Plant Disease • 2022 • 106:1944-1951 • https://doi.org/10.1094/PDIS-09-21-1877-RE

Comparison of the Infection Biology of *Teratosphaeria destructans* and *Teratosphaeria epicoccoides* on *Eucalyptus*

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Abstract

Leaf blight caused by *Teratosphaeria destructans* is one of the most important diseases of *Eucalyptus* planted in the subtropics and tropics. In contrast, the better-known *T. epicoccoides*, though also a primary pathogen of *Eucalyptus*, causes less damage to trees in these areas. Although *T. destructans* is an aggressive pathogen, nothing is known about its infection biology. In this study, the conditions for infection and disease development caused by *T. destructans* and *T. epicoccoides* were evaluated and compared on a *Eucalyptus grandis* × *E. urophylla* hybrid clone. The optimal temperature for germination ranged from 25 to 30°C for *T. destructans* and 15 to 20°C for *T. epicoccoides*. The germination of these pathogens was favored under conditions of light and high levels of RH. Penetration by *T. destructans* and *T. epicoccoides* occurred via stomata, and the hyphae colonized the intercellular spaces of infected leaves. Symptoms were clearly visible 3 weeks after inoculation by both pathogens, and reproductive structures started to develop in substomatal cavities at 4 weeks after inoculation. The results of this study will facilitate the establishment of rapid screening trials based on artificial inoculations aimed at reducing the impact of disease caused by *T. destructans.*

Keywords: *Eucalyptus*, infection biology, Teratosphaeria leaf blight, Teratosphaeria leaf spot

The most common pathogens associated with *Eucalyptus* leaf diseases are members of the Mycosphaerellaceae and Teratosphaeriaceae (Andjic et al. 2019; Carnegie 2007; Crous et al. 2007, 1998; Slippers et al. 2004). The genus *Teratosphaeria* includes a group of related species that cause a disease known as Teratosphaeria leaf blight (TLB). Some species can cause severe defoliation, which is particularly problematic in plantations where nonnative *Eucalyptus* spp. encounter accidental introductions of these fungi (Andjic et al. 2019; Burgess and Wingfield 2017; Wingfield et al. 2015, 2008).

Teratosphaeria species differ in their biology and relative aggressiveness toward their hosts. For example, *Teratosphaeria destructans*, *T. eucalypti*, *T. nubilosa*, and *T. pseudoeucalypti* are aggressive primary pathogens that infect young leaves and shoots and cause TLB (Andjic et al. 2019; Burgess and Wingfield 2017; Wingfield et al. 2015, 2008). In contrast, *T. epicoccoides* is commonly found on older *Eucalyptus* foliage, causing minor damage (Andjic et al. 2019; Carnegie 2007).

T. destructans is one of the most aggressive species causing TLB (Andjic et al. 2019). The first discovery and subsequent description of this pathogen were in North Sumatra, where it caused serious leaf and shoot blight in young plantations of *Eucalyptus grandis* (Wingfield et al. 1996). The pathogen was subsequently reported in tropical and subtropical areas of Southeast Asia (Burgess et al. 2006; Old et al. 2003). In 2015, it was found for the first time outside Asia on *E. grandis* × *E. urophylla* hybrids in South Africa (Greyling et al. 2016). The origin of this pathogen is unknown, but a recent population genetic study has revealed low genotypic diversity in isolates from Indonesia, China, Thailand, Vietnam, and a clonal population in South Africa (Havenga et al. 2020a, b). Both mating types of this heterothallic fungus have been found in Malaysia (Havenga et al. 2021), but structures linked to a sexual state have never been discovered, implying that infections are driven solely via asexual spores.

T. epicoccoides is known to be endemic to eastern Australia (Hansford 1957; Heather 1961; Taole et al. 2015; Walker et al. 1992), and it was later introduced to Western Australia (Jackson et al. 2008; Taole et al. 2015). This pathogen is the most widespread of all *Teratosphaeria* species, occurring in temperate areas where *Eucalyptus* spp. are planted (Park et al. 2000). It is generally considered to be only a mildly aggressive pathogen (Crous et al. 1988; Knipscheer et al. 1990; Old et al. 2003). However, when inoculum levels are high and plantations are established on marginal land or are poorly managed, *T. epicoccoides* can cause severe damage, resulting in defoliation and death of trees (Carnegie 2007; Knipscheer et al. 1990).

Knowledge of the infection biology of *Teratosphaeria* spp. on *Eucalyptus* comes mainly from studies on *T. cryptica* and *T. nubilosa* (Keane 2000; Park 1988; Park and Keane 1984, 1982). *T. cryptica* infects leaves directly through the leaf cuticle, whereas *T. nubilosa* establishes infections via stomata (Park and Keane 1982). A single study by Heather (1965) considered the infection biology for *T. epicoccoides* on *E. bicostata* and *E. tereticonis* and showed that it infects leaves via the stomata and that symptoms appear from 30 to 35 days after inoculation. In contrast, there have been no studies to elucidate the conditions necessary for infection by *T. destructans*. This knowledge gap precludes investigation of the molecular basis for infection and disease tolerance, which will also influence disease management.

The aim of this study was to determine the conditions necessary for infection by *T. destructans*. To achieve this, we compared this aggressive pathogen with the less aggressive *T. epicoccoides* on the same host. These studies considered the effects of temperature, RH, and light on spore germination and included various microscopic techniques to visualize host penetration and disease development on a susceptible *Eucalyptus* hybrid clone.

Materials and Methods

Fungal material. Isolations of *T. destructans* and *T. epicoccoides* were made from leaves of *E. grandis* × *E. urophylla* clones collected in the KwaZulu Natal region of South Africa. We made conidial

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Funding: This work was supported by Mondi Group, Tree Protection Co-operative Programme, Forestry South Africa, and the DST/NRF Centre of Excellence in Plant Health Biotechnology, South Africa, and by the Chilean Doctoral Fellowship Programme of the National Agency for Research and Development/Scholarship Program/DOCTORADO BECAS CHILE/2019–72200511, awarded to the first author.

The author(s) declare no conflict of interest.

Accepted for publication 3 December 2021.

isolates for each pathogen by collecting conidial masses exuding from the substomatal pycnidia with a sterile needle and transferring them to an Eppendorf tube containing 1 ml of sterile distilled water (SDW) and 0.01% Tween. An aliquot of 200 µl of the spore suspension was then spread onto the agar surface in Petri dishes containing 50% potato dextrose agar (19.5 g/liter of potato dextrose agar, 10 g/liter of agar; Merck) and incubated for 48 h at 25°C. Single germinating conidia were excised from the agar surface, transferred to 2% malt extract agar (20 g/liter of malt extract, 20 g/liter of agar; Biolab) and incubated for 4 weeks at 25°C in the dark. Isolates were preliminarily identified based on colony morphology and microscopic characters (e.g., conidial shape and dimensions) and further confirmed with DNA sequence data. DNA was extracted from isolates with the Prepman Ultra Sample Preparation Reagent (Thermo Fisher Scientific) according to the manufacturer's protocols. Sequences for three gene regions, the internal transcribed spacer region (ITS) (ITS1F [Gardes and Bruns 1993] and ITS4 [White et al. 1990]), the partial β-tubulin gene (BT2a and BT2b [Glass and Donaldson 1995]), and the elongation factor 1-alpha gene region (EF1-728F and EF1-986R [Carbone and Kohn 1999]) were sequenced according to the methods outlined by Havenga et al. (2019). Sequences were assembled in CLC Main Workbench version 8.0.1 and compared with sequences in the National Center for Biotechnology Information GenBank database (http://www.ncbi.nlm.nih.gov) with the BLASTn utility to confirm their identity. Two representative isolates, *T. destructans* (CMW56797) and *T. epicoccoides* (CMW56798), were chosen for use in subsequent studies and were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa.

Plant material. Ramets of a 1-year-old *E. grandis* × *E. urophylla* clone were kindly provided by the Mondi Group, South Africa, and were used in the study of symptom development and host colonization by *T. destructans* and *T. epicoccoides*. Plants were planted in 5-liter black polyethylene bags containing Culterra potting soil mix (Culterra [Pty] Ltd, South Africa) and maintained in a greenhouse under natural light, with temperature controlled with air conditioning. The day temperature ranged from 20 to 25°C, with an average night temperature of 20°C, for 4 months, until the experiments were performed.

Conditions for conidial germination. Several factors considered to be important for the germination of *T. destructans* and *T. epicoccoides* conidia were evaluated. These included temperature, RH, and light.

Effect of temperature. Two distinct sources of conidia were used to evaluate the effect of temperature on conidial germination. These were either from naturally infected leaves or from cultures. In the case of infected leaves, we prepared a conidial suspension by excising mature fruiting bodies with a needle and placing them into an Eppendorf tube containing SDW. The conidial concentration was adjusted to 1×10^6 conidia/ml of SDW amended with 0.01% of Tween 20 (Sigma-Aldrich) with a hemocytometer. The conidial suspension prepared from cultures was obtained for *T. destructans* (CMW56797) and *T. epicoccoides* (CMW56798) grown on MEA for 5 weeks at 25°C in the dark. We prepared the suspension by washing the plates with SDW and adjusting the concentration to 1×10^6 conidia/ml, as described previously. The influence of temperature on spore germination of *T. destructans* and *T. epicoccoides* was evaluated under a range of temperatures from 10 to 35°C at 5°C intervals, incubated simultaneously in independent incubators. From each conidial suspension, 100 µl was spread onto the agar surface in Petri dishes containing 2% water agar (agar 20 g/liter; Biolab). Four replicates of each suspension per pathogen were used at each temperature. Germination was evaluated after 6, 12, 24, 48, and 72 h, observed under a light microscope at 20× magnification.

Effect of RH. Conidia used to assess the effect of RH on germination were obtained from naturally infected plants. Six different RH levels were considered. The RH was established with saturated solutions of KAc (35.0% RH), D-glucose (50.5% RH), NaCl (70.3% RH), KCl (80.8% RH), and KH_2PO_4 (96.5%), and SDW was used to achieve 100% RH, according to the method described by Winston and Bates (1960). Aliquots of 100 µl of the conidial suspension of each pathogen were placed onto sterilized glass microscope slides and allowed to air dry in a laminar flow cabinet for 15 min or until the slide was free of water. Four slides were used per treatment, placed in plastic boxes on a rack suspended 5 cm above the saturated solutions. All boxes were sealed and placed at 25°C for 72 h.

Effect of light. The influence of light on the germination of *T. destructans* and *T. epicoccoides* conidia collected from naturally infected leaves was evaluated, with 100 µl of the conidial suspension placed on water agar, as described previously for evaluating the effect of temperature. The plates were incubated under three different light regimes: darkness, natural light (with a shade cloth to prevent direct sunlight), and fluorescent light at 25°C. The germination was evaluated after 72 h, and four replicates were used per treatment.

Statistical analyses. We evaluated spore germination by determining the number of germinated conidia per 100 plated conidia. Conidia were recorded as having germinated if a germ tube was clearly visible under 20× magnification. The data were analyzed statistically for each assay via a two-way analysis of variance. The data were arcsine square root transformed to normalize variances before the analysis. The Tukey post hoc test was used to identify significant differences between treatments ($P < 0.05$). All statistical analyses were performed in R statistical software, version 3.2.0 (R Foundation for Statistical Computing, Vienna, Austria).

Symptom development. To observe the disease development for *T. destructans* and *T. epicoccoides*, we performed an inoculation trial by using 1-year-old *E. grandis* × *E. urophylla* trees. Ten healthy plants were selected for inoculation with conidia from cultures of *T. destructans* (CMW56797) and *T. epicoccoides* (CMW56798). We prepared conidial suspensions by washing the cultures with SDW, as described previously, and the concentration was adjusted to 1×10^6 conidia/ml with a hemocytometer and amended with 0.01% Tween 20 (Sigma-Aldrich). New shoots, partially expanded leaves, and fully expanded leaves were inoculated on the upper and lower leaf surfaces with fine droplets of inoculum. Inoculum was collected and sprayed with a 50-ml glass bottle with atomizer spray cap onto the leaves until runoff. The plants were enclosed in clear plastic bags immediately after inoculation to maintain high humidity levels. Plastic bags were removed after 72 h. Control seedlings were sprayed with SDW and 0.01% Tween 20 (Sigma-Aldrich) and were also covered in transparent plastic bags for 72 h. The plants were maintained in a greenhouse under natural light, with a temperature ranging from 20 to 25°C and a night temperature of 20°C. The plants were monitored for the development of symptoms over a 6-week-period. Pycnidia of *T. destructans* and *T. epicoccoides* exuding conidia from the symptomatic tissue were placed on glass slides with SDW and examined under a light microscope to confirm the identity of the fungi.

Mode of infection, host colonization, and sporulation. Scanning electron microscopy was used to observe and compare the germination of conidia, mode of host penetration, and tissue colonization of *T. destructans* and *T. epicoccoides* on the *Eucalyptus* hybrid clone. Young leaves, new shoots, and expanded leaves were harvested 12, 24, 48, and 72 h and 14 days after inoculation. Samples were cut into 3-mm^2 pieces and placed in 2.5% glutaraldehyde/formaldehyde (50% vol/vol) for 24 h. The samples were dehydrated with a graded ethanol series (30, 50, 70, and 90%) for 15 min each, followed by four steps of dehydration in 100% ethanol, 15 min each and 30 min for the final ethanol dehydration. Samples were placed in hexamethyldisilazane and subsequently mounted on aluminum stubs, exposing either the abaxial or adaxial leaf surface. The samples were then coated with carbon with a Quorum Q150T Coating Unit (Quorum, United Kingdom). The samples were visualized under a Zeiss 540 Gemini Ultra Plus FEG SEM (Zeiss, Germany) scanning electron microscope at the Laboratory for Microscopy and Microanalysis, University of Pretoria, Pretoria, South Africa. To observe host tissue colonization, we harvested symptomatic samples 3 weeks after inoculation and visualized them via transmission electron microscopy. Samples were cut into 1-mm^2 sections and placed in 2.5% glutaraldehyde/formaldehyde for 24 h after the ethanol dehydration as described previously for scanning electron microscopy (SEM). Dehydrated samples were embedded in LR white resin in gelatin capsules and sectioned with a

Reichert Ultracut R microtome. Semithin sections (500 nm) were stained with 1% toluidine blue in 1% borax. Ultrathin sections (50 to 90 nm) were placed on 300 mesh copper grids and stained with saturated aqueous uranyl acetate and Reynolds lead citrate. Semithin sections stained with 1% toluidine blue in 1% borax were visualized with a Zeiss Axio Imager 2 light microscope (Carl Zeiss, Jena, Germany), and ultrathin sections were examined with a JEM 2100F transmission electron microscope (JEOL, Tokyo, Japan). To observe sporulation, we harvested samples 4 weeks after inoculation and confirmed spore formation with a hand lens (10× magnification). These samples were dehydrated as described previously, and semithin sections were stained with 1% toluidine blue in 1% borax and visualized with a Zeiss Axio Imager 2 light microscope.

Results

Conditions for spore germination. *Effect of temperature.* Conidia of *T. destructans* collected from naturally infected leaves germinated optimally at 25°C, with 100% germination at 24 h. After 72 h, 100% germination was also recorded at temperatures ranging from 20 to 30°C (Fig. 1A). The optimal temperature for germination of *T. epicoccoides* conidia was 20°C with 96% germination at 24 h. At 72 h, spores incubated at 15 to 25°C achieved 100% germination (Fig. 1B). At 10°C, the lowest tested temperature, neither pathogen displayed >10% germination. Up to 20% of the *T. destructans* conidia germinated at 35°C, but *T. epicoccoides* conidia did not germinate at this temperature (Fig. 1).

Germination of conidia from cultures was much lower than for those collected from infected leaves. For *T. destructans*, the optimal temperatures remained within the range of 20 to 30°C, both for spores from infected plant material and for those obtained from culture media. However, within this optimal range, 100% of the conidia from infected material germinated, whereas germination of conidia obtained from culture media did not exceed 15% germination (Fig. 1A and C). For *T. epicoccoides*, germination of conidia from culture media did not exceed 10% (Fig. 1D), but the optimal temperature range was similar to that for conidia from naturally infected leaves (Fig. 1B).

Effect of RH. Both pathogens displayed high conidial germination rates after 72 h at 25°C at 100% RH, with 97% germination for *T. destructans* and 92% for *T. epicoccoides* (Fig. 2A). Conidial germination was not observed at RH levels <70% for *T. destructans*, whereas *T. epicoccoides* conidia were able to germinate at the lowest level of RH evaluated (35%), but in this case the germination percentage was <1%. Both pathogens showed higher ($P < 0.001$) levels of germination,

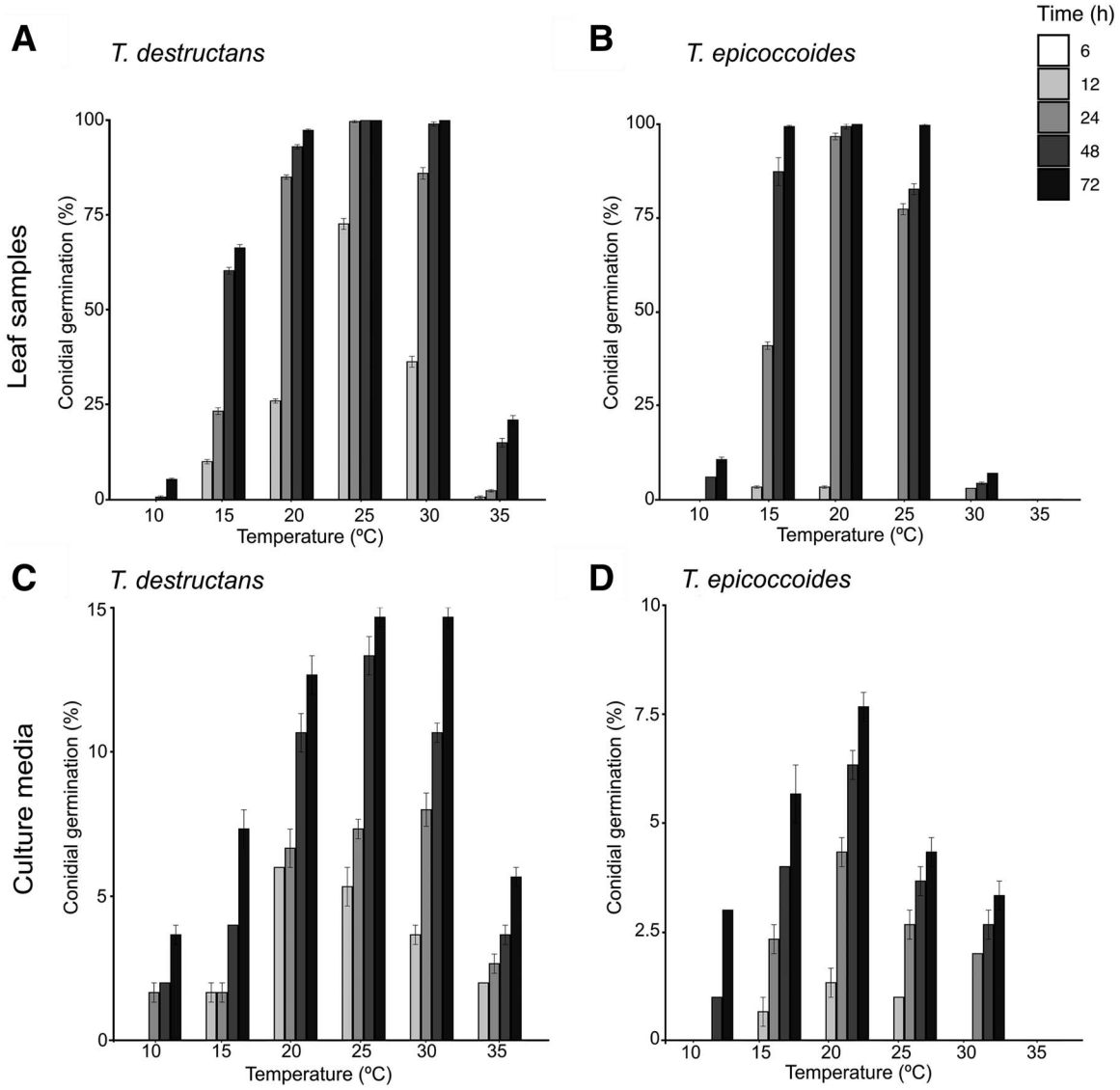

Fig. 1. Percentage of conidial germination of *Teratosphaeria destructans* and *T. epicoccoides* at temperatures ranging from 10 to 35°C at 5°C intervals. **A,** Germination of *T. destructans* with spores from infected leaves. **B,** Germination of *T. epicoccoides* with spores from infected leaves. **C,** Germination of *T. destructans* with spores grown on culture media (malt extract agar [MEA]). **D,** Germination of *T. epicoccoides* with spores grown on culture media (MEA). Spore germination evaluated at 6, 12, 24, 48, and 72 h. Error bars represent standard error of the mean.

when the RH increased, reaching maximum germination at 100% RH (Fig. 2A).

Effect of light. Both pathogens showed greater levels of germination when exposed to light after 72 h at 25°C ($P < 0.001$) (Fig. 2B). For *T. destructans*, conidia showed 99% germination under artificial as well as under natural light. Germination of *T. epicoccoides* conidia under natural light was 98% and under artificial light 96%. For both pathogens, germination was significantly reduced when retained in darkness, with 90% for *T. destructans* and 84% for *T. epicoccoides.* However, the germination percentages under different light sources did not differ significantly ($P > 0.05$) for either pathogen (Fig. 2B).

Symptom development on *E. grandis* × *E. urophylla*. Symptoms of *T. destructans* were visualized on young leaves and shoots 2 to 3 weeks after inoculation. Small chlorotic lesions with oily, diffuse borders appeared on the adaxial and abaxial leaf surfaces, and these became more evident 4 weeks after inoculation, forming yellow chlorotic lesions (Fig. 3A). After symptoms appeared, fruiting bodies appeared rapidly, mainly on the abaxial leaf surfaces (Fig. 3A), but some pycnidia were also present on adaxial leaf surfaces. For *T. epicoccoides* the first symptoms, including chlorotic spots delimited by leaf veins, appeared 3 to 4 weeks after inoculation on the abaxial and adaxial leaf surfaces (Fig. 3B). These were found mainly on expanded leaves and not on the most juvenile leaves. Pycnidia containing conidia appeared 5 weeks after inoculation on the abaxial leaf surfaces.

Infection and host colonization. Germinating conidia of both pathogens were visualized on leaves 24 h after inoculation (Fig. 4A and C). Germ tubes grew mostly from the terminal ends of the conidia, but lateral germ tubes were also observed. Penetration of the leaf tissue by both pathogens occurred only through the stomata, commencing at 48 h after inoculation, and this was seen on both leaf surfaces (Fig. 4B and D). After germination, growth of the hyphae occurred at multiple points on the leaves (Fig. 4E). Hyphal growth of *T. destructans* 14 days after inoculation was accompanied by apparent degradation of waxes on the leaf surfaces (Fig. 4E). In the case of *T. epicoccoides*, similar degradation of epicuticular wax was not visualized.

The growth of both *T. destructans* and *T. epicoccoides* within infected leaf tissue was exclusively intercellular (Fig. 5). However, in the case of *T. destructans*, hyphae were seen growing within the midrib tissues, and this was never seen for *T. epicoccoides*. In the interaction zone between host and pathogen cells, the cell walls were infiltrated with electron opaque deposits in response to hyphal presence (Fig. 5C to F). Haustoria were never observed in the infected leaf tissues, and cell rupture had not occurred 3 weeks after inoculation. However, cellular damage was evident 4 weeks after inoculation for both pathogens (Fig. 6). Cell damage was more pronounced on leaves inoculated with *T. destructans* (Fig. 6B) than on those inoculated with *T. epicoccoides* (Fig. 6E). Cell collapse was observed at the time when pycnidia formed and when sporulation commenced, predominantly in cells adjacent to recently developed fruiting bodies (Fig. 6B and E). Pycnidia of *T. destructans* and *T. epicoccoides* exuding conidia appeared mainly on the abaxial leaf surfaces. The development of the black pycnidia seen under a light microscope showed that the fruiting structures of both pathogens develop exclusively in substomatal cavities (Fig. 6) without producing stomatal rupture (Fig. 6C and F).

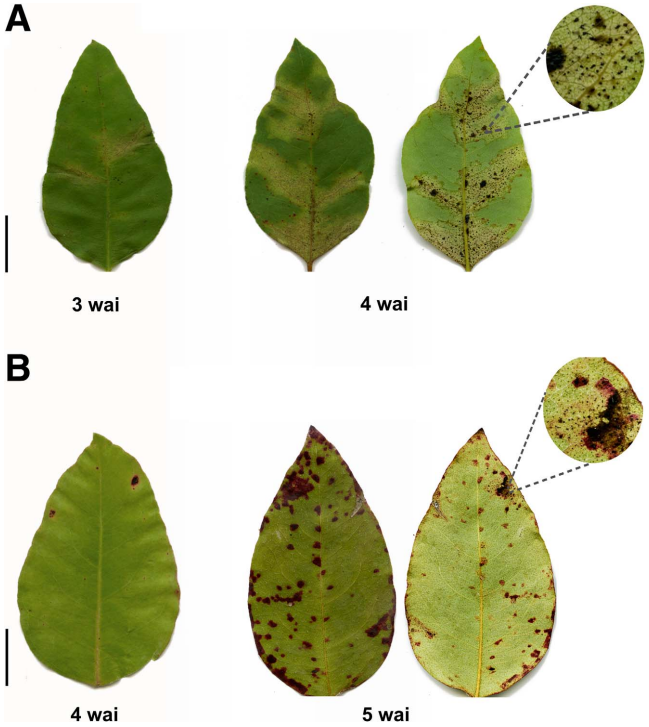

Fig. 3. Disease development on *Eucalyptus grandis* × *E. urophylla* leaves inoculated with *Teratosphaeria destructans* and *T. epicoccoides*. **A,** Leaves infected with *T. destructans* at 3 weeks after inoculation (wai) and 4 wai showing the leaf blight associated with the infection and sporulation on the abaxial leaf surface. **B,** Leaves infected with *T. epicoccoides* visualized at 4 wai and the symptoms and sporulation at 5 wai on the abaxial leaf surface. Scale bar = 10 mm.

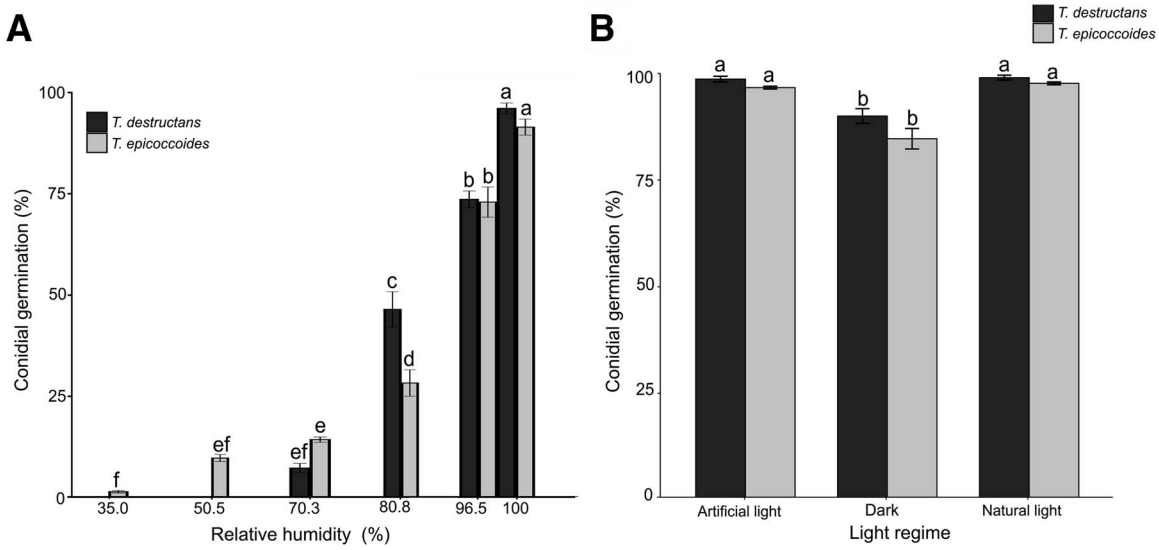

Fig. 2. Effect of RH and light on spore germination of *Teratosphaeria destructans* and *T. epicoccoides*. **A,** Germination under six RHs in presence of free water after 72 h. **B,** Germination under three light regimes (artificial light, dark, and natural light) after 72 h. Different letters at the tops of the bars indicate statistically significant differences between treatments and pathogen evaluated by Tukey test ($P < 0.05$). Error bars indicate standard error of the mean.

Discussion

This is the first detailed study of the conditions necessary for spore germination and infection by *T. destructans* and *T. epicoccoides*, two important foliar pathogens of *Eucalyptus* species. In addition, this research provides the first microscopic and histological observations of the infection process on *Eucalyptus* by *T. destructans* and *T. epicoccoides*. These observations will contribute to developing reliable inoculation protocols for both pathogens, facilitating future molecular genetic studies and rapid screening procedures for disease management.

Spore germination is the first crucial step needed by fungal pathogens to infect a host plant. Our conidial germination assessments under different environmental conditions showed that the optimal conditions for spore germination of *T. destructans* include higher levels of humidity and temperature than those for *T. epicoccoides*. This finding is consistent with the current epidemiological data available for these two pathogens. *T. destructans* occurs in tropical and subtropical areas with high temperatures and high humidity (Andjic et al. 2019; Burgess and Wingfield 2017). In contrast, *T. epicoccoides* has a wide distribution globally and is found on trees growing in temperate and subtropical environments (Park et al. 2000).

The *T. destructans* conidia germinated at all the temperatures evaluated in this study although at different levels. In contrast, *T. epicoccoides* conidia did not germinate at 35°C. This finding was consistent with the fact that conidia of other *Teratosphaeria* and *Mycosphaerella* species causing diseases in temperate areas, such as *Ramularia grevilleana*, *T. nubilosa*, *T. parva*, and *T. cryptica*, also could not germinate at temperatures >30°C (Carisse et al. 2000; Elliott 1988; Park and Keane 1982). The fact that *T. destructans* conidia were able to germinate at higher temperatures is consistent with its occurrence in hot, tropical environments (Burgess et al. 2006; Greyling et al. 2016; Old et al. 2003; Wingfield et al. 1996) and suggests that it is probably able to infect plants under a wider range of temperatures than *T. epicoccoides.*

Where conidia from naturally infected leaves and from culture media were compared, those of both *T. destructans* and *T. epicoccoides* from naturally infected material had higher levels of germination. The low viability of conidia from laboratory cultures can be explained by the fact that both fungi are known to have long biotrophic phases in the host and poor growth on culture media (Andjic et al. 2010; Aylward et al. 2019; Park and Keane 1984; Wingfield et al. 1996). Based on our observations, spores from naturally infected

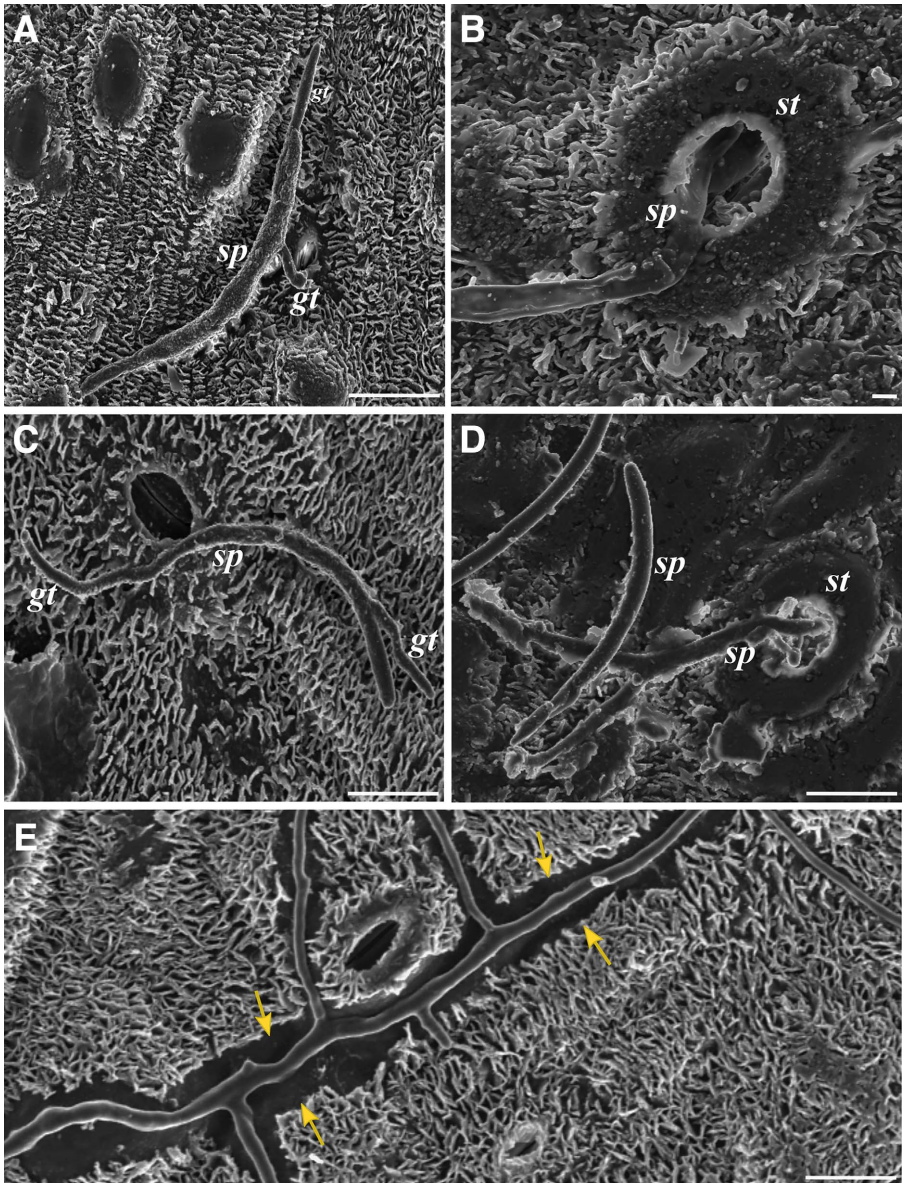

Fig. 4. Infection of *Teratosphaeria destructans* and *T. epicoccoides* on *Eucalyptus grandis* × *E. urophylla* leaves visualized by scanning electron microscopy. **A,** Spore germination of *T. epicoccoides* at 24 h after inoculation (hai), **B,** *T. epicoccoides* penetration through open stomata at 48 hai, **C,** Spore germination of *T. destructans* at 24 hai, **D,** *T. destructans* penetration through open stomata at 48 hai, **E,** Multiple branching of hyphae of *T. destructans* grew in an apparent direction toward the stomata; yellow arrows show the degradation of the epicuticular wax at 14 days after inoculation. Scale bars: 20 µm (A, C, D, and E), 2 µm (B). gt, germ tube; sp, spore; st, stomata.

plants should be used to develop a reliable inoculation protocol for interaction studies between *Eucalyptus* and these two pathogens. It will therefore be necessary to establish naturally infected plants under suitable greenhouse conditions to produce consistently viable conidia of a single genotype. Similar protocols are in place for the myrtle rust fungus, *Austropuccinia psidii* (McTaggart et al. 2018; Pegg et al. 2014; Roux et al. 2016).

After spore germination, many fungal pathogens produce specialized hyphae for infection of the host tissue directly through the leaf surface, natural openings such as stomata, or wounds. The infection of *T. destructans* and *T. epicoccoides* begins with penetration through the stomata, and there was no evidence of direct penetration of leaf tissues. This mode of infection is consistent with that of biotrophic foliar pathogens that develop and colonize plant tissues without causing loss of host cell viability to obtain nutrients (Almeida and Brand 2017; Szechyńska-Hebda et al. 2013; Wynn and Staples 1981).

After stomatal penetration, the hyphae of both pathogens grew in the intercellular spaces and continued to develop until host cell death occurred. Similar observations have been made for various other leaf pathogens with intercellular growth, such as *Quambalaria pitereka* and *Q. eucalypti* (Pegg et al. 2009) and *Mycosphaerella* (Simon et al. 2010). The contact between host and pathogen cells provides a zone of interaction, which probably includes a mechanism for nutrient acquisition by *T. destructans* and *T. epicoccoides*, as has been described, for example, in *Quambalaria* spp. on eucalypts (Pegg et al. 2009).

The switch from a biotrophic to a necrotrophic lifestyle of leaf diseases results in massive collapse of leaf tissue, leakage of nutrients from dying plant cells into apoplastic spaces, rapid increase in fungal biomass, and sporulation (Kema et al. 2008; Marshall et al. 2011; Shetty et al. 2003). For *T. destructans* and *T. epicoccoides*, sporulation occurred mainly on the abaxial leaf surfaces, where the majority of

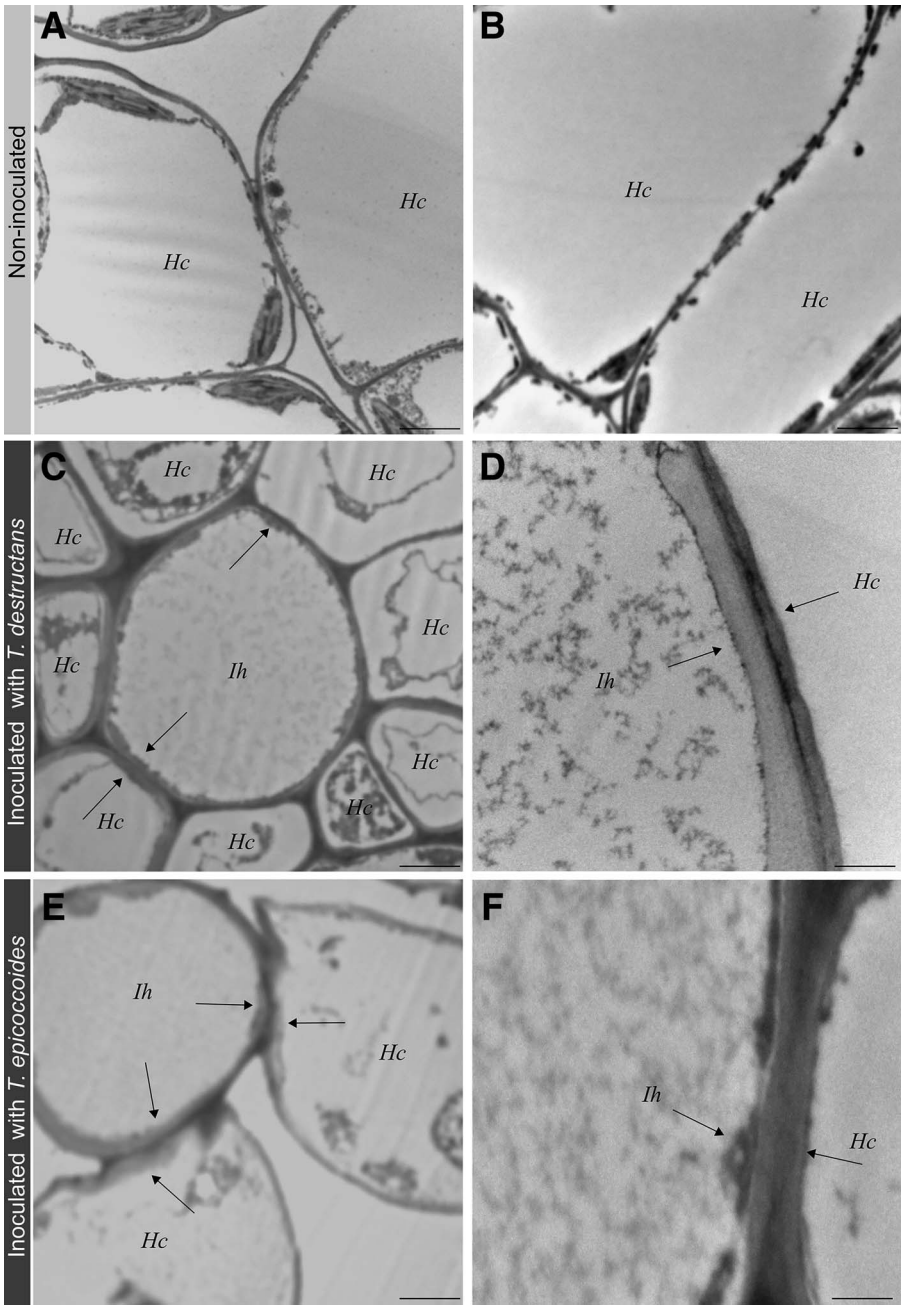

Fig. 5. Noninoculated and inoculated leaves showing the intercellular growth of *Teratosphaeria destructans* and *T. epicoccoides* hyphae in cells of *Eucalyptus grandis* × *E. urophylla* by transmission electron microscopy. **A and B,** Noninoculated cells of *E. grandis* × *E. urophylla*. **C and D,** Intercellular hyphae (*Ih*) of *T. destructans* in contact with host cells (*Hc*); the host cell presents a reaction zone infiltrated by electron-opaque material. **E and F,** Intercellular hyphae of *T. epicoccoides* and the reaction zone in contact with the host cell wall. Arrows show the interaction zone between fungal and host cell wall. Scale bars: 2,000 nm (A, C, and E); 1,000 nm (B, D, and F).

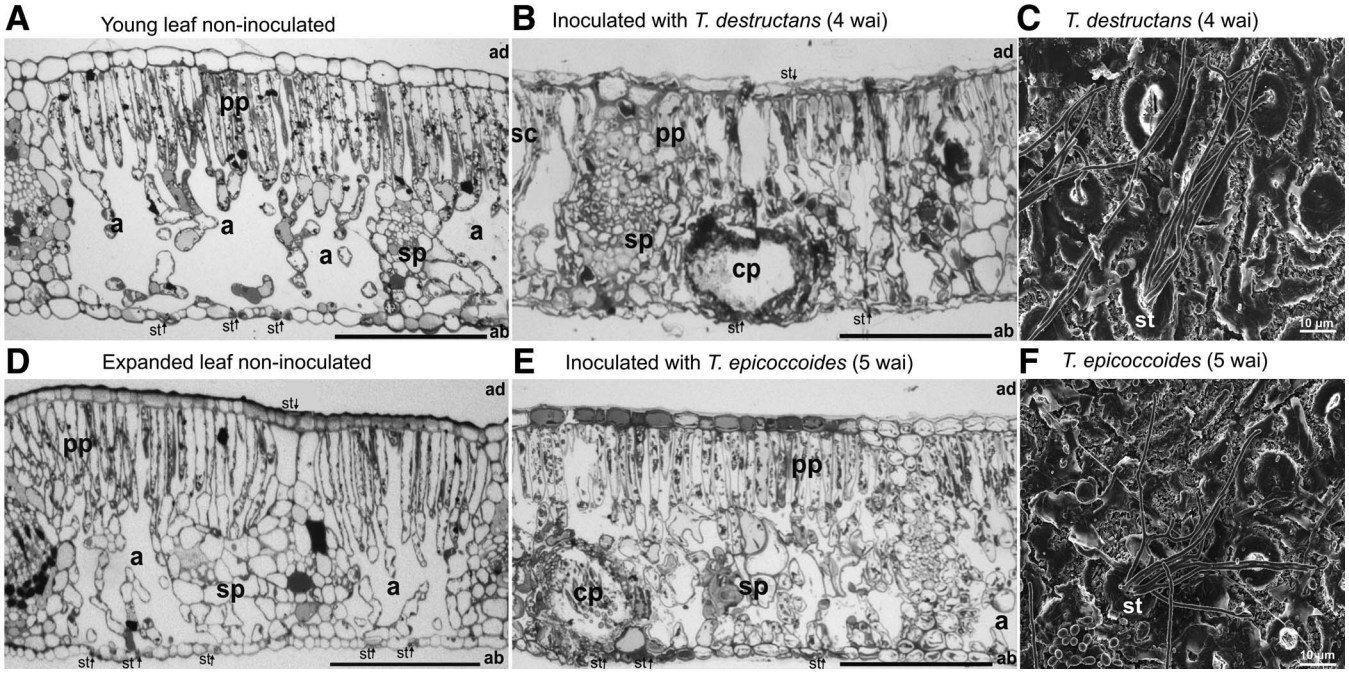

Fig. 6. Comparison of the cross-section of *Eucalyptus grandis* × *E. urophylla* leaves, noninoculated and inoculated with *Teratosphaeria destructans* and *T. epicoccoides*. **A,** Noninoculated young leaf control for *T. destructans*. **B,** Young leaf inoculated with *T. destructans* at 4 weeks after inoculation (wai) with evident cell collapse. **C,** Sporulation of *T. destructans* at 4 wai by scanning electron microscopy (SEM). **D,** Noninoculated expanded leaf control for *T. epicoccoides*. **E,** Expanded leaf inoculated with *T. epicoccoides* at 5 wai. **F,** Sporulation of *T. epicoccoides* at 5 wai by SEM. Scale bars: 20 µm. a, air space; ab, abaxial leaf surface; ad, adaxial leaf surface; cp, conidiomatal pycnidia; pp, palisade parenchyma; sc, secretory cavity; sp, spongy parenchyma.

stomata are located. Sporulation on the abaxial surface of the leaves has also been previously reported for various other *Teratosphaeria* spp. including *T. nubilosa*, *T. cryptica*, *T. parva*, and *T. epicoccoides* (Keane 2000; Park and Keane 1982). We observed large airspaces in the spongy parenchyma on the abaxial side of young leaves of a highly susceptible *E. grandis* × *E. urophylla* hybrid, leading to greater colonization of *T. destructans* in the foliar tissue. The reduced presence of airspaces in the palisade parenchyma has previously been associated with resistance of *E. globulus* to *T. nubilosa* (Smith et al. 2018).

Degradation of epicuticular waxes by *T. destructans* was visualized via SEM during the hyphal growth of the pathogen on the leaf surface. In general, epicuticular wax protects plants from infection by pathogenic fungi by reducing moisture on the leaf surface. The moisture is necessary for the germination of fungal spores (Neinhuis 1997). In some systems, pathogens are more likely to overgrow stomata on leaves with extensive wax coverage rather than penetrating and infecting the leaves (Rubiales and Niks 1992). Inhibition of pathogen penetration of leaf tissues by surface waxes has been observed for *Mycosphaerella fijiensis* on banana (Craenen et al. 1997) and *T. nubilosa* in *E. globulus* (Smith et al. 2018).

T. destructans has emerged as one of the most important pathogens of planted *Eucalyptus* globally (Andjic et al. 2019; Burgess and Wingfield 2017; Wingfield et al. 2015). For this reason, there is obvious need for a reliable and reproducible inoculation procedure that can be used to screen *Eucalyptus* genotypes for resistance to this pathogen. Such screening procedures have been adopted in the *Eucalyptus* forestry industry globally (Alfenas et al. 2016; Dianese et al. 1986; Mohan and Manokaran 2013). Relevant examples include screening for resistance to infection by the myrtle rust pathogen *Austropuccinia psidii* (Lee et al. 2015; McTaggart et al. 2018; Roux et al. 2016), the canker pathogen *Chrysoporthe cubensis* (van Heerden et al. 2005; van Heerden and Wingfield 2002) and the Ceratocystis wilt pathogen *Ceratocystis fimbriata* sensu lato (Harrington et al. 2015; Mafia et al. 2011). The results of the current infection biology study will guide the development of protocols to screen *Eucalyptus* genotypes for tolerance to *T. destructans* and to study the molecular interaction between *Eucalyptus* and these two pathogens. Both approaches will provide insights into managing these diseases more effectively.

Acknowledgments

We thank members of the Microscopy Centre, Department of Physics at the University of Pretoria, South Africa, particularly Mrs. Erna van Wilpe and Ms. Charity Maepa, for technical advice about the microscopy work conducted.

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