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Using metabolites as biomarkers for the identification of innate resistance to myrtle rust across the Myrtaceae

Final Report (PBSF037)

September 2022

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This document should be cited as:

Moffitt, Plett, Carnegie & Makinson, 2022, APBSF Project Final Report, Using metabolites as biomarkers for the identification of innate resistance to myrtle rust across the Myrtaceae

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1. Executive Summary

The objectives of this project are to employ metabolomics across a diverse selection of Myrtaceae species to establish a set of biomarkers (i.e. fingerprints) that rapidly identify resistant plants without infection trials or previous genomic knowledge. In this final report, we present the collection of species belonging to eight tribes of the Myrtoideae subfamily of Myrtaceae, including species listed as priority species in "Myrtle Rust in Australia – a National Action Plan" (Makinson et al., 2020). Where possible, twenty replicates of each species were inoculated with *Austropuccinia psidii* and their susceptibility to the infection was recorded. Species in our collection that displayed variation in susceptibility phenotype were analysed using metabolomics. Here we present that metabolomics can differentiate individuals within a species based on susceptibility phenotype. Based on our findings, **species-specific biomarkers can be developed for predicting resistant individual plants**. We have also trialled protocols to optimise the collection of leaf material from off-site locations which could be used for work with industry and breeders. We report that metabolic biomarkers separating resistant from susceptible genotypes can be identified in samples taken in a variety of scenarios to mimic possible methods of field collection. This confirms that **where industry or breeders are interested in employing this approach to identify resistant plants, leaf samples can be taken from either greenhouse or forest growing environments using a standardised protocol tailored to their needs**. Our project has been successful in the use of communication techniques to raise concern about the myrtle rust pandemic in Australia and have involved early career researchers, undergraduate and postgraduate students at Western Sydney University.

2. Introduction

Austropuccinia psidii is a fungal plant pathogen that exclusively infects plant species within the Myrtaceae, causing the disease myrtle rust (Carnegie and Pegg, 2018). Myrtle rust has had significant effects on natural and managed ecosystems, where it is causing declines in local populations and is expected to cause extinctions of several at-risk species within a generation (Fensham et al., 2020; Fensham and Radford-Smith, 2021). Additionally, myrtle rust continues to cause declines in the productivity of industrial plantations. **An encouraging discovery in our past APBSF grant (PBSF023) was that one myrtaceaeous species, *Melaleuca quinquenervia,* exhibited a range of disease levels from complete susceptibility to resistant and that these differences are linked to plant metabolism.** These metabolic differences have the potential to be exploited in the conservation space to identify resistant genotypes that could be used to prevent species loss in future, and be used in industry to boost plantation productivity.

The plant family Myrtaceae comprises seventeen tribes, 15 of them in the subfamily Myrtoideae (Wilson, 2011; Wilson et al., 2005). In Australasia, Myrtaceae predominate in most ecosystems, with more than 2,250 species recorded (Makinson *et al*., 2020). To date, worldwide 539 Myrtaceae species are known to be vulnerable to *A. psidii* infection (Fernandez Winzer et al., 2020; Soewarto J, Giblin F, Carnegie A. J, 2019).

Metabolomics is the profiling of molecules within a biological sample. This can be divided into targeted, focusing on the quantification of a subset of biomarkers, or untargeted, which assesses the full complement of molecules and their relative abundance (Hall et al., 2022). Untargeted metabolomics has shown promise in identifying resistance biomarkers present in a broad range of plant diseases, including those found early in *Phytophthora* infection of tomatoes (Garcia *et al.*, 2018), *Phakopsora pachyrhizi* infection of soybean (Silva *et al.*, 2020), and *Plasmopara viticola* defence response in a resistant grape variety (Chitarrini *et al.*, 2017). This technique profiles all small molecules that are present within a biological sample at a given time. Examples include substrates, intermediates and products of metabolic pathways, as well as signalling molecules, hormones, and secondary metabolites. The benefit of metabolomics over other omics-based strategies of identifying disease resistance markers is that no genomes are required and the method can be applied in non-model systems.

Our preliminary work in the APBSF funded project PBSF023 established that prior to infection, resistant germplasm from the species *Melaleuca quinquenervia* contain metabolic signatures that differentiate them from hypersensitive and susceptible phenotypes (Moffitt *et al.*, 2022). In particular, prior to infection, the resistant *M. quinquenervia* leaves contained increased concentrations of secondary metabolites, including flavonoids. Similar molecules are reported to aid in defence against pathogens in other plants by inhibiting germination of fungal spores or fungal growth (Gillmeister et al., 2019; Padmavati et al., 1997). **The results of our previous study had several constraints that limited the scope of their application to the broader forestry and conservation community** (Moffitt *et al.*, 2022).Firstly, metabolomic data was limited to a single species, and we were unsure as to how broadly applicable these results might be to other myrtaceaeous species. Secondly, leaf material used in the first project was taken from plants grown under controlled conditions,

conditions that did not reflect the wide variation a tree would experience under natural or plantation conditions. Finally, following collection, leaf material was stored immediately at -80°C to preserve metabolites in stasis, a workflow that would be infeasible in a plantation or natural environment. In this current study, we set out to overcome these drawbacks by further expanding on our previous work to understand the broader use of untargeted metabolomics as a tool that could be used in collaboration with conservation agencies, breeders and industry to protect our natural species diversity in the face of myrtle rust pressure.

3. Aim

The aims of project PBSF037 were to employ metabolomics across a diverse selection of species identified in the Myrtle Rust Action Plan to establish a set of biomarkers that rapidly identify resistant plants without infection trials or previous genomic knowledge. More specifically, the outputs proposed for this proposal are:

1. A curated set of biomarkers to assess individual plants within selected Myrtaceae species for levels of resistance
2. Optimised protocols for field collections to enable rapid metabolomic-based field surveys of *A. psidii* resistance
3. Improved understanding of the biochemistry responsible for resistance to *A. psidii*

4. Methods/Process

4.1. Plant growth and collection

Seedlings were obtained from commercial sources: Brush Turkey Enterprises, QLD, and Muru Mittigar nursery, NSW (Table 1). Plants were grown at the Western Sydney University (WSU) in controlled growth chambers with 16 h light/8 h dark cycle at 25˚C, 70% relative humidity and 500 µmol m^{-2} s^{-1} light intensity. We also collected leaf samples of *Rhodamnia rubescens* from the Royal Botanical Gardens Mount Annan nursery (Table 1) with assistance from Veronica Villier.

Table 1: List of species harvested for myrtle rust susceptibility testing and metabolomics.

Species	**Tribe**	**Source**	**Resistant individuals**	**Susceptible individuals**
Syzygium (Acmena) smithii	Syzygieae	Seedlings from seeds via Brush Turkey enterprises, QLD	20/20	0/20
Syzygium (Acmena) ingens	Syzygieae	Seedlings from seeds via Brush Turkey enterprises, QLD	19/20	1/20
Syzygium francisii	Syzygieae	Seedlings from seeds via Brush Turkey enterprises, QLD	16/20	4/20
Syzygium oleosum	Syzygieae	Seedlings from seeds via Brush Turkey enterprises, QLD	20/20	0/20
Decaspermum humile[1,2]	Myrteae	Seedlings from seeds via Brush Turkey enterprises, QLD	12/20	8/20
Decaspermum struckoilicum	Myrteae	Seedlings from seeds via Brush Turkey enterprises, QLD	20/20	0/20
Rhodamnia rubescens[1,3]	Myrteae	Royal Botanical gardens nursery cutting lineages stock[5]	3/7	4/7

Tristaniopsis laurina	Kanieae	Seedlings from seeds via Brush Turkey enterprises, QLD	17/20	3/20
Backhousia myrtifolia	Backhousieae	Seedlings from seeds via Muru Mittigar nursery, NSW	12/20	8/20
Syncarpia glomulifera	Syncarpieae	Seedlings from seeds via Muru Mittigar nursery, NSW	15/20	5/20
Lophostemon confertus	Lophostemoneae	Seedlings from seeds via Muru Mittigar nursery, NSW	20/20	0/20
Eucalyptus grandis	Eucalypteae	Seedlings from WSU APBSF 2020 project	6/11	5/11
Leptospermum scoparium[4]	Leptospermeae	via Peri Tobias and Alyssa Martino (USyd)	12/20	8/20
Melaleuca quinquenervia[4]	Melaleuceae	via Peri Tobias and Alyssa Martino (USyd)	12/20	8/20

[1]Listed as priority species (Makinson et al., 2020)
[2]Likely to belong to the southern metapopulation which is the priority
[3]Phenotyping was performed by Jason Bragg, Royal Botanic Sydney
[4]Phenotyping was performed by Peri Tobias and Alyssa Martino (USyd)
[5]Samples of *R. rubescens* were collected from cutting lineages which were confirmed to be genetically unique

4.2. **Austropuccinia psidii** *inoculation of plant material and phenotype assessment*

Cuttings consisting of stems bearing 4-6 immature leaves were taken from each of the following plant of species: *Syzygium (Acmena) smithii, Syzygium (Acmena) ingens, Syzygium francisii, Syzygium oleosum, Decaspermum humile, Decaspermum struckoilicum, Tristaniopsis laurina, Backhousia myrtifolia, Syncarpia glomulifera, Lophostemon confertus, Eucalyptus grandis*. Cuttings were placed in water agar (0.6 %) within 50 ml tubes and left overnight prior to infection with *A. psidii* spores was performed at the University of Sydney Plant Breeding Institute using the following protocol: Spores were collected from heavily infected *Syzygium jambos* plants by submerging the infected leaves in isopar oil. The inoculum was sprayed onto the leaf cuttings while maintained within the tubes. The *A. psidii* spores were allowed to settle for one minute and the inoculated leaves were then left in the dark for 12 hours at 18 ˚C inside a controlled growth chamber to ensure high relative humidity to assist with spore germination and infection. Infected cuttings were then maintained at WSU in a normal light cycle at 25˚C. 14 days following inoculation leaves were examined for *A. psidii* disease symptoms and scored as either resistant or susceptible.

Phenotyping of *M. quinquenervia* and *L. scoparium* plants was kindly performed by Ms. Alyssa Martino and Dr. Peri Tobias (USyd). *R. rubescens* phenotypes were provided by Dr Jason Bragg (RBG). Here, individual potted plants were inoculated directly using the University of Sydney Plant Breeding Institute protocol described above.

4.3 Comparison of field collection storage protocols and times at room temperature

Storage conditions of leaf material was compared to eliminate the requirement of freezing for the transport of samples prior to processing. Here, we sampled *Eucalyptus grandis* plants with known *A. psidii* infection phenotypes (resistant and susceptible, phenotyping performed in PBSF023) grown in the field at the WSU Hawkesbury campus. Leaf punches of immature leaves (avoiding veins) at the second position from the apical bud were collected and stored at room temperature for 1 hour, 3 hours and overnight, with or without extraction solvent in preparation for metabolite extraction.

4.4. Leaf metabolite extraction

Leaf samples were weighed and immediately ground in 200 µl extraction solvent (4:4:2 methanol:acetonitrile:deionised water) by bead-beating twice for 30 seconds each at 5.5 Hz (MP Bio FastPrep-24^{TM}). The leaf:solvent mixture was then combined with 300 µl additional extraction solvent, vortexed to mix and tubes placed in ice water and sonicated in a sonicating water bath for 25 min. Solid cellular material was removed by centrifugation at 14,000 xg for 10 min. The supernatants were diluted 3:1 in HPLC grade water and centrifuged to remove additional particulates.

4.3 Ultra Performance Liquid Chromatography High Definition Mass Spectrometry with Ion Mobility (UPLC HDMS^{E}) analysis:

Leaf extracts were analysed on a Waters Acquity I-Class Ultra Performance Liquid Chromatography (UPLC) system and a Waters Synapt G2-Si High Definition Mass Spectrometry (HDMS) with a Waters UniSpray Ionisation source. The metabolites were separated on a Waters ACQUITY UPLC HSS T3 1.8µm 100 x 2.1mm Column at 35° C. The injection volume was 2 µL. The mobile phases were A (Water + 0.1% Formic Acid) and B (Acetonitrile + 0.1% Formic Acid). The chromatographic flow rate was 0.5 mL/min with a 9 min gradient, with mobile phase A held at 99% for 1 min, decreased to 85% over 1 min, decreased to 50% over 2 mins, decreased to 5% over 2 min and increased to 99% over 2 mins. Leucine Enkephalin Lockspray solution (Waters, 1ng/mL) was used as a standard.

Data acquisition was performed with ion mobility separation followed by mass fragmentation and high resolution mass analysis. The mass range of metabolites acquired was 50–1200 m/z, the scan time was 0.2 seconds and the elevated energy transfer collision voltage was 20–50 eV. For this experiment, the instrument was run in positive ionisation mode with the following settings: Capillary: 0.5 kV, source temperature: 120 °C, sampling cone: 30 V, source offset: 80 V, desolvation temperature: 500 °C, desolvation gas flow: 800 L/Hr, cone gas flow: 20 L/Hr.

4.4 Analysis of results

Data from the mass spectrometer was exported to Progenesis QI for metabolomics for normalisation and preliminary analysis. Molecular features with low abundance or present in the blanks were removed. Putative identifications of molecular features was performed using the CHEBI and HDMB databases. Progenesis data was exported to the online tool MetaboAnalyst 5.0 for statistical analysis (https://www.metaboanalyst.ca/). Data was filtered using Inter Quartile Range and normalised by sample weight and auto-scaling. Partial Least Squares – Discriminant Analysis was used to separate metabolomic profiles based on phenotype. Variable Importance in Projection loadings were used to identify molecular features that are significantly different in the phenotypes. Biomarker analysis was performed using the MetaboAnalyst 5.0 suite of tools (https://www.metaboanalyst.ca/) to identify molecular features that can be used to predict the phenotype of unknown samples. Venn diagrams were created using the following webtool: http://bioinformatics.psb.ugent.be/webtools/Venn/.

5. Achievements, Impacts and Outcomes

In accordance with the APBSF services agreement, we have completed our milestone targets set out below.

5.1 - Curated set of biomarkers to assess individual plants within selected Myrtaceae species for levels of resistance/tolerance

5.1.1 Plant material infection and harvesting

We selected a diverse collection of Myrtaceae species phylogenetically belonging to 9 tribes (Appendix Table 1). Species were obtained as seedlings from nurseries, where the seedlings were grown from seed to represent variation in genotype, although it is not known if these were from a single or multiple maternal plant. In addition, cuttings of *Rhodamnia rubescens* were sampled from the Royal Botanical Gardens, Mount Annan nursery collections. The *R. rubescens* individuals tested were from plants sourced from cuttings, known to be genetically different (V. Villiers pers. comm.) *A. psidii* inoculations were performed and the rust-response phenotype of individuals was assessed (Appendix Table 1). Leaves were simultaneously collected before inoculation for metabolomics extraction and analysis, with the exception of *R. rubescens, Leptospermum scoparium* and *Melaleuca quinquenervia,* which were phenotyped separately by Jason Bragg or Peri Tobias and Alyssa Martino. Where a diversity of phenotypes was not represented amongst the seedlings, metabolomic analysis

was not performed. This included *Syzygium* (*Acmena*) *smithii*, *Syzygium* (*Acmena*) *ingens, Syzygium oleosum, Decaspermum struckoilicum* and *Lophostemon confertus.* As a result, metabolomic analysis was only able to be performed on members of 8 tribes.

5.1.2 Metabolomics differentiates susceptible and resistant plants in a species-specific manner

LC-MS has the ability to run both positive and negative mode. This allows the capture of metabolite with different chemistries based on their polarity. Here we expanded on our previous study and analysed both positive and negative mode data for *M. quinquenervia*. In order to determine if untargeted metabolomics is adaptable to the differentiation of susceptible and resistant plants of multiple species across the Myrtaceae, we performed statistical comparisons of the metabolomic profile. Our previous work identified that the multivariate analysis tool, Partial Least Squares – Discriminant Analysis (PLS-DA), was optimal for visualising the separation of susceptible and resistant metabolomes. PLS-DA analysis was performed on metabolomics samples with more than three individuals of differing susceptibilities. For simplicity, individuals were grouped as either resistant (zero symptoms or hypersensitive response) or susceptible (development of uredinial rust pustules). Variable Importance in Projection analysis identifies the most important features that differentiate the two phenotypes and the list of top 15 molecular features for each species was also analysed.

We compared the metabolomic profile of resistant and susceptible individuals from the species *Eucalyptus grandis* (Eucalypteae), *Syzygium francisii* (Syzygieae), *Decaspermum humile* (Myrteae), *Rhodamnia rubescens* (Myrteae) *Backhousia myrtifolia* (Backhousieae), *Tristaniopsis laurina* (Kanieae), *Syncarpia glomulifera* (Syncarpieae), *Melaleuca quinquenervia* (Melaleuceae) and *Leptospermum scoparium* (Leptospermeae). We analysed *Melaleuca quinquenervia* (Melaleuceae) plants that were different lineages to our previous study (APBSF PBSF023 and Moffitt *et al.*, 2022). In all cases**, PLS-DA differentiated the metabolomic phenotypes, indicating that the methodology that has been applied in our first APBSF project can now be used across a wide range of Myrtaceae species** (Figure 1). We were able to confirm that the susceptible and resistant phenotypes were distinguished using PLS-DA in both positive and negative mode data (Figure 2). Variable Importance in Projection analysis identified that most of the molecular features that were important in differentiating the phenotypes were higher in the susceptible individuals (Figure 3 and Figure 4).

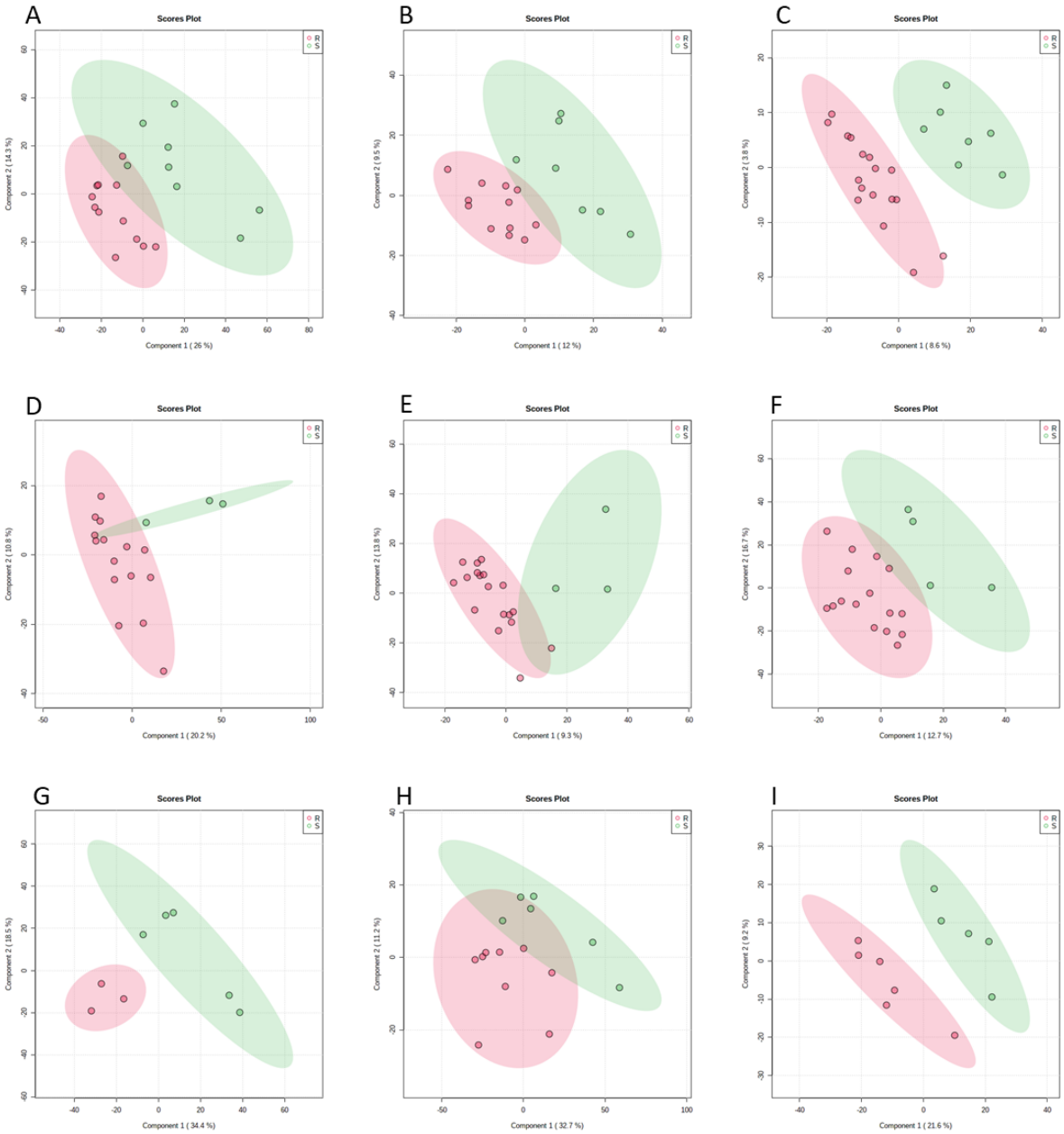

Figure 1: Partial Least Squares – Discriminant Analysis (PLS-DA) analysis of resistant (red) and susceptible phenotypes (green) in diverse Myrtaceae species. Analysis was performed using UPLC-HDMS positive mode features. A. *Melaleuca quinquenervia* B. *Backhousia myrtifolia* C. *Leptospermum scoparium* D. *Syncarpia glomulifera* E. *Syzygium francisii* F. *Tristaniopsis laurina* G. *Rhodamnia rubescens* H. *Decaspermum humile* I. *Eucalyptus grandis.*

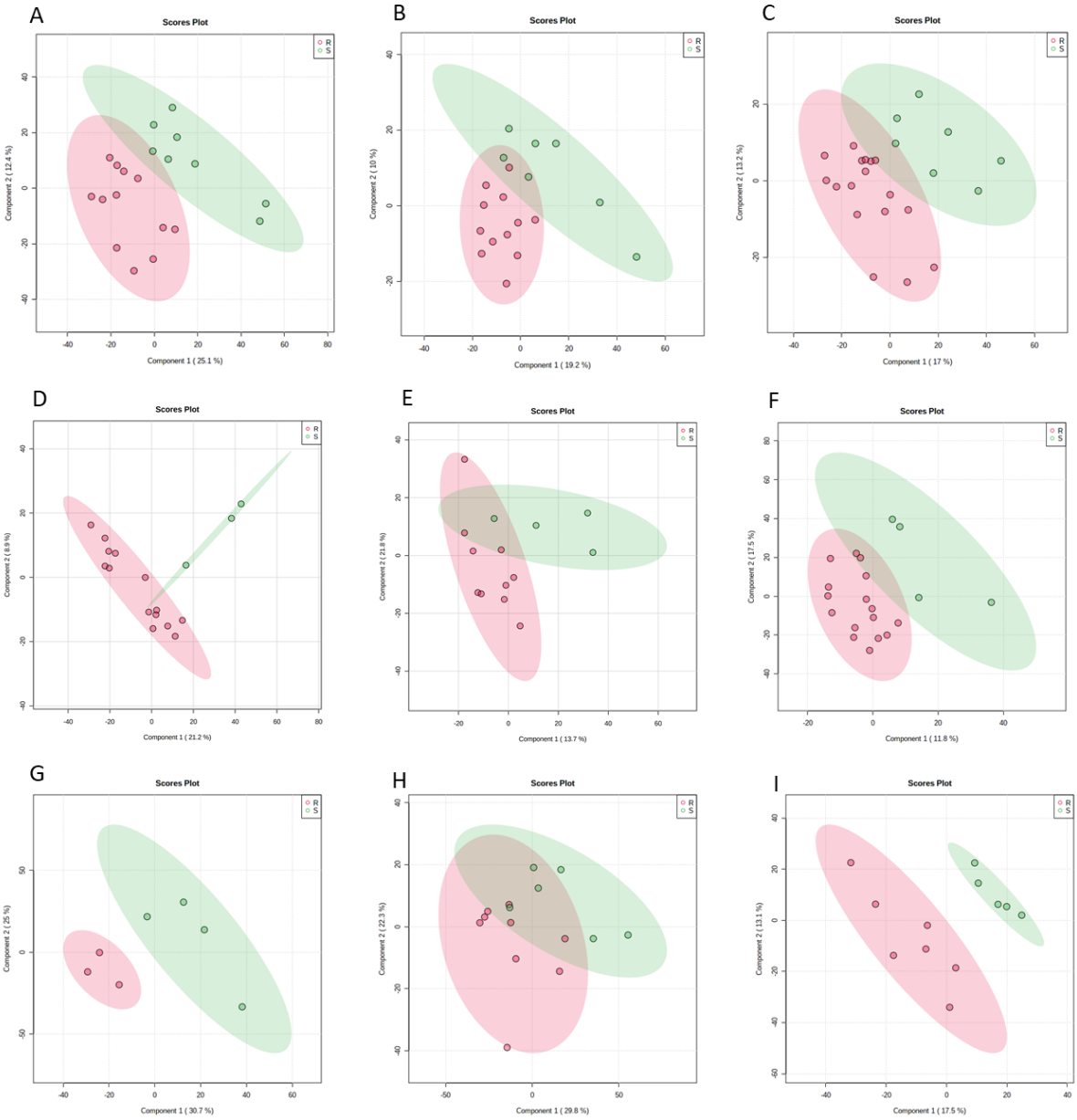

Figure 2: Partial Least Squares – Discriminant Analysis (PLS-DA) analysis of resistant (red) and susceptible phenotypes (green) in diverse Myrtaceae species. Analysis was performed using UPLC-HDMS negative mode features. A. *Melaleuca quinquenervia* B. *Backhousia myrtifolia* C. *Leptospermum scoparium* D. *Syncarpia glomulifera* E. *Syzygium francisii* F. *Tristaniopsis laurina* G. *Rhodamnia rubescens* H. *Decaspermum humile* I. *Eucalyptus grandis.*

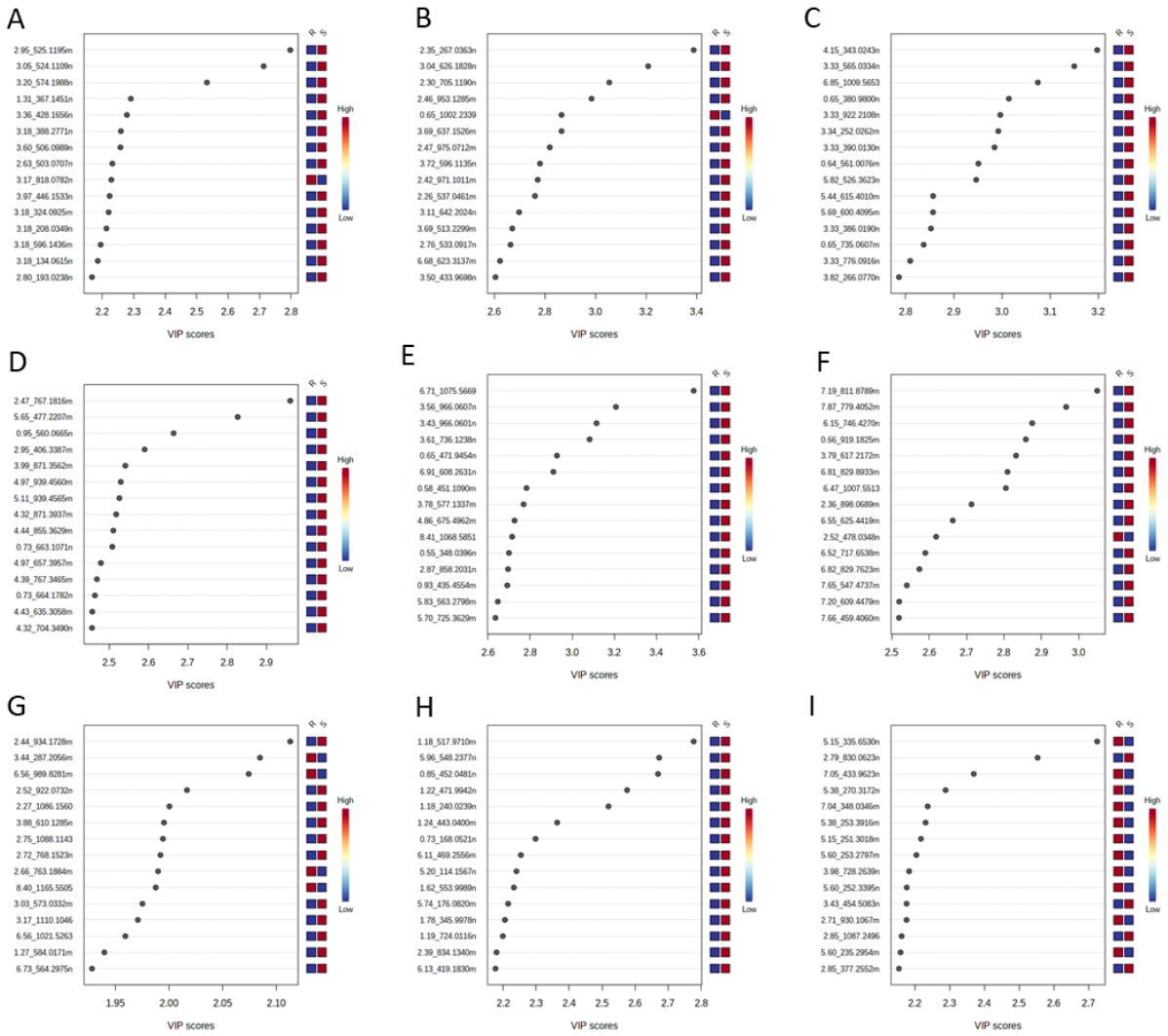

Figure 3: Variable Importance in Projection (VIP) 15 most important features that differentiate the two phenotypes, resistant (R) and susceptible (S). Metabolites that are at higher levels in the phenotype are indicated in red, while those that are lower are indicated in blue. Analysis was performed using UPLC-HDMS positive mode features. A. *Melaleuca quinquenervia* B. *Backhousia myrtifolia* C. *Leptospermum scoparium* D. *Syncarpia glomulifera* E. *Syzygium francisii* F. *Tristaniopsis laurina* G. *Rhodamnia rubescens* H. *Decaspermum humile* I. *Eucalyptus grandis.*

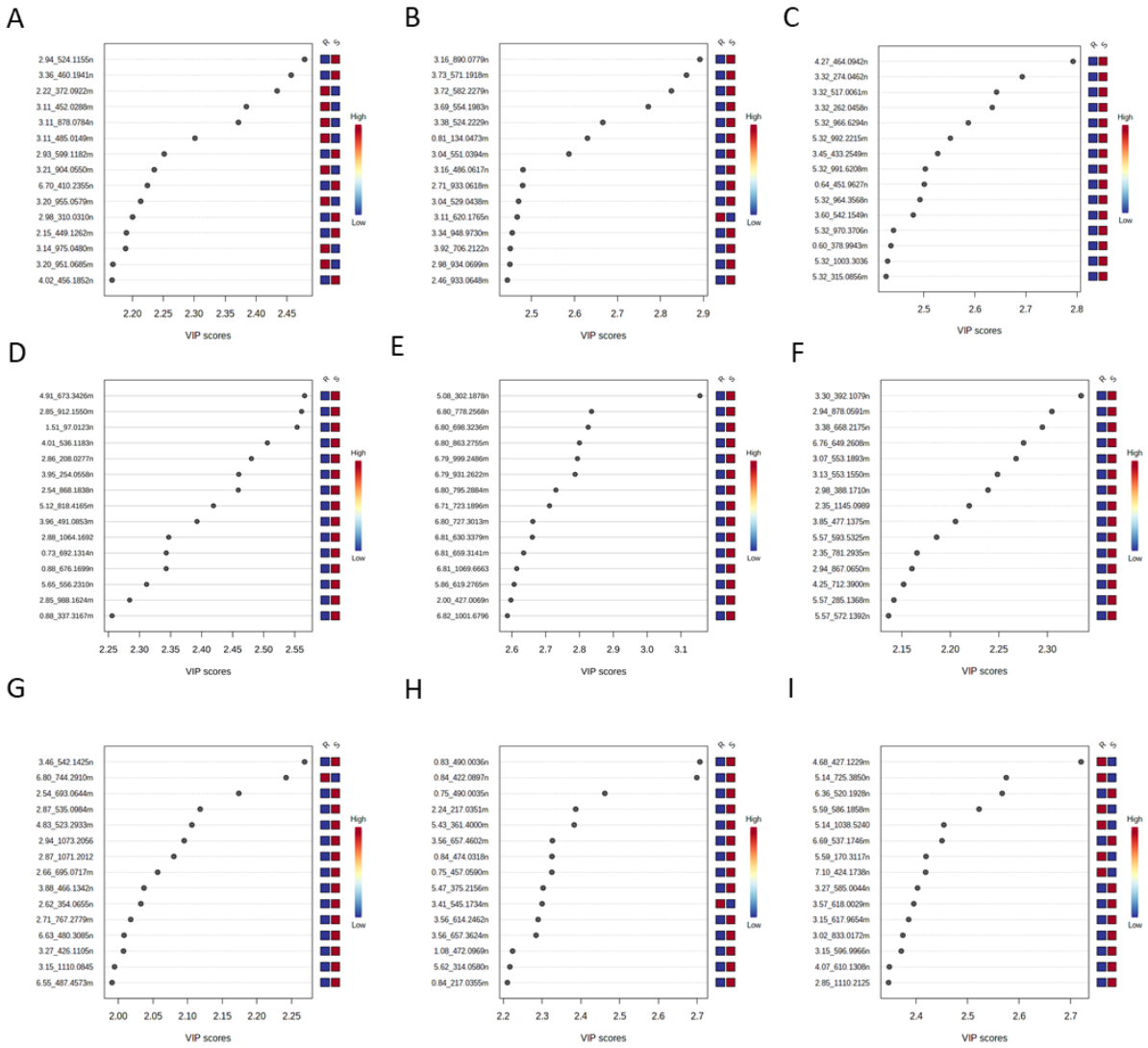

Figure 4: Variable Importance in Projection (VIP) 15 most important features that differentiate the two phenotypes, resistant (R) and susceptible (S). Metabolites that are at higher levels in the phenotype are indicated in red, while those that are lower are indicated in blue. Analysis was performed using UPLC-HDMS negative mode features. A. *Melaleuca quinquenervia* B. *Backhousia myrtifolia* C. *Leptospermum scoparium* D. *Syncarpia glomulifera* E. *Syzygium francisii* F. *Tristaniopsis laurina* G. *Rhodamnia rubescens* H. *Decaspermum humile* I. *Eucalyptus grandis.*

To determine whether a small subset of features could be used for biomarker identification across the Myrtaceae, the top 100 PLS-DA Variable Importance in Projection features for each species were compared. Cross species analysis was limited to two sets because identification of molecular features across mass spectrometry run times is difficult. Based on the comparisons that were performed, the chemistry of leaves across species appears to be different and a consistent set of biomarkers could not be identified across all species sampled (Figure 5). This would suggest that the basis for susceptibility and resistance to myrtle rust has evolved uniquely in even closely related species. Molecular features were shared between *B. myrtifolia* and *D. humile* (1), *B. myrtifolia* and *S. glomulifera* (2), *B. myrtifolia* and *R. rubescens* (3), *B. myrtifolia* and *S. francisii* (1), *D. humile* and *S. francisii* (1), *D. humile* and *R. rubescens* (3), *D. humile* and *S. glomulifera* (5), *S. francisii* and *R. rubescens* (2), *L. scoparium* and *M. quinquenervia* (4), *L. scoparium* and *E. grandis* (1), *T. laurina* and *E. grandis* (2). **This indicates that where this methodology is employed by breeders or industry, identification and characterisation of biomarkers for the species of interest will first need to be established before larger scale screening is performed.** Therefore, prompt action to secure samples and germplasm from fast-dwindling populations is needed to ensure this methodology can support conservation efforts into the future.

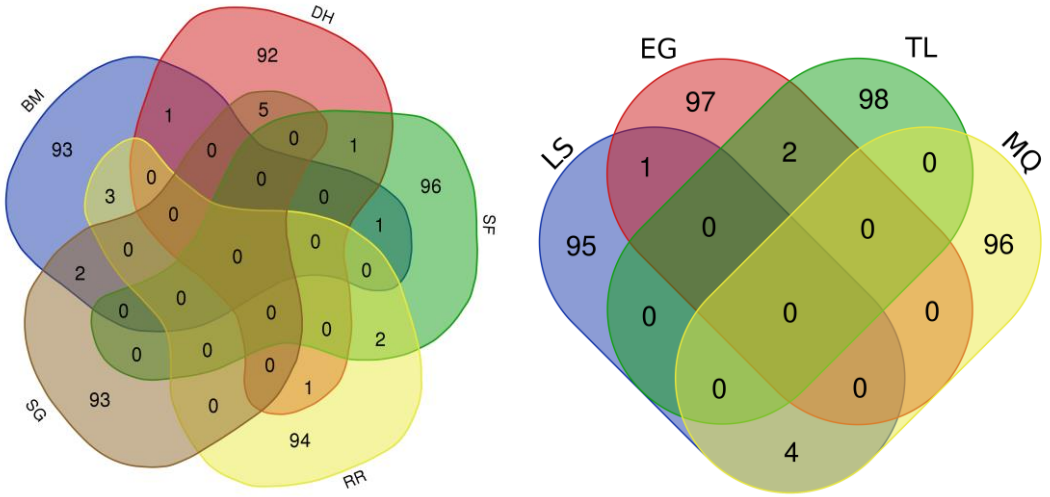

Figure 5: Venn diagrams indicating the number of Variable Importance in Projection features which are shared across species. Results are presented as two sets as they were run on the mass spectrometer at different times. A. Venn comparing top 100 VIP from *Syzygium francisii* (SF), *Decaspermum humile* (DH), *Rhodamnia rubescens* (RR) *Backhousia myrtifolia* (BM), *Syncarpia glomulifera* (SG) B. Venn comparing top 100 VIP from *Eucalyptus grandis* (EG), *Tristaniopsis laurina* (TL), *Melaleuca quinquenervia* (ML), *Leptospermum scoparium* (LS).

5.1.3 Biomarkers within a species can predict phenotypes of unknown individuals

To establish the robustness of the metabolomic biomarkers for phenotyping of unknown individuals, *L. scoparium* biomarkers were assessed. Fifteen biomarkers were selected based on the best receiver operating characteristic (ROC) curve, which is an indicator of false positive rate. These biomarkers of interest were used to assess the predictive power of metabolomics for individuals of unknown phenotype. Within the four *L. scoparium* individuals that were assessed, three were correctly predicted, with a probability of > 0.85 and one incorrectly predicted with a probability of <0.8 (Table 2). Here we show that biomarkers returning a high probability score accurately predict the phenotype of an individual.

Table 2: Prediction of the phenotype of four *Leptospermum scoparium* plants using biomarkers identified in this study

Plant ID	Probability	Predicted phenotype	Measured phenotype
LS-10	0.93264	Resistant	Resistant
LS-2	0.92932	Resistant	Resistant
LS-22	0.88017	Susceptible	Susceptible
LS-1	0.76723	Resistant	Susceptible

5.2 Optimised protocols for field collections to enable field-based metabolomic surveys for **A. psidii** *resistant genotypes*

5.2.1 Comparison of sampling protocols in **Eucalyptus grandis**

In order for the technique that we have been developing to be applicable for field surveys, without the requirement for freezing at ultra-low temperatures, or for use by breeders or industry, we have collected leaves from field grown *Eucalyptus grandis* of known infection phenotype. Leaf metabolite markers were assessed over time at room temperature (0, 1, 3 and 24 hours), with and without the addition of extraction solvent. The addition of solvent was expected to quench any biochemical reactions that may happen over time after sampling. Results show that **the resistant and susceptible phenotypes could be differentiated by PLS-DA, regardless of the sampling strategy (Figure 6).** Both positive and negative mode analysis are able to differentiate the resistance phenotype, however VIP analysis indicated that the most important biomarkers do change with length of storage. In the future, a standardised method would have to be used each time to

ensure accuracy. In addition, growing plants in the outdoor environment did not impact the robustness of metabolomics to differentiate the two phenotypes. This confirms that where industry, breeders, conservation agencies, council or forest management groups are interested in employing this approach to phenotype individuals, immature leaf samples from plants grown either in greenhouse conditions or outdoors could be sent via overnight shipping services without compromising the quality of the leaf chemistry for identifying phenotypes. **This significantly reduces the cost for analysis for stakeholders wishing to use this methodology for screening purposes**.

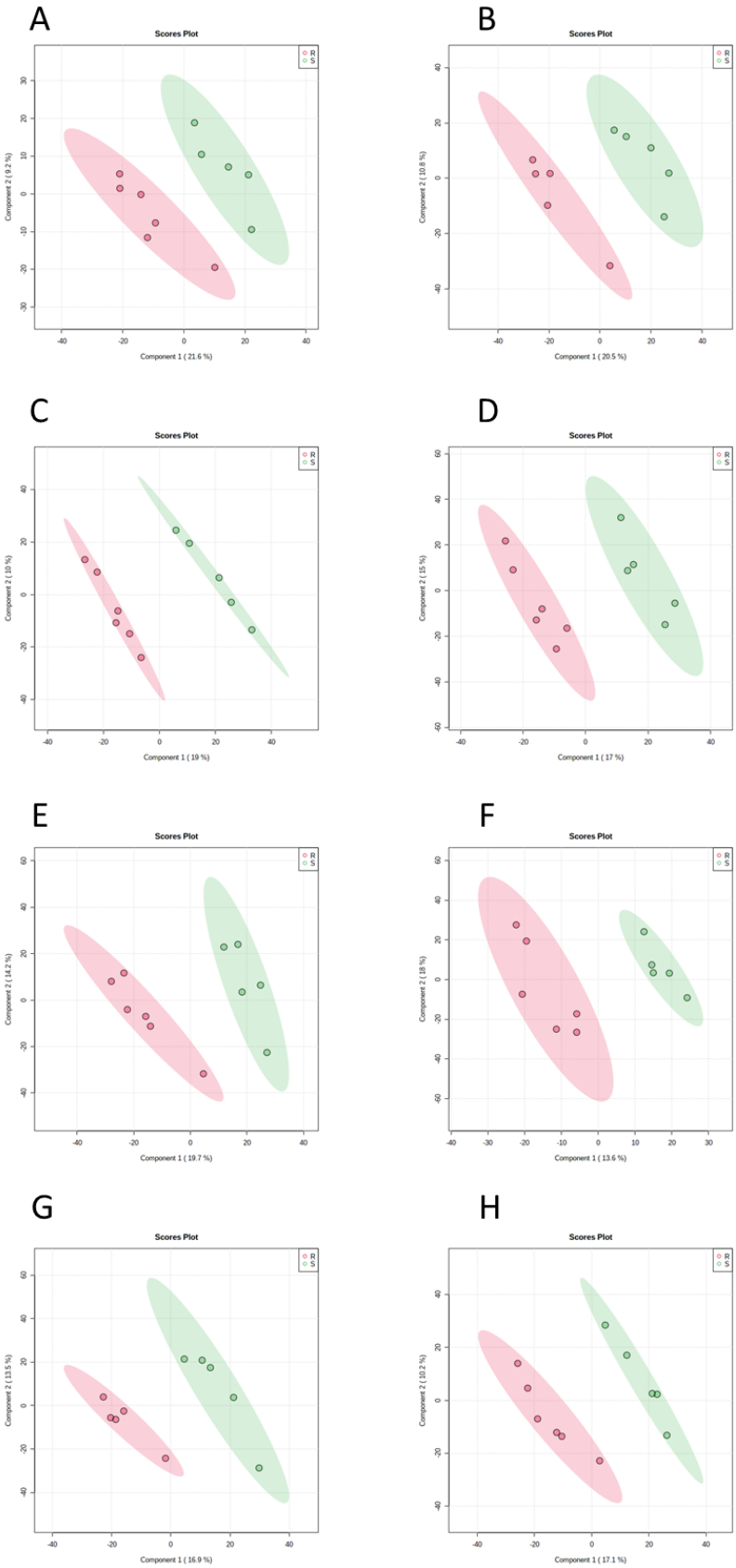

Figure 6: Effects of sample storage conditions over time on the analysis of susceptible (green) and resistant (red) field grown *Eucalyptus grandis*. Samples were collected from the second leaf from the apical tip and stored without solvent for 0 hours (A), 1 hour (B), 3 hours (C), 24 hours (D) and with extraction solvent for 0 hours (E), 1 hour (F), 3 hours (G), 24 hours (H). Metabolites were extracted and analysed by

UPLC-HRMS positive mode. Differentiation of phenotype is represented by PLS-DA analysis using MetaboAnalyst 5.0.

Variable Importance in Projection analysis was performed to identify molecular features that could be used as biomarkers that are consistent across all sampling times. To identify features that do not change over time, VIP features which differentiate phenotype at each time point were compared. Six and one features are important in differentiating phenotype at all time points in the absence and presence of solvent, respectively (Figure 7). Of those six features important in differentiating phenotype, the consistency of abundance in the samples according to phenotype were visualised using box and whiskers plots (Figure 8).

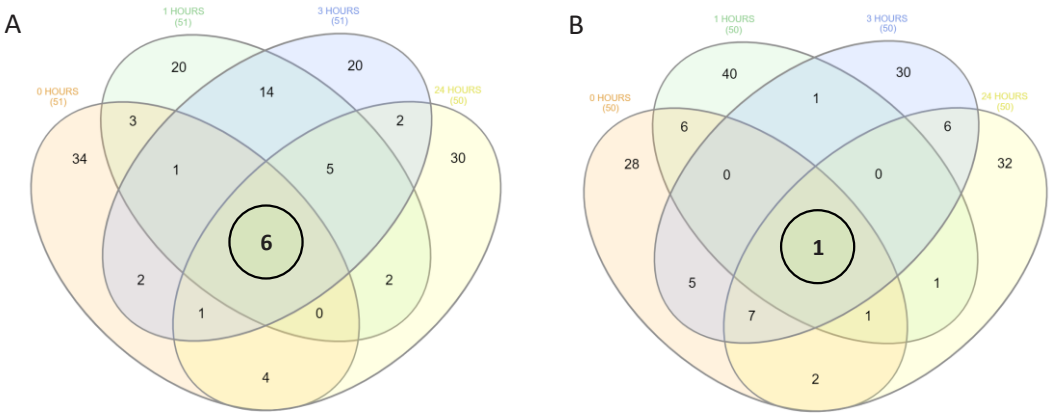

Figure 7: Venn diagram representing 50 most important features from VIP analysis at each storage time point. A. Six features were common to all treatment times in the absence of solvent. B. One feature was common to all treatment times in the presence of solvent.

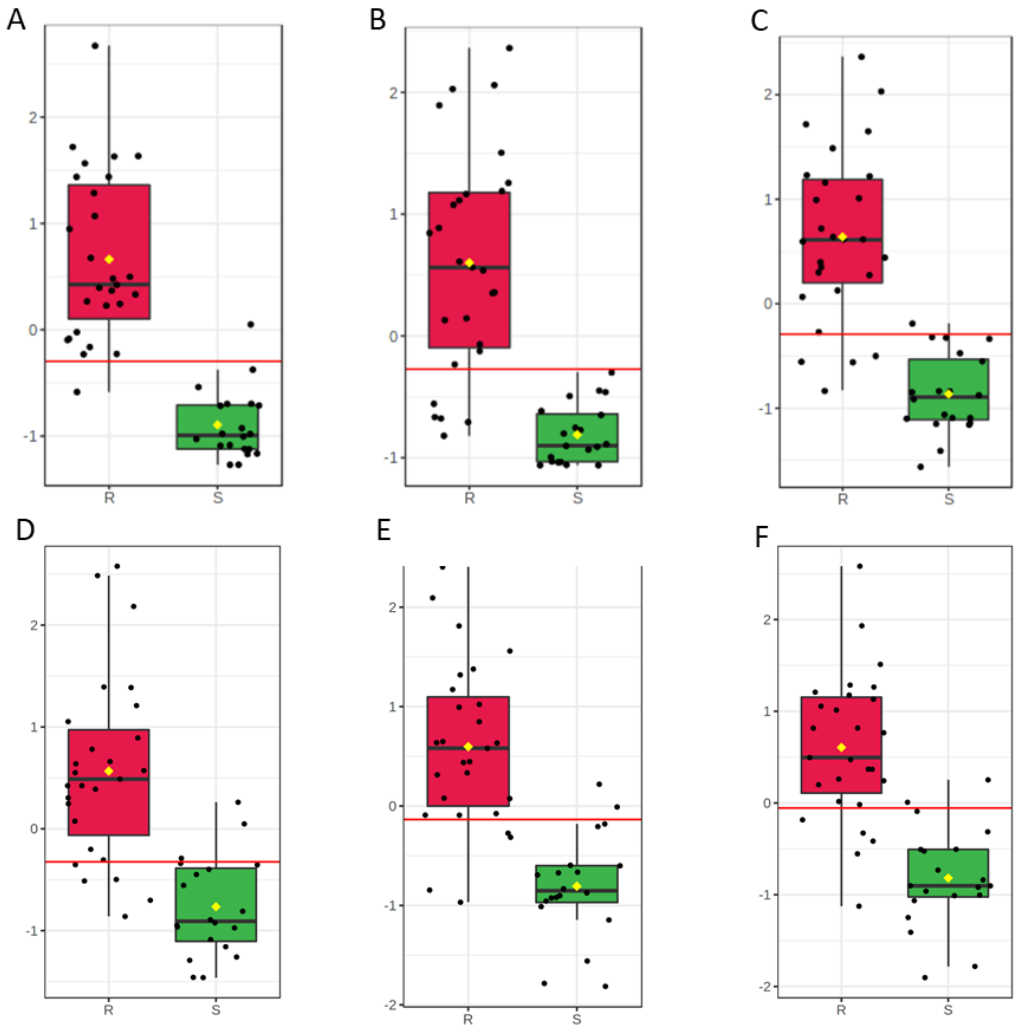

Figure 8: Box and whiskers plots showing the consistency of biomarker abundance in resistant and susceptible phenotypes, regardless of incubation time. A. 5.15_335.6530n B. 5.38_253.3916m/z C. 5.38_270.3172n D. 5.15_251.3328m/z E. 5.15_268.3772n F. 5.15_251.3018m/z

5.3 Improved understanding of the biochemistry responsible for resistance to A. psidii

Variable Importance in Projection analysis for each species identified the most important molecular features in the projection of the PLS-DA models (see Figure 3 and 4). In most cases, the most significant features were increased in abundance in the susceptible phenotype compared with resistant. The structural identification of the features was predicted using HMDB and CHEBI databases and the Progenesis QI program (Table 3). The structural predictions were classified into broad structural classifications and these included lipids, carbohydrates, phenylpropanoids.

Table 3: Examples of important features that can be structurally predicted based on UPLC HDMS data.

Feature ID	Score	Predicted structure	Superclass	Class
1.27_584.0171m/z	39.5	Caffeic acid 3-sulfate	Phenylpropanoid	Coumaric acid derivative
3.44_287.2056m/z	19.5	PC(16:0/22:1); [M+H]+ C46H91N1O8P1	Lipid	
3.88_610.1285n	38	Prodelphinidin B	Phenylpropanoid	Flavonoid
6.73_564.2975n	19.5	Khivorin	Phenylpropanoid	Limonoid

2.47_767.1816m/z	39.1	viscumneoside V	Phenylpropanoid	Flavanone glycoside
2.95_406.3387m/z	36.6	N,2,3-Trimethyl-2-(1-methylethyl)butanamide	Lipid	Fatty acyl
4.32_704.3490n	34.5	25-Acetyl-6,7-didehydrofevicordin F 3-glucoside	Lipid	Fatty acyl
4.32_871.3937m/z	50.2	27-O-demethylrifabutin	Lipid	Fatty acyl
4.39_767.3465m/z	37.9	5-(4'-Hydroxyphenyl)-gamma-valerolactone-4'-O-glucuronide	Benzenoid	Phenol ether
4.43_635.3058m/z	40	2,9-Dimethyl-2,9-diazatricyclo[10.2.2.25,8]octadeca-5,7,12,14,15,17-hexaene-3,10-diol, 9Cl	Nitrogen compound	
4.44_855.3629m/z	37.7	10-Hydroxymelleolide	Lipid	Prenol lipid
4.97_657.3957m/z	39.6	28-Glucosylpomolate	Lipid	Prenol lipid
4.97_939.4560m/z	39.7	Quillaic acid 3-[xylosyl-(1->3)-[galactosyl-(1->2)]-glucuronide]	Lipid	Prenol lipid
5.65_477.2207m/z	39.9	(x)-1,2-Propanediol 1-O-b-D-glucopyranoside	Organooxygen	Carbohydrate

5.4 Communication

A draft publication for the journal “Forest Ecology and Management” (Q1 journal, impact factor 4.384) is currently being prepared. This journal was chosen for its target audience which will likely include groups with an interest in management and conservation of natural forests and plantations.

In 2021, Dr Moffitt presented the results of PBSF023 as an oral presentation at the Myrtle Rust Symposium and the online Australasian Plant Pathology Symposium.

In 2022, Dr Moffitt and Dr Donovin Coles presented the results of PBSF023 at the Biosecurity conference in the myrtle rust session. Dr Moffitt also presented an online webinar in the Beyond Myrtle Rust series. The YouTube video currently has 65 views.

To increase interest in myrtle rust, we have used social media platforms, Twitter and Facebook, to highlight our work. We also follow and share myrtle rust related information to increase its exposure amongst the scientific groups and general public.

5.4 Engagement of Early Career Researchers

To assist in the engagement and training of early career researchers, we have employed former WSU Masters of Research student Elise Randall and former WSU PhD student Ahilya Singh. Elise and Ahilya were critical in the phenotypic evaluation of inoculated plants and metabolite extraction from leaves.

Undergraduate students were presented with learning opportunities related to biosecurity and specifically myrtle rust, in lectures and labs in the ‘Agricultural Biosecurity’ and ‘Microbiology’ units at Western Sydney University. Second year

microbiology students toured the “living laboratory” at WSU Hawkesbury campus to experience firsthand the importance of biosecurity and myrtle rust, although not present in the living labs, was discussed as a disease of concern during this event.

6. Discussion and Conclusion

In this study, we have extended upon APBSF project PBSF023 to assess the suitability of metabolomics for phenotyping a broad range of Myrtaceae species for susceptibility to myrtle rust infection. Our results indicate that species specific biomarkers of the susceptible phenotype can be established as a screening tool which were able to predict the rust-susceptibility phenotype of unknown individuals, grown both in the field and under the same greenhouse conditions. These genotypes could then be used to maintain or protect threatened species and could be used in industrial settings to improve productivity of plantations.

Hall et al. (2022) highlight some of the limitations of untargeted metabolomics for high-throughput plant phenotyping, including sample preparation which is time-consuming for large sample numbers, particularly weighing for standardisation and grinding, mass spectrometry run times and data analysis (Hall *et al.*, 2022). It is necessary to ensure that relevant biomarkers are stable with respect to leaf age, sampling time of day and enzymatic breakdown. Here we sampled leaves at the same position from the apical tip and confirmed that quenching of enzymatic reactions is not required within a 24-hour period at room temperature. We will require further experiments to determine if biomarkers fluctuate based on time of day. We avoided the issue associated with sample preparation weighing by taking standard leaf punches during our *E. grandis* field study. This will work for larger leaved species. Myrtaceae species with narrow leaves would need to have alternative sampling strategies trialled including a consistent length of leaf. Mass spectrometry run times have been decreased through the use of the ultra performance liquid chromatography (UPLC). While we used both positive and negative mode mass spectrometry for completeness, results indicate mass spectrometry time can be halved by running positive mode only, which is suitable for differentiation. Further, now that we have specific biomarkers for susceptibility, a targeted metabolomic procedure could be adopted which would screen only for specific metabolites thereby decreasing sample run time to a minimum and increasing simplicity and throughput.

We previously hypothesised that chemicals within resistant hosts may be inhibitory to the growth or germination of *A. psidii* spores (Moffitt *et al.*, 2022) and APBSF project PBSF023). Here we have identified that most of the molecular features that differentiate the two phenotypes are increased in the susceptible hosts. This is contrary to our hypothesis and may indicate that these metabolites act in stimulating the germination and growth of *A. psidii*. Hentriacontane is a molecule produced by the highly susceptible species *Syzygium jambos* which is present within the cuticle of leaves and increases *A. psidii* spore germination on water agar by 88% (Tessmann and Dianese, 2002). Metabolites such as lipids and carbohydrates are increased in susceptible hosts and may be important in their susceptibility. Given the stability of the products, these are likely to be terminal products of their pathways, and based on their structural classes, may be associated with the leaf cuticle layer (Skrzydeł et al., 2021).

In conclusion, our advanced studies enabled by APBSF project APBSF037 funding demonstrate that metabolomic screening should be explored and adopted as one of the tools for the identification of *A. psidii*-resistant plants belonging to a wide range of Myrtaceae species. We demonstrated that robust species-specific biomarkers can be identified that are stable over at least 24 hours at room temperature for transport of leaf material from remote locations. We propose that a number of key biomarkers may be located within the cuticle of susceptible leaves, indicating that these molecules may stimulate germination of *A. psidii* spores into the host. These findings will also generate a new series of research pathways for better understanding the mechanisms of disease establishment by *A. psidii* which will enable the development of novel preventative strategies in future.

7. Recommendations

Untargeted metabolomics as presented here is a sensitive method that can be used to differentiate individuals based on susceptibility phenotype. Future experimental studies could improve the applicability of the method for industry and breeders.

- Approach relevant industry and governmental bodies in the areas of conservation, forestry, and horticulture, and other Myrtle Rust stakeholders, to identify how this methodology may be translated for use. This could be

facilitated by the newly-formed National Myrtle Rust Working Group (Department of Agriculture, Forestry and Fisheries).

- Improve sensitivity of the untargeted metabolomics methodology by increasing sample size for species of interest based on stakeholder requirements.

- Ensure that selected biomarkers are not susceptible to fluctuations as a result of environmental factors including time of day or age of leaf.

- Develop a robust targeted metabolic approach based on currently identified biomarkers which can be implemented in multiple laboratories with a different instrumentation and improve downstream data analysis through the development of machine learning tools.

- Evaluate the role of molecules on the leaf cuticle which may stimulate *A. psidii* germination on susceptible hosts and factors that may be influencing the chemistry of the cuticle.

9. Appendices, References, Publications

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