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# A Robust Disease Scoring Method to Screen *Eucalyptus* for Resistance Against the Aggressive Leaf Pathogen *Teratosphaeria destructans*

**Myriam Solís,[1] Almuth Hammerbacher,[2] Michael J. Wingfield,[1] and Sanushka Naidoo[1,†]**

[1] Department of Biochemistry, Genetics and Microbiology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0028, South Africa
[2] Department of Zoology and Entomology, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria 0028, South Africa

## Abstract

Shoot and leaf blight caused by *Teratosphaeria destructans* is one of the most devastating foliar diseases on *Eucalyptus*. Therefore, breeding for resistance to this disease is considered urgent. Differences in susceptibility to *T. destructans* have been observed in the field but a robust inoculation protocol has, until recently, been unavailable and a disease scoring method for precise phenotyping has not been established. A first objective of this study was to determine the optimal conidial concentration for *T. destructans* inoculations on a susceptible *Eucalyptus* host. This concentration was then used to determine differences in susceptibility of six genotypes of *Eucalyptus grandis* × *E. urophylla* to the pathogen by assessing the percentage of infected stomata using electron microscopy and the percentage of leaf area covered by lesions (PLACL) using image processing. In addition, we developed a disease susceptibility index (SI) of six categories ranging from highly resistant (SI = 0) to highly susceptible (SI = 1.5 to 2). The more resistant genotypes were moderately resistant, with an SI value of 0.49 to 0.54 and a PLACL of 6.5 to 9%. In contrast, the more susceptible genotype scored an SI of 1.52 and PLACL of 48%. Host susceptibility was also assessed relative to the sporulation of the pathogen. This showed that the percentage of sporulation was not significantly correlated with host resistance. Overall, the results provide the basis for rigorous screening and selection of resistant genotypes to the disease caused by *T. destructans* using artificial inoculation.

*Keywords*: *Eucalyptus*, phenotyping, screening, *Teratosphaeria* leaf blight

*Eucalyptus* spp. are widely grown in plantations, due to their rapid growth and adaptability to different environmental and edaphic conditions (Coppen 2002). These traits have been sustained and promoted by traditional tree breeding programs to improve growth, wood quality, and resistance to biotic and abiotic stresses (Namkoong et al. 1980; White and Hodge 1988). However, pests and pathogens have increasingly emerged as serious threats to *Eucalyptus* productivity (Burgess and Wingfield 2017; Hurley et al. 2016; Wingfield et al. 2001, 2008).

Hybrids of *Eucalyptus* have been developed in many parts of the world to increase productivity and disease resistance (Assis 2011; Jacovelli 2009; Stanger et al. 2011). For example, in South Africa from the 1980s, *Eucalyptus grandis* × *E. urophylla* hybrids have been used as alternatives to planting pure *E. grandis* genotypes (Gardner 2001). One of the main objectives of this approach has been to exploit the disease resistance displayed by *E. urophylla* to canker diseases such those caused by *Chrysoporthe* spp. (Alfenas et al. 1983; Hodges et al. 1976; Wingfield 2003) and *Coniothyrium zuluense* (Cortinas et al. 2010) and to combine these traits with the rapid growth of *E. grandis* (Gardner 2001). However, since the commercialization of *E. grandis* × *E. urophylla* hybrids in South Africa, threatening new diseases have been reported in the country, including Ceratocystis wilt caused by *Ceratocystis eucalypticola* (Roux et al. 2020) and Teratosphaeria leaf blight (TLB) caused by *Teratosphaeria destructans* (Greyling et al. 2016).

Typical symptoms of the TLB disease include shoot and bud blight as well as leaf deformation (Andjic et al. 2019; Burgess et al. 2007; Wingfield et al. 1996). This destructive disease was first encountered in Indonesia (Wingfield et al. 1996) and spread rapidly through plantations in southeast Asia (Barber et al. 2012; Burgess et al. 2006; Havenga et al. 2021; Old et al. 2003). The first report outside southeast Asia was in the Zululand region of South Africa, where the disease was reported on *E. grandis* × *E. urophylla* hybrids (Greyling et al. 2016).

Successful marker-assisted breeding strategies for resistance to pathogens are based on robust phenotyping methods to identify resistant genotypes. Such assessments require a reproducible artificial inoculation protocol and a rigorous disease scoring method. Such methods have been successfully used for Eucalyptus diseases, including rust caused by *Austropuccinia psidii* (Junghans et al. 2003; Lee et al. 2015), Ceratocystis wilt caused by *C. fimbriata* sensu lato (Harrington et al. 2015; Mafia et al. 2011), and the canker pathogens *Chrysoporthe cubensis* (Chen et al. 2010; Guimaraes and Stotz 2004; Juárez-Palacios et al. 2013) and *C. austroafricana* (Van Heerden and Wingfield 2002; Van Heerden et al. 2005).

An artificial inoculation protocol for *T. destructans* was recently developed and this showed that the establishment of the pathogen on a susceptible host is initiated by stomatal penetration 48 h after inoculation (hai), followed by a biotrophic phase of intercellular growth of 2 to 3 weeks preceding the appearance of symptoms (Solís et al. 2022). Successful infection requires a high levels of humidity (approximately 100%) within the first 72 hai, and an optimal temperature of 25°C (Solís et al. 2022). However, the optimal concentration of inoculum necessary for disease development in artificial inoculations is still unknown. Due to the variety of symptoms, including leaf spot, leaf blight, and leaf deformation, displayed by hosts with different levels of susceptibility to the pathogen, a precise phenotyping method is required to assist breeding programs.

Observations in the area where *T. destructans* has appeared in South Africa suggest that there are differences in susceptibility

†Corresponding author: S. Naidoo; sanushka.naidoo@fabi.up.ac.za

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among clones in the field (Greyling et al. 2016). However, the performance of different host material under disease pressure has not been quantitatively assessed. The aim of this study was to develop a rigorous screening protocol to assess resistance in *E. grandis* × *E. urophylla* clones to infection by *T. destructans*. Because it was shown in previous studies that rust disease development is dependent on the inoculum concentration (Ireland and Pegg 2020), we assessed the effect of different *T. destructans* spore concentrations on symptom development in a susceptible host. After determining the optimal spore concertation for artificial inoculations, six *E. grandis* × *E. urophylla* clones were inoculated with the pathogen and a reliable method to evaluate their susceptibility to *T. destructans* was developed.

## Materials and Methods

### Determination of optimal conidial concentration to differentiate susceptible and resistant *Eucalyptus* genotypes

A genotype of *E. grandis* (G1), known to be susceptible to *T. destructans*, was used to determine the optimal conidial suspension concentration for successful pathogen infection and symptom development. Forty 1-year-old ramets of this genotype were planted in 5-liter black polyethylene bags containing Culterra potting soil mix (Culterra, Gauteng, South Africa). Plants were maintained for 3 months in a greenhouse under natural light, with an average daily temperature ranging from 20 to 25°C and an average night temperature of 20°C. A conidial suspension of *T. destructans* was prepared from pure cultures of *T. destructans* isolate CMW56797, grown on 2% malt extract agar (malt extract at 20 g/liter and agar at 20 g/liter; Biolab) and incubated for 3 weeks at 25°C in the dark. The suspension was prepared by washing the plates with 20 ml of sterile distilled water + 0.01% Tween 20 (Sigma-Aldrich, Taufkirchen, Germany). The suspension was then filtered through a double layer of sterile gauze and adjusted using a hemocytometer to yield seven different concentrations (i.e., $1 \times 10^2$, $1 \times 10^3$, $1 \times 10^4$, $1 \times 10^5$, $1 \times 10^6$, $1 \times 10^7$, and $1 \times 10^8$ conidia $ml^{-1}$). Four healthy plants were inoculated with each conidial concentration. The six youngest fully expanded leaves on the plants were sprayed until run-off with the conidial suspensions to cover both leaf surfaces, following the protocol described by Solís et al. (2022). Plants were enclosed for 72 h in clear plastic bags to maintain high humidity levels. Control plants were sprayed with sterile distilled water + 0.01% Tween 20 (Sigma-Aldrich). The plants were maintained in a greenhouse under natural light at a temperature ranging from 20 to 25°C.

Plants were evaluated for symptom development daily over a period of 35 days. Leaves showing typical chlorotic lesions with oily diffuse borders were harvested. Images of the leaves were captured using an Epson Perfection V700 flatbed scanner, using a resolution of 1,200 dots per inch (dpi). The percentage of leaf area covered by lesions (PLACL) was calculated using the open-source image processing software ImageJ (Ferreira and Rasband 2012), which been used previously to quantified the leaf area damage for different diseases such as southern corn leaf blight (Kumar et al. 2016; Zhang et al. 2022), bacterial blight disease on rice (Akbari et al. 2019), and early blight on potato caused by *Alternaria solani* (Ding et al. 2019). The color threshold parameters (hue, saturation, and brightness) were adjusted to separate the symptomatic tissue from the healthy tissue to quantify the pixels from the total leaf area, following the protocol described by Pride et al. (2020).

### Inoculation of GU genotypes to evaluate their susceptibility to *T. destructans* infection

Six different genotypes (GU1 to GU6) of 1-year-old *E. grandis* × *E. urophylla* hybrid clones provided by SAPPI Forests, South Africa, were used for susceptibility screening. The known susceptible genotype G1 used in the previous experiment was included as a control. In all, 20 ramets of each *E. grandis* × *E. urophylla* genotype and 10 ramets of G1 were planted and maintained as described above. Four healthy plants per genotype were selected as replicates for the inoculation trial with *T. destructans* (CMW56797). The conidial suspension and the inoculation were performed as described above. The 12 youngest fully expanded leaves per plant were inoculated by spraying both leaf surfaces until run-off. Control plants were sprayed with sterile distilled water + 0.01% Tween 20 (Sigma-Aldrich). The plants were maintained in a greenhouse under natural light with a temperature ranging from 20 to 25°C and were monitored for symptom development over an 8-week-period, and the number of leaves with symptoms was recorded every day.

**Percentage of infected stomata.** Three leaves per plant were harvested from each of the inoculated plants at 72 hai in order to determine the ability of the pathogen to infect. In addition, three leaves were harvested at 35 days postinoculation to determine the level of sporulation on plants having different levels of susceptibility, using scanning electron microscopy (SEM) and light microscopy to quantify the spore concentration. To determine the infection per genotype, the percentage of infected stomata per area was estimated using 12 leaves per genotype. The samples were cut into equal 1-$cm^2$ squares and placed in 2.5% glutaraldehyde/formaldehyde (50% vol/vol) for 24 h. These were dehydrated using a graded (30, 50, 70, and 90%) ethanol series for 15 min each, followed by four steps of dehydration in 100% ethanol, 15 min each, and 30 min for the final dehydration step. Samples were placed in hexamethyldisilazane and mounted on aluminum stubs, exposing the abaxial leaf surface. The samples were then coated with carbon using a Quorum Q150T Coating Unit (Quorum Emitech, London, U.K.). The samples were visualized under a Zeiss 540 Gemini Ultra Plus FEG SEM (Zeiss, Oberkochen, Germany) at the Laboratory for Microscopy and Microanalysis, University of Pretoria, Pretoria, South Africa.

**PLACL and susceptibility index.** To assess the disease susceptibility, four leaves per plant were harvested when the symptoms were evident at 4 weeks postinoculation. Images of the leaves were captured using an Epson Perfection V700 flatbed scanner and the PLACL values were calculated in the open-source image processing software ImageJ as described above. The susceptibility index (SI) was based on the proportion of leaves with each symptom type observed relative to the total number of leaves per tree (Fig. 1A). The 16 leaves per genotype, including the susceptible G1 genotype, were classified based on the observed symptoms: leaf spot (S), leaf blight (B), and leaf deformation (D) (Fig. 1A). To calculate the SI, the observed symptoms were weighted as follows: a weight of 2 was assigned to leaves displaying leaf deformation, because this symptom has a greater impact on the leaf physiology and early defoliation; and a weight of 1.5 was assigned to leaf blight symptoms, based on the greater degree of colonization by the pathogen in the leaf compared with the leaf spot, which was assigned a weight of 1, which indicated a defense host mechanism to restrict pathogen colonization. The equation used to calculate the SI value was the sum of the frequency of each symptom within a genotype, multiplied by its designated weight of importance divided by the total number of leaves (Fig. 1B). Subsequently, the SI values were used to rank host reaction by adopting the disease rating scale developed for rice bacterial leaf blight disease (Khan et al. 2015). The genotypes were classified as highly resistant (SI = 0), resistant (SI = 0.01 to 0.2), moderately resistant (SI = 0.21 to 0.6), moderately susceptible (SI = 0.61 to 1), susceptible (SI = 1.01 to 1.5) and highly susceptible (SI = 1.5 to 2; Fig. 1B).

### Sporulation on hosts with different levels of susceptibility

The degree of sporulation was determined by the number of spores per leaf area on each *Eucalyptus* genotype. To quantify the sporulation, 12 leaves per genotype were harvested at 35 days after inoculation and spores were scraped with a spatula into 1 ml of sterile distilled water in a 1.5-ml Eppendorf tube and agitated to release the conidia. The concentration of conidia was quantified using a hemocytometer. Conidial viability was evaluated by spreading 100 µl of the suspension onto the surface of potato dextrose agar in Petri dishes. The plates were then incubated at 25°C for 72 h. The conidial viability was quantified by counting the number of germinated conidia per 100 plated conidia. Germinated conidia were identified

by a germ tube that was clearly visible under ×20 magnification using an Axioskop 2 plus microscope (Zeiss).

### Data analyses

A completely randomized design was used for all of the experiments. All statistical analyses were performed using R version 3.5.1 (R Core Team 2020). Data manipulation, Spearman's rank correlation, and analysis were supported by the R software packages dplyr (Wickham et al. 2020) and agricolae (de Mendiburu 2013). To normalize the distributions of the residuals, the data for the PLACL values, infection, and sporulation were square-root transformed and all figures show untransformed data. The data were analyzed statistically for each assay using one-way analysis of variance test. Tukey's post hoc test was used to identify significant differences between treatments ($\alpha = 0.05$).

## Results

### Determination of optimal conidial concentration for differentiating susceptible and resistant *Eucalyptus* genotypes

Different concentrations of fungal inoculum were tested in order to determine the minimum conidial concentration required for the disease development in a susceptible genotype. Symptoms on the known susceptible genotype G1 were not visible at an inoculum concentration of $1 \times 10^2$ (Fig. 2). When the concentration was adjusted to $1 \times 10^6$, the damaged area increased significatively to 40% and did not continue to increase even at the highest concentration of $1 \times 10^8$ (Fig. 2). Based on this result on a highly susceptible host, a conidial concentration of $1 \times 10^6$ was used in the following experiments.

### Susceptibility of genotypes to infection

**Percentage of infected stomata.** The percentage of infected stomata visualized using electron microscopy (Fig. 3A and B) was significantly different among the evaluated genotypes (Fig. 3C). A greater percentage of infected stomata was recorded for the genotypes GU4 (12.7%) and GU5 (12.5%; Fig. 3C). In contrast, the percentage of infection was significantly lower in the genotype GU1 (3.7%) ($P = 0.0081$; Fig. 3A and C).

**PLACL and its correlation with pathogen infection.** PLACL values were determined by quantifying the leaf area affected by the pathogen using image analysis. The PLACL values were significantly

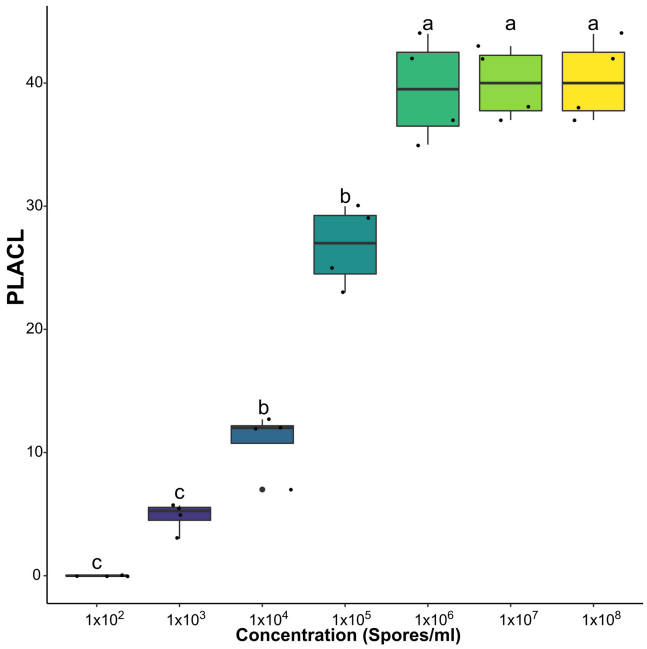
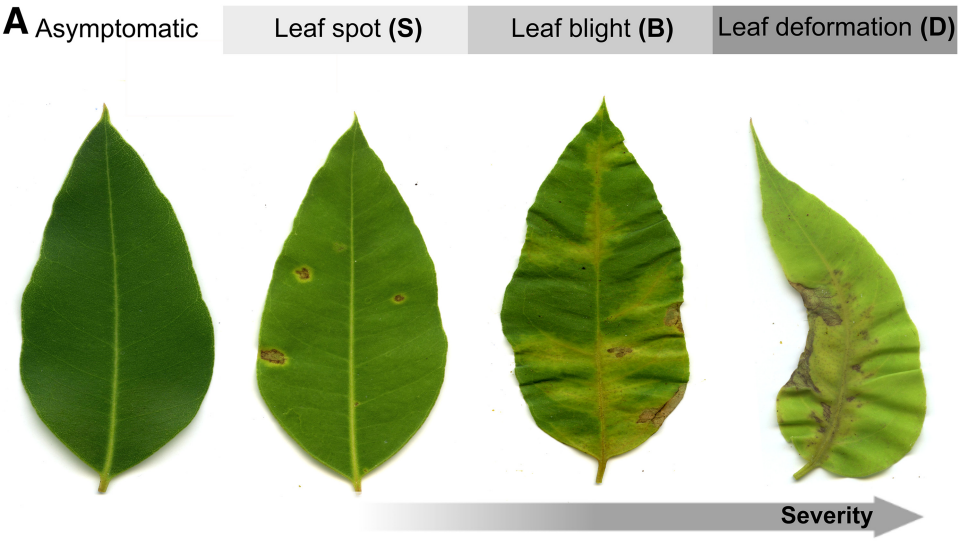



**Fig. 2.** Percentage of leaf area covered by lesions (PLACL) on *Eucalyptus grandis* G1 genotype using seven different inoculum concentrations of *Teratosphaeria destructans* estimated at 35 days after inoculation. PLACL was estimated using ImageJ; $N = 4$. Different letters above error bars (i.e., ± standard error) indicate significant differences ($P < 0.001$) among groups (Tukey's post hoc test).




**B**

$$SI = \frac{(nS) + 1.5(nB) + 2(nD)}{N}$$

***n*:** Number of leaves with symptoms
**N:** Total number of leaves

| SI | Description |
|---|---|
| 0 | Highly Resistant **(HR)** |
| 0.01 - 0.2 | Resistant **(R)** |
| 0.21 - 0.5 | Moderately Resistant **(MR)** |
| 0.51 - 1 | Moderately Susceptible **(MS)** |
| 1.01 - 1.5 | Susceptible **(S)** |
| 1.51 - 2 | Highly Susceptible **(HS)** |

**Fig. 1.** Symptoms visualized on Eucalyptus leaves, used to calculate the disease susceptibility index (SI). **A,** Typical symptoms observed on *Eucalyptus grandis* × *E. urophylla* genotypes inoculated with *Teratosphaeria destructans*. The arrow indicates the increase in severity; **B,** Formula to calculate the SI and the disease scale for determining level of resistance or susceptibility of each genotype against *T. destructans*. HR = highly resistant, R = resistant, MR = moderately resistant, MS = moderately susceptible, S = susceptible, and HS = highly susceptible.

different between the evaluated genotypes at 35 days after inoculation ($P < 0.05$) (Fig. 3D). The PLACL value was significantly higher for GU4, with a value of 48%, in comparison with the others genotypes evaluated (Fig. 3D). In contrast, the genotypes GU1 and GU3 showed significantly less damage compared with the other genotypes, with PLACL values of 5 and 6.5%, respectively (Fig. 3D). The percentage of stomatal infection and PLACL values were significantly positively correlated ($r = 0.71$, $P = 0.00011$; Fig. 3E). This result indicates that the genotypes with a higher number of infected stomata also displayed a greater area covered by lesions (Fig. 3E). There were significant

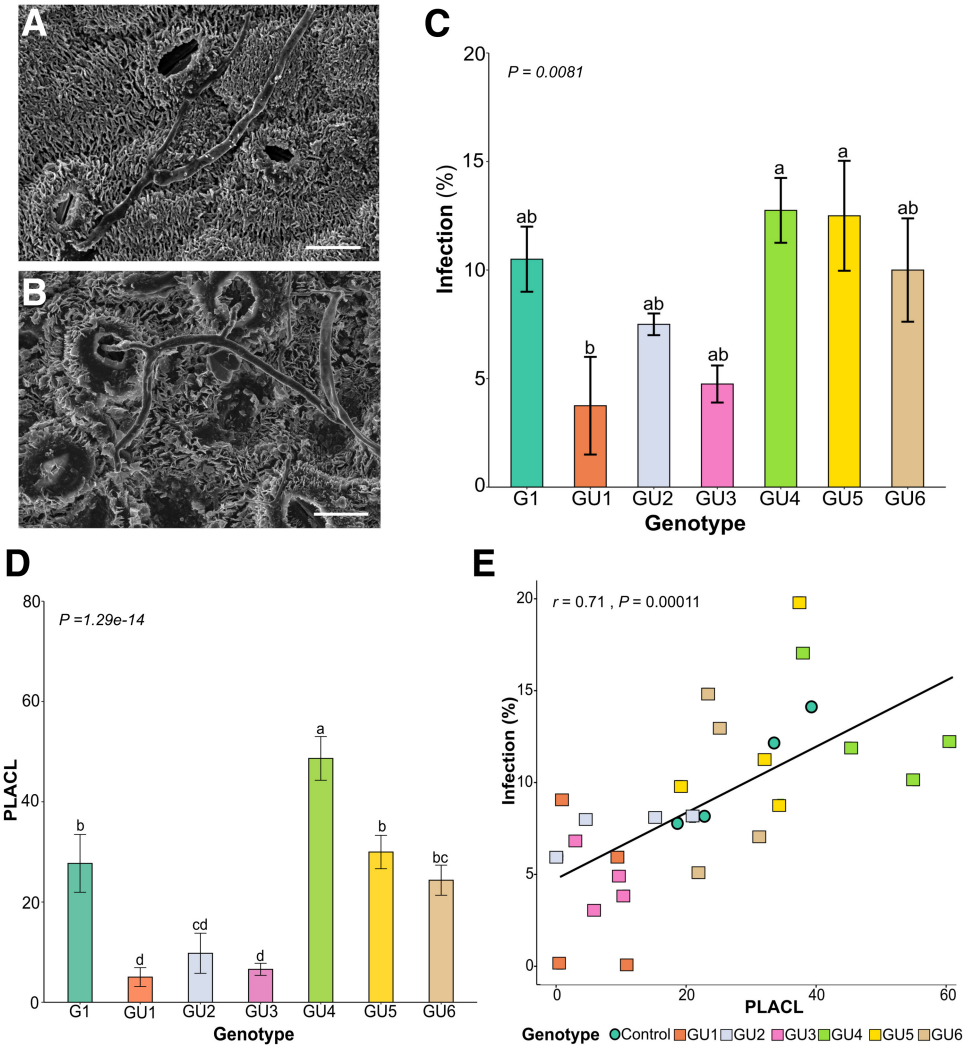



**Fig. 3.** Susceptibility of *Eucalyptus grandis* × *E. urophylla* genotypes to *Teratosphaeria destructans* infection. **A,** Scanning electron microscopy (SEM) of GU3 at 72 h after inoculation with *T. destructans*. **B,** SEM of GU4 at 72 h after inoculation showing the infection via stomata by *T. destructans*. **C,** Percentage of infected stomata by *T. destructans* on six genotypes of *E. grandis* × *E. urophylla* (GU1, GU2, GU3, GU4, GU5, and GU6) and in the control, *E. grandis* (G1). **D,** Percentage of leaf area covered by lesions (PLACL) for six genotypes of *E. grandis* × *E. urophylla* (GU) and the control, *E. grandis* (G1). **E,** Spearman's rank correlation between percentage of infection and PLACL. Data from four independent biological replicates. Different letters at the tops of the bars indicate statistically significant differences between genotypes evaluated using R software by performing one-way analysis of variance and posterior Tukey's test ($P < 0.05$). Error bars indicate standard error of the mean. Scale bar = 20 μm (A and B).

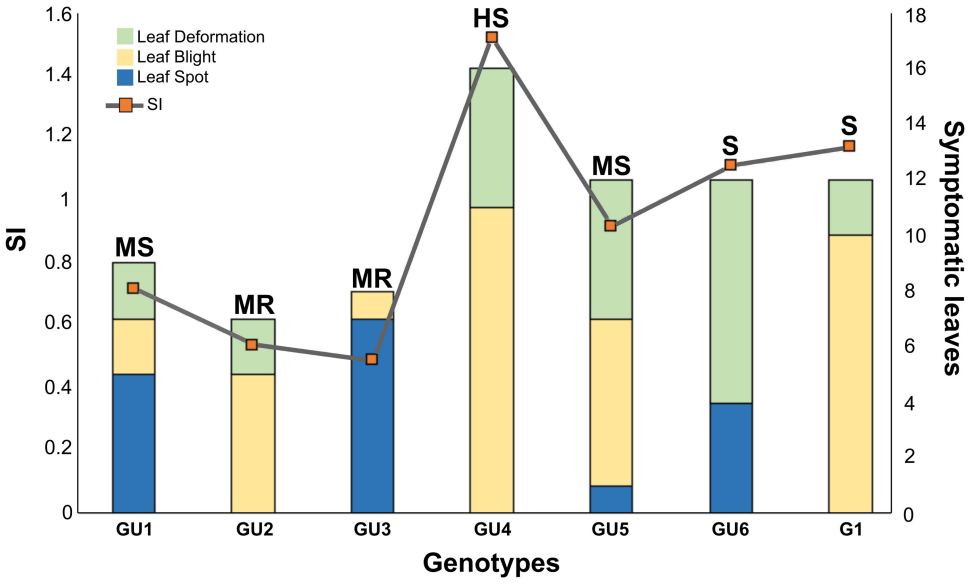



**Fig. 4.** Susceptibility index (SI) (main vertical axis) and number of leaves with symptoms (secondary vertical axis) of six genotypes of *Eucalyptus grandis* × *E. urophylla* and the control, *E. grandis* (G1), evaluated at 35 days after inoculation with *Teratosphaeria destructans*. Host categorization at the top of the bars: HR = highly resistant, R = resistant, MR = moderately resistant, MS = moderately susceptible, S = susceptible, and HS = highly susceptible; $N = 16$.

differences in days to symptom appearance in genotypes with different PLACL values (likelihood ratio = 47.4, degrees of freedom = 6, $P$ = 1.555e–08, $n$ = 812; Supplementary Fig. S1). Symptoms appeared earlier in genotypes with the highest PLACL values in comparison with those with less area covered by damage (Supplementary Fig. S1). This suggested a rapid colonization by the pathogen on the leaves in susceptible hosts.

**SI.** The formula and scoring scale developed based on the nature of the symptoms displayed by the infected leaves showed that the genotypes with the highest percentage of infected stomata and leaf area covered by lesions showed high SI values (Supplementary Fig. S2). The susceptible G1 genotype, with an SI value of 1.11, was categorized as susceptible. A similar value was obtained for GU6 (SI = 1.17), which was also classified as a susceptible host (Fig. 4; Supplementary Fig. S3). The genotype GU4 attained the highest SI value (SI = 1.52) and was classified as highly susceptible (Fig. 4; Supplementary Fig. S3). GU1 and GU5 were classified as moderately susceptible, with SI values of 0.72 and 0.92, respectively (Fig. 4; Supplementary Fig. S3). The lowest SIs were calculated for GU3 (SI = 0.49) and GU2 (SI = 0.54), which were classified as moderately resistant according to the predetermined scale (Fig. 4; Supplementary Fig. S3).

**Sporulation on hosts with different levels of susceptibility.** The sporulation was significatively higher in the moderately susceptible genotype, GU1 (90 spores/mm$^2$), compared with the other genotypes evaluated (Fig. 5A). The susceptible genotype G1 used as a control in this study produced 36 spores/mm$^2$ and the susceptible genotype GU6 produced 20 spores/mm$^2$ (Fig. 5A); similar levels of sporulation were shown in the moderately resistant genotypes GU3 (26 spores/mm$^2$) and GU2 (15 spores/mm$^2$) (Fig. 5A). In the highly susceptible genotype GU4, the level of sporulation was 69 spores/mm$^2$ and, interestingly, no sporulation was observed in the infected tissues of the moderately susceptible genotype GU5 (Fig. 5A). The level of sporulation did not provide a reliable correlation with the host susceptibility and was poorly correlated with the PLACL values ($r$ = 0.12, $P$ = 0.54) (Fig. 5B).

## Discussion

Tree breeding programs require reliable and precise phenotyping methods for the selection of genotypes with resistance to pathogens. Recently we developed an artificial inoculation method to screen *Eucalyptus* spp. for resistance to *T. destructans* (Solís et al. 2022). However, for this to be useful for quantitative studies, an accurate assignment of resistance and susceptibility needed to be achieved. In this study, we used various criteria to score disease susceptibility and resistance to *T. destructans*. We evaluated the number of infected stomata and correlated this to the PLACL. From these results, we developed a novel disease index to assign genotypes to specific susceptibility or resistance groups. Among all of the tested variables, sporulation of the pathogen, a characteristic rarely included in previous studies of this nature, gave the poorest reflection of resistance and did not correlate with the other variables tested.

When the percentage of infected stomata was evaluated on the different *Eucalyptus* genotypes, a higher percentage of infected stomata was observed in the hosts that were rated susceptible using PLACL or the disease index. In contrast, the hosts categorized as moderately resistant had a significantly lower percentage of infected stomata. The correlation between fungal infection of the stomata and susceptibility have been reported in previous studies. For example, for downy mildew caused by *Plasmopara viticola*, which infects through stomata on grapevine, the most susceptible hosts had the highest percentage of infected stomata (Boso Alonso and Kassemeyer 2008). Infection through stomata and host resistance have also been related to the presence of preformed barriers such as extensive wax covering the stomata. This has been reported for rust on *Hordeum chilense* (Rubiales and Niks 1996; Vaz Patto et al. 2003) and for *T. nubilosa* on *Eucalyptus* (Smith et al. 2018). The role of preformed barriers to infection by *T. destructans* are presently not known but the pathogen was observed to degrade epicuticular waxes in a compatible interaction with *Eucalyptus* spp. (Solís et al. 2022).

After assessing the percentage of infected stomata, two complementary approaches were used to develop a reliable disease screening protocol. These included the PLACL and an SI. PLACL values have previously been used as a disease severity index to indicate the damage caused by a pathogen on the host, mainly for foliar pathogens such as *Zymoseptoria tritici* (Karisto et al. 2018; Stewart et al. 2018; Zhan et al. 2005). Although this method provides accurate results, it is more laborious than using scoring scales (Stewart et al. 2018). For this reason, we developed an SI based on the main

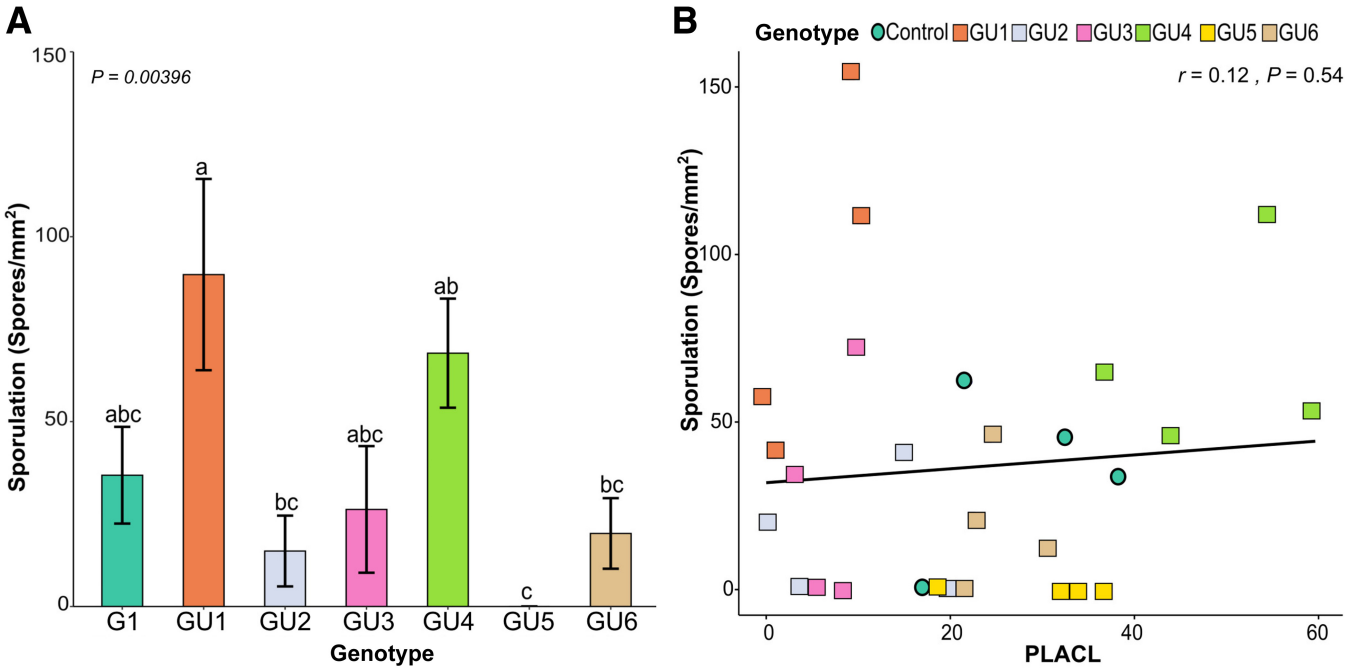



**Fig. 5.** Sporulation by *Teratosphaeria destructans* in *Eucalyptus* genotypes. **A,** Sporulation (spores per square millimeter) of *T. destructans* on six genotypes of *Eucalyptus grandis* × *E. urophylla* and the control, *E. grandis* (G1). **B,** Spearman's rank correlation between sporulation (spores per square millimeter) and percentage of leaf area covered by lesions (PLACL). Data from four independent biological replicates. Different letters at the tops of the bars indicate statistically significant differences between genotypes evaluated by Tukey's test ($P$ < 0.05). Error bars indicate standard error of the mean.

symptoms caused by *T. destructans* on *Eucalyptus*. The SI was significatively correlated with the PLACL values and, as a consequence, represents a reliable and rapid method to categorize the host reaction.

Symptoms appeared earlier on the susceptible genotypes, from 10 days after inoculation. In contrast, in genotypes with some degree of resistance, the symptoms were visualized approximately 20 days after inoculation. Later symptom appearance in moderately resistant genotypes could be related to physiological and molecular responses of the genotypes to restrict the pathogen colonization once infection has occurred. For example, at the cellular level, mesophyll cells may swell (hypertrophy) or divide (hyperplasia) in response to infection. These processes create a barrier termed "necrophylactic periderm" in leaves (Fink 1999). This form of barrier has been reported for *Eucalyptus* spp. in response to infection by *T. nubilosa*, *T. cryptica*, *T. parva*, and *T. molleriana*, containing lignin and suberin deposition (Smith et al. 2007). Differences in susceptibility to *T. nubilosa* have also been related to the tightness of cell packing in the palisade layer, where resistant genotypes had significantly less airspace between cells (Smith et al. 2018).

When *T. destructans* sporulation on hosts with different levels of susceptibility was assessed, there was no significant correlation with PLACL values. Some studies have shown the relation between the pathogen sporulation to the availability and acquisition of nutrients from the plant host (Keon et al. 2005). In the present study, differences in nutrient availability between the evaluated genotypes could explain the low correlation with resistance and the level of pathogen sporulation, as well as intrinsic physicochemical differences in the genotypes and different timelines of sporulation in each host. In this study, we conclude that sporulation is not a strong predictor for resistance to *T. destructans* under controlled conditions.

The previously reported lack of genetic variability in the *T. destructans* pathogen (Havenga et al. 2020) supports the use of a single isolate for screening, as was done in this study. However, this factor must be continuously monitored because other genotypes of the pathogen may appear in South Africa and in other countries. The screening method reported in this study allows researchers to test isolates with different genetic background and degrees of aggressiveness. This will help to accurately identify resistant hosts and also paves the way for identifying the genetic resistance mechanisms against *T. destructans*.

In this study, a reliable pipeline has been established for phenotyping of *Eucalyptus* susceptibility to one of its most aggressive foliar diseases. The results suggest that it is possible to use artificial inoculation under controlled conditions to select *Eucalyptus* genotypes that are resistant to *T. destructans*. This will contribute to reduce the time needed to select individuals for breeding programs which, at present, rely purely on field observations. Additionally, this protocol overcomes issues around disease escapes and variable inoculum levels experienced in field trials.

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