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Anatomical variation and defence responses of juvenile *Eucalyptus nitens* leaves to Mycosphaerella leaf disease

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Abstract. Under the same environmental conditions and inoculum load, a northern New South Wales provenance of *Eucalyptus nitens* (Deane and Maiden) Maiden is more resistant to Mycosphaerella leaf disease than a southern New South Wales provenance. Using histological methods, a comparison was made between one provenance from each distribution with respect to constitutive anatomy and cellular and histochemical changes after infection by *Mycosphaerella* species that cause Mycosphaerella leaf disease. Leaves from the resistant provenance were significantly thinner, had a higher proportion of palisade mesophyll and reduced intracellular airspace compared with those from the susceptible provenance. After infection, an increased level of cell division was observed in resistant leaves and the necrophylactic periderm formed was more organised, continuous, suberised and lignified than periderm formed in susceptible leaves. It is suggested that leaves with higher constitutive proportions of cell dense palisade layers and thinner leaves can partition infected leaf sections from healthy tissue more effectively as less cellular differentiation is required to form an effective necrophylactic periderm. Palisade layers with less intercellular airspace may also play a role in the slowing or prevention of infection as some *Mycosphaerella* species may not be able to penetrate tightly packed cells.

Additional keywords: defence chemicals, forest health management, leaf spot, necrosis, tolerance.

Introduction

Eucalyptus nitens is indigenous to south-eastern Australia and occurs naturally in populations ranging from northern New South Wales (NSW) to the Victorian Alps (Purnell and Lundquist 1986). Its success in plantation forestry has been due mainly to rapid growth, favourable wood properties and frost hardiness, making it suitable for planting in high altitude (600–1600 m) or cold or frosty areas where growth of *E. globulus* (Labill) and *E. grandis* (W. Hill) is unsatisfactory. *Eucalyptus nitens* was introduced into South Africa as a plantation species in 1926 (Poynton 1979) and is now a primary plantation species, grown for pulp and paper products (Hunter *et al.* 2004). However, the planting of *E. nitens* in the summer rainfall areas of South Africa has also increased the risk of disease epidemics such as Mycosphaerella leaf disease (MLD) (e.g. Mohammed *et al.* 2003), which is known to cause severe blighting and defoliation in warm, humid environments (18 and 24°C during the night and day, respectively) where there is ample leaf wetness (Park 1988*a*) to assist spore maturation, ejection and dispersal.

In South Africa, *Mycosphaerella* species were first discovered on an *E. globulus* plantation in the Eastern Cape Province in 1925 (Doidge 1950) and then again in 1933 near Capetown (Verwoerd and Du Plessis 1933). At this time, *E. nitens* appeared to be relatively tolerant to MLD. Research was commenced in 1973 to decide the most suitable provenances to grow on high altitude sites, with selection for growth rate, stem form and wood quality (Purnell and Lundquist 1986). The emergence of *Mycosphaerella* species on *E. nitens* resulted in additional selection for MLD resistance in the Victorian, southern NSW and northern NSW provenances (Lundquist and Purnell 1987). The results from this trial suggested that NSW provenances were considerably

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more resistant to MLD than Victorian provenances, with provenances from northern NSW demonstrating a higher tolerance to MLD than those from southern NSW.

Research into the response of *Eucalyptus* species to foliar pathogens at a cellular level is in its infancy. In *E. globulus*, there is some evidence that leaf density (influenced by internal leaf structure) may contribute to resistance to *Mycosphaerella* species (Smith *et al.* 2006). Increased cell density may enhance plant resistance by preventing infection of certain *Mycosphaerella* species due to their inability to penetrate closely packed palisade cells (Park 1984). This theory is supported by studies associating the density of palisade parenchyma with resistance to several foliar diseases (Basra *et al.* 1985; Mayee and Suryawanshi 1995; Yang 2000).

Parenchyma cells have various functions that include photosynthesis and the manufacture and transport of numerous chemicals used within the leaf and throughout the plant. They remain largely embryonic so that under the appropriate stimulation they may specialise further and develop into a large variety of other cell types (Weisz and Fuller 1962). On infection or injury, parenchyma cells undergo cellular division and abnormal cell swelling (hypertrophy) to fill airspaces in the leaf and form a continuous zone to delimit the pathogen. This is termed a necrophylactic periderm in leaves and a barrier zone in wood (Fink 1999). These cells may undergo secondary changes in cell walls whereby non-cellulosic polysaccharides in the normal cell wall are replaced by hydrophobic polymers (such as lignin or suberin). Periderm formation not only restricts pathogen spread but retains the hydraulic integrity of the surrounding healthy tissue. Effective pathogen restriction has been linked with resistance in tomato plants on infection with *Cladosporium fulvum* (Cooke) (Lazarovits and Higgins 1976*a*, 1976*b*) and *Fusarium oxysporum* (E.F. Sm. and Swingle) (Beckman *et al.* 1982). In addition, early lignification of cells after fungal attack has enhanced resistance in cucumber (Hammershmidt and Kuć 1982) and wheat (Southerton and Deverall 1990; Dushnicky *et al.* 1998).

The cellular response of *E. nitens* to infection by *Mycosphaerella* species has not been characterised. Plantings of both northern and southern NSW provenances of *E. nitens* in mixed plantations in the Mpumalanga province of South Africa have enabled a comparative study to test the working hypothesis that variations in leaf anatomy affect the formation of necrophylactic periderms and the effectiveness of pathogen restriction in *E. nitens*. The specific aims were to:

(i) quantify variations between provenances in leaf thickness, proportions of spongy and palisade mesophyll, cell size, density and relative proportion of airspace; and
(ii) at two stages of infection (initial and necrotic), characterise the response to infection by studying the cellular divisions and modifications required to form necrophylactic periderms.

Methods

Leaf material

Leaves with and without *Mycosphaerella* symptoms were sampled from both the Ebor provenance of northern NSW (herein referred to as resistant) and the Tallaganda provenance of southern NSW (herein referred to as susceptible) from a commercial plantation at Rooihoogte (26°22′S, 30°25′E) in the Mpumalanga province of South Africa. The leaves of trees from the resistant and susceptible provenances were distinctly different and could be easily distinguished visually. The resistant provenance had narrower, more vertically oriented leaves, whereas the susceptible provenance had wider, glaucous leaves. At the time of sampling, all trees in the plantation bore juvenile leaves. The plantation was infected naturally with three major species of *Mycosphaerella*, namely *M. nubilosa* (Cooke) Hansf., *M. marksii* (Carnegie and Keane) and *M. lateralis* (Crous and M.J. Wingf.). Species were identified using ascospore size, shape and germination patterns as described by Hunter *et al.* (2004).

Five trees from each population were chosen by strategic sampling, which included starting at an initial row, moving up two trees and along one row where the next tree was sampled. If that tree was not of the correct provenance then the next tree of the correct provenance in that row was chosen. The leaves sampled were from the fourth leaf pair and assumed to be of the same age. Five leaves with initial and necrotic lesions were sampled from each tree. Five asymptomatic control leaves that were from the same leaf stage and the same trees were also sampled. Initial symptoms of lesion development were identified as 1–4 mm diameter dispersed purple spots, visible on either the abaxial or adaxial surface and without necrotic tissue. Necrotic lesions were greater than 5 mm in diameter, light to dark brown-grey in colouration and without a purple margin. Initial symptoms were sampled to assess the type and quantity of induced cell division occurring after infection to form a necrophylactic periderm. Necrotic lesions were sampled to assess the integrity and accumulation of defence chemicals (lignin, suberin and flavonoids) in advanced necrophylactic periderm formation.

Fixed-sample preparation

Lesion sections of ~2 mm^2 were cut from the edge of each of the two lesion categories and from asymptomatic tissue. Asymptomatic tissue was taken from the middle section of uninfected leaves to the right of the midrib. Sections were fixed in 2.5% glutaraldehyde in 0.1 M Sörensen's phosphate buffer, pH 7.2, for 15 h at room temperature. Following two washes in buffer (each for 20 min), the samples were dehydrated in an ascending ethanol series in 20% increments, finishing with two changes (each for 20 min) of 100% ethanol before embedding in Quetol resin. Transverse sections 2–6 µm thick were cut on a Reichart OmU2 ultramicrotome fitted with a glass knife. Sections were expanded and gently heat-fixed onto clean glass microscope slides before staining.

Constitutive cell density

Three Quetol-embedded sections 50 µm apart were stained for 5 min in Toluidene Blue O stain (1 g Toluidene Blue O in 99 mL of 1% aqueous sodium borate). The stain was diluted 1 : 10 with distilled water before staining. After staining, sections were rinsed thoroughly with distilled water, destained for 50 s in 70% ethanol and then dehydrated in 100% ethanol for 1 min. Sections were photographed at 100× magnification. Three vertical transects through each leaf section (avoiding oil glands and vascular bundles) were used to assess the widths of cuticular, epidermal, palisade and spongy mesophyll cell layers, and the

proportion of palisade and spongy mesophyll of sample thickness measured per transect. Values for transects were averaged for each component before analysis. The same sections were then photographed at 200× magnification, at which cellular dimensions (length, width and area) and, using a horizontal transect, the number of palisade cells per 100 µm and the proportion of airspace in the palisade mesophyll layer were determined. All data were analysed by general analyses of variance using the statistical package GenStat® (VSN International, Hemel Hempstead, UK), with resistance as treatment and leaf as a block.

Pathogen restriction

Quetol-embedded sections of initial and necrotic disease symptoms were assessed. Phloroglucinol-hydrochloric acid (HCl) was used to stain for lignin deposition. Sections were immersed in 1% (w/v) phloroglucinol solution in 70% ethanol for 5 min. Excess phloroglucinol solution was poured off and sections were covered with two drops of concentrated HCl, covered with a glass coverslip and examined with a Zeiss compound light microscope. The sections were photographed immediately because the reaction faded with time. Bright red to orange areas appeared in the presence of lignin.

Suberin was observed on the same sections at 365 nm using a Zeiss Axiovert fluorescence microscope fitted with a mercury lamp where the phloroglucinol was used as a counter-stain to eliminate the autofluorescence of lignin (Biggs 1984). Naturstoffreagenz A (1% diphenylboric acid 2-aminoethyl ester in methanol) was used to detect flavonoids. Slides were flooded with the solution for 5 min. Flavonoids were detected at 365 nm, 450–490 nm and at >590 nm (Hutzler *et al.* 1998) using a Zeiss Axiovert fluorescence microscope fitted with a mercury lamp.

Results

Constitutive cell density

The resistant provenance was observed to have significantly thinner leaves ($P < 0.001$), thicker adaxial and abaxial cuticles ($P = 0.008$ and 0.002, respectively) and thinner adaxial and abaxial epidermal layers ($P = 0.002$ for both) than the susceptible provenance (Table 1). Leaves from the resistant provenance had a higher proportion of palisade cells ($P = 0.013$) and a lower proportion of spongy mesophyll ($P < 0.01$) (Table 1). Mean palisade cell dimensions (width and length) were not significantly different between provenances, but more palisade cells were recorded per mm of horizontal transects of leaves from the resistant provenance (Fig. 1*a*), compared with the susceptible provenance ($P < 0.001$) (Fig. 1*b*; Table 1). Palisade cells were more tightly packed in the resistant provenance as significantly less intercellular airspace was observed in the

Table 1. Quantitative anatomical features of *Eucalyptus nitens* leaves that are resistant (northern NSW) and susceptible (southern NSW) to Mycosphaerella leaf disease

Mean values, standard errors of the differences of means (s.e.) and their statistical significance (*P*-value) are given. Characteristics of susceptible and resistant leaves were statistically significant at $P < 0.05$

Character	Susceptible	Resistant	s.e.	*P*-value
Total leaf thickness (µm)	330	281	3.6	<0.001
Thickness of cuticle (µm)				
Adaxial	4.4	5.7	0.3	0.008
Abaxial	5.9	4.7	0.2	0.002
Thickness of epidermis (µm)				
Adaxial	12.7	11.4	0.3	0.002
Abaxial	13.3	11.3	0.5	0.002
Thickness of palisade mesophyll (µm)				
Adaxial	46.4	46.5	2.7	0.986
Abaxial	57.7	50.4	1.5	<0.001
Thickness of spongy mesophyll layer (µm)	191	150	1.8	<0.001
Total palisade as a proportion of leaf thickness (%)	31.6	34.5	5.3	0.013
Spongy mesophyll as a proportion of leaf thickness (%)	57.7	53.1	0.8	<0.001
Airspace in spongy mesophyll layer as a proportion of leaf thickness (%)	43.2	35.6	0.7	0.152
Proportion of airspace in palisade layer (%)				
Adaxial	54.1	35.8	3.05	0.002
Abaxial	38.0	28.8	5.36	0.026
No. palisade cells per mm				
Adaxial	17	21	1.7	<0.001
Abaxial	13	19	2.9	<0.001
Width of palisade cells (µm)				
Adaxial	9.7	9.4	0.2	0.243
Abaxial	9.3	9.2	3.9	0.880
Length of palisade cells (µm)				
Adaxial	46.1	42.8	1.7	0.295
Abaxial	33.4	31.7	0.8	0.345

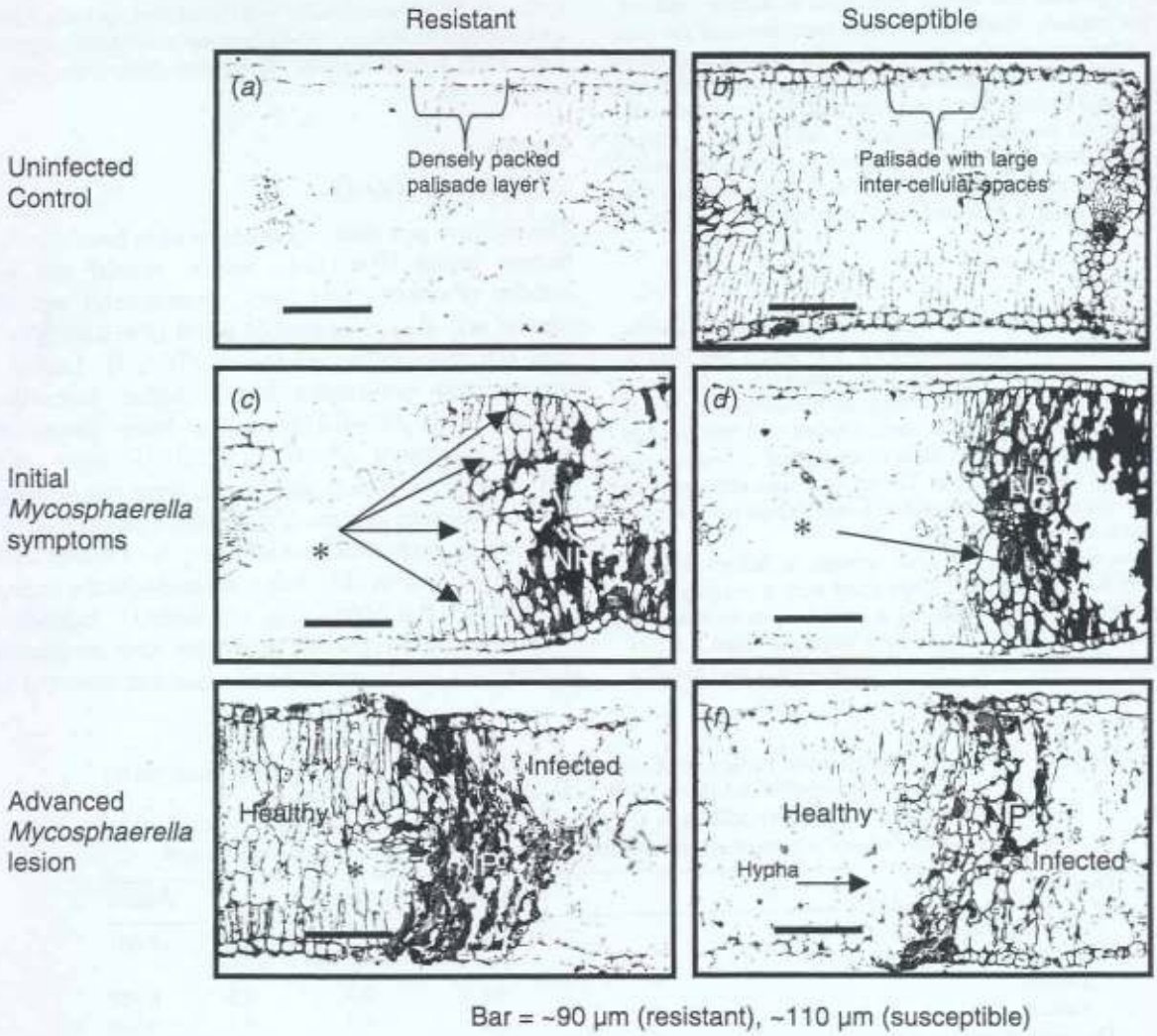

Fig. 1. Typical transverse sections through resistant and susceptible leaves that were healthy (*a*, *b*), displaying initial *Mycosphaerella* lesion symptoms (*c*, *d*) and advanced *Mycosphaerella* lesion symptoms (*e*, *f*). Densely packed palisade mesophyll and reduced intracellular airspace between palisade mesophyll cells was observed for leaves of resistant (*a*) and susceptible (*b*) trees. On infection, this was associated with large amounts of cell division and the coalescing of cells to form a necrophylactic periderm in resistant leaves (*c*) whereas only a small amount of cell division was observed in susceptible leaves (*d*). In advanced lesions on resistant leaves (*e*), cells coalesced to form an organised necrophylactic periderm (NP, >5 cells in thickness), which was infiltrated with defence chemicals (darker regions) such as lignin, suberin and other phenolics and cells continued to divide (*) on the healthy side of the NP. In advanced lesions on susceptible leaves (*f*), a distinct and organised NP was not observed. In some sections, fungal hyphae had already begun to penetrate cells on the healthy side of the periderm (*f*).

adaxial and abaxial palisade layers of the resistant provenance when compared with the susceptible provenance ($P = 0.002$ and 0.026, respectively).

Pathogen restriction

Transverse sections through initial lesions revealed a higher level of cell division adjacent to the infection site in the resistant (Fig. 1*c*) compared with the susceptible provenance (Fig. 1*d*) in all replicates. In resistant leaves, existing cells and divided cells coalesced to create a distinct zone that was often greater than five cell layers in necrotic samples. The zone was infiltrated heavily with suberin (Fig. 2*a*), lignin (not presented) and flavonoids (Fig. 2*c*, *e*). The necrophylactic periderm was continuous, organised and most probably impermeable to hyphae, water and toxins, as cells on the uninfected side of the barrier were healthy and had retained the capacity to continue cell division.

In the susceptible provenance, sections through initial lesions revealed that limited cell division occurred only in cells adjacent to the infection site (Fig. 1*d*) in all replicates. In advanced lesions, the arrangement of cells into a necrophylactic periderm was unorganised (Fig. 1*f*),

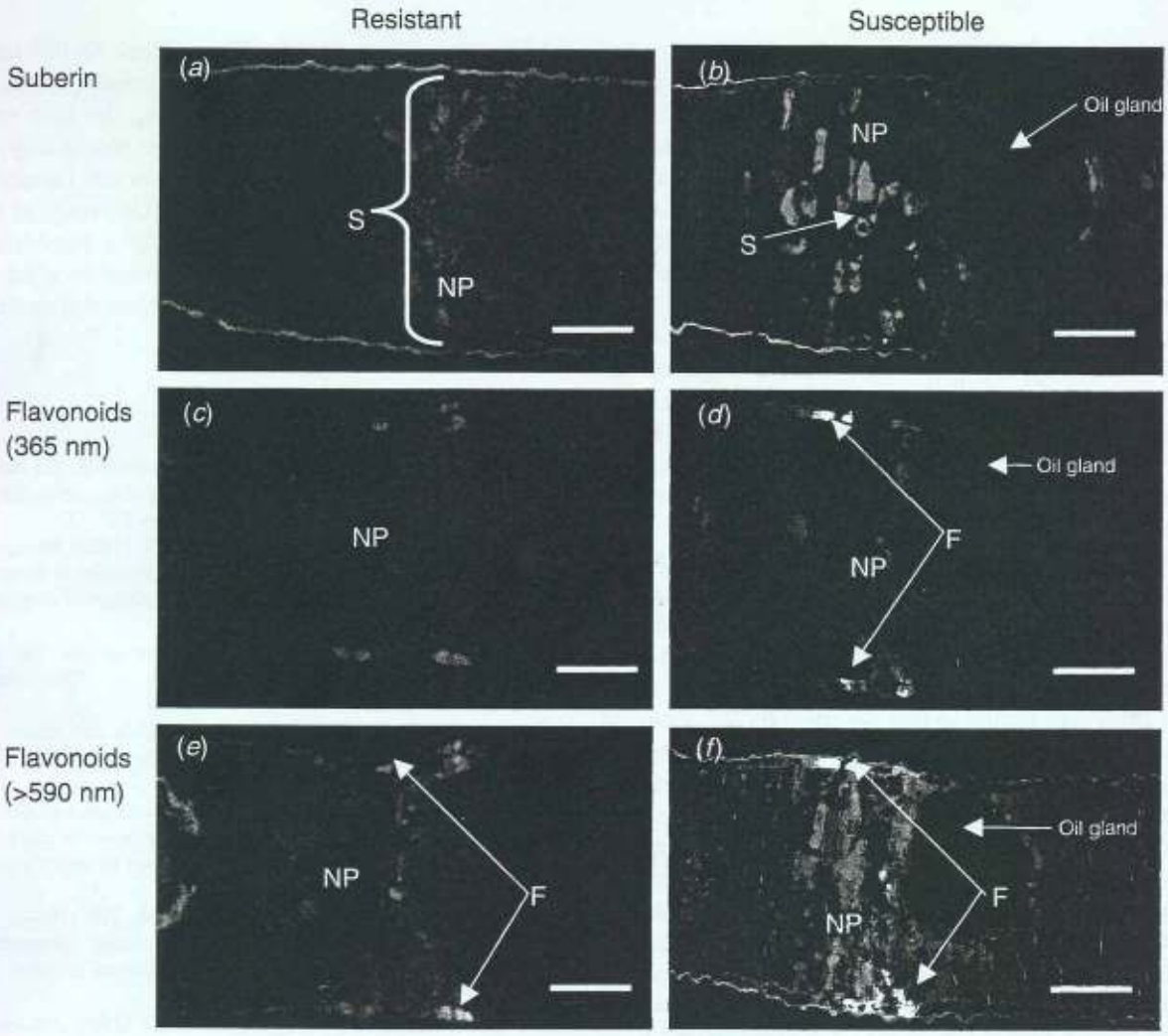

Fig. 2. Typical transverse sections through advanced *Mycosphaerella* lesions on resistant and susceptible leaves where the necrophylactic periderms (NP) have been histochemically stained for suberin (S) (blue fluorescence in *a* and *b*) and flavonoids (F) (yellow fluorescence in *c–f*). Bar = ~90 μm in resistant cells and ~110 μm in susceptible cells.

which resulted in a dispersed accumulation of lignin, suberin (Fig. 2*b*) and flavonoids (365 and >590 nm; Fig. 2*c*, *e*). In 40% of sections through necrotic lesions of susceptible leaves, the necrophylactic periderm was not continuous, allowing hyphae to progress into healthy tissue. Flavonoids were observed in epidermal cells on the infected side of the necrophylactic periderm in both resistant and susceptible leaves in all replicates.

Discussion

In eucalypts, variation in resistance to *Mycosphaerella* leaf spot pathogens has been observed between species (Carnegie *et al.* 1998), subspecies (*E. globulus*, Carnegie *et al.* 1994; Carnegie and Ades 2005) provenances (*E. nitens*, Purnell and Lundquist 1986) and families (*E. globulus*, Milgate *et al.* 2005). In addition, resistance to *Mycosphaerella* infection increases with ontogenetic transition from juvenile to adult foliage (Dungey *et al.* 1997; Carnegie and Ades 2002) and within the juvenile leaf stage, whereby newly formed, soft and expanding leaves have been identified as the most susceptible whereas older leaves are more resistant (Park 1988*b*). Although resistance to *Mycosphaerella* infection is likely to be a combination of several traits, there appears to be a relationship between resistance and the proportion of palisade mesophyll. For example, *E. nitens* (isobilateral palisade) has a higher tolerance to *Mycosphaerella* infection compared with *E. globulus* (unilateral palisade) (Smith *et al.* 2006), and palisade mesophyll layers increase in number from juvenile (one palisade layer) to adult foliage (~4–6 palisade layers) in *E. globulus* (James and Bell 2001).

Higher palisade densities (or volume fractions) have been associated with resistance to several leaf spot pathogens including *Cercospora* (Basra *et al.* 1985) and

Phaeoisariopsis leaf spot in groundnut (Mayee and Suryawanshi 1995), Frog-eye leaf spot in soybean (Yang 2000) and *Septoria* leaf spot in celery leaves (Edwards *et al.* 1999). A tighter packing of palisade cells was also observed in resistant families of *E. globulus* when compared with susceptible families; however, this was a qualitative assessment (A. Smith, unpublished data). For the first time we have quantitative evidence that a tighter packing of palisade mesophyll cells may be associated with resistance to MLD. Evidence from past and present studies suggests that compact palisade layers may slow or prevent hyphal development due to the inability of some *Mycosphaerella* species to penetrate and colonise tightly packed palisade cells (Park 1984; Smith *et al.* 2006). This, coupled with increases in cuticle thickness in resistant leaves compared with susceptible leaves, may act as a considerable barrier to hyphal penetration.

From histological examination, we can suggest that formation of necrophylactic periderms in *E. nitens* rely heavily on cell division and the incorporation and coalescing of cells to link the adaxial and abaxial leaf surfaces. The host response of *E. nitens* to *Mycosphaerella* species in the current study was similar to that observed for the Victorian provenance of *E. nitens* (Smith *et al.* 2006) and *E. globulus* (Park and Keane 1982; Smith *et al.* 2006), and, therefore, the production of highly lignified and suberised periderm is assumed to be a generalised host response to wounding and fungal invasion. However, the differences between resistant and susceptible provenances are likely to be exhibited in the speed and strength of pathogen restriction. Our images of initial and advanced infection show that resistant leaves are more proficient in cellular division and the formation of effective necrophylactic periderms. It is hypothesised from the current study that resistant leaves are thinner and have a higher proportion of dense palisade cell layers and reduced proportions of spongy mesophyll. Therefore, it is possible that resistant leaves would have a higher capacity to divide after infection and also require less cellular differentiation to form a necrophylactic periderm.

Although individual *Mycosphaerella* species can be detected within lesions using species-specific primers (Kularatne *et al.* 2004; Glen *et al.* 2006), the development of techniques such as real-time PCR will enable the hyphal mass within a lesion to be compared between resistant and susceptible provenances after infection. Future studies might also concentrate on comparing host pathogen interactions with the pathogenicity of *Mycosphaerella* species and biotypes on resistant and susceptible trees. Further testing of *E. nitens* provenances and families will determine the suitability of using palisade density and internal leaf structure as an indicator of resistance in *E. nitens*.

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