Final report

B.PAS.0006 Resistant grass varieties and endophytes

Project code: B.PAS.0006: Resistant grass varieties and endophytes

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Date published: June 2022

PUBLISHED BY

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NORTH SYDNEY NSW 2059

This is an MLA Donor Company funded project.

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government and contributions from the Australian Meat Processor Corporation to support the research and development detailed in this publication.

Abstract

Pasture dieback causes unhealthy growth and death in a range of introduced and native grasses across Queensland and into northern New South Wales, resulting in large losses in beef production areas. Pasture mealybug, *Heliococcus summervillei* Brookes, has been identified as the leading cause of pasture dieback.

Possible management strategies include the identification of resistant or tolerant pasture varieties, and the use of endophytic fungi (both seed born and facultative) to protect pasture grasses possibly in association with pasture mixes including legumes. In New Zealand, pasture dieback in rye grass caused by the mealybug *Balanococcus poae* has been effectively managed by selection of endophytic fungi and incorporation into commercial pasture varieties. Fungal endophytes may be transferred in seed, while others are acquired from healthy soils, primarily by colonising the roots.

Assay methods were developed and used. The relative susceptibility of 15 selected grass varieties to pasture mealybug was determined in screenhouse assays by infestation with the mealybug *H. summervillei*. Significant differences in susceptibility were detected. Mekong brizantha, Argentina bahia, curly Mitchel grass, Biloela buffel and Gatton panic were all less susceptible to mealybug than American buffel. Callide and Katambora Rhodes, *Paspalum nicorae*, green panic, and Gayndah buffel were all similar in susceptibility to American buffel. In sensitive, rapid and statistically robust laboratory assays, mealybugs on Gatton panic and Mekong brizantha died more rapidly and in higher proportions, and were less able to develop to maturity than on American Buffel.

Assays demonstrated that the seed-born fungal endophyte *Epichloë festucae* reduced the susceptibility of perennial ryegrass to the Australian pasture mealybug *H. summervillei*, and reduced the severity of dieback symptoms. This result provides ‘proof of concept’ of the use of endophytes, as used commercially in New Zealand against the pasture mealybug *Balanococcus poae*.

This work demonstrates a direct link between ‘pasture dieback’, pasture diversity and soil health. Analysis of soils sampled systematically across Queensland and New South Wales showed that severity of dieback was reduced in pastures with greater diversity of species in 5 key genera of beneficial rhizospheric-competent fungal endophytes. Abundance of these fungi was shown to be higher in trial plots planted with legumes in pasture mixes than in plots sown with pasture grasses alone. Management practices that increase diversity of beneficial rhizospheric fungi, such as inclusion of legumes, and impacts of management that reduces abundance and diversity of these fungi (such as, possibly, hot burning) requires investigation.

Further research to screen varieties for tolerance/ resistance and to determine tolerance / resistance mechanisms using a combination of rapid laboratory assay method and transcriptome analysis (B.PAS 0003) are strongly recommended. Further assays and detailed analysis of plant/endophyte/mealybug interactions in grasses should be conducted in combination with trials on pasture legume mixes is strongly recommended.

The project has significantly increased options to manage pasture dieback. Tolerant and resistant pasture varieties have been identified. The benefit of both seed-born and rhizospheric fungal endophytes in reducing mealybug and pasture dieback in the laboratory and in the field has been demonstrated. Rapid assays have been developed to reduce cost and increase speed and statistical power of future screening for development of new pasture varieties and endophytic symbionts for protection of tropical and subtropical pastures.

Executive summary

Background

Pasture dieback causes unhealthy growth and death in a range of introduced and native grasses across Queensland and into northern New South Wales, resulting in large losses in beef production areas. Pasture mealybug, *Heliococcus summervillei* Brookes, has been identified as the leading cause of pasture dieback. This mealybug was previously reported to have caused severe pasture dieback in Queensland in 1926 (Summerville 1928) and the 1930s (Brooks 1978), in New Caledonia in 1998 (Brinon et al 2004), and more recently in Puerto Rico and Barbados.

Possible management strategies include the identification of resistant or tolerant pasture varieties, and the use of endophytic fungi to protect pasture grasses. Endophytes are fungi that live within healthy plant tissue and can confer resistance to insects, including mealybugs. Some may be transferred in seed, while others are acquired from healthy soils, primarily by colonising the roots. In New Zealand, pasture dieback in rye grass caused by the mealybug *Balanococcus poae* has been effectively managed by selection of endophytic fungi and incorporation into commercial pasture varieties.

The project aims to develop methods and use them to identify resistant or tolerant pasture grasses, and soil or seed-born endophytes, that reduce severity of the mealybug causing pasture dieback, to provide new options to manage pasture dieback, and provide a greater understanding of the interactions between insects, plant and microbiota in tropical grasses.

Objectives

The project objectives, as outlined in the research agreement, are as follows:

1.Identify susceptible grass varieties to pasture dieback from field surveys.

2.Assess the relative susceptibility of selected grass varieties to pasture mealybug and/or other agents responsible for pasture dieback and their impact on mealybug biology.

3.Isolate and characterise endophyte species present in grasses collected during pasture dieback field surveys and determine those which are tolerant to the mealybug.

4. Develop procedures and use them to assess promising endophytes (both facultative and seed-born) as cost effective pasture pest control methods. Make recommendations on opportunity for an endophyte approach (via plant breeding/propagation) to address dieback.

5. Develop procedures and use them to assess endophytes and tolerant/resistant pasture mixes in field surveys, and laboratory and glasshouse assays. Make recommendations for field evaluation of prospective tolerant grasses and mixes.

6. Provide timely communication on research activities for use in industry briefings at least biannually throughout the project.

7. A 2-page summary outlining key facts and findings of tolerant and susceptible grass species and management created in consultation with MLA's communications team.

Methodology

The susceptibility of pasture grasses was initially determined from field surveys across central and southern Queensland and northern NSW. This work was successfully conducted despite border closures and lockdowns.

The relative susceptibility of selected grass varieties to pasture mealybug was determined in screenhouse assays of 18 pasture varieties and species infested with the mealybug *H. summervillei*. Improved assay methods were tested and adopted. These included using limited time counts of mealybugs compared to full destructive sampling, and rapid, robust and statistically powerful laboratory bioassays.

Fungal endophytes present in grasses and the rhizosphere were collected during pasture dieback field surveys and isolated on selective media. Fungi were characterised from DNA sequence 'barcodes'. Abundance and diversity of fungi from known beneficial (endophytic) genera, neutral genera, and the potentially pathogenic genus *Fusarium* at 35 sites were compared with severity of dieback determined from transect sampling.

Procedures were developed and used to assess promising endophytes (both facultative and seed-born). Screens used in variety testing were used to evaluate the impact of commercial and wild type endophytes in perennial ryegrass on mealybug abundance. Assays were also developed to determine the effect of inocula from single isolates of potentially beneficially fungi against the mealybug on grasses in laboratory assays. Finally, the impact of legumes in pasture mixtures on the abundance of beneficial fungi was determined in replicated field trials at 3 sites in trials established by collaborators at Applied Horticultural Research Pty Ltd (AHR).

Workshops, media outputs, farm visits, presentations at Beef Week, a webinar, panel discussions with NABRAC and other activities were used to provide information to MLA, the MLA communications team, dieback program participants and with graziers and others in livestock industries, and to communicate findings and implications for mealybug and dieback management throughout the project.

Results/key findings

The mealybug *H. summervillei* was found associated with symptoms of 'pasture dieback' on 24 varieties and species of pasture grasses across Queensland and New South Wales, and on sugar cane. Legumes and other broadleaf species were not hosts of the mealybug and do not show symptoms of 'pasture dieback'.

Methods to conduct screenhouse evaluation of pasture varieties with efficient sampling methods were developed and the relative susceptibility of 15 species and varieties of pasture grass to American buffel was determined. Significant differences in susceptibility were detected.

Mekong brizantha, Argentina bahia, curly Mitchel grass, Biloela buffel and Gatton panic were all less susceptible to mealybug than American buffel.

Callide and Katambora Rhodes, *Paspalum nicora*, green panic, and Gayndah buffel were all similar in susceptibility to American buffel.

There was an apparent correlation between varieties with thick, robust stems and tillers and reduced susceptibility. This was apparent in the Gatton Panic and Green Panic varieties, which are both varieties of *Panicum maximum* and genetically very similar (facultatively apomictic) but are significantly different in their susceptibility to mealybug. Further research on resistance mechanisms of strongly recommended.

Perennial rye grass (*Lollium perenne*) without endophytes was more susceptible to mealybugs than American Buffel.

Rapid (2 week) laboratory screening assays were developed and shown to determine sensitive and statistically robust differences in susceptibility between grass varieties.
American Buffel was shown to be statistically more susceptible than Callide Rhodes, while Gatton Panic and Mekong Brizantha were again statistically less susceptible than American buffel. Differences in development and survival of mealybugs were detected on different varieties.

The seed-born fungal endophyte *Epichloë festucae* var. *lolii* was shown to significantly reduce susceptibility of perennial ryegrass (*L. perenne*) to mealybug in comparison to ryegrass without endophytes and to American Buffel and Callide Rhodes in screenhouse assays.

Thirty nine (39) sites with differing severity of 'pasture dieback' were systematically sampled for plant and rhizospheric endophytes across Qld and NSW. Three sites included replicated trials of pasture mixes planted by AHR.

A total of 1,120 fungal isolates were isolated and identified by Sanger sequencing of the DNA 'barcode' ITS regions 1 and 2. Multiple species and variants of the broadly pathogenic genus *Fusarium* were identified. Five (5) genera of beneficial, rhizospheric-competent or endophytic fungi were isolated and identified from tillers, roots and/or soil: *Penicillium*, *Purpureocillium*, *Trichoderma*, *Clonostachys*, *Beauveria*, including at least one new species of *Clonostachys*.

A highly statistically significant correlation between diversity of species of beneficial endophytic fungi and severity of dieback was demonstrated. Where a high diversity of species of beneficial fungi were isolated, dieback severity was significantly reduced. In contrast, both diversity of *Fusarium* species and races, and diversity of 'neutral' fungi, were not significantly associated with dieback severity.

A statistically significant pattern of beneficial fungi was observed in the Gaeta AHR pasture mix trial: abundance of beneficial fungal was greater in plots that include legumes when compared to plots containing only grasses. The overall pattern at all three trials reflected a similar pattern of reduced mealybug abundance on grasses occurring in plots containing legumes recorded by AHR. Statistical analysis is continuing, with AHR data on mealybug abundance and percentage legume cover to be included.

This work demonstrates a direct link between 'pasture dieback', pasture diversity and soil health. Analysis of soils sampled systematically across Queensland and New South Wales showed that severity of dieback was reduced in pastures with greater diversity of key beneficial rhizospheric-competent fungal endophytes. Abundance of these fungi was shown to be higher in trial plots planted with legumes in pasture mixes than in plots sown with pasture grasses alone. Management practices that increase diversity of beneficial rhizospheric fungi, such as inclusion of legumes, and impacts of management that reduces abundance and diversity of these fungi (such as, possibly, hot burning) requires investigation.

Conclusions

This work has established methods to quantify the relative susceptibility of targeted grass varieties to pasture mealybug H. *summervillei*. Mekong brizantha, Argentina bahia, curly Mitchel grass, Biloela buffel and Gatton panic were all less susceptible to mealybug than American buffel. Callide and Katambora Rhodes, *Paspalum nicorae*, green panic, and Gayndah buffel were all similar in susceptibility to American buffel. In sensitive, rapid and statistically robust laboratory assays, mealybugs on Gatton panic and Mekong brizantha died more rapidly and in higher proportions and were less able to develop to maturity than on American Buffel.

There was an apparent correlation between varieties with thick, robust stems and tillers and reduced susceptibility. This was apparent in the Gatton Panic and Green Panic varieties, which are both varieties of *Panicum maximum* and genetically very similar (facultatively apomictic) but are significantly different in their susceptibility to mealybug. These more structurally robust varieties may be less palatable to livestock, however, appropriate management such as grazing and slashing to promote production of palatable leaf may improve productivity. Planting of Gatton panic and management with stock rotations and slashing to reduce aerial tillering has significantly improved returns in an area of severe mealybug/pasture dieback at Banana station.

This work demonstrates that the fungal endophytes *Epichloë festucae* reduce the susceptibility of perennial ryegrass to the Australian pasture mealybug *H. summervillei*, and reduced the severity of dieback symptoms. This result demonstrates the 'proof of concept' of the use of endophytes as used commercially in New Zealand against the pasture mealybug *Balanococcus poae*.

This result suggests that identification of endophytes for tropical pasture grasses in Australia may help to reduce incidence and severity of symptoms of *H. summervillei* and pasture dieback. However, development of commercial seed-born endophytes is costly. There is, however, significant recent investment in this area, with some promising fungi (such as *Acremonium strictum*) enjoying patent protection. Seed industries are unlikely to invest in development of seed-born endophytes soley for control of *H. summervillei*. Rapid screens such as laboratory assays may reduce some costs and time for development, and *H. summervillei* could be added to commercial screening panels for new varieties and strains using methods identified here.

This work demonstrates a direct link between 'pasture dieback', pasture diversity and soil health. Pastures with a higher diversity of 5 key genera of beneficial rhizospheric-competent endophytic fungi had significantly reduced severity of pasture dieback. Abundance of these species was shown to be higher in trial plots planted with legumes in pasture mixes than in plots sown with pasture grasses alone.

Legumes such as *Desmanthus*, lucerne, clovers, medic, and many other forage legumes, along with brassicas (rapa), chicories and other forbes, are not susceptible to mealybug. Several properties (including Banana station and farms in the Arcadia Valley) are now replacing buffel varieties with forage legumes (non-irrigated lucerne, Desmanthus).

Results in the AHR trials suggest that planting pasture mixes with legumes may offer benefit to graziers through improved populations of beneficial soil fungi (including rhizospheric endophytes). Assessment of beneficial fungi and pasture diversity may be an indicator of pasture resilience and inform future management decisions.

Finally, the methods developed in these trials could have significant benefits to graziers and seed companies. Field trials and infestation assays (including screenhouse assays) often have highly dispersed data, making determining significance in analysis of variance difficult without large numbers of replicates, and consequently cost of labour, materials and facilities. Field trials of varieties often take several months or years and even screenhouse assays take several weeks.

In contrast, laboratory bioassays are relatively rapid, approximately 7 to 10 days for the ST50 assays, can be assessed in one or two hours each day by one or two people, and are analysed by a regression analysis using continuous variables (proportion dead, time in hours), a much more statistically powerful analysis. Initial screens using laboratory bioassay methods (such as survival time) would significantly reduce costs of identifying bioactive endophytes and tolerant pasture varieties.

Benefits to industry

The project has significantly increased options to manage pasture dieback. Tolerant and resistant pasture varieties have been identified, the benefit of both seed-born and soil/rhizospheric fungal endophytes in reducing mealybug and pasture dieback in the laboratory and in the field has been demonstrated, and rapid assays have been developed to reduce cost and increase speed and statistical power of future screening for development of new pasture varieties and endophytic symbionts for protection of tropical and subtropical pastures.

This work demonstrates a direct link between 'pasture dieback', pasture diversity and soil health. Pastures with a higher diversity of 5 key genera of beneficial rhizospheric-competent endophytic fungi had significantly reduced severity of pasture dieback. Abundance of these species was shown to be higher in trial plots planted with legumes in pasture mixes than in plots sown with pasture grasses alone. Assessment of beneficial fungi and pasture diversity may be an indicator of pasture resilience and inform management decisions. Management practices that increase diversity of beneficial rhizospheric fungi, such as inclusion of legumes, and impacts of management that reduces abundance and diversity of these fungi (such as, possibly, hot burning) requires investigation.

Future research and recommendations

Further research to screen varieties for tolerance/ resistance and to determine tolerance / resistance mechanisms using a combination of rapid laboratory assay method and transcriptome analysis (B.PAS 0003) are strongly recommended.

Characterisation of the isolates of fungi in the 3 genera of the key beneficial endophyte genera (*Clonostachys, Trichoderma* and *Penecillium*) should be completed to determine their taxonomy. The beneficial impacts of these fungi on grasses and mealybugs should be a focus of further investigation using some of the rapid assay methods developed here.

Further assays and detailed analysis of plant/endophyte/mealybug interactions in grasses should be conducted in combination with trials on pasture legume mixes is strongly recommended. Management practices that increase diversity, such as inclusion of legumes, and management that reduces abundance and diversity of these fungi (such as, possibly, hot burning) should be investigated.

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1. Background

Pasture dieback causes unhealthy growth and death in a range of introduced and native grasses across Queensland and into northern NSW, resulting in large losses in beef production areas. Pasture mealybug, *Heliococcus summervillei* Brookes, is the primary cause of pasture dieback. This mealybug was previously reported to have caused severe pasture dieback in Queensland in 1926 (Summerville 1928) and the 1930s (Brooks 1978), in New Caledonia in 1998 (Brinon et al 2004), and more recently in Puerto Rico and Barbados.

Possible management strategies include the identification of resistant or tolerant pasture varieites, and the use of endophytic fungi to protect pasture grasses. Endophytes are fungi that live within healthy plant tissue and can confer resistance to insects, including mealybugs. Some may be transferred in seed, while others are acquired from healthy soils, primarily by colonising the roots. In New Zealand, pasture dieback in rye grass caused by the mealybug *Balanococcus poae* has been effectively managed by selection of endophytic fungi and incorporation into commercial pasture varieties.

The identification of tolerant grass varieties and endophytes in tropical grasses is an emerging area of research and might provide long-term management of dieback in tropical grasses. Very little is known about endophytes in tropical and sub-tropical grasses, but they are believed to be of importance in increasing tolerance of pasture grasses to insects' pests. The identification of endophytes against the causal agents of pasture dieback would provide new approaches to control of pasture pests, increase our understanding of pest biology in tropical grasses, and possibly reduce the impact of future pest attacks in tropical pastures.

The project builds on observations in the field by growers and agronomists of variation in susceptibility of grass species and varieties in the field, with some grass varieties and indeed individual plants or patches of plants with apparent resistance or tolerance to dieback. It builds on the successful use of endophytes in control of pasture mealybugs in temperate grasses in New Zealand. QUT will engage with AgResearch New Zealand to learn from their success in the use of endophytes to control pasture mealybug. This project proposes to use mealybug colonies and assay protocols developed by QUT in other funding proposals currently under consideration.

This project aims to identify grasses and soil of seed-born endophytes that reduce severity of the mealybug causing pasture dieback, to provide new approaches to control pasture pests, and provide a greater understanding of pest biology in tropical grasses.

2. Objectives

In this project we aimed to:

1.Identify susceptible grass varieties to pasture dieback from field surveys.

2.Assess the relative susceptibility of selected grass varieties to pasture mealybug and/or other agents responsible for pasture dieback and their impact on mealybug biology.

3.Isolate and characterise endophyte species present in grasses collected during pasture dieback field surveys and determine those which are tolerant to the mealybug.

4. Develop procedure and use them to assess promising endophytes (both facultative and seed-born) as cost effective pasture pest control methods. Make recommendations on opportunity for endophyte approach (via plant breeding/propagation) to address dieback.

5. Develop procedures and use them to assess endophytes and tolerant/resistant pasture mixes in field surveys, and laboratory and glasshouse assays. Make recommendations for field evaluation of prospective tolerant grasses and mixes.

6. Provide timely communication on research activities for use in industry briefings at least biannually throughout the project.

7. Produce a 2-page summary outlining key facts and findings of tolerant and susceptible grass species and management created in consultation with MLA's communications team.

3. Methodology

3.1 Identify susceptible grass varieties to pasture dieback from field surveys.

Field collections of mealybugs and grass samples were conducted by QUT staff across Queensland and Northern NSW, typically combined with seasonal monitoring, field trials, from elite lines of varieties at a grass breeding facility in Birkdale, Qld, and during on-farm extension activities. Samples of mealybugs were also sent by graziers and agronomists and field kits were provided for sample collection and return.

Initial mealybug samples were identified by comparison with reference specimens by Dr Mark Schutze, Biosecurity Qld. The majority of later samples were identified by QUT either using DNA sequencing or by physical inspection.

Grass species were identified by local agronomists and graziers. Dr. Don Loch provided identification of selected varieties at the grass breeding facility at Birkdale, Qld. In one case expert advice was sort to confirm identity of *Bothryochloa pertusa* (Indian couch) by the Qld Herbarium.

3.2 Assess the relative susceptibility of selected grass varieties to pasture mealybug and/or other agents responsible for pasture dieback and their impact on mealybug biology.

3.2.1 Screenhouse infestation assays

Seed of varieties of 18 pasture grass varieties were obtained and tested, including tolerant varieties (milestone 6.3) and 2 targeted grass varieties (milestone 6.4).

All seeds were obtained from PGW Seed with the exception of curly Mitchell grass, *Astrebbla lappacea*, which was purchased from Native Seeds (https://nativeseeds.com.au/?v=6cc98ba2045f).

Five assays were conducted in summer 2020/21 and 2021/22. The pasture variety 'American Buffel' was included in every assay as a standard for comparison across assays.

Assay 1: Callide Rhodes, Perennial ryegrass (*Lollium perenne* var. *lolli*) 'Commando' without endophytes

Assay 2: Biloela buffel, Gatton Panic (*Panicum maximum* var Gatton), Katambora Rhodes, Mekong brizantha

Assay 3: Argentina Bahia, Bambatsi panic, Callide Rhodes, Gayndah buffel

Assay 4: *Astrebla lappacae*, *Digitaria milanjana*, Green panic (*Panicum maximum*), *Panicum coloratum*, an elite *Paspalum* hybrid

Assay 5: *Paspalum nicorae*, *Urochloa mosambiscence*

All grasses were grown in 4 litre pots in potting mix in glasshouses (Redland Qld) and transferred to the QUT screenhouses at Samford Qld. (SERF) where they were inoculated with mealybugs.

Adaptation phase: 6 plants of each variety were infested with mealybugs that were allowed to replicate for 6 weeks to allow for any adaptation or adjustment in microbiome of the insect.

Screenhouse assays: Three uninfested plants of each variety were allocated to each of 6 blocks (replicates) on separate screenhouse benches, a total of 18 plants per variety. Each replicate was infested by placing a pre-infested plant of each variety between the 3 plants of the same variety.

Were observed until symptoms developed, typically 3 weeks after infestation. Plants were bagged and transferred to the laboratory for assessment (approx. 4 weeks after infestation)

Assessment

Two assessment methods were used in assays 1 to 3 to develop and evaluate more rapid assessment methods. Assays 4 and 5 were assessed only by the rapid assessment.

A rapid assessment using a limited time search of 10 minutes count was compared with full destructive sampling. Destructive sampling was then continued by removing and inspecting every leaf blade and destructive examination of the crown and roots until the plants had been fully inspected.

The number of mealybugs in each of the following categories were recorded: small, medium, large, pink adults (female), 3rd instar males, and male pupae.

The symptoms as a proportion of affected grass (yellow, red/purple and dead) were recorded for each plant using methods developed in field assessment (B.PAS 0004) and screenhouse assays (B.PAS 0003). These criteria are included in the dieback assessment sheet provided to all program participants in November 2020.

Analysis:
The effect of plant variety on total mealybug abundance was estimated using general linear modelling with poisson errors in R.

The proportion of mealybugs counted in the 10 minute sample was plotted against the number of mealybugs found in the full destructive method for each sample, again in R.

Further analysis of abundance and distribution on plant (crown, leaf, soil) and of different instars is continuing beyond the timeline for this project to determine if there is a difference in responses of mealybugs of different instars (early instars that feed on leaf, non-feeding adult females, males and pupae) and will be completed for publication.

3.2.2 Laboratory bioassays

Grasses selected from the screenhouse assays were used in laboratory assays to compare median survival time of mealybug nymphs on different grass varieties.

In the first assays, median survival time of nymphs on American buffel and Callide Rhodes was determined from life history assays. 10-day-old mealybug nymphs were reared in cohorts of 10 insects for 10 days (the age at which males begin to pupate) at 22°C on cut leaf in glass scintillation vials, 40 cohorts for Rhodes grass (400 insects total) and 41 cohorts for buffel grass (410 insects total) and observed twice a day for 10 days. The number alive in each cohort was recorded at each time point.

In the second assay and subsequent assay a method was developed and used to determine the survival time of nymphs on plants of American buffel, Gatton Panic and Mekong brizantha. Second instar nymphs were collected from grasses infested in the screenhouse assays, above. variety.

Sections of cut leaf with 10 nymphs were placed in a ventilated plastic clip cage attached to the leaf of uninfested plant of the test variety, a total of 10 clip cages per variety and 100 insects per variety. Plant and with cages/insects were then placed in an insect cage (bug dorm) in a temperature-controlled growth room at 26°C and 60% humidity and allowed 24 hours for neonates to move off the cut leaf and onto the plant. Mealybugs are then observed at 24-hour intervals and the number alive at each point recorded.

Median survival time (ST50) is calculated in R from the time at which 50% of the mealybugs were alive out of the total that died.

3.3 Isolate and characterise endophyte species present in grasses collected during pasture dieback field surveys and determine those which are tolerant to the mealybug.

Two types of fungal endophytes were sought: seed born / foliar endophytes in tillers and crown material, and rhizospheric-competent endophytes from roots and associated soil.

Sites were identified by discussion with graziers and agronomists in key dieback locations, often in conjunction with surveys of mealybugs and natural enemies in projects B.PAS.0003/CAS1 and B.PAS.0004/CAS2.

Potential survey sites for tolerant and resistant grasses are in 3 categories:

A: Dieback tolerant grasses (specific plants unaffected or tolerant of dieback within affected paddocks) and dieback tolerant pastures (paddocks or small areas of pasture at scale of a few square metres) in which dieback has either not occurred or occurred with limited impact despite significant dieback in surrounding paddocks/areas.

Grasses, soil and roots were also collected as single samples where specific plants or areas within dieback affected paddocks remained unaffected by dieback. These ‘tolerant’ plants, their roots and soil were identified by graziers and collected by digging, bagging and returning to the laboratory.

In tolerant paddocks or patches, samples of soil and plant material were collected at multiple points (5 or 10) at 5 m spacing along a transect. Severity of dieback (symptoms) and number of mealybugs was recorded at each point.

Samples were collected at 12 ‘type A’ sites (individual grasses and dieback-tolerant pastures) at Mulgildie, Roma, Rockhampton, Biggenden, Kin Kin, Banana Station, Wolvi (2 sites), Northern NSW, Theodore, Biggenden, and Moura.

B: ‘Recovered’ pastures: Paddocks in which dieback had been previously identified but had subsequently fully recovered or in which pastures recovered during project monitoring. Samples of soil and plant material were collected at multiple points (5 or 10) at 5 m spacing along a transect. Severity of dieback (symptoms) and number of mealybugs was recorded at each point.

Four ‘type B’ sites monitored repeatedly for mealybug and dieback symptoms at SERF (Samford), northern New South Wales, and 2 separate sites at Maudsland (Gold Coast). Where sites later appeared to have recovered from infestation and dieback symptoms a second plant, soil and root sample collection was conducted as above.

C: Chronic and severe dieback-affected sites. As above, samples of soil and plant material were collected at multiple points (5 or 10) at 5 m spacing along a transect. Severity of dieback (symptoms) and number of mealybugs was recorded at each point.

Type C sites with persistent and severe dieback were sampled at Brendale, Biggenden, Roma, Rockhampton, Mundubbera, Moura and Banana.

In addition, single samples of possibly 'dieback' affected grasses were sent by graziers from 2 sites: North Qld (Atherton) and Taroom.

Seven new Type C sites with persistent and severe dieback were sampled at Crows Nest, Arcadia valley (3 properties), Banana shire, Roma, and Taroom (14 samples).

3.3.1 Isolation and characterisation of endophytes

The two types of fungal endophytes were isolated from material collected using published methods:

- seed born / foliar endophytes in tillers and crown material using method adapted from Teasdale et al (2019) https://doi.org/10.1080/17550874.2019.1610913.
- rhizospheric-competent endophytes from roots and associated soil using methods described by Islam (2018) https://eprints.qut.edu.au/117674/.

Isolates were characterised by extraction of DNA, PCR amplification of the ITS marker and Sanger sequencing (by Macrogen). DNA sequences were compared with reference sequences by BLAST search in the NCBI database to determine probable isolate identity.

3.3.2 Impact of fungi diversity on mealybug and severity of 'dieback'

A comparison of diversity of fungi of 3 different types in sites of different severity was conducted. Isolates of fungi were categorised into 3 classes based on sequence identity: Species and races of the (broadly) pathogenic fungus genus *Fusarium*, 'neutral' fungi of unknown pathogenic or beneficial nature, and putatively beneficial fungi (and possible rhizospheric-competent endophytes) of 5 known genera: *Trichoderma*, *Clonostachys*, *Penicillium*, *Purpureocillium*, and *Beauveria*.

Severity of dieback was determined as the proportion of transect points at which symptoms of dieback and mealybugs were recorded. The number of species or races of fungi in the 3 classes was then compared to the severity of dieback at each site using GLM in R studio.

3.4 Develop procedures and use them to assess promising endophytes (both facultative and seed-born) as cost effective pasture pest control methods.

Experiments were conducted to develop and test methods to determine if the effects of rhizospheric endophytes isolated in this work (*Purpureocillium lilacinum*, and species of *Penicillium*, *Trichoderma*, and *Clonostachys*) could be detected conferring resistance to mealybug. Multiple assays and methods were tested. The most successful (in detecting efficacy) and most straight forward (for rapid processing) used a mycelial inoculum incorporated into compost in which grasses were subsequently grown, then infested with mealybugs, as below.

In an exemplar experiment, five species of Penicillium were selected: *P. ludwiii* (10), *P. clavistipitarum* (19), *P. paneum* (502), *P. pulvis* (585) and *P. citrinum* (505). Isolates in pure culture were grown in liquid medium (SAY) on shaker culture at 24°C for 1 week. The mycelium suspension was filtered to extract the mycelium, which was washed and then and homogenised in 0.05% Tween 80 to create an inoculum and stored refrigerated for 1 week. The concentration of inoculum was estimated by plating on SDAY agar in petri dishes, and the inoculum adjusted to an approximate concentration of 10^5 CFU/mL (CFU=colony forming units).

Fresh potting mix was inoculated with 10ml of the standardised inoculum or a 0.05% Tween 80 (control) and mixed well. Seeds of the test grass (Rhodes grass) were then sown into the inoculated potting mix, replicated with 6 plants/pots per treatment.

Plants were sorted into randomised blocks and placed into bug dorms in controlled environment cabinets at 26°C and watered twice weekly for 3 weeks. Each bug dorm was then infested by exposure to cut grass from plants infested with *H. summervillei* and incubated for a further 2 weeks (5 weeks total).

Mealybugs were counted by full destructive sampling. the number and size of each mealybug on the foliage of each replicate was recorded.

3.5 Develop procedures and use them to assess endophytes and tolerant/resistant pasture mixes in field surveys, and laboratory and glasshouse assays.

3.5.1 Seed-born endophytes in screenhouse assays

The perennial rye grass, *Lollium perenne*, var. ‘Commando’ without endophytes was evaluated in comparison to 3 lines containing different variants of the seed-born endophyte *Epichloë festucae* var *lolii*.

- Perennial ryegrass variety ‘Commando’ without endophytes
- Perennial ryegrass variety ‘Commando’ with *E. festucae* var *lolii* strain AR37
- Perennial ryegrass variety ‘Commando’ with *E. festucae* var *lolii* strain AR37
- Perennial ryegrass variety ‘Commando’ with an uncharacterised ‘wild type’ *E. festucae* endophyte

American buffel and Callide Rhodes grass (as above) were included as positive controls.

All grasses were grown in 4 litre pots in potting mix in glasshouses and transferred to the QUT screenhouses at SERF.

Preinoculation: 12 plants of American buffel were infested with medium and adult instar mealybugs. After 3 months, infestation was confirmed and the plants used to inoculate the assay.

Three uninfested plants of each variety were allocated to each of 6 blocks (replicates) on separate screenhouse benches, a total of 18 plants per variety. Each replicate was infested by placing pre-infested plants of American buffel between the 3 plants of the same variety. Plants were watered by drip irrigation for 20 minutes twice per day. Watering was reduced to 15 minutes during cooler or showery weather and stopped during heavy rain events to prevent waterlogging.

The number of mealybugs and the symptoms of dieback were assessed by 10 minute and full destructive sampling as above.

3.5.2 Diversity of beneficial fungi in replanted pasture mixes

Replicated field trials of pasture varieties were established by AHR on farms at Gaeta, Jambin and Biggenden. Trials were established 8 months before soil sampling. Pasture mixes included a monoculture (American buffel at Biggenden and Jambin, Bisset bluegrass at Gaeta), a grass mix (Reclaimer Rhodes, Callide Rhodes, Gatton Panic, and Purple Pigeon at Gaeta and Biggenden and Biloela buffel at Jambin), a legume mix (Lab lab, Butterfly-pea, Desmanthus, Stylo, and Cowpea), and a combination of the legume and grass mix. Full details of planting etc will be reported by AHR.

Soil samples were collected from the rhizosphere from 20 plots: 4 replicates in 5 treatments.

Soils were analysed for presence of fungi as previously described by culture on selective agar media.

Samples were sieved through 1mm-mesh laboratory sieves to separate roots and soil. Sieves were rinsed, soaked in Viraclean for 10 minutes, rinsed in 70% ethanol, and dried in a drying oven at 90°C for 5 minutes between uses. 5 grams of sieved soil were then taken from each sample

5g of sieved soil was placed in a clean ceramic mortar and pestle, wetted with sterile 0.05% Tween 80, and macerated into a smooth slurry. The slurry was transferred into a 50mL falcon tube and the total volume was made up to 50mL with sterile 0.05% Tween 80.

The tube was shaken for 30 seconds and allowed to settle for 30 seconds. Two aliquots of 100uL of supernatant were then removed and placed on to selective media plates and spread using a sterile t-spreader. Plates were sealed with parafilm and incubated inverted at 22°C for 7 days with a 12-hour photoperiod.

Unique morphologies were listed and counted for each isolated sample, with representative colonies subcultured individually onto new selective media plates and incubated as previously described. Single isolates were photographed and grouped into similar morphologies by sites. Up to 3 representative isolates were selected for DNA extraction and cold storage on agar slants.

DNA was extracted and the Internal Transcribed Spacer (ITS) region of the extracted DNA samples amplified using ITS1 and ITS4 primers (White, 1990) with MyTaq HS Red 2x (Meridian Bioscience) following the manufacturers protocol and published thermocycling parameters (White 1990). PCR products were Sanger sequenced by Macrogen.

Sequences were trimmed and quality-controlled using the sangeranalyseR package with default parameters in RStudio 4.2.0. Resulting sequences were compared to the NCBI ITS region fungal reference database using the blastn search tool. Representative species identifications were applied to the isolates grouped by morphology. Visual exploration and statistical analysis were conducted in RStudio using the tidyverse package suite.

Initial analysis has focused on analysis of variance using GLM and quasipoisson errors to compare the mean abundance (as colony forming units) of beneficial, neutral and *Fusarium* species and variants of fungi in each treatment. Analysis of this data is ongoing and will be further analysed once plant coverage data and mealybug data are available from AHR.

3.6. Provide timely communication on research activities for use in industry briefings at least biannually throughout the project.

Communications were provided frequently to MLA program leaders and comms team through reports, emails, a webinar, on-line participant meetings, material for specific media outputs, and a national science panel review in April 2022. A summary has been provided separately for all 3 QUT projects.

3.7. A 2-page summary outlining key facts and findings of tolerant and susceptible grass species and management created in consultation with MLA's communications team.

A summary of key findings is provided in results. An article on QUT research and management strategies for graziers in in preparation with MLA comms team and is attached as an appendix.

4. Results

4.1 Identify susceptible grass varieties to pasture dieback from field surveys.

The mealybug *H. summervillei* was found associated with symptoms of 'pasture dieback' on 31 varieties and species of pasture grasses across Queensland and New South Wales, and on sugar cane (Table 1).

There were differences in observed and reported susceptibility between these grass species and varieties. Within *C. cilliaris* (buffel grass) American buffel is widely reported to be more susceptible than Gayndah buffel, and Biloela to be less susceptible than both. Our field observations agree with this perception: Biloela growing next to American buffel did not appear to show symptoms of mealybug pressure to the same degree as the American buffel.

Within *P. maximum* varieties, the variety Gatton panic is reported by graziers to be less susceptible than Green panic. Our observations in the field surveys support the low susceptibility of Gatton panic, which often invades areas where American buffel and other local, more susceptible pasture grasses have died back, with few apparent symptoms and low numbers of mealybugs (one per plant). On the other hand, we observed green panic heavily infested with mealybugs (several hundred per plant) particularly in late summer, with frequent severe yellowing of leaves.

Species and varieties of genus *Urochloa* appear to show significant variation in susceptibility. *U. decumbens* (*Brachyaria decumbens*) was identified as a host, but appears to be highly tolerant, with low numbers of mealybug and few symptoms. *U. oligotricha*, in contrast, was observed to be highly susceptible, but two experimental varieties grown by a commercial breeder appear to show differences in severity of symptoms and abundance of mealybugs.

Some pasture grasses appear to be highly tolerant or not susceptible to the mealybug. Indian couch, *B. pertusa*, rapidly invaded pastures near Mundubbera (Qld) that had previously grown buffel grass. The invasion of previously productive pastures following mealybug and die-off of grasses by more tolerant but less palatable or productive varieties was widely reported by graziers and agronomists and observed in our sampling over several seasons in B.PAS 0004.

Sugarcane was reported by graziers near Ingham (Qld) to be a host for mealybug that would then invade nearby pastures, and supplied pictures to confirm that this was *H. summervillei* (Fig. 1). We observed rapid colonisation of sugarcane grown in screen cages at the QUT research facility at Samford (SERF) by *H. summervillei*, with severe yellowing symptoms on leaves. The mealybug identification was confirmed by Biosecurity Qld.

Figure 1: pasture mealybug infesting pasture grass with dieback symptoms, Ingham, Qld. Picture supplied by grazier.

Table 1: 26 species and varieties of pasture, turf grasses and crops (sugar cane) were observed as hosts of *H. summervillei* and that showed symptoms of dieback. Indian couch was identified invading pastures that had died and was not infested with *H. summervillei*.

Species	Common names and varieties
Dichanthium sericium	Bluegrass
Bothriochloa bladhii	Australian bluestem
Bothriochloa insculpta	Creeping blue-grass and var. Bisset bluegrass
Brachiaria decumbens (Urochloa decumbens)	Signal grass
Cenchrus ciliaris	Buffel grass (incl. American, Biloella, Gayndah)
Cenchrus clandestinus	Kikuyu
Chloris gayana	Rhodes grass
Cyperus rotundus	Nut grass (sedge)
Dichanthium sericeum	Queensland bluegrass
Digitaria didactyla	QLD Blue Couch
Digitaria eriantha	Pangola
Heteropogon contortus	Black spear grass
Lolium rigidum	Annual ryegrass
Megathyrsus maximus	Guinea grass
Melinis minutiflora	Molasses Grass
Melinis repens	Red Natal grass
Panicum maximum	Green panic and var. Gatton panic
Paspalum dilatatum	Dallis grass
Paspalum mandiocanum	Broad-leaved paspalum
Paspalum notatum	Bahia Grass
Saccharum officinarum	Sugacane
Setaria splendida	Setaria
Stenotaphrum secundatum	Buffalo turf grass
Themeda triandra	Kangaroo grass
Urochloa oligotricha	Signal grass incl. 2 research varieties
Not susceptible:	
Bothriochloa pertusa	Indian couch

Legumes, brassicas and other broadleaf species, including weeds such as *Parthenium* and balloon bush, were not hosts of the mealybug and do not show symptoms of 'pasture dieback'. Brigalow, lucaena, clover, lucerne and rapa were all observed not to host *H. summervillei*, though several other species were collected and identified on these species (Table 2).

Finally, the Rhodes grass mealybug, *Antonina graminis*, was also found on Rhodes grass, Setaria and *Urochloa*. In one location (Jambin, Qld) this was observed in very high levels and associated with yellow discolouration of leaves in an unidentified variety of *Urochloa* (probably *U. oligotricha*) similar to symptoms cause by *H. summervillei*. However, *H. summervillei* was also found at the same site (table 2).

Table 2: Other mealybugs recovered from pasture, weeds, and nearby vegetation and crops. Identifications of mealybugs were confirmed by Biosecurity Qld.

Mealybug species	Host plant
Saccharicoccus sacchari	Sugar Cane
Vryburgia brevicruris	Bluegrass
Antonina graminis	Rhodes grass, Setaria, *Urochloa sp.*
Trionymus ascripticius	Balloon bush
Phenacoccus solenopsis	Parthenium
Monophlebulus sp	Brigalow
Coccus longulus	Leucaena
Hypogeococcus festerianus	Harrisia cactus
Icerya aegyptiaca	*Urochloa sp.*

4.2 Assess the relative susceptibility of selected grass varieties to pasture mealybug and/or other agents responsible for pasture dieback and their impact on mealybug biology.

4.2.1 Screenhouse infestation assays

Methods to conduct screenhouse evaluation of pasture varieties with efficient sampling methods were developed. As might be expected, a comparison of 10 minute count versus full destructive sampling showed that 10 minute counts captured a higher proportion of total mealybugs, where total counts were under 100 mealybugs, but the proportion counted of the total declined at higher numbers (Fig. 2).

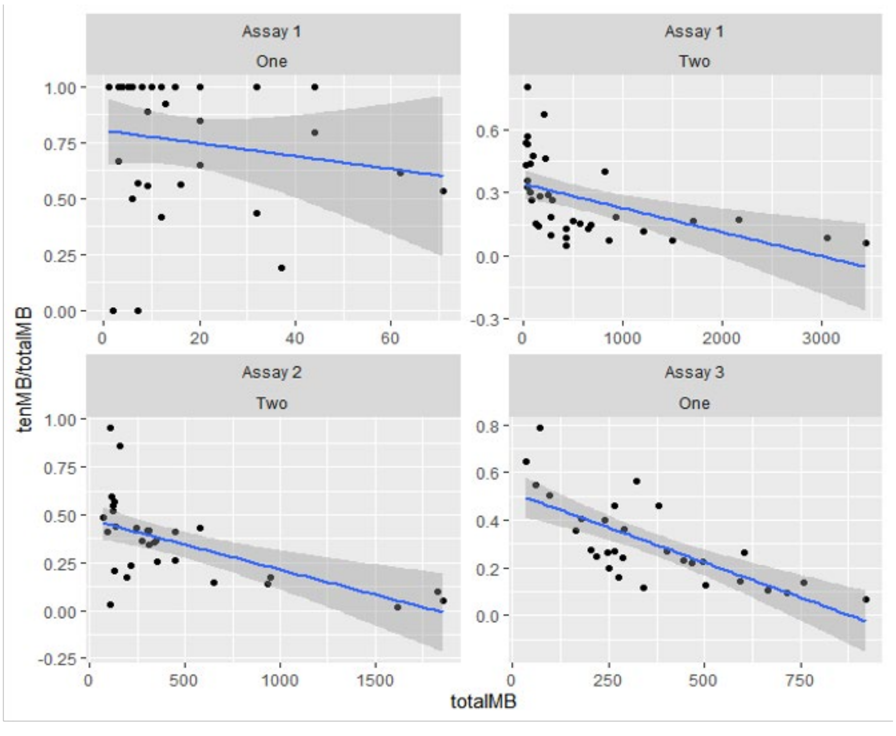


Figure 2: Proportion of mealybugs counted in 10 minutes as a proportion of total mealybugs counted in full destructive sampling of the same plant in 4 ranges of mealybug abundance.

Observations across a number of assays suggest that this decrease in proportion recorded by 10' counts at very high total numbers not to be important. Overall the 10' counts resulted in a higher degree of statistical significance in assays than the full counts (and are reported below).

Furthermore, multiple assays across all 3 projects suggest that there is little change in indicators such as symptoms at higher numbers of mealybugs. Only a few mealybugs are required to cause symptoms and induce changes in transcriptome making plants susceptible to infection.

Analysis and modelling of the data is continuing towards publication of final results.

Differences in susceptibility to mealybug.

Mekong brizantha and Gatton panic both supported significantly fewer mealybugs at the end of the experiment (Fig 3).

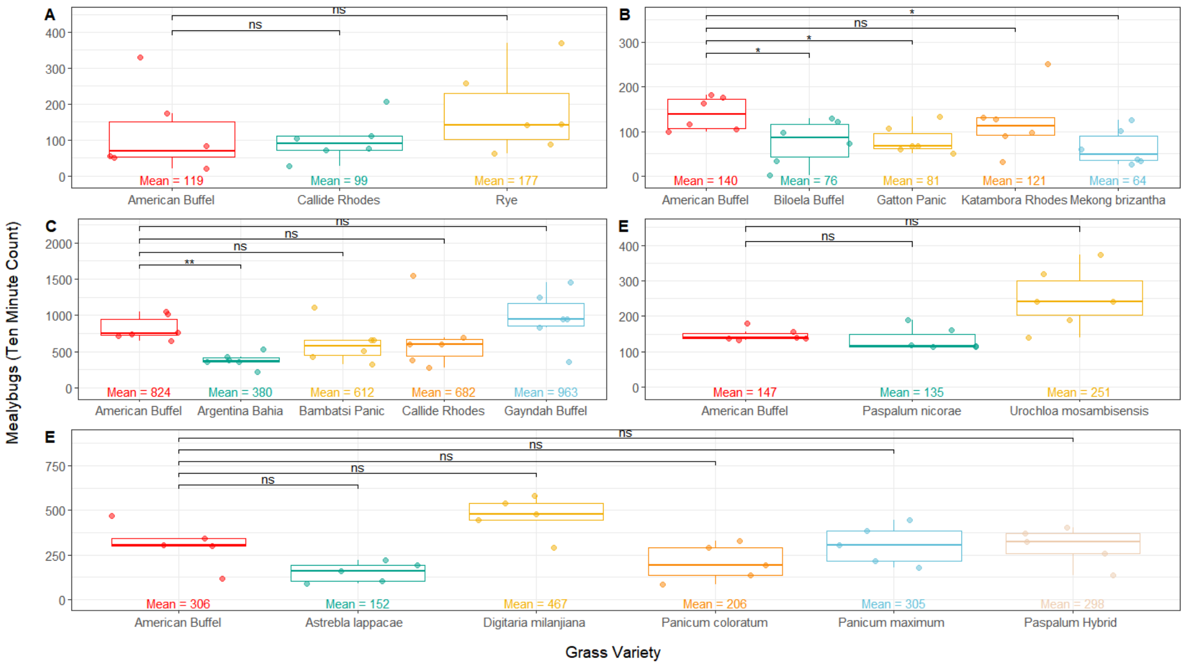


Figure 3: Box and whisker plot of mean number of mealybugs counted in 10 minutes on varieties of pasture grasses in 5 assays (1, 2A,2B,3A,3B) conducted in 2020/ 2021 and 2021/2022. Mean = bar in box, with 95% confidence intervals. Dots = mealybug count on replicate.

The relative susceptibility of 15 species and varieties of pasture grass to American buffel was determined (Fig.4). Mekong brizantha, Argentina bahia, curly Mitchel grass, Biloela buffel and Gatton panic were all less susceptible to mealybug than American buffel. Callide and Katambora Rhodes, *Paspalum nicora*, green panic, and Gayndah buffel were all similar in susceptibility to American buffel. Perennial rye grass (*L. perenne*) without endophytes, *Digitaria milanjiana* and *Urochloa mosambisensis* were all realtively more susceptible to mealybug than American buffel.

There was an apparent correlation between varieties with thick, robust stems and tillers and reduced susceptibility. This was most clear in the Gatton Panic and Green Panic varieties, which are both varieties of *Panicum maximum* and genetically very similar (facultatively apomictic) but are significantly different in their susceptibility to mealybug. Gatton panic is significantly less susceptible, while Green panic supported a similar number of mealybugs as American buffel. Further research to determine resistance mechanisms of strongly recommended.

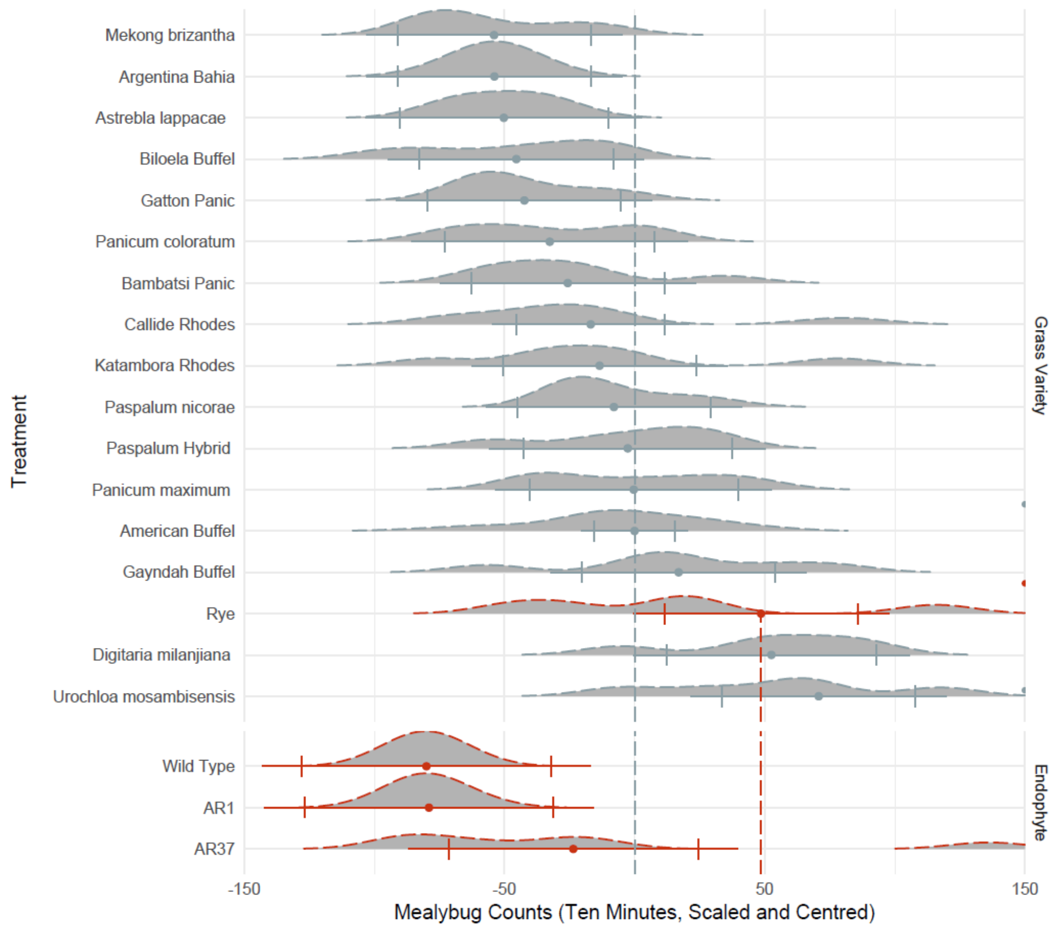


Figure 4: Relative susceptibility or a range of pasture grasses to the mealybug H. summervillei in 5 assays in screenhouses.
Blue outline (upper section): Mean mealybug count (blue dot) with 95% confidence intervals on 17 varieties of pasture grasses relative to the count on American buffel (vertical blue broken line) in each of 5 assays.
Red outline (lower section): Mean mealybug count (red dot) on perennial rye grass (*L. perenne*) variety 'Commando' with 3 different strains of the endophytic fungus *E. festuce* (wild type, AR1 and AR37) to the count on rye grass without endophytes (vertical red broken line) and American buffel (vertical blue broken line).

The pattern aligns with field reports from graziers and our field observations and variety trial data from AHR.

Further analysis of abundance and distribution on plant (crown, leaf, soil) and of different instars is continuing beyond the timeline for this project to determine if there is a difference in responses of mealybugs of different instars (early instars that feed on grass, non-feeding adult females, males and pupae) and will be completed for publication.

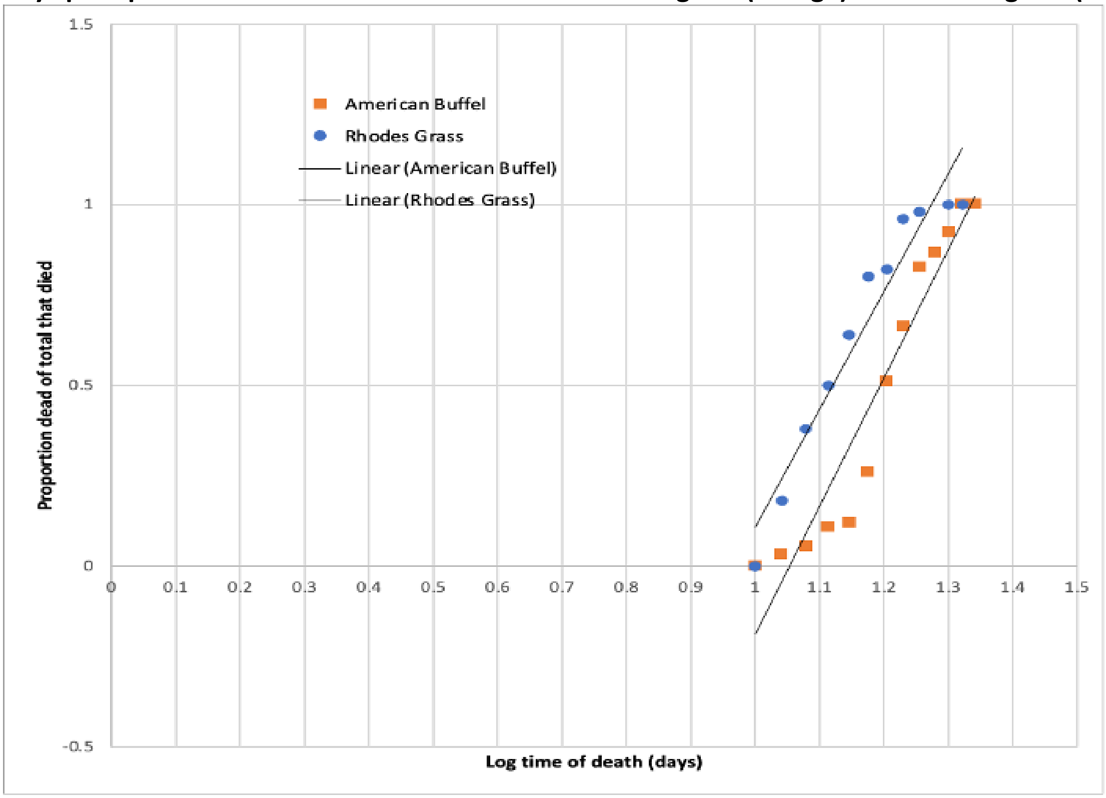
4.2.2 Laboratory Assays

Rapid (2 week) laboratory screening assays were developed and shown to determine sensitive and statistically robust differences in susceptibility between grass varieties.
Differences in development and survival of mealybugs were detects on different varieties.

The laboratory assays reflected the results in glasshouse tests, but with greater statistical power. In assay 1, survival of mealybugs was highest on American buffel, where the medium survival time

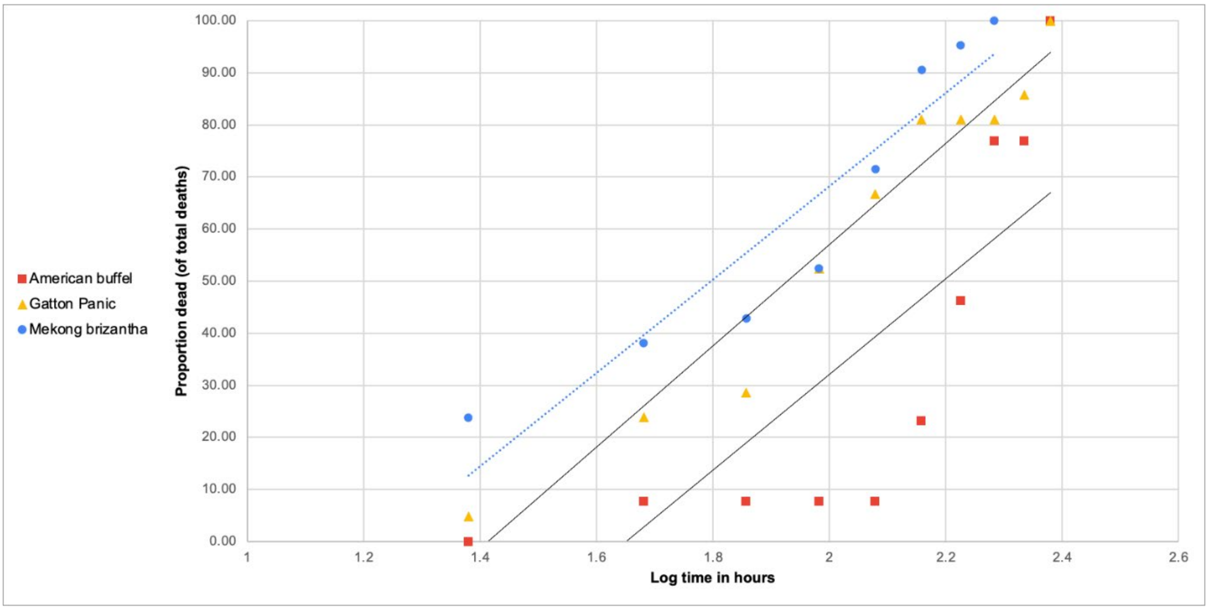
(ST50) was 16 hrs (95% CI = min. 16 hrs, max. 17 hrs). The ST50 on Callide Rhodes was lower at 12 hrs (CI 95% min. 12 hrs, max. 14hrs) (Fig. 5).

Figure 5: Plot of proportion mortality against log of time to death (in hours) between 10 and 30 days post-partum of cohorts raised on American buffel grass (orange) and Rhodes grass (blue).



In assay 2, survival of mealybugs was highest on American buffel (ST50 = 158 hours) reduced on Gatton Panic (ST50 = 83 hours) and even further reduced on Mekong Brizantha (ST50=63 hours) (Fig 6).

Figure 6: Plot of proportion mortality against log of time to death (in hours) of mealybugs raised on American buffel grass (red), Gatton panic (yellow) and Mekong brizantha (blue).



These assays demonstrate that modified bioassay techniques to determine median survival time can be used in laboratory populations as a rapid test (10 days to 2 weeks) to determine the relative impact of tolerant and susceptible grass varieties on mealybugs. This is a useful adjunct to more costly, seasonal and time-consuming screenhouse tests that determine the impacts of mealybugs on the grass.

4.3 Isolate and characterise endophyte species present in grasses collected during pasture dieback field surveys and determine those which are tolerant to the mealybug.

4.3.1 Isolation and characterisation of endophytes

Thirty nine (39) sites with differing severity of 'pasture dieback' were systematically sampled for plant and rhizospheric endophytes across Qld and NSW. Three sites included replicated trials of pasture mixes planted by AHR. A total of 1,120 fungal isolates were isolated and identified by Sanger sequencing of the DNA 'barcode' ITS regions 1 and 2.

Five (5) genera of beneficial, rhizospheric-competent or endophytic fungi were been isolated and identified from tillers, roots and/or soil: *Penicillium*, *Purpureocillium*, *Trichoderma*, *Clonostachys*, *Beauveria*.

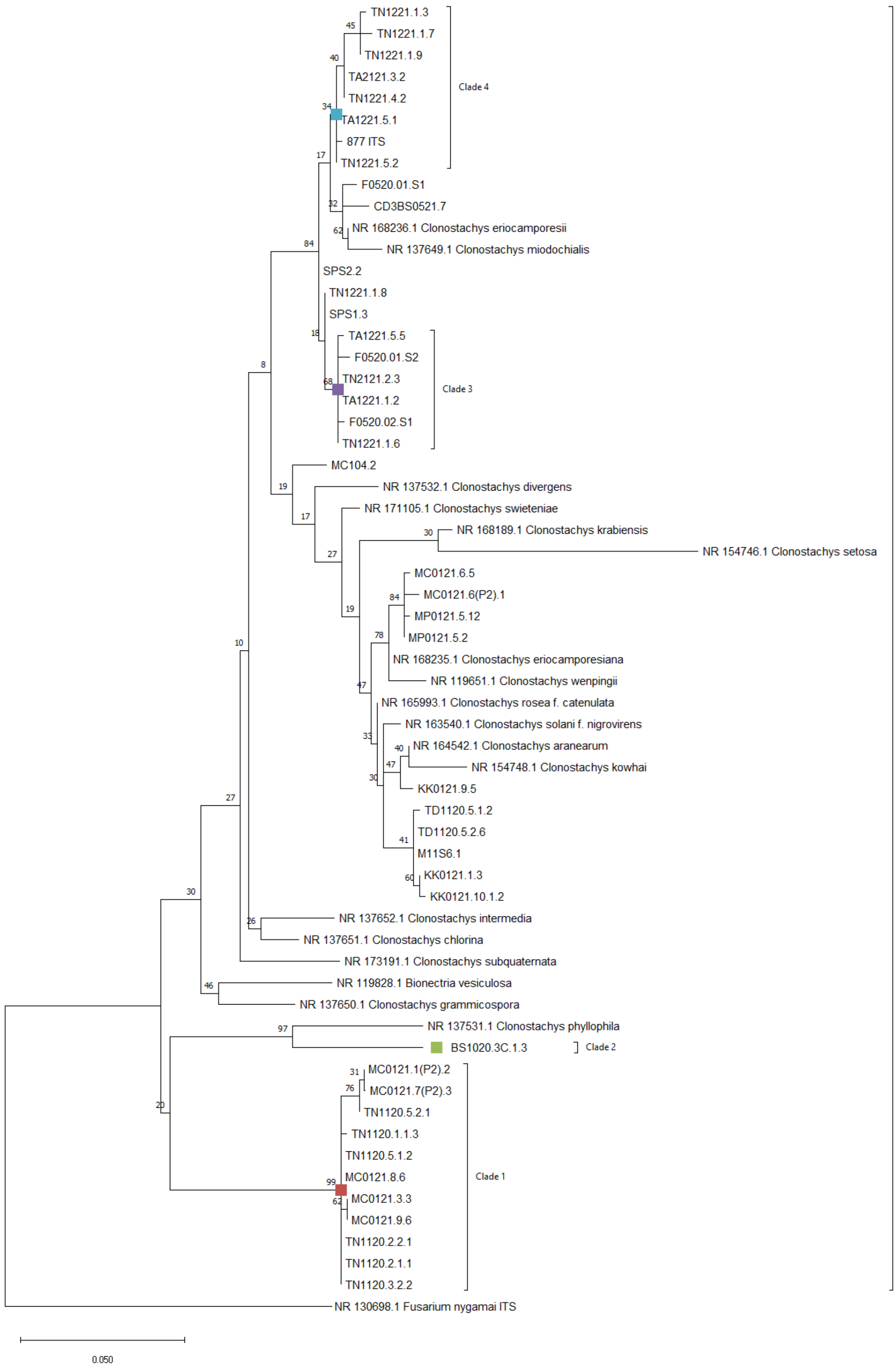
Endophytes were successfully isolated from 8 apparently-resistant plants (root, soil or tillers) of Biloela buffel, Green Panic, Bluegrass and Pigeon Pea collected in Mulgildie and Biggenden and *Urochloa* in Rockhampton. *P. lilacinum* was isolated from 6 samples, *Trichoderma sp.* from 4 samples, and *Penicillium citrinum,* a known beneficial endophyte, from 2 bluegrass plants (one from the grass tiller at Biggenden and one from soil associated with roots at Mulgildie). Soil from roots of 3 resistant plant samples (Biloela buffel, Bluegrass and Green Panic) from Mulgildie contained both *Trichoderma sp.* and *P. lilacinum,* and one plant (Bluegrass) both *P. lilacinum* and *P. citrinum.*

Twenty nine genera of other fungi of unknown pathology or benefit were also isolated. These included *Nigrospora pyriformis* and other *Nigrospora spp., Chaetomium* sp*., Aspergillus* sp*., Albifrimbia* sp*., Marasmiellus* sp., and *Paraconiothyrium estuarinum.* Multiple species and variants of the broadly pathogenic genus *Fusarium* were identified and described in detail in reports for B.PAS 0003.

The endophytic fungi isolated from grasses were broadly in the same genera of known beneficial rhizosperic-competent fungi isolated from rhizospheric soil: *Penicillium*, *Purpureocillium*, *Trichoderma*, *Clonostachys*, *Beauveria*. This included several new species of both *Clonostachys* and *Trichoderma*, which was confirmed by systematic comparison of ITS sequences with validated reference sequences in international databases (Fig. 7).

In the genus *Clonostachys*, and known endophyte and parasite of fungal pathogens, *C. roseus* was identified in several samples. However, sequence analysis also identified four and possibly more species within this genus that have not been previously identified (Fig. 7).
Characterisation of these fungi, their identity and impacts on mealybugs and grass health, requires further investigation.

Figure 7: Phylogeny of ITS loci of *Clonostachys* isolates from field sampling ITS sequence data from type material from NCBI. Isolates from MLA material are highlighted in colour.



4.3.2 Impact of fungi diversity on mealybug and severity of 'dieback'

Higher diversity of species and variants of beneficial rhizospheric-competent facultatively endophytic fungi in 5 genera (*Penicillium*, *Purpureocillium*, *Trichoderma*, *Clonostachys*, *Beauveria*) was significantly correlated with reduced dieback severity (P=0.0004). In contrast, both diversity of *Fusarium* species and races, and diversity of 'neutral' saprophytic fungi, were not significantly associated with dieback severity (Fig. 8).

This is an important finding, with significant implications for pasture management and reduction of the impacts of the mealybug.. It shows that absolute fungal diversity is not associated with resilience in pastures: it is specifically the increased diversity of beneficial, endophytic taxa that is associated with greater resilience of pastures against mealybug.

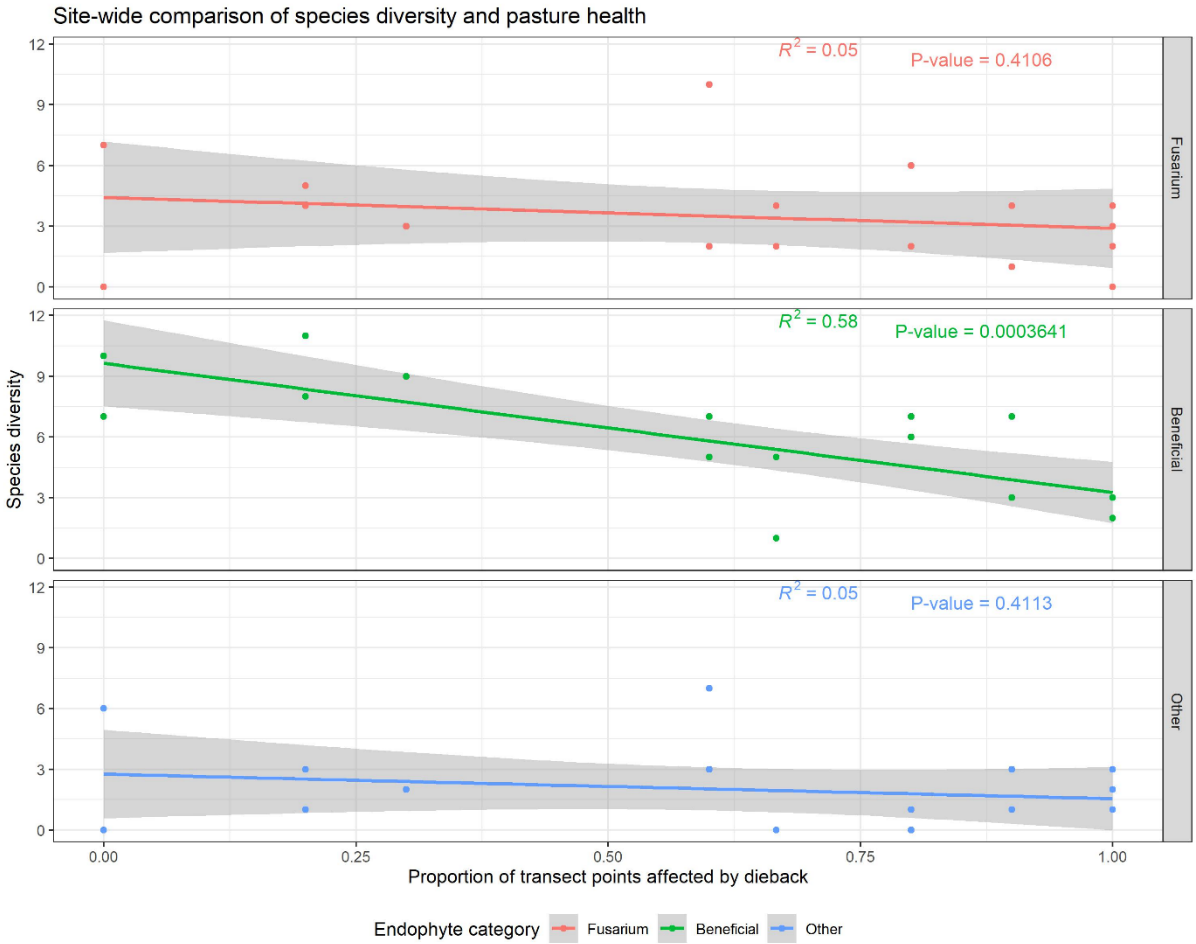


Figure 8: Diversity of species and variants of (upper) Fusarium, (middle, green) 5 beneficial genera, and (lower, blue) neutral fungi in comparison to severity of pasture dieback (as proportion of transect points affected by dieback) at 15 sites across Queensland and New South Wales. Severity of dieback increases significantly (P=0.0004) as diversity of beneficial fungi in soil decreases (middle panel, green). In contrast, dieback severity was not significantly correlated with diversity of either 'neutral' fungi or *Fusarium* species and variants, both of which were equally diverse across sites.

4.4 Develop procedure and use them to assess promising endophytes (both facultative and seed-born) as cost effective pasture pest control methods.

Tests of multiple methods of laboratory assays to evaluate endophytes were assessed. The most successful for facultative endophytes (inoculation of compost with mycelium of single isolates and infestation with mealybugs) was able to detect significant differences in impact of facultative endophytes on mealybug numbers. These experiments primarily demonstrate that small laboratory experiments with inocula can produce significant results in a short time (5 weeks from inoculation to assessment).

In an assay with *Penicillium* isolates isolate 585 (*P. pulvis*) and *P. clavistipitatum* had significantly (P >0.0001 and P=0.003) fewer mealybugs 2 weeks after exposure than controls with no inoculum. Mean number of mealybugs on plants inoculated with other isolates were not significantly different from the control (Fig.9).

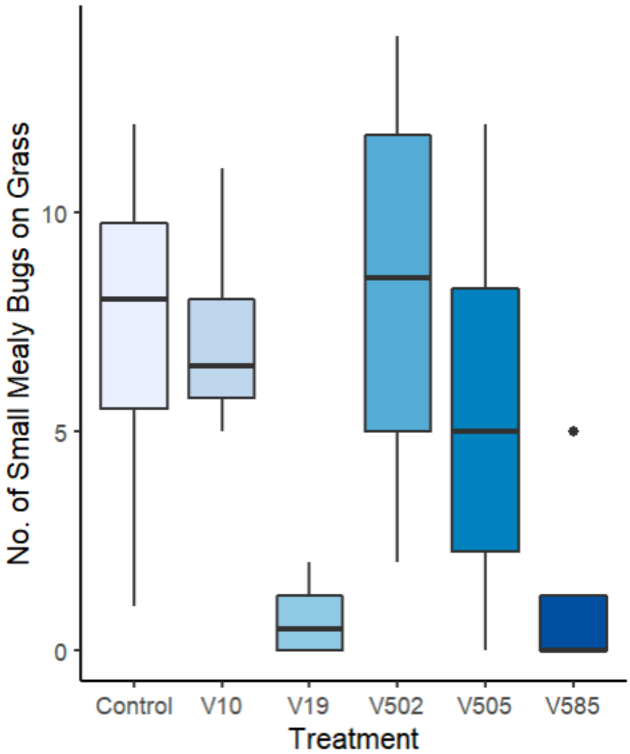


Figure 9: Abundance of mealybugs 2 weeks after infestation on Rhodes grass seedlings inoculated with a control (0.05% Tween80) or one of five species of *Penicillium*: *P. ludwiii* (10), *P. clavistipitarum* (19), *P. paneum* (502), *P. pulvis* (585) and *P. citrinum* (505).

Assays using infestation with mobile and variable populations of mealybugs have a tendency to over-dispersed data. Infestation assays for seed-born endophytes (4.5, below) produced significant results but again with dispersed data. In these experimental designs, significance using analysis of variance may be difficult without large numbers of replicates, and consequently cost of labour, materials and facilities.

In contrast, survival time assays are relatively rapid (10 days), can be assessed in one or two hours each day, and are analysed by a regression analysis using continuous variables (proportion dead, time in hours), a much more statistically powerful analysis.
Future assays will begin with a test survival analysis in the laboratory for screening of endophytes and grass varieties.

4.5 Develop procedures and use them to assess endophytes and tolerant/resistant pasture mixes in field surveys, and laboratory and glasshouse assays.

4.5.1 Seed born endophytes

In the variety screens, perennial rye grass (*L. perenne*) without endophytes was more susceptible to mealybugs than American buffel. The seed-born fungal endophyte *Epichloë festucae* var *lolii* was shown to significantly reduce susceptibility of perennial ryegrass (*L. perenne*) to mealybug in comparison to ryegrass without endophytes and to American buffel and Callide Rhodes in screenhouse assays (Fig. 10).

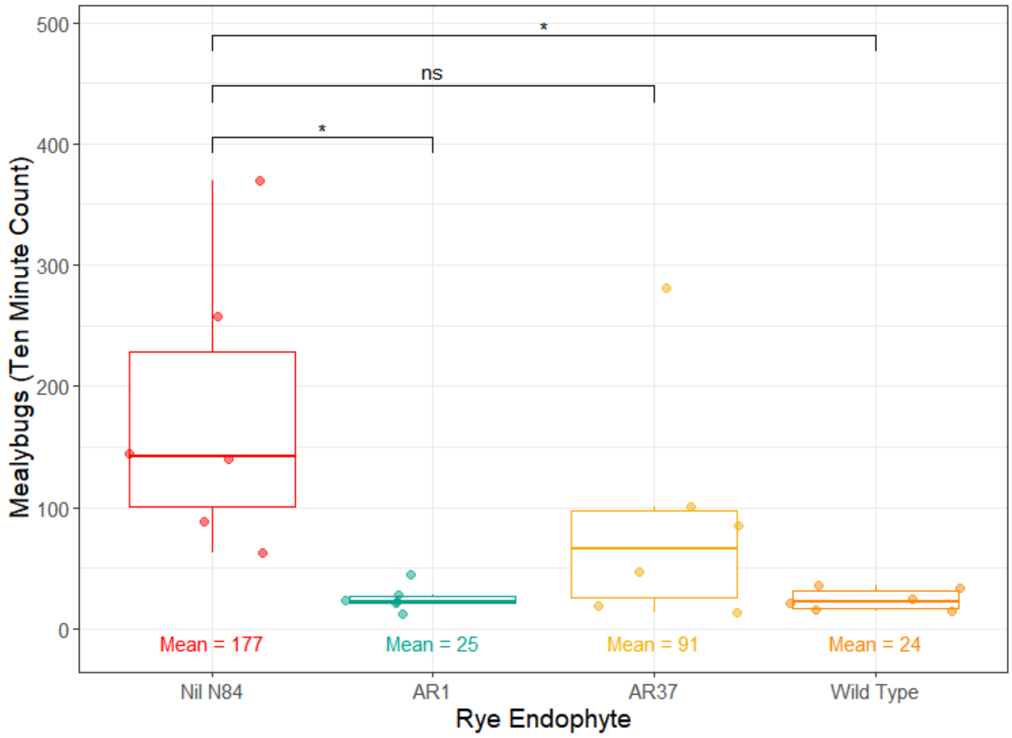


Figure 10: number of mealybugs recorded on 4 varieties of perennial rye grass 'Commando' with either no endophytes or one of 3 strains of *E. festuca*: the commercial strains AR1 and AR37, and a wild type strain. *: p < 0.05

There was a significant difference in the impact of the three *E. festuca* strains on mealybug abundance. Rye grass with fungal strains AR1 (a commercial endophyte strain) and a wild-type strain both had significantly fewer mealybugs than the ryegrass without endophyte ($p < 0.05$). The mean number of mealybugs in the ryegrass with AR37, another commercial fungal strain, was lower than in the ryegrass without endophyte but this was not significantly different.

4.5.2 Diversity of beneficial fungi in replanted pasture mixes

A pattern of abundance (colony forming units) of beneficial fungi in the 5 main genera was observed in the AHR pasture mix trial similar to that observed in the transect sampling across multiple sites. Abundance of beneficial fungal was significantly greater in plots that include legumes when compared to plots containing only grasses (P=0.0415) (Fig.11).

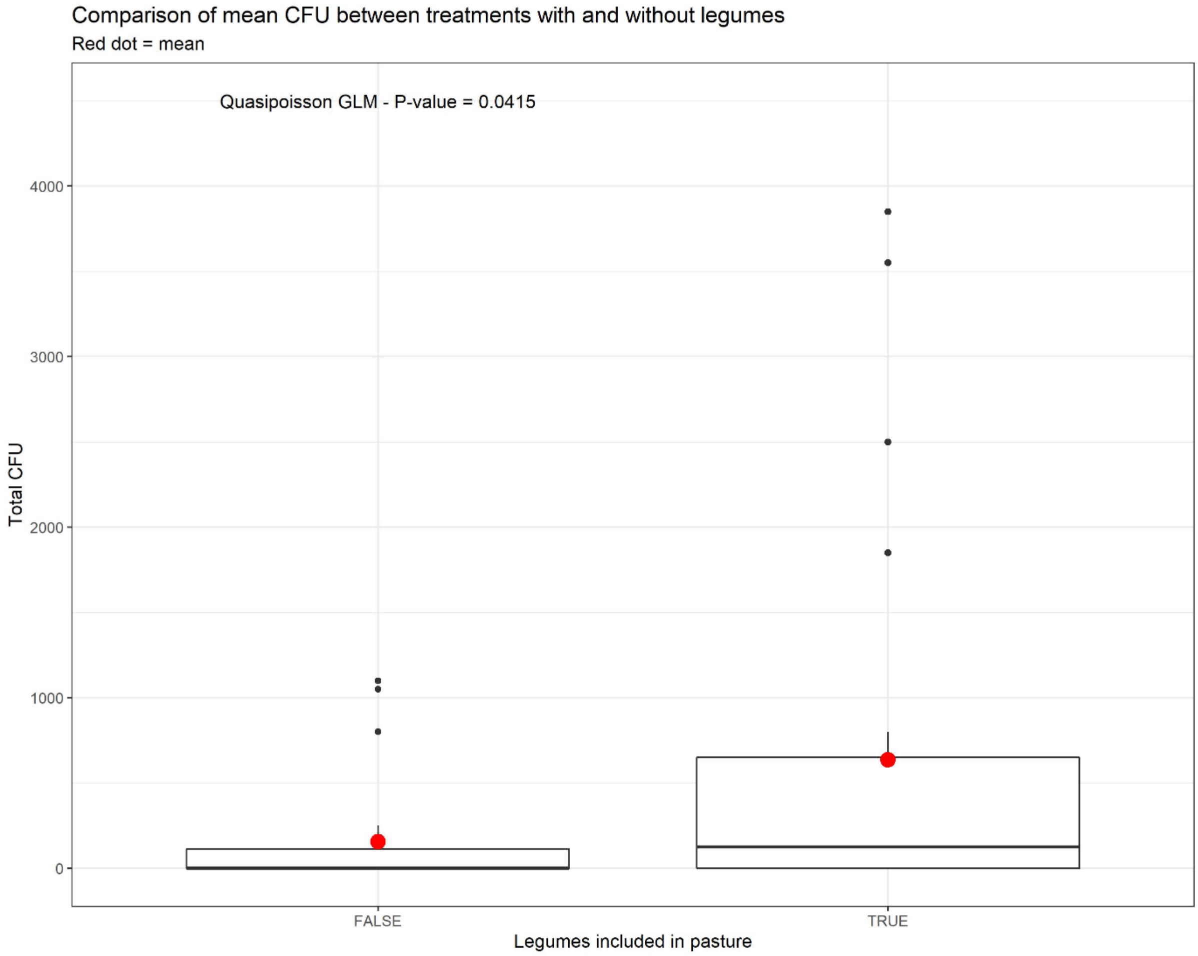


Figure 11: Mean abundance (as colony forming units, CFUs) of species of beneficial fungi in 5 genera in plots planted with legumes (2 treatments, 4 replicates each) or with grasses without legumes (8 plots of 2 treatments, 4 replicates each) in AHR pasture variety trials. Red dot = mean. P=0.0415.

This reflected a similar pattern of reduced mealybug abundance on grasses occurring in plots containing legumes recorded by AHR. Statistical analysis is continuing to incorporate continuous variables (proportion legume cover, mealybug abundance) from AHR as opposed to a categorial analysis. This is expected to increase the power of the analysis.

This result should be interpreted in conjunction with the result in 4.3.2, which shows that pastures with increased diversity of the same rhizospheric-competent endophytic fungi genera (*Penicillium*, *Purpureocillium*, *Trichoderma*, *Clonostachys*, *Beauveria*) are associated with greater resilience to mealybug (reduced severity of 'dieback') in on-farm sampling across Queensland and New South Wales.

4.5 Make recommendations for field evaluation of prospective tolerant grasses and mixes.

4.5.1 Assay methods

Field trials and infestation assays often have highly dispersed data, making significance in analysis of variance difficult without large numbers of replicates, and consequently cost of labour, materials and facilities. Field trials of varieties often take several months or years, and screenhouse assays take several weeks. Both are labour intensive to establish and assess, and dependent on work in summer months when mealybugs are active.

The laboratory survival time assays are relatively rapid: approximately 7 to 10 days, can be assessed in one or two hours each day, and are analysed by a regression analysis using continuous variables (proportion dead, time in hours), a much more statistically powerful analysis. Initial screens using laboratory bioassay methods (such as survival time) would significantly reduce costs of development of endophytes and tolerant pasture varieties.

4.5.2 Endophytes

Perennial ryegrasses with endophyte strains AR1 and AR37 are sold commercially in New Zealand to reduce incidence and damage in perennial ryegrass by the pasture mealybug *Balanococcus poae*.

This work demonstrates that endophytes of *Epichloë festucae* reduce the susceptibility of perennial ryegrass to the Australian pasture mealybug *H. summervillei*, and reduced the severity of dieback symptoms. This result suggests that identification of endophytes for tropical pasture grasses in Australia may help to reduce incidence and severity of symptoms of *H. summervillei* and pasture dieback.

Secondly this work demonstrates that there are significant differences between *E. festucae* strains in their impact on the mealybugs and damage to the grass, with AR1 and the wild type fungal strain reducing mealybug numbers more effectively than AR37. This is probably as a result of differences in production of secondary metabolites.

Wild type *E. festucae* are known to be more potent producers of secondary metabolites that impact mealybugs, but these strains are not used commercially because these metabolites are also more likely to cause staggers in livestock. Similar endophytes in tropical pasture grasses for Australia would need to be selected for efficacy against mealybugs and reduced impact on stock.

Development of commercial seed-born endophytes is costly. There is, however, significant recent investment in this area, with some promising fungi (such as *Acremonium strictum*) enjoying patent protection. Seed industries are unlikely to invest in development of seed-born endophytes soley for control of *H. summervillei*. However, rapid screens such as laboratory assays may reduce some costs and time for development, and *H. summervillei* could be added to commercial screening panels for new varieties and strains using methods identified here.

4.5.3 Rhizospheric beneficial fungi / endophytes

The endophytic fungi isolated from grasses were broadly in the same genera of known beneficial rhizosperic-competent fungi isolated from rhizospheric soil: *Penicillium*, *Purpureocillium*, *Trichoderma*, *Clonostachys*, *Beauveria*. This included several new species of both *Clonostachys* and *Trichoderma*, which was confirmed by systematic comparison of ITS sequences with validated reference sequences in international databases.

In the genus *Clonostachys*, and known endophyte and parasite of fungal pathogens, *C. roseus* was identified in several samples. However, sequence analysis also identified four and possibly more species within this genus that have not been previously identified.

At sites where a high diversity of species of these beneficial fungi were isolated, dieback severity was significantly reduced. In contrast, both diversity of *Fusarium* species and races, and diversity of ‘neutral’ fungi, were not significantly associated with dieback severity. This is an important finding, with significant implications for pasture management and reduction of the impacts of the mealybug.

The planting of legumes appears to increase abundance of the beneficial fungi associated with resilience and recovery. Management practices that increase diversity and abundance of these fungi, such as inclusion of legumes, should be practiced. In contrast, management that reduces abundance and diversity of these fungi should be avoided, and further work on the management practices such as burning should be conducted. Characterisation of these fungi, their identity and impacts on mealybugs and grass health, requires further investigation.

Recommendations to industry are based on 3 broad categories:

- Avoid highly susceptible varieties such as American buffel, broadleaf paspalum or bluegrass, or monitor and manage existing stands to reduce mealybug breeding in dense thatch during summer (results from B.PAS 0004).
- Where possible plant tolerant varieties such as Gatton panic, or Mekong brizantha, and manage these for productive foliage with grazing and slashing.
- Where possible (integrating with weed management), plant non-susceptible forage including legumes, brassicas and cereals (sorghum), or encourage resilient pasture mixtures that include legumes, forbes and a variety of more tolerant grass varieties. Avoid practices that may reduce beneficial soil fungi.

4.6. Provide timely communication on research activities for use in industry briefings at least biannually throughout the project.

Communications were provided frequently to MLA program leaders and comms team through reports, emails, a webinar, a presentation at an MLA panel event at Beef Week 2021, on-line participant meetings, material for specific media outputs, and a national science panel review in April 2022.

A summary of media outputs and extension activities has been provided separately for all 3 QUT projects.

4.7. A 2-page summary outlining key facts and findings of tolerant and susceptible grass species and management created in consultation with MLA's communications team.

An article on QUT research and management strategies for graziers in in preparation with MLA communications team.

4.7.1 Key findings

1. The mealybug *H. summervillei* was found associated with symptoms of 'pasture dieback' on 24 varieties and species of pasture grasses across Queensland and New South Wales, and on sugar cane. Legumes and other broadleaf species were no hosts of the mealybug and do not show symptoms of 'pasture dieback'.

2. Methods to conduct screenhouse evaluation of pasture varieties with efficient sampling methods were developed and the relative susceptibility of 15 species and varieties of pasture grass to American buffel was determined. Significant differences in susceptibility were detected.

3. Mekong brizantha, Argentina bahia, curly Mitchel grass, Biloela buffel and Gatton panic were all less susceptible to mealybug than American buffel.

4. Callide and Katambora Rhodes, *Paspalum nicora*, green panic, and Gayndah buffel were all similar in susceptibility to American buffel.

5. There was an apparent correlation between varieties with thick, robust stems and tillers and reduced susceptibility. This was apparent in the Gatton Panic and Green Panic varieties, which are

both varieties of *Panicum maximum* and genetically very similar (facultatively apomictic) but are significantly different in their susceptibility to mealybug. Further research on resistance mechanisms of strongly recommended.

6. Perennial rye grass (*Lollium perenne*) without endophytes was more susceptible to mealybugs than American buffel.

7. Rapid (2 week) laboratory screening assays were developed and shown to determine sensitive and statistically robust differences in susceptibility between grass varieties.
American buffel was shown to be statistically more susceptible than Callide Rhodes, while Gatton Panic and Mekong Brizantha were again statistically less susceptible than American buffel. Differences in development and survival of mealybugs were detects on different varieties.

8. The seed-born fungal endophyte *Epichloë festucae* var *lolii* was shown to significantly reduce susceptibility of perennial ryegrass (*L. perenne*) to mealybug in comparison to ryegrass without endophytes and to American buffel and Callide Rhodes in screenhouse assays.

9. Thirty nine (39) sites with differing severity of 'pasture dieback' were systematically sampled for plant and rhizospheric endophytes across Qld and NSW. Three sites included replicated trials of pasture mixes planted by AHR.

10. A total of 1120 fungal isolates were isolated and identified by Sanger sequencing of the DNA 'barcode' ITS region 1.

11. Multiple species and variants of the broadly pathogenic genus *Fusarium* were identified.

12. Five (5) genera of beneficial, rhizospheric-competent or endophytic fungi were been isolated and identified from tillers, roots and/or soil: *Penicillium*, *Purpureocillium*, *Trichoderma*, *Clonostachys*, *Beauveria*, including new species of *Clonostachys* and *Trichoderma*.

13. A highly statistically significant inverse correlation between diversity of species of beneficial endophytic fungi and severity of dieback was demonstrated. Where a high diversity of species of beneficial fungi were isolated, dieback severity was significantly reduced.
In contrast, both diversity of *Fusarium* species and races, and diversity of 'neutral' fungi, were not significantly associated with dieback severity.

14. A pattern of beneficial fungi was observed in all AHR pasture mix trials: abundance and diversity of beneficial fungal was greater in plots that include legumes when compared to plots containing only grasses. This reflected a similar pattern of reduced mealybug abundance on grasses occurring in plots containing legumes recorded by AHR. Statistical analysis is continuing.

This work demonstrates a direct link between 'pasture dieback', pasture diversity and soil health. Pastures with a higher diversity of 5 key genera of beneficial rhizospheric-competent endophytic fungi had significantly reduced severity of pasture dieback. Abundance of these species was shown to be higher in trial plots planted with legumes in pasture mixes than in plots sown with pasture grasses alone. Assessment of beneficial fungi and pasture diversity may be an indicator of pasture resilience and inform management decisions. Management practices that increase diversity of beneficial rhizospheric fungi, such as inclusion of legumes, and impacts of management that reduces abundance and diversity of these fungi (such as, possibly, hot burning) requires investigation.

5. Conclusion

5.1 Key findings

This work has established methods to quantify the relative susceptibility of targeted grass varieties to pasture mealybug H. *summervillei*. Broadleaf *Paspalum*, American buffel, Bluegrass and Rhodes grass are highly susceptible, as was observed in the field surveys. In contrast, some panic grasses (Gatton panic) appear to be more tolerant or able to recover from infestation by mealybug/dieback, as has also been observed in the field.

There was an apparent correlation between varieties with thick, robust stems and tillers and reduced susceptibility. This was apparent in the Gatton Panic and Green Panic varieties, which are both varieties of *Panicum maximum* and genetically very similar (facultatively apomictic) but are significantly different in their susceptibility to mealybug. These more structurally robust varieties may be less palatable to livestock, however, appropriate management such as grazing and slashing to promote production of palatable leaf may improve productivity. Planting of Gatton panic and management with stock rotations and slashing to reduce aerial tillering has significantly improved returns in an area of severe mealybug/pasture dieback at Banana station.

This work demonstrates that endophytes of *Epichloë festucae* reduce the susceptibility of perennial ryegrass to the Australian pasture mealybug *H. summervillei*, and reduced the severity of dieback symptoms. This result demonstrates the 'proof of concept' of the use of endophytes as used commercially in New Zealand against the pasture mealybug *Balanococcus poae*.

This result suggests that identification of endophytes for tropical pasture grasses in Australia may help to reduce incidence and severity of symptoms of *H. summervillei* and pasture dieback. However, development of commercial seed-born endophytes is costly. There is, however, significant recent investment in this area, with some promising fungi (such as *Acremonium strictum*) enjoying patent protection. Seed industries are unlikely to invest in development of seed-born endophytes solely for control of *H. summervillei*. However, rapid screens such as laboratory assays may reduce some costs and time for development, and *H. summervillei* could be added to commercial screening panels for new varieties and strains using methods identified here.

'Dieback' severity was reduced in pastures with greater diversity of key general of beneficial rhizospheric endophytes. This is in contrast to the many species and variants of *Fusarium* or 'neutral' fungi, whose diversity did not correlate with severity. Further analysis of this data set will focus on the impacts of pasture diversity (also assessed at the same samples points on the transect) on fungal diversity. Further work to assess the impacts of other management practices, such as burning, on these beneficial general should be investigated.

Legumes such as *Desmanthus*, lucerne, clovers, medic, and many other forage legumes, along with brassicas (rapa), chicories and other forbes, are not susceptible to mealybug. Several properties (including Banana station and farms in the Arcadia Valley) are now replacing buffel varieties with forage legumes (non-irrigated lucerne, Desmanthus).

Results in the AHR trials suggest that planting pasture mixes with legumes may offer benefit to graziers through improved populations of beneficial soil fungi (including rhizospheric endophytes). Assessment of beneficial fungi and pasture diversity may be an indicator of pasture resilience and inform future management decisions.

The methods developed in these trials could have significant benefits to graziers and seed companies. Field trials and infestation assays (including screenhouse assays) often have highly dispersed data, making determining significance in analysis of variance difficult without large numbers of replicates, and consequently cost of labour, materials and facilities. Field trials of varieties often take several months or years and even screenhouse assays take several weeks. In contrast, laboratory bioassays are relatively rapid, approximately 7 to 10 days for the ST50 assays, can be assessed in one or two hours each day by one or two people, and are analysed by a regression analysis using continuous variables (proportion dead, time in hours), a much more statistically powerful analysis. Initial screens using laboratory bioassay methods would significantly reduce costs of identifying bioactive endophytes and tolerant pasture varieties.

Summary

Tolerance and resistance to mealybug varies across a range of pasture grass species. Legumes and other broadleaf forages are not susceptible to the mealybug of symptoms of 'dieback.'

Highly susceptible varieties such as broadleaf *Paspalum*, American buffel and Bisset bluegrass should be avoided, or monitored and managed to reduce mealybug breeding in dense thatch in summer.

Where possible, replant with more tolerant varieties such as Gatton panic.

There was an apparent correlation between varieties with thick, robust stems and tillers and reduced susceptibility to mealybug. These varieties are potentially less palatable to cattle and require appropriate management (grazing, slashing) to maintain feed productivity.

Forage such as legumes, brassicas and cereals (sorghum), or resilient pasture mixtures that include legumes, forbes and a variety of more tolerant grass varieties, should be included in a farming system to provide diverse options and additional choices to marginal rangeland.

Both seed-born and rhizospheric-competent fungal endophytes are beneficial in reducing mealybug severity and farm management should aim to maximise these beneficial soil microbiota.

Legumes should be included in pasture mixes where possible to promote beneficial fungal diversity and provide forage that is not susceptible to mealybug.

Less diverse pastures and those with more susceptible varieties in monoculture should be monitored and managed to reduce mealybugs.

This work demonstrates a direct link between 'pasture dieback', pasture diversity and soil health. Pastures with a higher diversity of 5 key genera of beneficial rhizospheric-competent endophytic fungi had significantly reduced severity of pasture dieback. Abundance of these species was shown to be higher in trial plots planted with legumes in pasture mixes than in plots sown with pasture grasses alone. Assessment of beneficial fungi and pasture diversity may be an indicator of pasture resilience and inform management decisions. Management practices that increase diversity of beneficial rhizospheric fungi, such as inclusion of legumes, and impacts of management that reduces abundance and diversity of these fungi (such as, possibly, hot burning) requires investigation.

5.2 Benefits to industry

Methods of screening have been developed to provide rapid, high-throughput and statistically robust screening assays for grass varieties and beneficial microbiota. Laboratory bioassay are relatively rapid, approximately 7 to 10 days for the ST50 assays, can be assessed in one or two hours each day by one or two people, and are analysed by a regression analysis using continuous variables (proportion dead, time in hours), a much more statistically powerful analysis. Initial laboratory screens using *H. summervillei* in panel screens by seed companies could significantly reduce costs of identifying bioactive endophytes and tolerant pasture varieties.

Grasses with greater tolerance and reduced survival of mealybugs compared to susceptible varieties have been identified. Graziers should select more tolerant varieties for replanting, or more carefully monitor and manage more susceptible varieties to reduce mealybugs.

Seed-born endophytes have been shown to significantly reduce mealybugs and symptoms of dieback in perennial ryegrass, establishing proof of concept of the use of endophytes to protect from mealybug as is used in temperature pastures in New Zealand.

This work demonstrates a direct link between 'pasture dieback', pasture diversity and soil health. Pastures with a higher diversity of 5 key genera of beneficial rhizospheric-competent endophytic fungi had significantly reduced severity of pasture dieback. Abundance of these species was shown to be higher in trial plots planted with legumes in pasture mixes than in plots sown with pasture grasses alone. Assessment of beneficial fungi and pasture diversity may be an indicator of pasture resilience and inform management decisions. Management practices that increase diversity of beneficial rhizospheric fungi, such as inclusion of legumes, and impacts of management that reduces abundance and diversity of these fungi (such as, possibly, hot burning) requires investigation.

Recommendations to industry are based on 3 broad categories:

- Avoid highly susceptible varieties such as American buffel, broadleaf paspalum or bluegrass, or monitor and manage existing stands to reduce mealybug breeding in dense thatch during summer (results from B.PAS 0004).
- Where possible plant tolerant varieties such as Gatton panic, or Mekong brizantha, and manage these for productive foliage with grazing and slashing.
- Where possible (integrating with weed management), plant non-susceptible forage including legumes, brassicas and cereals (sorghum), or encourage resilient pasture mixtures that include legumes, forbes and a variety of more tolerant grass varieties. Avoid practices that may reduce beneficial soil fungi.

6. Future research and recommendations

6.1 Further research

Further research to screen varieties for tolerance/ resistance and to determine tolerance / resistance mechanisms using a combination of rapid laboratory assay method and transcriptome analysis (B.PAS 0003) are strongly recommended.

Characterisation of the isolates of fungi in the 3 of the key beneficial endophyte genera (*Clonostachys, Trichoderma* and *Penecillium*) should be completed to determine their taxonomy. The beneficial impacts of these fungi on grasses and mealybugs should be a focus of further investigation using some of the rapid assay methods developed here.

Further assays and detailed analysis of plant/endophyte/mealybug interactions in grasses should be conducted in combination with trials on pasture legume mixes is strongly recommended. Management practices that increase diversity, such as inclusion of legumes, and management that reduces abundance and diversity of these fungi (such as, possibly, hot burning) should be investigated.

6.2 Recommendations to industry

- Avoid highly susceptible varieties such as American buffel, broadleaf paspalum or bluegrass, or monitor and manage existing stands to reduce mealybug breeding in dense thatch during summer.
- Where possible plant tolerant varieties such as Gatton panic, or Mekong brizantha, and manage these for productive foliage with grazing and slashing.
- Where possible (integrating with weed management), plant non-susceptible forage including legumes, brassicas and cereals (sorghum), or encourage resilient pasture mixtures that include

legumes, forbes and a variety of more tolerant grass varieties. Avoid practices that may reduce beneficial soil fungi.

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