Susceptibility to the fungal plant pathogen *Austropuccinia psidii* is related to monoterpane production in Australian Myrtaceae species

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**Abstract** In 2010, the fungal plant pathogen that causes Myrtle rust, *Austropuccinia psidii*, which is native to South America, was first detected in Australia and has since had significant impacts on several Australian Myrtaceae species. Despite this, our understanding of the role secondary metabolites play in plant susceptibility to *A. psidii* is limited. This study aimed to determine: (1) whether secondary metabolite (phenolics, terpenes) production is induced after *A. psidii* inoculation and if so, (2) how their production relates to *A. psidii* susceptibility. To test these aims, we selected seven Myrtaceae species that have a wide range of within-species variability in their susceptibility to *A. psidii*. We found that five of the study species significantly increased either their phenolic or sesquiterpene production post-inoculation suggesting their pre-inoculation secondary metabolite levels were not sufficient to combat *A. psidii* infection. The two species (*Angophora costata* and *Corymbia citriodora*) that did not increase their secondary metabolite production post-inoculation tended to have the greatest pre-inoculation production levels amongst the species. Interestingly, across all species, monoterpenes were the only secondary metabolite found to reduce plant susceptibility to *A. psidii*. This study contributes to our limited understanding of the role that secondary metabolites play in plant susceptibility to *A. psidii*. In light of these findings, future research should aim to identify biomarkers (e.g. individual chemical compounds) that confer resistance to *A. psidii*, so that individuals with these biomarkers can be utilised in commercial and conservation projects.

**Keywords** Anti-fungal · Chemical defence · Myrtle rust · Phenolic · Resistance · Terpene

**Introduction**

Plant pathogens are a natural part of plant communities and often co-exist with host species that they have co-evolved with (Pagán and García-Arenal 2018). However, when plant pathogens are introduced to new hosts that they lack a co-evolutionary history, they can rapidly become invasive and cause significant reductions in host survival and fecundity, which may impact the structure and function of the plant communities in which the hosts occur (Dobson and Crawley 1994; Gilbert 2002; Mordecai 2011). It is becoming increasingly recognised that fungi represent the most important group of plant pathogens in terms of ecological and economic impacts (Dean et al. 2012; Fisher et al. 2012).
Some notable examples include Cryphonectria parasitica, an ascomycete fungus native to East Asia that causes Chestnut blight fungus on European chestnut trees, which have decimated chestnut tree populations in North America and Europe (Anagnostakis 1987) and Ophiostoma ulmi, an ascomycete fungus native to Western Europe that causes Dutch elm disease, which has devastated elm tree populations in North America (Karnosky 1979). The success of fungal plant pathogens as invaders is often due to their long-distance dispersal (both natural and anthropogenic) capabilities and accelerated evolutionary potential (due to high reproductive output and short generation times) (Brown and Hovmöller 2002; Anderson et al. 2004; Chakraborty 2013), which are both traits generally associated with invasive species (Whitney and Gabler 2008). However, despite their high invasive potential, plant fungal pathogens have received much less research attention in the biological invasion literature compared to their insect, plant and animal counterparts (Wingfield et al. 2017). Therefore, we must gain an improved understanding of the drivers that dictate plant-fungal pathogen interactions, so we can best manage the impacts of invasive plant fungal pathogens.

To minimise the significant threat posed by fungal pathogens, plants have evolved an intricate and vast array of secondary metabolites that act as chemical defences (Ferreira et al. 2006). Phenolics constitute one of the most common and diverse groups of plant secondary metabolites that have been shown to act as a defence mechanism against fungal pathogens (Lattanzio et al. 2006; Zaynab et al. 2018). They consist of aromatic benzene rings with one or more hydroxyl groups. Volatile organic compounds (VOCs) represent another group of secondary metabolites that are an important chemical defence employed by plants to prevent fungal pathogen infection. The most abundant class of VOCs in plants are the terpenes, which consist of two or more isoprene units (Dudareva et al. 2006; Rosenkranz and Schnitzler 2016). While the biological role of the vast majority of terpenes is still unknown (Gershenson and Dudareva 2007; Boutanaev et al. 2015), many have been shown to have antifungal properties (Freiesleben and Jäger 2014; Nazzaro et al. 2017). Both phenolics and terpenes act as either pre-formed (phytoanticipins) or induced (phytoalexins) anti-fungal compounds (Darvill and Albersheim 1984; VanEtten et al. 1994; Osbourn 1996). The pre-formed compounds are synthesised during normal growth of the plant but usually not at high enough concentrations to kill fungal pathogens (Shalaby and Horwitz 2015). If the levels of pre-formed compounds are not sufficient to prevent infection, increased amounts of these compounds or new compounds are synthesised (i.e. induced) to fight the infection (Shalaby and Horwitz 2015).

The Pucciniales (rust fungi) represent the most speciose (~ 8000 species) group of plant pathogens (Aime et al. 2017) with experts considering them to be in the top three most scientifically and economically important fungal pathogens (Dean et al. 2012). They are characterised by orange, brown or red coloured urediniospore masses on the host tissue surface (Callan and Carris 2004). In 2010, the fungal plant pathogen that causes Myrtle rust, Austropuccinia psidii, which is native to South America, was first detected in Australia (Carnegie et al. 2010). It has since become invasive, spreading rapidly through the eastern states (New South Wales, Queensland and Victoria) of mainland Australia as well as Tasmania and the Northern Territory (Carnegie and Pegg 2018). The high invasive potential of A. psidii is also evident from its rapid spread in many countries on several different continents (see Carnegie and Pegg 2018 for a complete list). Austropuccinia psidii poses a major threat to Australian vegetation communities in particular as it infects species from the Myrtaceae family, which is one of the dominant plant families in Australia (Glen et al. 2007). Despite its short residency time, A. psidii has already caused dramatic declines in several highly susceptible species in eastern Australia (Carnegie et al. 2016; Pegg et al. 2017; Fernandez-Winzer et al. 2020) with further species declines projected in the future (Berthon et al. 2018).

The relatively recent arrival of A. psidii in Australia means our understanding of the factors driving plant susceptibility to the pathogen in an Australian context is limited (Makinson 2018). Of these factors, plant defences are likely to be amongst the most important (Makinson 2018). To date, the majority of studies that have addressed this knowledge gap have focused on commercially important species such as Corymbia citrodera (Bonora et al. 2020a, b) and Eucalyptus grandis (Moon et al. 2007; Hantao et al. 2013; Xavier et al. 2015; Dos Santos et al. 2019), meaning there is significant scope for further research. The aim of this
study was twofold: (1) determine whether secondary metabolite (i.e. chemical defence compounds; e.g. phenolics, terpenes) production is induced after *A. psidii* inoculation and if so, (2) how their production relates to *A. psidii* susceptibility. To do this, we measured pre- and post-inoculation secondary metabolite production of seven species from five different Myrtaceae genera that are known to have within-species variability in susceptibility to *A. psidii* (Giblin and Carnegie 2014). We hypothesised that:

1. Phenolic and terpene production will be induced in plants that are inoculated with *A. psidii* but will not vary in uninoculated plants.
2. Susceptibility will be negatively related to the plant’s ability to induce phenolic and terpene production post-inoculation.

### Methods and materials

#### Study species and seed germination

We selected seven species from five different Myrtaceae genera that are known to have a wide range of within-species susceptibility to *A. psidii* (Giblin and Carnegie 2014; Table 1). Seeds for these Myrtaceae species were purchased from commercial seed suppliers (Nindethana, Albany, WA, Australia; Seed World, Nowra, NSW, Australia) and germinated on moist paper towels within aluminium trays that were sealed with plastic cling wrap.

### Experimental design and growth conditions

The study species were grown in a climate-controlled glasshouse at the Macquarie University Plant Growth Facility (North Ryde, NSW, Australia). For each species, 16 individuals were grown in 1 L pots (7 cm wide × 20 cm deep) filled with a commercially supplied 80% sand 20% soil blend (Australian Native Landscapes, Terrey Hills, NSW, Australia). Germinated seedlings were planted individually into the pots at the stage of first leaf emergence and grown for 12 weeks. Every week, the plants were randomly assigned new positions within the glasshouse. The temperature of the glasshouse was set for a maximum of 24 °C (day-time: 0600–1800 h) and a minimum of 19 °C (night-time: 1800–0600 h), which was continuously maintained by a fan coil unit using a water-cooling and heating system. The mean photosynthetically active radiation and relative humidity within the glasshouse at 1400 h were 655 μmol m⁻² s⁻¹ (range: 52–1071 μmol m⁻² s⁻¹) and 77% (range: 73–80%) respectively.

### Volatile organic compound measurements

After the 12-week growth period, the VOCs emitted by each plant were sampled. To sample the VOCs, a flat square piece of plywood (length = 30 cm, thickness = 0.5 cm) with space for a plant stem was covered with aluminium foil and placed on the lip of the pot being sampled. A 6.5 L glass beaker (h = 26 cm, Ø = 18 cm) was then placed over the top of the plant. The spout of the beaker was sealed

### Table 1

| Species                     | Distribution | Habitat              | Susceptibility category range |
|-----------------------------|--------------|-----------------------|------------------------------|
| *Angophora costata* (Gaertn.) Britten | NSW, QLD, VIC | Dry sclerophyll forest | Resistant to high            |
| *Callistemon citrinus* (Curtis) Skeels       | NSW, QLD, VIC | Swampy woodland       | Resistant to high            |
| *Callistemon viminalis* (Gaertn.) G.Don       | NSW, QLD     | Wet forest            | Resistant to high            |
| *Corymbia citriodora* Hook             | NSW, QLD     | Dry sclerophyll forest | Resistant to high            |
| *Eucalyptus moluccana* Roxb            | NSW, QLD     | Grassy woodland       | Resistant to high            |
| *Eucalyptus saligna* Sm                | NSW, QLD     | Wet forest            | Resistant to moderate        |
| *Leptospermum trinervium* (Sm.) Joy Thomps | NSW, QLD, VIC | Dry sclerophyll forest | Resistant to high            |

Information on species distribution and habitat was obtained from PlantNET (Royal Botanic Gardens and Domain Trust 2019) while susceptibility categories were obtained from the Australian *Austropuccinia psidii* host list (Giblin and Carnegie 2014). Distribution abbreviations are NSW, New South Wales; QLD, Queensland; VIC, Victoria
using aluminium foil and tape. The headspace within the beaker was then allowed to equilibrate for 20 min. After this equilibration period, a solid-phase micro-extraction (SPME) fibre (red 100 μm polydimethylsiloxane; Supelco, Bellefonte, PA, USA) enclosed within a 24 Ga manual holder (Supelco, Bellefonte, PA, USA) was introduced through the foil seal on the spout of the beaker and exposed to the headspace for 20 min until equilibrium between the headspace and fibre was reached. We used 100 μm polydimethylsiloxane SPME fibre because it is designed for the absorption of low molecular weight or volatile compounds that require a thick coating (Spietelun et al. 2010). These VOC sampling methods are adapted from widely used techniques to assess the composition and quantities of plant VOC emissions in ecological studies (Low et al. 2014; Stutz et al. 2016; Manea et al. 2019, 2021). All sampling was performed within an A1000 growth cabinet with CMP 6010 controller (Conviron, Winnipeg, MB, Canada) to ensure that temperature (25 °C), photosynthetically active radiation (1100 μmol m−2 s−1) and humidity (70%) were constant across samples. Sampling was conducted between 0800 and 1300 h when the plants were the most photosynthetically active.

We analysed samples using a single quadrupole gas chromatograph-mass spectrometer-QP 2010 (Shimadzu, Kyoto, Honshu, Japan) with a Rxi-5MS column (5% diphenyl 95% dimethylpolysiloxane, 30 m × 0.25 i.d., 0.25 μm film thickness; Restek Corporation, Bellefonte, PA, USA), and a split-splitless injector fitted with a Sky liner (1.8 mm × 5 mm × 95 mm; Restek Corporation, Bellefonte, PA, USA). The column was conditioned at 300 °C for 30 min at the start of every sampling day. Likewise, SPME fibres were conditioned in the injection port at 270 °C for 30 min at the start of every sampling day, and for 10 min between samples. The gas chromatograph was operated in split-less mode and used helium carrier gas (99.999%; BOC, North Ryde, NSW, Australia) at a constant flow rate of 1.5 mL per min. SPME fibres were manually inserted into the injection port and the gas chromatograph oven was held at 50 °C for 1 min. The temperature was then increased by 10 °C per min for 20 min to reach 250 °C, then held for 3 min (total run time = 24 min). The mass spectrometer was set to detect from 1 min onwards and to scan mass from 50 to 600 m/z. Post-run analysis software (Shimadzu, Kyoto, Honshu, Japan) was used to identify peaks with a slope of greater than 1000 total ion current/min. Peaks that had an 80% or greater similarity match to a terpene using the NIST 2002 spectral library were retained. These retained peaks were then separated into monoterpenes (C10H16) and sesquiterpenes (C15H24) and their total ion current were recorded.

**Phenolic measurements**

After measuring the VOCs, one new fully expanded leaf from each plant was selected and frozen immediately in liquid nitrogen. The total phenolic content for each of these leaves was then measured using a Folin-Ciocalteu assay with gallic acid as the quantification standard (8-point standard curve, 0–250 μg/mL). The method followed is outlined in Ainsworth and Gillespie (2007). Briefly, 20 mg of crushed leaf material was homogenised with 95% (vol/vol) methanol in centrifuge tubes and left to incubate in the dark at room temperature for 48 h. This process was also repeated for the methanol blanks and gallic acid standards. After 48 h, samples were centrifuged at 13,000 g for 5 min at room temperature. For each sample, standard or blank, 100 μl of the supernatant was collected into fresh centrifuge tubes and mixed thoroughly with 200 μl 10% (vol/vol) F–C reagent. We then added 800 μl 700 nM solution of sodium carbonate to each centrifuge tube and left them to incubate at room temperature for 2 h. Finally, 1 mL of each sample, standard or blank, was transferred into cuvettes and absorbance was read at 765 nm using a spectrophotometer (Mettler Toledo, Columbus, OH, United States). Total phenolic content was then calculated as mg gallic acid equivalent per g of leaf extract using the calculated standard curve.

All remaining leaves on each plant were then tagged to measure the pre-inoculation leaf area at the conclusion of the experiment.

**Austropuccinia psidii inoculation and susceptibility scoring**

After the pre-inoculation secondary metabolite measurements were completed (i.e. 14 weeks after the start of the experiment), 12 of the 16 individuals of each species were inoculated with A. psidii. Inoculation was carried out by suspending the urediniospores...
in light mineral oil (Australasian Solvents and Chemicals Company Ltd, Loganholme, QLD, Australia) at a concentration of 2 mg mL\(^{-1}\) and then spraying the solution evenly on the plants using a hydrocarbon propellant pressure pack. Individuals of a highly susceptible species, Syzygium jambos, were used as ‘positive controls’ to confirm that the inoculated uninfected plants were resistant (Giblin and Carnegie 2014). The control plants (remaining four plants per species) were sprayed with the mineral oil in absence of inoculum. After inoculation, plants were kept within an A1000 growth cabinet with CMP 6010 controller (Conviron, Winnipeg, MB, Canada) for 24 h to ensure ideal conditions for spore germination (95% relative humidity at 22 °C in darkness). After this procedure, the plants were transported back to a separate glasshouse from the uninoculated plants.

Two weeks after the plants were inoculated (i.e. 16 weeks after the start of the experiment), their susceptibility category was scored using the unified scale proposed by Berthon et al (2018). The scale has four categories: Resistant = no symptoms on new leaves and shoots, low = infection with minimal sporulation on new leaves and shoots (< 10%), moderate = infection with moderate sporulation on new leaves, shoots and stems (10–50%), high = infection with abundant sporulation on new leaves, shoots and stems (> 50%) with possible dieback. After susceptibility was scored, terpene and phenolic post-inoculation measurements were carried out on all plants using the same methods described above. In addition to the plants, we sampled the VOC emissions of the pure urediniospores and did not detect any terpenes. However, it should be acknowledged that fungi are capable of emitting similar VOCs to plants (Quintana-Rodriguez et al. 2018). Once the secondary metabolite measurements were completed, the leaves from each plant were removed and separated based on being pre- (tagged leaves only) and post-inoculation (all leaves). The pre- and post-inoculation leaf area of each plant was then measured using a LI-3100C Area Meter (Li-Cor, Lincoln, NE, United States) and used to calculate the amount of monoterpenes and sesquiterpenes emitted per cm\(^{2}\) of leaf area.

Data analysis

To determine if there was a significant difference in phenolic and terpene production between pre- and post-inoculation, paired t-tests or Wilcoxon signed-rank tests were used when a Shapiro–Wilk test showed that the differences between the pairs were or were not normally distributed, respectively. These analyses were performed at the species-level. To ensure any differences were a result of the A. psidii inoculation, paired t-tests were used to compare the pre- and post-inoculation phenolic and terpene production in the uninoculated control plants. These analyses were performed across all species.

To determine whether phenolic and/or terpene production induced after inoculation was related to A. psidii susceptibility across all species, ordinal logistic regressions were used. The data was first checked to ensure it fulfilled the no multi-collinearity and proportional odds assumptions of ordinal logistic regression. The predictor variable in the models was the change in the amount and number of phenolics, monoterpenes or sesquiterpenes produced by the pre- and post-inoculation plants. The response variable was susceptibility. These analyses were performed across all species.

The statistical analyses were performed in R (v. 3.5.1, R Development Core Team, 2014). All analyses used plant-level data and had the significance level set at 0.05. The ordinal logistic regressions were performed in the R package ‘MASS’ (Venables and Ripley 2002). The figures were produced using the R package ‘ggplot2’ (Wickham 2016).

Results

Phenolic (F = – 1.39, p = 0.177) and terpene production (monoterpene: F = 1.70, p = 0.101; sesquiterpene: F = 1.68, p = 0.105) did not significantly differ between pre- and post-inoculation in the uninoculated control plants. Therefore, we can conclude that any difference in phenolic and terpene production between pre- and post-inoculation in the inoculated plants is due to A. psidii inoculation.

Four of the seven study species (C. citrinus: F = – 2.90, p = 0.014; E. moluccana: X\(^2\) = 75.00, p = 0.005; E. saligna: F = – 3.34, p = 0.005; L. trinervium: F = – 4.06, p = 0.002) produced a significantly greater amount of phenolics post-inoculation (Fig. 1a; Table S1). None of the study species differed in the amount or number of monoterpenes they produced pre- and post-inoculation (Fig. 1b, c;
Fig. 1 Mean pre- and post-inoculation production of phenolics (a amount), monoterpenes (b amount, c number) and sesquiterpenes (d amount, e number) for each species. Asterisks indicate significant differences ($p < 0.05$) in secondary metabolite production between pre- and post-inoculation. Note that missing columns indicate that the species did not produce the secondary metabolite in question. Boxplot: the box displays the middle 50% of the data (interquartile range, IQR). Within the box the horizontal bar represents the mean value. Vertical bars extend to 1.5 IQR, while outliers beyond 1.5 IQR are indicated by dots.
Table S2). Two study species produced a significantly greater amount (*C. viminalis*: $F = -4.38, p = 0.001$; *E. saligna*: $F = -5.89, p < 0.001$) and number (*C. viminalis*: $X^2 = 66.00, p = 0.004$; *E. saligna*: $F = -5.16, p < 0.001$) of sesquiterpenes post-inoculation (Fig. 1d, e; Table S3).

Three (*A. costata, E. saligna, L. trinervium*) of the seven study species had individuals that were scored as resistant to *A. psidii* infection while three (*C. citriodora, C. viminalis, E. moluccana*) of the other study species had individuals that were scored as highly susceptible to *A. psidii* infection (Fig. 2). Overall, *E. saligna* had the most individuals that scored in the resistant or low susceptibility categories while *C. viminalis* had the most individuals that scored in the medium or high susceptibility categories (Fig. 2).
Susceptibility to A. psidii infection was negatively related to induced monoterpene production in terms of amount (z = 2.20, p = 0.028, Fig. 3b) but not number (z = 1.45, p = 0.146, Fig. 3c) across all species. In contrast, there was no relationship between induced phenolic (amount: z = −0.71, p = 0.480, Fig. 3a) and sesquiterpene (amount: z = −0.20, p = 0.842, Fig. 3d; number: z = 1.81, p = 0.071, Fig. 3e) production, and susceptibility across all species.

Discussion

In 2010, the fungal plant pathogen that causes Myrtle rust, Austropuccinia psidii, was first detected in Australia (Carnegie et al. 2010) and has since become a major threat to Australian vegetation communities (Carnegie et al. 2016; Pegg et al. 2017; Fernandez-Winzer et al. 2020). Despite this, little is known about the role secondary metabolites play in plant susceptibility to A. psidii. Therefore, the aim of this study was to determine whether secondary metabolite production (phenolics, terpenes) is induced after A. psidii inoculation and how this relates to plant susceptibility to A. psidii.

When pre-formed anti-fungal secondary metabolite production is insufficient to prevent infection, plants generally respond by inducing the biosynthesis of these compounds at the infection site (Kosuge 1969). We found this to be the case in five of our seven study species, with four species (C. citrinus, E. moluccana, E. saligna, L. trinervium) increasing their phenolic production and two species (C. viminalis, E. saligna) increasing their sesquiterpene production after inoculation with A. psidii. This result is unsurprising given the important anti-fungal activities performed by secondary metabolites in Myrtaceae family (Yong et al. 2019). This is exemplified by the fact that greater 1,8-cineole production is required for co-existence with A. psidii in Myrtaceae species (Yong et al. 2019). This may explain why relatively few Myrtaceae species (e.g. Rhodaninia rubescens, Rhodomyrtus psidioides) in Australia have been severely impacted by A. psidii despite not having co-evolved with it. It should be noted though that there is no universal secondary metabolite that confers resistance to A. psidii in Myrtaceae species (Yong et al. 2019).

The intention of this study was to have a broad focus by testing several species rather than concentrating on a single species. However, it is clear that the next step for future research is to examine individual chemical compounds at the species-level. Given the extraordinary diversity of secondary metabolites produced within the Myrtaceae family (Stefanello et al. 2011) and that our understanding of their function is poor (Naidoo et al. 2014; Padovan et al. 2014), the effort needed to conduct this research would not be trivial. As such, only a handful of studies have examined the role of individual secondary metabolite compounds (e.g. terpenes: Bustos-Segura et al. 2015; leaf wax: Dos Santos et al. 2019) in plant susceptibility to A. psidii. In addition, another research avenue worth
Fig. 3  The production of phenolics (a amount), monoterpenes (b amount, c number) and sesquiterpenes (d amount, e number) induced after inoculation for each susceptibility category across the seven study species. Note that figure b represent significant relationship between change in monoterpane amount and susceptibility. Boxplot: the box displays the middle 50% of the data (interquartile range, IQR). Within the box the horizontal bar represents the mean value. Vertical bars extend to 1.5 IQR, while outliers beyond 1.5 IQR are indicated by dots.
considering is the role of physical barriers (e.g. leaf hairs, toughness; wax layers; Xavier et al. 2015; Silva-Souza et al. 2017; Bonora et al. 2020a), proteins (e.g. enzymes; Boava et al. 2010; Hsieh et al. 2018) and physiological processes (Moon et al. 2007) in preventing A. psidii infection. At present, these factors have received very limited research attention with the majority of studies focusing on important forestry species (Naidoo et al. 2014). Overall, the desired outcome of these research efforts would be to identify biomarkers that confer resistance to A. psidii infection. This has already begun with 1,8-cineole and α-terpinyl acetate having been identified as biomarkers for A. psidii resistance in Eucalyptus grandis × E. urophylla hybrids (Hantao et al. 2013). Using these biomarkers, less susceptible individuals can be identified and utilised in resistance breeding efforts, which may have a profoundly positive effect on the success of both commercial and conservation projects (Makinson 2018).

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Conflict of interest None.

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