Final report

B.PAS.0505 – Rapid diagnosis of pasture dieback using SIFT-MS (Selected Ion Flow Tube Mass Spectrometry)

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Abstract

The accurate identification of pasture dieback has been rated as a very high priority. This project tests a portable gas collection tool, the analysis volatile organic compounds (VOCs), and mass spectrometry to identify 'pasture dieback' in laboratory and field samples.

A short-chain (C6) 'green leaf' plant volatile, (E)-2-hexen-1-ol, was associated with dieback. Green leaf volatiles are induced in response to abiotic and biotic stress such as attack by pests and pathogens, and water stress, and are involved in plant defenses including altering behavior of insect herbivores, attracting natural enemies of pests, and in plant-to-plant communication to reduce further attacks.

Results indicate that the use of the portable gas collection device is practical for use in the field. The work also demonstrates that VOC analysis can detect at least one induced plant volatile associated with early-stage dieback. However, the molecules identified so far are not specific to pasture dieback, and are produced in association with the appearance of early visible symptoms of dieback (leaf discoloration).

Further work is required to determine specific patterns of ions in SIFT analysis without the confounding effects of sites and grass species. This detailed laboratory analysis will be conducted and a revised report submitted.

Executive summary

Background

Pasture dieback causes unhealthy growth and death in introduced and native grasses across Queensland and northern NSW, resulting in large losses in key beef production areas.
The June 2020 Pasture Dieback Science Forum rated the development of accurate identification tools for pasture dieback to be very high priority to track the spread and impact of pasture dieback at an early stage and to implement appropriate management strategies. Diagnostic tools will assist researchers and producers to track the spread and impact of pasture dieback at an early stage and to implement appropriate management strategies. If successful this project would provide the basis for further development of a rapid identification test for pasture dieback that can be applied in the field.

Objectives

1. Detect and identify chemical markers associated with grass pasture dieback induced by mealybug in the laboratory (months 1-2)
2. Determine the potential for consistent detection of chemical markers associated with grass and early dieback and low density mealybug in the laboratory (months 2-4)
3. Determine the potential for detection of chemical markers associated with grass and early dieback and low-density mealybug in field samples (months 4-6)

Methodology

We used HS-SPME-GC-MS and SIFT-MS to generate profiels of volatile metabolites associated with dieback-affected grasses in the laboratory and in the field. We induced pasture dieback in lab-reared grasses by infesting them with the mealybug *Heliococcus summervillei*. We identified several field sites and sampled volatiles from asymptomatic and dieback-affected grasses in the same field. Sites with the same grass located near each other were likely to share the same pasture dieback status; we were unable to identify paired sites (dieback-affected and unaffected) that were sufficiently close to each other to have similar environmental conditions and grass species. Thus we were forced to analyse asymptomatic and affected plants from the same site, and from different sites with different grass species, possibly confounding results.

Results/key findings

We consistently identified statistically significant differences in volatile metabolome composition between healthy and diseased grasses, both in the laboratory and in the field. In controlled laboratory experiments, green leaf volatiles were significantly associated with dieback. One induced plant volatile, (E)-2-hexen-1-ol was identified and was clearly associated with dieback. Short-chain (C6) 'green leaf' plant volatiles such as 2-hexen-1-ol are are induced in response abiotic and biotic stress such as attack by pests and pathogens and water stress, and involved in plant defenses including altering behavior of insect herbivores, attracting natural enemies of pests, and in plant-to-plant communication to reduce further attacks.

Dieback-affected and grasses in the field produced a significantly different profile of VOCs from healthy grasses at the same location using SIFT analysis. However, VOC profiles were confounded by

both sites and grass species. Consistent markers or patterns that differentiate all affected from unaffected grasses could not be determined in the field and require further detailed laboratory tests that reduce the number of confounding factors.

Results indicate that use of the portable gas collection device is practical in the field, and that VOC analysis may have value in detecting early-stage dieback. However, the induced plant volatiles identified so far are not specific to pasture dieback, and are associated with the appearance of early svisible symptoms of dieback (leaf discoloration). A detailed laboratory analysis of VOCs generated over time in affected plants and that eliminates the confounding effects of site and grass species will be conducted. A revised report will be submitted later in 2021.

Benefits to industry

Results indicate that the use of the portable gas collection device and subsequent laboratory analysis is practical to use in the field. The work also demonstrates that VOC analysis can detect and identify at least one induced plant volatile associated with early-stage dieback. However, further work is required to determine specific patterns of ions in SIFT analysis that are more robust when collecting in different sites and grass species. We outline further steps that may clarify the rapid diagnosis of pasture dieback through the sensing of volatile chemicals.

Future research and recommendations

We suggest that further work include real-time monitoring of volatiles in the very early stages of pasture dieback to eliminate the confounding effects of site and grass species in the field. This detailed laboratory analysis will be conducted and a revised report submitted later in 2021.

Table of contents

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, 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 (Brookes 1978), in New Caledonia in 1998 (Brinon et al 2004), and more recently in Puerto Rico and Barbados.

For many producers and industry advisors the accurate identification of pasture dieback from other pasture conditions remains difficult. In June 2020 the Pasture Dieback Science Forum rated the development of accurate identification tools/technologies for pasture dieback to be a very high priority. Diagnostic tools will assist in the early identification of pasture dieback and allow for interventions preventing widespread pasture dieback.

This project tested the proof of concept of the practical collection and rapid analysis of Volatile Organic Compounds (VOCs) and chemical markers in both laboratory-induced dieback (using mealybugs) and in field samples as a basis for development of a rapid diagnostic test for pasture dieback and to provide an independent method of verification for modelling and prediction of pasture dieback risk and spread.

VOCs were collected using a portable device for gas collection in the field. The analytical techniques include Selected Ion Flow Tube Mass Spectrometry (SIFT-MS) and Head-Space Solid-Phase Micro-Extraction Gas Chromatography Mass spectrometry (HS-SPME-GC-MS).

2. Objectives

In this project we aimed to:

1. Detect and identify chemical markers associated with grass pasture dieback induced by mealybug in the laboratory (months 1-2)
2. Determine the potential for consistent detection of chemical markers associated with grass and early dieback and low-density mealybug in the laboratory (months 2-4)
3. Determine the potential for detection of chemical markers associated with grass and early dieback and low-density mealybug in field samples (months 4-6)

3. Methodology

3.1 Laboratory Trials

3.1.1 HS-SPME-GC-MS

We applied HS-SPME-GC-MS methods to healthy and diseased (mealybug infested grasses showing symptoms typical of ‘dieback’) grasses under laboratory conditions. Plant leaf was snap-frozen and milled in liquid nitrogen and transferred to glass vials for headspace (HS) gas extraction. Multiple SPME fibres with different coatings were used to extract and concentrate target compounds of different polarities, separated by column chromatography, followed by electron ionization of individual VOCs. Analytes were annotated against the NIST mass-spectral database. A list of compounds of interest was generated.

3.1.2 SIFT-MS

We performed a series of controlled experiments in the laboratory to compare the volatile metabolome associated with healthy and mealybug-infested pasture grasses in the lab. Gasses were collected from each plant using a Xitech 1060 portable vacuum sampler and Tedlar gas sampling bags, and the collected gasses were analysed with a Syft Voice200*ultra* SIFT-MS instrument. For this initial experiment we considered the raw ion abundances, but not processed to calculate target analyte abundances. We applied PERMANOVA tests using a Bray-Curtis distance to determine the proportion of VOC content correlated with mealybug infestation status in individual experiments, and with experiments combined.

3.2 Screenhouse Trials

We grew buffel and Rhodes grass plants in the DAF Qld Redlands glasshouse facility. Six plants of each species were treated with a 50 mL drench of 0.44% Confidor Guard soil insecticide in water as controls, preventing infestation by mealybugs. 6 plants were not treated in order to induce the early symptoms of dieback using mealybug infestation.

A week after the control grasses were treated, all the plants were transferred to screenhouses at the QUT Samford Ecological Research Facility (SERF). A visual inspection of plant health was performed to eliminate plant with symptoms of stress, and 2 buffel plants were excluded. Plants that were heavily infested with mealybugs were distributed among the untreated test plants (i.e. not treated with Confidor) in order to induce symptoms of dieback.

Plant health, and sampled gasses for two weeks after infestation. Plants were bagged for approximately two hours, before gasses were collected from each plant using a Xitech 1060 portable vacuum sampler and Tedlar gas sampling bags. In all cases the collected gasses were analysed with a Syft Voice200*ultra* SIFT-MS instrument within 48 hours of sampling.

We plotted the proportion of green (i.e. asymptomatic) foliage observed in each plant and fitted a generalised linear model with a binomial error distribution with 95% confidence intervals.

Concentrations for metabolites of interest were obtained from the SIFT-MS dataset, and this data was normalised with a centred-log ratio transformation. This normalised data was used to plot an RDA ordination. The unnormalized data was used to calculate Bray-Curtis dissimilarities for PERMAOVA testing for the influence of dieback status on metabolite profile. The effect of grass species was considered as a confounding variable.

Using a canonical correspondence analysis, we identified a possible compound of particular interest belonging to the category of ‘green leaf volatiles’. We reanalysed the SIFT-MS data to quantify a selection of other green leaf volatiles and repeated the ordination and PERMANOVA tests as above.

3.3 Field Sampling

We conducted field sampling at 7 sites: Brendale, SERF, Theodore, Maudsland Creek, Maudsland Paddock, Kin Kin, and Sutton Park. Gases were collected from healthy and sick plants as in previous trials: plants were bagged for approximately two hours and gasses were collected using a Xitech 1060 portable vacuum sampler and Tedlar gas sampling bags. Bags were returned to the laboratory and plant volatiles, ions and metabolites of interest were quantified using SIFT-MS.

Hierarchical clustering was applied to determine the grouping of samples by centered-log-ratio transformed ion abundance data. PERMANOVA tests using Bray-Curtis distance were used to determine whether field-sampled plants differed in volatile profile depending on pasture dieback status.

A t-test was used to determine whether the putative pasture dieback marker [(E)-2-hexen-1-ol], previously highlighted in our screenhouse trials, was effective in distinguishing between healthy and diseased plants, or in distinguishing between plants where mealybugs were or were not observed.

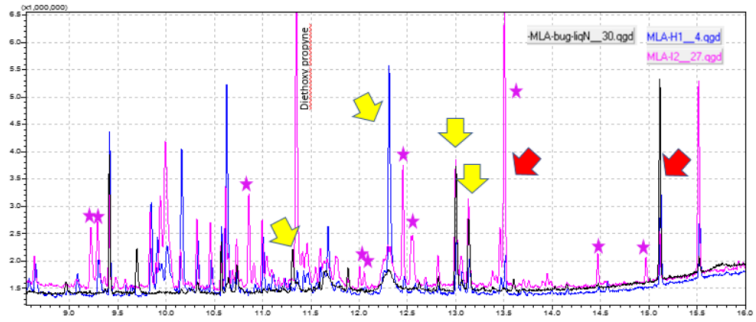
4. Results

4.1 Laboratory Trials

4.1.1 HS-SPME-GC-MS

Preliminary GC-MS analysis of VOCs associated with infested and healthy grasses were successfully conducted. These analyses generated a list of potential markers for pasture dieback indicated by yellow and red arrows (Fig 1.)

Figure 1: Representative chromatograms show VOC 'fingerprints' associated with mealybugs (black), healthy plants (blue), and mealybug-infested plants (pink). A list of putative compound annotations is given.

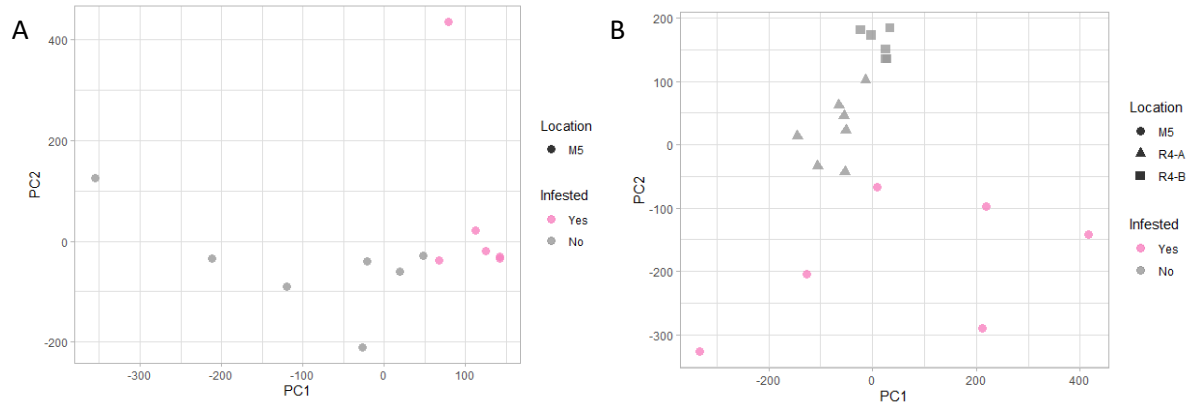


ID#	Name	Ret.Time
1	2-[(Trimethylsilyl)oxy]-2-[4-[(trimethylsilyl)oxy]phenyl]ethan	7.877
2	Acetoin	8.963
3	Cyclohexasiloxane, dodecamethyl-	9.413
4	2-Pentanone, 4-hydroxy-4-methyl-	9.698
5	RT:10.237	10.237
6	Acetic acid	10.328
7	Cycloheptasiloxane, tetradecamethyl-	10.572
8	RT:10.647	10.647
9	RT:10.733	10.733
10	RT:11.137	11.137
11	cis-.alpha.-Bergamotene	11.310
12	Bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pent	11.363
13	Silanediol, dimethyl-	11.652
14	RT:11.885	11.885
15	RT:12.267	12.267
16	Androst-4,6-diene-17β-ol-3-one	12.302
17	Hexanoic acid	12.820
18	Propanoic acid, 2-methyl-, hexyl ester	13.002
19	2,3-Hexanedione	13.060
20	Pentanoic acid, 2,2,4-trimethyl-3-hydroxy-, isobutyl ester	13.133
21	2-Ethyl-hexoic acid	13.380
22	ETHYL (S)-(-)-LACTATE	13.597
23	2,4-Di-tert-butylphenol	15.113
24	Diethyl Phthalate	15.518
25	RT:16.610	16.610

4.1.2 SIFT-MS

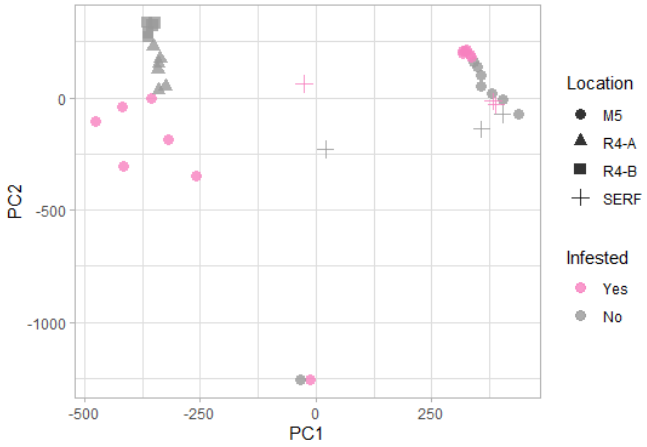
A significant difference in metabolite profile between healthy and infested/symptomatic grasses was identified in two separate laboratory trials. Figure 2 shows the results of two controlled experiments in which the VOCs associated with healthy and diseased plants were compared.

Figure 2: Ordination results using VOC fingerprints from healthy and pasture dieback grasses grown on vermiculite under laboratory condition. 58% of variation in VOC composition is explained by infestation status in Trial A (p = 0.002), while 33% is explained in Trial B (p = 0.001).



Tests for separation between VOC profiles in infested/symptomatic and healthy plants in the different environments (controlled temperature rooms and cabinets and grasses grown in screenhouses) were conducted. Figure 3 shows an ordination of both laboratory test depicted in Figure 2, but this time on the same axes. Differences between healthy and infested/symptomatic grasses are still evident, but (as expected) growth conditions (cabinets, rooms) are a major confounding factor.

Figure 3: Infestation status when the results of both controlled laboratory trials (conducted under different conditions) are combined is not significant.



SIFT-MS technology quantifies ions after soft chemical fragmentation of analytes. A combination of ions can be used to quantify target analytes, but our exploratory analyses showed that raw ion abundances are better-able to separate healthy and pasture dieback grass samples. This suggests that there is relevant variation in our dataset that is not captured by HS-SPME-GC-MS. This may be due to the fact that HS-SPME analyses are limited by the properties of the SPME fibre, the limited sample size used in the exploratory analysis, or the higher sensitivity of the FIST-MS method. It suggests that SIFT-MS has potential to identify different markers for pasture dieback from HS-SPME-GC-MS.

4.2 Screenhouse Trials

Analytes detected by HS-SPME-GC-MS were successfully quantified using SIFT-MS. Figure 4 shows the development of visible symptooms (leaf discoloration) over time from infestation. Buffel and Rhodes grass plants without treatment with imidacloprid and infested with mealybugs developed greater visible symptoms of dieback than imidacloprid-treated control plants after only one week.

Figure 4: Plant health over time as measured by proportion of green foliage. Shown with 95% confidence intervals from a GLM with binomial errors.

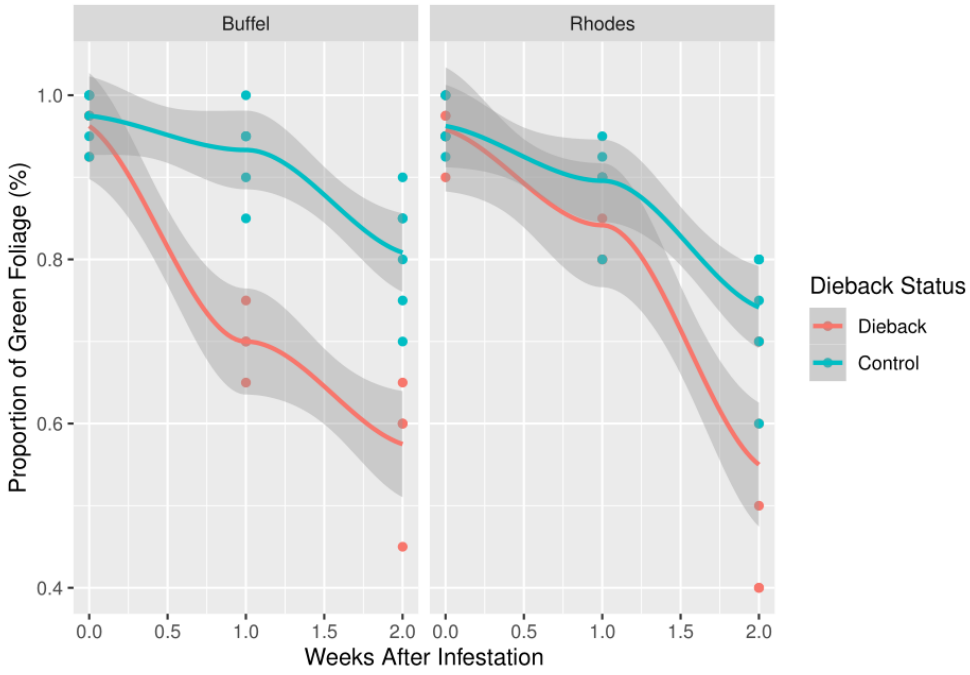
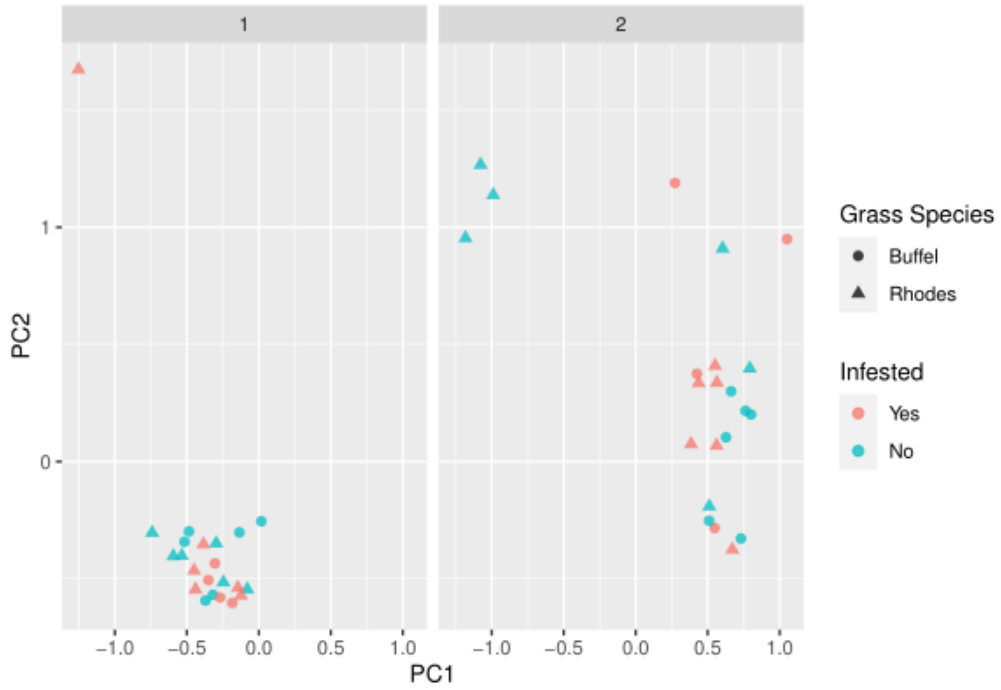


Figure 5 shows an RDA ordination using centred-log ratio transformed data from a targeted metabolite analysis. According to PERMANOVA results, disease status explains 24.2% of volatile metabolome variation between healthy and diseased plants one week after infestation ($p = 0.002$) and 15.2% two weeks after infestation ($p = 0.009$).

Figure 5: RDA ordination of SIFT-MS samples from plants sampled one and two weeks after infestation with mealybugs.



After performing a canonical correspondence analysis (not pictured) we identified (E)-2-hexen-1-ol as a possible marker for pasture dieback.

(E)-2-hexen-1-ol is a notable green leaf volatile. These short chain (C6) molecules are induced in plants in response to tissue damage (such as by insect herbivores), plant pathogens and biotic stress.

We reanalysed the data from this trial to quantify other green leaf volatiles. PERMANOVA tests showed a difference in a range of green leaf volatile composition between healthy and diseased plants (18.7%, p = 0.004), but this dataset did not provide any single confirmed markers.

There was a significant difference in the volatile metabolome between healthy and pasture dieback samples at one week after infestation. However, at this point, symptoms (leaf discoloration) were already visible in infested plants without Imidacloprid

Field Sampling

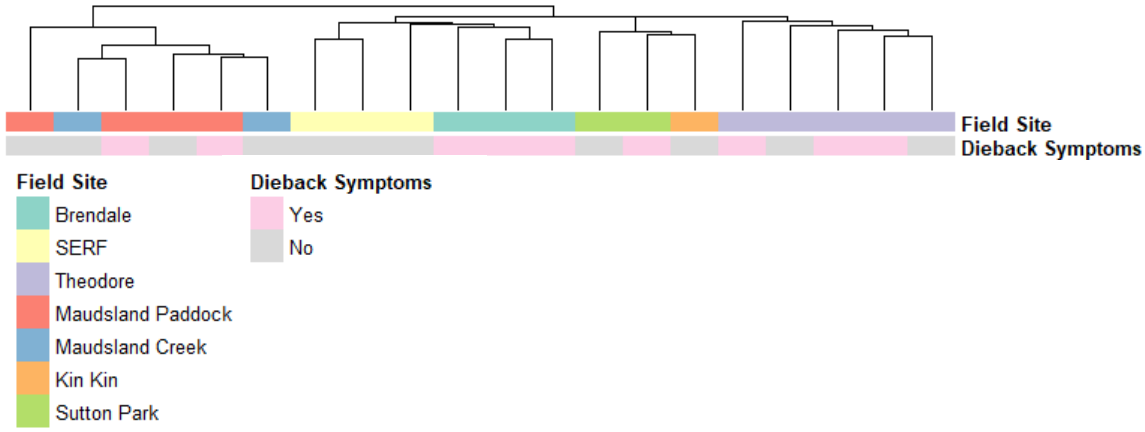
Bagging plants in the field for approximately two hours and sampling with the portable vacuum device was shown to generate sufficient concentration of plant volatiles to generate a SIFT-MS signal comparable to those generated with 12- and 24-hour bagging in laboratory experiments.

Sites with the same grass located near each other were likely to share the same pasture dieback status; we were unable to identify paired sites (dieback-affected and unaffected) that were sufficiently close to each other to have similar environmental conditions and grass species. Thus we were forced to analyse asymptomatic and affected plants from the same site, and from different sites with different grass species, possibly confounding results.

As with the laboratory experiments, we performed a PERMANOVA test. Results demonstrated that dieback status (healthy/affected) resulted in a significant difference (p = 0.001), and explained 14.7% of metabolite variability. Grass species and sampling site had significant effects (53.3%, p = 0.001,

and 21.9%, p = 0.001 respectively). This is illustrated in Figure 6, where hierarchical clustering based on metabolite profile clearly clusters samples by field site but not dieback status.

Figure 6: Field samples clustered by CLR-transformed ion fragment profiles.



T-tests revealed that detection of (E)-2-hexen-1-ol was not significantly different for healthy and pasture dieback grasses under field conditions (t = -0.61, p = 0.55), nor was it significantly different between grasses where mealybugs were present and absent (t = -1.27, p = 0.22) again under field conditions. This may be due to contamination between affected and healthy grasses sampled at the same site, and where apparently visually healthy grass might already be affected.

This confounding of healthy and affected grass within sites requires further controlled testing and analysis. We propose further test be conducted (below).

5. Conclusion

We demonstrated the utility of portable gas sampling and subsequent laboratory SIFT-MS analysis could differentiate sick from healthy grasses in any given pasture. Portable sampling with a vacuum unit for SIFT-MS was shown to be a highly effective and simple method of sampling volatiles in the field. We consistently identified statistically significant differences in volatile metabolome composition between healthy and diseased grasses, achieving Objective 1.

Our tests identified differences in the green leaf volatiles produced by grasses in the early stages of pasture dieback. In controlled laboratory experiments, (E)-2-hexen-1-ol was significantly associated with dieback, achieving Objective 2. Other green leaf volatiles, belonging to the same class of compounds as (E)-2-hexen-1-ol (Scala et al., 2013) were detected but not identified and were not significantly associated with dieback.

Dieback-affected and grasses in the field produced a significantly different profile of VOCs from healthy grasses at the same location using SIFT analysis. However, patterns differed between sites and grass species and consistent markers or patterns that differentiate all affected from unaffected grasses could not be determined in the field. Similarly, (E)-2-hexen-1-ol was not an effective marker of dieback in the field data where apparently asymptomatic plants may infact be affected by dieback.

There was a confounding effect of sampling apparently healthy and dieback-affected grass in the same field: healthy grass identified by lack of visual symptoms might in fact be already affected by dieback, or there may be some contamination of gasses from plants sampled in close proximity. Thus there was only partial success in completing Objective 3.

There is potential to improve the robustness of this analysis by collecting additional data in controlled laboratory environments to clarify consistent markers, and to confirm the consistency and identity of single markers such as (E)-2-hexen-1-ol. Real-time monitoring of volatiles in the very early stages of pasture dieback will eliminate the confounding effects of site and grass species in the field. This detailed laboratory analysis will be conducted and a revised report submitted later in 2021.

In summary, the results indicate that the the use of the portable gas collection device and subsequent laboratory analysis is practical to use in the field. The work also demonstrates that VOC analysis can detect and identify at least one induced plant volatile associated with early-stage dieback. However, further work is required to determine specific patterns of ions in SIFT analysis that are more robust when collecting in different sites and grass species. However, the green leaf volatiles identified so far are typically produced in response to a number of different biotic and abiotic stresses not specific to pasture dieback, and are produced in association with the appearance of early visible symptoms of dieback (leaf discoloration).

Further controlled, real-time analysis will be conducted and reported to clarify the diagnosis of pasture dieback through the sensing of volatile chemicals.

5.1 Key findings

- SIFT-MS technology paired with portable vacuum samplers are effective for surveying and quantifying metabolites associated with pasture diseases.
- The volatile metabolome differed significantly between healthy and diseased pasture grasses in both laboratory and field.
- In controlled laboratory experiments, (E)-2-hexen-1-ol is significantly associated with dieback.
- Other influences on the volatile metabolome include grass species and grass location across different field sites.
- We were able to induce symptoms of dieback in the glasshouse by infestation with mealybugs.
- The use of a chemical insecticide reduced both physical symptoms of dieback and resulted in significantly different VOC profiles.

5.2 Benefits to industry

We demonstrated the efficacy of SIFT-MS technologies, including field sampling methods, to detect patterns of ions associated with dieback-affected grasses in contrast to apparently healthy grasses in the same field. This suggests there is potential for further development of a rapid diagnostic test for pasture dieback that can be applied in the field and provide an independent method of verification for modelling and prediction of pasture dieback risk and spread. However, visual detection of symptoms and mealybugs are still the most clear and significant indicator of dieback.

6. Future research and recommendations

Small differences between the healthy and pasture dieback volatile metabolome are significant. With further sampling effort, it may be possible to generate a dataset sufficient to train a machine learning algorithm to classify samples. We will collect data from at least 3 additional field sites.

Finally, live, continuous monitoring via SIFT-MS could be used to track markers in real-time over the first hours or days of infestation, when changes might be more pronounced and patterns of ions can be more easily detected.

In summary, the results indicate that the the use of the portable gas collection device and subsequent laboratory analysis is practical to use in the field. The work also demonstrates that VOC analysis can detect and identify at least one induced plant volatile associated with early-stage dieback. However, further work is required to determine specific patterns of ions in SIFT analysis that are more robust when collecting in different sites and grass species. Further controlled, real-time analysis will be conducted and reported to clarify the diagnosis of pasture dieback through the sensing of volatile chemicals.

7. References

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