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Root disease constraints to pasture production

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Abstract

Yield of subterranean clover during autumn-winter is reduced when the plants roots are damaged by oomycete and fungal root rot pathogens. This project examined whether root damage limits yield indirectly by restricting nutrient (phosphorus) uptake, or directly by perturbing the shoot-root balance of the plant. It was found that root damage ("pruning" of roots) reduced shoot yield directly, in a manner analogous to the way "bonsai" plants are managed. Improved soil P fertility did not alleviate the yield constraint due to root damage. In contrast, clover growth in spring was not shown to be affected when fungicide(s) were injected into the root zone. It was concluded that root disease is unlikely to constrain spring growth or the response of clover to P fertiliser. Attempts to improve clover root morphology for improved P efficiency are unlikely to be negated by root rot pathogens. Root pathogen DNA concentrations associated with roots were examined and a tentative relationship with the occurrence of root damage was observed. Further work is needed to confirm the reliability of this observation. There was some evidence that root pathogen DNA levels may also be used to assess the efficacy of fungicide treatments and root disease resistance by subterranean clover.

Executive summary

Background

Damage to roots of subterranean clover is widespread in southern Australia during autumn-winter. A major cause is root rot due to oomycete and fungal pathogens. The damage takes two forms: (i) damage to the hypocotyl, cotyledons and root radicle when the seed is germinating resulting in death before emergence (often referred to as “damping off”) and (ii) damaged roots on seedlings. A survey across southern Australia (17 sites) found the median pre-emergence loss was 21% of germinating seeds (however, one site recorded a 93% loss), with 70% of the surviving plants having substantial damage to their roots. Root damage ranges from minor shortening of lateral roots, through substantial pruning of the primary laterals, to loss of the taproot. Root damage such as this is invariably accompanied by significant reductions in shoot yield. Estimates of the constraint to shoot yield of subterranean clovers during autumn-winter are typically about 20-40% but, occasionally, very high yield losses are also recorded.

It was unclear whether root damage limits yield ‘indirectly’ by restricting nutrient uptake, or as a ‘direct’ restriction of the shoot-root balance of the plant (i.e. a consequence of “root pruning”), or is due to both of these influences acting concurrently. If damaged roots are unable to forage adequately for phosphorus (P) in soil, it is possible that: (a) fertiliser applied at the time roots are being damaged may overcome pasture yield constraints, and (b) poor root growth may be increasing the amounts of P that farmers need to apply to achieve high pasture yield. Although it proved difficult to entirely separate the direct and indirect influences of root pruning on plant growth, the experiments provided evidence that direct restriction to shoot growth by root pruning was a major factor in poor autumn-winter growth. The impact was analogous to the creation of “bonsai”-like plants. It was concluded that adding additional P-fertiliser at this time of the year would not overcome poor growth associated with damaged roots unless soil P fertility was extremely low. Presently, there is interest in developing pasture legumes with long fine roots and long root hairs that can yield well at lower soil test P concentrations. Legumes such as these would reduce the P fertiliser required for maximum production and should also reduce risks of P loss from farmland to waterways. However, before any major investment is made in plant improvement, it is essential to know whether root damage by fungal pathogens is likely to negate the value of selecting plants with improved (P-efficient) root morphology. Injections of fungicide into the root zone of subterranean clover growing in low P soil during spring did not result in any improvement in clover growth in these experiments, indicating that access to P in soil had not been improved by application of the fungicide.

In recent years, DNA-based tests for the main root rot pathogens of subterranean clover have been developed by SARDI Plant and Soil Health. These tests enable the profile of root pathogens in pasture paddocks to be determined. In these experiments, the root pathogen DNA tests were used to test the levels of pathogen DNA that were directly associated with damaged root systems.

Method

During autumn-winter, field-based bioassays using subterranean clover as the test plant were deployed to assess the occurrence and severity of root damage, the impact of root damage on clover yield, and to examine associations between root damage and the pathogen DNA concentrations of roots. Each bioassay was planted with surface-sterilised clover seeds, pre-emergence seedling losses were recorded and the damage to roots was assessed at the 2-3 leaf stage of growth. Sites previously used for P fertiliser experiments on the southern tablelands of NSW were used for the experiments. The bioassays are a reasonable representation of autumn-winter clover growth in these locations. They were located across the P treatments of the earlier experiments to enable interactions between root damage and soil P fertility to be assessed.

For assessments of the impact of root disease on clover yield during spring, methods were developed to inject fungicide(s) into the root zone of established subterranean clover swards without soil disturbance. Clover yield in response to fungicide treatments and soil P fertility was examined.

Results

Root damage during autumn-winter: Most bioassays used subterranean clover cv. Woogenellup. Pre-emergence losses were often 20-30% of the germinating seeds. When growing in very P-deficient soil, young plants with undamaged roots responded moderately to improved soil P supply when temperature conditions permitted. However, in cool conditions (< 6^{o}C) or when roots were damaged, no response to soil P fertility was observed. In contrast, root damage consistently reduced the yield potential of the clover plants. Typically, up to 20% of the test plants had undamaged roots, 40% had lost secondary lateral roots (with a shoot yield loss of about 20%), 20% had substantial pruning of primary lateral roots (yield constraint ~40%) and the remaining 20% had damaged taproots (yield constraint ~50%). The impact of root damage on yield of a number of modern subterranean cover cultivars was very similar. However, cv. Riverina was notable with relatively high pre-emergence loss rates (40-60% of plants lost at germination).

Applications of extra P fertiliser did not alleviate the constraint to yield that was associated with root damage during autumn-winter. Damaged roots directly constrain yield. The yield constraint is analogous to the way that root pruning is used in horticulture to regulate shoot growth rates (e.g. bonsai plants, etc.).

Impact of root disease during spring: Fungicides (but mainly metylaxyl) were applied by injection into the root zone of subterranean clover monocultures and were shown to be taken up by the plants and to reduce oomycete root pathogen DNA concentrations associated with soil/root samples from the root zone. However, clover growth was not enhanced by fungicide treatment(s) and the response of clover to improved soil P fertility was also not affected by fungicide treatment. The short-term growth rates of treated and untreated subterranean clover in the experiments were very high and considered to be optimal for the prevailing conditions. Collectively, these observations indicated that root disease was neither constraining yield during spring, nor was it reducing the capacity of the clover to access P from soil.

There was no evidence to suggest that root disease would negate the selection of legumes with more “P-efficient” root morphology.

Root pathogen DNA tests: One of the field sites (“Sawyer’s Gully”) had a history of bioassays conducted over several years. Data from this site were collated and a tentative, negative relationship was observed between the proportions of test plants with only mild root damage and either the *Pythium* (clade F) or *Phytophthora clandestina* DNA concentrations associated with root dry matter. Such a relationship is an essential requirement for a “plant health test” using the DNA tools and this is the first time that such a relationship has been observed. However, the experiments were technically compromised because seasonal and pathogen effects were confounded. The observations will need to be confirmed within a single growing season and at a number of independent sites for the relationship to be regarded as more than just a chance observation.

The root pathogen DNA tests were found to be responsive to fungicide treatments (i.e. oomycete DNA concentrations were reduced when seed had been treated with metylaxyl) and, on this basis, it was surmised that DNA tests may prove to be useful for identifying/confirming plant-based "resistance" to root rot pathogens. This hypothesis will need to be proven using traditional plant pathology and plants with known genes for pathogen resistance. If correct, there may be potential to use DNA tests for rapid assessments of plant resistance genes. Pathogen DNA tests may also prove to be an effective way to test fungicide treatments and would be especially useful when yield and root damage symptoms are confounded by to the presence of other root rot pathogens.

Implications of the work

Root damage on subterranean clover is common in autumn-winter and reduces yields at this time of the year. Although the occurrence of damage is widespread and of concern, farmers are already dealing with this level of yield constraint. The work demonstrates, however, that if a solution to root disease(s) can be found it is likely that carrying capacity of pastures would be increased. Optimum soil P fertility management will ensure pastures are not constrained by P, but adding extra P-fertiliser to overcome the yield reductions associated with root damage during autumn-winter will not work.

A number of modern subterranean clover cultivars carry a useful level of root damage resistance and it is worthwhile to ensure that they are used when new pastures are being sown. It is also worthwhile using fungicide seed treatments when planting new subterranean clover pastures to protect seedlings from loss and root damage. However, our observations do indicate that the effectiveness of fungicide treatments may prove to be variable.

The experiments indicated that the field resistance of cv. Riverina to "damping off" (pre-emergence) losses was relatively poor and the fitness of this cultivar should be investigated further.

It is unlikely that root damage is a constraint to animal production during spring. There were no beneficial impacts observed when fungicides were applied directly to roots of subterranean clover swards at this time of the year and, consequently, no evidence that root disease was limiting yield or the ability of the plants to access soil P. Root damage was, therefore, considered unlikely to negate efforts to breed subterranean clovers for improved root morphology and nutrient foraging during spring.

A tentative relationship was established between the proportions of subterranean clover with only mild root damage in autumn-winter and the root pathogen DNA concentration associated with roots. However, further work is required to prove the relationship is real. The pathogen DNA tests of roots seemed to be able to detect the effects of fungicide treatments and possibly plant-based disease resistance. If correct, the application of the DNA tests to root disease ecology, plant improvement and fungicide testing could assist R&D in these areas.

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1 Background

1.1 Root damage on subterranean clover

The occurrence of damaged roots on subterranean clover is widespread in southern Australia during autumn-winter and is considered to be due primarily to oomycete and fungal root rot pathogens (Barbetti et al. 2007). A survey using a subterranean clover bioassay found substantial proportions of the test plants had damaged roots in every one of the 17 paddocks tested across NSW, SA and WA (Simpson et al. 2011). Damage to emerging seedlings was assessed in two phases: (i) damage to cotyledons and radicle resulting in death before emergence (sometimes referred to as “damping off”) and (ii) damage roots of the established seedling. The median pre-emergence loss of seedlings was 21% across the sites, but at one site 93% of germinating seedlings were lost. Post-emergence losses associated with damaged roots were considerably less (median 7%) but 70% of test plants (average all sites) had damaged roots. Root damage ranged from relatively minor shortening of lateral roots, through substantial pruning of the primary laterals, to loss of the taproot.

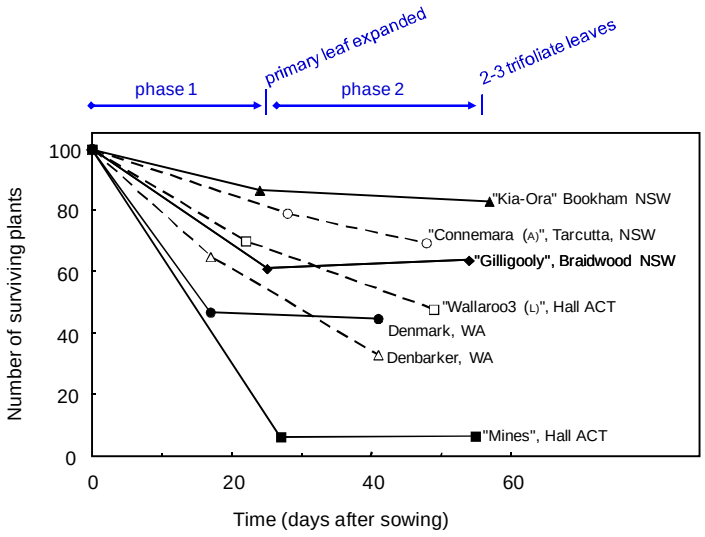


Fig. 1. Typical seedling loss profiles from root damage bioassays run at various sites in autumn-winter, 2006 (Simpson et al. unpublished).

1.2 Root damage and constraints to yield in autumn-winter

A subsequent study examined whether shoot yields of subterranean clovers was affected when plants had damaged roots. Data from four sites in NSW showed that damaged roots were consistently associated with substantial constraints to shoot growth (Fig. 2). The results of the subterranean clover bioassays and additional annual ryegrass bioassays at the same sites were used to estimate that damaged roots may constrain pasture production as a whole by 20%-40% during autumn-winter (Simpson and Richardson 2009). Constraints to pasture growth in autumn-winter of this magnitude were mimicked in a model of a sheep production system. The model predicated that damage to roots would reduce the livestock carrying capacity of the pasture system by a similar magnitude and would frustrate attempts by graziers to make productivity gains (Simpson and Richardson 2009).

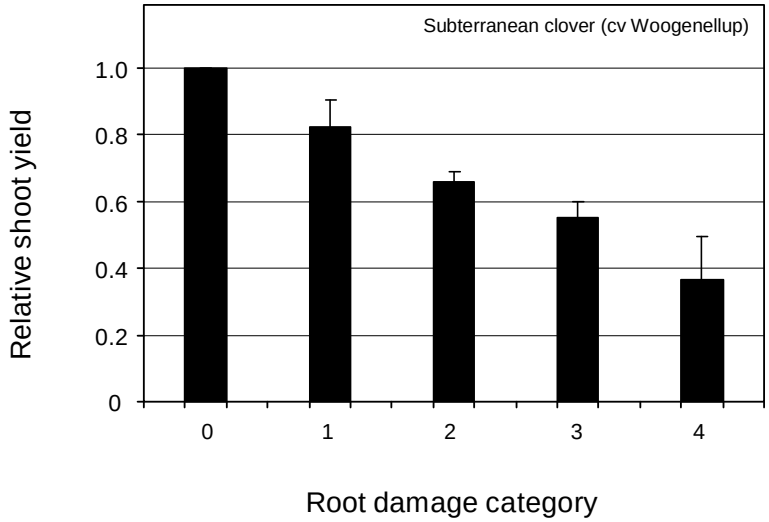


Figure 2. Relative yield of subterranean clover (cv. Woogenellup) in response to root damage (mean of bioassays conducted in 2007 at four NSW sites). Key to root damage categories: 0 = undamaged, 1 = secondary lateral roots damaged and/or tap root brown, 2 = primary lateral roots damaged, 3 = tap root damaged, 4 = severely damaged roots leading to plant death. Bars = 1xSD. Figure adapted from Simpson and Richardson (2009). Note: this version of the original Figure uses the revised numbering of root damage categories from: 0 (no damage) to 4 (most severe damage) as used in the current project (Table 2). At the time of the original report, the process of categorising root damage categories was still being developed and a slightly different numbering system was in use.

1.3 P fertiliser requirements of subterranean clover pastures

Root rot pathogens are also active in spring, but any impacts on pasture yield do not usually restrict carrying capacity because pasture growth rates almost always exceed the rate at which pasture can be utilised. However, the fertiliser requirements of grass-legume pastures are determined by the P requirements of the legume at this time of the year (Simpson et al. 2014). If root damage constrains root foraging for P in soil during spring, the cost of the root pathogens will be expressed as a higher P-fertiliser requirement for production than would be the case if plants were free of root disease. In reality, it is not known whether root damage constrains pasture nutrition during spring. It is known, for example, that root damage will be less of an issue in warmer soil temperatures (Wong et al. 1984; Wong et al. 1986) when root growth is more vigorous.

Gains in P-efficiency of crop species are being made by selecting for root traits that encourage better exploration in surface soil layers (Lynch 2007; Richardson et al. 2011; new MLA/AWI co-investment in PUE.0104: P Efficient Legume Pastures). However, this is also where soil pathogens damage clover roots. It is presently unknown whether root damage limits plant yield indirectly by restricting nutrient acquisition, or as a direct perturbation of the shoot-root balance of the plant as a consequence of “root pruning”. Two reasons why this needs to be clarified are:

(i) If damaged roots are unable to forage adequately for P in soil, it is possible that nutrients supplied at the time of root stress may help overcome pasture yield constraints.
(ii) There is a risk that the ubiquitous occurrence of damage to root foraging may negate attempts to select subterranean clover plants for root traits that improve soil exploration and confer P-use efficiency. Indeed, nutrient efficiency in this case may be achieved more effectively by selecting plants for resistance to root damage.

The experiments that follow were undertaken to guide pasture management options and to assess whether root damage will compromise attempts to breed subterranean clover for improved P-acquisition efficiency.

1.4 Root rot pathogen DNA assays for subterranean clover pastures

Quantitative DNA assays for a number of fungal and oomycete root rot pathogens of pastures have been available from SARDI Plant and Soil Health, Adelaide since their development and initial testing at field sites across southern Australia (Ophel-Keller et al. 2008, O'Rourke et al. 2009, Simpson et al. 2011).

Similar DNA assays of root pathogens are used under the brand name: Predicta B (http://www.sardi.sa.gov.au/diagnostic_services/predicta_b), to evaluate the risks of soil-borne diseases in crops. Early work conducted with MLA funding in the 2006 growing season (SHP017) and in 2007 (SHP0025), demonstrated that the pasture DNA assays were able to detect the presence of particular pathogens in soil and plant root samples, but relationships were not found between the pathogen DNA concentrations of soil under pasture and the extent or severity of root damage as measured in bioassays that used subterranean clover as the test species (Simpson et al. 2011).

In 2007, an attempt was made to assess the concentrations of pathogen DNA that were directly associated with damaged roots after the bioassay test plants had been sorted into their respective root damage categories. These data indicated that the DNA concentrations of some pathogens (e.g. *Pythium* clade F, *Phytophthora clandestina*) associated with the subterranean clover roots were elevated by worsening root damage (Fig. 3; Simpson and Richardson 2009). A positive association between the pathogen DNA concentration of the roots and the damage to the root system was observed at three of four field sites examined in NSW. None of the pathogen DNA tests were elevated by root damage at the fourth site ("Kia-Ora"). However, a comprehensive set of pathogen DNA tests was not available at the time (e.g. there was no test available for *Aphanamyces trifolii*).

It was also shown that the constraint to subterranean clover growth was always greater when root damage severity was higher (Simpson and Richardson 2009). It was hypothesised that if pathogen DNA concentrations of clover roots were reliably associated with the severity of root damage, the correlation between clover yield and root damage severity may enable root pathogen DNA assays be used to diagnose pasture yield constraints in autumn-winter due to fungal root rot (Simpson and Richardson 2009).

In the present study, pasture root pathogen DNA assays (SARDI Plant and Soil Health, Diagnostic Service) were used to monitor fungal and oomycete pathogen DNA

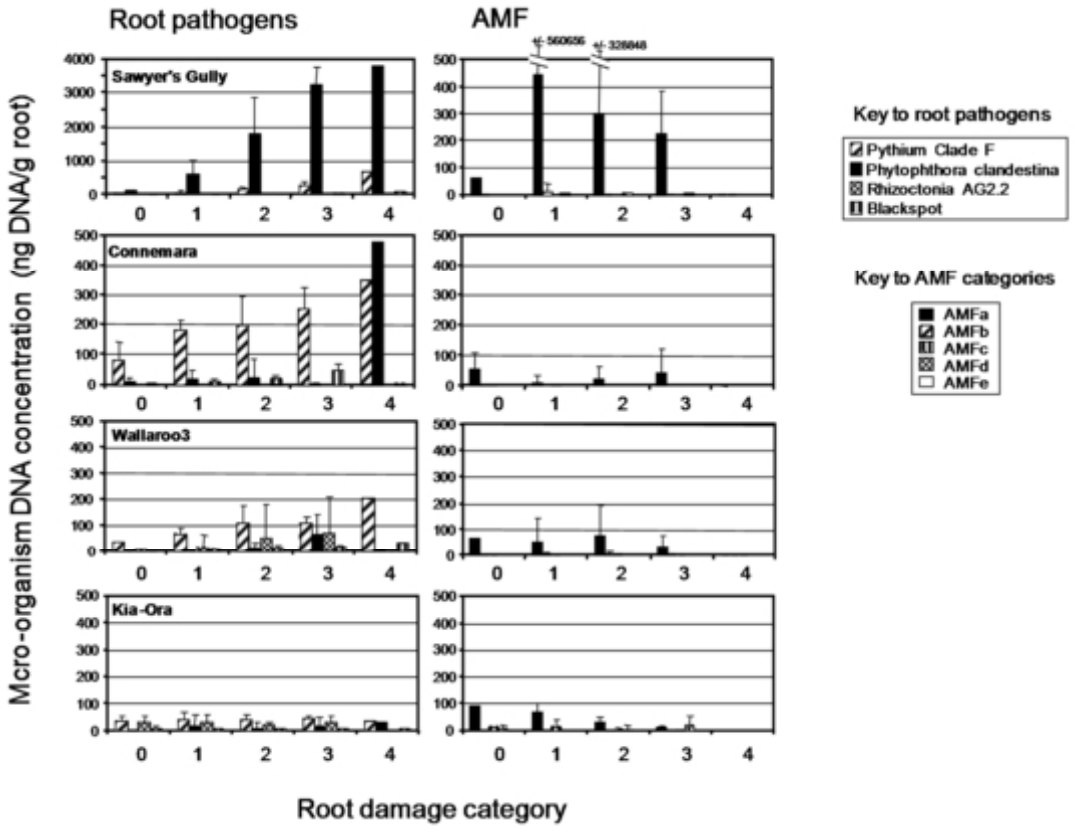


Fig. 3. Concentrations of the target DNA of some major root rot pathogens of subterranean clover and arbuscular mycorrhizal fungi (AMF) that were associated with the roots of cv. Woogenellup in each root damage category at four NSW pasture sites (autumn-winter, 2007). Key to root damage categories: 0 = undamaged, 1 = secondary lateral roots damaged and/or tap root brown, 2 = primary lateral roots damaged, 3 = tap root damaged, 4 = severely damaged roots leading to plant death. Bars = 1xSD. Figure adapted from Simpson and Richardson (2009). Note: this version of the original Figure uses the revised numbering of root damage categories from: 0 (no damage) to 4 (most severe damage), as is used in the current project (Table 2). At the time of the original report, the process of categorising root damage categories was still being developed and a slightly different numbering system was in use.

concentrations associated with damaged roots. This provided a collateral opportunity to explore whether fungicide treatments were effective against root pathogens, whether soil P fertility affected pathogen infection of roots, and whether pathogen DNA concentrations were related to the severity of root damage.

2 Projective Objectives

2.1 Specific objectives

1. To quantify the impact of damaged roots on the response of subterranean clover to soil P fertility improvement.

2. To assess whether P-nutrition of a pasture modifies the incidence and severity of damage to roots.

3. To determine whether breeding subterranean clover for improved root foraging traits will be an effective way of improving the P- efficiency of pastures systems.

4. To quantify the impact of soil P fertility on pathogen DNA levels in damaged roots and the relationship between damage and pathogen DNA concentration of roots.

3 Methodology

3.1 Subterranean clover (cv. Woogenellup) bioassays to examine the response of clover to root damage and soil P fertility in the field during autumn-winter.

3.1.1 Field sites used in the experiments

A major objective of is project was to understand the interaction of root damage on subterranean clover with soil P fertility. Assessments in the autumn-winter period cannot be done by simply applying P fertiliser to an existing pasture to create differences in soil fertility because when P fertiliser is applied a transient spike in soil P availability occurs (e.g. Simpson et al. 2010). This can temporarily exceed the P requirements of subterranean clover, even when only small quantities of fertiliser have been applied, and eliminates any difference in the P fertility that is experienced by the clovers in the "intended" P treatments. The transient increase in the availability of P can persist for a few months and is the reason why graziers are advised not to take soil tests for a few months after spreading fertiliser (Simpson et al. 2009). Therefore, autumn-winter bioassays were conducted at field sites used previously to assess the response of pasture to soil P fertility improvements (Table 1). The bioassays were established within previous soil P fertility treatments that had spanned the P response range of subterranean clover, from extremely deficient (unfertilised) through to supra-optimal for clover yield (as measured by growth during spring). P fertilisers were not applied to the bioassay experiment and soil P fertility levels in the original treatments will also have been modified by "scalping" the soil (0.5-1 cm depth removed) during bioassay establishment. P fertiliser is broadcast onto the soil surface when pastures are fertilised and scalping the soil removed the topmost layers of the soil which have the highest extractable P concentration. Consequently, the influence of soil P fertility was gauged by using soil extractable-P tests to quantify P availability.

Experiments during the spring growth period were also conducted at these sites. However, it was possible to established different P treatments for experiments during spring by applying P fertiliser early in the autumn-winter months.

Table 1. Location, pasture type, and root rot pathogens previously detected at the field sites using SARDI Plant Health root pathogen DNA assays. Additional soil analysis details are available in Simpson et *a*l. (2011).

Location (Paddock history)	Latitude/ Longitude	Main pasture species present (in order of abundance; most abundant [first] to less abundant)	Root rot pathogens known to be present based on previous assessment using SARDI DNA diagnostic tests of the soil.
"Sawyer's Gully, Wee Jasper, NSW Permanent sown pasture; >30 years	S 35°04.474' E 148°47.357'	Phalaris, subterranean clover & Patterson's curse (*Echium lycopsis*)	*Phytophthora clandestina* *Pythium* clade F *Rhizoctonia solani* AG2.2
"Wallaroo 3", Ginninderra Experiment Station; Hall, ACT Permanent sown pasture; >36 years	S 35°10.527' E 149°02.656'	Annual grasses (*Bromus molliformis*, *B. diandrus*, *Vulpia myuros, V. bromoides*), phalaris & subterranean clover	*Pythium* clade F *Rhizoctonia solani* AG2.2 *Gaeumannomyces graminis*# *Phytophthora clandestina* (detectable)
"Redmire", Taralga, NSW Permanent sown pasture; >10 years at least; prior history other than some use for potato production was unknown	S 34°21.330' E 149°47.415'	Phalaris, subterranean clover, ryegrass (*Lolium* sp.), annual grass (*Vulpia* spp.)	*Pythium* (clade F) *Phytophthora candestina* *Rhizoctonia solani* AG2.2 *Rhizoctonia solani* AG4 *Gaeumannomyces graminis*# *Fusarium culmorum*
"Gilligooly", Braidwood, NSW Permanent native and sown perennial grass pasture; >30 years	S 35°28.400' E 149°54.921'	Native grasses (mainly *M. stipoides*) with cocksfoot (*Dactylis glomerata*)	*Pythium* (clade F) *Gaeumannomyces graminis*#
"Kia-Ora", Bookham, NSW Grazing demonstration site (Permanent pasture with native grasses; >42 years	S 34°48.160' E 148°34.917'	Annual grasses (*Bromus molliformis*, *B. diandrus*, *Vulpia myuros, V. bromoides*), subterranean clover (*Trifolium subterraneum*), wallaby grass (*Austrodanthonia* sp.) & weeping grass (*Microlaena stipoides*)	*Pythium* clade F *Rhizoctonia solani* AG2.2
"Connemara" (Curse paddock) Tarcutta, NSW Permanent pasture sown to phalaris & subterranean clover in 1992	S 35°30.980' E 147°51.324'	Annual grasses (*Lolium rigidum*, *Bromus* spp., *Vulpia* spp.), phalaris, subterranean clover & naturalised clovers	*Pythium* clade F *Phytophthora clandestina* *Gaeumannomyces graminis*# *Rhizoctonia solani* AG2.2 (detectable)

pathogen of grass roots.

3.1.2 Bioassay protocol

Details of the bioassay protocol are now published (Simpson et al. 2011).

Seedling survival during germination and establishment was assessed after sowing *T. subterraneum* L. (cv. Woogenellup) with minimal disturbance into pasture sites in a bioassay based on a protocol used previously to assess the prevalence of damping off and root rot pathogens on *T. subterraneum* (Wong et al. 1985). However, causal organisms were not isolated from damaged roots in the present study. The *T. subterraneum* cultivar, Woogenellup, was specifically chosen for the bioassay because it is used commonly as the control cultivar in root disease studies for this species and this enables the occurrence and severity of root damage in the present study to be benchmarked against previous and future work in this field.

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. Seeds were surface-sterilised by washing in 70% ethanol for 30 seconds and dried, prior to planting. The germination percentage of the *T. subterraneum* seed exceeded 95%. Within about 4 weeks after the break of season at each site, each replicate bioassay area was prepared by removing the top 0.5-1 cm of soil and plant material from rows 1.2 m long x ~0.3 m wide using either a motorised "turf-cutter" or by hand with a flat, sharpened trenching shovel. This removed most naturally-occurring clover seed and reduced the potential for interference from weeds. Previous work with the bioassay has demonstrated that the root damage observed in bioassays prepared in this way does not differ from bioassays that had been prepared by hand-weeding without "scalping" the soil. However, the latter, were very labour-intensive and unable to be sustained when large number of bioassays were being used. A furrow (1 m long x ~4 mm wide x 5 mm deep) was formed by pressing a T-shaped furrow tool into the moist soil and the surface-sterilised seeds (100-germinable seeds) were sown at 1 cm spacings along the row before being covered lightly by brushing soil over the seed (Fig. 4). Development of the bioassay planting equipment shown in Fig. 4 was completed during this project and greatly enhanced the efficiency and accuracy of bioassay establishment. The seed number being planted was varied according to the predetermined germination percentage of each seed lot to ensure 100 germinable seeds were planted. This was done by closing an appropriate number of suction holes at either end of the planting tool (Fig. 4).

At sites where autumn-winter bioassays were overlaid on a previous soil fertility management experiment ("Sawyers Gully", 18 July-17 September 2012; "Redmire", sown 18 June 2013; "Gilligooly". 16 May – 5 August 2014), the 18 pre-existing plots were located, divided to create 36 unreplicated soil P treatments (each defined by its extractable soil test P concentration) and two bioassays were installed, one using surface-sterilised seed, the other using surface-sterilised seed that had been treated with Apron.

NOTE: *At "Sawyer's Gully", bioassays were initially sown on 24 May 2012 and failed due to poor seed germination (faulty seed). The area was resown with fresh seed on 18 July but it was soon noticed that mice had damaged some bioassays by eating buried seeds and/or nibbling the cotyledons from the emerging seedlings. Initial baiting*

with Ratsak (Yates) wax-based bait stations confirmed that mice were the problem, but proved insufficient to protect the experiment fully. The area was then baited using "Mouseoff" (see details below). Mouseoff baits were adopted routinely after this experience. However, the site at "Redmire" in 2013 was extensively damaged by mice despite baiting and could not be resown due to time constraints. The predation pressure from mice at this site also appeared to have affected the spring experiments (see Results).

The other autumn-winter bioassays referred to in this report (e.g. section 3.5.1; experiments examining root pathogen DNA concentrations of damaged roots) used eight replicate bioassays per site (when site was the treatment), or in a randomised complete block design (for subterranean clover cultivar comparisons).

Immediately after sowing, the area around and over all seed rows and a buffer area extending 2 m beyond was sprayed with bifenthrin (Talstar; FMC (chemicals) Pty Ltd) at 100 ml (10 g a.i.)/ha, to protect seedlings from insect damage. Bioassay rows were protected from large grazing animals by fencing or exclusion cages and (after an initial problem) from mice by spreading Mouseoff Zinc Phosphide (Animal Control Technologies [Australia] Pty Ltd) around the treatment areas.

The number of plants that emerged were usually counted when the primary leaf of the clover had expanded (about 2-4 weeks after sowing depending on site conditions; phase 1), and was always assessed when 2-3 trifoliate leaves were present (about 4-8 weeks depending on site conditions; phase 2). At this point, 15 soil cores (25 mm diam x 10 cm depth) were collected immediately around and between the paired bioassays in each plot. The soil cores were combined, oven dried at 40^{o}C and used subsequently to determine the extractable soil P concentration (Colwell 1963). Clods of soil containing all of the surviving *T. subterraneum* plants were then excavated carefully from each bioassay row and the 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 on the day that the plants were harvested, the clods of soil containing the test plants were kept intact and cool (4^{o}C) for up to 12-18 h prior to being washed.

3.1.3 Root damage scores and shoot dry weight assessments

The clover seedlings that had been washed from the soil with their roots intact were floated in shallow, black trays of water and scored using the 5-step rating scheme that is outlined in Table 2. The rating scheme was based on a root disease scoring system reported for *T. subterraneum* by Wong et al. (1985a) and was modified from the 4-step rating scheme reported originally by Simpson et al. (2011), when it was realised that score 0 plants (healthy roots) in the original scheme usually contained many plants with damage to their secondary lateral roots. The plants were sorted into their root damage categories (RDC) based on their root symptoms and the number of plants in each RDC was counted. Shoots of plants within each RDC were excised from the roots at the colour (white/green) junction between the shoots and roots and were combined and dried at 70^{o}C for subsequent shoot dry matter determination. The roots within each category were also combined and were frozen (-20^{o}C), and then dried by freeze-drying for subsequent dry matter determination and pathogen DNA analyses (Fig. 5).

3.1.4 Statistical analysis of the interaction between root damage, soil P fertility and shoot yield.

At sites where bioassays were overlayed on a previous soil fertility management experiment ("Sawyers Gully" in autumn-winter 2012, "Redmire" in 2013, "Gilligooly" in 2014), 18 pre-existing plots (6 P treatments, randomised complete block, n=3) at each site were located, divided to create 36 plots. The effects of (i) time since the last fertiliser application during which extractable soil P levels would be expected to decline, and (ii) soil "scalping" which would remove an unknown amount of extractable P from each bioassay location, were expected to result in 36 unreplicated soil P treatments. Each treatment would be defined by its extractable soil test P concentration. It was, therefore, anticipated that the bioassay experiments should be analysed predominantly using regression analysis.

The relationship between shoot yield and root damage was clearly shown by the regression analyses, but was also examined using bar graphs of average shoot yields for plants in each RDC. These data are subject to an issue of variable replication that arises because of the unpredictable numbers of plants that fall into each RDC as discussed in section 3.5.4. In this report, these data are discussed assuming that least significant differences (P = 0.05) are likely to be approximately 2xSE.

3.2 Root pruning experiment to simulate damage to roots by root rot pathogens

A method for pruning roots *in situ* was developed to distinguish the direct impact of a constricted root system on subterranean clover growth from any indirect effects of restricted root growth on P acquisition by the plants.

Subterranean clover (cv. Woogenellup) was grown in a glasshouse with temperatures maintained between a maximum of 22^{o}C (day) and minimum of 10 ^{o}C (night). The plants were grown in an unfertilised (low P; Colwell P = 5.4 mg P/kg) or a fertilised topsoil (high P; Colwell P = 28.1 mg P/kg) that had been steam pasteurised to remove root rot pathogens. The pasteurisation treatment reduces the likelihood of root disease but does not always eliminate the potential for root colonisation by AMF which in some experiments recolonise roots from spores that have survived pasteurisation. The soil was the 2-15 cm depth layer of a yellow chromosol from the Wallaroo 3 paddock at Ginninderra Experiment Station near Hall, ACT. Seeds were sown into small plastic "pots" (soil volume ranging from 4 - 85 cm^3) contained within a larger plastic pot (6.5 cm diameter x 11.0 cm depth; volume = 365 cm^3) (Fig. 6). The inner pot was drilled with many holes to allow unrestricted root growth into the larger pot. Two root and six soil volume treatments were imposed at each of the two soil P-fertility levels (Table 3, Fig. 6); each with 5 replicates.

The pruning of roots in "pruned root" treatments was achieved by regularly removing the inner pot from the soil in the outer pot and scraping the outside of the inner pot with a sharp scalpel blade to "shave" any protruding roots. The cavity in the soil of the outer pot created by the inner pot remained intact during this procedure and when the inner pot had been shaved it was replaced and gently pressed back into the soil of the outer

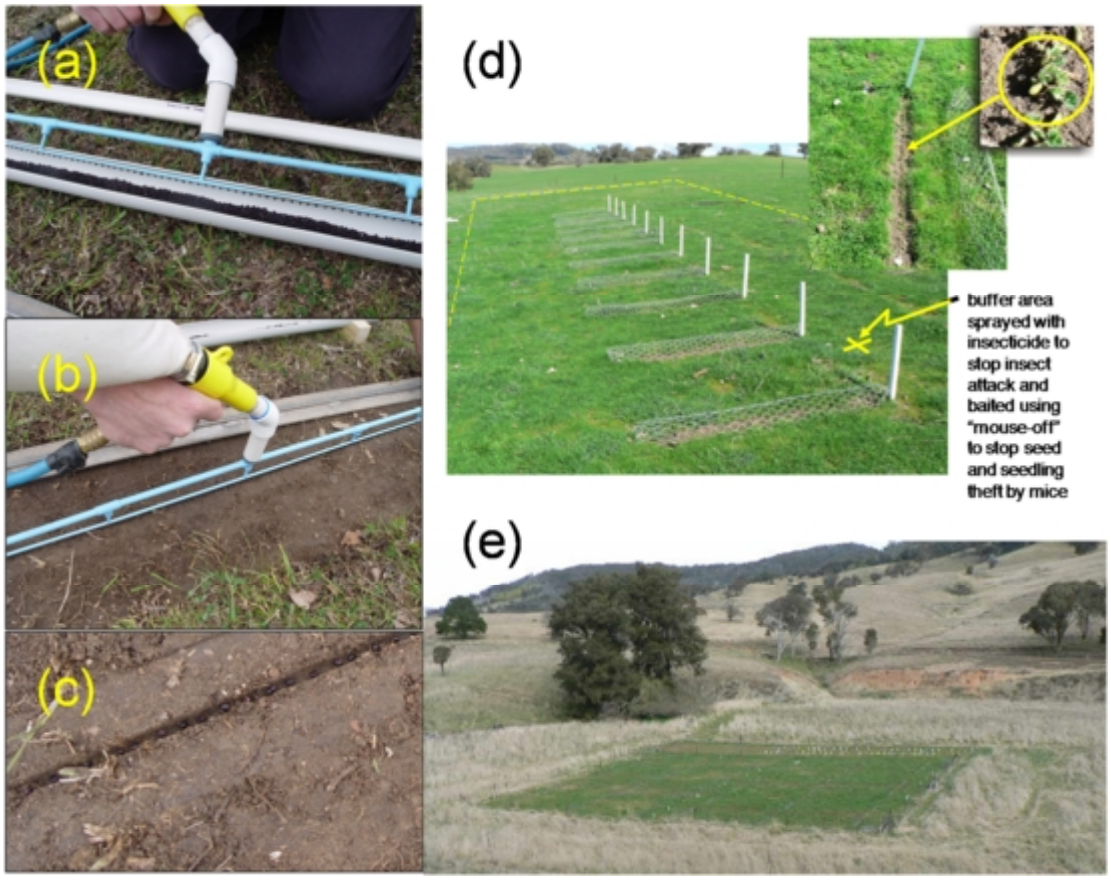
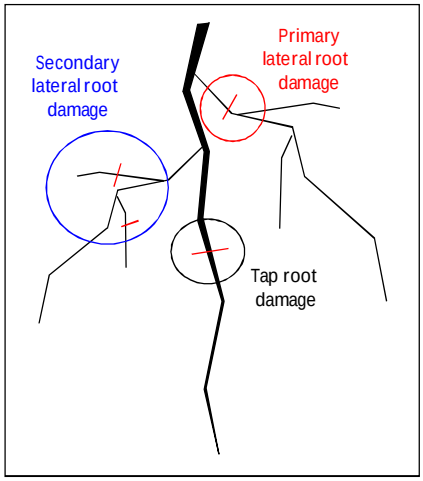


Fig. 4. (a, b, c) Each bioassay has 100 viable seeds planted (with and/or without fungicide treatments) in a single row. Development of the bioassay planting equipment shown here was completed during this project and used air suction to hold seeds at 1 cm spacings. Seeds were dropped accurately into the furrow by removing the suction. (d) Bioassay rows are protected against grazing, insect and mice attack as in this typical layout at 'Connemara' via Tarcutta. (e) The Sawyers Gully field site in winter 2012. Bioassays were laid out across a pre-existing fertiliser experiment to examine root damage and clover growth in relation to soil P fertility.

Table 2. Root damage in the clover bioassay is usually assessed on plants excavated at the 2-3 leaf stage of growth. The categories used to classify the extent of damage to roots of subterranean clover are described below.

Root damage category	Description of damage
0	Tap and lateral roots healthy and not discoloured.
1	Whole tap root light brown to brown, secondary lateral roots mostly absent.
2	Whole tap root light brown to brown, usually primary lateral roots affected.
3	Tap root stunted and brown to black, discrete lesions may be present.
4	Whole tap root rotted off, or seedling is dead.



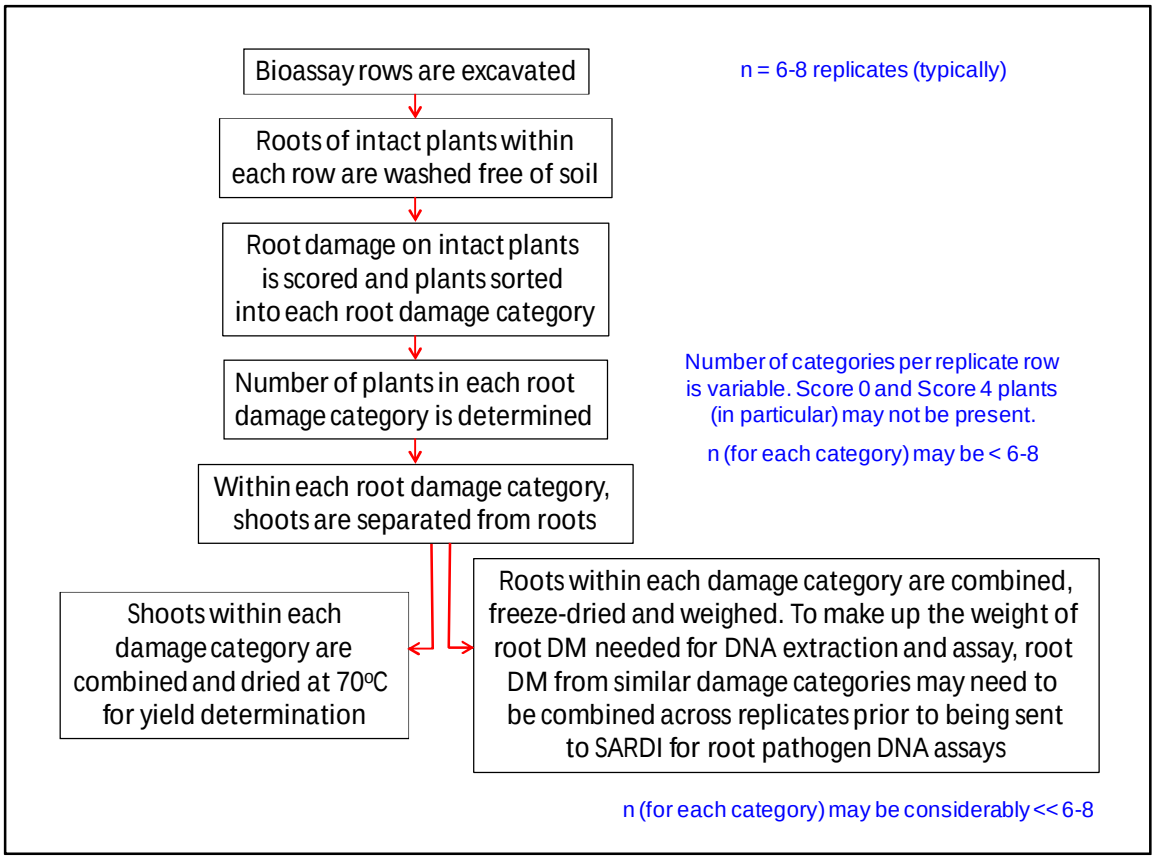


Fig. 5. Outline of protocol used to sort plants into root damage categories, prepare shoots for dry matter determination, and harvest the roots for DNA extraction and pathogen DNA analyses.

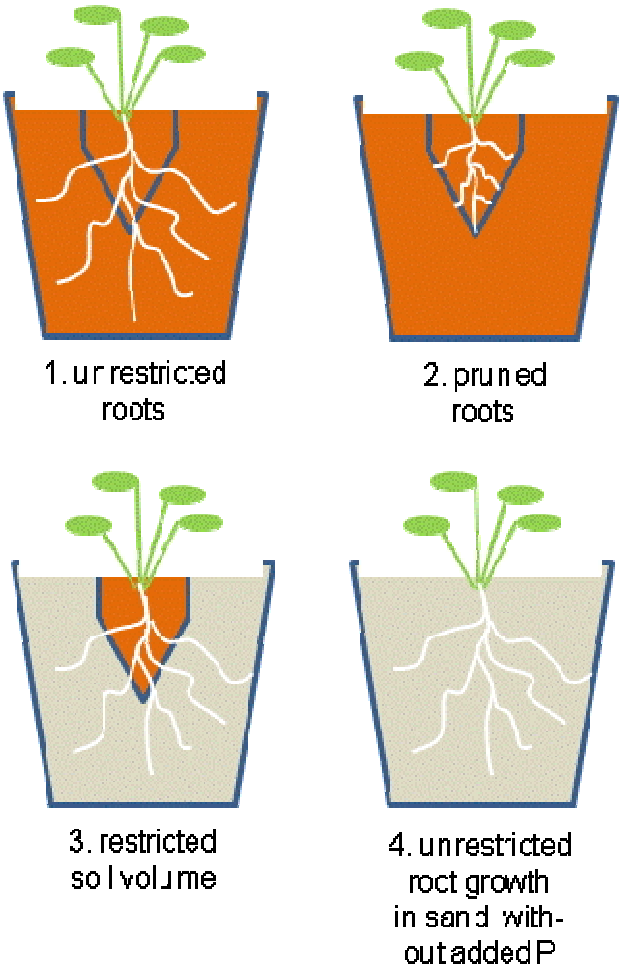


Fig. 6. Schematic representation of the root and restricted soil volume treatments implemented using a soil-filled, perforated, inner pot within an outer pot filled with either the same soil or fine silica sand. See Table 3 for more details.

Table 3. Root, soil volume and soil P fertility treatments

Treatment	Inner pot volumes & type	Soil P fertility	Replication
Unrestricted roots	Six inner pot volumes: 4.2, 6.0, 16.8, 23.7, 52.5, 85.1 cm^3 Inner pots were filled with either low-P or high-P soil and were perforated to allow unrestricted root growth into the same soil in the outer pot	Two soil P fertility levels: Colwell P = 5.4 or 28.1 mg P/kg.	5
Pruned roots	Six inner pot volumes: 4.2, 6.0, 16.8, 23.7, 52.5, 85.1 cm^3 Inner pots were filled with either low-P or high-P soil and were perforated, but the pots were gently removed from outer pot soil every few days for root pruning. Any extruding roots were excised using a scalpel blade (all root growth was consequently confined to the inner pot).	Two soil P fertility levels: Colwell P = 5.4 or 28.1 mg P/kg.	5
Restricted soil volume	Six inner pot volumes: 4.2, 6.0, 16.8, 23.7, 52.5, 85.1 cm^3 Soil was only present in the inner pots which were perforated to allow unrestricted root growth into the outer pot which was filled with a coarse silica sand.	Two soil P fertility levels: Colwell P = 5.4 or 28.1 mg P/kg. Sand in the outer pot was free of nutrients other than those introduced by watering with a P-free nutrient solution.	5
Root growth in P-free sand	No inner pot. Whole of pot contained coarse silica sand.	The sand was free of nutrients other than those introduced by watering with a P-free nutrient solution.	5

pot. All pots were watered daily with equal quantities of a P-free nutrient solution containing: 0.247 g/l $MgSO_4.7H_2O$, 0.430 g/l $CaSO_4.2H_2O$, 0.101 g/l KNO_3, 0.165 g/l $(NH_4)_2SO_4$ and micronutrients (0.711 mg/l H_3BO_3, 4.55 mg/l $MnCl_2.4H_2O$, 2.16 mg/l $ZnSO_4.7H_2O$, 0.200 mg/l $CuSO_4.5H_2O$, 0.433 mg/l $(NH_4)_2MoO_4$, 0.119 mg/l $CoCl_2.6H_2O$ and 9.2 mg/l FeNaEDTA) and were topped up when necessary with distilled water to maintain the soil moisture content at ~70-80% of field capacity. Plants were also grown in the larger pots in a nutrient-free sand that was also watered with an equal quantity of the P-free nutrient solution.

The plants were harvested after 33 days growth. Shoots were dried at 70^oC and weighed. Roots were stored in 50% ethanol until root length could be measured after scanning and analysis using WinRhizo software (Regent Instruments, Canada).

3.3 Responses of subterranean clover and root pathogen DNA to fungicide treatments injected into the root zone of established subterranean clover swards during spring

Root pathogens have their largest impact on the productivity of a grazing enterprise during autumn-winter when pasture growth rates are slow and any constraint to pasture yield will directly constrain the potential stocking rate of a farm (Simpson and Richardson 2009). Temperature is an important factor (Wong et al. 1984; 1986). It is hypothesized that fungal hyphae growth is relatively advantaged over root growth at this time of the year. By contrast, in spring it is likely that pasture growth can get ahead of the fungal attack on roots. Spring pasture growth also usually exceeds animal feed requirements and it is, therefore, less likely that root pathogens will directly constrain animal production at this time of the year. However, the “critical” soil P fertility requirement of pasture (the soil test P concentration or amount of P fertiliser necessary for near maximum growth) is determined by the rate of pasture growth in spring. Pasture that has restricted root growth may require a higher soil P concentration to achieve its maximum pasture growth rate.

The seedling bioassay used to assess impacts of root damage on autumn-winter growth does not adequately replicate the conditions likely to be experienced by established plants with large root systems growing under warm, moist spring conditions. For this reason, assessments of constraint to yield or ability to respond to P due to root rot pathogens were made by applying fungicide(s). A novel method for treating pasture with fungicide was necessary because fungicide applied topically to soil often may not penetrate to the root zone and can, as a result, be ineffective and/or phytotoxic (e.g. Lodge 2005). Even with careful use of control treatments it is very difficult to unravel the confounding effects of fungicide ineffectiveness, phytotoxicity and/or pathogen affects.

3.3.1 Fungicide injection into the root zone of subterranean clover swards

3.3.1.1 Fungicide dose rate determination

Metylaxyl was chosen as the fungicide for initial work as it often reduces root rot damage when used as a soil drench treatment in glasshouse pot experiments or when used as a seed treatment (Barbetti 1987a, 1987b). It is selectively active against

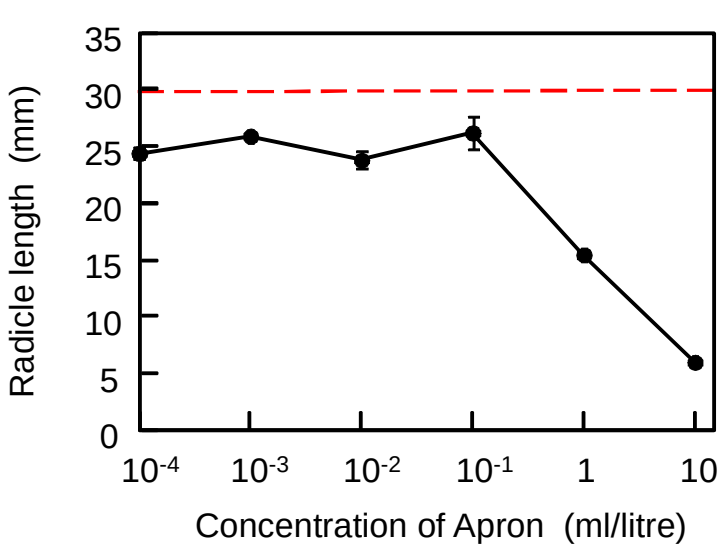


Fig. 7. Length of radicles grown by subterranean clover seedlings (cv Woogenellup), 4 days after germination in the presence of different concentrations of Apron (active ingredient: metylaxyl) in a petri dish assay. The length of radicles on seedlings grown in water is shown by the dashed red line. Bars represent 2xSE and where not shown were smaller than the symbol.

oomycetes and would be expected to reduce root disease associated with *Pythium* and *Phytophthora* infections. However, it was very unclear what dose rate should be used when injecting the fungicide into the root zone of an established clover sward. Industry representatives that market this fungicide product were consulted but it was clear that their recommendation for application in this way to this sort of pasture sward was, at best, an "educated guess". A laboratory bioassay was therefore devised to determine the dose rate range that would be used in the field experiment.

Fifty seeds of subterranean clover cv. Woogenellup per replicate were germinated in petri dishes in the dark at room temperature (22^{o}C) on filter papers that had been saturated with water or different aqueous concentrations of Apron XL 350ES (Syngenta) which contains 350 g/litre of metylaxyl as its active ingredient. Radicle length was reduced by high concentrations of Apron and this was assumed to indicate concentrations of the fungicide that would cause phytotoxicity when the fungicide was applied to subterranean clover (Fig. 7).

Even very low concentrations of Apron had a slight inhibitory effect on radicle growth when compared with growth in water, but substantial phytotoxicity only occurred at concentrations above 0.1 ml Apron/litre. On this basis 0.01, 0.1, and 1 ml/litre concentrations of Apron were selected for use in field experiments where the fungicide solution was to be injected into soil in the root zone of a pasture sward.

3.3.1.2 Fungicide injection via subsurface irrigation

A field trial was established in May 2012 at Sawyer's Gully (via Wee Jasper, NSW) by installing 4.5 m lengths of 13 mm diameter, Pressure-compensating Drip-Eze irrigation tubing (TORO) with outlets at 30 cm intervals. The irrigation tube was laid at 7 cm depth in narrow (5 cm) trenches created using a "Bull-Ant Irrigation Trencher" irrigation tube laying machine. This machine digs the soil and then replaces it over the tube in the trench. The loose soil was then pressed down manually with the aim of achieving a similar bulk density to the undisturbed soil. The area was subsequently prepared by applying a grass-selective herbicide (Select, Dow AgroSciences, rate 1000 ml/ha) to

Fig. 8. Subterranean clover (cv. Woogenellup) monoculture at Sawyer's Gully site on 5 October 2012 just prior to injection of fungicide treatments. The pasture sward was mown on this day to ensure that all treatments started with similar shoot dry matter yields. The ends of the subsurface irrigation tubes that were used to inject fungicide into the root zone at 7 cm depth can be seen to the left of the trial area

Table 4. Fungicide and control treatments applied in the field "soil drenching" trial. All tr*eatm*ents were replicated three times.

Fungicide treatment	Details/description	P treatment
Untreated	No treatment solution injected into the root zone (negative control treatment)	Low (P0)
Water	Water injected (negative control treatment)	
Nutrient	Nutrient solution containing 3.31 g ammonium nitrate/litre injected (positive control treatment) – i.e. equivalent to 35 kg N/ha	
Apron 1	Apron fungicide (0.01 ml/litre) injected	Intermediate (P1)
Apron 2	Apron fungicide (0.1 ml/litre) injected	
Apron 3	Apron fungicide (1 ml/litre) injected	
Apron surface	Apron fungicide (0.1 ml/litre solution sprayed 2.6 litres/strip) sprayed onto the pasture – i.e. 0.26 mls Apron over strip 1m² in area	High (P2)
Thiram surface	Thiram fungicide (120g / 50L water / 100m²; manufacturers recommended rate) sprayed onto the pasture - i.e. 1.2 g Thiram over strip 1m² in area	

remove phalaris and other grasses and was sown to subterranean clover cv. Woogenellup on 8 June 2012 using 1000 kg seed/ha to establish a high density sward that would reach canopy closure by early spring (e.g. Fig. 8).

Three soil P fertility treatments were applied to the pasture on 12 June 2012 by first applying basal nutrients (100kg potassium sulfate/ha, 60kg magnesium sulfate/ha, 0.07kg molybdenum trioxide/ha, 1.75 kg boric acid/ha, 1.75kg copper sulfate/ha and 3.5kg zinc sulfate/ha) over the whole area and then 0, 48 and 242 kg triple superphosphate/ha to achieve low (11.9±1.3 mg P/kg), intermediate (12.4±0.6 mg P/kg) and adequate P fertility (26.9±8.6 mg P/kg; Colwell 1963). The rates were chosen on the basis of an earlier P response trial run at this site and the variability recorded in some of the extractable P soil test outcomes was unexpected.

On 5 October, the irrigation tubes were used to deliver each fungicide treatment solution (Table 4). This was achieved by attaching a pressure chamber filled with the treatment solution to each irrigation tube and first filling the tube by gravitation until the treatment solution flowed from the open distal end of the tube which was then closed (Fig. 24c). Pressure (20 psi) was then applied to push 2.6 litres of the treatment solution through the irrigation line and out of the outlets which were pressure regulated to ensure equal volumes of solution would be delivered at each outlet.

The clover swards were harvested on 16 October 2012 by cutting at ground level with hand shears. An area 8.5 cm wide x 900 cm long was harvested from each plot directly above and centred on the irrigation/treatment line. Herbage was oven dried at 70^{o}C and weighed. Soil samples (0-10 cm depth) were taken from the soil in each treatment line and were freeze-dried for future root pathogen DNA analyses by SARDI (Plant and Soil Health) in Adelaide.

3.3.1.2 A new method for injecting fungicides into the root zone

Soil disturbance during the laying the subsurface irrigation lines, clearly interfered with assessments of the response of subterranean clover to fungicide injection (see Results). Consequently, a new method of injecting fungicide into the root zone of pasture without significant soil disturbance was developed.

The method is illustrated in Figs. 9 and 10. Once the injection tube has been installed, a standard volume of fungicide solution was injected under pressure. The blue dye used to demonstrate the distribution of the injected solution clearly indicated that roots of plants around the injection point would be in direct contact with the fungicide (Figs. 9d and 9f). After the fungicide had been applied, the injection tube was removed and the hole remaining in the soil was plugged with a flagged, wooden marker pin (Fig. 10) to indicate the treatment and accurately pin-point the site of injection for subsequent harvest of herbage grown in response to the root zone treatment.

Fig. 9. A new method developed for injecting fungicide into the root zone of subterranean clover pasture growing in undisturbed soil.

(a) injection tube, pin and "dolly" to protect the injection tube from hammer blows,

(b) unit is assembled and hammered gently to the injection depth,

(c) dolly and pin are removed,

(d) pump with solution to be injected is connected and solution is pumped into the soil (in this case the solution contains a blue dye),

(e) excavation of injection tube to reveal distribution of blue dye in the soil,

(f) the blue dye indicates how fungicide is expected to be delivered to the root zone.

Fig. 10. Initial test of the new protocol for fungicide injection at Sawyers Gully (August 2013):
(a) fungicide injection,
(b) injection hole was plugged with a site and treatment marker,
(c) at this site, a series of replicated treatments were applied including positive and negative controls across low, intermediate and high soil P treatments.

3.3.1.3 Fungicide injection into the root zone of undisturbed clover swards

Fungicide or control treatments (Table 5) were injected into the undisturbed, root zone of the subterranean clover monocultures on 4 and 5 September 2013 at the "Sawyer's Gully" and "Redmire" sites, respectively, using the new injection method (Figs. 9 and 10). The fungicide application rates were either determined on the basis of results from the "subsoil irrigation" experiment (i.e. Apron, see Section 4.3.1) or followed the manufacturers recommendation (i.e. Thiram). Water was used as a negative control (i.e. no treatment) and nitram (ammonium nitrate) was applied with the intention of having a positive control that should promote growth and allow comparisons with the results of the fungicide treatments.

A dressing of basal nutrients (100 kg potassium sulfate/ha, 60 kg magnesium sulfate/ha, 0.07 kg molybdenum trioxide/ha, 1.75 kg boric acid/ha, 1.75 kg copper sulfate/ha and 3.5 kg zinc sulfate/ha) had been applied over the whole experimental area at "Sawyer's Gully" on 12 June 2012 and was applied at "Redmire" on 5 July 2013. Low, intermediate and high P treatments were imposed by applying 0, 48 or 242 kg triple superphosphate/ha to the soil surface at "Sawyer's Gully" (14 August 2013) and 0, 300 and 800 kg triple superphosphate/ha at "Redmire" (25 July 2013). The experiments were laid out using a split plot design with fungicide treatments as the split plots and P treatments as the main plots; n=3 at "Sawyer's Gully" and n=6 at "Redmire".

Pasture was harvested by cutting at ground level from small quadrats (10 x 9 cm) centred on the treatment injection sites, 42 and 47 days after the treatments were applied at "Sawyer's Gully" and "Redmire", respectively. The herbage was dried at 70^{o}C for dry matter determination.

After the subterranean clover herbage had been harvested, 2 soil cores (25 mm diam, 0-10 cm depth) were removed from the harvested area of each replicate, enclosed in a

Table 5. Two hundred mls of each fungicide or control treatment was injected into the root zone of the subterranean clover swards at 7 cm depth in early spring; n = 3 replicates.

Fungicide treatment	Details/description	P treatment
Water	Water (negative control treatment)	Low (P0)
Nitram (ammonium nitrate)	3.31 g ammonium nitrate/litre; approximately equivalent to 35 kg N/ha (intended as a positive control treatment)	
Thiram (tetramethylthiuram disulfide)	Thiram DG (wettable powder) fungicide (0.15 g a.i./litre)	Intermediate (P1)
Apron+Thiram	Apron XL 350ES (1 ml [0.35 g a.i.]/litre) + Thiram (0.15 g a.i./litre)	High (P2)
Apron (metylaxyl)	Apron fungicide (1 ml [0.35 g a.i.]/litre)	

plastic bag and frozen on dry ice for subsequent freeze-drying prior to analysis for root pathogen DNA by the SARDI Plant and Soil Health diagnostic service.

Two additional soil cores were then taken immediately adjacent to the harvested area for extractable P analysis (Colwell 1963).

3.4 Impact of root pathogens on subterranean clover root morphology

Growth by subterranean clover during spring was shown to respond to P fertiliser in at least one experiment where the pasture was not compromised by other factors, but fungicide treatments (Thiram and Apron) failed to promote spring growth in any experiment despite being effective in reducing oomycete pathogen DNA concentrations in root zone soil. This prompted a change in direction for work during spring because it seemed unlikely that the fungicide treatments would resolve the question of whether root damage was affecting P acquisition by subterranean clover in spring.

Six cultivars of subterranean clover that differ in their specific root length (root length per gram of root system; Table 6) were planted at three field sites ("Wallaroo 3" [28 May 2014] , "Kia-Ora" [30 May 2014] and "Gilligooly" [17 June 2014]) in undisturbed soil that had been scalped (0.5 cm depth; as for a root damage bioassay) to remove native clover seeds, or in the same soil after cultivation to 15 cm depth with a rotary hoe.

Immediately after the site was prepared, PVC cylinders (10 cm diam. X 10 cm depth) were pushed into the soil to delineate each microsward and to facilitate subsequent harvesting of topsoil roots. One hundred seeds of each cultivar (sieved to provide a uniform size range (2-2.4 mm diam.) were planted per replicate sward (n=3) by sprinkling the seeds evenly across the soil surface of a cylinder. The seed was then covered with a thin layer of the same soil. The swards were protected against insect

Table 6. Subterranean clover cultivars used in a study of the impacts of root pathogens on root morphology. Root morphology traits are from project B.PUE 0104 (Phosphorus efficient legume pastures. Specific root length and average root diameter were determined on ~3 week old seedlings grown in thin layers of soil against a perspex sheet ("root boxes") and root hair length was determined on 4 week old plants grown in the same soil.

Cultivar	Specific root length (m/g)	Average root diameter (mm)	Root hair length (mm)
Goulburn	86.0	0.46	0.27
Izmir	94.6	0.48	0.37
Mt Barker	77.5	0.44	0.40
Riverina	68.5	0.52	0.22
Seaton Park	91.5	0.46	0.32
Woogenellup	76.1	0.48	0.33

damage by spraying the soil with bifenthrin (Talstar; FMC (chemicals) Pty Ltd) at 100 ml (10 g a.i.)/ha, from large grazing animals by exclusion cages, and from mice by spreading Mouseoff Zinc Phosphide (Animal Control Technologies [Australia] Pty Ltd) around the experiment.

Shoots were harvested by cutting herbage at ground level 141 ("Wallaroo 3"), 153 ("Kia-Ora" or 108 ("Gilligooly") days after sowing. Shoot material was rinsed free of any dirt and dried at 70°C for dry matter determination. The PVC cylinder was excavated intact and trimmed of soil and roots at 10 cm depth. The soil core containing the topsoil roots (0-10 cm depth) was pushed from the cylinder and divided exactly in half by cutting across the diameter with a sharp knife. Roots in one of the halves were washed free of soil on a fine sieve and dried at 70°C for dry matter determination. The other half was cut in half again, lengthwise. Roots in one of the quarters of the soil cylinder were washed free of soil and scanned for measurement of root length using WinRhizo software. These roots were then dried (70^{o}C) and weighed for determination of specific root length. Roots in the other subsample were washed free of soil, frozen at -20^{o}C and freeze-dried prior to analysis for the root pathogen DNA associated with the root dry matter (SARDI Plant and Soil Health, diagnostic service).

Topsoil (0-10 cm depth) was also collected adjacent to the "Wallaroo 3" field experiment. Half of the soil was steam pasteurised for 30 minutes from the time the soil temperature reached 60^{o}C. PVC pots (90 cm diam x 20 cm depth) were filled with either the pasteurised soil, or with soil that had not be pasteurised and the six cultivars of subterranean clover were planted on 15 August 2014 following the same procedure used in the field experiments. Group C rhizobium inoculant was applied directly to the soil after sowing. The pots were wrapped with reflective sleeves that were raised as the plants grew to equal the height of the leaf canopy. This simulates similar growth conditions to those experienced in a clover sward. The pot were arranged in a complete block design and remained outside in Canberra from 22 August - 22 October 2014. Shoots and roots were harvested at 68 days after sowing and were processed as described for the field experiments. Only topsoil roots (0-10 cm depth) were harvested.

3.5 Root pathogen DNA concentrations associated with damaged subterranean clover roots and the potential for using them as an indicator of root damage severity

Root pathogen DNA assays of clover roots (harvested as shown in Fig. 5) were conducted by SARDI Plant and Soil Health, Diagnostic Service (Adelaide) using the procedure outlined in Simpson et al. (2011) for soil samples. However, DNA was extracted from the freeze-dried root material after the dry material has been mixed with a standard amount of silica sand (Dr Alan McKay; *pers. commun.*).

DNA assays, for the root pathogens for which there are tests available (Table 7), were used in the present experiments to:

(i) assess which pathogens were present on the roots of subterranean clover at each field site,

(ii) determine whether the pathogen DNA concentrations associated with roots responded to treatments that were intended to reduce pathogen infections.

To contain the costs of DNA testing, a soil test from the sites were used initially to narrow the range of pathogen DNA tests to be used for routine testing of root material (see Table 7).

Table 7. Common root pathogens and mycorrhizal fungi groupings for which DNA assays were available from SARDI Plant and Soil Health, Diagnostic Service.

# indicates assays for organisms that are components of the commercial pathogen test package: Predicta B® (www.sardi.sa.gov.au/pestsdiseases/diagnostic_service/predicta_b; Ophel-Keller *et al*. 2003, Ophel-Keller *et al*. 2008, Heap and McKay 2009). For DNA sequence information concerning most other assays refer to Simpson et al. (2011). The DNA assay for *Aphanamyces trifolii* has been only recently developed by SARDI Plant and Soil Health and there is no sequence information published for it at present.

* indicates the subset of assays used routinely in the current experiments, after initially determining which pathogens and AMF could be detected at the field sites.

Fungal pathogens
Bipolaris sorokiniana (common root rot)
Gaeumannomyces graminis var. *tritici* and var *avenae* (take-all) #
Fusarium culmorum/graminearum #
Fusarium pseudograminearum #
Phoma medicaginis var. *pinodella*/*Mycospaerella pinodes* *
Rhizoctonia solani (AG2.1, AG2.2 *, AG4 * and AG8; Neate and Warcup, 1985)
Aphanamyces trifolii
Oomycete pathogens
Phytophthora clandestina *
Pythium spp. clade F (as defined by Levesque and de Cock, 2004) *
Arbuscular mycorrhizal fungi
*Group a *, a2 *;* Group b; Group c; Group d; Group e

The pathogen DNA concentrations of roots on subterranean clover (cv. Woogenellup) in root damage bioassays had been monitored previously for two years at "Sawyer's Gully" (2008, 2009) after the last MLA-funded study in 2007 (Fig. 3), and pathogen DNA concentrations associated with the roots of some alternative subterranean clover cultivars had also been examined. However, the data had not been analysed due to lack of resources for the work.

The current project provided an opportunity to analyse the data collected during the 2008 and 2009 growing seasons and to compare it with data collected in 2007 and in the current project. The objective was to explore the hypothesis that root pathogen DNA concentrations of subterranean clover roots may be indicative of the severity of clover root damage in an autumn-winter pasture, and if so could be used to monitor root damage and to quantify the cost to clover yield that is incurred in farm paddocks.

3.5.1 Background to root damage and pathogen DNA data collected in previous experiments

The experiments in 2008 and 2009 were conducted at four field sites in NSW that had been selected on the basis of previous work to provide contrasting suites of root rot pathogens in the soil ((Simpson et al. 2011; Table 1) and roots (Fig. 3). The core site used for the experiments was Sawyer's Gully because relatively high concentrations of DNA of both *Pythium* clade F and *P. clandestina,* two major oomycete pathogens, were detected in the soil at this site (Simpson et al. 2011).

Five cultivars (Woogenellup, Goulburn, Seaton Park LF, Riverina, and Denmark) and a field strain of subterranean clover collected from the "Sawyer's Gully" site early in 2008, or six cultivars of subterranean clover in 2009 (i.e. those above plus Mt Barker) were compared at "Sawyer's Gully". Only Woogenellup, Riverina and a field strain from the site in 2008, or Woogenellup, Riverina and Mt Barker in 2009 were compared at the other three field sites.

Fungicide treatments were not used to generate differences in root damage in the bioassay experiments until 2009 because previous work had shown that seed treatment with fungicides can have variable and unpredictable efficacy (e.g. Barbetti et al. 1987a) and fungicides applied topically to soil in the field (soil drenching) do not always penetrate the root zone. Poor penetration by the fungicide can directly cause it to be ineffective treatment and/or phytotoxic to the plants (e.g. Lodge 2005). These problems make fungicide treatments haphazard and unsuitable as "pathogen-free" controls. However, Woogenellup treated with Apron (metylaxyl) applied as a seed dressing was included in the 2009 experiment as an additional treatment (genotype being the main treatment) in a randomised, complete block design. Apron 350 SD (350g/l metalaxyl) was mixed with water (1:10 v/v) and surface sterilised seeds were soaked in the fungicide solution for 1 minute at a rate of 1 ml Apron/kg seed. The treated seeds were then air-dried prior to sowing.

Root damage bioassays were established about a month after the opening seasonal rainfall at each site by sowing eight replicate bioassay rows per treatment using the standard bioassay protocol (see sections 3.1.2 and 3.1.3).

3.5.2 Shoot yields and pathogen DNA concentrations associated with damaged roots in the current experiments

Roots and shoots were harvested from the autumn-winter, root damage bioassays conducted at "Sawyer's Gully" (18 July - 18 September 2012) and "Gilligooly" (6 May - 5 August 2014. A bioassay was also planted at "Redmire in 2013, but almost every seedling was lost at emergence due to predation by mice despite the use of Mouseoff baits to protect the experiment. The standard bioassay protocol was followed (see sections 3.1.2 and 3.1.3), but with fungicide seed-treatments applied as a split plot. Shoot dry matters and RDC's were determined as described previously. Root pathogen DNA was extracted from the root material and assayed by the SARDI Plant and Soil Health, Diagnostic Service (Adelaide).

3.5.3 An initial approach to assessing the statistical differences in the pathogen DNA concentrations of damaged roots

Preliminary analysis of the pathogen DNA concentrations associated with damaged roots (e.g. Fig. 8, etc.) has been conducted by calculating standard errors (SE) and the discussion of these results assumes that least significant differences ($P = 0.05$) are likely to be approximately 2xSE. The reason for this approach is that these assays exhibit variable replication as an inevitable consequence of the incidence and severity of root disease (see explanation below). We are seeking advice on how to handle this issue for statistical analyses. A similar statistical dilemma arises for the analysis of relative shoot yield.

Variable replication for shoot yield and the pathogen DNA concentration of root dry matter arises because the surviving bioassay plants (2-3 trifoliate leaf stage) in each replicate of the bioassay are sorted into RDC's. The number of plants in each RDC, depends on the incidence and severity of root disease. In some cases, there may be no plants in a particular RDC. Consequently, a missing replicate of shoot yield and DNA concentration of roots occurs. Replication can vary between treatments and between RDC within a treatment. This problem is most common for RDC = 0 (i.e. no healthy plants in a replicate bioassay row) and RDC = 4 (i.e. no surviving plants have roots that are so severely damaged that the plants are classed as dying). For RDC = 4, there is a further complication for interpreting pathogen DNA concentrations. These plants have the most severely damaged roots and are usually only seen in rare circumstances. In most instances, the plants have already died. Thus, the DNA assays are conducted on small amounts of root tissue, with low or non-existent replication and when reported per gram of tissue, the DNA results for RDC = 4 have a high chance of large errors. When there is DNA data for RDC = 4 it is reported for completeness, but none of the discussion in this report uses data for this RDC.

4 Results

4.1 Subterranean clover (cv. Woogenellup) bioassays to examine the response of clover to root damage and soil P fertility in the field during autumn-winter

4.1.1 Mouse damage

The resown autumn-winter bioassay at “Sawyers Gully” in 2012 was immediately subject to predation by field mice. The initial sign of a problem was excavation of seeds in some bioassay rows. Mouse baits (Ratsak wax blocks) were, consequently, deployed (within days of the experiment having been sown). Despite evidence that the baits had been killing mice, it was clear that the baits had not adequately protected the experiment from mouse damage. A more substantial mouse bating treatment (Mouseoff wheat grains) was immediately applied to stop further mouse damage and the experient was resown.

The data suggested that the mice were preferentially consuming seedlings in treatments where soil P fertility was improved (Fig. 11).

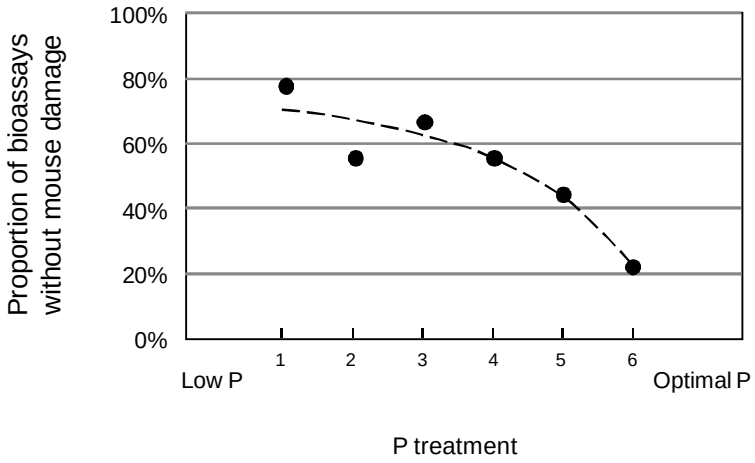


Figure 11. Proportion of bioassays in each of the soil P treatments that were not substantially affected by mouse damage at the end of Phase 1 (22 August 2012).

The mice would typically work their way along the bioassay rows and either eat the young seedlings (cotyledons are removed), or would occasionally excavate the germinating seeds. This sort of damage to bioassays had only occurred on one previous occasion over numerous bioassays conducted in the years leading up to the present experiments. However, damage due to mice proved to be very common during the present experiments. The bioassay established at “Redmire” in 2013 was entirely destroyed by mice despite the application and re-application of Mouseoff. Limited damage was also observed (with Mouseoff protection) at “Gilligooly” in 2014.

Definitive conclusions about seedling survival rates or the effectiveness of the seed treatment with metylaxyl on seedling survival by the end of Phase 1 in the experiments at “Sawyer’s Gully” (2012) and “Gilligooly (2014) could not be made because of the mouse damage and the fact that the mice had been attracted preferentially to high P-treatments.

Use of Mouseoff usually stabilised predation by mice and there was much less evidence of damage by mice after phase 1 of the experiments. It was, therefore, possible to assess the relationships between shoot yield or pathogen DNA concentrations and root damage at the end of phase 2 at “Sawyer’s Gully” and “Redmire” because these assessments are always conducted using the plants that are alive (and are not eaten) at the end of Phase 1. (The experiment at “Redmire” was, obviously, an exception).

4.1.2 Soil P fertility and experimental design

The extractable P concentrations (Colwell 1963) of the P-treatments was assessed at the end of Phase 1 (approx. midpoint of the experiment) at “Sawyer’s Gully” in 2012 by taking 15 soil cores (10 cm depth x 2.5 cm diam) adjacent to each paired bioassay. The Colwell P data indicated that soil scalping and absence of the annual maintenance fertiliser application had promoted variability in the soil P fertility of replicate plots within each soil fertility treatment (Fig. 12a). This confirmed the validity of the experimental design that had been planned for overlaying the present experiment on a previous soil fertility management experiment (previous design was randomised complete block, n=3). At “Gilligooly” in 2014, there was less evidence of disruption of the previous P treatment design, but there was still large overlaps in the soil P fertility of plots among the previous P treatments (Fig. 12b).

Each bioassay was subsequently treated at both sites as a unique data point for regression analyses when determining the interaction of soil P fertility and root damage.

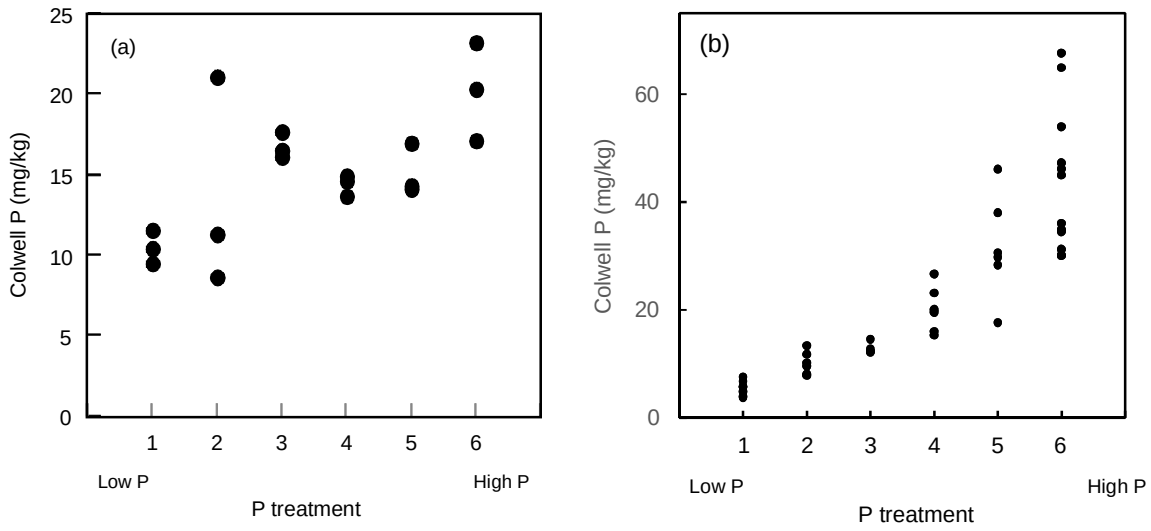


Fig. 12 (a) Extractable P concentrations (Colwell) of topsoil in the original replicated P treatments mid way through the bioassays at “Sawyer’s Gully” 2012. The original P treatments had been created to span very P deficient (treatment 1, unfertilised) through to supra-optimal soil P fertility (treatment 6, fertilised). Root damage bioassays were overlayed on the original design (i) without further application of P fertiliser during autumn, and (ii) after the top 0.5-1 cm of the soil had been “scalped” to remove most of the subterranean clover seed bank. These management requirements for the bioassays were expected to modify the original extractable P concentrations of the soil. (b) Extractable P concentrations (Colwell) of topsoil at the end of

phase 2 ("Gilligooly" 2014) in the 36 unreplicated plots. The plots are grouped by the original P treatments to which they had been allocated. The original P treatments had been created to span very P deficient (treatment 1, unfertilised) through to supra-optimal soil P fertility (treatment 6, fertilised).

4.1.3 Seasonal temperature regimes

The bioassay at "Sawyer's Gully" was sown initially in May 2012 but had to be resown. This pushed the bioassay period into the late winter with a mean daily average temperature for the bioassay period of 5.9 ^{o}C (Fig. 13). At "Gilligooly" 2014, the bioassay was conducted under autumn-winter conditions with a mean daily average temperature for the bioassay period of 7.6 ^{o}C.

4.1.4 Root damage

The impact of metalaxyl seed treatment on root damage was initially examined at Sawyer's Gully by grouping the paired assays according to their soil P fertility levels.

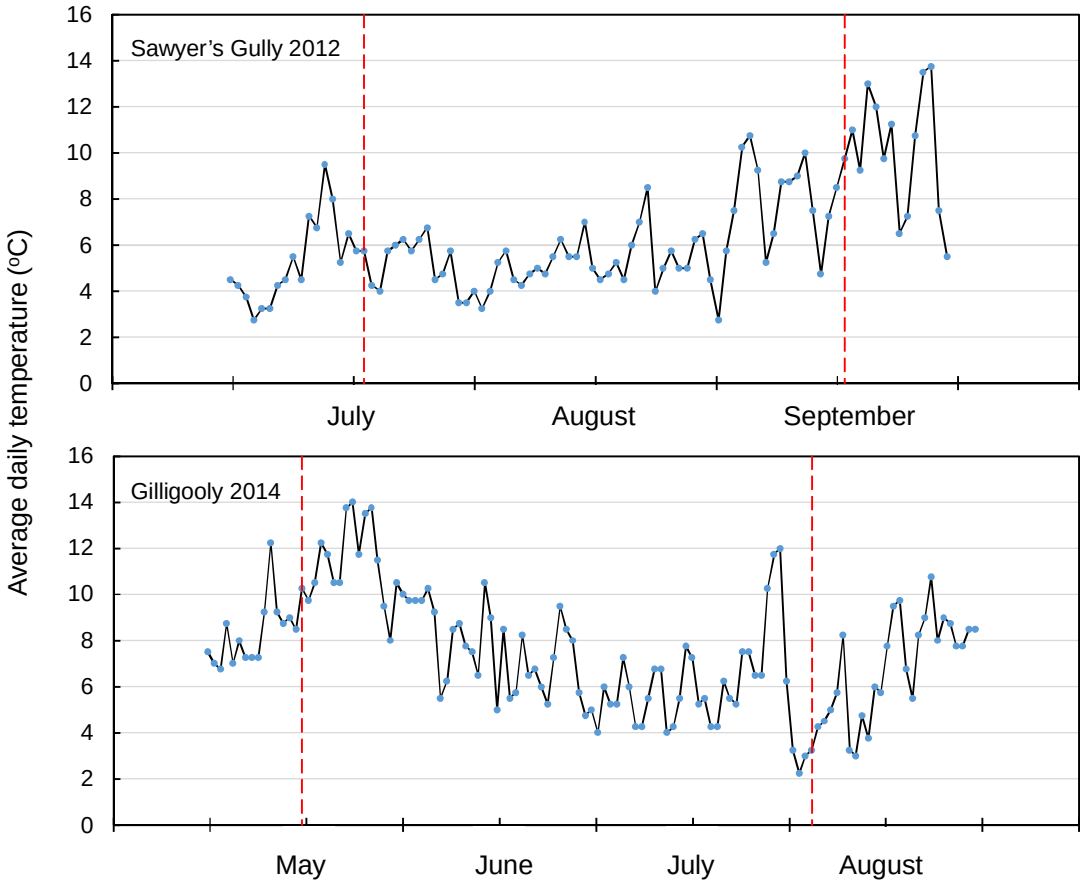


Fig. 13. Average daily temperatures at (a) "Sawyer's Gully" during the period of the 2012 bioassays and at (b) "Gilligooly" in 2014. Bioassay durations are indicated by the vertical dashed lines.

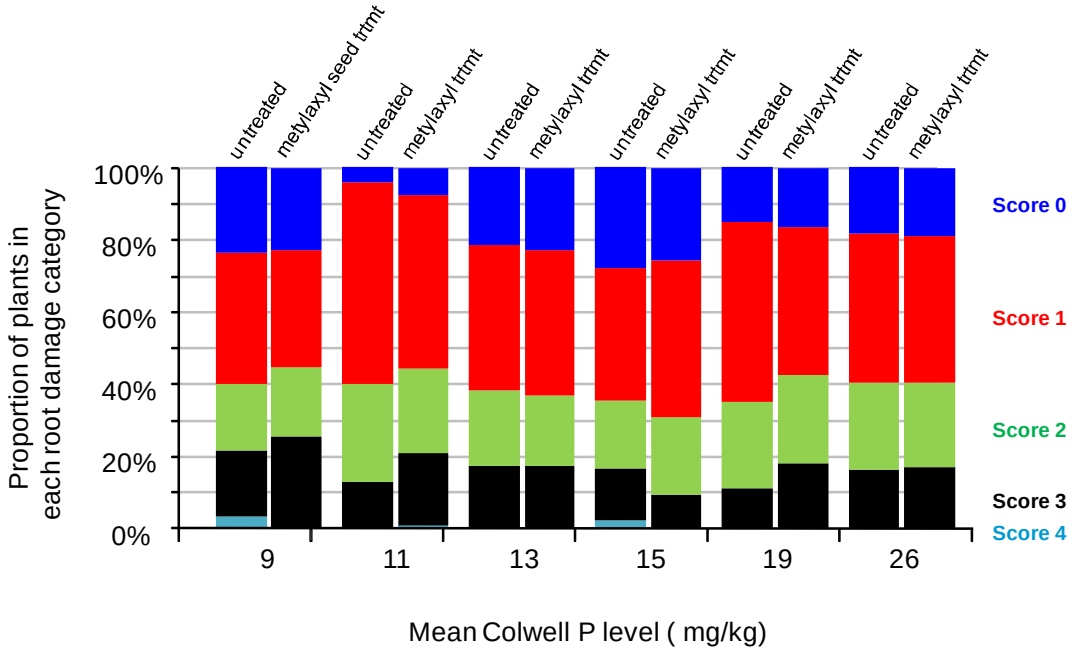


Fig. 14. Proportions of plants in each root damage category at various levels of soil P fertility (Sawyer's Gully (2012).

Regression analysis of shoot yields within each RDC plotted against the extractable P concentration of the soil in which the bioassays were grown (Fig. 15) showed there were no significant differences in the slope of the relationships measured for untreated and metylaxyl-treated bioassays. Consequently, further analyses used the combined data from the treated and metylaxyl-treated bioassays.

The soil fertility categories used were: Colwell P (mg/kg soil) 8-10, >10-12, >12-14, >14-16, >16-21, >21-31. However, there was no consistent evidence that metylaxyl treatment of the seeds had influenced the proportions of plants in each RDC (Fig. 14) or the relationship between root damage severity and shoot yields (Fig. 16). This outcome was repeated at "Gilligooly" in 2014: i.e. there was also no consistent evidence from either experiment that metylaxyl treatment had altered the proportions of plants in each RDC or relationship between root damage severity and shoot yields.

4.1.5 Response of subterranean clover to soil P fertility

The shoot yields of plants in each RDC were graphed against the soil P fertility of their bioassay location to assess whether the subterranean clover plants were responding to soil P fertility. At "Sawyer's Gully" in 2012 (Fig. 15a), there were no positive responses to soil fertility by plants in any of the RDC's. Plants in RDC = 3, in fact exhibited a very small but significant decline in shoot weight with increasing soil P fertility. The yield of these plants was severely compromised and it is speculated that high soil P levels were potentially toxic for the struggling seedlings. At "Gilligooly" in 2014 (Fig. 15b), only plants with health roots (RDC = 0) were able to respond to

improved soil P fertility. The yield increase was about 30% off a small base, with very high variability. The plants in all other RDC's did not show any response to improved soil P fertility.

Root damage itself, significantly constrained the yield of the subterranean clover plants in both experiments (Figs 15 and 16). Plants with more damage to their roots yielded less than those with healthier root systems. Indeed, the yield constraint at RDC = 1 (secondary lateral root damage) was about 30% and at RDC = 2 (primary lateral root damage) was ~60%. This is more severe than observed previously (e.g. Fig. 2). Yields were not further restricted by RDC = 3 (tap rot damage) as is usually observed. At "Gilligooly" the lack of further yield restriction at RDC = 3 was clearly associated with growth of fresh new laterals above the zone of tap root decay. Root recovery after damage is seen occasionally, but was noted on a number of samples during root damage assessments for this site.

4.1.6 Root pathogen DNA levels in soil, and associated with the damaged roots of subterranean clover in autumn-winter bioassays

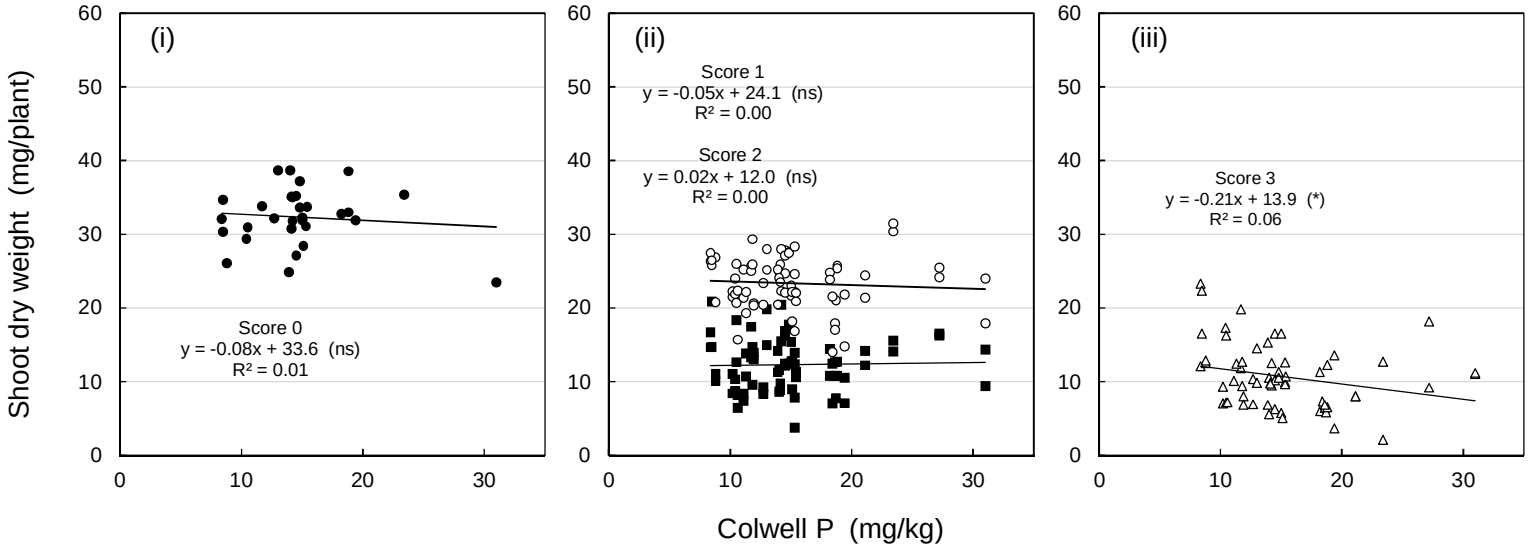
The winter bioassays at "Sawyer's Gully" were conducted with and without metylaxyl treatment of seed and over a range of soil P treatments. Metylaxyl is a specific oomycete fungicide and was expected to suppress *Pythium* spp. and *Phytophthora* spp. It is also one of the more reliable seed treatments for root rot (Barbetti et al. 1987a). However, metylaxyl treatment of the seed prior to sowing did not substantially alter the severity of damage to roots (Fig. 14), or the magnitude of the yield constraint associated with root damage severity (Fig. 16).

The DNA assays were initially used to assess which DNA assays were detecting pathogens in the soil at the Sawyers Gully site (Table 8). This information was used to narrow the number of DNA assays deployed in the subsequent experiment.

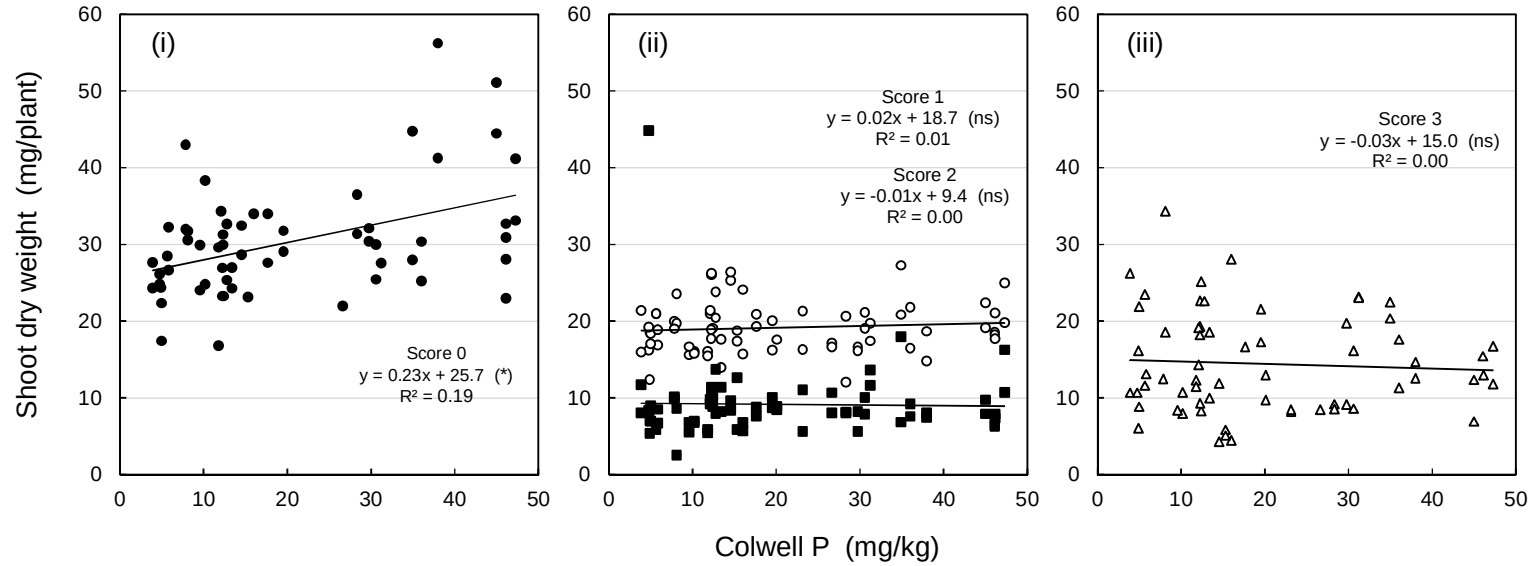
DNA analysis of subterranean clover roots from the bioassay were not influenced by soil P fertility levels (data not shown) and results were, therefore, combined from all P treatments. Pathogen DNA concentrations of damaged roots appeared to increase with increasing severity of root damage for *Pythium* clade F across most root damage categories and for Phoma (*Didymella pinodes/Phoma medicaginis* var *pinodella*) in plants with tap root damage (Fig. 17). In contrast to a previous observation (Fig. 3; Simpson and Richardson 2009), *P. clandestina* concentrations associated with root dry matter did not appear to increase with increasing severity of root damage.

Fig. 15. (following page) Shoot yields (3-4 leaf stage; the end of phase 2) of subterranean clover (cv Woogenellup) in each root damage category achieved at various levels of soil P-fertility at (a) "Sawyers Gully" (2102) and (b) "Gilligooly" (2014). Regressions for untreated and metylaxyl-treated bioassays were not significantly different (P = 0.05, LINEST, Excel) for any of the comparisons. Data from both treatments were, therefore, combined. The results of testing whether the slope of each regression was significantly different to a slope of zero are shown in brackets after each regression equation (ns = not significant; * indicates significance at P = 0.05).

(a) “Sawyer’s Gully” 2012



(b) “Gilligooly” 2014



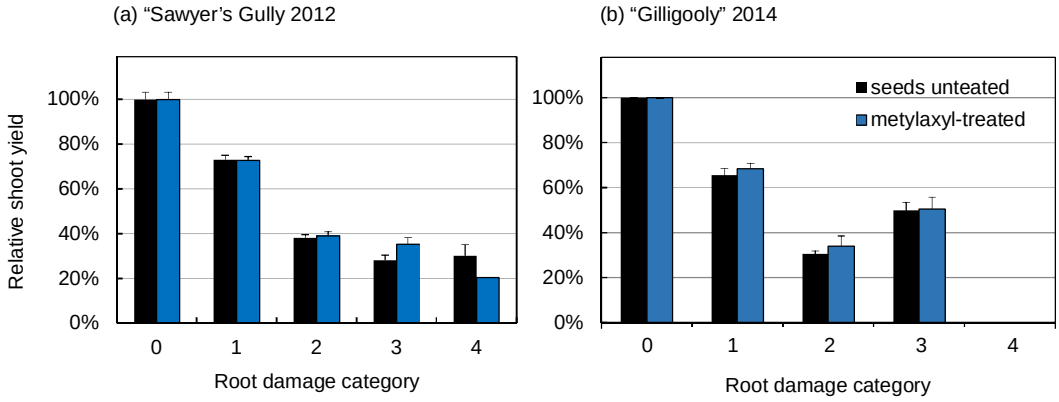


Fig. 16. Dry matter yields of shoots from plants in each root damage category at the 3-4 trifoliate leaf stage (end of phase 2 of the bioassay) at (a) "Sawyer's Gully" (2012) and "Gilligooly" (2014). Data were combined from untreated and metylaxyl-treated bioassays and across P levels and represent the main effects of root damage. Bars = 1xSE.

Table 8. Initial survey for detectable levels of fungal root pathogen DNA associated with roots of subterranean clover (cv Woogenellup) at "Sawyers Gully".

Root pathogen DNA assay	Result
Take-all (wheat + oat strains)	-
Rhizoctonia solani AG2.1	-
***R. solani* AG2.2**	**+**
R. solani AG4	-
R. solani AG8	-
Fusarium pseudograminearum test 1	-
F. pseudograminearum test 2	-
F. culmorum	-
Bipolaris	-
Pythium clade I	-
***Pythium clade* F**	**+**
M. javanica/incognita/arenaria	-
Didymella pinodes/Phoma medicaginis* var *pinodella	**+**
Phoma koolunga	-
Arbuscular mycorrhizal fungi (AMF)a	**+**
AMFa2	**+**
AMFb	-
AMFc2	-
AMFd	-
AMFe	-
Phytophthora clandestina	**+**
Aphanomyces trifolii	-

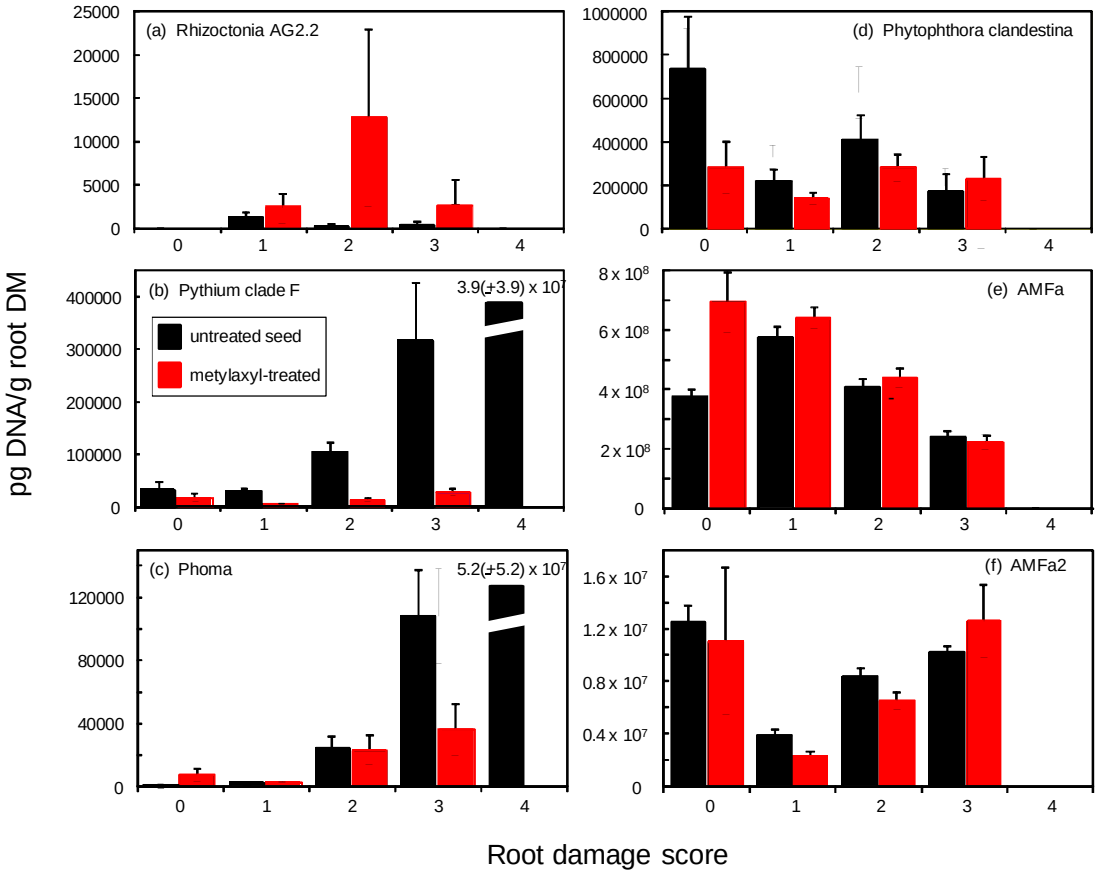


Figure 17. Concentrations of root pathogen DNA that were associated with damaged roots of plants in each root damage category: 0 - undamaged, 1- secondary lateral roots damaged, 2 - primary lateral roots damaged, 3 - tap root damage, 4 - dying plants. Note that category 4 results are often not available or are unreliable because few dying plants are found (most in this category are already dead) and when found are in low numbers (poorly-replicated or unreplicated) and of relatively small mass and, therefore, less reliably analysed for DNA content.

Metylaxyl treatment of seed prior to sowing reduced the *Pythium* clade F DNA detected per gram of clover root dry matter, but did not appear to affect DNA concentrations of any other pathogens including *P. clandestina*, or the AMF (Fig. 17).

4.1.7 Discussion

4.1.7.1 Constraints to yield of subterranean clover during autumn-winter

The topsoil at "Sawyers Gully" has a Phosphorus Buffering Index (PBI; Burkitt et al. 2002) value of 45, whilst that at "Gilligooly" was PBI = 281. On this basis, it would be recommended that optimum soil P fertility for pasture production was about 29-44 mg P/kg (critical Colwell P for 95% max yield), respectively. However, a previous soil fertility experiment found the critical soil P level for subterranean clover in a mixed pasture may be as low as 17 mg P/kg (critical Colwell P for 90% max yield) at "Sawyer's Gully" (Simpson et al., AWI Project EC664-2 Final Report). The soil P levels measured in the current experiments spanned a range that was expected to represent very P

deficient to optimal for subterranean clover growth despite the “scalping” of the soil and need to conduct the experiments without recent P fertiliser additions.

The critical values for soil P management actually reflect the requirements of rapidly growing clover-dominant pasture during spring when moisture and temperature conditions for pasture growth are at their seasonal optima. Monthly average pasture growth rates at that time of the year typically approach or exceed ~100 kg DM/ha/day. In autumn-winter, low temperatures are likely to be the limiting influence on pasture growth rates which may range from about 40, down to about 5 kg DM/ha/day over this period. The plant demand for P supply from soil at this time is consequently expected to be less than in spring and critical P levels for maximum growth will be lower than those used as targets for soil P management (Gourley et al. 2007). On this basis, it is perhaps not surprising that the subterranean clover plants were not limited in the present experiment by soil P fertility even at the lowest soil P fertility levels (Colwell P = about 10 mg/kg) at “Sawyer’s Gully” and was only limited significantly (P = 0.05) for plants with healthy roots at “Gilligooly”. It is possibly significant in this respect, that the prevailing temperatures during autumn-winter at “Gilligooly” were a few degrees warmer on average than at “Sawyer’s Gully”

When P-fertiliser is applied in the autumn period (the most common application time for temperate pastures) a transient spike in plant-available P during the autumn-winter period will push soil extractable P levels well above than the critical requirements of pasture growth (e.g. Simpson et al. 2010). Given the low P requirements observed in these experiments for growth during the cool autumn-winter period and the likelihood that recent P applications will mean P is highly available at this time of the year, it is unlikely that P will often be a limiting factor in pastures during autumn-winter.

In contrast, root damage constrained plant yield substantially, irrespective of soil P fertility. The impact of shoot yield was substantial with up to 30-60% yield reductions associated with the most commonly observed RDC’s (RDC = 1 or 2). Where a small response to soil P was possible (e.g. “Gilligooly”), damaged roots prevented the plants from responding. “Pruning” of the root system is the symptom used in these experiments to rate root damage severity. It is well established that plants moderate their shoot growth rates in response to root pruning (Poorter and Nagel 2000). In fact, the response to root pruning is used in ornamental horticulture (bonsai plants) and production horticulture (orchard management) to manage shoot growth rates. The constraint to yield associated with damaged roots is, consequently, also presumed to be a result of root pruning.

We conclude that in most instances P uptake by subterranean clover during winter will not be limited by damaged roots because a damaged (pruned) root system is more limiting for pasture growth than P supply and this is especially the case when temperatures low and and growth rates are relatively slow.

The present experiments were conducted under cold autumn-winter conditions on the NSW Tablelands. In some districts, autumn-winter growth occurs under relatively more favourable temperatures (e.g. e.g. coastal areas, W.A., etc). Where a response to P was observed in the current experiments, it was assumed to be associated with milder winter temperatures but only occurred when plants had undamaged roots. Even the

"mildest" category of root damage (RDC = 1) prevent subterranean clover from responding to higher soil extractable-P concentrations. We hypothesis that this is because shoot growth was constrained primarily by the "bonsai"-like effect of root pruning by root rot pathogens.

Consequently:
(i) additional P fertiliser will not overcome the constraint to pasture production caused by root disease;
(ii) control of root pathogens or plant resistance to root damage is likely to improve autumn-winter yields irrespective of soil P fertility conditions.

4.1.7.2 Root pathogen DNA concentrations associated with root dry matter

The root pathogen DNA concentration associated with root dry matter in the bioassays at "Sawyer's Gully" provided some new insights into how pathogen DNA assays respond to treatments and seasons. These indications are discussed in more detail later (section 4.5). However, two observations should be noted at this stage:

(i) *Pythium* clade F DNA concentrations were reduced when metylaxyl was applied providing a further indication that the fungicide treatments treated the root system effectively and, in particular, had been effective against this target oomycete pathogen despite there being no obvious impact of the fungicide on root damage. However, the uncoupling of *Pythium* clade F DNA concentrations and root damage severity was, on face value, a blow to the notion that the DNA assays might be used to diagnose root damage and constraints to yield in autumn-winter.

(ii) In contrast, *P. clandestina* DNA concentrations were (a) unresponsive to metylaxyl and (b) not elevated on roots from more damaged root systems. The latter observation being at odds with previous (limited) experience (see Fig. 3). However, the concentrations of *P. clandestina* DNA associated with roots in the 2012 bioassay were 10-fold lower than were recorded in 2007 (Fig. 3) and it was concluded that this may be indicating that *P. clandestina* was not actually present in 2012 at concentrations that were biologically meaningful. Critical pathogen DNA concentrations for all of the pathogen DNA assays are unknown and these two observations were the first indication that DNA assays may potentially be able to distinguish biologically-relevant root pathogen DNA concentrations.

4.2 Root pruning experiment to simulate damage to roots by root rot pathogens

4.2.1 Shoot growth

The low P soil was very limiting for subterranean clover growth with plants in the 'unrestricted root growth' treatment achieving only about 50% of the shoot yield of equivalent plants growing in the high P soil (Fig. 18). In retrospect, the severe growth limitation imposed by the very low soil P fertility of this treatment was not ideal for the experiment. Nevertheless, the following observations could be made. Plant yield was adversely affected as a result of restricting the soil volume that roots were able to access.

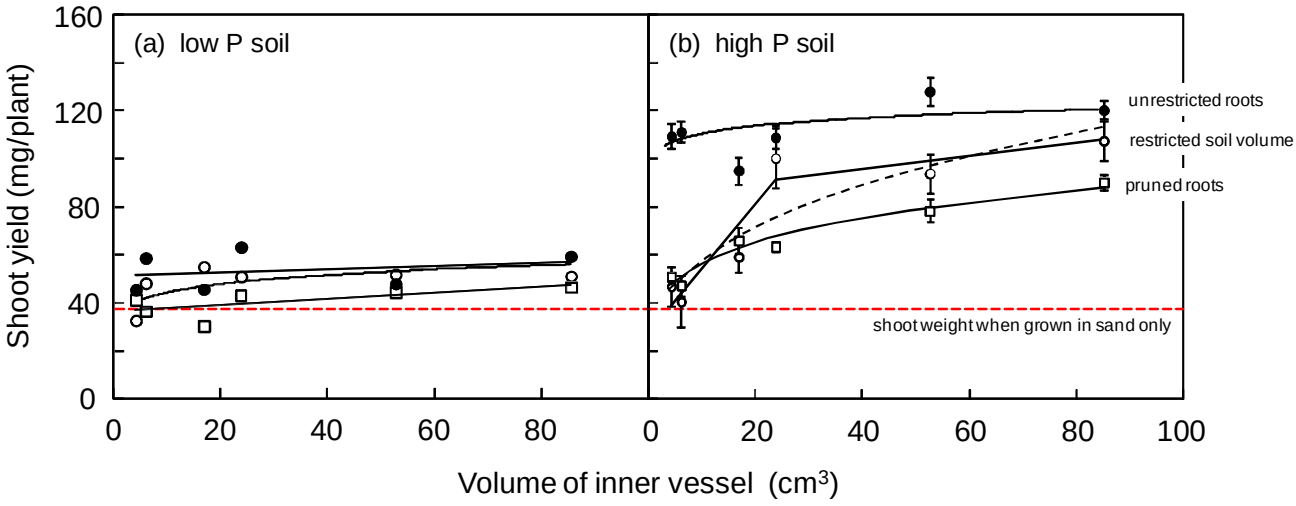


Fig. 18. Shoot yield after 33 days growth in (a) the low P soil and (b) the high P soil. The shoot weight of plants growth in sand without any added P is shown by the dashed red line. The yield of clover with unrestricted roots (solid circles), restricted soil volume (open circles) and pruned roots (open squares) is shown plotted against the volume of soil that was held by the six different sizes of perforated, inner pot. It was unclear whether data for the restricted soil volume treatment in high P soil should be fitted with a "broken stick" of asymptotic function so two options are shown using solid and dashed lines. Bars indicate 2xSE, and are not shown for the low P soil to improve the clarity of the Figure. The co-efficient of variation (COV = 100*(SE/mean)) for yield of plants grown in sand was 12%; average COV for plants grown in all low P soil treatments was 10%.

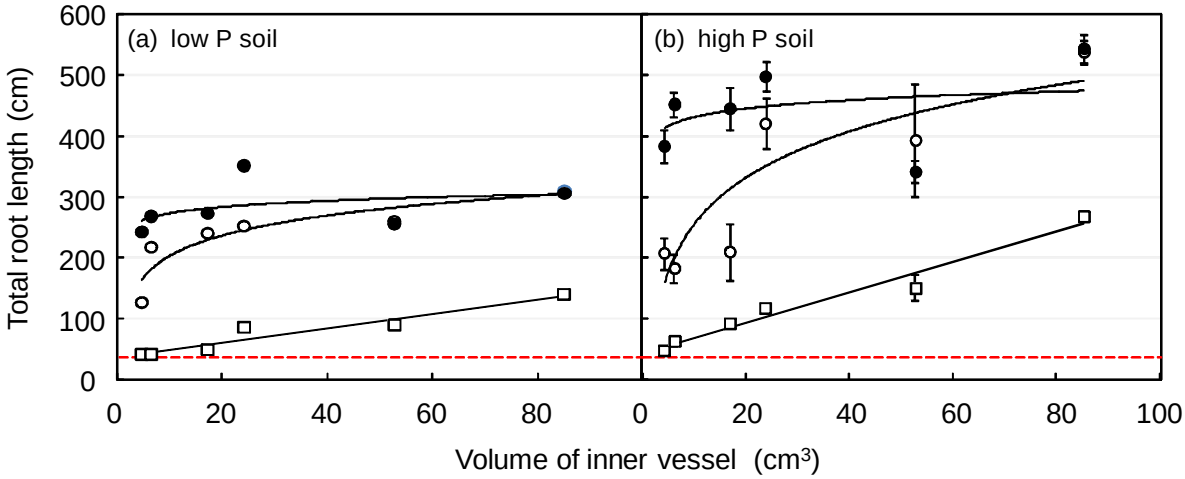


Fig. 19. Total length of roots after 33 days growth in (a) the low P soil and (b) the high P soil. The root length of plants growth in sand without any added P is shown by the dashed red line. Total root length of clover with unrestricted root growth (solid circles), restricted soil volume (open circles) and pruned roots (open squares) is shown plotted against the volume of soil that was held by the six different sizes of perforated, inner pot. Bars indicate 2xSE, and are not shown for the low P soil treatments to improve the clarity of the Figure. The co-efficient of variation (COV = 100*(SE/mean)) for total length of roots on plants grown in sand was 11%; average COV for plants grown in all low P soil treatments was 10%.

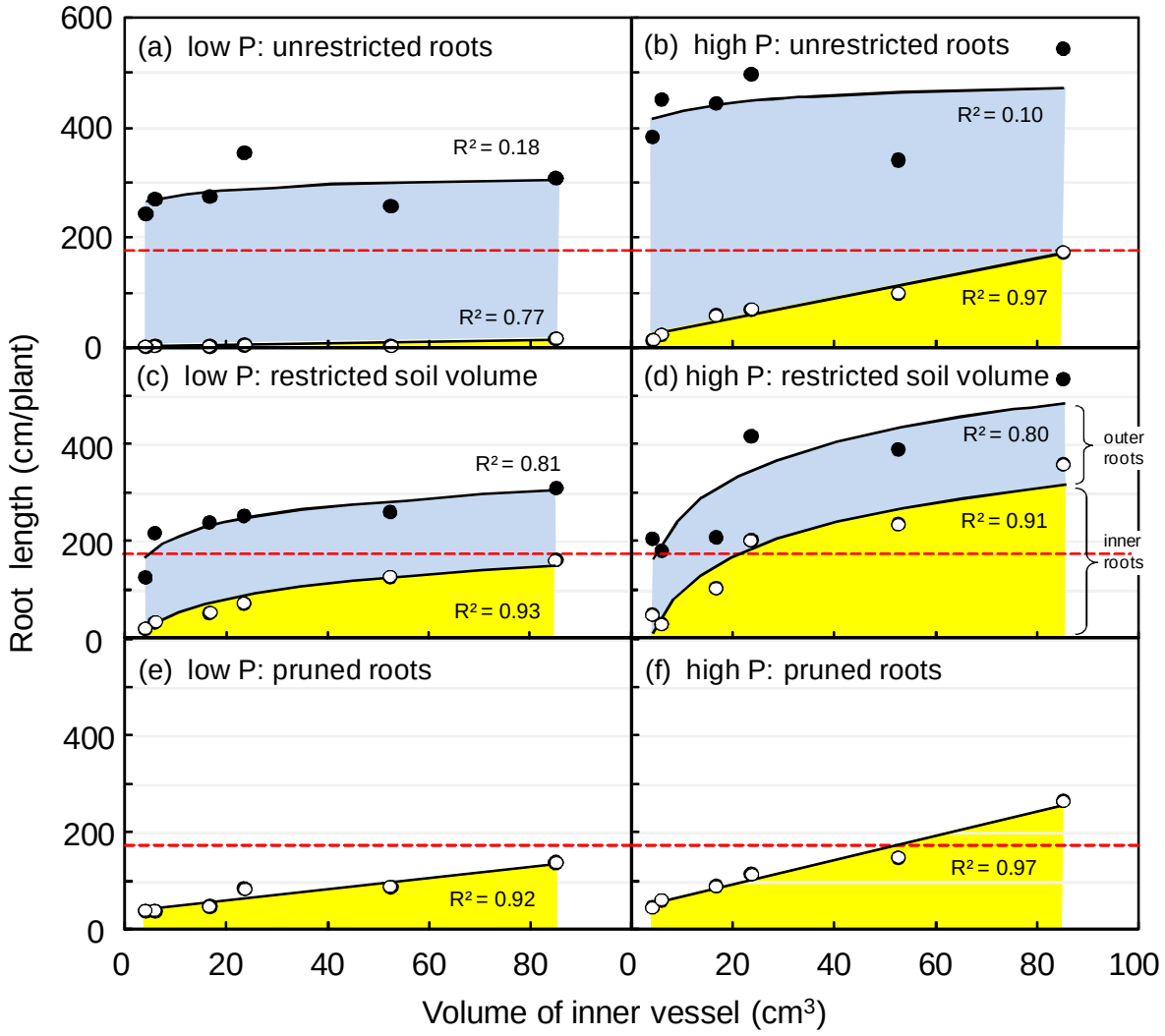


Fig. 20 Allocation of root length to soil contained within the perforated, inner pot (open circles and yellow shading) and to soil outside of the inner pot (blue shading) for plants with: unrestricted root growth in (a) low P soil, (b) high P soil; a restricted soil volume in (c) low P soil, (d) high P soil: and a pruned root system in (e) low P soil and (f) high P soil. Total root length data points are shown by the closed circles. The total root length of plants growth in sand without any added P is shown by the dashed red line.

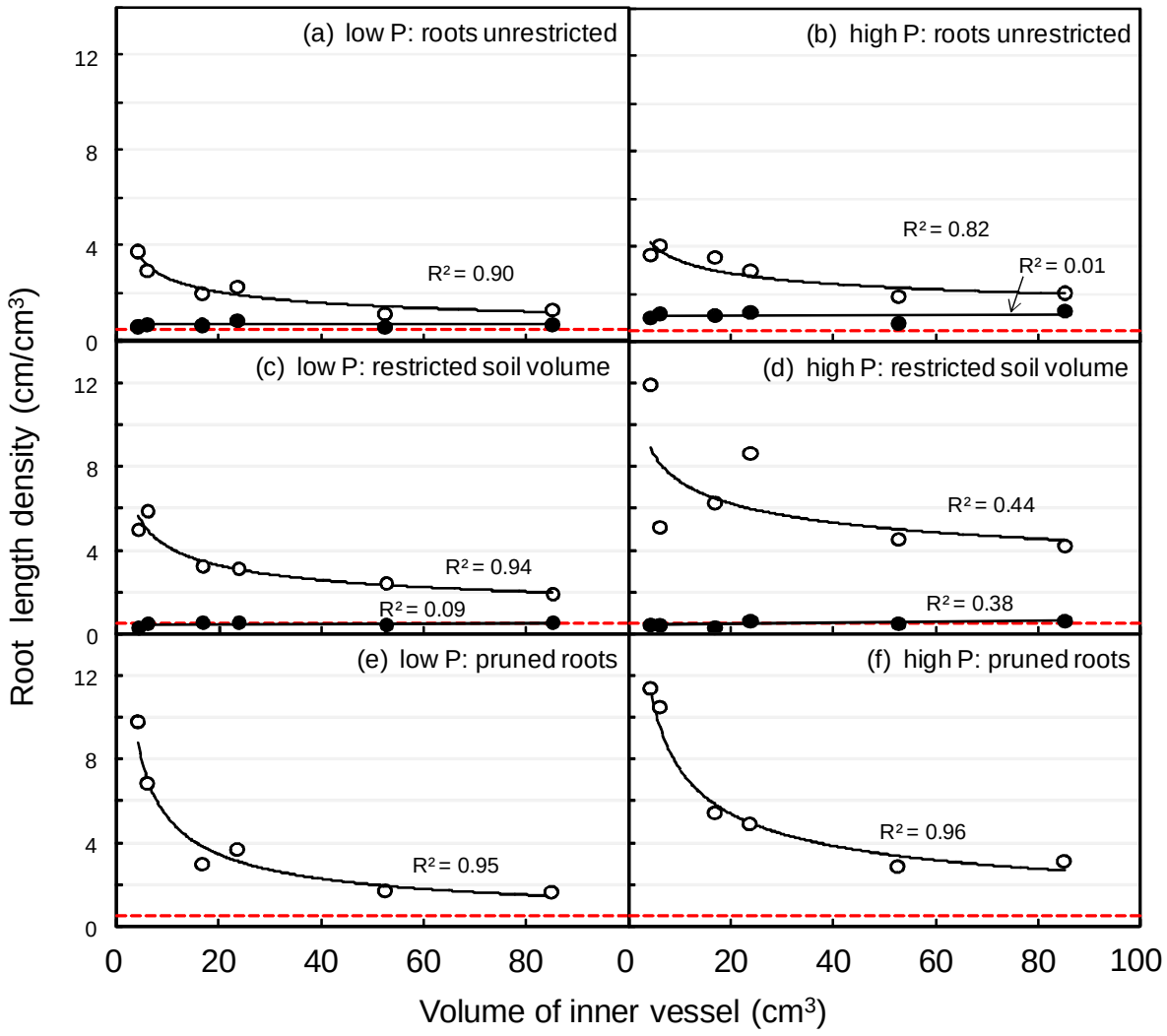


Fig. 21. Root length density (cm/cm^3 of soil) achieved by day 33 after sowing in the soil within the perforated, inner pot (open circles), and in soil outside of the inner pot (closed circles) for plants with: unrestricted root growth in (a) low P soil, (b) high P soil; a restricted soil volume in (c) low P soil, (d) high P soil: and a pruned root system in (e) low P soil and (f) high P soil. Total root length data points are shown by the closed circles. The density of roots grown by plants in sand without any added P is shown by the dashed red line.

When the access to soil was reduced to a very small volume, shoot yield equalled or approached that of plants grown in sand without P. As the 'restricted' soil volume was increased shoot yield equalled or approached that achieved by plants growing with unrestricted roots. Shoot yield was constrained substantially by all root pruning treatments.

4.2.2 Root length

Total root lengths generally reflected shoot yields across all treatments (Fig. 19) with the exception of the restricted soil volume treatments. In the restricted soil volume treatments, root length was increased and approached, then equalled the total length in the unrestricted root treatments as the soil volume in the 'restricted' treatments was increased. However, the increase in shoot yield in response to increasing soil volume lagged behind the increase in root length.

Unless the root system was being pruned, the plants grew roots in both the soil in the inner pot, and in the soil (unrestricted roots) or sand (restricted soil volume) in the outer pot. (Fig. 20). When root growth was unrestricted and soil was present in both inner and outer pots, more root length was grown in the outer pot irrespective of the soil P fertility. When soil was present only in the inner pot (restricted soil volume treatments), more root length was allocated to the soil in the inner pot than to the P-free sand outside the inner pot. However, the density of roots (root length/cm^3 soil, Fig. 21) was always highest within the inner pot and this was especially evident for restricted soil volume and pruned root treatments. Highest densities of roots in soil were observed when the inner pot volume was small; the density of roots declined as the inner pot size was increased.

4.2.3 Shoot-root relationships

The relationships between shoot and root growth in response to the root and fertility treatments were examined (Fig. 22). The yield and total root length of plants grown with unrestricted roots in high P soil was relatively stable. However, pruning roots and restricting the volume of nutrient-rich soil in a pot reduced total root length and shoot yield proportionately (Fig 22a). The slopes of the relationships between shoot yield and total root length of pruned and restricted soil volume treatments were the same indicating that similar absolute changes in root length in either treatment were associated with equivalent changes in shoot mass. The relationships were, however, displaced because the total length of roots (inner + outer roots) was greater in the restricted soil volume treatments when the volume of soil in the inner vessel exceeded ~20 cm^3 (compare Figs 20d and 20f; Fig. 23). When shoot yield was plotted against the length of roots that was in the nutrient-rich soil of the restricted root volume treatments (i.e. 'inner' root length), the relationship between shoot yield and root length was identical for pruned and restricted soil volume treatments except that longer 'inner' roots were grown in the restricted soil volume treatments when the inner vessel of soil exceeded ~20 cm^3. This enabled larger shoots to be grown in these particular restricted soil volume treatments than grown in the corresponding pruned root treatments (i.e. those with the same-sized inner pot) (Fig. 22b).

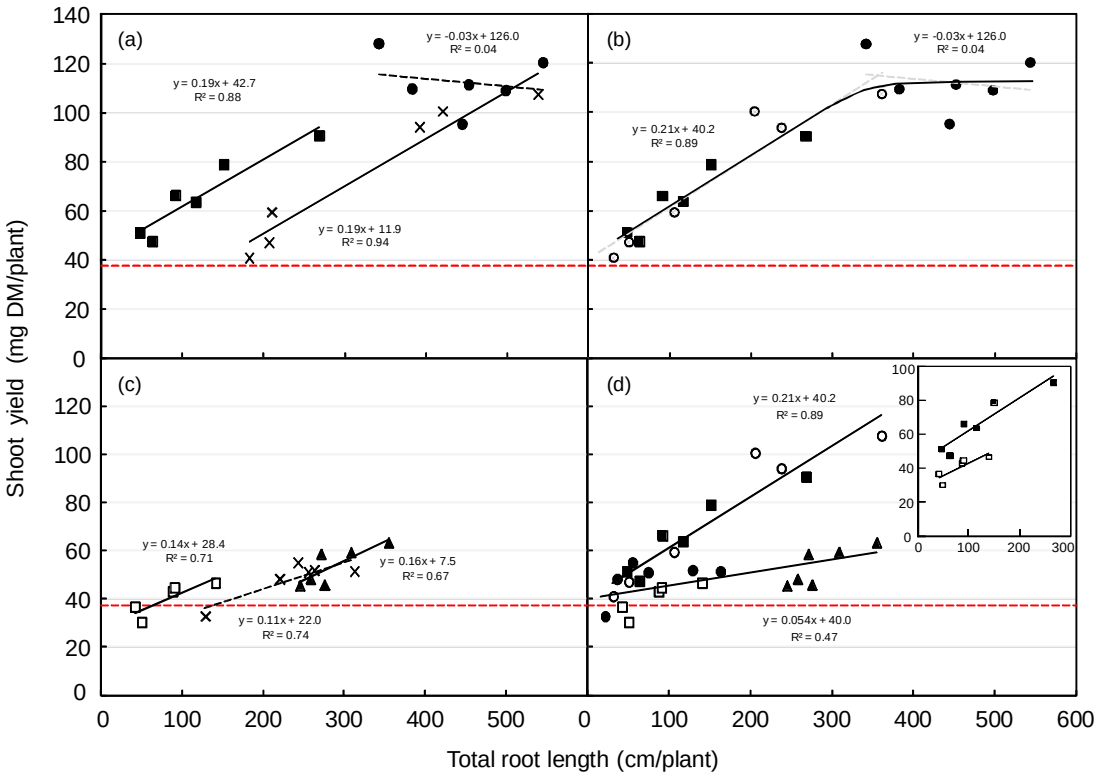


Fig. 22. Relationships between shoot yield and root length observed in various treatments. The shoot yield achieved by plants growing in sand with no added P is indicated by the dashed red line.

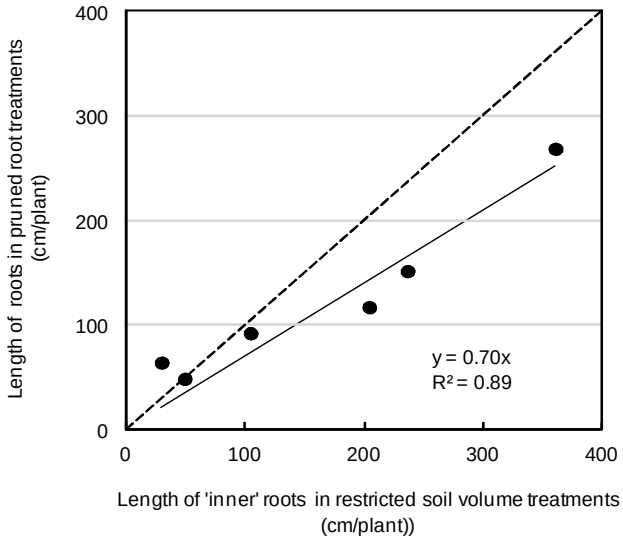
(a) Shoot yield plotted against total root length for plants grown in high P soil with unrestricted roots (closed circles), pruned roots (closed squares), or with a restricted soil volume (crosses).

(b) Shoot yield plotted against total root length for plants grown in high P soil with unrestricted roots (closed circles), pruned roots (closed squares), and shoot yield plotted against 'inner' root length of plants grown with a restricted soil volume (open circles) (i.e. root length in the inner pot only). The solid line shows the expected yield of the plants when the capacity of the plants to explore the high P soil is increased and is deduced from all of the data that is shown in the panel. On this basis, the 'critical" total root length for maximum plant growth in the high P soil was estimated to be ~360 cm/plant. The fitted linear regressions for (i) shoot yield plotted against total length of pruned roots and 'inner' root length of plants grown with a restricted soil volume, or (ii) shoot yield plotted against total root length for plants grown with unrestricted roots, are shown by the dashed lines.

(c) Shoot yield plotted against total root length for plants grown in low P soil with unrestricted roots (closed triangles), pruned roots (open squares), or with a restricted soil volume (crosses).

(d) Linear regressions describing P-limited relationships between shoot yield and root length in high P soil (total length of pruned roots [closed squares], and 'inner' roots, restricted soil volume [open circles]), and in low P soil (total length of pruned roots [open squares], 'inner' roots, restricted soil volume [closed circles], and total root length of unrestricted roots [closed triangles]). Inset shows only the relationships between shoot yield and total root length of pruned root treatments in high P soil (closed squares) and low P soil (open squares).

Fig. 23. Length of pruned roots plotted against the length of 'inner' roots of plants grown with restricted soil volumes grown in the same sized inner pot. Each point represents one of the 6 sizes of inner pot. The dashed line shows the 1:1 relationship expected if root length growth in both treatments were equivalent. The regression shown here is forced through the origin.



In low P soil, shoot yields were small irrespective of root treatments. The relationship between shoot yield and total root length for plants grown with unrestricted roots did not differ from that for plants grown with restricted soil volumes presumably because the soil P-fertility was so restrictive for plant growth. Yield of plants with pruned roots was in most cases not significantly greater than the yield of plants grown in sand without added P (Fig. 22c). Under these circumstances it was concluded that a common relationship would apply for shoots and total root length of plants with pruned roots or restricted roots, or shoots and the inner root length of plants grown with restricted soil volumes (Fig. 22d). Comparison of this shoot-root relationship with that for plants grown with pruned roots or restricted soil volumes showed that a response to P addition can be expected when plants with a limited length of roots are growing in a P-deficient soil. For clarity, the response to P addition by only the plants with pruned roots is also shown in the inset to Figure 22d.

When all data in Fig. 22b were considered together, it was evident that pruning roots and restricting root access to nutrient-rich soil had similar, adverse impacts on shoot yield by limiting access to soil (hence to P). However, continuous pruning of the clover plant's root system appeared to also restrict the plant's ability to develop root length and fully exploit the soil within the inner pot. The data indicate that up to 30% less root length was grown in the inner pot when roots were being pruned. Shoot growth by the plants with pruned roots consequently fell behind that of the corresponding restricted soil volume treatments except when the volume of the inner pot was relatively small.

4.2.4 Discussion

4.2.4.1 Constraints to shoot yield are associated with pruning of roots or soil volume-induced restriction to root length.

The shoot yield of subterranean clover plants was constrained when the roots were pruned mechanically. The yield of plants with pruned roots was directly related to the length of root that remained after pruning and consequently their ability to explore soil for nutrients.

The yield of plants with pruned roots growing in low P soil was increased by addition of P-fertiliser.

Plants grown in restricted volumes of soil but with root growth unconstrained, also demonstrated that shoot yield was correlated with the root length grown in nutrient-rich soil. However, these plants also grew more root length in the soil to which they had access, than plants with pruned roots with access to the same volume of soil (except when soil volume was very small). Consequently root pruning also constrained shoot yield more than expected given the soil volume to which the plants had access.

The effect of pruning roots on shoot yield was analogous to the "bonsai" effect in which the shoot growth of plants is constrained by growth in a small pot volume or by pruning irrespective of adequate water and nutrient supply (e.g. Richards and Rowe 1977). It is speculated elsewhere that roots impose "feed forward" control on shoot growth in such circumstances. This is also observed when root growth is constrained by a range of difficult soil conditions: hardness, dry soil, root disease and other adverse soil microbial activity (Passioura 2002). The mechanism by which fed forward control is imposed on shoot growth is unknown.

4.2.4.2 Implications for managing root disease (root damage) on subterranean clover

Possible implications of the results from this experiment for managing of the root damage that is caused by root rot pathogens on subterranean clover are:

(i) The adverse impacts of damaged roots may be partially counteracted by fertiliser application if the plants are growing in nutrient-poor soil. However, correcting nutrient deficiency in itself would be beneficial for pasture production and should be promoted for that reason alone, rather than as a partial cure for disease issues. This finding is, on face value, at odds with the results of field experiments in autumn-winter on the NSW Tablelands. However, in those experiments cool winter temperatures (i.e. slow pasture growth rates) will have significantly reduced the need for a high P supply to support subterranean clover for growth.

(ii) When environmental conditions for plant growth are favourable (e.g. in spring), damaged to roots that limits root exploration of soil, is likely to increase a plant's critical P requirement because shoot yield was linearly related to the length of roots developed in nutrient-rich soil.

(iii) The potential yield of clover plants that have damaged roots will not be achieved by correcting soil P deficiency alone, or by compensatory P applications when root length is suboptimal. This is because root damage also induces a constraint to

shoot yield (a bonsai-like effect) that (in the literature on this topic) is speculated to be a consequence of some form of “feed forward” regulation of plant growth.

(iv) When roots are damaged as a result of root rot organisms, the constraints on shoot yield may not just be a consequence of root pruning alone as disease organisms and other microbes in the rhizosphere may themselves exert feed forward control of plant growth rate (Kirkegaard et al.1995, 1999; Watt et al. 2003).

4.3 Responses of subterranean clover and root pathogen DNA to fungicide treatments injected into the root zone of established subterranean clover swards during spring

4.3.1 Growth of subterranean clover in response to topdressed P-fertiliser and fungicide treatments applied using subsurface irrigation during spring

The clover sward established well above the subsurface irrigation pipes that had been installed early in the growing season. A high and even density of seedlings established over the entire area, but differences in ground cover became progressively obvious with time. Poorer growth was associated with the low P (unfertilised) soil but only in the spaces between the irrigation/treatment lines (Figs 24b and 24c). Improved growth was observed along all irrigation/treatment lines and was assumed to be a consequence of cultivation when the irrigation pipe was laid (Figs 24a, 24b). The improved growth above the irrigation/treatment lines persisted throughout the experimental period (Figs 24a, 24b and 26).

Negative (untreated and water) and positive (nutrient) control treatments were included in the experiment to demonstrate the effectiveness of the treatment injection method: i.e. area of treatment influence on pasture growth; impact of introducing biocides at 30 cm intervals. However, improved growth above all irrigation/treatment lines did not permit any distinctions between treatments on the basis of these control treatments.

Phytotoxicity in the form of marginal leaf necrosis developed in the Apron1 treatment in which the highest concentration of fungicide (1 ml/litre) had been injected into the root zone (Fig. 25). This was present along the entire length of the irrigation/treatment line and affected plants in a band about 12 cm wide, i.e. the zone of improved growth above the irrigation/treatment lines), but did not significantly affect shoot yield (Fig. 26). This indicated that the upper concentration limit for using Apron (chosen on the basis of the laboratory bioassay) was appropriate for the experiment and that fungicide injection into the root zone had been an effective way of treating the plants with the biocide.

There were no significant differences in herbage yield associated with any of the treatments applied in this experiment (Fig. 26). Visual comparisons with plant growth in the area between the irrigation/treatment lines where growth was sometimes (especially in low P soil) less than in the irrigation/treatment lines (Fig. 24) indicated that cultivating the soil had removed all constraints to pasture growth. The average pasture growth rate for the experimental period (5-16 October) was 139 kg DM/ha/day. Monthly-average growth rates for October are typically expected to be ~100 kg

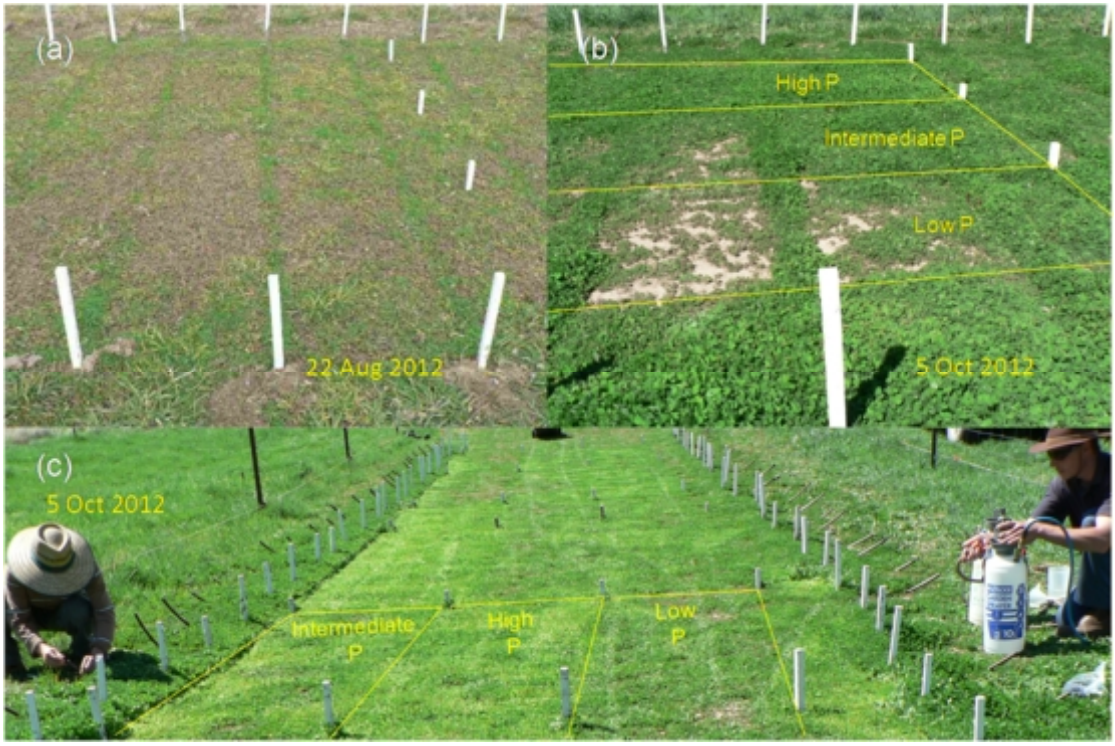


Fig. 24. The subterranean clover pasture at Sawyer's Gully (a) on 22 August 2012, shortly after establishment, showing the clear development of zones of improved growth where the soil had been cultivated to install the irrigation lines. The lines of improved growth were still very evident (b) by 5 October 2012, the date at which the fungicide treatments were to be injected into the root zone. Poorer clover growth was clearly evident in the uncultivated areas between the irrigation lines and, to some extent at least, reflected the P treatments (see panels b and c).

Fig. 25. Phytotoxicity symptoms (marginal leaf necrosis) were observed in the Apron 1 treatments. Symptoms persisted along the length of the irrigation line indicating that the treatment solution had spread away from outlets in the irrigation line which were 30 cm apart.

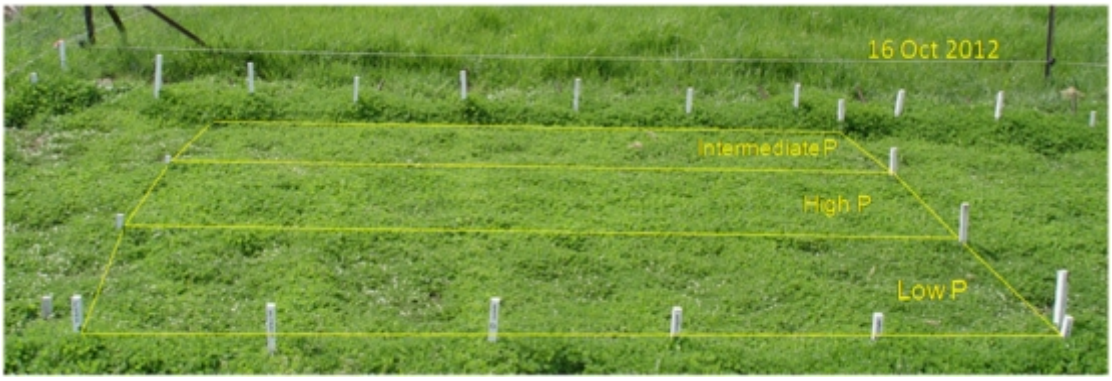


Fig. 25. Block 1 of the trial just prior to the pasture being harvested. Improved growth associated with cultivation in the treatment lines and a likely response to P treatments in the space between the lines can be seen.

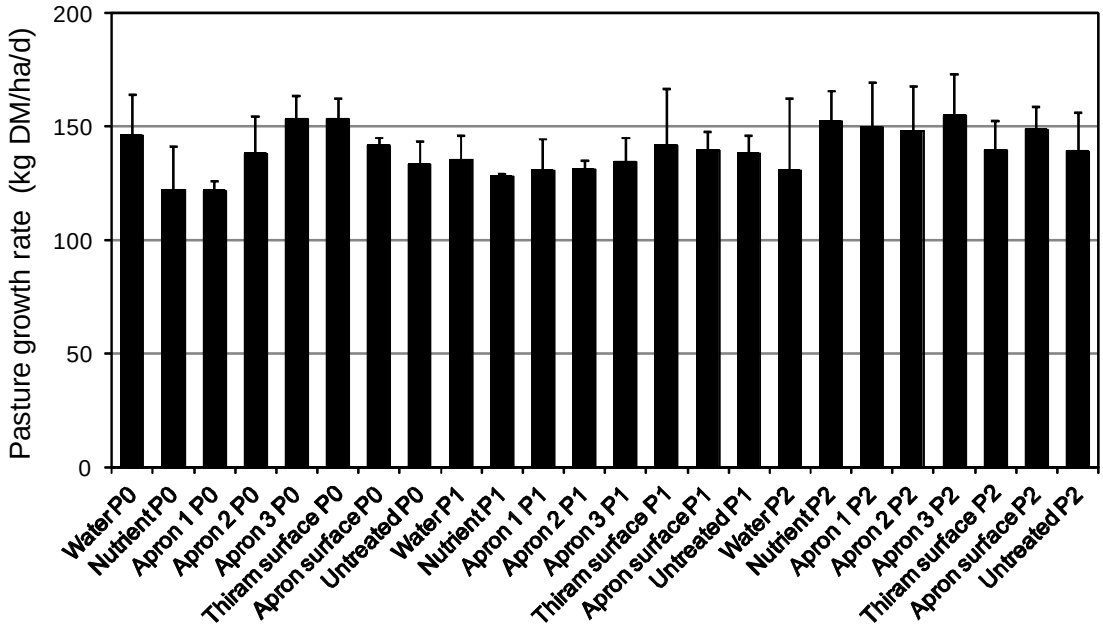


Fig. 26. Pasture growth rates achieved by subterranean clover growing in the treatments lines in the period from 5 – 16 October 2012. Bars = 1xSE.

DM/ha/day, indicating that the experiment had been conducted under ideal environmental conditions and had essentially achieved the peak spring pasture growth rate.

Although cultivation of soil is considered to disrupt fungal hyphae and to alleviate the impact of root pathogens (Barbetti and Macnish 1984), the effect is not permanent and it had been hoped that the fungal pathogens would re-invade the narrow cultivated trench used to install the irrigation/treatment lines. Either, this did not occur or, if it did, the root pathogens were not effective in causing root damage. Soil cultivation has many positive benefits for root and plant growth as it can stimulate mineralisation and release of nitrogen, P and other nutrients, and loose soil removes other constraints to root growth that are associated with soil hardness (Passioura 2002), so it is also possible that cultivation could have provided multiple benefits for pasture growth, alleviating any possibility of significant root damage by fungal pathogens.

4.3.2 Discussion - fungicide treatment via subsurface irrigation

The method used to inject fungicides into the root zone of established subterranean clover swards appeared to have worked as planned. Phytotoxic symptoms induced by the highest fungicide rate indicated that the plants had most likely been "treated" effectively by injecting fungicide into the root zone using subsurface irrigation pipes.

However, cultivation of the soil in the narrow trench that was necessary to install the sub-irrigation lines eliminated all potential impacts of root pathogens and soil P treatments on clover yield. As such the experiment was not successful and required a complete reassessment of how to inject fungicides into the root zone without disturbing the soil.

4.3.3 Fungicide injection into the root zone of undisturbed clover swards

4.3.3.1 Subterranean clover yield responses to P and root zone treatments

Soil P fertility was increased after application of P as shown in Fig. 27. Pasture growth rates were significantly affected only by application of P (Fig. 28). Main effects for P were significant at both sites with P2 > P1 > P0 at "Sawyers Gully" and P1 > P0 = P2 at "Redmire". No soil drench treatment had a significant effect on pasture growth.

Nitram was applied as a soil drench with the intention of creating a positive control treatment that would indicate that drench treatments had been effective even if a response to the fungicides was not observed. However, the yield improvement associated with Nitram was not statistically significant. Apron was also applied at a rate that had been predetermined to cause minor phytotoxicity without adverse impacts on herbage yield (see section 4.3.1). The expected symptoms were observed in Apron drench treatments (minor necrotic leaf margins; see inset to Fig. 29a) at "Sawyer's Gully", but were not seen at "Redmire". We speculate that this may be due to the "Redmire" soil having a much higher clay content and perhaps higher capacity to adsorb the fungicide. The mild phytotoxicity symptoms confirmed that the soil drench treatments (at "Sawyer's Gully" at least) had drenched the root zone and were able to be taken up by the clover, despite there being no impacts on pasture yield.

The increasing response to P observed for pasture growth at “Sawyer’s Gully” was expected given the low initial P fertility of the soil. Very high short-term growth rates were achieved in the P2 treatments reflecting favourable temperature and soil moisture conditions in September-October.

The pasture yield response to P application at “Redmire”, where the yield of P2 treatments was less than that of P1 treatments, was not expected and is not logical. It is believed (but cannot be proven) that the site has been compromised by field mice grazing clover in the high P treatments. The various observations that led to this conclusion were:

- The winter bioassay adjacent to the spring experiment had been completely destroyed by mice despite baiting the site with Mouseoff which we had used very effectively on a previous occasion at “Sawyer’s Gully to control mouse damage. Further baits were applied to protect the spring experiment, but it is possible that they were not entirely effective.

- Herbage P concentrations in the treatments were tested and found to be positively correlated with soil extractable P levels. This eliminated the possibility that shoot samples had been assigned erroneously to the wrong P treatment when samples were labelled at harvest.

- Ground cover by subterranean clover at the site was unexpectedly patchy (compare pasture cover achieved in Fig. 29b with that in Fig. 29a). To counter this, soil drench treatment location within the treated plots were selected carefully to ensure they each had complete clover cover but this did not prevent P2 treatments from having lower yields than P1 treatments. Pasture height in P2 treatments was similar to that in P1 treatments (see Fig. 29b), suggesting that differences in plant density between the P treatments may have been the cause of the unusually low yield in the P2 treatment.

- The reason why plant density would be unusually low in all P2 replicates was also perplexing, but we had found previously that mice preferentially graze higher P treatments (Fig. 11).

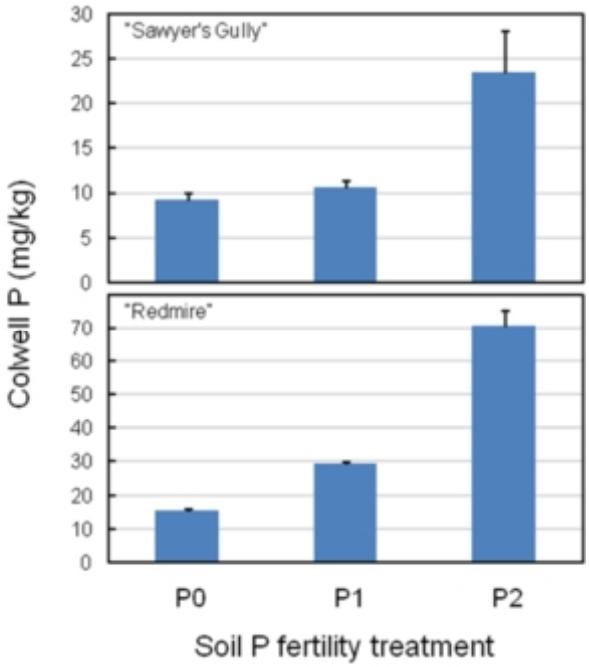


Fig. 27. Soil P fertility (Colwell P), 0-10 cm depth at the harvest date in spring 2013 at "Sawyer's Gully" and "Redmire".

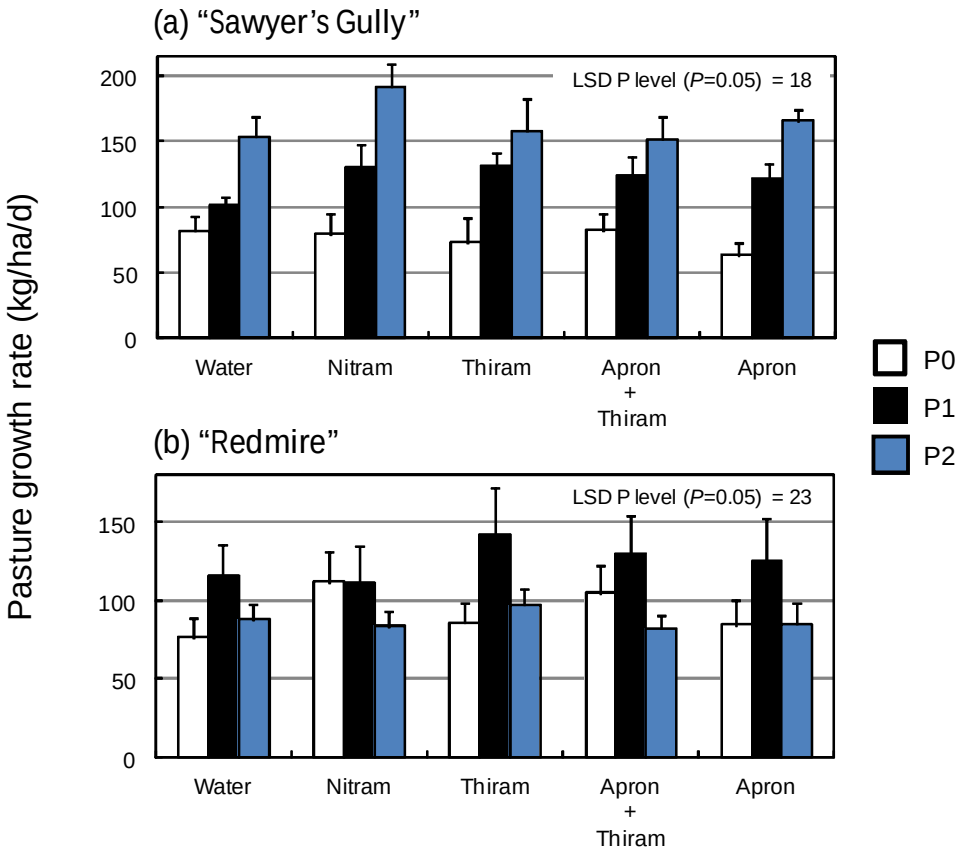


Fig. 28. Pasture growth rates (kg DM/ha) achieved after closure of the pasture for 42 days ("Sawyer's Gully") and 47 days ("Redmire") in September-October 2013. The LSD shown for each sites is for the main effect of P application as no other treatment effects with significant (P<0.05).

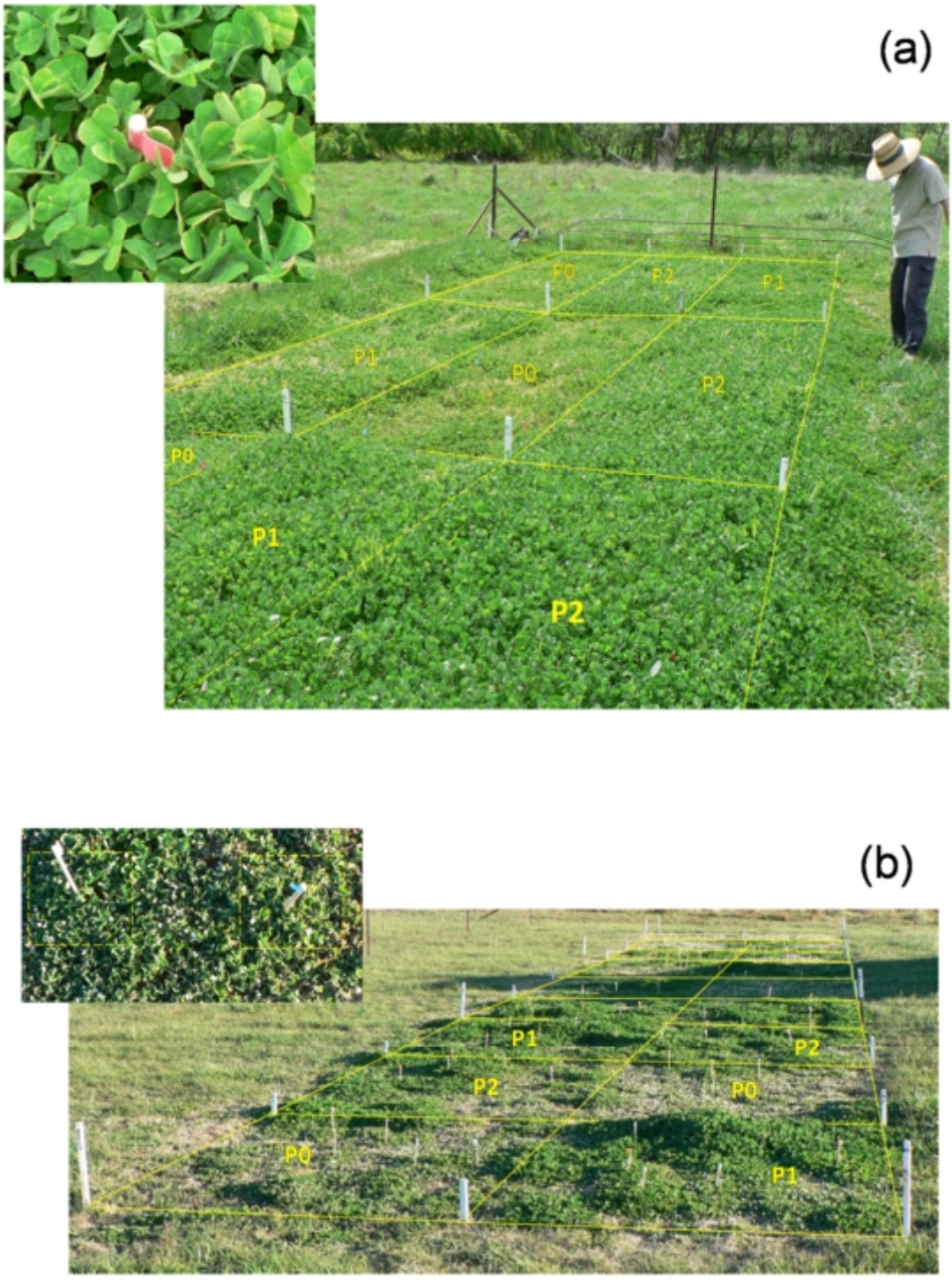


Fig. 29. Appearance of subterranean clover monocultures in spring immediately prior to harvest at: (a) “Sawyer’s Gully” (inset shows marginal necrosis [phytotoxity] in Apron treatments) and (b) “Redmire” (inset shows examples of treated areas marked with a flag that was pushed into the small hole left after fungicide injection to fill the hole and to mark the centre of the treated area).

4.3.3.2 Pathogen DNA concentrations in root zone soil samples

Soils samples for pathogen analysis were taken directly under the pasture sward that had been sampled for herbage yield and will reflect the DNA levels associated with soil and clover roots. Irrespective of the problems associated with interpreting the growth response of the pasture to the P applications at "Redmire", the effects of the soil treatments on the pathogen DNA concentrations in the soil from both sites were remarkably similar.

The DNA concentrations of all root pathogens except *Pythium* clade F and *P. clandestina*, were unaffected by P application, nitrogen application or by drenching the root zone with either of the fungicides used (Figs. 30 and 31).

The DNA concentrations for *Pythium* clade F were not influenced by P applications but were reduced by drenching with either Thiram or Apron and with Nitram as shown by significant (P>0.05) main effects after analysis of variance (Figs. 30 and 31; Table 9). Apron was the more effective fungicide against *Pythium* at "Sawyer's Gully" with Nitram and Thiram having a lower, but equivalent impact at this site. The suppressive effects of Apron, Thiram and Nitram were not significantly different at "Redmire".

The DNA concentrations of *P. clandestina* were, in contrast, elevated by P applications. They were reduced by Thiram, Apron and Nitram applications at "Sawyer's Gully", but were only reduced by Apron treatments at "Redmire" (Figs. 30 and 31, Table 3). It is assumed that the positive effect of soil P fertility on *P. clandestina* may be an indirect consequence of P promoting the growth of subterranean clover which is the specific host for *P. clandestina*. *Pythium* spp. are hosted on the roots of many plant species. Because the pastures were subterranean clover monocultures there is every reason to suspect that the *Pythium* DNA concentrations would also be positively influenced by increased clover growth, but this was not the case.

Apron is a fungicide with specific activity against oomycetes and it proved to be effective in suppressing the DNA concentration in soil of *Pythium* clade F and *P. clandestina* at both sites. It was often more effective than Thiram in reducing the oomycete DNA concentration of the soil.

Thiram is a broad spectrum fungicide used widely (but not exclusively) in the turf and vegetable industries and is claimed to be effective in controlling turf diseases such as Brown Patch (*Rhizoctonia solani*), Damping Off (*Pythium* spp.), Dollar Spot (*Sclerotonia homoecorpa*) and Fusarium Patch (*Fusarium* spp.) and *Phoma* as well as a number of other fungal diseases. However, in the present experiments Thiram was only effective against the oomycete pathogens that can be detected by the SARDI molecular diagnostic probes and was not consistently effective against *P. clandestina*. Combining Apron and Thiram in the soil drench was no more effective than drenching with Apron alone.

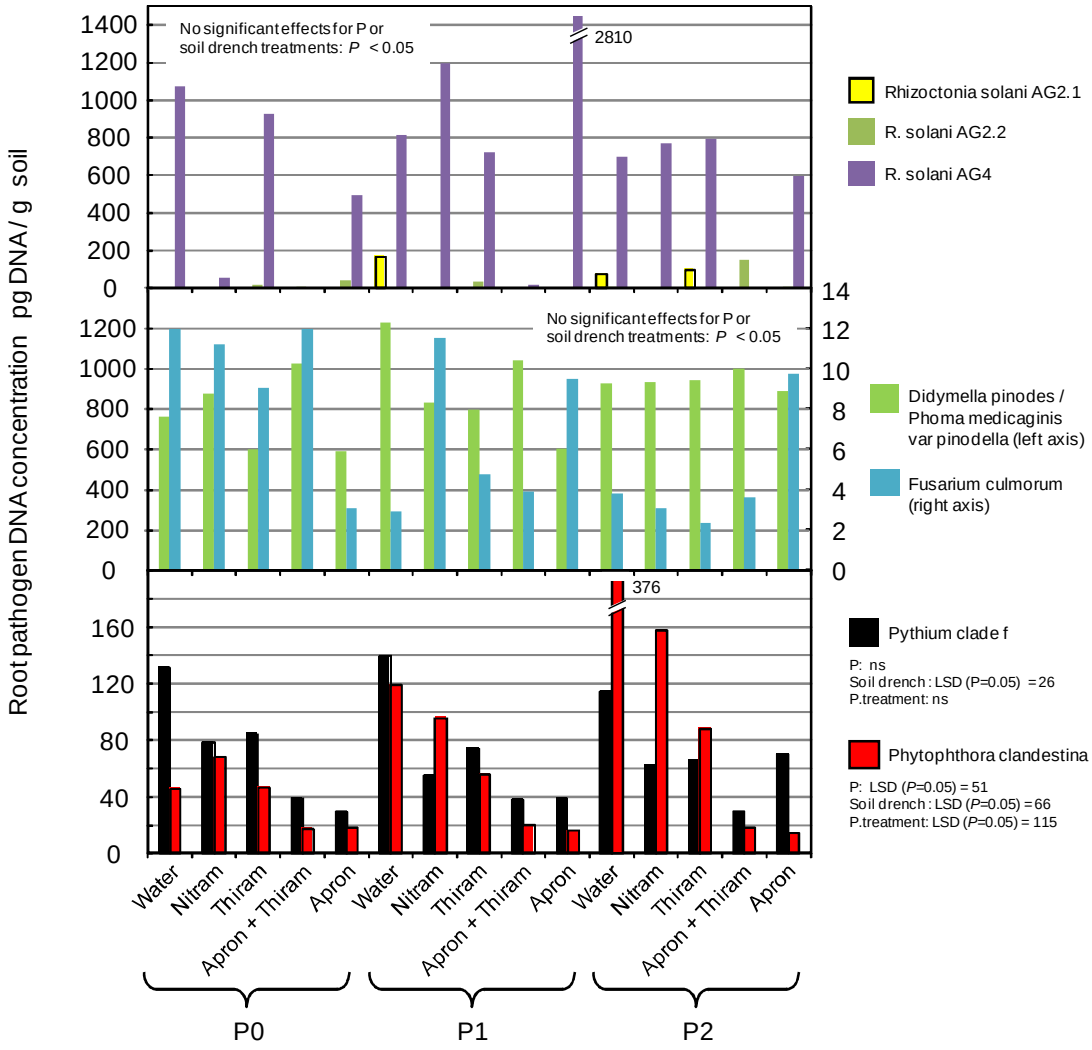


Fig. 30. Root pathogen DNA concentrations in soil sampled from the treated root zone of subterranean clover monocultures grown at “Sawyer’s Gully” in spring 2013. The relationships between pathogen DNA concentrations in soil and disease expression are unknown and comparisons between pathogen DNA concentrations are therefore invalid.

Fig. 31. (following page) Root pathogen DNA concentrations in soil sampled from the treated root zone of subterranean clover monocultures grown at “Redmire” in spring 2013. The relationships between pathogen DNA concentrations in soil and disease expression are unknown and comparisons between pathogen DNA concentrations are therefore invalid.

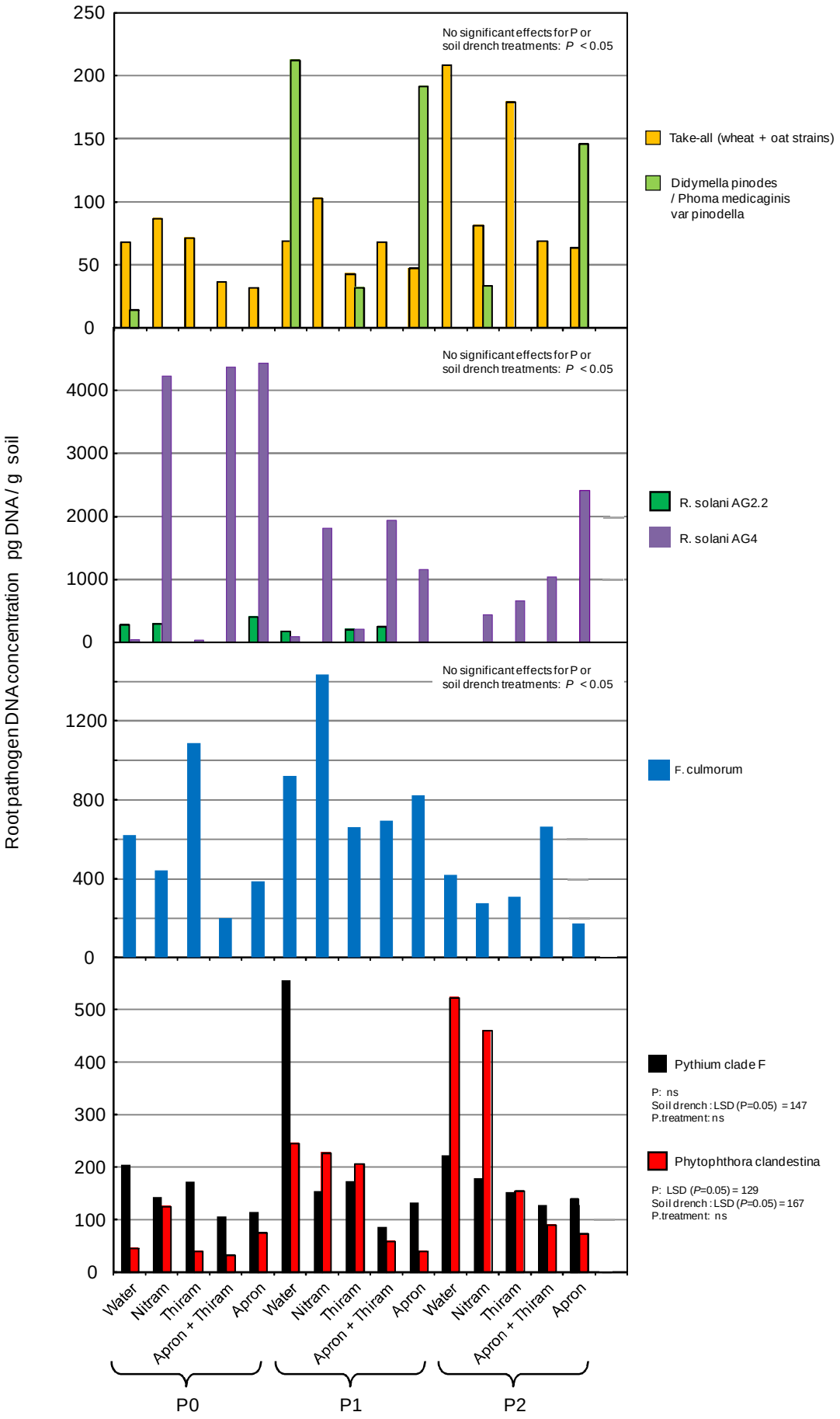

Root pathogen DNA concentration pg DNA / g soil
No significant effects for P or soil drench treatments: P < 0.05
Take-all (wheat + oat strains)
Didymella pinodes / Phoma medicaginis var pinodella
No significant effects for P or soil drench treatments: P < 0.05
R. solani AG2.2
R. solani AG4
No significant effects for P or soil drench treatments: P < 0.05
F. culmorum
Pythium clade F
P: ns
Soil drench : LSD (P=0.05) = 147
P.treatment: ns
Phytophthora clandestina
P: LSD (P=0.05) = 129
Soil drench : LSD (P=0.05) = 167
P.treatment: ns
Water
Nitram
Thiram
Apron + Thiram
Apron
P0
P1
P2

Table 9. Summary of the significant (*P*>0.05) effects of root-zone drench treatments on the DNA concentrations in soil of oomycete pathogens (spring 2013). Treatments in which pathogen DNA concentrations are least affected are denoted as > those that were significantly reduced by the treatment); ns = not significant.

Site	**"Sawyers Gully"**
Pythium clade F	
Main effects	Water > Nitram = Thiram > Apron = Apron+Thiram
Interactions	ns
Phytophthora clandestina	
Main effects	P2 > P1 = P0
	Water > Nitram = Thiram
	Nitram > Apron = Apron+Thiram
Interactions	Only significant within P2 treatments
Site:	**"Redmire"**
Pythium clade F	
Main effects	Water > Nitram = Thiram = Apron = Apron+Thiram
Interactions	ns
Phytophthora clandestina	
Main effects	P2 > P0
	Water = Nitram = Thiram > Apron = Apron+Thiram
Interactions	ns

Unexpectedly, Nitram (NH_4NO_3) which was applied in the hope of achieving a "positive" control treatment for soil drench effectiveness, was also effective in suppressing the oomycete pathogen DNA concentrations in the soil. Its effect was equivalent to Thiram in both suppression and consistency of impact against the oomycete pathogens. We are unaware of Nitram having any fungicidal activity, and speculate that it may be having an indirect effect on root infection by promoting plant growth. However, the net plant growth promotion by Nitram in this experiment was small and not statistically significant (e.g. Fig. 5, "Sawyer's Gully"). Its application would normally be expected to suppress legume nodule activity and nitrogen fixation, causing it to become the substitute N source for plant growth. A brief search of the literature indicates there are a number of reports (particularly in the turfgrass literature) where application of ammonium salts, including ammonium nitrate, is associated with reduced fungal disease severity, fungal suppression, and suppressed fungal spore germination (e.g. Leach and Davey 1942, Tsao and Oster 1981, Punja and Grogan 1982). However, some authors noted that the impacts of ammonium nitrate were not as effective as applying a fungicide (e.g. Liu et al. 1995).

4.3.4 Discussion – fungicide injection into the root zone

4.3.4.1 Experimental method

The new method of injecting fungicides into the undisturbed root zone of established subterranean clover monocultures was a successful way of avoiding the problems that confound conclusions from soil drenching experiments (drench penetration, phytotoxicity, etc). The evidence for success was: (a) the appearance of mild phytotoxic symptoms induced by Apron at a concentration proven previously to induce marginal necrosis on some leaves without reducing pasture yield; (b) suppression of root pathogen DNA by fungicide treatments (irrespective of their failure to increase pasture yield).

Apron and Thiram were effective in reducing the DNA concentrations of oomycete pathogens (*Pythium* clade F and *P. clandestina*) in the root zone, with Apron often, but not always the more effective treatment. The Apron concentration used in the experiment was chosen using a combination of bioassay and field experience and (for experiments at Sawyer's Gully) can confidently be regarded as the highest concentration feasible without adversely impacting clover yield. However, Thiram was used at a concentration based on label recommendations and it is not known whether this was an equivalent "optimal" rate of application. Combining the treatments (Apron+Thiram) was no more effective than Apron alone.

4.3.4.2 Does root damage constrain P acquisition in spring?

The experiments conducted during spring were specifically aimed at addressing whether breeding subterranean clover for improved root foraging traits would be counteracted by damage to roots from root rot pathogens. At this time of the year, pasture growth rates usually far exceed animal requirements and any potential impacts of root disease in spring do not limit animal production. However, they may have other impacts. For example, if clover content of pastures is depressed, less N will be fixed. If root damage increases the critical P requirement of subterranean clover because root "foraging" for nutrients is constrained, higher available-P concentrations in soil will be required to achieve maximum pasture growth. This in turn will mean higher fertiliser

costs and reduced P-balance efficiency because P accumulations in soil will be increased.

It was hypothesised that a response to fungicide at low and intermediate P supply could indicate that root damage was limiting P acquisition. This is turn would indicate that plants with improved root foraging would also be constrained by root damage, but it would only partially answer the question of whether breeding for improved root foraging would be ineffective because of the pressure from root pathogens.

Pasture yields were compromised at "Redmire", so the conclusions that involve yield analysis are based on results from "Sawyer's Gully". Phosphorus applications increased herbage yields but the fungicide treatments did not improve the yield of the clover monoculture despite suppressing the oomycete pathogen DNA levels in the root zone. There was no evidence of any interaction between fungicide treatment and soil P fertility on herbage yield. However, P and fungicide interactions were detected for *P. clandestina* DNA concentrations in the root zone.

Superficially, the lack of yield responses to fungicide combined with evidence that the fungicides reduced oomycete DNA in the root zone, might suggest that the oomycete root pathogens were not influencing plant P nutrition during spring. However, there are a number of interacting factors that make it impossible to be sure that this conclusion would be correct.

Observations that do not support this conclusion are:

(i) The fungicides were only partially effective against the suite of potential root pathogens detected by the DNA assays. All non-oomycete pathogens were unaffected by either of the fungicides and it is feasible that these potential pathogens could have increased their attack on the roots when the oomycetes were suppressed leading to no observable change as a result of fungicide treatment.

(ii) The relationships between pathogen DNA concentrations in soil and clover root disease incidence or severity are unknown and it is consequently unknown whether any of the root pathogens detected were actually causing root disease: i.e. the experiment may have been conducted under disease-free conditions. However, root damage and constraint to clover yield were detected using the subterranean clover bioassay in an adjacent area at this site in winter 2013 (see Milestone report #3). (Although we routinely assess root damage on young subterranean clover seedlings in autumn-winter, we do not believe this is feasible for large roots systems on older, well-established plants in spring.)

Observations that may support the conclusion are:

(i) Because relationships between pathogen DNA concentrations in soil and clover root disease are unknown and it is consequently unknown whether any of the root pathogens were actually causing root disease. Lack of response to fungicide may be indicating that this is the case and under these circumstances P nutrition in spring would not be influenced by the presence of root pathogens.

(ii) The peak growth rates achieved in the high P treatments in this experiment are very high and indicate pasture growth that is effectively equal to the highest rates of growth expected for short periods under optimum environmental conditions. This does not rule out a constraint to growth by soil pathogens, but it does limit the likelihood that they were important in this pasture system during spring 2013.

4.4 Impact of root pathogens on subterranean clover root morphology

4.4.1 Rationale

One objective of this project is to determine whether root pathogens damage roots of subterranean clover during spring growth in ways that are likely to affect nutrient (P) acquisition. Application of fungicides into the root zone were shown to reduce the DNA concentrations of target root rot pathogens in soil cores from the root zone but there were no concomitant beneficial impacts of fungicide application on herbage production at high or low soil P fertility. The results are not entirely conclusive for a range of reasons that have been discussed. In particular, because the fungicides selectively influence some of the potential root rot pathogens in soil, it is always possible that clover yield was not improved because any pathogens that were unaffected by the root treatment(s) may simply "take-over" the root damaging role of those that had been knocked out. The following experiment was conducted to determine whether root exploration of soil by subterranean clover could be shown to be altered by alternative soil treatments that are reputed to reduce pathogen loads in soil, and whether rankings of subterranean clover genotypes for "root foraging" traits (i.e. soil exploration for nutrients) would be altered by these soil treatments.

The soil treatments that were imposed was vigorous cultivation of the soil which is reported to provide temporary relief from root pathogens and reduced root damage (Barbetti and Macnish 1984) and steam pasteurisation of the soil which is often reported to boost herbage yield. Both treatments have collateral effects that are also likely to modify root growth. For example, cultivation loosens the soil and reduces physical resistance for root growth (Passioura 2002); pasteurisation is likely to temporarily release nutrients from the soil biomass that is "killed" by steaming the soil. Root pathogen DNA assays were used to examine whether there was any evidence that the treatments had altered the potential for root damage to subterranean clover roots.

4.4.2 Shoot and root responses to soil treatments

In every case, the soil treatments (soil cultivation at field sites; steam pasteurisation in the outdoor pot experiment) stimulated shoot growth significantly (Fig. 32).

Root exploration of topsoil (root length density [RLD]) was also stimulated in most cases. However, in some instances (Riverina, Mt Barker and Izmir at "Gilligooly", Mt Barker at "Wallaroo 3", Izmir in the pasteurised soil) shoot yields improvements were not accompanied by an increase in the RLD of the soil. Where RLD was increased, it was usually associated with an increase in specific root length ([SRL] i.e. length of root per unit root mass) (Fig. 33). However, in some instances improved root exploration of topsoil was also associated with increased allocation of dry matter for root growth (Fig. 34).

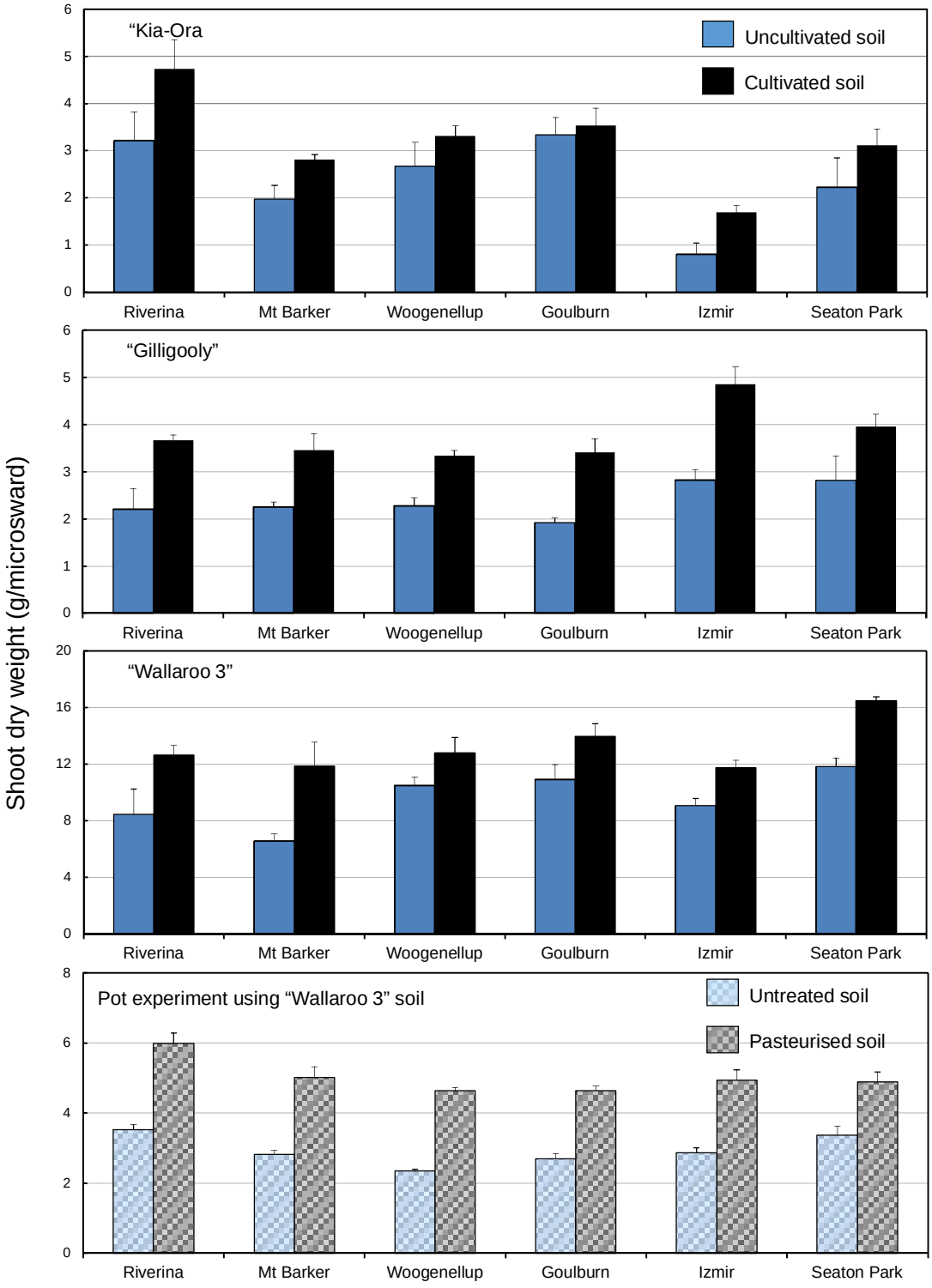
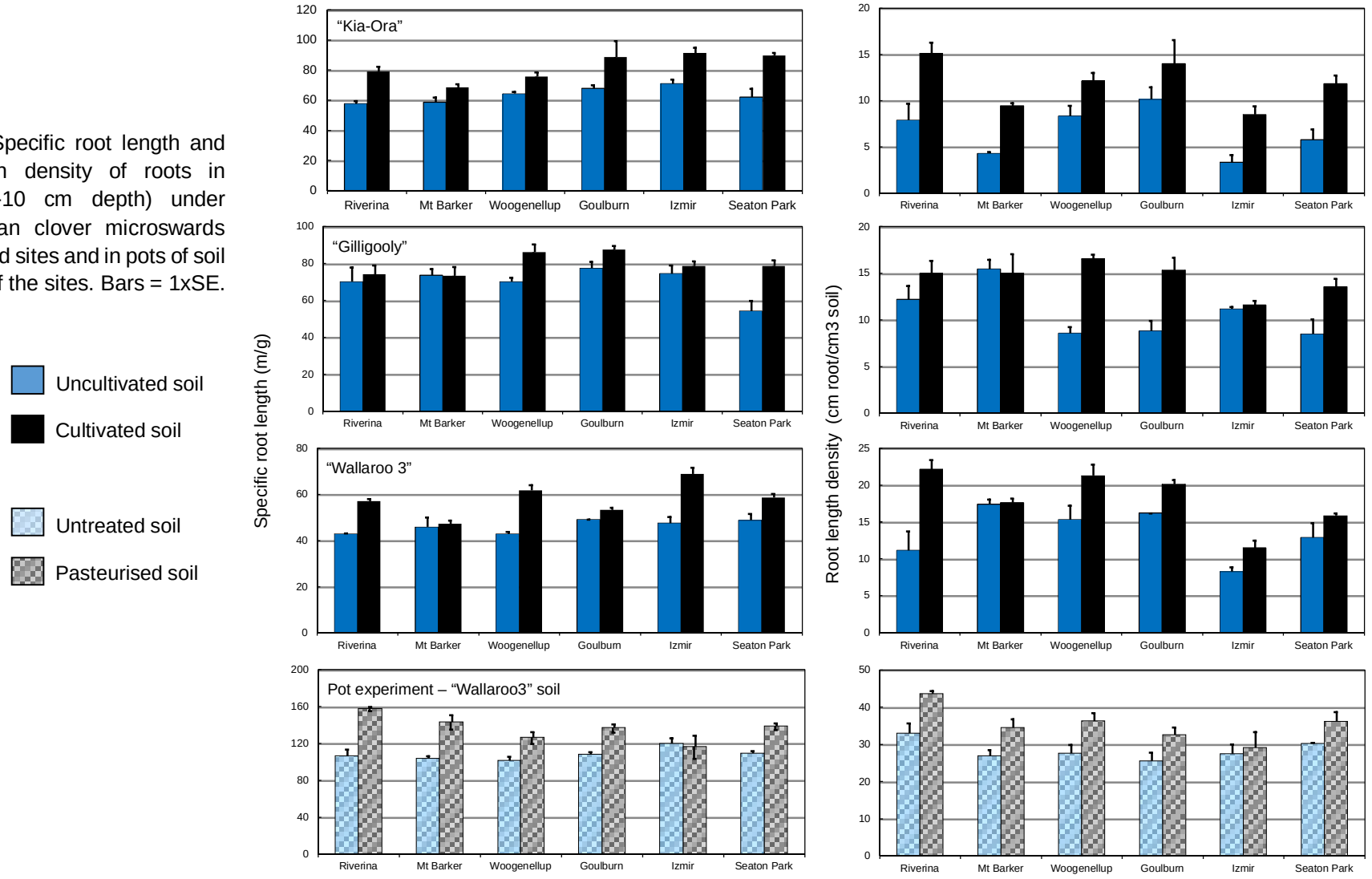


Fig. 32. Herbage yields of subterranean clover micro-swards at three field sites where the plants were grown in uncultivated soil or the soil after recent cultivation and in pots of topsoil from one of the sites which was collected and planted without treatment or planted after pasteurisation. Bars = 1xSE.

Fig. 33. Specific root length and root length density of roots in topsoil (0-10 cm depth) under subterranean clover microswards at three field sites and in pots of soil from one of the sites. Bars = 1xSE.



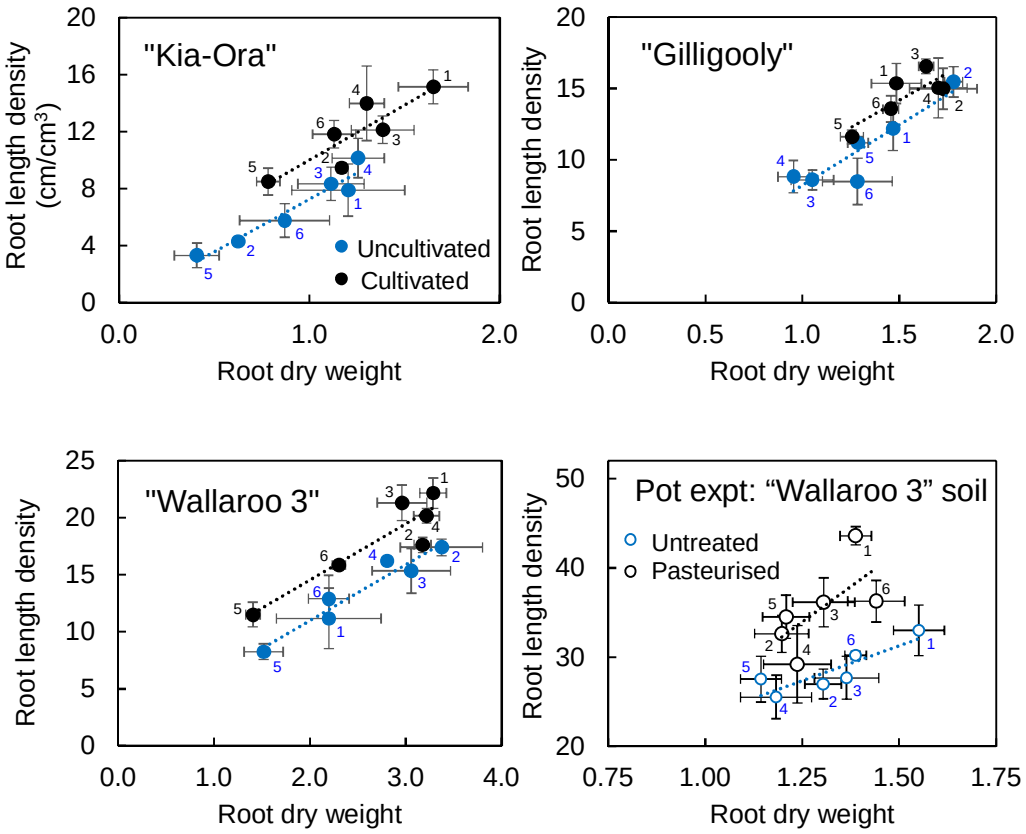


Fig. 34. Relationships between root length density and root dry matter in topsoil (0-10 cm depth) beneath micro-swards of subterranean clover grown at three field sites and in pots of topsoil collected at one of the field sites. Numbers indicate the cultivars of subterranean clover being grown: 1 = Riverina, 2 = Mt Barker, 3 = Woogenellup; 4 = Goulburn, 5 = Izmir, 6 = Seaton Park. Bars = 2xSE.

The emphasis on changes to SRL and dry matter allocation varied between the sites (and soil treatments). At “Kia-Ora”, soil exploration was increased in response to cultivation by allocation of more dry matter to roots and increased SRL, whilst at “Wallaroo 3” the response in root exploration of the soil was essentially only associated with increase in SRL. At “Gilligooly”, soil exploration by the subterranean clovers genotypes was either not changed in response to cultivation, or was increased as a result of higher dry matter allocation to roots and/or change in SRL (i.e. all options). In the pasteurised soil from “Wallaroo 3” , increases in RLD were associated mainly with increases in SRL and not increased root dry matter allocation, as had also been the case when the field soil at this site was cultivated.

The ranking of subterranean clover genotypes with respect to their ability to explore the topsoil before and after the soil treatments at field sites was often not changed by the treatments (Fig. 34). However, in every instance there were exceptions where the rank of one or two of the genotypes was changed by the soil treatment. In the comparison of pasteurised and untreated soil change in rank could not be examined adequately because only a few of the genotypes differed significantly.

4.4.3 Root pathogen and mycorrhiza DNA concentrations

Contrary to expectation, cultivation of soil at the field sites did not change the concentrations of root pathogen DNA or AMF DNA that was associated with topsoil roots (Fig. 35). There were only a few possible exceptions to this interpretation (e.g. *Pythium* associated with root of cv. Woogenellup at “Wallaroo 3”). Generally where numerical differences in the pathogen DNA concentration of roots were recorded, they were also associated with large errors and could not be regarded as reliable indications of significant differences.

Soil collected from the “Wallaroo 3” site for pasteurisation was tested for root pathogens before and after treatment. The results indicated that pasteurisation had reduced pathogen DNA concentrations below the level of detection (Fig. 36a). However, after eight weeks growth in the pasteurised soil (which had substantially stimulated shoot growth rates), the concentration of *Pythium* clade F DNA associated with roots was actually higher in the pasteurised soil than that associated with roots in the untreated soil (Figs. 36b). In contrast, AMF DNA concentrations associated with roots grown in the pasteurised soil were clearly less than those on roots in the untreated soil (Fig 36d).

4.4.4 Discussion

The *Pythium* DNA concentrations associated with root dry matter in the field experiments were of a similar order of magnitude to the levels that are associated with root damage and constraint to subterranean clover yield during autumn-winter (see section 4.5). However, there was no indication that pathogens were preventing plant root growth or shoot yields from responding positively to cultivation. Pathogen DNA concentrations on roots were also unaffected by cultivation of the soil, so it was considered more likely that the stimulation of plant growth and soil exploration by

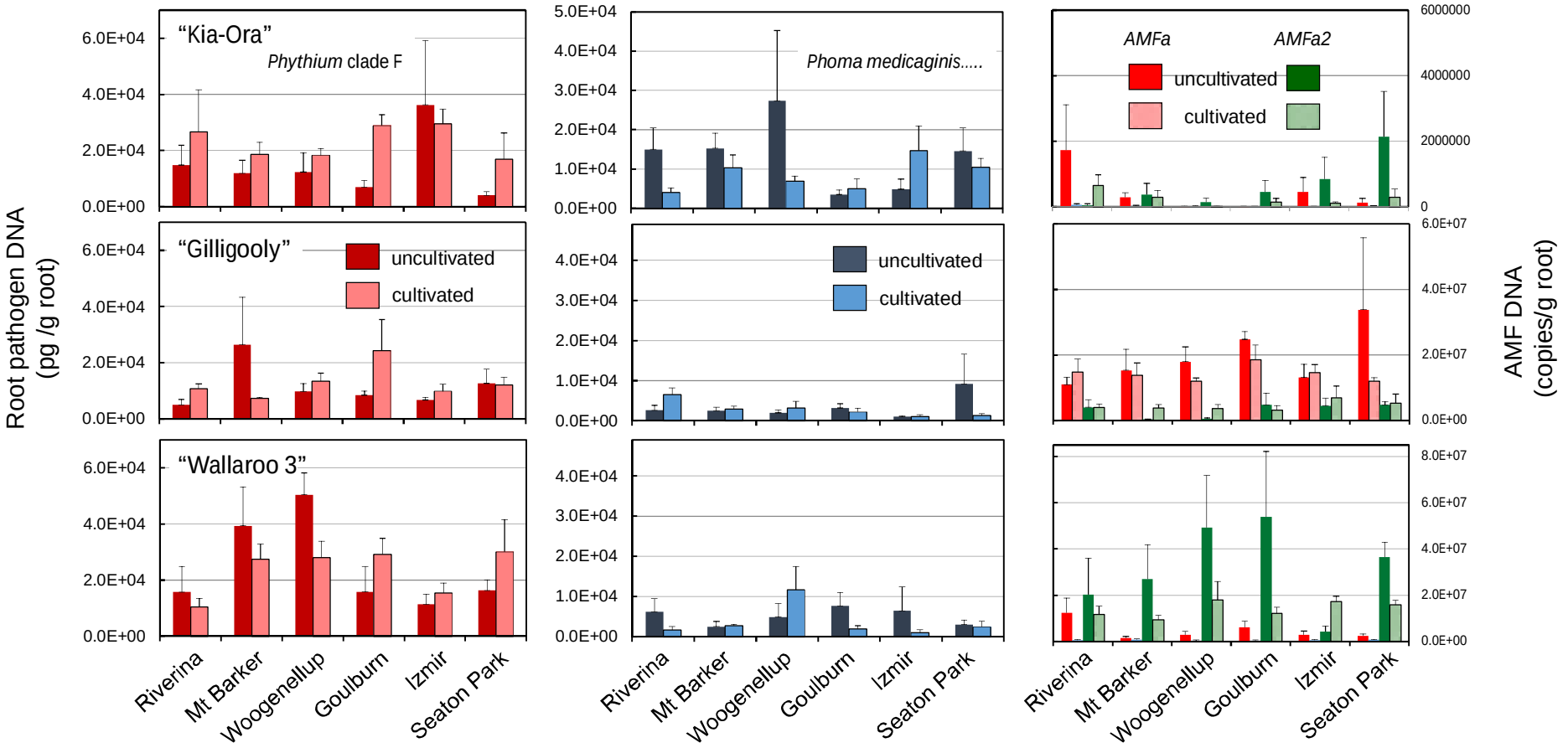
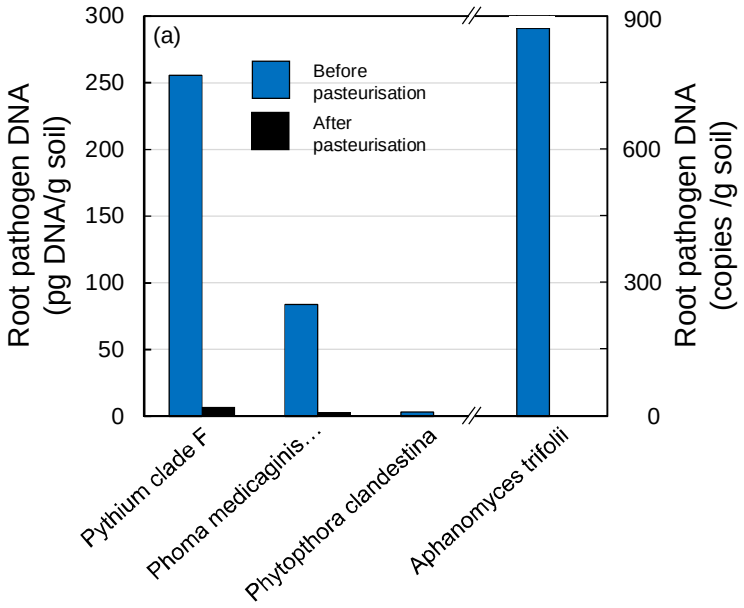


Fig. 35. Root pathogen and arbuscular mycorrhizal fungi (AMF) DNA associated with root dry matter in topsoil (0-10 cm) under microswards of subterranean clover cultivars grown at three field sites. Other root pathogen DNA tests were conducted for this experiment, but only those that returned "positive" results at each of the sites are shown here. Bars = 1xSE.



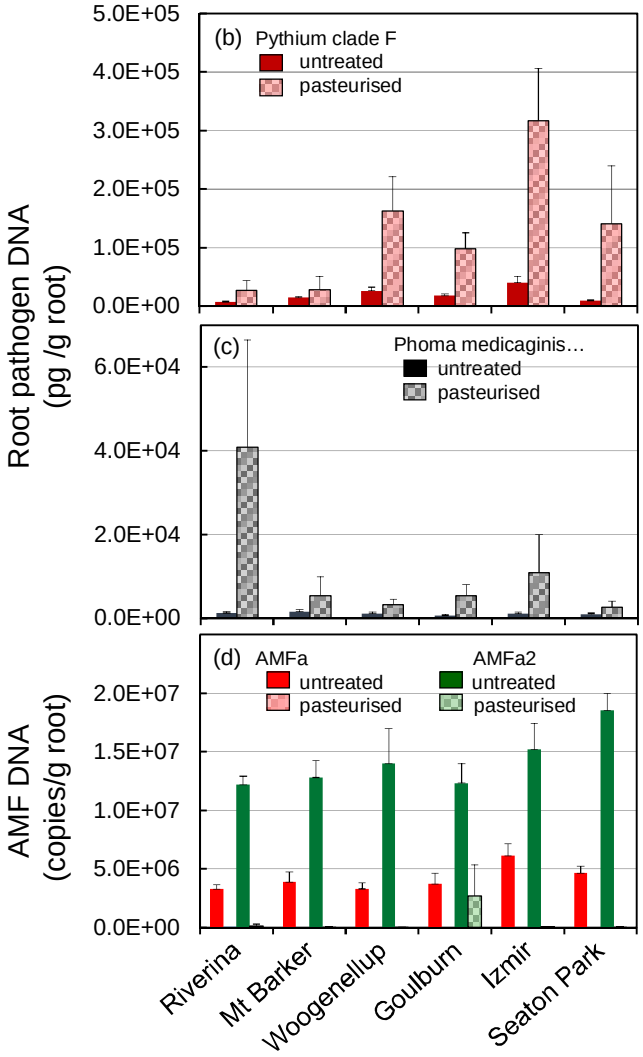


Fig. 36. Root pathogen DNA detected in topsoil (0-10 cm depth) collected from the "Wallaroo 3" site for a pot experiment (a) before and after the soil was steam pasteurised (unreplicated), and (b) associated with roots of subterranean clover plants grown in the pasteurised soil for eight weeks. Bars = 1xSE.

NB: The DNA test for *Aphanomyces trifolii* was not requested when the DNA tests of root dry matter were conducted because previous tests for "*Aphanomyces*" had been negative.

roots was due to loosening of the soil, rather than reductions in root pathogen activity. This is in contrast with findings that cultivation disrupts fungal hyphae and can provide relief (albeit short-lived) from root disease (Barbetti and Macnish 1984). Taken at face value, the present results may be indicating that relief from disease is not due to cultivation, or that it is not due to cultivation alone (i.e. removal of host plants may be as, or more important), or that cultivation provides relief by stimulating root and shoot growth because soil hardness and resistance to root growth is reduced (e.g. Masle and Passioura 1987).

Unfortunately, the present experiment does not provide a complete enough picture for definitive conclusion to be drawn.

- Threshold levels for biological significance of the DNA tests are unknown, so we are forced to speculate about their true meaning.
- We cannot rule out the possibility that cultivation did reduce root pathogen loads and the "snap-shot" assays of root dry matter only reflected recolonisation of the soil after the initial treatment. This was clearly the case in the pasteurised soil sourced from the "Wallaroo 3" site. When the soil was pasteurised, pathogen DNA concentrations were reduced to levels that were essentially undetectable. However, subterranean clover plants grown in the pasteurised soil had relatively high *Pythium* concentrations on their roots after eight weeks growth.

There were some important differences between the outcomes from cultivation and pasteurisation. *Pythium* DNA concentrations on roots in the pasteurised soil were considerably higher than those grown in untreated soil whilst AMF DNA did not recover after pasteurisation. These results hint at a substantive change in the microbial ecology of the pasteurised soil. By contrast, after cultivation pathogen DNA levels on roots were essentially similar to the levels on roots in untreated soil and AMF DNA levels were also unaffected. On balance we favour the view that cultivation did not have an immediate effect on root pathogens associated with the roots of the subterranean clover.

Overall the results indicate some potential constraints to growth by subterranean clover during spring, but the data (from pathogen DNA testing) does not indicate that the constraints can be associated with root pathogen activity. However, the evidence is also not strong enough to indicate that root pathogens do not influence plant growth rates during spring. Rankings of genotypes on the basis of root foraging in topsoils (i.e. the nutrient-rich soil layer under pasture) were reasonably stable when soil treatments were imposed at each site, but there were notable shifts in rank observed for some genotypes at some sites. The method by which plants varied root length density in response to treatments (root dry matter allocation, change in SRL) varied between sites.

4.5 Root pathogen DNA concentrations associated with damaged subterranean clover roots and the potential for using them as an indicator of root damage severity

4.5.1 Seedling survival in root damage bioassays

Subterranean clover genotypes were compared in root damage bioassays for the first time in 2008 and 2009. This had not been previously possible because the logistics and resources needed to compare genotypes are immense. Six lines x eight replicates x 100 potential surviving plants per line x 60%-80% survival (typical) results in up to 3400 plants to be counted, washed from soil and scored for root damage at any one site. Consequently, six subterranean clover lines were assessed at "Sawyer's Gully" (cv Woogenellup, 4 modern cultivars selected on the basis of their reputed resistance to root rot pathogen(s), and the strain of subterranean clover growing in the field at the site of the study), but only three (cvs Woogenellup, Riverina and the local field strain) were assessed at the other three sites.

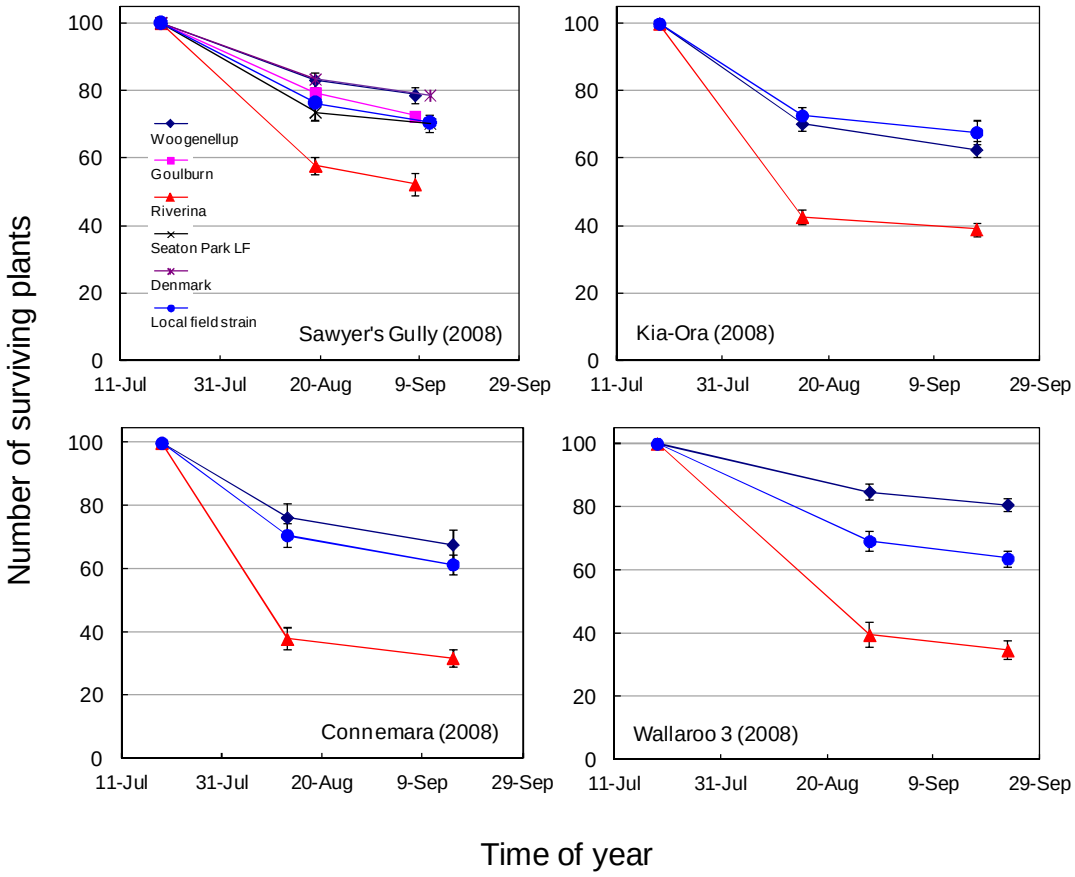


Fig. 37. Number of surviving plants in root damage bioassay rows conducted in 2008 at four sites in NSW for up to 6 lines of subterranean clover. Bars = 2xSE.

The patterns of seedling survival were essentially similar at all sites and were consistent with previous experience (Figs. 1 and 37). For most genotypes, losses of up to 30% of germinating seeds had occurred by the end of phase 1 (primary leaf expanded). The rate of seedling loss then slowed with losses of up to 40% recorded by the end of phase 2 (2-3 trifoliate leaf stage). However, cv. Riverina was exceptional suffering losses of 40%-60% during phase 1; subsequent losses (phase 2) were similar to the other genotypes. The relatively poor survival of cv. Riverina seedlings during phase 1 was also observed in 2009 (data not shown).

4.5.2 Impacts of root damage on subterranean clover yield during autumn-winter

The shoot yield of cv. Woogenellup had been observed to be negatively impacted by root damage with very consistent relationships between the constraint to yield and the extent of root damage at four NSW sites in 2007 (Fig. 2). The experiments conducted in 2008 and 2009 were intended to assess whether the relationship between clover yield and root damage differed between growing seasons, or was influenced by clover genotype. It was envisaged, for example, that if differences were found in the proportion of plants in each RDC it would indicate differences in "resistance" to root rot; differences between genotypes in yield achieved at each level of root damage would indicate differences in the ability of genotypes to "tolerate" root disease.

Damage to roots: Root damage is assessed on surviving clover plants at the 2-3 leaf stage (end of bioassay phase 2) and is not influenced by seedling losses experienced in phase 1 of the bioassay. A large change in the incidence (i.e. changes in proportions of plants in the RDC's) of root damage was observed between seasons at "Sawyer's Gully" in 2008 and 2009 (Fig. 38). This is most easily seen by examining the changes in the combined proportion of plants in the low damage categories (RDC 0 + RDC1). In 2008, cv. Woogenellup was superior to all other genotypes. However, all cultivars experienced a similar level of root damage severity in 2009.

The field strain of subterranean clover was the most severely affected line at "Sawyer's Gully" in 2008. This was also observed (data not shown) at "Kia-Ora" and "Wallaroo 3", but not at "Connemara" (the only site where pasture had been sown in recent years).

Shoot yield associated with root damage: Despite shifts in the severity of root damage across seasons, the relative constraint to subterranean clover yield associated with each RDC was essentially similar in 2008 and 2009 (Fig. 39). It was also essentially similar for all of the subterranean clover cultivars.

There were some exceptions to this assertion. For example, the relative yield of cv. Riverina was poorer than that of other cultivars in RDC 2 and 3 in 2009 at "Sawyer's Gully". It was also more tolerant of root disease at "Kia-Ora" in 2008. The relative yields of both cv. Woogenellup and cv. Riverina appeared to indicate less tolerance of root disease at "Connemara" than at the other sites in 2008. However, these differences were not observed consistently in both growing seasons.

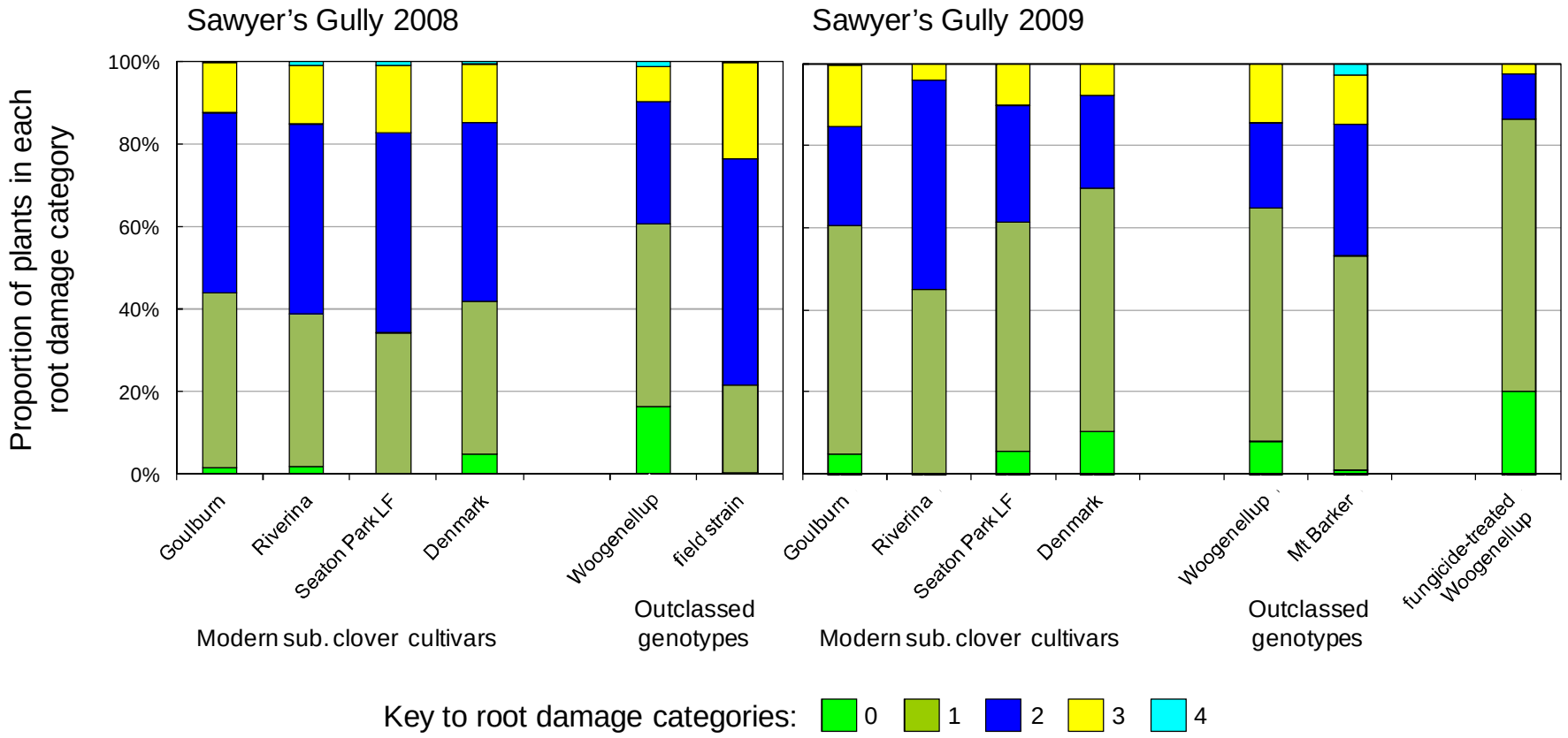


Fig. 38. The proportions of plants in each root damage category at the 2-3 leaf stage of development for a number of genotypes of subterranean clover grown at Sawyer's Gully in 2008 and 2009. Very few plants are ever ranked as having undamaged root systems, so it is more useful to look at the proportions of plants falling into combined RDC 0+1 categories when comparing the relative "health" of the cultivars, or cultivars between seasons.

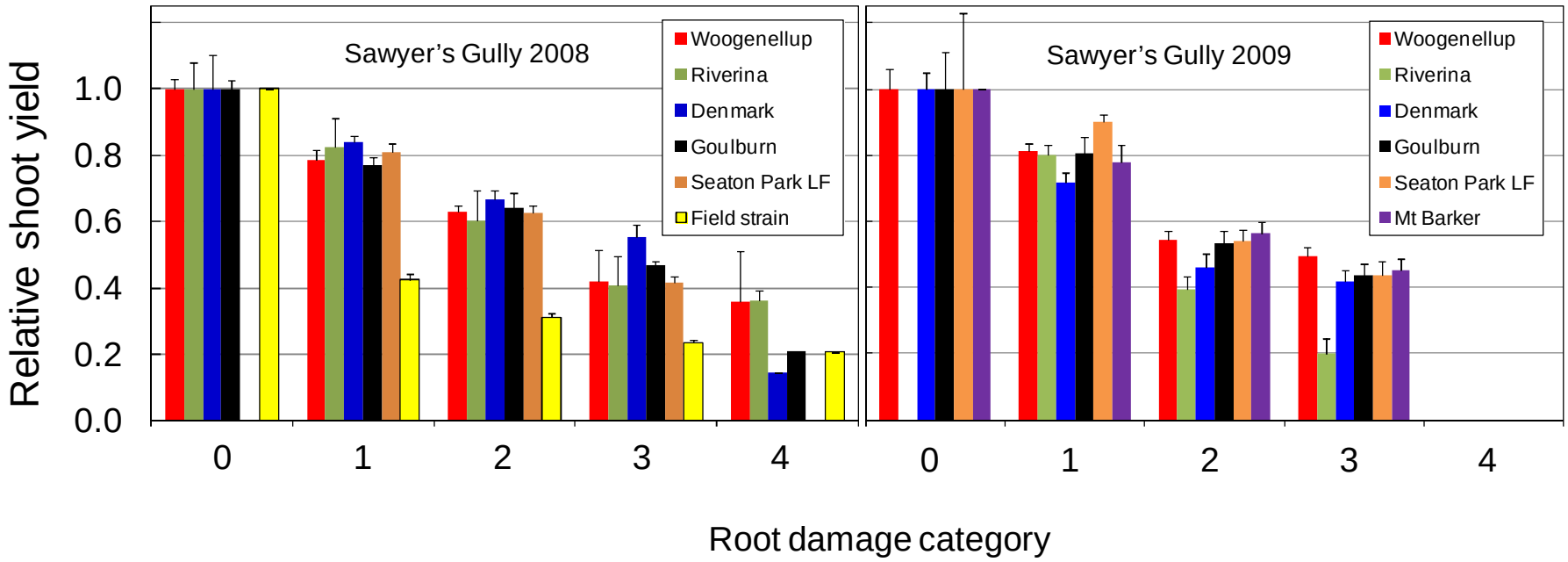


Fig. 39. Relative yield of the shoots of subterranean clover plants in each root damage category. Shoots of plants classed as RDC 0 (undamaged) were assumed to have achieved the maximum potential shoot growth and plants in other the RDC are ranked relative to the RDC 0 plants. For subterranean clover genotypes where no RDC 0 plant was found (e.g. cv. Seaton Park in 2008, cv. Riverina in 2009), the relative shoot yield of plants in RDC 1 was assumed to be equivalent to the mean relative shoot yield of all of the other genotypes and the plants in other the RDC were ranked relative to this. Bars = 1xSE.

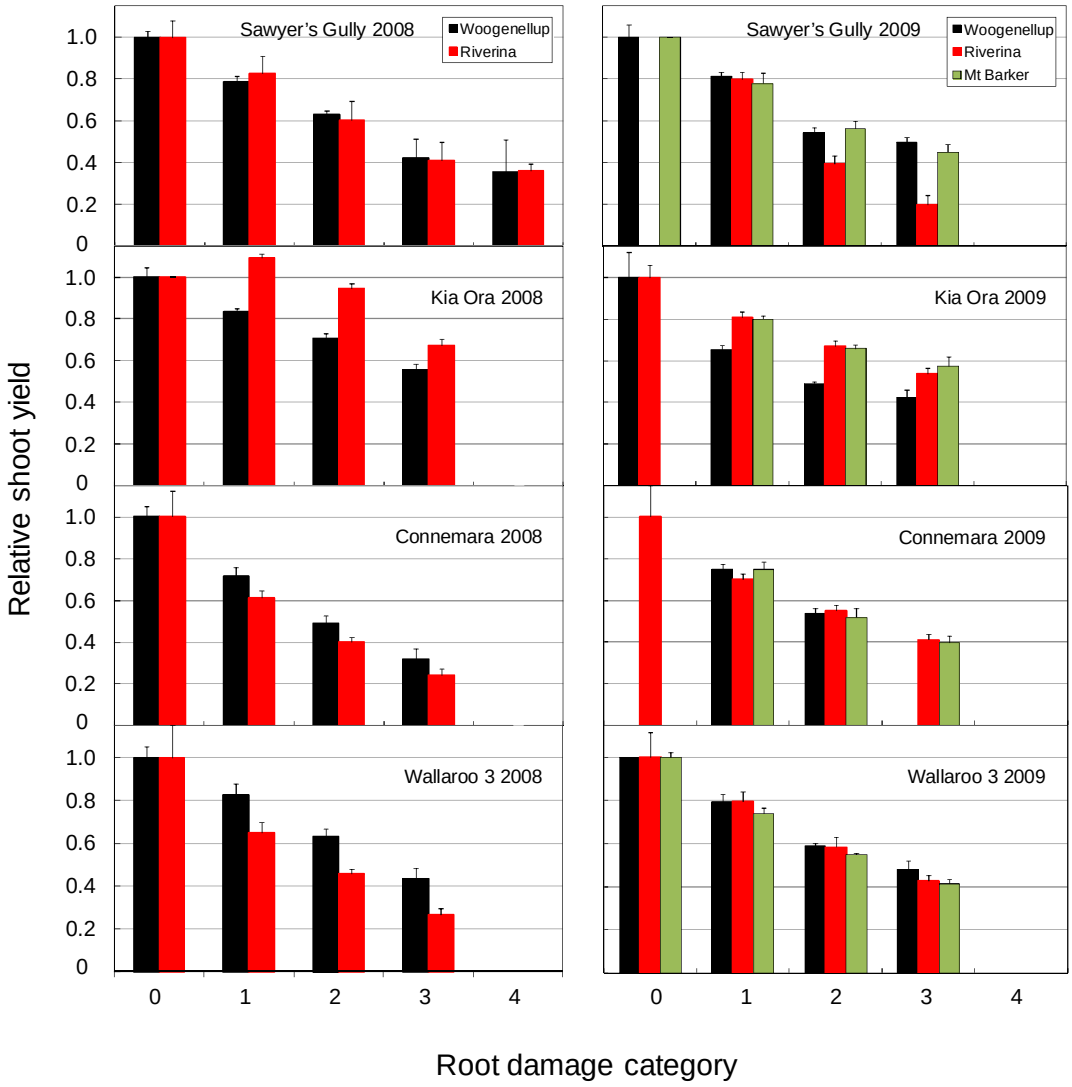


Fig. 40. Relative yield of the shoots of subterranean clover plants in each root damage category for up to three cultivars grown at four NSW sites in 2008 and 2009. Shoots of plants classed as RDC 0 (undamaged) were assumed to have achieved the maximum potential shoot growth and plants in other the RDC are ranked relative to the RDC 0 plants. For genotypes where no RDC 0 plant was found, the relative shoot yield of plants in RDC 1 was assumed to be equivalent to the mean relative shoot yield of the other genotypes and the plants in other the RDC were ranked relative to this. Bars = 1xSE.

A notable exception may be the strain of subterranean clover collected from the site in the previous year. The local field strain exhibited lower tolerance of root damage than the other genotypes grown at "Sawyer's Gully" in 2008. The identities of the field strain plants were keyed using leaf and flower markings (Dear et al. 1997) and were thought likely to be derived mainly from Mt Barker (with some Woogenellup-like plants also present) at all sites, except "Connemara". Plants grown from seeds collected at "Connemara" were tentatively identified as cv. Goulburn (56%) and cv. Denmark (35%) and cv. Karridale (8%). The farmer subsequently advised that he had sown the paddock with cvs. Goulburn, Leura, Denmark and some Karridale in 1992 (records of quantities used had not been kept). Because seed of the field strains was limited, cv. Mt Barker was sown in bioassays in 2009, but it did not exhibit higher levels of root damage or less tolerance of root damage when compared with the other subterranean clover cultivars (Figs 38, 39 and 40).

4.5.3 Root pathogen DNA associations with damaged subterranean clover roots

4.5.3.1 Relationships between pathogen DNA concentrations and root damage

The initial clue that pathogen DNA concentrations of roots might be used to measure root damage came from the observation that *Pythium* and *P. clandestina* DNA concentrations were positively associated with the extent of root damage on cv. Woogenellup at three of four NSW bioassay sites in 2007 (Fig. 3). However, at one site ("Kia-Ora") there was no relationship, and *P. clandestina* only infected roots at one site ("Sawyer's Gully").

The pattern of positive associations between *Pythium* DNA concentrations and root damage were repeated in 2008 and 2009 at "Sawyer's Gully" (Figs 41a and 42a) and was true in the majority of cases when associations with roots of different clover cultivars (Figs 41a, 4b, 41a and 42b) and at different sites (Figs 43a, 43b, 44a, 44b and 44c) were examined.

However, exceptions occurred:

- *P. clandestina* DNA concentrations were again positively associated with the extent of root damage on cv. Woogenellup at "Sawyer's Gully" in 2008, but this cultivar was apparently not infected by *P. clandestina* in 2009.
- *P. clandestina* did not appear to infect any of the other subterranean clover cultivars.
- Moderate concentrations of *Pythium* DNA were associated with of roots of cv. Woogenellup at "Kia-Ora" and "Connemara" in 2008 and 2009 but a positive correlation with root damage was not evident or weak. This followed the pattern observed in 2007 at "Kia-Ora" but was different to the pattern observed previously at "Connemara".
- There were no consistent patterns of association between the *Rhizoctonia solanii* AG2.2 or *Mycosporella/Phoma* DNA concentrations of roots and root damage.

4.5.3.2 Seasonal and site influences

- The largest differences in the pathogen DNA concentrations of roots occurred between seasons. For example, the *Pythium* DNA concentrations of roots of cv. Woogenellup were 100-fold higher in 2007 than in 2009 (Fig. 45). The concentrations of *P. clandestina* DNA

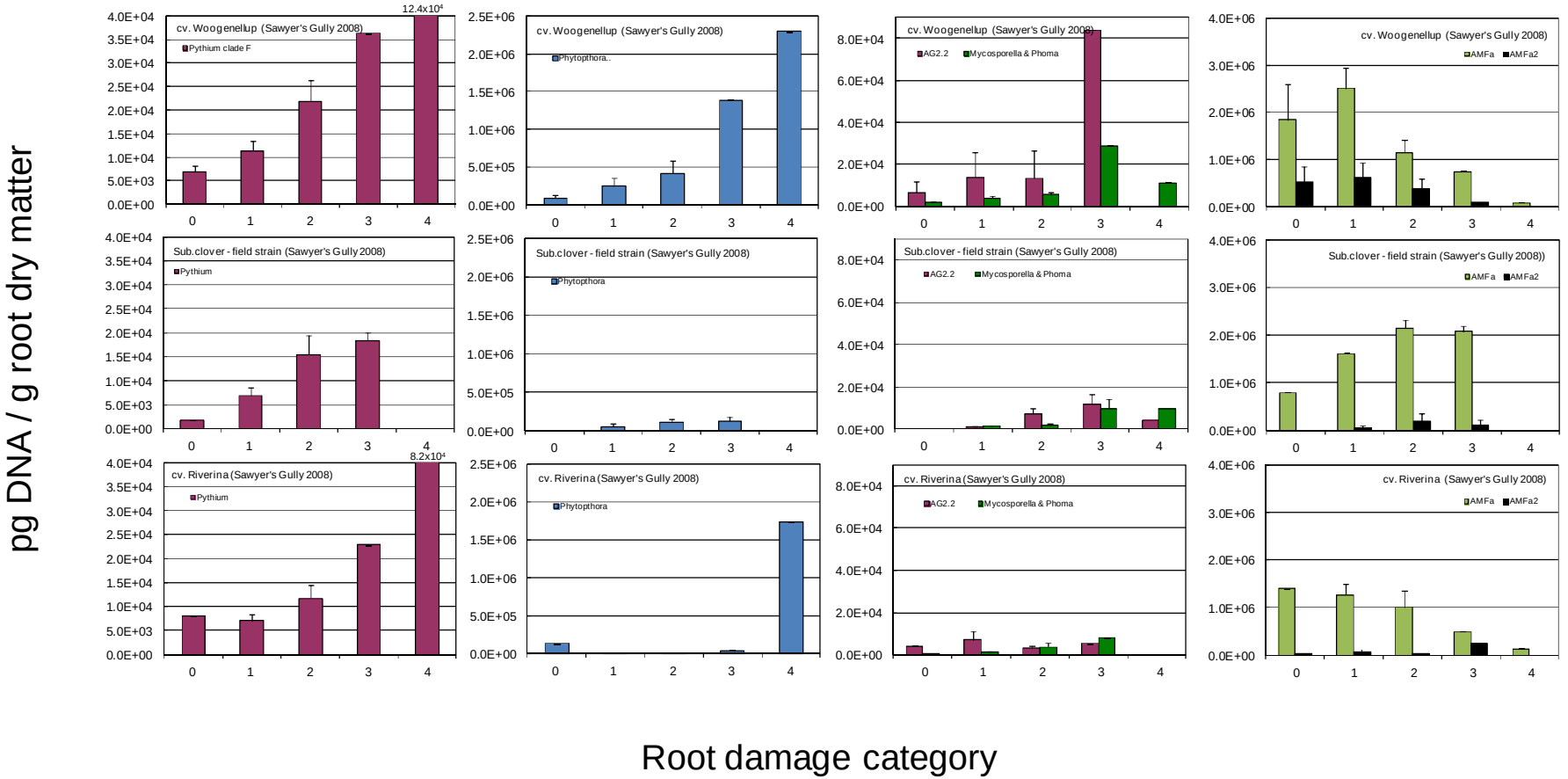


Fig. 41a. The concentrations of root pathogen DNA associated with roots of subterranean clover cultivars. Part (a) of two panels. See Fig. 41b for full explanation.

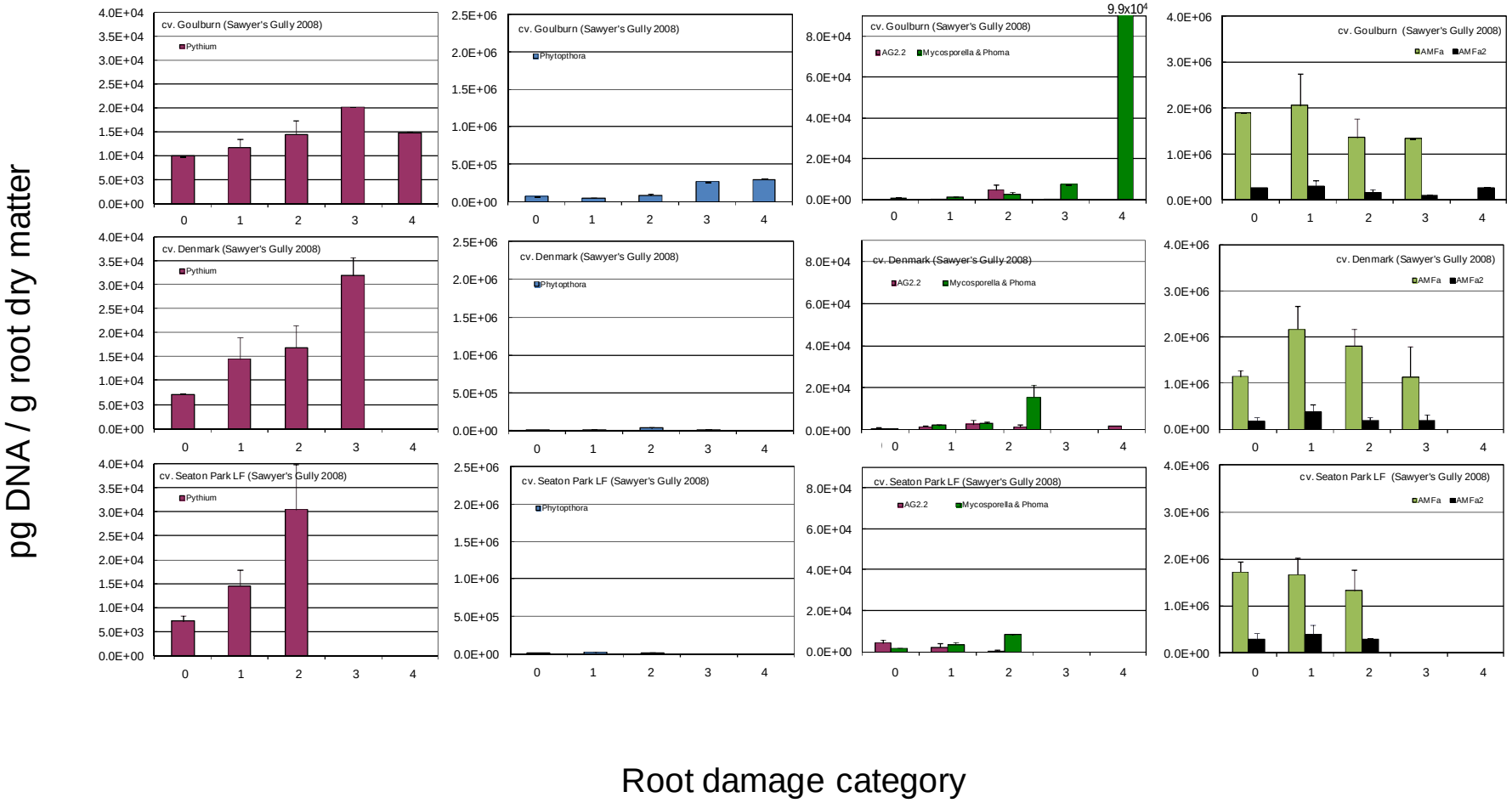


Figure 41b. Concentrations of root pathogen and arbuscular mycorrhizal fungi (AMF) DNA that were associated with roots of subterranean clover cultivars grown in root damage bioassays at “Sawyer’s Gully” during autumn-winter, 2008. The subterranean clover genotypes were: cultivars Woogenellup, Riverina, Goulburn, Denmark, Seaton Park LF, and a field strain of subterranean clover that had been collected at the site at the end of 2007. The field strain was keyed using leaf and floral characteristics and was most like the cultivar Mt Barker. The root fungi and oomycetes shown here were the main organisms detected at this site in an initial test of the soil using the complete suite of root pathogen DNA assays available from SARDI Plant Health, Adelaide. Bars = 1xSE.

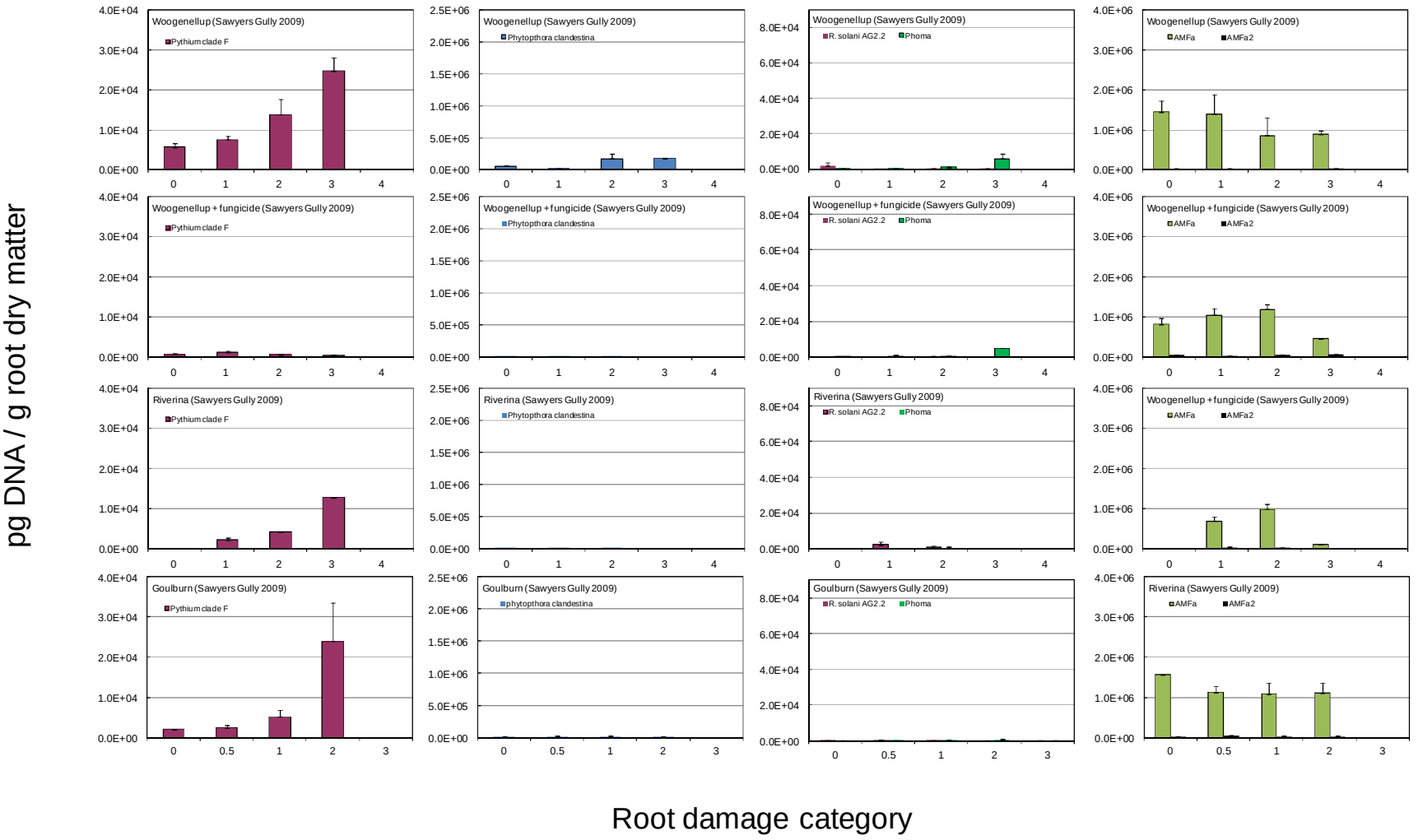


Fig. 42a. The concentrations of root pathogen DNA associated with roots of subterranean clover cultivars. Part (a) of two panels. See Fig. 42b for full explanation.

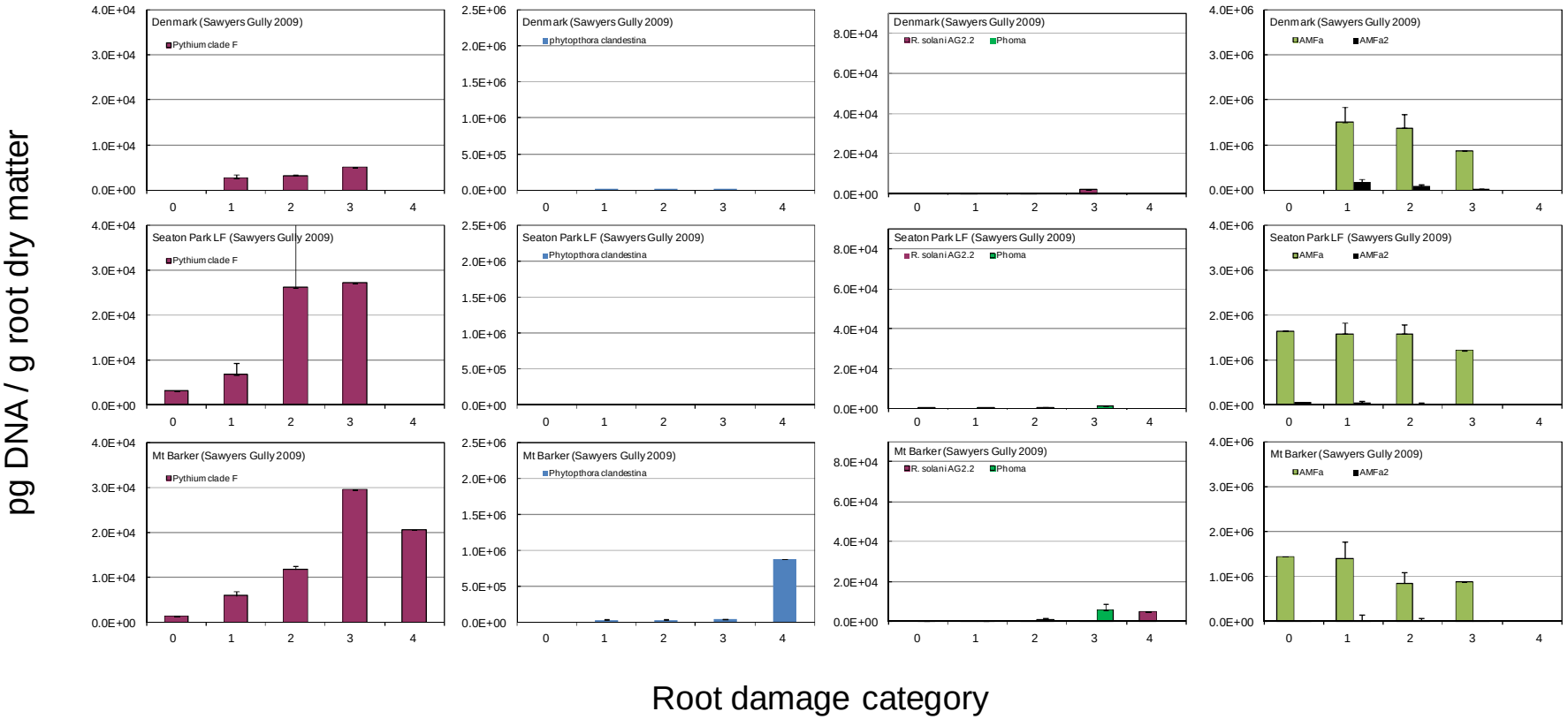


Fig. 42b. Concentrations of root pathogen and arbuscular mycorrhizal fungi (AMF) DNA that were associated with roots of subterranean clover cultivars grown in root damage bioassays at “Sawyer’s Gully” during autumn-winter, 2009. The subterranean clover genotypes were: cultivars Woogenellup, Riverina, Goulburn, Denmark, Seaton Park LF and Mt Barker. The concentration scales of the graphs were set by the levels of fungal DNA detected on roots of cv. Woogenellup in autumn-winter 2008. Bars = 1xSE.

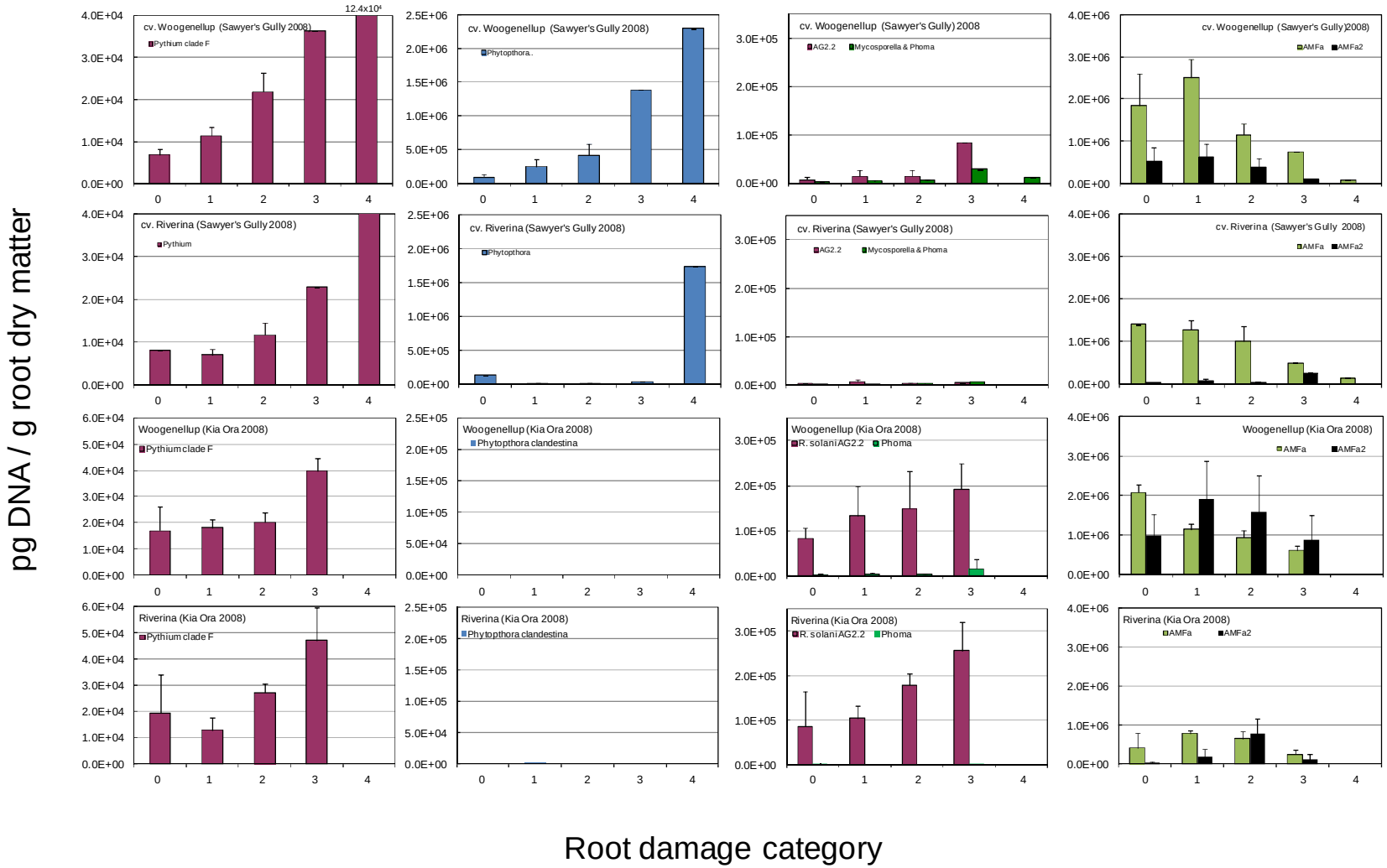


Fig. 43a. The concentrations of root pathogen DNA associated with roots of two subterranean clover cultivars at four sites in NSW. Part (a) of two panels. See Fig. 43b for full explanation.

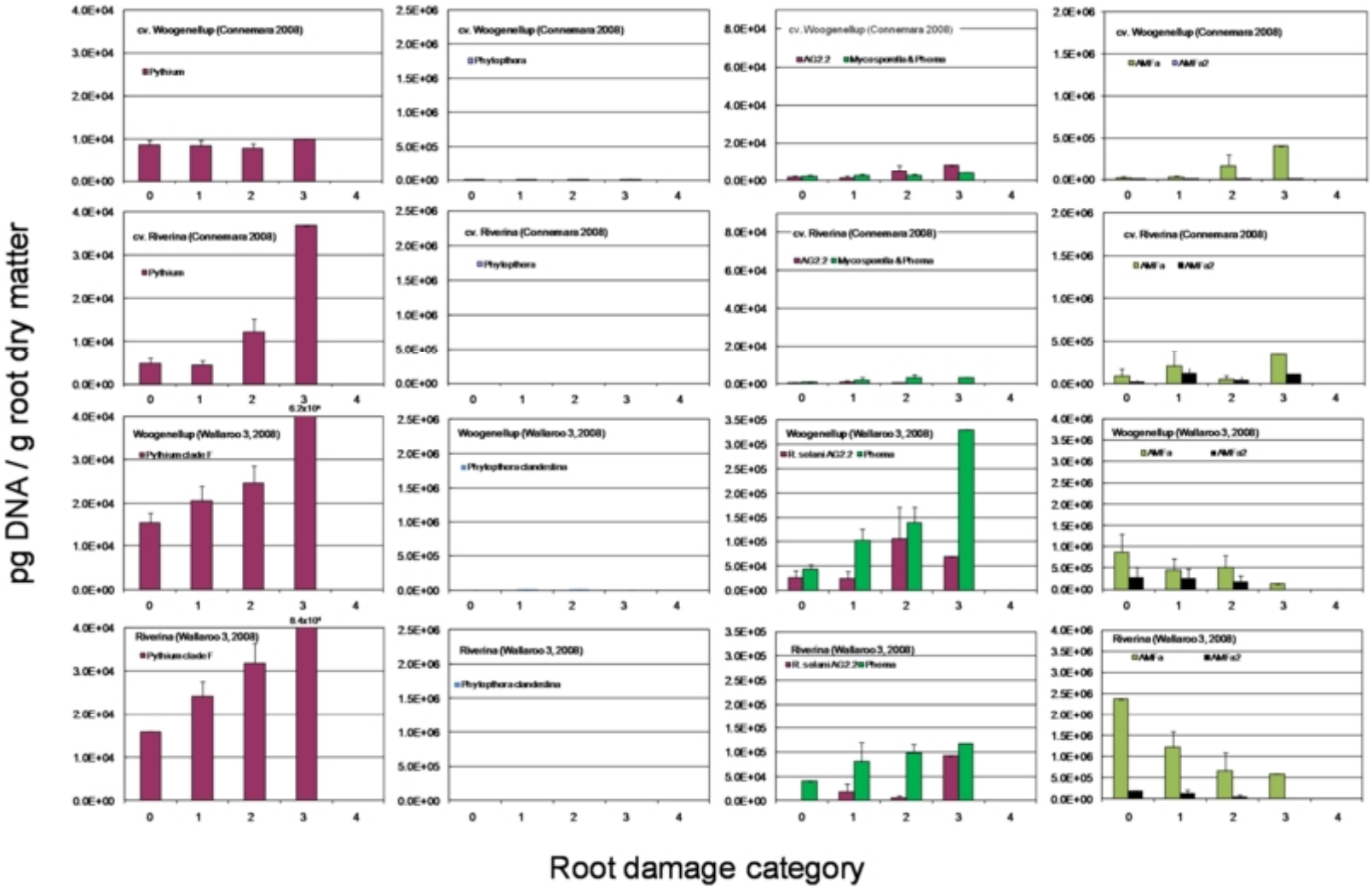


Fig. 43b. Concentrations of root pathogen and arbuscular mycorrhizal fungi (AMF) DNA associated with roots of subterranean clover cultivars (Woogenellup & Riverina) grown in root damage bioassays at four sites in NSW during autumn-winter, 2009. The concentration scales of the graphs were set by the levels of fungal DNA detected on roots of cv. Woogenellup at "Sawyer's Gully" in autumn-winter 2008. Bars = 1xSE

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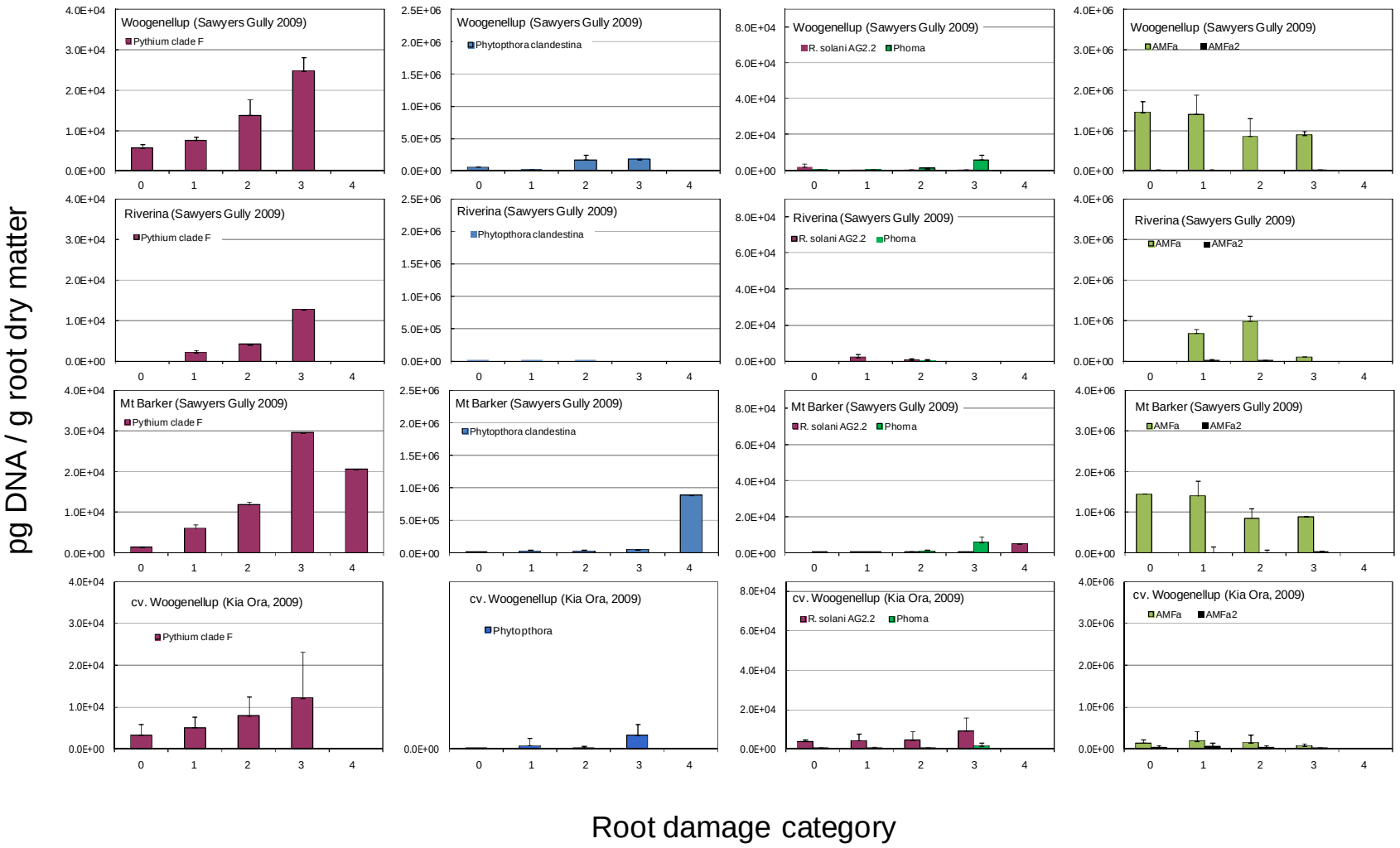

Fig. 44a. Concentrations of root pathogen DNA associated with roots of two subterranean clover cultivars at four sites in NSW. Part (a) of three panels. See Fig. 44c for full explanation

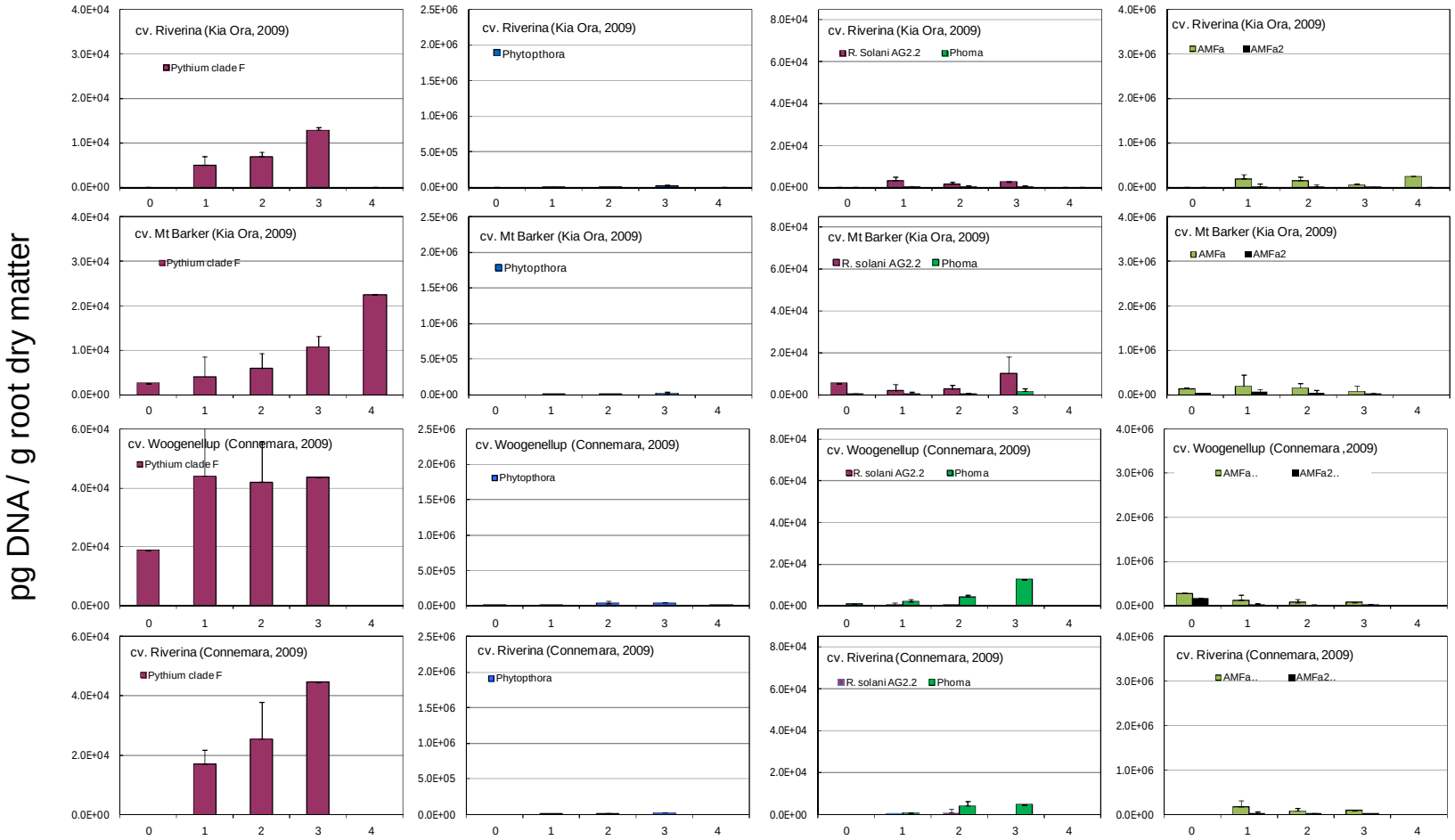

Fig. 44b. Concentrations of root pathogen DNA associated with roots of two subterranean clover cultivars at four sites in NSW. Part (b) of three panels. See Fig. 44c for full explanation.

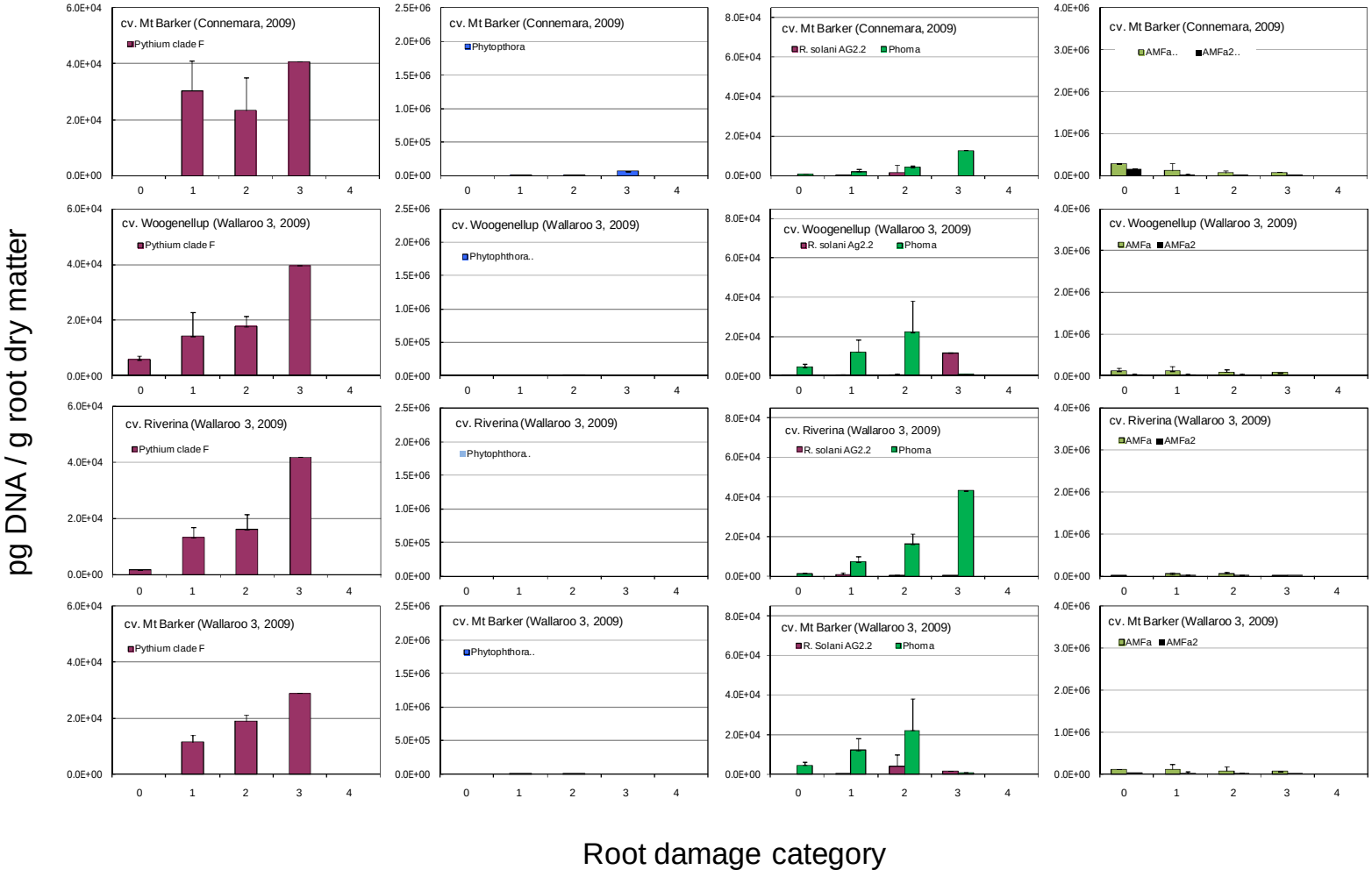

Fig. 44c. Concentrations of root pathogen and arbuscular mycorrhizal fungi (AMF) DNA associated with roots of subterranean clover cultivars (Woogenellup & Riverina) grown in root damage bioassays at four sites in NSW during autumn-winter, 2009. The concentration scales of the graphs were set by the levels of fungal DNA detected on roots of cv. Woogenellup at "Sawyer's Gully" in autumn-winter 2008. Bars = 1x SE.

also varied markedly between seasons. *P. clandestina* only infected the roots of cv. Woogenellup at Sawyer's Gully in 2007 and 2008, but appeared to absent from roots in 2009.

- *Pythium* clade F was present at all sites in all years, and its DNA concentration on roots was most commonly higher when root damage was greater. However, at two sites (as mentioned previously) the positive correlation between DNA concentration and damage was weak, or not evident.

- *P. clandestina* only infected the roots of Woogenellup and only when it was grown at Sawyer's Gully. *P. clandestina* had previously been detected in soil at Connemara (Table 1), but there was no evidence of root infection by this pathogen at Connemara in 2007, 2008 or 2009 (Figs 43b, 44b)

4.5.3.3 Effects of subterranean clover genotype and fungicide application on root pathogen DNA concentrations

- The roots of only one of the subterranean clover cultivars (Woogenellup) were infected by *P. clandestina*. It is assumed that the lack of *P. clandestina* DNA associated with the roots of the other genotypes indicated resistance to this pathogen. The cultivars used in this study were selected on the basis of claims that they were resistant to root rot. Riverina, in particular, is noted for resistance to certain race(s) of *P. clandestina* (Dear et al. 1996; You et al. 2005).

- Some of the subterranean clover cultivars appeared to exhibit lower *Pythium* clade F DNA concentrations of root dry matter than others in 2009. However, the apparent differences were not consistently observed in both years of the study, and no cultivars were free of infection (i.e. *Pythium* DNA below the detection threshold).

- Fungicide treatment of subterranean clover seed was used in 2009 for the first time. Prior to this, fungicide treatments were considered to be an unreliable way to generate disease-free controls. However, when disease-resistant cultivars appeared to resist infection by *P. clandestina,* it was hypothesized that pathogen DNA tests of plant roots should also enable direct tests of the effectiveness of fungicide treatments. This proved to be correct. Metylaxyl, applied as a seed treatment to cv. Woogenellup, reduced *Pythium* clade F DNA concentrations (Fig. 42a) to levels below the threshold we considered appropriate for biological significance (see Discussion). Metylaxyl is effective against oomycetes and would also be expected to affect *P. clandestina.* However, 2009 was the first year in which cv. Woogenellup was not infected by *P. clandestina* at Sawyer's Gully.

Woogenellup was also fungicide-treated in bioassays at "Kia-Ora", "Connemara" and "Wallaroo 3" and, in every case, was effective in reducing *Pythium* clade F DNA concentrations to negligible levels (data not shown).

4.5.4 Discussion: Pathogen DNA concentrations and root damage

4.5.4.1 Selection of DNA concentration scales used for graphing data in this report

The DNA tests for pasture root pathogens are a relatively new development (Ophel-Keller et al. 2008, O'Rourke et al. 2009, Simpson et al. 2011) and it is not known what functional or biological significance can be ascribed to the concentration of DNA measured in soil samples or in association with clover roots. The DNA concentration of

damaged roots became the focus of this work because it had been noted that there was no apparent association between pathogen DNA concentration of soil and root disease index (Simpson et al. 2011). However, this was a conclusion drawn from comparison of pathogen DNA concentrations and associated root disease scores at multiple sites in one season only.

It was noted in the course of the present work, that *Pythium* clade F and *P. clandestina* DNA concentrations of roots had varied by orders of magnitude between 2007 and 2009 and between cultivars of subterranean clover. The magnitude of pathogen DNA concentrations ranged between years as follows: 2007 >> 2008 = 2009 > 2009 with fungicide. To ensure that this range in pathogen DNA concentrations is easily appreciated and in an attempt to develop some idea of the biological relevance of the pathogen DNA concentrations that were associated with damaged roots, most graphs use the scales for pathogen DNA concentration observed on cv. Woogenellup in 2008, a year in which moderately severe root damage was observed on this cultivar. It should be noted that this scale was not set by the highest DNA concentration observed on roots (e.g. see Fig. 45).

Conclusions drawn in this report depend on the assumption that the DNA concentration scales that we have chosen are biologically relevant. For example, where it is concluded that a particular cultivar was not infected by a particular pathogen, the conclusion may be incorrect if we have misjudged the threshold for infection that is implicit in the chosen concentration scale (e.g. Fig. 46).

4.5.4.2 Can the pathogen DNA concentrations of roots be used as an indicator of "root health" and pasture productivity?

A number of conditions must be satisfied before root pathogen DNA tests can be used routinely to assess the root disease status of subterranean clover pasture:

- the adverse impact of root damage on clover shoot yield should ideally be consistent between seasons and across all clover genotypes. (Subterranean clover genotype(s) present in many paddocks will not be known and mixtures of cultivars are often sown.)
- the pathogen DNA concentration(s) of roots must be quantitatively related to the extent (proportions of damaged plants) and severity (RDC) of root damage.
- It must be possible to use the DNA of one pathogen or a suite of pathogens to quantify root damage under all (most?) field circumstances.

(i) *Relationships between plant yield and root damage.*

There was no reason to expect that the impact of root damage on clover yield would be consistent for all genotypes. In fact, it was hypothesized "tolerant" genotypes would be less yield constrained within each RDC. Different pathogens, or pathogen strains could also conceivably result in different levels of yield constraint.

Although there were occasional indications of differences in the yield constraint exhibited by a few of the clover genotypes, in most cases the constraint to yield in each category of root damage was remarkably similar for all cultivars, between sites and across seasons. There was no consistent evidence of differences in tolerance to root damage.

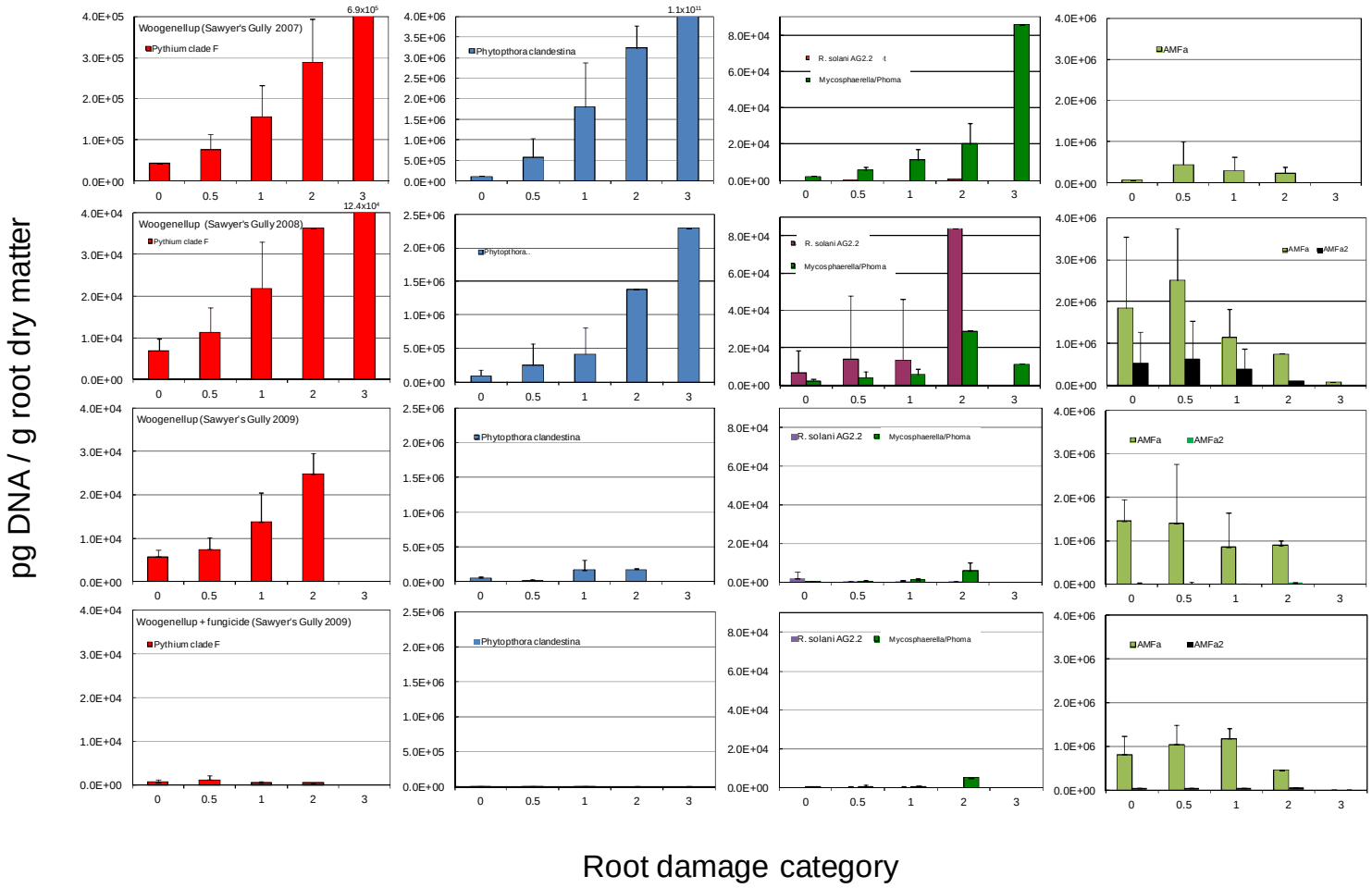

Fig. 45. Concentrations of pathogen DNA associated with roots of subterranean clover cv Woogenellup in bioassays conducted during autumn-winter 2007-2009. These data are drawn from previous graphs and are the data used to develop relationships shown in Fig. 14. Note: change of scale for *Pythium* DNA concentration for all years after 2007.

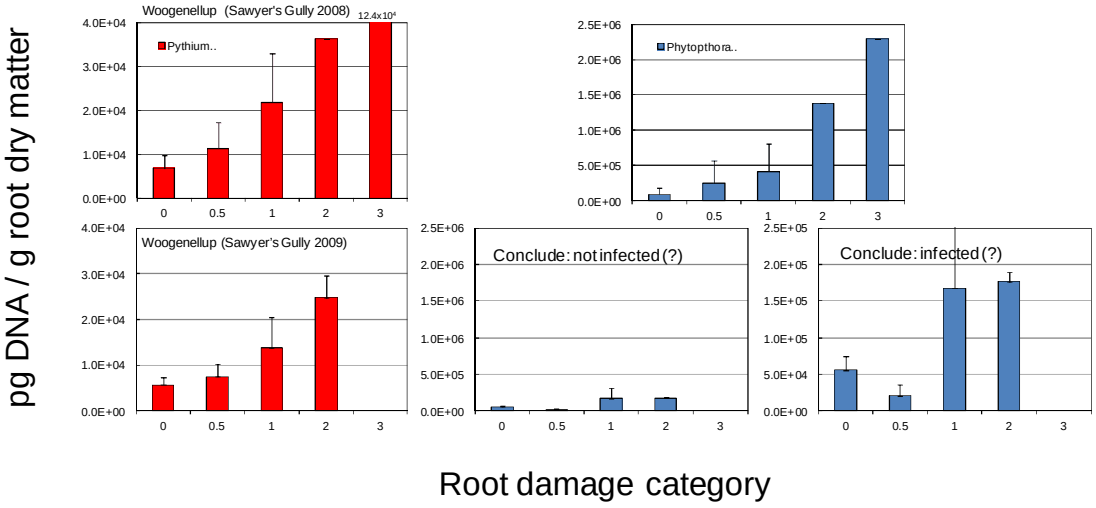

Fig. 46. *Pythium* clade F and *P. clandestina* DNA concentrations associated with the roots of subterranean clover cv. Woogenellup at Sawyer's Gully. This Figure illustrates the impact of viewing data at different scales by showing *P. clandestina* DNA in 2009 at two scales. On the left, the scale is that used for all *P. clandestina* graphs in this document and suggests that *P clandestina* infection is insignificant. The graph on the right has a scale that is 10-fold more sensitive. Without reference to a known threshold DNA concentration for biologically-significant infection, it might be concluded that roots were infected. Bars = 1xSE.

Considering all assessments of yield and RDC during autumn-winter (including the present experiment), the most frequent outcome was for subterranean clover yield to be reduced by about 20% at RDC = 1, about 40% at RDC = 2 and about 50%-60% at RDC = 3 (Figs 2, 16, 39 and 40).

(ii) *Relationships between pathogen DNA concentrations and root damage.*

Root damage severity: *Pythium* clade F and *P. clandestina* DNA concentrations of roots were usually elevated when associated with increased severity of root damage (i.e. DNA concentrations were positively correlated with RDC). However, there were also exceptions to this general observation, some associated with particular sites (as noted in the Results). The association between pathogen DNA concentration and root damage could only be tested at "Sawyer's Gully" for *P. clandestina* and only in two seasons (2007 and 2008).

Some clover genotypes also appeared to exhibit "resistance" to *P. clandestina* (i.e. all genotypes except Woogenellup had very low concentrations of *P. clandestina* DNA associated with their roots). However, resistance such as this should ideally translate into an improvement in the extent of root damage (i.e. lower proportions of damaged plants) caused by that organism.

Extent of root damage: Correlations between pathogen DNA concentrations and root damage are important because they indicate that DNA tests may flag pathogen infection and root damage. However, the tests will only be useful indicators of root health in subterranean clover pastures if they are also indicative of the proportions of plants that are damaged (or undamaged). The results of the bioassays conducted since 2007 have not always provided

an obvious answer to this question. Cultivars which appeared to resist *P. clandestina* (Fig. 41a) exhibited no less root damage (Fig. 39) nor lower proportions of plants with substantially damaged roots (Fig. 38) than cv. Woogenellup which had relatively high concentrations of *P. clandestina* DNA associated with its roots (Fig. 41a). However, root damage is widely thought to be caused by a suite of root pathogens, often acting in concert (Barbetti et al. 2007). Under these circumstances, resistance to only one of a suite of pathogens might be expected to be ineffective or, at best, only partially effective in protecting a plant from root damage.

When pathogen DNA concentrations of roots of cv. Woogenellup at "Sawyers Gully" were considered over the initial three years that the root DNA levels had been examined (2007-2009) and after seed treatment with a metylaxyl (2009), it was realised that changes in infection (DNA concentration of roots) had ranged over two orders of magnitude (Fig. 45). It was hypothesized that seeking relationships between the pathogen DNA concentrations of roots and the proportions of damaged plants within each season (i.e. between treatments) had proven futile because the investigations were conducted at the wrong scale. This was examined by combining data for all years and treatments in the period 2007-2009 using cv. Woogenellup at "Sawyer's Gully" (Fig. 45) and data from the current bioassays at Sawyers Gully (Fig. 47) (*NB. this was the only cultivar and site that had been tested consistently over several years*).

When an appropriate pathogen infection scale (log10 DNA concentration of roots) was used, it was first noted that roots in all RDC were often similarly infected (Figs 47). This was important because, for a farm paddock test of clover plant roots, it would be impractical to sort test plants into RDC; only a test of the whole root system would be feasible. A pathogen DNA test of the whole root system was then simulated by combining the data to obtain a whole of root system DNA concentration after weighting the pathogen DNA concentrations of each RDC using the proportions of plants recovered in each RDC (Figs 48).

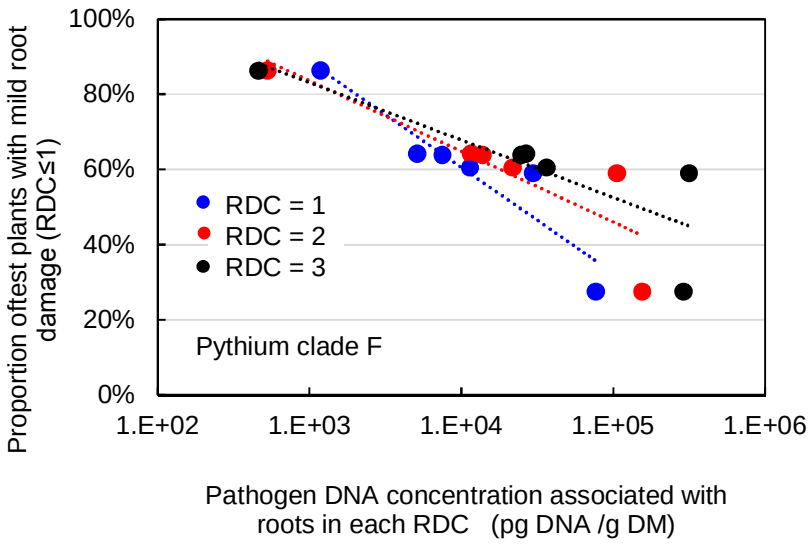

Fig. 47. Proportions of test plants (subterranean clover cv. Woogenellup) with mild root damage (RDC≤1) at "Sawyers' Gully" plotted relative to the *Pythium* clade F DNA concentrations that were associated with roots in each RDC from bioassays conducted in autumn-winter 2007-2009 and 2012, and on plants in bioassays where seed had been treated with metylaxyl in 2009 and 2012 (see Fig. 45 for further details from the 2007-2009 bioassays).

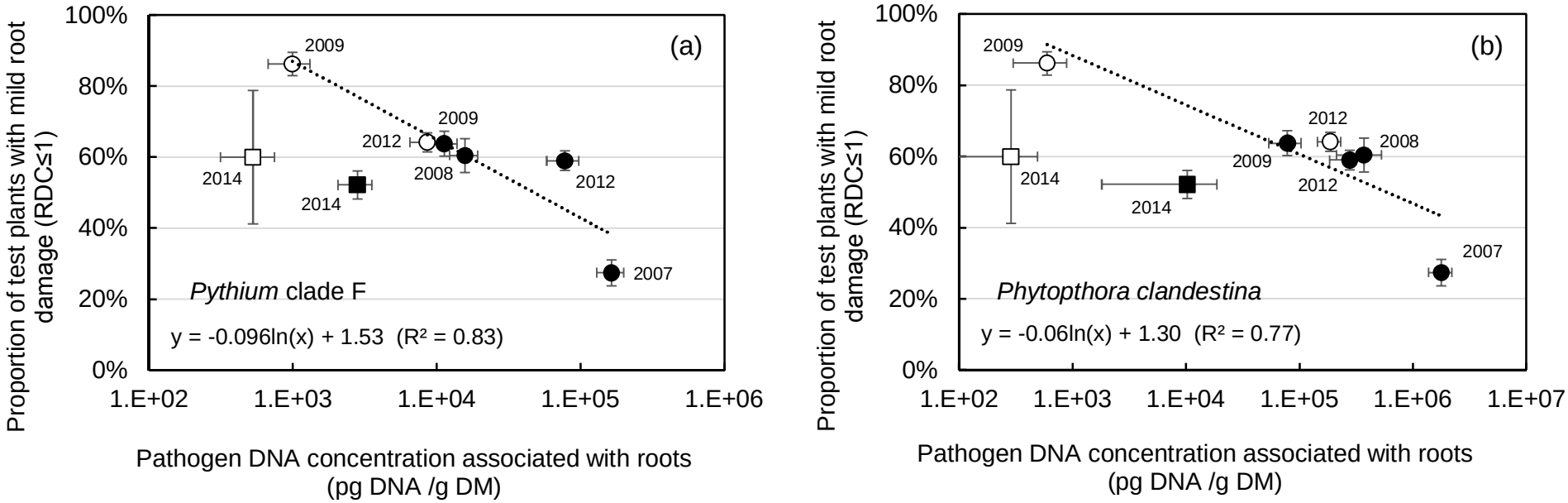

Fig. 48. Proportions of test plants (subterranean clover cv. Woogenellup) with mild root damage (RDC≤1) plotted relative to the estimated pathogen DNA concentrations that were associated with all root dry matter in bioassays conducted at "Sawyer's Gully" (circles) during autumn-winter 2007-2009 and 2012, and on plants in bioassays where seed had been treated with metylaxyl in 2009 and 2012. Results from bioassays in 2014 at "Gilligooly" are show using square symbols. Closed symbols indicate bioassays using surface-sterilised seeds, open symbols indicate bioassays using seeds that had also been treated with metylaxyl (fungicide). The pathogen DNA concentration associated with the roots of all plants in each bioassay (i.e. unsorted) was estimated by summing the weighted DNA concentration of roots from each RDC. Weightings were determined using the proportion of plants in each RDC. Bars = 2xSE.

These results suggested that there may be a potentially useful relationship between the relative root-health of subterranean clover and the concentration of pathogen DNA associated with root dry matter for bioassays conducted at "Sawyer's Gully'. Because shoot yield constraints in autumn-winter are correlated reasonably consistently with RDC, it is also possible to estimate the cost of the pathogen's presence in terms of plant yield. For example, subterranean clovers sampled from across a pasture with a pathogen DNA test at 10^3 pg *Pythium* clade F DNA per gram of root dry matter would be predicted to be relatively "healthy" with ~85% mildly damaged root systems (RDC≤1) with a yield constraint of about 9%[1]; those with 10^4 pg *Pythium* DNA/g would have ~55% mildly damaged roots and a yield constraint of about 20%; while plants with 10^5 pg *Pythium* DNA/g would only have ~30% mildly damaged root systems (RDC≤1) and a yield constraint of about 45%.

To put this in context, the range in the proportion of plants with RDC≤1 in the subterranean clover bioassay survey conducted across southern Australia in 2006 was only ~10% to ~60% (Note: RDC≤1 (present work) was equivalent to a root damage score = 0 in the original survey).

What are the flaws in this speculation?

- Results from the analysis of pathogen DNA concentration on roots at "Gilligooly" in 2014 did not line up with data from "Sawyer's Gully". This limited test of the hypothesis at only one other site is clearly not sufficient to draw conclusions, other than to observe that a robust test for "root health" should reliably give similar results at all sites.

- This assessment of how pathogen DNA tests of clover roots may be used to assess pasture health is scientifically flawed because the differences in pathogen DNA concentrations occurred over four growing seasons and the results cannot be ascribed unequivocally to pathogen affects alone (i.e. pathogen affects and seasonal affects are confounded).

- The assessment does not resolve the problem of root damage often being the result of a suite of the pathogens. The assertions made above could also have been argued using the results for *P. clandestina* alone. However, the general usefulness of this relationship is obscure because *P. clandestina* did not infect the roots of cv. Woogenellup at any of the other sites in this study. The situation is not clarified when some observations made during the present study also appear to conflict. For example, root damage on cultivars that appeared to resist *P. clandestina* was often as extensive as that on cv. Woogenellup infected with both *Pythium and P. clandestina.*

- At this stage it is unclear whether it would be possible/reliable to use only one pathogen DNA test to monitor pasture health because it is not known whether there is a "keystone" pathogen in the pathogen complex that results in clover root damage.

[1] This estimate assumes that yield during autumn-winter is reduced by about 20% at RDC = 1, about 40% at RDC = 2 and about 50%-60% at RDC = 3 (e.g. Figs 2, 16, 39 and 40), and that damaged roots in RDC >1 were distributed 80% in RDC = 2 and 20% in RDC = 3 (e.g. Fig. 39).

5 Discussion and Conclusions

5.1 Objectives 1 & 2:

What is the impact of damaged roots on the response of subterranean clover to soil P fertility improvement?

Can better P nutrition of a pasture modify the incidence and severity of damage to roots or overcome the constraint that root damage imposes on yield?

5.1.1 Field bioassays for root damage to subterranean clover during autumn-winter

The field bioassay demonstrated that P uptake does not limit growth by subterranean clover during winter when low temperatures were more limiting for pasture growth than P supply (e.g. "Sawyer's Gully" 2012, Fig. 15a). However, under milder autumn-winter temperatures, subterranean clover, growing in very low P soil, did respond moderately to improved soil P fertility (e.g. "Gilligooly" 2014). The response to soil P during autumn-winter was only observed when plants had undamaged roots (RDC = 0) and was limited because low temperatures inevitably constrain plant growth at this time of the year. Plants with damaged roots (even mild root damage, RDC = 1) were unable to respond to improved soil P fertility conditions.

Soil P fertility levels did not influence the *Pythium* (clade F) DNA concentrations associated with root damage, but *P. clandestina* DNA concentrations were elevated on roots of clover growing with increased soil P. There was no obvious consequence for shoot yield of the elevated *P. clandestina* DNA concentrations.

Root damage during autumn-winter was associated with large constraints to shoot growth (Figs 16 and 39) confirming earlier observations (Fig. 2). The constraint to growth was larger for plants with more severely damaged roots. It is concluded that damage to roots prevented plants from responding to improved soil P because damaged roots directly constrain plant yield. Our hypothesis was that root damage in autumn-winter is analogous to pruning the root system and that the constraint to shoot growth was mainly a consequence of roots being "pruned"`.

CONCLUSIONS:

(i) Control of root pathogens or plant resistance to root damage will improve autumn-winter yields irrespective of soil P fertility conditions.
(ii) Application of P to increase its availability to damaged plants does not overcome the constraint to yield associated with damaged roots.

5.1.2 Root pruning experiments

A root pruning experiment was conducted in a glasshouse, at warmer ambient temperatures more conducive for rapid plant growth, to examine how damaged roots constrain the shoot yields of subterranean clover plants (Figs 18 and 19).

The yield of plants with pruned roots was related to the length of root that remained after pruning indicating a direct effect of root pruning on shoot yield. However, shoot yield was also further constrained if the soil volume in which the remaining roots were grown was also restricted. This indicated that under favourable growth conditions, plants with damaged roots can potentially respond to resource (nutrient) availability (Fig. 22).

It is predicted from these data that, under conditions conducive for rapid plant growth (e.g. spring (and perhaps during autumn-winter in mild climates [e.g. coastal areas, Western Australia, etc.]), plants with damaged roots may potentially respond to application of P fertiliser. Importantly, these results do suggest that the critical P requirement (i.e. the soil test P level needed for near-maximum growth) of clover-based pastures may potentially be higher if root disease damages roots of subterranean clover during spring.

CONCLUSIONS:

(i) Damage to roots directly constrains shoot growth in subterranean clover; a response that is analogous to the "bonsai" effect of root pruning. This outcome is considered to be a consequence of the intrinsic balance that plants' maintain when allocating dry matter to shoot and root growth (Poorter and Nagel 2000).

(ii) The constraint to shoot growth associated with pruned roots was analogous to the shoot yield constraints that are associated with root damage in autumn-winter bioassays in the field. However, in autumn-winter, subterranean clover plants with damaged roots did not respond to improved soil P fertility. The root pruning experiments indicate that such a response is feasible. However, the root pruning experiment and autumn-winter bioassays were conducted under contrasting temperature conditions. The critical P requirement (i.e. the soil test P concentration of soil at which maximum growth is achieved) during autumn-winter will be much lower than that measured for pastures during spring (or the glasshouse experiment) because pasture growth rates are constrained by low temperature at this time of the year. In addition, if potential shoot growth rates are constrained further by damaged (pruned) roots the critical P requirement will be further reduced. Our conclusion is that that subterranean clovers growing in cool conditions with damaged roots do not respond to improved soil P fertility because they do not need to respond: i.e. even at low soil test P concentrations they have sufficient soil P for the growth rates they can potentially achieve.

(iii) If environmental conditions favour a response to improved soil fertility by clover plants with damaged roots (e.g. milder temperatures), the clover will still not achieve its true yield potential because the pruning of root length in itself induces a constraint to shoot yield that is not alleviated by access to more nutrients.

(iv) Pruning roots potentially limits a plant's access to soil nutrients because root exploration of soil is restricted. Thus, if significant root damage occurs when environmental conditions are favourable or rapid growth (e.g. spring), it is possible that the critical P requirements of clover pasture may be elevated as a result of poor soil exploration.

5.2 Objective 3: Will breeding subterranean clover for improved root foraging traits be an effective way of improving the P-efficiency of pastures systems?

5.2.1 Fungicide injection into the root zone of subterranean clover swards during spring

The validity of the hypothesis that root pathogens may increase the critical P requirement of a pasture depends on the incidence and severity of root damage during spring. This was unknown, however, root rot pathogens are usually considered to be more of an issue early in the growing season (Barbetti et al. 2007). The pathogens involved in rotting subterranean clover roots are known to be active over the temperature range from 10 to 25 ^{o}C, but most severe root rot occurs at the lower temperatures (10-15 ^{o}C). Soil moisture conditions interact with temperature to modify disease severity (Wong et al. 1984). Low moisture and flooding are not favourable for many pathogens, but there are exceptions (Wong et al. 1984; 1986)

A major problem for addressing the question of whether root damage was modifying P acquisition by clover in spring was determining how root damage, or its affects, could be assessed on established plants. The seedling bioassay provides a reasonable approximation of subterranean clover growth under autumn-winter conditions but does not adequately replicate the conditions experienced by established plants with large root systems during a warm, moist spring. For this reason, assessments of constraint to yield, or ability to respond to P due to root rot pathogens during spring were made by applying fungicide(s). A novel method for treating pasture with fungicide was necessary because fungicide applied topically to soil may not penetrate to the root zone and can, as a result, be ineffective and/or phytotoxic (e.g. Lodge 2005). It is also very difficult in these experiments to untangle the confounding effects of fungicide ineffectiveness, phytotoxicity and root pathogen affects. Consequently, the experiments included treatments intended as “positive” and “negative” controls against which responses to fungicide treatment could be compared.

The experiments investigated ways to inject fungicide directly into the pasture root zone during spring. Use of sub-irrigation lines proved unsuccessful because cultivating the soil to lay the irrigation pipes had an overriding and positive effect on plant growth rates that eliminated any treatment effects (i.e. soil P fertility and fungicide treatments).

A method for direct injection of fungicides into the root zone of undisturbed soil was developed. It was proven that fungicide was (i) delivered to, and absorbed by the subterranean clover plants (mild phytotoxicity symptoms on leaves; Fig. 29a) and (ii) was effective in reducing the DNA concentrations of susceptible pathogens in soil+root samples from the root zone (Fig. 30). However, the response to improved soil P fertility was not altered by fungicide treatment (Fig. 28a). Unfortunately, there is no fungicide that is effective against all potential root pathogens, so a neutral result cannot be relied on to indicate that root disease was not affecting pasture growth rates. However, the growth rates recorded in the fertilised treatments were exceptionally high (typical of short-term peak spring growth under the most favourable conditions). Together with the neutral response to fungicide, this observation provided reasonable evidence that pasture growth rates were most probably not constrained by root disease. In contrast, root damage was recorded at this site during autumn-winter over many years (*unfortunately bioassays were not conducted at this site in the year of this spring experiment*), and was shown at those times to consistently constrain shoot growth rate (e.g. Fig. 15).

CONCLUSIONS:

(i) No case was established for there being either: (a) a significant root disease problem during spring, or (b) any change in the ability of subterranean clover to acquire P for P-deficient soil that might be attributed to root disease.
(ii) However, the fungicide injection experiments do not provide unequivocal evidence for, or against there being problems associated with root disease in established subterranean clover pasture during spring. There were a number of experimental reasons why this was always a risk: (a) no fungicide is active against all of the potential root pathogens, (b) ensuring roots are adequately treated by the fungicide can be a problem, (c) fungicides can cause phytotoxicity and this may mask the beneficial effects of the fungicide treatment and (d) root pathogens may (by chance) not be active in the season in which the experiment was conducted. However, the new method for injecting fungicide into the root zone of plants growing in undisturbed soil did appear to eliminate risks (b) and (c).
(iii) The main observations that favour the argument that there were no significant constraints associated with root disease during spring were: (a) subterranean clover did not show either a growth response and/or a larger response to improved soil P fertility when treated with the fungicide (metylaxyl), (b) proof that an adequate dose of the fungicide had been taken up by the plants, and (c) the very high short-term growth rates achieved by the subterranean clover swards when soil P limitations were removed by applying P fertiliser.

5.2.2 Impact of root pathogens on subterranean clover root morphology

This experiment was an attempt to radically alter the pathogen loads on roots of subterranean clover during spring without resorting to biocides. Six cultivars of subterranean clover known to differ in specific root length (SRL: length of root per gram root mass) were chosen as the test plants. SRL is one of the key P-foraging traits examined in studies of plant P-acquisition efficiency. The aim was to disrupt root pathogen associations with roots and to determine if this altered root length density (RLD: length of roots per volume of soil) and SRL in the nutrient enriched topsoil of a pasture and/or stimulated shoot growth.

The main treatment was vigorous cultivation of the soil because this is being used in other work (PSP 0005: Managing soil-borne root disease in sub-clover pastures) to demonstrate how roots respond to reduced presence of root rot pathogens. However, a steam pasteurisation treatment was also established using topsoil from one of the three field sites. In nearly every case, the soil treatments stimulated shoot growth. At some sites, root growth in the topsoil was stimulated and, in most instances, RLD was increased in the cultivated and pasteurised soil. Unexpectedly, cultivation did not alter the root pathogen DNA concentrations associated with root dry matter. Pasteurisation clearly reduced pathogen DNA and AMF DNA concentrations of the soil and all of the subterranean clover cultivars subsequently grew substantially faster in the pasteurised soil. However, after 8 weeks the pathogen DNA concentrations associated with their roots were higher than for roots of plants growing in untreated soil. In contrast, AMF did not recover after pasteurisation and the roots of plants in the pasteurised soil had no associated AMF DNA.

The ranking of the subterranean clover cultivars with respect to their "nutrient foraging" root traits was often relatively stable. However, there were notable exceptions where the ranking of a few cultivars was shifted substantially as a consequence of a soil treatment or between field sites.

CONCLUSIONS:

(i) There are still uncertainties about what root pathogen DNA concentrations of roots are measuring (see section 5.3). If they are indicative of pathogen loads on plant roots, these data indicate that soil cultivation *per se* may not be reducing root damage by reducing pathogen levels in soil. Soil loosening allows roots to grow faster and was an important factor in the responses that were seen in the present experiments.
(NB: *in some instances cultivation treatments may also involved a fallow period (absence of host species); this may confound some of the conclusions about the perceived benefits of cultivation*.)

(ii) It is highly likely that soil hardness is restricting subterranean clover roots growth in field soils. Universally, these experiments have demonstrated large responses in shoot growth during spring after soil has been loosened by cultivation.

5.3 Objective 4: What is the impact of soil P fertility on pathogen DNA levels in damaged roots and the relationship between damage and pathogen DNA concentration of roots?

Soil P fertility levels did not influence the *Pythium* (clade F) DNA concentrations associated with root damage but the *P. clandestina* DNA concentrations were elevated on roots of clover growing with increased soil P. Our hypothesis was that the positive effect of soil P fertility on *P. clandestina* may be an indirect consequence of P promoting the growth of subterranean clover which is the specific host of this pathogen. However, there was just as much reason to suspect that the *Pythium* DNA concentrations would also be positively influenced by increased clover growth, but this was not the case.

Autumn-winter bioassays had been conducted at the "Sawyer's Gully" site over several years since 2006 and the pathogen DNA concentrations associated with damaged roots were available from four seasons (2007-2009 and 2012). The present experiment provided an opportunity to process and collate this data and especially to determine whether there was any evidence of a relationship between the incidence or severity of root damage and the pathogen levels associated with roots.

Using a relatively simple criterion (the proportion of plants with only mild root damage: i.e. RDC ≤1), a correlation was found between incidence of root damage and the estimated *Pythium* clade F or *P. clandestina* DNA concentrations associated with root dry mass. The magnitude of the relationship, in turn provided (for the first time) a clue to the scale that should be used when assessing the pathogen DNA concentrations. This, in turn, provided some insight into the likely DNA-test thresholds for "pathogenicity" by some of the root pathogens.

It is important to acknowledge that the analysis has significant experimental design flaws and there remain a number of unexplained observations. However, we contend that the results do provide important clues for future research with the root pathogen DNA assays.

Flaws and unexplained observations:

- The differences in pathogen DNA concentrations and incidence of root damage occurred over four growing seasons and pathogen affects and seasonal affects are confounded. For a statistical and scientific viewpoint, this does not permit the conclusions that have been drawn.

- The discussion of the DNA results so far has effectively assumed that there is a "keystone" pathogen (e.g. *Pythium*) in the suite of potential pathogens that may be acting together to cause root damage. Indeed, the assertions made could also have been argued using the results for *P. clandestina* which was also present on roots at "Sawyer's Gully". However, it is reported that root rot damage is typically greater when more than one pathogen is present (Wong et al. 1984) and it is not clear from the present results what the relationship between damage and pathogen DNA would look like for sites where only *Pythium* (for example) was present (e.g. most of other sites used in this project).

- In addition, some observations made during the present study appeared to conflict with expectations from the literature (such as in Wong et al. 1984). For example, root damage on other subterranean clover cultivars that appeared to "resist" *P. clandestina* infection was often as extensive as that on cv. Woogenellup infected with both *Pythium and P. clandestina.* Here it is assumed that pathogen DNA associated with roots was indicative of infection. This in itself may be a flawed assumption.

- Results from the analysis of pathogen DNA concentrations on roots at "Gilligooly" in 2014 did not line up with data from "Sawyer's Gully". This was a very limited test of the hypothesis that pathogen DNA concentrations associated with roots may have a predictive value. However, it was clearly not an encouraging observation for such an hypothesis.

Metylaxyl treatment of seeds (cv. Woogenellup) in the bioassays consistently reduced the oomycete pathogen DNA concentrations associated with root dry matter. The only apparent exceptions were instances where a pathogen DNA level was later judged to have already been below a suspected "biologically-relevant" threshold for pathogenicity (see Fig. 46 for discussion). In 2009, the fungicide also substantially increased the proportion of relatively healthy plants. However, this was not observed on subsequent occasions when seeds were fungicide treated. At the time, the lack of an obvious effect on root "health" was assumed to reflect the possibility that other root pathogens were present and had taken over the root-damaging role of the oomycetes. In retrospect, Fig. 48 shows that in 2012 and 2014 the differences in the pathogen DNA concentration between the fungicide-treated and untreated bioassays, although significant, were relatively small in magnitude. The observations that root damage had not be alleviated greatly, were consequently entirely consistent. In other word, fungicide effectiveness can be quite variable. This has also been reported previously (Barbetti et al 1987a).

Nevertheless, oomycete pathogen DNA concentrations were usually reduced when metylaxyl was applied to seeds. This observation suggested that absence of root pathogen DNA on roots of particular subterranean clover genotypes may indicate resistance to the pathogen in

question. For example, at “Sawyers Gully” cv. Woogenellup was the only cultivar with high *P. clandestina* DNA concentrations associated with its roots (Fig. 43).

CONCLUSIONS:

(i) The pathogen (*Pythium, P. clandestina*) DNA concentrations associated with roots of subterranean clover may be correlated with the incidence of root damage during autumn-winter. However, further work is required to confirm this speculation and to assess if relationships will hold at different locations.

(ii) Pathogen DNA concentrations associated with roots may be indicative of the effectiveness of fungicide treatments and potentially other suppressive treatments such as plant resistance. This is speculative and would need to be confirmed using a combination of traditional plant pathology, genotypes with known genetic resistance and the pathogen DNA tests. However, if true, the DNA tests offer the possibility of detecting resistance to pathogens even when the plant phenotype is confounded by other influences (e.g. presence of other pathogens).

6 Recommendations

6.1 Objectives 1 & 2:

What is the impact of damaged roots on the response of subterranean clover to soil P fertility improvement?

Can better P nutrition of a pasture modify the incidence and severity of damage to roots or overcome the constraint that root damage imposes on yield?

This project has demonstrated that alleviating damage to the roots of subterranean clover during autumn-winter will reduce constraints to shoot growth. Pasture growth at this time of the year is limited mainly by cool temperatures. However, root damage imposes a further constraint to production. When subterranean clover is growing in very P-deficient soil during autumn-winter, application of P fertiliser may improve its growth. The warmer the season, the more likely a response to P fertiliser will be observed. However, the critical P requirement of pasture at this time of the year is considerably lower than in spring when pasture growth rates are faster. As a consequence, the response to improved soil P levels recorded in the field experiments fell between zero and a modest improvement in growth. The presence of only mild root damage (RDC=1) eliminated the modest response to improved soil P fertility. We deduce that this is because mild root damage of this order imposes a further shoot yield constraint of about 20%, further reducing the critical P requirement of the plants.

Additional application of P fertiliser will not overcome the limitations imposed by root damage in autumn-winter. However, alleviation of root damage through control of root pathogens, or by developing cultivars with improved root rot resistance will increase autumn-winter yields by subterranean clover, irrespective of soil P fertility.

The following observations of root damage and seedling survival by a limited number of subterranean clover cultivars have been made:

- at three of four field sites, the “modern” cultivars tested had displayed less root damage than a strain selected from the field in which the autumn-winter bioassay was conducted. Subsequently, it was learned that the fourth site had been sown recently to modern cultivars (Fig. 38). This suggested there has been progress in improving resistance to root damage during the development of recent cultivars.

- cv. Woogenellup, was used most often as the test plant because it is a “control” cultivar used in root pathogen research. It is reputed to be a susceptible variety. However, it was not found to be necessarily worse or better than many other cultivars with respect to the incidence of root damage in the field. It did, however, have high *P. clandestina* DNA concentrations associated with its roots and this was not observed on the “modern” cultivars, some of which (e.g. cv. Riverina) are known to carry resistance genes to this pathogen (You et al. 2005).

- cv. Riverina was particularly susceptible to loss of seedlings at germination (Fig. 37). A syndrome commonly termed “damping off”. This cultivar is particularly valued for the

resistance it carries to *P. clandestina* but the potential for losses of up to 60% of seedlings at germination each year is a significant disadvantage.

Evaluation for yield and agronomic performance in several locations across southern Australia by new lines of subterranean clover has been part of the final stages of cultivar development and release. This practice should be extended to assessments of seedling survival and root damage to ensure new cultivars with genes for resistance to major pathogens also carry a reasonable degree of general "field resistance" to seedling loss and root damage.

6.2 Objective 3: Will breeding subterranean clover for improved root foraging traits be an effective way of improving the P-efficiency of pastures systems?

- The relatively high critical P requirement of subterranean clover during spring determines why legume-grass pastures must be fertilised to high soil test P concentrations to achieve high production. The present experiments were unable to demonstrate that root disease was limiting subterranean clover yield in spring or that root disease was interfering in the response of subterranean clover to P fertiliser during spring. Consequently, it was concluded that root disease was not elevating the critical P requirements of legume-based pastures and that there was no evidence to suggest that root disease would negate the selection of legumes with more "P-efficient" root morphology.

- Recent results in a parallel project (PUE 0104: P-efficient legume pastures) has now demonstrated in field experiments that some alternative legumes (e.g. French serradella, yellow serradella) with long fine roots and long root hairs can yield as well as subterranean clover at substantially lower soil test P concentrations. This adds weight to the conclusion that root disease is unlikely to prevent improvements in legume root system morphology.

Lack of interaction between root disease and response to P fertiliser in spring indicates that root disease is unlikely to negate efforts to breed clovers with more nutrient-efficient roots systems.

6.3 Objective 4: What is the impact of soil P fertility on pathogen DNA levels in damaged roots and the relationship between damage and pathogen DNA concentration of roots?

- A putative relationship between pathogen DNA concentration(s) of roots and root damage has been observed but would need to be confirmed within a single growing season and at a number of independent sites for the relationship to be regarded as more than just a chance observation. The current experiments provide clues as to how this may be achieved. Sites can be selected using soil and plant tests for wide and or narrow pathogen profiles and for high DNA concentrations. Treatments to vary the DNA concentrations can include: (i)

different cultivars, (ii) cultivation and removal of host species in the previous year(s), (iii) fungicide treatment, and (iv) combinations of these management interventions.

- The issue of multiple pathogen infections and whether they would negate use of DNA tests as indicators of "pasture health" is unresolved.
- Even if a "pasture health" test were available, there are presently few reliable management options available to farmers to counteract root disease problems in established pastures.

Other applications of pathogen DNA concentrations associated with roots?

Two potential applications of pathogen DNA technology were revealed by the present experiments:

It is possible that pathogen DNA tests can be used to identify/confirm plant-based "resistance" to root rot pathogens. This will require significant backup using traditional plant pathology techniques, but has the potential to be a rapid and cheap way to screen diverse genotypes and to identify cases of multiple resistances.

Pathogen DNA tests can be used to assess the effectiveness of fungicide treatments and will be especially effective at doing this when yield and root damage symptoms are confounded (i.e. not alleviated) by to the presence of other root rot pathogens.

6.4 Other observations: Losses associated with seed and seedling predation by mice

These experiments have seen unprecedented loss of bioassay seedlings to field mice. All experiments had to be protected using Mouseoff wheat grain baits (developed to protect crops) and, in one instance ("Redmire"), no amount of baiting prevented near complete loss of bioassay plants. In previous work (2006-2009), we had seen mice damage to an experiment on only one occasion where one replicate of an experiment was on a field boundary adjacent to a paddock with long grass. Two factors differed between the previous work and the current experiments. All of the early work was conducted during the "Millennium" drought period (2001-2010) which may have kept mice numbers in check, and all of the recent experiments were located adjacent to long grass paddocks (due to low stock numbers after the drought) or conservation areas with long grass. "Redmire" had been used as a site without incident on a previous occasion, but the farmer had in the interim period fenced the adjacent area as a conservation reserve and it supported unchecked growth of tall grasses during the current project. The experiment at "Redmire" could not be protected against mouse predation despite repeated applications of Mouseoff. It is unknown whether it was the drought or the subsequent long-grass pastures that was the more important mitigating factor. It must also be acknowledged that the bioassays, with seeds/seedling in neat isolated rows, are probably an easy and attractive target. (Note also, the "green target" appearance of the experiment at "Sawyer's Gully" on a farm with insufficient livestock after the Millennium drought (Fig. 4e).

A systematic examination may be warranted of the potential costs to pasture establishment and persistence due to seed/seedling predation by field mice, and the costs associated with conservation reserves if they are the source of the problem.

7 Key Messages

7.1 Messages for farmers

(i) Root damage on subterranean clover is common in autumn-winter (often due to fungal and oomycete pathogens) and reduces yields at this time of the year. The occurrence of root damage is widespread and farmers are already dealing with this level of yield constraint. However, if a solution to root disease(s) can be found, it is likely that carrying capacity of pasture would be increased.

(ii) It is unlikely that root damage is a constraint to animal production during spring.

(iii) Optimum soil P fertility management will ensure pastures are not constrained by P, but adding extra P-fertiliser to overcome the yield reductions associated with root damage does not work.

(iv) A number of modern subterranean clover cultivars carry a useful level of root damage resistance (as well as other yield advantages) and it is worthwhile to ensure that they are used when new pastures are being sown.

(v) It is worthwhile using fungicide seed treatments when planting new subterranean clover pastures to protect seedlings from loss and root damage. This is especially the case when it is remembered that seed of good cultivars is expensive and maximum germination and survival will help to ensure good establishment in the year of sowing. Unfortunately, our observations indicate that the effectiveness of fungicide treatments may prove to be variable.

7.2 Messages for researchers

(i) Root damage on subterranean clover is unlikely to negate efforts to breed subterranean clovers for improved nutrient foraging during spring.

(ii) A tentative relationship was established between the proportions of subterranean clover with only mild root damage in autumn-winter and the root pathogen DNA concentration associated with roots. However, further work is required to prove the relationship is real, repeatable and useful.

(iii) The pathogen DNA tests of roots seemed to be able to detect the effects of fungicide treatments and possibly plant-based disease resistance. This observation also needs further work involving traditional pathology and plants with known resistance genes to prove the concept. However, if correct, the application of the DNA tests to root disease ecology, plant improvement and fungicide testing could assist R&D in these areas.

(iv) We have significant reservations about the field resistance of cv. Riverina to “damping off” losses and believe the fitness of this cultivar should be investigated further.

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9 Appendix

NIL.