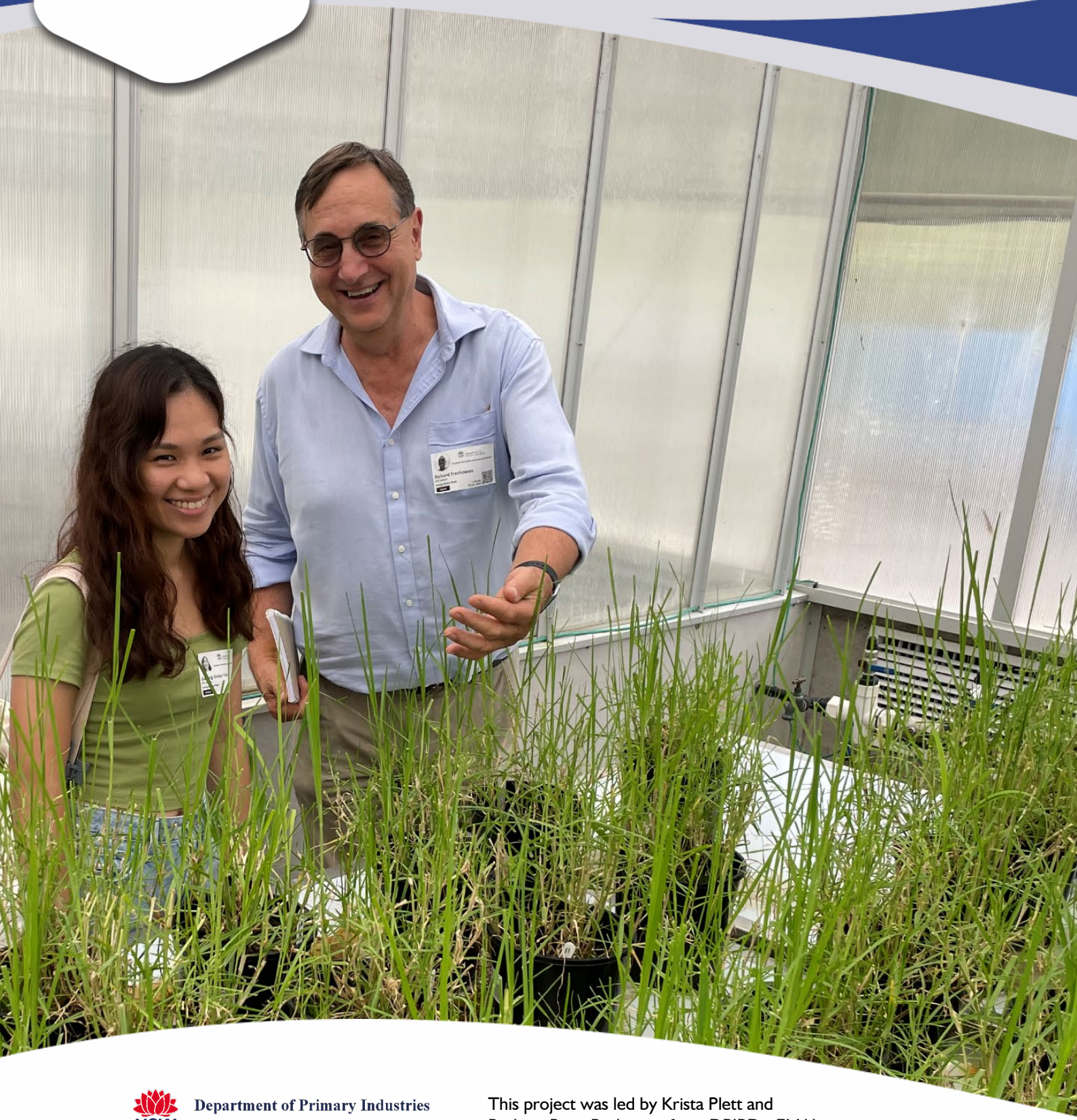
DairyUP
Unlocking potential

Final Report

P1b Antinutritional factors- Kikuyu Toxicity



Department of Primary Industries and Regional Development

This project was led by Krista Plett and Barbara Brito Rodriguez from DPIRD - EMAI

Dairy UP (Phase 1) was a $16 million, five-year industry driven project with a portfolio of 10 research, development and adoption projects collectively aiming to realise three primary objectives:

- Increase Productivity and Profitability by unlocking the potential of milk, the cow and water,
- De-risking the industry and
- Developing new markets.

A key part of Dairy UP was a coordinated network of partner farms across New South Wales (and beyond) providing valuable insights into real world application of new practices, including the challenges and benefits of new innovative technologies.

Dairy UP made a big contribution to dairy research and development rejuvenation, (attracting new researchers, PhD students and Industry investment).

Dairy UP was funded through the Australian and NSW Government's Bushfire Industry Package – Sector Development Grant (BIP-SDG) program, with cash co-contributions from Dairy Australia, The University of Sydney's Dairy Research Foundation, Local Land Services, Norco, Leppington Pastoral Co Ltd and Dairy NSW; and in kind contributions by all the above organisations plus NSW DPI (Biosecurity and Food Safety; Agriculture), Scibus, Australia Fresh Milk Holding Ltd, Dairy Connect and NSW Farmers.

This project was delivered jointly by University of Sydney's Dairy Research Foundation, Scibus, Department of Primary Industries and Regional development.

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1. Executive Summary

Kikuyu grass (Cenchrus clandestinus) is a vital forage species for Australian dairy and beef industries, particularly in coastal New South Wales and Queensland. However, its widespread adoption has been constrained by kikuyu poisoning, a sporadically occurring, potentially fatal syndrome in cattle characterised by forestomach necrosis and severe dehydration, with mortality rates up to 32%. Despite decades of reports, the causative agent has remained unknown.

This project, comprising three complementary subprojects, applied cutting-edge fungal microbiome sequencing and untargeted metabolomics to investigate the biological basis of kikuyu toxicity. Together, the studies examined five kikuyu genotypes under controlled drought and rehydration conditions, tracked the behaviour of the suspected fungal agent *Fusarium torulosum,* and compared the microbiome and metabolite profiles of pastures actively causing cattle poisoning with healthy farms across NSW.

Key outcomes across the three subprojects are:

- Sudden rehydration after drought, rather than drought itself, is the dominant trigger of biological change in kikuyu grass, consistent with field observations of poisoning events.
- *F. torulosum*, long implicated as the causative agent, was found to decline significantly after rehydration and was not consistently enriched in toxic pastures. The mycotoxin wortmannin, attributed to this fungus, was not detected in any sample across the pot studies.
- Specific metabolite signatures, including seven compounds uniquely elevated in both leaf and stem of actively toxic pastures, were identified in the field study. These represent the first candidate toxic compounds identified in kikuyu using untargeted metabolomics from real poisoning events.
- A distinct fungal species, *Acremonium polychromum*, was significantly elevated in leaf, stem, and soil of toxic pastures and is capable of producing toxic alkaloids, making it a strong candidate for further investigation.
- Kikuyu genotype significantly influenced metabolite profiles, suggesting that some cultivars may carry inherently lower toxicity risk, an important finding for future breeding programs.

These results collectively redirect the field away from *F. torulosum* as the primary causal agent and toward a more nuanced picture involving plant stress responses to overwatering and specific fungal interactions. The findings provide a scientific foundation for developing practical tools for farmers, including early-warning metabolite markers and evidence-based pasture management guidance.

2. Project Overview

Item	Description
Project Title	Antinutritional Factors- Kikuyu Toxicity
Funding Body	Dairy Australia, University of Sydney, NSW DPIRD
Dairy UP Project	P1c
Project Duration	
Subprojects	A Effect of Water Stress on Microbiome and Metabolite Profiles in Three Kikuyu Genotypes B Response of Fusarium torulosum to Water Stress in Five Kikuyu Genotypes C Fungal Profiles Associated with Kikuyu Exhibiting Toxicity Field Study
Lead Organisation	DPIRD- EMAI, USyd
Project Lead	Krista Plett, Barbara Brito Rodriguez
Key Collaborators	Vivien Tan (PhD), Percy Wong, Richard Trethowan, Pedro Pincowski, Aki Kawasaki

3. Abbreviations

CV — Coefficient of Variation
ASV — Amplicon Sequence Variant
DADA2 — Divisive Amplicon Denoising Algorithm 2
EMAI — Elizabeth Macarthur Agricultural Institute
ESI — Electrospray Ionisation
ITS — Internal Transcribed Spacer (fungal barcode region)
LC-MS/MS — Liquid Chromatography tandem Mass Spectrometry
OUT — Operational Taxonomic Unit
PCA — Principal Component Analysis
PCoA — Principal Coordinates Analysis
PERMANOVA — Permutational Multivariate Analysis of Variance
PLS-DA — Partial Least Squares Discriminant Analysis
WHC — Water Holding Capacity

4. Project Background and Rationale

Kikuyu grass is one of the most important pasture species for Australian dairy and beef producers, particularly along the coastal regions of New South Wales and Queensland. Its drought tolerance, rapid growth, and adaptability to acidic soils make it highly valued in grazing systems. However, the threat of kikuyu poisoning has long restricted farmer confidence in the species.

Kikuyu poisoning presents as a sudden-onset syndrome in cattle, characterised by forestomach necrosis, severe dehydration, salivation, incoordination, and ruminal fluid accumulation. Mortality rates can reach 32%, with significant economic consequences for affected farms. The syndrome typically occurs following rapid grass regrowth after heavy rain or irrigation that breaks a prolonged dry period, a pattern that is becoming more frequent with the increasing intensity of drought-and-flood cycles driven by climate change.

Despite reports of kikuyu poisoning dating back to the 1960s across Australia, New Zealand, South Africa, and Kenya, the causative agent has never been definitively identified. Earlier research using culture-based methods proposed *Fusarium torulosum*, a fungus capable of producing the mycotoxins wortmannin and butanolide, as a likely cause. However, this fungus has not been consistently found in toxic pastures across studies, and no definitive mechanistic link has been established.

This project was motivated by the need to apply modern, culture-independent genomic and metabolomic tools to comprehensively characterise the microbiome and chemistry of kikuyu grass under conditions that mirror real poisoning events. By combining controlled pot experiments with field sampling from active poisoning events, the project aimed to identify the biological signatures of kikuyu toxicity and test the *F. torulosum* hypothesis rigorously.

5. Project Objectives

The overarching objective of this project was to determine the biological basis of kikuyu poisoning in cattle by characterising the fungal microbiome and metabolite profiles of kikuyu grass under toxic and non-toxic conditions. Specific objectives were:

- To determine how drought stress followed by sudden rehydration affects the fungal community and metabolite profiles of different kikuyu genotypes under controlled conditions.
- To test whether *F. torulosum* abundance and wortmannin production are influenced by water stress and rehydration, and whether this is consistent with its proposed role as the causative agent.
- To compare the microbiome and metabolome of kikuyu grass collected from active kikuyu poisoning events with historically affected and unaffected pastures across NSW.
- To identify specific fungal taxa or metabolites consistently associated with confirmed toxic kikuyu, providing candidate targets for further mechanistic investigation.
- To assess whether kikuyu genotype influences toxicity-relevant biological responses.

6. Subproject P1b.1 – Effect of Water Stress on Microbiome and Metabolite Profiles in Three Kikuyu Genotypes

Research team: Vivien Tan, Percy Wong, Richard Trethowan, Barbara Brito & Krista L. Plett

6.1 Background

Kikuyu poisoning events are consistently reported following rapid grass regrowth after a prolonged dry period, suggesting the environmental trigger, drought followed by rehydration, plays a central role. However, it was unknown whether this pattern reflects a change in the fungal microbiome, a shift in plant chemistry, or both. Earlier research had also speculated that kikuyu poisoning may vary between different cultivars, but no systematic comparison had been made.

This subproject aimed to establish a baseline understanding of how drought stress and rehydration alter the fungal community and metabolite profiles in three kikuyu genotypes under controlled conditions. It was designed to test whether the environmental conditions associated with poisoning events produce measurable biological changes in kikuyu, and whether those changes differ by genotype.

6.2 Objectives

- To characterise shifts in fungal microbiome composition in soil, root, stem, and leaf tissues of three kikuyu genotypes following drought stress and rehydration.
- To identify metabolites significantly altered by water treatment and rehydration.
- To determine whether genotype influences microbial or metabolite responses to water stress.
- To test whether the conditions associated with kikuyu poisoning produce biological changes consistent with a toxicity mechanism.

6.3 Materials & Methods

A pot experiment was conducted at the Elizabeth Macarthur Agricultural Institute (EMAI), NSW, from November 2022 to June 2023. Three kikuyu genotypes: Whittet (commercial cultivar) and two breeding lines (lines 11 and 13), were established in 1.1 L pots using commercial potting mix with 1% soil from a kikuyu paddock with a history of poisoning to introduce a field soil microbiome. Six replicate pots per genotype were assigned to each of two treatments after four months of establishment.

Treatments consisted of a reduced-watering group maintained at 30% field capacity and a control group maintained at 80% field capacity. A gradual conditioning period was applied prior to treatment commencement to avoid physiological shock. After six weeks of treatment, pots were rehydrated to full saturation for one week to simulate drought-breaking rainfall. Grass was trimmed fortnightly throughout to maintain approximately 15 cm height.

Soil, root, stem, and leaf samples were collected before and after the rehydration week and stored at −80°C. Fungal community composition was analysed using ITS amplicon sequencing on the Illumina MiSeq platform, with taxonomy assigned against the UNITE fungal database. Metabolite profiling was conducted by untargeted LC-MS/MS using a Sciex ExionLC AD system coupled to a TripleTOF 6600+ mass spectrometer in both positive and negative electrospray ionisation modes. Data were processed using QIIME2/DADA2 for microbiome and MSDial/MetaboAnalystR for metabolomics, with statistical analysis including PERMANOVA, ANOVA with Tukey post-hoc, and PLS-DA.

6.4 Key Findings

- Based on the PERMANOVA results, rehydration, not drought, was the dominant driver of change in the fungal microbiome across leaf, stem, and root tissues. Timepoint (before vs. after rehydration) was the most significant factor in leaf and stem while genotype was the most significant factor in root and soil.
- Genotype was the strongest factor influencing metabolite profiles. Genotypes 11 and 13 were characterised by elevated amino acids, organic acids, and lipids, while genotype 15 showed higher cholines, dicarboxylic acids, and alpha-diketones.
- Water stress produced limited treatment-specific metabolite changes. In leaf tissue, alkaloids and nitrogen-containing compounds were slightly elevated under the reduced-watering treatment. In stem tissue, six metabolites (some only putatively identified), including amino acids, hydroxy fatty acids, and aldoximes were higher under drought conditions.
- A total of 174 metabolites were significantly elevated in genotypes 11 and 13 (leaf tissue), and 108 in genotype 15, suggesting a strong effect of the genetic background on the metabolome.

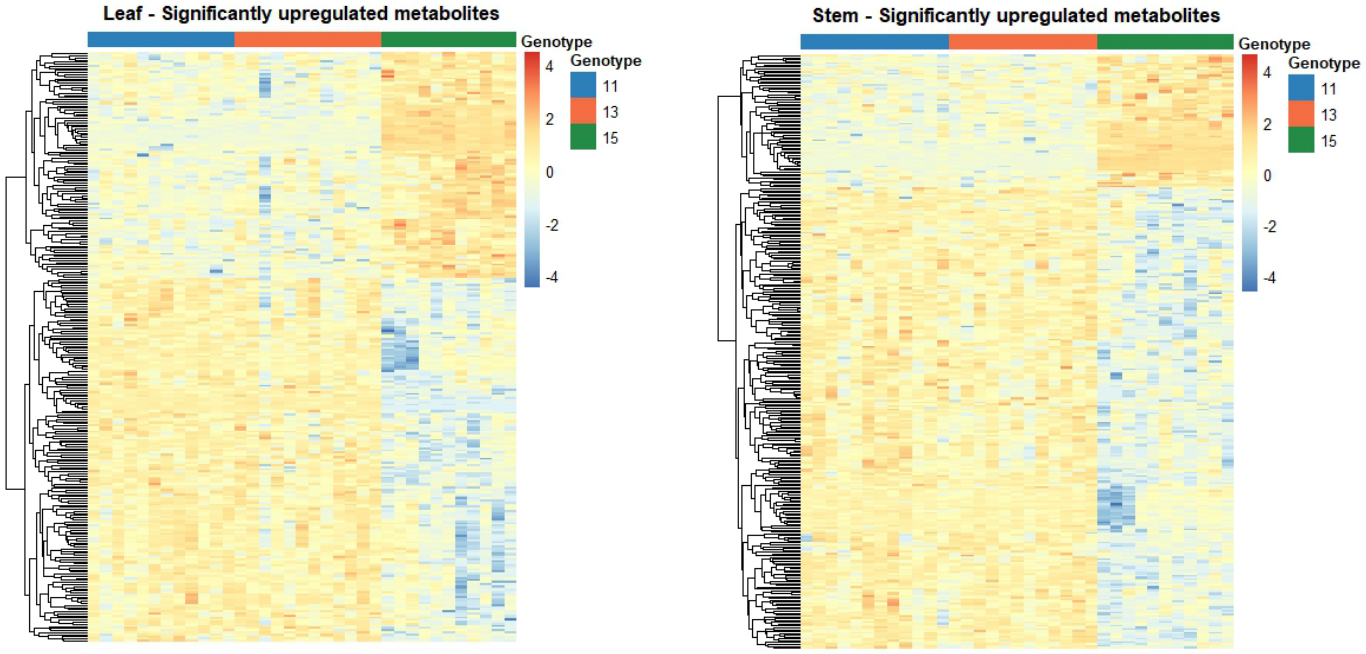

Figure 6.5. Abundance of significantly upregulated metabolites in leaf and stem across genotypes.

6.5 Outputs

- Dataset: ITS amplicon sequencing data and LC-MS/MS metabolomics dataset for three kikuyu genotypes under water stress and rehydration conditions.
- Manuscript in preparation for peer-reviewed publication.
- Contribution to project-wide database of kikuyu microbiome and metabolite profiles.

6.6 Applications & Impacts

The finding that rehydration, not the dry period, is the primary biological trigger is directly relevant to farming practice. It confirms that the critical risk window is in the days immediately following irrigation or rain after drought, rather than during the dry period itself.

The genotypic differences in metabolite profiles are significant for breeding programs. If specific chemical signatures are associated with toxicity risk, selecting against these profiles in breeding could yield safer cultivars. The absence of wortmannin across all conditions further calls into question management strategies or monitoring approaches that have been built around *F. torulosum* as the causal agent.

6.7 Future Research Opportunities and Actions

- Investigate the functional significance of the genotype-specific metabolite differences identified, particularly whether they are linked to susceptibility to toxin accumulation.
- Explore the mechanistic basis of the rehydration-triggered metabolite response, including gene expression analysis.
- Develop farmer-facing resources on safe reintroduction timeframes following drought-breaking rain, based on these and subsequent study findings.

7. Subproject P1b.2- Response of *Fusarium torulosum* to Water Stress in Five Kikuyu Genotypes

Research team: Vivien Tan, Percy Wong, Richard Trethowan, Barbara Brito & Krista L. Plett

7.1 Background

Fusarium torulosum has been the most frequently cited candidate cause of kikuyu poisoning, primarily because of its known capacity to produce wortmannin and butanolide, mycotoxins that produce symptoms in cattle resembling kikuyu poisoning when administered experimentally. However, *F. torulosum* has not been consistently found in toxic pastures, and prior studies were constrained by culture-based methods that may have missed or misrepresented fungal communities.

This subproject expanded on the findings of Subproject P1b.1 by directly inoculating all pots with a verified *F. torulosum* isolate and tracking its abundance alongside the broader microbiome and metabolome across five kikuyu genotypes. This allowed a direct test of whether drought and rehydration conditions influence *F. torulosum* abundance and wortmannin production in a way consistent with its proposed role as the causative agent.

7.2 Objectives

- To test the hypothesis that *F. torulosum* abundance is favoured by conditions associated with toxicity, namely a prolonged dry period followed by rehydration.
- To detect wortmannin or butenolide production under drought and rehydration conditions using metabolomics.
- To assess whether genotype influences *F. torulosum* colonisation or plant metabolite responses.
- To characterise fungal community and metabolite shifts across a broader set of five kikuyu genotypes.

7.3 Materials & Methods

A pot experiment was conducted at the University of Sydney Camden Campus, NSW, from July to November 2024. Five kikuyu genotypes, Whittet, Fulkerson, and three breeding lines (2, 8, and 10) were established in 1.1 L pots. At the start of the experiment, all 60 pots were inoculated with a spore suspension of *F. torulosum* (isolate accession number 11062, obtained from the Botanic Gardens of Sydney), confirmed by PCR amplification and Sanger sequencing of the ITS and Tef-1 gene regions. Spore concentration was 9.04×10^4 spores/mL.

After a two-week conditioning period at 80% field capacity, pots were assigned to either a reduced-watering treatment (30% field capacity) or a control (80% field capacity) in a randomised block design. After eight weeks, at which point reduced-watering plants had gone dormant, all pots were rehydrated to full saturation for one week to simulate drought-breaking rainfall. Soil, root, stem, and leaf samples were collected at two timepoints: immediately before rehydration and one week after.

F. torulosum abundance was quantified from ITS sequencing data. Fungal community composition was analysed using ITS sequencing on the Illumina MiSeq i100 Plus (QIIME2/DADA2 pipeline, UNITE database). Metabolomics was performed using LC-MS/MS in positive and negative ionisation modes, with data processed via MSDial and MetaboAnalystR. A correlation network analysis between microbial taxa and significantly upregulated metabolites was performed using Spearman rank correlation.

7.4 Key Findings

- Drought did not significantly reduce *F. torulosum* abundance over eight weeks. The organism persisted under prolonged dry conditions across all genotypes and tissue types.
- Rehydration caused a significant and consistent decline in *F. torulosum* abundance in both the control and reduced-watering treatments. This is the opposite of the pattern expected if *F. torulosum* were driving post-rehydration toxicity.
- Genotype had no significant effect on *F. torulosum* colonisation, but a significant interaction between genotype and treatment ($p = 0.04995$) was observed, suggesting that *F. torulosum* responds differently across genotypes under water stress.
- Neither wortmannin nor butanolide were detected in any sample. No metabolite fragment consistent with wortmannin or butenolide was identified under either dry or rehydrated conditions.
- As in Subproject 1, rehydration was the dominant driver of change in the fungal community across leaf, stem, and root tissues. Treatment (drought) had the greatest effect in soil only.
- Rehydration triggered upregulation of fatty acids, flavonoids, alkaloids, and nitrogen-containing compounds in leaf and stem tissue, consistent with a plant stress response to overwatering rather than a fungal toxin response.
- Correlation analysis between microbial taxa and upregulated metabolites was weak (maximum Spearman $r = 0.69$), indicating the metabolite changes are more likely driven by the plant itself than by the fungal community.
- A weak correlation ($r = 0.63$) was observed between the *F. torulosum* inoculum and hexadecanoic acid, a fatty acid with antimicrobial properties, in leaf control samples, possibly reflecting a plant defensive response.

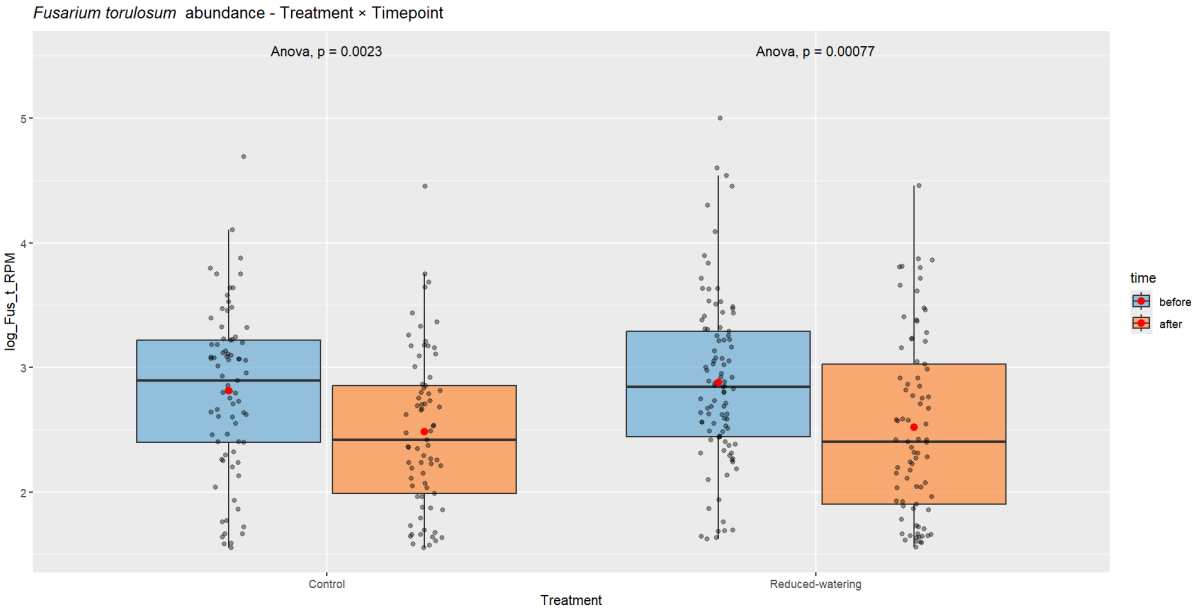

Figure 7.5. F. torulosum abundance across treatments and timepoints.

7.5 Outputs

- Dataset: ITS sequencing and LC-MS/MS metabolomics data for five kikuyu genotypes with *F. torulosum* inoculation under water stress and rehydration conditions.
- Quantified *F. torulosum* abundance data across genotypes, treatments, and tissue types.
- Manuscript in preparation for peer-reviewed publication.
- Contribution to the project-wide kikuyu microbiome and metabolite database.

7.6 Applications & Impacts

The results of this subproject substantially weaken the case for *F. torulosum* as the primary causative agent of kikuyu poisoning. Its decline, rather than proliferation, following rehydration, combined with the absence of wortmannin under any tested condition, suggests that monitoring or management strategies built around this fungus are unlikely to be effective.

These findings redirect attention toward alternate fungal species or the plant's own stress response as the source of toxic compounds, and open the possibility that overwatering, not merely drought, stresses kikuyu sufficiently to trigger the production of toxic metabolites. For farmers, this reinforces the importance of managing reintroduction of cattle to pastures after heavy rain and highlights the need for practical risk indicators that do not rely on *F. torulosum* detection.

7.7 Future Research Opportunities and Actions

- Examine the identity and biological activity of the alkaloids and nitrogen-containing compounds upregulated post-rehydration, to determine if any could cause the clinical signs of kikuyu poisoning.

8. Subproject P1b.3. Microbial and Metabolite Profiles Associated with Kikuyu Grass Exhibiting Kikuyu Toxicity

Research team: Krista L. Plett, Vivien Tan, Deirdre Hanrahan-Tan, Aki Kawasaki, Pedro Pinsczowski, Barbara Brito Rodriguez

8.1 Background

While the pot experiments (Subprojects P1b.1 and P1b.2) provided controlled evidence that rehydration drives the most significant biological changes in kikuyu, they could not directly identify which microbes or metabolites are present during actual poisoning events. Previous field studies had relied on culture-based methods, which detect only a subset of the fungal community and may miss the true causal organism or toxic compounds.

Subproject P1b.3 addressed this gap by sampling kikuyu from commercial farm pastures with confirmed, historical, suspected, and no history of kikuyu poisoning across multiple geographic regions and seasons in NSW. Using unbiased ITS and 16S sequencing and untargeted metabolomics, this study produced the first comprehensive molecular profiles of toxic kikuyu grass from confirmed field poisoning events.

8.2 Objectives

- To characterise the full fungal and bacterial microbiome of kikuyu pastures across active, historical, suspected, and unaffected toxicity classifications.
- To identify metabolites consistently and significantly elevated in actively toxic kikuyu compared to non-toxic pastures.
- To identify candidate fungal or bacterial taxa whose abundance is significantly associated with confirmed kikuyu toxicity events.
- To narrow down potential causative agents, microbial or chemical, for further targeted investigation.

8.3 Materials & Methods

Kikuyu pasture samples were collected from cattle farms across NSW between November 2021 and April 2024, spanning the Hunter Valley, Mid-Coast, North Coast, Shoalhaven, and Bega regions across multiple seasons. A total of 47 sampling sites were included, classified as: Toxic (active poisoning event confirmed by cattle necropsy, n = 9 sites from 1 farm), Historical (prior confirmed poisoning events, n = 12, from five farms), Suspected (symptoms consistent with poisoning but unconfirmed, n = 2; 1 farm), and Unaffected (no history of poisoning, n = 24, 17 farms).

At each site, intact swards (approximately 20 × 20 × 20 cm) were collected and sub-sampled within 24 hours into leaf, stem/stolon, fine root, and adhering soil fractions, then frozen at −80°C. Suspected samples were retained for comparative visualisation only and excluded from statistical analyses due to unconfirmed status.

Metabolomics was performed on stem and leaf samples using LC-MS/MS (Sciex ExionLC AD / TripleTOF 6600+) in positive and negative ionisation modes. Metabolite identification and peak alignment were conducted with MS-DIAL, and statistical analysis (PCA, ANOVA, heatmaps) with MetaboAnalyst 6.0. Microbiome profiling used ITS or 16S amplicon sequencing with taxonomy assigned against the UNITE database. Community diversity and composition were assessed using the vegan and ecodist R packages, and differential abundance of ASVs in toxic vs. non-toxic samples was determined using ALDEx2 with Benjamini-Hochberg correction.

8.4 Key Findings

Metabolomics:

- Toxic kikuyu leaf samples had a significantly different metabolite profile from both historical and unaffected samples, primarily in negative ionisation mode. Toxic samples separated on PC2 (11.7% of total variation) in PCA (Figure 1).

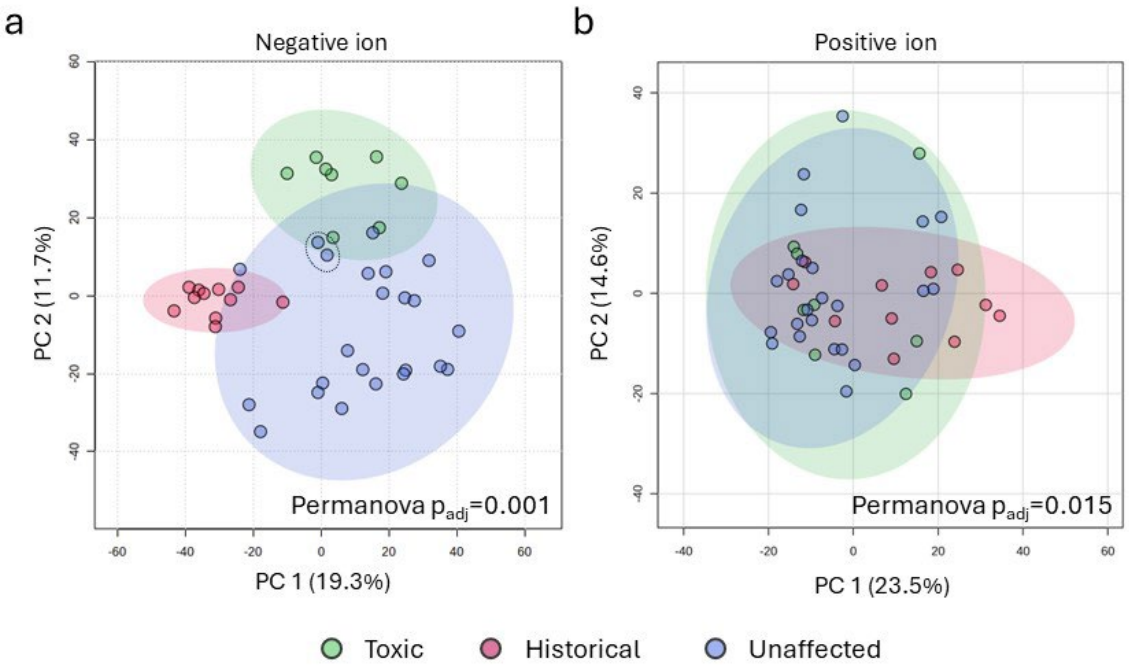

Figure 8.4 Actively toxic, historically toxic and unaffected Kikuyu leaf samples have significantly different metabolomic profiles. PCA of total metabolites present in Kikuyu leaf samples identified with negative (a) or positive (b) ionisation mode. Ellipses delineate the 95% confidence limit and results of PERMANOVA indicate significance of separation ($p<0.05$).

- ANOVA identified 259 negative-mode and 53 positive-mode metabolites significantly elevated in toxic kikuyu compared to both other groups.
- A refined subset of 17 (negative mode) and 6 (positive mode) metabolites showed consistently high abundance in toxic samples and low abundance in historical and unaffected samples. These represent the strongest candidate biomarkers of toxicity.
- Seven of these 23 metabolites were also significantly elevated in stem tissue, making them particularly robust candidates, a compound present in both leaf and stem during active toxicity is more likely to be causally relevant than one confined to a single tissue.
- Putative identities of the most elevated compounds include enterodiol, tridesacetoxykhivorin, a compound related to macrocyclic structures (resembling trichothecene-type mycotoxins), and several unidentified metabolites with high fold changes (up to 59-fold in leaf tissue; Table 1).

Table 1. Metabolites showing specific elevation in Toxic Kikuyu leaf and stem samples relative to non-toxic (unaffected) grass samples.

Metabolite ID	Ionisation Mode	Fold Change (Leaf/Stem)	Retention time	m/z	Putative ID (ontology/compound)
1927	Negative	40.5/11.1	10.00	303.15	Unknown
2482	Negative	27.3/3.5	14.78	347.15	Lignols/Enterodiol
4187	Negative	59.0/17.1	23.40	505.23	Hydroxybenzaldehydes/[(E,2R)-5-(3-chloro-5-formyl-2,6-dihydroxy-4-methylphenyl)-3-methyl-1-[(1S,2R,6R)-1,2,6-trimethyl-3-oxocyclohexyl]pent-3-en-2-yl] 3-methylbutanoate
4191	Negative	11.4/1.1	14.84	505.23	Hydroxybenzaldehydes/ [(E,2R)-5-(3-chloro-5-formyl-2,6-dihydroxy-4-methylphenyl)-3-methyl-1-[(1S,2R,6R)-1,2,6-trimethyl-3-oxocyclohexyl]pent-3-en-2-yl] 3-methylbutanoate
4199	Negative	20.6/4.5	12.41	506.23	Macrolactams/3,15-dibenzyl-1,4,12-trimethyl-1,4,7,10,13-pentazacyclopentadecane-2,5,8,11,14-pentone
4314	Negative	19.6/4.2	12.20	519.25	Liminoids/Tridesacetoxykhivorin
4143	Positive	14.4/5.0	3.11	423.81	Dicarboxylic acid derivative/2-[5-[2-[2-[5-(2-hydroxybutyl)oxolan-2-yl]propanoyloxy]propyl]oxolan-2-yl]propanoic acid

Microbiome:

- A total of 5,693 independent fungal ASVs were identified across soil, root, stem, and leaf tissue. Species richness was highest in soil and lowest in stem samples.
- Toxic pastures showed a trend toward lower fungal species richness compared to historical ($p = 0.096$) and unaffected ($p = 0.068$) sites, though not statistically significant.
- Six fungal taxa were significantly more abundant in leaves of toxic pastures (padj < 0.05), including Acremonium polychromum, Dothideomycetes sp., Sordariomycetes sp., Derxomyces longicylindricus, Dioszegia sp., and Neocucurbitaria ribicola.
- *Acremonium polychromum* was significantly elevated across leaf, stem, and soil of toxic pastures, a consistent multi-tissue signal that distinguishes it from organisms enriched in only one tissue. More detailed analysis of the ITS sequence associated with this ASV suggests that it may be more appropriately assigned to *Clonostachys rosea*, a common plant endophyte.

- An additional 12 fungi were significantly enriched in toxic stem and soil samples, including *Microascus chinensis, Amauroascus niger* (known producer of zaragozic acids), and *Hypomyces semicircularis*.
- *F. torulosum* was not identified as a significantly enriched fungus in toxic pasture samples.
- The bacterial community showed less variation in the toxic samples than the fungi with no species showing significant enrichment in toxic leaves.

8.5 Applications & Impacts

This subproject delivers the most directly actionable findings of the project. By identifying specific metabolite candidates and fungal taxa associated with confirmed poisoning events, it provides a starting point for developing a diagnostic toolkit for kikuyu toxicity risk. If the candidate metabolites or fungi can be validated as reliable markers, they could form the basis of a rapid field test or sampling protocol that allows farmers to assess pasture toxicity risk before introducing cattle.

The identification of *Acremonium polychromum* (or *Clonostachys rosea*) as a consistently enriched organism across multiple tissue types in toxic pastures is a significant finding. This organism belongs to a fungal group capable of producing toxic alkaloids. *C. rosea* is considered a beneficial endophyte promoting plant growth and disease suppression but also produces potent metabolites with known antifungal and insecticidal activity. This ASV's consistent presence, across leaf, stem, and soil, at toxic sites warrants targeted investigation into whether it produces compounds capable of causing kikuyu poisoning symptoms.

8.6 Outputs

- Dataset: ITS amplicon sequencing and LC-MS/MS metabolomics data from 47 kikuyu pasture sites across NSW, spanning confirmed toxic, historical, suspected, and unaffected classifications.
- Candidate list of 23 metabolite markers consistently elevated in toxic kikuyu (7 validated in both leaf and stem tissue).
- Candidate list of 18 fungal taxa significantly enriched in toxic pasture samples, with *Acremonium polychromum* (or *C. rosea*) identified as the highest-priority candidate for follow-up.
- Peer-reviewed manuscript in preparation (Plett et al.).
- Data contributed to cross-project kikuyu toxicity reference database.

8.7 Future Research Opportunities and Actions

- Expand field sampling to include more confirmed active poisoning events across a greater range of geographic locations and seasons, to strengthen the statistical power of metabolite and microbiome associations.
- Trial *A. polychromum* and *C. rosea* species on kikuyu in conditions associated with toxicity, and capture metabolites for *in vitro* trials to assess impact on cattle stomach cell lines.

9. Project-wide Dissemination

Table 9. Peer-reviewed publications.

Author	Title	Journal	Year Published	Citations (to April 26)
Jia Ling Vivien Tan, Barbara Brito, Percy T.W. Wong, Amit Singh, Richard Trethowan, and Krista L. Plett.	Effect of water-stress on the microbiome and metabolite profiles in three kikuyu (*Cenchrus clandestinus*) genotypes.	In preparation		
Jia Ling Vivien Tan, Barbara Brito, Percy T.W. Wong, Amit Singh, Richard Trethowan, and Krista L. Plett.	Response of *Fusarium torulosum* to water-stress in five kikuyu (*Cenchrus clandestinus*) genotypes. Microbial and metabolite profiles associated with *Cenchrus clandestinus* (Kikuyu) exhibiting Kikuyu toxicity	In preparation		

10. Conclusions and Recommendations

This project represents a comprehensive investigation of kikuyu toxicity, integrating controlled pot experiments with field sampling and applying modern, culture-independent sequencing and metabolomics tools that were unavailable to earlier researchers. Taken together, the three subprojects have substantially advanced understanding of the biological basis of kikuyu poisoning and meaningfully redirected the field toward productive new hypotheses.

The three subprojects converge on several clear conclusions:

The environmental trigger matters, and the risk window is rehydration, not drought. Across both pot experiments, rehydration, not the dry period itself, was the dominant driver of biological change in the kikuyu fungal microbiome and plant metabolome. This is consistent with the epidemiology of kikuyu poisoning, which is characteristically associated with rapid grass regrowth following drought-breaking rain or irrigation, and has direct implications for when cattle are most at risk.

Fusarium torulosum is not the primary causative agent. The controlled inoculation experiment (P1b.2) provided the most direct test of this hypothesis to date. *F. torulosum* did not proliferate under conditions associated with toxicity; instead, its abundance declined significantly following rehydration. Neither wortmannin nor butenolide was detected under any experimental condition. F. torulosum was also absent from the list of fungi significantly enriched in field-confirmed toxic pastures. Together, these findings make a compelling case that management or monitoring approaches built around this organism are unlikely to reduce poisoning risk, and that the field should move on from this hypothesis.

Candidate toxic compounds have been identified from real poisoning events for the first time. The field study (P1b.3) identified seven metabolites significantly elevated in both leaf and stem tissue of actively toxic pastures relative to historical and unaffected sites. These include putative lignols, limonoids, macrolactams, and hydroxybenzaldehyde-related compounds, some with fold changes as high as 59-fold in leaf tissue. Their consistent elevation across two tissue types in confirmed poisoning events makes them the strongest candidate biomarkers or causative compounds yet identified.

A specific fungal candidate has emerged. *Acremonium polychromum*, which may represent *Clonostachys rosea* based on refined phylogenetic analysis, was significantly enriched across leaf, stem, and soil of toxic pastures, making it the most consistently associated fungal organism identified to date. While *C. rosea* is broadly regarded as a beneficial plant endophyte, it is capable of producing potent secondary metabolites with antifungal and insecticidal activity, and its role in kikuyu poisoning warrants direct investigation.

Genotype shapes the chemical environment of kikuyu independently of water stress. Genotype was the strongest driver of metabolite profiles across both pot experiments, with substantial differences between commercial cultivars and breeding lines in the classes and quantities of metabolites produced. This raises the possibility that cultivar selection could be a practical long-term strategy for reducing toxicity risk.

Recommendations

Prioritise validation of the seven candidate toxic metabolites. Targeted chemical analysis and bioassays, including testing in bovine rumen or stomach cell lines, should be conducted to determine whether any of the identified compounds, particularly the macrolactam and limonoid candidates, are capable of causing the clinical signs of kikuyu poisoning.

Directly investigate *Acremonium polychromum* / *Clonostachys rosea*. Controlled inoculation

experiments on kikuyu under conditions associated with toxicity, combined with metabolite profiling of the fungus in culture, are the necessary next step to determine whether this organism produces compounds consistent with the toxicity syndrome.

Expand field sampling. The toxic sample set is currently limited to confirmed events from the mid coast (additionally, data is being analysed from two recent events). Expanding to additional farms, regions, and seasons will improve the statistical robustness of the metabolite and microbiome associations and determine whether the identified signatures generalise across NSW and beyond.

Move toward a practical on-farm diagnostic. The candidate metabolite markers identified in P1b.3 provide a foundation for developing a rapid field test, for example, a lateral flow assay or portable spectrometry approach, that would allow farmers and veterinarians to assess toxicity risk before cattle are introduced to recently rehydrated kikuyu pastures.

Update industry guidance on *F. torulosum* and rehydration risk. Current Dairy Australia and state veterinary guidance on kikuyu poisoning should be reviewed in light of these findings. In particular, the rehydration trigger and the lack of evidence for *F. torulosum* should be communicated to farmers, agronomists, and veterinary practitioners through extension channels.

Integrate genotype screening into breeding programs. The strong genotypic effect on metabolite profiles observed across both pot experiments should be incorporated into kikuyu cultivar development programs, with screening for toxicity-associated chemical signatures as a selection criterion alongside existing disease resistance traits.

11. Annexes

Table 2. Conference presentations, abstracts and other meetings

Authors	Title	Presentation Type	Conference/ Event	Location	Year	Audience
Jia Ling Vivien Tan, Barbara Brito, Percy T.W. Wong, Amit Singh, Richard Trethowan, and Krista L. Plett.	Effect of drought on microbiome community and metabolite profiles in kikuyu (Cenchrus clandestinus)	Oral	ADSA	Louisville,KY	2025	
Richard Trethowan, Krista Plett, Barbara Brito, Percy Wong, Amit Singh	Good grass gone bad – kikuyu poisoning	Oral	Annual District Veterinarians Conference	Wollongong	2024	
Krista Plett	Kikuyu Toxicity: A pasture out of balance?	Oral	DRF Symposium	Camden	2023	
Barbara Brito, Krista Plett, Vivien Tan.	DiaryUP Team meeting update	Oral	Virtual meeting		2022, 2025,	

Table 3. Technical reports, pamphlets, published material and other media engagements

Authors	Title	Place Published	Year
Lee-Ann Monks, Monks Communication	P1b Project Update: Investigating Kikuyu Toxicity	https://dairyup.com.au/wp-content/uploads/2026/03/Proj-Update-P1b-kikuyu-toxicity-2026.pdf	Feb 2026
Lee-Ann Monks, Monks Communication	Research aims to unlock Kikuyu grass benefits	https://www.dpi.nsw.gov.au/about-us/media-centre/releases/2026/general/research-aims-to-unlock-kikuyu-grass-benefits	May, 2026
McPherson Media Group	Testing kikuyu to solve an industry mystery	https://www.dairynewsaustralia.com.au/news/testing-kikuyu-to-solve-an-industry-mystery/	May, 2024

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