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

Progress Report (PBSF037)

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1. Executive Summary

Many species belonging to the Myrtaceae display variation in their susceptibility to the fungal plant pathogen, *Austropuccinia psidii*, causing the disease myrtle rust. Our previous work (project PBSF023) has identified that metabolomics could detect differences in small molecules in resistant and susceptible *Melaleuca quinquenervia* plants, before infection occurs (Moffitt et al, submitted) which led us to the hypothesis that the same methodology could be employed to differentiate resistant and susceptible individuals of other species. The objectives of this project are to employ the same metabolomics methodology across a diverse selection of Myrtaceae species to establish a set of biomarkers that rapidly identify resistant plants without infection trials or previous genomic knowledge. This project has been significantly delayed due to COVID lockdown in Sydney for 4 months and limited laboratory access at the university for the months following the easing of restrictions, however have continued to make progress in the project. In this progress report, we present the achievement of the three milestones outlined by APBSF:

1. Harvesting and infection of plant material

We have collected species belonging to eight tribes of the Myrtoideae subfamily of Myrtaceae, including species listed as priority species in the Myrtle Rust Action Plan (2020). Twenty replicates of each species were inoculated with *A. psidii* and their susceptibility to the infection was recorded. In some instances, additional field collections were obtained from the Royal Botanical Gardens Mount Annan living collection. We have confirmed our metabolomics methods from PBSF027 are suitable for differentiating resistant and susceptible phenotypes in *Eucalytpus grandis*. We have also trialled protocols to optimise the collection of leaf material from field sites without the loss of critical metabolomics biomarkers.

2. Use of social media

Our project has been successful in the use of communication techniques on social media, including Twitter and Facebook to raise concern about the myrtle rust pandemic in Australia.

3. Involvement of early career researchers

This project has employed the use of three early career researchers and has highlighted the issues of biosecurity and myrtle rust to undergraduate students at Western Sydney University.

2. Introduction

Austropuccinia psidii is a fungal plant pathogen that specifically infects plant species within the Myrtaceae, causing the disease myrtle rust. Myrtle rust has had significant effects on natural and managed ecosystems, where it is already causing declines in local populations and is expected to cause species extinctions within a generation. Additionally, myrtle rust continues to cause declines in the productivity of industrial plantations. Despite this, variation in response to *A. psidii* exists within some myrtaceaeous species, from complete susceptibility to resistance that prevents or limits infection by the pathogen. Identifying the mechanisms supporting improved resistance in these latter individuals will contribute significantly to conservation and management strategies for species at risk from this devastating disease.

The family of Myrtaceae, subfamily Myrtoideae consists of 15 tribes (Wilson et al. 2005). In Australasia, Myrtaceae predominate in most ecosystems, with more than 2,250 species from 14 tribes have been recorded (Makinson 2018). To date, worldwide 539 Myrtaceae species belonging to 14 tribes are known to be vulnerable to *A. psidii* infection (Fernandez Winzer, Cuddy, and Pegg 2020).

Recently, 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 has established that prior to infection, resistant germplasm from the species *Melaleuca quinquinervia* contain metabolic signatures that differentiate them from hypersensitive and susceptible phenotypes. In particular, prior to infection, the resistant *M. quinquenervia* leaves contained increased concentrations of secondary metabolites, including flavonoids (Moffitt et al., submitted). Similar molecules are reported to aid in defence against pathogens in other plants by inhibiting germination of fungal spores or fungal growth (Padmavati et al. 1997; Gillmeister et al. 2019).

3. Aim

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

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

The milestones for this progress report are:

1. *Harvesting and infection of plant material*
2. *Use of social media*
3. *Involvement of early career researchers*

4. Methods/Process

4.1. Towards Objective 1 and 2 - Improved understanding of the biochemistry responsible for resistance to A. psidii and Curated set of biomarkers to assess individual plants within selected Myrtaceae species for levels of resistance/tolerance

4.1.1 Plant growth and collection

Seeds were obtained from the Royal Botanical Gardens Mount Annan collection, germinated on agar plates and subsequently transferred to soil (Table 1). Seedlings were also 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. Field samples of selected species were collected from the Royal Botanical Gardens Mount Annan nurseries (Table 1) and the living collection (Table 2). Juvenile leaves at the second position from the apical bud and mature leaves were collected for solvent extraction.

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

Species	Tribe	Source	Material harvested and extracted for metabolomics	Susceptibility testing	Resistant individuals	Susceptible individuals
Syzygium (Acmena) smithii	Syzygieae	Seedlings from seeds via Brush Turkey enterprises, QLD	Yes[2]	Yes[4]	20/20	0/20
Syzygium (Acmena) ingens	Syzygieae	Seedlings from seeds via Brush Turkey enterprises, QLD	Yes[2]	Yes[4]	19/20	1/20
Syzygium hodgkinsoniae	Syzygieae	Seedlings from seeds via Brush Turkey enterprises, QLD	Yes[2]	Yes[4]	16/20	4/20
Syzygium francisii	Syzygieae	Seedlings from seeds via Brush Turkey enterprises, QLD	Yes[2]	Yes	16/20	4/20
Syzygium oleosum	Syzygieae	Seedlings from seeds via Brush Turkey enterprises, QLD	Yes[2]	Yes[4]	20/20	0/20
Syzygium anisatum[1]	Syzygieae	Royal Botanical gardens seed collection	No[3]	No[3]	-	-
Decaspermum humile[1]	Myrteae	Seedlings from seeds via Brush Turkey enterprises, QLD	Yes	Yes	12/20	8/20
Decaspermum struckoilicum	Myrteae	Seedlings from seeds via Brush Turkey enterprises, QLD	Yes[2]	Yes[4]	20/20	0/20
Archirhodomyrtus beckleri[1]	Myrteae	Royal Botanical gardens seed collection	No[3]	No[3]	-	-

Rhodamnia rubescens[1]	Myrteae	Royal Botanical gardens nursery stock	Yes	Yes[5]	-	-
Rhodomyrtus psidioides[1]	Myrteae	Royal Botanical gardens nursery stock	Yes	Yes[5]	-	-
Gossia fragrantissima[1]	Myrteae	Royal Botanical gardens nursery stock	Yes	Yes[5]	-	-
Lenwebbia sp. Main Range[1]	Myrteae	Royal Botanical gardens nursery stock	Yes	Yes[5]	-	-
Tristaniopsis laurina	Kanieae	Seedlings from seeds via Brush Turkey enterprises, QLD	Yes	Yes[4]	17/20	3/20
Backhousia myrtifolia	Backhousieae	Seedlings from seeds via Muru Mittigar nursery, NSW	Yes	Yes	12/20	8/20
Backhousia leptopetala[1]	Backhousieae	Royal Botanical gardens seed collection	No[3]	No[3]	-	-
Syncarpia glomulifera	Syncarpieae	Seedlings from seeds via Muru Mittigar nursery, NSW	Yes[2]	Yes	15/20	5/20
Lophostemon confertus	Lophostemoneae	Seedlings from seeds via Muru Mittigar nursery, NSW	Yes	Yes	20/20	0/20
Eucalyptus grandis	Eucalypteae	Seedlings from WSU APBSF 2020 project	Yes	Tested as part of the APBSF 2020 project		
Eucalyptus resinifera[1]	Eucalypteae	Royal Botanical gardens seed collection	No[3]	No[3]	-	-
Leptospermum trinervium[1]	Leptospermeae	Royal Botanical gardens seed collection	No[3]	No[3]	-	-

Melaleuca nodosa[1]	Melaleuceae	Royal Botanical gardens seed collection	No[3]	No[3]	-	-

[1]Listed as priority species. Makinson, Pegg and Carnegie (2020) Myrtle Rust in Australia – A National Action Plan.
[2]Both young and mature leaves of these species sampled for metabolomics to compare biomarker profiles
[3]Sufficient growth of seedlings was not available for analysis
[4]*A. psidii* inoculations will be repeated to confirm susceptibility of plants due to storm damage effecting causing loss of young leaves available for cutting

Table 2: List of individuals collected from the Royal Botanical Gardens Mount Annan living collection

Species	Tribe	Accession number
Syzygium (Acmena) smithii	Syzygieae	1986-1586
Syzygium (Acmena) smithii	Syzygieae	2018-0050
Syzygium (Acmena) smithii	Syzygieae	2015-0255
Syzygium (Acmena) smithii	Syzygieae	1998-1104
Syzygium (Acmena) ingens	Syzygieae	AA854-141
Syzygium (Acmena) ingens	Syzygieae	2006-0668
Syzygium (Acmena) ingens	Syzygieae	1986-1584
Syzygium francisii	Syzygieae	AB861-631
Syzygium oleosum	Syzygieae	2007-0094
Tristaniopsis laurina	Kanieae	2006-0367
Tristaniopsis laurina	Kanieae	1999-0386
Tristaniopsis laurina	Kanieae	1986-5660
Backhousia myrtifolia	Backhousieae	2005-0104
Backhousia myrtifolia	Backhousieae	AA865-448
Backhousia myrtifolia	Backhousieae	2015-0834
Lophostemon confertus	Lophostemoneae	1989-1977
Syncarpia glomulifera	Syncarpieae	1984-3023
Syncarpia glomulifera	Syncarpieae	1987-2708
Syncarpia glomulifera	Syncarpieae	1987-2708

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

Cuttings of the plants consisting of 4-6 juvenile leaves were taken and placed in water agar (0.6 %) within 50 ml tubes. 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 or jars. The *A. psidii* spores were allowed to settle for one minute and 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.

For phenotyping of each of the cuttings, leaves were examined for *A. psidii* disease symptoms, 14 days following inoculation and scored as either resistant or susceptible.

4.1.3. Leaf metabolite extraction

The frozen leaf samples were weighed and immediately ground into powder by bead-beating in 200 µl extraction solvent (4:4:2 methanol:acetonitrile:deionised water) twice for 30 seconds each. The leaf:solvent mixture was then combined with 300 µl additional extraction solvent, and then vortexed to mix for 10 seconds. The leaf:solvent mixture was then placed in icy water and sonicated in a sonicating water bath for 25 min. Solid cellular material was removed by centrifugation at 12,000 rpm for 10 min. The supernatants were diluted 3:1 in HPLC grade water and centrifuged to remove additional particulates.

4.1.4 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 UPLC system and a Waters Synapt G2-Si 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.

4.2 Towards objective 3 - Optimised protocols for field collections to enable rapid metabolomic-based field surveys of A. psidii resistance

Eucalyptus grandis plants with known *A. psidii* infection phenotype (resistant and susceptible) were grown in the field at the WSU Hawkesbury campus. Juvenile leaves at the second position from the apical bud were collected. To simulate possible field collection scenarios, leaves were stored for 1 hour, 3 hours and overnight in the following conditions before metabolite extraction:

- In tubes at room temperature for before placing in extraction solvent
- In tubes on ice before placing in extraction solvent
- Placed directly in extraction solvent at room temperature
- Placed directly in extraction solvent on ice

5. Achievements, Impacts and Outcomes

In accordance with the APBSF services agreement, we have completed our progress report milestone targets set out below as part of the three project objectives.

5.1 Towards Objective 1 - Improved understanding of the biochemistry responsible for resistance to A. psidii

5.1.1 Metabolomic comparison of Eucalyptus grandis *plants resistant and susceptible to* Austropuccinia psidii *infection*

To confirm that metabolites show the same pattern in defining *E. grandis* resistance to *A. psidii* before infection as previously seen in *M. quinquenervia* (PBSF023 and Moffitt et al, submitted), leaf samples were analysed and found to be significantly different in their metabolomic profile. Subsequently, PLS-DA and ANOVA analysis was applied to identify if any metabolites could significantly separate resistant and susceptible E. grandis plants. The heatmap of the top metabolites (ranked by the ANOVA FDR value) shows that resistant individuals were characterised a group of metabolites that increased in abundance, while another group of metabolites decreased in abundance (Figure 1). The enriched metabolites in the resistant *E. grandis* leaves included flavonoids which we previously found to be enriched in the metabolome of *M. quinquenervia* resistant leaves. This result supports the premise of our current project that other Myrtaceae plant susceptibilities to *A. psidii* can be predicted using metabolomics and that flavonoid secondary metabolites may play a role in resistance before and in the early stages of *A. psidii* infection.

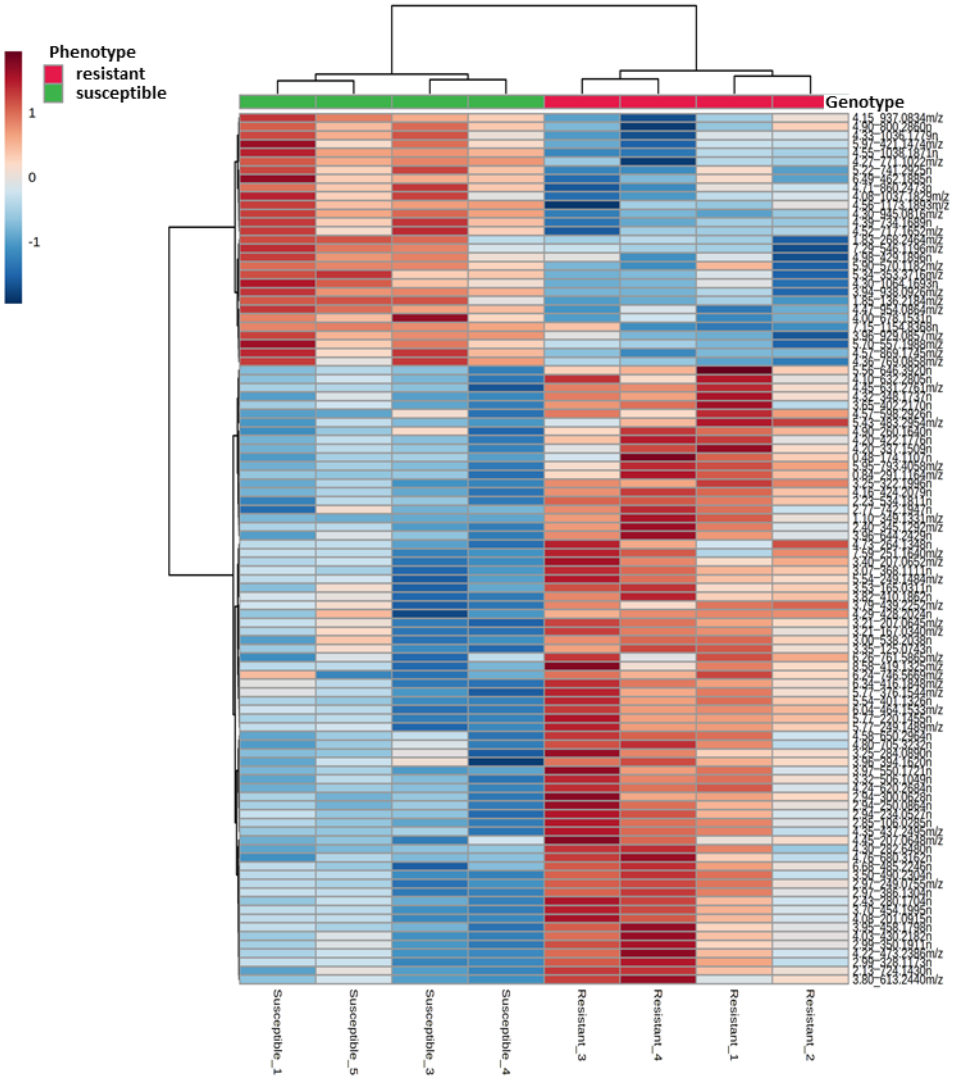

Figure 1. Heatmap of scaled peak intensity of the significant molecular features in *Eucalyptus grandis* plants that are resistant (red) and susceptible (green) to *Austropuccinia psidii* infection (ranked by adjusted p calculated with 2-way ANOVA). The heatmap is clustered by Euclidean distance and clustered with Ward's minimum variance method for both the columns and rows. Red and blue indicate the enrichment and reduction, respectively, of each significant molecular feature (rows) of each sample (columns).

5.2 Towards Objective 2 - Curated set of biomarkers to assess individual plants within selected Myrtaceae species for levels of resistance/tolerance

5.2.1 Interim report milestone 1 - Harvesting and infection of plant material

We selected a diverse collection of Myrtaceae species phylogenetically belonging to 8 tribes (Table 1). Species were selected for analysis based on Myrtle rust action plan priority listing or their reported varying susceptibility rating to *A. psidii* infection across individuals. To improve our resolution within two of these tribes, we selected a number of species within the tribes Myrteae and Syzygiae. The Myrteae are an important tribe consisting of a number of species that are listed as endangered. The Syzygiae are of interest due to many of the species displaying varying tolerance to myrtle rust infection.

Species were obtained as either seeds from the Royal Botanical Gardens, Mount Annan or as seedlings from nurseries. We used only seedlings (vs. clonal cuttings) to ensure a variation in genotype and, by extension, rust susceptibility phenotype. In addition, cuttings of *Rhodamnia rubescens, Rhodomyrtus psidioides, Gossia fragrantissim*a and *Lenwebbia* sp. Main Range were sampled from the Royal Botanical Gardens, Mount Annan nursery collections. *A. psidii* inoculations were performed and the phenotype of individuals was assessed (Table 1). Unfortunately, a storm event caused the loss of juvenile leaves from seedlings of six of the species analysed and phenotyping will need to be repeated to confirm the infection phenotype of each individual from these species. We will also perform phenotyping of seedlings that have been grown from seed.

5.3 Towards Objective 3 - *Optimised protocols for field collections to enable rapid metabolomic-based field surveys of A. psidii resistance*

In order for this technique to be applicable for the assessment of rapid field surveys (Action 3.2.3), we have collected leaves from field grown *Eucalyptus grandis* of known infection phenotype. Leaf metabolite markers will be monitored for changes over time at room temperature and on ice. The presence of biomarkers will be compared in both juvenile and mature leaves to assess importance of leaf type in the development. We will also compare biomarker profiles of leaves grown in growth chambers at WSU with those of leaves collected from cuttings from the living collection at the Royal Botanical Gardens, Mount Annan.

5.4 Interim report milestone 2 – Social media communication

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. We have also submitted an abstract to attend the Biosecurity conference which has been postponed to 2022.

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

5.5 Interim report milestone 3 - Engagement of ECRs

To assist in the engagement and training of ECRs, we have employed former WSU Masters of Research student Elise Randall and former PhD students Johanna Wong-Bajracharya and Donovin Coles. Elise was critical in the phenotypic evaluation of inoculated plants and metabolite extraction from leaves. Johanna, now an employee at DPI in the field of pathogen diagnostics and Donovin now a post doc in horticulture pathogen management have played a critical role in the metabolomics work in this study.

Undergraduate students were presented with learning opportunities related to biosecurity and specifically myrtle rust, in lectures and labs in the 'Plant Health and Biosecurity' and 'Microbiology' units at Western Sydney University. Second year microbiology students toured the "living laboratory" at WSU Hawkesbury campus to experience first hand the importance of biosecurity and myrtle rust, although not present in the living labs, was discussed as a disease of concern for Australia during this event.

6. Appendices, References, Publications

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