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Pasture plant interactions with soil organisms in the rhizosphere

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Abstract

Biological constraints may limit the productivity of Australian pastures. The objective of this project was to develop and apply new technologies to visualise and quantify the effects of soil microorganisms on the growth of pasture plants and to assess the impact of management options on soil biology and its subsequent effect on pasture productivity. Critical to this is greater understanding of how different microorganisms interact within the rhizosphere around plant roots. Results from the study showed that different groups of pathogenic and beneficial microorganisms could be quantified successfully using microbial DNA assays in bulk soil, rhizosphere soil and within plant roots. However, microbial DNA contents in soil and roots were subject to high variability and the correlation between the DNA content of specific pathogens in soil and incidence of disease on plants roots was poor. Further work to address this issue in field soils and greater understanding of the importance of environmental factors that contribute to the 'outbreak' of root diseases under field conditions is needed. Nevertheless, the DNA-based approach along with new techniques for the analysis of root exudates reported here, provides new insight into the response of microorganisms to soil and plant treatments in ways that could not be achieved using more conventional techniques. In the longer term, these technologies will allow growers to make more informed decision with regard to the health of their soils and to the productivity and sustainability of their pasture systems.

Executive Summary

This project on 'Pasture Plant Interactions with Soil Organisms in the Rhizosphere' was initiated as part of MLA's Pasture Soil Biology Initiative. The objective was to undertake basic science to explore new opportunities for investigating the interaction of soil microorganisms in the rhizosphere of pasture plants. The project aimed to develop and apply new technologies to visualise and quantify the effects of soil microorganisms on plant growth, with a view to identifying and developing management strategies that optimize the contribution of microorganisms to the productivity and sustainable of pasture systems.

Although it is widely recognised that biological constraints may limit the productivity of Australian pastures, there is relatively poor understanding of the actual contribution of soil microorganisms to the growth of pasture plants, which includes both pathogenic and beneficial microorganisms. However, the level of interest among producers in maintaining 'healthy' soils has increased significantly in recent years and there is widespread interest in better understanding the 'biology' of farming systems. Critical to this is greater understanding of how different microorganisms interact within the rhizosphere of plants and their subsequent effect on plant growth.

Major achievements of the project were:

- A survey of the literature showed that biological constraints are an important issue that can potentially reduce the productivity of Australian pastures and that microbial interactions in the rhizosphere may mitigate the severity of root diseases.

- The importance of disease on the growth of pasture plants was demonstrated in glasshouse experiments using intact cores of field soil. Whilst incidence of disease was highly variable, a number of potentially important root pathogens were identified. Controlled studies in sand culture showed that infection of roots of pasture plants by *Pythium* sp. significantly reduced root growth, whereas less consistent results were obtained in soil.

- New methods for the study of rhizosphere microorganisms were developed. This included a fluorescence-microscopy technique for direct visualisation of *Pythium* infection on pasture roots and procedures for 'tagging' different species of rhizobacteria with fluorescent proteins. Bacteria that inhibited the growth of *Pythium* in laboratory media were isolated from the rhizosphere of pasture plants.

- A system for the collection of root exudates from pasture plants was established for sand-grown plants and the C composition of the exudate was analysed by GC–MS. Defoliation of plants caused a significant change to the allocation of C to root growth and the quantity and composition of C in root exudates, which has important implication for root interactions with soil microorganisms. Attempts to isolate root exudates directly from soil-grown pasture plants were unsuccessful.

- The presence of various fungal (and oomycete) root pathogens and beneficial fungi in soil and on plant roots was investigated by quantitative DNA analysis (collaboration with SARDI). Although the presence of plant roots increased the DNA content of different fungal groups, no specific effects occurred in response to defoliation. Microbial DNA content of

soil was also generally not affected by P status, although higher mycorrhizal content of roots was evident in low P soil, with a possible interaction between the presence of mycorrhizal fungi and reduced presence of *Pythium*. However, in all cases there was high variability in the fungal DNA content of soil which was exacerbated by the presence of plants. No clear relationship was identified between changes in microbial DNA content in soils with changes in the rhizosphere, and to the extent of fungal infection on plant roots.

Results from the project showed that DNA-based technologies hold considerable promise for investigating the behaviour of specific groups of microorganisms in the rhizosphere of plants. DNA-based techniques provide insight into the response of microorganisms to soil and plant treatments in ways that could not be achieved using more conventional techniques. The study also showed that GC–MS has considerable potential for quantifying the C composition of root exudates and to the understanding of how plants respond to treatments that are indicative of management options for pastures.

Microbial DNA assays were successfully quantified groups of pathogenic and beneficial microorganisms, directly in bulk soil, rhizosphere soil and within plant roots. However, microbial DNA concentrations in soil were subject to high variability which was largely associated with intrinsic spatial variability in the distribution of the microbial species in field soil which were used as intact cores of soil. High intrinsic variability of different pathogens was further accentuated by the stimulatory presence of plant roots, which meant that it was difficult to interpret results from treatment effects that were imposed under glasshouse conditions. Whilst a number of measures were undertaken to limit this variability (i.e. increased replication, use of mixed and reconstituted soil cores rather than intact field cores, and controlled inoculation treatments), these also proved to be largely unsuccessful for root pathology studies in the glasshouse. In addition, it was evident from glasshouse studies that the presence of high DNA content of specific pathogens was not necessarily indicative of high incidence of root damage and/or clear evidence of root disease.

Further studies to correlate measured DNA content of specific pathogens in soil and incidence of disease on plants roots is required, as this relationship remains poor. Whilst it may be a consequence using glasshouse-grown plants, we are also aware of similar issues under field conditions. In addition to ensuring that the DNA probes used are representative of causative organisms, further investigation of the importance of environmental factors (e.g. soil type, climatic conditions, management practices, etc) that contribute to the ‘outbreak’ of root diseases is needed. As well as greater emphasis on use of field sites, this will require input from a plant pathologist. Future studies on the molecular ecology of soil-borne root pathogens, therefore, need to focus on field studies, with glasshouse studies limited to validation (e.g. pathogenicity tests in sand culture) and/or high-throughput germplasm screenings where artificially high inoculum levels can be used without compromising the outcome.

Improved understanding of how soil microorganisms interact with plant roots and respond to pasture management will, in the longer term, allow more informed decisions to be made in soil health and the productivity and sustainability of pasture systems. It is expected that such information will be readily available to growers and routinely used within the next 5–10 years.

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1 Background

1.1 Soil biological influences on pasture production in south-eastern Australia: Review of the impacts and processes of microbial interactions with plant roots.

Summary

This review and others indicate that soil biology can constrain the growth of pasture plants in Australia (21 of 23 studies analysed had improved plant growth when soil organisms were disturbed). However, it is unclear whether the published studies are broadly representative of pasture soil biology research because there is likely to be less opportunity and incentive to publish studies that did not record a response to soil treatments. Two types of soil biological constraints are evident; direct effects, where pathogens may invade roots and kill the plant host, and indirect effects, where detrimental microorganisms may reside around roots and cause slower plant growth ("low vigour"). The severity of soil biological constraints is influenced by soil pH, water, temperature, micro and macro-nutrients, and management factors such as choice of species and grazing regime. Bacteria, fungi and nematodes have been studied most, but are often applied singularly in controlled environments that do not reflect the physical and chemical complexities of permanent, long term pastures in the field where disease complexes are regarded as common occurrences. This review focuses on bacteria and fungi.

It is proposed that soil biological processes that influence plant growth in pastures take place in the rhizosphere, where plant roots and organisms exchange chemicals and signals (exudates) that influence how microorganisms interact with the roots. Permanent pastures are comprised of mixed populations of plants with roots of the different species having significant opportunity for interaction. Roots of differing age and species release a range of exudates and are likely to be associated with different populations of microorganisms. Root-soil and root-root interactions in pastures challenge the concept of a single rhizosphere around a single root. A new research framework is therefore required to account for overlapping rhizospheres, the wide temporal and spatial scales they occupy and the succession of organisms that reside there. The "balance" between organisms that positively and negatively influence plant growth will determine the extent to which the pasture community may be constrained by the soil biology. We hypothesise that whilst this "balance" may reduce the vigour of some species, it may also keep pathogens in check and maintain greater ecological stability. Better understanding of the factors that regulate rhizosphere interactions in pastures may lead to preferred management options for pastures. Indeed, research on "suppressive" soils and effects of plant rotations in cropping systems has given valuable insight into such processes. In terms of permanent pastures, key knowledge gaps identified in this review were:

● information on roots and rhizospheres from field conditions and the effects of above-ground management procedures (e.g. grazing and fertiliser practices) on rhizosphere processes
● understanding of the significance of mixtures of root systems (i.e. root–root interactions of different plant species) on root exudates, and the "balance" of soil microorganisms that have positive or negative influences on plant growth

● methods for *in situ* examination and quantification of roots, root exudates and soil microorganisms in pasture soils
● information on the longer term impacts of changes in the soil biology on permanent pasture productivity, nutrient acquisition and water use.

1.1.1 Introduction

"Soil biology" is the study of life in soil, and has been used to describe a wide range of above- and belowground processes, such as grazing, decomposition of organic matter by bacteria and fungi, predation or antagonism among soil microflora/fauna and population dynamics of these organisms as affected by root activity. The study of soil biology encompasses a range of scientific disciplines including plant pathology, soil-borne disease management and control, plant physiology and nutrition, agronomy and soil microbial ecology. Despite the broad use of the term, the main objective of soil biology research is to enhance soil biological functions through better understanding of root-microbe interactions in order to control pests and disease, improve nutrient cycling, soil quality and water use (United States Department of Agriculture 2004).

The ecological linkages between above- and belowground components of biota in a pasture are important and may be characteristic of the plant communities that occur in different ecosystems(Wardle et al. 2004). For perennial pasture systems, the coexistence of different plant species over relatively long periods of time distinguishes them from annual rotations of crops and pastures. The community structure and diversity of microorganisms, and dynamics under a perennial pasture is likely to be vastly different from those of an annual crop where a single species (or even genotype) is grown for shorter periods in soil that is often disturbed and subject to extensive agronomic management. Soil biology has been more extensively investigated in annual crops because diseases increase readily if crop sequences do not include alternative species that "break" disease cycles. The development of "suppressive soils" in which a biological balance is exerted on disease organisms has also been observed in crop sequences. However, these phenomena are relatively poorly studied in pasture systems, especially in permanent perennial pastures.

Perennial pastures

Grasses native to temperate south-eastern Australia include *Bothriochloa*, *Chloris* and *Austrodanthonia* spp., while native legumes include *Psoralea*, *Lespedeza*, *Glycine* and *Desmodium* spp. (Donald 1970). The native grasses, often in combination with naturalised exotic grasses and legumes, sustain livestock production in significant areas where artificial seeding with exotic species is uneconomic, or impractical. However, introduced grasses and legumes dominate pasture production in the more favoured grassland areas of southern Australia. The most widely sown perennial pastures in temperate south-eastern Australia comprise a suitable, persistent perennial grass in conjunction with exotic annual legume species such as *Trifolium subterraneum* (subterranean clover) or perennials such as *Trifolium repens* (white clover). Examples of introduced pasture grasses common to south-eastern Australia include *Lolium perenne* (perennial ryegrass), *Phalaris aquatica* (phalaris) and *Dactylis glomerata* (cocksfoot). Their distribution depends mainly on the length of growing

season at any particular location, with soil acidity also influencing the choice between phalaris and cocksfoot. Perennial pasture species are considered essential components of sustainable farming systems in southern Australia (Cransberg and McFarlane 1994). The deeper roots of perennial species (Ward et al. 2000) may help control some forms of land degradation by reversing hydrological imbalance, decreasing nitrate leaching, providing a root mat to lessen soil erosion, and creating biopores to improve soil structure and drainage (Cransberg and McFarlane 1994). Perennial species also assist in weed control and contribute to more uniform production of forage year-round (Wheeler 1987). Cransberg & McFarlane (1994) argue that the influence of perennial pastures on physical and chemical soil properties may help to reverse soil degradation. However, the impact of perennial species on soil biological properties and microbial population, diversity and structure, and their subsequent effect on soil fertility is poorly understood.

T. repens (white clover) and *L. perenne* are important species in perennial pastures in New Zealand and parts of south-eastern Australia (Victoria). Despite their economic importance, research on soil biological factors affecting perennial pasture production is scarce, with greater focus on annual species such as *Trifolium* and *Medicago*. Even scarcer is research on Australian native grass species such as *Austrodanthonia* which has experienced renewed interest in the 1980s as a species for areas where introduced species grow poorly (eg. low unreliable rainfall) or for revegetation (New South Wales Department of Primary Industries 2000).

Soil biology in pastures is also relevant to turf-grass production where perennial species such as *Poa annua* (annual bluegrass) and *L. perenne* (Uddin et al. 2004) are grown together over a long periods of time: the mixture of different species and longevity of production is analogous to use of mixed swards in pasture production. Occurrence, and management and control of disease (Uddin et al. 2004) have been investigated in turf grasses, whereas other soil biological factors such as microbial ecology affecting fertility and disease suppression in soils under turf production have received less attention.

The aim of this review is to address the question: Is there a soil biological constraint to the production of perennial pastures in south-eastern Australia? The review consists of four sections. First, the current knowledge of soil biological constraints on pasture production is discussed; second, the effects of several management practices relating to pasture production on soil biology is outlined; third, disease suppression in soil is examined; and fourth, rhizosphere mechanisms that may potentially control the interactions between beneficial and deleterious microorganisms on roots are discussed. Only bacteria and fungi are considered in this review. These include organisms living freely in soil, as well as those associated in beneficial and deleterious interactions with plant roots. Although other soil-borne organisms such as nematodes, collembola, earthworms and mites are likely important to rhizosphere processes in pasture production, they have not been included in this review.

1.1.2 Soil biological constraints to pasture production

Microorganisms ubiquitous to most soils include pathogenic fungal species such as *Pythium*, *Rhizoctonia*, *Fusarium*, *Phytophthora*, as well as beneficial species like arbuscular mycorrhizal fungi AMF. Common beneficial bacteria include rhizobia and some species of *Pseudomonas*

that promote plant growth (Brown et al. 1994). Major bacterial and fungal soil-borne plant pathogens affecting *T. subterraneum* and *L. perenne* have been identified and documented compared to *P. aquatica* and *Austrodanthonia fulva* (Table 1.1). Soil-borne pathogens that adversely affect growth and production of *P. aquatica* and *A. fulva* have not been specifically identified. However, this may reflect lack of study as it is possible that roots of these species may host microbes that affect the growth of other species. *Dreschlera*, a fungal species causing leaf spot, seedling blight, leaf stripe, or net blotch in cereals, was recently isolated from roots of *Austrodanthonia* growing in a pasture at Bookham, NSW, although symptoms of disease were not evident on the host plant (S. Wiebkin, pers. comm. June 2005).

Literature investigating the effect of soil biology on pasture production is summarised in Table 1.2. From the 12 studies analysed here, 10 report soil biological constraints to pasture species. This supports a recent analysis of Hannam (2003, Study Commissioned by MLA) of published and Industry reports of studies on soil biological constraints in pastures in Australia. All 11 studies found evidence of constraint due to soil organisms (range 10 to 200% increase in shoot biomass in response to soil drenches). The extent of constraint in the different studies often depended on soil physical and chemical factors (generally increased constraint with nutrient deficiency). At least two distinct groups of soil biological "constraints" to pasture growth can be identified from these analyses: (1) specific and identifiable soil-borne pathogens causing decrease in pasture production, establishment, decline or death of plants; and (2) non-specific "poor growth" situations where plant growth is improved following soil treatment that alters soil biology (eg. fumigation, pasteurisation, drenching). Of the two groups, constraints to plant growth posed by soil-borne pathogens are the most extensively investigated, primarily due to their obvious, specific and direct effects on plant growth. The majority of these are based on experimental plots or local sites where drenches for eliminating fungi or nematodes were applied, and there is poor extrapolation and documentation of their occurrence and relative importance to the production of perennial pastures in southern Australia. The significance of small scale studies, over short terms, for broad acre production has yet to be demonstrated. It may be that studies that did not find a response to soil biology perturbation have not been reported, and this makes it difficult to assess how wide spread real effects of soil organisms are in pasture systems.

Specific soil-borne pathogenic constraints

Use of *T. subterraneum* has allowed pastures in south-eastern Australia to have high stock carrying capacity, tolerance to heavy continuous grazing and ability to build up soil nitrogen and prevent soil erosion (Barbetti et al. 1986). The economic importance of *T. subterraneum* has resulted in extensive research on the soil-borne pathogenic constraints that limit its production (Barbetti and MacNish 1978; Barbetti et al. 1986; Barbetti et al. 1987). Important fungi associated with root rot in *T. subterraneum* include; *Fusarium avenaceum*, *Phytophthora clandestina*, *Pythium irregulare* and *Rhizoctonia* spp.. These species can interact with one another to form disease complexes (Flett and Clarke 1996). In a 4-year study of root rot in *T. subterraneum*, Barbetti et al., (1986) demonstrated that *P. clandestina* interacted with *F. oxysporum*, but not *F. avenaceum*, *P. medicaginis*, *P. irregulare* or *R. solani*, to produce more severe root rot than did either fungus alone. Disease severity is further complicated by the interaction between the pathogen and the environment. Root rot in *T. subterraneum* was particularly severe when heavier and more frequent rain occurred after planting (Barbetti et al. 1986). There was a significant interaction between temperature and moisture for the various

fungi and fungal combinations tested. The most severe root rot in *T. subterraneum* occurred at 65% water holding capacity, with less at 45% and even less under flooded conditions. Greatest seedling death caused by *P. clandestina* occurred at a soil temperature of 10 ºC (compared to 15 and 20 ºC) under saturated or flooded conditions (Barbetti et al. 1986). Using another *Trifolium* species, Sarathchandra et al. (2000) also showed that fungal pathogens alone did not contribute to decreased vigour of *T. repens*, but a combination of environmental factors (in this case, low moisture/high temperatures) made plants more susceptible to fungal infection. Although environmental factors can affect disease severity, their contribution to disease severity is situation-specific. For example, low vigour areas of *T. repens* in *T. repens*/*L. perenne* pastures in New Zealand were associated with higher levels of the fungal root pathogens *Codinaea fertilis* and *Fusarium* spp. compared to high vigour areas. However levels of these pathogens in low vigour areas were unrelated to topographical and nutritional factors (Sarathchandra et al. 2000).

A degree of "natural biological control" for pathogens may already exist in pastures (Brown et al. 1994). *In vitro* tests growing fungal pathogens (five *Pythium* spp. including *P. irregulare*, and *R. solani*) together with bacteria isolated from *T. repens* and *L. perenne* roots from 45 sites in New Zealand showed that 37-52% of bacterial isolates from roots of both species inhibited at least one pathogenic fungus. Although the presence of the pathogens was not determined in the pasture sites in this study, they are most likely to be present on *T. repens* and *L. perenne* roots due to their wide host range. A high incidence (72% and 63% for *T. repens* and *L. perenne*, respectively) of cyanogenic fluorescent *Pseudomonas* sp. was found at all sites, but it was not determined whether these were inhibitory to fungal root pathogens. Although this research is useful in indicating the levels of bacteria with potential beneficial effects, whether increasing or decreasing the numbers of beneficial and pathogenic organisms, respectively, would have significantly improved plant growth is left unanswered.

Care is needed when interpreting data from experiments in pasteurised soil done in the glasshouse (Flett and Clarke 1996). Experiments using disease isolates collected from the field and tested for pathogenicity in the glasshouse in pasteurised soil may exaggerate the actual severity of a pathogen in the field as sterilised or pasteurised soil exclude the role of other microorganisms in disease expression. Furthermore, pathogenicity of individual pathogens does not reflect the field situation where different fungal, bacterial or viral pathogens act together in disease complexes (Flett and Clarke 1996). For example, *F. avenaceum* and *P. irregulare* were pathogenic on *T. subterraneum* in pasteurised soil but did not cause disease in unpasteurised soil under the same controlled conditions. The authors did not speculate on the reasons for this, but it is likely that pasteurisation altered soil biological properties that confer suppression of these pathogens. Non-pathogenic *F. oxysporum* and *F. solani* have been suggested to play a role in suppressing pathogenic *Fusarium* spp. by occupying an ecological niche required by the pathogen (Rovira 1982). Pasteurisation may have removed non-pathogenic organisms, thus enabling pathogenic organisms to increase and cause disease. Differential conditions for pathogenicity have also been found between artificial and field conditions. In wheat plants grown in sterile soil inoculated with *Gaeumannomyces graminis* var. *tritici*, the fungus causing take-all (TA) disease in plants of the Graminae family, TA caused most severe disease symptoms at 20 ºC. In the field, however, disease was most severe between 12 and 20 ºC (Hornby 1990). In addition to studies where a specific soil-borne pathogen has been shown to affect pasture

growth adversely, there is also evidence that ill-defined biological constraints (which may include soil-borne pathogens) can also affect pasture production.

Table 1.1. Origin, growth pattern and major soil-borne pathogens affecting four pasture species in south-eastern Australia

Host species (common name)	Origin	Perenniality group	Identified pathogens
Trifolium subterraneum (subterranean clover)	Western Europe, Mediterranean Basin (Barbetti et al. 1986)	Annual	• *Phytophthora clandestina* (Flett and Clarke 1996) • *Pythium irregulare*, *P. ultimum* (Flett and Clarke 1996) • *Fusarium culmorum*, *F. avenaceum*, *F. oxysporum* (McGee and Kellock 1974; Barbetti and MacNish 1978; Barbetti et al. 1986; Flett and Clarke 1996) • *Rhizoctonia* spp. (Barbetti and MacNish 1978; Murray and Davis 1996; Flett and Clarke 1996) • *Phoma* sp. (Rovira 1982)
Lolium perenne (perennial ryegrass)	Europe, Mediterranean Basin (Balfourier et al. 1998; Bolaric et al. 2005)	Perennial	• *Bipolaris sorokiniana* (imperfect/asexual stage)/*Cochliobolus sativus* (perfect/sexual stage) (root rot) (Clarke and Eagling 1994) • *Fusarium culmorum* (Clarke and Eagling 1994)
Phalaris aquatica (phalaris)	Southern Europe, north-west Africa, Mediterranean Basin (Watson et al. 2000)	Perennial	• None identified (Watson et al. 2000)
Austrodanthonia fulva (wallaby grass)	Australia	Perennial	• None identified (New South Wales Department of Primary Industries 2000)

Table 1.2. Selected literature on soil biology and pasture production

Aim	Host species	Microorganism(s)	Treatments/details	Findings	Was there a specific soil biological constraint to pasture production?	Reference
Determined causes for pasture decline	*Trifolium subterraneum*	*Phytophthora clandestina*	• Multiple treatments including lime, P, Mo, Mg, co-planting with *Lolium rigidum* and fungicide (metalaxyl) in 4 field sites with poor pasture growth. • Measured herbage yield 3-4 times/year, plant density, root rot severity.	• *L. rigidum/T. subterraneum* plots had higher herbage yields than *T. subterraneum* only plots. • Fungicide application increased *T. subterraneum* seedling survival, growth and seed set. • Positive effect of fungicide in autumn and winter; consistent with soil temperature and moisture on pathogenicity of *P. clandestina*. • No effect of liming on disease severity and plant density.	Yes, fungicide treated plots yielded more herbage and seed suggesting soil-borne fungi constrained production. In the presence of disease, lime did not improve root health or seedling survival.	Hochman et al. (1990)

Assessed 5 fungicide drenches[1] to control root rot	*Trifolium subterraneum*	*Fusarium*, *Phytophthora*, *Pythium*, *Rhizoctonia*	• Fungicide drenches applied to intact soil cores over two years. • Measured % plants surviving, disease severity on tap/lateral roots, shoot/root dry weight.	• Pythiaceous fungi were the primary cause of pre- and post-emergence root rot. • Generally, drenches decreased disease severity in both tap and lateral roots, suggesting that the same or similar pathogens were responsible in both tap- and lateral roots.	Yes, but depends on site. Efficiency of each fungicide differed between sites suggesting different pathogens or disease complexes were involved.	Barbetti et al. (1987)
Compared fungal populations in infected and healthy areas; monitored fungal population changes in relation to soil water	*Trifolium subterraneum*	*Pythium*, *Fusarium*, *Rhizoctonia*	• Field roots assessed for disease severity from 4 irrigated sites (2 infected/2 healthy) • Isolates collected and inoculated onto *T. subterraneum* plants in sterilised soil in glasshouse. • Isolates tested for pathogenicity on tap and lateral roots.	• Wetter sub-sites of each site had more severe root rot. • *Pythium* spp. were major pathogens. • Rapid rise in *Pythium* spp. and overall decrease in *Fusarium* spp. following irrigation at both diseased and healthy sites. • Similar range of fungi isolated from visually healthy roots but with a lower frequency compared to diseased roots. • All fungi caused high levels of root rot in tap	Yes, high pathogenicity of fungi may explain poor stands in affected areas, caused by seedling death.	Barbetti and MacNish (1978)

[1] Benomyl, metalaxyl, iprodione, propamocarb, thiram

				and lateral roots causing decreased emergence, and shoot/root dry weights.		
Determined causes for low vigour (LV) areas in established pasture (*T. subterraneum* had declined to 3 % of pasture composition 2 years after establishment)	*Trifolium subterraneum*, *Lolium perenne*	*Codinaea fertilis*, *Fusarium* spp., deleterious bacteria, nematodes	• Field study on abundance of deleterious bacteria, fungi, nematodes in roots and rhizosphere soil. • Roots pieces from field placed on selective media. • Soil Pathogenicity Index (SPI)[2] determined for soils from LV and high vigour (HV) areas.	• Higher levels of *C. feritilis* and *Fusarium* spp. in LV compared to HV areas. • No relation between LV and total or fluorescent *Pseudomonas*, deleterious bacteria, topography, nutritional factors. • No difference in nematode numbers between LV and HV areas. • Seedling establishment higher in microwaved soil in both LV and HV areas. • No significant difference in SPI in LV and HV areas.	• Yes, fungal pathogens were higher in LV areas. However, cannot confirm association between *T. subterraneum* decline and high pathogen potential of soil due to insignificant differences in SPI between LV and HV •. areas.	Sarathchandra et al. (2000)

[2] $Soil Pathogenicity Index = 1 - \left(\frac{Plant DW untreated soil}{Plant DW microwaved soil} \right)$

Determined causes for decline in *Medicago* pastures	*Medicago* spp.	*Pythium*, *Fusarium*, *Phoma*, *Rhizoctonia*, nematodes	• Pathogens isolated from field soil and tested for pathogenicity in pasteurised soil mix	• 32 isolates of 3836 identified were tested for pathogenicity in glasshouse. • 56 % of these caused significant root damage. • Pathogenic fungi were main cause. • Nematodes were not related to these symptoms.	Yes, fungi may be cause for medic decline, but difficult to extrapolate glasshouse pathogenicity tests to field observations.	You et al. (2000)
Determined occurrence of rhizobacteria suppressive to root fungal pathogens	*Trifolium subterraneum*, *Lolium perenne*	*Pythium*, *Rhizoctonia*, cyanogenic *Pseudomonas*	• Bacteria isolated from roots of *T. subterraneum*, *L. rigidium*. • *In vitro* study of fungal suppression by isolated bacteria. • Effect of isolated bacteria on seedling root growth.	• Suppressive and pathogenic bacteria isolated from roots. • 32-57 % of isolated bacteria could inhibit at one fungus. • 0-40 % of isolated bacteria inhibited seedling root growth. • High incidence of cyanogenic fluorescent *Pseudomonas* was found in all sites. • No difference in fungal antagonism, root growth inhibition and cyanogenesis in bacteria isolated from *T. subterraneum* and *L. rigidium* rhizoplanes.	Yes, there is a natural occurrence of fungal suppressive bacteria in pastures. However, need to determine if cyanogenic *Pseudomonas* are causal organisms for fungal suppression or detrimental to root growth.	Brown et al. (1994)

Tested hypothesis that root exudation promoted by grazing increase plant growth by stimulating rhizosphere processes	*Poa pratensis* (grass native to north America)	Rhizosphere microbes	• Clipped plants to 6 cm height in sand-perlite mix. • Measured rhizosphere C, microbial biomass, N availability and plant N.	• Defoliation stimulated microbe and root respiration, root exudation, microbial C, soil N. • Clipped plants had higher N uptake, shoot N and photosynthesis.	Soil biological constraint was not tested. However, study showed possible mechanisms for grazing tolerance and recovery, and its possible effect on soil microbial activity. However, clipped plants in glasshouses oversimplify complexities of grazing systems.	Hamilton and Frank (2001)
Investigated cultivation on root rot levels	*Trifolium subterraneum*	*Pythium irregulare*	• Cultivated three continuous 3-year pastures with disc harrows at different times of year over 2 years.	• Cultivation decreased levels of tap and lateral root rot. • Disease decrease did not persist to 2nd year of treatment. • Cultivation before onset of autumn rains followed by reseeding gave best disease decrease.	Yes, cultivation provided short-term control of disease.	Barbetti and MacNish (1984)
Compared amoeba numbers in soils under pasture-pasture-wheat (PPW) rotation or continuous wheat (PPW	Not mentioned	*Gaeumannomyces graminis* var. *tritici* (take-all; TA), mycophagous amoeba	Measured numbers of mycophagous and other amoeba in PPW and wheat soil.	• Higher numbers of mycophagous and other amoeba in PPW compared wheat soil • Mycophagous amoeba are probably responsible for TA suppression in PPW soil. • Soil texture and water holding capacity were	Yes, mycophagous amoeba may be antagonistic TA pathogen.	Charkraborty and Warcup (1984)

known to be suppressive to TA)				not major influences on amoeba population. • Possible that more constantly moist soil of PPW supported more food organisms (bacteria) for amoeba.		
Determined growth responses to suppressive effects of AMF	10 temperate pasture species including *Trifolium subterraneum*, *Lolium rigidum*, *Phalaris aquatica*	Arbuscular mycorrhizal fungi (AMF)	1. **a.** Pasteurised soil was combined with γ-irradiated soil at 10 and 50%. P added at 3 levels; measured *T. subterraneum* shoot DW, mycorrhizal colonisation. **b.** Field soil was added to pasteurised soil at 10 and 50 %. P added at 3 levels; measured *T. subterraneum* shoot DW, mycorrhizal colonisation. 2. AMF sown with host species with no added P and 60 % P.	1. **a.** Pasteurisation did not eliminate AMF. **b.** AMF colonisation suppressed growth at high P. **c.** AMF colonisation decreased with increasing P. **d.** Complete and partial pasteurisation improved growth at high P, but this was not due to AMF(may be nutrient release). 2. **a.** *T. subterraneum* showed yield response with AMF colonisation at low P. **b.** AMF had no effect on shoot growth of grasses. **c.** AMF colonisation of all species decreased with P application.	No, but some other non-specific soil biological constraint was detected by partial and complete soil sterilisation. However, need to be careful when drawing conclusions from sterilisation/pasteurisation treatments as these treatments may alter other soil properties.	Hill (2003)

Determined effects of fungicide soil drenches on biomass production	*Trifolium subterraneum*	Fungal root pathogens	1. Fungicide drenches applied before sowing and after emergence. 2. Measure total herbage yield production	Fungicide drenching improved herbage production by 24-95% compared to undrenched control soil.	Fungal pathogens eliminated and plant production improved, but effect of drenches on other fungal species and overall soil biology is unknown.	Greenlagh and Clarke (1985)
Determined effect of fungicide soil drenches on biomass production	*Trifolium pratense*	Fungal root pathogens; *Fusarium* spp.	1. Fungicide drench applied to 2nd year pasture 2. Biomass production measured over 2 years	Fungicide drench treatment improved biomass production by 69% over 2 years after drenching	Fungal pathogens eliminated and plant production improved, but effect of drenches on other fungal species and overall soil biology is unknown.	Leath et al. (1973)

Non-specific soil biological "constraints"

Non-specific soil constraints are those where a soil biological property limiting plant growth is unidentified. Soil treatments such as pasteurisation or fumigation, or the application of soil drenches, may improve plant growth by altering the soil biology. However, such alteration is broad and non-specific and it is difficult to know what types of microorganisms, and what physical and chemical properties of the soil are affected. Fungicide soil drenches decreased root rot severity caused by pythiaceous fungi in *T. subterraneum* (Barbetti et al. 1987). As several fungal species may be responsible for root rot in *T. subterraneum* (Barbetti and MacNish 1978; Barbetti et al. 1986; Flett and Clarke 1996), the "sledgehammer" approach of drenching soil with a fungicide makes it to difficult to determine which beneficial and deleterious fungal species are also affected. Soil drenching also improved biomass production in *T. pratense* (red clover) (Leath et al. 1973) and *T. subterraneum* (Greenhalgh and Clarke 1985). Although fungicide drenches can be specific to target fungal species, their effect on the population of other soil microorganisms is unclear. What are the long- and short-term effects of removing the fungal population from a soil with drenching? Will bacteria dominate? If so, what types of bacteria and when will fungi re-colonise the soil?

Soil fumigation with methyl bromide (Sumner et al. 1997), and steam pasteurisation also target non-specific soil biological properties. These processes may also alter soil nutrient availability complicating the interpretation of results. Although complete and partial sterilisation of soil containing high levels of phosphorus improved growth of *T. subterraneum* compared to unsterilised soil (Hill 2003), it is difficult to rule out the possibility that improved plant growth may be due to nutrients released into the soil by lysed cells, rather than the elimination of deleterious soil microorganisms. Caution is needed when drawing conclusions from soil sterilisation treatments, as they may falsely indicate a soil biological constraint. On the other hand, it does not necessarily mean there is not a soil biological constraint. These experiments simply illustrate the complexity of soil microbiological processes on plant growth and such experiments need to be carefully controlled in order to interpret the myriad of possibilities after such drastic modifications to the soil biology.

1.1.3 Cultural practices that affect pasture soil biology

In contrast to fields that are cropped annually, management of pastures in southern Australia generally involves substantially less intervention in terms of soil disturbance and botanical species control. Although a range of management practices have been shown to affect the activity of deleterious and beneficial soil microorganisms in different soil types in cropping systems, this review will primarily consider agronomic practices that are applicable to the management of pasture systems: (1) grazing and defoliation; (2) soil disturbance; (3) soil amendment by fertilisers and liming; and (4) plant species resulting in differences in belowground root composition. These practices either impact directly on soil microorganisms, or alter soil physical and chemical properties, microclimate and energy sources through changes in root and shoot growth (Gupta and Ryder 2003). Some may have application in the management and control of disease, such as manipulating rhizosphere pH to control TA through the form of N fertiliser used.

Grazing and defoliation

Grazing is perhaps the most unique practice imposed onto pastures (although some annual crops such as winter wheat are also grazed). Research on defoliation of pasture plants has revealed root

physiological responses that include root exudation resulting in increased carbon and nitrogen flow in the rhizosphere. Bardgett *et al*. (1998) reviewed the effect of herbivory on root metabolism and the belowground activity of microorganisms. Grazing increased root respiration and exudation, efflux of organic acids, NO_3^- and NH_4^+ from roots, translocation of labile C to roots, root ^{14}C transfer and C allocation to roots. Grazing also increased nematode and collembolan numbers, rhizosphere microbial biomass and activity and numbers of *Pseudomonas* spp. (Bardgett et al. 1998). A hypothetical model was proposed on the effects of grazing on decomposition pathways in soils, where a grazed pasture ("disturbed" system) favoured a fast pathway dominated by labile root substrates (eg. C) and a dominance of bacterial species, while an ungrazed pasture ("undisturbed" system) favours a slow pathway dominated by more resistant root substrates (eg. root debris) and a dominance of fungal species (Bardgett et al. 1998). This is somewhat consistent with a previous proposal on the effect of cultivation and tillage on composition of the soil food web, where disturbance to the system favours bacteria, and no-till systems favour fungi (Gupta 1994).

Increased root carbon exudation occurred after clipping of *Poa pratensis* shoots when grown in a sand-perlite mixture inoculated with sieved soil from *P*. *pratensis* roots (Hamilton and Frank 2001). Increased root exudation after clipping was related to observed increases in microbial activity, nitrogen uptake by plants, and photosynthesis (Hamilton and Frank 2001). The greater "loading" of substrates (Welbaum et al. 2004) in the rhizosphere of clipped plants probably promoted higher numbers of microorganisms in the rhizosphere soil of clipped plants compared to of unclipped plants (Hamilton and Frank 2001). Organic C was released from *L*. *perenne* roots for a period of 3-5 days following defoliation (Paterson and Sim 1999). Although the effect on microbes was not measured, increased release of C from roots following defoliation would have a similar effect on promoting microbial activity. However, the short period of time (several days or weeks) over which these processes are measured in glasshouse studies with manually clipped plants do not account for the complexity of real grazing situations where the effects of animal trampling causing soil compaction, and animal excreta, affect the growth of both soil microorganisms and plants. Furthermore, preferential grazing of certain pasture species by herbivores may also affect overall rhizosphere properties and microbial activity. Indeed, a longer-term study found a negative effect of cutting field-grown *P. aquatica* swards on aboveground biomass production over a 20-month period (Volder et al. 2004), which may affect root growth and consequently the population of soil-borne microorganisms associated with those roots. The studies of Hamilton & Frank (2001) and Paterson & Sim (1999) assume that increased microbial activity after defoliation would have a positive effect on plant growth. Deleterious soil microorganisms could equally benefit from increased carbon flow in the rhizosphere, thus creating negative effects on plant growth. There appears to be no literature investigating the possibility of increased C flux in the rhizosphere and its effect on deleterious microorganisms and, importantly, what this means for pasture production.

Soil disturbance

Cultivation, tillage and other forms of soil disturbance affect the composition of soil microbial populations. Soil disturbance generally results in a predominance of bacteria in the food web; organisms with short generation times, high metabolic rates and mobility are favoured (Gupta 1994) as these characteristics allow rapid re-colonisation of the soil. In contrast, no-till systems favour fungi as it allows hyphal extension over soil residue (Gupta 1994; Gupta and Ryder 2003). This is evidenced by the disruption of the hyphal network of AMF with soil disturbance (Rovira 1982). In contrast, AMF populations may also decline following a period of fallow resulting in decreased AMF infection (Rovira 1982). In the northern wheat areas of NSW the incidence of infection by the fungal

pathogen *Bipolaris sorokiniana* which causes root rot of wheat, was greater after a long (18-month) fallow than after a shorter (6-month) fallow. This was considered to be due to absence of AMF host plants during the fallow, probably leading to a decline in numbers of *Glomus mosseae* propagules. Although a significant inverse relationship between root rot infection and root colonisation with AMF (mainly *G. mosseae*) was observed (Thompson & Wildermuth 1989), the mechanism by which *G. mosseae* may affect *B. sorokiniana* infection is not known.

Cultivation decreased the severity of root rot caused by *P. irregulare* in tap roots more than lateral roots in a continuous (3-year) *T. subterraneum* pasture in Western Australia (Barbetti and MacNish 1984). Cultivation may have decreased the impedance of tap roots penetrating the soil thus decreasing the accumulation of root rot organisms on these roots (Watt et al. 2003). Breaking up fungal hyphae and spores through cultivation or tillage can also decrease disease severity. However, cultivation could not provide long-term control of root rot as its effect does not persist beyond two years (Barbetti and MacNish 1978). This is probably due to the endemic and widespread occurrence of *P. irregulare* on *T. subterraneum* roots and in soils of south-western Australia.

Liming and fertilisers

In most production systems, fertilisers and other soil amendments such as liming are usually applied to correct plant nutrition problems and to increase production, rather than for the purpose of influencing soil microorganisms (Welbaum et al. 2004). The effect of fertilisers on soil microorganisms are probably related to improvements in root growth and exudation of substrates (Rovira 1982), which then provide conditions that are favourable for colonisation . Liming to ameliorate low soil pH generally increases microbial activity in soil. In the case of rhizobia, survival and growth of rhizobia, and nodulation of host legumes are sensitive to low pH (Coventry and Evans 1989). Because of the economic importance of take-all disease, there has also been extensive research on the effect of soil pH on the TA fungus.

Severity of TA is closely related to soil rhizosphere pH (Smiley and Cook 1973). Disease severity increases with liming (Rovira 1982), and soil acidification has been associated with increased growth of species of *Trichoderma*, a fungi that is antagonistic to TA (Duffy et al. 1997). Plant uptake of inorganic nitrogenous fertilisers (either as NH_4^+; rhizosphere more acidic, and NO_3^-; rhizosphere more alkaline) (Marschner and Römheld 1983) influence rhizosphere pH (Jaillard et al. 2003) and this can affect bacterial populations in the rhizosphere (Cheng et al. 2004; Cheng et al. 2005). Application of NH_4^+ fertilisers resulted in a decreased incidence of TA, which may be explained by rhizosphere acidification which made conditions unfavourable for the growth of TA and more favourable to *Trichoderma*. Fluorescent pseudomonads inhibitory to TA (Rovira 1982), and *Trichoderma* spp. suppressive to TA (MacNish 1988; Simon et al. 1988; Duffy et al. 1997) were also higher in the rhizosphere of wheat fertilised with NH_4^+.

Other soil-borne pathogens, such as root rot pathogens, respond differently to soil pH. For example, high levels of soil NO_3^- and possibly through its associated effect in increasing soil pH, encouraged the occurrence of *Pythium* and *F. oxysporum* in *Medicago* pastures (You et al. 2000). Although organisms antagonistic to *Pythium* and *F. oxysporum* were not specifically investigated, it is possible that their populations may have declined with increasing soil pH, favouring disease development. High levels of soil sulphur were associated with decreased frequency of *F. oxysporum* possibly through the acidifying effect of elemental sulphur on soil (You et al. 2000), although specific pH

values as a result of sulphur application were not reported. However, liming appeared to have no effect on the severity of root rot or plant density in *T. subterraneum* (Hochman et al. 1990).

Species composition

In cropping systems, rotations between different species in the same volume of soil offer disease breaks and improve plant nutrition (Gupta 1994). There is also evidence that soil under a pasture-pasture-wheat rotation contained higher numbers of mycophagous amoeba (suppressive to TA disease) than soil under continuous wheat cultivation (Charkraborty and Warcup 1984). Although "rotations" are not particularly relevant to permanent pastures, knowledge from single species grown in rotation with another species (e.g. wheat-subterranean clover rotation) provides some insight into the population dynamics of microorganisms in the rhizosphere of different host species and the mechanisms by which a plant may achieve benefits from other plant species. For example, wheat grown after legumes can capture nitrogen from the dead roots of legumes (McNeill et al., 2001; Khan et al., 2003) and phosphorus (Nuruzzaman et al., 2005). At least half of wheat roots are in contact with dead roots from a previous crop (Watt et al., 2005), and it is likely that nutrients are acquired at points of direct root-root contact.

Pastures are generally composed of different plant species growing together where roots of one species contact and interact with the roots of other species, as well as with soil microorganisms. The belowground species composition of pastures is vastly more complex than that of a crop grown in monoculture. Minimal soil disturbance under pastures compared to crops means that successive generations of roots will occupy a volume of soil, leading to a complex succession of microorganisms associated with those roots. Root-root interactions of the type now studied in crops are likely to be many times more important in permanent pastures.

Uninterrupted periods of plant growth allow soil microorganisms to respond to the presence of the plant species that are present (Sarathchandra et al. 1997). Indeed, plants and microorganisms may adapt to the presence of each other., Plant monocultures can lead to (i) build-up of disease and/or (ii) development of disease suppression. "Replant" diseases following continuous cultivation of one species which results in poor root health due to a build up in deleterious microorganisms, have been reported to decrease the productivity of sugar cane (Pankhurst et al. 2005), apple (Magarey 1999), *Medicago* pastures (You et al. 2000) and various other crops. Conversely, soil previously sown with wheat can develop suppressiveness to the fungal pathogen causing TA (Shipton et al. 1973). Permanent pastures are similar to monoculture crops in that there is continual production of above- and belowground biomass in a given volume soil, but differ in that multiple plant species are grown together, soil disturbance is less likely and the period over which plants co-exist is long. It is unknown if "replant" diseases or the development of suppressive soils occur in permanent pastures. Can the coexistence of several species in a pasture promote microbial diversity such that a "disease break" as those used in crop rotations can operate through a "balance" of beneficial and deleterious microorganisms? Can combinations of host species be manipulated to control deleterious microorganisms while favouring beneficial ones?

In situations where host and environmental conditions favour the survival and growth of soil-borne pathogens, disease is a clearly defined soil biological constraint to pasture production. Where disease symptoms are not as obvious, it is often difficult to identify a soil biological constraint to production. Negative soil biology influences may not always be noted because productivity of pastures is rarely measured directly and because there is considerable opportunity for

"compensatory" growth by companion plant species which may mask the influence of poor performance due to soil microorganisms. In the rhizosphere, physical, chemical and botanical factors can also affect microbial association with plant roots and expression of plant disease. For instance, similar root rot fungi were isolated from both healthy and diseased roots of *T. subterraneum* (Barbetti and MacNish 1978), and from low and high vigour areas of *T. subterraneum* (Sarathchandra et al. 2000). Similarly, cyanogenic *Pseudomonas* spp. that may be antagonistic to root rot pathogens were found in all sites from a survey of *T. subterraneum* and *L. perenne* pastures (Brown et al. 1994). These studies indicate that despite the presence in the rhizosphere of pathogenic microorganisms, plant disease may not always be manifest.

What keeps deleterious organisms under control given the apparent ubiquitous presence of both beneficial and deleterious microorganisms? "Community feedback" theory and experiments designed to modify microbial populations suggest that microorganisms associated with the roots of one host species growing in a mixed sward may sometimes affect the growth of another species more than the host plant (Bever 2003). Grass-legume mixtures can influence rhizosphere processes of each species synergistically, but the potential interactions may differ between different host species (Warembourg et al. 2004).

1.1.4 Disease suppression in soils

Despite the co-existence of deleterious and beneficial microorganisms in most soils, some soils are "suppressive" for plant disease because they limit the survival or growth of pathogens. Disease-suppressive soils can be defined as being "general" (decrease in fungal or bacterial attack) or "specific" (decrease in attack by one specific pathogen) (Garbeva et al. 2004). Further classification of suppressive soils relates to the longevity of the suppressive property: "long-standing suppression" where an ill-defined biological condition may decrease disease even in the absence of plants, and "induced suppression" where disease suppression is initiated and sustained by crop monoculture (Garbeva et al. 2004). Shipton et al. (1973) showed that the TA fungus was present in soil previously sown with wheat (field soil) and in soil recently cleared of native vegetation that has never been sown with wheat (native soil). However, more severe disease occurred in wheat growing in the native soil than wheat growing in field soil. Antagonists in the field soil may have suppressed disease (Rovira 1982). An interesting adjunct to the study by Shipton et al. (1973) was that addition of 1% of antagonistic field soil to methyl bromide-fumigated field soil restored antagonistic properties in fumigated soil. However, addition of up to 10 % antagonistic soil to native soil provided little or no antagonism. Therefore, it appears that the native soil may contain certain microorganisms that are antagonistic to TA-antagonists in the field soil. Severity of TA in wheat also varies with N source as a consequence of altered rhizosphere pH, because TA was controlled to a greater extent by antagonistic soil microorganisms in the rhizosphere at pH greater than 5.0 (Smiley and Cook 1973). These studies from crops highlight that disease incidence can be affected by the population of beneficial and deleterious microorganisms.

Brown et al. (1994) similarly reported that "natural biological control" can exist between plants and deleterious microorganisms in permanent pastures. It was found that bacteria isolated from the roots of *T. subterraneum* and *L. perenne* could inhibit the growth of at least one of four strains of *Pythium* spp, *P. irregulare* or *R. solani* (Brown et al. 1994). A high incidence of cyanogenic fluorescent *Pseudomonas* spp. was observed in these bacterial isolates. It is evident both beneficial and deleterious microorganisms coexist in the rhizosphere of crop and pasture plants, and that the

"balance" between these can affect disease incidence. Research with crop plants has extensively identified soil biological constraints on crop establishment, growth and yield. However, similar knowledge on the constraints imposed by soil biology on pasture production is lacking. The complexity of pasture systems where the roots of different species cohabit the same volume of soil, interact with one another and with their surrounding microflora makes it difficult to predict soil biological responses by extrapolating from monoculture crops. Whilst positive soil community feedback, where populations of beneficial and pathogenic microorganisms coexist, may apply to monoculture cropping systems, there is accumulating evidence of negative soil community feedback in agricultural, unmanaged, sand-dune and grassland communities (Bever 2003).

Negative soil community feedback in plant-microbe interactions has been described as a "diversifying force" in plant communities, while positive community feedback is a "homogenising force" (Reynolds et al. 2003). Negative community feedback may be especially relevant to pastures where several species grow together, and the presence of microorganisms on the roots of one plant species may affect the growth of another species. By assessing the benefits of AMF colonisation on the growth of different hosts, Bever (2002) showed that growth of *Plantago lanceolata* (a herb) was best promoted by mycorrhizal fungi that accumulated with another plant species *Panicum sphaerocarpon* (a grass). This is despite mycorrhizae growing better with *P. lanceolata*, but being a poor growth promoter of this species. Although the mutualistic benefit to *P. lanceolata* is less, the resulting community dynamic between plant species and mycorrhizae allowed the coexistence of two competing plant species. This study used an artificially created AMF community on only two species and its significance to wider plant communities with multiple host species and soil microorganisms needs to be verified. However, it does illustrate the interaction of different microbial species with plant roots. In pastures containing more than one species, a more diverse microbial community may be favoured. In contrast, the dominance of one plant species (as in a monoculture crop) would direct the composition of microorganisms towards those that are favoured by that species (Bever 2003). Soil microbial diversity is conventionally seen as an indicator of soil health and quality (Garbeva et al. 2004). Furthermore, a soil "rich" in microbiota is often considered to be important for reducing the severity of attack by a range of soil-borne plant pathogens (Rovira 1982). However, whether microbial diversity is indeed critical for the long-term integrity and sustainability of soil ecosystems has been questioned (Welbaum et al. 2004). Others have proposed that perhaps functional characteristics of the population is equally, if not more important, than microbial diversity (Bardgett and Shine 1999).

The "balance" of soil-borne microorganisms under pasture production is governed by the presence of different host species growing together, the root-root interaction between these species, the root-microbe interactions within and between host species, and community feedback in both plants and microorganisms. This "balance" may allow different plant species in a pasture to survive, persist or dominate at different times to create a dynamic system that keeps disease under control, thus enabling the pasture to exist. Whether or not the "balance" is actually important in driving and maintaining pasture productivity is unclear. If and how this "balance" may be manipulated to improve pasture production is also unclear. What is clear is that both plant and microbial mechanisms can influence the population of microorganisms in the rhizosphere.

1.1.5 Mechanisms affecting the "balance" of microorganisms in the rhizosphere

Plant mechanisms

Different species in mixed pasture communities compete for light and space aboveground, and water and nutrients, belowground. When nutrients are deficient for plant growth, their availability will be influenced by the soil microbial community, whose growth in turn depends on C sources. Carbon in soil comes from plant roots through exudation, and the decomposition of root tissues and incorporated organic matter. Microbial biomass and microbial activity are generally much greater in the rhizosphere than in the bulk soil because of the high availability of substrates from the root (Brimecombe et al. 2001). Under non-sterile conditions, up to 30% of plant photosynthate may be lost through the roots, resulting in a continual and readily usable source of organic substrates for microbial growth (Bowen 1991). Populations of microorganisms in the rhizosphere can reach between 10^{10} and 10^{12} cells g^{-1} rhizosphere soil (Foster 1988) and are largely supported by C and N released from roots. The microorganisms can influence the quality and quantity of root exudates. Furthermore, the rate of nutrient acquisition by plants depends on root architecture (spatial distribution, growth, proliferation, branching, fineness and root hairs) and interaction with neighbouring roots through competition, nutrient depletion or inhibitory compounds (Warembourg et al. 2004). Therefore, the presence of soil microorganisms and root morphology both affect nutrient availability and uptake by roots.

Microbial access to substrates in the rhizosphere depends on organisms developing a competitive advantage over other microorganisms, for example, by evolving specificity with a host plant. However, soil physical and chemical properties of the rhizosphere will also affect the availability of these substrates to microorganisms. Furthermore, the combination of plant species growing together affects nutrition. Although much of the research comes from field crops grown in mixed culture, it has particular relevance to pastures where different species grow together.

Different species in mixed-culture

Studies on the effect of different plant species grown together on microbial communities in the rhizosphere are scarce. In a study using a *Bromus madritensis–Trifolium angustifolium* (grass–clover) mix, Warembourg et al. (2004) showed that the two species influenced each other's rhizosphere activity. *T. angustifolium* was more affected than *B. madritensis*. In the first weeks of growth before the onset of N_2-fixation, roots of *T. angustifolium* exuded more C into the rhizosphere in the presence of *B. madritensis* than in monoculture, but the greater root exudation by *T. angustifolium* seemed to benefit only *B. madritensis* (Warembourg et al. 2004). Similarly, Gastine et al. (2003) showed that *T. angustifolium* produced 74% more aboveground biomass when grown in monoculture than in a mixture with *Lolium rigidum* (annual ryegrass) and *Centaurea solstitialis* (a forb). However, outcomes such as this are common and usually attributed primarily to competition for light and/or soil nutrients.

Roots of different species host distinct "microbial profiles" due to their differential production of root exudates (Grayston et al. 1998). Possible mechanisms by which soil microorganisms can enhance the nutrient status of host plants include biological N_2 fixation, increasing nutrient availability in the rhizosphere, enhancing other beneficial symbioses of the host, or a combination of these mechanisms (Vessey 2003). Although characteristics of the microbial population were not determined in the study by Warembourg et al. (2004), it is likely that the numbers, types and activity of microorganisms associated with the roots of *B. madritensis* were different to those associated with

T. angustifolium roots. Rhizosphere microorganisms can similarly restrict the availability of certain nutrients to plants. For example, field-grown plants of *Arachis hypogeae* (peanut) showed more iron-deficiency chlorosis symptoms when grown in monoculture compared to plants grown in mixture with *Z. mays* (Zuo et al. 2003). Increased abundance of Fe-siderophore producing bacteria probably competed for and decreased the availability of iron in the rhizosphere of monoculture-grown plants of *A. hypogeae* to those grown with *Z. mays* (Zuo et al. 2003). Chemical rhizosphere properties which affect nutrient availability are also altered by mixed- species culture. Mixed-culture of *T. aestivum* and *Lupinus albus* (white lupin) increased shoot growth and P uptake of *T. aestivum* without significantly affecting growth or P uptake of *L. albus* (Cu et al. 2005). When grown in mixed-culture, *L. albus* offset alkalinisation of the soil caused by *T. aestivum*, while *T. aestivum* offset acidification of the soil caused by *L. albus*. The effect of soil pH through proton efflux (Tang et al. 1997; McLay et al. 1997) and the exudation of citric acid from *L. albus* roots also affects the availability of soil P to both species (Cu et al. 2005).

Root exudates

Root exudates are considered as all substances produced by the root and released into the rhizosphere. Classes of organic compounds released in plant root exudates include carbohydrates (sugars, polysaccharides), amino acids, aromatic acids, fatty acids and enzymes. Water, gases (eg. O_2, CO_2), cell walls, root-cap cells, cell contents, mucilage and root debris are also released from roots (Brimecombe et al. 2001). Some of the important factors determining the relative and absolute amounts of root products released include plant species/genotype, age, growth nutrition and root architecture/morphology. Knowledge of the role of root exudates on pasture production is scarce, but the root exudation responses observed in crop species would also be relevant in pasture species.

Plant species and genotype

The type and amount of exudates released by roots vary between plant species and genotype, and probably relates to the adaptation of a species to a particular environment. For example, plants adapted to calcareous soils produce more oxalic and citric acids to solubilise P and Fe, respectively, compared to plants adapted to acid soils which exude smaller quantities of these acids (Ström et al. 1994). Research comparing the root exudates released by perennial and annual species is limited, although Brimecombe et al. (2001) suggested that perennials generally release more fixed C than annuals, which may be related to perennials investing more assimilates in root development in order to survive year round. The number of bacterial species isolated from the *T. repens* rhizosphere was higher than those isolated from *L. perenne*, *Agrostis capillaris* (bentgrass) and *T. aestivum* (Grayston et al. 1998). This is probably because the quality and quantity of root exudates produced by *T. repens* roots is more favourable for the growth and colonisation of bacteria compared to the other species. Populations of microorganisms in the rhizosphere of each species will be affected by root exudates produced by the host plant and those of neighbouring plants of the same or different species (Lawley et al. 1983). Also, processes such as allelopathy, where chemical retardants produced by the roots (or shoots) of one species may have a negative effect on another species (Wu et al. 2001), need to be considered.

Plant age and growth

Root exudation changes with plant age. Organic acids and fatty acids decreased in concentration from flowering to fruiting stages in *L. albus* and *L. luteus* (yellow lupin) (Lucas García et al. 2001). Total organic C in *Oryza sativa* (rice) root exudates was lowest at seedling stage, increased until flowering but decreased at maturity, exudation of organic acids substituted the exudation of sugars with increasing plant age (Aulakh et al. 2001). Changes in the quantity of certain compounds exuded probably correspond to differing requirements for these compounds during different stages of growth. For example, *L. albus* cluster roots, specialised to mobilise P through exudation of organic acids, release high amounts of citrate and other carboxylates (relative to other species, Veneklaas et al., 2003) at a specific stage of root development and biochemistry (Watt and Evans 1999). The bacterial and eukaryotic communities around cluster roots change with plant and cluster age. The type and quantity of root exudates is very likely a consequence of aging of different root tissues which release different compounds.

In a pasture, the quantity and quality of root exudates produced is also likely to differ depending on the age of plants. Of particular relevance is the growth of seedlings in an established pasture where root exudates produced by the roots of more mature plants may strongly influence the survival of younger plants. Also, the soil microbial "background" encountered by the seedling is important as both beneficial and detrimental microorganisms, and the balance between these, will affect the survival and growth of young plants. Despite the difficulty and complexity of experiments with soils, it would be instructive to compare the root exudates produced by soil-grown pasture species at different ages, and to determine their effect on microorganisms and the establishment of various plant species in soil.

Amounts of exudates released by roots are probably directly related to root growth. In *T. aestivum* plants almost no root exudation occurred when root growth was very slow, even though shoots were actively growing (Prikryl and Vancura 1980). A possible explanation for this is that exudation depends strongly on the physiological state of root tip cells, and that greatest exudation may occur from plants with growing root tips (Brimecombe et al. 2001). However, this may also depend on the type of exudate. For example, Jaeger et al. (1999) found the apical regions of grass roots released sucrose, while the more mature regions with branch roots, released the amino acid, tryptrophan. McCully and Canny (1985) showed that young and older maize roots released similar amounts of carbon, but the nature of the compounds differed.

From this it is assumed that in perennial pastures in south-eastern Australia, root exudation may be low in winter and summer months when root growth is slow, compared to autumn and spring months when root growth is increased. Such a temporal change in the pattern of root exudation throughout the year is likely to have significant effects on microbial populations in the rhizosphere.

Plant nutrition

Root exudation is important in plant nutrition in situations of both nutrient deficiency and toxicity. (Neumann and Römheld 2001). Roots of *L. albus* (Veneklaas et al. 2003) and *Cicer arietinum* (chickpea) (Veneklaas et al. 2003) released organic acids to enhance phosphorous uptake. Under Fe stress, the release of phytosiderophores increased Fe acquisition in *H. vulgare* (Takagi et al. 1984). For Al toxicity, extrusion of high concentrations of organic acids from the root tip by

T. aestivum roots chelated Al and thus limited the incorporation of Al into plant tissues (Christiansen-Weniger et al. 1992; Ryan et al. 2001).

Research into the role of root exudates and nutrition on pasture production is scarce. However with N nutrition at least, proton flux observed in crop plants in response to NH_4^+ or NO_3^- fertiliser also occurs in pasture species (Cheng et al. 2004), and may affect microbial numbers associated with the roots of *Medicago* spp. (Cheng et al. 2005).

Root architecture and morphology

The pattern of root growth is determined largely by the type of plant and its interaction with soil structure (Uren 2001). Properties of root morphology such as biomass, length, diameter, branching and the number of root tips affects the quantity of root exudates produced (Nielsen et al. 1994). Furthermore, these properties also affect the rate of nutrient acquisition by affecting the spatial distribution, growth, proliferation and branching of roots (Fitter 1987). Root hairs are particularly important for the production of substances that mediate many plant-microbe interactions, are important in anchoring the plant in the soil, and for the uptake of water and nutrients (Grierson et al. 2001; Bertin et al. 2003). Root hairs significantly affect the volume of soil explored by plant roots and thus the extent of contact between the root surface and soil microorganisms. As root hair production varies between different plant species, the volume of soil explored by roots will also differ between different plant species. Despite *Trifolium* spp. having a greater mean root diameter compared to grasses, roots of grass species such as *L. perenne*, *Dactylis glomerate* (cocksfoot) and *Phleum pratense* (timothy) explored approximately 10-times greater volume of soil than *Trifolium* spp. (Haynes 1980). Depending on species, the grasses achieved this by having longer, thinner roots, a higher percentage of roots with root hairs (95-97% for grasses; 57-68% for *Trifolium* spp.), and longer root hairs (0.51-0.77 mm for grasses; 0.20-0.23 mm for *Trifolium* spp.) (Haynes 1980).

Plants grown in mixed culture with other species develop different root morphology and physiology compared to plants grown in monoculture. For example, *A. hypogeae* grown in mixed culture with *Z. mays* produced more lateral roots, decreased root diameter, and greater branching and specific root length (root length/root weight) compared to plants grown in monoculture (Zuo et al. 2003). Greater numbers of lateral roots and root branches potentially create more root apices influencing numbers of microorganisms specifically associated with root tips (Jaeger et al. 1999). Reasons for the changes in root morphology are not clear, but may be related to differential nutrient depletion or to facilitate competition for nutrients. Root morphology is also influenced by the roots' growth medium. Groleau-Renaud et al. (1998) showed that changes in root morphology (as affected by mechanical impedance in hydroponic culture) was correlated to modified root exudation production in *Z. mays*. Increased mechanical impedance of the growth medium decreased root growth and increased root exudation (Boeuftremblay et al. 1995). This has implications for plants growing in soil, as would be the case for pastures where stock trampling may contribute to soil compaction.

Research on the role of root exudates in pasture production need to consider how the coexistence of different species and management situations will affect the root morphology and exudate production of individual species.

Microbial mechanisms

Microorganisms associated with plant roots can improve plant health by a number of mechanisms including suppressing disease, enhancing nutrient uptake, fixing atmospheric nitrogen and promoting plant growth. Just as the quality and quantity of root exudates affect the population of microorganisms on the root surface, the "balance" of beneficial and deleterious soil microorganisms will also affect plant growth. With the exception of disease complexes, most research on plant-microbe interactions involve only one host plant and one type of microorganism. However, interactions between different bacterial and fungal species can influence the growth and survival of other microorganisms as well as the host plant itself. Literature on the interaction between soil-borne microorganisms and possible mechanisms affecting plant growth is listed in Table 1.3. Specific microbial mechanisms that affect plant growth include competition for colonisation sites in the rhizosphere and the production of phytohormones and antagonistic metabolites.

Table 1.3. Selected literature on effect of soil-borne microbial interactions and possible mechanisms affecting plant growth

Microorganisms beneficial or deleterious to plant growth	Host species (common name)	Effect of microbial interaction	Possible mechanism(s)	Reference
Azospirillum brasilense, *A. lipoferum* (+bacteria)[3] *Bradyrhizobium japonicum* (+bacteria)	*Glycine max* (soybean)	*Azospirillum* spp. enhanced root growth and nodulation of *G. max* when co-inoculated with *B. japonicum*	Release of phytohormones by *Azospirillum* spp. increase root hair development and infection sites for *B. japonicum*	Molla et al. (2001)
Azospirillum brasilense (+bacteria); *Glomus mosseae*, *G. macrocarpum* (+fungi)	*Triticum aestivum* (wheat), *Zea mays* (maize)	Phytostimulatory effect of *A. brasilense* on root growth	Release of phytohormones did not affect root colonisation of *Glomus* spp.	Russo et al. (2005)
Pseudomonas fluorescens strains (+bacteria); *Pythium ultimum* (–fungus)	*Pisum sativum* (field pea)	*P. fluorescens* strains decreased root lesions, and root and soil populations of *P. ultimum*	Depending on *P. fluorescens* strain, *Pythium* suppression resulted from antifungal metabolites or greater colonisation of the rhizosphere	Naseby et al. (2001)

[3] + Beneficial bacteria or fungus with positive effect on plant growth
– Deleterious bacteria or fungus with negative effect on plant growth

Pseudomonas fluorescens strains, *Bacillus* spp., *Arthrobacter* spp., *Pantoea agglomerans*, *Erwinia rhapontici* (+bacteria); *Pythium* sp. (–fungus)	*Brassica rapa* (canola), *Carthamus tinctorius* (safflower), *Pisum sativum*, *Beta vulgaris* (sugar beet)	Seed treatment with bacteria decrease mycelial growth of *Pythium*. Effectiveness of control varied with bacterial strain and host species	Depending of bacterial species, *Pythium* suppression resulted from antifungal metabolites, greater colonisation of the rhizosphere or secretion of proteolytic enzymes	Bardin et al. (2003)
Pseudomonas fluorescens (+bacteria); *Glomus mosseae* (+fungus); *Rhizoctonia solani* (–fungus)	*Lycospersicon esculentum* (tomato)	Co-inoculation with *G. mosseae* and *P. fluorescens* decreased epiphytic and parasitic growth of *R. solani*, and increased root length and root tips of host plant	Antagonism of *R. solani* by *G. mosseae* and *P. fluorescens*, possibly at root tips which are most susceptible to *R. solani* infection	Berta et al. (2005)
Bradyrhizobium japonicum (+bacteria); *Glomus mosseae* (+fungus)	*Glycine max*	Roots nodulated with *B. japonicum* had enhanced colonisation by *G. mosseae*	Flavonoids released by roots in response to *B. japonicum* Nod factors also promote *G. mosseae* colonisation	Xie et al. (1995)
Rhizobium leguminosarum bv. *viciae* (+bacteria); *Pythium* sp. (–fungus)	*Pisum sativum*, *Beta vulgaris*	Seed treatment with *R. leguminosarum* increased seedling emergence compared to untreated control	Unknown	Bardin et al. (2004)
Glomus mosseae (+fungus); *Bipolaris sorokiniana* (–fungus)	Various, including *Phalaris aquatica* (phalaris) and *Lolium perenne* (perennial ryegrass)	Inverse relationship between *B. sorokiniana* infection and root colonisation by *G. mosseae*	Not determined, but possibly competition for colonisation sites in the rhizosphere	Thompson and Wildermuth (1989)

Pseudomonas putida (–bacteria); *Rhizobium leguminosarum* bv. *viciae* (+bacteria)	*Pisum sativum*	*P. putida* decreased nodule number and shoot N content of *P. sativum* when inoculated alone or with *R. leguminosarum* bv. *viceae*	*P. putida* highly colonised root hairs and significantly decreased root hair development, and nodule size	Berggren et al. (2005)

Competition for colonisation sites in the rhizosphere

The root surface presents a finite yet heterogeneous range of sites for soil microorganisms to colonise. Depending on growth rate and substrate requirements, microorganisms compete with one another for colonisation sites on the root. Among fungal species, Thompson and Wildermuth (1989) showed a significant inverse relationship between infection of root segments by the fungal pathogen *Bipolaris sorokiniana* ("common root rot" of wheat) and root colonisation with AMF, mainly *G. mosseae*, in a wide range of plant hosts. It appeared that root tissue colonised by *G. mosseae* becomes more resistant or less suitable for the growth of some root pathogens. For example, Newsham et al. (1995) demonstrated that colonisation by *Glomus* spp. protected *Vulpia ciliata* roots from root pathogenic fungi such as *F. oxysporum*. It is difficult to conclude whether the suppression of *B. sorokiniana* is a direct result of competition for colonisation sites between the fungal species *per se*, or due to the production of antagonistic metabolites by *Glomus* spp. that inhibited the growth of *B. sorokiniana*, although the latter was not in these studies. It may be possible that a combination of different mechanisms may be responsible for the suppression of *B. sorokiniana*. Colonisation of rhizosphere sites that would otherwise be occupied by a pathogen was demonstrated when a *Pseudomonas* strain antagonistic to the TA fungus was inoculated to field soil sown with *T. aestivum* (Gardner et al. 1998). In the inter-row space where *Pseudomonas* was not inoculated, roots of *T. aestivum* became infected with TA, while roots were uninfected in the furrows where *Pseudomonas* was inoculated (Gardner et al. 1998). This suggests that *Pseudomonas* may have occupied rhizosphere sites that would otherwise have been colonised by the TA fungus to cause disease in *T. aestivum*.

Occupation of rhizosphere sites may affect the populations of bacteria in the rhizosphere, resulting in altered plant growth and function. Berggren et al. (2005) examined the deleterious effect of a strain of *Pseudomonas putida* on the nodulation of *Pisum sativum* (field pea) with *Rhizobium leguminosarum* bv. *viceae*. Plants co-inoculated with *P. putida* and *R.l. viceae* had fewer root hairs, smaller nodules and lower shoot N content compared to plants inoculated only *R.l. viceae*. Using immunofluorescence microscopy, both *P. putida* and *R.l. viceae* colonised the root surface (Berggren et al. 2005), although the numbers of each bacterial species associated with the roots were not determined. Differential colonisation by microorganisms in the rhizosphere may be mediated by microbial processes such as production of antimicrobial compounds, quorum-sensing and molecular signalling between plants and microorganisms.

Results from numerous controlled environment studies suggest that some rhizosphere microorganisms can produce phytohormones that modify the morphology and growth pattern of roots to result in bigger roots, more branched roots, and/or roots with greater surface area (Vessey 2003). *Azospirillum* spp. stimulate root growth in different plant species (Molla et al. 2001). In

G. max, increased root hair development, and the regrowth of lateral roots provided infection sites for *B. japonicum* (Molla et al. 2001). However, the role of phytohormones produced by microorganisms on plant growth in the field has not been demonstrated. Knowledge on the role of phytohormones on plant growth comes from systems in which microorganisms are applied singly and in high numbers. In field conditions where soil and microbial factors are interacting in the rhizosphere, it is unclear how phytohormones affect plant growth. Similarly, differential behaviour (eg. degradation, decay) of bacterial quorum-sensing (Wang and Leadbetter 2005) and regulatory signals that affect fungal and bacterial associations with roots (Hirsch et al. 2003) under field and laboratory conditions may affect cellular communication between soil microorganisms and the consequent temporal and spatial patterns colonisation in the rhizosphere. In pastures, how these processes mediate microbial colonisation in the rhizosphere is further complicated by the presence of different plants species growing in mixture. The resulting "cocktail" of plant and microbial signals under a pasture in field conditions would definitely challenge any extrapolation of results obtained in laboratory studies.

1.1.6 Conclusions and knowledge gaps

Current knowledge of the interactions between roots and soil-borne microorganisms are based on studies done in controlled laboratory conditions. In situations where soil is used, it is often disturbed, amended through fumigation or pasteurisation, and plants and microorganisms of interest introduced as a single species. What is lacking is an understanding of these interactions in undisturbed field conditions where a multitude of different plants and microorganisms are present in a given volume of soil. Particularly relevant to pastures is the significance of mixed roots and rhizospheres on the "balance" between beneficial and deleterious soil microorganisms. Temporal and spatial distribution of roots from different pasture species needs to be examined to determine how this may affect the overall microbial "balance" in the pasture system. However, is the "balance" measurable in a given pasture, and what soil, plant and microbial indicators can be used to measure it? Environmental factors (eg. temperature, water) and pasture management practices (e.g. grazing) must also be considered. Simplification of an intricate system provides a "springboard" to unraveling the complexities of soil biological interactions under a pasture. However, the overall research approach needs to consider the complexity of the entire system under field conditions: above- and belowground production, different plants species growing in mixture, presence of both beneficial and deleterious microorganisms and their population dynamics, impact of grazing and trampling, and environmental factors such as water and nutrient availability. Only then can hypotheses such as an optimum "balance" of soil microorganisms that may keep pasture disease in check be investigated to better understand the longer term impacts of changes in the soil biology on permanent pasture productivity.

In understanding root–microbe interactions, research methodologies that reflect the complexities of the rhizosphere need to be developed, and existing techniques used to study these interactions in the rhizosphere may be adapted to more closely mimic real soil conditions, or even field conditions. For example, the design of rhizocylinders (Ortas et al. 2004) and rhizoboxes (Moritsuka et al. 2000; Casarin et al. 2003) used to culture plants for rhizosphere studies may need to be modified to cater for undisturbed rather than disturbed soil. Methods to collect root exudates from soil-grown plants also need to be developed. It is likely to be quite difficult to obtain sufficient amounts of exudates for analysis and bioassay with microorganisms. Other substances within soil solutions may also affect the potency of exudates collected from roots, especially if concentrations of exudates are low.

Combinations of selective culture methods, and molecular and microscope technologies are required to detect the presence and activity of rhizosphere microorganisms (Gamalero et al. 2003). Molecular techniques such as fluorescence *in situ* hybridisation (FISH) and fluorescence microscopy (Christensen et al. 1999) or confocal laser scanning microscopy (Lübeck et al. 2000) can be used to temporally and spatially quantify different microorganisms associated with roots from the field (Watt et al., 2006). Analysis of microbial communities using molecular-based techniques to assess diversity and to identify and isolate otherwise non-culturable microorganisms are also available (Allen and Banfield, 2005). However, significant challenges remain in the application of these technologies and their linkage to functional processes within soil and the rhizosphere.

2 Project Objectives

1. Discover interactions between pasture rhizosphere conditions and dominant soil organisms which affect pasture plant productivity, and collect data to elucidate potential mechanisms whereby rhizosphere–soil organism interactions can be influenced to enhance pasture plant performance.

2. Complete a comprehensive literature review of existing knowledge of plant root rhizosphere responses to external plant influences and interactions with soil organisms with special emphasis on pastures and soil borne diseases.

3. Evaluate and develop techniques to study rhizosphere exudates and microorganisms surrounding pasture plant roots in soil based systems.

4. Contribute to the development of rhizosphere based molecular assays of significant detrimental and beneficial organisms in pasture soils through collaboration with SARDI.

5. Evaluate pasture management options to influence rhizosphere and plant responses which reduce soil biological constraints to pasture productivity, and create a more beneficial rhizosphere.

3 Methodology

The project used a wide range of laboratory and glasshouse techniques (details are in the Results sections for each experiment undertaken).

4 Results and Discussion

4.1 Effects of soil biology, nutrients and defoliation on shoot and root growth of a pasture species in intact cores from the field, and characterisation of rhizosphere chemical and biological composition under the different treatments

Summary

Four pasture species were grown in undisturbed field soil from two permanent pasture locations at realistic autumn soil temperatures. Steam pasteurisation and methyl bromide fumigation treatments were used to perturb soil biology. Constraints to plant establishment and growth were observed. Soil temperature affected plant growth responses to soil microbial populations and this indicated that experiments need to be conducted at realistic field soil temperatures, rather than the warmer temperatures usually experienced in glasshouse studies. A strong interaction between species, soil type and soil treatment was evident. The effects of soil treatment differed depending on whether plants were germinating and establishing, or were in the subsequent phase of growth. In many cases, plant establishment was better in the untreated soil cores, whereas disturbing soil biology by pasteurisation or fumigation often reduced seedling survival and pasture basal cover. Despite early plant losses, pasteurisation improved growth by the remaining plants in 5 out of 8 cases. By contrast, fumigation had no further adverse or beneficial affects on plant growth in this phase. The results suggest that soil biology in these soils influenced the early stages of plant establishment differently to the influence it had on subsequent plant growth. The magnitude of the effects was species and soil dependent.

4.1.1 Glasshouse experiment with intact soil cores in temperature-controlled tanks

Introduction

Soil biology can constrain pasture growth in Australia. A literature review (section 1.1) showed that 21 of 23 studies found improved plant growth when soil organisms were disturbed, or when microbial populations were modified by soil treatment. Glasshouse studies using field soils have repeatedly shown improved growth of *Trifolium subterraneum* in disturbed soil that has been pasteurised or fumigated with methyl bromide (EC245 Milestone Report to Australian Wool Innovation, 1 Jun 2005). However, these studies used disturbed topsoil and elevated root temperatures. This experiment aimed to examine whether soil biological constraints on growth of four key pasture species occurred in undisturbed field soil (intact cores) when grown at soil temperatures that were realistic for autumn conditions in the field when root diseases are sometimes regarded as problematic.

Materials and methods

Experimental design: Intact soil cores were collected from two sites (Bookham, NSW; and Hall, ACT). In expt 1, soil cores were placed in root cooling tanks in a split-plot row–column design with five replications, with one replicate on each bench. Half the cores on each bench received complete nutrients, and cores within each +/–nutrient treatment were arranged in a 4×6 layout. Four pasture species (*T. subterraneum, Lolium perenne*, *Phalaris aquatica* and *Austrodanthonia fulva*) were sown in the cores. In expt 2, soil cores from Hall were placed on the glasshouse bench without cooling in a completely randomised block design with five replicates. *T. subterraneum* and *P. aquatica* were sown, and all bench-grown plants received complete nutrients.

Soil and site details: Intact soil cores were collected from two sites: (1) "Kia-Ora" (KO) in Bookham, NSW (148°34.782′E, 34°48.185′S); and (2) "Wallaroo 3" (W3) in Hall, ACT (149°02.547′E, 35°10.503′S). Cores were collected by driving a PVC tube (10cm diam., 20cm depth) into the ground with a jackhammer, extracted carefully so that soil in the tubes remained undisturbed (Fig. 4.1), and a plastic cap placed on the bottom of each tube.

Loose soil from the top 20cm from each site was also collected. Cores and loose soil were transported back to CSIRO's Black Mountain Laboratories, Canberra, where they were covered with a plastic sheet and stored in the dark at 10ºC for 2 weeks. Loose soil was air-dried in a glasshouse for 3 days, passed through a 2-cm sieve to remove coarse organic matter, and pasteurised by steaming at 65^{o}C for 3 h. This soil was used to cover seeds after sowing. The physical and chemical properties of the soils were analysed by Incitec Pivot Limited, Victoria, Australia (data not shown).

Fig. 4.1. Extracting intact field cores at Bookham, NSW. July, 2005.

Cores were pasteurised by steaming at 65^{o}C for 3 h, fumigated overnight with methyl bromide or left untreated. Immediately after treatment, cores were transferred to root cooling tanks installed in a glasshouse.

Temperature-controlled root chambers: Five tanks (700×2000×180 mm each) containing refrigerated water circulating around 54 water-jacketed sleeves were designed and constructed at CSIRO's Plant Industry Workshop, Canberra. Water temperature in the tanks was set at approximately 11ºC and at a depth of 140 mm (Fig. 4.2).

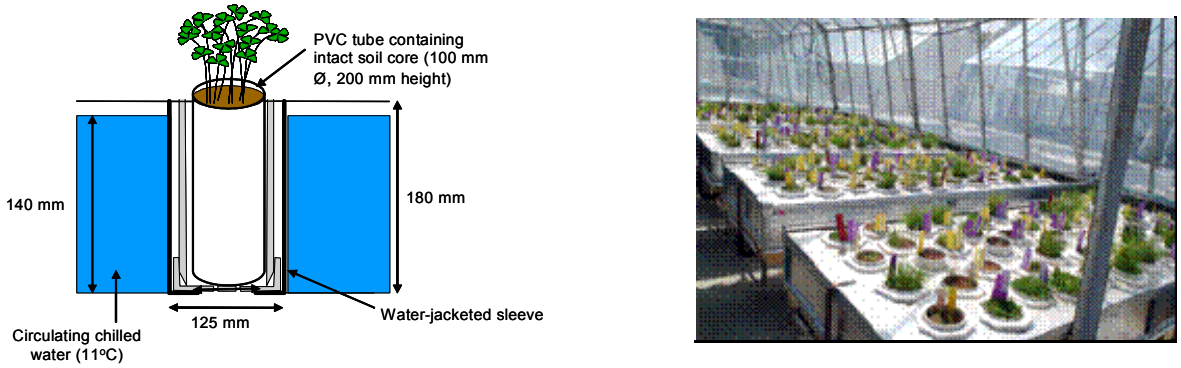

Fig. 4.2. *Left:* Design of water-jacketed sleeves containing intact soil cores to control root temperatures. *Right:* Intact soil cores inside tanks installed in a glasshouse.

Plants: Approximately 70 mg of viable seed of *T. subterraneum*, *L. perenne*, *P. aquatica* or *A. fulva* were sown in the cores. Complete nutrients were applied to half of the cores. Some cores of fumigated or untreated W3 soil were sown with *T. subterraneum* and *P. aquatica* and placed on a bench in the same glasshouse without root cooling. *T. subterraneum* was inoculated with a commercial inoculant containing *Rhizobium leguminosarum* bv. *trifolii* (strain WSM409). Soil moisture in the cores was maintained at 80% field capacity.

Soil temperature: Temperature data loggers (TinyTalk TK-0014, Gemini Data Loggers, UK) were placed in soil of randomly selected cores, water in cooling tanks and in the air of the glasshouse during a 53-day period (11-63 DAS).

Pasture basal cover & shoot growth: Pasture basal cover (PBC) provided a measurement of the germination, establishment and survival of plants. Percentage PBC of cores with uneven cover (<100%) was estimated by tracing area of shoot bases on the soil surface onto filter paper disks at harvest (64 DAS). An image of each disk was taken with a digital camera, and the %PBC was calculated using image analysis software (AnalySIS Pro, Version 3.2). Shoot dry weights were determined after drying shoots at 70^{o}C for 3 days.

Statistical analysis: All analyses were done using GenStat for Windows (8th edition, Lawes Agricultural Trust, Rothamsted Experimental Station, UK). In expt 1, ANOVA was used to determine significant differences in %PBC data. Shoot dry weight data were square root transformed to stabilise the residual variance. A linear mixed model was fitted to the transformed data using REML (residual maximum likelihood). Since the estimated variance components for the row, column and row.column effects were zero, the simpler split-plot ANOVA was used to test for the effects of species, soil type, soil treatment and nutrient application on shoot dry weight. In expt 2, ANOVA was used on untransformed shoot dry weight data.

Results and Discussion

Soil temperature: At 5 cm depth, temperatures of cores on the bench (12–23°C) fluctuated with air temperature in the glasshouse, while those in the cooling tanks fluctuated between 11–16°C (Table 4.1). Compared to cores on the bench, maximum temperature of cores at 10 cm depth in cooling tanks remained below glasshouse air temperature. Water temperature in cooling tanks fluctuated 11–11.6°C throughout the day. Relative to cores on the bench without cooling, the root cooling tanks were effective in keeping soil temperatures similar to those in the field during autumn in south-eastern Australia.

Table 4.1. Average temperature of air in glasshouse, water in root cooling tanks, and intact soil cores in cooling tanks and on the bench. Temperatures were recorded in a 53-day period (11–63 DAS).

Average temperature in 24-hour period (°C)	Air in glasshouse	Water in cooling tank	Soil cores in cooling tank		Soil cores on bench	
			5 cm	10 cm	5 cm	10 cm
Minimum	11.3	11.1	11.4	11.5	12.0	12.2
Maximum	22.4	11.6	16.1	14.1	23.2	23.4

Soil temperature & shoot growth: In the root cooling tanks, soil fumigation significantly decreased shoot growth in both *T. subterraneum* and *P. aquatica* (Table 4.2). However, there was no effect of fumigation on the growth of these species in cores on the bench. This suggested that soil temperature is important in the interaction between plants and soil microorganisms to affect shoot growth.

Table 4.2. Shoot dry weight (g/core) of *Trifolium subterraneum* and *Phalaris aquatica* in intact soil cores of Wallaroo 3 soil in root cooling tanks and on glasshouse bench without cooling. Differences were determined by LSD using either square root transformed data (cooling tanks) or untransformed data (bench). Values with different letters are significantly different (p<0.05). Comparisons are within cooling tanks or bench only.

	Cooling tanks		Bench	
	Untreated	Fumigated	Untreated	Fumigated
T. subterraneum	4.03[a]	2.38[b]	4.90[a]	5.29[a]
P. aquatica	3.27[a]	1.57[b]	4.75[a]	4.23[a]

Pasture basal cover: %PBC is a measure of the combined effects of seed germination and early seedling mortality. It was significantly lower in many cases after pasteurisation or fumigation treatments. %PBC was also lower in cores with added nutrients (Table 4.3). Nutrients were applied at higher rates than ideal and this was believed to have confounded the effects of the soil treatments and nutrient application on shoot dry weight (Table 4.4) . Therefore, data from the treatments to which nutrients were not applied are discussed below and are relied upon for interpreting the results of soil treatments.

Effect of soil treatment on %PBC varied with species and soil type: In most cases, there was no effect of pasteurisation or fumigation on PBC. However, pasteurisation decreased %PBC in *T. subterraneum* (W3 soil), *L. perenne* (W3 soil), *P. aquatica* (KO soil), while fumigation decreased % PBC in *L. perenne* (W3 soil) and *A. fulva* (KO soil) (Fig. 4.3).

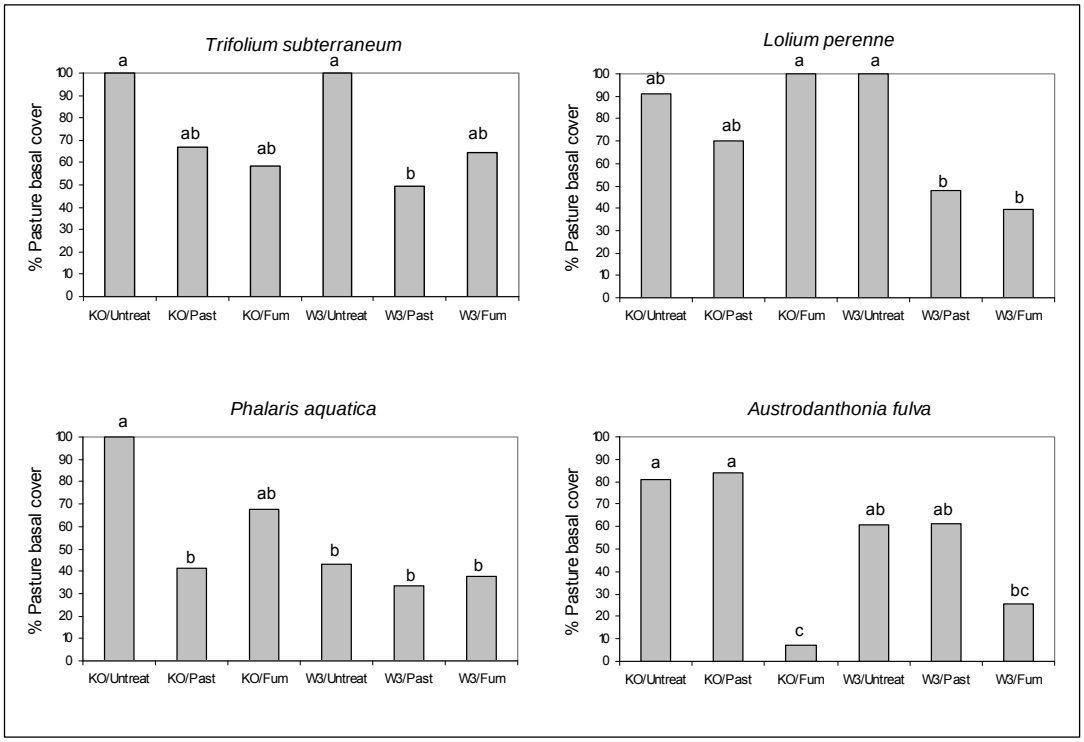

Figure 4.3. Mean % pasture basal cover of *Trifolium subterraneum*, *Lolium perenne*, *Phalaris aquatica* and *Austrodanthonia fulva* in intact cores of untreated (Untreat), pasteurised (Past) and fumigated (Fum) Wallaroo 3 (W3) and Kia-Ora (KO) soil. Differences between means were determined by LSD. Values followed by the same letter were not significantly different (P<0.05).

Shoot growth: Effect of soil treatment on shoot growth varied with species and soil type. In most cases, there was no effect of pasteurisation or fumigation on shoot growth. However, pasteurisation increased shoot growth in *L. perenne* (KO and W3 soil), *P. aquatica* (W3 soil) and *A. fulva* (KO and W3 soil) (Fig. 4.4).

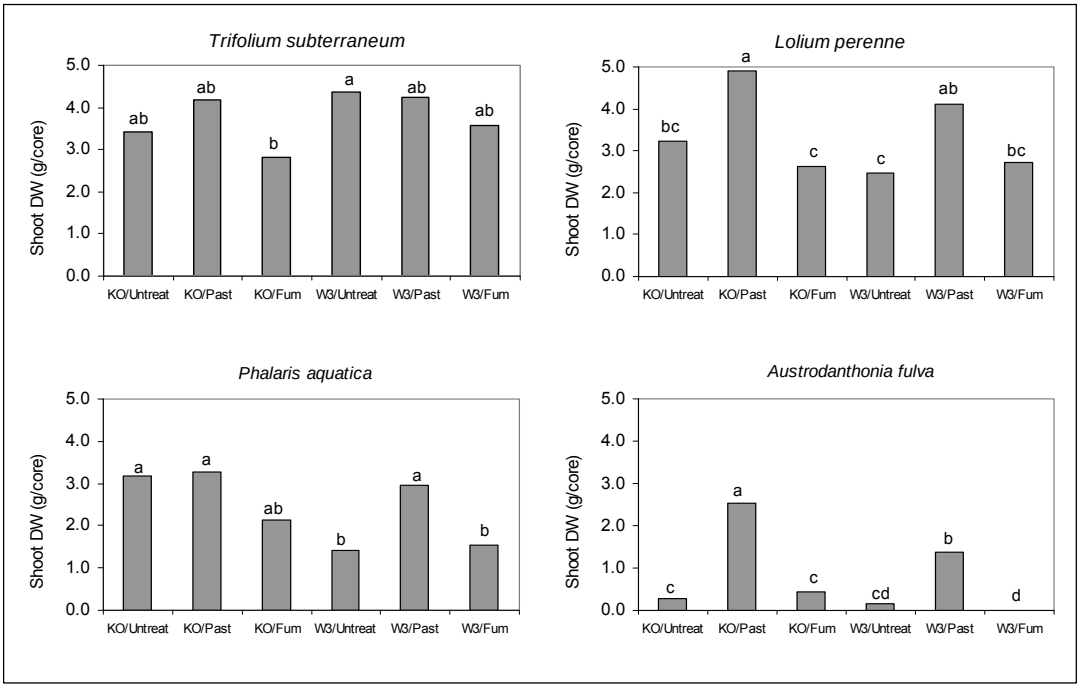

Fig. 4.4. Mean shoot growth (g/core) of *Trifolium subterraneum*, *Lolium perenne*, *Phalaris aquatica* and *Austrodanthonia fulva* in intact cores of untreated (Untreat), pasteurised (Past) and fumigated (Fum) Wallaroo 3 (W3) and Kia-Ora (KO) soil. Differences between means were determined by LSD on square root transformed values. Values followed by the same letter were not significantly different (P<0.05).

Plant response to soil biology: Species responded to pasteurisation or fumigation either by change in %PBC and/or shoot growth, or were unresponsive to the soil treatments (Table 4.5). Generally, pasteurisation or fumigation resulted in decreased %PBC, while pasteurisation increased shoot growth in 5 out of 8 cases. Fumigation did not alter shoot growth rate.

Table 4.3. Basal cover (% of pot area) of *Trifolium subterraneum*, *Lolium perenne*, *Phalaris aquatica* and *Austrodanthonia fulva* in untreated (Untreat), pasteurised (Past) and fumigated (Fum) intact cores of Wallaroo 3 (W3) and Kia-Ora (KO) soil (complete data set).

Nutrients	***T. subterraneum***					
	KO			**W3**		
	Untreat	**Past**	**Fum**	**Untreat**	**Past**	**Fum**
–	100	67	59	100	50	64
+	31	31	45	31	46	34
	L. perenne					
	KO			**W3**		
	Untreat	**Past**	**Fum**	**Untreat**	**Past**	**Fum**
–	91	70	100	100	48	39
+	75	38	41	90	26	52
	P. aquatica					
	KO			**W3**		
	Untreat	**Past**	**Fum**	**Untreat**	**Past**	**Fum**
–	100	41	67	43	33	38
+	81	47	40	85	51	7
	A. fulva					
	KO			**W3**		
	Untreat	**Past**	**Fum**	**Untreat**	**Past**	**Fum**
–	81	84	7	61	61	25
+	12	77	25	27	45	0

Table 4.4. Means of shoot mass (g/core) of *Trifolium subterraneum*, *Lolium perenne*, *Phalaris aquatica* and *Austrodanthonia fulva* in untreated (Untreat), pasteurised (Past) and fumigated (Fum) intact cores of Wallaroo 3 (W3) and Kia-Ora (KO) soil (complete data set).

Nutrients	***T. subterraneum***					
	KO			**W3**		
	Untreat	**Past**	**Fum**	**Untreat**	**Past**	**Fum**
–	3.43 bc	4.17 abc	2.83 c	4.36 ab	4.24 ab	3.59 bc
+	3.15 bc	1.36 d	0.47 e	5.34 a	1.39 d	3.63 bc
	L. perenne					
	KO			**W3**		
	Untreat	**Past**	**Fum**	**Untreat**	**Past**	**Fum**
–	3.24 ef	4.91 cd	2.64 f	2.48 f	4.12 de	2.71 f
+	7.73 ab	7.18 ab	5.43 cd	8.37 a	6.16 bc	7.02 ab
	P. aquatica					
	KO			**W3**		
	Untreat	**Past**	**Fum**	**Untreat**	**Past**	**Fum**
–	3.19 bcd	3.28 bcd	2.13 efg	1.41 g	2.95 cde	1.53 fg
+	4.80 a	2.95 cde	2.34 def	4.27 ab	3.94 abc	0.60 h
	A. fulva					
	KO			**W3**		
	Untreat	**Past**	**Fum**	**Untreat**	**Past**	**Fum**
–	0.28 cde	2.53 a	0.45 cd	0.14 de	1.38 b	0 f
+	0.69 c	2.19 ab	0.11 ef	0.50 c	2.46 a	0 f

* For each species, differences between means were determined by LSD using transformed data (square root transformation) and means not followed by the same letter are significantly different (P<0.05).

Table 4.5. Summary of the responses of *Trifolium subterraneum*, *Lolium perenne*, *Phalaris aquatica* and *Austrodanthonia fulva* to soil pasteurisation (Past) and fumigation (Fum) as measured by % pasture basal cover and shoot growth in intact cores of Wallaroo 3 (W3) and Kia-Ora (KO) soils relative to untreated (control) soil cores. (+) indicates increased cover or growth rate; (0) indicates no significant response; (–) indicates reduction in cover or growth rate relative to the untreated control. Growth rate responses were determined by considering both basal cover and final shoot yield data. For example, a soil treatment that reduced basal cover substantially compared to untreated soil but resulted in equivalent final shoot yield, was judged to have had a negative response in basal cover but a positive response in subsequent pasture growth rate.

Species	% Pasture basal cover				Shoot growth			
	KO		W3		KO		W3	
	Past	Fum	Past	Fum	Past	Fum	Past	Fum
T. subterraneum	0	0	–	0	0	0	0	0
L. perenne	0	0	–	–	+	0	+	0
P. aquatica	–	0	0	0	0	0	+	0
A .fulva	0	–	0	0	+	0	+	0

+ sig. increase compared to untreated; – sig. decrease compared to untreated; 0 same as untreated.

Conclusions

The responses of the pasture species to soil biology were considered to have occurred in two phases. In first phase of plant growth (germination, establishment, survival as measured by %PBC), the natural "balance" of soil microorganisms in untreated cores may have protected seedlings from disease(s) that affect germination and early establishment. This may explain why soil treatments expected to perturb soil biology in the intact cores often reduced %PBC compared to that in the untreated soil cores. In contrast, in the second phase of plant growth (as measured by shoot growth of surviving plants), the natural "balance" of soil microorganisms in untreated cores may inhibit the growth of shoots. This is concluded from the higher rates of shoot growth observed in many pots after pasteurisation compared with untreated soil. However, it was also evident that pasteurisation and fumigation were not equivalent in their effect on soil biology because fumigation failed to promote shoot growth rates. The results indicate that the natural soil biology of untreated soil may have a different role at different stages of plant growth.

In a previous growth cabinet study using disturbed soil and uncontrolled soil temperatures, disease was not observed in similar plant species and soil types (S Wiebkin, pers. comm. June 2005). In this experiment, we found diseased plants in some intact soil cores in the root cooling tanks. Diseased plants were sampled and sent to SARDI in an attempt to isolate and identify microorganisms that were present. Comparison of the outcomes from previous experiments using disturbed soil with those from the present experiment using intact and cooled soil cores, suggests that soil disturbance and temperature were critical in influencing soil-borne pathogens. Glasshouse studies in which soil is disturbed and without soil temperature control created conditions different to those experienced by plants and microorganisms in the field and expression of soil biological constraints to plant growth also differ. By simulating autumn soil conditions, possible constraints on plant establishment caused by soil microorganisms were observed in the glasshouse.

4.2 Development of new techniques to study rhizosphere exudates and micro organisms surrounding pasture plant roots in soil-based systems

Summary

Several methods for collecting root exudates (sand culture) and rhizosphere solutions (roots growing in soil) were assessed. Non-sterile, soil-based systems are intrinsically complicated because roots, soil properties and microbial activity all influence the composition of the rhizosphere solution. In these experiments, soil systems were found to also affect the quantity and repeatability of rhizosphere solution collection. One system, in which plants were grown in soil to develop a root mat which was then placed on a sand layer, was used for the collection of rhizosphere solutions and a more controlled system (e.g. sand culture followed by short term exudate collection) was used to to collect root exudates.

Interactions between *P. irregulare* and rhizosphere solutions collected from the roots of pasture plants were tested: There were no inhibitory or stimulatory effects of rhizosphere solutions on the hyphal growth of *P. irregulare*.

A new microscopic technique was developed to visualise the colonisation of *Pythium irregulare* on plant roots. The technique allowed visualisation of *Pythium* structures such as oospores and hyphae in and on epidermal and root hair cells of *Trifolium subterraneum*.

4.2.1 Collection and analysis of rhizosphere solutions from soil-grown pasture plants

Introduction

Root exudates contain a wide range of compounds which can affect microbial communities in the rhizosphere. Experiments reported here assessed various methods to collect rhizosphere solutions from soil-grown plants, and tested their effect on the growth of *Pythium irregulare*, a common soil-borne pathogen.

Materials and methods

Elution method: Moist coarse river sand (62g), Kia-Ora soil (KO) and Wallaroo 3 soil (W3) (50g each) were placed in 50 mL polypropylene test tubes with a small hole drilled at the bottom. Seed of *T. subterraneum* cv. Goulburn, *L. perenne* cv. Victorian and *P. aquatica* cv. Holdfast were sorted for uniformity in size and colour. Seeds were surface sterilised with absolute ethanol (1 min), rinsed in deionised water, and placed on water agar to imbibe and germinate for 2 days. Seven seeds were sown in each tube, with lids loosely screwed to allow for air exchange until germination. 'No plant' controls were included and there were four replicates.

Centrifuge method: Moist coarse river sand (38g) and W3 soil (28g) were placed in 50 mL centrifuge tubes containing special inserts for centrifugation. Seed of *T. subterraneum* cv. Goulburn and *L. perenne* cv. Victorian were sorted, sterilised, germinated and sown as described for the elution method, seven seedlings were sown in each tube. 'No plant' controls were included and there were six replicates. Tubes were placed in a growth cabinet maintained at 22^{o}C, and watered

every 3–4 days with a 1/10 complete Hoagland nutrient solution (Hill 2003). Exudates were collected at 14 DAS. For the elution method, 10mL of sterile deionised water was added to each tube until at least 5 mL of eluent was collected. For the centrifuge method, tubes were centrifuged at 5000 rpm for 20 min. Rhizosphere solutions were sterilised through 0.22 µm filters to remove bacterial cells and colloidal material and stored at –4^{o}C until analysis.

'Root mat' method: A total of 250 g of dried and sieved W3 soil was placed in root chambers for collection of rhizosphere solutions as described by Chen et al. (2002) and modified by George et al. (2006). Seed of *T. subterraneum* cv. Goulburn, *L. perenne* cv. Victorian, *P. aquatica* cv. Holdfast and *A. fulva* were sorted for uniformity in size and colour. Seeds of *T. subterraneum*, *L. perenne*, *P. aquatica*, and *A. fulva* (6, 7, 8, and 9 seeds/chamber, respectively) were grown for 6 weeks to form a planar mat of roots against a nylon mesh (25 µM) membrane. Plants were fertilised twice weekly with ½-strength complete nutrients. 'No plant' controls were included and there were six replicates. After 6 weeks, root mats were placed in contact with a 1.5 mm (30 g) disk of purified sand (Chem Supply, South Australia) for a further 7 days. At harvest, shoots and roots were separated, placed in oven at 70^{o}C for 3 days, and dry weights determined. Disks of purified sand were placed into tubes and centrifuged at 5000 rpm for 5 min to extract rhizosphere solutions, which were sterilised through 0.22 µm filters. Samples were then frozen at –80^{o}C, freeze-dried and reconstituted in 500 µL of sterile deionised water.

Analyses of rhizosphere solutions for C and N content: For the elution and centrifuge methods, 100 µL of each sample was analysed for C and N content by mass spectrometry. For the 'root mat' method, 100 µL of each reconstituted sample was dried overnight under vacuum.

Results and discussion

The elution and centrifuge methods proved to be unsuitable for collecting rhizosphere solutions with reproducibility. The amount of time taken to elute 5 mL of rhizosphere solution from plants grown in the elution method varied between 30 s and 2 min. The volume of samples collected by centrifugation was highly variable between tubes (range of 0 to 1mL/tube). In some cases, this resulted in some replicates where no solution was extracted. Differences in volumes extracted by either method depended on soil type (samples being easier to extract from sand than soil), but also varied very widely between replicates of the same soil type. The variability contributed to large differences in the concentrations of compounds in samples irrespective of treatments. For example, the N content of rhizosphere solutions collected by the elution method varied from 6.2–640.9 µg N/mL for *P. aquatica* in W3 soil; 13.3–679.6 µg N/mL for *L. perenne* in sand and from 183.5–907.0 µg N/mL for 'no plant' controls in W3 soil. The C contents in both the elution and centrifuge methods also varied considerably.

There was greater reproducibility in the concentration of C and N collected by the 'root mat' method. The amount of N in a rhizosphere solution is the net result of N additions in fertiliser, exudation and utilisation by roots and microorganisms, and adsorption to clay particles if N is present as ammonium or in an organic compound. In the rhizosphere solutions collected by the root mat method, the amounts of N on a per pot, or per sand collection layer basis were reduced, relative to the unplanted control, by the presence of plants. This was expected to occur mainly as a result of N utilisation in plant growth. Some differences between species were not evident on a per pot basis, but became so when N concentration per gram of roots was determined.

Differences between planted treatments and unplanted controls were not observed for concentrations of C on a per pot or per sand layer basis. Although plant roots are expected to be a source of C in exudates, the net amounts of C measured in rhizosphere solutions are determined by the balance between root exudation, microbial activity and adsorption or sequestration of C on soil particles and in adsorbed organic compounds. Consequently, lack of difference between planted treatments and controls was most probably indicative of microbial activity in the rhizosphere. Differences between some species were evident when C content of the rhizosphere solutions was expressed per gram of root. Differences in C:N ratio of planted treatments relative to unplanted controls were considered highly likely as a result of root and microbial activity. However, in this case, the differences observed were most likely to be mainly driven by plant growth and N uptake from the soil solutions. The result indicates a substantially different C:N environment in the rhizosphere as opposed to the bulk soil.

The final experiment in this series was damaged in a hail storm that destroyed the glasshouse in which it was situated. Plants that had been inoculated with *Pythium irregulare* showed essentially no infection in the plants that could be rescued, so all plants were used to collect root exudates by immersing the roots in 0.02 M $CaCl_2$ solutions over a 3-hour period. The amounts of C and N exuded per hour from subterranean clover and perennial ryegrass roots were a 1000-fold higher than observed in rhizosphere solutions collected from root mats after 7 days (Tables 4.6 and 4.7). This confirmed our expectations that the amounts of C and N found in the rhizosphere would be the net result of considerable microbial activity. Indeed, to achieve the low concentratons of C found in rhizosphere solutions, the microbial activity in the rhizosphere must consume almost all C exuded from roots. The C:N ratio of the root exudates was nearly two-fold that observed in the rhizoshere solutions.

The values for C and N in rhizosphere solutions are not a direct measure of the actual C and N content of root exudates. They were expected to represent the 'net' outcome of root exudation, microbial exudation and/or utilisation, and the sorption characteristics of the soil itself. The comparison of C and N content of exudates collected directly into $CaCl_2$ solutions with that of the rhizosphere solutions confirmed this notion. However, the overall objective of the project was to examine microbial activity and root exudation in soil rhizospheres. Consequently the next phase of examined: (1) collection of root exudates from ^{14}C-labelled plants grown in sterile media to establish (a) their composition and (b) their specific activity; and (2) collection of rhizosphere solutions from similarly labelled plants growing in soil. The combined information was to be used to estimate rate of exudation into the soil rhizosphere and to partially understand the fate of exuded C which results in the net amounts found in rhizosphere solutions.

The experiments would have been challenging due to the complex system involved. However, the project changed direction as a result of recommendations from the mid-term review and experiments using more controlled systems were used (e.g. inert media such as sterile sand or glass beads) under sterile conditions to collect root exudates and rhizosphere solutions.

Table 4.6. C and N contents of rhizosphere solutions collected by the 'root mat' method from four pasture species grown in soil. Values in parentheses are standard deviations; different letters represent significant difference between means.

	'No plant' control	*T.subterraneum*	*L. perenne*	*P. aquatica*	*A. fulva*	Significance
µg N/pot	2.93 (0.60)^{a}	1.58 (0.55)^{b}	1.19 (0.18)^{b}	1.70 (0.91)^{b}	1.33 (0.14)^{b}	**
µg N/g sand	0.098 (0.020)^{a}	0.053 (0.018)^{b}	0.040 (0.006)^{b}	0.057 (0.030)^{b}	0.044 (0.005)^{b}	**
µg N/g root	-	2.31 (2.19)^{a}	0.33 (0.09)^{b}	0.037 (0.026)^{ab}	2.13 (1.08)^{a}	*
µg C/pot	9.49 (1.72)^{a}	7.84 (3.17)^{a}	7.54 (1.84)^{a}	7.69 (2.84)^{a}	7.81 (1.67)^{a}	n.s.
µg C/g sand	0.317 (0.057)^{a}	0.261 (0.106)^{a}	0.251 (0.061)^{a}	0.256 (0.095)^{a}	0.260 (0.056)^{a}	n.s.
µg C/g root	-	11.07 (9.00)^{ab}	2.04 (0.60)^{c}	4.92 (2.79)^{bc}	11.76 (4.41)^{a}	**
C:N	3.37 (1.28)^{a}	5.16 (2.17)^{b}	6.30 (0.97)^{b}	4.92 (1.41)^{ab}	5.87 (1.00)^{b}	*

Table 4.7. C and N amounts exuded per hour by roots of intact subterranean clover and perennial ryegrass plants that were grown in soil, washed,and placed in solutions of 0.02 M $CaCl_2$ for 3 hours. Values in parentheses are standard deviations.

	T. subterraneum	*L. perenne*
mg N/g root/h	1.3 (0.4)	0.7 (0.1)
mg C/g root/h	10.9 (5.1)	7.1 (1.6)
C:N	8.4	10.1

4.2.2 Effect of rhizosphere solutions on *Pythium irregulare* growth

Introduction

Reconstituted rhizosphere solutions collected from the 'root mat' method (4.2.1) were tested for their ability to affect the growth of *P. irregulare*.

Materials and methods

The highest amount of C detected was 0.5 µg from a sample of rhizosphere solution collected from *T. subterraneum*. In order to standardise the amount of C in each sample, volumes of 0.1 M sucrose was added to each sample of rhizosphere solution such that 30 µL of each sample contained 0.5 µg of C. An agar plug (1 mm^2) of a 3-day-old culture of *P. irregulare* isolate BH40 grown on ½-corn meal agar (½CMA; Becton Dickinson & Company, MD, USA) was placed in the centre of 9 cm diameter Petri dishes containing water agar. Either 30 µL of rhizosphere solution adjusted to contain 0.5 µg of C, sterile deionised water, or 60 mM sucrose (equivalent to sucrose concentration in VP3 *Pythium*-selective medium) was placed 2.25 cm away from the plug of BH40. Three replicates were used and plates were incubated at 20^{o}C. Hyphal extension of BH40 was marked on plates over a period of 3 days. This was done in two directions: (1) directly towards the 30 µL sample; and (2) 180^{o} away from the 30 µL sample.

Results and Discussion

Hyphae of BH40 generally grew faster (P<0.05) "towards" the 30 µL sample than in the direction "away" from the sample. However, there was no difference between source of rhizosphere solution, deionised water and sucrose on the rate of hyphal extension. Therefore, there was no evidence for stimulation or inhibition of hyphal growth of *P. irregulare* by rhizosphere solutions.

4.2.3 Microscopic techniques for studying *Pythium*–root interactions

Introduction

A new protocol for visualising *P. irregulare* on the roots of pasture plants using microscopy was developed to be used in conjunction with work on rhizosphere solutions and interactions with other soil microorganisms.

Materials and methods

Seeds of *T. subterraneum* cvs. Goulburn and Woogenellup were sterilised in 100% ethanol (1 min), 12% NaOCl (v/v) (20 min), rinsed six times in sterile deionised water and left to imbibe overnight in the last rinse. Seeds were germinated on sterile, moist germination paper until radicals were approximately 1–2 mm long. Seedlings were then aseptically transferred to petri dishes containing water agar, one seedling/dish. A small of plug of agar (1 mm^2) containing hyphae of *P. irregulare* isolates BH40 and W3YC were placed next to the radical. Plates were sealed and placed upright in a growth cabinet maintained at 22^oC. Seven days after sowing, roots were carefully removed from plates and stained with 0.05% toluidine blue (pH 4.4) for 15 min, and examined by fluorescence microscopy (Leica DMR HC; excitation filters 360 ± 40nm; emission filter 470 ± 40nm). Hyphae of *P. irregulare* isolates BH40 and W3YC from ½CMA medium, *Rhizoctonia solani* AG8 and *Leptosphaeria maculans* (canola blackleg) from potato dextrose agar medium, were also stained with 0.05% toluidine blue and examined by fluorescence microscopy.

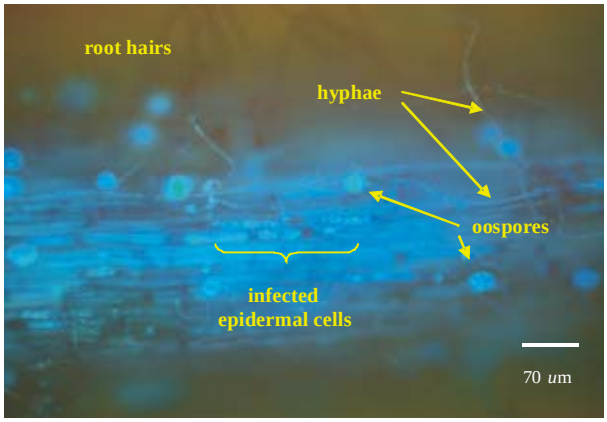

Fig. 4.5. *Pythium irregulare* BH40 on the root of *Trifolium subterraneum* cv. Woogenellup (roots and *Pythium* stained with 0.05% toluidine blue, pH 4.4, excitation filter 360±40 nm; emission filter 470±40 nm). Structures of *P. irregulare* were also seen within epidermal cells and root hairs of lateral roots (Fig. 4.6). Subsequently, infection caused significant damage to the epidermal cells of lateral roots.

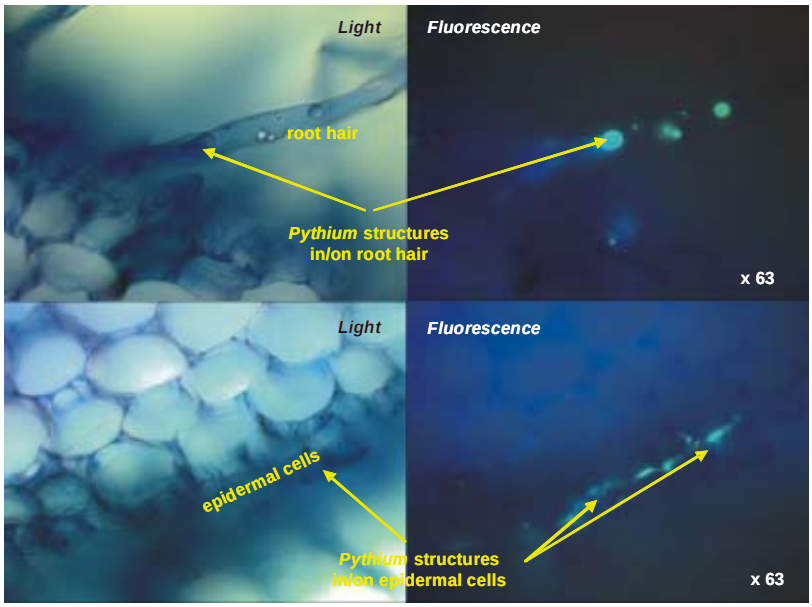

Fig. 4.6. *Pythium irregulare* BH40 in/on root hair and epidermal cells *Trifolium subterraneum* cv. Woogenellup (roots and *Pythium* stained with 0.05% toluidine blue, pH 4.4, excitation filter 360±40 nm; emission filter 470±40 nm). Structures not obvious under light microscopy (left panels) were clearly seen under fluorescence microscopy (right panels).

Results and Discussion

Structures of *P. irregulare* such as mycelia, oospores and zoospores on roots were clearly visible under fluorescence microscopy (Fig. 4.5). Compared with aseptate hyphae of *P. irregulare*, the septate hyphae of *R. solanii* AG8 and *L. maculans* did not fluoresce like those of BH40 and W3YC. This technique is therefore likely to be useful for visualising *Pythium* colonising roots in the presence of other organisms.

The experiments highlight the importance of a suitable system for collection of rhizosphere solutions from soil-grown plants that enable reproducible results. High variability in the samples collected by the elution and centrifuge methods cofounded results. This was improved by using the 'root mat' method and by collection of exudates into $CaCl_2$ solutions although C exudation by some species appeared to often be intrinsically variable. The root mat and direct collection methods indicated that C and N concentrations of exudates collected directly from roots were considerably higher than the C and N concentrations observed in rhizosphere solutions. The data support the notion that that microbial activity in the rhizosphere is utilizing a very high proportion of the exudate from roots. The hypothesis that rhizosphere solutions would reflect the net result of roots and microbial activity and soil influences was supported by the results obtained to this point. Consequently, to achieve the objective of studying microbial activity in the rhizosphere of plants growing in soil, further experiments would need to employ radiotracers to measure the flux of root-derived C exuded into the soil rhizosphere.

The rhizosphere solutions collected from the ‘root mat’ method had no inhibitory or stimulatory effect on the hyphal growth of *P. irregulare.*

The new microscopic technique allowed visualisation of *P. irregulare* on *T. subterraneum* roots. The staining and fluorescence techniques developed appear unique to *Pythium* spp., and in conjunction with work on rhizosphere solutions and interactions with other soil microorganisms, it provides a useful tool for studying interactions between *Pythium* and plant roots.

4.3 Experiments using a pasture plant-infection system to study the interaction of *Pythium irregulare* with roots

Summary

A plant-infection system in the glasshouse was developed to study the effect of *Pythium irregulare* on plants. It showed that infection of roots by *P. irregulare* in sand culture significantly decreased lateral root growth in both *Trifolium subterraneum* and *Lolium perenne,* but did not necessarily result in decreased shoot growth. Infection of *T. subterraneum* occurred with a range of *P. irregulare* isolates, including those from wheat, subterranean clover and an isolate obtained directly from soil. Root infection was independent of inoculum dose.

Several hundred isolates of bacteria were obtained from the rhizosphere of field-grown plants of *T. subterranean* and grasses. Approximately 25% of the isolates showed some inhibition to the growth of *P. irregulare* in laboratory media, with the best isolates showing up to 70% inhibition of growth. Techniques for labeling rhizobacteria with fluorescent markers (ie., based on green and red fluorescent proteins) were developed. *Rhizobium leguminosarum* bv *trifolii* and a root colonising strain of *Psuedomonas* species were transformed and shown to fluoresce when excited under microscopy. Transformants of *R. leguminosarum* were not compromised in their ability to grow, colonise and nodulate plants of *T. subterraneum*.

It was anticipated that fluorescent-labelled bacteria (including beneficials and isolates that were inhibitory to *P. irregulare*) would be used in conjunction with the plant-infection system and direct microscopic visualisation of *P. irregulare* to investigate microbial interactions on pasture roots in relation to root exudates. However, due to recommendations of the mid-term review, subsequent experiments were focussed on the use of DNA probes (in collaboration with SARDI) and molecular analysis of rhizosphere microbial communities.

4.3.1 Development of the plant-infection system for *Pythium*

Introduction

The objective was to develop a glasshouse-based plant growth system for studying the infection of roots of different pasture species with *Pythium irregulare*. *P. irregulare* was identified both in the review (section 1.1) and by the Soil Biology Group meeting (September 2005, Adelaide) as a significant pathogen of pasture species throughout southern and western Australia. A suitably developed plant-infection system for pasture plants would subsequently allow microbial interactions in the rhizosphere to be investigated using a range of techniques in fluorescence microscopy (developed as part of this project) and DNA probing (with the SARDI group). A collaboration with Paul Harvey (CSIRO Adelaide) who has studied *P. irregulare* infections of cereal crops, was developed. The effect of different isolates of *P. irregulare* and methods of inoculation and inoculum preparation on root infection were compared using a range of pasture species.

Materials and methods

Pythium: Four isolates of *P. irregulare*; BH40, KAP3, KAP4, YAB2 (isolated from either wheat [BH40] and various pasture plants; Harvey *et al*. 2001)) and an isolate (W3YC) obtained directly from pasture soil (Wallaroo soil, Hall, ACT) were used. Inoculum was prepared as described by (Harvey et al. 2001) whereby 3-day-old culture of each isolate were added to flasks of a sterile mix of cornmeal (8 g), washed coarse river sand (755 g) and deionised water (100 mL). Flasks were incubated at 20ºC for 14 days and numbers of viable propagules (oospores and mycelia) were determined using *Pythium*-selective VP3 medium (Ali-Shtayeh *et al*. 1986) before use in the glasshouse. For inoculation to pots, the inoculum was mixed with pasteurised (steamed twice at 65ºC for 1 h) washed coarse river sand (1:4 w/w) to achieve ~400 propagules/g sand. Pots contained a total of 800 g of media.

Inoculum of *P. irregulare* BH40 was also produced following the colonisation of sterilised wheat leaves. Sterilised leaf segments (7 cm) inoculated with BH40 mycelia were incubated at 20ºC for 7 days. Propagules (mainly oospores) were obtained by gently scaping from the leaf into the water and centrifugation of the suspension at 10,000 *g* for 10 min. The number of oospores was similarly determined as above using *Pythium*-selective VP3 medium. Oospore suspensions were inoculated to sand in pots in the glasshouse as a band ~5 cm below the pot surface. Inoculum was added at a rate of 0, 200, 800 and 1600 propagules/g sand in a total mass of 100 g of mixture per 700 g of coarse river sand per pot. Seeds of *T. subterraneum* cv. Goulburn were grown and harvested as described above.

Plants: Seeds of *T. subterraneum* cv Goulburn were surface sterilised and pre-germinated at 20ºC in the dark until radicals were 0.5 cm long. Seven seedlings were transplanted to each pot which was then covered with plastic beads to minimise any potential cross contamination to uninoculated controls. Plants were grown in temperature-controlled root cooling tanks as previously described. Temperature of the sand (5 cm depth) was maintained between 10–17ºC throughout the experiment, as it is known that the progression of *Pythium* infection on roots is temperature dependent. Pots were thinned to five plants per pot 7 days after sowing and pots were watered every second day with 100 mL of half-strength modified Hoagland nutrient solution.

*Assessment of plant dry weight, root length and infection frequency***:** Twenty one days after sowing, plants were carefully removed from pots and roots were washed in running tap water. Shoots were cut at the hypocotyl and shoot dry weight determined after 48 h at 70ºC. Intact, fresh root systems were stored overnight at 4ºC prior to scanning with Win RHIZO ProV Image Analysis software (Regent Instruments Inc, Quebec, Canada) to measure root length and branching. The extent of root infection by *P. irregulare* was determined using a plating method (P. Harvey, pers comm), whereby 1-cm segments were incubated at 20ºC for 48 h on VP3 medium. Twenty to 30 random segments were assessed for each pot. Infection frequency was determined by counting the number of segments with *P. irregulare* mycelia associated with them. Dry weights of the remaining roots were determined after 48 h at 70ºC.

Results and Discussion

Different isolates of P. irregulare: Compared with uninoculated plants, isolates BH40, KAP4 and YAB2 significantly decreased both root biomass (data not shown) and root length per plant (Fig.

4.7). Whilst the extent of root infection ranged from 80% (isolate BH40) to 100% (isolate YAB2), compared to ~5% for uninoculated controls, a similar degree of damage was evident for all isolates of *P. irregulare* irrespective of their origin of isolation (BH40 from wheat; KAP3, KAP5 and YAB2 from *Trifolium subterraneum*; and W3YC directly from pasture soil). This demonstrates promiscuity in host range across isolates, and as such BH40, was therefore used in further experiments due to its detrimental impact on root growth, relatively high infection frequency and previously published work on this isolate, including DNA sequence analysis and its known recognition by the SARDI DNA probes.

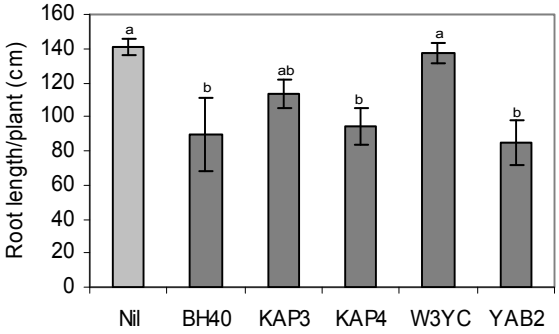

Fig. 4.7. Effect of *Pythium irregulare* isolates on root length of *Trifolium subterraneum* cv. Goulburn at 21 days after sowing. Bars represent SEM; different letters indicate significant differences by lsd (*P*=0.05)

Effect of inoculum dose on root infection: The effect of inoculum dose and its placement with respect to development of root symptoms on clover plants was assessed. Infective propagules of *Pythium* produced by the corn meal/coarse river sand method described above consisted of both oospores and mycelia. Unlike oospores, mycelia are more susceptible to damage and enumeration of both types of structures may overestimate the numbers of infective propagules. Therefore, an alternative procedure to generate inoculum was tested, whereby higher doses of only oospores were obtained. These were applied as a band of different densities below the roots of *T. subterraneum*.

The inoculum density affected the extent of root infection with a significantly higher % infection being observed only at the highest inoculum density (Figure 4.8). Irrespective of this, significant reductions in root length of *T. subterraneum* plants were observed across the various levels (Fig. 4.9). Although it was expected that higher infection frequency may cause increased root damage, this was not observed and suggests that a minimum inoculum dose is sufficient for disease development which is consistent with previous observations by Stovold (1974). Despite differences in root length, there was no effect of inoculum density on either shoot and root biomass (*P*>0.05; data not shown)

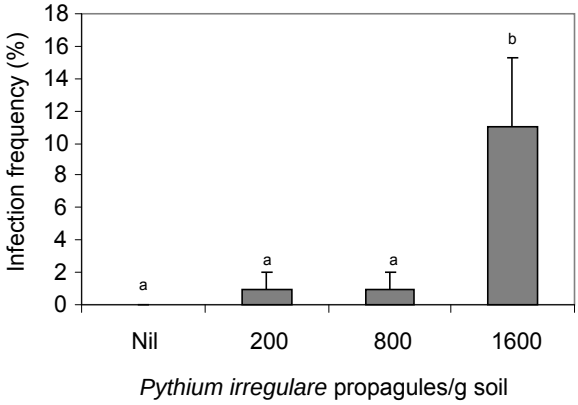

Fig. 4.8. Effect of *Pythium irregulare* propagule numbers (isolate BH40) on infection frequency of *Trifolium subterraneum* cv. Goulburn roots at 21 days after sowing. Bars represent s.e.m.; different letters indicate significant differences by l.s.d. (*P*=0.05).

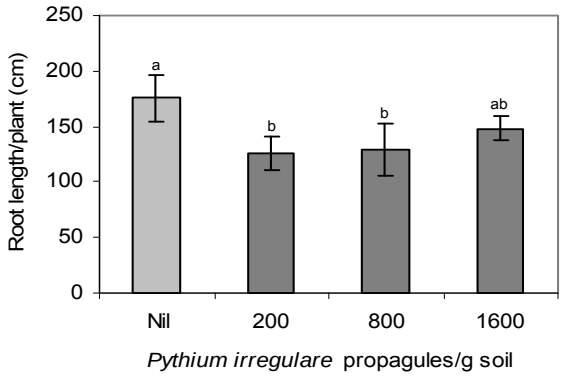

Fig. 4.9. Effect of *Pythium irregulare* propagule numbers (isolate BH40) on root length of *Trifolium subterraneum* cv. Goulburn roots at 21 days after sowing. Bars represent s.e.m.; different letters indicate significant differences by l.s.d. (*P*=0.05)**.**

Infection of different pasture species with *Pythium irregulare:* The infectivity of *Pythium irregulare* isolate BH40 was similarly investigated on *T. subterraneum* and *L. perenne*. Inoculum was prepared in corn meal/coarse river sand mix as previously outlined and used to inoculate plants as described above. At sowing, one seedling was planted per pot and root growth and *Pythium* infection was assessed.

Inoculation of plants with *P. irregulare* significantly decreased root length by 63 and 70% (and root mass; data not shown) in *T. subterraneum* and *L. perenne*, respectively (Table 4.8). The decrease in root length was particularly associated with a lower number of branch (lateral) roots in both species as illustrated in Fig. 4.10. Despite the effect on root length and mass, there was no difference in shoot biomass production between uninoculated and inoculated plants (data not shown), which may

be an artefact of plants grown in the glasshouse with otherwise sufficient water and complete nutrients. On the contrary, plants under field conditions with such root damage would be expected to be affected adversely in establishment and subsequent growth. Nodulation of *T. subterraneum* by rhizobia would also be expected to be compromised which combined with poor root growth, would have significant impact on pasture productivity. This is consistent with observations in MLA Project SHP.017 whereby post-emergent losses of *T. subterraneum* plants under field conditions was likely to be due to diseases such as *Pythium,* and that plants with severe root damage had no nodules (R. Simpson, Report to MLA 'Survey of root disease on subterranean clover (autumn/winter, 2006)'.

The plant-growth system described here was developed to investigate the infection of pasture roots by *P. irregulare* and its interaction with other rhizosphere microorgansims. This includes a range of potential beneficial microorganisms, such at *Rhizobium leguminosarum* bv. *trifolli*, and other root colonizing rhizobacteria (e.g. *Psuedomonas* spp.) that may competitively interact with *Pythium*, and/or inhibit its growth and pathogenicity. A number of experiments were therefore conducted to address this.

Table 4.8. Effect of *Pythium irregulare* (isolate BH40) incoualation on root length of *Trifolium subterraneum* and *Lolium perenne* grown for 21 days in coarse river sand. Numbers in parentheses indicate standard errors.

	***Trifolium subterraneum* cv. Goulburn**		***Lolium perenne* cv. Victorian**	
	Nil	*Pythium irregulare* BH40	Nil	*Pythium irregulare* BH40
Root length/plant (cm)	71.4 (6.1)	26.4 (5.4)	58.3 (5.4)	17.3 (2.4)
P-value	<0.001		<0.001	

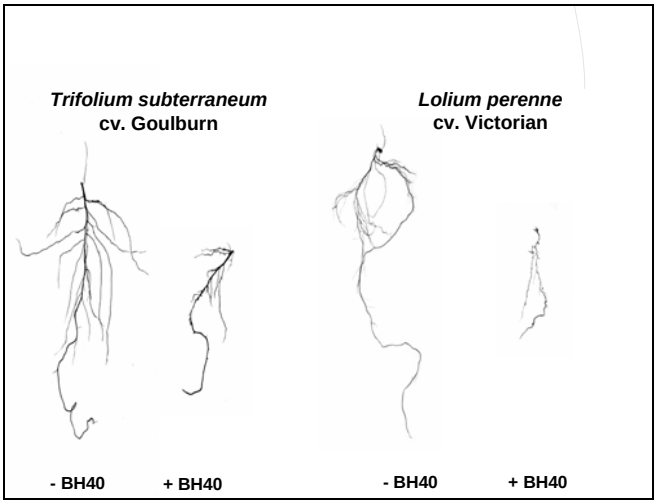

Figure 4.10. Roots of *Trifolium subterraneum* and *Lolium perenne* grown for 21 days in coarse river sand without and with infection by *Pythium irregulare* isolate BH40.

4.3.2 Interactions between bacteria isolated from the rhizosphere of pasture plant roots and *Pythium irregulare*

Introduction

The rhizosphere contains wide diversity of bacteria which can be either beneficial or detrimental to plant growth, and bacteria can inhibit the growth of pathogens such as *Pythium*. Bacteria were isolated from the rhizosphere of fertilised *T. subterraneum* plants and grass roots (predominantly *Phalaris aquatica*) growing in Wallaroo soil (Hall, ACT). Rhizobacteria were isolated from roots by placing the root sample in 0.89% sterile NaCl and shaking for 2 h at 20°C. Approximately 200 different bacterial colonies (based on colony morphology and growth rate) were randomly selected from various dilutions of soil suspensions plated on either ½-strength tryptone soy agar or Pseudomonas Agar F (Becton Dickinson & Company, MD, USA).

Materials and Methods

The effect of bacterial isolates on the growth of *P. irregulare* was determined by *in vitro* assays. Overnight cultures of the various isolates were adjusted to a standard optical density (OD 1 at 600nm) with sterile 0.89% NaCl. Twenty µL was then placed onto 9 cm Petri dishes containing ⅛-strength TSA at 2.25 cm away from the edge of the plate. Plates were incubated overnight at 20°C. The following day, an agar plug (1 mm^2) of a 3-day-old culture of *P. irregulare* isolate BH40 grown on ½-CMA was placed in the centre of each plate (4.5 cm from the edge) which were then incubated at 20°C (Fig. 4.12). Hyphal extension of BH40 was measured on the plates over 3 days in two directions: (1) directly towards the bacterial sample; and (2) 180° in the other direction. Appropriate plates (without samples) were used as controls.

Results and Discussion

Over 50 isolates (~25% of those tested) showed some inhibition towards the growth of BH40 compared with NaCl and 'nil' controls. The extent of inhibition of BH40 varied with different bacteria (Fig. 4.11) for 10 of the isolates that were inhibitory toward *P. irregulare*. Among these 10 isolates, there was a mean reduction of 47% growth of hyphae towards the bacterial isolates (20.6±10.0 mm over 3 days compared to 38–41 mm away from the bacterial isolates). Specific inhibition of hyphal growth is in Fig. 4.12, where isolate 50 was effectively inhibited *P. irregulare* compared to isolate 165. Bacteria that were antagonistic against *P. irregulare* were isolated equally from the rhizosphere of both clover and grasses.

It was anticipated that several of the isolates that showed specific inhibition toward *P. irregulare* would be identified by DNA analysis (for species verification) and transformed with fluorescent markers (as described below). This would enable further study of the interaction of these isolates with *P. irregulare* on plant roots using fluorescent microscopic techniques that have been developed for viusalisation of *P. irregulare* .

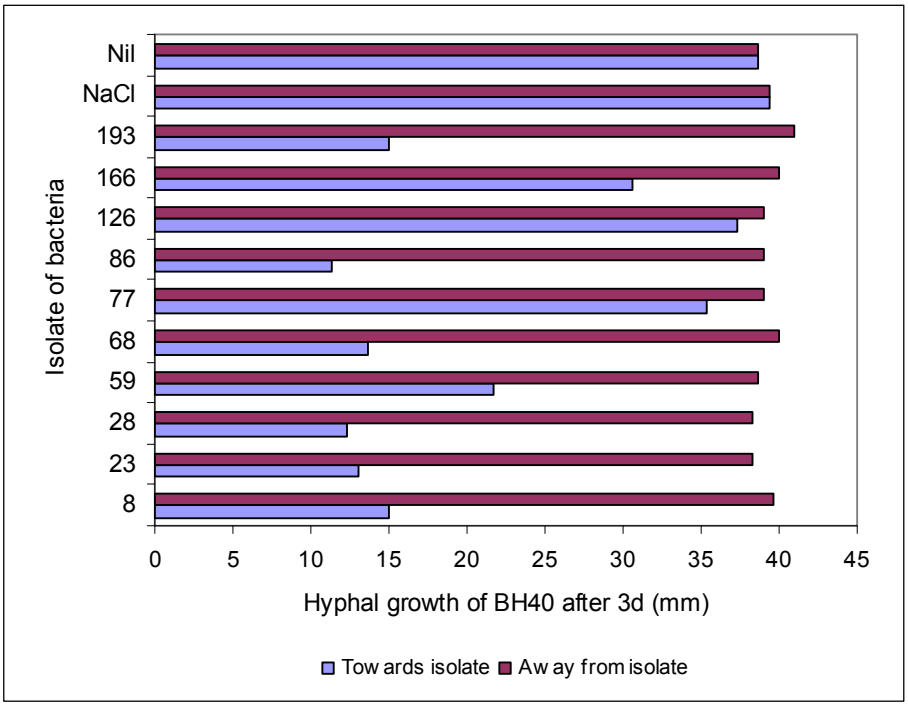

Fig. 4.11. Inhibition of hyphal growth of *Pythium irregulare* by different isolates of rhizobacteria. Hyphal growth after days of BH40 hyphae either towards or away from (lower and upper bar for each isolate, respectively) selected bacteria isolated from the rhizosphere of *Trifolium* spp. and grass roots in Wallaroo soil (Hall, ACT).

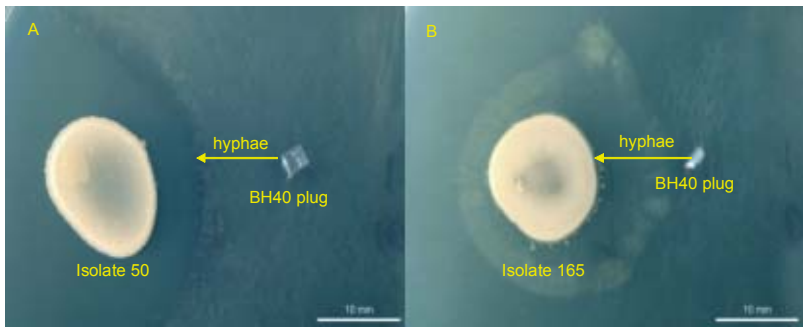

Fig. 4.12. Effect of bacteria isolated from roots of pasture plants on growth *of Pythium irregulare* BH40 *in vitro*: (A) isolate 50 showing specific inhibition of hyphal growth, and (B) isolate 165 showing no inhibition of BH40.

4.3.3 Techniques for fluorescent labelling of rhizobacterial isolates

Materials and methods

A protocol to insert broad-host range plasmids into Gram-negetive soil bacteria that allow expression of fluorescent proteins was developed. This was based on plasmid constructs described by Bloemberg *et al*. (2000) that express green, yellow, cyan and red fluorescent proteins (plasmids pMP4655, 4685, 4641 and 4662, respectively). When constructs were into bacteria, fluorescent proteins allowed visualisation of bacterial cells by fluorescence microscopy. This technique has wider application for studying the spatial and temporal distribution of different bacteria in the rhizosphere.

Results and Discussion

The fluorescent proteins were used to label a root-colonising strain of *Pseudomonas* spp. (isolate 266; Simpfendorfer *et al*., 2002) and *Rhizobium leguminosarum* bv. *trifolii* (strain WSM1325). Bacteria were transformed by either triparental mating or direct electroporation (details not shown). Expression of the fluorescent-proteins was verified by fluorescence microscopy (Fig. 4.13). The resulting transconjugants of WSM1325 were then assessed for growth rate in artificial medium and for ability to nodulate plants of *T. subterraneum*.

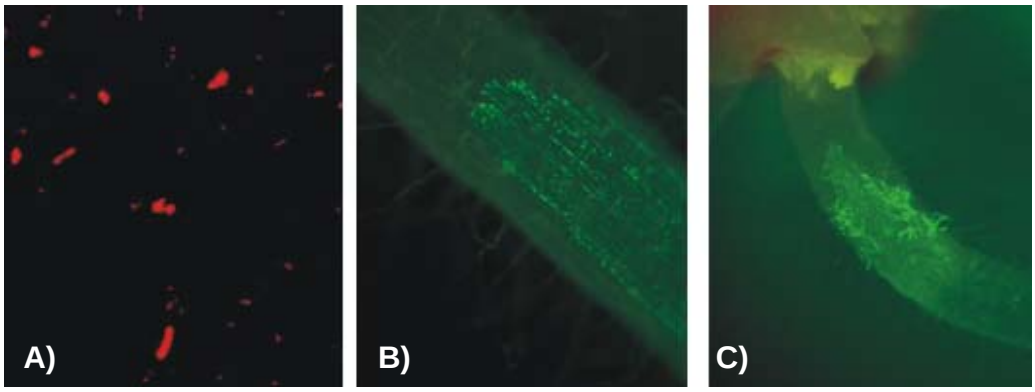

Fig. 4.13. Visualisation of fluorescently-labelled bacteria strains. (A) *Rhizobium leguminosarum* bv. *trifolii* strain WSM1325 (~x200) carrying plasmid pMP4662 (red fluorescent protein); and (B) and (C) root colonising *Pseudomonas* spp. isolate 266 carrying plasmid pMP4655 (green fluorescent protein) in association with a wheat root (~x25 and x10, respectively).

The fluorescent protein (plasmid) constructs were stably maintained in *Pseudomonas* and *Rhizobium* over several generations. The mean generation time of transformed *Rhizobium* was however increased between to 5.4 and 6.8 h (for WSM1325/pMP4655 and pMP4641, respectively) compared with 4.8 h for wild type WSM1325. The slower growth of the transformants compared to the wild type was likely due to the metabolic burden placed on the transformants by carrying a fluorescent marker. However, decreased growth rate did not affect the ability of the transformed rhizobia to nodulate plants of *T. subterraneum* (data not shown.

Conclusions

A plant-infection system to study the effect of *P. irregulare* on the roots of pasture plants was developed. Glasshouse studies showed that infection by *P. irregulare* significantly decreased root growth and in particular affected root length and the presence of branch (lateral) roots in both *Trifolium subterraneum* and *Lolium perenne,* Infection of *T. subterraneum* occurred with a range of *P. irregulare* isolates, including those from wheat, subterranean clover and an isolate obtained directly from soil. A wide range of bacterial isolates that showed inhibition to hyphal growth of *P. irregulare* were obtained from the rhizosphere of field-grown *T. subterraneum* and grass roots. Techniques were developed to successfully insert fluorescent markers (based on fluorescent proteins) into *Rhizobium* and *Pseudomonas* spp.

The plant-infection system could now be used in conjunction with fluorescently labelled bacteria (including beneficials and isolates that were inhibitory to *P. irregulare*), and direct microscopic visualisation of *P. irregulare* to investigate microbial interactions on pasture roots in relation to root exudates. However, this objective was not pursued in line with recommendations from the mid-term review (October 2007).

4.4 Effect of defoliation on the composition of root exudates

Summary

A plant culture system for collection and analysis of root exudates for major sugars and organic anions was developed and used to assess *Lolium perenne* and *Trifolium subterraneum* plants in response to defoliation. In summary, defoliation altered the allocation of C to root growth and release of exudates. Roots continued to grow in the control (undefoliated) plants but, as expected, root growth ceased after defoliation. In perennial ryegrass, loss of root weight also occurred. The amount of C exuded per plant per hour did not change through time in the controls but differed substantially between the plant species. Importantly, there was a rapid increase in C exudation from subterranean clover roots following defoliation, and then a decline in the rate of C exudation back to a level below that achieved by control roots 9 days later. The rate of C exudation from perennial ryegrass did not change on defoliation but declined during the regrowth period. The rate of C exudation per gram of root was initially stable but declined in control plants indicating that steady levels of C loss per plant over time were a result of increasing root size. After defoliation, C exudation rates per gram root were stable in perennial ryegrass but increased immediately upon leaf removal in the case of subterranean clover and then declined back as root growth began to recover achieving a similar rate of C exudation as that of control plant roots by 9 days.

Analysis of 21 selected compounds by GC–MS showed that sugars (predominantly fructose) were the major constituent (~90–99%) of the exudate compounds that were identified with significant differences being evident between the grass and clover in terms of the proportion of fructose. Significant changes in the sucrose, glucose and inositol contents were also evident between species and in response to defoliation. Large differences occurred in the composition of organic anions in the exudates with quinate and threonate being dominant for the grass and clover, respectively. Malate and shikimate were also common to both species and constituted a lower proportion of the total organic anions in clover exudates over time.

4.4.1 Collection of rhizosphere solutions

Introduction

There are two ways to collect solutions from the rhizosphere for the purpose of analysing the compounds in root exudates. The most desirable method is collection from plant roots grown in sterile culture to decrease the possibility of microbial modification of the compounds before analysis. Complete sterility is technically very difficult. Consequently, most researchers use a non-sterile system for plant culture, collect exudates for a short period of time (~2 h) and store samples under sterile conditions. This process minimises microbial modification of the compounds. Rhizosphere solutions from pasture plants grown in sand were collected using two methods: (1) “sterile culture” followed by vacuum suction; and (2) short time-frame collection into a root-bathing solution. Rhizosphere solutions were analysed for total C content, solutions from short-term collections were also assayed by GC–MS (in collaboration with Dr Ute Roessner, Australian Centre for Plant Functional Genomics, University of Melbourne) to determine C compound and organic anion profiles.

Materials and methods

Collection of exudates from plants in sterile culture: A number of preliminary experiments led to attempts to grow plants in a 3-layered jar using a washed coarse sand/glass bead mix that could be autoclaved from which rhizosphere solutions could be extracted using suction (Fig. 4.14). The system was autoclaved (121ºC, 20 min), planted with surface sterilised seeds and was closed except for air exchange via a foam filter. Sterile nutrient solution was supplied to plants via a cotton wick.

Batches of seed of *Trifolium subterraneum* cv. Goulburn and *Lolium perenne* cv. Victorian were sorted for uniformity in size and colour, sterilised with absolute ethanol (1 min), 12% (v/v) NaOCl (20 min), rinsed in six changes of sterile deionised water and left to imbibe in the final rinse overnight. The next day, seeds were washed in 3% (v/v) H_2O_2 (30 s) and then rinsed in two changes of sterile deionised water. A sample from each batch of sterilised seed was placed on ½-tryptone–soy agar (1.5%; TSA) plates and incubated at 28ºC to detect contamination. Only sterile seed batches were sown. 'No plant' controls were included.

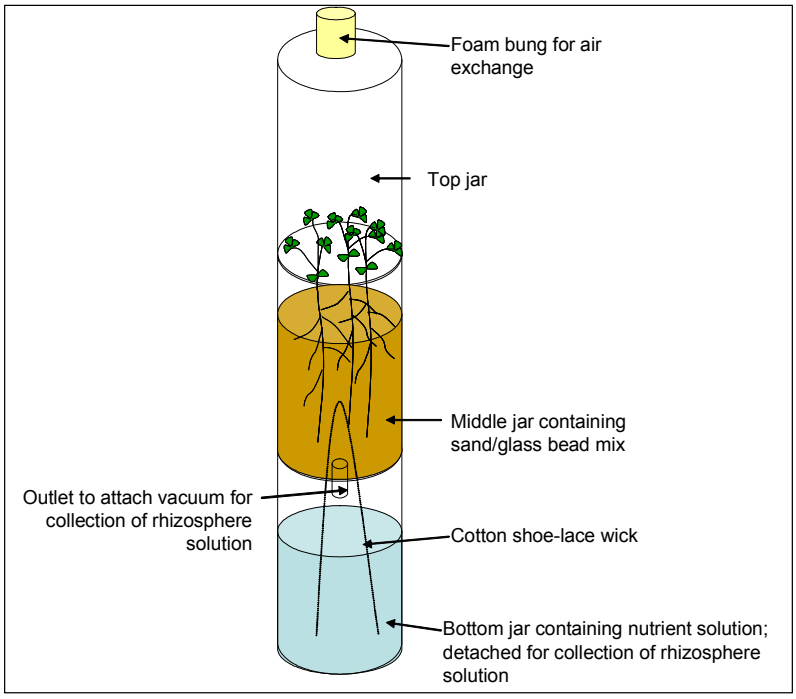

Fig. 4.14. Assembled 3-layered jar for collecting rhizophere solution using vacuum suction.

Four weeks after planting, rhizosphere solutions were extracted by vacuum. After removing the bottom jar containing nutrient solution, 40 mL of sterile deionised water was added to each middle jar and a vacuum pump (Dynavac, model 907CD, Melbourne, Australia) was attached through to the outlet at the bottom of the middle jar. Rhizosphere solutions were extracted at 15 psi for 10 min. Fifty mL of extracted solution was immediately filtered by passing through a 0.22 µm filter (Millex GP, Millipore, Cork, Ireland). A 1 mL-aliquot of the filtered solution was dried in rotor concentrator (Savant DNA Speed Vac, DNA 110, GMI, USA), reconstituted in 200 µL sterile deionised water and analysed for C content (CSIRO Analytical Services, Canberra, Australia). Roots were cut from shoots and were scanned for total root length with Win RHIIZO ProV Image Analysis software (Regent Instruments Inc., Quebec, Canada). Shoot and root dry weights were determined after drying at 70ºC for 2 days.

Sand/glass bead mix and nutrient solution in middle and bottom jars, respectively, of each assembly was tested for sterility at time of rhizosphere solution collection. For the sand/glass bead mix, approximately 5 g of the mix was suspended in 10 mL of sterilised 0.98% (v/v) NaCl and 100 µL was plated onto ½-TSA plates. For the nutrient solution, 100 µL was plated onto ½-TSA plates. All plates were incubated at 28ºC and examined every day for appearance of any contaminants.

Results and discussion

Plants grew well in this system with the grasses producing more root DW and longer roots (Table 4.9). However, there was no significant difference in the concentration of C exuded per gram of plant growth medium (i.e. per pot) by the two species or the unplanted control (Table 4.10) This indicated that the sand medium contained C that, although unable to be removed by initial washing, was released during the plant growth period. The background amounts of C were too high for any differences in C exudation per gram of root or per cm of root to be meaningfully interpreted (Table 4.10).

Colonies of bacteria and fungi also appeared on all ½-TSA plates within 1–5 days of incubation indicating that the 3-layered jars that had been autoclaved and into which exhaustively surface-sterilised seeds were planted, had become contaminated during the plant growth period.

Table 4.9. Root dry weight and total root length of *Trifolium subterraneum* and *Lolium perenne* grown in sand/glass bead mix. Mean values ± s.e.m.

Species	Shoot DW/jar (mg)	Root DW/jar (mg)	Total root length/jar (cm)
Trifolium subterraneum	102.4 ± 11.6	34.3 ± 6.7	243.8 ± 47.6
Lolium perenne	32.6 ± 3.5	70.3 ± 15.2	864.5 ± 135.2

Table 4.10. Total C content in rhizosphere solutions collected by vacuum extraction (15 psi, 10 min) from plants of *Trifolium subterraneum* cv. Goulburn and *Lolium perenne* grown in sand/glass bead mix. Mean values ± s.e.m.

Species	µg C/g medium
Unplanted control	64.3 ± 5.6
Trifolium subterraneum	62.6 ± 3.3
Lolium perenne	70.4 ± 3.1
l.s.d. (*P* = 0.05)	12.8

A number of attempts were made to improve the sterility of these systems but over time, almost all pots in any experiment became non-sterile for the system to be regarded as reliable. Highly purified quartz sand was obtained and used in alternative sand/glass bead growth medium trials but they became waterlogged and did not improve the management of this system of plant culture.

We believed that the background C problems were resolvable, and decided to use the root-bathing solution because checks on the system consistently showed that many pots became contaminated (non-sterile).

4.4.2 Short time-frame collection of exudates in a root-bathing solution

Materials and method

Root exudate collection: Plants were grown in coarse river sand and fertilised with ½-strength complete Hoagland nutrient solution. This medium permitted intact plants (shoots and roots) to be harvested easily and without damage. Roots of the intact plants were transferred to 100 mL of sterile 0.02 M $CaCl_2$ solution with their shoots supported above the solution by a ring of aluminium foil. Exudates were collected for 1 h in a plant growth cabinet so that they would continue to photosynthesise during the collection period. Fifty mL of the exudate collection solution was immediately sterilised by passing it through a 0.22 µm filter (Millipore). A 1 mL aliquot of the filtered solution was dried in rotor concentrator (GMI), reconstituted in 200 µL sterile deionised water and analysed for total C content.

Defoliation experiment: Twenty one days after sowing, plants in some treatments were defoliated with scissors whereby leaf material was removed to simulate 'grazing' treatments. The objective was to remove up to, but not exceeding, ~80% of the shoot material. At these levels of defoliation, root growth is slowed significantly. Approaching ~80% defoliation, root growth is reduced and eventually stops completely. More severe defoliation can also cause root death (Culvenor et al. 1989a, b). Control plants were not defoliated. At 0 h, 48 h and 9 days after defoliation, intact plants were carefully removed from the sand, roots washed gently in running tap water, and exudates collected. Roots were cut from shoots and were scanned for total root length with Win RHIIZO ProV Image Analysis software (Regent Instruments Inc). Roots harvested at 0 h were not scanned for root length. Shoot and root dry weights were determined.

Statistical analyses: Statistical analyses were conducted using one-way or two-way ANOVA (Genstat Version 9.2.0.152; Lawes Agricultural Trust (Rothamsted Experimental Station, UK).

Results and Discussion

Short time-frame exudate collection: Shoot weights indicated that control plants (uncut) continued to grow during the experimental period and that the defoliation treatments achieved 47% removal of shoot material in perennial ryegrass and 71% in subterranean clover (Fig. 4.15). No shoot regrowth had occurred by 48 h. However, by 9 days, shoots of the defoliated plants were regrowing at 62% of the rate of the control plants in perennial ryegrass, and 57% of subterranean clover. Roots continued to grow in the control plants but, as expected, root growth ceased after defoliation (Fig. 4.16). In the case of perennial ryegrass, some loss of root weight appeared to have occurred. Root growth was only just beginning to recover by 9 days, but the roots were still only growing at a fraction of the rate achieved by control plants.

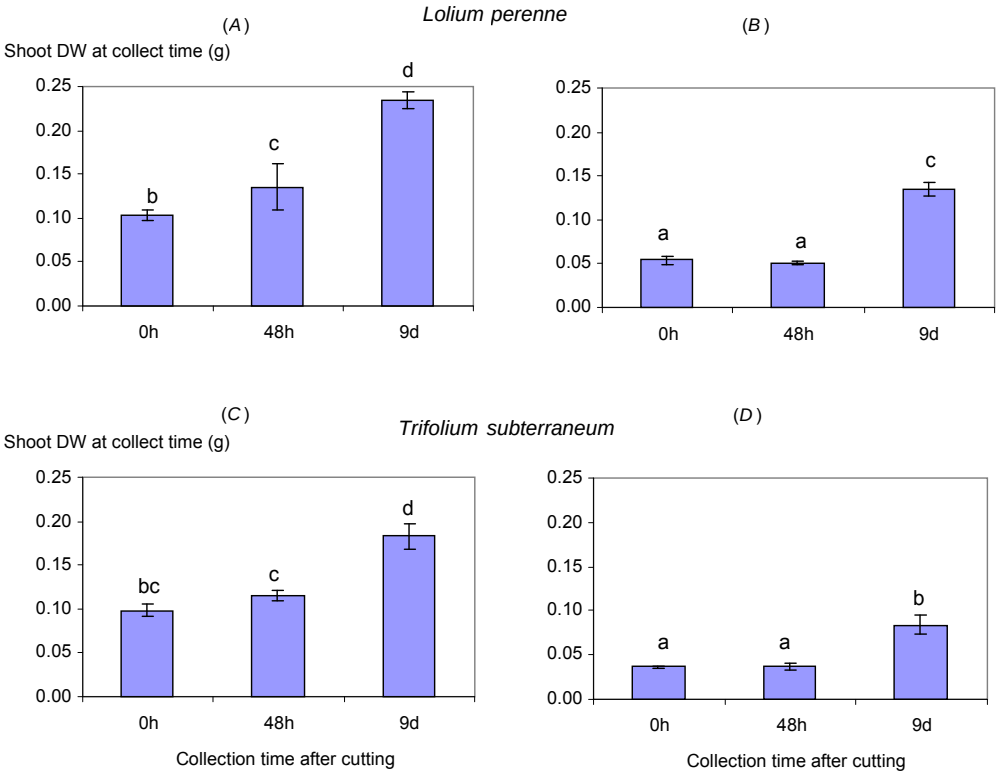

Fig. 4.15. Shoot dry weight of plants at time of rhizosphere solution collection 0 h, 48 h and 9 days after cutting. (*A*) *Lolium perenne* control plants, (*B*) *L. perenne* cut plants, (*C*) *Trifolium subterraneum* control plants, (*D*) *T. subterraneum* cut plants. Different letters indicate significant difference ($P < 0.05$) within each species.

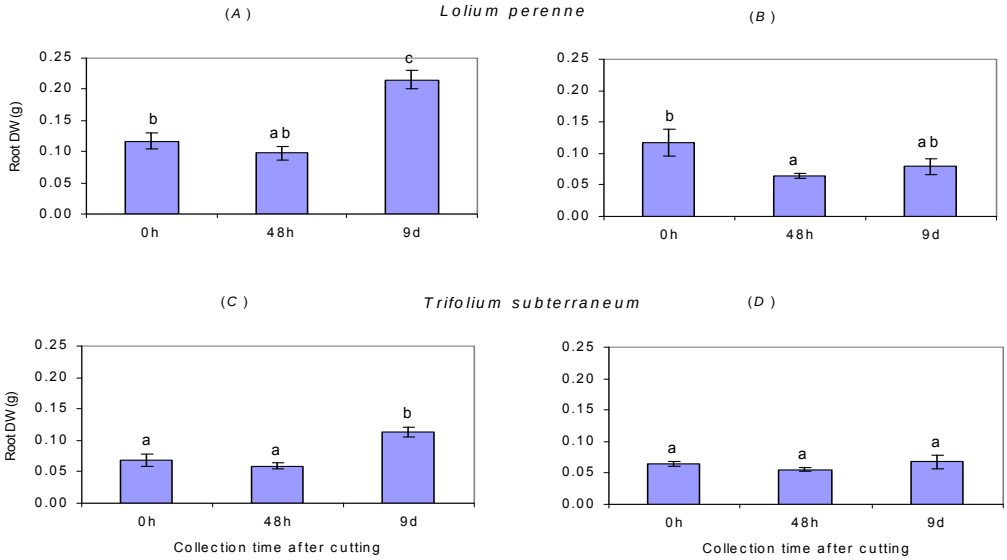

Fig. 4.16. Root dry weight at time of rhizosphere solution collection. (*A*) *Lolium perenne* control plants, (*B*) *L. perenne* cut plants, (*C*) *Trifolium subterraneum* control plants, (*D*) *T. subterraneum* cut plants. Different letters indicate significant difference ($P < 0.05$) within each species.

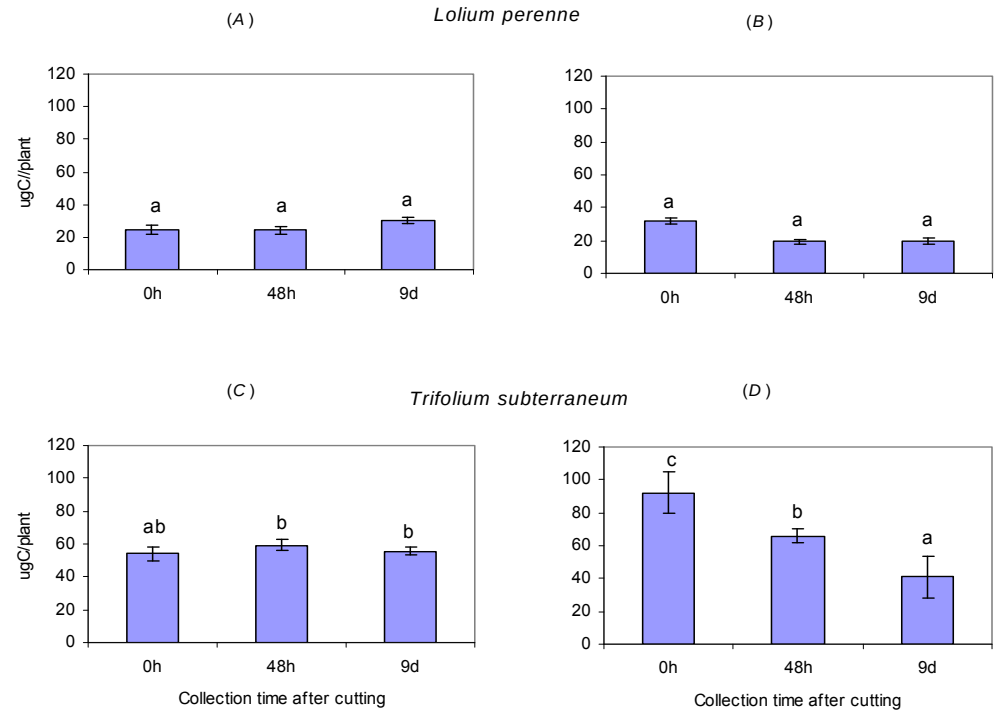

Fig. 4.17. C content (mg C/mL.plant) in rhizosphere solutions. (*A*) *Lolium perenne* control plants, (*B*) *L. perenne* cut plants, (*C*) *Trifolium subterraneum* control plants, (*D*) *T. subterraneum* cut plants. Different letters indicate significant difference ($P < 0.05$) within each species.

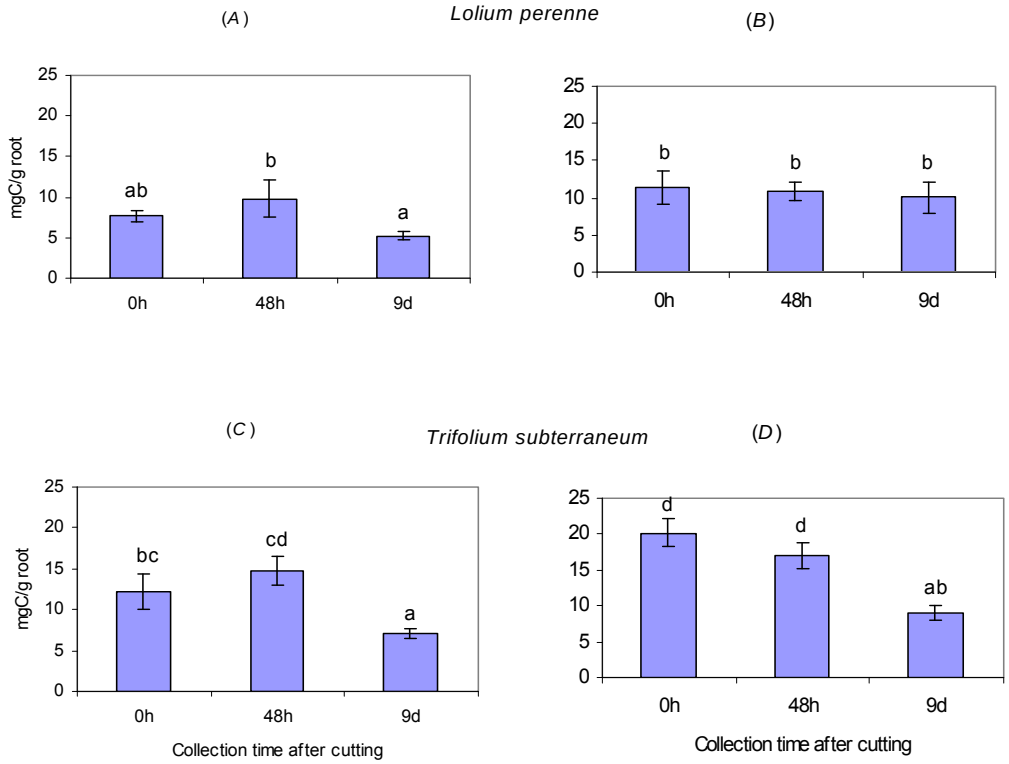

Fig. 4.18. C content (µg C/mL.g root) in rhizosphere solution at 0 h, 48 h and 9 days after defoliation. (*A*) *Lolium perenne* control plants, (*B*) *L. perenne* cut plants, (*C*) *Trifolium subterraneum* control plants, (*D*) *T. subterraneum* cut plants. Different letters indicate significant difference ($P < 0.05$) within each species.

The amount of C exuded per plant per hour did not change in the controls but differed substantially between the plant species (Fig. 4.17). On defoliation, there was a rapid increase in C exudation from subterranean clover roots and then a decline in the rate of C exudation back to a level below that achieved by control roots at 9 days. The rate of perennial ryegrass C exudation did not change on defoliation but also declined during the regrowth period. Differences between the species in the initial rate of C exudation after defoliation may reflect species differences, or may be due to the different levels of leaf area removal achieved by the defoliation treatments.

The rate of C exudation per gram of root (Fig. 4.18) was initially stable but declined in control plants over the experimental period indicating that steady levels of C loss per plant over time were a result of increasing root size which countered a decline in the average rate of C exudation of the root system. After defoliation, C exudation rates/g root were stable in perennial ryegrass but increased immediately upon leaf removal in the case of subterranean clover and then declined back as root growth began to recover achieving a similar rate of C exudation as that of control plant roots by 9 days.

The experiments indicated that a reliable method of exudate collection had been achieved and the collection method was applied to understanding how exudation of C was affected by defoliation. Root growth responses of the plants in these experiments also reflected the known physiology of root growth under defoliation. Differences between the species in the initial rate of C exudation after defoliation are very interesting, but it was not clear whether they reflected true species differences, or the different levels of leaf area removal achieved by the defoliation treatments. When cutting plants with very different leaf shapes and orientation, it was difficult to achieve similar levels of defoliation, and in this experiment, we achieved 71% shoot removal for clover (very close to the target of 80%) but only 47% in the case of the grass. The severity of the impact of treatment of C flows in the plants was therefore expected to have differed accordingly.

4.4.3 Composition of root exudates

Introduction

The effect of defoliation on the composition of root exudates from *T. subterraneum* and *L. perenne* was determined by utilising a new microanalysis service established primarily for metabalomics research by the Australian Centre for Plant Functional Genomics (University of Melbourne). Initial analysis of exudates collected from pine roots (Ms Shengjing Shi, PhD supervised by A. Richardson) using this approach indicated a wide range of potentially identifiable compounds (Table 4.11). However, as the service usually provides qualitative analyses so a more quantitative approach was negotiated for the analysis of selected compounds (predominantly organic anions, amino acids and phenolics and other secondary metabolites) in the root exudates.

Materials and methods

Exudate solutions were analysed for total C and N content by mass spectrometry (MS; CSIRO Analytical Services) and the relative composition of exudates for major sugars, organic anions and amino acids by gas chromatography–mass spectrometry (GC–MS; as reported here) was completed in collaboration with the Australian Centre for Functional Plant Genomics (Dr Ute Roessner, University of Melbourne).

Initial analysis of test samples indicated the presence of approximately 15 common monosaccarhide sugars, 12 organic anions, five amino acids, four fatty acids (Table 4.11) in addition to several unidentified disaccharide sugars (and a range of other putative compounds). On this basis, quantitative analysis using calibration standards for each of 11 sugars (fructose, sucrose, glucose, inositol, arabinose, mannitol, galactose, rhamose, xylose, maltose, raffinose), eight organic anions (citrate, malate, succinate, maleate, fumerate, threonate, quinate, shikimate) and five amino acids (glycine, proline, threonine, isoleucine, aspartate, phenyalanine) was undertaken (i.e., targeted compounds).

Table 4.11. Compounds that were reliably identified in exduate collected from pine roots, and those that were quantified in exudates from clover and grass roots subject to defoliation (in italics).

Sugars	Organic acids	Amino acids	Fatty acids	Phenolics and other 2° metabolites
fructose	*citric acid*	*aspartic acid*	C16:0	*catechin*
glucose	*fumaric acid*	*(oxo)proline*	C18:0	*quinic acid*
glycerol	*maleic acid*	*glycine*	C18:1	shikimic acid
sucrose	*oxalic acid*	*serine*	C20:0	*gallic acid*
xylose	*malic acid*	*leucine*		*threonic acid*
maltose	*malonic acid*			
mannitol	*succinic acid*			
galactinol	*tartaric acid*			
raffinose	*decanoic acid*			
myo-inositol				
scyllo-inositol				
(phenyl)pyruvic acid				
sorbitol[#]				
ononitol[#]				
pinitol[#]				

Results and discussion

Recovery of 'targeted' compounds: A comparison was made between the cumulative recovery of C and N (calculated as C released/hour.gram root DW) that was specifically associated with the 'targeted' compounds as measured by GC–MS, as compared to the total recovery of C and N (i.e. absolute recovery as determined by mass spectrometry). High variability was observed with the GC–MS method with mean recoveries of targeted C (across all 24 substrates) ranging from 2.4% (±1.7% s.e.) to 135% (±94% s.e.) of the total C pool across both species (Table 4.12). Amino acids ranged from 0.26% (±0.11% s.e.) to 18.2% (±13.9% s.e.) of the total N. This level of variability indicated that the quantification of the data through GC–MS was unreliable and that further refinement of the technique was required. Specifically, the data indicated that in addition to providing calibration standards for the targeted compounds and adjustment for differences in root system size, an internal standard of an otherwise novel C compound that does not occur in any of the samples to allow standardisation across samples is required.

Table 4.12. Recovery of C and N in targeted compounds that were present in exudates of *Lolium perenne* (ryegrass) and *Trifolium subterraneum* (subclover) plants that were defoliated or grown as controls. Targeted compounds (i.e. 11 identified sugars, eight organic anions and five amino acids) were measured by GC–MS. Values are expressed as a percentage of absolute recovery of total C and N as determined by mass spectrometer, and are derived from C and N release that was calculated (standardised) per h.g root DW. Data is the mean of five replicates with values in parenthesis show ± s.e.

	Recovery of C (%)		Recovery of N (%)	
Treatment*	*Lolium perenne*	*Trifoilum subterraneum*	*Lolium perenne*	*Trifoilum subterraneum*
control – day 21	13.8 (± 3.9)	123.2 (± 36.0)	0.61 (± 0.20)	8.01 (± 1.79)
control – day 30	11.5 (± 2.9)	73.0 (± 41.8)	0.33 (± 0.07)	2.95 (± 1.76)
defoliated – day 21	31.2 (± 11.6)	135.2 (± 94.0)	1.85 (± 0.55)	18.20 (± 13.98)
defoliated – day 30	2.4 (± 1.7)	4.9 (± 2.0)	0.11 (± 0.07)	0.26 (± 0.11)

* Defoliation removed 41% and 71% of shoot material for ryegrass and clover, respectively. Exudates were collected from each treatment either immediately after defoliation (on day 21), or after a further 9 days growth of both treatments (day 30).

Initially, it was considered that standardisation may be determined from either the total C recovery or from the calibration standards. However, neither proved appropriate because of the highly variable recovery of C in the GC–MS analysis and further investigation to address this issue is required. Irrespective of this, the data was valid for comparative analysis of the targeted compounds within each sample (see below).

Proportions of the 'targeted' compounds present in root exudates: Comparative analysis of the 21 compounds identified in the exudates (Table 4.13) indicated that sugars accounted for 93.7–97.7% of the total C in these compounds in ryegrass exudates, and 88.3–99.4% in clover exudates, with no significant differences ($P<0.05$) between species or defoliation treatments. Similarly, organic anions accounted for 0.5–11.0% of the total C identified, and amino acids 0.1–0.8%; again, with no significant differences between species or treatments.

Table 4.13. Comparative analysis of targeted compounds that were present in exudates of *Lolium perenne* (ryegrass) and *Trifolium subterraneum* (subclover) plants that were either subject to defoliation or grown as controls. Targeted compounds (i.e. 11 identified sugars, eight organic anions and five amino acids) were measured by GC–MS and for each group of compounds (sugars, organic anions and amino acids) are expressed as a percentage of the cumulative recovery of total C. Data is the mean of five replicates (± s.e.)

	Comparative analysis of targeted compounds (% of identified C)		
Treatment*	sugars	organic anions	amino acids
Lolium perenne			
control – day 21	97.7 (± 0.4)	1.5 (± 0.3)	0.8 (± 0.4)
control – day 30	93.7 (± 2.8)	6.1 (± 2.8)	0.1 (± 0.0)
defoliated – day 21	95.7 (± 2.2)	4.2 (± 2.2)	0.1 (± 0.0)
defoliated – day 30	94.6 (± 2.6)	4.6 (± 2.2)	0.8 (± 0.5)
Trifoilum subterraneum			
control – day 21	99.4 (± 0.2)	0.5 (± 0.2)	0.1 (± 0.0)
control – day 30	96.6 (± 1.2)	3.3 (± 1.2)	0.1 (± 0.0)
defoliated – day 21	97.9 (± 0.8)	1.9 (± 0.7)	0.1 (± 0.0)
defoliated – day 30	88.3 (± 4.4)	11.0 (± 4.3)	0.7 (± 0.1)

* Defoliation removed 41% and 71% of shoot material for ryegrass and clover, respectively. Exudates were collected from each treatment either immediately after defoliation (on day 21), or after a further 9 days growth of both treatments (day 30).

Sugars: Of the 11 sugars that were quantified in root exudates, fructose was the dominant sugar present in all cases (Fig. 4.19). Fructose accounted for 51.2% (±11.6%) to 72.4% (±1.8%) of the total sugars for ryegrass exudates, and 41.9% (±8.3%) to 53.4% (±4.5%) for clover exudates with the difference between species being significant ($P<0.05$), along with an interaction between species and the defoliation treatment. Sucrose, which is the major transport sugar in plants, only accounted for 4.4% (±1.3%) to 41.2% (±12.7%) and 18.8% (±9.6%) to 31.8% (±6.9%) of the identifiable sugars present in grass and clover exudates, respectively (Fig. 4.19). A significantly higher proportion of sucrose occurred in exudates (across both species) immediately after defoliation (i.e. day 21-defoliated) and a lesser percentage in exudates at 30 days relative to 21 days. Glucose accounted for 2.9% (±0.5%) to 19.8% (±1.5%) and 6.9% (±1.1%) to 29.4% (±7.4%) of the total sugars and was significantly higher in control plants of both species, at both harvest times, relative to those that were defoliated. Whilst inositol contributed only a small percentage of the total sugars in control plants (1.4% (±0.6%) to 3.6% (±1.1%) of the total), the proportion of inositol was significant higher (10.1% to 11.1% (±2.9% and ±2.1%, respectively)) in exudates collected from both species 9 days after the defoliation was imposed. In addition to these sugars minor, but not significantly different, amounts of mannitol (0.1–13.4%), arabinose (0.4–3.0%), and galactose (0.1–1.2%) were measured in the various extracts with trace amounts of xylose (<0.2%), maltose (<0.2%), rhamose (<0.1%) and raffinose (<0.1%).

Organic anions: Large differences were also observed in the relative amounts of different organic anions that were quantified in the root exudates. In particular, the proportion of quinate (quinic acid) was significantly higher in exduate collected from ryegrass roots where it accounted for 42.5% (±13.7%) to 61.0% (±9.5%) of the total pool of organic anions, but constituted less than 1.1% (±1.0%) for exudates collected from clover roots. The percentage of quinate in ryegrass exudate, however, was not significantly affected by time or by the defoliation treatment. In contrast, threonate (threonic acid) was the dominant organic anion found in clover root exudates (40.6–96.3% of the total (±4.7% and ±1.1%, respectively)) and constituted a significantly higher proportion of the organic anions at day 30 irrespective of the defoliation treatment (Fig. 4.20). In ryegrass, threonate contributed only 0.1– 2.0% (±0.7%) of the total pool of identified organic anions. For both species, malate (malic acid) was a dominant organic anion accounting for 19.0% (±9.5%) to 44.8% (±15.6%) and 0.3 (±0.1%) to 38.3% (±4.6%) for the ryegrass and clover exudates, respectively. A significantly lower proportion of malic acid occurred in exudates collected from clover plants at day 30 (Fig. 4.20). The percentage of shikimate (shikimic acid) in the exudate (ranging up to 17.5% (±4.7%) for defoliated ryegrass) similarly showed a significant interaction (reduction) with time for the clover roots but not for the grass. Citrate (citric acid) accounted for up to 5.3% (±2.3%) of the identified organic anions but showed little change in response to treatment. Only trace amounts of succinate (succinic acid, <4.3%), maleate (maleic acid, <1.8%) and fumerate (fumic acid, <0.8%) were present.

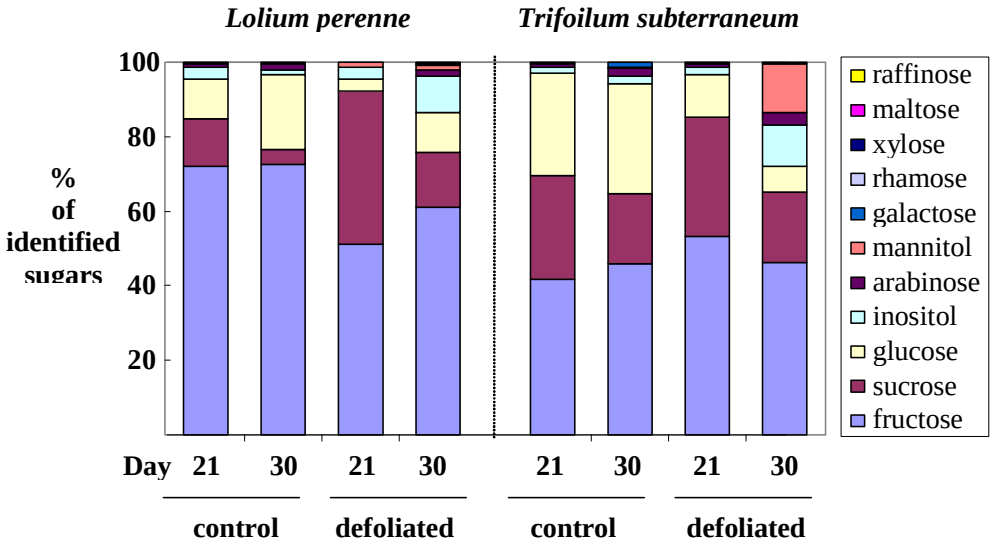

Fig. 4.19. Comparative quantitative analysis of identified sugars present in exudates of *Lolium perenne* (ryegrass) and *Trifolium subterraneum* (subclover) plants that were either subject to defoliation or grown as controls. Targeted compounds (i.e. 11 identified sugars) were measured by GC–MS and are expressed as a percentage of the total cumulative recovery of sugars. Each data column is the mean of five replicates.

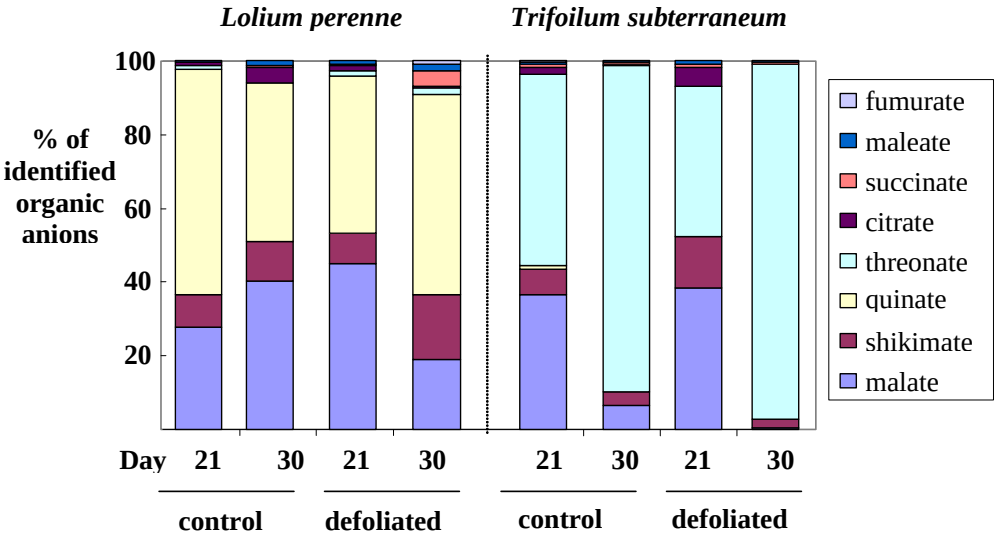

Fig. 4.20. Comparative quantitative analysis of identified organic anions present in exudates of *Lolium perenne* (ryegrass) and *Trifolium subterraneum* (subclover) plants that were either subject to defoliation or grown as controls. Targeted compounds (i.e. eight identified organic anions) were measured by GC–MS and are expressed as a percentage of the total cumulative recovery of organic anions. Each data column is the mean of five replicates.

Conclusions and implications for soil biology

Results from these experiments indicate that the defoliation influenced plant C metabolism in ryegrass and clover plants, both in terms of the total release of C to the rhizosphere and in the profile of sugars and organic anions that were identified within the exudates.

The study identified 21 compounds in exudates but the wide variation in C recovery by GC–MS analysis (2–135%) of the total C present meant that only relative differences in the compounds could be quantified. Nevertheless, it was clear that sugars (predominantly fructose) were a major constituent of the exudates with significant differences being evident between the ryegrass and subclover in terms of the proportion of fructose. Significant changes in the proportion of sucrose, glucose and inositol also occurred in response to defoliation. Large differences were also evident in the composition of organic acids in the exudates with quinate and threonate being dominant for the ryegrass and subclover, respectively. Malate and shikimate were common to both species and constituted a lower proportion of the total organic anions identified in clover exudates over time.

Differences in the content and composition of C in root exudates may influence interactions between roots and soil microorganisms. The defoliation strategy used here was replicated for ryegrass and subclover plants grown in intact and reconstituted cores of field soil which contained a natural population of root pathogens to assess whether pasture management practices (e.g. grazing/defoliation) might influence root–pathogen interactions and the potential impact of this for pasture production.

4.5 Effects of pasture management practices on rhizosphere microorganisms in plants grown in field soil

Summary

Microbial DNA assays provided insights into the behaviour of pathogenic and beneficial microbial species in a soil environment, and responses to soil and plant treatments that would have been impossible to achieve using traditional plant pathology methods. In these experiments, the microbial DNA concentrations in intact cores of soil responded predictably by declining in response to steam pasteurisation and cultivation treatments. However, host plant defoliation which was known to induce qualitative changes in the composition of C-compounds exuded from roots and, in the case of sub clover to promote a transient release of extra C from roots, had no impact of soil microbial DNA concentrations.

Improving P nutrition generally had no major impacts on microbial DNA concentrations in soil or roots with the exception of AMFa infection of subclover roots and their rhizosphere which was suppressed by application of P. Low AMF concentrations in soil and roots appeared to be accompanied by higher concentrations of *Pythium* DNA and a negative association between these organisms may occur.

The presence of plants and the species of plant host had measurable affects on microbial DNA in rhizosphere soil. In general, the presence of a plant stimulated soil colonisation, and led to root infection when the 'correct' host was present. However, take-all did not respond in this way and appeared to infect the roots of its host without a marked increase in soil colonisation. The level of soil DNA and root colonisation depended on the microorganism species or clade, and species of the host plant.

There were no clear correlations between the microbial concentrations observed in soil and the root infection of each pathogenic or beneficial microorganisms investigated.

All microbial DNA concentrations in soil were subject to high levels of variance. This was in part due to intrinsically variable spatial distribution of the microbial species in field soil, "hotspots" (e.g. *Rhizoctonia*) in the DNA concentrations of certain pathogens and intrinsically variable responses to the stimulatory presence of plant roots. These factors undoubtably contributed to the variability of microbial DNA seen in field research (e.g. MLA project SHP0025) and to the requirement for the minimum 30+ combined soil samples that is the standard procedure for sampling pathogen DNA from a field. Unfortunately, the intrinsic variability of the microbial DNA in soil makes glasshouse studies with natural populations of pathogens very difficult because space limits the replication that is required for a glasshouse study.

4.5.1 Impact of defoliation, nutrient management and soil treatments on rhizosphere microorganisms

Introduction

three experiments were done to examine effects of pasture management practices (e.g. defoliation, nutrient management, soil disturbance) on rhizosphere microorganisms in association with plants grown in field soil. Intact cores of soil were sourced from a field near Canberra after it had been established from subterranean clover bioassays and by sampling plants from the pasture, that clover roots growing in this soil often exhibited damage to primary and secondary laterals consistent with fungal root rot damage and roots of clovers in the bioassay tested positive for *Pythium* (clade F) DNA with variable amounts of *Rhizoctonia solani* AG2.2 and *Phytopthora clandestina* DNA also detected.

4.5.1.1 Experiment 1

Material and methods

Cores of field soil (8 cm diameter x 20 cm depth) were either left intact, or were tipped from the PVC coring sleeve (the "pot") and were "cultivated" by hand, before being replaced in the pot. Half of the pots of intact and disturbed soil were then steam pasteurised. Control pots were unplanted ('nil' treatment). Other pots were planted with either subterranean clover (cv. Goulburn) or perennial ryegrass (cv. Victorian) at densities of 2,200 and 5,400 viable seeds/m^2, respectively. Seeds were planted into a fine sand layer above the intact or disturbed soil core to avoid damping off and ensure uniform numbers of seedlings grew roots into the field soil.

Plants were grown for 20 days in a glasshouse with the pots maintained in a temperature-controlled chamber which simulated typical autumn soil conditions. An initial harvest of plant materials and soil was made at 20 days. Plants were either left intact or were defoliated 22 days from sowing. Final harvest was at 33 days after sowing. Each treatment had three replicates.

At each harvest, pots were cut longitudinally in half and each half analysed (Fig. 4.21). Soil below 16 cm (length of the longest roots) was discarded.

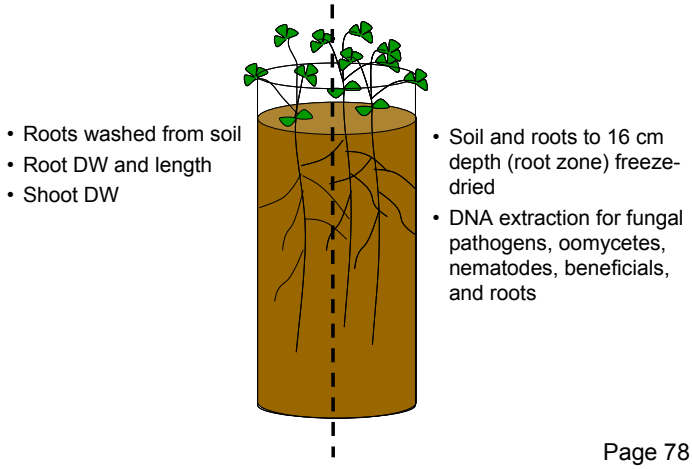

Fig. 4.21. Analyses and data collected for each half of each pot (*n* = 3).

Results

Of the soil organisms for which DNA assays were conducted, *Rhizoctonia solani* (AG2.1, AG8), *Fusarium* spp., nematodes (*Pratylenchus* spp.) and *Bipolaris* spp. were not detected in significant quantity (i.e. <1 pg DNA/g soil). Common root rot (*Bipolaris* spp.), *Phytophthora clandestina*, Blackspot (*Phoma medicaginis* var. *pinodes*) and AMF clades b, c & d were detected only at very low levels (<5 pg DNA/g soil). Relatively high levels of take-all, *R. solani* AG2.2, *Pythium* clade F and AMFa were detected in the soil cores and are, consequently, the focus of the analyses presented below.

Steam pasteurisation of the soil reduced the level of microorganism DNA to very low levels (e.g. Fig. 4.22a). Soil disturbance decreased take-all DNA levels in the soil but appeared to have little effect on other organisms (e.g. *Rhizoctonia* and *Pythium*; Fig. 4.22b). DNA assay indicated that take-all declined in concentration in soil planted with both clover and grass over time, but changes associated with some other microorganisms were not significant (Fig. 4.23). There were no significant differences in the microbial DNA concentrations in soil from the root zone of perennial ryegrass or subterranean clover in intact cores following defoliation (Fig. 4.24).

Discussion

Management interventions such as pasteurisation and cultivation of the soil reduced the DNA concentrations of one or more of the major microorganisms detected in this soil. These responses were significant and consistent with expected responses to such soil treatments. Change through time in DNA concentration was also detected for take-all. However, severe defoliation sufficient to temporarily stop root growth in both ryegrass and subterranean clover (section 4.3.1)), known to cause an initial release of C-exudate from subclover roots and qualitative changes in the C compound profile of exudates in both clover and ryegrass, did not significantly change microbia DNA concentrations of soil from the root zone of the plants.

Microbial DNA concentrations in soil at the latter harvests, and particularly those associated with defoliation treatments, were often characterised by high coefficients of variation. Combined with results from recent experiments from the Scottish Crops Research Institute (George and Richardson unpubl. data), prompted us to question whether sampling “bulk” soil in the root zone would have sufficiently diluted the influence of microbial activity in the rhizosphere or at the root surface, to have masked any real changes in soil microbial activity. The data was, therefore, also graphed per cm of root length in the root zone to assess whether roots themselves exerted a large influence on the amounts of microbial DNA being detected. For some microorganisms (e.g. AMFa and take-all on subclover), this resulted in a shift in the apparent response of the organisms to the defoliation treatment. However, variance in the data was not reduced substantially and the possible responses were not statistically significant.

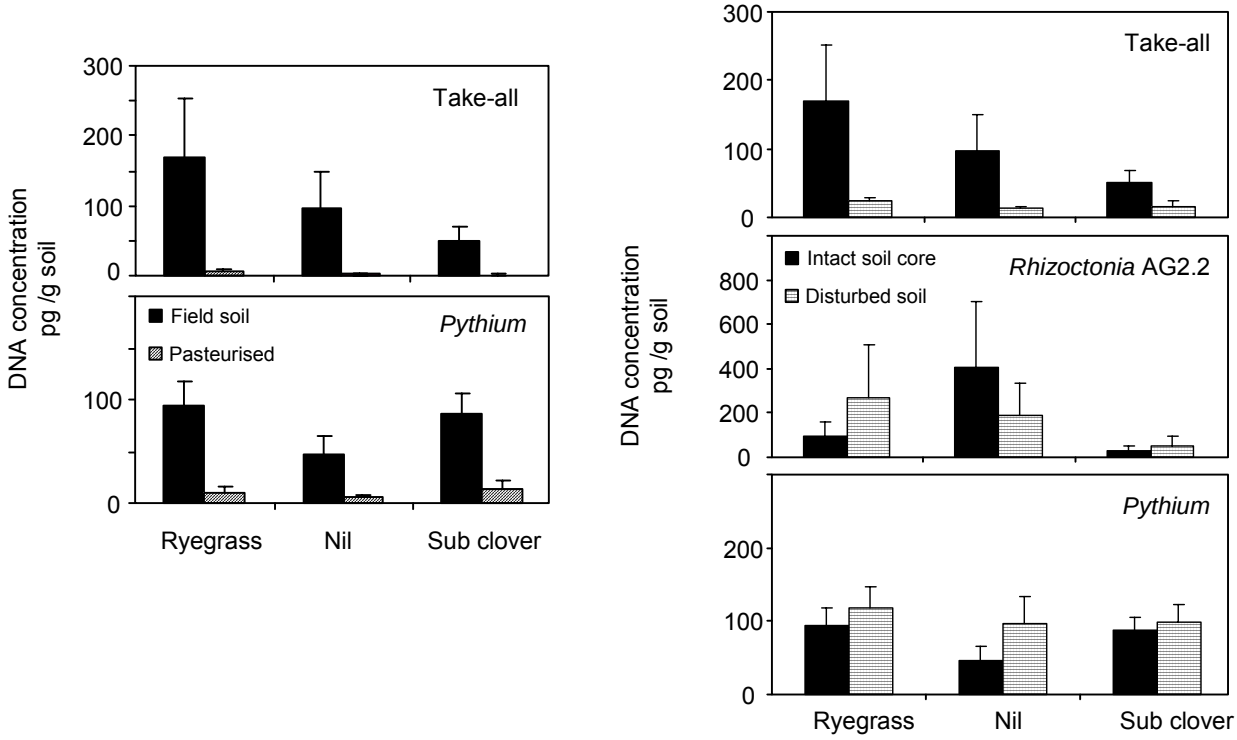

Fig. 4.22. *Left*: effect of soil pastuerisation on the DNA levels of pathogens 20 days after sowing. *Right*: effect of soil disturbance at 20 days after sowing. Bar, 1x s.e.m.

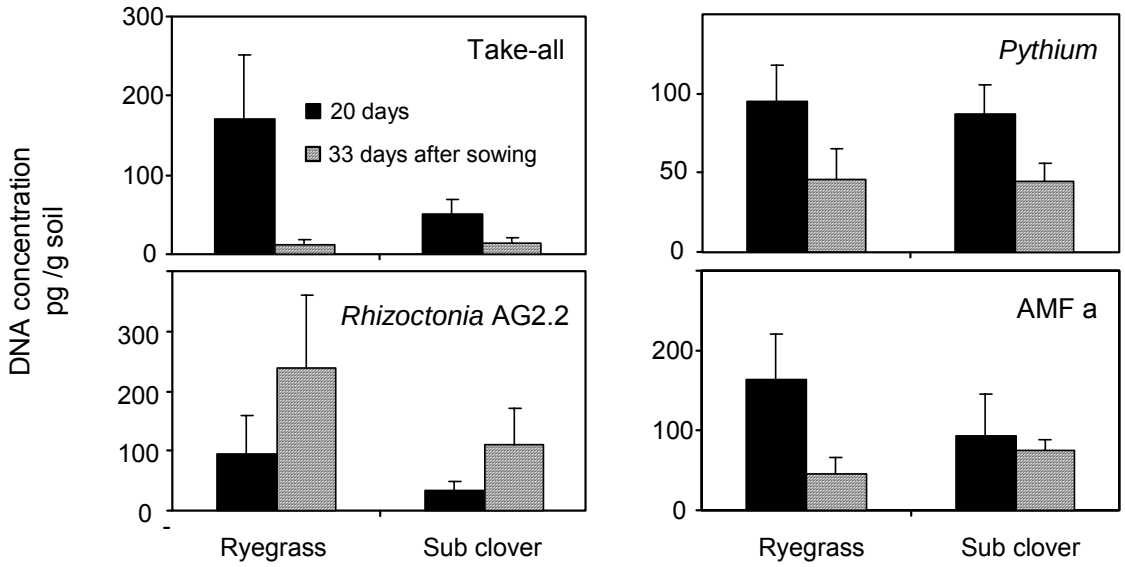

Fig. 4.23. Change in microbial DNA in intact soil cores planted to subterranean clover and perennial ryegrass which had not been defoliated at 20 and 33 DAS. Bar, 1x s.e.m

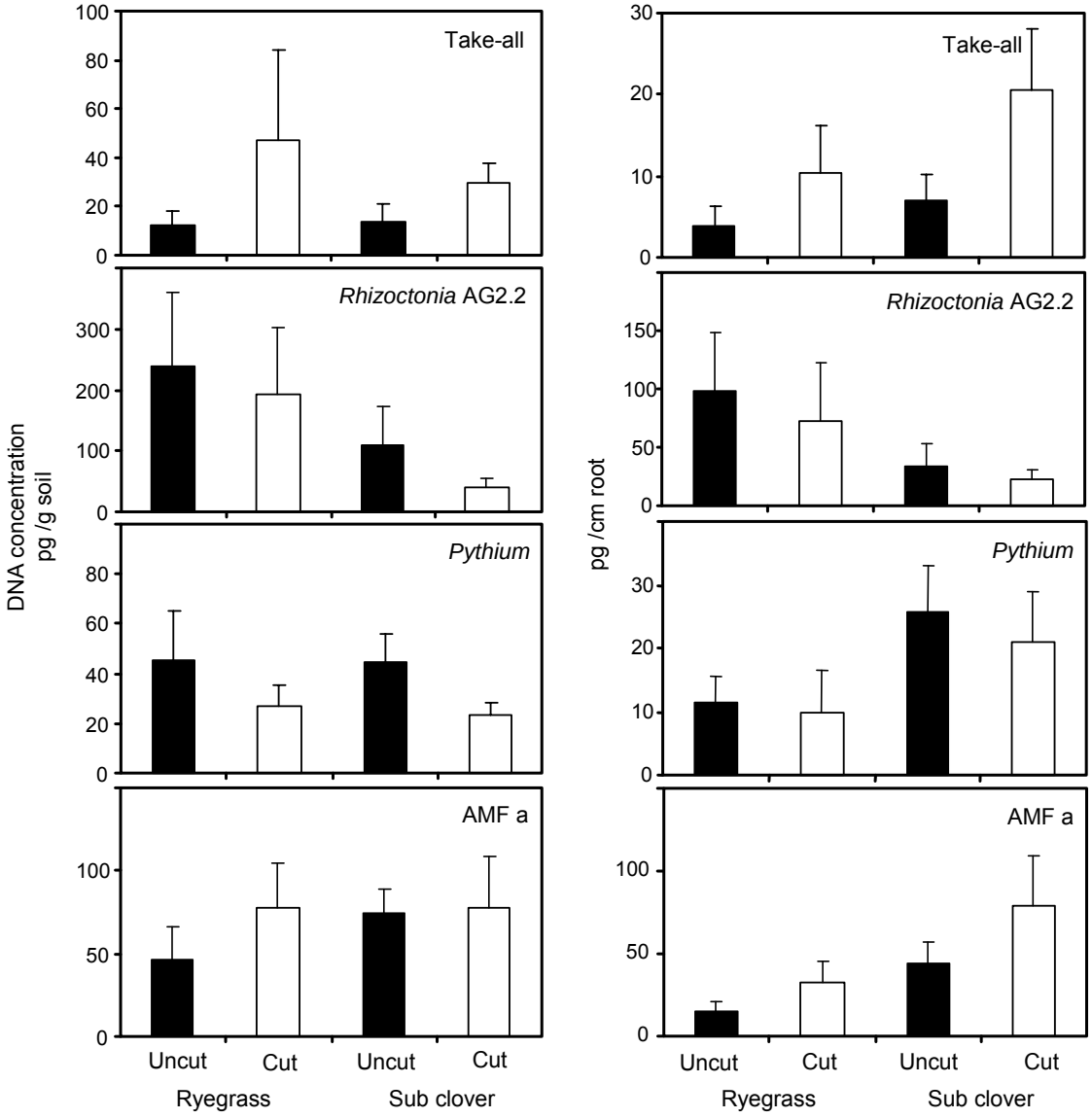

Fig. 4.24. Microbial DNA concentrations in soil from the root zone of intact cores of soil per gram of soil (left) or per cm of root (right) for cut and uncut perennial ryegrass and subterranean clover 33 days after sowing. Bar, 1x s.e.m.

4.5.1.2 Experiment 2

The influence of soil (P) fertility and defoliation on soil microbial DNA concentrations were examined in densely planted soil cores ("rhizosphere" soil) and unplanted soil cores ("bulk" soil). The microbial DNA associated with roots ("rhizoplane") was also quantified using roots washed from the soil. Replication was increased substantially in an attempt to counter the intrinsic variability in the microbial DNA being detected in intact soil cores from the field.

Material and methods

Intact cores of soil (8 cm diameter x 20 cm depth) were collected from the same field site as in Experiment 1. Any vegetation was removed by hand weeding. Control pots were left unplanted ("bulk" soil treatment). Other pots ("rhizosphere" soil and "rhizoplane" treatments) were planted with high densities of either subterranean clover (cv. Goulburn) or perennial ryegrass (cv. Victorian): 4,400 and 16,200 viable seeds/m^2, respectively. Seeds were planted into a fine sand layer on the intact soil core to avoid damping off and ensure uniform numbers of seedlings grew roots into the field soil. Each treatment had eight replicates. (-P) treatments did not receive P fertiliser and cut treatments were defoliated at 22 days after sowing.

At harvest, the top 10 cm of the core was taken to maximise the density of roots being sampled because the objective was for planted soil to reflect (as near as possible) rhizosphere soil. Roots for root mass–root DNA calibration were subsampled by cutting a longitudinal slice from the core, washing soil from the roots and gently disentangling them from old organic matter mats that were found in the soil. Roots for rhizoplane analyses (i.e. root-associated microbial DNA) were washed free of most soil but were not separated from old organic matter prior to further processing. Total root mass in the cores was estimated using root DNA amounts and the root mass–root DNA calibrations derived from the root subsamples.

Results

- Roots of subclover plants in this experiment were observed to have 0.5- (secondary lateral) and 1-score (primary lateral) root damage when they were being washed from the soil at harvest.

- The coefficients of variation for microbial DNA in intact field soil cores were again high (20–35% being common, *n*=8), with extremely high variablility recorded for some treatments and especially where relatively high mean microbial DNA concentrations occurred (e.g. Fig. 4.25).

- With one outlier value for *Rhizoctonia* removed, it was found that microbial DNA levels in 'nil' planted pots remained "relatively" stable over the duration of the experiment. However, AMFa was an exception and appeared to increase in 'nil' soil between the initial and final sampling dates (Fig. 4.25).

- Microbial DNA concentrations in the soil generally increased when the pots were planted with either subclover or perennial ryegrass (Fig. 4.26).

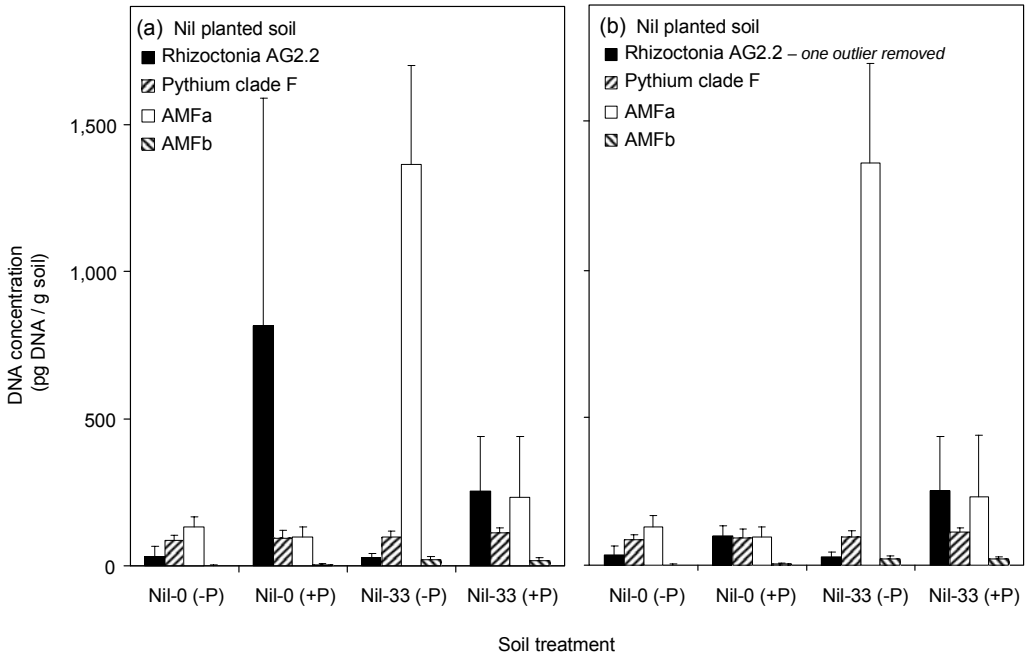

Fig. 4.25. Microbial DNA concentrations in the intact cores of soil which were not planted ("bulk" soil treatments). Bar, 1x s.e.m., *n*=8. In all cases, relatively high coefficients of variations 20–35% and greater were observed. Occasional "hot spots" for pathogen DNA were also encountered (e.g. compare *Rhizoctonia* in Figs 4.25a and 4.25b).

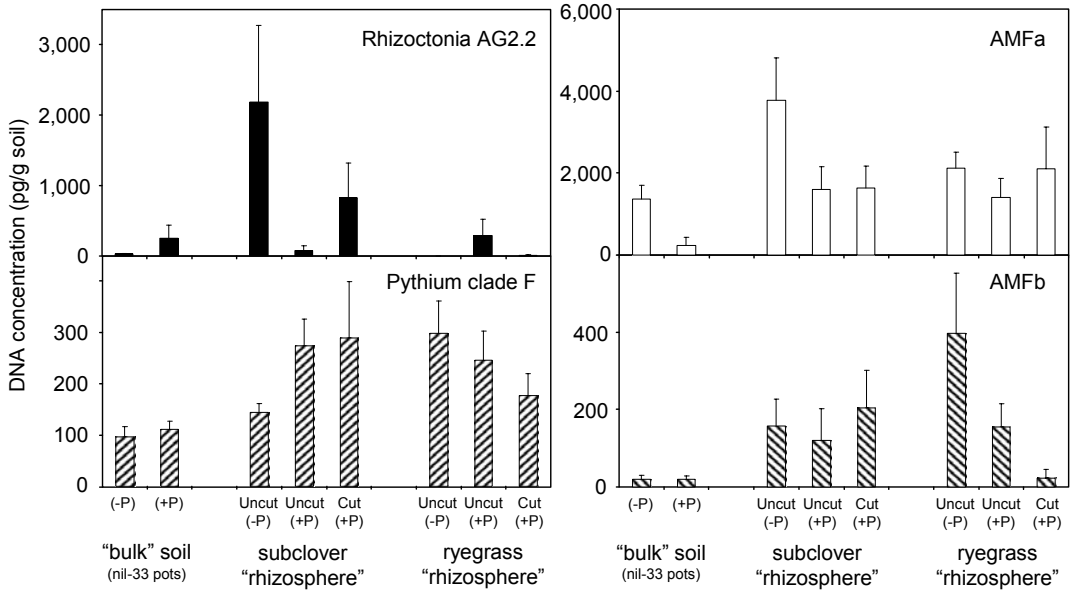

Fig. 4.26. Microbial DNA concentrations in "bulk" soil (unplanted pots) and "rhizosphere" soil (cores densely planted to either subterranean clover or perennial ryegrass). All data shown are from harvest at 33 DAS.

- Although high intrinsic variance in the data may have masked potential treatment effects, there was no consistent evidence that severe defoliation had influenced the concentrations of microbial DNA in the soil. Addition of P also did not consistently influence microbial DNA levels in the soil, but may have suppressed AMFa colonisation of the soil in sub clover treatments whilst enhancing colonisation of the soil by *Pythium*. The significant of this relationship is being analysed further. A similar effect was not observed in the perennial ryegrass treatments (Fig. 4.26).

- Microbial DNA concentrations in the soil cores were intrinsically very variable, so it was considered feasible to use the natural variability of the soil test levels to explore whether of relationships existed between soil microbial DNA concentrations and root infection by regressing soil DNA concentrations against root-associated DNA concentrations (e.g. Figs 4.28 and 4.29). No significant relationships were found.

Discussion

The concentration of microbial DNA in intact soil cores remained problematic and can only be dealt with by very high replication of treatments. In glasshouse trials where space is a limitation, high replication experiments are difficult. Plant growth clearly stimulated an increase in both pathogenic and beneficial microbial DNA generating differences between “rhizosphere” and “bulk” soil treatments.

By contrast, management interventions such as defoliation (grazing) and P fertilisation had little or no impact on soil microbial or plant root infection levels. The only potential exception was that P appeared to suppress stimulation of AMFa levels in soil planted to subterranean clover and promoted *Pythium* concentrations. It is not clear whether this response was a direct consequence of P addition on each of the organisms or was a reflection of a shift in the soil microbial population as a result of an affect on one or other of the organisms. Lower AMF infection of roots is, however, an expected outcome from P fertiliser applications. The shift toward increased *Pythium* when the AMF concentration was reduced in the soil is reminiscent of claims that AMF can protect grasses (*Vulpia ciliata*.) from root pathogens (*Fusarium oxysporum*; Newsham et al. 1995). However, the soil microbial DNA concentrations associated with the grass used in the present experiment (ryegrass) did not exhibit a similar response.

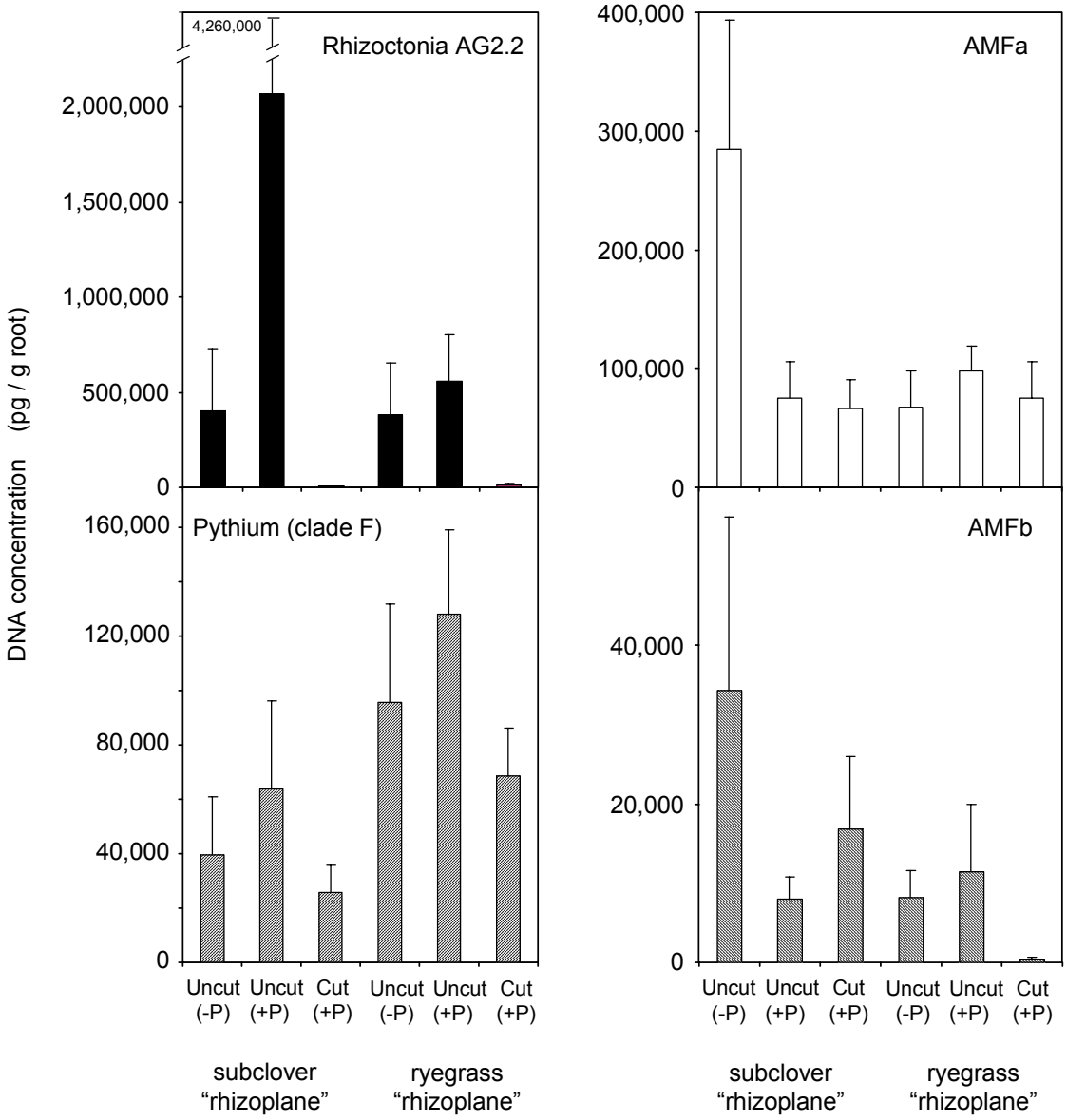

Fig. 4.27. Microbial DNA concentrations associated with roots of subterranean clover or perennial ryegrass partially washed free of soil from each intact soil core.

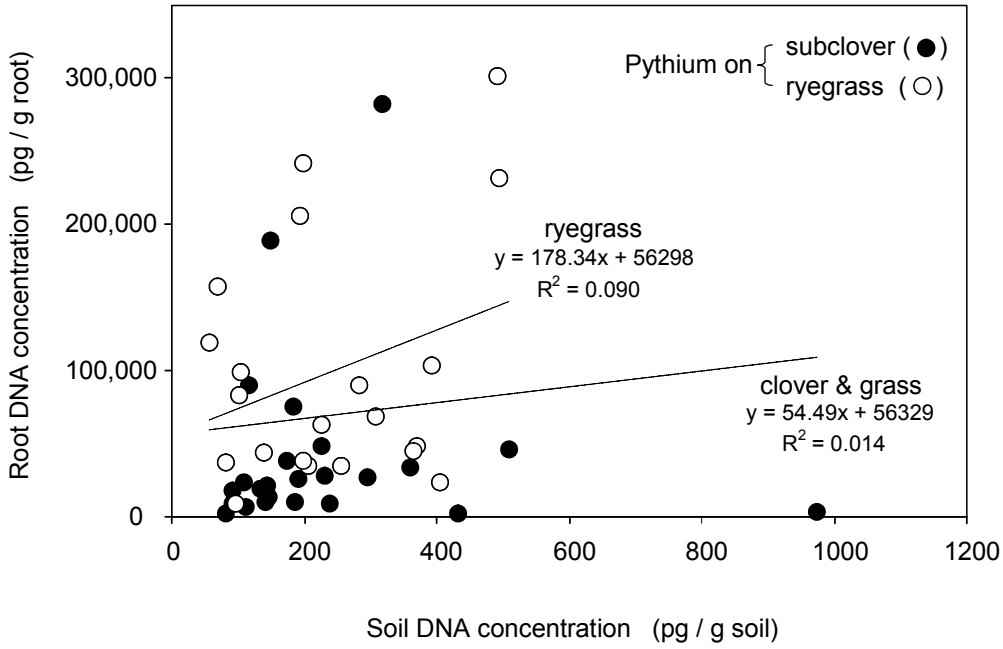

Fig. 4.28. Example of an attempt to relate soil microbial DNA concentrations (*Pythium* clade F) to infection by the same organism using the intrinsic variation in soil microbial DNA concentrations of soil cores from the field. Data are from the day 33 harvest.

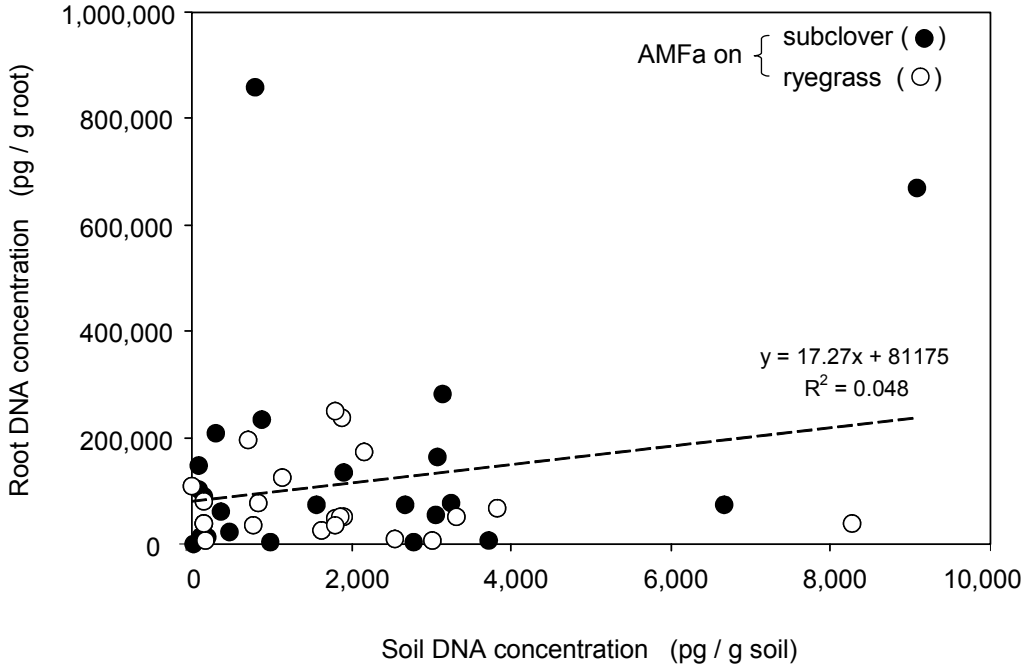

Fig. 4.29. Example of an attempt to relate soil microbial DNA concentrations (AMFa) to plant root infection by the same organism.

4.5.1.3 Experiment 3

The final experiment was conducted to determine whether the high variability of soil microbial DNA concentrations could be better managed and also to examine whether "rhizoplane" or "rhizosphere" were dominant influences on the stimulation of pathogenic and beneficial soil organisms DNA that was associated with plant growth.

Material and methods

Soil was collected from the same field, sieved fresh (2mm) and mixed to eliminate intrinsic variability in pathogen DNA concentrations. Pots of the soil were planted as above with subterranean clover (cv. Goulburn) and perennial ryegrass (cv Victorian) and were grown for 34 days. All pots were supplied nutrient including phosphorus. Each treatment had five replicates. Unplanted controls ("bulk" soil) were maintained under similar conditions. At the end of the plant growth period, pots were gently up-ended and the plants carefully shaken from the soil. Rhizosheaths (soil adhering to the roots) were present on roots of all plants and were washed from the roots into vials, frozen and freeze dried. Soil adhering to roots ("rhizosphere" soil) was then weighed. Roots were also frozen and freeze dried. Rhizosphere soil, bulk soil (unplanted), root-affected soil (whole pots from planted treatments) and roots ("rhizoplane") were assayed for microbial DNA.

Results

Disturbing, sieving and mixing the soil had the expected affect of reducing pathogen inoculum levels by 50–70% (Table 4.14). This is why intact soil cores were favoured in all previous experiments. However, variance in soil DNA concentrations was also considerably reduced at the start of the experiment and this was the main reason for used disturbed soil in this experiment.

Although pathogen DNA was associated with roots, root damage was not observed on the plants as they were washed free of soil. Levels of *Rhizoctonia* and *Pythium* appeared to increase in the presence of plant roots (Figs 4.30a and 4.30b). However, so too did the variance associated with these measurements. AMF DNA concentrations did not change significantly but this was expected given the plant nutrition regime used in the experiment (see also Fig. 4.26). This demonstrated that soil not only has intrinsically variable amounts of pathogen and AMF DNA, the proliferation of these pathogens in the presence of plant roots is also highly variable.

Take-all was unusual in that its DNA concentrations in soil did not change irrespective of the presence or absence of plant roots (Figs 4.30a and 4.30b). As expected, take-all infected ryegrass roots but had no effect on subclover roots (Figs 4.30c and 4.30d), but its DNA levels did not differ in the root-affected soil of either species.

Soil adhering to roots that had been shaken from the soil was washed gently from the roots and collected (rhizosphere soil) and analysed for pathogen DNA. When pathogen concentrations in whole-pot soil were observed to increase, the pathogen concentration also increased in this sample of rhizosphere soil but often not to the same extent that occurred in the whole pot, most likely because microbial DNA associated with root-infection was a significant additional source of the microbial DNA measured in root-affected soil.

Table 4.14. Concentration of soil microbial DNA in intact cores of soil from a field and in soil taken from the same field but disturbed, sieved and mixed. All data are from unplanted treatments at the end of the experiment (34 days).

	Concentration of microorganism DNA (pg/g soil)	
	Intact cores of field soil (Expt 2) mean ± s.d., *n*=16	Soil dug from field, sieved and mixed (Expt 3) Mean ± s.d., *n*=5
Take-all	150 (± 131)	76 (± 21)
Rhizoctonia AG2.2	63 (± 95)	22 (± 28)
Pythium clade F	90 (± 59)	26 (± 8)
AMFa	114 (± 92)	42 (± 32)
AMFb	3 (± 4)	2 (± 2)

The relative amounts of microbial DNA in bulk soil, rhizosphere soil and the rhizoplane were therefore compared by expressing root-associated DNA per gram of soil in a whole pot (Figs 4.30e and 4.30f). This indicated that root-associated microbial DNA (root infection) was a substantial but not dominant component of the microbial DNA in root-affected soil. Root infection and microbial colonization of rhizosphere soil were both concluded to be substantial components of the increase in the pathogen DNA concentration of root-affected soil for *Rhizoctonia* and *Pythium* but not for take-all where host root infection (ryegrass) occurred without substantial colonization of rhizosphere soil.

Discussion

This experiment further clarified why soil microbial DNA concentrations are highly spatially variable. Even when the initial spatial variability associated with sampling field soils was eliminated, pathogen DNA increases in the rhizosphere stimulated by the presence of plants were also very variable. For *Rhizoctonia* and *Pythium*, increase in DNA levels in root-affected soil was due to both root infection and soil colonisation. However, for take-all, roots of the host plant were infected but there was little evidence of soil colonisation.

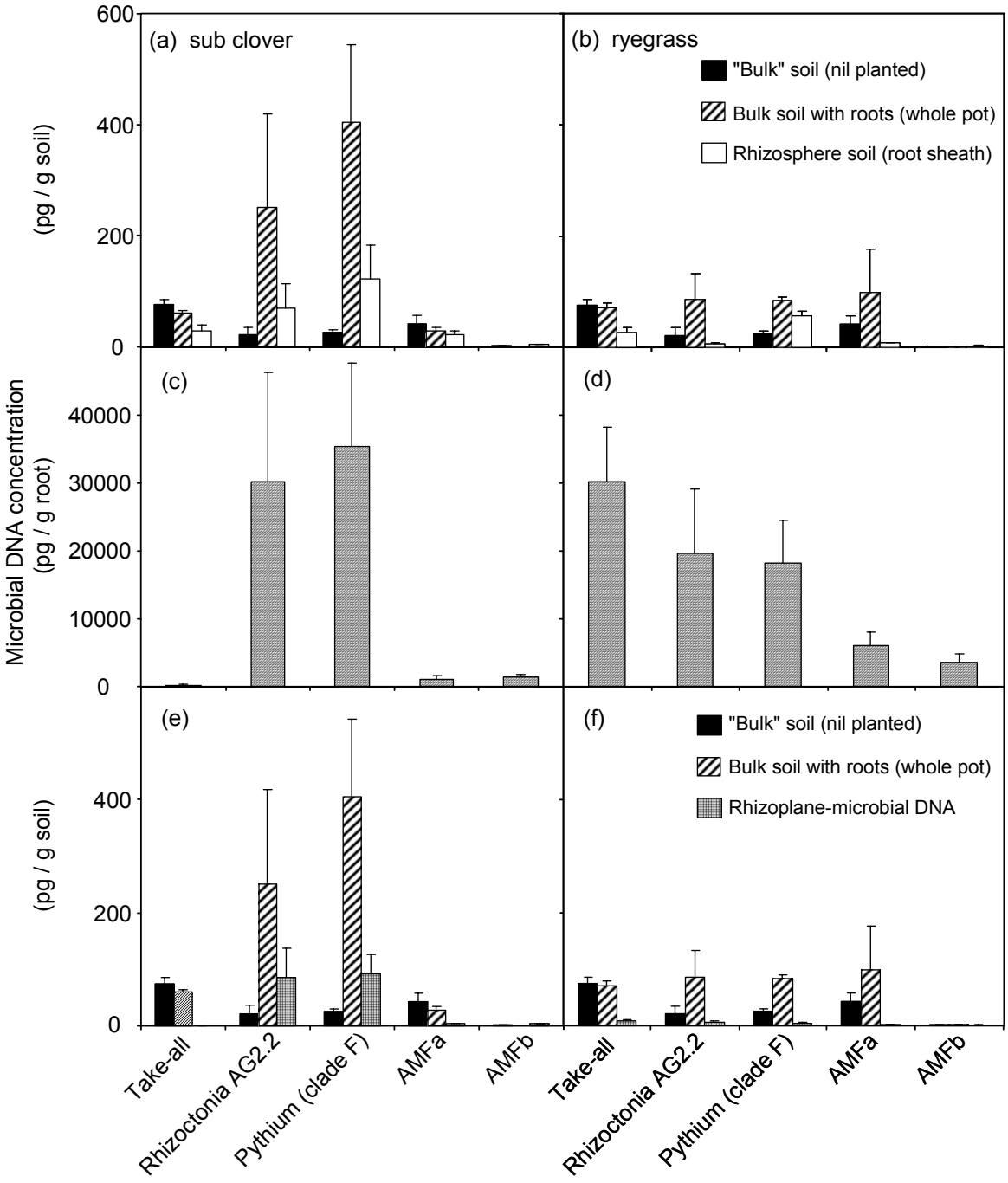

Fig. 4.30. Concentrations of microbial DNA in soil and roots from unplanted soil (bulk soil), root-affected soil (whole pots from planted treatments being the composite of bulk soil, rhizosphere soil and roots) and rhizosphere soil (root sheath soil washed gently from the roots). In panels (e) and (f) root-associated microbial DNA is expressed per gram of soil in the whole pot for direct comparison with microbial DNA concentrations in root-affected soil and unplanted soil.

Conclusions

Microbial DNA assays provided insights into the behaviour of pathogenic and beneficial microbial species in a soil environment and responses to soil and plant treatments that would have been impossible to achieve using traditional plant pathology methods. This technology continues to show great potential for opening the field of microbial ecology and management of soil biology. All microbial DNA concentrations in soil had high levels of variance. This was in part due to intrinsically variable spatial distribution of the microbial species in field soil, “hotspots” (e.g. *Rhizoctonia*) in the DNA concentrations of certain pathogens and intrinsically variable responses to the stimulatory presence of plant roots. These factors contributed to the variability of microbial DNA seen in field research (e.g. MLA project SHP0025) and to the requirement for the minimum 30+ combined soil samples that is the standard procedure for sampling pathogen DNA in a field. Unfortunately, the intrinsic variability makes glasshouse studies with natural populations of pathogens very difficult because space usually limits the replication that can be used in glasshouse experiments.

5 Success in Achieving Objectives

The project successfully met its objectives as highlighted by the following achievements:

1) Survey of the literature indicated that biological constraints are an important issue that have potential to reduce the productivity of Australian pastures. A number of important soil-borne fungal and oomycete diseases (or disease complexes) of possible economic impact can be identified. It is evident that the extent of incidence of root disease on pasture plants is influenced by a wide range of factors; with soil properties, climatic conditions and pasture management being of particular significance. Importantly, it is evident that microbial interactions in the rhizosphere may mitigate the severity of root diseases.

2) The importance of disease impact on the growth of pasture plants was illustrated by glasshouse experiments using intact and/or reconstituted soil cores that were subject to various treatments to manipulate soil biology. Whilst incidence of disease was highly variable, where evident it occurred as a disease complex, with both *Pythium* and *Fusarium* being isolated from affected soils and/or plant roots. Although subject to high variability, the results from glasshouse experiments were consistent with the incidence of disease (as indicated by extent of root damage) to pasture plants under field conditions across southern Australia (research conducted under MLA SHP025).

3) New methods were developed for the study of rhizosphere microorganisms. This included a staining and fluorescence microscopy-based technique for direct visualisation of *Pythium* infection on pasture roots and procedures for 'tagging' different species of rhizobacteria with fluorescent proteins (i.e. via plasmid-based DNA tags). A number of isolates of bacteria that showed inhibition toward the *in vitro* growth of *Pythium* in laboratory media were obtained from the rhizosphere of pasture plants. These strains along with the new techniques for their visualization provide future opportunity for more detailed investigation of microbial interactions in the rhizosphere.

4) Controlled growth studies using glasshouse conditions showed that infection of roots of pasture plants (*T. subterraneum* and *L. perenne*) by *Pythium* sp. significantly reduced root growth with loss of lateral roots being most evident. However, reliable infection assays only occurred for plants grown in sand culture, whereas inconsistent responses were observed in soils. Although measures were specifically taken to address this issue in soil (i.e. use of intact cores of field soil, controlled root temperature, controlled inoculation dose rates, etc) it was concluded that the glasshouse environment is somewhat compromised for the conduct of such studies, and that future work would need to focus to a greater extent on field conditions.

5) Attempts to isolate rhizosphere solutions and/or root exudates directly from various species of soil-grown pasture plants were unsuccessful. However, a reliable system for the collection and analysis of root exudates from pasture plants was established for plants grown in sand culture. This was used to collect and analyse exudate from plants subject to defoliation (i.e. imposed as a simulated grazing treatment) which caused a significant change to the allocation of carbon to root growth and the quantity of C present in root exudates. Comparative quantitative analysis of these exudates by GC–MS identified the presence of a range of major sugars and organic anions with some differences in composition occurring in response to defoliation, and large differences occurring between plant species. Changes to rhizosphere C flow may have important potential implication for root interactions with soil microorganisms.

6) The presence of various fungal (and oomycete) root pathogens and beneficial fungi was investigated by quantitative DNA analysis (in collaboration with SARDI) in soil and on plant roots assayed directly in soil. Whilst the presence of plant roots in all cases increased the DNA content of different fungal groups, no specific effects could be identified in response to defoliation treatments. Similarly, microbial DNA content of the soils was not affected by soil nutrient status (phosphorus), other than mycorrhizal content being increased in low P treatments. A possible interaction between the presence of mycorrhizal fungi and reduced presence of *Pythium* was identified. In all cases, there was high variability (and identified 'hot spots') in the fungal DNA content of soil, and when intact cores of field soil were used this variability was exacerbated by the presence of plants. However, no clear relationship could be established between changes in microbial DNA content in soils with changes in the rhizosphere and to the extent of fungal infection on plant roots. Further work to better understand the relationship between DNA content of different pathogens in soil and/or on plants roots and the extent of root disease is required.

6 Impact on Meat and Livestock Industry – now & in 5 years time

This project was instigated as basic research to explore new opportunities for investigating the interaction of soil microorganisms in the rhizosphere of pasture plants. The project aimed to develop and apply new technologies to visualise and quantify the effects of soil microorganisms on plant growth. As such the outputs of the project have little immediate impact on the Meat and Livestock Industry, but the technology developed and its application is important in the longer term.

Whether or not biological constraints limit the productivity of Australian pastures remains an important and topical issue. Evidence from the literature and anecdotal evidence from farmers and grower groups suggests that biological constraints are indeed significant. Results from the present study along with those from an accompanying MLA-funded project (MLA025) indicate that root diseases have the potential to significantly reduce pasture growth, through both plant mortality and restricted root growth. In addition to this, it is clear that the level of farmer interest in maintaining 'healthy' soils has increased dramatically in recent years and that there is strong (and ongoing) interest in developing a more 'biological' approach to farming systems. Critical to this is greater understanding of how different microorganisms interact within the rhizosphere and their subsequent effect on plant growth. Detailed compositional analysis of root exudates by GC–MS provides new opportunity for this to be investigated, as does the development of new technologies in fluorescent imaging and DNA-based diagnostics for the detection and quantification of specific groups of microorganisms in soil. Further development and application of such technologies is critical for future success. In particular, these techniques allow specific microorganisms to be monitored in relation to plant growth either *in situ* or without need for prior cultivation and often laborious (and technically difficult) taxonomic identification of target organisms. Improved understanding of how soil microorganisms interact with plant roots and respond to pasture management will allow more informed decisions to be made with regard to soil health and the productivity and sustainability of pasture systems. It is expected that such information will be readily available to growers and routinely used within the next 5–10 years.

7 Conclusions and Recommendations

Results from the project showed that DNA-based technologies hold considerable promise for investigating the behaviour of specific groups of microorganisms in the rhizosphere of plants. DNA-based techniques provide insight into the response of microorganisms to soil and plant treatments in ways that could not be achieved using more conventional techniques. The study also showed that GC–MS has considerable potential for quantifying the C composition of root exudates and to the understanding of how plants respond to treatments that are indicative of management options for pastures. However, further work to validate the use of GC–MS for quantification of root exudates is required. GC–MS was successfully used for determining the relative quantification of C profiles, whereas there is need to develop appropriate internal standards for absolute quantification of individual compounds. Nevertheless, these technologies collectively provide new opportunity for investigating the microbial ecology and management of soil biology in pasture systems.

Microbial DNA assays successfully quantified groups of pathogenic and beneficial microorganisms, both directly in soil, the rhizosphere and within plant roots. Microbial DNA contents in soil and the rhizosphere were clearly increased by the presence of plants which can be assumed to occur through the provision of root C. However, the response of microorganisms to imposed plant treatments that were indicative of pasture management techniques were not straightforward. For example, although defoliation altered both the content and composition of rhizosphere C, it had no consistent effect in changing the profile of individual soil pathogens. On the other hand, low P fertility promoted the presence of mycorrhizal fungal with some evidence for lesser content of root colonising pathogens such as *Pythium*. Indeed, the basis of this interaction and its implication for root health and pasture productivity warrants further investigation.

All microbial DNA concentrations in soil were, however, subject to high variability. This was largely associated with intrinsic spatial variability in the distribution of the microbial species in field soil (i.e. 'hot spots'; e.g. *Rhizoctonia*). High intrinsic variability of different pathogens was further exacerbated by the stimulatory presence of plant roots, which meant that it was difficult to interpret results from treatments effects that were imposed under glasshouse conditions. Whilst a number of measures were undertaken to limit this variability (i.e. increased replication, use of mixed and reconstituted soil cores rather than intact field cores, and controlled inoculation treatments), these also proved to be largely unsuccessful for the conduct of root pathology studies in the glasshouse. Moreover, it was determined that replication would need to be increased substantially (i.e. as much as 30 replicates per treatment, which is consistent with what is now accepted as minimum set of combined soil samples for the routine sampling of pathogen DNA in a field soil) to overcome the variability. In addition, it was evident from glasshouse studies that the presence of high DNA content of specific pathogens was not necessarily indicative of high incidence of root damage and/or clear evidence of root disease. Further work to investigate the basis of this relationship is needed.

Recommendations:

Further studies to correlate measured DNA content of specific pathogens and incidence of disease on plants roots is required, as this relationship remains poor. Whilst it may be a consequence of the glasshouse grown plants used in this study, we are also aware of similar issues under field conditions. It important that 'threshold' levels of microbial DNA or the interaction between levels of inoculum and its interaction with environmental conditions that lead to disease by established. In

addition to ensuring that the DNA probes being used are representative of causative organisms, there is clear need to further investigate the importance of environmental factors (e.g. soil type, climatic conditions, management practices, etc) that contribute to the 'outbreak' of root diseases. As well as greater use of field sites, this will require some input from a plant pathologist. Future studies on the molecular ecology of soil-borne root pathogens therefore need to focus more on field studies, with glasshouse studies being used for simple validation (e.g. pathogenicity tests conducted in sand culture) and/or high-throughput germplasm screenings only.

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