final report

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Root damage on subterranean clover in autumn-winter.

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Abstract

A “bioassay” was employed to assess claims that adverse soil biology may constrain pasture production. The study indicated that loss of seedlings and root damage on subterranean clover during autumn-winter was substantial and widespread. Clear costs for pasture renovation were indicated even when good practices are employed. Moderate to severe root damage was recorded at all sites. This was associated with poor shoot vigour. It is possible that sub-lethal damage to pasture roots constitutes a large, but underestimated cost to production because it was so widespread and because the damage occurs during autumn-winter when pasture yield limits stocking rate. DNA probes for root disease pathogens identified the pathogen profiles of paddocks and may be useful for indicating disease risks, guiding plant cultivar selection and appropriate use of pesticides. The probes enable novel insights into soil biology in farming systems.

Executive Summary

Why the work was done

Numerous studies report pasture growth responses to fungicides, nematicides and/or fumigation treatments suggesting that some aspects of soil biology potentially constrain pasture production. Root rot fungi are likely to cause such a constraint. They have been known since the 1960's to occur widely in subterranean clover pastures and large claims are made about the extent to which they reduce pasture yield or even cause legume failure. However, most studies that indicate yield increases have been under artificial or experimental conditions and it was unclear whether the results can be extrapolated confidently to production in grazed paddocks. Early work in the Pasture Soil Biology Program also demonstrated that there are substantial problems when using biocide treatments to explore soil biology constraints. Biocides were only partially effective against the target organisms, the efficacy of soil drenches was affected by soil type and/or the biocides were phytotoxic for plant growth. These results caste doubt on whether experiments reported in the literature reflected an unbiased sample of outcomes from studies of the impacts of soil biology.

An alternative "bioassay" approach was devised as a possible way to progress the assessment of potential for soil biology constraints in pastures. Seedling survival and damage to roots (similar to that expected of damping-off and root rot pathogens) was quantified after sowing a susceptible cultivar of subterranean clover (cv. Woogenellup) at 11 pasture sites/treatments in NSW, 4 in SA, 3 in WA. Lucerne (cv. SARDI 10) was the test species at 4 sites in the SA mallee. Annual ryegrass (cv. Safeguard) was also sown for comparison but grass root damage was not assessed.

New DNA probes for common root pathogens, some beneficial soil fungi and nematodes relevant to subterranean clover pastures were also employed to establish the prevalence of these soil micro-organisms at the sites.

What was achieved

The first objective of this project was to test the application of the bioassay of early-season root damage. The bioassay approach was successful and indicated there is a widespread and substantial potential in autumn-winter for subterranean clover seedlings to either: (a) fail to emerge, or (b) to germinate and establish, but with a high incidence of damaged roots.

Failure of seedlings to emerge: There was significant early loss of both subterranean clover and annual ryegrass seedlings. Between 20 and 35% of subterranean clover seedlings were lost prior to emergence at a majority of sites. In severely affected pastures, 50-90% of clover seedlings failed to emerge.

Root damage: At every site, substantial root damage occurred on the subterranean clover and lucerne test plants that survived emergence and managed to establish. On average, only 34% of subterranean clover plants established with undamaged roots and in the best case only 60% of established plants had undamaged roots. This was despite soil moisture conditions across southern Australia in the 2006 autumn-winter period that were not expected to be particularly favourable for most root rot pathogens. Shoot yield of the plants was also adversely affected by root damage but it was outside of the scope and resources of this project to quantify the impact of seedling loss and root damage on pasture yield.

The project also aimed to provide a preliminary assessment of how DNA probes for plant species, common root pathogens, nematodes and some beneficial soil fungi may be used to indicate site characteristics and the presence and relative abundance of particular soil microfauna groups.

Presence of plants, pathogens and potentially-beneficial organisms was successfully quantified using the DNA probes. For the first time, it is possible to rapidly identify the pathogens likely to cause problems at different sites and to explore the ecology of organisms such as AMF which are difficult to study. Indeed, the extent to which AMF clades vary in their relative abundance between sites is essentially impossible to study using current technologies. The results presented in this report demonstrate that this technology has the potential to revolutionise the study of AMF ecology, infection of plant roots and soil biology in general.

Soil micro-organisms varied considerably between sites and the DNA probes enabled the sites to be characterised by their soil micro-organism profiles. For instance, some sites appeared to have only a few pathogens whilst others possessed nearly all of the pathogens that can be detected currently. Some organisms, e.g. *Phytophthora clandestina*, were only present at particular sites; while others, e.g. *Pythium*, were universal. Developing a pathogen profile for a site by standard pathology methods alone would be difficult, time-consuming and potentially inaccurate because it is always possible to miss key species when trapping and culturing from infected roots. However, DNA probes only detect the organisms that they were developed for and will, therefore, be best employed in combination with standard pathology methods.

When, how and who can benefit from the work

This study indicates that loss of seedlings (putatively to damping off and root rots) presents a large financial risk during pasture establishment even when good sowing practices are employed and emphasizes the importance of treating pasture seeds with fungicide prior to sowing. This technology is available now, but greater emphasis of the benefits of treating seeds should improve uptake of the technology. Development and deployment of diagnostic tests, such as the DNA probes being trialled in this project, will be a medium term task as some further development and testing is desirable. However, these tools have the potential to further reduce establishment risks because paddocks at risk can be detected readily and cheaply and it is also possible for the information to be used to guide plant cultivar selection or appropriate use of pesticide treatments.

It was obvious from the poor shoot vigour of test plants, that reduced pasture yield will be associated with root damage. Indeed, it is likely that sub-lethal infection and damage to pasture roots constitutes a large, but underestimated cost to production because it was so widespread (moderate to severe root damage occurred at every site) and because the damage occurs during autumn-winter when pasture yield limits stocking rate. Although grasses were not the primary focus of the present experiment, the data indicate that they may be similarly affected and further investigation is warranted.

Damping-off and root rot on subterranean clover has been recognised across southern Australia for 50 years and some progress has been made in selecting cultivars with increased field resistance to root rot. However, the extent of the root constraints observed in this study was alarming and it is clear that the true cost of autumn-winter root damage to production systems in unknown. It is necessary to investigate whether field resistance to root rots has developed in paddocks exposed to root pathogens over long periods of time, and to quantify the extent to which modern cultivars resist root damage organisms. It appears possible that pasture production and stocking rates could be lifted if it proves feasible to reduce or eliminate autumn-winter root damage.

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1 Background

Numerous studies report pasture growth responses to fungicides, nematicides and/or fumigation treatments suggesting potential soil biological constraints to plant growth. Root rot fungi are highly likely to cause such a constraint. They are widespread in subterranean clover pastures and large claims are made about the extent to which they reduce pasture yield or even cause legume failure (e.g. Johnstone and Barbetti 1987; Barbetti *et al*. 2006). However, early work in the Pasture Soil Biology Program demonstrated the difficulties of using biocide treatments to explore soil biology constraints (Stirling and Lodge 2004). Biocides can be only partially effectiveness against the target organisms, the efficacy of soil drenches was affected by soil type and/or the biocides were phytotoxic for plant growth.

An alternative "bioassay" approach was devised as a possible way to progress the assessment of potential for soil biology constraints in pastures.

2 Project Objectives

(i) to test the use of a bioassay to assess potential for soil biological constraints to pasture growth;

(ii) to establish the prevalence and potential for root disease on subterranean clover during germination, establishment and early growth in autumn-winter (NSW, SA and WA). Soils in lucerne growing areas at some SA sites were assayed using lucerne instead of subterranean clover;

(iii) to collect soil from each site for analysis by the SARDI DNA probes for common root pathogens, some beneficial soil fungi and nematodes relevant to subterranean clover to give a preliminary assessment of how DNA probes may be used to indicate presence and relative abundance of soil microfauna.

3 Methodology

3.1 The research team

Richard Simpson, Alan Richardson (CSIRO Plant Industry, Canberra)
Ian Riley, Alan McKay, Ross Ballard, Suzanne McKay (SARDI, Adelaide)
Tiernan O'Rourke, Martin Barbetti, Krishnapillai Sivasithamparam, Hua Li (University of Western Australia, Perth).

Acknowledgements: Thanks are due to Adam Stefanski, David Marshall (CSIRO) for technical support and collaborating farmers at numerous locations on the southern tablelands NSW (B. Hazell, D. & T. Hewlett, P. Walker, P. Dunbar & B. Isaac, A. Campbell, J. Etcell) south-west slopes NSW (G. & A. Burbidge), south-east SA (G. Cunningham, S. Miller, J. Cooper), SA mallee (C. Swan, T. Freak, R. Spindley, A. Piggott) and south-west WA (K. Forbes, J. Rodgers, F. Knight).

3.2 Seedling survival and root damage bioassay

3.2.1 Sites and pasture systems examined

Subterranean clover-based pastures at six paddock locations on the southern tablelands and two on the south-west slopes of NSW, four in south-east SA and three in south-west WA were used to survey for presence of root pathogens and root damage symptoms in a subterranean clover bioassay. A further four paddock locations in the SA mallee were assayed using lucerne as the test plant (Fig.1 and Appendix 1).

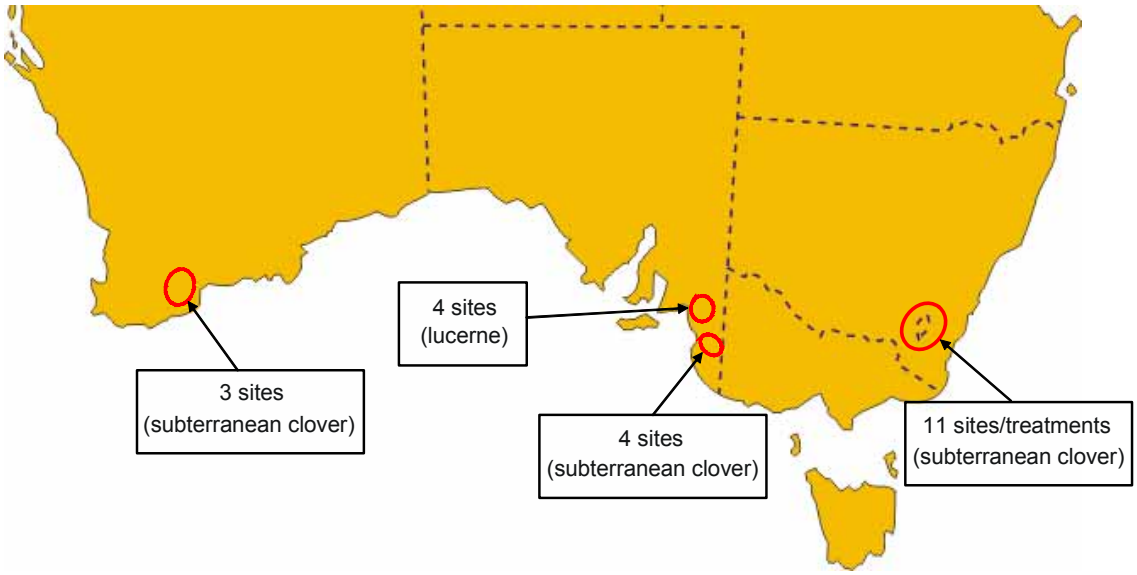

Figure 1. Locations of paddocks surveyed using the bioassay.

3.2.2 Bioassay

Seedling survival during germination and establishment was assessed after sowing subterranean clover (*Trifolium subterraneum* cv. Woogenellup) or lucerne (*Medicago sativa* cv. SARDI 10) and annual ryegrass (*Lolium rigidum* x multiflorum cv. Safeguard) with minimal disturbance into each pasture site in a bioassay based on the plant pathology protocol of Wong *et al*. (1985). The protocol was originally devised to assess the prevalence of damping off and root rot pathogens on clover. However, causal organisms were not isolated from damaged roots in the present survey. Damage to roots of the legumes that managed to establish was also assessed.

T. subterraneum seed was sourced from Cleanseeds Pty Ltd, (Bungendore, NSW) and was sieved to provide seed in the 2.2-2.8 mm diameter range. *Lolium rigidum x multiflorum* was sourced from Valley Seeds Pty Ltd and provided by David Kessell (Development Officer-ARGT, Centre for Cropping Systems, Northam WA) and *Medicago sativa* was from the South Australian Research and Development Institute. The germination of each seed batch was tested and lots of 100 germinable seeds were surface sterilised by washing in 70% ethanol for 30 seconds and dried prior to planting. Approximately 1 month after the break of season at each site, eight replicate areas for the legume

and three replicate areas for ryegrass were prepared by removing the top 0.5-1 cm of soil and plant material from rows 1.2 m long x ~0.25 m wide using a flat, sharpened trenching shovel (Fig. 2a). This removed most other subterranean clover seed and reduced the potential for interference from weeds. A furrow (~4 mm wide x 5 mm deep) was formed by pressing a 1m-long, Y-shaped iron bar gently into the exposed soil and the surface-sterilised seeds were sown evenly along the row and covered lightly by brushing soil over the seed (Figs 2b and c).

Figure 2a. Half to1 cm of soil was removed with a sharpened spade.

Figure 2b. A star-picket was pressed into soil to form a furrow into which seeds were sown.

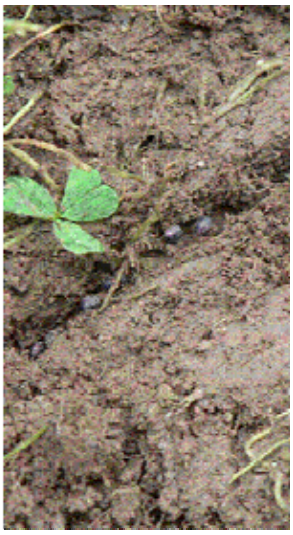

Figure 2c. Seeds sown along the furrow before being covered with soil.

Immediately after sowing, the whole area and a 2m barrier beyond the rows of sown seeds was sprayed with bifenthrin (Talstar) at 100 ml/ha, or an equivalent insecticide, to protect seedlings from insect damage. Where necessary the bioassay area was protected from grazing animals by fencing or exclusion cages.

The number of plants that emerged were counted typically when the primary leaf of the clover or lucerne had expanded (about 2-4 weeks after sowing depending on site conditions), and again when 2-3 trifoliate leaves were present (about 4-8 weeks depending on site conditions). At this time all surviving plants were carefully dug from each row and soil was washed from the roots to allow the severity of any root damage to be scored. If soil could not be removed from roots immediately the clods of soil were kept intact and cool (4^{o}C) for up to 12-18 h prior to being washed.

3.2.3 Root damage scores

Washed roots (intact seedlings) were floated in a shallow tray and scored using a 4-step rating scheme (the 4th group also comprises plants that have died and were not present). The number of plants in each group was counted.

Rating	Description
0	tap and lateral roots healthy and not discoloured
1	whole tap root light brown to brown, usually lateral roots affected
2	tap root stunted and brown to black, discrete lesions may be present
3	whole tap root rotted off, or seedling is dead.

Average root damage (disease) indices (%), based on the root damage ratings of surviving and dead seedlings were calculated using the method described by McKinney (1923).

(a) Total root damage index (damping off and root rot) = $\frac{\text{Sum of all numerical ratings x 100}}{\text{Number of germinable seeds x 3}}$

(b) Post-emergence root damage index (root rot) = $\frac{\text{Sum of all numerical ratings x 100}}{\text{Number of plants surviving the first assessment x 3}}$

3.2.4 Detection of common soil microfauna using DNA probes

The presence and relative abundance of a number of soil micro-organism groups representing common root pathogens, beneficial fungi and nematodes was assessed using new DNA probes developed by the SARDI team involved in this project.

Soil for DNA probe analyses was collected at sowing by combining eight, 2.5 cm diameter soil cores (0-10 cm depth) removed from the area around the furrow in which the test plants had been sown and at the final harvest, when eight cores were removed at 10 cm intervals along the furrow. The soils samples were frozen by placing them on dry ice and were either freeze dried or maintained at -20^{o}C until analysed.

DNA was extracted from soil samples using proprietary procedures developed by the SARDI Root Disease Testing Service and assayed by real-time PCR for the organisms listed in Table 1.

The test results were converted to picogram of target DNA per gram of oven-dried soil, except for the nematodes, where the concentration was estimated in numbers of nematode per gram of soil.

At the time of the analysis, standards had not been developed for the *Phalaris* and AMF assays. The number of PCR cycles taken to detect the target was converted to DNA concentrations for *Phalaris* based on the *Trifolium* test. The AMF assays were converted using a common standard curve to allow comparison of levels between treatments for the same group. However, quantitative comparisons cannot be made between AMF groups or with other tests.

Table 1. Plant species, common root pathogens, nematodes and beneficial fungi for which DNA probes were available.

Plants	*Lolium/Fescue* spp., *Trifolium subterraneum* and *Phalaris aquatica*
Fungal pathogens	*Bipolaris sorokinana* (Common root rot), *Gaeumannomyces graminis* var. *tritici* and var *avenae* (Take-all), *Fusarium culmorum/graminearum*, *Fusarium pseudograminearum* (two types), *Mycospaerella pinodes*/*Phoma medicaginis* var. *pinodes* (black spot complex), *Phoma* sp (associated with Black Spot) and *Rhizoctonia solani* (AG2.1, AG2.2, AG4 and AG8)
Oomycete pathogens	*Phytophthora clandestina* and *Pythium* Clade F (after Levesque and de Cock, 2004)
Nematode parasites	*Ditylenchus dipsaci, Heterodera avenae*, *Meloidogyne javanica/incognita/arenaria*, *Pratylenchus neglectus, P. penetrans, P. thornei*
Arbuscular mycorrhizal fungi	AMF groups a, b, c, d and e

4 Results and Discussion - Section

4.1 Loss of seedlings

All sites in the SA mallee were severely drought affected limiting the interpretation of their results. Consequently, less emphasis is placed on data from sites sown to lucerne except where interpretation of the data was not confounded by the dry conditions. All but three of the subterranean clover sites were considered to have had reasonable soil moisture conditions for germination and emergence. One site (Mines, NSW) was irrigated until rain occurred; plants emerged late at the other two sites (Willalooka & Kybybolite, SA) when rain occurred. Data from the unwatered sites is, therefore, not included in the analysis of seedling emergence.

4.1.1 Failure of seedlings to emerge

In moist soil conditions, seedling counts after emergence were expected to indicate losses due to "damping off". At 8 of 13 subterranean clover sites in this category, equivalent proportions of clover and annual ryegrass (9-52%) failed to emerge. At other sites (particularly Mines, NSW; Denmark and Denbarker, WA), more of one species failed. In the worst case (Mines, NSW), 93% of subterranean clover failed to emerge (Fig. 3).

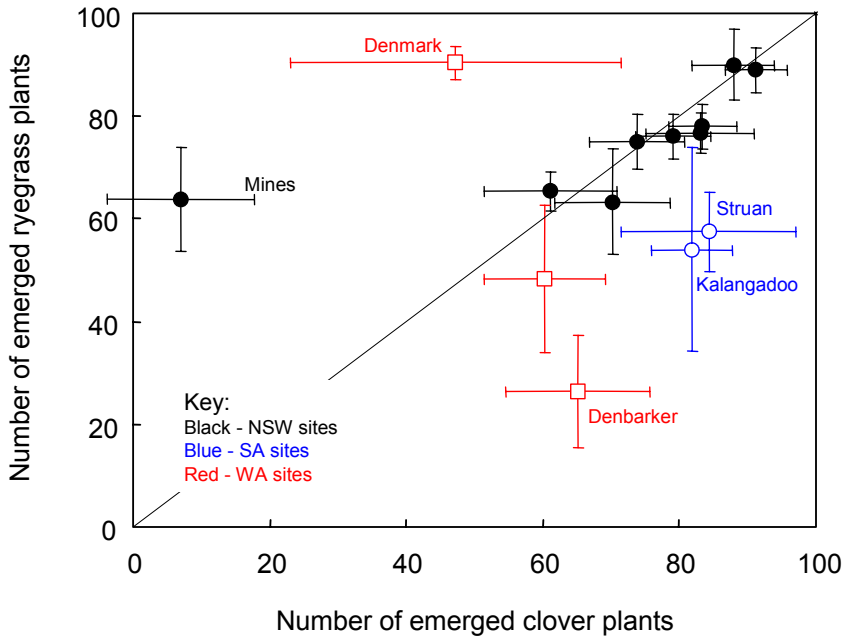

Figure 3. Numbers of annual ryegrass and subterranean clover seedlings that emerged at sites with adequate soil moisture. The diagonal line shows where points would fall if the losses of both species were equivalent. Bars = 2xSD.

4.1.2 Post-emergence clover losses

Loss of subterranean clover plants after emergence was considered likely to reflect the impacts of "root rot". On average, post-emergence losses were about 9% and generally appeared to be less than plant losses during germination and emergence (Fig. 4). However, the range was very wide (0-32%). Often the magnitude of post-emergence losses were similar for subterranean clover and annual ryegrass but at some sites (e.g. Mt Barker and Denbarker, WA), subterranean clover seedlings were lost without any apparent loss of annual ryegrass. Post-emergence loss of lucerne at one site was also estimated to be substantial (~40%; data not shown).

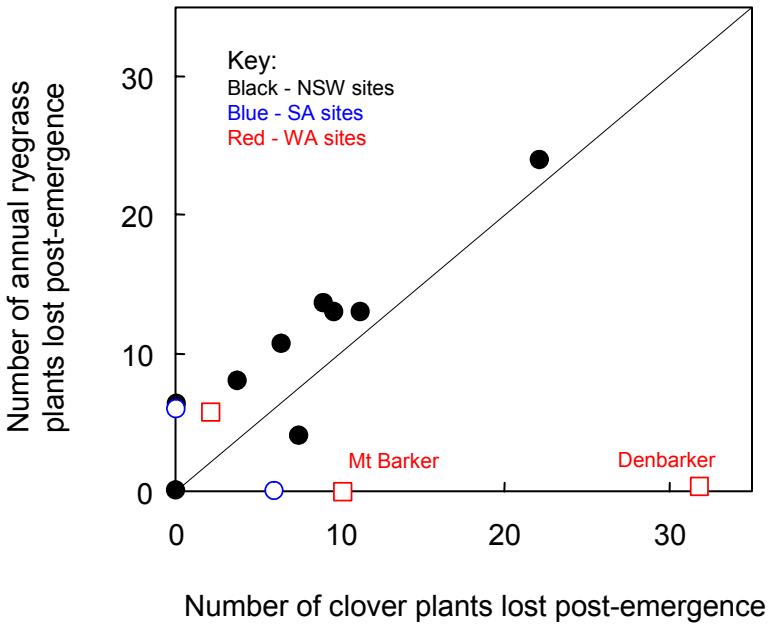

Figure 4. Numbers of annual ryegrass and subterranean clover seedlings lost after having emerged succesfully. The diagonal line shows where points would fall if the losses of both species were equivalent.

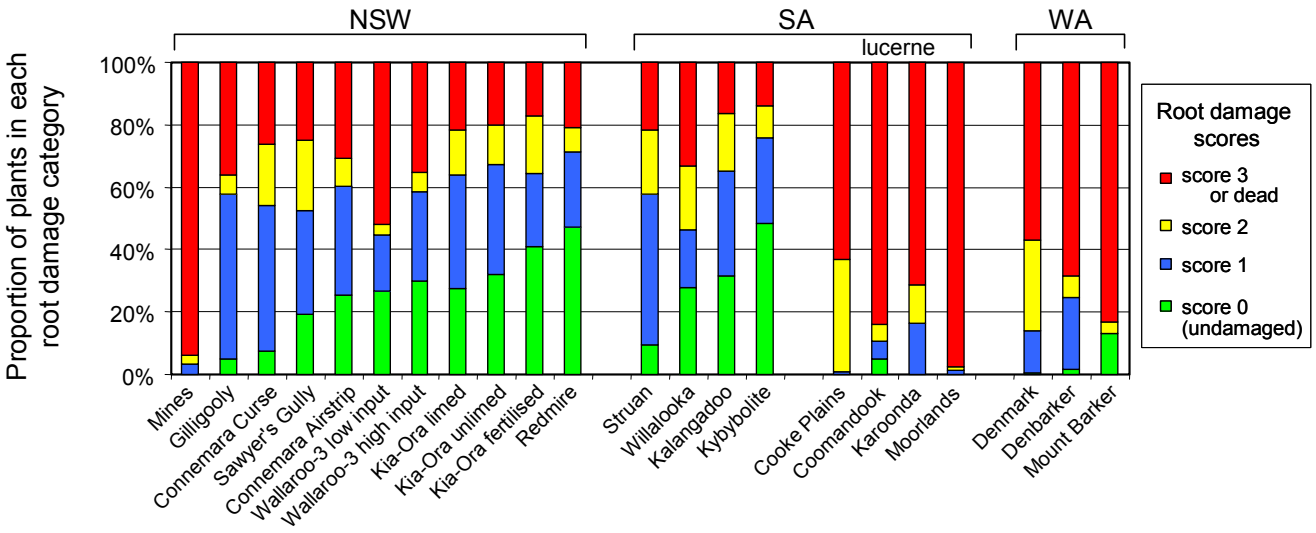

Figure 5. Root damage scores for subterranean clover and lucerne at the final harvest date (all sites).

The combined effect of pre- and post-emergence losses of subterranean clover was very large at some sites (Fig. 5; score 3 category). All WA sites and one NSW site experienced losses greater than or equal to 60% of seedlings. Total subterranean clover seedling loss at the majority of sites with adequate moisture for germination and establishment were nevertheless substantial (about 20-35% of seedlings). Note: Lucerne seedling losses also appear very high but this result was confounded by the drought conditions at these sites and is, therefore, not regarded as a reliable test of the potential for seedling loss due to root disease.

4.1.3 Effects of pasture treatments

Limited opportunities existed to compare the impacts of pasture management on seedling losses and clover root damage. Subterranean clover losses were not affected by application of lime to an acid soil (data not shown). However, loss of clover and ryegrass seedlings was significantly less in fertilised pasture grazed continuously by 18 sheep/ha compared with unfertilised pasture grazed by 9 sheep/ha (Fig. 6).

4.2 Root damage

Damage to subterranean clover roots was scored using a modification of the pathology scoring system reported by Wong *et al*. (1985) (e.g. Fig. 7). These data were also used to calculate root damage (disease) indices for (a) total root damage (damping-off and root rot) and (b) post-emergence root damage (root rot).

No site had more than 50% of plants germinating and establishing with undamaged roots. At a majority of sites less than 30% of plants were able to establish with undamaged roots. It was clear

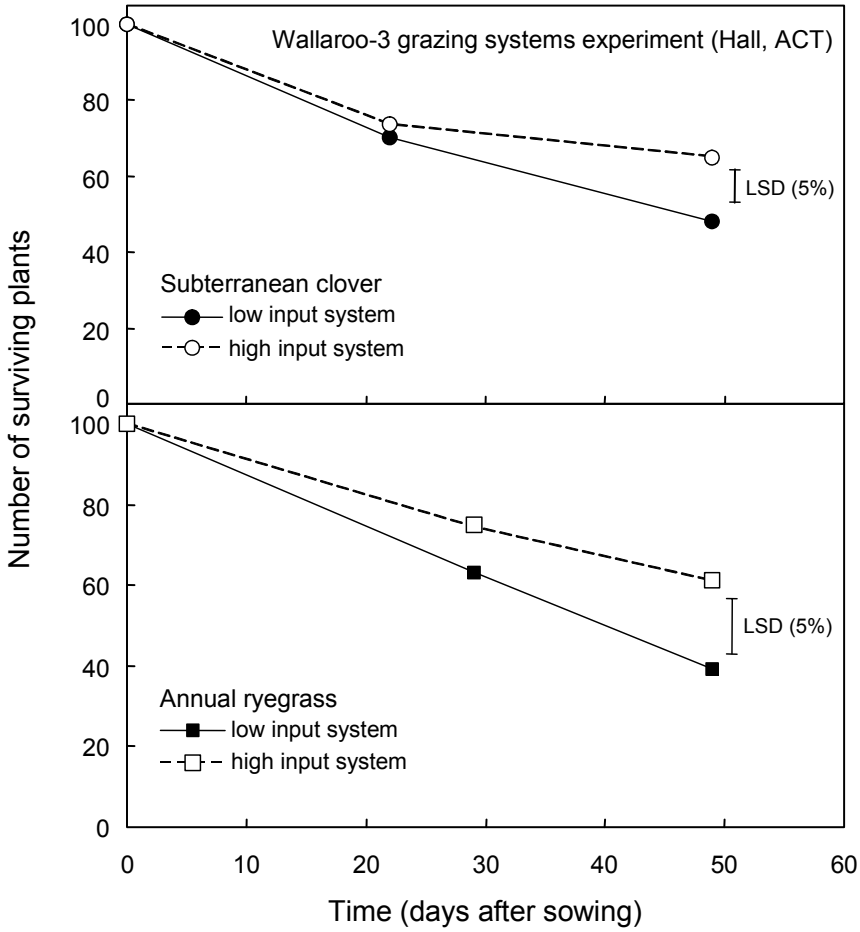

Figure 6. Effect of grazing system management on loss of annual ryegrass and subterranean clover seedlings. The low input system was not fertilised and was grazed continuously by 9 sheep/ha; the high input system was fertilised with phosphorus and grazed continuously by 18 sheep/ha.

during root scoring that root damage impacted adversely on shoot yield (Fig. 7) but this was not quantified.

If the extent of damage to roots of the subterranean clover plants that survived the emergence phase is considered, no site had better than 60% of plants with undamaged roots (Fig. 8). Across all sites, the average was only 34% of plants with undamaged roots. Lucerne emergence and establishment was adversely affected by drought in the SA mallee. However, moderate to severe damage was still recorded at all sites on the roots of lucerne plants that did manage to establish in the difficult conditions. Symptoms of damage on the roots of subterranean clover and lucerne were typical of those expected as a result of infection by root rot pathogens.

Root damage indices which summarise the overall extent of root damage to a root system were calculated for “total damage” and “post-emergence damage” in an attempt to estimate the likely effects of “damping-off and root rot” and “root rot alone”, respectively (Figs 9 and 10). Moderate to high indices were recorded across all sites with particularly high seedling loss and/or root damage evident at one NSW site and all WA sites.

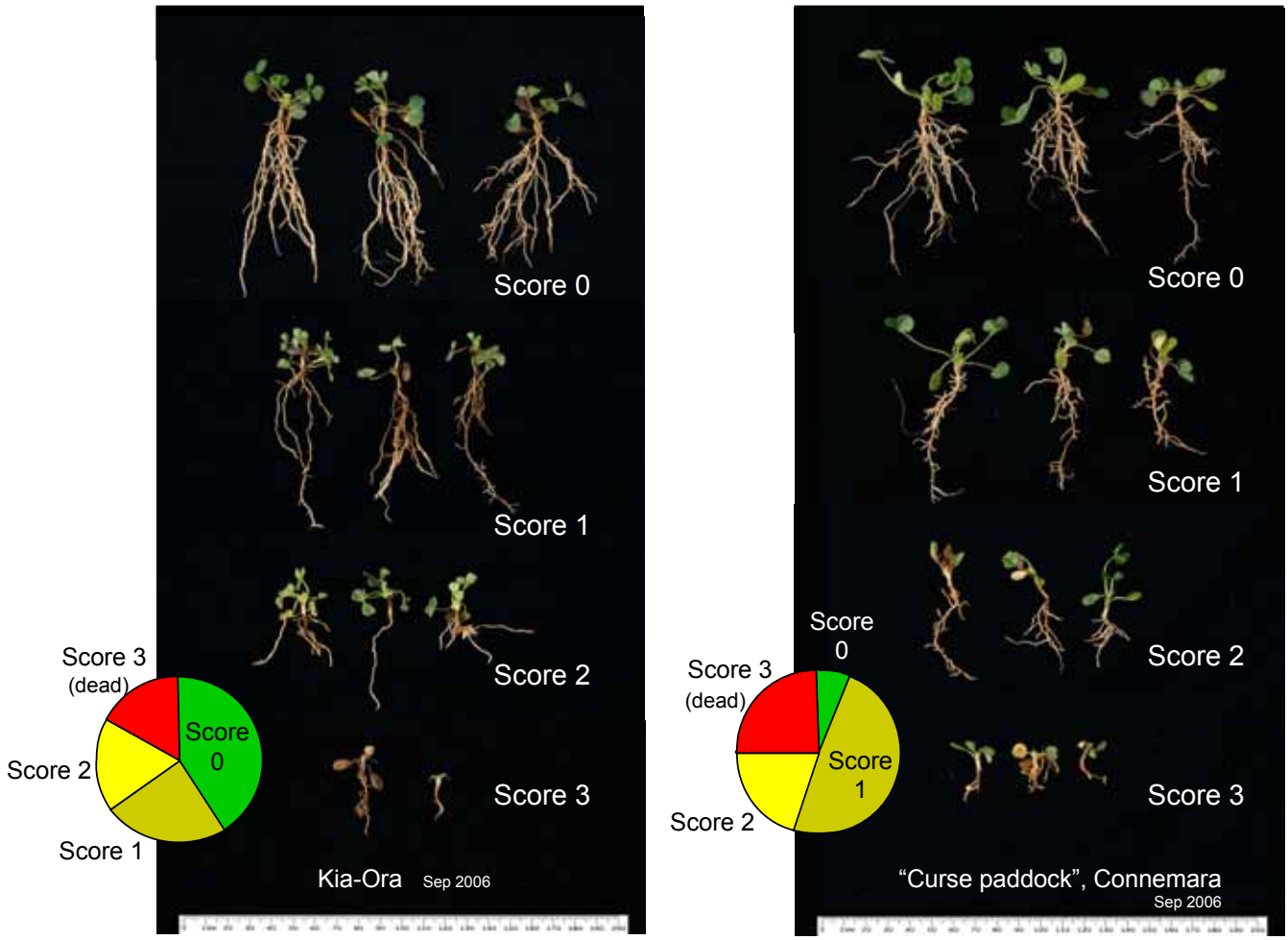

Figure 7. Examples of the scoring system applied to root damage on subterranean clover plants (cv. Woogenellup) grown at "Kia-Ora", Bookham NSW, a site where a relatively high proportion of plants had undamaged roots, and "Connemara", Tarcutta NSW, where a very low proportion of plants had undamaged roots. The pie graphs indicate the proportions of plants observed in each root damage category.

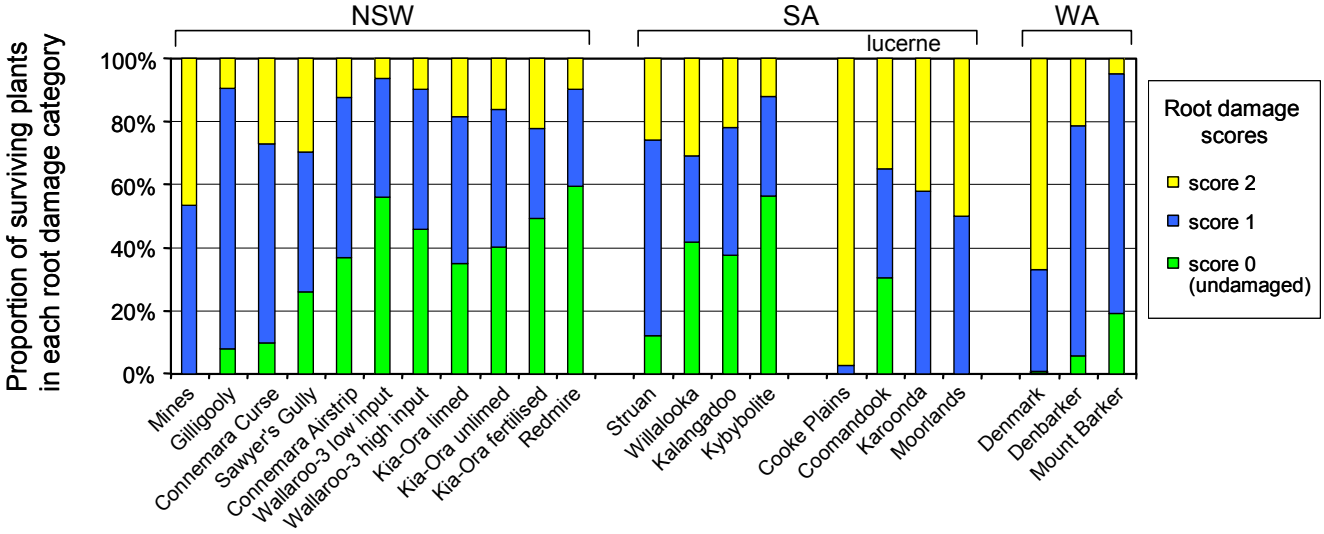

Figure 8. Proportions of surviving plants (i.e. those that had managed to germinate, emerge and survive to the final harvest) that were recorded in each root damage category.

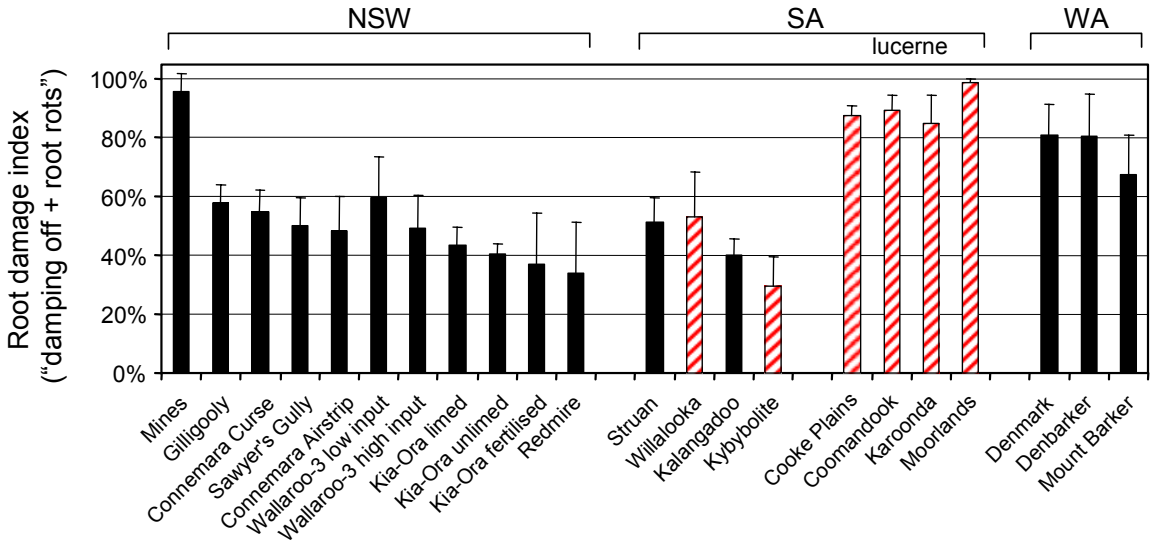

Figure 9. Total root damage index which was calculated using post-emergence damage data and all plants losses during emergence and post-emergence phases. This index is assumed to reflect losses due to damping-off and rootrot pathogens. Results for sites subject to moisture stress (hatched bars) are not be reliable indicators of disease influences alone.

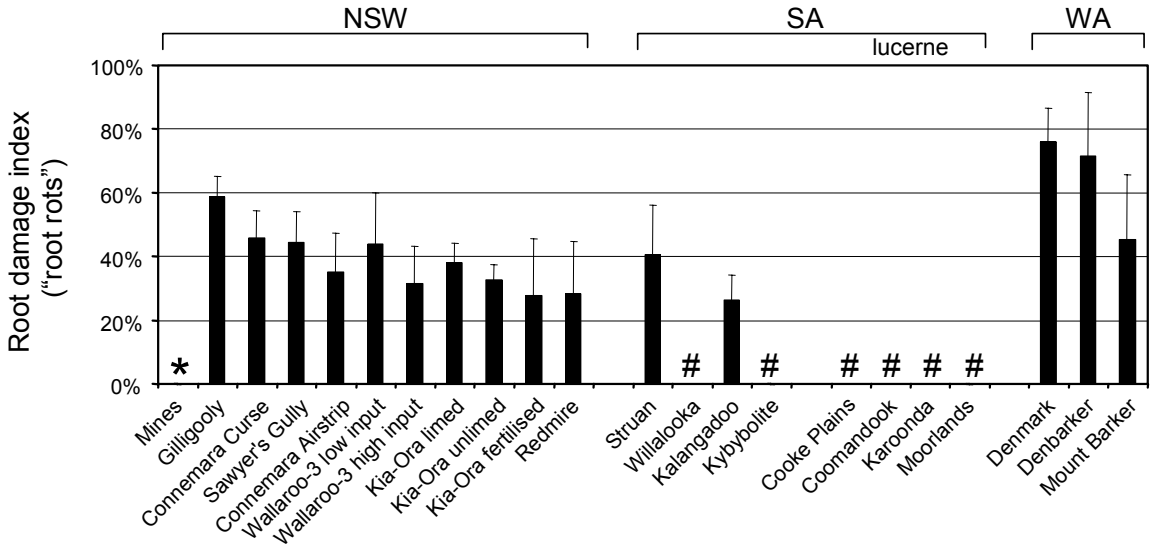

Figure 10. Post-emergence root damage index was calculated using root damage data and post emergence plant losses only and is assumed to reflect losses and damage due to root rot pathogens. Although root damage was present at sites subject to early moisture stress (#), the post-emergence index was not calculated for these sites as it will be confounded by abiotic stresses during emergence. An index was not calculated for the Mines site (*) because almost all seedlings were lost during the emergence phase at this site.

4.3 Detection of plant species and common micro-organisms using DNA probes

DNA probes for major plant types, clover and grass root pathogens, arbuscular mycchorhizal fungi (AMF) and nematodes developed by SARDI as part of the larger soil biology project were applied in the bioassay experiment. In some cases, this was their first use in a field trial. The probe results reveal substantial differences in the plant and microbial profiles of sites and regions as illustrated by Figure 11 (south-east SA vs SA mallee), Figure 12 (NSW vs WA) and Figure 13 (selected clover pathogens across all subterranean clover sites).

4.3.1 Plant probes

Subterranean clover occurred at all sites (with the exception the SA Mallee, where it was not expected). The concentration of its DNA generally remained stable or increased (except at WA sites), as expected given the experimental design and sampling protocol. *Lolium/Fescue* DNA was not a significant part in most swards other than at 2 sites in WA and 1 in NSW. *Phalaris* was present at 10 sites in NSW and the SE of SA. For NSW sites, at least, this probe accurately identified all pastures sown to phalaris.

4.3.2 Nematodes

Overall nematode detection by DNA was low. Stem nematode was present in low numbers at 4 sites in NSW. *Pratylenchus thornei* and *Meloidogyne javanica* were not detected. *Pratylenchus penetrans* was only detected in 1 sample from 1 site in WA. *Pratylenchus neglectus* was found in 4

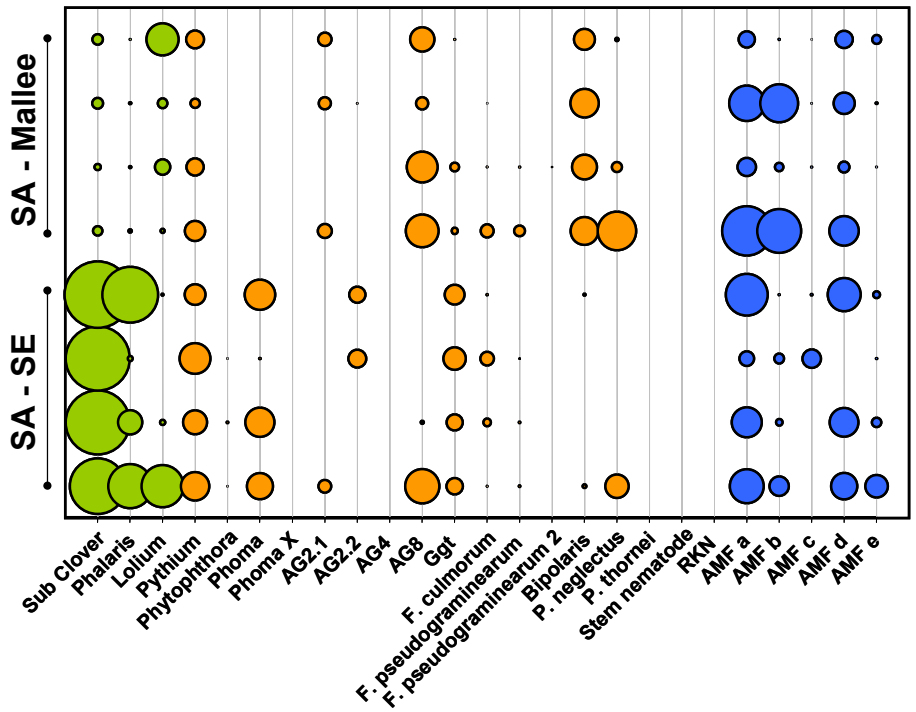

Figure 11. Relative abundance of plant species and micro-organisms at sites in the south-east of SA and in the SA mallee, illustrating some of the contrasts between sites and regions. Diameter of circles is proportional to log(pg DNA/g soil).

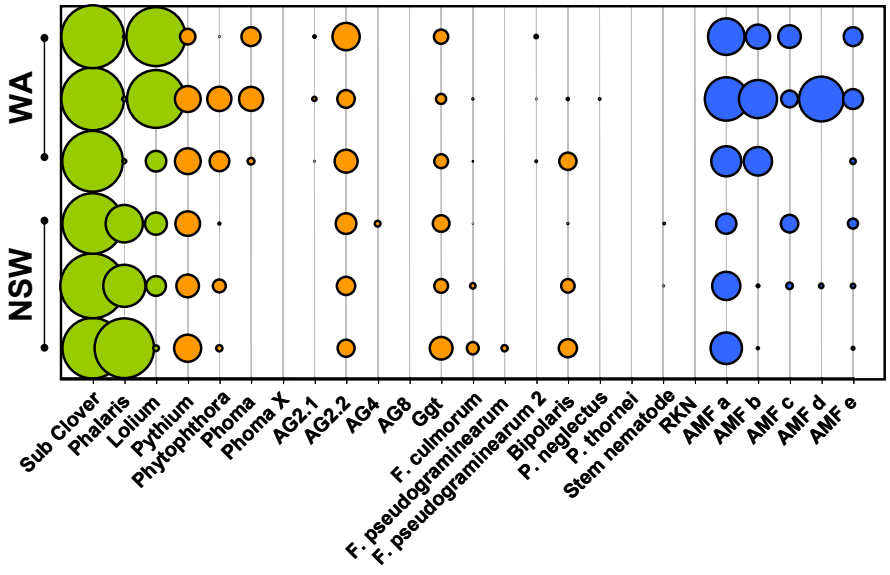

Figure 12. Relative abundance of plant species and micro-organisms at a subset of sites in NSW and WA sites. Diameter of circles is proportional to log(pg DNA/g soil).

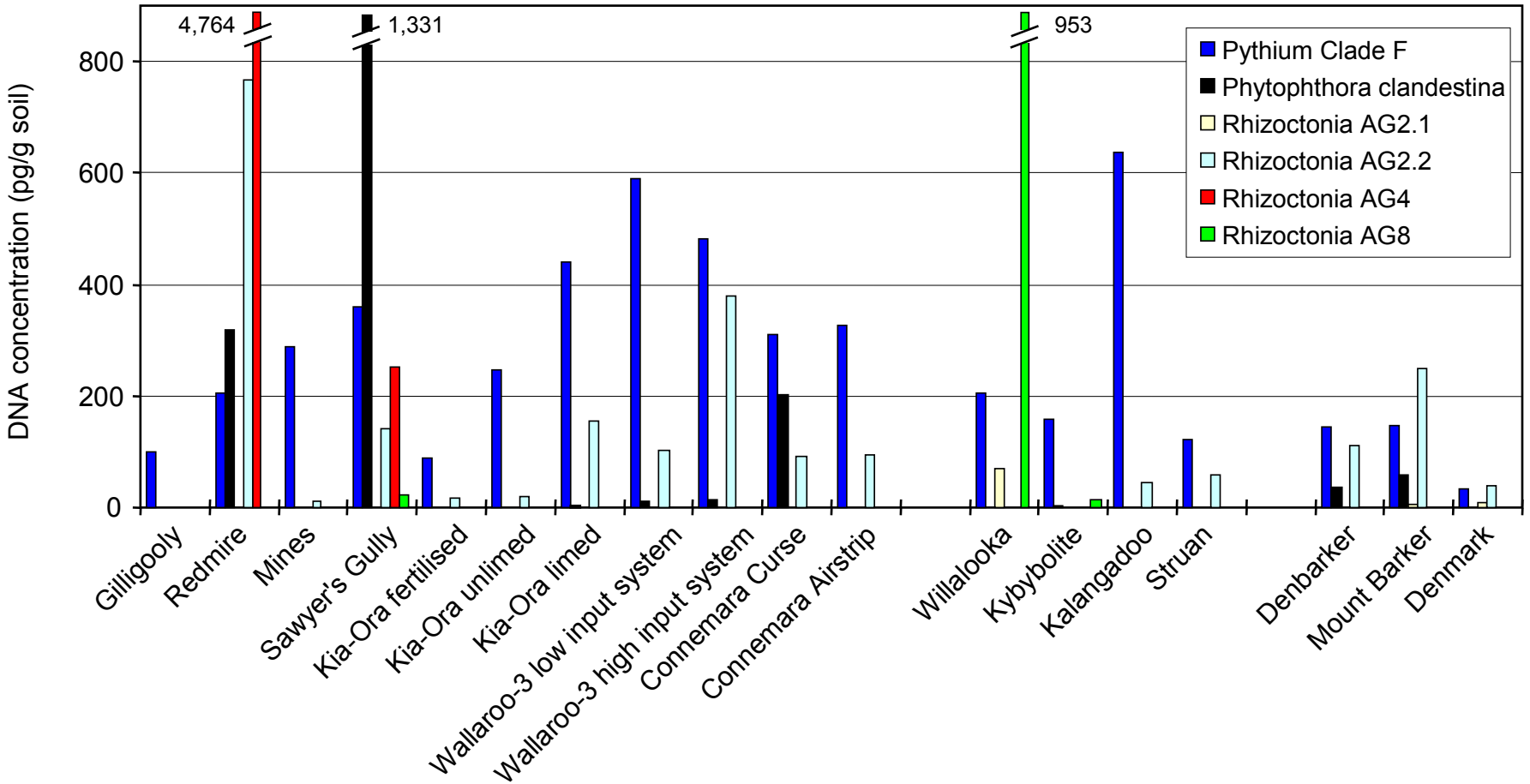

Figure 13. Selected soil-borne pathogens of clover present in soil at the final harvest at all subterranean clover sites to illustrate the diversity of the fungi and oomycete profiles of the sites. Note that only one pathogen of those for which DNA probes were available may occur at a site (e.g. Gillygooly, NSW) while up to 5 of the 6 tested occurred at Sawyer's Gully, NSW. Paddocks on the same farm may have different profiles (cf. Connemara Airstrip and Curse paddocks). Some pathogen groups (e.g. *Pythium-clade F*) were very widely distributed, whilst others (e.g. *Phytophthora clandestine*, *Rhizoctonia* AG4 or *Rhizoctonia* AG8) occurred at discrete locations.

sites in SA and 1 in WA. Plant-feeding nematodes are almost certainly important in pastures, so it is likely that the results indicate that an alternative set of nematode tests are needed.

4.3.3 Oomycetes

Pythyium clade F stood out as the most common pathogen and was found at all sites. At sites where moisture was adequate, the concentration of *Pythium* increased substantially during the bioassay period. Given the observed root damage symptoms and these results it is suspected that *Pythium* had significant impacts on subterranean clover seedling health. *Phytophthora clandestina* was only detected in significant concentrations at 3 sites in NSW and 2 in WA. Although not as common as *Pythium*, *Phytophthora* DNA also increased substantially during the clover bioassay.

4.3.4 Pathogenic fungi

Some of these probes target cereal/grass pathogens. *Gaeumannomyces graminis* var *tritici* was common especially at sites with phalaris and probably other sites with moderate to high grass components. *Biploris* (common root rot) was common in the SE Mallee sites and at sites in NSW and WA but in all cases DNA concentrations declined in concentration. Detection of *Fusarium* species was either low or sporadic.

Rhizoctonia solani groups were more common. Patterns of distribution AG2.1 and AG2.2 appeared to be distinct although they also occasionally occurred together. AG4 only occurred in significant concentrations at 1 site in NSW and AG8 only at the Mallee sites or the most northerly of the SE sites in SA. AG2.1 and AG8 was the only Rhizoctonia types to show a trend towards increased concentration during the bioassay.

Phoma (black spot) occurred in moderate concentrations in the SE of SA and was also present in WA. It is possible that at the SA sites it was contributing to the poor condition of the subterranean clover plants.

4.3.5 Mycorrhizal fungi

AMF occurred at all sites at varying concentrations and combinations of clades. There was a range in clade diversity and abundance between sites and regions. It seems evident that the DNA assays provide a means to examine diversity and abundance of AMF in pasture ecosystems that would otherwise be impossible.

4.3.6 Ecology of soil biology and plant infection

The experiments provided some limited opportunities to explore how the DNA probes might be applied to understanding the interactions of soil biology and plant roots. Figure 14 illustrates the relative abundance of a selection of plant pathogens and AMF in the soil at sowing and in the soil and clover roots at the final harvest at two sites in SA. Relatively high concentrations of pathogen DNA and AMF DNA were often found in roots relative to the soil in which they were growing. However, there were also indications of differences in the interactions between roots and pathogens or AMF between the sites.

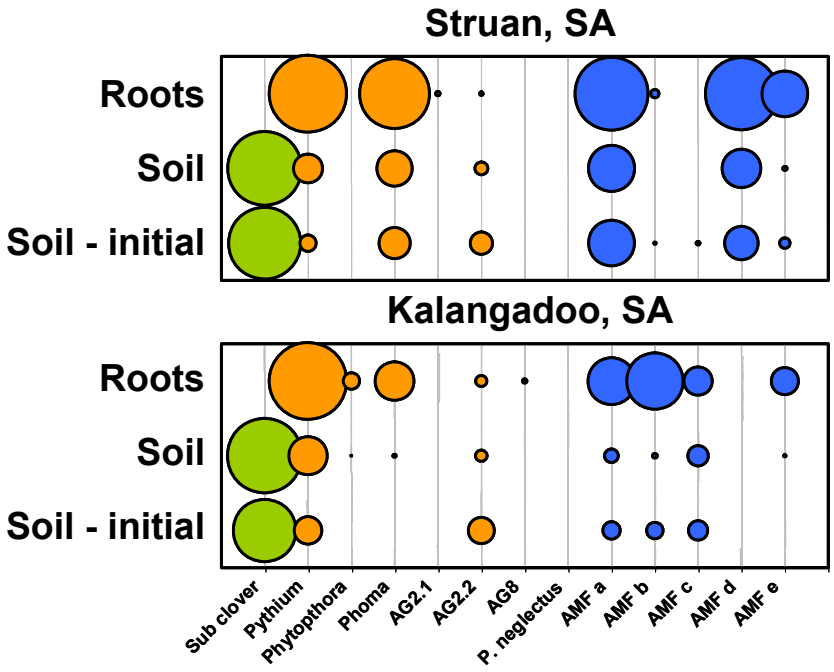

Figure 14. Relative abundance of selected pathogens and AMF in soil at sowing and in subterranean clover roots and the surrounding soil at the completion of the experiment. Diameter of circles is proportional to log(pg DNA/g soil). Note: DNA content of root samples was not measured.

4.3.7 Relationships between root damage and pathogen presence

Root damage indices were combined with DNA probe data to determine whether there was any evidence of relationships between the presence of pathogens and the extent of damage to subterranean clover roots. At most sites *Pythium*-clade F and *Phytophthora clandestina* DNA concentrations increased during the bioassay so the relationships between the probe data for these organisms and post-emergence root damage (i.e. damage most likely to be associated with root rot organisms) was explored. However, it should be recognised that although the DNA probes will indicate presence and therefore risk of infection by a pathogen, disease outbreaks also require the occurrence of suitable environmental conditions and these cannot be predicted by a DNA probe.

Phytophthora clandestina occurred at only a limited number of sites and no relationship with root damage was evident (data not shown). *Phytophthora clandestina* infection is favoured by very wet conditions with free water (Greenhalgh and Taylor 1985). The situation may be very different in a wet year.

Pythium-clade F occurred at every site. However, there was no relationship between the concentrations of *Pythium*-clade F DNA at sowing or at the final harvest, and post-emergence root damage index (Fig. 15). Based on data which indicates that active growth of *Pythium* mycelia is an important means of disease spread and progression (Green and Jensen, 2000), the root damage index was also regressed against the proportional increase in *Pythium* DNA from sowing to final

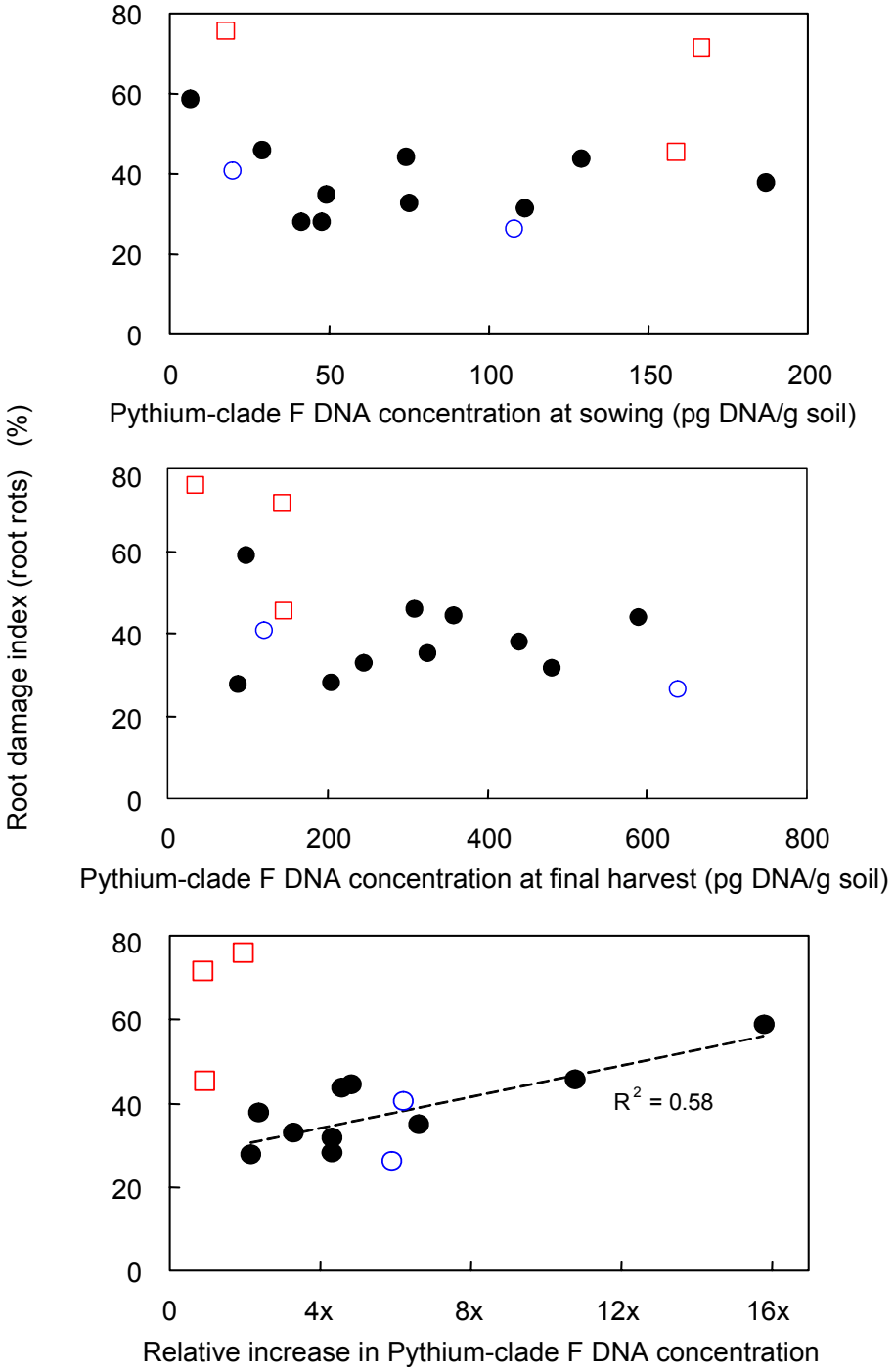

Figure 15. Post-emergence root damage index of subterranean clover at moist sites were it was possible to calculate the index, graphed in relation to the concentrations of Pythium-clade F DNA at sowing and final harvest, and in relation to the relative increase in Pythium DNA concentration at each site. Closed black circles (NSW sites), open blue circles (SA sites) and open red squares (WA sites)

harvest. This appeared to indicate a relationship between relative rate of increase in *Pythium* and root damage was possible for NSW and SA sites. WA sites clearly had developed more root damage for equivalent relative rates of *Pythium* increase and did not fit the relationship. These indications are too preliminary to be treated with anything other than extreme caution but they may indicate that soil environmental conditions or the presence of unknown pathogens differ substantially between WA and eastern Australian sites.

5 Success in Achieving Objectives

5.1 Adverse impact of root pathogens

5.1.1 Bioassay indicates high potential for seedling losses and root damage

The first objectives of this project were to test the application of a bioassay of early-season root damage (disease) to assess potential for soil biological constraints to pasture growth. In particular, to assess the prevalence and potential for root disease on subterranean clover during germination, establishment and early growth in autumn-winter. The bioassay was developed to avoid problems that had stalled earlier attempts (using soil drenches) to assess impacts of soil biology on pastures.

The bioassay approach was successful and indicated there is a widespread and substantial potential for subterranean clover seedlings to either: (a) fail to emerge, or (b) to germinate and establish, but with a high incidence of damaged roots. There was significant loss of seedlings and substantial root damage on subterranean clover test plants (or lucerne) at every site examined (on average only 34% of plants established with undamaged roots). This was despite the fact that conditions across southern Australia in the 2006 autumn-winter period were not excessively wet and were not expected to be particularly favourable for most root rot pathogens. Shoot yield of the test plants was also adversely affected by root damage but it was outside of the scope and resources of this project to quantify the impact of seedling loss and root damage on pasture yield.

5.1.2 DNA probes reveal plant, pathogen and beneficial fungi profiles of sites

The project also aimed to provide a preliminary assessment of how DNA probes for plant species, common root pathogens, nematodes and some beneficial soil fungi developed by SARDI may be used to indicate site characteristics and the presence and relative abundance of particular groups of soil microfauna.

The DNA data showed that plants, pathogens and potentially-beneficial organisms vary considerably between sites. Plant probes (e.g. for phalaris) were highly accurate in identifying sites with and without the plant species.

For the first time, it was possible to rapidly identify the pathogens likely to cause problems at different sites and to explore the ecology of organisms such as AMF which are difficult to study. Indeed, the extent to which AMF clades vary in their relative abundance between sites is essentially impossible to study using current technologies and the results presented in this report potentially represent a major breakthrough in the study of AMF ecology and infection of plant roots.

It was also possible to characterise sites by the organisms present. For instance, some sites appeared to have only a few major pathogens present whilst others possessed nearly all of the pathogens that can be detected currently. Some organisms, e.g. *Phytophthora clandestina*, were only present at particular sites; while others, e.g. *Pythium*, were universal. Developing a pathogen

profile for a site by standard pathology methods alone would be difficult, time-consuming and potentially inaccurate because it is always possible to miss key species when trapping and culturing from infected roots. However, DNA probes only detect known organisms and will, therefore, be best employed in combination with standard pathology methods.

6 Impact on Meat and Livestock Industry

6.1 Pasture renovation costs

Loss of seedlings (putatively to damping off and root rots) was typically in the range 20-35% across the majority of sites. If it is assumed that clover and grasses are equally affected, the cost of plant losses for pasture renovated by sowing 4kg subterranean clover with 4 kg grass seed would typically be $13-$22/ha. This result emphasises the importance of treating pasture seeds with fungicide (cost: ~$5.20/ha), even if the fungicide treatment were to prove only partially effective in controlling root disease.

Seedling loss problems were not uniform across the sites tested. At particular sites (e.g. some southern tableland paddocks and all WA paddocks), seedling losses were extremely high (50-90%) and indicate that the cost due to seedling failure would be $32-$58/ha in seed cost alone, and higher if forgone grazing were to be factored into the analysis.

DNA-based tests that can predict which paddocks are at risk and/or the extent of seedling losses, or that can be for guiding plant cultivar selection or appropriate use of pesticide treatments through knowledge of the pathogens that are present, have potential to reduce some of the significant financial risks that are associated with pasture renovation.

6.2 Adverse consequences for pasture yield and stocking rates

It was obvious from the poor shoot vigour of test plants, that reduced pasture yield will be associated with root damage. Indeed, it is highly likely that sub-lethal infection of pasture roots constitutes a very large, hidden cost to production because it is so widespread (moderate to severe root damage occurred at every site) and because the damage occurs during autumn-winter when pasture yield limits stocking rate. Damaged subterranean clover plants will also fix less nitrogen, will be less competitive and will be susceptible to moisture stress during autumn when rainfall reliability is usually very low. Although the comparisons with annual ryegrass in the present experiment were rather preliminary, the data indicate that grasses may also be similarly affected and further investigation is warranted.

The occurrence of damping-off and root rot on subterranean clover has been noted across southern Australia since 1960's (Barbetti *et al*. 2006). Reduced root damage, and yield increases of 50-95% have been recorded after application of fungicide treatments to subterranean clover. However, most information concerning the impacts of soil-borne pathogens on yield comes from glasshouse, controlled environment, spaced plant or single row field plot experiments which demonstrate the potential of a pathogen to cause damage, but may be unrealistic in terms of the impacts on yield in grazed pastures. Other indirect evidence also adds weight to the argument that root damage may be imposing a significant cost to pasture production. In recent simulation experiments using the GrassGro pasture-systems model to estimate land capability at the "Kia-Ora" site on the southern tablelands of NSW (Simpson *et al*. 2005), it was noted that the model consistently over estimated autumn-winter pasture yields by 300-1200 kg DM/ha depending on the year being simulated. The

reason was not determined but it was noted that soil-borne disease, which was not explicitly modelled, could be involved. Increases in autumn-winter pasture availability of this order had a substantial affect on animal production.

Progress has been made in selection of subterranean clover cultivars with increased field resistance to root rot and some cultivars are in current use (Barbetti *et al*. 2006). Gains have also been made in selecting for resistance to specific soil-borne pathogens. For example, the subterranean clover cultivars Denmark, York and Goulburn were all released in the 1990's with resistance to the most commonly occurring race of *Phytophthora clandestina*. However, major gaps in the levels of resistance to root pathogens are strongly suspected. Goulburn, for example, appears to be very sensitive to *Pythium irregulare* (Y. Cheng, *pers. comm*.) a *Pythium* species in clade-F, a pathogen group detected at all sites in the present experiment. Much less is known about the levels of root damage and/or resistance in pasture grasses. The timing and widespread nature of the root constraints observed in this study are such that it is highly likely that increased production and stocking rates would be achieved if it were feasible to reduce or eliminate autumn-winter root damage in temperate pastures.

7 Conclusions and Recommendations

7.1 Conclusions

New DNA-probes for soil pathogens and beneficial fungi employed in the study demonstrated that the pathogen and fungal profile of a site can be quickly and cheaply determined. This is a considerable advance on using standard pathology tests alone. In this study it was clear which groups of pathogens were broadly distributed at the test sites, and which pathogens were confined to specific locations. These tools have the potential to be very useful in reducing the risks and costs associated with pasture renovation, although more work is required to understand how they should be applied. It was clear that the tools will also open completely novel territory in the study of soil and root system biology and ecology.

This study also revealed significant seedling losses for legumes and a grass at pasture sites across southern Australia and substantial root damage to subterranean clover (cv. Woogenellup) and lucerne (cv. SARDI 10) at every site examined. The autumn-winter period in which the experiments were conducted was not excessively wet, and did not present conditions recognised as favourable for most root diseases. Nevertheless, the occurrence and severity of root damage was substantial. Using records of distribution and incidence collected since 1953, Murray *et al*. (1993) estimated that root rots of subterranean clover probably caused widespread "moderate to severe (5->15%)" losses in 1-3 years in 5 in NSW. However, they cautioned that their assignment of a severity index may imply "greater knowledge than is available". The indications from the present experiment are that the estimate by Murray *et al.* (1993) may indeed understate the true cost of root diseases for pasture production and stocking rates because, in years where disease incidence is recorded as low, it is likely that substantial and widespread sub-lethal disease and root damage will still be occurring in autumn-winter pastures.

The fact remains that insufficient is known about the impacts of soil-borne pathogens on pasture production in temperate Australian pastures. The levels of field resistance to soil-borne pathogens in our key pasture species are generally not known and the distribution and races of key pathogens in southern Australian pasture systems is poorly understood.

7.2 Recommendations

The present study used subterranean clover cv. Woogenellup, a root rot-susceptible cultivar, with the intention of assessing root damage (disease) "potential". The bioassay approach avoided many problems encountered in earlier research and can now be adapted to:

(a) quantify the costs to pasture yield of root disease during autumn-winter;
(b) determine whether plants persisting in established pastures have developed resistance to root damage organisms;
(c) assess whether modern subterranean clover cultivars and key pasture grasses possess reasonable resistance to root damage organisms; and to
(d) confirm the identity of the suspected causal organisms and/or organism complexes.

Estimating yield penalties due to root damage organisms will be difficult because few (if any) pesticides have broad spectrum action, often do not penetrate soils adequately, and fumigation treatments cannot be applied without killing the existing pasture and are imperfect because disease organisms reinvade fumigated soil. However, by combining the bioassay approach with use of the DNA probes to monitor pathogen reinvasion in fumigated soil, it should be possible to estimate the cost to yield of early-season root damage.

The DNA data show that plants, pathogens and potentially-beneficial organisms vary considerably between sites. For the first time, it is possible to rapidly identify the organisms likely to cause problems at different sites and to explore the ecology of organisms such as AMF which are difficult to study. At the very least it is possible to characterise sites by the organisms present.

Further work with the DNA probes is required to develop an understanding of how they should be applied and interpreted. Additional probes for pathogens, such as *Aphanomyces euteiches*, which are difficult to isolate but are, nevertheless, implicated in severe root disease should also be developed.

A limitation of DNA probe technology is that probes can only be developed for known organisms and sometimes only for specific groups or subgroups of organisms. Causal organisms must, therefore, still be identified by standard pathology methods after isolation from diseased roots. However, the combination of DNA-probing of infected plant roots and standard pathology will vastly improve our ability to detect transient fungal complexes and organisms that are difficult to isolate. It will give novel insight into the occurrence and ecology of soil organisms. Indeed, the DNA tools are now the best method we have for quantifying temporal and spatial aspects of soil micro-organisms, their involvement in disease development and their management. The tools will improve our capacity to study interactions between organisms and disease complexes. The efficiencies that DNA tools offer mean they are also very suitable for examining larger scale impacts of soil biology on farming systems, changes in management practice, and for exploring the relationships between root development, root function and soil biology. These topics are logistically difficult to study using standard technologies alone.

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9 Appendices

9.1 Appendix 1: Sites and site characteristics

Paddock/farm name/treatment	Location	State	Main pasture species present in paddock	TEXTURE GROUP	PBI	COLWELL PHOSPHORUS	COLWELL POTASSIUM	KCl40 - SULPHUR	ORGANIC CARBON	PH_CACL2	PH_H2O	EXC_CA	EXC_MG	EXC_NA	EXC_K	EXC_AL	Al%	CEC	CHLORIDE
					Index	mg/kg	mg/kg	mg/kg	%	pH	pH	meq/100g	meq/100g	meq/100g	meq/100g	meq/100g			mg/kg
Gilligooly	Braidwood	NSW	Native grasses (Microlaena)	3	221	8	182	8.9	3.7	4.3	5.2	2.27	0.85	0.08	0.5	1.22	24.8%	4.9	22
Redmire	Taralga	NSW	Phalaris-sub clover-ryegrass-vulpia	3	281	16	94	10.1	4.9	4.5	5.1	6.96	2.11	0.22	0.26	0.97	9.2%	10.5	30
Mines	Hall	ACT	Phalaris-annual grasses-storksbill-capeweed-subclo	3	48	10	332	7.6	2.7	4.2	5	1.56	0.55	0.05	0.83	0.65	17.9%	3.6	15
Sawyer's Gully	Wee Jasper	NSW	Phalaris-sub clover & Pattersons Curse	3	40	9	404	7.1	3.2	4.7	5.2	5.68	1.08	0.05	0.95	0.31	3.8%	8.1	21
Kia-Ora fertilised	Bookham	NSW	Danthonia-Microlaena-annual grasses-sub clover	2.5	57	7	78	6.6	2.4	4.2	4.9	1.51	0.39	0.06	0.19	0.58	21.2%	2.7	10
Kia-Ora unlimed	Bookham	NSW	Sub clover (cv. Goulburn)	3	57	11	58	8.8	2.1	4.2	4.8	1.17	0.3	0.08	0.14	0.63	27.2%	2.3	15
Kia-Ora limed	Bookham	NSW	Sub clover (cv. Goulburn)	3	49	11	71	7.8	2.4	4.4	5.1	2.26	0.47	0.07	0.15	0.37	11.1%	3.3	11
Wallaroo-3 low input system	Hall	ACT	Annual grasses-phalaris-sub clover	3	33	14	353	9.7	2.5	4.5	5.3	2.55	0.87	0.08	0.89	0.20	4.3%	4.6	24
Wallaroo-3 high input system	Hall	ACT	Annual grasses-phalaris-sub clover	3	41	53	314	9.2	2.7	4.6	5.4	3.06	0.90	0.13	0.78	0.14	2.8%	5.0	34
Connemara Curse	Tarcutta	NSW	Annual grasses-phalaris-naturalised and sub clover	3	52	19	83	20.8	2.5	4.4	5.1	3.36	0.47	0.05	0.2	0.24	5.6%	4.3	7
Connemara Airstrip	Tarcutta	NSW	Annual grasses-phalaris-subclover	3	36	29	73	9.5	2.3	4.2	5	1.91	0.26	0.03	0.15	0.34	12.6%	2.7	5
Cunningham	Willalooka	SA		1	14	11	87	8.7	2.1	5	5.9	4.34	0.78	0.15	0.23	0.08	1.4%	5.6	21
PIRSA	Kybybolite	SA		1.5	32	30	81	7.5	3.3	4.2	5.4	2.4	0.66	0.14	0.18	0.21	5.8%	3.6	8
Miller	Kalangadoo	SA		2	39	59	212	10.2	2.2	4.7	5.8	2.58	0.4	0.09	0.55	0.16	4.2%	3.8	15
PIRSA	Struan	SA		2.5	47	26	76	7.9	2.0	4.4	5.4	1.86	0.36	0.13	0.18	0.2	7.3%	2.7	8
Swan	Cooke Plains	SA		2	30	26	260	10.3	1.8	7.5	7.5	10.59	1.02	0.1	0.62	0	0.0%	12.3	44
Freak	Coomandook	SA		1.5	13	36	74	5.1	0.6	6.2	6.6	2.78	0.43	0.03	0.18	0	0.0%	3.4	6
Spindley	Karoonda	SA		1.5	13	30	103	4.8	0.6	6.5	6.9	3.13	0.58	0.06	0.23	0	0.0%	4.0	9
Piggott	Moorlands	SA		1.5	11	28	131	4.3	1.2	5.5	6.4	4	0.68	0.1	0.34	0	0.0%	5.1	18
Forbes	Mount Barker	WA	Annual grasses-sub clover-dock	2		42	126	29.1	7.2	5.4	5.8	12.72	1.26	0.63	0.42	0.11	0.7%	15.1	96
Denmark Ag	Denmark	WA	Sub clover-annual grasses-flat weed-cape weed	1.5		13	32	9.8	4.7	4.6	5.1	10.55	0.54	0.33	0.09	0.17	1.5%	11.7	31
Rogers	Denbarker	WA	Dock-annual grasses-sub clover-erodium	1.5		44	116	15.9	5.0	5	5.5	8.52	1.2	0.24	0.35	0.08	0.8%	10.4	63