



New Zealand Myrtaceae are susceptible to a strain from the *Eucalyptus* biotype of *Austropuccinia psidii* present in South America

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Abstract The international spread of the myrtle rust pathogen, *Austropuccinia psidii*, can be largely attributed to the “pandemic” biotype that has more than 450 host species. However, within South America, the putative native range of *A. psidii*, multiple biotypes have been characterised, each with a restricted known number of hosts. These biotypes may pose

a significant biosecurity threat to countries already affected by the pandemic biotype. Here, we report the susceptibility of four species of Myrtaceae from New Zealand, pōhutukawa (*Metrosideros excelsa*), mānuka (*Leptospermum scoparium*), kānuka (*Kunzea ericoides*), and rawiri mānuka (*Kunzea ericoides* ‘gumland ecotype’), to a strain of the *Eucalyptus* biotype of *A. psidii*. Symptoms and signs developed on inoculated plants of all species. Qualitative resistance phenotypes, with no disease development, were

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observed for all four species. However, no hypersensitive responses were observed. As seen for other biotypes, pōhutukawa had the greatest susceptibility, while kānuka had the lowest. These findings are consistent with prior work, showing that the *Eucalyptus* biotype can infect a broader range of species than its field host association implies. As well as uredinia (asexual spores), telia (sexual spores) developed on several plants, indicating that these species could provide a universal host for sexual reproduction and outcrossing between biotypes. Knowledge that the *Eucalyptus* biotype of *A. psidii* is virulent on several indigenous New Zealand Myrtaceae will inform future biosecurity risk assessments. These findings highlight the need to develop diagnostics tools to differentiate between biotypes and allow rapid responses to potential future incursions.

Keywords Biological invasion · Biosecurity risk assessment · Disease resistance and susceptibility screening · Host adaptation · Pathogen virulence

Introduction

Myrtle rust is caused by the invasive fungal pathogen *Austropuccinia psidii* (G. Winter) Beenken (Sphaerophragmaceae). With a putative native range in South America, the pathogen is now established in all continents except Europe and Antarctica. The pathogen infects a wide range of commercially and ecologically important species of Myrtaceae, with at least 480 hosts recorded throughout its expanding geographic range (Soewarto et al. 2019). *Austropuccinia psidii* primarily spreads by windborne asexual urediniospores that infect young actively growing vegetative and reproductive host tissues (Glen et al. 2007). The pathogen can also reproduce sexually through the development of teliospores (Pegg et al. 2014b; Roux et al. 2016) that produce basidiospores able to infect and complete the life cycle on single Myrtaceae species (McTaggart et al. 2018). Symptoms on susceptible hosts range from minor leaf spots through to severe shoot dieback. Severe infection

can directly and indirectly restrict host fecundity and result in plant mortality (Carnegie et al. 2016; Soewarto et al. 2018; Sutherland et al. 2020). In Australia, local extinction of highly susceptible species has been observed and further extinctions are predicted (Pegg et al. 2017; Fensham et al. 2021).

A total of ten genetic clusters of *A. psidii* have been identified, with most diversity in the centre of origin in South America (Graça et al. 2013; Roux et al. 2016; Stewart et al. 2018). Seven clusters (C2, C3, and C5–C9) are restricted to South and Central America. These clusters are each associated with a narrow range of hosts and may have specific host associations. For example, cluster C2 represents strains from *Eucalyptus* species and rose apple (*Syzygium jambos*) collected in Brazil and Uruguay, while cluster C6 was associated with guava (*Psidium guajava* and *P. guineense*), and C9 was associated with jaboticaba (*Myrciaria cauliflora*) (Stewart et al. 2018). While strains from field sampling had clear host associations, this does not preclude the ability of these strains to cause disease on a wider range of hosts in controlled laboratory inoculations, although there is evidence of significant variability in aggression and virulence towards different hosts (Aparecido et al. 2003; Morales et al. 2023). For example, controlled inoculation studies found that an *A. psidii* strain from *Eucalyptus grandis* was virulent on several *Melaleuca* species from Southeast Asia and Australia (Zauza et al. 2010) and strains from *Eucalyptus* and jaboticaba were virulent on ‘ōhi’a (*Metrosideros polymorpha*) native to Hawaii (Costa da Silva et al. 2014). In contrast, strains from guava caused only very minor symptoms on ‘ōhi’a (Costa da Silva et al. 2014). Indeed, strains from guava (C6 cluster) appear to have a strong degree of host specificity and are more aggressive on guava than other hosts (Aparecido et al. 2003). In a recent cross-inoculation study Morales et al. (2023) found that strains from rose apple and guava were able to infect but did not sporulate on the other host.

The remaining clusters (C1 and C4) represent the pandemic biotype, that is associated with a diverse range of hosts and is found in Colombia (Granados et al. 2017) and throughout much of the pathogen’s invasive range, including Hawaii (Graça et al. 2013; Stewart et al. 2018), Florida (Stewart et al. 2018), Mexico (Stewart et al. 2018), Australia (Machado et al. 2015), China (Machado et al. 2015), Indonesia

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(McTaggart et al. 2016), New Caledonia (Machado et al. 2015; Soewarto et al. 2018), Singapore (du Plessis et al. 2019), and New Zealand (du Plessis et al. 2019; Ho et al. 2019). An additional distinct genetic cluster has been identified on multiple hosts in South Africa (Roux et al. 2016; du Plessis et al. 2019; McTaggart et al. 2020).

A strain of *A. psidii* belonging to the pandemic biotype was first detected in New Zealand in autumn 2017 (du Plessis et al. 2019; Ho et al. 2019; Toome-Heller et al. 2020). In the first year following arrival, *A. psidii* was detected throughout much of the North Island and the top of the South Island (Toome-Heller et al. 2020) and is now established throughout most of its predicted climatically suitable range (Beresford et al. 2018). New Zealand is home to 18 native species of Myrtaceae in six genera, as well as over 100 non-native species across 43 genera (Buys et al. 2016; Heenan et al. 2023). These species hold cultural significance for Māori (the Indigenous people of New Zealand (Aotearoa)), who regard many as taonga (treasures) (Black et al. 2019), and play critical roles in the country's ecological health and economy, contributing to biodiversity, habitat restoration, and commercial sectors, such as honey production (Ballingall and Pambudi 2017; Jo et al. 2022; McCarthy et al. 2024). Reported hosts in New Zealand include 19 native taxa (14 species and five hybrids), and 17 exotic taxa (Toome-Heller et al. 2020; Ministry for Primary Industries 2024). Endemic ramarama (*Lophomyrtus bullata*), rōhutu (*L. obcordata*), and maire tawake (*Syzygium maire*) appear to be the most affected species to date, with records on pōhutukawa (*Metrosideros excelsa*) rising recently, and only a few reports on mānuka (*Leptospermum scoparium*), and none on kānuka (*Kunzea ericoides*). Smith et al. (2020) assessed the susceptibility of pōhutukawa, ramarama, rōhutu, mānuka, kānuka, and rawiri mānuka (*K. ericoides* 'gumland ecotype', *Kunzea linearis* sensu de Lange 2014) to the pandemic biotype. Many open-pollinated families of New Zealand mānuka, kānuka, and rawiri mānuka contained both individuals with either qualitative (i.e. no symptom development) or quantitative resistance (i.e. lower severity of symptom development relative to other individuals) and susceptible phenotypes, and the proportion of resistant seedlings varied among families and provenances. Conversely, qualitative resistance phenotypes were not found for either *Lophomyrtus*

species and only a single pōhutukawa seedling was fully resistant, although small numbers of seedlings were tested for these three species.

While the pandemic biotype is now well established in New Zealand, other exotic strains and biotypes are considered a biosecurity risk. Incursions from exotic strains of pathogens already present in a region can lead to shifts in virulence and aggression, with examples of this phenomena seen for wheat rust fungi (Ellis et al. 2014). In Florida, *A. psidii* was largely recorded on allspice (*Pimenta dioica*) for nearly 20 years before sudden expansion of host range, with the introduction of additional strains hypothesised as a driver (Loope and La Rosa 2008). Testing the susceptibility of native New Zealand Myrtaceae to exotic strains and biotypes can support risk assessment. For example, Soewarto et al. (2021) assessed the susceptibility of four species of cultural, environmental and/or economic importance for New Zealand: pōhutukawa, mānuka, kānuka, and rawiri mānuka to the South African biotype. Pōhutukawa is an iconic tree regarded as "taonga" (treasured) in Māori culture, while mānuka and kānuka are ecologically important and valued for their roles in honey production and ecosystem restoration (Bylsma et al. 2014; Teulon et al. 2015; Jo et al. 2022; McCarthy et al. 2024). Rawiri mānuka, although less prominent, is highly endemic and critical for maintaining soil stability in coastal shrublands (de Lange 2014). All four species were susceptible to the South African biotype under controlled conditions, highlighting the potential threat of this strain to New Zealand Myrtaceae-based ecosystems and industries.

Like the pandemic biotype, the South African biotype of *A. psidii* has been isolated from multiple hosts. In comparison, biotypes within the putative native range of the pathogen were identified from a narrower range of hosts (Stewart et al. 2018). To date, two biotypes have been described in Uruguay: one associated with exotic *Eucalyptus* species and another associated with the native sweet flower (*Myrrhinium atropurpureum* var. *octandrum*) (Pérez et al. 2011; Stewart et al. 2018). The pathogen has also been isolated from another native host in Uruguay, the guaviyu (*Myrcianthes pungens*), however these strains have not been genotyped (Pérez et al. 2011). There is significant concern about strains from the *Eucalyptus* biotype and their potential to cause high impact in Australia where these hosts are native.

Should a *Eucalyptus* biotype strain arrive in Australia it would likely disperse to New Zealand, and whether strains of the *Eucalyptus* biotype are virulent towards New Zealand's native Myrtaceae is currently unknown.

To support the risk assessment of exotic *A. psidii* strains to New Zealand Myrtaceae, and building on previous studies with these host species and different strains, we screened the susceptibility of four New Zealand species, pōhutukawa, mānuka, kānuka, and rawiri mānuka, to a strain of the *Eucalyptus* biotype of *A. psidii* isolated from *Eucalyptus globulus* in Uruguay.

Materials and methods

Plant material and growth conditions

Seeds of four New Zealand species: pōhutukawa (*M. excelsa*), mānuka (*L. scoparium*), rawiri mānuka (*K. ericoides* 'gumland ecotype') and kānuka (*K. ericoides*) were harvested from open-pollinated mother plants, with permission from mana whenua (indigenous Māori with territorial authority). The mother plants were in natural populations across twelve locations in the northern half of the North Island of New Zealand. Detailed GPS locations cannot be publicly shared, in line with mana whenua cultural agreements, but seed batches were uniquely numbered to maintain seed family identification and the region of origin is reported (Table S2).

Seeds were weighed and pre-packed in small paper envelopes. They were then fungicide treated with Mancozeb® (c. 0.1–1 g depending on seed weight) inside a Cytotoxic Drug Safety Cabinet. This was carried out at The New Zealand Institute for Plant and Food Research Limited facilities at Palmerston North under the examination of an AsureQuality inspector to obtain phytosanitary certificates before postage to the Facultad de Agronomía, Paysandú, Uruguay (permit no. 1341772; 1341773; 1341774; 1365029), where all experimental work was undertaken.

Seeds were germinated in October 2019 and March 2020 in plastic trays with potting mix (Table S3) and plants maintained in a greenhouse under natural light (no supplementary light) with temperatures held between 20 and 25 °C. Plants were watered as necessary and no pesticides were applied. After 4 months,

a total of 322 seedlings, 29 rawiri mānuka (5 families), 113 kānuka (22 families), 84 mānuka (17 families), and 96 pōhutukawa (15 families) were selected for inoculation (Table S2).

Inoculum sourcing

A single pustule strain from *Eucalyptus globulus* (strain #151), conserved in the fungal collection of National Institute of Agricultural Research (INIA), was used to inoculate highly susceptible rose apple (*Syzygium jambos*) to mass-produce the spore inoculum for the inoculation experiments. Urediniospores from fresh uredinia were harvested using a vacuum pump and sterile Pasteur pipette, desiccated with silica gel for at least 48 h and stored at -80 °C until needed.

Genetic characterisation of strain #151

Four microsatellite loci described in Graça et al. (2013) and Stewart et al. (2018) were scored for the *A. psidii* strain #151, including PpSSR012, PpSSR014, PpSSR018, PpSSR087. These four microsatellites have been selected as suitable markers for identifying the stains to biotypes. PCR amplifications were performed using a Mastercycler ProS thermocycler (Eppendorf, Hamburg, GE) with an initial denaturation period of 95 °C for 5 min, followed by three cycles at 95 °C for 30 s, 40–55 °C (depending on the locus) for 30 s, 72 °C for 80 s, 35 cycles at 94 °C for 15 s, 40–55 °C (depending on the locus) for 15 s and 45 s at 72 °C, followed by a final extension period of 72 °C for 5 min, ending with a 4 °C hold. Fragment analysis was conducted at the Genewiz, Azenta Life Sciences, via capillary electrophoresis using an ABI 3700 DNA automated sequencer (Life Technologies Corporation, Carlsbad, CA, USA). Positive and negative controls were included. Allele sizes were estimated using marker standards (ROX GeneFlo 625; CHIMERx, Milwaukee, WI, USA) and scored using Geneious Prime 2022.0.2 and the associated Microsatellite Plugin. Population genetic analysis to compare strain #151 with known *A. psidii* reference genotypes of the *Eucalyptus*, pandemic, and guava biotypes was performed using GenAlEx v6.501 (Peakall and Smouse 2006). Principal coordinates analysis (PCoA), based on a covariance matrix with data standardisation (Smouse and Peakall 1999),

was performed ($n=25$) with reference strains for each of the other biotypes, as documented in Stewart et al. (2018).

Inoculation protocol

Frozen urediniospores were removed from $-80\text{ }^{\circ}\text{C}$ storage and allowed to warm to room temperature prior to being added to 0.05% Tween 20 (Sigma®) solution in sterile distilled water. Urediniospore concentration was determined using a haemocytometer and adjusted to 1×10^5 spores ml^{-1} (Pegg et al. 2014a). Seedlings were watered at their bases before inoculation. Inoculum was applied to both the upper and lower surfaces of young leaves with an airbrush, ensuring an even coating of fine mist while avoiding run-off. After inoculation, seedlings were covered with a plastic box that was sprayed with water on the inside to create a moist environment and kept at $20\text{ }^{\circ}\text{C}$ in the dark for 24 h. The seedlings were then maintained in a growth chamber under controlled conditions ($25\text{ }^{\circ}\text{C}$, 75–85% relative humidity, 12 h photoperiod) and watered daily. Seedlings of rose apple and *E. grandis* with known susceptibility were used as positive controls, to confirm inoculum was viable and that environmental conditions were suitable for infection and disease expression. Two negative-controls for each tested species were mock-inoculated with sterile distilled water. Inoculation trials were laid out in an incomplete randomised block due to the unbalanced numbers of seedlings tested for each species.

Disease assessment

All seedlings were examined individually to evaluate disease severity at 14 and 21-days post inoculation (DPI). We were unable to record the first appearance of different signs and symptoms due to COVID-19 related restrictions limiting access to the experiment during the assessment period. Assessment of disease severity was conducted independently on the leaves and on the stems, using both a variation of the infection scales developed by Smith et al. (2020) and visually estimating the percentage of leaf or stem tissue area covered by rust pustules and the percentage of dying-blackening shoots, leaves or stems. A 5-point ordinal scale was used to score disease severity on leaves, stem severity was scored on a 4-point ordinal scale, and dieback was scored on a 5-point ordinal scale (Table S1). After the experiment, all plants were

destroyed, as agreed with mana whenua (indigenous Māori with territorial authority).

Statistical analysis

All statistical computations were performed using R version 3.6.1 (R Core Development Team 2019). Mixed-effects models were used to analyse four response variables: disease severity on leaves assessed on a 5-point ordinal scale, percentage of leaf area covered by lesions and pustules assessed on a scale of 0–100, stem severity score assessed on a 4-point ordinal scale, and dieback incidence classing seedlings as either with or without dieback (i.e. a binary response). Ordinal scoring of dieback was not used because there were no frequency counts in categories 4 and 5 and very low frequency counts in categories 2 and 3. The total number of seedlings for rawiri mānuka was not large enough to provide a reliable estimate of the effect of species on the response variables of interest. This species was therefore excluded from the analysis comparing species. Separate mixed-effects models were used to test the effect of days past inoculation on the response variables of interest for rawiri mānuka.

Cumulative link mixed models (CLMMs) (Agresti 2002; Christensen 2022) with a logit link and flexible thresholds fitted using Laplace approximation were applied to analyse the disease severity scores on leaves and stems. Percentage of leaf area covered by lesions and pustules was analysed using a generalised linear mixed model (GLMM) assuming a beta distribution based on the parameterisation of Ferrari and Cribari-Neto (2004) and logit link function. Prior to analysis, the percentage of symptoms of leaf area covered by lesions and pustules on a scale of 0–100 was converted into the open standard unit interval (0,1) to satisfy conditions of the beta distribution by using $(y \times (n-1) + 0.5)/n$, where y is the proportion of leaf area covered by lesions and pustules on a scale of 0–1 and n is the number of observations, as recommended by Smithson and Verkuilen (2006). The incidence of dieback was analysed using GLMMs with a binomial distribution and logit link function. All GLMMs were fitted by maximum likelihood via Template Model Builder (TMB), using automatic differentiation to estimate model gradients and the Laplace approximation for handling random effects [R package glmmTMB (Brooks et al. 2017)].

For all models testing the effect of species and/or days post inoculation on the response variables, ‘species’ (factor with three groups: ‘kānuka’, ‘mānuka’, and ‘pōhutukawa’) and ‘days post inoculation (DPI)’ (factor with two levels: ‘14 DPI’ and ‘21 DPI’) and their interaction were included as fixed effects. The random model comprised of random effects for ‘seedling’ nested in ‘family’ and ‘family’. For the analysis of the effect of days post inoculation on the response variables for rawiri mānuka, ‘days post inoculation’ was modelled as the sole fixed effect and ‘seedling’ nested in ‘family’ were incorporated as random effects.

For model validation, plots of Pearson residuals against the fitted values and versus each explanatory variable in the model, and residual diagnostics based on a simulation-based approach [R package DHARMA, (Hartig 2022)] were used. For all models, the significance of the fixed terms was assessed via a backward variable selection using a likelihood-ratio test and Akaike’s Information Criterion (AIC) (Zuur et al. 2009). Significant two-way interactions were sliced (partitioned analysis) by applying a multiple-comparison procedure based on the significance of estimated marginal mean differences [R package emmeans, (Lenth et al. 2023)]. To account for multiple testing, the resulting *p*-values were adjusted using the Tukey method.

Results

Genetic characterisation

Austropuccinia psidii strain #151 had identical allele sizes to the reference strains of the *Eucalyptus* biotype and grouped with these strains in the principal coordinate analysis (PCoA) (Figure S1; Figure S2).

Symptom and sign development

No symptoms and signs developed on mock-inoculated negative control plants. Symptoms and signs developed on inoculated plants of all species (Fig. 1). The first symptoms consisted of red- or purple-coloured spots. Later, uredinia developed on foliage and juvenile stems. Although in most cases only uredinia formed, on some seedlings both uredinia and telia were observed; in some cases, only telia developed. Telia were observed on all four species but were more common on pōhutukawa and mānuka than rawiri mānuka and kānuka (Table S4). Telia (co-occurring with uredinia) were more common at 21 days post inoculation (DPI) compared to 14 DPI. Some degree of stem and foliar blight resulting in tip/shoot die-back was also observed on all four species. Symptom development and disease severity on rose apple and *E. grandis* seedlings used as positive controls in this



Fig. 1 Symptoms and signs caused by the *Eucalyptus* biotype of *Austropuccinia psidii* on leaves and stems of four New Zealand Myrtaceae species: A–B: pōhutukawa (*Metrosideros*

excelsa), C: mānuka (*Leptospermum scoparium*), D: kānuka (*Kunzea ericoides*), E: rawiri mānuka (*Kunzea ericoides* ‘gumland ecotype’)

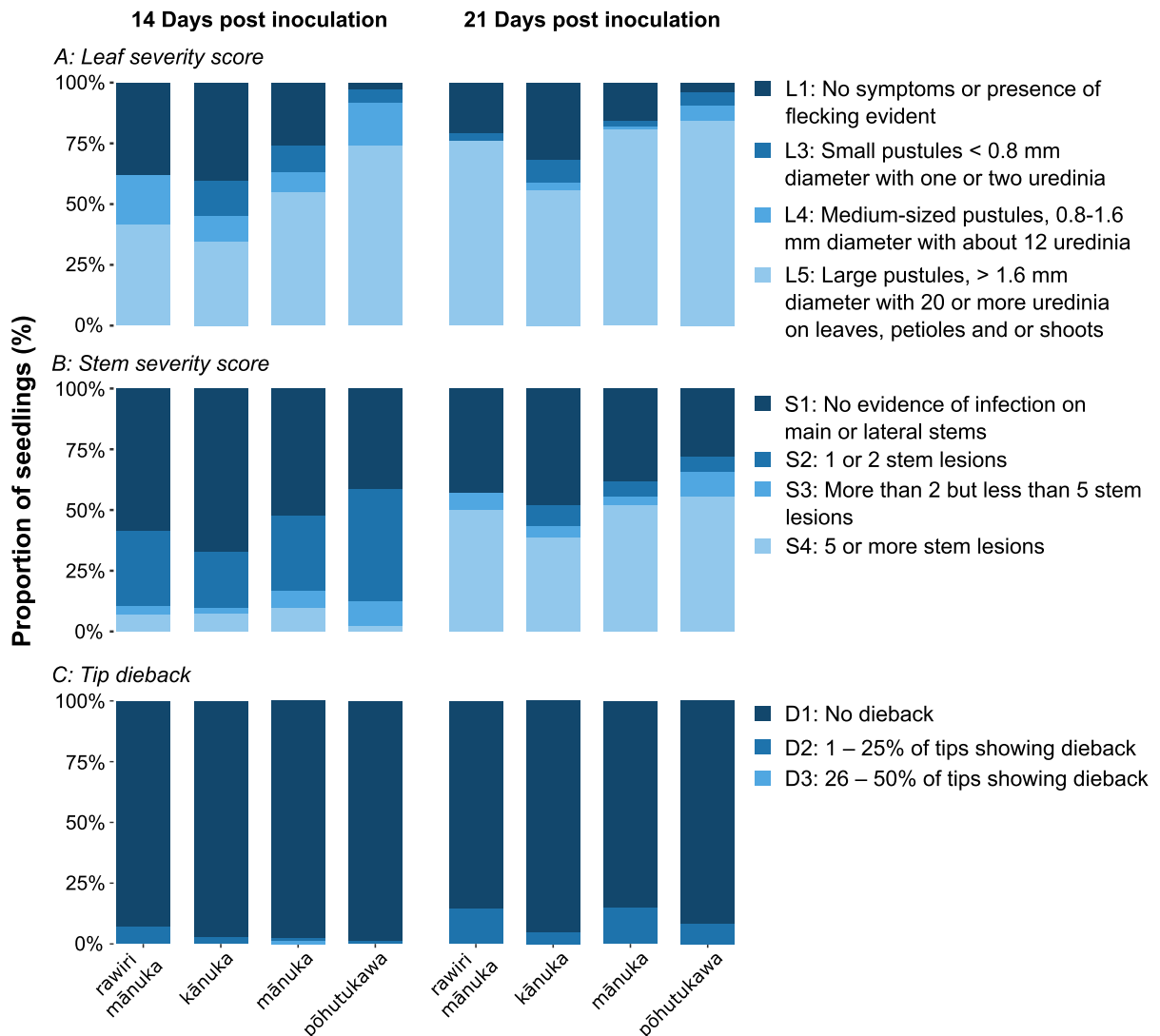


Fig. 2 Proportion of seedlings with each disease severity score (Table S1) for A: leaves, B: stems and C: dieback for rawiri mānuka (*Kunzea ericoides* ‘gumland ecotype’), kānuka

(*K. ericoides*), mānuka (*L. scoparium*), and pōhutukawa (*M. excelsa*) at 14- and 21-days post inoculation

study (data not shown) were comparable with those published by Pérez et al. (2011).

At 14 DPI, 62%, 59%, 74% and 97% of rawiri mānuka, kānuka, mānuka, and pōhutukawa seedlings showed symptoms on leaves (Fig. 2A), while evidence of infection on main or lateral stems was recorded on 41%, 33%, 48% and 58% of rawiri mānuka, kānuka, mānuka and pōhutukawa seedlings respectively (Fig. 2B). Tip dieback was recorded on 7%, 3%, 2% and 1% of rawiri mānuka, kānuka, mānuka, and pōhutukawa seedlings respectively at

14 DPI (Fig. 2C). The average percentage of leaf area covered by lesions and pustules was 20%, 8%, 18%, and 30% for rawiri mānuka, kānuka, mānuka, and pōhutukawa respectively at 14 DPI (Fig. 3).

Generally, there was an increase in symptom severity and incidence between 14 and 21 DPI. Seven kānuka and two mānuka seedlings died between 14 and 21 DPI and were removed from the analysis. At 21 DPI, 79%, 68%, 84% and 96% of rawiri mānuka, kānuka, mānuka, and pōhutukawa seedlings showed symptoms on leaves (Fig. 2A), while evidence of

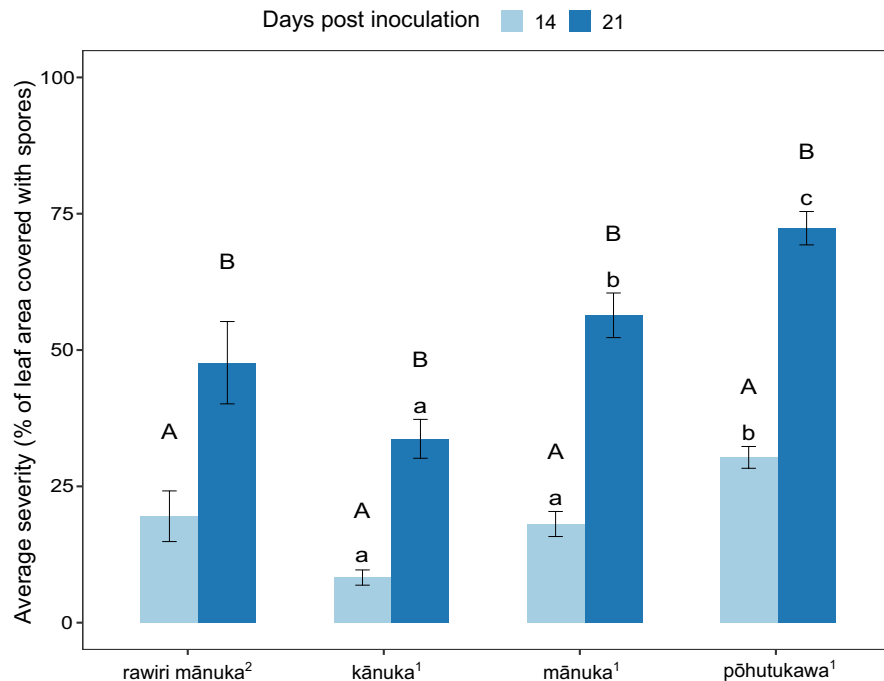


Fig. 3 Average severity (% of leaf area covered by lesions and pustules) $\pm$ standard errors (SE) of four species by days post inoculation. Different lower-case letters indicate statistically significant differences between species within days post inoculation (rawiri mānuka was not included in this analysis), whereas different upper-case letters denote statistically significant differences between days post inoculation within species (multiple comparison procedures were performed using

Tukey-adjusted method at $\alpha=0.05$). ¹The beta GLMM analysis of percentage of leaf area covered by lesions and pustules of the three species data as a function of species and/or days post inoculation with seedling nested in family included as random effects. ²The beta GLMM analysis of percentage of leaf area covered by lesions and pustules of rawiri mānuka data as a function of days post inoculation with seedling nested in family included as random effects

infection on main or lateral stems was recorded on 57%, 52%, 62% and 72% of rawiri mānuka, kānuka, mānuka and pōhutukawa seedlings respectively (Fig. 2B). Tip dieback was recorded on 14%, 4%, 14% and 8% of rawiri mānuka, kānuka, mānuka, and pōhutukawa seedlings respectively at 21 DPI (Fig. 2C). The average percentage of leaf area covered by lesions and pustules was 47.7%, 33.7%, 56.4%, and 72.3% for rawiri mānuka, kānuka, mānuka, and pōhutukawa respectively at 21 DPI (Fig. 3).

Disease severity on leaves on a 5-point ordinal scale

The percentage of seedlings that developed disease on leaves was significantly greater at 21 DPI than

14 DPI for rawiri mānuka, kānuka and mānuka (all $p < 0.05$; Table 1 and Figure S3) but did not significantly vary for pōhutukawa ($p = 0.088$; Table 1). For disease severity on leaves of kānuka, mānuka and pōhutukawa, there was a significant interaction between host species and DPI ($p < 0.01$; Table 2). The percentage of seedlings that developed disease on leaves was significantly lower for kānuka than the other species at both time points (Table 1 and Figure S3). At 14 DPI, for mānuka the percentage of seedlings with disease was significantly lower than for pōhutukawa (Tukey-adjusted method: $p < 0.01$; Table 1 and Figure S3) but did not differ significantly after 21 days ($p = 0.482$; Table 1 and Figure S3).

Table 1 Multiple comparison tests based on the Tukey-adjusted method at $\alpha=0.05$ for the analysis of disease severity on leaves using CLMMs for kānuka, mānuka, and pōhutukawa data and rawiri mānuka separately. The multiple comparison was performed using the latent (or link) scale

Comparison	Estimate	SE	z-value	p^z
<i>14 days past inoculation¹</i>				
Kānuka–mānuka	−3.03	1.15	−2.64	<0.05*
Kānuka–pōhutukawa	−6.21	1.09	−5.69	<0.001***
Mānuka–pōhutukawa	−3.18	0.90	−3.54	<0.01**
<i>21 days past inoculation¹</i>				
Kānuka–mānuka	−3.82	1.25	−3.07	<0.01**
Kānuka–pōhutukawa	−4.96	1.02	−4.87	<0.001***
Mānuka–pōhutukawa	−1.14	0.99	−1.15	0.482
<i>Rawiri mānuka²</i>				
14 days–21 days	−3.43	1.73	−1.98	<0.05*
Kānuka ¹				
14 days–21 days	−2.07	0.46	−4.45	<0.001***
Mānuka ¹				
14 days–21 days	−2.86	0.66	−4.33	<0.001***
Pōhutukawa ¹				
14 days–21 days	−0.82	0.48	−1.71	0.088

Results are given on the latent (logit) scale

¹The CLMM analysis of leaf score of the three species data as a function of species and/or days post inoculation with seedling nested in family included as random effects

²The CLMM analysis of leaf score of rawiri mānuka data as a function of days post inoculation with seedling nested in family included as random effects

^zSignificant values are denoted as follows: * $p<0.05$, ** $p<0.01$, and *** $p<0.001$

Percentage of leaf area covered with lesions and pustules

The average percentage of leaf area covered with lesions and pustules was greater at 21 DPI than at 14 DPI for all four species (all $p<0.01$; Table 3 and Fig. 3). There was a significant interaction between host species and DPI for the percentage of leaf area covered with lesions and pustules for kānuka, mānuka

and pōhutukawa ($p<0.05$; Table 2). At 14 DPI, percentage of leaf area covered by lesions and pustules was significantly greater on pōhutukawa than kānuka and mānuka (Tukey-adjusted method: $p<0.001$ and $p<0.05$ for kānuka and mānuka, respectively; Table 3 and Fig. 3) and almost significantly lower for kānuka compared to mānuka ($p=0.068$; Table 3). At 21 DPI, percentage of leaf area covered by lesions and pustules was significantly lower on kānuka than the other two species ($p<0.001$; Table 3 and Fig. 3) and significantly lower on mānuka than pōhutukawa ($p<0.01$; Table 3 and Fig. 3).

Stem severity score on a 4-point ordinal scale

The percentage of seedlings that developed disease on stems was significantly greater at 21 DPI than 14 DPI for all four species (all $p<0.001$; Table 4 and Fig. 2B). There was no significant interaction effect ($p=0.731$; Table 2) of DPI and host species on stem severity, but both individually had significant effects ($p<0.05$; Table 2). The percentage of seedlings with disease on stems was significantly greater on pōhutukawa than kānuka (Tukey-adjusted method: $p<0.01$; Table 4) but did not differ significantly between kānuka and mānuka ($p=0.100$; Table 4) or between mānuka and pōhutukawa ($p=0.690$; Table 4).

Dieback incidence

Dieback incidence was significantly greater at 21 DPI than 14 DPI for kānuka, mānuka and pōhutukawa ($p<0.001$; Table 5; Fig. 2C) and almost significantly greater at 21 DPI than 14 DPI for rawiri mānuka ($p=0.062$; Table 5; Fig. 2C). There was no significant interaction effect of host species and DPI ($p=0.474$; Table 2) and no significant effect of host ($p=0.880$; Table 2).

Evidence of qualitative resistance

Plants with qualitative resistance phenotypes (L1 and S1—see Table S1) were observed for all four species (Table 6). A hypersensitive response (L2) was not recorded for any of the inoculated seedlings. Symptom development could be observed on leaves only,

Table 2 Results of backwards selection procedures based on likelihood ratio tests and Akaike's Information Criterion (AIC), applied to mixed-effects models of the analysis of the four responses for kānuka, mānuka, and pōhutukawa data and rawiri mānuka separately

Response	Dropped term	Three species data				Rawiri mānuka data			
		AIC	L	df	p^z	AIC	L	df	p^z
Disease severity on leaf on a 5-point ordinal scale ^{1,2}	None	993.8				997.5			
	Species x days post inoculation	997.5	7.6	2	<0.01**				
	Species	1017.4	24.0	2	<0.001***				
	Days post inoculation	1055.4	40.0	1	<0.001***	1039.6	44.1	1	<0.001***
% of leaf area covered with lesions and pustules ^{3,4}	None	−1186.7				−55.7			
	Species x days post inoculation	−1182.1	8.6	2	<0.05*				
	Species	−1173.9	12.2	2	<0.01**				
	Days post inoculation	−1052.5	123.4	1	<0.001***	−49.6	8.1	1	<0.01**
Stem severity score ^{1,2}	None	1208.7				105.4			
	Species x days post inoculation	1205.4	0.6	2	0.731				
	Species	1209.4	8.0	2	<0.05*				
	Days post inoculation	1361.2	153.8	1	<0.001***	133.5	30.1	1	<0.001***
Dieback incidence ^{5,6}	None	126.8				19.9			
	Species x days post inoculation	124.3	1.5	2	0.474				
	Species 7	120.6	0.3	2	0.880				
	Days post inoculation	194.9	76.3	1	<0.001***	27.5	9.6	1	<0.01**

¹The CLMM analysis of leaf score of the three species data as a function of species and/or days post inoculation with seedling nested in family included as random effects. ²The CLMM analysis of leaf score of rawiri mānuka data as a function of days post inoculation with seedling nested in family included as random effects. ³The beta GLMM analysis of leaf area covered with lesions and pustules of the three species data as a function of species and/or days post inoculation with seedling nested in family included as random effects. ⁴The beta GLMM analysis of leaf area covered with lesions and pustules of rawiri mānuka data as a function of days post inoculation with seedling nested in family included as random effects. ⁵The binomial GLMM analysis of dieback incidence of the three species data as a function of species and/or days post inoculation with seedling nested in family included as random effects. ⁶The binomial GLMM analysis of dieback incidence of rawiri mānuka data as a function of days post inoculation with seedling nested in family included as random effects

L likelihood ratio statistic, df=degrees of freedom of the L statistic

^z Significant values are denoted as follows: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$

stems only, or both leaves and stems. The percentage of plants that developed symptoms on leaves or stems was lowest for kānuka (71%), followed by rawiri mānuka (79%), then mānuka (84%) and pōhutukawa (96%) (Table 6). Among the four species there were a similar percentage of plants that developed symptoms on leaves but not on stems (a combination of L3-5 and S1); rawiri mānuka (24%), pōhutukawa (24%), mānuka (22%) and kānuka (19%) (Table 6). Development of symptoms on stems when no symptoms developed on leaves (a combination of L1 and S2-4) was far less common and only observed for kānuka (3%). All seed families for each species containing resistant phenotype seedlings (L1 and S1) also contained seedlings with the highly susceptible phenotype (L5 and S4).

Discussion

Seedlings from all four of the New Zealand Myrtaceae tested were susceptible to a strain of *A. psidii* from *E. globulus* from Uruguay. This is the first assessment of the susceptibility of New Zealand Myrtaceae to an *A. psidii* strain that is only found in the native range of the pathogen and one that is from the *Eucalyptus* biotype. Results suggest that strains from South America isolated from specific hosts may not necessarily have narrow host ranges and may pose a significant biosecurity risk to New Zealand and other countries. We report similar relative species level resistance to that seen in previous studies with strains from other biotypes (Smith et al. 2020; Soewarto et al. 2021); pōhutukawa (*M. excelsa*) had the greatest susceptibility, kānuka (*K. ericoides*) the

Table 3 Multiple comparison tests based on the Tukey-adjusted method at $\alpha=0.05$ for the analysis of percentage of leaf area covered by lesions and pustules using beta GLMMs for kānuka, mānuka, and pōhutukawa data and rawiri mānuka separately

Comparison	Estimate	SE	t-value	p^z
<i>14 days post inoculation</i> ¹				
Kānuka—mānuka	-0.51	0.23	-2.22	0.068
Kānuka— pōhutukawa	-1.14	0.23	-4.95	<0.001***
Mānuka— pōhutukawa	-0.63	0.24	-2.56	<0.05*
<i>21 days post inoculation</i> ¹				
Kānuka—mānuka	-0.96	0.22	-4.39	<0.001***
Kānuka— pōhutukawa	-1.77	0.22	-7.95	<0.001***
Mānuka— pōhutukawa	-0.81	0.23	-3.51	<0.01**
<i>Rawiri mānuka</i> ²				
14 days–21 days	-1.06	0.37	-2.91	<0.01**
Kānuka ¹				
14 days–21 days	-1.10	0.17	-6.64	<0.001***
Mānuka ¹				
14 days–21 days	-1.55	0.19	-8.10	<0.001***
Pōhutukawa ¹				
14 days–21 days	-1.74	0.19	-9.21	<0.001***

Results are given on the logit scale

¹The beta GLMM analysis of leaf area covered by lesions and pustules of the three species data as a function of species and/or days post inoculation with seedling nested in family included as random effects

²The beta GLMM analysis of leaf area covered by lesions and pustules of rawiri mānuka data as a function of days post inoculation with seedling nested in family included as random effects

Degrees of freedom of the t-statistic are 566 and 52 (df=566 and df=52) for the Tukey-adjusted method of ¹ and ² respectively

^zSignificant values are denoted as follows: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$

lowest, and the other species, mānuka (*L. scoparium*) and rawiri mānuka (*K. ericoides* 'gumland ecotype') intermediate.

Using artificial inoculation to assess qualitative resistance (no symptom development or hypersensitive response on leaves or stems), we found that a low proportion (4%) of pōhutukawa seedlings were putatively fully resistant. This is similar to results seen in previous studies with other biotypes. For

Table 4 Multiple comparison tests based on the Tukey-adjusted method at $\alpha=0.05$ for the analysis of stem severity score using CLMMs for kānuka, mānuka, and pōhutukawa data and rawiri mānuka separately

Comparison	Estimate	SE	z-value	p^z
<i>Species</i> ¹				
Kānuka—mānuka	-1.21	0.59	-2.05	0.100
Kānuka— pōhutukawa	-1.68	0.57	-2.95	<0.01**
Mānuka— pōhutukawa	-0.47	0.58	-0.82	0.690
<i>Days post inoculation</i> ¹				
14 days–21 days	-3.27	0.38	-8.63	<0.001***
<i>Days post inoculation</i> ²				
14 days–21 days	-38.53	0.001	<-1000	<0.001***

Results are given on the latent (logit) scale

¹The CLMM analysis of leaf score of the three species data as a function of species and/or days post inoculation with seedling nested in family included as random effects

²The CLMM analysis of leaf score of rawiri mānuka data as a function of days past inoculation with seedling nested in family included as random effects

^zSignificant values are denoted as follows: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$

example, against the pandemic biotype only a single individual pōhutukawa showed qualitative resistance (Smith et al. 2020), while 3% of seedlings tested did not develop symptoms following two rounds of inoculation with the South African biotype (Soewarto et al. 2021). The proportion of resistant mānuka seedlings was 16% and comparable to testing with the pandemic biotype (15%; Smith et al. 2020), but much higher than only 1% of seedlings tested against the South African biotype (Soewarto et al. 2021). Percentages of putatively resistant seedlings for both rawiri mānuka (21%) and kānuka (29%) were higher than those recorded previously, at 6% and 13% respectively against the South African biotype (Soewarto et al. 2021), and 15% for both rawiri mānuka and kānuka combined against the pandemic biotype (Smith et al. 2020). Ideally, inoculation should be repeated to confirm resistant response phenotypes as in Soewarto et al. (2021), therefore our percentage estimates of resistant seedlings should be treated conservatively without secondary inoculations to confirm these results. A similar trend is also reflected in the disease severity data, with the lowest

Table 5 Multiple comparison tests based on the Tukey-adjusted method at $\alpha=0.05$ for the analysis of dieback incidence using binomial GLMMs for kānuka, mānuka, and pōhutukawa data and rawiri mānuka separately

Comparison	Estimate	SE	<i>t</i> -value	<i>p</i> ^z
<i>Species</i> ¹				
Kānuka—mānuka	−1.29	2.70	−0.48	0.8813
Kānuka— pōhutukawa	−0.65	2.87	−0.228	0.9718
Mānuka— pōhutukawa	0.638	2.31	0.277	0.9587
<i>Days post inoculation</i> ¹				
14 days – 21 days	−11.0	1.65	−6.67	<0.001***
<i>Days post inoculation</i> ²				
14 days – 21 days	−15.4	8.12	−1.90	0.062

Results are given on the latent (logit) scale

¹The binomial GLMM analysis of dieback incidence of the three species data as a function of species and/or days post inoculation with seedling nested in family nested included as random effects

²The binomial GLMM analysis of dieback incidence of rawiri mānuka data as a function of days post inoculation with seedling nested in family included as random effects

Degrees of freedom of the *t*-statistic are 573 and 54 (df=573 and df=54) for the Tukey-adjusted method of ¹ and ² respectively

^zSignificant values are denoted as follows: **p*<0.05, ***p*<0.01, and ****p*<0.001

percentage of area on leaves with symptoms recorded on kānuka, followed by mānuka and the greatest on pōhutukawa (Fig. 3). While there are broadly similar trends, it is important to note that the number of seedlings and families screened in each study differed (Table S5). Differences in response phenotypes may be of particular interest for future work investigating

Table 6 Distribution of resistant phenotypes in plants grown from New Zealand provenance seed for four species—rawiri mānuka, kānuka, mānuka and pōhutukawa, at 21-days post

Phenotype	Rawiri mānuka	Kānuka	Mānuka	Pōhutukawa
Plants that did not develop symptoms on leaves (L1)	6 (21%)	34 (32%)	13 (16%)	4 (4%)
Plants that did not develop symptoms on stems (S1)	13 (45%)	51 (48%)	31 (38%)	27 (28%)
Plants that did not develop symptoms on leaves or stems (L1 and S1)	6 (21%)	31 (29%)	13 (16%)	4 (4%)
Plants that developed symptoms on leaves but not on stems (S1 and L3-5)	7 (24%)	20 (19%)	18 (22%)	23 (24%)
Plants that developed symptoms on stems but not on leaves (L1 and S2-4)	0 (0%)	3 (3%)	0 (0%)	0 (0%)
Plants that developed symptoms on leaves and stems (not L1 or S1)	16 (55%)	52 (49%)	51 (62%)	69 (72%)
Total number of seedlings	29	106	82	96

the genetic basis of resistance and the interactions between host and pathogen genotype.

Artificial inoculations under controlled conditions can efficiently provide important information about genetic resistance to specific pathogen genotypes. However, the high inoculum loads applied and the ideal environment for the pathogen may overestimate field vulnerability (Raffa et al. 2023). Should an incursion occur, field trials are needed to understand disease epidemiology, how this varies in the environment and contributes to observed impacts on particular hosts. Importantly, the broad pattern of susceptibility with pōhutukawa>mānuka>kānuka from artificial inoculation, now against strains of three biotypes, follows observations from the field in New Zealand where currently only the pandemic biotype is present. Many records of myrtle rust in New Zealand are from *Lophomyrtus* species and pōhutukawa (Toome-Heller et al. 2020). While seedlings of mānuka, kānuka, and rawiri mānuka are susceptible to the pandemic biotype (Smith et al. 2020), to date no field infection records have been reported from kānuka or rawiri mānuka and there are few for mānuka. Other factors such as environmental conditions or inoculum load may play a key role in mediating the development of disease for these species.

In addition to threatening indigenous Myrtaceae in New Zealand, introduction of additional strains or biotypes of *A. psidii* could pose a threat to economically important exotic species of Myrtaceae. The pandemic biotype of *A. psidii* currently appears to be a relatively low threat to *Eucalyptus*. In New Zealand, while reported on *Eucalyptus globoides*, *E. fastigata* and *E. regnans* in nurseries (MPI 2024; Toome-Heller et al. 2020), myrtle rust has yet to cause significant damage to *Eucalyptus* species. Similarly, surveillance

inoculation with *Austropuccinia psidii* isolated from *Eucalyptus globulus* in Uruguay

in Australia has found the disease only sporadically at very low incidence in eucalypt plantations (Carnegie 2014; Carnegie and Pegg 2018). Conversely in South America where the *Eucalyptus* biotype is present, the disease can be a significant problem in nurseries and young plantations (Coutinho et al. 1998; Passos dos Santos et al. 2020; Hakamada et al. 2022). In Uruguay, myrtle rust is not severe on native Myrtaceae but has been reported causing severe damage on one-year-old plants of *E. globulus*, *E. grandis* and *E. dunii*, both in the field and on clonal material in nurseries (Telechea et al. 2003; Pérez et al. 2011, 2014; Simeto et al. 2020). The methods applied here could be used to screen the relative susceptibility of *Eucalyptus* species, provenances, families, and clones of interest to the New Zealand forestry industry to provide a clearer picture of this risk.

Further to the direct threat that different *A. psidii* biotypes pose to New Zealand Myrtaceae, is the potential for their outcrossing through sexual reproduction. Increased genetic and genotypic diversity in the pathogen population would increase its adaptive potential (Drenth et al. 2019; McTaggart et al. 2020). McTaggart et al. (2018) found that basidiospores of *A. psidii* were able to infect Myrtaceae resulting in the production of recombinant sori and the completion of the life cycle. Telia of *A. psidii* have been reported in Australia (Pegg et al. 2014b), South Africa (Roux et al. 2016), and New Zealand (Toome-Heller et al. 2020) on several hosts. There is evidence from population genetic analysis that recombination and therefore sexual reproduction has occurred in the New Zealand *A. psidii* population (McTaggart, et al. 2020; Bartlett et al. unpublished data). However, the host, pathogen and environmental drivers of telia production are not well understood for *A. psidii*. Telia were observed on all four New Zealand species following inoculation with a strain of the *Eucalyptus* biotype of *A. psidii* from Uruguay. Similar observations on the same hosts with strains of the pandemic and South African biotypes (Smith et al. 2020; Soewarto et al. 2021), suggest hybridisation of these strains infecting the same hosts in New Zealand is plausible. Whether genetically distinct strains belonging to different biotypes are reproductively compatible has not been confirmed, however related mating genes present in genomes from pandemic, South African and *Eucalyptus* strains suggest outcrossing on a universal host is theoretically possible (Ferrarezi et al. 2022).

Virulence towards some Australian *Melaleuca* species (Zauza et al. 2010), Hawaiian ‘Ōhi’a (Costa da Silva et al. 2014) and now several New Zealand Myrtaceae has been demonstrated for South American *Eucalyptus* associated strains, showing that strains of the *Eucalyptus* biotype are able to infect a broader range of species than their known field host association implies. Further work to identify host ranges for different biotypes could be used to investigate the connection between host associations and virulence and improve our understanding of the evolution of virulence towards or away from multiple hosts. Telia have now been recorded following inoculations on New Zealand Myrtaceae for strains belonging to the *Eucalyptus*, pandemic and South African biotypes, indicating that these species could provide a universal host for sexual reproduction and outcrossing of strains of different biotypes if they happen to co-occur, further increasing the risk posed by invasion of additional strains to New Zealand. Relative susceptibility of the four New Zealand Myrtaceae tested against the *Eucalyptus*, pandemic and South African biotypes was similar, and is reflected in field observations to date in New Zealand. This suggests we would not necessarily see an immediate shift in the host species affected or change in symptom expression to indicate the arrival of a strain of the *Eucalyptus* biotype in New Zealand. These findings further highlight the need for development of tools to differentiate between strains and biotypes for rapid diagnostics to monitor for, and respond to, incursions. The knowledge that a strain of the *Eucalyptus* biotype of *A. psidii* is virulent on several indigenous Myrtaceae in New Zealand should be considered in future biosecurity risk assessments.

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request. Detailed GPS locations of origin of the New Zealand mother plants cannot be publicly shared, in line with mana whenua cultural agreements.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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