctonia barepatch decline the *Rhizoctonia* populations in decline and non-decline soils need to be compared quantitatively. Further, qualitative comparison may be useful to explain any lack of correlation between pathogen population and disease severity. Isolates of *R. solani* can vary greatly in morphological and pathogenic characteristics (O'Brien, 1994), and high levels of genetic heterogeneity can occur in groups (ie ZG, AG) both between and within geographic locations (Duncan *et al.*, 1993). Hence, even in barepatch dominated by a single ZG (MacNish and Sweetingham, 1993b; MacNish *et al.*, 1993) considerable qualitative differences may exist between isolates.

### 1.7 General aims of project

The general aims of this project were to characterise the observed decline of Rhizoctonia barepatch at the Avon trial site by comparison of the decline soil with a similar soil still exhibiting disease symptoms and to determine likely mechanisms by which the disease was being reduced.

This project was initiated following the observation of declining severity of Rhizoctonia barepatch in a long-term tillage and rotation trial at Avon in South Australia. Preliminary experiments indicated suppression was due to biological factor(s) but little else was known. A description of the trial site and soils used are given in Chapter 2. The first question to be answered related to the underlying basis of this suppression, that is, was it biological, chemical or physical in nature? Chapter 3 details the use of existing methods to establish the biological nature of suppression.

The concept of soil receptivity has previously been used to identify and compare suppressive soils. This approach was used to compare soils

suppressive and non-suppressive to Rhizoctonia barepatch on the basis of receptivity / suppressiveness to the causal agent, *R. solani* AG-8, and a range of other common cereal pathogens (Chapter 4).

It is necessary to quantify the pathogen population in suppressive soils to further understand the underlying mechanisms. Chapter 5 deals with the quantification of the *Rhizoctonia* populations in the two test soils and discusses the relative amounts of the different propagule types quantified. Further quantification of *Rhizoctonia* is described in Chapter 6 with experiments designed to determine the likely site of action of suppression.

An initial comparison of microbial populations in suppressive and non-suppressive soil was carried out (Chapter 7). Experiments to compare specific groups of organisms and the whole soil biota in suppressive and non-suppressive soils were conducted.

A general discussion of results and implicated mechanisms of suppression forms Chapter 8.

# Chapter 2: General Materials and Methods

## 2.1 Field site and soil sampling

Soils were collected from the Avon field site 100 km north of Adelaide in South Australia (34° 14' S, 138° 18' E). Two field trials were used from this site, "Phase II" (PhII) and "Rhizoctonia monitoring" (RSM) trials. The trials were approximately 50 m apart. Soil type was a solonized brown soil (Stace *et al.*, 1968), classified as mixed calcic Palexeralf (Soil Survey Staff, 1975), with winter-dominant rainfall averaging 350 mm per year (Rovira, 1986).

PhII was established in 1978 and consisted of 6 replicate blocks of a 2 x 4 factorial tillage x rotation trial. Tillage treatments were conventional cultivation (CC) or direct drilling (DD). Rotation treatments were wheat-wheat (w-w), wheat-annual volunteer pasture (w-p), wheat-peas, and wheat-sown medic pasture. Plots were 100 m long and 1.5 m wide. These treatments have been fully described previously (Rovira, 1986; Roget, 1995).

RSM was established in 1990 and consisted of 5 replicate blocks of a 2 x 3 factorial direct drilling method x rotation trial. Direct drilling was done with either 10 cm shares or deep narrow points, and the rotations were w-p, p-w, and p-p-pw. Plots were 50 m long and 1.5 m wide.

Suppressive soil was collected from PhII w / p DD plots and non-suppressive soil from RSM w / p DD (10 cm share) plots. The replicates used are described for each experiment in the text. Throughout the thesis soil from PhII is referred to as "suppressive" and soil from RSM as "non-suppressive".

Soils were sampled from the top 10 - 15 cm of field plot replicates 1 - 5 unless stated otherwise in the text. Sampling dates and water contents used for experiments are listed in Table 2.1. Soils were adjusted to the listed water content by adding distilled water prior to any pre-incubation steps. To reduce water loss due to evaporation during pre-incubation, plastic lids were placed on all pots. After sowing, approximately 1 cm depth of white plastic beads (3 mm dia.) added to the soil surface to reduce evaporation. Soils were watered to weight every 2 - 3 days to maintain the initial water content.

### 2.2 Production of *Rhizoctonia* inoculum

Artificial inoculum of *Rhizoctonia* was prepared on millet seed (*Panicum miliaceum* L.) using the method of McDonald and Rovira (1985). Millet seed was soaked in distilled water (overnight), drained (1 hour), and placed in conical flasks. Flasks were plugged with non-absorbent cotton wool and autoclaved at 121°C for 60 minutes on each of 3 successive days. Agar plugs of *Rhizoctonia* were added to the sterile millet which was then incubated for approximately 2 weeks at 25°C. Flasks were shaken every 3-4 days to ensure thorough colonisation of the millet seed by the fungus. Colonised millet seed was dried in a laminar flow and stored at -20°C until use.

| Section | date of soil sampling | water content used (% w/w) |
|---|---|---|
| 3.2.1 | 21/5/93 | 10 |
| 3.2.2.1 | 3/8/94 | 10 |
| 3.2.2.2 | 7/8/95 | 12 |
| 3.2.3.1 | 10/1/95 | 10 |
| 3.2.3.2 | 10/1/95 | 10 |
| 4.2.1.1 | 14/9/94 | 10 |
| 4.2.1.2 | 10/1/95 | 12 |
| 4.2.1.3 | 3/8/95 | 12 |
| 4.2.2.1 | 10/1/95 | 12 |
| 4.2.2.2 | 7/9/95 | 12 |
| 4.2.3 | 10/1/95 | 12 |
| 4.2.4 | 10/1/95 | 15 |
| 4.2.5 | 3/8/95 | 12 |
| 4.2.6 | 3/8/95 | 12 |
| 5.2.1.1 | 21/5/93 | 10 |
| 5.2.1.2 | 18/8/93 | 12 |
| 5.2.1.3 | 10/1/95 | 12 |
| 5.2.3.1.1 | 30/3/93 | NA |
| 5.2.3.1.2 | 30/3/93, 21/5/93 | NA |
| 5.2.3.2 | 18/8/93 | NA |
| 6.2 | 11/4/95 | 12 |
| 7.2.1 | 7/1/95 | NA |
| 7.2.2 | 7/1/95 | NA |
| 7.2.4 | 7/1/95 | NA |

Table 2.1: Sampling dates for PhII and RSM soils and soil water contents used in experiments
(NA = not applicable)

## 2.3 Plant species and seed treatments

The plant species and cultivars used in pot experiments were: wheat (*Triticum aestivum* L. cv 'Spear', cv 'Machete' for Section 4.2.5), barley (*Hordeum vulgare* L. cv 'Chebec'), oats (*Avena sativa* L. cv 'Echidna'), peas (*Pisum sativum* L. cv 'Dundale'), lupin (*Lupinus angustifolias* L. cv 'Warrah'), subterranean clover (*Trifolium subterranean* L. cv 'Mt. Barker'), medic (*Medicago littoralis* Loisel. cv 'Harbinger') and tomato (*Lycopersicum esculentum* Mill cv 'Grosse Lisse', cv 'Quick Pick' for Section 4.2.2).

All seeds were surface disinfested prior to sowing. For surface disinfestation seeds were soaked in 1 % sodium hypochlorite with or without 10 % ethanol for 10 min and rinsed 2-3 times with sterile distilled water. Legume seeds were then coated in a slurry of commercial *Rhizobium* inoculum (Inoculant Services, Nitri-Life, Victoria, Australia; Bio-Care Technology Pty Ltd, New South Wales, Australia).

## 2.4 Sowing and inoculation of pre-incubated soils and intact soil cores

To minimise the disturbance of soil prior to seeding a thin metal spatula was inserted into the soil and the seed or fungal inoculum placed into the resulting slit then covered with soil.

## 2.5 Harvesting and processing of pot experiments

Plants were washed free of soil with a strong jet of tap water. Roots were stored at 4°C until disease severity ratings were made (up to 4 days). Dry weights were determined after approximately 4 days at 60°C.

## 2.6 Rhizoctonia disease assessment

### 2.6.1 Cereals

The 0-5 scale of McDonald and Rovira (1985) was used to score the severity of Rhizoctonia root rot for all cereal plants (wheat, oats and barley). The categories corresponded to:

| | | |
|---|---|---|
| 0 | = | no disease |
| 1 | = | <25 % of primary roots with infected root tips/cortical rot |
| 2 | = | 25-50 % of primary roots with infected root tips/cortical rot |
| 3 | = | 50-75 % of primary roots with cortical rot and truncation |
| 4 | = | >75 % of primary roots with cortical root and severe truncation |
| 5 | = | 100 % primary roots severely truncated, top stunted and moribund. |

2.6.2 Non-cereals

The root disease index (RDI) of Sweetingham and MacNish (1994) was used to score the level of Rhizoctonia root rot for all non-cereals (lupin, medic, pea, sub-clover and tomato). The RDI was the mean lesion severity of the first six 1 cm segments of tap root. Lesions were scored on a 0-5 scale and the categories corresponded to:

| | | |
|---|---|---|
| 0 | = | no lesion |
| 1 | = | shallow cortical necrosis (but lesion greater than 1 mm diameter) |
| 2 | = | necrosis penetrating the cortex to the stele |
| 3 | = | cortex completely rotted girdling the stele |
| 4 | = | cortex completely rotted and some stellar necrosis |
| 5 | = | cortex and stele completely rotted to sever the tap root (where a root is completely severed by disease the remaining segments are scored as 5) |

## 2.7 Soil water content determination

Soil water content was determined on a mass basis after drying of 20-30 g samples at 105°C to constant weight in aluminium trays.

## 2.8 Statistical analysis

Results were subjected to single or multi-factor analysis of variance using the GENSTAT 5 computer package. Sources of significant difference between means were determined using Fisher's protected LSD at $P = 0.05$.

# Chapter 3: Nature of the suppression

## 3.1 Introduction

The first hypothesis tested was whether the suppression observed at Avon was biological, chemical or physical in nature. Methods to demonstrate a biological involvement in the suppression are based on the transfer of suppressive characteristics to another soil (Rovira and Wildermuth, 1981; Rovira, 1982), and on the elimination of suppressive characteristics by application of biocidal treatments (Alabouvette, 1986).

Two approaches were used to test for presence of biological suppression and one for chemical suppression.

The transfer of suppression test described by Rovira and Wildermuth (1981) and Rovira (1982) was used to determine if suppressive characteristics could be transferred to a sterile soil by addition of suppressive soil (10 % w / w) to the sterile soil. Addition of non-suppressive soil was used as a comparison.

An alternative method for establishing a biological involvement in suppression (Alabouvette, 1986) was used in two subsequent experiments, to allow conclusions on the general and / or specific nature of the suppression to be made. In this method biocidal treatments are applied to determine if suppressive characteristics are removed. In the first experiment, complete destruction of the soil biota was attempted using gamma irradiation or steam pasteurisation at 80°C, and in the second partial sterilisation with steam pasteurisation temperatures ranging from 50 - 80°C was used. Isolation and quantification of selected microbial groups following steam pasteurisation

was done to determine groups potentially involved in the suppression. Results of the microbial isolations are presented and discussed in Chapter 7.

The potential chemical involvement in suppression was tested using the effect of water soluble extracts from suppressive soil and non-suppressive soil on the *in vitro* growth of *R. solani* AG-8 in solid and liquid media . A role of soil chemical characteristics would be assumed to exist if addition of soil extracts inhibited the *in vitro* growth of *R. solani* AG-8.

**3.2 Materials and Methods**

3.2.1 Transfer of suppression

Suppressive and non-suppressive soil was mixed together in equal proportions and either autoclaved at 121°C for 50 min on 2 successive days or steam pasteurised at 80°C for 30 min.

Suppressive or non-suppressive soil was thoroughly mixed with either the autoclaved or steam pasteurised soil. Unsterilised soil was added at a rate of 10 % (w/w) to the cooled, heat-treated soils. Following mixing, the soil-mix (300 g) was added to plastic 300 ml capacity pots and inoculum was added. Two millet seeds of *R. solani* AG-8 (isolate RS-21) inoculum (Section 2.2) were added to each pot, one approximately 4 cm and one 8 cm below the soil surface. Pots were pre-incubated for 2 weeks prior to sowing to allow colonisation of the disturbed soil by *R. solani*. Controls were either 100 % steam pasteurised or autoclaved soil, with and without inoculum of *R. solani* AG-8. The four treatments were: suppressive soil + *R. solani*, non-suppressive soil + *R. solani*, heat treated soil + *R. solani*, and heat treated

soil - *R. solani*. There were 5 replications of each treatment in a randomised complete block design (RCBD).

Following the 2 week pre-incubation, five surface disinfested wheat seeds were sown with minimum soil disturbance in each pot. Pots were incubated for a further 5 weeks at 15°C with a 9 hour day and photon irradiance of 335 μmols $m^{-2}$ $s^{-1}$. At harvest, plants were assessed for disease severity, shoot dry weight and root fresh weight.

3.2.2 Removal of suppression with gamma irradiation or steam pasteurisation

3.2.2.1 Experiment 1 - Gamma irradiation and steam pasteurisation (80°C)

Intact soil cores (10 cm dia.) were taken from the top 10 - 15 cm of suppressive and non-suppressive soil and treated with one of two biocidal treatments (steam pasteurisation at 80°C for 30 min or gamma irradiation at 25 kgys, "Steritech" Victoria, Australia) or left untreated. The experiment was a 2 x 3 x 2 factorial of field soil type (suppressive or non-suppressive) x soil sterilisation treatment x +/- *R. solani* AG-8 (isolate RS-21) inoculum with 5 replicates of each treatment combination in a RCBD. Inoculum preparation was as described in Section 2.2. In inoculated cores half of the inoculum (2 millet seeds) was added at 4 cm depth and half at 2 cm depth. Cores were pre-incubated for 2 weeks to allow the fungus to colonise the soil before sowing with 5 surface disinfested wheat seeds. Pots were incubated for a further 5 weeks at 15°C with a 9 hour day and light intensity of 180 μmols $m^{-2}$ $s^{-1}$. Plant nutrient solution (5 ml half strength Hoaglands solution without manganese (Mn)) was added weekly. Manganese was not added due to a known interaction with plant defence mechanisms and the possibility of increased Mn availability following heat treatment of the soil

(Cawse, 1975; McCay-Buis *et al.*, 1995). At harvest, plants were assessed for disease severity, shoot dry weight and root dry weight.

3.2.2.2 Experiment 2 - Steam pasteurisation (50 - 80°C)

Intact cores were taken as for Experiment 1 (Section 3.2.2.1) and steam pasteurised at either 50, 60, 70 or 80°C for 30 min or left untreated. The experiment was a 2 x 5 x 2 factorial of field soil type (suppressive or non-suppressive) x steam pasteurisation temperature x +/- *R. solani* AG-8 inoculum with 4 replicates of each treatment combination in a RCBD. Inoculation, sowing and incubation were carried out as described for Experiment 1.

3.2.3 Effect of soil extracts on the *in vitro* growth of *R. solani* AG-8

To compare the effects of water soluble soil extracts on the *in vitro* growth of *R. solani* AG-8, extracts were made from suppressive soil and non-suppressive soil which had been gamma irradiated or left untreated. The effect of adding extracts to solid and liquid media inoculated with *R. solani* AG-8 was assessed and compared with a control of sterile water.

3.2.3.1 Experiment 1 - Solid medium

The experiment was a 2 x 2 x 2 factorial of soil type (suppressive or non-suppressive) x + / - sterilisation x inoculation method (liquid or filter paper) with 2 replicate plates of each treatment combination.

Each soil (150 g) was shaken thoroughly by hand in 150 ml sterile water for 5 min and left to settle for approximately 10 min. The solution was filtered through a sterile 9 cm Whatman No. 1 filter paper, left to settle for 10 min, filtered through two sterile 9 cm Whatman No. 1 filter papers, then a sterile 0.2 μm Millipore filter. Controls consisted of filtered sterile distilled water.

An agar plug (ca. 4 mm dia.) from a culture of *R. solani* AG-8 (RS-21) on potato dextrose agar (PDA) (Appendix 1) was inoculated near the edge of a 9 cm petri plate containing PDA. Soil extract was added to the centre of the PDA plate as 50 μl of liquid or as a 1 $cm^2$ piece of sterile Whatman No. 50 filter paper that had been dipped in the extract. Approximately 30 μl of liquid was retained in each 1 $cm^2$ of filter paper. Petri plates were sealed with parafilm (American National Can™, Greenwich) and incubated at 25 °C in the dark. Colony radii of *R. solani* AG-8 were measured 3, 7 and 9 days after inoculation. The point of fungal inoculation was approximately 3.3 cm from the soil extract addition.

3.2.3.2 Experiment 2 - Liquid medium

The treatments were the addition of 1 ml of soil extract from suppressive or non-suppressive soil that had been prepared in Experiment 1 (Section 3.2.3.1). Controls were the addition of 1 ml of sterile distilled water or no addition. There were three replicate plates for each treatment.

An agar plug (ca. 4 mm dia.) from a PDA culture of *R. solani* AG-8 (RS-21) was inoculated into a 9 cm petri plate containing 20 ml of sterile neutral dox yeast (NDY) (Appendix 1) broth. Plates were sealed with parafilm and incubated at 20°C in the dark. Colony diameter of *R. solani* AG-8 was measured 4 and 6 days after inoculation.

**3.3 Results**

3.3.1 Transfer of suppression

Disease severity was significantly less ($P < 0.001$) when 10 % suppressive soil was added to either autoclaved or steam pasteurised soil compared to when 10 % non-suppressive soil was added (Fig. 3.1). Addition of either suppressive or non-suppressive soil resulted in significantly less disease compared to the inoculated 100 % autoclaved or pasteurised soil. Soil treatment method (autoclaving or steam pasteurisation) did not affect disease severity. No disease occurred in the uninoculated pots.

The response of root fresh weight and shoot dry weight to the four treatments was similar (Table 3.1). Plant growth was significantly higher ($P < 0.001$) in uninoculated 100 % autoclaved or steam pasteurised soil compared to inoculated 100 % autoclaved or steam pasteurised soil. Plant growth was similar following addition of either 10 % suppressive or non-suppressive soil. Steam pasteurised soil was more favourable ($P = 0.05$) for plant growth than autoclaved soil.

### 3.3.2 Removal of suppression with gamma irradiation or steam pasteurisation

#### 3.3.2.1 Experiment 1 - Gamma irradiation and steam pasteurisation (80°C)

In the untreated field soil, there was significantly ($P = 0.01$) less disease due to *R. solani* AG-8 in suppressive soil compared to non-suppressive soil for both the inoculated and uninoculated treatments (Fig. 3.2). No disease occurred in uninoculated $\gamma$-irradiated or pasteurised soil. In both pasteurised and $\gamma$-irradiated soil, there was no significant difference between the suppressive and non-suppressive soils in the inoculated and uninoculated treatments. The method of soil treatment (pasteurisation or irradiation) was not significant.

Plants grown in untreated suppressive and non-suppressive soil had the same shoot dry weight for both inoculated and uninoculated soil (Table 3.2). Shoot dry weight per plant was significantly ($P = 0.01$) reduced by inoculation of pasteurised or $\gamma$-irradiated soil. Shoot dry weight per plant was significantly ($P = 0.01$) higher in inoculated field soils compared to inoculated pasteurised or $\gamma$-irradiated soils. For uninoculated soils shoot dry weight per plant was similar in pasteurised and $\gamma$-irradiated soils and higher in pasteurised soil compared to field soil.

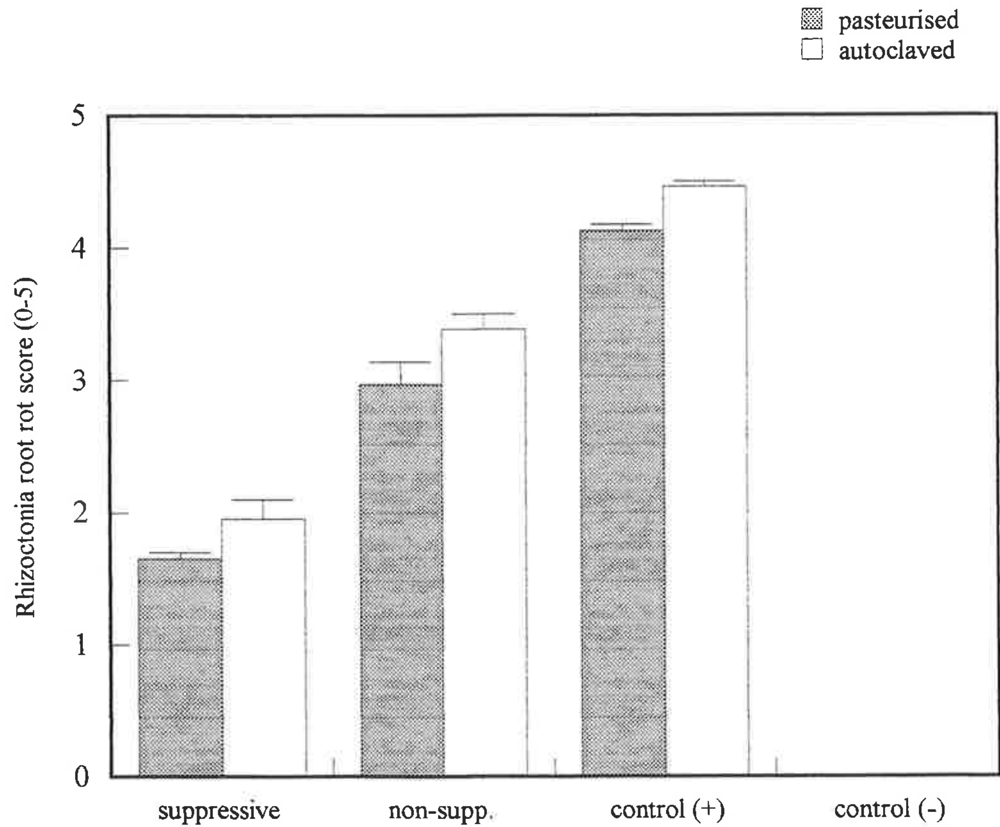


Figure 3.1: Transfer of suppression to autoclaved or steam pasteurised soil. Treatments were plus 10 % suppressive soil + *R. solani* AG-8, plus 10 % non-suppressive soil +*R. solani* AG-8, no soil addition plus *R. solani* AG-8 (control (+)) and no soil addition minus *R. solani* AG-8 (control (-)). Bars are means of 5 replicate pots and lines indicate standard error of the data.

| soil type | soil treatment | root fresh weight (mg/plant) | shoot dry weight (mg/plant) |
|---|---|---|---|
| suppressive soil + AG-8 | autoclaved | 401 (17) | 64 (5) |
| | pasteurised | 587 (14) | 72 (5) |
| non-supp. soil + AG-8 | autoclaved | 359 (32) | 58 (5) |
| | pasteurised | 656 (71) | 82 (8) |
| control + AG-8 | autoclaved | 90 (4) | 23 (1) |
| | pasteurised | 119 (7) | 25 (1) |
| control - AG-8 | autoclaved | 1214 (28) | 93 (2) |
| | pasteurised | 1349 (44) | 117 (2) |
| soil treatment | | ** [108] | ** [12] |
| soil type | | * [153] | * [17] |
| soil type.treatment | | NS | NS |

Table 3.1: Root fresh weight and shoot dry weight per plant for wheat plants grown in autoclaved or pasteurised soil mixed with 10 % suppressive soil plus *R. solani* AG-8 (suppressive soil + AG-8), 10 % non-suppressive soil plus *R. solani* AG-8 (non-supp. soil + AG-8) , no soil addition plus *R. solani* AG-8 (control + AG-8) or no soil addition minus *R. solani* AG-8 (control - AG-8). Values are means of 5 replicates and standard errors are given in parentheses. Significance of individual treatments and interaction are indicated by ** (1 % level), * (5 % level) or NS (non-significant) and values for LSD at P = 0.05 are given in square parentheses

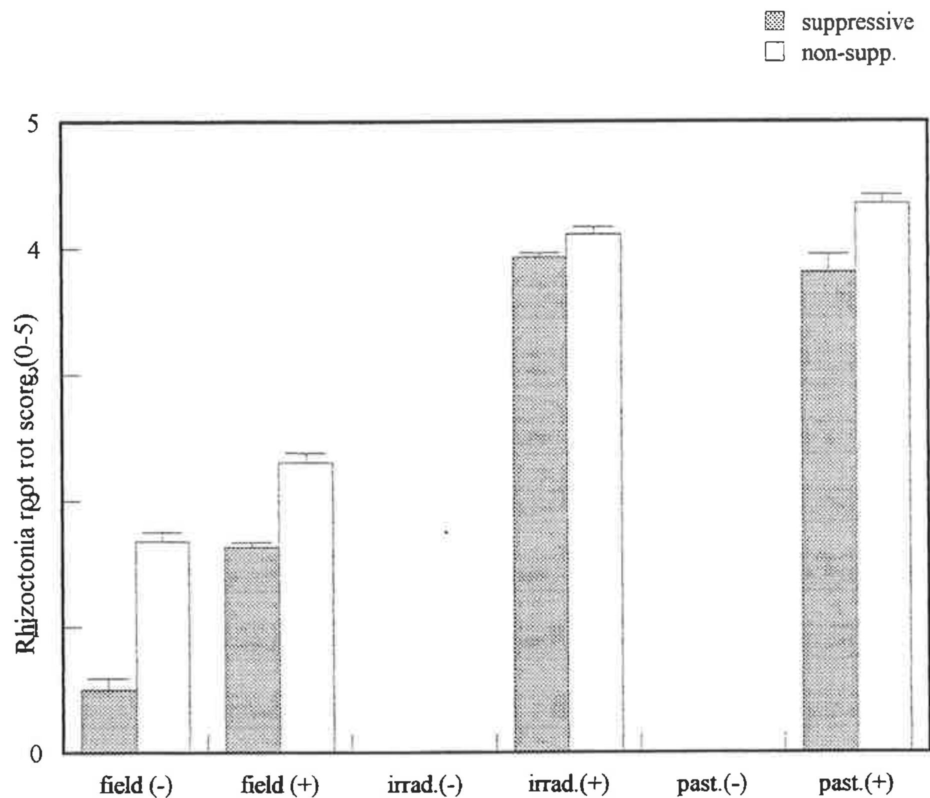


Figure 3.2: Removal of suppression with gamma irradiation or steam pasteurisation at 80°C for 30 min. Treatments were suppressive or non-suppressive (non-supp.) soil either untreated (field), gamma irradiated (irrad.) or steam pasteurised (past.), inoculated with *R. solani* AG-8 (+) or not inoculated (-). Bars are means of 4 replicate pots and lines indicate standard error of the data.

The overall trend for root dry weight was similar to that for shoots with a significant (P = 0.01) reduction following inoculation of pasteurised or γ-irradiated cores but not untreated cores and no difference between suppressive and non-suppressive soil (Table 3.2).

3.3.2.2 Experiment 2 - Steam Pasteurisation (50 - 80°C)

Disease severity was significantly (P = 0.01) less in suppressive soil compared to non-suppressive soil for the untreated and 50°C steam pasteurised treatments (Fig. 3.3). Following 60, 70 or 80°C pasteurisation, disease severity was similar in suppressive and non-suppressive soil. Inoculation significantly (P = 0.01) increased disease severity of pasteurised soil but not untreated soil.

Root and shoot dry weight per plant were significantly (P = 0.05) reduced following inoculation of 60, 70 or 80°C pasteurised cores but not 50°C or untreated soil (Table 3.3). Root dry weight was significantly higher (P = 0.01) for plants grown in non-suppressive compared to suppressive soil.

3.3.3 Effect of soil extracts on *in vitro* growth of *R. solani* AG-8

3.3.3.1 Experiment 1 - Solid medium

There were no clear trends in the response of growth of *R. solani* AG-8 *in vitro* to addition of soil extracts from suppressive soil or non-suppressive soil (Table 3.4). There was a significant (P < 0.001) interaction between soil type (suppressive or non-suppressive) and inoculation method (liquid or filter paper).

| soil type | soil treatment | inoculation dose | root dry weight (mg/plant) | shoot dry weight (mg/plant) |
|---|---|---|---|---|
| suppressive | field | + | 82 (3) | 85 (1) |
| | | - | 79 (1) | 87 (4) |
| | irradiated | + | 16 (1) | 25 (1) |
| | | - | 79 (3) | 100 (5) |
| | pasteurised | + | 17 (1) | 21 (3) |
| | | - | 73 (3) | 104 (4) |
| non-supp. | field | + | 73 (1) | 75 (2) |
| | | - | 87 (3) | 97 (3) |
| | irradiated | + | 18 (1) | 17 (1) |
| | | - | 103 (4) | 145 (9) |
| | pasteurised | + | 17 (1) | 12 (2) |
| | | - | 95 (3) | 130 (7) |
| soil type | | | NA | NS |
| inoculation | | | NA | NA |
| soil treatment | | | NA | NA |
| type.inoculation | | | * [10] | NS |
| type.treatment | | | NS | NS |
| inoc.treatment | | | * [10] | * [23] |
| type.inoc.treatment | | | NS | NS |

Table 3.2: Root and shoot dry weight per plant for wheat plants grown in suppressive or non-suppressive (non-supp.) soil that was gamma irradiated, steam pasteurised (80°C for 30 min) or left untreated (field) and inoculated (+) or uninoculated (-) with *R. solani* AG-8. Values are means of 4 replicates and standard errors are given in parentheses. Significance of individual treatments and interactions are indicated by * (5 % level), NS (non-significant) or NA (not applicable) and values for LSD at P = 0.05 are given in square parentheses.

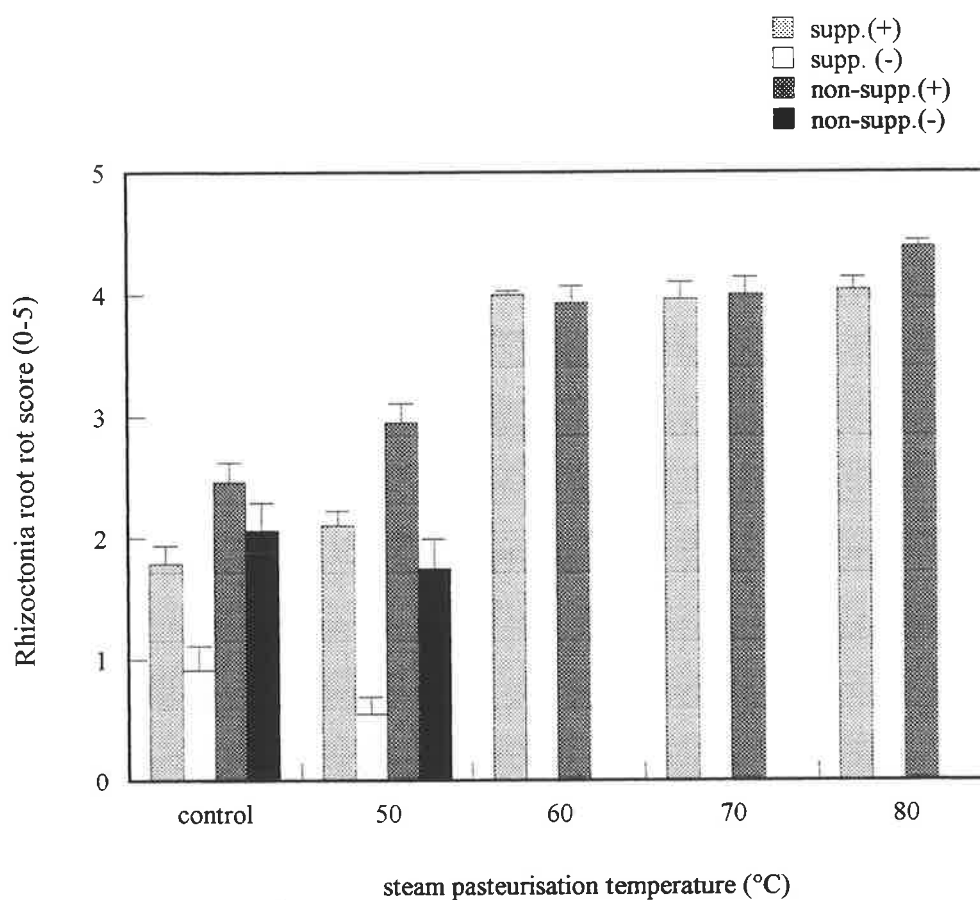


Figure 3.3: Removal of suppression with steam pasteurisation at different temperatures. Treatments were suppressive (supp.) soil or non-suppressive (non-supp.) soil either untreated (field) or steam pasteurised at 50, 60, 70 or 80 °C for 30 min and with (+) or without (-) *R. solani* AG-8 inoculation. Bars are means of 4 replicate pots and lines indicate standard error of the data.

| soil type | pasteurisation temperature | inoculation level | root dry weight (mg/plant) | shoot dry weight (mg/plant) |
|---|---|---|---|---|
| suppressive | control | + | 58 (4) | 64 (3) |
| | | - | 76 (8) | 55 (4) |
| | 50°C | + | 74 (9) | 76 (9) |
| | | - | 99 (7) | 98 (11) |
| | 60°C | + | 18 (1) | 17 (1) |
| | | - | 104 (7) | 176 (12) |
| | 70°C | + | 15 (1) | 11 (1) |
| | | - | 88 (11) | 150 (14) |
| | 80°C | + | 18 (1) | 83 (10) |
| | | - | 101 (6) | 117 (10) |
| non-supp. | control | + | 87 (15) | 76 (8) |
| | | - | 86 (7) | 85 (3) |
| | 50°C | + | 70 (7) | 65 (7) |
| | | - | 88 (10) | 77 (5) |
| | 60°C | + | 17 (1) | 23 (5) |
| | | - | 163 (6) | 151 (6) |
| | 70°C | + | 23 (2) | 22 (7) |
| | | - | 131 (7) | 163 (6) |
| | 80°C | + | 15 (2) | 16 (2) |
| | | - | 137 (7) | 179 (9) |
| inoculation | | | NA | NA |
| soil type | | | ** [13] | NS |
| temperature | | | NA | NA |
| inoculation.soil type | | | NS | NS |
| inoculation.temperature | | | * [29] | * [29] |
| soil type.temperature | | | NS | NS |
| inoc.type.temperature | | | NS | NS |

Table 3.3: Root and shoot dry weight per plant for wheat plants grown in suppressive or non-suppressive (non-supp.) soil steam pasteurised at 50, 60, 70 or 80°C or left untreated (control) and with (+) or without (-) inoculation with *R. solani* AG-8. Values are means of 4 replicates and standard errors are given in parentheses. Significance of individual treatments and interactions are indicated by ** (1 % level), * (5 % level), NS (non-significant) or NA (not applicable) and values for LSD at P = 0.05 are given in square parentheses.

| soil | treatment | inoculation method | day 3 | day 7 | day 9 |
|---|---|---|---|---|---|
| suppressive | none | liquid | 1.80 (0.02) | 5.05 (0.11) | 6.05 (0.04) |
| | | paper | 1.85 (0.01) | 4.90 (0) | 5.90 (0.07) |
| | irradiation | liquid | 1.63 (0.09) | 5.00 (0.04) | 5.95 (0.04) |
| | | paper | 1.47 (0.12) | 4.70 (0.14) | 5.75 (0.18) |
| non-supp. | none | liquid | 1.87 (0) | 5.00 (0.07) | 6.10 (0) |
| | | paper | 1.78 (0.04) | 4.95 (0.04) | 5.95 (0.04) |
| | irradiation | liquid | 1.85 (0.08) | 5.05 (0.04) | 6.10 (0) |
| | | paper | 1.82 (0.01) | 5.00 (0) | 6.05 (0.04) |
| water | | liquid | 1.93 (0.07) | 5.40 (0.07) | 6.45 (0.04) |
| | | paper | 1.60 (0.09) | 4.75 (0.04) | 5.80 (0.07) |
| time | | | | | * [0.089] |
| soil | | | | | NA |
| inoculation | | | | | NA |
| time.soil | | | | | NA |
| time.inoc | | | | | NS |
| soil.inoc | | | | | * [0.163] |
| time.soil.inoc | | | | | NS |

Table 3.4: Solid medium. Colony radius of *R. solani* AG-8 3, 7 and 9 days after inoculation with soil extracts from suppressive or non-suppressive (non-supp.) soil that had been gamma irradiated or left untreated or a sterile water control. Soil extracts were added directly (liquid) or as filter paper dipped in the extract (paper). Values are means of 2 replicate plates and standard errors are given in parentheses. Significance of individual treatments and interactions are indicated by * (5 % level), NS (non-significant) or NA (not applicable) and values for LSD at P = 0.05 are given in square parentheses.

For liquid inoculations, colony radii were reduced following addition of all extracts from untreated and γ-irradiated suppressive and non-suppressive soil compared to the sterile water control. For filter paper inoculations, colony radii were similar following inoculation with suppressive soil extract, non-suppressive soil extract or γ-irradiated non-suppressive soil extract and higher compared to inoculation with γ-irradiated suppressive soil extract or sterile water.

3.3.2.2 Experiment 2 - Liquid medium

As in Experiment 1 (Section 3.3.2.1), there were no clear trends in the response of growth of *R. solani* AG-8 *in vitro* to addition of soil extracts from suppressive soil or non-suppressive soil (Table 3.5). Colony diameters were larger at day 6 compared to day 4. At day 4, colony diameter was larger when untreated non-suppressive soil had been added compared to all other treatments except for γ-irradiated non-suppressive soil.

Colony diameters when both suppressive soil extracts were added were similar to the water and blank controls. At day 6, colony diameters for both untreated soil extracts were similar to the water and blank controls. Addition of both γ-irradiated soil extracts increased colony diameter compared to the water control and the blank control for γ-irradiated suppressive soil extract.

| soil | treatment | day 4 | day 6 |
|---|---|---|---|
| suppressive | none | 4.58 (0.13) | 7.00 (0) |
| | irradiated | 4.42 (0.10) | 7.83 (0.10) |
| non-supp. | none | 5.00 (0) | 6.83 (0.29) |
| | irradiated | 4.67 (0.05) | 7.50 (0) |
| water | | 4.58 (0.10) | 6.83 (0.10) |
| blank | | 4.50 (0) | 7.17 (0.10) |
| time | | | NA |
| treatment | | | NA |
| time.treatment | | | * [0.397] |

Table 3.5 Liquid medium. Colony diameter of *R. solani* AG-8 4 and 6 days after addition of soil extract to the culture solution from suppressive or non-suppressive (non-supp.) soil that had been gamma irradiated or left untreated or two sterile controls (water, blank). Values are means of 3 replicate plates and standard errors are given in parentheses. Significance of individual treatments and interactions are indicated by * (5 % level) or NA (not applicable) and values for LSD at P = 0.05 are given in square parentheses.

**3.4 Discussion**

Experiments described in this chapter indicate that the decline in Rhizoctonia barepatch at Avon in South Australia is biologically based. The objectives of these experiments were to test the nature of the disease suppression, by determining if the suppressive characteristics could be transferred to a sterile soil, if they could be removed by biocidal treatments, and the effect of sterile soil extracts on the growth of *R. solani* AG-8 *in vitro* .

The successful transfer of suppressive characteristics to a sterile soil and removal by gamma irradiation or steam pasteurisation at 60°C provides evidence for the biological nature of this suppression. Either of these approaches can be used to demonstrate the biological nature of suppression and when combined, conclusions with respect to the specific or general nature of the suppression can be made using the definitions of Gerlagh (1968). Results obtained in this chapter suggest both general and specific forms of suppression are important in suppression of disease caused by *R. solani* AG-8. The suppressive characteristics were established in a sterile soil following addition of suppressive soil, resulting in lower disease severity on wheat following inoculation with *R. solani* AG-8 compared to when non-suppressive soil was added. However, addition of either suppressive or non-suppressive soil resulted in lower disease severity compared to inoculated pasteurised or gamma ($\gamma$) irradiated soil. A level of general suppression was therefore present in both suppressive and non-suppressive soil, inhibiting disease development. Reduced disease severity in inoculated intact cores of non-suppressive soil compared to pasteurised or $\gamma$-irradiated soil also indicated a natural level of suppression in the non-suppressive soil. Using the definitions of Gerlagh (1968) this natural level of suppression should be non-

transferable. In the decline of Rhizoctonia barepatch observed at Avon, a level of natural suppression that is transferable exists in all soils. Lower disease severity following addition of suppressive soil compared to non-suppressive soil indicates the presence of specific (sensu Gerlagh) suppression in the suppressive soil which inhibits disease development in addition to the general suppression present in both soils.

Further evidence for a natural level of suppression occurring in both suppressive and non-suppressive soil was found in the transfer of suppression experiment. Greatest inhibition of plant growth occurred in inoculated sterile or steam pasteurised soil and addition of either suppressive or non-suppressive field soil resulted in increased plant growth. Improved growth may have been due to addition of the organisms that suppressed disease, and possibly addition of purely growth promoting organisms. The increased plant growth in the uninoculated pasteurised and autoclaved soil compared to those with added field soil may be the results of increased nutrient levels following heat treatment or irradiation due to release of nutrients from lysis of dead microbial cells (eg carbon and nitrogen) and alteration of the soil particles (eg manganese) (Cawse, 1975). Fumigation of soil can result in increased plant growth (Warcup, 1957; Rovira, 1976)

Plant growth was observed to be similar in suppressive and non-suppressive soils. These parameters (root and shoot weight per plant) were selected to provide additional information on disease severity of plants grown in the two soils. It was initially thought these parameters may illustrate trends not shown by disease severity results, or show good correlation with disease severity. This was not the case and plant growth results are only reported in subsequent chapters when meaningful significant differences between soils were found. Large differences in disease severity correlated with plant growth results, but not smaller ones such as those between the two field

soils. This may be due to the short duration of the experiments not allowing full expression of symptoms as would occur over the growing season.

Although it can be concluded that soil biological characteristics are involved in this disease suppression this does not preclude the physico-chemical characteristics of the soil also being involved. However, addition of sterile soil extracts did not influence the growth of *R. solani* AG-8 *in vitro* and this does not support a hypothesis of chemical inhibition of pathogen growth. Results differed somewhat between the two experiments on soil extract but no major *in vitro* inhibition of growth of *R. solani* AG-8 occurred. Colony growth in liquid medium was increased following addition of either of the soil extract compared to sterile water, indicating a positive effect of the nutrients contained in the soil extracts. Water soluble soil extracts of the suppressive and non-suppressive soils do not have any inhibitory effect on growth of *R. solani* AG-8 *in vitro* and in some cases enhance fungal growth.

Detection of inhibitory compounds may have been masked by growth enhancement of *R. solani* AG-8 *in vitro* by the addition of soil extract, or it may relate to the mode of action of the compounds potentially inhibiting disease. For example, zinc nutrition is inversely related to severity of Rhizoctonia root rot but the suggested mode of action is through effects on the stability or integrity on host plant membranes rather than fungitoxicity (Thongbai *et al*., 1993 a ; b). Suppression of disease due to adequate or high zinc nutrition would therefore be undetectable with assessment of growth of *R. solani* AG-8 *in vitro*. Characteristics of potentially inhibitory compounds, such as volatility or instability, may also prevent detection with this method.

Further investigation of the role of soil chemical characteristics in suppression of disease due to *R. solani* AG-8 would need to include *in vivo*

as well as *in vitro* methods. Refinements to the *in vitro* method include modifications to the extraction and inoculation procedures. More vigorous suspension of the soil (eg using sonication) may release additional compounds into the solution. Incorporation of the soil extracts into nutrient rich media may overcome the compounding effect of growth enhancement following addition of soil extract. Investigation of the effect of temporal and physical separation of *R. solani* AG-8 inoculum and soil extract may provide evidence for stability and mobility of potentially inhibitory compounds.

Assessment of the effects of soil extracts on *R. solani* AG-8 *in vivo* could be based on the transfer of suppression test (Rovira and Wildermuth, 1981; Rovira, 1982). The transfer of suppression experiment described in this chapter involved adding a small amount (10 % w / w) of field soil to autoclaved or pasteurised soil. An assumption of this method is that chemical components of the soil would be diluted beyond active concentrations, whereas biological components could grow and colonise the entire soil mix. A modification of this experiment would be to add filter-sterilised soil extract in place of untreated soil. Chemical components of the soil, including those of biological and inorganic origin, would be added, but living organisms would not. Use of extract from untreated soil rather than sterilised soil would avoid complications due to increased nutrient concentrations in sterilised soil following the release of nutrients from dead microbes. Preliminary experiments would be necessary to determine application rates able to provide similar nutrient concentrations to those in the soil used in preparing the extract.

In summary, using established methods, a biological basis to the observed decline in Rhizoctonia barepatch at Avon is now known. Severity of disease due to *R. solani* AG-8 is lower in suppressive field soil compared to non-

suppressive field soil with or without inoculation of the pathogen. Subsequent experiments are now described with the objectives of determining : whether other diseases are suppressed by the soil suppressive to disease caused by *R. solani* AG-8 (Chapter 4); whether there is a correlation between populations of *Rhizoctonia* and disease severity (Chapters 5 and 6); and, whether there are detectable differences in the microbial populations in suppressive soil and non-suppressive soil (Chapter 7).

# Chapter 4: Receptivity of suppressive and non-suppressive soils to a range of fungal pathogens

## 4.1 Introduction

The aim of this section was to compare the suppressive and non-suppressive soils in terms of soil receptivity to *R. solani* AG-8 and to assess receptivity to a number of other fungal pathogens. Results from these experiments may be helpful in understanding the mechanisms of the suppression by showing which diseases are suppressed by the soil suppressive to Rhizoctonia barepatch and which are not. Soil-borne pathogens of importance to southern Australian cereal production were selected to represent a range of taxonomic groups, modes of action, and types of symptoms.

The diseases and pathogens selected were: root rot caused by R. solani AG-8 (Section 4.2.1), a range of diseases caused by *Rhizoctonia* (*R. solani* AG-2 and AG-4, *W. circinata, C. cornigerum*) (Section 4.2.2), root rot caused by *G. graminis* var. *tritici* (Section 4.2.3), seedling damping-off caused by *Pythium irregulare* Buisman (Section 4.2.4), crown and sub-crown rot caused by *Bipolaris sorokiniana* (Sacc.) Shoem. (Section 4.2.5), and crown and sub-crown rot caused by *Fusarium graminearum* Schwabe (Section 4.2.6). Details of isolates used are given in Table 4.1.

Field soil collected at three different times was tested to determine if the receptivity of the suppressive and non-suppressive soils to disease caused by *R. solani* AG-8 changed over the season. In the third experiment (Section 4.2.1.3) an additional soil from the Avon site was tested to determine if this type of bioassay could be used to compare the suppressiveness of different

field soils. This soil was selected due to its considerably higher carbon content than the suppressive and non-suppressive soils to test the hypothesis that disease suppression develops due to high residue inputs.

A range of *Rhizoctonia* spp. was used to determine if the suppression was limited to AG-8 or was active against other species and intraspecific groups within the genus. The range of *Rhizoctonia* isolates was chosen to represent three levels of "similarity" to *R. solani* AG-8. The most similar were isolates from different zymogram groups within AG-8, the least similar were isolates with perfect states other than *T. cucumeris* (*Ceratobasidium* spp. and *W. circinata*). The intermediately similar isolates were from AG's other than 8 (AG-2 and AG-4). The initial experiment was carried out to determine the inoculum doses required so that each *Rhizoctonia* isolate could be added at a dose which would produce similar disease severities. Differences in the *in vitro* growth of the isolates indicated that millet seed inoculum may have varied in inoculum potential. Once equivalent inoculum levels had been determined, the second experiment was carried out to determine the relative disease severity on plants grown in suppressive and non-suppressive soil inoculated with a range of *Rhizoctonia* spp.

Receptivity to the diseases caused by other pathogens was tested in a similar way to disease caused by *R. solani* AG-8. Soils was sterilised in all experiments by gamma irradiation (25 kgys, "Steritech", Victoria, Australia).

## 4.2 Materials and Methods

### 4.2.1 Soil receptivity to *R. solani* AG-8

Soil (300 g) added to plastic 300 ml capacity pots and pre-incubated in a 15° C airbath in a glasshouse for 7 weeks. Millet seed inoculum of *R. solani* AG-8 was then added. The inoculum doses used were 1, 2, 4 or 8 millet seeds per pot and an uninoculated control treatment. The experimental design was a 2 x 5 factorial of soil type (suppressive or non-suppressive) x inoculum dose with 4 replicate pots of each treatment combination in a RCBD.

Two weeks after fungal inoculation, pots were sown with 5 surface disinfested wheat seeds. Plants were harvested 5 weeks later and assessed for disease severity (Experiment 1).

The experiment was repeated with different samples of suppressive and non-suppressive soil, using the same methods except the pre-incubation was for 2 weeks and the *R. solani* AG-8 inoculation doses were 0, 1, 2 or 4 millet seeds per pot (Experiment 2).

A third receptivity experiment was done (Experiment 3) using suppressive, non-suppressive and "haystack soil". The methodology used was as the first two experiments except there was 250g soil per pot and the *R. solani* AG-8 inoculation doses were 0, 2 , 4 or 6 millet seeds per pot. The haystack soil had been underneath a haystack for approximately 30 years at the Avon site and as a result had a higher organic carbon content than the other 2 soils (suppressive soil 2.7 %, non-suppressive soil 2.0 %, haystack soil 5.7 %). Other changes likely to have occurred to the haystack soil include nutritional,

microbiological and physical characteristics. At harvest, plants were assessed for disease severity, and shoot and root dry weight per plant.

### 4.2.2 Range of *Rhizoctonia* spp.

#### 4.2.2.1 Determination of inoculum dose

The experiment had 1 treatment factor (fungal isolate) and 4 replications of each treatment in a RCBD. A mixture of sterilised suppressive and non-suppressive soil was used so the average disease severity in these two soils could be determined.

Fungal inoculum of the range of *Rhizoctonia* spp. was prepared on millet seed as previously described for *R. solani* AG-8. The fungal isolates and host plants used are listed in Table 4.1.

Plastic 300 ml capacity pots were prepared by adding 160 g soil, millet seed fungal inoculum, 110 g soil, 5 surface disinfested seeds, then 30 g soil. The inoculum doses used were 2, 4 or 6 colonised millet seeds per pot plus an uninoculated control treatment.

Plants were grown in a 15°C airbath in a glasshouse for 5 weeks then harvested and assessed for disease severity. The method of disease assessment varied for cereal and non-cereal host plants, as described in the Section 2.3.

4.2.2.2 Soil receptivity to a range of *Rhizoctonia* spp.

The experimental design was a 2 x 11 factorial of soil type (suppressive or non-suppressive) x *Rhizoctonia* inoculation with 4 replicate pots for each treatment combination in a RCBD. The 11 *Rhizoctonia* inoculations included 7 different fungal isolates and 4 uninoculated treatments (lupin, sub-clover, tomato, and wheat).

Soil (250 g) was added to plastic 300 ml capacity pots and pre-incubated in a 15°C airbath in a glasshouse for 2 weeks. Millet seed inoculum was added as in Section 4.2.2.1. Following a further 2 weeks of pre-incubation the pots were sown with 5 surface disinfested plant seeds. Plant host-fungal isolate combinations are listed in Table 4.1.

Plants were harvested 5 weeks later and assessed for disease severity. Disease severity was analysed separately for wheat and for lupin / sub-clover / tomato because two different assessment methods were used (Section 2.6).

4.2.3 Soil receptivity to *Gaeumannomyces graminis* var. *tritici*

The experiment was a 2 x 2 x 4 factorial of soil type (suppressive or non-suppressive) x +/- sterilisation x inoculum dose with 4 replications of each treatment combination in a RCBD.

Plastic 300 ml capacity pots were prepared by adding 166 g soil, a thin layer of ryegrass inoculum, 104 g soil, 5 surface disinfested wheat seeds, then 30 g soil. The inoculum doses used were 0.02, 0.05 and 0.25 g of ryegrass inoculum per pot plus an uninoculated control treatment. *G. graminis* var. tritici (isolate Ggt8) inoculum was prepared using the method described for

*Rhizoctonia* except sterile ryegrass seed was used instead of millet seed. After preparation the ryegrass inoculum was stored in a plastic container at -20°C until use.

Pots were incubated in a 15°C airbath in a glasshouse for 4 weeks. At harvest plants were assessed for disease severity (% root length infected).

4.2.4 Soil receptivity to *Pythium irregulare*

The experiment was a 2 x 2 x 4 factorial of soil type (suppressive or non-suppressive) x +/- sterilisation x inoculum dose with 4 replications of each treatment combination in a RCBD.

*P. irregulare* (isolate BH16) inoculum consisted of 4 mm diameter plugs of colonised potato dextrose agar (PDA) (Appendix 1). Plugs were taken from the edge of colonies incubated overnight at 25°C. Sterile PDA plugs were also used so the total number of plugs (colonised plus sterile) was the same for each inoculation level.

Plastic 150 ml capacity pots were prepared by adding 57 g soil, followed by the PDA plugs, 57 g soil, 5 surface disinfested medic seeds, then 36 g soil. The inoculum doses used were 2, 4 or 6 colonised PDA plugs per pot plus a control treatment of 6 sterile PDA plugs.

Plants were grown under controlled conditions (15°C, 9 hour day, photon irradiance of 236 $\mu mol\ m^{-2}\ s^{-1}$) for 4 weeks. At harvest, plants were assessed for emergence.

### 4.2.5 Soil receptivity to *Bipolaris sorokiniana*

The experiment was a 2 x 2 x 4 factorial of soil type (suppressive or non-suppressive) x +/- sterilisation x inoculum dose with 4 replications of each treatment combination in a completely randomised design (CRD).

*B. sorokiniana* inoculum was prepared using the method described for *Rhizoctonia,* except that sterile ryegrass seed was used instead of millet seed. After preparation the ryegrass inoculum was stored in a plastic container at room temperature until use.

Plastic 300 ml capacity pots were prepared by adding 150 g soil, 5 surface disinfested wheat seeds, then 150 g soil with the *B. sorokiniana* inoculum thoroughly mixed through it. The inoculum doses used were 0.7, 1.4 and 2.1 g ryegrass inoculum per pot plus an uninoculated control treatment.

Plants were grown under controlled conditions (15°C, 9 hour day, photon irradiance of 136 $\mu m\ m^{-2}\ s^{-1}$) for 12 weeks. Plant nutrient solution (5 ml, half strength Hoaglands solution without Mn) was added weekly. Nutrient solution was added to avoid deficiencies developing during the longer incubation period (12 weeks). Manganese was not added due to a known interaction with plant defence mechanisms (McCay-Buis *et al*., 1995). At harvest, plants were assessed for disease severity. The disease index of Ledingham *et al* (1973) was used to score the symptoms on the sub-crown internode as 0, 1, 2 or 3. These categories corresponded to clean, slight, moderate and severe disease symptoms respectively. Mean disease scores for each pot were calculated using the following formula (Tinline *et al*., 1975; Tinline and Ledingham, 1979):

$$\frac{\Sigma\ (\text{category value x number of subcrown internodes in category})}{\text{total number of subcrown internodes in sample x 4}}$$

4.2.6 Soil receptivity to *Fusarium graminearum*

The experiment was a 2 x 2 x 4 factorial of soil type (suppressive or non-suppressive) x +/- sterilisation x inoculum dose with 4 replications of each treatment combination in a CRD.

*F. graminearum* (isolate F11767) inoculum was prepared on autoclaved cereal (oat) chaff using the method of Liddell *et al.* (1986). Chaff was added to 1 L conical flasks and moistened with distilled water. The flasks were plugged with non-absorbent cotton wool and autoclaved for 60 min at 121°C on two successive days. The sterile chaff was inoculated with approximately ten 0.5 $cm^2$ plugs of half strength PDA colonised with *F. graminearum*. The cultures were incubated at 25°C in the dark for 4 weeks. After 1 week the cultures were shaken and this was repeated every 2-3 days for the remainder of the incubation. Approximately 3 times a week the cultures were left on the laboratory bench in incident sunlight for 6-8 hours. The inoculum was dried in a laminar flow overnight and stored in a plastic container at room temperature until use.

Plastic 300 ml capacity pots were prepared by adding 150 g soil, 5 surface disinfested wheat seeds, then 150 g soil with the *F. graminearum* inoculum thoroughly mixed through it. The inoculum doses used were 0.01, 0.03 and 0.15 g inoculated chaff per pot plus an uninoculated control treatment.

Pots were incubated under controlled conditions (15°C, 9 hour day, photon irradiance of 236 µmol $m^{-2}$ $s^{-1}$) for 12 weeks. Plant nutrient solution (5 ml, half strength Hoaglands solution without manganese) was added weekly (as for Section 4.2.5). At harvest, plants were assessed for disease severity. Crown rot symptoms on the sub-crown internode, leaf sheaths and stems were scored as 0, 1, 2, 3 or 4 (Nelson and Burgess, 1994). These categories corresponded to no symptoms, small lesions on sub-crown internode, sub-crown internode or leaf sheaths brown, sub-crown internode and leaf sheaths brown, plant dead. The mean disease severity for plants from each pot was determined using the following index:

$$\frac{\Sigma\ (\text{disease class x no. plants in class})}{\text{total no. of plants}}$$

| species | teleomorph[a] | isolate number | AG[b] | ZG[c] | host[d] | origin | source |
|---|---|---|---|---|---|---|---|
| *Rhizoctonia solani* | *Thanatephorus cucumeris* | RS-21 | 8 | 1-1 | wheat | wheat root, Avon, South Australia | H.McDonald |
| | " | R444 | 8 | 1-2 | wheat | | M. Sweetingham |
| | " | R1824 | 8 | 1-3 | wheat | | M. Sweetingham |
| | " | 1344 | 2 | | sub-clover | wheat root, Bow Hill, South Australia | S. Neate |
| | " | 1661 | 4 | | tomato | cotton soil, Narrabri, New South Wales | S. Neate |
| | *Ceratobasidium cornigerum* | 1484 | | CZG3 | tomato | wild turnip root, Bow Hill, South Australia | S. Neate |
| | " | 755 | | CZG5 | tomato | wheat root, Avon, South Australia | S. Neate |
| | *Waitea circinata* | R65 | | | lupin | | M. Sweetingham |
| *Gaeumannomyces graminis var. tritici* | | Ggt8 | | | wheat | wheat roots, Avon, South Australia | H. McDonald |
| *Bipolaris sorokiniana* | | | | | wheat | | H. Wallwork |
| *Fusarium graminearum* Group 1 | | F11767 | | | wheat | wheat crown, South Australia | D. Backhouse |
| *Pythium irregulare* | | BH16 | | | medic | soil, Spalding, South Australia | B. Hawke |

[a]where known for *Rhizoctonia* isolates
[b] anastomosis group of *Rhizoctonia* isolates where known
[c]zymogram group of *Rhizoctonia* isolates where known
[d]plant host used in reported experiments

Table 4.1: Fungal isolates, source, and plant hosts used in soil receptivity experiments on a range of fungal pathogens in soil suppressive and non-suppressive to *Rhizoctonia solani* AG-8.

## 4.3 Results

### 4.3.1 Soil receptivity to *R. solani* AG-8

In all experiments disease severity was significantly higher in non-suppressive soil compared to suppressive soil (Figures 4.1, 4.2 and 4.3). When a third soil with a high carbon content (haystack soil) was used (Experiment 3) disease severity was similar in suppressive soil and this soil, and less than in non-suppressive soil. There was a significant effect of soil type ($P = 0.05$) and inoculation dose ($P = 0.01$) but the interaction was non-significant in all three experiments. Therefore, disease severity was significantly lower in suppressive soil compared to non-suppressive soil regardless of inoculation dose. In all experiments increased inoculation doses led to increased disease severity compared to the uninoculated control.

In Experiment 3 shoot dry weight was similar in suppressive and non-suppressive soil, but significantly ($P = 0.01$) higher in haystack soil (Table 4.2).

### 4.3.2 Range of *Rhizoctonia* spp.

#### 4.3.2.1 Determination of inoculum dose

There was a significant ($P = 0.05$) difference in disease severity of wheat caused by isolates of *R. solani* AG-8 isolates (Table 4.3a). Disease severity for isolate R1824 increased with each increase in inoculation dose. Disease severity for isolate R444 increased up to a dose of 4 millet seeds per pot with no further increase at a dose of 6 seeds per pot. Disease severity for isolate RS-21 was similar at all inoculation doses. All inoculations with AG-8

isolates resulted in disease severity scores of approximately 3 or above on the 0-5 scale of McDonald and Rovira (1985). Disease severity was similar for all three isolates at a dose of 2 millet seeds per pot. At doses of 4 and 6 seeds per pot disease severity was similar for R444 and R1824 but higher than RS-21.

All inoculations of non AG-8 isolates also resulted in significantly (P = 0.01) higher disease severity compared to the sterile control, but in all cases with averages of less than 2 on the 0-5 scale of Sweetingham and MacNish (1994) (Table 4.3b). Disease severity was similar following inoculation with 2, 4 or 6 millet seeds per pot for all non AG-8 isolates. Disease severity for isolate 1484 (*C. cornigerum*) was significantly (P = 0.01) less than for 1344 (AG-2) and R65 (*W. circinata*) and reduced compared to isolate 1661 (AG-4).

An inoculation dose of 2 millet seeds per pot was chosen for Experiment 2 for all AG-8 isolates and 8 millet seeds per pot for non AG-8 isolates. Isolate 1484 (*C. cornigerum*) was replaced with isolate 755 in Experiment 2 due to the negligible disease severity resulting from inoculation with 1484.

4.3.2.2 Soil receptivity to a range of *Rhizoctonia* spp.

For *R. solani* AG-8 isolates, disease severity was significantly (P = 0.05) higher in non-suppressive soil compared to suppressive soil (Table 4.4). All inoculations significantly (P = 0.01) increased disease severity compared to the uninoculated control. Disease severity due to isolate R444 was greater than for isolate R1824 and equal to isolate RS-21.

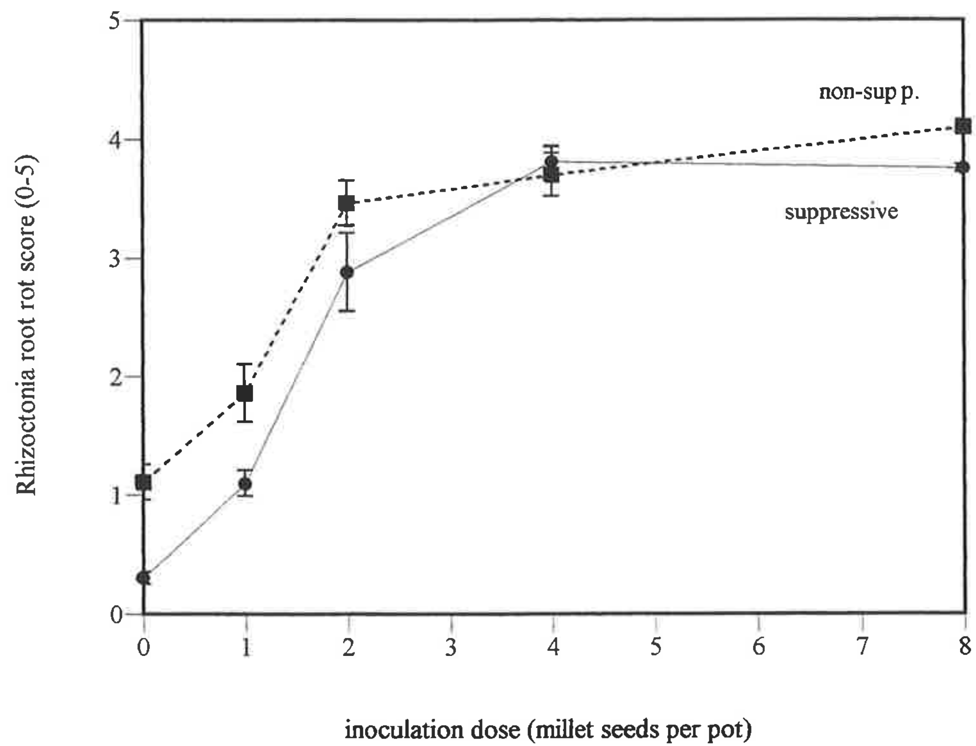


Figure 4.1: Experiment 1. Rhizoctonia root rot scores for wheat plants grown in suppressive and non-suppressive (non-supp.) soil inoculated with increasing doses of *R. solani* AG-8. Values are means of 4 replicates and lines indicate standard error of the data

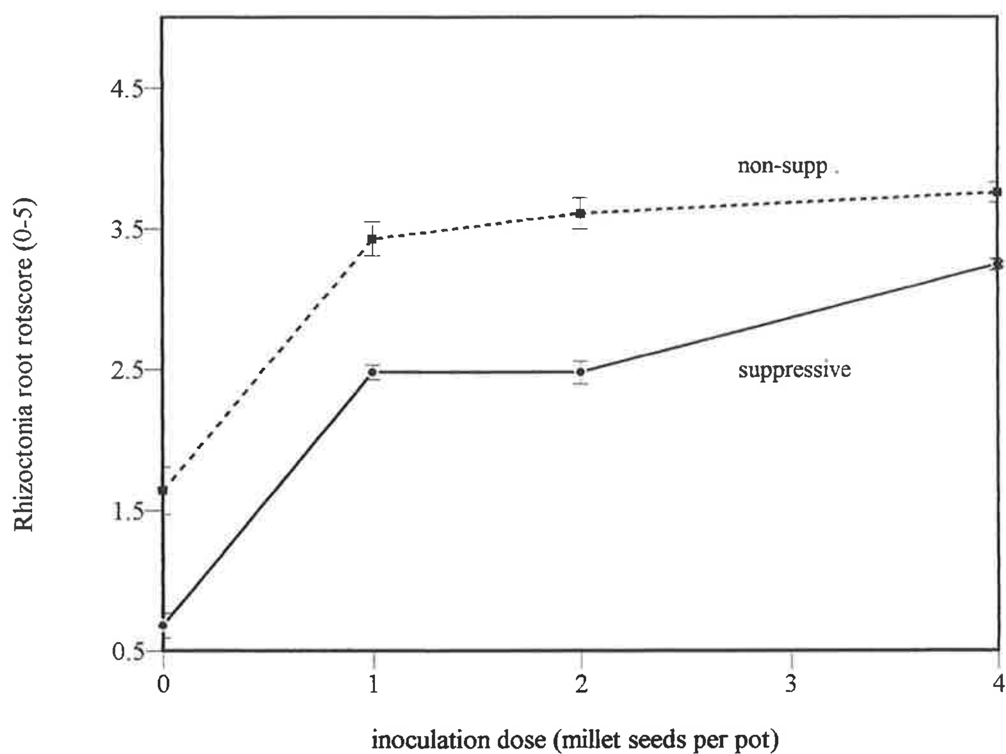


Figure 4.2: Experiment 2. Rhizoctonia root rot scores for wheat plants grown in suppressive and non-suppressive (non-supp.) soil inoculated with increasing doses of *R. solani* AG-8. Values are means of 4 replicates and lines indicate standard error of the data.

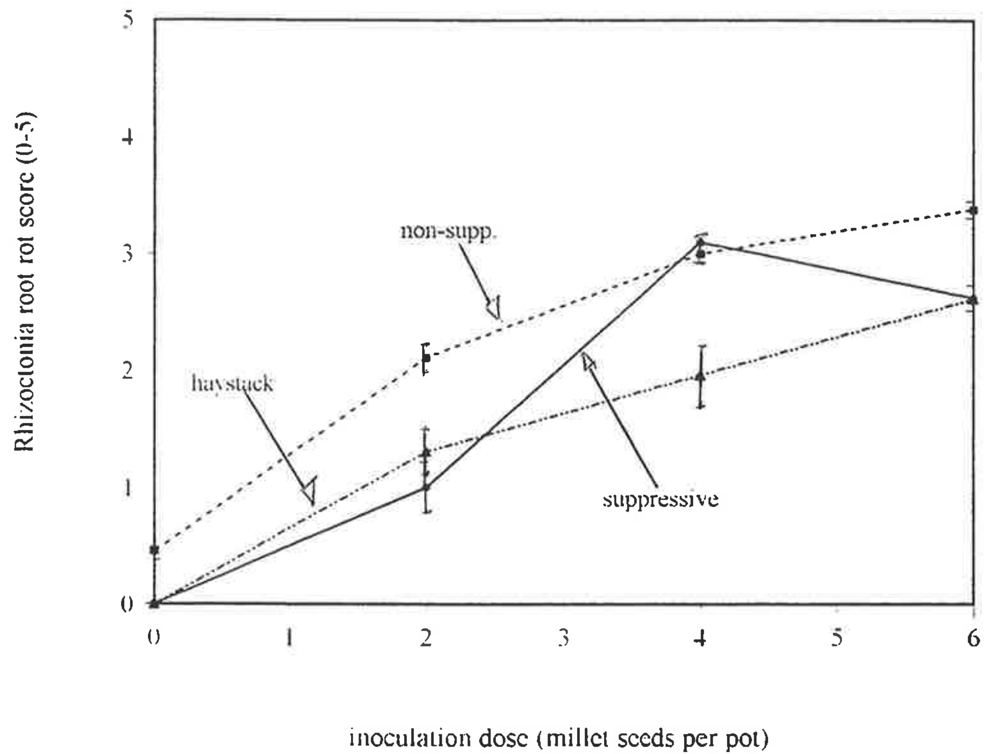


Figure 4.3: Experiment 3. Rhizoctonia root rot scores for wheat plants grown in suppressive, non-suppressive (non-supp.) andhaystack soil inoculated with increasing doses of *R. solani* AG-8. Values are means of 4 replicates and lines indicate standard error of the data.

| soil | inoculation dose (millet seeds / pot) | root dry weight (mg/plant) | shoot dry weight (mg/plant) |
|---|---|---|---|
| suppressive | 0 | 80 (3) | 59 (6) |
| | 2 | 75 (5) | 65 (5) |
| | 4 | 42 (1) | 33 (2) |
| | 6 | 67 (7) | 54 (5) |
| non-suppressive | 0 | 107 (12) | 80 (11) |
| | 2 | 71 (3) | 67 (2) |
| | 4 | 55 (2) | 52 (3) |
| | 6 | 43 (4) | 55 (6) |
| haystack | 0 | 78 (7) | 139 (26) |
| | 2 | 62 (3) | 110 (10) |
| | 4 | 58 (4) | 80 (4) |
| | 6 | 72 (4) | 105 (7) |
| inoculation dose | | ** [18] | NS |
| soil | | NS | * [26] |
| dose.soil | | NS | NS |

Table 4.2: Experiment 3 Root and shoot dry weight per plant for wheat plants grown in suppressive, non-suppressive or haystack soil inoculated with increasing doses of *R. solani* AG-8. Values are means of 4 replicate pots and standard errors are given in parentheses. Significance of individual treatments and interaction are indicated by ** (1 % level), * (5 % level) or NS (non-significant) and values for LSD at P = 0.05 are given in square parentheses.

a

| *Rhizoctonia* | isolate no. | inoculation dose (millet seeds / pot) | Rhizoctonia root score (0-5) |
|---|---|---|---|
| AG-8, ZG 1-1 | RS-21 | 2 | 3.1 (0.03) |
| | | 4 | 3.2 (0.01) |
| | | 6 | 3.3 (0.1) |
| AG-8, ZG 1-2 | R444 | 2 | 3.0 (0.1) |
| | | 4 | 3.7 (0.1) |
| | | 6 | 3.7 (0.1) |
| AG-8, ZG 1-3 | R1824 | 2 | 3.0 (0.01) |
| | | 4 | 3.6 (0.1) |
| | | 6 | 3.9 (0.01) |
| none | | 0 | 0 (0) |
| inoculation dose | | | NA |
| soil | | | NA |
| dose.soil | | | * [0.3] |

b

| *Rhizoctonia* | isolate no. | inoculation dose (millet seeds / pot) | plant | Rhizoctonia root score (0-5) |
|---|---|---|---|---|
| AG-2 | 1344 | 2 | sub-clover | 0.7 (0.1) |
| | | 4 | | 0.7 (0.01) |
| | | 6 | | 1.1 (0.1) |
| none | | 0 | sub-clover | 0 (0) |
| AG-4 | 1661 | 2 | tomato | 0.4 (0.1) |
| | | 4 | | 0.7 (0.1) |
| | | 6 | | 0.9 (0.1) |
| *C. cornigerum* | 1484 | 2 | tomato | 0.1 (0.01) |
| | | 4 | | 0.3 (0.01) |
| | | 6 | | 0.4 (0.01) |
| none | | 0 | tomato | 0 (0) |
| *W. circinata* | R65 | 2 | lupin | 0.7 (0.1) |
| | | 4 | | 1.0 (0.1) |
| | | 6 | | 1.4 (0.1) |
| none | | 0 | lupin | 0.02 (0.01) |
| inoculation dose | | | | ** [0.2] |
| soil | | | | ** [0.2] |
| dose.soil | | | | NS |

Tables 4.3 a and b: Rhizoctonia root scores for wheat (a), and sub-clover, tomato and lupin (b) grown in sterile soil inoculated with increasing doses of *Rhizoctonia* spp. Values are means of 4 replicate pots and standard errors are given in parentheses. Significance of individual treatments and interaction are indicated by ** (1 % level), * (5 % level), NS (non-significant) or NA (not applicable) and values for LSD at P = 0.05 are given in square parentheses.

| soil | plant | *Rhizoctonia* | isolate no. | Rhizoctonia root score (0-5) |
|---|---|---|---|---|
| suppressive | wheat | none | | 0.1 (0.1) |
| | wheat | AG-8, ZG 1-1 | RS-21 | 3.1 (0.2) |
| | wheat | AG-8, ZG 1-2 | R444 | 3.5 (0.2) |
| | wheat | AG-8, ZG 1-3 | R1824 | 2.5 (0.1) |
| non-supp. | wheat | none | | 0.9 (0.1) |
| | wheat | AG-8, ZG 1-1 | RS-21 | 3.2 (0.3) |
| | wheat | AG-8, ZG 1-2 | R444 | 4.0 (0.1) |
| | wheat | AG-8, ZG 1-3 | R1824 | 3.1 (0.2) |
| suppressive | sub-clover | none | | 0.1 (0.01) |
| | sub-clover | AG-2 | 1344 | 0.2 (0.1) |
| | tomato | none | | 0.1 (0.01) |
| | tomato | AG-4 | 1661 | 0.1 (0.03) |
| | tomato | *C. cornigerum* | 755 | 0.3 (0.2) |
| | lupin | none | | 0.1 (0.02) |
| | lupin | *W. circinata* | R65 | 0.1 (0.1) |
| non-supp. | sub-clover | none | | 0.7 (0.1) |
| | sub-clover | AG-2 | 1344 | 0.5 (0.03) |
| | tomato | none | | 0.1 (0.04) |
| | tomato | AG-4 | 1661 | 0.3 (0.1) |
| | tomato | *C. cornigerum* | 755 | 0.2 (0.03) |
| | lupin | none | | 0.2 (0.03) |
| | lupin | *W. circinata* | R65 | 0.2 (0.01) |
| soil | | | | AG-8 * [0.5]<br>non AG-8 NA |
| *Rhizoctonia* | | | | AG-8 ** [ 0.7]<br>non AG-8 NA |
| soil.*Rhizoc.* | | | | AG-8 NS<br>non-AG-8 * [0.2] |

Table 4.4: Rhizoctonia root scores for plants grown in suppressive or non-suppressive soil inoculated with one of a range of *Rhizoctonia* spp. or left uninoculated (none). Values are the means of 4 replicate pots and standard errors are given in parentheses. Significance of individual treatments and interaction are indicated by ** (1 % level), * (5 % level), NS (non-significant) or NA (not applicable) and values for LSD at P = 0.05 are given in square parentheses.

There were no clear trends between suppressive and non-suppressive soil for disease severity caused by non AG-8 isolates, although the interaction between soil type and isolate was significant (P = 0.05) (Table 4.6). In general, inoculated plants showed no higher disease severity than inoculated plants except for tomato and isolate 755 (*C. cornigerum*). Disease severity was significantly higher in non-suppressive compared to suppressive soil for clover (uninoculated and inoculated with isolate 1344, AG-2) and tomato inoculated with isolate 1661 (AG-4). Disease severity for all other treatments was similar in suppressive and non-suppressive soil.

4.3.3 *Gaeumannomyces graminis* var. *tritici*

Disease severity was significantly (P = 0.01) higher in non-suppressive soil compared to suppressive soil (Fig. 4.4). Uninoculated γ-irradiated and field soils had similar disease severity, and for all inoculated soils disease severity was significantly (P = 0.01) higher in γ-irradiated soil compared to field soil. Disease severity in γ-irradiated soil increased with each increase in inoculation dose but only following inoculations of 0.05 or 0.25 g ryegrass in field soils. The three-way interaction between soil type, soil treatment and inoculation dose was non-significant, but the interaction between soil treatment and inoculation dose was significant (P = 0.01) implying that field soils and γ-irradiated soils responded to inoculation dose and soil type similarly. The trend for lower disease severity for suppressive soil compared to non-suppressive soil was clear for all inoculated treatments for field soil, but only at a dose of 0.25 g ryegrass for γ-irradiated soils (Fig. 4.4).

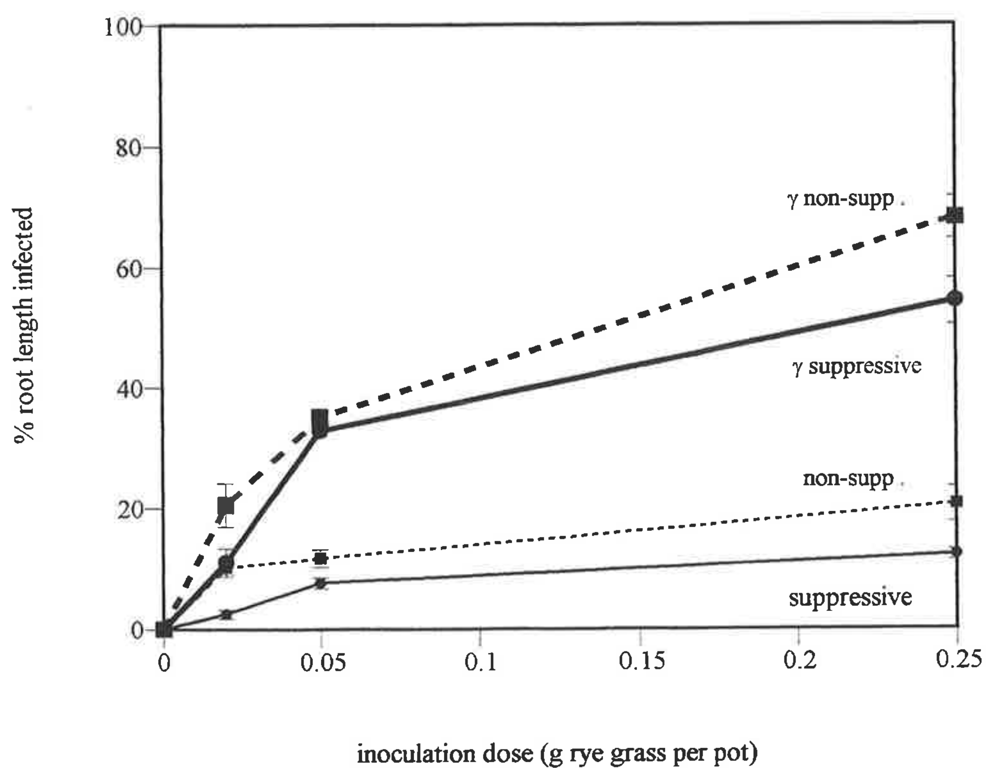


Figure 4.4: Percentage root infection for wheat plants grown in suppressive and non-suppressive (non-supp.) soil either gamma irradiated (γ) or left intreated and inoculated with increasing doses of *Gaeumannomyces graminis* var. *tritici.* Values are means of 4 replicates and lines indicate standard error of the data.

4.3.4 *Pythium irregulare*

There were no differences in disease severity between suppressive soil and non-suppressive soil (Fig. 4.5). Plant emergence was significantly (P = 0.01) higher in inoculated field soils than inoculated γ-irradiated soils and similar in all uninoculated soils. Plant emergence in γ-irradiated soils was significantly reduced at all inoculation doses compared to the uninoculated control but only with an inoculation dose of 4 colonised PDA plugs in field soils.

4.3.5 *Bipolaris sorokiniana*

There was no clear trend in disease severity between suppressive soil and non-suppressive soil, although there was a significant (P = 0.01) interaction between soil type and soil treatment (Fig. 4.6). All inoculations significantly (P = 0.01) increased disease severity. For suppressive soil, there was no difference between field and γ-irradiated soils, but for non-suppressive soil, disease severity was higher in field soil compared to irradiated soil. For field soil, disease severity was higher in non-suppressive soil compared to suppressive soil and there was no difference between the two irradiated soils.

4.3.6 *Fusarium graminearum*

Disease severity was significantly (P = 0.01) lower in suppressive field soil compared to non-suppressive field soil at inoculation doses of 0.03 and 0.15 g chaff per pot (Fig. 4.7). Disease severity was similar in the two field soils at inoculation doses of 0 and 0.01 g chaff per pot. For γ-irradiated soils, disease severity was similar for suppressive and non-suppressive soil for

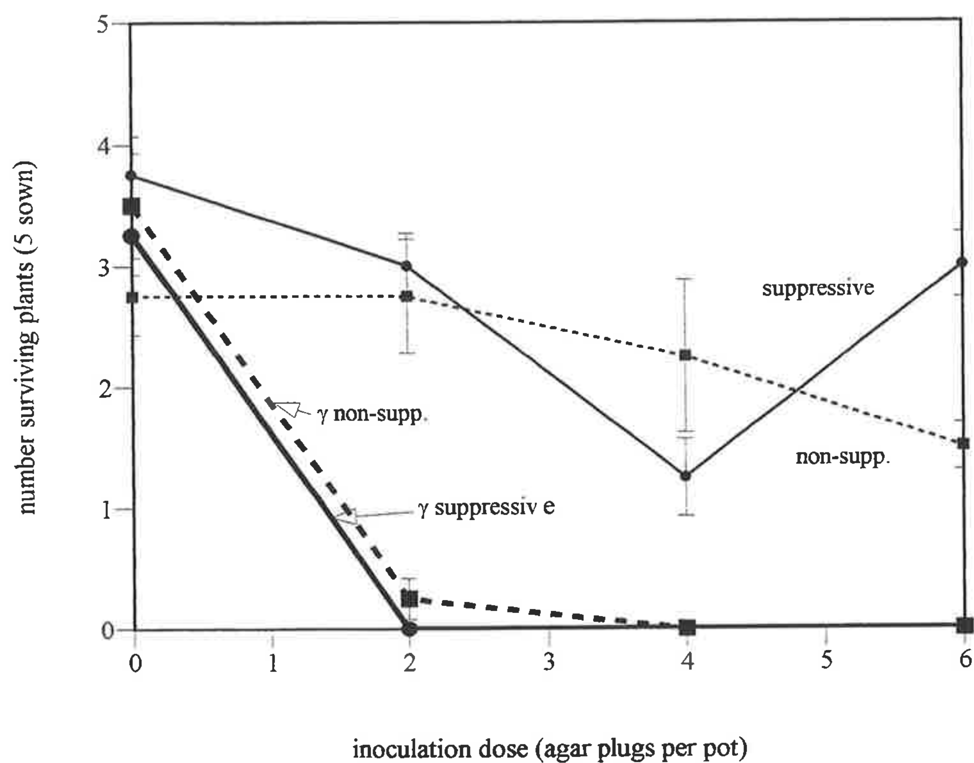


Figure 4.5: Number of surviving medic plants grown in suppressive or non-suppressive (non-supp.) soil either gamma irradiated ($\gamma$) or left untreated and inoculated with increasing doses of *Pythium irregulare*. Values are means of 4 replicates and lines indicate standard error of the data.

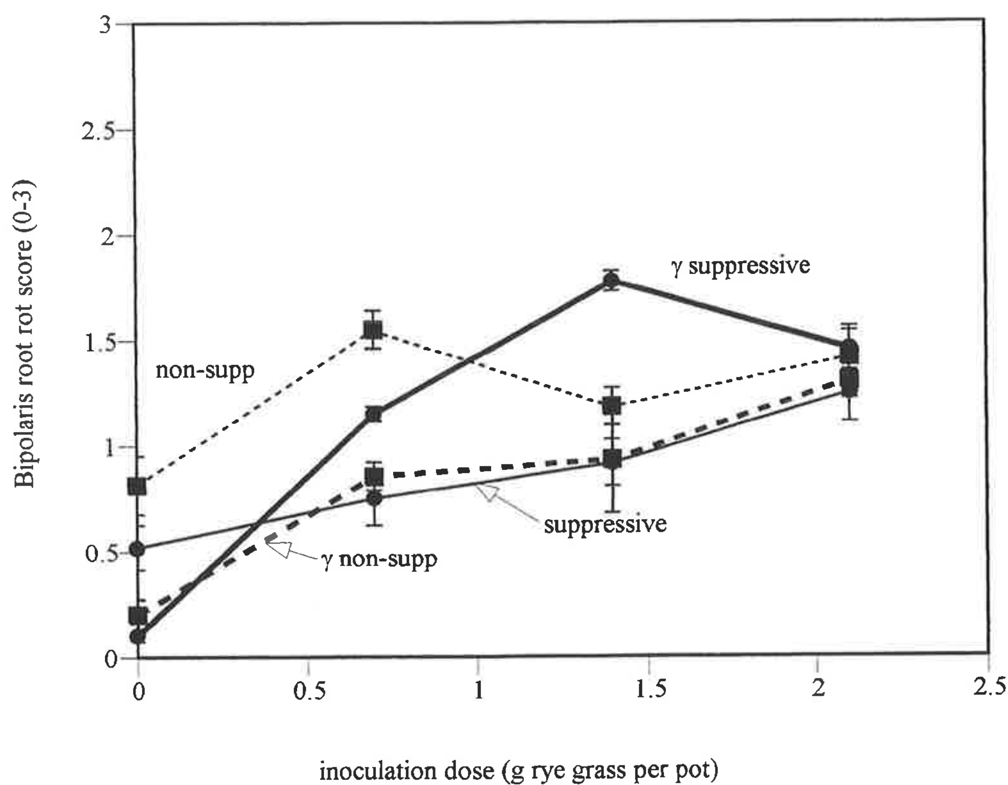


Figure 4.6: Bipolaris root rot scores for wheat plants grown in suppressive and non-suppressive (non-supp.) soil either gamma irradiated (γ) or left untreated and inoculated with increasing doses of *Bipolaris sorokiniana* Values are means of 4 replicates and lines indicate standard error of the data

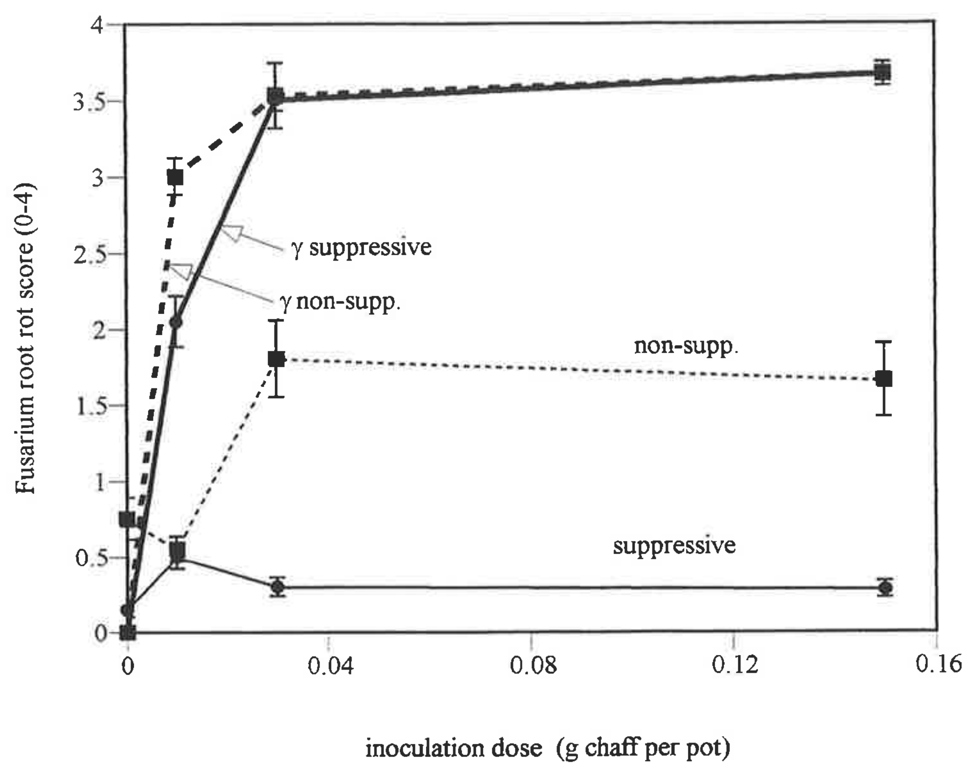


Figure 4.7: Fusarium root rot scores for wheat plants grown in suppressive and non-suppressive (non-supp) soil either gamma irradiated (γ) or left untreated and inoculated with increasing doses of *Fusarium graminearum*. Values are means of 4 replicates and lines indicate standard error of the data.

inoculation doses of 0, 0.03 and 0.15 g chaff per pot, and higher in suppressive soil for 0.01 g per pot. For all inoculated treatments disease severity was higher in γ-irradiated soils compared to field soils. Significant differences were due to a significant (P = 0.01) three way interaction between soil type, soil treatment and inoculation dose.

### 4.4 Discussion

Results presented in this chapter indicate that the soil suppressive to Rhizoctonia barepatch is suppressive against *R. solani* AG-8 at relatively high and low inoculum doses, and also several other fungal pathogens. In soil receptivity experiments, the suppressive soil was less receptive to diseases due to *R. solani* AG-8, *F. graminearum*, and *G. graminis* var. *tritici* compared to the non-suppressive soil. There was no evidence of suppression of damping-off caused by *P. irregulare* and results for *B. sorokiniana* were inconclusive.

Initial results (Sections 3.3.1 and 3.3.2) demonstrated that severity of disease due to *R. solani* AG-8 was significantly lower in suppressive soil compared to non-suppressive soil at one inoculation dose. Results presented here indicate that disease severity is consistently lower in suppressive soil compared to non-suppressive soil regardless of inoculation dose of *R. solani* AG-8 (except for one experiment). Hence, the suppressive soil is not receptive to disease development even at very high inoculation doses. This finding has implications for the persistence of this suppression in the field, because disease would be reduced even if the *R. solani* AG-8 population increased over time. An additional soil with a high level of organic carbon (haystack soil) was tested for receptivity. Disease severity in this soil at a range of inoculation doses was similar to the suppressive soil. This soil was

chosen due to the high residue inputs it had received in the past (R. Manley, pers. comm.). These results provided correlative evidence for the role of residue inputs in the development of suppression. However, the role of plant nutrition in the suppression occurring in this soil can not be discounted because improved plant nutrition in haystack soil was indicated by significantly higher shoot dry weight per plant compared to both suppressive soil and non-suppressive soil. Soil receptivity experiments have proved to be a useful way of comparing soils for existing disease severity, and for response to inoculation. Future experiments involving the screening of soils for suppression will clearly have to take the nutrient status of the soil into account. Severity of disease due to *F. graminearum* differed between suppressive and non-suppressive soil (Section 4.3.6) even though nutrient solution was added, so nutritional differences were not responsible for differences in suppressiveness in this case.

The second conclusion from these experiments is that the organisms involved in this suppression are present in the soil, and can become active during the two week pre-incubation period. Soils were collected from under an established pasture (September 1994), during the dry summer period (January 1995), and during the wheat growing season (August 1995). Environmental and soil conditions at these times would be distinctly different due to the different seasons (spring, summer and winter respectively) and different plants growing (mixed grass pasture, no plants, and wheat respectively). The existence of suppressive characteristics in this soil is therefore not temporal or dependent on the presence of particular plant species, and can be expressed (given suitable plant growing conditions) throughout the year.

The receptivity of suppressive and non-suppressive soil to *G. graminis* var. *tritici* and *F. graminearum* was similar to that for *R. solani* AG-8. The reason for increased disease severity due to *G. graminis* var. *tritici* in irradiated non-suppressive soil compared to irradiated suppressive soil is not known. This difference was only expressed at the highest inoculum dose, so a biologically significant difference between these two irradiated soils is unlikely. Further experimentation may be necessary to establish whether there are additional non-biological factors involved in suppression of disease caused by *G. graminis* var. *tritici* in the soils. An important finding was the level of suppression against disease due to *G. graminis* var. *tritici* and *F. graminearum* in both suppressive and non-suppressive field soils compared to the irradiated soils which was similar to that reported in Chapter 3 for *R. solani* AG-8.

The response to *P. irregulare* inoculation was much more variable than for the other pathogens, and suppressive and non-suppressive field soils showed similar receptivity. No conclusion on relative receptivity of the soils to *B. sorokiniana* could be made. Low disease severity, and absence of any difference between field and irradiated soils indicated that the pathogenicity of the inoculum was too low to give reliable results. The experiment was repeated (data not presented) and a similar response was observed.

The response to inoculation of suppressive and non-suppressive field soils with a range of *Rhizoctonia* spp. was unexpected. Firstly, the suppressive and non-suppressive soils did not differ greatly in disease development for the *R. solani* AG-8 treatments. Furthermore, disease severity was low when soils were inoculated with *Rhizoctonia* spp. from other AG's. When all data was pooled, disease severity on wheat was significantly lower in suppressive soil compared to non-suppressive soil, but differences between all inoculated

treatments were not obvious. There were clear-cut differences between only two of the four treatments (uninoculated and inoculated with R1824, AG-8). These results were in contrast to those from a soil collected approximately one month earlier (3/8/95). Inoculation of suppressive soil and non-suppressive soil collected on 3/8/95 with two millet seeds of *R. solani* AG-8 (RS-21) resulted in mean disease severities of 1.0 and 2.1 respectively. However, in the latter experiment (soil collected on 7/9/95) the same inoculation dose resulted in disease severities of 3.1 and 3.2 respectively. These experiments were conducted under similar conditions, using the same batch of inoculum and reasons for the differences are not known. Although inconsistent with other disease severity results for the two soils, it is unlikely these results represent more than experimental variability, and perhaps undetected experimental differences. The lack of clear differences between the two soils in terms of disease severity due to *R. solani* AG-8 means that conclusions on the relative receptivity to other *Rhizoctonia* spp can not be made. Few differences in disease severity were found when the two soils were inoculated with non AG-8 *Rhizoctonia* isolates. An explanation for this was that a much higher inoculation rate was necessary to detect any differences (similar to that for the *B. sorokiniana* experiment). Disease severity due to *R. solani* AG-2 (isolate 1344) and AG-4 (isolate 1661) was significantly higher in non-suppressive compared to suppressive soil, so it appears the two soils show a similar response to disease caused by non AG-8 *Rhizoctonia* spp. as those caused by *R. solani* AG-8 in these two soils.

Differing results for soil receptivity to the range of pathogens tested were not due to time of soil collection. Soil samples used for the *G. graminis* var *tritici* and *P. irregulare* experiments, and the *B. sorokiniana* and *F. graminearum* experiments were also used in one of the *R. solani* AG-8 receptivity experiments. For both of these soil samples (10/1/95 and 3/8/95

respectively), disease severity due to *R. solani* AG-8 was higher in non-suppressive soil compared to suppressive soil so the suppressive mechanisms were active.

The pathogens used in these experiments were selected to have differences in a number of characteristics, these were : chemical characteristics of cell wall components; nature of infective propagules; and nature of survival structure and its site in the soil. These characteristics were chosen with the following possibilities in mind : antagonism to cell walls with specific chemical composition only; inhibition of particular modes of infection and hence propagule type; and antagonism to survival structures, resulting in reduced survival and / or successful growth of infective propagules from these sites.

A relationship between fungal cell wall components and taxonomic groups is established (Bartnicki-Garcia, 1968). Taxonomic classes of fungi can be divided into eight groups based on the primary polysaccharides in their mycelial cell walls. Group II (cellulose - glucan) contains the Oomycetes and group V (chitin - glucan) contains the Ascomycetes, Basidiomycetes and Chytridiomycetes. The fungi used in the soil receptivity experiments all fall into groups II and V. These fungi are classified into the following classes : *R. solani* AG-8 (teleomorph *T. cucumeris*), Basidiomycetes; *G. graminis* var *tritici*, Ascomycetes; *F. graminearum* (teleomorph *Gibberella zeae* (Schw.), Petch), Ascomycetes; *B. sorokiniana* (teleomorph *Cochliobolus sativus* (Ito & Kurib.) Dreschler ex. Dastur), Ascomycetes; *P. irregulare*, Oomycetes (von Arx, 1987). Suppressive soil was less receptive to disease development when inoculated with fungi in group V (*R. solani* AG-8. *G. graminis* var. *tritici* and *F. graminearum*) compared to non-suppressive soil and the two soils were equally receptive to the fungus from group II (*P. irregulare*). *B. sorokiniana* is in group V, so in terms of this characteristic,

suppressive soil should be less receptive to this pathogen than non-suppressive soil. Larger populations of *Trichoderma* spp. in suppressive soil compared to non-suppressive soil (Chapter 7) suggest a role of specific antagonists such as these. This type of direct antagonism involves enzymic breakdown of cell walls so chemical compositions of the walls will be of key importance. *Trichoderma* spp. produce both cellulase and chitinase (Elad *et al.*, 1982 ; 1984; Wood and McCrae, 1986; Ridout *et al*, 1986). The presence of particular cell wall components can "hinder access of lytic enzymes" to other susceptible components, probably due to physical limitations (Wessels and Sietsma, 1981). Hence, the antagonist may either not produce the necessary lytic enzyme, or the substrate in the walls may be physically protected from degradation by the other cell wall components. To test the hypothesis directly that fungi with chitin - glucan walls are suppressed, soil receptivity to disease due to fungi from other groups (I - VIII, Bartnicki-Garcia, 1968) needs to be assessed. In summary the walls types are : Group I cellulose - glycogen, II cellulose - glucan, III cellulose - chitin, IV chitosan - chitin, V chitin - glucan, VI mannam - glucan, VII mannan - glucan, and VIII polygalactosamine - galactan. As well, the antagonistic activity of *Trichoderma* spp. and other antagonists isolated from the site could be tested against a range of pathogens.

Grouping of the fungi used in the receptivity experiments in terms of infective propagules provides further correlative evidence of mechanisms of suppression. *R. solani* AG-8 and *G. graminis* var. *tritici* infect via hyphae only, *F. graminearum* and *B. sorokiniana* infect via hyphae and spores (conidia, chlamydospores and ascospores, and conidia respectively), and *P. irregulare* infects with motile zoospores (Wiese, 1991). Hence, a clear difference between *P. irregulare* and the other pathogens exists in terms of their epidemiology. Diseases that can be suppressed in this soil involve

growth of hyphae towards the root, or germination of spores and growth of the resulting germ-tube towards the root. The disease that is not suppressed by this soil (damping-off caused by *P. irregulare*) involves active movement of zoospores towards the root, followed by germination and rapid, direct infection. However, it must be noted that in the experiment described in this chapter, infection by either zoospores or mycelium was possible as myelium of *P. irregulare* (colonised PDA plugs) was used as inoculum. Grouping of these pathogens in terms of infective propagules suggests that diseases involving growth of the pathogen through the soil prior to infection can be suppressed, but those involving active movement directly towards the root prior to germination and infection may not be suppressed by the soil suppressive to Rhizoctonia barepatch. To test this hypothesis directly, soil receptivity to disease due to fungi with motile spores (eg *Phythophthora* spp., other *Pythium* species), and without (eg *Pseudocercosporella herpotrichoides*, *Rhizoctonia cerealis*) (Wiese, 1991).

A third classification of these fungi is in terms of survival structure and its site in the soil. *R. solani* AG-8 and *G. graminis* var. *tritici* both survive as hyphae in particles of debris (Wiese, 1991). Ascospores of *G. graminis* var. *tritici* are formed but are not important propagules (Wiese, 1991). *F. graminearum* and *B. sorokininana* survive as spores in the soil or hyphae in particles of debris (Wiese, 1991). *P. irregulare* survives as spores in the soil and in particles of debris (Wiese, 1991). All fungi that are suppressed in this study survive as hyphae in particles of debris. If antagonism to the survival structure is an important mechanism of suppression, presence of hyphae in particles of debris may be a requirement for suppression to occur. Furthermore, the antagonists may require the organic matter to survive saprophytically.

Of these three groupings of fungi (cell-wall components; infective propagules; survival site and structure), only cell-wall components may be important in the pot experiments reported, but all three may have a role in field soil. These pot experiments were short term (maximum of 12 weeks) and artificial inoculum was used. Therefore, disease severity would not have been influenced by infection from propagules resident in the soil or organic matter, or the relative survival of the fungi in suppressive soil or non-suppressive soil. However, disease severity in pot experiments may have been influenced by cell-wall components of the causal agents due to the requirement for growth out from the inoculum to cause disease.

In summary, several important and novel results are presented in this chapter. The suppressive soil from Avon is active against disease caused *R. solani* AG-8 at high or low inoculum doses, and against disease caused by two other important pathogens of wheat (*G. graminis* var. *tritici* and *F. graminearum*). These findings also showed that presence in pathogens of chitin - glucan cell walls, infection by non-motile propagules, and survival as hyphae in particles of debris may all correlate with suppression by the soil suppressive to Rhizoctonia barepatch.

# Chapter 5: Quantification of *Rhizoctonia* in suppressive and non-suppressive soil

## 5.1 Introduction

Chapters 3 and 4 have established that Rhizoctonia barepatch decline is biological in nature and that the suppression is active against high and low inoculum doses of *R. solani* AG-8, and *G. graminis* var. *tritici* and *F. graminearum*. However, the size of the population of resident pathogens in suppressive soil compared to non-suppressive is not known. As absence of disease symptoms does not always imply absence of the causal agent, it needs to be determined if the two soils differ in amount of pathogen as well as in disease severity. The aim of the experiments described in this chapter was to quantify the *Rhizoctonia* populations in suppressive soil and non-suppressive soil and determine if a correlation with disease severity exists. To elucidate possible sites of suppression further and to determine which growth phase of *Rhizoctonia* is being suppressed, *Rhizoctonia* populations were quantified in soil with and without living plant roots (Chapter 6). Differences in soil populations of *Rhizoctonia* would support the hypothesis that *R. solani* AG-8 is being suppressed in the saprophytic phase.

Different techniques were used in an attempt to quantify the range of propagule types of *Rhizoctonia* that may exist in the Avon soils used. Different propagule types of *Fusarium oxysporum* f. sp. *lini* are known to have differing abilities to cause disease (Couteaudier and Alabouvette, 1990b), and this may also be the case for *R. solani* AG-8. Distinct morphological structures as found in *F. oxysporum* f. sp. lini (microconidia and microchlamydospores) do not occur in *R. solani* AG-8, however

separation of fungal units into total, viable, active and pathogenic is possible (S. Neate, pers. comm.). Techniques to quantify pathogenic units (pot bioassays and assessment of field-grown plants), viable units (isolation on agar plates) and total units (*R. solani* AG-8 specific DNA probe) were used.

Wheat plants from the field plots growing during the 1994 season were assessed for plant growth and disease severity. Soil was collected for bioassays when wheat was not growing.

Quantitative isolation of *Rhizoctonia* has been reported using methods that require colonisation of living host plants (Davey and Papavizas, 1962; Sneh *et al.*, 1966), saprophytic colonisation of filter paper immersed in nutrient solution and antibiotics (Herr, 1973), growth on nutrient agar in tubes (Mueller and Durrell, 1957) and plant segments (Blair, 1945; Papavizas and Davey, 1959; Sneh *et al.*, 1966), and growth *in vitro* on selective agar media. A number of *Rhizoctonia*-selective agar media have been reported based on the medium of Ko and Hora (1971) (Ferris and Mitchell, 1976; Flowers, 1976; Gangopadhyay and Grover, 1985; Castro *et al.*, 1988), and a medium based on ethanol and potassium nitrate has also been described (Trujillo *et al.*, 1987; Vincelli and Beaupre, 1989). These media are selective for *Rhizoctonia* and not *R. solani* AG-8 specifically. Isolation *in vitro* can be from organic matter sieved from soil (Weinhold, 1977; Neate, 1987), clumps of soil (Ko and Hora, 1971) or soil formed into uniform pellets (Henis *et al.*, 1978b).

Isolations of *Rhizoctonia* from suppressive and non-suppressive soil were attempted using whole soil and organic matter sieved from the soil and plated onto selective and non-selective media. A non-selective agar medium (modified PDA) and two selective agar media (modifed media of Ko and

Hora, 1971 and Trujillo *et al*., 1987)) were used. This range of media was used to avoid selecting against any section of the *Rhizoctonia* population by using one particular medium only.

Recent developments in molecular based detection procedures have resulted in a DNA probe specific to *R. solani* AG-8 (Matthew *et al.*, 1995) and a procedure for extraction of DNA from soil organic matter (Whisson *et al.*, 1995 after Raeder and Broder, 1985). The amount of DNA of *R. solani* AG-8 present in soil organic matter can be determined and compared with disease severity in the growing crop or used as a diagnostic tool for prediction of disease levels (Whisson *et al.*, 1996). This approach has also been successfully applied for quantification of *G. graminis* var. *tritici* DNA in organic matter and infected plant roots (Herdina *et al.*, in press; Ophel Keller *et al*., in press).

The DNA assay was used to quantify the amount of *R. solani* AG-8 present in suppressive and non-suppressive soils 4, 6 and 8 weeks after sowing of the field plots in the 1994 season (coinciding with assessment of field plants).

## 5.2 Materials and Methods

### 5.2.1 Bioassay of suppressive and non-suppressive field soils for Rhizoctonia root rot

#### 5.2.1.1 Experiment 1 - Bioasssay on wheat

Soil (300g) was added to plastic 300 ml capacity pots and pre-incubated in a 15°C water bath for 4 weeks. Five surface disinfested wheat seeds were then sown. Plants were harvested after a further 5 weeks incubation in the water bath and assessed for disease severity, shoot dry weight and root fresh weight.

The experiment had 1 treatment factor (soil type : suppressive or non-suppressive) and 4 replicate pots of each in a RCBD.

#### 5.2.1.2 Experiment 2 - Bioassay on a range of plant hosts

The methods used were similar to Experiment 1 (Section 5.2.1.1) except there was 3 weeks pre-incubation and plants were harvested 4 weeks after sowing and assessed for disease severity, shoot dry weight and root dry weight.

To assess the effect of plant host on disease severity, pots were sown with surface disinfested seeds of wheat, barley, oats, peas or sub-clover. Plant cultivars and disease assessment procedures are described in Sections 2.3 and 2.6.

The experimental design was a 2 x 5 factorial of soil type (suppressive or non-suppressive) x host plant with 4 replications of each treatment combination in a RCBD. Results on disease severity were analysed separately for cereals and non-cereals due to the different assessment procedures used.

5.2.1.3 Experiment 3 - Bioassay on a range of plant hosts

The methods used were similar to Experiment 2 (Section 5.2.1.2) except there was 2 weeks pre-incubation, plants were harvested 5 weeks after sowing and two additional plant hosts (lupin and tomato) were used.

The experimental design was a 2 x 7 factorial of soil type (suppressive or non-suppressive) x plant host with 4 replications of each treatment combination in a RCBD.

5.2.2 Assessment of field-grown plants from suppressive and non-suppressive soils for Rhizoctonia root rot

Five plants were sampled from field plot replicates 1-5 of the suppressive and non-suppressive soils 4, 6 and 8 weeks after sowing during 1994. Plants were assessed for shoot height, shoot dry weight, root dry weight, root length and severity of Rhizoctonia root rot (Section 2.6). Root length was measured using the grid intercept method of Tennant (1975). Intact root systems were spread out in a 15 cm dia. glass petri dish with grid (1cm) markings. The number of intercepts between roots and the grid lines was counted under ca. 20x magnification and converted to root length with the following formula: root length (cm) = number of intercepts x 0.7857

### 5.2.3 Isolation of *Rhizoctonia* from suppressive and non-suppressive soil

#### 5.2.3.1 Isolation of *Rhizoctonia* from soil pellets

The pellet soil-sampler described by Henis *et al.* (1978b) was used to make cylindrical pellets of suppressive and non-suppressive soil approximately 0.5 g in weight, 5 mm in diameter and 5 - 7 mm high.

##### 5.2.3.1.1 Experiment 1

Soil sub-samples were placed in 9 cm petri dishes and a pellet sampler used to prepare the pellets. Pellets were placed on a non-selective medium (PDA + 100 ppm streptomycin sulphate and 100 ppm tetracycline hydrochloride) or a *Rhizoctonia*-selective medium. Two *Rhizoctonia*-selective media were used, a modified form of the ethanol potassium nitrate (EPN) media of Trujillo *et al.* (1987) that was effective for the Avon soil type (S. Neate, pers. comm.) and Ko and Hora's (1971) selective medium as modified by Vincelli and Beaupre (1989) ($KH_{pm}$). Approximately fifteen pellets were placed in each 9 cm petri dish (5-8 per media) and incubated at 25°C for 4 days. After incubation, fungi growing from pellets were transferred onto water agar (WA) for further identification. Media are described in Appendix 1.

##### 5.2.3.1.2 Experiment 2

The methods used in Experiment 1 (Section 5.2.3.1.1) were modified to include pre-incubation of the soils prior to pellet sampling. Soil sub-samples were placed in 9 cm petri dishes, sealed with parafilm and incubated in the dark at 15°C for 7 days. Twelve pellets of each soil were added to five 9 cm

petri dishes containing modified EPN media and incubated at room temperature for 2 weeks. This was repeated using soil pre-incubated at 15°C for 5 days.

5.2.3.2 Isolation of *Rhizoctonia* from organic matter

Organic matter was sieved from suppressive and non-suppressive soils and mixed with cooled agar media in 9 cm petri dishes (Weinhold, 1977; Neate, 1987). Soil subsamples (100 g) were washed through a stack of three sieves (2 mm, 710 µm and 250 µm) with a jet of tap water and the plant debris retained on the sieves was floated off in water. Debris from each sieve was kept separate.

To determine which agar medium was suitable for growth of *Rhizoctonia* from organic matter pieces, a sub-sample of the sieved organic matter was added to cooled modified EPN media or cooled neutral dox yeast media (NDY/6) (Appendix 1) with 100 ppm streptomycin sulphate and 50 ppm tetracycline hydrochloride (NDY/6 + strep. + tet.). Fifty pieces of organic matter from each size range (250 - 710 µm, 710 µm - 2 mm, and > 2 mm) were mixed with cooled modified EPN media and 20 in cooled NDY/6 + strep. + tet. media. The EPN plates were incubated for 5 days at 25°C and the NDY/6 + strep. + tet. plates for 1 day at 25°C.

After selecting the most suitable medium, 100g of each soil was sieved as described above and all pieces of organic matter from each size range mixed in cooled NDY/6 + strep. + tet. media and incubated at 25°C for 1 day. Fungal isolates were transferred onto WA + strep. + tet. for further identification, based on characteristic morphology (Sneh *et al.*, 1991).

5.2.4 *R. solani* AG-8 specific DNA probe

Soils were collected from field plot replicates 1-4 on 20/7/94, 3/8/94 and 18/8/94 (4, 6 and 8 weeks after sowing respectively). Replicate samples for each soil were combined and washed through 2 mm and 710 μm sieves with a jet of water. Organic matter was floated off from the >2 mm and 710 μm-2 mm fractions and air-dried.

DNA was extracted from the air-dried organic matter using a modification of the method of Raeder and Broder (1985) as described by Whisson *et al.* (1996). Solutions are described in Appendix 2. Organic matter (0.2 g) was ground in liquid nitrogen and mixed with SDS extraction buffer (1 ml) to form a slurry. The slurry was divided between two 1.5 ml eppendorf tubes and an equal volume of phenol / chloroform added to form an emulsion. After centrifugation (1 hour at 13 000 g) the upper phase was transferred into a new tube and RNase A added (0.1 mg / ml). The tubes were incubated (37°C for 30 min) then an equal volume of chloroform / isoamyl alcohol (24:1) added. Tubes were then centrifuged (10 min at 13 000 g), the upper phase transferred to a new tube and cold isopropanol (0.54 vol.) added. The precipitated DNA was spun down, washed with 70 % cold ethanol and dried under vacuum. The dry pellet was resuspended in 50 μl TE buffer (pH 8).

The DNA concentration was determined by running a small sample on a 1 % agarose gel, staining in ethidium bromide (0.5 μg / ml TAE) and comparing the resulting band intensity with a known concentration of marker (λ phage DNA restricted with *Hind*III or *Hind*III/*Eco*RI).

The DNA was purified by eluting through a Sepharose CL6B column. The purified DNA was precipitated with 2 x volumes of cold 100 % ethanol

overnight at -20°C, washed with 70 % cold ethanol, dried under vacuum and resuspended in 50 μl TE (pH 8). The purified DNA concentration was determined as for the unpurified sample.

A DNA probe specific to *R. solani* AG-8 (pRAG-12; Matthew *et al.*, 1995) was labelled with $^{32}P$ [α-$^{32}P$]dCTP using random oligonucleotides as a primer (Feinberg and Vogelstein, 1983). The specific activity of the labelled probe was approximately 1.8 x 108 cpm/μg. A Sephadex G-100 column was used to separate the labelled probe from unincorporated nucelotides.

To quantify the amount of *R. solani* AG-8 DNA in soil organic matter approximately 1000 ng of purified DNA from soil organic matter was loaded into a slot blot apparatus (Bio-Dot SF Microfiltration Apparatus, BioRad 170-6542). DNA extracted from a culture of *R. solani* AG-8 (isolate RS-21) by the method of Raeder and Broder (1985) was serially diluted and used as a positive control. The negative control was a mixture of TE buffer and 1 M NaOH. Samples were denatured with 1 M NaOH. The slot blot apparatus was assembled with 3 layers of filters (Bio-Dot slot format filter papers, BioRad 162-0161) and a nylon membrane (Zeta Probe Blotting Membrane, BioRad 162-0165) soaked in sterile nanopure water. The slots were rinsed with 0.5 ml sterile nanopure water then the samples loaded. A vacuum was applied then 0.5 ml 0.4 M NaOH was added to each well. The membrane was removed, rinsed briefly in 2 x SSC and baked (80°C for 1 hour).

Pre-hybridisation of the membrane was carried out in a glass bottle at 65°C overnight using standard hybridisation buffer (ca. 12 ml) (Appendix 2). The membrane was then soaked in 2 x SSC. The labelled pRAG12 probe was denatured in boiling water for 5 min and cooled immediately in an ice / water mix. Hybridisation was carried out in a glass bottle at 65°C overnight with

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standard hybridisation buffer (ca. 12 ml). Low stringency washing conditions were then used : two washes each of 2 x SSC, 0.1 % SDS (65°C for 20 min); 1 x SSC, 0.1 % SDS (65°C for 20 min, shaken) (Sambrook *et al.*, 1989). The washed membrane was exposed to Cronex X-ray film with intensifying screens (Dupont) at -80°C for 4 days. Hybridisation signals from the purified organic matter samples were compared in their intensity to the fungal control.

## 5.3 Results

### 5.3.1 Bioassay of suppressive and non-suppressive field soil

In Experiment 1, disease severity was significantly lower ($P = 0.05$, $LSD = 0.76$) for wheat grown in suppressive soil (average root score of 0.9) compared to non-suppressive soil (2.1).

In Experiment 2, disease severity was similar for all three cereals (wheat, barley and oats), and was significantly lower ($P < 0.001$) in suppressive compared to non-suppressive soil (Table 5.1). Disease severity for the two non-cereals (pea and sub-clover) was similar in suppressive and non-suppressive soil (Table 5.1). Disease severity for sub-clover was significantly ($P < 0.001$) higher than for pea.

In Experiment 3, disease severity for all three cereals was again significantly ($P < 0.001$) lower in suppressive compared to non-suppressive soil (Table 5.2). There was a significant ($P = 0.05$) effect of type of cereal, with lower disease severity for oats compared to wheat and barley. As in Experiment 2, disease severity of the four non-cereals (lupin, pea, sub-clover and tomato)

was similar in suppressive soil and non-suppressive soil (Table 5.2). Disease severity did not differ between the non-cereals.

5.3.2 Assessment of field-grown plants from suppressive and non-suppressive soil for Rhizoctonia root rot

Disease severity was similar in suppressive and non-suppressive soil 4 weeks after sowing, but significantly ($P = 0.05$) higher in non-suppressive soil 6 and 8 weeks after sowing (Table 5.3). Disease severity was low at all sampling times. Between 6 and 8 weeks after sowing, disease severity increased in non-suppressive soil but remained similar in suppressive soil.

Root dry weight, shoot dry weight and root length were similar in the two soils at all sampling times (Table 5.3). Shoot height was significantly ($P = 0.05$) higher in suppressive soil compared to non-suppressive soil (Table 5.3). Root and shoot dry weight were similar 4 and 6 weeks after sowing and higher 8 weeks after sowing ($P < 0.001$ for both). Root length increased between 4 and 6 weeks then decreased after 8 weeks ($P < 0.001$), indicating a sampling inconsistency or an effect of disease. Shoot height increased between 4 and 6 weeks then was similar after 8 weeks ($P = 0.007$).

| soil | plant | Rhizoctonia root score (0-5) |
|---|---|---|
| suppressive | wheat | 0.5 (0.1) |
| | barley | 1.1 (0.1) |
| | oat | 0.4 (0.1) |
| | pea | 1.0 (0.1) |
| | sub-clover | 3.2 (0.1) |
| non-suppressive | wheat | 1.4 (0.1) |
| | barley | 2.1 (0.2) |
| | oat | 2.1 (0.2) |
| | pea | 1.0 (0.1) |
| | sub-clover | 3.3 (0.1) |
| soil | | ** [0.5] |
| plant | | NS |
| soil.plant | | NS |

Table 5.1: Experiment 2. Rhizoctonia root score for plants grown in suppressive or non-suppressive soil. Values are the means of 4 replicate pots and standard errors are given in parentheses. Significance of individual treatments and interactions is indicated by ** (1 % level) or NS (non-significant) and values for LSD at P = 0.05 are given in square parentheses.

| soil | plant | Rhizoctonia root score (0-5) |
|---|---|---|
| suppressive | wheat | 0.7 (0.1) |
| | barley | 0.6 (0.04) |
| | oat | 0.2 (0.1) |
| | pea | 0.1 (0.01) |
| | sub-clover | 0.8 (0.1) |
| | lupin | 0.02 (0.1) |
| | tomato | 0.3 (0.1) |
| non-suppressive | wheat | 1.9 (0.2) |
| | barley | 2.2 (0.2) |
| | oat | 1.0 (0.2) |
| | pea | 0.2 (0.1) |
| | sub-clover | 0.7 (0.1) |
| | lupin | 0.9 (0.4) |
| | tomato | 0.7 (0.1) |
| soil | | ** [0.5] |
| plant | | * [0.6] |
| soil.plant | | NS |

Table 5.2: Experiment 3. Rhizoctonia root score for plants grown in suppressive or non-suppressive soil. Values are the means of 4 replicate pots and standard errors are given in parentheses. Significance of individual treatments and interactions is indicated by ** (1 % level), * (5 % level) or NS (non-significant) and values for LSD at P = 0.05 are given in square parentheses.

| soil | time (weeks) | Rhizoctonia root score (0-5) | root dwt. (mg/plant) | root length (cm/plant) | shoot dwt. (mg/plant) | shoot ht. (cm) |
|---|---|---|---|---|---|---|
| suppressive | 4 | 0 (0) | 13 (7) | 22 (1) | 29 (1) | 21 (0.2) |
| | 6 | 0 (0) | 17 (1) | 86 (9) | 72 (2) | 23 (0.6) |
| | 8 | 0.1 (0.03) | 47 (3) | 65 (7) | 244 (21) | 25 (0.7) |
| non-supp. | 4 | 0.4 (0.08) | 16 (1) | 37 (3) | 28 (1) | 17 (0.7) |
| | 6 | 0.7 (0.1) | 25 (1) | 110 (3) | 81 (8) | 21 (0.8) |
| | 8 | 1.7 (0.2) | 44 (5) | 72 (5) | 192 (23) | 23 (0.8) |
| soil | | NA | NS | NS | NS | ** [2] |
| time | | NA | * [11] | * [23] | * [62] | * [2] |
| soil.time | | ** [0.6] | NS | NS | NS | NS |

Table 5.3: Rhizoctonia root rot score, root dry weight per plant (root dwt.), root length, shoot dry weight per plant (shoot dwt.) and shoot height (shoot ht.) of wheat plants collected from suppressive and non-suppressive (non-supp.) field plots 4, 6 and 8 weeks after sowing in 1994. Values are the means of 5 replicates of 5 plants each and standard errors are given in parentheses. Significance of individual treatments and interaction is indicated by ** (1 % level), * (5 % level), NS (non-significant) or NA (not applicable) and values for LSD at P = 0.05 are given in square parentheses.

5.3.3 Isolation of *Rhizoctonia* from suppressive and non-suppressive soil

Attempts to quantify the viable population of *Rhizoctonia* in suppressive and non-suppressive soils using the soil pellet sampler were unsuccessful. Only one *Rhizoctonia* isolate was recovered from both soil pellet experiments (1 isolate from 75 pellets of non-suppressive soil cultured on $KH_{pm}$, Experiment 1).

NDY/6 was selected as the more suitable medium for fungal isolations from organic matter as EPN plates became contaminated with bacteria and yeast before isolation of fungi was possible. More *Rhizoctonia* was isolated from organic matter sieved from non-suppressive compared to suppressive soil (Table 5.4). There were approximately five times as many *Rhizoctonia* isolates from size fractions > 2 mm and 710 µm - 2 mm from non-suppressive soil compared to suppressive soil. No *Rhizoctonia* was isolated from the 250 - 710 µm size fraction.

5.3.4 *R. solani* AG-8 specific DNA probe

The unadjusted intensity of the hybridisation signal indicated that there was more *R. solani* AG-8 DNA in organic matter sieved from suppressive compared to that from non-suppressive soil 4 and 6 weeks after sowing, and the opposite after 8 weeks (Fig. 5.1). Signals were compared with the positive control and adjusted for unequal loading of DNA on the membrane (Table 5.5). Following adjustment there were few clear differences between the two soils. Approximately half of the samples for both soils were estimated to contain 0.5 ng DNA of *R. solani* AG-8. Entire DNA samples were loaded due to low sensitivity with smaller samples. Variability in

| soil | size fraction | no. particles incubated | no. particles with fungal growth | no. particles with *Rhizoctonia* isolated |
|---|---|---|---|---|
| suppressive | > 2 mm | 162 | 34 | 1 |
| | 710 µm - 2 mm | 1273 | 154 | 4 |
| | 250 - 710 µm | 1559 | 98 | 0 |
| non-suppressive | > 2 mm | 125 | 16 | 5 |
| | 710 µm - 2 mm | 909 | 149 | 21 |
| | 250 - 710 µm | 2049 | 120 | 0 |

Table 5.4: Number (no.) of organic matter particles sieved from suppressive or non-suppressive soil, number with fungal growth, and number from which *Rhizoctonia* was isolated from following incubation in NDY/6 + strep. + tet..

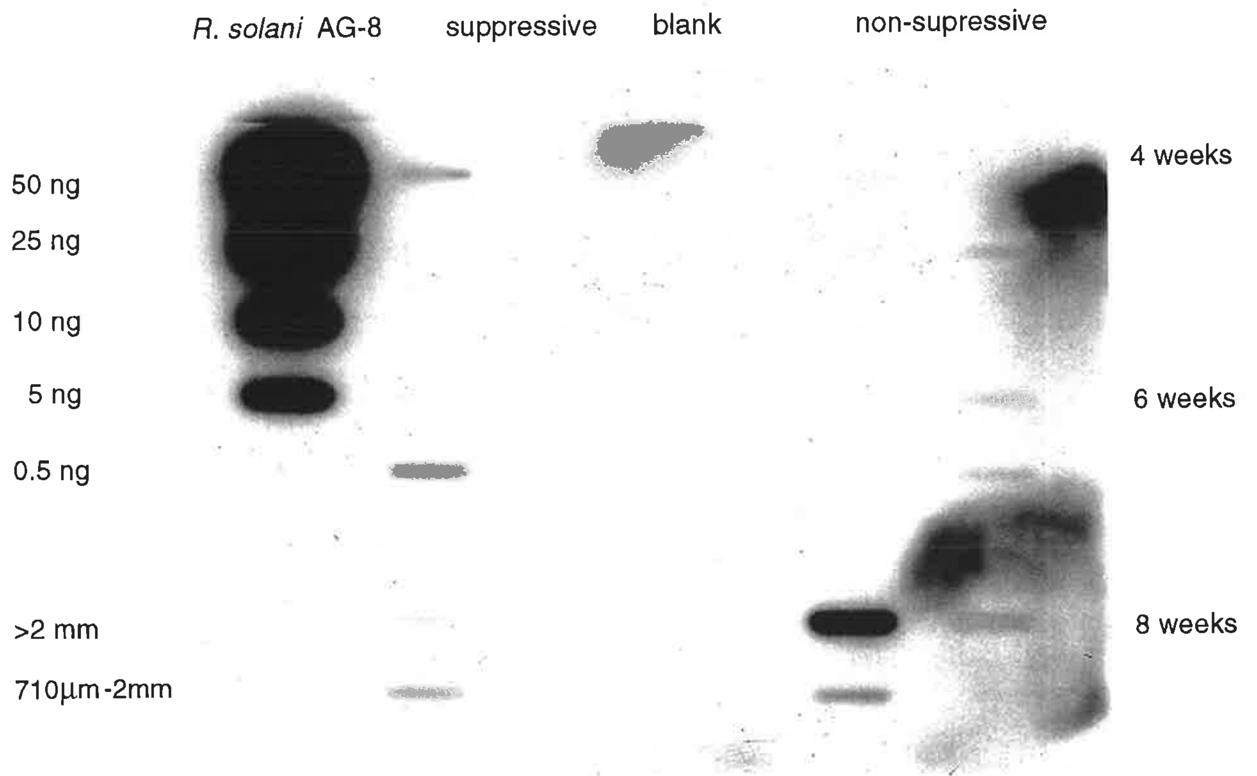



Figure 5.1: Slot blot of DNA extracted from *R. solani* AG-8 culture (isolate RS-21) and suppressive and non-suppressive soil organic matter (>2mm and 710µm-2mm fractions) collected 4, 6, and 8 weeks after sowing in 1994 and hybridised with a *R. solani* AG-8 specific DNA probe (pRAG12).

| weeks after sowing | soil | size fraction | adjusted *R. solani* AG-8 DNA (ng / 1000ng total DNA) |
|---|---|---|---|
| 4 | suppressive | > 2mm | 2.6 |
| | | 710 µm - 2 mm | 0.5 |
| | | | 0.5 |
| | non-supp. | > 2mm | 0.5 |
| | | 710 µm - 2 mm | 0.5 |
| | | | 0.5 |
| 6 | suppressive | > 2mm | 1.8 |
| | | 710 µm - 2 mm | 2.3 |
| | non-supp. | > 2mm | 1.1 |
| | | | 1.5 |
| | | 710 µm - 2 mm | 0.5 |
| | | | 0.5 |
| 8 | suppressive | > 2mm | 0.5 |
| | | 710 µm - 2 mm | 16 |
| | non-supp. | > 2mm | 17 |
| | | | 0.7 |
| | | 710 µm - 2 mm | 2.3 |

Table 5.5: Estimated amount of DNA of *R. solani* AG-8 detected in organic matter from suppressive and non-suppressive soil with a specific DNA probe (pRAG12).

amounts of DNA extracted from 0.2 g organic matter samples meant the maximum amount able to be loaded was not consistent.

No DNA of *R. solani* AG-8 was detected in the negative controls. DNA of *R. solani* AG-8 was detected in all samples of organic matter sieved from suppressive and non-suppressive soil (Table 5.5). Adjusted amounts of DNA of *R. solani* AG-8 were similar in DNA from organic matter sieved from suppressive and non-suppressive soil 4 weeks after sowing (ca. 0.5 ng per 1000 ng total DNA). Amount of DNA of *R. solani* AG-8 tended to be higher for both soils after 6 and 8 weeks compared to 4. There were two strong signals for DNA from organic matter sieved from non-suppressive soil organic matter 8 weeks after sowing (estimated as 17 and 0.7 ng for replicated > 2 mm samples).

**5.4 Discussion**

Results obtained in this chapter highlight the need to define the form of *Rhizoctonia* being quantified in investigations. The separation of *Rhizoctonia* into functional types has proved to be a useful means of quantifying populations in suppressive and non-suppressive soil. Pathogenic and viable *Rhizoctonia* populations were found to be higher in non-suppressive soil compared to suppressive soil, but this was not the case for the total *Rhizoctonia* population. While supporting the use of these techniques for quantification studies, these results show the biology of the organism needs to be taken into account in the planning and interpretation of such studies.

Following the trend of previous work (Roget, 1995; Sections 1.3, 3.3 and 4.3.1), disease severity was higher for cereal plants grown in non-suppressive soil compared to those in suppressive soil. Populations of pathogenic

*Rhizoctonia* may therefore be higher in non-suppressive soil compared to suppressive soil. Qualititave differences in the population of pathogenic *Rhizoctonia* in the two soils may also exist. Results presented in this chapter indicate low viable populations of *Rhizoctonia* in suppressive soil, but negligible disease severity may also result from low pathogenicity of these isolates. Pathogenicity tests of *Rhizoctonia* isolated from these soils in the future is warranted. Disease severity on non-cereals grown in suppressive and non-suppressive soils indicated populations of *Rhizoctonia* pathogenic on these plants are similar in the two soils and lower than populations pathogenic on cereals. *R. solani* AG-8 is known to be pathogenic on both cereals and non-cereals (Section 1.2.3), but in these soils pathogenicity must be greater on cereals. Disease severity for clover plants in bioassay Experiment 2 was very high compared to that for peas and compared to clover in bioassay Experiment 3. This may have been due to incorrect assessment of disease on clover plants in Experiment 2 due to interference of the soil type with disease assessment.

Severity of disease in field plants followed the same trend as in previous experiments (Sections 3.3.1, 3.3.2 and 4.3.1), with higher severity for wheat grown in non-suppressive soil compared to suppressive soil. Similar disease severity in both soils 4 weeks after sowing is likely to be because there was insufficient time for symptom expression. Assessment of disease severity had previously been done 5 weeks after sowing for controlled environment experiments (Sections 3.3.1, 3.3.2 and 4.3.1), and 8 - 10 weeks after sowing for field plants (Roget, 1995).

Methods to quantify viable propagules of *Rhizoctonia* using isolation of fungi from organic matter and soil incubated on semi-selective media were found to be tedious and often unsuccessful. Whether this was due to relatively low

population of the fungus in both soils or the methodology itself is not known. Results from isolations from organic matter provided more definite evidence of a reduced viable population of *Rhizoctonia* in suppressive compared to non-suppressive soil. The viable population (number of isolates) in organic matter from non-suppressive soil was approximately five times that in suppressive soil. In both cases most isolations were from the 710 µm - 2 mm fraction which is in agreement with results of a similar study by Neate (1987) on a non-suppressive soil at the same field site.

Quantification of pathogenic and viable *Rhizoctonia* isolates suggested a good correlation between disease severity and pathogen population. However, results for the total *Rhizoctonia* population, as determined with the *R. solani* AG-8 specific DNA probe, did not support such a conclusion. Positive detection of *R. solani* AG-8 with the specific probe (Matthew *et al.*, 1995) occurred for total DNA extracted from organic matter from both suppressive and non-suppressive soil at all three sampling times (4, 6 and 8 weeks after sowing, 1994) for both size fractions (> 2 mm and 710 µm - 2 mm). Estimation of the amount of *R. solani* AG-8 DNA indicated a slight increase after 6 and 8 weeks compared to 4 but no differences between the two soils.

The organic matter samples used for DNA estimation and fungal isolations were not taken at the same time, but the lack of correlation between results from these two methods of quantification warrants further discussion. Similar amounts of *R. solani* AG-8 DNA were detected in DNA extracted from organic matter in the 710 µm - 2 mm size fraction in suppressive soil and non-suppressive soil but the number of fungal isolations from this fraction were different. Fewer *Rhizoctonia* isolations were made from organic matter from suppressive soil and disease severity was lower, but similar amounts of

*R. solani* AG-8 DNA were detected in organic matter from the two soils. Explanation of the different relative amounts of total, viable and pathogenic *Rhizoctonia* in suppressive soil and non-suppressive soil may be possible by consideration of the biology of this fungus.

Sieved material greater than 2 mm may contain plant roots and larger pieces of organic matter, and material in the 710 µm - 2 mm range may contain plant roots, smaller organic matter, and hyphae previously growing through the soil. It is suggested that roots contain pathogenic *Rhizoctonia*, whereas organic matter contains viable, non-viable, pathogenic, and non-pathogenic *Rhizoctonia* (S. Neate, pers. comm.). Disease severity of Rhizoctonia root rot (pathogenic *Rhizoctonia*), and the viable population of *Rhizoctonia* are lower in suppressive soil compared to non-suppressive soil. Hence, results from the AG-8 specific probe (pRAG12) indicate the population of *Rhizoctonia* that is non-viable and / or non-pathogenic is higher in the suppressive soil compared to the non-suppressive soil.

Disease severity was higher in field grown wheat in non-suppressive soil than in suppressive soil except for one assessment time (4 weeks after sowing). Organic matter samples for fungal isolations were collected approximately 8 weeks after sowing of adjacent plots in 1993 but the field plots used were under grass pasture. The higher number of *Rhizoctonia* isolates from organic matter from non-suppressive soil compared to from suppressive soil correlated with higher disease severity measured at a similar time the following season. It appears that if a propagule of *Rhizoctonia* can become active and successfully grow out of colonised organic matter, it can potentially incite disease. However, positive detection of *R. solani* AG-8 DNA in organic matter with the pRAG12 probe (Matthew *et al.*, 1995) gives no indication of the ability of the fungus to incite disease. This may be

because the probe can detect non AG-8 DNA or non-viable DNA. The pRAG12 probe cross-hybridises moderately with binucleate *Rhizoctonia* spp. (Herdina, pers. comm.). So the higher signal for organic matter from suppressive soil may be at least partially due to binucleate *Rhizoctonia* spp. It is not likely that the probe is detecting dead mycelium. Preliminary experiments (Herdina, pers. comm.) using *G. graminis* var. *tritici* indicate detection of killed mycelium using this technique is unlikely. Soon after (ca. <5 days) artificially killing mycelium, positive detection of *G. graminis* var. *tritici* DNA is markedly reduced or absent. Suppression may be due to reduced total population and increased non-pathogenic *Rhizoctonia* (such as binucleate *Rhizoctonia* spp.).

When disease is being initiated, hyphae of *R. solani* AG-8 can be in three possible places: in the infection court; in the bulk soil; or in pieces of organic matter. Presence in the infection court is clearly necessary for disease incitement, and presence in organic matter is necessary for survival. However, continued presence in organic matter may not be necessary for disease incitement. Nor, as mentioned above, does presence in organic matter implicate subsequent disease incitement. Monitoring of *R. solani* AG-8 DNA levels in organic matter over time may not be suitable for correlation with disease severity due to: a) temporal separation of the phases of disease development, and the associated changes in the pathogen population structure (viable, active, and / or pathogenic), and b) the influence of the level of soil suppressiveness and the subsequent variation in disease severity resulting from a particular total amount of pathogen. The former point refers to the three possible sites of *R. solani* AG-8 during disease initiation and the different forms of *Rhizoctonia* possible at each. Initially the population of *Rhizoctonia* in organic matter will include viable, non-viable, pathogenic, and non-pathogenic forms (S. Neate, pers. comm.). After the break of the season

only *Rhizoctonia* that is viable will begin to grow, and when living roots appear only *Rhizoctonia* that is pathogenic will cause disease. Quantification of the total population of *Rhizoctonia* in organic matter includes all four forms, but disease is only initiated by *R. solani* AG-8 that is viable, pathogenic, and in the infection court. The latter point refers to the concept of soil receptivity described by Alabouvette (1986) where the at a given inoculum dose disease severity is lower in soils with higher levels of suppressiveness. A correlation between disease suppression and soil fungistatis has been found in soils suppressive to Fusarium wilt (Alabouvette, 1986), and may also exist in the soil suppressive to Rhizoctonia barepatch.

In conclusion, pathogenic and viable *Rhizoctonia* populations are higher in non-suppressive soil compared to suppressive soil. Positive detection with a *R. solani* AG-8 specific probe (pRAG12) showed that AG-8 DNA was present in organic matter from suppressive soil. As *R. solani* AG-8 is present in suppressive soil, even though there is negligible disease severity, the fungus must be existing saprophytically rather than both pathogenically and saprophytically. Detection of DNA from *R. solani* AG-8 that is non-viable and / or non-pathogenic, and DNA from *Rhizoctonia* not in AG-8 can not be ruled out, and these fungi may have a role in the suppression.

It is important to use a combination of methods to quantify *Rhizoctonia* populations. This is particularly crucial in suppressive soils because information about the presence and absence of both pathogen and disease is required, neither of which can be quantified with a single measurement.

# Chapter 6: Identification of the site of suppression via quantification of hyphal length

## 6.1 Introduction

It has been established that viable and pathogenic *Rhizoctonia* populations are lower in suppressive soil compared to non-suppressive soil (Chapter 5). The aim of this chapter was to further quantify the *Rhizoctonia* population by direct observation of hyphae in the two soils. This approach was used for two reasons; to confirm the results using techniques outlined in Chapter 5, and, to quantify *Rhizoctonia* populations in soil with and without the presence of living plant roots.

Suppression of disease due to *R. solani* AG-8 has been established in earlier Chapters (3-5) but the likely sites of suppression (bulk and / or rhizosphere soil) are not known. Two general mechanisms for reduced disease severity exist. Reduced amounts of *R. solani* AG-8 may reach the rhizosphere (zone of soil influenced by the root; Bowen and Rovira, 1991) in suppressive soil compared to non-suppressive soil. Alternatively, *R. solani* AG-8 may reach the rhizosphere of both soils but inhibition of disease development may be greater in the rhizosphere of plants growing in suppressive soil compared to non-suppressive soil. Quantification of *Rhizoctonia* in suppressive and non-suppressive soils with or without living roots will indicate which of these mechanisms is occurring. Suppression in either the rhizosphere or bulk soil would be indicated by smaller *Rhizoctonia* populations in suppressive soil compared to non-suppressive soil.

*Rhizoctonia* was directly quantified by measuring length of all hyphae and *Rhizoctonia*-like hyphae in soil. Quantification was done by extracting the hyphae using a membrane filtration technique (Hanssen *et al.*, 1974) and determining hyphal length with the grid interception method. This approach has been used for quantification of external hyphae of vesicular arbuscular mycorrhizal (VAM) fungi by a number of authors (Abbott *et al.*, 1984; Abbott and Robson, 1985; Jakobsen *et al.* 1992a, b). It may be possible to distinguish *Rhizoctonia* hyphae from other soil fungi due to their characteristic wide diameter and branching pattern.

Fine mesh was used to exclude roots to separate soil into that with, or without living roots. Physical exclusion of roots has previously been reported in investigations of the growth and phosphorus translocation of VAM fungi (eg Jasper *et al.*, 1989a ; 1989b; Jakobsen *et al.*, 1992a).

In addition to distinguishing hyphae by morphological characters, hyphae can be examined for metabolic activity using microscopic observations following vital and non-vital stains. Vital stains, such as fluorescein diacetate (FDA), stain only metabolically active hyphae whereas non-vital stains, such as acid fuchsin, stain all hyphae regardless of their metabolic state. FDA is non-fluorescent and it is taken up by metabolically active cells where it is hydrolysed, resulting in the accumulation of fluorescein which can be detected by fluorescence microscopy (Rotman and Papermaster, 1961; Söderström, 1977; Ingham and Klein, 1984). Acid fuchsin is a commonly used fungal stain and the use of fluorescence microscopy can enhance its resolution (Merryweather and Fitter, 1991).

To quantify hyphal length in suppressive and non-suppressive soils the following experiment was performed. Wheat plants were grown enclosed in a

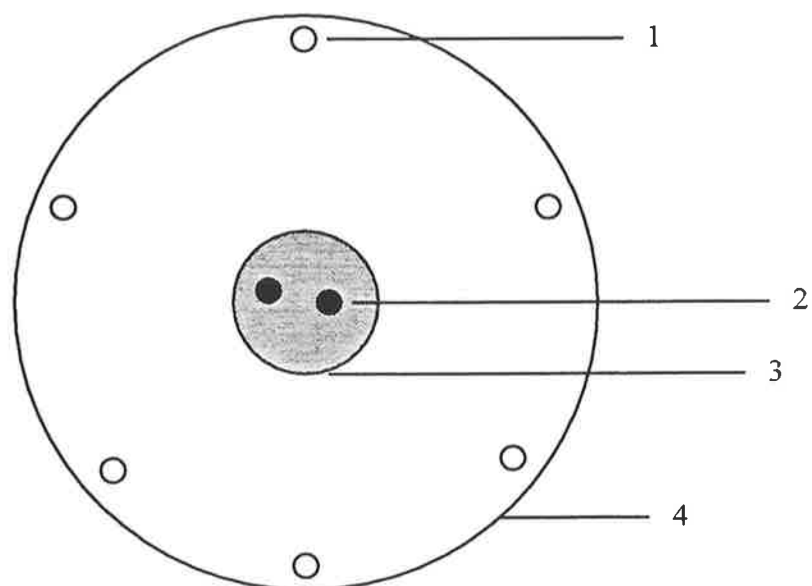
mesh bag in a pot of suppressive or non-suppressive soil inoculated with *R. solani* AG-8 or left uninoculated. Uninoculated treatments allowed quantification of background *Rhizoctonia* populations. Inoculated treatments allowed quantification of *Rhizoctonia* growth from point sources in the bulk soil section of the pots, and detection of differences in suppressive and non-suppressive soils.

## 6.2 Materials and Methods

The experiment was a 2 x 2 factorial of soil type (suppressive or non-suppressive) x inoculum level with 4 replications of each treatment combination in a RCBD.

Tubular mesh bags were prepared from screen printing mesh (pore size 33 μ m) and were approximately 10 cm long with a 3 cm diameter at the top and the base pinched closed. Solvent Cement® (methyl ethyl ketone 58 % w/v, Hardie Iplex) was used to seal the side and base of the bags (D. Jasper, pers. comm.)

The bags were filled with soil and placed in plastic 950 ml capacity pots containing 150 g soil. Soil was then added around the bag giving a total soil weight of 1000 g. Pots were then pre-incubated under controlled conditions (15°C, 9 hour day, photon irradiance 236 $\mu mol\ m^{-2}\ s^{-1}$) for 1 week. Half the pots were then inoculated at their outer edge with 6 millet seeds colonised with *R. solani* AG-8 (isolate RS-21) (Fig. 6.1). One week after inoculation two pre-germinated surface disinfested wheat seeds were sown in the soil contained in the bag. Plants were harvested after a further 4 weeks incubation and assessed for disease severity. At harvest, soil sub-samples (ca. 5 g) were taken with a soil core (13 mm dia.) at the outer edge of the




1. inoculation point
2. wheat seed
3. mesh bag (3 cm dia.)
4. plastic 950 ml pot (11 cm dia.)

Figure 6.1: Inoculation and sowing points for identification of site of suppression experiment

pot (position A), approximately half way between position A and the edge of the mesh bag (position B), and inside the mesh bag (position C) (Fig. 6.2). Water content at the time of sampling was determined so final results could be expressed on a per gram of soil dry weight basis.

A modification of the method of Abbott *et al.* (1984) as described by Sukarno (1994) was used to extract and quantify the soil hyphae. Soil (2 g from the 5 g sub-sample) was suspended in distilled water (200 ml) using a magnetic stirrer (5 min). A sample (6 ml) was taken using a glass pipette and rubber bulb and filtered through a Millipore filter (2.5 cm dia., 8 μm). A sampling manifold for vacuum filtration was used which allowed simultaneous filtration of up to 10 samples (Carbon 14 Centralen sampling manifold, The International Agency for $^{14}C$ Determination, Denmark) (Fig. 6.3)

The soil suspension retained on the filters was stained with FDA and observed under a Zeiss Standard Lab 16 compound microscope equipped for epifluorescence (excitation filter BP 450-490, barrier filter LP 520) at 160x magnification for quantification of metabolically active hyphal length. Filters were observed within 30 min of staining to avoid problems due to loss of fluorescence over time. Hyphae stained with FDA were termed "FDA-active hyphae". A concentrated solution of FDA (2 mg in 1 ml acetone) was prepared and stored at -20°C. The working solution of FDA was diluted in phosphate buffer (60 mM $K_2HPO_4$, pH 7.4) giving a final concentration of 10 μg $ml^{-1}$. The filters were then counter-stained with 180 μl acid fuchsin 0.01 % solution) and observed again for quantification of "total hyphae" (including both metabolically active and inactive hyphae). Metabolically active hyphae fluoresce yellow after staining with FDA and all hyphae stain red after counter staining with acid fuchsin.

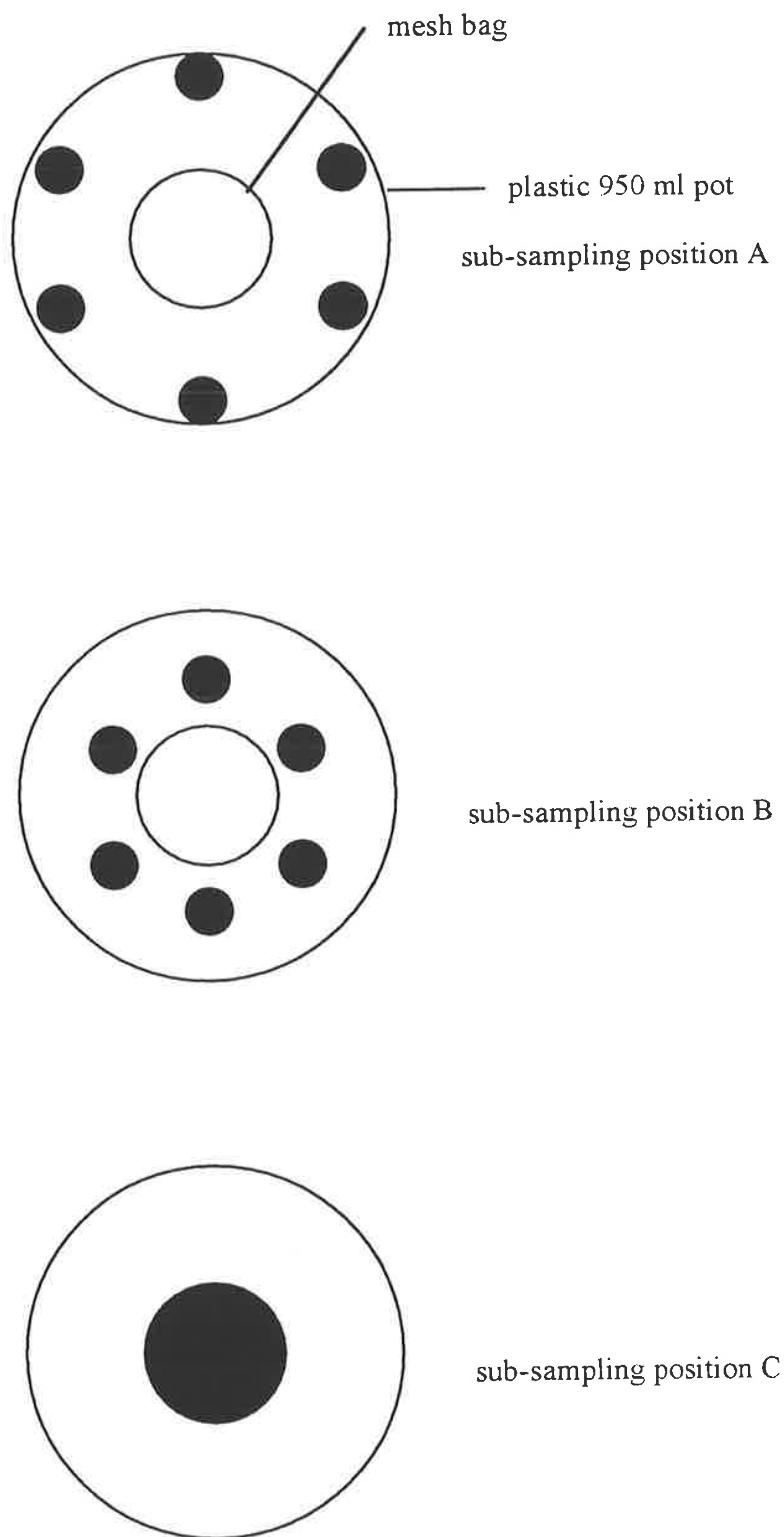



Figure 6.2: Soil sub-sampling positions (A, B and C) in 950 ml plastic pots for identification of site of suppression experiment

Figure 6.3: Multiple sample vacuum filtration sampling manifold (Carbon 14 Centralen sampling manifold, The International Agency for $^{14}C$ Determination, Denmark)

Hyphal lengths were determined by counting the number of intersections between hyphae and a grid eyepiece micrometer. The sum of intercepts in 25 random fields of view was recorded. The modified formula of Tennant (1975) was used to calculate the hyphal length (Section 5.2.2).

*Rhizoctonia*-like hyphae were subjectively distinguished from non-*Rhizoctonia*-hyphae based on diameter, straightness, branching pattern and position of septa. Straight hyphae with constantly large diameters (approximately 10 µm) with characteristic branching and septation were counted as *Rhizoctonia*-like hyphae and all other hyphae as non-*Rhizoctonia*. The total length of hyphae was thus the sum of *Rhizoctonia*-like and non-*Rhizoctonia* lengths.

**6.3 Results**

Disease severity was significantly (P = 0.01) lower in suppressive compared to non-suppressive soil, and significantly (P = 0.01) higher in inoculated soil compared to the uninoculated soil (Table 6.1).

There was a significant (P = 0.01) interaction between position (A, B or C) and soil type (suppressive or non-suppressive) for all four hyphal measurements, indicating that hyphal length responded differently in the two soils at the 3 positions (Table 6.2). Also, total length of hyphae and total length of *Rhizoctonia*-like hyphae were significantly (P = 0.01) higher in uninoculated compared to inoculated soil.

Length of FDA-active *Rhizoctonia*-like hyphae was significantly (P = 0.01) higher in non-suppressive soil compared to suppressive soil at all three

positions and not affected by inoculation (Fig. 6.4). In suppressive soil length of FDA-active *Rhizoctonia*-like hyphae was similar at all three positions. In non-suppressive soil length of FDA-active *Rhizoctonia*-like hyphae was higher at position A compared to B and C.

Total length of *Rhizoctonia*-like hyphae was significantly (P = 0.01) higher in non-suppressive soil compared to suppressive soil at positions A and B, similar in the two soils at position C (Fig. 6.5), and higher in uninoculated (0.98 m per g soil) compared to inoculated soil (0.75 m per g soil). In suppressive and non-suppressive soil total length of *Rhizoctonia*-like hyphae was similar at positions A, B and C.

FDA-active hyphal length was significantly (P = 0.01) higher in non-suppressive soil compared to suppressive soil at position A, similar in the two soils at positions B and C, and not affected by inoculation (Fig. 6.6). In suppressive soil FDA-active hyphal length was similar at positions A, B and C. In non-suppressive soil FDA-active hyphal length was higher at position A compared to B and C.

Total hyphal length was significantly (P = 0.01) higher in non-suppressive soil compared to suppressive soil at position B, similar in the two soils at positions A and C (Fig. 6.7). Total hyphal length was higher in uninoculated (2.0 m per g soil) compared to inoculated (1.6 m per g soil) soil. In suppressive soil, total hyphal length was similar at positions A and B, and A and C, but higher at position C than B. In non-suppressive soil, total hyphal length was also similar at positions A and B, but similar at C and B, and higher at position A than C.

| soil | inoculation (6 millet seeds/pot) | Rhizoctonia root score (0-5) |
| --- | --- | --- |
| suppressive | + | 2.9 (0.03) |
| | - | 1.4 (0.2) |
| non-suppressive | + | 3.8 (0.1) |
| | - | 2.0 (0.1) |
| soil type | | ** [0.5] |
| inoculation | | ** [0.5] |
| soil.inoculation | | NS |

Table 6.1. Rhizoctonia root score for wheat plants grown in suppressive or non-suppressive soil with (+), or without (-) *R. solani* AG-8 inoculation. Values are means of 4 replicate pots and standard errors are given in parentheses. Significance of individual treatments and interaction is indicated by ** (1 % level) or NS (non-significant) and values for LSD at P = 0.05 are given in square parentheses.

| | total hyphal length | FDA-active hyphal length | total *Rhizoctonia*-like length | FDA-active *Rhizoctonia*-like length |
|---|---|---|---|---|
| position | NA | NA | NA | NA |
| soil | NA | NA | NA | NA |
| inoculation | ** [0.21] | NS | ** [0.18] | NS |
| pos.soil | ** [0.36] | ** [0.17] | ** [0.31] | ** [0.15] |
| pos.inoc | NS | NS | NS | NS |
| soil.inoc | NS | NS | NS | NS |
| pos.soil.inoc | NS | NS | NS | NS |

Table 6.2: Significance of individual treatments and interactions on hyphal lengths at positions A, B or C (position / pos) in suppressive and non-suppressive soil (soil) with or without inoculation of *R. solani* AG-8 (inoculation / inoc). Significance levels indicated by ** (1 % level), NS (non-significant) or NA (not applicable) and values for LSD at P = 0.05 are given in square parentheses. Abbreviations: hyphae stained with FDA as "FDA-active"' and hyphae stained with acid fuchsin as "total".

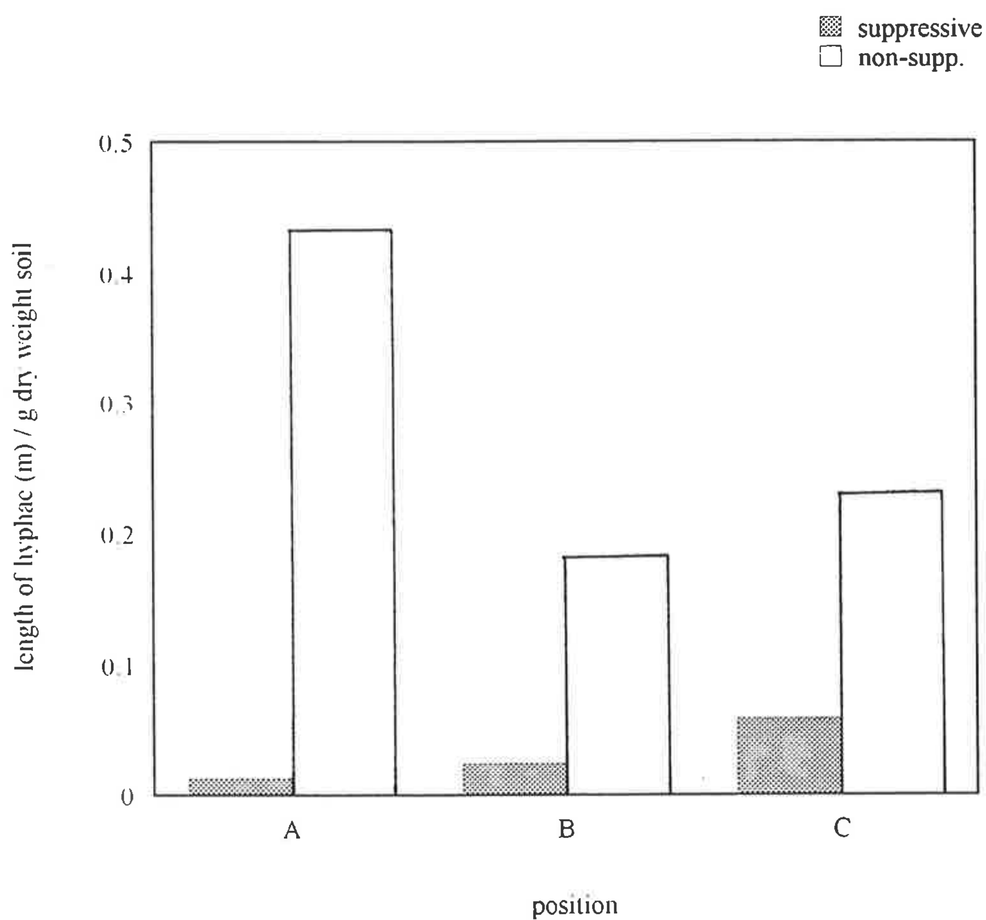



Figure 6.4: Length of FDA-active *Rhizoctonia*-like hyphae per g dry weight soil at three positions (A, B and C) in suppressive and non-suppressive (non-supp.) soil. Bars are means for the significant (P = 0.01) interaction between the position and soil type treatments.

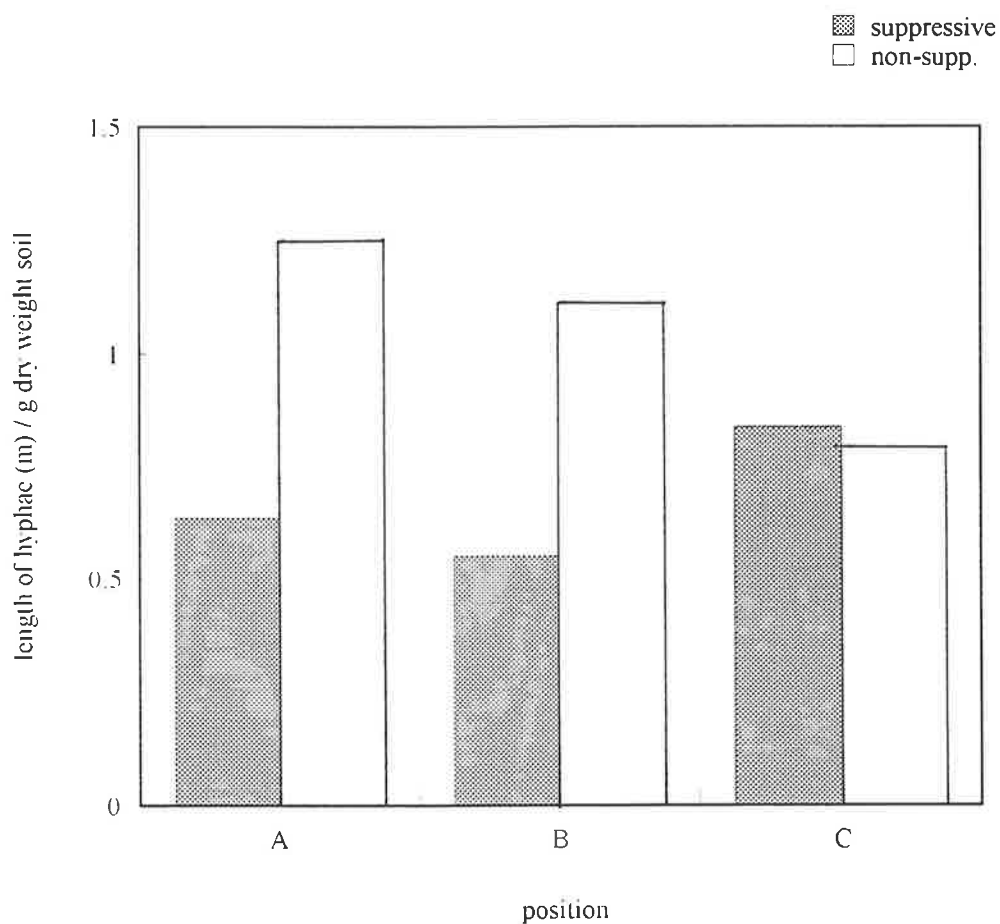



Figure 6.5: Length of *Rhizoctonia*-like hyphae per g dry weight soil at three positions (A, B and C) in suppressive and non-suppressive (non-supp.) soil. Bars are means for the significant (P = 0.01) interaction between the position and soil type treatments.

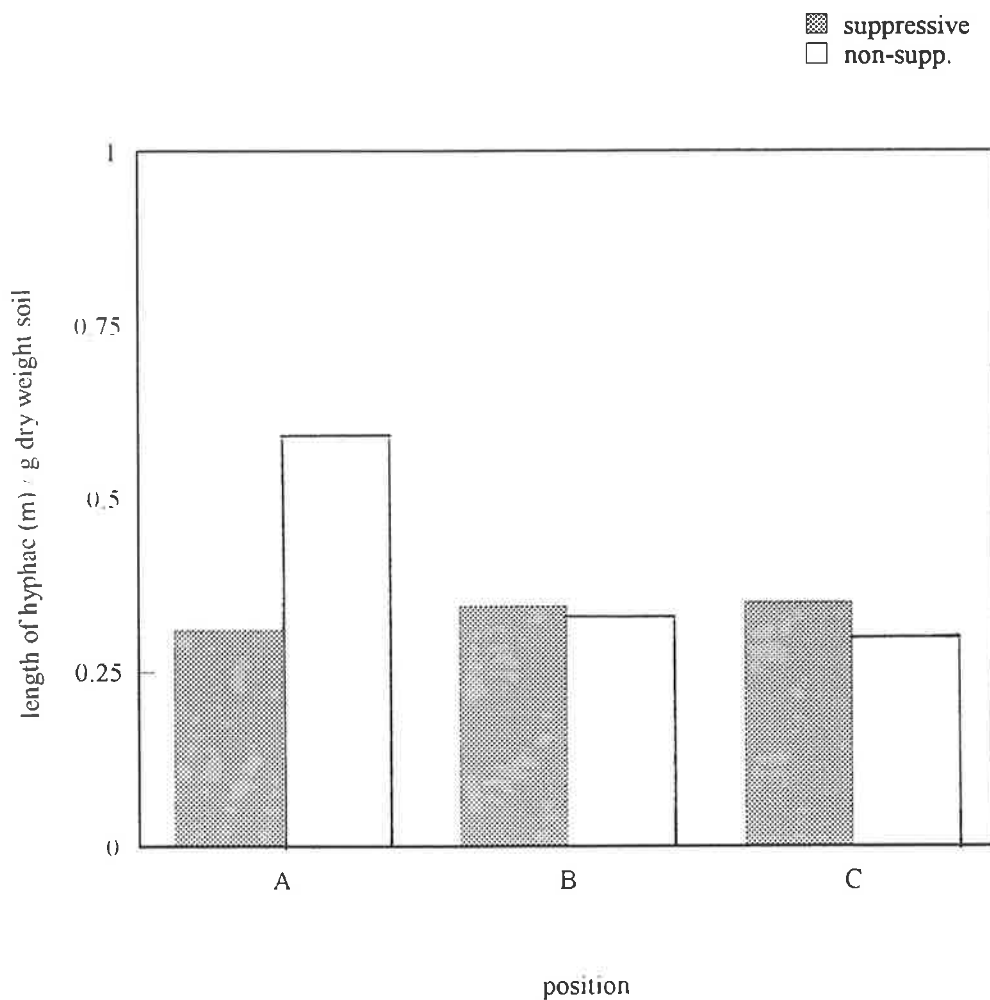



Figure 6.6: Length of FDA-active hyphae per g dry weight of soil at three positions (A, B and C) in suppressive or non-suppressive (non-supp.) soil. Bars are means for the significant (P = 0.01) interaction between the position and soil type treatments.

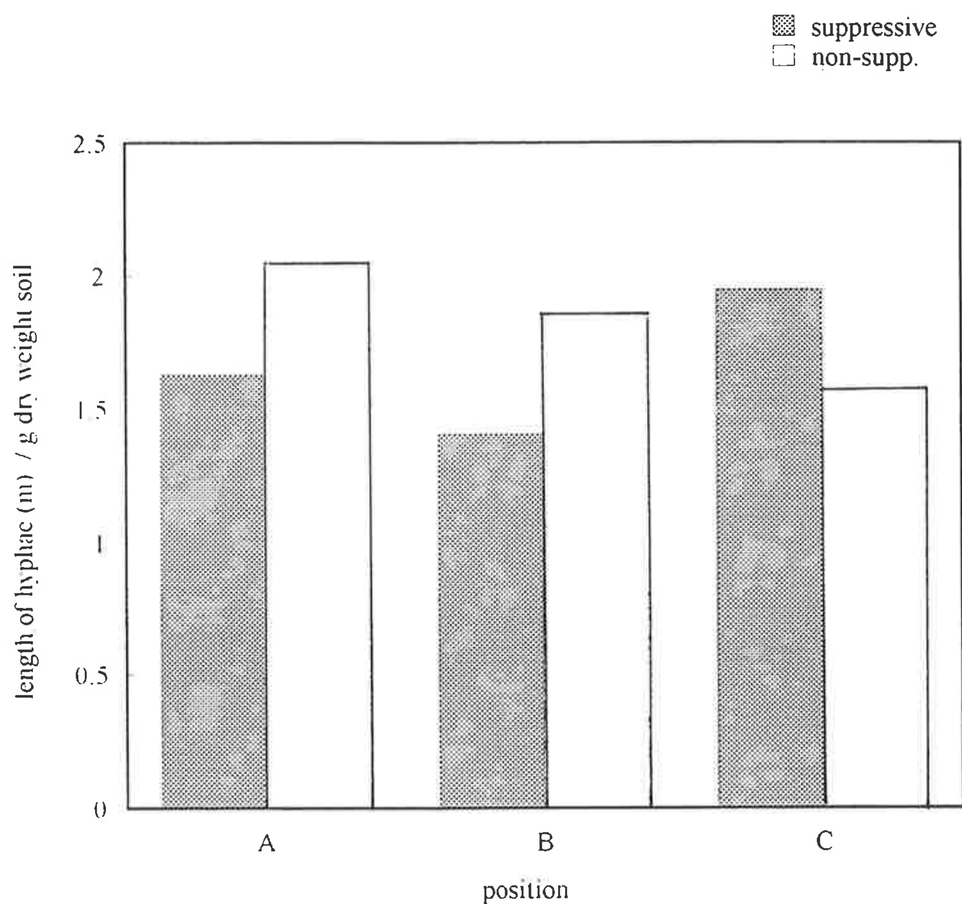



Figure 6.7: Length of total hyphae per g dry weight of soil at three positions (A, B and C) in suppressive and non-suppressive (non-supp.) soil. Bars are means for the significant (P = 0.01) interaction between the position and soil type treatments.

In summary, there was no trend in hyphal length in suppressive soil compared to non-suppressive soil. FDA-active and total length of *Rhizoctonia*-like hyphae tended to be higher in non-suppressive compared to suppressive soil. Hence, the *Rhizoctonia*-like hyphae represented a smaller proportion of hyphal length in suppressive compared to non-suppressive soil. Length of *Rhizoctonia*-like hyphae was similar at all 3 positions in suppressive soil, but higher at the point of inoculation compared to other positions in non-suppressive soil.

**6.4 Discussion**

Quantification of *Rhizoctonia* using a membrane filtration technique proved to be a successful approach for comparing populations in suppressive soil and non-suppressive soil, and determining likely sites of suppression. With some experience, it is possible to distinguish *Rhizoctonia*-like hyphae from other hyphae subjectively. However, it is not possible to distinguish *R. solani* AG-8 hyphae from other *Rhizoctonia* populations as hyphal characteristics are similar. Automated image analysis systems capable of distinguishing hyphae from other filamentous structures and reducing subjectivity are available (Morgan *et al.*, 1991; Daniel *et al*, 1995). However, these techniques are currently unable to distinguish between hyphae of different species. The main finding of this experiment was that populations of viable (metabolically active) *Rhizoctonia* were lower in suppressive soil compared to non-suppressive soil. This confirmed the results from the soil isolations (Chapter 5).

Results presented in this chapter provide evidence of suppression of *R. solani* AG-8 in bulk soil. Length of total and FDA-active *Rhizoctonia*-like

hyphae were consistently lower in suppressive soil compared to non-suppressive soil at positions A (inoculation point) and B (ca. 2 - 3.5 cm from inoculation point). This simplest explanation of these data is hyphal growth of *R. solani* AG-8 is inhibited in suppressive soil at the point of inoculation (position A), and during subsequent growth (position B). Antagonistic organisms must therefore be present, and active, in suppressive soil at some distance from living plant roots. Soil 1.25 mm away from living plant roots may be unaffected by the roots and the extent of the rhizosphere is estimated at 20 µm ("outer" rhizosphere) (Bowen and Rovira, 1991). Hence, it appears suppression is occurring in this zone which is unaffected by plant roots.

There is evidence for suppression in the rhizosphere as well as in the bulk soil. Suppression in rhizosphere soil was indicated by these results, with lower length of FDA-active *Rhizoctonia*-like hyphae in suppressive soil compared to non-suppressive soil at position C. Similar total length of *Rhizoctonia*-like hyphae at this position indicates a higher proportion of inactive *Rhizoctonia*-like hyphae in suppressive soil compared to non-suppressive soil.

Non-*Rhizoctonia* fungal populations in the rhizosphere (position C) were higher in suppressive than non-suppressive soil. Length of FDA-active *Rhizoctonia*-like hyphae was higher in the rhizosphere of plants growing in non-suppressive compared to suppressive soil. FDA-active hyphal length was similar in the two soils, indicating that FDA-active populations of other types of fungi (non-*Rhizoctonia*) were higher in the rhizosphere in suppressive compared to non-suppressive soil.

The results presented in this chapter confirm the observation of the correlation between viable *Rhizoctonia* and pathogenic *Rhizoctonia*, but not

total *Rhizoctonia* and pathogenic *Rhizoctonia* described in Chapter 5. In the rhizosphere (position C), length of FDA-active *Rhizoctonia*-like hyphae was higher in non-suppressive compared to suppressive soil, correlating with disease severity in the two soils. However, length of total (active plus inactive) *Rhizoctonia*-like hyphae was similar in the two soils in the rhizosphere. Fungal viability and metabolic activity are required for disease incitement. The higher non-viable (FDA inactive) *Rhizoctonia* populations in suppressive soil need to be investigated further.

As expected from results in previous chapters (Chapters 3-5), inoculation of soil with *R. solani* AG-8 increased disease severity in this experiment. However, inoculation did not increase the length of *Rhizoctonia*-like hyphae (total length of *Rhizoctonia*-like hyphae reduced by inoculation, length of FDA-active *Rhizoctonia*-like hyphae not affected by inoculation). An explanation for this lack of correlation between disease severity and length of *Rhizoctonia*-like hyphae is not known.

In summary, quantification of *Rhizoctonia* populations in soil with and without living plant roots has shown that suppression of *R. solani* AG-8 in suppressive soil definitely occurrs in bulk soil. In the root zone of plants grown in suppressive soil there was less FDA-active *Rhizoctonia* compared to non-suppressive soil. Whether this is due to microbial antagonism in the root zone is not known. Differences in the fungal populations of suppressive and non-suppressive soil were indicated. Initial investigation of the microbial populations in suppressive soil and non-suppressive soils was carried out and is reported in Chapter 7.

# Chapter 7: Characterisation of microbial populations in suppressive soil and non-suppressive soil

## 7.1 Introduction

The biological basis of the observed decline in Rhizoctonia barepatch was established in Chapter 3. Initial characterisation of the microbial populations in suppressive soil and non-suppressive soil was done to compare communities and identify organisms that may be involved in this suppression. The aim of this chapter was to determine if taxonomic or functional differences in the microbial populations in suppressive and non-suppressive soils could be detected and related to established differences in suppressiveness.

Previous work has correlated soil suppressiveness to *Ophiobolus* (now *Gaeumannomyces graminis*) with the number of bacteria in soil (Garrett, 1933). The number of bacteria, the number of fluorescent pseudomonads and higher substrate-induced respiration have been correlated with suppressiveness to *Fusarium oxysporum* (Höper *et al.*, 1995). This chapter describes characterisation of taxonomic groups of micro-flora and protozoa in suppressive and non-suppressive soil based on isolations using various selective media (Sections 7.2.1 and 7.2.2). The microbial communities were compared *in situ* using analysis of fatty acid methyl esters (FAME) of whole soil and organic matter extracts (Section 7.2.5). In addition, groups of organisms were enumerated because they have been previously implicated in disease suppression. A general comparison of biomass in the suppressive soil and non-suppressive soil was made. Protozoa, fluorescent pseudomonads, actinomycetes, and *Trichoderma* spp. have all been implicated in cases of

disease suppression or biological control, and so were selected for quantification (Cook and Rovira, 1976; Sivasithamparam and Parker, 1978; Chakraborty, 1983a; b; Weller and Cook, 1983; Wong and Baker, 1984; Coley-Smith *et al.*, 1991; Maplestone *et al.*, 1991).

To examine the role of specific microflora in disease suppression the approach described by Alabouvette (1986) was used (Section 3.2.2). In addition to assessing disease severity of wheat grown in steam pasteurised suppressive soil and non-suppressive soil, dilution plating of the soils on selective media was done to determine which components of the soil micro-flora were removed by each temperature treatment.

To test the hypothesis that carbon inputs induce suppression (D. Roget, pers. comm.) (eg due to increased numbers of competitive saprophytes), carbon utilisation in suppressive soil and non-suppressive soil was investigated (Section 7.2.4). Utilisation of carbon *in vivo* was assessed using dry weight loss of plant material in litterbags buried in suppressive and non-suppressive field soil. To determine if there had been a shift towards a soil biota that could readily decompose the types of plant material normally entering the soil, a range of types of plant material were used. Wheat roots represented one of the normal input of plant material into the soils. Wheat shoots, and pea roots and shoots represented inputs uncommon to these soils. Utilisation of carbon *in vitro* was assessed using a most probable number technique to quantify number bacteria and fungi with cellulolytic activity.

A low proportion of the soil microbial community are culturable (Zuberer, 1994), and populations growing on artificial media reflect the specific *in vitro* conditions used as well as microbial distribution in the soil (Cavigelli *et al.*, 1995). To compare suppressive soil and non-suppressive soil without

relying on culturing of organisms, the microbial biomass and activity was measured. These techniques provide measures of microbial population size and activity. FAME profiles of whole soil communities allow comparison in terms of quality and quantity of fatty acids. Many types of fatty acids are present in the lipids of microbes, with each organism having a different combination (Cavigelli *et al.*, 1995). FAMEs are quantified and identified using gas chromatography, and there is a linear relationship between retention time on the chromatogram and the number of carbon atoms in the FAME with the microbial FAME range being 12 to 24 carbon atoms (Wander *et al.*, 1995). Fatty acid markers unique to some organisms can be useful in interpreting profiles (Vestel and White, 1989; Cavigelli *et al*, 1995) but many organisms do not have unique markers identified.

## 7.2 Materials and Methods

### 7.2.1 Dilution plating of suppressive and non-suppressive field soil

Suppressive and non-suppressive soil was serially diluted in sterile phosphate buffer (Appendix 1) and added to various semi-selective media to quantify protozoa (amoebae, flagellates, ciliates), and total bacteria, total fungi and total actinomycetes. For quantification of populations of protozoa, soils were pre-incubated at 23°C for 3 days before serially diluting to induce activity in organisms in resting states (V. V. S. R. Gupta, pers. comm.).

7.2.1.1 Protozoa

7.2.1.1.1 Experiment 1

For quantification of bacterial feeding protozoa, diluted soil suspension (100 μl) was inoculated into 5 wells of a sterile 24 well plate (6 x 4, Sarstedt, USA 24 well plate flat base, No. 83.1836) containing Neff's solution (1 ml) (Appendix 1) and *Enterobacter* suspension (E64) (100 μl) as a food source for the protozoa. For quantification of fungal feeding protozoa, four 50 μl drops of serially diluted soil suspension were added to 17 day old *R. solani* AG-8 cultures on water agar (Appendix 1) in 9 cm plastic petri dishes with a central divider. Three replica well plates and split-petri dishes were set up for each dilution-soil combination. Controls consisted of sterile phosphate buffer only.

Counts were made after 3, 7, 10 and 14 days at 23°C using an inverted compound microscope at 100x magnification. Counts were made over several days as different protozoa may be detected over time (V. V. S. R. Gupta, pers. comm.).

7.2.1.1.2 Experiment 2

Bacterial feeding protozoa were quantified using the method described for Experiment 1, the soil being stored moist at 4°C between experiments (approximately 8 weeks). Three replicate well plates were set up for each soil type-dilution combination. Counts were made after 3, 7, 10 and 14 days at 23°C.

7.2.1.2 Fungi, bacteria and actinomycetes

7.2.1.2.1 Experiment 1

Populations were quantified using a dilution plate counting technique. Total bacteria were quantified using 9 cm plastic petri dishes containing 0.3% TSA inoculated with serially diluted soil suspension (100 µl). Total actinomycetes were quantified using AGS with 5 x $10^3$ µg $ml^{-1}$ $NaN_3$, 1 x $10^3$ µg $ml^{-1}$ chloramphenicol and 1 x $10^3$ µg $ml^{-1}$ cyclohexamide, and total fungi with DRBC. All media are described in Appendix 1. Two replicate plates were set-up for each soil type-media combination. Counts were made after 14 days at 25°C.

7.2.1.2.2 Experiment 2

The soil samples, preparation and dilution as described in Experiment 1 (Section 7.2.1.2.1) were used, soils being stored moist at 4°C between experiments (11 days).

Total bacteria were quantified as in Experiment 1. Total actinomycetes were quantified using AGS with 5 x $10^3$ µg $ml^{-1}$ $NaN_3$, 1 x $10^3$ µg $ml^{-1}$ chloramphenicol, 1 x $10^3$ µg $ml^{-1}$ cyclohexamide, 100 x $10^3$ µg $ml^{-1}$ streptomycin sulphate and 100 x $10^3$ µg $ml^{-1}$ tetracycline hydrochloride. Additional antibiotics were added to AGS due to the growth of bacteria on this media in Experiment 1. Total fungi were quantified with DRBC, and NDY with 100 x $10^3$ µg $ml^{-1}$streptomycin sulphate and 100 x $10^3$ µg $ml^{-1}$ tetracycline hydrochloride. An additional medium for fungi (NDY) was used in this experiment in an attempt to apply less selection pressure and isolate a

wider range of fungal types. Two replicate plates were set up for each soil type-media combination. Counts were made after 10 days at 25°C.

7.2.2 Dilution plating of steam pasteurised suppressive and non-suppressive soils

The collection and treatment of intact cores of suppressive and non-suppressive soil was described in Section 3.2.2. Populations were quantified using dilution plate counts, as described in Section 7.2.1.2

Soil (ca. 100 g) was sampled from the edge of cores of suppressive and non-suppressive soil 2 days after steam pasteurisation. One core was sampled for each soil type-temperature treatment combination and from the untreated control cores.

Soil was serially diluted in sterile phosphate buffer (Appendix 1) and 100 µl spread plated onto 9 cm plastic petri dishes containing 0.3 % TSA (total bacteria and actinomycetes), DRBC (total fungi), AGS (actinomycetes), TSM (*Trichoderma* spp.) and S1 (fluorescent pseudomonads). Three replicate plates were set up for each dilution-media combination. Counts of number of colonies on 0.3 % TSA and S1 were made after 3 days incubation of plates at 25°C, and after 7 days at 25°C for DRBC, AGS and TSM.

### 7.2.3 Microbial activity and biomass of suppressive and non-suppressive soils

#### 7.2.3.1 Microbial activity

Microbial activity was estimated by measurement of respiration of soil following incubation at 25°C (Anderson, 1982).

Loose soil was collected from field plot replicates 1 and 2 on the 28th of April and the 5th, 12th and 19th of May 1995. Soil was placed in a plastic bag, loosely sealed with a rubber band and processed on the day of collection. Soil water content was determined so that results could be expressed on a per gram dry weight soil basis.

On the day of collection a sub-sample of the moist soil (40 g) was placed into a plastic vial (size 20, 70 ml capacity) which was then placed into a fume hood. KOH (0.5 M) was freshly prepared using distilled water and 10 ml pipetted into a plastic vial (size 6, 20 ml capacity). Both vials were placed into a 1 L capacity glass screw top jar, distilled water (3 ml) pipetted onto the base of the jar and the lid tightly sealed. Four replicate jars were set up for each soil plus 4 blanks containing KOH but no soil. Jars were incubated at 25°C for 7 days in darkness.

Respired $CO_2$ absorbed by the KOH was estimated by back titration of the remaining alkali against HCl using an automated titration apparatus (Radiometer Copenhagen TTT 85 titrator and ABU 80 autoburette).

7.2.3.2 Microbial biomass

Microbial biomass was estimated using the technique of Amato and Ladd (1988) where ninhydrin-reactive nitrogen (nin-N) in fumigated and unfumigated soils is measured and converted to a biomass carbon value. Biomass carbon was estimated as the nin-N flush (fumigated nin-N - unfumigated nin-N) multiplied by 21 (M. Amato, pers. comm.).

The soil used for microbial activity measurements was stored at 4°C until all samples had been collected. Moist soil (20 g) was chloroform-fumigated in a glass dessicator for 7 days at 25°C. Moisture was removed from the chloroform (analytical grade) prior to use by passing it through a 40 cm glass column containing oven dry $Al_2O_3$ powder.

Fumigated and unfumigated controls soils were then extracted with 2 M KCl (60 ml, shaken end-over-end for 30 min) and filtered (Whatman No. 42, 12.5 cm dia.). Three replicates for each soil type (suppressive or non-suppressive, fumigated or unfumigated) and sampling date were used.

Extract (500 µl) was reacted with freshly prepared ninhydrin reagent (2 ml) (2 g ninhydrin and 0.2 g hydrindantin dissolved in 50 ml methyoxy ethanol, 50 ml 4 N sodium acetate added) then placed in a boiling water bath for 15 min. The absorbance at 570 nm of cooled, diluted samples (plus 5 ml 50 % ethanol in water) was measured using a Beckman DV 640 spectrophotometer with a batch sampler attached. L-leucine and ammonium sulphate were used as standards.

### 7.2.4 *In vitro* and *in vivo* carbon utilisation

#### 7.2.4.1 Cellulolytic fungi and bacteria

The most probable number technique for cellulolytic fungi and bacteria described by Gupta and Roper (1994) was used with slight modification. Solutions used are described in Appendix 3.

A 10-fold serial dilution of suppressive and non-suppressive soil was prepared in phosphate buffer (Appendix 3) and 100 μl added to each well.

Components A, B and C (Appendix 3) of the medium for the enumeration of cellulolytic fungi and bacteria were prepared, mixed and 1.5 ml dispensed into the wells of sterile 6 x 4 well tissue culture plates. Four replicates of each dilution were used for each medium. Controls were inoculated with sterile phosphate buffer only. Plates were incubated at 28°C for 48 hours then the number of wells with bacterial or fungal growth recorded. Carboxy-methyl cellulose (CMC) overlay (0.5 ml) was added to all wells and the plates incubated at 28°C overnight. CMC utilisation was detected by flooding the wells with congo-red solution and shaking at room temperature for 15 min. The congo-red solution was removed, wells rinsed twice with SDW and 1 M NaCl (0.75 ml) was added. Plates were shaken at room temperature for 30 min then the NaCl was removed. Wells without cellulolytic activity were stained red and those with cellulolytic activity were yellow.

The most probable numbers of total bacteria and fungi were determined from counts after the initial 48 hour incubation using the tables of Cochran (1950). The most probable numbers of cellulolytic bacteria and fungi were

determined from counts of the number of yellow wells after staining. The proportion of fungi and bacteria able to utilise CMC was calculated by dividing the number of cellulolytic fungi or bacteria by the total number of fungi or bacteria for each replicate.

The soil samples were stored at 4°C and the procedure repeated after one week to confirm the results.

7.2.4.2 Total cellulolytic micro-organisms

CMC spread plates (Appendix 1) were prepared using 9 cm plastic petri dishes and inoculated with 100 µl soil suspension prepared by serially diluting suppressive or non-suppressive soil in phosphate buffer (Appendix 1). The soil used was the same as in Section 7.2.3 and had been stored dry at room temperature for approximately 16 weeks. The petri dishes were incubated at 23°C for 7 days. The total number of colonies was counted then the plates were flooded with congo-red solution to detect cellulolytic colonies. Clear, non-staining zones appeared around colonies with cellulolytic activity (due to the utilisation of the surrounding CMC).

7.2.4.3 Decomposition of plant material in field soil

Wheat and pea seeds were surface disinfested and sown in 15 cm dia. plastic pots containing steam pasteurised U.C. potting mix (Matkin and Chandler, 1985) (2/3 $m^3$ washed coarse sand, 1/3 $m^3$ peatmoss, 700 g $Ca(OH)_2$, 480 g $CaCO_3$, 600 g Nitrophoska 15-4-12). After 7 weeks growth in the glasshouse, plants were harvested, the roots and shoots separated and dried at 60°C for 3 days.

Nylon mesh bags (hole diameter ca. 2 mm) were prepared by heat sealing three sides then adding the dried plant material and sealing the fourth side. Shoot material (2.0 g) was placed in bags measuring 10 x 10 cm and root material (1.0 g) placed in 5 x 5 cm bags.

Four bags of each type of plant material (wheat roots / shoots and pea roots / shoots) were buried 5-10 cm deep in suppressive and non-suppressive field plot replicates 1-4 during early spring of 1994. One bag of each type of plant material was collected from each field plot replicate 9, 20 and 32 weeks after burial. Dry weights were determined after drying for 3 days at 60°C.

Loss of marker pegs meant a full set of replicates could not be collected at each sampling date.

7.2.5 FAME

FAME extraction and analysis was done on organic matter (710 µm - 2 mm) and rhizosphere soil collected 7/9/95 (15 samples per soil). Samples were from continuous wheat plots rather than wheat - pasture plots as in all other experiments. Continuous wheat plots were used to avoid variation in FAMEs due to different grass pasture plant communities (D. Kluepfell, pers. comm.).

Soils used for microbial biomass and activity measurements were stored at 4 °C for approximately 3 months before FAME extraction. Moist soil (2 g) was weighed into glass tubes (13 mm dia, teflon capped) for FAME extraction.

Organic matter was removed from suppressive soil and non-suppressive soil by washing through 1.18 mm and 104 µm sieves. Organic matter retained on

the 104 µm sieve was air-dried then FAMEs extracted (7/9/95), or stored at -70 °C for 3 weeks prior to extraction (3/8/95). Organic matter (0.2 g) was ground in liquid nitrogen then transferred to glass tubes (13 mm dia, teflon capped) for FAME extraction.

Rhizosphere soil was collected by shaking 8 g fresh weight of wheat roots in 100 ml of sterile distilled water (SDW) for 25 min, sub-sampling the suspension (25 ml), centrifuging (20 min at 7000 rpm), removing supernatant, adding 1 ml SDW, vortexing (20 s), sub-sampling the suspension (1.5 ml; ca. 75 % of volume), centrifuging (15 min at 14 000 rpm), then transferring the pellet to a glass tube (13 mm dia, teflon capped) for FAME extraction (D. Kluepfell, pers. comm.). This procedure was used to allow separation of rhizosphere soil from the roots and removal of as much water as possible to ensure the FAME extraction procedure was not inhibited.

FAME extraction was carried out according to the Microbial Identification System (MIS) (1993) standard protocol for pure cultures with minor modification. Samples were saponified by adding 1 ml of reagent 1 (NaOH 45 g, methanol 150 ml, deionised distilled water 150 ml), vortexing (10 s), heating (100 °C water bath, 5 min), vortexing (10 s), heating (100 °C water bath, 25 min), then cooling in tap water. Methyl esters were formed by adding 2 ml of reagent 2 (6 N HCl 325 ml, methanol 275 ml), vortexing (10 s), heating (80 °C water bath, 10 min), then cooling in tap water. Fatty acids were transferred from the aqueous phase to an organic phase by adding 1.25 ml of reagent 3 (HPLC grade hexane 200 ml, HPLC grade methyl-tert butyl ether 200 ml), shaking (end-over-end, 10 min), centrifuging (4 min at 2600 rpm), then transferring the upper phase to a new tube. The organic extract was washed prior to chromatographic analysis by adding 3 ml of reagent 4

(NaOH 10.8 g, deionised distilled water 900 ml), shaking (end-over-end, 5 min), centrifuging (4 min at 2600 rpm), then transferring to a GC vial (ca. 100 μl). Modifications to the standard procedure were the transfer of the upper phase to a new glass tube rather than removal of the lower phase, and the two centrifugations (at 2600 rpm) (D. Kluepfell, pers. comm.). Extracts were stored for up to 10 days at -20°C prior to analysis on the GC.

Extracted FAME samples were analysed on a Hewlett Packard 5890 Series II gas chromatograph fitted with a Hewlett Packard 7673 automatic sampler. Results were analysed with MIS software in conjunction with the Hewlett Packard 3365 gas chromatograph ChemStation software.

## 7.3 Results

### 7.3.1 Dilution plating of suppressive and non-suppressive field soil

#### 7.3.1.1 Protozoa

Protozoa numbers in suppressive soil and non-suppressive soil were similar in both experiments (Table 7.1). The few significant effects of soil type or observation day differed between the two experiments. Attempts to quantify fungal feeding protozoa were unsuccessful.

Numbers of amoebae (Expt. 1) and total protozoa (Expt. 2) were significantly (P = 0.05) higher in suppressive soil compared to non-suppressive soil. Number of ciliates in experiment 1 was significantly (P = 0.05) higher in non-suppressive soil compared to suppressive soil at day 14 and similar in the two soils at days 3, 7 and 10. Number of total protozoa in Experiment 2 was significantly (P = 0.05) higher at days 10 and 14 compared

to days 3 and 7, and similar in suppressive soil and non-suppressive soil. The number of flagellates per gram of soil was not affected by soil type (suppressive or non-suppressive) or observation time in either experiment. Numbers of amoebae and ciliates were not affected by soil type or observation day in Experiment 2.

Circular holes were observed in hyphae of *R. solani* AG-8 after 10 days incubation following addition of suppressive soil suspension but no protozoa were observed. Mycophagous amoebae (V. V. S. R. Gupta, pers. comm.) were observed following addition of non-suppressive soil suspension but no hyphal damage was observed.

7.3.1.2 Fungi, bacteria and actinomycetes

Numbers of actinomycetes and fungi per gram of soil were similar in the two soils in Experiments 1 and 2 (Table 7.2). There were significantly more bacteria and fungi per gram of soil in Experiment 1 than 2. Numbers of bacteria were significantly ($P = 0.05$) higher in suppressive soil compared to non-suppressive soil in Experiment 1, and similar in the two soils in Experiment 2. These differences may be explained by storage of soils at 4 °C between experiments.

a) Experiment 1

| soil | day | total protozoa | amoebae | flagellates | ciliates |
|---|---|---|---|---|---|
| suppressive | 3 | 148 (20) | 43 (1) | 98 (22) | 7 (4) |
| | 7 | 229 (42) | 87 (24) | 89 (6) | 54 (14) |
| | 10 | 373 (32) | 65 (19) | 125 (30) | 183 (30) |
| | 14 | 371 (10) | 22 (8) | 126 (15) | 223 (2) |
| non-supp. | 3 | 128 (32) | 13 (4) | 114 (31) | 0 (0) |
| | 7 | 219 (19) | 37 (5) | 102 (34) | 81 (19) |
| | 10 | 306 (76) | 13 (4) | 229 (76) | 64 (6) |
| | 14 | 662 (107) | 22 (8) | 137 (27) | 537 (78) |
| soil | | NS | ** [31] | NS | NA |
| day | | ** [193] | NS | NS | NA |
| soil.day | | NS | NS | NS | ** [159] |

b) Experiment 2

| soil | day | total protozoa | amoebae | flagellates | ciliates |
|---|---|---|---|---|---|
| suppressive | 3 | 120 (38) | 0 (0) | 120 (38) | 0 (0) |
| | 7 | 296 (78) | 64 (6) | 226 (76) | 7 (4) |
| | 10 | 225 (24) | 92 (11) | 133 (32) | 0 (0) |
| | 14 | 203 (22) | 70 (18) | 127 (5) | 0 (0) |
| non-supp. | 3 | 35 (4) | 7 (4) | 28 (5) | 0 (0) |
| | 7 | 127 (8) | 51 (4) | 67 (13) | 0 (0) |
| | 10 | 127 (25) | 20 (7) | 107 (26) | 0 (0) |
| | 14 | 144 (39) | 80 (35) | 64 (6) | 0 (0) |
| soil | | ** [96] | NS | NS | NS |
| day | | NS | NS | NS | NS |
| soil.day | | NS | NS | NS | NS |

Tables 7.1 a and b: Number of protozoa (total, amoebae, flagellates or ciliates) per gram dry weight of suppressive or non-suppressive (non-supp.) soil after incubation of 3, 7, 10 and 14 days (day) in two experiments using the same soil sample. Values are means of 3 replicate plates and standard errors are given in parentheses. Significance of individual treatments and interaction is indicated by ** (1 % level), NS (non-significant) or NA (not applicable) and values for LSD at $P = 0.05$ are given in square parentheses.

| soil | expt | bacteria (cfu / g soil) | actinomycetes (cfu / g soil) | fungi (cfu / g soil) |
|---|---|---|---|---|
| suppressive | 1 | 1.7 (0.11) x $10^5$ | 2.1 x $10^5$ (0) | 4.2 x $10^3$ (0) |
| | 2 | 4.0 (0.27) x $10^5$ | 2.1 (0) x $10^5$ | 2.9 (0.10) x $10^2$ |
| non-suppressive | 1 | 9.8 (0.38) x $10^5$ | 1.0 (0.73) x $10^5$ | 8.2 (0.22) x $10^3$ |
| | 2 | 2.4 (0.51) x $10^5$ | 2.2 (0.36) x $10^5$ | 3.3 (0.1) x $10^2$ |
| soil | | NA | NS | NS |
| expt | | NA | NS | ** [4.3 x $10^3$] |
| soil.expt | | ** [2.9 x $10^5$] | NS | NS |

Tables 7.2 Colony forming units (cfu) of bacteria, actinomycetes and fungi per gram dry weight of suppressive or non-suppressive (non-supp.) soil in two experiments using the same soil sample. Values are means of 2 replicate plates and standard errors are given in parentheses. Significance of individual treatments and interaction is indicated by ** (1 % level), NS (non-significant) or NA (not applicable) and values for LSD at $P = 0.05$ are given in square parentheses.

### 7.3.2 Dilution plating of steam pasteurised suppressive and non-suppressive soil

Microbial counts after pasteurisation revealed differences between suppressive soil and non-suppressive soil (Table 7.3). The microbial populations in suppressive and non-suppressive soil responded differently to steam pasteurisation at 50°C. Following 50°C steam pasteurisation, bacterial numbers were reduced in both suppressive soil and non-suppressive soil whereas numbers of fluorescent pseudomonads were reduced in non-suppressive soil and increased in suppressive soil. Fungal and *Trichoderma* spp. numbers were decreased by 50°C steam pasteurisation in suppressive soil but unaffected in non-suppressive soil while actinomycete numbers were increased in non-suppressive soil and unaffected in suppressive soil.

There were significantly fewer bacteria, fluorescent pseudomonads and fungi and more *Trichoderma* spp. in untreated suppressive soil compared to untreated non-suppressive soil. Actinomycete numbers were similar in both untreated soils. Following 50°C steam pasteurisation there were significantly more bacteria, fluorescent pseudomonads, fungi and *Trichoderma* spp. and fewer actinomycetes in suppressive soil compared to non-suppressive soil. The relative populations were therefore different before and after steam pasteurisation at 50°C. Numbers of *Trichoderma* spp. were always higher in suppressive soil compared to non-suppressive soil while the relative numbers of bacteria, fungi and fluorescent pseudomonads became higher in suppressive soil following 50°C steam pasteurisation.

| soil | temp. (°C) | fungi | *Trichoderma* spp. | bacteria | fluorescent pseudomonads | actinomycetes |
|---|---|---|---|---|---|---|
| supp | control | $1.78 \times 10^3$a | $1.39 \times 10^2$a | $1.29 \times 10^6$a | $9.67 \times 10^3$a | $1.03 \times 10^5$ad |
| | 50 | $6.51 \times 10^3$b | 71.1b | $6.37 \times 10^5$b | $3.06 \times 10^4$b | $9.61 \times 10^4$a |
| | 60 | 25.3c | 3.62c | $5.50 \times 10^5$c | 0c | $3.25 \times 10^4$c |
| | 70 | 3.68c | 0c | $9.74 \times 10^4$c | 0c | $6.62 \times 10^3$c |
| | 80 | 7.25c | 0c | $3.12 \times 10^5$c | 0c | $2.90 \times 10^4$c |
| ns | control | $3.53 \times 10^3$d | 32.1d | $1.81 \times 10^6$d | $4.81 \times 10^4$d | $1.07 \times 10^5$d |
| | 50 | $3.62 \times 10^3$d | 28.4d | $1.10 \times 10^6$e | $1.75 \times 10^4$e | $1.49 \times 10^5$e |
| | 60 | 14.4c | 3.59c | $3.36 \times 10^5$c | 0c | $1.65 \times 10^4$c |
| | 70 | 7.13c | 0c | $2.14 \times 10^4$c | 0c | $7.14 \times 10^3$c |
| | 80 | 10.8c | 0c | $8.67 \times 10^4$c | 0c | $1.81 \times 10^4$c |
| | LSD (P=0.05) | $1.26 \times 10^3$ | 20.1 | $3.00 \times 10^5$ | $1.01 \times 10^4$ | $3.29 \times 10^4$ |

Table 7.3: Treatment means of colony forming units per g dry weight soil of total fungi, *Trichoderma* spp., total bacteria, fluorescent pseudomonads and actinomycetes. Suppressive (supp) soil or non-suppressive (ns) soil steam pasteurised at 50, 60, 70 or 80°C or left untreated (control) and a soil suspension plated onto selective agar media and incubated at 25°C. Values are means of 3 replicate plates and those with the same letter are not significantly different (P = 0.05)

Following steam pasteurisation at 60, 70 or 80°C the number of each organism per gram of soil was similar in suppressive and non-suppressive soil and significantly less than in untreated or 50°C steam pasteurised soils.

7.3.3 Microbial biomass and activity

Microbial biomass was significantly (P = 0.01) higher in suppressive compared to non-suppressive soil at all sampling dates (Fig 7.1). For both soils, microbial biomass was lowest in the April 28 sample, significantly higher in the May 12 and 19 samples and highest in the May 5 sample. Microbial biomass increased following the first major rainfall of the year between the 28th of April and the 5th of May then decreased and remained steady on the 12th and 19th of May. Soil water content (w / w) increased from 3.8 to 13.1 % in suppressive soil and 2.9 to 9.9 % in the non-suppressive soil between the 28th of April and the 5th of May.

Microbial activity ($CO_2$ respiration) was significantly (P = 0.01) higher in suppressive soil compared to non-suppressive soil at the first 3 of the 4 sampling times (Fig 7.2). The response of microbial activity over time was similar to that for microbial biomass with the lowest level at the 28th of April, highest at the 5th of May and lower for the 12th and 19th of May. When treatment means were converted to microbial activity per unit of biomass the response of the two soils over time was similar (Fig 7.3).

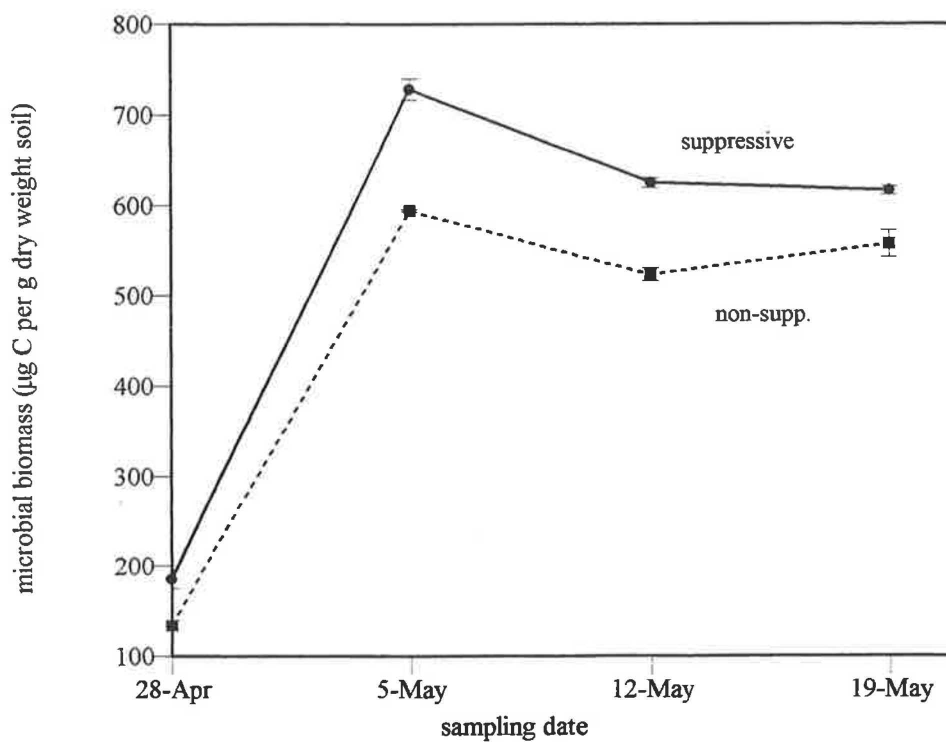



Figure 7.1: Microbial biomass (µg C per g dry weight soil) of suppressive and non-suppressive (non-supp.) soils sampled during 1995. Values are means of 3 replicates and lines indicate standard error of the data.

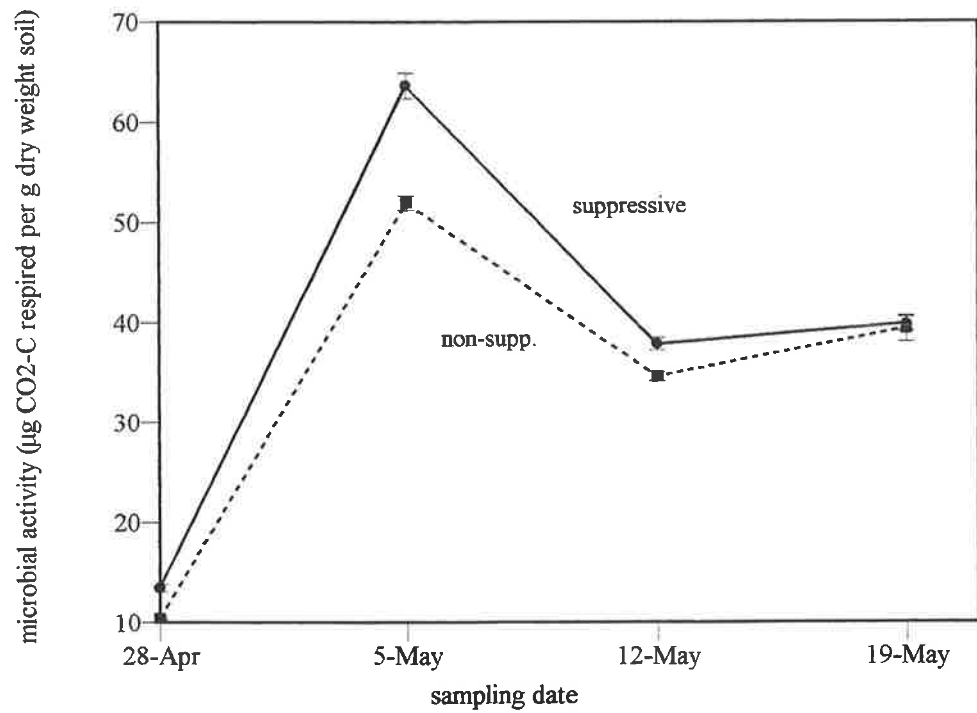



Figure 7.2: Microbial activity (µg CO2-C respired) of suppressive and non-suppressive (non-supp.) soils sampled during 1995. Values are means of 4 replicates and lines indicate standard error of the data.

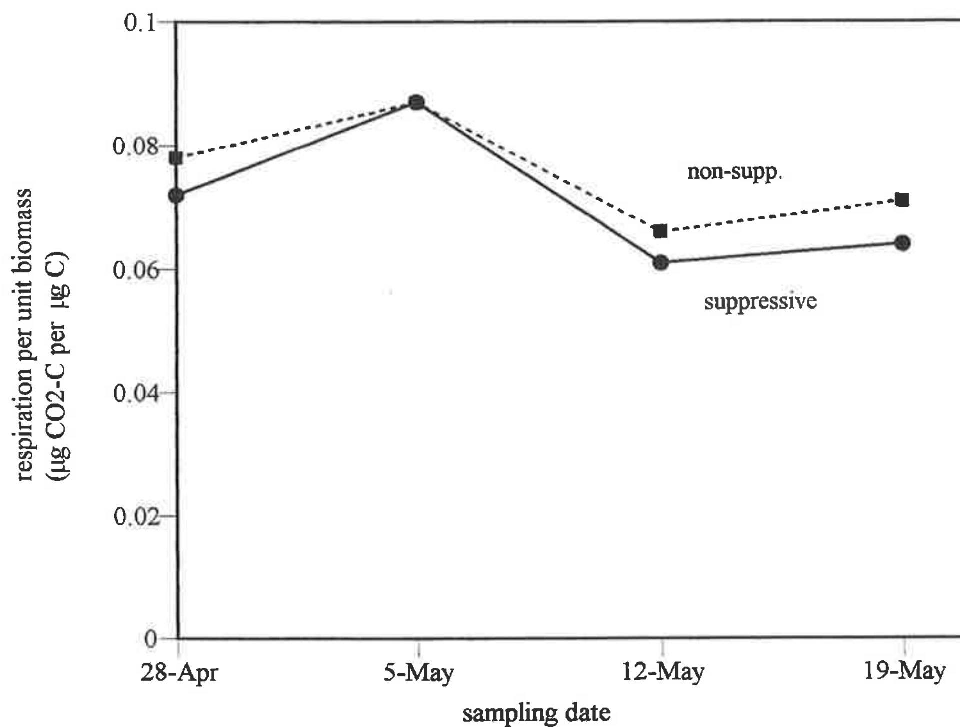



Figure 7.3: Respiration per unit of biomass in suppressive and non-suppressive (non-supp.) soils sampled during 1995. Values are from treatment means of biomass (3 replicates) and respiration (4 replicates) determinations.

### 7.3.4 *In vitro* and *in vivo* carbon utilisation

#### 7.3.4.1 Cellulolytic fungi and bacteria

Numbers of total fungi, cellulolytic fungi and the proportion of total fungi with cellulolytic ability were similar in the two soils in both Experiment 1 and 2 (Table 7.4a). The trend was for a higher proportion of cellulolytic fungi in suppressive soil but this was not significant. Bacterial counts were similar in both soils in Experiments 1 and 2 (Table 7.4b). The proportion of cellulolytic organisms decreased approximately ten fold from Experiment 1 to 2 while total counts were similar. This may be explained by storage of the soils at 4 ° C between experiments.

#### 7.3.4.2 Total cellulolytic micro-organisms

Total numbers of micro-organisms, cellulolytic micro-organisms and the proportion of the total number of micro-organisms with cellulolytic ability were similar in the two soils when assessed using spread plates (Table 7.5). Total counts were similar to those determined using well plates (7.3.4.1) but the proportion of cellulolytic organisms on spread plates was approximately 50 and 500 times higher than in the well plates in Experiments 1 and 2 respectively.

#### 7.3.4.3 Decomposition of plant material in field soil

Dry weight loss of shoot material was similar for wheat and pea in both soil types. Dry weight per litterbag significantly ($P = 0.01$) decreased between weeks 0 and 9 and between weeks 9 and 20, but no further decrease was observed at week 32 (Fig. 7.4a)

a) Experiment 1

| | soil | total (cfu / g soil) | cellulolytic (cfu / g soil) | proportion cellulolytic |
|---|---|---|---|---|
| fungi | suppressive | 4.9 (0.5) x $10^3$ | 8.6 (1.2) x $10^2$ | 0.22 (0.04) |
| | non-supp. | 3.0 (0.4) x $10^3$ | 3.0 (0.5) x $10^2$ | 0.12 (0.02) |
| bacteria | suppressive | 6.9 (0.4) x $10^4$ | 6.2 (1.3) x $10^3$ | 0.09 (0.02) |
| | non-supp. | 6.8 (0.9) x $10^4$ | 3.7 (0.6) x $10^3$ | 0.09 (0.01) |
| soil (fungi) | | NS | NS | NS |
| soil (bacteria) | | NS | NS | NS |

b) Experiment 2

| | soil | total (cfu / g soil) | cellulolytic (cfu / g soil) | proportion cellulolytic |
|---|---|---|---|---|
| fungi | suppressive | 5.4 (0.6) x $10^3$ | 3.2 (0.8) x $10^2$ | 0.05 (0.01) |
| | non-supp. | 4.9 (0.7) x $10^3$ | 83 (9) | 0.02 (0.01) |
| | suppressive | 1.6 (0.1) x $10^5$ | 1.4 (0.1) x $10^3$ | 0.01 (0.001) |
| bacteria | non-supp. | 1.1 (0.1) x $10^5$ | 8.9 (0.4) x $10^2$ | 0.01 (0.001) |
| soil (fungi) | | NS | NS | NS |
| soil (bacteria) | | NS | NS | NS |

Table 7.4 a and b: Number of total and cellulolytic, and proportion of total with cellulolytic activity, fungi and bacteria in suppressive and non-suppressive (non-supp.) soil in two experiments using the same soil sample measured with well plates. Values are means of 4 replicates and standard errors are given in parentheses. Significance of individual treatment is indicated by NS (non-significant).

| soil | total organisms (cfu / g soil) | cellulolytic organisms (cfu / g soil) | proportion cellulolytic |
|---|---|---|---|
| suppressive | 4.0 (0.08) x $10^4$ | 1.6 (0.1) x $10^4$ | 0.41 (0.03) |
| non-suppressive | 3.7 (0.3) x $10^4$ | 1.8 (0.3) x $10^4$ | 0.52 (0.02) |
| soil | NS | NS | NS |

Table 7.5: Total colony forming units (cfu), cfu showing cellulolytic activity and proportion of total cfu showing cellulolytic activity (proportion cellulolytic) per gram dry weight of suppressive or non-suppressive soil measured with spread plates. Values are means of 3 replicate plates and standard errors are given in parentheses. Significance of treatment is indicated by NS (non-significant).

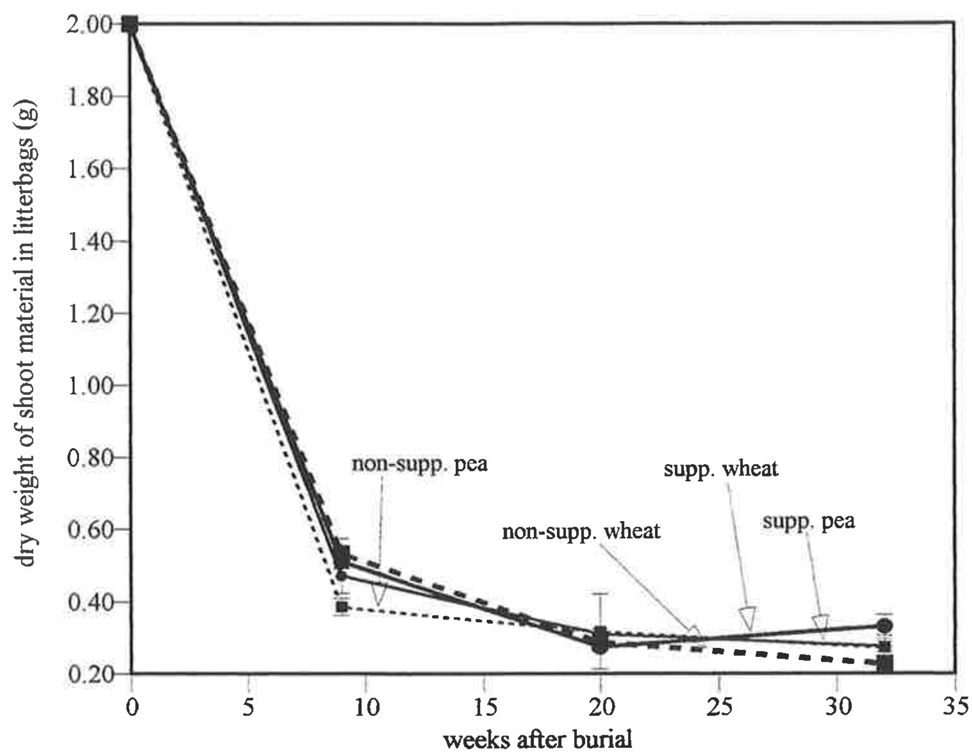



Figure 7.4a: Dry weight loss of wheat and pea shoot material buried in suppressive (supp.) and non-suppressive (non-supp.) soil. Values are means of 2-4 replicates and lines indicate standard error of the data.

Dry weight loss of wheat roots differed significantly (P = 0.05) between the two soils but no there was no clear trend (Fig 7.4b). Dry weight loss of pea roots was similar in the two soils.

The trend for average dry weight loss for pea roots in suppressive soil was not as expected with a higher weight measured at week 32 compared to week 20 after a decrease between weeks 0 and 9. Incomplete replication at the second and third harvests may explain this variation.

7.3.5 FAME

Qualitative and quantitative differences were observed in the FAME profiles of organic matter sieved from suppressive and non-suppressive soils and rhizosphere soil from the two soils. Qualitative differences were shown by grouping of suppressive and non-suppressive samples following principal component analysis and representation in dendograms (Figs. 7.5 a/b and 7.6 a/b). The trend for separation of suppressive and non-suppressive samples indicates the individual FAMEs of the two soils differ.

Peaks of bacterial and fungal origin were separated from the total peak area and compared between the two soils. These specific peaks have been reported previously (Bååth *et al.*, 1993; Fröstegård *et al.*, 1995). Total peak area was significantly (P = 0.01) higher for organic matter samples compared to rhizosphere soil samples (Table 7.6). Size of organic matter and rhizosphere samples was not calibrated so this was expected. Bacterial peak area for organic matter samples was similar in the two soils, whereas fungal peak area was significantly (P = 0.01) higher in rhizosphere soil from non-suppressive soil (Table 7.6).

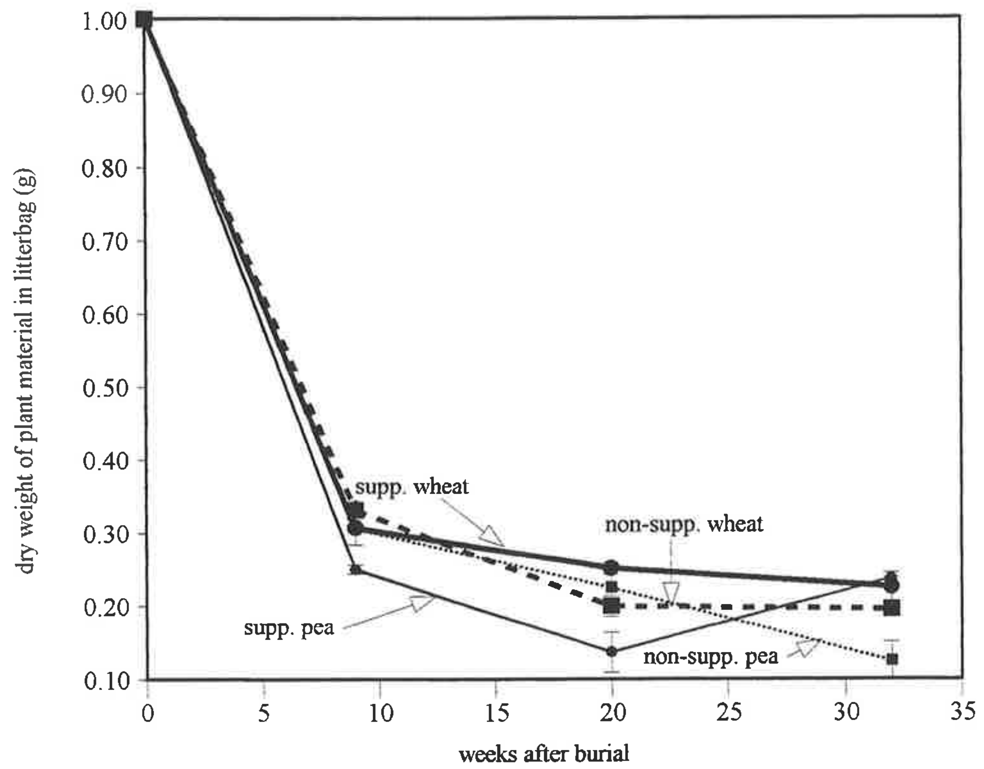



Figure 7.4b: Dry weight loss of wheat and pea root material buried in suppressive (supp.) and non-suppressive (non-supp.) soil. Values are means of 2-4 replicates and lines indicate standard error of the data.

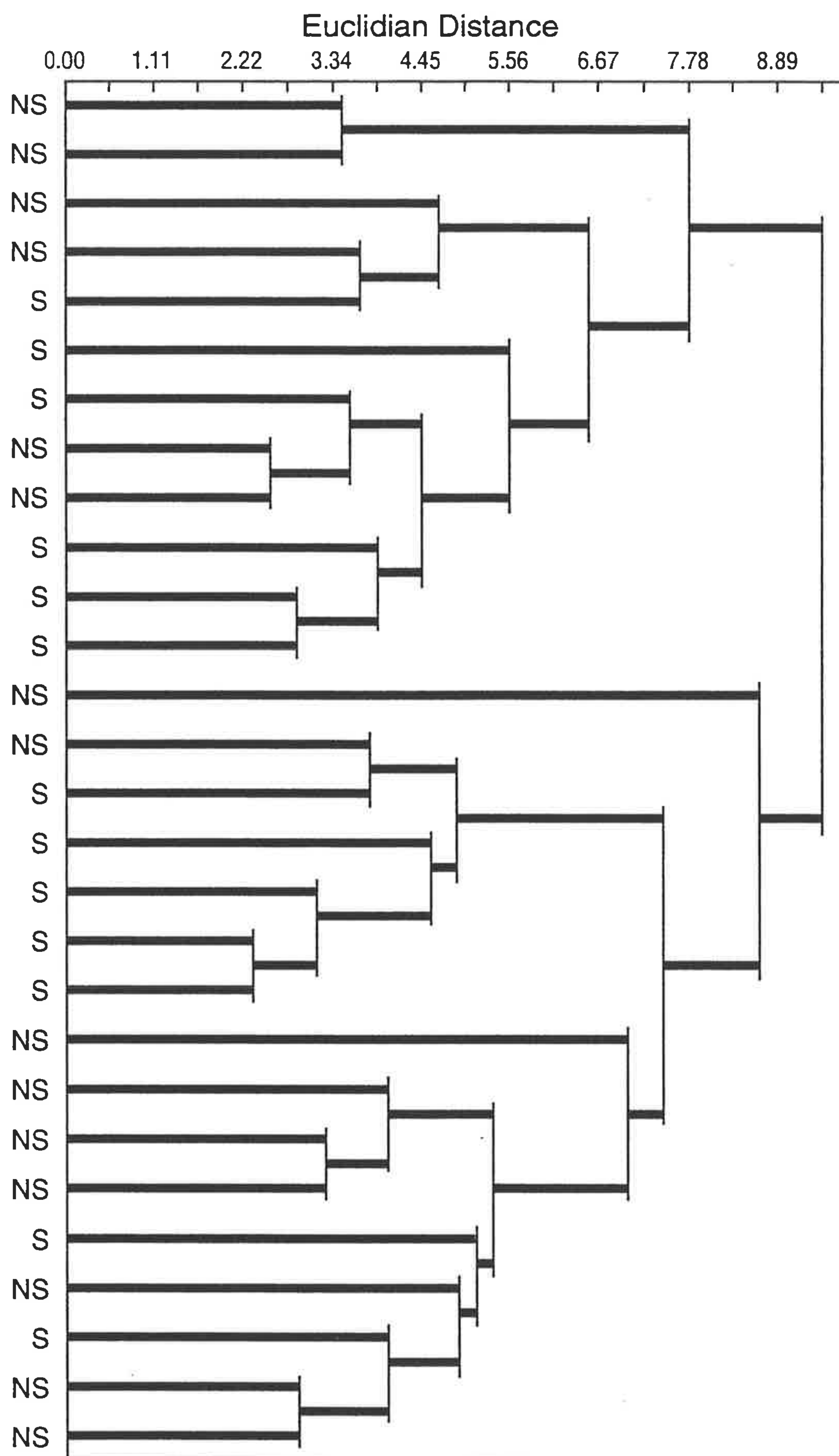



Figure 7.5a: Dendrogram of similarity of FAME profiles of organic matter from suppressive (S) and non-suppressive (NS) soil. Samples with outlying profiles removed.

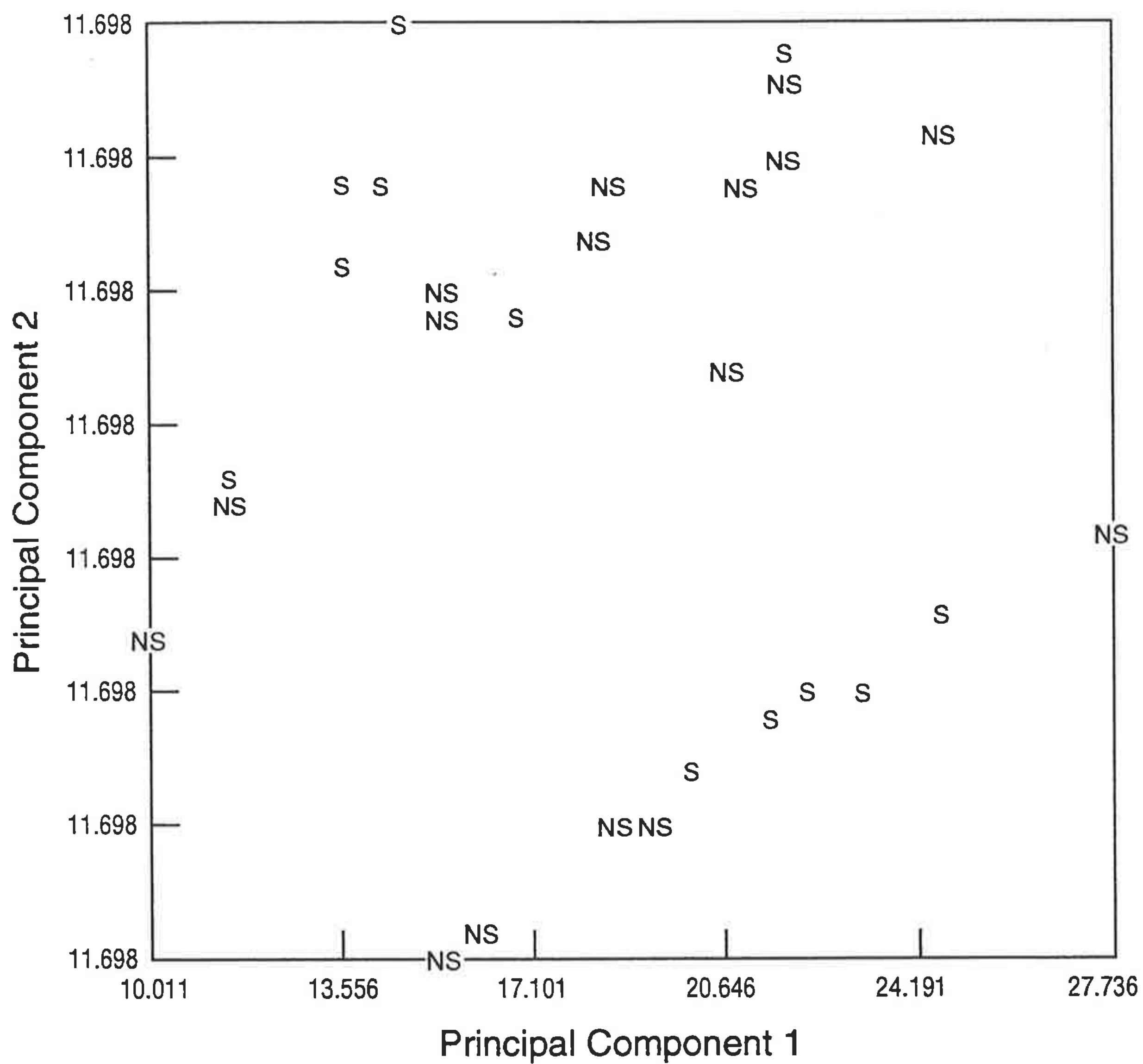



Figure 7.5b: Principal component (PC) analysis (PC 1 versus PC 2) for FAME profiles of organic matter from suppressive (S) and non-suppressive (NS) soil. Samples with outlying profiles removed.

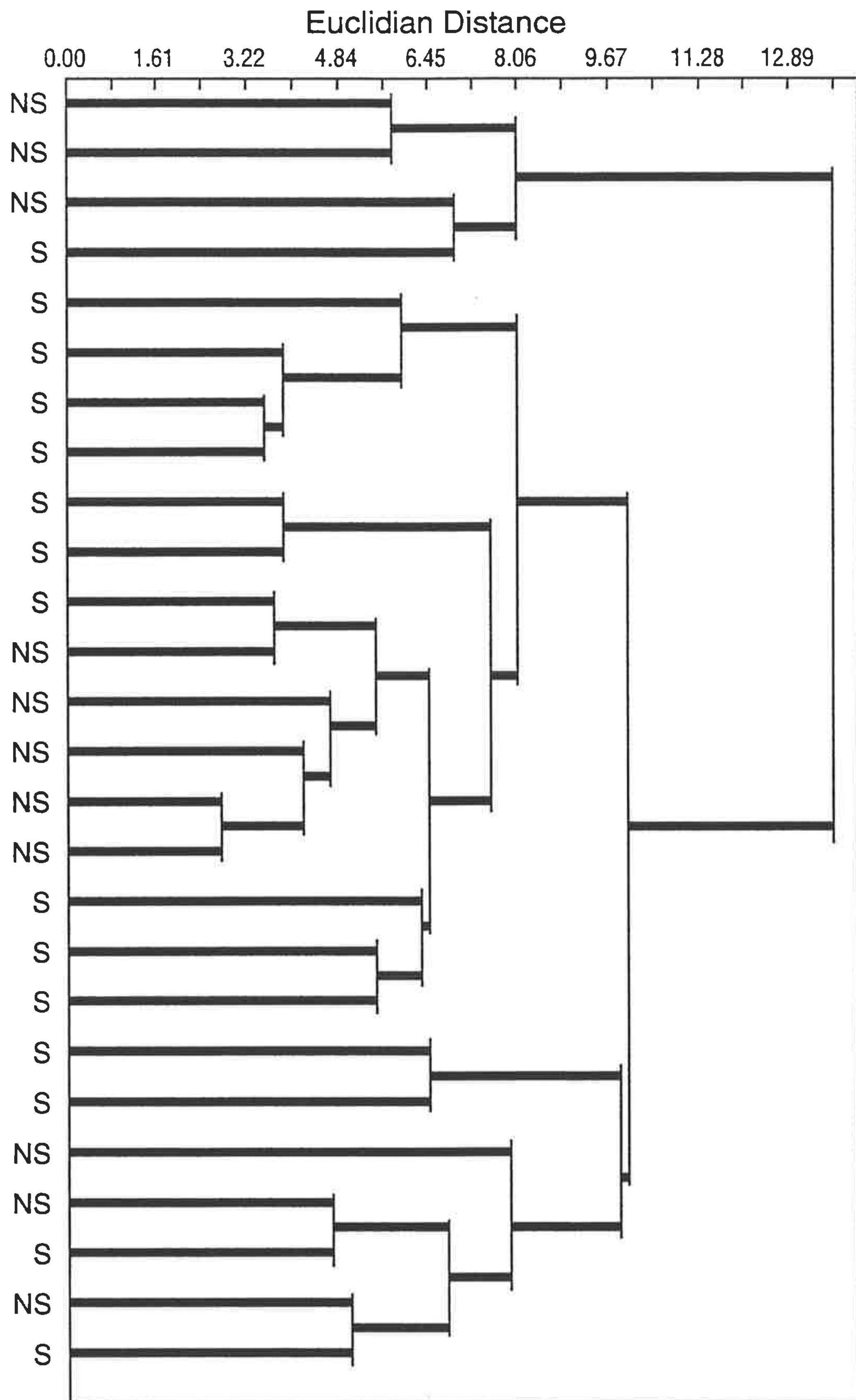



Figure 7.6a: Dendrogram of similarity of FAME profiles of rhizosphere soil from suppressive (S) and non-suppressive (NS) soil. Samples with outlying profiles removed.

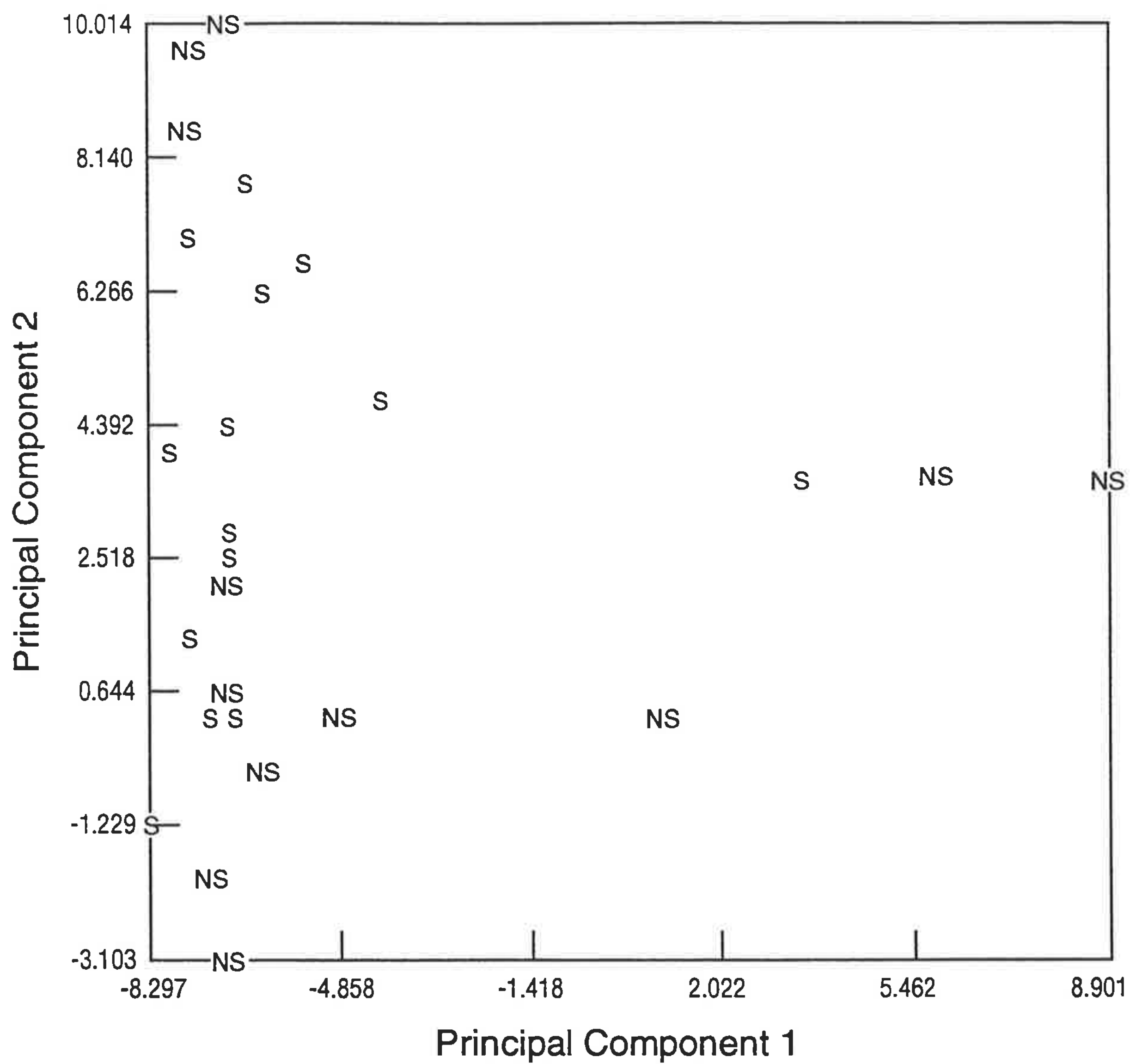



Figure 7.6b: Principal component (PC) analysis (PC 1 versus PC 2) for FAME profiles of rhizosphere soil from suppressive (S) and non-suppressive (NS) soil. Samples with outlying profiles removed.

| position | soil | total peak area | bacterial peak area | fungal peak area |
|---|---|---|---|---|
| organic matter | supp. | 3.90 (0.07) x $10^5$ | 1.13 (0.03) x $10^5$ | 0.14 (0.001) x $10^5$ |
| organic matter | non-supp. | 4.50 (0.1) x $10^5$ | 0.99 (0.03) x $10^5$ | 0.12 (0.001) x $10^5$ |
| rhizosphere soil | supp. | 1.96 (0.04) $10^5$ | 0.35 (0.001) x $10^5$ | 0.05 (0.001) x $10^5$ |
| rhizosphere soil | non-supp. | 3.68 (0.09) x $10^5$ | 0.72 (0.01) x $10^5$ | 0.11 (0.003) x $10^5$ |
| soil | | ** [0.56 x $10^5$] | NA | NA |
| position | | ** [0.56 x $10^5$] | NA | NA |
| soil.pos. | | NS | ** [0.03 x $10^5$] | ** [0.22 x $10^5$] |

Table 7.6: Area of total, bacterial and fungal FAME peaks for organic matter and rhizosphere soil from suppressive (supp.) and non-suppressive (non-supp.) soil. Values are means of 15 replications and standard errors are given in parentheses. Significance of individual treatments and interaction is indicated by ** (1 % level), NS (non-significant) or NA (not applicable) and values for LSD at P = 0.05 are given in square parentheses.

## 7.4 Discussion

Increased microbial activity and biomass, as well as increases in populations of particular groups of organisms, were found in suppressive compared to non-suppressive soil. Few differences were found when the soils were compared using dilution plate techniques. In contrast, clear functional differences were found in total microbial biomass and activity measurements, and differences in community structure were indicated by comparison of FAME profiles of suppressive soil and non-suppressive soil. Comparison of populations of five microbial groups following steam pasteurisation provided evidence of differences in community structure in suppressive soil and non-suppressive soil, and an implicated a role for *Trichoderma* spp. in the disease suppression.

Quantification of fungi, bacteria and actinomycetes using semi-selective media illustrated the limitations of dilution plate techniques for characterising microbial populations. Few differences were detected between suppressive soil and non-suppressive soil even though clear differences were found in microbial biomass and activity. The dilution plate technique is useful when highly selective media can be used in a targeted approach to study specific groups of organisms and groups that are implicated in particular activities. For example, the selective media used in this chapter for quantification of *Trichoderma* spp. (S. Dyer, pers. comm.) and fluorescent pseudomonads (Gould *et al.*, 1985) allowed highly selective culturing of single groups of organisms. In contrast, the use of dilution plating for groups such as total fungi or total bacteria was less useful due to the small proportion of the total population which is culturable.

Quantification of a range of microbial groups in untreated and pasteurised suppressive soil and non-suppressive soil provided further support for the

biological basis of Rhizoctonia barepatch decline as described in Chapter 3. When treated at temperatures above 60°C the two soils did not differ in disease severity on wheat (Chapter 3) or numbers of a range of microbial groups. Of the five microbial groups quantified, *Trichoderma* spp. was the only one that was higher in suppressive soil than non-suppressive soil when untreated or pasteurised at 50°C. The ability of *Trichoderma* spp. as biological control agents has been widely reported (Maplestone *et al.*, 1991; Benhamou and Chet, 1993; Manzali *et al.*, 1993) and it may also be involved in this case of suppression of *R. solani* AG-8. This hypothesis could be tested by isolating *Trichoderma* from soils at Avon and checking for antagonism of *R. solani* AG-8.

Further evidence for differences in the biological community structure was provided by the different response of suppressive soil and non-suppressive soil to steam pasteurisation and FAME profiles. In untreated non-suppressive soil there were higher numbers of bacteria, fluorescent pseudomonads and fungi compared to suppressive soil. The reverse was the case when the soils were steam pasteurised at 50°C, implying that the composition of these broad groups was different in the two soils. Analysis of FAME profiles of soils allows comparison of the structure of the microbial community to be made. FAME profiles are useful in comparisons of microbial communities due to their capacity to extend information based on only culturable members of the microbial population. Using methods not relying on culturing of organisms, such as FAME analysis, more realistic comparisons of microbial communities can be made. Marker FAMEs for fungi and bacteria are known (Bååth *et al.*, 1993; Fröstegård *et al.*, 1995), and in rhizosphere soil the amount of these particular FAMEs was higher in non-suppressive compared to suppressive soil. Such differences were not found in organic matter samples, which supports the results for plate counts of fungi and bacteria from the two soils. In addition to these quantitative differences, qualitative

differences in the microbial communities in suppressive and non-suppressive soils were found. With the existing knowledge of FAME profiles these differences can not be attributed to particular populations but they do indicate that the composition of the microbial communites in the two soils differ. Differences in the compositions of microbial communities of suppressive and non-suppressive soil indicated by FAME analysis support the earlier conclusion of different microbial communites existing in suppressive and non-suppressive soil (Chapter 3) : the microbial population of the non-suppressive soil causing general suppression of disease, and the population in the suppressive soil causing both general and specific suppression of disease. More detailed identification of the microbial groups involved in suppression of Rhizoctonia barepatch is needed before the roles of particular groups can be ascertained. This has been done for *Fusarium* suppressive soils of the Châteaurenard region and Salinas Valley, with correlations of soil suppressiveness with populations of non-pathogenic *Fusarium* (Alabouvette, 1986), and fluorescent pseudomonads (Kloepper *et al.*, 1980; Scher and Baker, 1980) being identified.

There was no evidence for a role for protozoa in suppression of Rhizoctonia barepatch. Unlike the increased populations of mycophagous amoebae and hyphal damage observed in soils suppressive to take-all (Chakraborty, 1983a; b; Chakraborty and Warcup, 1983 / 1984), no differences in populations sizes of protozoa or hyphal damage due to protozoa were observed between suppressive soil and non-suppressive soil.

Higher microbial biomass and activity in suppressive soil compared to non-suppressive soil correlated with previous reports (Garrett, 1933; Gerlagh, 1968; Alabouvette, 1986; Höper *et al.*, 1995). Further characterisation of the microbial activity with short-term, substrate induced respiration (SIR) would be useful to clarify what stage(s) of the decomposition of organic matter are different in the two soils. Investigations of microbial activity over periods of

hours can be done with SIR, rather than days and weeks for the incubation methods used in this chapter. Assessment of SIR may also be useful for explaining the results of increased microbial activity and biomass in suppressive soil compared to non-suppressive soil but similar rates of *in vitro* and *in vivo* carbon utilisation reported in this chapter. A role of inputs of organic matter in development of suppression of Rhizoctonia barepatch seems likely, but little strong evidence to support this was found. One explanation for this is the time scale used when assessing carbon utilisation. Experiments reported in this chapter lasted for periods of days or weeks. In comparison, differences observed between soils of varying suppressiveness to *Fusarium oxysporum* were detected in SIR experiments of up to 52 hours (Alabouvette, 1986; Amir and Alabouvette, 1993; Höper *et al.*, 1995). In these SIR experiments microbes in suppressive soil reacted quicker, and to a greater extent, to added glucose compared to microbes in conducive soil.

Total carbon levels in Rhizoctonia barepatch suppressive soil and non-suppressive soil were similar (Section 4.2.1.3) and have not changed significantly over time (D. Roget, pers. comm.) even though stubble retention has been performed for longer with the suppressive soil. Hence, the role of inputs of organic matter in suppression may be through short term affects at the time of addition rather than long-term increases in organic matter levels in the soil. This conclusion is supported by results for the medium-term decomposition of organic matter in suppressive soil and non-suppressive soil presented in this chapter. No major differences were observed for decomposition of pea or wheat material between suppressive soil and non-suppressive soil over a 32 week period.

Total hyphal lengths were similar in suppressive and non-suppressive soil (Chapter 6), but microbial biomass tended to be higher in suppressive soil. This lack of correlation is not unexpected as hyphal length gives approximate estimates of only fungal biomass, mainly due to variation in hyphal diameter

(Hanssen *et al.*, 1974). Similarly, a correlation between FDA-stained hyphae and total fungal biomass does not always exist (Ingham and Klein, 1984). In addition, total microbial biomass is clearly composed of many non-fungal organisms, which would influence any correlations between biomass and hyphal lengths. Bacterial counts also gave slightly inconsistent results. Counts varied from being similar in the two soils in some experiments, higher in suppressive soil, or higher in non-suppressive soil. A similar quantification procedure was used in all cases so variation is likely to be due to fluctuations in soil bacterial populations over time and uneven distribution of colonies in the soil.

The composition of the microbial community in suppressive and non-suppressive soil is different. This has been determined by the initial characterisation of microbial populations in the two soils presented in this chapter as well as results presented in earlier chapters (3-6). Biological suppression of Rhizoctonia barepatch may therefore be based on two forms of suppression. These two forms of suppression are: specific antagonism of *R. solani* AG-8 by particular components of the biological community (eg *Trichoderma* spp.) (specific suppression, after Gerlagh, 1968), and general antagonism and increased competition due to increased microbial biomass and activity (general suppression, after Gerlagh, 1968). Support for this conclusion is given by the level of suppression found in non-suppressive soil compared to sterile or partially sterile soil (Chapter 3), higher microbial biomass and activity in the non-suppressive soil presumably making it more suppressive than soil with lower or absent microbial biomass and activity. Further discussion of possible mechanisms with respect to results presented in this thesis and previous reports from the literature is given in Chapter 8.

## Chapter 8: General Discussion and Conclusions

The general aims of this project were to characterise an observed decline in Rhizoctonia barepatch severity and determine likely mechanisms by which the disease was being controlled.

Rhizoctonia barepatch occurred at the Avon site from the initiation of the trial in 1978 through to 1990 (Roget, 1995). Disease severity initially rose with the introduction of reduced tillage, but began declining until there were no detectable symptoms eight years later. In a suppressive soil, either the pathogen does not establish, it establishes but does not produce disease, or it establishes and initially produces disease which then declines (Baker and Cook, 1974). Suppression of Rhizoctonia barepatch at the Avon site involved establishment of the pathogen which initially caused disease and then disease severity declined.

The work described in this thesis shows that the observed decline in Rhizoctonia barepatch is due, at least in part, to changes in the characteristics of the soil biota over time. The suppressive characteristics of the suppressive soil are transferable, and removed by gamma irradiation or steam pasteurisation at 60°C for 30 min. General and specific (Gerlagh, 1968; Cook and Rovira, 1976) mechanisms of suppression are acting in this soil. General suppression is due to the effect of the entire soil biota on disease severity and is found to a certain extent in all soils. The higher microbial biomass and activity in suppressive soil compared to non-suppressive soil indicates increased general suppression in suppressive soil compared to non-suppressive soil. Specific suppression is due to the antagonism of particular groups of the soil biota. A role of specific antagonists at Avon was suggested

by larger populations of *Trichoderma* in suppressive soil compared to non-suppressive soil. A role of chemical suppression, such as inhibitory levels of micro-nutrients or antibiotic production, is not supported by results presented here but further investigation of this is necessary.

The effects of high and low doses of inoculum of *R. solani* AG-8 are suppressed in this soil, as are diseases caused by *G. graminis* var. *tritici* and *F. graminearum*. The presence in pathogens of chitin - glucan cell walls correlates with suppression of these pathogens in pot experiments by the soil suppressive to Rhizoctonia barepatch. This correlation needs to be investigated by extended screening for suppression of disease caused by pathogens with other primary cell wall components. Specialisation of types of cell walls able to be attacked is possible due to the need for antagonistic organisms to produce specific enzymes for breakdown of cell walls. In further investigations pathogens could be screened using the classification system of Bartnicki-Garcia (1968) which separates taxonomic groups based on their cell wall components (eg Group I : cellulose - glucan, Oomycetes; Group IV : chitosan - chitin, Zygomycetes).

There may be a correlation between suppression and infection by non-motile propagules and survival of hyphae in particles of debris. This could only be observed under field conditions when the entire disease cycle of the pathogens occurs.

Disease on cereals not used in the field trial (oats and barley) is also suppressed. This is both the first documented case of suppression developing without monoculture of the host, and also of suppression being active on a range of cereal hosts. Suppression of *R. solani* AG-8 has been induced previously (Lucas *et al.*, 1993), as has suppression of *R. solani* AG-2, AG-2-

2 and AG-4 (Henis *et al.*, 1978a; Liu and Baker, 1980; Davik and Sundheim, 1984; Hyakumachi *et al.*, 1990). However, in all cases monoculture of the host was necessary for development of suppression. Lack of requirement for monoculture for development of suppression of Rhizoctonia barepatch, and suppression of disease on a range of cereals indicates that specific root exudates / secretions / lysates / mucilages are not required for suppression to occur. These compounds are important determinants of the composition of the microbial population in the rhizosphere (Bowen and Rovira, 1991). The conclusion must be that a collection of organisms is responsible for this suppression, rather than a particular one promoted by specific rhizosphere conditions.

Take-all suppressive soil also suppressed other diseases (Wildermuth, 1977), but soils suppressive to Fusarium wilts did not (Alabouvette, 1986). Soil suppressive to take-all suppressed disease due to *R. solani*, and reduced (not always significantly) disease due to *P. irregulare*, *Gibberella zeae* (*F. graminearum*) and *F. culmorum* (W. G. Smith) Sacc.. Variation in suppressiveness of different diseases suggested that different organisms were involved in suppression of the causal agents (Wildermuth, 1977), or that there was general suppression. Importantly, the variation suggested the pathogens responded differently in the same soil. If there is a correlation between disease suppression with cell wall type, and possibly also with infection propagules, mode of action, and survival, suppression of different diseases at Avon is probably not due to different organisms. What may vary at Avon is the effectiveness with which the organisms suppress disease development. If suppression is the result of several mechanisms, being carried out by a collection of organisms, some variability in suppression between each particular host - pathogen combination would be expected. It would be possible to test whether a collection of organisms is responsible for

suppression of several diseases by identifying potentially antagonistic organisms (such as *Trichoderma* spp.), and demonstrating antagonism to the pathogens which are suppressed in the Avon soil.

Preliminary evidence indicates that it is the saprophytic phase and possibly the parasitic phase of *R. solani* AG-8 that are suppressed in this soil. Quantitative assessment of *Rhizoctonia* in soil suggests that survival is appears reduced in suppressive soil, as is hyphal growth through soil and in the rhizosphere. Little is known about the surviving populations of *G. graminis* var. *tritici* in suppressive soils, but there is a negative correlation between length of runner hyphae on the root surface following inoculation and disease suppression (Wildermuth and Rovira, 1977). This provides some evidence for suppression of the parasitic phase of *G. graminis* var. *tritici*. Decline of take-all is attributed to breakdown of inoculum during the saprophytic phase (Gerlagh, 1968), but without accurate assessment of pathogen populations, rather than resultant disease severity, firm conclusions can not be made. For soils suppressive to *Fusarium*, evidence indicates that the pathogen is present but does not produce disease (Scher and Baker, 1980; Alabouvette, 1990) and that suppressiveness of soils is independent of their ability to support populations of the pathogen (Alabouvette, 1986).

Populations of viable *Rhizoctonia* and disease severity due to *R. solani* AG-8 are lower in suppressive soil compared to non-suppressive soil. This implies survival of *Rhizoctonia*, and successful infection of living roots was lower in suppressive soil compared to non-suppressive soil. Following inoculation with *R. solani* AG-8, the length of metabolically active and inactive *Rhizoctonia*-like hyphae was lower in suppressive soil compared to non-suppressive soil in the absence of living plant roots. This indicates growth of the pathogen has been inhibited during saprophytic growth through bulk

suppressive soil compared to bulk non-suppressive soil. Suppression is therefore active in bulk soil. This may partially explain the suggested lack of a role for specific root exudates / secretions / lysates / mucilages in the rhizosphere in suppression. In the presence of living roots, length of active *Rhizoctonia*-like hyphae is lower in suppressive soil compared to non-suppressive soil, and length of active non-*Rhizoctonia*-like hyphae is higher. There may be inhibition of the *Rhizoctonia* in the infection court in suppressive soils, a suggestion supported by the observation of increased amounts of inactive *Rhizoctonia*-like hyphae in the rhizosphere in suppressive compared to non-suppressive soil.

The different techniques used to quantify populations of *Rhizoctonia* in this investigation highlighted not only differences between suppressive and non-suppressive soil, but also the need to use different techniques when comparing populations and define clearly what form of *Rhizoctonia* is being quantified. Separation of the population of *Rhizoctonia* into viable, active, pathogenic and total forms is possible. As only viable, pathogenic forms will cause disease, interpretation of individual sets of results in terms of disease potential is difficult. For example, a correlation with disease severity was found with the number of isolations of *Rhizoctonia* from organic matter (viable population) and length of metabolically active *Rhizoctonia*-like hyphae in soil. But, the total population of *Rhizoctonia*, as quantified with an AG-8 specific DNA probe (Matthew *et al.*, 1995; pRAG12), did not correlate with disease severity. Results using this DNA assay indicate that further refinement of this technique is necessary before it can be reliably used in quantification studies. Areas to be investigated are the possibilities of the AG-8 specific probe detecting dead, and non-AG-8 (eg binucleate *Rhizoctonia* spp.) DNA.

The higher microbial biomass and activity found in soil suppressive to Rhizoctonia barepatch is similar to that found in soil suppressive to *Fusarium* (Alabouvette, 1986; Höper *et al.*, 1995). Organic carbon levels have not significantly increased in the suppressive soil (D. Roget, pers. comm.). As the organic inputs have been higher the early stages of breakdown and colonisation of organic matter may be important in inducing suppression, rather than increased total amounts of organic matter. The finding that decomposition of plant material in suppressive and non-suppressive soil was not different 2 - 8 months after burial supports the conclusion that any differences in rate of breakdown of organic matter which are occurring between the two soils during the early stages of colonisation.

The role of specific antagonists needs to be tested to determine which organisms are involved in the suppression, and if there is any variation temporally or for different diseases. Numbers of total bacteria and fluorescent pseudomonads are higher in soil suppressive to *Fusarium* and soil suppressive to take-all (Garrett, 1933; Cook and Rovira, 1976; Lemanceau and Alabouvette, 1993), but not (as shown in this thesis) in soil suppressive to Rhizoctonia barepatch. A role for pseudomonads in take-all suppression was suggested partly due to removal of suppressive characteristics by 60°C moist heat which kills pseudomonads (Cook and Rovira, 1976). Suppressive characteristics of the soil suppressive to Rhizoctonia barepatch were removed with steam pasteurisation at 60°C, but enumeration of fluorescent pseudomonad populations did not indicate any correlation with suppressiveness. *Trichoderma* populations are higher in suppressive soil, indicating a likely role for this organism in suppression of Rhizoctonia barepatch, as suggested also for suppression of *Fusarium* (Sivan and Chet, 1986).

The role of non-pathogenic *Fusarium* spp. in suppression of pathogenic *Fusarium* is well established (Alabouvette, 1986; Alabouvette, 1990; Alabouvette *et al.*, 1993). The ratio of pathogenic to non-pathogenic *Fusarium*, rather than the density of the pathogen population, is important in determining disease severity (Alabouvette, 1986). A similar situation may exist for suppression of Rhizoctonia barepatch with the ratio of pathogenic *R. solani* AG-8 and non-pathogenic / binucleate *Rhizoctonia* spp. The relative amounts of binucleate and multinucleate *Rhizoctonia* at different positions within a barepatch varies, and this may relate to the "sharp and abrupt" edge of barepatches (Roberts and Sivasithamparam, 1986). Control of disease caused by non-AG-8 *R. solani* has been found (Burpee and Goulty, 1984; Cardoso and Echandi, 1987; Herr, 1988; Escande and Echandi, 1991; Harris *et al.*, 1993a; 1994), so antagonism of *R. solani* AG-8 by non-pathogenic / binucleate *Rhizoctonia* in barepatches is possible. The use of fungal isolation methods will allow differentiation of *Rhizoctonia* into binucleate and multinucleate populations, but the existing DNA assay may not because of complications due to cross-hybridisation with non-AG-8 DNA. However, it would be a relatively simple matter to construct a more specific probe, or even a range of probes specific to a range of *Rhizoctonias*, that could be used with the current soil DNA assay.

A potentially important topic not considered in this thesis is temporal variation in suppression. Soils were sampled at a number of different times over a three year period, but were generally used for only one or two experiments. Bulk sampling of soil for use throughout the investigation was not done because of the potential changes in soil characteristics that might occur with long term storage. A number of mechanisms and organisms are likely to occur in the soil suppressive to Rhizoctonia barepatch and these may not all act at any given time. Temporal effects could be investigated by

assessment of the same parameters at various times in the year. This approach was taken for the soil bioassay experiments reported, but assessment of functioning and identity of the microbial community and other potentially important characteristics needs to be coordinated with disease severity assessment.

The work presented in this thesis provides a preliminary investigation on which future investigations of soils suppressive to Rhizoctonia barepatch and to other diseases can be based. The suggested mechanisms of suppression require further experimental evidence and clarification. There are two major areas of future work -

a.) development of a technique to see how extensive suppression of Rhizoctonia barepatch is, and

b.) identification of soil characteristics leading to suppression.

This investigation was based on the comparison of only two soils. Further investigation of suppression of disease due to *R. solani* AG-8, and other pathogens, depends on the ability to effectively screen and compare soils for suppressiveness. As described by Alabouvette (1990) "...the first tool to study a pathogen-suppressive soil is an accurate method for assessing the degree of suppressiveness...". This type of procedure is required for both large scale screening of field soils, and more detailed studies under controlled conditions to further understand mechanisms of suppression.

Soils can be suppressive to either pathogens or disease, or both (Hornby, 1983), so any screening procedure must assess both the size of the pathogen population and the disease severity. A correlation between the size of the pathogen population and disease severity may not always exist, so quantification of both is necessary. In cases of low disease severity

quantification will determine if the pathogen is being suppressed, or is in fact absent. Screening for suppressiveness using assessment of disease severity only (Lester and Shipton, 1967; Andrade *et al.*, 1994), may identify suppressive soils but conclusions on the type(s) of suppression acting can not be made. Bioassay methods using inoculated and uninoculated soils do not differentiate between soils of similar levels of suppressiveness (Alabouvette, 1990), but this may be overcome by combination with the DNA assay. For example, disease severity could be related to total amount of pathogen in the soil with and without inoculation, and before and after a period of incubation. Information on saprophytic colonisation and survival of the pathogen in soil, plus data on disease severity resulting from a known amount of pathogen would be gained.

The suggested approach for future work is a combination of a (possibly improved) DNA assay (Matthew *et al.*, 1995; Whisson *et al.*, 1996), and a soil bioassay / pathogenicity test (McDonald and Rovira, 1985; Carling and Sumner, 1992; Sweetingham and MacNish, 1994). The DNA assay estimates the total amount of *R. solani* AG-8 DNA and the bioassay estimates the disease severity. Quantification of pathogen populations using methods relying on fungal isolation or colonisation of organic material should be avoided due to their tedious and unreliable nature, and lack of specificity to *R. solani* AG-8. Methods for the soil bioassay would be similar to those described in this thesis. The DNA assay would be used to assess the resident amount of *R. solani* AG-8 DNA, and the amount of *R. solani* AG-8 DNA following incubation of the soil (with and without inoculation with *R. solani* AG-8).

For each soil the screening method would determine : a) the resident amount of pathogen in the soil, b) the amount of pathogen in the soil following

incubation of inoculated and uninoculated soil, and c) the disease severity. Low disease severity may then be explained in terms of failure of the pathogen to develop in the soil, or of inhibition of disease development by an established population. These options represent two of the possible types of suppression (Baker and Cook, 1974). Expression of disease severity in terms of per unit DNA of *R. solani* AG-8 would identify soils suppressive to disease, but which still contained a large resident AG-8 population. In addition, soils exhibiting high disease severity, but capable of suppressing increased severity would be identified.

There are several limitations to this screening method that still need to be overcome. As mentioned earlier, clarification of what the AG-8 specific DNA probe (pRAG12) actually detects is vital for its successful use in an assay. Similar total populations of *R. solani* AG-8 were found in suppressive soil and non-suppressive soil using this DNA assay. The lack of correlation of these results with those for viable forms of *Rhizoctonia* and disease severity caused by *R. solani* AG-8 raises some questions on the accuracy of the current assay. The possibility of detection of dead and non-AG-8 DNA needs to be investigated. Detection of dead DNA appears unlikely but detection of non-AG-8 DNA such as that from binucleate *Rhizoctonia* spp. is possible. The possible antagonistic role of binucleate *Rhizoctonia* spp. in suppression makes separation of their population from the AG-8 population crucial in quantification studies. Assuming these limitations can be overcome, the DNA assay is the most promising quantification method to be used in conjunction with assessment of disease severity. Methods for assessment of disease severity are well established and a standardised approach could be designed from current knowledge.

Once detection of viable DNA and specificity of the probe to AG-8 has been established, an investigation of the relationship between amount of DNA and amount of fungus (ie number of cells; length of hyphae) may be useful. The mechanism of control of Rhizoctonia barepatch using tillage has been suggested as fragmentation of particles of debris and disturbance of the hyphal network, which reduces viability and / or pathogenicity of propagules of *R. solani* AG-8 (Rovira, 1986). Detection of small amounts of *R. solani* AG-8 and correlation with the size of propagule from which it was extracted may allow the minimum size of a viable, pathogenic propagule to be determined.

Characteristics of soils with identified degrees of suppressiveness may be compared to determine likely characteristics predisposing them to, or inducing, suppression. An initial investigation of two soils is reported in this thesis, but more extensive comparisons are needed for likely characteristics to be identified. Soils suppressive to *Fusarium* have been characterised in this way (Amir and Alabouvette, 1993; Duijff *et al.*, 1995; Höper *et al.*, 1995), resulting in strong correlative evidence for the role of several biotic and abiotic characteristics. Strong evidence has also been found for a correlation between soil type and suppressiveness to black root rot of tobacco (Stutz *et al.*, 1985). Experimental evaluation of abiotic factors may be difficult (Alabouvette, 1990), so a correlative approach is required.

The most obvious starting point for such a potentially wide investigation would be factors resulting from increased stubble retention, due to the likely role of organic inputs and decomposition in inducing suppression of Rhizoctonia barepatch decline at Avon (D. Roget, pers. comm.). There is potential overlap between these factors and those with a known involvement in other cases of suppression. Characteristics including faster and increased

response of soil respiration to glucose addition (Höper *et al.*, 1995), increased competition for carbon (Couteaudier and Alabouvette, 1990a), and increased microbial biomass (Alabouvette, 1986) or counts of specific microbial groups (Garrett, 1933) are all potential points of investigation.

This thesis describes the occurrence of natural, biologically based suppression of Rhizoctonia barepatch at Avon in South Australia. Suppression of other diseases by this soil has also been established. Initial investigation of the microbial populations in suppressive soil and non-suppressive soil indicate differences in their functioning and composition. The development of this suppression is of great importance to the control of Rhizoctonia barepatch, a disease of major importance but with no effective control strategy except tillage. The experiments reported here build on the initial description of the disease decline phenomenon by Roget (1995). Disease control such as this, developed through manipulation of the existing soil biota by adoption of appropriate farming practices, is clearly successful and represents a significant breakthrough in the search for effective, long term reduction of losses due to disease.

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## Appendix 1: Culture media and solutions

### Arginine glycerol salt medium (AGS)

El-Nakeeb and Lechevalier (1962)

| | |
|---|---|
| arginine | 1.0 g |
| glycerol | 12.5 g |
| $K_2HPO_4$ | 1.0 g |
| NaCl | 1.0 g |
| mineral stock | 10 ml |
| agar | 15 g |
| water | 990 ml |

Mineral Stock

| | |
|---|---|
| $MgSO4.7H_2O$ | 10 g |
| $Fe_2(SO_4)_3.6H_2O$ | 0.2 g |
| $CuSO_4.5H_20$ | 0.02 g |
| $ZnSO_4.7H_2O$ | 0.02 g |
| $MnSO_4.H_2O$ | 0.02 g |
| water | 200 ml |

### Carboxy-methyl cellulose (CMC) hydrolysis medium

Modified from Wollum (1982)

| | |
|---|---|
| CMC | 12 g |
| $NaNO_3$ | 0.5 g |
| $K_2HPO_4$ | 1.0 g |
| $MgSO_4.7H_2O$ | 0.5 g |
| $FeSO_4.7H_2O$ | 0.01 g |
| agar | 15 g |
| water | 1000 ml |

**Dichloran rose bengal chloramphenicol agar (DRBC)**

King *et al.* (1979)

| | |
|---|---|
| glucose | 10 g |
| bacteriological peptone | 5 g |
| $KH_2PO_4$ | 1.0 g |
| $MgSO_4.7H_2O$ | 0.5 g |
| agar | 15 g |
| water | 1000 ml |
| rose bengal | 25 mg |
| dichloran | 2 mg |
| chloramphenicol | 100 mg |

**Ethanol potassium nitrate medium (EPN)**

Trujillo *et al.* (1987) as modified by S. Neate (pers. comm.)

| | |
|---|---|
| $KNO_3$ | 0.2 g |
| agar | 14 g |
| ridomil (250 g / L a.i metalaxyl) | 800 µl |
| prochloraz | 17.6 µl |
| ethanol | 21 ml |
| streptomycin sulphate | 100 mg |
| tetracycline hydrochloride | 50 mg |
| water | 979 ml |

Fungicide, antibiotics and ethanol mixed, added to cooled medium

**Ko and Hora's *Rhizoctonia* selective media (KH)**

Ko and Hora (1971)

| | |
|---|---|
| $K_2HPO_4$ | 1 g |
| $MgSO_4.7H_2O$ | 0.5 g |
| KCl | 0.5 g |
| $FeSO_4$.7H2O | 0.01 g |
| $NaNO_2$ | 0.2 g |
| gallic acid | 0.4 g |
| (dexon | 0.09 g) |
| chloramphenicol | 0.05 g |
| streptomycin sulphate | 0.05 g |
| agar | 20 g |
| water | 1000 ml |

Gallic acid and antibiotics added to cooled medium.
(Dexon omitted as no longer available)

**KHp**

Vincelli and Beaupre (1989)

KH + 50 µl prochloraz per litre

**KHpm**

Vincelli and Beaupre (1989)

KH + 50 µl prochloraz + 100 mg metalaxyl per litre

**Modified Neff's amoeba saline**

V.V.S.R.Gupta (pers. comm.)

Working solution:

| | |
|---|---|
| A - E | 10 ml each |
| water | 950 ml |

Stock solutions (in 100 ml):

| | | |
|---|---|---|
| A | NaCl | 1.2 g |
| B | $MgSO_4.7H_2O$ | 0.04 g |
| C | $Na_2HPO_4$ | 1.42 g |
| D | $CaCl_2.2H_2O$ | 0.04 g |
| E | $KH_2PO_4$ | 1.36 g |

**Neutral Dox Yeast broth (NDY)**

| | |
|---|---|
| czapek-dox broth (Difco, 0338-01-2) | 35 g |
| water | 1000 ml |

**Neutral Dox Yeast medium (NDY/6)**

Warcup (1955)

| | |
|---|---|
| $NaNO_3$ | 0.33g |
| $KH_2PO_4$ | 0.17 g |
| $MgSO_4.7H_2O$ | 0.08 g |
| KCl | 0.08 g |
| $FeSO_4$ (0.1 % solution) | 1.7 ml |
| yeast extract | 0.08 g |
| sucrose | 5.0 g |
| agar | 14 g |
| water | 1000 ml |

**Phosphate saline buffer**

Wollum (1985)

| | |
|---|---|
| NaCl | 8 g |
| $K_2HPO_4$ | 1.21 g |
| $KH_2PO_4$ | 0.34 g |
| water | 1000 ml |

Adjust pH to 7.3 with 0.1 N NaOH or HCl, autoclave

**Potato dextrose agar (PDA)**

| | |
|---|---|
| potato dextrose agar (Oxoid, CN139) | 39 g |
| water | 1000 ml |

***Rhizoctonia* liquid medium**

De Beer (1965)

| | |
|---|---|
| $KH_2PO_4$ | 0.6 g |
| $K_2HPO_4$ | 0.87 g |
| $MgSO_4.7H_2O$ | 0.133 g |
| sucrose | 17 g |
| bacteriological peptone | 7 g |
| water | 1000 ml |

**S1 (fluorescent pseudomonads selective medium)**

Gould *et al.* (1985)

| | |
|---|---|
| agar | 18 g |
| sucrose | 10 g |
| glycerol | 10 ml |
| casamino acids | 5.0 g |
| $NaHCO_3$ | 1.0 g |
| $MgSO_4.7H_2O$ | 1.0 g |
| $K_2HPO_4$ | 2.3 g |
| sodium lauryl sarcosine | 1.2 g |
| water | 1000 ml |
| trimethoprim | 20 mg |

Trimethoprim added to cooled medium.

**0.3% Tryptic Soy Agar (TSA)**

Martin (1975)

| | |
|---|---|
| tryptic soy broth (Difco, 0370-01-1) | 3 g |
| agar | 12 g |
| water | 1000 ml |

**Water Agar (WA)**

agar 15 g
(Difco Bacto-Agar 0140-01 / Difco BiTek agar 0138-01-4)
water 1000 ml

## Appendix 2: Solutions for use with specific DNA probe

**20 X SSC**

| | |
|---|---|
| NaCl | 175.3 g |
| sodium citrate | 88.2 g |
| water | 800 ml |

Dissolve NaCl and sodium citrate in water, adjust pH to 7.0 with 10 N NaOH solution. Adjust the volume to 1000 ml with water and sterilise by autoclaving

**TE pH 8.0**

10 mM Tris-Cl (pH 8.0)
1 mM EDTA (pH 8.0)

**TAE**

Stock solution (per litre):
50 X: 242 g Tris base
57.1 ml glacial acetic acid
100 ml 0.5M EDTA (pH 8.0)

Working solution:
1 X: 0.04 M Tris-acetate
0.001 M EDTA

**Elution Buffer**

| | |
|---|---|
| 1.5 M | NaCl |
| 100 mM | tris pH 7.2 |
| 10 mM | EDTA |

Sambrook *et al.* (1989)

**SDS Extraction Buffer**

200 mM Tris-HCl pH 8.5
250 mM NaCl
25 mM EDTA
0.5 % sodium dodecyl sulphate (SDS)

Raeder and Broder (1985)

**Standard Hybridisation Buffer**

| | |
|---|---|
| nanno-pure water | 2 ml |
| 5 x HSB | 3 ml |
| 10 x Denhardt's III solution | 1 ml |
| 25 % dextran sulphate | 4 ml |
| 5 µg/ml salmon sperm DNA | 0.2 ml |

<u>5 X HSB</u>
3 M NaCl
100mM PIPES
25mM $Na_2EDTA$

pH 6.8 with 4 M NaOH

<u>10 x Denhardt's III solution</u>

| | |
|---|---|
| 2 % | gelatin |
| 2 % | Ficoll |
| 2 % | polyvinyl pyrollidone |
| 10 % | SDS |
| 5 % | tetrasodium pyrophosphate |

after Sambrook *et al.* (1989) (Herdina, pers. comm.)

## Appendix 3: Most Probable Number (MPN) of cellulolytic bacteria and fungi solutions and media

Gupta and Roper (1994)

BACTERIAL ENUMERATIONS

Solution A

| | |
|---|---|
| CMC | 5.0 g |
| water | 780 ml |
| $(NH_4)_3SO_4$ | 4.0 g |
| NaCl | 2.5 g |
| $MgSO_4.7H_2O$ | 0.1 g |
| $CaCl_2.2H_2O$ | 0.1 g |
| yeast extract | 0.5 g |
| FeEDTA (1.64 % solution) | 2 ml |
| agar | 15 g |

Dissolve CMC in water, cool, add remaining ingredients. Adjust pH to 6.8-7.0.

SolutionB

0.5 M $KH_2PO_4$ - NaOH pH 7.0 120 ml

Solution C

| | |
|---|---|
| glucose | 10 g |
| water | 100 ml |

Antibiotic

100 µg $ml^{-1}$ cyclohexamide

FUNGAL ENUMERATIONS

Solution A

| | |
|---|---|
| CMC | 5.0 g |
| water | 500 ml |
| $(NH_4)_3SO_4$ | 4.0 g |
| NaCl | 2.5 g |
| $MgSO_4.7H_2O$ | 0.1 g |
| $CaCl_2.2H_2O$ | 0.1 g |
| yeast extract | 0.5 g |
| FeEDTA (1.64 % solution) | 2 ml |
| agar | 15 g |

Dissolve CMC in water, cool, add remaining ingredients. Adjust pH to 6.8-7.0.

Solution B

0.2 M potassium hydrogen phthalate pH 5.0

Antibiotic

400 $\mu g\ ml^{-1}$ streptomycin sulphate

Overlay

| | |
|---|---|
| CMC | 1.0 g |
| solution B | 60 ml |
| agar | 4.0 g |
| water | 500 ml |