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# Susceptibility of *Eucalyptus* trees to defoliation by the *Eucalyptus* snout beetle, *Gonipterus* sp. n. 2, is enhanced by high foliar contents of 1,8-cineole, oxalic acid and sucrose and low contents of palmitic and shikimic acid

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## Abstract

*Gonipterus* sp. n. 2 (Coleoptera, Curculionidae) is an invasive, commercially important weevil that causes large-scale defoliation of *Eucalyptus* trees. The weevil specifically feeds on young leaves and new shoots, thus reducing tree growth. The weevil displays a very strong preference for certain *Eucalyptus* genotypes, however, this behaviour and the chemistry underlying it is poorly understood, thereby complicating the selection of resistant trees. To elucidate the feeding preference of *Gonipterus* sp. n. 2, we assessed the relative levels of susceptibility of 62 *Eucalyptus* genotypes from 23 species using a laboratory choice assay. This revealed large intraspecific variation in susceptibility to weevil feeding, which for certain species, exceeded the interspecific variation. A semiquantitative metabolite profile analysis on 13 genotypes revealed strong correlations of 10 metabolites to feeding damage. The behavioural effects of the identified compounds were assessed through an in vitro feeding preference assay using artificial diets as well as under field conditions. This revealed three phagostimulants (1,8-cineole, oxalic acid and sucrose) and two feeding deterrent compounds (shikimic acid and palmitic acid) for *Gonipterus* sp. n. 2. These chemical markers can be applied to tree breeding programmes for the selection of resistant genotypes to reduce damage caused by *Gonipterus* weevils.

**KEYWORDS**
bioassay-guided compound identification, insect feeding inhibitor, insect feeding preference, phagostimulant, plant–insect interactions

## 1 | INTRODUCTION

The *Eucalyptus* snout weevil (*Gonipterus* spp.) is a devastating pest of *Eucalyptus*, which over the last 100 years was introduced from South East Australia and Tasmania to nearly every continent with commercial *Eucalyptus* timber plantations (Paine et al., 2011; Withers, 2001). The *Gonipterus scutellatus* species complex contains eight cryptic species, with *Gonipterus platensis*, *Gonipterus pulverulentus* and *Gonipterus* sp. n. 2 being invasive pests. In South Africa, *Gonipterus* spp. identified as *G. scutellatus* is now identified as a different but still undescribed species, *Gonipterus* sp. n. 2 (Mapondera et al., 2012). Damage is caused by feeding of both the adult and larval stages of *Gonipterus* sp. n. 2. Adult weevils feed on leaves, young shoots, buds and tips resulting in scalloped leaf edges and die-back of shoot tips. Larval feeding occurs on the entire epidermis of young leaves leaving behind fibrous leaf tissue (Tooke, 1955). Feeding occurs mostly on young and tender developing leaves which may lead to complete defoliation and a loss in apical dominance (Reis et al., 2012). Severe *Gonipterus* spp. outbreaks, which annually consume up to 50% of the tree's crown, are thought to reduce wood production by an estimated 85% over a 10-year growth period (Reis et al., 2012).

*Gonipterus* sp. n. 2 is primarily controlled by *Anaphes nitens*, an egg parasitoid, which was introduced from the weevils' native range into countries with significant weevil damage, such as South Africa and Spain (Rivera et al., 1999; Tooke, 1955). While historically *A. nitens* reduced *Gonipterus* sp. n. 2 damage enough to be considered under economic control, many large-scale outbreaks have been recorded over the last two decades in South Africa (Schröder et al., 2020; Verleur, 2012). The reason for these outbreaks is unknown, but it is hypothesized that the efficacy of *A. nitens* as a biocontrol agent may have decreased (Schröder et al., 2020). Therefore, alternative management strategies, such as planting resistant trees, should be considered.

The inherent resistance mechanisms of *Eucalyptus* against feeding by *Gonipterus* sp. n. 2 have not been studied in detail. *Gonipterus* sp. n. 2 shows a very strong host preference, specifically targeting *Eucalyptus globulus* (Mally, 1924; Tooke, 1955), *Eucalyptus viminalis* (Mally, 1924; Newete et al., 2011; Tooke, 1955) and *Eucalyptus smithii* (Newete et al., 2011) while avoiding species like *Eucalyptus citriodora* and *Eucalyptus saligna* (Mally, 1924; Newete et al., 2011; Tooke, 1955). Tooke (1955) investigated this preference by grouping the different *Eucalyptus* species known in 1927 into four groups, based on their level of susceptibility and compared these groups to the chemical composition of their essential oils as determined by Baker and Smith (1920). Although no clear patterns emerged, this analysis did show that most of the species which contained high levels of 1,8-cineole were susceptible (Tooke, 1955). Bouwer (2013) expanded on this by linking host preference to host volatiles, showing that *Gonipterus* sp. n. 2 females could detect *Eucalyptus* volatiles, with a greater response in preferred hosts (*E. globulus*) compared to nonhosts (*E. citriodora*).

The positive responses of *Gonipterus* sp. n. 2 to volatile essential oils demonstrate a potential link between the chemical composition of *Eucalyptus* trees and the weevils' feeding preference. However, little is known about the specific compounds responsible for this preference. Therefore, we studied the feeding preference of *Gonipterus* sp. n. 2 by conducting an in-depth analysis of the foliar chemistry of *Eucalyptus* and comparing resistant and susceptible genotypes. Our analysis revealed that *Gonipterus* sp. n. 2 feeding was enhanced by high foliar contents of 1,8-cineole, oxalic acid and sucrose and reduced by palmitic and shikimic acid, both on leaves and a recently developed artificial medium amended with pure chemicals. The robustness of these chemical markers was confirmed through an independent field trial conducted to screen *Eucalyptus* genotypes against natural infestation by *Gonipterus* sp. n. 2. Furthermore, palmitic acid and oxalic acid acted as markers of leaf age, while shikimic acid concentration significantly altered phytohormone response.

## 2 | METHODS AND MATERIALS

### 2.1 | Plant materials and insect collections

Adult *Eucalyptus* snout weevils were collected from commercial *Eucalyptus* plantations in the KwaZulu-Natal (Richard's Bay, Zululand, Midlands and New Hanover) and Mpumalanga (Piet Retief) provinces of South Africa. The adult weevils were maintained on young *Eucalyptus dunnii* leaves (Dun00) in a Memmert IN110 incubator (Memmert) at 20°C with a 14:10 day:night cycle. Dun00 was used throughout as a positive feeding control. This genotype is susceptible to all life stages of *Gonipterus* sp. n. 2, and is used for rearing *Gonipterus* sp. n. 2 in the FABI biocontrol centre to maintain its egg parasitoid, *A. nitens*.

Hybrid *Eucalyptus* saplings were obtained from the South African Pulp and Paper Industries Limited and maintained under shade netting at the experimental farm of the University of Pretoria (25.7472°S, 28.2588°E). Saplings were planted in 2.5 L pots and watered twice daily. Leaves from pure *Eucalyptus* species were obtained from the Tom Jenkins plantation (25°44′07.7″S 28°14′17.9″E) in Pretoria (Supporting Information: Table S1). Harvested leaves from each genotype were either used within 1 day for bioassays or immediately frozen upon collection and stored at −80°C for chemical analysis.

### 2.2 | Feeding preference trial

The feeding preference of *Gonipterus* sp. n. 2 was assessed for 62 *Eucalyptus* genotypes using a choice bioassay. These genotypes are either currently planted or were historically planted in commercial *Eucalyptus* plantations in South Africa. The choice arena was prepared by modifying a Petri dish (65 diameter × 14.5 mm height) (Jplast) by inserting four thumbtacks equidistantly through the

bottom, 1 cm from the edge, in a square arrangement with each tack 90° to the previous (Supporting Information: Figure S1). The floor of the arena was covered with a moist filter paper disk, which was secured in place by allowing the exposed tacks to pierce through the paper. Young leaves were harvested at the distal end of branches (leaf position (LP) 3–6) and cut into disks with a 1.5 cm diameter, straight-edged cork borer. Two leaf disks of the genotype of interest and two leaf discs of the control genotype (*E. dunnii*, Dun00) were alternately fastened to the tacks in the choice arena. Eight adult weevils of mixed gender and unknown age were starved for 3 days and placed into the centre of the choice arena. This arena was placed in a Memmert IN110 incubator at 20°C with a 14:10 day: night cycle for 2 days. Each bioassay was photographed from a height of 25 cm using a Sony Xperia XA1 smartphone, before and after the 2-day incubation. This trial was replicated four times for each genotype.

The number of coloured pixels (leaf surface area) in each leaf disk before and after feeding was quantified using Adobe Photoshop CC 2015.

The average percentage feeding on control disks (Dun 00) per replicate was calculated as:

$$\text{AvrControl} = \frac{\frac{CA1 - CB1}{CB1} + \frac{CA2 - CB2}{CB2}}{2},$$

where, CA1 is the total number of coloured pixels in control disk 1 after feeding, CA2 is the total number of coloured pixels in control disk 2 after feeding, CB1 is the total number of coloured pixels in control disk 1 before feeding and CB2 is the total number of coloured pixels in control disk 2 before feeding.

The average percentage feeding on the genotype of interest disk per replicate was calculated as:

$$\text{AvrGenotype} = \frac{\frac{GA1 - GB1}{GB1} + \frac{GA2 - GB2}{GB2}}{2},$$

where, GA1 is the total number of coloured pixels in genotype of interest disk 1 after feeding, GA2 is the total number of coloured pixels in genotype of interest disk 2 after feeding, GB1 is the total number of coloured pixels in genotype of interest disk 1 before feeding and GB2 is the total number of coloured pixels in genotype of interest disk 2 before feeding.

The relative feeding (RF) amount for each replicate was calculated as $\text{RF} = \frac{\text{AvrGenotype}}{\text{AvrControl}}$.

The software R v. 4.0.3 (R Core Team, 2017) was used to perform a Kruskal–Wallis test between AvrControl and AvrGenotype values, to test for significant differences in feeding between the control and genotype of interest. This approach was utilized to reduce variation in the data brought about by the variation in weevil behaviour, that is, between replicates, the weevils would consume the same ratio of material between the control and genotype of interest, however, at a much higher or lower rate, thus adding large variation to the data set.

A second set of bioassays was conducted, following the above protocol, where all possible combinations of resistant and susceptible genotypes were compared against each other, this approach allowed the genotypes to be ranked by the feeding preference of *Gonipterus* sp. n. 2.

## 2.3 | Gas chromatography-mass spectrometry (GC-MS) analysis of polar metabolites

Thirteen *Eucalyptus* genotypes (seven *E. dunnii*, six *Eucalyptus grandis* × *Eucalyptus urophylla* hybrids genotypes) were selected for further semiquantitative chemical analysis (Supporting Information: Table S3). These species were selected due to the large intraspecific variation in beetle feeding between the selected genotypes, which spanned the full range of resistance against *Gonipterus* sp. n. 2.

Young leaves were harvested at the distal end of branches (LP 3–6) from four trees/saplings of each genotype and stored at −80°C. These leaves were frozen in liquid nitrogen and hand-ground using a mortar and pestle. Subsamples of the ground leaves were freeze-dried using a Virtis adVantage lyophilizer (SP Scientific) for 24 h at a pressure of 13.33 kPa and then stored at room temperature. A total of 26 mg of each sample was suspended in 1.8 mL absolute methanol (Sigma) and incubated at room temperature for 4 h, then centrifuged at 1200 rpm for 5 min. A volume of 1.2 mL of the supernatants were transferred to glass vials. This methanol extract underwent chemical derivatization to mask the polar functional groups, thus allowing for GC-MS analysis.

The samples were dried under ambient temperature using nitrogen airflow and resuspended in 100 µL pyridine (Sigma, Germany) containing 20 mg $ml^{-1}$ methoxamine HCl (Sigma). This solution was incubated at 30°C for 90 min and then centrifuged at 1200 rpm for 20 min. A total of 30 µL of the supernatant was added to 30 µL MS-TFA (N-Methyl-N-(trimethylsilyl)trifluoroacetamide) (Sigma) in a glass vial and incubated at 37°C for 30 min, then stored at 4°C until use. A total of 1 µL of the supernatant was analysed on an Agilent 7890 GC-MS (Agilent) (GC-MS) using an HP5 column with a linear temperature programme starting at 70°C increasing at a rate of 5°C $min^{-1}$ until a maximum temperature of 300°C, then held for 2 min. The parameters of the GC-MS were a solvent delay of 6 min, a split inlet with a split ratio of 100:1 and a flow rate of 120 mL $min^{-1}$ leading to a 1.2 mL $min^{-1}$ flow rate on the column. The mass spectrometer was set to scan mode with a low mass of 40 m $z^{-1}$ and a high mass of 650 m $z^{-1}$ and the ion source was maintained at 70 eV (Supporting Information: Figure S2).

Serial dilutions of pure standards were analysed to confirm the identity and concentration of the compounds of interest. Standard concentration was determined from literature to match their natural concentration in leaves. The standards were: 39 mg $mL^{-1}$ fructose, 40.5 mg $mL^{-1}$ oxalic acid, 7.5 mg $mL^{-1}$ palmitic acid, 4.5 mg $mL^{-1}$ shikimic acid and 26 mg $mL^{-1}$ sucrose suspended in 100 µL pyridine (Sigma) containing 20 mg $mL^{-1}$ methoxamine HCl and derivatized as described above. A total of 100 µL of each solution was added to 900 µL methanol (Sigma). Following this, a series of dilutions were made ($10^{-1}$, $10^{-2}$ and $10^{-3}$) for each solution and analysed as described above. Compound quantities in leaf samples were

calculated as mg $g^{-1}$ dry sample weight using the external standard curves and the sample volume as factors in the calculation (Supporting Information: Figure S5).

## 2.4 | GC-MS analysis of nonpolar metabolites

A total of 41.1 mg (fresh weight) of each ground leaf sample was weighed and extracted with 1 mL hexane (Sigma). This solution was incubated at 200 rpm at 24°C for 1 h and then centrifuged at 1200 rpm for 20 min. 800 µL of the supernatant was transferred into a glass vial. 1 µL of the supernatant was analysed on an Agilent 7890 GC-MS (Agilent) (GC-MS) using an HP5 column with a linear temperature programme starting at 40°C increasing at a rate of 4 °C $min^{-1}$ until a maximum temperature of 200°C then held for 2 min. The parameters of the GC-MS were a solvent delay of 3.5 min, split-less inlet and a flow rate of 1.2 mL $min^{-1}$. The mass spectrometer was set to scan mode with a low mass of 40 m $z^{-1}$ and a high mass of 450 m $z^{-1}$ and the ion source was maintained at 70 eV (Supporting Information: Figure S3).

Serial dilutions of pure standards were analysed to confirm the identity and concentration of the compounds of interest. Standard concentration was determined from the literature to match their natural concentration in leaves. The standards were: 0.92 mg $mL^{-1}$ 1,8-cineole (Sigma), 0.858 mg $mL^{-1}$(+) $\alpha$-pinene (Sigma), 0.85 mg $mL^{-1}$ $\gamma$-terpinene (Sigma), 0.8 mg $mL^{-1}$ *trans*-$\beta$-ocimene (Sigma) suspended in n-hexane (Sigma), after which a series of dilutions were made ($10^{-1}$, $10^{-2}$ and $10^{-3}$) and analysed as described above. Compound quantities in leaf samples were calculated as mg $g^{-1}$ fresh sample weight using the external standard curves and the sample volume as factors in the calculation (Supporting Information: Figure S6).

## 2.5 | Analysis of GC-MS results

MassHunter Unknowns Analysis software was used to integrate and deconvolute peaks of the chromatograms. The compounds were then tentatively identified utilizing the 2017 NIST library (Information Services Office). The parameters for the deconvolution were as follows: left m $z^{-1}$ delta of 0.3, right m $z^{-1}$ delta of 0.7, sharpness threshold of 25%. For the library search, a minimum match factor of 30 was used and no peak filters were applied to maximize peak detection. The compound name, retention time, mass and peak area was exported. Following this, the software R (R Core Team, 2017) was used to conduct a general linear regression of peak area to the average RF value from the first bioassay (genotype vs. Dun00). A summary of the regression was generated and correlations showing a significant F-statistic were selected for further identification and testing.

## 2.6 | Artificial diet

An artificial diet for *Gonipterus* sp. n. 2 was developed using the protocol described by Wheeler and Zahniser (2001). The protocol was modified by using ground freeze-dried *Eucalyptus* leaves instead of *Melaleuca* leaves. The artificial diet comprised of two mixtures (Supporting Information: Table S4). Mixture 1 was autoclaved at 121°C for 15 min. Mixture 2 was added to 40 mL water and filter (0.2 µm pore size) sterilized. The two solutions were mixed and poured into Petri dishes (65 mm diameter × 14.5 mm height) (Jplast) and stored at 4°C.

To test insect viability on the artificial diet, we compared beetle performance when fed on the diet versus young leaves of Dun00. Ten field-collected adult weevils were each placed in six Petri dishes modified in the same manner as described in the feeding preference trial and stored in a Memmert IN110 incubator (Memmert) at 20°C with a 14:10 day:night cycle. These beetles were fed every 4 days, with two Petri dishes receiving four artificial diet disks (cut using a 1.5 cm diameter, straight-edged cork borer), two Petri dishes receiving four young leaves (Dun00) harvested at the distal end of branches (leaf position (LP) 3–6) and two Petri dishes receiving no food. The total number of living beetles and eggs was recorded every 4 days for 32 days (Supporting Information: Figure S4).

## 2.7 | Behavioural assay—polar metabolites

The behavioural effects of the polar metabolites of interest were assessed using a choice bioassay, following the above protocol for the feeding preference trial. However, we replaced the genotype of interest with an artificial diet disk (cut using a 1.5 cm diameter, straight-edged cork borer) amended with either a high, medium or low concentration of the compounds of interest. The control genotype was replaced with an artificial diet disk amended with a different concentration of the same compound. This experiment was repeated using different concentration combinations until all possible combinations were tested. Furthermore, the level of feeding was measured by weighing the artificial diet disks before and after feeding. Pairwise *t*-tests, conducted using R, were used to determine if the mean weight change of the artificial diet disks was significantly different between concentrations.

The artificial diets amended with either high, medium or low quantities of metabolites were prepared by taking 25 mL of artificial diet and adding a filter sterilized solution (0.2 µm pore size) of 2 mL water containing specific quantities of each metabolite. Media either contained 37, 20.4 and 9.3 mg $mL^{-1}$ fructose (Sigma) or sucrose (Huletts); 10.9, 6.2 and 1.6 mg $mL^{-1}$ oxalic acid dehydrate (Sigma); 0.42, 0.28 and 0.14 mg $mL^{-1}$ palmitic acid (Sigma); 0.028, 0.014 and 0.006 mg $mL^{-1}$ shikimic acid (Sigma). These concentrations were chosen to reflect the high, medium and low concentrations of metabolites naturally present in leaves.

## 2.8 | Behavioural assay—nonpolar metabolites

To make a volatile extract of *E. dunnii* (Dun00), leaves were frozen in liquid nitrogen then hand-ground using a mortar and pestle. The

ground leaves were added to 150 mL hexane (Sigma) until the leaves reached the 75 mL mark on a measuring cylinder and incubated for 60 min. This mixture was then poured through filter paper and stored at 4°C.

The behavioural effects of the nonpolar metabolites of interest were assessed using a choice bioassay, following the above protocol for the behavioural bioassay of polar metabolites. However, we used an unamended artificial diet throughout, while the nonpolar metabolite treatment was applied via filter paper circles. This was conducted by fastening filter paper circles to all four tacks of the feeding arena, then 5 µL *Eucalyptus* leaf extract was added to their centre, simulating the normal volatile emissions of susceptible *Eucalyptus* leaves (Dun00 genotype)). Five µL of the nonpolar metabolite of interest was added to two oppositely fastened filter paper circles, while the two remaining oppositely located paper circles received 5 µL hexane (control). Unamended artificial diet disks (cut using a 1.5 cm diameter, straight-edged cork borer), were fastened to all four tacks covering the filter paper circles. Therefore, two control artificial diet disks emitted the volatile blend of susceptible *Eucalyptus* leaves, and two treatment artificial diet disks emitted the volatile blend of susceptible *Eucalyptus* leaves with higher/unique levels of the nonpolar metabolite of interest. This trial was repeated twice changing the nonpolar metabolite treatment with a serial dilution of the same compound at a 10- and 100- fold dilution. Trials, for all concentrations, were replicated four times.

The nonpolar metabolites used were standard solutions of 0.92 mg $ml^{-1}$ 1,8-cineole (Sigma), 0.858 mg $ml^{-1}$ $\alpha$-pinene (Sigma), 0.85 mg $ml^{-1}$ $\gamma$-terpinene (Sigma) and 0.8 mg $ml^{-1}$ *trans*-$\beta$-ocimene (Sigma) suspended in n-hexane (Sigma).

## 2.9 | Field trial

In 2016, 26 clones of 8 *E. grandis* × *Eucalyptus nitens*, 13 *E. grandis* × *E. urophylla*, 1 *E. saligna* × *E. urophylla*, 1 *Eucalyptus benthamii*, 1 *E. dunnii*, 1 *E. grandis* and 1 *E. saligna* genotypes/species were used to establish a field trial at Hodgsons (29.2053S, 30.5532E) plantation in the Midlands of KwaZulu-Natal in South Africa. This trial was implemented to determine relative clone performance and to measure the impact of pests and diseases by comparing the growth of trees treated and untreated with pesticide protection. At 8-, 11- and 18-months postplanting, trees were assessed for damage caused by *Gonipterus* sp. n. 2. *Gonipterus* sp. n. 2 damage was assessed by counting the number of adults, larvae and egg capsules observed in 1 min, per tree, and roughly estimating the percentage tree damage caused by adult and larval feeding.

In 2020, branches of 14 *Eucalyptus* genotypes were harvested from plots untreated with pesticides at the Hodgson's trial. These included one *E. dunnii*, one *E. grandis*, three *E. grandis* × *E. nitens* hybrids, six *E. grandis* × *E. urophylla* hybrids, two *E. nitens* × *E. grandis* hybrids and one *E. saligna* × *E. urophylla* hybrid. Branches were stored in a cooler box at 4°C and transported to the University of Pretoria, where they were immediately stored at −80°C. Young leaves at the distal end of branches (LP 3–6) were removed, frozen in liquid nitrogen and hand-ground using a mortar and pestle. Subsamples of the ground leaves were extracted and analysed using the above protocol for the GC-MS analysis of polar and nonpolar metabolites. The software R (R Core Team, 2017) was used to conduct a general linear model of the extracted peak areas of the three phagostimulants (1,8-cineole, oxalic acid and sucrose) and two feeding deterrent compounds (shikimic acid and palmitic acid) to the feeding damage recorded at 8- and 11-months postplanting. A summary of the regression was generated, and the F-statistic was recorded.

## 2.10 | Effect of leaf age on organic acid content

*Gonipterus* sp. n. 2 prefers to feed on young emerging leaves (Tooke, 1955). Due to the significant effect of oxalic and palmitic acid on beetle feeding, we wanted to assess if the concentration of these important organic acids differs in young and old leaves. Therefore, both young and old leaves of eight *E. dunnii* trees from two genotypes Dun07 (resistant) and Dun09 (susceptible) were harvested. The young leaves were harvested at the distal end of branches (LP 3–6) and the mature leaves were harvested near the proximal end of branches (LP 9–12). These leaves were immediately frozen and stored at −80°C. The leaves were processed following the protocol detailed for the GC-MS analysis of polar metabolites. The software R (R Core Team, 2017) was used to conduct a Tukey HSD test for differences in leaf age and peak area for oxalic and palmitic acid in each genotype.

## 2.11 | Phytohormone analyses

Due to the significant effect of the phytohormone precursor, shikimic acid, on insect feeding, phytohormones in a resistant and susceptible *Eucalyptus* genotype were analysed. Since phytohormones are at a significantly lower concentration in the constitutive metabolome an induction experiment was performed, where sixteen *E. dunnii* trees of 2 genotypes (Dun07 (high shikimic acid concentration) and Dun09 (low shikimic acid concentration)) were planted in 2.5 L pots and individually placed inside a steel wire mesh bag (US mesh size 12). Twenty adult weevils of mixed gender and unknown age were placed into eight bags per genotype with the bags enclosing the remaining eight trees per genotype left empty as controls. The mesh bags containing the trees and weevils were placed outside and maintained under shade netting at the experimental farm of the University of Pretoria (25.7472°S, 28.2588°E) for 1 week, and watered twice daily. Young leaves from each tree were frozen in liquid nitrogen and hand-ground using a mortar and pestle. The ground leaves were freeze-dried using a Virtis adVantage lyophilizer (SP Scientific) for 24 h at a pressure of 13.33 kPa and then stored at room temperature. Fifty mg of each sample was weighed and phytohormones were extracted and analysed following the protocol described in Chen et al. (2021). The software R (R Core Team, 2017) was used to test for significant

differences in phytohormone concentrations between beetle-exposed trees and control trees using Pairwise Wilcoxon Rank Sum Tests.

## 3 | RESULTS

### 3.1 | Intraspecific variation plays a major role in the feeding preference of *Gonipterus* sp. n. 2

We conducted bioassays to rank 62 different *Eucalyptus* genotypes by their relative level of susceptibility to *Gonipterus* sp. n. 2 (Table 1). The first round of the feeding preference trial compared weevil feeding on a genotype of interest to a control, *E. dunnii* genotype (Dun00) (Supporting Information: Table S2). The genotypes separated into groups of 16 highly resistant ($p < 0.05$ and RF < 0.7), 17 moderately resistant ($p < 0.2$ and RF < 0.9), 12 similar to control ($p > 0.4$ or 0.9 < RF < 1.1), eight moderately susceptible ($p < 0.2$ and RF > 1.1) and nine highly susceptible genotypes ($p < 0.05$ and RF > 1.25).

In the second round of feeding preference trials, species were ranked by their level of resistance to feeding by *Gonipterus* sp. n. 2 relative to each other. The three most resistant genotypes were an *E. grandis* × *camaldulensis* (GC01) genotype, an *E. nitens* (NIT04) genotype and an *E. dunnii* (DUN10) genotype. The three most susceptible genotypes were an *E. saligna* × *urophylla* (SU01), an *E. nitens* (NIT09) and an *E. dunnii* (DUN08) genotype (Table 2).

The 62 genotypes contained 23 species, for six of these species more than one genotype was tested. *E. grandis* × *urophylla* contained two highly resistant, seven moderately resistant, one similar to control, one moderately susceptible and three highly susceptible genotypes. *E. dunnii* contained three highly resistant, three moderately resistant, four similar to control, two moderately susceptible and three highly susceptible genotypes. *E. nitens* contained one highly resistant, two moderately resistant, four similar to control, two moderately susceptible and three highly susceptible genotypes. *Eucalyptus macarthurii* contained one moderately resistant and moderately susceptible genotype. *E. grandis* × *camaldulensis* contained one highly resistant and two moderately resistant genotypes. *E. grandis* contained two highly resistant genotypes. This data led us to conclude, that feeding preference was not based on host species but might be driven by differences in leaf chemistry and that leaf chemistry varied as much or more among genotypes within a species as it did among species.

### 3.2 | The feeding behaviour of *Gonipterus* sp. n. 2 correlated strongly with specific chemical markers

Thirteen genotypes of one pure species and one hybrid (Supporting Information: Table S3) that displayed large intraspecific variation were selected for chemical analysis, to identify chemical markers of resistance. RF (where the percent leaf surface area lost by the control was subtracted from the percent leaf surface area lost from the genotype of interest) was correlated by linear regressions to compound concentrations determined by GC-MS analyses (Figures 1 and 2). Regressions with all 47 tentatively identified polar metabolites (Supporting Information: Figure S2) and 39 tentatively identified nonpolar metabolites (Supporting Information: Figure S3) revealed 10 compounds (Supporting Information: Table S4) with statistically significant ($p < 0.05$) correlations. 1,8-Cineole, γ-terpinene, lapachone, oxalic acid, palmitic acid, sucrose, terpinen-4-ol and trans-β-ocimene showed a significant positive correlation to relative weevil feeding. α-Pinene and shikimic acid showed a significant negative correlation. Strong correlations ($r^2$ => 0.6) were obtained for α-pinene, 1,8-cineole, oxalic acid, sucrose, terpinen-4-ol and trans-β-ocimene in all tested genotypes. Palmitic acid correlated strongly with feeding in the tested *E. grandis* × *urophylla* genotypes and γ-terpinene and lapachone showed strong correlations to feeding in *E. dunnii* genotypes. Weaker correlations between feeding preference and compound concentrations ($0.4 > r^2 > 0.6$) were obtained for γ-terpinene and lapachone in the *E. grandis* × *urophylla* genotypes tested and palmitic acid and shikimic acid also showed weaker correlations in the *E. dunnii* genotypes tested.

### 3.3 | Artificial diet disks are a suitable alternative to leaf feeding

A no-choice feeding assay was conducted for 32 days comparing the survival of 20 beetles (per group) on artificial diet disks, Dun00 leaves and no food (starvation). After 8 days, all beetles in the starvation group were dead. After 12 days, one beetle had died in the artificial diet group. No beetles in the Dun00 treatments died. Eleven egg capsules were obtained in the leaf-feeding group, while no other group produced eggs (Supporting Information: Figure S4).

### 3.4 | Behavioural assays revealed three phagostimulants (1,8-cineole, oxalic acid and sucrose) and two feeding deterrents (palmitic acid and shikimic acid) for *Gonipterus* sp. n. 2

A bioassay was conducted where weevils were offered a choice between an artificial diet amended with different concentrations of 1,8-cineole, α-pinene, γ-terpinene and trans-β-ocimene and a nonamended control diet. The weevils displayed a significant preference ($p < 0.05$) for the diet amended with high, medium and low concentrations of 1,8-cineole (Figure 3). No behavioural differences were observed between the different concentrations of α-pinene, γ-terpinene and trans-β-ocimene. A significant ($p < 0.05$) preference for the nonamended control diet when compared to the lowest concentration of α-pinene and a preference for the amended diet when compared to the lowest concentration of trans-β-ocimene was observed.

A similar bioassay was conducted where weevils were offered the choice between an amended artificial diet (oxalic acid, sucrose, shikimic acid and palmitic acid) and another artificial diet amended with the same compound at either higher or lower concentrations (Figure 4). In all cases, weevils showed a significant preference

**TABLE 1** Genotypes separated into five columns based on their resistance relative to *Eucalyptus dunnii* with the *p value* obtained from the *Kruskal–Wallis* test (RF = the average percentage feeding on genotype of interest/the average percentage feeding on control disks per replicate) (See Section 2.2).

| Highly susceptible | | | Moderately susceptible | | | Similar to control | | | Moderately resistant | | | Highly resistant | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Genotype | RF | *p* value | Genotype | RF | *p* value | Genotype | RF | *p* value | Genotype | RF | *p* value | Genotype | RF | *p* value |
| BEN01 | 1.72 ± 0.55 | 0.05 | DUN01 | 1.57 ± 0.88 | 0.17 | DOR01 | 4.19 ± 4.06 | 0.38 | BOT01 | 0.87 ± 0.14 | 0.01 | CIT01 | 0.55 ± 0.04 | 0.00 |
| DUN02 | 1.20 ± 0.32 | 0.03 | DUN08 | 1.73 ± 0.75 | 0.10 | DUN03 | 0.93 ± 0.30 | 0.06 | DUN10 | 0.70 ± 0.11 | 0.01 | DUN05 | 0.61 ± 0.16 | 0.03 |
| DUN09 | 1.70 ± 0.29 | 0.01 | GU08 | 1.59 ± 0.79 | 0.14 | DUN04 | 0.23 ± 0.15 | 0.22 | DUN11 | 0.76 ± 0.32 | 0.10 | DUN07 | 0.47 ± 0.11 | 0.03 |
| DUN14 | 1.32 ± 1.32 | 0.06 | MAC02 | 1.57 ± 0.65 | 0.10 | DUN06 | 3.22 ± 2.69 | 0.32 | DUN15 | 0.19 ± 0.09 | 0.13 | DUN13 | 0.30 ± 0.06 | 0.01 |
| GU04 | 1.28 ± 0.39 | 0.05 | MIC01 | 8.30 ± 4.56 | 0.17 | DUN12 | 0.97 ± 0.06 | 0 | GC01 | 0.73 ± 0.27 | 0.08 | GC03 | 0.13 ± 0.03 | 0.04 |
| GU12 | 1.66 ± 0.34 | 0.02 | NIT06 | 2.09 ± 1.28 | 0.20 | GU05 | 1.46 ± 1.10 | 0.28 | GC02 | 0.71 ± 0.10 | 0.01 | GON01 | 0.44 ± 0.09 | 0.02 |
| GU14 | 1.27 ± 0.21 | 0.01 | PIL01 | 6.30 ± 3.60 | 0.18 | NIT02 | 01.00 ± 0.19 | 0.02 | GU01 | 0.79 ± 0.09 | 0.00 | GRA01 | 0.11 ± 0.04 | 0.02 |
| NIT09 | 1.53 ± 0.47 | 0.05 | SU01 | 2.45 ± 1.19 | 0.13 | NIT04 | 0.22 ± 0.19 | 0.33 | GU02 | 0.68 ± 0.09 | 0.00 | GRA02 | 0.50 ± 0.11 | 0.02 |
| PAN01 | 1.26 ± 0.39 | 0.05 | | | | NIT05 | 16.89 ± 15.79 | 0.36 | GU03 | 0.84 ± 0.27 | 0.05 | GU07 | 0.61 ± 0.16 | 0.03 |
| | | | | | | NIT08 | 1.05 ± 0.15 | 0.01 | GU06 | 0.83 ± 0.14 | 0.01 | GU11 | 0.47 ± 0.12 | 0.03 |
| | | | | | | PUN01 | 1.13 ± 0.19 | 0.01 | GU09 | 0.37 ± 0.14 | 0.07 | NIT03 | 0.26 ± 0.05 | 0.01 |
| | | | | | | ROB01 | 1.10 ± 0.48 | 0.11 | GU10 | 0.77 ± 0.29 | 0.08 | OBL01 | 0.21 ± 0.07 | 0.05 |
| | | | | | | | | | GU13 | 0.77 ± 0.27 | 0.07 | OVA01 | 0.44 ± 0.06 | 0.00 |
| | | | | | | | | | MAC01 | 0.63 ± 0.21 | 0.06 | PRO01 | 0.31 ± 0.07 | 0.02 |
| | | | | | | | | | NIT01 | 0.68 ± 0.05 | 0.00 | SID01 | 0.19 ± 0.03 | 0.01 |
| | | | | | | | | | NIT07 | 0.68 ± 0.23 | 0.06 | VIM01 | 0.19 ± 0.05 | 0.04 |
| | | | | | | | | | SAL01 | 0.85 ± 0.19 | 0.02 | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| BEN = *Eucalyptus benthamii* | DOR = *Eucalyptus dorriengoenis* | GON = *Eucalyptus goniocalyx* | MAC = *Eucalyptus macarthurii* | OBL = *Eucalyptus obliqua* | PIL = *Eucalyptus pilularis* | SID = *Eucalyptus sideroxlon* |
| BOT = *Eucalyptus botryoides* | DUN = *Eucalyptus dunnii* | GRA = *Eucalyptus grandis* | MIC = *Eucalyptus microcorys* | OVA = *Eucalyptus ovata* | PRO = *Eucalyptus propinqua* | SU = *Eucalyptus saligna* × *urophylla* |
| CIT = *Eucalyptus citriodora* | GC = *E. grandis* × *camaldulensis* | GU = *E. grandis* × *urophylla* | NIT = *Eucalyptus nitens* | PAN = *Eucalyptus paniculate* | SAL = *Eucalyptus saligna* | |

Abbreviation: RF, relative feeding.

**TABLE 2** Table ranking genotypes of Table 1 by RF = (the average percentage feeding on genotype of interest/the average percentage feeding on control disks per replicate) (See Section 2.2) by *Gonipterus* sp. n. 2.

| More resistance | | Less resistance | |
|---|---|---|---|
| Rank | Genotype | Rank | Genotype |
| 1 | GC01 | 1 | SU01 |
| 2 | NIT04 | 2 | NIT09 |
| 3 | DUN10 | 3 | DUN08 |
| 4 | NIT07 | 4 | DUN09 |
| 5 | GRA01 | 5 | PIL01 |
| 5 | GU10 | 5 | NIT08 |
| 6 | GU09 | 5 | GRA03 |
| 6 | GU11 | 6 | BEN01 |
| 7 | GRA02 | 6 | GU07 |
| 7 | BOT01 | 7 | GU12 |
| 7 | MAC01 | Species | |
| 8 | OVA01 | BEN = *Eucalyptus benthamii* | |
| 8 | SID01 | BOT = *Eucalyptus botryoides* | |
| 9 | GON01 | CIT = *Eucalyptus citriodora* | |
| 9 | PRO01 | DUN = *Eucalyptus dunnii* | |
| 10 | CIT01 | GC = *Eucalyptus grandis* × *camaldulensis* | |
| 10 | OBL01 | GON = *Eucalyptus goniocalyx* | |
| 11 | GU02 | GRA = *E. grandis* | |
| 12 | DUN11 | GU = *E. grandis* × *urophylla* | |
| 12 | GC02 | MAC = *Eucalyptus macarthurii* | |
| 13 | GU05 | NIT = *Eucalyptus nitens* | |
| 14 | DUN05 | OBL = *Eucalyptus obliqua* | |
| 14 | DUN06 | OVA = *Eucalyptus ovata* | |
| 14 | NIT03 | PIL = *Eucalyptus pilularis* | |
| 14 | DUN15 | PRO = *Eucalyptus propinqua* | |
| 15 | GU05 | SID = *Eucalyptus sideroxlon* | |
| | | SU = *Eucalyptus saligna* × *urophylla* | |

Abbreviation: RF, relative feeding.

($p < 0.05$) for a higher concentration of oxalic acid and sucrose, while showing a significant avoidance of shikimic acid. Palmitic acid inhibited weevil feeding, however, this was only significant at the highest concentrations.

## 3.5 | Laboratory assays were confirmed under field conditions

To determine if the concentration of these compounds influenced feeding preferences in the field, we utilized an independent field trial, planted with a different set of *Eucalyptus* genotypes (one *E. dunnii*, one *E. grandis*, three *E. grandis* × *E. nitens* hybrids, six *E. grandis* × *E. urophylla* hybrids, two *E. nitens* × *E. grandis* hybrids and one *E. saligna* × *E. urophylla* hybrid). The concentration of the compounds of interest within selected trees were correlated to the natural *Gonipterus* sp. n. 2 feeding damage at 8- and 11-months postplanting (Figure 5). Feeding damage at 8 months postplanting was significantly correlated with leaf concentrations of 1,8-cineole, palmitic acid and shikimic acid ($p < 0.05$), while oxalic acid and sucrose showed a near-significant ($p < 0.06$) correlation at 8 months postplanting. At 11 months postplanting, statistically significant ($p < 0.05$) correlations, matching the laboratory assays, were obtained for shikimic acid and palmitic acid, while 1,8-cineole and sucrose showed a near-significant ($p < 0.06$) correlation and oxalic acid showed a nonsignificant correlation ($p = 0.12$). However, at 11 months postplanting on the selected trees, the adult population decreased by 25%, larval population decreased by 65% and the egg population increased by 628%, which might have contributed to the decrease in the significance of the correlations.

## 3.6 | Resistant genotypes contain higher concentrations of phytohormones

The level of several key phytohormones associated with plant defence (abscisic acid (ABA), jasmonic acid (JA), jasmonyl isoleucine (JA-Ile), carboxy-jasmonyl-isoleucine (COOH-JA-Ile), hydroxy-jasmonic acid (OH-JA), hydroxy-jasmonyl-isoleucine (OH-JA-Ile), sulfo-jasmonic acid, oxo-phytodienoic acid (OPDA), salicylic acid (SA) and salicylic acid glucoside (SA-Glu-mz137)) was assessed in both a resistant (Dun07) and susceptible (Dun09) *E. dunni* genotype (Figure 6).

The phytohormones ABA, JA and OPDA showed significantly higher constitutive concentrations in the resistant genotype compared to the susceptible genotype (230%, 390% and 590%, respectively). Furthermore, OH-JA, SA and SA- Glu-mz137 showed near significantly ($0.05 < p < 0.08$) higher concentrations in the resistant genotype compared to the susceptible genotypes (165%, 140% and 220%, respectively). Sulfo-JA showed a nearly significantly ($p = 0.082$) higher concentration in the susceptible than in resistant genotype (158%). No significant constitutive differences were found for COOH-JA-Ile and OH-JA-Ile.

After feeding by *Gonipterus* sp. n. 2, ABA showed a significant ($p = 0.007$) 158% increase in the susceptible genotype and a significant ($p = 0.021$) decrease in the resistant genotype dropping to 46% of its original value. However, the postfeeding concentration of the susceptible genotype was only 68% of the constitutive concentration of ABA in the resistant genotype. JA showed a significant ($p = 0.007$) 182% increase in concentration in the susceptible genotype and a significant ($p = 0.002$) decrease in the resistant genotype falling to 29% of its original value. Furthermore, the postfeeding concentration of the susceptible genotype was only 71% of the constitutive concentration of the resistant genotype. OH-JA and OPDA showed a significant ($p < 0.05$) decrease in the resistant genotype, with their concentration falling to 35% and 27% of their initial concentration, respectively. Furthermore, there was no

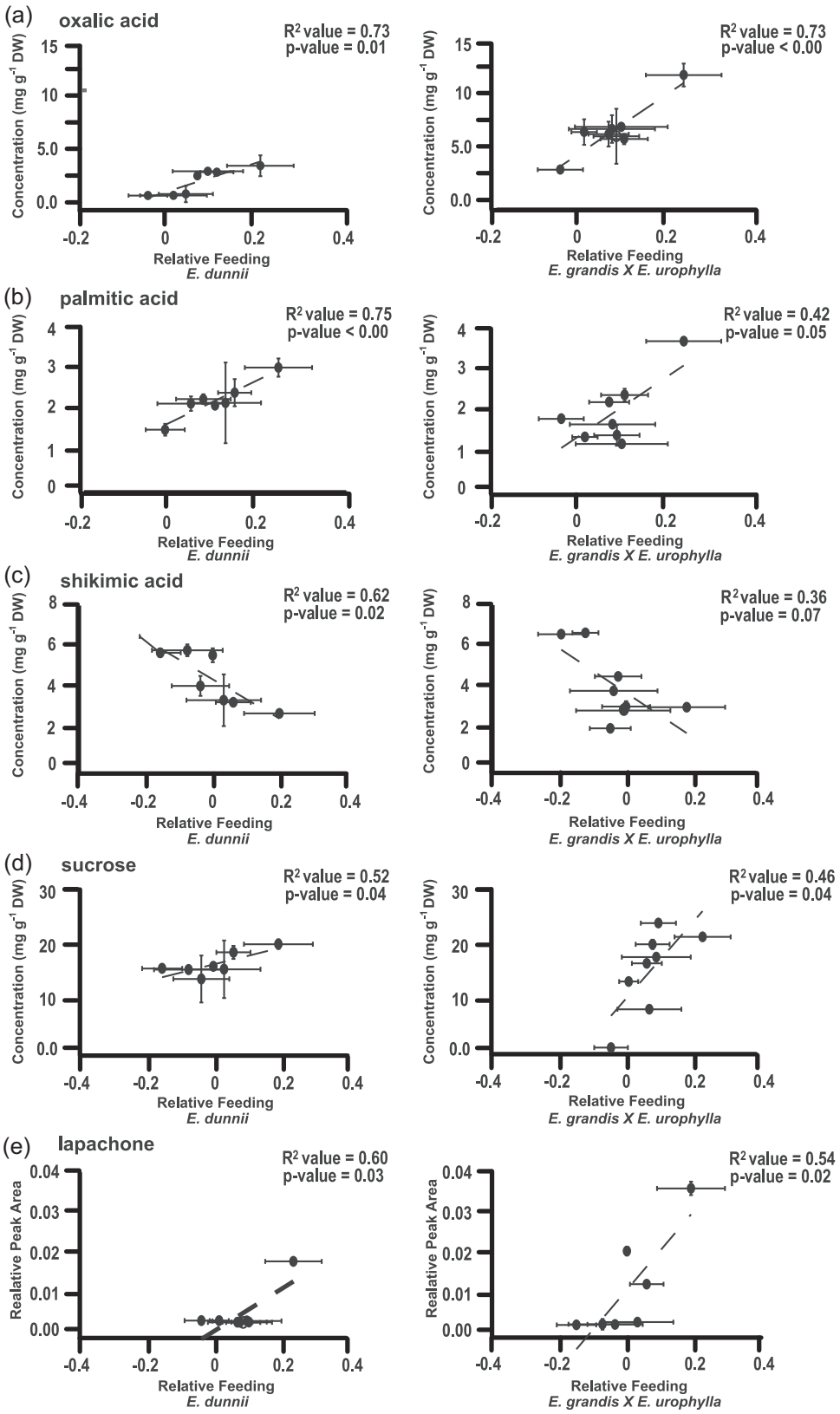


**FIGURE 1** (See caption on next page).

significant change in the susceptible genotype. OH-JA-Ile showed a significant ($p = 0.035$) 210% increase in concentration in the resistant genotype, which was 194% higher than the concentration in the susceptible genotype. Furthermore, no significant change in the susceptible genotype was observed. COOH-JA-Ile showed a near-significant ($p = 0.06$) 185% increase in concentration in the resistant genotype, which was 229% higher than the concentration in the susceptible genotype, Furthermore, no significant change in the susceptible genotype was observed. SA-Glu-mz137 showed a near-significant ($p = 0.061$) decrease in the resistant genotype falling to 47% of its original concentration, which was 98% of the concentration of the susceptible genotype. Furthermore, no significant change in the susceptible genotype was observed. JA-Ile, SA and Sulfo-JA showed no significant changes in concentration after feeding in both genotypes.

### 3.7 | Chemical markers of leaf age affect weevil feeding

To determine how leaf age influences weevil feeding behaviour, we analysed the difference in concentrations of palmitic and oxalic acid between old and young leaves of a highly resistant (Dun07) and a highly susceptible (Dun09) *E. dunnii* genotype (Figure 7). The young leaves of the susceptible genotype had a significant ($p < 0.05$) 170% higher oxalic acid concentration and 284% lower palmitic acid concentration compared to the old leaves. The young leaves of the resistant genotype had a significant ($p < 0.05$) 381% higher oxalic acid concentration and a nonsignificant difference in palmitic acid concentration compared to the old leaves.

We also observed a 231%, and 459% higher oxalic acid concentration in young and old leaves, respectively, when comparing the susceptible genotype to the resistant genotype. Furthermore, the palmitic acid concentration was 1093% and 584% lower in young and old leaves, respectively, when comparing the susceptible genotype to the resistant genotype.

## 4 | DISCUSSION

*Gonipterus* sp. n. 2, a major defoliator of *Eucalyptus*, has strong feeding preferences. Several studies have tried to characterize the host range of *Gonipterus* sp. n. 2 (Mally, 1924; Newete et al., 2011; Tooke, 1955); however, these studies have had contradictory results. We studied this host preference using bioassays to compare the level of resistance against feeding of 62 *Eucalyptus* genotypes from 23 species. We observed that the intraspecific variation in host resistance could match and sometimes even exceed interspecific variation, which could explain these contradictions. Therefore, we investigated the chemical basis of this intraspecific variation and identified three phagostimulants (1,8-cineole, oxalic acid and sucrose) and two feeding deterrent compounds (shikimic acid and palmitic acid) which significantly influenced the feeding behaviour of *Gonipterus* sp. n. 2 on both an artificial medium and at the tree level in a plantation (Figure 8).

### 4.1 | Intraspecific variation plays a major role in the host range of *Gonipterus* sp. n. 2

The current host range of *Gonipterus* sp. n. 2 has not been clearly delimited. Mally (1924) identified *E. punctata* as highly susceptible, whereas Newete et al. (2011) reported it to be resistant in plantation trials, Tooke (1955) reported that it was susceptible in certain locations and tolerant in others, and our findings show it to be similar to our susceptible control. Furthermore, *E. propinqua* was classified by Tooke (1955) as susceptible, while Mally (1924), Newete et al. (2011) and our findings report this species to be resistant. When we compare our results to these previous reports, we also find matching levels of susceptibility for *E. citriodora*, *Eucalyptus dorriengoenis*, *Eucalyptus paniculata* and *Eucalyptus obliqua* (Mally, 1924; Newete et al., 2011; Tooke, 1955). However, we also observed conflicting results for *Eucalyptus goniocalyx*, *Eucalyptus pilularis* and *E. saligna*.

Comparison of different genotypes of a single species provided our most interesting results. Of the 23 species tested, six contained more than one genotype (*E. dunnii*, *E. grandis*, *E. grandis* × *urophylla*, *E. grandis* × *camaldulensis*, *E. macarthurii* and *E. nitens*). Within these six species, we observed that the weevil's preference varied considerably among genotypes. For example, in a range of pure *E. grandis* genotypes, we identified genotypes with both considerably higher and lower feeding compared to our control. This large intraspecific variation in host susceptibility might explain the contradictory data reported by previous studies, as these studies only used single genotypes per species (Mally, 1924; Newete et al., 2011; Tooke, 1955).

**FIGURE 1** Feeding preference of *Gonipterus* sp. n. 2 on leaves of *Eucalyptus* varieties containing different concentrations of compounds with polar functional groups. Linear regressions show chromatogram peak areas or concentrations (*y* axis) of compounds, extracted from the leaves of seven varieties of *Eucalyptus dunnii* and six hybrids of *Eucalyptus grandis* × *Eucalyptus urophylla* (GU), to the average relative feeding value of *Gonipterus* sp. n. 2 (average percentage feeding on genotype of interest/average percentage feeding on control) (*x* axis). Significant correlations were observed between beetle feeding and the relative leaf content of (a) oxalic acid, (b) palmitic acid, (c) shikimic acid, (d) sucrose, (e) lapachone. The beetle's feeding preference for each variety was assayed using laboratory assays and compounds were analysed using gas chromatograph-mass spectrometer (GC-MS) and quantified using external standard curves of pure compounds, where available (Supporting Information: Figure S5). Feeding assays were conducted using four biological replicates and GC-MS analysis was conducted using four replicates per variety. Only correlations with significant $R^2$-values are shown.

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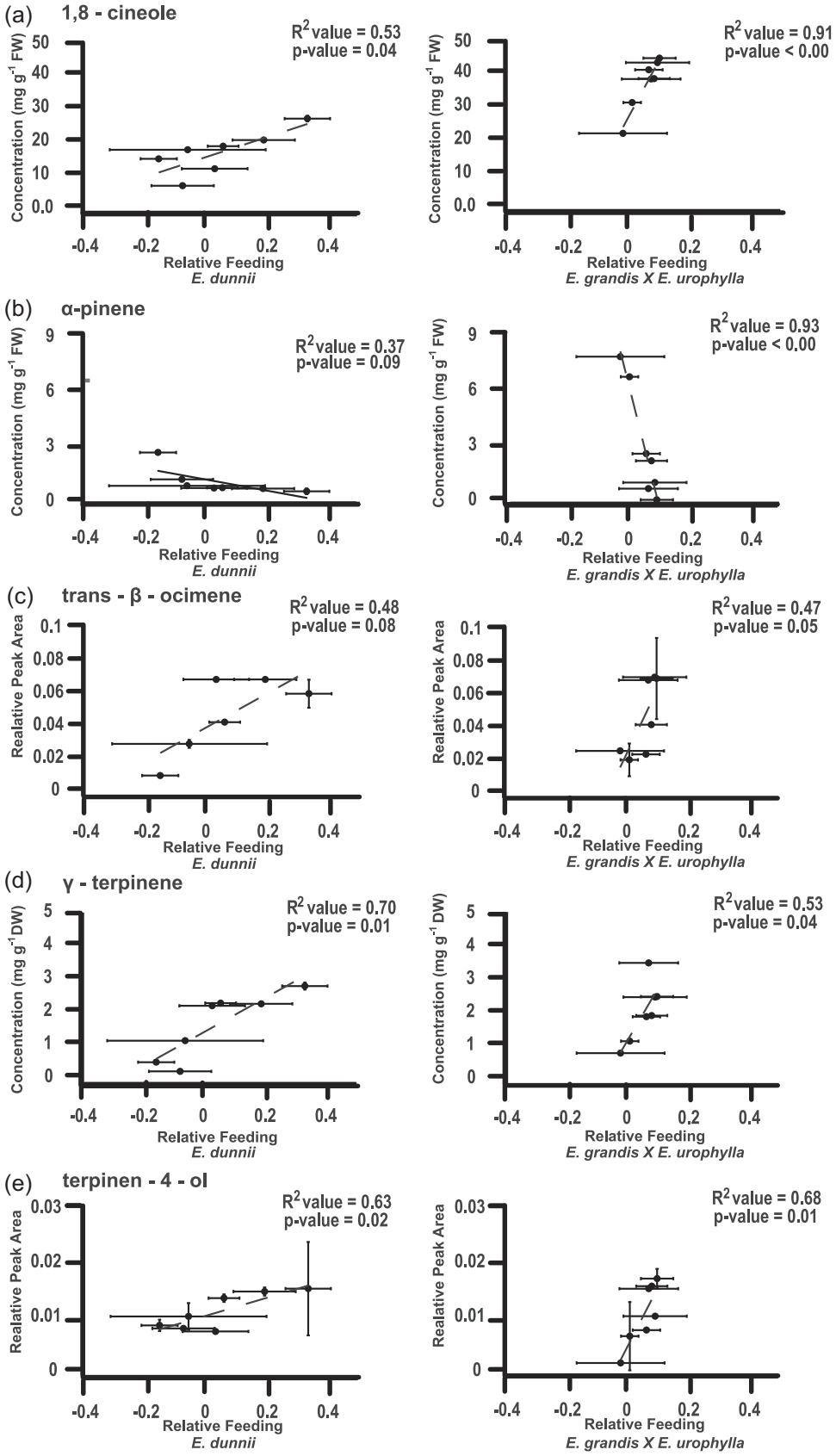



**FIGURE 2** (See caption on next page).

### 4.2 | Effect of foliar chemistry on tree susceptibility

We documented high intraspecific variation in the susceptibility of *Eucalyptus* trees against *Gonipterus* sp. n. 2, and linked this to variation in genotype chemical profile. This type of intraspecific chemical variation has been observed in other plants such as *Melaleuca alternifolia* (*Myrtaceae*) (Butcher et al., 1994) and *Populus tremuloides* (Hemming & Lindroth, 1995). Furthermore, Hemming and Lindroth (1995) theorized that, *Lymantria dispar L. (Erebidae)* and *Malacosoma disstria Hbn. (Lasiocampidae)*, preferentially feed on *P. tremuloides* genotypes based on their chemical profiles. Our results suggest that markers for resistance to help improve selective breeding can be identified using leaf chemistry. In this study, we identified five compounds (1,8-cineole, oxalic acid, sucrose, shikimic acid and palmitic acid) that could potentially be used as markers for resistance to weevil feeding in *Eucalyptus*.

#### 4.2.1 | The role of essential oils in *Gonipterus sp. n.* 2 host choice and pheromone production

We found 1,8-cineole to be a strong phagostimulant for *Gonipterus* sp. n. 2, confirming earlier observations by Tooke (1955) and Bouwer (2013). This monoterpene is a major constituent of *Eucalyptus* essential oils and can comprise up to 79% of the total foliar terpene content in *Eucalyptus* (*Eucalyptus polybractea* (King et al., 2004)). Furthermore, 1,8-cineole has been observed to be repellent (*Anoplognathus* spp. [Matsuki et al., 2011]), insecticidal (*Aedes aegypti* [Lucia et al., 2007]) or an attractant (*Cosmopolites sordidus* [Ndiege et al., 1996]). Furthermore, 1,8-cineole is sometimes sequestered by insects for defence against predation (*Perga affinis* [Morrow et al., 1976]). Interestingly Branco et al. (2019) identified several potential pheromones for *G. platensis* (2-α-hydroxy-1,8-cineole and 2-oxo-1,8-cineole), which are thought to be derived from 1,8-cineole. Sequestering plant volatiles for pheromone production has been reported in other beetles such as the mountain pine beetle (*Dendroctonus ponderosae*) which utilizes a host defence metabolite, α-pinene, to produce its aggregation pheromone, trans-verbenol (Chiu et al., 2018, 2019). However, while Branco et al. (2019) also identified α-pinene-derived *cis*-verbenol, *trans*-verbenol and verbenone as potential pheromones in the headspace of *G. platensis*, neither this study nor Tooke (1955) found any evidence that α-pinene significantly affects the feeding behaviour of *Gonipterus* sp. n. 2. Similarly, γ-terpinene and *trans*-β -ocimene which can be detected by female *Gonipterus* sp. n. 2 (Bouwer, 2013) did not significantly affect their feeding behaviour in our assays. While these compounds did not have any effect in isolation, they may have additive or synergistic effects when used in combination with 1,8-cineole. Alternatively, these compounds could be sufficiently abundant that they are not a limiting resource for the weevil. Furthermore, these compounds might have a behavioural effect that does not affect feeding, such as aggregation or dispersal, which was not studied in our feeding bioassays.

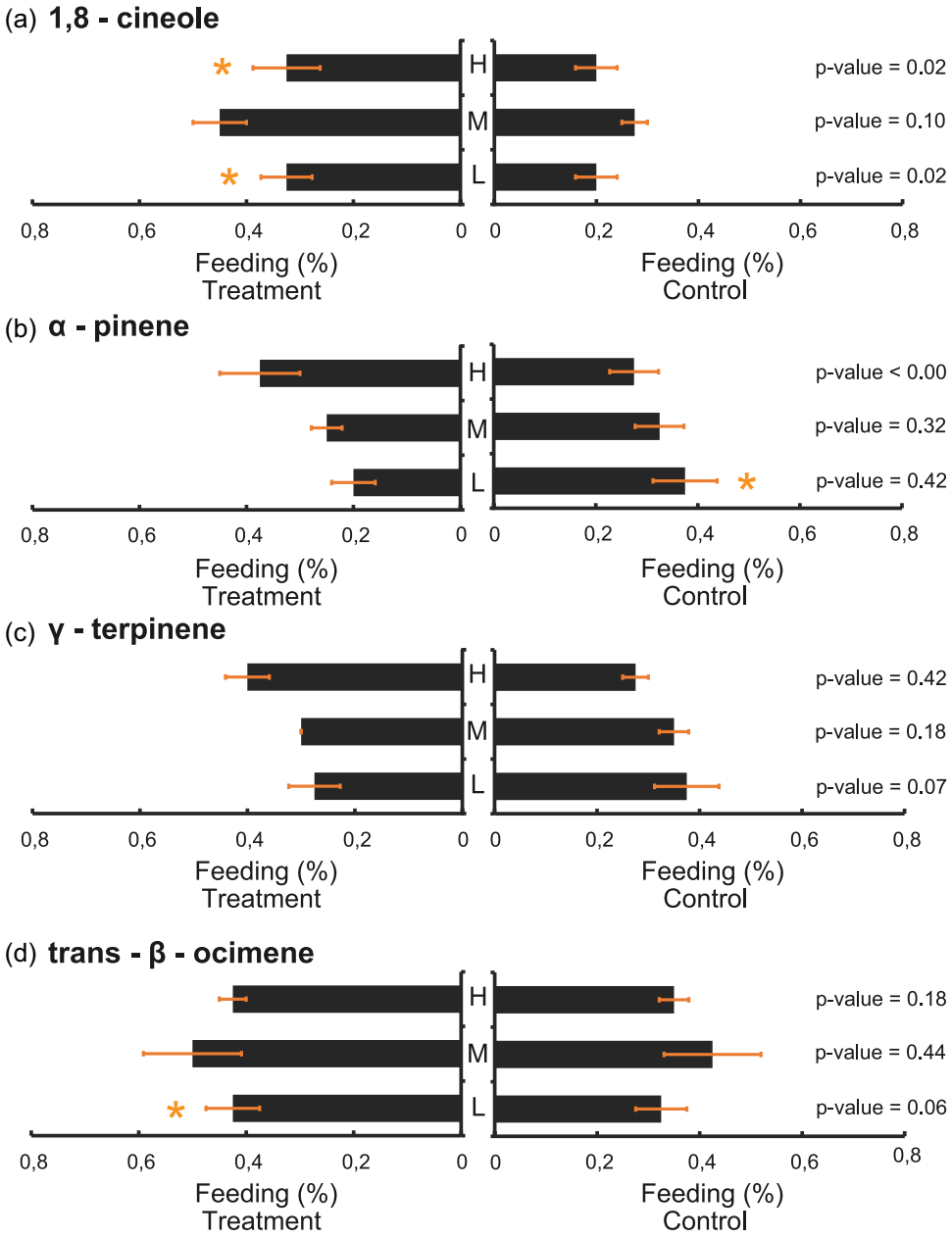
#### 4.2.2 | The role of sugars in selective feeding patterns of *Gonipterus* sp. n. 2

Sugar content, particularly sucrose, is an important phagostimulant for many beetles (*Sitona cylindricollis* [Akfson et al., 1970], *Oulema melanopus* [Panella et al., 1974]). We found that sucrose also acted as a phagostimulant for *Gonipterus* sp. n. 2. In plants, sucrose is formed through the coupling of two monosaccharides, D-(-)- glucose and D-(-)-fructose. Glucose and fructose are produced by plants in their leaves via photosynthesis (in source tissue), however, they are primarily required in the plant's growing areas or storage tissue (sink tissue). Therefore, plants need to transport these sugars, in the form of sucrose, via their vascular tissue. This results in high sucrose concentrations in vascular bundles, which might explain why weevils are often found feeding on the soft bark of young branches of *Eucalyptus* trees (Tooke, 1955).

#### 4.2.3 | *Gonipterus* sp. n. 2 could detect preferred leaf age through leaf chemical content

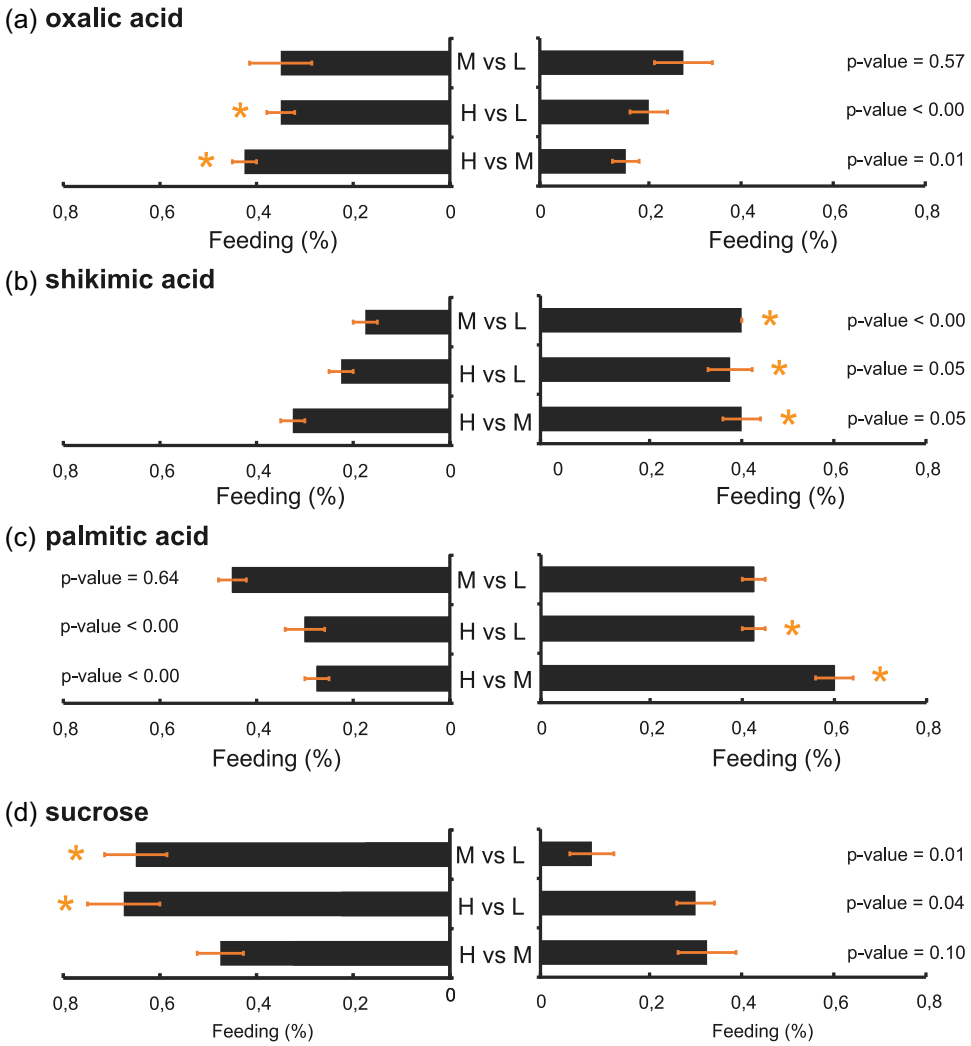
Oxalic acid, the simplest dicarboxylic acid, is a strong plant toxin capable of forming calcium oxalate crystals. This compound has been well-documented to protect against herbivory. For example, a study by Hudgins et al. (2003) showed that calcium oxalate crystals in addition to fibre rows can be an effective barrier in conifer trees against bark beetles. Yet our study finds that oxalic acid acts as a phagostimulant of *Gonipterus* sp. n. 2. Little is known about oxalic

**FIGURE 2** Feeding preference of *Gonipterus* sp. n. 2 on leaves of *Eucalyptus* varieties containing different concentrations of compounds with nonpolar functional groups. Linear regressions show chromatogram peak areas or concentrations (*y* axis) of compounds extracted from the leaves of seven varieties of *Eucalyptus dunnii* and six hybrids of *Eucalyptus grandis* × *Eucalyptus urophylla* (GU) to the average relative feeding value of *Gonipterus* sp. n. 2 (average percentage feeding on genotype of interest/average percentage feeding on control) (*x* axis). Significant correlations were observed between beetle feeding and the relative leaf content for (a) 1,8-cineole, (b) α-pinene, (c) *trans*-β-ocimene, (d) γ-terpinene, (e) terpinen-4-ol. The beetle's feeding preference for each variety was assayed using laboratory assays and compounds were analysed using gas chromatograph-mass spectrometer (GC-MS) and quantified using external standard curves of pure compounds. Feeding assays were conducted using four biological replicates and GC-MS analysis was conducted using four replicates per variety. Only correlations with significant $R^2$-values are shown.




| Concentration (mg ml$^{-1}$) | | | |
|---|---|---|---|
| Compound | High (H) | Medium (M) | Low (L) |
| 1,8 - cineole | 92.67 | 9.27 | 0.93 |
| α - pinene | 85.8 | 8.58 | 0.86 |
| γ - terpinene | 850 | 85 | 8.5 |
| trans - β - ocimene | 800 | 80 | 8 |

**FIGURE 3** Feeding preference of *Gonipterus* sp. n. 2 on an artificial diet amended with different concentrations of compounds with nonpolar functional groups. Bar graphs representing the percentage of artificial diet disk consumed by *Gonipterus sp. n.* 2 between either high (H), medium (M) or low (L) and null concentration of the amended compound (* represent significant differences found by pairwise *t*-test, indicating the preferred treatment) (a) 1,8-cineole, (b) α-pinene, (c) γ-terpinene, (d) trans-β-ocimene. The beetle's feeding preference for each compound was assayed using a choice bioassay in which beetles could choose between artificial diet disks amended with various concentrations of the compounds. Feeding assays were conducted using four biological replicates. [Color figure can be viewed at wileyonlinelibrary.com]




**Concentration (mg ml$^{-1}$)**

| Compound | High (H) | Medium (M) | Low (L) |
|---|---|---|---|
| oxalic acid | 196 | 112 | 28 |
| shikmic acid | 7.5 | 5 | 2.5 |
| palmitic acid | 0.5 | 0.25 | 0.1 |
| sucrose | 700 | 350 | 150 |

**FIGURE 4** Feeding preference of *Gonipterus* sp. n. 2 on an artificial diet amended with different concentrations of compounds with polar functional groups. Bar graphs representing percentage of artificial diet disk consumed by *Gonipterus sp. n.* 2 between either medium and low (MvsL); high and low (HvsL) and high and medium (HvsM) concentration of the amended compound (* represent significant differences found by pairwise *t*-test, indicating the treatment preferred by the weevil) (a) oxalic acid, (b) shikimic acid, (c) palmitic acid, (d) sucrose. The beetle's feeding preference for each compound was assayed using a choice bioassay in which beetles could choose between artificial diet disks amended with various concentrations of the compounds. Feeding assays were conducted using four replicates. [Color figure can be viewed at wileyonlinelibrary.com]

acid acting as an attractant or feeding stimulant in beetles. However, it has been hypothesized that oxalic acid levels may be higher in young leaves to protect them from insects before they can fully mature into tougher leaves (Finley, 1999). For example, the number of calcium oxalate crystals in *Cyclanthus subpalmata*, *Pandanus leram*, *Crinum amabile*, *Heliconia longiflora* and *Guzmania zahnii* were inversely correlated to leaf age and leaf toughness (Finley, 1999). We tested this hypothesis by comparing the oxalic acid concentration between old and young leaves of one highly resistant (Dun07) and one highly susceptible (Dun09) *E. dunnii* genotype. Interestingly, we found that young leaves contained overall higher concentrations of oxalic acid and the difference between young and old leaves was

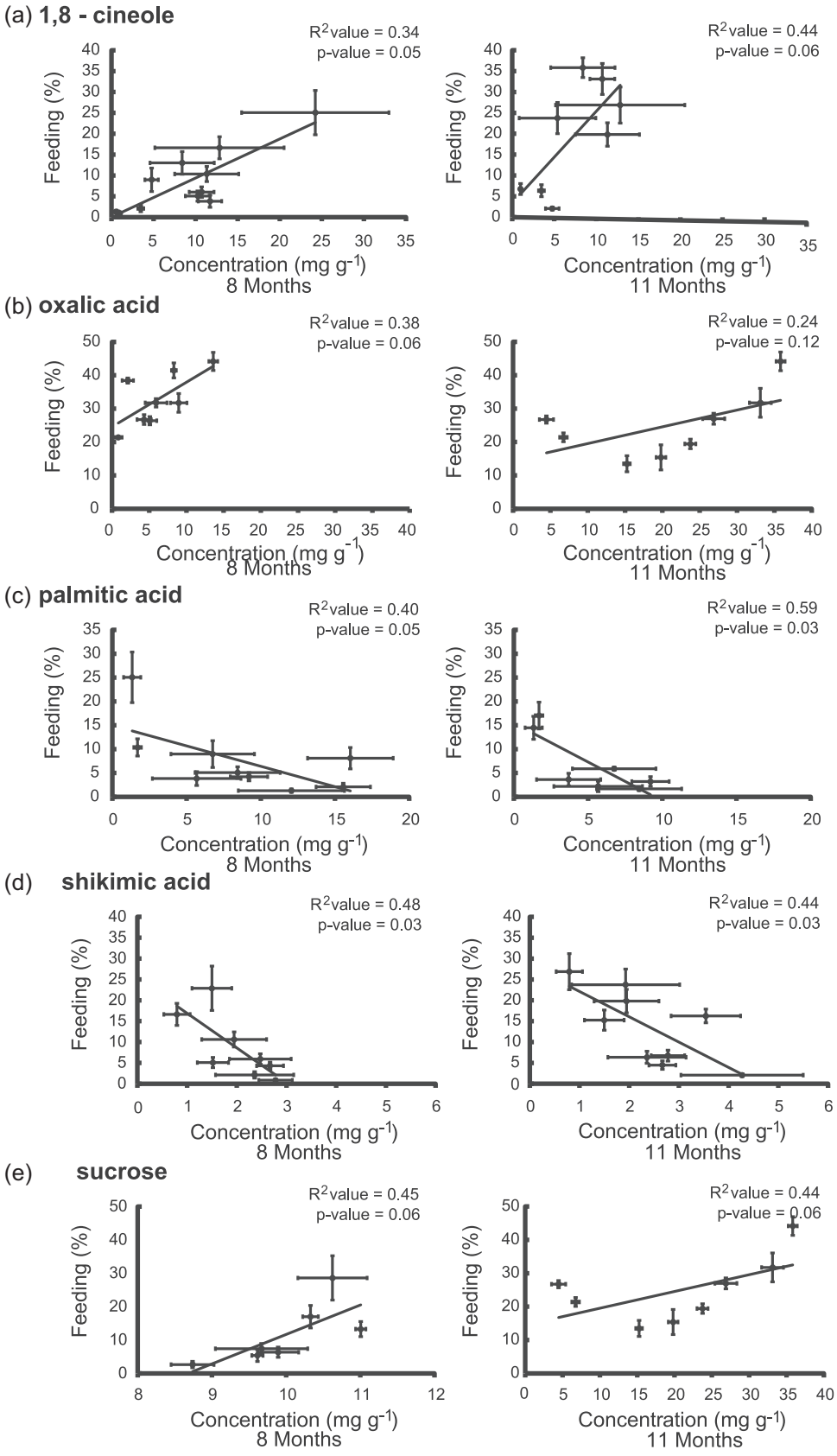



**FIGURE 5** (See caption on next page).

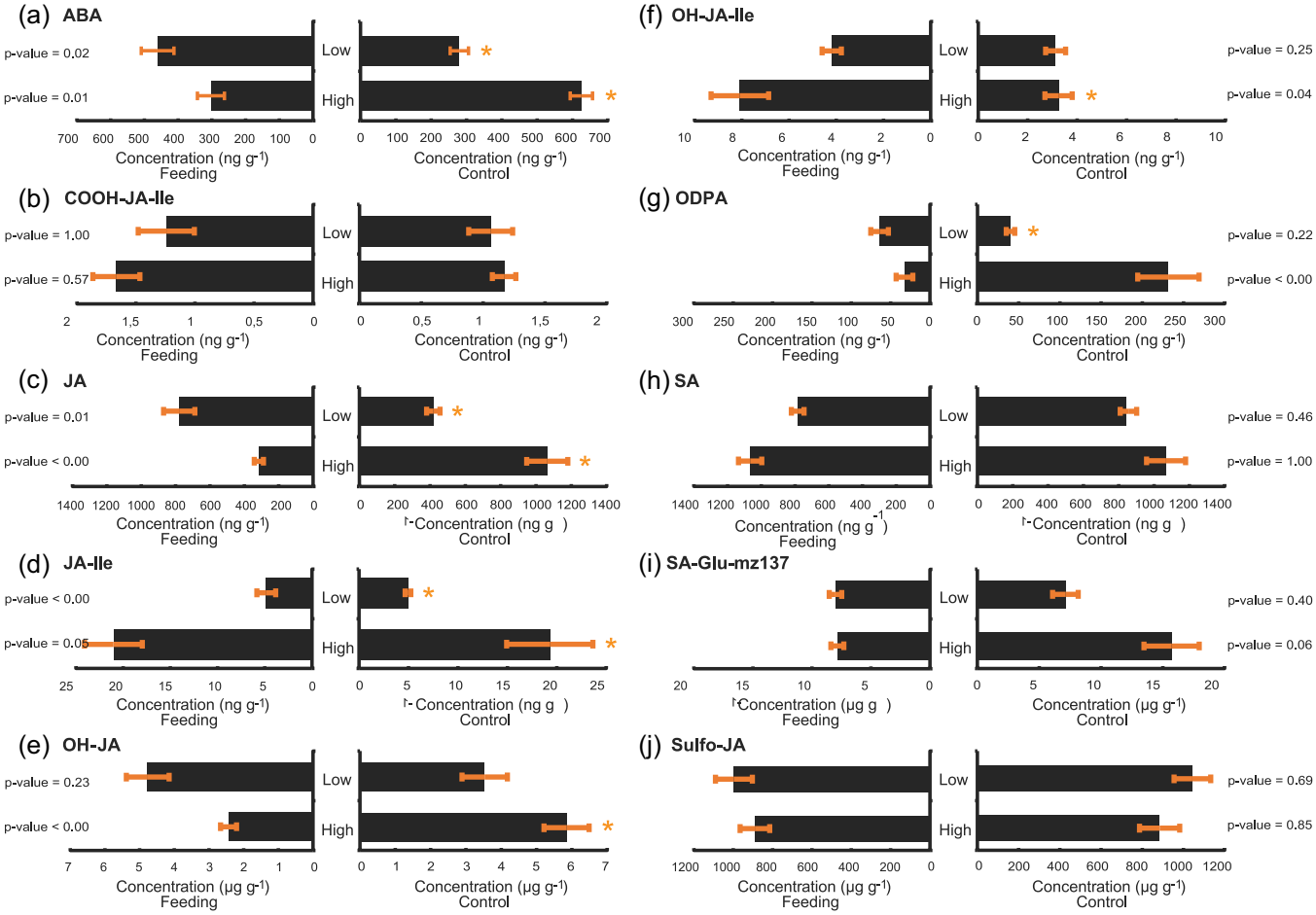



**FIGURE 6** The role of phytohormones in the feeding preference of *Gonipterus* sp. n. 2. Bar graphs representing the concentration of (a) abscisic acid (ABA), (b) carboxy-jasmonyl-isoleucine (COOH-JA-Ile), (c) jasmonic acid (JA), (d) jasmonyl isoleucine (JA-Ile), (e) hydroxy-jasmonic acid (OH-JA), (f) hydroxy-jasmonoyl-isoleucine (OH-JA-Ile), (g) oxophytodienoic acid (OPDA), (h) salicylic acid (SA), (i) salicylic acid glucoside (SA-Glu-mz137) and (j) sulfo-jasmonic acid (SulfoJA) which were either fed upon by 20 *Gonipterus sp. n.* 2 adults of mixed age and gender for 1 week (right) or Control plants (left). (* represent significant differences found by Pairwise Wilcoxon Rank Sum test). High: Resistant Dun7 genotype with high shikimic acid concentration. Low: Susceptible Dun09 genotype with low shikimic acid concentration. High and low shikimic acid leaves were harvested from eight replicates per genotype and treatment, and analyzed by gas chromatograph-mass spectrometer. [Color figure can be viewed at wileyonlinelibrary.com]

higher in the susceptible genotypes. Therefore, it seems that oxalic acid is a defence of *Eucalyptus* to protect its younger leaves. *Gonipterus*. sp. n. 2 seems to overcome this defence, and now utilizes it as a chemical marker for its preferred leaf type. This behaviour could be exploited by planting trees that lack this particular defence trait, which renders them less palatable to the weevils. However, this may render them more susceptible to other phytophagous insects that are negatively affected by this defence trait. Therefore, more studies of the effects of oxalic acid in other phytophagous *Eucalyptus* pests may be needed.

In contrast to oxalic acid, we found that palmitic acid, a 16-carbon, saturated long-chain fatty acid, is a feeding deterrent for *Gonipterus* sp. n. 2. Palmitic acid is mostly documented in insects as a phagostimulant, yet we observed the opposite effect in *Gonipterus* sp. n. 2. Interestingly,

**FIGURE 5** Feeding preference of *Gonipterus* sp. n. 2 on leaves of *Eucalyptus* varieties in the field containing different concentrations of the five compounds of interest. Linear regressions show the concentration of compounds (mg $g^{-1}$; *y* axis) extracted from the leaves of 12 varieties of *Eucalyptus* and the percentage feeding done by *Gonipterus* sp. n. 2 in the field at 8- and 11-months postplanting (*x* axis). Significant correlations were observed between beetle feeding in the field and the relative leaf content of (a) 1,8-cineole, (b) oxalic acid, (c) palmitic acid, (d) shikimic acid, (e) sucrose. The beetle's feeding preference for each variety was assayed by assessing beetle damage on tree canopies and the compounds were analysed using gas chromatograph-mass spectrometer (GC-MS). Feeding damage was assessed on nine biological replicates and GC-MS analysis was conducted using five replicates per variety.

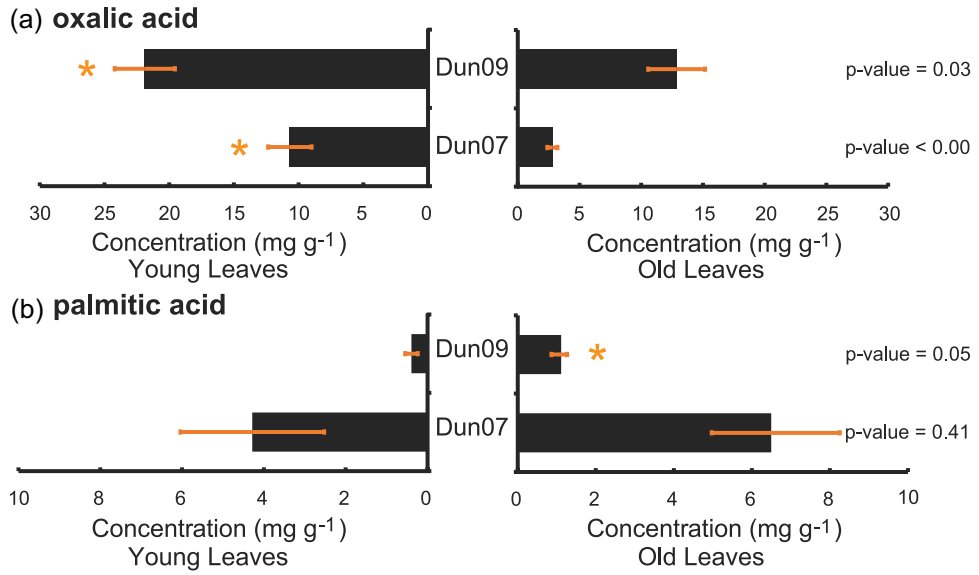



**FIGURE 7** Effect of leaf age on organic acid content between a susceptible and resistant *Eucalyptus dunnii* genotype. Bar graphs representing concentration (mg $g^{-1}$) of (a) oxalic acid and (b) palmitic acid between young and old leaves of a susceptible (Dun09) and resistant (Dun07) *E. dunnii* genotype (* represent significant differences found by a one-way analysis of variance and Tukey test). Old and young leaves were harvested from four replicates per genotype and analyzed by gas chromatograph-mass spectrometer. [Color figure can be viewed at wileyonlinelibrary.com]

palmitic acid shows the inverse distribution of oxalic acid and is found in higher concentrations in the older leaves of several plants (*Luffa cylindrical* [Cucurbitales], *Luffa acutangular* [Cucurbitales] and *Mimosa scabrella* [Fabaceae] (Annadurai, 1987). We compared the palmitic acid concentration between old and young leaves of two *E. dunnii* genotypes, one highly resistant (Dun07) and one highly susceptible (Dun09) to feeding by *Gonipterus* sp. n. 2. This revealed the exact opposite response of oxalic acid, with young leaves showing an overall lower concentration of palmitic acid, with a greater difference between young and old leaves in the resistant genotypes. Therefore, it seems that leaf age preference of *Gonipterus* sp. n. 2 might be partially explained by oxalic acid as phagostimulant and by palmitic acid as a feeding deterrent. Furthermore, the magnitude of the difference in concentration of these compounds between young and old leaves may play a major role in the host preference of the weevil.

### 4.2.4 | The role of phytohormones in the feeding preference of *Gonipterus* sp. n. 2

Phytohormones play a major role in regulating many processes, such as plant defences. A major phytohormone associated with plant defence is SA, which can induce the expression of multiple plant defence-related genes (Bennett & Wallsgrove, 1994). SA can be derived from shikimic acid which we found to be a feeding deterrent for *Gonipterus* sp. n. 2. Therefore, we tested the phytohormone response induced by feeding between a susceptible (low shikimic acid concentration, Dun09) and resistant (high shikimic acid concentration, Dun07) genotype of *E. dunni* (Figure 8b). ABA, JA, OPDA and SA each interact with each other to regulate plant responses against necrotrophic stressors. SA can repress JA and enhance ABA accumulation (Zhao et al., 2017). However, ABA can inhibit the accumulation of SA, and enhance the accumulation of JA (Zhao et al., 2017). We observed a drastic decrease in ABA in the resistant genotype and an increase in the susceptible genotype after feeding. JA accumulated in the susceptible genotype while decreasing in the resistant genotype. JA regulates genes via two distinct pathways, the MYC branch and the ERF branch with ABA acting as an important co-regulator in the activation of the MYC branch (Sánchez-Vallet et al., 2012; Vos et al., 2013). It appears that these two pathways are employed by the susceptible genotypes, which the weevils may have developed some resistance against. The resistant genotypes, instead, converted their abundant JA, OH-JA and OPDH into OH-JA-Ile and COOH-JA-Ile. OH-JA-Ile has been observed to induce mild protection in *Arabidopsis thaliana* against herbivory, by activating a subset of JA-Ile co-receptors, COI1-JAZ (Jimenez-Aleman et al., 2019). It is hypothesized that following a strong immune response mediated by JA-Ile, the JA-Ile is converted into OH-JA-Ile to modulate induced processes and improve plant resilience (Jimenez-Aleman et al., 2019). Furthermore, OH-JA-Ile and COOH-JA-Ile have been shown to deactivate the JA-signalling pathway in *A. thaliana* (Miersch et al., 2008). Therefore, the resistant genotype appears to be activating a completely different pathway from the susceptible genotype while actively repressing the JA pathway utilized by the susceptible genotype. This might explain why the weevils show such a large difference in preference between the different genotypes. Furthermore, the resistant genotype also possessed higher constitutive levels of SA and lower levels of Sulfo-JA which remain unchanged by feeding.

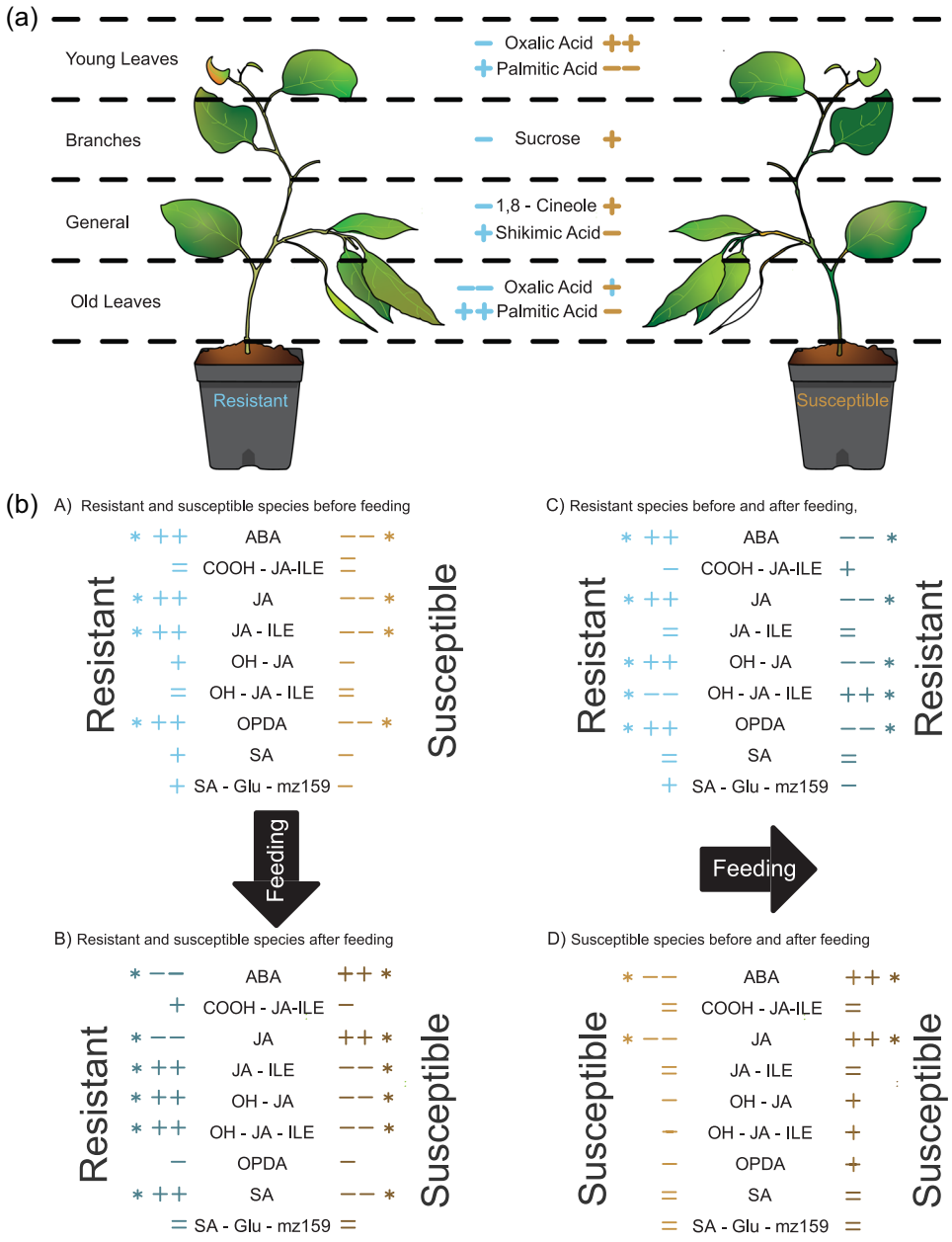



**FIGURE 8** (a). Overview of foliar chemistry's effect on weevil behaviour. Overview of 1,8-cineole, oxalic acid, sucrose, shikimic acid and palmitic acid in different plant tissues between resistant and susceptible species. + indicates a higher concentration, while – indicates lower concentration. Blue, resistant; brown, susceptible. (b) Overview of *Eucalyptus* phytohormone response to weevil feeding. Overview of abscisic acid (ABA), carboxy-jasmonyl-isoleucine (COOH-JA-Ile), jasmonic acid (JA), jasmonyl isoleucine (JA-Ile), hydroxy-jasmonic acid (OH-JA), hydroxy-jasmonoyl-isoleucine (OH-JA-Ile), oxophytodienoic acid (OPDA), salicylic acid (SA) and salicylic acid glucoside (SA-Glu-mz137) concentration between (A) resistant (Dun07, high shikimic acid) and susceptible (Dun09, low shikimic acid) species before feeding, (B) resistant (Dun07, high shikimic acid) and susceptible (Dun09, low shikimic acid) species after feeding, (C) resistant (Dun07, high shikimic acid) species before and after feeding, (D) susceptible (Dun09, low shikimic acid) species before and after feeding. + indicates an increase in concentration, – indicates a decrease in concentration, = indicated same concentration and *indicates significant differences. Blue, resistant; brown, susceptible. [Color figure can be viewed at wileyonlinelibrary.com]

## 5 | CONCLUSION

We observed significant interspecific variation in the amount of feeding by *Gonipterus* sp. n. 2 on different genotypes of different *Eucalyptus* species. We also observed patterns of intraspecific variation in the chemical composition of these genotypes consistent with the observed feeding preferences. To facilitate breeding of resistant genotypes, we propose the use of chemical markers of resistance to determine the level of resistance of individual genotypes. By utilizing a bioassay-guided mass-spectrometry approach coupled with laboratory behavioural assays and field trials, we identified five chemical markers of resistance for *Gonipterus* sp. n.

2, three phagostimulants (1,8-cineole, sucrose and oxalic acid) and two feeding deterrents (shikimic acid and palmitic acid). Furthermore, we provide preliminary evidence that the differing pathways used in the phytohormone response in *Eucalyptus* genotypes, through either the accumulation of OH-JA-Ile and COOH-JA-Ile or the accumulation of ABA and JA plays a major role in *Gonipterus* sp. n. 2 host preference. Understanding the effects of chemical defence and phytohormone response on *Gonipterus* sp. n. 2 host preference and its implementation in tree breeding programmes provides an opportunity to reduce damage caused by these weevils.

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## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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