Final report

Comprehensive diagnostic analysis of pastures affected by dieback

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Abstract

Pasture dieback is a complex condition likely involving a range of pre-disposing biotic and abiotic factors. The role and impact of these factors are poorly understood. This project aimed to clarify the range of pathogenic organisms associated with pasture dieback across multiple regions in Queensland, as well as northern New South Wales (NSW), to assess for pathogenicity.

The project was conducted through three concurrent activities; 1. Field sampling of plants and soil at paired sites; 2. Laboratory analysis of these samples; 3. Pathogenicity testing of specific pathogenic organisms determined from the analysis of field collected samples.

The knowledge of multiple potential causal agents or co-factors of pasture dieback has improved. Limited regional variation was detected in the suite of pathogens isolated from material collected in Queensland surveys. Microbiome research demonstrates very similar microbiomes across single point sampled symptomatic and non-symptomatic plants and that there is no clear, bacterial or fungal population strongly associated with symptomatic plants that indicates a single causal microbial pathogen for dieback. Virology research conducted during the project indicate a range of viruses present in both symptomatic and non-symptomatic plants. Some of these are novel i.e. have not been recorded previously. Others represent new host or geographic records for known viruses. Grass velariviruses warrant further investigation as it is possible these viruses play a role in pasture dieback. Research into the pasture mealybug provides evidence of direct and persistent impact on the productivity and survival of Bisset creeping blue grass. However, as the precise mechanism of plant death was not investigated there is a possibility that the impact, and subsequent plant death, could be the result of factors in addition to direct feeding by pasture mealybug e.g. a disease vectored by mealybug. Gaining an understanding of the mechanism is important in predicting the medium-long term viability of the infected pasture and the selection of appropriate management tactics.

The outcomes of this project have improved the knowledge of potential causal agents including the impact these can have on pasture productivity and will guide future research into effective management solutions to restore pasture productivity and business profitability.

Executive summary

Background

Pasture dieback is a complex condition likely involving a range of pre-disposing biotic and abiotic factors. The role and impact of these factors are poorly understood. This project aimed to clarify the range of pathogenic organisms associated with pasture dieback across multiple regions in Queensland, as well as northern New South Wales (NSW), to assess for pathogenicity.

Due to the complexities and likely range of pre-disposing biotic and abiotic factors of this condition, this project investigated multiple potential causal agents including fungi, bacteria, viruses and insects. To our knowledge no other project has examined this range of potential causal agents for pasture dieback at one time, in one project. This project aimed to identify causal agents of pasture dieback and the impact of these agents on pasture productivity and provide guidance for future research into effective management solutions to restore pasture productivity and business profitability.

Objectives

The objectives of this project are:

1. Improved knowledge of the potential causal agents or co-factors of pasture dieback using pathogenicity testing.
2. Gain knowledge of pathogenic organisms present in the pasture affected by dieback from geographical areas where plant and soil samples have not been previously collected.

Knowledge of multiple potential causal agents or co-factors of pasture dieback has improved through the analysis of plant and soil samples from affected and unaffected areas, and the pathogenicity testing of multiple fungi, viruses and the pasture mealybug.

Multiple extensive field surveys across pasture dieback affected regions in Queensland were undertaken. These field surveys provided samples for the pathogenicity testing of potential pathogenic organisms. Results to date show that there are limited differences in the suite of pathogens (viruses, bacteria, fungi) found in association with pasture dieback in different regions of Queensland. Observations during surveys indicate an association of pasture dieback with the presence of pasture mealybug.

All project objectives have been met.

Methodology

This project was conducted by undertaking three activities.

1. Field sampling to collect additional plant and soil samples for analysis.
    a. Sampling occurred mainly in southern Queensland as the central and northern Queensland areas were sampled just prior to the project starting.
2. The analysis of samples collected just prior to the project starting, and during the project.
    a. Plant and soil samples were analysed for multiple pathogen organisms including fungi, viruses and insects. The microbiome of plants and rhizosphere was also analysed.
3. Pathogenicity testing of specific pathogenic organisms determined from the analysis of field collected samples.
    a. Pathogenicity testing of two fungi and the pasture mealybug were conducted during the project.

Results/key findings

- The knowledge of multiple potential causal agents or co-factors of pasture dieback has improved through the analysis of plant and soil samples from affected and unaffected areas, and the pathogenicity testing of multiple fungi and the pasture mealybug.
- Multiple field surveys across pasture dieback affected regions in Queensland (southern, central and northern) and subsequent sample analysis indicate it is unlikely that different casual agents of pasture dieback occur in different regions of Queensland
- No evidence was found for a link between fungal pathogens and pasture dieback. However, some known fungal pathogens do occur in conjunction with pasture dieback e.g. buffel grass blight (*Pyricularia grisea)*.
- Microbiome research demonstrates very similar microbiomes across single point sampled symptomatic and non-symptomatic plants and that there was not a clear, bacterial or fungal population strongly associated with symptomatic plants to indicate a possible single causal agent for dieback.
- Virology research conducted during the project indicate a range of viruses present in both symptomatic and non-symptomatic plants. Some of these are novel i.e. have not been recorded previously. Others represent new host or geographic records for known viruses. Grass velariviruses warrant further investigation as it remains possible that these viruses play a role in pasture dieback.
- Analysis of soil chemistry and nutrient levels, and investigation of ground pearl and nematodes, do not provide clear evidence of a causal link with pasture dieback.
- Replicated field research demonstrated that uncontrolled, high-density infestations of the pasture mealybug can cause the rapid onset of dieback symptoms, decline in plant growth and plant death. Control of pasture mealybug infestations at early onset of symptoms resulted in recovery of pasture productivity. These results apply only to Bisset creeping blue grass, it is unclear if these can be reliably extrapolated to other species/varieties. Due to the precise mechanism of plant death not being investigated, there is a possibility that the impact, and subsequent plant death, could be the result of factors in addition to direct feeding by pasture mealybug e.g. a disease vectored by mealybug.

Benefits to industry

The outcomes of this project provide empirical evidence of the role of pasture mealybug on one variety of pasture and indicate no evidence of a single fungal, bacterial or viral pathogen associated with pasture dieback. Current project outcomes will guide future research into effective management solutions to restore pasture productivity and business profitability.

Future research and recommendations

Despite the significant research effort and improvement in knowledge of pathogenic organisms associated with pasture dieback, further research is required to overcome the limitations encountered and to answer new questions that emerged during the project. These include:

1. New field-based sampling experiments to track impacts of pasture dieback over time.

Building on knowledge already generated from selected sites, new experiments are needed where repeated plant and soil samples are collected from set locations to determine changes in pathogenic (and beneficial) organisms and the plant and soil microbiome over time (1 – 2 years).

2. Investigation of the mechanism/s of pasture dieback, with focus on pasture mealybug as the primary causal agent.

While there is evidence of direct and persistent impact of pasture mealybug infestations on the productivity and survival of Bisset creeping bluegrass (i.e. pasture mealybugs can cause pasture dieback), it cannot be concluded that mealybug alone are producing the effects observed. A critical need is to investigate the potential involvement of pathogens transmitted by the pasture mealybug, including viruses and other micro-organisms. Also, further research is needed to fully explore the range of pathogens present, especially viruses, and continue through to pathogenicity testing.

3. Undertake wider screening of sown/improved and natives grass species to the pasture mealybug.

Due to the association of pasture mealybugs and pasture dieback on multiple grass species, research is needed to screen a broad range of introduced (and potentially native) pasture species. Attention to the suitability of the different grasses for pasture mealybug population establishment, build up and persistence is as important as the relative impact of mealybug. Understanding the likely population dynamics of pasture mealybugs in the different pasture grasses will make a valuable contribution to management of mealybug.

4. Undertake additional analysis of soil sample data.

Additional analysis is needed to include pasture mealybug as a factor (as done with diversity measures) in nematode and nutrient analyses. While it is unlikely there is an association or correlation between mealybugs, nematodes and soil nutrient levels, analysis is required to complete the understanding of the relationship between these factors. Further multivariate analysis combining the ground pearl, nematode, soil nutrient, alpha diversity and eukaryote data is also required.

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1. Background

Pasture dieback is a condition causing premature death of tropical and sub-tropical grass pastures across Queensland and New South Wales. Sown grass pasture species are mainly affected by pasture dieback in southern, central and northern districts of eastern Queensland. Pasture productivity is severely affected by pasture dieback and cattle do not graze these areas resulting in beef production losses. Surveys and discussions with graziers indicate pasture dieback can affect properties for multiple years. Economic analysis undertaken in the Meat and Livestock Australia (MLA) project B.PAS.0511 (Buck et. al., 2022) indicated that a short-term pasture dieback scenario (stocking rate reduction for 4 years) reduced the profitability of an average sized central Queensland property by $66,320, compared to a reduction in profitability of $181, 701 in a long-term pasture dieback scenario (stocking rate reduction for 8 years). Due to the magnitude of these economic losses, graziers are anxious to understand the cause(s) of pasture dieback and implement management solutions that are cost-effective to restore pasture productivity.

Pasture dieback is a complex condition likely involving a range of pre-disposing biotic and abiotic factors, and the role and impact of these factors are poorly understood. This project aimed to clarify the range of pathogenic organisms associated with pasture dieback across multiple regions in Queensland, as well as northern New South Wales (NSW), to assess for pathogenicity.

This project expanded the identifications already conducted on existing samples collected by the Department of Agriculture and Fisheries (DAF) during the first half of 2020 and undertook additional micro-eukaryotic diversity profiling of these samples. Likely causal agents or co-factors from this and other analysis were tested for pathogenicity in controlled (e.g. glasshouse) and field environments. Experienced pasture agronomists and scientists in fungal plant pathology, entomology, virology and bacteriology conducted further field inspections comprising surveys and sample collection in geographical areas where samples have not previously been collected.

Due to the complexities and likely range of pre-disposing biotic and abiotic factors of this condition, this project investigated multiple potential causal agents including fungi, bacteria, viruses and insects. To our knowledge no other project has examined this range of potential causal agents at one time in one project. The outcomes of this project will improve the knowledge of potential causal agents including the impact these can have on pasture productivity, and guide future research into effective management solutions to restore pasture productivity and business profitability.

2. Objectives

The objectives of this project were:

1. Improved knowledge of the potential causal agents or co-factors of pasture dieback using pathogenicity testing.
2. Gain knowledge of pathogenic organisms present in the pasture affected by dieback from geographical areas where plant and soil samples have not been previously collected.

All project objectives have been met.

Knowledge of multiple potential causal agents or co-factors of pasture dieback has improved through the analysis of plant and soil samples from affected and unaffected areas, and the pathogenicity testing of multiple fungi and viruses and the pasture mealybug.

Multiple extensive field surveys across pasture dieback affected regions in Queensland (southern, central and Northern) were undertaken. Despite plans to do so, no field surveys were undertaken in New South Wales due to the impact of the Covid-19 pandemic (staff travel restrictions, work from

home orders, and closed state boarder for extended periods) during the project period. Field surveys provided samples for the pathogenicity testing of potential pathogenic organisms. Results to date indicate it is unlikely that there are different casual agents of pasture dieback in different regions of Queensland i.e. it is likely grasses exhibiting pasture dieback symptoms across Queensland are impacted by the same (or similar) pathogenic organism(s).

3. Methodology

This project was conducted by undertaking three activities.

1. Field sampling to collect additional plant and soil samples
2. The analysis of plant and soil samples collected just prior to the project starting, and during the project
3. Pathogenicity testing of specific pathogenic organisms determined from the analysis of field collected samples

3.1 Field sampling

DAF project staff conducted field sampling trips across central and northern Queensland between February and July 2020. These were conducted just prior to the project commencing with MLA and were undertaken due to the presence of suitable pasture conditions and the availability of staff time. The first trip was in March 2020 in central Queensland. This trip was one of the larger trips conducted due to the number of properties affected by dieback at that time. Diagnostic scientific staff gained experience and a better understanding of pasture dieback expression across a range of pasture situations. Also, the trip enabled the fine tuning of sampling methodology for future trips. Subsequent sampling trips were conducted during the project including trips to a small number of individual sites. A summary of these trips is outlined in Table 1. A collection of photos from these trips are shown in Figs. 1 – 5.

Table 1. Summary details of field sampling trips before and during the project.

Region	Nearest Town	Number of properties/locations sampled	Number of sites sampled	Date
Central Qld	Bajool	2	3	March 2020
	Thangool	2	3	March 2020
	Biloela	1	5	March 2020
	Banana	3	9	March 2020
	Rannes	1	1	Feb 2020
	Dululu	1	1	Feb 2020
	Moura	2	6	March 2020
	Rolleston	1	2	March 2020
	Anakie	1	4	March 2020
	Emerald	1	1	October 2020
	Comet	2	4	March-April 2020
	Moranbah	1	1	June 2020

Region	Nearest Town	Number of properties/locations sampled	Number of sites sampled	Date
Southern Qld	Coulson	1	4	March 2020
	Gayndah	1	4	April 2020
	Kalbar	3	6	May 2020
	Amamoor	1	1	April 2021
	Mooloo	1	2	April 2021
	Calico creek	1	1	April 2021
	Kaimkillenbun	1	2	March 2021
	Glastonbury	1	2	April 2021
	Owanyilla	1	1	April 2021
Region	Nearest Town	Number of properties/locations sampled	Number of sites sampled	Date
Northern Qld	Mt Garnet	1	2	June 2020
	Ingham	1	2	June 2020
	Ravenshoe	1	2	July 2020
	Jaggan	1	2	July 2020
	Julatten	1	2	August 2020
	Malanda	1	2	August 2020
Total		35	75	

Figure 1. Using hand lens to inspect leaves for pathogenic organisms.

Figure 2. Surveying a pasture affected by pasture dieback.

Figure 3. Undertaking pasture surveying and sampling by mobile phone spotlight.

Figure 4. Inspecting and sampling a pasture affected by dieback.

Figure 5. Discussions, surveying and sampling in the paddock.

To ensure robust field sampling methodologies, protocols were designed by project staff across multiple disciplines. This included project staff knowledge of field sampling procedures for pathogenic organisms (i.e. DAF project staff experience), inputs from DAF staff with knowledge of pastures and sampling protocols (i.e. experience from other DAF specialists), and from a sampling strategy document collaboratively compiled by project staff and other organisations (New South Wales DPI) including those funded by MLA (University of Queensland, Applied Horticulture Research) to undertake pasture dieback research.

To ensure effective use of the time in the field, all sites were visited prior to ensure pasture dieback was present (as opposed to other stress factors such as moisture or nutrient stress), plan which locations on the property to sample so a paired sampling strategy could be accomplished, and to determine the most time efficient property visit order. Depending on the situation a paired sampling strategy was undertaken which entailed:

- Locating a pasture site with dieback (symptomatic) close to a pasture site without dieback (non-symptomatic) i.e. paired site
- Each site contains the same pasture species, or mix of
- Each site be on a similar or the same soil type
- Each site be sampled at the same time
- The recording of site demographic data including grazing and paddock management
- Taking photos at each recording site.

If obvious pasture dieback symptoms were observed on road-sides, these were also sampled however these made up a low percentage of the total number of sites. Some of the road-side sites were not paired, that is no samples from an unaffected area was collected. At each site multiple samples were collected as outlined in Table 2.

Table 2. Details of plant and soil samples collected at field sampling sites

Type of sample	Plant parts; depth of soil sample	Samples analysed for….
Plant	Leaves	Viruses; microbiome; fungi
	Stems	Viruses; microbiome; fungi
	Roots	Microbiome; fungi
	Rhizosphere soil	Microbiome; micro-eukaryote
Soil	0-10cm	Soil chemistry and nutrients
		Nematodes
		Ground pearls
	10-30cm	Soil chemistry and nutrients
		Nematodes
		Ground pearls

The presence of insects, especially the pasture mealybug in above ground plant parts and leaf litter/thatch, were also recorded at all sites visited. Observations that were recorded at each site are outlined in Table 3.

Table 3. Details of recordings at each site sampled.

Property and pasture demographic information	Location/nearest town	Grazing and paddock management
	GPS location of each site	When dieback was first noticed
	Date of sampling	Soil type
	Owner's details	Weather conditions
	Predominant pasture type	A record of samples collected
Individual site details	Grass species	Presence of insects including pasture mealybug
	Stage of dieback	Leaf disease symptoms
	Dieback symptoms	Sample types, reference numbers

3.2 Analysis of field collected samples

All samples collected were prepared and analysed according to standard scientific protocols for the sample type. Details of these methodologies are outlined in the Results section pertaining to the type of pathogen or soil analysis undertaken.

3.3 Pathogenicity testing of specific pathogenic organisms

Pathogenicity testing of specific organisms occurred throughout the project as these were discovered. Pathogenicity testing occurred with fungi, viruses and pasture mealybug, results of which are outlined in the Results section pertaining to the specific pathogen.

4. Results

4.1 Plant pathology analysis and pathogenicity testing

Plant pathology assessments of grasses affected by pasture dieback in northern Queensland recorded that two pathogenic fungal organisms, *Gaeumannomyces* sp. and *Colletotrichum* sp. were commonly present on two grasses, Digit and Rhodes grass. As these fungi are known to be pathogenic on pasture grasses, pathogenicity testing under glasshouse conditions was undertaken to determine if these can cause pasture dieback symptoms i.e. leaf discolouration and plant death.

4.1.1 Fungal inoculation methodology

4.1.1.1 Inoculum production

A range of field and glasshouse pathogenicity testing techniques have been successfully used in the production of *Gaeumannomyces* inoculum (Singleton *et al.* 1992). Two methods were selected and assessed against isolates of both *G. graminicola* and *Colletotrichum* sp. for glasshouse experiments conducted at the Department of Agriculture and Fisheries (DAF), Mareeba Research Facility.

1. Steel-cut organic oats (Red Tractor Foods) – 500g were soaked overnight in sterile distilled water, and excess water decanted the following day. The oats (100g) were placed into 500ml volumetric flasks and sealed with cottonwool bungs covered with aluminium alfoil before being autoclaved 3 times at 121°C to ensure sterility.

2. Sand–cornmeal-water medium. Dry sand (500g) and 15g of coarse polenta (Siena) was mixed with 65 mL of sterile distilled water. The same weight and process as with method 1 was followed.

Cultures of *G. graminicola* and *Colletotrichum* sp. grown on malt extract agar (MEA) were used to inoculate the two different media. Four (5mm) plugs were cut from the actively growing margin of both cultures and placed on the surface of the media. Flasks were kept at 25°C and shaken on a weekly basis for five weeks to evenly distribute the fungus throughout the flask.

4.1.1.2 Glasshouse experiment 1

Soil was collected from a local housing development and sieved to remove rocks and large clods before being moistened with water containing water crystals to allow water absorption.

Moistened paper towel was placed over the drainage holes before adding 2 cm of coarse sand at the bottom of 200mm diameter pots, then filled with soil to within 5 cm of the top. Each pot was covered with aluminium foil for seven days to allow the germination of weeds which were removed before applying the inoculum and sowing with pasture seed. Inoculum (oats) of each isolate (5g) was spread evenly across the soil surface then covered with 1 cm of soil before sprinkling seed of Digit grass (*Digitaria eriantha*) cv. Premier and Rhodes grass (*Chloris gayana)* cv Epica. The seed was again covered with 1 cm of soil then watered to allow germination. The experiment consisted of five replicates plus two control pots for each pasture species, 24 pots in total.

Assessments were conducted visually, microscopically and through fungal isolations at two and four months (post inoculation). Confirmation of *G. graminicola* was determined by the presence of hyphopodia and not the presence of fungal hyphae as other fungal organisms have similar characteristics.

4.1.1.3 Glasshouse experiment 2

Soil was again sourced from the same location as was used in Experiment 1. In addition, the soil was autoclaved (121°C) three times to minimise any naturally occurring fungal organisms or insects that may affect the experiment.

The setup for Experiment 2 was identical to that of Experiment 1, but with the addition of Buffel grass (*Cenchris ciliaris)* cv. Gayndah, as this is one of the most dominant species affected by pasture dieback. The only isolate used in Experiment 2 was *G. graminicola* as *Colletotrichum* sp. was not recovered from any plants in Experiment 1. The number of inoculated pots was increased from five to seven, whilst the number of control pots remained at two, resulting in a total of 27 pots. The experiment was initiated in April 2021 and situated in the pathology glasshouse at the Mareeba DAF Research Facility. Assessments were conducted at two and seven months (post inoculation) by visual, microscopic inspection and fungal isolations (where appropriate). As with experiment one, the presence of *G. graminicola* was based on the presence of hyphopodia.

4.1.2 Sample assessment and fungal identification methodology

Samples collected during surveillance activities, in most instances, were divided into two portions and assessed at two separate laboratories (Ecosciences Precinct, Dutton Park Brisbane and Mareeba). This allowed for comparison of fungal organisms recovered. Samples were assessed using a dissecting microscope and observations noted on the presence of fungal organisms. Material was subsequently washed to remove soil particles before surface sterilizing in 1% sodium hypochlorite for 1-2 minutes, then blotted dry. Small sections (1-2mm of discoloured root or stem material) were placed onto malt extract agar (MEA), incubated at 25°C and assessed for evidence of

fungal growth. Cultures were placed under black-light to induce the development of sporing structures, then identified microscopically to genera level. Where appropriate, single spore cultures were prepared and sent to Ecosciences Precinct for molecular identification to species level.

4.1.3 Fungal inoculation results

4.1.3.1 Inoculum production

After one week of incubation, the growth of *G. graminicola* and *Colletotrichum* sp. established well on both the oats and sand-cornmeal media. However, the weekly shaking of the flasks had a negative impact and resulted in limited to no growth of *G. graminicola* and *Colletotrichum* sp. after five weeks on the sand-cornmeal media. The sand-cornmeal media was discarded as a result, leaving the oats as the only source of inoculum used for both glasshouse experiments. Inoculated oats were removed from the flasks and airdried on blotting paper for 3 days before being crushed into small fragments (1-3mm).

4.1.3.2 Glasshouse experiment 1

Symptoms of blackening were observed (one month, post inoculation) at the crown level of Digit grass plants inoculated with *G. graminicola,* but symptoms were not observed on the Rhodes grass. Symptomatic plants were removed together with asymptomatic control plants and assessed microscopically. Hyphopodia (plant attachment structures of the fungus) were observed at the base of symptomatic plants only and isolations were successful in the recovery of the organism. Isolates were sent to ESP for verification and *G. graminicola* (BRIP 71996) was confirmed. Microscopic assessments and isolations conducted from the control plants did not recover *G. graminicola*.

Two months post inoculation and all pots were visually inspected, prior to the plants being cut back. Symptomatic plants were removed from the pots and assessed microscopically, and isolations conducted. Control plants were also assessed using the same method. All treatments exhibited some evidence of leaf chlorosis and death of old leaves. Basal stem browning was observed in three of the five replicates of Digit grass inoculated with *Colletotrichum* sp., but similar symptoms were not observed in the Rhodes grass inoculated pots. Isolations were not successful in the recovery of *Colletotrichum* sp., indicating the organism was not pathogenic.

Four of the five replicates of both Rhodes and Digit grass inoculated with *G graminicola,* had evidence of stem browning. Lobed hyphopodia were consistently observed (microscopically) at the base of plants and isolations conducted from this region as well as roots were successful in the recovery of *G. graminicola.* The recovery of the organisms was more consistent from the stem portions than the roots. No fungal organisms were recovered from any of the control plants.

The second assessment was conducted at four months post inoculation (January 2021), and all pots were again visually inspected, prior to being cut back. Symptomatic plants were removed, then washed to remove soil, prior to microscopic examination and fungal isolations. Plants from the control pots were also assessed using the same method. Regardless of the inoculation treatment or the host species, most plants exhibited varying levels of leaf chlorosis, and death of old leaves. In addition, basal stem browning and complete death of plants and tillers (where present) was also observed. Similar symptoms were also observed in one of the Digit grass control pots after the experiment was moved to an area in the glasshouse with automatic irrigation. On microscopic examination, it was evident that plants in some of the inoculated pots had produced perithecia, the sexual state of *G. graminicola*. In this phase, spores are ejected from the mature perithecia

(triggered by rain), but in this instance initiated by the overhead irrigation. The ejection of these spores has been responsible for the unfortunate infection of one of the control pots.

Samples were taken from each of the inoculation treatments and the pasture species. Visual assessments were also conducted, noting the presence or absence of mealybugs in individual pots. In all but one instance, the microscopy assessment for *G. graminicola* was backed up with a positive recovery through isolations (Table 4). The anomaly was that of control pot 1 of Digit grass, whereby typical symptoms were observed microscopically, however isolations were not successful in the recovery of the organism. It is hypothesised that this was due to the low incidence of the organism in comparison to the inoculated pots.

Table 4. Microscopy, isolation results and visual assessments conducted at the second assessment.

Digit grass (cv. Premier)	***G. graminicola***			***Colletotrichum* sp.**		
	Microscopy	Isolation	Mealybugs (Visual)	Microscopy	Isolation	Mealybugs (Visual)
Rep 1	✓	✓	✘	✘	✘	✓
Rep 2	✓	✓	✘	✘	✘	✓
Rep 3	Sample not taken		✓	✘	✘	✓
Rep 4	✓	✓	✓	✘	✘	✓
Rep 5	Sample not taken		✓	✘	✘	✓
Control 1	✓	✘	✘	✘	✘	✘
Control 2	Sample not taken		✘	✘	✘	✘
Rhodes grass (cv. Epica)	***G. graminicola***			***Colletotrichum* sp.**		
	Microscopy	Isolation	Mealybugs (Visual)	Microscopy	Isolation	Mealybugs (Visual)
Rep 1	✓	✓	✓	✘	✘	✓
Rep 2	✓	✓	✘	✘	✘	✓
Rep 3	Sample not taken		✘	✘	✘	✓
Rep 4	Sample not taken		✓	✘	✘	✘
Rep 5	✓	✓	✓	✘	✘	✓
Control 1	✘	✘	✓	✘	✘	✓
Control 2	✘	✘	✓	✘	✘	✓

✓ Positive assessment ✘ Negative assessment

Low levels of mealybugs were evident in most pots and numbers increased prior to the experiment being terminated at the end of February 2021. Samples were collected and sent to Biosecurity Queensland for identification and were confirmed as *Heliococcus summervillei,* the paspalum mealybug (Brooks, 1978) which has been associated with pasture dieback since the early 1920's.

4.1.3.3 Glasshouse experiment 2

The germination rate of the Digit and Rhodes grass seed was consistent, however the seed of the Buffel grass (cv. Gayndah) had to be resown twice due to the low germination rate. Therefore, the growth of the Buffel grass was behind that of the other grasses by about two weeks.

Whole plants were removed from the pots at two (June 2021) and seven months post inoculation (November 2021), examined microscopically and fungal isolations conducted where necessary.

Based on the microscopic examination, *G. graminicola* was observed in 5 of the 7 replicates of Rhodes grass, and 1 of the 7 replicates of Digit grass. At this assessment, there was no evidence of

the fungus on any of the Buffel grass replicates. Isolation results were similar to the microscopic observations in the Rhodes grass, with only one replicate (6) not confirmed. However, there was a higher recovery in both the Digit and Buffel grass as shown in Table 5. Even though hyphopodia were not seen, similar fungal hyphae were observed in 6 of the 7 replicates of both the Digit and Buffel grass pots. There was no evidence of mealybugs at the first assessment, indicating the pre-sterilization process of the soil was successful.

Table 5. Comparison of microscopic assessment and the recovery of *G. graminicola* from fungal isolations at two months post inoculation.

Replicate	Digit grass cv. Premier		Rhodes grass cv. Epica		Buffel grass cv. Gayndah	
	Microscopy	Isolations	Microscopy	Isolations	Microscopy	Isolations
1	Negative	Positive	Negative	Negative	Negative	Negative
2	Negative	Positive	Positive	Positive	Negative	Negative
3	Positive	Positive	Positive	Positive	Negative	Positive
4	Negative	Positive	Positive	Positive	Negative	Negative
5	Negative	Positive	Negative	Negative	Negative	Positive
6	Negative	Negative	Positive	Negative	Negative	Negative
7	Negative	Negative	Positive	Positive	Negative	Positive
Control 1	Negative	Negative	Negative	Negative	Negative	Negative
Control 2	Negative	Negative	Negative	Negative	Negative	Negative

The second assessment was extended to seven months (post-inoculation) due to the cooler weather conditions experienced, compared to the timing of Experiment 1. Only visual and microscopic assessments were conducted. The number of replicates of all of grass species increased over time (Table 6), however, other than obvious death of leaf sheaths and some leaf chlorosis and necrosis, plant death was not observed as with Experiment 1.

Table 6. Visual observations for *G. graminicola* at seven months post inoculation

Replicate	Digit grass cv. Premier	Rhodes grass cv. Epica	Buffel grass cv. Gayndah
1	Negative	Positive	Positive
2	Positive	Positive	Positive
3	Positive	Positive	Positive
4	Negative	Positive	Positive
5	Positive	Positive	Positive
6	Positive	Positive	Positive
7	Positive	Positive	Positive
Control 1	Negative	Negative	Negative
Control 2	Negative	Negative	Negative

4.1.4 Field sample assessment and identification results

A total of 18 samples were received for fungal diagnostics from the Atherton Tablelands (9), in north Queensland and Boonah (7) and Dalby (2) in South-East Queensland. Pasture species consisted of Kikuyu (*Pennisetum clandestinum - 4*), Setaria (*Setaria sphacelata - 4*), Rhodes grass (*C. gayana – 4),* Buffel grass (*C. ciliaris - 3*), and individual samples of Digit grass (*Digitaria eriantha*), Broadleaf carpet grass (*Axonopus compressus)* and a sample consisting of various species. The latter two samples were received from residential properties.

A range of fungal organisms were identified and are listed in Table 7, however not all were identified by molecular sequencing due to time and money constraints. Those identified by molecular

sequencing have a Queensland herbarium number associated with them (BRIP) and cultures have been lodged and stored with the herbarium. As a result, additional species of both *Gaeumannomyces* (*G. californicus*) and Fusarium (*F. caatingaense*) were identified, however, there is limited information available in the literature on these fungi. The only known report of *G. californicus* is on buffalo turf (*Stenotaphrum secundatum*) in California (Hernández-Restrepo et al. 2016), whereas *F. caatingaense* forms part of the *Fusarium incarnatum-equiseti* species complex and has been reported as an entomopathogen (Santos *et al*. 2019). Of the other identifications, *Pyricularia grisea*, *Curvularia* sp., *Nigrospora* sp. and *Rhizoctonia* sp. are well known as common grass or turf pathogens. Of the samples split between Mareeba and the Ecosciences Precinct laboratories, a similar range of fungal organisms were recovered.

Table 7. List of fungal organisms identified microscopically and by molecular sequencing.

Host	Symptoms	Location	Identification
Broadleaf carpet grass (*Axonopus compressus)*	Dieback	Mareeba#	*Curvularia* sp. *Rhizoctonia* sp.
Buffel grass (*C. ciliaris*)	Dieback	Dalby^	*Epicoccum* sp. *Fusarium* spp.
Buffel grass (*C. ciliaris*)	Dieback	Dalby^	*Curvularia* sp. *Fusarium* spp. *Nigrospora* sp.
Buffel grass (*Cenchrus ciliaris*) cv. Gayndah	Dieback	Boonah^	*Fusarium* sp.
Digit (*D. eriantha*)	Dieback	Mareeba glasshouse	*G. graminicola* (BRIP 71996) *
Kikuyu (*P. clandestinum*)	Dieback	Ravenshoe	*G. graminicola* (BRIP 71565a) * *G. californicus* (BRIP 71565b) *
Kikuyu (*P. clandestinum*)	Healthy	Ravenshoe	*G. graminicola* (BRIP 71566a) * *G. californicus* (BRIP 71566b) *
Kikuyu (*P. clandestinum*)	Dieback	Evelyn Central*	*Gaeumannomyces* sp. *Pyricularia grisea*
Kikuyu (*P. clandestinum*)	Leaf spot	Ravenshoe*	*Pyricularia grisea*
Rhodes grass (*C. gayana*)	Dieback	Boonah^	Fusarium sp.
Rhodes grass (*C. gayana*)	Dieback	Boonah^	*Fusarium caatingaense* (BRIP 71992a) * *Gaeumannomyces* sp. (BRIP 71993a) *
Rhodes grass (*C. gayana*) cv. Epica	Dieback	Boonah^	*Fusarium* sp.
Setaria (*S. sphacelata*)	Dieback	Julatten	*G. graminicola* (BRIP 71567) *
Setaria (*S. sphacelata*)	Healthy	Julatten	*Gaeumannomyces* sp.
Setaria (*S. sphacelata*)	Dieback	Boonah^	*Fusarium* sp.
Setaria (*S. sphacelata*)	Dieback	Boonah^	*Fusarium incarnatum-equiseti* species complex (BRIP 71994) *
Various species	Dieback	Atherton#	*Gaeumannomyces* sp.

*BRIP – represents identifications conducted by molecular sequencing

^Samples assessed at ESP (Ecosciences Precinct) and Mareeba

Samples received from residential properties

4.2 Microbiome diagnostic analysis including micro-eukaryotic testing

4.2.1 Diagnostic experiment methodology

4.2.1.1 Microbiome

4.2.1.1.1 Sample collection and receipt

Pasture dieback samples collected on sampling trips attended by DAF's Microbial Ecology Group (MEG) members had details recorded on field collection sheets including the site, sample site GPS location, sample number, grass species, growth stage, plant dieback symptoms, property dieback history and where possible, mealybug and ground pearl status. The samples were placed on ice and either processed in the field or returned to the laboratory. Processing involved examining the collected plant and recording visible dieback symptoms such as reddening or yellowing of leaves and taking a photo of selected affected leaves. The plant was then sub-sampled into 5 mL tubes as leaf (L), root (R) and soil (S) and if processed in the field, frozen on dry ice for transport back to the laboratory. Samples were stored frozen at -20 °C until processing.

From the bio-geographical transect established on site AJ sample site 1, three 30 x 30 cm quadrats were sampled along the transect, which contained dead and live pasture. Quadrat 1 (Q1) was within the dead patch; Quadrat 2 (Q2) spanned the edge of the dead patch and live pasture; and Quadrat 3 (Q3) was within the live pasture. In each quadrat, two categories of samples were collected; i) all aerial plant material which was divided into pasture grass (Grass) or forb (Plant); and living (Aerial) or dead material (Dead Aerial); or ii) all plant roots with associated soil (Root & Soil) for pasture grass and the soil to a depth of 10 cm (Soil). At the time of collection, the soil was mixed well and sub-sampled for microbiome (Soil), nutrient, ground pearl and nematode analyses.

Additional pasture dieback samples, collected by other DAF staff in the field and sent to the MEG laboratory, Ecosciences Precinct, were examined and processed like those collected on sampling trips. Any visible symptoms such as reddening or yellowing of leaves were noted and a photo of affected leaves taken. The plant material was sub-sampled into 5 mL tubes as leaf (L), root (R) and soil (S) and stored at 20 °C until processing.

Each sample was logged into an Excel spreadsheet and given a unique identifier utilising the location code and site number, the sample type and the number allocated in the field. For example, 'DB-A1-L-01' identifies a sample from the Pasture Dieback project (DB), site 'A', sample site '1', 'L' leaf, field data sheet sample number '01'. The labelled sub-samples were then stored at 20 °C until processed. A number of mealybug samples were collected from grasses, and ground pearls from soils at sample sites. These were either immediately frozen or placed into ethanol and stored frozen at -20°C until processing. Scanned copies of the field collection sheets were stored on the DAF server. A complete listing of the samples collected are contained within section 9.1.1 as a separately attached spreadsheet, *Master_Pasture_Dieback_Diagnostic_Sample_Data.*

4.2.1.1.2 Cryo-grinding

To obtain a representative sample of the collected leaf, root or soil material, each sample was ground in a mortar and pestle under liquid nitrogen to ensure the sample remained frozen. Once ground to a homogeneous fine powder, it was transferred, whilst still frozen, into a new 5 mL tube labelled with its unique sample ID, the date, and a laboratory reference number for tracking purposes. The ground samples were stored at -20 °C until genomic DNA (gDNA) extraction was undertaken. The full cryo-grinding procedure is detailed in section 9.1.2.

4.2.1.1.3 Genomic DNA extraction

Genomic DNA (gDNA) extractions were undertaken from:

- leaf and root samples using the DNeasy Plant Pro kit (Qiagen, catalogue # 69206) with 0.1 g of the cryo-ground sample extracted following the manufacturer's instructions,
- soil samples using the DNeasy PowerLyzer PowerSoil kit (Qiagen, catalogue #12855-100) using up to 0.25 g of cryo-ground soil following the manufacturer's instructions, and
- individual mealybugs or ground pearls using either the QIAamp DNA Mini Kit (Qiagen, catalogue # 51306) or an adaptation of the method of Phillips and Simon (1995).

Briefly, all DNA extraction methods utilised the addition of beads (zirconia or ballcone) to the sample in a bead beating tube along with a buffer and physical disruption using a Mini beadbeater 16 (Biospec products) for 3 min. The methods utilising a commercial kit involved the binding of the total gDNA within the sample onto the kit's spin column where it was washed and finally eluted off into a new tube and frozen at -20 °C. The adaptation of the published method utilised lysis buffer containing sodium dodecyl sulphate (SDS) and ß-mercaptoethanol and isopropanol precipitation of the gDNA in the sample. The quality and quantity of the extracted gDNA was measured using a Nanodrop Microvolume Spectrophotometer (Thermo Fisher Scientific, USA). The full protocols for the DNA extraction methods are detailed in section 9.1.3.

4.2.1.1.4 PCR Amplicon preparation and sequencing

The extracted gDNA from the samples were used as template in PCR reactions within the:

- MEG laboratory, using primers targeting either the V3/V4 variable region of the 16S rRNA gene (bacteria) or internal transcribed spacer (ITS) region (fungi), and
- laboratory of Dr Paul Dennis (School of Earth and Environmental Sciences, The University of Queensland), using primers targeting the V8-V9 variable region of the 18S rRNA gene (micro-eukaryotes).

Details of primer sets used are listed in Table 8. and detailed methodologies of the PCR reaction components and volumes, and the PCR running conditions, are detailed in section 9.1.4.

The bacterial and fungal PCR amplicons prepared at the MEG laboratory were loaded onto 96 well plates and sent to the external sequencing provider, the Australian Genomic Research Facility (AGRF). The amplicons were barcoded to enable sample differentiation, pooled and sequenced on an Illumina MiSeq Platform to obtain 300 bp paired end reads. The eukaryotic PCR amplicons, prepared at Dr Paul Dennis' laboratory were purified, barcoded and sequenced in-house on an Illumina MiSeq using a MiSeq Reagent Kit v3 (600 cycles; Illumina) using 8 pM libraries spiked with 30% PhiX Control v3 (Illumina) according to the manufacturer's instructions. The detailed protocols used are contained within section 9.1.4.

Table 8. Details of the PCR Primer sets used in PCR reactions, with or without the addition of the Illumina sequence tag required for further sample indexing and library preparation.

Primer name	Target group	Primer sequence (5'-3')	Reference
799F	Bacteria	5' **AACMGGATTAGATACCCKG** 3'	Chelius and Triplett, 2001
1391R	Bacteria	5' **GACGGGCGGTGWGTRCA** 3'	Walker & Pace, 2007
NXT-967F	Bacteria	5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**CAACGCGAAGAACCTTACC** 3'	Sogin et al., 2006
NTX-1391R	Bacteria	5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GACGGGCGGTGWGTRCA**'3	Walker & Pace, 2007
ITS1Ftagged	Fungi	5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**CTTGGTCATTTAGAGGAAGTAA** 3'	Gardes & Bruns, 1993
ITS2Rtagged	Fungi	5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GCTGCGTTCTTCATCGATGC** 3'	White et al., 1990
V8F	Micro-eukaryotes	5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**ATAACAGGTCTGTGATGCCCT** 3'	Bradley et al., 2016
1510R	Micro-eukaryotes	5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**CCTTCYGCAGGTTCACCTAC** 3'	Amaral-Zettler et al., 2009

Illumina sequence tags are underlined, and gene-specific primer sequences are coloured with the first round bacterial PCR primers (**blue**), second round bacterial PCR primers (**green**), fungal ITS primers (**red**) and micro-eukaryotic primers (**purple**).

4.2.1.1.5 Sequence analysis

4.2.1.1.5.1 Bacterial and fungal microbiomes

The sequence data were received from AGRF as 300 bp paired end reads, from four Illumina MiSeq lanes, for both the bacterial 16S rRNA gene (for samples from leaf and root material) and the fungal ITS region (also for samples from leaf and root material). These data sets were initially analysed using the same approach with the sequence reads de-multiplexed, quality filtered, paired and size trimmed (>200 bp in length remaining) to remove primer/barcode sequences using Trimmomatic version 0.36 (Bolger et al., 2014). The paired reads were imported into the Quantitative Insights Into Microbial Ecology 2 (QIIME 2) software pipeline package, either version 2019.10 or 2021.4 (Caporaso et al., 2010, Caporaso et al., 2012) where the DADA2 software (Callahan et al., 2016) was used to model and correct any remaining Illumina sequencing errors. In this way the reads were further quality filtered, the forward and reverse reads merged, unique sequences (sequence variants) grouped, and chimeras removed.

Unique sequences were identified, and the numbers of each unique sequence in samples was determined with a representative sequence (Feature or sequence variant, similar to the Operational Taxonomic Unit determined by previous versions of QIIME) for each of these were selected. A phylogenetic tree was created to relate the Features to one another before the taxonomy (identity) of each Feature was determined. For the bacterial 16S rRNA representative sequences, taxonomy was assigned using a pre-trained Naïve Bayes classifier trained on the SILVA database December 2019 update, version 138 (Yilmaz et al., 2014). For the fungal ITS representative sequences, taxonomy was assigned using the UNITE database version 7, 10.10.2017 update (Nilsson et al., 2019). Following taxonomic classification of bacterial microbial Features classified as plant chloroplasts or mitochondria were removed. Similarly, any fungal microbial Features sequences classified within the kingdom Plantae, were removed.

Analysis focused on microbiome data relating to plant samples (leaf and root material) collected from non-trial sites as the plants samples from the DAF agronomy trials (collection sites R and O) were found to show growth retardation, atypical of pasture dieback. Statistical analysis of microbiome data was also undertaken, on the basis of biological factors such as pasture dieback symptoms (Symptomatic or Non-symptomatic plant samples) and mealybugs (presence or absence of mealybugs within plant samples).

4.2.1.1.5.2 Micro-eukaryotic microbiomes

The micro-eukaryotic 18S rRNA gene sequences were processed by Dr Paul Dennis' group using a modified UPARSE workflow (Edgar, 2013). Briefly, demultiplexing and primer removal were performed, and the sequences were trimmed to (250 bp) forward reads and quality filtered. These sequences were mapped against representative sequences to create an operational taxonomic unit (OTU) table. The OTUs were assigned SILVA 138 (Quast et al., 2012) and PR2 taxonomy (Guillou et al., 2013) in QIIME 2. Variation in the composition of microbial communities between samples was investigated from a taxonomic and phylogenetic perspective.

The detailed bioinformatic methods and analyses undertaken are contained in section 9.1.5.

4.2.1.1.5.3 Microbiome data storage

Copies of the electronic microbiome sequence data have been archived on the DAF server, which is backed up daily and the Department of Environment and Science high performance computing

clusters, Athena and Apollo. Copies of micro-eukaryote sequence data are stored on the University of Queensland servers and high performance computers.

4.2.2 Diagnostic Experiment results

4.2.2.1 Microbiome

Plants possess complex micro-ecosystems harbouring abundant microbial populations. Research into the diversity and structure of the microbiota of plants and soils has dramatically increased in recent years with the identification of some bacteria and fungi as beneficial plant growth-promoting bacteria (PGPB) that have micronutrient (phosphate, zinc) solubilising capabilities and pathogen antagonists. In this project we investigated the epiphytic (surface) and endophytic (internal) microbiomes associated with the phyllosphere (leaves) and rhizosphere (roots and associated soil) of a range of different species of pastures grasses that were either showing signs of pasture dieback (symptomatic) or appeared healthy (non-symptomatic). In total, 60 individual plants, encompassing nine different pasture grass species and consisting of 48 symptomatic and 12 non-symptomatic plants, had their microbiomes sequenced. A trial of extracting gDNA from individual mealybug and ground pearl specimens, preserved by either immediately freezing or placing in ethanol and storing frozen, was undertaken. The bacterial microbiomes associated with three individual mealybugs and three ground pearl samples were analysed.

The microbiomes, specifically the bacterial and fungal populations of plant material (from both leaf and root samples), bacterial populations of mealybug and ground pearls, and micro-eukaryotic of soil, were determined using a culture-independent, sequence-based approach using gDNA purified from samples collected from producer properties and DAF trial sites in central and south-east Queensland. Bioinformatic analyses of the resulting sequence data determined the distinct microbial populations associated with each of the samples and their role, if any, with pasture dieback.

4.2.2.1.1 Sample collection and processing

A total of 405 samples, consisting of 133 sets of three (plant leaf, root and soil) and three sets of two (plant leaf and root), were collected from central, southeast and north Queensland for microbiome analysis. To date a further 36 (sets of three) samples as well as eight samples of preserved mealybug and seven samples of preserved ground pearl have been collected for future microbiome analysis within this project. A total of 133 of the 136 collected plant leaf and root samples and 132 soil samples, have been cryoground to ensure a homogenous range of representative samples for gDNA extraction. One soil sample was lost during the cryogrinding process. For the microbiomes, 94 sample sets were selected as an initial set of phyllosphere (leaf) and rhizosphere consisting of plant root/associated soil (root) samples to be sequenced for bacterial and fungal diversity sequencing and soil sequenced for micro-eukaryote (Table 9).

Extensive metadata were collected alongside the pasture dieback samples including soil nutrients, nematode numbers and ground pearl numbers. Several workshops were held with key project staff including pasture agronomists, the project statistician and bioinformatics experts to define the research questions considered to be the most important and refine which metadata parameters to use for the interrogation of the microbiome datasets. An extensive data cleansing and consolidation of all the sample data was undertaken to enable statistical analysis of the data set to be undertaken and metadata parameters to be refined. The cleansed and consolidated sample data are provided within section 9 and as a separately attached spreadsheet, named *Master_Pasture_Dieback_Diagnostic_Sample_Data.*

To generate the bacterial and archaeal microbial amplicons for the bacterial microbiome sequencing, nested PCR assays were used for all samples to avoid the non-specific amplification of plant-based genetic material (chloroplast or mitochondrial DNA) which previously contaminated the original plant leaf microbial sequence dataset (reported in B.PAS.0509 Milestone Report 2). This approach successfully generated amplicons of the correct size and in sufficient quantity, to enable sequencing on the Illumina MiSeq platform. The amplicons for fungal and eukaryotic microbiomes were generated using a single PCR assay and sequenced on the Illumina MiSeq platform by AGRF and Dr Paul Dennis' laboratory respectively. Details of the various microbiome sequence datasets are summarised in Table 10.

Table 9. Details of the plant species, number of samples and sites selected for microbiome sequencing.

Common name	Species	# of samples	# of sites
Buffel grass	*Cenchrus cilaris*	37	20
Rhodes Grass	*Chloris gayana*	18	5
Bluegrass	*Bothriochloa insculpta*	12	3
Green Panic	*Megathyrsus maximus*	10	8
Sabi grass	*Urochloa mosambicensis*	7	4
Angleton grass	*Dichanthium aristatum*	4	3
Panic	*Panicum coloratum*	4	3
Indian couch	*Bothriochloa pertusa*	1	1
Massai Panic	*Panicum effusum*	1	1

Table 10. Summary of sequence datasets obtained for determining the bacterial microbiome and fungal populations (ITS), of leaf and root sub-samples from the same original plant sample, as well as mealybug and ground pearl microbiome test samples.

Sample type	Microbiome target	Number of samples[B]	Number of sequence reads per sample (Mean ± STDEV)	Total Number of sequence reads and dataset size
Leaf	Bacterial	95	106,176 ± 49,202	10,086,752 reads; 6.07Gb
Root	Bacterial	96	129,547 ± 49,799	12,436,514 reads; 7.49Gb
Leaf[A]	Fungal	95	80,150 ± 18,058	7,614,204 reads; 4.58Gb
Root	Fungal	97	118,646 ± 26,489	11,508,626 reads; 6.93Gb
Mealybug	Bacterial	4	566,005 ± 266,477	2,264,022 reads; 1.36 Gb
Ground Pearl	Bacterial	4	317,997 ± 114,403	1,271,991; 0.77 Gb
Soil	Micro-eukaroyote	130[C]	n/a[D]	n/a

[A]Dataset received and preliminary description included in Milestone Report 2.
[B]Includes negative control (blank) samples.
[C]Including soil samples associated with the 94 leaf and root samples.
[D]n/a – not available

4.2.2.1.2 Bacterial microbiome

The bacterial microbiomes of plant samples (leaf and root) were determined from the amplicon sequence data generated throughout the project. Bioinformatics analysis focused on determining the classification (taxonomy) of the bacterial populations present as well as the diversity of microbial populations occurring on individual plant samples. The bacterial populations associated with groups

of plants e.g. plants which were showing signs of pasture dieback (Symptomatic) and plants which were not showing signs of pasture dieback (Non-symptomatic), were also compared, in order to determine whether there were any highly abundant, dominant types of bacteria associated with pasture dieback symptoms. In addition, samples were grouped according to whether mealybugs were present or absent from the plant material collected, to determine whether this insect contributed to any differences occurring in the diversity of plant-associated bacterial populations.

4.2.2.1.3 Diversity of plant associated bacterial populations

The extent of within-sample microbial diversity, based on four measures of alpha diversity (Faith PD, Shannon entropy, Pielou evenness and Observed Features), was determined for leaf and root bacterial microbiomes, from plants that were either Symptomatic or Non-symptomatic for pasture dieback. The 60 samples of each plant sample type were analysed by Residual Maximum Likelihood (REML) to assess the impact of dieback symptoms on each of these measures. Models included the random effect of *Site* and the fixed effect of *Dieback symptoms* (Non-symptomatic, Symptomatic).

No differences in the bacterial population diversity were evident between samples of non-symptomatic and symptomatic leaf and root material for all four diversity measures (P>0.05; Table 11).

Table 11. The effect of pasture dieback symptoms on microbial diversity, based on four measures of alpha diversity, for bacterial populations of leaf and root material.

Treatment	Faith's PD index	Shannon entropy	Pielou evenness	Observed features
	Leaf Bacteria			
Dieback symptoms	P=0.791	P=0.101	P=0.192	P=0.982
Non-symptomatic	10.2	3.07	0.48	85.91
Symptomatic	9.9	2.58	0.42	85.54
s.e.d.	1.2	0.29	0.04	16.53
	Root Bacteria			
Dieback symptoms	P=0.081	P=0.868	P=0.674	P=0.272
Non-symptomatic	20.5	7.14	0.86	325.70
Symptomatic	18.4	7.10	0.87	296.10
s.e.d.	1.2	0.22	0.02	26.70

The predicted means and standard error of differences (s.e.d.) are shown and significance levels indicated by the P value, with significance at the 5% threshold.

The bacterial populations of the 45 plant (leaf and root) samples where mealybug data were available, i.e. presence (yes) or absence (no) of mealybugs, were not evenly distributed across pasture dieback symptoms and presence/absence of mealybug (Table 12). A Chi-square analysis however, determined a significant relationship between the occurrence of pasture dieback symptoms and presence of mealybugs with 86% of symptomatic samples having mealybug compared with 33% of non-symptomatic samples.

Table 12. Uneven distribution of available diversity records occurring between samples of plant material (leaf and root) which were either Symptomatic or Non-symptomatic for pasture dieback and the presence (yes) or absence (no) of mealybugs.

	Dieback Symptom	
Mealybug	**Non-symptomatic**	**Symptomatic**
no	6 (67%)	5 (14%)
yes	3 (33%)	31 (86%)
Total No. (n)	9	36

Pearson chi-square value of 10.86 with 1 d.f.
Probability level (under null hypothesis) $P < 0.001$

The 45 plant samples (leaf and root) which had mealybug data available were analysed by REML to assess the impact of the presence or absence of dieback symptoms and the occurrence of mealybugs on each of the within-sample microbial diversity (alpha diversity) measures. The models undertaken included the random effect of *Site* and the fixed effects of *Dieback symptoms* (Non-symptomatic, Symptomatic) and *Mealybug* (no, yes). Although the variance component for *Site* was not always significant it was retained in the model. A parsimonious model was achieved by sequentially removing non-significant fixed effects but always retaining the main effect terms.

There was no significant interaction between pasture dieback symptoms and presence of mealybug on the extent of leaf sample bacterial microbial diversity ($P>0.05$; Table 13). Further, whether plants had pasture dieback symptoms or not, or if mealybugs were present or not, did not have a significant effect on leaf microbial diversity.

Similar effects were observed for three of the four alpha diversity measures (Faith PD, Shannon entropy and Observed Features) for root sample bacterial microbial diversity with no interaction or differences due to dieback symptoms or mealybug. In contrast, the Pielou evenness measure was less in the roots of plants non-symptomatic to pasture dieback in the presence of mealybug than in the roots of symptomatic plants when mealybugs were present, and in both symptomatic and non-symptomatic plants without mealybug (0.754 vs 0.876; $P=0.035$; Table 14). The Pielou evenness measure is a measurement of the "evenness" component of diversity (Pielou, 1966), with evenness described as the ratio of the observed diversity to the maximum possible in a collection having the same number of species. In the current study, the ratio was higher in root samples without mealybug and symptomatic samples in the presence of mealybug, suggesting an increased relative abundance, or dominance, of a few bacterial populations within the total bacterial community. While this effect was significant ($P < 0.05$), it did not occur consistently across all of the microbial alpha diversity measures determined, therefore these effects can only be considered to be relatively minor.

Table 13. The effect of pasture dieback symptoms (Symptomatic, Non-symptomatic) and the presence of Mealybugs (yes, no) on microbial diversity, based on four measures of alpha diversity, for bacterial populations of leaf samples.

Treatment	Faith PD	Shannon entropy	Pielou evenness	Observed features
Dieback symptoms (D)	P=0.464	P=0.194	P=0.470	P=0.603
Non-symptomatic	10.1	2.89	0.45	80.1
Symptomatic	8.9	2.38	0.41	70.4
s.e.d.	1.6	0.39	0.06	18.4
Mealybug (M)	P=0.835	P=0.630	P=0.594	P=0.953
no	9.4	2.73	0.45	74.7
yes	9.7	2.54	0.41	75.8
s.e.d.	1.5	0.41	0.06	17.2
D x M	P=0.862	P=0.576	P=0.611	P=0.602

The predicted means and standard error of differences (s.e.d.) are shown and significance levels indicated by the P value, with significance at the 5% threshold.

Table 14. The effect of pasture dieback symptoms (Symptomatic, Non-symptomatic) and the presence of Mealybugs (yes, no) on microbial diversity, based on four measures of alpha diversity, for bacterial populations of root samples.

Treatment	Faith PD	Shannon entropy	Pielou evenness	Observed features
Dieback symptoms (D)	P=0.410	P=0.201	P=0.035[A]	P=0.510
Non-symptomatic	20.0	6.81	0.83	322
Symptomatic	18.5	7.21	0.89	296
s.e.d.	1.7	0.31	0.03	39
Mealybug (M)	P= 0.540	P=0.101	P= 0.030 [A]	P=0.782
no	19.8	7.25	0.88	314
yes	18.7	6.77	0.83	303
s.e.d.	1.7	0.29	0.02	39
D x M	P= 0.602	P=0.063	P=0.035 [A]	P=0.221

The predicted means and standard error of differences (s.e.d.) are shown and significance levels indicated by the P value, with significance at the 5% threshold. [A] Significant effect, $P < 0.05$.

The extent of between-sample microbial diversity (beta-diversity) was also determined for leaf and root bacterial microbiomes including those which were either symptomatic or non-symptomatic for pasture dieback. Principal component analysis (PCA; Joliffe and Cadima, 2016) was used to explore and identify the largest sources of variation occurring between each respective group of bacterial microbiome samples. The dimensionality of the dataset was then reduced using the Sparse Principal Component Analysis (sPCA) method (http://mixomics.org/methods/spca/; Shen and Huang, 2008) and a differential abundance analysis undertaken to determine which microbes were driving, or contributing, to the differences occurring between samples (sPLSDA; sparse Partial Least Squares Discriminant Analysis; Le Cao et al. (2011).

The PCA results indicated that the majority of the observed bacterial populations associated with samples of symptomatic leaf material were very similar to those associated with non-symptomatic leaf samples, as indicated by the relatively low percentage of variation observed (≤ 10%) and the clustering of samples and overlapping ellipses depicted in Fig. 6. Further analysis to reduce the

dimensionality of the dataset also showed few differences in the bacterial populations. Of the differences observed to occur between symptomatic and non-symptomatic leaf material, the bacterial genus *Paenibacillus*, family Erwiniaceae, family Xanthobacteraceae, and the genera *Kineosporia* and *Sphingomonas* were found to contribute to these differences (section 9.2.1; Fig. 38, Table 46).

For the root microbiomes, as indicated by PCA, the majority of the observed bacterial populations associated with root samples collected from plants that were either symptomatic or non-symptomatic for pasture dieback, were also very similar. The percentage of variation captured in the PCA of respective root sample bacterial microbiomes, was again relatively low (< 8%) with the majority of samples clustering together and the ellipses describing the two groups of samples, overlapping (Fig. 7). Further analysis to reduce the dimensionality of the dataset also showed few differences occurring between root-associated bacterial populations. Of the differences observed to occur between symptomatic and non-symptomatic root material, the bacterial family Kineosporiaceae, genus *Streptomyces*, family Comamonadaceae, order Solirubrobacterales, genus *Acinetobacter*, family Enterobacteriaceae, genus *Acinetobacter* were found to contribute to these differences (section 9.2.1; Fig. 39, Table 47).

Figure 6. Principal components analysis (PCA) indicating similar, diverse bacterial populations associated with leaf samples, where samples are represented by a single point and grouped according to pasture dieback symptoms (Non-symptomatic leaf samples (O) and Symptomatic leaf samples (Δ)). PCA shown on the basis of three components (A) Components 1 vs 2 (PCA comp 1-2); and (B) Components 1 vs 3 (PCA comp 1-3).

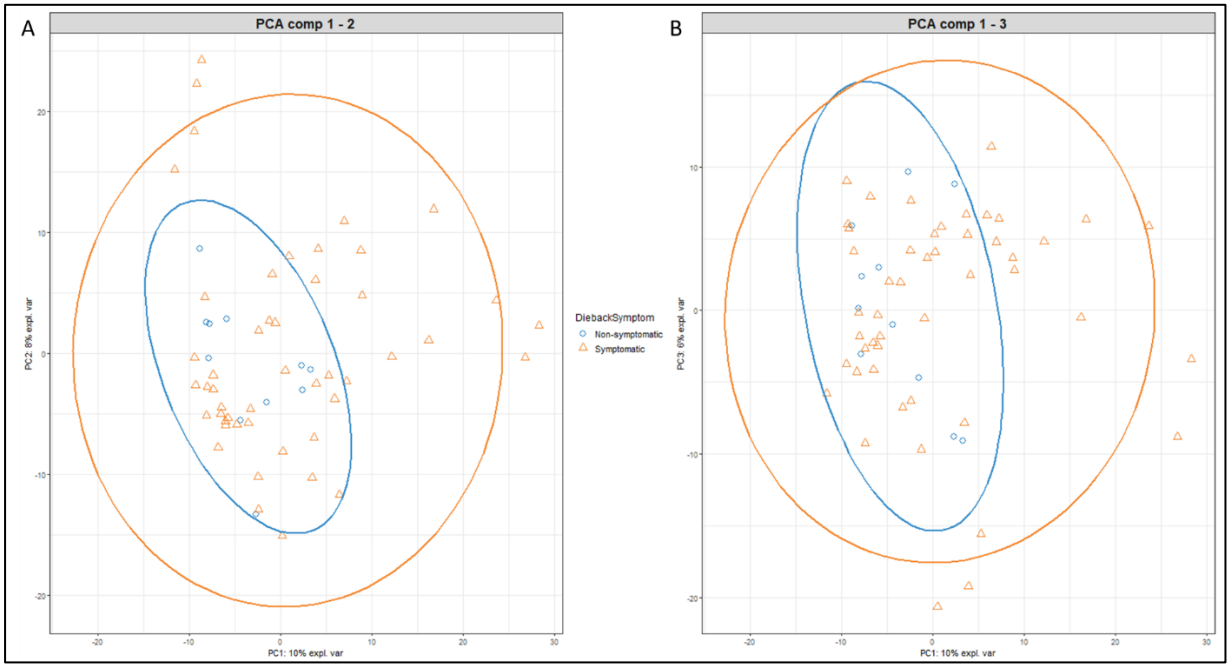
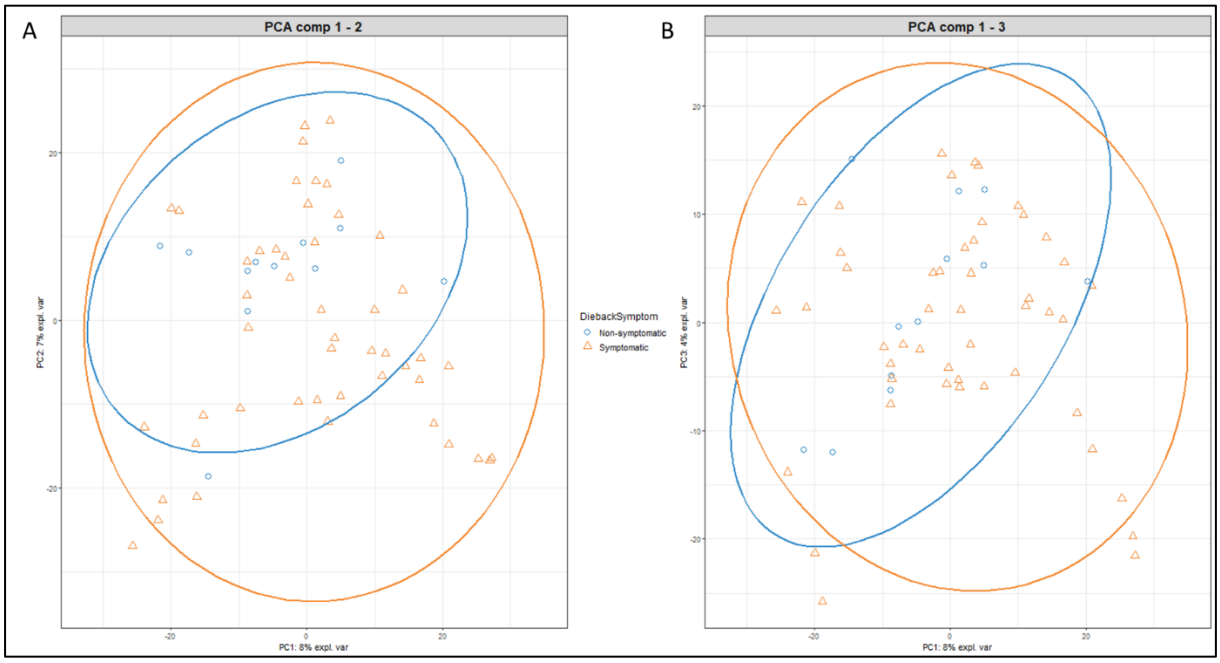


Figure 7. Principal components analysis (PCA) indicating similar, diverse bacterial populations associated with root samples, where samples are represented by a single point and grouped according to pasture dieback symptoms (Non-symptomatic root samples (O) and Symptomatic root samples (Δ)). PCA shown on the basis of three components (A) Components 1 vs 2 (PCA comp 1-2); and (B) Components 1 vs 3 (PCA comp 1-3).



4.2.2.1.3.1 Taxonomy of plant-associated bacterial populations

The classification or taxonomy, of the dominant populations of bacteria associated with leaf samples (phyllosphere) collected from non-trial sites was determined (Fig. 8). Because of the large number of bacterial types identified within the leaf sample dataset (337 bacteria classified to genus level) only the highly abundant, core bacterial populations, i.e. those bacteria types present in > 80% of samples, found to be associated with leaf material from plants showing signs of pasture dieback (Leaf, Symptomatic), and healthy plants (Leaf, Non-symptomatic) are tabulated (Table 15).

Bacterial populations classified within the phyla Proteobacteria and Actinobacteriota were highly abundant in all plant samples and the presence of these are usually reported in plant phyllospheres (Johnston-Monje et al 2021). Specific bacterial populations classified with the genera *Pantoea, Ralstonia*, and *Pseudomonas* were found to dominate the leaves of both symptomatic and non-symptomatic plants. Members of the genus *Pantoea* have been associated with plants either as epiphytes or endophytes with the genera *Pantoea* and *Pseudomonas* recently identified as part the core shoot microbiome across 17 plant species (Johnston-Monje et al. 2021). The genus Pantoea contains both beneficial and pathogenic species with isolates being identified in rice as either beneficial plant growth-promoting (Lu et al 2021) or causal agents in diseases such as stem necrosis and leaf blight (Doni et al., 2021). The genus *Pseudomonas* also contains both pathogenic and beneficial members with the plant pathogenic *Pseudomonas syringae* species complex identified as the causal agent of diseases across hundreds of species of monocots, herbaceous dicots and woody dicots worldwide (Lamichhane et al. 2015). Other Pseudomonas are known as biocontrol agents active against bacterial and fungal phytopathogens, nematodes and different insects (Dimkić et al. 2022). Members of the genus *Ralstonia* are known as major phytopathogens that cause bacterial wilt in many crops (Genin and Denny, 2012). However, of note, the genus *Ralstonia* was also found to be present in the sequencing negative control samples and it is also known as a common

contaminant of DNA extraction kit or PCR reagents, which may lead to it appearing in amplicon sequence datasets (Salter et al 2014). This genus was not present in all samples within the sequence dataset, suggesting it was not necessarily a laboratory-derived contaminant. For this genus, further quantitative PCR assays should be undertaken to validate the presence of *Ralstonia* in the microbiomes of plant samples.

When all the bacterial populations classified to genus level in symptomatic and non-symptomatic leaf microbiomes were compared (Fig. 9; A, B), approximately 43% of bacteria were found to be present in both groups and similarly, there was a high percentage of highly abundant, core bacteria present in both groups (41.7%). This finding supports the taxonomy-independent analysis (e.g. PCA) which also showed that there was little variation occurring in the overall bacterial populations associated with the phyllosphere (leaf) of symptomatic and non-symptomatic plants.

The taxonomy of the dominant populations of bacteria associated with rhizosphere (root/associated soil) samples collected from non-trial sites were also determined (Fig. 10). Overall, more genera of bacteria were identified in the root sample dataset (451 bacteria classified to genus level), than for leaf samples, as would be expected, as soil is known to harbour diverse populations of bacteria (Luchibia et al. 2020). Because of the large number of bacterial types observed within each root sample, the highly abundant, core bacterial populations (bacteria present in > 80% of samples) found to be associated with root material from plants showing signs of pasture dieback (Root, Symptomatic), and healthy plants (Root, Non-symptomatic) were tabulated (Table 16). Bacterial populations classified within the phyla Actinobacteriota and Proteobacteria were highly abundant in all plant root samples. The roots of both symptomatic and non-symptomatic plant samples were dominated by bacterial populations of the genus *Streptomyces.* Other bacteria found to be highly abundant, core bacteria associated with the roots of symptomatic plants included the genus *Amycolatopsis*, an unclassified genus from the order Solirubrobacterales, and the genus *Actinoplanes*. The highly abundant, core bacteria associated with the roots of non-symptomatic plants also included an unclassified genus from the order Solirubrobacterales, the genus *Amycolatopsis* as well as the genus *Rubrobacter*.

When all the root-associated bacterial populations classified to genus level for symptomatic and non-symptomatic plant groups were compared (Fig. 9; C, D), over half (53.4%) of the genus-level classified bacteria were found to be present in both groups and similarly, there was a high percentage of highly abundant, core bacterial genera present in both root sample groups (66.7%). This finding also supports the taxonomic-independent analysis (PCA) which indicated that there were very similar, diverse bacterial populations associated with root samples from both symptomatic and non-symptomatic plants.

Figure 8. Highly abundant bacterial populations associated with Leaf samples, classified to order level taxonomy. Samples are sorted within the stacked bar graph according to the location from which they were collected (e.g. site A), whether the leaf samples were Symptomatic or Non-symptomatic for pasture dieback, and the common name of the plant from which the leaf samples were obtained (e.g. Sabi grass). Bacteria shown had an abundance ≥ third quartile threshold (37 sequences per feature). Taxonomic rank is indicated as d (domain); p (phylum); c (class); and o (order).

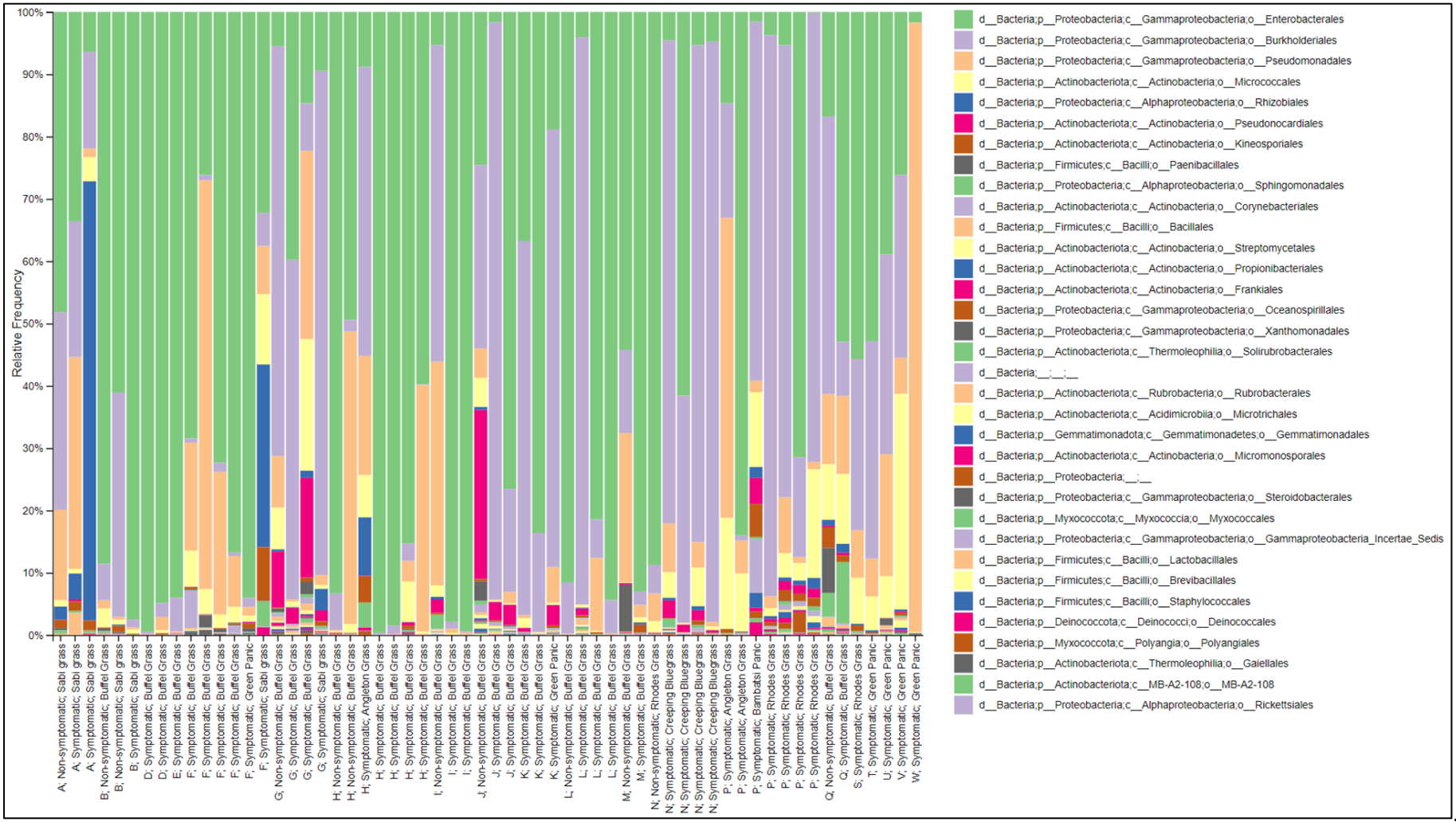


Table 15. Core bacterial populations found to be associated with leaf material from plants showing signs of pasture dieback (Leaf, Symptomatic), and healthy plants (Leaf, Non-symptomatic). The bacterial taxonomy is shown at the lowest level of classification achieved, usually Family or Genus level with the core populations designated as those present in ≥ 80% of the total number of leaf samples analysed (n). The relative abundance for each core bacterial population is expressed as a percentage of the total number of sequences obtained for each leaf sample group.

Leaf, Symptomatic (n = 48)		**Leaf, Non-symptomatic (n = 12)**	
Core bacteria	**Relative abundance (%)**	**Core bacteria**	**Relative abundance (%)**
Family Erwiniaceae; Genus *Pantoea*	58.47	Family Erwiniaceae; Genus *Pantoea*	53.71
Family Burkholderiaceae; Genus *Ralstonia*	18.53	Family Burkholderiaceae; Genus *Ralstonia*	19.66
Family Pseudomonadaceae; Genus *Pseudomonas*	9.22	Family Pseudomonadaceae; Genus *Pseudomonas*	9.95
Family Erwiniaceae; Genus Unclassified	4.65	Family Erwiniaceae; Genus Unclassified	3.40
Family Microbacteriaceae; Genus *Curtobacterium*	1.89	Family Microbacteriaceae; Genus *Curtobacterium*	1.57
Family Kineosporiaceae; Genus *Quadrisphaera*	0.11	Family Paenibacillaceae; Genus *Paenibacillus*	0.92
		Family Beijerinckiaceae; Genus *Methylobacterium-Methylorubrum*	0.26
		Family Kineosporiaceae; Genus *Kineococcus*	0.19
		Order Enterobacterales; Family Unclassified	5.51
		Family Sphingomonadaceae; Genus *Sphingomonas*	0.41
		Family Pseudonocardiaceae; Genus *Amycolatopsis*	0.27

Figure 9. Venn diagrams indicating the numbers of bacterial populations associated with either Leaf material (A and B) or Root material (C and D) that were unique or shared between samples Symptomatic or Non-symptomatic for pasture dieback. Bacterial populations were assigned to genus level taxonomy and core populations were defined as those present in ≥ 80% of samples within each sample group (Symptomatic [n = 48], Non-symptomatic [n = 12]).

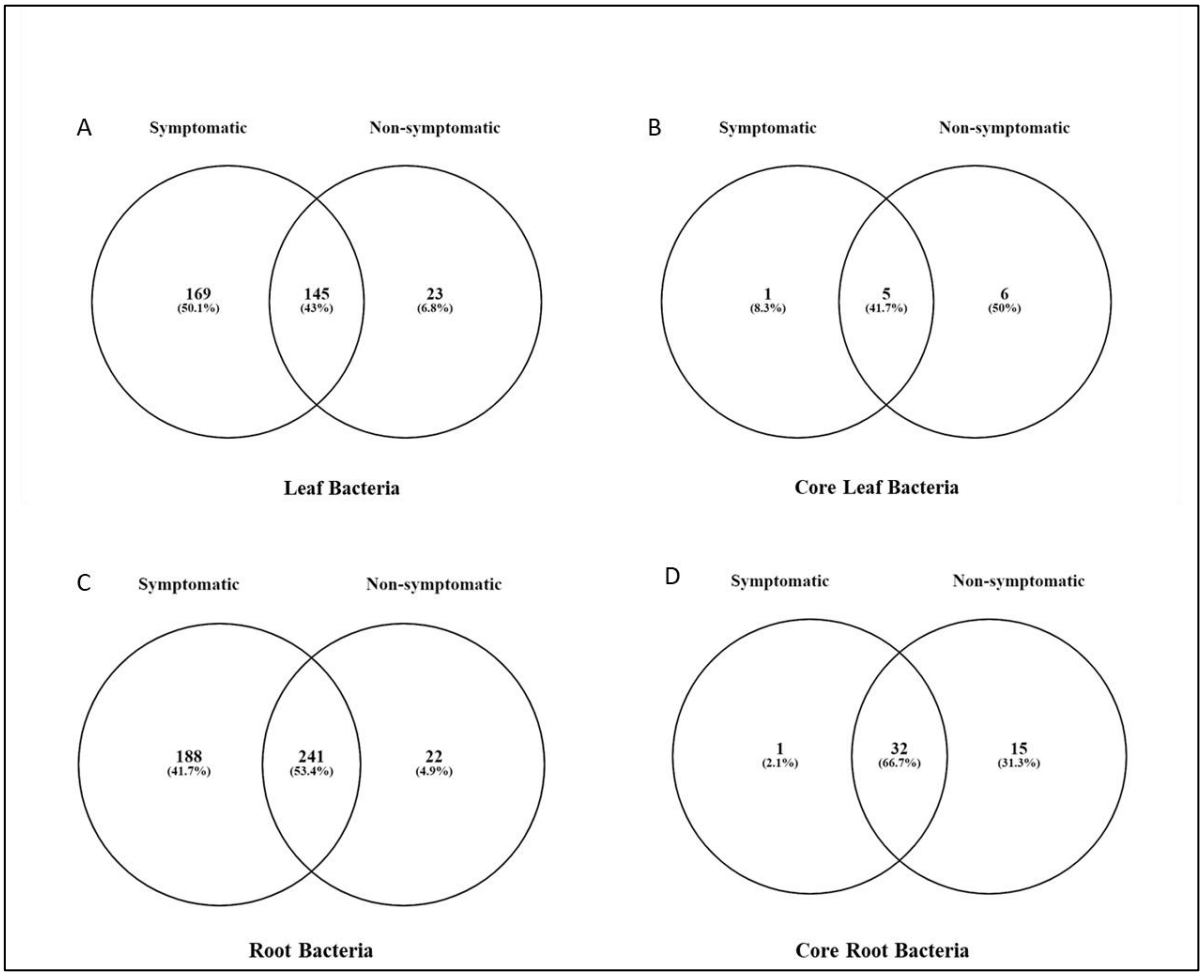


Figure 10. Highly abundant bacterial populations associated with Root samples, classified to order level taxonomy. Samples are sorted within the stacked bar graph according to the location from which they were collected (e.g. site A), whether the plant root samples were Symptomatic or Non-symptomatic for pasture dieback, and the common name of the plant from which the root samples were obtained (e.g. Sabi grass). Bacteria shown had an abundance ≥ third quartile threshold (49 sequences per feature) and the top 24 bacteria are shown. Taxonomic rank is indicated as d (domain); p (phylum); c (class); and o (order)

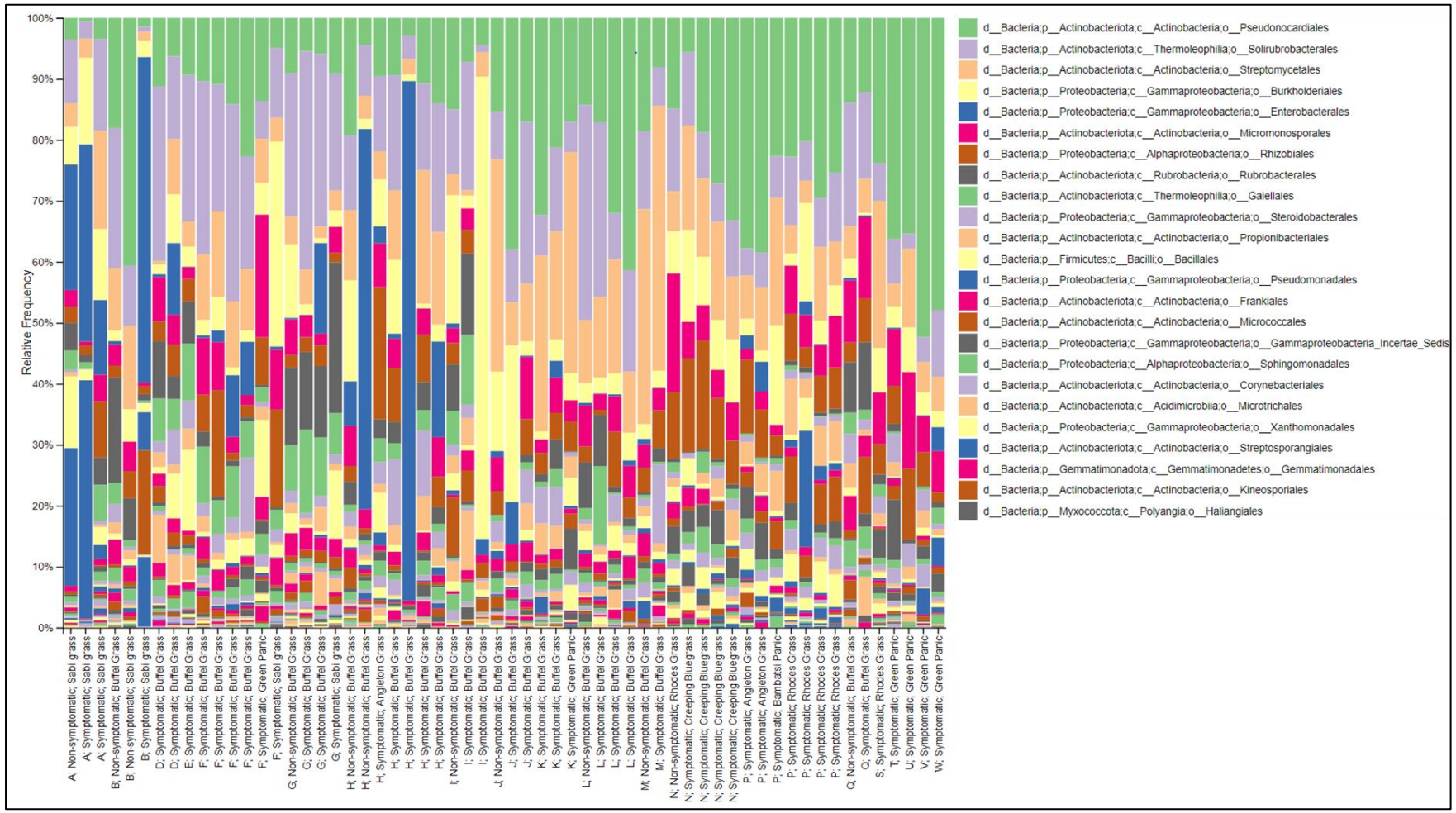


Table 16. Core bacterial populations found to be associated with root material from plants showing signs of pasture dieback (Root, Symptomatic), and healthy plants (Root, Non-symptomatic). The bacterial taxonomy is shown at the lowest level of classification achieved, usually Family or Genus level with the core populations designated as those present in ≥ 80% of the total number of root samples analysed (n). The relative abundance for each core bacterial population is expressed as a percentage of the total number of sequences obtained for each root sample group.

Root, Symptomatic (n = 48)		**Root, Non-symptomatic (n = 12)**	
Core bacteria	**Relative abundance (%)**	**Core bacteria**	**Relative abundance (%)**
Family Streptomycetaceae; Genus *Streptomyces*	9.84	Family Streptomycetaceae; Genus *Streptomyces*	11.37
Family Pseudonocardiaceae; Genus *Amycolatopsis*	7.75	Order Solirubrobacterales; Family 67-14	7.12
Order Solirubrobacterales; Family 67-14	6.66	Family Pseudonocardiaceae; Genus *Amycolatopsis*	6.99
Family Micromonosporaceae; Genus *Actinoplanes*	3.06	Family Rubrobacteriaceae; Genus *Rubrobacter*	5.11
Family Rhizobiaceae; Genus *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*	2.99	Family Micromonosporaceae; Genus *Actinoplanes*	4.02
Family Solirubrobacteraceae; Genus Unclassified	2.71	Family Solirubrobacteraceae; Genus Unclassified	3.48
Family Nocardioidaceae; Genus *Nocardioides*	1.86	Family Bacillaceae; Genus *Bacillus*	2.35
Family Micromonosporaceae; Genus Unclassified	1.28	Order Gaiellales; Family Unclassified	2.24
Family Mycobacteriaceae; Genus *Mycobacterium*	1.15	Family Solirubrobacteraceae; Genus *Solirubrobacter*	1.86
Family Sphingomonadaceae; Genus *Sphingomonas*	0.70	Order Solirubrobacterales; Family Unclassified	1.75
Family Rubrobacteriaceae; Genus *Rubrobacter*	2.89	Family Steroidobacteraceae; Genus *Steroidobacter*	1.71
Family Steroidobacteraceae; Genus *Steroidobacter*	2.56	Family Pseudonocardiaceae; Genus Unclassified	1.67
Family Solirubrobacteraceae; Genus *Solirubrobacter*	1.96	Family Rhizobiaceae; Genus *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*	1.64
Family Bacillaceae; Genus *Bacillus*	1.92	Family Pseudonocardiaceae; Genus *Pseudonocardia*	1.61
Family Xanthobacteraceae; Genus *Bradyrhizobium*	0.85	Family Burkholderiaceae; Genus *Ralstonia*	1.34
Family Pseudonocardiaceae; Genus *Pseudonocardia*	2.30	Family Nocardioidaceae; Genus *Nocardioides*	1.34
Family Burkholderiaceae; Genus *Ralstonia*	0.78	Family Micromonosporaceae; Genus Unclassified	1.28
Family Gemmatimonadaceae; Genus Unclassified	0.50	Family Sphingomonadaceae; Genus *Sphingomonas*	1.06
Order Gaiellales;Family uncultured	2.72	Family Mycobacteriaceae; Genus *Mycobacterium*	0.92
Order Gammaproteobacteria Incertae Sedis;Family Unknown_Family; Genus *Acidibacter*	1.56	Family Frankiaceae; Genus *Jatrophihabitans*	0.91

Family Pseudonocardiaceae; Genus Unclassified	1.45	Order Gammaproteobacteria Incertae Sedis; Family Unknown Family; Genus *Acidibacter*	0.85
Family Haliangiaceae; Genus *Haliangium*	0.51	Family Xanthobacteraceae; Genus *Bradyrhizobium*	0.79
Family Comamonadaceae; Genus Unclassified	3.90	Phylum Chloroflexi; Class TK10	0.72
Family Pseudonocardiaceae; Genus *Lechevalieria*	2.85	Family Gemmatimonadaceae; Genus Unclassified	0.68
Family Pseudonocardiaceae; Genus *Actinophytocola*	1.27	Family Haliangiaceae; Genus *Haliangium*	0.52
Order Solirubrobacterales; Family Unclassified	1.54	Family Pseudonocardiaceae; Genus *Actinophytocola*	0.51
Family Gaiellaceae; Genus *Gaiella*	0.69	Family Pseudonocardiaceae; Genus *Lechevalieria*	2.08
Phylum Chloroflexi; Class TK10	0.48	Family Comamonadaceae; Genus Unclassified	2.07
Family Rhizobiaceae; Genus Unclassified	0.39	Family Oxalobacteraceae; Genus *Massilia*	1.16
Family Frankiaceae; Genus *Jatrophihabitans*	0.57	Family Sphingomonadaceae; Genus Unclassified	0.44
Family Nocardioidaceae; Genus *Kribbella*	0.52	Family Geodermatophilaceae; Genus Unclassified	0.42
Order Polyangiales; Family BIrii41	0.31	Family Longimicrobiaceae; Genus YC-ZSS-LKJ147	0.41
Class Acidimicrobiia; Order IMCC26256	0.16	Family Nocardioidaceae; Genus *Kribbella*	0.26
		Family Pseudomonadaceae; Genus *Pseudomonas*	1.88
		Family Erwiniaceae; Genus *Pantoea*	1.10
		Family Geodermatophilaceae; Genus *Geodermatophilus*	0.69
		Family Gaiellaceae; Genus *Gaiella*	0.68
		Family Solirubrobacteraceae; Genus *Conexibacter*	0.46
		Actinobacteriota; Class MB-A2-108	0.43
		Order Polyangiales; Family BIrii41	0.26
		Family Pseudonocardiaceae; Genus *Kibdelosporangium*	0.25
		Family Rhizobiaceae; Genus Unclassified	0.22
		Family Caulobacteraceae; Genus *Caulobacter*	0.18
		Family Cryptosporangiaceae; Genus *Cryptosporangium*	0.18
		Family Micromonosporaceae; Genus *Dactylosporangium*	0.17
		Family Chitinophagaceae; Genus *Niastella*	0.14
		Family Thermomonosporaceae; Genus *Actinomadura*	0.13

4.2.2.1.4 Fungal microbiome

The fungal populations of plant samples (leaf and root) were determined from the amplicon sequence data generated throughout the project. Bioinformatics analysis focused on determining the classification (taxonomy) of the fungal populations present, as well as the diversity of the fungal populations occurring on individual plant samples. The fungi associated with groups of plants e.g. plants which were showing signs of pasture dieback (Symptomatic) and plants which were not showing signs of pasture dieback (Non-symptomatic) were also compared, in order to determine whether there were any highly abundant, dominant types of fungi associated with pasture dieback symptoms.

4.2.2.1.5 Diversity of plant-associated fungal populations

Within-sample microbial diversity, based on four measures of alpha diversity (Faith PD, Shannon entropy, Pielou evenness and Observed Features), was determined for leaf and root fungal microbiomes, from plants that were either Symptomatic or Non-symptomatic for pasture dieback. The 60 samples of each plant sample type (leaf and root) were analysed by REML to assess the impact of dieback symptoms on each of these measures. Models included the random effect of *Site* and the fixed effect of *Dieback symptoms* (Non-symptomatic, Symptomatic).

For leaf fungal communities, the alpha diversity measures of Shannon entropy and Pielou evenness were significantly higher in plants showing symptoms of pasture dieback than in non-symptomatic plants ($P<0.05$). There was also some evidence ($P=0.067$) that the diversity measure of Observed features was higher in plants showing symptoms than in non-symptomatic plants. In contrast, for root fungal communities, all four diversity measures tended to be higher in plants which were non-symptomatic for pasture dieback than in plants symptomatic for pasture dieback, although these differences were not statistically significant ($P > 0.05$; Table 17).

Table 17. The effect of pasture dieback symptoms on microbial diversity, based on four measures of alpha diversity, for the fungal populations of leaf and root material. The predicted means and standard error of differences (s.e.d.) are shown and significance levels indicated by the P value, with significance at the 5% threshold.

Treatment	Faith PD	Shannon entropy	Pielou evenness	Observed features
	Leaf Fungi			
Dieback symptoms	P=0.166	P=0.015 [A]	P=0.013[A]	P=0.067
Non-symptomatic	5.7	2.52	0.53	28.9
Symptomatic	6.3	2.94	0.59	34.1
s.e.d.	0.4	0.17	0.03	2.8
	Root Fungi			
Dieback symptoms	P=0.065	P=0.060	P=0.055	P=0.078
Non-symptomatic	12.8	3.22	0.57	52.5
Symptomatic	10.2	2.56	0.47	41.7
s.e.d.	1.4	0.35	0.05	6.1

[A]Significant effect, $P < 0.05$

The 45 plant samples (leaf and root) which had mealybug data available were analysed by REML to assess the impact of the presence or absence of dieback symptoms and the occurrence of mealybugs on each of the within-sample fungal diversity (alpha diversity) measures. The models undertaken included the random effect of *Site* and the fixed effects of *Dieback symptoms* (Non-symptomatic,

Symptomatic) and *Mealybug* (no, yes). Although the variance component for *Site* was not always significant it was retained in the model. A parsimonious model was achieved by sequentially removing non-significant fixed effects but always retaining the main effect terms.

There was no significant interaction between pasture dieback symptoms and presence of mealybug on leaf sample fungal diversity measures of Shannon entropy, Pielou evenness and Observed features (P>0.05; Table 18). Further, whether plants had pasture dieback symptoms or not, or if mealybugs were present or not, did not have a significant effect on these diversity measures. However, there was a significant interaction for Faith PD (P=0.012) with no difference due to dieback symptoms in the absence of mealybug (ave. 6.2) while Faith PD was lower in non-symptomatic plants than symptomatic plants when mealybug were present (4.7 vs 6.4). No logical explanation for this effect was evident, so the interaction was removed from the model and just main effects fitted. There was no effect of dieback symptoms or of mealybug on Faith PD in leaf fungal populations (P>0.05; Table 18).

For root fungal communities there was no significant interaction between pasture dieback symptoms and presence of mealybug, and no effect of dieback symptoms or of mealybug on the diversity measures of Faith PD and Observed features (P>0.05; Table 19). However, there was a significant interaction with both the Shannon entropy and Pielou evenness diversity measures, with lowest measures in symptomatic plants and highest measures in non-symptomatic plants in the absence of mealybug, while dieback symptoms did not affect the diversity measures when mealybug were present (P<0.05; 1.6, 3.6, 2.8, and 2.3 for Faith PD ; 0.31, 0.61, 0.51 and 0.44 for Pielou evenness, respectively). Again, no logical explanation for these effects was evident, so the interaction was removed from the model and just main effects fitted. There was no effect of dieback symptoms or of mealybug on either Shannon entropy or Pielou evenness in root fungal populations

Table 18. The effect of pasture dieback symptoms (Symptomatic, Non-symptomatic) and the presence of Mealybugs (yes, no) on microbial diversity, based on four measures of alpha diversity, for fungal populations of leaf samples. The predicted means and standard error of differences (s.e.d.) are shown and significance levels indicated by the P value, with significance at the 5% threshold.

Treatment	Faith PD	Shannon entropy	Pielou evenness	Observed features
Dieback symptoms (D)	P=0.337	P=0.166	P=0.163	P=0.215
Non-symptomatic	5.9	2.61	0.54	29.8
Symptomatic	6.4	2.98	0.59	34.6
s.e.d.	0.5	0.26	0.04	3.9
Mealybug (M)	P=0.721	P=0.860	P=0.727	P=0.992
no	6.2	2.82	0.56	32.2
yes	6.0	2.77	0.57	32.2
s.e.d.	0.5	0.27	0.04	3.8
D x M	P=0.012[A]	P=0.069	P=0.121	P=0.064

[A]Significant effect, P < 0.05

Table 19. The effect of pasture dieback symptoms (Symptomatic, Non-symptomatic) and the presence of Mealybugs (yes, no) on microbial diversity, based on four measures of alpha diversity, for fungal populations of root samples. The predicted means and standard error of differences (s.e.d.) are shown and significance levels indicated by the P value, with significance at the 5% threshold.

Treatment	Faith PD	Shannon entropy	Pielou evenness	Observed features
Dieback symptoms (D)	P=0.369	P=0.161	P=0.091	P=0.328
Non-symptomatic	12.3	3.21	0.58	50.5
Symptomatic	10.6	2.51	0.46	42.3
s.e.d.	1.9	0.49	0.07	8.2
Mealybug (M)	P=0.600	P=0.409	P=0.256	P=0.566
no	11.9	2.67	0.48	48.6
yes	11.0	3.05	0.55	44.2
s.e.d.	1.8	0.45	0.07	7.7
D x M	P=0.132	P=0.009 [A]	P=0.005 [A]	P=0.101

[A]Significant effect, P < 0.05

The extent of between-sample fungal diversity (beta-diversity) was also determined for leaf and root fungal microbiomes, including those which were either symptomatic or non-symptomatic for pasture dieback. As performed for the bacterial dataset, PCA was used to explore and identify the largest sources of variation occurring between each respective group of fungal microbiome samples. The dimensionality of the dataset was then reduced using the sPCA method and a differential abundance method undertaken to determine which microbes were driving, or contributing, to the differences occurring between samples (sPLSDA).

The PCA indicated that the majority of the fungal populations associated with samples of symptomatic leaf material were very similar to those associated with non-symptomatic leaf samples. In contrast to the bacterial PCA, the percentage of variation observed (≤ 15%) was higher indicating a more statistically robust effect, with the clustering of each sample type and the overlapping ellipses indicating that the fungal populations showed little variation (Fig. 11). Further analysis to reduce the dimensionality of the dataset (sPLSDA) also showed few differences in the leaf fungal populations. Of the differences observed between symptomatic and non-symptomatic leaf material, the fungal genera *Naganishia, Curvularia, Strelitziana* and *Pseudoseptoria* were found to contribute to these differences (section 9.2.1; Fig. 40, Table 48).

For the root microbiomes, as indicated by PCA, the majority of fungal populations associated with root material collected from plants that were either symptomatic or non-symptomatic for pasture dieback, were also very similar. The percentage of variation captured in the PCA of respective root fungal populations, was again relatively low (≤ 10%) with the majority of samples clustering together and the ellipses, describing the two groups of samples, overlapping (Fig. 12). Further analysis to reduce the dimensionality of the dataset (sPLSDA) also showed few differences occurring between root-associated fungal populations. Of the differences observed to occur between symptomatic and non-symptomatic root material, the fungal family Marasmiaceae, genera *Acrophialophora* and *Curvularia*, family Ceratobasidiaceae, genera *Botryosphaeria* and *Erythrobasidium* and family Teratosphaeriaceae were found to contribute to these differences (section 9.2.1; Figure 40, Table 49).

Figure 11. Principal components analysis (PCA) indicating similar, diverse fungal populations associated with leaf samples, where samples are represented by a single point and grouped according to pasture dieback symptoms (Non-symptomatic leaf samples (O) and Symptomatic leaf samples (Δ)). PCA shown on the basis of three components (A) Components 1 vs 2 (PCA comp 1-2); and (B) Components 1 vs 3 (PCA comp 1-3).

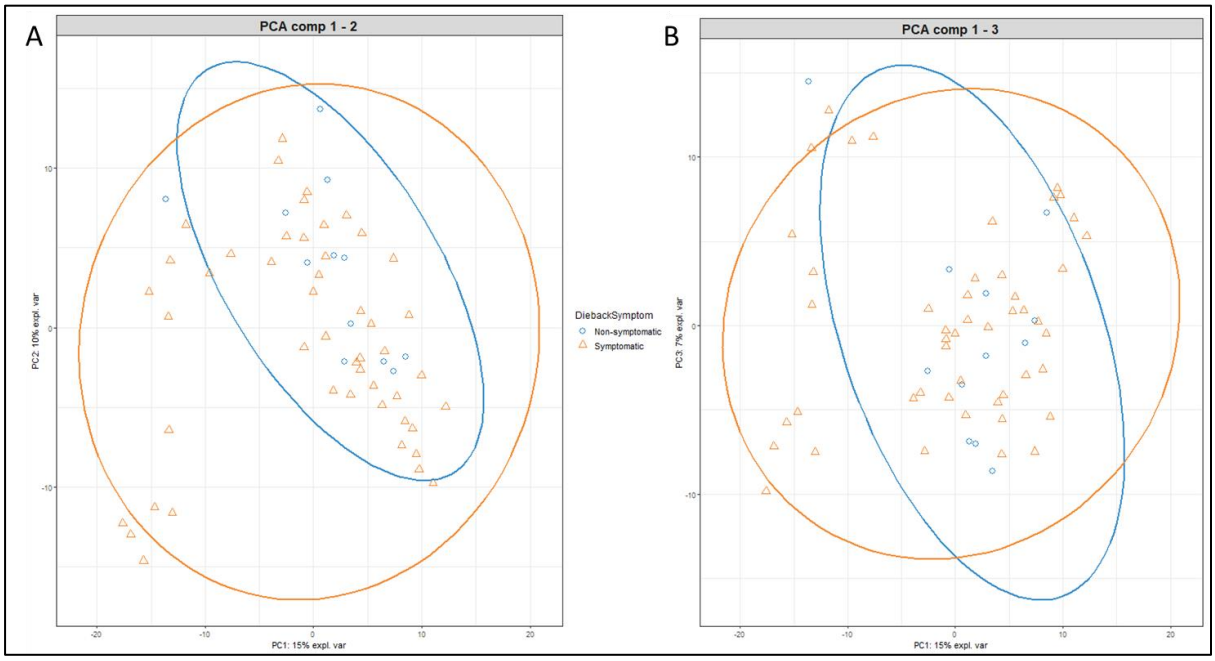
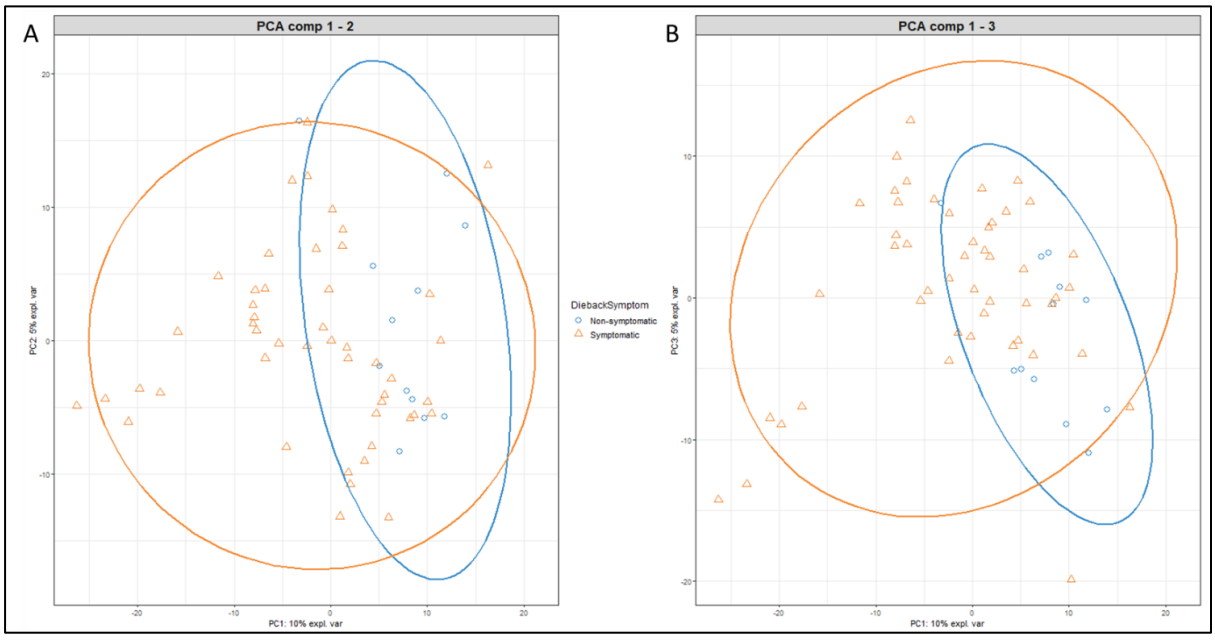


Figure 12. Principal components analysis (PCA) indicating similar, diverse fungal populations associated with root samples, where samples are represented by a single point and grouped according to pasture dieback symptoms (Non-symptomatic root samples (O) and Symptomatic root samples (Δ)). PCA shown on the basis of three components (A) Components 1 vs 2 (PCA comp 1-2); and (B) Components 1 vs 3 (PCA comp 1-3).



4.2.2.1.5.1 Taxonomy of fungal populations

The classification or taxonomy, of the dominant populations of fungi associated with leaf samples (phyllosphere) collected from non-trial sites was determined (Fig. 13). Because of the large number of fungal types observed within each leaf sample dataset (142 fungi classified to genus level) only the highly abundant, core fungal populations (fungi present in > 80% of samples) found to be associated with leaf material from plants showing signs of pasture dieback (Leaf, Symptomatic), and healthy plants (Leaf, Non-symptomatic) are tabulated (Table 20).

Fungal populations classified within phylum Ascomycota and phylum Basidiomycota were highly abundant in all plant samples. Within the dataset, there were a number of sequence Features that were either only able to be assigned to the kingdom Fungi or were unassigned. There are still many unknown fungi, with a recent study of fungal communities in grassland plant species (Francioli et al., 2020) reporting nearly 4.5% of the fungal sequences in their data could not be assigned to a fungal phylum.

Specific, highly abundant core fungal populations which could be classified, and were associated with symptomatic plants (core fungi present in > 80% samples) included the genera *Cladosporium, Alternaria, Aureobasidium, Didymella* and *Nigrospora*. Highly abundant core fungal populations which could be classified and were associated with non-symptomatic plants (core fungi present in > 80% samples) included the genera, *Exserohilum, Aureobasidium, Cladosporium, Fusarium* and *Didymella* (Table 20). Several of these core genera (e.g. *Cladosporium, Aureobasidium*, and *Didymella*) were found to be highly abundant on the leaves of both symptomatic and non-symptomatic plants.

When all the leaf-associated fungal populations classified to genus level for symptomatic and non-symptomatic plant groups were compared (Fig. 14; A,B), approximately half (51%) of the fungi identified to genus level were found to be present in both groups. When the classified, core populations were compared, 77% of the core fungi were shared or present, in both the symptomatic and non-symptomatic sample groups. This finding supports the taxonomy-independent analysis (e.g. PCA) which also showed that there was little variation occurring in the overall fungal populations associated with the phyllosphere (leaves) of symptomatic and non-symptomatic plants.

The leaf fungal microbiome dataset included many fungal genera, known to contain members which are plant pathogens, as well as members well known as naturally occurring epiphytes or endophytes, of a wide range of plant species. For example, species within the genus *Cladosporium* are widespread and commonly encountered on all types of plants, with some species known to be common endophytes (Brown et al., 1998, El-Morsy, 2000), whilst other species of this genus are plant pathogens, causing leaf spots and other lesions (Bensch et al., 2012). The genus *Alternaria* is a widely distributed fungi containing 300 species, some of which are common saprophytes found in soil and air, whilst other species are known as plant pathogens (Nowicki et al., 2012). The genus *Aureobasidium* has a worldwide distribution, and some species are being investigated for potential use in the biological control of plant diseases (Romeralo et al., 2015; Wachowska et al., 2020). The genus *Didymella* contains species that are known to be plant pathogens causing foliar diseases in commercially important crops such as chickpea (Pande et al., 2005) and field pea (Tran et al., 2014), resulting in yield losses. The genus *Nigropsora* is widespread, particularly in the tropics and whilst many species are saprotrophic, some are plant endophytes with some being phytopathogenic (Hao et al., 2020). The genus *Fusarium* are widely distributed in soil and are often associated with plants. Most are harmless saprophytes, but some are plant pathogens, causing root and stem rot, vascular wilt or fruit rot (O'Donnell et al., 2015). Whilst not a core fungal group within the leaf microbiomes,

the genus *Pyricularia*, identified as causing blight of buffel grass (*Cenchrus ciliaris*) in Queensland (Perrott & Chakraborty, 1999) was present in 27% of the buffel grass leaf microbiome samples.

Table 20. Core fungal populations found to be associated with leaf material from plants showing signs of pasture dieback (Leaf, Symptomatic), and healthy plants (Leaf, Non-symptomatic). The fungal taxonomy is shown at the lowest level of classification achieved, usually Family or Genus level with the core populations designated as those present in ≥ 80% of the total number of leaf samples analysed (n). The relative abundance for each core fungal population is expressed as a percentage of the total number of sequences obtained for each leaf sample group.

Leaf, Symptomatic (n = 48)		**Leaf, Non-symptomatic (n = 12)**	
Core fungi	**Relative abundance (%)**	**Core fungi**	**Relative abundance (%)**
Family Cladosporiaceae; Genus *Cladosporium*	10.88	Family Pleosporaceae; Genus *Exserohilum*	1.53
Family Pleosporaceae; Genus *Alternaria*	10.57	Kingdom Fungi; Phylum Unclassified	1.40
Family Aureobasidiaceae; Genus *Aureobasidium*	10.06	Family Aureobasidiaceae; Genus *Aureobasidium*	8.74
Family Didymellaceae; Genus *Didymella*	8.67	Family Cladosporiaceae; Genus *Cladosporium*	2.39
Family Trichosphaeriaceae; Genus *Nigrospora*	6.51	Family Nectriaceae; Genus *Fusarium*	17.36
Kingdom Fungi; Phylum Unclassified	2.23	Family Didymellaceae; Genus *Didymella*	15.17
Family Pleosporaceae; Genus *Exserohilum*	0.91	Family Pleosporaceae; Genus *Alternaria*	3.32
Family Nectriaceae; Genus *Fusarium*	5.01		
Order Pleosporales; Family Unclassified	6.97		

Figure 13. Highly abundant fungal populations associated with leaf samples, classified to order level taxonomy. Samples are sorted within the stacked bar graph according to the location from which they were collected (e.g. site A), whether the plant leaf samples were Symptomatic or Non-symptomatic for pasture dieback, and the common name of the plant from which the leaf samples were obtained (e.g. Sabi grass). Fungi shown had an abundance ≥ third quartile threshold (455 sequences per feature). Taxonomic rank is indicated as k (kingdom); p (phylum); c (class); and o (order).

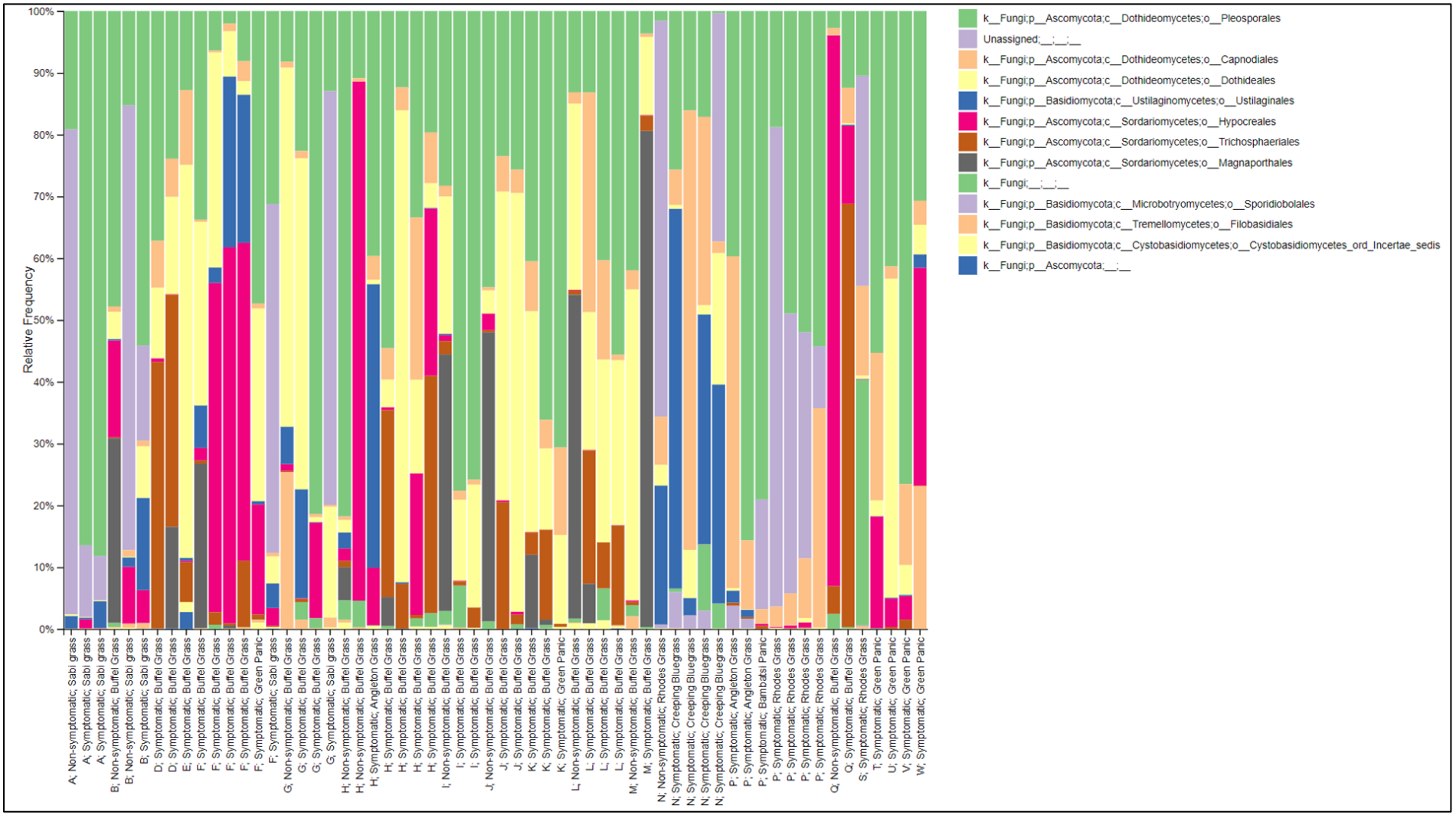
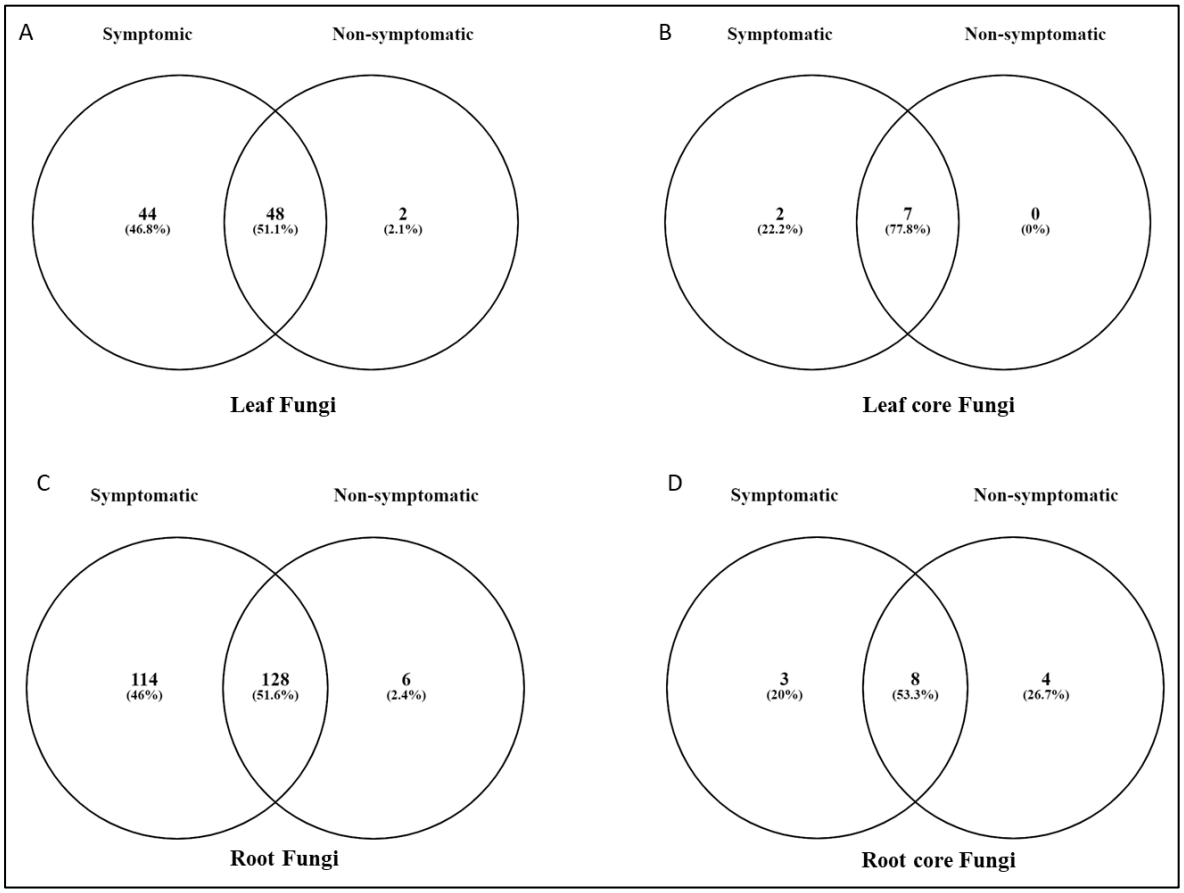


Figure 14. Venn diagrams indicating the numbers of fungal populations associated with either Leaf material (A and B) or Root material (C and D) that were unique or shared between samples Symptomatic or Non-symptomatic for pasture dieback. Fungal populations were assigned to genus level taxonomy and core populations were defined as those present in ≥ 80% of samples within each sample group (Symptomatic [n = 48], Non-symptomatic [n = 12]).



The classification or taxonomy, of the dominant populations of fungi associated with rhizosphere (root/associated soil) samples collected from non-trial sites were also determined (Fig. 15). Overall, more genera of fungi were identified in the root sample dataset (367 fungi classified to genus level) than in the leaf microbiomes and again this was expected as the rhizosphere is known to harbour more diverse populations of fungi (Fierer, 2017). Because of the large number of fungal types observed within each root sample, only the highly abundant, core fungal populations (fungi present in > 80% of samples) found to be associated with root and associated soil from plants showing signs of pasture dieback (Leaf, Symptomatic), and healthy plants (Leaf, Non-symptomatic) are tabulated (Table 21).

Fungal populations classified within the phylum Basidiomycota and phylum Ascomycota were highly abundant in all plant root samples. Specific, highly abundant core fungal populations which could be classified, and were associated with symptomatic plants (core fungi present in > 80% samples), included the unclassified genera from the family Nectriaceae, the genera *Fusarium* and *Nigrospora*, an unclassified genus from the class Sordariomycetes, and the genus *Alternaria*. Highly abundant core fungal populations which could be classified and were associated with symptomatic plants (core fungi present in > 80% samples) included two genera (*Fusarium* and unclassified genus) from the family Nectriaceae, the genera *Poaceascoma, Curvularia* and *Acrophialophora* (Table 21). Several of

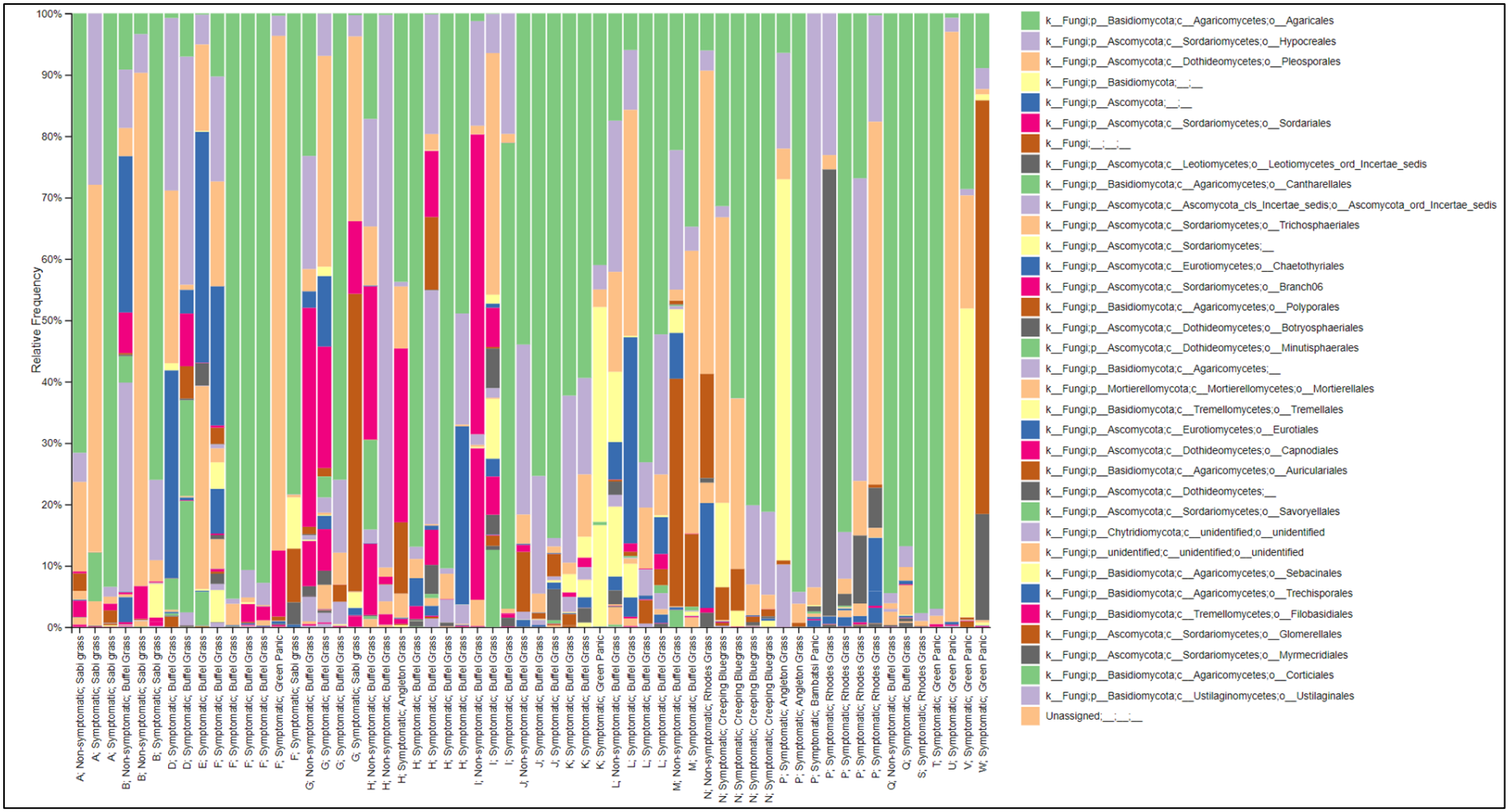
these core fungi (e.g. those classified within the family Nectriaceae), were found to be highly abundant on the roots of both symptomatic and non-symptomatic plants.

When all the root-associated fungal populations classified to genus level for symptomatic and non-symptomatic plant groups were compared (Fig. 14; C, D), approximately half (51.6%) of the fungi identified to genus level were found to be present in both groups. When the classified, core populations were compared, 53.3% of the core fungi were shared or present, in both the symptomatic and non-symptomatic sample groups. This finding supports the taxonomy-independent analysis (e.g. PCA) which also showed that there was little variation occurring in the overall fungal populations associated with the rhizosphere (roots) of symptomatic and non-symptomatic plants.

Table 21. Core fungal populations found to be associated with root material from plants showing signs of pasture dieback (Root, Symptomatic), and healthy plants (Root, Non-symptomatic). The fungal taxonomy is shown at the lowest level of classification achieved, usually Family or Genus level with the core populations designated as those present in ≥ 80% of the total number of root samples analysed (n). The relative abundance for each core fungal population is expressed as a percentage of the total number of sequences obtained for each root sample group.

Root, Symptomatic (n = 48)		**Root, Non-symptomatic (n = 12)**	
Core fungi	**Relative abundance (%)**	**Core fungi**	**Relative abundance (%)**
Family Nectriaceae; Genus Unclassified	5.72	Family Nectriaceae; Genus *Fusarium*	12.23
Kingdom Fungi; Phylum Unclassified	2.38	Family Nectriaceae; Genus Unclassified	9.07
Family Nectriaceae; Genus *Fusarium*	2.11	Family Lentitheciaceae; Genus *Poaceascoma*	7.67
Family Trichosphaeriaceae; Genus *Nigrospora*	1.00	Kingdom Fungi; Phylum Unclassified	1.87
Class Sordariomycetes; Order Unclassified	0.74	Kingdom Fungi; Phylum Ascomycota	3.22
Family Pleosporaceae; Genus *Alternaria*	0.24	Family Pleosporaceae; Genus *Curvularia*	0.26
Family Cladosporiaceae; Genus *Cladosporium*	0.13	Family Ascomycota fam Incertae sedis; Genus *Acrophialophora*	4.31
Order Pleosporales; Family Unclassified	1.22	Family Pleosporaceae; Genus *Exserohilum*	1.49
Phylum Ascomycota; Class Unclassified	3.06	Family Periconiaceae; Genus *Periconia*	0.38
Family Pleosporaceae; Genus *Exserohilum*	1.90	Order Pleosporales; Family Unclassified	0.35
Family Periconiaceae; Genus *Periconia*	1.73	Family Didymellaceae; Genus *Didymella*	0.35
		Family Cladosporiaceae; Genus *Cladosporium*	0.30

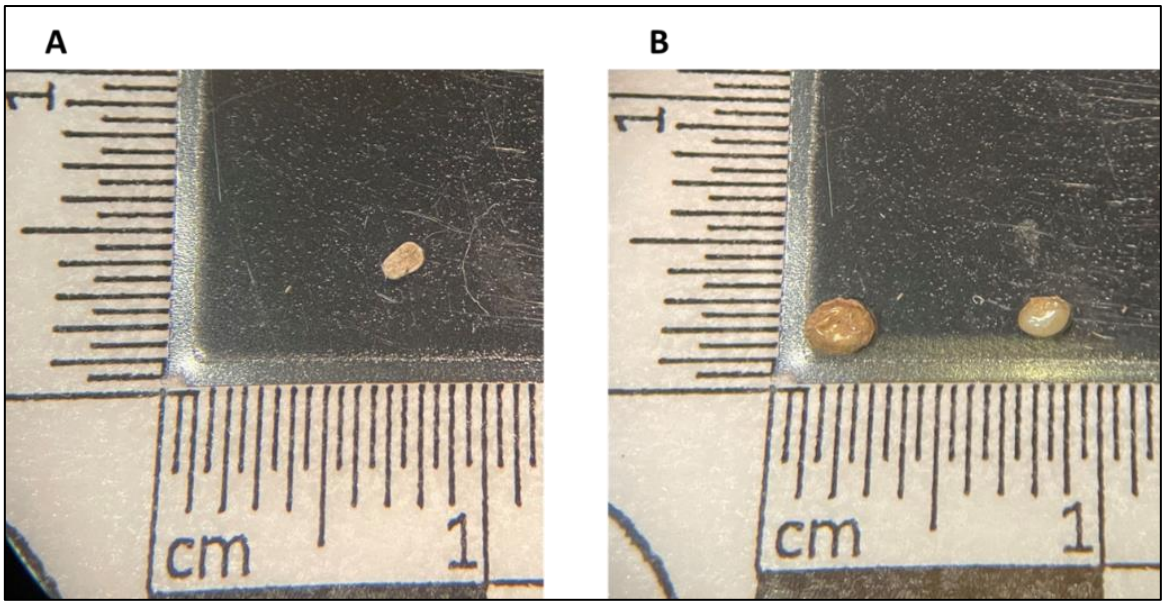
Figure 15. Highly abundant fungal populations associated with root samples, classified to order level taxonomy. Samples are sorted within the stacked bar graph according to the location from which they were collected (e.g. site A), whether the plant samples were Symptomatic or Non-symptomatic for pasture dieback, and the common name of the plant from which the root samples were obtained (e.g. Sabi grass). Fungi shown had an abundance ≥ third quartile threshold (287 sequences per feature). Taxonomic rank is indicated as k (kingdom); p (phylum); c (class); and o (order).



4.2.2.1.6 Mealybug and ground pearl microbiome

Mealybugs (*Heliococcus* sp.) and white ground pearls (*Margarodes australis*) are both pest insects known to attack plants and have been implicated in pasture dieback. To allow comparison of the microbiomes present within and on these insects to the microbiomes of plant leaf, root and soil samples in this project, methodologies were developed for the preservation of the insect, extraction, and purification of gDNA from individual specimens (Fig. 16) collected in this project and preserved either in ethanol or immediately frozen. Two different methods for the extraction and purification of gDNA were evaluated and both techniques successfully extracted gDNA from a single insect specimen preserved either in ethanol or frozen. However, the gDNA extracted using the published gDNA extraction of Phillips and Simon (1995), resulted in gDNA containing PCR inhibitors affecting the amplification of the bacterial amplicon needed for sequencing. The commercial kit protocol produced gDNA which amplified well in the PCR assays and may therefore be preferred for future DNA extractions from ground pearl and mealybug specimens. The extracted gDNA from mealybugs and ground pearls from both extraction methods were used as template in the fungal ITS PCR assay but no amplification occurred. Three mealybug and ground pearl bacterial gDNA samples were selected, along with two blank samples, and prepared for bacterial microbiome sequencing with the details on preservation and gDNA extraction method used contained in section 9.1.3 (Table 43).

Figure 16. Frozen (A) mealybug specimen (B) ground pearl specimens used for gDNA extraction method testing, both with a 1 cm scale bar to indicate the size of specimens.



The initial analysis of the extent of within-sample microbial diversity (alpha diversity) was determined for mealybug and ground pearl bacterial microbiomes, generated from individual insect specimens that were preserved either by immediately freezing or stored in ethanol and then frozen and had their gDNA extracted by one of two different methods. Four measures of alpha diversity were calculated (Table 22) and showed that both insect types, possessed diverse bacterial populations. However, the microbiomes generated using gDNA prepared by the commercial kit contained higher microbial diversity compared to those generated using gDNA prepared by the published method of Phillips and Simon (1995). The preservation method used also had a slight impact on the microbiome diversity with immediately freezing resulting in higher levels of within-sample microbial diversity compared to the ethanol preserved samples. The bacterial microbiome data generated using gDNA extracted using the published method of Phillips and Simon (1995) were

removed from further analysis of the datasets. Despite the very limited number of samples examined, distinct microbial populations were found to be associated with each of these insect types.

Table 22. Microbial diversity observed within ground pearl (GP) and mealybug (MB) samples obtained during method development experiments, described using four measures of microbial alpha diversity (Faith pd, Shannon, Pielou evenness and Observed Features).

Sample Type	DNA Extraction method	Preservation method	Sample No.	Faith pd	Shannon	Pielou evenness	Observed features
GP	Commercial kit	ethanol	522	3.04	2.08	0.44	27
GP	Commercial kit	frozen	523 (1)	8.11	4.14	0.64	88
GP	Commercial kit	frozen	523 (2)	8.37	4.15	0.62	100
GP	Published method[A]	frozen	523 (3)	4.87	0.58	0.10	52
MB	Commercial kit	ethanol	485	2.73	1.29	0.27	26
MB	Commercial kit	frozen	488 (1)	4.95	1.13	0.20	48
MB	Commercial kit	frozen	488 (2)	4.12	1.01	0.19	38
MB	Published method[A]	frozen	488 (3)	2.48	0.24	0.06	20

[A]SDS-based DNA extraction method published by Phillips and Simon (1995).

The classification of the dominant populations of bacteria associated with three individual mealybugs was classified to genus level (Fig. 17). The highly abundant, bacterial populations, i.e. those bacteria types present at ≥ 0.05% relative abundance are tabulated (Table 23). Mealybugs are plant sap-sucking insects that rely on symbiotic micro-organisms to help them with their nutritional requirements with a study of six mealybugs belonging to the Phenacoccinae sub-family revealing they host only one type of symbiotic bacteria, *Tremblaya phenacola* (Michalik et al., 2019). Of note, all three of the mealybug microbiomes were dominated by a bacterial population previously associated with a mealybug symbiont (Lopez-Madrigal, 2015; C. Hauxwell, pers. comm. 2020), classified within the family Burkholderiaceae and the candidate genus, *Tremblaya* with a relative abundance of 73%. Whilst the plant (leaf, root/associated soil, soil) samples from the site that the mealybugs were obtained from have not had microbiome sequencing undertaken, the genus *Tremblaya* was present in the leaf microbiomes of 16 (33%) of the symptomatic plant samples and only 1 (8%) of the non-symptomatic plants. While some bacteria found associated with mealybug samples were also found in soil samples, including root samples where mealybugs were not detected (Fig. 18), the genus *Tremblaya* was not detected within the sequence dataset describing the root-associated microbiota. The second most dominant bacterial population was the genus Ralstonia which contains members known as plant pathogens, however, as discussed previously, further quantitative PCR assays should be undertaken to validate the presence of *Ralstonia* in the microbiomes of mealybugs rather than a laboratory-derived contaminant (Salter et al 2014). There were more bacterial populations present in the frozen mealybugs and this may be due to the preservation in ethanol resulting in the loss of bacteria located on the mealybug's external body surface.

Figure 17. Highly abundant bacterial populations associated with three individual mealybugs collected from a single site (AG) but preserved either in ethanol then stored frozen (sample 485) or immediately frozen (sample numbers 488) classified to genus level. Bacteria shown had an abundance ≥ third quartile threshold (24 sequences per feature). Taxonomic rank is indicated as d (domain); p (phylum); c (class); o (order); f (family) and g (genus).

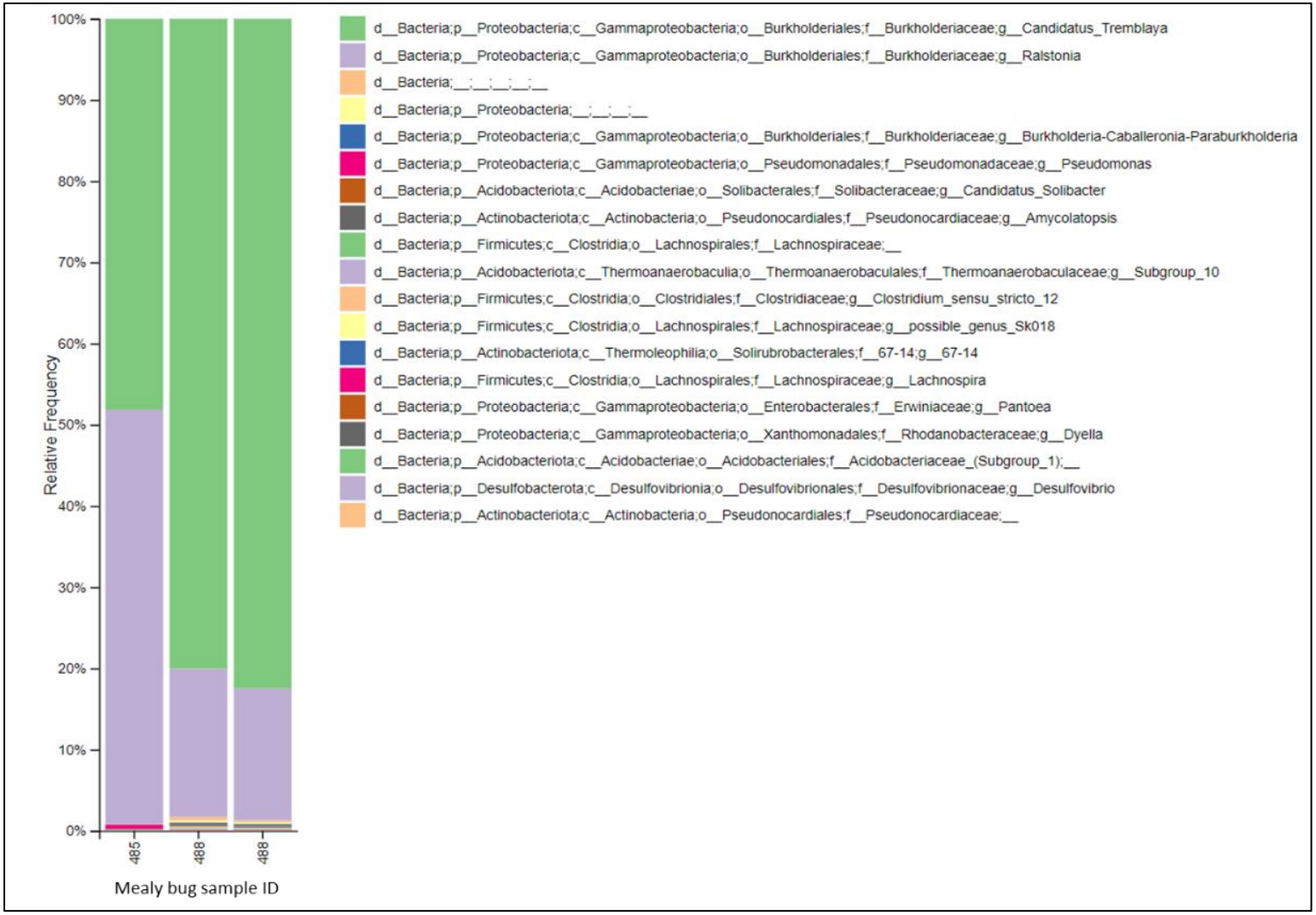
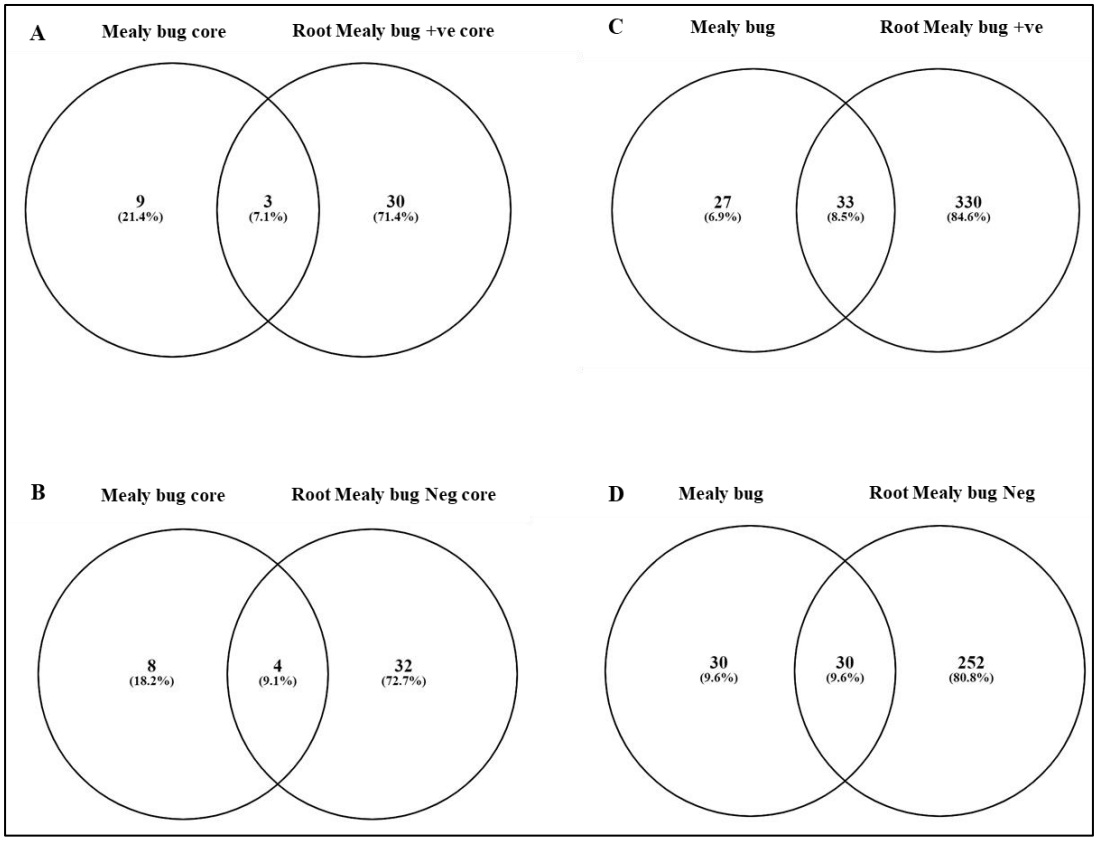


Table 23. Bacterial populations found to be associated with the three individual mealybug samples. The bacterial taxonomy is shown at the lowest level of classification achieved, usually Family or Genus level, The bacteria listed were designated as highly abundant, being present at ≥ 0.05% relative abundance, where the relative abundance for each bacterial population is expressed as a percentage of the total number of sequences obtained for all the mealybug samples. The number of mealybug samples each bacterial population was found in, is also listed.

Mealybug, bacteria	**Relative abundance (%)**	**No. of samples**
Family Burkholderiaceae; Genus Candidatus *Tremblaya*	73.21	3
Family Burkholderiaceae; Genus *Ralstonia*	24.72	3
Domain Bacteria; Phylum Unclassified	0.46	3
Phylum Proteobacteria; Class Unclassified	0.24	3
Family Burkholderiaceae; Genus *Burkholderia-Caballeronia-Paraburkholderia*	0.17	2
Family Pseudomonadaceae; Genus *Pseudomonas*	0.14	3
Family Solibacteraceae; Genus Candidatus *Solibacter*	0.12	2
Family Pseudonocardiaceae; Genus *Amycolatopsis*	0.11	3
Family Lachnospiraceae; Genus Unclassified	0.10	3
Family Thermoanaerobaculaceae; Genus Subgroup 10	0.06	2
Family Bryobacteraceae; Genus *Bryobacter*	0.05	2
Family Lachnospiraceae; Genus *Lachnospira*	0.05	3

Figure 18. Venn diagrams showing the number and percentage of bacterial populations found to be either unique or shared, between mealybug samples and plant root samples either positive (+ve) or negative (Neg) for mealybugs. (A) Core bacteria detected in mealybug samples and mealybug positive root samples; (B) Core bacteria detected in mealybug samples and mealybug negative root samples; (C) All bacteria detected in mealybug samples and mealybug positive root samples; (D) All bacteria detected in mealybug samples and mealybug negative root samples. Core populations of bacteria were those found in ≥80% of samples.



Ground pearls are insect pests which can form a shiny cyst stage of the second instar, from which they get their name (Thompson et al 2021). There is little information on the biology of these cryptic, root-feeding insects or their microbiomes. The classification of the dominant populations of bacteria associated with three individual ground pearls were determined classified to genus level (Fig. 19). The highly abundant, bacterial populations, i.e. those bacteria types present at ≥ 0.05% relative abundance are tabulated (Table 24). The bacterial populations found associated with the ground pearl samples were also often found in the rhizosphere (root/associated soil) samples (Fig. 20). The most abundant bacteria population in all three ground pearl microbiomes was the genus *Ralstonia* and as mentioned previously further quantitative PCR assay will need to be used to validate it as real member of the microbiome. The second most abundant bacteria present in all three ground pearl microbiomes which was also present in all of the root microbiomes (approx. 7% relative abundance) was the genus *Amycolatopsis* which has been identified as a beneficial rhizobacterial species associated with positive effects on plant growth in other plant species such as stylo (Zhou et al 2017) and cucumber (Alipour et al 2021). Two of the ground pearl microbiomes were dominated by a bacteria identified as genus *Burkholderia-Caballeronia-Paraburkholderia* with a relative abundance of 39%. Members of the genus *Caballeronia* are found as symbionts in other insects such as squash bugs but are thought to be environmentally acquired (Acevedo et al 2021; Mendiola et al 2022). Whilst the plant (leaf, root/associated soil, soil) sample that the ground pearls

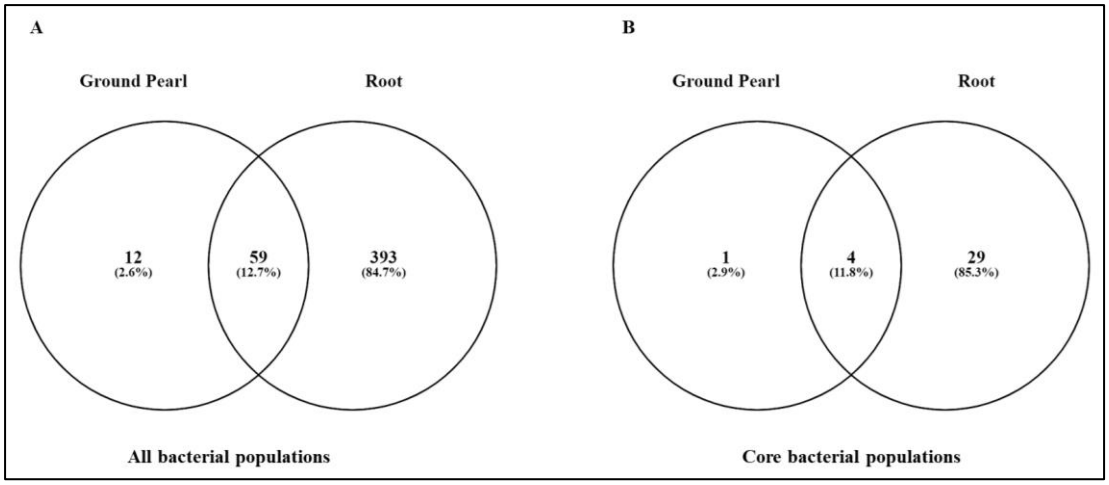
were obtained from has not had microbiome sequencing undertaken, the genus *Burkholderia-Caballeronia-Paraburkholderia* was present at a low relative abundance in the microbiomes of 24 (50%) of the symptomatic plant samples and 8 (67%) of the non-symptomatic plants and was not present in any of the leaf microbiomes. The genus *Paraburkholderia* is commonly found in the rhizosphere with plant growth promoting species isolated from *Paspalum* with the capacity to solubilise phosphate (Amaral et al 2022). As was seen in the mealybugs, there were more bacterial populations present in the microbiomes generated from frozen ground pearl samples and this may be due to the preservation in ethanol resulting in the loss of bacteria located on the external surface of the ground pearl cyst.

Figure 19. Highly abundant bacterial populations associated with three individual ground pearl samples, collected from two different sites and preserved either in ethanol then stored frozen (sample 522, site AJ) or immediately frozen (sample number 523, site AK), classified to genus level. Bacteria shown had an abundance ≥ third quartile threshold (24 sequences per feature). Taxonomic rank is indicated as d (domain); p (phylum); c (class); o (order); f (family) and g (genus).

Table 24. Bacterial populations found to be associated with ground pearl samples. The bacterial taxonomy is shown at the lowest level of classification achieved, usually Family or Genus level. The bacteria listed were designated as highly abundant, being present at ≥ 0.5% relative abundance, where the relative abundance for each bacterial population is expressed as a percentage of the total number of sequences obtained for all the ground pearl samples. The number of ground pearl samples each bacterial population was found in, is also listed.

Ground Pearl, bacteria	Relative abundance (%)	No. of samples
Family Burkholderiaceae; Genus *Burkholderia-Caballeronia-Paraburkholderia*	39.60	2
Family Pseudomonadaceae; Genus *Pseudomonas*	10.46	1
Family Burkholderiaceae; Genus *Ralstonia*	10.08	3
Family Solibacteraceae; Genus Candidatus *Solibacter*	7.48	2
Family Pseudonocardiaceae; Genus *Amycolatopsis*	5.69	3
Family Clostridiaceae; Genus *Clostridium* sensu stricto 12	3.18	2
Family Thermoanaerobaculaceae; Genus Subgroup 10	3.02	2
Order Solirubrobacterales; Family 67-14	2.14	3
Family Bryobacteraceae; Genus *Bryobacter*	1.94	2
Family Acidobacteriaceae (Subgroup 1); Genus Unclassified	1.84	2
Family Mycobacteriaceae; Genus *Mycobacterium*	1.55	2
Family Pseudonocardiaceae; Genus Unclassified	1.42	2
Family Xanthobacteraceae; Genus *Bradyrhizobium*	1.20	3
Family Rhodanobacteraceae; Genus *Dyella*	0.96	2
Family Solirubrobacteraceae; Genus *Conexibacter*	0.86	2
Order Gammaproteobacteria Incertae Sedis; Family Unclassified; Genus *Acidibacter*	0.69	2
Family Rhizobiaceae; Genus *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*	0.62	2
Family Oxalobacteraceae; Genus Unclassified	0.58	1

Figure 20. Venn diagrams showing the number and percentage of bacterial populations found to be either unique or shared, between the three ground pearl samples and plant root samples. (A) All bacteria detected; (B) core populations of bacteria found in ≥80% of samples.



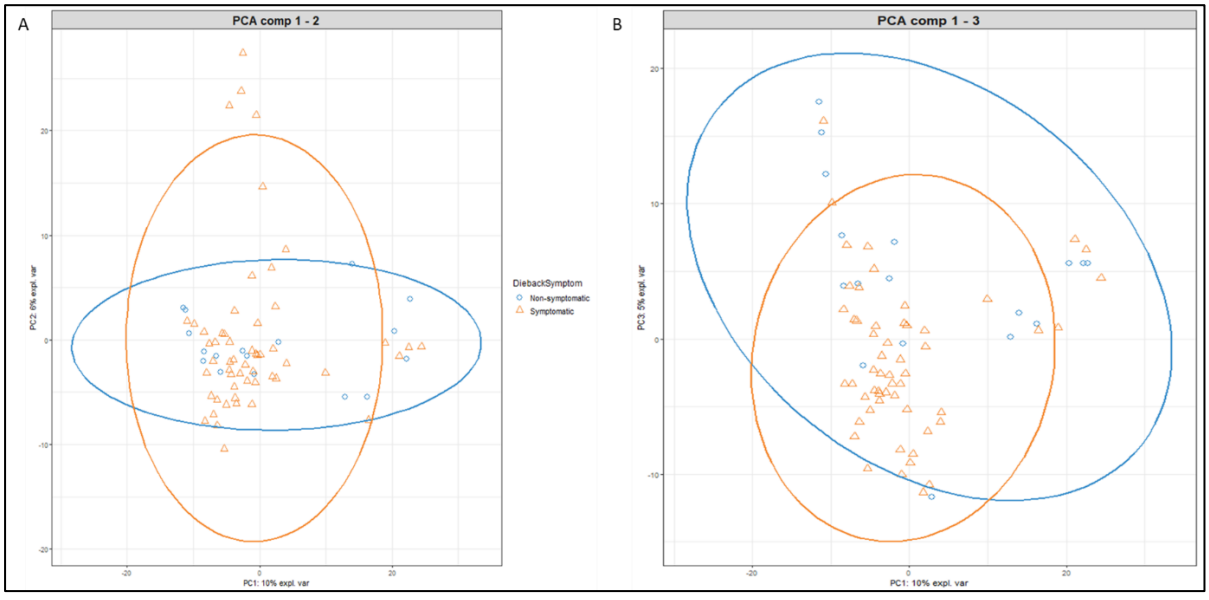
4.2.2.1.7 Soil micro-eukaryote microbiome

The soil samples were the only sample type to have the eukaryotic microbiome sequencing undertaken, as the leaf and root samples amplified mostly plant host genes and the amount of plant DNA in soil is relatively low. The micro-eukaryote microbiome amplicon preparation, sequencing and taxonomic assignment work was done by Dr Paul Dennis' group at his UQ laboratory. Further analysis of the taxonomically assigned micro-eukaryote dataset was undertaken by the DAF MEG group at the Ecosciences Precinct.

4.2.2.1.7.1 Diversity of soil eukaryote populations

The extent of between-sample micro-eukaryote diversity (beta-diversity) was determined for the samples of soil collected from around plants which were either symptomatic or non-symptomatic for pasture dieback. Principal component analysis (PCA; Joliffe and Cadima, 2016) was used to explore and identify the largest sources of variation occurring between each respective group of bacterial microbiome samples. The PCA results indicated that the majority of the eukaryote populations found associated with the soil samples from symptomatic plants were very similar to those associated with non-symptomatic plants, as indicated by the relatively low percentage of variation explained (≤ 10%) and the clustering of samples and overlapping ellipses as depicted in Fig. 21. The within-sample variation (alpha diversity) is yet to be determined for this dataset.

Figure 21. Principal components analysis (PCA) indicating similar, diverse eukaryote populations in soil associated with plants, where the soil samples are represented by a single point and grouped according to the pasture dieback symptoms of the plants from which the soil was collected (Non-symptomatic plants (O) and Symptomatic plants (Δ)). PCA shown on the basis of three components (A) Components 1 vs 2 (PCA comp 1-2); and (B) Components 1 vs 3 (PCA comp 1-3).



4.2.2.1.7.2 Taxonomy of soil-associated eukaryote populations

The taxonomy of the dominant, highly abundant populations of eukaryotes associated with soil samples collected from non-trial sites were determined (Fig. 22). For each soil sample examined, a highly diverse population of different eukaryote types were observed, as would be expected, as soil is known to harbour diverse populations of eukaryotes (Fierer, 2017; Dopheide et al., 2021). Because of the large number of eukaryote types observed within each soil sample, the highly abundant, core eukaryote populations (eukaryotes present in 100% of samples) found to be associated with soil

collected with plants showing signs of pasture dieback (Table 25), and healthy plants (Table 26) were tabulated.

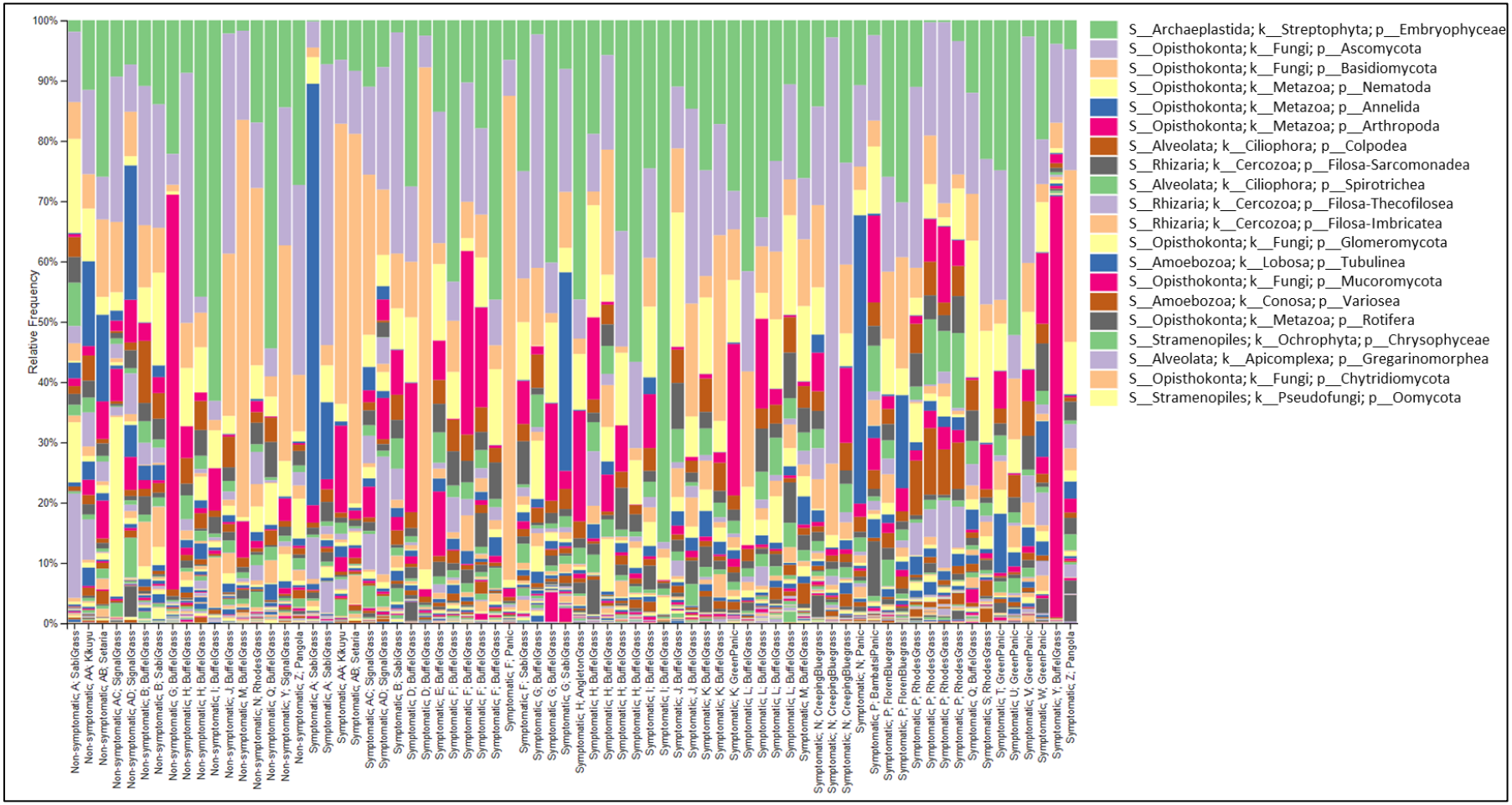
Table 25. Core, highly abundant populations of eukaryotes found to be associated with soil material collected with plants showing signs of pasture dieback (Symptomatic). The eukaryote taxonomy is shown three levels of taxonomy (Supergroup (S__); kingdom (k__); phylum (p__). Core populations were designated as those present in 100% of the total number of soil samples analysed (n). The relative abundance for each core bacterial population is expressed as a percentage of the total number of sequences obtained for each soil sample group.

Soil, Symptomatic Core eukaryotes (n = 55)	**Relative abundance (%)**
S__Archaeplastida; k__Streptophyta; p__Embryophyceae	17.64
S__Opisthokonta; k__Fungi; p__Ascomycota	14.98
S__Opisthokonta; k__Fungi; p__Basidiomycota	12.19
S__Opisthokonta; k__Metazoa; p__Nematoda	7.38
S__Opisthokonta; k__Metazoa; p__Arthropoda	5.68
S__Rhizaria; k__Cercozoa; p__Filosa-Sarcomonadea	3.50
S__Alveolata; k__Ciliophora; p__Colpodea	2.69
S__Alveolata; k__Ciliophora; p__Spirotrichea	2.57
S__Rhizaria; k__Cercozoa; p__Filosa-Imbricatea	2.07
S__Rhizaria; k__Cercozoa; p__Filosa-Thecofilosea	2.00
S__Amoebozoa; k__Conosa; p__Variosea	2.00
S__Amoebozoa; k__Lobosa; p__Tubulinea	1.95
S__Opisthokonta; k__Fungi; p__Glomeromycota	1.72
S__Opisthokonta; k__Metazoa; p__Rotifera	1.33
S__Opisthokonta; k__Fungi; p__Mucoromycota	1.29
S__Stramenopiles; k__Ochrophyta; p__Chrysophyceae	1.19
S__Opisthokonta; k__Fungi; p__Chytridiomycota	0.98
S__Excavata; k__Discoba; p__Heterolobosea	0.84
S__Alveolata; k__Ciliophora; p__Oligohymenophorea	0.73
S__Alveolata; k__Ciliophora; p__Litostomatea	0.72
S__Amoebozoa; k__Lobosa; p__Lobosa_X	0.49
S__Hacrobia; k__Cryptophyta; p__Cryptophyceae	0.37
S__Rhizaria; k__Cercozoa; p__Endomyxa	0.32
S__Alveolata; k__Dinoflagellata; p__Syndiniales	0.21
S__Stramenopiles; k__Sagenista; p__Labyrinthulomycetes	0.21

Table 26. Core, highly abundant populations of eukaryotes found to be associated with soil material collected with plants showing no signs of pasture dieback (Non-symptomatic). The eukaryote taxonomy is shown three levels of taxonomy (Supergroup (S__); kingdom (k__); phylum (p__). Core populations were designated as those present in 100% of the total number of soil samples analysed (n). The relative abundance for each core bacterial population is expressed as a percentage of the total number of sequences obtained for each soil sample group.

Soil, Non-symptomatic **Core eukaryotes (n = 17)**	**Relative abundance (%)**
S__Archaeplastida; k__Streptophyta; p__Embryophyceae	18.77
S__Opisthokonta; k__Fungi; p__Ascomycota	15.87
S__Opisthokonta; k__Fungi; p__Basidiomycota	13.36
S__Opisthokonta; k__Metazoa; p__Arthropoda	7.34
S__Opisthokonta; k__Metazoa; p__Nematoda	5.48
S__Rhizaria; k__Cercozoa; p__Filosa-Sarcomonadea	3.23
S__Alveolata; k__Ciliophora; p__Colpodea	2.87
S__Stramenopiles; k__Pseudofungi; p__Oomycota	2.65
S__Opisthokonta; k__Fungi; p__Chytridiomycota	2.59
S__Rhizaria; k__Cercozoa; p__Filosa-Thecofilosea	2.18
S__Opisthokonta; k__Fungi; p__Glomeromycota	1.97
S__Alveolata; k__Ciliophora; p__Spirotrichea	1.74
S__Amoebozoa; k__Lobosa; p__Tubulinea	1.68
S__Opisthokonta; k__Fungi; p__Mucoromycota	1.54
S__Amoebozoa; k__Conosa; p__Variosea	1.41
S__Rhizaria; k__Cercozoa; p__Filosa-Imbricatea	1.21
S__Excavata; k__Discoba; p__Heterolobosea	1.12
S__Stramenopiles; k__Ochrophyta; p__Chrysophyceae	1.08
S__Opisthokonta; k__Metazoa; p__Rotifera	0.86
S__Alveolata; k__Ciliophora; p__Litostomatea	0.65
S__Opisthokonta; k__Fungi; p__Fungi_X	0.49
S__Rhizaria; k__Cercozoa; p__Filosa-Granofilosea	0.41
S__Amoebozoa; k__Lobosa; p__Lobosa_X	0.34
S__Hacrobia; k__Cryptophyta; p__Cryptophyceae	0.29
S__Rhizaria; k__Cercozoa; p__Endomyxa	0.25
S__Stramenopiles; k__Sagenista; p__Labyrinthulomycetes	0.23
S__Alveolata; k__Ciliophora; p__Oligohymenophorea	0.22
S__Amoebozoa; k__Lobosa; p__Discosea-Flabellinia	0.20
S__Hacrobia; k__Centroheliozoa; p__Centroheliozoa_X	0.14

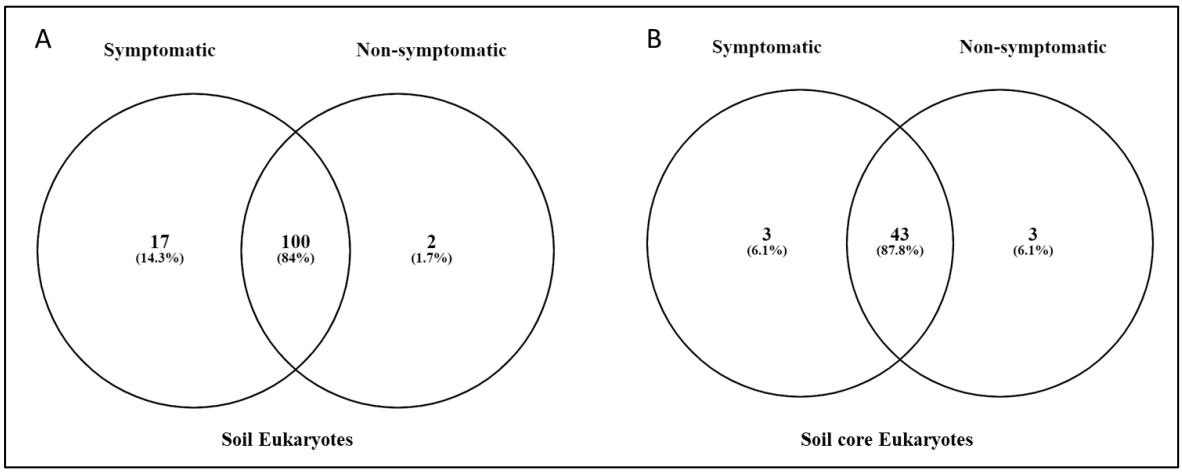
Figure 22. Highly abundant eukaryote populations associated with soil samples classified to three levels of taxonomy (top 20 phyla shown). Samples are sorted within the stacked bar graph according to the location from which they were collected (e.g. site A), whether the associated plant samples were Symptomatic or Non-symptomatic for pasture dieback, and the common name of the plant from around which the soil samples were obtained (e.g. Sabi grass). Eukaryotes shown had an abundance ≥ third quartile threshold (112 sequences per feature). Taxonomic rank is indicated as S (Supergroup); k (kingdom); and p (phylum).



The soil associated with both symptomatic and non-symptomatic plant samples were dominated by eukaryote populations classified within the supergroups Opisthokonta (fungi); Archaeplastida (Streptophyta -plants and other algae); other Opisthokonta (Metazoa animals such as earthworms, nematodes and insects). When all the soil-associated eukaryote populations, classified to three levels of taxonomy, for symptomatic and non-symptomatic plant groups were compared (Fig. 23; A), the majority of the eukaryotes identified (84%) were found to be present in both groups. A higher proportion of unique taxonomic groups were found associated with symptomatic plants (14.3% of all identified taxa), however the number of samples examined for this group was considerably higher (55 soil samples from symptomatic plants vs. 17 soil samples from non-symptomatic plants), which may have contributed to the overall, increased numbers of eukaryote types associated with this group.

When the core populations (defined for the purposes of the Venn analysis as the eukaryote populations identified in ≥80% of samples) of symptomatic and non-symptomatic samples were compared (Fig. 23; B), an even higher proportion of eukaryote populations (87.8%) were shared between both sample types with an equal number of eukaryote populations found to be unique to either sample group (3 unique eukaryote populations representing 6.1% of the total core populations). Core eukaryote populations found to be unique to the symptomatic soil samples include those classified with the phyla Apusomonadidae Group-1 (protozoan zooflagellates); Zygnemophyceae (green algae); and Bangiophyceae (red algae). The core eukaryote populations found to be unique to the non-symptomatic soil samples also included algae and protozoa, classified instead within the phyla Bacillariophyta (microalgae, diatoms); Ichthyosporea (fungus-like protists); and Heterotrichea (ciliate protozoa). This taxonomic comparison supported the taxonomy-independent analysis (PCA) which also indicated that there were very similar, diverse eukaryote populations associated with the soil samples associated with both symptomatic and non-symptomatic plants.

Figure 23. Venn diagrams indicating A. the numbers of eukaryote populations associated with soil material that were unique or shared between samples grouped as Symptomatic or Non-symptomatic for pasture dieback, and B. comparing the core eukaryote populations, defined as those present in ≥ 80% of samples within each sample group (Symptomatic [n = 55], Non-symptomatic [n = 17]). Eukaryote populations were assigned to three levels of taxonomy.



The most dominant population identified in the soil micro-eukaryotic microbiomes was Embryophyceae (land plants). Whilst this relative dominance within the data set may be attributed to the presence of contaminating plant root and leaf/stem material within the soil samples

collected, it may also be from the detection of mosses, hornworts, liverworts and algae, which are often found associated with soil. The next most abundant populations were the fungi. The diversity of these fungi is not described here, as the rhizosphere fungal microbiome is detailed in the section titled *Fungal Microbiome*. Nematode populations were present in all soil micro-eukaryotic microbiomes at high abundance but a lack of publicly available, curated taxonomic sequences meant they were only classified taxonomically to class level, such as the classes Enoplea and Chromadorea. Within this project, bulk soil samples taken from sample sites, were examined for plant-parasitic nematodes (section 4.3.2) whereby a number were identified from the Class Enoplea, including dagger nematodes (*Xiphinema* sp.) and stubby-root nematodes (*Paratrichodorus* sp.). Members of the Phylum Annelida (earthworms) were present in over 90% of the soil micro-eukaryotic microbiomes. Populations of earthworms (Phylum Annelida) were present in over 90% of the soil micro-eukaryotic microbiomes.

Eukaryotic populations belonging to the Phylum Arthropoda, which includes insects and spiders, were highly abundant and present in all the soil micro-eukaryotic microbiomes. Insect populations included springtails, scale, ants, wasps, thrips, termites and lady beetles. No mealybugs were found to be present and again this is because of very few DNA sequences from mealybugs available in public sequence databases that can be downloaded into specialist curated reference databases for taxonomic assignment. As an example, there are over 60 known species of mealybug within the genus *Heliococcus,* however there are only DNA sequences for 11 *Helicoccus s*pp. within the NCBI GenBank database. The majority of these are sequences from other nuclear genes (elongation factor, 28S rRNA) used for phylogenetic classification (Downie & Gullan, 2004) with only two partial 18S rRNA gene sequences available which don't cover the variable regions used in this study. A similar situation exists for ground pearls with 30 known species within the genus *Margarodes,* and currently no corresponding DNA sequences available in the GenBank public database. Potentially the gDNA isolated from the individual mealybug or ground pearls could be used as template in a PCR assay to amplify the 18S rRNA gene and the mealy-bug or ground pearl specific DNA sequence determined. These sequences could then be used to examine the micro-eukaryotic microbiome dataset more thoroughly, and enable the presence of mealybug and ground pearls to be determined using this sequence-based approach.

Protists are eukaryotes that are not a land plant, fungus or animal and the diversity of protists in soils is extremely high but this diversity and their role and interactions within the plant microbiome is still mostly unknown (Geisen et al. 2018). Diverse populations of protists (Rhizaria, Alveolata, Amoebozoa, Stramenopiles, Hacrobia) were present in all soil microbiomes and were highly diverse, with over 164 members present in the symptomatic and 111 members in the non-symptomatic samples.

Across the soil micro-eukaryotic microbiomes, a lack of curated taxonomic 18S rRNA sequence data limited the identification of many of the micro-eukaryotic populations to higher levels such as family or even phylum. However, research into soil eukaryotic microbiome is rapidly increasing which will lead to the taxonomic gaps being filled. A recent molecular study of 193 soil eukaryotic microbiomes found the community structure of microbial (fungal, protist) groups was affected more strongly by environmental conditions such as soil pH, whereas the animal groups (Annelida, Athropoda and Nematoda) were affected more strongly by mean annual precipitation, soil moisture, and fire history (Aslani et al. 2022).

4.3 Analysis of soil samples

The number of ground pearl (GP) and nematodes were recorded, and soil nutrients measured, in the 0-10 and 10-30 cm soil layers at 27 sites (properties/locations) each with one of more sample sites (paddocks, areas). Dieback symptoms (symptomatic, non-symptomatic) at each sample site was noted and the presence of mealybugs (present, absent) was recorded at a sub-set of the sample sites. Not all data were recorded at each sample site resulting in an incomplete data set.

Ground pearl determination was undertaken by Melody Thomson, University of Queensland, Gatton campus. Ground pearl cysts were sieved from soil samples collected by DAF project staff and live or empty numbers were recorded. No other soil preparation was undertaken. The nematode assessment was undertaken by the DAF Nematology Diagnostic Laboratory at Ecosciences Precinct, Brisbane. Soil samples collected by DAF project staff were sent as collected to the laboratory and analysed using the Whitehead Tray method over three days. The number of plant-parasitic nematodes / 200 mL soil (corrected for extraction efficiency) were recorded. Soil nutrients were assessed through the Nutrient Advantage laboratory in Victoria (division of Incitec-Pivot). All samples were sent as collected via courier then air dried at 40°C, ground to 2mm sieve size, and analysed by the laboratory using test code E39.

4.3.1 Ground Pearl

The relationship between pasture dieback symptom (non-symptomatic; symptomatic) and the presence of ground pearl (GP) (live, empty or overall) in the 0-10 and 10-30 cm soil layers was tested by chi-square analysis for contingency tables. The impact of dieback symptoms on the number of live and empty ground pearl (GP), and the overall number of GP, in the 0-10 and 10-30 cm soil layers was investigated. Models including the random effect of *Site* and the fixed effect of dieback symptom (non-symptomatic, symptomatic) were fitted by REML. Data were log-transformed prior to analysis to satisfy variance and normality assumptions. Predicted means and standard errors of difference were obtained and back-transformed means calculated. Analyses were then repeated considering only sample sites where GP were present.

No relationship between dieback symptom and presence of GP (live, empty or overall) was evident in the 0-10 or 10-30 cm soil layers ($P>0.05$) (Table 27).

There was no difference between non-symptomatic and symptomatic sites in the number of live GP, empty GP or GP overall in the 0-10 cm soil layer or in the number of live GP or GP overall ($P>0.05$; Table 27). However, in the 10-30 cm soil layer, there was evidence of more empty GP in non-symptomatic sites than symptomatic sites ($P=0.059$; 2.0 vs 1.3 empty GP) and when just including sites with GP ($P=0.042$; 42.5 vs 18.9 empty GP).

Table 27. Number of Ground Pearl (GP) in dieback symptomatic and non-symptomatic areas in the 0-10 and 10-30 cm soil layers. Data were log-transformed prior to analysis to account for heterogeneity of variance. Back-transformed means are given in parentheses.

		0-10 cm					
Attribute	**n**	**Non-symptomatic**		**Symptomatic**		**s.e.d.**	**Prob**
No. Ground Pearl (GP)	30	1.57	(3.8)	1.43	(3.2)	0.23	P=0.552
No. GP if GP present	14	3.37	(28.1)	2.96	(18.3)	0.45	P=0.393
No. live GP	30	0.92	(1.5)	0.89	(1.4)	0.12	P=0.819
No. live GP if GP present	10	3.19	(23.2)	3.14	(22.0)	0.36	P=0.896
No. empty GP	30	1.52	(3.6)	1.29	(2.6)	0.27	P=0.400
No. empty GP if GP present	14	3.27	(25.3)	2.68	(13.6)	0.53	P=0.307
		10-30 cm					
Attribute	**n**	**Non-symptomatic**		**Symptomatic**		**s.e.d.**	**Prob**
No. Ground Pearl (GP)	42	1.54	(3.7)	1.36	(2.9)	0.30	P=0.547
No. GP if GP present	19	3.39	(28.7)	2.95	(18.2)	0.60	P=0.482
No. live GP	32	0.75	(1.1)	0.62	(0.9)	0.19	P=0.517
No. live GP if GP present	8	2.42	(10.2)	2.71	(14.1)	0.88	P=0.754
No. empty GP	32	1.09	(2.0)	0.85	(1.3)	0.12	P=0.059
No. empty GP if GP present	9	3.77	(42.5)	2.99	(18.9)	0.23	P=0.042

4.3.2 Nematodes

Ten nematode species were identified and counted and the total across all species was calculated. However, due to limited numbers, only three species groups (Spiral *Rotylenchus brevicaudatus/Helicotylenchus dihystera,* Reniform *Rotylenchulus* spp. + 3rd spp, and Lesion *Pratylenchus* spp.) and the total of all species had sufficient data for analysis.

The relationship between dieback symptom and the presence/absence of nematodes in the spiral, reniform, or lesion groups, or across all species, in the 0-10, 10-30 or 0-30 cm soil layers was tested by chi-square analysis for contingency tables. The impact of dieback symptoms on the number of nematodes, where nematodes were present, in each species group and for the total overall in the 0-10 and 10-30 cm soil layers was investigated. Models including the random effect of *Site* and the fixed effect of dieback symptom (non-symptomatic, symptomatic) were fitted by REML. Data were log-transformed prior to analysis to satisfy variance and normality assumptions. Predicted means and standard errors of difference were obtained and back-transformed means calculated.

No relationship between dieback symptom and presence of nematodes was evident in the 0-10, 10-30 or 0-30 cm soil layers for any of the species groups or the overall total (P>0.05) (Table 28). There was no difference between non-symptomatic and symptomatic sites in the number of nematodes in the spiral or reniform groups, or in the total number of nematodes, in either the 0-10 or 10-30 cm soil layers (P>0.05; Table 28). However, there was some evidence of less nematodes in the lesion group in the 0-10 cm soil layer for non-symptomatic sites than symptomatic sites (29 vs 59; P=0.052) but not for the 10-30 cm soil layer (26 vs 22; P=0.734).

Table 28. Number of nematodes (Spiral, Reniform, Lesion and total), when nemotodes present, in dieback symptomatic and non-symptomatic areas in the 0-10 and 10-30 cm layers. Data were log-transformed prior to analysis to account for heterogeneity of variance. Back-transformed means are given in parentheses.

				0-10 cm		
Attribute	**n**	**Non-symptomatic**		**Symptomatic**	**s.e.d.**	**Prob**
No. Spiral	22	4.79	(120)	4.75 (115)	0.43	P=0.927
No. Reniform	35	5.52	(248)	4.61 (99)	0.58	P=0.131
No. Lesion	30	3.40	(29)	4.09 (59)	0.33	P=0.052
Total number of nematodes	43	6.18	(480)	5.85 (346)	0.42	P=0.448
				10-30 cm		
Attribute	**n**	**Non-symptomatic**		**Symptomatic**	**s.e.d.**	**Prob**
No. Spiral	20	4.64	(103)	4.35 (76)	0.63	P=0.655
No. Reniform	30	4.96	(141)	5.01 (148)	0.76	P=0.952
No. Lesion	24	3.29	(26)	3.15 (22)	0.40	P=0.734
Total number of nematodes	39	5.92	(373)	5.88 (358)	0.43	P=0.926
				0-30 cm		
Attribute	**n**	**Non-symptomatic**		**Symptomatic**	**s.e.d.**	**Prob**
No. Spiral	20	5.05	(155)	5.14 (170)	0.47	P=0.843
No. Reniform	33	5.98	(396)	5.49 (242)	0.62	P=0.436
No. Lesion	30	3.85	(46)	4.36 (78)	0.32	P=0.128
Total number of nematodes	39	6.95	(1042)	6.79 (886)	0.39	P=0.681

4.3.3 Nutrients

Soil nutrient attributes in dieback symptomatic and non-symptomatic areas in the 0-10 and 10-30 cm soil layers were compared. Models including the random effect of *Site* and the fixed effect of dieback symptom (non-symptomatic, symptomatic) were fitted by REML. Variance and normality assumptions were assessed by inspection of normal probability and residual plots, and appropriate log transformations applied to the data where necessary. Predicted means and standard errors of difference were obtained, and back-transformed means calculated where necessary.

Ca (Amm. Acet.) levels were greater for symptomatic than non-symptomatic sample sites in the 0-10 cm layer (8.6 vs 6.4; *P*=0.037) (Table 29) and in the 10-30 cm layer (10.2 vs 7.7; *P*=0.056) (Table 30). Similarly, the Ca/Mg ratio was greater for symptomatic than non-symptomatic sample sites in both the 0-10 cm (2.78 vs 2.18; *P*=0.028) and 10-30 cm (2.82 vs 1.98; *P*=0.018) layers, and for Ca % of Cations in both the 0-10 cm (59.4 vs 54.3; *P*=0.026) and 10-30 cm (57.2 vs 46.8; P=0.017) layers (Tables 29 and 30). There were few other differences of note.

Nutrient attributes of Chloride, Aluminium (KCl), and Aluminium (KCl) % of Cations were not analysed due to insufficient data.

Table 29. Soil nutrients in dieback symptomatic and non-symptomatic areas in the 0-10 cm layer. Where necessary to account for heterogeneity of variance, data were log-transformed prior to analysis. Back-transformed means are given in parentheses.

		0-10 cm					
Attribute	**n**	**Non-symptomatic**		**Symptomatic**		**s.e.d.**	**Prob**
pH (1:5 water)	48	6.59		6.73		0.12	P=0.268
EC (1:5 water) - log(x * 100)	48	1.99	(0.073)	2.25	(0.095)	0.14	P=0.065
EC Sat index - log (x+1)	48	0.50	(0.65)	0.57	(0.78)	0.06	P=0.262
Nitrate N - log (x+1)	48	1.89	(5.6)	1.92	(5.8)	0.16	P=0.832
Ammonium N - log(x+1)	48	1.73	(4.6)	1.90	(5.7)	0.12	P=0.178
P (Colwell) - log(x+1)	48	3.15	(22.2)	3.16	(22.5)	0.17	P=0.934
P buffer (Colwell) - log(x+1)	48	4.62	(101)	4.78	(118)	0.13	P=0.227
Ca (Amm Acet) - log (x+1)	48	2.00	(6.4)	2.26	(8.6)	0.12	P=0.037
K (Amm Acet) - log (x * 100)	48	4.35	(0.78)	4.22	(0.68)	0.13	P=0.325
Mg (Amm Acet) - log (x+1)	48	1.40	(3.0)	1.48	(3.4)	0.12	P=0.506
Na (Amm Acet) - log (x * 100)	48	2.09	(0.081)	2.38	(0.108)	0.18	P=0.114
Ca/Mg (Amm Acet) - log (x+1)	48	1.16	(2.18)	1.33	(2.78)	0.07	P=0.028
CEC (Al; Amm Acet) - log(x+1)	47	2.46	(10.7)	2.66	(13.3)	0.12	P=0.118
Na% of Cations (Al) - log(x+1)	48	0.67	(0.95)	0.73	(1.07)	0.05	P=0.253
Cu (DTPA)	47	1.80		1.98		0.16	P=0.276
Fe (DTPA) - log(x+1)	48	4.22	(66.8)	4.09	(58.9)	0.085	P=0.158
Mn (DTPA) - log(x+1)	48	3.37	(28.2)	3.33	(26.9)	0.16	P=0.774
Zn (DTPA) - log(x+1)	47	0.90	(1.46)	0.93	(1.52)	0.073	P=0.722
Boron (CaCl2)	48	0.74	(1.10)	0.78	(1.18)	0.052	P=0.511
S (MCP) - log(x+1)	48	2.25	(8.5)	2.44	(10.4)	0.08	P=0.018
Organic Carbon - log(x+1)	47	1.22	(2.4)	1.25	(2.5)	0.067	P=0.653
P (BSES) - log(x+1)	48	3.32	(26.5)	3.50	(32.2)	0.13	P=0.173
Silicon – CaCl	40	85.4		81.6		6.5	P=0.565
Organic Matter- log(x+1)	48	1.62	(4.1)	1.65	(4.2)	0.08	P=0.725
Avail. K (Amm Acet) - log(x+1)	48	5.72	(304)	5.59	(266)	0.13	P=0.307
Ca % of Cations	32	54.3		59.4		2.1	P=0.026
Mg% of Cations	32	29.7		27.2		2.2	P=0.254
K% of Cations	32	9.75		6.80		1.40	P=0.050

Table 30. Soil nutrients in dieback symptomatic and non-symptomatic areas in the 10-30 cm layer. Where necessary to account for heterogeneity of variance, data were log-transformed prior to analysis. Back-transformed means are given in parentheses.

		10-30 cm					
Attribute	**n**	**Non-symptomatic**		**Symptomatic**		**s.e.d.**	**Prob**
pH (1:5 water)	35	6.89		7.15		0.16	P=0.123
EC (1:5 water) - log(x * 100)	35	1.92	(0.068)	2.20	(0.090)	0.15	P=0.074
EC Sat index - log (x+1)	35	0.42	(0.52)	0.52	(0.68)	0.06	P=0.111
Nitrate N - log (x+1)	35	1.24	(2.5)	1.40	(3.1)	0.16	P=0.351
Ammonium N - log(x+1)	35	1.48	(3.4)	1.55	(3.7)	0.11	P=0.523
P (Colwell) - log(x+1)	35	2.08	(7.0)	2.07	(6.9)	0.23	P=0.961
P buffer (Colwell) - log(x+1)	35	4.91	(135)	5.09	(161)	0.12	P=0.180
Ca (Amm Acet) - log (x+1)	35	2.17	(7.7)	2.42	(10.2)	0.12	P=0.056
K (Amm Acet) - log (x * 100)	35	3.74	(0.42)	3.70	(0.40)	0.19	P=0.834
Mg (Amm Acet) - log (x+1)	35	1.61	(4.0)	1.64	(4.2)	0.12	P=0.779
Na (Amm Acet) - log (x * 100)	35	2.90	(0.181)	2.92	(0.186)	0.19	P=0.897
Ca/Mg (Amm Acet) - log (x+1)	35	1.09	(1.98)	1.34	(2.82)	0.10	P=0.018
CEC (Al; Amm Acet) - log(x+1)	34	2.69	(13.7)	2.84	(16.1)	0.12	P=0.232
Na% of Cations (Al) - log(x+1)	35	1.01	(1.75)	0.89	(1.44)	0.11	P=0.304
Cu (DTPA)	34	1.68		1.70		0.14	P=0.897
Fe (DTPA) - log(x+1)	35	3.91	(48.7)	3.75	(41.5)	0.078	P=0.064
Mn (DTPA) - log(x+1)	35	3.09	(20.9)	2.96	(18.3)	0.22	P=0.576
Zn (DTPA) - log(x+1)	34	0.36	(0.44)	0.40	(0.49)	0.042	P=0.388
Boron (CaCl2)	34	0.83	(1.30)	0.86	(1.36)	0.085	P=0.759
S (MCP) - log(x+1)	34	2.31	(9.1)	2.32	(9.2)	0.10	P=0.932
Organic Carbon - log(x+1)	34	0.91	(1.5)	0.90	(1.5)	0.063	P=0.844
P (BSES) - log(x+1)	35	2.69	(13.8)	2.73	(14.4)	0.19	P=0.832
Silicon – CaCl	27	59.7		58.6		4.1	P=0.789
Organic Matter- log(x+1)	35	1.31	(2.7)	1.24	(2.5)	0.07	P=0.358
Avail. K (Amm Acet) - log(x+1)	34	5.04	(153)	5.06	(157)	0.16	P=0.868
Ca % of Cations	19	46.8		57.2		3.5	P=0.017
Mg% of Cations	19	31.8		27.5		4.4	P=0.363
K% of Cations	19	7.28		4.63		1.72	P=0.157

4.4 Impact of *Heliococcus sumervillei* (pasture mealybug) on Bisset creeping bluegrass

4.4.1 Background

Heliococcus summervillei (pasture mealybug) has been identified as a primary causal agent of pasture dieback (MLA, C Hauxwell, pers comm 2020). When the research reported here commenced in 2020, this relationship had not been empirically described and was largely based on correlations between the incidence of pasture mealybug and plant symptoms attributed to pasture dieback. *H. summervillei* is a native species (Williams 1985) with an apparently wide distribution. Reports of dieback are more frequent in introduced (non-native) pasture species than in native species (Buck 2017), which suggests that *H. summervillei* is not in itself a highly damaging pest or persists at sub-

damaging levels in these native pastures. It is possible that introduced pasture species are more susceptible to direct feeding by this mealybug, or that the mealybug populations are larger and more damaging in these species.

The research reported here was designed to test the impact of a persistent, high density mealybug infestation on actively growing pasture. The primary objective of this work was to determine empirically the nature and scale of the impact on pasture productivity.

Following two seasons working with pasture mealybug in the glasshouse, it was apparent that reliable, quantitative trial data could not be obtained from pot trials because of the enormous inter-plant variation that existed in commercial grass lines. The amount of variation observed between plants/pots in the glasshouse trials, based on plant growth parameters (e.g. rate of biomass production, leaf:stem ratio, height), made it impossible to find statistical differences between applied treatments. In addition, the watering regime required to maintain pasture grasses in pots precluded the population of roots by mealybugs. Observations of mealybug distribution in the field indicated that infestation of the roots comprised a significant component of the overall population at some periods during the year. It was considered possible that without infestation of the roots, the full impact of mealybug on the plants may not be realised. As a result, the decision was made to establish a replicated, small plot trial at Brian Pastures Research Facility (BPRF), where mealybug populations and pasture growth could be manipulated. The small plots represent a population of plants, reducing the effect of the inter-plant variation experienced in pot trials. Bisset creeping blue grass (*Bothiochloa insculpta*) was selected for this trial as it is a variety for which there are many producer reports of dieback.

4.4.2 Materials and methods

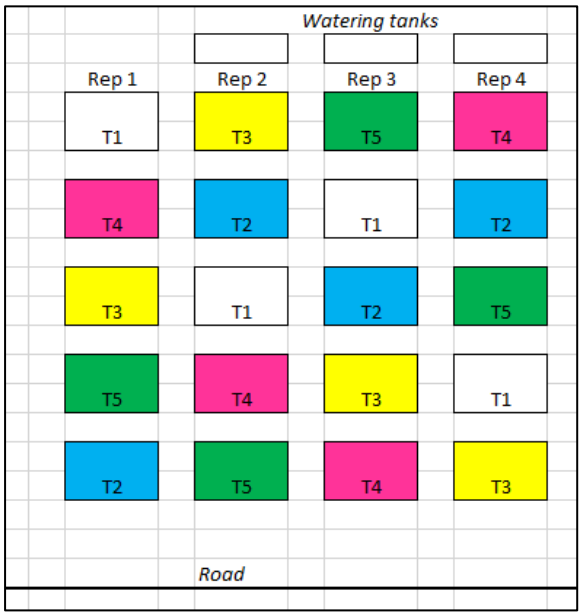
A replicated cage trial was conducted on a single pasture variety as a proof of concept. A stand of Bisset creeping blue grass (*Bothiochloa insculpta*) was identified at the BPRF, Ban Springs, Qld (-25.661006, 151.742909), where previous monitoring had identified persistent mealybug infestations. Caged, rather than open plots, were used to provide some protection from natural enemy impacts on the mealybug populations during the trial. Because of the influence that the cages may exert on growth of the grass, all plots were caged.

The site was originally selected in July 2020 for its uniform stand of creeping blue grass. The grass was slashed on 7 September 2020 to even out the stand across all plots. On the 10th September, 20 cage frames were set up in a randomised block design of 5 treatments x 4 replications. Each cage measured 1.8 m (H) x 1.8 m (W) x 1.8 m (D). Up to that point, BPRS had had insufficient rainfall to stimulate grass growth, but as temperatures were deemed sufficiently warm for growth irrigation (35 mm equivalent) was applied across the trial site to stimulate growth. Each plot was left uncovered to encourage a natural infestation of mealybugs across the trial site.

On 8 October 2020, a small population of mealybugs was observed in all cage areas, and the trial commenced. Cage covers were put on all plots and treatments assigned to each cage, according to the design described (Figures 24 and 25). Uninfested control treatments (no mealybugs) were sprayed with 400 mL/ha Movento® 240 SC (240g/l Spirotetramat) plus Hasten® spray adjuvant at 1 L/ha as per APVMA permit 88482. Spirotetramat was selected as the insecticide to minimise mealybug infestations because it has systemic activity which includes translocation of insecticide to the roots of the plant. Consequently, this product is most likely to control mealybug infestations that occur above and below ground.

Figure 24. Layout of the pasture mealybug impact trial BPRS, October 2020 – November 2021.

T1= treated control (no mealybug), T2= no mealybug + artificial grazing, T3 = Untreated control (mealybug), T4 = mealybug + artificial grazing, T5 = Untreated until clear symptoms evident (late control of mealybug).



Whilst the trial design incorporated a total of five treatments, not all treatments were implemented from the commencement of the trial. For the full complement of treatments to be implemented, the trial needed the following conditions (i) enough pasture growth to apply the simulated grazing stress (hand cutting to remove biomass) and (ii) emergence of plant symptoms consistent with early stages of pasture dieback. These conditions were not fully met until 4 December 2020 (see section 9.3).

Persistent dry conditions meant that supplementary irrigation was necessary to maintain grass growth, and cages were irrigated on 10 September, 8 October, 20 October and 18 November 2020. Post November 2020, there was sufficient rainfall, and no additional irrigation was applied for the remainder of the growing season. In 2021, when temperatures were deemed sufficiently warm to stimulate growth, supplementary irrigation was applied to commence regrowth in the plots in August. Further supplementary irrigation was applied as required, to November 2021.

Figure 25. Cage frames (top) defining plots prior to the commencement of the trial in September 2020. Cages with covers in place in October 2020 (bottom).

The timetable on which treatments were applied, and assessments made is presented in Table 31.

Table 31. Staged application of treatments to the pasture mealybug impact trial. October 2020 - November 2021.

Treatment	Treatment Description	Date applied	Notes
T1	Treated control (no mealybug)	8 October 2020	Insecticide applied at approximately 3-4 week intervals throughout the trial. 8 October, 30 October, 24 November, 5 January 2021.
T2	Treated + artificial grazing (no mealybug + grazing stress)	Grazing: 4 December 2020	Treated for mealybug as per T1. Artificial grazing = whole plot cut close to ground with hand shears. Cut material removed from plot.
T3	Untreated Control (mealybug infestation)	8 October 2020	
T4	Untreated + artificial grazing (mealybug + grazing stress)	Grazing: 4 December 2020	Grazing as per T2.

T5	Sprayed when dieback symptoms emerge (Late spray to control mealybug)	4 December 2020	Treated as per T1 from 4 Dec.
From August 2021, insecticide (spirotetramat) was applied to all plots at 3-4 week intervals to exclude mealybug from potentially impacting the regrowth of the plots.			

All cages were sprayed with Dicamba on 30 October 2020, 24 November 2020 and 5 January 2021 to control broadleaf weeds within the cages. The area between the cages was mown on 11 January 2021 to minimise invasion of grass into caged plots and competition with plots.

4.4.3 Data collection and analysis

Site visits for data collection and trial maintenance were made on 30/9/20, 30/10/20, 24/11/20, 4/12/20, 5/1/21, 19/2/21, 6/5/21 and 5/8/21, 16/9/21 and 25/11/21.

At site visits, the following data were collected:

1. Photographs were taken of each cage to document the progression of symptoms in December-February 2020 and recovery of plots in the spring of 2021.
2. Mealybug density was assessed by suction DVAC. The end of DVAC was covered by a fine gauze sock and for 30 seconds at full throttle was moved through the grass stand. The resulting collection was transferred into a plastic bag and mealybug density assessed in the laboratory. These collections were made on 30/9/20, 30/10/20, 24/11/20, 4/12/20, 5/1/21, 19/2/21, 6/5/21 and 5/8/21.
3. Biomass cuts were made to assess the decline and recovery of the pasture on 4/12/20, 6/5/21, 25/11/21. No biomass cut was taken in January 2021 because there had been insufficient regrowth across the plots to provide meaningful data.

Differences between treatments (mealybug density and biomass) were analysed by ANOVA (Genstat 19th edition).

4.4.4 Results and discussion

The progression of symptoms and impact on pasture growth with and without mealybug is presented in section 9.3 through a time series of plot photographs.

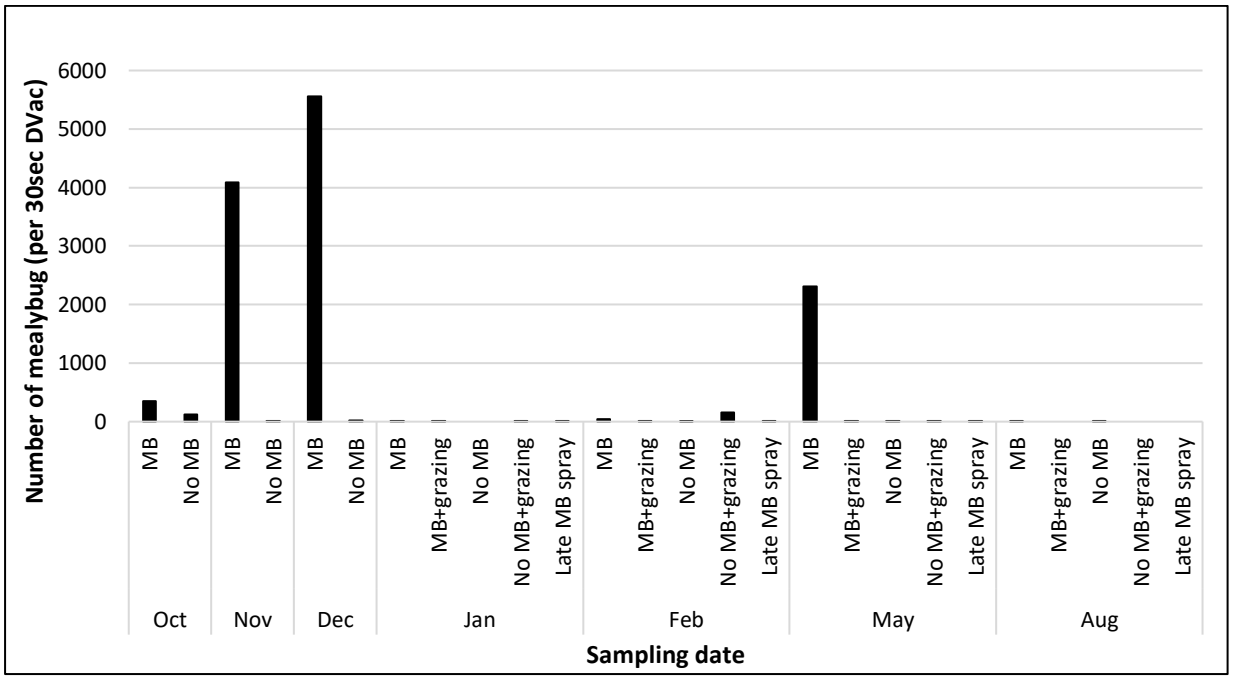
4.4.4.1 Mealybug density

Mealybug densities in the unsprayed plots (MB) were significantly higher across all dates than densities in the sprayed plots (no MB) (Fig. 26). Whilst data in Figure 26 are averaged across the trial, and provide a relative indication of mealybug infestation levels, it was clear from individual sampling events that there was significant variation over time in the mealybug pressure in each treatment (Fig. 27). The most likely explanation for the overall decline in the mealybug densities is the death of the pasture in the mealybug-infested plots, and consequently limited host availability.

Figure 26. Mean mealybug density by treatment across the trial. Means are not significantly different if followed by the same letter (p=0.05, Fisher's protected LSD test). These density estimates are combined estimates of small, medium and large mealybug.

Treatment	**Mean mealybug density (per 30 sec suction sample)**
T2= no mealybug + artificial grazing	42 a
T1= treated control (no mealybug)	186 a
T5 = Untreated until clear symptoms evident (late control of mealybug).	866 ab
T3 = Untreated control (mealybug)	2465 bc
T4 = mealybug + artificial grazing	2675 c

Figure 27. Mean mealybug density across treatments for each sampling event throughout the trial, prior to the period of regrow (August-November 2021) when all plots were sprayed to allow for unimpeded evaluation of regeneration.



4.4.4.2 Quantifying the impact of mealybug on pasture growth

Pasture productivity was measured in terms of biomass production at the end of the first growing season (6/5/2021) and then post regeneration the following spring (25/11/25) (Fig. 28).

The results from the assessment on 6/5/21 provides insight into the immediate impact of the treatments on productivity during the growing season. The November 2021 assessment looks to determine the longer-term impacts of the treatments on pasture productivity.

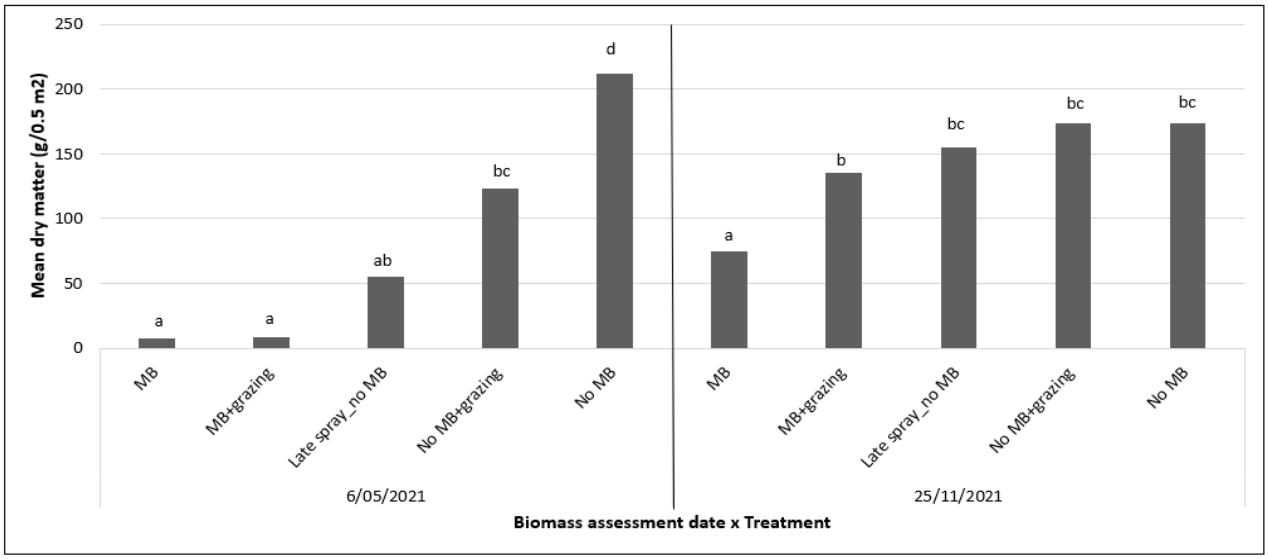
4.4.4.2.1 Immediate impact of treatments

The results show that mealybug infested treatments accumulated significantly lower biomass than treatments where mealybug were excluded by spraying for all of the growing season. Late spraying (after symptoms were evident in the plots) did not prevent a depression of biomass, compared with the treatment where mealybug were excluded all season. This suggests that prolonged mealybug infestation can lead to major reductions in pasture productivity, but even relatively short periods of exposure early in the season, 6-8 weeks in this trial, can have a persisting negative impact on productivity.

Where mealybug infested plots were also 'grazed' there was no significant difference in mealybug numbers between this treatment (T4) and the 'ungrazed' mealybug infested treatment (T3). However, the biomass yields in the mealybug-infested treatments were very low, so the impact of grazing (if there was one) would have been difficult to detect. Alternatively, mealybug infestation could have imposed such a significant stress that the addition of grazing would not have been an additive stress.

The "late spray" treatment showed increased productivity over the unsprayed treatments but was still significantly less productive than the sprayed treatments. This result suggests that an extended period of mealybug infestation could have season-long impact from which the exposed plots wouldn't recover, even after being sprayed to remove mealybug.

Figure 28. Pasture productivity (biomass production, g DM/0.5 m^2) assessed at the end of the first growing season (6/5/2021) and once pasture had regenerated in the second growing season (25/11/21). Mealybug were excluded from all plots from August 2021 to allow for assessment of regenerating crowns and seedling recruitment. Means with the same superscript letters are not significantly different (LSD p=0.05).



4.4.4.2.2 Regeneration of plots – longer term impacts of mealybug infestation

Assessment of regeneration was made on 25/11/2021. Regeneration, where it occurred, was primarily from plants (crowns) that persisted from the first growing season. Very little recruitment, from seed, was observed in any of the plots. What is very clear from these data is the persistent impact of earlier mealybug infestations on regeneration. The majority of crowns that were present in mealybug-infested plots in May 2021 did not regenerate by November 2021. However, those that did were productive, resulting in the MB+ grazing treatment being as productive as the treatments without mealybug. It is possible that mealybug density plays a role in the extent of plant death, or that the removal of plant material in the artificial grazing also removed a major proportion of the mealybug population.

Figure 29 provides a visual representation of the treatments for three dates during the growing season. The differences in the biomass of the plots under the different treatment regimes is clearly evident.

Visual representation of the regeneration across plots is presented in Fig. 30.

Figure 29. Visual representation of pasture growth from representative plots for each of the treatments applied. On December 4, 2020 and January 5, 2021 there were only two treatments – no mealybug (No MB) and mealybug-infested (MB). At the end of the season, when the final biomass assessment was made (6/5/2021) a range of responses to the treatments was evident. These images show the variation between plots, which is an ongoing challenge for replicated trials in pasture. Bisset creeping blue grass at BPRF.

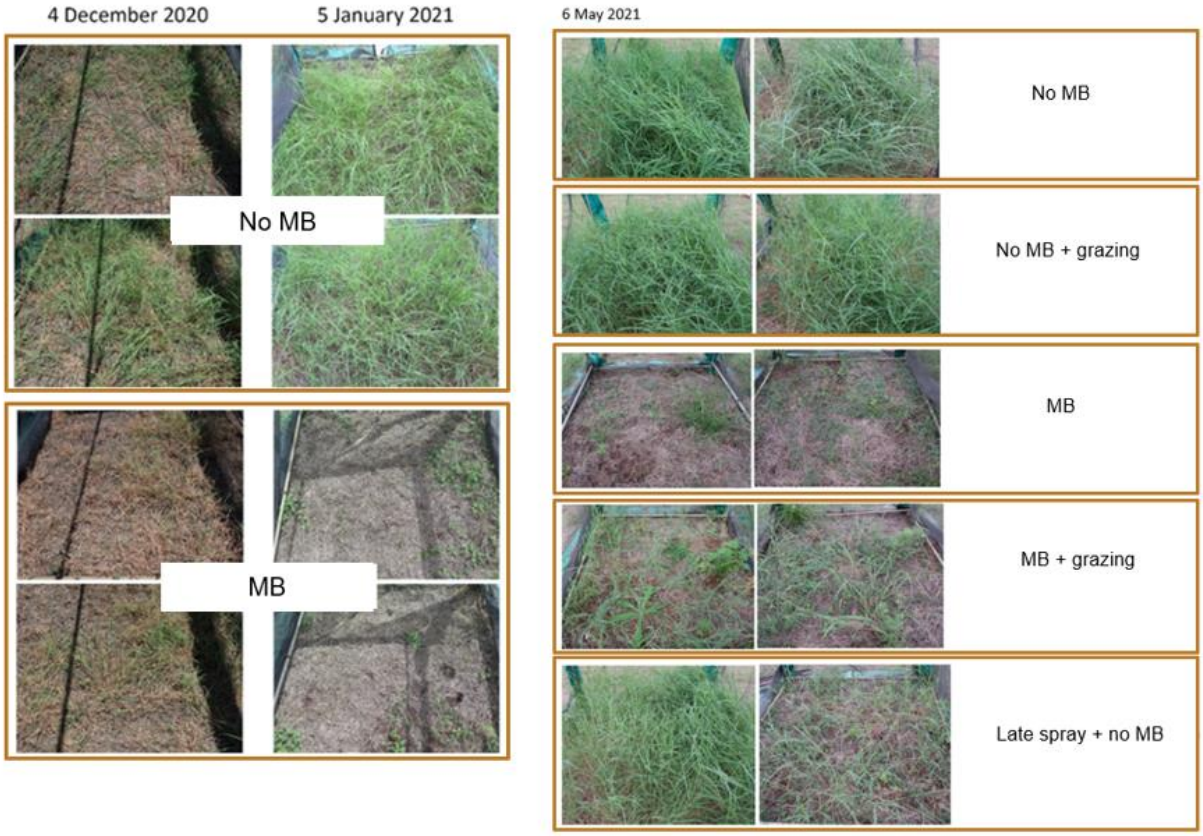
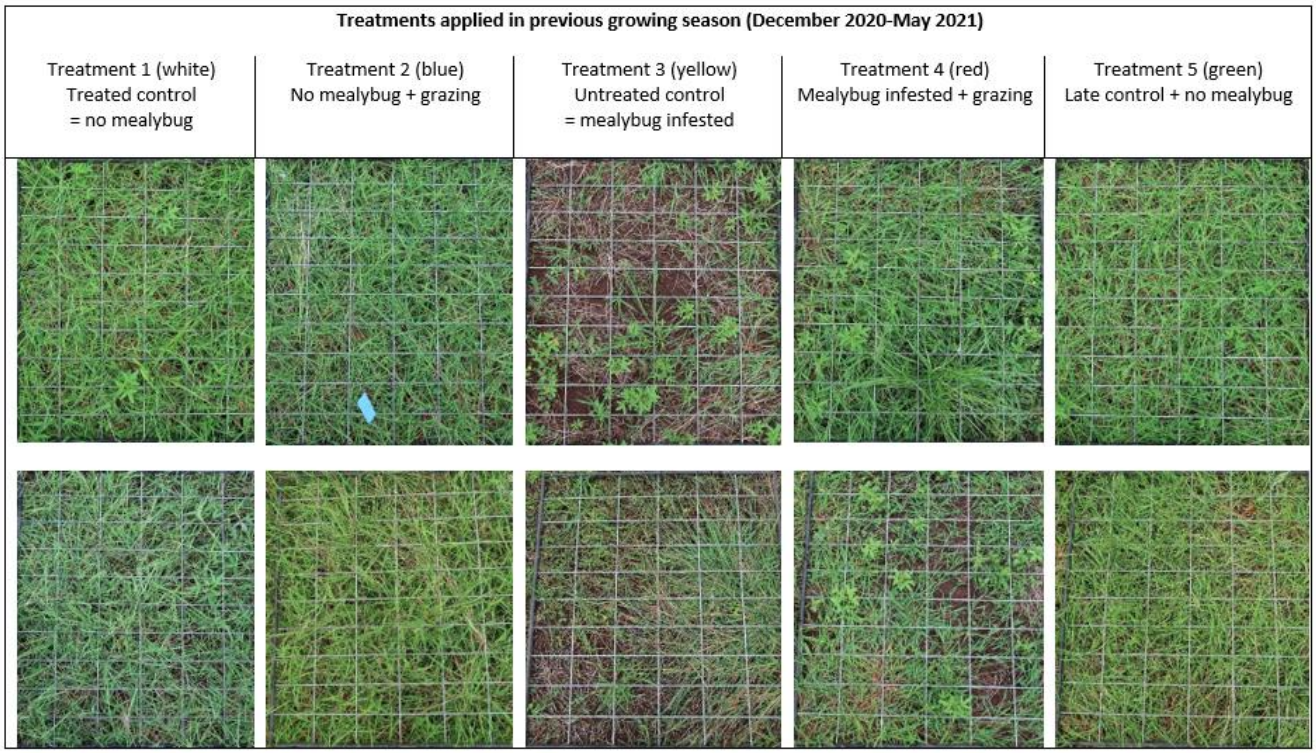


Figure 30. Visual representation of regeneration of plots at 25/11/21. Mealybug excluded from all plots from August 2021 when pasture regeneration commenced. Bisset creeping blue grass, Brian Pastures Research Station, Gayndah.



4.4.5 Conclusions

These data provide evidence of direct and persistent impact of pasture mealybug infestations on the productivity and survival of Bisset creeping blue grass. Mealybug-infested plots exhibited symptoms consistent with those of pasture dieback within four weeks of the first mealybug being detected. Reduced pasture growth and plant death occurred rapidly after the appearance of leaf symptoms. There was very limited regeneration of heavily impacted plots the following spring, despite excluding mealybug during the period of regeneration. In this trial, pasture dieback was terminal for many of the plants directly infested by mealybug. Plots in which mealybug were controlled once symptoms were established remained productive during the summer and regenerated the following spring.

It is important to acknowledge that we cannot discount the possibility that the impact of mealybug infestations on Bisset productivity and plant death could be the result of a disease vectored by mealybug, not simply a function of direct mealybug feeding activity. The persistence of impacts on productivity, including plant death, are indicative of severe and permanent damage to the plant (e.g. the vascular system) from mealybug feeding, or possibly the transmission of pathogen/s.

The rapid and terminal decline of Bisset blue grass in this trial cannot be extrapolated further than for this pasture variety. Previous observations of mealybug infestations (M Miles, DAF Qld) on a range of introduced pasture species have identified differing responses in terms of:

- symptoms (colour of leaves can vary from yellow to red)
- the rate at which symptoms appear after the introduction of mealybug, and
- the severity of the symptoms and resulting impact on plant growth characteristics.

A valuable extension of this preliminary trial with Bisset blue grass would be broader screening of native and introduced pasture species. This work would provide more robust recommendations around the need for, and timing of, mealybug control/management to minimise loss of pasture productivity and persistence. Also, sharing of methodology for working with this challenging pest, and with highly variable pasture grasses, would be of value in ensuring efficiency in achieving outcomes across research groups. The collaboration between entomologists and pasture specialists has greatly benefited this research effort.

4.5 Virology diagnostic analysis

4.5.1 Background

Plant virus involvement in pasture dieback has not been examined until recently. Many plant viruses are known to cause reddening and yellowing of leaves, which leads to reduced vigor, plant decline, and sometimes death. Cereal yellow dwarf viruses and maize yellow dwarf virus (genus Polerovirus, family *Solemoviridae*) and barley yellow dwarf viruses (unassigned species, family *Solemoviridae*) have small isometric particles and affect wheat, barley, oat, maize and rice crops. Panicum mosaic virus (PMV, syn. St Augustine's decline virus, Panicovirus, family *Tombusviridae*) also has small isometric particles and infects *Stenotaphrum secundatum* causing decline and death of turf. Maize is also a known host of PMV. Wheat streak mosaic virus (Potyvirus, family *Potyviridae*) has long, flexuous rod-shaped particles and affects wheat, corn, rye, oats, barley, sorghum, millet and some grassy weeds. Several other potyviruses, including sugarcane mosaic virus, are also known to affect cereal crops. A wide range of mastreviruses (family *Geminiviridae*) with geminate particles are known to infect grasses of Australia. Rice tungro disease is caused by synergism between two viruses with spherical (Waikavirus, family *Secoviridae*) and bacilliform (Tungrovirus, family *Caulimoviridae*) particles respectively. Additionally, some viruses with very broad host ranges are also reported to infect cereals and grasses. And, as with other less well studied pathosystems, the possible existence of novel viruses is high.

Transmission of plant viruses may be through insect vectors, mechanical means, seed, pollen and rarely fungi. Badnaviruses (family *Caulimoviridae*), ampeloviruses and velariviruses (family *Closteroviridae*) and vitiviruses (family *Betaflexiviridae*) are known to have mealybug vectors. Additionally, symptoms may have more than one cause e.g. weakened plants may be more susceptible to one or more mealybug-transmitted viruses or other pathogens. Environmental conditions may also play a role in plant susceptibility or symptom expression.

Electron microscopy of pasture dieback samples has detected only small isometric virus particles of a range of sizes (26-40nm, spherical and hexagonal); no rod-shaped or bacilliform particles have been observed. This excludes some taxonomic groupings e.g. plant pathogenic viral taxa with rod-shaped particles usually found at moderate to high concentrations if present (e.g. tobamoviruses, potyviruses, potexviruses). However, virus concentration in a plant is not always high (particularly for phloem limited viruses) and consequently electron microscopic examination of a sample may not detect virus particles present at only low concentrations.

High throughput sequencing of pasture grass samples was undertaken as a non-specific method for identifying plant viruses present in dieback affected pastures.

4.5.2 Methods

4.5.2.1 Sample collection and receipt

Leaf samples for virology analysis were collected at the same time and manner as described in Section 4.2.1.1. Leaf samples from either single plants or bulks of multiple plants with the same symptoms were dried and stored over silica gel at -20 °C. From late 2021, stem samples were included with the leaf samples.

The samples used for targeted analyses are included in each section below.

4.5.2.2 Phytoplasmas

Generic phytoplasma testing was conducted on the samples listed in Table 1 using a nested PCR for the 16S rRNA gene (Makarova et al., 2012). Products were Sanger sequenced as described in section 4.5.2.2.

4.5.2.3 Poleroviruses

Representative pasture dieback samples (Table 32) were tested for poleroviruses using the Pol3870F/AS3 primer pair (Table 2) with sample extraction and RT-PCR protocol similar to that described in section 4.5.2.5.

Five sets of RT-PCR primers (Table 33) were used on 12 samples (Table 34) from Mt French to identify the individual samples from which the barley virus G sequence was assembled from HTS pool 17 / KCNGS028. Sample extraction and RT-PCR protocol was similar to that described in section 4.5.2.5. When required, PCR amplicons were sent for direct Sanger sequencing by AGRF (Brisbane, Australia).

Table 32. Representative symptomatic pasture dieback samples tested for phytoplasmas and poleroviruses using generic primers. Pasture grass species displayed typical leaf yellowing and reddening; the rattle pod sample displayed typical phytoplasma little leaf symptoms.

Sample	Common name	Scientific name	Location	Collection date	HTS pool
20	Buffel grass cv. Gayndah	*Cenchrus ciliaris*	Thangool	9/03/2020	1
37	Buffel grass cv. Biloela	*Cenchrus ciliaris*	Thangool	9/03/2020	4
65	Green Panic	*Panicum maximus*	Orange Creek	10/03/2020	9
66	Sabi grass cv. Nixon	*Urochloa mosambicensis*	Baralaba	10/03/2020	10
70	Forest Bluegrass	*Bothriochloa bladhii*	Baralaba	10/03/2020	14
93	Silk Sorghum	*Sorghum halepense*	Kianga	11/03/2020	15
116	Buffel grass cv. Gayndah	*Cenchrus ciliaris*	Lochington	12/03/2020	2
153	Angleton grass	*Dicanthium aristatum*	The Gemfields	12/03/2020	7
199	Creeping bluegrass cv. Bisset	*Bothriochloa insculpta*	Coulson	30/03/2020	6
275	Rattlepod	*Crotalaria sp*	Mt French	30/04/2020	-
337	Setaria	*Setaria sphacelata var. anceps*	Boonah	1/05/2020	16
1/11/19-06	Rhodes grass	*Chloris gayana*	Kalbar	1/11/2019	12

Table 33. Polerovirus primers used for polerovirus and barley virus G diagnostic testing.

Primer name	Sequence (5' - 3')	Product size (bp)	Reference
PLF	ACDGAYTGYTCYGGTTTYGACTGG	1058	Corrêa et al., 2005
PLR	TCTGAWARASWCGGCCCGAASGTGA		Corrêa et al., 2005
Pol3628F	TAATGAATACGGYCGYGGBTAG	354	Sharman et al., 2015
Pol3982R	CGAGGCCRCGGAGATGAACT		Sharman et al., 2015
Pol3870F	ATCACBTTCGGGCCGWSTYTWTCAGA	370	Sharman et al., 2015
AS3	CACGCGTCIACCTATTTIGGRTTITG		Abraham et al., 2008
BVG3646up	AACACTTCAGGAGGATCTGGA	602	Gavrili et al., 2021
BVG4247down	AACTCGGAATTCTTGCGTGA		Gavrili et al., 2021
BVG proso millet F	GTGAGTTGCAAGTACTGGAT	988	Park et al., 2016
BVG proso millet R	GTACCCTGCCGAAAGTGTT		Park et al., 2016

Table 34. Samples from Mt French tested individually for barley virus G.

Sample	Common name	Cultivar	Scientific name	HTS pool
272	Buffel grass	Gayndah	*Cenchrus ciliaris*	17
278	Bread grass	Mekong	*Brachiaria brizantha*	17
288	Green panic	NuCal	*Panicum maximus*	17
290	Angleton grass	Floren	*Dicanthium aristatum*	17
292	Rhodes grass	Reclaimer	*Chloris gayana*	17
295	Rhodes grass	Epica	*Chloris gayana*	17
267	Kikuyu		*Pennisetum clandestinum*	18
274	Narok Setaria	Narok	*Setaria sphacelata var. sericea*	18
279	Kleingrass	ATF714	*Panicum coloratum var. coloratum*	18
282	Green panic	MegaMax059	*Panicum maximus*	18
283	Signal grass	Basilisk	*Brachiaria decumbens*	18
286	Buffel grass	Tarwinnabar	*Cenchrus ciliaris*	18

4.5.2.4 Sequencing

Twelve pooled RNA samples were prepared and sent to AGRF (Melbourne, Australia) for high throughput sequencing (Table 35). Total plant nucleic acid was extracted from pooled dried leaf pieces using the Invitrogen™ TRIzol™ Plus RNA Purification Kit (ThermoFisher Scientific) according to the manufacturers' instructions. Contaminating DNA was removed using DNaseI (New England Biolabs) following the protocol of Asif et al. (2000) but omitting the polysaccharide removal step. RNA extracts were then sent to AGRF for ribosomal RNA removal and library preparation using a TruSeq Stranded Total RNA with Ribo-Zero Plant kit (Illumina), followed by sequencing. Total library size averaged 300 base pairs (bp) and 150-bp paired-end sequencing was performed with an Illumina NovaSeq sequencer and 300 cycle kit. Data output from the flowcell (ID: HM3J7DRXX) are presented in Table 5.

Sanger sequencing was conducted by AGRF (Brisbane, Australia) on selected PCR amplicons.

4.5.2.5 Bioinformatics analysis

High throughput sequencing (HTS) analysis was conducted through Galaxy Australia. De-multiplexed sequence read data sets were initially quality filtered, paired and size trimmed to remove sequencing primers and low-quality bases using Trimmomatic version 0.36.6 (Bolger et al., 2014). *De novo* assembly was conducted using the MEGAHIT algorithm v1.1.3 (Li et al., 2015) using k-mers of either 27 or 25,35,45,55,65,75; output was restricted to contigs of at least 500 nt. The NCBI BLAST suite v2.7.1 (Camacho et al., 2009) was used to identify contigs with viral similarity, using the Virus RefSeq nucleic acid and protein databases (downloaded on 11 July 2020) for reference (Pruitt et al., 2005). Additional bioinformatics analysis was conducted using Geneious v10.0.3 (Biomatters Inc, New Zealand).

Table 35. Pooled leaf RNA samples sent for high throughput sequencing for viral sequence analysis.

RNA pool	Sample details	Number of samples	Sites	RNA A260/280 ratio	RNA Concentration
1 / KCNGS012	Buffel grass cv. Gayndah - Moura to coast	6	5	1.953	170.50
2 / KCNGS013	Buffel grass cv. Gayndah - west of Moura	6	6	2.078	237.50
3 / KCNGS014	Buffel grass cv. Biloela - yellowing	5	5	2.096	391.60
4 / KCNGS015	Buffel grass cv. Biloela - yellowing and reddening	5	4	2.088	340.90
5 / KCNGS016	Red Natal grass, QLD bluegrass, Native millet/Yabila grass, nut grass	3	1	2.091	358.70
6 / KCNGS017	Creeping bluegrass cv. Bisset	5	4	2.026	347.60
7 / KCNGS018	Angleton grass (2 Floren)	5	5	1.997	189.10
8 / KCNGS019	Green panic (3*) & Bambatsi panic (2)	5	2	2.064	322.80
9 / KCNGS020	Green panic - interveinal chlorosis	5	5	2.097	362.40
10 / KCNGS021	Sabi grass - yellowing	5	4	1.979	121.30
11 / KCNGS022	Rhodes grass* - spherical 26nm isometric virus particles	1	1	2.027	164.20
12 / KCNGS023	Rhodes grass* - yellowing/reddening	6	4	2.053	238.60
13 / KCNGS024	Rhodes grass - strong striations	3	3	2.083	295.40
14 / KCNGS025	Bothriochloa (5) and Urochloa (1)	6	5	2.027	88.42
15 / KCNGS026	Silk sorghum	4	1	2.035	350.80
16 / KCNGS027	Red Natal grass, scented top grass, black spear grass, Setaria sp., Digit grass, Strickland fingergrass	6	3	2.023	161.40
17 / KCNGS028	Mount French site - no virus symptoms	6	1	1.990	381.10
18 / KCNGS029	Mount French site – virus symptoms	6	1	2.067	371.90
19 / KCNGS030	*Megathyrsus maximus* - virus symptoms†	3	2	2.106	514.80
20 / KCNGS031	#2871 BYDV-notMAV (Oat yellow dwarf virus) CONTROL‡	1	1	2.045	180.00

* Virus particles had previously been detected in these leaf samples.

† Isolates 5174, 5578, 5579 were collected from Kenilworth and Brisbane.

‡ Isolate 2871 was included as a Polerovirus control for the experiment.

Table 36. Data yield from high throughput sequencing run for virology leaf samples.

Lane	Sample Name	Paired Reads	Data Yield (bp)
2	KCNGS012	19,490,846	5.89 Gb
	KCNGS013	16,845,239	5.09 Gb
	KCNGS014	19,799,694	5.98 Gb
	KCNGS015	18,142,676	5.48 Gb
	KCNGS016	19,370,171	5.85 Gb
	KCNGS017	23,286,587	7.03 Gb
	KCNGS018	20,067,200	6.06 Gb
	KCNGS019	21,970,832	6.64 Gb
	KCNGS020	20,038,866	6.05 Gb
	KCNGS021	20,933,952	6.32 Gb
	KCNGS022	18,187,623	5.49 Gb
	KCNGS023	29,687,155	8.97 Gb
	KCNGS024	22,854,980	6.90 Gb
	KCNGS025	19,760,038	5.97 Gb
	KCNGS026	18,147,816	5.48 Gb
	KCNGS027	22,700,734	6.86 Gb
	KCNGS028	19,214,821	5.80 Gb
	KCNGS029	18,967,427	5.73 Gb
	KCNGS030	20,061,130	6.06 Gb
	KCNGS031	18,759,824	5.67 Gb
Total		408,287,611	123.30 Gb

4.5.2.6 Tobacco streak virus

To investigate the detection of tobacco streak virus (TSV) in five HTS pools, diagnostic screening using an enzyme linked immunosorbent assay (ELISA) was undertaken on 24 individual samples from these pools. These samples are listed in Table 37. A double antibody sandwich ELISA specific for tobacco streak virus (Agdia Inc, USA; catalogue number SRA 25500) was used as per manufacturer's protocol. Positive controls were included from the DAF virology isolate collection: #1972, sunflower from Clermont, QLD in 2006 and #2074, chickpea from Emerald, QLD in 2007. An uninfected grass and extraction buffer only negative control were also included in the assay.

Inoculation experiments were conducted in the glasshouse at the Ecosciences Precinct, Dutton Park. Buffel grass was opportunistically inoculated in February 2021with TSV isolate #1973 in *Nicotiana glutinosa* (originally from a sunflower field sample from Emerald). A planned inoculation experiment was conducted with Buffel grass cv. Gayndah, Buffel grass cv. Biloela, Creeping bluegrass cv. Bisset, Rhodes grass cv. Epica, Rhodes grass cv. Reclaimer, Gatton panic, Bambatsi panic, Indian couch cv. Keppel, Red Natal grass and *N. glutinosa* as a susceptible control. Seeds were planted in trays in the glasshouse. Ten seedlings of each species were potted on into individual pots and mechanically inoculated with isolate #1973 (symptomatic petals). Plants were monitored for the development of symptoms with the intention of using the diagnostic assay to confirm virus transmission.

Table 37. List of pasture dieback samples indexed for TSV.

Sample	Scientific name	Common name	Collection date	Site	HTS pool
26/02/20-07	*Cenchrus ciliaris*	Buffel grass cv. Biloela	20/02/20	Orange Creek	3
19	*Cenchrus ciliaris*	Buffel grass cv. Biloela	9/03/20	Thangool	3
99	*Cenchrus ciliaris*	Buffel grass cv. Biloela	11/03/20	Kianga	3
112	*Cenchrus ciliaris*	Buffel grass cv. Biloela	11/03/20	Coorumbene	3
222	*Cenchrus ciliaris*	Buffel grass cv. Biloela	27/04/20	Gayndah	3
12	*Urochloa mosambicensis*	Sabi grass	9/03/20	Bajool	10
13	*Urochloa mosambicensis*	Sabi grass	9/03/20	Bajool	10
32	*Urochloa mosambicensis*	Sabi grass	9/03/20	Thangool	10
62	*Urochloa mosambicensis*	Sabi grass	10/03/20	Orange Creek	10
66	*Urochloa mosambicensis*	Sabi grass cv. Nixon	10/03/20	Baralaba	10
31	*Bothriochloa pertusa*	Indian couch	9/03/20	Thangool	14
92	*Bothriochloa pertusa*	Indian couch	11/03/20	Kianga	14
184	*Bothriochloa pertusa*	Indian couch	10/03/20	Baralaba	14
70	*Bothriochloa bladhii*	Forest bluegrass	10/03/20	Baralaba	14
183	*Bothriochloa ewartiana*	Desert bluegrass	13/03/20	Blackwater	14
251	*Brachiaria decumbens*	Signal grass cv. Basilisk	28/04/20	Gayndah	14
93	*Sorghum halepense*	Silk Sorghum	11/03/20	Kianga	15
96	*Sorghum halepense*	Silk Sorghum	11/03/20	Kianga	15
97	*Sorghum halepense*	Silk Sorghum	11/03/20	Kianga	15
98	*Sorghum halepense*	Silk Sorghum	11/03/20	Kianga	15
94	*Melinis repens*	Red Natal grass	11/03/20	Kianga	5
100	*Dichanthium sericeum*	Queensland bluegrass	11/03/20	Kianga	5
102	*Panicum decompositum*	Native millet/Yabila Grass	11/03/20	Kianga	5
105	*Cyperus rotundus*	Nut grass	11/03/20	Kianga	5

4.5.2.7 Velariviruses

Diagnostic primers for each of the novel velariviruses detected in Rhodes grass HTS pools were designed to conserved genomic regions (Table 38, Fig. 31). These primers were used to index a range of Rhodes grass samples (Table 39) using the following protocol.

Total nucleic acid was extracted using a BioSprint 15 workstation (Qiagen) and BioSprint 15 Plant DNA kit (catalogue number 941514), as per the manufacturer's instructions but without the use of RNase A. SuperScript III reverse transcriptase (Invitrogen) was used to prepare cDNA as per the manufacturer's instructions. PCR was performed using Taq DNA Polymerase (Invitrogen) and products were separated electrophoretically prior to staining and visualisation. Direct Sanger sequencing of PCR amplicons was performed to confirm amplification of viral sequence and check the identity with the HTS assembled sequence.

Table 38. Diagnostic primers for the two velariviruses detected in Rhodes grass.

Virus	Primer name	Sequence (5′ - 3′)	Tm (°C)	Product size (bp)
Grass velarivirus 1	6688F	TCTTCGTATGCGGCATGTTC	58.7	501
	7188R	TTCAGCTTTCACACCTACCC	57.4	
Grass velarivirus 2	4287F	TGTTGAAGATTTTGCGGCCG	60.0	485
	4775R	TCTCGAACACACTTCCACCG	60.0	

Figure 31. Position of diagnostic primers designed against assembled velarivirus sequences from Rhodes grass.

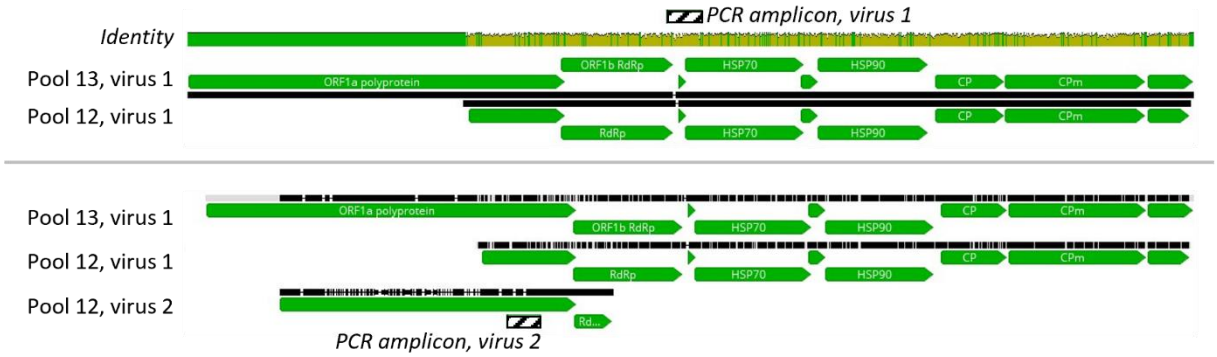


Table 39. Rhodes grass (*Chloris gayana*) sample list for velarivirus testing.

Sample	Cultivar	Collection date	Location	HTS pool
10/09/2019-02	Callide	10/09/2019	Kalbar	11
10/09/2019-05	Callide	10/09/2019	Kalbar	-
1/11/2019-01	Callide	1/11/2019	Kalbar	-
1/11/2019-02	Callide	1/11/2019	Kalbar	-
1/11/2019-03	Callide	1/11/2019	Kalbar	-
1/11/2019-04	Callide	1/11/2019	Kalbar	-
1/11/2019-05	Callide	1/11/2019	Kalbar	-
1/11/2019-06	Callide	1/11/2019	Kalbar	12
1/11/2019-11	Callide	1/11/2019	Kalbar	-
"CSMV"	Callide	12/12/2019	Kalbar	13
"less affected"	Callide	12/12/2019	Kalbar	-
"older runners"	Callide	12/12/2019	Kalbar	-
QG7451	Callide	12/12/2019	Kalbar	-
QJ5883	Callide	12/12/2019	Kalbar	-
QJ5884	Callide	12/12/2019	Kalbar	-
QJ5888	Callide	12/12/2019	Kalbar	-
QJ5890	Callide	12/12/2019	Kalbar	12
192	Callide	30/03/2020	Coulson	12
193	Callide	30/03/2020	Coulson	13
194	Callide	30/03/2020	Coulson	12
198	Callide	30/03/2020	Coulson	-
227	Callide	28/04/2020	Gayndah	12
228	Reclaimer	28/04/2020	Gayndah	-
229	Reclaimer	28/04/2020	Gayndah	-
264	Tolgar	28/04/2020	Gayndah	-
265	Tolgar	28/04/2020	Gayndah	-
277	Tolgar	30/04/2020	Mt French	-
281	Epica	30/04/2020	Mt French	-
284	Sabre	30/04/2020	Mt French	-
285	Mariner	30/04/2020	Mt French	-
291	Callide	30/04/2020	Mt French	-
292	Reclaimer	30/04/2020	Mt French	17
293	Callide	30/04/2020	Mt French	13
294	Epica	30/04/2020	Mt French	-
295	Epica	30/04/2020	Mt French	17
338	unknown	1/05/2020	Boonah	12

4.5.3 Results and discussion

4.5.3.1 Phytoplasmas

The *Crotolaria* sp sample with typical phytoplasma symptoms (275; Table 32) was positive in the diagnostic assay. Four symptomatic pasture grass samples (20, 66, 116, 337) and the healthy control produced bands of the expected size, however as these primers are known to also amplify *Bacillus* sp., PCR products were sent for Sanger sequencing. The amplicon for sample 275 was confirmed as *Candidatus* Phytoplasma australiense. No phytoplasma matches were obtained for the other samples. Further phytoplasma testing was not conducted.

4.5.3.2 High throughput sequencing

De novo assembly and BLAST (BLASTn, megaBLAST and BLASTx) analysis was undertaken for each of the 20 pooled samples. Assemblies with each of the k-mer values gave similar results but the 25-75 stepped k-mer values tended to result in longer contigs. MegaBLAST analysis tended to provide the most informative analysis of assembled contigs. Many contigs with matches to non-plant viruses were identified; these matched viruses of bacteria (phage), fungi, insects and vertebrates. Matches with plant virus species or genera are listed in Table40.

In one case (HTS sample 11 / KCNGS022), additional bioinformatic strategies to remove host genomic reads prior to de novo assembly were used as a high concentration of spherical 26 nm isometric virus particles had been observed under the electron microscope in a partially purified preparation but the virions of the assembled mastrevirus sequences were known to be geminate in shape. These strategies are described further in section 4.5.3.4. This instance does highlight those viruses with relatively low read numbers, or for which the assembly has not worked well, or with significant variation from known reference sequences may not be identified through this approach. Unfortunately, the additional strategies used for HTS sample 11 were not available for all other HTS samples as not all host genomes or the genomes of closely related hosts have been sequenced.

Contigs with matches to the mastreviruses (family *Geminiviridae*) Chloris striate mosaic virus (CSMV) and Digitaria ciliaris striate mosaic virus (DCSMV) were identified in HTS pools 11/KCNGS022 and 13/KCNGS024. CSMV is known to cause striate mosaic symptoms in *Chloris gayana* and a full circular ssDNA genomic sequence of 2750 nt was obtained from pool 13, which was 98% identical to an Australian isolate (GenBank accession JQ948058.1). The 704 nt contig from pool 13 was 92% identical to an Australian isolate of DCSMV (GenBank accession JQ948091.1) and is a new host record for this virus. Neither SCMV nor DCSMV cause pasture dieback, however their detection in the sequencing lends confidence that the methodology for this run will detect systemic viruses of higher titre.

Further results and discussion pertaining to tobacco streak virus, grass velariviruses, the deltapartitivirus, poleroviruses and the badnavirus are described in separate sections below.

Table 40. Summarised results of high throughput sequencing of leaf RNA samples sent for viral sequence analysis.

RNA sample	Sample details	Plant virus similarity
1 / KCNGS012	Buffel grass cv. Gayndah - Moura to coast	NVD
2 / KCNGS013	Buffel grass cv. Gayndah - west of Moura	NVD
3 / KCNGS014	Buffel grass cv. Biloela - yellowing	tobacco streak virus
4 / KCNGS015	Buffel grass cv. Biloela - yellowing and reddening	NVD
5 / KCNGS016	Red Natal grass, QLD bluegrass, Native millet/Yabila grass	tobacco streak virus
6 / KCNGS017	Creeping bluegrass cv. Bisset	NVD
7 / KCNGS018	Angleton grass (2 Floren)	NVD
8 / KCNGS019	Green panic (3*) & Bambatsi panic (2)	NVD
9 / KCNGS020	Green panic - interveinal chlorosis	NVD
10 / KCNGS021	Sabi grass - yellowing	tobacco streak virus
11 / KCNGS022	Rhodes grass* - spherical 26nm isometric virus particles	mastrevirus, deltapartitivirus†
12 / KCNGS023	Rhodes grass* - yellowing/reddening	velarivirus
13 / KCNGS024	Rhodes grass - strong striations, yellowing	velarivirus, mastrevirus
14 / KCNGS025	Bothriochloa (5) and Urochloa (1)	tobacco streak virus
15 / KCNGS026	Silk sorghum	tobacco streak virus
16 / KCNGS027	Red Natal grass, scented top grass, black spear grass, Setaria sp., Digit grass, Strickland fingergrass	NVD
17 / KCNGS028	Mount French site - no virus symptoms	polerovirus
18 / KCNGS029	Mount French site – virus symptoms	NVD
19 / KCNGS030	*Megathyrsus maximus* - virus symptoms	badnavirus
20 / KCNGS031	#2871 BYDV-notMAV (Oat yellow dwarf virus) CONTROL	polerovirus

NVD, no virus detected

* Virus particles had previously been detected in these leaf samples

† The deltapartitivirus sequences were assembled and analysed differently, see section 4.5.3.6 for details.

4.5.3.3 Poleroviruses

Poleroviruses and unassigned species of the family *Solemoviridae* are phloem-limited, aphid-transmitted viruses with ssRNA genomes that are known to cause reddening and yellowing of grain crops. A range of pasture grass species (Table 6) were tested with generic polerovirus primers to investigate the association of these viruses with pasture dieback. Multiple amplicons were obtained for many of the samples, but analysis of the Sanger sequenced amplicons did not return matches for plant viruses (data not shown).

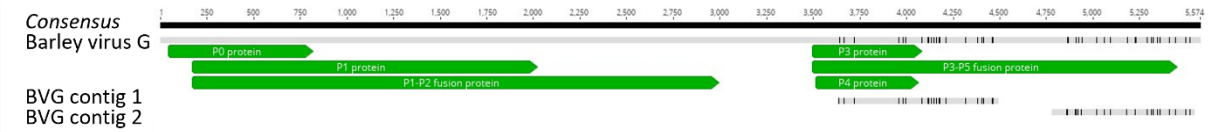
HTS sample 20 was included to assist with developing the *de novo* assembly methodology and to confirm that poleroviruses could be detected from total RNA extracts of leaf samples. A near complete genome for the novel Oat yellow dwarf virus was obtained.

Two polerovirus-like contigs of 861 and 765 nt were identified in HTS sample 17, which was a pool of asymptomatic young plant samples from Mt French. Both contigs were 97% identical to an Australian isolate of barley virus G (BVG; GenBank accession LC500835.1) and mapped to different regions at the 3' end of the genome (Fig. 32). This is an extension of the known geographic range for BVG. At this stage the sequence has not been extended to obtain a whole genome, but this is advised to confirm the species identification and rule out recombination with a related species, as is

known for viruses in this taxon. Polerovirus-like contigs were not identified in other HTS samples, suggesting this taxon is not associated with pasture dieback.

To identify the BVG positive sample from Mt French, a range of generic polerovirus primers were tried on the samples from HTS sample 17 (Table 34). These primers have a range of targets that they amplify but no one set amplifies all species in this genus (with or without the related but unassigned family members). A range of amplicons has been generated but very few are of the expected size for the respective primer pair; Sanger sequencing has not yet identified polerovirus matches for amplicons of the expected size. An alternative RNA extraction technique will be tried on stem tissue to improve the detection limit for these assays.

Figure. 32. Alignment of assembled barley virus G contigs with their closest GenBank match (accession LC500835.1). Grey lines represent the nucleic acid sequence of each sequence with single nucleotide mismatches in black; green arrows represent open reading frames.



4.5.3.4 Tobacco streak virus

Tobacco streak virus (TSV) is an ilarvirus (family *Bromoviridae*; Bujarski et al., 2019) and has three ssRNA components to its genome. Particles are either spherical or quasi-spherical with a diameter of 26–35 nm. TSV is transmitted mechanically by thrips feeding on pollen grains containing the virus. Contigs matching all three genomic components were assembled in HTS pools 3 / KCNGS014, 5 / KCNGS016 and 10 / KCNGS021, while contigs that matched RNA3 were assembled in HTS pools 14 / KCNGS025 and 15 / KCNGS026 (Table 40).

When individual samples from these five HTS pools were tested individually for TSV, only five of the 24 samples were positive by ELISA: two samples of Indian couch, two samples of silk sorghum and one sample of nut grass (Table 41).

The initial opportunistic inoculation of a Buffel grass plant did not produce virus symptoms and the plant was negative when checked by ELISA. Plants inoculated in the planned experiment also did not develop symptoms, however, the susceptible control failed to become infected. Plants were unfortunately disposed of before samples were taken for diagnostic testing.

Given the limited number of positive samples by ELISA compared with that detected by HTS, several explanations may account for the presence of TSV in these pasture dieback samples. It is possible that the difference in detection limit between HTS and ELISA means that false negatives may be observed by ELISA. More likely though, is that the samples which were positive by HTS but negative by ELISA have environmental cross-contamination from TSV-positive parthenium pollen, which is common is central Queensland (Sharman et al., 2009). The known range of TSV-positive parthenium is likely to have expanded since 2009 to cover the areas from which TSV was detected in the HTS samples. Samples positive by ELISA as well as HTS also carry the risk of TSV-positive Parthenium pollen contamination but may also represent true infections. It is unfortunate that the transmission test which included Indian couch failed. This test should be repeated to confirm host status of at least Indian couch, silk sorghum and nut grass.

Table 41. Results of ELISA diagnostic testing of individual samples comprising TSV positive HTS pools.

Sample	Common name	Chlorosis	Reddening	TSV ELISA	HTS pool
26/02/20-07	Buffel grass cv. Biloela	1	0	-	3
19	Buffel grass cv. Biloela	1	0	-	3
99	Buffel grass cv. Biloela	1	0	-	3
112	Buffel grass cv. Biloela	1	0	-	3
222	Buffel grass cv. Biloela	1	0	-	3
12	Sabi grass	1	1	-	10
13	Sabi grass	1	0	-	10
32	Sabi grass	1	0	-	10
62	Sabi grass	1	0	-	10
66	Sabi grass cv. Nixon	1	0	-	10
31	Indian couch	0	1	+	14
92	Indian couch	0	1	-	14
184	Indian couch	0	1	+	14
70	Forest bluegrass	0	1	-	14
183	Desert bluegrass	0	1	-	14
251	Signal grass cv. Basilisk	1	1	-	14
93	Silk Sorghum	0	1	+	15
96	Silk Sorghum	0	1	-	15
97	Silk Sorghum	0	1	-	15
98	Silk Sorghum	1	1	+	15
94	Red Natal grass	0	1	-	5
100	Queensland bluegrass	0	1	-	5
102	Native millet/Yabila Grass	0	1	-	5
105	Nut grass	1	0	+	5

4.5.3.5 Velariviruses

Novel velarivirus sequences (family *Closteroviridae*; Fuchs et al., 2020) were assembled from two pooled Rhodes grass samples (Table 42). Velariviruses have ssRNA genomes approximately 16-17 kb in length, encapsidated to form long very-flexuous filamentous particles. Velariviruses are close relatives of the mealybug-transmitted ampeloviruses which cause economically important diseases in pineapple and grapevine. Some velariviruses are known to cause disease in their hosts, but until recently, their insect vector was not known. This year, areca palm velarivirus 1 has been reported to be transmitted by the mealybug species *Ferrisia virgata* and *Pseudococcus cryptus* to cause the fatal yellow leaf disease in betel palm (Zhang et al., 2022).

Symptoms caused by species within the family *Closteroviridae* include reddening or yellowing of leaves and while the infections are systemic, virus particles tend to be limited to the phloem (vascular) tissue. Electron microscopy detection of these viruses can be difficult because even as part of a virus purification as they are often at low concentration and their long particles tend to break up.

Multiple related *Closteroviridae* species are common in the same host species, and mixed infections of these species are common within individual plants. Sequence variation between and within the species can be high, posing issues for assembly and identification of these viral sequences and diagnostic detection of distant variants. Association of host symptoms with a particular viral species has also been difficult in some crops (e.g. pineapple mealybug wilt disease).

This section presents analysis of the HTS sequences, test results for a range of samples and interpretation of these findings and recommendations for further work.

Sequence analysis

Three contigs with velarivirus matches were identified during BLAST analysis (Fig. 33). One contig of 14,038 nt covering almost the full coding sequence of the virus was assembled from one HTS sample (pool 13/KCNGS24; virus 1). A large contig (10,133 nt) was assembled from pool 12/KCNGS23; this was 85% identical to the first contig at the nucleotide level. Identities between translated key open reading frames (ORFs) for these sequences were 93.3%, 93.8% and 94.6% for the RdRp, CP and HSP70h sequences respectively. This contig appears to represent a variant of virus 1.

The third velarivirus contig (4,370 nt) was also assembled from poot 12/KCNGS23 but was only 52% identical to the first contig at the nucleotide level. Alignment of the truncated translated RdRp sequences (183 amino acids) found 94% identity between the two sequences of virus 1 but only 53% identity between this third contig and virus 1 sequences, strongly suggesting that the third contig represents a second velarivirus species (virus 2).

The near complete genome of virus 1 was further analysed to confirm placement within the velarivirus genus. All main open reading frames (ORFs; polymerase, coat protein and HSP70h) were identified, however the first ORF was truncated, and this sequence lacked the final ORF present in other velarivirus species (Fig. 34). Phylogenetic analysis of the polymerase, coat protein and HSP70h translated sequences with reference sequences of the other viruses in this family clearly grouped virus 1 with other species of the velarivirus genus (Fig. 35).

Figure. 33. Velarivirus contigs assembled from pooled samples of Rhodes grass. Black lines represent the nucleic acid sequence of each contig; green arrows represent open reading frames.

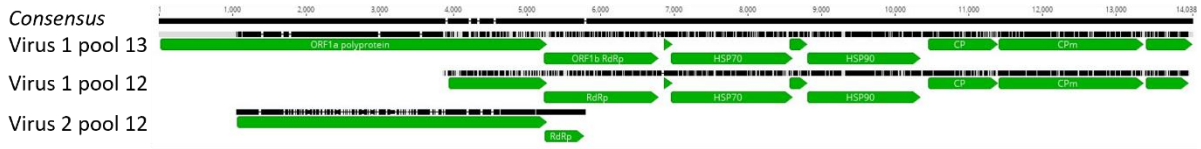


Figure. 34. Whole genome nucleotide alignment of the grass velarivirus 1 with other velarivirus species genomes. Black lines represent the nucleic acid sequence of each genome; green arrows represent open reading frames.

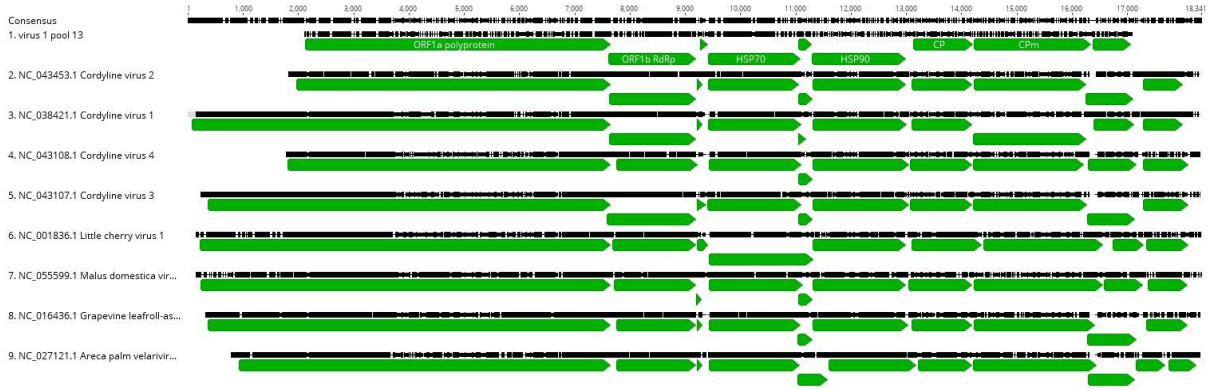
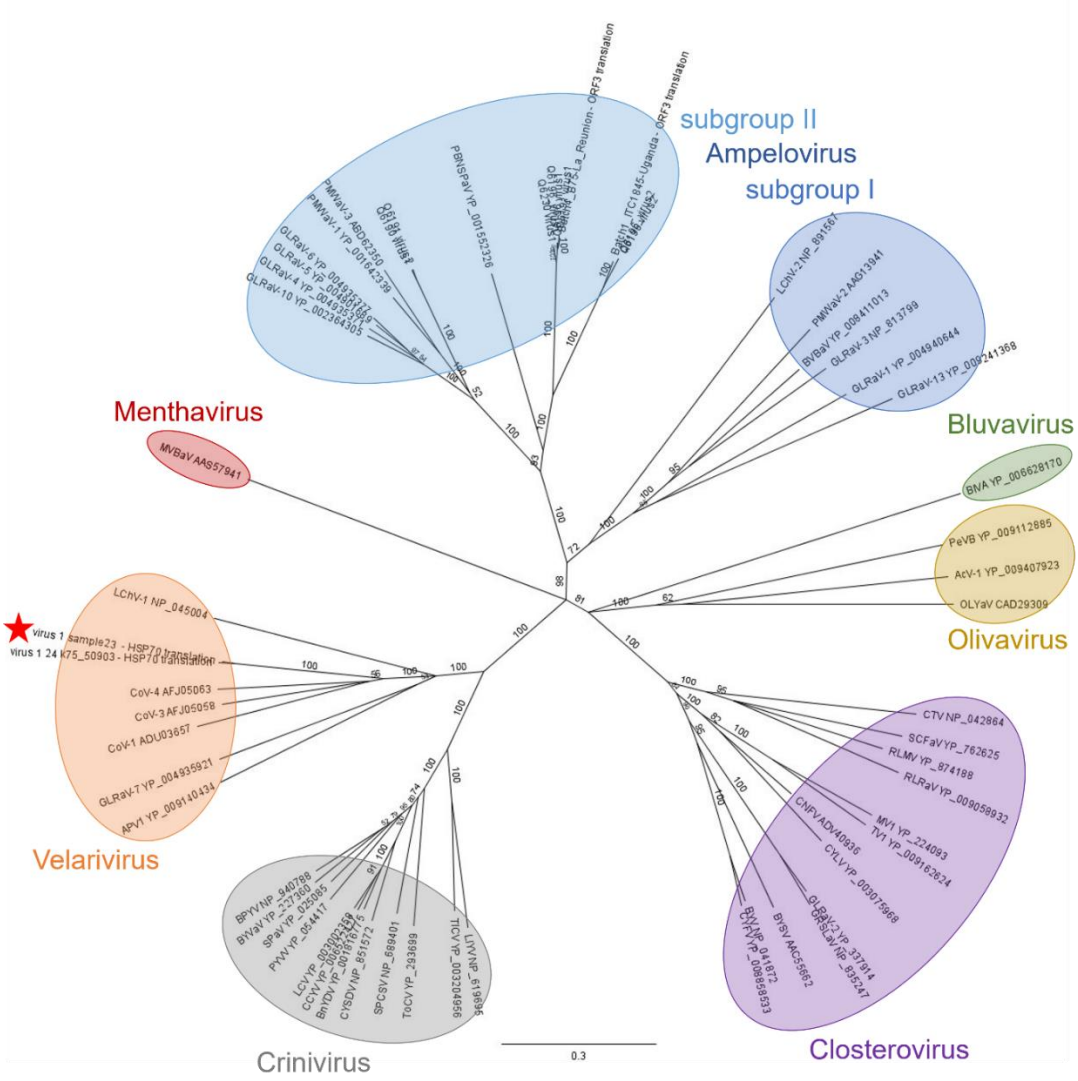


Figure. 35. Phylogenetic placement of the grass velarivirus 1 based on the HSP70h translated amino acid sequences. Virus 1 is indicated by the red star. Virus acronyms and reference sequence accession GenBank numbers are as per the ICTV listing (https://www.ncbi.nlm.nih.gov/genomes/GenomesGroup.cgi?taxid=69973) with the addition of sequences from three novel banana ampelovirus (*pers. comm.* K. Crew).



Diagnostic testing

Separate RT-PCR diagnostic assays for virus 1 and virus 2 were developed in conserved sequence regions and a range of Rhodes grass samples were tested for the presence of these viruses. Sanger sequencing found that the amplicons from samples 3 and 4 were 100% identical to the near complete genome of virus 1 from HTS pool 13, while amplicons from samples 5 and 18 were near identical (99% and 94% respectively) to the variant of virus 1 from HTS pool 12 (Fig. 36). Three sequenced amplicons of virus 2 were 100% (one amplicon) and 99.7% (two amplicons) to the virus 2 de novo assembly from HTS pool 12 (data not shown).

Figure. 36. Comparison of de novo assembled sequences for virus 1 with Sanger sequence of four RT-PCR amplicons. Colours represent differing nucleic acid residues. Samples 1/11/2019-11 (amplicon 1-18), 192 (amplicon 1-04), 194 (amplicon 1-05) and 198 (amplicon 1-03) were sequenced.

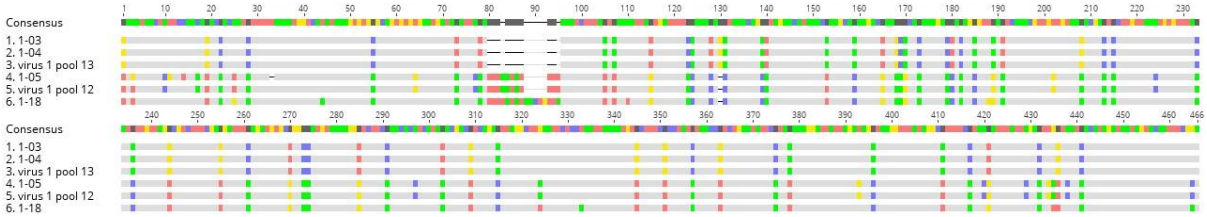


Table 42. Diagnostic testing results for grass velarivirus in pasture dieback samples*.

Site	Number of samples tested	Virus 1	Virus 2	Comments
Kalbar	18	1	1	Mixed infection
Coulson	4	4	4	All mixed infections
Mt French	9	1	0	
Gayndah	5	0	0	
Total	36	6	5	

* Note that not all samples tested were clearly symptomatic.

At the time of testing, Rhodes grass samples from four localities were available: Kalbar, Coulson and Mt French in the Fassifern Valley, and Gayndah in the Burnett Region (Table 42). Six of 36 samples tested were positive for at least one velarivirus; five of these samples had mixed infections of both viruses. Four of the positive samples had yellowing leaves, one was a mixed infection with mastreviruses which masked symptoms with a very strong striate mosaic, and the sixth plant (with a single infection) was a relatively young seedling.

Interpretation and further work

The detection of two velarivirus species in pasture dieback affected Rhodes grass samples is potentially quite exciting. Additional strongly symptomatic Rhodes grass samples collected since this diagnostic testing from Mt French, Kalbar and Gympie as well as a range of clearly affected samples from a range of pasture species (particularly buffel grass, creeping bluegrass, panics and sabi grass) need to be tested for the presence of these viruses to establish whether there is a link between the grass velarivirus(es) and pasture dieback.

4.5.3.6 Deltapartitivirus

A single Rhodes grass sample from a pasture dieback affected paddock but which displayed atypical colouring (one-sided leaf yellowing with an orange tinge and a fine striate mosaic) had been observed to contain a moderate concentration of small spherical virus particles. However, the standard *de novo* assembly of all trimmed and paired reads used in this study did not assemble any contigs with matches to a virus with this particle shape (HTS sample 11/KCNGS022, Table 40). Instead, contigs matching two mastrevirus sequences were detected, which the likely cause of the fine striate mosaic symptom.

Hence, removal of host sequences by mapping reads to first the *Sorghum bicolor* genome (GenBank genomic sequence GCF_000003195.3) and then a *Chloris virgata* chloroplast genome (GenBank sequence NC_032034.1) was undertaken prior to de novo assembly. Two contigs assembled from 69,222 and 39,262 of 28,136,866 reads respectively were identified, with matches to the family *Partitiviridae* (genus Deltapartitivirus and unclassified family members). Each contig encoded a single ORF; dsRNA1 (1574 bp) encoded the RNA dependent RNA polymerase and dsRNA2 (1554 bp) encoded the coat protein.

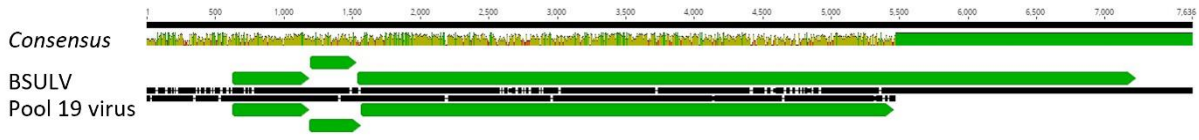
Deltapartitivirus genomes typically consist of two dsRNA segments, with lengths of 1563–1696 bp and 1415–1575 bp for dsRNA1 and dsRNA2 respectively (Vainio et al., 2018). They cause persistent but often cryptic infections of plants and are seed transmitted. Virus particles are isometric and 25–43 nm in diameter, which fits within the size range observed for this sample. Currently only dicot hosts have been reported for this genus, making a grass-infecting species a notable record.

A diagnostic assay for this virus is needed as it may account for some of the spherical particles observed by electron microscopy in other samples. However, at this stage it does not appear likely to cause pasture dieback.

4.5.3.7 Badnavirus

Three samples of panic grass with chlorotic flecking were pooled for HTS sample 19 / KCNGS030, and following *de novo* assembly, a 5,404 nt contig with high similarity to monocot-infecting episomal badnavirus sequences was identified. The contig encodes three ORFs, although the ORF3 appeared truncated when compared to the closest reference sequence, banana streak UL virus (Fig. 37). The aspartic protease domain is present in the translated ORF3 sequence, however the reverse transcriptase domain required for phylogenetic placement of this contig is missing. BLAST matches and the structure of this contig suggest this represents an episomal rather than endogenous viral sequence. No other plant viral sequences were found in this sample, so it is likely this badnavirus is the cause of the chlorotic flecking symptoms. However, other badnavirus-like contigs were not identified in other samples, suggesting that species in this genus are not the causal agent of pasture dieback.

Figure. 37. Alignment of the panic badnavirus contig with the reference sequence for the nearest match banana streak UL virus. Black lines represent the nucleic acid sequence of each contig; green arrows represent open reading frames. Banana streak UL virus is GenBank accession NC_015504.1.



4.5.4 Acknowledgements

High throughput sequencing analysis was conducted through Galaxy Australia, a service provided by the Australian Biocommons and its partners. The service receives National Collaborative Research Infrastructure Strategy (NCRIS) funding through Bioplatforms Australia and the Australian Research Data Commons (https://doi.org/10.47486/PL105), as well as The University of Melbourne and Queensland Government Research Infrastructure Co-investment Fund (RICF) funding.

5. Conclusion

5.1 Plant pathology - fungi

5.1.1 Glasshouse Experiment 1

Results from both glasshouse experiments concluded that *G. graminicola* can infect Digit and Rhodes grass. Plant decline can be influenced by weather conditions and appeared to progress more rapidly in the presence of mealybugs (*H. summervillei*). Inoculations with the *Colletotrichum* sp. isolate did not prove to be pathogenic on either of the Digit or Rhodes cultivars.

The low infestation of mealybugs, observed in Experiment 1, manifested over time, and had an impact on plant death, even though *G. graminicola* was consistently recovered from inoculated plants. Therefore, it is essential that studies should be initiated using sterilised soil and not assume that mealybugs are not present.

There was also evidence that the sexual state of *G. graminicola* could be produced in the glasshouse under certain conditions (overhead irrigation), and this was the reason for the recovery of this organism from the control pots. This also resulted in segregation of control pot from the inoculated treatments and avoiding the use of overhead irrigation, instead relying on hand watering.

5.1.2 Glasshouse Experiment 2

All grass species can host *G. graminicola,* however there was a lack of evidence in the glasshouse experiment to conclude that the fungal organism is the primary cause of dieback, although it is associated with pasture dieback symptoms, particularly in north Queensland.

No further inoculation studies are required in relation to *G. Gaeumannomyces.*

5.2 Microbiomes

The plant phyllosphere (leaf) and rhizosphere (root/associated soil) microbiomes were very similar across the entire dataset and there was not a clear, single bacterial or fungal population strongly associated with symptomatic plants, to indicate a possible causal agent for dieback. The soil micro-eukaryotic microbiomes were also very similar across the entire data set. A lack of curated taxonomic 18S rRNA sequence data limited the identification of many of the micro-eukaryotic populations to higher levels and the presence of the two insects often implicated with pasture dieback, mealybugs and ground pearls, was not able to be determined due to the sequence databases available, which lacked the reference sequences for these genera.

There were also several limitations associated with the samples reported on in this study, including:

- the samples representing only a single time point collected at each site,

- the inconsistent recording of field data (e.g. presence absence of mealybugs, ground pearls) which affected the metadata tables and analysis,
- a low number of non-symptomatic samples available i.e. often a matching healthy plant of the same species and growth stage was not able to be collected at or near the same site, and
- the variation in the growth stage of the plants sampled, with plants ranging from seedlings through to mature with spent seed heads collected, as plants were chosen based on presence or absence of physical symptoms thought to be active dieback.

In regard to the last point above, a recently published study, Grady et al. (2019) sampled two perennial grasses (switchgrass and miscanthus) every three weeks from pre-emergence through to senescence. Their analysis of the core phyllosphere microbiota found changes in the microbial community, with younger plants possessing what they termed early microbial colonisers, which were found to be rare or absent by the end of the growing season (Grady et al., 2019). Another study in wheat found that the biodiversity in the roots varied with a succession of bacteria and fungi within the wheat root environment from those present in the seed changing to include recruits from the rhizosphere soil communities over time (Araujo et al. 2020). Recent research has also started to indicate that microbiome community amplicon sequencing data needs to be interpreted carefully as there can be biases. Beule et al. 2021 undertook a comparison of relative (microbiome) and absolute (quantitative PCR) abundances of selected fungal groups in a soil fungal community. Their results found large differences between the two methods suggesting that amplicon sequencing alone may not be able to assess population size and dynamics adequately. Further tests, such as species-specific quantitative PCR may therefore need to be incorporated into future work, to validate the findings of sequence-based microbiome analyses.

5.3 Analysis of soil samples

The analysis of soil samples for ground pearl, nematodes and soil chemistry and nutrients suggests that none of these factors have any relationship to the presence of pasture dieback.

5.4 Mealybug research

The data outlined in this report provides evidence of direct and persistent impact of pasture mealybug infestations on the productivity and survival of Bisset creeping blue grass however we cannot discount the possibility that the impact of mealybug infestations on Bisset productivity and plant death could be the result of a disease vectored by mealybug, not simply a function of direct mealybug feeding activity. The persistence of impacts on productivity, including plant death, are indicative of severe and permanent damage to the plant (e.g. the vascular system) from mealybug feeding, or possibly the transmission of pathogen/s. The rapid and terminal decline of Bisset blue grass in this trial cannot be extrapolated further than for this pasture variety. Previous observations of mealybug infestations (M. Miles, DAF Qld) on a range of introduced pasture species have identified differing responses in terms of:

- symptoms (colour of leaves can vary from yellow to red)
- the rate at which symptoms appear after the introduction of mealybug, and
- the severity of the symptoms and resulting impact on plant growth characteristics.

5.5 Viruses

Although present in pasture weeds, phytoplasmas were not detected in pasture grass species in this study.

From the current HTS analysis based on total leaf RNA samples, new host or geographic records for known viruses (barley virus G, tobacco streak virus, Digitaria ciliaris striate mosaic virus) were identified. Novel virus genomic sequences for badnaviruses, deltapartitivirus and velarivirus species were assembled. Although detected, poleroviruses, tobacco streak virus, badnaviruses and partitiviruses were not found to be widespread in pasture dieback affected samples. Grass velariviruses warrant further investigation to determine host range and gain a better understanding of the geographic distribution of these viruses. Mealybug (*Heliococcus summervillei*) transmissions of the velariviruses should also be investigated.

Additionally, a second HTS run is needed using nucleic acids from purified virus preparations in which small isometric viruses have been detected by electron microscopy. The identity of these viral particles has not been resolved through the current HTS run, likely because of the low concentration of viral reads amongst host sequences. Nucleic acid extractions from mealybugs and ground pearls could also be included as they are likely to contain plant viruses from the plants they have fed on.

5.6 Key findings

- The knowledge of multiple potential causal agents or co-factors of pasture dieback has improved through the analysis of plant and soil samples from affected and unaffected areas, and the pathogenicity testing of multiple fungi and the pasture mealybug.
- Multiple field surveys across pasture dieback affected regions in Queensland (southern, central and northern) and subsequent sample analysis indicate it is unlikely that different casual agents of pasture dieback occur in different regions of Queensland i.e. it is likely grasses exhibiting pasture dieback symptoms across Queensland are impacted by the same pathogenic organism(s).
- It is highly unlikely pasture dieback is caused directly by fungal pathogens. However, these can be commonly associated with pasture dieback, most pastures affected by dieback also are infected with multiple fungal diseases some of which can be pathogenic (e.g. buffel grass blight caused by *Pyricularia grisea*). Results obtained in this project corroborates findings from other fungal studies commissioned by MLA during 2018 (MLA pers. comm. 2018) and supports the notion that no further fungal pathogenicity testing is needed unless new compelling evidence indicates otherwise.
- Microbiome research demonstrates very similar microbiomes across single point sampled symptomatic and non-symptomatic plants and that there was not a clear, bacterial or fungal population strongly associated with symptomatic plants to indicate a possible single causal agent for dieback.
- Virology research conducted during the project indicate a range of viruses present in both symptomatic and non-symptomatic plants. Some of these are novel i.e. have not been recorded previously. Others represent new host or geographic records for known viruses. Grass velariviruses warrant further investigation as it remains possible that these viruses play a role in pasture dieback. Additional work using modified methodology is required to identify small isometric virus particles found in pasture dieback affected samples.

- Analysis of soil chemistry and nutrient levels together with ground pearl and nematode presence suggests none of these are likely to cause pasture dieback. Ground pearl and nematodes are known plant pathogens however data generated by this project suggest the presence of these organisms in pastures affected by pasture dieback is likely to be coincidental.
- Replicated field research demonstrated that uncontrolled, high-density infestations of the pasture mealybug can cause the rapid onset of dieback symptoms, decline in plant growth and plant death. Control of pasture mealybug infestations at early onset of symptoms resulted in recovery of pasture productivity. These results apply only to Bisset creeping blue grass, it is unclear if these can be reliably extrapolated to other species/varieties. Due to the precise mechanism of plant death not being investigated, there is a possibility that the impact, and subsequent plant death, could be the result of factors in addition to direct feeding by pasture mealybug e.g. a disease vectored by mealybug.

5.7 Benefits to industry

The outcomes of this project will improve the knowledge of potential causal agents including the impact these can have on pasture productivity. There are specific pathogenic organisms that are unlikely to cause pasture dieback, these include fungi, nematodes and ground pearls. Unless new and compelling evidence is found, we suggest no further research is warranted into these and so research funds could be directed to other activities.

Project outcomes will also guide future research into effective management solutions to restore pasture productivity and business profitability. Data generated by the project suggest the pasture mealybug can negatively impact pasture productivity. Management practices that either directly control this pest, boost predatory or parasitic insects, or manage impacts through more tolerant and resistant pasture species (e.g. annual forages, legumes), need to be investigated.

Benefits of this project to the wider red meat industry are substantial. This condition has the potential to spread further and cause significantly higher productivity losses in districts that are currently affected. Despite reports of past dieback incidences disappearing by natural means, there is no guarantee this will happen again; there are multiple anecdotal reports from central Queensland that indicate pasture dieback can re-emerge after a period of healthy pasture growth. Pasture dieback also has the potential to spread into other beef producing regions where it doesn't occur currently, such as coastal and adjacent inland areas of New South Wales where tropical grass pastures are utilised for beef, dairy and sheep production systems. Project findings will be valuable for improving the understanding of the causes of pasture dieback and subsequent management solutions to stakeholders both familiar with and naive to this condition.

6. Future research and recommendations

This project has generated a significant amount of rigorous research data on a range of potential pathogenic organisms that may have a direct and/or indirect role in pasture dieback. To our knowledge no other project has examined this range of potential causal agents at one time in one project. Despite the significant research effort and improvement in knowledge of pathogenic organisms associated with pasture dieback, further research is required to overcome the limitations that were encountered and generate answers to new questions that emerged during the project. These include:

1. New field-based sampling experiments to track impacts of pasture dieback over time.

Field sampling conducted prior and during the project were primarily taken at individual locations at one point in time. Very limited opportunity occurred to undertake repeated sampling at multiple set locations to determine changes to plant health and organisms over time. Building on knowledge already generated from selected sites, new experiments are needed where repeated plant and soil samples are collected from set locations over a 1 – 2 year period to determine changes in pathogenic (and beneficial) organisms and the plant and soil microbiome, over time. Also, evaluation of the impact of pasture mealybug density and persistence through the season will contribute to the refinement of recommendations around pasture monitoring, the need for and timing of mealybug control and/or management. However, these experiments will be dependent on dieback continually impacting the pasture for longer than one season, which (from experience) might not occur in all situations. Further, experience in sampling methodologies will provide guidance to fine tune which growth stage of the plant to be sampled and improve the standardisation of data recording sets to maximise the number of samples to be included in analyses. Combining frequency data based on amplicon sequencing with absolute quantification through use of quantitative PCR assays should be considered to more accurately assess changes in population size and dynamics in the microbiota present in samples taken across the progression of dieback in plants. Whilst there was no single causative agent for pasture dieback identified, the microbiota of pasture grasses may reveal potential beneficial bacterial targets to use as biocontrol or plant growth-promoting bacteria to assist pasture grasses in overcoming the effects of pasture dieback. Sequencing of pasture mealybug and ground pearl 18S rRNA gene using DNA extracted from field samples will enable the interrogation of the soil micro-eukaryotic microbiome sequence datasets for the presence of these organisms.

2. Investigation of the mechanism/s of pasture dieback, with focus on pasture mealybug as the primary causal agent.

The combined plant pathology, virology, microbiome and pasture mealybug research conducted by the project is unique; a large volume of data has been generated by collecting and analysing hundreds of plant samples through to pathogenicity testing. While there is evidence of direct and persistent impact of pasture mealybug infestations on the productivity and survival of Bisset creeping bluegrass (i.e. pasture mealybugs can cause pasture dieback), it cannot be concluded that mealybug alone are producing the effects observed. A critical need is to investigate the potential involvement of pathogens transmitted by the pasture mealybug, including viruses and other micro-organisms. This is due to management recommendations for the pasture mealybug as the direct cause of pasture dieback being quite different to recommended management in the event of being primarily vectors of pathogens. If pathogens are detected, the potential for seed transmission and alternative hosts in pastures, will be important management considerations. Further research is needed to fully explore the range of pathogens present, especially viruses, and continue through to pathogenicity testing.

3. Undertake wider screening of sown/improved and natives grass species to the pasture mealybug.

The pasture mealybug research reported here was conducted on just one pasture species, Bisset creeping bluegrass. Due to the association of mealybugs and pasture dieback on multiple grass species, an expansion of this research is needed to screen a broader range of introduced (and potentially native) pasture species. Attention to the suitability of the different grasses for mealybug population establishment, build up and persistence is as important as the relative impact of

mealybug. Understanding the likely population dynamics of mealybugs in the different pasture grasses will make a valuable contribution to management of mealybug. This work would provide more robust recommendations around the need for, and timing of, mealybug control/management to minimise loss of pasture productivity and persistence. Due to the importance of this type of research, project staff have already taken the opportunity to start an experiment at the DAF field site near Boonah at the end of 2021 where the pasture mealybug has been introduced to 30 grass species in replicated plots. Plant impacts (leaf discolouration, plant health, biomass etc) will initially be recorded over a 4 - 6 month period. Also, plant and mealybug samples were collected prior to mealybug infestation, and regular sampling will occur during infestation until the end of the pasture growing season including assessment of micro-organisms present, especially viruses. Mealybug population will be recorded to benchmark symptom expression with mealybug presence/numbers.

4. Undertake additional analysis of soil sample data

While valuable insights have been generated from the assessment of soil, additional analysis is needed to complete the dataset:

- Include mealybug factor (as done with diversity measures) in nematode and nutrient analyses. While it is unlikely there is an association or correlation between the pasture mealybug, nematodes and soil nutrient levels, analysis is required to complete the understanding of the relationship between these factors.
- Multivariate analysis combining the ground pearl, nematode and nutrient data.
- Multivariate analysis combining ground pearl, nematode, nutrient, alpha diversity measures and eukaryote samples.

As the data sets have been processed and are currently in a form to enable these types of analyses, minimal effort would be required to complete these activities.

Detailed costings of the proposed research activities have not been conducted. Further discussion is needed to understand the current progress of the virology research. Discussions are required with MLA to determine the need for these activities based on outcomes from other organisations undertaking pasture dieback research.

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- DAF biometricians including David Reid, Kerri Chandra and Miranda Li who undertook statistical analysis of data and assisted with bioinformatic analysis of microbiome data.
- DAF plant pathology staff including Kathy Grice, Dr Fiona Giblin and Peter Trevorrow who undertook sample collection, analysis, and pathogenicity testing for fungal pathogens.

- DAF virology staff Dr Kathy Crew and Visnja Steele who conducted sample analysis and pathogenicity testing for viruses.
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9. Appendix

9.1 Microbiome – detailed methodologies

9.1.1 Collected sample details

From the bio-geographical transect established on property AJ site 1, three 30 x 30 cm three quadrats were sampled along the transect. Quadrat 1 (Q1) was sampled within the dead patch, quadrat 2 (Q2) spanning the edge of the dead patch and live pasture and quadrat 3 (Q3) within the live pasture. In each quadrat the following samples were collected – all aerial plant material which then divided into pasture grass (Grass) or forb (Plant) and then living or dead material (Aerial and Dead Aerial), all plant root with associated soil (Root & Soil) for pasture grass and the soil to a depth of 10 cm (Soil). At time of collection the soil was mixed well and sub-sampled for microbiome (Soil), nutrient, ground pearl and nematode analyses. These samples were placed on ice, returned to the laboratory and stored at 30 C, until sub-sampled with each bulk sample mixed in a clean tray and five random pick samples of leaf and stem of the pasture grass (both live and dead material), five picks from root and associated soil material, five random samples of soil material and half of the forb (Plant) samples taken and cryo-ground using liquid nitrogen as detailed in section 9.1.2. Details of the collected samples are outlined in the attached spreadsheet document.

9.1.2 Cryo-grinding protocol

The frozen plant sample to be cryo-ground was transferred to an aluminium foil boat, weighed and the sample weight recorded. The sample was then transferred into a clean mortar and pestle that had been pre- chilled using liquid nitrogen. The sample was ground until it was a fine powder ensuring that the liquid nitrogen did not completely evaporate. Once ground the sample was transferred whilst frozen into a new 5 mL tube using a disposable wooden probe and placed -20 °C with the lid loosely screwed on until all liquid nitrogen had evaporated. The lids were tightened, and the samples stored at -20 °C until gDNA extraction.

Between sample processing the mortars and pestles, along with the forceps and paint brush were all decontaminated by cleaning thoroughly and immersion in a 10% bleach for 15 min. The equipment was then rinsed with reverse osmosis water followed by spraying with 80% ethanol and allowed to dry before reuse.

9.1.3 Genomic DNA extraction protocols

Genomic DNA (gDNA) extraction from cryoground leaf and root samples was undertaken using the DNeasy Plant Pro kit (Qiagen, catalogue # 69206) with 0.1 g of the cryo-ground plant material extracted following the manufacturer's instructions. Genomic DNA from soil samples was extracted using the DNeasy PowerLyzer PowerSoil kit (Qiagen, catalogue #12855-100) using up to 0.25 g of cryo-ground soil following the manufacturer's instructions. Briefly, both methods physically disrupted with the samples added to a tissue disruption tube containing a specially shaped bead and buffer for rapid homogenisation using a Mini beadbeater-16 (Biospec Products) for three minutes. The kit contains inhibitor removal to enable the removal of inhibitors and proteins before the released total gDNA from the plant, microbes and fungi onto a membrane spin column where it was washed and finally eluted off the column in a Tris buffer in a fresh tube and frozen at -20°C. The extracted gDNA extraction success determined by 1% agarose gel electrophoresis in Tris Borate EDTA (TBE) buffer along with a 5.0 µL aliquot of GeneRuler 1Kb DNA ladder (Thermo Fisher

Scientific) visualising the DNA using GelRed® stain (Biotium, USA). The quality and quantity of the extracted gDNA was measured using a Nanodrop Microvolume Spectrophotometer (Thermo Fisher Scientific, USA).

Two methods for the extraction and purification of microbial DNA associated with collected mealybug and ground pearl specimens which had been preserved by freezing directly after collection and stored at -20 °C were tested and compared. Details of the specimens, their preservation and gDNA extraction method are detailed in Table 43. The first method employed a commercial kit for the extraction of DNA (QIAamp DNA Mini Kit, Catalogue number 51306; Qiagen). Briefly, either a single mealybug or ground pearl specimen was placed in a 2 mL screw cap tube with a ballcone-shaped bead (Qiagen) and 200 µL of ATL buffer (Lysis buffer, Qiagen) added. This mixture was then physically disrupted in a Mini beadbeater-16 (Biospec Products) for three minutes. A 20 µL volume of Proteinase K (20mg/mL; Qiagen) was then added and the sample was incubated for 40 min at 65 °C. A 400 µL volume of an equal parts mixture of ethanol and AL buffer was added to the homogenised sample, which was then vortexed and transferred to a QIAamp mini spin (MS) column (Qiagen). The DNA bound to the resin of the MS column, which was washed according to manufacturer's instructions (Qiagen), was eluted in 50 µL of AE buffer (10 mM Tris-Cl, 0.5 mM EDTA; Qiagen) and quality checked by agarose gel electrophoresis and Nanodrop as described above before being stored at -20 °C.

The second method involved an adaptation of a published DNA extraction method which utilised a lysis buffer containing Sodium dodecyl sulphate (SDS) (Phillips and Simon, 1995). Either a single mealybug or ground pearl specimen was placed in a 2 mL screw cap tube with 0.25 g of 1.0 mm Zirconia/Silica beads (Daintree Scientific) and 200 µL of DNA extraction buffer (100 mM NaCl, 10 mM Tris-HCl, 50 mM EDTA, 0.5% SDS, 0.2% ß-mercaptoethanol, pH 8.0) was added. The samples were physically disrupted for three minutes using a Mini beadbeater-16 (Biospec Products). A 5 µL volume of Proteinase K (20 mg/mL; Qiagen) was then added to the homogenised sample and incubated for 40 min at 65 °C. The DNA was then precipitated using ice cold 5 M NaCl and isopropanol, pelleted by centrifugation at 14, 000 x g for 15 min using a 5702R Centrifuge (Eppendorf), washed in 70% ethanol, and then air dried at room temperature. The extracted DNA pellet was resuspended in 40 µL ultra-pure water and quality checked as detailed above before being stored at -20 °C.

Table 43. Details of mealybug and ground pearl samples, sample preservation and genomic DNA extraction.

Sample type	Preservation method	DNA Extraction method
Ground pearl 1	Frozen	Ballcone beads/QIAamp DNA Mini Kit
Mealybug 1	Frozen	Ballcone beads/QIAamp DNA Mini Kit
Ground pearl 2	Frozen	Zirconia-Silica beads/ Phillips and Simon, 1995
Mealybug 2	Frozen	Zirconia-Silica beads/ Phillips and Simon, 1995
Ground pearl 3	EtOH	Ballcone beads/QIAamp DNA Mini Kit
Mealybug 3	EtOH	Ballcone beads/QIAamp DNA Mini Kit
Ground pearl 1	Frozen	Ballcone beads/QIAamp DNA Mini Kit
Mealybug 1	Frozen	Ballcone beads/QIAamp DNA Mini Kit

9.1.4 Amplicon PCR and sequencing protocols

The production of the DNA amplicons for sequencing were prepared by PCR amplification using the extracted gDNA from samples as template. The reaction components and volumes used in all the PCR assays are detailed in Table 44 and all PCRs were carried out in a C1000 Thermal Cycler PCR machine (Bio-Rad Laboratories Pty, USA) with the hot lid set to 105 °C.

For the bacterial microbiome, a nested PCR approach was undertaken. The primers 799F and 1391R, used in the first PCR assay, are detailed in Table 8 and the amplification conditions used are detailed in Table 45. Following the first PCR reaction, the PCR amplicons were separated on the size by agarose gel electrophoresis (2% TAE agarose gel with 0.01% Gel Red nucleic acid gel stain Biotium), using a Sub-Cell GT Gel electrophoresis unit (Biorad). For each sample, the DNA amplicon band corresponding to the PCR product amplified from bacterial populations was excised from the agarose gel and purified using the QIAquick Gel Extraction Kit (Qiagen). The purified DNA was then used as template in a second PCR assay, using primers NXT-967F and NXT-1391R, which had the addition of an adapter (overhang) sequence, and these are detailed in Table 8 and the amplification conditions used are detailed in Table 45. The PCR products from this second PCR reaction were checked for specificity by agarose electrophoresis and Nanodrop for quantity.

For the fungal microbiome the ITS region was amplified by PCR using the primers ITS1-tagged and ITS2-tagged which had the addition of an adapter (overhang) sequence, and these are detailed in Table 8 and the amplification conditions used are detailed in Table 45. The PCR products were checked for specificity by agarose electrophoresis and Nanodrop for quantity.

Table 44. PCR reaction components and volumes used in each reaction for bacterial and fungal PCRs.

PCR reaction component	Volume (µL)
Ultra-pure water	13.0
Super Fi buffer (5x)	5.0
1.25 mM dNTP	4.0
12.5 mM Forward primer	1.0
12.5 mM Reverse primer	1.0
Super Fi DNA polymerase	0.5
gDNA Template	0.5
Total volume of reaction	25.0

Table 45. Amplification conditions (temperature, time, and number of cycles) used in PCR reactions - A. Bacterial microbiome first PCR and B. Bacterial microbiome nested PCR with adapter sequence and C. Fungal microbiome ITS PCR with adapter sequence.

A. Bacterial microbiome first PCR

Temperature (°C)	Time (sec)	Cycles
98°C	60	1
98°C	10	30
53°C	10	

B. Bacterial microbiome nested PCR with adapter sequence

Temperature (°C)	Time (sec)	Cycles
98 °C	60	1
98 °C	10	30
53 °C	10	

C. Fungal microbiome PCR with adapter sequence

Temperature (°C)	Time (sec)	Cycles
98 °C	60	1
98 °C	10	30
50 °C	10	
72 °C	30	
72 °C	300	1
4 °C	forever	1

The PCR products for bacterial and fungal microbiomes were then sent to the Australian Genomic Research Facility (AGRF) where the adapter sequence was used for the attachment of sample-specific, index adaptors (dual index adaptors, Nextera, Illumina), library preparation and sequencing using the MiSeq platform (Illumina).

For the micro-eukaryotic microbiome, the V8-V9 regions of 18S rRNA genes were PCR amplified by the Paul Dennis laboratory using 1510R: CCTTCYGCAGGTTCACCTAC (Amaral-Zettler et al., 2009) modified on the 5' end to contain a unique 8 bp molecular identifier (MID) barcode and an Illumina adapter for compatibility with the i5 Nextera XT index; and V8F: ATAACAGGTCTGTGATGCCCT (Bradley et al 2016), modified on the 5' end to contain the Illumina overhang adapter for compatibility with i7 Nextera XT index. Complete primer sequences are detailed in Table 8. Each PCR was performed using 2.5 µL DNA with 5X Phire Green Reaction Buffer (Thermo Scientific), 200 µM of each dNTP, 0.25 µM of each primer, and 0.4 µL Phire Hot Start II DNA Polymerase, made up to a total volume of 20 µL with water. Thermocycling conditions were as follows: 98°C for 45 s; followed by 30 cycles of 98°C for 5 s, 56°C for 5 s, 72°C for 6 s; then by 72°C for 1 min. Amplifications were performed using a Simpliamp® 96-well Thermocycler (Applied Biosystems). No-template control reactions were included and confirmed to be contamination-free using gel electrophoresis. Amplicons were purified using an 18% suspension of Sera-Mag Speed-beads Carboxyl Magnetic Beads (GE Healthcare). The magnetic beads were added to the PCR products in a ratio of 1.8:1.0. The amplicons were then washed twice with 75% ethanol and resuspended in 25 µl water.

Pools of up to 24 MID-barcoded amplicons were combined in equimolar concentrations and will be subjected to dual indexing using the Nextera XT Index Kit (Illumina) according to the manufacturer's instructions. Briefly, PCRs will comprise 5 ng pooled DNA, 1X Phire Green Reaction Buffer (Thermo Fisher), 200 µM of each dNTP (Invitrogen), 1 µL Phire Green Hot Start II DNA Polymerase, and 1 µL of each primer, made up to a total volume of 50 µL with water. Thermocycling conditions will be as follows: 98 °C for 45 s; then 8 cycles of 95 °C for 5 s, 55 °C for 5 s, 72 °C for 6 s; followed by 72 °C for 1 min. Amplicons will then be purified using an 18% suspension of Sera-Mag Speed-beads Carboxyl Magnetic Beads (GE Healthcare) as described above. Purified Illumina-indexed pools will be mixed

and sequenced on an Illumina MiSeq using a MiSeq Reagent Kit v3 (600 cycles; Illumina) using 8 pM libraries spiked with 30% PhiX Control v3 (Illumina) according to the manufacturer's instructions.

9.1.5 Bioinformatic processing of the microbiome sequence data

The sequence data were received from AGRF as 300 bp paired end reads obtained for both the bacterial 16S rRNA gene and the fungal ITS region. These data sets were initially analysed using the same approach. S with the sequence reads de-multiplexed, were initially quality filtered, paired and size trimmed (>200 bp in length remaining) to remove primer/barcode sequences using Trimmomatic version 0.36 (Bolger et al., 2014). The paired reads were then imported into the Quantitative Insights Into Microbial Ecology 2 (QIIME 2) software pipeline package, either version 2019.10 or 2021.4 (Caporaso et al., 2010, Caporaso et al., 2012) where and the DADA2 software (Callahan et al., 2016) was used to model and correct any remaining Illumina sequencing errors. In this way the reads were further quality filtered, the forward and reverse reads merged, unique sequences (sequence variants) grouped, and chimeras removed.

Unique sequences were identified, and the numbers of each unique sequence in samples was determined with a representative sequence (Feature or sequence variant, similar to the Operational Taxonomic Unit determined by previous versions of QIIME) for each of these were selected. A phylogenetic tree was created to relate the Features to one another before the taxonomy (identity) of each Feature was determined A Feature table containing the counts (frequencies) of each unique sequence in each sample in the dataset (Feature or sequence variant, similar to the Operational Taxonomic Unit determined by previous versions of QIIME), a representative sequences file (rep set) and a FeatureData file which maps Feature identifiers in the Feature table to the sequences they represent, was were then created. A multiple sequence alignment was done in the 'Multiple Alignment using Fast Fourier Transform' software (Katoh and Standley, 2013) and a phylogenetic tree was created to relate Features to one another and assign phylogenetic groups to the Feature table.

For the bacterial 16S rRNA representative sequences (rep set), taxonomy was assigned using a pre-trained Naïve Bayes classifier trained on the SILVA database December 2019 update, version 138 (Yilmaz et al., 2014). For the fungal ITS representative sequences (rep set), taxonomy was assigned using the UNITE database version 7, 10.10.2017 update (Nilsson et al., 2019) which was formatted and imported into QIIME 2 using the q2-feature classifier software (Bokulich et al., 2018). Following taxonomic classification, any bacterial microbial Features and rep set sequences classified as plant chloroplasts or mitochondria were removed. Similarly, any fungal Features and corresponding rep set sequences classified within the kingdom Plantae, were removed from the dataset.

General micro-eukaryotic 18S rRNA gene sequences were processed by Dr Paul Dennis' laboratory using a modified UPARSE workflow (Edgar, 2013). Briefly, demultiplexing and primer removal was performed using cutadapt in QIIME 2 (Bolyen et al., 2019). Then in USEARCH (v10.0.240) (Edgar, 2010), fastx_truncate was used to trim (250 bp) forward reads, which were quality filtered using fastq_filter (−fastq_maxee = 1.0), and then mapped against representative sequences, generated using fastx_uniques and cluster_otus (sequence similarity = 0.97), to create an operational taxonomic unit (OTU) table using otutab. OTUs were assigned SILVA 138 (Quast et al., 2012) and PR2 taxonomy (Guillou et al., 2013) using BLASTN (v2.3.0+) (Zhang et al., 2000) in QIIME 2. Representative OTU sequences were aligned using MAFFT (v7.221) (Katoh et al., 2013) and masked using QIIME 2 to calculate phylogenetic distance and generate a midpoint-rooted phylogenetic tree using FastTree (v2.1.9) (Price et al., 2010). Samples were rarefied to 1,000 reads, and the mean

numbers of observed (Sobs) and predicted (Chao1) (Chao, 1984) OTUs, as well as Shannon's Diversity Index (Shannon, 1948), and Faith's Phylogenetic Diversity Index (Faith's PD) (Faith, 1992) were calculated using QIIME 2.

For the leaf and root microbiomes, four sequence datasets were obtained (1) Leaf bacteria; (2) Leaf fungi (3) Root bacteria and (4) Root fungi. All datasets were initially analysed using the Quantitative Insights into Microbial Ecology (QIIME 2) software package (Version 2020.4) (Bokulich et al., 2018; Boylen et al., 2019). Sequence datasets were imported into QIIME 2 and the DADA2 software used for modelling and correcting Illumina-sequenced amplicon errors (Callahan et al., 2016). In this way the input sequences were further quality filtered, the forward and reverse reads merged, unique sequences (sequence variants) grouped, and chimeras removed. A Feature table (the equivalent of the QIIME 1 OTU or BIOM table) containing the counts (frequencies) of each unique sequence (Feature) in each sample within the dataset. A representative sequences file (rep set) and a FeatureData file which maps Feature identifiers in the Feature table to the sequences they represent, was then created. The Feature table was further filtered to remove Features representing < 5 sequences and to remove negative sequencing control samples. A multiple sequence alignment using MAFFT v7 (Katoh and Standley, 2013) and a phylogenetic tree was created to relate Features to one another and assign phylogenetic groups to the Feature table. The 16S rRNA gene bacterial taxonomy was assigned using a pre-trained Naïve Bayes classifier trained on the SILVA database (update 138, released December 16, 2019; downloaded from QIIME 2 Resources) (Yilmaz et al., 2014; https://www.arb-silva.de/). The ITS2 fungal taxonomy was assigned using the UNITE database version 7, 10.10.2017 update (Nilsson et al., 2019).

The taxonomy of specific samples was depicted using taxonomic bar plots generated using QIIME 2, with samples ordered on the x-axis on the basis of specific metadata categories of interest (e.g. Symptomatic vs Non-symptomatic samples; Sample collection site; plant common name). Alpha diversity analysis (microbial diversity within a sample) was determined on the basis of three measures: (1) counts of observed species (Observed Species); (2) Faith phylogenetic diversity (Faith-PD); (3) Shannon entropy of counts (Shannon); and (4) Peilou evenness. The four alpha diversity measures were analysed in Genstat Release 21.1 (27 January 2022; VSN International Ltd., 2022) using a repeated measures residual maximum likelihood (REML) method. Predicted means and standard error differences (s.e.d.) were calculated.

For determination of the differences in the microbial communities occurring between samples (Beta diversity), the respective metadata files, as well as the table, representative sequence (rep set), and unrooted phylogenetic tree (.tre) files generated using QIIME2, were imported into the R packages, Phyloseq (version 1.30.0; McMurdie and Holmes, 2013; https://joey711.github.io/phyloseq/index.html) and MixOmics (version 6.10.6; Rohart et al. 2017; http://mixomics.org/methods/pls-da/). Statistical exploration and microbial community analysis used a multivariate projection-based approach with repeated measures. For the identification of indicator species and determination of microbial signatures, a sparse Partial Least Squares Discriminant Analysis (SPLSDA) was undertaken. This method was conducted using the MixOmics R package.

Briefly, an unsupervised analysis with Principal Component Analysis (PCA) (Jolliffe, 2005) was conducted using the Feature table data generated using QIIME 2, transformed using the centred log ratio (CLR). To determine the most discriminative Features or OTUs (Features being referred to as OTUs within the MixOmics package), that best characterised factors of interest (e.g. days of each fermentation or each sampling period within the animal trial), a supervised analysis and selection of discriminative OTUs was undertaken with a multivariate analysis SPLSDA on three components (Shen

and Huang, 2008; Le Cao et al., 2011). Contribution plots showing the most discriminative OTUs were generated based on the coefficient derived from the component analysis. This indicated the importance of the respective OTUs in determining the microbial signature, with the sign indicating the positive of negative correlations between the OTUs, relative to the proportions of the others. Due to the high numbers of OTUs within the microbial signatures, results were presented as tables of the top 10 most important OTUs with respective assigned taxonomy, for each of the three components of the sPLSDA.

Core microbial communities were determined following taxonomic classification of Features identified using QIIME2. Features which were present in 80% of samples according to the metadata category of interest (e.g. Symptomatic vs Non-symptomatic for pasture dieback; with or without mealybugs), were designated as "core" microbial communities. For comparison of the numbers of core and overall microbial communities present in fermentations maintained on different Leucaena cultivars, the on-line tool Venny was used (https://bioinfogp.cnb.csic.es/tools/venny/; Oliveros, J.C., 2007-2015). This method was used to generate Venn diagrams and lists of microbial populations which were designated as either shared or unique, according to the metadata category of interest (e.g. Symptomatic vs Non-symptomatic for pasture dieback).

9.2 Microbiome analysis – additional results.

9.2.1 Differential abundance analysis for bacterial and fungal populations.

To determine which microbial populations were contributing to the differences occurring between symptomatic and non-symptomatic plant material (leaf and root samples), a sparse Partial Least Squares Discriminant Analysis (sPLSDA; Le Cao et al. 2011) was undertaken. The sPLSDA plots are provided together with lists of the top 10 microbial populations contributing to the variation seen between symptomatic and non-symptomatic plant material. This analysis was undertaken for leaf and root samples, where the bacterial and fungal populations were determined. SPLSDA results shown for Leaf bacteria (Fig. 38; Table 46), Root bacteria (Fig. 39, Table 47); Leaf fungi (Fig. 40, Table 48); Root fungi (Fig. 41, Table 49).

Figure 38. **Differences in variation occurring between the bacterial populations associated with pasture dieback symptoms (Non-symptomatic leaf samples (O) and Symptomatic leaf samples (Δ)), determined by sPLSDA.** Results shown on the basis of three components (A) Components 1 vs 2 (sPLSDA comp 1-2); and (B) Components 1 vs 3 (sPLSDA comp 1-3).

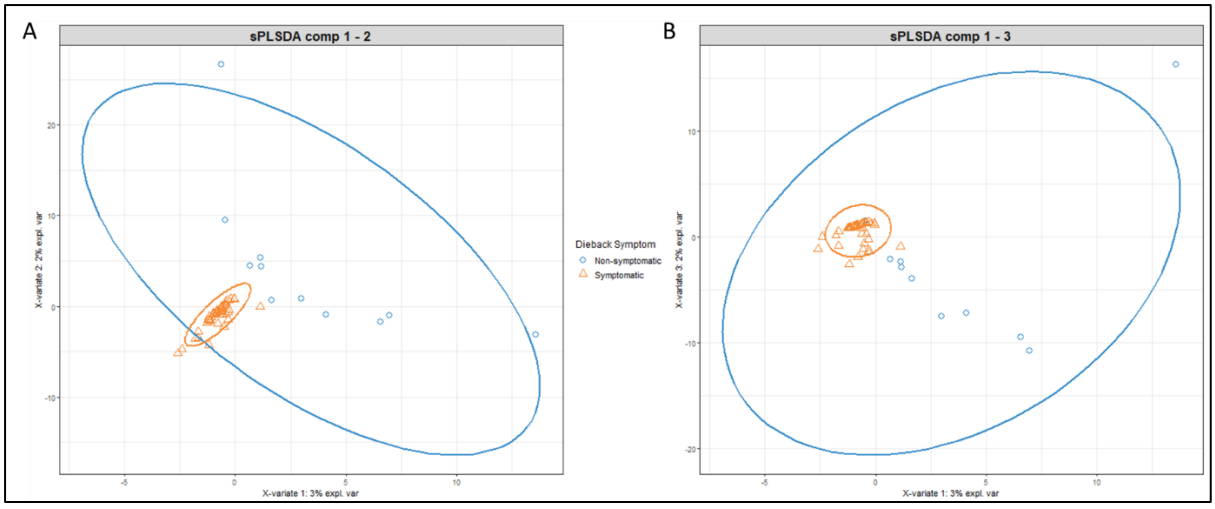

Table 46. Table of bacteria contributing to the differences in variation occurring between the bacterial populations associated with non-symptomatic and symptomatic leaf material, determined by sPLSDA. The top 10 bacteria are listed, with the respective positive or negative correlation value (Importance). All bacterial taxons identified in this analysis were from non-symptomatic leaf samples. Taxonomy is listed according to the taxonomic levels of phylum (p); class (c); order (o); family (f); and genus (g)

Contribution to sPLSDA component 1 - Bacterial taxon	**Importance**
p__Firmicutes; c__Bacilli; o__Paenibacillales; f__Paenibacillaceae; g__*Paenibacillus*[A]	0.43
p__Firmicutes; c__Bacilli; o__Paenibacillales; f__Paenibacillaceae; g__*Paenibacillus*	0.36
p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacterales; f__Erwiniaceae	0.29
p__Proteobacteria; c__Alphaproteobacteria; o__Rhizobiales; f__Xanthobacteraceae	0.29
p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__*Bacillus*	0.29
p__Actinobacteriota; c__Actinobacteria; o__Pseudonocardiales; f__Pseudonocardiaceae; g__Pseudonocardia	0.28
p__Actinobacteriota; c__Actinobacteria; o__Micromonosporales; f__Micromonosporaceae	0.27
p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacterales; f__Erwiniaceae	0.24
p__Proteobacteria; c__Alphaproteobacteria; o__Reyranellales; f__Reyranellaceae; g__Reyranella	0.22
p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae; g__Sphingomonas	0.21
Contribution to sPLSDA component 2 - Bacterial taxon	**Importance**
p__Firmicutes; c__Bacilli; o__Paenibacillales; f__Paenibacillaceae; g__Paenibacillus	0.21
p__Actinobacteriota; c__Actinobacteria; o__Kineosporiales; f__Kineosporiaceae; g__Kineosporia; s__uncultured_bacterium	0.18
p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__Bacillus	0.18
p__Actinobacteriota; c__Actinobacteria; o__Micromonosporales; f__Micromonosporaceae; g__Actinoplanes	0.18
p__Actinobacteriota; c__Actinobacteria; o__Corynebacteriales; f__Mycobacteriaceae; g__Mycobacterium	0.18
p__Actinobacteriota; c__Actinobacteria; o__Streptosporangiales; f__Thermomonosporaceae; g__Actinoallomurus	0.18
p__Actinobacteriota; c__Thermoleophilia; o__Solirubrobacterales; f__67-14; g__67-14	0.18
p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacterales; f__Erwiniaceae	0.18
p__Firmicutes; c__Bacilli; o__Paenibacillales; f__Paenibacillaceae; g__Paenibacillus	0.18
p__Firmicutes; c__Bacilli; o__Paenibacillales; f__Paenibacillaceae; g__Paenibacillus	0.18
Contribution to sPLSDA component 3 - Bacterial taxon	**Importance**
p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae; g__Sphingomonas	-0.48
p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacterales; f__Erwiniaceae	-0.29
p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacterales; f__Erwiniaceae	-0.22
p__Actinobacteriota; c__Actinobacteria; o__Frankiales; f__Geodermatophilaceae; g__Klenkia	-0.20
p__Gemmatimonadota; c__Gemmatimonadetes; o__Gemmatimonadales; f__Gemmatimonadaceae	0.19
p__Actinobacteriota; c__Actinobacteria; o__Pseudonocardiales; f__Pseudonocardiaceae; g__Actinomycetospora; s__Actinomycetospora_sp.	0.19
p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__Bacillus	0.19
p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__Burkholderiaceae	0.19
p__Acidobacteriota; c__Acidobacteriae; o__Solibacterales; f__Solibacteraceae; g__Candidatus_Solibacter; s__uncultured_Solibacter	0.16
p__Firmicutes; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae	0.16

* Where bacteria with the same taxonomy are listed multiple times, they represent different features (or OTUs) identified within the sequence dataset that could not be classified beyond the lowest taxonomic level listed.

Figure 39. Differences in variation occurring between the bacterial populations associated with pasture dieback symptoms (Non-symptomatic root samples (O) and Symptomatic root samples (Δ)), determined by sPLSDA. Results shown on the basis of three components (A) Components 1 vs 2 (sPLSDA comp 1-2); and (B) Components 1 vs 3 (sPLSDA comp 1-3).

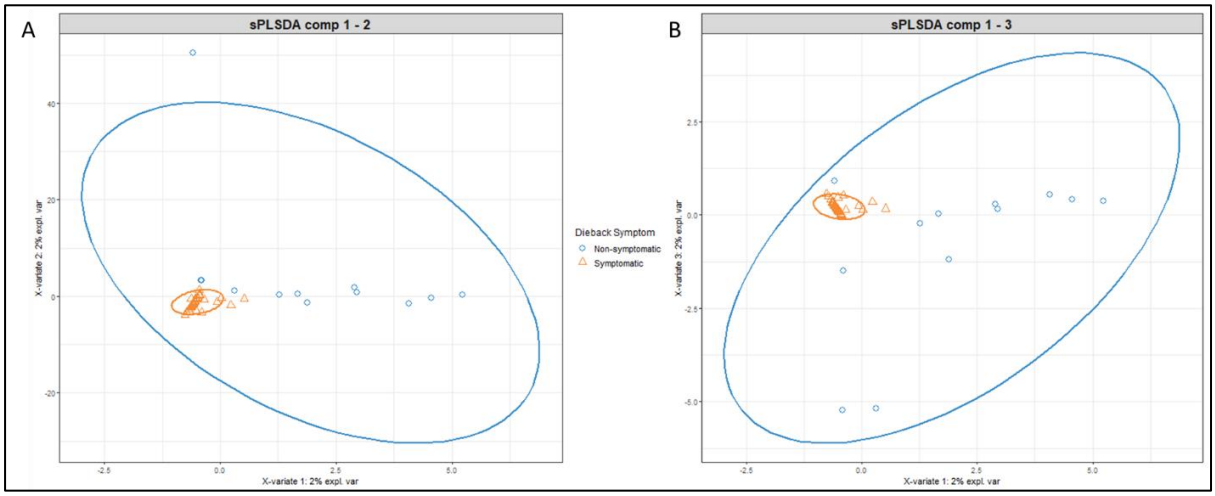

Table 47. Table of bacteria contributing to the differences in variation occurring between the bacterial populations associated with non-symptomatic and symptomatic root material, determined by sPLSDA. The top 10 bacteria are listed, with the respective positive or negative correlation value (Importance). All bacterial taxons identified in this analysis were from non-symptomatic leaf samples. Taxonomy is listed according to the taxonomic levels of phylum (p); class (c); order (o); family (f); and genus (g).

Contribution to sPLSDA component 1 - Bacterial taxon	**Importance**
p__Actinobacteriota; c__Actinobacteria; o__Kineosporiales; f__Kineosporiaceae	0.91
p__Actinobacteriota; c__Actinobacteria; o__Streptomycetales; f__Streptomycetaceae; g__Streptomyces	0.36
p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__Comamonadaceae	0.18
p__Actinobacteriota; c__Actinobacteria; o__Pseudonocardiales; f__Pseudonocardiaceae; g__Crossiella; s__uncultured_actinobacterium	0.12
p__Actinobacteriota; c__Thermoleophilia; o__Gaiellales; f__Gaiellaceae; g__Gaiella; s__uncultured_Rubrobacteria	0.05
Contribution to sPLSDA component 2 - Bacterial taxon	**Importance**
p__Actinobacteriota; c__Thermoleophilia; o__Solirubrobacterales; f__67-14; g__67-14	0.41
p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Moraxellaceae; g__Acinetobacter	0.40
p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacterales; f__Enterobacteriaceae	0.35
p__Proteobacteria; c__Alphaproteobacteria; o__Rhizobiales; f__Beijerinckiaceae; g__Microvirga	0.35
p__Actinobacteriota; c__Actinobacteria; o__Pseudonocardiales; f__Pseudonocardiaceae	0.08
p__Proteobacteria; c__Gammaproteobacteria; o__Steroidobacterales; f__Steroidobacteraceae; g__Steroidobacter	0.07
p__Chloroflexi; c__TK10; o__TK10; f__TK10; g__TK10; s__bacterium_Ellin6519	0.07
p__Bacteroidota; c__Bacteroidia; o__Chitinophagales; f__Chitinophagaceae; g__Chitinophaga; s__Flexibacter_sp.	0.07
p__Actinobacteriota; c__Thermoleophilia; o__Gaiellales; f__Gaiellaceae; g__Gaiella	0.07
p__Actinobacteriota; c__Actinobacteria; o__Micrococcales; f__Micrococcaceae	0.07
Contribution to sPLSDA component 3 - Bacterial taxon	**Importance**
p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Moraxellaceae; g__Acinetobacter	-0.97
p__Myxococcota; c__Polyangia; o__Polyangiales; f__BIrii41; g__BIrii41; s__uncultured_Sorangiineae	-0.23
p__Fibrobacterota; c__Fibrobacteria; o__Fibrobacterales; f__Fibrobacteraceae; g__possible_genus_04; s__uncultured_bacterium	-0.07
p__Actinobacteriota; c__Actinobacteria; o__Micromonosporales; f__Micromonosporaceae; g__Actinoplanes	-0.06
p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__Comamonadaceae	-0.01

Figure 40. Differences in variation occurring between the fungal populations associated with pasture dieback symptoms (Non-symptomatic leaf samples (O) and Symptomatic leaf samples (Δ)), determined by sPLSDA. Results shown on the basis of three components (A) Components 1 vs 2 (sPLSDA comp 1-2); and (B) Components 1 vs 3 (sPLSDA comp 1-3).

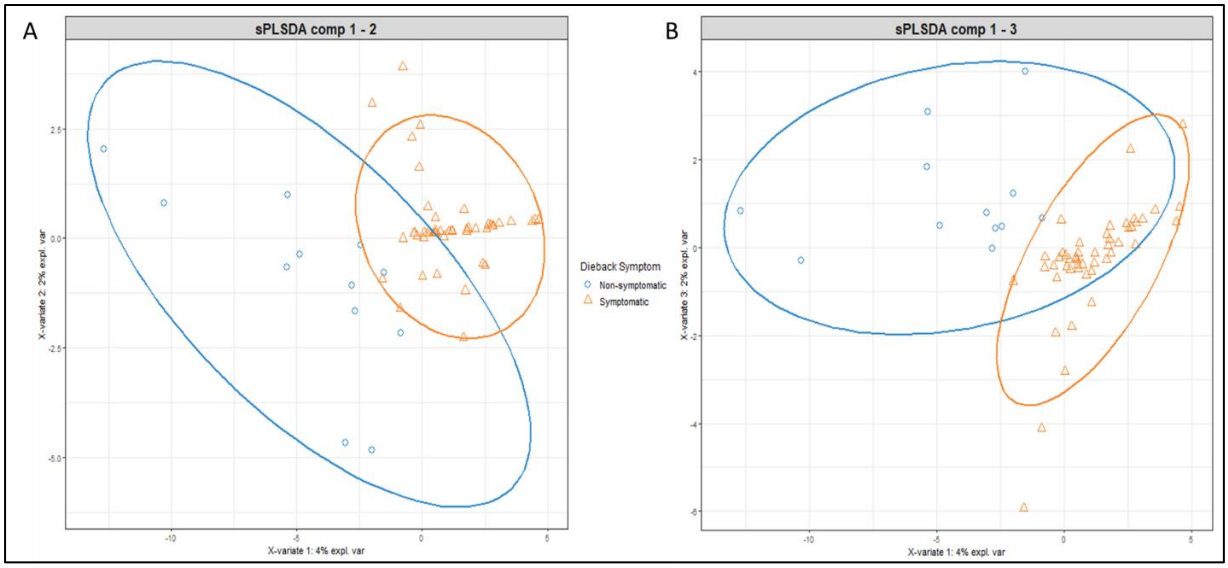

Table 48. Table of fungi contributing to the differences in variation occurring between the fungal populations associated with non-symptomatic and symptomatic leaf material, determined by sPLSDA. The top 10 fungi are listed, with the respective positive or negative correlation value (Importance). Features contributing to differences in variation, for which taxonomy could not be assigned (Unclassified) were not listed although the number of these Unclassified features indicated for each sPLSDA component. Taxonomy is listed according to the taxonomic levels of phylum (p); class (c); order (o); family (f); and genus (g).

Contribution to sPLSDA component 1 - Fungal taxon*	**Importance**	**Symptoms**
k__Fungi;p_Unclassified	-0.28	Non-symptomatic
k__Fungi;p__Basidiomycota;c__Tremellomycetes;o__Filobasidiales;f__Filobasidiaceae;g__Naganishia;s__Naganishia_diffluens	-0.24	Non-symptomatic
k__Fungi;p_Unclassified	-0.21	Non-symptomatic
k__Fungi;p_Unclassified	-0.20	Non-symptomatic
k__Fungi;p_Unclassified	-0.20	Non-symptomatic
k__Fungi;p__Basidiomycota;c__Ustilaginomycetes;o__Ustilaginales;f__Ustilaginaceae	-0.19	Non-symptomatic
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Pleosporaceae;g__Curvularia;s__Curvularia_eragrostidis	-0.18	Non-symptomatic
k__Fungi;p_Unclassified	-0.16	Non-symptomatic
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Phaeosphaeriaceae;g__Phaeosphaeria;s__Phaeosphaeria_podocarpi	-0.16	Non-symptomatic
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Pleosporaceae;g__Curvularia;s__Curvularia_trifolii	-0.16	Non-symptomatic
Contribution to sPLSDA component 2 - Fungal taxon**	**Importance**	**Symptoms**
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Pleosporaceae;g__Curvularia;s__Curvularia_eragrostidis	-0.58	Non-symptomatic
k__Fungi;p__Ascomycota;c__Eurotiomycetes;o__Chaetothyriales;f__Chaetothyriales_fam_Incertae_sedis;g__Strelitziana;s__Strelitziana_eucalypti	0.40	Symptomatic
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Pleosporaceae;g__Bipolaris;s__Bipolaris_drechsleri	-0.36	Non-symptomatic
k__Fungi;p_Unclassified	-0.34	Non-symptomatic
k__Fungi;p_Unclassified	0.25	Non-symptomatic
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Didymellaceae	0.19	Symptomatic
k__Fungi;p_Unclassified	0.01	Non-symptomatic
k__Fungi;p_Unclassified	0.01	Symptomatic
Contribution to sPLSDA component 3 - Fungal taxon***	**Importance**	**Symptoms**
k__Fungi;p_Unclassified	-0.46	Symptomatic
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Dothideales;f__Dothioraceae;g__Pseudoseptoria;s__Pseudoseptoria_obscura	0.40	Non-symptomatic

k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Xylariales;f__Microdochiaceae;g__Microdochium	-0.37	Non-symptomatic
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Capnodiales;f__Cladosporiaceae;g__Toxicocladosporium;s__Toxicocladosporium_cacti	-0.34	Symptomatic
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Sporormiaceae;g__Preussia	0.24	Non-symptomatic
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Capnodiales;f__Cladosporiaceae;g__Cladosporium;s__Cladosporium_exasperatum	0.22	Symptomatic
k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Hypocreales;f__Nectriaceae	-0.12	Non-symptomatic
k__Fungi;p__Basidiomycota;c__Ustilaginomycetes;o__Ustilaginales;f__Ustilaginaceae	0.11	Non-symptomatic
k__Fungi;p__Basidiomycota;c__Cystobasidiomycetes;o__Cystobasidiomycetes_ord_Incertae_sedis;f__Cystobasidiomycetes_fam_Incertae_sedis;g__Symmetrospora	-0.10	Symptomatic
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Didymosphaeriaceae;g__Paraconiothyrium;s__Paraconiothyrium_cyclothyrioides	0.09	Symptomatic

*Five Unclassified features contributing to differences see for sPLSDA component 1 removed from list;**Two Unclassified features contributing to differences see for sPLSDA component 2 removed from list;***Two Unclassified features contributing to differences see for sPLSDA component 3 removed from list.

Figure 41. Differences in variation occurring between the fungal populations associated with pasture dieback symptoms (Non-symptomatic root samples (O) and Symptomatic root samples (Δ)), determined by sPLSDA. Results shown on the basis of three components (A) Components 1 vs 2 (sPLSDA comp 1-2); and (B) Components 1 vs 3 (sPLSDA comp 1-3).

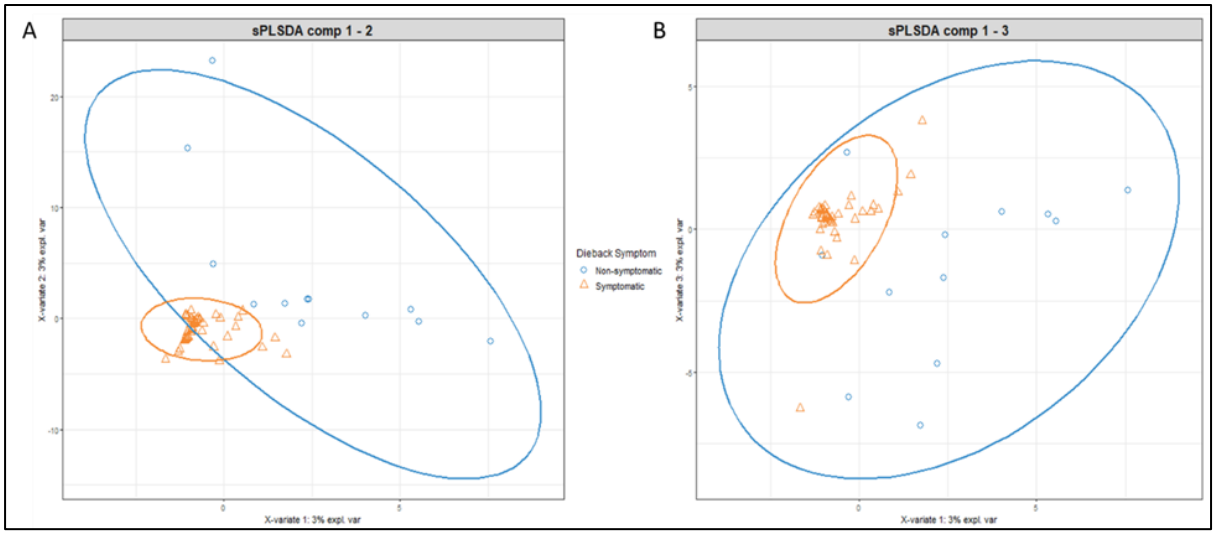

Table 49. Table of fungi contributing to the differences in variation occurring between the fungal populations associated with non-symptomatic and symptomatic root material, determined by sPLSDA. The top 10 fungi are listed, with the respective positive or negative correlation value (Importance). Taxonomy is listed according to the taxonomic levels of phylum (p); class (c); order (o); family (f); and genus (g).

Contribution to sPLSDA component 1 - Fungal taxon	**Importance**	**Symptoms**
k__Fungi;p_Unclassified	0.55	Non-symptomatic
k__Fungi;p_Unclassified	0.51	Non-symptomatic
k__Fungi;p__Basidiomycota;c__Agaricomycetes;o__Agaricales;f__Marasmiaceae	0.39	Non-symptomatic
k__Fungi;p__Ascomycota;c__Ascomycota_cls_Incertae_sedis;o__Ascomycota_ord_Incertae_sedis;f__Ascomycota_fam_Incertae_sedis;g__Acrophialophora;s__Acrophialophora_fusispora	0.35	Non-symptomatic
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Pleosporaceae;g__Curvularia;s__Curvularia_lunata	0.23	Non-symptomatic
k__Fungi;p__Basidiomycota;c__Agaricomycetes;o__Agaricales;f__Lycoperdaceae;g__Calvatia;s__Calvatia_fragilis	0.18	Non-symptomatic
k__Fungi;p__Ascomycota;c__Eurotiomycetes;o__Chaetothyriales;f__Herpotrichiellaceae;g__Cladophialophora	0.17	Non-symptomatic
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Lentitheciaceae;g__Poaceascoma;s__Poaceascoma_helicoides	0.15	Non-symptomatic
k__Fungi;p__Ascomycota	0.12	Non-symptomatic
k__Fungi;p__Chytridiomycota	0.12	Non-symptomatic
Contribution to sPLSDA component 2 - Fungal taxon	**Importance**	**Symptoms**
k__Fungi;p__Basidiomycota;c__Agaricomycetes;o__Cantharellales;f__Ceratobasidiaceae	0.28	Non-symptomatic
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Botryosphaeriales;f__Botryosphaeriaceae;g__Botryosphaeria	0.26	Non-symptomatic
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Periconiaceae;g__Periconia;s__Periconia_macrospinosa	0.21	Non-symptomatic
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Capnodiales	0.19	Non-symptomatic
k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Branch06	0.19	Non-symptomatic

k__Fungi;p__Basidiomycota;c__Agaricomycetes;o__Agaricales;f__Psathyrellaceae;g__Parasola	0.19	Non-symptomatic
k__Fungi;p__Chytridiomycota	0.19	Non-symptomatic
k__Fungi;p_Unclassified	0.18	Non-symptomatic
k__Fungi;p__Basidiomycota;c__Agaricomycetes;o__Agaricales;f__Psathyrellaceae;g__Psathyrella;s__Psathyrella_parva	0.18	Non-symptomatic
k__Fungi;p__Chytridiomycota;c__Rhizophlyctidomycetes;o__Rhizophlyctidales;f__Rhizophlyctidaceae;g__Rhizophlyctis	0.18	Non-symptomatic
Contribution to sPLSDA component 3 - Fungal taxon	**Importance**	**Symptoms**
k__Fungi;p__Basidiomycota;c__Cystobasidiomycetes;o__Erythrobasidiales;f__Erythrobasidiaceae;g__Erythrobasidium;s__Erythrobasidium_hasegawianum	-0.46	Non-symptomatic
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Capnodiales;f__Teratosphaeriaceae	-0.42	Non-symptomatic
k__Fungi;p__Basidiomycota	-0.38	Non-symptomatic
k__Fungi;p__Ascomycota;c__Eurotiomycetes;o__Chaetothyriales;f__Herpotrichiellaceae;g__Cladophialophora	-0.35	Non-symptomatic
k__Fungi;p__Basidiomycota	0.24	Symptomatic
k__Fungi;p__Basidiomycota;c__Agaricomycetes;o__Cantharellales;f__Ceratobasidiaceae	-0.24	Non-symptomatic
k__Protista;p__Cercozoa	0.21	Non-symptomatic
k__Fungi;p_Unclassified	-0.18	Non-symptomatic
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Phaeosphaeriaceae	-0.17	Non-symptomatic
k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Hypocreales;f__Bionectriaceae	-0.16	Non-symptomatic

9.3 Pasture mealybug experiment at Brian Pastures research station, Gayndah.

Photographic timeline of each plot in the pasture mealybug impact trial. The images are presented as per the assigned treatments so that the condition of each cage/plot/rep can be followed over time). The development of symptoms through November-December is evident, as is the pasture death by January.

Figure 42. Photos of plots treated for mealybugs (treated control) over time.

Figure 43. Photos of plots not treated for mealybugs (untreated control) over time.