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RESEARCH ARTICLE

First line of defence: *Eucalyptus* leaf waxes influence infection by an aggressive fungal leaf pathogen

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ABSTRACT

- Leaf epicuticular waxes provide important anatomical and chemical defences against fungi that infect leaves. In this study we analysed the leaf wax composition of *Eucalyptus grandis* × *Eucalyptus urophylla* hybrids with contrasting susceptibilities to Teratosphaeria leaf blight (TLB) caused by *Teratosphaeria destructans*, one of the most important foliar diseases of *Eucalyptus*.
- The *Eucalyptus* cuticular wax was extracted from non-inoculated and inoculated genotypes with different levels of susceptibility to TLB and analysed by gas chromatography–mass spectrometry.
- The results showed that a triterpenoid, cycloartenol (CAS), was abundant in a resistant genotype and that hexanedioic acid content increased in the resistant genotypes in response to *T. destructans* infection. In contrast, palmitic acid was significantly more abundant in the inoculated highly susceptible genotype. *In-vitro* and *in-planta T. destructans* spore germination assays with pure compounds, showed that CAS and hexanedioic acid significantly inhibited spore germination. Application of these two compounds to the leaves of a susceptible host also significantly increased resistance to infection. In contrast, palmitic acid promoted spore germination and, when applied to the leaves of a resistant genotype, increased colonization by the pathogen.
- This is the first study providing insights into differences in the leaf wax composition of hosts with different levels of susceptibility to *T. destructans*. It also showed that leaf wax compounds can modulate spore germination and, ultimately, host resistance to infection.

INTRODUCTION

Leaves, young shoots and fruits of higher plants possess a hydrophobic cuticle layer, formed by the deposition of cutin and occasionally wax on the outer epidermal cell walls (Juniper & Jeffree 1983). Cuticular waxes are chemically diverse, and contain unique mixtures of primary and secondary alkanes, alcohols, aldehydes, ketones, triterpenes and esters derived from very-long fatty acid chains (C20–C34) (Raffaele *et al.* 2009; Malinovsky *et al.* 2014). Physically, wax layers are embedded within the cuticle (intracuticular wax) and are also deposited predominantly as crystalloids on leaf surfaces (Martin & Juniper 1970; Barthlott *et al.* 1998).

The chemodiversity of cuticular waxes has a key function in the adaptation of terrestrial plants to abiotic and biotic challenges (Martin & Juniper 1970; Ziv *et al.* 2018). For example, drought-tolerant plants generally have thicker cuticles, enriched with long-chain alkanes (Seufert *et al.* 2022; Sanjari *et al.* 2001; Xue *et al.* 2017). In addition, epicuticular waxes play an important role in the host choice of piercing/-sucking insects (Begum *et al.* 2016; Makunde *et al.* 2023) as well as chewing herbivores (Eigenbrode & Espelie 1995). The most dramatic effects of cuticular waxes can be seen in plant–fungus interactions. Foliar pathogens use the chemical composition of leaf waxes for host recognition, which triggers fungal germination (Feng *et al.* 2009; Uppalapati *et al.* 2012), but pathogen-generated breakdown products from the cuticular layer can also facilitate recognition of the pathogen by the host (Fauth *et al.* 1998). In addition, toxic substances embedded in the cuticle, such as glucosinolates and oxygenated fatty acids, can form a chemical defence barrier against fungal penetration (Ahuja *et al.* 2016; Santos *et al.* 2019; Dubey *et al.* 2020).

On *Eucalyptus* leaves, the wax crystalloids occur as plates, tubes, or a mixture of plates and tubes (Hallam 1964), but their arrangements and distribution differ significantly between species (Hallam & Chambers 1970; Knight *et al.* 2004). Interestingly, differences in leaf wax morphology on *Eucalyptus* leaves have been shown to determine the success of infection by foliar pathogens (Hansjakob *et al.* 2011; Xavier *et al.* 2015). For example, urediniospores, germ tubes and appressoria of the rust pathogen, *Austropuccinia psidii*, had lower viability on *Eucalyptus grandis* leaves with thick cuticular wax layers (Xavier *et al.* 2015). However, some *Eucalyptus* foliar pathogens, such as *Quambalaria eucalypti* and *Teratosphaeria destructans*, appear to degrade leaf cuticular waxes during the early infection process prior to stomatal penetration (Pegg *et al.* 2009; Solís *et al.* 2022).

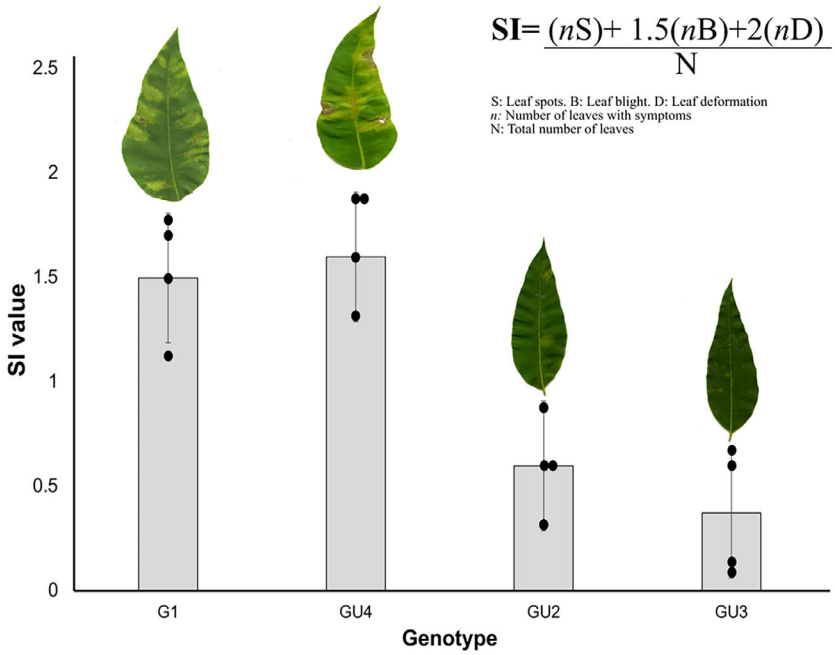

Fig. 1. Susceptibility of *Eucalyptus grandis* × *E. urophylla* genotypes (GU4, GU2 and GU3) and *Eucalyptus grandis* (G1) to *Teratospaheria destructans* infection 35 days after inoculation with 1×10^6 spores·ml^{-1}. The susceptibility index (SI) was calculated using the equation developed by Solís *et al.* (2023), where the SI values classified the genotypes as follows: GU4: Highly susceptible, G1: Susceptible, GU2: Moderately resistant and GU3: Moderately resistant. Error bars indicate ±SE, for N = 4 plants per genotype. No symptoms were visualized in control plants.

Teratosphaeria destructans (Capnodiales, Teratosphaeriaceae) is an aggressive pathogen that causes leaf and shoot blight disease on *Eucalyptus* (Wingfield *et al.* 1996; Andjic *et al.* 2019). The disease was first reported in 1995 in Northern Sumatra, Indonesia, affecting young *E. grandis* trees (Wingfield *et al.* 1996). Since then, the pathogen has spread rapidly in *Eucalyptus* plantations throughout tropical and subtropical South East Asia (Andjic *et al.* 2011; Andjic *et al.* 2019), including Thailand, East Timor, Vietnam, China, Laos, and Malaysia (Old *et al.* 2003; Burgess *et al.* 2006; Barber *et al.* 2012; Havenga *et al.* 2021). In 2015, *T. destructans* was also reported from South Africa on *E. grandis* × *E. urophylla* (Greyling *et al.* 2016) and is now established in *Eucalyptus* nurseries and plantations in subtropical parts of the country. Despite the spread of *T. destructans* to many locations in recent years, and the substantial economic losses that it causes in *Eucalyptus* plantations, the molecular and biochemical mechanisms underlying resistance to the pathogen have not been studied.

The aim of this study was to consider the role of leaf-surface wax composition of *Eucalyptus* genotypes with different levels of resistance or susceptibility to *T. destructans*. In addition, the effect of wax compounds on pathogen germination and leaf colonization was evaluated, focusing on compounds that were unique in resistant and susceptible hosts.

MATERIALS AND METHODS

Inoculum preparation

The inoculum was prepared from pure cultures of *T. destructans* isolate CMW5679 from a *E. grandis* × *E. urophylla* host (Solís *et al.* 2022), grown on 2% Malt Extract Agar (MEA, 20 g·l^{-1}) for 3 weeks at 25 °C in the dark, after which the conidial suspension was prepared as described in Solís *et al.* (2022). The suspension was obtained by washing the plates with 20 ml sterile distilled water +0.01% Tween 20 (Sigma-Aldrich, Taufkirchen, Germany). The concentration of the spore suspension was adjusted to 1×10^6 using a haemocytometer.

Plant material and inoculation

Three genotypes of a *E. grandis* × *E. urophylla* hybrid (GU2, GU3 and GU4) and a single genotype of *E. grandis* (G1) were used in this study (kindly provided by SAPPI and MONDI S.A, South Africa). Forty-one-year-old ramets of each of these four genotypes were maintained for 3 months in a greenhouse with an average daily temperature from 20 to 25 °C and average night temperature of 20 °C. Eight healthy plants per genotype were selected for inoculation and leaf epicuticular wax analysis. Four plants per genotype were selected as controls.

The inoculum was sprayed onto both leaf surfaces of the top ten apical leaves, with four replicates per genotype, until run-off. Control plants were sprayed with sterile distilled water +0.01% Tween 20 (Sigma-Aldrich). Plants were maintained in a greenhouse, under natural light with temperatures from 20 to 25 °C. After 10 days, six leaves per replicate of inoculated and non-inoculated (control) plants were harvested for cuticular wax analysis. The remaining four leaves per plant were used to evaluate susceptibility after 35 days using a susceptibility index (SI). SI values were calculated following the scale described in Solís *et al.* (2023). The SI considers the number of leaves affected as well as the severity of the disease (spots, blights, or distortion) on individual leaves (Fig. 1). The susceptibility

indices for the four genotypes were as follows: G1 SI = 1.5, GU2 SI = 0.6, GU3 SI = 0.4 and GU4 SI = 1.6 (Fig. 1). Following this SI scale, genotype G1 was classified as susceptible (S), GU2 and GU3 as moderately resistant (MR), and GU4 as highly susceptible (HS) (Fig. 1). G1, GU2 and GU4 showed leaf blight symptomatology, in contrast GU3 was the only genotype that showed isolated leaf spots (Fig. 1). No symptoms were seen on leaves of control plants.

Cuticular wax extraction

The six leaves harvested at 10 days after inoculation from each of the four replicates of *E. grandis* × *E. urophylla* genotypes and the *E. grandis* genotype, either inoculated or non-inoculated, were used to extract cuticular wax, as described in Viana *et al.* (2010) and modified by Bini (2016). Approximately 1 mg wax was obtained by immersing the six leaves of each replicate in 5 ml chloroform (JT Baker, Phillipsburg, NJ, USA amended with 0.4 $\mu l \cdot ml^{-1}$ of the internal standard, 2-phenylethanol, and gently agitating the samples for 30 s. Four biological replicates of non-inoculated and inoculated plants were used for the chemical analyses. The total leaf area was calculated by capturing images of the selected leaves using an Epson Perfection V700 scanner with a resolution of 1200 dots per inch (dpi).

Chemical derivatization and GC–MS analysis of cuticular waxes

The chloroform extract for each of the four inoculated and four non-inoculated samples per genotype were dried at 37 °C under constant air flow and resuspended in 100 µl pyridine containing 20 $mg \cdot ml^{-1}$ methoxamine HCl. The solution was incubated at 30 °C for 90 min, then centrifuged at 1200 rpm for 20 min. An aliquot of 30 µl of the supernatant was transferred into a glass insert in a glass vial (VWR, Germany). Then, 30 µl MS-TFA (Sigma, USA) were added to the supernatant and incubated at 37 °C for 30 min. A 1 µl sample of the supernatant was analysed on an Agilent 7890 gas chromatograph-mass spectrometer (Agilent, USA) (GC–MS) using a HP5 column with a linear temperature program starting at 70 °C, increasing at a rate of 5 $°C \cdot min^{-1}$ until a maximum temperature of 300 °C was reached, and then maintained for 2 min. The parameters of the GC–MS: were a solvent delay of 6 min, split inlet with a split ratio of 10:1 and a flow rate of 12 $mg \cdot ml^{-1}$ leading to a 1.2 $ml \cdot min^{-1}$ flow rate on the column. The mass spectrometer was set to scan mode, with a low mass of 40 $m \cdot z^{-1}$ and a high mass of 650 $m \cdot z^{-1}$ and the ion source was maintained at 70 eV.

Chromatograms were analysed using Agilent MassHunter® Qualitative Analysis software, build 8.0.598.0. Compounds were tentatively identified utilizing the 2017 NIST library (Information Services Office). Data exploration and multivariate principal components analysis (PCA) were conducted using MetaboAnalyst 4.0.

Effect of CAS, hexanedioic acid and palmitic acid on germination *in-vitro* and *in-planta*

A bioassay was performed using commercially available cycloartenol (CAS), palmitic acid (hexadecanoic acid) and hexanedioic acid (all Sigma Aldrich). Serial dilutions of derivatized pure standards were analysed to confirm the identity and concentration of the compounds of interest. The starting concentrations were 5.0 ppm, diluted down to 2.0, 1.0 and 0.1 ppm. Compound quantities in leaf wax mixtures were calculated as $mg \cdot cm^{-2}$ leaf surface area.

The effect of each compound on pathogen germination was evaluated at four different concentrations; 0 (control), 0.1, 1.0 and 5.0 ppm. For the *in-vitro* assay, each compound was diluted in ethanol (JT Baker) at different concentrations. An aliquot of 1 ml of each solution was evenly distributed on the surface of a 50-mm diameter Petri dish and allowed to air dry in a laminar flow cabinet until the surface was free of visible moisture. A *T. destructans* conidial suspension (150 µl), prepared as described above, was homogenously distributed onto the base surface of Petri dishes amended with the compounds of interest at each concentration, with four replicates. Because of their hydrophobic nature, the compounds remained fixed to the hydrophobic plastic surface in the presence of an aqueous spore suspension. The Petri dishes were placed into plastic boxes on a rack, suspended 5 cm above sterile distilled water,and sealed for 72 h at 25 °C to maintain a high humidity, after which spore germination was assessed. The germinated conidia were identified when the germ tubes were clearly visible under 20× magnification using an Axioskop 2 plus microscope (Zeiss, Oberkochen, Germany).

For the *in-planta* assay, each compound was diluted in sterile distilled water to 0 (control), 0.1, 1.0 and 5.0 ppm from an ethanol stock solution of 100 ppm. Approximately 5 ml of each CAS and hexanedioic acid dilution were applied to the adaxial and abaxial surfaces of the eight youngest leaves of four replicate plants of the highly susceptible variety GU4. Similarly, dilutions of palmitic acid were applied to the young leaves of the moderately resistant host, GU3. Subsequently, the leaves were allowed to dry for 2 h. Then, a *T. destructans* spore suspension of 1×10^6 $spore \cdot ml^{-1}$ was prepared as described above and applied to the leaf surfaces; the spore suspension was sprayed until run-off onto both the adaxial and abaxial surfaces of the leaves of each experimental plant. Four replicates per treatment were treated as controls, where the eight youngest leaves were sprayed with sterile distilled water. Plants were maintained in a greenhouse, under natural light at temperatures ranging from 20 to 25 °C. Four leaves per individual were harvested at 72 h to determine percentage of pathogen germination and to examine changes in the abundance of the leaf epicuticular wax using scanning electron microscopy (SEM).

For SEM, four leaves per plant were cut into equal 1 cm^2 squares and placed in 2.5% glutaraldehyde/formaldehyde for 24 h, as described by Solís *et al.* (2022). Samples were dehydrated using an ethanol series from 30% to 100%, and later placed in hexamethyldisilazane (HMDS) and mounted on aluminium stubs. The samples were then coated with carbon using a Quorum Q150T Coating Unit (Quorum Emitech, London, UK). Conidial germination percentage was estimated in an area of 1 cm^2 under a Zeiss 540 Gemini Ultra Plus FEG SEM (Zeiss, 167) at the Laboratory for Microscopy and Microanalysis, University of Pretoria, Pretoria, South Africa. The remaining four leaves in the treatment and controls were maintained for 30 days in the greenhouses under natural light, with a temperature ranging from 20 to 25 °C to determine the new SI values.

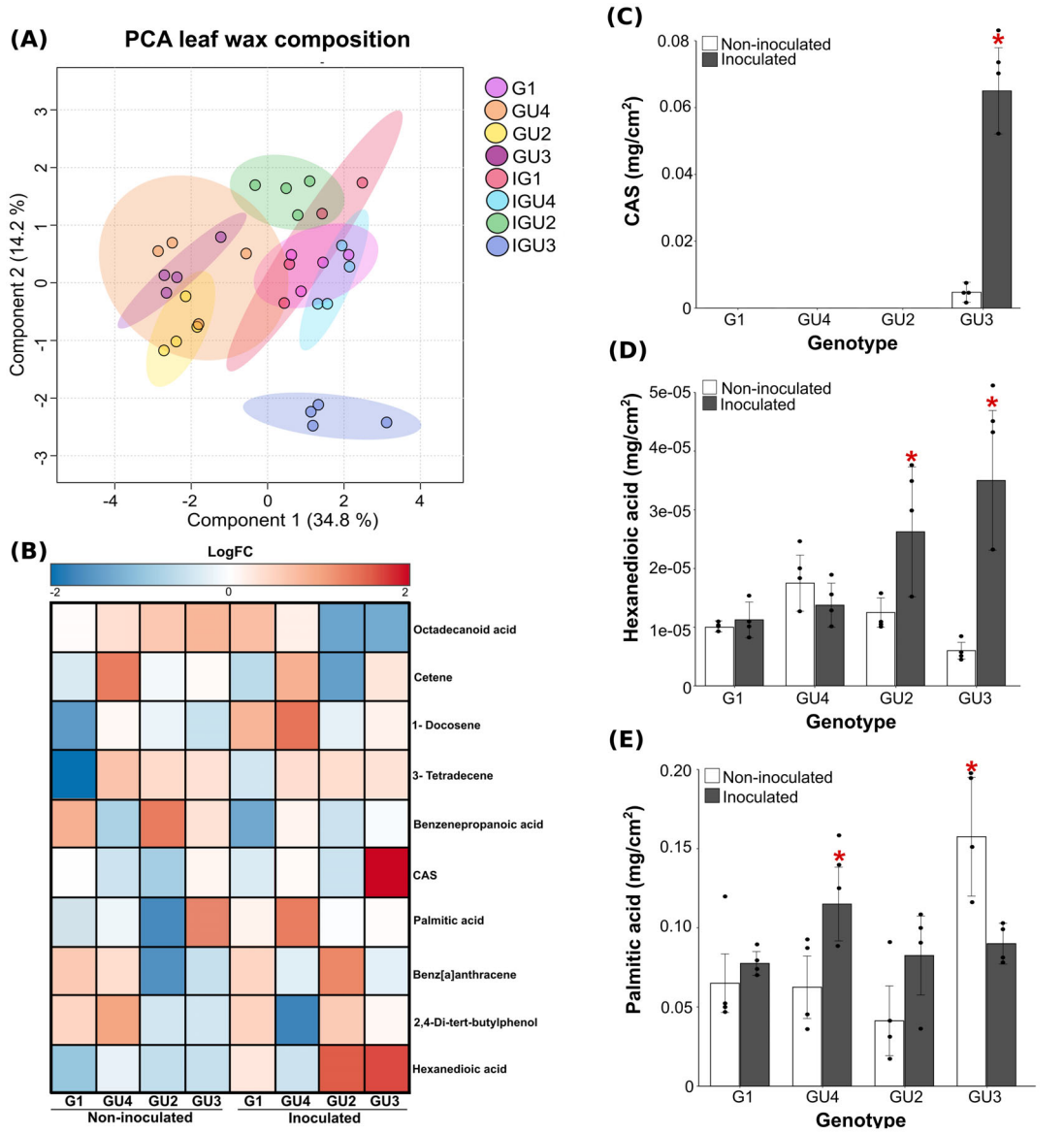

Fig. 2. Analysis of the leaf-epicuticular wax composition of *Eucalyptus grandis* × *E. urophylla* genotypes (GU4, GU2 and GU3) and *Eucalyptus grandis* (G1) genotype inoculated and non-inoculated with *Teratospaheria destructans* at 10 days after inoculation. (A) PCA of the 10 main leaf wax compounds detected by GC–MS on the leaf surface of the *Eucalyptus* genotypes, Non-inoculated: G1, GU4, GU2 and GU3, Inoculated with *T. destructans*: IG1, IGU4, IGU2 and IGU3. (B) Heatmap of the log-transformed peak areas (relative abundance) of the major compounds identified by GC–MS in the non-inoculated and inoculated genotypes. (C–E) Absolute abundance of selected leaf wax metabolites in the four different *E. grandis* × *E. urophylla* genotypes, N = 4. Red* indicates statistically significant differences between treatments evaluated by ANOVA and Tukey test ($P < 0.05$). Error bars indicate ±SE. Absolute quantifications in mg per leaf area of compounds are based on external standard curves.

Statistical analyses

A completely randomized design was used for all the experiments. Statistical analyses were performed using MetaboAnalyst V.4 and R version 3.2.0 (R Foundation for Statistical Computing, Vienna, Austria). Data were normalized using square-root transformations. The data were analysed statistically for each assay using ANOVA, and Tukey's post-hoc test was used to determine the significance of differences between all treatments at a 5% confidence level ($P < 0.05$).

RESULTS

Cuticle wax characterization

The GC–MS analysis revealed more than 300 compounds from the leaf surfaces of the four studied *Eucalyptus* genotypes. The normalized peak areas were analysed using PCA. The PCA for the ten major compounds showed that the sum of the first two principal components explained 49% of the total variance (Fig. 2A). The inoculated genotypes, GU2, G1, and GU4, and non-inoculated plants clustered together (Fig. 2A). In contrast, the inoculated moderately resistant genotype GU3 formed a

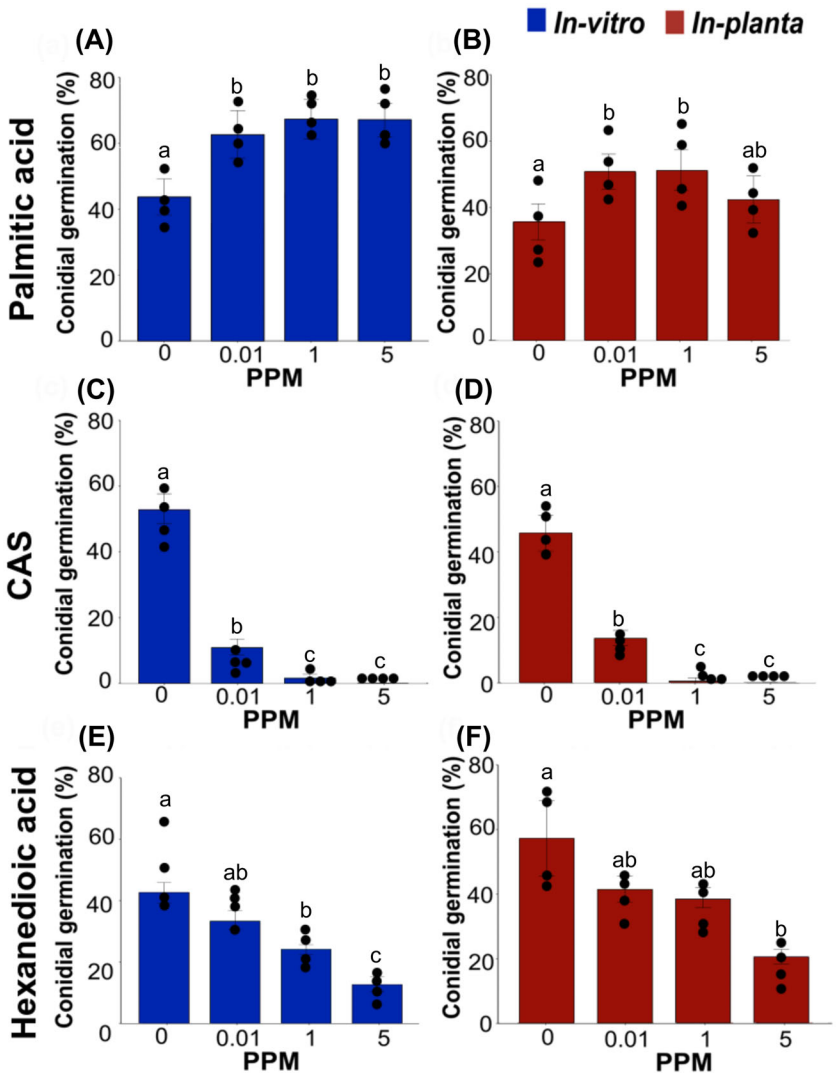

Fig. 3. *In-vitro* and *in-planta* effect of different concentrations of palmitic acid, CAS and hexanedioic acid on the spore germination of *Teratospaheria destructans*. (A, C, E) *In-vitro*, (B, D, F) *In-planta* evaluation of the effect of palmitic acid in the moderately resistant genotype GU3 and the effect of CAS and hexanedioic acid evaluated in the highly susceptible genotype GU4. Different letters at the top of bars indicate statistically significant differences between treatments evaluated by ANOVA and Tukey test ($P < 0.05$). Error bars indicate ±SE; N = 4.

separate cluster (Fig. 2A). The main compounds included alkenes (tetradecane, cetene, docosene), esters (benzenepropanoic acid), aromatic hydrocarbons (benzanthracene derivatives), phenols (*2,4-di-tert-butylphenol*) and fatty acids (octadecanoic; hexadecanoic acid) (Fig. 2B).

Among the 10 compounds showing major differences, 9,19-cyclolanostan-3-ol (CAS), a triterpenoid phytosterol, was only found in the GU3 genotype (Fig. 2B), and the concentration increased significantly from 0.01 to 0.08 mg·cm^{-2} in response to pathogen challenge (Fig. 2C), with a fold change value of 22 ($P < 0.01$) (Fig. 2B). The concentration of hexanedioic acid, was 1.5- to 2-fold higher in resistant plants inoculated with *T. destructans*, and accumulated significantly in the inoculated resistant genotypes GU3 and GU2 ($P < 0.05$) (Fig. 2D). In contrast, the concentration of palmitic acid was significantly lower after pathogen inoculation in the moderately resistant genotype GU3 (non-inoculated: 0.20 mg·cm^{-2}, inoculated: 0.11 mg·cm^{-2}) and significatively higher in the highly susceptible genotype GU4 (non-inoculated: 0.07 mg·cm^{-2}, inoculated: 0.12 mg·cm^{-2}) (Fig. 2E). The identities of these three compounds were verified by comparisons with published retention indices, as well as with retention times and mass spectra of pure standards.

Effect of CAS, hexanedioic acid and palmitic acid on germination *in-vitro* and *in-planta*

Hexanedioic acid and the triterpenoid CAS were selected for further study based on their higher accumulation in resistant genotypes under pathogen challenge (Fig. 2C, D). Palmitic acid was selected because of its increased concentration in the inoculated susceptible genotypes and its lower concentration in inoculated resistant genotypes (Fig. 2E).

The spore germination of *T.* destructans, assessed *in-vitro* under different concentrations of palmitic acid, was significantly higher when applied as a solution of 0.01 ppm (35.75%), 1.00 ppm (44.7%) and 5.00 ppm (42.7%),

compared to the control with 0 ppm (21.7%) (Fig. 3A). *In-planta*, the spore germination in the control was 35.5%. At higher concentrations of palmitic acid, spore germination increased significantly to 50% at 0.01 and 1.00 ppm ($P < 0.05$) (Figs 3B and 4B). Palmitic acid also increased the susceptibility of the host. When applied to a resistant host (GU3) with an SI of 0.4 (moderately resistant) at 0 ppm the SI increased to 0.6 at 5 ppm, thus changing its status to moderately susceptible (Fig. 5A, D). These results show that palmitic acid promotes *T. destructans* germination and increases the success of its establishment.

The *in-vitro* assessment of cycloartenol (CAS) resulted in a greater decrease in pathogen germination compared to the control (0 ppm). The percentage of germination at 0 ppm was 50.75%, and decreased to 10.5% at a concentration of 0.01 ppm of the compound (Fig. 3C). Higher CAS concentrations of 1 and 5 ppm further decreased the rate of germination to 1.5% and 0%, respectively. Similar results were obtained from the *in-planta* assay, where *T. destructans* germination decreased significantly from 45% at 0 ppm to 13.5% at 0.01 ppm and 0.5% and 0% at 1 ppm and 5 ppm (Figs 3D and 4A). The SI values, scored in the highly susceptible host GU4 at 30 days treatment with CAS, were 1.6 at 0 ppm (highly susceptible), 0.5 and 0.32, at 0.1 and 1 ppm, respectively (moderately resistant), and 0.05 at 5 ppm (resistant) (Fig. 5B, E). These results show that CAS inhibited *T. destructans* spore germination significantly and consequently inhibits development of the disease.

In-vitro treatment with hexanedioic acid resulted in 55.7% spore germination in the control (0 ppm), and this decreased with increased concentrations of the compound to 43% at 0.01 ppm, and was significantly lower at 1 ppm (31%) and 5 ppm (16%) (Fig. 3E). The effect of hexanedioic acid on spore germination *in-planta* was consistent with results from the *in-vitro* assay. In this case, the germination at 0 ppm of the compound was 58% and was significantly lower at 0.01 ppm (42%), 1 ppm (39%) and at 5 ppm (20%) (Fig. 3F). Treatment with hexanedioic acid lowered the SI values from 1.6 (0 ppm) to 0.9, 0.85 and 0.4 at 0.1, 1 ppm and 5 ppm, respectively, thereby reducing susceptibility from highly susceptible (1.6), to moderately resistant (SI = 0.4) (Fig. 5C, E). Thus, the *in-vitro* and *in-planta* assays both showed that hexanedioic acid reduced *T. destructans* spore germination and expression of disease symptoms.

Effect of the compounds on epicuticular wax abundance

Scanning electron microscopy of leaves showed that the epicuticular wax on *E. grandis* × *E. urophylla* leaves is predominantly deposited in the form of platelets. However, the highly susceptible GU4 genotype had a more glabrous surface morphology (Fig. 4). An incremental increase in platelets was observed in this genotype with increasing CAS concentrations (Fig. 4D). At higher concentrations of CAS, spores in contact with the wax surface remained inert (Fig. 4C, D). In contrast, in the resistant GU3 genotype, the leaves treated with palmitic acid showed a reduction in platelet abundance at the highest concentration of the compound (Fig. 4H). Here, mycelial growth of the pathogen was evident on the leaf surface and stomatal penetration was observed (Fig. 4F–H).

DISCUSSION

This study showed that the composition of the leaf wax surface of *E. grandis* × *E. urophylla* genotypes and an *E. grandis*

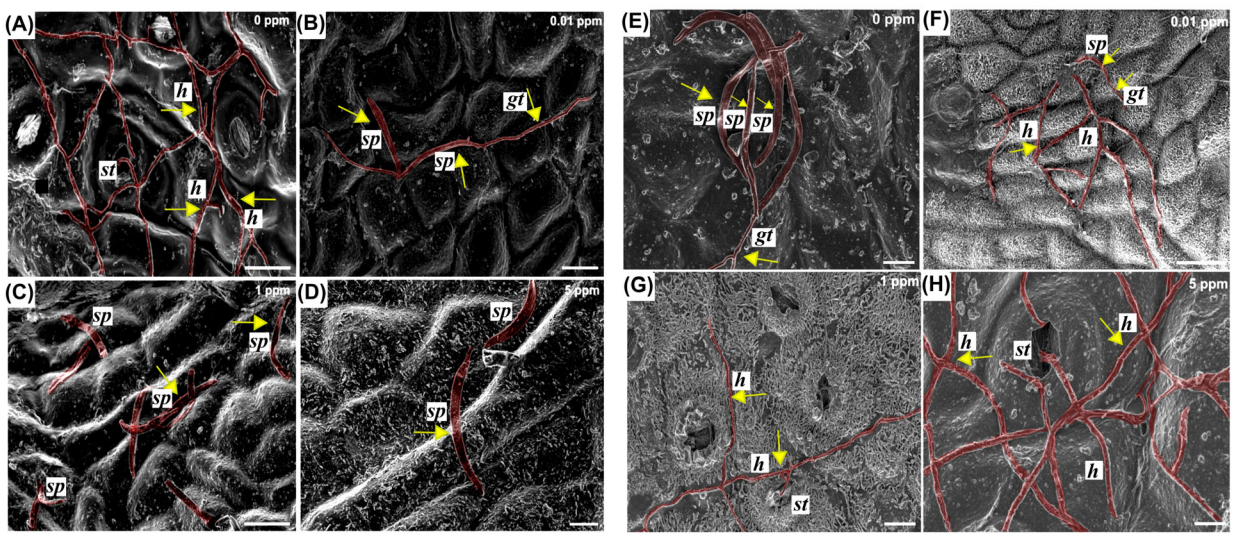

Fig. 4. Scanning Electron Micrographs (SEM) of *Eucalyptus grandis* × *E. urophylla* leaf surfaces including genotypes highly (GU4) and moderately (GU3) susceptible to *Teratospaheria destructans* 72 h after inoculation. Spores and mycelium are shown in red. (A–D) Leaf surfaces of susceptible genotype (GU4) treated with different concentrations of CAS (cycloarstenol). (A, B) Leaf surfaces of GU4 treated with 0 and 0.01 ppm CAS, respectively, showing hyphal growth (A) and spore germination (B). (C, D) Leaf surfaces of GU4 treated with concentrations of 1 and 5 ppm CAS, respectively, showing no fungal growth and thus inhibition at high concentrations of the compound. (E–H) Leaf surfaces of the moderately resistant *Eucalyptus* genotype (GU3) treated with different concentrations of palmitic acid. (E, F) Leaf surfaces of GU3 treated with 0 and 0.01 ppm palmitic acid, showing spore germination (E) and hyphal growth (F). (G, H) Leaf surfaces of GU3 treated with high concentrations (1 and 5 ppm, respectively) palmitic acid, showing hyphal growth and pathogen penetration through stomata on the moderately resistant GU3. Scale bars 10 μm, ***sp***: spore, ***h***: hyphae, ***st***: stomata, ***gt***: germ tube. All yellow arrows point to fungal structures.

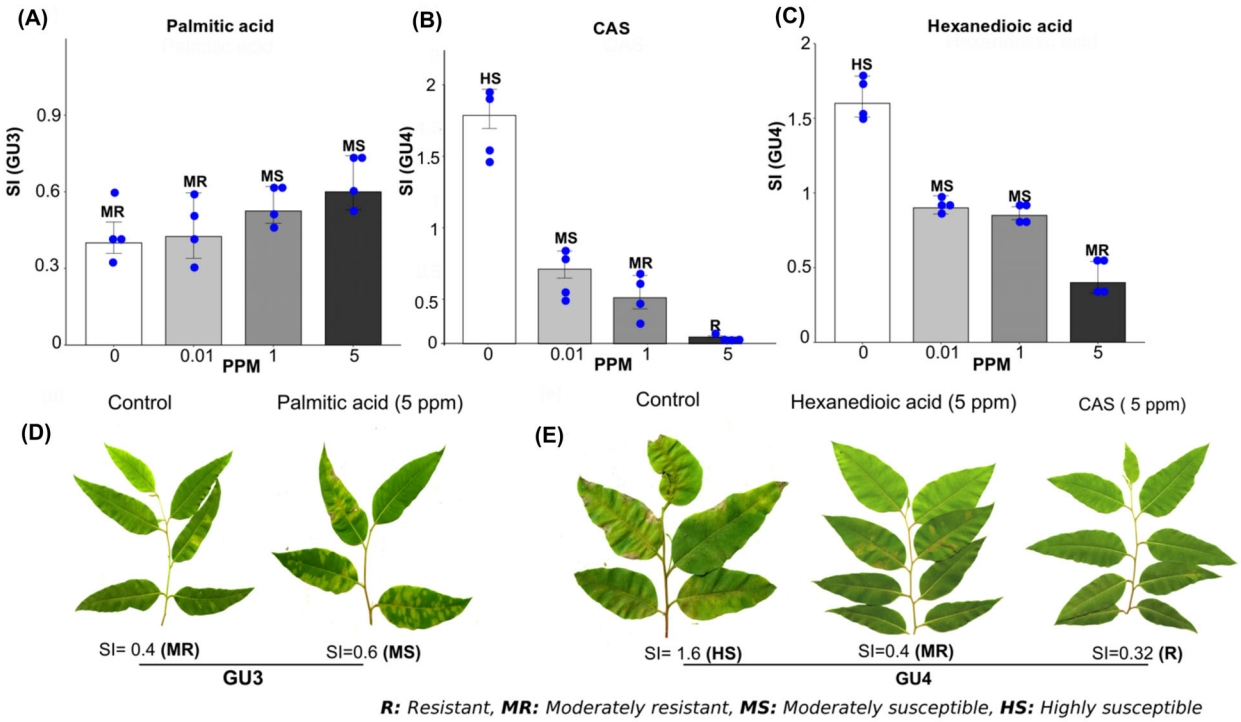

Fig. 5. Susceptibility Index (SI) to *Teratosphaeria destructans* of a resistant genotype (GU3) treated with palmitic acid, and a susceptible genotype (GU4) treated with CAS and hexanedioic acid 30 days after inoculation. The compounds were evaluated at four concentrations (0, 0.01, 1 and 5 ppm). SI categories, **R:** Resistant, **MR:** Moderately Resistant, **MS:** Moderately Susceptible, **HS:** Highly Susceptible. (A) SI results for resistant genotype GU3 treated with different concentrations of palmitic acid, from 1 to 5 ppm; this genotype was classified as **MS**. (B) SI results for the highly susceptible genotype GU4 treated with different concentrations of CAS at 5 ppm classified as **R**. (C) SI results for the highly susceptible genotype GU4 treated with different concentrations of hexanedioic acid at 5 ppm classified as **MR**. Error bars indicate SE, N = 4. (D) Leaves of the moderately resistant genotype GU3 inoculated with *T. destructans* treated with palmitic acid at 0 ppm (SI = 0.4, **MR**) and 5 ppm (SI = 0.6, **MS**) with evident presence of leaf blight symptoms. (E) Leaves of the highly susceptible genotype GU4 (SI = 1.6, **HS**) inoculated with *T. destructans* treated with 5 ppm hexanedioic acid (SI = 0.4, **MR**) and CAS (SI = 0.32, **R**), CAS reduced the leaf blight symptoms in leaves and small necrotic spots were the main localized symptoms visible.

genotype influence their susceptibility to infection by the aggressive leaf blight pathogen *T. destructans*. We identified three major chemical compounds on the leaf surfaces, including CAS, hexanedioic acid and palmitic acid, which modulated the spore germination of *T. destructans* both *in-vitro* and *in-planta*. Additionally, our SEM results showed that CAS increased the abundance of leaf wax platelets in a susceptible host. In contrast, palmitic acid reduced platelet abundance. We also demonstrated that these compounds altered host susceptibility, especially in the case of leaves treated with CAS, where a susceptible genotype treated with this compound became resistant to *T. destructans* infection after treatment.

Evaluation of leaf epicuticular wax composition showed that in all four tested *Eucalyptus* genotypes, the major leaf wax compounds were fatty acids, such as octadecanoic acid, as well as straight-chain alkenes, such as docosene. This is similar to previous findings in *Eucalyptus*, where the major components of leaf surface waxes were identified as fatty acids and straight-chain alkenes (Santos *et al.* 2019; Makunde *et al.* 2023). Our results showed significant variation between the wax metabolite profiles of different hybrid genotypes. This illustrates the metabolic plasticity of wax synthesis in *Eucalyptus* and the different putative roles that waxes might have in the recognition of the host by *T. destructans* and its attachment to the hydrophobic surface of the leaves. Similar effects have also been found for the biotrophic pathogens *Blumeria graminis* on barley (Zabka *et al.* 2008) and *Magnaporthe grisea* on rice (Lee *et al.* 1994).

Palmitic acid was a highly abundant compound in the inoculated susceptible genotypes included in this study, and its concentration was lower in inoculated leaves of the more resistant genotype. We demonstrated that this compound induced the spore germination of *T. destructans in-vitro*, as well as on leaves of a resistant genotype *in-planta*. This fatty acid has been reported previously to induce germination and appressorium differentiation in the rice pathogen *Magnaporthe grisea* (Gilbert *et al.* 1996). It is also known to promote conidial germination and cutinase expression of *Botrytis cinerea* (Leroch *et al.* 2013). Particularly relevant to this study, it has recently been reported as the major component on the leaf wax surface of a susceptible *E. grandis* genotype, where it induced germination of urediniospores of the myrtle rust pathogen *Austropuccinia psidii* (Santos *et al.* 2019). The mechanism by which this compound enhances host susceptibility is unknown, but it might play a role in host recognition, as has been shown for *Blumeria graminis* f.sp. *tritici* (Kong *et al.* 2020) As a fatty acid, it could also be utilized by fungi as a nutrient source during germination on leaf surfaces, as has been reported for *Aspergillus nidulans* (Dashti *et al.* 2008).

The triterpenoid CAS was identified on *E. grandis* × *E. urophylla* leaves by GC–MS to be present only in the moderately resistant genotype GU3, and was also more abundant after

T. destructans infection. This compound inhibited germination of the pathogen both *in-vitro* and *in-planta*. The antimicrobial activity of CAS has been studied in stem bark extracts of the tree *Garcinia lucida*, and tested against various bacteria and *Candida albicans* (Momo *et al.* 2011). CAS is also a major compound of leaves of *Garcinia mangostana*, and leaf extracts from this plant have antimicrobial activity against the bacterial plant pathogens, *Pseudomonas syringae* pv. *tomato* and *Xanthomonas oryzae* pv. *oryzae* (Alsultan *et al.* 2016). The antifungal activity of CAS could relate to the capacity of triterpenoids to interact with the fungal membrane, leading to increased membrane permeability and favouring the entrance of extracellular substances and the leakage of cell constituents, as reported in *Candida albicans* cells (Haraguchi *et al.* 1999).

Hexanedioic was found in higher amounts in the wax layer of moderately resistant *E. grandis* × *E. urophylla* genotypes after pathogen inoculation. This adipic acid was also shown to reduce *T. destructans* germination *in-vitro* and *in-planta* in a susceptible host. Hexanedioic has also been extracted from leaves of *Ficus sycomorus* and was linked with insecticidal and acaricidal activity (Romeh 2013). The antifungal activity of hexanedioic acid has been reported from leaf extracts of *Melia azedarach* against the soil-borne fungal pathogen, *Sclerotium rolfsii* (Sana *et al.* 2016). This adipic acid has previously been identified as one of the main compounds in wood extracts of *Eucalyptus globulus* (Freire *et al.* 2002). Although the mechanisms of antifungal action are still unknown, it has recently been reported that adipic acids can alter the plasma membrane of *Saccharomyces cerevisiae* (Fletcher *et al.* 2021), and this may be related to the mode of action of this compound that inhibited the germination of *T. destructans* in our study.

The results of this study demonstrate the chemodiversity of the epicuticular wax surface of *Eucalyptus* leaves, and that it influences susceptibility to infection by *T. destructans*, as well as spore germination of the pathogen. The results could be used to develop rapid screening methods, such as biomarkers for predicting *Eucalyptus* susceptibility or resistance to TLB disease. Future work should expand on studying leaf wax composition in different *Eucalyptus* species, including a larger number of replicates and genotypes. This would lead to a better understanding of the role they could play as preformed and induced defence barriers against pathogens. In addition, studies on the genetic and molecular mechanisms involved in wax metabolite biosynthesis and mode of action of these compounds will contribute to the development of effective molecular tools to breed and select disease-resistant *Eucalyptus* genotypes.

AUTHOR CONTRIBUTIONS

MS, SN, MJW, and AH conceived the study. MS selected the plant material and pathogen, did the inoculation trials, inhibition experiments, and microscopy. JCJ and MS performed the leaf wax extraction and analysed the samples with GC-MS. AH and MS performed the analysis of GC-MS results and identified the compounds. MS and AH designed the inhibition trials. MS wrote the first draft of the manuscript, which was edited by SN, MJW, and AH. All the authors read and approved the final manuscript.

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