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# Galling of genotypes of *Eucalyptus camaldulensis* resistant to *Leptocybe invasa* is unlikely to be altered by edaphic factors but is not immune to climatic influences

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**Research Paper**

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## Abstract

The blue gum chalcid (*Leptocybe invasa*) is a serious invasive, galling insect pest of eucalypts grown outside Australia. Variability in resistance of species and genotypes of *Eucalyptus* to the pest is widely reported but without consideration of the influence of silviculture on the severity of galling. We assessed the variability of gall expression by 29 genotypes of *E. camaldulensis* by *L. invasa* in common nursery experiments and in 5 common garden arboreta planted in diverse climatic zones and soil types around Kenya. We quantified variation in growth and the concentrations of defensive chemical compounds (namely polyphenolic compounds) to assess possible genotype × environment interactions which we also relate to the climate of the parent seed trees in Australia. Generally, genotypes endemic to low latitude regions of Australia were more resistant to the pest while the concentration of quinic acid derivatives (QUIN) exhibited an interaction with arboretum location in Kenya. The concentration of QUIN in potted plants did not vary significantly with nitrogen supplementation. However, growth rates and total polyphenolic concentrations varied with arboretum location. Since QUIN, which have been previously shown to confer resistance against *L. invasa*, did not vary in different arboreta, resistant subspecies and genotypes of *E. camaldulensis* can be deployed in novel habitats and will not be galled. Our findings support the critical need to plant stock of known genotype(s) rather than planting stock grown from locally collected seed. This will require the establishment of eucalypt seed orchards if clonal production of planting stock is not possible.

## Introduction

In the space of just 15 years, *Leptocybe invasa* Fisher & La Salle (Hymenoptera: Eulophidae) spread to over 35 countries and became a serious insect pest of *Eucalyptus* species (Myrtaceae) grown in plantations outside Australia (Otieno *et al.*, 2019). Consequently, research has been conducted to identify trees resistant to galling by this tiny wasp including testing of variable genotypes. *Eucalyptus camaldulensis*, one of the most widely planted species of this economically important but preferred host species, is used in many tree breeding programmes to confer drought and/or salinity tolerance to hybrid eucalypts. Variability in the incidence of galling by *L. invasa* has been reported in some published literature (Mendel *et al.*, 2004; Nyeko *et al.*, 2010; Otieno *et al.*, 2022), emphasising the potential to identify resistance mechanisms. Subspecies of *E. camaldulensis* have been found to exhibit variable susceptibility to *L. invasa* but genotype × environment interactions have not been considered (Andrew *et al.*, 2010; O'Reilly-Wapstra *et al.*, 2010). For example, Otieno *et al.* (2022) found that the incidence of galling of *E. camaldulensis* was inversely correlated with concentrations of quinic acid derivatives (QUIN) in different subspecies. This suggests that these compounds adversely impact agents of gall induction, which can be either eggs or neonate larvae (Giron *et al.*, 2016; Isaias *et al.*, 2018), prior to gall induction.

These studies however do not give clarity on whether traits evolved in a particular region of endemism will remain constant when the progeny of that population are grown elsewhere and where growing conditions and/or the mosaic of herbivores they are exposed to differ in composition and abundance (Rapley *et al.*, 2004; Henery *et al.*, 2009). Moreover, other studies have found that constitutive resistance traits in other plant species are influenced by genetic and environmental factors (Andrew *et al.*, 2010; O'Reilly-Wapstra *et al.*, 2010). This indicates that environmental influences could alter expression of genetically determined traits when eucalypts are deployed in novel environmental gradients, i.e. counter-gradient variation (Conover *et al.*, 2009).

Despite the belief that the expression of many plant traits are shaped by interactions between genotypes and the environment (Underwood and Rausher, 2000; Hochwender and Fritz, 2004; Johnson and Agrawal, 2005), mechanisms are often not well understood (Sultan, 2000). Other than genes, the environmental factors at play are many and diverse. Soil nutrient availability may influence plant growth such that those endemic to environments with limited resources may grow more slowly and/or may invest more in traits conferring resistance (Haukioja *et al.*, 1998; Fine *et al.*, 2006). For example, some species of conifer and ericaceous plants produce high foliar concentrations of phenolic compounds when grown in nutrient poor soils (Northup *et al.*, 1995; Kraus *et al.*, 2004). Nitrogen availability can affect plant secondary metabolite expression and hence influence plant defences against some pests and diseases (Sakakibara *et al.*, 2006) (Sakakibara *et al.*, 2006). Larvae of galling insects, such as *L. invasa*, induce the formation of and develop within, highly modified plant tissues (Kaiser *et al.*, 2010; Agrawal, 2011; Oliveira *et al.*, 2016) and maybe least affected by changes in host plant quality (Hartley, 1998). Nevertheless, galling by some insects has been shown to follow soil fertility gradients (Blanche and Westoby, 1995; Cuevas-Reyes *et al.*, 2003).

Some plant genera and species from lower latitudes have been found to invest more in resistance than those at higher latitudes (Rasmann and Agrawal, 2011), or plant phenotypes can exhibit plasticity of expression in which responses may be either continuous or interactive (Singh *et al.*, 1999; Des Marais *et al.*, 2013). Although phenotypic plasticity may enhance plant fitness in heterogeneous environments (Sultan, 2000), it may not be desirable in an applied context where consistency of expression of constitutive resistance is needed for consistent pest management. Variability in the abundance of such galling insect taxa with environmental conditions implies that host suitability is important to their success (Fernandes and Price, 1992; Blanche and Westoby, 1995).

We investigated the evolutionary influence on the defensive characteristics of *E. camaldulensis* genotypes to the invasive galling wasp *L. invasa*. The influence of place of origin in Australia and its possible effects in shaping defensive traits was used as our indicator of the evolutionary influence on extant characteristics. The influence of growing conditions in Kenya on the expression of resistance when grown in common garden arboreta as well as in experiments with potted plants was studied to quantify phenotypic plasticity. Since constitutive resistance is mediated by 'always present defences' such traits are difficult to manipulate (Wittstock and Gershenzon, 2002). Genotype × environment (G × E) experiments provide a means to address this problem because plants exhibit tremendous phenotypic plasticity. Common garden arboreta permit an insect herbivore to choose between numerous genotypes which may, due to differences in their vegetative growth in a specific space and time, provide for marked variation in relative resource availability and suitability. Since galling insects are essentially specialists of rapidly differentiating plant tissues, common garden arboreta may provide situations in which populations of species such as *L. invasa* have the opportunity to overwhelm putative resistance mechanisms of some genotypes prior to the expression of specific harmful compounds or the full suite of compounds (see Khanal *et al.*, 2025).

## Materials and methods

### Seed source locations and information

We selected 29 genotypes representative of all seven subspecies of *E. camaldulensis* from their endemic populations (fig. 1). These were identified into their respective subspecies by McDonald *et al.* (2009) and Butcher *et al.* (2009). Within each subspecies we chose genotypes representative of as wide a geographical distribution as possible so as to present as much of the diversity within the species as possible. The seedlots selected were those that originated from as few parent trees as possible (typically a single parent tree), i.e. to minimise diversity within genotypes and hence variability in expression of traits. Seeds of selected genotypes were purchased from the Australia Tree Seed Centre (ATSC), Canberra, Australia.

### Arboreta establishment and data collection

Seeds were sown and raised in a common nursery at KEFRI, Muguga, Kenya in October 2012. A nursery trial was conducted in Embakasi (−1.287995, 36.977557), a suburb of Nairobi, where *L. invasa* infestation had been confirmed while five common garden arboreta were established in four agroecological zones (see table 1). The trials were laid out in nursery and field arboreta in April 2013, when the seedlings about 30 cm and well hardened off. In each arboretum, the genotypes were planted in 12-plant line plots replicated in three blocks. Seedlings from the Muguga nursery were also planted out in a common garden reserve comprising ten plants of each genotype at KEFRI, Muguga where there was no *L. invasa* infestation. Additional seed from the same seedlots was germinated and 10 individual plants of each were grown in native plant potting mix in 15 cm diameter pots in the open in the Agriculture Reserve at La Trobe University, Melbourne. A potting mix suitable for native Australian plants comprising composted pine bark, river sand, trace elements, professional-grade wetting agent, and two types of slow-release fertiliser sufficient for 4–6 months of growth was used for the plants maintained in 15 cm diameter plastic pots in the Agriculture Reserve at La Trobe University.

### Environmental and soil data

Climatic data for seedlot collection locations were extracted from the CliMond dataset (Kriticos *et al.*, 2012) in ArcMap 10.2.2. Soil data for the genotype source location was obtained from CSIRO website at http://www.clw.csiro.au/aclep/soilandlandscapegrid/index.html.

### Soil collection and analysis

Nine soil samples per site were collected using soil auger, along two transects of the whole plot. The soil samples were collected from the top 30 cm layer, at the time of arboretum establishment, packed and sent to the laboratory for analysis of their nitrogen, phosphorus, potassium, calcium, magnesium, organic carbon, pH, and electrical conductivity. These were quantified using methods described by Okalebo *et al.* (2002) after the soil samples were air dried at room temperature, milled and passed through 2 mm sieve. A portable glass electrode was used to measure soil pH and electrical conductivity (EC) while available phosphorus (P) and magnesium (Mg) were determined using a UV spectrophotometer. Potassium (K) and calcium (Ca) were determined using a flame photometer. Organic carbon (C) was determined using

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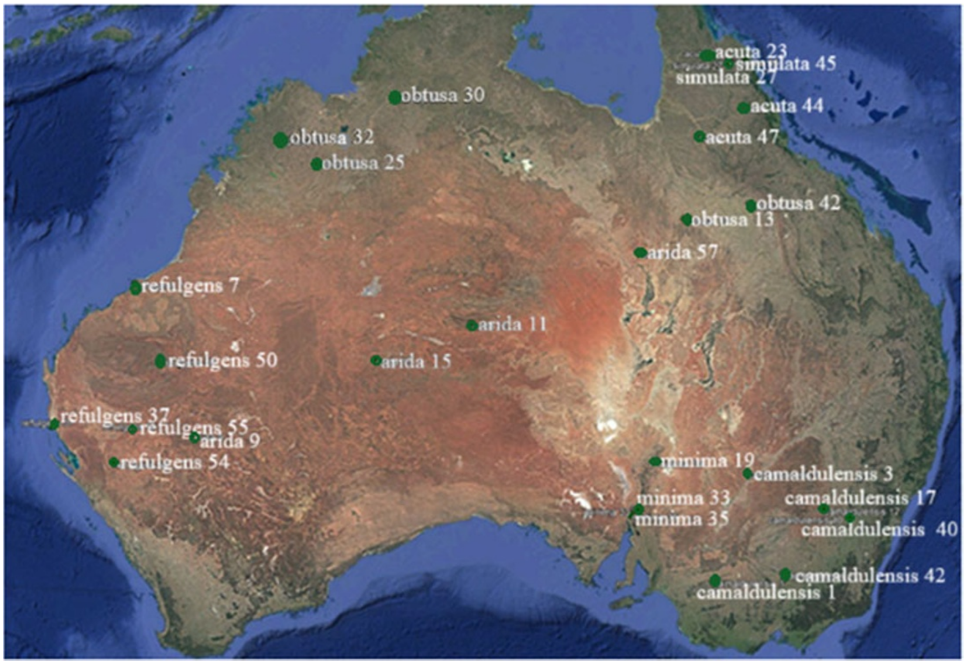

**Figure 1.** Location of collection of seedlots of endemic populations of *Eucalyptus camaldulensis* genotypes used in this study. Seeds sourced from by Australian Tree Seed Centre (ATSC), Canberra, Australia.

**Table 1.** Locations of common garden arboreta in Kenya and their climatic characteristics. Zones are agroecological zones as recognised by Orodho (2006)

| Site name | Zone | Description | Annual rainfall (mm) | Latitude | Longitude |
|---|---|---|---|---|---|
| Turbo | II | Sub-humid | 1000–1600 | 0.639477 | 35.066645 |
| Akala | III | Semi-humid | 800–1400 | −0.025737 | 34.451497 |
| Maranda | IV | Semi-humid to semi-arid | 600–1100 | −0.083958 | 34.229365 |
| Mitumbiri | V | Semi-arid | 450–900 | −0.999813 | 37.173070 |
| Yatta | V | Semi-arid | 450–900 | −1.119748 | 37.377775 |

the Walkley–Black method and total nitrogen (N) was determined using the Kjeldahl method.

### Analysis of plant growth performance, insect infestation, and foliar phenolic compounds

From the experimental plots in the arboreta, we measured the height of plants and apical bud length (our indicator of plant vigour, see Steinbauer (1999)), leaf toughness, insect oviposition intensities, and the intensity of gall development. Specific leaf weight, as determined using the method of Steinbauer (2001), was used as our indicator of leaf toughness. These data were collected every 6 months within the first 2 years of the trials because damage was more pronounced on seedlings and saplings compared to mature trees (Nyeko *et al.*, 2010).

To quantify the intensity of oviposition in the common arboreta, a sample of one apical bud was collected from each sapling. This was done in the third month of their establishment. Since the eggs are laid inside soft plant tissue and not visible, the samples were taken to the laboratory and ovipuncture marks counted under a stereo microscope (Olympus SZ, Japan). Assessments of galling intensity were undertaken twice during the year for 2 years using a 4-point scale (Nyeko *et al.*, 2010), i.e. 1 = not infested, 2 = 1–25% of canopy infested (minor infestation), 3 = 26–50% infestation (moderate infestation), and 4 = >50% canopy infested (severe infestation).

Phenolic compounds in actively growing shoots (rather than in mature leaves) were analysed for this study. Shoot collection was carried out mid-morning from all the experimental sites during the same sampling season. Samples were collected from selected genotypes representing highly susceptible, moderately susceptible, and resistant genotypes as previously determined in the nursery experiments (Otieno *et al.*, 2022). The shoots were collected in the second year of the common arboreta in four locations in Kenya, from plants at a protected nursery in Muguga and Agriculture Reserve at La Trobe University. Shoots samples were harvested by pinching off actively growing tips of each plant and pooling into a common zip-lock bag by genotype and site. In the laboratory, the identity and quantity of phenolic compounds in the genotypes were determined from freeze dried samples ground into a fine powder in a mortar and pestle. The samples were analysed for extracted phenolic compounds analysed, using methods described in Steinbauer *et al.* (2016) as adapted from Engström *et al.* (2014), in the Natural Chemistry Research Group, University of Turku, Finland. An amount of 20 mg of plant powder was extracted twice for 3 h with 1.4 ml of acetone/water (80/20, v/v). The pooled extract was concentrated to water phase by an Eppendorf concentrator then freeze-dried. The extracts were dissolved in 1 ml of H2O, filtered with 0.2 µm PTFE syringe filters and diluted five times with $H_2O$ before subsequent ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) analyses. The UPLC system was connected to a Xevo TQ triple-quadrupole mass spectrometer (Waters Corp., Milford, MA, USA) with electrospray ionisation and the UPLC system utilised a 100 mm × 2.1 mm i.d., 1.7 µm, Acquity UPLC BEH Phenyl column (Waters Corp., Wexford, Ireland). The polyphenol groups were quantified from the extracts by UPLC-MS/MS, considering the specific fragmentation of different types of polyphenols in the ion source and their subsequent specific detection and quantitation by sensitive and selective multiple reaction monitoring methods (Engström *et al*., 2015). The following standards were used for quantitation: proanthocyanidins (PA) were quantified using standard curves from stock solutions of procyanidin (PC) and prodelphinidins purified by Sephadex LH-20 gel chromatography. Ellagitannins and gallic acid derivatives were quantified as tellimagrandin I and pentagalloylglucose, respectively. QUIN and three classes of flavonols (kaempferol, quercetin, and myricetin derivatives) were quantified using chlorogenic acid, kaempferol, quercetin, and myricetin glycosides as quantitation standards. Before each set of runs, a flavonoid mix stock solution was injected twice to determine the systems performance at the start of analysis. Additionally, prior to and after each 10 samples, 5 samples of a catechin stock solution were run to determine the stability in the systems ionisation efficiency for polyphenols over the course of the analysis. Rapid fingerprint UPLC-MS/MS was used to determine the abundance of QUIN and total polyphenols.

### *Nitrogen supplementation studies*

A principal component analysis of soil characteristics found nitrogen to be the macronutrient that accounted for the greatest site variability. Hence, the effect of nitrogen supplementation on host susceptibility was investigated under glasshouse conditions at KEFRI, Muguga. Seedlings of three susceptible genotypes (17, 19, and 35) (selected based on Otieno *et al*., 2022) were transplanted into 15 cm diameter pots in forest soil and gravel mix and allowed to establish for 2 months. The plants were then separated into groups of 60 pots per genotype and given urea at the rate of 0, 1, 2, or 4 g and watering to field capacity. Single apical shoots from 15 randomly selected plants were harvested 2 weeks after urea application for analysis of polyphenolic compounds. Using 13 mm diameter, 18 mm deep clip cages (sourced from BioQuip, California, USA), one newly emerged female wasp was caged onto a shoot of each of the remaining plants and left for 24 hours. The wasps were from field collected galls that were caged in the insectary for adult wasp emergence. Locations where wasps had been caged were marked with thread and monitored for gall development.

### *Data analyses*

To determine the variability of genotype performance in different growing sites, analysis of variance and principal component analysis was used (Crossa, 1990). Measurements pertaining to host quality/vigour (latter indicated by increase in height), oviposition intensity, galling incidence, and intensity from common garden arboreta were analysed to consider the responses of the genotypes in different subspecies to the environmental conditions under which they were grown. Linear and log-linear regression modelling was used depending on the distribution of the data.

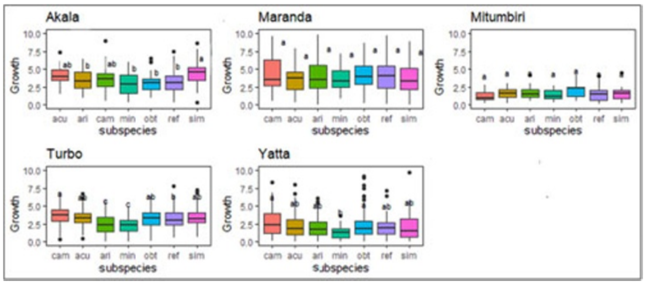

**Figure 2.** Mean growth rate (in cm/month) of subspecies of *Eucalyptus camaldulensis* in the first 6 months after arboretum establishment. Box and whisker plots show 25th and 75th percentiles (shaded), mean (solid line inside box). Letters indicate similarities of means. Details relating to the location of each arboretum are given in table 2. The subspecies are abbreviated as: sim = *simulata*, ref = *refulgens*, acu = *acuta*, obt = *obtusa*, ari = *arida*, cam = *camaldulensis*, min = *minima*.

Analysis of the effect of place of origin in Australia on the performance of genotypes was by principal component, canonical correlation analysis, and discriminant analyses followed by regression analysis using environmental and performance data from nursery experiments (Crossa, 1990; Anderson and Willis, 2003). Relationships among the environmental variables of host plant origin were modelled using linear regression with their level of infestation, growth, physical, and chemical characteristics. ANOVA was used to compare log transformed data on composition of phenolic compounds on leaves.

## Results

### *Influence of site and tree growth on galling*

The genotypes of subspecies *camaldulensis* were generally the fastest growing except in Maranda and Mitumbiri, while those of subspecies *minima* were the slowest (fig. 2). While tree growth rate was positively correlated with oviposition incidence ($r = 0.48$; $F_{1,52} = 14.99$; $P < 0.001$), it did not have a significant correlation with galling incidence.

Differences in apical shoot length and leaf toughness were dependent on interactions between subspecies and arboretum location. Although there was variability in the incidence of galling both among subspecies and according to arboretum location, there was no interaction between genotype and arboretum location (table 2).

### *Influence of soil nutrients on leaf galling incidence and leaf phenolic composition*

Soil nutrients of locations in Australia from where the seed was sourced were not correlated with susceptibility to gall formation, growth rate, or the abundance polyphenols of the trees grown in Kenya. However, galling index and the concentration of hydrolysable tannins were inversely correlated with soil pH (table 3).

Arboreta differed significantly in soil macronutrients and physical properties (Appendix 1). Principle component analysis of soil nutrients and characteristics showed that the variability among sites was influenced by pH (component 1) and nitrogen + phosphorus concentrations (component 2; Appendix 2). These two components explained 52.2% of the variation. Although discriminant analysis grouped soils according to the location of arboreta ($\chi^2 = 25.79$, $df = 8$, $P = 0.001$), there was no correlation between individual soil nutrients and galling incidence or severity.

**Table 2.** Interactions among subspecies/genotype and arboretum on leaf physical characteristics and galling

| | | Grouping | | Arboretum | | Interaction | |
|---|---|---|---|---|---|---|---|
| Grouping | Plant variable & response to wasp | *F* | *P* | *F* | *P* | *F* | *P* |
| Subspecies | Bud length | 18.85 | <**0.001** | 58.32 | **0.001** | 1.93 | **0.01** |
| | Leaf toughness | 2.30 | **0.033** | 16.28 | <**0.001** | 1.49 | 0.20 |
| | Galling incidence | 8.04 | <**0.001** | 5.39 | **0.001** | 0.64 | 0.84 |
| Genotype | Bud length | 4.95 | <**0.001** | 58.32 | <**0.001** | 0.99 | 0.5 |
| | Leaf toughness | 4.41 | <**0.001** | 20.13 | <**0.001** | 2.75 | <**0.001** |
| | Galling incidence | 2.95 | <**0.001** | 6.05 | <**0.001** | 1.0 | 0.5 |

*Note:* Significant values ($P < 0.05$) are shown in bold.

**Table 3.** Correlations among soil attributes, growth rate, concentrations of phenolics and galling index across all genotypes

| | N | | P | | C | | pH | |
|---|---|---|---|---|---|---|---|---|
| Plant variable and response to wasp | *r* | *P* | *r* | *P* | *r* | *P* | *r* | *P* |
| Growth rate | 0.30 | ns | −0.04 | ns | −0.02 | ns | −0.15 | ns |
| HT | 0.24 | ns | 0.26 | ns | 0.24 | ns | 0.39 | **0.038** |
| PA | −0.20 | ns | −0.10 | ns | −0.22 | ns | −0.21 | ns |
| QUIN | −0.28 | ns | −0.03 | ns | −0.19 | ns | −0.28 | ns |
| TP | 0.20 | ns | 0.25 | ns | 0.18 | ns | 0.34 | ns |
| Galling index | 0.06 | ns | 0.19 | ns | 0.21 | ns | 0.59 | <**0.001** |

*Notes:* ns, not statistically significant.
Significant values ($P < 0.05$) are shown in bold.

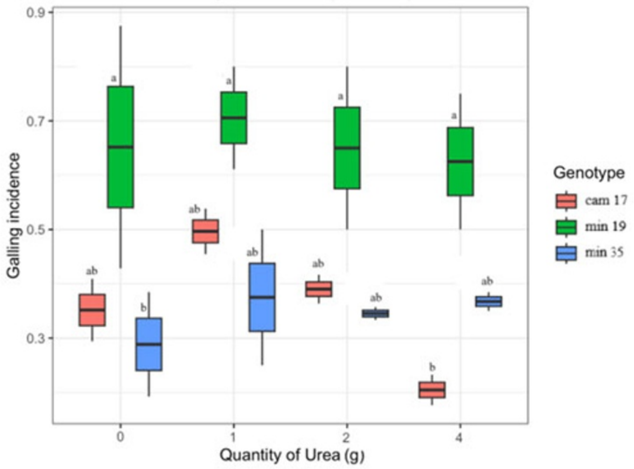

**Figure 3.** Galling incidence on potted plants treated with different quantities of urea. Box and whisker plots show 25th and 75th percentiles (shaded), mean (solid line inside box). Letters indicate similarities of means. The subspecies are abbreviated as: cam = *camaldulensis* and min = *minima*.

In the glasshouse, plants treated with urea had lower concentrations of total phenolics ($F_{1,4} = 614.70$, $P < 0.001$) and hydrolysable tannins ($F_{1,4} = 589.20$, $P < 0.001$) than control plants. The concentrations of QUIN were also reduced but differences among genotypes were not statistically significant ($F_{1,4} = 0.65$, $P = 0.95$). There was variability in galling incidence between genotypes given different treatments of urea, but differences between treatments were not statistically significant and did not follow any obvious pattern (fig. 3).

### Variability in leaf composition of phenolics in different growing sites

Different sites didn't show clear differences in the concentrations of QUIN in plants of same subspecies despite variability in the climatic zones where they were grown (table 1, fig. 4A) except in one from, which was not consistent with plants from the same climatic zone. The abundance of total polyphenols differed between sites and damage classes within sites (fig. 4B).

### Correlation between the environmental variables of host endemism with host plant characteristics and infestation

Using principal components analysis, the environmental variables of source locations within Australia grouped the genotypes closely according to subspecies (fig. 5A) but not by resistance status (fig. 5B).

In stepwise discriminant analyses, the environmental variables grouped the genotypes into subspecies ($\chi^2 = 65$; $P < 0.001$; reclassification error rate 17.25) and resistance status ($\chi^2 = 17.18$; $P < 0.001$; reclassification error rate 11.44). Wilk's lambda criterion identified precipitation of the wettest week, precipitation seasonality, radiation of warmest quarter, radiation of driest quarter, minimum temperature of the coldest week, radiation of coldest quarter, and radiation seasonality as the most important variables influencing the grouping of genotypes into subspecies while precipitation seasonality and minimum temperature of the coldest week grouped them into resistance status.

Galling incidence was inversely correlated with seasonality of precipitation ($r = -0.74$; $F_{1,27} = 32.65$; $P < 0.001$), mean annual temperature ($r = -0.75$; $F_{1,27} = 35.40$; $P < 0.001$), and minimum temperature of the coldest week ($r = -0.73$; $F_{1,27} = 30.60$;

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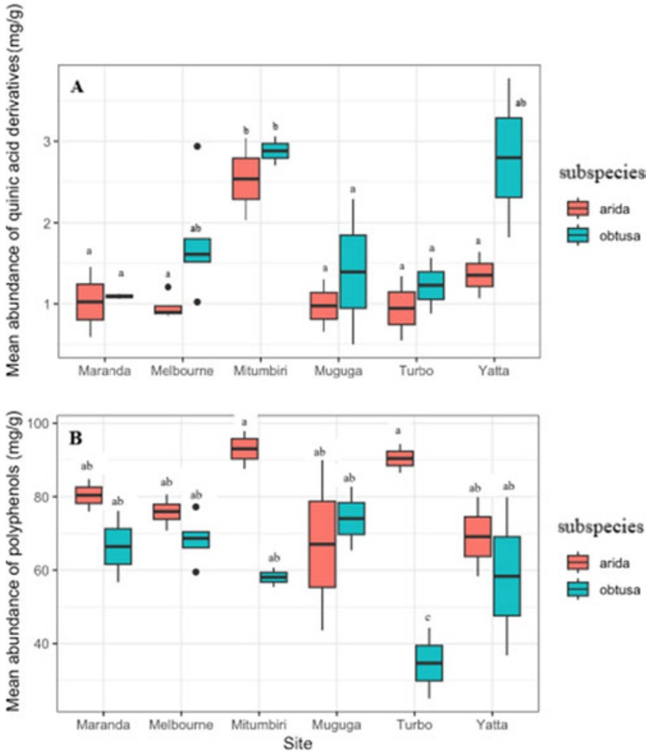

**Figure 4.** Concentrations of quinic acid derivatives (A) and total polyphenols (B) from plants in field arboreta (Maranda, Mitumbri, Muguga, Turbo, and Yatta, $n = 7$ each) and in pots (Melbourne, $n = 7$). Subspecies arida represent highly susceptible genotype while obtuse represent moderately susceptible genotype. Box and whisker plots show 25th and 75th percentiles (shaded), mean (solid line inside box). Letters indicate similarities of means.

$P < 0.001$). These climatic variables influenced hydrolysable tannins in a manner opposite to that of QUIN and PC (table 4).

### *Latitudinal influence on leaf galling and polyphenol composition*

The intensity of galling increased with increasing latitude while oviposition incidence and intensity displayed the inverse relationship (table 5). The concentration of QUIN and PC decreased inversely with latitude while that of hydrolysable tannins, quercetin, and total polyphenols increased with increasing latitude (table 6).

## Discussion

We have shown that the growth of genotypes of *E. camaldulensis* can vary according to geographic location while the expression of chemical resistance was invariable across locations. In addition, patterns of resistance observed under nursery conditions were maintained in field arboreta. The plant growth attributes we measured, such as the growth rate, leaf toughness, and bud length, exhibited strong G × E interactions. Growth rate is a genetically regulated characteristic for which plasticity is desirable in selection programmes targeting performance (Lambers and Poorter, 1992). The positive correlation between growth rate and oviposition indicates that faster growing modules offered more soft parenchyma tissue for egg deposition though this did not translate into higher susceptibility which has been shown to be more controlled by plant chemistry (Otieno *et al.*, 2022). While leaf toughness exhibited plasticity due to both genetic and environmental factors as also observed by Read *et al.* (2005) and Read *et al.* (2009), it showed no effect on galling.

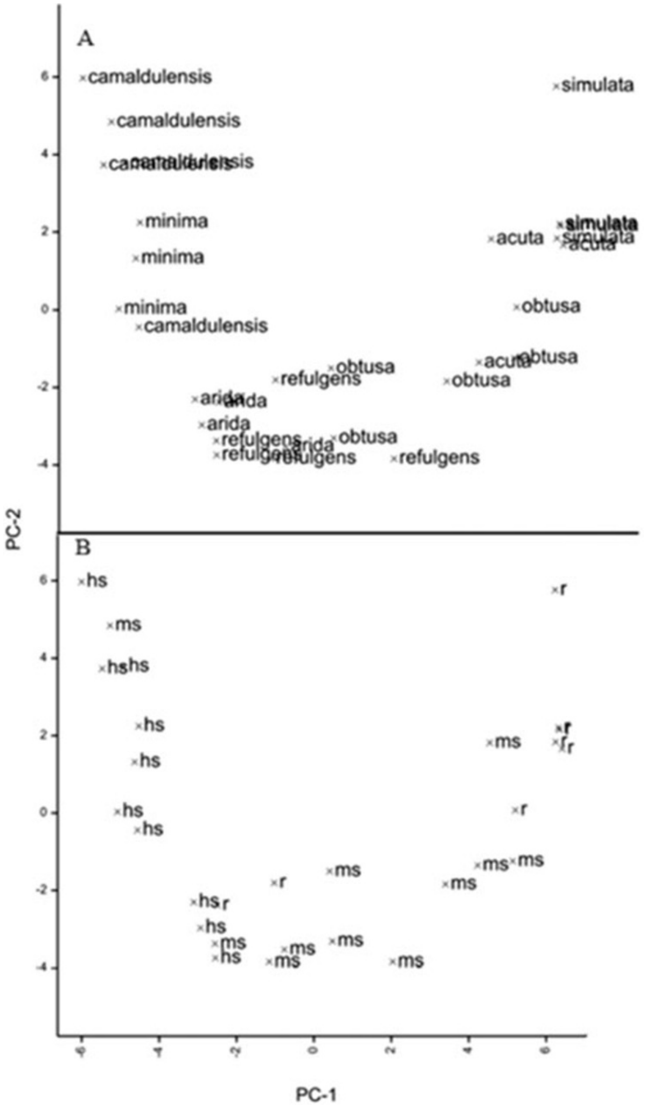

**Figure 5.** Principal components biplots of climatic variables of seed source of genotypes grouped according to subspecies (A) and according to level of resistance (B).

The absence of any interaction between the genotypes and the environment on susceptibility to galling indicates stability of genotypic resistance – at least at the population densities of *L. invasa* that our trees were exposed to and the age of tissues when exposed. This is an important attribute for the purposes of plant breeding (Becker and Leon, 1988), especially in the case of *L. invasa* because previous research has found that the wasp will oviposit in all *E. camaldulensis* hosts (Otieno *et al.*, 2022). Plasticity of host resistance could have been detrimental since it may be exploited by *L. invasa* and increase the fitness of the wasp (Fordyce, 2006) and considering that the wasp has shown tolerance of diverse environments (Otieno *et al.*, 2019).

Although levels of plant defence and herbivory have been related to nutrient availability (Coley *et al.*, 1985; Fine *et al.*, 2006; Shrivastav *et al.*, 2020), our study did not show any influence of

**Table 4.** Correlations among climatic variables in region of endemism and concentrations of foliar phenolics

| | Annual mean temperature (°C) | | | Min temperature of coldest week (°C) | | | Seasonality of precipitation | | |
|---|---|---|---|---|---|---|---|---|---|
| Type of polyphenol | *r* | *F* | *P* | *r* | *F* | *P* | *r* | *F* | *P* |
| HT | −0.71 | 27.7 | **<0.001** | −0.71 | 27.2 | **<0.001** | −0.76 | 36.02 | **<0.001** |
| PC | 0.46 | 7.4 | **0.01** | 0.36 | 3.99 | 0.06 | 0.50 | 8.9 | **0.006** |
| QUIN | 0.76 | 37.9 | **<0.001** | 0.64 | 18.7 | **<0.001** | 0.75 | 35.0 | **<0.001** |

*Notes:* HT, hydrolysable tannins; PC, procyanidins; QUIN, total quinic acid derivatives.
Significant values ($P < 0.05$) are shown in bold.

**Table 5.** Correlations among latitude of seed source, oviposition by wasps and galling

| | Nursery | | | Arboretum | | |
|---|---|---|---|---|---|---|
| Wasp and plant responses | *r* | *F* | *P* | *r* | *F* | *P* |
| Oviposition incidence | −0.25 | 1.78 | 0.19 | −0.34 | 10.77 | **0.002** |
| Ovipuncture intensity | −0.34 | 3.3 | 0.08 | −0.24 | 5.22 | **0.025** |
| Galling incidence | 0.67 | 21.98 | **<0.001** | 0.50 | 34.48 | **<0.001** |
| Galling intensity | 0.58 | 13.52 | **0.001** | - | - | - |

*Note:* Significant values ($P < 0.05$) are shown in bold.

**Table 6.** Correlations among latitude of seed source and concentrations of polyphenols

| Type of polyphenol | *r* | *F* | *P* |
|---|---|---|---|
| HT | 0.66 | 20.82 | **<0.001** |
| PC | −0.49 | 8.40 | **0.007** |
| KAEM | −0.09 | 0.24 | 0.63 |
| QUER | 0.72 | 29.27 | **<0.001** |
| Caffeoyl quinic acid 1 | −0.74 | 33.42 | **<0.001** |
| Caffeoyl quinic acid 2 | −0.59 | 14.58 | **<0.001** |
| Coumaroyl quinic acid 1 | −0.69 | 24.30 | **<0.001** |
| Coumaroyl quinic acid 2 | −0.70 | 25.97 | **<0.001** |
| Other quinic acid derivatives | −0.45 | 6.71 | **0.015** |
| Total polyphenols | 0.60 | 15.33 | **<0.001** |

*Notes:* HT, hydrolysable tannins; PC, procyanidins; KAEM, kaempferol; QUER, quercetin derivatives.
Significant values ($P < 0.05$) are shown in bold ($df = 27$ each).

soil nutrients on resistance to the wasp. The absence of any correlations with soil nutrients in the place of origin and arboretum of growth indicates that edaphic factors may not have influenced the evolution of putative defences against *L. invasa*. Variation in nutrient availability to potted plants also did not have an influence on QUIN, although the overall phenolic profile changed in these conditions.

The presence of high concentrations of QUIN was shown to confer resistance to genotypes of *E. camaldulensis* by Otieno *et al.* (2022). Therefore, the presence of these compounds in the shoots of plants growing in locations where plants are likely to have experienced galling by *L. invasa*, as well as in locations where no galling was observed (e.g. in Melbourne and the protected nursery in Muguga), again confirms that these metabolites are constitutive components of the chemistry of leaves. According to the optimal defence hypothesis, constitutive defensive compounds are prevalent in plants or plant parts that experience frequent insect attack (McKey, 1979; Zangerl and Bazzaz, 1992; Zangerl and Rutledge, 1996) and should occur in eucalypts (Heatwole *et al.*, 1999). Nevertheless, only one study has presented evidence linking a group of eucalypt secondary metabolites to defence against an individual species of insect (Steinbauer and Tanha, 2023).

Our study found that higher susceptibility was associated with the lower temperatures of the higher latitudes in seed source locations. Many authors have observed that plants in the tropics are better defended against herbivores than those in higher latitudes (Rasmann and Agrawal, 2011; Woods *et al.*, 2012; Więski and Pennings, 2014). Nevertheless, we cannot rule out the possibility that QUIN could be adaptations for protecting leaves against photodamage in cold environments (Moore *et al.*, 2004). The genotypes of *E. camaldulensis* from northern Australia (regions with warmer climates) are believed to be evolutionarily older lineages than those from higher latitude, colder regions (Butcher *et al.*, 2009). Consequently, the evolutionary age of the interaction between the wasp and its hosts could also explain latitudinal variability in resistance because younger plant taxa have been observed to support more galling insect species (Fernandes and Price, 1992). This finding corroborates distribution modelling results which have revealed that the most suitable region for origin of the invasive population of *L. invasa* in Africa lies in southern Australia (Otieno *et al.*, 2019). Our research, however, could not explain an increase in hydrolysable tannins with increasing latitude which could point to their role in providing protection against other herbivore pests or photodamage.

The linkage of the phenolic defensive metabolites to the environmental conditions of their respective regions of endemism in Australia, as seen in the groupings in the multivariate analyses, agrees with the phylogenetic conservatism hypothesis (Balagawi *et al.*, 2013). However, it is unlikely that they have been shaped by galling by *L. invasa* alone but rather in response to endemic insect herbivore community pressure in Australia (Agrawal *et al.*, 2002; Johnson and Agrawal, 2005) or possibly maladaptations on coevolution (Thompson *et al.*, 2002).

Irrespective of whether the resistance we have identified is specific or not, the identification of genes controlling resistance

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conferring traits and analysis of their performance in progeny trials could provide a better understanding of the direction of evolutionary processes involved in this system as well as opportunities to develop improved genotypes for planting in seed orchards by breeders and farmers. The genotypes identified to be resistant to the wasp can be adopted for growing in infested areas, as has been a common silvicultural practice for the mitigation of outbreaks of key insect pests and diseases for many decades (Henery, 2011; Naidoo *et al.*, 2019), while breeding research continues.

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## Appendix 1 Chemical properties of arboretum soil samples. Significant values ($P < 0.05$) are shown in bold

| Zone | Mg (ppm) | Ca (ppm) | K (ppm) | P (ppm) | N (%) | C (%) | pH $H_2O$ | EC mS $cm^{-1}$ | pH $CaCl_2$ |
|---|---|---|---|---|---|---|---|---|---|
| Turbo | 376.9 | 1015.4 | 447.9 | 3.8 | 0.3 | 2.0 | 6.2 | 0.0 | 5.1 |
| Akala | 500.9 | 2295.0 | 232.8 | 3.1 | 0.3 | 0.5 | 6.0 | 0.0 | 5.1 |
| Maranda | 413.3 | 1621.9 | 380.4 | 2.9 | 0.3 | 1.0 | 6.4 | 0.1 | 5.6 |
| Mitumbiri | 796.7 | 2544.9 | 282.6 | 7.8 | 0.3 | 1.6 | 6.6 | 0.0 | 5.5 |
| Yatta | 448.5 | 1168.4 | 800.9 | 1.5 | 0.1 | 1.4 | 5.8 | 0.1 | 5.3 |
| $F_{4,54}$ | 19.92 | 1.14 | 3.85 | 2.86 | 7.18 | 18.62 | 6.18 | 7.19 | 0.17 |
| $P$ | $<$**0.001** | 0.35 | **0.008** | **0.032** | $<$**0.001** | $<$**0.001** | $<$**0.001** | $<$**0.001** | 0.99 |

## Appendix 2 PCA loadings for soil chemical properties

| Variable | PC1 | PC2 | PC3 | PC4 | PC5 | PC6 |
|---|---|---|---|---|---|---|
| C | −0.068 | 0.157 | 0.817 | 0.002 | 0.113 | 0.174 |
| Ca | 0.228 | 0.289 | −0.077 | −0.786 | −0.005 | −0.171 |
| K | 0.240 | −0.405 | 0.478 | −0.167 | 0.167 | 0.093 |
| Mg | 0.391 | 0.257 | −0.153 | −0.171 | 0.545 | 0.368 |
| P | 0.169 | 0.403 | 0.010 | 0.489 | 0.501 | −0.345 |
| pH $CaCl_2$ | 0.574 | −0.106 | −0.019 | 0.159 | −0.305 | 0.031 |
| pH $H_2O$ | 0.544 | 0.106 | 0.022 | 0.234 | −0.377 | 0.218 |
| N | 0.004 | 0.531 | 0.267 | −0.048 | −0.378 | −0.407 |
| EC (mS/cm) pH $CaCl_2$ | 0.282 | −0.443 | 0.052 | −0.053 | 0.185 | −0.680 |
| % of variance | 28.01 | 24.2 | 13.85 | 10.77 | 8.87 | 6.29 |

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