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Infection and disease development of *Quambalaria* spp. on *Corymbia* and *Eucalyptus* species

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Quambalaria spp. are eucalypt leaf and shoot pathogens of growing global importance, yet virtually nothing is known regarding the manner in which they infect and colonize their hosts. A study of the infection process of *Q. pitereka* and *Q. eucalypti* on *Corymbia* and *Eucalyptus* species was thus undertaken using light, scanning and transmission electron microscopy after artificial inoculation. Conidial germination was triggered when relative humidity levels exceeded 90% and commenced within 2 h in the presence of free water. Light reduced germination but did not prevent germination from occurring. Conidial germination and hyphal growth occurred on the upper and lower leaf surfaces with penetration occurring via the stomata or wounds on the leaf surface or juvenile stems. There was no evidence of direct penetration of the host. Following penetration through the stomata, *Q. pitereka* and *Q. eucalypti* hyphae grew only intercellularly without the formation of haustoria or interaction apparatus, which is characteristic of the order Microstromatales. Instead, the presence of an interaction zone is demonstrated in this paper. Conidiophores arose through stomatal openings producing conidia 7 days after infection.

Keywords: *Corymbia* spp., *Eucalyptus* spp., quambalaria shoot blight, symptomatology

Introduction

Australian native eucalypts, including spotted gums (*Corymbia citriodora* subsp. *variegata* [hereafter referred to as *C. variegata*], *C. citriodora* subsp. *citriodora* [hereafter referred to as *C. citriodora*], *C. henryi* and *C. maculata*) and species of *Eucalyptus*, are a source of valuable commercial timber, widely used in construction, furniture and pulping industries. Due to the rapid decline in access to native stands of spotted gum, there is an increasing reliance on timber resources from plantations to meet consumer demands. The hardwood plantation area has expanded rapidly in Australia in recent years with over 800 000 ha currently planted. In subtropical and tropical regions of eastern Australia, spotted gum, *E. dunnii* and *E. grandis* hybrids are widely planted. However, the selection of rapidly growing germplasm deployed extensively in genetically uniform plantations has increased the threat of pests and diseases (Drenth, 2004). This is especially poignant with the deployment of species in large scale monoculture stands that are native to the region. Unlike elsewhere in the world where eucalypts are exotic, monocultures in Australia are under huge pressure from pests and pathogens moving out of the mixed native stands (Wingfield *et al*., 2001; Burgess & Wingfield, 2002).

Quambalaria shoot blight caused by the fungus *Quambalaria pitereka* has become an important biological constraint to the success of spotted gum plantations in eastern Australia (Pegg *et al*., 2008). Taxonomic research by Bauer *et al*. (1997) identified monophyletic groups within the smut fungi and related taxa which lack interaction apparatus, a key feature of the Tilletiales, Georgefisheriales, Microstromatales and Entorrhizomycetidae. De Beer *et al*. (2006) provided the first phylogenetic data to consider the relationship of *Quambalaria* spp. to each other and to other fungi. It was shown in this study that *Quambalaria* spp. form a monophyletic clade in the family Microstromatales, which is an order of the Ustilaginomycetes and, based on this, de Beer *et al*. (2006) described a new family Quambalariaceae.

The first record of *Q. pitereka* causing damage to eucalypts was on *C. maculata* (then *Eucalyptus maculata*) to nursery seedlings in New South Wales, Australia, in the 1950s (Walker & Bertus, 1971). Surveys conducted by Old (1990) determined that *Q. pitereka* was endemic to the coastal forests of New South Wales, where seedlings

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and young spotted gum trees could be severely damaged. During wet weather, infected shoots and stems were reported to become coated with white patches of spore-bearing mycelium. Subsequent surveys have shown that the disease is specific to *Corymbia* species and commonly present in spotted gum plantations in subtropical and tropical regions of eastern Australia (Carnegie, 2007b; Pegg *et al*., 2008).

Quambalaria eucalypti was first discovered on *E. grandis* in South Africa (Wingfield *et al*., 1993). It has subsequently been found in Brazil, causing stem girdling on seedlings of *E. globulus* and leaf and shoot blight on mini-stumps of *E. saligna* × *E. maidenii* hybrids (Alfenas *et al*., 2001), and in Uruguay from twig lesions on *E. globulus* (Bettucci *et al*., 1999). Recent surveys have revealed that *Q. eucalypti* is specific to *Eucalyptus* species and is present in commercial plantations as well as species and taxa trials of *Eucalyptus* species in Australia (Carnegie, 2007a; Pegg *et al*., 2008).

Lesion development on immature leaves and stems starts on average 5 days after infection by *Q. pitereka* and is visible as discrete chlorotic spots with necrotic centres (Self *et al*., 2002; Pegg *et al*., 2005). These may develop into large, sporulating lesions 10 to 14 days after infection under favourable conditions. Roux *et al*. (2006) reported disease development, including the presence of conidiophores and conidia, 14 days after inoculation with *Q. eucalypti* on species of *Eucalyptus* in South Africa. While it is apparent that the disease cycle is rapid for these pathogens, very little is known regarding the mode of infection of *Q. pitereka* and *Q. eucalypti* on *Corymbia* spp. and *Eucalyptus* spp., respectively. Pegg *et al*. (2008) reported an association with insect damage and the presence of *Q. eucalypti*, suggesting wounds may play a role in infection. The influence of leaf age on disease development has been documented, with older leaves showing decreased disease development after infection by *Q. pitereka* and *Q. eucalypti* (Pegg *et al*., 2008).

The aim of this study was to elucidate the infection process and disease development of two *Quambalaria* species on host and non-host tissue. In particular, to determine: (i) the effect of relative humidity, temperature and light on conidial germination; (ii) the mechanism of conidial germination, fungal growth and penetration *in planta* of *Q. pitereka* and *Q. eucalypti* on *Corymbia* and *Eucalyptus* spp., identifying any differences; (iii) the nature and process of colonization and growth of *Q. pitereka* and *Q. eucalypti* within susceptible and non susceptible host tissue; and (iv) describe symptom and disease development on *Corymbia* and *Eucalyptus* spp.

Materials and methods

Conidial germination

The effect of relative humidity (RH) levels on germination of conidia was determined for *Q. pitereka* (BRIP 48385) and *Q. eucalypti* (BRIP 48508). To establish different levels of humidity, saturated solutions of $MgCl_2 \cdot 6H_2O$ (32·5% RH), Ca $(NO_3) \cdot 4H_2O$ (47% RH), $(NH_4)_2SO_4$ (80% RH), KCl (84·5% RH), KNO_3 (91% RH), KH_2PO_4 (93·5% RH) and K_2SO_4 (96·5% RH) were placed in sealed containers. Sterile distilled water (SDW) was used to achieve 100% RH following the method of Sheridan (1968).

Quambalaria pitereka and *Q. eucalypti* cultures were grown on potato dextrose agar (PDA) for 2 to 3 weeks at 25°C in the dark prior to spore collection. A conidial suspension was made to a concentration of 1×10^6 conidia mL^{-1} by washing the cultures with SDW. A 100 μL droplet of the suspension was placed onto a sterilized glass microscope slide and allowed to air dry in a Laminar flow for 15 min or until the slide was free of any visible moisture. Slides were placed on wire stands suspended 5 cm above the liquid levels in containers representing each RH treatment, with four replicates per treatment. All containers were sealed to ensure air-tightness and placed in the dark at 25°C. Germination of conidia was assessed after 72 h by determining the number of germinated spores out of 100. Conidia were recorded as having germinated if a germ tube was clearly visible at 400 × magnification.

To determine the effect of temperature on spore germination, *Q. pitereka* conidia were examined for germination in the presence of free water at a range of temperatures from 10°C to 35°C at 5°C intervals. Spore suspensions were produced as described previously with a 100 μL droplet placed onto the surface of cleaned and sterilized glass slides placed in the dark at different temperatures in sealed containers with SDW to maintain a constant level of free water. Six slides, with four droplets of spore suspension on each slide, were placed at each temperature and 100 conidia counted from each slide after 1, 3, 6 and 24 h.

The influence of light on germination of *Q. pitereka* conidia was examined by preparing spore suspensions on glass slides as for the temperature and relative humidity tests. The slides were placed in sealed containers at 100% RH and 25°C. Containers were placed in three light treatments: (i) in the dark, (ii) under natural light (under shade cloth to avoid direct sunlight), and (iii) under fluorescent lighting at 25°C. Germination levels were examined after 6 h, with 100 conidia counted and observed for the presence of germ tubes. Each treatment was replicated four times. All proportion data was arcsine square root transformed prior to analysis using ANOVA and compared using Fishers PLD post hoc test (StatView®).

Fungal growth and host penetration

Scanning electron microscopy (SEM) was used to determine and compare the infection process of *Q. pitereka* and *Q. eucalypti* on *Corymbia* and *Eucalyptus* spp. Spore germination and host penetration by *Q. pitereka* was examined on three provenances of *C. variegata* known to have differing susceptibility to quambalaria shoot blight: Woondum (low susceptibility), Presho (high susceptibility) and Richmond Range (moderate susceptibility). Other species examined were *C. torelliana*, *C. torelliana* × *C. variegata* hybrids, *E. grandis, E. cloeziana, E. dunnii,*

E. microcorys, *E. resinifera* and *E. pilularis*. *Quambalaria eucalypti* germination and host penetration was observed on *E. grandis* and *C. variegata*. Six trees of each species were inoculated and sampled to observe fungal growth and penetration.

Corymbia and *Eucalyptus* seedlings were grown in steam sterilized soil mix and fertilized with slow release Osmocote® (Native Trees) as required and irrigated twice a day for 10 min each using overhead sprinklers. Glasshouse temperatures were maintained at 25–28°C during the day and 20–22°C overnight. Pruning was conducted when necessary to induce the development of new shoots. After 4 months, seedlings were selected for inoculation.

Isolates of *Q. pitereka* (BRIP 48385, BRIP 48349) and *Q. eucalypti* (BRIP 48508) were obtained from single lesions and grown on PDA for 2 to 3 weeks in the dark at 25°C. A spore suspension (1×10^6 spores mL^{-1}) was obtained by washing plates with SDW to which two drops of Tween 20 had been added prior to inoculation. Seedlings were inoculated using a fine mist spray (2·9 kPa pressure) generated by a compressor driven spray gun (Iwata Studio series 1/6 hp; Gravity spray gun RG3), to the upper and lower leaf surfaces of the seedlings until runoff was achieved. All seedlings were covered with plastic bags immediately after inoculation to maintain high humidity levels and to increase the period of leaf wetness. Bags were removed after 48 h and plants watered using overhead irrigation systems twice a day for a period of 10 min. Sub-samples of the spore suspension applied to the trees were placed onto PDA and incubated at 25°C for 48 h to ensure that the spores were viable.

To examine the germination of conidia and host penetration process of *Q. pitereka* and *Q. eucalypti*, juvenile leaves, including new shoots, expanding and fully expanded leaves and photosynthetically active stems, were removed from inoculated plants after 12, 24, 48 and 72 h. Samples were also collected from *C. variegata* leaves 5 and 8 days after inoculation with *Q. pitereka*. To observe the influence of wounding on the *Q. eucalypti* infection process, leaves were wounded with sterilized pins just prior to inoculation and samples collected 48 h after inoculation. Leaf and shoot samples were cut into 3 mm^2 pieces and prepared for observation under scanning electron microscope (van den Berg *et al*., 2003) with specific modification described by Pegg *et al*. (2008). Leaf and stem samples of *C. variegata* trees from plantations showing symptoms of *Q. pitereka* infection were also examined using SEM.

Infection and colonization

In order to observe the infection and subsequent colonization process of *Q. pitereka* and *Q. eucalypti* following penetration of the leaf surface, leaf samples were collected 5 days after inoculation. Samples were processed for examination under SEM as previously described. However, prior to platinum coating, double-sided adhesive tape was used to peel away cell layers to expose the underside of the cuticle and epidermal cell layers. Each sample was then placed onto a metal stub and sputter coated with platinum and examined with SEM.

Mode of infection and colonization of cells following penetration was examined using transmission electron microscopy (TEM). Fresh foliage and juvenile stem samples of *E. grandis* and *C. variegata* exhibiting symptoms of quambalaria shoot blight were collected and cut into 1 mm^2 sections under a drop of 3% glutaraldehyde in 0·1 M phosphate buffer at a pH of 6·8. Samples were fixed in 3% glutaraldehyde in 0·1 M phosphate cacodylate buffer at a pH of 6·8 under vacuum in a Pelco Biowave at 80 W for a pre-programmed 2 min on, 2 min off, 2 min on cycle. Samples were washed in 0·1 M phosphate buffer at 80 W under vacuum for a cycle of 1 min on, 1 min off and 1 min on. Subsequently the phosphate buffer was removed and samples placed in 1% osmium tetroxide in 0·1 M cacodylate buffer at 80 W under vacuum for a pre-programmed 2 min on, 2 min off, 2 min on cycle. Osmium tetroxide was removed and samples dehydrated using a range of acetone concentrations: 50, 60, 70, 80, 90% and twice in 100%. All samples were processed at 250 W without vacuum with a cycle of 1 min on, 1 min off, 1 min on. Samples were embedded in Spurr's plastic and sectioned using a diamond knife, Leica Ultracut UC6 ultramicrotome. Semi-thin (500 nm) sections were stained with 1% Toluidine Blue, 1% borax. Ultra-thin sections (60 nm) were lifted and placed on pioliform coated slot copper grids and stained with 5% uranyl acetate in 50% methanol and Reynolds lead citrate. Semi-thin sections were examined under a light microscope and images captured using a microscope mounted digital camera. Ultra-thin sections were examined under TEM JEOL 1010 operated at 80 kV with images captured using a Soft Imaging Megaview III digital camera.

Symptom development

The development of symptoms on new shoots and expanding and fully expanded leaves was observed on *C. variegata* and *E. grandis* inoculated with *Q. pitereka*, and *E. dunnii*, *E. grandis* and *C. variegata* inoculated with *Q. eucalypti*. *Corymbia* and *Eucalyptus* seedlings were grown in steam sterilized soil mix and fertilized and inoculated with isolates of *Q. pitereka* (BRIP 48385) and *Q. eucalypti* (BRIP 48508) as described before. Uninoculated seedlings, sprayed with SDW, were also covered with plastic bags and along with inoculated plants, were monitored for the development of symptoms over a 20 day period. The presence of conidiophores of *Q. pitereka* and *Q. eucalypti* on lesions was confirmed using a 10× magnification hand lens. Each treatment was replicated 10 times.

Results

Conidial germination

Conidial germination, *in vitro*, after 72 h at 25°C increased significantly (Two Way ANOVA $F_{7,48} = 160{\cdot}6$; $P < 0{\cdot}0001$)

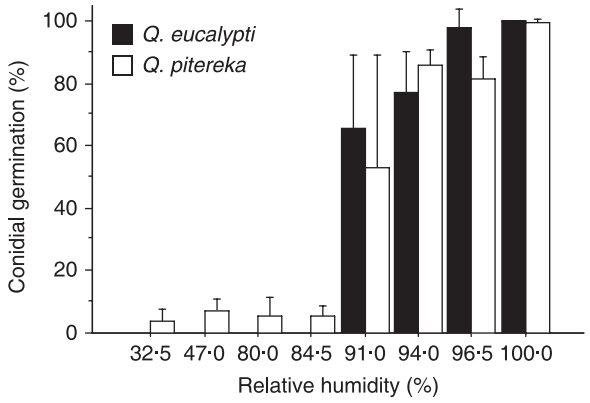

Figure 1 Mean germination (+1 standard error) of *Quambalaria pitereka* and *Quambalaria eucalypti* conidia after 72 h at a range of relative humidity levels.

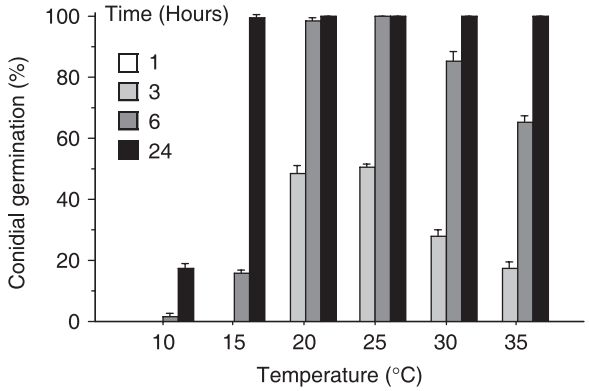

Figure 2 Mean germination of *Quambalaria pitereka* conidia (+1 standard error) in the presence of free water over time at temperatures ranging from 10°C to 35°C at 5°C intervals.

when RH levels exceeded 90% for both *Q. pitereka* and *Q. eucalypti* (Fig. 1). Below 90% RH conidia germination was limited for *Q. pitereka* and did not occur for *Q. eucalypti*. There were no significant differences in germination levels between *Q. pitereka* and *Q. eucalypti* ($F_{1,48}$ = 5·5; P = 0·0535).

Quambalaria pitereka conidia germinated optimally in the presence of free water at 20–25°C, with germ tube development starting within 3 h and 100% conidia germination attained within 6 h (Fig. 2). At temperatures below 20°C, conidial germination rates were reduced, with germination occurring only after 5 h at 15°C and 6 h at 10°C. At all temperatures investigated, other than 10°C, 100% conidial germination was recorded after 24 h. Conidial germination was significantly (Two Way ANOVA $F_{5,72}$ = 3020·5; P < 0·0001) reduced at 10°C with less than 20% of the conidia germinating after 24 h. Studies on the effect of temperature on *Q. eucalypti* were not included as it was found that at low and high temperatures, i.e. 10, 15, 30 and 35°C, secondary conidia were produced making it difficult to identify germ tube production.

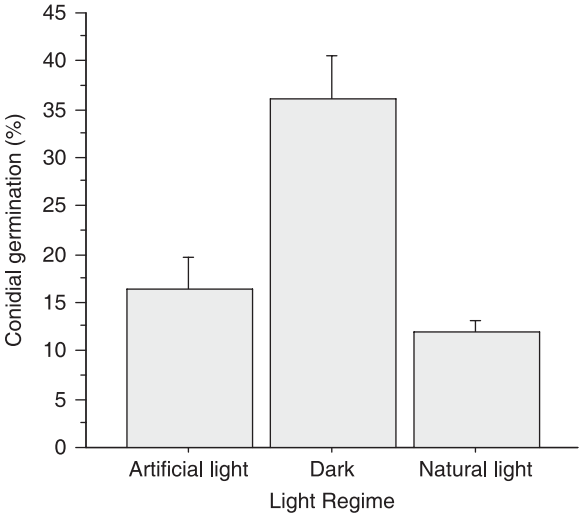

Figure 3 Germination of *Quambalaria pitereka* conidia (+1 standard error) after 6 h in free water in different light and dark conditions.

Light did not prevent germination of *Q. pitereka* spores in the presence of free water. However, germination was significantly reduced when the conidia were exposed to natural light (One Way ANOVA $F_{2,21}$ = 16·2; P < 0·0001) and artificial light (P = 0·0003) in comparison to conidia allowed to germinate in the dark (Fig. 3).

Fungal growth and host penetration

Germination of conidia and hyphal growth of *Quambalaria* spp. on leaf and stem surfaces occurred within 12 h of plants being inoculated on both the abaxial and adaxial leaf surface. Neither spotted gum provenance (Woondum, Presho, Richmond Range) nor leaf morphology (hairy or smooth) (Fig. 4), influenced spore germination or hyphal growth. Germination occurred from one or both ends of conidia (Fig. 4) with no obvious pattern of hyphal growth. Lateral branching of the emerging mycelium was observed at various points with some occurring at the point of emergence from the conidium (Fig. 4). Multiple branching was observed as the hyphae grew over the leaf surface. Some branches also appeared to grow only short distances and then cease further development. Hyphal swellings were also noted on the leaf surface with branching occurring from the swellings (Fig. 4). However, this was not observed in all cases. These swellings were not considered to be appressorial structures as there was no evidence of direct penetration through the leaf surface. Conidia and hyphae were not observed from leaf samples that were collected 5 and 8 days after plants were inoculated.

Evidence of enzymatic activity associated with *Q. eucalypti* and *Q. pitereka* was observed on the surface of *E. grandis* and *C. variegata*, respectively. Here the cuticle appeared to be eroded in areas where fungal hyphae were present (Fig. 4). This was not observed in all instances.

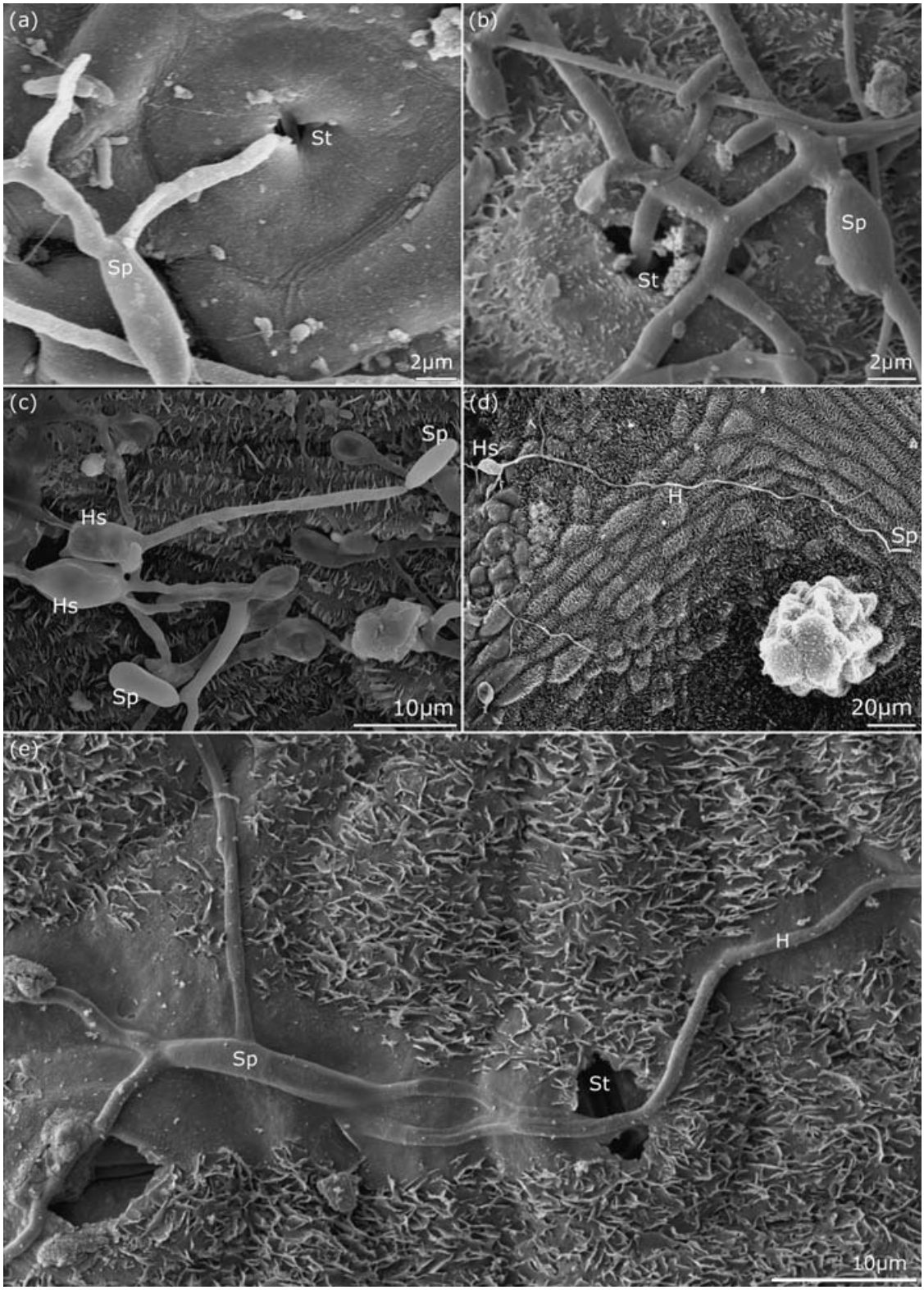

Figure 4 *Quambalaria pitereka* (a, c, d) and *Quambalaria eucalypti* (b) spore germination occurred within 12 h of inoculation with germ tubes produced from either end (a, b) or from a single end (c, d) of the conidia (Sp). Germination of *Quambalaria pitereka* conidia was not affected by leaf texture, occurring on both smooth textured (a) and hairy leaves (c, d). Multiple branching of hypha (H) was observed with growth towards stomata (St) apparent. Hyphal swellings (Hs) were observed in some instances (c, d). Degradation of the waxy layer of *Eucalyptus grandis* leaf associated with germination of *Quambalaria eucalypti* spore (Sp) and growth of hyphae (H) on the epidermis (e).

Germination of *Q. pitereka* conidia and growth of hyphae occurred on the surfaces of *E. dunnii* and *E. grandis* (Fig. 5) and *E. pilularis*, which are not considered hosts. Likewise, when *Q. eucalypti* was inoculated onto *C. variegata*, conidial germination and hyphal growth was observed. However, one marked difference observed in the case of *Q. eucalypti* was the accumulation of ungerminated conidia on the surface of *C. variegata* leaves (Fig. 5). This accumulation of conidia occurred from the production of secondary conidia directly from primary conidia or from short germ tubes arising from primary conidia. Primary and secondary conidia were present on the leaf surface with evidence of denticles directly on primary conidia and short germ tubes (Fig. 5).

Penetration of photosynthetically active *Corymbia* and *Eucalyptus* leaves and shoots by *Q. pitereka* and *Q. eucalypti* hyphae occurred via the stomata (Fig. 5). Hyphal penetration also occurred through artificial wounds. Penetration occurred within 24 h after inoculation on *Eucalyptus* and *Corymbia* species. There was no evidence of direct penetration or the production of structures such as appressoria that are required for direct penetration. Germ tubes were often observed to grow over the stomata without evidence of penetration.

Leaf age did not influence the germination of *Q. pitereka* conidia and penetration occurred through stomata of *C. variegata* leaves of all stages of development including new shoots, expanding foliage and fully expanded juvenile foliage. Penetration through stomata was also observed when *Q. pitereka* was inoculated onto *Eucalyptus* species, which are considered non-hosts, and when *C. variegata* was inoculated with *Q. eucalypti* (Fig. 5).

Infection and colonization

Quambalaria pitereka and *Q. eucalypti* grew exclusively intercellularly after penetration of the stomata or wounds while haustoria were not observed in any of the samples analysed. Hyphae were detected on the underside of stomata and in the intercellular spaces between epidermal, spongy and palisade mesophyll cells but not in the vascular tissue in leaves (Figs 6, 7) and pre-cambium, xylem and phloem of juvenile stems. *Quambalaria* spp. were not observed entering the cells nor was there evidence that the cell walls were ruptured, but the cells did collapse following infection.

Following contact between the host and pathogen cells, a change in the fungal cell wall occurred, with fungal material appearing to be transferred into the host intercellular space (Fig. 8). A change in the composition of the intercellular space was observed along with the development of a reaction zone, vesicles and a change in the cell wall in the host cell closest to the intercellular hypha (Fig. 8).

At the point of contact between host cell and the intercellular hyphae, the host cell walls were infiltrated by electron-opaque deposits of variable shape and size at the interface of the host-pathogen interaction (Fig. 8). Transfer traces were also observed in the host cell wall at the point of contact with the intercellular hyphae and vesicles present in the matrix. Electron opaque material was evident inside the host cells in reaction to contact with fungal hypha (Fig. 8).

Primary interactive vesicles, which accumulate in the fungal cytoplasm at interaction areas (Bauer *et al*., 1997), were observed at the interface between host and pathogen. These primary interactive vesicles fuse with the fungal plasma membrane where their contents are deposited and can be seen as a secretion profile (Fig. 8). No interaction apparatus were observed. Cell collapse and death was restricted to areas where the *Q. pitereka* and *Q. eucalypti* hyphae were physically present. Cell death and colonization of intercellular spaces by *Q. pitereka* and *Q. eucalypti* was not observed on non-host species.

Fungal reproduction

Conidiophores bearing conidia appeared abundantly on the leaf and stem surfaces 10–14 days after inoculation. Conidiophores arose through stomatal openings on leaves and juvenile stems (Fig. 9). Conidiophores were also observed rupturing through the epidermal layer on immature stems (Fig. 9). These were never found when *Q. eucalypti* was inoculated onto *Corymbia* spp. or when *Q. pitereka* was inoculated onto *Eucalyptus* spp.

Symptom development

Chlorotic spots surrounding stomata were evident on new shoots and expanding juvenile leaves of *C. variegata* leaves 5 days after inoculation with *Q. pitereka*. Necrotic lesions, some showing evidence of conidiophores and conidia, were present on expanding new shoots and juvenile leaves after 7 days. Chlorotic spots were present on fully expanded juvenile foliage. Ten days after inoculation, necrotic lesions were present on all foliage types and sporulation, including conidiophores and conidia, were evident on new shoots and expanding leaves. Sporulation was less frequent on fully expanded foliage. Small necrotic lesions were noted on juvenile stems. Fourteen days after inoculation new shoots and expanding foliage were severely buckled with some premature senescence observed. Lesions expanded and often coalesced forming large necrotic areas on the leaf surfaces. Lesions on fully expanded juvenile foliage were restricted and rarely coalesced.

Similar stages of disease development were observed on *E. grandis* and *E. dunnii* foliage inoculated with *Q. eucalypti*. No symptoms were observed on *C. variegata* inoculated with *Q. eucalypti* nor when *Eucalyptus* spp. were inoculated with *Q. pitereka*.

Discussion

Results of this study represent the first histopathological observations of the interaction between *Q. pitereka* and *Q. eucalypti* and species of *Corymbia* and *Eucalyptus*. The infection process of the pathogens on susceptible and

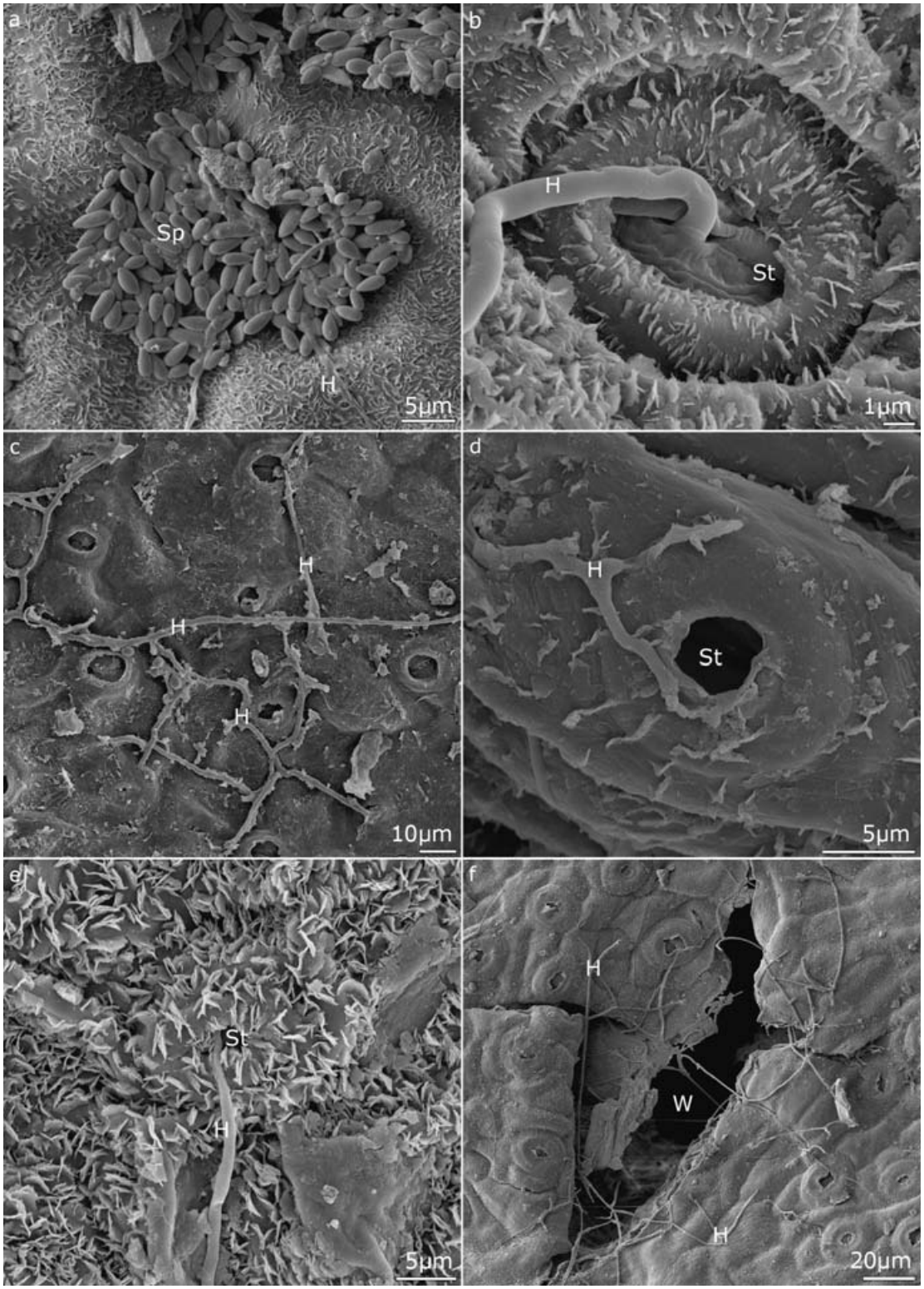

Figure 5 Germination of *Quambalaria eucalypti* (a) and *Quambalaria pitereka* (b, c, d) conidia (Sp) and growth of hyphae (H) occurred on host species outside their known range. (a) *Quambalaria eucalypti* produced an accumulation of secondary conidia directly from primary conidia or from short germ tubes on *Corymbia variegata* leaves. *Quambalaria pitereka* hypha (H) penetrating a stomata (St) on juvenile *Corymbia torelliana* leaf (b), *Quambalaria pitereka* conidia germinated producing hyphae that grew on the surface of a *Eucalyptus grandis* leaf (c), juvenile stem of *Corymbia variegata* (d) and penetration of the stomata (St) of *Eucalyptus dunnii* (e). *Quambalaria eucalypti* hypha (H) penetrating an artificially created wound (W) on the surface of a *Eucalyptus grandis* leaf (f).

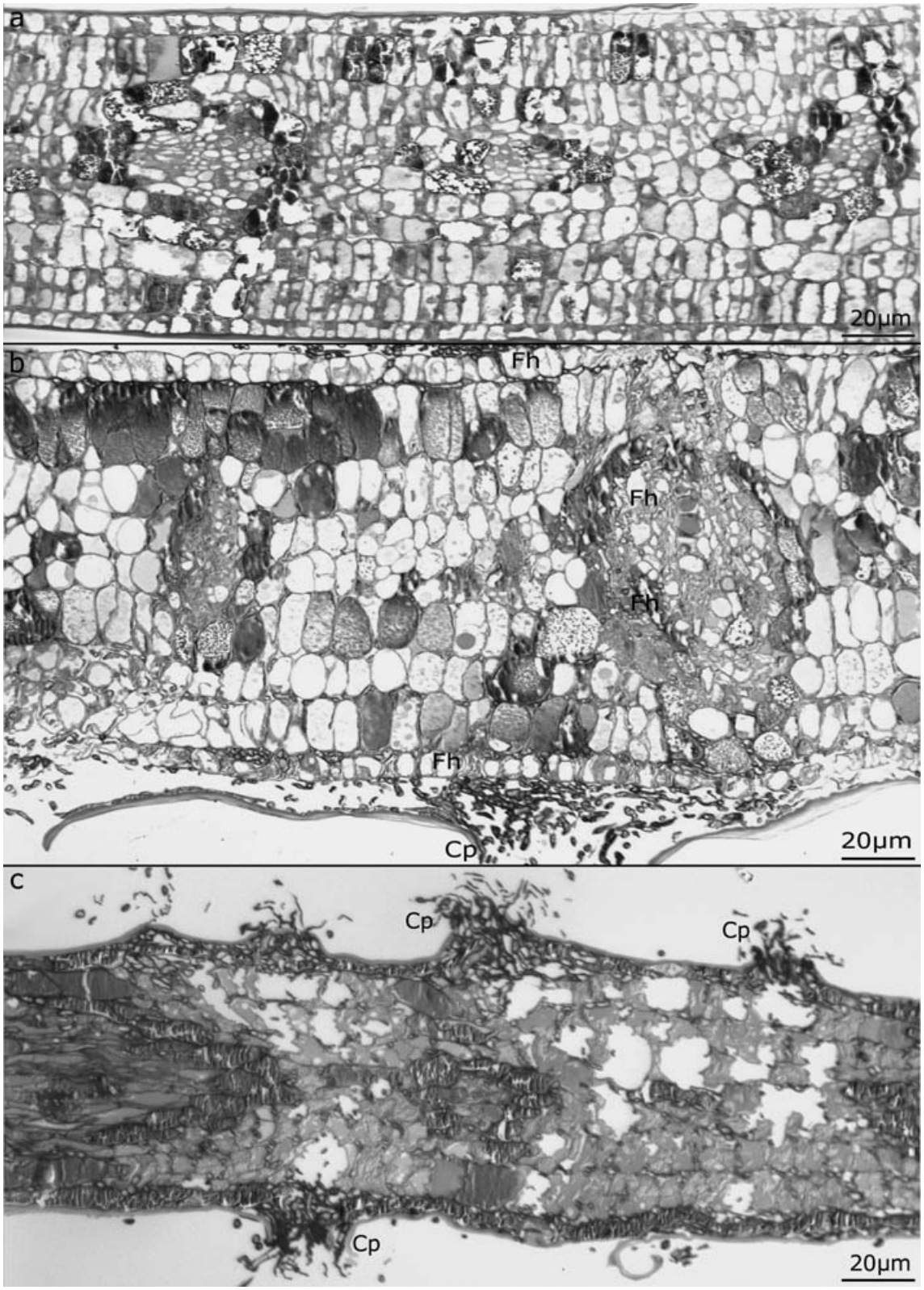

Figure 6 Semi-thin transverse sections comparing cell structure of uninfected leaves in comparison to leaves at different stages of infection by *Quambalaria pitereka* and symptom development. (a) Uninfected leaf and (b) early stages of infection with *Quambalaria pitereka* hyphae (Fh) colonizing the intercellular spaces and rupturing the cuticle. (c) Cell death and collapse in the infected part of the leaf with conidiophores (Cp) and conidia rupturing through stomata.

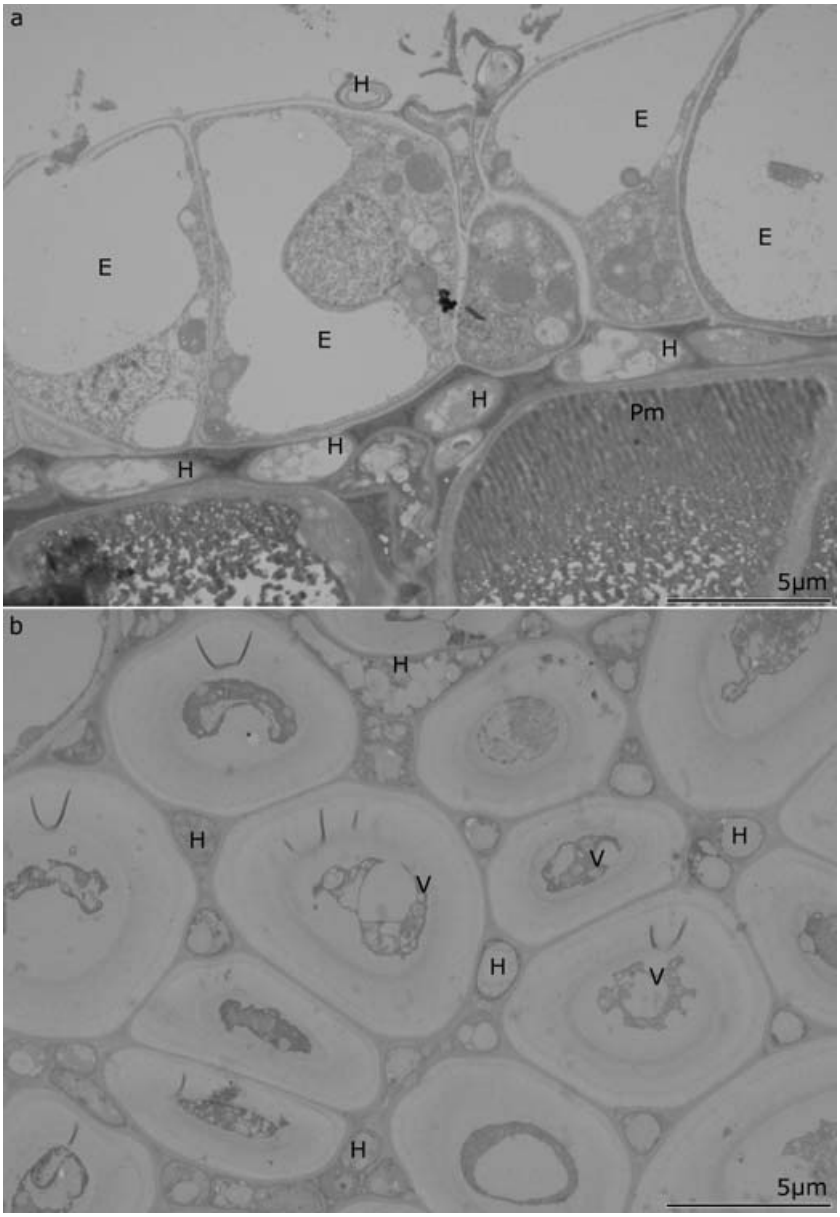

Figure 7 Ultra thin sections of (a) *Quambalaria pitereka* hyphae (H) growing in the intercellular spaces between epidermal (E) and palisade mesophyll (Pm) cells of *Corymbia variegata*. (b) *Quambalaria pitereka* hyphae (H) within the intercellular spaces of vascular cells (V) within a juvenile *Corymbia variegata* stem.

non-susceptible hosts is also presented for the first time. Penetration was shown to preferentially occur on juvenile tissues and this occurred via stomata or through freshly made wounds.

Similarities can be drawn between *Eucalyptus* rust, caused by *Puccinia psidii*, and *Quambalaria* spp., with conidia requiring high humidity levels or the presence of free water at temperatures above 15°C and below 30°C. However, unlike *P. psidii*, germination of *Q. pitereka* and *Q. eucalypti* conidia is only retarded by light and not prevented. *Puccinia psidii* requires a minimum 8 h darkness to induce spore germination (Coutinho *et al.*, 1998).

The conditions observed for germination and fungal growth pre-penetration of the host frequently occur during the warmer spring and summer months in subtropical and tropical regions of Australia. During these times, rainfall events are common and day and night temperatures optimal for spore germination. Storm activity is elevated during this period with rainfall occurring predominantly late in the afternoon and evening, increasing the chance of extended leaf wetness periods. Minimum temperatures rarely fall below 20°C. Increased rainfall and a rise in temperature also induces frequent growth flushes with young, susceptible leaves and shoots becoming abundant on *Corymbia* and *Eucalyptus* spp.

Examination of artificially inoculated *Corymbia* and *Eucalyptus* spp. with *Q. pitereka* and *Q. eucalypti* using SEM showed that spores adhere to the leaf and stem surface, and that penetration occurs by hyphae through stomata and wounds. Degradation of the cuticle of

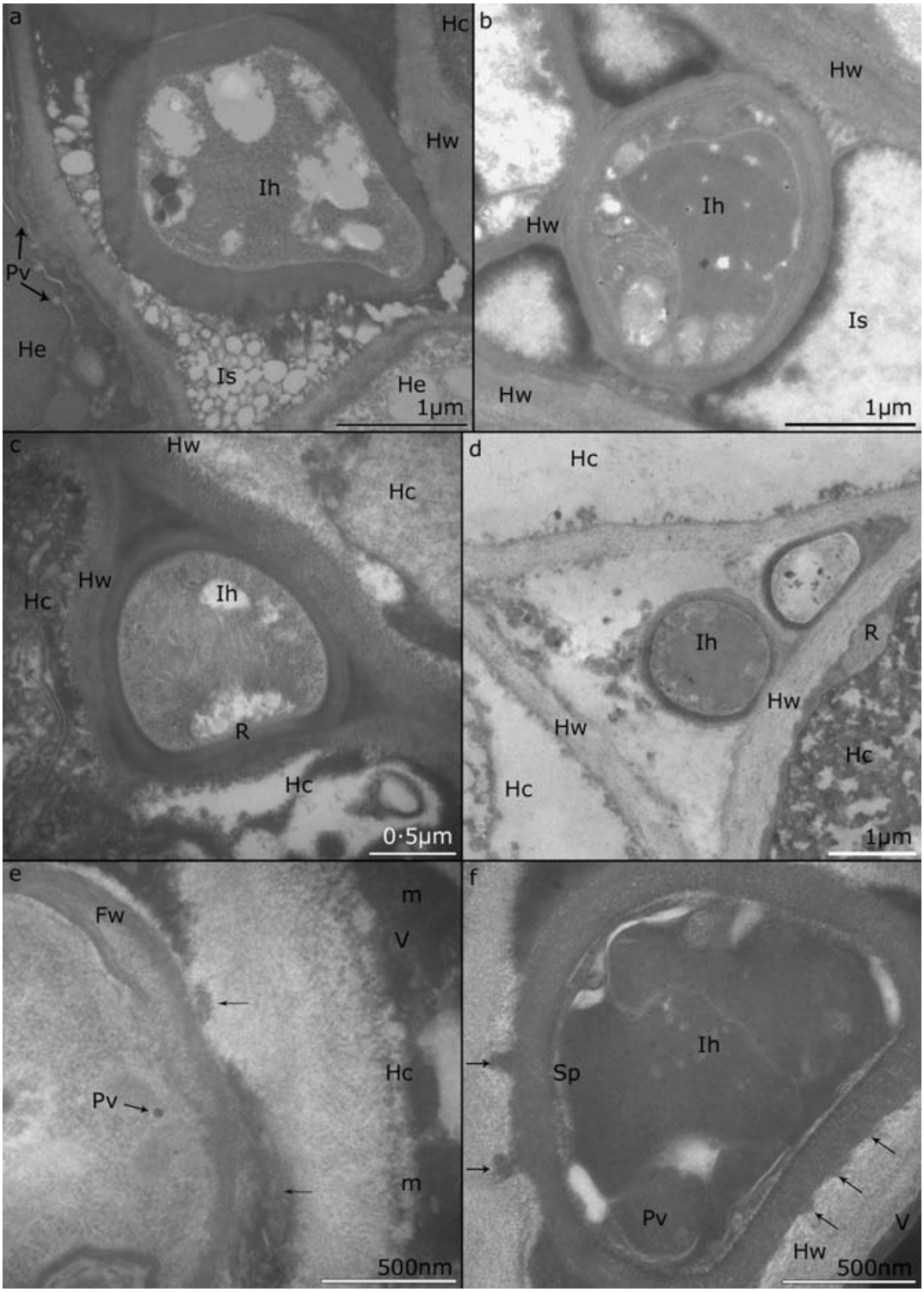

Figure 8 Transmission electron micrograph showing the interaction between intercellular fungal hypha of *Quambalaria pitereka* and the host cells of *Corymbia variegata* with a reaction zone (R) and primary interactive vesicles (Pv) in the host cell (Hc) where contact with hypha is evident. (a) TEM of intercellular hypha (Ih) showing an alteration in the host and fungal cell wall prior to contact and reaction within the intercellular space (Is). (b) TEM of intercellular hypha of *Quambalaria pitereka* in contact with palisade mesophyll cells of juvenile *Corymbia variegata* leaves. (c, d) TEM of intercellular hyphae of *Quambalaria pitereka* in contact with cells in juvenile stems of *Corymbia variegata*. The host cell wall (Hw) infiltrated by electron-opaque material at the contact area and the presence of primary interaction vesicles and secretion profiles at the point of contact between host and pathogen. (e, f) Alteration of the fungal cell wall (Fw) at the point of interaction with the host cell and the presence of vesicles (V) within the matrix (m) forming in the host cell. (e) & (f).

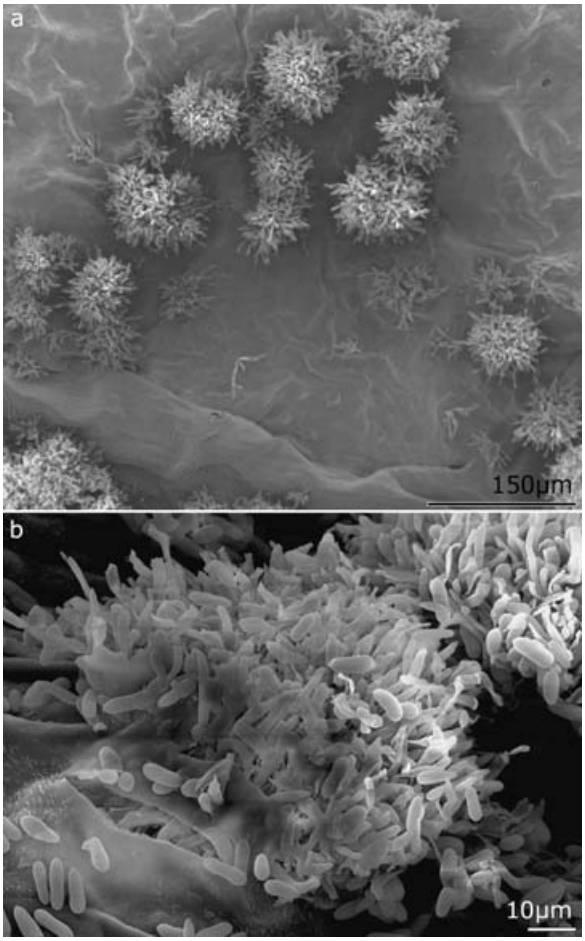

Figure 9 Scanning electron micrograph showing conidiophores and conidia of *Quambalaria pitereka* (a) arising from leaf stomata and (b) rupturing through the epidermal layer on juvenile stem of *Corymbia variegata*.

E. grandis by *Q. eucalypti* and *C. variegata* by *Q. pitereka* suggests that *Quambalaria* spp. produce an adhesive material containing degrading enzymes. The nature of adhesion of *Quambalaria* spores to the leaf and stem surface is unknown but it is likely that a mucilaginous coating is activated in the presence of moisture. Adequate moisture during the infection process is important for the hydration of fungal propagules leading to a rapid release of mucilage to aid in adhesion to a variety of substrates as described by Struck (2006) for the rust *Uromyces fabae*. The composition of this mucilaginous product is unknown for *Quambalaria* spp. While fungal adhesives are typically water-insoluble glycoproteins, their composition varies (Tucker & Talbot, 2001).

An interesting observation in this study was that both *Quambalaria* spp. are able to penetrate the stomata of eucalypts that are considered non-hosts. Thus, germ tube production, hyphal growth and stomatal penetration was observed for *Q. eucalypti* inoculated onto the non-host *C. variegata*. Likewise *Q. pitereka* spores germinated and penetrated the stomata on a range of *Eucalyptus* spp. However, hyphal growth appeared to cease within host tissues and disease symptoms failed to appear. This suggests that host resistance does not occur early and that a specific host pathogen interaction takes place only after penetration of stomata or wounds.

Quambalaria pitereka and *Q. eucalypti* were shown to penetrate directly through stomata or wounds without specialized penetration structures. While many fungi respond to contact stimuli resulting in directional change, hyphal growth of *Q. pitereka* and *Q. eucalypti* following germination did not appear to be specific to any pattern or stimulus of leaf cell structures. This is unlike hyphae of rusts such as *Puccinia graminis* f.sp. *tritici* on wheat and *Uromyces appendiculatus* on bean, which grow at right angles to the parallel arrangement of anticlinal plant cell walls, providing an increased chance of encountering stomata (Allen *et al*., 1991).

It is not possible to tell from this study whether a tactile stimulus alone pre-empts penetration of the stomata by *Q. pitereka* and *Q. eucalypti* germ tubes. The fact that these structures were commonly seen growing over the leaf and stem surfaces, appearing to show no preferential growth toward stomata, suggests that contact with stomata occurs at random. Branching also continued to occur even if stomatal penetration was achieved. A thigmotropic response by *Q. pitereka* and *Q. eucalypti* to leaf and stem topography, in particular to cuticular ridges, which generally surround stomata, or the presence of the guard cell, seems likely to play a role. However, hyphae often grew past or over stomata without penetration. It is possible that stomata need to be open for germ tubes to penetrate and that many stomata could be closed at the time of spore germination. Yet various authors have shown that penetration by germ tubes of other fungi can occur even where stomata are closed (De Wit, 1977; Rathaiah, 1976).

The importance of wounds in the development of quambalaria shoot blight within plantations is not fully understood. Pegg *et al*. (2008) indicated that wounding is not commonly associated with *Q. pitereka* on foliage of *Corymbia* species. However, in comparison, *Q. eucalypti* in Australia has been found both independent of and in association with insect damage. The association with insect activity has not been reported in South Africa (Wingfield *et al*., 1993; Roux *et al*., 2006) or Brazil (Alfenas *et al*., 2001) although *Q. eucalypti* has been associated with mechanical wounding during nursery operations in Brazil (Ferreira *et al*., 2007). Clearly both pathogens are able to infect freshly made wounds on young tissues and such wounding is likely to influence the impact of the associated diseases.

Infection and colonization of *Corymbia* spp. by *Q. pitereka* and *Eucalyptus* spp. by *Q. eucalypti* occurs through intercellular growth of hyphae following penetration and remains intercellular until host cell death. A localized interaction zone is apparent at points of interaction between hyphae and host cell walls. The absence of an interaction apparatus, a feature of species within the Exobasidiales, is characteristic of fungi within the order

Microstromatales (Bauer *et al*., 1997) to which *Quambalaria* belongs (de Beer *et al*., 2006). Hyphae were found to occur in intercellular spaces between all cell types of leaves and juvenile stems resulting in the collapse of cells. It is assumed that *Q. pitereka* and *Q. eucalypti* obtain nutrients from the host cells through these interaction zones.

The mechanism of host-pathogen interaction identified for *Q. pitereka* and *Q. eucalypti* in this study is hypothesized to be ancestral and plesiomorphic for the Ustilaginomycetes (Bauer *et al*., 1997). Furthermore, it is believed that the establishment and operation of the interaction zone is a vital and important feature that allows the fungus to obtain nutrients from the host cell and become pathogenic. The exact nature of the establishment of the interaction zone for *Q. pitereka* and *Q. eucalypti*, which are specific pathogens of *Corymbia* and *Eucalyptus* species, respectively, deserves further attention.

Sporulation of *Q. pitereka* and *Q. eucalypti* can occur within 7 to 10 days after inoculation of susceptible host plants under optimal conditions. Large numbers of individual spores are produced from conidiophores arising from stomata on leaves and stems or rupturing directly through the cuticle. Under ideal climatic conditions, shoot and leaf blight disease caused by *Q. pitereka* and *Q. eucalypti* has the potential to build-up rapidly and complete the cycle within 7 days, especially within a plantation situation where there is a high concentration and large area of uniformly developing susceptible hosts.

Field observations have identified variability in susceptibility amongst *C. variegata* provenances and families to infection by *Q. pitereka* (Self *et al*., 2002; Dickinson *et al*., 2004; Lee, 2007; Johnson *et al*., 2009). The use of *Corymbia* hybrids to reduce the impact of quambalaria shoot blight was based on the reported resistance of *C. torelliana* to infection by *Q. pitereka* (Lee, 2007). The results of this study indicate that variation in susceptibility is not through retarded germination or growth of the spores or hyphae or the inability of *Q. pitereka* to penetrate the host. Differences in susceptibility should be compared at the level of the interaction zones using plants displaying different levels of resistance within a single host species.

Current management strategies have focused on the identification of tolerance within seed provenances of spotted gum with little understanding of the mechanisms of this tolerance and how it influences disease development. Similarly, the development of hybrids between *C. torelliana* and *C. variegata*/*C. citriodora* was based on the assumption that *C. torelliana* was resistant to *Q. pitereka* (Lee, 2007) which was later shown to be incorrect (Pegg *et al*., 2008). Hence, understanding the infection biology of *Q. pitereka* and *Q. eucalypti* on different species of *Corymbia* and *Eucalyptus* is paramount to developing effective disease management strategies.

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