Limonene, a Chemical Compound Related to the Resistance of *Eucalyptus* Species to *Austropuccinia psidii*

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Abstract

The fungus *Austropuccinia psidii* is a major pathogen of *Eucalyptus* spp. that damages mainly early-stage leaves. Resistant clones are the most widely used control measure for the rust disease caused by *A. psidii*. Essential oils produced in the plant-host cells are associated with resistance. However, the chemical characteristics of *Eucalyptus* leaves at different stages of maturity, associated with resistance or susceptibility to *A. psidii*, need to be determined. The aims of this study were to characterize the chemical composition of essential oils in *Eucalyptus* leaves at three developmental stages of clones of three *E. urophylla* × *E. grandis* hybrids that exhibit different resistance levels to rust and to identify probable resistance-related compounds from them. The rust severity following inoculation and the quantity and quality of the essential oil extracted by hydrodistillation were determined at the first, third, and fifth leaf stages of the three clones. Identification of the compounds present in the essential oil was determined by gas

chromatography coupled with mass spectrometry. No rust was observed on the three leaf stages of the resistant clone or on the fifth leaf stage of the susceptible clones. Limonene was found at high percentages in essential oils from the three leaf stages of the resistant clone and at low percentages in the susceptible clones. In vitro and in vivo tests indicated that low limonene percentages stimulated *A. psidii* ure-diniospore germination and were not fungitoxic, whereas high percentages caused complete inhibition of germination and degenerative changes in fungal reproductive structures. The limonene present in *Eucalyptus* leaves can be considered a chemical compound related to the rust resistance of *Eucalyptus* spp.

Keywords: *Eucalyptus* essential oils, eucalyptus rust, gas chromatography, phenology stage, *Puccinia psidii*, resistance, secondary metabolism

The fungus *Austropuccinia psidii* (G. Winter) Beenken, family Sphaerophragmiaceae (Pucciniales) [Beenken, L. (2017)], is native to South and Central America (Alfenas et al. 2005). *A. psidii* causes eucalyptus rust, one of the most harmful diseases and a major threat to Myrtaceae biodiversity and especially to the *Eucalyptus* forestry industry worldwide (Coutinho et al. 1998; Pérez et al. 2011). This pathogen has been reported on four continents (Machado et al. 2015). Together with the wide host range of this fungus (*Eucalyptus*

spp., *Eugenia cambucae*, *Eugenia koolauensis*, *Eugenia uniflora*, *Melaleuca* spp., *Metrosideros polymorpha*, *Myrcianthes fragrans*, *Myrciaria cauliflora*, *Psidium guajava*, and *Syzygium jambos*, among other Myrtaceae species), this pathogen is of considerable global importance (Coutinho et al. 1998; da Silva et al. 2014a; Glen et al. 2007). This rust can occur in nursery cuttings and younger trees in the field, causing damage and crop losses (Coutinho et al. 1998; Silva et al. 2013). Planting of elite clones of *Eucalyptus* species (e.g., *E. grandis*, *E. cloeziana*, *E. dunnii*, *E. benthamii*, *E. phaeotricha*, *E. globulus*, *E. viminalis*, and *E. nitens*) has been limited because of their high susceptibility to this pathogen (Alfenas et al. 2009; Glen et al. 2007).

Genetic host resistance is the main strategy for managing *A. psidii* (Mamani et al. 2010). Several rust-resistant genetic materials have been identified (Dianese et al. 1986; Santos et al. 2014; Silva et al. 2013), but little is known about the resistance mechanisms of *Eucalyptus* plants to this disease. Genetic (Junghans et al. 2003; Laia et al. 2015; Mamani et al. 2010), chemical (Boava et al. 2010; Taylor and Mims 1991), and structural (Santos et al. 2019; Silva et al. 2017; Xavier et al. 2015) factors associated with plant resistance to *A. psidii* have been reported, but there are currently no studies showing an interaction between the essential oils present in the *Eucalyptus* leaf and resistance to this rust.

Plants produce secondary metabolites useful in their interaction with the environment, such as the development of resistance against

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pathogens and tolerance to various abiotic stresses (Sudha and Ravishankar 2002). Compounds derived from secondary metabolism, including essential oils, have impressive biological activities ranging from antibiotic, insecticidal, to pharmaceutical activity (Stockigt et al. 1995). Awareness of the functions of these compounds in plants has led to new studies in the last decade (Sudha and Ravishankar 2002).

Essential oils are composed of various chemical compounds (Batish et al. 2006). The essential oil of *Eucalyptus* leaves has volatile organic acids, alcohols, aldehydes, esters, hydrocarbons, and ketones, which are predominantly mono- and sesquiterpenes (Batish et al. 2008; Elaissi et al. 2010). The chemical composition of plant essential oils is genetically determined and may vary according to species, individual, organ of synthesis, phenological stage, and leaf age (Batish et al. 2006; Brooker and Kleinig 2006). Moreover, essential oil bioactivity depends on the type, nature, and concentration of its constituents (Batish et al. 2008). Therefore, *Eucalyptus* essential oils and their main constituents could be used in the management of phytopathogens such as fungi (Pereira et al. 2011; Vilela et al. 2009), bacteria (Amorim et al. 2011), and nematodes (Andrés et al. 2012). Regarding fungal pathogens, *Eucalyptus* essential oils have been reported to reduce mycelial growth (Fiori et al. 2000) and inhibit spore production (Fiori et al. 2000; Oluma and Garba 2004).

A. psidii infects only young tissues of susceptible *Eucalyptus* plants (Silva et al. 2017; Xavier et al. 2001). There is a gradual reduction in the process of penetration, establishment, and reproduction of *A. psidii* in *Eucalyptus* leaves as the leaf ages (Silva et al. 2017; Xavier et al. 2001, 2015). The relationship between the essential oil composition in *Eucalyptus* clones and differential resistance to rust, as well as between leaf phenological stages and resistance to *A. psidii*, needs to be clarified. In addition, essential oil compounds in different *Eucalyptus* clones and in leaf phenological stages may be associated with resistance to rust disease.

Our objectives in this study were to characterize the chemical composition of essential oils in leaves of clones of three *E. urophylla* × *E. grandis* hybrids with different levels of resistance to *A. psidii*, at three developmental stages, and to identify putative resistance-related compounds in these hybrids.

Materials and Methods

Plant material and evaluation of leaf-stage resistance. Clones of three *E. urophylla* × *E. grandis* (Myrtales, Myrtaceae) hybrids were selected based on their response to *A. psidii* in field tests at Votorantim Celulose and Papel S.A. Company (Jacareí, SP, Brazil; 23°17'S and 45°54'W). Cuttings of clones C041A, VR3748, and TC31 were considered to be resistant, susceptible, and highly susceptible to *A. psidii*, respectively, and were supplied by Votorantim Celulose and Papel S.A. Cuttings of the three hybrid clones were grown in 2-liter-capacity pots containing Carolina Soil (Carolina Soil do Brasil, Santa Cruz do Sul, RS, Brazil) substrate enriched with simple superphosphate (6 kg/m³) and Osmocote (1.5 kg/m³, NPK 19:06:10; Tecnutri do Brasil, Tietê, SP, Brazil) and kept in a greenhouse at 20 to 32°C for 50 days. Plants were then transferred to the growth chamber (23 ± 3°C temperature, 12-h light/12-h dark photoperiod) and inoculated with an isolate of *A. psidii* (FCA-PP2303) obtained from the mycological collection of the Universidade Estadual Paulista Laboratory of Forest Pathology. To increase the concentration of urediniospores for inoculation tests, young leaves of *Syzygium jambos* (L.) Alston were inoculated until runoff with a suspension of 1 × 10⁶ urediniospores/ml using a manual sprayer with a conical ejection nozzle. The inoculated plants were incubated at 23 ± 3°C in a growth chamber under continuous darkness for 24 h, followed by a 12-h photoperiod. After 12 days, newly formed urediniospores were collected using a fine brush and stored at -80°C. We then used a hemocytometer to prepare an inoculum of 1% Tween 20 in water at a concentration of 1 × 10⁶ urediniospores/ml and sprayed the plants as previously described. After inoculation, the plants were kept in darkness for 24 h, followed by a 12-h light/dark photoperiod. Eight days after inoculation, disease severity (number of pustules/2 cm² of leaf area previously marked on the middle third of each leaf) was

evaluated on the first, third, and fifth leaf pairs from the apex to the base of each *Eucalyptus* clone. The experiment was carried out using a completely randomized design with 10 replications (one cutting per replication).

Extraction and analysis of essential oils. We selected 1-year-old plants from clones C041A, VR3748, and TC31 grown in the municipality of Jacareí, São Paulo, Brazil (23°17'S and 45°54'W) under the same edaphoclimatic and silvicultural conditions to evaluate the chemical composition of their essential oils. Healthy leaves of the first, third, and fifth pairs of leaves at the same phenological stage were collected from 20 plants per clone per replication in the morning during July 2010. Essential oils were extracted from 200 g of fresh leaves per leaf stage, clone, and replication. The leaves were cut and transferred to a Clevenger apparatus for hydrodistillation. The essential oil collected after 2 h of hydrodistillation was separated and stored at -40°C in flasks covered with aluminum foil (da Silva et al. 2014b). The oil yield was expressed as oil volume (in milliliters) per 100 g of sample. The experimental design consisted of completely randomized blocks with three replications per clone (20 plants per clone per replication).

Oil samples were analyzed by gas chromatography (GC) with flame ionization detection (FID) using a Shimadzu model 17A chromatograph (Kyoto, Japan) and by GC-mass spectrometry (MS) in a Shimadzu model QP5000 instrument with a quadrupole detector. All analyses were carried out using a DB-5 fused-silica column (30 m × 0.25 mm i.d., 0.25 µm film thickness; J & W Scientific Inc.). The chromatographic conditions were as follows: initial temperature of 60°C increasing to 240°C at 3°C min⁻¹, helium carrier gas at a flow rate of 1.0 ml min⁻¹, injector temperature at 220°C, injection volume of 1.0 µl, flow ratio of 1:20, interface temperature of 230°C, ionization potential of 70 eV, mass range of 40 to 550 m/z, and scan rate of 0.84 scans s⁻¹. The compounds were identified by comparing their MS profiles with those of the National Institute of Standards and Technology (1998) online library and literature databases (Adams 2007). The retention indices (RIs) were obtained with a series of *n*-alkanes (C₈ to C₃₂) chromatographed under conditions analogous to those of the samples using the equation proposed by Van den Dool and Kratz (1963). Retention values were then compared with those reported in the literature (Adams 2007). We used GC/FID to detect the relative concentrations of essential oil components by the area normalization method without considering specific response factors.

Fungitoxicity of limonene on urediniospores of *A. psidii*. The toxic effect of the main constituent found in the essential oil from each of the three phenological stages of the clone resistant to *A. psidii* was evaluated in a spore germination bioassay test. Infected *Eucalyptus* leaves were collected 20 days after inoculation with the same isolate (FCA-PP2303) of *A. psidii* used before, and a spore suspension in water containing 1% Tween 20 was prepared at a concentration of 1 × 10⁵ spores/ml. The GC analysis identified a high concentration of limonene in the *Eucalyptus* essential oil of the resistant clone. Pure samples of (*R*)-(+)-limonene (183164; Sigma-Aldrich, St. Louis, MO) and (*S*)-(-)-limonene (218367-50G; Sigma-Aldrich) were then tested at concentrations of 1, 10, 100, 1,000, and 10,000 ppm. A limonene/water premix was prepared using Tween 20 emulsifier at 1% to produce the concentrated emulsions. One milliliter of the emulsion was mixed with 4 ml of water agar previously autoclaved at 121°C for 20 min and poured into 5-cm-diameter Petri dishes (da Silva et al. 2012). Medium for the negative control was prepared in the same manner but omitting the essential oils. After the agar solidified, 70 µl of the spore suspension was spread on the agar medium in each dish. The samples were incubated in the dark for 48 h at 23°C. The spore germination percentage was then evaluated with a light microscope. Each Petri dish was divided into four quadrants; 50 spores per quadrant, totaling 200 spores per dish, were scored as germinated or nongerminated. The experiment was a completely randomized design with three replications (one Petri dish per replication) and repeated once.

Fungitoxicity of limonene on rust in *Eucalyptus* plants. Cuttings of the rust-susceptible clone (VR3748) were grown in 2-liter-capacity

pots and kept in a greenhouse at 20 to 30°C for 50 days as previously described. The plants were then transferred to the growth chamber (23 ± 3°C temperature, 12-h/12-h light/dark photoperiod) where they were inoculated with the FCA-PP2303 isolate in a suspension in water containing 1% Tween 20 at a concentration of 1 × 10⁶ urediniospores/ml. After inoculation, the plants were kept in the dark for 24 h followed by a 12-h photoperiod. Fifteen days after inoculation, the plants (one plant per treatment) were sprayed with either (R)-(+)- or (S)-(-)-limonene at concentrations of 1, 10, 100, 1,000, and 10,000 ppm. In the control treatment, plants were sprayed with only water containing 1% Tween 20. Seventy-two hours after the treatment application, five leaves inoculated with the pathogen per treatment were collected for analysis by scanning electron microscopy (SEM). Sample preparation and analysis followed previous methods (Silva et al. 2017).

Statistical analysis. Data from the evaluation of resistance of the leaf stages of the *Eucalyptus* clones were submitted to factorial analysis constituted by the combination of three clones × three leaf stages. The data were transformed ($x + 0.5$)^{1/2} following the procedure described by Box and Cox (1964) to obtain data normality and were then submitted to analysis of variance (ANOVA). Mean values, when significant, were compared using the Tukey test. The percentage data of the essential oil composition for the first, third, and fifth leaf stages from the three clones were submitted to multivariate statistical tests of cluster and principal component analyses (Sneath and Sokal 1973) to correlate them with resistance or susceptibility to *A. psidii*. The multivariate statistical clustering and principal component analyses were carried out for the most abundant substances for better visualization and to carry out Pearson's product-moment correlation between the factors. Performance data were submitted to ANOVA, followed by the Tukey test. The germination test was performed in a factorial analysis consisting of the combination of the two limonenes (R and S) × six concentrations. A regression analysis was also performed on the limonene concentrations against spore germination, with the standard error of the regression coefficient also being calculated. The normality and homogeneity of variance was evaluated on the residuals and no

deviations from normality were found, based on the presuppositions applied to the analysis. The statistical analyses were performed using R software (R Development Core Team).

Results

Natural *Eucalyptus* infection by *A. psidii* was evaluated in young trees up to 1 year old in field tests at Votorantim Celulose and Papel S.A. Disease scores were based on the scale by Zamprognio et al. (2008) as follows: S0, no pustules or healthy plants; S1, isolated punctate pustules on the limbs and young leaves; S2, rust pustules generally sparse or occasionally abundant on the limbs and young leaves; and S3, pustules abundant on the limbs, petioles and leaves, at the tips of branches and the primary stem, and apical necrosis). The C041A clone was resistant to rust (S0), the VR3748 clone was susceptible (S2), and the TC31 clone was highly susceptible (S3). In the evaluation of resistance according to the leaf stages of the three *Eucalyptus* clones, no pustules were observed on the three phenological leaf stages of the resistant clone (Table 1). In the highly susceptible and susceptible clones, a decrease in the number of pustules per square centimeter of leaf area was observed with increasing leaf age. The disease was not observed at the fifth leaf stage of any of the three clones tested. The first leaf stage of the highly susceptible and susceptible clones had higher infection levels than the third and fifth leaf stages of their clones. The first and third leaf stages of the highly susceptible clone had the highest densities of rust pustules.

The resistant clone had higher essential oil yields at the first and third leaf stages than the susceptible and highly susceptible clones (Table 2). At the fifth leaf stage, oil production was similar among the three *Eucalyptus* clones. The resistant clone produced the highest oil yield at the third leaf stage, the susceptible clone had similar yields in the third and fifth leaf stages, and the highly susceptible clone exhibited an increasing essential oil yield from the first to the fifth phenological stages.

Chemical analysis of the essential oils extracted from the three leaf stages of the three *Eucalyptus* clones showed that monoterpenes were the main constituents, followed by sesquiterpenes (*trans*-caryophyllene and viridiflorene) (Table 3). Moreover, limonene (29 to 33.87%), α-pinene (19 to 20.62%), and 1,8-cineole (18 to 24.45%) were the major essential oil compounds in all three phenological leaf stages of the resistant clone (Table 3). In the susceptible clone, 1,8-cineole (35 to 40.47%), α-pinene (33 to 41.31%), and α-terpineol (4 to 5.54%) were the major compounds. The highly susceptible clone showed 1,8-cineole (21 to 35.14%), γ-terpinene (15 to 19.91%), and α-pinene (15 to 18.08%) as the principal compounds. For the susceptible and highly susceptible clones, α-pinene and 1,8-cineole were the major compounds at all three leaf phenological stages. Limonene, however, had the highest concentrations in the three leaf phenological stages of the resistant clone.

The first principal component analysis (Table 4) with the graphic dispersion (Fig. 1) showed higher γ-terpinene, α-terpinyl acetate, ortho-cymene, 4-terpineol, and terpinolene percentages and lower α-pinene levels in the three leaf stages of the clone highly susceptible to the disease. For the second principal component, the resistant clone was distinguished by higher percentages of limonene and lower 1,8-cineole levels in the essential oils from its three leaf stages. The correlation between the 13 major and two main (Y1 and Y2) compounds of the essential oils showed that the resistant clone was separated from the susceptible and highly susceptible ones along axis Y2, which accounted for approximately 25% of the variation in oil composition (Fig. 1; Table 4). Limonene was the major influence on axis Y2, consistent with the hypothesis that limonene concentration is important for rust resistance (Table 4) and could potentially be selected for resistance tests. The highly susceptible clone was separated from the susceptible and resistant clones along axis Y1, which accounted for approximately 55% of the variation, yet the major discriminant along axis Y1 was ortho-cymene; the resistant and susceptible clones overlap along this axis (Fig. 1; Table 4).

The graphic dispersion (Fig. 1) and dendrogram resulting from cluster analyses of the 13 compounds of the essential oils (Supplementary Fig. S1) showed differences among both clones and the leaf

Table 1. Severity of *Austropuccinia psidii* on leaves of the first, third, and fifth phenological stages (E1, E3, and E5) from the resistant (C041A), susceptible (VR3748), and highly susceptible (TC31) clones of *Eucalyptus urophylla* × *E. grandis*^y

Clone	Leaf phenological stage		
	E1 ^z	E3	E5
TC31	18.3 aA	9.3 aB	0 aC
VR3748	4.8 bA	0.8 bB	0 aB
C041A	0 cA	0 bA	0 aA

^y Data are presented as the mean number of pustules/2 cm² of leaf area. Means followed by the same small letter per column or capital letter per line do not differ by the Tukey test at 5%. For statistical analysis, data were transformed to ($x + 0.5$)^{1/2}. Data are presented in their actual values. Significant minimum difference = 0.3976.

^z Mean E1, E3, and E5 values were obtained from 10 replicates.

Table 2. Total yield of essential oils per 100 g of leaves of the first, third, and fifth phenological stages (E1, E3, and E5) from the resistant (C041A), susceptible (VR3748), and highly susceptible (TC31) clones of *Eucalyptus urophylla* × *E. grandis* determined by gas chromatography/mass spectrometry analysis^y

Clone	Leaf phenological stage		
	E1 ^z	E3	E5
C041A	1.61 aB	2.28 aA	1.88 aB
VR3748	1.44 aB	1.60 bAB	1.85 aA
TC31	0.85 bC	1.35 bB	1.82 aA

^y Data are presented as means in milliliters per 100 g of leaves. Means followed by the same small letter per column or capital letter per line do not differ by the Tukey test at 5%. Significant minimum difference = 0.277.

^z Mean E1, E3, and E5 values were obtained from three replicates.

phenological stages. The chemical composition and relative proportions of compounds in the oils grouped the three clones into different clades (Fig. 1 and Supplementary Fig. S1). The highly susceptible clone showed marked dissimilarity compared with the susceptible and resistant clones (Fig. 1). A similar effect was also identified among the phenological leaf stages. Leaves of the first phenological stage (most susceptible) differed from those of the third and fifth stages (less susceptible) in all clones (Fig. 1). This supported the hypothesis of a relationship between the essential oil composition and *Eucalyptus* resistance to the fungus *A. psidii*.

No difference was detected between experiments conducted to evaluate the fungitoxicity of limonene on urediniospore germination, so the data obtained were combined. The in vitro test evaluated limonenes at concentrations of 1, 10, 100, 1,000, and 10,000 ppm. There was no difference between the *R* and *S* enantiomers, nor was there any interaction between the limonene enantiomers and their concentrations in terms of spore germination. A difference was observed only at doses of both compounds. (*R*)-(+)-limonene and the (*S*)-(–)-limonene at concentrations of 1 or 10 ppm stimulated fungus germination to rates of 146 and 164% and 55 and 84%, respectively, in relation to germination in the control treatment (Fig. 2). The limonene enantiomers *R* and *S* at concentrations of 100, 1,000, or 10,000 ppm produced 100% inhibition of urediniospore germination.

The fungitoxic effects of *R* and *S* limonene enantiomers on eucalyptus rust were confirmed using SEM (Figs. 3 and 4). Limonene at concentrations >100 ppm caused morphological changes to the fungus. Degeneration in the urediniospore cell wall and collapse of the urediniospores, teliospores, and paraphyses were the main changes observed in the pustules. No changes in fungus morphology were observed in the control treatment and in limonene tested at concentrations of 1 and 10 ppm.

Discussion

Our analysis of rust severity, yield of essential oils, and oil composition emphasized the differences in resistance among the three *Eucalyptus* clones and their leaf stages. Multivariate statistical analysis differentiated among the three *Eucalyptus* clones and the first leaf stage, indicating possible relationships among the essential oil concentrations and their constituents as well as the incidence and severity of *A. psidii*. The youngest (first leaf stage) *Eucalyptus* leaves were the most susceptible to *A. psidii* (Silva et al. 2017; Xavier et al. 2001). The susceptible and highly susceptible clones had a lower essential oil yield in the first and third leaf pairs. The resistant clone with no symptoms of rust at all leaf stages tested had higher essential oil yield in the first and third leaf stages than the susceptible and highly susceptible ones. Oil yield was similar among the three *Eucalyptus* clones at the fifth leaf stage. The fifth leaf stage is resistant to *A. psidii* independent of intrinsic resistance or susceptibility to the

fungus (Silva et al. 2017; Xavier et al. 2015). The lower pathogen occurrence and similar oil yield at the fifth leaf stage of all three of the clones suggests that high quantities of essential oils may be involved in plant defense, as reported for the leaf disease pathosystem *Eucalyptus globulus*–*Mycosphaerella* (Hantao et al. 2014).

The essential oil constituents of the resistant, susceptible, and highly susceptible *Eucalyptus* clones in the three phenological leaf stages were similar but with variations in their relative concentrations. This may be attributable to their common genetic parentage, *E. urophylla* × *E. grandis*. Therefore, resistance or susceptibility to *A. psidii* may be related to the percentage of each compound in the essential oil in different clones and different leaf phenological stages. Limonene was found at high concentrations in the essential oil extracted from the three-leaf stages of the resistant clone and at low concentrations in the leaves of the susceptible and highly susceptible clones. Limonene is a compound with a broad action spectrum against fungi and bacteria (Aggarwal et al. 2002; Regnier et al. 2008; Singh et al. 2010), although its low ability to inhibit some pathogens has been reported (Combrinck et al. 2011).

The effectiveness of limonene depends on its concentration and on the target pathogen (Combrinck et al. 2011; Droby et al. 2008; Eckert et al. 1984). Limonene at low concentrations (1 and 10 ppm) in the culture medium stimulated spore germination. This was probably attributable to the perception of biochemical signals by *A. psidii* urediniospores, since limonene is a component of the *Eucalyptus* leaf. Oil

Table 4. Correlation coefficients between 13 chemical compounds of essential oils present in three leaf stages (E1, E3, and E5) of three *Eucalyptus urophylla* × *E. grandis* clones and their two principal components (Y1 and Y2)

Chemical compound	Y1	Ordination ^z	Y2	Ordination
α-pinene	–0.7118	6	–0.6984	3
α-phellandrene	–0.6315	8	0.6486	4
Ortho-cymene	0.9711	1	0.1510	11
Limonene	–0.4220	10	0.9000	1
1,8-cineole	–0.1454	12	–0.8474	2
cis-β-ocimene	0.1102	13	–0.4328	5
γ-terpinene	0.9698	2	–0.1207	12
Terpinolene	0.7963	5	0.0881	13
4-terpineol	0.8766	4	0.2169	10
α-terpineol	–0.6402	7	–0.4092	6
α-terpinyl acetate	0.9174	3	0.3870	8
trans-caryophyllene	0.2697	11	0.3930	7
Viridiflorene	0.5939	9	–0.2320	9
Retained information (%)	54.88		24.53	
Accumulated information	54.88		79.41	

^z Ordination of substances as the discriminatory power.

Table 3. Chemical compounds of essential oils present in three leaf stages (E1, E3, and E5) of *Eucalyptus urophylla* × *E. grandis* clones C041A (resistant), VR3748 (susceptible), and TC31 (highly susceptible) to *Austropuccinia psidii*^y

Chemical compound	Clone C041A			Clone VR3748			Clone TC31			Retention index	
	E1	E3	E5	E1	E3	E5	E1	E3	E5	Calculated	Lit. ^z
α-pinene	20.62	19.88	20.24	33.55	39.64	41.31	15.33	17.29	18.08	932	939
α-phellandrene	2.88	4.41	2.77	2.97	1.44	1.13	0.81	0.67	0.49	1004	1005
Ortho-cymene	0.96	1.35	1.79	0.83	0.49	0.43	3.89	3.28	3.17	1021	1022
Limonene	29.37	33.87	33.22	4.77	4.65	4.16	2.94	3.45	3.53	1027	1031
1,8-cineole	18.56	22.0	27.45	35.08	38.97	40.47	21.93	33.91	35.14	1028	1033
cis-β-ocimene	2.19	1.37	0.85	3.80	2.10	1.65	2.93	2.13	1.45	1034	1040
γ-terpinene	0.40	0.51	0.46	0.18	0.21	0.24	15.14	19.31	19.91	1056	1062
Terpinolene	0.88	0.63	0.60	0.97	0.40	0.35	1.77	1.14	0.90	1086	1088
4-terpineol	0.74	0.75	0.90	0.22	0.29	0.34	1.18	1.63	2.04	1172	1177
α-terpineol	5.10	3.54	2.73	4.55	5.54	5.16	3.93	2.35	2.71	1186	1189
α-terpinyl acetate	3.19	3.40	3.21	0.64	0.27	0.18	6.79	5.69	6.03	1345	1350
trans-caryophyllene	1.92	1.22	0.50	1.23	0.67	0.37	1.70	1.08	0.71	1417	1418
Viridiflorene	1.29	0.41	0.28	2.24	0.88	0.52	3.59	1.71	1.04	1493	1493

^y Mean values are given in percentages and were obtained from three replicates.

^z Literature, Adams (2007).

from the peels of several citrus species and volatiles emitted from injured oranges were responsible for stimulating germination of *Penicillium digitatum* conidia, and this was also found to be related to the limonene component of the citrus essential oil (Droby et al. 2008; Eckert et al. 1984; French et al. 1978).

Chemical substances released on the leaf surface can stimulate fungal germination (Parbery and Blakeman 1978). However, the stimulation of fungal germination by volatile compounds present in essential oils depends on their concentration (Droby et al. 2008). Citral strongly stimulated *P. digitatum* germination at concentrations

equivalent to 0.06 to 0.15 ppm but completely inhibited it at higher concentrations (Droby et al. 2008). *P. italicum* germination was stimulated over a wider range of concentrations (from 0.06 to 1.5 ppm) and complete inhibition was not evident until the citral concentration reached 15 ppm (Droby et al. 2008). In in vitro testing, limonene inhibited 100% of *A. psidii* urediniospore germination at concentrations >100 ppm. In the in vivo test, concentrations of limonene >100 ppm caused degenerative changes in the morphology of the urediniospores, teliospores, and paraphyses present in rust pustules, confirming the fungitoxic capacity of limonene when present at higher concentrations. No changes in fungal morphology were observed at lower concentrations of this compound. Essential oils can alter the morphology of a fungus by their fungitoxic action, such as oils from *Aloysia gratissima*, *Cordia verbenacea*, and *Hyptis marruboides* on Asian soybean rust (da Silva et al. 2014b) and on eucalyptus powdery mildew (da Silva et al. 2014c). Our study is the first report showing the degenerative effect of limonene on fungal reproductive structures.

Although the fifth leaf stage of the susceptible and highly susceptible clones had no signs or symptoms of rust, only a low concentration of limonene was found. However, there was a high concentration of essential oil. Little is known about how optimum concentrations, and activity in general, may be affected (additively, synergistically, or negatively) by other compounds present in essential oils (Droby et al. 2008). The increased resistance of these leaves to disease may be linked to a set of interrelated factors, such as the high concentration of essential oil, where compounds at high concentrations may exhibit synergistic effects (Droby et al. 2008), or by physical (Silva et al. 2017; Xavier et al. 2015) or biochemical barriers present in leaves (Boava et al. 2010; Taylor and Mims 1991) as previously reported.

The effect of limonene in the clone resistant to *A. psidii* may also have been synergistically intensified by other leaf components, increasing its inhibitory capacity (Romagnoli et al. 2005; Sharma

Fig. 1. Graphic dispersion of the three *Eucalyptus* clones and the three leaf phenological stages using the two first principal components (Y1 and Y2) for the group of 13 major chemical compounds of the essential oils. C041A, resistant clone; VR3748, susceptible clone; and TC31, highly susceptible clone. E1, E3, and E5 are the first, third, and fifth leaf stages, respectively.

Fig. 2. Percentage of *Austropuccinia psidii* urediniospore germination on agar medium containing different concentrations of (R)-(+)-limonene and (S)-(-)-limonene. Germination percentage was assessed from 200 spores in each of three replicate Petri dishes for each treatment.

and Tripathi 2006). The components of oil from *Melaleuca alternifolia*, except β -myrcene, have antifungal activity and 4-terpineol, α -terpineol, linalool, and α and β -pinene, followed by 1,8-cineole, inhibit the growth of various fungal species (Hammer et al. 2003). D-limonene, cineole, β -myrcene, α -pinene, β -pinene, and camphor exhibited high levels of inhibitory activity against *Botrytis cinerea* (Wilson et al. 1997). The monoterpenes, E-anethole, p-anisaldehyde,

carvacrol, carvone, 1,8-cineole, limonene, myrcene, α -phellandrene, and α -pinene inhibit the germination of the fungi *B. cinerea*, *Monilinia laxa*, *Mucor piriformis*, *Penicillium expansum*, *P. digitatum*, *P. italicum*, and *Rhizopus stolonifer* (Caccioni and Guizzardi 1994). Our in vitro and in vivo tests showed that limonene concentrations above a certain threshold were damaging to *A. psidii* urediniospores, but testing additional compounds present in high concentrations

Fig. 3. Scanning electron microscopy images of *Austropuccinia psidii* pustules on *Eucalyptus* leaves treated with **A**, water containing 1% Tween 20 used as a control, **B**, (S)-(+)-limonene at 1 ppm, **C**, 10 ppm, **D**, 100 ppm, **E**, 1,000 ppm, and **F**, 10,000 ppm. At concentrations above 100 ppm, degeneration was observed in the urediniospore cell wall and collapse of the urediniospores, teliospores, and paraphyses. In the control treatment and at dosages of 1 and 10 ppm, no morphological changes were observed in the pustules of *A. psidii*.

in essential oils such as ortho-cymene, α -pinene, and 1,8-cineole could clarify their roles in resistance and help in developing rapid screening tests for rust resistance. Testing additional clones would also be of great value in validating this technique as a screening test. *Eucalyptus* plants with a high limonene concentration in their essential oils could be used in breeding to obtain rust-resistant genetic material. *E. grandis* is considered to be highly susceptible to *A. psidii*

(Alfenas et al. 2009) and limonene was not present in its essential oil (Estanislau et al. 2001). *E. urophylla*, considered to be resistant to fungi (Alfenas et al. 2009), has limonene as one of the main constituents in its essential oil (Shieh 1998). Because clones C041A, VR3748, and TC31 are derived from the *E. urophylla* $\times$ *E. grandis* cross, the resistant clone may have inherited a higher quantity of limonene from *E. urophylla* than did the susceptible and highly

Fig. 4. Scanning electron microscopy images of *Austropuccinia psidii* pustules on *Eucalyptus* leaves treated with **A**, water containing 1% Tween 20 used as a control, **B**, (R)-(+)-limonene at 1 ppm, **C**, 10 ppm, **D**, 100 ppm, **E**, 1,000 ppm, and **F**, 10,000 ppm. At concentrations above 100 ppm, degeneration was observed in the urediniospore cell wall and collapse of the urediniospores, teliospores, and paraphyses. In the control treatment and at dosages of 1 and 10 ppm, no morphological alteration was observed in the pustules of *A. psidii*.

susceptible clones. The hybrid of *E. grandis* and *E. urophylla* may have increased or decreased concentrations of these compounds and even other embedded constituents that are not present within each of the parental plant species. However, we cannot rule out the hypothesis of synergism of limonene and other compounds present in the essential oil on the resistance of *Eucalyptus* plants to *A. psidii*.

The essential oil composition of the three *Eucalyptus* clones analyzed was similar to that extracted from *E. cinerea* leaves with 1,8-cineole, α -pinene, limonene, and α -terpineol (Franco et al. 2005) and from *E. urophylla* with these compounds plus 4-terpineol and ocimene (Shieh 1998). The γ -terpinene, o-cymene, β -pinene, and 1,8-cineole are the main oil compounds of *E. grandis* leaves (Estanislau et al. 2001). This species, in Australia, has three monoterpenes, α -pinene (22%), 1,8-cineole (6%), and α -terpineol (5%), and β -triketones, leptospermone, and flavosone as its major compounds (Boland et al. 1991), whereas 1,8-cineole, α -pinene, β -pinene, *p*-cymene, α -terpineol, and limonene were in general some of the principal oil compounds for 10 *Eucalyptus* species (*E. alba*, *E. camadulensis*, *Corymbia citriodora*, *E. deglupta*, *E. globulus*, *E. propinqua*, *E. robusta*, *E. saligna*, *E. tereticornis*, and *E. urophylla*) (Cimanga et al. 2002).

Differences in the essential oil compounds and yield from *E. urophylla* $\times$ *E. grandis* are also important in identifying leaf pairs useful for oil extraction. Knowing the variations in concentration of chemical compounds in essential oil by clone is important to the various purposes of using these oils.

This research identified the variability in the quantity and quality of the essential oils of three *Eucalyptus* clones based on their genetic material and leaf stage. These findings may be associated, in conjunction with other factors, with resistance to *A. psidii* in the three hybrid *E. urophylla* $\times$ *E. grandis* clones and their leaf phenological stages. This study provides evidence that limonene is fungitoxic to *A. psidii* urediniospores and, when found at high percentages in *Eucalyptus* leaves, is associated with rust resistance. This is the first work to demonstrate the relationship between the amount of limonene and essential oils in *Eucalyptus* leaves with rust resistance and the age-related resistance of their leaves. This biochemical marker may be important in identifying rust-resistant plant material in a genetic improvement program to develop *Eucalyptus* plants resistant to this major disease of *Eucalyptus* species.

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